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Preface

Gastrointestinal cancers account for more than one third of all deaths from cancer. Peritoneal dissemination is considered as an advanced stage in the natural history of these malignancies and a frequent finding in the recurrent condition. As a curative approach to peritoneal surface malignancies confined to the peritoneal cavity, cytoreductive surgery combined with perioperative intraperitoneal chemotherapy has brought about long-term benefits in selected patients with peritoneal carcinomatosis (PC) of gastrointestinal origin and pseudomyxoma peritonei (PMP) syndrome. However, intraperitoneal chemotherapy fails to maintain the surgical complete response in a proportion of patients with gastrointestinal PC and PMP. In this context, mucins aberrantly expressed by tumor cells are believed to play key roles in tumor biology and pathogenesis, contributing to the tumor growth and progression, resistance to chemotherapy, evasion of immune surveillance, and formation of mucinous ascites.

While providing the reader with a detailed overview of peritoneal surface malignancies and an in-depth understanding of the role of mucin in the pathogenesis of mucin-producing tumors, this monograph introduces a novel locoregional approach to these challenging entities. This experimental treatment is capable of enhancing microscopic cytoreduction, eliminating mucin, and maximizing cytotoxic effects of chemotherapeutic agents on tumor cells while minimizing their toxic effects on host cells. Such a treatment has thus the potential to improve therapeutic benefits of the current multidisciplinary strategies in patients with mucin-producing peritoneal malignancies. The first chapter outlines the classification of peritoneal surface malignancies and discusses gastrointestinal PC as well as PMP syndrome. Each section describes the incidence and natural history of the disease, pathogenesis, predicting and predisposing factors, diagnostic and prognostic evaluation, and the state-of-the-art treatment. Results from studies using cytoreductive surgery and perioperative intraperitoneal chemotherapy along with pros and cons of this curative approach are summarized at the end of each section. The second chapter introduces the diverse family of mucin glycoproteins with emphasis on their classification, regulation, and role in health and cancer. This is followed by two chapters devoted to bromelain and *N*-acetylcysteine, the two natural agents used in our experimental

treatment. These chapters include a brief history of the evolution of the drugs over the past decades and provide an overview of their biochemical properties, pharmacokinetics, pharmacodynamics, potential and actual applications, safety, and tolerability. Of particular interest are their potential benefits in the management of cancer that have been reviewed in further detail. Eventually, the last chapter summarizes our findings on the combined use of bromelain and *N*-acetylcysteine in treating the experimental models of gastrointestinal PC and PMP, indicating (a) inhibitory effects on malignant cell and tumor growth; (b) chemosensitizing effects on cancer cells treated with commonly used chemotherapeutic agents; and (c) mucin-depleting effects observed as a decrease in mucin produced/secreted by cancer cells both in vivo and in vitro.

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Abbreviations

2AAF	2-Acetylaminofluorene
5-FU	5-Fluorouracil
°C	Degrees Celsius
ACEC	Animal Care and Ethics Committee
ACF	Aberrant crypt foci
ADAM17	Disintegrin and metalloprotease domain containing protein 17
AGE	Advanced glycation end products
AIDS	Acquired immunodeficiency syndrome
AIHW	Australian Institute of Health and Welfare
AJCC	American Joint Committee on Cancer
ALDH1A1	Aldehyde dehydrogenase 1A1
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AOM	Azoxymethane
APAF1	Apoptotic protease-activating factor 1
ATM	Ataxia telangiectasia mutated
AUC	Area under the time-concentration curve
AUC IP/IV	Area under the curve ratios of intraperitoneal to intravenous exposure
B[a]P	Benzo(a)pyrene
Bad	B-cell lymphoma 2-associated agonist of cell death
BAK	Bcl2 homologous antagonist/killer
BAX	Bcl2-associated X protein
Bcl2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra-large
BCS	Body condition scoring
BH3	Bcl2 homology 3
BID	BH3-interacting domain death agonist
BR	Bromelain
BSA	Bovine serum albumin
BTG1	B-cell translocation gene 1

C/EBP β	CAAT/enhancer binding protein β
CA125	Carbohydrate antigen 125
CA19.9	Carbohydrate antigen 19.9
CC	Completeness of cyto reduction
CCS	Completeness of cyto reduction score
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CDX2	Caudal-type homeobox protein 2
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
CGI	Clinical Global Impression
CI	Combination index
CIN	Contrast-induced nephropathy
CIN85	Cbl-interacting protein of 85 kDa
Cis	Cisplatin
CK	Cysteine-knot domain
CK(7, 18, 20)	Cytokeratin (7, 18, 20)
CLS	Capillary-like structures
cm	Centimeter
cm ²	Square centimeter
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CRCPC	Peritoneal carcinomatosis from colorectal cancer
CRD	Complete redistribution
CREB	Cyclic AMP response element-binding protein
CRS	Cytoreductive surgery
CSCs	Cancer stem cells
CSE	Cigarette smoke extract
CT	Computed tomography
CTGF	Connective tissue growth factor
CTL	Control
DAB	Diaminobenzidine
DDR	DNA damage response
DDT	<i>p,p'</i> -Dichlorodiphenyltrichloroethane
DEN	Diethylnitrosamine
DMBA	7,12-Dimethylbenz(a)anthracene
DMF	<i>N,N</i> -Dimethylformamide
DMH	1, 2-Dimethylhydrazine
DPAM	Disseminated peritoneal adenomucinosis
DSS	Dextran sulfate sodium
dUTP	Deoxyuridine triphosphate
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EIPL-IPC	Extensive intraoperative peritoneal lavage followed by intraperitoneal chemotherapy
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EPIC	Early postoperative intraperitoneal chemotherapy
ErbB	Erythroblastic leukemia viral oncogene homolog
ERK	Extracellular signal-regulated kinase
EUS	Endoscopic ultrasonography
FADD	FAS-associated death domain protein
FASL	FAS ligand
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FIP	Fédération Internationale Pharmaceutique
FLAP	5-Lipoxygenase activating protein
g	Gram
GC	Gastric cancer
GCPC	Peritoneal carcinomatosis from gastric cancer
GGT	Gamma-glutamyl transpeptidase
GIST	Gastrointestinal stromal tumor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Reduced glutathione
H&E	Hematoxylin and eosin
HB-EGF	Heparin-binding EGF-like growth factor
HBP1	HMG box-containing protein 1
HCC	Hepatocellular carcinoma
HCR-Gli	Highly conserved region containing a Gli-binding sequence
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HIPEC	Hyperthermic intraperitoneal chemotherapy
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinoma
HP	<i>Helicobacter pylori</i>
I/R	Ischemia–reperfusion
IC50	Fifty percent (half-maximal) inhibitory concentration
ICAM-1	Intercellular adhesion molecule 1
ICC	Immunocytochemistry
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IM	Intestinal metaplasia
IMS	(Mitochondrial) intermembrane space
iNOSnf	Inducible nitric oxide synthase
ip	Intraperitoneal
IPMN	Intraductal papillary mucinous neoplasm
LD50	Median lethal dose

LDL	Low-density lipoproteins
LMWT	Low-molecular-weight thiol
LPS	Lipopolysaccharide
LS	Lesion size score
LUS	Laparoscopic ultrasonography
MAPK	Mitogen-activated protein kinases
MCP	Mucinous carcinoma peritonei
MCP-H	High-grade mucinous carcinoma peritonei
MCP-L	Low-grade mucinous carcinoma peritonei
MDR	Multidrug resistance
MEFs	Murine embryonic fibroblasts
MMN	Mismatch negativity
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
MPM	Malignant peritoneal mesothelioma
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MUC1	Mucin 1
MUC2	Mucin 2
MUC5AC	Mucin 5AC
NAC	<i>N</i> -Acetylcysteine
NAPQI	<i>N</i> -Acetyl- <i>p</i> -benzoquinone imine
NCI	National Cancer Institute
NEAA	Non-essential amino acids
NHMRC	National Health and Medical Research Council
NHTBE	Normal human tracheobronchial epithelial
NIPS	Neoadjuvant intraperitoneal/systemic (bidirectional) chemotherapy
NSCLC	Non-small cell lung carcinoma
Nup62	Nucleoprotein 62
OCD	Obsessive-compulsive disorder
PAF	Platelet-activating factor
PANSS	Positive and Negative Symptoms Scale
PARP	Poly ADP ribose polymerase
PAS	Periodic acid-Schiff
PC	Peritoneal carcinomatosis
PCI	Peritoneal cancer index
PCNA	Proliferating cell nuclear antigen
PDA	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PFCCs	Peritoneal free cancer cells
PGE2	Prostaglandin E2
PGK1	Phosphoglycerate kinase 1
PI3K	Phosphatidylinositol 3-kinase

PK (A, B, C, G)	Protein kinase (A, B, C, G)
PMA	Phorbol 12-myristate 13-acetate
PMCA	Peritoneal mucinous carcinomatosis
PMCA-D	Peritoneal mucinous carcinomatosis with discordant features
PMCA-I	Peritoneal mucinous carcinomatosis with intermediate features
PMCs	Peritoneal mesothelial cells
PMP	Pseudomyxoma peritonei
PPP	Pentose phosphate pathway
PSC	Primary sclerosing cholangitis
PSM	Peritoneal surface malignancy
PSS	Prior surgical score
PTBD	Percutaneous transhepatic biliary drainage
PTS	Proline, threonine, and serine
PTX	Paclitaxel
PVDF	Polyvinylidene fluoride membrane
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RPD	Random proximal distribution
rTdT	Recombinant terminal deoxynucleotidyl transferase
SAA3	Serum amyloid A3 protein
SCE	Shark cartilage extracts
SDS	Sodium dodecyl sulfate
SEA	Sea urchin sperm protein, enterokinase, and agrin domain
SEM	Standard error of the mean
SIP1	Smad-interacting protein 1
SLE	Systemic lupus erythematosus
sLe ^A	Sialyl Lewis ^A antigen
sLe ^x	Sialyl Lewis ^x antigen
SMAC	Second mitochondria-derived activator of caspase
SNP	Single-nucleotide polymorphism
SPCI	Simplified peritoneal cancer index
SRB	Sulforhodamine B
SRCC	Signet-ring cell carcinoma
SSC	Saline–sodium citrate
STn	Sialyl-Tn antigen
TA	Tumor-associated
TACA, TCA	Tumor-associated (carbohydrate) antigen
TACE	TNF- α converting enzyme
TBST	Tris-buffer saline containing Tween 20
TCA	Tricarboxylic acid (cycle)
TCF/LEF	T-cell factor/lymphoid enhancer factor
TdT	Terminal deoxynucleotidyl transferase
TF	Thomsen–Friedenreich antigen
TFF3	Trefoil factor family peptide 3

TGF	Transforming growth factor
Th1	T helper cell type 1
TLR2	Toll-like receptor 2
TNF	Tumor necrosis factor
TPA	12- <i>O</i> -Tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	TdT-mediated dUTP nick-end labeling
TXB2	Thromboxane B2
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid
UICC	International Union Against Cancer
US	Ultrasonography, ultrasound
VCR	Vincristine
VEGF	Vascular endothelial growth factor
VM	Vasculogenic mimicry
VNTR	Variable number tandem repeat
vs.	Versus
vWF	von Willebrand factor
w/v	Weight/volume
WCD	Widespread cancer distribution
WHO	World Health Organization
Wnt	Wingless-type (pathway)
XIAP	X-linked inhibitor of apoptosis protein

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Chapter 1

Peritoneal Dissemination of Gastrointestinal Tumors

1.1 Peritoneal Carcinomatosis from Colorectal Cancer

1.1.1 Incidence and Natural History

According to GLOBOCAN 2012 (Torre et al. 2015), colorectal cancer (CRC) is the third most common cancer in men (746,300 new cases, 10.0 % of the total) and the second in women (614,300 new cases, 9.2 % of the total) worldwide. With respect to mortality, it accounts for 693,900 cancer deaths (8.4 % of the total) occurring in 2012. Almost 60 % of the cases occur in developed regions, with the highest rates being estimated in Australia/New Zealand (Torre et al. 2015). In Australia, CRC is the second most common cancer in both men and women, with a total of 15,151 new cases diagnosed in 2011. It represents the second cause of cancer deaths after lung cancer, accounting for 9.2 % of the total cancer mortality in 2012 (AIHW 2015a, b).

The development of PC from CRC (CRCPC) is a common mode of the disease progression and a frequent finding in the recurrent CRC (Table 1.1). Three principal studies on the natural history of CRCPC have consistently indicated that PC can occur along with lymph node and liver metastases or as isolated peritoneal surface dissemination (Chu et al. 1989; Jayne et al. 2002; Sadeghi et al. 2000). In the largest study, 349 out of 3019 CRC cases (13 %) were identified as having PC, either metachronous (5 %) or synchronous (8 %). In 57 % of those with synchronous PC, the peritoneum was the only metastatic site (Jayne et al. 2002). Moreover, a minimal peritoneal disease might be present in CRC patients at the time of initial surgery (Ceelen and Bracke 2009). As such, peritoneal free cancer cells (PFCCs) are found on the peritoneal surfaces (Baskaranathan et al. 2004; Solomon et al. 1997) or in peritoneal washes (Guller et al. 2002; Hara et al. 2007; Lloyd et al. 2006) in

Table 1.1 Classification of peritoneal surface malignancies (PSM) (Di Giorgio 2015)

Type	Malignant	Borderline/low grade	
Primary PSM	Diffuse malignant peritoneal mesothelioma (DMPM)	Well-differentiated papillary mesothelioma, multicystic peritoneal mesotheliomas	
	Primary peritoneal serous papillary carcinoma		
	Desmoplastic small round cell tumor		
Secondary PSM	Intra-abdominal origin		
	Colorectal cancer		
	Gastric cancer		
	Ovarian and fallopian tube cancer		
	Borderline ovarian cancer		
	Mucinous adenocarcinoma of the appendix (corresponding to peritoneal mucinous carcinomatosis (PMCA))	Low-grade mucinous adenocarcinoma of the appendix (corresponding to disseminated peritoneal adenomucinosis (DPAM))	
	Adenocarcinoid of the appendix (goblet cell adenocarcinoma or mixed adenoneuroendocrine carcinoma (MANEC))		
	Adenocarcinoma of the small bowel		
	Gastrointestinal stromal tumor (GIST)		
	Retroperitoneal origin		
	Pancreatic cancer		
	Kidney, ureter, adrenal, and bladder cancer		
	Sarcomas		
	Extra-abdominal origin		
	Breast cancer		
	Lung cancer		

10–15 % and 18–25 % of CRC patients, respectively. Intraoperative detection of these cells is associated with a higher risk of recurrence (Rekhraj et al. 2008). Metachronous PC is identified in 2–19 % of patients following curative resection, in 21–44 % of recurrent CRC, and in up to 40 % of autopsies (Klaver et al. 2012b). PC has always been regarded as a terminal condition (Harmon and Sugarbaker 2005). Natural history studies have revealed the rapidly fatal course of CRC following peritoneal dissemination, where survival is adversely affected by the extent of PC and the T stage of the primary tumor (Sugarbaker 2005a). In the absence of curative approaches, the median survival of CRCPC cases ranges from 3 to 7 months (Chu et al. 1989; Jayne et al. 2002; Sadeghi et al. 2000).

1.1.2 Pathogenesis

As with hematogenous and lymphatic metastases, the development of CRCPC involves well-defined steps, including cancer cell shedding and transport, adhesion to the mesothelial layer, invasion of and proliferation into the submesothelial stroma,

tumor-peritoneal angiogenesis, and potential access to the systemic circulation [reviewed by (Ceelen and Bracke 2009; Jayne 2007)]. The process starts with the detachment of tumor cells from the primary cancer followed by spontaneous shedding of loose cells caused by either an increase in the interstitial fluid pressure of tumors invading the serosal bowel surface or spontaneous bowel perforation. Once detached, PFCCs are transported by peritoneal fluid flow through the peritoneal cavity along predictable directions and adhere to the mesothelial lining of the peritoneal surfaces. As shown by Sugarbaker et al., the peritoneal distribution of PFCCs is affected by tumor type, the intraperitoneal environment, and the physiology of the peritoneal surface tissues, where peritoneal surface motion (peristalsis) and intraperitoneal fluids are prominent mechanisms controlling the patterns of spread. In contrast to tumor cells within a mucinous fluid that are freely redistributed on the abdominopelvic surfaces by peritoneal flow governed by intraperitoneal hydrodynamics, those in the absence of a fluid vehicle are more likely to adhere, implant, and progress proximal to the primary site (Carmignani et al. 2003; Sugarbaker 1996b). Through a number of adhesion molecules, including CD43, CD44, CXCR4, and MUC16, PFCCs can adhere to mesothelial cells. In areas of absent or contracted mesothelial cells, interaction between cancer cells and the underlying extracellular matrix components (laminin and fibronectin) occurs. Specialized structures, such as the omentum and the diaphragmatic peritoneum, represent preferential location for mesothelial adhesion. Common sites for peritoneal implants of CRC are the omentum, mesentery, bowel surface, pouch of Douglas, right paracolic gutter, and diaphragm (Klaver et al. 2012b). Postoperative inflammation and wound healing may also contribute to tumor cell adhesion and growth. The submesothelial tissue can then be accessed by tumor cells at areas of peritoneal discontinuity, or alternatively exposed by the induction of apoptosis or contraction of mesothelial cells and disruption of intercellular junctions. Invasion of the submesothelial tissue is accompanied by adhesion of integrins to the extracellular matrix and subsequent degradation by proteases (Ceelen and Bracke 2009). The peritoneal stromal tissue appears to be a favorable host for tumor proliferation, providing a rich source of growth factors and chemokines known to be involved in tumor progression. As the peritoneal deposits grow, they need to develop a blood supply to meet their increasing metabolic demands. The deeper layer of the peritoneum contains a rich capillary network and is ideally suited to this function. However, the angiogenic events specific to PC remain to be elucidated (Jayne 2007). An underestimated aspect of PC is the potential role of the peritoneal implants in lymphatic and hematogenous spread. To this end, tumor cells have the capability to access systemic circulation via submesothelial lymph channels draining into substernal, parasternal, mediastinal, paraaortic, and renal hilum lymph nodes. For this purpose, subperitoneal lymphatic lacunae located between the muscle fibers of the diaphragm can be reached through openings (stomata) between cuboidal mesothelial cells (Ceelen and Bracke 2009). Moreover, the blockage of lymphatic channels by clumps of tumor cells prohibits the efflux of protein and peritoneal fluid, which is apparently the major pathophysiologic mechanism behind the formation of malignant ascites (Garrison et al. 1987). Immunomodulators, vascular permeability factors, and metalloproteinase also

contribute significantly to this process (Parsons et al. 1996). Following tumor growth on the intestinal surfaces and associated fluid accumulation, partial bowel obstruction and massive ascites will develop eventually (Klaver et al. 2012b).

Technical mishaps during surgery can similarly give rise to peritoneal cancer spread (Ceelen and Bracke 2009). According to the “tumor cell entrapment” hypothesis advanced by Sugarbaker (1996a), the surgical procedure per se substantially contributes to the natural history of gastrointestinal cancer through traumatic dissemination of tumor emboli within the peritoneal cavity. Sourced from severed lymphatic channels, disrupted tissue interstices at the lateral margins of tumor dissection, and backflow of venous blood, these tumor emboli are then implanted (entrapped) within the fibrinous exudate that accumulates at the resection site and on abraded peritoneal surfaces. This phenomenon explains disease recurrence confined to the resection site and peritoneal surfaces in patients who undergo treatment using surgery alone compared to those treated with surgery plus the addition of intraperitoneal anti-neoplastic therapies (Sugarbaker 1999a; Sugarbaker et al. 1990).

PC may progress more rapidly than parenchymal metastases and, because of the intimate relationship with the bowel, rapidly disrupts host function. As the cancer nodule enlarges, cancer cells are programmed to exfoliate from their attachment to the basement membrane. Thus, peritoneal implants may exfoliate cancer cells in great numbers into the free peritoneal space. With no growth inhibition applied to these newly implanted cells, peritoneal nodules rapidly progress and subsequently create additional peritoneal implants. The exponential progression of disease to multiple sites on the abdominal and pelvic peritoneal surfaces may rapidly cause the patient’s demise. Without curative interventions, PC’s progression and expansion compared to those of other metastases elsewhere are overwhelming (Sugarbaker 2012).

1.1.3 Predicting and Predisposing Factors

All gastrointestinal cancers have the propensity to metastasize to the peritoneum. However, a number of factors have been shown to predict or predispose to the development of CRCPC [reviewed by (Chua et al. 2012d)]. Depth of tumor invasion is the strongest predictor of PC. Higher T stage, representing tumor invasion of the serosal layer (T3) or adjacent organ and peritoneum (T4), often contributes toward transcoelomic spread of cancer (Jayne et al. 2002; Yang et al. 2004). Histopathological features, including mucinous histology (Chen et al. 2010; Nozoe et al. 2000) and signet ring morphology (Chen et al. 2010; Pande et al. 2008), have been shown to be associated with PC. Clinically, tumors that present with obstruction or perforation increase the risk of PC, owing to their extensive growth potential and intraoperative seeding in the peritoneal cavity. The presence of PFCCs reflects shedding of cancer cells into the peritoneal cavity and is a predictor of recurrence. In a meta-analysis by Rekhraj et al., the presence of PFCCs before and after resection of the primary tumor was found to be associated with a significantly higher risk of overall recurrence, and, if present before resection, resulted in a significantly higher risk of

local recurrence as well (Rekhraj et al. 2008). Ovarian metastases from a primary gastrointestinal malignancy, known as Krukenberg tumors, are often associated with PC (Fujiwara et al. 2010; Sugarbaker and Averbach 1996). Genomic profiling studies suggest that differences in gene expression determine whether CRC spreads toward the peritoneal surfaces, toward the liver, or both (Varghese et al. 2007; Ceelen and Bracke 2009; Kotanagi et al. 1998).

1.1.4 Diagnostic and Prognostic Evaluation

1.1.4.1 Diagnosis and Preoperative Assessment

CRCPC often remains unknown until the disease becomes symptomatic or a laparotomy or laparoscopy is performed. Initial symptoms are nonspecific and include abdominal discomfort, nausea, weight loss, cachexia, and fatigue. Those with more advanced tumor burdens may present with massive ascites, signs of partial bowel obstruction, and generalized inanition (Klaver et al. 2012a; Royal and Pingpank 2008). However, the gold standard in diagnosing PC is direct peritoneal visualization, by either laparotomy or laparoscopy (Coccolini et al. 2013). Although regular follow-up and serial imaging is the rule in patients with resected gastrointestinal malignancies, early diagnosis of small-volume PC is rarely possible. The preoperative staging of peritoneal disease is limited by the insensitivity of imaging modalities such as computed tomography (CT), ultrasound (US), magnetic resonance imaging (MRI), and positron emission tomography (PET) (Royal and Pingpank 2008). For both CT and US, detection of peritoneal implants 1 cm or less in size approximates 25 % (Archer et al. 1996). In addition, the accuracy of the CT is considerably reduced with intestinal histologic type of CRC (Jacquet et al. 1993). Using peritoneal cancer index (PCI) for quantification of the disease extent in CRCPC (see next section), our department previously reported a statistically significant difference between radiologically and intraoperatively visualized lesion sizes, where preoperative CT underestimated clinical PCI (Koh et al. 2009). These modalities are most sensitive for locating and quantifying mucinous adenocarcinomas (Jacquet et al. 1995; Archer et al. 1996) and detecting omental metastases or indirect evidence of tumor such as ascites, mucin collections, mesenteric thickening, or matting of loops of bowel (Royal and Pingpank 2008).

Tumor histopathology and the extent of prior resections can be useful in the preoperative assessment of the peritoneal disease. In CRCPC, the importance of histopathology of the primary tumor is less definitive and tumor grade is not a dominant prognostic factor (Mohamed et al. 2011). As such, no significant difference in survival based on tumor differentiation was reported (Elias et al. 2010a). This stands in contrast with mucinous appendiceal malignancies with widespread peritoneal dissemination wherein peritoneal mucinous carcinomatosis (PMCA) is an invasive disease with aggressive histology whereas disseminated peritoneal adenomucinosis (DPAM) and hybrid type are minimally invasive and amenable to complete cytoreduction

(see Sect. 1.3). The extent of prior resections can be quantified by a scoring system similar to PCI (see below). In this system, a prior surgical score (PSS) of 0 is applied when no prior surgery or only a biopsy has been performed, PSS-1 denotes one region, and PSS-2 indicates 2–5 regions with prior surgery. PSS-3 refers to more than five regions of previous resection, which is equivalent to a prior attempt at complete cytoreduction in the absence of perioperative intraperitoneal chemotherapy. Higher PSS has a negative impact on survival, resulting from the cancer cell entrapment. Surgical resection provides raw surfaces for cancer cell adherence, vascularization, and progression. Tumor implants invaded deep to the peritoneal surfaces and embedded in scar tissue are difficult or impossible to remove by peritonectomy or to eradicate by intraperitoneal chemotherapy (Harmon and Sugarbaker 2005).

1.1.4.2 Intraoperative Assessment

In CRCPC, the extent of carcinomatosis has been shown to be the most important prognostic factor determining survival even when complete cytoreduction is achieved (da Silva and Sugarbaker 2006). Thus, attempts have been made to quantify the extent of the peritoneal disease during surgical exploration and the following three systems have been established for CRCPC [reviewed by (Harmon and Sugarbaker 2005)]:

Lyon (Gilly) Peritoneal Carcinomatosis Staging

First introduced by Gilly et al. in 1994, the Lyon Peritoneal Carcinomatosis Staging is a simple and reproducible tool used as a prognostic indicator in gastrointestinal PC (Gilly et al. 1994). As shown in Table 1.2, this system takes into account the size of the peritoneal implants and, in part, their distribution to quantify the extent of PC (Gilly et al. 2006).

The predictive value of this method for survival of patients with PC from non-gynecologic malignancies was demonstrated in the multicentric prospective EVOCAPE study (Sadeghi et al. 2000). Similarly, it has been validated as a predictor of survival in curative approaches to PC (Glehen et al. 2003). The main criticism of the Lyon (Gilly) staging is that it fails to incorporate distribution of peritoneal surface implants in high-stage categories, despite the fact that PC implants confined

Table 1.2 Lyon (Gilly) peritoneal carcinomatosis staging

Stage	Description
0	No macroscopic disease
1	Malignant implants <5 mm in diameter localized in one part of the abdomen
2	Malignant implants <5 mm in diameter diffuse to the whole abdomen
3	Malignant implants 5 mm to 2 cm in diameter
4	Large malignant nodules (>2 cm in diameter)

to one portion of the abdomen, even those of large size, may portend good prognosis (Harmon and Sugarbaker 2005).

Peritoneal Cancer Index

The PCI devised by Sugarbaker et al. quantitatively combines the distribution of PC implants throughout 13 abdominopelvic regions with the size of lesions (Jacquet and Sugarbaker 1996a). Two transverse and two sagittal planes divide the abdomen into nine regions (0–8). The upper transverse plane is located at the lowest aspect of the costal margin, and the lower transverse plane crosses the anterior superior iliac spines. The sagittal planes divide the abdomen into three equal sectors. The remaining four regions relate to upper and lower jejunum (9 and 10) and upper and lower ileum (11 and 12). After complete lysis of all adhesions and complete inspection of all parietal and visceral peritoneal surfaces within the abdominopelvic regions, the greatest diameter of tumor implants is measured and scored, yielding a lesion size score (LS) of 0 (no visible implants), 1 (<2.5 mm), 2 (from 2.5 mm to 2.5 cm), or 3 (>2.5 cm) (Harmon and Sugarbaker 2005). Please note that according to the original scoring, LS1, LS2, and LS3 correspond to lesions up to 0.5 cm, up to 5 cm, and larger than 5 cm in size, respectively (Sugarbaker 1999a).

Primary tumors or localized recurrences at the primary site are excluded from the assessment. LS for all abdominopelvic regions are then combined and PCI is demonstrated as total score values ranging from 0 to 39. PCI was first shown in 1995 to be a meaningful assessment tool for CRCPC (Sugarbaker and Jablonski 1995). Since then, the utility of PCI as a prognostic indicator and a guide for estimating the likelihood of a complete cytoreduction at re-operative surgery has been established (da Silva and Sugarbaker 2006; Elias et al. 2001; Sugarbaker 1999b; Verwaal et al. 2003; Yan et al. 2006b). According to this system, palliation is the goal of treatment for patients with CRCPC scoring greater than 20. This scoring method, however, is utilized with the caveat that a low PCI score could be recorded in the presence of invasive cancer at a crucial anatomic site where a complete cytoreduction may not be possible (Harmon and Sugarbaker 2005).

Simplified Peritoneal Cancer Index

The Simplified Peritoneal Cancer Index (SPCI) was established at the Netherlands Cancer Institute (Witkamp et al. 2001a). Similar to PCI, SPCI incorporates both tumor size and distribution in the assessment of the tumor burden. For practical convenience, however, the abdomen is divided into seven anatomical regions. Tumor size is similarly scored 0–3, yet with different cutoff points. This system thus yields SPCI values ranging from 0 to 21 (Table 1.3).

SPCI has shown predictive values for survival (Verwaal et al. 2004b) and incidence of complications (Verwaal et al. 2004a) in CRCPC patients treated with a curative approach. A major criticism of this system is that the epigastric region, very important in determining the completeness of cytoreduction in some diseases, is not designated separately (Harmon and Sugarbaker 2005).

Table 1.3 Simplified peritoneal cancer index

Regions		Lesion size score (LS)
I	Pelvis	0–3
II	Right lower abdomen	0–3
III	Greater omentum, transverse colon, and spleen	0–3
IV	Right subdiaphragmatic area	0–3
V	Left subdiaphragmatic area	0–3
VI	Subhepatic and lesser omental area and stomach	0–3
VII	Small bowel and small bowel mesentery	0–3
SPCI		0–21

LS 0, nil; LS 1, <2 cm; LS2, 2–5 cm; LS 3, greater than 5 cm or confluence

Post-cytoreduction Assessment

Based on the above-mentioned prognostic indicators, CRCPC patients who most benefit from cytoreduction are selected. In these patients, however, the assessment and quantitative scoring of the residual disease is sought once a maximal surgical effort is completed. The so-called completeness of cytoreduction score (CCS) described by Jacquet and Sugarbaker is known to be a major predictor of survival (Jacquet and Sugarbaker 1996a). Completeness of cytoreduction (CC) is scored 0 when no peritoneal seeding is evident within the operative field; CC-1 indicates tumor nodules less than 2.5 mm remaining after cytoreduction; CC-2 corresponds to nodules between 2.5 mm and 2.5 cm in size; and CC-3 represents the residual disease as nodules greater than 2.5 cm or a confluence of unresectable tumor nodules at any site within the abdomen or pelvis. According to this system, CC-0 and CC-1 are considered as complete cytoreduction whereas CC-2 and CC-3 indicate incomplete cytoreduction. Since CC-1 tumor nodules are believed to be penetrable by locoregional chemotherapy, they are considered as complete cytoreduction if treatment is combined with perioperative intraperitoneal chemotherapy. The requirement of complete cytoreduction for the long-term survival of CRCPC patients treated with such a combination therapy is well documented in the literature (Harmon and Sugarbaker 2005; Chua et al. 2011c, 2012d; Elias et al. 2001, 2009, 2010a; Glehen et al. 2004a; Sugarbaker and Jablonski 1995; Witkamp et al. 2001b; Sugarbaker 2012).

1.1.5 Treatment

Owing to a grim natural history and poor prognosis, CRCPC has been historically regarded as a universally fatal condition traditionally treated with palliative intent. In the past, neither systemic chemotherapy nor intraperitoneal chemotherapy alone had any significant impact on survival. Palliative debulking surgery was almost always associated with disease recurrence within a few months. As the number of repeated debulking procedures increased, patients were more likely to suffer from

intestinal obstruction and fistula formation. Eventually, failure of the treatments would lead to death. However, by virtue of two treatment innovations, namely cytoreductive surgery (CRS) [referred to as peritonectomy procedures] and perioperative intraperitoneal chemotherapy, a curative approach to PC was devised by Sugarbaker (2005a) (Yan et al. 2006d). When combined together, these innovative modalities serve as a treatment option with long-term benefits for appropriately selected patients (Sugarbaker 2012). Considering the aims of the present project, this multimodal strategy is addressed here.

1.1.5.1 Cytoreductive Surgery

CRS includes six peritonectomy procedures that are used to resect cancer on visceral intra-abdominal surfaces or to strip cancer from parietal peritoneal surfaces. Peritonectomy procedures are used in the areas of visible cancer progression and combined as needed with different visceral resections in an attempt to leave the patient with only microscopic residual disease. Using high-voltage electrosurgery with a ball tip and a thorough knowledge of the distribution patterns of PC, the surgeon is able to strip away the lining of the abdomen and pelvis at all sites where there is visible evidence of cancerous implants. Peritonectomy procedures include greater omentectomy-splenectomy; left upper quadrant peritonectomy; right upper quadrant peritonectomy; lesser omentectomy-cholecystectomy with stripping of the omental bursa; pelvic peritonectomy with sleeve resection of the sigmoid colon; and antrectomy. Depending on the distribution and volume of PC, one or all six of these procedures may be required. Small tumor nodules are removed using electroevaporation. Involvement of visceral peritoneum frequently requires resection of a portion of the stomach, small intestine, or colorectum (Sugarbaker 1995, 1999a). This radical approach requires the knowledge and technical expertise of the peritonectomy procedures in specialized centers that allow delivery of intraperitoneal chemotherapy (Chua et al. 2012d).

1.1.5.2 Perioperative Intraperitoneal Chemotherapy

With increasing response rates for gastrointestinal cancers, systemic chemotherapy has become a standard treatment for unresectable metastatic disease and an adjuvant treatment after successful resection of the primary tumor. In the context of PC, however, intravenous chemotherapy has offered minimal benefits. Hence, in recent attempts toward the curative management of CRCPC, conceptual changes in the route and timing of chemotherapy, along with knowledgeable selection of patients, have been considered, and intraperitoneal administration of certain chemotherapeutic agents in a perioperative setting has been established as an adjunct to CRS (Sugarbaker 2005a). Intraperitoneal chemotherapy gives high response rates within the abdomen because the “peritoneal-plasma barrier”—a diffusion barrier consisting of the mesothelium, interstitium, and submesothelial capillary wall—provides

dose-intensive therapy (Jacquet and Sugarbaker 1996b). Thus, regional dose intensity is the major advantage of intraperitoneal therapy. Drugs selected for intraperitoneal administration are usually hydrophilic and have large molecular size so that they pass slowly through the peritoneal-plasma barrier. For these agents, the “area under the curve” (AUC) ratios of intraperitoneal to intravenous exposure (AUC IP/IV) are favorable and are therefore more effectively sequestered in the peritoneal cavity. As a result, the peritoneal cavity is exposed to higher concentrations than are the other parts of the body (Sugarbaker 1999a). The penetration of intraperitoneal chemotherapy into PC nodules is limited to between 2 and 5 mm, even when combined with heat. For this reason, CRS to reduce intraperitoneal tumor volume is essential before locoregional chemotherapy (Mohamed et al. 2011). On the other hand, post-operative adhesion process interferes with uniform distribution of chemotherapy and also sequesters cancer cells within the scar tissue (Sugarbaker 2005a). Therefore, perioperative intraperitoneal chemotherapy should start in the operating room immediately following cytoreduction, through a procedure called hyperthermic intraperitoneal chemotherapy (HIPEC), and may be followed by early postoperative intraperitoneal chemotherapy (EPIC) for up to 6 days after surgery, during which formation of postoperative adhesions is minimal (Yan et al. 2006d). For HIPEC, the peritoneal cavity is filled with a heated chemotherapy solution. Certain chemotherapeutic agents used for HIPEC have rapid cytotoxic effects and are amenable to heat synergy. Heat also increases the penetration of these drugs into tumor nodules and can also exert stress on tumor cells. Drugs that are most frequently used include mitomycin C, cisplatin, 5-fluorouracil, oxaliplatin, and doxorubicin, usually administered for 30–120 min with moderate heat (42 °C) (Mohamed et al. 2011; Sugarbaker 2005a). Drugs selected for EPIC require cell contact for a longer time to cause cytotoxic effects and their activity is dependent on cell division. Hence, the chemotherapy solution dwells for 23 h and then drains for 1 h prior to the next instillation. Drugs frequently used include 5-fluorouracil, paclitaxel, and docetaxel (Sugarbaker et al. 1990; Sugarbaker 2005a). Perioperative intraperitoneal chemotherapy methods and regimens used for CRCPC and survival benefits reported are summarized in Table 1.4.

1.1.5.3 Selection of Patients for Treatment with CRS and HIPEC

The efficacy of CRS and HIPEC as a treatment option for CRCPC is an established part of the oncologic literature (Sugarbaker 2012). However, the initiation of treatment must occur as early as possible in the natural history of the disease, particularly in GC and CRC. In other words, the less the peritoneal surfaces involved, the greater the achievable benefit. In this regard, the lesion size and distribution of the peritoneal implants are of crucial importance. This multidisciplinary strategy is not likely to produce a lasting benefit when PC from a colorectal primary is extensive. In this context, rapid recurrence of PC combined with progression of lymph nodal, liver, or systemic disease interrupts long-term benefit. In contrast, patients with small tumor nodules of limited distribution within the abdomen and pelvis and amenable to

Table 1.4 Perioperative intraperitoneal chemotherapy used after cytoreductive surgery of CRCPC and reported survival rates

Investigator	Year	Number	Treatment	Duration (min)	Temp (°C)	Median Survival (months)	Survival (%) (year)
HIPEC regimen							
Loggie et al. (2000)	2000	38 ^a	Mitomycin C	60–120	40.5	14.6	60 (1), 39 (2), 24 (3)
		22 ^b				31.1	74 (1), 52 (2), 52 (3)
Piso et al. (2001)	2001	17	Cisplatin	90	41–42	39 [†]	75 (4)
Pilati et al. (2003)	2003	34	Mitomycin C and cisplatin	90	41.5	18	68 (1), 31 (2)
Shen et al. (2004)	2004	77	Mitomycin C [•]	60–120	38.5–43	16	56 (1), 25 (3), 17 (5)
Rouers et al. (2006)	2006	21	Mitomycin C	–	–	34	–
		13	Oxaliplatin				
Zanon et al. (2006)	2006	25	Mitomycin C	60	42	30.3	64 (1), 40 (2)
Kiamanesh et al. (2007)	2007	43	Mitomycin C and cisplatin	90–120	41–43	38.4	72 (2), 44 (4)
Gusani et al. (2008)	2008	28	Mitomycin C	100	40	15.2	78 (1), 36.7 (2), 36.7 (3)
Hagendoorn et al. (2009)	2009	49*	Mitomycin C	90	42	–	88 (1), 75 (2)
Varban et al. (2009)	2009	142	Mitomycin C	120	40.5–42.5	23	43.3 (2), 14.4 (4)
		128 ^{HM} –				15.8	36.8 (2), 17.4 (4)
Franko et al. (2010)	2010	67	Mitomycin C 30 mg then 10 mg	60 then 40	42	34.7	–
Hill et al. (2011)	2011	62	Mitomycin C	90	40	18	71.3 (1), 45.4 (2)
Cavalieri et al. (2011)	2011	146	Cisplatin ± mitomycin C	60–90	41.5–43	21	45 (2)
			Oxaliplatin (HIPEC) + systemic 5-fluoruracil*				
Passot et al. (2012)	2012	120	MMC + irinotecan or oxaliplatin	–	–	36.2	77 (1), 51 (2), 33 (5)
Haslinger et al. (2013)	2013	38	Mitomycin C	60–120	41	45.2	38.2 (5)
Yonemura et al. (2013)	2013	142	Mitomycin C and cisplatin	60	42–43	24.4	23.4 (5)

(continued)

Table 1.4 (continued)

Investigator	Year	Number	Treatment	Duration (min)	Temp (°C)	Median Survival (months)	Survival (%) (year)
Berger et al. (2014)	2014	42	Mitomycin C	90	41	20.2	–
Braam et al. (2014)	2014	32 ^c	Mitomycin C	90	42	42.9	–
Desantis et al. (2015)	2014	74	Mitomycin C	60	43	45.9	60.3 (3), 37 (5)
Levine et al. (2014)	2014	248 ^a	Mitomycin C or oxaliplatin	60–120	40–43	16.4	–
		472 ^b				63.5	
SPIC or HIPEC or EPIC ± adjuvant chemotherapy							
Sugraber and Jablonski (1995)	1995	51	ip 5-fluoruracil + systemic mitomycin C (adjuvant)	–	–	N/A	36 (3)
			5-fluoruracil + mitomycin C (EPIC + adjuvant)				
			5-fluoruracil + mitomycin C (EPIC) + ip 5-fluoruracil + systemic mitomycin C (adjuvant)				
Portilla et al. (1999)	1999	18	Mitomycin C (HIPEC) + 5-fluoruracil (EPIC) + systemic mitomycin C	–	–	20	–
Cavaliere et al. (2000)	2000	14 ^a	Mitomycin C and cisplatin (HIPEC) or 5-fluoruracil (EPIC)	90	41.5–42.5	–	63.5 (2)
		2 ^b					100 (2)
Beaujard et al. (2000)	2000	27	Mitomycin C (HIPEC or SPIC) ± systemic 5-fluoruracil	90	46–49	12	50 (1)
Pestieu and Sugarbaker (2000)	2000	104	Mitomycin C (HIPEC) + 5-fluoruracil (EPIC)	90	42	–	100 (5)
		4				24	30 (5)
		44 ^c				12	0 (5)
		55 ^d					
Verwaal et al. (2003, 2008)	2003, 2008	49	Mitomycin C (HIPEC) + systemic 5-fluoruracil	90	41–42	22.4 (2003)	45 (5) [‡]
						48 (2008)	
Verwaal et al. (2005)	2005	117	Mitomycin C (HIPEC) + systemic adjuvant	90	41–42	21.8	75 (1), 28 (3), 19 (5)
Mahteme et al. (2004)	2004	17	5-fluorouracil (EPIC)	–	–	32	60 (2), 28 (5)

Carnignani et al. (2004b)	2004	17	10	Mitomycin C (HIPEC) + 5-fluorouracil (EPIC) + systemic adjuvant	90	42	15.2	–
			7	5-fluorouracil (EPIC) + systemic adjuvant				
Glehen et al. (2004a)	2004	506	271	HIPEC	30–90	40–43	19.2	72 (1), 39 (3), 19 (5)
			123	EPIC	–	–	19.2	
			112	HIPEC+EPIC	–	–	21.6	
Kecmanovic et al. (2005)	2005	18		Mitomycin C (HIPEC) + 5-fluorouracil (EPIC)	120	42	15	–
Cavaliere et al. (2006)	2006	120		Mitomycin C and cisplatin	60–90	41.5–43	19	25.8 (3)
				Oxaliplatin + systemic 5-fluorouracil*	30	43		
da Silva and Sugarbaker (2006)	2006	70		Mitomycin C (HIPEC) + 5-fluorouracil (EPIC)	90	41–40	33	88 (1), 44 (3), 32 (5)
				Mitomycin C + 5-fluorouracil (EPIC) ± systemic adjuvant	–	–		
Yan and Morris (2008)	2008	50		Mitomycin C + 5-fluorouracil (EPIC)	90	42	29	79 (1), 39 (3)
Ceelen et al. (2008)	2008	32		Oxaliplatin (HIPEC) + systemic 5-fluorouracil*	30	41	–	80 (1)
Bijelic et al. (2008)	2008	70	36	Mitomycin C + 5-fluorouracil (EPIC)	–	–	30	17 (5)
			34	Mitomycin C (HIPEC)	90	42		
Chua et al. (2009c)	2009	55□	39	Mitomycin C (HIPEC)	90	42	36	89 (1), 74 (2), 60 (3)
			44	5-fluorouracil (EPIC)				
			33	HIPEC+EPIC				
Elias et al. (2009)	2009	48		Oxaliplatin (HIPEC) + systemic 5-fluorouracil*	30	43	62.7	81 (2), 51 (5)
Bretcha-Boix et al. (2010)	2010	20		Mitomycin C (HIPEC) or oxaliplatin (HIPEC) + 5-fluorouracil (EPIC)	40–90	42	–	36 (5)
Váira et al. (2010)	2010	40	23	Mitomycin ± cisplatin (HIPEC)	60	41.5	16.6	–
			17	Oxaliplatin (HIPEC) + systemic 5-fluorouracil	30	42	24.6	

(continued)

Table 1.4 (continued)

Investigator	Year	Number	Treatment	Duration (min)	Temp (°C)	Median Survival (months)	Survival (%) (year)
Elias et al. (2010a)	2010	443	Mitomycin ± cisplatin (HIPEC) ± adjuvant	60–120	41	30.1	81 (1), 41 (2), 27 (5)
			Oxaliplatin ± irinotecan (HIPEC) + systemic 5-fluoruracil * ± adjuvant	30	43		
		84	Mitomycin C and 5-fluoruracil (EPIC)	–	–		
		9	HIPEC+EPIC ± adjuvant	–	–		
Elias et al. (2010c)	2010	440	Mitomycin ± cisplatin (HIPEC) ± mitomycin C and 5-fluoruracil (EPIC)	60–90	41	–	33 (5)
			Oxaliplatin ± irinotecan (HIPEC) + 5-fluoruracil (adjuvant) ± mitomycin C and 5-fluoruracil (EPIC)	30	43	–	
Glehen et al. (2010b)	2010	523 ^a	Mitomycin C ± cisplatin or Oxaliplatin ± irinotecan (HIPEC) ± systemic 5-fluoruracil ± mitomycin C and 5-fluoruracil (EPIC)	30–120	40–43	30	41 (3), 26 (5)
		50 ^b				77	56 (3), 56 (5)
Quenet et al. (2011)	2011	103	Oxaliplatin + irinotecan (HIPEC) + systemic 5-fluoruracil*	30	43	47	42.4 (5)
		43	Oxaliplatin (HIPEC) + systemic 5-fluoruracil*			40.83	41.8
Tentes et al. (2011)	2011	107	Mitomycin C or Oxaliplatin (HIPEC)	60–90	42.5–43	–	100 (3)
		67	5-fluoruracil (EPIC)	–	–		69 (3)
Chua et al. (2011e)	2011	110	Mitomycin C (HIPEC) + 5-fluoruracil (EPIC) + systemic adjuvant (standard or modern)	90	42	14 ^s , 38 ^m	64 (1), 24 (3), 12 (5) ^s 96 (1), 52 (3), 33 (5) ^m
Turrini et al. (2012)	2012	26	Oxaliplatin (HIPEC) + systemic 5-fluorouracil*	30	43	39	100 (1), 51 (3), 37 (5)
Hompes et al. (2012)	2012	48	Oxaliplatin (HIPEC) + systemic 5-fluorouracil* ± 5-fluoruracil plus oxaliplatin or irinotecan (adjuvant)	30	41–42		97.9 (1), 88.7 (2)

Klaver et al. (2012a)	2012	23	12	Mitomycin C or Oxaliplatin (HIPEC)	90	42	35	83 (1), 68 (1.5)
				5-fluoruracil (EPIC)				
			6	Mitomycin C or Oxaliplatin (HIPEC) + 5-fluoruracil (EPIC)				
Cashin et al. (2012)	2012	2	44	Mitomycin C (HIPEC)	90	41–42	34	40 (5)
				Oxaliplatin (HIPEC) + systemic 5-fluoruracil ⁺	30			
				Oxaliplatin + irinotecan (HIPEC) + systemic 5-fluoruracil ⁺	30			
			57	5-fluoruracil (SPIC)	–			
Fajardo et al. (2012)	2012	23	13 ^a	5-fluoruracil [¶] (SPIC) + systemic adjuvant	60	–	22.3	64.9 (2), 44.5 (3)
			10 ^b					
Ung et al. (2013a)	2013	96	25	Mitomycin C (HIPEC)	–	42	46.8	87 (1), 58 (3) and 42 (5)
				5-fluoruracil (EPIC)	–			
			90	HIPEC + EPIC	–			
de Cuba et al. (2013)	2013	139		HIPEC or EPIC	–	–	6–36	–
Klaver et al. (2013)	2013	13 ^a	5 ^b	Mitomycin C or Oxaliplatin (HIPEC) ± 5-fluoruracil (EPIC)	–	–	–	74 (1), 50 (2)
Gervais et al. (2013)	2013	25		Oxaliplatin + systemic 5-fluoruracil [¶] ± 5-fluoruracil plus oxaliplatin and/or irinotecan ± bevacizumab (adjuvant)	30	42–44		61 (3), 36 (5)

HIPEC hyperthermic intraperitoneal chemotherapy, *EPIC* early postoperative intraperitoneal chemotherapy, *SPIC* sequential postoperative intraperitoneal chemotherapy, ⁺before starting HIPEC, [¶]during HIPEC, [‡]for those with complete cytoreduction, [†]mean survival (months), ^{*}Five patients received systemic adjuvant, [□]Some individuals repeated in more than one treatment group, [‡]including two cases with gastric cancer, ^{HM}with hepatic metastases, ^{HM}–without hepatic metastases, [‡]standard chemotherapy including 5-fluoruracil, ^mmodern chemotherapy including 5-fluoruracil or capecitabine with oxaliplatin or irinotecan and biological therapy (modern chemotherapy with bevacizumab, cetuximab, or panitumumab), [¶]repeated every 2 weeks for up to a total of 9 cycles, ^acolorectal cancer, ^bappendiceal cancer, ^crecurrence with prior resection of primary tumor, complete cytoreduction, ^drecurrence with prior resection of primary tumor, incomplete cytoreduction, ^{N/A}not available

complete cytoreduction will achieve the greatest benefit (Sugarbaker 1999a). Thus, a knowledgeable selection of patients who most benefit from this combination therapy is necessary. To this end, PCI represents the most important selection tool. PCI is available at the time of abdominal exploration and serves as a meaningful assessment tool for CRCPC (Sugarbaker and Jablonski 1995). PCI is useful not only as a prognostic indicator but also as a guide for estimating the likelihood of CC at reoperative surgery (Portilla et al. 1999). Sugarbaker reported that 5-year survival for patients with PCIs of less than 10, 11–20, and greater than 20 were 50 %, 20 %, and 0 %, respectively (Sugarbaker 1999b). More recently, Elias et al. in the French collaborative study of 523 patients reported a 44 % survival at 5 years with a PCI of 6 or less, 22 % 5-year survival with PCI between 7 and 12, 29 % 5-year survival with PCI between 13 and 19, and 7 % 5-year survival with PCI greater than 19 (Elias et al. 2010a). For patients with PCI greater than 20, palliation is the goal of treatment (Harmon and Sugarbaker 2005). Unlike PCI, CC score is not available unless cytoreduction is attempted, hence less valuable in prior planning of the treatment. Nevertheless, it is the most definitive assessment of prognosis (Sugarbaker 2005a). According to Sugarbaker, there is a 40 % chance of 5-year survival in those who undergo complete cytoreduction (CC-0 and CC-1) versus 0 % chance in those with incomplete cytoreduction (CC-2 and CC-3) (Sugarbaker 1999b). In the French study, chance of 5-year survival in CC-0 and CC-1 groups was 29 % and 14 %, respectively, as compared with 0 % in patients undergoing incomplete cytoreduction (Elias et al. 2010a).

1.1.5.4 Pros and Cons of CRS and HIPEC for CRCPC

Because of a large amount of mature data gathered over 30 years, one can begin to formulate the advantages and disadvantages associated with CRS and HIPEC for CRCPC (Sugarbaker 2012). As summarized in Table 1.4, the most important advantage is the long-term survival of a highly selected group of patients on surgical series. As such, a large, multi-institutional registry study (Glehen et al. 2004a) and a systematic review (Yan et al. 2006a) have reported a median survival ranging from 13 to 32 months. More recently, with the use of modern, more effective chemotherapeutic (e.g., oxaliplatin or irinotecan) and biological (bevacizumab or cetuximab) agents, the outcomes of patients with CRCPC have further been improved (Chua et al. 2012d). In agreement, median survival of 63 months and 5-year survival of 51 % were reported by Elias et al. in patients with isolated, resectable CRCPC who underwent CRS and simultaneously received intravenous 5-fluorouracil and leucovorin before exposure to oxaliplatin-based HIPEC (Elias et al. 2009). In a multi-center study on the influence of modern systemic therapies as adjunct to CRS & HIPEC, Chua et al. reported median and 5-year survivals of 14 months and 12 % for patients receiving 5-fluorouracil-based chemotherapy, 38 months and 33 % for patients receiving modern chemotherapy (oxaliplatin- or irinotecan-based), and 46 months and 29 % for patients receiving modern chemotherapy and biological therapy (bevacizumab or cetuximab) (Chua et al. 2011e). Secondly, the degree of PC as

measured by CC and PCI scores allows selection of individuals who are most likely to benefit. On the other hand, there are disadvantages that remain. The procedure is complex and requires an extended learning curve. The maturity of the treatment, however, is associated with the improvement of perioperative outcomes, surgical results, and long-term survival (Chua et al. 2011c). Mortality from the operation appears to be less than 5 % in centers of excellence and even less than 1 % in some. However, treatment-related morbidity is significant. There is lack of uniformity of the patients entered into surgical databases. As such, surgical series contain patients who have received many different HIPEC regimens at many different timepoints during their treatment. Lack of uniformity also exists among service providers in the technical aspects of CRS and HIPEC. The relative roles of CRS and HIPEC in the causation of long-term survival are yet to be determined. According to the literature, 70 % of patients went on to die of CRCPC, usually because HIPEC did not maintain the surgical complete response (Sugarbaker 2012).

1.2 Peritoneal Carcinomatosis from Gastric Cancer

1.2.1 *Incidence and Natural History*

According to GLOBOCAN 2012, gastric cancer (GC) is the fourth most common cancer in men (631,300 cases, 8.5 % of the total) and the fifth in women (320,300 cases, 4.8 % of the total) worldwide, with an estimated new cases of 951,600 in 2012. GC remains one of the deadliest gastrointestinal malignancies. With 723,100 (8.8 % of the total) deaths, it precedes CRC to represent the third most frequent cause of cancer death in 2012. Incidence rates are highest in Eastern Asia (particularly in Korea, Mongolia, Japan, and China), Central and Eastern Europe, and South America (Torre et al. 2015). In 2011, 2093 Australians were diagnosed with GC. Following the general trend, it affects twice as many men as women in Australia, with age-standardized rates per 100,000 of 11.9 versus 5.5 in 2011. It caused 1143 deaths in 2012, accounting for 2.6 % of the total cancer mortality (AIHW 2015a, c).

Peritoneal dissemination is the most frequent pattern of metastasis and recurrence of GC (Deraco et al. 2011a). PFCCs are identified in peritoneal washes of up to 24 % of stage IB and up to 40 % of stage II or III GC (Juhl et al. 1994). As a highlight and key feature of the seventh edition of the TNM classification guidelines published and recommended by American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC), positive peritoneal cytology is now classified as metastatic disease (M1) in the GC staging (Washington 2010; Sobin et al. 2009). Approximately 50 % of GC patients present with PC developed from a primary GCPC (Glehen et al. 2004b). Synchronous peritoneal implants are present at abdominal exploration in 20–30 % of patients investigated for potentially curative resection of GC (Goldstein et al. 2005). GCPC is also the most frequent pattern of recurrence after curative surgery of the primary tumor (Boku et al. 1990). After

radical resection, the peritoneum is the only site of recurrence in 10–34 % of cases, and one of the recurrence sites in 29–44 % of cases (Coccolini et al. 2013). PC is the most important prognostic factor and most frequent cause of death from GC (Boku et al. 1990). It accounts for 20–40 % of GC deaths (Yonemura et al. 2003). In GC with positive PFCC, but no macroscopic peritoneal metastases, median survival of 20 months (Lee et al. 2012) and 5-year survival rate of 15.3 % (Saito et al. 2011) have been reported. According to the EVOCAPE study on the natural history of gastrointestinal PCs, GCPC prognosis is better than that of pancreatic PC, but worse than that of CRCPC, with mean and median survivals of 6.5 months (range, 0.1–48.0 months) and 3.1 months, respectively (Sadeghi et al. 2000). The chance of 5-year survival is lower than 3 % (Yonemura 1996).

1.2.2 Pathogenesis

Peritoneal dissemination of GC starts with the detachment and infiltration of PFCCs from the invaded serosa or metastatic lymph nodes (Boku et al. 1990). As with CRCPC, PFCCs then move through the peritoneal cavity, some attach through adhesion molecules to the surface of the peritoneum, subsequently invade into the subperitoneal tissue, and finally proliferate to form peritoneal implants (Yonemura et al. 1996a; Glehen et al. 2004b). When the gastric serosa is infiltrated by tumor, PC becomes very frequent (Coccolini et al. 2013). The unfavorable prognosis of GC patients with serosal invasion, even following curative gastrectomy, is probably related to both the presence and biological behavior of viable PFCCs (Boku et al. 1990). Through comparative analysis between GC and CRC patients, Hara et al. demonstrated that although GC and CRC similarly exfoliate PFCCs into the peritoneal cavity in terms of incidence and cell number, clinical outcomes are completely different, and concluded that the metastatic potential of PFCCs from GC are far higher than their CRC counterparts (Hara et al. 2007). Moreover, iatrogenic spread of cancer cells can result from surgical manipulation of the cancer-bearing organ, or in case of lymphatic and venous invasion, from lymphatic transection and hemorrhage. As such, peritoneal lavage of patients with non-serosa-invasive GC at laparotomy and immediately after lymphadenectomy proved that lymph node dissection resulted in peritoneal spread of viable cancer cells (Marutsuka et al. 2003). According to the tumor cell entrapment hypothesis, cancer cells shed into the peritoneal cavity can be entrapped in fibrin during the wound-healing process that eventually form tumor implants on the peritoneal surfaces (Sugarbaker et al. 2003). Common sites for the GC peritoneal implants include the greater omentum, pelvic peritoneum, Morrison's pouch, paracolic gutter, transverse colon, mesentery of the small intestine, and splenic capsule (Yonemura et al. 2010b). Subsequent to the invasion of the visceral or parietal peritoneum, malignant ascites may develop; largely resulting from mechanical interference with venous or lymphatic drainage and increased capillary permeability in response to biologically active peptides,

such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Tamsma 2007; Enck 2002).

Using the MKN45 gastric cancer cell line, Yonemura et al. developed a murine model of human GCPC and investigated mechanisms of the GCPC formation by intraperitoneally inoculated cells mimicking PFCCs (Yonemura et al. 1996a). This study clearly demonstrated the site specificity of GCPC and identified three major processes and routes for peritoneal dissemination of PFCCs. The investigators found that among the various parts of the peritoneum, the greater omentum acted as an initial site for tumor take. Milky spots—the omentum-associated lymphoid tissues analogous to regional lymph nodes (Shimotsuma et al. 1993)—were the main route to be invaded through which cancer cells can access the subperitoneum and metastasize directly to omental nodes via efferent lymphatics. Cancer cell infiltration through intercellular stomata between mesothelial cells covering diaphragm and subsequent invasion to subdiaphragmatic lymphatic lacunae were described as the second route of dissemination. Numerous stomata are detected on the undersurface of the diaphragm, small bowel mesentery, greater omentum, appendix epiploicae of the large bowel and the pelvic peritoneum. When determining the PCI in GC, the surgeon should carefully and meticulously examine the peritoneal surfaces containing stomata or milky spots (Yonemura et al. 2006a). The third route of dissemination was observed through the attachment of cancer cells onto the naked area of the submesothelial connective tissue, following the shrinkage of the mesothelial cells and hence the exposure of the basement membrane and extracellular matrix (Yonemura et al. 1996a). As described earlier, the pattern of peritoneal dissemination is also conditioned by random-proximal distribution, gravity, associated fluids (ascites and mucin), and intrinsic biological aggressiveness (Montori et al. 2014).

Moreover, interaction of cancer cells with peritoneal mesothelial cells (PMCs) (Satoyoshi et al. 2015; Tsukada et al. 2012) and tumor-associated macrophages (Yamaguchi et al. 2014) has been shown to contribute to peritoneal dissemination of GC. Interestingly, cancer-activated PMCs were recently found to form an invasion front that controls and guides PFCCs during the establishment of GCPC (Satoyoshi et al. 2015). Molecular mechanisms causing GCPC remain to be elucidated. In this regard, signaling pathways, including p38 mitogen-activated protein kinase (MAPK) (Graziosi et al. 2012), Akt (Zhu et al. 2014), MET (Graziano et al. 2014; Zhao et al. 2013), Src kinase/Tks5 (Satoyoshi et al. 2015), and phosphoglycerate kinase 1 (PGK1) (Zieker et al. 2008), Smad interacting protein 1 (SIP1) (Okugawa et al. 2013), DJ1 protein (Zhu et al. 2014), matrix metalloproteinases (Yonemura et al. 2001b; Zhu et al. 2014), chemokines, in particular CXCL12/CXCR4 axis (Zieker et al. 2008; Yasumoto et al. 2006), and growth factors, such as VEGF (Sako et al. 2004; Yasumoto et al. 2006), hepatocyte growth factor (HGF) (Zhao et al. 2013), transforming growth factor β (TGF- β) (Li et al. 2012; Lv et al. 2012), heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (Yasumoto et al. 2011), have been implicated.

1.2.3 Risk Factors

Serosal invasion and lymph node metastasis are risk factors of relapse in all patterns of GC recurrence (Yoo et al. 2000). Peritoneal cytology was shown to be the most important factor for predicting peritoneal recurrence (Bando et al. 1999). In addition, size, stage (T3/T4), grade (G3/G4), gross appearance and histological features of the primary tumor, including infiltrative, diffuse or scirrhous (linitis plastica) type and signet-ring cell differentiation, have been reported as independent risk factors for the development of metachronous PC (Seyfried et al. 2015; Huang et al. 2009; Maehara et al. 2000; Roviello et al. 2003; Yoo et al. 2000; Dittmar et al. 2015; Kim et al. 2012a; Thomassen et al. 2014; Lee et al. 2003).

1.2.4 Diagnostic Evaluation

Although useful for preoperative diagnosis of GCPC, imaging techniques, including CT, MRI, US, PET, and PET-CT, have variable limitations incurred by low accuracy and insensitivity, in particular in diagnosing PC of low-volume density. This drawback is further highlighted by the fact that diameter of GCPC nodules tends to be smaller than those of CRCPC (Yonemura et al. 2010a). CT and MRI are important mainly in evaluating unresectable disease and cancer staging (Montori et al. 2014). The availability and lower incidence of movement artifacts make multi-sliced CT the most widely used imaging tool (Yonemura et al. 2010a). Current CT techniques cannot consistently identify low-volume macroscopic metastases that are 5 mm or less in size. However, CT is sensitive for the detection of omental metastases or indirect evidence of tumor such as the presence of ascites, mesenteric thickening, or matting of loops of bowel (Bozzetti et al. 2008). A small amount of fluid on CT or US may alert the clinician to the presence of peritoneal seeding (Sugarbaker and Yonemura 2000). Lee et al. found that endoscopic US (EUS) was more sensitive than combined US and CT scan examinations in diagnosing ascites (Lee et al. 2005). Although there is enhanced accuracy in the assessment of extra-gastric disease with high-speed spiral CT, sensitivity declines as the size of metastases decreases (Bozzetti et al. 2008). PET-CT shows a better sensitivity than high-speed spiral CT for the detection of GCPC. In this regard, Yang et al. reported that high-speed spiral CT has an accuracy of 78 %, a sensitivity of 39 %, and a specificity of 94 %, compared with accuracy, sensitivity, and specificity rates of 87 %, 72.7 % and 93.6 %, respectively, achieved by PET-CT (Yang et al. 2006).

Given the limitations of imaging techniques, diagnostic laparoscopy or laparotomy and peritoneal cytology are used as the main methods for definitive diagnosis and evaluation of GCPC (Yonemura et al. 2010a). Diagnostic laparoscopy is recommended in all stage IB-III GC, considered to be potentially resectable, to exclude metastatic disease (Waddell et al. 2014). Laparoscopy also allows for the assessment of peritoneal cytology and intraperitoneal evaluation with adjunctive diagnostic techniques, such as laparoscopic ultrasonography (LUS) (Bozzetti et al. 2008).

1.2.5 Staging and Prognostic Tools

As with CRCPC, a quantitative system is needed for the objective evaluation of the distribution and volume of GCPC. However, one should take into consideration the more aggressive biological behavior of GC for staging and treatment planning of GCPC (Yonemura et al. 2010a). The following scoring systems have been used in GCPC for staging and prognostic purposes:

1.2.5.1 The Japanese Staging System

Specifically developed for GCPC, this system was introduced by Japanese Research Society for Gastric Cancer as part of Japanese classification of gastric carcinoma (Table 1.5) (Japanese Research Society for Gastric Cancer 1995). According to this prognostic tool, GCPC is classified based solely on the presence of PFCCs or peritoneal implants, regardless of size (Aiko and Sasako 1998). In the original version, a “P stage” indicates the extent of the peritoneal dissemination, wherein P0 means no macroscopic disease; P1 indicates implants immediately adjacent to the stomach and above the transverse colon; P2 means several scattered implants within the peritoneal cavity; and P3 represents numerous peritoneal implants (Japanese Research Society for Gastric Cancer 1995). In the revised versions, this four-tier categorization was abandoned and peritoneal metastasis was instead scored P0 or P1, indicating the absence or presence of macroscopic peritoneal implants, respectively (Japanese Gastric Cancer Association 1998, 2011). At the same time, however, peritoneal cytology, described as CY0 (negative) and CY1 (positive), was included in the staging. Despite the fact that the Japanese classification does not take into account the size and location of PC, it benefits from peritoneal cytology as a unique feature. In GC, PFCCs have a high proliferative activity as indicated by Ki-67 index (Yonemura et al. 2010a). The median survival of patients with a positive cytology is 6 months, and the survival curve of patients with P0/CY1 is not significantly different from that of patients with P1, P2, or P3 (Bando et al. 1999). Thus, in accord with AJCC/UICC guideline, P0/CY1 is categorized as M1 disease (Stage IV). As an important prognostic tool, peritoneal cytology is recommended in patients with GC scheduled to undergo curative resection (Yonemura et al. 2010a).

Table 1.5 The Japanese staging of GCPC (revised)

Peritoneal metastasis		Peritoneal cytology	
Px	Peritoneal metastasis is unknown	CYx	Peritoneal cytology not performed
P0	No peritoneal metastasis	CY0	Negative peritoneal cytology
P1	Peritoneal metastasis	CY1	Positive peritoneal cytology

1.2.5.2 The Lyon (Gilly) Staging System

As discussed earlier, Gilly et al. proposed a simple, reproducible staging system for PC that takes into account the size of the peritoneal nodules and their distribution (localized or diffuse) (Table 1.2). In all the phase II prospective studies reported from Lyon on PC treated by intraperitoneal chemotherapy, there were significant differences between the prognosis of stages 1 and 2 ($PC < 5$ mm), and that of stages 3 and 4 ($PC > 5$ mm) (Gilly et al. 2006). For resectable GC with stage 1 or 2 PC, 1-year survival rate was reported to be 80 % versus 10 % in stages 3 and 4 (Sayag-Beaujard et al. 1999).

1.2.5.3 The Peritoneal Cancer Index

It was reviewed earlier that Sugarbaker et al. devised PCI to more precisely quantitate PC through accurately measuring and locating the peritoneal nodules throughout the abdominopelvic cavity (Table 1.3). PCI is a prognostic indicator that allows for an estimate of the probability of complete cytoreduction (Montori et al. 2014). Of a total of 28 GC cases with peritoneal involvement, Yang et al. reported that median survival of 18 patients with a $PCI \leq 20$ was significantly better than that of 10 patients with a $PCI > 20$ (27.7 months versus 6.4 months, respectively) (Yang et al. 2010). Yonemura et al. reported that a complete cytoreduction was done in 91 % (42/46) of GC patients with a $PCI \leq 6$, but in only 42 % (12/29) of those with a PCI score ≥ 7 , with significantly better survival in the former (Yonemura et al. 2010a). Because GC has a more aggressive biological behavior, the threshold value of PCI for a favorable prognosis is less than that of CRC (Yonemura et al. 2010b). While the best indication for multidisciplinary therapy of GCPC is $PCI \leq 6$ from a resectable tumor that can be completely removed during a peritonectomy, those with PCI scores greater than 7 should be treated with palliative intent without peritonectomy (Yonemura et al. 2010a).

1.2.5.4 The Completeness of Cytoreduction Score

CCS described by Jacquet and Sugarbaker is an independent prognostic factor to be used after cytoreduction. In GC, CRC and appendiceal cancer, complete cytoreduction (CC-0/1) is an independent predictor of favorable survival after peritonectomy (Yonemura et al. 2010b). Through a systematic review of the efficacy of CRS and HIPEC in GCPC, Gill et al. reported that median survival of 7.9 months was improved to 15 months by virtue of complete cytoreduction (Gill et al. 2011). According to one of the largest series published by Yonemura et al., median survival after complete and incomplete cytoreduction was 15.5 and 7.9 months, respectively, with 5-year survival rates of 13 % and 2 %, respectively (Yonemura et al. 2005). This was improved to 27 % in patients treated with CRS and HIPEC after complete cytoreduction. In a retrospective French multi-institutional study on 159 GCPC

patients treated with CRS combined with perioperative intraperitoneal chemotherapy, Glehen et al. observed that when complete cytoreduction was achieved, 5-year survival rate (a median of 13 %) reached 23 % (Glehen et al. 2010a). In both studies, CCS proved to be an independent prognostic indicator.

1.2.6 Treatment

Peritoneal metastasis has traditionally been considered as the terminal stage of GC. The presence of peritoneal implants and even a positive peritoneal cytology are considered poor prognostic signs and indicators of incurable disease. In agreement, AJCC and UICC classify positive peritoneal cytology as M1 disease and treatment guidelines from both Europe (ESMO) and the USA (NCCN) call for palliative treatment only with either systemic chemotherapy or supportive care (Bijelic and Sugarbaker 2012). Conventional surgery alone has minimal effects on survival since residual PC is a stronger indicator of postoperative survival (Yonemura et al. 2010b). Chu et al. noted a median survival of only 1 month for GCPC cases treated with surgery alone (Chu et al. 1989). Results of a patient care study by the American College of Surgeons were similarly disappointing (Wanebo et al. 1993). Likewise, systemic chemotherapy for GCPC has been uniformly disappointing because peritoneal implants of GC are accessed and penetrated less efficiently than the metastatic disease elsewhere (Canbay et al. 2014). Preusser et al. published a response rate to aggressive chemotherapy of 50 % in patients with advanced GC, among which GCPC cases demonstrated the worst response (Preusser et al. 1989). Ajani et al. identified PC as the most common cause of intensive neoadjuvant treatment failure in GC (Ajani et al. 1991). A phase II trial by the Swiss Group for Clinical Cancer Research using new systemic chemotherapy regimens in metastatic GC reported overall response rate and survival of up to 36.6 % and 11 months, respectively (Roth et al. 2007). Many efforts have been made to develop adjuvant therapies for resectable GC, but large randomized trials of intravenous chemotherapy (Hermans et al. 1993; Lise et al. 1995) or radiotherapy (Hallissey et al. 1994) have failed to demonstrate improved survival (Sugarbaker et al. 2003).

A renewed interest and paradigm shift in the treatment of GCPC using intraperitoneal hyperthermic chemotherapy as an adjunct to gastrectomy with or without CRS developed in the late 1980s (Fujimoto et al. 1988) and the 1990s (Yonemura et al. 1991, 1995, 1996b). The new paradigm rests in the concept that PC is a locoregional disease and can thus be treated with locoregional approaches (Yonemura et al. 2010b). With the advent of the peritonectomy procedures for peritoneal surface malignancy (PSM), a combination of palliative gastrectomy, peritonectomy, and perioperative intraperitoneal chemotherapy was later proposed by Sugarbaker and Yonemura as a treatment option in selected patients with GCPC (Sugarbaker and Yonemura 2000). Considering the aims of the present work, current knowledge on the utility of CRS plus perioperative intraperitoneal chemotherapy as a new trend of multidisciplinary therapy for GCPC is reviewed here.

1.2.6.1 Gastrectomy and Cytoreductive Surgery

In this multidisciplinary approach to GCPC, a combination of palliative gastrectomy and peritonectomy is attempted. The rationale for palliative gastrectomy rests in assumptions that the primary tumor will cause catastrophic complications, including gastric obstruction, perforation, bleeding, or debilitating ascites, and that the more residual cancer, the less choice of response to subsequent chemotherapy. Gastrectomy should include the resection of as much gross disease as possible, including other organs involved (Sugarbaker and Yonemura 2000). For patients with a P0/CY1 status (a positive cytology with no macroscopic PC), gastrectomy is believed to improve survival (Lee et al. 2012; Yonemura et al. 2010a). Peritonectomy is used to further reduce or, if possible, eliminate all visual evidence of disease and optimally prepare the patient for intraperitoneal chemotherapy. The peritonectomy procedures for GCPC include epigastric peritonectomy, anterolateral peritonectomy, subphrenic peritonectomy, omental bursa peritonectomy, and pelvic peritonectomy (Sugarbaker et al. 2003). During peritonectomy, the peritoneal zones affected by the tumor are removed. These include greater omentum, pelvic peritoneum (including the sigmoid colon and rectum), Morrison's pouch, paracolic gutter, transverse colon, mesentery of the small intestine, and splenic capsule. In contrast, the incidence of involvement on the parietal peritoneum is relatively low (Yonemura et al. 2010b). After the introduction of peritonectomy techniques, the incidence of complete cytoreduction increased significantly, which in turn, as an independent prognostic factor, improved survival (Yonemura et al. 2005, 2009, 2010b). The factors which limit a complete cytoreduction are an unresectable primary tumor and the involvement of the small bowel mesentery (Yonemura et al. 2010b).

1.2.6.2 Perioperative Intraperitoneal Chemotherapy

Patients with GCPC cannot be cured using CRS alone because viable cancer cells persist at sites distant from the surgical field (Yamamura et al. 2007). The rationale for the use of perioperative intraperitoneal chemotherapy in PC was reviewed in the previous section. Generally, intraperitoneal chemotherapy offers potential therapeutic advantages over systemic chemotherapy by generating high local concentrations of chemotherapeutic drugs in the peritoneal cavity. Intraperitoneal chemotherapy can target remnants of widely distributed PFCCs, as well as cancer cells growing on mesothelial cells, in the lymphatic vessels, and in the superficial layer of submesothelial tissue (Yonemura et al. 2010b). In GC, intraperitoneal chemotherapy is administered preoperatively (neoadjuvant intraperitoneal/systemic (bidirectional) chemotherapy (NIPS)), intraoperatively (HIPEC and extensive intraoperative peritoneal lavage followed by intraperitoneal chemotherapy (EIPL-IPC)), and postoperatively (EPIC).

The aims of neoadjuvant chemotherapy are to downstage the tumor, to eliminate micrometastasis outside the surgical field, and to improve tumor resectability and complete cytoreduction incidence, preoperatively (Yonemura et al. 2003).

Cunningham et al. reported that perioperative chemotherapy consisting of three preoperative and three postoperative cycles of intravenous epirubicin and cisplatin along with a continuous intravenous infusion of 5-FU (ECF regimen) in patients with resectable gastroesophageal cancer decreased tumor size and stage and significantly improved progression-free and overall survival (Cunningham et al. 2006). However, neoadjuvant systemic chemotherapy has minimal effects on GCPC (Yonemura et al. 2010a). Yonemura et al. developed NIPS that combines intraperitoneal chemotherapy with systemic chemotherapy to attack PC from both sides of the peritoneum, hence known as bidirectional chemotherapy (Yonemura et al. 2006b). They indicated that a combination of oral S-1 (a fluorinated pyrimidine agent, containing tegafur, 5-chloro-2, 4-dihydroxypyridine (CDHP), and potassium oxonate) and peritoneal docetaxel and cisplatin using a port system is a safe and effective treatment for eliminating PFCCs (Yonemura et al. 2009). In 37 out of 55 patients, positive cytology turned negative after two or more cycles of NIPS. Two cycles of NIPS is thus recommended to achieve a negative cytology status. They also reported NIPS-induced histological changes in the primary and PC tumors, graded from 1 (degeneration of cancer in less than two third of the tumor tissue) to 3 (complete disappearance of the cancer cells), in 30 patients with GC (Yonemura et al. 2007a). While grade 3 change of the primary tumor was found only in one case, that of PC was evident in 15 patients. Moreover, tumor downstaging (from stage 4 to stage 1, 2, or 3) occurred in 10 patients (Yonemura et al. 2010a). In a similar approach by Ishigami et al., a combination chemotherapy regimen of oral S-1, plus intravenous and intraperitoneal paclitaxel resulted in negative cytology in 24 out of 28 patients (Ishigami et al. 2012). This NIPS regimen, followed by gastrectomy in 60 patients after the disappearance or obvious shrinkage of peritoneal nodules, yielded median survival of 34.5 months. Therefore, NIPS appears to be a viable option that may help with better selection of patients that should go on to aggressive cytoreduction. Also, it seems to be useful for patients with synchronous carcinomatosis by clearing PFCCs in the peritoneal cavity and allowing a high rate of complete cytoreduction in this subgroup (Bijelic and Sugarbaker 2012). PHOENIX-GC is an ongoing phase III study that aims to evaluate the effect of an NIPS regimen (S-1 plus intravenous and intraperitoneal paclitaxel) against systemic chemotherapy (S-1 plus cisplatin) in GC patients with minimal or gross peritoneal metastasis (Ishigami et al. 2012).

In HIPEC for GC, mitomycin C and cisplatin, which have synergistic effects when used with hyperthermia, are typically used. According to the pharmacokinetic studies, approximately 70–75 % of cisplatin (Panteix et al. 2002) and up to 90 % of mitomycin C (van Ruth et al. 2003) administered through HIPEC are absorbed within 90 min and low plasma levels of the drugs reduce the risk of toxicity. Accordingly, 90–120 min HIPEC might be more beneficial (Yonemura et al. 2010a). The efficacy of HIPEC in the prevention of peritoneal recurrence after curative resection of GC (Hamazoe et al. 1994; Yonemura et al. 2001a) and improvement of overall survival after CRS (Yan et al. 2007b) has been shown in a number of clinical trials. EIPL-IPC is a novel treatment of GC advocated by Kuramoto et al. in which the peritoneal cavity is extensively and repeatedly ($\times 10$) washed with saline

(Kuramoto et al. 2009). Extensive lavage is believed to reduce the number of PFCCs to potentially zero and followed by intraperitoneal chemotherapy. This treatment modality was reported to significantly improve 5-year survival and reduce peritoneal recurrence in P0/CY1 patients (Kuramoto et al. 2009). Also, HIPEC may similarly improve the survival of patients with positive cytology (Yonemura et al. 2010b). Therefore, interest has been expressed in the prophylactic administration of HIPEC during primary tumor resection to prevent peritoneal dissemination (Ung et al. 2013b). EPIC starts as soon as possible during the early postoperative period to eliminate minimal residual disease. In this phase, a large number of residual cancer cells are in a proliferative cycle that can be targeted before being entrapped in postoperative fibrin deposits (Jeung et al. 2002; Sugarbaker et al. 1990). In a clinical trial on 248 patients with advanced GC, Yu et al. reported that surgery plus EPIC offered a superior advantage over surgery alone with respect to survival rate (54 % versus 38 %) and locoregional recurrence risk (a reduction of 49 %) (Yu et al. 2001). EPIC is recommended for patients of good performance status (Jeung et al. 2002) with gross serosal invasion of the primary tumor (Yu et al. 2001). Perioperative intraperitoneal chemotherapy methods and regimens used for GCPC and survival benefits reported are summarized in Table 1.6.

1.2.6.3 Selection of Patients for Multidisciplinary Treatment

As with other malignancies, particularly those with such an aggressive biological behavior, treatment of GC should start as early as possible in the natural history of the disease. Since PC is a characteristic feature of the advanced stage GC with a grim prognosis and controversial therapeutic approaches, a knowledgeable selection of patients who most benefit from this multimodal therapy is necessary. Surgeons have to judge the balance of the postoperative risk associated with the magnitude of the peritonectomy and the treatment benefits with respect to long-term survival and quality of life. To avoid futile aggressive treatments, such selection tools as peritoneal cytology and PCI are of particular value (Yonemura et al. 2010a). For patients with a P0/CY1 status, gastrectomy (Lee et al. 2012), EIPL-IPC (Kuramoto et al. 2009), and HIPEC (Yonemura et al. 2010b) are believed to improve survival. In those with macroscopic peritoneal implants, completeness of cytoreduction is the most important predictor of outcome (Glehen et al. 2004c, 2010a; Yonemura et al. 2005). Accordingly, the best indication for multidisciplinary therapy is a localized PC ($PCI \leq 6$) from a resectable primary tumor that can be completely removed (CC-0) by peritonectomy. PCI is determined during laparoscopic examination and exploratory laparotomy, in which all 13 peritoneal regions should be meticulously observed and palpated. Yonemura et al. reported that while complete cytoreduction was achieved in 86 % of patients with a $PCI \leq 6$, it was achievable in 39 % with a $PCI \geq 7$ and in only 7 % with a PCI greater than 13 (Yonemura et al. 2010a). Patients with $PCI \geq 7$ and liver, distant lymph node, or extraperitoneal metastasis are not candidates for an extensive peritonectomy (Yonemura et al. 2010b).

Table 1.6 Perioperative intraperitoneal chemotherapy combined with surgery in advanced GC/GCPC and reported survival rates

Investigator	Year	No.	Treatment	Duration (min)	Temp (°C)	Median survival (months)	Survival (%) (year)
HIPEC							
Yonemura et al. (1991)	1991	41	Mitomycin C + cisplatin	40–60	41–43	14.6	28.5 (3)
Yonemura et al. (1996b)	1996	83	Mitomycin C + cisplatin + etoposide	60	42–43	13.9 (CR), 6.8 (RD)	43 (1), 11 (5) [CR: 61 (1), 17 (5); RD: 30 (1), 2 (5)]
Fujimoto et al. (1997)	1997	48	Mitomycin C	120	43–45	–	54.0 (1), 41.5 (3), 31.0 (5), 25.4 (8)
Loggie et al. (2000)	2000	19	Mitomycin C	60–120	40.5	10.1	37 (1), 21 (2), 14 (3)
Hall et al. (2004)	2004	34	Mitomycin C	120	40–41	11.2 (R0/1), 3.3 (R2)	27 (1), 23 (2), 6 (5) [R0/1: 45 (2), R0: 21 (5)]
Glehen et al. (2004c)	2004	49	Mitomycin C	90	46–48	10.3	48.1 (1), 19.9 (2), 16 (5) [CC-0/1: 29 (5)]
Yonemura et al. (2005)	2005	107	Mitomycin C + cisplatin + etoposide	–	42–43	11.5	6.7 (5)
Yang et al. (2010)	2010	28	Mitomycin C + hydroxycamptothecin or cisplatin	90–120	42.5–43.5	43.4 (CC-0/1), 9.4 (CC-2/3)	50 (1), 42.8 (1.5), 42.8 (2)
Yang et al. (2011a)	2011	34	Mitomycin C + cisplatin (HIPEC)	60–90	42.5–43.5	11	41.2 (1), 14.7 (2), 5.9 (3)
Levine et al. (2014)	2014	46	Mitomycin C ± mitoxantrone	60–120	40–43	6.1	–
Magge et al. (2014)	2014	26	Mitomycin C	100	42	9.5	50 (1), 18 (3)
NIPS/HIPEC ± EPIC ± adjuvant chemotherapy							
Beaujard et al. (2000)	2000	42	Mitomycin C (HIPEC or SPIC)	90	46–49	–	48 (1), 33 (2)
Yonemura et al. (2006b)	2006	61*	Docetaxel + carboplatin + systemic methotrexate and 5-fluorouracil (NIPS)	–	–	14.4	67 (1)
Cheong et al. (2007)	2007	154	5-fluorouracil + cisplatin (EPIC)	–	–	11.4	12.2 (5)
Yonemura et al. (2009)	2009	41**	Docetaxel + cisplatin + systemic S-1	–	–	20.4	67.4 (1), 40 (2), 30 (3)
		38	(NIPS)			10.5	35.9 (1), 20.4 (2), 0 (3)

(continued)

Table 1.6 (continued)

Investigator	Year	No.	Treatment	Duration (min)	Temp (°C)	Median survival (months)	Survival (%) (year)
Glehen et al. (2010a)	2010	159	Mitomycin C ± cisplatin (HIPEC) ± mitomycin C & 5-fluorouracil (EPIC)	60–120	41–42.5	9.2	43 (1), 18 (3), 13 (5)
			Oxaliplatin ± irinotecan (HIPEC) ± systemic 5-fluorouracil ± mitomycin C & 5-fluorouracil (EPIC)	30	43	15 (CC-0)	[CC-0: 61 (1), 31 (3), 23 (5)]
			Intraperitoneal and systemic paclitaxel + S-1 (NIPS)	–	–	23.6	80 (1)
Kitayama et al. (2012)	2012	100	Intraperitoneal and systemic paclitaxel + S-1 (NIPS)	–	–	34.5	–
Ishigami et al. (2012)	2012	60	Intraperitoneal and systemic paclitaxel + systemic S-1 (NIPS)	–	–	34.5	–
Desantis et al. (2015)	2014	14	Cisplatin	60	43	13.3	21.6 (3), 21.6 (5)

NIPS neoadjuvant intraperitoneal and systemic chemotherapy, SPIC sequential postoperative intraperitoneal chemotherapy, S-1 a fluorinated pyrimidine agent (a combination of tegafur, 5-chloro-2, 4-dihydropyridine (CDHP), and potassium oxonate), CR complete resection, RD residual disease, CC completeness of cytoreduction, CC-0 no residual peritoneal disease after CRS, CC-1 residual disease less than 2.5 mm, CC-2 residual tumor between 2.5 mm and 2.5 cm, CC-3 residual tumor greater than 2.5 cm or the presence of confluence, R0 complete removal and negative cytology, R1 complete removal and positive cytology or microscopic margin, R2 macroscopic residual disease

*Of a total of 61 patients, 30 underwent CRS with median survival of 18 months. Median survival for those without surgery and patients who received a complete resection was 9.6 and 20.4 months, respectively. **Of a total 79 patients, 41 received subsequent CRS, but 38 did not

1.2.6.4 Pros and Cons

CRS plus perioperative intraperitoneal chemotherapy has emerged as a standard treatment and state of the art for selected patients with peritoneal dissemination from colorectal and appendiceal tumors. Nevertheless, a much smaller body of evidence can be found in the literature on the efficacy of this strategy in patients with GCPC, owing to the common perception that peritoneal spread of GC is indicative of incurability. This is despite the fact that, as summarized in Table 1.6, this strategy has brought about modest improvement in the outlook for select patients with advanced GC (Bijelic and Sugarbaker 2012). As with other areas of research on GC, the majority of studies on the efficacy of this multidisciplinary therapy come from Japan. Yonemura et al. were the first to report that long-term survival was possible in GCPC (Yonemura et al. 1991, 1996b). Results from the US (Hall et al. 2004) and European (Glehen et al. 2004c, 2010a) studies that followed have confirmed the initial promise. In agreement, a meta-analysis of 10 randomized studies (including a total of 1474 patients) by Yan et al. showed that adjuvant intraperitoneal chemotherapy (HIPEC with or without EPIC) of patients with locally advanced, resectable GC is associated with a significant improvement of overall survival. Recently, Coccolini et al. published the results of an updated meta-analysis of 20 randomized controlled trials (a total of 2145 patients randomly assigned to surgery plus intraperitoneal chemotherapy (1152) or surgery alone (993)). Likewise, they reported that adjuvant intraperitoneal chemotherapy improved overall survival of patients with GCPC after curative resection, where improved survival was not affected by locoregional nodal metastasis or serosal infiltration (Coccolini et al. 2014). In addition, adjuvant intraperitoneal chemotherapy was found to reduce the incidence of peritoneal recurrence and distant metastases. Thus, it appears that accumulating data support the use of CRS and intraperitoneal chemotherapy (NIPS and HIPEC, with or without EPIC) in select patients with GCPC.

On the other hand, a number of drawbacks remain. The use of this multimodal therapy in GCPC remains a matter of considerable debate. Current data shows that its long-term benefits for GCPC are inferior to those achieved for PC from appendiceal cancer and CRCPC. CRS is a morbid and complex procedure. Until now, a precise description of peritonectomy techniques for GCPC has not been published. Glehen et al. reported a mean operation time of 5.2 h (range 1.5–9.5 h), a 30-day mortality rate of 4 % (2/49), a major complication rate of 27 % (13/49), and a higher complication rate (47 %) in patients who underwent extensive CRS (gastrectomy combined with the removal of more than two peritoneal zones) (Glehen et al. 2004c). NIPS might increase the morbidity. In their consecutive series of 96 patients with PCGC, Yonemura et al. reported major postoperative complications in 30 patients (32 %) and a second operation in 4, as well as two hospital deaths (2 %) from pancreatic fistula and sepsis (Yonemura et al. 2010a). The magnitude of surgery, the number of resected organs, the number of anastomoses, and the operation time are considered to have contributed to the significantly higher complication rate. Meta-analysis studies reported that intraperitoneal chemotherapy is associated with an increased risk of morbidity (Coccolini et al. 2014), intra-abdominal abscess,

and neutropenia (Yan et al. 2007b). Technically, surgeons should have an extensive experience with the procedure. Yan et al. reported the existence of a learning curve with this procedure and recommended the accumulation of experience to achieve an acceptable morbidity rate (Yan and Morris 2008). They suggested that at least 70 peritonectomy procedures are needed to obtain a reliable level of surgical proficiency and postoperative care. Taken together, renewed research interest in the optimal utilization of CRS plus perioperative intraperitoneal chemotherapy, using new anticancer agents, in appropriately selected patients with GCPC is warranted (Bijelic and Sugarbaker 2012; Yonemura et al. 2010a). Randomized controlled studies may provide higher levels of evidence in the future and help to determine the significance of CRS with perioperative chemotherapy as a standard treatment strategy in these patients (Yonemura et al. 2010b). As such, GASTRICHIP is an ongoing multicentric phase III study (2011–2019) that investigates the effect of HIPEC with oxaliplatin on GC patients with serosal invasion and/or lymph node involvement and/or positive cytology who undergo perioperative systemic chemotherapy and D1–D2 curative gastrectomy (Glehen et al. 2014).

1.3 Pseudomyxoma Peritonei

1.3.1 Incidence and Natural History

According to the NIH Office of Rare Diseases Research (ORDR 2015), National Organization for Rare Disorders [Reg. No. 843, (NORD 2015)], and the European reference portal for rare diseases and orphan drugs [ORPHA26790, (Orphanet 2015)], pseudomyxoma peritonei (PMP), also known as adenomucinosis or gelatinous ascites, is a rare disease with an estimated incidence of 1–2 per million population per year (Smeenk et al. 2008b; Sugarbaker et al. 1996). It is 2–3 times more common in females than males and is said to be present in two of every 10,000 laparotomies (Hinson and Ambrose 1998; Mann et al. 1990). PMP is a PSM with the characteristic feature of copious mucin production by tumor cells. While the neoplastic cells and mucous ascites occupy predictable anatomic sites within the peritoneal cavity, PMP is an indolent, minimally aggressive epithelial tumor which has limited capability of invading the peritoneum (Yan et al. 2006d). Nevertheless, the natural history of PMP is slow progression to death (Spiliotis et al. 2012). Owing to unspecific manifestations, PMP tends to be misdiagnosed, or discovered at advanced stages. Although PMP is the paradigm for the successful treatment of PSM (Sugarbaker 2001), it remains challenging and debilitating, yet poorly understood (Sugarbaker 2006a). Despite the multidisciplinary approach, PMP frequently recurs and increasingly jeopardizes quality of life. To enhance outcomes of the current standard of care, novel approaches based on in-depth understanding of the pathological processes and biological events in the pathogenesis of the disease are warranted. Since PMP and mucin are inextricably linked, any therapeutic intervention needs to properly target the mucin ectopy. In this section, we will review the current knowledge on PMP, with

emphasis on the crucial role of mucin in the pathogenesis of the disease and its potential as a therapeutic target (Amini et al. 2013b, 2014b, 2015c).

1.3.2 Definition and Etiology

PMP is characterized by dissemination of mucinous tumor implants on peritoneal surfaces and progressive accumulation of mucinous ascites throughout the peritoneal cavity. The term “jelly belly” has been used to characterize the large accumulations of mucinous ascites that may be encountered at laparotomy (Hinson and Ambrose 1998). Since the initial descriptions of PMP as a syndrome in association with an ovarian tumor (Werth 1884) or an appendiceal mucocele (Fraenkel 1901), a pre-existing intraperitoneal mucinous neoplasm has been implicated as the primary cause of PMP. As follows, emerging evidence supports the appendiceal origin of the disease.

1.3.3 Nomenclature and Classification

Historically, PMP has been used as a nonspecific, poorly defined term to describe a heterogeneous group of different peritoneal pathologies with similar clinical presentation. In the broadest sense, PMP represents a syndrome of mucin accumulation that most commonly rises from an underlying low-grade appendiceal neoplasm, but it can be associated with high-grade appendiceal adenocarcinoma, other gastrointestinal cancers (including those of colon and pancreas), and mucinous ovarian malignancies. The confusing, ambiguous nomenclature has obscured the clinico-pathological characterization of the condition. In an attempt to precisely define the condition, Ronnett et al. first suggested a three-tiered classification system based solely on pathological features (Ronnett et al. 1995). According to this system, multifocal peritoneal mucinous tumors were classified into DPAM with histologically benign features; PMCA with malignant features; and a hybrid group, including PMCA with intermediate (PMCA-I) or discordant (PMCA-D) features. They defined PMP as a syndrome of mucinous ascites accompanied by a characteristic distribution of peritoneal mucinous tumors with the pathological features of DPAM. In this classification, the intermediate tumors represent a hybrid of DPAM and PMCA in both the primary tumors and the peritoneal lesions, that is to say the coexistence of adenoma and invasive carcinoma in the primary tumors, and a combination of bland mucinous tumor and well-differentiated mucinous carcinoma as the peritoneal lesions. Ronnett et al. also separated the discordant group from PMCA on the grounds of the presence of identifiable precursor lesions. Nevertheless, they later reported that although the pathology of the intermediate tumors closely resembles DPAM, their behavior more closely mimics that of PMCA (Ronnett et al. 2001). Moreover, since the peritoneal lesions in the discordant tumors were comprised uniformly of carcinoma, they also suggested that these tumors are considered

a variant of PMCA. Later on, Bradley et al. suggested the term “mucinous carcinoma peritonei” (MCP) to describe all cases of bona fide PMP, complying with the notion that adenocarcinoma includes any evidence of independent growth of neoplastic cells outside the wall of the appendix (Bradley et al. 2006). Accordingly, PMP cases were classified by Bradley et al. as either low-grade (MCP-L) or high-grade (MCP-H) carcinoma. In this classification, MCP-L includes those originally identified as DPAM and PMCA-I whereas MCP-H applies to the moderately-to-poorly differentiated adenocarcinomas, including PMCA and those with a signet-ring cell component. More recently, Shetty et al. advocated a new histology-based, three-tiered classification for PMP, with emphasis on the biological importance of signet-ring cells (Shetty et al. 2013). In this study, the cohort was initially analyzed using the two aforementioned systems and tumors were then graded according to the histological criteria. Following survival analysis, three distinct categories analogous to tumor grades were identified: PMP1 and PMP2, representing peritoneal lesions without and with cytological atypia, respectively, and PMP3, including all cases with a signet-ring cell component. As regards the histopathological description of the PMP tumor, such descriptive terms as “mucinous neoplasm of low malignant potential” and “low-grade appendiceal mucinous neoplasm” are also found in the literature (Buell-Gutbrod and Gwin 2013).

1.3.4 Pathogenesis

A primary mucinous tumor arising from the appendiceal goblet cells, usually characterized as an appendiceal adenoma, is believed to be the source of PMP (O’Connell et al. 2002b). The primary tumor causes peritoneal dissemination early in the natural history of the disease (Sugarbaker 2001) which, according to Sugarbaker, occurs based on a redistribution phenomenon (Sugarbaker 1994). The pathological process starts when goblet cells undergo neoplastic transformation and proliferate, and yet maintain their constitutive level of mucin expression. As a result, the overall secretion of mucin dramatically rises, the narrow lumen of the appendix is clogged by tumor, and a mucocoele develops. After the walls of the mucocoele are stretched to bursting point, tumor cells are released and gain access into the peritoneal cavity through a small perforation or rupture. Surrounded by the mucin coat, the exfoliated cells lacking cell surface adhesion molecules passively circulate and redistribute with the peritoneal fluid. As a result, tumor cells and secreted mucin, with no way to degrade or drain away, accumulate at the peritoneal fluid reabsorption sites, including the undersurface of the right hemidiaphragm and the greater omentum, and within the dependent areas of the peritoneal cavity, such as pelvis, right retrohepatic space, the left abdominal gutter, and the ligament of Treitz (Sugarbaker 1994, 2001, 2006a). While the continuous peristaltic motion prevents tumor implantation on the small bowel surfaces, quiescent surfaces such as liver, gall bladder, and parietal peritoneum and, to a lesser extent, the stomach and large bowel are invariably involved (Sugarbaker 2001). This widespread formation of voluminous gels and

peritoneal surface implants increases the intra-abdominal pressure, compresses visceral organs, and triggers inflammatory and fibrotic responses, with major contribution to morbidity and eventual development of fatal complications, including bowel obstruction (Sugarbaker 1996c; Yan et al. 2006d). Secreted mucin plays important roles in the biology of PMP tumor. The mucin coat not only allows tumor cells to freely disseminate and redistribute throughout the peritoneal cavity but also provides protection from adverse growth conditions and confers control of tumor microenvironment. As such, it is believed that mucin enhances tumor cell growth and survival and builds a molecular shield against chemotherapy and immune recognition (Hollingsworth and Swanson 2004). As shown in Table 2.3 in the following section, the expression of three secretory, gel-forming types of mucin, including MUC2, MUC5AC, and MUC5B, has been reported in PMP. MUC2 and MUC5AC are the predominant mucins secreted by PMP tumor. MUC2, however, represents the PMP-specific mucin and plays the key role in the pathophysiology of PMP. O'Connell et al. provided evidence that PMP is in fact a disease of MUC2-secreting goblet cells (O'Connell et al. 2002a, b). Under physiological conditions, MUC2 is secreted specifically by the intestinal goblet cells and comprises the substantial component of the intestinal double-layered mucus. In the context of PMP, MUC2 is abundantly, and ectopically, produced by peritoneally disseminated MUC2-secreting goblet cells originated from the primary appendiceal tumor. Since MUC2 is more extensively glycosylated, and thus more voluminous on an equimolar basis, than is MUC5AC, it accounts for the high degree of gelation in PMP (O'Connell et al. 2002b). In addition, the contributory role of MUC5B in the formation of semisolid material in some patients has been suggested by Mall et al. (2007, 2011). The role of mucins in cancer and PMP will be discussed in the next section.

1.3.5 Clinical Presentation

The average age at diagnosis for PMP is 53 (Mann et al. 1990). As summarized in Table 1.7, PMP usually presents with nonspecific manifestations. These signs and symptoms can be roughly classified based on the disease progression (Smeenk et al. 2008a). In advanced disease, increased abdominal girth and complaints of abdominal pain are the most common symptoms present in 30–50 % of patients. In less advanced disease, local signs and symptoms are seen in 50–80 % of patients and might correspond to the location of the primary tumor, such as appendicitis-like symptoms in 25 % of cases, or those related to the peritoneal implants, including lower abdominal pain, pelvic pressure, and gynecological complaints in females due to the ovarian deposits of the mucinous tumor in 20–30 % of the patients (Sugarbaker et al. 1996; Esquivel and Sugarbaker 2000). Finally, coincidental discovery of PMP has been reported in up to 20 % of patients during laparotomy or diagnostic procedures for other medical conditions and complaints, including hernia (Ben-Hur et al. 1996; Sugarbaker et al. 1996; Young et al. 1997; Edwards and Scott 1998; Esquivel and Sugarbaker 2000, 2001; Rezkalla et al. 2006; Shinohara

Table 1.7 Common presentations or incidental discovery of PMP on the basis of the disease progression

Disease status at diagnosis			Presenting or incidentally diagnosed with	
Advanced disease			Abdominal distension, ascites, obstruction (Esquivel and Sugarbaker 2000; Smeenk et al. 2007b)	
Less-advanced disease	Localized disease		Abdominal pain (Esquivel and Sugarbaker 2000)	Appendicitis-like syndrome
				Presumed cholecystitis
				Vague nonspecific pain
				Lower abdominal pain/pelvic mass
	Incidentally diagnosed disease	Non-surgical procedures	Gynecological conditions	Pelvic pain/mass (Esquivel and Sugarbaker 2000; Kalu and Croucher 2005; Gortchev et al. 2010)
				Infertility investigation (Esquivel and Sugarbaker 2000)
				Postmenopausal bleeding (Esquivel and Sugarbaker 2000; Khan et al. 2002)
				Abnormal Pap test (Esquivel and Sugarbaker 2000)
			Others (Esquivel and Sugarbaker 2000)	Deep vein thrombosis, rectal bleeding, anaemia
			Laparoscopy or laparotomy	Hernia repair, fibroids, CRC, tubal ligation, nephrectomy, abdominal aortic aneurysm repair (Esquivel and Sugarbaker 2000)

et al. 2006; Campbell et al. 2009; Ghidirim et al. 2011; Morris-Stiff et al. 2011), bladder tumor (Skaane et al. 1985), urological problems (Gandhi and Nagral 2012), total uterovaginal prolapse (Snyder and Vandivort 1992), recurrent rectal cancer (Newman and Moran 2011), pregnancy (Koyama et al. 2011) and cesarean section (Abdu et al. 2009), ulcerated skin fistula (Cakmak et al. 2009), and subcutaneous umbilical nodule (Srinivasaiah et al. 2009).

1.3.6 Diagnosis

1.3.6.1 Imaging

Ultrasound (US) combined with parallel fine needle biopsy has been used as a diagnostic method in PMP. US is accessible and inexpensive, and cytological study of the mucin biopsies seems to be a useful accompanying procedure. However, unspecific findings, sampling errors, dry taps, and false negative results due to low amount

of mucin or low cellular density are considered as the disadvantages of this method (Smeenk et al. 2008a). CT remains the most widely used imaging modality in PMP. It distinguishes mucinous ascites with higher densities from nonmucinous collections (Kreel and Bydder 1980), recognizes the characteristic pattern of the mucinous accumulation (Sulkin et al. 2002), and evaluates the extent of the disease for preoperative planning and prognostic purposes (Smeenk et al. 2007b). MRI shows the location of mucocoele and its morphologic criteria identically to CT. T1- and T2-weighted MRI are more sensitive in distinguishing between mucin and fluid ascites (Buy et al. 1989; Matsuoka et al. 1999; Fairise et al. 2008). Despite the reported benefits of PET for the prediction of peritoneal dissemination of abdominal malignancies (Yang et al. 2006) and preoperative evaluation of the pathological grade and potential for complete cytoreduction (Passot et al. 2010), its value in PMP remains controversial (Stewart et al. 2005; Rohani et al. 2010).

1.3.6.2 Circulating Tumor Markers

Carcinoembryonic antigen (CEA) (Carmignani et al. 2004a; Alexander-Sefre et al. 2005) and carbohydrate antigen 19.9 (CA19.9) (van Ruth et al. 2002; Carmignani et al. 2004a; Baratti et al. 2007; Chua et al. 2012a; Koh et al. 2013) have been reported to be of diagnostic and prognostic values in PMP. The gynecological tumor marker carbohydrate antigen 125 (CA125), also known as MUC16, has also been suggested as a marker with diagnostic sensitivity for PMP (Baratti et al. 2007). However, it is not widely used as a tumor marker for PMP and is recommended for the exclusion of an ovarian neoplasm instead (Smeenk et al. 2008a). Although relatively nonspecific for diagnostic purposes, these markers are also used as baseline values for postoperative follow-up and predictors of the completeness of cytoreduction, a significant prognostic factor for PMP (Alexander-Sefre et al. 2005; Baratti et al. 2007; Chua et al. 2012a; Kusamura et al. 2013; Taflampas et al. 2014; Koh et al. 2013).

1.3.6.3 Histopathological Analysis

PMP tumor is characterized with acellular to paucicellular pools of mucin with variable amounts of neoplastic mucinous epithelium. In addition, the following immunohistochemical markers are used for the identification of PMP: positive cytokeratin 20 (CK20), CEA, caudal-type homeobox protein 2 (CDX2) and MUC2, as well as negative cytokeratin 7 and CA125 (Smeenk et al. 2008a). Of particular interest with extensive positive staining in PMP specimens is MUC2. MUC2 has been suggested as a biological marker of PMP (O'Connell et al. 2002a; Bibi et al. 2006; Semino-Mora et al. 2008; Flatmark et al. 2010; Guo et al. 2011), with controversial significance as a prognostic factor (Baratti et al. 2009).

1.3.6.4 Differential Diagnosis

PMCA originating from any primary mucinous carcinoma is the main entity to be ruled out. Other conditions reported in the literature include endometriosis with myxoid change (Clement et al. 1994), melioidosis (a lethal infectious disease caused by *Burkholderia pseudomallei*) (Sugi Subramaniam et al. 2013), and entities with abdominal CT resemblance, including extensive abdominal plexiform neurofibromatosis (Mirich et al. 1989).

1.3.7 Treatment

PMP has been traditionally treated with repetitive surgical debulking. Due to the presence of tumor deposits after the first debulking surgery, this approach could result in short-term palliation with imminent recurrence or progression; hence redo procedures and a shorter 5–10-year overall survival rate of approximately 50 % (Gough et al. 1994; Miner et al. 2005; Jarvinen et al. 2010). PMP is generally considered resistant to systemic chemotherapy. This treatment modality has thus been used as a palliative option in patients with unresectable or relapsed disease. A phase II trial by Farquharson et al. evaluating the use of concurrent mitomycin C and capecitabine was the first to demonstrate an apparent benefit of systemic chemotherapy in patients with advanced unresectable PMP (Farquharson et al. 2008). Recently, Pietrantonio et al. reported the results of a single-center, observational study wherein systemic 5-FU plus oxaliplatin (FOLFOX-4 regimen) was found tolerable and active in patients with unresectable disease or relapse after the standard treatment (Pietrantonio et al. 2014).

Since the advent of CRS and perioperative peritoneal chemotherapy, PMP and PC from the appendiceal mucinous tumors have been shown to be a paradigm for this multidisciplinary approach, owing to six distinctive features (Sugarbaker 1996b). Firstly, these tumors are usually of low biological aggressiveness. Secondly, peritoneal dissemination occurs early in the natural history of the disease, prior to the involvement of lymph channels or venules in the appendiceal wall or in the mesoappendix. As a result of these two, lymph node and liver metastases are very rarely found. Thirdly, the mucinous tumor accumulates at anatomic sites resectable by the peritonectomy procedures. Fourthly, the small bowel, active in peristalsis, becomes compartmentalized as a tumor-free area. This sparing of the small bowel surfaces makes CRS a possibility. Fifthly, the texture of the implants allows greater penetration by chemotherapy agents than is possible with solid tumors. Finally, the disease is confined to the peritoneal cavity and all of its components are within the regional chemotherapy field (Sugarbaker 1999a, 2001). Thus, the use of peritonectomy procedures, even in bulky tumors, can eradicate all macroscopic disease and when combined with perioperative intraperitoneal chemotherapy leads to long-term survival (Goldstein et al. 2005). CRS and perioperative peritoneal is currently the state of the art for curative treatment of PMP (Sugarbaker 1991, 2001), with

well-documented benefits reported by our Center (Hadi et al. 2006; Yan et al. 2006c; Chua et al. 2009a, b, 2010a, b, c, 2011b, d, 2012a, b, c; Saxena et al. 2010; Kirby et al. 2013; Koh et al. 2013) and others (Sugarbaker and Chang 1999; Deraco et al. 2004; Loungnarath et al. 2005; Murphy et al. 2007; Smeenk et al. 2007a; Moran et al. 2008; Elias et al. 2010b; Andreasson et al. 2012; Dayal et al. 2013; Kuijpers et al. 2013; McBride et al. 2013).

1.3.7.1 CRS

CRS is to reduce the tumor volume to microscopic disease or tiny tumor implants prior to locoregional chemotherapy (Sugarbaker 2005c). The generic surgical approach involves peritonectomy procedures and visceral resections as described by Sugarbaker (1995). This includes between one and six peritonectomy procedures utilizing electro-evaporative surgery to gain a minimal but adequate margin of excision. Widespread tumor implants accumulated at specific anatomic sites within the abdominopelvic cavity are resected. These are the surfaces which absorb peritoneal fluid and those that are dependent, especially the right retrohepatic space and the pelvis. Peritoneal fluid is absorbed on the undersurface of the right hemidiaphragm through the lymphatic lacunae and on the greater and lesser omental surfaces through lymphoid aggregates. Dependent portions of the abdomen and pelvis become a site for mucinous tumor accumulations. Therefore, tumor volume will be greatest within the greater and lesser omentum, within the pelvis, beneath the right lobe of the liver, in the right retro-hepatic space, at the ligament of Treitz, and in the abdominal gutters (Sugarbaker 2001).

1.3.7.2 Perioperative Intraperitoneal Chemotherapy

In patients with peritoneal dissemination of a primary appendiceal mucinous tumor, including PMP, the response achieved by the intraperitoneal chemotherapy following a complete cytoreduction determines the outcome. If the intraperitoneal chemotherapy is successful in eradicating the residual tumor on peritoneal surfaces, the patient will be a long-term survivor. If disease persists after chemotherapy, the peritoneal malignancy will recur (Sugarbaker 1999a). According to the current standard of care, HIPEC is the procedure of choice for chemotherapy in PMP. HIPEC is usually performed with 10–12.5 mg/m² mitomycin C administered at 40–42 °C over a 90-min period or, alternatively, 460 mg/m² oxaliplatin delivered at 43 °C for 30 min. This can be followed by EPIC delivered to patients who are deemed clinically stable after surgery without any evidence of early postoperative complications. EPIC is performed with 650 mg/m² 5-fluorouracil (5-FU), intraperitoneally administered at room temperature, for the first 5 post-operative days (Chua et al. 2012c). A number of studies, including meta-analyses, reporting the use and long-term benefits of this multidisciplinary strategy in PMP are summarized in Table 1.8.

Table 1.8 Perioperative intraperitoneal chemotherapy used after cytoreductive surgery for PMP and reported survival rates

Investigators	Year	Number of patients	Chemotherapy method and regimen	Duration (min)	HIPEC temperature (°C)	Median survival (month)	Survival % (year)
HIPEC							
Elias et al. (2010b)	2010	255	Mitomycin C	60–120	41–42	>100	89.4 (1)
			Oxaliplatin	30	43		72.6 (5)
Arjona-Sanchez et al. (2011)	2011	30	Mitomycin C or paclitaxel	60	41–43	111	67 (5)
Austin et al. (2012)	2012	282	Mitomycin C 30 mg then 10 mg	40 then 60	42	81	52.7 (5)
Arjona-Sanchez et al. (2013)	2013	38	Mitomycin C	60	41–43	36	58.7 (5)
Marcotte et al. (2014)	2014	58	Oxaliplatin	30	43	–	77 (5)
Desantis et al. (2015)	2014	36	Mitomycin C	60	43	–	88.6 (3)
							83.1 (5)
HIPEC and/or EPIC							
Yan et al. (2007a)	2007	213	Mitomycin C (HIPEC)	–	–	51–156	53–60 (5)
		60	Mitomycin C + cisplatin (HIPEC)				52–96 (5)
		28	Mitomycin C or cisplatin (HIPEC)				–
		551	Mitomycin C (HIPEC) + 5-fluorouracil (EPIC)				69–72 (5)
		11	Mitomycin C + 5-fluorouracil (EPIC)				60 (3)
Chua et al. (2011a)	2011	46	Mitomycin C (HIPEC) ± 5-fluorouracil (EPIC)	90	42	56.4	45 (5)
Chua et al. (2012c)	2012	2298	Mitomycin C (HIPEC)	90	40–42	196	78 (5)
			Oxaliplatin (HIPEC)	30			66 (10)
			Mitomycin C or oxaliplatin (HIPEC) + 5-fluorouracil (EPIC)	–		–	82 (5)
							78 (10)
							84 (5)
							73 (10)

Sorensen et al. (2012)	2012	45	Mitomycin C (HIPEC)	90	39.5–41.6	154 [†]	79 (7)
		48	Mitomycin C + 5-fluorouracil (EPIC)	–	–		75 (7)
McBride et al. (2013)	2013	1624	HIPEC ± EPIC (not specified)	30–120	–	–	77 (5)
HIPEC/EPIC ± adjuvant							
Smith et al. (1992)	1992	17	EPIC + adjuvant (not specified)	–	–	–	75 (5)
							60 (10)
Sugarbaker et al. (1993)	1993	38	Mitomycin C* and 5-fluorouracil (EPIC) and mitomycin C (adjuvant)	–	–	–	89.5 (3)
Witkamp et al. (2001b)	2001	46	Mitomycin C (HIPEC) ± 5-fluorouracil (adjuvant)	90	40–41	–	91 (2)
							81 (3)
Glehen et al. (2010b)	2010	301	Mitomycin C ± cisplatin (HIPEC) ± mitomycin C and 5-fluorouracil (EPIC)	60–120	41–42.5	–	85 (3)
			Oxaliplatin ± irinotecan (HIPEC) ± systemic 5-fluorouracil ± mitomycin C and 5-fluorouracil (EPIC)	30	43		73 (5)

HIPEC hyperthermic intraoperative intraperitoneal chemotherapy, EPIC early postoperative intraperitoneal chemotherapy
†Results reported as mean survival (median was not reached). *Administered on the first preoperative day

1.3.7.3 Pros and Cons

Long-Term Benefits

As summarized in Table 1.8, the efficacy of CRS combined with perioperative intraperitoneal chemotherapy in the treatment of PMP and the resultant long-term benefits are well established in the literature. Through a retrospective, multi-institutional study on 2298 patients treated at 16 specialized centers affiliated with the Peritoneal Surface Oncology Group International, Chua et al. reported a median survival rate of 196 months (16.3 years) and a median progression-free survival rate of 98 months (8.2 years) as well as 10- and 15-year survival rates of 63 % and 59 %, respectively (Chua et al. 2012c). Of the previously described prognostic indicators for PSMs, tumor histopathology, PSS, and CCS, but not PCI, are of relevance for PMP. Among PSMs of appendiceal origin, the minimally invasive mucinous tumors are amenable to complete cytoreduction. According to the Ronnett's classification (Ronnett et al. 1995), these tumors include DPAM and the hybrid type, collectively identified and classified as low-grade tumors by Bradley et al. (2006). Thus, more definitive treatment and improved survival using the combined approach is expected with these histopathological types (Sugarbaker 2006a; Bradley et al. 2006). In contrast, PMCA is a predictor of poor survival (Baratti et al. 2009; Chua et al. 2012c). PSS has proved to have a good correlation with prognosis in patients with PMP (Sugarbaker and Chang 1999). Higher PSS has a negative impact on survival, resulting from the tumor cell entrapment (Harmon and Sugarbaker 2005; Spiliotis et al. 2012). CCS has proven to be the strongest quantitative prognostic indicator in patients with peritoneal dissemination of gastrointestinal malignancies who were treated with CRS and perioperative peritoneal chemotherapy, independent of the primary site of origin (Goldstein et al. 2005). As such, CCS has been identified as an independent prognostic factor in PMP (Chua et al. 2012c; Sugarbaker and Chang 1999; Koh et al. 2013). With respect to PCI, however, PMP is an exception and caveat to the utility of this prognostic tool (Goldstein et al. 2005; Harmon and Sugarbaker 2005). In other words, a PCI of 39 in a patient with PMP can be converted to an index of 0 by cytoreduction (Sugarbaker 1999a). Access to the current state of the art at specialized centers with a PSM program and a proficient team is also an important determinant of the disease outcome (Spiliotis et al. 2012).

Learning Curve

As mentioned before, CRS combined with perioperative intraperitoneal chemotherapy is a complex approach that can potentially lead to postoperative failure and morbid complications. Teams undertaking this treatment strategy are to minimize morbidity and mortality by learning from the experience of established centers and using the “global learning curve” (Mohamed and Moran 2009). According to a multicenter study of the performance of 33 international centers offering this multidisciplinary treatment to PMP patients between 1993 and 2012, the learning curve

in different centers turned out to be extremely long (Kusamura et al. 2014). Only eight of the 33 centers and six of 47 surgeons achieved proficiency after a median of 100 (range 78–284) and 96 (86–284) procedures, respectively. Rates of optimal cytoreduction, severe postoperative morbidity, and early oncological failure were 84.4 %, 25.7 %, and 29 %, respectively. However, reports from specialized centers with an established PSM program, including the one led by David Morris, clearly show that technical maturity and improvement of perioperative outcomes and long-term benefits along with an acceptable morbidity rate can be achieved by virtue of accumulated experience with the use of this procedure (Yan et al. 2007c; Chua et al. 2009b, 2011c).

Chapter 2

Mucins and Tumor Biology

2.1 Classification

Mucins, also known as MUC glycoproteins, belong to a gene family of over 20 members that are expressed on a tissue-specific basis by specialized epithelial cells at mucosal and secretory surfaces throughout the body (Corfield et al. 2001; Desseyn et al. 2008; Gipson 2005; Govindarajan and Gipson 2010; Hattstrup and Gendler 2008; Kreda et al. 2012; Linden et al. 2008; McGuckin et al. 2011; Thornton et al. 2008; Voynow et al. 2006). The classification and distribution of mucin family is summarized in Table 2.1. Based on distinct structural and functional features, mucins are categorized into “membrane-associated” and “secreted” types, with the latter being divided into gel-forming and non-gel-forming subtypes (Rose and Voynow 2006; Williams et al. 2006). The membrane-associated mucins include typical monomeric glycoproteins that are anchored to the cell membrane whereas secreted mucins form extracellular homo-oligomeric structures that are secreted at mucosal and secretory surfaces (Corfield 2015). Both types contribute to the protection of epithelial cells from extracellular insults. The membrane-associated mucins possess a short cytoplasmic tail which participates in intracellular signal transduction (Brayman et al. 2004; Gendler 2001). Hence, these mucins mediate signaling cascades, communicate information about extracellular conditions, and contribute to morphological and behavioral characteristics of the epithelial cells (Hollingsworth and Swanson 2004; Rachagani et al. 2009). Secreted mucins provide a physical barrier for epithelial cells lining the respiratory and gastrointestinal tracts and form the ductal surfaces of organs such as liver, breast, pancreas, and kidney (Kufe 2009). Moreover, they are part of a defensive system at the mucosal surfaces, including intestinal mucosa (Corfield et al. 2000).

Table 2.1 Mucin family: classification and distribution (Amini et al. 2014b; Corfield 2015)

Designation	Chromosomal location	Tandem repeat size (amino acids)	Main tissue expression
Membrane-associated			
MUC1	1q21	20	Breast, stomach, duodenum, ileum, colon, pancreas, trachea, bronchi, cornea, conjunctiva, middle ear, salivary gland, fallopian tubes, uterus, endometrium, endocervix, ectocervix, vagina
MUC3A/B	7q22	17	Small intestine, colon, gall bladder
MUC4	3q29	16	Breast, respiratory tract, stomach, small intestine, colon, conjunctiva, cornea, endocervix, ectocervix, vagina, endometrium, prostate
MUC11	7q22	28	Gastrointestinal, respiratory, reproductive and urinary tract, thymus, middle ear
MUC12	7q22	28	Colon, stomach, pancreas, prostate, uterus
MUC13	3q21.2	27	Colon, small intestine, trachea, kidney, middle ear
MUC15	11p14.3	None	Colon, small intestine, esophagus, respiratory tract, salivary gland, thyroid gland, kidney, prostate, testis, placenta
MUC16	19p13.2	156	Cornea, conjunctiva, respiratory tract, endometrium, ovary, middle ear
MUC17	7q22	59	Stomach, duodenum, colon, conjunctiva, fetal kidney
MUC20	3q29	18	Kidney, placenta, colorectum, esophagus, liver, respiratory tract, prostate, middle ear
MUC21	6p21	15	Respiratory tract, thymus, colon, testis
Secreted, gel-forming mucins			
MUC2	11p15.5	23	Jejunum, ileum, colon, endometrium
MUC5AC	11p15.5	8	Respiratory tract, stomach, conjunctiva, lacrimal glands, endocervix, endometrium
MUC5B	11p15.5	29	Respiratory tract, submandibular salivary glands, esophagus, pancreatobiliary epithelia, endocervix
MUC6	11p15.5	169	Stomach, duodenum, ileum, hepatobiliary tract, pancreas, endocervix, endometrium
MUC19	12q12	19	Salivary glands, submucosal gland of the tracheal tissue, cornea, conjunctiva, lacrimal glands
Secreted, non-gel-forming mucins			
MUC7	4q13-q21	23	Oral cavity, sublingual and submandibular salivary gland, respiratory tract, submucosal glands of the bronchus, conjunctiva, pancreas
MUC8	12q24.3	13/41	Normal Human Nasal epithelial (NHNE) cells, middle ear, endocervix, endometrium
MUC9	1p13	15	Fallopian tubes

2.2 Molecular Structure

Attempts to characterize the molecular nature of mucins have been complicated by biophysical properties such as a relatively large mass (over 10^6 Da), a complex biochemical composition (50–80 % O-linked oligosaccharides), and a tendency to form higher-order structures through polymerization (Carlstedt et al. 1985). Through the cloning of mucin complementary DNAs (cDNAs) in the late 1980s, it was confirmed that some membrane-associated mucins were integral membrane proteins, that mucins contained both O-linked and N-linked oligosaccharides, and that the glycosylation of mucins produced by normal epithelial cells and their malignant counterparts were significantly different (Gendler et al. 1990; Gum et al. 2002; Lan et al. 1990a; Ligtenberg et al. 1990; Pallesen et al. 2002; Williams et al. 1999). Mucins are flexible macromolecular polypeptides identified by the characteristic organization of their monomeric peptide domains. Functions of each domain are described in Table 2.2 (Hollingsworth and Swanson 2004; Corfield 2015). The structural feature common to all mucins is the tandem-repeat domain, which contains tandem repeats of identical or highly similar sequences rich in serine, threonine, and proline residues (Gendler et al. 1987; Gupta and Jentoft 1989; Timppte et al. 1988). The specific sequence and number of tandem repeats is highly variable among different mucins and among orthologous mucins from different species. The tandem repeat provides a scaffold on which cells build oligosaccharide structures. These domains are highly O-glycosylated on serine and threonine residues. Mucin core protein contains from 5 to 500 repeats, and each repeat typically contains from 5 to 100 potential glycosylation sites. O-glycosylation with complex oligosaccharides is crucial to mucin structure and function. Mucin-type oligosaccharides are involved in specific ligand–receptor interactions (McDermott et al. 2001), confer hydropscopic properties (Carlstedt et al. 1985), and might bind various small molecules and proteins. Arrays of tandem repeats provide a high degree of multivalency for oligosaccharide structures, thereby providing a significant degree of stoichiometric power (McDermott et al. 2001). The largest mucins contain over 22,000 amino acids, 50 % of which might be O-glycosylated, which corresponds to a potential stoichiometric amplification of greater than 7500-fold for associated oligosaccharide side chains.

Different normal mucosal tissues within the same individual attach different oligosaccharides to the same mucin core proteins which reflect the distinct requirements of the epithelia (Lan et al. 1990a). Tumors also express oligosaccharide structures that are distinct from the normal epithelia and account for many of the tumor-associated carbohydrate antigens (TACAs) found on adenocarcinomas (Lan et al. 1990a, b). The density of glycosylation of tandem repeats among different normal and tumor cells is also highly variable and is believed to contribute significantly to the normal or aberrant functions observed (Hanisch and Muller 2000).

Table 2.2 Specific mucin domains and their function (Corfield 2015; Hollingsworth and Swanson 2004)

Peptide domain	Mucin	MUC type	Domain features and function
PTS-tandem repeat sequences (VNTR)	All MUCs	Secreted and membrane-associated	Heavily O-glycosylated domains rich in serine, threonine, and proline. Characteristic of mucin core protein. Can be highly polymorphic for length and sequence variability
Signal sequence	All MUCs	Secreted and membrane-associated	Directs insertion to the endoplasmic reticulum and mediates secretion or membrane delivery
Cysteine Rich, CYS domains	MUC2, 5AC, 5B and 19	Secreted	Non-glycosylated multiple copy domains adjacent to or inserted within tandem repeat domains. Important for various mucin-mucin interactions
Cysteine knot	MUC2, 5AC, 5B, 6, and 19	Secreted	Conserved with von Willebrand factor and the cysteine knot of TGF- β . Involved in dimerization
D domain (D1, D2, D', D3)	MUC2, 5AC, 5B, C6 and 19	Secreted	Shows homology to the dimerization domain of von Willebrand factor and mediates oligomerization
D domain (D4)	MUC2, 4, 5AC, 5B and 6	Secreted and membrane-associated	Next to the VNTR domain, shows homology to D4 dimerization domain of von Willebrand factor and contains the GDPH autocatalytic cleavage site
Cytoplasmic tail	MUC1, 3, 4, 12, 13, 16, 17 and 21	Membrane-associated	Located on the cytoplasmic side of the cell surface membrane. Contains phosphorylation sites involved in signaling and might mediate association with cytoskeletal elements
SEA domain	MUC1, 3, 12, 13, 17 and 21	Membrane-associated	Widely distributed among heavily O-glycosylated cell surface proteins. Involved in protein binding to carbohydrate moieties. Contains autocatalytic proteolytic cleavage site
Epidermal growth factor (EGF)-like domains	MUC3, 4, 12, 13 and 17	Membrane-associated	Shows homology to EGF and related growth factors and cytokines and mediates interactions between mucin subunits and ErbB receptors
Transmembrane domain	MUC1, 3, 4, 12, 13, 16, 17, 20 and 21	Membrane-associated	Membrane-spanning sequence typical for membrane-associated mucins

(continued)

Table 2.2 (continued)

Peptide domain	Mucin	MUC type	Domain features and function
GDPH autocatalytic proteolytic site	MUC2, 4 and 5AC	Secreted and membrane-associated	Autocatalytic proteolysis site that cleaves between GD and PH residues, prior to formation of a unique covalent bond by which mucin subunits are linked to other secreted molecules
Proteolytic cleavage site	MUC1, 3, 4, 12, 13, 16 and 17	Membrane-associated	Found within the SEA domains of some mucins and outside of the SEA domains in others. Facilitates the creation of mucin subunits that remain associated

ErbB (Erythroblastic Leukemia Viral Oncogene Homolog): a protein family containing four receptor tyrosine kinases structurally related to epidermal growth factor receptor (EGFR), *MUC* mucin, *PTS* proline, threonine, serine, *SEA* (sea-urchin sperm protein, enterokinase and agrin): a domain named after the first three proteins in which it was identified (sperm protein, enterokinase, and agrin), *VNTR* variable number tandem repeat

2.3 Membrane-Associated Mucins

Mucins anchored to the apical cell surface form the largest group of mucins (Table 2.1). In contrast to the secreted mucins, membrane-associated mucins do not form oligomers and gels. These monomeric mucins contain characteristic membrane peptide domains (Table 2.2) and have properties typical of the membrane glycoproteins (Corfield 2015). They are bound to cells by an integral transmembrane domain and have relatively short cytoplasmic tails at the C-terminus that associate with cytoskeletal elements, cytosolic adaptor proteins and/or participate in signal transduction (Carraway et al. 2003). There are also common features that are seen in the extracellular juxtamembrane portions of the membrane-associated mucins. One common feature is a specific proteolytic cleavage that occurs during the intracellular post-translational processing on the juxtamembrane part of the protein that is destined to be expressed on the extracellular surface (Parry et al. 2001). This creates two subunits that remain associated during cellular transport through the endoplasmic reticulum and Golgi complex and at the cell surface. In several membrane-associated mucins, this cleavage is mediated by an unidentified intracellular protease in the SEA domain (Bork and Patthy 1995; Wreschner et al. 2002). Most membrane-associated mucins have juxtamembrane domains with homology to the epidermal growth factor (EGF) family (Gum et al. 1997b). These EGF-like domains are postulated to allow interaction with members of the EGF receptor (*ErbB*) family, thereby participating in the intracellular pathways related to growth, motility, differentiation, inflammation, or other higher-order functions (Carraway et al. 2000; Jepson et al. 2002). The number and general arrangement of EGF domains shows some conservation among membrane-associated mucins. Several mucins, including MUC3A, MUC3B, MUC4, MUC12, MUC13, and MUC17, have

two or three EGF domains. The EGF domains of MUC3A, MUC12, MUC13, and MUC17, but not that of MUC4, are separated by the SEA domain. One EGF domain is located on the extracellular subunit that contains the tandem-repeat domain, and a second (and, in some cases, third) EGF domain is located on the extracellular side of the membrane-associated subunit, proximal to the cell surface (Hollingsworth and Swanson 2004). MUC1 contains a SEA domain (Bork and Paththy 1995) and has been found to be associated with lipid rafts. However, it has no clearly defined extracellular EGF-like domains. Interestingly, it has been co-immunoprecipitated with ErbB1 (also known as EGFR) from human breast cancer cells (Li et al. 2001c) and all four ErbB members in mammary glands of MUC1-transgenic mice (Schroeder et al. 2001), indicating that they are associated directly or indirectly in molecular complexes. MUC1 also co-localizes with ErbB1 in lactating mammary glands and the stimulation of breast cancer cell lines with EGF, amphiregulin, or transforming growth factor- α (TGF- α) leads to phosphorylation of the MUC1 cytoplasmic tail on tyrosine and to its association with tyrosine phosphorylated proteins of 180 kDa (presumably one or more ErbB family members). It has been postulated that altered extracellular pH, ionic concentration, and hydration or other adverse conditions might lead to release of the extracellular domains, which might facilitate rapid clearance of cell surface-associated material (Hollingsworth and Swanson 2004). Autocatalytic peptide cleavage within the SEA domain leads to the formation of a non-covalent complex (Macao et al. 2006) that allows the release of the large extracellular mucin component into the mucus gel layer, while the membrane-specific domain is retained in the membrane (Thathiah et al. 2003; Thathiah and Carson 2004; Williams et al. 2001). The prototypical MUC1 is the membrane-associated mucin of relevance to the present project which is further discussed here.

2.3.1 *MUC1*

MUC1 (also known as episialin, PEM, H23Ag, EMA, CA15-3, and MCA) is a heterodimeric type I transmembrane protein with a heavily glycosylated extracellular domain that extends up to 200–500 nm from the cell surface (Nath and Mukherjee 2014). MUC1 is encoded by *MUC1* gene located on the long arm (q) of chromosome 1 at position 21. The human *MUC1* gene spans 4–7 kb and is comprised of 7 exons that can be alternatively spliced to form transcripts from 3.7 to 6.4 kb (Gendler and Spicer 1995; Lagow et al. 1999). In humans, there are several isoforms of MUC1 that result from alternative splicing, exon skipping, and intron retention. A recent study identified 78 isoforms of MUC1 (Zhang et al. 2013), with the most common isoforms being MUC1/A, MUC1/B, MUC1/C, MUC1/D, MUC1/X (or MUC1/Z), MUC1/Y, and MUC1/ZD. MUC1/A, MUC1/B, MUC1/C, and MUC1/D, encoding “full-length” MUC1, arise from alternative splicing between sites located in intron I and exon 2 and vary only by VNTR length (Ligtenberg et al. 1990; Obermair et al. 2001). MUC1/B is the so-called normal MUC1 mRNA. MUC1/X (or MUC1/Z), MUC1/Y, and MUC1/ZD isoforms are generated from alternative

splice acceptor sites located within exon 2, where VNTR encoding exon 2 is skipped (Oosterkamp et al. 1997; Zrihan-Licht et al. 1994). The MUC1/Y isoform is 54 bp shorter than MUC1/X and is highly expressed in breast, ovarian, and prostate cancer cells (Baruch et al. 1997; Hanisch and Muller 2000; Schut et al. 2003). MUC1/ZD also lacks the VNTR region and the flanking degenerate sequence, but contains a unique C terminal domain (43 amino acids) that results from a shift in the reading frame (Levitin et al. 2005a). A secreted isoform of MUC1 called MUC1/SEC that lacks both the TMD and CT binds to MUC1/Y causing phosphorylation of the tyrosine residues of MUC1/Y [38]. Presently, there is a lack of clear understanding of the functional significance of each of these spliced MUC1 variants [reviewed by (Nath and Mukherjee 2014)].

The MUC1 gene encodes a single polypeptide chain which, due to conformational stress, is autoproteolytically cleaved immediately after translation at the GSVVV motif, located within the SEA domain, into two peptide fragments: the longer N-terminal subunit (MUC1-N) and the shorter C-terminal subunit (MUC1-C) (Hattrup and Gendler 2008; Levitin et al. 2005b). Extracellularly, the two subunits remain associated through stable hydrogen bonds. MUC1-N is composed of the proline, threonine, and serine-rich (PTS) domain and the SEA domain. The PTS domain, also designated as the variable number tandem repeat (VNTR) region, is encoded by a highly polymorphic exon encoding for multiple 20–21 amino acid sequence repeats (Gendler et al. 1990). In northern Europeans, the VNTR is composed of 20–120 repeats, with 40–80 repeats being the most common (Hanisch and Muller 2000). The amino acid sequence of the VNTR region can vary in different cancer cell lines, consistent with the highly polymorphic nature of this motif (Muller et al. 1999). The VNTR region is flanked on both ends by a short degenerate sequence which bears subtle sequence similarity to the VNTR region (Hanisch and Muller 2000). MUC1 is extensively O-glycosylated and moderately N-glycosylated to yield mature functional mucin (Gendler 2001). MUC1 core protein and the mature glycosylated form have an estimated weight of 120–225 kDa and 250–500 kDa, respectively (Gendler and Spicer 1995; Lagow et al. 1999). The full-length protein contains three domains: short cytoplasmic (72 amino acids) and transmembrane (28 amino acid) domains that are highly conserved among species (Spicer et al. 1995), as well as a large extracellular domain (1000–2200 amino acids). The proline residues and glycosylation give rise to a rigid, extended structure that protrudes 200–500 nm above the cell surface, much farther than the distance spanned by most cell surface proteins, including syndecans and integrins [reviewed by (Brayman et al. 2004)]. Under normal conditions, MUC1 exists on the plasma membrane as a heterodimeric complex. However, the complex dissociates following stimulation with the proinflammatory cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), and this is catalyzed by the sheddase activities of the enzymes including TNF- α converting enzyme (TACE, also called disintegrin and metalloprotease domain containing protein 17 (ADAM17)) and matrix metalloproteinases (MMPs). These enzymes cause release of MUC1-N from MUC1-C, and also catalyze the cleavage of the 58 amino acid ECD of MUC1-C, thereby generating smaller peptide fragments MUC1* and MUC1-CTF₁₅ [reviewed by (Nath and

Mukherjee 2014)]. It has been reported that MUC1* can promote tumor growth (Mahanta et al. 2008) and also function as a growth factor receptor for a metastasis-associated protein (NM23-H1) in human embryonic stem cells (hESCs) (Smagghe et al. 2013).

2.4 Secreted Mucins

Lacking a transmembrane domain, these mucins are secreted into the extracellular space, remain at the apical surface, and form oligomers and gels (Table 2.1), with an evolutionary history going back to early metazoans (Lang et al. 2007). Genes that encode the gel-forming mucins are believed to have arisen by duplication from a common ancestor. They share some sequence homology and are clustered in the order *MUC6/MUC2/MUC5AC/MUC5B* on chromosome 11p15 (Pigny et al. 1996; Desseyn et al. 1998a). Secreted mucins show patterns of expression that are restricted to the secretory organs and cell types (Hollingsworth and Swanson 2004). The 5' genomic regions of MUC2, MUC5AC, and MUC5B are composed of 29 or 30 exons that encode for cysteine-rich domains that are similar to structural domains, termed D1, D2, D', and D3 domains, within von Willebrand factor (vWF) (Desseyn et al. 1998b; Escande et al. 2001; Gum et al. 1992). These D domains are important for the disulfide-mediated polymerization of this blood glycoprotein. Likewise, the 3' genomic region of MUC2, MUC5AC, and MUC5B is composed of 18 exons that also code for cysteine-rich vWF-like domains (D4, B, C, and cysteine-knot (CK)) (Buisine et al. 1998; Desseyn et al. 1997; Gum et al. 1992). Each secretory mucin has a central region with a VNTR, but there is little similarity, among the different mucins, in the sequences of the VNTR-encoded threonine-, serine- and proline-rich repeat peptides. Furthermore, the exact sequence of the tandem repeats is poorly conserved between species, suggesting that it is the high content of Thr, Ser, and Pro, rather than the arrangement of these amino acids, that is most important for mucin function. Two structural features that are conserved are the presence of sequences homologous to vW D domains, thought to be involved in mucin oligomerization to form gels, and the C terminal CK motif, with likely involvement in the initial dimerization of apomucin monomers. MUC2, MUC5AC, and MUC5B also have sequences homologous to von Willebrand factor C domains involved in binding of trefoil factors (Tomasetto et al. 2000) and two to seven conserved 108-amino acid cysteine-rich domains (Byrd and Bresalier 2004). The C-terminal region of MUC6, in contrast, does not contain the vW D4, B, and C domains (Rousseau et al. 2004).

The core proteins of secreted gel-forming mucins are very large (typically greater than 5000 amino acids) and their overall structure is predicted to be complex. The ability to form mucin-type gels that are commonly found in the aerodigestive tract results from oligomerization of mucin core proteins. Oligomerization is mediated by D domains (Gum et al. 1994). The oligomeric secreted mucins show a characteristic linkage of monomers through disulphide bridges located in cysteine-rich CK

and vW C and D domains at the N- or C-terminus of the monomers. These domains flank centrally located VNTR sequences which are unique to each MUC gene and which are also PTS rich and serve to carry the glycan chains (Ambort et al. 2011). The molecular weights of mucins are characteristically very high, reflecting a large carbohydrate content, extensive oligomerization, and very large apomucin proteins. The complex, branching network of covalently linked mucin molecules is responsible for their gel-forming properties, which can be destroyed by reduction of the disulphide bonds. It is not known at present if intermolecular bonds are formed between different mucin core-protein backbones; however, it would not be surprising if these did occur and contribute to higher-order structures (Hollingsworth and Swanson 2004).

2.4.1 MUC2

MUC2 is the major structural molecule of the intestinal mucus. The assembly of this large and complex molecule is a major task for the intestinal goblet cell. The human MUC2 is still not fully sequenced, but recent next-generation sequencing suggests that MUC2 is 5100 amino acids long (Pelaseyed et al. 2014). Recent work on MUC2 (Ambort et al. 2011, 2012a, b; Johansson and Hansson 2012; Round et al. 2012) has established the detail of the peptide domain organization and its relation to mucin function and gel formation. MUC2 has two PTS domains and shows the following arrangement: **N-terminus**, vW D1, D2, D', D3, cysteine-rich D, small PTS, cysteine-rich D, large PTS, vW D4, vW B, vW C, CK, **C-terminus** (Corfield 2015). Rapid dimerization of the translated MUC2 peptide via the cysteine knot (CK) disulphide bridges occurs in the endoplasmic reticulum. Subsequent migration to the Golgi apparatus enables glycosylation of the PTS domain serine and threonine residues with mucin type O-linked glycans. In the trans-Golgi network, the third vW D domain in its N-terminal part is responsible for trimer formation and the macromolecules are concentrated in goblet cell vesicles. This process is analogous to the oligomerization and packing of vWF and is pH and Ca^{2+} ion concentration dependent. The creation of MUC2 trimers is necessary to permit the production of mucus networks at the cell surface and also provides a possible mechanism to account for the dramatic increase in volume seen during mucin secretion (Ambort et al. 2012a, b; Johansson and Hansson 2012; Corfield 2015). MUC2 is arranged in bundles having an association of N-terminal trimer rings linked at right angles to dimers stabilized by C-terminal CK and vW domains. On secretion and hydration of the condensed vesicular mucus granules, stacked planar networks are formed with a volume increase of approximately 3000-fold relative to the cellular granules (Ambort et al. 2011, 2012a, b; Johansson and Hansson 2012; Johnson et al. 2009; Verdugo 2012). The secreted mucins are packed in vesicles where a pH of 5.2, together with a high intragranular Ca^{2+} level, is found. A MUC2 isoform lacking the long TR2 tandem repeat portion designated MUC2.1 has been reported to be generated through alternative splicing (Sternberg et al. 2004).

2.4.2 *MUC5AC*

MUC5AC is one of the major structural molecules of the gastric (Ho et al. 2004) and respiratory tract mucus (Hovenberg et al. 1996a, b). *MUC5AC* gene is clustered with *MUC2*, *MUC5B*, and *MUC6* on chromosome 11p15.5. The 5' region reveals high degree of sequence similarity with *MUC2* and *MUC5B* and codes for 1336 amino acids organized into a signal peptide, four N-terminal vWF domains (D1, D2, D' and D3), and a short domain which connects to the central repetitive region. In the central region, coded by a single large exon, 17 major domains have been identified. Nine domains are cysteine-rich domains (Cys-domains 1–9) and exhibit high sequence similarity to the cysteine-rich domains described in the central region of *MUC2* and *MUC5B*. Cys-domains 1–5 are interspersed by four PTS domains and Cys-domains 5–9 are interspersed by four *MUC5AC*-specific TR domains (TR1-TR4). The C-terminal region of *MUC5AC* has the cysteine-rich vWF-like domains D4, B, C, and CK (Escande et al. 2001). The CK domain mediates the formation of disulfide-linked dimers by a pH-dependent, autocatalytic process. This cleavage may be important in pathological conditions, in which changes in pH within cells or at the epithelial surface may result in cross-linking of the mucins, potentially contributing to the aberrant properties in mucus (Desseyn 2009; Thornton et al. 2008). Similar to other secreted mucins, the biosynthesis of *MUC5AC* must ensure the gene translation, proper folding of the peptide, dimerization, appropriate O-glycosylation, polymerization, and storage. The initial stages of MUC peptide translation include N-glycosylation. The N-linked oligosaccharides direct the precursor peptides to their correct subcellular compartments for dimerization and subsequent O-glycosylation and oligomerization (Dekker and Strous 1990; van Klinken et al. 1998). *MUC5AC* dimerizes in the rough endoplasmic reticulum, similarly to *MUC2* (Asker et al. 1998). However, these two structurally similar secretory mucins seem to have different chaperone requirements in the ER since no interaction of *MUC5AC* with ER lectins calnexin and calreticulin was detected at the stage of folding and oligomerization (McCool et al. 1999). Monomers and dimers are then transferred to the Golgi apparatus and undergo O-glycosylation (Asker et al. 1998; van Klinken et al. 1998). Once they reach the acidic trans-Golgi compartments, mucins are assembled into large covalent disulfide-linked oligomers/multimers. The process of *MUC5AC* packing and release is not completely known. It seems that the combination of low pH and high calcium ion concentration allows the packing of the mucin macromolecules in the vesicles and links with the remarkable volume expansion which occurs during secretion (Corfield 2015; Paz et al. 2003; Perez-Vilar et al. 2005).

2.5 Regulation of Mucin Expression

The expression of mucin genes is cell- and tissue-specific but is submitted to variations during cell differentiation and inflammatory process and altered during carcinogenesis. The molecular mechanisms responsible for the control of mucin transcription

and expression are beginning to be understood as mucin gene promoters and regulatory regions are characterized (Van Seuning et al. 2001).

2.5.1 Regulation of MUC1 Expression

The regulation of MUC1 expression can be transcriptional or post-transcriptional (Nath and Mukherjee 2014). Studies on epigenetic regulation have shown that methylation of histone H3-K9 and the CpG islands in the MUC1 promoter (close to the transcriptional start site; -174 to -182 bp) causes transcriptional repression (Yamada et al. 2008). By contrast, H3-K9 acetylation is permissive of MUC1 expression. Thus, demethylation of CpG and H3-K9, and the acetylation of H3-K9 in the 50 flanking region lead to elevated MUC1 expression in cancer cells (Yamada et al. 2008). The MUC1 promoter contains several putative transcription start sites (Zaretsky et al. 1999) and several cis-acting elements such as binding sites for Sp1, AP1-4, NF-1, NF- κ B, an E-box, GC boxes, peroxisome proliferator-activated receptor (PPAR) responsive region, and estrogen and progesterone receptor sites [reviewed by (Gendler 2001)]. Proinflammatory cytokines such as TNF- α and IFN- γ also induce strong MUC1 induction through the independent actions of NF- κ B p65 and STAT1a (Lagow and Carson 2002). Furthermore, MUC1 expression is regulated post-transcriptionally. MUC1 mRNA contains the seed sequence for microRNA (miR)-125b in the 3' untranslated region (UTR), and loss of miR-125b expression in breast cancer cells contributes to MUC1 overexpression (Rajabi et al. 2010). Navabi et al. demonstrated that infection with *Helicobacter pylori* (HP) reduces the rate of mucin turnover and decreases the levels of Muc1 in the murine gastric mucosa (Navabi et al. 2013).

2.5.2 Regulation of MUC2 and MUC5AC Expression

It appears that *MUC2* and the *MUC5AC* genes have much in common both at the level of sequence homology and in molecular mechanisms responsible for the regulation of the expression (Van Seuning et al. 2001). The expression of secreted mucins can be altered by methylation of the promoter (Gratchev et al. 2001; Hanski et al. 1997; Mesquita et al. 2003b). At the transcriptional level, 11p15 mucin genes are also regulated by different transcription factors, including ATF-1, cyclic AMP response element-binding protein (CREB), RAR- α (Van Seuning et al. 2001), and Sp1/Sp3 family (Aslam et al. 2001; Gum et al. 1997a; Perrais et al. 2002), as well as by growth factors (EGF, TGF- α), proinflammatory cytokines (interleukin (IL)-1 β , IL-6, TNF- α , INF- γ), pleiotropic cytokines (IL-4, IL-13, IL-9), bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), platelet-activating factor (PAF), retinoids, and hormones (Thai et al. 2008). In this regard, different intracellular signaling pathways, including MAPK, protein kinase A (PKA), PKC, PKG, NF- κ B, and Ca²⁺ signaling, have been shown to mediate the regulation of mucin

expression in response to an extracellular insult or during carcinogenesis (Van Seuning et al. 2001).

Kageyama-Yahara et al. reported that MUC5AC expression is regulated by combination of multiple regulatory mechanisms such as universal transcription factors and epigenetic modulations. They found that Gli, a universal transcription factor, regulates MUC5AC gene expression via direct protein-DNA interaction through a highly conserved region containing a Gli-binding sequence (HCR-Gli) in the promoter of MUC5AC gene in gastrointestinal cells (Kageyama-Yahara et al. 2014). The induced MUC5AC expression was also observed after treatments with DNA demethylation reagent and/or histone deacetylase inhibitor in several cell lines that were deficient in MUC5AC expression. This epigenetic regulation of MUC5AC was in line with another study by Yamada et al. who indicated CpG methylation and histone H3-K9 modification of the MUC5AC promoter distal region as a regulatory mechanism in different cancer cells (Yamada et al. 2010). Perrais et al. showed that while Sp3 is a strong inhibitor of 11p15 mucin gene transcription, transcription factor Sp1 could not only bind and activate MUC2 and MUC5AC promoters, but also contributed to their EGF- and TGF α -mediated upregulation (Perrais et al. 2002). They reported that MUC2 and MUC5AC are two target genes of EGFR ligands in lung cancer cells, and up-regulation of these two genes goes through concomitant activation of the EGFR/Ras/Raf/Erk pathway and Sp1 binding to their promoters. Jonckheere et al. showed an important role for two transcription factors, GATA-4/-6 and HNF-1/-4 families of transcription, as regulators of expression of the murine MUC5AC mucin during stomach development and in epithelial cancer cells (Jonckheere et al. 2012). Kim et al. demonstrated that CREB activation via nonclassical retinoic acid (RA) signaling pathway may play an important role in regulating the expression of MUC2 and MUC5AC mucin genes and mediating the early biological effects of RA during normal mucous differentiation in normal human tracheobronchial epithelial (NHTBE) cells (Kim et al. 2007). Mesquita et al. indicated that human MUC2 mucin gene is transcriptionally regulated by the intestine-specific transcription factor CDX2 in gastrointestinal carcinoma cells (Mesquita et al. 2003a). It has also been described that MUC2 is transcriptionally activated by p53 in human CRC cells (Ookawa et al. 2002). Yang et al. showed that the cell fate determinant Numb promotes MUC2 protein expression and intestinal cell secretion of mucins and modulates intestinal epithelial cell differentiation toward goblet cell phenotype by inhibiting the Notch signaling pathway (Yang et al. 2011b).

In a study by Ho et al., the stimulation of PKA pathway appeared to upregulate MUC5AC (Ho et al. 2002). Hong et al. showed that MUC2 and MUC5AC gene expressions were stimulated by phorbol 12-myristate 13-acetate (PMA), an activator of PKC, in human colonic cell lines (Hong et al. 1999). The induced expression of MUC5AC protein and gene by PMA or EGF has also been confirmed in airway epithelial cells (Kim et al. 2012b). Investigating the regulatory role of IL-1 β in human pulmonary epithelial cells, Kim et al. demonstrated that IL-1 β activates extracellular signal-regulated kinase (ERK) or p38 to induce cyclooxygenase 2 (COX-2) production, which in turn induces MUC2 and MUC5AC expressions at both the mRNA and protein levels (Kim et al. 2002). IL-1 β induction of MUC5AC

gene expression mediated by CREB and NF- κ B has also been observed by Chen et al. in respiratory cells (Chen et al. 2014). The role of pro-inflammatory cytokines such as TNF- α in the induction of MUC5AC gene and protein expression in airway epithelial cells (Fischer et al. 1999; Shao et al. 2003; Song et al. 2003) through NF- κ B signaling pathway has also been reported (Seo et al. 2014). In a study by Iwashita et al., MUC2 was upregulated at mRNA level by IL-4, IL-13, or TNF- α through a MAPK pathway in the human CRC cell lines (Iwashita et al. 2003). Upregulation of MUC2 gene expression by IL-4 and IL-13 in goblet cells has also been shown by Blanchard et al. (2004). In another study, Iwashita et al. also reported that cell attachment regulates MUC5AC production, which is upregulated by low adhesion to the ECM, and MUC5AC production is inversely proportional to the function of integrin β 1, a major adhesion molecule between cells and the ECM (Iwashita et al. 2013). It has also been described that MUC2 is transcriptionally activated by LPS from *Pseudomonas aeruginosa* in tracheobronchial epithelial cells (Li et al. 1997, 1998). The downstream cascade known to activate mucin gene transcription was reported to be the Src/Ras/MAPK/pp90rsk cascade, which leads to the activation of the transcription factor NF- κ B. In another study by the same group, the similar mechanism of regulation by LPS was also found for MUC5AC (Dohrman et al. 1998). Induction of serum amyloid A3 protein (SAA3), an acute-phase protein, by *Escherichia coli* and LPS is also capable of upregulating MUC2 mucin production in colonic epithelial cells (Shigemura et al. 2014). Perrais et al. indicated that infection of GC cells by *Helicobacter pylori*, a causative agent in GC, alters 11p15 mucin gene transcription and induces MUC5AC expression (Perrais et al. 2014). Raja et al. has showed that *Shigella dysenteriae*-induced expression of interleukin-1 β (IL-1 β) upregulates MUC2 expression and the differential expression of MUC5AC through a cross talk between IL-1 β and Akt wired by trefoil factor family peptide 3 (TFF3) in colonic epithelial cells (Raja et al. 2012). In an in vivo investigation on homozygous MUC1-deficient mice, Phillipson et al. found a thinner, firmly adherent mucus layer in both gastric and colon mucosa. These observations suggested a regulatory rather than structural role of MUC1 in the formation of the colonic and gastric mucus mainly composed of the gel-forming MUC2 and MUC5AC, respectively (Phillipson et al. 2008).

2.6 Mucins in Health and Cancer

2.6.1 Mucins and Gastrointestinal Physiology

Membrane-associated mucins are believed to serve as cell-surface receptors and sensors, and hence participate in signal transduction in response to changes in extracellular microenvironment and external stimuli that lead to coordinated cellular responses, including cell proliferation, differentiation and apoptosis, or secretion of specialized cellular products. They also associate with the secreted mucin layer by covalent and non-covalent bonds and contribute to physicochemical protection of

the epithelial cell surface from adverse conditions (Gipson et al. 2014; Hollingsworth and Swanson 2004). MUC1, MUC3, MUC4, MUC12, MUC13, and MUC17 are all found in the gastrointestinal tract (Pelaseyed et al. 2014). As the first to be characterized, MUC1 is the most extensively studied membrane-associated mucin. In the gastrointestinal tract, MUC1 is expressed abundantly in the stomach and only in small amounts in the intestine (Linden et al. 2007). MUC1 is found in the surface foveolar cells in the entire stomach, in mucous neck cells and in chief cells of the gastric fundus and antrum, as well as in the pyloric gland. In normal gastric mucosa, MUC1 is believed to protect gastric epithelial cells from a variety of external insults that cause inflammation and carcinogenesis [reviewed by (Saeki et al. 2014)].

Secreted mucins are expressed at mucosal surfaces with secretory and/or absorptive functions, including gastrointestinal tract. They have a central role in maintaining homeostasis in these sites and providing protection against insults by endogenous and exogenous agents in a relatively harsh environment with diverse, variable conditions. In the gastrointestinal tract, MUC2 and MUC5AC are the major components of mucus in the stomach and intestines, respectively. Secreted mucins not only protect and lubricate the lining of the alimentary canal for enhanced digestive functionality (Cone 2009), but also contribute to the specialized tasks of these organs. In the stomach, the mucous layer consists primarily of MUC5AC extending in layered sheets with MUC6 in between (Ho et al. 2004). MUC5AC along with MUC6 forms a protective layer over the surface epithelium and acts as a selective diffusion barrier for HCl (Bhaskar et al. 1992). The intestinal mucin MUC2 participates in the front line of the enteric host defense generated by the alliance of the epithelial cells, immune cells, and resident microbiota (Lievin-Le Moal and Servin 2006). This interactive ecosystem is essential for the maintenance of intestinal homeostasis and the normal function and activity of digestive system (McCracken and Lorenz 2001). Colonic mucus is composed of two layers. The outer, loose layer is the habitat of the microbial flora. The inner, dense layer is bacteria-free and firmly attached to the epithelium. This organization keeps the flora well separated from the mucosal surface. The gel-forming MUC2 comprises the substantial component of this double-layered mucus compartment. MUC2 is uncleaved in the inner layer and undergoes proteolytic cleavage to allow expansion of the polymeric structure, hence formation of the outer layer (Johansson et al. 2011).

The first stage in the biosynthesis of MUC2 is the formation of MUC2 monomer as an N-glycosylated apoprotein in the endoplasmic reticulum. Subsequently, MUC2 dimers are formed when intermolecular disulfide bonds bridge between the C-terminal cysteine knot domains. During transit through the Golgi apparatus, MUC2 dimers become heavily O-glycosylated. Complete glycosylation of the dimers occurs in the Golgi where trimerization through disulfide bonds at the N-terminus forms protease-resistant trimers. The fully glycosylated and processed MUC2 mucin is densely packed and stored in secretory granules/vesicles and released through constitutive or stimulated secretory mechanisms. Once released, MUC2 is organized into the firmly adherent inner layer. At a certain distance from the epithelium, this layer is converted into the loose outer layer through proteolytic cleavage and expansion. Mucus also contains immunoglobulins and other proteins (Amini et al. 2014b).

2.6.2 *Mucins in Cancer*

Mucins have been implicated in the pathophysiology of cancer. Malignant tumors, especially adenocarcinomas, express aberrant forms and/or amounts of mucins. At the simplest level, cancer cells use mucins in much the same way as normal epithelia to control their local microenvironment and to protect themselves from adverse growth conditions. Aberrant production, composition, and structure of tumor-associated (TA) mucins enhance growth and survival of tumor cells in otherwise inhospitable conditions and provide them with an effective means for invasion, metastasis, and immune evasion. In addition, many lines of evidence support the involvement of TA mucins in diverse biological mechanisms underlying resistance to chemotherapy, including their implications in physical barrier formation, resistance to apoptosis, drug metabolism, cell stemness, and epithelial–mesenchymal transition (EMT) (Jonckheere et al. 2014; Nath and Mukherjee 2014; Singh and Settleman 2010). Role of membrane-associated and secreted mucins in cancer is reviewed here (Byrd and Bresalier 2004; Hollingsworth and Swanson 2004; Nath and Mukherjee 2014).

2.6.2.1 Membrane-Associated Mucins

Membrane-associated mucins of cancer cells differ from those expressed by normal cells in both the expression status (amount and arrangement) and biochemical features. Overexpression, redistribution, and aberrant glycosylation of membrane-associated mucins contribute to the invasive and metastatic properties of adenocarcinomas by simultaneously configuring the adhesive and anti-adhesive properties of tumor cell surface (Hollingsworth and Swanson 2004). Upon loss of polarity associated with transformation, overexpressed TA-MUC1 is redistributed over the entire surface (Gendler 2001) and inhibits integrin-mediated cell adhesion to extracellular matrix components, thereby promoting cell detachment and increasing cancer cell invasiveness (Wesseling et al. 1995). The anti-adhesive properties of the overexpressed MUC1 also prevent tumor cells from conjugating with the effector cells of the immune system and allow them to evade immune surveillance (van de Wiel-van Kemenade et al. 1993). Aberrant glycosylation of TA-MUC1, on the other hand, exposes some epitopes otherwise masked in the normal mucin, resulting in the expression of a number of glycans serving as tumor-associated carbohydrate antigens (TACA) and potential ligands for interaction with other receptors. These antigens, such as Thomsen-Friedenreich (TF), Tn, sialyl-Tn (STn), sialyl Lewis^A (sLe^A, also termed CA19.9), and sialyl Lewis^X (sLe^X), are believed to facilitate tumor invasion and metastasis. In CRC cells, for example, MUC1 overexpresses sLe^X and sLe^A epitopes, resulting from a decrease in O-acetylation (Mann et al. 1997).

With loss of the MUC1 restricted localization to the apical membrane along with the redistribution of cell surface growth factors normally restricted to the basolateral

surface of epithelial cells, TA-MUC1 forms complexes with EGFR and other members of the ErbB family. Growth factors juxtaposed to MUC1 and intracellular kinases, such as ZAP-70, PKC-g, GSK-3b, and c-Src, phosphorylate serine, tyrosine, and threonine residues on MUC1. It is also thought that hypoglycosylation unmasks the core peptide and allows MUC1-N cleavage and release by extracellular proteases. MUC1-N release induces conformational changes in MUC1-C that alter its ligand status and subsequently activates downstream cell signaling pathways such as MAPK, phosphatidylinositol 3-kinase (PI3K)/Akt, and wingless-type (Wnt) pathways (Hollingsworth and Swanson 2004; Nath and Mukherjee 2014).

In addition, hypoglycosylation impacts the stability and subcellular localization of MUC1 (Altschuler et al. 2000). Compared with fully glycosylated MUC1, hypoglycosylated MUC1 shows increased intracellular uptake by clathrin-mediated endocytosis, without any enhanced degradation. Thus, hypoglycosylation may potentiate MUC1 oncogenic signaling by decreasing its cell surface levels and increasing intracellular accumulation (Altschuler et al. 2000). TA-MUC1-C can generate functional homodimers that translocate to the nucleus via importin- β and nucleoprotein 62 (Nup62) and act as a co-transcription factor (Raina et al. 2012; Kufe 2013). Several studies have indicated that MUC1 plays a critical role in the transcriptional regulation of genes associated with tumor cell proliferation, survival, invasion, metastasis, angiogenesis, drug resistance, inflammation, and immune regulation (Ahmad et al. 2009; Behrens et al. 2010; Cascio et al. 2011; Hattrup and Gendler 2006; Kufe 2009; Nath et al. 2013; Roy et al. 2011; Sahraei et al. 2012). Evidence also indicates that MUC1 causes transcriptional alterations that result in metabolic reprogramming in cancer cells (Mehla and Singh 2014). MUC1 interacts with both p53 and hypoxia-inducible factor 1 α (HIF-1 α), two key transcription factors that directly regulate metabolic gene expression. Serving as a transcriptional co-activator, MUC1 directly regulates expression of genes involved in multiple nutrient uptake and metabolic pathways (Chaika et al. 2012; Wei et al. 2005). MUC1 expression leads to changes in metabolic flux during glycolysis, as well as in the pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle, and fatty acid biosynthesis pathways (Chaika et al. 2012; Pitroda et al. 2009). PPP leads to the production of ribose, an essential building block for de novo DNA and RNA synthesis. As a consequence of MUC1 expression, the production of biosynthetic intermediates required for cell growth (i.e., biomass) is increased in cancer cells and cell proliferation is enhanced (Chaika et al. 2012). In addition to the transcriptional co-activator functions, MUC1 also directly modulates enzymatic functions of metabolic enzymes to regulate carbon flux (Kosugi et al. 2011). Metabolic alterations are a hallmark feature of cancer and provide tumorigenic properties to cancer cells (Hanahan and Weinberg 2011). Additionally, by modulation of autophagy, levels of reactive oxygen species, and metabolite flux, MUC1 facilitates cancer cell survival under hypoxic and nutrient-deprived conditions [reviewed by (Mehla and Singh 2014)]. Moreover, it has been hypothesized that the sugar branches sequester proinflammatory factors, such as transforming growth factor α (TGF- α), IL-1, IL-4, IL-6, IL-9, and IL-13, which are released upon MUC1-N shedding, thereby triggering inflammation (Hollingsworth and Swanson 2004). Smoldering inflammation in the

tumor microenvironment enhances proliferation and survival of malignant cells, promotes angiogenesis and metastasis, suppresses adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (Mantovani et al. 2008).

2.6.2.2 Secreted Mucins

The mucus layer covering tumor cells is believed to serve as an impenetrable physicochemical barrier that helps them evade immune and inflammatory responses. In so doing, it is hypothesized that the viscous mucin coating, equipped with several ligands for adhesion molecules as well as with sequestered suppressive cytokines, prevents the approach of antigen-presenting and effector cells and suppresses their motility and activation. This mucus layer is also thought to capture biologically active molecules, including growth factors or cytokines, which might contribute to tumor growth (Hollingsworth and Swanson 2004). Secreted mucins are also implicated in the development of tumor chemoresistance. With the contribution of the aberrant membrane-associated mucins, they form a physical barrier that can act as either a size filter allowing entrance of particles smaller than the mucus network porosity, or an interaction filter via electrostatic or hydrophobic forces (Shaw et al. 2005; Sigurdsson et al. 2013). Secreted mucins can largely contribute to the biological behavior of cancer cells. As such, MUC2 has been identified as a major carrier of STn and sLe^x antigens, with implications in tumorigenesis and metastasis of gastrointestinal cancer (Conze et al. 2010; Izumi et al. 1995; Mann et al. 1997). Similarly, Bara et al. provided evidence that M1 antigen, an early oncofetal marker of colonic carcinogenesis, is indeed the product of the MUC5AC gene (Bara et al. 1998). They consistently reported later that M1/MUC5AC mucin is abnormally expressed by colonic goblet cells during colon carcinogenesis (Bara et al. 2003). In agreement, *de novo* expression of MUC2 and MUC5AC (Conze et al. 2010; Wakatsuki et al. 2008; Walsh et al. 2013) or a mucinous phenotype (Koseki et al. 2000; Wakatsuki et al. 2008; Thota et al. 2014; Tung et al. 1996) can be indicative of a more aggressive phenotype. In addition, overproduction or ectopic secretion of gel-forming mucins may largely contribute to tumor pathogenesis and clinicopathological features observed. A typical example is the peritoneal adenomucinosis or mucinous carcinomatosis from different primary sites, including the appendix, stomach, small and large bowel, urachus, pancreas, gallbladder, and ovary. In this regard, PMP syndrome is a paradigm (Sugarbaker 2006a; O'Connell et al. 2002b). Role of secreted mucins in PMP is discussed here [also reviewed elsewhere (Amini et al. 2014b, 2015c)].

2.6.2.3 Role of Secreted Mucins in PMP

Under normal conditions, metabolic turnover of intestinal mucin is maintained by the constitutive expression against enzymatic degradation, and, elimination. In PMP, however, mucin is ectopically secreted and increasingly deposited in the

peritoneal cavity where it is unable to degrade or drain away. Accumulating mucin causes a major part of the PMP morbidity. The typical syndrome develops after secreted mucin forms voluminous gels over months and years. Mucin also plays a key role in the biology of the PMP tumor. Most of the tumor cells are surrounded by a mucin coat that allows them to freely move, disseminate, and redistribute within the peritoneal cavity to create the distinctive feature of PMP (Sugarbaker 1994). As mentioned earlier, this coating also appears to protect tumor cells against extracellular insults, immune recognition, and chemotherapy. MUC2, MUC5AC, and MUC5B are the gel-forming mucins reportedly found in the PMP secretions (Mall et al. 2007; O'Connell et al. 2002b). MUC2, however, is known as the PMP-specific mucin. According to O'Connell et al. (2002a, b), primary ovarian mucinous tumors essentially express MUC5AC whereas solitary appendiceal mucinous tumors and different categories of PMP express MUC2 along with MUC5AC. This finding also supports the notion that PMP is a neoplasm of the appendiceal origin. MUC2 plays the key role in the pathogenesis of PMP. In their studies, O'Connell et al. showed that MUC2 is behind the high degree of gelation formed in PMP. Since MUC2 becomes more extensively glycosylated, it sterically occupies a greater volume than MUC5AC on an equimolar basis. Thus, ectopic production and accumulation of MUC2 lead to the formation of copious mucinous collections (O'Connell et al. 2002a). Widespread collection of massive gels increases the intra-abdominal pressure, compresses visceral organs, and triggers inflammatory and fibrotic responses, with major contribution to morbidity and eventual development of fatal complications, including bowel obstruction (Sugarbaker 1996c). PMP inflammatory microenvironment with a unique profile of cytokines (Lohani et al. 2014) has been shown to upregulate MUC2 expression and thus increase mucin secretion (Enss et al. 2000; Kim et al. 2000a; Iwashita et al. 2003). Apart from MUC2 and MUC5AC with definitive roles in PMP pathogenesis, Mall et al. reported that MUC5B is also present in the PMP material (Mall et al. 2007, 2011). Based on the investigations by Sheehan et al. implicating a low-charge glycoform of MUC5B in the production of a tenacious respiratory mucus plug (Sheehan et al. 1995, 1999), Mall et al. speculated that it may be MUC5B that is responsible for the semisolid material found in some PMP patients. Given the high protein content of the PMP secretions, they also raised the possibility that interactions between mucin and non-mucin proteins could contribute to the viscous nature of the PMP exudates (Mall et al. 2007, 2011). The expression status of MUC2 and other mucins reported by a number of investigators are summarized in Table 2.3.

Table 2.3 Expression of MUC2 and other mucins in PMP

Study	Year	Number of PMP cases	Percentage of cases exhibiting the expression of mucins	
			MUC2	Other forms of mucins
O'Connell et al. (2002b)	2002	100	98 %	MUC5AC 95 %
O'Connell et al. (2002a)	2002	25	96 %	MUC5AC 92 %
Mohamed et al. (2004)	2004	33	97 %	MUC1 57.5 %
Nonaka et al. (2006)	2006	42	100 %	MUC5AC 100 %
Mall et al. (2007)	2007	1	100 %	MUC5AC 100 %
				MUC5B 100 %
Ferreira et al. (2008)	2008	7	100 %	MUC1 28.6 %
				MUC5AC 100 %
				MUC6 28.6 %
Semino-Mora et al. (2008)*	2008	16	N/A [‡]	N/A ^{‡‡}
Baratti et al. (2009)**	2009	85	100 %	MUC5AC 87.5 %
Flatmark et al. (2010)	2010	5	100 %	MUC1 0 %
				MUC5AC 40 %
				MUC4 100 %
Guo et al. (2011)	2011	35	94.3 %	MUC1 0 %
Mall et al. (2011)	2011	1	100 %	MUC1 0 %
				MUC4 100 %
				MUC5AC 100 %
				MUC5B 100 %
				MUC6 0 %
Chang et al. (2012)***	2012	4	64 %	MUC5AC 43 %

*This study reports the expression of MUC2 and MUC5A in DPAM and PMCA tissues as apomucin volumetric density ($V_{vi}/10^4 \mu m$) in epithelium, lymphoid aggregates, stroma vessels and free mucin compartments, respectively, as follows

[‡]MUC2, DPAM: 264 ± 60 , 47 ± 16 , 31 ± 14 and 261 ± 51 ; PMCA: 356 ± 90 , 170 ± 26 , 117 ± 25 and 1043 ± 282

^{‡‡}MUC5AC, DPAM: 90 ± 13 , 345 ± 20 , 65 ± 17 , 37 ± 6 ; PMCA: 56 ± 12 , 246 ± 17 , 50 ± 15 and 48 ± 9

^{‡‡‡}The percentages shown are numerical estimations of data originally presented by a column graph

^{‡‡‡‡}Four out of 14 patients with mucinous adenocarcinoma were PMP cases. With no individual data reported for PMP, results shown are indicative of MUC2 and MUC5AC expressions in the whole group. N/A not available

Chapter 3

Bromelain

3.1 History

The history of bromelain is linked to pineapple. *Ananas comosus* var. *comosus* (pineapple) is a member of the family *Bromeliaceae*. Pineapples were first domesticated from *A. comosus* var. *ananassoides* by the Tupi-Guaraní Indian tribe and discovered by Christopher Columbus in 1493 on Guadeloupe Island off the coast of Mexico (Bartholomew et al. 2002). Pineapples have been used for centuries as a folk medicine by the indigenous inhabitants of Central and South America to treat a range of ailments (Taussig and Batkin 1988). In 1891, the Venezuelan chemist Vicente Marciano discovered the existence of a proteolytic principle in the pineapple, which he named “Bromelin.” However, he did not live to publish the results of his last investigation (Asenjo 1946). Soon after, while addressing the initial discovery by Marciano (N/A 1891), Chittenden reported the isolation and characterization of “the proteolytic ferment of the pineapple juice” for which he kept the original name, bromelin (Chittenden 1891, 1893). This preparation is currently known as fruit bromelain. In the early 1950s, the Pineapple Research Institute in Hawaii studied a large number of varieties of *Ananas comosus* var. *comosus* and a large number of species of the family *Bromeliaceae* and produced similar proteolytic preparation on a pilot plant scale. Finally, Heinecke and Gortner found in 1957 that mature pineapple stem is a rich source of an enzyme mixture similar to bromelin which they named “Stem Bromelain” (Heinicke and Gortner 1957). Since then, the extracts of the pineapple stem, commonly referred to as “Bromelain,” have been produced on a commercial scale and used in pharmaceutical preparations.

3.2 Manufacturing Process Summary

The commercially available product is most often made from stem bromelain, whereby the extract is removed from cooled pineapple juice through centrifugation, ultra filtration, and lyophilization. After the extraction processes, the crude extract containing the enzyme of interest is then subjected to various purification operations in order to remove contaminants that may interfere with the application of bromelain as well as to increase the specific activity of the enzyme. Figure 3.1 represents various strategies employed for extraction and purification of bromelain. Techniques used by researchers for the characterization of bromelain in order to standardize the enzyme have been shown in Fig. 3.2. Recently, functional recombinant bromelain has been produced using an *Escherichia coli* expression system (Amid et al. 2011). For this purpose, a gene encoding the *Ananas comosus* stem bromelain was amplified using polymerase chain reaction. The protein expression was then conducted in the BL21-AI *E. coli* strain and the recombinant bromelain was subsequently purified using a single step immobilized metal affinity chromatography, specifically a Ni-NTA spin column.

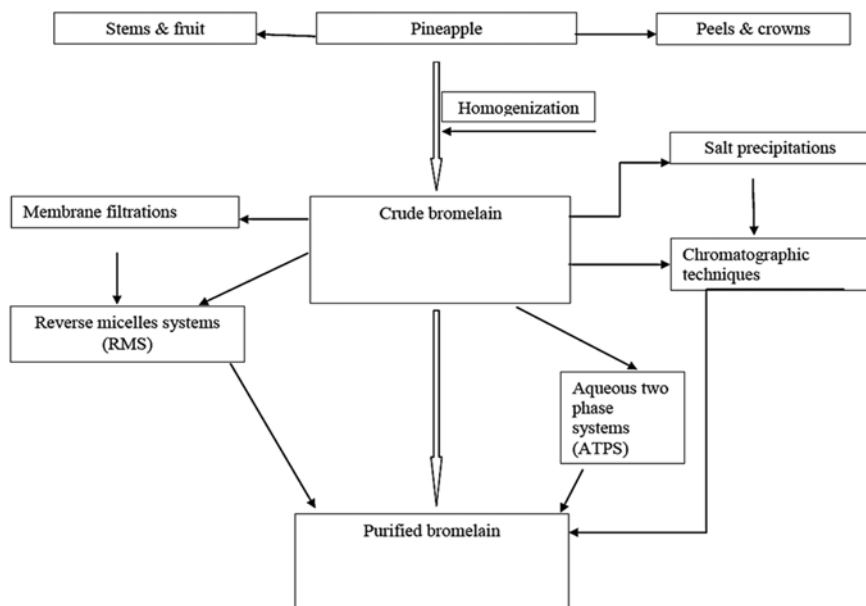


Fig. 3.1 Schematic presentation of extraction and purification strategies of bromelain (Bala et al. 2012)

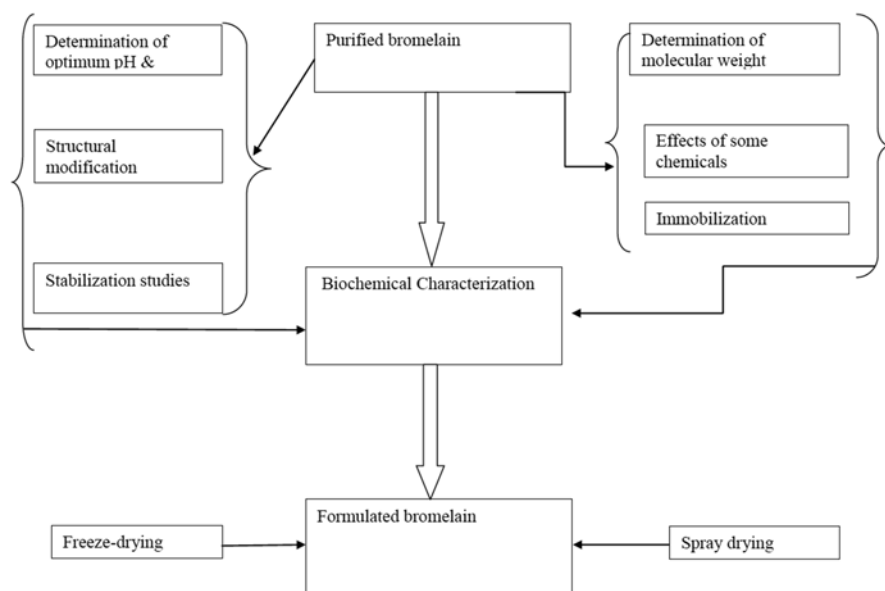


Fig. 3.2 Techniques used for biochemical characterization of bromelain (Bala et al. 2012)

3.3 Biochemical Properties

As the pineapple stem crude extract, bromelain is a mixture of different thiol endopeptidases and non-protease components. Proteases are the major constituents of bromelain and include stem bromelain (80 %), fruit bromelain (10 %), ananain (5 %), and comosain. It has been noted that stem bromelain (EC.3.4.22.32) is different from fruit bromelain (EC.3.4.22.33) (Rowan and Buttle 1993). Escharase, phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates, and several protease inhibitors are among non-protease components (Chobotova et al. 2010). Escharase is a non-proteolytic component which contributes to debridging effects of bromelain (Houck et al. 1983). As a glycoprotein with one oligosaccharide moiety and one reactive sulfhydryl group for each molecule, the optimal activity of this enzyme is between pH 5.0 and 8.0 (Thornhill and Kelly 2000; Yoshioka et al. 1991). Basic (stem bromelain, ananain, comosain) and acidic thiol-proteinases have been isolated from crude bromelain. They mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kDa, and isoelectric points between >10 and 4.8 have been extracted from crude bromelain. Among the basic proteinases, F4 and F5 are the two main components and the nonglycosylated F9 (Ananain) is the most active one (Harrach et al. 1995). Bromelain is capable of hydrolyzing esters and amides such as *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-arginine amide (Brocklehurst et al. 1972; Inagami and Murachi 1963; Pillai et al.

2014a). Although not very specific in action, bromelain preferentially cleaves glycol, anayl, and leucyl bonds. This drug is now prepared from cooled pineapple juice by centrifugation, ultrafiltration, and lyophilization. The process yields a yellowish powder, the enzyme activity of which is determined with different substrates such as casein (FIP unit), gelatin (gelatin digestion units), or chromogenic tripeptides [reviewed by (Maurer 2001)]. At room temperature (21 °C), aqueous proteolytic activity of bromelain declines rapidly. In concentrated forms (>50 mg/mL), it is stable for a week at room temperature, with minimal inactivation by multiple freeze-thaw cycles or exposure to the digestive enzyme trypsin (Hale et al. 2005b). Commercially, bromelain is used for hydrolysis of chitosan (a complex carbohydrate) into simpler forms. It enables this hydrolysis by cleavage of the glycosidic linkages (Wang et al. 2008).

3.4 Pharmacokinetics

Bromelain is absorbed from the intestine. In a study in adult rats, bromelain was absorbed from the gastrointestinal tract in a functionally intact form, approximately up to 40 % of labeled bromelain in high molecular form (Seifert et al. 1979). It is believed that α_2 -macroglobulin, the main complexing agent for bromelain, leaves the proteolytic activity of bromelain intact but reduced (Streichhan et al. 1995). In a clinical study by Castell et al., oral bromelain was detected to retain its proteolytic activity in plasma and was also found linked with α_2 -macroglobulin and α_1 -anti-chymotrypsin, the two antiproteinases of blood. The estimated plasma half-life was 6–9 h. After oral multidosing (3 g/day), plasma concentration reached as much as 5000 pg/mL by 48 h. Moreover, the enzyme retained, at least in part, its biological activity. Results of this work confirmed the existence of a small but significant intestinal transport of undegraded proteins in healthy men (Castell et al. 1997). In another clinical study, the bioavailability of Phlogenzym®, a combination of bromelain, trypsin, and the flavonoid rutoside (rutin), was examined in 21 healthy males (Maurer 2001). Following oral administration of 400-mg and 800-mg tablets (corresponding to 1.94 and 3.88×10^4 FIP units) four times daily up to 4 days, the specific activities of bromelain and trypsin were determined in plasma. The activities and AUC values proportionally correlated with the respective dosage. In addition, quantitative studies by means of enzyme immunoassays and Western blot analyses confirmed these findings. Moreover, plasma concentrations of trypsin and specific proteinase activities correlated as well. In another study, it was demonstrated that 3.66 mg/mL of bromelain was stable in artificial stomach juice after 4 h of reaction and also 2.44 mg/mL of bromelain remained in artificial blood after 4 h of reaction (Shiew et al. 2010). These results support the notion that the enzymes are absorbed from the gastrointestinal tract in a functionally intact form (Maurer 2001).

3.5 Pharmacodynamics

Bromelain belongs to a group of proteolytic enzymes that demonstrate—in vitro and in vivo—antiedematous, anti-inflammatory, antithrombotic, and fibrinolytic activities. These enzymes, including both plant cysteine proteinases (such as bromelain and papain) and proteinases from animal organs (such as trypsin and chymotrypsin), are used in the United States and Europe as an alternative or complementary medication to glucocorticoids, nonsteroidal antirheumatics, and immunomodulatory agents. Their very low toxicity ensures their safe use as remedies for chronic inflammatory diseases, as well as adjuvants to chemoradiotherapy and perioperative care. Nevertheless, clinical evidence to support preclinical findings is limited (Maurer 2001). Figure 3.3 is a representative illustration of the bromelain effects on inflammation and cancer.

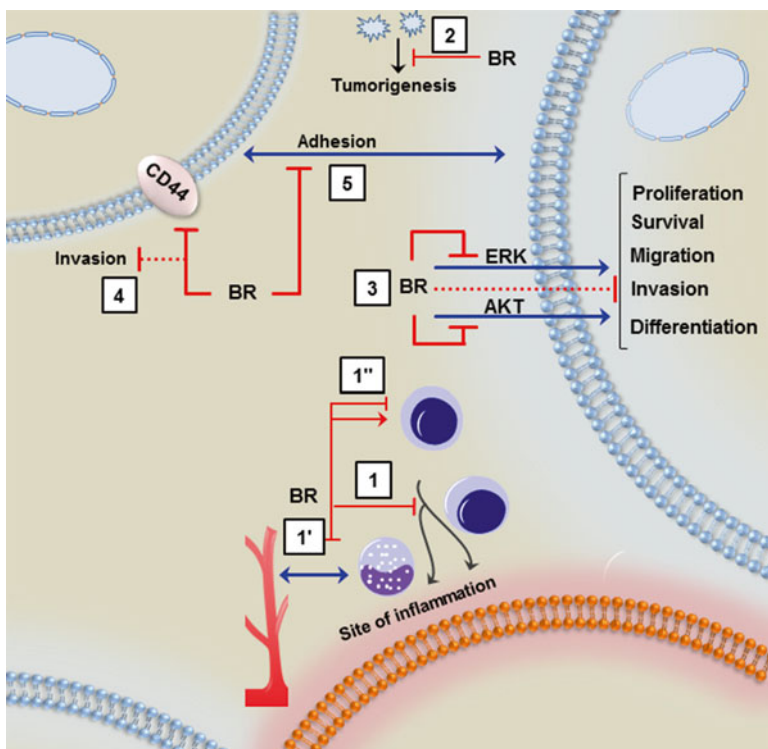


Fig. 3.3 Bromelain in inflammation and cancer. Anti-inflammatory and immunomodulatory effects of bromelain include inhibition of leukocyte migration (*I*) and their adhesion to blood vessels (*I'*) at the inflammation site, as well as T cells activation or inhibition (*I''*). Bromelain's anticancer benefits include prevention of tumorogenesis (2), inhibition of malignant cell differentiation, proliferation, migration, and survival (3), and prevention of cancer cell invasion (4) and adhesion (5)

Based on the results from different studies, including clinical observations, bromelain's functions may be categorized as follows ([No Authors Listed] 2010; Maurer 2001).

3.5.1 Anti-inflammatory Effects

Bromelain diminishes inflammation, prevents edema formation, ameliorates existing edema, and alleviates pain. It reduces leukocyte migration into the inflamed areas and, by removal of cell surface molecules including CD44 and CD128, prevents firm adhesion of leukocytes to blood vessels at the site of inflammation (Fitzhugh et al. 2008; Munzig et al. 1994). Plasmakinins and prostaglandins play important roles as mediators of pain and vascular phenomena associated with acute inflammation. Animal experiments demonstrated that bromelain lowers the plasmakinin level (Oh-ishi et al. 1979). Similarly, it caused a dose-dependent decrease in bradykinin levels at inflammatory sites and a parallel decrease in the prekallikrein levels in sera (Kumakura et al. 1988). Bromelain might also be a specific inhibitor of cyclooxygenase-2 (Cox-2) expression, inducing a significant decrease in the production of two key mediators of inflammation, substance P and prostaglandin E2 (PGE2) (Gaspani et al. 2002). Studies of prostaglandin metabolism during acute inflammation showed that orally administered bromelain reduces the level of PGE2 and of thromboxane B2 in a dose-dependent manner (Vellini et al. 1986). Bromelain may selectively inhibit the proinflammatory thromboxane generation and shift the ratio of thromboxane/prostacyclin in favor of the anti-inflammatory prostacyclin (Taussig and Batkin 1988). Bromelain increases tissue permeability by fibrinolysis and promotes reabsorption of edema fluid into blood circulation (Maurer 2001; Smyth et al. 1962).

3.5.2 Immunomodulatory Effects

In vitro observations provide evidence for the immunomodulatory role of bromelain. Hale and Haynes indicated that bromelain treatment of T cells removed CD44, CD45RA, E2/MIC2, CD6, CD7, CD8, and Leu 8/LAM1 surface molecules and enhanced CD2-mediated T cell activation (Hale and Haynes 1992). Selective cleavage of CD44, a co-stimulator of T cells that also increases bioavailability of cytokines and contributes to leukocyte migration, was similarly reported by others (Kleef et al. 1996; Munzig et al. 1994). Bromelain also inhibited the activation of CD4+ T cells and reduced the expression of CD25 (Secor et al. 2009). Mynott et al. reported that bromelain proteolytically blocked ERK2 activation in the stimulated T cells (Mynott et al. 1999). Bromelain also inhibited the activation of CD4+ T cells and reduced the expression of CD25 (Secor et al. 2009). On the other hand, there is evidence showing that bromelain activates natural killer cells and increases the production of tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), interleukin

1 (IL-1), IL-2, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Barth et al. 2005; Dessler et al. 1994; Engwerda et al. 2001a, b). Thus, bromelain appears to induce complex immune responses that might be adaptogenic in nature. In agreement, bromelain was shown in vivo to simultaneously enhance and inhibit T cell responses via a stimulatory action on accessory cells and a direct inhibitory effect on T cells (Engwerda et al. 2001a). In a human study, bromelain supplementation increased monocytic cytotoxicity in individuals with decreased activity (Eckert et al. 1999). It also stimulated the secretion of IL-1 β from monocytes and reduced the expression of CD44.

3.5.3 Antithrombotic and Fibrinolytic Activities

Bromelain decreases thrombin-induced platelet aggregation in vitro (Glaser and Hilberg 2006; Heinicke et al. 1972). It also reduces the adhesion of platelets to endothelial cells, suggesting that anti-adhesion might contribute to the prevention of platelet aggregation (Metzig et al. 1999). Bromelain treatment causes an increase in prothrombin and activated partial thromboplastin time as well as a decrease of ADP-induced platelet aggregation (Livio et al. 1978). Bromelain is also an effective fibrinolytic agent in vitro and in vivo. The fibrinolytic activity of bromelain has been attributed to the enhanced conversion of plasminogen to plasmin, which in turn cleaves fibrin (De-Giuli Morghen and Pirotta 1978; Taussig and Batkin 1988). This property is shared with streptokinase. Unlike streptokinase, however, bromelain is not able to dissolve fibrin. In addition, bromelain inhibits the thrombin-induced formation of fibrin (Maurer 2001). In agreement with these findings, isolation from bromelain of active fractions as platelet aggregation inhibitor (Morita et al. 1979) or fibrinolysis activator (Ako et al. 1981) has been reported.

3.5.4 Protection Against Ischemia-Reperfusion Injury

In vivo, bromelain was shown to trigger an Akt-dependent survival pathway in the myocardium, thereby inducing cardioprotection against ischemia-reperfusion (I/R) injury (Juhasz et al. 2008). Similarly, Phlogenzym® had a protective effect on skeletal muscles during I/R injury (Neumayer et al. 2006).

3.5.5 Protective Effects on Gastrointestinal Physiology

In vitro and in vivo evidence indicates that bromelain inhibits NF- κ B pathway activation and inducible nitric oxide synthase (iNOS) overexpression that can lead to gastrointestinal motility restoration after postoperative or LPS-induced ileus

(Wen et al. 2006). Bromelain can also reverse some of the effects of certain intestinal pathogens through interaction with intestinal secretory signaling pathways, including adenosine 3':5'-cyclic monophosphate, guanosine 3':5'-cyclic monophosphate, and calcium-dependent signaling cascades. As such, bromelain counteracts the enterotoxin-induced increase in the intestinal secretions caused by *Vibrio cholera* (Mynott et al. 1997). In *E. coli* infection, bromelain has anti-adhesion effects, preventing the bacteria from attaching to specific glycoprotein receptors located on the intestinal mucosa by proteolytically modifying the receptor attachment sites (Chandler and Mynott 1998; Mynott et al. 1996).

3.5.6 Potentiation of Antibiotics Absorption

Bromelain is capable of enhancing the absorption and tissue permeability of antibiotics after oral, subcutaneous, or intramuscular application (Bradbrook et al. 1978; Luerti and Vignali 1978; Renzini and Varengo 1972). As a result, it can maintain a higher serum and tissue levels of the drug, thus potentiating the efficacy and reducing side effects (Maurer 2001; Renzini and Varengo 1972; Tinozzi and Vengoni 1978).

3.5.7 Effects on Malignant Growth

Since the initial, anecdotal reports in the 1970s (Gerard 1972; Nieper 1976), different investigators have argued for the potential roles that bromelain could play in the management of cancer. This concept has been underpinned by in vitro and in vivo observations as well as by the known pharmacodynamic profile of bromelain. According to the literature, one can classify bromelain's effects on malignant growth into the following (Maurer 2001; Chobotova et al. 2010):

3.5.7.1 Chemoprevention

A number of preclinical studies have reported on the benefits of bromelain treatment in cancer prevention. Two initial studies by Goldstein et al. (1975) and Taussig and Goldstein (1976) showed that bromelain inhibited UV-induced tumorigenesis in nude mice skin. In agreement, Kalra et al. and Bhui et al. observed the inhibitory effects of topical pre-treatment and treatment with bromelain on tumor initiation and promotion in another murine model of skin carcinogenesis (Kalra et al. 2008; Bhui et al. 2009). They found that bromelain inhibited tumorigenesis through induction of p53 and caspase system, shift in Bax/Bcl-2 ratio, and inhibition of NFκB-mediated Cox-2 expression by blocking MAPK and Akt/protein kinase B (PKB) signaling pathways. Recently, Romano et al. reported that intraperitoneal treatment

of the azoxymethane (AOM) mouse model of colon carcinogenesis with bromelain (1 mg/kg, three times a week for 3 months, starting 1 week prior to the commencement of AOM exposure) offers chemoprevention by inhibiting the AOM-induced development of aberrant crypt foci (ACF), polyps, and tumors (Romano et al. 2014).

3.5.7.2 Effects on Cancer Cells

Table 3.1 summarizes in vitro and in vivo studies reporting on the effects of bromelain on a variety of cancer cells. As seen, different aspects of tumor biology, including cell survival, growth, proliferation, differentiation, migration, adhesion, and invasion, can be affected by bromelain treatment. In so doing, bromelain has been shown to regulate key cellular pathways responsible for invasive behavior of cancer cells, to induce cell growth arrest or apoptosis, to alter cell surface molecules of adhesion and invasion, and to inhibit the expression/production of other pathophysiological factors.

3.5.7.3 Effects on Cancer-Related Inflammation

Regardless of whether inflammation in tumor microenvironment is cause or consequence of malignant transformation, it contributes to tumor growth, angiogenesis, progression, and metastasis (Mantovani et al. 2008). This aspect of tumor biology can thus be influenced by anti-inflammatory and immunomodulatory activity of bromelain. To this end, bromelain was shown to inhibit NF κ B/Cox-2 pathway in a skin carcinogenesis model (Bhui et al. 2009; Kalra et al. 2008). Bromelain-induced inhibition of NF κ B/Cox-2/PGE2 was also observed in vitro (Hou et al. 2006; Huang et al. 2008). Among bromelain-sensitive regulators of inflammation that are connected to NF κ B pathways and can be regulated by bromelain at tumor microenvironment are IFN- γ , TNF- α , IL-1 β , and IL-6 (Huang et al. 2008). It is also postulated that bromelain can prevent formation of intracellular reactive oxygen species (ROS) and resultant genotoxicity through interrupting the interaction between advanced glycation end (AGE) products and their receptor (RAGE) (Stopper et al. 2003). RAGE is a multiligand receptor that regulates activation of NF κ B and its target genes (Bierhaus et al. 2005). Thus, bromelain-induced degradation of RAGE can also mediate NF κ B inhibition.

3.5.7.4 Immunomodulation

A number of tumor cell- and lymphocyte-expressed surface molecules, including CD44, are bromelain-sensitive. Bromelain reduces CD44 on cancer cells that in turn diminishes cell invasion (Grabowska et al. 1997; Tysnes et al. 2001; Guimaraes-Ferreira et al. 2007). Lymphocyte CD44 cleavage in response to bromelain resulted in the enhancement of impaired cytotoxicity of monocytes

Table 3.1 Cellular and molecular targets of bromelain related to its anticancer activity

Study type	Treatment	Effect
<i>In vitro</i>		
Cell		
LLC, YC, MCA-1	BR	↓ Cell growth (Taussig et al. 1985)
KATOIII	BR	↓ Cell growth [Tausig et al., Proceedings of Southwest Oncology Group Meeting (1985); Oishi et al., Proceedings of Coulter Electronic Flow Cytometry Meeting (1985); referenced in (Taussig and Batkin 1988)]
M1-T22, HL-60, K562	BR	↓ Cell growth, ↑ differentiation, ↑ phagocytosis (Maurer et al. 1988)
<i>PBMC</i>	BR	↓ Cell surface molecules involved in migration (CD44, CD45RA, E2/MIC2, CD6, CD7, CD8, and Leu 8/LAM1 surface molecules) and ↑ CD2-mediated T cell activation (Hale and Haynes 1992)
MCF-7, KB, SK-MEL-28	F9	↓ Cell growth (Garbin et al. 1994)
Molt-4/8, SK-MEL-28	BR, F4, F9	↓ CD44 expression and adhesion (Harrach et al. 1994)
<i>PBL</i>	F9	↓ adhesion to HUVEC cells through ↓ CD44 expression, ↔ LFA-1 (Munzig et al. 1994)
<i>PMN</i>	WE*	↑ ROS, ↑ cytotoxicity toward tumor cell lines (Zavadova et al. 1995)
B16F10	F9	↓ Cell growth, ↓ invasion, ↓ CD44 expression (Grabowska et al. 1997)
RAW 264.7, <i>BMM</i>	BR	↑ TNFα, ↑ IFN-γ-mediated NO production (Engwerda et al. 2001b)
<i>NK</i>		↑ IFN-γ (Engwerda et al. 2001b)
AN1/lacZ, U-251/lacZ, U-251/GFP, A-172, U-87/GFP	BR	↓ Adhesion, ↓ migration, ↓ invasion, ↓ integrin and CD44 expression, ↔ cell viability, ↓ CRE-mediated signaling (Tysnes et al. 2001)
<i>PBMC</i>	BR	↓ Cell surface molecules involved in lymphocyte migration and activation (e.g. CD44, CD7, CD8alpha, CD14, CD16, CD21, CD41, CD42a, CD45RA, CD48, CD57, CD62L, CD128a and CD128b) (Hale et al. 2002)
<i>Neutrophils (human)</i>	BR	↓ Chemotaxis and neutrophil migration toward IL-8 (Fitzhugh et al. 2008)
THP-1, <i>PBMC</i>	BR	↓ NF-κβ, ↓ Cox-2, ↓ PGE2, ↓ LPS-induced TNFα, ↓ IL-1β and IL-6 (Huang et al. 2008)
GI101A	BR	↑ cytotoxicity, ↑ apoptosis (Paroule et al. 2009)
MCF-7	BR	↑ Autophagy, ↑ apoptosis, ↓ p-ERK1/2, ↑ p-JNK and p-p38 kinase (Bhui et al. 2010)
A431, A375	BR	↓ Cell growth, ↓ proliferation, ↑ cell cycle arrest, ↑ apoptosis, ↓ NF-κβ translocation, ↑ ROS (Bhui et al. 2012)
GI101A	BR	↑ Apoptotic cell death (Dhandayuthapani et al. 2012)

(continued)

Table 3.1 (continued)

Study type	Treatment	Effect
MCF-7	BR (recombinant and commercial)	↓ Cell viability, ↓ cell proliferation (Fouz et al. 2014a)
MCF-7	BR (recombinant)	↓ Cell growth, effects on cytotoxicity or cell death mechanism through alteration of the diverse gene networks and metabolic and cell regulatory pathways (Fouz et al. 2014b)
Caco-2	BR	↓ Cell proliferation, ↑ apoptosis, ↓ p-ERK1/2/total, ERK, and p-AKT/AKT expression, ↓ ROS (Romano et al. 2014)
DLD-1		
PET, YOU	BR	↓ Cell proliferation, ↑ apoptosis, ↑ autophagy, ↓ MUC1, p-Ikkb, p-NF-κβ and p-AKT (Pillai et al. 2014b)
<i>In vivo</i>		
No cancer cell, UV-induced mouse skin tumors	BR ^o	↑ Skin cancer prevention (Goldstein et al. 1975)
LLC	BR ^o	↓ Lung metastasis (Batkin et al. 1988a, b)
B16F10	F9	↓ Lung colonization and metastasis (Grabowska et al. 1997)
P-388, S-37, EAT, LLC, B16F10, ADC-755	BR ^{ip}	↑ Survival except for MB-F10, ↓ number of lung metastasis (LLC), ↓ number of tumor cells in the ascitic fluid (EAT) (Baez et al. 2007)
No cancer cell, DMBA and TPA-induced mouse skin tumors	BR	↑ Apoptosis, ↓ NF-κβ-driven Cox-2 expression, ↓ ERK1/2 and AKT activity (Kalra et al. 2008)
<i>Neutrophils (mice)</i>	BR	↓ Migration toward inflammatory stimulus (intraperitoneal thioglycollate) (Fitzhugh et al. 2008)
No cancer cell, DMBA and TPA-induced mouse skin tumors	BR ⁱ	↓ Onset of tumorigenesis, ↓ cumulative number of tumors and average number of tumors/mouse in regard to DMBA-induced mouse skin tumors, ↑ apoptosis, ↓ NF-κβ-driven Cox-2 expression, ↓ ERK1/2, p38 mitogen-activated protein kinase and AKT activity (Bhui et al. 2009)
	BR ^o	↑ Prevention, ↓ tumor volume in regard to DMBA-induced mouse skin tumors (Bhui et al. 2009)
Caco-2	BR ^{ip}	↓ Development of aberrant crypt foci, polyps, and tumors induced by azoxymethane (Romano et al. 2014)
Clinical		
Target		
Breast and ovarian cancer	BR ^o	↑ Remissions of malignant tumors with negligible side effects in patients with ovarian and breast cancers (Gerard 1972)

(continued)

Table 3.1 (continued)

Study type	Treatment	Effect
Breast cancer	BR ^o in combination with 5-fluorouracil and vincristine	↑ Remissions of malignant tumors with negligible side effects (Nieper 1974; Nieper 1976)
Advanced pancreatic cancer	Multimodal treatment [‡] including WE*	↑ Patients survival [(Hager et al. 1996): referenced in (Boik 2001)]
Blood sample (breast cancer)	BR ^o	↑ Monocytic cytotoxicity [†] , ↓ CD44 expression on lymphocytes, (Eckert et al. 1999)
Blood sample (RA, OMF, HZ)	OET [†]	↓ Serum TGF-β1 (Desser et al. 2001)
PMN (healthy donors)	WE ^o	↑ ROS production in polymorphonuclear neutrophils (Guimaraes-Ferreira et al. 2007)

AKT protein kinase B, *BR* bromelain, *Cox-2* cyclooxygenase-2, *CRE* cAMP response element, *DMBA* 7,12-dimethyl-benzanthracene, *ERK1/2* extracellular signal regulated protein kinase1/2, *F9* highly purified bromelain proteinase, *HZ* herpes zoster, *IFNγ* interferon gamma, *IL-1β* interleukin-1β, *IL-6* interleukin-6, *IL-8* interleukin-8, *LFA-1* lymphocyte function-associated antigen 1, *LPS* lipopolysaccharide, *MUC1* mucin1, *NF-κβ* nuclear factor kappa-β, *NO* nitric oxide, *OMF* osteomyelofibrosis, *p-AKT* phosphorylated protein kinase B, *p-NF-κβ* phosphorylated nuclear factor kappa-β, *p-ERK1/2* phosphorylated extracellular signal-regulated kinase1/2, *PGE2* prostaglandin E2, *p-JNK* phosphorylated-c-jun N-terminal kinase, *p-Ikkb* phosphorylated nuclear factor kappa-β inhibitor kinase subunit b, *RA* rheumatoid arthritis, *ROS* reactive oxygen species production, *TNFα* tumor necrosis factor alpha, *TGF-β1* transforming growth factor beta 1, *TPA* 12-O-tetradecanoylphorbol-13-acetate, *WE* Wobenzym

^{ip}: intraperitoneal, ^o: oral, ^t: topical

Cell lines: *A-172* human glioma cells, *A375* human melanoma cells, *A431* human epidermoid carcinoma, *ADC-755* mammary adenocarcinoma cells, *AN1/lacZ* human glioma cells, *B16F10* mouse melanoma cells, *BMM* mouse bone marrow-derived Macrophages, *Caco-2* colon adenocarcinoma, *DLD-1* colorectal adenocarcinoma, *EAT* Ehrlich ascitic tumor cells, *GI101A* human breast cancer cells, *HL-60* human promyelocytic leukemia, *HUVEC* human umbilical vein endothelial cells, *K562* human leukemia cells, *KATOIII* gastric carcinoma, *KB* squamous carcinoma, *LLC* mouse Lewis lung carcinoma, *MI-T22* myeloid mouse leukemia, *MCA-1* mouse ascitic tumor cells, *MCF-7* breast cancer cells, *MDA-MB-231* human breast cancer cells, *Molt-4/8* human molt-4/8 leukemia cells, *NK* mouse natural killer cells, *P-388* mouse lymphocytic leukemia, *PBL* peripheral blood lymphocytes, *PBMC* healthy human peripheral blood mononuclear cells, *PET* human malignant peritoneal mesothelioma cells, *PMN* polymorphonuclear neutrophils, *S-37* mouse Sarcomas cells, *RAW 264.7* mouse macrophage, *SK-MEL-28* melanoma cells, *THP-1* human monocytic leukemia, *U-251/GFP* human glioma cells, *U-87/GFP* human glioma cells, *U-251/lacZ* human glioma cells, *YC* mouse YC-lymphoma cells, *YOU* human malignant peritoneal mesothelioma cells

Non-cancerous cells or diseases are shown in *italics*

*Wobenzym (WE) is a polyezyme preparation containing pancreatin, papain, bromelain, trypsin, and chymotrypsin used in adjuvant therapy

[†]Oral proteolytic enzymes therapy with combination drug products containing papain, bromelain, trypsin, and chymotrypsin

[†]Immunocytotoxicity of blood monocytes and lymphocytes against the leukemic K562 and MDA-MB-231 mammary carcinoma target cells

[‡]Combination of hyperthermia, immunostimulants, hormone therapy, and Wobenzym

derived from patients with breast cancer (Eckert et al. 1999). Based on the observation that TGF- β , known as a major regulator of cancer-induced immune suppression, was reduced by bromelain in patients with excessive blood levels (Desser et al. 2001), TGF- β inhibition can similarly be postulated as another mechanism through which bromelain might boost immune response against cancer. Bromelain was also shown to stimulate ROS production and cytotoxicity of neutrophils against tumor cells (Zavadova et al. 1995). Following repeated oral exposure, bromelain can stimulate both systemic and mucosal immune responses (Hale et al. 2006). While leaving the proteolytic activity of bromelain unaffected, the generated antibodies were cytotoxic/lytic to cancer cells (Guimaraes-Ferreira et al. 2007).

3.5.7.5 Effects Mediated by Fibrinolysis and Platelet Disaggregation

Fibrin is directly involved in inhibiting lymphocyte adherence and cytotoxicity against tumor cells (Biggerstaff et al. 2008). Additionally, tumor cells are believed to form a protective coat by polymerizing fibrin and human serum albumin (Lipinski and Egyud 2000). Besides, there is a reciprocal stimulatory relationship between platelets and tumor cells. Tumor cells initiate platelet activation and production of multiple factors facilitating angiogenesis and tumor-platelet aggregates protect tumor cells from immune recognition. During metastasis, these aggregates can facilitate endothelial adhesion and tissue invasion (McNicol and Israels 2008). Thus, it is hypothesized that bromelain-induced fibrinolysis and platelet disaggregation can decrease soluble fibrin in circulation, uncover tumor cells, make them visible to the immune system, and lessen their invasion (Chobotova et al. 2010).

3.5.7.6 Chemosensitization

The capability of BR in potentiating the cytotoxic effects of anticancer agents has been shown in a limited number of studies. According to the anecdotal clinical studies in early 1970s, oral administration of BR in doses of over 1000 mg daily in combination with chemotherapeutic agents, such as 5FU and VCR, resulted in tumor regression (Gerard 1972; Nieper 1974). Oishi et al., however, were the first to observe in vitro that cytotoxicity on KATO-III cells of 5FU, mitomycin-C, doxorubicin, and cisplatin was enhanced by the addition of BR [(Oishi et al. 1985) in (Batkin et al. 1988a) and (Taussig and Batkin 1988)]. Similarly, BR was found to enhance cisplatin cytotoxicity on MPM cells PET and YOU (Pillai et al. 2014b).

3.6 Potential and Actual Applications

3.6.1 *Gastrointestinal Health and Disease*

As a digestive aid, bromelain was shown to improve intestinal motility and defecation in rats post-laparotomy (Wen et al. 2006). In humans, it has been used in combination therapy to alleviate signs and symptoms of pancreatic insufficiency (Wen et al. 2006), steatorrhea (Balakrishnan et al. 1981), and dyspepsia (Pellicano et al. 2009), and to enhance protein utilization in elderly patients on tube feeding (Glade et al. 2001). In experimental models, bromelain has shown protective effects against microbial invasion. As such, bromelain supplementation helped protect animals against diarrhea caused by bacterial enterotoxins from *Escherichia coli* and *Vibrio cholera* (Chandler and Mynott 1998; Mynott et al. 1997) and exhibited antihelminthic activity against the gastrointestinal nematodes *Trichuris muris* and *Heligmosomoides polygyrus* (Stepik et al. 2005, 2006).

3.6.2 *Infections*

Some investigators have reported on the usefulness of bromelain in single agent or combination therapy of a number of infectious diseases ([No Authors Listed] 2010). Several studies conducted in the 1960s reported a benefit of bromelain for sinusitis (Ryan 1967; Seltzer 1964; Taub 1966). For example, in patients with sinusitis who were not receiving antibiotic treatment, 85 % receiving bromelain had complete resolution of inflammation of the nasal mucosa and complete resolution of breathing difficulties, compared with the placebo group with complete resolution of inflammation and breathing difficulty in only 40 % and 53 % of patients, respectively (Ryan 1967). In a more recent study in children with acute sinusitis, treatment with bromelain shortened the duration of symptoms and speeded recovery compared with usual care (Braun et al. 2005). In a double-blind trial, patients with urinary tract infections received antibiotics plus either bromelain/trypsin in combination or a placebo. All patients who received the enzymes had complete resolution of infection, compared with only 46 % of those given the placebo (Mori et al. 1972). In a phase III study, Phlogenzym® was used as an adjuvant to antibiotic therapy for children with sepsis. Phlogenzym® supplementation resulted in earlier improvements in fever and on the Glasgow Coma Scores (Shahid et al. 2002). In another study, 3-month treatment with bromelain was found an effective therapeutic option for patients with pityriasis lichenoides chronica (Massimiliano et al. 2007). In these combination therapies, beneficial effects of bromelain can be postulated to result, at least in part, from its role in antibiotic potentiation.

3.6.3 *Inflammatory Diseases*

The beneficial role of bromelain in inflammatory diseases has been reported by some investigators ([No Authors Listed] 2010). Daily treatment with oral bromelain decreased the incidence and severity of spontaneous colitis and significantly decreased the clinical and histological severity of colonic inflammation in mice with established colitis (Hale et al. 2005a). Bromelain treatment decreased secretion of pro-inflammatory cytokines and chemokines by colon biopsies from patients with inflammatory bowel disease (Onken et al. 2008). In case reports of two patients who were not responding to conventional medical therapy, addition of bromelain to usual drug regimen resulted in rapid improvement of symptoms, which was confirmed by endoscopy (Kane and Goldberg 2000). In an animal model of rheumatoid arthritis, Phlogenzym® plus cyclosporin showed superior efficacy compared with either agent on its own (Rovenska et al. 1999). One uncontrolled study conducted in humans in the 1960s suggested that bromelain might be of benefit in rheumatoid arthritis wherein addition of bromelain reportedly resulted in significant to complete decrease in soft tissue swelling in most cases (Cohen and Goldman 1964). In an open-labeled study, bromelain treatment of patients with knee osteoarthritis resulted in significant decrease in pain and stiffness (Walker et al. 2002). Likewise, treatment with Phlogenzym® or diclofenac similarly reduced pain and inflammation (Akhtar et al. 2004). In another 6-week trial, diclofenac or Phlogenzym® showed equal efficacy in reducing pain and joint stiffness of patients with osteoarthritis of the hip (Klein et al. 2006). In an open-labeled study of men with category III chronic prostatitis (nonbacterial chronic prostatitis and prostatodynia), 1 month of treatment with a combination of bromelain, papain, and quercetin resulted in an improvement of at least 25 % in symptom score in 14 out of 17 patients (Shoskes et al. 1999).

3.6.4 *Musculoskeletal Injuries*

Bromelain has been shown to speed healing from bruises and hematomas ([No Authors Listed] 2010). In one controlled study of boxing injuries, 58 out of 74 boxers who took bromelain had lost all signs of bruising within 4 days, compared to only 10 out of 72 in the placebo group (Blonstein 1969). In another study of musculoskeletal blunt injuries resulting in strains and torn ligaments, bromelain produced a reduction in swelling, pain at rest and during movement, and tenderness (Masson 1995). Bromelain, in combination with papain and fungal-derived proteases, seemed to reduce the damaging effects of unaccustomed exercise and accelerate recovery of muscle tissue (Buford et al. 2009). Likewise, a combination of bromelain, papain, trypsin, pancreatic enzymes, and other proteolytic substances attenuated soft tissue injury and soreness resulting from intense exercise (Miller et al. 2004).

3.6.5 Surgical Trauma

Administration of bromelain can alleviate postoperative complaints and complications ([No Authors Listed] 2010). Prophylactic use of bromelain can reduce the average number of days for complete disappearance of pain and inflammation post surgery (Tassman et al. 1965). Bromelain was reported to reduce post-operative swelling and edema after rhinoplasty (Seltzer 1962). Two trials suggested that bromelain might be effective in reducing swelling, bruising, and pain in women following episiotomy (Howat and Lewis 1972; Zatuchni and Colombi 1967).

3.6.6 Thrombotic and Ischemic Disorders

Evidence shows that bromelain decreases platelet aggregation (Glaser and Hilberg 2006; Heinicke et al. 1972), promotes fibrinolysis (De-Giuli Morghen and Pirota 1978), and inhibits thrombus formation (Metzig et al. 1999). In an animal model of ischemia/reperfusion injury, bromelain treatment increased aortic flow and reduced both the infarct size and the degree of apoptosis (Juhasz et al. 2008). In agreement, bromelain was helpful in reducing platelet aggregation (Heinicke et al. 1972) and managing angina pectoris [(Taussig and Batkin 1988)] and acute thrombophlebitis (Seligman 1969).

3.6.7 Burn Debridement

Topical bromelain has been used successfully for debridement of eschar tissues and acceleration of wound healing in burns (Houck et al. 1983; Rosenberg et al. 2004). Rapid debridement of third-degree burns considerably reduces the morbidity and mortality of severely burned patients. It permits early skin grafting and lessens the risk of sepsis. Chemical debridement, as opposed to surgical debridement, selectively removes the burned, denatured skin (Maurer 2001). A synergistic interaction between escharase and two proteolytic enzymes (ananain and comosain) is thought to be responsible for debriding activity of bromelain, resulting in an “enzymatic dissection” between the viable, native tissue and the non-viable, denatured tissue (Houck et al. 1983; Rowan et al. 1990). Such minimally invasive debridement leaves behind enough non-injured dermis that can epithelialize spontaneously, decreasing the need for excisional debridement and autografting. This efficacy laid the basis for the development of a bromelain-based preparation, named NexoBrid™ or Debrase™, for burn wound management (Rosenberg et al. 2012, 2014; Singer et al. 2010a, b). Following approval by Committee for Medicinal Products for Human Use (CHMP), the European Commission granted in 2012 a marketing authorisation valid throughout the European Union for NexoBrid™ (European

Medicines Agency 2012). Through a multi-center, open-label, randomized controlled trial, Rosenberg et al. recently reported that debridement with NexoBrid™ reduced the need for and the extent of surgery compared with the standard of care and offered comparable long-term results in patients with deep burns (Rosenberg et al. 2014).

3.6.8 Cancer

Initial studies by Gerard in 1972 (Gerard 1972) and Nieper in 1976 (Nieper 1976) reported on beneficial effects of oral bromelain in cancer patients. After long-term treatments with relatively high doses, they noted remarkable remissions of malignant tumors with negligible side effects. These reports, however, are considered anecdotal. Since then, anticancer properties of bromelain have been investigated in a variety of in vitro studies and preclinical settings (Table 4.1). Emerging evidence suggests that bromelain affects multiple molecular and cellular targets and has the potential to interrupt malignant growth as a result of direct impact on cancer cells and tumor micro-environment, as well as of regulatory effects on immune and haemostatic systems. These effects will be discussed in the following chapters. Experimental and anecdotal evidence, however, still need to be further investigated and are yet to be confirmed in clinical studies (Chobotova et al. 2010).

3.7 Safety and Tolerability

Bromelain is considered to have very low toxicity. Significant amount of bromelain, about 12 g/day, can be consumed without any major side effects (Castell et al. 1997) and daily doses from 200 up to 2000 mg (500–5000 FIP units) can be taken for long periods (Kelly 1996).

3.7.1 Acute Toxicology

According to Moss et al., no LD50 could be determined with oral doses up to 10 g/kg in mice, rats, and rabbits (Moss et al. 1963). LD50 after intraperitoneal administration to mice and rats were 37 and 85 mg/kg, respectively. After intravenous administration to mice and rabbits, LD50s of 30 and 20 mg/kg were reported. No immediate toxic reactions were observed (Maurer 2001). In humans, there is no evidence of toxicity at oral doses up to 12 g/day (Castell et al. 1997).

3.7.2 *Chronic Toxicology*

In rats, oral bromelain at a dosage of 500 mg/kg/day did not provoke any alteration in food intake, growth, histology of the heart, spleen, kidney, or hematological parameters in rats (Moss et al. 1963), and at 1500 mg/kg/day showed no carcinogenic or teratogenic effects. In dogs, doses up to 750 mg/kg/day showed no toxic effects after 6 months. In humans, no significant changes in blood coagulation parameters have been reported after giving bromelain (3000 FIP unit/day) for 10 days (Eckert et al. 1999).

3.7.3 *Side Effects*

In human clinical tests, side effects are generally not observed; however, caution is advised if administering bromelain to individuals with hypertension, since one report indicated individuals with pre-existing hypertension might experience tachycardia following high doses of bromelain (Gutfreund et al. 1978). Bromelain, as well as other proteolytic enzymes, can cause IgE-mediated respiratory allergies of both the immediate type and the late-phase of immediate type (Gailhofer et al. 1988).

Chapter 4

N-Acetylcysteine

4.1 History

N-acetylcysteine has been in clinical practice for several decades. Studies in the early 1960s demonstrated that thiol compounds had potent mucolytic properties (Ziment 1986). One of the most effective agents was L-cysteine, which is derived from hair and skin. L-cysteine, however, readily undergoes rapid oxidation, generating the inactive disulfide, cysteine. Acetylation of the L-cysteine N-terminus was found to confer sufficient stability to the molecule (Rushworth and Megson 2014). Except in very high concentrations, this derivative did not precipitate upon oxidation and indicated superior mucolytic activity, without toxicity (Reas 1963a). Accordingly, *N*-acetyl-L-cysteine, commonly known as *N*-acetylcysteine or NAC, was introduced in the 1960s as a mucolytic to reduce the viscosity of abnormal respiratory tract secretions in various tracheobronchial and bronchopulmonary diseases, including cystic fibrosis (CF) (Suddarth 1963; Zollinger and Williams 1964; Reas 1963a, b; Hurst et al. 1967). Following the commercial availability of a 20 % intravenous solution, its best known use as an antidote to paracetamol poisoning was revealed (Prescott et al. 1977). Since the 1980s, there has been a growing interest in the therapeutic potential of NAC as an antioxidant in a range of diseases where oxidative stress is seen to be a driver (Rushworth and Megson 2014). It is also sometimes used as a dietary supplement (nutraceutical), by athletes in particular. The widespread use of NAC is partly a function of its ready availability. Oral preparations are widely available without prescription from pharmacies and health food stores (Dodd et al. 2008).

Oral and aerosol preparations are licensed for use in some countries, but not worldwide (Rushworth and Megson 2014).

4.2 Manufacturing Process Summary

N-acetyl-L-cysteine ($\text{HSCH}_2\text{CH}(\text{NHCOCH}_3)\text{CO}_2\text{H}$) is the acetylated derivative of the naturally occurring amino acid L-cysteine with a molecular weight of 163.2 (Ziment 1988). It is manufactured by acetylation of L-cysteine hydrochloride monohydrate with acetic anhydride in an alkaline aqueous medium (Anton et al. 2003). NAC is also synthesized endogenously, with an average circulating concentration of 80 nM (Gabard and Mascher 1991).

4.3 Biochemical Properties

The biological activity of NAC is attributed to its sulfhydryl (thiol) group, while its acetyl-substituted amino group affords it protection against oxidative and metabolic processes (Bonanomi and Gazzaniga 1980; Sjodin et al. 1989). The acetyl group makes cysteine more water-soluble, and functions to speed absorption and distribution of orally ingested cysteine. The acetyl group also reduces the reactivity of the sulfhydryl group, making NAC less toxic and more resistant to oxidation than cysteine (Dekhuijzen 2004). NAC is thus a membrane-permeable aminothiols that can serve as a sulfhydryl group donor and a precursor to intracellular cysteine and glutathione. The concept for the historical use of NAC as a mucolytic was derived from the need to deliver reduced sulfhydryl moieties to effect the disruption of disulfide bridges within the glycoprotein matrix of mucus. After decades, NAC remains the treatment of choice for *N*-acetyl-*p*-aminophenol (paracetamol) toxicity. Likewise, the founding principle that underpinned this indication was NAC's potential as a sulfhydryl donor. Cleavage of the acetyl group is thought to reveal free, reduced Cys, which is available for incorporation into the highly abundant intracellular antioxidant, glutathione. Reduced glutathione (GSH) is a linear tripeptide (γ -glutamylcysteinylglycine) that is synthesized and maintained at high concentrations in cells. In its reduced form, GSH has a wide variety of functions, from antioxidant protection to protein thiolation and drug detoxification, often supported by specific enzymes (Meister and Anderson 1983). GSH is a critical intracellular antioxidant that helps to limit the impact of oxidative stress and to protect vital cellular components (lipids, proteins, DNA) against harmful peroxidation. The antioxidant effects of GSH rely on the presence of the free sulfhydryl group as a ready source of reducing equivalents to quench radical species. Hence, NAC replenishes hepatic GSH that has become depleted during drug detoxification process (Rushworth and Megson 2014).

4.4 Pharmacokinetics

NAC is rapidly absorbed after oral administration; however, significant first-pass metabolism by the small intestine and liver results in the incorporation of NAC into protein peptide chains and the formation of a variety of metabolites (Borgstrom et al. 1986; Rodenstein et al. 1978; Sheffner et al. 1966; Kelly 1998). Only 3 % of radioactively labeled NAC is excreted in the feces following oral administration, indicating an almost complete absorption of NAC and its metabolites. NAC's volume of distribution ranges from 0.33 to 0.47 L/kg (Holdiness 1991). Peak plasma levels occur 1–3 h after administration (Holdiness 1991; Borgstrom et al. 1986; De Caro et al. 1989). The plasma half-life of free NAC is estimated to be about 2.15 h, and virtually no NAC is detectable 10–12 h post-administration (De Caro et al. 1989). Although extensive hepatic metabolism results in a low bioavailability of 4–10 % for the unchanged molecule, oral administration of NAC appears to be clinically effective (Borgstrom et al. 1986). Rodenstein et al. administered labeled NAC orally to patients with respiratory disorders (Rodenstein et al. 1978). Their results indicated that concentrations of radioactivity in lung tissue were comparable with those of plasma. However, the percentage of free NAC, metabolites of NAC, and NAC bound in labile disulfide bridges to proteins accounted for 95 % of the radioactivity in lung tissue, whereas the majority of radioactivity in plasma (about 64 %) was firmly bound to proteins. Additionally, concomitant increases in non-protein and protein SH groups, and small molecular weight protein-bound thiols, are found in human plasma following oral administration of NAC (Kelly 1998). As such, although free NAC and GSH could not be identified in bronchoalveolar lavage (BAL) fluid after oral administration (Bridgeman et al. 1991; Cotgreave et al. 1987), an increase in cysteine and GSH levels in plasma and lung was reported (Bridgeman et al. 1991). Serum concentrations after intravenous administration of an initial loading dose of 150 mg/kg over 15 min are about 500 mg/L. A steady state plasma concentration of 35 mg/L (10–90 mg/L) was reached in about 12 h following the loading dose with a continuous infusion of 50 mg/kg over 4 h and 100 mg/kg over the next 16 h (Goldfrank et al. 1998).

4.5 Pharmacodynamics

NAC is a low-molecular-weight thiol (LMWT) with diverse effects (Fig. 4.1). It has been shown to interact with numerous signaling pathways. The molecular mechanisms by which NAC exerts its complex effects are not fully understood. As a source of SH groups, NAC can stimulate GSH synthesis, enhance glutathione-S-transferase activity, promote detoxification, and act directly on reactive oxidant radicals (De Vries and De Flora 1993). In comparison with other thiols, the uniqueness of NAC lies most probably in its capability to serve as a precursor of cysteine, to efficiently

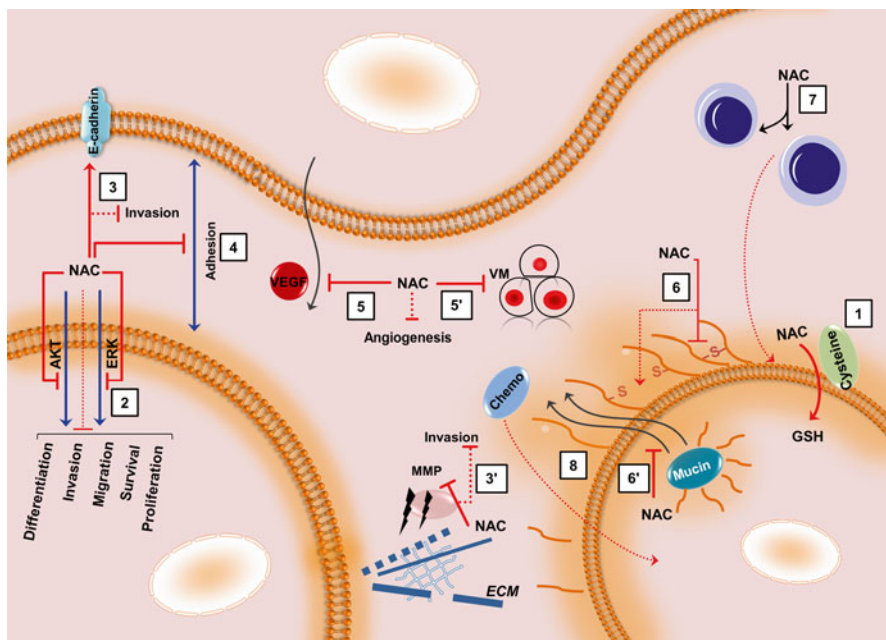


Fig. 4.1 NAC in cancer. NAC can affect different aspects of the pathophysiology of cancer. These include the role of NAC as an antioxidant (1), as well as in the inhibition of cancer cell survival, differentiation, proliferation, migration (2), invasion (3, 3'), adhesion (4), and tumor angiogenesis (5). It also exerts mucin-depleting effects by the cleavage of disulfide bonds (6) and decrease in mucin production (6'). NAC can also activate T cell-mediated cytotoxicity

reduce disulfide bonds in proteins and disrupt their structures, and to compete with larger reducing molecules in sterically less accessible spaces.

Biological effects of NAC can be classified as follows (Samuni et al. 2013; Rushworth and Megson 2014; Dodd et al. 2008):

4.5.1 Antioxidant activity

NAC exhibits direct and indirect antioxidant properties. NAC is a scavenger of reactive oxygen species (ROS), in particular $\cdot\text{OH}$, $\cdot\text{NO}_2$, $\text{CO}_3^{\cdot-}$, and thiyl radicals. However, it reacts neither with O_2 nor with NO , and relatively slowly with $\text{O}_2^{\cdot-}$, H_2O_2 and peroxynitrite. The principal role of NAC as a therapeutic antioxidant stems from its role as a precursor of cysteine. NAC administration is the most enduring delivery mode for cysteine (Rushworth and Megson 2014). Cysteine incorporation in GSH is the rate-limiting step in synthesis and replenishment of GSH cellular levels (Samuni et al. 2013). GSH is a critical intracellular antioxidant that helps to limit the impact of oxidative stress and to protect vital cellular

components (lipids, proteins, DNA) against harmful peroxidation. As well as acting as a direct “sacrificial” scavenger of potentially harmful ROS, GSH provides reducing equivalents to support the antioxidant activity of GSH peroxidases. The antioxidant effects of GSH, however, rely on the presence of the free sulfhydryl group as a ready source of reducing equivalents to quench radical species. On the other hand, the antioxidant potential of NAC is primarily in the provision of substrate for synthesis of intracellular GSH under conditions of oxidative stress, thus ineffectual once GSH has been replenished (Rushworth and Megson 2014).

4.5.2 Protein Modification

NAC is a potent reducing agent, stronger than GSH, cysteine, and cysteamine (Noszal et al. 2000). Hence, it can reduce disulfide bonds in proteins, thereby disrupting their ligand bonding and altering their structures (Samuni et al. 2013). As such, NAC cleaves disulfide bonds that crosslink glycoproteins in mucus (Dekhuijzen 2004). The cleavage of glycoprotein cross-linkages reduces viscosity by producing greater mucosal fluidity which in turn facilitates clearance of bronchial passages (Dodd et al. 2008). Other examples of NAC-induced protein modification are the following (Samuni et al. 2013): decrease in the angiotensin II receptor binding in vascular smooth muscle cells (Ullian et al. 2005); blocking TNF-induced signaling by lowering the cytokine affinity to the receptor (Hayakawa et al. 2003); reducing ligand binding capacity of betaglycan (Meurer et al. 2005); increasing c-Src cysteine reduced thiol content in cells, which primed the shift of the enzyme from the membrane into perinuclear endolysosomes (Krasnowska et al. 2008); and modifying the redox state of functional membrane proteins with exofacial SH critical for their activity (Laragione et al. 2003). Elevated levels of the amino acid homocysteine have been identified as a risk factor for cardiovascular (Trabetti 2008) and psychiatric disorders (McCaddon et al. 2001; Dittmann et al. 2007). NAC can act as a methyl donor in the conversion of homocysteine to methionine (Dodd et al. 2008).

4.5.3 Detoxification and Chelation

The toxicity of most quinones is attributed to their reduction to the corresponding semiquinone radicals, which are readily oxidized by oxygen forming $O_2^{\cdot-}$, and/or to their reaction with GSH leading to GSH depletion (O’Brien 1991). Hence, the effect of NAC on the detoxification of paracetamol (Lauterburg et al. 1983), doxorubicin (Powell and McCay 1988), and paraquat (Hoffer et al. 1996) might be attributed to NAC addition, in place of GSH, to *N*-acetyl-*p*-benzoquinone imine (NAPQI) (the toxic metabolite of paracetamol) and doxorubicin, to the reduction of the various semiquinone radicals to their corresponding

hydroquinones, and/or to an enhancement of GSH synthesis (Samuni et al. 2013). NAC is also known to have metal-chelating properties. Thiol groups present in NAC act to reduce free radical and provide chelating site for metals. Thus, NAC has a strong ability to restore the impaired prooxidant/antioxidant balance in metal poisoning (Flora 2009). NAC is capable of binding transition metal ions, such as Cu(II) and Fe(III) (Zheng et al. 2010), and heavy metal ions, such as Cd(II) (Jalilehvand et al. 2011), Hg(II) (Trumpler et al. 2009) and Pb(II) (Chen et al. 2012), primarily through its thiol side chain. Thus, by chelating toxic metal ions NAC forms complex structures, which are readily excreted from the body (Samuni et al. 2013).

4.5.4 Regulatory Effects on Cell Biology

4.5.4.1 Cell Cycle Progression and Apoptosis

Several studies have indicated regulatory effects of NAC on mitogenic activity and survival of eukaryotic cells in different contexts (Samuni et al. 2013). NAC was found to inhibit induction of cyclin D and DNA synthesis and to induce G1 arrest in response to phorbol ester in NIH 3 T3 cells (Huang et al. 1995). NAC also induced cyclin-dependent kinase inhibitors such as p16 and p21, independent of p53, which resulted in G1 arrest (Liu et al. 1999). NAC also inhibited DNA synthesis by and proliferation of pheochromocytoma PC12 cells (Yan and Greene 1998; Ferrari et al. 1995). Treatment of hepatic stellate cells with NAC resulted in sustained activation of ERK, Sp1 phosphorylation, induction of p21 expression, and G1-growth arrest (Kim et al. 2001). In cardiac fibroblasts, NAC inhibited ERK mitogenic activation and EGFR transactivation mediated by angiotensin II (Wang et al. 1998, 2000). In addition, a large body of evidence indicates that NAC plays an antiapoptotic role. NAC was shown to prevent apoptosis of serum-deprived neuronal cells (Ferrari et al. 1995), glutamate-induced apoptosis of oligodendrocytes, and TNF- α -induced apoptosis of fibroblasts (Mayer and Noble 1994) and U937 myelomonocytic cells (Cossarizza et al. 1995). Similar protective effect of NAC was also shown against O₂⁻-mediated apoptosis of selenite-treated HepG2 cells (Shen et al. 2001). NAC was also shown to protect against peroxynitrite-induced apoptosis by modulating levels of O₂⁻ and H₂O₂ (Lin et al. 1997), and to afford protection against cocaine-induced apoptosis by upregulating anti-oxidative enzymes such as manganese superoxide dismutase (Mn-SOD), Cu/Zn-SOD, glutathione peroxidase (Zaragoza et al. 2000), and catalase (Oh and Lim 2006). However, evidence has shown that the modulation of apoptosis afforded by NAC depends on both cell type and stimulus specificity and is thus very complex (Nargi et al. 1999). As such, NAC is also capable of inducing apoptosis in different cancer cell lines (Table 4.1).

Table 4.1 Cellular and molecular targets of NAC related to its anti-cancer activity

Study type	Treatment	Effect
<i>In vitro</i>		
Cells		
A2058, K1735, B16F10, C87LLC	NAC	↓ Invasion, ↓ gelatinase activity (Albini et al. 1995)
<i>P-3 T3, N-3 T3</i>	NAC	↓ TPA-mediated induction of cyclin D1 and DNA synthesis in P-3 T3 cells, ↔ TPA-induced inhibition of the cyclin E-associated kinase in N-3 T3 cells (Huang et al. 1995)
<i>RASMC, HASMC, HAEC</i>	NAC	↓ Cell viability, ↑ apoptosis (except for HAEC) (Tsai et al. 1996)
P308	NAC	↓ Cell viability, ↑ p53-mediated apoptosis (Liu et al. 1998)
<i>NIH 3 T3</i>	NAC	↓ Cell proliferation, ↓ cyclin D1 and DNA synthesis, ↑ blockage of cell cycle progression, ↓ MAP kinase pathway activation, ↔ PCNA (Sekharam et al. 1998)
<i>EAhy926, HUVEC</i>	NAC	↓ Chemotaxis, ↓ invasion, ↓ gelatinolytic activity (Cai et al. 1999)
RKO, RC10.1, SW480, HCT-116, 80S4	NAC	↓ Proliferation in a p53-independent manner, ↑ cell death, ↓ ROS (Nargi et al. 1999)
U373-MG	NAC	↓ Serum- and ROS-induced proliferation (Arora-Kuruganti et al. 1999)
P308	NAC	↑ Expression of the CDK inhibitors and prolonged G1 phase (Liu et al. 1999)
HeLa	NAC	↓ TNF-induced NF-κβ activity (Oka et al. 2000)
KS-Imm	NAC	↓ Chemotaxis, ↓ invasion, ↓ gelatinase activity, ↓ VEGF production, ↓ total VEGF mRNAs, ↔ MMP-2, or MMP-9 mRNA levels (Albini et al. 2001)
SGC-7901	NAC	↓ Cell growth (Li et al. 2001b)
T24	NAC	↓ Invasion, ↓ MMP-9 production, and activity (Kawakami et al. 2001)
C8161	NAC	↑ UV radiation-mediated apoptosis (Rieber and Rieber 2003)
MIA PaCa-2, A549	NAC	↑ Hypoxia-induced apoptosis (Qanungo et al. 2004)
<i>NHEK, Caco-2, OVCAR-3</i>	NAC	↓ Cell proliferation, ↑ differentiation (Parasassi et al. 2005)
<i>NHEK, Caco-2</i>	NAC	↓ Cell proliferation, ↑ differentiation with differentially expressed related genes (Gustafsson et al. 2005)
Jurkat	NAC	↑ MK886-induced apoptosis (Deshpande and Kehrler 2006)
SJ-89	NAC	↓ Cell growth, ↑ apoptosis, ↑ DNA synthesis arrest (Li et al. 2007b)

(continued)

Table 4.1 (continued)

Study type	Treatment	Effect
T24	NAC	↓ Cell proliferation, ↓ adhesion, ↓ migration, ↓ invasion (Supabphol et al. 2009)
PC3	NAC	↓ Cell proliferation, ↓ NF- κ B activity, ↑ transient activation of PI3K- and/or ERK-related intracellular signaling pathways, ↑ Cyr61 expression (Lee et al. 2011)
B16F10	NAC	↓ Growth, ↓ migration (Im et al. 2012)
DU145, PC3	NAC	↓ Cell proliferation, ↓ migration, ↓ invasion, ↓ adhesion (Supabphol and Supabphol 2012)
HSC-3, SCC-4	NAC	↓ Cell growth, ↑ apoptosis, ↑ cell cycle arrest, ↓ EGFR/AKT signaling activation (Lee et al. 2013a)
H1650, A549, H1792, H2106, H460, H358	NAC	↓ Cell proliferation, ↓ PDK1 expression, ↑ PPAR α expression (Hann et al. 2013)
Oncogene-expressing mouse fibroblasts, A549, H460, H838, H1975, H1299, H23, H411	NAC	↑ Cell proliferation (except for mutant p53 expressing cells including H1299, H23, H411 and H358), ↓ ROC, ↓ p53 (Sayin et al. 2014)
<i>In vivo</i>		
No cancer cell, urethane-induced lung carcinogenesis	NAC ^o	↓ Frequency of tumor-bearing animals and mean number of tumors (De Flora et al. 1986)
No cancer cell, DMH-induced colon carcinogenesis	NAC ^o	↓ Incidence of intestinal tumors and tumor yield (Wilpart et al. 1986)
No cancer cell, 2AAF-induced hepatocarcinogenesis	NAC ^o	↑ Delay in the development of GGT-positive foci, prevention of sebaceous squamocellular carcinomas of Zymbal glands (Cesarone et al. 1987)
B16F10	NAC pretreated cells	↓ Lung metastases (Albini et al. 1995)
B16F10, B16BL6, LLC	NAC ^o	↓ Weight of the locally formed primary tumor, ↑ delay in tumor formation, ↓ spontaneous metastasis by B16-F10 and B16-BL6 (Albini et al. 1995)
B16F10	NAC ^o	↓ Lung metastases (alone), synergistic effects with DOX in ↓ the frequency and weight of primary tumors and local recurrences, and ↓ lung metastases (De Flora et al. 1996)
L1210	NAC ^o	↓ Peritoneal tumor formation, ↑ TNF α -dependent T-cell cytotoxicity, resistance to re-inoculation (Delneste et al. 1997)
<i>Endothelial cells</i>	NAC ^o	↓ Neovascularization of the matrigel sponges in response to Kaposi's sarcoma cell products while preserving endothelial cells (Cai et al. 1999)
KS-Imm	NAC ^o	↓ Tumor growth, ↑ tumor regression, ↑ median survival time, ↓ VEGF production, ↓ Ki-67 proliferation marker (Albini et al. 2001)

(continued)

Table 4.1 (continued)

Study type	Treatment	Effect
HCT-15	NAC ^{ip}	↑ Efficacy of 5-FU, ↑ apoptosis, ↑ tumor necrosis, ↔ cell cycle kinetics, ↔ neovascularization (Bach et al. 2001)
B16F10	NAC ^{ip}	↓ Peritoneal tumor formation, ↓ intracellular ROS in peripheral blood mononuclear cells (Im et al. 2012)
No cancer cells, DEN-induced liver cancer model	NAC pretreated animals	↓ Carcinogenesis and progression of HCC, ↓ ROS/ER stress, ↓ the unfold protein response and inflammatory response in liver tissue, ↓ aggregation of p62 and Mallory-Denk bodies in the liver tissue (Lin et al. 2013)
HSC-3	NAC ^{ip}	↓ Colony formation and tumor size (Lee et al. 2013a)
No cancer cell, mouse models of B-RAF- and K-RAS-induced lung cancer	NAC ^o	↑ Tumor growth, ↓ survival, ↓ ROC, ↓ p53 (Sayin et al. 2014)
Clinical		
Head and neck cancer or NSCLC	NAC ^{o*}	↔ Survival, ↔ event-free survival, ↔ second primary tumors (van Zandwijk et al. 2000)

5-FU 5-fluorouracil, AKT protein kinase B, B-RAF proto-oncogene B-RAF, CDK cyclin-dependent kinase, Cyr61 cysteine-rich angiogenic inducer 61, DEN diethylnitrosamine, DMH dimethylhydrazine, DOX doxorubicin, ERK extracellular signal-regulated protein kinase, GGT gamma-glutamyl transpeptidase, HCC hepatocellular carcinoma, Ki-67 proliferating cell nuclear antigen, K-RAS proto-oncogene K-RAS, MAP kinases mitogen-activated protein kinases, MMP-2 or -9 matrix metalloproteinase-2 or -9, mRNAs messenger ribonucleic acid, NF- κ B nuclear factor kappa- β , NSCLC non-small-cell lung cancer, p53 tumor suppressor p53, PCNA proliferating cell nuclear antigen, PDK1 3-phosphoinositide-dependent protein kinase 1, PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase, PPAR α peroxisome proliferators activated receptor alpha, ROS reactive oxygen species, VEGF vascular endothelial growth factor

Cell lines: 80S4 human colorectal carcinoma, A2058 human melanoma cells, A549 human non-small-cell lung carcinoma cells, B16BL6 highly metastatic mouse melanoma cells, B16F10 mouse melanoma cells, C8161 human melanoma cells, C87LLC mouse Lewis lung carcinoma, Caco-2 colon adenocarcinoma, DU145 human prostate cancer cells, EAhy926 human endothelial cells, H1299 human non-small-cell lung carcinoma cells, H1650 human non-small-cell lung carcinoma cells, H1792 human non-small-cell lung carcinoma cells, H1975 human non-small-cell lung carcinoma cells, H2106 human non-small-cell lung carcinoma cells, H23 human non-small-cell lung carcinoma cells, H358 human non-small-cell lung carcinoma cells, H411 human non-small-cell lung carcinoma cells, H460 human non-small-cell lung carcinoma cells, H838 human non-small-cell lung carcinoma cells, HAEC human aortic endothelial cells, HASMC human aortic smooth muscle cells, HCT-15 human colorectal adenocarcinoma cells, HCT-116 human colorectal carcinoma cells, HeLa human cervical cancer, HSC-3 human tongue squamous carcinoma cells, HUVEC human umbilical vein endothelial cells, Jurkat human acute T-cell leukemia cells, K1735 mouse melanoma cells, KS-Imm human Kaposi's sarcoma cells, L1210 human lymphoma cells, MIA PaCa-2 human pancreatic cancer cells, N-3T3 subclone of the NIH 3T3 mouse embryonic fibroblasts, NHEK human normal keratinocytes, NIH 3T3 mouse embryonic fibroblasts, OVCAR-3 human epithelial ovarian cancer cells, P308 mouse papilloma cells, P-3T3 subclone of the NIH 3T3 mouse embryonic fibroblasts, PC3 human prostate cancer cells, RASMC rat aortic smooth muscle cells, RC10.1 human colorectal carcinoma, RKO human colon carcinoma, SCC-4 human tongue squamous carcinoma cells, SGC-7901 human gastric cancer cells, SJ-89 human signet ring (continued)

Table 4.1 (continued)

gastric cancer cells, *SW480* human colorectal adenocarcinoma cells, *T24* human bladder cancer cells, *U373-MG* human astrocytoma cells

Non-cancerous cells are shown in *italics*

^{ip}: intraperitoneal, ^o: oral *: in combination with vitamin A

4.5.4.2 Signal Transduction and Gene Expression

NAC affects redox-sensitive signal transduction and gene expression and directly modulates the activity of common transcription factors (De Flora et al. 2001). For example, while oxidative stress is an effective inducer of NF- κ B, NAC treatment suppressed NF- κ B activation and subsequent cytokine production (Kim et al. 2000b; Paterson et al. 2003). Likewise, NAC was shown to inhibit P450-dependent production of intracellular adhesion molecule-1 (ICAM-1), MMP-2, platelet-derived growth factor (PDGF), and VEGF (Zangar et al. 2011).

4.5.4.3 Cytoskeleton and Trafficking

NAC can also modulate cytoskeleton-dependent processes, including cell–cell interaction and intracellular trafficking. As such, NAC was found to improve adhesion properties of epithelial cells (Malorni et al. 1995) and restore tubulin dynamics and nuclear transport of NF- κ B in cultured neurons and developing fetal rat brain (Mackenzie et al. 2011). NAC was also reported to affect trafficking of intracellular proteins. Moreover, NAC treatment has been shown to improve mitochondrial functionality (Samuni et al. 2013).

4.5.5 Immunomodulation

A variety of experimental and clinical observations support the immunomodulatory activity of NAC (Samuni et al. 2013). These include reports on the enhancement of natural killer and T-cell function and delay in the CD4⁺ reduction in HIV patients (Akerlund et al. 1996; Breitzkreutz et al. 2000), improvement of phagocytic capacity, leukocytes chemotaxis, and natural killer cell function, and decrease in TNF- α and interleukin-8 (IL-8) levels in post-menopausal women (Arranz et al. 2008), and blockade of the mammalian target of rapamycin (mTOR) in T cells of patients with systemic lupus erythematosus (SLE) (Lai et al. 2012). Similar in vitro enhancement of T-cell growth and function (production of IL-2) was demonstrated when peripheral blood T cells were treated with NAC (Eylar et al. 1993). It also modulated both cellular and humoral immunity through downregulating the T-cell dependent B cell activation and inducing T helper cell type 1 (Th1) polarization, favoring cell-mediated immunity (Giordani et al. 2002).

4.5.6 *Effects on Malignant Growth*

By virtue of divergent biological functions, NAC has proven to be capable of affecting neoplastic growth in preventative, pre-neoplastic, and treatment stages (Rushworth and Megson 2014; De Flora et al. 1991b).

4.5.6.1 Chemoprevention

NAC has long been proposed as a chemopreventive agent in cancer. Early investigations by De Flora et al. demonstrated that NAC possesses antimutagenic and anticarcinogenic properties and is thus a promising chemopreventive agent (Cesarone et al. 1987; De Flora et al. 1985, 1986). Since then, extensive evidence of such a role of NAC has been accumulated. In playing this role, NAC blocks electrophilic metabolites and direct-acting compounds of either endogenous or exogenous source, attenuates several xenobiotic-metabolizing pathways, protects DNA and DNA-dependent nuclear enzymes, and prevents carcinogen-DNA adduct formation (De Flora et al. 1991b).

4.5.6.2 Effects on Cancer Cells

According to in vitro and in vivo investigations (Table 4.1), evidence for the regulatory effects of NAC on cancer cell biology in favor of malignant growth inhibition is well documented in the literature. To this end, NAC treatment of different cancer cell lines has been found to inhibit growth and proliferation, to regulate differentiation and adhesion, and to limit migration and invasion. Moreover, despite a large body of evidence supporting an anti-apoptotic role by which NAC can protect normal cells against cytotoxic stimuli, an increasing number of studies have indicated that NAC has the potential to exert opposite effects on cell survival, in particular promoting cancer cell apoptosis. All these cellular processes are key tasks underpinning tumor growth, progression, and metastasis. Inhibitory effects of NAC on cancer cell biology and their mechanistic basis will be discussed in Chap. 5.

4.5.6.3 Effects on Tumor Microenvironment and Angiogenesis

Inhibitory effects of NAC on malignant growth can also result from regulation of the tumor milieu and angiogenesis. As such, NAC decreases tumor invasiveness by inhibiting extracellular matrix degrading enzymes. For example, NAC was shown to reduce activity and production of matrix metalloproteinases, including MMP-2 and MMP-9 (Albini et al. 1995; Kawakami et al. 2001). NAC was also shown to inhibit angiogenesis through hampering endothelial cell activation, invasion, chemotaxis and gelatinolytic activity (Cai et al. 1999), and VEGF production (Albini et al. 2001; Redondo et al. 2000).

4.5.6.4 Immunomodulatory Effects

It is postulated that NAC, as an immunomodulator, has the potential to enhance immune response against cancer. In this regard, Delneste et al. identified a mechanism of action for NAC where it induced an early and sustained increase in the membrane expression of TNF- α on stimulated peripheral blood T-cells and also upregulated membrane TNF-RI and TNF-RII on tumoral cell lines and T-cells after stimulation (Delneste et al. 1997). As a result, NAC enhanced T-cell cytotoxicity against cancer cells both in vitro and in vivo. In agreement, Mantovani et al. reported that impaired functions of T-cells derived from cancer patients, including defective proliferative response to anti-CD3 and reduced expression of CD25 (IL-2R) and CD95 (Fas), was restored by NAC treatment in vitro which significantly enhanced T-cell response/function (Mantovani et al. 2000).

4.5.6.5 Enhancement of Chemotherapy

Evidence also shows that NAC improves the utility of chemotherapy through enhancing the cytotoxic effects of chemotherapeutic agents and/or protecting the host tissues against their toxic effects. This feature will be discussed in Chap. 5.

4.6 Potential and Actual Applications

4.6.1 Respiratory Diseases

NAC was first used in clinical medicine in the 1960s as a mucolytic agent in aerosolized formulations (Ziment 1986). Initial studies revealed that NAC is capable of reducing the viscosity of abnormal respiratory tract secretions in various tracheo-bronchial and bronchopulmonary diseases, with several applications in respiratory diseases, including CF (Hurst et al. 1967; Reas 1963a, b). Since GSH was shown to be elevated in CF sputum (Dauletbaev et al. 2004), NAC can also enhance antioxidant activity of GSH and afford protection against the neutrophil-driven generation of ROS that mediate the longer-term tissue damage and fibrosis in CF (Ratjen and Grasemann 2012). The potential benefits of NAC have also been evaluated in a number of other respiratory diseases, including chronic obstructive pulmonary disease (COPD) (Dekhuijzen 2004), which may be characterized, in part, by chronic mucus production, leading to an increased risk of infection (Rushworth and Megson 2014). NAC treatment was found to normalize the alterations in inflammatory cell activity induced by smoking (Eklund et al. 1988; Linden et al. 1988). Treatment with NAC has resulted in symptomatic improvement in COPD patients, reflected by decreased sputum viscosity and purulence and improved sputum expectoration, reduced the number of exacerbations and sick leave days in some, and decreased the

number of viral infections and airway bacterial colonization (Repine et al. 1997; [No Authors Listed] 1980, 1985; Boman et al. 1983). Mucolytic and antioxidant agents, including NAC, are mentioned in the guidelines of the American Thoracic Society and the European Respiratory Society, although their use is not formally recommended (Pierson 2006; Dodd et al. 2008). The presence of an imbalance between oxidants and antioxidants has laid the basis for the evaluation of NAC treatment in idiopathic pulmonary fibrosis (IPF). The IFIGENIA trial reported improvements in the 12-month vital capacity and carbon monoxide diffusing capacity in IPF patients prescribed high-dose oral NAC (600 mg three times a day) in addition to prednisolone and azathioprine (Demedts et al. 2005). Due to safety concerns, however, this combination therapy was not recommended in a more recent study (Raghu et al. 2012).

4.6.2 Poisoning

NAC is known as the specific antidote for paracetamol (acetaminophen) overdose. Paracetamol is one of the most frequent medications recognized in both accidental and intentional overdoses (Lai et al. 2006) and the leading cause of acute liver failure in hospitalized patients (Ostapowicz et al. 2002). Oral NAC has traditionally been used in the United States in the treatment of paracetamol toxicity. However, since oral administration of NAC is often associated with several difficulties, it has been filtered to generate an intravenous solution. This preparation, approved by the Food and Drug Administration (FDA) in 2004, has been used openly and safely with similar efficacy (Whyte et al. 2010). NAC has also regulatory authority approval as a second line agent for the treatment of acrylonitrile (Buchter et al. 1984) and methacrylonitrile (Peter and Bolt 1985) poisonings. Moreover, NAC has been used in metal poisoning on account of its strong ability to restore the impaired prooxidant/antioxidant balance as well as its metal-chelating properties (Flora 2009). NAC has shown chelating and detoxing activity for chromium, boron (Banner et al. 1986), arsenic (Flora 1999; Modi et al. 2006; Santra et al. 2007), lead (Neal et al. 1998; Ottenwalder and Simon 1987; Pande et al. 2001), gold, cadmium (Odewumi et al. 2011; Ottenwalder and Simon 1987), copper (Ozcelik et al. 2012), and mercury (Lash et al. 2005).

4.6.3 Contrast-Induced Nephropathy

Contrast-induced nephropathy (CIN) is a serious adverse condition that develops in response to contrast media administered to patients during radiological procedures. Apart from hemodynamic changes induced by contrast media, toxic effects on the renal tubules and resultant inflammation and necrosis have been implicated in the pathogenesis of CIN. In 2000, positive results from a clinical trial using NAC as

prophylaxis for CIN initiated a burst of research activity. Of 13 meta-analyses, seven reported beneficial effects of NAC, five determined that the data are inconclusive, and one concluded that NAC is ineffective in preventing renal damage (Millea 2009).

4.6.4 Circulatory Diseases

NAC appears to have several possible therapeutic roles in cardiovascular diseases (Kelly 1998; Rushworth and Megson 2014; Samuni et al. 2013). Oxidative stress is a key component in the atherogenic process resulting in the oxidation of lipids in low density lipoproteins (LDL) and rendering them recognizable to macrophages prior to ingestion and formation of resident foam cells in the vessel wall (Griendling and Alexander 1997). Oxidative stress also contributes to atherosclerotic plaque vulnerability and destabilization, thus converting chronic atherosclerosis into an acute thrombo-embolic disorder (Cominacini et al. 2015). As well as GSH depletion associated with atherosclerotic plaques, there is also evidence that GSH is depressed in platelets in conditions associated with increased risk of thrombosis, in particular diabetes (Mazzanti and Mutus 1997). NAC was shown to suppress the severity of experimental atherosclerosis in apolipoprotein E-deficient mice by decreasing $O_2^{\cdot-}$ levels and macrophage aggregation (Shimada et al. 2009). It also inhibited NF- κ B, MMP-2, and MMP-9 and suppressed the atherosclerotic plaque destabilization in the same model (Lu et al. 2011). In ischemia–reperfusion injury models, NAC showed protective effects, by direct scavenging of hydroxyl radicals and enhancing the coronary flow (Brunet et al. 1995), and improved cardiac function (Forman et al. 1988). Likewise, NAC was found to be neuroprotective in animal models of acute brain injury, promoting vascular dilation and restoring cerebrovascular responsiveness through inhibition of endothelin-1 (Ellis et al. 1991; Sury et al. 2006). NAC also potentiated nitroglycerin-induced reversal of platelet aggregation (Chirkov and Horowitz 1996). Clinically, NAC was found to reduce lipoprotein(a) levels (Gavish and Breslow 1991) and/or homocysteine (Bostom et al. 1996; Wiklund et al. 1996) and inhibited platelet-monocyte conjugation, a surrogate marker of cardiovascular risk, in patients with type 2 diabetes (Treweweke et al. 2012). In these patients, long-term treatment with a combination of L-arginine and NAC reduced blood pressure (Martina et al. 2008). Addition of NAC to nitroglycerin and/or streptokinase resulted in reduction of oxidative damage and improved left ventricular function in patients suffering from myocardial infarction (Arstall et al. 1995; Sochman et al. 1996).

4.6.5 Viral Infections

In general, low cysteine and GSH levels are found in human immunodeficiency virus (HIV)-positive individuals (Kelly 1998). Two key papers in 1990s reported that low GSH (Herzenberg et al. 1997) or thiol (Marmor et al. 1997) levels may be

used as a predictor of decreased survival in patients with HIV. A double-blind, placebo-controlled clinical trial reported that oral NAC could safely replenish whole blood GSH and T-cell GSH in patients with HIV infection (de Rosa et al. 2000). Studies on lymphocytes from patients with HIV infection or acquired immunodeficiency syndrome (AIDS) indicated that NAC has the potential to enhance antibody-dependent cellular cytotoxicity (Roberts et al. 1995) and T-cell colony formation from (Wu et al. 1989) in vitro. However, the therapeutic efficacy of NAC administration in these patients is still equivocal. In a double-blind, placebo-controlled trial by Akerlund et al., administration of NAC (800 mg) increased plasma cysteine levels to normal, and slowed the decline of the CD4+ lymphocyte count (Akerlund et al. 1996). In a subsequent study, however, they found that NAC combined with co-trimoxazole for primary *Pneumocystis carinii* prophylaxis in HIV sero-positive patients failed to replenish plasma cysteine or glutathione levels and did not significantly decrease the risk of adverse reactions to antibiotic therapy (Akerlund et al. 1997). Spada et al. randomized HIV-infected patients to antiretroviral treatment with adjunctive NAC or adjunctive placebo and found that NAC was superior to placebo as regards the stability of the hematocrit and CD4 cell count (Spada et al. 2002). Two years later, however, a similar trial conducted by the same group reported no significant difference between the two treatment groups (Treitinger et al. 2004). The usefulness of NAC for the treatment of influenza in a population of frail older adults was investigated in a double-blind, placebo-controlled study over a 6-month period (De Flora et al. 1997). Although frequency of seroconversion toward A/H1N1 Singapore 6/86 influenza virus was similar in the two groups, NAC treatment reduced both the frequency and severity of influenza-like episodes. Cell-mediated immunity continually improved in the NAC group as a whole, whereas immunity in the placebo group remained unchanged.

4.6.6 Neuropsychiatric Disorders

A number of small clinical studies and case reports have evaluated the utility of NAC in a variety of neuropsychiatric conditions (Rushworth and Megson 2014; Dodd et al. 2008). GSH depletion is a feature of a wide range of neurodegenerative disorders, including Alzheimer's disease (Johnson et al. 2012). In a small, 24-week trial of NAC in patients with Alzheimer's disease, outcome was not significantly different between NAC and placebo, but favored NAC (Adair et al. 2001). Oxidative stress has also been implicated in the pathology of several psychiatric diseases, including schizophrenia (Akyol et al. 2002), bipolar disorder (Andreazza et al. 2007), major depression (Bilici et al. 2001), and obsessive-compulsive disorder (OCD) (Kuloglu et al. 2002). In double-blind, placebo-controlled trials in schizophrenia, NAC was superior to placebo for improvement in Positive and Negative Symptoms Scale (PANSS) total, PANSS negative, PANSS general, Clinical Global Impression (CGI) severity, CGI improvement scores, akathisia (Berk et al. 2008a), and mismatch negativity (MMN) (Lavoie et al. 2008). In a 24-week, double-blind, placebo-controlled

trial, NAC was superior to placebo for improvement in depressive symptoms in bipolar disorder (Berk et al. 2008b). In a report of two cases of patients with severe monoamine oxidase inhibitor-resistant major depression, NAC augmentation of tranylcypromine was observed (Carvalho et al. 2013). In another case report, NAC similarly augmented the treatment of serotonin reuptake inhibitor-refractory OCD (Lafleur et al. 2006). In addition, evidence from a randomized controlled pilot trial supported the potential usefulness of NAC for treating irritability in children with autistic disorder (Hardan et al. 2012). Some studies have suggested that NAC is effective for the treatment of addictive behaviors. Baker et al. found that repeated cocaine treatment and withdrawal reduced extracellular glutamate in the nucleus accumbens of rats by decreasing the exchange of extracellular cysteine for intracellular glutamate (Baker et al. 2003). In this study, NAC, as a potential glutamatergic agent, restored extracellular glutamate concentration in the nucleus accumbens and therefore offers promise in reducing addictive behavior. In agreement, a double-blind placebo-controlled trial reported that NAC is well tolerated in healthy, cocaine-dependent individuals and may reduce cocaine-related withdrawal symptoms and craving (LaRowe et al. 2006). NAC was also shown in a pilot study to improve symptoms of pathological gambling in a majority of subjects (Grant et al. 2007).

4.6.7 Other Potential Applications

Some anecdotal studies have also reported the beneficial effects of NAC on ocular symptoms of Sjogren's syndrome (Walters et al. 1986), the clinical course of patients with progressive myoclonus epilepsy of the Unverricht-Lundborg type (Hurd et al. 1996), ovulation and pregnancy rates in polycystic ovary syndrome (Badawy et al. 2007; Rizk et al. 2005), the eradication of *Helicobacter pylori* infection (Gurbuz et al. 2005), and the prevention of postoperative complications (Sisillo et al. 2008; Zingg et al. 2007). The divergent biological functions of NAC have been drawing attention to its potential utility in the management of cancer. As discussed in the following chapters, a substantial body of experimental work has validated this assumption. Evidence supports anticancer activity of NAC both at the preventive and intervention stages. However, clinical data are still limited and results from few trials examining the efficacy of NAC in reducing the risk of cancer (Pendyala and Creaven 1995) or providing outcome benefits (van Zandwijk et al. 2000) have not been conclusive. Moreover, NAC has been shown to improve the utility of chemotherapy through enhancing the cytotoxic effects of chemotherapeutic agents and/or affording protection against their toxicity. This feature will be discussed in Chap. 5.

There are no recommendations for oral dosing with NAC and consequently a broad range of doses have been used in clinical trials. Mild nausea, vomiting, and diarrhea have been reported as dose-dependent side effects of oral NAC, suggesting that an upper dosage limit is readily achieved with oral administration and that this affects tolerability. In Australia, regulatory authorities have recommended a maxi-

mum dose of 1000 mg/day for “over-the-counter” preparations. Interestingly, in a 4-week open label trial, Mardikian et al. found no significant difference in side-effect profile between 1200, 2400, and 3600 mg/day oral doses of NAC, with all three doses tolerated equally well (Mardikian et al. 2007). In two studies, Berk et al. administered a daily dose of 2 g of NAC (1000 mg twice daily) with few adverse effects (Berk et al. 2008a, b).

4.7 Safety and Tolerability

NAC is of low toxicity and a naturally occurring compound in human and animal bodies. When administered orally, it undergoes extensive first-pass metabolism to cysteine and other endogenous substances without toxicological relevance (The European Agency for the Evaluation of Medicinal Products).

4.7.1 Acute Toxicology

LD50 of NAC is >10 g/kg in mice and rats when administered orally. LD50 of parenteral NAC is 4.6 g/kg in mice and 2.8 g/kg in rats (Johnston et al. 1983). Probable oral lethal dose in humans is 5–15 g/kg (Gosselin et al. 1984).

4.7.2 Subacute and Chronic Toxicology

NAC oral doses of up to 2 g/kg/day for 4 weeks and up to 1 g/kg/day for 12–28 weeks in rats, and a dose of 300 mg/kg/day for 52 weeks in dogs did not affect behavior, body weight gain, hepatic and renal function, hematology, prothrombin, and bleeding time. Necropsy findings and histological examinations revealed no evidence of pathological lesions (Bonanomi and Gazzaniga 1980; Johnston et al. 1983). Likewise, a daily oral dose of 1 g/kg for 18–24 months was devoid of detrimental effects in both rats and mice (Johnston et al. 1983). No teratogenic or mutagenic activity or detrimental effects on delivery, physical development, or lactation were reported (Bonanomi and Gazzaniga 1980; Johnston et al. 1983).

4.7.3 Side Effects

NAC has a benign side effect profile that does not differ significantly from placebo in most clinical trials. The most common side effects are mild gastrointestinal symptoms (Dodd et al. 2008). At doses lower than 2.5 g/day, side effects are unusual,

but may include nausea, vomiting, diarrhea, transient skin rash, flushing, epigastric pain, and constipation (Atkuri et al. 2007). Side effects have been more noticeable at doses higher than 3 g/day and with intravenous administration. Anaphylactoid reactions, including rash, pruritus, angioedema, bronchospasm, tachycardia, and hypotension, are among the most commonly reported adverse effects of intravenous NAC in paracetamol overdose (Heard 2008). Deaths have been reported from accidental overdose of intravenous NAC (Mant et al. 1984) due to the reduction in hemostatic proteins (Knudsen et al. 2005). During the synthesis of NAC some related substances, including L-cysteine, L-cysteine, *N*, *N'*-diacetylcysteine, and *N,S*-diacetylcysteine are formed and may contribute to the adverse effect profile (Dodd et al. 2008).

Chapter 5

A Novel Approach to Peritoneal Dissemination of Mucin-Expressing Malignancies of Gastrointestinal Origin

5.1 Cytotoxic Effects of Bromelain and *N*-acetylcysteine in Single Agent and Combination Treatment of Human Gastrointestinal Carcinoma Cell Lines, In Vitro

5.1.1 Introduction

Colorectal (CRC) and gastric cancer (GC), respectively, are the third and fourth most commonly diagnosed cancers in males, and the second and fifth in females, accounting for 8.4 and 8.8 % of all deaths from cancer worldwide (Torre et al. 2015). In Australia, CRC represents the second most common cancer in both men and women, accounting for 9.3 % of all cancer deaths (AIHW 2015a, b) and 12–13 % of the burden of disease due to cancer (AIHW and AACR 2012). Gastrointestinal carcinomas have the potential to disseminate throughout the peritoneal cavity. Peritoneal implants are present in 20–30 % of patients with GC and 10 % of CRC cases at diagnosis (Goldstein et al. 2005). Peritoneal carcinomatosis is considered as an advanced stage in the natural history of gastrointestinal cancers and also a frequent finding in the recurrent disease, with median survival of 3–7 months for patients treated with palliative intent (Chu et al. 1989; Sadeghi et al. 2000). In contrast to lymphatic and hematogenous metastasis, however, peritoneal

dissemination can remain confined to the peritoneal cavity where the peritoneum is believed to serve as the first line of defense against tumor progression (Sugarbaker 2007). Hence, a curative approach using cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) (Sugarbaker 1991, 2001) has brought about long-term benefits to select patients with peritoneal surface malignancies (PSMs), including PMP (Chua et al. 2012c). This combined therapy is also a promising approach to peritoneal carcinomatosis of gastrointestinal origin (Sugarbaker 2012). With an established Peritoneal Surface Malignancy Program since 1996, St George Hospital (University of New South Wales, Sydney, Australia) is among the centers that specialize in administering this strategy (Chua et al. 2009a, 2010c). Nevertheless, despite this multidisciplinary approach, peritoneal malignancies recur and their management remains challenging. In this regard, evidence shows that HIPEC often fails to maintain the surgical complete response (Sugarbaker and Bijelic 2012). Moreover, it is believed that disruption of the peritoneal barrier and iatrogenic implantation of cancer cells also contribute to recurrence (Sugarbaker 2007). Thus, novel modalities are required for the enhancement of microscopic cytoreduction. To this end, our research group at St George Hospital has sought innovative ways to improve current therapeutic benefits in patients with peritoneal malignancies. In an attempt to develop a safe, novel formulation with cytotoxic effects on cancer cells, we studied in the present project the potential value of two natural agents with good safety profiles, bromelain (BR) and *N*-acetylcysteine (NAC), for prospective locoregional strategies. In so doing, cytotoxic and growth-inhibitory effects of these agents were evaluated in *in vitro* and *in vivo* models. As described in this section, we first explored whether and how single agent and combination therapy with BR and NAC affect growth, proliferation, and survival of a panel of human gastrointestinal cell lines, *in vitro*.

5.1.2 Results

5.1.2.1 BR and NAC, on Their Own, Significantly Inhibited Proliferation of Human Gastric and Colon Carcinoma Cells

First, using a range of concentrations of BR (5–600 $\mu\text{g/mL}$) and NAC (1–100 mM), antiproliferative effects of each agent, individually, after 72 h treatment of a panel of human gastrointestinal carcinoma cell lines were evaluated by sulforhodamine B (SRB) assay. Our data showed that BR significantly inhibited proliferation of MKN45 ($p=0.0018$, 0.0010, 0.0002, and <0.0001 for concentrations of 100, 200, 400, and 600 $\mu\text{g/mL}$, respectively) and KATO-III ($p<0.0001$ for concentrations ≥ 100 $\mu\text{g/mL}$) human gastric carcinoma cells, as well as of HT29-5 F12, HT29-5 M21 ($p<0.0001$ for concentrations of 40 and 50 $\mu\text{g/mL}$), and LS174T ($p<0.0001$ for concentrations ≥ 30 $\mu\text{g/mL}$) human colon carcinoma cells in a concentration-dependent manner. Similarly, NAC was found to significantly inhibit proliferation of MKN45 ($p=0.0006$ and 0.0037 for concentrations of 5 and 10 mM, respectively,

and $p < 0.0001$ for concentrations of 25, 50, and 75 mM) and KATO-III ($p = 0.0071$ and 0.0004 for concentrations of 25 and 50 mM, respectively, and $p < 0.0001$ for concentrations of 75 and 100 mM) cells. It also exerted significant antiproliferative effects on HT29-5 F12 ($p = 0.0003$ for concentration of 10 mM and $p < 0.0001$ for concentrations ≥ 25 mM), HT29-5 M21 ($p = 0.0091$ and 0.0008 for concentrations of 5 and 10 mM, respectively, and $p < 0.0001$ for concentrations ≥ 25 mM), and LS174T ($p = 0.0026$ for concentration of 10 mM and $p < 0.0001$ for concentrations ≥ 20 mM) cells, all in a concentration-dependent manner (Amini et al. 2014a).

5.1.2.2 Fifty Percent Inhibitory Concentration Analysis

Based on the results from the single agent treatment, the concentration-response curves were then constructed and 50 % inhibitory concentration (IC₅₀) values for BR and NAC were accordingly calculated. The highest BR IC₅₀ values were recorded for KATO-III and MKN45 gastric cancer cells (142.90 and 94.20 $\mu\text{g/mL}$, respectively). By comparison, BR IC₅₀ values for the three CRC cells were much lower (27.76, 30.02, and 34.60 $\mu\text{g/mL}$ for LS174T, HT29-5F12, and HT29-5M21 cells, respectively). Whereas the highest NAC IC₅₀ value (57.74 mM) was also revealed for KATO-III cells, our data indicated similar, relatively low NAC IC₅₀ values for the remaining cells (15.92, 15.40, 15.99, and 22.49 mM for MKN45, HT29-5F12, HT29-5M21, and LS174T cells, respectively).

5.1.2.3 Combined Use of BR and NAC Resulted in Significantly More Potent Growth-Inhibitory Effects

Subsequently, the effect of BR and NAC on cell proliferation was examined in combination therapy. Based on the results from the single agent study, we treated each cell line with three selected concentrations of each agent individually as well as with nine possible combinations of the two. Our data revealed that in most combination groups, growth-inhibitory effects resulted were significantly more potent than those induced by single agent BR or NAC. As such, BR 50 $\mu\text{g/mL}$ + NAC 10 mM, BR 75 $\mu\text{g/mL}$ + NAC 5 and 10 mM, and BR 100 $\mu\text{g/mL}$ + NAC 5 and 10 mM inhibited MKN45 cell proliferation more significantly than did BR and NAC on their own. Also, NAC 1 mM more significantly inhibited the cell proliferation when combined with BR 75 and 100 $\mu\text{g/mL}$. Similarly, BR 50 $\mu\text{g/mL}$ + NAC 10 mM, BR 75 $\mu\text{g/mL}$ + NAC 5 and 10 mM, and BR 100 $\mu\text{g/mL}$ + NAC 1, 5 and 10 mM inhibited proliferation of KATO-III cells more significantly than did BR and NAC, individually (Amini et al. 2014a).

In HT29-5F12 cells, as compared to single agent BR and NAC, BR 5 $\mu\text{g/mL}$ + NAC 5 and 10 mM, BR 10 $\mu\text{g/mL}$ + NAC 10 mM, and BR 20 $\mu\text{g/mL}$ + NAC 5 and 10 mM more significantly inhibited the cell proliferation. Also, BR 10 $\mu\text{g/mL}$ more significantly inhibited the cell proliferation when combined with NAC 5 mM. Similarly, BR 5, 10, and 20 $\mu\text{g/mL}$ in combination with NAC 5 and 10 mM

more significantly inhibited HT29-5M21 cell proliferation than did the single agent BR and NAC. Finally, BR 20 $\mu\text{g/mL}$ + NAC 10 and 20 mM, and BR 30 $\mu\text{g/mL}$ + NAC 20 mM inhibited LS174T cell proliferation more significantly than did BR and NAC on their own. Also, NAC 5 and 10 mM more significantly inhibited the cell proliferation when combined with BR 30 $\mu\text{g/mL}$, so did NAC 20 mM when used in combination with BR 10 $\mu\text{g/mL}$.

5.1.2.4 Synergy Was the Predominant Pattern of BR-NAC Interaction in Combination Therapy

By the median effect analysis, we next investigated how BR and NAC interacted with each other in combination therapy. To this end, the combination indices (CIs) were calculated based on the drug concentration and cell viability. In MKN45 cells, the interaction between the drugs changed from antagonism, observed only with the lowest concentration of BR (BR 50 $\mu\text{g/mL}$ in combination with NAC 1, 5 and 10 mM), to additivity and synergism as the bromelain concentration increased. In KATO-III cells, synergistic effects were evident in all treatment groups, except for two groups with the lowest concentration of NAC (additive and antagonistic effects when NAC 1 mM used in combination with bromelain 50 and 100 $\mu\text{g/mL}$, respectively) whereby consistent strengthening of the synergy accompanied higher concentrations of BR and NAC. Apart from the antagonistic effects observed in two groups with the lowest concentration of NAC (NAC 1 mM in combination with BR 5 and 10 $\mu\text{g/mL}$), the interaction between the two drugs in the treatment of HT29-5F12 cells was revealed to be synergistic, with a slight strengthening in higher concentrations. With no antagonism and only one additive pattern observed with the lowest concentrations of BR and NAC, drug-drug interaction in the treatment of HT29-5M21 cells was mainly synergistic, with a fairly slight fluctuation in the potency following rises in concentrations. Finally, in the LS174T treatment groups, increases in BR concentrations were associated with progressing interaction with NAC, from antagonism (BR 10 $\mu\text{g/mL}$ combined with NAC 10 and 20 mM, and BR 20 $\mu\text{g/mL}$ combined with NAC 5 mM) to additivity and slight synergism. On the other hand, with the exception of one instance (BR 20 $\mu\text{g/mL}$ with NAC 5 mM), rises in NAC concentrations resulted in the weakening of interaction with a given BR concentration.

5.1.2.5 Antiproliferative Effects of BR/NAC Are Mediated by Caspase-Dependent Apoptosis, with Likely Contribution of Autophagy and Cell Cycle Arrest

To explore the mechanistic basis for the antiproliferative effects of BR and NAC, we hypothesized that BR/NAC treatment promotes cell death and/or interferes with cell cycle progression in gastrointestinal carcinoma cells. To test this hypothesis, we first investigated the presence of apoptosis in BR/NAC-treated MKN45

cells by fluorometric TdT-mediated dUTP nick-end labeling (TUNEL) assay. Untreated and DNase I-treated cells were included in the experiment as the negative and positive controls, respectively. Cells were treated for 48 h with two concentrations of BR, two concentrations of NAC, and four combinations of the two. As anticipated, apoptotic bodies as an indication of DNA fragmentation were detected in treated cells. When we conducted the same experiment using LS174 cells, similar results were observed.

To further explore the underlying mechanisms of effect, we then analyzed the expression of a variety of proteins involved in the initiation, execution, and regulation of apoptosis. In so doing, after single agent and combination treatment of MKN45 and LS174T cells with different concentrations of BR and NAC for 48 h, we performed Western blot analysis of the protein expression on treated cells, as well as on their untreated counterparts used as the negative control. Treatment was found to induce caspase-dependent apoptosis evident as the emergence of the immunoreactive subunits of caspase-3, caspase-7, and caspase-8, withering or cleavage of procaspase-9, and the overexpression of cytochrome c. The functionality of activated caspase-3 was also confirmed by the emergence of the cleaved poly ADP ribose polymerase (PARP). Meanwhile, the expression of anti-apoptotic Bcl-2 and phosphorylation of prosurvival Akt were shown to be attenuated. Next, to explore the likely activation of autophagy as a contributor to cell death, we examined the expression of a number of the related proteins in response to the treatment. The results indicated an increase in the expression of the autophagosomal marker LC3-II along with the deregulation of the expression of other autophagy-related proteins, in particular Atg3, Atg7, Atg12, and Beclin-1. Finally, we evaluated the influence of the treatment on the expression of cyclins, the regulatory proteins that are expressed in, and control the progression of, different phases of cell cycle. As detected by Western blot, the diminished expression of cyclins D, A, and B were revealed in the treated cells. In general, the above findings were more prominent in combination therapy. Collectively, it became evident that caspase-dependent apoptosis is activated in response to BR/NAC to inhibit the proliferation of the cancer cells studied. In this regard, autophagy and cell cycle arrest appeared to contribute to the inhibitory effects observed.

5.1.3 Discussion

BR is a crude, aqueous extract from the pineapple plant comprised of sulfhydryl-containing proteolytic enzymes as well as of non-proteolytic constituents. Pineapples and BR have a history of folk and modern medicinal use. BR supplements have been promoted as an alternative or complementary medicine and are thought to have anti-inflammatory, immunomodulatory, and anti-coagulative properties. Benefits of BR in a range of health problems, including inflammation, edema, injuries, infections and burns, have been reported in the literature (Chap. 3). NAC is the acetylated derivative of the naturally occurring amino acid L-cysteine.

As a membrane-permeable aminothiols and sulfhydryl group donor, NAC is recognized as a precursor to intracellular cysteine and glutathione that functions as a nucleophilic reactive oxygen species (ROS) scavenger and antioxidant. It was historically described as a mucolytic and an antidote for acetaminophen overdose hepatotoxicity. More recently, the potential utility of NAC, mainly based on its antioxidative or ROS-scavenging properties, has been studied in a variety of pathological conditions as reviewed in Chap. 4.

As inexpensive, natural products with good safety profiles, BR and NAC have been investigated for their potential use in cancer therapy, too. In this regard, early, anecdotal observations reported the use of orally administered BR in cancer patients (Gerard 1972; Nieper 1976), and chemoprotective (Kline et al. 1973) or chemopreventive (De Flora et al. 1984, 1985) potential of NAC. Since then, anticancer properties of BR and NAC have been variably documented. Nevertheless, not much is known about their effects on divergent types of human cancer cells of gastrointestinal origin, as well as on mucins produced by MUC-expressing gastrointestinal cells. More importantly, to the best of our knowledge, combined use of these two agents in cancer research has not been reported by other groups. We aimed in the first part of this project to examine the efficacy of BR and NAC in both single agent and combination treatments of the *in vitro* models of human gastrointestinal cancer. To provide a more comprehensive and reliable overview of the treatment efficacy and spectrum, we selected a panel of cell lines with distinctive histopathological features and differential chemosensitivity. MKN45 and KATO-III represent two different types of the GC cells. While MKN45 has been isolated from a poorly differentiated adenocarcinoma of medullary type with the nature of both ordinary gastric and metaplastic intestinal mucosa, KATO-III has been derived from a unique subtype classified as signet-ring cell carcinoma (SRCC) (Sekiguchi et al. 1978; Motoyama et al. 1986). As regards the colon cancer cell lines used, HT29-5F12 (Leteurtre et al. 2004) and HT29-5M21 (Lesuffleur et al. 1998) represent two subpopulations of the HT29 colon adenocarcinoma cell line that are resistant to 5-fluorouracil (5FU) and methotrexate, respectively, and LS174T is an adenocarcinoma cell line with a goblet cell-like phenotype (Kuan et al. 1987; Tom et al. 1976). Moreover, these cells exhibit different expression phenotypes with respect to the gastrointestinal secreted mucins. MKN45 and KATO-III both represent a predominantly gastric phenotype and specifically express MUC5AC (Matsuda et al. 2008). Of the two HT29 clones, HT29-5F12 mainly expresses the intestinal mucin MUC2 whereas HT29-5M21 expresses MUC5AC and MUC5B (Leteurtre et al. 2004). Finally, LS174T cells express the three goblet cell-specific secreted mucins MUC2, MUC5AC, and MUC6 (van Klinken et al. 1996). The mucin-expressing profile is of particular significance in the pathophysiology of the peritoneally disseminated gastrointestinal malignancies and represents another aspect of the present research that will be addressed later.

It was found in the present study that BR and NAC, individually, are capable of inducing growth-inhibitory and cytotoxic effects in different human gastrointestinal carcinoma cell lines. IC₅₀ values ranged between 21 and 147 $\mu\text{g/mL}$ for BR, and between 11 and 60 mM for NAC, with the highest IC₅₀ values of either being

recorded for KATO-III cells. This indicates phenotype-dependent sensitivity of the cancer cells studied to the BR/NAC treatment. More interestingly, BR and NAC were found to interact in a predominantly synergistic or additive manner when used in combination, resulting in the enhancement of their efficacy and thus left-sided shift of the concentration-response curves. With regard to BR, growth-inhibitory activity of BR on cancer cells was documented for the first time by Taussig et al. (1985). According to their reports, BR had induced the concentration-dependent inhibition of cell growth, apparently independent of its proteolytic properties, in mouse tumor cell lines, including Lewis lung carcinoma, YC-lymphoma, and MCA-1 ascitic tumor cells (Taussig et al. 1985), as well as in KATO-III cells [Taussig et al., Proceedings of Southwest Oncology Group Meeting (1985); Oishi et al., Proceedings of Coulter Electronic Flow Cytometry Meeting (1985): referenced in (Batkin et al. 1988a; Taussig and Batkin 1988)]. Meanwhile in a separate study, Maurer et al. found that BR was capable of inducing the differentiation of both murine and human leukemia cells and proposed this phenomenon as an explanation for cytostatic effects of BR (Maurer et al. 1988). Later, Garbin et al. indicated growth-inhibitory effects of BR, in a concentration-dependent manner, on various human cancer cell lines, including MCF-7 breast cancer, KB squamous carcinoma, and SK-MEL-28 melanoma cells (Garbin et al. 1994). Grabowska et al. reported later that BR not only inhibited the proliferation of B16F10 mouse melanoma cells but also suppressed invasive and metastatic capacities of the cancer cells (Grabowska et al. 1997). In a study on a panel of human glioma cell lines, Tysnes et al. found that BR significantly, but reversibly, reduced glioma cell adhesion, migration, and invasion, yet did not affect cell viability (Tysnes et al. 2001). This is an indication that BR differentially influences key biological processes in different cancer cell types. In these two studies, the inhibitory effect of BR on invasive behavior of cancer cells was correlated with CD44 attenuation. Growth-inhibitory and cytotoxic effects of BR have been confirmed in more recent studies on breast carcinoma (Bhui et al. 2010; Paroulek et al. 2009; Dhandayuthapani et al. 2012), melanoma, epidermoid carcinoma (Bhui et al. 2012), malignant peritoneal mesothelioma (MPM) (Pillai et al. 2014b), and CRC cells, too. With respect to the gastrointestinal cancers, our preliminary results from BR treatment of different gastric and colon carcinoma cell lines (Amini et al. 2013a) were recently confirmed by a study on Caco-2 colon adenocarcinoma cells (Romano et al. 2014). As an agent with anticancer activity, BR targets cancer cells and their microenvironment per se and also exerts modulatory effects on the immune, inflammatory, and hemostatic systems (Chobotova et al. 2010). It has also shown to play chemopreventive roles in skin (Bhui et al. 2009; Goldstein et al. 1975; Kalra et al. 2008) and colon (Romano et al. 2014) tumorigenesis. Further, there is evidence that it has the potential to sensitize cancer cells to cytotoxic effects of chemotherapeutic agents. Chemosensitizing and chemopreventive properties of BR will be revisited in the next section (Sect. 5.2).

Evidence shows that NAC is also capable of inhibiting cancer cell growth, viability, and invasiveness, *in vitro*. In an initial study using human (A2058) and murine (K1735 and B16-F10) melanoma cell lines and murine Lewis lung carcinoma cells (C87 variant), Albini et al. indicated that NAC inhibited chemotactic and invasive

activities of the cancer cells, probably due to inhibition of matrix metalloproteinase (MMPs), including gelatinases (Albini et al. 1995). In a separate study, they showed that NAC is also capable of inhibiting angiogenesis through hampering endothelial cell activation, invasion, chemotaxis, and gelatinolytic activity (Cai et al. 1999). In agreement, they later reported that NAC reduced chemotaxis and invasion of KS-Imm cells, an immortalized cell line isolated from the highly vascularized human tumor Kaposi's sarcoma, and inhibited matrix metalloproteinase-2 (MMP-2) activity and vascular endothelial growth factor (VEGF) production (Albini et al. 2001). Similarly, NAC inhibition of VEGF production by melanoma cell lines was reported by Redondo et al. (2000). Through an initial study on human CRC cell lines HCT-116 and HCT-15, Chinery et al. indicated that antioxidants have the potential to inhibit malignant cell growth and viability, in which p53-independent induction of the cyclin-dependent kinase (CDK) inhibitor p21^{WAF1/CIP1} via CAAT/enhancer binding protein β (C/EBP β) was implicated (Chinery et al. 1997). Using another CRC cell line (HCA-7), they consistently reported later the NAC-induced inhibition of cell growth in a concentration-dependent manner (Chinery et al. 1998). Similarly, Nargi et al. demonstrated antiproliferative effects of NAC on a range of CRC cell lines where it either inhibited cell growth or induced cell death (Nargi et al. 1999). Arora-Kuruganti et al. found that NAC inhibited serum- and H₂O₂-induced proliferation of human U373-MG astrocytoma cells (Arora-Kuruganti et al. 1999). In two separate reports, Li et al. have documented growth-inhibitory and proapoptotic effects of NAC on human GC cell lines SGC-7901 (Li et al. 2001b) and SJ-89 (Li et al. 2007b). Kawakami et al. observed in T24 human bladder cancer cells that NAC was capable of limiting the cancer cell invasion by inhibiting the MMP-9 production and activity (Kawakami et al. 2001). Likewise, Fujiyama et al. found that NAC treatment of an invasive human bladder cancer cell line (EJ28) significantly inhibited cell invasion into the rat stroma (Fujiyama et al. 2001). The inhibitory effects of NAC on human bladder cancer cells were also reported by Supabphol et al. in which NAC was found to reduce cell proliferation, adhesion, migration, and invasion (Supabphol et al. 2009). In a study by Parasassi et al., NAC treatment of human normal keratinocytes (NHEK) and human cancer cell lines of colon (Caco-2) and ovarian origin (OVCAR-3) resulted in a dose-dependent inhibition of cell proliferation along with the induction of cell differentiation (Parasassi et al. 2005). In parallel, global gene expression analysis of the NAC-treated NHEK and Caco-2 cells revealed a number of differentially expressed transcripts with connections to the inhibition of cell growth and the induction of cell differentiation (Gustafsson et al. 2005). Using ARCaP human prostate cancer cells, Barnett et al. reported that NAC treatment partially reversed Snail-mediated epithelial-mesenchymal transition (EMT), characterized by increased E-cadherin levels and decreased ERK activity, thereby reducing prostate cancer cell aggressiveness (Barnett et al. 2011). In agreement, Supabphol and Supabphol reported that NAC inhibited proliferation, adhesion, migration, and invasion of human prostate cancer cells DU145 and PC3 (Supabphol and Supabphol 2012). Recently, NAC was found to inhibit growth and viability of human hepatocellular (HCC) (Kretzmann et al. 2012) and tongue squamous carcinoma cells (Lee et al. 2013a), too.

As with BR, while the overall body of evidence supports the inhibitory effects of NAC on cancer cell growth and progression, it appears that different cancer cell types or those of the same type but with different phenotypes or genetic backgrounds respond differentially, or even selectively, to NAC. In the study by Parasassi et al., the three cell types used were differentially influenced by anti-proliferative and pro-differentiating effects of NAC, and apoptotic cell death was not evident (Parasassi et al. 2005). In another study on human prostate cancer cells, Lee et al. reported antiproliferative effects of NAC on PC3 cells (Lee et al. 2011). However, NAC treatment caused an early, transient activation of Akt and Erk1/2 along with an increase in the expression of Cyr61, an extracellular matrix-associated signaling protein with pro-survival activity (Lee et al. 2013b). In two independent studies, NAC treatment of human lung cancer cells yielded opposite results. In the first study, Hann et al. reported antiproliferative effects of NAC on non-small cell lung carcinoma (NSCLC) cells (Hann et al. 2013). In contrast, Sayin et al. reported thereafter that growth and proliferation of K-RAS- and B-RAF-mutated mouse lung tumor cells and human lung cancer cell lines with wild-type p53, but not their counterparts with mutant p53, were enhanced in response to NAC (Sayin et al. 2014). These findings support the notion that NAC functions in a cell- and/or context-dependent manner.

In addition to its inhibitory effects on cancer cell growth, survival and behavior, NAC is believed to exert protective effects at both preventive and treatment levels (Rushworth and Megson 2014). As such, NAC has long been proposed as a chemopreventive agent with antimutagenic and anticarcinogenic properties (De Flora et al. 1991b), as well as a chemoprotectant providing protection against chemotherapy-induced toxicity (Martinez and Domingo 1991). Moreover, NAC has the potential to enhance cytotoxic effects of chemotherapeutic drugs (Sect. 5.2), as well as cytotoxic or growth-inhibitory effects of such substances and agents as garcinol (Hong et al. 2007), epigallocatechin-3-gallate (EGCG) (Lambert et al. 2008), gossypol (Sikora et al. 2008; Ni et al. 2013), fisetin (Wu et al. 2014), shark cartilage extract (SCE) (Simard et al. 2011), copper (Zheng et al. 2010), the drug interferon α -2A (Kretzmann et al. 2012), the 5-lipoxygenase activating protein (FLAP) inhibitor MK886 (Deshpande and Kehrer 2006), the tyrosine kinase inhibitor imatinib (Rakshit et al. 2009), and the epidermal growth factor receptor (EGFR) inhibitor AG1478 (Lee et al. 2013a).

Mechanistically, treatment-induced cytotoxicity was identified as a mechanism underlying the antiproliferative effects of BR/NAC observed in the present study. In this regard, caspase-dependent apoptosis was found to be activated through both extrinsic and intrinsic pathways. Caspase-8 and caspase-9 are the initiators of the extrinsic and intrinsic pathways, respectively. Our results indicated that the both caspases were activated in response to BR/NAC. The execution of the apoptotic program was evident by the overexpression of cytochrome c and the activation of the executioner caspases 3 and 7. The functionality of the activated caspase-3 was also confirmed by the emergence of the cleaved PARP. Bcl-2, as an important anti-apoptotic protein, prevents mitochondrial outer membrane permeabilization (MOMP), which is the trigger for the intrinsic pathway (Tait and Green 2010). Thus, the decrease in the expression of Bcl-2 further supports the activation of

apoptotic processes in the treated cells. These events were found in parallel with the inhibition of the Akt activity. The activation of Akt pathway provides cells with a survival signal that allows them to withstand apoptotic stimuli (Song et al. 2005), with well-documented contribution to CRC carcinogenesis, progression, and metastasis (Agarwal et al. 2013). The presented data led us to conclude that the activation of apoptotic pathways and the inhibition of anti-apoptotic/pro-survival processes collectively reduce cell viability in response to the BR/NAC treatment.

Moreover, the deregulated expression of a number of autophagy-related proteins not only indicates the likely contribution of autophagy to cell death, but also implies a likely interplay with apoptosis. In this regard, the overexpression of Atg3, Atg7, and Atg12, and more characteristically, an increase in the expression of LC3-II, known as the most reliable marker of autophagosomes (Kabeya et al. 2000), are indicators of autophagic activity in the treated cells. Besides, evidence shows that some autophagy-related proteins can serve as mediators of cross talk between apoptosis and autophagy. As such, Atg12 has been recently identified as a positive regulator of apoptosis by binding and inactivating Bcl-2 (Rubinstein et al. 2011). The key pro-autophagic protein Beclin 1, on the other hand, has been shown to play an anti-apoptotic role in several settings (Kang et al. 2011). Hence, differential expression of Atg12 and Beclin 1 observed in MKN45 cells could signal the promotion of cell death in favor of apoptosis.

The mechanistic basis of antiproliferative effects of BR and NAC on malignant cells has been explored by a number of investigators. Kalra et al. and Bhui et al. have demonstrated in their investigations on mouse skin tumors that BR treatment is associated with the upregulation of p53 and Bax, the activation of caspase 3 and caspase 9, the inhibition of Erk and Akt phosphorylation, and a decrease in Bcl-2 (Kalra et al. 2008; Bhui et al. 2009). Consistently, they later reported BR-induced apoptosis through modulation of Bax-Bcl-2 ratio, apoptotic protease activating factor 1 (Apaf-1), caspase-9 and caspase-3 in A431 human epidermoid carcinoma, and A375 human melanoma cell lines (Bhui et al. 2012). In a separate report, they documented the induction of apoptosis and autophagy in MCF-7 human breast cancer cells in response to BR where autophagy preceded and facilitated apoptotic response (Bhui et al. 2010). In this study, BR induced the expression of LC3-II and Beclin 1 which was found to be regulated negatively by ERK(1/2) and positively by JNK and p38 kinase pathways. Also, investigating the effects of BR on human breast cancer cells, Paroulek et al. and Dhandayuthapani et al. consistently reported that BR treatment of GI-101A cells increased the activities of caspase-9 and caspase-3 which was associated with the elevated levels of caspase-cleaved cytokeratin 18 (CK18) (Dhandayuthapani et al. 2012; Paroulek et al. 2009). In line with the present study, a recent report by Romano et al. demonstrated antiproliferative and pro-apoptotic effects of BR on Caco-2 human colon cancer cell line which were attributed to the increased caspase3/7 activity and reduced activation of ERK(1/2) and Akt (Romano et al. 2014). It is worth noting that despite a proapoptotic activity in malignant cells, BR has shown a protective, prosurvival role in normal cells (Juhasz et al. 2008; Neumayer et al. 2006).

In relation to NAC, despite a large body of evidence supporting an anti-apoptotic role by which it can protect normal cells against cytotoxic stimuli, an increasing number of scholars have argued that NAC is also capable of exerting opposite effects on cell survival. As an initial observation, Tsai et al. reported that NAC induced apoptosis in aortic smooth muscle cells but not in endothelial cells (Tsai et al. 1996). In agreement, Liu et al. (1998) and Havre et al. (2002) indicated that NAC selectively induced p53-mediated apoptosis in several oncogenically transformed fibroblasts, but not in normal cells. In their study on a range of CRC cell lines, Nargi et al. reported antiproliferative and pro-apoptotic effects of NAC wherein p21^{WAF1/CIP1}, functional p53, Ras status, and basal levels of ROS were identified as important determinants of CRC cell susceptibility to NAC-induced apoptosis (Nargi et al. 1999). ROS have shown to paradoxically influence cancer cell biology by promoting cell proliferation or inducing apoptosis (Kardeh et al. 2014). In their study, Nargi et al. found that growth-inhibitory potential, but not anti-apoptotic activity, of NAC correlated with its ability to reduce ROS. Likewise, Arora-Kuruganti et al. observed that ROS induced the proliferation of astrocytoma cells, which was abolished by NAC (Arora-Kuruganti et al. 1999). In agreement, Hong et al. reported that the inhibitory effects of garcinol on CRC cell growth were significantly enhanced in the presence of NAC, which was found to abort growth-promoting effects of ROS (Hong et al. 2007). These findings have been confirmed by recent studies. Wang et al. found that ROS enhanced HeLa cervical cancer cell growth both in vitro and in vivo in an IgG-dependent manner, and that NAC further suppressed the growth and proliferation of IgG-deficient cells in a dose-dependent manner by decreasing the levels of intracellular ROS (Wang et al. 2013). Recently, Rose et al. demonstrated that the growth-promoting effect of high selenium levels on some mesothelioma cell lines was mediated by ROS and reverted by NAC (Rose et al. 2014). Similarly, Song et al. recently reported that *p,p'*-dichlorodiphenyltrichloroethane (DDT) exposure promoted DLD1 human CRC cell growth through Wnt/ β -catenin signaling, which was mediated by oxidative stress and suppressed by NAC (Song et al. 2014). Moreover, Jung et al. showed that NAC treatment of Lewis lung carcinoma cells suppressed glucose uptake by malignant cells that closely paralleled the resultant reductions in ROS levels (Jung et al. 2013). On the other hand, Nargi et al. found in their study that cell lines undergoing NAC-induced apoptosis had higher baseline levels of ROS than their counterparts in which proliferation was blocked. Surprisingly, Lepri et al. found that NAC pretreatment of 3DO murine lymphocytic hybridoma cells even enhanced H₂O₂-induced apoptosis. Cho et al. reported that NAC treatment of BT-474 human breast cancer cells reduced ROS production and AKT phosphorylation, yet induced apoptotic cell death (Cho et al. 2014). Taken together, it is concluded that NAC-induced apoptosis is a mechanism independent of its ROS-scavenging function. Furthermore, NAC has been shown to sensitize tumor cells to other apoptotic stimuli. As such, Rieber and Rieber showed that NAC synergized with UV radiation to promote apoptosis of C8161 human melanoma cells, evidenced as the enhanced activation of caspase-3 and fragmentation of the retinoblastoma protein and the E2F-4 transcription factor (Rieber and Rieber 2003). In agreement, Qanungo et al. reported that NAC enhanced hypoxia-induced

apoptosis in murine embryonic fibroblasts (MEFs), MIA PaCa-2 human pancreatic and A549 human lung carcinoma cells by inhibiting the hypoxia-induced transactivation of NF κ B and its anti-apoptotic targets such as XIAP (Qanungo et al. 2004). In their later study, glutaredoxin-dependent S-glutathionylation of p65-NF κ B was implicated in the NAC-mediated inactivation of NF κ B and enhancement of hypoxic apoptosis (Qanungo et al. 2007). The authors recently reported the observation of such an effect in other human pancreatic cancer cell lines, including PANC-1, ASPC-1, and Capan-2, and showed consistently that this mechanism enables NAC to sensitize MIA PaCa-2 xenografts to gemcitabine (Qanungo et al. 2014). Consistently, Kretzmann et al. observed that NAC induced apoptosis and potentiated the apoptotic response to interferon α -2A of HepG2 and Huh7 human HCC cells through inhibition of NF κ B (Kretzmann et al. 2012). Lambert et al. demonstrated that NAC interacts and forms an adduct with EGCG that results in an 8.8-fold increase in apoptosis of CL13 mouse lung cancer cells (Lambert et al. 2008). In a study by Zheng et al., NAC, known as a metal-binding agent, was found to interact with copper to induce apoptosis of A2780 ovarian cancer cells (Zheng et al. 2010). Similarly, NAC was shown to increase gossypol-induced apoptosis in both cisplatin sensitive and resistant head and neck squamous cell carcinoma (HNSCC) cells (Sikora et al. 2008) as well as in Burkitt lymphoma cells (Ni et al. 2013). Rakshit et al. reported that NAC enhanced imatinib-induced apoptosis of chronic myeloid leukemia cells by endothelial nitric oxide synthase-mediated production of nitric oxide (Rakshit et al. 2009). In their study on invasive oral cancer cells, Lee et al. found that NAC induced apoptosis in EGFR-overexpressing cells, with the involvement of HMG box-containing protein 1 (HBP1), and, in doing so, exhibited synergy with the EGFR inhibitor AG1478 (Lee et al. 2013a). In a recent study, Wu et al. demonstrated that NAC boosted cytotoxic effects of fisetin on CRC cells COLO-205, HCT-116, HT-29, and HCT-15 through inducing apoptosis, which, in line with our results, was evident as an increased number of apoptotic bodies, the expression of cleaved caspase-3, caspase 9, and PARP, as well as the formation of DNA ladders (Wu et al. 2014). In conclusion, evidence shows that NAC has the potential to induce a preferential or selective apoptosis in malignant cells in a cell- and context-dependent, but ROS-independent, manner.

Our results indicating the diminution of cyclin D with consistent decreases in cyclins A and B suggest that the treatment also interferes with cell cycle progression to induce growth-inhibitory effects. Cyclins are recognized as the key components of the cell cycle regulatory machinery. Orderly progression of the cell cycle from one phase to another is coordinated by cyclins sequentially activating their partner proteins, CDKs (Satyanarayana and Kaldis 2009). This is initiated by the expression of cyclin D in early G1 which drives the cell cycle through to late G1 and followed by subsequent induction of cyclin E, cyclin A and cyclin B at late G1, early S and late S phases, respectively. Effects of BR or NAC on cell cycle progression have been explored in a number of studies. According to an initial report by Taussig and Batkin, DNA perturbation was found to be induced in KATO-III cells in response to BR (Taussig and Batkin 1988). Bhui et al. reported that BR treatment of human epidermoid carcinoma and melanoma cells resulted in cell cycle arrest at G2/M checkpoint (Bhui et al. 2012). In a recent gene expression profiling study on

MCF-7 breast cancer cells, B cell translocation gene 1 (BTG1), a member of a new family of anti-proliferative genes that regulates cell cycle progression, was identified among the genes with significantly altered expression after BR treatment (Fouz et al. 2014b). With regard to NAC, Huang et al. demonstrated the efficacy of NAC in inhibiting the mitogenic effects of phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) using P-3T3 and N-3T3 subclones of the NIH 3T3 mouse embryonic fibroblasts (Huang et al. 1995). TPA is a potent activator of protein kinase C that can either stimulate or inhibit cell proliferation, depending on the cell type. As such, Huang et al. observed mitogenic and anti-mitogenic effects of TPA on P-3T3 and N-3T3 cells, respectively, inducing the expression of cyclin D1 and DNA synthesis in the former and inhibiting cyclin E-associated kinase at the G1/S transition in the latter. This cell context-dependent response was differentially affected by NAC where TPA-mediated induction of cyclin D1 and DNA synthesis in P-3T3 was inhibited by NAC, but TPA-induced inhibition of the cyclin E-associated kinase in N-3T3 cells was left unaffected. In agreement, Irani et al. reported antimitogenic effects of NAC on an H-Ras-expressing clone of NIH 3T3 fibroblasts, where NAC treatment effectively inhibited DNA synthesis and mitogenic signaling in a dose-dependent manner (Irani et al. 1997). In line with these reports, Sekharam et al. observed in NIH 3T3 cells that NAC inhibited the expression of cyclin D1 and DNA synthesis and induced the blockage of cell cycle progression in early to mid-G1 (Sekharam et al. 1998). Liu et al. reported that NAC was capable to induce the expression of the CDK inhibitors p16-INK4a and p21^{WAF1/CIP1} and to prolong G1 phase in murine papilloma cells (Liu et al. 1999). In the study by Arora-Kuruganti on U373-MG human astrocytoma cells, NAC was found to inhibit both serum- and H₂O₂-induced DNA synthesis in the malignant cells (Arora-Kuruganti et al. 1999). DNA synthesis arrest in response to NAC was also observed by Li et al. in SJ-89 human GC cells (Li et al. 2007b). Using the estrogen-dependent human breast cancer cell line MCF7, Felty et al. showed that NAC inhibited estrogen-induced cell growth, cyclin D1 expression, and DNA synthesis (Felty et al. 2005). In their recent study on human oral squamous carcinoma cells, Lee et al. found that NAC treatment of HSC-3 cells caused G1 arrest with a concomitant increase in the expression of HBP1—a regulator of cell cycle exit and cell differentiation—and reduction of the cyclin D1 expression (Lee et al. 2013a).

In sum, we observed in the present study that BR and NAC inhibit growth, proliferation, and survival of gastrointestinal carcinoma cells. BR/NAC-induced cytotoxicity was found to be more potent in combination therapy, where they appeared to interact with each other in a predominantly synergistic or additive manner. Apoptotic cell death with the contribution of autophagy and cell growth arrest was identified as the underlying mechanism of effect. Given the role of the aberrantly produced mucins in cancer cell growth, proliferation, and survival, mucin-depleting effects of BR/NAC on mucin-expressing gastrointestinal carcinoma cells might contribute to their cytotoxicity. This feature will be explored and discussed in Sect. 5.3. Here, we conclude that an optimized combination of BR and NAC is a potential candidate for utility in novel locoregional approaches to peritoneal dissemination of gastrointestinal malignancies aimed to enhance microscopic cyto-reduction following conventional therapies.

5.2 Effects of BR/NAC on Chemosensitivity of Gastrointestinal Cancer Cells in Sequential and Combination Therapy In Vitro

5.2.1 Introduction

Chemotherapeutic agents are widely administered intravenously in cancer therapy. Nevertheless, it has been shown in the context of the PSMs that disease control may be significantly improved when chemotherapy is used through the intraperitoneal route (Sugarbaker and Bijelic 2012). In agreement, a combination of CRS and HIPEC, with or without early postoperative intraperitoneal chemotherapy (EPIC), has offered long-term benefits in selected patients with PSM (Mohamed et al. 2011). This multimodal strategy is now considered as the standard of care for patients with PMP (Sugarbaker 2006b) and advocated as a promising approach to other primary or secondary peritoneal malignancies, including peritoneal carcinomatosis (PC) of gastrointestinal origin (Sugarbaker 2012) and MPM (Yan et al. 2009). Owing to the limited penetration of chemotherapy into tumor nodules, patients with minimal residual disease on both the parietal and visceral peritoneal surfaces are likely to benefit most from this combined therapy (Sugarbaker 2005b). However, evidence shows that HIPEC often fails to maintain the surgical complete response achieved by CRS (Sugarbaker and Bijelic 2012). In addition, chemotherapy-induced toxicity even at low plasma levels is always an issue of concern. Thus, HIPEC needs to be supplemented by novel treatments capable of targeting the residual disease. In this regard, locoregional use of safe agents with cytotoxic effects on cancer cells represents a potentially efficacious strategy for the enhancement of microscopic cytoreduction. As a potential candidate for such a strategy, we intended to find out if BR/NAC treatment has the capability to sensitize gastrointestinal cancer cells into chemotherapy. To this end, we used a number of chemotherapeutic agents of different classes and variable utility in both peritoneal and systemic chemotherapy, including cisplatin, 5-fluorouracil, paclitaxel, and vincristine, and evaluated the influence of the BR/NAC pretreatment on cancer cell response to chemotherapy in sequential therapy and analyzed the interaction between BR/NAC and chemotherapeutic agents in combination therapy. In the present section, the results obtained from this in vitro study are reviewed and discussed (Amini et al. 2015b).

5.2.2 Results

Using escalating concentrations of cisplatin (Cis), 5-fluorouracil (5FU), paclitaxel (PTX), and vincristine (VCR), we first performed cytotoxicity assay of these chemotherapeutic agents on KATO-III, MKN45, and LS174T cells using sulforhodamine B assay. Possible chemosensitizing effects of BR/NAC on two cell lines of different origin, including KATO-III gastric and LS174T colon cancer cell lines, were then explored in sequential treatment.

5.2.2.1 BR/NAC Pretreatment Sensitizes KATO-III Cells to Chemotherapy with Cisplatin, 5-Fluorouracil, Paclitaxel, or Vincristine

We initially investigated whether and how pretreatment with single agent BR for three different periods could affect chemosensitivity of KATO-III cells. To this end, based on the results from the cytotoxicity assays, KATO-III cells in four chemotherapy groups were pretreated with three selected concentrations of BR (100, 200, and 300 $\mu\text{g/mL}$) for 2, 4, or 8 h, and subsequently treated with three selected concentrations of Cis (0.5, 1, and 5 μM), or two selected concentrations of 5FU (10 and 50 μM), PTX (1 and 5 nM), or VCR (1 and 2.5 nM) for 72 h. Positive chemosensitization was observed in all treatment groups after 2 h pretreatment with BR, except for the two sequentially treated with 100 $\mu\text{g/mL}$ BR and either VCR concentration. However, when BR was used at concentrations of 100 and 200 $\mu\text{g/mL}$, chemosensitizing effects, with the exception of one instance (BR 200 $\mu\text{g/mL}$ and Cis 0.5 μM ; $p=0.0252$), were not statistically significant (Amini et al. 2015b).

In contrast, the highest concentration of BR (300 $\mu\text{g/mL}$) induced significant enhancement of response to Cis ($p=0.0053$, 0.0041 and 0.0042), 5FU ($p=0.0496$ and 0.0270), and VCR ($p=0.0276$ for VCR 1 nM). When pretreatment was applied for a longer period, significant results appeared at lower concentrations of BR, too. Significant enhancement of response to all chemotherapeutic agents used was evident after 4 h pretreatment with 200 or 300 $\mu\text{g/mL}$ BR. In this regard, significant sensitization to both concentrations of Cis was also found with 4 h BR pretreatment at the concentration of 100 $\mu\text{g/mL}$ ($p=0.0128$ and 0.0188). Finally, when KATO-III cells were pretreated for 8 h, BR at all the three concentrations used significantly enhanced response to the four chemotherapeutic agents.

To explore the effect of combined use of BR and NAC, we then pretreated KATO-III cells with two selected combinations of BR and NAC (BR 50 $\mu\text{g/mL}$ +NAC 5 mM or BR 100 $\mu\text{g/mL}$ +NAC 10 mM) for 4 or 8 h and subsequently treated them with single agent Cis (1, 5 and 10 μM), 5FU (50 and 100 μM), PTX (1 and 5 nM), or VCR (1 and 2.5 nM) for 72 h.

Both 4- and 8-h pretreatment with BR/NAC positively sensitized KATO-III cells to all the four cytotoxic agents. Statistical analysis of the results indicated that, with the exception of two instances (4 h pretreatment with 50 $\mu\text{g/mL}$ BR plus 5 mM NAC followed by 72 h treatment with 10 μM Cis or 1 nM VCR), the chemosensitizing effects observed were all significant.

5.2.2.2 BR/NAC Pretreatment Sensitizes LS174T Cells to Chemotherapy with Cisplatin, 5-Fluorouracil, Paclitaxel, or Vincristine

Next, we evaluated the capability of short-term pretreatment with BR or BR+NAC in enhancing response to chemotherapy of LS174T cells. For this purpose, different concentrations of BR (10, 20, and 30 $\mu\text{g/mL}$), BR+NAC (BR 10 $\mu\text{g/mL}$ +NAC 20 mM and BR 20 $\mu\text{g/mL}$ +NAC 10 mM), Cis (10 and 20 μM), 5FU (10 and 50 μM),

PTX (10 and 50 nM), and VCR (10 and 50 nM) were selected based on the results from the cytotoxicity assays. LS174T cells were then sequentially exposed to 4 h pretreatment with BR or BR+NAC and 72 h single agent chemotherapy. Our data indicated that pretreatment differentially affected the cancer cell response to chemotherapy. All the pretreatment protocols significantly enhanced cancer cell sensitivity to 10 μ M Cis. Response to the both 5FU concentrations was enhanced by pretreatment which was statistically significant for the higher 5FU concentration (50 μ M) after BR pretreatment (20 and 30 μ g/mL) as well as for the both concentrations of 5FU (10 and 50 μ M) after BR+NAC pretreatment. With the exception of one instance (10 μ g/mL BR pretreatment for 50 nM PTX), pretreatment enhanced cancer cell sensitivity to the both concentrations of PTX used, of which response to 10 nM PTX was significantly improved by all protocols. Of the four cytotoxic agents, response to VCR was least affected by BR/NAC pretreatment. In this regard, although pretreatment of cancer cells with 20 μ g/mL BR and both combinations of BR+NAC apparently enhanced sensitivity to 10 nM VCR, the results were not statistically significant.

5.2.2.3 Concomitant Treatment of MKN45 Cells with BR + NAC Differentially Affects Response to Cisplatin, 5-Fluorouracil, Paclitaxel, or Vincristine

At the first stage, we treated MKN45 cells using nine possible combinations of three selected concentrations of BR (50, 75, and 100 μ g/mL) and NAC (1, 5, and 10 mM) in conjunction with two different concentrations of Cis (0.5 and 1 μ M), 5FU (5 and 10 μ M), PTX (1 and 5 nM), or VCR (0.5 and 2.5 μ M) for 72 h. This created four different chemotherapy groups and 72 (4 $\times$ 18) individual treatment subgroups. Our data indicated that BR/NAC treatment differentially affect cancer cell response to concomitant chemotherapy with individual agents. As regards Cis group, BR/NAC enhanced the effect of chemotherapy in 10 out of 18 subgroups, statistically significant in seven subgroups, including BR 50+NAC 5+Cis 0.5, BR 50+NAC 10+Cis 0.5, BR 75+NAC 10+Cis 0.5, BR 100+NAC 5+Cis 0.5, BR 100+NAC 10+Cis 0.5, BR 100+NAC 5+Cis 1, and BR 100+NAC 10+Cis 1 (concentrations as per μ g/mL, mM and μ M for BR, NAC and Cis, respectively). BR/NAC also enhanced 5FU-induced cytotoxicity in 13 subgroups, which was significant in 10, including BR 50+NAC 10+5FU 5, BR 75+NAC 5+5FU 5, BR 75+NAC 10+5FU 5, BR 100+NAC 5+5FU 5, BR 100+NAC 10+5FU 5, BR 75+NAC 5+5FU 10, BR 75+NAC 10+5FU 10, BR 100+NAC 1+5FU 10, BR 100+NAC 5+5FU 10, and BR 100+NAC 10+5FU 10 (concentrations as per μ g/mL, mM and μ M for BR, NAC, and 5FU, respectively). Finally, cytotoxic effects of PTX and VCR were found to be enhanced by BR/NAC in all treatment subgroups. Statistically, results in these two groups were all significant, except for three PTX (BR 50+NAC 1+PTX 1, BR 50+NAC 5+PTX 1, and BR 50+NAC 1+PTX 5) and one VCR (BR 100+NAC 5+VCR 2.5) subgroups (concentrations as per μ g/mL, mM, μ M, and nM for BR, NAC, VCR, and PTX, respectively).

5.2.2.4 Concomitant Treatment of LS174T Cells with BR+NAC Enhances Response to Cisplatin, 5-Fluorouracil, Paclitaxel, or Vincristine

Using similar experimental design, we then examined how BR+NAC influence cytotoxic effects of the four chemotherapeutic agents in combination treatment of LS174T cells. We used nine possible combinations of three selected concentrations of BR (10, 20, and 30 $\mu\text{g/mL}$) and NAC (5, 10, and 20 mM) in conjunction with three different concentrations of Cis (5, 10, and 20 μM), 5FU (10, 50, and 100 μM), PTX (10, 50, and 100 nM), or VCR (10, 50, and 100 nM). Hence, LS174T cells were treated in four different chemotherapy groups and 108 (4×27) individual treatment subgroups. Our data indicated that BR/NAC treatment enhanced cancer cell response to concomitant chemotherapy in 104 out of 108 treatment subgroups. Except for four subgroups of Cis group (BR 20+NAC 5+Cis 5, BR 30+NAC 10+Cis 5, BR 20+NAC 5+Cis 20, and BR 30+NAC 20+Cis 20) and two subgroups of PTX (BR 10+NAC 5+PTX 50 and BR 10+NAC 5+PTX 100) (concentrations as per $\mu\text{g/mL}$, mM, μM , and nM for BR, NAC, Cis, and PTX, respectively), BR+NAC-induced enhancement of chemotherapy was statistically significant.

5.2.2.5 Drug–Drug Interaction Analysis of the Combination Treatments

Using CalcuSyn software, we analyzed how BR+NAC interact with each cytotoxic agent at concentrations used for combination treatment of MKN45 and LS174T cells and compared the results in individual chemotherapy groups. The outcome of this interaction in combination treatment of MKN45 cells with Cis was antagonistic, with the lowest concentrations of BR+NAC (BR 50+NAC 1 and 5) indicating the weakest antagonism with Cis. As regards LS174T cells, synergy and additivity appeared at given concentrations. Synergistic interactions were found with BR 10+NAC 5, 10 and 20 in combination with Cis 5, BR 10+NAC 5 and 20 in combination with Cis 10, and BR 10+NAC 5 in combination with Cis 20 (concentrations as per $\mu\text{g/mL}$, mM, and μM for BR, NAC, and Cis, respectively). The strongest synergism was observed when any of the three Cis concentrations was combined with the lowest concentrations of BR and NAC. When BR 20+NAC 5 and BR 20+NAC 10 were used in combination with Cis 5 and 10, respectively, additive interaction resulted. In addition, Cis 10 in combination with BR 10+NAC 10 and BR 20+NAC 5 showed a borderline interaction.

As regards the 5FU group, drug–drug interaction in the majority of the combination formulations used for the treatment of MKN45 cells was synergistic or additive. Formulations with synergistic interaction included BR 75+NAC 10 with 5FU 5 and 10, BR 100+NAC 1 with 5FU 5 and 10, BR 50+NAC 10 with 5FU 5, and BR 75+NAC 5 with 5FU 10 (concentrations as per $\mu\text{g/mL}$, mM, and μM for BR, NAC, and 5FU, respectively). Additive interactions were present between BR 50+NAC 10 and 5FU 10, BR 75+NAC 5 and 5FU 5, as well as between BR 100+NAC 5 and 5FU 5 and 10. When BR and NAC were used at the lowest concentrations

(BR 50+NAC 1), the strongest antagonism with 5FU appeared. With respect to LS174T cells, drug–drug interaction in all formulations used was synergistic. In this regard, an increase in the concentration of NAC in combination with a given concentration of BR weakened the resultant interaction with 5FU, following a similar pattern in combination with different concentrations of 5FU.

With regard to PTX group, our results indicated that synergy and, less frequently, additivity are the predominant models of drug–drug interaction in both cell lines. BR 50+NAC 5 and BR 100+NAC 10 in combination with PTX 1 and 5, respectively, were the only formulations with antagonistic interaction in MKN45 cells (concentrations as per $\mu\text{g/mL}$, mM, and nM for BR, NAC, and PTX, respectively). Formulations with additivity included BR 50+NAC 1, BR 75+NAC 1 and BR 100+NAC 10 in combination with PTX 1, as well as BR 50+NAC 10 in combination with PTX 5. The remaining formulations all showed synergistic interaction, among which BR 75+NAC 5 had the strongest synergy with PTX. In LS174T cells, when the lowest concentrations of BR+NAC (BR 10+NAC 5) were used, the weakest interaction with PTX resulted. This was present as two additive patterns (in combination with PTX 10 and 50) and the only antagonistic interaction (in combination with PTX 100). The interaction between BR+NAC and PTX in all the remaining formulations was synergistic which followed a similar pattern for different concentrations of PTX.

VCR group indicated the most favorable drug–drug interaction, with synergistic interaction found in 7 and 9 out of 9 combination formulations used for the treatment of MKN45 and LS174T cells, respectively. The only antagonistic interactions with VCR (0.5 and 2.5 μM) were present in combination with the highest concentrations of BR+NAC (BR 100 $\mu\text{g/mL}$ +NAC 5 and 10 mM). In MKN45 cells, the remaining patterns were all synergistic and similar for both concentrations of VCR. As with 5FU, the interaction between BR+NAC and VCR in all formulations used for LS174T cells was synergistic. BR 20+NAC 20 represented the weakest interaction with both VCR concentrations.

5.2.3 Discussion

The peritoneal component of malignancies is often a major source of morbidity and mortality. In the context of PSM, surgery per se has shown limited curative effectiveness and thus needs to be combined with chemotherapy. On the other hand, the existence of the peritoneal-plasma barrier, a diffusion barrier consisting of the mesothelium, interstitium, and submesothelial capillary wall (Jacquet and Sugarbaker 1996b), and the paucity of subperitoneal blood vessels prevent systemic chemotherapy from delivering therapeutic concentrations to the superficial tumor deposits on the peritoneal lining. Hence, systemic chemotherapy has proven to be minimally effective in this context. In contrast, one can take advantage of the peritoneal-plasma barrier to achieve a much higher drug concentration in the peritoneal cavity by intraperitoneal administration of chemotherapeutic agents [reviewed

in (Rubino et al. 2012)]. In this regard, slow output from the peritoneal cavity of drugs with high plasma clearance is associated with pharmacokinetic advantages (Gladieff et al. 2009; Trimble et al. 2008). This advantage of intraperitoneal chemotherapy can be expressed by the area under the curve (AUC) ratios of intraperitoneal to intravenous (IP/IV) exposure penetration (Sugarbaker et al. 2005). By this approach, not only tumor deposits and peritoneal free cancer cells (PFCCs) are targeted, but also tumor cells growing in the submesothelial lymphatic sinus are exposed to high concentrations of drugs absorbed from the lymphatic orifices (Yonemura et al. 2007b; Rubino et al. 2012). Therefore, use of intraperitoneal chemotherapy in conjunction with surgery is rational in PSM. For an enhanced treatment efficacy, efforts should be made to maximize cytotoxic effects of chemotherapeutic agents on tumor cells while minimizing their toxic effects on host cells. Since the penetration of intraperitoneally administered agents into peritoneal nodules, even with hyperthermia, is limited to 2–5 mm, CRS is essential to reduce the tumor volume to minimum (Mohamed et al. 2011). In addition, locoregional chemotherapy after complete dissection of an adhesive process and before the onset of wound healing and organization of fibrinous deposits minimizes nonuniform distribution of chemotherapeutic agents and facilitates their access to residual disease and PFCCs (Sugarbaker 2005b). It has been demonstrated that the capillary wall and the surrounding interstitial matrix, but not the mesothelial lining, are the principal barriers for clearance of molecules from the abdominopelvic space (Sugarbaker et al. 2011; Jacquet and Sugarbaker 1996b). Thus, the extent of the peritoneal resection aimed in CRS only minimally affects the pharmacokinetics of the intraperitoneally administered agents (de Lima Vazquez et al. 2003). Finally, hyperthermia is believed to enhance cytotoxic effects of selected agents (Urano et al. 1999) and to improve drug penetration (Jacquet et al. 1998). On this basis, HIPEC is advocated as the standard, or preferable, chemotherapy in selected patients with peritoneal dissemination of malignancies. For clinically stable patients without any evidence of early postoperative complications, HIPEC might be followed by EPIC. As follows, evidence also suggests that intravenous chemotherapy administered simultaneously with intraperitoneal perfusion gains pharmacokinetic advantages. In this regard, it was shown that perfused peritoneal solution rapidly became saturated by intravenously administered cytotoxic agent through large peritoneal and subperitoneal surface blood circulation. This “sink” phenomenon in the absence of enzymatic metabolism provides persistently high levels of intraperitoneal drug (Van der Speeten et al. 2010). Hence, adjuvant and neoadjuvant bidirectional chemotherapy, too, has been proposed as a treatment option following a major cytoreductive procedure (Sugarbaker and Bijelic 2012; Yonemura et al. 2009).

Although CRS combined with HIPEC has brought about long-term benefits in selected patients with PSM, this multimodal curative approach remains associated with treatment failures attributed to the inadequacy of HIPEC to maintain the surgical complete response. This indicates the need for the development of supplementary strategies (Sugarbaker and Bijelic 2012). In this regard, our preliminary findings on cytotoxic effects of BR/NAC on gastrointestinal cancer cells provided evidence in support of potential utility of this compound in microscopic cytoreduction for

PSM of gastrointestinal origin. As described in the present section, we subsequently investigated whether BR/NAC also has the capability to enhance chemotherapy-induced cytotoxicity if used on their own as pretreatment or in combination with individual chemotherapeutic agents of different families, including cisplatin, 5FU, PTX, and VCR. Cisplatin is a platinum-containing, alkylating-like metal salt that binds to and causes cross-linking of DNA, ultimately triggering apoptosis. 5FU is a pyrimidine analog that irreversibly inhibits thymidylate synthase and thus prevents the formation of thymidine monophosphate required for DNA replication. PTX belongs to the taxane family of cytoskeletal drugs that targets tubulin. Through stabilizing the microtubule polymer, it prevents a metaphase spindle configuration, resulting in apoptosis or reversion of cell cycle to the G phase (G2/M arrest). VCR is a vinca alkaloid that binds to tubulin dimers, inhibiting assembly of microtubule structures and arresting mitosis in metaphase. With the exception of cisplatin, all these chemotherapeutic agents are cell cycle-specific [reviewed in (Espinosa et al. 2003)]. Cisplatin, 5FU, and PTX are among the commonly used intraoperative agents in intraperitoneal chemotherapy of PSM (Mohamed et al. 2011). HIPEC with cisplatin is particularly employed for PCs from gastric (Yang et al. 2011a) and ovarian cancer (Deraco et al. 2011b). When administered via hyperthermic peritoneal perfusion, cisplatin gains pharmacological advantages that result from not only higher peritoneal concentration and lower systemic absorption and toxicity (Cho et al. 1999), but also enhanced penetration to peritoneal tumors (Los et al. 1989, 1991), rapid absorption (Cashin et al. 2013), and heat synergy (Urano et al. 1999; Barlogie et al. 1980). 5FU and PTX display relatively high AUC IP/IV ratios (Sugarbaker et al. 2005). 5FU is considered as the cornerstone of the perioperative chemotherapy for peritoneal carcinomatosis of gastrointestinal origin (Van der Speeten et al. 2010). Due to its large particle size and prolonged retention in the peritoneal cavity, PTX is considered to be suitable for intraperitoneal chemotherapy (Soma et al. 2009). Moreover, the bidirectional administration was shown to maintain effective concentrations of PTX in the peritoneal cavity for over 72 h (Ishigami et al. 2009). Intraperitoneal and bidirectional administration of PTX has been reported to be clinically safe and effective in patients with GCPC (Emoto et al. 2014; Ishigami et al. 2010). 5FU and PTX are also frequently used in EPIC for PSM (Mohamed et al. 2011). VCR is also a widely used intravenous chemotherapeutic agent in human oncology, including combination therapy of CRC (Fleischer et al. 1983) and primary colonic lymphoma (Tang et al. 2011). VCR has been recently shown to demethylate and restore the expression of the tumor suppressor gene runt-related transcription factor-3 (RUNX3) and other methylated genes in CRC cells (Moon et al. 2014a, b). As with the aforementioned agents, intraperitoneal administration of VCR has been shown *in vivo* to provide good clinical results and high bioavailability of the drug with no specific side effects and suggested as a safe and effective alternative for VCR chemotherapy (Bairy et al. 2003; Voorhorst et al. 2014; Teske et al. 2014).

Our data indicated that BR/NAC pretreatment has the potential to sensitize KATO-III and LS174T cells to chemotherapy. In this regard, different pretreatment protocols and cytotoxic concentrations were tested. As a result, with the exception

of VCR chemotherapy group of LS174T cells, evidence was found in all groups of statistically significant enhancement of response to chemotherapy after pretreatment. With regard to combination therapy, too, our results provided evidence in support of statistically significant potentiation of chemotherapeutic effects induced by BR/NAC in MKN45 and LS174T chemotherapy groups. At the concentrations used, BR/NAC and individual chemotherapeutic agents were found to differentially interact with one another in combination treatment of either cell line, with resultant interaction ranging from synergy to additivity to antagonism. The most favorable interactions were observed in 5FU group of LS174, as well as in PTX and VCR groups of both MKN45 and LS174T. Synergistic and additive interactions were also evident in other groups, except for Cis group of MKN45. Even in this group, treatment subgroups with minimal antagonism were present. The capability of BR in potentiating the cytotoxic effects of anticancer agents has been shown in a limited number of studies. According to the anecdotal clinical studies in early 1970s, oral administration of BR in doses of over 1000 mg daily in combination with chemotherapeutic agents, such as 5FU and VCR, resulted in tumor regression (Gerard 1972; Nieper 1974). Oishi et al., however, were the first to observe *in vitro* that cytotoxicity on KATO-III cells of 5FU, mitomycin-C, doxorubicin, and cisplatin was enhanced by the addition of BR [(Oishi et al. 1985) in (Batkin et al. 1988a) and (Taussig and Batkin 1988)]. Similarly, BR was found to enhance cisplatin cytotoxicity on MPM cells PET and YOU (Pillai et al. 2014b).

Evidence also shows that NAC improves the utility of chemotherapy through enhancing the cytotoxic effects of chemotherapeutic agents and/or protecting the host tissues against their toxic effects. Initially, Kline et al. reported that NAC enhanced therapeutic effects of ifosfamide in prolonging the survival of mice with early L1210 leukemia while protecting against chemotherapy-induced toxicity (Kline et al. 1973). Using murine models of experimental and spontaneous lung metastasis by malignant melanoma cells, De Flora et al. reported that NAC not only on its own but also in synergy with doxorubicin prevented tumorigenicity and metastases (De Flora et al. 1996). Consistently, they later showed that NAC interacted with doxorubicin to inhibit B16-BL6 melanoma cell tumorigenicity and metastasis in mice and prevented doxorubicin-induced toxicity (D'Agostini et al. 1998). In agreement, Adeyemo et al. reported that NAC and vitamin E enhanced the susceptibility of Colo201 and Colo205 colon carcinoma cells to 5FU, *in vitro*, by augmenting the expression of the proapoptotic protein Bax (Adeyemo et al. 2001). These results were supported by a separate study *in vivo* wherein NAC increased activity of 5FU against HCT-15 colorectal cancer xenografts in nude mice, which was accompanied by a sustained elevation in p53-independent apoptosis (Bach et al. 2001). Exploring the role of DNA damage response (DDR) defects and ataxia telangiectasia mutated (ATM)/p53 inactivation in lymphomagenesis and chemoresistance in Eμ-myc transgenic mice model of B-cell lymphomas, Reimann et al. found that tumors developed under NAC therapy not only retained a functional ATM-governed DDR but also maintained sensitivity to chemotherapy (cyclophosphamide and doxorubicin) and indicated a profoundly improved long-term outcome (Reimann et al. 2007). In line with this, Brum et al. recently reported that NAC

pretreatment of CaOV3 ovarian cancer cells potentiates doxorubicin-induced activation of p53 and ATM, leading to reorganization of cytoskeletal networks, inhibition of mTOR activity, and inhibition of cell proliferation and migration (Brum et al. 2013). In a study of the underlying role of CXCL12/CXCR4 signaling in chemoresistance to gemcitabine in first-line therapy of pancreatic cancer, Arora et al. found that gemcitabine promotes chemoresistance, migration, and invasion of MiaPaCa and Colo357 pancreatic cancer cells through NF κ B- and HIF1 α -mediated upregulation of CXCR4, a mechanism which was abrogated by NAC pretreatment (Arora et al. 2013). In this connection, a recent study by Qanungo et al. consistently revealed that gemcitabine failed to inhibit the growth of MIA PaCa-2 xenografts in nude mice, individually. However, combination treatment with NAC resulted in a reduction of approximately 50 % in tumor growth, where NAC markedly enhanced tumor apoptosis (Qanungo et al. 2014). In so doing, NAC was found to block p65-NF κ B activation and anti-apoptotic XIAP expression induced by gemcitabine. As a chemoprotectant, NAC has been shown to provide protection against toxic effects of a variety of chemotherapeutic agents, including cisplatin (Luo et al. 2008; Muldoon et al. 2015; Riga et al. 2013; Yoo et al. 2014; Dickey et al. 2005), 5FU (Numazawa et al. 2011; Al-Hamdany and Al-Hubaity 2014), cyclophosphamide (Berrigan et al. 1980; Palma et al. 1986; Botta et al. 1973; Christophidis et al. 1984), ifosfamide (Kline et al. 1973; Chen et al. 2008; Hanly et al. 2013), oxaliplatin (Lin et al. 2006), methotrexate (Caglar et al. 2013), doxorubicin (Kockar et al. 2010; D'Agostini et al. 1998; Doroshov et al. 1981), bleomycin (Serrano-Mollar et al. 2003; Berend 1985; Hagiwara et al. 2000), and combined carboplatin, melphalan, and etoposide phosphate (Neuwelt et al. 2004). In this regard, dose, route and timing of administration are believed to influence the protective properties of NAC (Dickey et al. 2008; Muldoon et al. 2015).

In vitro models used in this study represent mucin-expressing carcinoma cell lines with gastric (MKN45 and KATO-III) or intestinal (LS174T) mucin phenotype. While MKN45 and KATO-III cells express the prototypical membrane-associated mucin MUC1 along with the secreted mucin MUC5AC, LS174T expresses the secreted mucins specific to the intestinal goblet cells, primarily MUC2. Evidence shows that both membrane-associated and secreted mucins are involved in diverse biological mechanisms that underlie resistance to chemotherapy. To this end, mucins are thought to form a physical barrier to cellular drug uptake, to alter drug metabolism, to promote resistance to apoptosis, and to contribute to cell stemness and EMT (Jonckheere et al. 2014). These mechanisms will be explained in Chap. 6, where mucin-depleting effects of BR/NAC treatment will also be discussed. Collectively, chemosensitizing effects of BR/NAC treatment on mucin-expressing gastrointestinal carcinoma cells may be justified in part by their role in depriving tumor cells of their mucins.

In conclusion, our findings supported by results from the aforementioned studies suggest that optimized protocols of BR/NAC adjuvant therapy could give rise to an improved chemotherapeutic index in therapeutic approaches to PSM of gastrointestinal origin. This represents a promising area for future research. Taking into consideration the aberrant expression of mucins in carcinomas with contributory roles

in the development of resistance to chemotherapy, chemosensitizing effects of this novel treatment might be resulted, at least in part, from its mucin-depleting potential. This interesting feature will be discussed in Sect. 5.3.

5.3 Mucin-Depleting Effects of BR/NAC on Mucin-Expressing Gastrointestinal Carcinoma Cells

5.3.1 Introduction

Mucins, also known as MUC glycoproteins, are a diverse family of high molecular weight, heavily glycosylated proteins that are differentially expressed by specialized epithelial cells of mucosal and secretory surfaces throughout the body in a relatively organ- and cell type-specific manner (Gum 1992). Under physiological conditions, mucins are anchored to the apical surface of mucosal epithelial cells or secreted by epithelial goblet cells and mucosal glands and involved in signal transduction, the protection and lubrication of luminal epithelial surfaces (Singh and Hollingsworth 2006). As such, the luminal surface of the gastrointestinal tract is covered by a viscoelastic mucous gel layer composed of the high-molecular-weight mucins that act as a protective barrier against the harsh luminal environment (Corfield et al. 2000). In this context, the specific molecular composition and higher-order structures of mucins in the gastric mucosa contribute to such specialized functions as allowing secretion of hydrochloric acid (HCl) from gastric glands but protecting the epithelium from it (Hollingsworth and Swanson 2004). By comparison, intestinal mucins participate in the front line of the enteric host defense generated by the alliance of the epithelial cells, immune cells, and resident microbiota (Lievin-Le Moal and Servin 2006) while facilitating the transit of intestinal contents (Cone 2009). The structural characteristics of this barrier are primary indicators of its physiological function and changes to its composition have been identified in gastrointestinal pathologies, including GC and CRC (Corfield et al. 2000). Moreover, mucins have long been implicated in the pathogenesis of cancer, particularly adenocarcinomas. As sites of tumor growth are often hypoxic, acidic, and laden with proteases and other biologically active factors, it is possible that tumors use mucins to configure the local microenvironment during invasion, metastasis, and growth in sites and conditions that might be inhospitable (Hollingsworth and Swanson 2004). Furthermore, mucins evidently make individual contributions to the biology and clinical features of some peritoneal malignancies, including PC from mucinous gastrointestinal adenocarcinomas and PMP.

In Sect. 5.2, we reported the capability of BR and NAC, in particular in combination, in significantly inhibiting the growth, proliferation, and survival of a panel of human gastrointestinal cancer cell lines and their potential in enhancing the cytotoxic effects of chemotherapeutic agents when used prior to or concomitant with chemotherapy. The cancer cells used include gastric and colon carcinoma

cell lines that differentially express MUC glycoproteins. Taking into consideration the significance of the expression of mucins as contributory factors in the pathophysiology of cancer, we intended in the next step to explore another feature of the treatment, that is to say whether and how BR/NAC treatment could alter the expression of mucin by gastrointestinal cancer cells. For this purpose, the expression status of three major mucins with known or highly suspected contributions to PC tumor biology and pathogenesis were evaluated using three different in vitro models of gastrointestinal carcinoma. MKN45 and KATO-III are two gastric models derived from metastatic carcinomas of undifferentiated and signet-ring cell type, respectively, and LS174T is a colon adenocarcinoma model with goblet cell-like phenotype. These cell lines represent experimental models for the expression of the major MUC glycoproteins. While MKN45 and KATO-III express both membrane-associated MUC1 and secreted MUC5AC mucins (Byrd et al. 2000; Linden et al. 2007), LS174T is a MUC1-negative cell line (Blockzijl et al. 1998) that expresses goblet cell-specific secreted mucins, including MUC2 (Bu et al. 2011; van Klinken et al. 1996). Results of this study are presented and discussed here (Amini et al. 2015a).

5.3.2 Results

5.3.2.1 Effect of BR/NAC Treatment of MKN45, KATO-III, and LS174T Cells on PAS-Stained Mucosubstances

First, we used Periodic Acid-Schiff (PAS) staining, known as the quintessential mucin histochemical technique (Corfield 2000), to investigate the effect of treatment on the production of mucosubstances. MKN45 cells were treated with two selected concentrations of single agent BR (100 and 200 $\mu\text{g/mL}$) and NAC (5 and 10 mM), and four combinations of the two for 48 h. Subsequently, untreated and BR/NAC treated cells were subjected to PAS staining. As a result of treatment, the amount of the PAS-positive substances was dramatically reduced in response to BR and NAC. The decrease in PAS-stained area was more prominent in BR and combination treatment groups, in particular in the latter where the amount of the remaining mucosubstances was minimal.

In a similar experiment, KATO-III were treated with two selected concentrations of single agent BR (50 and 100 $\mu\text{g/mL}$) and NAC (50 and 100 mM), and four combinations of the two for 48 h, and stained, along with their untreated controls, with PAS. As with MKN45, a decrease in the PAS-stained areas was evident after treatment, in particular in combination groups.

This experiment was also carried out on LS174T cells after BR (20 and 50 $\mu\text{g/mL}$) and NAC (5 and 10 mM) single agent and combination treatment and, as expected, similar results were obtained.

5.3.2.2 Effect of BR/NAC on Immunostained MUC1 Protein Expressed by MKN45 and KATO-III Cells

Next, we benefitted from the immunocytochemical staining technique to specify how the expression of different types of mucin probed by specific antibodies is affected by treatment. For this purpose, BR/NAC treated MKN45, KATO-III, and LS174T cells, as well as their untreated controls, were subjected to immunocytochemical analysis. In these experiments, positive (CTL+) and negative (CTL-) control groups were used for immunostaining, representing untreated cells exposed to or devoid of the primary antibody, respectively.

MUC1 is the most extensively studied membrane-associated mucin and the first to be characterized. Initially, we used anti-MUC1 specific antibody to probe the protein of interest expressed by the MUC1-expressing models (MKN45 and KATO-III) with or without treatment. In so doing, we treated MKN45 cells with two concentrations of single agent BR (100 and 200 $\mu\text{g/mL}$) and NAC (5 and 10 mM) and their combinations for 48 h before immunostaining for MUC1. MUC1 was strongly expressed in control group with cytoplasmic localization. Immunocytochemical expression of MUC1, however, was found to be reduced after BR/NAC treatment. This feature was found to be more prominent in association with combination therapy.

Next, we performed similar experiments on KATO-III and LS174T cells. KATO-III cells were specifically stained for MUC1 after treatment groups were treated with two concentrations of either single agent, BR (50 and 100 $\mu\text{g/mL}$) and NAC (50 and 100 mM), or their combinations. Untreated cells demonstrated strong expression of MUC1 with cytoplasmic localization. BR/NAC treatment, however, reduced the immunocytochemical expression of MUC1, in particular in combination treatment groups. As anticipated, LS174T cells did not appear to express MUC1 in a similar experiment.

5.3.2.3 Effect of BR/NAC on Immunostained MUC5AC Protein Expressed by MKN45, KATO-III, and LS174T Cells

MUC5AC is a gastric-type secreted mucin. All of the three cell lines used in the present study are known to express MUC5AC. In this regard, however, MKN45 and KATO-III are preferable to LS174T since they constitutively express MUC5AC at higher levels. Using anti-MUC5AC specific antibody, we then examined the expression of MUC5AC in BR/NAC-treated cells as compared to their untreated control. To this end, MKN45 cells were first to be immunohistochemically stained, with or without treatment with single agent or combined BR (100 and 200 $\mu\text{g/mL}$) and NAC (5 and 10 mM). As anticipated, untreated cells strongly expressed MUC5AC. In contrast, decreased expression of MUC5AC was evident in treated cells. Consistent with our earlier observations, an even more remarkable decrease in MUC5AC expression resulted from combined use of BR and NAC.

Finally, we treated LS174T cells with BR (20 and 50 $\mu\text{g/mL}$), NAC (5 and 10 mM) and their combinations, and similarly compared the immunocytochemical expression of MUC5AC in treated and untreated cells. LS174T expressed MUC5AC at much lower levels than did the other two models. BR/NAC treatment, however, consistently reduced the expression of MUC5AC. As a result, this mucin was barely detected in treatment groups.

5.3.2.4 Effect of BR/NAC on Immunostained MUC2 Protein Expressed by LS174T Cells

LS174T represents an excellent model for the expression of MUC2, known as an intestinal-type secreted mucin. To find out how MUC2 expression is affected by BR/NAC treatment, we examined the immunocytochemical expression of MUC2 in LS174T cells after treatment with BR (20 and 50 $\mu\text{g/mL}$), NAC (5 and 10 mM) and their combinations, and compared the resultant expression status with that in control groups. The results indicated that the strong expression by LS174T of MUC2 protein was attenuated by BR/NAC treatment. These findings are in agreement with our earlier results from a similar immunocytochemical study on the effect of the treatment on the expression of MUC1 and MUC5AC in the gastric models.

5.3.2.5 Western Blot Analysis of MUC1, MUC5AC, and MUC2 Proteins Expressed by Untreated and BR/NAC Treated MKN45, KATO-III, and LS174T Cells

Following the immunocytochemical study of MUC glycoproteins expressed by the three models, we intended to confirm our results by Western blot analysis of the expression of MUC1, MUC2, and MUC5AC. For this purpose, each model was first categorized into individual groups as per eight treatment regimens used in the previous mucin studies (PAS and ICC), and accordingly treated for 48 h. Then, Western blot detection of the proteins of interest was performed on treatment and control groups. As seen, BR/NAC treatment reduced the expression of MUC1 and MUC5AC in MKN45 and KATO-III cells, as well as of MUC2 and MUC5AC in LS174T. Results were then subjected to densitometric quantification and statistical evaluation by one-way ANOVA.

BR/NAC treatment resulted in statistically significant decreases in the expression of the proteins of interest in all cases, except for MUC1 in KATO-III cells in response to BR 50 $\mu\text{g/mL}$, and MUC5AC in LS174T cells treated with BR 20 and 50 $\mu\text{g/mL}$. A substantial majority of single agent regimens (18/24) and all combination regimens induced extremely significant decreases in mucin expression. In all three models, reductions in densitometric values of mucin expression induced by low-concentration BR and high-concentration BR+NAC represented the lowest and highest level of significance, respectively. The only exception was MUC2 expression in LS174T, on which the lowering effects of low-concentration NAC (5 mM) alone and in combination with high concentration BR (50 $\mu\text{g/mL}$) were associated with the lowest and highest level of significance, respectively.

In MKN45 model, 3 out of 4 combination regimens (BR 100 $\mu\text{g/mL}$ +NAC 10 mM, and BR 200 $\mu\text{g/mL}$ +NAC 5 and 10 mM) reduced the MUC5AC expression to the minimum. A drastic effect on MUC2 expression was also evident in LS174T model. Next, we used ANOVA test to figure out if and how statistically different from each other single agent and combination treatment affect the mucin expression. In MKN45 model, the statistical analysis of densitometric values for MUC1 and MUC5AC revealed significant differences between 12 out of 16 compared pairs in favor of combination therapy. In four pairs, including BR 200 $\mu\text{g/mL}$ with and without NAC 5 mM, NAC 5 mM with and without BR 100 $\mu\text{g/mL}$, NAC 5 mM with and without BR 200 $\mu\text{g/mL}$, and NAC 10 mM with and without BR 100 $\mu\text{g/mL}$, although combination therapy appeared to more effectively reduce MUC1 expression, the differences were not statistically significant.

The statistical difference between single agent and combination therapy values in 9 out of 12 pairs with significant change, including all eight pairs compared for their MUC5AC-lowering effect and one MUC1-lowering pair (NAC 10 mM with and without BR 200 $\mu\text{g/mL}$) received an extreme level of significance. As with MKN45, combined use of BR and NAC in KATO-III model reduced the expression of MUC1 and MUC5AC more than did either agent individually. The statistical advantage of combination therapy over single agent therapy in this model was significant, except for the following three pairs: BR 100 $\mu\text{g/mL}$ +NAC 50 mM vs. BR 100 $\mu\text{g/mL}$, BR 100 $\mu\text{g/mL}$ +NAC 100 mM vs. BR 100 $\mu\text{g/mL}$, and BR 50 $\mu\text{g/mL}$ +NAC 50 mM vs. NAC 50 mM. The statistical difference between single agent and combination therapy values in 10 out of 13 pairs with significant change, including all eight pairs compared for their MUC1-lowering effect and two MUC5AC-lowering pairs (BR 50 $\mu\text{g/mL}$ $\pm$ NAC 100 mM, and NAC 50 mM $\pm$ BR 100 $\mu\text{g/mL}$) reached an extreme level of significance.

In the third model, the lowering effect of treatment on LS174T expression of MUC2 and MUC5AC was found to be more significant in all combination treatment groups when compared with their single agent counterparts. As the only exception, however, the addition of NAC 10 mM apparently enhanced the MUC2-lowering effect of BR 50 $\mu\text{g/mL}$, but this was not found statistically significant. Extremely significant differences between the effect of single agent and combination therapy on the expression of MUC2 and MUC5AC were found in six (BR 20 $\mu\text{g/mL}$ $\pm$ NAC 5 mM, BR 20 $\mu\text{g/mL}$ $\pm$ NAC 10 mM, NAC 5 mM $\pm$ BR 20 $\mu\text{g/mL}$, NAC 5 mM $\pm$ BR 50 $\mu\text{g/mL}$, NAC 10 mM $\pm$ BR 20 $\mu\text{g/mL}$, NAC 10 mM $\pm$ BR 50 $\mu\text{g/mL}$) and four pairs (BR 20 $\mu\text{g/mL}$ $\pm$ NAC 5 mM, BR 20 $\mu\text{g/mL}$ $\pm$ NAC 10 mM, BR 50 $\mu\text{g/mL}$ $\pm$ NAC 5 mM, BR 50 $\mu\text{g/mL}$ $\pm$ NAC 10 mM), respectively.

5.3.2.6 Effect of Treatment on LS174T Secretion of MUC2 and MUC5AC Into Culture Media by ELISA

We detected and compared culture media levels of MUC2 and MUC5AC secreted by treated and untreated LS174T cells. In so doing, we initially categorized and treated LS174T cells according to eight treatment regimens, comprised of single agent BR (20 and 50 $\mu\text{g/mL}$), single agent NAC (5 and 10 mM) and their

combinations, for 48 h. Subsequently, the culture media of both treatment and control groups were collected and subjected to ELISA for detection of MUC2 and MUC5AC levels secreted by treated and untreated cells. The concentration values were then analyzed and statistically compared using ANOVA test. BR/NAC treatment dramatically decreased culture media levels of both secreted mucins. With only one exception indicating a lower level of significance (MUC5AC in response to NAC 5 mM), reductions in the secreted levels of MUC2 and MUC5AC were all extremely significant. This drastic effect was maximal in response to combination treatment, reducing MUC2 and MUC5AC levels to undetectable levels. Single agent treatment with high-concentration BR (50 µg/mL) and NAC (10 mM) also made MUC5AC levels undetectable.

5.3.3 Discussion

Mucins are physiologically expressed by various epithelial cells at mucosal, glandular, or luminal surfaces throughout the body. Mucins are categorized into membrane-associated and secreted types. Membrane-associated mucins are expressed at the apical surface glycocalyx of all wet-surfaced mucosal epithelia. These mucins lack N- and C-terminal cysteine-rich domains that allow homo-oligomerization. Instead, they contain an integral transmembrane domain and a short cytoplasmic tail through which they are tethered to the apical surface and associated with cytoplasmic molecules, including cytoskeletal elements and cytosolic adaptor proteins. Membrane-associated mucins are believed to serve as cell-surface receptors and sensors, and hence participate in signal transduction in response to changes in extracellular microenvironment and external stimuli that lead to coordinated cellular responses, including cell proliferation, differentiation and apoptosis, or secretion of specialized cellular products. They also associate with the secreted mucin layer by covalent and noncovalent bonds and contribute to physicochemical protection of the epithelial cell surface from adverse conditions (Gipson et al. 2014; Hollingsworth and Swanson 2004). As the first to be characterized, MUC1 is the most extensively studied membrane-associated mucin. In the gastrointestinal tract, MUC1 is expressed abundantly in the stomach and only in small amounts in the intestine (Linden et al. 2007). MUC1 is found in the surface foveolar cells in the entire stomach, in mucous neck cells and chief cells of the gastric fundus and antrum, as well as in the pyloric gland. In normal gastric mucosa, MUC1 is believed to protect gastric epithelial cells from a variety of external insults that cause inflammation and carcinogenesis [reviewed by (Saeki et al. 2014)].

Secreted mucins are divided into gel-forming and non-gel-forming subtypes. Gel-forming mucins contain N- and C-terminal cysteine-rich domains that are both involved in homo-oligomerization mediated by intermolecular disulfide bonds. Based on this capacity, gel-forming mucins comprise the major component of and impart viscoelastic properties to mucus. In contrast, non-gel-forming mucins are not able to oligomerize, and their structural and functional properties are not well

described (Linden et al. 2008). Secreted mucins are expressed by specialized cells of the epithelial tissues at such sites with secretory or absorptive functions as respiratory, digestive, and genitourinary system, as well as salivary glands, lacrimal glands, and eye (Forstner 1978). Secreted mucins have a central role in maintaining homeostasis in these sites and providing protection against their relatively harsh environments with diverse, variable conditions. They also contribute to the specialized functions of these organs. As such, gastrointestinal mucins allow secretion of the gastric acid and participate in the enteric defense system. Secreted mucins have also been shown to capture and hold biologically active molecules. As indicators of molecular or physical breach of the mucin layer, these molecules might trigger inflammatory or immune responses or incite repair or healing processes (Hollingsworth and Swanson 2004). MUC2, MUC5AC, and MUC6 are recognized as the classical gel-forming mucins. In the gastrointestinal tract, MUC2 and MUC5AC are the major components of mucus in the intestines and stomach, respectively. MUC2 and MUC5AC are synthesized by intestinal goblet cells and gastric foveolar cells in a site-specific manner. As a result, MUC5AC is expressed at low levels in the intestine, and MUC2, on the other hand, is absent in the stomach (Linden et al. 2007). MUC6 is another gel-forming mucin synthesized in the gastric mucosa by neck and gland cells. The mucous layer on the gastric surface consists primarily of MUC5AC extending in layered sheets with MUC6 in between (Ho et al. 2004). In other words, MUC6 is not a component of the surface mucus, and instead passes through transient channels formed in the surface mucus together with the gastric acid. As such, MUC5AC and MUC6 form a protective layer over the surface epithelium and act as a selective diffusion barrier for HCl (Bhaskar et al. 1992). Intestinal mucins, including membrane-associated and secreted mucins expressed by enterocytes and intestinal goblet cells, provide the first defense line of the gastrointestinal tract and interact with the immune system (Pelaseyed et al. 2014).

Mucins have been implicated in the pathophysiology of cancer. Malignant tumors, especially adenocarcinomas, express aberrant forms and/or amounts of mucins. At the simplest level, cancer cells use membrane-associated and secreted mucins in much the same way as normal epithelia to control their local microenvironment and to protect themselves from adverse growth conditions. Moreover, aberrant composition and structure of tumor-produced mucins enhance their growth and survival in otherwise inhospitable conditions. Aberrantly expressed mucins confer on tumor cells potential ligands for interaction with other receptors at the cell surface and contribute to their survival during invasion and metastasis. Through ligand–receptor interactions and morphogenetic signal transduction, membrane-associated mucins are believed to regulate differentiation and proliferation of malignant cells and to provide signals about adhesion status and presumably other cell-surface conditions. Membrane-associated mucins contribute to the invasive and metastatic properties of adenocarcinomas by simultaneously configuring the adhesive and anti-adhesive properties of tumor cell surface. Moreover, the mucus layer covering tumor cells is believed to serve as an impenetrable physicochemical barrier that helps them evade immune and inflammatory responses. In so doing, it is

hypothesized that the viscous mucin coating, equipped with several ligands for adhesion molecules as well as with sequestered suppressive cytokines, prevents the approach of antigen-presenting and effector cells and suppresses their motility and activation. This mucus layer is also thought to capture biologically active molecules, including growth factors or cytokines, which might contribute to tumor growth [reviewed in (Hollingsworth and Swanson 2004)]. In addition, many lines of evidence support the involvement of both membrane-associated and secreted mucins in diverse biological mechanisms underlying resistance to chemotherapy, including their implications in physical barrier formation, resistance to apoptosis, drug metabolism, cell stemness and EMT. This aspect has been recently revisited (Jonckheere et al. 2014; Nath and Mukherjee 2014; Singh and Settlemann 2010) and will be discussed in a separate section.

In the present study, three *in vitro* models of gastrointestinal carcinoma were employed with respect to the expression of membrane-associated and secreted mucins. Our two gastric models represent two distinct types of gastric carcinoma. MKN45 has been established from a poorly differentiated adenocarcinoma of medullary type. Linden et al. reported that MKN45 is a gastric model expressing a combination of gastric mucins that are physiologically expressed by individual cell types (Linden et al. 2007). They concluded that MKN45 cells most closely mimic gastric mucin expression, as they express MUC1 and MUC5AC, but not MUC2 and MUC3 normally expressed in the intestinal mucosa. KATO-III has been derived from a gastric SRCC. SRCC is a unique subtype of mucin-producing adenocarcinoma that primarily arises in the stomach, followed in order by the colorectum and lung. SRCC cells are characterized by abundant intracytoplasmic mucins, ample and clear cytoplasm, and eccentrically located nuclei compressed by intracytoplasmic mucins (Terada 2013). Similar to MKN45, KATO-III cells were reported to express MUC1 and MUC5AC, but not MUC2 (Byrd et al. 2000; Matsuda et al. 2008). The third model, LS174T, is a colon adenocarcinoma cell line shown to be MUC1-negative (Blockzijl et al. 1998). Van Klinken et al. and, more recently, Bu et al. have investigated the mucin expression profile of LS174T at mRNA and protein levels, and concluded that LS174T is as an excellent model for the expression of the intestinal goblet cell-specific secreted mucins, of which MUC2 is most prominently expressed (Bu et al. 2011; van Klinken et al. 1996). We used two different techniques, ICC and Western blot, to evaluate the expression of MUC1, MUC2, and MUC5AC in these models. In line with earlier studies, our immunocytochemical and Western blot analyses demonstrated that MKN45 and KATO-III express both MUC1 and MUC5AC. MUC1, which is expressed at the apical surface of normal cells, indicated a cytoplasmic localization in both models. This aberrant localization results from the loss of cell polarity and redistribution of MUC1 over the cell surface and within the cytoplasm (Gendler 2001). While LS174T expressed MUC2 and MUC5AC at high and low levels, respectively, it did not express MUC1. This observation is compatible with previous descriptions of this model.

A large body of evidence indicates that the prototypical membrane-associated and secreted mucins MUC1, MUC2, and MUC5AC are implicated in the pathogenesis of human carcinomas. The current data provides support for an oncogenic role

of MUC1 (Hatstrup and Gendler 2006; Wei et al. 2007). Taking into account its apparent involvement in such key biological processes as proliferation, survival, differentiation, invasion, adhesion, angiogenesis, apoptosis inhibition, chemoresistance, tumor metabolism, and inflammation, MUC1 is believed to play critical roles in carcinogenesis and, more likely, in cancer progression and metastasis [reviewed in (Nath and Mukherjee 2014)]. MUC1 is a key modulator of several signaling pathways that affect biological behavior of malignant cells. It mediates production of growth factors such as connective tissue growth factor (CTGF) (Behrens et al. 2010) and platelet-derived growth factor (PDGF) (Hatstrup and Gendler 2006), and activates mitogen-activated protein kinase (MAPK) (Besmer et al. 2011) and phosphoinositide 3-kinase (PI3K)/Akt (Raina et al. 2004) signaling and hence potentiates proliferation and survival of tumor cells. It mediates activation and translocation to the nucleus of EGFR, promoting G1/S phase gene expression (Bitler et al. 2010). MUC1 also regulates hypoxia-inducible factor 1 α (HIF-1 α)-mediated expression of PDGF-A to induce proliferation and invasion of malignant cells (Sahraei et al. 2012). In addition, it is evident that a fragment of the MUC1 cytoplasmic tail can be transported to the nucleus in association with β -catenin, where it might facilitate activation of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors through Wnt signaling pathway which in turn regulate several genes related to cell proliferation and differentiation (Wen et al. 2003; Hollingsworth and Swanson 2004). As such, MUC1 has been shown to promote transcriptional (Roy et al. 2011; Horn et al. 2009) and posttranscriptional (Mohr et al. 2013; Rajabi et al. 2014) induction of EMT, the biological process by which cancer cells acquire invasive and metastatic potential (Kalluri and Weinberg 2009). Similarly, it confers self-renewal capacity and inhibits terminal differentiation of acute myeloid leukemia (AML) cells (Yin et al. 2011). Recent data also suggests that MUC1 regulation of miRNAs, including miR-145, helps cancer stem cells (CSCs), a subset of tumor cell population characterized by an extensive self-renewing capacity (Greaves and Maley 2012), remain in a dedifferentiated state, thereby contributing to tumor recurrence and metastasis [reviewed in (Nath and Mukherjee 2014) and (Jonckheere et al. 2014)]. In agreement, MUC1 was shown to upregulate the expression of the breast CSC marker aldehyde dehydrogenase 1A1 (ALDH1A1) (Alam et al. 2013), and to be expressed by CD34+ CD38- AML cells which have been associated with CSCs (Stroopinsky et al. 2013). Furthermore, the association of MUC1 with β catenin (Singh et al. 2007) and Cbl-interacting protein of 85 kDa (CIN85) (Cascio et al. 2013) and resultant enhancement of the migratory and invasive potential of cancer cells has been reported. Furthermore, overexpressed MUC1 can also inhibit integrin-mediated cell adhesion to extracellular matrix components and thus increase cancer cell invasiveness (Wesseling et al. 1995). The anti-adhesive properties of the overexpressed MUC1 also prevents tumor cells from conjugating with the effector cells of the immune system and allows them to evade immune surveillance (van de Wiele-van Kemenade et al. 1993). On the other hand, aberrantly glycosylated MUC1 exposes some epitopes otherwise masked in the normal mucin and expresses a number of aberrant glycans. These antigens, including TF, Tn, STn, and sLe^x, are believed to facilitate tumor invasion and metastasis (Taylor-Papadimitriou et al. 1999).

This process, for example, provides tumor cells with new ligands for adhesion molecules, including selectins (Mann et al. 1997) and intercellular adhesion molecule 1 (ICAM-1) (Hayashi et al. 2001). MUC1 also assists cancer cells in evading cell death by preventing the activation of the intrinsic apoptotic pathway. As such, it can decrease intracellular ROS levels (Yin et al. 2003), inhibit hypoxia-induced cell death (Yin et al. 2007), selectively upregulate the expression of the anti-apoptotic protein B-cell lymphoma extra-large (Bcl-xL), and inactivate the proapoptotic protein Bcl2-associated agonist of cell death (Bad) (Raina et al. 2004). MUC1 also increases resistance to chemotherapeutic drugs by participating in the formation of a cellular barrier, with overexpressed MUC1 covering the entire cell surface (Kalra and Campbell 2007), as well as by upregulating multidrug resistance (MDR) genes (Nath et al. 2013) and contributing to cancer cell stemness and EMT (Jonckheere et al. 2014). Through inducing the expression of growth factors with proangiogenic effects (Kitamoto et al. 2013), including VEGF (Woo et al. 2012) and CTGF (Behrens et al. 2010), MUC1 has been shown to enhance tumor angiogenesis. Moreover, direct and indirect influences of MUC1 on tumor metabolism—and hence cancer cell growth and proliferation—through regulation of glucose and lipid metabolism, carbon flux, cellular energy state, and survival under hypoxic and nutrient-deprived conditions, have been reported in the literature [reviewed in (Mehla and Singh 2014)]. Finally, it is postulated that MUC1, serving as an anti-inflammatory molecule in gastric mucosal cells, plays an opposite role in cancer-related inflammation (Nath and Mukherjee 2014), where smoldering inflammation in the tumor microenvironment enhances proliferation and survival of malignant cells, promotes angiogenesis and metastasis, suppresses adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (Mantovani et al. 2008). In a study by the National Cancer Institute (NCI) for prioritization of cancer vaccine target antigens, MUC1 was ranked as the second best potential target among 75 tumor-associated antigens for immunotherapy (Cheever et al. 2009).

The role of MUC1 in GC and CRC has been explored in a number of studies. Contrary to its protective function in normal gastric mucosa, MUC1 is believed to act as an oncogene/oncoprotein in GC. The association between single-nucleotide polymorphisms (SNPs) in *MUC1* gene and GC in different ethnic populations strongly supports the notion that *MUC1* is a GC susceptibility gene (Saeki et al. 2014). Moreover, while *MUC1* is silenced in the complete type of the intestinal metaplasia (IM), the precursor lesion for GC, it is strongly expressed in incomplete IM, in which progression to dysplasia and carcinoma is more common (Reis et al. 1999), and frequently reactivated in GC (Saeki et al. 2014; Ho et al. 1993). This is consistent with the observation that growth and invasiveness of GC cells and xenografts are increased after MUC1 transfection (Suwa et al. 1998). In addition, *MUC1* polymorphism was found to be associated with the expression of the cancer-associated TF antigen (Santos-Silva et al. 2005), a marker of progression and prognosis in early GC (Baldus et al. 2001). Role of MUC1 as an oncoprotein has been specifically explored in *Helicobacter pylori*-associated GC wherein MUC1 was shown to interact with β -catenin, resulting in modulation of Wnt signaling pathway and upregulation of cyclin D1 (Udhayakumar et al. 2007). Despite the fact that

MUC1 expression in GC decreases with higher tumor stage (Boltin and Niv 2013; Ilhan et al. 2010) and immunohistochemical studies relate MUC1 expression to a better differentiation status of tumor cells since it can be considered as a differentiation marker, association of MUC1 expression with invasive features and worse prognosis has been reported in most of these studies (Kocer et al. 2004; Lee et al. 2001; Reis et al. 1998; Utsunomiya et al. 1998; Wang et al. 2003; Tamura et al. 2012). In the context of CRC, increased expression of MUC1, along with differential expression of MUC2, in premalignant adenomas is associated with an increased risk for malignant transformation (Ajioka et al. 1997; Ho et al. 1996; Li et al. 2001a). In addition, the carbohydrate epitope sLe^x located on MUC1 (Hanski et al. 1993), as well as on MUC2 (Hanski et al. 1995), has been identified as a marker of dysplasia in the colonic adenoma-carcinoma sequence that is overexpressed in 90 % of carcinomas (Hanski et al. 1990; Hanisch et al. 1992). As a ligand for E selectin and P selectin adhesion molecules, sLe^x was found to be overexpressed during, and contribute to, the CRC progression and metastasis (Izumi et al. 1995; Mann et al. 1997). Moreover, MUC1 was found to promote survival of colon cancer cells under hypoxia (Yin et al. 2007). In line with these findings, immunohistochemical studies have shown that MUC1 expression is as a marker of progression and metastasis of human CRC (Hiraga et al. 1998; Nakamori et al. 1994). MUC1 is under investigation as a potential target of immunopreventive approaches in CRC (Kimura et al. 2013).

Known as the prototypical secreted mucin specifically expressed in the small intestine and colon, MUC2 apparently plays a tumor-suppressing role in normal mucosa. In so doing, MUC2 is postulated to prevent colonization of the intestinal tract with pro-carcinogenic microbial flora, to provide a physical barrier to dietary carcinogens, to protect against a pro-carcinogenic wound-repair phenotype, and to cooperate with membrane-associated mucins in the regulation of the differentiation and proliferative status of the epithelia (Hollingsworth and Swanson 2004). In agreement, *Muc2*-knockout mice frequently developed tumors of the small intestine, colon, and rectum (Velcich et al. 2002). Nevertheless, MUC2 expression has been variably implicated in gastrointestinal cancers. Constitutional genetic variability analysis of *MUC1*, *MUC2*, and *MUC6* in patients with GC precursor lesions followed up for more than 12 years has revealed that genetic variation in *MUC2*, but not in *MUC1* and *MUC6*, is significantly associated with evolution of these lesions, in particular in *H. pylori*-infected patients (Marín et al. 2012). In agreement, while MUC1, MUC5AC, and MUC6 are markedly reduced in complete IM, the strong, uniform, de novo expression of MUC2 is evident in both complete and incomplete types of IM (Reis et al. 1999). Similarly, de novo expression of MUC2 is frequently seen in both diffuse and intestinal types of GC (Ho et al. 1993; Ilhan et al. 2010). MUC2 expression in GC was reported to be associated with a favorable outcome (Utsunomiya et al. 1998) and progressively lost with increasing tumor stage (Boltin and Niv 2013; Ilhan et al. 2010). Nevertheless, the intestinal mucin phenotype of GC, characterized by isolated MUC2 expression, was found to be correlated with metastatic disease and the worst survival (Wakatsuki et al. 2008). In agreement, MUC2 expression in peritoneal washings was identified as a marker for peritoneal

recurrence of GC following surgery in the absence of overt metastasis (Satoh et al. 2012). In colorectal tumors, MUC2 was found to be expressed more frequently, and at higher levels, in adenomas than in carcinomas. As regards the role of MUC2 expression in malignant transformation of colorectal neoplasia, different studies have come to contradictory conclusions. Ajioka et al. characterized high-grade atypia in both flat and polypoid adenomas by reduced MUC2 and increased MUC1 expression (Ajioka et al. 1997). Similarly, Li et al. related this phenotype to malignant transformation of colorectal neoplasia where decreased MUC2 expression was correlated with increased Ki-67 and tumor cell proliferative activity, and implicated in the progression of colorectal adenomatous change (Li et al. 2001a). In contrast, Ho et al. reported that increased expression of both MUC1 and MUC2 occurs in adenomatous polyps and is associated with an increased risk for malignant transformation (Ho et al. 1996). MUC2 is overexpressed in colorectal adenocarcinomas of all histological subtypes, with the greatest increase observed in colloid (mucinous) tumors (Ho et al. 1993). The current data collectively supports the notion that higher expression of MUC2 and MUC5AC, but a lower expression of MUC1, confers genetic and molecular features on CRC that incurs adverse consequences in relation to treatment response and clinical outcome (Conze et al. 2010). In the most comprehensive study of mucin protein expression in CRC by Walsh et al., overexpression of MUC2 was found in 33 % of cases. This also included tumors without mucinous differentiation. The investigators reported that the expression of MUC2, as well as *de novo* expression of MUC5AC and MUC6, was strongly associated with molecular somatic events involved in the serrated neoplasia pathway, including methylator positivity, somatic BRAF p.V600E mutation, and mismatch repair deficiency, as well as with clinicopathological features such as proximal location, poor differentiation, lymphocytic response, and increased T stage. MUC2 has also been identified as a major carrier of the cancer-associated sialyl Tn antigen, which is aberrantly expressed in premalignant lesions and carcinomas of the gastrointestinal tract and serves as an independent indicator of poor prognosis (Conze et al. 2010). Similarly, MUC2 is known to carry sLe^x antigen, overexpression of which is implicated in CRC metastasis.

The major gastric-secreted mucin MUC5AC, too, is differentially expressed and variably implicated in gastrointestinal neoplasms. While the complete type of gastric IM exhibits markedly reduced expression of MUC5AC, its strong, uniform expression is found in incomplete IM (Reis et al. 1999). As a marker of the gastric phenotype, MUC5AC is also expressed in GC, reportedly at higher levels in intestinal type than in diffuse type (Ilhan et al. 2010). Nevertheless, the clinicopathological relevance and prognostic significance of the gastric mucin phenotype remain a matter of controversy. In this regard, MUC5AC expression was reported to progressively decrease with the loss of tumor differentiation, as well as with increases in the tumor invasion depth and the number of metastatic lymph nodes (Ilhan et al. 2010). In agreement, MUC5AC expression was found to be associated with a favorable prognosis (Kim et al. 2013; Wang et al. 2003). In contrast, other investigators reported that the expression of a gastric phenotype is associated with increased risk of invasion and metastasis (Koseki et al. 2000), and a poorer outcome (Tajima et al. 2001).

In addition, although gastric phenotype is commonly indicative of a well- or moderately differentiated cancer, tumors with this mucin phenotype often change histologically into a SRCC or a poorly differentiated adenocarcinoma (Namikawa and Hanazaki 2010). MUC5AC is rarely expressed, and only by a minority of goblet cells, in the normal colon. However, it is frequently expressed in colorectal adenomas and carcinomas (Byrd et al. 1998). Bara et al. provided evidence that M1 antigen, an early oncofetal marker of colonic carcinogenesis, is indeed the product of the MUC5AC gene (Bara et al. 1998). They consistently reported later that M1/MUC5AC mucin is abnormally expressed by colonic goblet cells during colon carcinogenesis (Bara et al. 2003). The *de novo* expression of MUC5AC, together with low levels of MUC6, is added to the normal pattern of high MUC2 and low MUC5B during the adenoma–carcinoma sequence in CRC (Corfield 2015; Buisine et al. 1996; Myerscough et al. 2001; Bartman et al. 1999). In their recent study on a large series of CRC tumors, Walsh et al. demonstrated the ectopic (*de novo*) expression of MUC5AC in around 50 % of all cases (Walsh et al. 2013). In this study, the expression of MUC5AC, along with MUC2 and MUC6, was strongly associated with tumorigenic features and adverse clinicopathological characteristics.

With a mucin production pattern distinct from other mucinous adenocarcinomas, SRCCs of the stomach and colorectum indicate differential mucin expression profiles and clinicopathological associations. By contrast with mucinous carcinomas characterized by the abundant production of extracellular mucins, SRCC demonstrates a unique cytological feature in which the so-called signet-ring cells, comprising more than 50 % of tumor cells, are identified by abundant intracytoplasmic mucin and hence peripherally displaced nuclei (Bosman et al. 2010). Up to 99 % of gastrointestinal SRCCs are found in the stomach (Maehara et al. 1992). With respect to mucin expression profile, Terada found that primary gastric SRCC expressed MUC5AC (67 %) and MUC6 (70 %) more frequently than MUC1 (10 %) and MUC2 (13 %), whereas primary colorectal SRCC tended to primarily express MUC2 (92 %) and less frequently MUC1 (42 %) and MUC5A (33 %), but did not express MUC6 (Terada 2013). Clinicopathological relevance of signet-ring cell differentiation has been reported in the literature. In this regard, gastric SRCC has long been thought to have a worse prognosis than other forms of GC. As such, when the clinicopathological features and prognosis of advanced SRCC were compared with those of non-signet-ring cell adenocarcinoma, Li et al. concluded that SRCC tends toward deeper tumor invasion, more lymph node and peritoneal metastasis, and worse prognosis (Li et al. 2007a). In agreement, Piessen et al. provided evidence that signet-ring cell histology is a major and independent predictor of poor prognosis due to specific characteristics such as more infiltrating tumors showing affinity for lymphatic tissue accompanied by a higher rate of peritoneal carcinomatosis (Piessen et al. 2009). Similarly, when mucinous gastric carcinomas were divided into two histological subtypes based on the predominant differentiation of tumor cells in mucin pool, Choi et al. indicated that signet-ring cell subtype more frequently metastasized to distant sites, and that signet-ring cell differentiation was associated with poorer disease-specific survival (Choi et al. 2011). This notion, however, has been debated by some investigators, reporting that gastric SRCC

presents with more advanced disease, yet, when stratified for stage, fails to predict a poorer outcome (Taghavi et al. 2012; Zhang et al. 2010). Colorectal SRCC has also been shown to behave more aggressively than ordinary adenocarcinoma of the colon (Thota et al. 2014; Tung et al. 1996). Supported by gene expression studies, the clinicopathological relevance and epidemiologic distinction of signet-ring cell differentiation have led to the notion that SRCC may be a completely distinct entity wherein signet-ring cells arise from a separate genetic pathway (Borger et al. 2007; Shah et al. 2011; Taghavi et al. 2012).

In addition, the secretion of gel-forming mucins, in particular MUC2 and MUC5AC, is believed to influence the pathogenesis of neoplastic conditions in a context-dependent manner. Of these two, MUC2 is more extensively glycosylated and therefore sterically occupies a greater volume on an equimolar basis (O'Connell et al. 2002b). High levels of MUC2 are expressed in indolent pancreatobiliary neoplasms with a favorable prognosis (Yonezawa et al. 2011) as well as in indolent mucinous carcinomas of the breast (Matsukita et al. 2003). The expression of highly viscous MUC2 in these tumors may act as a barrier to tumor progression, confining tumor growth to the primary site and restricting its invasion and metastasis (Yonezawa et al. 2011). In contrast, MUC2 can be a major contributor to the pathogenesis of mucin-secreting tumors of gastric and colorectal origin and their peritoneal spread, among which PMP is prototypical. PMP is a disease of intestinal goblet cells originated from a primary appendiceal mucinous adenoma that gain access and ectopically secrete MUC2, as well as MUC5AC, to the peritoneal cavity (O'Connell et al. 2002b). MUC2 is the PMP-specific mucin that is responsible for the high degree of gelation. In this context, the copious secretion of MUC2 not only gives rise to the formation of the appendiceal mucocoele and subsequent release of tumor cells, and allows them to freely move, circulate, and redistribute within the peritoneal cavity (Sugarbaker 1994), but also plays the key role in the development of morbidity and major complications in this condition. The abundant formation of multifocal, viscous gels increases the intra-abdominal pressure, compresses visceral organs, and triggers inflammatory and fibrotic responses with fatal consequences, including bowel obstruction (Sugarbaker 1996c). Since MUC2 expression in PMP is apparently independent of the degree of malignant transformation and subject to epigenetic regulation, it has been suggested as a biological marker and a therapeutic target in this condition (O'Connell et al. 2002a, b). The involvement of the secreted mucins in the pathophysiology of PMP, with emphasis on the role of MUC2, has been reviewed in the first chapter as well as in our relevant published article (Amini et al. 2014b).

Both membrane-associated and secreted mucins have been implicated in the development of chemoresistance through involvement in the formation of a physical barrier, resistance to apoptosis, drug metabolism, cell stemness, and EMT [reviewed in (Jonckheere et al. 2014)]. The outstanding web formed by the secreted mucus as well as the extraordinary size of the heavily glycosylated membrane-associated mucins suggest that mucins are capable of limiting drug absorption. For this purpose, mucins act as either size filters allowing entrance of particles smaller than the mucus network porosity, or interaction filters via electrostatic or hydrophobic forces

(Shaw et al. 2005; Sigurdsson et al. 2013). Mucins may also reduce the sensitivity of cancer cells to genotoxic drugs. In this sense, the role of MUC1 as an apoptosis inhibitor was explained earlier. In agreement, MUC1 was shown to attenuate release of mitochondrial apoptotic factors, activation of caspase-3, and subsequent induction of apoptosis in cisplatin-treated CRC cells (Ren et al. 2004). Moreover, mucins can alter the metabolism of the chemotherapeutic agents. As such, MUC1 was found to upregulate a number of MDR genes, product of which function as ATP-dependent drug efflux pumps (Nath et al. 2013). A growing body of evidence suggests a relationship between EMT, CSCs, and drug resistance, wherein mesenchymal differentiation of tumor cells or stem cell-like features of CSCs gives rise to the emergence of tumor cells with de novo or acquired chemorefractoriness [reviewed in (Singh and Settleman 2010)]. MUC1 is able to trigger the molecular process of EMT, which in turn contributes to acquisition or selection of stem/progenitor cell-like features and ultimately promotes drug resistance and recurrence. Similarly, it is extrapolated from recent studies that MUC1 somehow enables CSCs to maintain their “stemness” and hence contribute to tumor relapse after therapy (Nath and Mukherjee 2014; Jonckheere et al. 2014).

Of particular interest in the present study was the effect of BR/NAC treatment on mucin production and specific mucin expression profiles in the aforementioned models of gastric and colon carcinoma. In this regard, mucosubstances produced by treated and untreated cells were initially visualized using PAS staining, known as the quintessential mucin histochemical technique (Corfield 2000). We observed that BR and NAC significantly reduced the amount of PAS-stained substances in all the three models. This effect was most pronounced in combination treatment where PAS-positive areas were reduced to the minimum. To specify how the expression of individual mucin glycoproteins in each model is altered in response to the treatment, we used specific antibodies to probe MUC1, MUC2, and MUC5AC. Results from ICC and Western blot expression analyses consistently showed that BR and NAC, on their own and, more remarkably, in combination, reduced the expression of MUC1 and MUC5AC in the two gastric models. Adenocarcinoma cell line MKN45 and signet-ring cell line KATO-III both express MUC1 and MUC5AC and represent gastric carcinoma models with a gastric mucin phenotype. As discussed earlier, emerging evidence shows that MUC1 functions as an oncogene/oncoprotein and induces a chemoresistant phenotype. In addition, a gastric phenotype, identified by the expression of specific markers, including MUC5AC, and a signet-ring cell differentiation could per se portend more invasive features. Likewise, the drastic reductions of MUC2 and MUC5AC in response to BR/NAC treatment were evident in the colorectal model LS174T. Consistently, ELISA revealed decreases in the levels of MUC2 and MUC5AC mucins secreted by treated cells to the culture media. The current data suggests that increased expression of MUC2 and de novo expression of MUC5AC are of clinicopathological relevance in CRC. With a goblet cell phenotype, LS174T also represents a PMP-like model in which MUC2 is a key pathophysiological component. As the principal gastrointestinal mucins capable of forming viscous gels, MUC2 and MUC5AC can similarly play important roles in the pathogenesis and resilience of other mucin-secreting gastrointestinal tumors.

Besides, they participate in the formation of an efficient barrier against chemotherapeutic drug absorption. Thus, mucin-depleted tumor cells are in fact deprived of a key biological infrastructure and a protective framework. Given the critical roles of mucins in the biology of epithelial tumors, the mucin-depleting feature of BR/NAC treatment is postulated to largely contribute to the induction of cytotoxicity (Sect. 5.1) and chemosensitivity (Sect. 5.2) in our *in vitro* models. Meanwhile, this capability supports the use of this formulation in locoregional approaches for reducing the adverse effects of the aberrantly secreted gel-forming mucins, as in PMP and similar pathologies with ectopic production of mucin.

In agreement with such a mucin-depleting effect, BR and, more extensively, NAC have been reported to reduce the expression and/or secretion of mucins/mucus in different pathological conditions. Both agents are known as natural mucolytics. Mucolytic activity of BR is thought to be driven by its proteolytic enzymes breaking down complex proteins or directly acting on the naked peptide region of mucus glycoproteins (Hechtman 2012). To this end, although the enzymatic activity and substrate spectrum of BR are broad, it preferentially cleaves glycyl, alanyl, and leucyl bonds (Maurer 2001). BR's effectiveness in respiratory tract diseases, including pneumonia, bronchitis, and sinusitis, has been attributed in part to its mucolytic activity (Rimoldi et al. 1978). Suzuki et al. reported viscosity-lowering effect of BR on rabbit sputum *in vitro* and *in vivo*, thereby supporting the utility of BR as a mucoactive agent (Suzuki et al. 1983). Bernkop-Schnurch et al. observed high mucolytic activity of BR on porcine gastric mucin *in vitro* (Bernkop-Schnurch et al. 2000). Similarly, Bara et al. used BR for enzymatic proteolysis of the native gastric M1 mucin (Bara et al. 1998). Likewise, incubation of cervical mucus with BR results in complete liquefaction of the gel (Ingerslev and Poulsen 1980; Shulman et al. 1980). In support of our results, there is evidence that BR has the potential to inhibit the expression and production of secreted mucins as well. As an anti-inflammatory agent, BR has been shown to inhibit the production of such eicosanoids as prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) (Gaspani et al. 2002; Vellini et al. 1986). On the other hand, the inflammatory milieu of the intestinal tract is highly conducive to mucin promoter upregulation (Choudry et al. 2012). As such, COX-2/PGE2 pathway is known to mediate MUC2 and MUC5AC expressions at both the mRNA and protein levels (Gray et al. 2004; Kim et al. 2002). In agreement, Choudry et al. showed that anti-inflammatory drugs dexamethasone and celecoxib significantly reduced the MUC2 mRNA levels in LS174T cells (Choudry et al. 2012). It is thus postulated that BR-induced inhibition of MUC2 and MUC5AC observed in the present study is linked to its anti-inflammatory effects, including COX-2/PGE2 inhibition. This aspect needs to be addressed in future research.

NAC is a mucoactive agent with both mucolytic and mucoregulatory functions. Initial studies in the early 1960s reported clinical benefits of NAC as an effective mucolytic for the liquefaction of tenacious tracheobronchial secretions in suppurative pulmonary conditions, in particular cystic fibrosis (Hurst et al. 1967; Webb 1962). Since then, mucolytic activity of NAC has been evaluated in a variety of respiratory diseases with mucus hypersecretion, including chronic obstructive pulmonary disease (COPD) [reviewed in (Sadowska 2012)]. NAC has shown similar

effects on mucins secreted by the gastrointestinal tract (Chen et al. 2013; De Lisle et al. 2007; Iiboshi et al. 1996; Corne et al. 1974; Kistler et al. 2012; Hong et al. 2014; Hu et al. 2012; Ozdil et al. 2011) and eye (Thermes et al. 1991; Urashima et al. 2004; Anderton and Tragoulias 1998). As a mucolytic, NAC works through depolymerization of the mucin glycoprotein oligomers by hydrolyzing the disulfide bonds that link the mucin monomers (Sheffner 1963). Hence, NAC treatment results in decreased viscoelasticity (Sheffner et al. 1964; Seagrave et al. 2012; Sun et al. 2002), and increased transportability (Sun et al. 2002) and penetrability (Suk et al. 2011) of the tracheobronchial secretions and sputum. NAC also proved to exhibit viscoelasticity-lowering effects on porcine gastric mucin, *in vitro* (Misawa and Imamura 1988). Besides, NAC is known as a mucoregulatory agent that controls mucin secretion at mucosal surfaces. In this regard, and in line with our results, NAC reportedly reduces mucin hypersecretion and goblet cell hyperplasia in the airways in response to the cigarette smoke (Rogers et al. 1988, 1989), ovalbumin (Park et al. 2009), and bleomycin exposure (Mata et al. 2003), and decreases mucin production and retention in the eye (Urashima et al. 2004). In so doing, NAC appears to inhibit EGFR-induced MAPK activity (Takeyama et al. 2000; Urashima et al. 2004) and NF κ B (Mata et al. 2011; Blesa et al. 2003). With respect to the secreted, gel forming mucins, NAC treatment was shown to hamper the expression of MUC5AC, the predominant mucin in the airways, by MUC5AC-expressing human lung carcinoma cells NCI-H292 (Takeyama et al. 2000) and Calu-3 (Sprenger et al. 2011). Similarly, NAC prevented cigarette smoke extract (CSE)-induced expression of MUC5AC by NCI-H292 cells (Baginski et al. 2006). Further, Mata et al. indicated that NAC reduced Muc5ac expression, at mRNA and protein levels, and secretory epithelial hyperplasia in the rat model of bleomycin-induced pulmonary fibrosis (Mata et al. 2003). Consistently, increased expression of Muc5ac induced by allergen challenge in rats was abolished by NAC (Blesa et al. 2003). In accord with these observations, NAC has been reported to inhibit the expression of airway mucins, particularly MUC5AC, induced by viral (Mata et al. 2011, 2012) or bacterial infections (Sprenger et al. 2011; Hauber et al. 2007), as well as by benzo[a]pyrene (B[a]P) (Chiba et al. 2011), acrolein (Choi et al. 2011), IL-9 (Hauber et al. 2008), and IL-13 stimulation (Seagrave et al. 2012). As a mucoregulator, on the other hand, NAC was shown by Amrouche-Mekkioui et al. to restore mucin depletion of colonic mucosa in a murine model of chronic colitis, with potential benefits in inflammatory bowel diseases and a protective role in CRC (Amrouche-Mekkioui and Djerdjouri 2012). Together, it is concluded that NAC is a mucolytic agent that also has the potential to regulate the expression of mucins in a content-dependent manner.

Since BR and NAC use different mechanisms to disrupt molecular structure of the mucin glycoproteins, combined use of the two was hypothesized to give rise to the enhancement of mucolysis. In this regard, a parallel study carried out in our Department consistently revealed that an optimized combination of BR and NAC is mucolytically more potent than either agent individually and has the potential to effectively disintegrate PMP-secreted mucin gels (Akhter et al. 2014; Pillai et al. 2014a). The resultant mucolysis was evident not only *ex vivo*, but also after intraperitoneal treatment of the peritoneally implanted mucins in nude rats. In agreement,

sparse evidence from case reports supports the benefits of NAC-induced mucolysis in the treatment of hepatobiliary conditions, such as primary sclerosing cholangitis (PSC) (Ozdil et al. 2011) and intraductal papillary mucinous neoplasm (IPMN) of the bile duct (Hu et al. 2012; Hong et al. 2014). Taken together, mucolytic activity of BR and NAC, enhanced in combination therapy, holds promise for use in locoregional strategies for the management of pathological or ectopic mucin production.

In conclusion, aberrant expression of membrane-associated and secreted mucins, as evident in epithelial tumors, is known to facilitate tumor growth, progression, and metastasis, and to provide protection against adverse growth conditions, chemotherapy, and immune surveillance. Emerging evidence provides support for the oncogenic role of MUC1 in gastrointestinal carcinomas and relates its expression to an invasive phenotype. Similarly, mucinous differentiation of gastrointestinal tumors, in particular increased or de novo expression of MUC2 and/or MUC5AC, is widely believed to imply an adverse clinicopathological feature. Through forming viscous gels, too, MUC2 and MUC5AC significantly contribute to the biology and pathogenesis of mucin-secreting gastrointestinal tumors. BR/NAC combination treatment shows potent mucin-depleting activity, effectively reducing the expression of MUC1, MUC2, and MUC5AC mucins in mucin-expressing models of GC and CRC, *in vitro*. Taken together, results from our *in vitro* studies suggest that a combination of BR and NAC with dual effects on mucin-expressing tumor cells and their mucin products is a promising candidate toward the development of novel approaches to gastrointestinal malignancies with the involvement of mucin pathology. The efficacy of this treatment was subsequently tested in two relevant animal models, which will be discussed in Sect. 5.4.

5.4 Efficacy of Intraperitoneal Administration of BR/NAC in Two Animal Models of Peritoneal Dissemination of Human Gastric and Colon Carcinoma

5.4.1 Introduction

Peritoneal dissemination results from the propensity to neoplastic growth on peritoneal surface of a group of primary or secondary tumors that eventually gives rise to the development of PSMs. Three models of pathogenesis have been described for peritoneal dissemination (Kusamura et al. 2010): (1) dissemination from a primary tumor (e.g. GC, CRC, ovarian cancer, and pseudomyxoma peritonei (PMP)); (2) primary tumor of the peritoneum (e.g. MPM, serous papillary peritoneal adenocarcinoma); and, (3) independent origins of the primary tumor and peritoneal implants (e.g. ovarian tumors with low malignant potential, extraovarian serous papillary adenocarcinoma of the peritoneum). In the last model, a field defect allows polyclonal, multifocal origins, and independent pathogenesis of primary tumor and peritoneal implants (Kusamura et al. 2010). PSMs include a variety of malignancies that

are challenging for both clinicians and patients. They all follow a locally progressive process that can lead to significant morbidity and ultimately death (Rubino et al. 2012). Of different PSM entities, peritoneal dissemination of gastrointestinal primary tumors, in particular PMP and peritoneal carcinomatosis of gastric (GCPC) and colorectal cancer (CRCPC), comprise the focus of the current project.

PMP is believed to originate from a primary neoplasm of the appendix. It seems that approximately 10–20 % of patients with an epithelial appendiceal neoplasm, in particular those with mucinous adenoma, develop PMP. This clinical syndrome is a low-grade malignancy characterized by a distinctive pattern of the peritoneal spread and multifocal mucin collections in the abdominopelvic cavity (Smeenk et al. 2008a). Despite its rarity and indolent neoplastic nature, PMP poses unique clinical challenges. It runs a protracted course wherein progressive accumulation of mucin and development of morbid complications, including fibrous adhesions and obstructive disease, drastically impact the quality of life. Owing to its unspecific manifestations, PMP tends to be diagnosed at advanced stages when recurrent episodes limit treatment options and increase mortality (Buell-Gutbrod and Gwin 2013). Peritoneal carcinomatosis (PC) of gastrointestinal origin is considered as an advanced stage in the natural history of the primary cancer and a frequent finding in the recurrent disease (Davies and O’Neil 2009). Almost 15 % of patients with CRC and approximately 40 % of patients with stage II–III GC present with PC at abdominal exploration. Moreover, in 10–35 % of patients with CRC and up to 50 % of patients with GC, tumor recurrence is confined to the peritoneal cavity. However, patients ultimately die from complications of locoregional tumor spread, in most cases without occurrence of metastases in other sites (Coccolini et al. 2013).

CRS plus HIPEC is advocated as the current standard of care for PMP. This multidisciplinary approach has brought about 10- and 15-year survival rates of 63 % and 59 %, respectively (Chua et al. 2012c). The prognosis of patients with peritoneal carcinomatosis from gastric (GCPC) and colorectal cancer (CRCPC) is very poor. With supportive care only, median survival time ranges from 3 to 6 months, with GCPC showing a worse prognosis (Sadeghi et al. 2000; Chu et al. 1989). Despite the fact that PC of gastrointestinal origin is traditionally regarded as a terminal condition for which no standard treatments have been established yet, the management of this challenging entity has progressed over the last two decades. In this regard, the literature supports the evolving role of CRS combined with HIPEC (Canbay et al. 2014; Yan and Sugarbaker 2007), prolonging overall survival in selected patients with GCPC (Coccolini et al. 2014; Yan et al. 2007b) and CRCPC (Sugarbaker 2012; Yan and Morris 2008). Nevertheless, this multimodal strategy is associated with treatment failure and recurrence, most commonly seen on the peritoneal surface. In order to maintain a disease-free peritoneal surface after complete cytoreduction, additional efforts should be made to optimize, and even customize, HIPEC and to improve locoregional treatment. For this purpose, the efficacy of HIPEC needs to be enhanced not only by determining optimal agents and hyperthermia, but also by developing novel locoregional treatments (Bijelic et al. 2008). To this end, we explored and observed previously the efficacy of BR/NAC combination therapy in inducing cytotoxicity and mucin depletion in *in vitro* models of

mucin-expressing gastrointestinal carcinomas. In the final part of this project, preclinical evaluation of the treatment efficacy was attempted through intraperitoneal treatment of two nude mice models of human PSM of gastrointestinal origin, using MKN45 gastric and LS174T colon adenocarcinoma cell lines. Results of this study are reviewed and discussed in the present chapter (Amini et al. 2015a).

5.4.2 Results

5.4.2.1 Effect of BR/NAC Treatment on Tumor Burden in MKN45 Model of PC

We first developed an MKN45 model of PC for the treatment phase. For this purpose, 54 nude mice were randomly assigned to one control and eight treatment groups and intraperitoneally inoculated with 2×10^6 MKN45 cells. Animals were treated with intraperitoneal administration of two different doses of single agent BR (3 and 6 mg/kg) or NAC (300 and 500 mg/kg), or four possible combinations of the two. The control group received intraperitoneal injection of the drug-free vehicle (0.9 % Saline) instead. Treatment started 2 weeks post-inoculation and continued for 12 days on an alternate day basis. Mice were then euthanized and peritoneal tumors were weighed and counted. BR/NAC treatment reduced intraperitoneal tumor burden. Mean tumor weights in control, BR 3 mg/kg, BR 6 mg/kg, NAC 300 mg/kg, NAC 500 mg/kg, BR 3 mg/kg+NAC 300 mg/kg, BR 3 mg/kg+NAC 500 mg/kg, BR 6 mg/kg+NAC 300 mg/kg, and BR 6 mg/kg+NAC 500 mg/kg groups were 1195, 706, 688, 770, 764, 488, 444, 428, and 433 mg, respectively. Thus, different treatment regimens resulted in decreases in tumor weight of 35 % (with low-dose NAC) to 64 % (with combination of high-dose BR and low-dose NAC). With regard to the number of peritoneal nodules, mean values were 136.75, 52.5, 47.2, 53.16, 40.4, 39.16, 34.8, 35.2, and 36.5 in control, BR 3 mg/kg, BR 6 mg/kg, NAC 300 mg/kg, NAC 500 mg/kg, BR 3 mg/kg+NAC 300 mg/kg, BR 3 mg/kg+NAC 500 mg/kg, BR 6 mg/kg+NAC 300 mg/kg, and BR 6 mg/kg+NAC 500 mg/kg groups, respectively. This denotes decreases between 61 % (with low-dose NAC) and 74 % (with combination of low-dose BR and high-dose NAC) in the number of tumor nodules in response to treatment. Statistically, one-way ANOVA confirmed that all treatment regimens significantly reduced the peritoneal tumor weight and number when results were compared with control. As regards the levels of significance, the declines in weight and number of tumors resulted from single agent and combination therapy were all “extremely significant,” except for single agent NAC with “very significant” effect on tumor weight. ANOVA analysis also indicated that tumor weight differences from control in combination therapy groups reached a higher level of significance than those in single agent counterparts. In order to figure out how exactly these values are different between single agent and combination therapy, we analyzed relevant paired values by ANOVA test. Despite the fact that combination therapy reduced tumor weight more than did low-dose and

high-dose BR individually, the differences shown were not statistically significant. Likewise, low-dose NAC appeared to induce a greater reduction in tumor weight when used in combination with low-dose BR, but the difference was not statistically significant. However, apart from this exception, the remaining combination regimens (low-dose NAC+high-dose BR, high-dose NAC+low-dose BR, and high-dose NAC+high-dose BR) were found to reduce tumor weight more significantly than did NAC alone. Finally, when single agent and combination therapy groups were compared in relation to the decreases induced in the number of the peritoneal tumor nodules, no statistically significant differences were found.

5.4.2.2 Effect of BR/NAC Treatment on Tumor Burden in LS174T Model of PC

LS174T is a human colon adenocarcinoma cell line with tumorigenicity in nude mice (Tom et al. 1976, 1977) that presents a goblet cell-like phenotype similar to that of PMP tumor cells (Choudry et al. 2012). Thus, we used this cell line for the development of a nude mice model of CRCPC and a surrogate model for PMP as well.

Subsequent to the set-up experiment, LS174T model of PC was developed for the efficacy study using 54 nude mice. For this purpose, animals randomly assigned to one control and eight treatment groups were intraperitoneally inoculated with 1×10^6 LS174T cells. One week post-inoculation, intraperitoneal treatment started and then continued for 17 days, on an alternate day basis, according to the treatment protocol, composed of 8 treatment regimens using two different doses of single agent BR (3 and 6 mg/kg) or NAC (300 and 500 mg/kg), or their four possible combinations. The drug-free vehicle (0.9 % saline) was used for the intraperitoneal treatment of the control group. At the end of the treatment period, the extent of the peritoneal disease was explored post-euthanasia and tumor nodules were weighed and counted. BR/NAC treatment dramatically inhibited intraperitoneal tumor development. Our results showed that mean tumor weights in control, low-dose BR, high-dose BR, low-dose NAC, high-dose NAC, low-dose BR + low-dose NAC, low-dose BR + high-dose NAC, high-dose BR + low-dose NAC, and high-dose BR + high-dose NAC groups were 460, 300, 292, 332, 72, 22, 21, 28, and 20 mg, respectively. In other words, decreases in tumor weight ranging from 27 % (with low-dose NAC) to 95 % (with combination of high-dose BR and NAC) resulted from BR/NAC treatment. With respect to the number of peritoneal nodules, mean values of 77.5, 31.6, 32.5, 44, 10.75, 3, 1.5, 3.8, and 3.6 were recorded for control, low-dose BR, high-dose BR, low-dose NAC, high-dose NAC, low-dose BR + low-dose NAC, low-dose BR + high-dose NAC, high-dose BR + low-dose NAC, and high-dose BR + high-dose NAC groups, respectively. Thus, BR/NAC treatment resulted in decreases between 43 % (with low-dose NAC) and 98 % (with combination of low-dose BR and high-dose NAC) in the number of tumor nodules. The treatment-induced decreases in weight and number of the peritoneal tumor nodules were then analyzed and compared to control by ANOVA test. The statistical analysis revealed that all

results were statistically significant, except for tumor weight-lowering effect of low-dose NAC. Apart from two (effects of single agent BR on tumor weight), all significant effects statistically reached an extreme level. These levels of significance were even higher in combination therapy. To comprehend how significantly combination therapy offers advantage over single agent therapy, we performed ANOVA analysis to statistically compare the relevant values in pairs. The results indicated that differences between paired values of single agent and combination therapy were significant in favor of combination therapy, with the exception of high-dose NAC. In this instance, despite the fact that the addition of BR apparently enhanced the lowering effects of high-dose NAC on weight and number of peritoneal tumor nodules, these results were not statistically significant. Apart from this exception, the levels of statistical difference between the paired results of single agent and combination therapy were very significant for two and extremely significant for the others.

5.4.2.3 Effect of BR/NAC Treatment on the Expression of Ki-67

Ki-67 protein is a cellular marker for proliferation (Gerdes et al. 1984). To find out how tumor cell proliferation is affected by BR/NAC treatment, we employed immunohistochemistry (IHC) to compare and contrast the expression of Ki-67 protein in different groups of either model. For this purpose, paraffin-embedded tumor tissue sections were stained with Ki-67 specific antibody where a nuclear stain was considered indicative of positive staining. We found that BR/NAC treatment reduced Ki-67 expression, with the lowest expression observed in the combination therapy groups. This effect in LS174T model was drastic. To quantitatively analyze our results, we calculated Ki-67 index for each group as the percentage of the positively stained cells. In MKN45 model, mean Ki-67 index ranged from a minimum of 26.33 % (high-dose BR + high-dose NAC) to a maximum of 55.57 % (low-dose BR) in the treatment groups, compared with 75.33 % in the untreated control group. As regards LS174T model, mean Ki-67 index of the treatment groups ranged between 4.9 % (low-dose BR + high-dose NAC) and 27 % (low-dose BR), indicating a dramatic reduction in the Ki-67 expression of the treatment groups compared with control (82.6 %).

We then performed ANOVA test to statistically analyze and compare the values in different groups. In MKN45 model, while all treatment regimens were found to significantly decrease Ki-67 index, results of combination therapy reached a higher level of significance. In this regard, one single agent (high-dose BR) and three combination regimens (low-dose BR + high-dose NAC, high-dose BR + low-dose or high-dose NAC) were capable of inducing an extremely significant reduction in Ki-67 index. When these results were compared in single agent and combination therapy groups, statistically significant differences in favor of combination therapy were revealed for three pairs, including low-dose BR + high-dose NAC vs. low-dose BR, high-dose BR + low-dose NAC vs. low-dose NAC, and high-dose BR + high-dose NAC vs. high-dose NAC. In LS174T model, both single agent and combination therapies resulted in extremely significant falls in Ki-67 expression indices.

In three pairs, including low-dose BR + low-dose or high-dose NAC vs. low-dose BR, and high-dose BR + high-dose NAC vs. high-dose BR, combination therapy appeared significantly more effective than single agent therapy.

5.4.2.4 Effect of Treatment on Mucosubstances Produced by MKN45 Peritoneal Tumors

Mucins and their role in gastrointestinal cancers and relevant peritoneal entities comprise a substantial part of the present work. Following our studies on mucin-depleting effects of BR/NAC on in vitro models of gastrointestinal malignancies and as a key part of our animal study, we intended to examine the mucin content of the peritoneal tumors developed by different groups of either model. In so doing, we obtained and fixed tumor tissue specimens from both animal models post-euthanasia and prepared paraffin-embedded slides for PAS analysis of mucosubstances and immunohistochemical detection of MUC glycoproteins.

PAS is the quintessential mucin histochemical technique (Corfield 2000). To investigate the effect of BR/NAC treatment on the mucosubstance content of the peritoneal tumors developed by MKN45 cells in the murine model of PC, we initially performed and compared PAS staining of paraffin-embedded tumor tissue sections obtained from treated and untreated animals. As a result, the amount and intensity of mucosubstances, stained rose to magenta, decreased in response to treatment, more remarkably with combination therapy.

5.4.2.5 Effect of Treatment on Mucosubstances Produced by LS174T Peritoneal Tumors

Likewise, we performed and compared PAS staining of paraffin-embedded slides prepared from tumor tissue samples of treated and untreated animals in LS174T model. The results showed that the amount and intensity of mucosubstances decreased in response to treatment, with combination therapy producing the most drastic effect. There was also a profound difference between control and treated tumors with respect to the amount and intensity of mucosubstances accumulated in “mucin pools,” as well as the presence of apoptotic bodies after treatment.

5.4.2.6 Effect of Treatment on Tumor Expression of MUC1 and MUC5AC Proteins in MKN45 Model of PC

As with our previous study on in vitro models (Sect. 5.3), we next investigated how BR/NAC treatment of nude mice models of mucin-expressing gastrointestinal carcinomas alters the expression of different types of mucins immunostained by specific antibodies. To this end, we evaluated the immunohistochemical expression status of the proteins of interest in the paraffin-embedded tumor tissues obtained

from nine different groups of either model. First, we detected the expression of MUC1 and MUC5AC proteins in the peritoneal tumors of the MKN45 model. The expression of MUC1 with mild-to-moderate intensity and both apical and cytoplasmic localizations was evident in the control tumor tissue. MUC1 expression, however, decreased with single agent therapy and withered away with combination therapy. Our results indicated strong expression of MUC5AC with both cytoplasmic localizations in MKN45 peritoneal tumors. In response to BR/NAC treatment, however, MUC5AC expression was dramatically reduced by single agent therapy and barely detectable with combination therapy.

In order to compare the expression status of the proteins of interest in different groups, we then analyzed our results according to a semiquantitative IHC scoring method (Liu et al. 2007), wherein a combination of intensity and quantity values yields 8-point total scores from 0 (no staining) to 7 (extensive, strong staining). The resultant expression scores for either mucin ranged from 0 to 5. Mean IHC scores of control and four single agent groups were 4, 3, 2.5, 3 and 2.5, respectively, for MUC1, and 4.8, 0.3, 0.3, 0.4 and 0.3, respectively, for MUC5AC. This denotes the decreases in MUC1 expression of 25, 37.5, 25, and 37.5 %, along with the reductions of 93.75, 93.75, 91.6, and 93.75 % in the expression of MUC5AC, induced by low-dose BR, high-dose BR, low-dose NAC, and high-dose NAC, respectively. All four combination regimens were associated with IHC score of 0 for both MUC1 and MUC5AC. Statistically, ANOVA analysis revealed an “extremely significant” reduction in MUC5AC expression in response to all single agent and combination regimens. Similar analysis on treatment-induced alterations in MUC1 expression indicated a statistically significant change resulting from single agent therapy with high-dose NAC, and, more importantly, “extremely significant” effects of all combination regimens.

5.4.2.7 Effect of Treatment on Tumor Expression of MUC2 and MUC5AC Proteins in LS174T Model of PC

Subsequently, we performed similar IHC study on our second animal model to detect the expression of MUC2 and MUC5AC proteins in LS174T peritoneal tumors. LS174T tumor tissue demonstrated mild immunohistochemical expression of MUC5AC with cytoplasmic and extracellular reactivity. Nevertheless, decreases in the expression of this secreted mucin resulted from both single agent and combination therapy, with the latter inducing a more prominent effect. As regards MUC2, our results indicated strong MUC2 expression in tumor tissue with both cytoplasmic and extracellular localizations, which was reduced by single agent treatment and, more dramatically, by combination therapy.

Using the same intensity- and quantity-based, semiquantitative method employed for MKN45 model, we then quantified and statistically compared the expression status of the proteins of interest in different groups of LS174T model. The resultant expression scores of MUC2 and MUC5AC ranged from 1 to 7 and 1 to 4, respectively. MUC2 received mean IHC scores of 7, 4.75, 4.2, 5.4, 5.2, 3.5, 3.5, 2.8, and

1.8 for control, low-dose BR, high-dose BR, low-dose NAC, high-dose NAC, low-dose BR + low-dose NAC, low-dose BR + high-dose NAC, high-dose BR + low-dose NAC, and high-dose BR + high-dose NAC groups, respectively. As regards MUC5AC, the corresponding values included 3.8, 3, 2.8, 3, 2.8, 2.3, 2.3, 1.6, and 1.6, respectively. These denote treatment-induced decreases of 22–74 % and 21–57 % in the expression of MUC2 and MUC5AC, respectively. The lowest IHC scores of both mucins were associated with combination therapy. Statistically, ANOVA analysis revealed “extremely significant” reduction in MUC2 expression in response to all single agent and combination regimens. Moreover, when MUC2-lowering effects of all single agent and combination therapy regimens were compared in pairs, statistically significant difference in favor of combination therapy resulted, with differences in 5 out of 8 compared pairs reaching an “extreme” level of significance. Similar analysis on treatment-induced alterations in MUC5AC expression indicated a statistically significant change resulting from single agent therapy with high-dose BR and NAC, and highly or extremely significant effects of all combination regimens. Despite the fact that combination therapy appeared to reduce MUC5AC expression more than did single agent treatment, the differences were not statistically significant.

5.4.2.8 Toxicological Evaluation of Intraperitoneal Treatment with BR/NAC in Nude Mice Models of PC Using Relevant Clinicopathological Criteria

In the final part of this animal study, and the present project, we evaluated in both models a number of clinicopathological criteria used for toxicological purposes. Since eight different single agent and combination regimens comprised of two different doses of either agent were administered for a treatment period of 2 weeks or so, results of this comparative study can provide a preliminary insight into the treatment safety. To this end, we analyzed clinical data obtained from regular monitoring of animals throughout the study along with the findings of gross necropsy and histopathological examination of organ tissue samples post-euthanasia.

5.4.2.9 Evaluation of Clinical Parameters of Animal Health and Well-Being

In the present study, we monitored health and well-being of animals from acclimatization to euthanasia at least three times a week. For this purpose, we not only checked their body weight, but also scored them according to a method standardized for monitoring mice with peritoneal tumors (Paster et al. 2009) that examines a combination of general appearance, body condition, and behavioral parameters. To evaluate the effect on body weight of treatment as well as of time, as an important cofactor for toxicological assessments, we statistically compared the differences in values recorded at different time points between treated and untreated

(control) groups using two-way ANOVA, post-hoc Dennett's multiple comparison test. The results yielded no significant difference between control and any treatment group with respect to body weight measured at any time point.

Then, we analyzed in different groups of either model four clinical parameters of health, including appearance, body condition, natural behavior, and provoked behavior, as well as the combined health scores received by each group according to the health assessment method standardized for peritoneal tumor-bearing mice. When these values were analyzed using Chi-square test, statistically significant ($p=0.0028$) and insignificant ($p=1.00$) associations resulted for MKN45 and LS174T models, respectively. For a more accurate analysis with the inclusion of time, the combined health score values were subjected to Dunnett's test. No statistically significant differences between control and individual treatment groups of LS174T model with regard to health score recorded at any time point were revealed. In MKN45 model, statistically significant differences in favor of treatment groups appeared on the last days. In this regard, on days 23 and 25, all single agent groups as well as high-dose BR + low-dose NAC group received significantly higher scores than did control group. On day 23, high-dose BR + high-dose NAC group also showed a significantly higher health score.

5.4.2.10 Gross Necropsy and Histopathological Examination of Organ Tissue Samples Post-euthanasia

At the scheduled necropsy, we observed no treatment-related gross findings in any of the treated animals of either model. The external surface of the liver was smooth. The color was brown at the external surface and the cut surface. There was no gross fibrosis or necrosis in the macroscopic observation. Treated mice also showed no gross evidence of damage in small intestine and colon tissues, such as inflammation, ulceration, wall thickening, and necrosis. Using hematoxylin & eosin (H&E) staining of paraffin-embedded sections as the standard method in preclinical toxicology studies (Haschek et al. 2013), we then examined organ tissue samples obtained from different groups of either model with respect to the relevant histopathological criteria of toxicity. Normal liver architecture and components of hepatic lobules are evident in all micrographs. No features of toxic change and hepatopathy, including degenerative, inflammatory, and proliferative changes, such as fibrosis, hepatocyte necrosis, fatty change, cholestasis, macrovesicular or microvesicular steatosis, centrilobular hepatocellular hypertrophy, Kupffer cells pigmentation and proliferation, and mononuclear cell infiltration, were observed.

5.4.3 Discussion

Peritoneal dissemination was first described in 1931 as the regional spread of ovarian carcinoma (Sampson 1931). Since then, the following three patterns of spread have been identified for peritoneal dissemination of different primary malignancies

[reviewed in (Coccolini et al. 2013)]: (1) Random proximal distribution (RPD), in which early peritoneal implantation occurs, even when ascites is present. This is due to the presence of adherence molecules on the cancer cell surface. This pattern is typical of moderate-grade and high-grade cancers, such as adenocarcinoma and carcinoid of the appendix, non-mucinous CRC, GC, and serous ovarian cancer. (2) Complete redistribution (CRD), in which no adhesion to the adjacent peritoneal surface is present. This is due to the low biologic aggressiveness of tumor cells and/or lack of adherence molecules. This pattern is typical of PMP and diffuse malignant mesothelioma. (3) Widespread cancer distribution (WCD), where adherence molecules are present on the surface of cancer cells, but the great amount of mucus secreted by cancer cells interferes with cell adherence. This biological behavior is found in aggressive and undifferentiated tumors such as G2-G3 cystadenocarcinoma of the appendix, mucinous CRC, and mucinous ovarian cancer. Peritoneal dissemination of gastrointestinal cancers is a multistep process that starts with the detachment of malignant cells from the serosal surface of the primary tumor and results in the formation of tumor implants on the peritoneum via trans-mesothelial or trans-lymphatic pathways. The former occurs when PFCCs directly adhere to the mesothelial cells and penetrate the submesothelial tissue whereas the latter involves the migration of PFCCs into subperitoneal lymphatic sinus through lymphatic orifices and subsequent progression to the peritoneal surface. These processes are finalized by tumor-induced neovascularization. In addition, iatrogenic seeding of tumor might happen during an operation if blood or lymphatic fluid contaminated with cancer cells spill into the peritoneal cavity (Yonemura et al. 2007b).

For preclinical evaluation of the efficacy of BR/NAC as a candidate for locoregional treatment of gastrointestinal PSMs, we selected two cell lines from our initial *in vitro* models to develop two relevant nude mice models. MKN45 cell line, established from liver metastasis of a 62-year-old Japanese woman with poorly differentiated gastric adenocarcinoma (Hojo 1977), was shown to develop tumors in nude mice while preserving the histological characteristics of the original tumor (Motoyama et al. 1986; Yokozaki 2000). Nude mice models developed by MKN45 and its highly metastatic variant (MKN45-P) have been used as excellent models of GCPC (Kaneko et al. 2000; Yonemura et al. 1996a; Tsunemitsu et al. 2004). Using this model, Yonemura et al. explored the pathways of peritoneal dissemination and identified lymphatic structures (milky spots and the diaphragmatic stomata) and the naked submesothelial connective tissue (exposed after shrinkage of the mesothelial cells) as the major metastatic routes involved in this process (Yonemura et al. 1996a). In addition, MKN45 most closely mimics a gastric mucin phenotype (Linden et al. 2007). Thus, nude mice bearing peritoneal xenografts of MKN45 can be an ideal model for drug development studies on GCPC, in particular mucin-expressing adenocarcinomas. LS174T cell line was derived from a 58-year-old Caucasian female with Dukes type B adenocarcinoma of the colon (Tom et al. 1976). When grafted to nude mice, it grew as a mucinous adenocarcinoma microscopically resembling the original tumor (Tom et al. 1977). Therefore, LS174T has been used to develop nude mice models of CRCPC (Hyams et al. 1987; Ito et al. 1991; Jie et al. 2007). LS174T is also an excellent model for the expression of the intestinal goblet cell-specific secreted mucins, of which MUC2 is most prominently

expressed (Bu et al. 2011; van Klinken et al. 1996). MUC2 mRNA was found to comprise approximately 98 % of the overall gel-forming mucin mRNAs encoded by LS174T (Choudry et al. 2012). Since PMP is a disease of MUC2-expressing goblet cells (O'Connell et al. 2002b), one can take advantage of the PMP-like mucin secretory phenotype of LS174T in the preclinical evaluation of new approaches to this challenging entity (Choudry et al. 2012). Hence, LS174T can be utilized for the development of an ideal model of CRCPC and a surrogate model of PMP in the absence of an established primary cell line.

In this study, intraperitoneal treatment of MKN45 tumor-bearing nude mice with BR/NAC caused significant reductions in the peritoneal tumor nodule weight and count of up to 64 % and 74 %, respectively. Statistically, these effects received high, and more frequently, extreme levels of significance. Moreover, differential effect of single agent and combination therapy on tumor weight, in favor of the latter, was statistically significant in 3 out of 8 compared pairs. These results were obtained despite the fact that the 12-day-long treatment of this animal model of GCPC started 2 weeks post-inoculation, when peritoneal tumor nodules were readily visible in the primarily set up model. The depth of the drug penetration into peritoneal tumors depends on both drug and tumor type. Nevertheless, even with selected cytotoxic agents, the finite penetration depth limits the advantage of the intraperitoneal chemotherapy to patients with minimal tumor volume. In the context of GCPC, the tumor penetrability by chemotherapeutic agents is 1–3 mm that corresponds to the complete cytoreduction scores of CC-0 or CC-1 (Yonemura et al. 2010a). Therefore, early commencement of the intraperitoneal treatment with BR/NAC, similar to intraoperative or early postoperative use of chemotherapy, is expected to offer even better results. As regards the CRCPC model, the intraperitoneal administration of same regimens, starting 1 week earlier (day 7 post-inoculation) and ending 5 days later, dramatically inhibited the development of the peritoneal tumors by LS174T cells. Our results indicated the treatment-induced declines of up to 95 % and 98 % in tumor weight and number, respectively, with combination therapy holding advantage over single agent therapy. Statistically, when effects of single agent and combination regimens were compared in pairs, apart from one exception, highly or extremely significant differences were revealed. Based on the experience with the LS174T model setup, 1-week inoculation period corresponds to the early phase of the tumor development when small peritoneal nodules form. Thus, a smaller “pre-existing tumor burden at the treatment onset” in LS174T model appears to be a major contributor to the superior efficacy of the treatment in this model in comparison with the other. This feature mimics HIPEC/EPIC targeting of “residual disease post CRS.” Besides, a more penetrable and/or sensitive tumor type (consistent with our earlier *in vitro* findings showing the differential sensitivity of MKN45 and LS174T cells to BR/NAC treatment) and a longer treatment period are postulated to play additional roles.

A number of investigators have provided preclinical evidence in support of different implications of BR or NAC for cancer prevention and therapy. Initial studies by Goldstein et al. (1975) followed by Taussig and Goldstein (1976) showed that BR feeding (80 mg/kg/day for 6 months in the former study, and 20 mg/kg/day for

1 year in the latter) had inhibitory effects on UV-induced skin tumor development in nude mice. In two successive studies on a 2-stage mouse skin carcinogenesis model (initiated and promoted by 7,12-dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), respectively), Kalra et al. and, subsequently, Bhui et al. observed the inhibitory effects of topical BR pretreatment and treatment (1 mg/animal, three times a week for 24 weeks) on tumor initiation and promotion (Kalra et al. 2008; Bhui et al. 2009). With respect to the mechanisms of effect, they concluded that BR inhibited tumorigenesis through induction of p53 and caspase system, shift in Bax/Bcl-2 ratio, and inhibition of NF κ B-mediated Cox-2 expression by blocking MAPK and Akt/protein kinase B (PKB) signaling pathways. Cox-2 is involved in the synthesis of prostaglandin E2 (PGE2), a pro-inflammatory lipid that can also act as an immunosuppressant and promoter of cancer progression. Both Cox-2 and PGE2 are key players in cancer-related inflammation (Wang et al. 2007). Cancer-related inflammation is believed to contribute to tumor growth, angiogenesis, progression, and metastasis (Mantovani et al. 2008). Therefore, BR-induced blockage of NF κ B/Cox-2/PGE2 pathway may suppress inflammatory responses in tumor microenvironment, resulting in the inhibition of tumor progression (Chobotova et al. 2010). Recently, Romano et al. reported that intraperitoneal treatment of the azoxymethane (AOM) mouse model of colon carcinogenesis with BR (1 mg/kg, three times a week for 3 months, starting 1 week prior to the commencement of AOM exposure) offers chemoprevention by inhibiting the AOM-induced development of aberrant crypt foci (ACF), polyps, and tumors (Romano et al. 2014). In addition to the above experimental models of carcinogenesis, animal models developed by cancer cell lines have been employed to explore the effects of BR on tumor growth and progression. As an initial attempt in this regard, Batkin et al. found that oral BR (140 or 400 mg/kg/day for 26 days) decreased lung metastasis of Lewis murine lung carcinoma cells from the primary subcutaneous tumors (Batkin et al. 1988a, b). Similarly, Grabowska et al. reported that BR pretreatment of B16F10 mouse melanoma cells prior to intravenous inoculation prevented lung colonization of cancer cells and reduced metastatic tumor weight to about three times (Grabowska et al. 1997). Using a range of murine cancer cell lines inoculated subcutaneously (B16F10 melanoma and ADC-755 mammary adenocarcinoma), intramuscularly (Lewis lung carcinoma), and intraperitoneally (P-388 leukemia, S-37 sarcoma, and Ehrlich ascites tumor), Baez et al. evaluated the effect of intraperitoneal administration of BR (1–25 mg/kg, starting one day post-inoculation and continuing 5 times a week for 3 weeks) on animal survival (Baez et al. 2007). They used similar treatment, but with doses ranging from 12.5 to 50 mg/kg, to study antimetastatic activity of BR in a Lewis lung carcinoma model. In response to treatment, the survival index of all models, except for melanoma, significantly increased, with the ascites tumor model treated with 12.5 mg/kg BR indicating the best survival. This was even superior to the effect of 20 mg/kg 5FU. Further, BR treatment significantly reduced the number of lung metastases and tumor cells in Lewis lung carcinoma and Ehrlich ascites tumor models, respectively.

Likewise, anticancer activities of NAC have been explored in a number of pre-clinical studies. Early investigations by De Flora et al. demonstrated that NAC

possesses antimutagenic and anticarcinogenic properties and is thus a promising chemopreventive agent (De Flora et al. 1991b). Through intraperitoneal treatment (500 mg/kg) of rats cotreated with an enzyme inducer and/or reduced glutathione (GSH) depletors, they monitored a number of parameters relevant to the metabolism of carcinogens and indicated protective effects of NAC through modulation of glutathione metabolism and the biotransformation of mutagenic/carcinogenic compounds (De Flora et al. 1985). Consistently, they later showed in a mice model of urethane-induced lung carcinogenesis that NAC supplementation of the diet (0.2 %, approximately equal to an average intake of 120 mg/kg/day) over 15 days before and 4 months after the intraperitoneal injection of the pulmonary carcinogen urethane resulted in a significant decrease in the frequency of tumor-bearing animals and, in particular, a drastic drop in the mean number of tumors (De Flora et al. 1986). In a separate study, dietary NAC (0.1 %) was found to delay the development of gamma-glutamyl transpeptidase (GGT)-positive foci, known as an early event during hepatocarcinogenesis, and prevented sebaceous squamocellular carcinomas of Zymbal glands in rats treated with the carcinogen 2-acetylaminofluorene (2AAF) (Cesarone et al. 1987). Wilpart et al. reported antimutagenic activity of NAC in 1, 2-dimethylhydrazine (DMH) model of colon carcinogenesis in rats wherein NAC supplementation of drinking water (1 % w/w) for 15 weeks reduced the incidence of intestinal tumors and lowered tumor yield (Wilpart et al. 1986). Using the same model, Fragoso et al. consistently found in a recent study that NAC supplementation of the standard diet (0.2 %) for 10 weeks suppressed the development of CRC preneoplastic lesions (Fragoso et al. 2013). In a murine model of chronic ulcerative colitis (UC)-associated CRC developed by the dextran sulfate sodium (DSS)-induced chronic UC plus iron supplementation, Seril et al. similarly indicated that long-term dietary NAC (200 mg/kg/day) significantly reduced tumor incidence and multiplicity, possibly via inhibition of cell proliferation, induction of apoptosis, and suppression of nitrosative cellular damage (Seril et al. 2002). The protective effect of oral NAC in this model was recently confirmed by Amrouche-Mekkioui and Djerdjouri (2012). Using mammary gland organ culture, Mehta et al. observed that NAC was effective in preventing the development of mammary lesions in response to DMBA exposure (Mehta et al. 1991). In two consistent studies by De Flora et al. (1991a) and Balansky et al. (1996), oral administration of NAC (1 g/kg) was consistently shown to prevent cytogenetic changes and adducts induced in rat organs by benzo(a)pyrene (B[a]P), 2AAF, and cigarette smoke. They later showed that similar NAC regimen protected mice against B[a]P-induced formation of preneoplastic lesions and/or tumors in mouse stomach and lung (Balansky et al. 2006). Using the Mutatect MN-11 model, a mouse fibrosarcoma-derived cell line engineered for sensitive detection of mutations by clastogenic agents, Birnboim and Privora found that NAC pretreatment blocked mutations induced by nitric oxide-donating agents (Birnboim and Privora 2000). Reliene et al. indicated the inhibition of oxidative DNA damage and DNA deletions in ATM-deficient embryos and offspring of NAC-fed mouse dams (1 g/kg/day) (Reliene et al. 2004). They later reported that lifelong dietary NAC (1 g/kg/day, starting with regular feeding of dams with NAC-supplemented water during pregnancy and nursing, and continuing in the same way

for offspring throughout a 2-year study) reduced incidence and multiplicity of lymphoma in ATM-deficient mice (Reliene and Schiestl 2006). Consistently, Rassool et al. found that similar treatment (1 g/kg/day for a period of 6 weeks post-gestation) can decrease or even reverse DNA damage and error-prone repair in a mouse model of myeloid leukemia (Rassool et al. 2007). Likewise, Reimann et al. reported that lifelong exposure of a E μ -myc transgenic mice model of B-cell lymphomas to NAC (0.5 % w/v supplementation of the drinking water starting at a mid-embryonic stage) prevented acquisition of DDR defects, maintained sensitivity to chemotherapy and provided a profoundly improved long-term outcome (Reimann et al. 2007). Employing a transgenic model of hepatocellular carcinoma (HCC), Gao et al. similarly reported that oral administration of NAC (1 g/kg/day) inhibited hepatocarcinogenesis (Gao et al. 2007). Consistently, Hanczko et al. indicated that lifelong administration of NAC (10 g/L drinking water of breeding pairs and their offspring) blocked hepatocarcinogenesis in transaldolase-deficient mice (Hanczko et al. 2009). Using a diethylnitrosamine (DEN)-induced model of hepatocarcinogenesis in toll-like receptor 2 (TLR2)-deficient mice, Lin et al. similarly reported that NAC treatment (100 mg/kg as pretreatment plus boosters on alternate days for 3 months) attenuated carcinogenesis and HCC progression (Lin et al. 2013). In a study by Cotter et al., NAC treatment not only protected melanocytes against oxidative stress/damage *in vitro* but also delayed onset of UV-induced melanoma in a highly penetrant mouse model when orally administered at a maternal dose of 1.9 g/kg/day during gestation and lactation up to 2 weeks post-irradiation (Cotter et al. 2007). The chemopreventive potential of NAC has been ascribed to its roles as a nucleophile, an antioxidant, an ROS scavenger, and a glutathione precursor, hence participation in such protective mechanisms as inhibition of exogenous and endogenous mutagens, deactivation of ROS, detoxification of xenobiotics, protection of DNA and DNA-dependent nuclear enzymes, prevention of carcinogen-DNA adduct formation (De Flora et al. 1991b).

In addition to antimutagenic and anticarcinogenic properties, NAC has shown inhibitory effects on growth and progression of tumors developed by a variety of cancer cell lines *in vivo*. In a study by Albini et al., the number of lung metastases resulting from intravenous injection of B16-F10 murine melanoma cells decreased sharply when malignant cells used were pretreated with 10 mM NAC (Albini et al. 1995). Besides, oral treatment of mice throughout the experiment (0.025–4 g/kg/day, starting 48–72 h before subcutaneous or intramuscular inoculation with murine melanoma (B16-F10 and B16-BL6) or Lewis lung carcinoma (C87) cells, respectively) delayed primary tumor formation and decreased tumor weights in all groups, and also induced a slight, yet significant, reduction in spontaneous metastasis from the melanoma tumors. In a subsequent study on these models, NAC consistently exhibited similar effects in single agent therapy (namely in both pretreatment of B16-F10 cells (10 mM) before intravenous inoculation and oral treatment of mice (2 g/kg/day) starting 48–72 h before B16-BL6 cell injection) and demonstrated synergism with doxorubicin in combination therapy (De Flora et al. 1996). In agreement, Im et al. reported that NAC inhibited growth and migration of B16-F10 cells *in vitro*, and diminished B16F10 tumor growth in mice after intraperitoneal treatment

(32 mg/kg/day) (Im et al. 2012). In an interesting study by Delneste et al., oral administration of NAC (5 g/L drinking water) inhibited peritoneal tumor formation in more than a third (18 out of 50) of mice intraperitoneally inoculated with L1210 lymphoma cells (Delneste et al. 1997). Intriguingly, protected mice were resistant to a second inoculation of malignant cells without further treatment with NAC, and spleen cells obtained from these mice were able to kill L1210 cells. Mechanistically, NAC was found to induce TNF α -dependent cytotoxic pathways and thereby enhanced T-cell cytotoxicity. In this connection, Kesarwani et al. recently reported that NAC-pretreated T cells exhibited key antitumor properties such as low glycolysis, increased persistence, and controlled tumor growth (Kesarwani et al. 2014). Moreover, Goldman et al. found that NAC enhanced the immunogenicity of a low-immunogenic clone of Lewis lung carcinoma (D122) (Goldman et al. 2000). As such, immunotherapy with NAC-treated tumor cells combined with intravenous NAC (367 mg/kg) in mice with established lung metastases provoked an antitumor response capable of eradicating the metastatic nodules. Taking into account the critical role of angiogenesis in tumor biology, Cai et al. explored a likely antiangiogenic role for NAC and indicated its capability in hampering endothelial cell invasion *in vitro* and inhibiting neovascularization of the matrigel sponges in response to Kaposi's sarcoma cell products *in vivo* (Cai et al. 1999). While acting as antiangiogenic agent, NAC was found to prevent apoptosis and oxygen-related genotoxicity in endothelial cells (Aluigi et al. 2000). Consistently, they later showed that daily administration of oral NAC (2 g/kg), initiated after the development of detectable tumor mass in nude mice subcutaneously inoculated with immortalized Kaposi's sarcoma (KS-Imm) cells, resulted in a dramatic inhibition of tumor growth, markedly prolonged median survival time, and a significant reduction in tumor production of VEGF (Albini et al. 2001). In line with these findings, Agarwal et al. observed that NAC treatment (subcutaneous, 10 mg/kg/day) of nude mice bearing MDA-MB-435 human breast carcinoma xenografts resulted in tumor vascular collapse and significant tumor cell apoptosis/necrosis (Agarwal et al. 2004). In contrast, however, they reported that NAC treatment resulted in angiostatin-induced endothelial cell apoptosis. Simard et al. found that cotreatment with intraperitoneal NAC (7.5 mg/kg/day) synergistically enhanced antiangiogenic and antitumoral activity of SCEs in mice orthotopically implanted with the GL26 mouse glioblastoma cell line and improved survival (Simard et al. 2011). Apart from angiogenesis, vasculogenic mimicry (VM)—defined as *de novo* generation of capillary-like structures (CLS) and matrix-rich patterned network without participation of endothelial cells—has been implicated in the perfusion and metastasis of aggressive tumors, including highly aggressive and metastatic melanomas (Maniotis et al. 1999). In this regard, a study by Vartanian et al. showed that NAC treatment of melanoma cells not only abolished the generation of peroxide but also blocked formation of CLS (Vartanian et al. 2007). In another study, Gao et al. challenged the paradigm that antioxidants suppress tumorigenesis primarily through decreasing DNA damage and mutations (Gao et al. 2007). Using xenograft (P493 B) models of human B lymphoma along with a transgenic model of HCC, the investigators indicated that oral NAC (1 g/kg/day) dramatically inhibited tumorigenesis, which was found to be resulted from

destabilization of HIF, rather than diminution of genomic instability. In vitro, NAC treatment of P493 B cells consistently resulted in a significant reduction in media levels of VEGF, a target of HIF-1 regulation. In this connection, Sceneay et al. later reported that NAC treatment of murine breast cancer cells similarly prevented HIF-1 α stabilization under hypoxia in vitro (Sceneay et al. 2013). However, using the same in vivo treatment protocol as the precedent, this did not translate to tumor growth inhibition in the relevant orthotopic syngeneic models. Investigating the utility of antioxidants as adjuncts to chemotherapy in CRC, Bach et al. found that intraperitoneal administration of NAC (200 mg/kg) enhanced the efficacy of 5-FU (120 mg/kg) against HCT-15 tumor xenografts in nude mice, which was accompanied by extensive tumor necrosis and a sustained elevation in p53-independent apoptosis (Bach et al. 2001). However, no significant impairment of neovascularization was observed in this study. In a similar attempt toward the management of invasive, EGFR-overexpressing oral cancer, Lee et al. demonstrated that intraperitoneal treatment of mice bearing HSC-3 human tongue squamous carcinoma xenografts with NAC (100 mg/kg/day for 20 days) significantly reduced mean tumor volume by 33 % (Lee et al. 2013a). In so doing, NAC was found to inhibit HSC-3 cell growth and survival through decreasing EGFR/Akt activation and increasing the HBP1 expression. In a recent study by Qanungo et al., while single agent chemotherapy with the first-line agent gemcitabine (intraperitoneal, 100 mg/kg, once a week) failed to inhibit the growth of MIA PaCa-2 pancreatic cancer xenografts in nude mice, adjunct treatment with NAC (intraperitoneal, 100 mg/kg, three times a week for 35 days) reduced tumor growth by approximately 50 % and markedly enhanced tumor apoptosis by targeting NF κ B pathway (Qanungo et al. 2014). Sayin et al., however, reported that supplementing the diet with NAC (1 g/L drinking water) and vitamin E (0.1 and 0.5 g/kg chow) increased tumor progression in, and reduced survival of, mouse models of lung cancer induced by B-RAF and K-RAS (Sayin et al. 2014). Since this effect was evident only in tumor cells with wild-type p53, but not in their counterparts with mutant or inactivated p53, the authors postulated that antioxidant-induced reduction in oxidative DNA damage removes the stimuli for DDR pathway, namely ATM/p53 cascade, thereby accelerating tumor growth by preventing p53 activation. This is an in vivo example of context dependency of the NAC's proapoptotic activity. Although NAC appears to mediate preferential apoptosis in cancer cells independent of its ROS scavenging function, inhibition of ROS-initiated and -mediated processes by NAC with paradoxical effects on cancer biology could justify contradictory reports on the role of ROS and antioxidants in cancer (Kardeh et al. 2014). This further highlights the necessity of in-depth understanding of the relevant molecular mechanisms and signaling pathways for the development of customized and even personalized strategies.

In the present study, consistent with the inhibitory effects of BR/NAC treatment on cell proliferation and tumor growth in our gastrointestinal cancer models in vitro (Sect. 5.1) and in vivo (Sect. 5.4) is the significant reduction in the Ki-67 expression indices of the peritoneal tumors of either model in response to treatment. Ki-67 is a nuclear protein that is found in growing, dividing cells but is absent in the quiescent or resting cells. Gerdes et al. were the first to identify Ki-67 as a cell

proliferation-associated human nuclear antigen present in the G1, S, and G2 phases of the cell division cycle as well as in mitosis, but not in the G0 phase (Gerdes et al. 1983, 1984). Since then, this protein has been extensively used as a proliferation marker, the expression index of which is often correlated with the clinical course of human tumors (Scholzen and Gerdes 2000). Our immunohistochemical study revealed that combination therapy with BR and NAC caused decreases of up to 65 % and 94 % in Ki-67 indices of MKN45 and LS174T tumors, respectively. Statistical analysis of data obtained from different treatment groups confirmed extremely significant effect of combination treatment in reducing tumor cell proliferation. These findings are in line with the observations by others that NAC treatment alters the expression of proliferation markers, including Ki-67 and proliferating cell nuclear antigen (PCNA) (Keim and Hanash 1992), in the neoplastic tissues. In a clinical study by Estensen et al. on patients with history of adenomatous colonic polyps, oral treatment with NAC (800 mg/day) for 12 weeks significantly reduced the PCNA index of colonic crypts (Estensen et al. 1999). In agreement, Seril et al. found that NAC treatment of a murine model of UC-associated CRC resulted in a reduction in PCNA index (Seril et al. 2002). Likewise, Albini et al. observed that Ki-67 and PCNA markers were significantly lower in Kaposi's sarcomas from NAC-treated mice compared to the untreated control (Albini et al. 2001). In the study of B[a]P-induced tumorigenesis by Balansky et al., NAC significantly decreased PCNA in mouse lung tumors (Balansky et al. 2006). More recently, Poncin et al. demonstrated that intraperitoneal NAC treatment (100 mg/kg, 1 day or 4 days) of animal (mice and rats) models of propylthiouracil (PTU)- or perchlorate-induced goitrogenesis significantly decreased the thyroid weight and PCNA indices in both types of goiter (Poncin et al. 2010).

Apart from revealing the inhibitory effects of BR/NAC on tumor growth, this animal study also indicated, consistent with our *in vitro* observations (Sect. 5.3), that BR/NAC therapy remarkably diminished the tumor production of mucins in both models. As a typical finding, pools of the extracellular mucin in LS174T tumors became mucin-depleted. These mucin pools are a characteristic feature of PMP tumor (O'Connell et al. 2002a). More specifically, the treatment proved to influence the expression of the membrane-associated mucin MUC1, as well as of the secreted mucins MUC2 and MUC5AC. As mentioned earlier, MKN45 with typical expression of MUC1 and MUC5AC is a prototype for gastric mucin-expressing adenocarcinomas, and peritoneal development of MKN45 tumors in nude mice is an ideal model of GCPC. Besides representing an animal model of CRCPC, LS174T is an excellent model for the expression of the intestinal goblet cell-specific secreted mucins, in particular MUC2 and MUC5AC, and a surrogate model of PMP. Our immunohistochemical study revealed that combination therapy with BR and NAC completely inhibited the expression of MUC1 and MUC5AC in the MKN45 peritoneal tumors, and induced decreases of up to 57 % and 74 % in the expression of MUC5AC and MUC2, respectively, in the peritoneal tumors developed by the LS174T model. Given the critical roles played by tumor-associated mucins in the pathophysiology of cancer and pathogenesis of cancer-induced complications, with specific implications in gastrointestinal cancers, PC and PMP,

mucin depletion achieved by this experimental approach apparently contributes to the inhibition of malignant growth evidenced in our gastrointestinal cancer models in vitro and in vivo.

In support of our results, there is evidence that BR has the potential to inhibit the expression and production of secreted mucins. As an anti-inflammatory agent, BR inhibits the production of substance P, PGE₂, and TXB₂ in vivo (Gaspani et al. 2002; Vellini et al. 1986). COX-2/PGE₂ pathway is known to mediate MUC2 and MUC5AC expressions at both the mRNA and protein levels (Gray et al. 2004; Kim et al. 2002). In agreement, Choudry et al. showed that dexamethasone treatment decreased the MUC2 mRNA levels in LS174T cells in vitro and reduced the MUC2 protein volume in a PMP xenograft model (Choudry et al. 2012). It is thus postulated that BR-induced inhibition of MUC2 and MUC5AC expression/production observed in the present study might be linked to its anti-inflammatory effects, including COX-2/PGE₂ inhibition. Also in keeping with the present data, mucoregulatory activity of NAC has been demonstrated in different experimental models. In two initial studies, Rogers et al. consistently observed that oral NAC (1 % w/v drinking water) inhibited cigarette smoke-induced goblet cell hyperplasia (Rogers et al. 1988) and mucin hypersecretion (Rogers et al. 1989) in the rat airways. In a similar study on the bleomycin-induced pulmonary fibrosis model, Mata et al. found that orally administered NAC (490 mg/kg/day by gavage, starting 1 week before bleomycin exposure and continuing for 3 weeks) diminished Muc5ac expression at both mRNA and protein levels and reduced the goblet cell hyperplasia in the rat airway epithelium (Mata et al. 2003). Inhibition of *Muc5ac* expression was also evident in a rat model of asthma treated similarly (Blesa et al. 2003). Consistent with these observations, Dharajiya et al. found that coadministration of NAC and ascorbic acid (intranasal instillations of 120 and 130 mg/kg, respectively) reduced pollen challenge-induced mucin production in mice by 10 times (Dharajiya et al. 2007). Likewise, NAC pretreatment (intraperitoneal, 200 mg/kg) of ovalbumin-sensitized and challenged mice significantly decreased bronchial mucin production (Park et al. 2009). Mata et al. later indicated in two successive studies that MUC5AC expression and goblet cell hyperplasia induced by viral infections of the human respiratory epithelial cells were inhibited by NAC in vitro (Mata et al. 2011, 2012). Similarly, Urashima et al. found that topical administration of NAC (six instillations of 10 % (w/v) solution at 2-h intervals) significantly lowered the amounts of conjunctival and corneal mucins in the rabbit eyes (Urashima et al. 2004). The authors concluded that NAC not only dissolved mucins but also reduced mucin production and retention. In order to reduce the expression of MUC5AC, NAC appears to inhibit p44/42 (Takeyama et al. 2000) and p38 (Jang et al. 2010) MAPK pathways, EGFR (Takeyama et al. 2000), and NFκB (Mata et al. 2011). On the other hand, mucoregulatory activity of NAC has the potential to restore pathological mucin depletion with a protective purpose. As such, Amrouche-Mekkioui et al. observed in a murine model of chronic colitis that oral NAC (150 mg/kg for 45 days) improved the hallmarks of DSS-induced colitis, including crypt alterations, mucin depletion, and epithelial cell hyperplasia, with potential benefits in inflammatory bowel diseases and CRC (Amrouche-Mekkioui and Djerdjouri 2012).

In accord with tumor mucin-depleting activity of the treatment documented in the present study, BR/NAC was found in a parallel study in our Department to effectively disintegrate PMP-secreted mucin gels. In this regard, an optimized formulation combined of BR and NAC (BR 300 $\mu\text{g/mL}$ + NAC 40 mg/mL in Tris buffer, pH 7) proved mucolytic effects on PMP gels *ex vivo*, as well as on the peritoneally implanted mucins in nude rats (intraperitoneal injection of 500 μL of the formulation twice a day for 2 days) (Pillai et al. 2014a). Further investigation on mucin samples obtained from 36 patients with disseminated peritoneal adenomucinosis (DPAM) or peritoneal mucinous carcinomatosis (PMCA) revealed that absolute disintegration *in vitro* and *in vivo*, respectively, was 100 % and 100 % for soft, 57.38 % and 48.67 % for semi-hard, and 50 % and 28.67 % for hard mucin (Akhter et al. 2014). In agreement, a number of case reports provide evidence that supports the benefits of NAC-induced mucolysis in hepatobiliary conditions. Studying two cases with PSC, Ozdil et al. reported that oral NAC (800 mg/kg/day), alone or combined with ursodeoxycholic acid (UDCA), yielded markedly positive effects on the clinical course of the disease (Ozdil et al. 2011). To this end, NAC appeared to facilitate the bile drainage in partial obstructions by decreasing the bile mucin viscosity. In agreement, Hu et al. observed that continuous infusion of NAC by nasobiliary catheter (4 mg/mL , 50 mL/h) as a palliative treatment in a patient with advanced IPMN of the bile duct was associated with remarkable improvement of biliary obstruction (Hu et al. 2012). In a similar approach to another case with IPMN of the bile duct, Hong et al. recently reported that while endoscopic suction failed to remove thick mucoid impaction and alleviate abdominal pain and ongoing jaundice, the condition was successfully managed with NAC irrigation (300 mg , three times daily for 10 days) through percutaneous transhepatic biliary drainage (PTBD) (Hong et al. 2014). Taken together, mucoregulatory and mucolytic properties of BR/NAC combination therapy represent a hallmark of this treatment that could be utilized to improve the efficacy and practicability of the conventional therapies for mucin-expressing tumors. Hence, utility of BR/NAC as a dual action adjunct in novel locoregional modalities could not only enhance microscopic cytoreduction attempted by chemotherapy, but also holds promise for *in situ* mucolysis in the context of mucin-secreting tumors.

Lastly, safety assessment of this experimental treatment according to the clinicopathological indicators of toxicity revealed no evidence of adverse effects in any of the eight treatment groups of either model. Despite the fact that a standard toxicology study is yet to be conducted, the present study using different doses of single agent BR (3 and 6 mg/kg) and NAC (300 and 500 mg/kg) and their combinations in two individual models mimics a dose escalation study and provides a preliminary insight into the safety of the treatment. Clinically, we monitored animals' well-being on a regular basis using a method described by Paster et al. (2009). This method is tailor-made for mice with peritoneal tumors where visual evaluation of tumor is not feasible and body weight *per se* is not an accurate indicator of well-being since it may plateau or increase despite a decline in health. Moreover, although body condition scoring (BCS) is a routinely used technique in veterinary medicine for assessing health and nutritional status, it alone does not give a complete picture

of animal health. Hence, this method utilizes a combination of body weight, BCS, appearance, and behavioral assessments to evaluate morbidity in murine models with peritoneal tumors. There were no treatment-related mortalities in this study. Statistical analysis of body weight changes with the inclusion of time as an important cofactor showed no significant difference between control and any treatment group. Similarly, when animals in different groups were compared with respect to the combined scores received for body condition, appearance, natural behavior, and provoked behavior, results were statistically either insignificant or significant in favor of treatment. For pathological assessment, macroscopic observation of abdominal organs along with histopathological study of tissue specimens was performed. At necropsy, we observed no gross evidence of toxicity. In agreement, microscopic examination of the paraffin-embedded sections of the liver, small intestine, and colon stained with H&E—the standard, extensively used method in preclinical toxicology studies (Haschek et al. 2013)—was not indicative of any toxic change.

In a dose escalation study, our Department reported recently that intraperitoneal administration to nude rats of 10, 20, and 30 mg/kg of BR in combination with 500 mg/kg of NAC, four times over 48 h, yielded no adverse effects observed over 50 days of monitoring (Pillai et al. 2014a). Our literature review consistently confirmed that BR and NAC are known to be generally safe agents. BR has very low acute or chronic toxicity. No median lethal dose (LD50) could be determined with oral doses up to 10 g/kg in mice, rats, or rabbits. LD50s of intraperitoneal BR in mice and rats are 37 mg/kg and 85 mg/kg, respectively, and those of intravenous administration to mice and rabbits are 30 mg/kg and 20 mg/kg, respectively. When orally administered to rats at 500 mg/kg/day, BR did not provoke any alteration in food intake, growth, hematological parameters, and histology of heart, spleen, and kidney (Maurer 2001; Moss et al. 1963). With increasing doses up to 750 mg/kg/day, oral BR showed no toxic effects on dogs after 6 months. At 1.5 g/kg/day, no carcinogenic or teratogenic effects were observed in rats (Kelly 1996; Taussig et al. 1975). In humans, no evidence of toxicity has been reported with oral administration of BR up to 12 g/day (Castell et al. 1997). With respect to the safety of the intraperitoneal treatment with BR in animal models of abdominal cancers, our results are consistent with earlier reports of similar treatments in mice. As such, Baez et al. reported no visible side effects after intraperitoneal administration of BR at doses ranging from 1 to 50 mg/kg, five times a week for 3 weeks (Baez et al. 2007). Similarly, clinical and gross pathological evaluation by Romano et al. in a murine model of colon cancer revealed no toxic effects following intraperitoneal treatment with BR at 1 mg/kg, three times a week for 3 months (Romano et al. 2014). Similar to BR, NAC is of low toxicity in both animals and humans. LD50 of NAC is 4.6 g/kg in mice and 2.8 g/kg in rats when used parenterally, and >10 g/kg in both when administered orally. A daily oral dose of 1 g/kg for 18–24 months was devoid of detrimental effects in both rats and mice (Johnston et al. 1983). Further, NAC has not been shown to induce teratogenic or mutagenic effects (Bonanomi and Gazzaniga 1980; Johnston et al. 1983). On the contrary, as reviewed earlier, a large body of evidence supports antimutagenic and anticarcinogenic activities of NAC.

In humans, NAC has been in clinical practice for several decades. As an extensively used mucolytic, it has been well tolerated in high dosages and long-lasting schedules, and its clinical safety is thus well established (De Flora et al. 1991a). As such, it has been used at an oral dose of 400 mg/kg in the treatment of patients with chronic bronchitis for over 6 months, and daily oral or intravenous doses as high as 500 mg/kg and 300 mg/kg, respectively, have been administered without any obvious side effects (De Flora et al. 1986). Oral administration of NAC at doses up to 8 g/day was not found to cause clinically significant adverse reactions (de Rosa et al. 2000). As with BR and in line with our experience, NAC has been used safely for intraperitoneal treatment of different murine models of cancer by other investigators. In their experiment with B16-F10 murine melanoma model of lung metastasis, De Flora et al. treated their nude mice with intraperitoneal injections of NAC at a dose of 1 g/kg/day (De Flora et al. 1996). In the study by Lee et al. on mice bearing HSC-3 human tongue squamous carcinoma xenografts, animals were treated with intraperitoneal NAC at a dose of 100 mg/kg/day for 20 days (Lee et al. 2013a). Likewise, in the recent study by Qanungo et al., a similar dose of NAC (100 mg/kg) was used three times a week for 35 days as an adjunct to gemcitabine therapy of MIA PaCa-2 pancreatic cancer xenografts (Qanungo et al. 2014).

Taken together, the preclinical evaluation of the BR/NAC efficacy in two models of peritoneal dissemination of gastrointestinal cancers revealed the relevance and translatability of our *in vitro* findings in *in vivo* settings. In other words, this experimental treatment showed preclinical promise for locoregional treatment of these malignancies, representing a modality with dual effects on cancer cells and their mucin synthesis. In addition, the present study provided preliminary safety evidence in favor of the peritoneal use of BR/NAC, which needs to be confirmed in a separate toxicology study. Finally, with the inclusion of the *in vitro* data, our results lay the basis for further evaluation of this treatment as an adjunct in combination with chemotherapy.

Chapter 6

Summary and Future Directions

6.1 Summary of the Study

This project consists of in vitro and in vivo parts. To explore the efficacy of the treatment in a more comprehensive way in vitro, we selected a panel of human gastrointestinal cancer cells with differential sensitivities to cytotoxic agents. These include two gastric (MKN45 and KATO-III) and three colorectal (HT29-5 F12, HT29-5 M21, and LS174T) carcinoma cell lines. For in vivo studies, we selected MKN45 and LS174T cells for the development of GCPC and CRCPC models. With a PMP-like phenotype, LS174T was also considered as a surrogate model of PMP. The select cell lines and their peritoneal growth in nude mice are well-established models of gastrointestinal cancer and PC. The experimental treatment of these models included the use of different concentrations or doses of BR and NAC as single agent or in combination. Initially, we examined the effect of the treatment on growth, proliferation, and survival of the cancer cells, in vitro. Our results indicated that BR and NAC, on their own, inhibited proliferation of all cancer cell lines used. When used in combination, BR and NAC showed more potent antiproliferative activity as a result of additive or synergistic interaction. Mechanistically, caspase-dependent apoptosis and cytotoxicity were found to underlie the inhibitory effects of BR/NAC, with autophagy and cell cycle arrest serving as contributory factors. Since intraperitoneal chemotherapy is fundamental to the curative treatment of PC and PMP, we next intended to find out if the treatment has the potential to enhance the efficacy of chemotherapy. For this purpose, we selected a number of commonly used

chemotherapeutic agents, including cisplatin, 5-fluorouracil, paclitaxel, and vincristine. Our data showed that BR/NAC pretreatment sensitized KATO-III and LS174T cells to chemotherapy. Likewise, BR/NAC combination therapy potentiated cytotoxic effects of chemotherapeutics on MKN45 and LS174T cells. In the majority of the combination treatment groups, additivity or synergy represented the pattern of drug-drug interaction. Taking into account the significance of tumor-associated mucins in the pathophysiology of gastrointestinal carcinomas and PMP, we then explored whether and how BR/NAC treatment could alter the expression of mucin in our *in vitro* models. Three relevant models included MKN45, KATO-III, and LS174T. With respect to mucin expression, MKN45 and the signet-ring cell KATO-III both represent a gastric phenotype and express MUC1 and MUC5AC. In contrast, LS174T with a PMP-like phenotype is an excellent model for the expression of the intestinal goblet cell-specific mucins, in particular MUC2. In all three models, we found that BR and NAC significantly reduced the amount of PAS-stained substances, indicative of secreted mucins. This effect was most pronounced in combination treatment where PAS-positive areas were reduced to the minimum. These results were confirmed by immunocytochemical and Western blot analyses, consistently showing MUC1 and MUC5AC inhibition in both gastric models, as well as the drastic reductions of MUC2 and MUC5AC in the colorectal model LS174T. ELISA also revealed decreases in the levels of MUC2 and MUC5AC secreted to culture media by this model.

Subsequent to these *in vitro* studies with promising results, preclinical evaluation of the treatment efficacy through intraperitoneal treatment of two nude mice models was attempted. Nude mice models developed by MKN45 have been used as excellent models of GCPC. Since MKN45 most closely mimics a gastric mucin phenotype, nude mice bearing MKN45 peritoneal tumors can be an ideal model for drug development studies on GCPC, in particular mucin-expressing adenocarcinomas. LS174T, by contrast, has been used to develop nude mice models of CRCPC. Moreover, in the absence of an established primary cell line, one can take advantage of the PMP-like mucin secretory phenotype of LS174T to develop a surrogate model of PMP. Intraperitoneal treatment of MKN45 model with BR/NAC resulted in significant reductions in the peritoneal tumor nodule weight and count of up to 64 % and 74 %, respectively, with therapeutic benefits favoring the combination therapy. A more dramatic effect was observed with LS174T model. In this model, the intraperitoneal administration of same regimens, starting 1 week earlier (day 7 post-inoculation) and ending 5 days later, induced reductions in tumor weight and number of up to 95 % and 98 %, respectively, with combination therapy holding advantage over single agent therapy. Moreover, the peritoneal tumors of either model exhibited a consistent reduction in Ki-67 proliferation indices. In addition, BR/NAC therapy remarkably diminished the tumor production of mucins in both models. As a typical finding, extracellular mucin pools, a characteristic feature of PMP tumor, became mucin-depleted in LS174T tumors. Our immunohistochemical study of the peritoneal tumors developed by MKN45 and LS174T models consistently revealed that combination therapy with BR and NAC completely inhibited the expression of MUC1 and MUC5AC in the former, and induced decreases of up to

57 % and 74 % in the expression of MUC5AC and MUC2, respectively, in the latter. Testing different doses of BR and NAC as single agent or in combinations in two different murine models, this animal study mimics a dose escalation study and can thus provide a preliminary insight into the safety of the treatment. No treatment-related mortalities were found in this study. Statistical analysis of changes in body weight—with the inclusion of time as an important cofactor—showed no significant difference between control and treatment groups. With respect to the clinical scores received for body condition, appearance, natural behavior, and provoked behavior, the differences were either insignificant or significantly better in favor of treatment. In agreement, the examination of abdominal organs revealed no gross or histopathological evidence of toxicity. In sum, our findings indicate that BR/NAC significantly inhibits proliferation and survival of gastrointestinal cancer cells *in vitro* and, when administered intraperitoneally, represents a safe treatment that hampers peritoneal growth of gastrointestinal cancer *in vivo*. This experimental treatment also has the potential to enhance the efficacy of chemotherapy, to induce mucin-depleting effects on mucin-expressing cancer cells, and to reduce tumor-associated mucins.

BR and NAC are safe, naturally occurring agents that have been in clinical practice for decades. Both agents have long been evaluated for their divergent effects and utilities under normal and pathological conditions. As such, inhibitory effects of BR or NAC on malignant growth have been reported in the literature. In this regard, NAC has been more widely investigated. However, their combined use in cancer research has not been reported by other groups. Employing the experimental models of GCPC and CRCPC, we observed that intraperitoneal administration of BR+NAC is a promising locoregional treatment for PC. In the state-of-the-art approach to PC, CRS attempts to reduce the tumor volume to minimum and locoregional chemotherapy targets residual disease and PFCCs. Evidence shows that complete cytoreduction represents the most important prognostic factor in patients with GCPC, CRCPC, or PMP treated with this multimodal strategy. Since the penetration of intraperitoneally administered agents into peritoneal nodules, even with hyperthermia, is limited to 2–5 mm, CRS is also essential for enhanced success with subsequent chemotherapy. Locoregional chemotherapy starting immediately after complete dissection of an adhesive process, and before the onset of wound healing and organization of fibrinous deposits, minimizes nonuniform distribution of chemotherapeutic agents and facilitates their access to residual disease and PFCCs. In our LS174T model, early commencement of the treatment, and hence a smaller “pre-existing tumor burden at the treatment onset,” appears to be a major contributor to the superior efficacy of the treatment in this model in comparison with the other. This protocol mimics “post-CRS targeting of minimal residual disease” by perioperative intraperitoneal chemotherapy. In terms of differential response to treatment in these two models, a more penetrable and/or sensitive tumor type (consistent with evidently more aggressive biological behavior of GC as well as with differential sensitivity of MKN45 and LS174T cells to BR/NAC treatment observed in the present study *in vitro*), and a longer treatment period are postulated to play additional roles. According to the literature and our *in vitro* results, BR and NAC might also potentiate chemotherapy. Taken together, our findings suggest that

perioperative use of BR/NAC has the potential to play a role as monotherapy in its own right after CRS, as an adjunct to intraperitoneal chemotherapy, or even as preconditioning prior to CRS.

Both membrane-associated and secreted mucins largely contribute to the pathophysiology of human carcinomas. Through ligand–receptor interactions and morphogenetic signal transduction, membrane-associated mucins are believed to regulate survival, proliferation, and differentiation of malignant cells and to provide signals about adhesion status and presumably other cell-surface conditions. Membrane-associated mucins are aberrantly expressed in cancer. MUC1 is overexpressed and, upon loss of cell polarity, redistributed over the entire cell surface. Tumor-associated MUC1 (TA-MUC1) is believed to play critical roles in carcinogenesis and, more likely, in cancer progression, invasion, and metastasis. TA-MUC1 also contributes to tumor angiogenesis, chemoresistance, metabolism, and inflammation. In GC, MUC1 is believed to act as an oncogene/oncoprotein. Likewise, MUC1 expression correlates with an increased risk of carcinogenesis and serves as a marker of progression and metastasis in CRC. Secreted mucins, too, play different roles in cancer. The mucus layer secreted by and associated with tumor cells serves as an impenetrable physicochemical barrier that helps them evade immune and inflammatory responses and resist chemotherapy. This mucus layer also captures biologically active molecules, including growth factors or cytokines, which might contribute to tumor growth. Furthermore, it is an important factor in determining the pattern of peritoneal dissemination. In contrast to PFCCs cells lacking a fluid vehicle, those coated by secreted mucins are freely redistributed on the abdominopelvic surfaces by peritoneal flow governed by intraperitoneal hydrodynamics. MUC2 and MUC5AC are gel-forming mucins with specific contributions to tumor biology. MUC2 has been identified as a major carrier of tumor-associated antigens, including STn and sLe^x, with implications in tumorigenesis and metastasis of gastrointestinal cancer. M1 antigen, an early oncofetal marker of colonic carcinogenesis, is indeed the product of the MUC5AC gene. M1/MUC5AC mucin is abnormally expressed by colonic goblet cells during colon carcinogenesis. In agreement, *de novo* expression of MUC2 and MUC5AC or a mucinous phenotype can be indicative of a more aggressive phenotype in gastrointestinal malignancies. Moreover, overproduction or ectopic secretion of gel-forming mucins can be a major determinant of tumor pathogenesis. A typical example is the peritoneal adenomucinosis or mucinous carcinomatosis from different primary sites, including the appendix, stomach, small and large bowel, urachus, pancreas, gallbladder, and ovary. The presence of mucus ascites produced by all grades of mucinous adenocarcinoma results in a wider distribution of cancer cells throughout the abdomen and pelvis. As a result, mucinous tumors reliably occupy spaces within the abdominopelvic cavity that are rarely involved when non-mucinous cancer occurs. In this regard, PMP is a paradigm wherein gel-forming mucins, in particular MUC2, are a major cause of morbidity. Given the critical roles of MUC1, MUC2, and MUC5AC mucins in the biology and pathogenesis of epithelial tumors, the mucin-depleting activity of BR/NAC is an eminent virtue of the treatment. With depriving tumor cells of a key biological infrastructure and a protective framework, mucin depletion apparently con-

tributes to BR/NAC-induced cytotoxicity and chemosensitivity, too. In accord with this striking feature, BR/NAC was found in a parallel study in our Department to effectively disintegrate PMP-secreted mucin gels. This capability can be utilized to solubilize, completely or partially, intraperitoneal mucinous material, thus enabling the drainage of solubilized matter or facilitating surgical removal. Therefore, BR/NAC treatment of gastrointestinal cancer cells and tumors not only inhibits the synthesis of both membrane-associated and secreted mucins, but also dissolves the secreted mucins. The resultant depletion and lysis of tumor-associated mucins further justify the use of this modality in peritoneal dissemination of mucin-expressing tumors. Taken together, this experimental formulation shows preclinical promise for intraperitoneal treatment of gastrointestinal PC and PMP, representing a dual-function modality affecting tumor cells and their associated mucins both.

6.2 Future Directions

In the multidisciplinary approach to PC, the pattern of recurrence after an initial optimal treatment may be suggestive of the underlying cause of failure and thus indicate the potential directions for improvement in the standard of care. A localized form of recurrence within the abdomen may be a result of “surgical failure” to completely eradicate the disease burden despite complete adhesiolysis before the administration of intraperitoneal chemotherapy. Tumor cells entrapped in scar tissue are less likely to be eradicated with intraperitoneal chemotherapy than PFCCs. Another potential cause for surgical failure is the involvement of the small bowel during initial cytoreduction as electroevaporative surgery cannot be used in the same manner as in other anatomic locations in the abdomen. As such, the progression of disease on the small bowel surface can be encountered at reoperation of patients with the primary appendiceal neoplasms. Disruption of the peritoneal barrier and iatrogenic implantation of cancer cells may also contribute to surgical failure. On the other hand, a diffuse intra-abdominal recurrence probably suggests a failure of intraperitoneal chemotherapy to eradicate tumor cells remaining after initial cytoreduction. This is an important type of failure since it is associated with worse outcomes. Thus, novel locoregional methods are needed to help maintain a disease-free peritoneal surface after complete cytoreduction. This should include efforts to improve hyperthermic intraperitoneal chemotherapy (HIPEC) by determining optimal agents, maximal doses, and optimal levels of hyperthermia to be used (Bijelic et al. 2008). Given the high propensity of PC for recurrence, especially in high-grade cancers, the development of adjuvant modalities is necessitated.

According to the pharmacokinetic studies, while orally administered NAC is almost completely absorbed from the gastrointestinal tract (Holdiness 1991), only a very small amount of oral BR (0.01–0.05 %) is absorbed (Izaka et al. 1972). Hence, oral NAC, but not oral BR, can achieve the cytotoxic concentrations. Pharmacological evidence also shows that intravenous drugs can be targeted to the peritoneal surface if administered in the presence of artificial ascites, for example

simultaneously with a large volume of intraperitoneal chemotherapy solution (Van der Speeten et al. 2010). Thus, a combination of intraoperative intraperitoneal and intravenous chemotherapy (bidirectional chemotherapy) is another direction for optimization of HIPEC. According to this pharmacological phenomenon, it can also be concluded that intravenous application of BR/NAC might be of therapeutic benefits in patients with malignant ascites. Issues with drug incompatibility can further justify the use of bidirectional chemotherapy (Sugarbaker and Bijelic 2012). Results from the present work suggest that an optimized combination of BR and NAC is a promising candidate for locoregional strategies. According to the literature and our *in vivo* data, intraperitoneal administration of BR and NAC appears to be a safe treatment option. However, safety of this treatment needs to be validated preclinically. For this purpose, a toxicology study has been designed and approved by The University of New South Wales Animal Care and Ethics Committee (ACEC). Our *in vitro* results suggest that BR/NAC has the potential to enhance cytotoxic effects of chemotherapeutic agents on gastrointestinal cancer cells. Using similar murine models, we thus intend to evaluate the efficacy and chemosensitizing potential of BR/NAC in adjuvant intraperitoneal chemotherapy. In this regard, treatment-induced depletion of mucin is postulated to be a contributory factor to chemotherapy potentiation. This notion and other possible mechanisms underlying chemosensitizing activity of the treatment need to be further investigated. Finally, the beneficial effects of this experimental treatment can also be evaluated in other peritoneal surface malignancies or primary tumors of the gastrointestinal tract, as well as in mucinous tumors or other pathological conditions with overproduction or ectopic secretion of mucin glycoproteins.

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