STUDIES ON THE ANTICANCER PROPERTIES OF PLEUROCIDINS: A
PRECLINICAL EVALUATION

by

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For my wonderful husband and kindred spirit

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Abstract

Cationic antimicrobial peptides (CAPs) are small peptides that constitute an important defence against microbial pathogens. Certain CAPs also possess anticancer properties. NRC-03 and NRC-07 are pleurocidins derived from winter and yellowtail flounder, respectively. The purpose of this investigation was to study the anticancer properties of NRC-03 and NRC-07. NRC-03 and NRC-07 killed breast cancer cells, including P-glycoprotein-overexpressing cells, in a time-dependent manner that peaked at 4 h. NRC-03 and NRC-07 lysed breast cancer cells by a mechanism that involved cell binding, mitochondrial destabilization, nuclear localization, and significant membrane damage. Interestingly, NRC-07, but not NRC-03, caused DNA fragmentation. NRC-03 and NRC-07 killed normal human epithelial cells, but did not kill endothelial cells or fibroblasts, or lyse human erythrocytes. NRC-03, and to a lesser extent NRC-07, had chemo-sensitizing properties, suggesting promise for their inclusion in combinational treatment regimens. Importantly, intratumoural injections of NRC-03 or NRC-07 inhibited tumour growth in a mouse model of breast cancer. Fetal bovine serum dose-dependently reduced cell killing by NRC-03. NRC-03 was degraded in human and mouse serum, which limited its potency. NRC-03- and NRC-07-induced cytotoxicity correlated with expression of several different negatively-charged molecules, rationalizing the generation of [D]-NRC-03, which carries the same positive charge as NRC-03, and was more potent but less selective for cancer cells than NRC-03. [D]-NRC-03 was also cytolytic and exhibited *in vivo* anticancer properties. To further test the clinical potential of NRC-03- and NRC-07-resistant cells were generated. NRC-03 and NRC-07 bound to resistant cells to a lesser extent than parental cells and were phenotypically distinct. Importantly, NRC-03- and NRC-07-resistant cells were killed by chemotherapeutic drugs, as well as [D]-NRC-03. These studies demonstrate that NRC-03, NRC-07, and [D]-NRC-03 are cytolytic peptides that kill breast cancer cells *in vitro* and *in vivo*. While more potent than NRC-03, [D]-NRC-03 requires further modification to minimize its toxicity toward normal cells. Although cancer cells may become resistant to NRC-03 and NRC-07 over time, resistant cells are still killed by other cytotoxic drugs, thereby reinforcing the value of adding these peptides to combinational regimens for the treatment of breast cancer.

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List of Abbreviations and Symbols Used

^{3}H-TdR		tritiated thymidine
aa		amino acid
AIF		apoptosis inducing factor
Amino acids		
	A	alanine
	D	asparatic acid
	E	glutamic acid
	F	phenylalanine
	G	glycine
	H	histidine
	I	isoleucine
	K	lysine
	L	leucine
	Q	glutamine
	R	arginine
	S	serine
	T	threonine
	V	valine
	W	tryptophan
	Y	tyrosine
ANOVA		one way analysis of variance
APS		ammonium persulfate
ATP		adenosine triphosphate

bcl-2	B cell lymphoma 2
biotin-NRC-03	biotinylated-NRC-03
biotin-NRC-07	biotinylated-NRC-07
BNIP3	bcl-2 and nineteen kilodalton interacting protein-3
BSA	bovine serum albumin
C	carboxy
CAP	cationic antimicrobial peptide
caspase	cysteine-aspartic acid specific protease
cFBS	control not heat-inactivated FBS
CFU	colony forming units
Ci	Curie
CO_2	carbon dioxide
CPM	counts per minute
CS	chondroitin sulfate
CSP	chondroitin sulfate proteoglycan
d	day
DHE	dihydroethidium
$DiOC_6$	3,3'-dihexyloxacarbocyanine iodide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EC_{50}	half maximal effective concentration

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGM	endothelial cell growth medium
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EIA	enzyme immunoassay
ER	estrogen receptor
FBS	fetal bovine serum
FGM	fibroblast growth medium
FLZ	flunarizine
g	gram
GSH	reduced glutathione
h	hour
H_2SO_4	sulphuric acid
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HDF	human dermal fibroblast
HER2	human epidermal growth factor receptor 2
HeNe	helium neon
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMEC	human mammary epithelial cell
HRP	horseradish peroxidase
HeS	heparan sulfate
HS	human serum
Hsp	heat shock protein

HSP	heparan sulfate proteoglycan
HUVEC	human umbilical vein endothelial cell
IC_{50}	half maximal inhibitory concentration
l	litre
LDH	lactate dehydrogenase
LfcinB	bovine lactoferricin
LfcinM	murine lactoferricin
m	milli
mm	millimetres
M	molar (moles/l)
mAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
MEGM	mammary epithelial cell growth medium
MFI	mean fluorescence intensity
min	minute
MS	mouse serum
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	amino
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaF	sodium fluoride
Na_2HPO_4	disodium hydrogen phosphate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide

NaVO$_4$	sodium orthovanadate
NMR	nuclear magnetic resonance
NOD SCID	non-obese diabetic severe combined immunodeficient
NS	not statistically significant
OD	optical density
o/n	overnight
OSGE	O'-sialoglycoprotein endopeptidase
OsO$_4$	osmium tetroxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	propidium iodide
PMSF	phenylmethanesulfonyl fluoride
P-gp	P-glycoprotein
PR	progesterone receptor
RBC	red blood cell
RGD	arginine-glycine-aspartic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RNA	ribonucleic acid
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SE	standard error of the mean
SEM	scanning electron microscopy

sfDMEM	serum-free Dulbecco's modified Eagle's medium
StDev	standard deviation
TBST	tris buffered saline-Tween 20
Tdt	terminal deoxynucleotidyl transferase
TEAB	triethylammonium bicarbonate
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
TUNEL	Tdt dUTP nick end labeling
UV	ultraviolet
v	volume
VEGF	vascular endothelial growth factor
w	weight
wk	week
~	approximately
°C	degrees Celsius
α	alpha
α-carbon	chiral carbon
β	beta
μ	micro
$\Delta\Psi_m$	change in mitochondrial membrane electrochemical gradient

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Despite many advances in cancer treatment, cancer remains a growing health concern, primarily due to the aging demographic. Based on GLOBOCAN 2008 estimates, approximately 12.7 million cancer diagnoses and 7.6 million cancer deaths occurred in 2008. The Canadian Cancer Society estimates that 177,800 Canadians will be diagnosed with cancer and 75,000 will die from cancer in 2011 (1). The word cancer is a general term that describes a multitude of individual diseases that affect different types of cells and tissues. Cancers are alike in that they all involve abnormal cellular growth as a consequence of a small number of genetic mutations (2). Hanahan and Weinberg argue that cancer development is characterized by six biological capabilities. These "hallmarks of cancer" are: [1] sustaining proliferation; [2] resisting antiproliferation signals; [3] resisting programmed cell death; [4] enabling replicative immortality; [5] stimulating angiogenesis; [6] invading tissues and metastasizing throughout the body (3). In addition to these hallmarks, two additional hallmarks are emerging, i.e., reprogramming of energy metabolism, and evading detection and elimination by the immune system (4). Whereas localized tumours are treated with surgery and/or radiotherapy, chemotherapy continues to be the treatment of choice for advanced or metastatic cancers (5).

This investigation focuses on two types of cancer: breast cancer and multiple myeloma. Breast cancer is the most frequently diagnosed cancer and second leading cause of cancer-related deaths in Canadian women (1). An estimated 23,600 Canadians will be diagnosed with breast cancer, and 5,100 will die from breast cancer in 2011 (1). Three main types of breast cancer exist: lobular carcinoma, ductal carcinoma, and inflammatory breast cancer (6). Breast cancer is typically treated with a combination of surgery, radiotherapy, and systemic therapy, which includes both hormonal therapy and chemotherapy. The choice of systemic therapy is dependent on the stage of the disease, estrogen receptor (ER), progesterone receptor (PR) and/or human epidermal growth

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factor receptor 2 (HER2) expression levels, lymph node involvement, patient age, and menopausal status. Hormonal therapies, which include the selective estrogen receptor modulators and selective aromatase inhibitors, are indicated for patients with ER-positive PR-positive tumours (6). Trastuzumab (Herceptin®) is offered to patients with HER2-expressing tumours (7, 8). Combinational chemotherapy (e.g., cyclophosphamide, methotrexate, and 5-fluorouracil) is offered to patients with ER-negative PR-negative tumours, as well as patients with tumours larger than 1 cm (6). Doxorubicin, methotrexate, mitoxantrone, cyclophosphamide, docetaxel, paclitaxel, 5-fluorouracil and cisplatin are chemotherapeutic agents that may be used to treat breast cancer (9-12). Side effects from these drugs are numerous and include fatigue, changes in taste, headache, nausea, fluid accumulation, muscle pain, joint pain, weight gain, vomiting, hair loss, ovarian failure, hypersensitivity reactions, kidney damage, cardiotoxicity, myelosuppression, nerve damage, and the potential to cause secondary cancers (10-12).

Multiple myeloma is a hematological malignancy that is characterized by the proliferation of neoplastic plasma cells in the bone marrow (13). In comparison to breast cancer, multiple myeloma is relatively rare. The Canadian Cancer Society estimates that 2,300 Canadians will be diagnosed with multiple myeloma, of which 1,350 will die from multiple myeloma in 2011 (1). The prognosis of patients with multiple myeloma has improved in recent years with the introduction of autologous stem cell transplantation and treatment with the drugs thalidomide, lenalidomide, and bortezomib (14-16). The treatment for multiple myeloma is dependent on the biological age of the patient, whether the patient can tolerate stem cell transplantation, and whether the patient has any coexisting conditions (13). These factors determine the drugs the patient will receive, as well as the dose and duration of therapy. Patients diagnosed with multiple myeloma are typically treated with combinational treatment regimens consisting of thalidomide, lenalidomide, bortezomib (Velcade®), dexamethasone, cyclophosphamide, melphalan, and prednisone (13). Side effects from these therapies include infection, neutropenia, anemia, hyperglycemia, lymphopenia, deep vein thrombosis, peripheral sensory neuropathy, and thrombocytopenia. The 10-year survival rate for patients under 60 years of age is approximately 30%, which highlights the need for more effective anticancer agents for the treatment of multiple myeloma (14).

Cisplatin and docetaxel are two chemotherapeutic agents used in this investigation. Cisplatin is a platinating chemotherapeutic agent that kills cells by causing intra- and inter-strand deoxyribonucleic acid (DNA) crosslinks, which interfere with transcription and translation (17, 18). Cisplatin also reacts with phospholipids, ribonucleic acid (RNA) and proteins, which may also contribute to cisplatin-induced cytotoxicity (19, 20). Cancer cells can become resistant to cisplatin by increasing their synthesis of glutathione (GSH), which reacts with cisplatin and forms a complex that is pumped out of the cell by the adenosine triphosphate (ATP)-dependent GSH S-conjugate export pump (21, 22). Cancer cells can also become resistant to cisplatin by increasing DNA repair of cisplatin/DNA lesions (23). Docetaxel belongs to the taxane family of chemotherapeutic agents. Docetaxel (Taxotere®) is a semisynthetic derivative of paclitaxel (Taxol®), which is derived from the bark and needles of the Pacific (*Taxus brevifolia*) and European (*Taxus baccata*) yew trees, respectively (24). Docetaxel is believed to inhibit microtubule disassembly, which interferes with the formation of the mitotic spindle, thereby causing cell death (25). Cancer cells can become resistant to docetaxel by increasing their expression of the drug efflux pump P-glycoprotein (P-gp) (26, 27), or by altering β-tubulin isotype expression (28).

Traditional chemotherapeutic agents target cells that rapidly divide. As a result, chemotherapeutic agents cannot discriminate between proliferating cancer cells and proliferating normal cells (29). Consequently, chemotherapy is associated with the many side effects listed above. Moreover, slow growing or dormant cancer cells often fail to respond to chemotherapy (30). In these instances, patients experience negative side effects without any decrease in tumour burden. Drug resistance further reduces the therapeutic utility of cytotoxic chemotherapeutic agents (31). Furthermore, certain anticancer drugs are associated with the development of secondary malignancies. For example, tamoxifen use in patients with breast cancer has been associated with the development of endometrial cancer (32), whereas treatment with cyclophosphamide, methotrexate and 5-fluorouracil has been associated with the development of acute myeloid leukemia (33). Therefore, significant efforts have been made to develop anticancer agents that specifically target and kill cancer cells without harming normal proliferating cells. These efforts have resulted in the development of novel "targeted"

therapies that selectively kill cancer cells by interfering with specific molecules that are required for tumour growth. Trastuzumab is a humanized anti-HER2 monoclonal antibody that blocks HER2 signaling in HER2-expressing breast cancers (34). Unfortunately, trastuzumab-resistance can result from receptor mutations, receptor masking by bulky mucin proteins, and/or alterations in dominant signaling pathways (35, 36). Therefore, despite many advances in the development of novel anticancer agents, the need for a drug that can specifically kill cancer cells, including those that are slow growing and/or multidrug-resistant, remains a focus in the development of novel therapeutic agents for the treatment of cancers.

1.2 Cationic Antimicrobial Peptides

Cationic antimicrobial peptides (CAPs) represent a promising alternative to conventional chemotherapeutic agents for the treatment of cancers. CAPs are small positively charged peptides that are typically fewer than 40 amino acid (aa) residues in length (37). CAPs are predominantly composed of basic (e.g., lysine [K] and arginine [R]) and hydrophobic (e.g., tryptophan [W]) aa. CAPs typically adopt random coil arrangements in aqueous environments and amphipathic secondary structures when they encounter biological membranes (38-40). CAPs are classified on the basis of their secondary structure, of which four main classes have been identified: [1] α-helical; [2] β-sheet; [3] loop; [4] extended peptides (41). CAPs play an important role in the innate immune system of virtually all organisms, including bacteria, fish, and mammals, because they are able to kill microorganisms at low concentrations. Various CAPs are able to modulate immune cell function (42-44), as well as kill both Gram-negative and -positive bacteria (45-48). In addition, certain CAPs exhibit antiviral (49), antifungal (45, 50), antiparasitic (45, 51), and/or anticancer properties (46, 52-57).

1.3 Pleurocidin-derived CAPs

Fish are a particularly rich source of CAPs because they rely heavily on their innate immune system (40). For example, mucous secretions of certain species of fish exhibit antimicrobial properties that protect the fish from pathogens (58). Pleurocidin (GWGSFFKKAAHVGKHVGKAALTHYL) is a 25 aa CAP that was originally isolated

from the skin secretions of *Pleuronectes americanus*, or winter flounder (59). Pleurocidin was predicted to adopt an α-helical secondary structure when in contact with biological membranes. The authors also predicted that pleurocidin would kill bacteria by forming pores in the membrane resulting in cell lysis. Experiments revealed that pleurocidin is produced in epidermal mucous-producing cells, as well as goblet cells of the proximal portion of the small intestine, but not in the heart, striated muscle, gills, stomach, liver, or spleen of the fish (60). Pleurocidin inhibited the growth of *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus,* and *Candida albicans* isolated from patients. Interestingly, low concentrations of pleurocidin interfere with DNA replication and gene expression, whereas high concentrations lyse bacteria (47). This finding suggests that the concentration of pleurocidin determines the mechanism by which it kills bacteria.

The conserved flanking regions of pleurocidin were used to amplify potentially novel CAPs from various Atlantic flatfish species, including winter flounder (*P. americanus*), yellowtail flounder (*P. ferruginea*), American plaice (*Hippoglossoides platessoides*), witch flounder (*Glyptocephalus cynoglossus*), and others (48). These investigators amplified 35 sequences and predicted those that encode functional CAPs based on the charge, hydrophobicity, and sequence similarity to known biologically active CAPs. Of the 35 sequences that were amplified, 20 were synthesized (NRC-01-NRC-20) and tested for antimicrobial activity against various pathogens, including *P. aeruginosa, Salmonella enterica* serovar Typhimurium, *E. coli*, *S. epidermidis*, *C. albicans*, and methicillin-resistant *S. aureus*. Of these CAPs, the inhibitory effects of NRC-03, NRC-12 and NRC-13 were equal to or greater than those of the original pleurocidin against all of the pathogens evaluated, whereas the inhibitory properties of NRC-06, NRC-07, NRC-11, NRC-15, NRC-16, and NRC-17 were equal to or greater than pleurocidin in *almost* all of the pathogens examined. These findings suggested that CAPs from Atlantic flatfish, which are herein referred to as pleurocidins on the basis of the conserved flanking regions, may be a promising novel treatment option for bacterial infections; however the anticancer properties of these CAPs were not evaluated. The purpose of my research was to determine whether these CAPs possess anticancer properties. Of the 20 CAPs identified, the anticancer potential of NRC-03, NRC-07,

NRC-13 and NRC-16 was evaluated. These CAPs were selected on the basis of charge, length, number of hydrophobic aa, as well as antibacterial activity. In addition, our collaborators speculated that the antibacterial core of NRC-03 resided in the first 6 aa residues (GRRKRK) (48). Furthermore, our research group showed that the anticancer properties of the CAP bovine lactoferricin (LfcinB) resided in a sequence of only 10 aa (55). Based on these findings, the anticancer potential of the first 10 aa of NRC-03 (NRC-03$_{10 \text{ mer}}$) was also evaluated. Table 1.1 indicates the sequence, length and charge of these peptides.

1.4 CAPs with Anticancer Properties

CAPs with anticancer properties can be direct-acting and/or indirect-acting (61). Direct-acting CAPs bind to cells and cause significant and irreversible membrane damage, which leads to cell lysis. Therefore, direct-acting CAPs induce cell death by necrosis rather than apoptosis. Certain CAPs, such as PR-39 and pleurocidin, kill bacteria in the absence of membrane damage, which suggests that these CAPs can gain access to the cytosol of bacteria without directly causing cell lysis (47, 62, 63). Certain CAPs may also kill cancer cells by an indirect mechanism. In this regard, indirect-acting CAPs are defined as CAPs that bind to cancer cells and enter the cytoplasm where they initiate a signaling cascade that leads to cell death. Indirect-acting CAPs may trigger cysteine-aspartic acid specific protease (caspase)-dependent or -independent apoptosis, which is typically mitochondrial-dependent (61). Alternatively, cell death may involve autophagy (64). Mechanisms of action of direct- and indirect-acting CAPs, as well as specific examples of each will be discussed in detail in sections 1.4.2 and 1.4.3, respectively.

Both direct- and indirect-acting CAPs must bind to the target cell in order to cause cell death (61). As described in section 1.4.1, there are many fundamental differences between cancer cell membranes and normal cell membranes, which are thought to contribute to the selective binding of certain CAPs to cancer cells (37, 38, 65-67). Most experts agree that electrostatic interactions between positively charged CAPs and negatively charged molecules on the outer membrane leaflet of cancer cells mediates selective binding of certain CAPs to cancer cells (37, 38, 66, 67). Direct-acting CAPs that bind to cancer cells on the basis of charge are of particular interest as novel anticancer

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Table 1.1 Pleurocidins with potential anticancer activities

CAP	Sequence[a]	Charge[b]	Length (aa)
NRC-03	GRRKRKWLRRIGKGVKIIGGAALDHL-NH$_2$	+9.5	26
NRC-03$_{10mer}$	GRRKRKWLRR-NH$_2$	+8.0	10
NRC-07	RWGKWFKKATHVGKHVGKAALTAYL-NH$_2$	+8.0	25
NRC-13	GWRTLLKKAEVKTVGKLALKHYL-NH$_2$	+6.5	23
NRC-16	GWKKWLRKGAKHLGQAAIK-NH$_2$	+7.5	19

[a]Basic or cationic aa are shown in red, whereas blue indicates hydrophobic aa.
[b]K and R are estimated to have a charge of +1, H a charge of + 0.5, D and E a charge of -1 and C-terminal amidation a charge of +1.

agents because they are predicted to target a large number of cancer cells, including those that are slow growing and multidrug-resistant, without harming normal cells. Furthermore, certain direct-acting CAPs are likely attracted to a number of negatively charged surface molecules. Consequently, cancer cell resistance to these CAPs is unlikely because the cell would have to significantly change the composition of its membrane rather than mutate a unique receptor. Furthermore, because direct-acting CAPs do not cause cell death by initiating a signaling cascade in the target cell (61), resistance cannot be achieved by mutations in the dominant signaling pathway. Certain CAPs also enhance cell killing by traditional cytotoxic chemotherapeutic agents. Cecropin A, which kills leukemia cells (68), synergizes with 5-fluorouracil and cytarabine against leukemia cells (69). Magainin A and B, which are synthetic derivatives of magainin, kill small cell lung cancer cells and enhance cell killing by etoposide and cisplatin (70). Additionally, certain CAPs kill multidrug-resistant cancer cells, e.g., gaegurin 6 and its derivative PTP7 (71), as well as the mammalian-derived extended-helical CAPs (OLP-1, -4), and insect-derived alpha-helical CAPs (OLP-7, -8) (72). Together these findings suggest that certain CAPs may be useful candidates to add to combinational treatment regimens for the treatment of certain cancers. Furthermore, as described above, certain CAPs kill bacteria by different mechanisms, depending on the concentration used. A similar finding was recently reported in cancer cells by Chang *et al* who showed that the hepcidin-like CAP TH1-5 induces apoptosis in HeLa cells at 50 μM, whereas 100 μM TH1-5 kills HeLa cells by lysis (57). Importantly, neither dose was significantly toxic for normal human cells. The dual mechanism of action of certain CAPs gives them an additional advantage over traditional cytotoxic chemotherapeutic agents.

1.4.1 Suggested Basis for Anticancer Activities by CAPs

Although researchers have speculated for many years why certain CAPs preferentially kill cancer cells (37, 38, 55, 73, 74), few experiments have been done to determine why certain CAPs selectively bind to and kill cancer cells. Regardless, there are many fundamental differences between cancer cell membranes and normal cell membranes that likely account for differences in CAP binding, and consequently CAP-mediated cell killing. One of the most significant differences between cancer cell membranes and normal cell membranes is that the former carry a net negative charge. It

is expected that positively charged CAPs will be attracted to cells with negatively charged membranes (37, 38, 61, 67, 75). Normal cell membranes are zwitterionic and therefore neutral in charge due to the presence of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine (76). In contrast, the outer membrane leaflet of cancer cells contains 3- to 7-fold more negatively charged phosphatidylserine residues than normal healthy cells (77). Differences in lipid composition between cancer cells and normal cells may contribute to the selective binding of CAPs to cancer cell membranes. This hypothesis is supported by experiments conducted in model membrane systems, which show that cecropin and magainin bind to acidic model membranes to a greater extent than those composed of zwitterionic phospholipids (78-80). These findings suggest that the lipid composition of the target cell membrane is crucial for determining whether the cell will be susceptible to CAP-mediated killing.

In addition to differences in lipid composition between normal cells and cancer cells, the negative charge of cancer cell membranes is further augmented by increased expression of negatively charged O-glycosylated mucins (81), heparan sulfate proteoglycans (HSPs) (82), and sialylated glycoproteins (83). Paradoxically, negatively charged HSPs, and to a lesser extent chondroitin sulfate proteoglycans (CSPs), inhibit LfcinB-induced cell death (84). The authors speculated that this was a consequence of HSPs sequestering LfcinB from the plasma cell membrane, thereby inhibiting the ability of LfcinB to induce cell lysis (84). In contrast, the same authors showed that certain 9 aa derivatives of LfcinB kill HSP- and CSP-overexpressing cells to a greater extent than control cells (85). The authors propose that whereas all CAPs studied are attracted to HSPs and CSPs, there is inadequate steric hindrance by HSPs and CSPs to sequester truncated CAPs away from the plasma membrane.

Cancer cell membranes have a greater surface area than normal cell membranes, which is a consequence of greater numbers of microvilli on the surface of the cell (86). Chan *et al* have found a correlation between microvilli numbers and cecropin-B-induced cell death (87). The authors speculated that a threshold concentration of cecropin-B must bind to the target cell to initiate cell death. Although this is an attractive hypothesis to explain why CAPs selectively kill cancer cells, it has not yet been proven.

Membranes of certain cancer cells are more fluid than normal cell membranes (88, 89). In 2007, Leuschner and Hansel suggested that cholesterol may protect cells from CAP-mediated cell killing by decreasing membrane fluidity (90). Other studies demonstrated that the cholesterol content of the membrane is inversely correlated with CAP-induced cell death (44, 91, 92). CAPs may preferentially kill cancer cells with more fluid membranes because the hydrophobic aa side chains of the CAP may integrate more easily into the lipid core of the membrane thus anchoring the CAP to the cell. Furthermore, once bound, CAPs may more easily aggregate and destabilize membranes with higher membrane fluidity, thus enhancing CAP-mediated cell death. Finally, research suggests that the presence of a negative transmembrane potential enhances cell killing by the CAP magainin (93). Other groups have shown that killing by magainin and its synthetic analogues is dependent on the presence of a polarized membrane (94). Taken together, these findings show that the composition and characteristics of the target cell membrane are crucial for determining whether the cell will be susceptible to CAP-mediated cell killing.

1.4.2 Direct-Acting CAPs

Direct-acting CAPs are defined as CAPs that directly kill target cells by causing membrane damage and cell lysis (61). Many CAPs directly kill cancer cells, including the LfcinB derivative LTX-302 (95), gomesin (56), magainin II, (52), the hybrid CAP P18 (96), TH1-5 (57), and epinecidin-1 (97). Direct-acting CAPs have different toxicity profiles toward both cancer cells and normal cells. Moreover, the concentration required for cytotoxic activity differs depending on the CAP. I will now discuss the anticancer properties of three recently characterized direct-acting CAPs. These CAPs were selected based on the organism(s) from which they were derived, the evidence for a cytolytic mechanism of action, and/or their antitumour properties *in vivo*.

P18 is an 18 aa hybrid peptide that consists of the amino (N)-terminal region of cecropin A and the carboxy (C)-terminal portion of magainin II, which were isolated from the pupa of the cecropia moth *Hyalaphora cecropia* (98), and the skin secretions of the African clawed flog *Xenopus laevis* (99), respectively. P18 has antibacterial properties, but lacks hemolytic activity (100). P18 kills melanoma cells in a dose-

dependent manner that exceeds P18 killing of normal mouse fibroblasts (96). P18 binds to melanoma cells within 2 min and rapidly depolarizes cellular membranes. By 24 h, nearly 100% of P18-treated cells undergo cytolysis (96). Interestingly, despite the damage that P18 causes to the plasma membrane, the nuclear membrane largely remains intact.

Gomesin is an 18 aa CAP isolated from the hemocytes of the tarantula spider *Acanthoscurria gomesiana* (45). Gomesin kills melanoma, breast, colon, and cervical cancer cells within 12 h with IC_{50} values ranging from 1.36-8.13 μM (56). Importantly, gomesin also kills human umbilical vein endothelial cells (HUVECs), suggesting that modification of the peptide may be necessary to improve its clinical potential. Gomesin binds to the surface of melanoma cells, causing membrane permeabilization within 10 min, which suggests that gomesin rapidly causes direct cell death (56). Importantly, gomesin derivatives composed exclusively of D-aa maintain their anticancer properties (56), suggesting that gomesin-induced membrane damage is not a consequence of chiral-dependent interactions between gomesin monomers and the cell membrane. Interestingly, cancer cell killing by gomesin requires at least one disulfide bond, whereas two disulfide bonds are necessary for enhanced serum stability *in vitro*.

Epinecidin-1 is a 21 aa CAP isolated from the grouper *Epinephelus coioides* (101). Epinecidin-1 kills lung carcinoma, cervical carcinoma, hepatocellular carcinoma, and fibrosarcoma cell lines in a dose-dependent manner (97). Higher doses of epinecidin-1 are also toxic for normal cells, including fibroblasts, kidney cells, and hepatocytes. Epinecidin-1 (2 μg/ml) kills fibrosarcoma and normal kidney cells within 1 h and does not induce DNA fragmentation (97). These findings suggest that epinecidin-1 kills cells by lysis.

Many theories have been proposed to explain how CAPs destabilize cellular membranes. These theories include the barrel-stave, carpet, toroidal pore (two-state), and detergent models (38). To date, most of the data supporting or rejecting these theories has been performed on model membranes. Consequently, mechanisms of membrane disruptions in cancer cells have not been well defined. Nonetheless, these theories help

investigators visualize how CAPs enter eukaryotic cells and/or cause extensive membrane damage that leads to cell death.

The "barrel-stave" model, which was first proposed in 1974, describes a process whereby aggregated peptides form transmembrane pores in the target cell membrane in a manner that resembles staves in a barrel (102). In the barrel-stave model, pore formation is initiated by the recruitment of CAP monomers to the target cell membrane via electrostatic interactions between the basic aa of the CAP and the acidic molecules on the surface of the target cell (Fig. 1.1A). A conformational change in the CAP following its binding to the cell surface leads to the exposure of hydrophobic aa side chains, which subsequently insert into the lipid core of the plasma membrane securing the CAP in place. Over time, CAP monomers aggregate and penetrate deeper into the lipid core of the membrane until a transmembrane pore is formed. The length and secondary structure of the CAP are instrumental in determining whether the CAP can damage cell membranes by this mechanism because the CAP must be long enough to span the membrane of the target cell. A minimal length of ~20 and ~8 aa is required for membrane spanning by α-helical and β-sheet CAPs, respectively (38). Therefore, shorter direct-acting CAPs cannot damage cell membranes by the barrel-stave model. Alamethicin is a 22 aa non-cationic peptide that is predicted to form transmembrane pores, as described by the barrel-stave model (103). Pores formed by alamethicin are ~1 nm in diameter (104) and are composed of ~11 molecules (103).

As the name suggests, the "carpet" model describes a mechanism by which CAPs bind to the target cell in a parallel manner covering the membrane of the target cell like a carpet (Fig. 1.1B). As with the barrel-stave model, CAP monomers bind to the surface of the target cell by electrostatic interactions. CAP binding causes the displacement of phospholipids, leading to membrane thinning and curvature stress, which is predicted to cause the formation of transient pores (37). The formation of transient pores could explain how certain CAPs gain access to the cytosol in the absence of any significant membrane damage. Alternatively, membrane disruption may occur when a threshold concentration of peptide is reached. In this regard, excessive CAP binding and phospholipid dislocation may cause cell lysis as a consequence of the inability of the cell
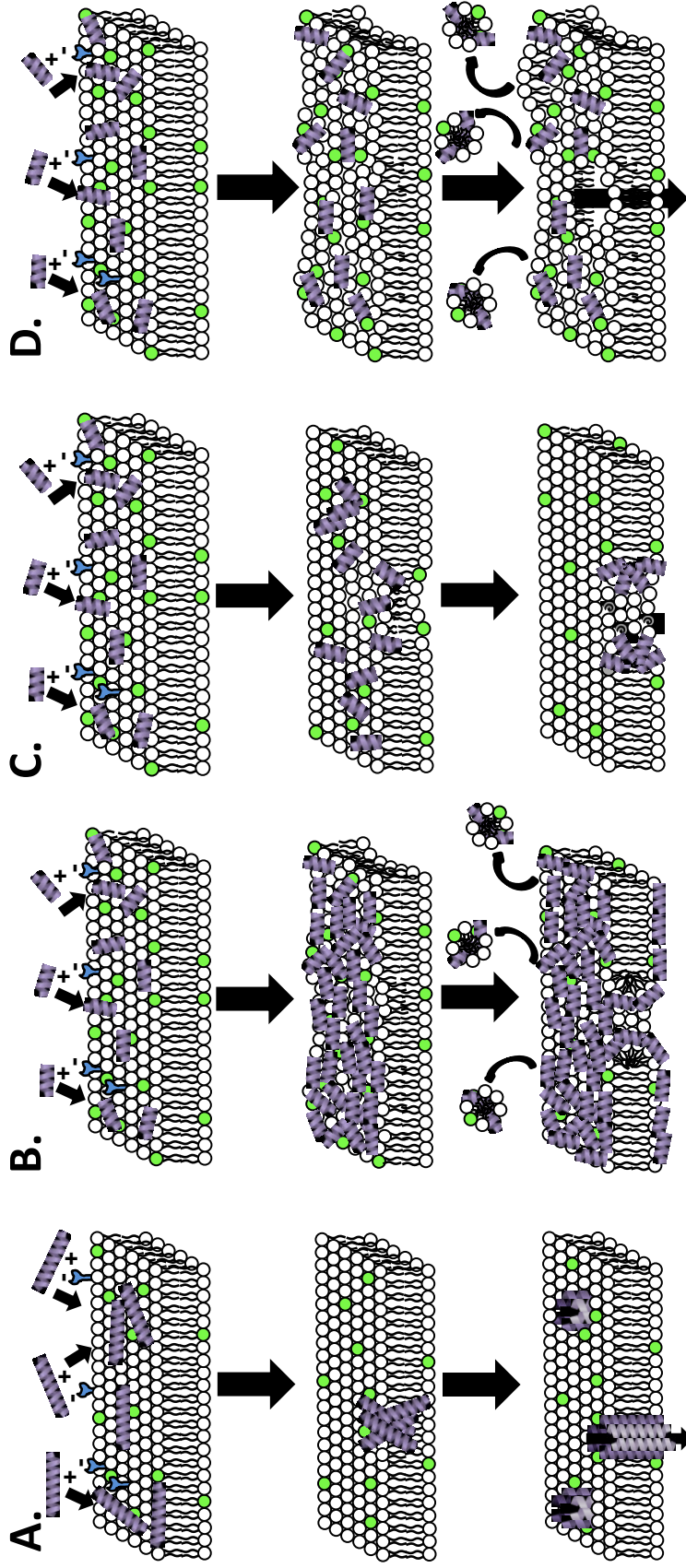
Figure 1.1. Models of CAP-mediated membrane disruption. (A) The barrel-stave model suggests that membrane damage is initiated by CAP binding to the surface of the target cell via electrostatic interactions. Following peptide binding, the hydrophobic aa side chains insert into the lipid core of the plasma membrane. CAP monomers then aggregate and insert deeper into the membrane, causing the formation of transmembrane pores composed solely of CAP molecules. (B) In the carpet model, CAP monomers cover the surface of the target cell like a carpet, which results in significant phospholipid displacement. Upon reaching a threshold concentration, transient pores lined with CAPs are formed. (C) The toroidal pore model suggests that membrane damage occurs as described by the barrel-stave model, except that the resulting torus shaped pore is composed of CAP and lipid molecules. (D) Like the other models, the detergent model proposes that membrane damage is initiated by peptide binding; however, CAP binding results in membrane miscellization rather than the formation of discrete pores. Adapted from Hilchie and Hoskin, 2010.

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to withstand the internal osmotic pressure of the cell. Early studies suggested that low concentrations of magainin I function as described by the carpet model, whereas higher concentrations lead to membrane lysis as described by the barrel-stave model (105).

Recently, magainin II was reported to cause cell lysis by the "toroidal pore" or "two-state" model, which combines elements of the carpet model and the barrel stave model (104). In this regard, CAP monomers bind to the surface of the target cell in a parallel manner via electrostatic interactions (Fig. 1.1C). This state is referred to as the "S" state, and is a functionally inactive state. CAP monomers continue to bind to the membrane, causing membrane thinning that is directly proportional to the concentration of membrane-bound CAPs (104). As peptide binding reaches a threshold level, transient pores may be formed (106). In this regard, CAP monomers may gain access to the cytosol in the absence of significant membrane damage as described by the carpet model. When the transient pores close, CAP monomers reabsorb in the head group region of the phospholipid bilayer; however, the CAP molecules may surface on either side of the membrane. In this instance, CAPs may gain access to the cytosol without causing significant membrane damage. When the threshold concentration is reached, CAP monomers reorient themselves such that they become perpendicular to the lipid bilayer (104). This state is referred to as the "I" state. During the "I" state, a torus-shaped pore is formed through the lipid bilayer, which is composed of CAP and lipid molecules. The lipid molecules stabilize the pore by reducing the electrostatic repulsion forces between highly basic CAPs. Toroidal pores are predicted to contain ~4-7 monomers (106) and have a diameter of ~3 nm (104), which is large enough for the passage of small ions and molecules (106).

Finally, the detergent model predicts that membrane damage is initiated by peptide binding to the membrane of the target cell via electrostatic interactions as described above (Fig. 1.1D); instead of forming pores through the membrane, the detergent model predicts that peptide binding leads to the formation of micelle-like structures composed of CAPs and lipid molecules, which are then released from the cell. It is noteworthy that there is currently insufficient evidence to conclude that any CAP causes cell lysis by this mechanism (37).

1.4.3 Indirect-Acting CAPs

Certain CAPs kill cancer cells indirectly. For the purpose of this investigation, indirect cell killing is defined as cell death that is characterized by the induction of apoptosis, inhibition of macromolecular synthesis, and/or alterations in signal transduction pathways. CAPs that are thought to kill cancer cells by an indirect mechanism include LfcinB (55, 107), BMAP-27 and -28 (108), the gaegurin 6-derivative PTP7 (71), pentadactylin (54) and brevinin-2R (64). It is worth noting that the actions of direct- and indirect-acting CAPs are not necessarily mutually exclusive. For example, BMAP-27 and -28 induce DNA laddering in lymphoma cells, indicating the induction of apoptosis; however, both peptides also cause lactate dehydrogenase (LDH)-release in lymphoma cells, indicating significant membrane damage (108). Therefore, BMAP-27 and -28 have both direct and indirect anticancer properties. These results suggest that certain direct-acting CAPs may also possess indirect properties that have yet to be elucidated. Alternatively, CAPs may kill different cell types by different mechanisms of action. The mechanism(s) of action of four indirect-acting CAPs will now be discussed. These CAPs were selected based on their mechanism(s) of action, as well as evidence that supports indirect anticancer properties.

Perhaps the best studied example of an indirect-acting CAP is LfcinB, which is a 25 aa CAP derived from the acid-pepsin hydrolysis of bovine lactoferrin from cow's milk (109). Our research group showed that LfcinB is cytotoxic for leukemia, lymphoma, breast, colon and ovarian carcinoma cell lines (55). Importantly, LfcinB did not kill endothelial cells, fibroblasts, or resting or activated T cells. LfcinB induces caspase-2, -3, and -9-dependent and caspase-8-independent apoptosis in leukemia cells. These findings suggest that LfcinB induces mitochondrial-dependent apoptosis. The same study showed that LfcinB causes changes in the mitochondrial membrane electrochemical gradient ($\Delta\Psi_m$) by a mechanism that is dependent on the generation of reactive oxygen species (ROS), as well as caspase-2 activation. Our research group subsequently showed that LfcinB-induced apoptosis involves damage to the plasma membrane, which is followed by mitochondrial membrane damage (107). Importantly, damage to the plasma membrane precedes DNA fragmentation. These findings suggest that LfcinB causes sufficient membrane damage to allow LfcinB monomers to enter the cell, thereby allowing LfcinB

to target mitochondria and cause mitochondrial-dependent apoptosis. LfcinB also targets the plasma membrane and mitochondria of neuroblastoma cells (53). Interestingly, whereas LfcinB induces apoptosis in leukemia cells, as evidenced by DNA laddering (55), LfcinB kills neuroblastoma and fibrosarcoma cells by cell lysis (53, 110). These results suggest that the mechanism of action of LfcinB differs depending on the cell type.

Arginine-glycine-aspartic acid (RGD) is an integrin binding motif used by many adhesion molecules, including fibronectin and collagen (111, 112). Tachyplesin is a 17 aa CAP isolated from the horseshoe crab *Tachypleus tridentatus* (113). RGD was conjugated to tachyplesin to form RGD-tachyplesin, which induces mitochondrial-dependent and mitochondrial-independent apoptosis in prostate cancer, melanoma, and endothelial cells (114). It is noteworthy that the importance of either pathway was not evaluated by the addition of specific caspase inhibitors. Interestingly, the anticancer properties of RGD-tachyplesin are dependent on the sequence of the peptide, because scrambled RGD-tachyplesin is not toxic for cancer or endothelial cells. These findings suggest that the structure of the peptide is vital for its anticancer properties.

Pentadactylin, which is a 25 aa CAP isolated from the South American bullfrog *Leptodactylus pentadactylus*, possesses antimicrobial properties against Gram positive and Gram negative bacteria without causing hemolysis (115). Pentadactylin is cytotoxic for melanoma cells, and to a lesser extent, normal fibroblasts (54). Pentadactylin damaged the plasma and mitochondrial membrane in 17% and 10% of melanoma cells, respectively, at the IC_{75}. Furthermore, 29% of melanoma cells exhibited fragmented DNA. Cell cycle analysis showed a block at the S phase in pentactylin-treated melanoma cells (54). Although the mechanism of pentadactylin-induced cell death was not fully elucidated, these data suggest that pentadactylin may kill cancer cells by both a direct and indirect mechanism.

Brevinin-2R, which is a 25 aa CAP isolated from the skin of the marsh frog *Rana ridibunda*, kills leukemia, lymphoma, colon carcinoma, fibrosarcoma, breast adenocarcinoma, and lung carcinoma cells to a greater extent than peripheral blood mononuclear cells, T cells, or fibroblasts (64). Brevinin-2R-induced cell death is sequence specific because scrambled brevinin-2R is not toxic for cancer cells. Brevinin-

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2R-induced cell death involves ROS generation and $\Delta\Psi_m$, which are not a consequence of any direct interaction between brevinin-2R and the mitochondria. Furthermore, brevinin-2R-induced cell death is not associated with the release of cytochrome *c* or apoptosis inducing factor (AIF). Cell death is decreased when the anti-apoptotic protein B cell lymphoma-2 (Bcl-2) is overexpressed or the proapoptotic protein Bcl-2 and 19-kilodalton interacting protein-3 (BNIP3) is inhibited; however, brevinin-2R does not cause caspase activation. Brevinin-2R localizes to early and late endosomes, permeabilizes lysosomes, and induces the formation of autophagosomes. These findings suggest that brevinin-2R-induced cell death occurs by the lysosomal-mitochondrial death pathway and involves autophagy.

It should also be noted that while many of these CAPs have cytotoxic effects on cancer cells, certain CAPs also possess other activities that may influence their use as anticancer agents. For example, in addition to its cytotoxic properties, LfcinB inhibits basic fibroblast growth factor- and vascular endothelial growth factor (VEGF)-induced angiogeneis (116). The antiangiogenic properties of LfcinB are lost when the aa sequence of LfcinB is scrambled (116). These findings show that certain CAPs possess several additional properties that may impact on their clinical potential as novel anticancer agents.

1.4.4 In Vivo Studies

Many naturally-occurring CAPs and their derivatives exhibit anticancer properties *in vivo*. For example, studies conducted by our research group show that linear LfcinB kills lymphoma cells by an indirect mechanism and is able to extend the long-term survival of mice bearing disseminated B-cell non-Hodgkin's lymphoma, as well as prevent weight loss in comparison to vehicle-treated mice (117). Another research group established that LfcinB inhibits lung metastasis of melanoma cells, as well as liver and spleen metastasis of lymphoma cells (118). Other studies show that 3 intratumoral injections (d 1-3) of cyclic LfcinB inhibits the growth of fibrosarcoma tumours, but not melanoma or colon tumours, in wild-type mice (110). Interestingly, linear LfcinB, cyclic murine lactoferricin (LfcinM), and linear LfcinM do not prevent the growth of fibrosarcoma, melanoma, or colon tumours *in vivo* (110). The same group later showed

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that 1 mg LfcinB inhibits the growth of established neuroblastoma tumours grown as xenografts in immune-deficient rats after only 3 injections on d 1-3 (53). LfcinB also kills neuroblastoma cells by a direct mechanism. Together, these findings suggest that LfcinB has potential as a novel anticancer agent for the treatment of lymphoma, melanoma and neuroblastoma. However, LfcinB-induced cell killing is reduced by the presence of serum (119), which currently limits its clinical usefulness.

Magainin II is a direct-acting α-helical CAP that kills a variety of cancer cells, including lung and bladder cancer cells (52, 120, 121). Derivatives of magainin also kill lung and stomach cancer cells, as well as melanoma, lymphoma and leukemia cells *in vitro* (70, 121-123). MSI-511 is a magainin derivative in which all L-aa are replaced with D-aa. MSI-511 has potent antitumour properties in a mouse model of human melanoma (123). In this study, melanoma tumours grown as xenografts in nude mice were injected at the base of the tumour with 2.5 mg MSI-511 once tumours reached 75 mm^3. In contrast to tumours treated with control peptide, MSI-511-treated tumours disappeared in 6 out of 9 mice after only 1 injection. The remaining 3 tumour-bearing mice were reinjected with MSI-511; one of these tumours completely disappeared after a total of 3 injections, whereas the other tumours did not resolve. Tumours from "cured" mice did not reappear within the 90 d observation period. MSI-511 caused tumour ulceration that healed within 3 wk with minimal scarring. In order to test whether this phenomenon was tumour specific, human newborn foreskins grafted onto severe combined immunodeficient (SCID) mice were injected with equal concentrations of MSI-511 (123). In this capacity, MSI-511-treated grafts showed demarcated areas of eschar (piece of dead tissue cast off from the surface of skin) within 24 h, which were completely healed within 25 d. Another study showed that all-D magainin significantly increases the life span of mice harbouring leukemia, sarcoma or ovarian ascites (121). However, it should be noted that these animals were treated the day after tumour cells were injected into the peritoneal cavity. Together, these results suggest that the magainin peptidomimetic composed solely of D-aa holds considerable potential for the treatment of cancers.

As discussed earlier, gomesin is a direct-acting CAP that kills many cancer cell lines, as well as endothelial cells *in vitro* (45). This study was also the first *in vivo* study to administer CAPs topically using cream as a delivery system. Topical applications of

gomesin significantly delay the growth of melanoma tumours grown in the hind flanks of syngenic mice (45). Moreover, repeated applications of gomesin do not cause any adverse effects. These findings suggest that gomesin, and perhaps other CAPs, can be used as topical agents for the treatment of melanoma.

LTX-302, which is a highly selective 9 aa direct-acting CAP derived from LfcinB, inhibits the growth of A20 (lymphoma) tumours grown as xenografts in immune-deficient mice (95). Interestingly, LTX-302 caused complete tumour regression in 5 out of 8 wild-type mice that was characterized by tumour necrosis and leukocyte infiltration. Moreover, "cured" mice were protected against rechallenge with A20 cells, but not other cancer cell types. Furthermore, tumour-resistance could be adoptively transferred to recipient mice by injecting them with T cells from "cured" donor mice prior to being challenged with A20 cells (95). This study was the first to show that CAPs can generate a tumour-specific immune response. Together these studies show that certain CAPs hold considerable potential as novel therapeutic agents for the treatment of cancers.

1.4.5 Limitations as Therapeutic Agents

Despite many advances, the use of CAPs as anticancer agents remains limited by several disadvantages, including the cost of production. Although the cost to produce CAPs continues to decline (67), research grade CAPs still cost ~$50 per aa for ~100 mg at >95% purity. Several research groups have attempted to deal with this issue by producing CAPs using recombinant expression methods (124). Others are attempting to deal with this problem by identifying shorter derivatives of native CAPs with equal potency *in vitro* and *in vivo* (95, 125).

Although many CAPs kill cancer cells to a greater extent than normal cells, most CAPs are toxic to certain types of normal cells. Furthermore, many CAPs exhibit reduced toxicity in the presence of serum (119, 126, 127), which is often attributed to peptide degradation by proteases. Trypsin cleaves the peptide bond on the C-terminal side of lysine and arginine residues, and chymotrypsin cleaves the peptide bond on the C-terminal side of bulky hydrophobic residues, such as tryptophan (128). Therefore, CAPs are highly susceptible to proteolytic degradation, which would limit their half-life *in vivo*. Many researchers have investigated novel ways to modify CAPs to enhance their

selectivity for cancer cells and decrease their susceptibility to protease degradation (125, 129-131). These modification strategies will be discussed in detail in section 1.5.

There is often concern that CAPs will be immunogenic *in vivo*; however, melittin is the only CAP with documented immunogenic properties (132). Certain CAPs have been shown to modulate immune cell function (42, 133, 134), which may influence the antitumour properties of the CAP *in vivo*. Other reports show that certain CAPs initiate an antitumour immune response (95), which may be a consequence of the CAP lysing cancer cells, thereby generating the "danger" signal that is required to initiate an effective antitumour immune response. These findings highlight the need to thoroughly evaluate the biological properties of the CAP before it can be considered for use in a clinical setting.

1.5 CAP Modification Strategies

Many investigators have attempted to improve the clinical potential of CAPs for use as novel antimicrobial agents by designing peptidomimetics with similar biological properties to the existing CAP. Peptidomimetics are peptide-like molecules that can be created by modifying an existing peptide, or by engineering a molecule that mimics the existing CAP. These modification strategies are typically aimed at improving the stability of the peptide by reducing its susceptibility to protease-mediated degradation (131). In contrast, the aa sequence of the CAP is often modified to improve the selectivity of the CAP for cancer cells (130). While there has been some success in this field, the science of modifying CAPs to enhance their anticancer properties is still in its infancy. A few examples of each modification strategy will be discussed herein to highlight the different mechanisms by which CAPs can be modified to improve their potential as anticancer agents.

1.5.1 Improving the Anticancer Properites and/or Cancer Cell Selectivity of CAPs

Amino acid substitutions can influence the anticancer properties of the CAP, as well as the selectivity of the CAP for cancer cells. As discussed in section 1.4, cationic and hydrophobic aa are both necessary for the anticancer properties of CAPs. Therefore, it seems intuitive that greater anticancer effects will result from an increase in the positive

charge and/or hydrophobicity of the native CAP. To date, only one study evaluated how changes in the charge and hydrophobicity of a CAP influences its anticancer properties. This study was performed on the N-terminal helical region of lactoferrin that corresponds to aa positions 17-41 (135). Helical wheel diagrams of the native CAP suggest that the positively charged aa are clustered into two spatially separated sections, termed the major and minor sectors, which consist of 4 and 2 cationic aa, respectively. Moving the 2 cationic aa from the minor sector to the major sector results in increased cancer cell killing but a loss in cancer cell selectivity, suggesting that the presence of a minor sector may improve cancer cell selectivity. This study also evaluated whether cancer cell killing could be increased by adding a charge of +2 to the major sector, thereby increasing the net charge from +6 to +8. The potency of the modified CAP was reduced in comparison to the unmodified CAP; however, both were selective for cancer cells. The loss in potency was likely due to the loss of two bulky tryptophan aa, which are essential for the anticancer properties of certain CAPs (136). This hypothesis is supported by findings from Strom *et al,* who reported that the antibacterial properties of a 15 aa LfcinB derivative is abolished when either tryptophan residue is replaced with alanine (137). Other aa may also be essential for CAP-induced cytotoxicity. For example, bulky aa side chains may protect the peptide backbone from protease degradation. Proline and glycine are helix-breaking aa, which in some cases are required for the antibacterial and/or hemolytic properties of certain CAPs (138, 139). These aa may also be required for the anticancer properties of certain CAPs. Furthermore, the secondary structure can be manipulated by changing the aa sequence of the peptide. In the event that the anticancer properties of certain CAPs is mediated by CAP binding to a receptor (64, 114), manipulating the aa sequence could interfere with receptor binding and thereby eliminate the anticancer properties of the CAP. Together, these findings suggest that while the anticancer potential of CAPs can conceivably be enhanced by modifying their aa sequence, it may not be the most effective strategy.

Another strategy used to enhance CAP selectivity for cancer cells involves coupling a cancer cell-targeting sequence to the CAP of interest. Targeting sequences that selectively recognize cancer cells can be identified using phage-display libraries. The sequence leucine-threonine-valine-serine-proline-tryptophan-tyrosine (LTVSPWY) was

identified by this method and has been shown to selectively deliver antisense oligonucleotides to breast cancer cells (140). However, studies conducted in our laboratory suggest that coupling LTVSPWY to a LfcinB derivative does not result in selective breast cancer cell killing (Vale and Hoskin, unpublished data). Bomesin is a 14 aa tumour homing peptide that binds receptors that are overexpressed on many types of cancer cells (141-143). Recently, magainin II was conjugated to bomesin to increase the peptide's anticancer properties (130); the IC_{50} of the modified CAP was 10-fold lower than magainin II. Furthermore, the IC_{50} of the modified peptide for cancer cells was 6-10 times lower than the IC_{50} for normal cells, suggesting that the increase in potency of the modified CAP was not at the expense of cancer cell selectivity. The integrin-binding motif RGD also enhances the potency of certain peptides (114, 144). Other researchers established that CAPs conjugated to a 15 aa segment of the β-chain of human chorionic gonadotropin possess *in vitro* and *in vivo* anticancer properties (90, 145). The targeted peptides also possess the ability to destroy tumour metastases in lymph nodes, bones, and other organs (145). Importantly, organs from peptide-treated mice were normal except for the ovaries, which contained involuted ovarian follicles and no recently formed corpora lutea (145). Although these data show promising results, they are dependent on the expression of the receptor that is recognized by the targeting sequences. As a result, it may be easier for cancer cells to develop resistance to the peptide. The cost to produce the modified peptide would also increase as a consequence of adding the targeting sequence. Furthermore, it is imperative that the targeting sequence is accessible to its receptor in an aqueous environment. Moreover, the targeting sequence should not obstruct the membrane active portion of the CAP, or the anticancer properties will be lost. Therefore, an understanding of the secondary structure of the modified CAP would help predict whether the modified peptide will be more or less potent than the existing CAP.

For some time we have known that the solid tumour microenvironment is more acidic than that of normal tissues. Tumour acidity is a consequence of lactic acid buildup, inadequate washout of acidic products, and a lack of adequate vascularization (146-148). An elegant study by Yechiel Shai's group modified the CAP [D]-K_6L_9 by replacing lysine residues (pKa ~10.5) with histidine residues (pKa ~6.1) (131). The authors predicted that

the modified CAPs, called [D]-$K_3H_3L_9$ and [D]-H_6L_9, would become protonated, and therefore activated, in acidic environments. Interestingly, only [D]-H_6L_9 showed pH-dependent cytotoxicity *in vitro*. The authors reported that unlike [D]-K_6L_9, which was toxic when delivered systemically, neither [D]-$K_3H_3L_9$ nor [D]-H_6L_9 were toxic when administered to mice intravenously. Importantly, both [D]-$K_3H_3L_9$ and [D]-H_6L_9 reduced the volume of prostate cancer tumours grown as xenografts in immune deficient mice when injected intratumourally or systemically (131). Although similar findings have not been reported with other CAPs, this study suggests that CAP selectivity for solid tumours may be increased by replacing lysine or arginine residues with histidine residues.

1.5.2 Increasing CAP Stability

The potential value of CAPs as therapeutic agents is limited by their susceptibility to proteolytic degradation. For example, trypsin and chymotryspin recognize basic and hydrophobic aa, respectively (128). One strategy to reduce CAP degradation by proteases involves changing the chirality of the chiral (α)-carbon, thereby eliminating the protease recognition site. Enantiomers are peptide mimics in which all of the naturally-occurring L-aa are replaced with D-aa, whereas diastereomers are peptide mimics that are composed of D-aa in addition to L-aa. Studies show that the antifungal properties of all-D pleurocidin are 2-fold greater than that of all-L pleurocidin (149). Furthermore, the pleurocidin enantiomer is resistant to degradation by trypsin, plasmin, and carboxypeptidase. Other studies show that all-D magainin II is as potent as all-L magainin II against bacteria (150). CAPs with anticancer properties have also been successfully modified by changing the stereochemistry of the peptide. As discussed in section 1.4.4, enantiomers of magainin analogues possess potent *in vitro* and *in vivo* anticancer properties (123). Other studies show that diastereomers of magainin II analogues are potent anticancer agents *in vitro* and *in vivo* (121). Studies by Papo and colleagues supported this finding by showing that a synthetic diasterermeric CAP possesses *in vitro* and *in vivo* anticancer properties (74). These findings suggest that replacing only selected L-aa with D-aa may have the same effect as replacing all L-aa with D-aa.

CAP digestion can also be eliminated by creating peptoids. Peptoids are peptidomimetics that are resistant to proteolytic degradation because the aa side chain is positioned on the nitrogen atom rather than the α-carbon (151). Certain peptoids possess antibacterial activities (152, 153), while others possess anticancer properties (151). However, to my knowledge the anticancer properties of CAPs and the peptoid equivalent have never been compared *in vitro* or *in vivo*.

Lysine and arginine residues are recognized by trypsin (128). Certain groups have removed trypsin recognition sites by alkylating or acylating lysine residues (154, 155). Other research groups have synthesized peptide mimics with novel unnatural aa that are bulkier and more positively-charged than lysine or arginine (156). These molecules are toxic to bacteria and have longer half lives *in vitro*. The addition of bulky, highly cationic aa could compensate for a lack of positively charged residues. Similarly, novel aa with bulky hydrophobic side groups could compensate for an inherent lack of hydrophobicity. Therefore, in addition to removing trypsin recognition sites, this modification strategy could also influence the potency of the peptide to cancer cells.

End capping involves chemically modifying the N- and/or C-terminus of the CAP (157). Typically, end capping refers to N-acetylation and/or C-amidation. End capping can also compensate for a lack of hydrophobicity and/or positive charge, respectively, thereby influencing the biological properties of the CAP (158-160). End capping may also protect the CAP from aminopeptidase and/or carboxypeptidase digestion, which are involved in alimentary digestion and could limit the bioavailability of CAPs delivered orally (161). Therefore, in the right context, end capping may be a useful strategy for enhancing CAP stability.

The above modification strategies represent novel ways to enhance the stability of CAPs *in vivo*, although the success of these modification strategies is dependent on the mechanism of action of the CAP. For example, modifying the stereochemistry of the CAP would be of no value if the CAP killed cancer cells by a chiral-specific mechanism. In contrast, chemically altering specific aa may be sufficient to reduce the susceptibility of the CAP to proteolytic degradation, provided that the altered aa are not needed for

interaction with a specific receptor. Therefore, an understanding of the mechanism of action should precede CAP modification by these methods.

1.6 Objectives and Hypotheses

1.6.1 Objectives 1-3: Identify Pleurocidin-like CAPs with Anticancer Properties

Previous studies conducted by our collaborators identified pleurocidins with antibacterial properties (48). Certain CAPs with antibacterial properties also possess anticancer properties (56, 64, 119). A subset of CAPs with anticancer properties also kill multidrug-resistant cancer cells (71, 72). It is currently not known whether pleurocidins possess anticancer properties. Therefore, the first three objectives of this investigation were to: [1] identify pleurocidins that kill human breast cancer cells; [2] determine whether pleurocidins kill untransformed cells; [3] determine whether pleurocidins kill multidrug-resistant breast cancer cells. These objectives, which test the hypothesis that certain pleurocidins selectively kill human breast cancer cells, including multidrug-resistant breast cancer cells, are described in Chapter 3.

1.6.2 Objective 4: Identify Pleurocidins with Chemosensitizing Properties

Cytotoxic chemotherapeutic agents with independent mechanisms of action are often combined to enhance the clinical response in patients with breast cancer (162). Furthermore, certain CAPs with anticancer properties enhance cancer cell killing by traditional cytotoxic chemotherapeutic agents (69, 70). Thus, the fourth objective of this investigation was to determine whether sublethal concentrations of pleurocidins are able to enhance breast cancer cell killing by a conventional cytotoxic chemotherapeutic agent. The aim was to test the hypothesis that sublethal levels of pleurocidins enhance breast cancer cell killing by other cytotoxic drugs. This objective is described in Chapter 3.

1.6.3 Objective 5: Determine the Mechanism of Action of Pleurocidins

CAPs with anticancer properties kill cancer cells by a direct or indirect mechanism (61). Many α-helical CAPs directly lyse cancer cells (52, 95, 96). Nuclear magnetic resonance (NMR) studies conducted by others suggest that NRC-03 and NRC-07 adopt α-helical conformations in membrane-mimicking environments (Syvitski, R., personal communication). Therefore, the fifth objective of this investigation was to

determine the mechanism of action of pleurocidins, in order to test the hypothesis that pleurocidins are direct-acting CAPs that kill breast cancer cells by a mechanism that involves peptide binding, pore formation, and extensive membrane damage that is followed by cell lysis. This objective is described in Chapter 3.

1.6.4 Objective 6: Determine the Antitumour Properties of Pleurocidins

Several studies show that CAPs possess antitumour properties *in vivo* (53, 56, 95, 114, 118, 145, 163). However, the majority of these experiments involve treating the tumour-bearing animal during the earliest stages of tumour growth. Thus, the sixth objective of this experiment was to determine the antitumour properties of pleurocidins in xenograft models of human breast cancer. These studies were designed to test the hypothesis that pleurocidins inhibit the growth of established tumours grown as xenografts in immune-deficient mice and are also described in Chapter 3.

1.6.5 Objectives 7-10: Identify Pleurocidin Derivatives with Enhanced Stability

CAPs are limited by their sensitivity to proteolytic degradation (37, 38, 61). Many research groups have successfully modified CAPs by creating enantiomers or diastereomers (74, 121, 123, 149). Therefore, my next four objectives were to: [7] determine whether pleurocidins are susceptible to protease degradation; [8] create an all-D form of the most potent pleurocidin and test whether it is selectively cytotoxic for breast cancer cells, including multidrug-resistant breast cancer cells; [9] determine whether the modified pleurocidin kills breast cancer cells by the same mechanism as the existing pleurocidin; [10] determine whether the modified pleurocidin possesses antitumour properties *in vivo.* These objectives, which test the hypothesis that enantiomeric pleurocidins are as potent in terms of anticancer properties as unmodified pleurocidins *in vitro* and *in vivo*, are described in Chapter 4.

1.6.6 Objective 11-13: Determine Whether Cancer Cells Can Become Resistant to Direct-acting CAPs

Many researchers predict that direct-acting CAPs will have many advantages over traditional cytotoxic chemotherapeutic agents owing to their unique mechanism of action (37, 38, 61, 67). In contrast to normal cells, cancer cell membranes carry a net negative

charge due to the increased expression of several different negatively charged surface molecules (77, 81-83). Researchers speculate that certain CAPs will have fewer side effects than cytotoxic chemotherapy because CAPs preferentially kill cancer cells based on the net negative charge of the outer membrane leaflet (37, 38, 61, 67). CAPs are also predicted to kill slow growing cancers because they do not kill cells based on their rate of proliferation (37, 61). Moreover, CAPs are also predicted to kill multidrug-resistant cancer cells because they do not require access to the inside of the cell (37, 61). Furthermore, most researchers agree that resistance to direct-acting CAPs is unlikely because the cancer cell would likely have to substantially alter the composition of its entire membrane. In fact, to date cancer cell resistance to direct-acting CAPs has never been reported. Therefore, the last three objectives of this investigation were to: [11] generate breast cancer cells that are resistant to pleurocidins; [12] evaluate mechanism(s) of cancer cell resistance to direct-acting CAPs; [13] determine whether pleurocidin-resistant cells are also resistant to other cytotoxic drugs. The aim of these studies was to test the hypothesis that breast cancer cells cannot become resistant to direct-acting CAPs. These objectives are described in Chapter 5. Collectively, the results from these studies would determine the clinical potential of pleurocidin-like CAPs with anticancer properties.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

MDA-MB-231 human breast cancer cells were a kind gift from Dr. S. Drover (Memorial University of Newfoundland, St. John's, NL, Canada). MDA-MB-468 and T47D human breast cancer cells were generously provided by Dr. P. Lee and Dr. J. Blay, respectively (Dalhousie University, Halifax, NS, Canada). SKBR3 cells were kindly provided by Dr. G. Dellaire (Dalhousie University). MCF7 and paclitaxel-resistant MCF7 (MCF7-TX400) breast cancer cells were a kind gift from Dr. K. Goralski (Dalhousie University). HEY, OCC-1, OVCA-429 and SKOV-3 ovarian cancer cells were generously provided by Dr. M. Nachtigal (Dalhousie University). Dr. C. MacCormick (Dalhousie University) kindly provided L cells (parental immortalized cells), gro2C cells (L cells lacking HSPs) and sog9 cells (HSP and CSP deficient) (164, 165). Cells were maintained at 37°C in a 10% CO_2 humidified atmosphere in Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich Canada, Oakville, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), which were all purchased from Invitrogen (Burlington, ON, Canada). This medium will hereafter be referred to as complete DMEM. Stock flasks were passaged as required to maintain optimal cell growth. Cells were routinely tested for mycoplasma contamination using the MycoAlert® mycoplasma detection kit (Lonza Walkersville Inc., Walkersville, MD).

Dr. D. Waisman kindly provided 4T1 mouse breast cancer cells (Dalhousie University). U266 and KMS11 human multiple myeloma cells were a generous gift from Dr. A. Reiman (Saint John Regional Hospital, St. John, NB, Canada). Jurkat T leukemia cells were purchased from American Type Culture Collection (Manassas, VA). These cells were maintained at 37°C in a 5% CO_2 humidified atmosphere in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 5 mM HEPES,

hereafter referred to as complete RPMI. Stock flasks were passaged as required to maintain optimal growth and were routinely tested for mycoplasma contamination.

Human red blood cells (RBCs) collected from healthy volunteers were generously provided by Dr. J. Marshall. HUVECs, human mammary epithelial cells (HMECs) and adult human dermal fibroblasts (HDFs) were purchased from Lonza Walkersville Inc. and were maintained at 5% CO_2 in Clonetics® endothelial cell growth medium (EGM)-2, mammary epithelial cell growth medium (MEGM), and fibroblast growth medium (FGM)-2, respectively, which were also purchased from Lonza. Normal cell cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere for no more than 6 passages.

2.2 Peptides

NRC-13 (aa sequence: GWRTLLKKAEVKTVGKLALKHYL-NH$_2$), NRC-16 (aa sequence: GWKKWLRKGAKHLGQAAIK-NH$_2$), NRC-03$_{10 \text{ mer}}$ (aa sequence: GRRKRKWLRR-NH$_2$), biotinylated (biotin)-NRC-03, and biotin-NRC-07 were synthesized by Dalton Pharma Services (Toronto, ON, Canada). NRC-03 (aa sequence: GRRKRKWLRRIGKGVKIIGGAALDHL-NH$_2$) and NRC-07 (aa sequence: RWGKWFKKATHVGKHVGKAALTAYL-NH$_2$) were synthesized by Dalton Pharma Services and American Peptide Company (Sunnyvale, CA). [D]-NRC-03 (aa sequence: $_D$[GRRKRKWLRRIGKGVKIIGGAALDHL]-NH$_2$) was purchased from American Peptide Company. Peptide stocks (500 µM) were prepared in serum free (sf)DMEM unless otherwise indicated, and stored at -80°C until required. All experiments using peptides were conducted in medium containing 2.5% FBS unless otherwise indicated.

2.3 Reagents

Cisplatin, tamoxifen and docetaxel were kindly provided by Dr. C. Giacomantonio (Dalhousie University). TO-PRO®-3 iodide was generously provided by Dr. P. Lee, and Coomassie Blue stain (R-250) was kindly provided by Dr. C. MacCormick (Dalhousie University). Reduced GSH, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), human serum (HS), mouse serum (MS), poly-L-lysine, bovine serum albumin (BSA), phosphate buffered saline (PBS), dimethyl

sulfoxide (DMSO), propidium iodide (PI), triethylammonium bicarbonate buffer (TEAB), trifluoroacetic acid (TFA), trypan blue solution (0.4%), crystal violet, phosphatase assay substrate, flunarizine (FLZ), paclitaxel, leupeptin hemisulfate salt, sodium orthovanadate ($NaVO_4$), pepstatin A, aprotinin (from bovine lung), phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), sodium fluoride (NaF), Nonidet™ P 40, disodium hydrogen phosphate (Na_2HPO_4), heparan sulfate (HeS) sodium salt, chondroitin sulfate (CS) sodium salt, phenylarsine oxide and sodium deoxycholate were purchased from Sigma-Aldrich (Oakville, ON, Canada). Cytotox 96® non-radioactive cytotoxicity assay (LDH-release assay) kit and sequencing grade trypsin and chymotrypsin were from Promega (Madison, WI). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) matrix solution was purchased from Agilent Technologies (Palo Alto, CA). Streptavidin-conjugated Texas Red was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Avidin-conjugated horseradiash peroxidase (HRP) was from BD Biosciences (San Jose, CA). Boc-D-FMK was from EMD Biosciences (San Diego, CA). Concentrated hydrochloric acid (HCl), concentrated sodium hydroxide (NaOH), concentrated sulphuric acid (H_2SO_4), and methanol were from Fisher Scientific (Ottawa, ON, Canada). Glacial acetic acid was from VWR (Mississauga, ON, Canada). O-sialoglycoprotein endopeptidase (OSGE) was purchased from Cedarlane Laboratories (Hornby, ON, Canada). Dihydroethidium (DHE), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) and Alexa Fluor® 488 phalloidin were from Molecular Probes (Eugene, OR). Osmium tetroxide (OsO_4), glutaraldehyde, sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), and ethylenediaminetetraacetic acid (EDTA) were purchased from Electron Microscopy Sciences (Hatfield, PA). Gibco® trypsin (0.25% with EDTA) was from Invitrogen. Paraformaldehyde (PFA), acrylamide, glycine, tricine, sodium dodecyl sulfate (SDS), ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), sucrose, sodium chloride (NaCl), Tris-Base, tetramethylethylenediamine (TEMED), Tween-20, and ammonium persulfate (APS) were from Bioshop® Canada Inc. (Burlington, ON, Canada). Hank's balanced salt solution (HBSS) was from Invitrogen (Burlington, ON). TMB substrate solution ($1\times$) was from eBioscience Inc (San Diego, CA). Tritiated-thymidine (^3H-TdR) was purchased from MP Biomedicals (Irving, CA). Rabbit anti-P-glycoprotein (P-gp) was a kind gift from Dr. G.

Dellaire (Dalhousie University). Mouse anti-cytochrome *c* monoclonal antibody (mAb) was from Upstate Biotechnology (Charlottesville, VA) and mouse anti-mitochondrial heat shock protein (Hsp)-70 mAb was from Affinity BioReagents (Golden, CO). Goat anti-actin mAb, HRP-conjugated goat anti-mouse IgG, bovine anti-goat IgG and donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4 Cell Seeding Conditions

In experiments conducted in 96-well flat-bottomed tissue culture plates, breast and ovarian cancer cell lines and normal cell cultures were seeded, in quadruplicate, at a density of 2×10^4 cells per well (100 µl) except 4T1 cells and HUVECs, which were seeded at a density of 1×10^4 cells per well. Cell seeding numbers were selected on the basis of the proliferative capacity of the cell. In experiments evaluating the chemosensitizing properties of sublethal doses of NRC-03 and NRC-07, MDA-MB-231 cells were seeded, in quadruplicate, at a density of 5×10^3 cells/well and were treated at 0 h and retreated at 48 h. Cancer cells and normal cell cultures were seeded in complete DMEM and incubated overnight (o/n) at 37°C in a 10% CO_2 humidified atmosphere to promote cellular adherence, with the exception of 4T1 cells, which were seeded in complete RPMI and incubated o/n in a 5% CO_2 humidified atmosphere to promote cellular adherence. Finally, KMS11, U266 and Jurkat cells were seeded in 96-well flat-bottomed tissue culture plates, in quadruplicate, at a density of 5×10^4 cells per well (100 µl). Cells were seeded in complete RPMI medium and were treated immediately.

SEM experiments were conducted in 24-well flat-bottomed tissue culture plates containing sterile coverslips. MDA-MB-231 cells and HDFs were seeded at a density of 2×10^5 cells per well (1 ml), and were incubated o/n as described above to promote cellular adherence. U266 cells were seeded at a density of 5×10^5 cells per well (500 µl) in plates containing sterile poly-L-lysine-coated coverslips. Cells were treated after a 2 h incubation at 37°C in a 5% CO_2 humidified atmosphere to promote cell adhesion.

Clonogenic assays were conducted in 6-well flat-bottomed tissue culture plates, whereas fluorescence-based experiments were conducted in 6-well plates containing sterile coverslips. All adherent cells were seeded at a density of 4×10^5 cells per well (2

ml) except MCF7-TX400, which were seeded at a density of 8×10^5 cells per well. In fluorescence-based experiments, U266 cells were seeded at a density of 1×10^6 cells/well (1 ml) in 6-well flat-bottomed tissue culture plates containing sterile poly-L-lysine-coated coverslips. U226 cells were treated following a 2 h incubation to promote cell adhesion as described above. In flow cytometry experiments, all cells were seeded (500 μl) at a density of 1×10^6 cells/ml. In all experiments, cells were only used when in exponential growth phase and >95% viable by trypan blue exclusion. All plastics were purchased from Sarstedt Inc. (St. Laurent, QC, Canada), except enzyme immunoassay (EIA) plates, which were from Corning Life Sciences (Lowell, MA).

2.5 Animals

Adult (6-7 weeks old) female non-obese diabetic severe combined immunodeficient (NOD SCID) mice were purchased from Charles River Canada (Lasalle, QC, Canada) and were housed in the Carleton Animal Care Facility. Mice were maintained on a diet of sterilized rodent chow and water *ad libitum*. Animal use was approved by the Dalhousie University Committee on Laboratory Animals and was in agreement with the Canadian Council on Animal Care guidelines.

2.6 MTT Assay

Cell viability in response to NRC-03 or NRC-07 treatment was determined using the MTT viability assay (166). Cells seeded in 96-well flat-bottom tissue culture plates as described in section 2.4 were cultured under the indicated conditions for the designated period of time. MTT (100 μg) was added to the cells for the final 2 h. Formazan crystals generated in metabolically active cells were solubilized in DMSO (100 μl) and absorbance (490 nm) was measured using a Bio-Tek microplate reader (Bio-Tek Instruments, Winooski, VT). Percent cytotoxicity, relative to vehicle-treated cells (100% viable) was calculated using the formula ([1-E/C] × 100), where E and C denote the optical density of peptide- and medium-treated cells, respectively.

2.7 Acid Phosphatase Assay

The acid phosphatase assay was used *in lieu* of the MTT assay to measure the viability of MCF7-TX400 (P-gp-overexpressing) cells and MDA-MB-231 cells exposed to BSA because drug efflux pumps and albumin interfere with the MTT assay (167, 168). Cells seeded in 96-well flat-bottomed tissue culture plates as described in section 2.4 were cultured under the indicated conditions for the designated period of time. Cell monolayers were then thoroughly washed with PBS and incubated in 100 µl assay buffer (0.1M sodium acetate, 0.1% [v/v] Triton X-100, 4 mg/ml phosphatase substrate [1 tablet]) for 1.5 h. The reaction was stopped by adding 1M NaOH (10 µl). Absorbance (405 nm) was measured and percent toxicity relative to vehicle-treated cells (100% viable) was calculated as described in section 2.6.

2.8 ^{3}H-TdR-Release Assay

DNA fragmentation was measured in suspension cells as an indicator of cell death (169). Jurkat cells labeled with 5 µCi/ml ^{3}H-TdR for 2 h at 37°C in a 5% CO_2 humidified atmosphere were extensively washed in complete RPMI medium and seeded in 96-well flat-bottomed tissue culture plates as described in section 2.4. ^{3}H-TdR-labeled cells cultured under the indicated conditions for the indicated periods of time were harvested onto glass fiber filter paper mats using a multiple sample harvester (Skatron Instruments, Sterling, VA). Radioactivity (counts per minute [CPM]) was quantified by liquid scintillation and percent DNA fragmentation was calculated using the formula ([C_{cpm}-E_{cpm}/ C_{cpm}] x 100), where C and E refer to intact DNA (CPM) from vehicle control- and experimentally-treated cells, respectively.

2.9 Clonogenic Assay

In addition to the acid phosphatase assay, the clonogenic assay was used to compare NRC-03- and NRC-07-induced killing of paclitaxel-resistant MCF7-TX400 cells to parental MCF7 cells. MCF7 and MCF7-TX400 cells were cultured in 6-well flat-bottomed tissue culture plates as described in section 2.4. Cells were cultured under the indicated conditions for 4 h, lifted by mild trypsinization, counted, diluted (40- to 1600-

33

fold), and replated in fresh 6-well flat-bottomed tissue culture plates in complete DMEM. Cells were maintained at 37°C in a 10% CO_2 humidified atmosphere and were fed every 3 days for 10 d to allow for colony formation. Colonies were washed with PBS, fixed and stained with crystal violet (0.4% [w/v] in methanol), washed with deionized distilled water, dried, and counted to determine the number of colony forming units (CFU) following treatment.

2.10 Hemolysis Assay

RBC lysis in response to NRC-03 or NRC-07 was determined by the hemolysis assay. Human RBCs were collected from healthy volunteers, separated by centrifugation at 500 × g for 10 min, washed twice with PBS, and cultured in the presence or absence of the indicated concentrations of NRC-03 or NRC-07 (prepared in PBS) at a final concentration of 5% RBCs [v/v]. Maximum hemolysis was achieved by culturing RBCs in an equal volume of water. Following an 8 h incubation at 37°C in a 10% CO_2 humidified atmosphere, RBCs were pelleted (1400 × g) and supernatants were collected for analysis. Absorbance (490 nm) was measured and percent hemolysis was calculated using the formula ([(E/S)/(M/S)] × 100), where E, S and M denote experimental, spontaneous and maximum hemolysis, respectively.

2.11 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to visualize changes in cell ultrastructure following peptide exposure. Cells were seeded in 24-well flat-bottom tissue culture plates containing sterile circular coverslips as described in section 2.4. Cells cultured under the indicated conditions for the indicated periods of time were washed with 0.1M sodium cacodylate and fixed with glutaraldehyde (2.5% [v/v] in 0.1M sodium cacodylate) for 2 h. Following extensive washing with sodium cacodylate, cells were fixed with OsO_4 (1% [w/v] in 0.1M sodium cacaodylate) for 30 min, washed again in 0.1M sodium cacodylate, and dehydrated in increasing concentrations of ethanol (50%, 75%, 95%, 100% × 2). Cells were then dried to their critical point using a Polaron E3000 Critical Point Dryer (Quorum Technologies, Guelph, ON, Canada), mounted onto stubs, and coated with gold using a Polaron SC7620 Mini Sputter Coater (Quorum

Technologies). Samples were viewed on a Hitachi S4700 scanning electron microscope (Hitachi High Technologies, Rexdale, ON, Canada) at the Institute for Research in Materials at approximately 500, 7000, and 40,000 × magnification.

2.12 Peptide Binding Assays

Binding of NRC-03 to breast cancer cells was determined using the peptide binding assay. MDA-MB-231 and HDFs grown on coverslips in 6-well flat-bottom tissue culture plates as described in section 2.4 were cultured under the indicated conditions for 10 min. Cells were fixed with PFA (4% [w/v] in PBS) for 1 h, washed 3 times with PBS, stained with 1 ml Texas Red-conjugated streptavidin (1.54 µg/ml in PBS) for 45 min, washed 3 times with PBS, dried, and mounted on slides with Dako fluorescent mounting medium (Dako Canada, Missisauga, ON, Canada). Cells were visualized under white and ultraviolet (UV) light at 400 × magnification. Fluorescence per cell was quantified using NIS-elements software (Nikon Canada, Mississauga, ON, Canada).

2.13 Solid Phase Heparan Sulfate- or Chondroitin Sulfate-binding Assay

Biotin-NRC-03 and -NRC-07 binding to plastic immobilized heparan sulfate (HeS) or chondroitin sulfate (CS) was determined using a modification of a method described by Silvestri and Sundqvist (170). Briefly, 10 µg/ml (200 µl) HeS or CS in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.2]) was incubated in EIA plates o/n at 4°C. Following extensive washing with PBS, plates were blocked with 200 µl 10% FBS ([v/v] in PBS) for 2 h at 4°C, washed again with PBS, and incubated in the presence or absence of 50 µM NRC-03 or NRC-07 (100 µl) for 2 h at 23°C. Following another round of washing, plates were incubated with avdin-HRP (1:1000 [v/v] in PBS) for 1 h at 23°C, washed extensively with PBS, and incubated with 1× TMB substrate solution (100 µl). The reaction was almost immediately stopped by the addition of 50 µl 0.3 M H_2SO_4. Data is represented as optical density (OD) at 450 nm.

2.14 Confocal Microscopy

Confocal microscopy was used to determine whether NRC-03 or NRC-07 enter the cytoplasma of breast cancer cells and/or multiple myeloma cells. MDA-MB-231 and

U266 cells seeded in 6-well flat-bottom tissue culture plates containing sterile coverslips as described in section 2.4 were cultured in the presence or absence of biotin-NRC-03 or -NRC-07 for 30 sec. Cells were washed twice with PBS, fixed with 4% PFA for 1 h, washed 3 times with PBS and stained with 500 µl Alexa Fluor® 488 phalloidin (10 units/ml in 0.1% Triton-X 100 [v/v]). Stained cells were washed 3 times with PBS, stained with 1 ml Texas Red-conjugated streptavidin (1.54 µg/ml in PBS) for 45 min, washed 3 times with PBS, dried, and mounted on slides with Dako fluorescent mounting medium. Images (1000 × magnification) were acquired with an LSM-510 META laser scanning confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) using the appropriate excitation lasers (Argon 488 and Helium Neon [HeNe] 543 for Alexa Fluor® 488 phalloidin and Texas Red-conjugated streptavidin, respectively).

Confocal microscopy was also used to elucidate whether NRC-03 or NRC-07 enter the nucleus of breast cancer cells. MDA-MB-231 cells seeded in 6-well flat-bottom tissue culture plates containing sterile coverslips as described in section 2.4 were cultured in the presence or absence of biotin-NRC-03 or NRC-07 as described above. Cells were washed with PBS, fixed with 4% PFA for 1 h, washed with PBS, and stained with Texas Red-conjugated streptavidin for 1 h as described above. Stained cells were washed 3 times with PBS and stained with TO-PRO®-3 (1:1000 [v/v] in PBS) for 10 min. Cells were washed twice, dried and mounted on slides with Dako fluorescent mounting medium. Images (1000 × magnification) were acquired with an LSM-510 META laser scanning confocal microscope using the appropriate excitation lasers (HeNe 543 and 633 for Texas Red-conjugated streptavidin and TO-PRO®-3, respectively).

2.15 Measurement of $\Delta\Psi_m$ and ROS

ROS generation and $\Delta\Psi_m$ were determined by staining cells with $DiOC_6$ or DHE, respectively. MDA-MB-231 cells seeded in 5 ml tubes as described in section 2.4 were cultured under the indicated conditions for the indicated periods of time. DHE (2.5 µM) or $DiOC_6$ (40 nM) were added to cells 10 min before analysis on a FACSCalibur™ flow cytometer (BD Biosciences).

2.16 LDH Release Assay

The LDH release assay was used to quantify extensive membrane damage and was conducted as per the manufacturer's instructions. Cells plated in 96-well flat-bottomed tissue culture plates as described in section 2.4 were cultured under the indicated conditions for the indicated periods of time. Cells were separated by centrifugation (500 × g) and supernatants (50 µl) were transferred to fresh 96-well flat-bottomed tissue culture plates. Complete cytolysis was achieved by 3 freeze/thaw (-80°C/37°C) cycles. Supernatants were incubated with 50 µl substrate solution for 30 min and the reaction was stopped by adding 50 µl stop solution. Absorbance (490 m) was used to calculate percent cytotoxicity using the equation ([(E/S)/(M/S)] × 100), where E and S and M denote experimental, spontaneous (vehicle-treated) and maximum LDH release, respectively.

2.17 PI Uptake

PI is a small molecule (MW, 668.4) that is excluded from cells with intact membranes (171). PI uptake was used to assess NRC-03- and NRC-07-induced membrane damage in multiple myeloma cells. U266 cells seeded in 5 ml tubes as described in section 2.4 were cultured in the presence or absence of NRC-03 or NRC-07 for 10 min. PI (10 µg/ml) was added to samples 5 min prior to analysis by flow cytometry.

2.18 Tricine Gel Electrophoresis

To evaluate the effect of FBS, HS and MS on NRC-03 degradation, biotin-NRC-03 was incubated at 37°C under the indicated conditions for 30 min. Samples were diluted in 4× tricine sample buffer (500 nM Tris-HCl [pH 6.8], 48% glycerol [v/v], 16% SDS [w/v], 10% β-mercaptoethanol [v/v] and 0.04% Coomassie Brilliant Blue 6-250 [w/v]), and were boiled for 5 min. Denatured proteins were separated on a 16.5% tricine gel (1M Tris-HCl [pH 8.45], 0.1% SDS [w/v], 10.5% glycerol [v/v], 0.05% APS [w/v], 0.5% TEMED [v/v]) for 4.5 h at 100 volts, transferred to nitrocellulose membranes using the iBlot® dry blotting system, and blocked for 1 h with Tris buffered saline-Tween 20

(TBST) (0.2 M Tris [pH 7.5], 1.5 M NaCl, 0.2% Tween 20) containing 5% fat free powdered skim milk (w/v). Membranes were incubated o/n in avidin-HRP (1:500 [v/v] in TBST containing 5% powdered skim milk) and were washed 3 times with TBST. Biotin-NRC-03 was visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and pixel intensity was determined using Scion Image Software (Scion Corporation, Frederick, MD) to calculate NRC-03 degradation.

2.19 Mitochondrial Isolation

Mitochondria were isolated from breast cancer cells to determine whether NRC-03 or NRC-07 interact directly with mitochondria. MDA-MB-231 cells (10×10^6) were washed with PBS and incubated in 900 µl ice-cold mitochondrial isolation buffer (0.2 mM EDTA, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.8) containing protease inhibitors (5 µg/ml leupeptin, 5 µg/ml pepstatin A, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM DTT, 100 µM NaVO$_4$, 10 mM NaF and 10 µM phenylarsine oxide) for 10 min. Cells were homogenized using an ice-cold glass tissue homogenizer and cleared by centrifugation ($1000 \times g$) for 10 min at 4°C. Mitochondria-containing supernatants were collected and the remaining pellet was homogenized and clarified as described above using 400 µl mitochondrial isolation buffer containing protease inhibitors. Mitochondria were collected from the supernatant by centrifugation ($12,000 \times g$) for 10 min at 4°C and were resuspended in 180 µl mitochondrial isolation buffer containing protease inhibitors. Mitochondria (50 µl) were cultured in the presence or absence of the indicated concentration of NRC-03 or NRC-07 for 10 min at which point intact mitochondria were collected by centrifugation at $12,000 \times g$ for 10 min at 4°C. Supernatants were collected (supernatant fraction) and intact mitochondria were lysed by incubating for 15 min on ice in 100 µl lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na$_2$HPO$_4$, 0.25% sodium deoxycholate [w/v], 0.1% Nonidet P-40 [v/v], 5 mM EDTA, and 5 mM EGTA) containing protease inhibitors. Supernatants containing mitochondrial proteins (mitochondria fraction) were separated from mitochondrial debris by centrifugation at $12,000 \times g$ for 10 min at 4°C.

2.20 Western Blottting

Western blotting was used to compare P-gp expression in paclitaxel-resistant MCF7-TX400 cells to parental MCF7 cells. MCF7 and MCF7-TX400 cells (1×10^6) were washed with PBS and resuspended in 0.1 ml ice-cold lysis buffer for 30 min at 4°C. Cellular proteins were clarified by centrifugation at $10,000 \times g$ for 10 min. Western blotting was also used to detect cytochrome c release from NRC-03- and NRC-07-treated mitochondria. Proteins were isolated from supernatants and mitochondrial fractions as described in section 2.19. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories Ltd.). Samples were diluted in 3× SDS polyacrylamide gel electrophoresis (PAGE) sample buffer (200 nM Tris-HCl [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], 15% β-mercaptoethanol [v/v] and 0.01% bromophenol blue [w/v]) and were boiled for 5 min. Equal amounts of protein (5 µg for cytochrome c and 15 µg for P-gp detection) were separated on a 12 and 7.5% polyacrylamide gel (375 mM Tris-HCl [pH 8.8], 0.1% [w/v] SDS, 0.1% APS, 0.15% TEMED) to detect cytochrome c and P-gp, respectively. Proteins were transferred to nitrocellulose membranes as described in section 2.20, and blots were blocked for 1 h in TBST containing 5% powdered skim milk. Membranes were washed 3 times with TBST, incubated o/n with the appropriate primary antibody (1:1000 [v/v] in TBST containing 5% powdered skim milk or BSA for anti-cytochrome c and anti-P-gp, respectively), washed extensively with TBST, probed for 1 h with the appropriate HRP-conjugated secondary antibody (1:1000) and washed extensively with TBST. Protein bands were visualized using an ECL detection system. Blots were subsequently stripped and re-probed with anti-mitochondrial Hsp70 or anti-actin (1:1000) to confirm equal protein loading.

2.21 Coomassie Blue Staining

BSA was added to NRC-03- and NRC-07-treated cells to determine whether soluble anionic molecules inhibit NRC-03- or NRC-07-induced cell killing. To verify that BSA was not degraded over time, supernatants were collected from samples cultured in the presence or absence of BSA (2.5% [w/v]) at 0, 4 and 24 h. Supernatants were diluted (1:10 [v/v] in PBS containing freshly added protease inhibitors) and separated on a 12% polyacrylamide gel. Gels were subsequently stained with Coomassie Blue (45% methanol

[v/v], 10% glacial acetic acid [v/v], 0.25% Coomassie® Brilliant Blue [w/v]) for 1 h, destained with destain solution (40% methanol [v/v] and 10% glacial acetic acid [v/v] in water) until background staining was minimal, and scanned. Grayscale images are shown.

2.22 TUNEL Staining

Terminal deoxynucleotidyl transferase (Tdt) deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) was used to determine whether NRC-03 or NRC-07 cause DNA fragmentation in cancer cells. MDA-MB-231 breast cancer and U266 multiple myeloma cells seeded in 6-well flat-bottom tissue culture plates containing sterile coverslips as described in section 2.4 were cultured in the presence or absence of NRC-03 or NRC-07 for 30 min. Cells were washed twice with PBS, fixed with 4% PFA for 1 h, washed twice with PBS and incubated, on ice, for 2 min in permeabilization solution (0.1% Triton X-100 [v/v] in 0.1% sodium citrate [w/v]). Permeabilized cells were washed twice with PBS, dried, and stained with TUNEL reaction mixture for 1 h at 37°C in a 10% CO_2 humidified atmosphere. Stained samples were washed 3 times with PBS, dried and mounted on slides using Dako fluorescent mounting medium, and visualized under white and UV light (400 ×).

2.23 Mass Spectrometry

Mass spectrometry was used to confirm that NRC-03 and NRC-07, but not [D]-NRC-03, are susceptible to degradation by trypsin. NRC-03, NRC-07, and [D]-NRC-03 (100 μg) reconstituted in 50 mM TEAB buffer were incubated in the presence or absence of trypsin (2 μg) o/n at 37°C . Samples were divided in two and dried by centrifugation in a vacuum at 23°C. Samples were reconstituted in 50 μl TFA (0.1% [w/v] in water) and diluted in Matrix Solution (1:1). Samples were spotted (500 ng) on a MALDI plate, dried, and analyzed on a MALDI-TOF mass spectrometer (Waters Corp., Milford, MA) by Mr. Ken Chisholm at the National Research Council of Canada Institute for Marine Biosciences (Halifax, NS).

2.24 Xenograft Model of Breast Cancer

A xenograft model of human breast cancer was used to determine whether NRC-03, NRC-07 or [D]-NRC-03 exhibit anticancer properties *in vivo*. NOD SCID mice were injected subcutaneously with 5×10^6 MDA-MB-231cells in the right hind flank. Cells were freshly thawed and confirmed to be free from mycoplasma contamination. No more than 4 mice were injected at once to maintain optimal cell viability and to achieve a high (90%) tumour take. Tumour growth was monitored every other day and tumour volume was determined using the equation $(L \times P^2)/2$, where L and P denote the longest diameter and the diameter perpendicular to the longest diameter, respectively. Tumour-bearing mice were randomized into 3 groups once tumours reached a volume greater than or equal to 120 mm^3 (day 1, approximately 35 d after tumour cell implantation). Tumours were injected with 20 µl of the HBSS vehicle control, 25 mg/ml NRC-03 or NRC-07 (500 µg) or 6.25 mg/ml [D]-NRC-03 (125 µg) on days 1, 3, and 5. Tumour-bearing mice were sacrificed 1 wk after the last injection (d 12) or earlier if the animal exhibited signs of distress or tumour ulceration. Sacrificed mice were photographed and tumours were excised, photographed, sectioned, stained with hematoxylin and eosin and visualized under brightfield microscopy (40 × magnification).

2.25 Statistical Analysis

All data were analyzed by the Student's *t*-test, the Bonferroni multiple comparison test and the one way analysis of variance (ANOVA), as appropriate, using InStat version 3.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

CHAPTER 3

NRC-03 AND NRC-07 ARE DIRECT-ACTING CAPS THAT INHIBIT THE GROWTH OF BREAST CANCER XENOGRAFTS

3.1 NRC-03 and NRC-07 Kill Breast Cancer Cells and HMECs, but are not Cytotoxic to Other Normal Cell Types.

Traditional chemotherapeutic drugs that are currently on the market kill rapidly dividing cancer cells. These drugs fail to kill indolent or slow-growing cancer cells and are limited by their toxicities toward rapidly dividing normal cells (29, 30, 61). To this end, research has focused on developing novel anticancer agents with the capacity to kill cancer cells without causing undue harm to normal tissues. In this capacity, CAPs represent a promising alternative to traditional cytotoxic chemotherapy as they kill cancer cells on the basis of charge rather than the rate of growth of the target cell (37, 38). The purpose of this investigation was to determine whether pleurocidins possess anticancer properties, and to determine the clinical potential of those identified as promising anticancer agents both *in vitro* and *in vivo*. In 2003, a panel of novel pleurocidins isolated from various Atlantic flatfish species was identified (48). Based on the net charge and length of the peptide, I chose to investigate 5 pleurocidins as potential anticancer agents: NRC-03, NRC-07, NRC-03$_{10\,mer}$, NRC-13, and NRC-16. LDH-release assays demonstrated that NRC-03 and NRC-07, but not NRC-13, NRC-16, or NRC-03$_{10mer}$, killed human breast cancer cell lines at 50 μM by 24 h (Fig. 3.1A). NRC-03 killed 58 ± 7%, 63 ± 13% and 98 ± 19% MDA-MB-231, T47D and MCF7 human breast cancer cells, respectively, whereas NRC-07 killed 45 ± 2%, 57 ± 14% and 98 ± 19, respectively.

Figure 3.1B and 3.1C depict MTT assay results showing that NRC-03 and NRC-07 killed T47D, MCF7, MDA-MB-468, MDA-MB-231 and SKBR3 human breast cancer cells, as well as 4T1 mouse mammary carcinoma cells in a dose-dependent manner (*p* < 0.005). Interestingly, NRC-03 and NRC-07 were nearly equally cytotoxic to all breast cancer cells, with the exception of MDA-MB-468 and 4T1 at 5 and 10 μM. MCF7, SKBR3, MDA-MB-468 and 4T1 cells were most susceptible to 50 μM NRC-03 (73 ± 1%, 75 ± 3%, 86 ± 7% and 94 ± 1% cytotoxicity, respectively) and NRC-07 (73 ± 4%, 87
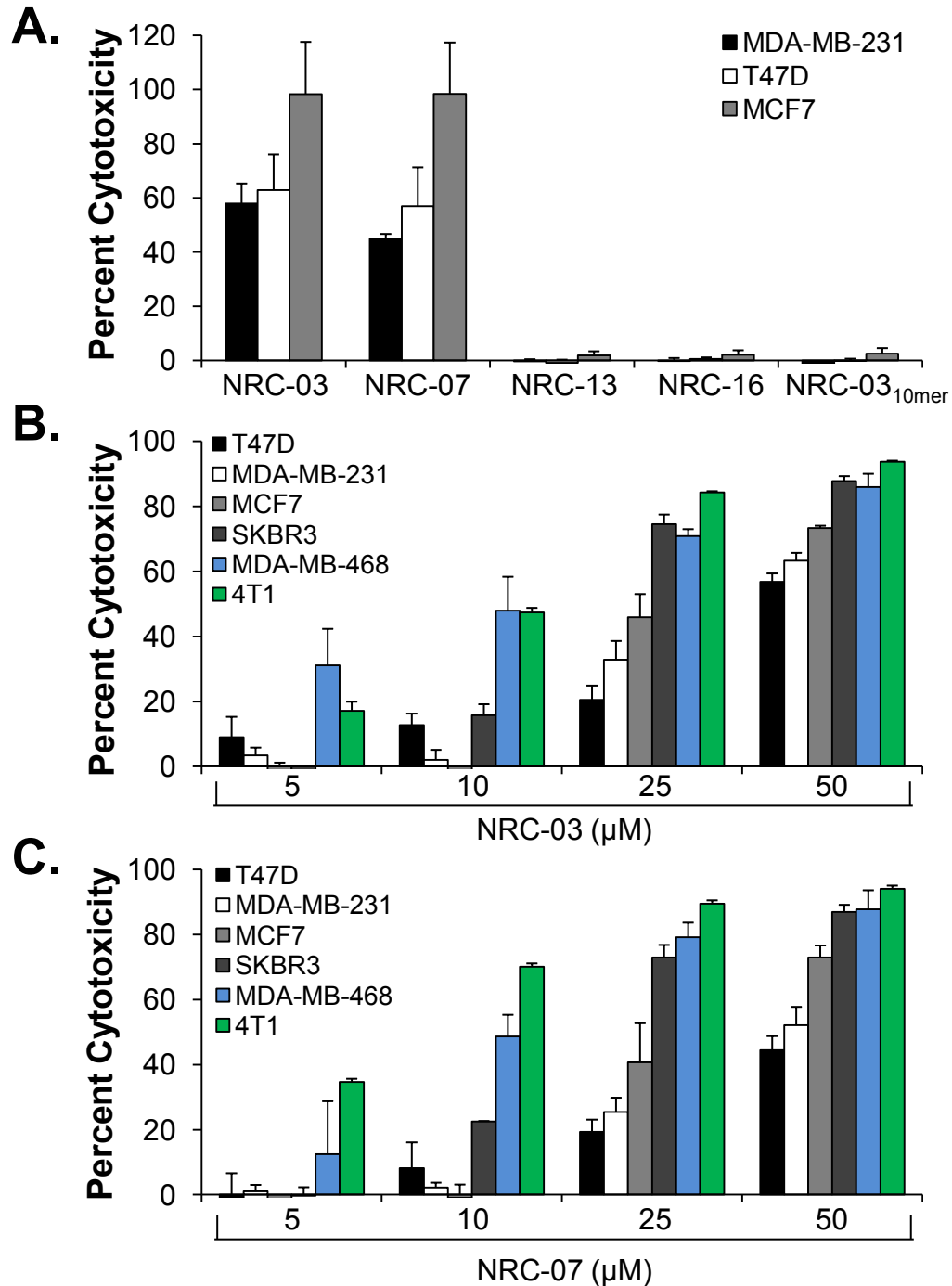
Figure 3.1. NRC-03 and NRC-07 are CAPs with anticancer activities. (A) MDA-MB-231, T47D, and MCF7 breast cancer cells were exposed to medium alone or to 50 μM NRC-03, NRC-07, NRC-13, NRC-16, or NRC-03$_{10mer}$. Cell viability was determined by LDH-release assay after 24 h. T47D, MDA-MB-231, MCF7, SKBR3, and MDA-MB-468 human breast cancer cells, and 4T1 murine mammary carcinoma cells were exposed to medium alone or the indicated concentrations of (B) NRC-03 or (C) NRC-07. Cell viability was determined by MTT assay after 24 h. Data shown are statistically significant by ANOVA ($p < 0.005$).

± 2%, 88 ± 10% and 94 ± 1% cytotoxicity, respectively), whereas T47D and MDA-MB-231 breast cancer cells were least susceptible to 50 µM NRC-03 (57 ± 3% and 63 ± 2% cytotoxicity, respectively) and NRC-07 (44 ± 4% and 52 ± 6% cytotoxicity, respectively). Importantly, NRC-03- and NRC-07-induced cell killing was comparable between LDH-release and MTT assays (compare Fig. 3.1A to 3.1B-C). Therefore, the MTT assay was used to evaluate NRC-03- and NRC-07-induced cell killing in future experiments unless otherwise indicated. MDA-MB-231 cells were used as a model breast cancer cell line because they represented a worst case scenario due to their relative resistance to killing by NRC-03 and NRC-07, and because they form solid tumours in immune-deficient mice (172-174).

The development of multidrug-resistant cancer cells limits the therapeutic utility of traditional anticancer drugs (29). MCF7-TX400 cells express 2.6-fold more P-gp than parental MCF7 cells and are resistant to 400 ng/ml paclitaxel (Appendix Fig. 1). Clonogenic assays were used in place of MTT assays to determine whether NRC-03 or NRC-07 kill MCF7-TX400 cells because drug efflux proteins interfere with MTT assays (168). After only 4 h, 50 µM NRC-03 and NRC-07 significantly reduced the number of MCF7 and MCF7-TX400 CFUs (Fig. 3.2B). In addition, colonies from NRC-03- and NRC-07-treated samples were significantly smaller than colonies from control-treated samples (Fig. 3.2A). Moreover, MCF7-TX400 CFUs were smaller than MCF7 CFUs. Acid phosphatase assays conducted in parallel with clonogenic assays confirmed that 50 µM NRC-03 and NRC-07 killed MCF7 and MCF7-TX400 cells to an equal extent (Fig. 3.2C). Importantly, NRC-03 and NRC-07 also killed cisplatin-resistant ovarian cancer cells (Appendix Fig. 2), suggesting that these peptides kill cancer cells that develop drug resistance by mechanisms other than increasing P-gp expression.

MTT assays were conducted on normal cells (HMECs, HDFs, HUVECs) cultured in the presence or absence of NRC-03 or NRC-07 to determine whether NRC-03 or NRC-07 selectively kill cancer cells. NRC-03 and NRC-07 were toxic to HMECs, albeit to a lesser extent than they were to MDA-MB-231 cells (Table 3.1). NRC-03 and NRC-07 were minimally toxic to HUVECs, and were not toxic to HDFs. Importantly, neither NRC-03 nor NRC-07 were hemolytic, which is a problem for certain α-helical CAPs,

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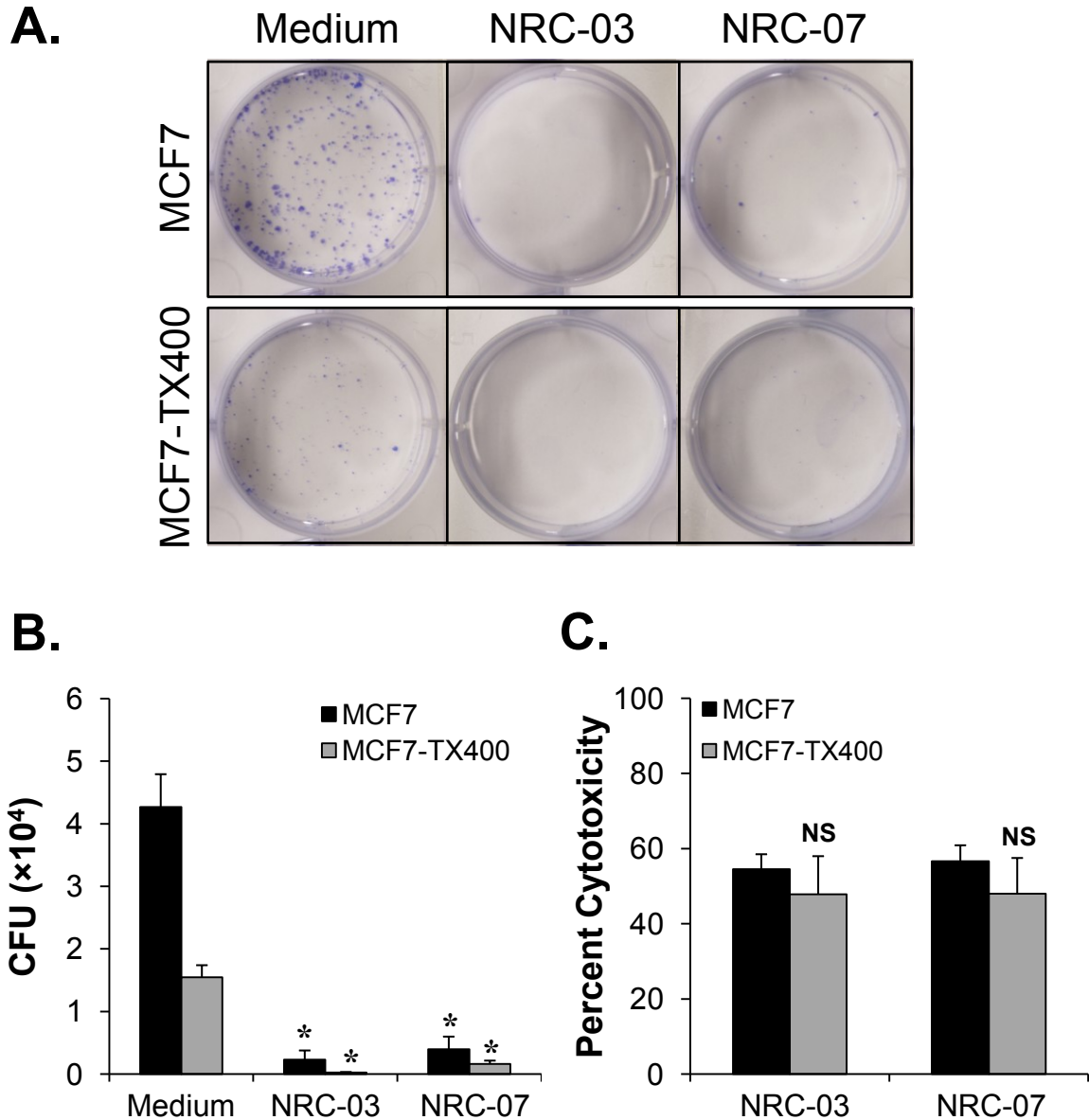
Figure 3.2. NRC-03 and NRC-07 kill multidrug-resistant breast cancer cells. (A) MCF7 and MCF7-TX400 breast cancer cells were cultured in the presence or absence of 50 μM NRC-03 or NRC-07. After 4 h, the cells were diluted and cultured for 10 d. (A) Representative images of medium-, NRC-03- and NRC-07-treated cells (800 × dilution) are shown. (B) Colony formation by medium- NRC-03-, and NRC-07-treated cells was determined by clonogenic assay. Data shown are statistically significant by the Bonferroni multiple comparison test in comparison to medium-treated cells; * $p < 0.05$. (C) MCF7 and MCF7-TX400 cells were cultured in the presence or absence of 50 μM NRC-03 or NRC-07. Cell viability was determined by acid phosphatase assay after 4 h. Data shown are not statistically significant (NS, $p \geq 0.05$) by the two-tailed student's t test in comparison to MCF7-treated cells. All data shown represent the mean of 3 independent experiments ± SE.

Table 3.1. NRC-03- and NRC-07-induced cytotoxicity toward a panel of normal human cells compared to breast cancer cells

Treatment	% Cytotoxicity[a,c]				% Hemolysis[b,c]
	HMECs	HDFs	HUVECs	MDA-MB-231	Erythrocytes
25 µM NRC-03	17 ± 5	0 ± 2	3 ± 3	46 ± 6	2 ± 1
50 µM NRC-03	46 ± 3	2 ± 1	19 ± 2	74 ± 4	3 ± 2
25 µM NRC-07	20 ± 4	1 ± 1	7 ± 1	29 ± 8	1 ± 1
50 µM NRC-07	47 ± 9	0 ± 3	17 ± 8	62 ± 5	1 ± 1

[a]NRC-03- and NRC-07-mediated cytotoxicity toward cultures of normal cells was determined by MTT assay after 24 h.
[b]Hemolytic activity toward human erythrocytes was determined by hemolysis assay after 8 h.
[c]Data shown represent the mean of 3 independent experiments ± SE.

such as melittin (175). HDFs were selected as the negative control cell line because they were least susceptible to killing by NRC-03 or NRC-07.

3.2 NRC-03, and to a Lesser Extent NRC-07, Enhance Breast Cancer Cell Killing by Cisplatin

Cytotoxic chemotherapies with independent mechanisms of action are often combined to enhance the clinical response in patients with breast cancer (162). NRC-03 and NRC-07 were hypothesized to kill cells with negatively charged membranes by causing irreversible membrane damage. This unique mechanism of action could make these peptides useful candidates for inclusion in combinational treatment regimens. Therefore, I tested whether NRC-03 or NRC-07 could enhance cell killing by cisplatin at 72 or 96 h by calculating the half maximal effective concentration (EC_{50}) of cisplatin toward MDA-MB-231 cells in the presence or absence of sublethal doses of NRC-03 or NRC-07. As shown in Figure 3.1, 10 µM NRC-03 or NRC-07 did not kill MDA-MB-231 cells ($2 \pm 3\%$ and $2 \pm 2\%$ cytotoxicity, respectively). When cisplatin was combined with NRC-03 or NRC-07, the EC_{50} for MDA-MB-231 cells decreased by 5.5- and 1.6-fold, respectively, by 72 h (Fig. 3.3). By 96 h, NRC-03 and NRC-07 decreased the EC_{50} of cisplatin for MDA-MB-231 cells by 4- and 1.6-fold, respectively. This finding suggests that NRC-03 or NRC-07 may be useful candidates for inclusion in combinational treatment regimens; however, additional experiments are required to determine whether this effect is additive or synergistic.

3.3 NRC-03 and NRC-07 Bind to Breast Cancer Cells and Localize to the Nucleus, but Only NRC-07 Causes DNA Fragmentation

CAPs kill cancer cells directly by causing significant membrane damage that leads to cytolysis, or indirectly by inducing apoptosis (61). Understanding the mechanism of action of NRC-03 and NRC-07 is essential for rational peptide modification to improve potential clinical utility. CAPs are predicted to initiate cell death by first binding to the target cell (37). The peptide binding assay was used to quantify biotin-NRC-03 and -NRC-07 binding to MDA-MB-231 cells and HDFs. Importantly, biotin-NRC-03 and -NRC-07 were as potent as NRC-03 and NRC-07 to MDA-MB-231 breast cancer cells
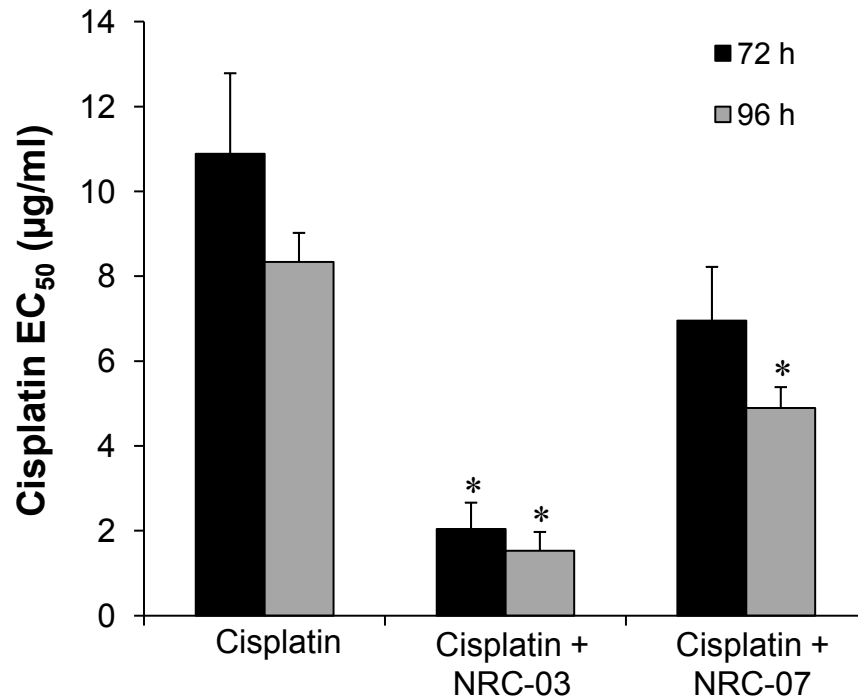
Figure 3.3. NRC-03, and to a lesser extent NRC-07, reduce the EC$_{50}$ of cisplatin. MDA-MB-231 breast cancer cells were cultured in increasing concentrations of cisplatin in the presence or absence of 10 μM NRC-03 or NRC-07 for 72 and 96 h. Cell viability was determined by MTT assay to determine the EC$_{50}$ of cisplatin. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test; * $p < 0.05$ in comparison to cisplatin-treated MDA-MB-231 cells.

(Appendix Fig. 3). Biotin-NRC-03 and -NRC-07 bound to MDA-MB-231 cells, but not HDFs, within 10 min (Fig. 3.4A). Peptide binding was quantified to determine differences between biotin-NRC-03 and -NRC-07 binding to MDA-MB-231 breast cancer cells and HDFs, as well as differences between MDA-MB-231 and HDFs. Compared to HDFs, binding of biotin-NRC-03 and -NRC-07 to MDA-MB-231 breast cancer cells was increased by 56- and 98-fold, respectively (Fig. 3.4B). Biotin-NRC-03 and -NRC-07 were equally potent in MDA-MB-231 cells (Appendix Fig. 3). Interestingly, in comparison to biotin-NRC-07 binding, biotin-NRC-03 binding to MDA-MB-231 cells and HDFs was increased by 8- and 14-fold, respectively.

CAPs are thought to preferentially bind to cancer cells because the outer membrane leaflet of cancer cells carries a net negative charge, whereas the outer membrane leaflet of normal cells is zwitterionic and therefore neutral in charge (37, 38, 61). Many molecules contribute to the negative charge of cancer cell membranes including phosphatidylserine, O-glycosylated mucins and sialylated glycoproteins (77, 83, 176, 177). HSPs and CSPs have been shown to influence CAP-mediated cancer cell killing (84, 85). I chose to investigate whether sialylated glycoproteins, HSPs, and/or CSPs influence NRC-03- and/or NRC-07-mediated breast cancer cell killing. MDA-MB-231 breast cancer cells incubated in OSGE prior to peptide treatment were used to evaluate whether NRC-03 or NRC-07 are attracted to sialylated glycoproteins. Compared to vehicle-treated cells, marginal reductions in cell killing were observed when OSGE-treated MDA-MB-231 cells were exposed to 10 μM NRC-03 or NRC-07; however, significant reductions in cell death were not observed when cells were treated with 50 μM NRC-03 or NRC-07 (Fig. 3.5A). NRC-03- and NRC-07-induced cell death was investigated in L cells, gro2C cells (HSP-deficient) and sog9 cells (HSP- and CSP-deficient) to determine whether HSPs and/or CSPs influence NRC-03- and/or NRC-07-induced cell killing. Gro2C and sog9 cells were less susceptible to 25 or 50 μM NRC-03 or NRC-07 than were L cells. Sog9 cells were most resistant to NRC-03 and NRC-07; in fact, killing nearly decreased to baseline when sog9 cells were treated with 25 μM NRC-03 or NRC-07 (Fig. 3.5B). Interestingly, gro2C and sog9 cells were equally resistant to 25 μM NRC-07. Compared to L cell killing, decreases in NRC-03- and NRC-07-induced cytotoxicity were also evident when gro2C and sog9 cells were treated with 50 μM NRC-
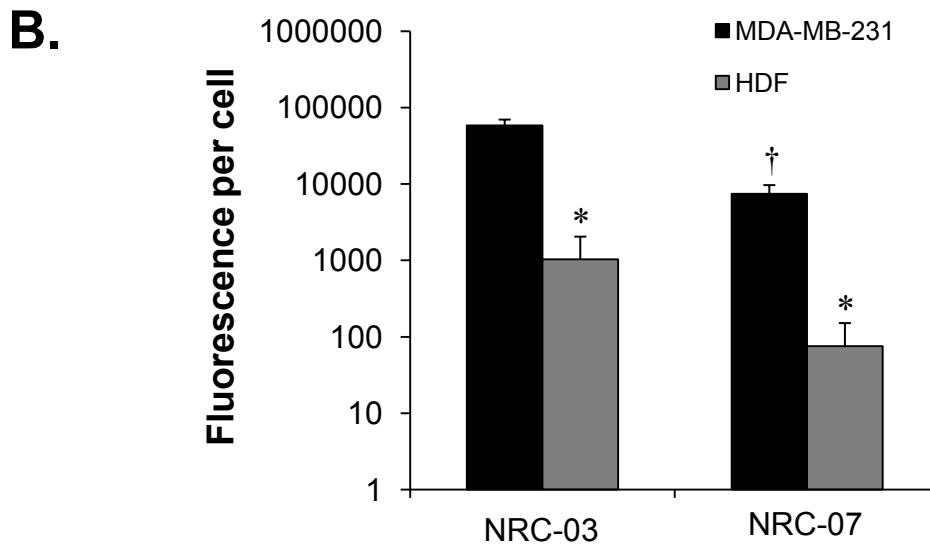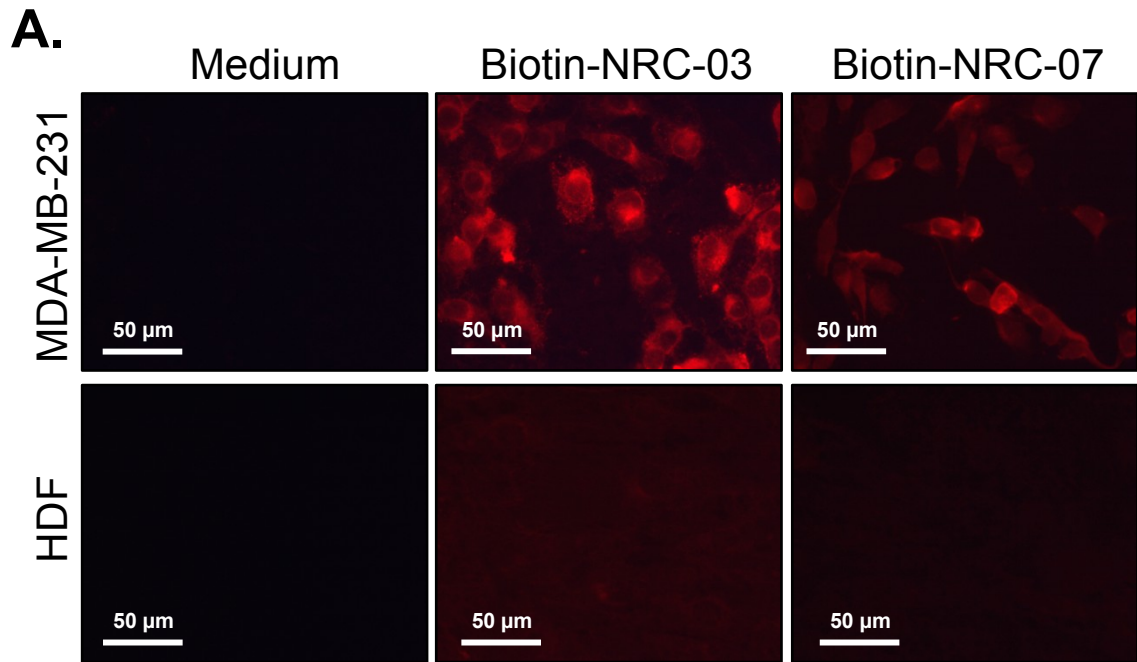
Figure 3.4. NRC-03 and NRC-07 bind to breast cancer cells but not normal HDFs. (A) MDA-MB-231 breast cancer cells and HDFs were cultured in the absence or presence of 50 μM biotin-NRC-03 or -NRC-07 for 10 min, stained with Texas Red-streptavidin, and visualized by fluorescence microscopy. (B) Peptide binding was quantified using NIS-Elements. Data shown represent the mean of 3 independent experiments ± SE. Statistical significance was determined by the Bonferroni multiple comparisons test; * $p < 0.05$ relative to NRC-03- and NRC-07-treated MDA-MB-231 cells, † $p < 0.05$ relative to NRC-03-treated MDA-MB-231 cells.
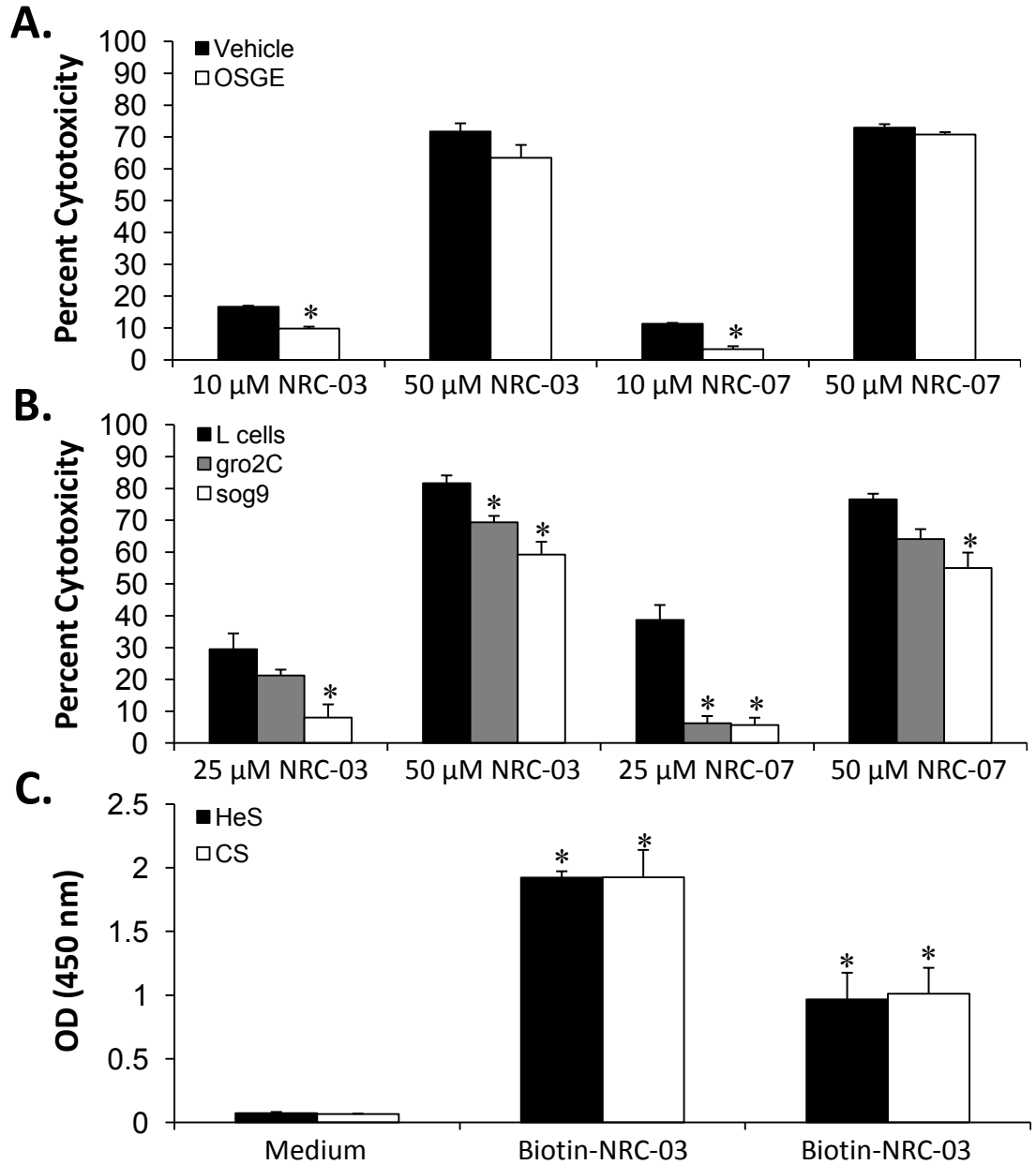
Figure 3.5. NRC-03 and NRC-07 are attracted to several different negatively charged molecules. (A) MDA-MB-231 cells pretreated with OSGE for 30 min were cultured in the presence or absence of the indicated concentrations of NRC-03 or NRC-07. Cell viability was determined by MTT assay after 24 h. Data is statistically significant by the Student's *t* test in comparison to vehicle-treated cells; * *p* < 0.05. (B) L, gro2C and sog9 cells were cultured under the indicated conditions for 24 h. Cell viability was determined by MTT assay. Statistical significance was determined by the Bonferroni multiple comparison test; * *p* < 0.05 relative to NRC-03- or NRC-07-treated L cells. (C) Biotin-NRC-03 and -NRC-07 binding to HeS and CS was determined using the solid phase HeS- and CS-binding assay. Data are significant by the Bonferroni multiple comparison test; * *p* < 0.05 relative to medium OD. Data represent the mean of 3 independent experiments ± SE.

03 or NRC-07; however, cell killing was not reduced to baseline levels as it was when cells were treated with 25 μM NRC-03 or NRC-07. Together, these findings suggest that NRC-03 and NRC-07 are likely attracted to many negatively charged surface molecules rather than a unique receptor. The solid phase HeS- and CS-binding assay was then used to confirm that NRC-03 and NRC-07 bound to HeS and CS. Biotin-NRC-03 and -NRC-07 bound to HeS and CS 28- and 14-fold more than the medium control, respectively, suggesting that NRC-03 and NRC-07 may bind HSPs and CSPs (Fig. 3.5C).

Certain CAPs pass through the plasma membrane of cancer cells and target specific organelles (37). To better understand the mechanism of NRC-03- and NRC-07-induced cell death, confocal microscopy was used to determine whether NRC-03 or NRC-07 enter the cytoplasma of breast cancer cells. Biotin-NRC-03 and -NRC-07 gained access to the cytosol of MDA-MB-231 breast cancer cells within 30 sec (Fig. 3.6). Interestingly, both biotin-NRC-03 and -NRC-07 appeared to localize to the nucleus of MDA-MB-231 breast cancer cells. Nuclear localization was confirmed by staining for double-stranded DNA using TO-PRO®-3 (Fig. 3.7). Interestingly, biotin-NRC-03 and NRC-07 also appeared to localize to the nuclear. TUNEL staining was then used to determine whether NRC-03 and/or NRC-07 cause DNA fragmentation in breast cancer cells. Interestingly, after 30 min NRC-07, but not NRC-03, caused DNA fragmentation in MDA-MB-231 cells (Fig 3.8).

3.4 NRC-03- and NRC-07-Induced Cell Death is Caspase-independent and Involves Mitochondrial Damage, but not ROS Generation

Certain direct- and indirect-acting CAPs cause $\Delta\Psi_m$ in cancer cells (54, 107). To determine whether NRC-03 or NRC-07 cause $\Delta\Psi_m$ in human breast cancer cells, MDA-MB-231 cells were treated with NRC-03 or NRC-07 and were stained with the dye $DiOC_6$, which localizes to intact mitochondria (178). Both NRC-03 and NRC-07 caused significant $\Delta\Psi_m$ by 1 h (Fig. 3.9A). ROS generation, which is associated with LfcinB-induced apoptosis and can result from $\Delta\Psi_m$ (107), was not observed following treatment with NRC-03 or NRC-07 (Fig. 3.9B). Mitochondria isolated from MDA-MB-231 cells were treated with NRC-03 or NRC-07 to determine whether either peptide could directly damage mitochondria. Cytochrome c was released after only 10 min, showing that NRC-
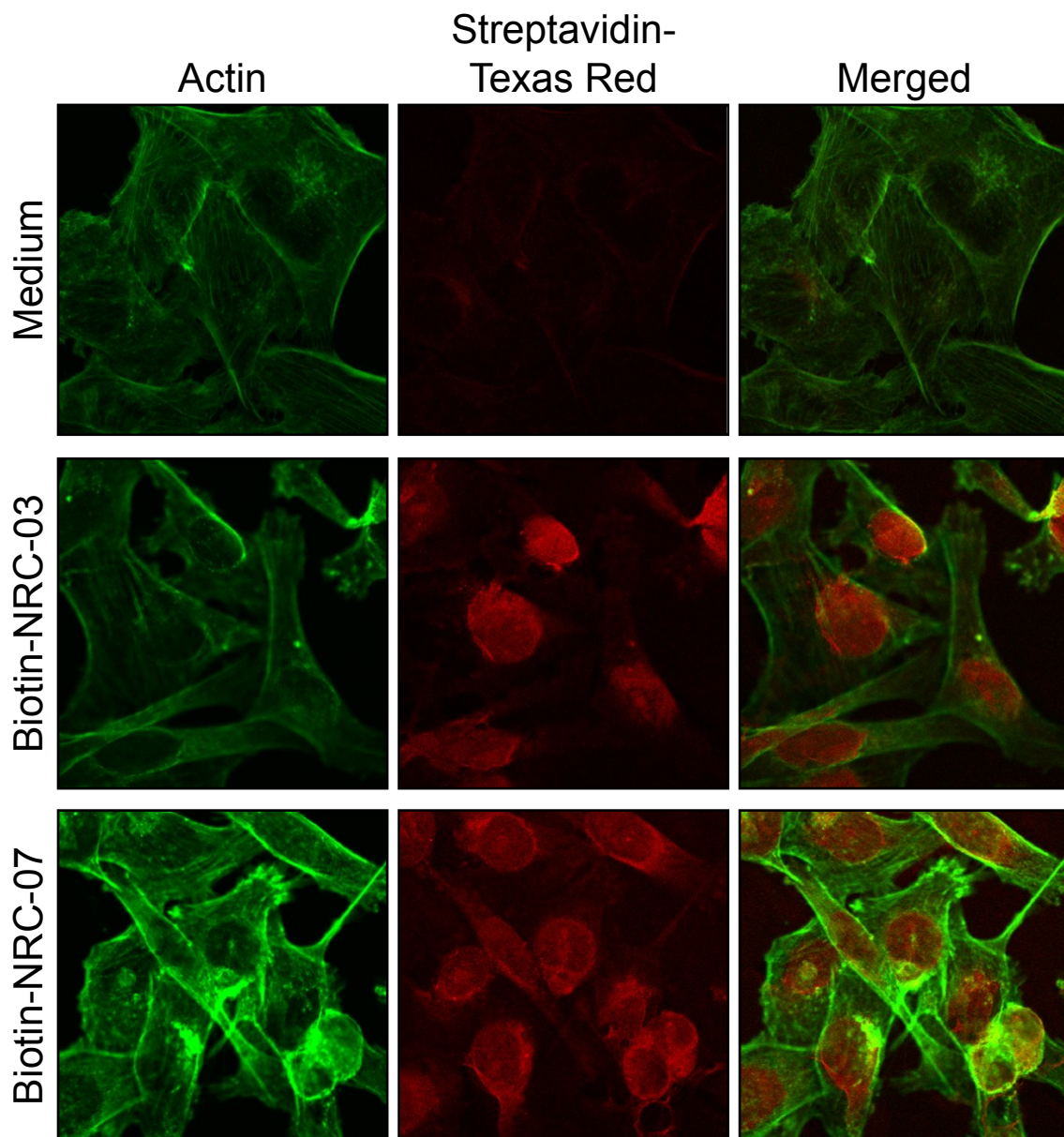
Figure 3.6. NRC-03 and NRC-07 rapidly enter breast cancer cells. MDA-MB-231 breast cancer cells were cultured in the presence or absence of 50 μM biotin-NRC-03, or -NRC-07 for 30 sec. NRC-03, NRC-07, and the actin cytoskeleton were visualized by confocal microscopy through the addition of Texas Red-conjugated streptavidin, and Alexa-Fluor 488-phalloidin, respectively (1000 ×). Data shown are from a representative experiment (n = 3).
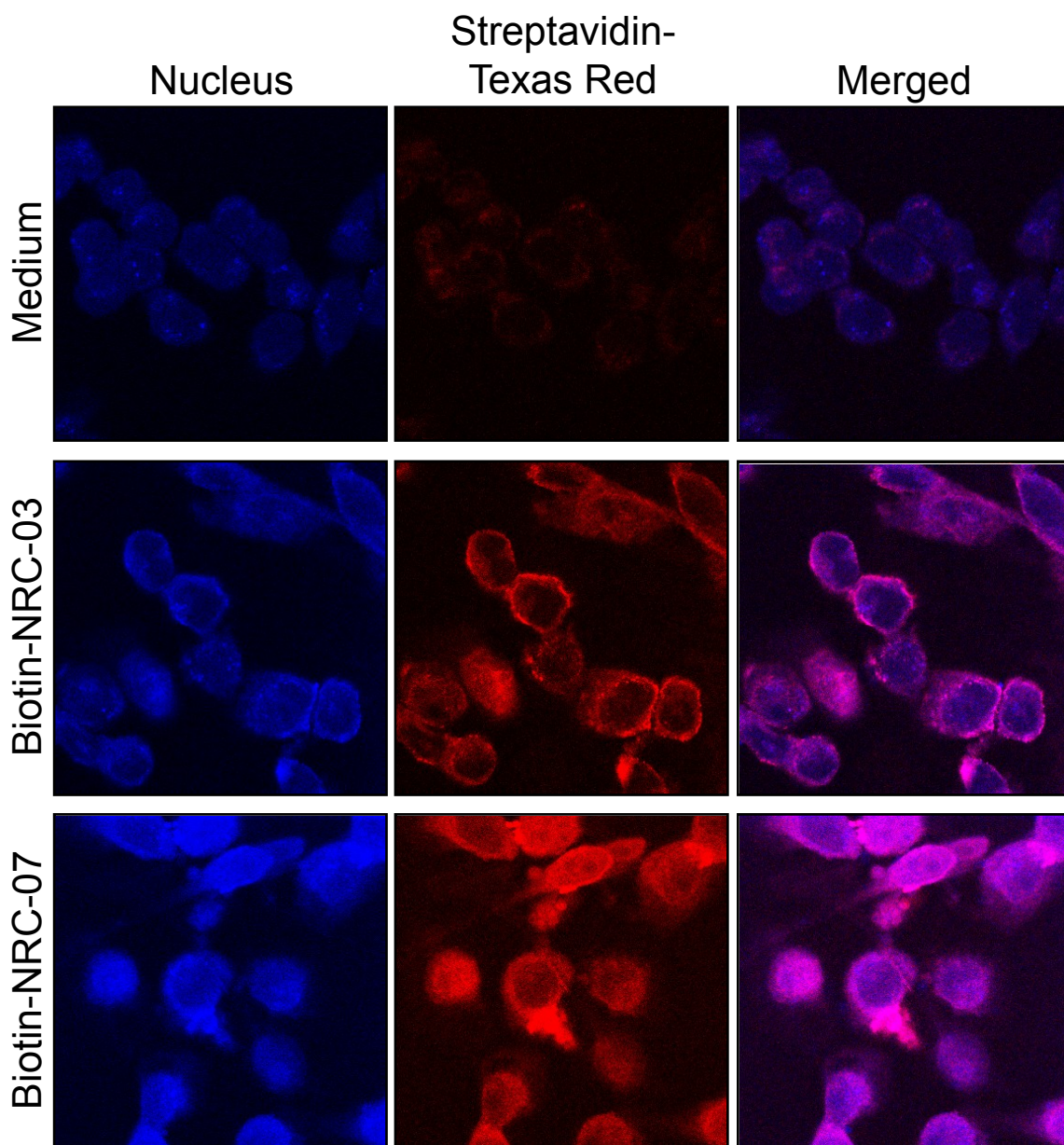
Figure 3.7. NRC-03 and NRC-07 rapidly enter the nucleus of breast cancer cells. MDA-MB-231 breast cancer cells were cultured in the presence or absence of 50 μM biotin-NRC-03, or -NRC-07 for 30 sec. NRC-03, NRC-07, and the nucleus were visualized by confocal microscopy through the addition of Texas Red-conjugated streptavidin, and TO-PRO®-3 iodide, respectively (1000 ×). Data shown are from a representative experiment (n = 3).
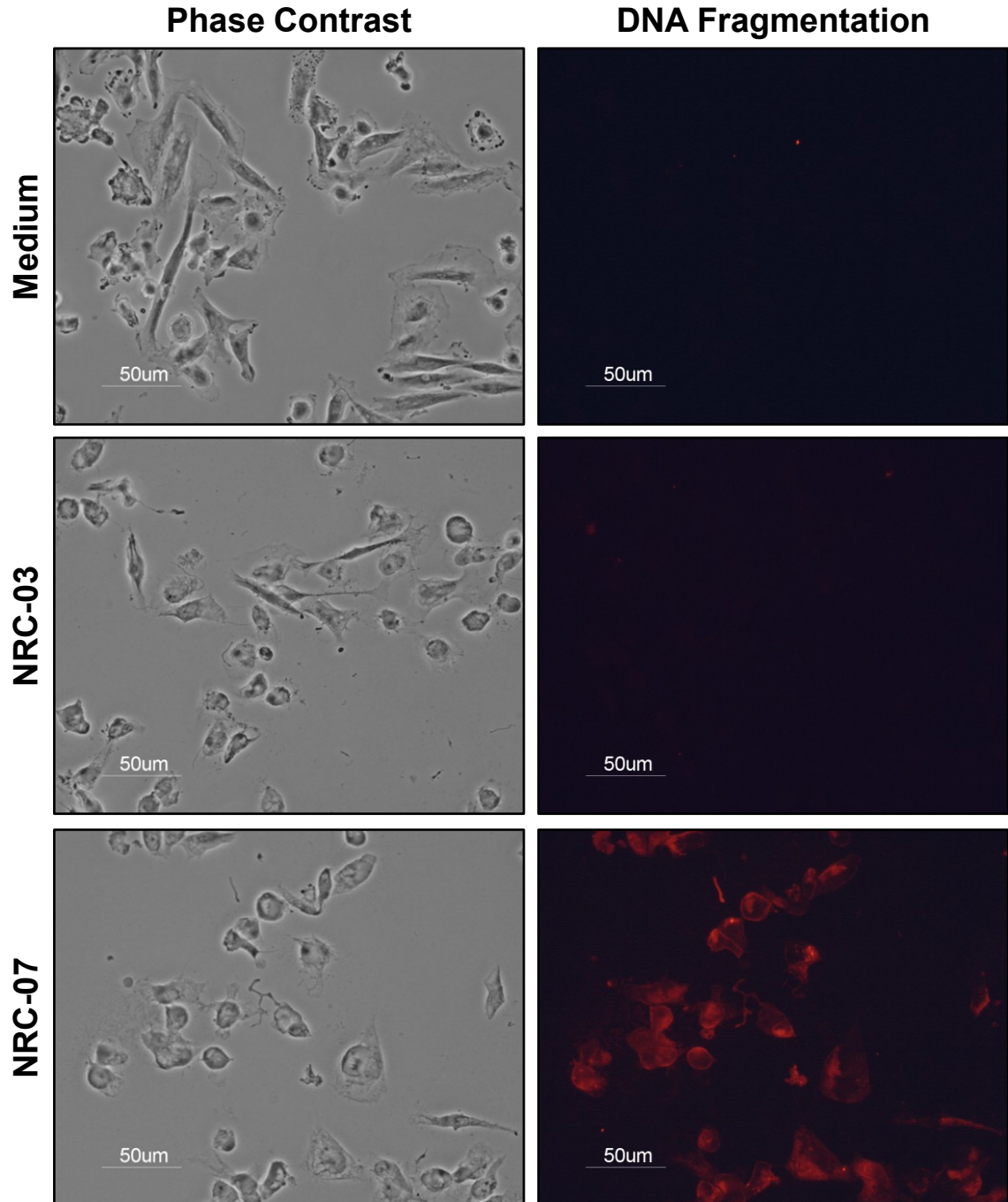
Figure 3.8. NRC-07, but not NRC-03, causes DNA fragmentation in breast cancer cells. MDA-MB-231 breast cancer cells were cultured in the presence or absence of 50 μM NRC-03, or NRC-07 for 30 min. DNA fragmentation was detected by TUNEL assay and visualized by fluorescence microscopy. Data shown are from a representative experiment (n = 3).

A.

B.

C.
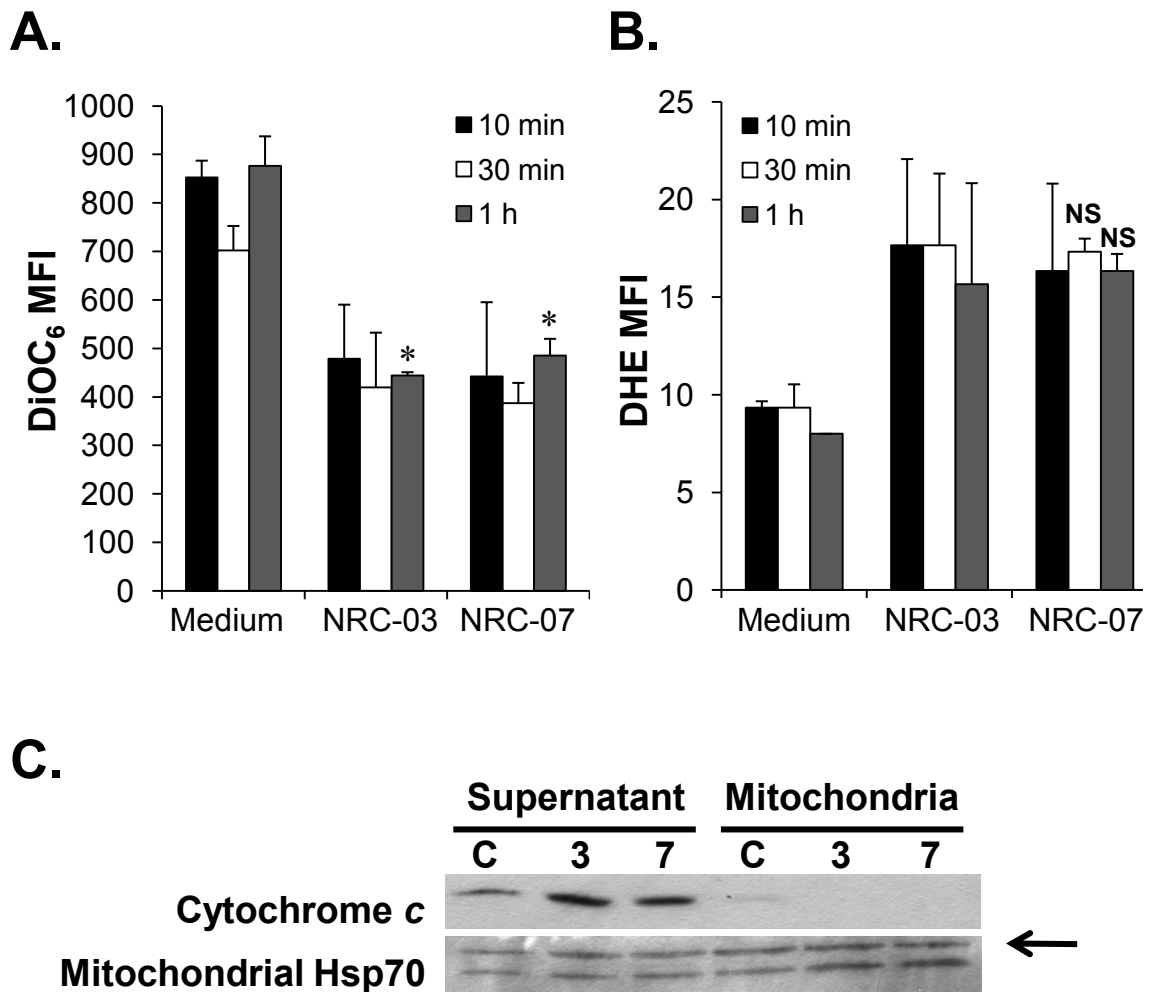
Figure 3.9. NRC-03- and NRC-07-induced cytotoxicity is associated with mitochondrial membrane damage, but not ROS generation. MDA-MB-231 breast cancer cells were cultured in the absence or presence of 50 μM NRC-03 or NRC-07 for 10 min, 30 min, or 1 h. (A) DiOC$_6$ or (B) DHE were added to cells to detect ΔΨ$_m$ and ROS generation, respectively. Data shown represent the average MFI of 3 independent experiments ± SE, and are statistically significant by the Bonferroni multiple comparison test in comparison to medium-treated cells; * $p < 0.05$. (C) Mitochondria isolated from MDA-MB-231 cells were treated as described above for 10 min and cytochrome c was detected in cytosolic and membrane fractions by western blot analysis. Mitochondrial Hsp70 was detected to confirm equal protein loading. Data shown are from a representative experiment (n = 3). C: control. 3: NRC-03. 7: NRC-07.

03 and NRC-07 directly damage mitochondria (Fig. 3.9C). In order to determine whether NRC-03 or NRC-07 induce caspase-dependent apoptosis in breast cancer cells, MDA-MB-231 breast cancer cells were pretreated with the pancaspase inhibitor Boc-D-fmk prior to exposure to NRC-03 or NRC-07. Boc-D-fmk reversed flunarizine (FLZ)-induced apoptosis in Jurkat T leukemia cells, which served as a positive control, but did not inhibit NRC-03- or NRC-07-induced cell death (Fig. 3.10). This data suggests that NRC-03 and NRC-07 do not trigger caspase-dependent apoptosis; however, this finding does not rule out caspase-independent apoptosis.

3.5 NRC-03 and NRC-07 Lyse Breast Cancer Cells but not Normal HDFs

Many CAPs are directly lytic to cancer cells (61). The LDH-release assay was used to quantify NRC-03- and NRC-07-induced breast cancer cell lysis over time. Both NRC-03 and NRC-07 lysed MDA-MB-231 breast cancer cells in a time-dependent manner (Fig. 3.11). NRC-03- and NRC-07-induced cytolysis peaked at 4 h. As before, the potency of NRC-07 mirrored that of NRC-03. SEM was then used to visualize changes in the ultrastructure of MDA-MB-231 breast cancer cells and HDFs following exposure to NRC-03 or NRC-07. Figure 3.12, which represents approximately 30% of peptide-treated cells, shows that 50 μM NRC-03 and NRC-07 caused extensive damage to breast cancer cells after only 10 min. Images at lower magnification show that NRC-03- and NRC-07-treated cells were largely intact; however, cell fragments were visible. Images at intermediate magnification show pores that have formed in the plasma membrane, swollen and reduced numbers of microvilli, membrane blebs, and exposed intracellular organelles. High magnification images clearly show altered microvilli and the presence of pore structures in the plasma membrane. Importantly the non-cytotoxic CAP NRC-13 did not cause any changes in the ultrastructure of MDA-MB-231 cells. Furthermore, neither NRC-03 nor NRC-07 changed the ultrastructure of HDFs (Fig. 3.13). These findings, taken with the results from the binding assays described in section 3.3, suggest that NRC-03- and NRC-07-induced membrane damage is a consequence of peptide binding to breast cancer cells.
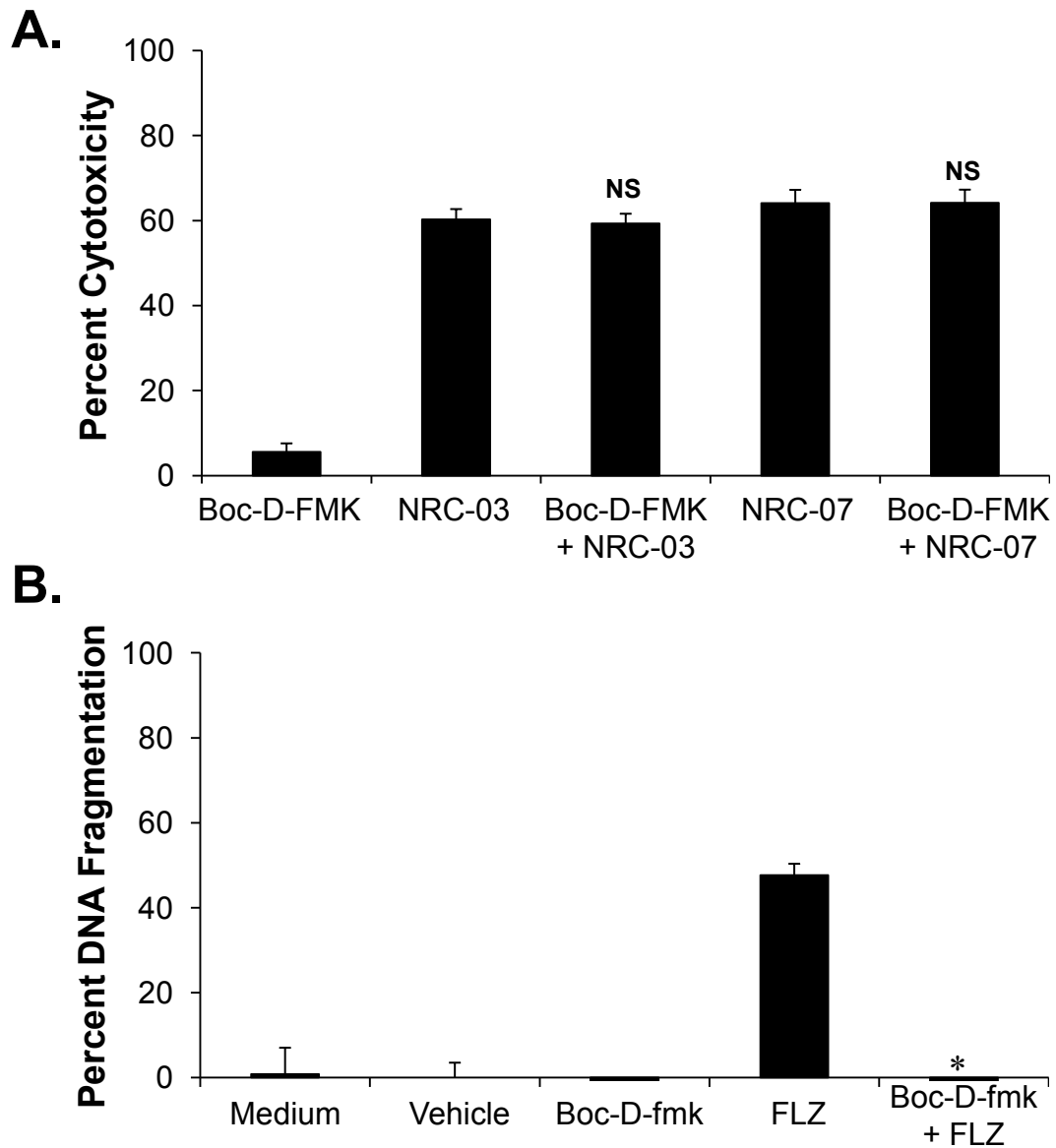
Figure 3.10. NRC-03 and NRC-07 do not kill cells by the caspase-dependent apoptotic pathway. (A) MDA-MB-231 breast cancer cells pretreated with 40 μM Boc-D-fmk were cultured in the absence or presence of 50 μM NRC-03 or NRC-07 for 24 h. Percent cytotoxicity was determined by MTT assay. Data shown represent the mean of 6 independent experiments ± SE and are not statistically significant ($p \geq 0.05$) by the Bonferroni multiple comparson test in comparison to NRC-03- or NRC-07-treated cells. (B) Jurkat T leukemia cells pretreated with 40 μM Boc-D-fmk were cultured in the presence or absence of 80 μM FLZ. Percent DNA fragmentation was determined by ^{3}H-TdR-release assay after 18 h. Data shown is of a representative experiment (n = 4) ± StDev, and is statistically significant by Bonferroni multiple comparison test in comparison to FLZ-treated cells; *$p < 0.05$.
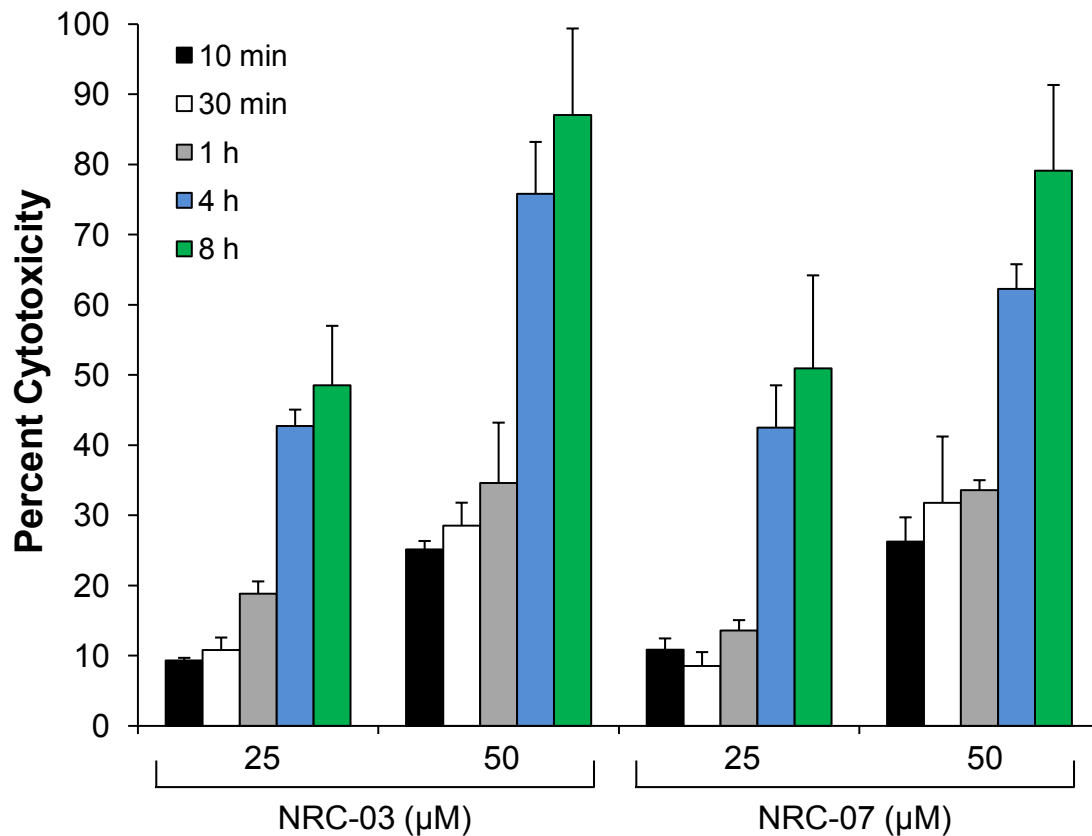
Figure 3.11. NRC-03 and NRC-07 induce cell lysis that peaks at 4 h. MDA-MB-231 breast cancer cells were cultured in the presence or absence of the indicated concentrations of NRC-03 or NRC-07. Relative cytotoxicity was measured by the LDH-release assay after 10 min, 30 min, 1 h, 4 h and 8 h. Data shown represent the mean of 3 independent ± SE.
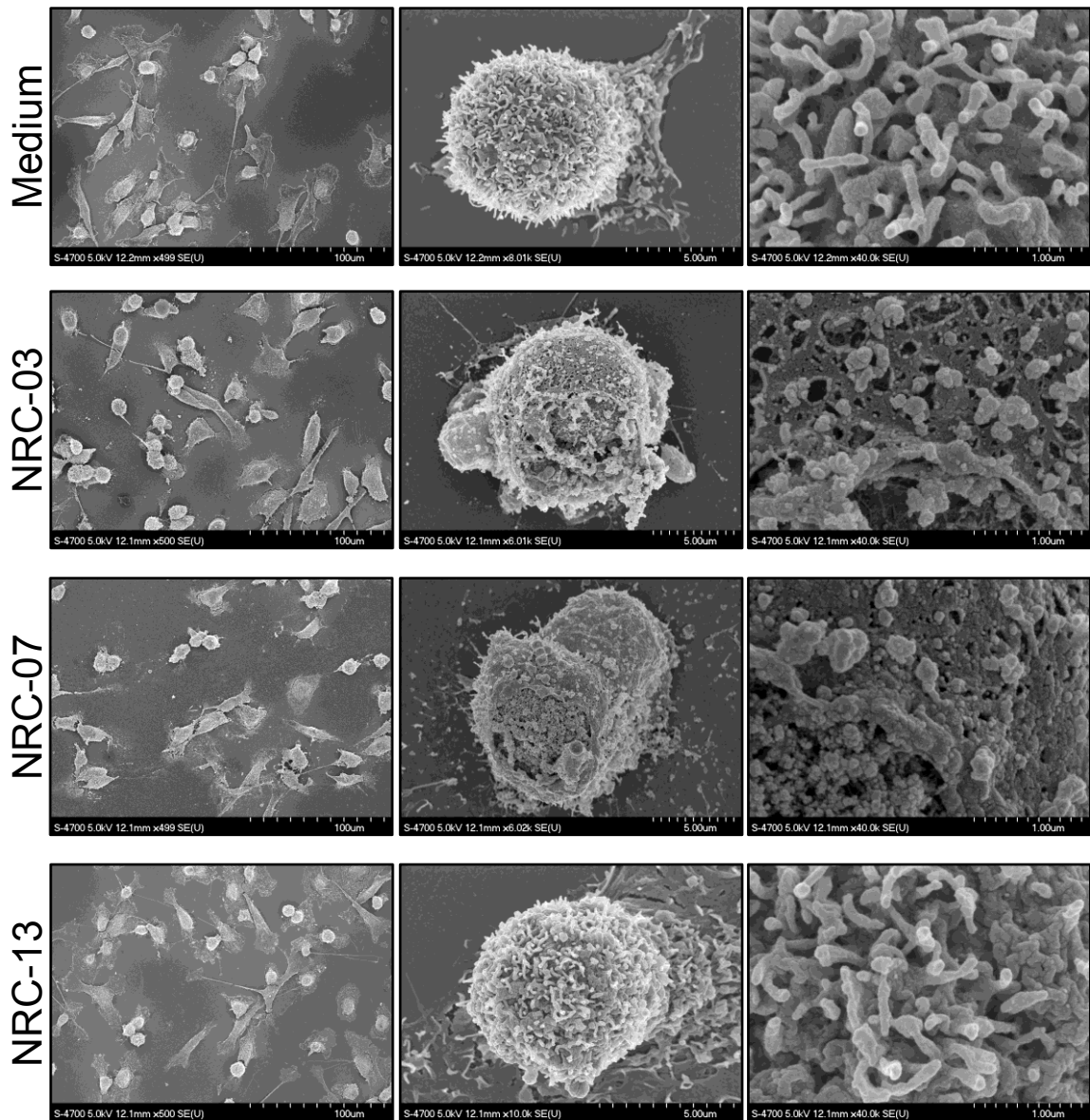
Figure 3.12. NRC-03 and NRC-07 cause extensive damage to breast cancer cells.
MDA-MB-231 breast cancer cells were cultured in the presence or absence of 50 µM
NRC-03, NRC-07, or NRC-13 for 10 min. Membrane damage was visualized by
SEM. Data shown are representative images of 2 independent experiments.
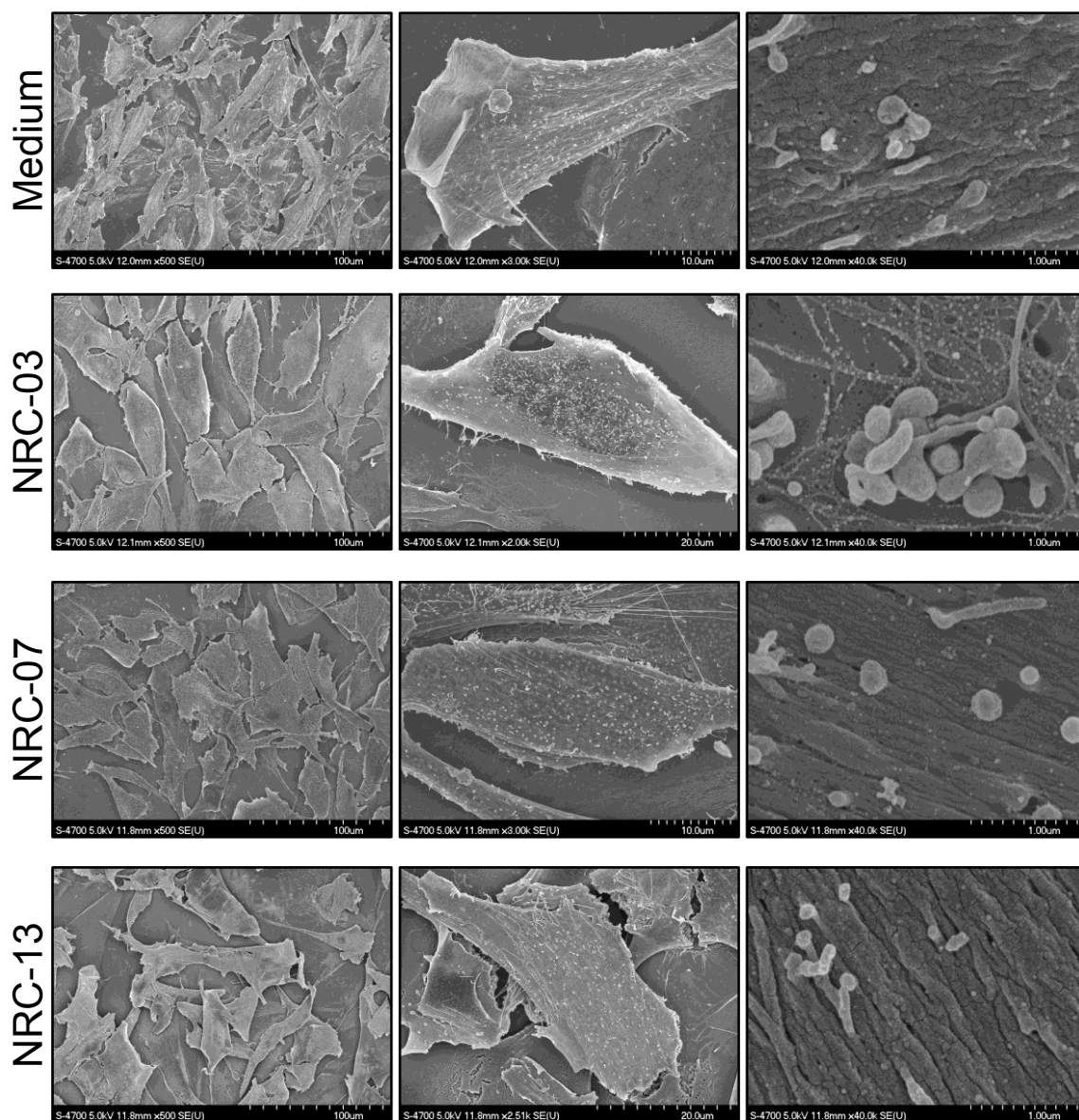
Figure 3.13. NRC-03 and NRC-07 do not damage HDFs. HDFs were cultured in the presence or absence of 50 μM NRC-03, NRC-07, or NRC-13 for 10 min. Membrane damage was visualized by SEM. Data shown are from a representative experiment (n = 2).

3.6 NRC-03 and NRC-07 Kill Breast Cancer Xenografts

A xenograft model was used to determine whether NRC-03 or NRC-07 possessed antitumour properties *in vivo*. MDA-MB-231 cells were grown in the hind flanks of NOD SCID mice and the resulting tumours were injected with the HBSS vehicle, or with 500 μg NRC-03 or NRC-07 on d 1, 3, and 5, after tumours reached a volume >120 mm^3 (d 1). Whereas HBSS-treated tumours grew significantly beyond their starting volume ($p <$ 0.05), NRC-03- and NRC-07-treated tumours were not significantly larger at the end of the experiment; however, only NRC-03-treated tumours were significantly smaller ($p <$ 0.05) than HBSS-treated tumours (Fig. 3.14A). NRC-03- and NRC-07-treated tumours were visibly smaller, both *in situ* (Fig. 3.14B) and after excision (Fig. 3.14C). Microscopic analysis of tumour sections stained with hematoxylin and eosin showed that NRC-03- and NRC-07-treated tumours had larger areas of necrosis than HBSS-treated tumours (Fig. 3.14D). Although not quantified, the number of infiltrating leukocytes appeared comparable between HBSS-, NRC-03-, and NRC-07-treated tumours. Necropsies showed the absence of metastasis in all mice with the exception of 1 HBSS-treated mouse, which developed a metastatic nodule in a regional lymph node. Major organs (lungs, kidneys, stomach, large and small intestine, liver, and reproductive organs) from all animals appeared healthy, with the exception of 1 NRC-03-treated mouse that appeared to have had tumour cells injected into the kidney, and several mice from all treatment groups that appeared to have vascularized oviducts. Tumours from NRC-03- and NRC-07-treated mice were no more ulcerated than those from control-treated mice, and there was no evidence of weight loss in any treatment group throughout the course of the experiment.

3.7 NRC-03 and NRC-07 Kill Human Multiple Myeloma Cells by a Mechanism that Involves Significant Membrane Damage and DNA Fragmentation

To determine whether the cytotoxic properties of NRC-03 and/or NRC-07 are unique to breast cancer cells, NRC-03- and NRC-07-induced cell killing was also investigated in KMS11 and U266 human multiple myeloma cell lines. Figure 3.15 shows that NRC-03 and NRC-07 killed KMS11 and U266 myeloma cells in a dose-dependent

A.

B.

C.

D.
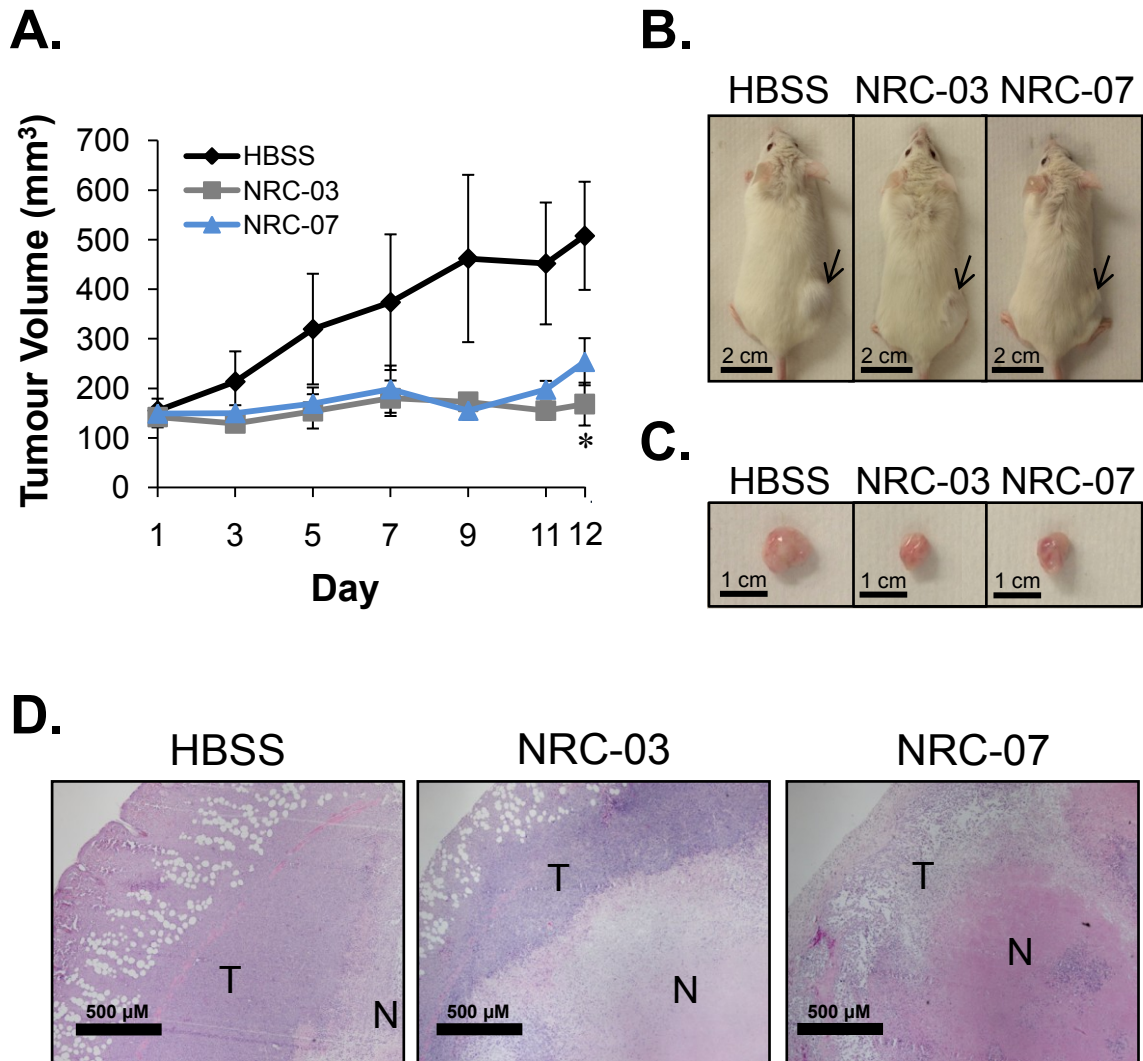
Figure 3.14. NRC-03 and NRC-07 kill breast cancer cells *in vivo*. MDA-MB-231 breast cancer cells grown in the hind flanks of NOD SCID mice formed tumours that were injected intratumorally with the HBSS vehicle control, or with 500 μg NRC-03 or NRC-07 on days 1, 3, and 5. (A) Caliper measurements were recorded on days 1, 3, 5, 7, 9, 11, and 12 to determine tumour volume. Data shown represent the mean of 3 independent experiments ± SE. (B) On day 12, the animals were sacrificed and photographed and (C) the tumours were excised and photographed. (D) Tumour sections were stained with hemotoxylin and eosin and photographed. Images shown are from representative animals (n = 9). T: tumour. N: necrotic.
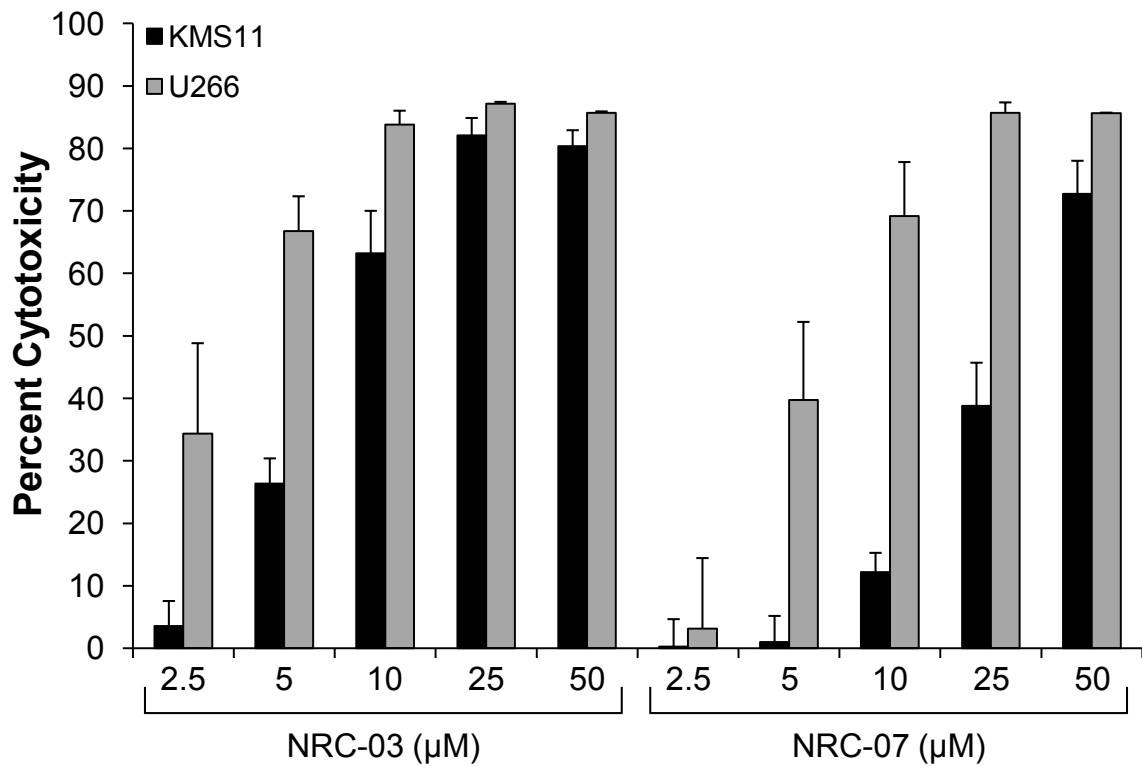
Figure 3.15. NRC-03 and NRC-07 kill human myeloma cell lines. KMS11 and U266 human myeloma cells were grown in the presence or absence of the indicated concentrations of NRC-03 or NRC-07. Cell viability was determined by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE.

manner. Whereas NRC-03 and NRC-07 were equally potent between breast cancer cell lines (Fig. 3.1B-C), NRC-03 was more potent than NRC-07 in both U266 and KMS11 myeloma cells. KMS11 cells were least susceptible to NRC-03 and NRC-07, and showed greater differences between NRC-03- and NRC-07-induced cell killing; therefore, U266 cells were subsequently used as a model cell line. A concentration of 50 μM NRC-03 and NRC-07 was used in subsequent experiments to compare peptide killing of U266 myeloma cells to MDA-MB-231 breast cancer cells.

NRC-03 and NRC-07 killed breast cancer cells by cell lysis. Consistent with this finding, PI uptake studies showed that NRC-03 and NRC-07 caused significant membrane damage in U266 cells after only 10 min (Fig. 3.16A). SEM was then used to visualize the extent of NRC-03- and NRC-07-induced membrane damage (Fig. 3.16B). Low magnification images show a reduction in myeloma cell number after 10 min exposure to NRC-03 or NRC-07. Large cellular fragments and cellular debris covered the majority of the coverslip. Images taken under intermediate magnification showed that medium-treated cells lacked membrane damage and had an abundance of microvilli, whereas membranes of NRC-03- and NRC-07-treated cells were significantly damaged, showing organelles and/or exposed cytoskeleton, which was consistent with findings in breast cancer cells. Peptide-treated U226 cells possessed reduced numbers of microvilli and membranes blebs. High magnification images showed damaged microvilli on NRC-03- and NRC-07-treated cells, and the presence of a stringy molecule extruded from certain cells (e.g., NRC-07-treated cells, indicated with a white arrow).

Confocal microscopy showed that both biotin-NRC-03 and -NRC-07 enter U266 myeloma cells within 30 sec (Fig. 3.17). Interestingly, certain peptide-treated cells were not encircled by an actin cytoskeleton (indicated with blue arrows), while other cells appeared to extrude a peptide-bound substance from the cell (indicated with white arrows). TUNEL staining showed that NRC-07 fragmented DNA in U266 cells, which was consistent with findings in breast cancer cells; however, NRC-03 also caused DNA fragmentation in U266 cells, albeit to a far lesser extent than NRC-07 (Fig. 3.18). Collectively, these data suggest that NRC-03 and NRC-07 kill cancer cells of epithelial and hematopoietic origin by a similar mechanism of action.

A.

B.
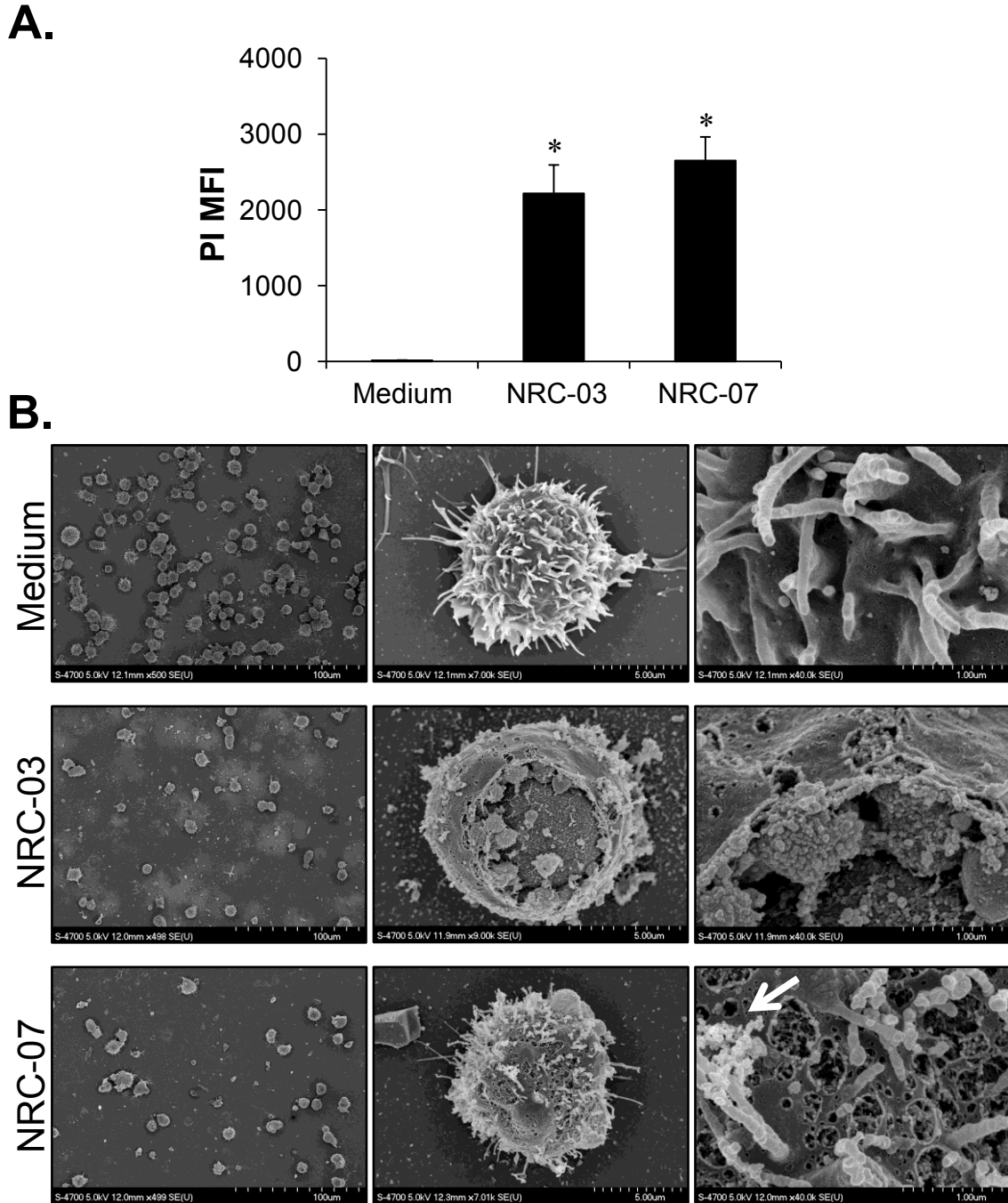
Figure 3.16. NRC-03 and NRC-07 cause extensive damage to the cell membrane of myeloma cells. (A) U266 myeloma cells were cultured in the presence or absence of 50 μM NRC-03 or NRC-07 for 10 min. PI was added to cells to detect membrane damage. Data shown represent the average MFI of 3 independent experiments ± SE, and are statistically significant by the Bonferroni multiple comparison test in comparison to medium-treated cells; * $p < 0.05$. (B) U266 myeloma cells were cultured in the presence or absence of 50 μM NRC-03 or NRC-07 for 30 min. Membrane damage was visualized by SEM. Data shown are from a representative experiment (n = 2).
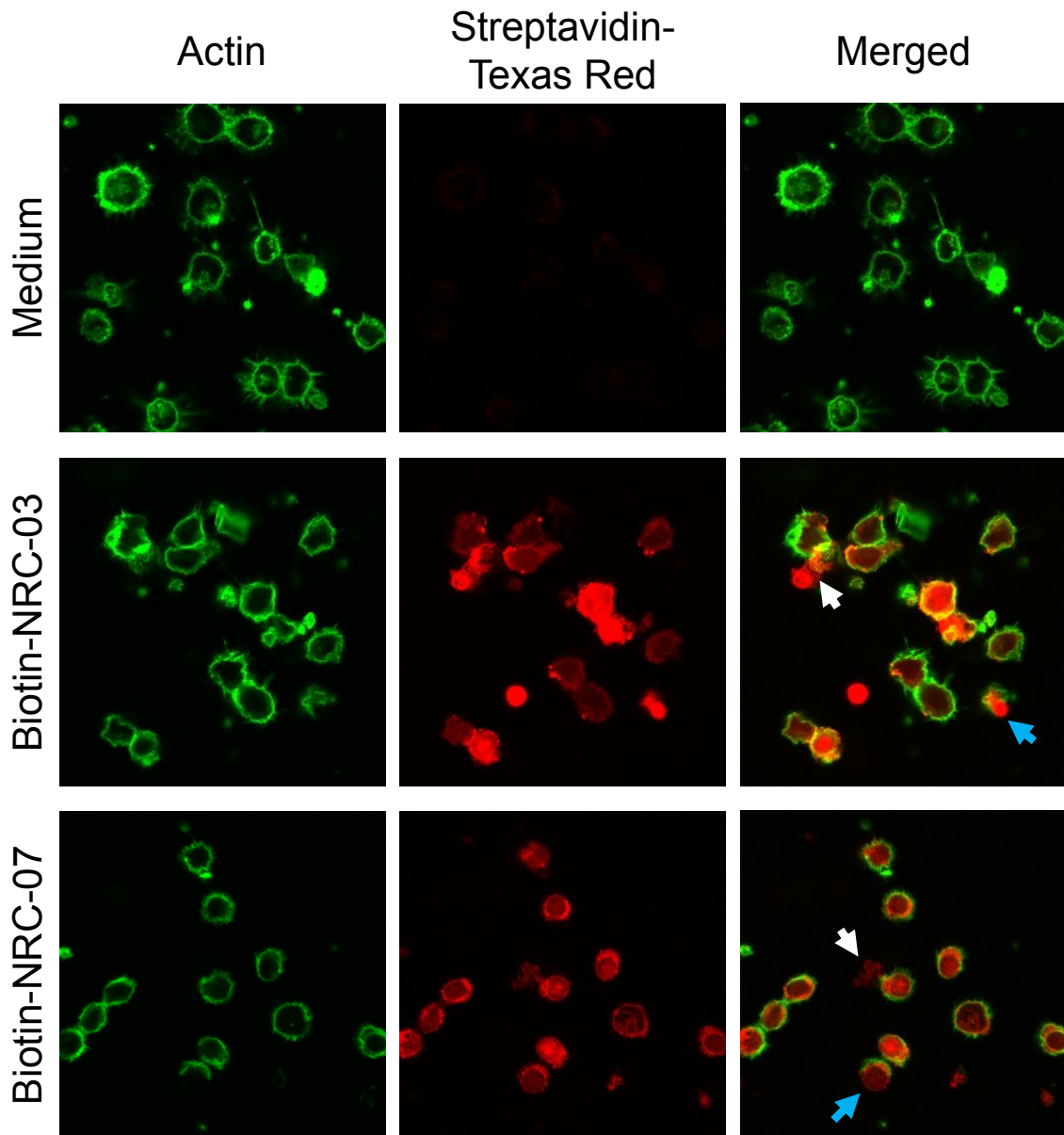
Figure 3.17. NRC-03 and NRC-07 rapidly enter myeloma cells. U266 cells were cultured in the presence or absence of 50 μM biotin-NRC-03, or -NRC-07 for 30 sec. NRC-03, NRC-07, and the actin cytoskeleton were visualized by confocal microscopy through the addition of Texas Red-conjugated streptavidin, and Alexa-Fluor 488-phalloidin, respectively (1000 ×). Data shown are from a representative experiment (n = 3). White arrows indicate peptide-bound molecules leaking from treated cells; blue arrows indicate cells lacking a complete cytoskeleton.
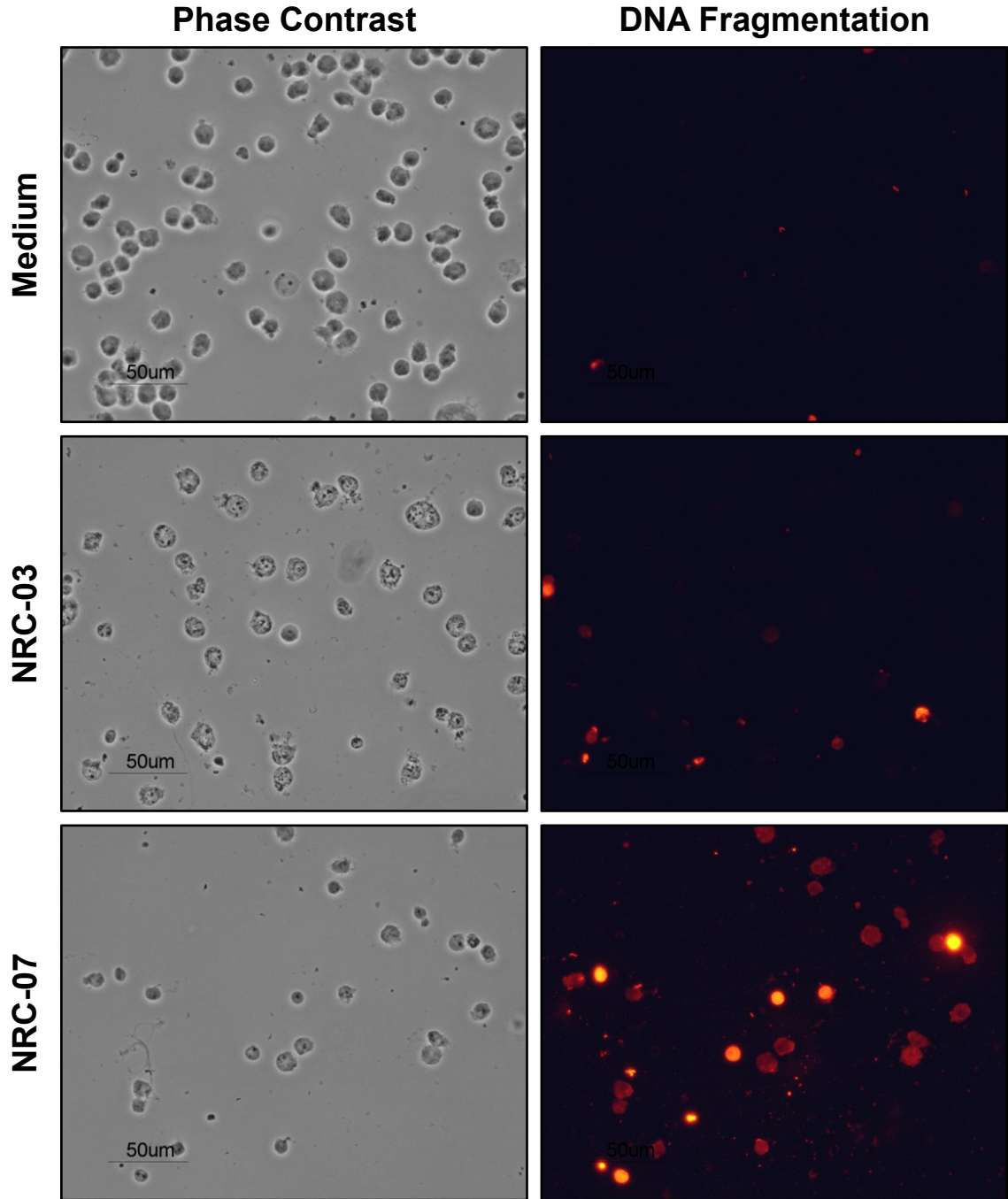
Figure 3.18. NRC-07 and NRC-03 cause DNA fragmentation in human myeloma cells. U266 cells were cultured in the presence or absence of 50 µM NRC-03 or NRC-07 for 30 min. DNA fragmentation detected by TUNEL assay was visualized by fluorescence microscopy. Data shown are from a representative experiment (n = 3).

CHAPTER 4

[D]-NRC-03 IS A POTENT CYTOTOXIC DERIVATIVE OF NRC-03

4.1 Cell Killing by NRC-03, but not NRC-07, is Reduced in Increasing Concentrations of FBS

The majority of research evaluating CAPs as novel anticancer agents is conducted in low serum concentrations because CAP-induced cytotoxicity is often reduced in the presence of serum (119, 126, 179). Serum-dependent decreases in CAP-mediated cell killing are thought to be due to either peptide degradation by serum proteases or peptide sequestering by soluble anionic molecules such as albumin. To determine whether NRC-03- or NRC-07-induced cell killing was reduced in the presence of FBS, MDA-MB-231 cells were cultured in increasing concentrations of FBS in the absence or presence of 5, 10, 25, or 50 µM NRC-03 or NRC-07 for 24 h. Increasing concentrations of FBS inhibited NRC-03 killing of MDA-MB-231 breast cancer cells in a dose-dependent manner (Fig. 4.1). Although NRC-07-mediated cell death was consistently reduced in increasing concentrations of FBS, the results were not significantly different at any concentration of NRC-07 tested. In order to establish whether the reduction in NRC-03- and NRC-07-induced cell killing was a consequence of the peptides being sequestered by soluble anionic molecules, MDA-MB-231 cells were pretreated with 2.5% BSA prior to their exposure to NRC-03 or NRC-07. Interestingly, NRC-03- and NRC-07-mediated cell death was significantly reduced by exogenous BSA at 4 h, but not 24 h (Fig. 4.2A). To confirm that this effect was not due to BSA degradation over time, supernatants were collected from cells at 0 and 24 h, and albumin was detected by Coomassie blue staining. As shown in Figure 4.2B, BSA levels did not decrease at 24 h compared to 0 h. These data suggest that the FBS-mediated decrease in NRC-03-induced cell death was not a consequence of NRC-03 being sequestered by anionic molecules.

4.2 NRC-03 is Degraded by Proteases in HS and MS

Both NRC-03 and NRC-07 are degraded by trypsin (Appendix Fig. 4). To investigate whether NRC-03 or NRC-07 are susceptible to degradation by serum
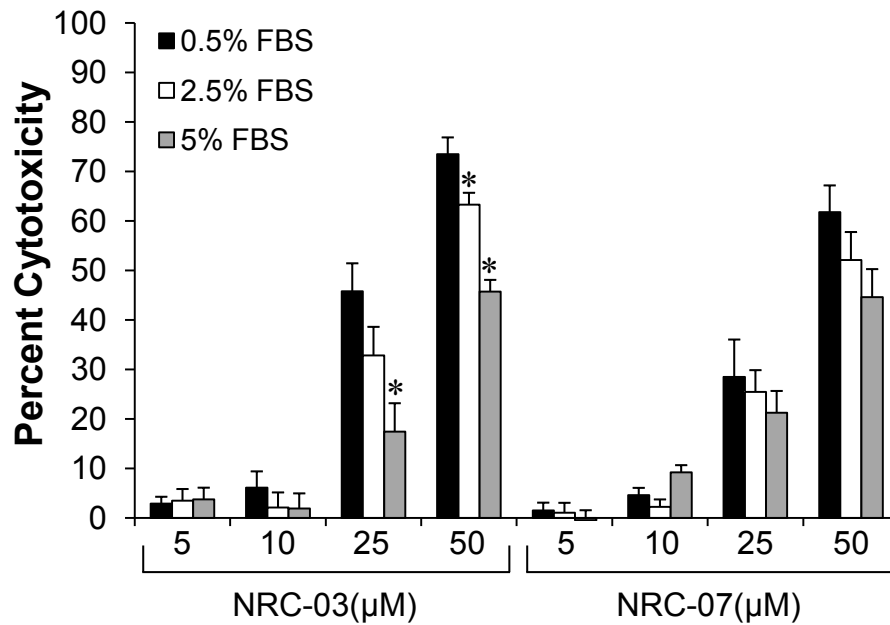
Figure 4.1. Breast cancer cell killing by NRC-03 is significantly reduced in the presence of FBS. MDA-MB-231 breast cancer cells were cultured in 0.5, 2.5, or 5% FBS in the presence or absence of the indicated concentrations of NRC-03 or NRC-07. Cell viability was assessed by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test in comparison to peptide-treated cells cultured in 0.5% FBS; * $p < 0.01$.
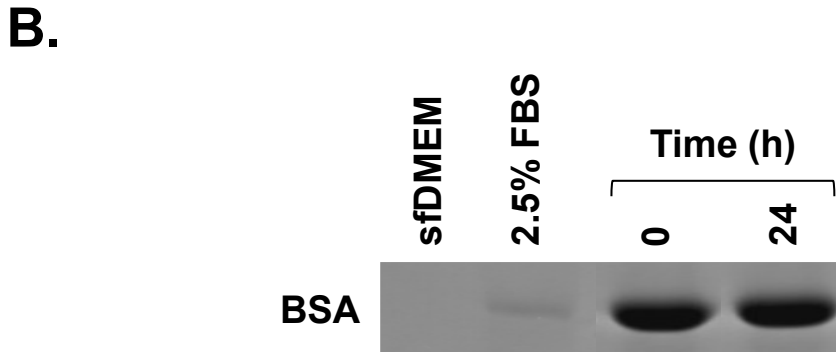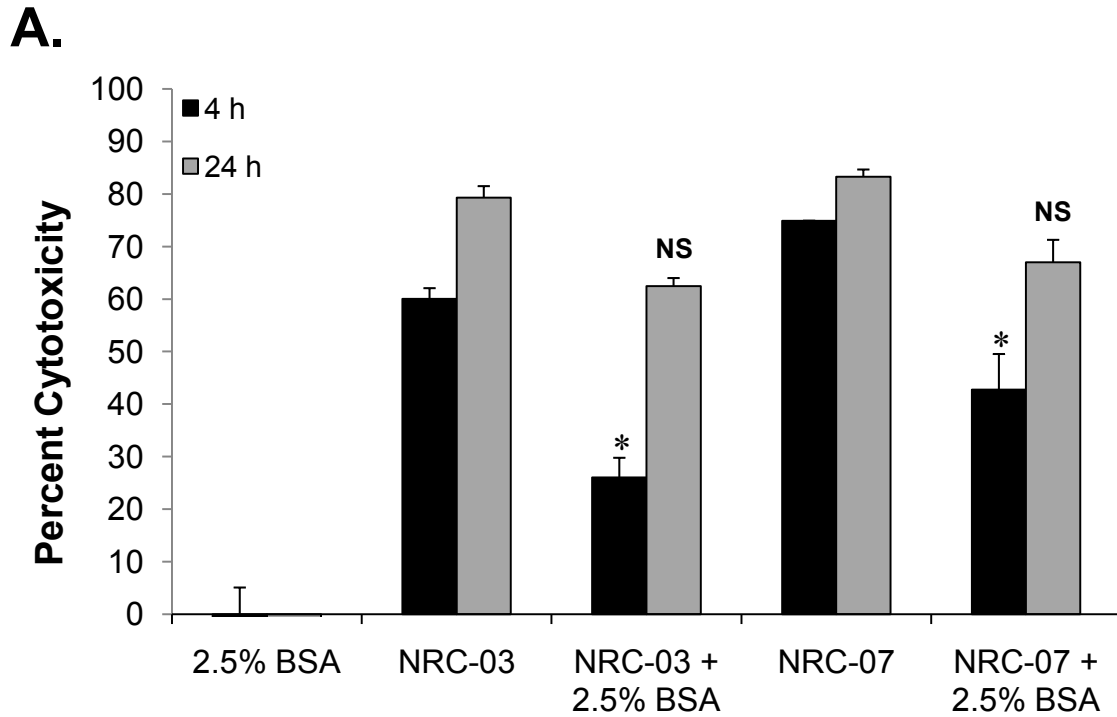
Figure 4.2. BSA reduces NRC-03- and NRC-07-induced cytotoxicity at 4, but not 24 h. (A) MDA-MB-231 breast cancer cells pretreated with 2.5% BSA were cultured in the presence or absence of 50 µM NRC-03 or NRC-07. Cell viability was determined by acid phosphatase assay after 4 and 24 h. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant at 4 h by the Bonferroni multiple comparison test in comparison to NRC-03- or NRC-07-treated cells; * $p < 0.05$. (B) Supernatants were collected, diluted, and separated on a reducing gel to evaluate BSA degradation overtime. The gel shown is from a representative experiment (n = 3).

proteases, NRC-03 or NRC-07 were incubated in sfDMEM (traditional setup) or DMEM containing FBS, MS, or HS (modified setup) for 1 h prior to their addition to MDA-MB-231 breast cancer cell cultures (Fig. 4.3). A final concentration of 5% serum and 50 μM NRC-03 or NRC-07 was achieved in all samples. NRC-03- and NRC-07-induced cytotoxicity in cells cultured under the modified experimental setup (i.e., NRC-03 and NRC-07 incubated with DMEM containing FBS, MS, or HS before exposure to MDA-MB-231 cells [in sfDMEM]) was compared to NRC-03- and NRC-07-induced cytotoxicity in cells cultured under the traditional experimental setup (i.e., NRC-03 and NRC-07 incubated with sfDMEM prior to exposure to MDA-MB-231 cells [in serum-containing medium]). Surprisingly, prior exposure of NRC-03 to FBS did not affect subsequent NRC-03-mediated cell killing, whereas MS treatment significantly reduced NRC-03-induced cell killing; however the effect was marginal. In contrast, HS treatment significantly inhibited cell killing by NRC-03. Interestingly, pretreatment of NRC-07 with FBS, MS or HS did not affect subsequent NRC-07-induced killing of MDA-MB-231 cells.

To determine whether NRC-03 was degraded in the presence of MS or HS, biotin-NRC-03 was incubated in the presence or absence of FBS, FBS that was not heat-inactivated (cFBS), HS, or MS for 30 min. Complete degradation of biotin-NRC-03 was achieved by incubating biotin-NRC-03 in trypsin. Samples were then separated on a tricine gel, and biotin-NRC-03 was detected using streptavidin-HRP. Biotin-NRC-03 was degraded in HS, and to a lesser extent MS, but not FBS or cFBS (Fig. 4.4A). Densitometry was used to calculate biotin-NRC-03 degradation relative to undigested biotin-NRC-03. These data show that biotin-NRC-03 is degraded in HS and MS, but not FBS.

4.3 [D]-NRC-03 is More Cytolytic than NRC-03 and has Delayed Kinetics

[D]-NRC-03 is a peptidomimetic of NRC-03 in which all L-aa are replaced with D-aa, making [D]-NRC-03 the enantiomer of NRC-03. Certain enantiomeric peptides, which are resistant to proteolytic degradation, retain their biological activities (149). The purpose of this study was to determine whether [D]-NRC-03 retains cytotoxicity for breast cancer cells, and whether it kills cells by the same mechanism of action as native
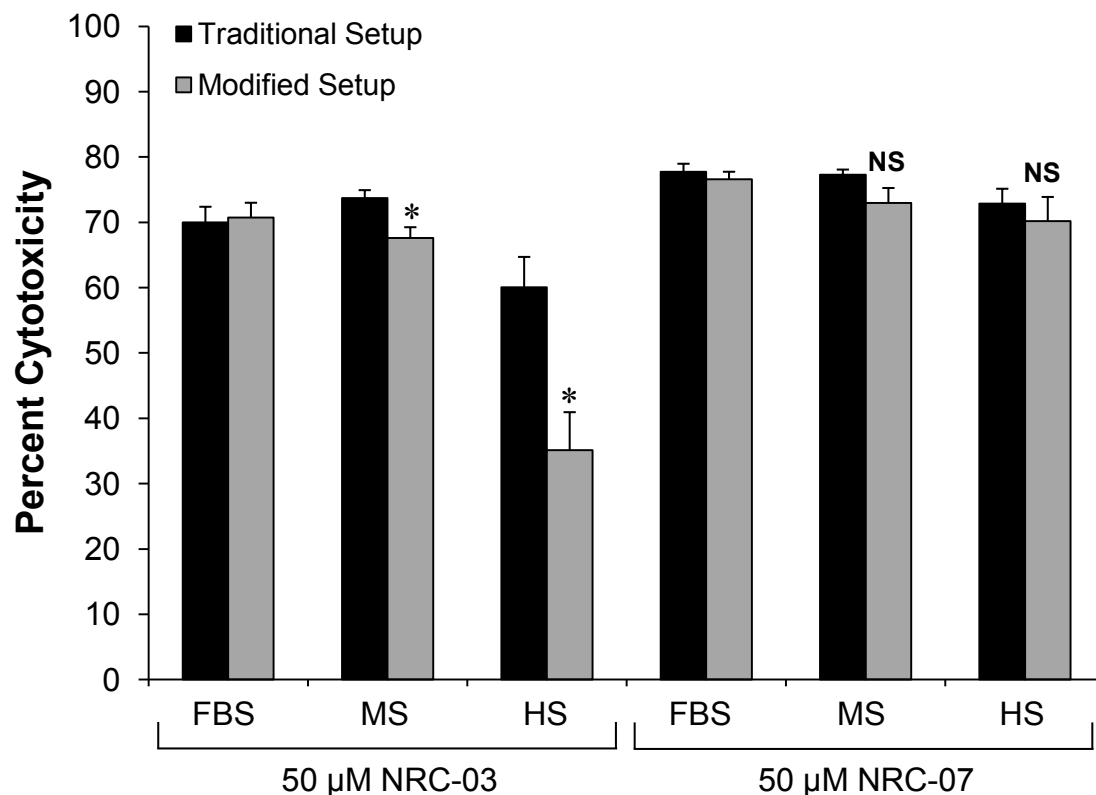
Figure 4.3. Cell killing by NRC-03, but not NRC-07, is reduced when NRC-03 is pretreated with HS or MS, but not FBS. 100 μM NRC-03 and NRC-07 were incubated with sfDMEM for 1 h at 37°C prior to exposure to MDA-MB-231 cells containing 10% FBS, MS, or HS (traditional setup). Alternatively, 100 μM NRC-03 and NRC-07 were incubated with 10% FBS, MS, or HS for 1 h at 37°C prior to exposure to MDA-MB-231 cells containing sfDMEM (modified setup). A final concentration of 5% serum and 50 μM NRC-03 and NRC-07 was attained. Cell viability was assessed by MTT assay after 24 h. Data shown represent the mean of 4 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test in comparison to cells treated under traditional conditions; * $p < 0.05$.

A.
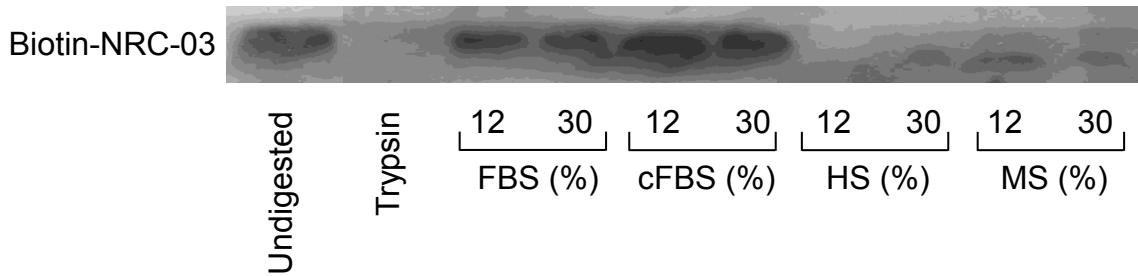
B.
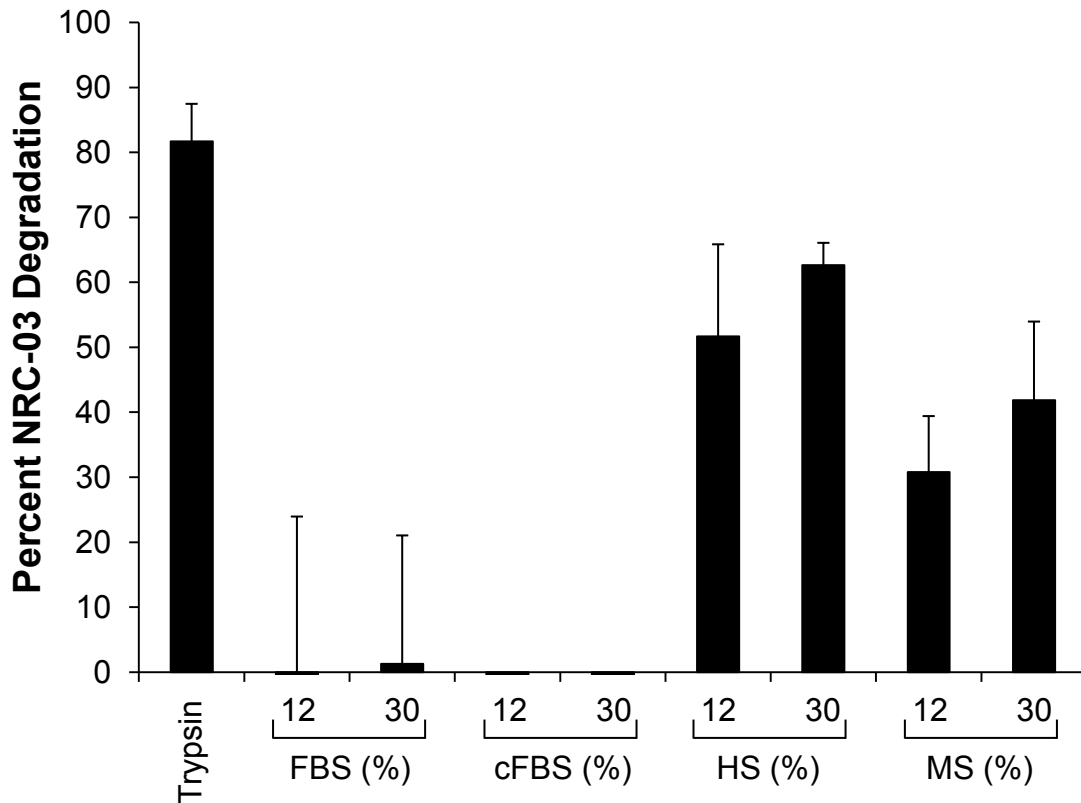
Figure 4.4. NRC-03 is degraded in HS and MS. 30 μg biotin-NRC-03 was incubated for 30 min under the indicated conditions at 37°C. (A) The resulting digests were separated on a 16.5% tricine gel. Biotinylated-NRC-03 was detected by ECL using streptavidin-HRP. Data shown are from a representative experiment (n=3). (B) Pixel intensity was calculated to quantify NRC-03 degradation. Data shown represent the mean of 3 independent experiments ± SE.

NRC-03. Like NRC-03, [D]-NRC-03 killed MDA-MB-231, MCF7, SKBR3, T47D, 4T1 and MDA-MB-468 breast cancer cells in a dose-dependent manner at 24 h (Fig. 4.5). Interestingly, [D]-NRC-03 appeared to be more potent than NRC-03 with doses as low as 5 µM being cytotoxic to T47D, 4T1 and MDA-MB-468, whereas 10 µM NRC-03 was required to achieve similar levels of cell killing in the same cell lines (Fig. 4.5). For subsequent experiments, 25 µM was chosen as a representative concentration of NRC-03 and [D]-NRC-03 because [D]-NRC-03-induced cell killing peaked at 25 µM in all breast cancer cell lines tested.

LDH-release assays showed that while both NRC-03 and [D]-NRC-03 induce time-dependent cell lysis, NRC-03-mediated cell killing peaked at 4 h, whereas [D]-NRC-03-mediated cell killing peaked at 8 h (Fig. 4.6). Consistent with the findings from the MTT assays (Fig. 4.5), [D]-NRC-03 caused greater cell lysis than did NRC-03, suggesting that [D]-NRC-03 is more potent than NRC-03.

To determine whether [D]-NRC-03-induced cell killing is reduced by increasing concentrations of FBS, as is the case with NRC-03, an MTT assay was conducted in which MDA-MB-231 cells were incubated in increasing concentrations of FBS prior to exposure to NRC-03 or [D]-NRC-03. Unlike NRC-03-induced cytotoxicity, [D]-NRC-03-mediated cell killing was not affected by FBS (Fig. 4.7). Mass spectrometry analysis confirmed that unlike NRC-03 and NRC-07, [D]-NRC-03 was not degraded by trypsin (Appendix Fig. 4).

The capacity of [D]-NRC-03 to kill chemoresistant MCF7-TX400 breast cancer cells was next evaluated. Clonogenic assays showed that 25 µM [D]-NRC-03 significantly reduced colony formation after only 4 h treatment, despite peak cytotoxicity being achieved at 8 h. Consistent with previous findings, colonies from peptide-treated cells were smaller than colonies from medium-treated cells. Furthermore, colonies from [D]-NRC-03-treated cells were smaller than those from NRC-03-treated cells (Fig. 4.8A). Colony formation was significantly reduced in [D]-NRC-03-treated samples in comparison to medium-treated MCF7 and MCF7-TX400 cells (Fig. 4.8B). Interestingly, the number of MCF7-TX400 CFUs following exposure to [D]-NRC-03 was significantly less than the number of MCF7 CFUs following exposure to [D]-NRC-03 (p=0.0266).
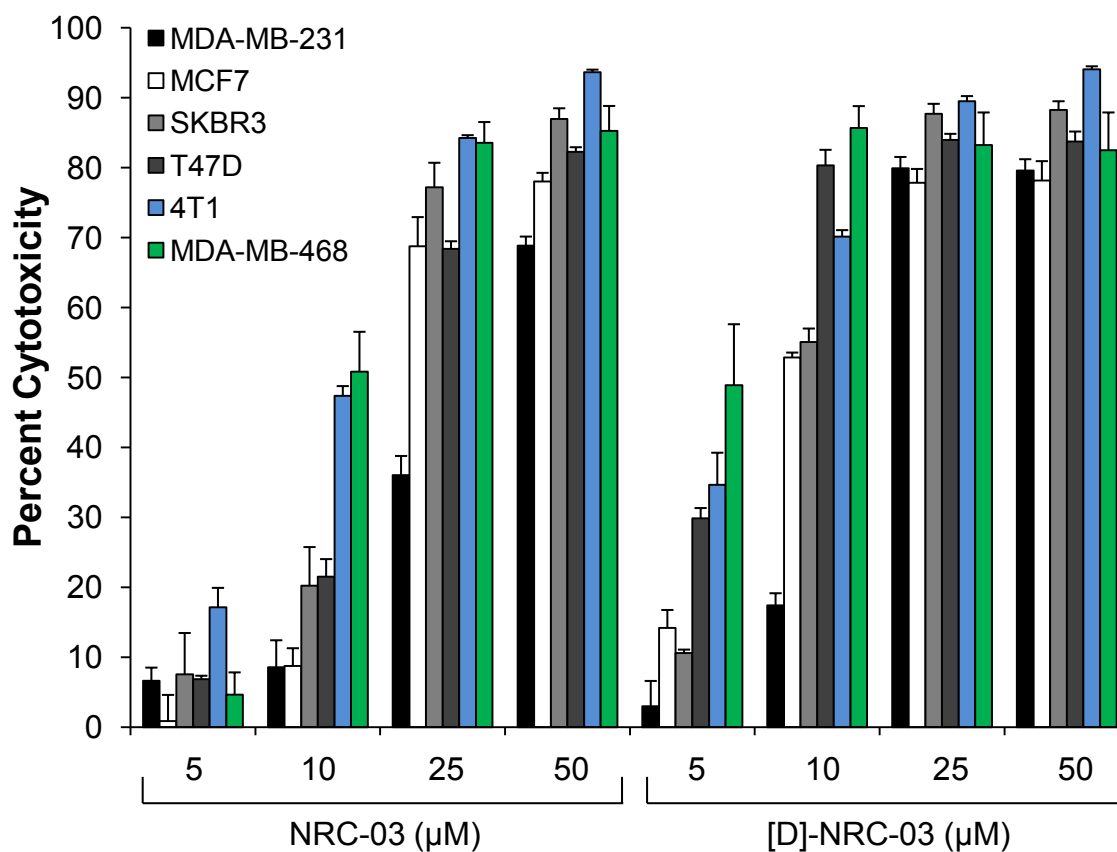
Figure 4.5. [D]-NRC-03 kills human breast cancer cells in a dose-dependent manner. MDA-MB-231, MCF7, SKBR3, T47D or MDA-MB-468 human breast cancer cells, or 4T1 murine mammary carcinoma cells were exposed to medium alone or the indicated concentrations of NRC-03 or [D]-NRC-03. Cell viability was determined by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE.
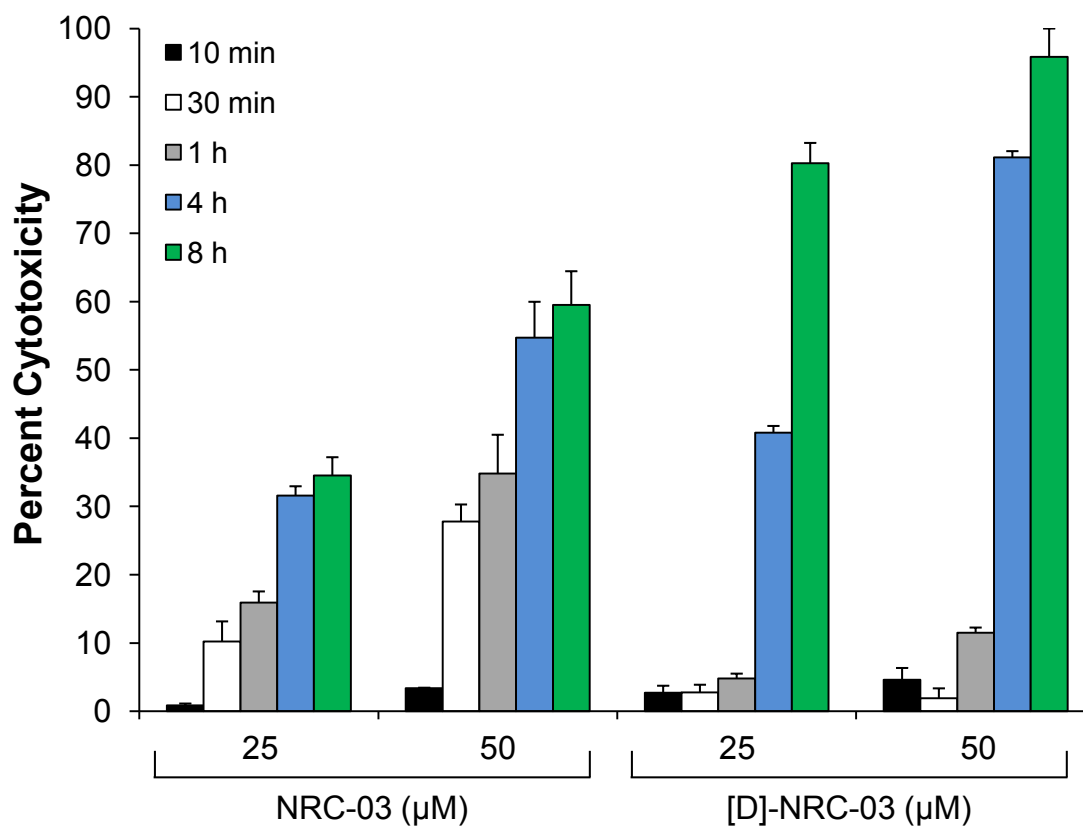
Figure 4.6. [D]-NRC-03 induces time-dependent cytolysis that peaks at 8 h. MDA-MB-231 cells were cultured in the presence or absence of the indicated concentrations of NRC-03 and [D]-NRC-03. Relative cytotoxicity was measured by the LDH-release assay after 10 min, 30 min, 1 h, 4 h and 8 h. Data shown represent the mean of 3 independent ± SE.
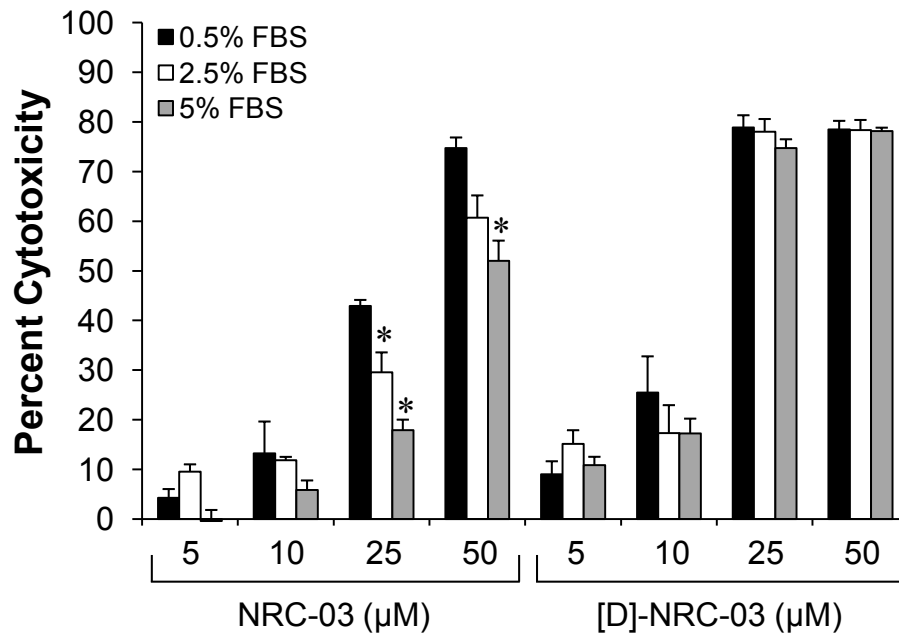
Figure 4.7. Cell killing by [D]-NRC-03 is not reduced by increasing concentrations of FBS. MDA-MB-231 breast cancer cells were cultured in 0.5, 2.5, or 5% FBS in the presence or absence of the indicated concentrations of NRC-03 or [D]-NRC-03. Cell viability was assessed by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test in comparison to NRC-03-treated cells cultured in 0.5% FBS; * $p < 0.01$.
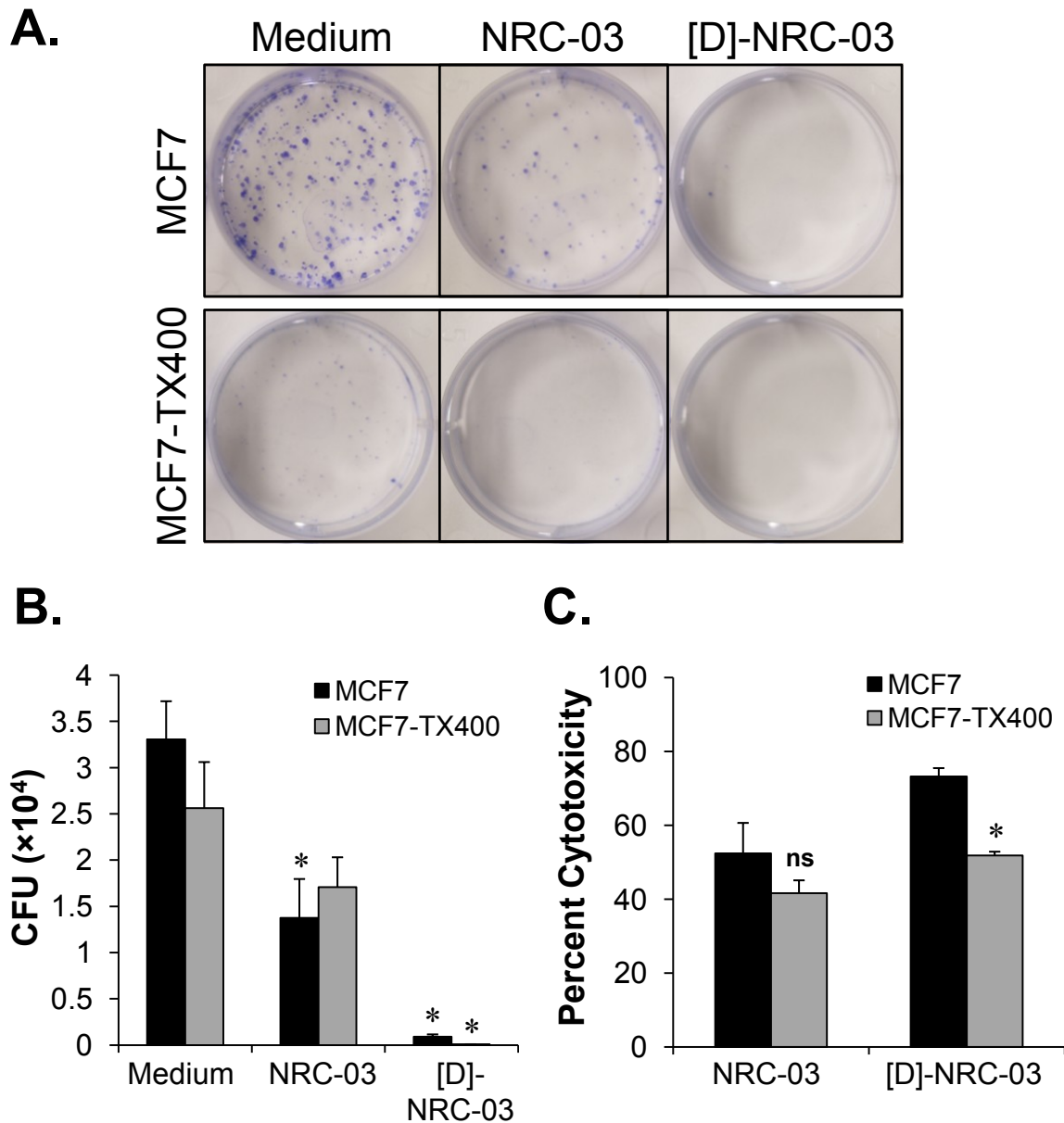
Figure 4.8. [D]-NRC-03 kills multidrug-resistant breast cancer cells. (A) MCF7 and MCF7-TX400 breast cancer cells were cultured in the presence or absence of 25 μM NRC-03 or [D]-NRC-03. After 4 h, the cells were diluted and cultured for an additional 10 d. (A) Representative images of medium-, NRC-03- and [D]-NRC-03-treated cells (800× dilution) are shown. (B) Colony formation by medium-, NRC-03-, and [D]-NRC-03-treated cells was determined by clonogenic assay. Data shown are statistically significant by the Bonferroni multiple comparison test in comparison to medium-treated cells; * $p < 0.05$. (C) MCF7 and MCF7-TX400 cells were cultured in the presence or absence of 25 μM NRC-03 or [D]-NRC-03. Cell viability was determined by acid phosphatase assay after 4 h. Data shown are statistically significant by the two-tailed Student's t test in comparison to MCF7-treated cells; * $p < 0.05$. All data shown represent the mean of 3 independent experiments ± SE.

Colony formation in NRC-03-treated samples was significantly reduced in comparison to medium-treated samples in MCF7, but not MCF7-TX400 cells, which is in contrast to experiments conducted with 50 μM NRC-03 (Fig. 3.2 vs. 4.8). Confirmatory acid phosphatase assays were conducted in parallel with clonogenic assays. Like the clonogenic assay, there was not a significant difference between NRC-03-induced killing of MCF7 and MCF7-TX400 cells (Fig. 4.8C). Paradoxically, the acid phosphatase assay suggested that [D]-NRC-03 was significantly more potent toward MCF7 cells than MCF7-TX400 cells.

Finally, the cytotoxic effect of [D]-NRC-03 on normal cells was compared to normal cell killing by NRC-03 to determine whether [D]-NRC-03 was more or less selective for cancer cells than NRC-03. Importantly, neither NRC-03 nor [D]-NRC-03 were hemolytic. Due to the limited lifespan of HMECs, HUVECs and HDFs, new cultures of each cell type had to be acquired for this experiment. Surprisingly, whereas NRC-03 was as toxic to HUVECs as it was in the experiment that evaluated NRC-03- and NRC-07-induced killing of normal cells, NRC-03 was significantly more toxic to HMECs and HDFs in this experiment (compare tables 3.1 and 4.1). Moreover, [D]-NRC-03 was more toxic to HMECs and HDFs than was NRC-03.

SEM was used to visualize changes in cellular ultrastructure following exposure to 25 μM NRC-03 or [D]-NRC-03 for 10 min, 1 h, and 4 h. As in previous experiments, the membranes of medium-treated breast cancer cells were completely intact with an abundance of smooth microvilli (Fig. 4.9A-C). After 10 min, NRC-03-treated cells possessed fewer microvilli that appeared to be detached from the cell exposing the cell membrane, which was minimally damaged (Fig. 4.9A). Interestingly, [D]-NRC-03-treated cells showed an intermediate number of microvilli, an exposed membrane, and the development of membrane blebs. By 1 h, many NRC-03-treated cells had completely lysed while those cells that remained intact exhibited damaged membranes (Fig. 4.9B). Interestingly, one cell appeared to repairing its cell membrane by forming membrane blebs. [D]-NRC-03-treated cells appeared more damaged at 1 h than at 10 min. Microvilli numbers appeared to be further reduced and/or truncated, and membrane blebs appeared greater in size than they did at 10 min. By 4 h the majority of NRC-03-treated cells were lysed and the remaining cells appeared significantly damaged (Fig. 4.9C). [D]-NRC-03-

Table 4.1. NRC-03- and [D]-NRC-03-induced cytotoxicity toward a panel of normal human cells compared to breast cancer cells

Treatment	% Cytotoxicity[a,c]				% Hemolysis[b,c]
	HMECs	HDFs	HUVECs	MDA-MB-231	Erythrocytes
25 µM NRC-03	47 ± 1	5 ± 2	1 ± 4	36 ± 3	3 ± 1
50 µM NRC-03	77 ± 2	21 ± 3	27 ± 3	69 ± 2	2 ± 1
25 µM [D]-NRC-03	81 ± 1	67 ± 4	20 ± 4	80 ± 2	1 ± 1
50 µM [D]-NRC-03	80 ± 1	75 ± 2	24 ± 2	80 ± 2	2 ± 1

[a]NRC-03- and [D]-NRC-03-mediated cytotoxicity toward cultures of normal cells was determined by MTT assay after 24 h.
[b]hemolytic activity toward human erythrocytes was determined by hemolysis assay after 8 h.
[c]Data shown represent the mean of 3 independent experiments ± SE.
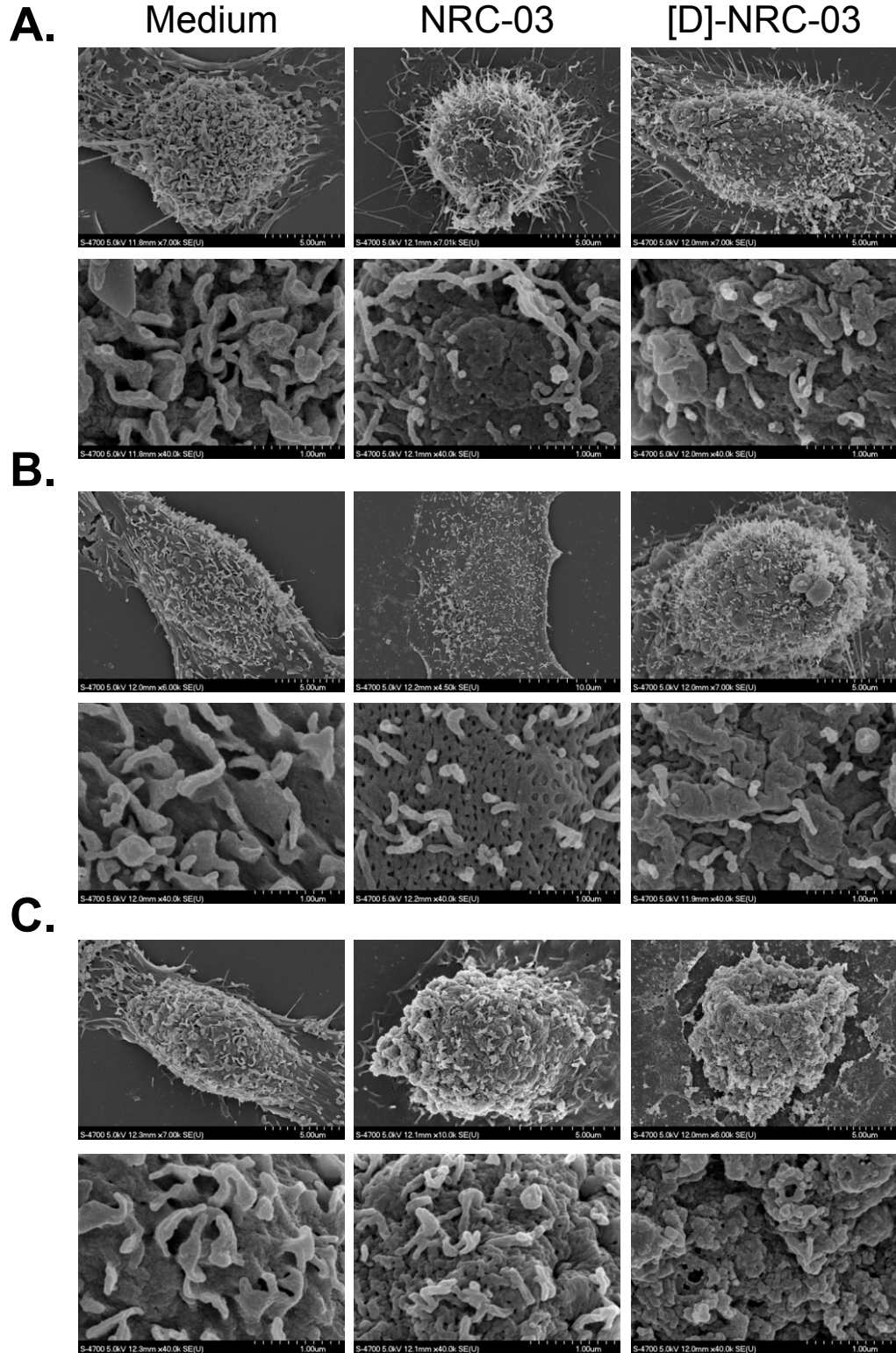
Figure 4.9. [D]-NRC-03 lyses breast cancer cells. MDA-MB-231 breast cancer cells were cultured in the presence or absence of 25 μM NRC-03 or [D]-NRC-03 for (A) 10 min, (B) 1 h, or (C) 4 h. Membrane damage was visualized by SEM. Data shown are from a representative experiment (n = 2).

treated cells appeared lysed and cellular attachment fibers were detached from the cell, which caused many cells to lift from the coverslip.

4.4 [D]-NRC-03 Kills Breast Cancer Xenografts

A xenograft model was used to compare the antitumour properties of [D]-NRC-03 to NRC-03. MDA-MB-231 cells were grown in the hind flanks of NOD SCID mice as described in section 3.6 and were injected with the HBSS vehicle, 500 µg NRC-03, or 125 µg [D]-NRC-03. As shown in Figure 4.10A, NRC-03- and [D]-NRC-03-treated tumours were significantly smaller than HBSS-treated tumours at the end of the experiment (407 ± 87 mm^3, 233 ± 79 mm^3, and 219 ± 52 mm^3, respectively). Tumours from NRC-03- and [D]-NRC-03-treated mice were visibly smaller than HBSS-treated tumours both *in situ* (Fig. 4.10B) and after excision (Fig. 4.10C). Microscopic analysis of tumour sections stained with hematoxylin and eosin showed that NRC-03- and [D]-NRC-03-treated tumours had larger necrotic areas than HBSS-treated tumours (Fig. 4.10D). Consistent with previous findings, the number of infiltrating leukocytes appeared comparable between HBSS-, NRC-03-, and [D]-NRC-03-treated tumours. Necropsies showed the absence of metastasis in all mice and all major organs appeared healthy, with the exception of 1 HBSS-treated mouse that appeared to have had tumour cells injected into the kidney, and several mice from all treatment groups that had vascularized oviducts. Tumours from NRC-03- and NRC-07-treated mice were no more ulcerated than those from control-treated mice and there was no evidence of weight loss in any of the mice throughout the course of the experiment.
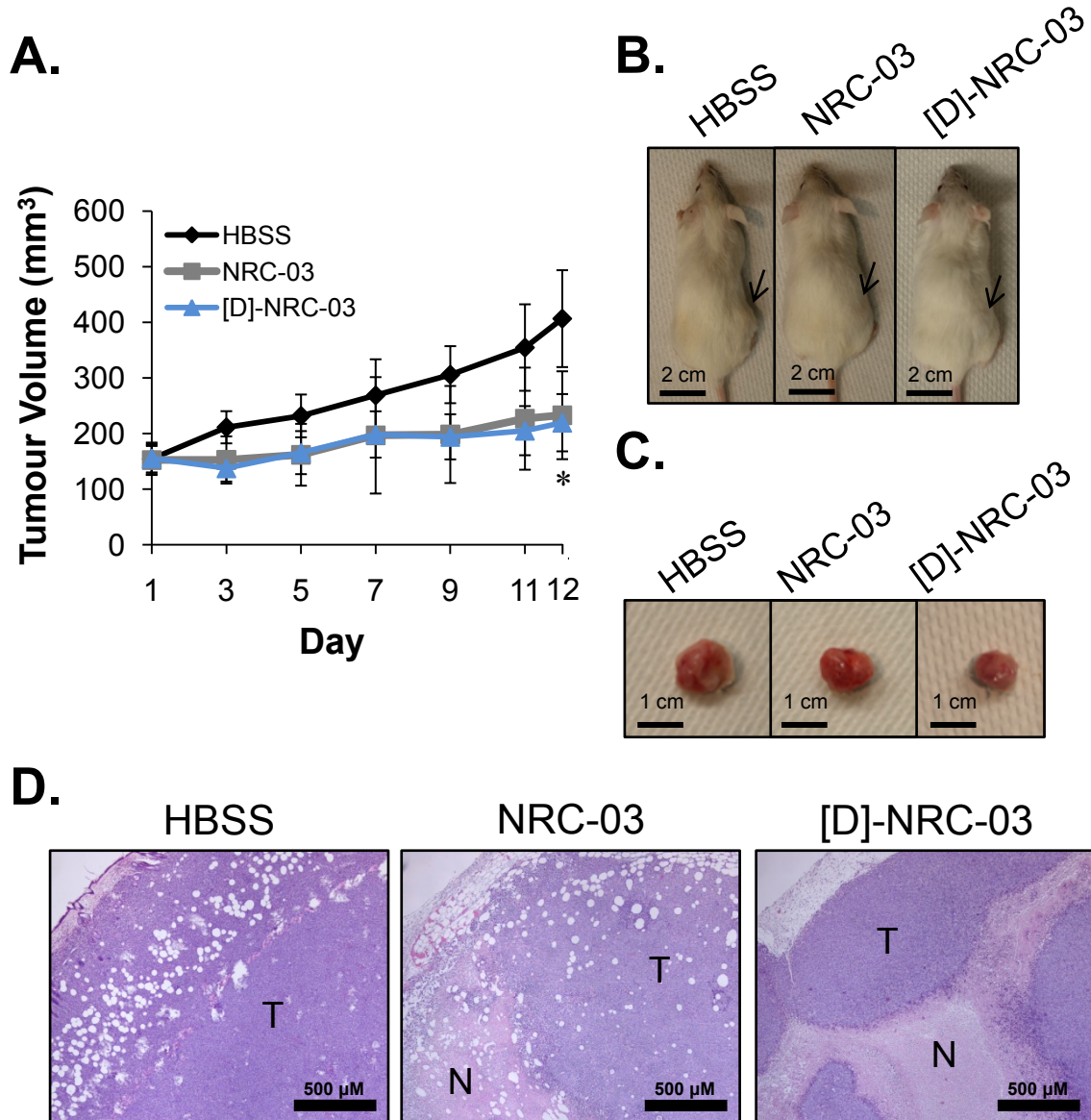
Figure 4.10. [D]-NRC-03 kills breast cancer cells *in vivo*. MDA-MB-231 breast cancer cells grown in the hind flanks of NOD SCID mice formed tumours that were injected intratumorally with the HBSS vehicle control (n=12), or with 500 µg NRC-03 (n=10) or 125 µg [D]-NRC-03 (n=11) on days 1, 3, and 5. (A) Caliper measurements were recorded on days 1, 3, 5, 7, 9, 11, and 12 to determine tumour volume. Data shown represent mean tumour volume ± SD. (B) On day 12, the animals were sacrificed and photographed and (C) the tumours were excised and photographed. (D) Tumour sections were stained with hemotoxylin and eosin and photographed. Images shown are from representative animals. T: tumour. N: necrotic.

CHAPTER 5

BREAST CANCER CELL RESISTANCE TO NRC-03 AND NRC-07

5.1 Breast Cancer Cells Can Become Resistant to NRC-03 and NRC-07 by a Mechanism that Involves Reduced Peptide Binding

One of the greatest problems with cytotoxic chemotherapies is the development of drug-resistant cancer cells (29). However, cancer cell resistance to CAPs has never been investigated. To determine whether breast cancer cells can develop resistance to CAPs, I cultured MDA-MB-231 breast cancer cells in the presence of gradually increasing concentrations of NRC-03 and NRC-07. After roughly 100 generations, NRC-03- and NRC-07-resistant breast cancer cells that were refractory to 50 µM NRC-03 or NRC-07 were isolated. Both NRC-03- and NRC-07-resistant breast cancer cells were killed by 100 µM NRC-03 or NRC-07 (Fig. 5.1A and B, respectively). To determine the magnitude of resistance to NRC-03 and NRC-07, the EC_{50} of NRC-03 and NRC-07 toward parental breast cancer cells and NRC-03- and NRC-07-resistant breast cancer cells was calculated. The EC_{50} of NRC-03 and NRC-07 for NRC-03-resistant breast cancer cells was 3.2- and 4.3-fold greater than that of parental MDA-MB-231 cells (Fig. 5.1C). Similarly, the EC_{50} of NRC-03 and NRC-07 for NRC-07-resistant breast cancer cells was 3.8- and 3.6-fold greater than that of parental cells, respectively.

Because NRC-03 and NRC-07 are lytic peptides that rapidly bind to and enter breast cancer cells, it is reasonable to hypothesize that resistance to NRC-03 and NRC-07 is due to a reduction in peptide binding to cancer cell membranes. Peptide binding assays were therefore conducted in order to correlate cancer cell killing with peptide binding. Consistent with previous findings, biotin-NRC-03 and -NRC-07 bound to parental breast cancer cells, whereas biotin-NRC-03 and -NRC-07 bound poorly to NRC-03- and NRC-07-resistant cancer cells (Fig. 5.2A). Peptide binding was quantified to determine differences in NRC-03 and NRC-07 binding to resistant and parental breast cancer cells (Fig. 5.2B). In comparison to biotin-NRC-03 binding to parental cells, biotin-NRC-03 binding to NRC-03- and NRC-07-resistant breast cancer cells decreased by 14.2- and 8.1-
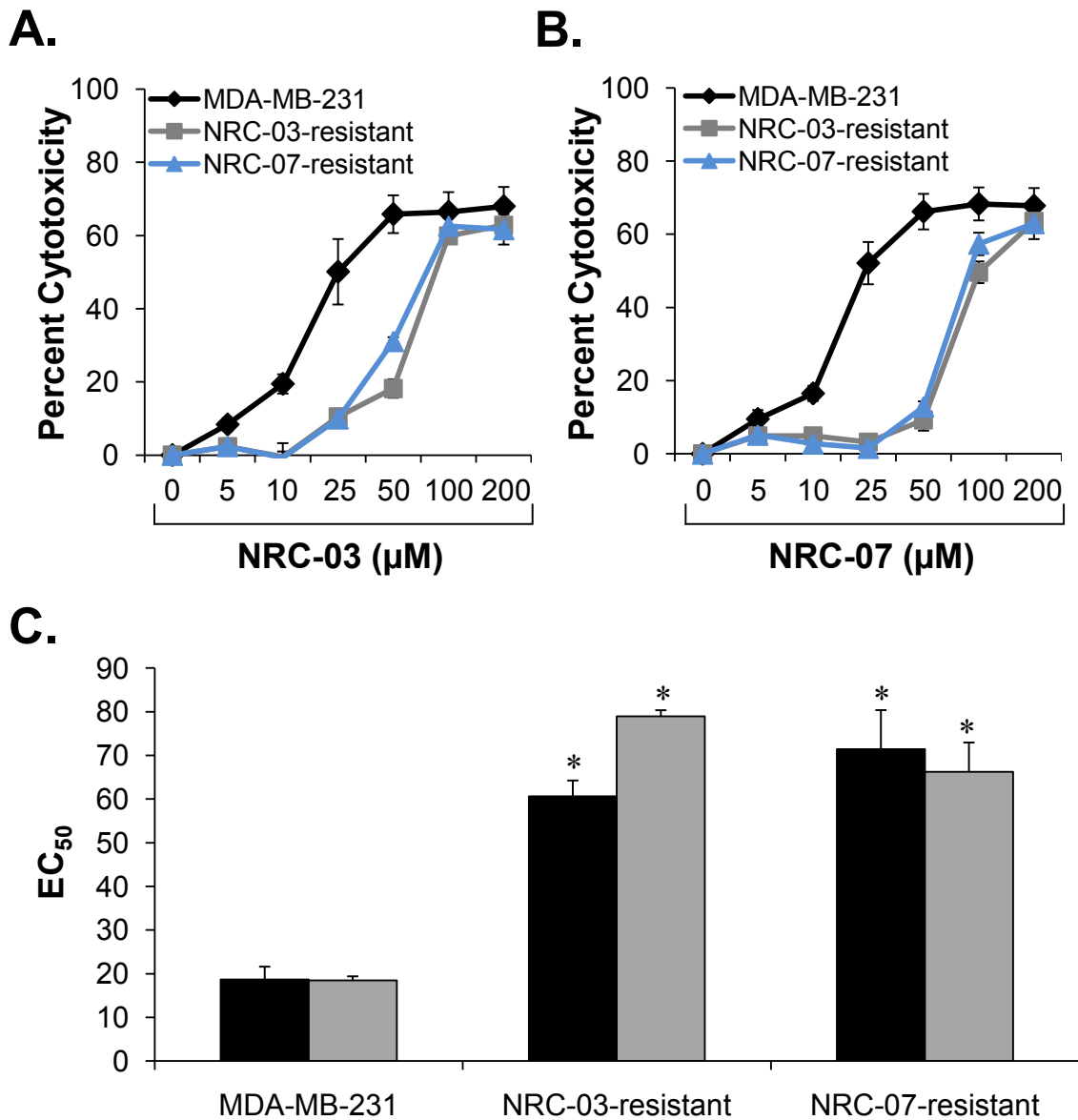
Figure 5.1. NRC-03- and NRC-07-resistant breast cancer cells are less susceptible to NRC-03 and NRC-07 than parental MDA-MB-231 cells. MDA-MB-231, NRC-03-resistant MDA-MB-231, and NRC-07-resistant MDA-MB-231 cells were cultured in increasing concentrations of (A) NRC-03 or (B) NRC-07 for 24 h. Cell viability was determined by MTT assay. Data are significant ($p < 0.0001$) by ANOVA. (C) Cell viability was used to calculate the EC_{50} of NRC-03 and NRC-07. Data shown represent the mean of 3 independent experiments $\pm$ SE and are statistically significant by the Bonferroni multiple comparison test in comparson to NRC-03- or NRC-07-treated parental MDA-MB-231 cells; * $p < 0.01$.
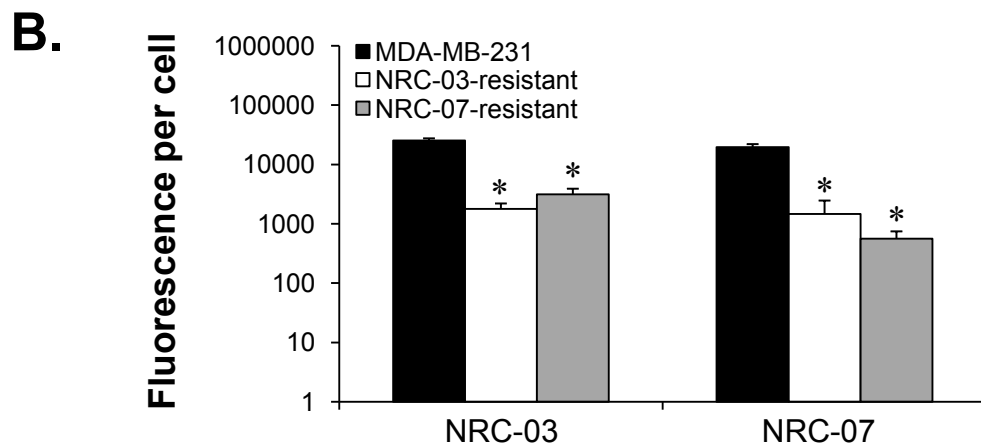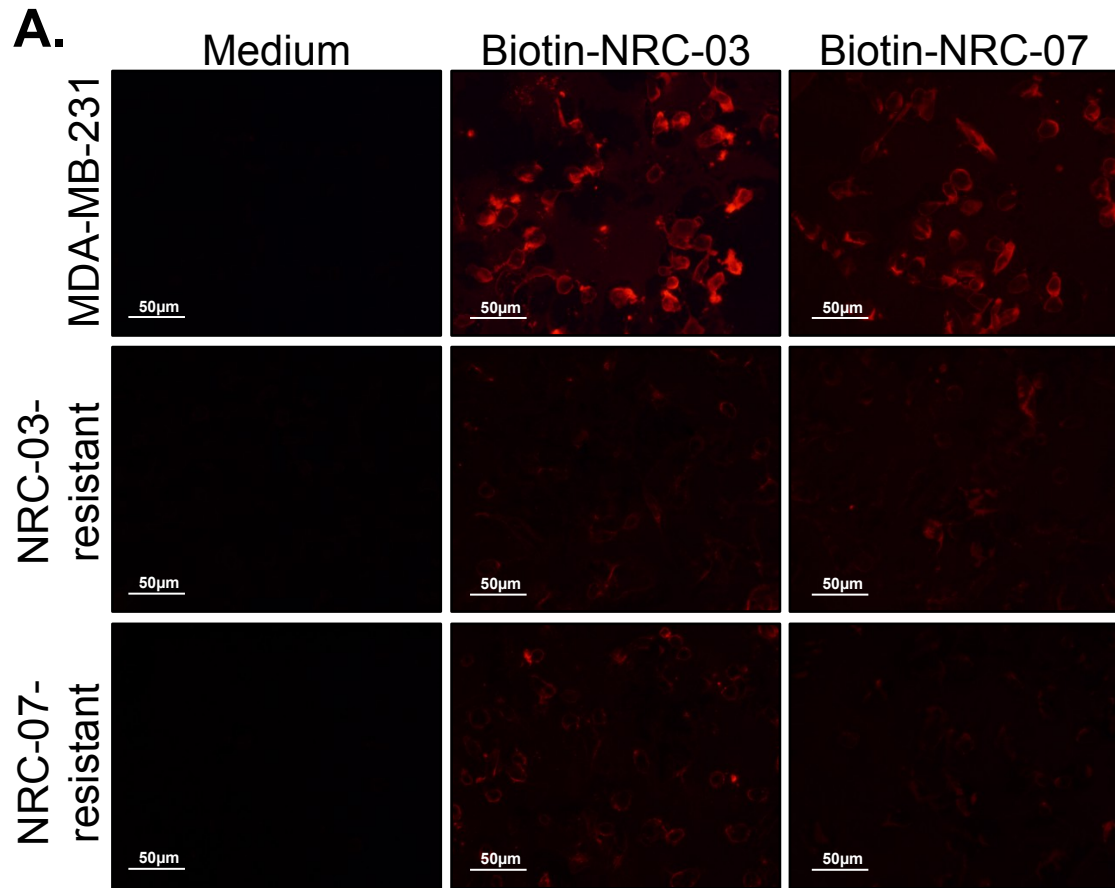
Figure 5.2. NRC-03 and NRC-07 bind poorly to NRC-03- and NRC-07-resistant breast cancer cells. (A) MDA-MB-231, NRC-03-resistant MDA-MB-231, and NRC-07-resistant MDA-MB-231 cells were cultured in the absence or presence of 50 µM biotin-NRC-03 or -NRC-07 for 10 min, stained with Texas Red-streptavidin, and visualized by fluorescence microscopy. (B) Peptide binding was quantified using NIS-Elements. Data shown represent the mean of 3 independent experiments ± SE. Statistical significance was determined by the Bonferroni multiple comparisons test in comparison to MDA-MB-231 cells; * $p < 0.001$.

fold, respectively. Similarly, biotin-NRC-07 binding to NRC-03- and NRC-07-resistant breast cancer cells was reduced by 13.5- and 34.9-fold in comparison to parental cells.

5.2 NRC-03- and NRC-07-Resistant Cells are Phenotypically Different From Parental Cells

Breast cancer cells treated with NRC-03 and NRC-07 exhibit distinct morphologies. Therefore, SEM was used to identify possible phenotypic differences between parental, NRC-03- and NRC-07-resistant breast cancer cells. Low magnification images showed that NRC-07-resistant cancer cells appeared similar to parental cells, whereas NRC-03-resistant cancer cells appeared flattened in comparison to parental cells (Fig. 5.3 top panel). Images at intermediate magnification show that the flattened appearance of NRC-03-resistant cells appeared to be due to an expanded plasma membrane and/or cellular protrusions, whereas NRC-07-resistant cancer cells appeared to have reduced numbers of microvilli and an abundance of membrane blebs, some of which were rather large (Fig. 5.3 middle panel). Images taken at high magnification showed that membrane blebs were apparent on NRC-03-resistant cells, whereas membrane damage was observed on blebs coming off of NRC-07-resistant cells (Fig. 5.4 bottom panel).

5.3 Peptide-Resistant Cells are Killed by Traditional Chemotherapeutic Agents and [D]-NRC-03

To determine whether NRC-03- or NRC-07-resistant breast cancer cells are also resistant to other cytotoxic drugs, parental MDA-MB-231, NRC-03- and NRC-07-resistant breast cancer cells were exposed to cisplatin or docetaxel. Both cisplatin and docetaxel were equally cytotoxic to the 3 cell lines (Fig. 5.4 A and B, respectively). NRC-03- and NRC-07-mediated killing of parental and resistant cancer cells was also compared to [D]-NRC-03-mediated cell killing. Interestingly, both NRC-03- and NRC-07-resistant cancer cells were killed by 50 μM [D]-NRC-03, albeit to a significantly lesser extent than parental cells (Fig. 5.5). Additional experiments are required to determine whether cisplatin, docetaxel, and/or [D]-NRC-03 kill NRC-03- and/or NRC-07-resistant cancer cells by apoptosis or necrosis.

MDA-MB-231   NRC-03-resistant MDA-MB-231   NRC-07-resistant MDA-MB-231
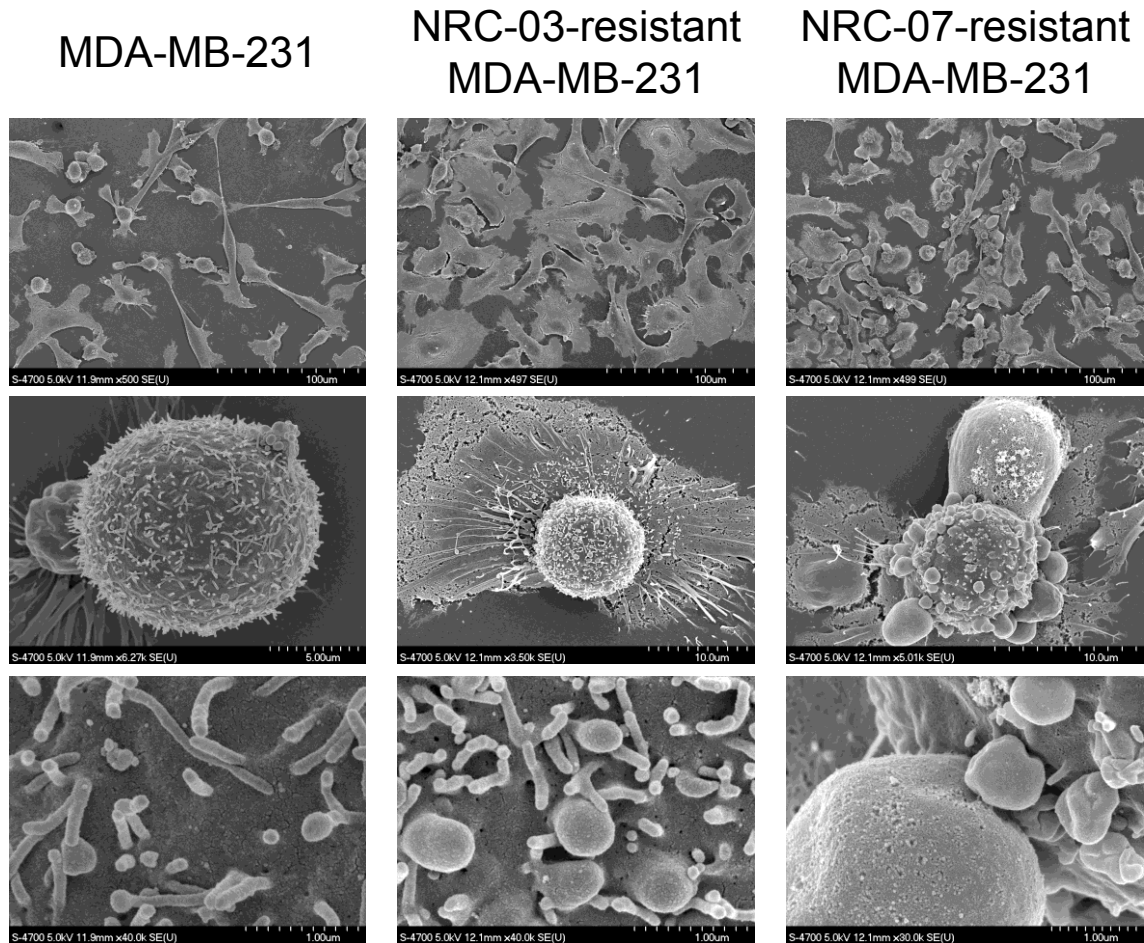
Figure 5.3. Morphological features of NRC-03- and NRC-07-resistant MDA-MB-231 cells in comparison to parental MDA-MB-231 cells. NRC-03-resistant, NRC-07-resistant, and parental MDA-MB-231 cells were examined by SEM. Data shown are from a representative experiment (n = 2).
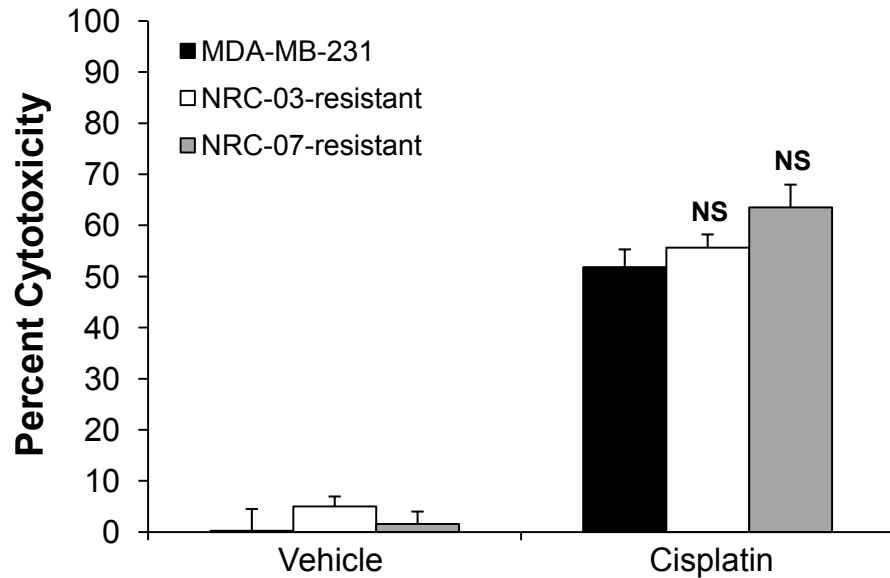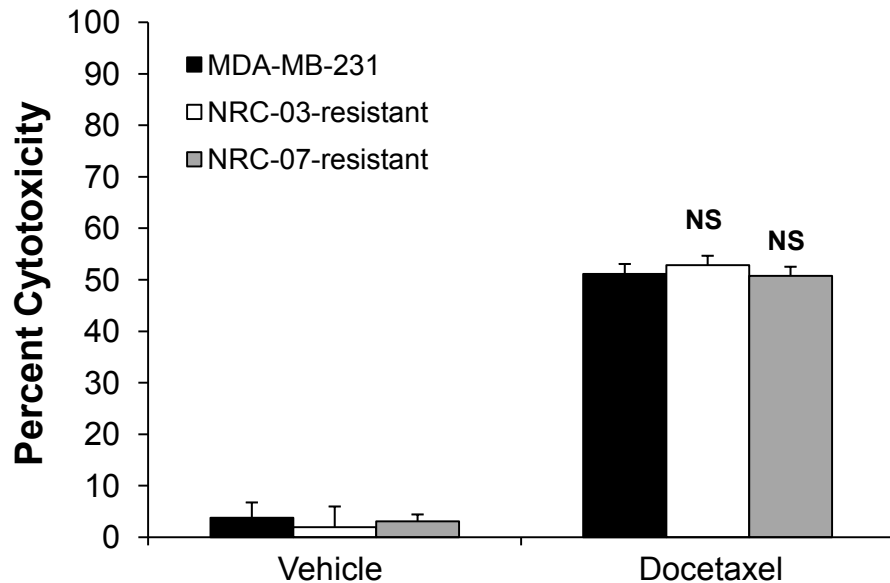
Figure 5.4. NRC-03- and NRC-07-resistant breast cancer cells are killed by cisplatin and docetaxel. MDA-MB-231, NRC-03-resistant MDA-MB-231 and NRC-07-resistant MDA-MB-231 cells were cultured in the presence or absence of (A) 16 μg/ml cisplatin, or (B) 20 ng/ml docetaxel. Cell viability was determined by MTT assay after 72 h. Data shown represent the mean of 3 independent experiments ± SE and are not statistically significant ($p \geq 0.05$) by the Bonferroni multiple comparson test in comparison to cisplatin- or docetaxel-treated parental MDA-MB-231 cells.
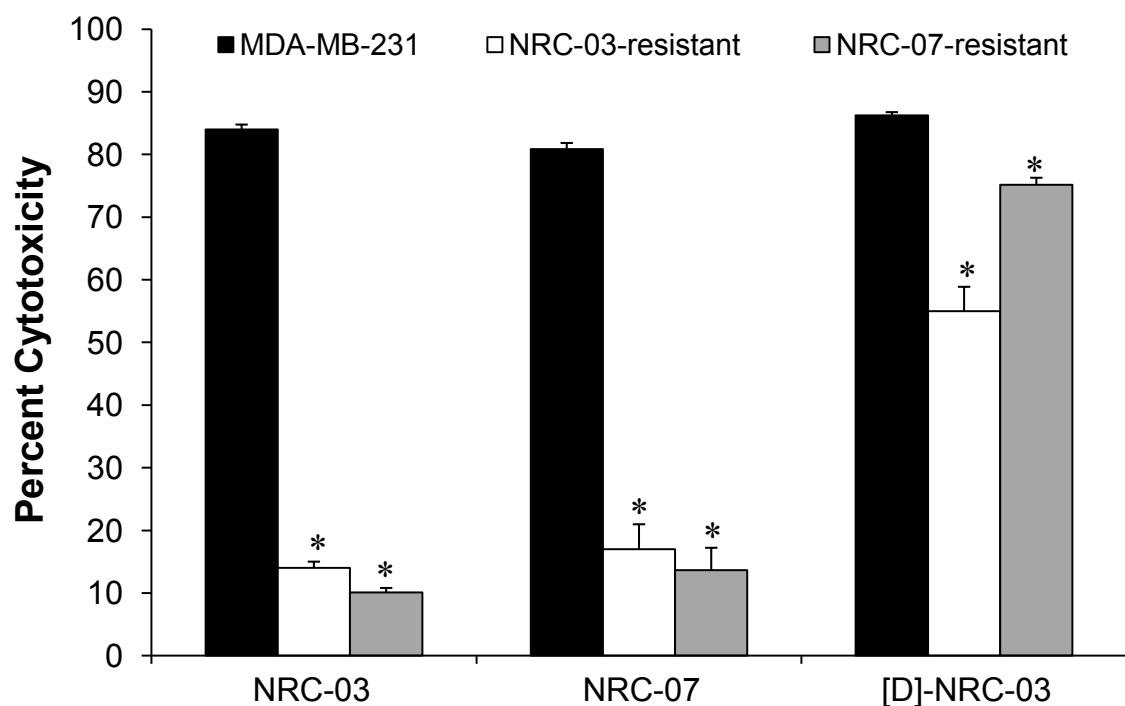
Figure 5.5. [D]-NRC-03 kills NRC-03- and NRC-07-resistant breast cancer cells.
MDA-MB-231, NRC-03-resistant MDA-MB-231, or NRC-07-resistant MDA-MB-231 cells were cultured in the presence or absence of 50 μM NRC-03, NRC-07, or [D]-NRC-03. Cell viability was determined by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test in comparison to NRC-03-, NRC-07-, or [D]-NRC-03-treated parental MDA-MB-231 cells; * $p < 0.05$.

CHAPTER 6

DISCUSSION

Cancer is often treated with chemotherapy, which is limited by non-specific toxicities, development of multidrug-resistance, the potential for secondary malignancies, and the inability of chemotherapeutic agents to kill slow growing or dormant cancer cells (29-31, 33). Significant efforts led to the development of targeted therapies, e.g. trastuzumab, which cause cancer cell death by interfering with specific molecules that are required for tumour growth and/or survival. While targeted therapies have significantly improved patient outcomes, they are also limited by the eventual development of drug resistance (8, 35, 36). Therefore, the search continues for novel anticancer drugs that kill cancer cells, including those that are slow growing and multidrug-resistant, without causing undue harm to normal cell types.

6.1 Cytotoxic Properties of Pleurocidins

Pleurocidins exhibit potent antibacterial activities (48); however, their anticancer properties had not been well studied. The purpose of this investigation was to determine whether certain pleurocidins, specifically NRC-03, NRC-07, NRC-13, NRC-16, and/or NRC-03$_{10mer}$, have potential as novel agents for the treatment of breast cancer. My study showed for the first time that NRC-03 and NRC-07 killed breast cancer cells, whereas NRC-13, NRC-16, and NRC-03$_{10mer}$ were not cytotoxic for breast cancer cells. NMR studies have not been performed on NRC-13, NRC-16, or NRC-03$_{10\ mer}$; therefore, it is difficult to explain why these pleurocidins did not kill cancer cells. Assuming that NRC-13 adopts a rigid α-helical secondary structure when bound to cancer cell membranes, its length should be sufficient to span the plasma membrane (38). On the other hand, NRC-16 and/or NRC-03$_{10mer}$ may not be long enough to span the cancer cell membrane, which may explain why these pleurocidins did not kill breast cancer cells. Note that this explanation is only valid if pleurocidins damage cellular membranes as described by the barrel-stave model, which is impossible to ascertain with the existing data; however, this hypothesis could be tested by aa addition and extending NRC-16 and/or NRC-03$_{10mer}$ to a length that is sufficient to span the plasma membrane of the cancer cell.

Alternatively, the lack of cancer cell killing by NRC-13, NRC-16, and/or NRC-$03_{10\,mer}$ may be due to the distribution of basic and hydrophobic aa. Studies conducted by others suggest that the basic and hydrophobic aa of NRC-03 and NRC-07 are separated into two spatially separated domains in membrane mimicking environments (Syvitski, R., personal communication), which may not be the case for NRC-13, NRC-16, or NRC-03_{10mer}. Consequently, NRC-13, NRC-16, and/or NRC-$03_{10\,mer}$ may be unable to bind to and/or destabilize cancer cell membranes. NMR studies on NRC-13, NRC-16, and NRC-$03_{10\,mer}$ would clarify whether this is a likely explanation for the lack of cancer cell killing by these pleurocidins.

Since, hydrophobicity, specifically the number of tryptophan residues, is essential for the antibacterial properties of certain CAPs (137), NRC-13 and/or NRC-03_{10mer} may not be sufficiently hydrophobic to kill breast cancer cells. While this hypothesis may explain why the highly cationic NRC-03_{10mer} lacks anticancer properties, it is unlikely to account for the lack of NRC-13-mediated killing because NRC-03 only possesses one tryptophan residue and is highly cytotoxic for breast cancer cells, as well as multiple myeloma cells. To determine whether NRC-03_{10mer} is non-toxic due to a lack of hydrophobicity, one of the cationic aa could be replaced with hydrophobic tryptophan (e.g., GRRWRKWLRR-NH_2). Modified NRC-03_{10mer} could then be tested for cytotoxicity toward breast cancer cells.

It is noteworthy that the LDH-release assay was used to assess whether pleurocidins possess anticancer properties. It is therefore possible that NRC-13, NRC-16, and/or NRC-$03_{10\,mer}$ might kill cancer cells by an indirect mechanism of action; however, this explanation is unlikely because most indirect-acting anticancer CAPs are still somewhat membranolytic (54, 107). Nevertheless, additional experiments are required to confirm that NRC-13, NRC-16, and NRC-03_{10mer} are devoid of anticancer potential.

6.1.1 Cytotoxic Properties of NRC-03 and NRC-07

NRC-03 and NRC-07 killed MDA-MB-231, MDA-MB-468, MCF7, SKBR3, and T47D human breast cancer cells, 4T1 mouse mammary carcinoma cells, and U266 and KMS11 multiple myeloma cells in a dose-dependent manner. NRC-03 and NRC-07 were not equally cytotoxic between all of the breast cancer cell lines tested. For example,

MDA-MB-468 cells were highly susceptible to killing by NRC-03 and NRC-07, whereas MDA-MB-231 breast cancer cells were less susceptible to NRC-03 and NRC-07. This finding suggests that breast cancer cell lines may have different membrane compositions that determine whether the cell will be susceptible to NRC-03- or NRC-07-induced cell death. Differences in NRC-03- and NRC-07-induced breast cancer cell killing were not associated with the absence of HER2, ER, or PR expression, which was evidenced by NRC-03- and NRC-07-induced killing of triple negative MDA-MB-468 breast cancer cells (180). Interestingly, NRC-03 and NRC-07 were equally potent killers of the breast cancer cell lines tested. In contrast, NRC-03 was more cytotoxic than NRC-07 for human multiple myeloma cells. Unlike breast cancer cells, which are all adherent cell lines, U266 and KMS11 cells grow in suspension. Consequently, there may be subtle differences in the properties of the cellular membranes that may influence the membrane binding capacity of NRC-03 and NRC-07, thereby determining the susceptibility of the cancer cell type to NRC-03- and NRC-07-induced cell death.

It is important to note that NRC-03 and NRC-07 were similarly cytotoxic when cell death was quantified by LDH-release or MTT assay. Cytotoxicity quantified using these methods approximated what was observed microscopically, i.e., number of lysed cells. Therefore, despite the fact that these assays do not technically measure cytotoxicity, but rather quantify enzymatic activity (166, 181), the output of these assays was referred to as percent cytotoxicity because there was no evidence to suggest that NRC-03 or NRC-07 affect the function of these enzymes, nor was there evidence to suggest that these CAPs inhibited proliferation rather than caused direct cell death. It is also noteworthy that I was required to change my peptide supplier midway through this investigation. Importantly, NRC-03 and NRC-07 synthesized by Dalton Pharma Services and American Peptide Company were equally potent (Appendix Fig. 5). However, I cannot rule out the possibility that there are subtle differences between the mechanism of action of NRC-03 and NRC-07 obtained Dalton Pharma Services versus NRC-03 and NRC-07 synthesized by American Peptide Company.

Importantly, NRC-03 and NRC-07 were cytotoxic to P-gp overexpressing MCF7-TX400 cells. In fact, 50 μM NRC-03 and NRC-07 were equally potent killers of MCF7-TX400 cells and parental MCF7 cells. This finding suggests that NRC-03 and NRC-07

are not substrates for P-gp. As an alternate explanation, NRC-03 and/or NRC-07 may kill cancer cells by causing significant and irreversible membrane damage rather than causing indirect cell death that is dependent on the peptides entering the cell. Additional experiments are required to determine whether this phenomenon holds true at lower concentrations of NRC-03 or NRC-07. NRC-03 and NRC-07 also killed cisplatin-resistant ovarian cancer cells, which suggests that these peptides kill cancer cells that develop drug resistance by increasing DNA repair and/or GSH production.

NRC-03 and NRC-07 were not cytotoxic for HDFs or HUVECs. HDFs are often used as a negative control in studies evaluating the anticancer properties of CAPs (52, 53, 64), possibly because fibroblast membranes do not contain as many negatively charged molecules as cancer cell membranes. In contrast, certain CAPs are cytotoxic to HUVECs (56). In this study cytotoxicity assays using normal cell cultures were conducted in DMEM, with the exception of hemolysis assays, which were conducted in PBS. Cytotoxicity assays were conducted in DMEM so that the concentration of FBS could be controlled, and so that NRC-03- and NRC-07-induced killing of normal cells could be compared to NRC-03- and NRC-07-induced cancer cell killing. HDFs and HMECs grew very well in DMEM, whereas HUVECs did not grow as well in DMEM as they do in EGM-2. Therefore, the lack of NRC-03- and NRC-07-mediated HUVEC killing may be due to a decrease in the viability of DMEM-cultured HUVECs, because percent cytotoxicity is calculated under the assumption that control cells are 100% viable. Additional experiments should be conducted to ascertain whether NRC-03 and NRC-07 are cytotoxic to HUVECs growing in EGM-2 supplemented with FBS. Hemolysis assays are typically conducted in PBS (115, 132, 149); however, others report differences in antifungal and hemolytic properties of certain CAPs in PBS versus other culture media (182). NRC-03 and NRC-07 were not hemolytic; however, this experiment was conducted in PBS. To confirm that NRC-03 and NRC-07 lack hemolytic properties, additional hemolysis assays should be conducted in DMEM lacking phenol red, which would allow for a more reasonable comparison between the hemolytic and anticancer properties of NRC-03 and NRC-07. Finally, NRC-03 and NRC-07 were cytotoxic to HMECs, albeit to a lesser extent than they were to cancer cells. This finding suggests that HMEC membranes are more negatively charged than the membranes of other normal

cells, which is not surprising considering that the breast cancer cells used in this investigation are of epithelial origin. This finding also suggests that epithelial cells should be routinely added to the panel of normal cells used in other studies to ascertain whether their CAP is selective for cancer cells. This finding also suggests that NRC-03, NRC-07, and/or their derivatives require modification to improve their selectivity for cancer cells.

Sublethal doses of NRC-03, and to a lesser extent NRC-07, enhanced cell killing by cisplatin. Cisplatin is a platinating agent, which functions by binding to DNA causing inter- and intra-strand DNA crosslinkages (17, 18). Since NRC-03 and NRC-07 likely bind to DNA (discussed in section 6.2), NRC-03 and/or NRC-07 may work with cisplatin to cause sufficient DNA damage to kill the cell. Cisplatin-resistance can result from the conjugation of cisplatin to intracellular GSH, which can pumped out of the cell by GSH S-conjugate export pump (21, 22). As an alternative explanation for these findings, sublethal doses of NRC-03 and NRC-07 may cause the formation of transient pores that may facilitate the re-uptake of cisplatin-GSH complexes, thereby enhancing cisplatin-induced cell death. Additional experiments could be conducted to determine whether either of these hypotheses explain these findings. For example, membrane damage could be quantified in MDA-MB-231 cells treated with 10 μM NRC-03 or NRC-07. If transient pores are formed, PI uptake should occur without a loss in cellular viability, which would support the latter hypothesis. Alternatively, similar experiments could be conducted in breast cancer cells with silenced GSH S-conjugate export pump expression. If knocking down GSH S-conjugate export pump expression does not enhance cisplatin-induced cell killing, the latter hypothesis would be rejected. Future experiments are also required to determine whether this effect is additive or synergistic. In either case, this finding suggests that NRC-03, NRC-07, and/or their derivatives may prove useful agents in combinational cancer treatment regimens.

6.2 Mechanism of NRC-03- and NRC-07-induced Cytotoxicity

Anticancer CAPs kill cancer cells by a direct and/or indirect mechanisms (61). After elucidating that NRC-03 and NRC-07 are pleurocidins with anticancer properties, the next aim of this investigation was to determine the mechanism of action of NRC-03

and NRC-07, which would test the hypothesis that NRC-03 and NRC-07 are direct-acting CAPs that kill cancer cells by cytolysis.

6.2.1 NRC-03 and NRC-07 are Attracted to Several Different Negatively Charged Molecules

The data collected in this study showed that NRC-03 and NRC-07 bind to breast cancer cells, but not HDFs. Importantly, non-biotinylated NRC-03 or NRC-07 did not cause an increase in fluorescence (Appendix Fig. 6), which indicates that the fluorescence observed in biotin-NRC-03- and -NRC-07-treated breast cancer cells was not a consequence of non-specific streptavidin-conjugated Texas Red binding to molecules inside the cell. Many negatively charged molecules, including O-glycosylated mucins, sialylated glycoproteins, and HSPs, contribute to the net negative charge of cancer cell membranes (81-83). NRC-03- and NRC-07-induced breast cancer cell killing was only marginally reduced when cells were incubated with OSGE prior to exposure to NRC-03 or NRC-07, suggesting that sialylated glycoproteins do not contribute to NRC-03- and/or NRC-07-mediated cytotoxicity. It is noteworthy that OSGE was removed from OSGE-treated cells prior to their being incubated o/n to promote cellular adherence. OSGE was removed from the cells because the manufacturer indicated that OSGE treatment would likely interfere with cellular attachment. Therefore, sialylated glycoprotein expression may have been restored to normal levels by the time that NRC-03 and NRC-07 were added to OSGE-treated breast cancer cells. Consequently, sialylated glycoproteins may play a greater role in NRC-03- and/or NRC-07-induced cytotoxicity than these data suggest. The importance of sialylated glycoproteins for NRC-03- or NRC-07-induced cancer cell killing could be better elucidated by using multiple myeloma cells rather than breast cancer cells as a model, because myeloma cells grow in suspension and could therefore be continuously exposed to OSGE.

HSPs and CSPs are negatively charged molecules that decrease LfcinB-induced cell killing (84). I have shown that NRC-03 and NRC-07 kill cells that lack HSPs and CSPs to a lesser extent than wildtype cells. This finding suggests that NRC-03 and NRC-07 are attracted to HSPs and CSPs, and that these molecules enhance cell killing by NRC-03 and NRC-07. This finding was more pronounced at lower concentrations of

NRC-03 and NRC-07, which suggests that NRC-03 and NRC-07 are attracted to other negatively charged cell surface molecules. Subsequent experiments determined that NRC-03 and NRC-07 bind to HeS and CS. Collectively, these findings contradict those reported by another group (84), who showed that LfcinB-induced cell killing is inhibited by the presence of HSPs, and to a lesser extent CSPs. The authors subsequently developed truncated LfcinB derivatives that were equally or more toxic to HSP- and CSP-expressing cells (85). The authors suggested that the arginine residues in LfcinB are responsible for LfcinB binding to HSPs and CSPs, and that HSPs and CSPs tether LfcinB away from the cell surface, thereby preventing LfcinB from interacting with the cellular membrane. The authors postulated that truncated LfcinB derivatives cannot be inhibited by CSPs and HSPs in this manner, and that the truncated peptides are able to gain access to, and subsequently destabilize the cellular membrane. Like LfcinB, NRC-03 is arginine rich. Moreover, the length of NRC-03 (26 aa) is comparable to the length of LfcinB (25 aa). Studies conducted by our research group suggest that the sequence of LfcinB, rather than its positive charge, determines whether LfcinB will bind to heparin-like structures (116). Therefore, it is possible that, unlike LfcinB, NRC-03 and NRC-07 are attracted to HSPs and CSPs via elecrostatic interactions, which is determined by the cationic nature of these peptides rather than by their sequence. Moreover, the interactions between pleurocidins and HSPs or CSPs may be reversible, which would allow at least some NRC-03 or NRC-07 to gain access to the membrane of the cell. Although this study does not confirm why CSPs and HSPs have inhibitory effects on LfcinB-induced cell killing and enhance cell killing by NRC-03 and NRC-07, the hypothesis that the interaction between the pleurocidins and CSPs and/or HSPs is due to elecrostatic interactions rather than a sequence-specific mechanism could be tested by scrambling the aa sequence of NRC-03 and NRC-07. The toxicity of the scrambled pleurocidins to L, gro2C and sog9 cells, as well as the ability of these peptides to bind to CSPs and HSPs could be determined by cytotoxicity assays and solid phase HeS- and CS-binding assays, respectively.

Collectively, these findings suggest that susceptibility of the target cell to NRC-03- or NRC-07-induced cell killing is at least partly determined by the charge of the target cell membrane. NRC-03 and NRC-07 are likely attracted to several different types

of negatively charged surface molecules that are present on cancer cell membranes. The interaction between NRC-03 or NRC-07 and anionic surface molecules is likely not dependent on the aa sequence of the pleurocidin. Therefore, the anticancer potential of NRC-03 and/or NRC-07 could be improved upon by modifying the aa sequence and/or chirality of either NRC-03 or NRC-07. These modification strategies could improve the stability and/or selectivity of NRC-03 and/or NRC-07 for cancer cells.

6.2.2 NRC-03 and NRC-07 Enter Target Cells where they Localize to the Nucleus and Damage Mitochondria

Many direct- and indirect-acting CAPs have effects on mitochondria (37). Certain CAPs directly interact with mitochondria and cause $\Delta\Psi_m$ (107), whereas other CAPs cause $\Delta\Psi_m$ without localizing to mitochondria (64). The theory of endosymbiosis proposes that mitochondria arose from ancient bacteria (183, 184). In keeping with this theory, experts argue that certain CAPs selectively permeabilize mitochondrial membranes because the membrane composition of mitochondria is comparable to the membrane composition of bacteria (37). Importantly, NRC-03 and NRC-07 have antibacterial properties (48). The data presented here show that NRC-03 and NRC-07 caused $\Delta\Psi_m$ by 1 h, which was indicated by the release of $DiOC_6$ from NRC-03- and NRC-07-treated breast cancer cells. Furthermore, mitochondria isolated from breast cancer cells released cytochrome c when exposed to NRC-03 or NRC-07, suggesting that NRC-03 and/or NRC-07 may interact with mitochondria inside the cell, thereby causing $\Delta\Psi_m$. However, NRC-03 and/or NRC-07 may have interacted with isolated mitochondria because it is more energetically favorable for these pleurocidins to integrate into mitochondrial membranes than remain in solution. Consequently, NRC-03 and/or NRC-07 may not directly interact with mitochondria inside the cell, but rather initiate a signaling pathway that leads to $\Delta\Psi_m$. Furthermore, to my knowledge, the membrane composition of each organelle has not been elucidated. Therefore, it is possible that NRC-03 and NRC-07 may interact with other organelles within cancer cells. Additional experiments, e.g., transmission electron microscopy using biotinylated pleurocidins and streptavidin-conjugated colloidal gold, should be conducted to determine whether NRC-03 and/or NRC-07 target mitochondria and/or other organelles in breast cancer cells. Additional experiments should also be conducted to determine whether NRC-03 and/or

NRC-07 trigger the release of other pro-apoptotic factors, such as AIF, from the mitochondria of breast cancer cells.

Certain CAPs that cause $\Delta\Psi_m$ also generate ROS and activate caspases, which are involved in CAP-induced cell death (55). The data collected in my investigations show that NRC-03- and NRC-07-mediated cell death was not associated with significant ROS generation, specifically superoxide generation, which was determined by staining NRC-03- and NRC-07-treated breast cancer cells with DHE. Due to the kinetics of NRC-03- and NRC-07-induced cell death, DHE and $DiOC_6$ staining were performed on breast cancer cells that were treated with peptide while in suspension, rather than cells that were adherent. Consequently, NRC-03- and NRC-07-treated cells may have behaved differently in suspension than they would when adherent. Moreover, NRC-03 and/or NRC-07 may cause the generation of other ROS that are not detected by DHE staining. Despite these limitations, NRC-03- and NRC-07-mediated breast cancer cell killing is likely ROS-independent because the antioxidant GSH did not inhibit NRC-03 or NRC-07 killing of breast cancer cells (Appendix Fig. 7). Moreover, the pancaspase inhibitor Boc-D-fmk did not inhibit NRC-03- or NRC-07-mediated cell death, suggesting that NRC-03 and NRC-07 do not kill breast cancer cells by caspase-dependent apoptosis.

Confocal microscopy was used to determine whether NRC-03 and/or NRC-07 enter breast cancer cells. Surprisingly, NRC-03 and NRC-07 entered breast cancer and multiple myeloma cells after only 30 sec. Moreover, NRC-03 and NRC-07 localized to the nucleus of breast cancer cells within 30 sec. Electrostatic attraction to negatively charged DNA likely accounts for the rapid localization of NRC-03 and NRC-07 to the nucleus; however, additional experiments are required to determine whether NRC-03 and/or NRC-07 actually bind DNA. Interestingly, NRC-07, but not NRC-03, caused DNA fragmentation in breast cancer cells. NRC-03 caused DNA fragmentation in multiple myeloma cells; however, NRC-03-induced DNA fragmentation was significantly less than NRC-07-induced DNA fragmentation. Studies conducted by others show that NRC-03 and NRC-07 adopt α-helical arrangements in membrane mimicking environments (Syvitski *et al*, unpublished data); however, there are subtle differences between the secondary structures of NRC-03 and NRC-07. Both NRC-03 and NRC-07 have random coil arrangements at the N-terminus, although the random coil N-terminal

region of NRC-03 is longer. Moreover, NRC-03 possesses 2 glycine residues that kink the α-helix, whereas NRC-07 adopts a more rigid α-helix. It is therefore tempting to speculate that the structural differences between NRC-03 and NRC-07 contribute to the observed differences in NRC-03- and NRC-07-induced DNA fragmentation. For example, NRC-07 may more easily intercalate into DNA, thereby causing mechanical stress that leads to DNA shearing. This hypothesis could be tested by replacing the glycine residues in NRC-03 with a non-helix-breaking aa such as alanine. Modified NRC-03 may then adopt the rigid α-helical secondary structure required to fragment DNA, which could then be measured by TUNEL staining. As an alternative explanation, NRC-07, and to a lesser extent NRC-03, may trigger the release of AIF from the mitochondria of breast cancer cells. AIF may then localize to the nucleus causing DNA fragmentation. Additional experiments should determine whether NRC-03 or NRC-07 trigger the release of AIF or other caspase-independent pro-apoptotic factors.

6.2.3 NRC-03 and NRC-07 Lyse Breast Cancer and Multiple Myeloma Cells

CAPs with anticancer properties can directly kill cancer cells by causing significant and irreversible membrane damage, while other CAPs kill cancer cells indirectly by triggering a signaling cascade that leads to cell death by apoptosis (61). The next objective of this investigation was therefore to determine whether NRC-03 and/or NRC-07 are direct- or indirect-acting CAPs. NRC-03- and NRC-07-induced cell death was caspase-independent. Moreover, NRC-03 and NRC-07 caused LDH-release from MDA-MB-231, T47D, and MCF7 human breast cancer cells. LDH-release assays also showed that NRC-03 and NRC-07 caused time-dependent breast cancer cell lysis that peaked at 4 h. This experiment also demonstrated that approximately 30% of NRC-03- and NRC-07-treated cells exhibit significant membrane damage at 10 min. PI uptake studies showed that NRC-03 and NRC-07 damaged cell membranes of multiple myeloma cells within 10 min, suggesting that NRC-03 and NRC-07 kill other cancer cell types by a similar mechanism of action. SEM studies revealed that NRC-03 and NRC-07 damaged breast cancer cell membranes within 10 min. The NRC-03- and NRC-07-treated cells shown represent approximately 30% of the population. These cellular membranes are likely damaged enough to allow LDH-release. The remaining 70% of peptide-treated cells lack microvilli and show the initial stages of pore formation. This data supports the

data collected using the LDH-release assay. SEM analysis also revealed that NRC-03 and NRC-07 damage multiple myeloma cell membranes within 30 min. These images represent the majority of NRC-03- and NRC-07-treated multiple myeloma cells. Importantly, NRC-03 and NRC-07 did not bind to or damage HDF membranes, suggesting that membrane binding and damage is associated with peptide-mediated cell death. It is noteworthy that control HDFs had fewer microvilli than control breast cancer and multiple myeloma cells. This finding suggests that the surface area of the target cell influences peptide binding and cell killing. Although this hypothesis cannot be concluded with the data presented here, this finding has been reported by others (87). NRC-03- and NRC-07-treated cells had decreased numbers of microvilli that appeared damaged, suggesting that NRC-03 and/or NRC-07 may first attack the microvilli of the target cell; however, the significance of microvilli in CAP-mediated cancer cell death remains to be elucidated. Perhaps microvilli membranes have an abundance of highly anionic surface molecules. Alternatively, it may be easier for CAPs to reach the threshold concentration required for membrane lysis when microvilli are more numerous. Regardless, these findings suggest that NRC-03 and NRC-07 are direct-acting CAPs that kill breast cancer and multiple myeloma cells by a necrotic mechanism of action.

6.2.4 Proposed Mechanism of NRC-03- and NRC-07-Induced Cell Death

I propose the following model for the mechanism of NRC-03- and NRC-07-mediated breast cancer cell killing (Fig. 6.1). NRC-03- and NRC-07-mediated cell death is likely initiated by electrostatic interactions between the positively charged aa in the pleurocidins and negatively charged surface molecules present on the surface of the target cell membrane. This interaction is likely not dependent on the chirality or sequence of the pleurocidins. These electrostatic interactions likely bring NRC-03 and NRC-07 close enough to the cellular membrane to allow the hydrophobic aa of the pleurocidins to be exposed and subsequently insert into the hydrophobic core of the membrane, thereby anchoring the peptides to the cancer cell membrane. CAP monomers then aggregate and form pores that allow other CAP monomers to enter the cytoplasm. Pore formation most likely occurs as described by the toroidal pore model, although this conclusion cannot be made with the data obtained in this investigation. Cytoplasmic pleurocidins likely then
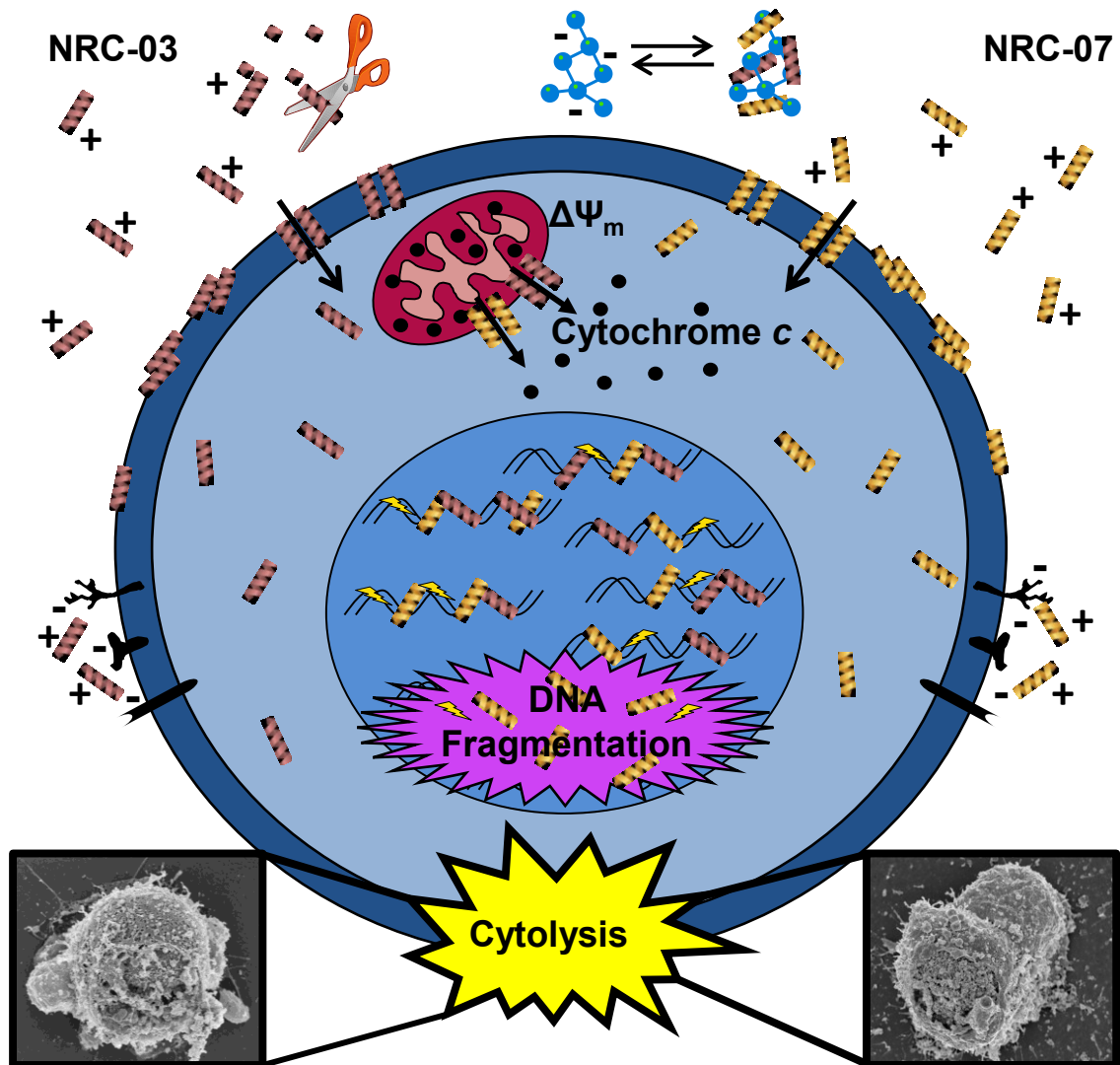
Figure 6.1. Proposed model of NRC-03- and NRC-07-mediated cell killing. NRC-03 and NRC-07 lacking secondary structures (not shown) are attracted to negatively charged molecules present on the surface of the target cell. Electrostatic interactions between positively charged NRC-03 and NRC-07 and negatively charged surface molecules consequently allows insertion of the hydrophobic side chains into the lipid core of the cytoplasmic membrane. NRC-03 and NRC-07 then become anchored to the cell membrane, forming stable secondary structures. Peptide monomers then aggregate and traverse the membrane forming stable pores, allowing NRC-03 and NRC-07 to enter the cytoplasm where they interact with mitochondria resulting in $\Delta\Psi_m$ and cytochrome *c* release. NRC-03 and NRC-07 also localize to the nucleus; however, only NRC-07 causes DNA fragmentation. NRC-03-induced cell death is likely a consequence of overwhelming membrane damage, whereas NRC-07-induced cytotoxicity may also depend on DNA fragmentation. Soluble NRC-03 and NRC-07 may be temporarily sequestered by negatively charged soluble molecules, such as albumin, which may delay cell death; however, only NRC-03 is degraded by serum proteases.

localize to mitochondria causing $\Delta\Psi_m$, which is most likely not required for cell death. NRC-03 and NRC-07 also localize to the nucleus where NRC-07, and to a lesser extent NRC-03, cause DNA fragmentation. DNA fragmentation may be caused by the release of pro-apoptotic molecules from peptide-treated mitochondria, or it may be a consequence of peptide monomers intercalating into the major and/or minor groves of DNA leading to DNA shearing; however, the importance of DNA fragmentation for NRC-03- and NRC-07-induced cytotoxicity remains to be elucidated. NRC-03- and NRC-07-induced cell death is most likely a consequence of significant and irreversible membrane damage that leads to cell death by necrosis, which is dependent on peptide binding to the cellular membrane; however, additional experiments should be conducted to determine whether other cellular processes, e.g., autophagy, are also involved in NRC-03- and/or NRC-07-induced cell death.

6.3 Antitumour properties of NRC-03 and NRC-07

Anticancer drugs must show antitumour properties *in vivo* in order to justify clinical evaluation. Several studies show that CAPs possess antitumour properties *in vivo* (53, 56, 95, 114, 117, 118, 145, 163); however, the majority of these studies treated tumour-bearing animal during the earliest stages of tumour growth (5-75 mm^3) or immediately after tumour cell inoculation. Arguably, these studies evaluated the chemoprotective properties of CAPs rather than their potential for the effective treatment of established cancer. In this investigation, established tumours ($\geq$120 mm^3, or about the size of a pea) were injected thrice with NRC-03 or NRC-07. Both pleurocidins significantly delayed the growth of breast cancer cell xenografts in immune-deficient mice; however, tumours in NRC-03- and NRC-07-treated mice failed to be completely eliminated, which was likely a consequence of inadequate tissue penetration, peptide degradation, and short duration of therapy. Major organs isolated from control- and peptide-treated mice appeared normal, with the exception of one kidney, which was likely injected with tumour cells, and several oviducts. The abnormal oviducts exhibited increased vascularization, which may have been a consequence of the secretion of VEGF by MDA-MB-231 cells (185-187); however, the cause and significance of oviduct vascularization remains to be elucidated. Histological analysis revealed that the necrotic

core of peptide-treated tumours was larger than that of control-treated tumours, suggesting that NRC-03 and NRC-07 killed breast cancer xenografts; however, additional experiments are required to determine whether NRC-03 and/or NRC-07 also inhibit the proliferation of tumour cells. Collectively, these findings indicate that NRC-03 and NRC-07 have antitumour properties *in vivo*. Importantly, this study showed that NRC-03 and NRC-07 are able to kill cells of established tumours, which suggests that these pleurocidins may be useful agents for the treatment of cancer, rather than the prevention of cancer.

The most significant limitation of this study is that NRC-03 and NRC-07 were delivered by intratumoural injection, which is not an ideal route of administration for cancer patients. NRC-03 and NRC-07 were administered in this manner because CAPs, including pleurocidin, are substrates for proteolytic degradation (149). Consequently, the next aim of this investigation was to determine the susceptibility of NRC-03 and NRC-07 to proteolytic degradation in serum, and to identify peptide derivatives with enhanced stability *in vitro* and *in vivo*.

6.4 Susceptibility to Protease Degradation

CAP-mediated cancer cell killing is often inhibited by serum (119, 126, 127), which may be a consequence of proteolytic degradation of the CAP by serum proteases, or CAP binding to negatively charged molecules that sequester the CAPs and prevent them from interacting with cellular membranes (37, 38, 67). The data collected in this study showed that NRC-03-mediated breast cancer cell killing was inhibited by FBS in a dose-dependent manner. NRC-07-mediated cell killing was also inhibited by FBS; however, the data did not achieve statistical significance. BSA is a highly negatively charged molecule found in FBS that inhibits cell killing by defensins (127). Breast cancer cells cultured in the presence or absence of BSA were treated with NRC-03 or NRC-07 to determine whether the FBS-mediated decrease in NRC-03- and/or NRC-07-mediated cytotoxicity was due to BSA binding to pleurocidins, thereby sequestering them from breast cancer cell membranes. Interestingly, BSA significantly inhibited NRC-03- and NRC-07-mediated breast cancer cell killing at 4, but not 24 h, which was not a consequence of BSA degradation. This finding suggests that the interaction between

pleurocidins and BSA is reversible. Many drugs bind albumin (188), and albumin binding may enhance the pharmacokinetics of protein-based drugs (189); however, the importance of albumin binding in the pharmacokinetics of NRC-03 and NRC-07 was not elucidated in my study. Moreover, BSA binding to pleurocidins does not explain why FBS reduced NRC-03- or NRC-07-mediated breast cancer cell killing in a dose-dependent manner. Pleurocidins may bind to other serum proteins that limit their ability to kill breast cancer cells. For example, the direct-acting CAPs C18G and 399 are also inhibited by high serum concentrations; however, low-density lipoproteins potently inhibited peptide-mediated cytolysis, whereas albumin and high-density lipoproteins had only slight inhibitory properties (126). Additional experiments should therefore be conducted to determine whether NRC-03 and/or NRC-07 bind to other serum molecules, and whether this interaction inhibits NRC-03- and/or NRC-07-mediated cell killing.

CAPs can be degraded by proteases (149). Experts argue that the anticancer properties of CAPs are limited by their sensitivity to proteolytic degradation by serum proteases (37, 38, 61). Moreover, the serum of breast cancer patients has greater proteolytic activities than serum from healthy individuals (190, 191). Therefore, the next objective of this investigation was to determine whether NRC-03 and/or NRC-07 were susceptible to proteolytic degradation by serum proteases. NRC-03 that was incubated in the presence of HS or MS prior to being added to breast cancer cells was significantly less potent than NRC-03 that was not incubated in serum-containing medium. Interestingly, this phenomenon was not observed when NRC-03 was incubated in FBS. Moreover, NRC-07-mediated cell killing was not inhibited when NRC-07 was incubated with FBS, MS, or HS. These findings suggest that proteases in HS and MS degrade NRC-03 and thereby inhibit NRC-03-mediated cell death. It is noteworthy that while pretreating NRC-03 with MS prior to adding the peptide to cancer cells significantly limited its ability to kill breast cancer cells, the results were not as dramatic as those obtained when NRC-03 was exposed to HS. These findings suggest that HS contains more proteases than MS or FBS. Alternatively, HS may contain more anionic molecules capable of sequestering NRC-03, but not NRC-07, from breast cancer cell membranes. I therefore evaluated NRC-03 degradation in FBS, MS and HS. As predicted, NRC-03 was degraded in MS and HS, but not FBS. This finding suggests that NRC-03 requires

modification to improve its stability in serum before it will be considered for possible clinical use. Importantly, these findings did not reveal why NRC-03-mediated cytotoxicity was reduced by FBS in a dose-dependent manner. My current hypothesis is that other non-albumin molecules present in FBS bind to NRC-03, thereby reducing its capacity to bind to and kill breast cancer cells. As an alternate hypothesis, breast cancer cells incubated in low concentrations of FBS may express fewer negatively charged cell surface molecules, thereby making them less susceptible to NRC-03-mediated cell death. Alternatively, 30 min exposure to FBS may not be sufficient to cause NRC-03 degradation. Moreover, it is possible that cells grown in 2.5% FBS are less healthy than cells grown in higher concentrations of FBS, and are therefore easier to kill. Further experiments are required to determine which, if any, of these explanations is most likely.

6.5 Cytotoxic Properties of [D]-NRC-03

NRC-03 degradation in HS limits its therapeutic potential. Therefore, the next objective of this investigation was to identify a derivative of NRC-03 that is resistant to proteolytic degradation. Changing the chirality of anticancer CAPs by creating the enantiomer represents one promising strategy to overcome proteolytic degradation (61). All-D CAPs are non-immunogenic (132), which gives them an additional advantage over naturally-occurring CAPs. Importantly, several enantiomeric CAPs maintain potent anticancer properties (121, 123, 150). The data discussed in section 6.2.1 suggests that NRC-03 is likely attracted to several different negatively charged molecules present on the surface of cancer cells. Consequently, NRC-03-induced cell death is probably not dependent on a chiral-specific interaction with a unique receptor. Collectively, these findings suggest that [D]-NRC-03 would be expected to maintain anticancer properties, whilst being resistant to proteolytic degradation.

[D]-NRC-03 killed MDA-MB-231, MDA-MB-468, MCF7, T47D, and SKBR3 human breast cancer cells, as well as 4T1 mouse mammary carcinoma cells, in a dose-dependent manner. These data also indicated that [D]-NRC-03 was more potent than NRC-03 as a cytotoxic peptide, which confirmed that NRC-03-mediated cell death was not dependent on a chiral-specific interaction between NRC-03 and a specific receptor in the target cell membrane. Like NRC-03, [D]-NRC-03 killed MCF7-TX400 cells that

overexpressed P-gp, which was shown using clonogenic assays. NRC-03 significantly reduced the number of MCF7 CFUs after only 4 h, albeit to a lesser extent than [D]-NRC-03; however, NRC-03 did not significantly reduce the number of MCF7-TX400 CFUs. This data contrasts with data collected using 50 μM NRC-03. Therefore, it is possible that lower concentrations of [D]-NRC-03 may be less toxic to MCF7-TX400 cells than parental cells. It is also noteworthy that data collected by acid phosphatase assays suggest that NRC-03 was equally cytotoxic for MCF7 and MCF7-TX400 cells, whereas [D]-NRC-03 was significantly more toxic to MCF7 cells than MCF7-TX400 cells. The discrepancies between clonogenic and acid phosphatase assays are likely due to the difficulty in determining what constitutes a colony in the clonogenic assay. Therefore, although these data suggest that NRC-03 and [D]-NRC-03 are toxic to P-gp-overexpressing cells, additional experiments should be conducted to elucidate whether or not multidrug-resistant cells are less susceptible to cell killing by these peptides than parental cells.

Importantly, [D]-NRC-03 was not degraded by trypsin. Moreover, [D]-NRC-03-mediated cell killing was not reduced in increasing concentrations of FBS. However, the studies discussed in section 6.4 suggest that the inhibitory properties of FBS on NRC-03-mediated cell killing was likely not a consequence of NRC-03 degradation in FBS, but were rather caused by NRC-03 binding to negatively charged serum proteins other than albumin. Perhaps prolonged exposure of NRC-03 to FBS, i.e., longer than 30 min, would lead to NRC-03 degradation. Alternatively, [D]-NRC-03 may not bind to these unidentified serum proteins to the same extent as NRC-03. Additional experiments are therefore required to determine why NRC-03-induced cytotoxicity is reduced by FBS in a dose-dependent manner.

[D]-NRC-03-mediated killing of normal cells was evaluated to determine whether [D]-NRC-03 was more or less selective than NRC-03 for cancer cells. Neither NRC-03 nor [D]-NRC-03 were hemolytic. Additionally, NRC-03 and [D]-NRC-03 were not as cytotoxic for HUVECs as they were for cancer cells. These findings were consistent with the studies that evaluated the selectivity of NRC-03 and NRC-07 for cancer cells. Surprisingly, both NRC-03 and [D]-NRC-03 were able to kill HMECs, which was in contrast to the studies described in section 6.1.1. Moreover, NRC-03, and to a greater

extent [D]-NRC-03, killed HDFs, which was also in contrast to the results described in section 6.1.1. Importantly, there was a significant chronological delay between experiments evaluating the toxicity of NRC-03 and NRC-07 to normal cells, and experiments comparing the selectivity of [D]-NRC-03 for cancer cells to NRC-03. Because HUVECs, HMECs and HDFs are normal cell cultures, they can only be used for a finite number of passages. For this reason, the experiment comparing the selectivity of [D]-NRC-03 for cancer cells to NRC-03 was conducted on new stocks of each normal cell culture. The HUVECs purchased for both studies were pooled, whereas HDFs and HMECs were not, suggesting that the differences in NRC-03-mediated cytotoxicity may have been caused by variations between donors. It is tempting to speculate that differences in normal cell killing by NRC-03, and likely NRC-07, were due to individual differences in human leukocyte antigen expression. This hypothesis is supported by studies showing that the direct-acting CAPs BMAP-27 and BMAP-28 kill activated, but not resting, human lymphocytes (108). However, activated lymphocytes increase their expression of other negatively charged molecules (192). Moreover, to my knowledge, the net charge of different HLA serotypes, as well as differences in HLA glycosylation, have not been reported. Consequently, it is premature to draw this conclusion. However, if future studies identify that the selectivity of CAPs for cancer cells is dependent on the individual patient, the therapeutic utility of anticancer CAPs would be significantly limited. Consequently, this hypothesis warrants further investigation.

LDH-release assays showed that [D]-NRC-03 killed breast cancer cells in a time-dependent manner with activity that peaked at 8 h. This finding was interesting because NRC-03-mediated cell death peaked earlier at 4 h. This study also confirmed that [D]-NRC-03 was a more potent killer of MDA-MB-231 cells than NRC-03. SEM was subsequently used to confirm that [D]-NRC-03 killed breast cancer cells by a cytolytic mechanism of action. Microscopic examination confirmed that [D]-NRC-03-induced membrane damage lagged behind NRC-03-induced membrane damage; however, by 4 h the majority of [D]-NRC-03-treated cells had undergone lysis. These data suggest that [D]-NRC-03 has a similar mechanism of action as NRC-03. These data suggest that NRC-03 may bind to target cell membranes faster than [D]-NRC-03; however, the secondary structure of [D]-NRC-03 should be identical to NRC-03 except that [D]-NRC-

03 likely forms a left-handed α-helix rather than a right-handed α-helix. Further studies examining the kinetics of [D]-NRC-03 binding to breast cancer cells, as well as [D]-NRC-03 intracellular localization should be conducted to compare the kinetics of [D]-NRC-03-mediated cell killing to NRC-03-mediated cell killing.

6.6 Antitumour Properties of [D]-NRC-03

NRC-03 and NRC-07 are anticancer CAPs that killed breast cancer cells grown as xenografts in immune deficient mice; however, the clinical potential of NRC-03 is limited by its susceptibility to proteolytic degradation. [D]-NRC-03 is a potent, protease-resistant derivative of NRC-03. *In vivo* studies demonstrated that 125 µg [D]-NRC-03 administered by intratumoural injection of breast cancer xenografts elicited the same antitumour response as 500 µg NRC-03 injected in the same manner, suggesting that [D]-NRC-03 is also more potent that NRC-03 *in vivo*. Note that the apparent difference in tumour growth in the xenograft study evaluating the antitumour properties of NRC-03 and NRC-07 (Fig. 3.14) and the study evaluating the antitumour properties of NRC-03 and [D]-NRC-03 (Fig. 4.10) is likely due differences in the experimental design, where the former was repeated thrice and the latter was only performed once. Necropsies of control- and peptide-treated mice, and histological examination of control- and peptide-treated tumours, showed identical findings to the xenograft model discussed in section 6.3. Like the xenograft study that evaluated the antitumour properties of NRC-03 and NRC-07, this study is limited by the route of administration of NRC-03 and [D]-NRC-03. Furthermore, the clinical potential of [D]-NRC-03 is limited by its toxicity to normal cells. Therefore, future experiments aim to identify derivatives of [D]-NRC-03, herein referred to as [Dmod]-NRC-03, with improved selectivity for cancer cells. This might be achieved by replacing D-lysine and/or D-arginine residues with D-histidine, by adding a targeting motif, or by using nanotechnology delivery systems (90, 129-131). The anticancer potential of [Dmod]-NRC-03 should then be evaluated *in vivo* as described in section 6.8.

6.7 Mechanisms of Cancer Cell Resistance to NRC-03 and NRC-07

Chemoresistance represents one of the most significant limitations to chemotherapy-based treatments of cancer. The mechanism(s) by which cancer cells become resistant to chemotherapy depend on the drug in question. For example, resistance to cisplatin can occur by increasing DNA repair of cisplatin/DNA lesions, or by increasing the synthesis of GSH, which binds cisplatin and forms a complex that is pumped out of the cell (21-23). In contrast, resistance to taxanes may occur by increasing the expression of drug efflux proteins, or by altering β-tubulin isotype expression (26-28). Experts argue that cancer cell resistance to CAPs with anticancer properties would be a rare occurrence because CAPs are thought to be attracted to many different negatively charged molecules present on the surface of the cancer cell (38, 61, 67). Therefore, the cancer cell would have to change the expression of several molecules rather than a single receptor or downstream effecter molecule in order to acquire resistance to CAPs. Moreover, research suggests that many CAPs likely do not require access to the inside of the cell in order to mediate cytotoxicity (56, 95, 96). In these instances, cancer cells cannot become resistant to the cytolytic peptide by eliminating it from the cytosol. To date melittin is the only CAP with anticancer properties with documented resistance by eukaryotic cells. Melittin is a 26 aa direct-acting CAP that is the principal active component in bee venom (193). Melittin is an immunogenic and hemolytic CAP that selectively kills ras-transformed NIH-3T3 cells (mouse embryonic fibroblasts), which become resistant to melittin by decreasing Ras expression (132, 175, 194). Interestingly, melittin-resistant Ras-transformed NIH-3T3 cells were morphologically normal (194). While these results are interesting, they do not adequately address the issue of cancer cell resistance to direct-acting CAPs.

The data collected throughout this study suggest that NRC-03 and NRC-07 are direct-acting CAPs that kill cancer cells by causing significant and irreversible damage to the cellular membrane. NRC-03- and NRC-07-induced cytolysis correlated with NRC-03 and NRC-07 binding to cancer cells. Surprisingly, prolonged exposure to increasing concentrations of NRC-03 or NRC-07 generated NRC-03- and NRC-07-resistant breast cancer cells. This finding rejects the hypothesis that cancer cells cannot become resistant to direct-acting CAPs. NRC-03- and NRC-07-resistant breast cancer cells were also

resistant to both NRC-07 and NRC-03, respectively, which is not surprising considering that NRC-03 and NRC-07 have similar anticancer properties and mechanisms of action. However, both NRC-03- and NRC-07-resistant cancer cells could be killed by higher concentrations of NRC-03 or NRC-07; albeit this is unlikely to be clinically useful because these peptide concentrations are likely to be significantly cytotoxic for normal cells. Not surprisingly, NRC-03 and NRC-07 bound to resistant cells to a lesser extent than parental cells, which supports the hypothesis that peptide binding is required for cell death; in fact, NRC-03 and NRC-07 binding to resistant cells was comparable to NRC-03 and NRC-07 binding to HDFs, which were not killed by NRC-03 or NRC-07. A morphological comparison of NRC-03- and NRC-07-resistant breast cancer cells to parental cells showed that NRC-03-resistant cells resembled epithelial cells, whereas NRC-07-resistant cells exhibited membrane blebbing. Although unlikely, prolonged exposure to NRC-03 may have caused genetic changes that caused the cell to become more like a normal epithelial cell. This hypothesis is consistent with the study that examined melittin-resistant ras-transformed NIH-3T3 cells, and could be tested using a bioinformatics approach. The significance of the membrane blebs observed in NRC-07-resistant cells, and to a lesser extent NRC-03-resistant cells, has not been elucidated in this study. Importantly, membrane blebs were also seen in NRC-03- and NRC-07-treated MDA-MB-231 cells. Membrane blebbing has been documented in melittin-treated liposomes, as well as latarcin 2a-treated erythroleukemia cells (195, 196). However, to my knowledge the significance of CAP-induced membrane blebbing has not been elucidated. It is noteworthy that in many cases the membrane blebs consisted of damaged membrane, whereas the rest of the cellular membrane appeared normal. Future studies could use transmission electron microscopy and straptavidin-colloidal gold staining to compare the amount of biotinylated peptide molecules bound to the membrane blebs versus molecules bound to the cell body. Importantly, the data obtained in this study do not identify the mechanisms responsible for the alterations in phenotype of NRC-03- or NRC-07-resistant cells, nor do they adequately address mechanism(s) of cancer cell resistance to NRC-03 or NRC-07. Ongoing microarray and metabolic labeling experiments aim to identify potential mechanisms of cancer cell resistance to NRC-03

and NRC-07. These data may also provide additional information on the mechanism of action of NRC-03 and/or NRC-07

Cancer is often treated with combinational treatment regimens that are selected based on evidence collected in clinical trials (162). Generally, therapies with different mechanism(s) of action and toxicities are combined to decrease the likelihood of cancer cells developing resistance to these regimens, increase the clinical response, and limit the side effects experienced by the patient. The data collected in this study suggest that NRC-03 and NRC-07 have different mechanisms of action than cisplatin and docetaxel. Moreover, NRC-03, and to a lesser extent NRC-07, enhance cell killing by cisplatin. Furthermore, NRC-03 and NRC-07 kill taxane-resistant breast cancer cells. NRC-03- and NRC-07-resistant breast cancer cells are also killed by cisplatin and docetaxel. Collectively, these findings suggest that NRC-03 and/or NRC-07 may be useful candidates for addition to combinational treatment regimens for the treatment of breast cancer. Future studies should identify whether NRC-03 and/or NRC-07 enhance docetaxel- and/or cisplatin-induced breast cancer cell killing *in vivo*. Importantly, NRC-03- and NRC-07-resistant breast cancer cells were killed by [D]-NRC-03, albeit it to a lesser extent than parental cells. Killing of resistant cells by [D]-NRC-03 is likely mediated by its reduced selectivity for cancer cells; i.e., [D]-NRC-03 likely kills all cells indiscriminately. Future studies should identify whether NRC-03- or NRC-07-resistant breast cancer cells can be killed by other direct- and/or indirect-acting CAPs. Results from this study could clarify whether all direct-acting CAPs function by a similar mechanism, or whether there are subtle differences between different cytolytic peptide families.

6.8 Future Directions

The next objective of this investigation should be to identify a derivative of [D]-NRC-03 with improved selectivity for cancer cells ([Dmod]-NRC-03). The toxicity of systemically-delivered [Dmod]-NRC-03, i.e., LD_{50}, should be determined to identify a dose that can safely be delivered systemically to tumour-bearing mice. Ideally, pharmacokinetic and pharmacodynamic studies should also be conducted on [Dmod]-NRC-03. These studies may, for example, identify potential drug-related problems such

as drug interactions. Once an appropriate dose of [Dmod]-NRC-03 is identified, *in vivo* studies could be conducted to determine whether systemically-delivered [Dmod]-NRC-03 reduces tumour growth in mice bearing breast cancer xenografts. Additional *in vivo* studies could also determine whether [Dmod]-NRC-03 enhances the antitumour properties of cisplatin and/or docetaxel. The ability of [Dmod]-NRC-03 to kill breast cancer metastasis could then be evaluated. In this regard, mouse mammary cancer cells could be injected into the tail vein or mammary fat pad of syngeneic mice, which would evaluate the antitumour properties of [Dmod]-NRC-03 in immune-competent mice. These studies would determine whether [Dmod]-NRC-03 can elicit an antitumour immune response as described by Berge and colleagues (95). Alternatively, breast cancer tissues isolated from patients could be surgically implanted into immune-deficient mice.

Studies should also be conducted to determine whether NRC-03 and/or NRC-07 selectivity for cancer cells is dependent on the individual patient. Microarray and metabolic-labeling experiments should be completed to elucidate mechanisms of cancer cell resistance to NRC-03 and NRC-07. Additionally, the tumourgenicity of NRC-03- and NRC-07-resistant breast cancer cells should be determined. This study would further elucidate the clinical implications of cancer cell resistance to NRC-03 and/or NRC-07. Collectively, these studies would determine the clinical potential of pleurocidins and their derivatives.

6.9 Conclusions

The data collected throughout this investigation suggest that NRC-03 and NRC-07 are direct-acting CAPs that kill breast cancer cells by causing significant and irreversible membrane damage, which leads to cell death by necrosis. NRC-03- and NRC-07-induced cytolysis was likely dependent on peptide binding to several different types of negatively charged molecules present on the surface of breast cancer cells. NRC-03 and NRC-07 killed multidrug-resistant breast cancer cells but caused minimal damage to normal cells, although this may vary between individuals. NRC-03, and to a lesser extent NRC-07, enhanced breast cancer cell killing by cisplatin, which suggests that these peptides may be a valuable addition to combinational treatment regimens for the treatment and/or management of breast cancer. Importantly, both NRC-03 and NRC-07

exhibited antitumour properties *in vivo*; however, the clinical use of NRC-03 is limited by its susceptibility to proteolytic degradation. Proteolytic degradation of NRC-03 was overcome by creating [D]-NRC-03, which was more potent than NRC-03, both *in vitro* and *in vivo*. Like NRC-03, [D]-NRC-03 was membranolytic; however, [D]-NRC-03 was slower-acting than NRC-03, and was less selective for cancer cells. Additional studies are therefore needed to develop derivatives of [D]-NRC-03 with improved selectivity for cancer cells.

This study found that resistance to membranolytic CAPs is possible and is most likely a consequence of reduced peptide binding to cancer cell membranes. Further experiments are required to determine mechanisms of cancer cell resistance to pleurocidins. Importantly, NRC-03- and NRC-07-resistant cancer cells were killed by other cytotoxic chemotherapeutic agents, as well as by [D]-NRC-03. Collectively, these data suggest that derivatives of [D]-NRC-03 and/or NRC-07 with enhanced selectivity for cancer cells may hold potential as novel therapeutic agents for the treatment of breast cancer.

REFERENCES

1.   Canadian Cancer Society's Steering Committee on Cancer Statistics. *Canadian Cancer Statistics 2011.* Toronto, ON: Canadian Cancer Society; 2011.

2.   Renan, M. J. 1993. How many mutations are required for tumorigenesis? Implications from human cancer data. *Mol Carcinog* 7:139-146.

3.   Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100:57-70.

4.   Hanahan, D., and R. A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell* 144:646-674.

5.   Espinosa, E., P. Zamora, J. Feliu, and M. Gonzalez Baron. 2003. Classification of anticancer drugs--a new system based on therapeutic targets. *Cancer Treat Rev* 29:515-523.

6.   Maughan, K. L., M. A. Lutterbie, and P. S. Ham. 2010. Treatment of breast cancer. *Am Fam Physician* 81:1339-1346.

7.   Romond, E. H., E. A. Perez, J. Bryant, V. J. Suman, C. E. Geyer, Jr., N. E. Davidson, E. Tan-Chiu, S. Martino, S. Paik, P. A. Kaufman, S. M. Swain, T. M. Pisansky, L. Fehrenbacher, L. A. Kutteh, V. G. Vogel, D. W. Visscher, G. Yothers, R. B. Jenkins, A. M. Brown, S. R. Dakhil, E. P. Mamounas, W. L. Lingle, P. M. Klein, J. N. Ingle, and N. Wolmark. 2005. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 353:1673-1684.

8.   Smith, I., M. Procter, R. D. Gelber, S. Guillaume, A. Feyereislova, M. Dowsett, A. Goldhirsch, M. Untch, G. Mariani, J. Baselga, M. Kaufmann, D. Cameron, R. Bell, J. Bergh, R. Coleman, A. Wardley, N. Harbeck, R. I. Lopez, P. Mallmann, K. Gelmon, N. Wilcken, E. Wist, P. Sanchez Rovira, and M. J. Piccart-Gebhart. 2007. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet* 369:29-36.

9.   Pal, S. K., and J. Mortimer. 2009. Triple-negative breast cancer: novel therapies and new directions. *Maturitas* 63:269-274.

10.   Greene, D., L. M. Nail, V. K. Fieler, D. Dudgeon, and L. S. Jones. 1994. A comparison of patient-reported side effects among three chemotherapy regimens for breast cancer. *Cancer Pract* 2:57-62.

11.   Shapiro, C. L., and A. Recht. 2001. Side effects of adjuvant treatment of breast cancer. *N Engl J Med* 344:1997-2008.

12. Smith, I. E., and D. C. Talbot. 1992. Cisplatin and its analogues in the treatment of advanced breast cancer: a review. *Br J Cancer* 65:787-793.

13. Palumbo, A., and K. Anderson. 2011. Multiple myeloma. *N Engl J Med* 364:1046-1060.

14. Brenner, H., A. Gondos, and D. Pulte. 2008. Recent major improvement in long-term survival of younger patients with multiple myeloma. *Blood* 111:2521-2526.

15. Kristinsson, S. Y., O. Landgren, P. W. Dickman, A. R. Derolf, and M. Bjorkholm. 2007. Patterns of survival in multiple myeloma: a population-based study of patients diagnosed in Sweden from 1973 to 2003. *J Clin Oncol* 25:1993-1999.

16. Kumar, S. K., S. V. Rajkumar, A. Dispenzieri, M. Q. Lacy, S. R. Hayman, F. K. Buadi, S. R. Zeldenrust, D. Dingli, S. J. Russell, J. A. Lust, P. R. Greipp, R. A. Kyle, and M. A. Gertz. 2008. Improved survival in multiple myeloma and the impact of novel therapies. *Blood* 111:2516-2520.

17. Jamieson, E. R., and S. J. Lippard. 1999. Structure, recognition, and processing of Cisplatin-DNA Adducts. *Chem Rev* 99:2467-2498.

18. Yang, X. L., and A. H. Wang. 1999. Structural studies of atom-specific anticancer drugs acting on DNA. *Pharmacol Ther* 83:181-215.

19. Speelmans, G., R. W. Staffhorst, K. Versluis, J. Reedijk, and B. de Kruijff. 1997. Cisplatin complexes with phosphatidylserine in membranes. *Biochemistry* 36:10545-10550.

20. Fuertes, M. A., J. Castilla, C. Alonso, and J. M. Perez. 2003. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. *Curr Med Chem* 10:257-266.

21. Godwin, A. K., A. Meister, P. J. O'Dwyer, C. S. Huang, T. C. Hamilton, and M. E. Anderson. 1992. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci U S A* 89:3070-3074.

22. Ishikawa, T., and F. Ali-Osman. 1993. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem* 268:20116-20125.

23. Parker, R. J., A. Eastman, F. Bostick-Bruton, and E. Reed. 1991. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J Clin Invest* 87:772-777.

24.   Cragg, G. M., and D. J. Newman. 2005. Plants as a source of anti-cancer agents. *J Ethnopharmacol* 100:72-79.

25.   Dumontet, C., and B. I. Sikic. 1999. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* 17:1061-1070.

26.   Hill, B. T., R. D. Whelan, S. A. Shellard, S. McClean, and L. K. Hosking. 1994. Differential cytotoxic effects of docetaxel in a range of mammalian tumor cell lines and certain drug resistant sublines in vitro. *Invest New Drugs* 12:169-182.

27.   Liu, B., E. D. Staren, T. Iwamura, H. E. Appert, and J. M. Howard. 2001. Mechanisms of taxotere-related drug resistance in pancreatic carcinoma. *J Surg Res* 99:179-186.

28.   Shalli, K., I. Brown, S. D. Heys, and A. C. Schofield. 2005. Alterations of beta-tubulin isotypes in breast cancer cells resistant to docetaxel. *FASEB J* 19:1299-1301.

29.   Donnelly, J. G. 2004. Pharmacogenetics in cancer chemotherapy: balancing toxicity and response. *Ther Drug Monit* 26:231-235.

30.   Naumov, G. N., J. L. Townson, I. C. MacDonald, S. M. Wilson, V. H. Bramwell, A. C. Groom, and A. F. Chambers. 2003. Ineffectiveness of doxorubicin treatment on solitary dormant mammary carcinoma cells or late-developing metastases. *Breast Cancer Res Treat* 82:199-206.

31.   Bush, J. A., and G. Li. 2002. Cancer chemoresistance: the relationship between p53 and multidrug transporters. *Int J Cancer* 98:323-330.

32.   Smith, L. L., K. Brown, P. Carthew, C. K. Lim, E. A. Martin, J. Styles, and I. N. White. 2000. Chemoprevention of breast cancer by tamoxifen: risks and opportunities. *Crit Rev Toxicol* 30:571-594.

33.   Curtis, R. E., J. D. Boice, Jr., M. Stovall, L. Bernstein, R. S. Greenberg, J. T. Flannery, A. G. Schwartz, P. Weyer, W. C. Moloney, and R. N. Hoover. 1992. Risk of leukemia after chemotherapy and radiation treatment for breast cancer. *N Engl J Med* 326:1745-1751.

34.   Dean-Colomb, W., and F. J. Esteva. 2008. Her2-positive breast cancer: herceptin and beyond. *Eur J Cancer* 44:2806-2812.

35.   Nagy, P., E. Friedlander, M. Tanner, A. I. Kapanen, K. L. Carraway, J. Isola, and T. M. Jovin. 2005. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res* 65:473-482.

36.   Nahta, R., D. Yu, M. C. Hung, G. N. Hortobagyi, and F. J. Esteva. 2006. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 3:269-280.

37.   Mader, J. S., and D. W. Hoskin. 2006. Cationic antimicrobial peptides as novel cytotoxic agents for cancer treatment. *Expert Opin Investig Drugs* 15:933-946.

38.   Hoskin, D. W., and A. Ramamoorthy. 2008. Studies on anticancer activities of antimicrobial peptides. *Biochim Biophys Acta* 1778:357-375.

39.   Bulet, P., R. Stocklin, and L. Menin. 2004. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev* 198:169-184.

40.   Patrzykat, A., and S. E. Douglas. 2003. Gone gene fishing: how to catch novel marine antimicrobials. *Trends Biotechnol* 21:362-369.

41.   Powers, J. P., and R. E. Hancock. 2003. The relationship between peptide structure and antibacterial activity. *Peptides* 24:1681-1691.

42.   Easton, D. M., A. Nijnik, M. L. Mayer, and R. E. Hancock. 2009. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol* 27:582-590.

43.   Hancock, R. E. 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect Dis* 1:156-164.

44.   Hancock, R. E., and H. G. Sahl. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 24:1551-1557.

45.   Silva, P. I., Jr., S. Daffre, and P. Bulet. 2000. Isolation and characterization of gomesin, an 18-residue cysteine-rich defense peptide from the spider *Acanthoscurria gomesiana* hemocytes with sequence similarities to horseshoe crab antimicrobial peptides of the tachyplesin family. *J Biol Chem* 275:33464-33470.

46.   Rozek, T., K. L. Wegener, J. H. Bowie, I. N. Olver, J. A. Carver, J. C. Wallace, and M. J. Tyler. 2000. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis* the solution structure of aurein 1.2. *Eur J Biochem* 267:5330-5341.

47.   Patrzykat, A., C. L. Friedrich, L. Zhang, V. Mendoza, and R. E. Hancock. 2002. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. *Antimicrob Agents Chemother* 46:605-614.

48.   Patrzykat, A., J. W. Gallant, J. K. Seo, J. Pytyck, and S. E. Douglas. 2003. Novel antimicrobial peptides derived from flatfish genes. *Antimicrob Agents Chemother* 47:2464-2470.

49. Chattopadhyay, S., N. K. Sinha, S. Banerjee, D. Roy, D. Chattopadhyay, and S. Roy. 2006. Small cationic protein from a marine turtle has beta-defensin-like fold and antibacterial and antiviral activity. *Proteins* 64:524-531.

50. Lopez-Garcia, B., P. H. Lee, K. Yamasaki, and R. L. Gallo. 2005. Anti-fungal activity of cathelicidins and their potential role in *Candida albicans* skin infection. *J Invest Dermatol* 125:108-115.

51. Aley, S. B., M. Zimmerman, M. Hetsko, M. E. Selsted, and F. D. Gillin. 1994. Killing of *Giardia lamblia* by cryptdins and cationic neutrophil peptides. *Infect Immun* 62:5397-5403.

52. Lehmann, J., M. Retz, S. S. Sidhu, H. Suttmann, M. Sell, F. Paulsen, J. Harder, G. Unteregger, and M. Stockle. 2006. Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines. *Eur Urol* 50:141-147.

53. Eliassen, L. T., G. Berge, A. Leknessund, M. Wikman, I. Lindin, C. Lokke, F. Ponthan, J. I. Johnsen, B. Sveinbjornsson, P. Kogner, T. Flaegstad, and O. Rekdal. 2006. The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo. *Int J Cancer* 119:493-500.

54. Liberio, M. S., G. A. Joanitti, R. B. Azevedo, E. M. Cilli, L. C. Zanotta, A. C. Nascimento, M. V. Sousa, O. R. Pires Junior, W. Fontes, and M. S. Castro. 2011. Anti-proliferative and cytotoxic activity of pentadactylin isolated from *Leptodactylus labyrinthicus* on melanoma cells. *Amino Acids* 40:51-59.

55. Mader, J. S., J. Salsman, D. M. Conrad, and D. W. Hoskin. 2005. Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. *Mol Cancer Ther* 4:612-624.

56. Rodrigues, E. G., A. S. Dobroff, C. F. Cavarsan, T. Paschoalin, L. Nimrichter, R. A. Mortara, E. L. Santos, M. A. Fazio, A. Miranda, S. Daffre, and L. R. Travassos. 2008. Effective topical treatment of subcutaneous murine B16F10-Nex2 melanoma by the antimicrobial peptide gomesin. *Neoplasia* 10:61-68.

57. Chang, W. T., C. Y. Pan, V. Rajanbabu, C. W. Cheng, and J. Y. Chen. 2011. Tilapia (*Oreochromis mossambicus*) antimicrobial peptide, hepcidin 1-5, shows antitumor activity in cancer cells. *Peptides* 32:342-352.

58. Austin, B. a. M., D. 1988. Natural antimicrobial compounds on the surface of rainbow trout, *Salmo gairdneri* Richardson. *J Fish Dis* 11:275-277

59. Cole, A. M., P. Weis, and G. Diamond. 1997. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem* 272:12008-12013.

60. Cole, A. M., R. O. Darouiche, D. Legarda, N. Connell, and G. Diamond. 2000. Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. *Antimicrob Agents Chemother* 44:2039-2045.

61. Hilchie, A. L., and Hoskin, D.W. 2010. The application of cationic antimicrobial peptides in cancer treatment: laboratory investigations and clinical potential. In *Emerging Cancer Therapy: Microbial Approaches and Biotechnological Tools*. A. Chakrabarty, ed. John Wiley & Sons, Inc., Hoboken, NJ. 309-332.

62. Oh, J. T., Y. Cajal, E. M. Skowronska, S. Belkin, J. Chen, T. K. Van Dyk, M. Sasser, and M. K. Jain. 2000. Cationic peptide antimicrobials induce selective transcription of micF and osmY in *Escherichia coli*. *Biochim Biophys Acta* 1463:43-54.

63. Cabiaux, V., B. Agerberth, J. Johansson, F. Homble, E. Goormaghtigh, and J. M. Ruysschaert. 1994. Secondary structure and membrane interaction of PR-39, a Pro+Arg-rich antibacterial peptide. *Eur J Biochem* 224:1019-1027.

64. Ghavami, S., A. Asoodeh, T. Klonisch, A. J. Halayko, K. Kadkhoda, T. J. Kroczak, S. B. Gibson, E. P. Booy, H. Naderi-Manesh, and M. Los. 2008. Brevinin-2R(1) semi-selectively kills cancer cells by a distinct mechanism, which involves the lysosomal-mitochondrial death pathway. *J Cell Mol Med* 12:1005-1022.

65. Yeaman, M. R., and N. Y. Yount. 2003. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* 55:27-55.

66. Bhutia, S. K., and T. K. Maiti. 2008. Targeting tumors with peptides from natural sources. *Trends Biotechnol* 26:210-217.

67. Giuliani, A., Pirri, G., Nicoletto, S.F. 2007. Antimicrobial peptides: an overview of a promising class of therapeutics. *Central European Journal of Biology* 2:1-33.

68. Chen, H. M., W. Wang, D. Smith, and S. C. Chan. 1997. Effects of the anti-bacterial peptide cecropin B and its analogs, cecropins B-1 and B-2, on liposomes, bacteria, and cancer cells. *Biochim Biophys Acta* 1336:171-179.

69. Hui, L., K. Leung, and H. M. Chen. 2002. The combined effects of antibacterial peptide cecropin A and anti-cancer agents on leukemia cells. *Anticancer Res* 22:2811-2816.

70. Ohsaki, Y., A. F. Gazdar, H. C. Chen, and B. E. Johnson. 1992. Antitumor activity of magainin analogues against human lung cancer cell lines. *Cancer Res* 52:3534-3538.

71.   Kim, S., S. S. Kim, Y. J. Bang, S. J. Kim, and B. J. Lee. 2003. In vitro activities of native and designed peptide antibiotics against drug sensitive and resistant tumor cell lines. *Peptides* 24:945-953.

72.   Johnstone, S. A., K. Gelmon, L. D. Mayer, R. E. Hancock, and M. B. Bally. 2000. In vitro characterization of the anticancer activity of membrane-active cationic peptides. I. Peptide-mediated cytotoxicity and peptide-enhanced cytotoxic activity of doxorubicin against wild-type and p-glycoprotein over-expressing tumor cell lines. *Anticancer Drug Des* 15:151-160.

73.   Papo, N., and Y. Shai. 2005. Host defense peptides as new weapons in cancer treatment. *Cell Mol Life Sci* 62:784-790.

74.   Papo, N., M. Shahar, L. Eisenbach, and Y. Shai. 2003. A novel lytic peptide composed of DL-amino acids selectively kills cancer cells in culture and in mice. *J Biol Chem* 278:21018-21023.

75.   Schweizer, F. 2009. Cationic amphiphilic peptides with cancer-selective toxicity. *Eur J Pharmacol* 625:190-194.

76.   Zachowski, A. 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem J* 294 (Pt 1):1-14.

77.   Utsugi, T., A. J. Schroit, J. Connor, C. D. Bucana, and I. J. Fidler. 1991. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res* 51:3062-3066.

78.   Gazit, E., A. Boman, H. G. Boman, and Y. Shai. 1995. Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry* 34:11479-11488.

79.   Gazit, E., W. J. Lee, P. T. Brey, and Y. Shai. 1994. Mode of action of the antibacterial cecropin B2: a spectrofluorometric study. *Biochemistry* 33:10681-10692.

80.   Matsuzaki, K., M. Harada, T. Handa, S. Funakoshi, N. Fujii, H. Yajima, and K. Miyajima. 1989. Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles. *Biochim Biophys Acta* 981:130-134.

81.   Bafna, S., S. Kaur, and S. K. Batra. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. 2010. *Oncogene* 29:2893-2904.

82.   Koo, C. Y., Y. P. Sen, B. H. Bay, and G. W. Yip. 2008. Targeting heparan sulfate proteoglycans in breast cancer treatment. *Recent Pat Anticancer Drug Discov* 3:151-158.

83.   van Beek, W. P., L. A. Smets, and P. Emmelot. 1973. Increased sialic acid density in surface glycoprotein of transformed and malignant cells--a general phenomenon? *Cancer Res* 33:2913-2922.

84.   Fadnes, B., O. Rekdal, and L. Uhlin-Hansen. 2009. The anticancer activity of lytic peptides is inhibited by heparan sulfate on the surface of the tumor cells. *BMC Cancer* 9:183.

85.   Fadnes, B., L. Uhlin-Hansen, I. Lindin, and O. Rekdal. 2011. Small lytic peptides escape the inhibitory effect of heparan sulfate on the surface of cancer cells. *BMC Cancer* 11:116.

86.   Zwaal, R. F., and A. J. Schroit. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89:1121-1132.

87.   Chan, S. C., L. Hui, and H. M. Chen. 1998. Enhancement of the cytolytic effect of anti-bacterial cecropin by the microvilli of cancer cells. *Anticancer Res* 18:4467-4474.

88.   Sok, M., M. Sentjurc, and M. Schara. 1999. Membrane fluidity characteristics of human lung cancer. *Cancer Lett* 139:215-220.

89.   Kozlowska, K., J. Nowak, B. Kwiatkowski, and M. Cichorek. 1999. ESR study of plasmatic membrane of the transplantable melanoma cells in relation to their biological properties. *Exp Toxicol Pathol* 51:89-92.

90.   Leuschner, C., and W. Hansel. 2004. Membrane disrupting lytic peptides for cancer treatments. *Curr Pharm Des* 10:2299-2310.

91.   Lodish, H., Berk, A., Kaiser, M., Scott, M.P., Bretscher, A., Ploegh, H., Matsudaira, P. 2008. *Molecular Cell Biology*.

92.   Yeagle, P. L. 1985. Cholesterol and the cell membrane. *Biochim Biophys Acta* 822:267-287.

93.   Vaz Gomes, A., A. de Waal, J. A. Berden, and H. V. Westerhoff. 1993. Electric potentiation, cooperativity, and synergism of magainin peptides in protein-free liposomes. *Biochemistry* 32:5365-5372.

94.   Cruciani, R. A., J. L. Barker, M. Zasloff, H. C. Chen, and O. Colamonici. 1991. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. *Proc Natl Acad Sci U S A* 88:3792-3796.

95.   Berge, G., L. T. Eliassen, K. A. Camilio, K. Bartnes, B. Sveinbjornsson, and O. Rekdal. 2010. Therapeutic vaccination against a murine lymphoma by intratumoral injection of a cationic anticancer peptide. *Cancer Immunol Immunother* 59:1285-1294.

96.   Huang, W., L. Lu, X. Shao, C. Tang, and X. Zhao. 2010. Anti-melanoma activity of hybrid peptide P18 and its mechanism of action. *Biotechnol Lett* 32:463-469.

97.   Lin, W. J., Y. L. Chien, C. Y. Pan, T. L. Lin, J. Y. Chen, S. J. Chiu, and C. F. Hui. 2009. Epinecidin-1, an antimicrobial peptide from fish (Epinephelus coioides) which has an antitumor effect like lytic peptides in human fibrosarcoma cells. *Peptides* 30:283-290.

98.   Steiner, H., D. Hultmark, A. Engstrom, H. Bennich, and H. G. Boman. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292:246-248.

99.   Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* 84:5449-5453.

100.   Shin, S. Y., S. H. Lee, S. T. Yang, E. J. Park, D. G. Lee, M. K. Lee, S. H. Eom, W. K. Song, Y. Kim, K. S. Hahm, and J. I. Kim. 2001. Antibacterial, antitumor and hemolytic activities of alpha-helical antibiotic peptide, P18 and its analogs. *J Pept Res* 58:504-514.

101.   Pan, C. Y., J. Y. Chen, Y. S. Cheng, C. Y. Chen, I. H. Ni, J. F. Sheen, Y. L. Pan, and C. M. Kuo. 2007. Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*), and its role in protecting fish against pathogenic infection. *DNA Cell Biol* 26:403-413.

102.   Baumann, G., and P. Mueller. 1974. A molecular model of membrane excitability. *J Supramol Struct* 2:538-557.

103.   He, K., S. J. Ludtke, D. L. Worcester, and H. W. Huang. 1996. Neutron scattering in the plane of membranes: structure of alamethicin pores. *Biophys J* 70:2659-2666.

104.   Huang, H. W. 2000. Action of antimicrobial peptides: two-state model. *Biochemistry* 39:8347-8352.

105.   Ludtke, S. J., K. He, Y. Wu, and H. W. Huang. 1994. Cooperative membrane insertion of magainin correlated with its cytolytic activity. *Biochim Biophys Acta* 1190:181-184.

106.   Ludtke, S. J., K. He, W. T. Heller, T. A. Harroun, L. Yang, and H. W. Huang. 1996. Membrane pores induced by magainin. *Biochemistry* 35:13723-13728.

107.   Mader, J. S., A. Richardson, J. Salsman, D. Top, R. de Antueno, R. Duncan, and D. W. Hoskin. 2007. Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria. *Exp Cell Res* 313:2634-2650.

108. Risso, A., M. Zanetti, and R. Gennaro. 1998. Cytotoxicity and apoptosis mediated by two peptides of innate immunity. *Cell Immunol* 189:107-115.

109. Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita. 1992. Identification of the bactericidal domain of lactoferrin. *Biochim Biophys Acta* 1121:130-136.

110. Eliassen, L. T., G. Berge, B. Sveinbjornsson, J. S. Svendsen, L. H. Vorland, and O. Rekdal. 2002. Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Res* 22:2703-2710.

111. Takada, Y., X. Ye, and S. Simon. 2007. The integrins. *Genome Biol* 8:215.

112. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* 238:491-497.

113. Nakamura, T., H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao, and Y. Shimonishi. 1988. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Isolation and chemical structure. *J Biol Chem* 263:16709-16713.

114. Chen, Y., X. Xu, S. Hong, J. Chen, N. Liu, C. B. Underhill, K. Creswell, and L. Zhang. 2001. RGD-Tachyplesin inhibits tumor growth. *Cancer Res* 61:2434-2438.

115. King, J. D., N. Al-Ghaferi, B. Abraham, A. Sonnevend, J. Leprince, P. F. Nielsen, and J. M. Conlon. 2005. Pentadactylin: an antimicrobial peptide from the skin secretions of the South American bullfrog *Leptodactylus pentadactylus*. *Comp Biochem Physiol C Toxicol Pharmacol* 141:393-397.

116. Mader, J. S., D. Smyth, J. Marshall, and D. W. Hoskin. 2006. Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor165-induced angiogenesis by competing for heparin-like binding sites on endothelial cells. *Am J Pathol* 169:1753-1766.

117. Furlong, S. J., J. S. Mader, and D. W. Hoskin. 2011. Bovine lactoferricin induces caspase-independent apoptosis in human B-lymphoma cells and extends the survival of immune-deficient mice bearing B-lymphoma xenografts. *Exp Mol Pathol* 88:371-375.

118. Yoo, Y. C., S. Watanabe, R. Watanabe, K. Hata, K. Shimazaki, and I. Azuma. 1998. Bovine lactoferrin and lactoferricin inhibit tumor metastasis in mice. *Adv Exp Med Biol* 443:285-291.

119. Yoo, Y. C., R. Watanabe, Y. Koike, M. Mitobe, K. Shimazaki, S. Watanabe, and I. Azuma. 1997. Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species. *Biochem Biophys Res Commun* 237:624-628.

120. Dennison, S. R., M. Whittaker, F. Harris, and D. A. Phoenix. 2006. Anticancer alpha-helical peptides and structure/function relationships underpinning their interactions with tumour cell membranes. *Curr Protein Pept Sci* 7:487-499.

121. Baker, M. A., W. L. Maloy, M. Zasloff, and L. S. Jacob. 1993. Anticancer efficacy of Magainin2 and analogue peptides. *Cancer Res* 53:3052-3057.

122. Park, Y., D. G. Lee, S. H. Jang, E. R. Woo, H. G. Jeong, C. H. Choi, and K. S. Hahm. 2003. A Leu-Lys-rich antimicrobial peptide: activity and mechanism. *Biochim Biophys Acta* 1645:172-182.

123. Soballe, P. W., W. L. Maloy, M. L. Myrga, L. S. Jacob, and M. Herlyn. 1995. Experimental local therapy of human melanoma with lytic magainin peptides. *Int J Cancer* 60:280-284.

124. Greenshields, A. L., L. C. Knickle, R. Syvitski, and S. E. Douglas. 2008. Strategies for recombinant expression of small, highly disulphide-bonded, cationic antimicrobial peptides. *Protein Pept Lett* 15:985-994.

125. Richardson, A., R. de Antueno, R. Duncan, and D. W. Hoskin. 2009. Intracellular delivery of bovine lactoferricin's antimicrobial core (RRWQWR) kills T-leukemia cells. *Biochem Biophys Res Commun* 388:736-741.

126. Peck-Miller, K. A., R. P. Darveau, and H. P. Fell. 1993. Identification of serum components that inhibit the tumoricidal activity of amphiphilic alpha helical peptides. *Cancer Chemother Pharmacol* 32:109-115.

127. Lichtenstein, A. K., T. Ganz, T. M. Nguyen, M. E. Selsted, and R. I. Lehrer. 1988. Mechanism of target cytolysis by peptide defensins. Target cell metabolic activities, possibly involving endocytosis, are crucial for expression of cytotoxicity. *J Immunol* 140:2686-2694.

128. Vajda, T., and T. Szabo. 1976. Specificity of trypsin and alpha-chymotrypsin towards neutral substrates. *Acta Biochim Biophys Acad Sci Hung* 11:287-294.

129. Soman, N. R., S. L. Baldwin, G. Hu, J. N. Marsh, G. M. Lanza, J. E. Heuser, J. M. Arbeit, S. A. Wickline, and P. H. Schlesinger. 2009. Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth. *J Clin Invest* 119:2830-2842.

130. Liu, S., H. Yang, L. Wan, H. W. Cai, S. F. Li, Y. P. Li, J. Q. Cheng, and X. F. Lu. 2011. Enhancement of cytotoxicity of antimicrobial peptide magainin II in tumor cells by bombesin-targeted delivery. *Acta Pharmacol Sin* 32:79-88.

131. Makovitzki, A., A. Fink, and Y. Shai. 2009. Suppression of human solid tumor growth in mice by intratumor and systemic inoculation of histidine-rich and pH-dependent host defense-like lytic peptides. *Cancer Res* 69:3458-3463.

132. King, T. P., D. Wade, M. R. Coscia, S. Mitchell, L. Kochoumian, and B. Merrifield. 1994. Structure-immunogenicity relationship of melittin, its transposed analogues, and D-melittin. *J Immunol* 153:1124-1131.

133. Coffelt, S. B., and A. B. Scandurro. 2008. Tumors sound the alarmin(s). *Cancer Res* 68:6482-6485.

134. Oppenheim, J. J., and D. Yang. 2005. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* 17:359-365.

135. Yang, N., W. Stensen, J. S. Svendsen, and O. Rekdal. 2002. Enhanced antitumor activity and selectivity of lactoferrin-derived peptides. *J Pept Res* 60:187-197.

136. Schibli, D. J., R. F. Epand, H. J. Vogel, and R. M. Epand. 2002. Tryptophan-rich antimicrobial peptides: comparative properties and membrane interactions. *Biochem Cell Biol* 80:667-677.

137. Strom, M. B., O. Rekdal, and J. S. Svendsen. 2000. Antibacterial activity of 15-residue lactoferricin derivatives. *J Pept Res* 56:265-274.

138. Nilsson, I., A. Saaf, P. Whitley, G. Gafvelin, C. Waller, and G. von Heijne. 1998. Proline-induced disruption of a transmembrane alpha-helix in its natural environment. *J Mol Biol* 284:1165-1175.

139. Chi, S. W., J. S. Kim, D. H. Kim, S. H. Lee, Y. H. Park, and K. H. Han. 2007. Solution structure and membrane interaction mode of an antimicrobial peptide gaegurin 4. *Biochem Biophys Res Commun* 352:592-597.

140. Shadidi, M., and M. Sioud. 2003. Identification of novel carrier peptides for the specific delivery of therapeutics into cancer cells. *FASEB J* 17:256-258.

141. Anastasi, A., V. Erspamer, and M. Bucci. 1971. Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians *Bombina* and *Alytes*. *Experientia* 27:166-167.

142. Reubi, J. C., S. Wenger, J. Schmuckli-Maurer, J. C. Schaer, and M. Gugger. 2002. Bombesin receptor subtypes in human cancers: detection with the universal radioligand (125)I-[D-TYR(6), beta-ALA(11), PHE(13), NLE(14)] bombesin(6-14). *Clin Cancer Res* 8:1139-1146.

143. Cornelio, D. B., R. Roesler, and G. Schwartsmann. 2007. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. *Ann Oncol* 18:1457-1466.

144. Zitzmann, S., V. Ehemann, and M. Schwab. 2002. Arginine-glycine-aspartic acid (RGD)-peptide binds to both tumor and tumor-endothelial cells in vivo. *Cancer Res* 62:5139-5143.

145.   Hansel, W., F. Enright, and C. Leuschner. 2007. Destruction of breast cancers and their metastases by lytic peptide conjugates in vitro and in vivo. *Mol Cell Endocrinol* 260-262:183-189.

146.   Newell, K., A. Franchi, J. Pouyssegur, and I. Tannock. 1993. Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc Natl Acad Sci U S A* 90:1127-1131.

147.   Vaupel, P., F. Kallinowski, and P. Okunieff. 1989. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 49:6449-6465.

148.   Tannock, I. F., and D. Rotin. 1989. Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* 49:4373-4384.

149.   Jung, H. J., Y. Park, W. S. Sung, B. K. Suh, J. Lee, K. S. Hahm, and D. G. Lee. 2007. Fungicidal effect of pleurocidin by membrane-active mechanism and design of enantiomeric analogue for proteolytic resistance. *Biochim Biophys Acta* 1768:1400-1405.

150.   Bessalle, R., A. Kapitkovsky, A. Gorea, I. Shalit, and M. Fridkin. 1990. All-D-magainin: chirality, antimicrobial activity and proteolytic resistance. *FEBS Lett* 274:151-155.

151.   Mas-Moruno, C., L. J. Cruz, P. Mora, A. Francesch, A. Messeguer, E. Perez-Paya, and F. Albericio. 2007. Smallest peptoids with antiproliferative activity on human neoplastic cells. *J Med Chem* 50:2443-2449.

152.   Song, Y. M., Y. Park, S. S. Lim, S. T. Yang, E. R. Woo, I. S. Park, J. S. Lee, J. I. Kim, K. S. Hahm, Y. Kim, and S. Y. Shin. 2005. Cell selectivity and mechanism of action of antimicrobial model peptides containing peptoid residues. *Biochemistry* 44:12094-12106.

153.   Chongsiriwatana, N. P., J. A. Patch, A. M. Czyzewski, M. T. Dohm, A. Ivankin, D. Gidalevitz, R. N. Zuckermann, and A. E. Barron. 2008. Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proc Natl Acad Sci U S A* 105:2794-2799.

154.   Pethe, K., P. Bifani, H. Drobecq, C. Sergheraert, A. S. Debrie, C. Locht, and F. D. Menozzi. 2002. Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. *Proc Natl Acad Sci U S A* 99:10759-10764.

155.   Radzishevsky, I. S., S. Rotem, D. Bourdetsky, S. Navon-Venezia, Y. Carmeli, and A. Mor. 2007. Improved antimicrobial peptides based on acyl-lysine oligomers. *Nat Biotechnol* 25:657-659.

156. Oh, J. E., and K. H. Lee. 1999. Synthesis of novel unnatural amino acid as a building block and its incorporation into an antimicrobial peptide. *Bioorg Med Chem* 7:2985-2990.

157. Svenson, J., W. Stensen, B. O. Brandsdal, B. E. Haug, J. Monrad, and J. S. Svendsen. 2008. Antimicrobial peptides with stability toward tryptic degradation. *Biochemistry* 47:3777-3788.

158. Lockwood, N. A., J. R. Haseman, M. V. Tirrell, and K. H. Mayo. 2004. Acylation of SC4 dodecapeptide increases bactericidal potency against Gram-positive bacteria, including drug-resistant strains. *Biochem J* 378:93-103.

159. Zweytick, D., G. Pabst, P. M. Abuja, A. Jilek, S. E. Blondelle, J. Andra, R. Jerala, D. Monreal, G. Martinez de Tejada, and K. Lohner. 2006. Influence of N-acylation of a peptide derived from human lactoferricin on membrane selectivity. *Biochim Biophys Acta* 1758:1426-1435.

160. Eliassen, L. T., B. E. Haug, G. Berge, and O. Rekdal. 2003. Enhanced antitumour activity of 15-residue bovine lactoferricin derivatives containing bulky aromatic amino acids and lipophilic N-terminal modifications. *J Pept Sci* 9:510-517.

161. Bernkop-Schnurch, A., and T. Schmitz. 2007. Presystemic metabolism of orally administered peptide drugs and strategies to overcome it. *Curr Drug Metab* 8:509-517.

162. Carter, S. K. 1972. Single and combination nonhormonal chemotherapy in breast cancer. *Cancer* 30:1543-1555.

163. Yoo, Y. C., S. Watanabe, R. Watanabe, K. Hata, K. Shimazaki, and I. Azuma. 1997. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn J Cancer Res* 88:184-190.

164. Banfield, B. W., Y. Leduc, L. Esford, K. Schubert, and F. Tufaro. 1995. Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. *J Virol* 69:3290-3298.

165. McCormick, C., Y. Leduc, D. Martindale, K. Mattison, L. E. Esford, A. P. Dyer, and F. Tufaro. 1998. The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nat Genet* 19:158-161.

166. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55-63.

167. Funk, D., H. H. Schrenk, and E. Frei. 2007. Serum albumin leads to false-positive results in the XTT and the MTT assay. *Biotechniques* 43:178, 180, 182 passim.

168. Vellonen, K. S., P. Honkakoski, and A. Urtti. 2004. Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity. *Eur J Pharm Sci* 23:181-188.

169. Matzinger, P. 1991. The JAM test. A simple assay for DNA fragmentation and cell death. *J Immunol Methods* 145:185-192.

170. Silvestri, M. E., and V. A. Sundqvist. 2001. An investigation into the heparin-binding properties of a synthetic peptide deduced from the antigenic domain 2 of human cytomegalovirus glycoprotein B. *Scand J Immunol* 53:282-289.

171. Shapiro, H. M. 1994. Cell membrane potential analysis. *Methods Cell Biol* 41:121-133.

172. Khalili, P., A. Arakelian, G. Chen, M. L. Plunkett, I. Beck, G. C. Parry, F. Donate, D. E. Shaw, A. P. Mazar, and S. A. Rabbani. 2006. A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo. *Mol Cancer Ther* 5:2271-2280.

173. Hawkins, W., C. Mitchell, R. McKinstry, D. Gilfor, J. Starkey, Y. Dai, K. Dawson, V. Ramakrishnan, J. D. Roberts, A. Yacoub, S. Grant, and P. Dent. 2005. Transient exposure of mammary tumors to PD184352 and UCN-01 causes tumor cell death in vivo and prolonged suppression of tumor regrowth. *Cancer Biol Ther* 4:1275-1284.

174. Fraker, L. D., S. A. Halter, and J. T. Forbes. 1984. Growth inhibition by retinol of a human breast carcinoma cell line in vitro and in athymic mice. *Cancer Res* 44:5757-5763.

175. Tosteson, M. T., S. J. Holmes, M. Razin, and D. C. Tosteson. 1985. Melittin lysis of red cells. *J Membr Biol* 87:35-44.

176. Dobrzynska, I., B. Szachowicz-Petelska, S. Sulkowski, and Z. Figaszewski. 2005. Changes in electric charge and phospholipids composition in human colorectal cancer cells. *Mol Cell Biochem* 276:113-119.

177. Yoon, W. H., H. D. Park, K. Lim, and B. D. Hwang. 1996. Effect of O-glycosylated mucin on invasion and metastasis of HM7 human colon cancer cells. *Biochem Biophys Res Commun* 222:694-699.

178. Koning, A. J., P. Y. Lum, J. M. Williams, and R. Wright. 1993. DiOC6 staining reveals organelle structure and dynamics in living yeast cells. *Cell Motil Cytoskeleton* 25:111-128.

179. Hamamoto, K., Y. Kida, Y. Zhang, T. Shimizu, and K. Kuwano. 2002. Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiol Immunol* 46:741-749.

180. Xu, L., S. Yin, S. Banerjee, F. Sarkar, and K. B. Reddy. 2011. Enhanced anticancer effect of the combination of cisplatin and TRAIL in triple-negative breast tumor cells. *Mol Cancer Ther* 10:550-557.

181. Korzeniewski, C., and D. M. Callewaert. 1983. An enzyme-release assay for natural cytotoxicity. *J Immunol Methods* 64:313-320.

182. Helmerhorst, E. J., I. M. Reijnders, W. van 't Hof, E. C. Veerman, and A. V. Nieuw Amerongen. 1999. A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. *FEBS Lett* 449:105-110.

183. Gray, M. W. 1988. Organelle origins and ribosomal RNA. *Biochem Cell Biol* 66:325-348.

184. Gray, M. W., G. Burger, and B. F. Lang. 1999. Mitochondrial evolution. *Science* 283:1476-1481.

185. Masuda, M., M. Suzui, J. T. Lim, A. Deguchi, J. W. Soh, and I. B. Weinstein. 2002. Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. *J Exp Ther Oncol* 2:350-359.

186. Kurebayashi, J., T. Otsuki, H. Kunisue, Y. Mikami, K. Tanaka, S. Yamamoto, and H. Sonoo. 1999. Expression of vascular endothelial growth factor (VEGF) family members in breast cancer. *Jpn J Cancer Res* 90:977-981.

187. Scott, P. A., J. M. Gleadle, R. Bicknell, and A. L. Harris. 1998. Role of the hypoxia sensing system, acidity and reproductive hormones in the variability of vascular endothelial growth factor induction in human breast carcinoma cell lines. *Int J Cancer* 75:706-712.

188. Tajmir-Riahi, H. A. 2007. An overview of drug binding to human serum albumin: protein folding and unfolding. *Scientia Iranica* 14:87-95.

189. Dennis, M. S., M. Zhang, Y. G. Meng, M. Kadkhodayan, D. Kirchhofer, D. Combs, and L. A. Damico. 2002. Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J Biol Chem* 277:35035-35043.

190. Stankovic, S., G. Konjevic, K. Gopcevic, V. Jovic, M. Inic, and V. Jurisic. 2010. Activity of MMP-2 and MMP-9 in sera of breast cancer patients. *Pathol Res Pract* 206:241-247.

191. Roth, C., K. Pantel, V. Muller, B. Rack, S. Kasimir-Bauer, W. Janni, and H. Schwarzenbach. 2011. Apoptosis-related deregulation of proteolytic activities and high serum levels of circulating nucleosomes and DNA in blood correlate with breast cancer progression. *BMC Cancer* 11:4.

192.   Piller, F., V. Piller, R. I. Fox, and M. Fukuda. 1988. Human T-lymphocyte activation is associated with changes in O-glycan biosynthesis. *J Biol Chem* 263:15146-15150.

193.   Kreil, G. 1973. Biosynthesis of melittin, a toxic peptide from bee venom. Amino-acid sequence of the precursor. *Eur J Biochem* 33:558-566.

194.   Sharma, S. V. 1992. Melittin resistance: a counterselection for ras transformation. *Oncogene* 7:193-201.

195.   Park, S. C., M. H. Kim, M. A. Hossain, S. Y. Shin, Y. Kim, L. Stella, J. D. Wade, Y. Park, and K. S. Hahm. 2008. Amphipathic alpha-helical peptide, HP (2-20), and its analogues derived from *Helicobacter pylori*: pore formation mechanism in various lipid compositions. *Biochim Biophys Acta* 1778:229-241.

196.   Vorontsova, O. V., N. S. Egorova, A. S. Arseniev, and A. V. Feofanov. 2011. Haemolytic and cytotoxic action of latarcin Ltc2a. *Biochimie* 93:227-241.
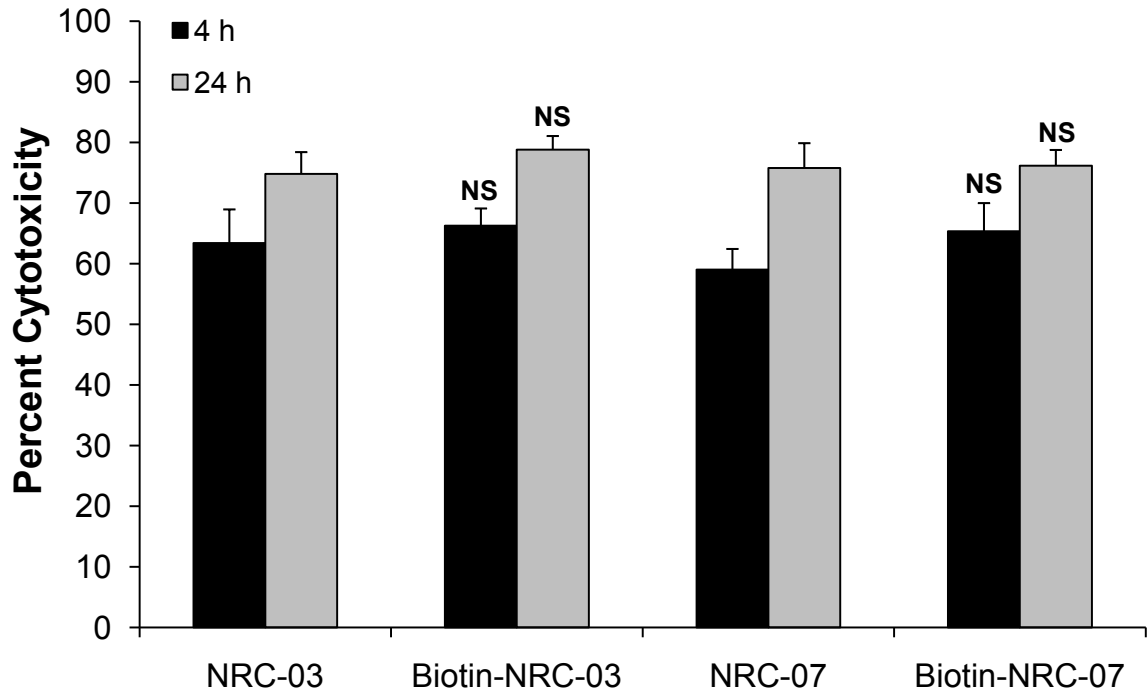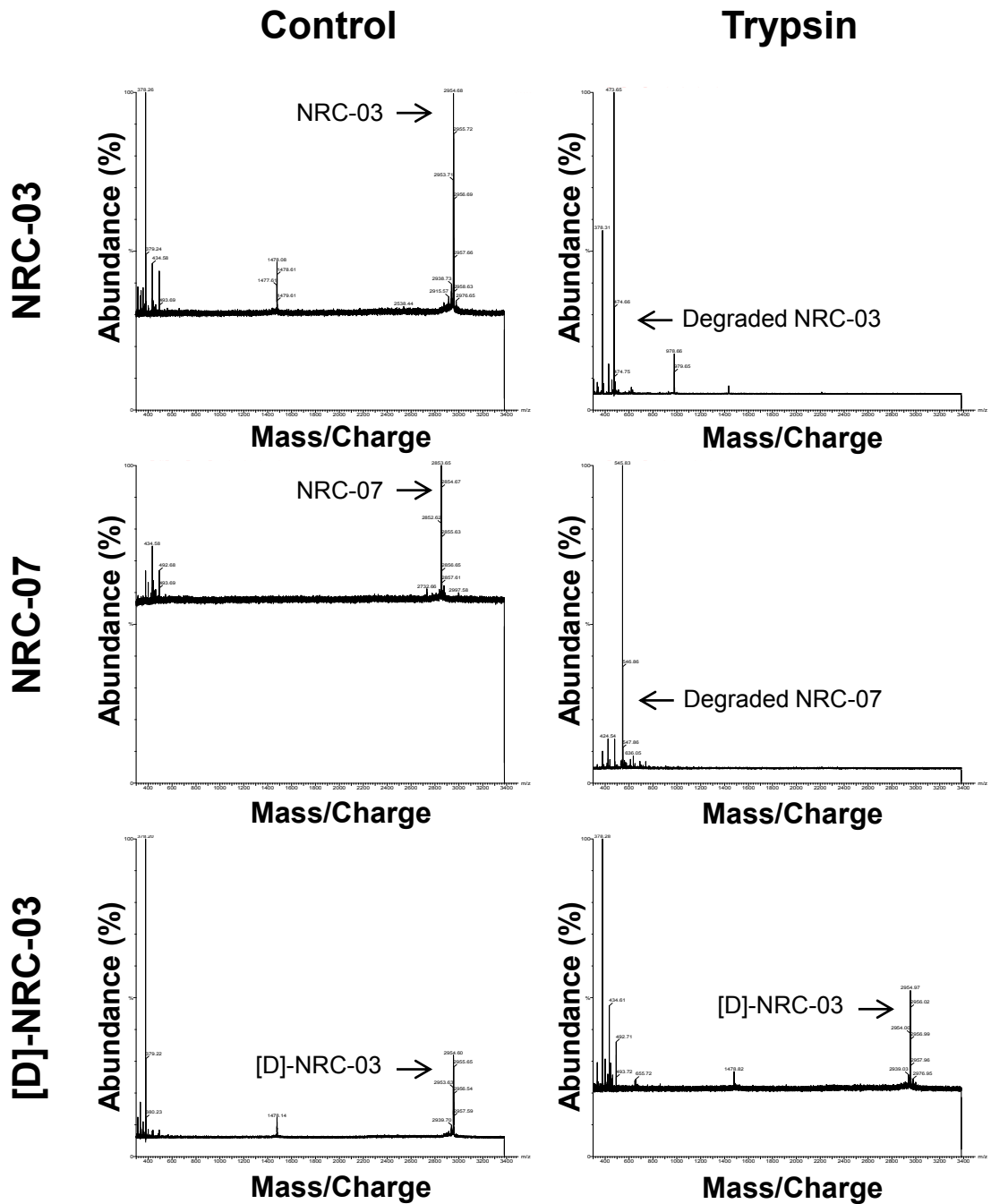
APPENDIX
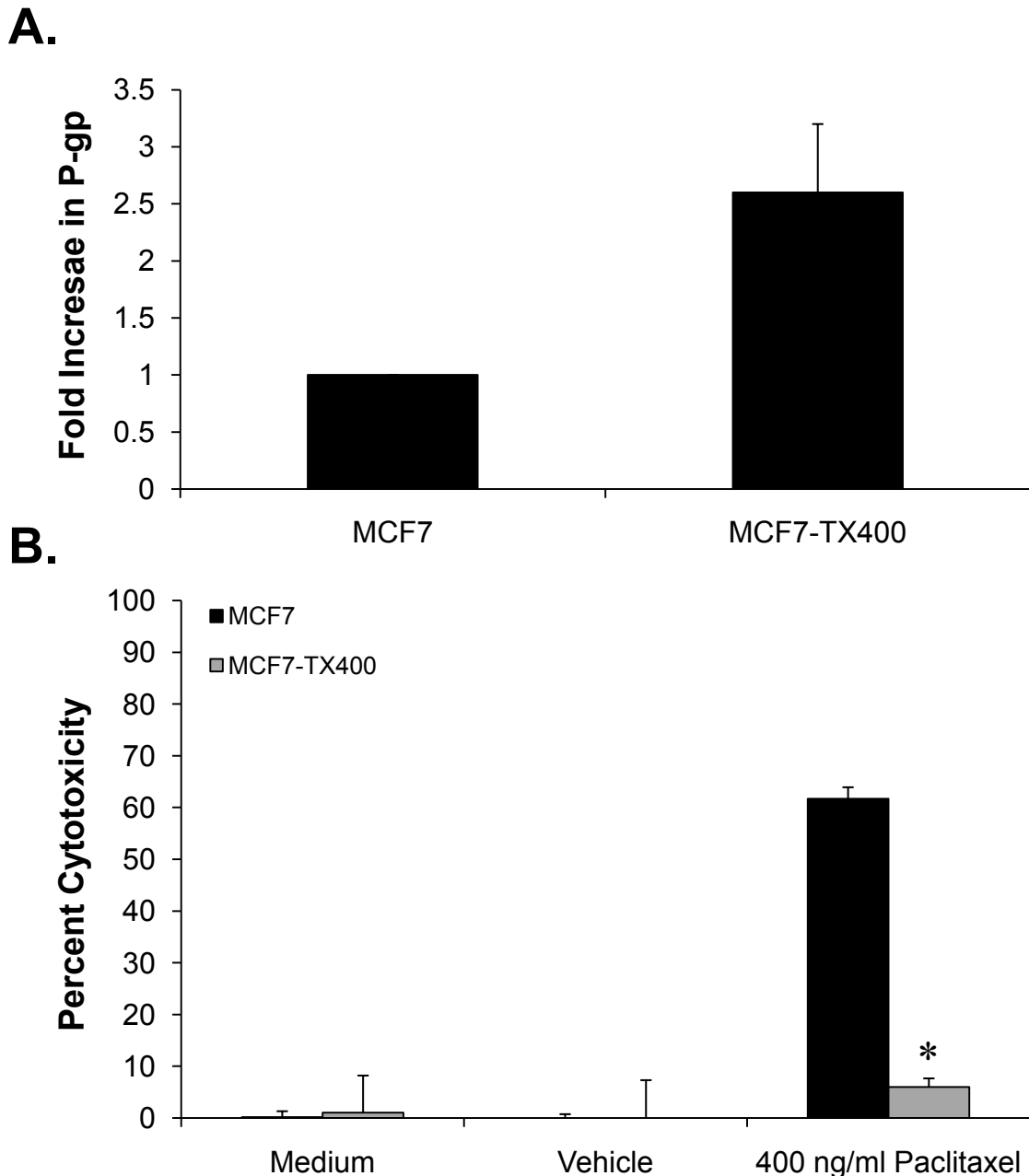
Appendix Figure 1. MCF7-TX400 breast cancer cells overexpress P-gp and are resistant to paclitaxel. (A) P-gp was detected in MCF7 and MCF7-TX400 cells by western blot analysis. Pixel intensity was used to quantify fold-increase in P-gp relative to actin. Data shown represent the mean of 3 independent experiments ± SE and were performed in collaboration with Anna Greenshields and Dr. Matthew Smith. (B) MCF7 and MCF7-TX400 cells were cultured in the absence or presence of 400 ng/ml paclitaxel. Cell viability was determined by acid phosphatase assay after 72 h. Data were collected in collaboration with Dr. Matthew Smith. Data shown are from a representative experiment (n = 3) ± SD and are statistically significant by the Bonferroni multiple comparison test in comparison to paclitaxel-treated MCF7 cells; * $p < 0.05$.

A.
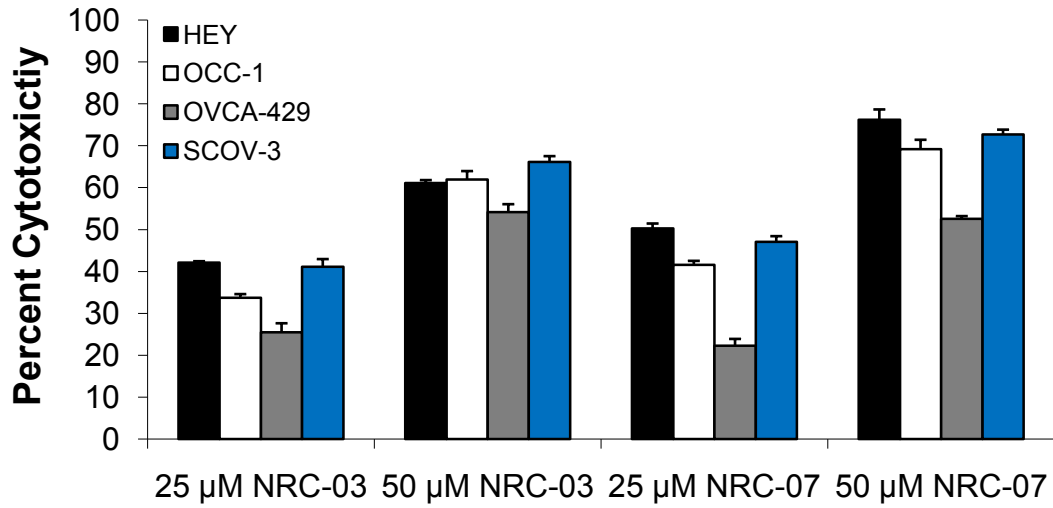
B.
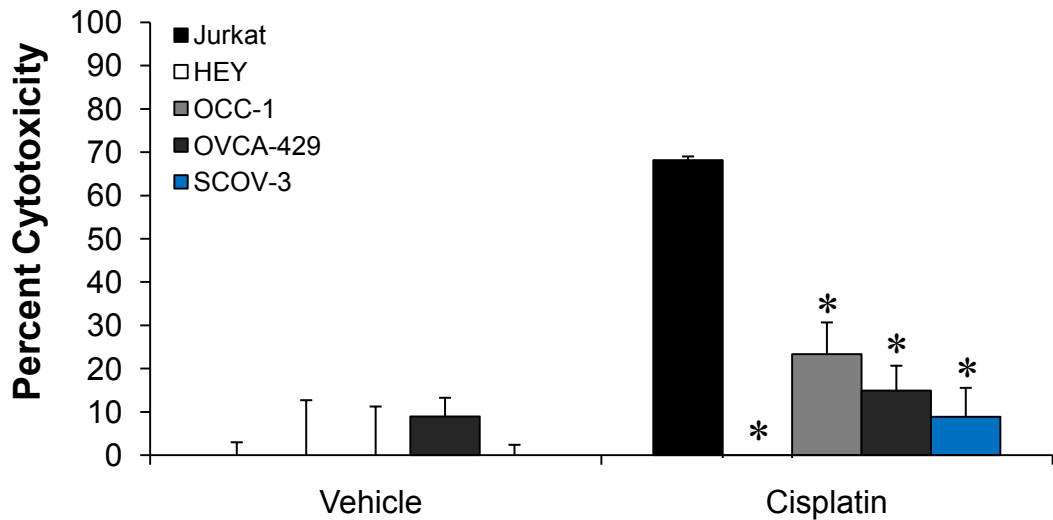
Appendix Figure 2. NRC-03 and NRC-07 kill cisplatin-resistant ovarian cancer cells. (A) HEY, OCC-1, OVCA-429 and SKOV-3 ovarian cancer cells were cultured in the presence or absence of 25 or 50 μM NRC-03 or NRC-07. Cell viability was determined by MTT assay after 24 h. Data shown are from a representative experiment (n=3) ± SD and were collected in collaboration with Anna Greenshields. (B) Jurkat T leukemia cells, and HEY, OCC-1, OVCA-429 and SCOV-3 ovarian cancer cells were cultured in the presence or absence of 8 μg/ml cisplatin. Cell viability was determined by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE and are statistically significant by the Bonferroni multiple comparison test in comparison to cisplatin-treated Jurkat cells; * $p < 0.01$.
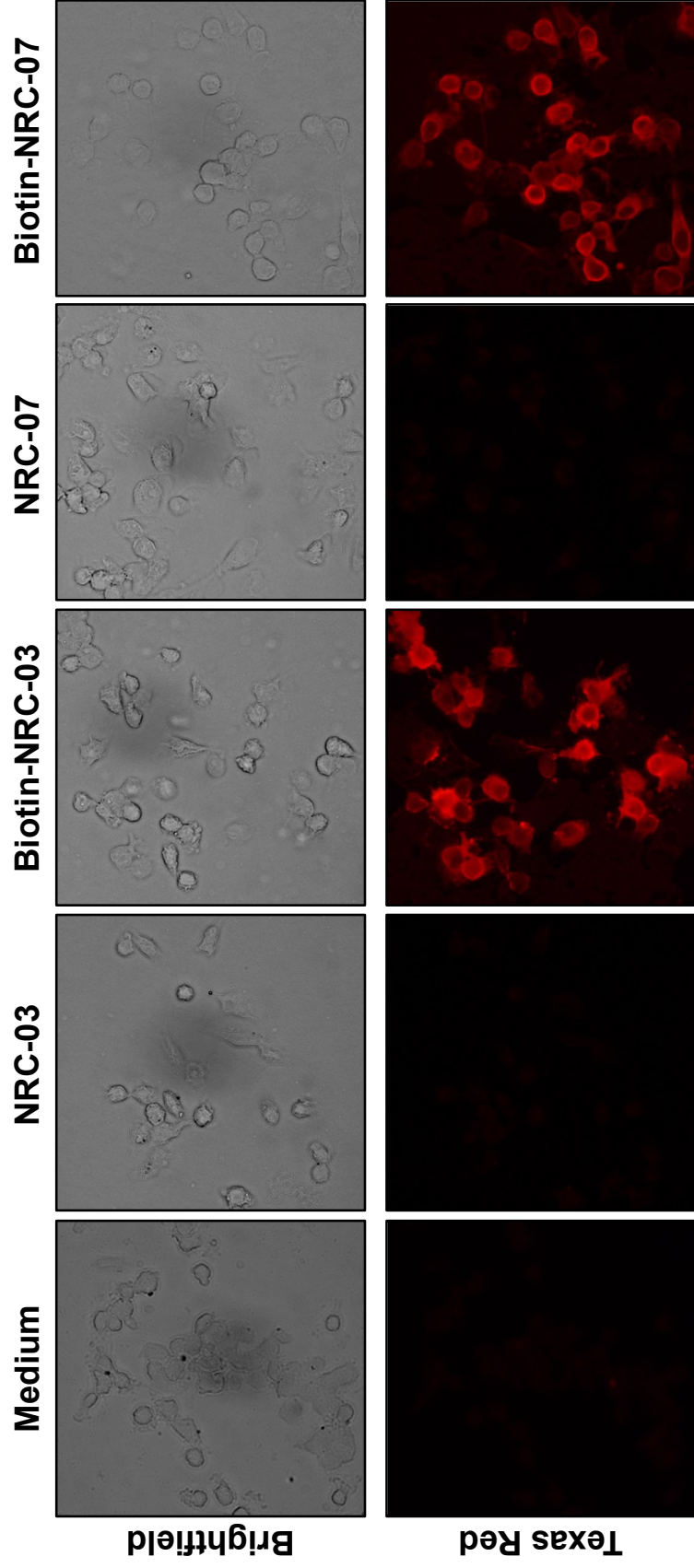
Appendix Figure 3. Biotin-NRC-03 and -NRC-07 are as potent as NRC-03 and NRC-07 in terms of cytotoxicity. MDA-MB-231 cells breast cancer cells were cultured in 50 μM NRC-03, biotin-NRC-03, NRC-07, or biotin-NRC-07. Cell viability was determined by MTT assay after 4 or 24 h. Data shown represent the mean of 3 independent experiments ± SE. Biotin-NRC-03- and -NRC-07-induced cell killing is not significantly different ($p \geq 0.05$) than killing by NRC-03 and NRC-07, respectively.
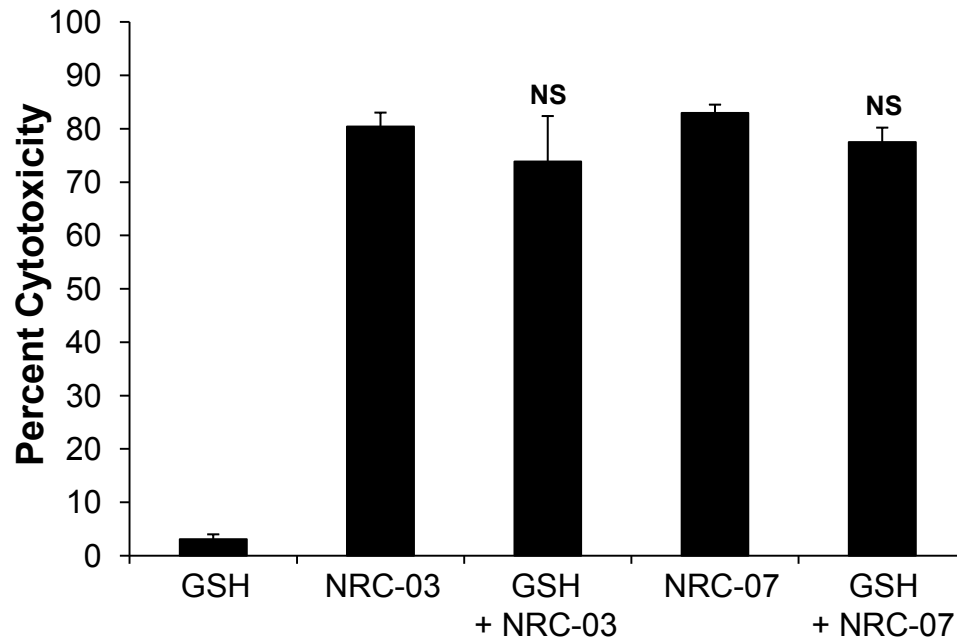
Appendix Figure 4. NRC-03 and NRC-07, but not [D]-NRC-03, are degraded by trypsin. 50 μg NRC-03, NRC-07 or [D]-NRC-03 were incubated in 1 μg trypsin o/n at 37°C. Intact or fragmented peptides were detected by MALDI-TOF mass spectrometry. The X and Y axes are identical in every plot (400 to 3400 atomic mass/charge, and 0 to 100%, respectively). Data shown is from 1 experiment and was collected by Mr. Ken Chisholm at the Institute for Marine Biosciences
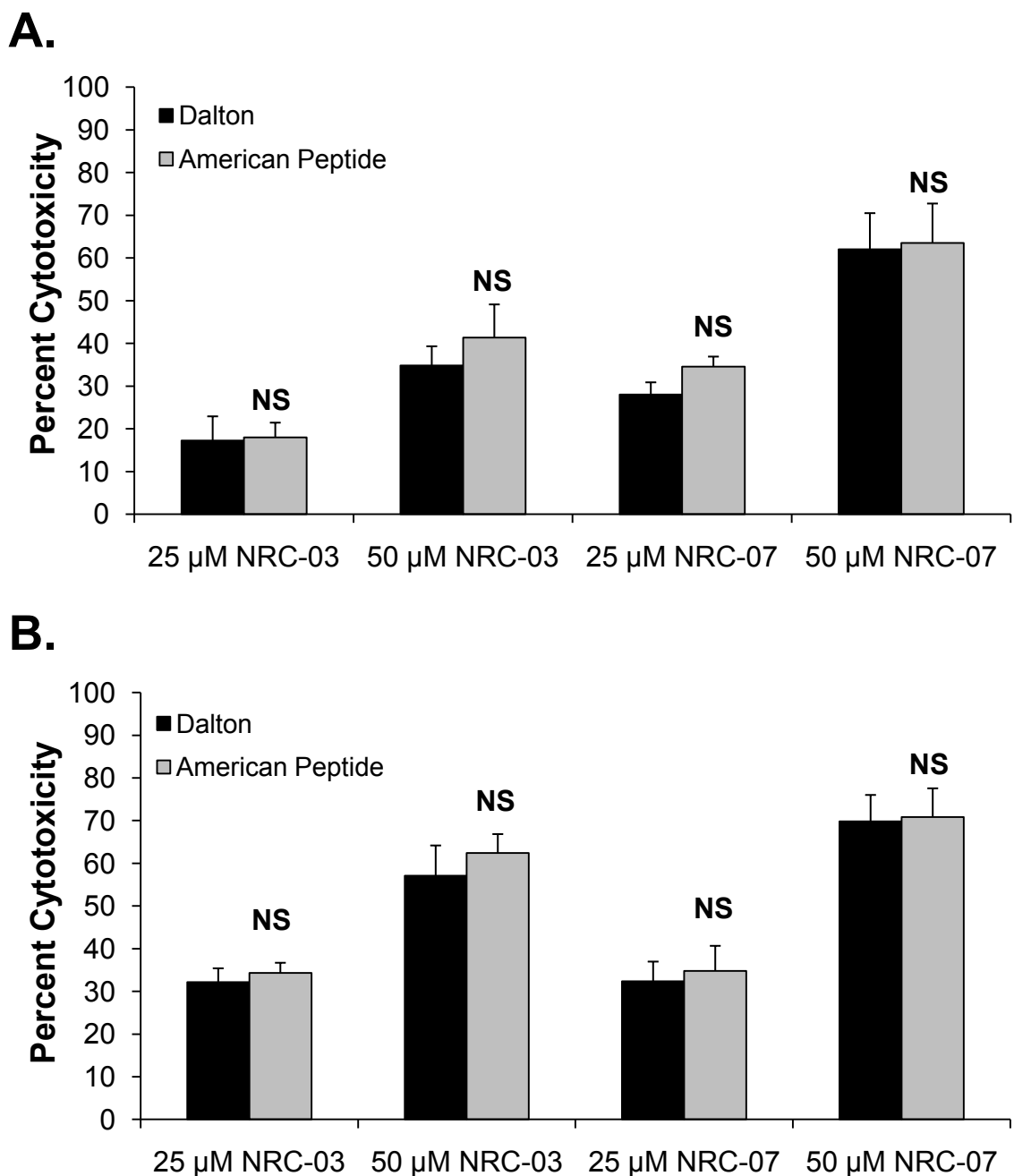
Appendix Figure 5. NRC-03 and NRC-07 from American Peptide are as potent cytotoxic agents as NRC-03 and NRC-07 from Dalton Pharma Services. MDA-MB-231 breast cancer cells were cultured in the absence or presence of the indicated concentrations of NRC-03 and NRC-07 from Dalton Pharma Services or Americal Peptide Company. Cell viability was determined by MTT assay after (A) 4h or (B) 24 h. Data shown represent the mean of 3 independent experiments ± SE and are not statistically significant ($p \geq 0.05$) by the Bonferroni multiple comparison test in comparison to NRC-03 or NRC-07 from Dalton Pharma Services.

Appendix Figure 6. Texas Red-streptavidin staining is a consequence of its interaction with biotin-NRC-03 and -NRC-07, and not a result of NRC-03- or NRC-07-mediated membrane damage. MDA-MB-231 breast cancer cells were cultured in the absence or presence of 50 μM NRC-03, biotin-NRC-03, NRC-07, or biotin-NRC-07 for 10 min, stained with Texas Red-streptavidin, and visualized by fluorescence microscopy (400 ×). Data shown are from a representative experiment (n = 3).

Appendix Figure 7. NRC-03- and NRC-07-mediated cell death is ROS-independent. MDA-MB-231 breast cancer cells pretreated for 20 min with 2.5 mM GSH were cultured in the presence or absence of 50 µM NRC-03 or NRC-07. Percent cytotoxicity was determined by acid phosphatase assay after 24 h. Data shown represent the mean of 3 independent experiments ± SE and are not statistically significant ($p \geq 0.05$) by the Bonferroni multiple comparson test in comparison to NRC-03- or NRC-07-treated cells.