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Enhanced killing of breast cancer cells by a D-amino acid analog of the winter flounder-derived pleurocidin NRC-03

Ashley L. Hilchie [a,b], Evan F. Haney [b], Devanand M. Pinto [c,d], Robert E.W. Hancock [b], David W. Hoskin [a,e,f,*]

[a] Department of Microbiology and Immunology, Dalhousie University, Halifax B3H 4R2, Canada
[b] Department of Microbiology and Immunology, University of British Columbia, Vancouver V6T 1Z4, Canada
[c] Department of Chemistry, Dalhousie University, Halifax B3H 4R2, Canada
[d] National Research Council Institute for Marine Biosciences, Halifax B3H 3Z1, Canada
[e] Department of Pathology, Dalhousie University, Halifax B3H 4R2, Canada
[f] Department of Surgery, Dalhousie University, Halifax B3H 4R2, Canada

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ABSTRACT

Cationic antimicrobial peptides (CAPs) defend against pathogens and, in some cases, exhibit potent anticancer activities. We previously reported that the pleurocidin NRC-03 causes lysis of breast cancer and multiple myeloma cells. NRC-03 also reduces the EC_{50} of other cytotoxic compounds and prevents tumor growth in vivo. However, the therapeutic utility of NRC-03 may be limited by its susceptibility to degradation by proteases. The goal of this study was to characterize the anticancer activities of a D-amino acid analog of NRC-03 ([D]-NRC-03) that was predicted to be resistant to proteolytic degradation. Unlike NRC-03, [D]-NRC-03 was not degraded by human serum or trypsin and, in comparison to NRC-03, showed increased killing of breast cancer cells, including multidrug-resistant cells; however, [D]-NRC-03 was somewhat more cytotoxic than NRC-03 for several types of normal cells. Importantly, [D]-NRC-03 was more effective than NRC-03 in vivo since 4-fold less peptide was required for an equivalent inhibitory effect on the growth of breast cancer cell xenografts in immune-deficient mice. These findings demonstrate that a D-amino acid analog of NRC-03 overcomes a major limitation to the therapeutic use of NRC-03, namely peptide stability. Further modification of [D]-NRC-03 is required to improve its selectivity for cancer cells.

1. Introduction

In the last two decades, breast cancer death rates have dropped by 34% (DeSantis et al., 2014); nevertheless, in 2014, an estimated 232,670 American women were expected to be diagnosed with breast cancer and 40,000 were expected to die, which makes breast cancer the second leading cause of cancer-related deaths in American women (Siegel et al., 2014). Consequently, continued progress in breast cancer screening, diagnosis, and treatment are required to further reduce mortality. While the treatment of breast cancer varies considerably between individuals, a combination of surgery, radiotherapy, chemotherapy, endocrine therapies, and/or HER-2-targeted treatments are typically employed (Maughan et al., 2010). Surgery is effective in the treatment of localized breast cancer but more advanced disease requires chemotherapy, endocrine therapies, and/or HER-2-specific trastuzumab (Herceptin). Unfortunately, most chemotherapeutic agents indiscriminately kill all rapidly dividing cells and may therefore cause significant adverse side effects without a sufficient reduction in tumor burden (Donnelly, 2004; Naumov et al., 2003). In addition, endocrine therapies may result in the development of secondary malignancies (Smith et al., 2000). Trastuzumab selectively kills HER-2-expressing breast cancer cells and addresses the shortcomings of chemotherapy and endocrine therapies (Dean-Colomb and Esteva, 2008); however, resistance has been reported in some HER-2-expressing breast cancer patients (Nagy et al., 2005; Nahta et al., 2006). Therefore, the search continues for therapeutic agents that selectively kill breast cancer cells, regardless of their rate of growth, reliance on specific signal-transduction pathways, and expression of multidrug-resistance proteins. In this regard, certain members from the class of cationic antimicrobial peptides (CAPs) show potential for adjunct use in the treatment of breast cancer and other malignancies.

CAPs are small peptides that are an important component of innate immunity in virtually all organisms (Hilchie et al., 2013a). CAPs are predominantly composed of basic and hydrophobic amino acids (typically fewer than 50 residues), and are classified on the basis of the secondary structures they adopt upon contact with biological membranes (Hoskin and Ramamoorthy, 2008). Compared to normal cells, which have membranes composed of zwitterionic lipids that carry a neutral charge, cancer cell membranes are characterized by a net negative charge due to the abundance of anionic molecules such

* Corresponding author at: Department of Microbiology and Immunology, Dalhousie University, 5850 College Street, PO Box 15000, Halifax, Nova Scotia B3H 4R2, Canada.
E-mail address: d.w.hoskin@dal.ca (D.W. Hoskin).

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as phosphatidylserine and heparan sulfate proteoglycans. Consequently, certain CAPs also selectively bind to cancer cells and kill by disrupting the membrane or by inducing apoptosis (Hilchie and Hoskin, 2010; Hoskin and Ramamoorthy, 2008). These CAPs are cytotoxic for many types of cancer cells, including slow-growing and/or multidrug-resistant cells, and are active against both the primary tumor and metastases without causing undue harm to normal tissues (Chen et al., 2001; Hansel et al., 2007; Hoskin and Ramamoorthy, 2008; Kim et al., 2003; Leuschner et al., 2003). Resistance to cell membrane-disrupting CAPs has not been documented, most likely because these peptides interact with several different anionic molecules rather than a unique receptor. Interestingly, some membranolytic CAPs also initiate a T cell-dependent anti-tumor immune response in mice, thus protecting the animal from cancer recurrence (Berge et al., 2010).

NRC-03, a pleurocidin-family CAP from winter flounder (Patrzykat et al., 2003), is cytotoxic for human breast cancer, multiple myeloma, and leukemia cells (Hilchie et al., 2011; Hilchie et al., 2013b; Morash et al., 2011). In addition to killing slow-growing and multidrug-resistant breast cancer cells via a direct lytic mechanism, subcytotoxic concentrations of NRC-03 reduce the EC_{50} of several chemotherapeutic drugs (Hilchie et al., 2011). Furthermore, NRC-03 inhibits the growth of breast cancer and multiple myeloma cell xenografts in immune-deficient mice (Hilchie et al., 2011; Hilchie et al., 2013b). Unfortunately, the clinical potential of NRC-03 may be diminished by its susceptibility to proteolytic degradation, i.e., NRC-03 is completely degraded by trypsin and NRC-03-mediated cytotoxicity is reduced in the presence of serum (Hilchie et al., 2011). It is therefore of interest to develop a biologically active version of NRC-03 that is resistant to proteases. The substitution of one or more L-amino acids with D-amino acids yields CAPs that are resistant to proteolytic degradation but retain biological activities (Bessalle et al., 1990; Makovitzki et al., 2009; Papo et al., 2004). Peptides composed of D-amino acids are also predicted to be less immunogenic because they cannot be processed by antigen-presenting cells (Wang et al., 1996). We therefore investigated the anti-cancer activities of a version of NRC-03 in which all of the L-amino acid residues were replaced with D-amino acids ([D]-NRC-03). Our findings suggest that [D]-NRC-03 may have utility as a novel adjuvant treatment for breast cancer and other malignancies, although additional modification to improve its selectivity for cancer cells will be necessary.

2. Materials and methods

2.1. Cell culture and conditions

MDA-MB-231 breast cancer cells were a gift from Dr. S. Drover (Memorial University of Newfoundland, St. John's, NL, Canada). SKBR3, T-47D, 4T1, and MDA-MB-468 mammary carcinoma cells were kindly provided by Drs. G. Dellaire, J. Blay, D. Waisman, and P. Lee, respectively (Dalhousie University, Halifax, NS, Canada). MCF7 and paclitaxel-resistant MCF7-TX400 breast cancer cells were obtained from Dr. K. Goralski (Dalhousie University). All breast cancer cells were maintained at 37 °C in a 10% CO_2 humidified atmosphere in DMEM (Sigma-Aldrich Canada, Oakville, ON, Canada). DMEM was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, 2 mM L-glutamine, and 5 mM HEPES (Invitrogen, Burlington, ON, Canada). Stock flasks were passaged as required to maintain optimal growth for no more than 40 passages, and were routinely confirmed to be free from mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza Inc., Mississauga, ON, Canada). Human mammary epithelial cells (HMEC), human dermal fibroblasts (HDF), and human umbilical vein endothelial cells (HUVEC) were from Lonza Inc., and were maintained in Clonetics MEGM, FGM-2, and EGM-2, respectively. Normal cell cultures were cultured at 37 °C in a 5% CO_2 humidified atmosphere for a maximum of six passages. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over LymphoPrep (Stemcell Technologies, Vancouver, BC, Canada) from the venous blood of healthy volunteers, with the approval of the University of British Columbia Research Ethics Board, and were used immediately. Human erythrocytes from healthy volunteers were provided by Dr. R. Duncan (Dalhousie University), and were used immediately.

2.2. Reagents

NRC-03 (GRRKRKWLRRIGKGVKIIGGAALDHL-NH_2) and [D]-NRC-03 (composed of D-amino acids) were synthesized by American Peptide Company at >95% purity by HPLC (Sunnyvale, CA, USA). Lyophilized peptides were reconstituted in serum-free DMEM, and were stored at −80 °C until use. All experiments were conducted in 2.5% FBS unless otherwise indicated. Crystal violet dye and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich Canada.

2.3. MTT assay

The MTT assay was used to quantify breast cancer cell and normal cell viability. Briefly, 2×10^4 breast cancer cells, HMECs, HDFs, or HUVECs were seeded, in quadruplicate, into 96-well flat-bottom tissue culture plates (Sarstedt, St. Leonard, QC, Canada). Cells were incubated overnight to promote cellular adhesion, at which point the cells were cultured under the indicated experimental conditions. PBMCs were seeded, in quadruplicate, into 96-well flat-bottom tissue culture plates at 2×10^5 cells/well. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at a final concentration of 0.5 μg/ml was added to each well for the final 2 h of culture, the plates were centrifuged (1400 *g* for 5 min), the supernatants discarded, and the formazan crystals were solubilized by the addition of dimethyl sulfoxide (100 μl/well). Absorbance at 490 nm was measured using a Bio-Tek microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Percent cytotoxicity was calculated using the formula (1-E/C) × 100, where E and C denote the optical density of peptide- and medium-treated cells, respectively.

2.4. Peptide stability studies

The stability of NRC-03 and [D]-NRC-03 in human serum was assessed by incubating 100 μM peptide in 30% human serum at 37 °C. Aliquots were removed at time intervals of 0, 5, 10, 15, 30, 60 and 120 min and immediately flash frozen in liquid nitrogen for later analysis by tricine-SDS-PAGE. Gels consisting of a 16% separating gel (49.5% T, 6% C) overlaid with a 10% spacer gel (49.5% T, 3% C) and a 4% stacking gel (49.5% T, 3% C) were used to resolve the low molecular weight peptides in the serum-digested samples. Protein bands were stained with 0.025% Coomassie Brilliant Blue dye in 10% acetic acid overnight, followed by destaining in 10% acetic acid.

Mass spectrometry was used to assess NRC-03 and [D]-NRC-03 degradation by trypsin. NRC-03 or [D]-NRC-03 (100 μg) reconstituted in 50 mM TEAB buffer were incubated in the presence or absence of trypsin (2 μg) overnight at 37 °C. Samples were dried in TFA (0.1% [*w*/*v*]) and diluted in matrix solution (1:1). Samples (500 ng) were spotted on a MALDI plate, dried, and analyzed on a MALDI-TOF mass spectrometer (Waters Corp., Milford, MA).

2.5. Clonogenic assay

MCF7 (4×10^5) and P-glycoprotein-overexpressing paclitaxel-resistant MCF7-TX400 (8×10^5) cells were cultured in 6-well flat-bottom tissue culture plates overnight to promote cell adhesion. The cells were then cultured under the indicated conditions for 4 h, lifted by mild trypsinization, diluted (40- to 1600-fold), and seeded into fresh 6-well plates. Cells were maintained at 37 °C in a 10% CO_2 humidified atmosphere, and were fed every 3 d for 10 d to allow for colony formation. Colonies were washed with phosphate buffered saline

(PBS), fixed and stained with crystal violet (0.4% [*w*/*v*] in methanol), washed with deionized water, dried, and counted in order to determine the number of colony forming units (CFUs).

2.6. Lactate dehydrogenase-release assay

Peptide-induced cytolysis was quantified using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit as per the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Complete cell lysis was obtained by repeated freeze/thaw cycles. Absorbance at 490 nm was used to quantify cell lysis using the equation [(E/S)/(M/S) × 100], where E, S, and M indicate peptide-induced, spontaneous, and maximal cell lysis, respectively.

2.7. Hemolysis assay

Peptide-induced hemolysis was quantified by culturing human erythrocytes (5% [*w*/*v*] in PBS) in the presence or absence of the indicated concentration of peptide in 96-well round-bottom tissue culture plates (Sarstedt). Maximum lysis was achieved by treating erythrocytes with an equal volume of water. After 8 h incubation at 37 °C in a 5% CO_2 humidified atmosphere, erythrocytes were pelleted by centrifugation (1400 *g*), and supernatants were collected. Absorbance at 490 nm was measured, and percent hemolysis was calculated using the formula [(E/S)/(M/S) × 100], where E, S, and M indicate peptide-induced, spontaneous, and maximal hemolysis, respectively.

2.8. Scanning electron microscopy

Cellular ultrastructure was visualized by scanning electron microscopy. MDA-MB-231 cells were plated in 24-well flat-bottom tissue culture plates containing sterilized circular coverslips, and were incubated overnight to promote cellular adhesion. Cells on coverslips were then cultured in the presence or absence of the indicated peptide for the indicated periods of time, then washed with 0.1 M sodium cacodylate, fixed with gluteraldehyde (2.5% [*v*/*v*] in sodium cacodylate) for 2 h, washed again, and fixed with osmium tetroxide (1% [*w*/*v*] in sodium cacodylate) for 30 min. Following extensive washing, cells were dehydrated in increasing concentrations of ethanol, and were dried to their critical point using a Polaron E3000 critical point dryer (Quorum Technologies, Guelph, ON, Canada). Dried samples were mounted onto stubs, and were coated with gold using a Polaron SC7620 mini sputter coater (Quroum Technologies). The samples were viewed on a Hitachi S4700 scanning electron microscope (Hitachi High Technologies, Resdale, ON, Canada) at the indicated magnifications.

2.9. In vivo studies

Adult (6- to 7-wk) female NOD SCID mice were purchased from Charles River Laboratories (Lasalle, QC, Canada). Mice were housed in the Carleton Animal Care Facility, maintained on a diet of sterilized rodent chow, and had access to water *ad libitum*. Animal use was approved by the Dalhousie University Committee of Laboratory Animals, and was in accordance with the Canadian Council of Animal Care Guidelines. To compare the anti-tumor effects of [D]-NRC-03 to NRC-03, NOD SCID mice were engrafted with 5×10^6 MDA-MB-231 human breast cancer cells in the rear flank by subcutaneous injection. Tumor size was monitored every other day using caliper measurements, and tumor volume was calculated using the equation (L × P)/2, where L and P denote the longest diameter and the diameter perpendicular to the longest diameter, respectively. Mice were randomized into 3 groups and tumors were injected with the Hank's balanced salt solution (HBSS) vehicle, NRC-03 (0.5 mg), or [D]-NRC-03 (0.125 mg) once tumors reached a volume equal to or greater than 120 mm^3 (approximately 21 d following tumor cell implantation). Day 1 denotes the day of the first treatment. Tumor-bearing mice received another treatment on d 3 and 5. The first treatment consisted of 2 injections at opposing ends of the longest diameter of the tumor, the second treatment was 2 injections perpendicular to the first injection sites, and the third treatment was 2 injections at opposing ends of the longest diameter of the tumor. Mice were sacrificed 7 d following the last treatment (d 12), and the tumors were excised, photographed, sectioned, and stained with hematoxylin and eosin. Stained tumor sections were visualized under brightfield microscopy (400× magnification).

2.10. Statistical analysis

All data were analyzed using the unpaired Student's *t* test or one-way analysis of variance coupled with the Bonferroni multiple comparisons test, as appropriate. Data were significant when *p* was less than, or equal to, 0.05.

3. Results

3.1. [D]-NRC-03 shows increased potency and stability but is less selective than NRC-03

We first determined whether [D]-NRC-03 was resistant to serum proteases. As shown in Fig. 1A, [D]-NRC-03-mediated cytotoxicity was not affected by FBS whereas NRC-03-mediated killing of breast cancer cells was reduced in the presence of higher concentrations of FBS. Furthermore, in contrast to NRC-03, [D]-NRC was not degraded by proteases present in human serum (Fig. 1B). [D]-NRC-03 also remained intact in the presence of trypsin (Fig. 1C), unlike NRC-03, which is completely degraded (Hilchie et al., 2011). Importantly, [D]-NRC-03 was more cytotoxic than NRC-03 to all breast cancer cell lines tested; including slow-growing SKBR3 cells (compare Fig. 2A with Fig. 2B). In most cases, 10 μM [D]-NRC-03 was sufficient to kill at least 50% of breast cancer cells (Fig. 2A) whereas 25 μM NRC-03 was needed to achieve a similar cytotoxic effect (Fig. 2B). Furthermore, in comparison to NRC-03, [D]-NRC-03 was a more effective killer of multidrug-resistant breast cancer cells. Peptide-induced cytotoxicity in cultures of P-glycoprotein-overexpressing paclitaxel-resistant MCF7-TX400 cells was determined using a clonogenic assay since drug efflux pumps interfere with the MTT assay (Funk et al., 2007). A 4-h exposure to NRC-03 caused a 2.4- and 1.5-fold decrease in colony formation by MCF7 and MCF7-TX400 cells, respectively, whereas equimolar concentrations of [D]-NRC-03 reduced MCF7 and MCF7-TX400 colony formation by 36- and 320-fold, respectively (Fig. 2C). [D]-NRC-03 was therefore 15 to 200 fold more cytotoxic than NRC-03. We also compared the cytotoxic effect of [D]-NRC-03 to NRC-03 on a panel of normal cell types versus MDA-MB-468 breast cancer cells (Table 1). Compared to NRC-03, [D]-NRC-03 was more cytotoxic for PBMCs, HMECs, and HDFs, but in all cases cytotoxicity was considerably less than observed with the breast cancer cells. HUVECs were relatively resistant to the cytotoxic effect of both [D]-NRC-03 and NRC-03. Neither [D]-NRC-03 nor NRC-03 caused lysis of erythrocytes.

3.2. [D]-NRC-03 has delayed kinetics of action and kills by a lytic mechanism

Subsequent experiments were conducted using MDA-MB-231 breast cancer cells since they were the least susceptible to killing by NRC-03 and [D]-NRC-03, and therefore represented a worst case scenario. A comparison of the kinetics of cell death showed that [D]-NRC-03-mediated killing peaked at 8 h following peptide treatment, whereas the cytotoxicity of NRC-03 peaked after 4 h exposure to the peptide (Fig. 3). As before, [D]-NRC-03 was more potent than NRC-03. Lactate dehydrogenase release by peptide-treated MDA-MB-231 cells suggested induction of necrosis. Scanning electron microscopy was used to visualize changes in the cellular ultrastructure of MDA-MB-231 cells

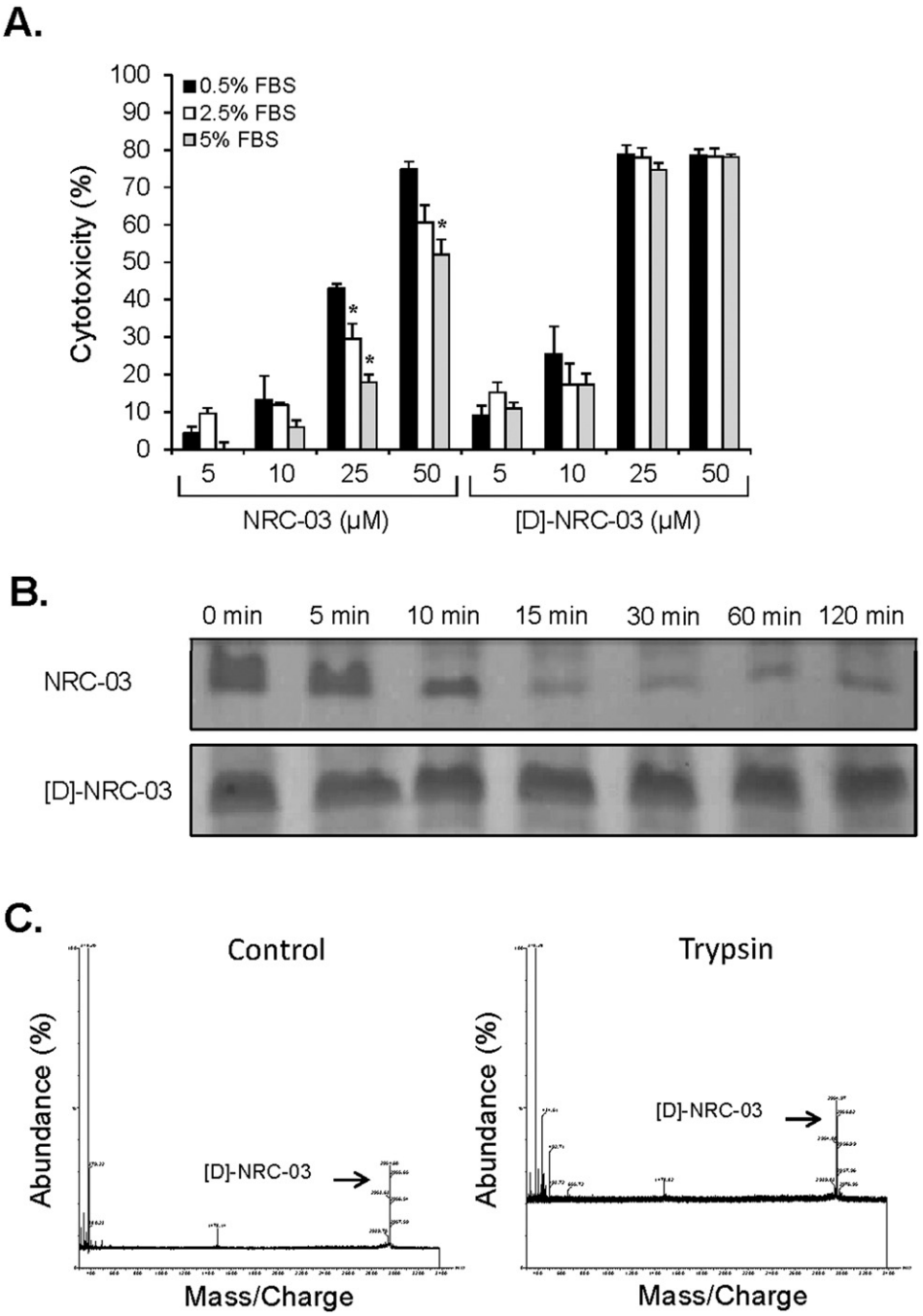

Fig. 1. [D]-NRC-03 is not neutralized by FBS and is resistant to proteases. (A) MDA-MB-231 breast cancer cells were cultured in medium containing 0.5, 2.5, or 5% FBS in the absence or presence of the indicated concentrations of NRC-03 or [D]-NRC-03. Cell viability was assessed by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SEM and are statistically significant by the Bonferroni multiple comparisons test in comparison to NRC-03-treated cells cultured in 0.5% FBS; $^{*}p < 0.01$. (B) NRC-03 or [D]-NRC-03 (100 μM) were incubated in the presence of 30% human serum for the indicated periods of time. The resulting digests were analyzed by Tricine-SDS-PAGE. Proteins were visualized using Coomassie Brilliant Blue staining. The images shown are from a representative experiment (n = 3). (C) [D]-NRC-03 was incubated in 1 μg trypsin overnight at 37 °C. Intact and fragmented peptide was detected by MALDI-TOF mass spectrometry.

following exposure to NRC-03 or [D]-NRC-03. After 10 min in the presence of NRC-03, MDA-MB-231 cells exhibited fewer microvilli and an exposed membrane (Fig. 4A). In contrast, the membranes of [D]-NRC-03-treated cells showed less overt damage. By 1 h post exposure, most NRC-03-treated cells had lysed while those that remained exhibited membrane damage due to pore formation (Fig. 4B). In contrast, [D]-NRC-03-treated cells appeared similar to NRC-03-treated cells at the 10 min mark. Finally, by 4 h the remaining NRC-03- or [D]-NRC-03-treated cells were shrunken with badly damaged membranes (Fig. 4C). These data indicate that [D]-NRC-03, like NRC-03, kills target cells by causing extensive membrane damage.

3.3. [D]-NRC-03 is more potent than NRC-03 in vivo

We used a mouse xenograft model of human breast cancer to compare the therapeutic potential of [D]-NRC-03 with NRC-03. We chose intratumoral rather than systemic administration of [D]-NRC-03 and NRC-03 because of the potential for toxicity, as suggested by the

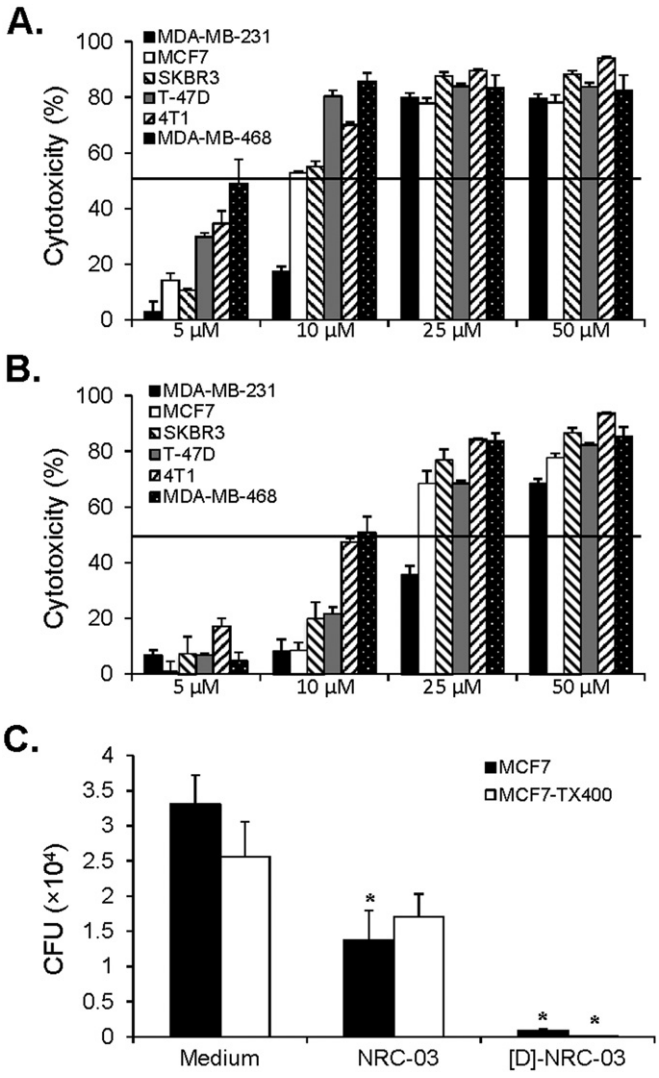

Fig. 2. [D]-NRC-03 is more cytotoxic than NRC-03 to breast cancer cells. MDA-MB-231 (black), MCF7 (white), SKBR3 (light gray), T-47D (dark gray), 4 T1 (hatched), and MDA-MB-468 (speckled) breast cancer cells were exposed to medium alone or to the indicated concentrations of (A) [D]-NRC-03 or (B) NRC-03. Cell viability was determined by MTT assay after 24 h. Data shown represent the mean of 3 independent experiments ± SEM and are significant by ANOVA ($p < 0.0001$). (C) NRC-03 and [D]-NRC-03-induced cytotoxicity in MCF7 and paclitaxel-resistant MCF7-TX400 cells was determined by clonogenic assay. Colony formation was assessed in cells treated with medium or with 25 μM NRC-03 or [D]-NRC-03 for 4 h. Data shown are statistically significant by the Bonferroni multiple comparisons test; *indicates $p < 0.05$ in comparison to medium-treated cells.

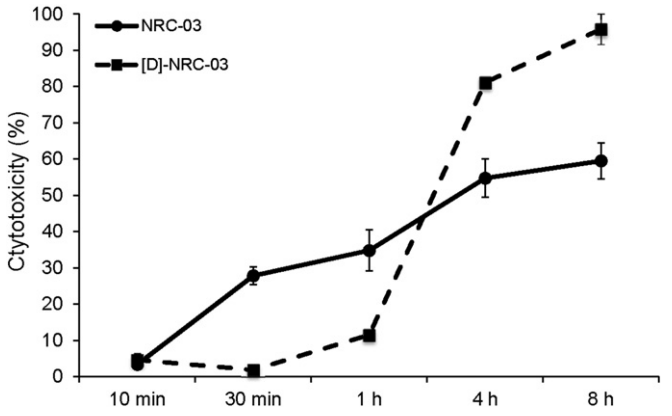

Fig. 3. [D]-NRC-03 has delayed kinetics of killing in comparison to NRC-03. MDA-MB-231 cells were cultured in the absence or presence of 50 μM NRC-03 or [D]-NRC-03. Relative cytotoxicity was measured by the lactate dehydrogenase-release assay after 10 min, 30 min, 1 h, 4 h and 8 h. Data shown represent the mean of 3 independent experiments ± SEM.

findings detailed in Table 1. Fig. 5A shows that a 4-fold reduction in [D]-NRC-03 prevented the growth of MDA-MB-231 tumors to the same extent as NRC-03. A representative tumor from each treatment group is also shown (Fig. 5B). Hematoxylin and eosin staining revealed increased necrosis in peptide-treated tumors; this effect was most pronounced in tumors treated with [D]-NRC-03 (Fig. 5C) and corresponded with tumor growth inhibition. Taken together, these data indicate that [D]-NRC-03 had greater *in vivo* efficacy than NRC-03. There were no signs of overt toxicity such as altered behavior or weight loss in peptide-treated mice, and necropsies showed no signs of damage to major organs (uterus, colon, stomach, spleen, kidneys, lungs, and heart) of mice treated with [D]-NRC-03 or NRC-03, suggesting that intra-tumoral administration of these peptides was well tolerated.

4. Discussion

Current strategies to treat metastatic breast cancer are limited by drug-resistance, toxicity to normal tissues, and the development of secondary malignancies (Donnelly, 2004; Nagy et al., 2005; Nahta et al., 2006; Naumov et al., 2003; Smith et al., 2000). CAPs with anticancer properties represent a potential reservoir of agents that lack these shortcomings and might therefore be used in the treatment of breast cancer and other malignancies. In this regard, we previously demonstrated that the pleurocidin NRC-03 has therapeutic potential for the treatment of breast carcinomas and multiple myeloma (Hilchie et al.,

Table 1
NRC-03- and [D]-NRC-03-induced cytotoxicity against normal human cells [peripheral blood mononuclear cells (PBMC), human mammary epithelial cells (HMEC), human dermal fibroblasts (HDF), and human umbilical vein endothelial cells (HUVEC)] in comparison to activity versus MDA-MB-468 breast cancer cells.

	% Cytotoxicity[a,c]					% Hemolysis[b,c]
Treatment	PBMCs	HMECs	HDFs	HUVECs	MDA-MB-468	Erythrocytes
5 μM NRC-03	1 ± 6	2 ± 1	1 ± 1	1 ± 1	5 ± 3	1 ± 1
10 μM NRC-03	23 ± 7	11 ± 5	1 ± 1	1 ± 1	51 ± 6	2 ± 1
25 μM NRC-03	67 ± 5	47 ± 1	5 ± 2	1 ± 1	84 ± 3	2 ± 1
50 μM NRC-03	88 ± 1	77 ± 2	21 ± 3	27 ± 3	85 ± 4	2 ± 1
5 μM [D]-NRC-03	34 ± 1	19 ± 7	15 ± 3	1 ± 1	50 ± 9	1 ± 1
10 μM [D]-NRC-03	45 ± 2	65 ± 2	28 ± 7	1 ± 3	86 ± 3	1 ± 1
25 μM [D]-NRC-03	82 ± 2	81 ± 1	67 ± 4	20 ± 4	83 ± 5	1 ± 1
50 μM [D]-NRC-03	90 ± 2	80 ± 1	75 ± 2	24 ± 2	83 ± 5	2 ± 1

[a] The cytotoxic effect of NRC-03 and [D]-NRC-03 on normal cells and breast cancer cells was determined by MTT assay after 24 h exposure to the indicated concentrations of peptide in medium containing 2.5% FBS.
[b] Hemolytic activity toward human erythrocytes was determined by hemolysis assay after 8 h exposure to the indicated concentrations of peptide.
[c] Data shown represent the mean of 3 independent experiments ± SEM.

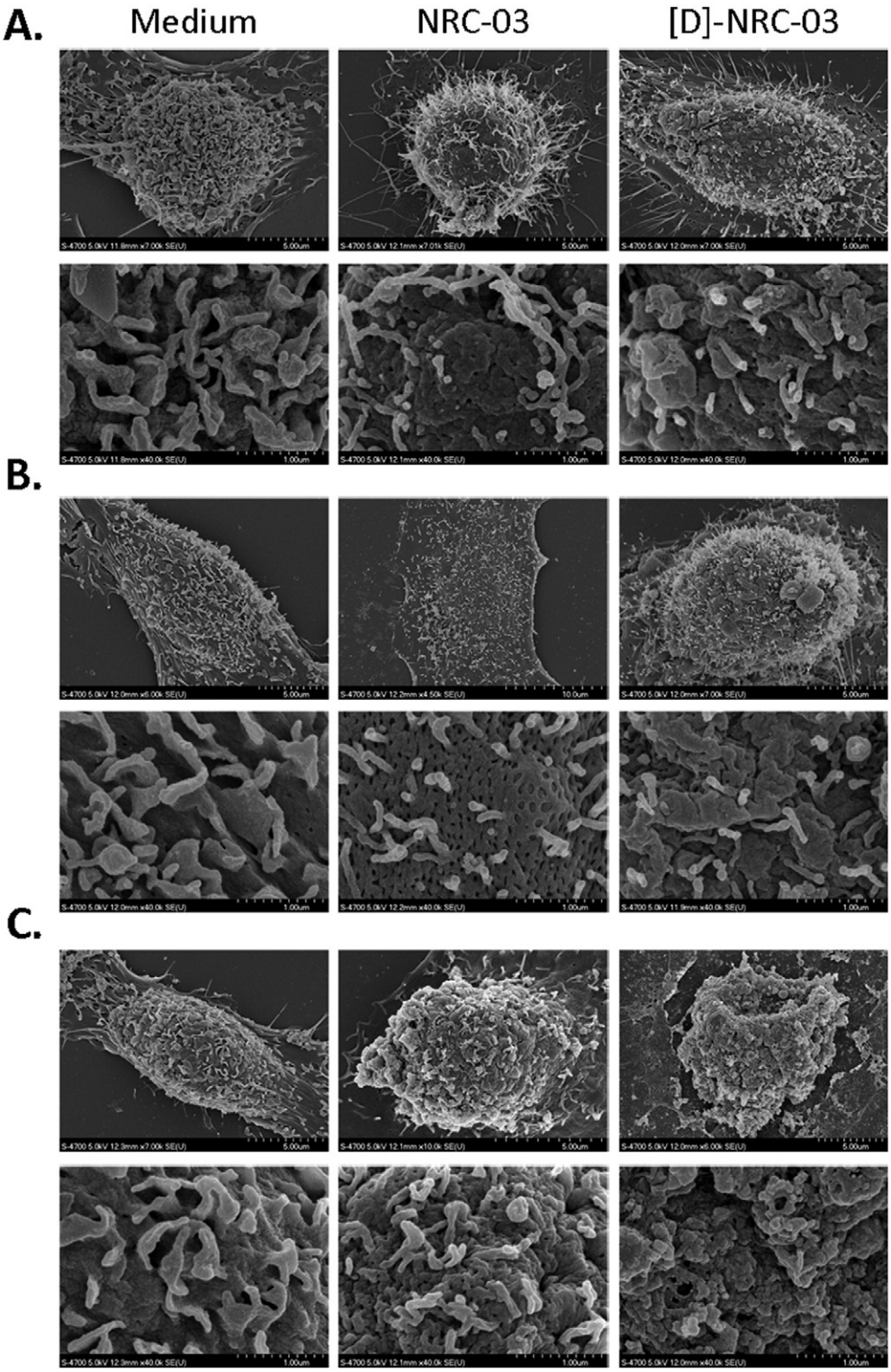

Fig. 4. [D]-NRC-03 damages the cell membrane of breast cancer cells. MDA-MB-231 breast cancer cells were cultured in the absence or presence of 25 μM NRC-03 or [D]-NRC-03 for (A) 10 min, (B) 1 h, or (C) 4 h. Membrane damage was visualized by scanning electron microscopy at 7000× (upper panels) and 40,000× (lower panels). Data shown are from a representative experiment (n = 2).

2011; Hilchie et al., 2013b); however, the anticancer potential of NRC-03 may be limited by its susceptibility to proteolytic degradation (Hilchie et al., 2011). In line with previous reports that replacing L-amino acids with D-amino acids allows CAPs to resist degradation by proteases (Bessale et al., 1990; Makovitzki et al., 2009; Papo et al., 2004), we show here that a D-amino acid analog of NRC-03 ([D]-NRC-03) did not exhibit reduced cytotoxicity in the presence of FBS and resisted degradation by human serum proteases, including trypsin. This finding is consistent with a report that a related pleurocidin composed of D-amino acids is refractory to degradation by trypsin, plasmin, and carboxypeptidase (Jung et al., 2007). It is noteworthy that [D]-NRC-03 contains several glycine residues, which might serve as cleavage sites for proteases since glycine is achiral; however, the presence of these glycines did not have any noticeable negative impact

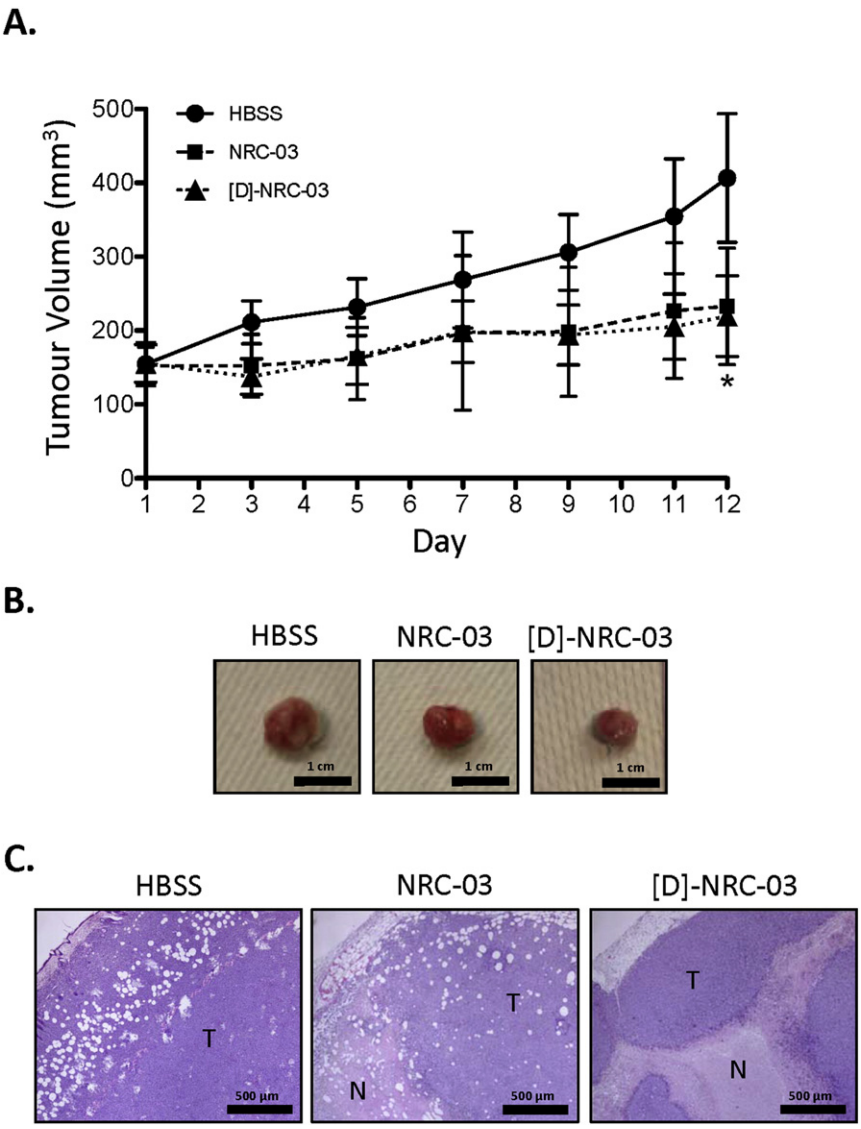

Fig. 5. [D]-NRC-03 kills breast cancer cells *in vivo*. MDA-MB-231 breast cancer cells grown in the hind flanks of NOD SCID mice formed tumors that received intra-tumoral injections of HBSS (n = 12), or 500 μg NRC-03 (n = 10) or 125 μg [D]-NRC-03 (n = 11) on days 1, 3, and 5. (A) Caliper measurements were recorded on days 1, 3, 5, 7, 9, 11, and 12 to determine tumor volume. Data shown represent mean tumor volume ± SD and are statistically significant by the Bonferroni multiple comparisons test in comparison to HBSS-treated tumors; $^{*}p < 0.05$. (B) Tumors from each treatment group were photographed and an image representing the median is shown. (C) Tumor sections were stained with hematoxylin and eosin and photographed. Images shown are from representative tumor sections; T: tumor tissue. N: necrotic tumor tissue.

on the stability of [D]-NRC-03 in human serum. Indeed, peptides consisting of a tumor-targeting domain coupled via a glycine-glycine linker to a pro-apoptotic domain composed of D-amino acids have been reported to be stable *ex vivo* and in mice (Ellerby et al., 1999). Importantly, compared to NRC-03, [D]-NRC-03 had increased cytotoxicity for breast cancer cells, including both slow-growing SKBR3 cells and multidrug-resistant MCF7-TX400 cells. These attributes should allow for more economical use of smaller quantities of [D]-NRC-03.

[D]-NRC-03, like NRC-03, caused extensive damage to the cell membrane of breast cancer cells leading to cell death by necrosis, albeit with delayed kinetics that may be a consequence of differences in peptide-membrane interactions such that [D]-NRC-03 was not able integrate into (and therefore disrupt) the lipid bilayer as efficiently as NRC-03. CAPs such as [D]-NRC-03 that kill cells by causing membrane damage have several advantages over apoptosis-inducing peptides. It is unlikely that cancer cells will easily develop resistance to membranolytic peptides because the interactions are with a number of different negatively-charged molecules rather than a unique receptor that might be altered by a single mutation (Hoskin and Ramamoorthy, 2008). For example, NRC-03, and presumably [D]-NRC-03, binds strongly to both heparan sulfate and chondroitin sulfate proteoglycans (Hilchie et al., 2011). In addition, recent studies show that cytolytic peptides can trigger antitumor immunity, which may aid in the elimination of metastatic lesions and, by generating immunologic memory, reduce the chances of cancer recurrence (Berge et al., 2010). Furthermore, NRC-03 and [D]-NRC-03 are effective against solid cancers, as well as hematological malignancies (Hilchie et al., 2011; Hilchie et al., 2013b; Morash et al., 2011), whereas the cytotoxic action of bovine lactoferricin, for example, is dependent on cancer cell type (Eliassen et al., 2002; Eliassen et al., 2006; Mader et al., 2005).

Although [D]-NRC-03 did not lyse erythrocytes and was minimally cytotoxic for HUVECs, for reasons that are not clear at this time [D]-NRC-03 was less selective than NRC-03 for cancer cells. The somewhat greater cytotoxicity of [D]-NRC-03 than its L-analog for normal HMECs, HDFs, and PBMCs might indicate that systemic administration of [D]-NRC-03 could have adverse effects on healthy tissues, which might be exacerbated by the increased stability of the peptide, although there was no overt evidence of this in the mouse model. Kidneys may be

at particular risk since CAPs can undergo rapid renal clearance (Meléndez-Alafort et al., 2004). Additional modification is therefore needed to render [D]-NRC-03 more selective for breast cancer and other neoplasms. One possibility is to eliminate or reduce [D]-NRC-03 interactions with healthy cells by replacing positively-charged arginine and lysine residues with histidine residues, which have a neutral charge but develop a positive charge under the acidic conditions that prevail in the microenvironment of a solid tumor (Tannock and Rotin, 1989). In this regard, histidine-substituted cytolytic peptides consisting of D-amino acids show pH-dependent cytotoxicity and are effective *in vivo* for the treatment of solid tumors (24). Alternatively, a targeting peptide that binds to a molecule such as EphA2, which is overexpressed in breast, lung, colon, and prostate cancer (Ireton and Chen, 2005), could be coupled to [D]-NRC-03 to increase its selectivity for neoplastic cells. This strategy has already been used to successfully target apoptosis-promoting peptides to the tumor vasculature in models of breast and prostate cancer, as well as to the inflamed synovium in a mouse model of rheumatoid arthritis (Ellerby et al., 2008). However, this approach has some drawbacks. The targeting domain is typically composed of L-amino acids in order to ensure optimal receptor binding and will therefore be susceptible to degradation by proteases. Furthermore, mutation of the gene(s) coding for the target structure on cancer cells may reduce selective binding of the targeted peptide to cancer cells. Addition of a targeting motif might also decrease tumor penetration due to the increased size of the peptide. Finally, the addition of a targeting motif will increase cost. For these reasons, we are currently developing a histidine-substituted form of [D]-NRC-03 for use in future studies.

Consistent with our *in vitro* data, *in vivo* studies using MDA-MB-231 breast cancer cells xenografted into immune-deficient NOD SCID mice indicated that [D]-NRC-03 was more potent than NRC-03. In comparison to NRC-03, 4-fold less [D]-NRC-03 was required for an equivalent inhibitory effect on tumor growth. Histological analysis confirmed that, in comparison to NRC-03, [D]-NRC-03 caused more tumor cell death, as indicated by larger areas of necrosis. Increased potency addresses another important limitation to the clinical use of therapeutic peptides, namely the cost of production. Despite the moderately increased cytotoxicity for HMECs, HDFs, and PBMCs, intra-tumoral injections of [D]-NRC-03 were well tolerated with no obvious toxicity to healthy tissues. Furthermore, [D]-NRC-03 is not expected to induce an anti-peptide immune response since antigen-presenting cells are not able to process and present peptides composed of D-amino acids to T lymphocytes (Wang et al., 1996). Collectively, these data indicate that [D]-NRC-03 is superior to its parent peptide in terms of both stability and potency, in spite of the somewhat increased toxicity of [D]-NRC-03 for normal cells. Development of a variant with increased selectivity for cancer cells is therefore required in order to advance [D]-NRC-03 as a possible novel adjunct treatment for breast cancer and other malignancies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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