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Cary N. Robertson · Kathryn M. Roberson · Aaron Pinero · Jesse M. Jaynes · David F. Paulson

Peptidyl membrane-interactive molecules are cytotoxic to prostatic cancer cells in vitro

Abstract Cytotoxic membrane disruption via lytic peptides is a well-recognized mechanism of immune surveillance for antifungal and antibacterial host protection. Naturally occurring lytic peptides were shown to exhibit antitumor activity as well. Peptidyl membrane-interactive molecules (MIMs) are synthetic lytic peptides specifically designed to maximize antitumor activity. We tested nine novel Peptidyl MIMs for activity against four androgen-insensitive prostate-cancer cell lines using a standard microculture tetrazolium (MTT) assay. Five Peptidyl MIMs known to form alpha-helical secondary structures were active against prostate carcinoma and were chosen for further study. Three peptides configured in beta-pleated sheets were noticeably less effective. Concentrations lethal to 50% of the prostate-cancer cell lines treated (D_{50} values) with the five chosen Peptidyl MIMs ranged from 0.6 to 1.8 μM. For comparison, two alpha-helically structured peptides, D2A21 and DP1E, were tested on several other cancer types: breast ($n = 2$), colon ($n = 2$), bladder, cervical and lung carcinomas ($n = 1$ each). Resulting LD_{50} values obtained in breast carcinoma cells were significantly higher ($P < 0.05$) than those observed in prostate cancer cells. LD_{50} values recorded for D2A21 and DP1E in cervical, colon, bladder, and lung cancer lines were similar to those obtained in prostate cancer cells. As compared with cisplatin, a standard chemotherapeutic drug, the LD_{50} values recorded for D2A21 were significantly lower ($P < 0.04$) in prostate-cancer cell lines, suggesting the therapeutic efficacy of Peptidyl MIMs. These data demonstrate for the first time the cytotoxic potential of Peptidyl MIMs against prostate cancer cells and suggest a dependence on a specific secondary alpha-helical structure of the peptide.

Prostatic carcinoma is the most commonly diagnosed cancer in men and is the second most common cause of cancer death in western civilization [4]. Current therapeutic options available for cancer patients include surgery, radiation therapy, and hormonal therapy. Nevertheless, a significant number of patients, especially when diagnosed at a more advanced stage, relapse and develop incurable disseminated metastatic disease [4]. Therefore, effective treatment modalities for patients with residual disease are clearly needed.

Peptidyl membrane-interactive molecules (MIMs) are laboratory-synthesized lytic peptides similar to the naturally occurring peptides that are involved in primitive immune systems of a number of invertebrates, lower vertebrates, and mammals [11]. Naturally occurring lytic peptides are active in innate immunological responses in diverse species [3], range from 23 to 39 amino acids in length, and fall into distinct structural classes (melittins, cecropins, magainins, and defensins) based on the arrangement of amphipathic and positive-charge densities. A number of naturally occurring peptides, such as cecropins produced by *Hyalophora cecropia* (the giant silk moth [11], magainins found in the skin of *Xenopus laevis* [6, 13], and tachyplesins isolated from the horseshoe crab *Tachylleus tridentatus* [7, 9], exhibit antimicrobial and other biological properties. A similar type of bioactive protein, melittin, is found in bee venom [5, 11, 14], and defensins, which were originally found in human neutrophilis [8], have also been described in insects [8]. Their role in nature may be that of a primitive immune system, protecting the host against bacterial

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C. N. Robertson (✉) · K. M. Roberson · A. Pinero · D. F. Paulson
Division of Urology, Department of Surgery, Box 3062, Duke University Medical Center, Durham, NC 27710, USA
Tel.: +1-919-684-8436; Fax: +1-919-684-3805

J. M. Jaynes
Demegen Inc., Durham, North Carolina, USA

infection, or, in the case of melittin in bee venom, as a defense against predators.

Naturally occurring cecropin, magainin, defensin, and melittin peptides differ significantly in amino acid sequence, but they retain a characteristically positively charged and potentially amphipathic alpha-helical or beta-pleated sheet structure [12, 13], indicating that the specific amino acid sequence is irrelevant to peptide function as long as certain physical properties of the peptide are maintained.

Recently magainins were shown to exhibit antitumor activity in human lung-cancer cell lines [11]. Concentrations lethal to 50% of the cell lines (LD_{50} values) of 1–5 μM were obtained in preliminary studies of 26 novel synthetic lytic peptides in human breast cancer cells, which demonstrates a potential for anti-tumor activity [7]. In the current study, nine novel Peptidyl MIMs were tested for in vitro cytotoxicity against four androgen-insensitive prostate-cancer cell lines. Comparison was made between these peptides and cisplatin, a chemotherapeutic agent of known antitumor activity. Breast, cervical, lung, and colon cancer lines were also tested for purposes of comparison.

Materials and methods

Cell lines and culture media

Cancer cell lines were maintained in RPMI 1640[1] supplemented with 5% fetal bovine serum (FBS)[2] and were passed with 0.25% trypsin/7 mM ethylenediaminetetraacetic acid (EDTA, Gibco) twice weekly. Human cancer cell lines included in this study were: androgen-insensitive prostatic cancer cell lines (1-LN, PC-3, DU145, ALVA-41); a transitional-cell carcinoma of the bladder (J82); an epidermoid carcinoma of the cervix (ME 180); a lung adenocarcinoma (A549); two colon adenocarcinomas (SW480, CoCo320); and two breast-cancer cell lines (MCF7, MDA-MB-453). All cell lines were obtained from the American Type Tissue Collection[3].

Novel lytic peptides

Peptidyl MIMs were provided by Demgen Inc.[4]. Peptides were solubilized in sterile H_2O diluted in culture media, and added to cells immediately. Their designations are D1A21, D2A21, D4E, D4E-1 D4B, DP1E, D5C, D5C-1, and D5F. Six of the Peptidyl MIMs (D1A21, D2A21, DP1E, D5C, D5C-1, and D5F) form alpha-helical secondary protein structures. Three of the Peptidyl MIMs (D4E, D4E-1, and D4B) form beta-pleated sheets. Their molecular weights range from 2000 to 5500 and they are 17–37 amino acids in length.

Cytotoxicity assays

Cytotoxicity was evaluated using the microculture tetrazolium (MTT) assay, which provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells [1]. Cultures were initiated in 96-well plates at 5×10^3 cells/well. This number of cells yields an optical density within the linear range of the relationship between cell numbers and formazan crystal production. For standard cytotoxicity assays, cells were allowed to reattach overnight and were exposed to Peptidyl MIMs (concentrations 1.0–10.0 μM; Demegen for 72 h. A 50-µl volume of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (12 μM) in phosphate-buffered saline (PBS)] was added to each well, and the plates were reincubated for 4 h. Supernatant media were aspirated, and formazan crystals were solubilized with 150 µl diethylsulfoxide (DMSO) per well at 37 °C for 10 min with gentle agitation. The quantity of solubilized formazan per well was determined spectrophotometrically at 540 nm using an EL311 Microplate Reader[5] linked to a Macintosh computer. Each assay was performed at least three times with six replicates per data point.

Data analysis and statistical methods

Raw data from the microplate reader were exported to Microsoft Excel for data analysis. All data were normalized to the mean optical density value recorded for the six control wells on each plate. Replicate wells of each peptide treatment concentration (six per concentration per plate) were then averaged. The resulting data were represented graphically as the mean treatment survival expressed as a percentage of the control value (control = 100% survival) ± standard error. An LD_{50} value was obtained from each plate by interpolation of the averaged data. At least three LD_{50} values were obtained for each cell line, and these values were averaged and presented as the mean LD_{50} value ± standard error. Comparisons between mean LD_{50} values were performed using Instat version 2.00 (GraphPad Software). A two-tailed Student's t-test was performed to establish significant differences between the results. This test assumes equal standard deviations in each data set. Where differences in the standard deviations became significant, a nonparametric (Mann-Whitney) test was substituted.

Results

Nine novel Peptidyl MIMs were tested against four human androgen-insensitive prostate-cancer cell lines (DU145, 1-LN, PC-3, Alva-41) using a standard MTT assay. Of the nine peptides tested, five (D1A21, D2A21, D5C, D5C-1, DP1E) demonstrated cytotoxicity at low concentrations in prostate cancer cells (Fig. 1). These peptides are known to form secondary alpha-helical structures. Four of the peptides were less effective against the prostate cell lines (Fig. 1). Three of these peptides (D4E, D4E-1, D4B) are thought to form beta-pleated sheets in their secondary structures.

Five peptides (D1A21, D2A21, D5C, D5C-1, DP1E) displaying antitumor activity against prostate-cancer cell lines were chosen for further study. Mean LD_{50} values ranged from 0.635 ± 0.008 to 1.800 ± 0.478 μM (Table 1). Four of the five peptides (DP1E, D1A21, D5C, D5C-1) had average LD_{50} values of >1.00 μM in each prostate-cancer cell line. D2A21 was most effective in the prostate-cancer cell lines, producing a group average LD_{50} of 0.882 μM.

[1] Sigma Chemical Co., St. Louis, Mo., USA

[2] Gibco, Grand Island, N. Y., USA

[3] Rockville, Md., USA

[4] Durham, N.C., USA

[5] Bio Tek Instruments, Inc., Winooski, Vt., USA

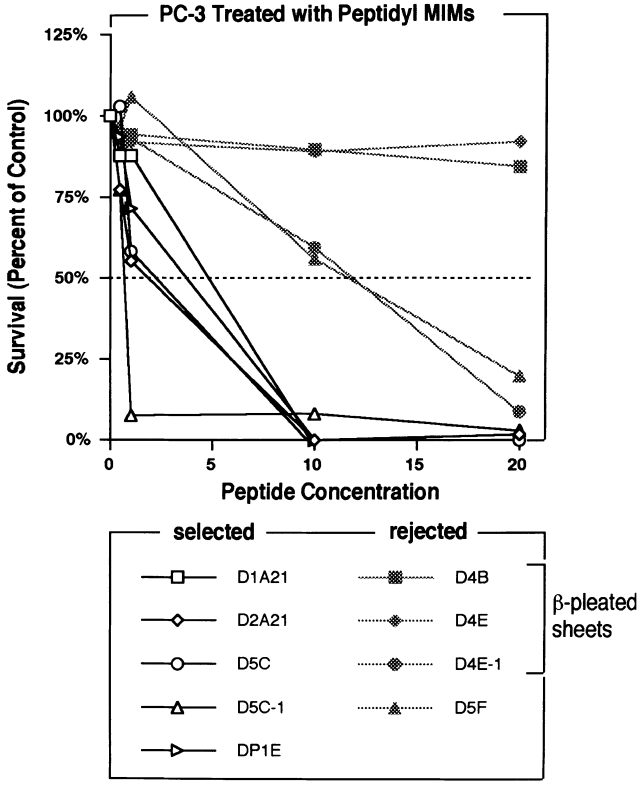

Fig. 1 Cytotoxic response of PC-3 to Peptidyl MIMs. Numbers of viable cells were determined by a standard MTT assay. The percentage of survival is given in the *ordinate* for the corresponding peptide concentration on the *abscissa*. Percentage of survival values represent the average of data obtained in at least 3 experiments. In all experiments, each concentration of peptide had 6 replicate survival values, which were averaged and normalized to calculate the percentage of survival of each peptide concentration

A standard chemotherapeutic drug, cisplatin, was assayed for comparison with D2A21 in prostate cancer cells. Cisplatin LD_{50} values ranged from 2 to 17 μM (Table 1). LD_{50} values recorded for peptides were significantly lower ($P < 0.04$) than those determined for cisplatin prostate-cancer cell lines 1-LN, DU145, and PC-3 (Fig. 2).

We then assayed the cytotoxicity of D2A21 and DP1E in two breast-cancer cell lines (MCF-7, MDA-MB-453). Mean LD_{50} values ranged from 1.818 ± 0.123 to 3.117 ± 0.112 μM for D2A21 and from 1.555 ± 0.105 to 1.945 ± 0.097 μM for DP1E. These values were significantly higher ($P < 0.05$) than those obtained in prostate cancer lines with the same peptides (Tables 1, 2).

A series of cancer cell lines were assayed with D2A21 and DP1E for comparison and the specificity of cytotoxicity. Bladder, colon, cervical, and long cancer lines were responsive to D2A21 and DP1E, with LD_{50} values ranging from 0.566 ± 0.019 to 1.334 ± 0.029 μM and from 0.950 ± 0.270 to 1.361 ± 0.136 μM, respectively (Table 2). These values were not significantly different from those observed for prostate cancer cells.

Discussion

This study demonstrates that Peptidyl MIMs (1) forming an alpha-helical secondary structure are cytotoxic to prostate cancer cells in vitro, (2) are significantly more effective than cisplatin in prostate cancer cells, (3) are significantly more effective in prostate cancer cells than in breast cancer cells in vitro, and (4) are equally cytotoxic to cervical, bladder, and lung cancer cells in vitro.

That Peptidyl MIMs forming alpha-helices are more active in prostate cancer cells than those forming beta-pleated sheets is a novel finding. Naturally occurring peptides are highly heterologous in their amino acid sequence, but at least a portion of the sequence has the capacity to form an amphipathic alpha-helix [2]. Amphipathy, therefore, appears to be important in maximizing the interaction of the peptide with the cell membrane, with the ultimate result being cell death. Formation of an alpha-helix may confer an advantage for membrane intercolation and, therefore, enhance membrane disruption. Of the five Peptidyl MIMs that form alpha-helices, D2A21 was consistently more effective than the other four peptides in each prostate-cancer cell line (difference not statistically significant). Interestingly, D2A21 is identical to D1A21 in terms of composition, but D1A21 is the mirror image of D2A21. Spatial orientation may therefore be important in anti-

Table 1 Mean LD_{50} values obtained in prostate-cancer cell lines[a]

Cell line	D2A21 (*n*)	DP1E (*n*)	D1A21 (*n*)	D5C (*n*)	D5C-1(*n*)	Cisplatin
1-LN	0.99 μM ± 0.132 (6)	1.34 μM ± 0.245 (6)	0.928 μM ± 0.201 (4)	1.25 μM ± 0.248 (4)	1.09 μM ± 0.478 (3)	4.31 μM ± 0.710
ALVA-41	1.11 μM ± 0.332 (4)	0.969 μM ± 0.143 (6)	1.73 μM ± 0.388 (4)	1.80 μM ± 0.477 (3)	1.53 μM ± 0.421 (3)	5.15 μM ± 1.59
DU 145	0.635 μM ± 0.088 (6)	0.864 μM ± 0.112 (6)	0.803 μM ± 0.149 (3)	1.46 μM ± 0.069 (3)	0.989 μM ± 0.374 (2)	2.17 μM ± 0.018
PC-3	0.700 μM ± 0.103 (5)	1.45 μM ± 0.162 (6)	1.03 μM ± 0.205 (4)	1.17 μM ± 0.055	1.23 μM ± 0.434 (3)	16.4 μM ± 0.496
Group mean value	0.845 μM	1.16 μM	1.14 μM	1.39 μM	1.23 μM	7.01 μM

[a] Mean values ± SE

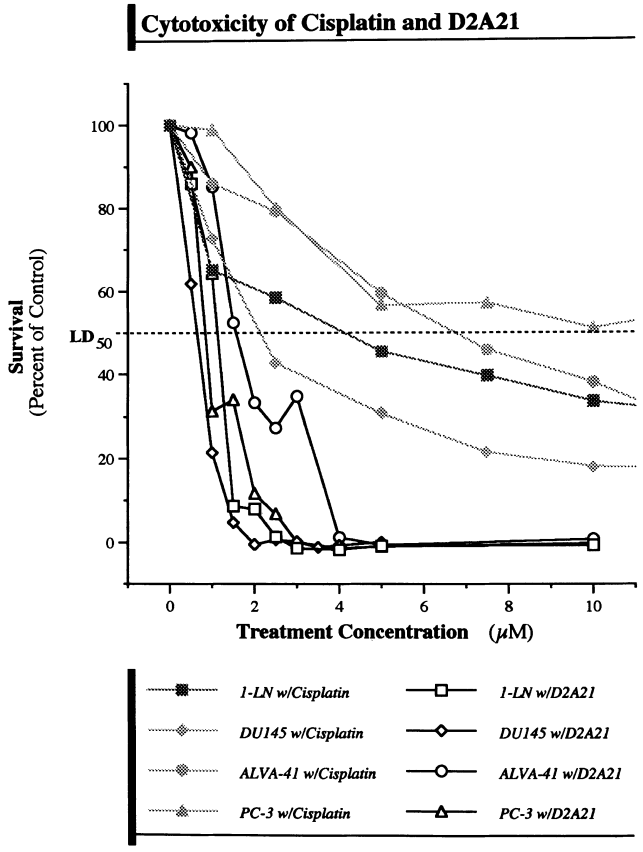

Fig. 2 Cytotoxic response of prostate-cancer cell lines treated with cisplatin and D2A21. The percentage of survival is given in the *ordinate* and represents the percentage of survival relative to the control value. The treatment concentration is given in the *abscissa*. Each *data point* represents a percentage of survival calculated as the mean value for at least 3 separate MTT experiments. There were 6 replicates per experiment per concentration

tumor activity. Also, the degree of hydrophobicity may be important in terms of antitumor activity because D5F forms an alpha-helix but is less hydrophobic than the other alpha-helical formin peptides tested.

Peptidyl MIMs were specifically designed to maximize antitumor effects in human cancer cells. LD_{50} values ranged from 0.635 to 1.800 μM. In similar studies using naturally occurring peptides (magainins), human lung-cancer cell lines were inhibited with average LD_{50} values of 8.82–8.64 μM [11, 13]. Peptidyl MIMs were 8 to 10-fold more effective in prostate cell lines. We also compared the activity of D2A21 and cisplatin in prostate-cancer cell lines. LD_{50} values recorded for D2A21 were significantly lower than those obtained for cisplatin ($P < 0.04$). Taken together, these data make Peptidyl MIMs, especially D2A21, a therapeutically reasonable option for prostate cancer patients. We are currently assessing the administration, dosage, and efficacy of D2A21 in human prostate cancer cells (PC-3) implanted in nude mice. A subsequent publication will present the findings.

Prostate cancer cells were significantly more responsive than breast cancer cells to D2A21 ($P < 0.05$). Whether differences in the membranes of prostate cancer cells account for the differential interaction with these peptides is unknown. It has been suggested that Peptidyl MIMs may have an affinity for membranes that contain accessible acidic phospholipids [9].

The Peptidyl MIMs tested were not, however, specific for prostate cancer cells. Cervical, lung, bladder, and colon cancer cells were sensitive to D2A21 with similar LD_{50} values. Increased specificity for prostatic cells attained through targeting strategies is under investigation in our laboratory. DU 145 was the most sensitive of the prostate-cancer cell lines, responding the best to four of the 5 alpha-helix-forming peptides.

Although the mechanism of action is unknown, lytic peptides are thought to cause disruption of the cell membrane secondary to micropore enlargement, resulting in lysis due to loss of osmotic integrity [10]. However, several mechanisms have been proposed for similar compounds. Cytolysis due to membrane disruption may occur via any of several modes: (1) the amphipathic helix may intercolate into the head group region of the membrane lipid bilayer [8], (2) phospholipids may be released from the bilayer [12], or (3) ion channels [8] may form due to aggregation of native proteins [8]. Further study of the mechanism of action of Peptidyl MIMs is warranted and is in progress in our laboratory.

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Table 2 Mean LD_{50} values obtained in breast-cancer cell lines and other cancer cell lines[a]

Cell line (origin)	D2A21 (*n*)	DP1E (*n*)
Breast cancer cell lines:		
MDA-MB-453	1.818 ± 0.123 μM (4)	1.945 ± 0.097 μM (4)
MCF 7	3.117 ± 0.112 μM (3)	1.555 ± 0.105 μM (4)
Group mean value	2.370 μM	1.750 μM
Other cancer cell lines:		
J82 (Baldder)	0.566 ± 0.019 μM (4)	1.254 ± 0.090 μM (4)
COCO320 (colon)	0.860 ± 0.082 μM (4)	0.966 ± 0.070 μM (4)
ME 180 (cervix)	0.807 ± 0.143 μM (4)	0.950 ± 0.270 μM (5)
A549 (lung)	0.981 ± 0.146 μM (4)	1.272 ± 0.080 μM (5)
SW480 (colon)	1.334 ± 0.029 μM (3)	1.361 ± 0.136 μM (4)
Group mean value	0.897 μM	1.166 μM

[a] mean values ± SE

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