Suppression of Human Prostate Tumor Growth in Mice by a Cytolytic d-, l-Amino Acid Peptide: Membrane Lysis, Increased Necrosis, and Inhibition of Prostate-Specific Antigen Secretion

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ABSTRACT

Gene-encoded host defense peptides are used as part of the innate immunity, and many of them act by directly lysing the cell membrane of the pathogen. A few of these peptides showed anticancer activity in vitro but could not be used in vivo because of their inactivation by serum. We designed a 15-amino acid peptide, composed of d- and l-amino acids (diastereomer), which targets both androgen-independent and androgen-dependent human prostate carcinoma cell lines (CL1, 22RV1, and LNCaP). Most importantly, we observed a complete arrest of growth in CL1 and 22RV1 xenografts treated intratumorally with the diastereomer. This was also accompanied by a lowering of prostate-specific antigen serum levels secreted by the 22RV1 xenograft. Furthermore, the diastereomer synergized with conventional chemotherapeutics. In contrast, the parental all l-amino acids peptide was highly active only in vitro and could not discriminate between tumor and nontumor cells. Fluorescent confocal microscopy, histopathologic examination, and cell permeability studies (depolarization of transmembrane potential and release of an encapsulated dye) suggest a necrotic mechanism of killing, after a threshold concentration of peptide has been reached. Its destructive killing effect and the simple sequence of the diastereomer make it an attractive chemotherapeutic candidate possessing a new mode of action, with potential to be developed additionally for the treatment of prostate carcinoma.

INTRODUCTION

Prostate carcinoma is considered the most common nonskin cancer in America. It accounts for ~30% of all of the major cancers in men, which is more than twice the next most common cancer (1–3). Prostate carcinoma is also the cancer with the largest expected increase in the next decade, with a projected annual incidence rising to 300,000 cases and an annual death rates reaching 50,000 by the year 2015 (reported by the Prostate Cancer Foundation). The approaches currently used to combat prostate carcinoma are surgery, irradiation, or chemotherapeutic (4–6). Chemotherapeutic drugs damage cancer cells by a variety of mechanisms (e.g., DNA cleavage/alkylation and topoisomerase II inhibition) that are eventually translated into apoptotic signals. Unfortunately, prostate carcinoma, in contrast to several other cancers, does not respond well to single or multiple drug regimens, especially in the case of androgen-independent cancer (7–10). In addition, chemical anticancer agents are nonspecific and, consequently, damage healthy tissues as well. This has stimulated the search for new drugs with new modes of action and with a potential to overcome the inherent resistance. Examples include the development of polypeptides that control apoptosis (11–13), or alternatively, peptides that lyse cells. Indeed, cell lytic antimicrobial peptides have been shown to act in vitro against different types of cancer cells (14–17). These peptides have a central role in the innate immunity of all of the organisms, including insects, amphibians, and mammals (18). Examples include human defensins (19–21), cecropins (22), cecropins-magainin hybrids (23, 24), magainins (14), peptides conjugated to homing domains (15–17), propeptides (25), and others (26, 27). These peptides preferentially bind and disrupt negatively charged phospholipid membranes, the major component of the bacterial cytoplasmic membrane. However, it is not clear why some of them bind better and kill cancer cells compared with normal cells (28, 29).

Despite the potent anticancer activity of these peptides in vitro, studies in vivo are very limited, mainly because of their inactivation in serum, partially because of their binding to serum components and their enzymatic degradation. Examples include the following data: (a) apoptotic peptides targeted to specific tissues by conjugating them to homing domains (15, 16); and (b) i.p.-injected antimicrobial peptides derived from magainin and its all l-amino acid analog against ovarian cancer (14).

We have shown recently that incorporation of l-amino acids into noncell-selective lytic peptides made them selective toward bacteria (30). Some of these peptides were also lytic to cancer cells (29). Most importantly, this family of peptides preserved their activity in serum, and their enzymatic degradation could be controlled. One peptide, a 15-amino acids diastereomer composed of leucines, arginines, and lysines, was shown recently to act against the mouse melanoma and lung carcinoma cell lines and to significantly inhibit lung metastasis in mice with no detectable side effects (31). Here, we show that a 15-mer all l-amino acids lytic peptide and its diastereomer, both of which are composed of only lysines and leucines, are highly active preferentially toward both androgen-dependent and androgen-independent human prostate carcinoma cells. Most importantly, the diastereomer, but not its all l-amino acids parental peptide, completely inhibited the growth of several human prostate tumor xenografts and markedly reduced the secretion of the prostate-specific antigen (PSA) produced by the 22RV1 xenografts. In contrast, a 12-amino acids diastereomer and two other native antimicrobial peptides, with antimicrobial activity similar to that of the 15-amino acids diastereomer, were either inactive or nonselectively active toward cancer cells compared with noncancer cells. The 15-amino acids diastereomer was additionally evaluated for its ability to act synergistically with other chemotherapeutic agents and for its plausible mode of action, by using confocal fluorescence microscopy, fluorescently labeled peptide, and cell-permeable markers. The results are discussed with respect to a necrotic-like mode of action for the diastereomer and its advantages over all l-amino acids lytic peptides for future development as a new chemotherapeutic agent with a new mode of action.

MATERIALS AND METHODS

Cell Culture

The CL1 human prostate carcinoma cell line is an androgen-independent subclone of LNCaP, which was generated by culturing androgen-dependent LNCaP cells in charcoal-stripped, androgen-de-
pendent serum as described previously (32). The 22RV1 human prostate carcinoma cells are androgen-independent subclones of the androgen-dependent prostatic adenocarcinoma CWR22 xenograft (33). CL1 and 22RV1 (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 10% FCS (Biological Industries, Beit Haemek, Israel). Androgen-dependent LNCaP human prostate carcinoma cells (34) were grown similarly to CL1 but with the addition of 5 μg/ml insulin and 10 nm testosterone (Sigma Chemical Co.). NIH-3T3 mouse fibroblast cell lines (American Type Culture Collection) were grown in DMEM supplemented with 10% FBS. OL human foreskin fibroblasts (American Type Culture Collection; a generous gift from Professor Menachem Rubinstein, Weizmann Institute of Science, Rehovot, Israel) were maintained in 10% FCS and 90% DMEM.

Peptide Synthesis, Rhodamine Labeling, and Purification

Peptides were synthesized by a F-moc solid-phase method on Rink amide 4-methyl benzhydrylamine resin (Calbiochem-Novabiochem, La Jolla, CA; Ref. 35), using an ABI 433A automatic peptide synthesizer, and purified as described previously in detail (31). Labeling of the NH2 terminus of the peptides with rhodamine was done on the resin-bound peptide (36). The purified peptides were shown to be homogeneous (>99%) by analytical high-performance liquid chromatography. Finally, the peptides were subjected to amino acid analysis and electrospray mass spectroscopy to confirm their composition and molecular weights.

In Vitro Cytotoxicity Assays

Aliquots of medium containing 1 × 10^4 cells were distributed into a 96-well plate (Falcon). The following day, the media were replaced with 90 μl of fresh media and 10 μl of a solution containing different concentrations of the peptides. The plate was then incubated for 24 h before adding to each well 50 μl of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) reaction solution (Biological Industries); viability was determined as described previously (31). The LC50 (concentration at which 50% of the cells die) for each peptide was obtained from the dose-dependent cell viability curves. The kinetics of cell killing was also monitored by using the LC100 (concentration at which 100% of the cells die) of the peptide (50 μM, 80 μM, 300 μM, and 400 μM, for CL1, 22RV1, OL, and 3T3 cells, respectively).

The toxicity of the peptides against human red blood cells (4% v/v) was also investigated as described previously (30). Controls for zero hemolysis (blank) and 100% hemolysis consisted of human red blood cells suspended in PBS and 1% Triton (Sigma), respectively.

Detection of Cell Permeability Induced by the Peptide

Depolarization of Transmembrane Potential. Cells were incubated with 1 μM diS-C5-5 (Molecular Probes, Eugene, OR), and the fluorescence intensity was recorded until a plateau was reached (λex = 620 nm; λem = 670 nm). The peptide (final concentration of 0.1–100 μM), dissolved in PBS, was then added to 50 μl of the fluorescently labeled cells to make a final volume of 100 μl (the number of cells/well was similar to those in the XTT viability assay). The resulting suspension was incubated with agitation for 60 min at 37°C. Membrane depolarization was monitored by observing the change in the intensity of fluorescence emission of the membrane-potential-sensitive dye. The control for zero membrane depolarization (blank) consisted of fluorescently labeled cells suspended in PBS. One hundred percent membrane depolarization was set as the difference in fluorescence intensity between zero and 60 min after adding the dye to the cells.

Calcein Leakage from Cells

Calcein fluorophore (Hach Chemical Co., Loveland, CO; 1 μM, a self-quenching concentration) was entrapped into the above cells by incubation for 1 h (0.4 × 10^6 cells/ml in 10 ml of PBS). The nonencapsulated calcein was removed from the cells by centrifugation. Cells were resuspended in PBS, and the peptide (final concentration 0.1–100 μM) was added to the cell suspensions. Peptide-induced calcein leakage resulted in an increase of fluorescence (37); it was monitored at room temperature (λex = 485 nm; λem = 515 nm). Complete dye release (100% activity) was obtained after disrupting the cells with Triton X-100 (0.1% final concentration). Under the experimental conditions, in the absence of peptide, the leakage rate was <1% in 5 h. The kinetics of transmembrane potential depolarization and calcein leakage was also monitored. For this assay we used the LC100 of the peptide (50, 80 μM, 300 μM, and 400 μM, for CL1, 22RV1, OL, and 3T3 cells, respectively).

Confocal Fluorescence Microscopy Studies

Confocal images were obtained using an Olympus IX70 FV500 confocal laser scanning microscope. Briefly, cells were placed on a coverslip, and a series of images were taken before and 3 min after the addition of rhodamine-labeled peptide (5 μM in PBS), using oil immersion. The kinetics of peptide binding and cell lysis was also followed using the rhodamine-labeled peptide at its LC100 for each cell. The setting of the photomultipliers (gain and black level) was constant for the series of images. Care was taken so that any existing photobleaching did not compromise the interpretation and laser irradiation, and other means of illumination were prevented between images. The confocal images were obtained at a 12-bit resolution.

Measurements of Synergism by Using Checkerboard Titrations

Synergistic activities were assayed by checkerboard titrations with RPMI 1640. Fractional lethal concentration (FLC) indices were calculated by using the following formula:

\[
\left[\frac{(A)/LC_{100A}}{LC_{100B}} + \left[\frac{(B)/LC_{100B}}{LC_{100A}}\right]\right] = FLC_{A} + FLC_{B} = FLC_{index},
\]

where LC100A and LC100B are the LC100 of drugs A and B when used alone, and (A) and (B) are the LC100 of drugs A and B when used in combination. Drug A stands for the peptide, and drug B stands for doxorubicin or cyclophosphamide (Sigma); the cells used were CL1, 22RV1, and LNCaP human prostate carcinoma cells (grown and treated as described previously). The interaction between the peptide and the chemotherapeutic agent was defined as synergistic if the FLC index was 0.5 or less, additive if the FLC index was >0.5–1.0, indifferent if the FLC index was >1.0–2.0, and antagonistic if the FLC index was >2.0 (38).

Studies with Prostate Carcinoma Xenografts

Inhibition of Tumor Growth in Human Prostate Carcinoma Xenografts. The s.c. implantation of human prostate carcinoma in mice was done as described previously (39). Briefly, 0.1 ml of androgen-independent CL1 and 22RV1 human prostate carcinoma cells (5 × 10^6 cells) in Matrigel (Biological Industries) was inoculated s.c. into the dorsal side of 5–6-week-old nude male mice weighing 20–25 g (Harlan Co., Jerusalem, Israel). Two weeks after cell implantation, when the tumor diameter reached ≥5 mm (we denoted this day as day 1), the all t-amino acids peptide and its diastereomer (at 1 mg/kg; 0.1 mm) or vehicle (PBS: pH = 7.4) were injected intratumorally (dosing volume of 2.5 ml/kg) three times a week for a total of 9 doses. Tumor size was measured by a caliper and recorded twice a week during a period of 28 days. Mice were weighed, and tumor weight (mg) was estimated by using the formula of length × width × depth × 0.52 in mm^3, assuming the specific gravity to be 1. At the end of the treatment, the mice were killed, and the
tumors were removed, photographed, and weighed. The animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee.

Serum PSA Levels

Four weeks after the first treatment, blood was withdrawn from the 22RV1-inoculated mice to determine the level of PSA. The blood samples were taken directly to heparin-containing tubes, centrifuged, and the supernatants were stored at −20°C. The CanAg PSA ELISA kit (CanAg Diagnostics) was used to determine the total PSA in the mice plasma (39). Tumor weight and PSA levels, represented as the mean ± SE, were calculated from the raw data and then subjected to Student’s t test. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

We synthesized an amphipathic all $L$-amino acids peptide and its diastereomer, designated as amphipathic-$L$ (LKLLKKLLKLKLKLKH$_2$), and amphipathic-$D$ (LKLLKKLLKLKLKLKLKH$_2$, underlined and italic letters are $D$-amino acids). The data presented below demonstrated that only amphipathic-$D$ could arrest the growth of human prostate carcinoma xenografts, and, therefore, most experiments regarding mode of action were performed only with this peptide.

In Vitro Activity of the Peptides toward Androgen-Dependent and Androgen-Independent Human Prostate Carcinoma Cells.

The activity of amphipathic-$L$ and -$D$ was tested against various cell lines. We used as controls a 12-mer diastereomer (LKLLKKLLKLKLKLKLKH$_2$, underlined and italic letters are $D$-amino acids), the bee venom melittin and its diastereomer (melittin-$D$; Ref. 30, 40), and the human chemotherapeutics doxorubicin and mitomycin C (Sigma Chemical Co.) were used as additional controls. The LC$_{50}$ of the peptides are shown in Table 1. The data reveal that amphipathic-$D$ is the most selective peptide toward prostate carcinoma cells. In addition, amphipathic-$D$ is not hemolytic toward human erythrocytes at the maximal concentration tested, whereas amphipathic-$L$, LL-37, and melittin are highly hemolytic (Table 1). Note that the kinetics of cell killing using LC$_{100}$ values (shown in Fig. 1A, right panel) is very rapid and is similar in all cell types.

Depolarization of Transmembrane Potential and the Release of Entrapped Calcein from Cells Induced by Amphipathic-$D$. Amphipathic-$D$ induced depolarization of transmembrane potential (Fig. 1B) and leakage of entrapped calcein (Fig. 1C) from cells. Importantly, the data revealed a direct correlation between the LC$_{50}$ values of the peptide toward all of the cells (Table 1; Fig. 1A, left panel) and the dose-dependent potential depolarization (Fig. 1B, left panel) and calcein leakage (Fig. 1C, left panel). This suggests that the cell membrane is one of the targets. As expected, the peptide could not dissipate the transmembrane potential of erythrocytes, in agreement with its nonhemolytic activity (data not shown).

We also followed the kinetics of membrane depolarization (Fig. 1B, right panel) and calcein release (Fig. 1C, right panel) as a function of time at the LC$_{100}$ of the peptide. The data revealed that the kinetics of cell killing (Fig. 1A, right panel) is similar to the kinetics of the two membrane disruption assays. These results additionally suggest that membrane disruption is the lethal event for all of the cells tested.

The Addition of Amphipathic-$D$ Triggers Necrosis of Prostate Carcinoma Cells. Amphipathic-$D$ was labeled with rhodamine at its NH$_2$ terminus (without affecting its anticancer activity) and then incubated (5 $\mu$m peptide) with the cells. The cells were then washed and observed for residual rhodamine. The data revealed that the peptide bound strongly and preferentially to the CL1 (Fig. 2A, lower panel) and 22RV1 (Fig. 2B, lower panel). Prostate carcinoma cells and significantly less to OL foreskin fibroblasts (Fig. 2C, lower panel).

The peptide was additionally incubated with the cells at its LC$_{100}$ values, and images were taken. Fig. 2D shows examples of images of CL1 cells after treatment for 2, 3, and 5 min with 50 $\mu$m of the rhodamine-labeled amphipathic-$D$. The cells died as a result of acute injury, swelling (after 3 min), and bursting (after 5 min), leaving only the nucleus intact. This suggests a necrotic mode of action.

The kinetics of binding of the peptide (at its LC$_{100}$ value) to cells was also monitored. The data reveal >80% binding to all of the cells already after ~3 min and complete binding after ~5 min. Note that ~80% of membrane leakage (depolarization of transmembrane potential and calcein release experiments) took place a few minutes after the binding of the peptide (Fig. 1, B and C, right panels).

Synergistic Effect between Amphipathic-$D$ and Doxorubicin against Androgen-Dependent and Androgen-Independent Prostate Carcinoma Cells. Checkerboard titrations were performed using amphipathic-$D$ in combination with doxorubicin and cyclophosphamide against the CL1, 22RV1, and LNCaP cell lines (Table 2). The LC$_{100}$ values of doxorubicin against CL1 and LNCaP cells was found to be 200 $\mu$m and 30 $\mu$m, respectively, and the LC$_{100}$ values for cyclophosphamide against CL1, 22RV1, and LNCaP cells were 1000 $\mu$m, 3000 $\mu$m, and 5000 $\mu$m, respectively. The peptide was incubated for 2 min with the cells before adding the drugs. The peptide showed a significant synergism with doxorubicin against the three cell lines (FLC < 0.5). For example, the FLC value of 0.44 (Table 1) corresponds to a combination of .25 and .20 of the LC$_{100}$ values of amphipathic-$D$ and doxorubicin, respectively. In contrast, only an additive effect was found when the peptide was combined with cyclophosphamide (0.5 < FLC < 1.0). The results were similar when the peptide was incubated for 60 min before adding the drugs or when the order of the addition was reversed. Note that in all of the cases,

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### Table 1 Lethal concentrations (LC$_{50}$ in $\mu$m)* of the peptides detected by XTT assay

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>NIH-3T3 mouse fibroblasts</th>
<th>OL foreskin fibroblasts</th>
<th>CL1 prostate carcinoma</th>
<th>22RV1 prostate carcinoma</th>
<th>LNCaP prostate carcinoma</th>
<th>% Hemolytic activity at 100 $\mu$m</th>
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<tr>
<td>12-mer</td>
<td>&gt;100</td>
<td>50</td>
<td>100</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0</td>
</tr>
<tr>
<td>Amphipathic-$D$</td>
<td>100</td>
<td>35</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Amphipathic-$L$</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>LL-37</td>
<td>28</td>
<td>14</td>
<td>6</td>
<td>12</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>Melittin</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Melittin-$D$</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0</td>
</tr>
<tr>
<td>Doxorubicin†</td>
<td>ND</td>
<td>ND</td>
<td>120</td>
<td>ND</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are the mean of three independent experiments each performed in duplicate, with SD not exceeding 10%. Amphipathic-$D$ is similarly potent against other cancer cell lines (29).

† LC$_{50}$ values for doxorubicin were taken from An et al. (51) and repeated in this study.
neither indifference (1.0 < FLC ≤ 2.0) nor antagonism (FLC > 2.0) was observed. Experiments were repeated three times with a SD of 5%.

Amphipathic-D but not Amphipathic-L Inhibits Prostate Tumor Growth in Xenografts and Lowers the PSA Level. CL1 and 22RV1 cells were implanted s.c. in mice, and the peptides were injected intratumorally at a dose of 1 mg/kg (stock solution of 0.1 mM), starting two weeks after tumor cell implantation. Dose response studies with 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, 3 mg/kg, and 5 mg/kg revealed 1 mg/kg as the optimal dose. A significant reduction in tumor weight was observed only with mice treated with amphipathic-D (see Figs. 3 and 4A for 22RV1; and Fig. 4B for CL1 xenografts). Most importantly, the tumor completely disappeared in 40% of the mice. A significant reduction in tumor weights was observed from day 13 until day 28, reaching 4-fold in the mean weight of both left tumors (Fig. 4, A and B). Furthermore, in the PSA-secreting 22RV1 xenografts (33), the reduction in tumor weight was accompanied by a marked lowering of the PSA serum levels (Fig. 4C). Note also that treatment with amphipathic-D resulted in an increase in the body weight of the animals compared with the vehicle-treated control group (data not shown). In contrast, amphipathic-L was inactive in both xenograft models. Moreover, the peptide caused severe necrosis on the skin of the animal in the area of injection (Fig. 3A, right panel).

Tumors contain extracellular matrix (ECM), therefore, we studied its interactions with the peptides. Briefly, amphipathic-D and amphipathic-L were mixed with Matrigel matrix for 1 h, and the solution was analyzed by using reverse phase-high-performance liquid chromatography and mass spectroscopy. The data revealed that amphipathic-L was fully inactivated by the Matrigel, in contrast to amphipathic-D, which preserved 50% of its activity (data not shown).

Preliminary acute toxicity of the peptide was also examined by i.v. injecting mice (n = 5) with a single dose of a 0.9-ml solution containing 9 mg/kg/day for 5 days. No mortality and no long-lasting side effects were observed. All of the differential and biochemistry tests were in the range of normal values.

Histological Examination. Tumor sections taken 6 weeks after cell implantation revealed that amphipathic-D but not amphipathic-L inhibited the growth of prostate carcinoma cells. Fig. 3C shows the histological sections only for 22RV1 cells. The data revealed that in both the control xenografts (left panel) and those treated with amphipathic-L (right panel) most of the ECM was replaced by tumor cells. This is in contrast with mice treated with amphipathic-D, in which the number of tumor cells was reduced dramatically (middle panel).
The most important result is that amphipathic-D but not its all L-amino acids parental peptide, amphipathic-L, markedly reduced tumor weights in both CL1 and 22RV1 prostate carcinoma xenografts, and the tumor completely disappeared in 40% of the mice. The histological evaluation showed necrotic debris in the sections taken from the remaining tumors treated with amphipathic-D, and the density of the cells was very low (Fig. 3C). This is in contrast with the significantly higher amount of mitotic cells in the untreated tumor or tumors treated with amphipathic-L. These results demonstrate that the remaining tumor does not proliferate normally. Furthermore, the reduction in the tumor size was accompanied by a drastic lowering of the PSA level in the 22RV1 xenografts, which secrete this antigen (33). Note that amphipathic-L did not inhibit PSA synthesis, suggesting that the reduction in the PSA level was probably due to the reduction in tumor size. Therefore, prolonged treatment with amphipathic-D is not likely to interfere with the validity of PSA as an indicator of prostate carcinoma progression, in contrast to other drugs, such as antiandrogen or antiestrogen chemotherapeutics (42).

Interestingly, despite being similarly active on cancer cells (Table 1) and bacteria (44), amphipathic-D but not amphipathic-L, is selective toward prostate carcinoma cells (Table 1; Fig. 1A, left panel). A plausible explanation for this selective activity could be as follows: A common feature found in amphipathic-L and all of the other noncell-selective peptides is their stable amphipathic α-helical structure in membranes as well as their similar affinity to negatively charged and zwitterionic membranes. Structural studies revealed that both amphipathic-L and amphipathic-D have unordered structures in solution (31). However, whereas amphipathic-L strongly binds and becomes fully helical in any type of membrane, amphipathic-D binds 15-fold better negatively charged membranes compared with zwitterionic membranes (45). This binding is governed mainly by electrostatic interactions (45). Membrane binding forces the peptide to adopt a functional structure, which allows destabilization of the membrane (31). In agreement with this, many cancer cells have approximately

### Table 2 Checkerboard titrations of combinations of amphipathic-D with different chemotherapeutic agents

<table>
<thead>
<tr>
<th>Chemotherapeutic agent</th>
<th>Cell type</th>
<th>FLC index*</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>CL1 prostate carcinoma</td>
<td>0.44</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>22RV1 prostate carcinoma</td>
<td>0.49</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>LNCaP prostate carcinoma</td>
<td>0.49</td>
<td>Synergism</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>CL1 prostate carcinoma</td>
<td>0.57</td>
<td>Additivity</td>
</tr>
<tr>
<td></td>
<td>22RV1 prostate carcinoma</td>
<td>0.66</td>
<td>Additivity</td>
</tr>
<tr>
<td></td>
<td>LNCaP prostate carcinoma</td>
<td>0.63</td>
<td>Additivity</td>
</tr>
</tbody>
</table>

* FLC index = FLC_A + FLC_B = [(A)/(LC_{100A})] + [(B)/(LC_{100B})], where LC_{100A} and LC_{100B} are the LC_{100} values of drugs A and B when used alone, and (A) and (B) are the LC_{100} of drugs A and B when used in combination. Experiments were repeated three times with an SD of 5%.
2–4% more acidic phospholipids in their outer leaflet, and together with O-glycosylated mucines [high molecular weight glycoproteins consisting of a backbone protein to which oligosaccharides are attached via the hydroxylic groups of serine or threonine (46)] they create the additional negative charge. Nevertheless, we cannot rule out the possibility that the higher negative potential inside cancer cells, compared with noncancer cells, also contributes to the selective lytic activity of amphipathic-D.

Although a necrotic rather than an apoptotic mechanism of killing is suggested, the details by which amphipathic-D kills prostate carcinoma cells is still not fully understood. Among the hundreds of antimicrobial peptides isolated thus far, only a few were investigated for their mode of action on cancer cells (27).³ Most of these studies included many biophysical techniques conducted mainly with model phospholipid membranes (29, 47). To better understand the killing mechanism, we used predominantly living cells and evaluated the data from killing curves, binding studies, fluorescent dye efflux, and membrane potential experiments. All of these experiments were conducted under identical experimental conditions. We observed a necrotic process that involves four major steps in the following order: (a) amphipathic-D binds initially to distinct sites on the cytoplasmic membrane of the cell, probably due to the nonhomogeneous distribution of


anionic phospholipids or other acidic components in its outer leaflet (29, 48), and then it reaches a threshold concentration; (b) the peptide induces marked membrane depolarization; (c) the kinetics of membrane permeation is fast followed by an equal distribution of the peptide in the cytoplasm; and (d) the cells die. These steps are similar to those observed with the cytolytic perforin produced by killer lymphocytes, which kill its target cells by puncturing their membranes (49).

Finally, we show that amphipathic-D acts synergistically with doxorubicin toward all types of cells including androgen-independent and androgen-dependent, and, therefore, the status of hormone-dependency does not seem to affect this synergism. This is in contrast to other chemotherapeutics, such as the proapoptotic tumor necrosis factor-related apoptosis-inducing ligand that acts synergistically with the proteasome inhibitor bortezomib on LNCaP prostate carcinoma but has an antagonistic effect on CL1 prostate carcinoma. The mechanism of the synergism by amphipathic-D still remains largely unknown. Nevertheless, one possibility is its ability to increase the permeability of the cell to other chemotherapeutics.

In summary, this study shows that amphipathic-D, but not its parental amphipathic-I, peptide, can be administered intratumorally, and it dramatically reduces the tumor growth of various human prostate carcinoma xenografts. Note, however, that membrane-active peptides are known to be toxic when injected i.v. at high doses. Here,
Furthermore, amphipathic-D, together with other chemotherapeutic agents, represents a promising drug combination that should be explored additionally for therapeutic use.

ACKNOWLEDGMENTS

We thank Arik Makovitzki and Tova Waks for their help in the in vivo experiments. We are also grateful to Menachem Rubinstein for providing us human OL foreskin fibroblasts, Dr. Alon Harmelin for his assistance in the histologic evaluation, and Vladimir Kiss for his technical support in the confocal microscopy studies.

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