A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity

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Abstract

A potent cytotoxic peptide (r7-kla) was synthesized by incorporating a mitochondrial membrane disrupting peptide, kla (klaklaklaklak), with a cell-penetrating domain, r7 (rrrrrrr). The IC50 of r7-kla (3.54 ± 0.11 μmol/L) was more than two orders of magnitude lower than that of kla. r7-kla induced cell death in both in vitro and in vivo environments, and showed rapid kinetics. Within minutes, the morphologic changes in cells and mitochondrial leakage were apparent by microscopy and was consistent with rapid apoptosis. Our results suggested that r7-kla is an apoptosis inducer and can be potentially used as an anticancer agent, especially when combined with the appropriate systemic delivery systems. [Mol Cancer Ther 2006;5(8):1944–9]

Introduction

Molecularly targeted anticancer agents are rapidly emerging; however, many cancers ultimately show drug resistance. Consequently, combination therapies are common places to improve treatment outcomes. The essential role of mitochondria to mediate programmed cell death has led to the recent attention towards antitumor agents that target mitochondrial functions (1, 2).

Certain cationic amphipathic peptide sequences are able to interact with the negatively charged prokaryotic plasma membrane and cause lipid matrix distortion (3, 4). These peptides are normally nontoxic to eukaryotic cells, as the mammalian plasma membrane is mainly composed of zwitterionic phospholipids. Recently, an L-configuration amphipathic antimicrobial peptide sequence, KLAKLAKLAKLAKLAKLAKLAK (KLA), was reported as an antibacterial agent (5). This nontoxic peptide can be further modified by fusion of a transduction domain to enhance its cellular uptake (6). Once inside the cell, KLA is cytotoxic and was shown to disrupt the negatively charged mitochondrial membrane. Subsequent apoptosis was triggered via the release of cytochrome c (7, 8). Other studies have also shown that the attachment of a tumor homing motif (RGD-4C) to KLA reduced the growth of breast carcinoma in nude mice (9).

Recently, a bifunctional peptide has been designed, combining an anti-HER-2 peptide and KLA, in order to simultaneously perturb the growth factor receptor signaling and the mitochondrial activity (10). This peptide showed selective internalization into HER-2-overexpressing human breast cancer cells both in vitro and in vivo.

A major obstacle for therapeutic peptides is that amide bonds are subject to protease digestions. The metabolic stability of different peptides have been studied in detail (11–13) and their half-lives in vitro vary from minutes to days, depending on the peptide sequences and the cells studied. In the present study, we have created a fusion of the D-forms of KLA (kla) and an arginine-rich cell-penetrating peptide (D-hepta-arginine) to increase cellular uptake. Two unstructured glycine residues were inserted as a spacer. The advantage of using hepta-arginine (r7) as a delivery vector is that the D-configuration has better membrane-crossing properties (14), and at the same time, is more likely to resist protease digestion. The resulting conjugate, r7-kla (Fig. 1A; Table 1), shows enhanced kinetic of cellular uptake, and efficient apoptosis in cells. In vivo evaluation also suggests that r7-kla could lead to a new approach for anticancer drug design.

Materials and Methods

All solvents were purchased from Fisher Scientific (Fair Lawn, NJ). 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyldiisopropylethylamine, and N,N,N,N-tetramethylpropyleneamine were the products of Applied Biosystems (Foster City, CA). AnaSpec (San Jose, CA) supplied all the amino acids for peptide synthesis. Rink amide resin was from Novabiochem (San Diego, CA). Annexin V FITC conjugate (AnxV-FITC) solution, anisole, cyclophosphamide monohydrate, cis-diammineplatinum (II) dichloride (cisplatin), doxorubicin, ethanedithiol, methotrexate, piperidine, propidium iodide solution (PI), thioanisole, and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 and paclitaxel were purchased from Molecular Probes (Eugene, OR). DMEM and RPMI 1640 were purchased from Mediatech (Herndon, VA).

Peptide Synthesis

Peptide synthesis was done on an automated peptide synthesizer (FastMoc, 0.1 mmol, ABI 433A; Applied Biosystems) employing the traditional Nα-Fmoc strategy.
The Rink amide resin (162 mg, 0.1 mmol) by stepwise acid building blocks (10 equivalents) were attached to the lidone (15 mL) was used as the coupling cocktail. All amino propylethylethylamine (20 equivalents) in to synthesize r7-kla. R7-KLA represents the L-configuration of r7-kla. The proapoptotic domain (kla). All D-configuration amino acids were used in the covalent linkage of the cell-penetrating domain (r7) and the mitochondrial disruption domain (kla). The N-tertbutyl protecting groups were removed by 20% (v/v) of piperidine in N,N-diisopropylethylamine (20 equivalents) in N-methylpyrrolidone (15 mL). After synthesis, the peptides were cleaved in a mixture of trifluoroacetic acid (1 mL), thioanisole (250 (15 mL). After synthesis, the peptides were cleaved in a mixture of trifluoroacetic acid (1 mL), thioanisole (250 μL), and tert-butyl ether (15 mL) at 4°C for 3 hours at. All amino acid building blocks (10 equivalents) were attached to the Rink amide resin (162 mg, 0.1 mmol) by stepwise elongation. The Nα-Fmoc protecting groups were removed by 20% (v/v) of piperidine in N-methylpyrrolidone (15 mL). After synthesis, the peptides were cleaved in a mixture of trifluoroacetic acid (1 mL), thioanisole (250 μL), ethanedithiol (150 μL), and anisole (100 μL) for 3 hours at room temperature. The resins were filtered out and the filtrates were collected. The peptides were then precipitated by methyl-tert-butyl ether (15 mL) at 4°C, dried, and further purified with reversed phase high-pressure liquid chromatography (Ranin, Worburn, MA). The purified fractions were lyophilized, yielding white foams with >95% homogeneity. All final products were characterized by analytic high-pressure liquid chromatography and MALDI-TOF mass spectrometry (Tufts Protein Chemistry Facility, Boston, MA; Table 1).

Cell Culture
All tumor cells lines [HT-1080, HCT-116, HT-29, MDA-MB-468, HeLa, PC-3, MIA PaCa-2, and LL/2 (LLC1)] were obtained from American Type Culture Collection (Rockville, MD) and were cultured in 5% CO₂ at 37°C. HT-1080 fibrosarcoma cells were incubated in DMEM supplemented with 10% (v/v) fetal bovine serum, glucose (4.5 g/L), 1-glutamine (4 mmol/L), nonessential amino acids (0.1 mmol/L), sodium bicarbonate (1.5 g/L), penicillin (50,000 units/L), and streptomycin (0.05 g/L). Jurkat T lymphocytes (clone E6-1) were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1-glutamine (4 mmol/L) in the absence of antibiotics.

Cell Viability Assay
The cytotoxicity of peptides and drugs was monitored by MTS assay (CellTiter 96 AQueous; Promega, Madison, WI). HT-1080 cells (5,000/well) were seeded on a 96-well cell culture cluster plate (Costar, Corning, NY) for 12 hours. Cells were incubated with different concentrations (ranging from 0 to 500 mmol/L) of peptides, cyclophosphamide, cisplatin, doxorubicin, methotrexate, and paclitaxel in culture medium (100 μL) for different time points (1, 4, 8, 16, and 24 hours) at 37°C in 5% CO₂. MTS solution (20 μL) was then added to individual wells and allowed to further incubate for 1 hour at 37°C. Samples were prepared in triplicate and the absorbance was measured at 490 nm. IC₅₀ values were calculated using Prism (version 4.0a, GraphPad Software, Inc., San Diego, CA). Paired t test analysis (one-tailed) was done by comparing the data (at specific concentrations) with the cells treated with kla.

Fluorescence Microscopy Studies
HT-1080 cells (10,000/well) were seeded on microscope cover glasses (Fisher Scientific) in 24-well cell culture cluster plates (Costar) for 24 hours at 37°C in 5% CO₂. Cells were then treated with peptide r7-kla (10 μmol/L) in culture medium (1 mL) for 2 minutes at room temperature and washed once with PBS (1 mL). For staining, a solution of Hoechst 33342 (1 μmol/L), AnxV-FITC (125 ng), and PI (500 ng) in HEPES/NaOH binding buffer (10 mmol/L, pH 7.5; 1 mL) containing NaN₃ (140 mmol/L) and CaCl₂ (2.5 mmol/L) was added to the samples. The cells were incubated for 10 minutes at 37°C and washed once with culture medium (1 mL). For mitochondrial staining, DePsipher kit (Treviron, Gaithersburg, MD) was used according to the manufacturer’s instruction. Cover glasses were then transferred to microscope slides (Superfrost, Pittsburgh, PA) and observed under the epifluorescence microscope (Nikon Eclipse 80i, Melville, NY). Images were recorded on a CCD camera (Photometrics Cascade-512B, Tucson, AZ) interfaced with a computer. Data was analyzed by IPLab software (Scanalytics, Inc., Fairfax, USA).}

![Diagram](image-url)

**Figure 1.** Peptide sequences and cytotoxicity. A, r7-kla is the result of the covalent linkage of the cell-penetrating domain (r7) and the proapoptotic domain (kla). All D-configuration amino acids were used to synthesize r7-kla. R7-KLA represents the L-configuration of r7-kla. A in vitro impairment of cell viability by r7-kla and kla. Peptides were added to HT-1080 cell lines (5,000/well) for 24 h at concentrations ranging from 0 to 500 mmol/L. Cell viability was determined with the MTS assay, measuring absorbance at 490 nm. Mean values were calculated from three independent experiments. P < 0.05, t test.

![Graph](image-url)

**Table 1. Molecular weight of different peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Expected molecular weights</th>
<th>Actual mass ion [M + H]⁺</th>
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</thead>
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<tr>
<td>kla</td>
<td>1,522.96</td>
<td>1,523.80</td>
</tr>
<tr>
<td>r7</td>
<td>1,110.28</td>
<td>1,111.48</td>
</tr>
<tr>
<td>r7-kla</td>
<td>2,730.32</td>
<td>2,731.55</td>
</tr>
<tr>
<td>R7-KLA</td>
<td>2,730.32</td>
<td>2,731.55</td>
</tr>
</tbody>
</table>
VA). Images from the same excitation and emission channels were adjusted to the same window settings.

**Flow Cytometry (Fluorescence-Activated Cell Sorting)**

Jurkat cells were concentrated by centrifugation for 5 minutes at 1,000 rpm to 2 × 10⁶ cells/mL. Cells (200 µL) were then incubated in culture medium containing r7-kla (6 µmol/L) for different time points (in minutes), washed with RPMI 1640 (10 mL) and further centrifuged for 5 minutes. Cell pellets were resuspended in 400 µL of HEPES/NaOH buffer (10 mmol/L, pH 7.5) containing NaCl (140 mmol/L) and CaCl₂ (2.5 mmol/L). AnxV-FITC HEPES/NaOH buffer (10 mmol/L, pH 7.5) containing benzidine chromogenic substrate system from the EnVision kits (DAKO, Carpinteria, CA). Primary rabbit polyclonal antibody turer's instructions. Primary rabbit polyclonal antibody against human cleaved caspase-3 (Asp175; Cell Signaling, Beverly, MA) was used for the detection of activated caspase-3. Staining was visualized by using 3,3'-diaminobenzidine chromogenic substrate system from the EmVision kits (DAKO, Carpinteria, CA).

**Results and Discussion**

**Toxicity of r7-kla Conjugate**

Cell-penetrating peptides have been widely used for the delivery of biologically active compounds in *in vitro* and *in vivo* disease models (15). Among different cell-penetrating peptides, arginine-rich sequences are known to have high cellular uptake (16, 17), and their internalization mechanism was recently proposed to involve binding to the cell surface heparan sulfate, and subsequently by endocytosis (18). To determine the cytotoxic effects of r7-kla, we initially screened eight different tumor cell lines for proliferation studies by MTS assay. Cell survival rates were determined 24 hours following peptide treatments. We observed that the killing effects of r7-kla to different tumors were nonselective (Table 2). The IC₅₀ values are within the range of 3 to 25 µmol/L.

HT1080 fibrosarcoma was then chosen to confirm the toxicity of r7-kla. This cell line could develop multiple drug resistance to doxorubicin and paclitaxel (19). The determined IC₅₀ value of r7-kla was 3.54 µmol/L (Fig. 1B), whereas individual kla and r7 sequences both had IC₅₀ values of >100 µmol/L. Equal amounts of kla and r7 were separately added to the cells, and the obtained IC₅₀ value remained at >100 µmol/L, indicating that the cytotoxicity of r7-kla was not caused by the synergistic effect of mitochondrial-disrupting and cell-penetrating domains.

The D-form r7-kla has a slightly lower IC₅₀ value than its L-counterpart, R7-KLA (IC₅₀ = 7.21 µmol/L). The differences in the IC₅₀ values obtained for r7-kla and R7-KLA could be attributed to (a) the higher cellular uptake of r7 when compared with the R7 (14), (b) the degradation of R7-KLA by protease digestions, and (c) the difference of mitochondrial membrane disruption properties. Further detailed experiments are required to confirm these hypotheses.

We next studied the kinetics of cell deaths over 24 hours. As expected, r7-kla showed a very fast kinetic of cell killing, especially in the first 8 hours of drug treatment (Table 3). In contrast, other clinically used anticancer agents exhibited cytotoxic effects after 8 hours of incubation and at higher doses. The IC₅₀ values of all screened compounds at 24 hours were: r7-kla < doxorubicin < paclitaxel < methotrexate. Other anticancer drugs such as cisplatin and cyclophosphamide were unable to kill HT1080 cells at the tested conditions.

**r7-kla Induced Apoptosis In vitro**

Next, we did a series of experiments to elucidate the mechanism of cell death. After incubation with r7-kla (10 µmol/L), as shown in Fig. 2A, the treated cells detached from the slide within 5 minutes and showed typical signs of apoptosis. These cells shrank in size and had altered plasma membranes including blobbing. Their DNA showed “comet” patterns with Hoechst staining. To distinguish early apoptosis from late apoptosis/necrosis, r7-kla-treated and nontreated cells were costained with

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Table 2. Comparison of IC₅₀ (µmol/L) of r7-kla in different cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>Types</th>
<th>IC₅₀ (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
<td>3.54 ± 0.11</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colorectal carcinoma</td>
<td>7.37 ± 0.16</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colorectal carcinoma</td>
<td>15.7 ± 0.11</td>
</tr>
<tr>
<td>MDX-MB-468</td>
<td>Breast adenocarcinoma</td>
<td>4.74 ± 0.14</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix adenocarcinoma</td>
<td>4.95 ± 0.12</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate adenocarcinoma</td>
<td>24.8 ± 0.10</td>
</tr>
<tr>
<td>MIA PaCa-2</td>
<td>Pancreas adenocarcinoma</td>
<td>10.3 ± 0.13</td>
</tr>
<tr>
<td>LL/2 (LLC1)</td>
<td>Lewis lung carcinoma</td>
<td>3.17 ± 0.14</td>
</tr>
</tbody>
</table>

NOTE: Results are mean ± SD of three independent experiments.
AnxV-FITC and PI. Without r7-kla treatment, cells were predominantly AnxV negative and PI negative (AnxV⁻/PI⁻). Both early and late apoptosis was induced by incubation with r7-kla (Fig. 2A). The single positive (AnxV⁺/PI⁻) cells were identified as early apoptotic, because they exposed phosphatidylserine on the outer leaflet of the plasma membrane for AnxV binding. AnxV⁺/PI⁻ cells were considered late apoptotic/necrotic because their membrane was permeable to PI.

The mitochondrial-disruptive ability of r7-kla was further evaluated using a commercially available cationic dye, DePsipher, which is commonly used to monitor mitochondrial membrane potential losses during apoptosis (20). This dye enters cells readily and gives a bright red fluorescence within healthy mitochondria, whereas its fluorescence turns green when the mitochondrial membrane is damaged. After 5 minutes of r7-kla incubation, unhealthy HT-1080 cells showed primarily green fluorescence, and the disappearance of red aggregates suggested that DePsipher no longer accumulated onto the mitochondrial membrane in which the electrochemical gradient had collapsed (Fig. 2B).

Kinetic Transition from Viable to Apoptotic Cells

As mentioned above, r7-kla exhibited its full cytotoxicity in cell culture in <1 hour and few differences were seen at later time points. To further study the kinetics of cell death, flow cytometry (fluorescence-activated cell sorting) was used to collect real-time information in Jurkat T lymphocyte. After staining with AnxV-FITC and PI, three distinct populations (Fig. 3A) were identified: double negative cells (AnxV⁻/PI⁻), single positive cells (AnxV⁺/PI⁻), and double positive cells (AnxV⁺/PI⁺), corresponding to viable, early apoptotic, and late apoptotic/necrotic cells, respectively. Prior to r7-kla addition, ~93% of cells were healthy. Sixteen minutes after incubation with r7-kla (8 μmol/L), the number of viable cells had decreased to 6%. Conversely, the apoptotic and necrotic populations had increased to 48% and 42%, respectively. After 30 minutes, the majority of cells had become necrotic. Dose-dependent cell death was studied by using the same method (Fig. 3B). After 20 minutes of incubation with 2 μmol/L r7-kla, >70% of cells were viable. On the other hand, >70% of cells had turned necrotic with 12 μmol/L r7-kla. Therefore, 8 μmol/L r7-kla was chosen to follow the cell kinetics during 45 minutes. After 2 minutes of r7-kla treatment, most cells were nonviable and the apoptotic distribution had increased to 63% (Fig. 3C). At longer times, the apoptotic population started declining and reached a minimum of 5% at 45 minutes. During this experimental time frame, the necrotic population increased inversely with the kinetic profile of apoptotic cells. At a r7-kla concentration of 8 μmol/L, the transition from the early apoptotic to the necrotic state was completed within 30 minutes of peptide incubation.

### Table 3. Comparison of the IC₅₀ (μmol/L) of r7-kla with other antitumor agents

<table>
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<th>8</th>
<th>16</th>
<th>24</th>
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</thead>
<tbody>
<tr>
<td>r7-kla</td>
<td>5.24 ± 0.14</td>
<td>3.90 ± 0.15</td>
<td>3.56 ± 0.19</td>
<td>3.45 ± 0.14</td>
<td>3.54 ± 0.11</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>15.1 ± 0.13</td>
<td>11.3 ± 0.12</td>
<td>9.80 ± 0.14</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>55.1 ± 0.22</td>
<td>16.2 ± 0.12</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>76.1 ± 0.17</td>
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<tr>
<td>Cisplatin</td>
<td>&gt;100</td>
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<td>Cyclophosphamide</td>
<td>&gt;100</td>
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</tbody>
</table>

NOTE: Results are mean ± SD of three independent experiments. P < 0.05, t test was compared with kla.
**In vivo** Evaluation of r7-kla

We next tested whether the new fusion peptide could be used in vivo to treat human HT1080 fibrosarcoma xenografts. Tumors were implanted into both hind legs of each mouse (n = 4). When tumors reached ~5 mm in size, a single dose of r7-kla (100 μg) or saline was injected into each tumor site. The mice were then sacrificed and tumors were removed for immunohistologic studies (Fig. 4). The treated tumor showed significant tissue loss and extensive necrotic areas. Moreover, ~80% of the cell residuals at the injection sites were stained positive to terminal nucleotidyl transferase–mediated nick end labeling and caspase-3.

**Conclusion**

Our data confirm that the cytotoxicity of KLA can be significantly enhanced through cellular internalization. Unlike the traditional lock-and-key approach, the antitumor effect of KLA is based on physical disruption of the membrane bilayer and is therefore expected to be less susceptible to multiple drug resistance. With the D-configuration of KLA (kla) attached to a hepta-arginine cell-penetrating domain (r7), the synthetic peptide, r7-kla, showed better cytotoxicity against the HT1080 human fibrosarcoma cell line compared with the clinically used antitumor agents evaluated in the current study. The peptide induces apoptosis both in vitro and in vivo. In addition, we showed the distinct cytotoxic property of r7-kla. The instant toxicity of r7-kla could be an advantage, especially by early inhibition of cell proliferation. However, the fusion of cell-penetrating domain r7 to kla had compromised the killing kinetic with cellular selectivity.

**Figure 4.** r7-kla mediates apoptosis in vivo. Nude mice (n = 4) bearing HT-1080-derived human fibrosarcoma xenografts were administrated with r7-kla (100 μg) in saline (50 μL) by intratumor injection. The controls (n = 2) were treated with saline only. The mice were sacrificed 16 h after injection and the tumor tissues were excised, paraffin-embedded, and sectioned into 7 μm and stained with H&E staining (A and B), terminal nucleotidyl transferase–mediated nick end labeling (C and D), and caspase-3 staining (E and F). Insets, magnification, ×40.

Recently, we have encapsulated this sequence into a peptide-based biomaterial system for functional drug delivery (21). Overall, we believe that the encapsulation of this fusion peptide into nanoparticles containing specific targeting moieties may be useful for the systemic treatment of tumors.

**Acknowledgments**

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**References**

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