Apoptotic Anticancer Effect of Alvaradoin E Isolated from Alvaradoa haitiensis

QIUWEN MI1, DANIEL LANTVIT1, EULENIA REYES-LIM1, HEEBYUNG CHAI1, SHARNELLE S. PHIFER2, MANSUKH C. WANI2, MONROE E. WALL2**, GHEE T. TAN1, GEOFFREY A. CORDELL1, NORMAN R. FARNSWORTH1, A. DOUGLAS KINGHORN1* and JOHN M. PEZZUTO1*

1Program for Collaborative Research in the Pharmaceutical Sciences, and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, 60612; 2Natural Products Laboratory, Research Triangle Institute, P. O. Box 12194, Research Triangle Park, North Carolina 27709, U.S.A.

Abstract. Two anthracenone C-glycosides, alvaradoins E and F, isolated from the leaves of Alvaradoa haitiensis Urb. (Simaroubaceae), were found to have potent inhibitory activities with cultured cancer cells. Using the in vivo hollow fiber model, these compounds demonstrated significant growth inhibition at the i.p. site when tested with KB, LNCaP, and Col2 cells. To determine if these anthracenone C-glycosides mediated anticancer activity through an apoptotic pathway, a series of assays were performed with the 10S isomeric compound, alvaradoin E. With a DAPI assay, treatment of LNCaP cells with alvaradoin E at concentrations of 0.4, 2, 10, or 50 μM for 24 or 48 h showed chromatin condensation, a morphological characteristic of apoptosis. Mitochondrial membrane potential, analyzed with a DiOC6 uptake assay, showed that treatment of LNCaP cells with 0.07, 0.14, 0.28, 0.56, 0.86, and 1.12 μM alvaradoin E for 12 h caused dose-dependent membrane depolarization, another indication of early apoptosis. Also, with an annexin V-FITC assay system, treatment of HL-60 cells with 0.07 μM alvaradoin E for 24 h increased annexin V-FITC binding from 3 to 25.9% (8.6-fold). Finally, with the TUNEL assay system, treatment of HL-60 cells with 1.12 μM alvaradoin E for 32 h increased FITC-dUTP binding from 1.2 to 12.1% (10-fold). These data suggest alvaradoin E is an effective anticancer agent that induces apoptosis. Additional studies to establish clinical utility should be of interest.

In a search for potential anticancer agents of plant origin conducted through a National Cooperative Drug Discovery Group program (1), a crude chloroform extract from the leaves of Alvaradoa haitiensis Urb. (Simaroubaceae) was found to have significant inhibitory activity with cultured KB cells. Through bioassay-guided fractionation, two new isomeric anthracenone C-glycosides, alvaradoins E (10S) and F (10R), along with several structural analogs were isolated (2). Alvaradoin E exhibited activity in the in vivo P388 mouse leukemia model (2).

Key Words: Alvaradoin E, alvaradoin F, annexin V-FITC, anthracenone C-glycosides, apoptosis, DAPI, DiOC6, hollow fiber test, mitochondrial membrane potential analysis, propidium iodide, TUNEL assay.
elimination of tumor cells (3-6). Apoptosis, or programmed cell death, is a general mechanism for removal of unwanted cells from the immune system. The process is characterized by membrane blebbing, nuclear and cytoplasmic shrinkage, chromatin condensation, and endonuclease cleavage of DNA into oligonucleosomal length fragments (7-11). Apoptosis is also accompanied by a loss of mitochondrial membrane potential (MMP/Δψm) (12-15) and exposure of phosphatidylserine (PS) at the surface of the cell (16-18).

Based on morphological and biochemical criteria, various methods have been developed for the characterization of apoptosis (19, 20). For example, DAPI (6,4'-diamidino-2-phenylindole) is a DNA-specific probe that forms a fluorescent complex by attaching to the minor groove of A-T rich sequences (21). This reagent is used to visualize DNA fragmentation, a process that is diagnostic for apoptosis (22). In addition, DNA of apoptotic cells is cleaved by endogenous endonucleases into multimers of 180-200 bp fragments (23-26). The TUNEL assay (27-30) can be employed to measure the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT); the fluorescein-12-dUTP-labeled DNA can then be quantified by flow cytometry.

Changes in MMP are indicated by 3',3'-dihexyloxacarbocyanine iodide (DiOC6) uptake (12), and expression of PS at the cell surface can be monitored using annexin V, which is a 35-36 kDa Ca2+-dependent, phospholipid-binding protein with high affinity for PS. Finally, flow cytometry with simultaneous dye exclusion of propidium iodide (PI) allows the discrimination of intact, apoptotic, and necrotic cells (31, 32).

In the present study, we report the cytotoxicity of alvaradoins E and F in a tumor panel (33), and their effects in an in vivo hollow fiber assay (34, 35). Alvaradoin E was investigated for its apoptotic effects using LNCaP human prostate cells and HL-60 human promyelocytic leukemia cells.

Materials and Methods

Test material. All general chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. Polyvinylidene fluoride (PVDF) hollow fibers (500,000 Da molecular weight cut-off, 1.0 mm ID) were purchased from Spectrum Medical Industries (Luguan Hills, CA, USA). Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY, USA). An annexin V-FITC kit (65874X) was purchased from BD Pharmingen (San Diego, CA, USA), and a DeadEnd™ Fluorometric TUNEL System kit (G3250) was purchased from Promega (Madison, WI, USA). Alvaradoins E and F were isolated from *Alvaradoa haitiensis* Urb. (Simaroubaceae) as described elsewhere (2). For hollow fiber studies, compounds were coprecipitated with PVP to increase solubility, and then suspended in PBS (36).

Cell culture. Human promyelocytic leukemia HL-60 cells, human oral epidermoid carcinoma KB cells, and human prostate carcinoma LNCaP cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human colon carcinoma Co2 cells, and human lung carcinoma Lu1 cells were obtained from the Department of Surgical Oncology, University of Illinois at Chicago, Chicago, IL, USA. Human telomerase reverse transcriptase hTERT-RPE1 cells were purchased from Clontech (Clontech, Palo Alto, CA, USA). Co2 and Lu1 cells were maintained in MEME medium, HL-60 cells were maintained in RPMI medium, and KB cells were maintained in DMEM medium. LNCaP cells were maintained in DMEM/F-12 medium. In each case, PSF (100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, 250 ng/mL amphotericin B) was added. All media were supplemented with 10% heat-inactivated FBS.

Evaluation of cytotoxic potential. The cytotoxic potential of the test substances with cultured cells was determined as described previously (33, 37). For adherent cells, various concentrations of test compounds (dissolved in 10 µl of 10% DMSO) were transferred to 96-well plates, and 190 µl of the cell suspensions were added to each
well. The plates were then incubated for 72 h at 37 °C (100% humidity with a 5% CO₂ atmosphere in air), and 50-100 μl of cold 10-50% aqueous trichloroacetic acid were added to the growth medium in each well to fix the cells. The cultures were incubated at 4 °C for 30 min, washed, air-dried, stained with sulforhodamine B solution, and washed with 1% acetic acid. Finally, 200 μl of 10 mM Tris base were added to each well and the optical densities were determined at 515 nm utilizing an ELISA plate reader. In each case, a zero-day control was analyzed by adding an equivalent number of cells to several wells and incubating at 37 °C for 30 min, and processing as described above. Optical density values obtained with the zero-day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was calculated. For HL-60 suspension cells, cytotoxic potential was determined using the previously described MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] microculture tetrazolium assay with some modification (37). Briefly, MTT was prepared at 5 mg/ml in PBS and stored at 4 °C. Next, the drug and cell suspensions were incubated for 72 h at 37 °C, MTT was diluted 5-fold in medium without serum, and 50 μl of diluted solution were added to each microwell culture well. After 4 h incubation at 37 °C, plates were centrifuged at 2,000 rpm for 30 min, the medium was aspirated, and 150 μl of 100% DMSO were added to solubilize the MTT-formazan product in the dry plates. After thorough mixing, absorbance at 540 nm was measured with an ELISA plate reader.

In vivo hollow fiber test. The in vivo hollow fiber test was performed as described previously with some modifications (34, 35). Confluent monolayers of the cells were harvested, collected by centrifugation and resuspended in conditioned medium at a concentration of 10⁶ cells/ml. Fibers filled with cells were incubated in 6-well plates overnight at 37 °C in a 5% CO₂ atmosphere. Female athymic NCr nu/nu mice (5-6 weeks-old) were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Each mouse hosted up to six fibers, which were cultured in 6-well plates containing the hollow fiber samples, was inserted caudally through the subcutaneous tissues, and fibers were deposited during withdrawal of the trocar. The incision was closed with skin staples.

For treatment protocols, drugs were co-precipitated with PVP (MW 360,000, Sigma) to increase solubility, and then dissolved in PBS (36). Mice were randomized into PBS vehicle control groups (six mice per group) and drug treatment groups (three mice per group for each dose tested). Test compounds were administrated once daily by intraperitoneal injection from days 3-6 after implantation. Body weights were determined daily.

On day 7, mice were sacrificed by cervical dislocation and fibers were retrieved. The fibers were placed into 6-well plates, each well containing 2 ml of fresh, pre-warmed culture medium (with 20% calf serum or FBS), and allowed to equilibrate for 30 min at 37°C. To define the viable cell mass contained within the intact hollow fibers, a MTT dye conversion assay was used. Briefly, 1 ml of pre-warmed culture medium (with 20% calf serum or FBS) containing 1 mg MTT/ml was added to each dish. After incubating at 37°C for 4 h, the culture medium was removed by aspiration, 2 ml of normal saline containing 2.5% proteinate sulfate solution were added to each well, and the plates were stored at 4°C for 24 h. Prostate specific antigen solution was then removed by aspiration, 2 ml of fresh proteinate sulfate solution were added for a second wash, and the plates were stored at 4°C for at least 2 h. To assess the optical density of the samples, the fibers were transferred to 24-well plates, cut in half, and allowed to dry overnight. The formazan was extracted from each sample with DMSO (250 μl/well) for 4 h at room temperature on a rotation platform. Aliquots (150 μl) of extracted MTT formazan were transferred to individual wells of 96-well plates and assessed for optical density at a wavelength of 540 nm. The effect of the treatment regimen was determined by the net growth percentage of the cells relative to changes in body weight.

**DAPI nuclear staining.** DAPI staining was performed as previously described (21). LNCaP cells were grown in 6-well microwell plates (10⁶ per well) and incubated overnight. Cells were treated with DMSO control, or alvaradoin E at 0.4, 2, 10 or 50 μM, for 24 or 48 h. After drug treatment, cells were harvested, washed twice with PBS, and stored at –20 °C until inspection.

**Mitochondrial membrane potential analysis.** Mitochondrial membrane potential was measured as previously described (38). Fifteen min prior to collection of LNCaP cells after drug treatment, 40 nM DiOC₆ was added to the cells. Detached cells were pooled together with trypsinized cells and centrifuged at 1,000 rpm for 8 min at 4°C. The cell pellets were washed once with PBS before resuspending in 500 μl PBS containing 40 nM DiOC₆. Fluorescence intensities of DiOC₆ were analyzed by flow cytometry with excitation and emission settings of 484 and 500 nm, respectively. Immediately before flow cytometric analysis, PI (final concentration 30 μg/ml) was added to gate out dead cells.

**Annexin V-FITC analysis.** Procedures were performed using the manufacturer’s instructions. Briefly, after drug treatment, HL-60 cells were washed twice with cold PBS and resuspended in 1 x binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. Following a transfer of the cells to 5-ml centrifuge tubes, 10 μl of annexin V-FITC (5 μl) and PI (10 μl, 50 μg of PI in 1 ml of PBS buffer) were added, and the cells were gently vortexed and incubated at room temperature for 15 min in the dark. Flow cytometric analysis was performed within 1 h after 1 x binding buffer (400 μl) was added to each tube.

**TUNEL assay.** Procedures were performed following the manufacturer’s instructions. After drug treatment, HL-60 cells were harvested, washed twice with PBS, and resuspended in 0.5 ml of PBS. After adding 5 ml of 1% methanol-free formaldehyde, cells were fixed for 20 min at 0-4°C, and centrifuged at 300 x g for 2 min. The slides were stored at –20 °C until inspection.

**Mitochondrial membrane potential analysis.** Mitochondrial membrane potential was measured as previously described (38). Fifteen min prior to collection of LNCaP cells after drug treatment, 40 nM DiOC₆ was added to the cells. Detached cells were pooled together with trypsinized cells and centrifuged at 1,000 rpm for 8 min at 4°C. The cell pellets were washed once with PBS before resuspending in 500 μl PBS containing 40 nM DiOC₆. Fluorescence intensities of DiOC₆ were analyzed by flow cytometry with excitation and emission settings of 484 and 500 nm, respectively. Immediately before flow cytometric analysis, PI (final concentration 30 μg/ml) was added to gate out dead cells.

**Annexin V-FITC analysis.** Procedures were performed using the manufacturer’s instructions. Briefly, after drug treatment, HL-60 cells were washed twice with cold PBS and resuspended in 1 x binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. Following a transfer of the resulting solution (100 μl) to a 5 ml culture tube, annexin V-FITC (5 μl) and PI (10 μl, 50 μg of PI in 1 ml of PBS buffer) were added, and the cells were gently vortexed and incubated at room temperature for 15 min in the dark. Flow cytometric analysis was performed within 1 h after 1 x binding buffer (400 μl) was added to each tube.

**TUNEL assay.** Procedures were performed following the manufacturer’s instructions. After drug treatment, HL-60 cells were harvested, washed twice with PBS, and resuspended in 0.5 ml of PBS. After adding 5 ml of 1% methanol-free formaldehyde, cells were fixed for 20 min at 0-4°C, and centrifuged at 300 x g for 2 min. The slides were stored at –20 °C until inspection.

**Mitochondrial membrane potential analysis.** Mitochondrial membrane potential was measured as previously described (38). Fifteen min prior to collection of LNCaP cells after drug treatment, 40 nM DiOC₆ was added to the cells. Detached cells were pooled together with trypsinized cells and centrifuged at 1,000 rpm for 8 min at 4°C. The cell pellets were washed once with PBS before resuspending in 500 μl PBS containing 40 nM DiOC₆. Fluorescence intensities of DiOC₆ were analyzed by flow cytometry with excitation and emission settings of 484 and 500 nm, respectively. Immediately before flow cytometric analysis, PI (final concentration 30 μg/ml) was added to gate out dead cells.

**Annexin V-FITC analysis.** Procedures were performed using the manufacturer’s instructions. Briefly, after drug treatment, HL-60 cells were washed twice with cold PBS and resuspended in 1 x binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. Following a transfer of the resulting solution (100 μl) to a 5 ml culture tube, annexin V-FITC (5 μl) and PI (10 μl, 50 μg of PI in 1 ml of PBS buffer) were added, and the cells were gently vortexed and incubated at room temperature for 15 min in the dark. Flow cytometric analysis was performed within 1 h after 1 x binding buffer (400 μl) was added to each tube.

**TUNEL assay.** Procedures were performed following the manufacturer’s instructions. After drug treatment, HL-60 cells were harvested, washed twice with PBS, and resuspended in 0.5 ml of PBS. After adding 5 ml of 1% methanol-free formaldehyde, cells were fixed for 20 min at 0-4°C, and centrifuged at 300 x g for 2 min. The slides were stored at –20 °C until inspection.
Table I. Cytotoxic activity of alvaradoin E and F with cultured cells a.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lu1</th>
<th>Col2</th>
<th>HL-60</th>
<th>KB</th>
<th>LNCaP</th>
<th>hTERT-RPE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alvaradoin E</td>
<td>0.21</td>
<td>0.23</td>
<td>1.23</td>
<td>0.23</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>Alvaradoin F</td>
<td>0.18</td>
<td>0.23</td>
<td>0.30</td>
<td>0.23</td>
<td>0.12</td>
<td>0.084</td>
</tr>
</tbody>
</table>

a Results are expressed as IC_{50} values (concentration required to inhibit cell growth by 50%) in μM. Data represent the means of the two independent experiments, with each concentration tested in triplicate. Assays were performed as described in the "Materials and Methods" section.

Figure 2. The effect of alvaradoin F and alvaradoin E on the growth of KB (Panels A and B, respectively), LNCaP (Panels C and D, respectively), and Col2 (Panels E and F, respectively) cells implanted at the i.p. (solid columns) and the s.c. (open columns) compartments of NCr nu/nu mice. The animals were treated with PBS (control) or the indicated doses once daily by intraperitoneal injection from day 3-6 after implantation. On day 7, mice were sacrificed, fibers were retrieved and analyzed as described in the "Materials and Methods" section. Results are shown as the average percentage of cell growth relative to control values, ± SE (bars). Changes in mouse body weight at the end of the experiment are listed at the bottom of the figure. The *** and ** treatment groups were significantly different from the control group (p<0.0001, and p<0.001, respectively) using the Student’s t-test, with n = 6 for the control group and n = 3 for treatment groups.
10 min at 4°C. Cell pellets were resuspended in 5 ml of PBS, centrifuged, and resuspended in 0.5 ml of PBS. Subsequently, 70% ice-cold aqueous ethanol was added and the cell suspension was kept at −20 °C for at least 4 h, followed by centrifugation at 300 x g for 10 min. Cell pellets were washed with PBS twice, resuspended in 1 ml of PBS, transferred into a 1.5 ml microcentrifuge tube, and centrifuged at 300 x g for 10 min. After being resuspended in 80 µl of equilibration buffer, cell pellets were incubated at room temperature for 5 min, centrifuged at 300 x g for 10 min, and resuspended in incubation buffer (45 µl of equilibration buffer, 5 µl of nucleotide mix and 1 µl of TdT enzyme) (for negative control, 1 µl of autoclaved, deionized water was substituted for TdT enzyme). Next, cells were incubated in a water bath for 60 min at 37°C, and resuspended at 15-min intervals. After the reaction was terminated by adding 1 ml of 20 mM EDTA, cells were vortexed, centrifuged at 300 x g for 10 min, and resuspended in 1 ml of 0.1% Triton X-100 solution in PBS containing 5 mg/ml of bovine serum albumin. The process was repeated once and 0.5 ml of PI solution (freshly diluted to 5 µg/ml in PBS) was added to the cell pellet followed by incubation at room temperature for 30 min in the dark. Cells were analyzed by flow cytometry assessing the green fluorescence of fluorescein-12-dUTP at 520 nm and the red fluorescence of PI at 620 nm.

Results

Evaluation of cytotoxic potential. Alvaradoins E and F showed potent cytotoxic activity against a panel of tumor cells (Table I), with IC50 values ranging from 0.084 to 0.30 µM. The cytotoxic potency of the two enantomeric compounds was comparable with each of the cell lines, aside from HL-60, where alvaradoin F appeared more active.

In vivo hollow fiber test. Alvaradoins E and F were evaluated in the hollow fiber model at doses of 0.195, 0.39, 0.78, and 1.56 mg/kg, using KB (Panel A), LNCaP (Panel B) and Col2 cells (Panel C) (Figure 2). For KB cells, the two compounds inhibited cell growth by 31.9-57.5 and 40.0-58.1% at the i.p. site, but only 1.5-17.9 and 4.8-11.4% at the s.c. site, respectively. With LNCaP cells, 59.3-72.4% growth inhibition was observed at the i.p. site, and 3.1-14.7% at the s.c. site, for alvaradoin F. The corresponding values were 60.3-79.3 and 9.8-18.6% for alvaradoin E. The corresponding values were 60.3-79.3 and 9.8-18.6% for alvaradoin E. With Col2 cells, alvaradoin E and F mediated respective cell growth
Table II. Percentage of apoptotic LNCaP cells induced by treatment with Alvaradoin Ea.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>DMSO control</th>
<th>Alvaradoin E (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>0.33 (± 0.01)</td>
<td>17 (± 2.6)</td>
</tr>
<tr>
<td>48</td>
<td>2.7 (± 0.4)</td>
<td>15 (± 2.0)</td>
</tr>
</tbody>
</table>

a Results are expressed as % values. Data represent the means (± SD) of the two independent experiments, with each concentration tested in triplicate. Assays were performed as described in the 'Materials and Methods' section.

DAPI nuclear staining. LNCaP cells were treated with alvaradoin E at concentrations of 0.4, 2, 10 or 50 µM for 24 or 48 h. Compared with the negative control, alvaradoin E treatment caused membrane blebbing and chromatin condensation, at both 24 and 48 h. At 24 h, only 0.3% of the control cells demonstrated nuclear fragmentation, whereas cells treated with alvaradoin E exhibited 17, 30, 43, or 84.7% apoptosis, respectively, at concentrations of 0.4, 2, 10, or 50 µM. At 48 h, only 2.7% of the control cells demonstrated nuclear fragmentation, whereas cells treated with alvaradoin E exhibited 15, 32, 42.3, and 76.7% apoptosis at concentrations of 0.4, 2, 10, or 50 µM, respectively. There were no significant differences in the percentages of apoptotic cells at 24 and 48 h (Table II).

Mitochondrial membrane potential analysis. AEm-related fluorescence was examined in cell populations by measuring their ability to accumulate DiOC6. When gated cells were examined for DiOC6 uptake, cells treated with alvaradoin E clearly differed in DiOC6 fluorescence compared with the DMSO control. Treatment of LNCaP cells with 0.07, 0.14, 0.28, 0.56, 0.86, and 1.12 µM alvaradoin E for 12 h showed dose-dependent membrane depolarization with LNCaP cells, as an indication of early apoptosis (Figure 3).

Annexin V-FITC analysis. With the use of flow cytometry, various cell populations were simultaneously measured by staining with fluorescein isothiocyanate (FITC)-labeled annexin V (green fluorescence) and exclusion of the non-vital dye PI (red fluorescence negative) (Figure 4). In this manner, discrimination between intact, apoptotic, and necrotic cells was facilitated (32). The lower left quadrant (quadrant 2) of the cytograms shows viable cells, which excluded PI and were negative for FITC-annexin V binding (FITC-/PI-). The upper right quadrant (quadrant 3) represents non-viable, necrotic cells, positive for FITC-annexin V binding (FITC+/PI-). The lower right quadrant (quadrant 4) represents apoptotic cells, with FITC-annexin V binding and cytoplasmic membrane integrity (FITC+/PI-) (32). As shown in Figure 4, alvaradoin

inhibition by 21.0-71.6 and 31.2-73.8% at the i.p. site, and 0.7-10.1 and 5.5-15.7% at the s.c. site. A dose of 1.56 mg/kg was lethal to two mice with alvaradoin F, and lethal to one mouse with alvaradoin E. No significant weight loss was observed with other doses.
E treatment (0.07 μM for 24 h) increased annexin V-FITC binding 8.6-fold; from the DMSO control of 3% (Figure 4A) to alvaradoin E treatment of 25.9% (Figure 4B).

TUNEL assay. As shown in Figure 5, alvaradoin E treatment (1.12 μM for 32 h) of HL-60 cells increased fluorescence-dUTP binding 10-fold, from 1.2% in the DMSO control (Figure 5A) to 12.1% with alvaradoin E treatment (Figure 5B).

Discussion

Part of our NCDDG natural product drug discovery program involves monitoring the potential of plant extracts to mediate cytotoxic effects against a tumor cell panel, and cell culture has proven to be an invaluable tool for the discovery and characterization of anticancer agents (39). Two anthracenone C-glycoside enantomers, alvaradoins E and F were isolated from the leaves of *Alvaradoa haitiensis* through bioassay-guided fractionation (2). Since both of these compounds exhibited cytotoxic activity (33), their efficacy was further tested with the *in vivo* hollow fiber model, which provides a preliminary assessment of therapeutic efficacy in a cost-and time-effective manner (34, 35). The hollow fiber studies were designed to determine if pharmacologically active concentrations of test compound could reach tumor cells growing in two distinct physiologic compartments. Alvaradoins E (10S) and F (10R) demonstrated comparable levels of cytotoxic potency (Table I), and similar growth inhibitory responses were observed in the hollow fiber model with the same respective cell lines (Figure 2). When alvaradoins E and F were evaluated for *in vivo* effectiveness with the P388 mouse leukemia model in previous work (2), alvaradoin E exhibited threshold activity, and alvaradoin F was inactive (data not shown). Therefore, the anticancer mechanism of alvaradoin E was further explored.

Among KB, LNCaP, and Col2 cells, alvaradoin E demonstrated greatest growth inhibitory activity with LNCaP cells in the hollow fiber model (Figure 2). Therefore, LNCaP cells were selected for analysis. Apoptosis is accompanied by various morphological changes, including nuclear condensation, DNA fragmentation, and cell surface changes. DAPI has been used successfully as a DNA-specific probe for flow cytometry, chromosome staining, DNA visualization and quantitation in histochemistry and biochemistry (32, 40). Based on this method, treatment of cells with alvaradoin E showed clear apoptotic nuclear condensation at concentrations of 0.4, 2, 10, or 50 μM at both 24 or 48 h (Table II).

Recent studies have demonstrated that mitochondrial alterations constitute critical events of the apoptotic cascade (41). Reduction of MMP is among the changes encountered during the early reversible stages of apoptosis, and is preceded by cytochrome-c release in several cell types. A decrease in MMP in apoptotic cells is associated with a reduction of DiOC₆(3) uptake as demonstrated by flow cytometric analysis (42), and ΔΨm analysis seems to be a reliable method for studying apoptosis (43). As shown in Figure 3, LNCaP cells treated with 0.07, 0.14, 0.28, 0.56, 0.86, and 1.12 μM alvaradoin E for 12 h showed a dose-dependent reduction in the incorporation of the ΔΨm-sensitive dye DiOC₆(3), as indicated by the left shift of the open peaks compared with the DMSO control.

Annexin V was initially discovered as a vascular protein with strong anticoagulant properties. It belongs to a multigene family of proteins defined by a repeated sequence motif, originally termed the endonexin loop (44). This protein binds preferentially to phospholipid species such as PS, which is normally absent in the outer leaflet of the plasma membrane, and shows minimal binding to phospholipid species such as phosphatidylcholine and sphingomyelin, which are constitutively present in the outer leaflet of plasma membranes (16-18, 32). When cell death occurs, PS is translocated to the outer layer of the
membrane, i.e., the external surface of the cell. This occurs in the early phases of apoptotic cell death during which the cell membrane itself remains intact. Necrosis, on the other hand, is accompanied by loss of cell membrane integrity and leakage of cellular constituents into the environment. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane loses its integrity and begins leaking. Therefore, measurement of annexin V binding, executed simultaneously with a dye exclusion test, provides a suitable means of discriminating intact cells (FITC-/PI-), executed simultaneously with a dye exclusion test, provides a suitable means of discriminating intact cells (FITC-/PI-), apoptotic cells (FITC+/PI-), and necrotic cells (FITC+/PI+) (31, 32, 42). However, the annexin V-FITC-based assay cannot be applied readily with adherent cell types, since damage to these cells during collection for flow cytometry would allow access of annexin V-FITC to internally located PS and, hence, afford false-positive results. Also, harvesting with trypsin and EDTA can influence the measurable amount of bound annexin V, since trypsin and EDTA remove bound annexin V from the surface by proteolysis and chelation of Ca2+ ions, respectively (31, 32, 42). Therefore, suspension HL-60 cells, rather than adherent LNCaP cells, were selected to perform the annexin V-FITC-based assay. As shown in Figure 4, treatment with alvaradin E (0.07 µM) for 24 h enhanced the number of apoptotic (FITC+/PI-) (lower right quadrant 4, Figure 4B for alvaradin E treatment) HL-60 cells by 8.6-fold.

In many cell types, apoptosis is characterized by the generation of DNA fragments through the action of endogenous endonucleases. The DeadEND™ fluorometric TUNEL system is designed for the specific detection of apoptotic cells within a cell population. As shown in Figure 5, for the DMSO control (Figure 5A), only 1.2% of the cells demonstrated fluorescein-dUTP binding, but when treated with alvaradin E (1.12 µM for 32 h), a 10-fold increase in fluorescein-dUTP binding was observed (Figure 5B). Therefore, consistent with the previous analysis, alvaradin E treatment of HL-60 cells induced apoptosis.

Among the methods that were employed, DiOC6 uptake and annexin-V-PI co-labeling techniques target different compartmental changes in early apoptotic cells. Externalization of PS in the cell membrane occurs prior to nuclear condensation and DNA fragmentation, but it is probably a downstream event relative to reduction of MMP in the apoptotic process (22). Therefore, lower drug concentrations and shorter incubation times were used for the DiOC6 uptake assay relative to the annexin-V-PI co-labeling assays, but higher drug concentrations and longer incubation times were required for DAPI and TUNEL assays. Taken together, these data indicate that alvaradin E facilitates apoptosis and functions as an effective anticancer agent in the hollow fiber model. Structural aspects responsible for these activities remain to be defined, and the results of more advanced in vivo tests will be pivotal in contemplating further development as a clinically useful pharmaceutical agent.

Acknowledgments

Support of this work was provided by grant U19 CA52956, funded by the National Cancer Institute.

References


