Composition and Preliminary Pharmacology Studies with Anvirzel™: An Extract of Nerium oleander

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ABSTRACT. Initial identification and characterization of the major biochemical and biological properties of Anvirzel™, a patented hot-water extract of Nerium oleander, were undertaken using HPLC, LC/MS, and in vitro cell growth inhibition assays. Analyses using high pH anion exchange chromatography with electrochemical detection consistently revealed seven major polysaccharide peaks which were subsequently designated as a “carbohydrate fingerprint.” Analyses of monomer sugar composition revealed glucose and galacturonic acid as major carbo-

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hydrate residues, while carbohydrate linkage studies identified glucopyranosyl and 6-glucopyranosyl as major terminal carbohydrate residues. Nonpolar compounds were separated from polar components through solid phase extraction and analyzed by both reverse phase HPLC and LC/MS methods. Two nonpolar cytotoxic components, oleandrin and its aglycone, oleandrigenin, were detected. Quantitative analysis showed that the oleander extract contained oleandrin and oleandrigenin at concentrations of 2.5 and 4.4 µg/mg extract, respectively. Five proteins with molecular weights of 6, 20, 35, 68, and 150 kD were also identified in the oleander extract although their functions remain unknown. Cytotoxicity studies showed oleandrin to be a potent growth inhibitory compound against human melanoma B16 cells with an IC50 of 4.0 ng/ml. In the same test system, the IC50 values for oleandrigenin and the complete oleander extract against human melanoma cells were 17.0 ng/ml and 1.6 µg/ml, respectively. This initial characterization and pharmacology research has served as a basis for quality control studies for the production and subsequent clinical Phase I trial of Anvirzel™.

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KEYWORDS, Nerium oleander, oleandrin, oleandrigenin, cytotoxicity, polysaccharides, cardiac glycosides, Na,K-ATPase

INTRODUCTION

Nerium oleander, an ornamental plant from the Apocynaceae family, is widely distributed in tropical and subtropical countries. Both water and lipid extract preparations derived from this plant continue to be used as folk medicine remedies for the treatment of a wide variety of maladies and conditions including abscesses, corns, asthma, dysmenorrhea, eczema, epilepsy, epitheliomas, herpes, malaria, psoriasis, ringworm, scabies, sores, warts and tumors. Oleandrin, a cardiac glycoside derived from this plant, has been used for the treatment of congestive heart failure in China for years. A unique, proprietary hot water extract of oleander, Anvirzel™ has been used in Europe and the United States with regulatory approval on a limited, compassionate basis to treat small numbers of patients with malignant disease. A Phase I trial of Anvirzel™ has also recently been initiated in the United States.
While the presence and identification of specific toxic compounds in organic solvent extracts of oleander have been reported, knowledge of the content of cardiac glycosides as well as other constituents contained within water-based extracts of oleander is poorly documented.\textsuperscript{3-7} Two brief reports described water-soluble extracts of *Nerium oleander* with polysaccharides having immunomodulatory potential, although no attempts were described to identify specific components.\textsuperscript{8-9}

The present study has determined major components in the aqueous oleander extract therefore aiding the development of analytical assays that can be applied during the pharmaceutical production and quality control of Anvirzel™. In addition, a better understanding of the pharmacology of this plant extract has been achieved by \textit{in vitro} studies of the relative cytotoxicity against selected human and rodent tumor cell lines.

**MATERIALS AND METHODS**

**Identification of Components in Oleander Extract**

Anvirzel™, supplied as a lyophilized powdered extract, was obtained from Ozelle Pharmaceuticals, Inc. (San Antonio, TX, USA). Oleandrin, oleandrigenin, and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Details of the preparation of the hot water extract of oleander are described in U.S. patent \#5,135,745.

A method for fingerprinting the complex polysaccharides within Anvirzel™ was developed. Aqueous solutions of oleander extract (containing 40 mg) were applied to an anion exchange HPLC column (CarboPak PA-100, 250 × 2.6 mm, Dionex, Sunnyvale, CA).\textsuperscript{10-11} Polysaccharides were eluted from the column using a ternary solvent system consisting of water, 1.0 M sodium acetate and 0.5 M sodium hydroxide. The chromatography system consisted of a Model 5040 Analytical Cell with a Gold electrode (ESA Inc., Chelmsford, MA) and ESA Coulachem II electrochemical detector. Waters 600S gradient pumps with Model 717 autosampler (Water Assoc., Milford, MA) were used. The detector was set at potentials E1, +100 mV; t1, 500 mS; E2, +600 mV, t2 166 mS; E3, −600 mV, t3, 83 mS; AD, 283 mS with a gain of 20 uA and output at +1.0 V. Elution patterns were used to "fingerprint" the complex polysaccharides. To further determine the
nature of the oleander carbohydrates, samples of oleander extract were submitted to the Complex Carbohydrate Research Center (Univ. GA, Athens, GA) for analyses of individual sugar composition from hydrolyzed polysaccharides, and carbohydrate linkage. Individual sugar content within oleander extract was determined by hydrolysis of a sample of extract in freshly prepared 1 M methanolic-HCl for 16 hours at 80°C. The released methylglycosides were dried and N-acetylated using methanol and acetic anhydride (1:1, V:V) for 15 min at 45°C. The acetylated sample was derivatized with Tri-Sil reagent (Pierce Chemical, Rockford, IL) and analyzed by gas chromatography with a 30 m DB-1 column (0.25 × 0.25 mm, i.d.; J&W Scientific, Folsom, CA). The following temperature conditions were used: an initial temperature of 160°C, followed by 200°C raised at 2°C/min, and then to 260°C at 10°C/min. An internal standard of derivatized methylglycosides was also run with the oleander samples for identification.12

Polysaccharide linkage analysis. Analysis was performed by methylating the sample. Methylated material was isolated by extraction into dichloromethane, dried and then subjected to sequential hydrolysis using trifluoroacetic acid (2 M, 120°C), reduction with NaBD₄ and then acetylation (acetic anhydride, pyridine). The resulting partially methylated alditol acetates were analyzed by GC-MS using a 30 m fused silica column in a Hewlett-Packard Model 5985 GC-MS system with myoinositol as an internal standard. The following temperature conditions were used: 2 min at initial temperature of 80°C, then 170°C at 30°C/min, then to 240°C at 40°C/min and then held at 240°C for 5 min.13

Nonpolar compounds: Identification of cytotoxic species. The presence of nonpolar compounds in water extracts of oleander plant material was determined by passing an aqueous solution (1 ml) of oleander extract (1 mg/ml) through a prepared tC₁₈ solid phase extraction cartridge (Waters Assoc., Milford, MA). The cartridge was then washed with water (2 ml) and the retained compounds were eluted with 2 ml ethyl acetate. The eluant was dried at 22°C under nitrogen. The dried samples were then reconstituted each in 100 µl 100% methanol and transferred to 1 ml amber glass vials. Aliquots (10 µl) were injected onto a Spherisorb 5 (ODS) HPLC column (Phenomenex, Torrance, CA) and the column materials were eluted with 100% acetonitrile at a flow rate of 1 ml/min. Compounds were detected on a Waters Photo-diode Array detector set at 220 nm.
The presence of oleandrin and oleandrigenin in oleander extract was confirmed by LC/MS analyses of the corresponding peaks with co-elution of authentic standard compounds. LC/MS was performed using a HP (Hewlett-Packard) 1100 HPLC system and a Micromass Platform (Wythenshawe, Manchester, UK) mass spectrometer with an electrospray ion source. The separation of oleandrin and oleandrigenin was achieved using a Luna 3\(\mu\)m phenyl-hexyl (2 x 150 mm) analytical column maintained at 40°C (Phenomenex, Torrance, CA). The solvent system used for column elution was: solvent A, 2 mM ammonium formate with 0.1% formic acid, pH 3.0 and solvent B, acetonitrile. Mobile phase was delivered at 250 \(\mu\)l/min starting at 60% A and 40% B, followed by 20% A and 80% B at 8 min and continued until 13 min, then programmed back to starting conditions until 20 min. An aliquot (50 \(\mu\)l) of the nonpolar compounds isolated by solid phase extraction was applied to the column. Oleandrin and oleandrigenin in oleander extracts and as reference standards were detected using electrospray ionization in positive ion mode (M+H).

Validated analytical HPLC assays for quantitative determinations of oleandrin and oleandrigenin were developed to determine batch-to-batch reproducibility of new extract preparations. Briefly, an aliquot of oleander extract dissolved in water was applied to a Waters tC18 solid phase extraction cartridge and then eluted with water followed by ethyl acetate. The ethyl acetate fraction was collected, dried and reconstituted in 100 \(\mu\)l of methanol. The same extraction procedure was applied to the reference standards. The samples and standards were subjected to analyses on a reverse phase HPLC column (Phenomenex Spherisorb 5 ODS, 250 x 4.5 mm) preceded by a \(\mu\)Bondapak C18 guard column (Waters Assoc., Millford, MA). Oleandrin and oleandrigenin were eluted with 50% acetonitrile at a flow rate of 1.0 ml/min with a photodiode array detector (220 nm).

For investigation of relative cytotoxicity of eluted species, fractions (5 ml) were collected, brought to dryness under nitrogen, reconstituted in 50% ethanol, and filter-sterilized in preparation of analysis of relative cytotoxicity against human BRO melanoma cells. Carbohydrate material was not retained by the column and passed through in the water wash of :C18 cartridge; this carbohydrate fraction was also collected and assessed for relative ability to inhibit the growth of human BRO cells.

**Proteins.** Proteins in the oleander extract were examined by appl-
ing 500 μg of oleander extract directly onto a prepared 12% polyacrylamide gel containing a 4% stacking gel. The oleander extract was dissolved in buffer (0.0625 M Tris HCl pH 6.8, 2% SDS, 5% glycerol, and 2% 2-mercaptoethanol). The sample was boiled for 2 min in a 90°C water bath prior to leading the sample onto the gel. Electrophoresis was carried out at a constant voltage (60 V) for 3 hr. The separated proteins were detected on the gel by silver staining.15

**Pharmacology**

*Sample preparation.* To determine the relative cytotoxicity of eluted species derived from oleander extract, fractions (5 ml) of separated nonpolar compounds were collected, brought to dryness under nitrogen, reconstituted in 50% ethanol and filter sterilized before testing in human and murine cell lines. Carbohydrate material that was not retained by the tC18 cartridge was also collected and assessed for relative ability to inhibit cell growth.

*Cytotoxicity studies.* Human BRO melanoma cells and murine B16 melanoma cells were grown in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (Hyclone Labs, Logan, UT), 1 mM L-glutamine, 1 mM sodium pyruvate, 0.5 mM MEM non-essential amino acids, and antibiotics (penicillin, 5 μg/ml; streptomycin, 5 μg/ml and neomycin 10 μg/ml) all obtained from Cellgro Mediatech, Inc. (Herndon, VA). Cell cultures were maintained at 37°C with 5% CO₂ in a humidified atmosphere. For determination of relative growth inhibition, Anvirzel™ or a specific cardiac glycoside standard was added to cells in logarithmic phase of growth. Cytotoxicity tests were conducted in quadruplicate. Cells were exposed to drugs for 72 hr prior to addition of MTT, a vital dye, according to the method of Mossman.16 The resulting formazan product was dissolved in DMSO and the absorbance of the samples read at 570 nm (reference wavelength of 650 nm) on a microplate reader.

**RESULTS**

**Composition**

*Plant polysaccharides.* High pH anion exchange column chromatography with electrochemical detector assays revealed seven major polysaccharide peaks (Figure 1). These “fingerprint” peaks were re-
producbly detected in ten separate batch preparations of oleander extracts. This method has been accepted by the FDA as one of a series of quality control criteria for the development of this plant extract product. Results of analysis of monomer sugar composition of the polysaccharides determined by GC/MS following acid hydrolysis are presented in Table 1. Hydrolysis of the polysaccharides indicated that

FIGURE 1. Chromatogram "fingerprint" of polysaccharides in oleander extract. Oleander extract (40 μg) was applied to an anion-exchange HPLC column and a ternary solvent system used to separate the components (see Methods). Separated polysaccharides were detected using an ESA Coulachem II electrochemical detector equipped with a gold electrode. The peaks denoted as A through G on the chromatogram are the fingerprint with which all batches of oleander extract are compared.

![Chromatogram](image)

TABLE 1. Compositional monosaccharide analysis showing the mole percentage of each carbohydrate residue in oleander extract.

<table>
<thead>
<tr>
<th>Carbohydrate residue</th>
<th>Mole % present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>68.3</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>10.6</td>
</tr>
<tr>
<td>Arabinose</td>
<td>7.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>5.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>5.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.7</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.3</td>
</tr>
</tbody>
</table>
they contained 68% glucose and 10% acidic galacturonic residues. The smaller components were arabinose, rhamnose, xylose and galactose residues. The percentage of soluble carbohydrate was calculated in the sample by measuring the peak area of the added internal standard (myoinositol) and comparing it to the peak area of all carbohydrate peaks in the sample. The calculation of the peak areas of all carbohydrate peaks indicated that about 40% of the sample was soluble in methanolic-HCl. GC analysis showed the presence of some of the non-carbohydrate peaks. The remainder of the sample was presumed to be insoluble carbohydrates.

Methylation analysis was used to indicate the manner in which carbohydrate residues are linked together. Methylation analysis also indicates the presence of branching in the polysaccharide. As shown in Table 2 the presence of a large amount of terminal glucose (60%) suggested the presence of a large glucose polymer. The main branching of oleander polysaccharides are 6-linked glucopyranosyl and 4-linked glucopyranosyl residues. Arabinose was present as terminal arabinofuranosyl residue (4.1%) and 4-linked arabinofuranosyl or 5-linked arabinofuranosyl residues (2.2%).

**Nonpolar compounds.** Nonpolar compounds were separated from polysaccharides in aqueous oleander extracts by passage through a solid phase extraction cartridge. Polysaccharides not bound by the reverse phase resin were eluted with water and evaluated for relative cytotoxicity against human melanoma cells. Compounds retained on the column were eluted with ethyl acetate, concentrated and reana-

**TABLE 2. Analyses of carbohydrate linkages performed using partially methylated alditol acetates of the oleander polysaccharide.**

<table>
<thead>
<tr>
<th>Carbohydrate residue</th>
<th>Mole % present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal glucopyranosyl</td>
<td>60.9</td>
</tr>
<tr>
<td>6-glucopyranosyl</td>
<td>14.6</td>
</tr>
<tr>
<td>4-glucopyranosyl</td>
<td>6.6</td>
</tr>
<tr>
<td>Terminal arabinofuranosyl</td>
<td>4.1</td>
</tr>
<tr>
<td>Terminal galactopyranosyl</td>
<td>2.7</td>
</tr>
<tr>
<td>4-arabinopyranosyl or 5-arabinopyranosyl</td>
<td>2.2</td>
</tr>
<tr>
<td>3-glucopyranosyl</td>
<td>1.8</td>
</tr>
<tr>
<td>Terminal rhamnopyranosyl</td>
<td>1.7</td>
</tr>
<tr>
<td>2-glucopyranosyl</td>
<td>1.2</td>
</tr>
<tr>
<td>6-galactopyranosyl</td>
<td>1.0</td>
</tr>
<tr>
<td>4,6-glucopyranosyl</td>
<td>1.0</td>
</tr>
</tbody>
</table>
lyzed on an analytical reverse phase HPLC column. As shown in Figure 2, at least 14 separate peaks were observed. Fractions of the eluant were collected and prepared for analysis of relative cytotoxicity against human melanoma cells. Only two major peaks were identified as having the potential to inhibit tumor cell growth. Analyses of these individual peaks by LC/MS demonstrated that they were, in fact, oleandrín and its aglycone product, oleandrigenin (Figure 3). Peak identity was confirmed by co-elution of authentic standards on a reverse phase HPLC column. Other compounds contained within the oleander extract that have been identified by LC/MS include odoroside A and neritaloside. By using reverse phase HPLC with UV detection, the amount of oleandrín and oleandrigenin in lyophilized oleander extract was estimated to be 2.5 μg/mg and 4.4 μg/mg, respectively, with only minor variations in cardiac glycoside content between extract batches.

Proteins. Oleander extract buffer solution was applied to 12% SDS-PAGE and the proteins were identified by silver nitrate staining. Oleander water extract contained five proteins with molecular weights

FIGURE 2. Reverse-phase HPLC analysis of non-polar components in oleander extract. The solid phase extraction procedure used to obtain the non-polar compounds is described in Methods. Two peaks have been identified as oleandrín and oleandrigenin using LC/MS.
FIGURE 3. Identification of oleandrin and oleandrinogenin in oleander extract by LC/MS. Oleander extract (1 mg/ml) was applied to a Waters tC18 extraction cartridge and washed with water. Nonpolar compounds were then eluted from the column using ethyl acetate, dried and prepared for analysis by LC/MS as described in Methods. The precursor ion and fragments ions of m/z = 576, 517 and 373 were selected to monitor oleandrin (A); whereas, ions of m/z 433 and 373 were used to identify oleandrinogenin (B).
ranging from 6 to 150 kD. No attempt was made to determine function or amino acid composition.

**Pharmacology**

*Cytotoxicity.* Aliquots of solutions of oleander extract, reference oleandrin, or reference oleandrigenin were incubated with human and mouse melanoma cells in tissue culture. As shown in Table 3, there are marked differences in the relative cytotoxicity amongst the compounds. Reference oleandrin was clearly the most cytotoxic compound with an \( IC_{50} \) of 0.004 \( \mu \)g/ml against human melanoma cells. Reference oleandrigenin was less cytotoxic with an \( IC_{50} \) of 0.017 \( \mu \)g/ml. Oleander extract was also cytotoxic against human BRO cells although less so (\( IC_{50} \) of 1.6 \( \mu \)g/ml) than either pure oleandrin or oleandrigenin. Reference ouabain, a cardiac glycoside structurally related to oleandrin, was also a potent inhibitor of cell growth (\( IC_{50} \) of 0.016 \( \mu \)g/ml). In contrast to the growth of human melanoma cells, growth of mouse melanoma cells was markedly less affected by either the oleander extract or any of the reference cardiac glycoside compounds. As seen in Table 3, the \( IC_{50} \) values for these cardiac glycosides ranged from 137 to 287 \( \mu \)g/ml which was approximately 50,000 fold higher than \( IC_{50} \) values determined using human cell lines.

**TABLE 3. Relative cytotoxicity of oleander extract and extract components against human and murine cell lines.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Compound</th>
<th>( IC_{50} ) (( \mu )g/ml) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human BRO melanoma</td>
<td>Oleander extract</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Oleandrin</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>Oleandrigenin</td>
<td>0.017 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>Ouabain</td>
<td>0.016 ± 0.004</td>
</tr>
<tr>
<td>Mouse melanoma B16</td>
<td>Oleander extract</td>
<td>2424 ± 291</td>
</tr>
<tr>
<td></td>
<td>Oleandrin</td>
<td>137 ± 46</td>
</tr>
<tr>
<td></td>
<td>Oleandrigenin</td>
<td>287 ± 18</td>
</tr>
<tr>
<td></td>
<td>Ouabain</td>
<td>171 ± 17</td>
</tr>
</tbody>
</table>
DISCUSSION

The search for novel, safe and effective compounds from plants that have therapeutic potential for the treatment of cancer is a major goal of anticancer research. Unfortunately, many natural products under development for treatment of cancer, including those available through nutraceutical markets, have been poorly characterized with regard to either their composition or their relative pharmacologic activity. The present report represents the initial publication describing the specific composition of oleander extract used in the preparation of Anvirzel™.

While many bioactive polysaccharides from medicinal plants and microorganisms possess immunomodulatory and anti-tumor activities, most are poorly characterized due in part to the difficult analytical procedures required to accomplish this task. In the present studies, an anion exchange HPLC/electrochemical detection method for determination of carbohydrate “fingerprint” analyses was established. The use of “fingerprints” for complex carbohydrate products has become established as an important means of identifying and standardizing botanical products.18 This, in part, provided a reliable method for control of quality of manufactured lots of oleander extract.

Many compounds within oleander have been identified using organic solvent extraction; however, the presence and concentration of these chemicals within hot water extracts of this plant is unknown.19 By mass spectrometry analysis, two cardiac glycoside compounds have been specifically identified as oleandrin and oleandrigenin. The presence of oleandrin is of particular interest in that it has been used as a treatment for congestive heart failure. The recommended daily dose (400 µg/day; in press20) of oleandrin for this therapeutic use is 20-fold higher than the amount of oleandrin (2.5 µg/mg, 18.75 µg/day) present in a recommended “therapeutic dose of Anvirzel™”. Given the absence of cardiac problems cited to date in the use of Anvirzel™ (personal communication), the relatively low concentration (on a weight basis) of oleandrin in the hot water oleander extract, as opposed to the intact plant, appears to account for the lack of toxicity of this product in humans.21

The cytotoxicity of oleander extract was markedly reduced with the removal of the nonpolar compounds suggesting that relative direct cellular cytotoxicity was due mainly to the presence of oleandrin and oleandrigenin and not polysaccharides. In fact, when polysaccharides themselves were tested in the human melanoma cells, they were found to be non-toxic.
Determination of the pharmacology and immunology of complex plant extracts such as oleander extract contained within the product, Anvirzel™, is, of course, difficult due to the complex nature of the product. Our working hypothesis, however, states that the complex negatively charged polysaccharides may be responsible for positive immune modulation while specific nonpolar components, such as oleandin, are responsible for more direct antitumor activity.

The data in this report clearly demonstrate the potent cytotoxicity of oleandin and oleandrin against a number of human malignant cell lines. Their relative inactivity against murine cell lines is of interest although this phenomenon has been previously reported for related cardiac glycosides. An explanation for this species-dependent difference in sensitivity is believed to be due to the relative insensitivity of murine Na,K-ATPase to cardiac glycosides. A direct demonstration of oleandrin-mediated inhibition of Na,K-ATPase has previously been reported by Jortani et al. While it is clear that inhibition of Na,K-ATPase by itself can produce cell death, of considerable interest has been a recent report demonstrating that the export of FGF-2 (bFGF), a potent angiogenic growth factor for tumors, is mediated by Na,K-ATPase. This suggests that cardiac glycosides, such as oleandrin may, in fact, have an anti-angiogenic component to their mechanism of action. The potential role of cardiac glycosides for treatment of cancer has also recently been suggested by Stenkvist who reported a lower death rate of breast cancer patients on digitalis therapy compared to those patients not being treated with cardiac glycoside. Present studies are being conducted to determine the relative importance of oleandrin and related cardiac glycosides as inhibitors of cellular export of FGF-2. Other studies examining the mechanisms of apoptosis in human cancer cells as well as demonstrating oleandrin-mediated inhibition of activation of NF-κB have recently been published.

REFERENCES


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