Elenoside, a New Cytotoxic Drug, with Cardiac and Extracardiac Activity

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This paper deals with the effects of elenoside, (3-hidroxymethyl-1-methoxy-5,6-methylene-dioxy-4-(3,4-methylenedioxyphenyl)-2-naftoic acid lactone-β-D-glucoside) an arylnaphthalene lignan with broad spectrum cytotoxicity in a human tumor cell line panel, isolated from Justicia hyssopifolia (Acanthaceae) grown in the Canary Islands (Spain), on isolated cardiac auricle of rabbits, urinary excretion of rats, and on isolated rat ileum. These effects, using a vehicle (propylene glycol–ethanol–plant oil–Tween 80 (40 : 10 : 50 : 2) as a standard, are presented. Elenoside at concentrations of 3.2×10⁻⁴, 6.4×10⁻⁴, and 1.2×10⁻³ M produced an increase in the contraction force of auricles in a concentration-dependent way. At doses of 25 and 50 mg/kg, an antidiuretic effect and a decrease in sodium excretion were observed. Elenoside at concentrations of 3.2×10⁻⁴, 6.4×10⁻⁴ and 1.2×10⁻³ M produced an increase in the contraction force of ileum in a concentration-dependent manner. Elenoside produced the concentration dependent inhibition of ⁸⁶Rb uptake. These results indicate that elenoside has digitalis-like activity similar to mammalian lignans. Moreover, this lignan has an irritant effect on the gastrointestinal tract.

Key words elenoside; lignan; Justicia hyssopifolia; isolated auricle

A great number of arylnaphthalene lignans have been isolated from different species of Justicia, many of them exhibiting diverse biological activities, of which the most noteworthy are their antitumor, antiviral, insecticidal, anti-inflammatory and anti-hypertensive properties. Also, the lignans on the central nervous system (CNS) are capable of acting both as depressants and antidepressants. On the other hand, some lignans are shown to be present in human and certain animal urine or semen and blood plasma. These mammalian lignans show an inhibitory effect on Ca²⁺ channels, or digitalis-like action. Thus, 2,3 dibenzilbutane-1,4-diol is noted to inhibit K⁺ channels, or digitalis-like action. Thus, 2,3 dibenzilbutane-1,4-diol is noted to inhibit K⁺ depolarization-induced and receptor-mediated contractions, so as to block Ca²⁺ entry into the smooth muscle of rabbit aorta, and it blocks the insensitive Ca²⁺ channels to organic Ca²⁺ blockers, as well as the sensitive channels in rabbit femoral artery. Also, some lignans (enterolactone, presteganol B and 3-O-methylenterolactone) inhibited Na⁺/K⁺-pump activity in human red cells, with IC₅₀ ranging from 5 to 9×10⁻⁴ mol/l. The IC₅₀ for ouabain (7×10⁻⁷ mol/l) was not modified by the addition of lignans, suggesting non-competitive inhibition. Enterolactone inhibited Na⁺/K⁺ ATPase activity in human and guinea pig heart membrane. It also displaced [3H]-ouabain binding from the human heart with IC₅₀ 1.5×10⁻⁵ M. The apparent dissociation rate constant (K₅₀) of [3H]-ouabain was not different in the presence of digoxin or enterolactone. Those results suggest that elenoside (lignan) could inhibit Na⁺/K⁺ ATPase activity in red cells and produce inotropic and chronotropic effects, similar to cardiac glycosides. In the present paper, its effects on the isolated cardiac auricle of rabbits and the urinary excretion of rats (an other effect of cardiac glycosides) are studied.

In a previous paper we reported the isolation from Justicia hyssopifolia L. of an arylnaphthalene lignan and its aglycone, now called elenoside and elenin, respectively, the first as a β-D-glucoside. This lignan has a central depressant effect in animals and cytotoxic activity when studied in a human tumor cell line panel of the US National Cancer Institute (NCI). Cytotoxic compounds produced alterations in the intestinal tract. In this work the effect of elenoside on isolated rat ileum is studied.

MATERIAL AND METHODS

Reagents Elenoside: Phytochemical study of elenoside was realized. Air-dried and ground leaves were extracted in a Soxhlet with EtOH, with heating and under reflux, to afford an extract that was concentrated under reduced pressure. Column chromatography using solvents of increasing polarity (n-hexane, EtOAc, EtOH) gave different groups of fractions. Elenoside was recovered as needles from the most polar fraction of the ethanolic extract and identified by NMR and MS methods and by comparison with reported data. This lignan was determined to have the molecular formula: C₂₉H₄₄O₁₂·8 H²O, with a molecular weight of MW=498. The elenoside was suspended in a mixture of propylene glycol–ethanol–plant oil–Tween 80 (40 : 10 : 50 : 2).

Ouabain: Ouabain octahydrate (Janssen Chimica, Belgium) had the molecular formula C₂₅H₃₂O₁₂·8H₂O and a molecular weight of MW=728.79.

Animals Male albino rabbits (1000—1300 g) and male Sprague-Dawley rats (Leticia, Barcelona, Spain; 250—300 g) were used. The animals were housed under normal laboratory conditions at 22°C on a standard light–dark schedule (12 : 12; lights on: 0800 to 2000) and were given free access to standard laboratory diet and water. The animals were assigned to randomized groups of 10 each. The control group received the vehicle. Animal care complied with the Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, D.C., 1996, and the study plan was approved by the local ethical committee for animal experimentation of University of La Laguna.

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Isolated Auricle of Rabbit The preparation was mounted according to the usual procedures. Albino male rabbits weighing 1000—1300 g were anesthetized and sacrificed. The chest was opened, then the heart removed as quickly as possible and placed in Krebs–Ringer solution. The auricles were dissected free from all other tissues, and threads were tied to the tips of each auricle. The preparations were mounted in Krebs–Ringer solution, at 31 °C and pH 7.4, through which a stream of a 5% CO2–95% O2 mixture was continuously bubbled. An initial tension of 1 g was applied, and after a period of 30 min of stabilization, spontaneous isometric contractions were recorded in a Grass Model 7D Polygraph through a Statham force–displacement transducer. The control group comprised ten auricles whose contractions were recorded for 30 min in the presence of the vehicle used for dissolving elenoside. The effect of elenoside was studied for 30 min at the following concentrations: 3.2×10⁻³, 6.4×10⁻⁴ and 1.2×10⁻⁴ M. Each concentration was tested on ten auricles.

Effect on ⁸⁶Rb⁺ Uptake by Human Erythrocytes Cardiac glycosides are known to produce an inhibitory action on Na⁺; K⁺-ATPase. The procedure described by Berstein and Israel[11] was used. Red cells were separated from heparinized blood by centrifugation, then washed twice with potassium-free Krebs–Henseleit solution containing ⁸⁶Rb⁺ at 4 °C. Next, 0.5 ml of red cells was mixed with potassium-free Krebs–Henseleit solution at 4 °C. After 40 min of incubation at 37 °C, the suspension was centrifuged and the erythrocytes washed with choline chloride (150 mM) and several concentrations of ouabain. An aliquot was removed for counting the total radioactivity using Becton Dickinson equipment. The effect of elenoside was studied at concentrations of 0.25; 0.5; 1; 2; 4 and 8×10⁻⁴ M.

Urinary Excretion in Rats The effect of elenoside on urinary volumetric excretion was studied according to the procedure described by Colo[12]. The animals were starved during a period of 18 h prior to experiments. Following this period, the animals received a solution of 0.9% NaCl (50 ml/kg) by an intragastric route, followed by the administration of elenoside, 25 and 50 mg/kg. Elenoside was dissolved in the vehicle mentioned above. The control group received only the NaCl solution and a volume of vehicle similar to that used for dissolving the drugs. The animals were then individually placed in metabolism cages. Urine was collected during six hours, and urinary excretion was calculated. The concentration of Na⁺ and K⁺ was measured on samples from the total urine of six hours. Each dose was tested in ten animals.

Electrolyte Analyses in Urine The electrolytes Na⁺ and K⁺ were analyzed by the flame photometry method without recourse to chemicals and without waiting periods on a Flame Photometer-Standard II. The method of operation for sodium and potassium is as follows: place 1 ml of urine in a 25 ml measuring flask, fill it up with distilled water, and pour the contents into a measuring cup. The diluted urine was taken up by suction and sprayed into the flame. The measurement was immediately read on the instrument scale, and the required concentration was obtained from a table of values.

Isolated Rat Ileum The rats used were anesthetized and then sacrificed, similarly to the rabbits. The abdomen was opened, and a length of ileum was removed and placed in Tyrode’s solution. Pieces of ileum (1—2 cm) were dissected free from surrounding tissues and mounted in Tyrode’s solution at 37 °C, pH 7.4, through which a mixture of 5% CO2–95% O₂ bubbled continuously. The isotonic contractions of the preparation were recorded on a Grass Model 7D Polygraph through a Statham force–displacement transducer. The load applied to the lever was 1 g. After stabilization, the effect of elenoside was studied for 5 min at the following concentrations: 3.2×10⁻⁴, 6.4×10⁻⁴ and 1.2×10⁻³ M. Ten pieces of ileum for each concentration were used.

Statistical Analysis Statistical analysis was performed using the Prism program containing two-way analysis of variance (ANOVA) (between group factor: dose repeated measures factor: time). The response exhibited a clear concentration–effect relationship, and between 10—15 min after

RESULTS

Isolated Auricle of Rabbit The inotropic and chronotropic effects of elenoside are depicted in Fig. 1. Significant differences were found in the contraction force for the control and elenoside at concentrations of 3.2×10⁻⁴, 6.4×10⁻⁴ and 1.2×10⁻³ M during time of registered. Two-way ANOVA (between group factor: dose; repeated measures factor: time) produced F(3,32,13)=9.75, p=0.0005 Dose; F(6,48.08)=7.291, p=0.0005 Time.

Elenoside at a concentration of 1.2×10⁻³ M produced a significant increase in the contraction force vs. control group (Tukey’s test, p<0.05). The response exhibited a clear concentration–effect relationship, and between 10—15 min after
its administration the peak effect appeared to have been reached (Fig. 1A). The beating rate of the auricles was decreased by all concentrations of elenoside used and during the experimental period. Two-way ANOVA (between group factor: dose; repeated measures factor: time) produced $F(3, 61.89)=18.61$, $p=0.0001$ Dose; $F(6, 18.16)=2.73$, $p=0.045$ Time. Elenoside produced a significant decrease in heart rate (Tukey’s test, $p<0.001$ control vs. 1.2×10⁻³ M elenoside; Tukey’s test, $p<0.01$ control vs. 3.2×10⁻⁴ and 6.4×10⁻⁴ M elenoside) (Fig. 1B).

**Effect on ⁸⁶Rb⁺ Uptake by Human Erythrocytes**

The effect of elenoside and ouabain on ⁸⁶Rb⁺ uptake by red cells is shown in Table 1. Elenoside produced a concentration-dependent inhibition of ⁸⁶Rb⁺ uptake. A similar effect was obtained with ouabain, but at a smaller concentration.

### Table 1. Effect of Elenoside and Ouabain on the ⁸⁶Rb⁺ Uptake by Red Cells

<table>
<thead>
<tr>
<th>Concentration of elenoside</th>
<th>% uptake inhibition of ⁸⁶Rb⁺</th>
<th>Concentration of ouabain</th>
<th>% uptake inhibition of ⁸⁶Rb⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.25 \times 10^{-4} \text{M}$</td>
<td>0.00</td>
<td>$1 \times 10^{-7} \text{mm}$</td>
<td>13</td>
</tr>
<tr>
<td>$0.5 \times 10^{-4} \text{M}$</td>
<td>3.25</td>
<td>$5 \times 10^{-7} \text{mm}$</td>
<td>28</td>
</tr>
<tr>
<td>$1 \times 10^{-4} \text{M}$</td>
<td>6.34</td>
<td>$1 \times 10^{-6} \text{mm}$</td>
<td>53</td>
</tr>
<tr>
<td>$2 \times 10^{-4} \text{M}$</td>
<td>10.12</td>
<td>$5 \times 10^{-7} \text{mm}$</td>
<td>79</td>
</tr>
<tr>
<td>$4 \times 10^{-4} \text{M}$</td>
<td>19.45</td>
<td>$1 \times 10^{-6} \text{mm}$</td>
<td>95</td>
</tr>
<tr>
<td>$8 \times 10^{-4} \text{M}$</td>
<td>27.38</td>
<td>$5 \times 10^{-6} \text{mm}$</td>
<td>99</td>
</tr>
</tbody>
</table>

Percent of uptake inhibition as compared with ouabain. Each value represents the mean±S.D. of five experiments.

**Urinary Excretion in Rats**

Elenoside, at doses of 25 and 50 mg/kg intraperitoneally, produced a steady decrease in the urinary excretion of water, compared with the control, at all hours following its administration. Two-way ANOVA (between group factor: dose; repeated measures factor: time) produced $F(2, 26.35)=23.26$, $p<0.0001$ Dose; $F(6, 66.85)=19.67$, $p<0.0001$ Time. No differences were found between the control and 25 mg/kg of elenoside. A dose of 50 mg/kg caused a decrease at all hours following administration without statistical significance (Fig. 2A). Elenoside produced a decrease in the contraction force of ileum between 0—1 min and an increase in the contraction force at 2, 3 and 5 min following administration (Fig. 3A).

**Isolated Rat Ileum**

Significant differences were found in the contraction force of ileum with elenoside at concentrations of 3.2×10⁻³, 6.4×10⁻⁴, 1.2×10⁻³ M and during the 30 min of experimental period. Two-way ANOVA (between group factor: dose; repeated measures factor: time) produced $F(2, 20.69)=6.66$, $p=0.0145$ Dose; $F(5, 63.77)=8.21$, $p=0.0026$ Time. Elenoside produced a decrease in the contraction force of ileum between 0—1 min and an increase in the contraction force at 2, 3 and 5 min following administration (Fig. 3A).

The intestinal frequency was decreased by all concentra-
tions of elenoside used, and during the register time. Two-way ANOVA (between group factor: dose; repeated measures factor: time) produced $F(2, 15.83) = 10.49, p = 0.0035$ Dose; $F(5, 76.63) = 20.32, p = 0.0001$ Time. No statistical differences among concentrations of elenoside were observed. (Fig. 3B)

DISCUSSION

There is a certain structural analogy between aryl-naphthalene lignans and digitalis, since these two compounds have a common lactone cycle, butanolide and butenolide, respectively. On the other hand, the presence of a lactone ring (butanolide) in mammalian lignans suggests that these compounds could interact at the digitalis receptor site of the Na\textsuperscript{+}K\textsuperscript{-}pump. In fact, some of the tested lignans (enterolactone, prestegane B and 3-O-methylenterolactone) inhibited the Na\textsuperscript{+}K\textsuperscript{-}pump activity with IC\textsubscript{50} ranging 5 to 9×10\textsuperscript{-4} M, and the IC\textsubscript{50} for ouabain (7×10\textsuperscript{-7} M) was not modified by the addition of lignans, suggesting a non-competitive inhibition. Therefore, lignans may contribute to the digitalis-like activity found in the tissues, blood and urine of several mammals, including man.\textsuperscript{5} Elenoside, an aril-naphthalene lignan, at concentrations of: 3.2×10\textsuperscript{-4}, 6.4×10\textsuperscript{-4}, and 1.2×10\textsuperscript{-3} M, gave rise to an increase in the contraction force of the rabbit atriun, which was accompanied by significant changes in chronotropy. Increases in the contraction force of rapid establishment and short duration were typical of strophantoside compounds.\textsuperscript{13} The results obtained clearly indicate that elenoside possesses pharmacological activity similar to that of ouabain, although its positive inotropic potency is still much lower than that exerted by ouabain.\textsuperscript{13}

On the other hand, it is a well-known fact that the aglycones of digitalic drugs are far less active than their corresponding heterosides, and that aglycones of the lignans studied were slight inhibitors of Na\textsuperscript{+}K\textsuperscript{-}ATPase.\textsuperscript{14} Thus, it is logical to believe that elenine, a lignan aril-naphthalene in the form of an aglycone, possesses lower positive inotropic potency than elenoside, a β-D-glucoside.

Although prestegane B, a mammalian lignan in the form of aglycone, possesses diuretic and natriuretic properties,\textsuperscript{15} elenoside produced an antidiuretic effect with a dose–effect relationship at doses of 25 and 50 mg/kg of elosnide. At these doses, elenoside caused a significant and dose–dependent decrease in sodium excretion, and did not modify the excretion of potassium. Moreover, MDT indicated significant differences between the control and elenoside at a dose of 50 mg/kg. The antidiuretic effect can be attributable to the cathartic action, obtained by two doses of elenoside (unpublished observations) and characteristic of antineoplastic compounds.\textsuperscript{10}

On isolated ileum rat, elenoside produced a decrease in contraction force and posterior increase, accompanied by no significant changes in intestinal frequency. Similar results were found with lignans extracted from Podophyllum species, on isolated smooth muscle preparations.\textsuperscript{17} Increased peristaltic and cathartic activity are parameters of irritant cathartics, according to Fingl classification.\textsuperscript{18}

In a recent paper, currently available evidence of the cardiovascular benefits and risks associated with phyto-estrogens has been reported.\textsuperscript{19} Thus, pretreatment with flaxseed attenuated the endotoxin-induced cardiac dysfunction and cellular damage.\textsuperscript{20} Also, a traditional Asiatic phytoestrogen-rich diet is associated with a reduction of cholesterol. Therefore, the estrogenic effects of phyto-estrogens can be useful in preventing cardiovascular disease. These compounds may protect low density lipoprotein (LDL) cholesterol from oxidation, and inhibit cyclooxygenase and lipoxigenase enzymes.\textsuperscript{21,22} Moreover, a 1-arylnaphthalene lignan was disclosed as a new structural class of 5 phosphodiesterase inhibitor.\textsuperscript{23}

There is now an increased awareness that plants contain many phytoprotectants. Lignans and isoflavones represent two of the main classes of phytoestrogens of current interest in clinical nutrition.\textsuperscript{24} They are present in many human foodstuffs, including beans, sprouts, cabbage, spinach, soybeans, grains and hops. Epidemiological studies suggest that dietary phytoestrogens may play a role in the prevention of several types of chronic diseases, including atherosclerosis and cardiovascular disease.\textsuperscript{25}

In conclusion, we have found that elenoside has digitalis-like activity similar to mammalian lignans. Moreover, there are similarities and differences between elenoside and strophantoside compounds, such as ouabain, regarding pharmacological power due to structural similarities and differences. Findings from previous laboratory and clinical studies, as well as ours, indicate the need for further research to clarify the biological activities of elenoside.

REFERENCES AND NOTES

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