Inhibition of Na,K-ATPase by oleandrin and oleandrigenin, and their detection by digoxin immunoassays

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Ingestion of oleander plant, containing the cardiac glycoside oleandrin, has been reported to induce fatal poisonings. Derivatives of oleandrin are structurally similar to digoxin. We investigated the cross-reactivities of oleandrin and its aglycone metabolite, oleandrigenin, in several commercially available digoxin immunoassays; assessed their ability to inhibit Na,K-ATPase catalytic activity; and measured their binding to proteins in serum. As assayed with ACS:180, Stratus, RIA, On-Line, and TDx digoxin assays, oleandrin at 100 μmol/L in digoxin-free serum gave apparent digoxin values of 0, 0.83, 2.24, 2.37, and 5.34 nmol/L, respectively, whereas oleandrigenin at that concentration gave results of 0, 0.52, 0.77, 4.94, and 1.40 nmol/L. Study of Na,K-ATPase inhibition showed IC₅₀ values (μmol/L) of 0.22 for ouabain, 0.62 for oleandrin, 1.23 for oleandrigenin, and 2.69 for digoxin. At 25 °C, 96% of oleandrin and 48% of oleandrigenin were bound to serum proteins. Because detection of oleandrin and oleandrigenin by digoxin immunoassays is variable between assays as well as between congeners, assessment of cross-reactivity is warranted for each assay. The inhibition of Na,K-ATPase by oleandrin and oleandrigenin confirms that they likely exert their toxic effects through inhibition of sodium pump activity. In cases of digitalis-like poisoning with suspicion of oleander ingestion, a combination of digoxin immunoassays may be useful to effectively rule out the presence of oleander.

INDEXING TERMS: oleander • digitalis • ouabain • toxicology • cardiac glycosides • sodium pump • cardiolides

Nerium oleander (oleander) is an ornamental shrub that grows in warm climate such as the southern parts of the US from Florida to California [1]. All parts of the plant, including the leaves, flowers, and twigs, are poisonous and contain cardiac toxins such as oleandrin, nerin, folinierin, digitoxigenin, and nergioside [2, 3]. Fatal oleander poisonings have been reported in humans [2, 4, 5], cows [6], cats [7], and geese [8]. The American Association of Poison Control Centers received 3873 reports of oleander exposures in humans during 1991–1995 (Rose Ann G. Soloway, American Association of Poison Control Centers, personal communication, 1996), and many incidents of exposure to oleander have been reported in other countries worldwide [2, 9–11]. Modes of exposure to the plant include accidental ingestions by children [2] as well as intentional administration in foods, drinks, or medicinal preparations prepared from its leaves [5, 10, 12]. Oleander has also been used in suicide attempts [4, 9], in criminal poisonings, and in rat poison [13].

Symptoms of oleander intoxication are very similar to those of digoxin, in that nausea and vomiting are followed by potentially lethal cardiotoxicity manifested by tachyarrhythmia, bradyarrhythmia, premature ventricular beats, and various types of atrioventricular blocks [14]. Other toxic effects of oleander include burning of the mucous membranes of the eyes, mouth, and gastrointestinal tract; bloody diarrhea; xanthopsia (yellow vision); convulsions; respiratory paralysis; and loss of consciousness [2, 15].

The structural similarity of oleander glycosides to the cardiac glycoside digoxin has led to the use of digoxin-specific Fab fragments ("Digibind"); Glaxo Wellcome, Research Triangle Park, NC) for treatment of oleander poisoning [14, 16, 17]. Oleandrin is one of the major cardiac glycosides present in the common oleander [2, 12, 18]; similar to digoxin, it contains a steroid nucleus and an unsaturated lactone ring (Fig. 1). The steroid ring of oleandrin has an additional substitution of an acetyloxy group at C-16 and lacks a hydroxyl group at position 12; also, instead of the dideoxy ribose sugars present in digoxin, oleandrin has a dideoxy arabinose. Oleandrigenin, the deglycosylated congener of oleandrin, is not usually included in the list of oleander plant glycosides [2]. This aglycone has been synthesized [19] and has been reported to be present in Adenium obesum [20] and Beaulonita brevituba [21] plants.

Liquid chromatographic–mass spectrometric analyses of oleandrin and oleandrigenin in decayed human tissues have been described [22]. Recently, we have also reported on the detection of both of these glycosides by HPLC in the serum of a child.
suspected of exposure to oleander plant. Although oleandrin and digoxin have some structural differences, oleander glycosides have been reported to cross-react in digoxin immunoassays [4, 23]. This cross-reactivity has been the basis for recommended screening of suspected oleander poisoning by immunoassays, e.g., RIA [12].

Here, we have characterized the cross-reactivities of oleandrin and its deglycosylated congener, oleandrinigen, in several commercially available digoxin immunoassays. Using tissue from porcine cerebral cortex, which contains all three α-isomers of Na,K-ATPase [24], we compared the inhibitory potency of oleandrin and oleandrinigen with that of digoxin and ouabain. We also assessed the extent of binding of oleandrin and oleandrinigen to serum proteins.

**Materials and Methods**

**Materials.** All chemicals used were reagent grade. Digoxin, ouabain, oleandrin, oleandrinigen, porcine cerebral cortex Na,K-ATPase, ATP ammonium molybdate, Tween80, human albumin (purity 98%), goat anti-rabbit antiserum, and acetonitrile and methanol (both HPLC-grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Nonsterile 96-well polystyrene microtiter plates were purchased from Evergreen Scientific (Los Angeles, CA). A Multiskan (MCC/340, MK II) microtiter plate reader (Flow Laboratories, McLean, VA) was used. Centricon concentrators (30-kDa cutoff) were purchased from Amicon (Beverly, MA).

**Methods.**

Kits for TDx Digoxin II assay (TDx) and Stratus Digoxin Fluorometric Enzyme Immunoassay (Stratus) were purchased from Abbott Labs. (Abbott Park, IL) and Baxter Diagnostics (Deerfield, IL), respectively. The Roche On-Line digoxin assay (On-Line) was performed on a Roche Cobas Far A II analyzer provided by Roche Diagnostic Systems (Branchburg, NJ). ACS Digoxin assay (ACS) kits were provided by Ciba Corning Diagnostics (Medfield, MA). The antidigoxin polyclonal antibody and 125I used in the digoxin RIA (NEN) were obtained from NEN Research Products (Boston, MA). The ACS and On-Line digoxin assays use monoclonal antibodies, whereas the others use polyclonal antisera. The ACS, TDx, Stratus, and On-Line digoxin assays were performed as recommended by the manufacturers. The NEN digoxin assay was performed according to a previously described method [25].

**Immunoeassay cross-reactivity studies.** Primary stock solutions (1 g/L) of oleandrin and oleandrinigen were prepared in methanol. Various concentrations of the two glycosides (from 0 to 200 μmol/L) were prepared in digoxin-free serum. Measurement of digoxin-free serum used for cross-reactivity studies gave digoxin concentration of less than the detection limits of all assays except Stratus, which yielded an apparent reading of 0.43 μg/L. The %B/B₀ for each assay at a given concentration of cross-reactant was calculated by dividing the assay signal at that concentration by the assay signal for the digoxin-free serum.

**Na,K-ATPase catalytic activity inhibition assay.** We used the method of Chan and Swaminathan [26], with several modifications to make the method suitable for very small volumes (so that absorbance could be read with an automated microplate reader). Briefly, 20 μL of sample containing the desired concentration of glycoside was pipetted into a well of a microtiter plate placed in a 37 °C water bath [Tris buffer (see below) was used as the no-inhibitor control]. After addition of 20 μL of the porcine Na,K-ATPase solution (60 U/L), the mixture was preincubated for 10 min; then 20 μL of the ATP solution was added and allowed to react for 15 min. The final concentrations were: potassium 2.33 mmol/L, sodium 134.2 mmol/L, magnesium 1.67 mmol/L, and ATP 4.2 mmol/L, in Tris-HCl buffer, 82 mmol/L, pH 7.8. After the incubation period, we added 150 μL of molybdate solution: per liter, 1.4 mmol of molybdate, 560 mmol of sulfuric acid, and 23.8 mL of Tween 80:methanol solution (12:88 by vol). After 30 min of incubation, 150 μL of the reaction mixture from each well was pipetted onto the corresponding wells on another microtiter plate. The absorbances of all wells were read simultaneously at 340 nm with a microtiter plate reader.

The percentage of Na,K-ATPase activity inhibition represents the proportion of ouabain activity that is inhabitable by the glycosides. Curves were fit by logit regression, performed on SPSS for Windows advanced statistical program version 6.0 (SPSS, Chicago, IL).

**Protein binding.** The extent of serum protein binding of oleandrin and oleandrinigen was evaluated by adding each glycoside at 50 μmol/L to digoxin-free serum in 30-kDa-cutoff Centricon
concentrator tubes at 4, 25, and 30 °C. For a control, the same additions were made to water. The mixtures were then vortex-mixed for 20 s, incubated for 10 min, and centrifuged at 1800g for 5 h. All parts of experimentation (e.g., incubation and centrifugation) were performed at the above temperatures. The apparent digoxin concentrations of the samples before ultrafiltration and of the ultrafiltrates after centrifugation were determined by the Roche On-Line assay. We chose to use this assay because it had cross-reactivity to both oleandrin and oleandrigenin.

**Results**

*Cross-reactivity in digoxin immunoassays.* Cross-reactivities of oleandrin and oleandrigenin in various digoxin immunoassays are shown in Fig. 2. The cross-reactivities of oleandrin and oleandrigenin in the ACS and Stratus digoxin assays were negligible. However, these glycosides cross-reacted in the On-Line, TDx, and RIA digoxin assays.

*Inhibition of Na,K-ATPase catalytic activity.* The extent of inhibition of porcine cerebral cortex Na,K-ATPase activity by ouabain, oleandrin, oleandrigenin, and digoxin is presented in Fig. 3. The 50% inhibition concentration (IC₅₀) values (µmol/L) of these compounds in this material were 0.22, 0.62, 1.23, and 2.69 µmol/L, respectively. Addition of human albumin (40 g/L) to oleandrin raised its IC₅₀ for Na,K-ATPase from 0.62 to 2.63 µmol/L (424% increase).

*Serum protein binding.* The extent of oleandrin and oleandrigenin binding to protein in serum is presented in Table 1. For oleandrin only 8% (at most) of the added amount was measured in the ultrafiltrate. The extent of binding of oleandrigenin was less: As much as 52% of its added concentration was recovered in the ultrafiltrate. To assess possible differences in matrices on the apparent digoxin measurements presented in Table 1, we added 50 µmol/L of oleandrin or oleandrigenin to digoxin-free serum—and to its ultrafiltrate after ultrafiltration at 25 °C—and analyzed the samples by On-Line digoxin assay. The unfiltered serum yielded apparent digoxin equivalents of 1.72 and 3.40 nmol/L for oleandrin and oleandrigenin, respectively. Addition of the glycosides to the serum ultrafiltrate gave respective concentrations of 1.54 and 3.26 nmol/L. Therefore, matrix differences of serum and ultrafiltrate of serum did not significantly bias the results of the serum protein binding experiments.

**Discussion**

The structural similarity of digoxin and oleandrin (Fig. 1) has several clinical implications. One might expect antibodies developed against digoxin to cross-react with oleandrin derivatives. Indeed, interference of digoxin measurements after oleander ingestion has been reported [4, 23], and Digibind is suggested as an effective antidote for the treatment of oleander intoxication.
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<sup>a</sup> Each specimen was incubated and centrifuged at the same temperature.

<sup>b</sup> Values reported are in digoxin equivalents (nmol/L) determined by the On-Line digoxin assay.

UF, ultrafiltrate.

Table 1. Binding of oleandrin and oleandrin (50 μmol/L each) to serum protein.

Therefore, rapid identification of individuals with oleandrin intoxication is important. This clinical need also underscores the importance of characterizing cross-reactivities of oleandrin and its derivatives in digoxin immunoassays. In this study we have demonstrated three important findings: The cross-reactivities of oleandrin and its aglycone metabolite oleandrin are highly variable between digoxin immunoassays; oleandrin and oleandrinogenin inhibit the sodium pump (Na,K-ATPase) with potencies between those of ouabain and digoxin; and both oleander compounds have markedly different binding affinities for proteins in serum.

Because oleandrin has been described as a deglycosylated metabolite of oleandrin [22], we constructed cross-reactivity curves for both glycosides in several commercially available digoxin immunoassays (Fig. 2). Although the cross-reactivities of oleandrin and oleandrinogenin in the ACS and Stratus digoxin assays were negligible, both glycosides cross-reacted markedly differently in the TDX, On-Line, and RIA digoxin assays. For example, oleandrin was less cross-reactive than oleandrinogenin in the On-Line digoxin assay, whereas oleandrinogenin was less cross-reactive than oleandrin in the TDX assay. Assuming that the relative concentrations of these two compounds in serum change with time after ingestion, this suggests that measurable apparent digoxin after exposure to oleander may show up in earlier specimens when measured by some digoxin immunoassays (e.g., TDX) and in later specimens when measured by others (e.g., On-Line). These results point to the importance of selection of appropriate antibodies in preparing reagents for use in digoxin immunoassays as well as the importance of assay selection according to its intended use (e.g., routine TDM vs emergency screening). For example, because neither oleandrin nor oleandrinogenin cross-reacts in the ACS or the Stratus digoxin assays, one might use such assays along with a cross-reacting assay (e.g., On-Line) to resolve a discrepancy in digoxin results when interference by the presence of oleander is suspected.

Inhibition of Na,K-ATPase activity by cardiac glycosides, including digoxin, is known to result in increased intracellular sodium concentrations and subsequently increased calcium. This leads to the positive inotropic effects of these compounds on the heart and potentially to a poisoning of the cells [27]. Although not proved, other toxic manifestations of cardiac glycosides might also be mediated by inhibition of Na,K-ATPase. Symptoms of oleander intoxication include effects on the gastrointestinal, cardiac, and central nervous system in a manner similar to digoxin [14]. Using commercially available isolates of Na,K-ATPase from porcine cerebral cortex, which contain all three isoforms of the alpha subunit of the enzyme [26], we evaluated the relative abilities of oleandrin and oleandrinogenin to inhibit the catalytic activity of Na,K-ATPase and compared them with digoxin and ouabain. Oleandrin and its aglycone metabolite had IC<sub>50</sub> values greater than that of ouabain but less than that of digoxin (Fig. 3). These results are in agreement with cellular toxicity studies of human HeLa cells by cardiac glycosides, in which oleandrin was shown to be more cytotoxic than digoxin [28].

The actual concentration of oleandrin in serum during oleander-induced toxicity is not well established. For example, the total concentration of immunoreactive cardenolides in serum measured by digoxin RIA in a patient after ingestion of ~4 g of oleander leaves was estimated at 530 mg/L [4]—a value derived by comparing the measured serum cross-reactivity (5.8 μg/L digoxin equivalents) with that of a crude oleander leaf extract measured with the same digoxin RIA. Therefore, the apparent oleander concentrations in plasma seen in cases of oleander-toxicity would appear to be in the micromolar concentration range, whereas digoxin and ouabain are toxic at low nanomolar concentrations. Given that our data demonstrate a sodium-pump inhibitory potency of oleandrin and oleandrinogenin between those of ouabain and digoxin, what might explain the disparate plasma concentrations associated with toxicity? Differential binding of these glycosides to proteins in serum may help account for differences in observed toxic concentrations. Our ultrafiltration experiments showed that oleandrin is >92% bound to proteins (Table 1), whereas the bound fraction of oleandrinogenin is considerably lower (~50%). In comparison, the serum protein-bound fraction of digoxin at temperatures between 4 °C and 37 °C reportedly ranges from 4% to 30% [29–31]. Thus, protein-binding may well explain the requirements for higher doses of oleander glycosides to generate toxic effects similar to those of digoxin.

Furthermore, addition of albumin (40 g/L) to the oleander sample before analysis of Na,K-ATPase activity increased its...
This confirms the results of our protein-binding experiments that oleandrin binds strongly to albumin. Thus, because of protein binding, the concentrations of oleandrin needed in plasma to elicit toxic effects at the sodium-pump receptors are likely higher than those of digoxin or ouabain. Other effects, e.g., solubility differences and availability of the compounds at or near the receptors, may also contribute to differences in biological activity.

Laboratorians and clinicians therefore must be aware of the extent of cross-reactivities of oleandrin and oleandrinigen in the digoxin assays used in their clinical setting. In a clinical presentation with symptoms of digitalis poisoning, the finding of low digoxin concentrations in cases of oleander intoxication may prevent the patient from getting effective antidotal therapy (e.g., Digibind). This could occur when the immunoassays used are highly specific for digoxin. On the other hand, having cross-reactivity for the oleander derivatives or other nondigoxin substances in the immunoassays may compromise analytical specificity of one immunoassay vs the other. In cases when digoxin results are clinically or analytically discrepant or in cases when oleander exposure is suspected, one might consider the use of a combination of digoxin immunoassays to resolve such discrepancies or use HPLC instead. Furthermore, because Digibind has been recommended for the treatment of oleander poisoning /14, 17/, knowing the concentrations of oleandrin and its metabolite in serum (either quantitatively or as apparent digoxin equivalents) would be helpful in estimating the amount of Digibind to use. Further studies are needed to determine the exact doses of Digibind needed for successful antidotal therapy in cases of oleander poisoning. Understanding the effects of oleander ingestion on digoxin immunoassay measurements, the effects on sodium pump activity, and the effects of protein-binding will help in design of those studies.

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References