Characterization of the Neutralizing Activity of Digoxin-Specific Fab toward Ouabain-Like Steroids

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ABSTRACT

Digoxin-specific Fab (Digibind) is a mixture of antidigoxin Fab fragments prepared from sheep sera and is used as a treatment for digoxin poisoning. Digoxin-specific Fab has been shown to neutralize an endogenous Na\(^+\)/K\(^+\) ATPase inhibitor (endogenous digoxin-like Na\(^+\)/K\(^+\) ATPase regulatory factor; EDLF) in rats and humans and to lower blood pressure. Although the exact structure of EDLF is unknown, compounds identical to or structurally related to ouabain, bufalin, and marinobufagenin have been detected in mammalian plasma. In this study, some structural characteristics of EDLF were inferred from the ability of digoxin-specific Fab to neutralize the Na\(^+\)/K\(^+\) ATPase inhibitory activity of several known cardenolides and bufodienolides. Additional structural information was obtained from \(^{3}H\)ouabain binding and enzyme-linked immunosorbent assay experiments. Digoxin-specific Fab had the ability to interact to some extent with all of the cardenolides and bufodienolides tested. However, digoxin-specific Fab was more than 20-fold more potent in neutralizing ouabain and bufalin than marinobufagenin. The antihypertensive effect of digoxin-specific Fab seen in preeclampsia and animal models of hypertension may therefore be due to a molecule identical to or structurally similar to ouabain or bufalin.

The existence of an endogenous digoxin-like Na\(^+\)/K\(^+\) ATPase regulatory factor (EDLF) or factors have been known for some time and has been reviewed previously (Schoner, 2002). They are thought to play a major role in the cardiovascular system (Ke, 2001) in part by affecting the contractility of heart and vascular smooth muscle tissue by elevating intracellular calcium (Levi et al., 1995). The exact structure(s) of EDLF is unknown, but there have been reports that it is closely related to or identical to the cardenolide ouabain, which is produced by adrenal cells in culture (El-Masri et al., 2001) in vitro. Both digoxin-specific Fab and intact antidigoxin or antiouabain antibodies have been shown to neutralize the effect of ouabain (Krep et al., 1996) and EDLF tested. However, digoxin-specific Fab was more than 20-fold more potent in neutralizing ouabain and bufalin than marinobufagenin. The antihypertensive effect of digoxin-specific Fab seen in preeclampsia and animal models of hypertension may therefore be due to a molecule identical to or structurally similar to ouabain or bufalin.

Digoxin-specific Fab (Digibind) is a mixture of Fab fragment produced by papain digestion of antibodies raised in sheep against a digoxin-human albumin conjugate (Curd et al., 1971). The Fab fragments are purified by affinity chromatography. The main therapeutic indication for this product is digoxin overdose. The utility of this agent has also been studied in the neutralization of similar cardiotoxins from plants and toad venom (Dasgupta and Emerson, 1998; Brubacher et al., 1999). The ability of digoxin-specific Fab to interact with an endogenous EDLF has also been measured in animal studies of corticotropin-induced hypertension (Li et al., 1997) and 5/6 nephrectomized hypertensive rats (Kaide et al., 1999). Digoxin-specific Fab has been shown to neutralize the effect of ouabain (Krep et al., 1996) and EDLF (Balzan et al., 1991) in vitro. Both digoxin-specific Fab and intact antidigoxin or antiouabain antibodies have been shown to have hemodynamic and cardiac effects (Ke, 2001).

EDLF has been shown to increase significantly during pregnancy-induced hypertension and preeclampsia (Goodlin, 2001).

ABBREVIATIONS: EDLF, endogenous digoxin-like factor; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
1988; Adair et al., 1996; Lopatin et al., 1999). Digoxin-specific Fab lowered blood pressure and increased urine output in preeclamptics (Goodlin, 1988; Adair et al., 1996), suggesting a role for EDLFS in the pathogenesis of preeclampsia.

Because the actual structure of EDLFS remains controversial and elusive, a systematic study of the ability of digoxin-specific Fab to neutralize the Na⁺/K⁺ ATPase inhibitory activity of 15 different digoxin-like molecules in vitro was conducted. The purpose of this study was to gain knowledge of the structure-activity relationship concerning the ability of digoxin-specific Fab to neutralize the functional activity of these molecules in inhibiting Na⁺/K⁺ ATPase. Additional structure-activity information was obtained by cross-reactivity ELISA experiments using digoxin-specific Fab as the primary antibody and competition experiments of [³H]Ouabain binding to digoxin-specific Fab.

Materials and Methods

All Na⁺/K⁺ ATPase inhibitors, ammonium molybdate, polyvinyl alcohol, and malachite green were obtained from Sigma-Aldrich (St. Louis, MO). Human heart membranes were obtained from Biological Services Inc. (Wilmington, DE). [³H]Ouabain was from PerkinElmer Life and Analytical Sciences (Boston, MA). Immunosorb 96-well plates were from Nunc (Rochester, NY). Dignibit was from GlaxoSmithKline (Research Triangle Park, NC). All experiments were performed with a single lot, 2C3451.

**Na⁺/K⁺ ATPase Assay.** ATPase activity was measured by detecting the inorganic phosphate produced from the hydrolysis of ATP by a reaction with malachite green and ammonium molybdate under acidic conditions in the presence of polyvinylalcohol. Briefly, 0.4 to 0.7 µg of human heart membrane protein was added to 50 µl of HEPES buffer, pH 7.5, consisting of 3 mM ATP, 4 mM MgCl₂, 144 mM NaCl, 5 mM KCl, and 0.1 mM EDTA. Because potassium shifts Na⁺/K⁺ ATPase to a conformation that does not bind ouabain (Lingrel and Kuntzweiler, 1994), potassium was added last. After 1-h incubation at 37°C with shaking, 200 µl of color reagent was added. Color reagent was made 1 h before use and consisted of 0.8 mM malachite green base, 8.0 mM ammonium molybdate tetrahydrate, 1 N HCl, and 4 mg/ml polyvinylalcohol. Polyvinylalcohol was dissolved in boiling water, and ammonium molybdate tetrahydrate was dissolved in 6 N HCl at 6 times their final concentrations. Assays were conducted in 96-well plates, which were read in a Molecular Devices (Sunnyvale, CA) Thermomax spectrophotometer at 650 nm after 4 min.

Human cardiac membranes were chosen for these studies because of the sensitivity of cardiac tissue to cardioglycosides and ouabain in vivo, whereas rat ATPase, particularly in kidney, is relatively resistant to ouabain. Initial experiments with the human cardiac membrane showed almost no ouabain sensitivity, so the membranes were treated with sodium dodecyl sulfate buffered with bovine serum albumin (BSA) as described previously (Forbush, 1983). The ideal conditions for treating the cardiac membranes involved the addition of membrane protein and SDS at the same concentration of 0.36 mg/ml in 50 mM HEPES, pH 7.5. After 10 min of SDS treatment at room temperature, the mixture was diluted 6-fold with 0.3% BSA in 50 mM HEPES. The ATPase activity was about 80% ouabain sensitive and stable upon freezing. Ouabain sensitivity of the cardiac membrane was maintained when the final SDS concentration in the ATPase assay was 0.01 mg/ml.

**Neutralization of Cardioactive Steroids.** The ability of digoxin-specific Fab to neutralize the inhibition of ATPase activity of several cardenolides and bufodienolides was measured by its ability to cause a rightward shift in the inhibition curves of these compounds. Ten microliters of digoxin-specific Fab diluted in 50 mM HEPES, 0.3% BSA was incubated for 1 h at 37°C with 10 µl of increasing concentrations of cardenolide or bufodienolide. Twenty microliters of cardiac membranes with sodium, magnesium, EDTA, and ATP were added 5 min before adding potasium. The assay was stopped 1 h later with color reagent and read at 650 nm after 4 min. IC₅₀ values were determined using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Schild analysis was used to calculate the concentration of digoxin-specific Fab needed to shift the cardenolide inhibition curve 2-fold to the right. Because the ATPase enzyme, which competes with the digoxin-specific Fab for binding to the inhibitor, was held constant, these values are not true pA₂ values. The values reflect the concentration of digoxin-specific Fab needed to neutralize the ATPase inhibitors at their IC₅₀ concentration.

**ELISA Assay Using Digoxin-Specific Fab as the Primary Antibody.** Nunc Immunosorb plates were coated with digoxin-BSA conjugate by adding 200 µl of conjugate at a concentration of 1 µg/ml in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.5, to each well. Plates were stored at 4°C for up to 1 week. Plates were blocked with 1% BSA in phosphate-buffered saline (PBS) for 1 h before the assay. Plates were washed three times with PBS with 0.02% Tween 20. Fifty microliters of PBS-0.2% BSA followed by 50 µl of increasing concentrations of cardioactive steroids were added to the plates. One hundred microliters of 0.5 µg/ml digoxin-specific Fab in PBS-0.1% BSA was added to each well. After 2-h incubation with shaking at 37°C, the mixture was removed from the plate, and the plate was washed three times with PBS-0.02% Tween 20. Digoxin-specific Fab that bound to the digoxin-BSA conjugate was detected by adding 200 µl of peroxidase-labeled donkey anti-sheep antibody diluted 1:5000 in PBS-0.1% BSA to each well and incubating for an additional hour at 37°C. The plates were then washed three times with PBS-0.02% Tween 20 to remove unbound anti-sheep antibody. Two hundred microliters of peroxidase substrate, OPD-fast from Sigma-Aldrich, was added to each well, and color was allowed to develop for 12 min. Plates were read at 450 nm in a Thermomax (Molecular Devices) spectrophotometer. Preliminary experiments determined the saturating concentration of digoxin-specific Fab and donkey antibody dilution to produce an absorbance of 1.0 at 14 min. Background was measured with 10 µM digoxin and was less than 10% of total.

[³H]Ouabain Binding to Digoxin-Specific Fab. Digoxin-specific Fab was coated onto Packard (PerkinElmer Life and Analytical Sciences) 96-well Optiplates at a concentration of 2 µg/ml in 200 µl of 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9.5, at least overnight. Plates were blocked with 1.0% BSA in PBS for 1 h. [³H]Ouabain (666 GBq/mmol) diluted in PBS-0.1% BSA. After 1-h incubation at 37°C, unbound [³H]Ouabain was removed by washing the plate three times with PBS-0.02% Tween 20 before the addition of 100 µl of PBS 0.15% BSA. Fifty microliters of several concentrations of ATPase inhibitors were added to each well immediately before the addition of 50 µl of 6.4 nM [³H]Ouabain (666 GBq/mmol) diluted in PBS-0.1% BSA. After 1-h incubation at 37°C, [³H]Ouabain was removed by washing the plate three times with PBS-0.02% Tween 20. Two hundred microliters of Packard Microscint 20 scintillation cocktail was added to each well, and plates were counted in a Packard TopCount NXT (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Statistical Analysis.** All IC₅₀ values and apparent pA₂ values presented were calculated using GraphPad Prism software from data of experiments performed at least three times. Statistical analysis of IC₅₀ values was performed by two-tailed t test of log IC₅₀.
glycosylated in position 3. The steroid substitutions are summarized in Table 1.

Of the various cardenolides and bufodienolides used in this study, neriifolin, convallatoxin, and ouabain were the most potent inhibitors of human cardiac Na\(^+\)/H\(^+\)/K\(^+\) ATPase (Table 2). In general, glycosylation had little effect on the potency of cardenolides without a 5-hydroxyl group. Digoxin was not statistically different from digoxigenin, whereas the difference between digitoxin and digitoxigenin was significant (\(p < 0.005\)) but small (Fig. 2A; Table 2). In contrast, cardenolides with a 5-hydroxyl and a sugar group such as convallatoxin and ouabain were at least 10-fold more potent than their corresponding aglycones, strophanthidin and ouabagenin, respectively (\(p < 0.005\)) (Fig. 2B; Table 2).

Digoxin-specific Fab was found to shift the inhibition curves of these compounds to the right when preincubated with the cardenolide or bufodienolide before Na\(^+\)/H\(^+\)/K\(^+\) ATPase assay (Table 2). For example, concentrations of digoxin-specific Fab ranging from 100 to 2500 nM shifted the marinobufagenin curve severalfold (Fig. 3A). The apparent \(pA_2\) values for digoxin-specific Fab against this bufodienolide were calculated by Schild plots (Fig. 3B). The slopes of the Schild plots for all of the compounds were not significantly different from \(-1.0\) (average \(\pm\) S.E.M., \(-1.1 \pm 0.05, n = 16\)) and linear (\(R^2 = 0.955 \pm 0.01\)), indicating that the inhibitory effect of digoxin-specific Fab toward these compounds was competitive. Digoxin-specific Fab was able to neutralize all of the inhibitors to some extent (Table 2).

Because the neutralization of ouabain by digoxin-specific Fab was observed, experiments were conducted to study the ability of the cardenolides and bufodienolides to compete with \(^{3}H\)ouabain for binding to digoxin-specific Fab. Preliminary experiments showed that \(^{3}H\)ouabain bound with a reasonably high affinity of 0.8 nM (data not shown). Because digoxin-specific Fab is a mixture of many different Fabs from a large population of sheep, binding studies with \(^{3}H\)ouabain should give information about the subpopulation of antibodies that have a high affinity for ouabain. Both digoxin and convallatoxin, which are not hydroxylated in position 11, competed better for \(^{3}H\)ouabain binding than ouabain itself, which is hydroxylated in position 11 (\(p < 0.005\)) (Fig. 4). This would indicate that although the binding sites of certain antibodies can recognize ouabain, they recognize the antigen, digoxin, better. The rhamnoside form of strophanthidin, convallatoxin, was more potent than the aglycone (\(p < 0.005\)). Bufodienolides with 14–15 epoxides were the least potent competitors (Table 2).

ELISA experiments were performed to determine the structural characteristics of cardenolides and bufodienolides that can compete against digoxin-specific Fab binding to digoxin BSA conjugate (Table 2). Digoxin and digitoxin, which differ by one hydroxyl group in the steroid 12 position, had no statistical difference in potency (Fig. 5; Table 2). Convallatoxin, which has more differences in the number and position of hydroxyl groups in the steroid nucleus, loses binding ability. The importance of the glycoside in antibody interaction is again observed with the decreased binding ability of strophanthidin relative to convallatoxin (\(p < 0.005\)). Marinobufagenin was a rather poor competitor (Fig. 5; Table 2).

**Discussion**

The existence of an EDLF(s) involved in blood pressure control has been known for many years (for review, see...
Although the exact chemical structure of this factor(s) is still debated, current evidence suggests that it is closely related to or identical to ouabain (Hamlyn et al., 1991), marinobufagenin (Bagrov and Fedorova, 1998), proscillardin A (Sich et al., 1996), or bufalin (Hilton et al., 1996). Digoxin-specific Fab has been shown to neutralize the effects of exogenous ouabain on central nervous system-mediated changes in blood pressure (Teruya et al., 1997), Rb uptake in erythrocytes (Balzan et al., 1991), and vascular smooth muscle contraction (Krep et al., 1995). Additional studies have shown that digoxin-specific Fab has the ability to neutralize an endogenous factor with Na+/K+ ATPase inhibitory activity similar to ouabain produced in human peritoneal dialysate (Krep et al., 1995; Tao et al., 1996), cord blood of neonates (Balzan et al., 1991), and rat brain (Huang and Leenen, 1996). Digoxin-specific Fab has also been shown to reduce blood pressure in a rat corticotropin (Li et al., 1997) and 5/6 nephrectomy models (Kaide et al., 1999). Finally, digoxin-specific Fab has activity against endogenous Na+/K+ ATPase inhibitors involved in hypertension associated with pregnancy. A substance that cross-reacts with digoxin immunoassays has been reported in patients with preeclampsia and pregnancy-induced hypertension (Kaminski and Rechberger, 1991; Graves et al., 1995). This has been further characterized as both an ouabain-like and marinobufagenin-like factor (Lopatin et al., 1999). There have been two reported clinical cases of digoxin-specific Fab treatment of preeclampsia (Adair et al., 1996) and eclampsia (Goodlin, 1988).

### Table 2

<table>
<thead>
<tr>
<th>ATPase IC50</th>
<th>Digoxin-Specific Fab Neut.</th>
<th>[3H]Ouabain IC50 nM</th>
<th>ELISA IC50</th>
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<tr>
<td>Digoxigenin</td>
<td>160 ± 9</td>
<td>200 ± 52</td>
<td>0.19 ± 0.07</td>
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<tr>
<td>Digoxin</td>
<td>120 ± 20</td>
<td>15 ± 1</td>
<td>0.51 ± 0.16</td>
</tr>
<tr>
<td>Digitoxigenin</td>
<td>130 ± 53</td>
<td>78 ± 5</td>
<td>0.60 ± 0.21</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>78 ± 18</td>
<td>8.4 ± 2.1</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Neriifolin</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
<td>0.66 ± 0.16</td>
</tr>
<tr>
<td>Strophanthidin</td>
<td>180 ± 7</td>
<td>190 ± 5</td>
<td>6.7 ± 2.0</td>
</tr>
<tr>
<td>Convalatoxin</td>
<td>11 ± 1</td>
<td>6.9 ± 1</td>
<td>0.36 ± 0.1</td>
</tr>
<tr>
<td>Acetylstrophanthidin</td>
<td>140 ± 20</td>
<td>60 ± 9</td>
<td>0.64 ± 0.16</td>
</tr>
<tr>
<td>Ouabagenin</td>
<td>640 ± 90</td>
<td>580 ± 52</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Ouabain</td>
<td>21 ± 1</td>
<td>20 ± 2</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>Bufalin</td>
<td>53 ± 6</td>
<td>13 ± 1</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Proscillardin</td>
<td>130 ± 15</td>
<td>30 ± 2</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>Marinobufagenin</td>
<td>520 ± 49</td>
<td>470 ± 30</td>
<td>53 ± 12</td>
</tr>
<tr>
<td>Cinobufagen</td>
<td>50 ± 4</td>
<td>420 ± 14</td>
<td>200 ± 6</td>
</tr>
<tr>
<td>Cinobufatolin</td>
<td>160 ± 4</td>
<td>1400 ± 48</td>
<td>4800 ± 600</td>
</tr>
</tbody>
</table>

![Fig. 2](image1.png)

**Fig. 2.** Concentration related inhibition of Na+/K+ ATPase by cardenolide inhibitors. A, activity of glycosides and aglycones of steroids lacking a hydroxyl in position 5. B, 5-hydroxyl cardenolides with (closed symbols) and without (open symbols) a rhamnose in position 3 (n = 3).

![Fig. 3](image2.png)

**Fig. 3.** Eleven concentrations of digoxin-specific Fab ranging from 0.1 to 2.5 μM shift the Na+/K+ ATPase inhibition curve of marinobufagenin toward the right (A). A Schild plot is generated from the IC50 values (B).
A key unanswered question is the identity of the molecule(s) that digoxin-specific Fab neutralizes in vivo. In the present study, we evaluated the ability of digoxin-specific Fab to neutralize the activity of several cardenolides and bufodienolides to provide additional information as to the structural identity of EDLF. Interestingly, digoxin-specific Fab demonstrated the ability to neutralize cardenolides that are glycosylated in position 3 (digitoxin, neriifolin, convallatoxin, and ouabain) with a similar potency compared with its ability to neutralize digoxin regardless of substitutions in the steroid portion of the molecule. Not only could digoxin-specific Fab neutralize glycosylated inhibitors but also their respective aglycones. However, the effect of digoxin-specific Fab could only be observed when the ATPase inhibitor concentration was close to its IC₅₀ value in the cardiac membrane system being studied. Higher concentrations of aglycones were used in the neutralization assays because they displayed lower potency than the glycosylated forms as characterized previously (Melero et al., 2000). In virtually every case, when the inhibitor was present at its IC₅₀ concentration, an equal concentration of digoxin-specific Fab was effective. Digoxin-specific Fab can neutralize the activity of a variety of inhibitors. Because the neutralizing affinity of digoxin-specific Fab toward the inhibitors could not be observed below the IC₅₀ value of the inhibitor in this system, additional studies were conducted to elucidate the interaction of digoxin-specific Fab with the inhibitors.

Binding studies with [³H]ouabain and ELISA experiments showed a decrease in potency with greater changes in the steroid nucleus. The aglycones digoxigenin, strophanthidin, and ouabagenin differ only in the number and position of hydroxyl groups and lose potency as they diverge in structure from digoxigenin. Rhamnosides of 5-hydroxy cardenolides are more potent than their respective aglycones in both assays. These data show that there is a population of Fab fragments in the mixture of Fabs that recognizes several different positions on the steroid nucleus in addition to the glycosylated position 3. This is in contrast to the neutralization data with digoxin-specific Fab that demonstrated little difference between the steroid substitutions of inhibitors glycosylated in position 3.

Bufodienolides behave in a similar manner to the cardenolides in some respects. The least substituted, bufalin, has the same structure as digitoxigenin except for the six-membered lactone ring in the 17 position. This does not negatively affect Na⁺/K⁺ ATPase activity. Bufalin is also neutralized by digoxin-specific Fab to a greater degree than would be expected based on its aglycone structure. Some data exist for endogenous bufalin-like Na⁺/K⁺ ATPase inhibitor that reacts with bufalin-specific antibodies and is elevated in the serum of salt-sensitive Dahl rats (Oda et al., 2001). A bufalin-like molecule lacking a 14-hydroxyl and an unsaturated bond in the lactone ring have been identified in human placenta (Hilton et al., 1996). This molecule, when prepared from androsterone, has the ability to inhibit Na⁺/K⁺ ATPase. Our results suggest that digoxin-specific Fab may have some neutralizing activity toward these types of molecules.

Some of the bufodienolides have 14–15 epoxydized instead of a 14-hydroxyl found in all of the cardenolides tested. This, along with acetylation of position 16, seems to have additional negative effects on digoxin-specific Fab's ability to neutralize these compounds and their potency in [³H]ouabain and ELISA assays. These positions do not seem to be as important as the steroid position 3 that is the site of glycosylation on both the cardenolides and bufodienolides. This observation supports the lack of efficacy of digoxin-specific Fab in a mouse model of toad venom poisoning (Brubacher et al., 1999) and in clinical settings involving voluntary ingestion of toad venom extract (Brubacher et al., 1996). Digoxin-specific Fab was only 55% effective in preventing death and 30% effective in preventing seizures in mice. When digoxin-specific Fab was used clinically to treat toad venom poisoning, one patient died and two recovered after receiving up to 20 vials of digoxin-specific Fab. Clearly, digoxin-specific Fab is less effective in treating toxicity of toad venom than digoxin toxicity. This is in agreement with the relatively poor neutralizing ability of digoxin-specific Fab toward cinobufagenin and cinobufotalin, which were among constituents of the toad venom in both studies by Brubacher et al. (1996, 1999). These two bufodienolides are the only inhibitors that required digoxin-specific Fab concentrations severalfold higher than their IC₅₀ concentrations to produce neutralization. Other factors in toad venom unrelated to bufodienolides may also be involved in toxicity.

The source of Na⁺/K⁺ ATPase used in this study was human cardiac membranes, which demonstrated a more potent inhibition by ouabain than marinobufagenin. This relative potency is similar to that observed for the sarcolemma fraction of the rat thoracic aorta and is the reverse of the relative activity seen in the nerve ending plasmalemma of this tissue (Fedorova and Bagrov, 1997). Comparison of the ability of several cardenolides to inhibit cardiac Na⁺/K⁺ ATPase activity shows that digoxin and digitoxin gain a slight amount of activity over their respective position 3 nonglycosylated forms. This is not the case for the cardenolides that
are hydroxylated at position 5 where the rhamnosides of strophanthidin and ouabagenin are 16-fold for convallatoxin and 30-fold for ouabain more potent, respectively, than their aglycones. The area around position 3, where glycosylation occurs, and the adjacent position 5 that is sometimes hydroxylated seem to be important regions for Na\(^+\)/K\(^-\) ATPase inhibitory activity. This is consistent with the recently generated three-dimensional structure-activity relationship study using comparative molecular field analysis alignment (Farr et al., 2002a). Using 28 steroid Na\(^+\)/K\(^-\) ATPase inhibitors, it was found that adding steric bulk and/or electronegativity around the sugar attached to position 3 increased inhibitory activity. The importance of hydroxylation of position 5 in affecting the spatial orientation of gycosides in position 3 has recently been shown also using molecular field analysis (Farr et al., 2002b). A greater range of inhibitory activity was seen in this study compared with Farr et al. (2002b). Perhaps this is because potassium, as opposed to ATP, was added last in these studies, which increased ouabain activity greater than 30-fold (data not shown) consistent with current models of the ATPase (Lingrel and Kuntzweiler, 1994; Yingst et al., 1998).

An unexpected finding in these studies was that the structure-activity relationship for the cross-reactivity of digoxin-specific Fab toward the ATPase inhibitors was not always predictive of neutralization activity. ELISA type assays are commonly used to test cross-reactivity and represent a global predictive of neutralization activity. ELISA type assays are specifically Fab toward the ATPase inhibitors was not always

demonstrated three-dimensional structure-activity relationship study of the inhibition of Na\(^+\)/H\(^+\) -ATPase by cardiotonic steroids using comparative molecular field analysis.

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