Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 6062–6070
Bioorganic & Medicinal Chemistry

Identification and characterization of novel sodium/potassium-ATPase inhibitors by virtual screening of a compound database

David T. Stanton,[a] Julie Ankenbauer,[b] David Rothgeb,[b] Matthew Draper[b] and Stefan Paula[b,*]

[a]*Miami Valley Innovation Center, Procter & Gamble, Cincinnati, OH 45252, USA*
[b]*Department of Chemistry, Northern Kentucky University, Highland Heights, KY 41099-1905, USA*

Received 8 May 2007; revised 19 June 2007; accepted 20 June 2007
Available online 29 June 2007

Abstract—The medicinal value of cardiac glycoside inhibitors for the treatment of congestive heart failure symptoms stems from their ability to specifically inhibit the ion transport activity of the transmembrane enzyme sodium/potassium-ATPase (Na/K-ATPase) in myocardial cells. In this study, we used the inhibitory potencies of 39 cardiac glycoside analogues for the development of a quantitative structure–activity relationship (QSAR) model for Na/K-ATPase inhibition. In conjunction with a substructure and similarity search, the QSAR model was used to select ten potential inhibitors from a commercial compound database. The inhibitory potencies of these compounds were measured and four were found to be more active than the commonly used inhibitor ouabain. The results of the bioassays were incorporated into a second QSAR model, whose physical interpretation suggested that the nature of substituents in positions 10, 12, and 17 at the cyclopentanoperhydrophenanthrene core of the inhibitors was critical for enzyme inhibition. All descriptors of the QSAR models were conformation-independent, making the search protocol a suitable tool for the rapid virtual screening of large compound databases for novel inhibitors.

1. Introduction

Even after over 200 years of use, cardiac glycosides (digitalis) are still frequently prescribed for the treatment of congestive heart failure symptoms (CHF).[1–3] They exert their therapeutic effect by inhibiting the activity of the ion-transporting transmembrane enzyme sodium/potassium ATPase in the membrane of heart muscle cells. As a result of the inhibition, the transmembrane sodium concentration gradient decreases which slows the exchange of intracellular calcium ions against extracellular sodium ions. The consequence is a rise in intracellular calcium concentration, which is the main cause of the so-called positive inotropic effect. The latter constitutes an increase in the contractile force of the heart muscle and cardiac output, which alleviates symptoms of CHF. Despite their effectiveness, cardiac glycosides such as digoxin or digitoxin have the disadvantage of a relatively low therapeutic index, which increases the likelihood for digitalis toxicity that can trigger life-threatening heart arrhythmias.[2] In severe cases of digitalis toxicity, sheep polyclonal antibodies (DigiBind and DigiFab) can be administered to bind free digitalis in the patient's blood and accelerate its excretion.[4–6] Since these antibodies are non-human, repeated use bears the risk of adverse reactions of the human immune system, including anaphylactic shock.

As a potential alternative to detoxification by antibodies, the development of a small molecule digitalis antagonist has been proposed.[7] Such a compound would display no or only a greatly reduced inhibitory potency while still being able to bind to the digitalis receptor site of Na/K-ATPase with high affinity. Thus, the antagonist would successfully compete with digitalis for the digitalis binding site and thereby protect it from excess digitalis binding. The development of such an antagonist would start with searches for molecules with low inhibitory potency but high binding affinity for Na/K-ATPase. As proof of principle, a previous study has identified several compounds that show the required inhibition/binding disparity, demonstrating that the design of a competitive digitalis antagonist should be feasible.[7] Since digitalis are structurally complex molecules

Keywords: Sodium pump; Digitalis; Cardiac glycosides; Quantitative structure–activity relationship; Congestive heart failure; Compound library.
* Corresponding author. Tel.: +1 859 572 6552; fax: +1 859 572 5162; e-mail: paulas1@nku.edu

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.06.050

and the pool of potential compound candidates is large, the identification of candidate compounds could greatly benefit from computational search protocols capable of rapidly and accurately identifying molecules with specified inhibitory potencies and binding affinities.

By correlating the molecular structure of compounds with their biological activity, QSAR models frequently play a major role in the virtual high-throughput screening of large compound databases. Moreover, close examination of the molecular descriptors employed by QSAR models can reveal the structural requirements for bioactivity. Despite the large volume of structure–activity relationship (SAR) studies on hundreds of naturally occurring and synthetic cardiac glycosides and their analogues, only few quantitative models have been published and the analysis of the molecular requirements for bioactivity has been restricted frequently to mere qualitative interpretation.[8] For instance, a series of studies by Melloni and coworkers have systematically explored the effects of replacing the lactone ring of digitoxigenin by a large variety of linear, nitrogen-containing substituents, but only in one case a QSAR model was developed.[9–14] The same applies to the numerous studies by the groups of Repke[15–19] and of Templeton and LaBella,[20–26] who investigated the effects of modifying the cyclopentanoperhydrophenanthrene backbone and its substituents. More recently, two 3D-QSAR studies based on comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA) have been employed to the Na/K-ATPase/cardiac glycoside system.[7,27] Whereas these 3D-QSAR methods are excellent tools for the identification and localization of crucial inhibitor/enzyme interactions, their requirement for structural alignment of inhibitors in their presumed bioactive conformation makes them not amendable for rapid searches of large, structurally diverse compound databases for new inhibitors. This task is more likely achieved by conventional QSAR methods based on conformation-independent descriptors that are faster and do not require any hypotheses about the bioactive conformation. Although this type of QSAR modeling continues to be used routinely in pharmaceutical research for numerous ligand/receptor systems, it has been greatly underutilized for the Na/K-ATPase/digitalis system.

This study describes a virtual search protocol and the development of two conventional QSAR models for Na/K-ATPase inhibition derived from the potencies of 39 and 43 cardiac glycosides, respectively. Both models employ six conformation-independent descriptors that were identified from an initial set of 189 descriptors. Using a substructure and a similarity search for steroid-like molecules similar to a known, potent inhibitor, a small subset of compounds was filtered from a sizeable vendor database as potential Na/K-ATPase inhibitors and the QSAR model-predicted inhibitory potencies for these compounds were calculated. Then, a selection of ten molecules was subjected to experimental testing in ATPase activity assays. These tests assessed the virtual screening protocol's usefulness for finding novel compounds with well-defined properties regarding the inhibition of Na/K-ATPase. Moreover, a physical interpretation of the descriptors in the final QSAR model permitted the identification of the structural requirements for effective Na/K-ATPase inhibition.

2. Results

2.1. Development of an initial QSAR model for digitalis-mediated Na/K-ATPase inhibition

An initial QSAR model for Na/K-ATPase inhibition was developed based on the previously published relative inhibitory potencies (RIP, the IC_{50} value of Na/K-ATPase inhibition of a given compound divided by that of the commonly used inhibitor ouabain) of 38 compounds (activity data provided in 'Supplementary materials) and the experimentally determined bioactivities of compounds **1**–**5**, which had been obtained commercially (see Fig. 1 for molecular structures, Fig. 2 for representative bioassay data, and Table 1 for RIP values of compounds **1**–**5**). The rationale for including **1**–**5** was to broaden the structural diversity of the compound set for QSAR modeling by including molecules with bulky substituents at C-10 (**1**–**3**), which are not typically found in cardiac glycosides (see Scheme 1 for carbon numbering). Likewise, the inclusion of **4** added more structural variability to the compound set in the position normally occupied by the second sugar of digoxin and **5** displayed an unusual system of five condensed rings.

After modeling the molecular structure of the inhibitors and minimizing their conformational energy, several rounds of model development were performed with the program ADAPT, which operates with a generalized simulated annealing approach.[28–30] The compounds evomonoside, helveticoside, and chlormadinone acetate were classified as outliers, and **5** was omitted because it turned out to be a partial inhibitor with very low potency. Thus, the initial QSAR model was developed based on the activities of the remaining 39 compounds and employed the six following descriptors: VCH5, VCH9, SsOH, SHBint3, BEHv3, and Belv2.[31] Several statistical parameters such as cross-validated and conventional correlation coefficients, *t*-values, and variance inflation factors indicated that the obtained model was of good quality (Table 2). A graphical comparison of the QSAR-computed and observed RIP values on a logarithmic scale (Fig. 3) revealed good correlation between the two quantities (see Table in Supplementary materials for numerical values).

It should be noted that all six descriptors were insensitive to compound conformation, which eliminated any bias with respect to postulating a bioactive conformation. Moreover, the model was well suited for the rapid prediction of the activities of a large number of compounds since no time-consuming conformational analysis was required prior to descriptor calculation. During the preparation of this manuscript, it was discovered that in the early stages of QSAR model development the biological activity for **4** had been entered erroneously as the raw RIP value (RIP = 0.870) instead of the inverse logarithm ($\log \mathrm{RIP}^{-1} = 0.0600$). This error

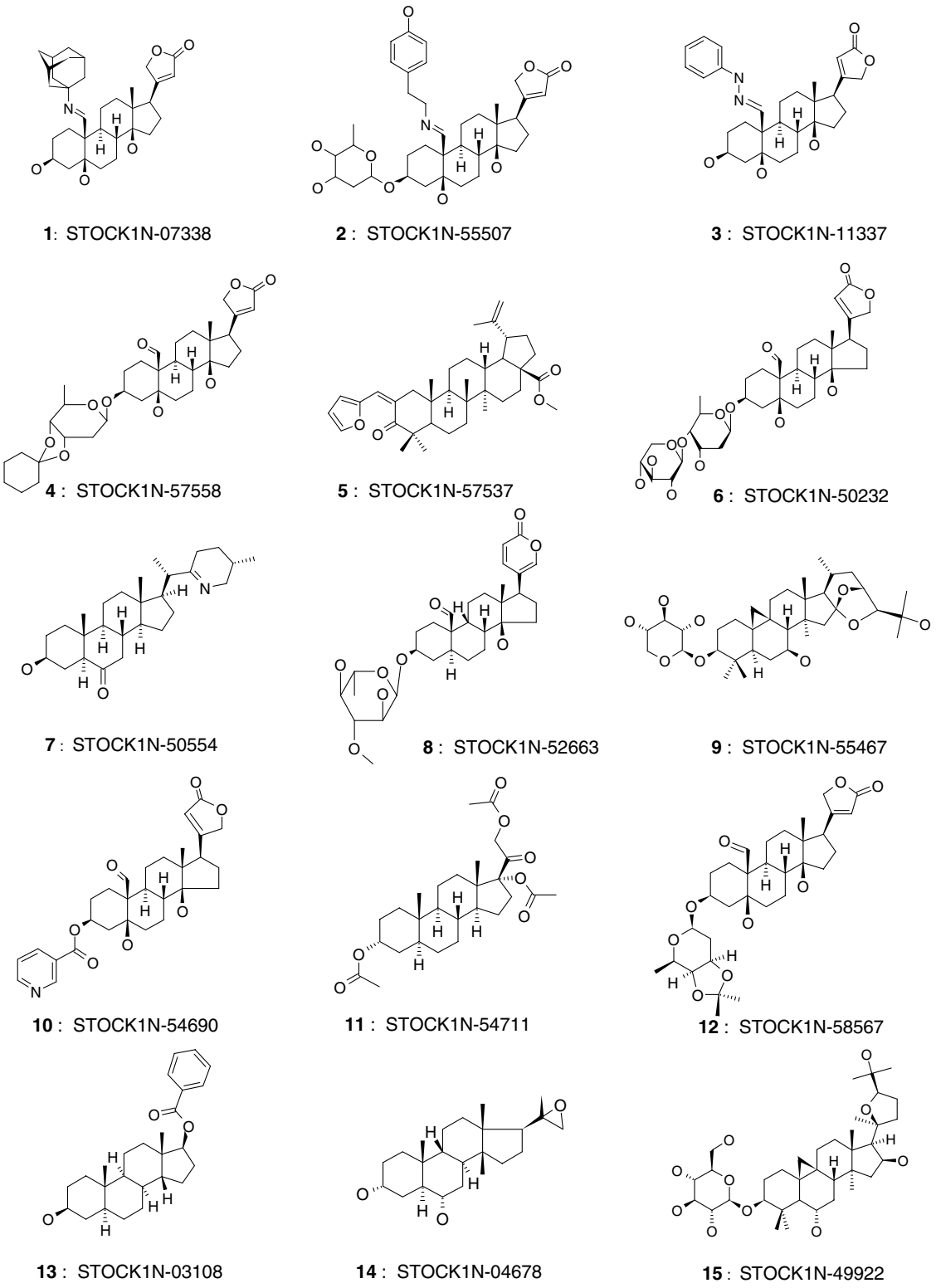


Figure 1. Chemical structure of the 15 compounds evaluated in bioassays.

was corrected during the development of the final model (see below) and it did not seem to affect the quality of the initial model significantly, which could be validated based on conventional regression statistics.

For the virtual screening for novel inhibitors, the natural products compound database from InterBioScreen containing 37,000 structures was used. Six hundred and sixty of these compounds were selected by a substructure search that filtered compounds that possessed the cyclopentanoperhydrophenanthrene skeleton composed of the four condensed rings typical for steroids. In order to increase the likelihood of identifying active compounds, this set was further reduced to a subset of

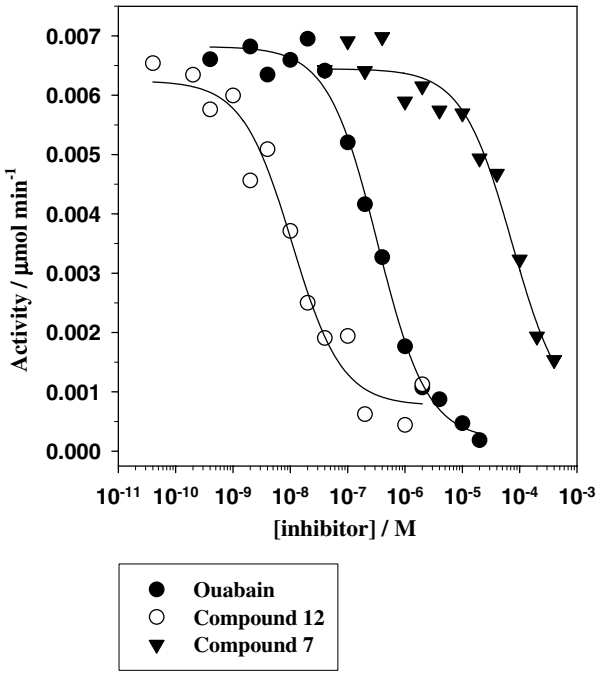
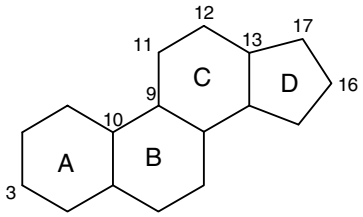


Figure 2. Typical ATPase activity inhibition curves in the presence of inhibitors showing varying bioactivity.

233 compounds by a similarity search with the tool ChemFinder (version 9.0, CambridgeSoft, Boston, MA) that searched for structural features found in potent Na/K-ATPase inhibitors. The compound evomonoside was selected as a reasonable target for the similarity search (similarity greater than 60%) because it exhibited the greatest potency among all compounds evaluated thus far. For all compounds in the subset, the six descriptors relevant for the QSAR model were calculated, allowing the prediction of their inhibitory potencies (Table 1).

2.2. Selection of compounds for experimental testing

With the assistance of the initial QSAR model, ten compounds were selected from the set of 233 for experimental evaluation. The criteria for choosing the test compounds were high QSAR-predicted inhibitory potencies and unique structural features. Even though all compounds conserved the connectivity of the steroid-typical cyclopentanoperhydrophenanthrene moiety, the *cis*/*trans* stereochemistry of some bonds in the ring system was not always the same. Many compounds displayed structural variations of the lactone ring at C-17, which had been identified previously as crucial for modulating inhibitory potency.[27] For instance, **7**, **8**, and **13** had six- rather than five-membered rings attached to C-17, whereas **14** and **15** featured a much smaller epoxide and a non-lactone five-membered ring, respectively. Due to its relatively low predicted inhibitory potency, **15** served as a negative control. The potency of **11** was predicted to be about two orders of magnitude greater than that of ouabain and carried at C-17 a linear chain that was similar in size and chemical composition to a five-membered lactone. Compound **9** had a unique bicyclic system condensed to the D ring at C-16 and C-17, while **12** was selected for its unique bicyclic substituent at C-3. Compound **6** was a rare representative of cardiac glycosides with two sugar moieties, and **10** carried a non-carbohydrate moiety at C-3.



Scheme 1. Numbering of carbon atoms and identity of rings in the cyclopentanoperhydrophenanthrene skeleton.

2.3. Experimentally observed potencies of inhibitors and final QSAR model development

Five out of the ten test compounds from the InterBioScreen database were capable of inhibiting Na/K-ATPase activity, albeit with greatly varying inhibitory potencies. Typical inhibition curves obtained from the ATPase activity inhibition assay are shown in Figure 2

Table 1. Experimentally determined and QSAR model-computed inhibitory potencies of compounds

Compound	IC_{50} (nM)	Measured RIP	Predicted RIP, initial model	Fitted RIP, final model
1	313 ± 85	0.668	0.752[a]	0.906
2	2640 ± 920	4.92	3.91[a]	2.77
3	4230 ± 2260	7.89	8.91[a]	8.54
4	409 ± 87	0.872	9.20[a]	—
5	Partial inhibitor	—	—	—
6	160 ± 44	0.341	0.644	0.461
7	143,000 ± 75,000	305	0.103	—
8	55.6 ± 16.0	0.118	0.111	0.101
9	Inactive	—	0.117	—
10	443 ± 259	0.944	3.37	1.19
11	Inactive	—	0.00933	—
12	14.6 ± 9.7	0.0310	4.17	0.0351
13	Partial inhibitor	—	0.183	—
14	Inactive	—	0.786	—
15	Inactive	—	2.92	—

[a] Included in training set for initial model, fitted value reported.

Table 2. Summary of the regression analysis equation for the initial Na/K-ATPase inhibition model

Descriptor	Regression coefficient	Standard error of coefficient	*t*-Value	Variance inflation factor
VCH5[a]	−15.7	2.28	−6.89	1.5
VCH9[a]	116	26.8	4.34	1.5
SsOH[a]	−0.00710	0.00214	−3.32	2.9
SHBint3[a]	0.0151	0.00357	4.23	2.4
BEHv3[b]	−6.79	0.845	−8.04	4.6
BELv2[b]	8.12	1.23	6.59	4.7
Y-Intercept	9.49	1.65	5.75	—

R^2 = 86.1%, Q^2 = 80.7%, s = 0.182, N = 39, F-value = 33.0.
[a] Computed using MC364.
[b] Computed using Dragon.

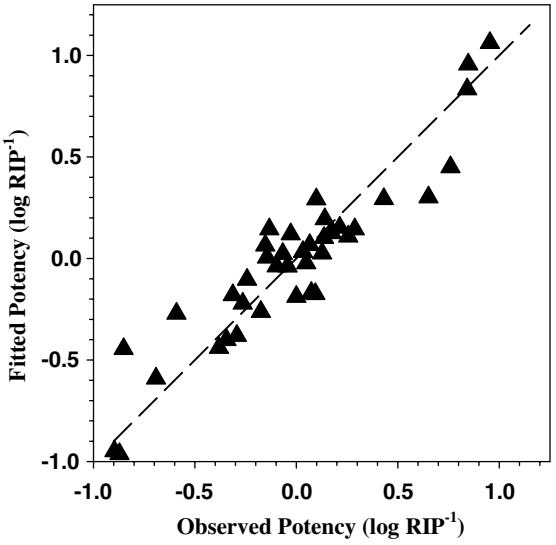


Figure 3. Fit plot showing the correlation between QSAR-computed and experimentally determined inhibitory potencies for the initial QSAR model based on the activities of 39 compounds.

and the assay results for all compounds tested are summarized in Table 1. Some compounds were one (**8**) or more (**12**) orders of magnitude more potent than the standard inhibitor ouabain whereas another one (**7**) required concentrations in the upper micromolar range to be effective. Despite high predicted inhibitory potencies, compounds **9**, **11**, and **14** were found to be inactive at concentrations up to 1 mM and **15**, as predicted, was not active either. Compound **13** was unique in that it displayed partial inhibition (approximately 30%) with an IC_{50} value of approximately 1 μM. Its activity gradually disappeared and was entirely lost after several hours of storage in DMSO, which was attributed to chemical decomposition. Therefore, **13** was omitted from further consideration. Overall, the most significant finding of the assays was the discovery of four novel Na/K-ATPase inhibitors (**6**, **8**, **10**, and **12**) with potencies comparable to or better than that of ouabain that had not been characterized previously.

The experimentally determined potencies of the tested compounds (Table 1) were included in the development of a final QSAR model for the physical interpretation of the underlying SAR. This final model was generated using the same procedure described above and was based on a total of 43 instead of 39 observations since in addition to the active compounds **6**, **8**, **10**, and **12**, the initially excluded evomonoside and chlormadinone acetate could be included whereas the previously included anthroylouabain and **4** were statistical outliers (see Table in Supplementary materials). Like the initial model, the final model employed six conformation-independent descriptors (xch9, nelem, C-027, dxvp6, SHBint3, and Ui[31]) and the statistical parameters were similar to those of the initial model. Details of the final QSAR model are provided in Table 3, and a graphic comparison of QSAR-computed and observed inhibition values is shown in Figure 4.

3. Discussion

3.1. Qualitative interpretation of the SAR for Na/K-ATPase inhibition by cardiac glycosides

Due to the complexity of cardiac glycoside structure, a correct prediction of bioactivity based on a single structural property is unfeasible. Nonetheless, a close inspec-

Table 3. Summary of the regression analysis equation for the final Na/K-ATPase inhibition model

Descriptor	Regression coefficient	Standard error of coefficient	*t*-Value	Variance inflation factor
nelem[a]	−1.57	0.147	−10.7	2.07
xch9[a]	98.3	13.2	7.44	1.05
dxvp6[a]	0.673	0.0736	9.15	2.10
SHBint3[a]	0.0241	0.00369	6.52	2.07
C-027[b]	0.407	0.0978	4.16	1.48
Ui[b]	0.590	0.112	5.29	3.01
Y-Intercept	0.675	0.396	1.71	N/A

R^2 = 87.4%, Q^2 = 81.7%, s = 0.215, N = 43, F-value = 41.7.
[a] Computed using MC364.
[b] Computed using Dragon.

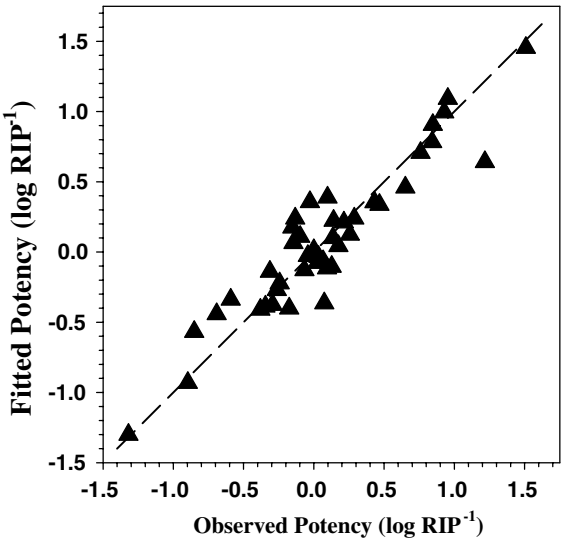


Figure 4. Fit plot showing the correlation between QSAR-computed and experimentally determined inhibitory potencies for the final QSAR model based on the activities of 43 compounds.

tion of molecular structure reveals several structural features that are, at least qualitatively, indicative of Na/K-ATPase inhibition. For instance, the stereochemistry of the bonds that connect the four cyclopentanoperhydrophenanthrene rings certainly exerts an influence on a compound's activity. A *cis* fusion of steroid rings A and B (Scheme 1) as found in the active compounds **1**–**4**, **6**, **10**, and **12** appears to be favorable for inhibition. Likewise, a *cis* fusion of rings C and D which is present in compounds **1**–**4**, **6**, and **10** also seems to concur with inhibitory potency. On the other hand, the stereochemistry of the linkage between rings B and C, which is typically *trans*, is less predictive of bioactivity since a *cis* bond is present both in the highly active compound **8** as well as in the inactive compounds **9**, **13**, and **15**.

Another important determinant is the nature of the substituent at position **17**, which is a monounsaturated five-membered furanone in most active glycosides. In certain naturally occurring compounds such as bufalin or cinobufagin, however, this ring is replaced by a six-membered pyranone with two double bonds, which is accompanied by a noticeable increase in inhibitory potency. This trend is verified by the high potency of **8**. Interestingly, the six-membered nitrogenous ring in **7** seems to be partially capable of assuming the role of these lactones whereas the tetrahydrofuran-based group in **15** is not. As predicted in an earlier study,[7] a reduction in ring size to an epoxide as present in **14** is detrimental to inhibitory potency, although in this particular instance, modifications of the cyclopentanoperhydrophenanthrene moiety may also contribute to the compound's inactivity. Overall, the identification of structural features that are predictive of Na/K-ATPase inhibition is complex and therefore accomplished best by a rigorous analysis of QSAR descriptors as described in the following section.

3.2. Physical interpretation of the descriptors used in the final QSAR model

A close examination of QSAR models and their descriptors frequently reveals which structural features of compounds contribute most to their bioactivities. Here, the final QSAR model for Na/K-ATPase inhibition was used for this purpose since it contained the newly tested compounds and was therefore more comprehensive and informative than the initial QSAR model. A method described previously employing PLS regression was used to examine the model which contained six validated PLS components.[32] Since the first three of these components account for over 93% of the SAR captured by the model, the following discussion will be limited to these.

3.2.1. Component-1. Component-1 is dominant and accounts for 75.8% of the SAR. Three descriptors provide 84.3% of the information in this component. The most important of these is the 9th-order chain molecular connectivity index (xch9, 33.6%), followed by a simple count of element types (nelem, 30.8%), and the descriptor C-027 (19.9%), which is a count of features focusing on an sp^2-hybridized carbon atom bonded to a heteroatom and any other group. The xch9 descriptor captures the presence and characteristics of a nine-membered ring or chain whose members are chemical bonds. The particular substructure relevant for xch9 is illustrated in Figure 5. The primary instance of a nine-membered chain occurs in the C and D rings of the cyclopentanoperhydrophenanthrene skeleton. An additional occurrence of such a nine-membered chain is shown in compound **12**, which contains the 2,2,4-trimethyltetrahydro-3aH-[1,3]dioxolo[4,5-c]pyran group in place of the usual carbohydrate moiety at the A ring. The descriptor xch9 takes a positive coefficient in component-1, indicating that larger values of xch9 are correlated with increased inhibition. Factors that alter the value of this descriptor in the context of cardiac

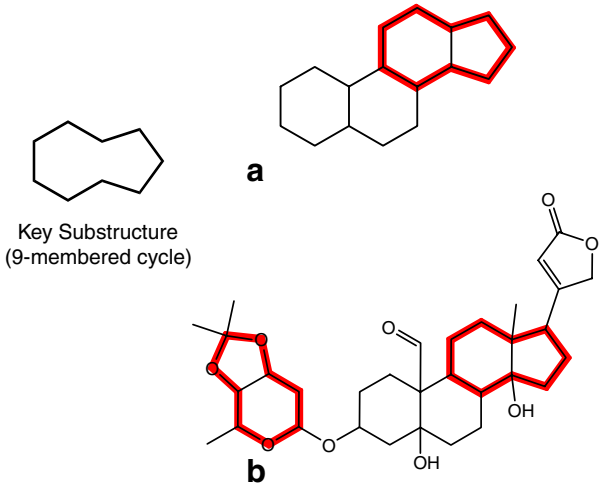


Figure 5. Instances of the occurrence of the substructure feature that is the focus of the 9th-order, chain molecular connectivity index (xch9). The primary instance in all structures is the C and D rings of the cyclopentanoperhydrophenanthrene core (a). Another instance is found in the group replacing the carbohydrate moiety at C-3 on the A ring (b) of compound **12**.

glycoside analogues are the number of instances of these nine-membered chains and the degree of substitution on the atoms involved. The primary role of xch9 is to discriminate between members of the training set that possess a hydroxyl group at C-12 of the C ring and those that do not. The model suggests that presence of the hydroxyl group in this position adversely affects Na/K-ATPase inhibition. This is consistent with observations made by others that demonstrate that the presence of a hydrophilic group at C-12 reduces potency[33] and is further supported by another report that suggests that the cyclopentanoperhydrophenanthrene core in the region of atoms 9, 10, 11, 12, and 13 interacts with the Na/K-ATPase through hydrophobic interactions.[34] The nelem descriptor in component-1 takes a negative weight indicating that increasing values of this descriptor are correlated with decreasing potency. The main role of this descriptor is to identify the structures containing large aromatic substituents at C-10, all of which carry the heteroatom nitrogen atom in the linker and therefore affect the value of nelem (see compounds **2** and **3**). These materials show diminished potency even though the rest of the structure is very similar to compounds with higher potencies. This suggests that there are limits to the size of the substituents allowed at this position, since smaller groups are tolerated (e.g., **8** and **12**). The last descriptor in component-1, C-027, takes a positive weight and discriminates between the five-membered furanone and the six-membered pyranone substituents at C-17. This descriptor suggests that, all other aspects of the structure being equal, the pyranone is preferred over the furanone (see bufalin and **8**).

3.2.2. Components 2 and 3. The second PLS component accounts for an additional 9.9% of the SAR (85.7% cumulative). The majority of the information in this component is supplied by a single descriptor, SHBint3 (70.3%), which is the sum of the hydrogen atom-type electrotopological-state index values for potential internal hydrogen bonds separated by three edges.[35] The structural feature that is the focus of this descriptor is illustrated in Figure 6. SHBint3 takes a positive weight in component-2, indicating that increased values of this descriptor correlate with increased potency. The role of SHBint3 is to provide a correction for structures whose potency is underestimated by component-1, which focuses primarily on the steroid core. In the context of the present training set, SHBint3 codes specifically for the presence and configuration of carbohydrate groups at C-3. Examples for structures most affected include evomonoside, digitoxigenin-bisdigitoxoside, digitoxigenin-monodigitoxoside, proscillaridin A, and compound **6**. It is frequently assumed that the primary benefit of the carbohydrate groups is improved bioavailability,[36] but this property is not accounted for by the bioassay used in this study (purified enzyme), suggesting that the carbohydrate portion indeed plays a small but noticeable role in the interactions between the molecule and the enzyme. This notion is in agreement with observations made by others.[34]

The third PLS component accounts for another 7.6% of the SAR (93.3% cumulative) and makes use mainly of

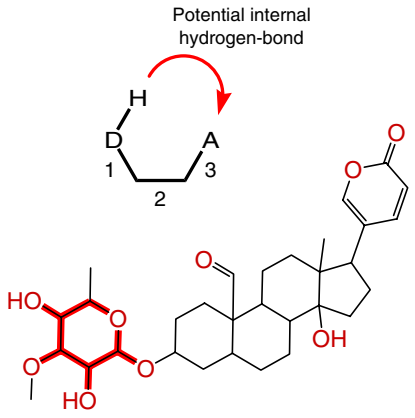


Figure 6. Illustration of the occurrence of the substructure feature that is the focus of the sum of the hydrogen atom-type electrotopological-state index values for potential internal hydrogen bonds separated by three edges (SHBint3). There are five instances of this substructure in the carbohydrate moiety in compound **8**, highlighted in red.

two descriptors, dxvp6 and Ui, which together provide 87.6% of the information contained in this component (34.2 and 53.4%, respectively). The dxvp6 descriptor is the differential 6th-order valence-corrected path connectivity index,[37] and the Ui descriptor is the unsaturation index.[31] The differential connectivity index focuses on the π and lone pair electrons in the structure, and the unsaturation index captures information regarding the presence of hetero atoms and bond types. The purpose of this component is to make further refinements of the SAR captured in component-1. For instance, the potency of **10** is underestimated in component-1 due to the presence of the nitrogen in the pyridine ring, which is corrected in component-3 by recognizing this particular group through the conjugated link to the aromatic ring. As can be seen for **2** and **3**, the presence of a large aromatic substituent containing a nitrogen atom does not always have a negative effect on potency, which is accounted for in this component. Another correction is made for dihydrodigoxin, which is missing the double bond typically present in the furanone ring on C-17 causing a decrease in potency.

3.3. Summary and future directions

The usefulness of the virtual screening protocol outlined in this study was demonstrated by the identification of four novel, highly potent Na/K-ATPase inhibitors from an initial pool of more than 37,000 compounds. In addition, the developed QSAR models provided informative structure–property trends for Na/K-ATPase inhibition, which can be of great value for the future development of new digitalis-based drugs for the treatment of CHF symptoms or for the design of a competitive digitalis antagonist. Since the latter requires compounds with distinct properties – high affinity for the enzyme's digitalis binding site and low inhibitory potency – the findings of this study suggest that future efforts could focus on cardiac glycoside analogues with substituents at C-10, C-12, and C-17 that interfere with inhibitory potency. To be of practical use, however, the present

search protocol for Na/K-ATPase inhibitors needs to be complemented by an analogous one capable of finding compounds with high binding affinity to the enzyme.

Finally, it should be noted that the predictive strength of the presented QSAR models for Na/K-ATPase inhibition is likely limited to molecules that bear a certain degree of structural similarity to those used to train the models. Since the training sets employed in this study represent only a small fraction of a rather large, structurally diverse group of potential inhibitors, the models could be improved and made more versatile by including a larger number of compounds in an iterative process. In each iteration, a small set of selected molecules would be tested and the newly obtained information could be incorporated in new, more comprehensive QSAR models.

4. Experimental

4.1. Materials

Inhibitors were obtained from InterBioScreen (IBS, Moscow, Russia) and used without further purification. Substrates and enzymes for the ATPase activity assays were received from Sigma–Aldrich (St. Louis, MO). All other compounds were from Fisher Scientific (Pittsburgh, PA).

4.2. Molecular modeling and descriptor calculation

The molecular structures of the compounds received from InterBioScreen were obtained in electronic form from the vendor database whereas the structures of cardiac glycosides used by Paula et al. were modeled as described in the original study.[7] Initial 3D atomic coordinates were generated with the Sybyl module Concord (Tripos, St. Louis, MO), followed by strain-energy optimization using the Tripos force field with the electrostatic terms included and partial atomic charges computed by the Gasteiger–Hückel method. The strain energy of the structures was then reoptimized in Spartan (version '02, Wavefunction, Irvine, CA) using molecular mechanics in conjunction with the MMFF force field. The resultant conformations were further refined by semi-empirical quantum mechanics (PM3) and all structures with calculated Mulliken partial atomic charges were exported from Spartan and stored in a Sybyl database for further use. It should be noted that the efforts to generate high quality 3D conformations were necessary for the calculation of the charged partial surface area (CPSA) descriptors, even though this particular descriptor class was eliminated later in the descriptor selection process.

A set of 25 CPSA descriptors was computed in Sybyl (Tripos, St. Louis, MO).[38] 173 additional descriptors such as atom centered fragments, empirical descriptors, BCUT descriptors, and other molecular connectivity indices were calculated with the program Dragon (version 3, Milano Chemometrics) and 363 additional descriptors were obtained with MC364 (Hall and Associates, Boston, MA), yielding a total of 561 descriptors for further analysis.

4.3. QSAR model development

Minitab (release 14.20, Minitab Inc., State College, PA) was used to identify and eliminate any descriptor from the set of 561 that yielded zero values for more than 90% (35) of the compounds. The remaining set of 189 descriptors was used for several rounds of QSAR model development with the program ADAPT employing a generalized simulated annealing approach.[28–30] Partial-least-squares analysis (PLS) with Minitab was used to ensure that the model was not overfitted, in which case the number of validated PLS components was lower than the number of descriptors used by the model. This protocol was followed for the development of both the initial and the final QSAR model. Once the final model had been obtained, PLS was used again to facilitate the physical interpretation of the underlying SAR.[32]

Similarity searches of the Natural Compound Database from IBS and database management were conducted with ChemFinder (version 9.0, CambridgeSoft, Boston, MA). Bioactivities of compounds that were considered potential inhibitors were calculated using the six descriptors relevant for the initial QSAR model.

4.4. Na/K-ATPase activity assays

The inhibitory potencies of cardiac glycosides were determined in a coupled ATPase activity assay with Na/K-ATPase from porcine cerebral cortex (Sigma–Aldrich) as described previously.[39] Even though Na/K-ATPase from this source is known to contain multiple isoforms of the enzyme, their variation in digitalis sensitivity was minor on the large inhibitor concentration range used in this study (up to six orders of magnitude).[40] Briefly, 88 μg of lyophilized Na/K-ATPase was incubated at 37 °C for at least 30 min in the assay buffer (100 mM NaCl, 45 mM histidine, 10 mM KCl, 10 mM $MgCl_2$, 1 mM phosphoenol pyruvate, and 225 μM NADH, pH 7.3) and a mixture of pyruvate kinase/lactate dehydrogenase (12 and 8.4 U/mL, respectively) at varying (10–14 data points) inhibitor concentrations (final volume: 1.2 mL). In a few instances, exposure of the enzyme to inhibitors for more than 30 min affected the measured inhibitory potency, in which case the samples were equilibrated overnight at room temperature before incubation at 37 °C. The reactions were initiated by adding 5 mM ATP to the reaction mixture and the velocity of the rate-limiting NADH oxidation was determined spectroscopically at 340 nm for five minutes with an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA) equipped with an automatic sample changer and thermostat (37 °C). A three-parameter logistic fit of the measured rates as a function of inhibitor concentration yielded the IC_{50} value which served as a quantitative measure for inhibitory potency. By definition, the IC_{50} was the concentration of inhibitor that reduced the enzyme activity to half of the activity observed in the absence of inhibitor. All experiments were performed at least in triplicate. For QSAR modeling,

the negative logarithm of RIP rather than the IC_{50} value was used. RIP was defined as the IC_{50} value of a compound divided by the IC_{50} value of the standard inhibitor ouabain obtained in reference measurements (469 nM for all compounds except for the measurements involving **2** and **3**, in which a slightly higher reference IC_{50} of 536 nM was obtained).

Acknowledgments

This work was supported by the Center for Integrated Natural Sciences and Mathematics at Northern Kentucky University (Grant 2005-12), Grant RSF-014-03 from Kentucky EPSCoR, and Grant 4800000011 from NSF.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.06.050.

References and notes

1. Li-Saw-Hee, F. L.; Lip, G. Y. *QJM-Mon. J. Assoc. Phys.* **1998**, *91*, 259.
2. Lip, G. Y.; Metcalfe, M. J.; Dunn, F. G. *Postgrad. Med. J.* **1993**, *69*, 337.
3. Erdmann, E. *Basic Res. Cardiol.* **2000**, *95*(Suppl. 1), I3.
4. Azrin, M. A. *Am. Heart J.* **1992**, *124*, 753.
5. Martiny, S. S.; Phelps, S. J.; Massey, K. L. *Crit. Care Med.* **1988**, *16*, 629.
6. Hickey, A. R.; Wenger, T. L.; Carpenter, V. P.; Tilson, H. H.; Hlatky, M. A.; Furberg, C. D.; Kirkpatrick, C. H.; Strauss, H. C.; Smith, T. W. *J. Am. Coll. Cardiol.* **1991**, *17*, 590.
7. Paula, S.; Tabet, M. R.; Ball, W. J., Jr. *Biochemistry* **2005**, *44*, 498.
8. Gupta, S. P. *Prog. Drug Res.* **2000**, *55*, 235.
9. Quadri, L.; Cerri, A.; Ferrari, P.; Folpini, E.; Mabilia, M.; Melloni, P. *J. Med. Chem.* **1996**, *39*, 3385.
10. Quadri, L.; Bianchi, G.; Cerri, A.; Fedrizzi, G.; Ferrari, P.; Gobbini, M.; Melloni, P.; Sputore, S.; Torri, M. *J. Med. Chem.* **1997**, *40*, 1561.
11. Cerri, A.; Almirante, N.; Barassi, P.; Benicchio, A.; De Munari, S.; Marazzi, G.; Molinari, I.; Serra, F.; Melloni, P. *J. Med. Chem.* **2002**, *45*, 189.
12. Cerri, A.; Almirante, N.; Barassi, P.; Benicchio, A.; Fedrizzi, G.; Ferrari, P.; Micheletti, R.; Quadri, L.; Ragg, E.; Rossi, R.; Santagostino, M.; Schiavone, A.; Serra, F.; Zappavigna, M. P.; Melloni, P. *J. Med. Chem.* **2000**, *43*, 2332.
13. Cerri, A.; Serra, F.; Ferrari, P.; Folpini, E.; Padoani, G.; Melloni, P. *J. Med. Chem.* **1997**, *40*, 3484.
14. Gobbini, M.; Barassi, P.; Cerri, A.; De Munari, S.; Fedrizzi, G.; Santagostino, M.; Schiavone, A.; Torri, M.; Melloni, P. *J. Med. Chem.* **2001**, *44*, 3821.
15. Repke, K. R.; Megges, R.; Weiland, J.; Schon, R. *FEBS Lett.* **1995**, *359*, 107.
16. Repke, K. R.; Schon, R.; Megges, R.; Weiland, J.; Nissen, E.; Matthes, E. *Anticancer Drug Des.* **1995**, *10*, 177.
17. Repke, K. R.; Sweadner, K. J.; Weiland, J.; Megges, R.; Schon, R. *Prog. Drug Res.* **1996**, *47*, 9.
18. Repke, K. R.; Weiland, J.; Megges, R.; Schon, R. *Prog. Med. Chem.* **1993**, *30*, 135.
19. Repke, K. R.; Weiland, J.; Megges, R.; Schon, R. *J. Enzyme Inhib.* **1996**, *10*, 147.
20. Templeton, J. F.; Kumar, V. P.; Bose, D.; LaBella, F. S. *J. Med. Chem.* **1989**, *32*, 1977.
21. Templeton, J. F.; Kumar, V. P.; Bose, D.; Smyth, D. D.; Kim, R. S.; LaBella, F. S. *Can. J. Physiol. Pharmacol.* **1988**, *66*, 1420.
22. Templeton, J. F.; Kumar, V. P.; Kim, R. S.; LaBella, F. S. *Steroids* **1987**, *49*, 383.
23. Templeton, J. F.; Ling, Y.; Kumar, V. P.; LaBella, F. S. *Steroids* **1993**, *58*, 518.
24. Templeton, J. F.; Ling, Y.; Marat, K.; LaBella, F. S. *J. Med. Chem.* **1997**, *40*, 1439.
25. Templeton, J. F.; Ling, Y.; Zeglam, T. H.; LaBella, F. S. *J. Med. Chem.* **1993**, *36*, 42.
26. Templeton, J. F.; Setiloane, P.; Kumar, V. P.; Yan, Y. L.; Zeglam, T. H.; LaBella, F. S. *J. Med. Chem.* **1991**, *34*, 2778.
27. Farr, C. D.; Burd, C.; Tabet, M. R.; Wang, X.; Welsh, W. J., ; Ball, W. J., Jr. *Biochemistry* **2002**, *41*, 1137.
28. Jurs, P. C.; Chou, J. T.; Yuan, M. In *Computer-Assisted Drug Design*; Olson, R. C., Christoffersen, R. E., Eds.; American Chemical Society: Washington, DC, 1979; p 103.
29. Luke, B. T. In *Genetic Algorithms in Molecular Modeling*; Devillers, J., Ed.; Academic Press: New York, 1996.
30. Stuper, A. J. J. P. C. *J. Chem. Inf. Comput. Sci.* **1976**, 99.
31. Todeschini, R. C. v. *Handbook of Molecular Descriptors*; Wiley-VCH: Weinheim, 2000.
32. Stanton, D. T. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 1423.
33. Brown, L.; Erdmann, E.; Thomas, R. *Biochem. Pharmacol.* **1983**, *32*, 2767.
34. Balzan, S.; D'Urso, G.; Ghione, S.; Martinelli, A.; Montali, U. *Life Sci.* **2000**, *67*, 1921.
35. Contrera, J. F.; Maclaughlin, P.; Hall, L. H.; Kier, L. B. *Curr. Drug Discov. Technol.* **2005**, *2*, 55.
36. Middleton, D. A.; Rankin, S.; Esmann, M.; Watts, A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13602.
37. Kier, L. B. H. L. H. *Quant. Struct.-Act. Relat.* **1991**, *10*, 134.
38. Stanton, D. T. *Anal. Chem.* **1990**, *62*, 2323.
39. Wallick, E. T.; Schwartz, A. *Methods Enzymol.* **1988**, *156*, 201.
40. Rose, A. M.; Qazzaz, H. M.; Zolotarjova, N.; Mellett, B. J.; Martin, A. W.; Valdes, R., Jr. *Clin. Chem.* **2000**, *46*, 234.