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Structure-based virtual screening for novel inhibitors of the sarco/endoplasmic reticulum calcium ATPase and their experimental evaluation

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ABSTRACT

A public compound library with 260,000 compounds was screened virtually by computational docking for novel inhibitors of the transmembrane enzyme sarco/endoplasmic reticulum calcium ATPase (SERCA). Docking was performed with the program GOLD in conjunction with a high resolution X-ray crystal structure of SERCA. Compounds that were predicted to be active were tested in bioassays. Nineteen novel compounds were discovered that were capable of inhibiting the ATP hydrolysis activity of SERCA at concentrations below 50 μM. Crucial enzyme/inhibitor interactions were identified by analyzing the docking-predicted binding poses of active compounds. Like other SERCA inhibitors, the newly discovered compounds are of considerable medicinal interest because of their potential for cancer chemotherapy.

1. Introduction

Due to their ability to trigger apoptosis, specific inhibitors of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) are of potential therapeutic value for the treatment of cancer.[1,2] SERCA is a calcium ion transport enzyme located in the membrane of intracellular storage compartments, such as the sarco- and endoplasmic reticulum (SR/ER).[3,4] Powered by the energy gained from the hydrolysis of ATP, SERCA transports calcium ions into intracellular stores. These stores act as sources for rapid calcium release through calcium-specific channels and ryanodine receptors, which constitutes an integral part of various signaling pathways. Inhibitors of SERCA's calcium transport function interfere with calcium homeostasis in cells, a condition known to induce apoptosis. The mechanism that ultimately causes apoptosis is complex, but most of the experimental evidence implicates the escape of calcium ions from the intracellular stores (SR/ER) as the main cause. This depletion leads to an increase in cytosolic calcium concentration and a concomitant decrease in calcium concentration in the SR, but only the latter appears to be responsible for triggering apoptotic pathways.[1]

The natural compound thapsigargin (TG) from *Thapsia garganica* is by far the most frequently used SERCA inhibitor,[5,6] but other compounds such as di-*tert*-butylhydroquinone (BHQ),[7–10] the fungal metabolite cyclopiazonic acid (CPA),[11–15] the antifungal drug clotrimazole,[16,17] thiouronium benzene derivatives,[18–20] terpenolides,[21,22] the endocrine disruptor 4-nonyl-phenol,[23] and the polyphenol curcumin[24,25] are also capable of inhibiting the enzyme. The inhibitory potencies of these compounds cover a broad range and are—in some cases—dependent on the particular isoform of SERCA they interact with.[25] To date, the most potent inhibitor is TG, which inhibits SERCA at subnanomolar concentrations, followed by CPA and BHQ, whose reported IC_{50} values of inhibition are approximately 120 nM and 400 nM, respectively. Among these compounds, derivatives of the small hydroquinone BHQ offer the advantage of being structurally simple and therefore relatively easily synthesized from inexpensive starting materials.[7] For medicinal chemists striving to synthesize new compounds with improved properties, this constitutes a considerable advantage over TG analog syntheses which require protection and deprotection of a fairly large number of functional groups.[26–29]

It has been demonstrated that both cancerous and healthy cells undergo apoptosis after exposure to TG at remarkably low concentrations, making this SERCA inhibitor a highly potent but rather nonselective cytotoxic agent. Whereas this lack of selectivity is thought to be advantageous when targeting prostate cancer cells with low proliferation rates,[30,31] the concomitant toxicity to healthy cells constitutes a major problem. This obstacle has been circumvented by connecting a specific peptide (His-Ser-Ser-Lys-Leu-Gln-Leu) to a linker attached to TG, which renders the inhibitor inactive.[30,32,33] However, prostate cancer cells produce on their

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surface the serine protease prostate-specific antigen (PSA), a well-known tumor marker, in concentrations much higher than found in healthy cells. PSA is capable of cleaving the peptide bond between Gln and Leu, thereby producing a TG analog that is an active SERCA inhibitor and thus toxic to the cancer cell. No other major proteases share PSA's hydrolytic specificity, which prevents premature inhibitor activation in healthy tissues.[34,35] Studies with PSA-producing human prostate cancer cell lines showed that treatment with a TG-based prodrug lead to complete arrest of tumor growth while healthy cells were spared, thereby underscoring the remarkable potential of SERCA inhibitors as novel anticancer drugs.[30]

In principle, the strategy of inactivating TG by attachment of a peptide should be applicable to the other classes of SERCA inhibitors as well. In fact, the availability of SERCA inhibitors other than TG may be a considerable advantage since they have different chemical structures and physicochemical properties, which will likely affect bioavailability once injected into an organism. Thus, having an arsenal of SERCA inhibitors with different pharmacokinetic and pharmacodynamic properties could become an important asset in potential in vivo trials. For this reason, the discovery of SERCA inhibitors that are either derivatives of known inhibitors or completely novel molecules will likely enhance the chances of developing this inhibitor class into useful anticancer drugs.

For the search for new bioactive compounds, high throughput screening (HTS) is a commonly employed method.[36] Almost exclusively used by pharmaceutical companies, HTS relies on high capacity, automated testing systems that allow for the rapid evaluation of millions of compounds in a short amount of time. Due to the requirements for highly specialized equipment, control software, large compound repositories, and the inherent high cost, HTS is typically not a viable option for academic research laboratories. In the recent past, however, virtual high throughput screening (vHTS) has emerged as a valuable alternative to HTS.[37,38] In vHTS, compounds are evaluated computationally prior to testing, typically by ligand docking (structure-based screening), pharmacophores, or quantitative structure–activity relationship models.[39] A prerequisite for structure-based screening is the availability of the three-dimensional structure of the target receptor, usually in the form of a high resolution X-ray crystal structure. The screening process often starts with a prefilter that eliminates from the library those molecules whose physical properties (solubility, size, etc.) likely prevent them from being bioactive. Next, the remainder of the library is docked computationally into the receptor binding site and the most favorable orientation and conformation of each molecule is predicted by scoring functions. The computed docking score provides a numerical measure for the predicted binding affinity, thereby permitting the identification of the most active compounds *in silico*.[40] As a result, experimental testing can be restricted to a relatively small selection of compounds that are expected to have the highest bioactivities. Since vHTS does not require the extensive resources of HTS, it is accessible for academic laboratories or small biotech companies with low- or medium-throughput screening capabilities.

In a recent study, we examined the molecular determinants of BHQ-mediated SERCA inhibition using computational docking in conjunction with a high resolution crystal structure of the enzyme.[7] We showed that docking routines can predict correctly the binding pose of BHQ in SERCA and that the correlation between docking score and experimentally determined bioactivity is sufficiently high to allow the distinction between active and inactive compounds in most cases. However, the study was limited to only 22 commercially available or synthesized compounds whose structures were closely related to that of BHQ. In the present study, we broadened the search for novel inhibitors by applying the developed docking protocol for vHTS to a compound library with approximately 260,000 entries maintained by the National Cancer Institute. Samples of the highest scoring molecules were requested for experimental testing. Nineteen novel SERCA inhibitors with potencies in the micromolar range were identified and their interactions with SERCA were analyzed at the molecular level.

2. Results

2.1. Initial screen of a compound library

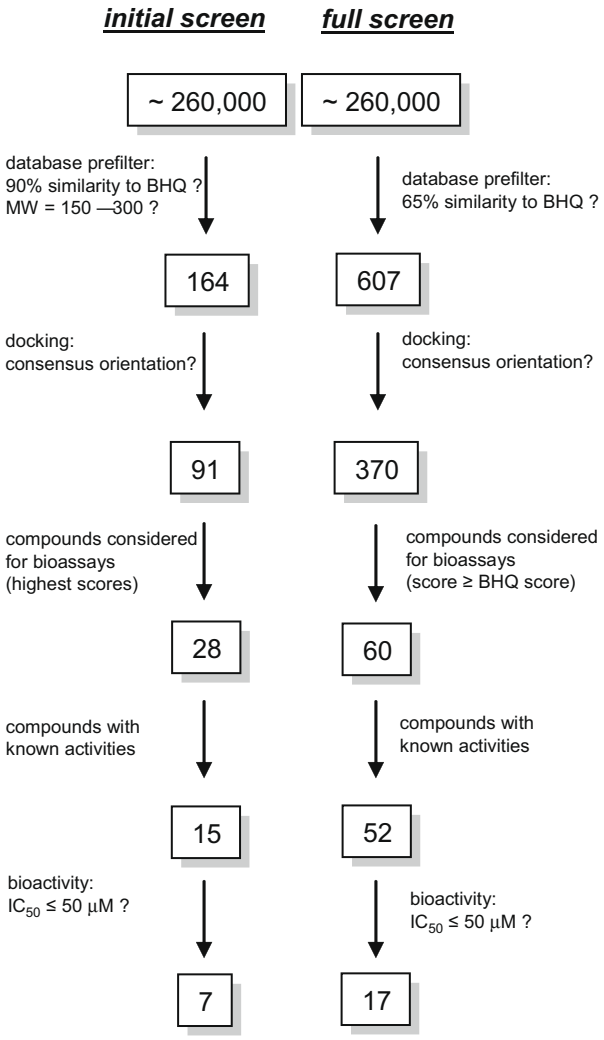
In order to test the usefulness of a previously developed computational docking protocol for structure-based virtual screening, it was applied to an initial screen of the National Cancer Institute's (NCI) 'Cactus' (Computer Assisted Drug Design Group's Chemoinformatics Tools and User Services) compound library. As the target receptor, the BHQ binding site in the crystal structure of the BHQ/TG/SERCA complex was used. This particular structure is representative of the enzyme in the E_2 conformation, which is known to be the relevant one for binding of BHQ and other inhibitors.[41] Since the pool of compounds (approximately 260,000 compounds at the time of access in 2008) was too large for docking each molecule individually within a reasonable amount of time, its size was narrowed significantly before applying the docking routine. After a similarity search (90% similarity to BHQ according to Tanimoto coefficients) and applying a molecular weight filter to account for the finite size of the binding site (MW = 150–300), the remaining 164 compounds were docked into the SERCA structure with the docking tool GOLD in conjunction with the scoring function ChemScore[42,43] as described previously.[7] Only docking runs that gave consensus orientations were considered reliable and considered for further analysis (91 compounds, see Supplementary data). A consensus orientation was defined as at least half of the docking-predicted poses deviating from each other by an RMSD value of no more than 2 Å. Among the 30 top-ranked compounds (14 scored higher than BHQ), two were redundant duplicate entries for BHQ and therefore eliminated and two others corresponded to active inhibitors that had been characterized previously (di-amylhydroquinone and 2,5-di-*tert*-butylphenol). Samples of the remaining 26 compounds were requested from NCI and 13 were received for experimental evaluation in inhibition assays. The overall selection process is summarized in Scheme 1.

2.2. Full virtual library screen

Next, we attempted to broaden the structural diversity within the set of SERCA inhibitors by widening the pool of compounds to be evaluated by docking. For this purpose, a second, more comprehensive screen was conducted in which the similarity threshold in the prefilter was reduced from 90% to 65% and the molecular weight restriction was omitted (Scheme 1). Subsequent docking was conducted with the resultant pool of now 607 compounds using exactly the same protocol as the one in the initial screen. Among the 370 compounds (see Supplementary data for identities) with consensus poses, 60 scored better than BHQ (ChemScore $\geqslant$ 27 kJ/mol) and were considered for further analysis. Among these compounds, one had been characterized previously as active (di-amylhydroquinone) and three others had been already identified in the initial screen (compounds **1a**, **1b**, and 2-phenyl-2′-*para*-phenolpropane). The remaining 56 materials were requested and 48 were received for experimental evaluation.

2.3. Determination of inhibitory potencies in ATPase activity assays

The inhibitory potencies of compounds were determined in a commonly used ATPase activity assay that couples the rate of SERCA-catalyzed ATP hydrolysis to the oxidation of NADH. Typical



Scheme 1. Flow chart describing the selection procedure employed for the two library screen.

inhibition curves obtained at varying inhibitor concentrations for BHQ, **6a**, and **7a** are depicted in Figure 1 (left panel) and the obtained inhibitory potencies are listed in Table 1. By convention, only compounds with IC_{50} values below 50 μM were labeled as 'active' because potencies below this threshold were considered not useful for practical purposes. According to this criterion, five of the thirteen compounds from the initial screen were active (not including the two previously identified inhibitors) and 14 compounds from the second screen were active (not including di-amylhydroquinone and compounds **1a** and **1b**; Table 1). It should be noted that some of the 'inactive' compounds showed measureable SERCA inhibition activity at concentrations above 50 μM, but were excluded from further consideration due to their low potencies and their potential for non-specific interactions.

In order to assure that an inhibitor was truly interfering with the ATPase activity of SERCA and not with the two other assay enzymes (pyruvate kinase and lactate dehydrogenase), a second assay was employed that monitored directly the production of inorganic phosphate via its reaction with the dye malachite green. Since this second assay required more materials and was more time-consuming, it was conducted only at three inhibitor concentrations to confirm or rule out SERCA inhibition by a compound that had tested positive in the coupled assay (Fig. 1, right panel). A compound's inhibitory activity was considered to be confirmed if the SERCA ATPase activity was inhibited clearly (more than 50%) at the highest of the three inhibitor concentrations. With one exception (NCI compound number 310835), the inhibitory activities of all compounds classified as 'active' according to the coupled assay were confirmed by the results of the malachite green based assay.

An inspection of the structures tested after the initial screen (Fig. 2) revealed that this set of compounds could be divided into one subgroup and two structurally unique molecules. The subgroup comprised three molecules with a relatively high resemblance to BHQ, with at least one hydroxyl group and at least one bulky, hydrocarbon side chain at the central phenyl ring. All three compounds displayed high inhibitory potencies, particularly the mono-phenylated hydroquinone **1c**, which had an IC_{50} of 2.3 μM. Compound **2** possessed two phenol moieties linked via a central quaternary carbon atom whereas compound **3** consisted of two condensed rings of five and six carbon atoms, respectively.

As expected, the compounds identified by the full screen (Fig. 3) displayed a lower resemblance to the parent compound BHQ and were structurally more diverse. Groups 4 and 5 were similar to group 2 from the initial screen by featuring two connected phenyl rings whereas the hydroquinone derivatives present in group 6 resembled those in group 1. SERCA inhibitors with completely novel molecular scaffolds were represented by group 7, which consisted of molecules with two connected naphthalene rings, and by compound **8**, a somewhat larger version of groups 2, 4, and 5 compounds which featured two substituted phenyl rings connected by an ethylene bridge. Finally, compounds **9**–**11** were structurally unique and could not be assigned to any of the larger groups for that reason. Among them was **10**, an isomer of 4-*n*-nonylphenol, which is a hormonally active agent and known SERCA inhibitor with moderate potency (IC_{50} = 7 μM).[23] It should be emphasized that—overall—the chemical structures of many of these compounds displayed relatively little structural resemblance to BHQ or any other known class of SERCA inhibitors.

3. Discussion

3.1. Molecular interactions crucial for SERCA inhibition

For a comprehensive account of SERCA inhibition by small molecules, detailed knowledge of the intermolecular interactions between the enzyme and the inhibitors is required. This is usually accomplished by analysis of X-ray crystal structures of SERCA/inhibitor complexes, provided that such structures have been determined. Even though SERCA is one of the few transmembrane proteins for which high quality crystal structures are available in multiple conformations, only three structures with co-crystallized inhibitors (TG, BHQ, and CPA) have been solved to date and none of them contain the inhibitors tested in this study.[41,44,45] In the absence of such crystallographic information, docking-predicted inhibitor poses are a viable alternative that can provide the much-needed insight into the molecular details of inhibitor binding to SERCA.[7,46,47]

For the sake of simplicity, the following discussion of docking-predicted enzyme/inhibitor interactions will be restricted largely to a few compounds that represent larger groups with similar chemical structures. As representatives of groups 1 and 6, both of which contain compounds with a single phenol ring, compounds **1a** and **6b** were chosen. Compound **5a** represents the compound groups with two phenyl rings connected by a methylene or an ethylene bridge (groups 4, 5, and compound **8**) and **7a** is one of the two materials with two naphthalene groups.

In order to facilitate a straightforward and precise description of crucial enzyme/inhibitor interactions, the binding pocket of SERCA

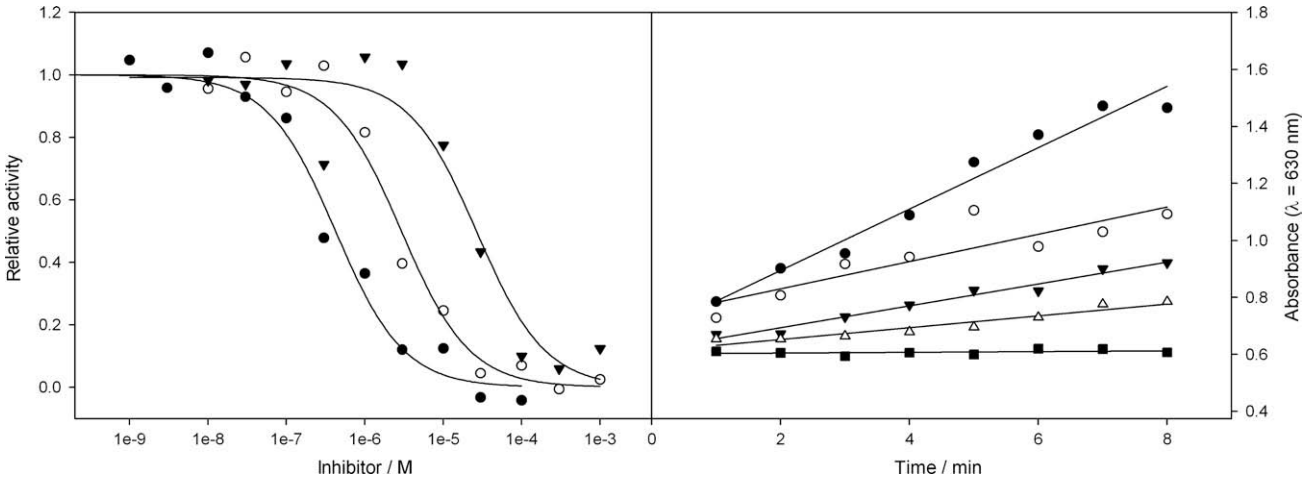


Figure 1. Representative results of inhibition assays. Left panel: results of a coupled ATPase activity assay in the presence of inhibitors at varying concentrations (●: BHQ; ○: compound **6a**; ▼: compound **7a**). Right panel: results of an ATPase activity assay with the dye malachite green at three BHQ concentrations (●: no inhibitor; ○: 40 nM; ▼: 400 nM; △: 4 μM; ■: control/no enzyme).

Table 1
Identities and experimentally determined inhibitory potencies of 19 novel SERCA inhibitors

Compound #	Cactus ID	IC_{50} (μM)
1a	68832	18.8 ± 11.6
1b	99308	11.5 ± 4.6
1c	407988	2.29 ± 1.79
2	73730	19.5 ± 15.9
3	33084	28.1 ± 8.9
4a	321572	33.8 ± 13.9
4b	321575	35.3 ± 16.5
4c	321583	45.4 ± 18.5
5a	48161	11.8 ± 8.1
5b	56592	49.7 ± 15.4
5c	73728	39.3 ± 35.0
6a	86636	9.4 ± 7.7
6b	99303	20.2 ± 7.9
7a	143535	27.3 ± 15.5
7b	5992/402620	15.0 ± 8.6
8	35752	28.3 ± 16.3
9	362474	23.8 ± 4.5
10	664154	17.2 ± 2.6
11	284506	21.3 ± 10.8

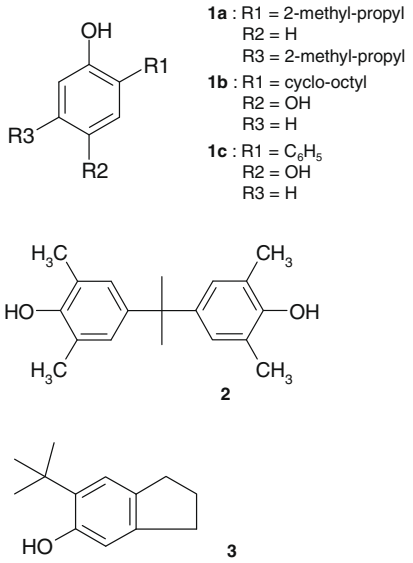


Figure 2. Chemical structure of active SERCA inhibitors identified by the initial library screen.

was subdivided into four regions (Fig. 4A and B). Area S1 has a predominantly hydrophobic character that is lined by residues Ile97, Leu98, Asn101, Val62, Leu65, and Asp59. In the SERCA/BHQ complex, S1 is occupied by one of BHQ's *tert*-butyl groups. S2 is the region located in the center of the binding site and formed by residues Leu61, Glu309, Pro308 and Pro312. Its moderately hydrophobic character is well-suited to accommodate the phenyl ring of BHQ. Residues Ile315, Leu311, Phe256, and Leu253 form the hydrophobic area S3, which is the location at which the second *tert*-butyl group of BHQ binds. The small area S4 is technically not part of the BHQ binding pocket since it is located at the site's entrance from the solvent-exposed area. S4 is only of significance for some of the larger inhibitors which protrude from the binding pocket and can form a hydrogen bond with residues Asp254 or Gln250.

Compounds **1a** and **6b** bear considerable similarity to the parent compound BHQ in that they both possess one or two hydroxyl groups and bulky, hydrophobic substituents at the phenyl ring, respectively. Not surprisingly, the binding poses predicted for these compounds strongly resemble that observed for BHQ (Fig. 4C). For example, the two alkyl substituents of **1a** occupy S1 and S3, thereby placing the central phenyl ring in S2. In addition to extensive hydrophobic contacts, this pose is stabilized by a hydrogen bond between the hydroxyl group of the inhibitor and Asp59. Compared to **1a**, the binding pose of the monoalkylated compound **6b** is somewhat shifted. Due to the large size of the benzyl moiety residing in S3, the central phenyl ring is pushed partially out of S2 towards S1 (Fig. 4D). As a result, this compound can form a second hydrogen bond to Asp309 in addition to the one with Asp59. An almost identical scenario is encountered with **1b**, which forms the same hydrogen bond pattern and whose cyclooctyl group is positioned at the site of the benzyl group of **6b**.

A second, quite different binding mode is predicted for compounds with two or more phenyl groups, such as **5a**. This pose can be described as the two phenyl groups being located in S2 and S3, while the area around S1 is occupied by the *tert*-butyl group (Fig. 4E). The finite size of the binding pocket forces the planes of the two phenyl rings to be approximately perpendicular to each other. This peculiar 90° bend is also predicted for compound **8** and almost all molecules from groups 4 and 5. The large

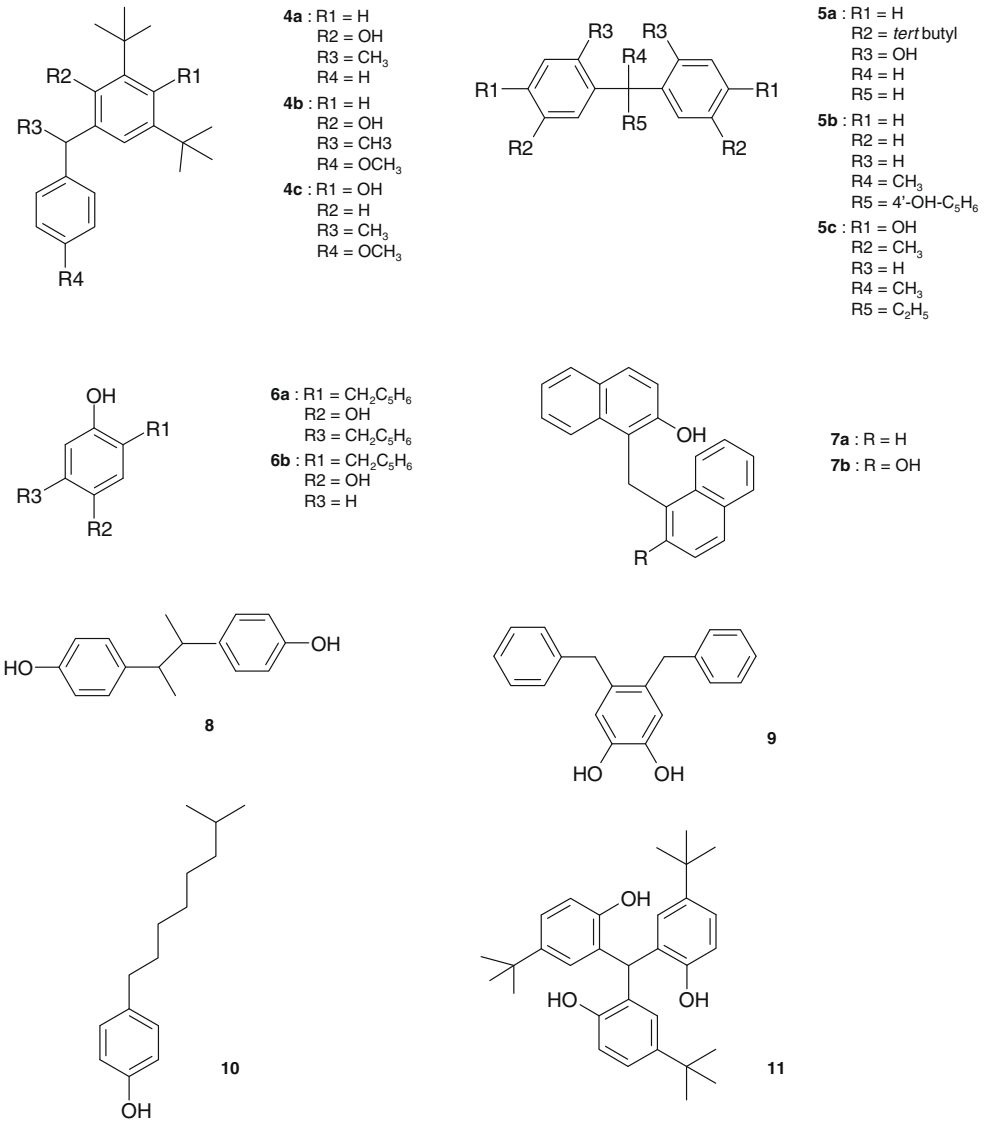


Figure 3. Chemical structure of active SERCA inhibitors identified by the full library screen.

size of these inhibitors pushes the second phenyl ring out of the BHQ binding site toward the solvent-exposed area and permits the formation of a hydrogen bond with Asp245 in S4 at the binding site entrance.

Compound **7a**, one of the two dinaphthalenes, is predicted to bind to SERCA via hydrophobic interactions between one of its naphtyl groups and S2 and S3. In addition, the second naphtyl group of **7a** occupies the area at the binding site entrance and its hydroxyl group is engaged with Asp254 in S4 (Fig. 4F). Among the set of structurally unique molecules, **10** may be of the greatest interest since it is very similar to a known SERCA inhibitor *n*-nonylphenol. The entire hydrophobic tail of **10** is predicted to occupy the binding site whereas its hydroxyl group is engaged in a single H-bond with Asp254 (Supplementary data).

Even though the inhibitors described above are structurally diverse, they appear to employ a similar overall binding strategy that matches their hydrogen bonding and hydrophobic profiles with that of the binding site. According to the breakdown of individual binding energy terms as predicted by the ChemScore scoring function, hydrophobic interactions between bulky hydrophobic moieties and the regions S1 and S3 provide most of the binding energy. In many cases, this large and favorable hydrophobic term is complemented by one to two hydrogen bonds that involve almost exclusively residues Asp59, Asp 254, Pro308, and Glu309.

3.2. Evaluation of the docking protocol for structure-based searches for novel SERCA inhibitors

The discovery of a total of 19 novel SERCA inhibitors, some of which have potencies in the low micromolar range, demonstrates the potential of structure-based vHTS protocols for the purpose of drug discovery. In particular, the discovery of new inhibitor scaffolds may be of value for future synthetic work aimed at modifying inhibitor structure to enhance bioactivity. In this context, the identification of compound groups 4, 5, and 7 as potent SERCA inhibitors is significant because their structural similarity to BHQ is only remote. Most likely, these classes would have been overlooked in a manual search for BHQ-based inhibitors, thus illustrating the value of unbiased computational methods for database searches.

In light of a previously proposed preliminary pharmacophore,[7] the observation that the monosubstituted BHQ analogs **1b** and **1c** are inhibitory was somewhat surprising. We previously postulated that one hydroxyl group and two *para*-disposed bulky substituents at the phenyl ring were a minimal requirement for potency.

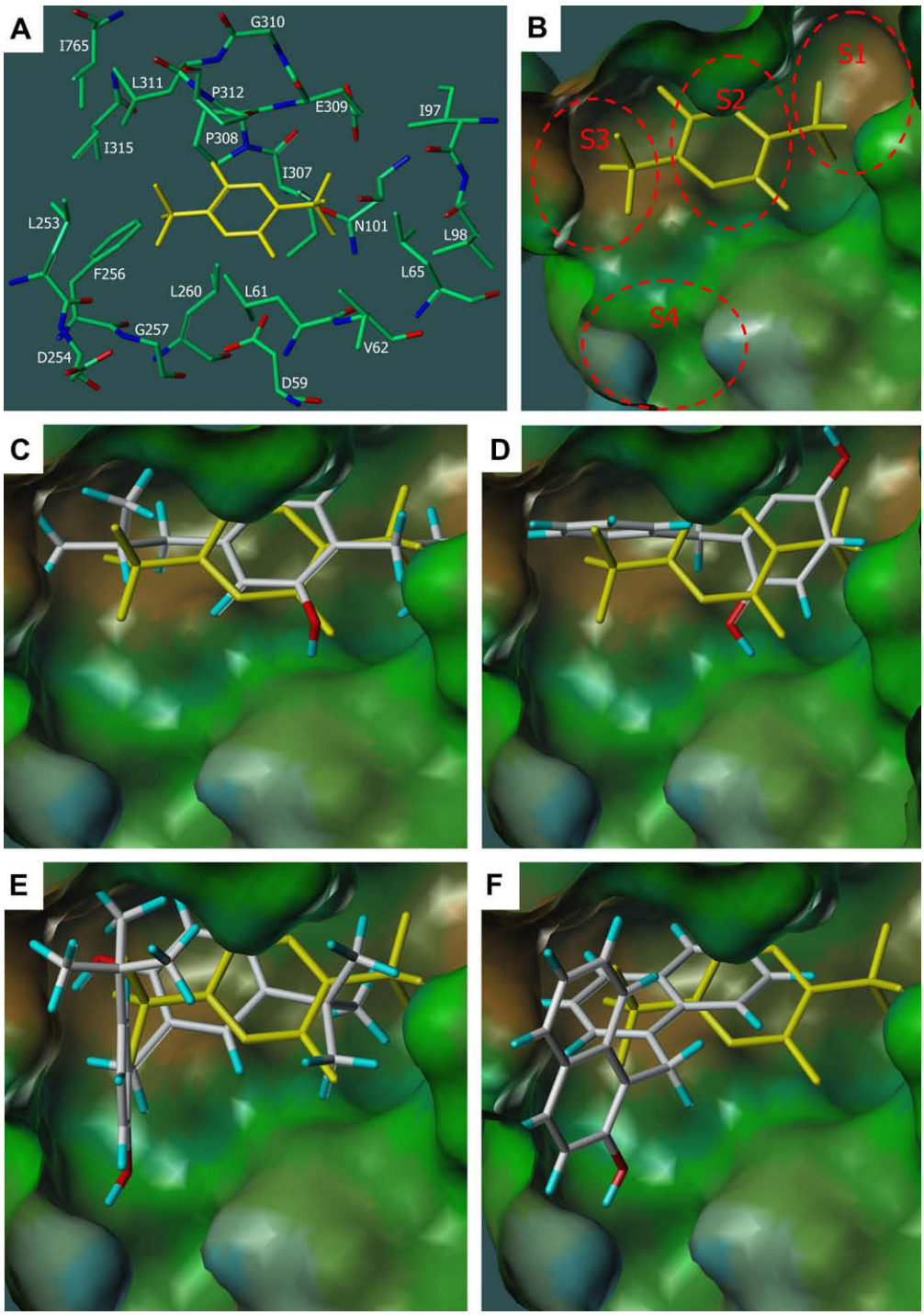


Figure 4. Structure of the BHQ binding site and docking-predicted inhibitor poses of representative inhibitors in the SERCA structure. (A) Stick representation of residues in direct proximity of BHQ (6 Å). (B) Hydrophobic potential (brown: most hydrophobic, blue: most hydrophilic) mapped onto the MolCad (Connolly) surface of the binding site. The binding site regions S1–S4 are specified by red circles. C–F. Selected inhibitors are shown as stick models and the pose of BHQ as seen in the crystal structure is shown as a reference (yellow). Displayed inhibitors are compounds **1a** (C), **6b** (D), **5a** (E), and **7a** (F).

Apparently, this requirement is not as stringent as originally assumed and it appears plausible that the presence of a single, cyclic substituent is sufficient to convey inhibitory potency.

Among the 60 compounds that were classified as 'interesting' in the full virtual screen, three were previously described active SERCA inhibitors, one was known to be inactive, and 19 of the 48 tested were classified as 'active' in the bioassays. This formally translates into a success rate of 33% (14 + 3 'actives' out of 48 + 4 total compounds with known activities), but since some of the compounds requested from NCI for testing were unavailable, this number should be considered as an estimate. A similar calculation for the initial screen yields a success rate of 47% (7 'actives' among 15 compounds with known activities). Even though traditional HTS procedures and some vHTS protocols cover much more chemical space, the achieved hit rates of over 30% are certainly remarkable.

For the improvement of future virtual screens, the current protocol could be altered in several ways. For instance, the prefilter

could be conducted using criteria such as solubility or drug-likeness rather than being based on structural similarity to BHQ. Such an approach would likely further increase the structural diversity of compounds selected for testing and would have the potential of including molecules whose structure is completely unrelated to BHQ. In addition, lowering the cutoff threshold of the prefilter and thereby increasing the number of compounds to be docked from several hundred to thousands would allow for a more thorough exploration of chemical space (at the expense of computation time). In fact, the number of compounds evaluated computationally in the present study is relatively small compared to other studies in which a less stringent prefilter was employed[48,49] or was omitted altogether.[50–52] In order to preserve time and resources, the compounds in the screening library could be clustered in groups of similar structure prior to docking and only a fraction of each group with a few representative molecules would need to be subjected to computational evaluation in the subsequent step. Another area for potential improvement relates to the docking algorithm itself. Until recently, even the most sophisticated docking routines allowed complete conformational flexibility of the ligand, but only limited flexibility of the receptor. For example, the settings of GOLD used in this study permitted only the torsion angles of terminal hydroxyl and amino groups of Ser, Tyr, Thr, and Lys to be conformationally flexible for optimizing hydrogen bond geometry while the rest of the binding site was static. The latest versions of GOLD and Autodock[53,54] show great improvement with regard to receptor flexibility in that they now permit almost complete flexibility of the binding site. Even though the enhanced conformational flexibility requires greatly increased computation times, it has the considerable advantage of being able to account for so-called 'induced fits'.[55] An induced fit is a phenomenon that causes the receptor in the presence of a ligand to adopt a conformation that differs from that observed in the absence of the ligand. In some cases, these conformational changes are of considerable magnitude so that their neglect can severely impact the accuracy of docking-based predictions.

3.3. Future design of SERCA inhibitors with high potencies

The virtual or experimental screening of compound libraries for molecules with desired bioactivities often constitutes the first major step in drug discovery programs. After the identification of lead compounds, their properties are improved upon by synthetic modifications. In this context, the SERCA inhibitors identified by the vHTS protocol of this study can be regarded as molecules whose potential as therapeutic agents would certainly benefit from a further enhancement of their bioactivities, even though some of them already are remarkably potent. Whereas the 2,5-disubstituted hydroquinones in groups 1 and 5 display the largest potencies, the relatively small size of these molecules somewhat limits opportunities for structural modifications aimed at further improving their binding affinity to SERCA. In contrast to the hydroquinones, some of the larger compounds in groups 4, 5, and 7 offer more options for synthetically tractable structural modifications. Some of these compounds already have good potencies against SERCA activity (IC_{50} values in the low micromolar range) that could be improved further by increasing the strength of the inhibitor/enzyme interactions. For instance, the creation of one additional hydrogen bond by the attachment of a hydroxyl or carbonyl group would add an increment of 10–20 kJ/mol to the binding energy, which is expected to decrease the IC_{50} value into the nanomolar range. Based on the analysis of the docking poses, residues Asp59, Asp 254, Pro308, and Glu309 are the most promising candidates as potential partners for additional hydrogen bonds. In particular Asp254 positioned at the entrance of the binding site seems to be capable of interacting with the larger compounds, a possibility that is not available for the aforementioned BHQ analogs which are too small to reach out to this area.

4. Computational and experimental procedures

4.1. Receptor modeling and computational ligand docking

The three-dimensional molecular structures of compounds were downloaded from the NCI/'Cactus' library (Computer Assisted Drug Design Group's Chemoinformatics Tools and User Services) at http://cactus.nci.nih.gov/ and used without further manipulation. The crystal structure of the SERCA/BHQ complex was obtained from the Protein Databank (2AGV) and prepared for docking as described in a previous study.[7] Docking was performed with version 3.1 of the program GOLD (Genetic Optimisation for Ligand Docking, Cambridge Crystallographic Data Centre, UK) using the ChemScore scoring function.[43] The genetic algorithm was executed at the default settings and the docking sphere had a radius of 15 Å centered at the (deleted) C-1 phenyl carbon (atom number 15395) of BHQ in the SERCA/BHQ complex.

For the analysis of intermolecular interactions, the descriptor calculation feature of Hermes, a GOLD utility, was used. Using the default settings, residues involved in hydrogen bonds, close steric contacts, and hydrophobic interactions were identified.

4.2. Materials

SERCA microsomes from rabbit hind leg tissue were prepared by a standard procedure.[3,7] Pyruvate kinase and lactate dehydrogenase were received from Sigma–Aldrich (St. Louis, MO) and all other reagents required for the assays were from Fisher Scientific (Pittsburgh, PA). Upon request, small samples of potential inhibitors to be tested in bioassays were kindly supplied by Dr. Ven Narayanan at the National Cancer Institute Developmental Therapeutics Program Open Chemical Repository (http://dtp.nci.nih.gov). The chemical identities of the materials were confirmed by ^{1}H NMR using deuterated chloroform or DMSO as a solvent. Only samples whose NMR spectra were in agreement with their chemical structures were used in bioassays without further purification.

4.3. Coupled ATPase activity assay

The SERCA-catalyzed rate of ATP hydrolysis coupled to the oxidation of NADH by the action of the enzymes pyruvate kinase and lactate dehydrogenase was measured spectroscopically at a wavelength of 340 nm. The technical details of this assay have been described in a previous study[7] and the only modification was the reduction of the total sample volume from 1.25 mL to 225 μL to conserve sample materials. Samples were prepared in a 96-well polystyrene plate that was read by a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) for a duration of 5 min. Inhibitory potency was expressed in terms of the IC_{50} value, which was the inhibitor concentration required to reduce SERCA activity by 50%. IC_{50} values were obtained from fitting the experimental data to a three parameter logistic equation and are the averages of at least three independent repeats.

4.4. ATPase activity assay with malachite green

In order to confirm inhibitory potency, a second independent ATPase activity was conducted that was based on the colorimetric reaction between inorganic phosphate and the dye malachite green. In a 96-well polystyrene plate, 10 μL of a 0.1 mg protein/mL SERCA solution (in 10 mM EGTA) was added to a buffer solution

composed of 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 4.5 μM calcimycin, 0.7 mM $CaCl_2$, and 20 mM Trizma (pH 7.5) to give a total volume of 112.5 μL. SERCA-catalyzed ATP-hydrolysis was initiated by the addition of 112.5 μL 0.45 mM ATP (total sample volume: 225 μL) and stopped after well-defined delay times (1 min intervals up to 10 min) by the addition of a solution containing malachite green (3 mM), sodium molybdate (10 mM), Triton-X-100 (0.05%), and 0.7 M HCl. The formation of the dye/phosphate complex was detected by reading the absorbances of the samples at a wavelength of 630 nm.[56] Reaction rates were obtained by linear regression. Since this assay was more time-consuming and required more material than the coupled assay, ATP hydrolysis rates were measured only at three inhibitor concentrations to confirm or rule out SERCA inhibition. The three concentrations for each inhibitor were centered about the IC_{50} value from the coupled assay and differed from each other by approximately one order of magnitude.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.010.

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