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Design, synthesis, and biological evaluation of hydroquinone derivatives as novel inhibitors of the sarco/endoplasmic reticulum calcium ATPase

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ABSTRACT

Analogues of the compound 2,5-di-*tert*-butylhydroquinone (BHQ) are capable of inhibiting the enzyme sarco/endoplasmic reticulum ATPase (SERCA) in the low micromolar and submicromolar concentration ranges. Not only are SERCA inhibitors valuable research tools, but they also have potential medicinal value as agents against prostate cancer. This study describes the synthesis of 13 compounds representing several classes of BHQ analogues, such as hydroquinones with a single aromatic substituent, symmetrically and unsymmetrically disubstituted hydroquinones, and hydroquinones with ω-amino acid tethers attached to their hydroxyl groups. Structure–activity relationships were established by measuring the inhibitory potencies of all synthesized compounds in bioassays. The assays were complemented by computational ligand docking for an analysis of the relevant ligand/receptor interactions.

1. Introduction

Among known inhibitors of the enzyme sarco/endoplasmic reticulum calcium ATPase (SERCA), the natural product thapsigargin (TG) has found widespread use, primarily as a tool for the study of the enzyme's physiological functions but also as the parent molecule for the synthesis of prodrugs capable of specifically targeting prostate cancer cells.[1–5] Inhibition of the ion transport activity of SERCA interferes with calcium homeostasis in cells, which ultimately leads to apoptosis. Due to its high potency and specificity, TG is by far the most commonly used SERCA inhibitor. As a natural product of considerable structural complexity, TG is not easily synthesized, even though its total synthesis has been reported.[6–8] As a result, the main source of TG remains the Mediterranean plant *Thapsia garganica*, from which TG can be isolated in relatively small quantities, making it a rather expensive agent.[9]

Besides TG, other compounds are also capable of inhibiting SERCA. Examples include the fungal metabolite cyclopiazonic acid,[10,11] the antifungal drug clotrimazole,[12] thiouronium benzene derivatives,[13] terpenolides,[14,15] the flame retardant tetrabromobisphenol,[16,17] and polyphenols such as curcumin.[18,19] In addition, the small compound 2,5-di-*tert*-butylhydroquinone (BHQ) and some of its derivatives have been shown to inhibit SERCA at concentrations in the upper nanomolar and low micromolar concentration ranges.[20–23] Because of their small size, these compounds can be prepared from inexpensive starting materials in only a few synthetic steps. This constitutes a considerable advantage over TG-based inhibitors, whose complex total synthesis entails 42 steps, has an overall yield of 0.6%, and requires the use of multiple protecting groups.[7]

Whereas a number of structure/activity relationship (SAR) studies are available for SERCA inhibition by TG,[24–26] this type of information for BHQ-mediated inhibition is far more limited. In a few previous studies, we and others started investigating systematically the molecular determinants of SERCA inhibition by BHQ and its analogues.[20,27–29] To this end, the most potent compounds capable of inhibiting in the submicromolar concentration range are hydroquinones with two identical bulky alkyl residues in positions 2 and 5. In a recently conducted structure-based virtual screen, we identified several novel monosubstituted hydroquinones carrying aryl groups that displayed inhibitory potencies in the low micromolar range.[27] This was a somewhat surprising finding since previous evidence had suggested that two substituents were an absolute requirement for inhibitory potency.[20] Even though the SAR obtained in the study above[27] provided valuable insights into SERCA/inhibitor interactions, it was limited by the availability of compounds.

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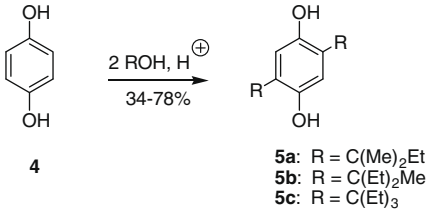
In an effort to overcome this limitation and to gain further knowledge about the structural requirements for effective SERCA inhibition by hydroquinones, we utilized several synthetic routes to prepare a total of 13 hydroquinone derivatives representative of several structurally distinct classes. For instance, we synthesized a series of symmetrical, di-alkylated hydroquinones in order to explore the effects of size and chemical composition of the substituents attached to the central phenyl group on inhibitory potency. Prompted by the abovementioned discovery that monosubstituted hydroquinones are also capable of inhibiting SERCA, we prepared hydroquinones with single, aromatic substituents of varying size and polarity. In addition, we synthesized several unsymmetrical 2,5-disubstituted hydroquinones, a compound class whose ability to act as SERCA inhibitors is completely unexplored to date. An interesting aspect of this class of compounds relates to their reduced structural symmetry, a property that might translate into enhanced specificity for SERCA and reduce the likelihood of interactions with other target receptors. Finally, we synthesized BHQ derivatives with one or two tethers attached to their hydroxyl groups. These compounds are of particular interest since the BHQ hydroxyl groups can become anchor points for the attachment of moieties that could convey specific properties to the inhibitor. For example, the inhibitor could be tethered to a short peptide that creates specificity for prostate cancer cells in a fashion already established for TG-based prodrugs.[4] Alternatively, a BHQ derivative could be linked to a fluorescent group, yielding a relatively inexpensive marker that would facilitate SERCA localization and imaging studies similar to the ones now routinely conducted with labeled TG analogues.[30,31] Finally, the close proximity of the BHQ and TG binding sites has lead to the suggestion to design dual inhibitors capable of binding to both sites, yielding novel compounds with potentially superior inhibitory properties.[32]

2. Results and discussion

2.1. Synthesis of hydroquinone derivatives

Monosubstituted hydroquinones (**2a**–**d**) were prepared following the procedure of Ozaki et al.[33] Reaction of 1,4-cyclohexanedione and an appropriate aldehyde in the presence of lithium chloride either in 1,3-dimethyl-2-imidazolidinone (DMI) at 160–170 °C or in refluxing pyridine gave **2a**–**d** in low to moderate yields (Scheme 1). Not unexpectedly, the hydroquinones prepared in this study darkened after several hours of exposure to the atmosphere due to oxidation. All new compounds were characterized by ^{1}H- and ^{13}C-NMR and by high resolution mass spectrometry. Of the four monosubstituted hydroquinones prepared, compound **2b** was the most active in bioassays (vide infra) and was therefore selected to be the starting material to react with alcohols under acidic conditions to furnish unsymmetrically disubstituted hydroquinones (**3a**,**b**). In addition to the unsymmetrically disubstituted hydroquinones, three symmetrically disubstituted hydroquinones (**5a**–**c**) were prepared from hydroquinone (**4**) by reaction with



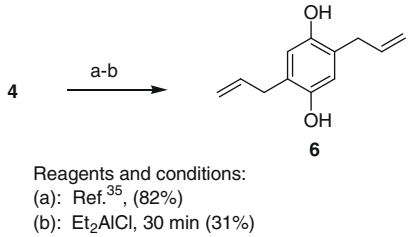
Scheme 2. Synthesis of symmetrically di-alkylated hydroquinones.

slightly more than 2 equiv of alcohol under acidic conditions (Scheme 2). A preliminary account of the synthesis of this compound class has recently been published.[20] The known 2,5-diallylhydroquinone **6**[34] was prepared by a modified procedure. Reaction of 1,4-bis-allyloxybenzene[35] with diethyl aluminum chloride in hexane–heptane for 30 min at room temperature, followed by quenching with diluted aqueous HCl,[36] gave a 31% yield of **6** (Scheme 3).

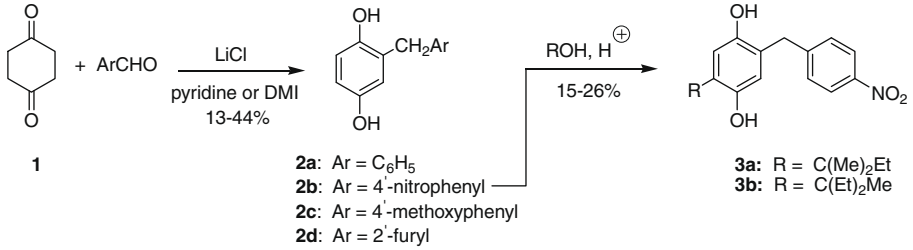
The next goal was to test the feasibility of the attachment of a linker to BHQ and/or its analogues that could potentially facilitate the conversion of non-specific SERCA inhibitors into prodrugs with specificity for prostate cancer cells in a fashion similar to work done with TG-based compounds.[4] Coupling of BHQ (**7**, Scheme 4) with 1 M equiv of 4-(*tert*-butoxycarbonylamino)butyric acid in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) gave as the major product the bis-acylated compound **8a**. When the ratio of **7** to amino acid, DCC, and DMAP was changed to 3:1, the desired mono-acylated product **8b** was obtained, albeit in low yield. Removal of the *tert*-butoxycarbonyl (Boc) protecting group from **8b** to afford **9** was accomplished in 78% yield using trifluoroacetic acid (TFA) in methylene chloride.

2.2. Potencies and structure–activity relationships of synthesized inhibitors

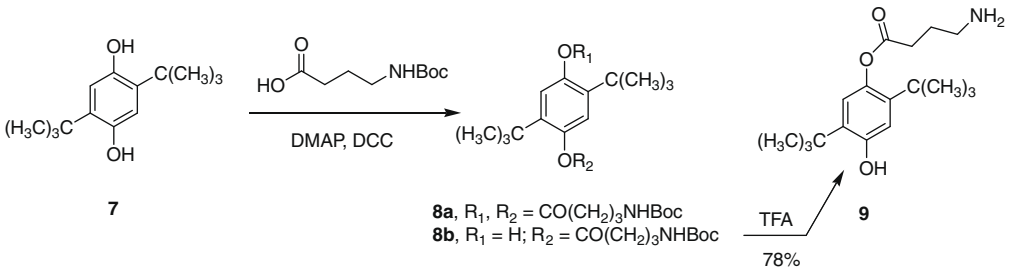
The ability of synthesized molecules to inhibit SERCA was evaluated in a well-established assay that measures the rate of SERCA-catalyzed ATP hydrolysis coupled to the oxidation of NADH via the action of the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH).[20,25,37] Inhibitory potency is reported as the IC_{50} value,



Scheme 3. Synthesis of di-allylhydroquinone.



Scheme 1. Synthesis of monosubstituted and unsymmetrically disubstituted hydroquinones.



Scheme 4. Synthesis of BHQ analogue with linker.

the inhibitor concentration at which the enzyme's activity is reduced by half (Table 1 and Fig. 1). To rule out false positives caused by compounds inhibiting the auxiliary enzymes PK and/or LDH instead of SERCA, a second assay was employed that was based on the direct chromogenic reaction of the dye malachite green with inorganic phosphate liberated from ATP by SERCA.[38,39] Among the compounds tested, the symmetrically disubstituted hydroquinones displayed the highest potencies, ranging from 340 nM (**5a**) to 3.3 μM (**5c**). In agreement with previous reports,[20,28] the presence of five carbon atoms in the side chains of these compounds maximized potency (**5a**) whereas the presence of fewer (**7**) or more (**5b** and **5c**) carbon atoms reduced potency slightly. The introduction of allyl groups (**6**) caused inhibitory potency to drop by about one order of magnitude in relation to BHQ, which is consistent with the smaller size of this substituent.

Compared to the disubstituted hydroquinones, activity assays with monosubstituted hydroquinones with an aromatic side group revealed somewhat lower potencies. Within this series of compounds (**2a**–**d**), the presence of a polar group such as a nitro or methoxy group attached to a phenyl ring enhanced inhibitory potency considerably. Although the monosubstituted hydroquinones were in general somewhat less potent than their disubstituted analogues (IC_{50} between 5.7 and 120 μM), their potencies were still remarkable, particularly since it had been suggested earlier that two substituents were necessary for activity.[20]

In an attempt to improve potency further, we reacted the most active monosubstituted hydroquinone (**2b**) with 2-methyl-2-butanol and 3-methyl-3-pentanol under acidic conditions to furnish unsymmetrically disubstituted hydroquinones **3a** and **3b**, respectively (Scheme 1). To our knowledge, these compounds are the first unsymmetrically substituted hydroquinones that have been evaluated as SERCA inhibitors. Somewhat disappointingly, however, the alkylation of **2b** increased its potency only modestly. Following the trend observed in the symmetrically disubstituted hydroquinone series, compound **3a** was slightly more active than **3b**.

The attachment of a tether in the form of 4-aminobutyric acid to a single hydroxyl group of BHQ (**8b**) preserved potency, albeit at a lower level, whereas esterification of both hydroxyl groups rendered the compound inactive (**8a**). Compound **9** with a free terminal amino group had an IC_{50} value of 2.6 μM, which was about one order of magnitude lower than that of **8b**, in which the amine was blocked by the protecting Boc group. As indicated above, tethers can serve as potential attachment points for inactivating moieties such as a short peptides, which can only be removed by proteases present on the surface of prostate cancer cells, thereby rendering the inhibitor active.[4,40,41] In this context, the observed preservation of potency in **8b** despite the presence of a sizeable tether is encouraging, since the latter would remain attached to the inhibitor after proteolytic cleavage. Moreover, recent crystallographic work has lead to the proposition to create dual inhibitors that bind simultaneously to the BHQ and the nearby TG binding site.[32] Thus, the connection of a hydroquinone to a TG analogue could be made using a tether similar to the one present in **9**.

Table 1
Inhibitory potencies of synthesized SERCA inhibitors with detectable activities

Compound no.	IC_{50} (μM)
2a	120 ± 30
2b	5.7 ± 2.9
2c	7.3 ± 2.5
2d	52 ± 3
3a	3.8 ± 1.9
3b	12 ± 3
5a	0.34 ± 0.04
5b	0.61 ± 0.23
5c	3.3 ± 0.6
6	6.0 ± 1.6
8b	29 ± 27
9	2.6 ± 1.3

2.3. Enzyme/inhibitor interactions prediction by computational docking

For an analysis of enzyme/inhibitor interactions on the molecular level, the modeled structures of the synthesized inhibitors were computationally docked into the crystal structure of the BHQ binding site of SERCA in a conformation representative of the enzyme's E_2 state, which is relevant for the binding of most inhibitors, including BHQ.[29] This was accomplished by using a previously established protocol based on the program GOLD and the scoring function ChemScore.[20] In the case of disubstituted hydroquinones (compounds **3a** and **b**, **5a**–**c**, and **6**), excellent consensus orientations were obtained, implying that the repeated docking runs under identical conditions generated the same inhibitor pose. Analogous to the orientation of BHQ in complex with SERCA, the

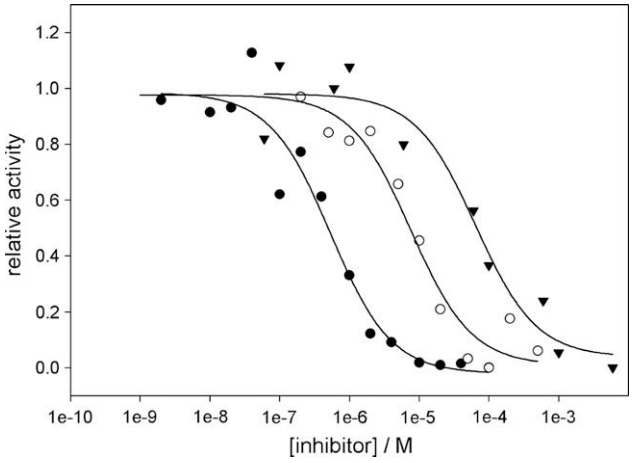


Figure 1. Representative SERCA inhibition assays. Relative enzyme activity as a function of inhibitor concentration (●: **5a**; ○: **6**; ▼: **2d**).

poses showed the inhibitor hydroxyl groups forming two hydrogen bonds with the SERCA residues Pro308 and Asp59 (Fig. 2, panels A and D). Additional binding energy was provided by extensive hydrophobic contacts between hydrophobic parts of the inhibitors and nonpolar residues in the binding site. With the exception of the nitro compound **2b**, the docking results obtained for the monosubstituted hydroquinones (**2a**, **2c**, and **2d**) differed somewhat from those obtained for the disubstituted compounds in that a few more deviations from the overall consensus pose occurred. Also, in contrast to the disubstituted hydroquinones, we observed that the docking-predicted poses of these three compounds (**2a**, **2c**, and **2d**) displayed a slight shift in position, which had been recognized before for other monosubstituted hydroquinones.[27] Interestingly, this particular pose allowed the inhibitors to maintain two hydrogen bonds, one involving the side chain of Asp59 and a second one involving the backbone carbonyl group of Glu309, which assumed the function of the neighboring Pro308 (Fig. 2, panels B and D). In the case of **2b**, the charged nitro group presumably prohibited the side group of **2b** from entering the predominantly hydrophobic area accessible to the other three monosubstituted hydroquinones. Consistent with the smaller size of the monosubstituted hydroquinones, an inspection of the breakdown of the ChemScore energy terms suggested that the somewhat lower potencies of these compounds were caused by a lowered binding affinity due to a reduction of the hydrophobic contact area. Docking results for compounds **8b** and **9** showed the position of the central phenyl ring rotated by 60° around its central axis (compared to all other hydroquinones), and the single hydroxyl group of these molecules forming a hydrogen bond with Pro308. The loss of the hydrogen bond to Asp59 or Glu309 appeared to be compensated by the formation of a new hydrogen bond between Gln250 and the amide oxygen of compound **8b**, whereas in the case of the unprotected **9**, a bifurcated hydrogen bond between the amino nitrogen atom and Asp59 and Asp254 stabilized the enzyme/inhibitor complex (Fig. 2, panels C and D). Most likely, the involvement of these additional hydrogen bonds was the reason for the surprisingly high inhibitory potency of **9**.

It should be noted that docking with the program GOLD was conducted in a manner that allowed for only partial flexibility of the inhibitor binding site.[42–44] More precisely, the torsion angles of the hydroxyl groups of threonine, serine, and tyrosine and of the amino group of lysine were permitted to be flexible whereas the rest of the protein was kept static. The fact that docking runs with all active hydroquinones were successful suggests that the protein conformation in all SERCA/inhibitor complexes is essentially the same and that none of the inhibitors is capable of inducing major conformational changes that would cause SERCA to adopt a conformation different from that seen in the X-ray crystal structure.[29] With regard to the inhibition mechanism, the docking results are compatible with a previously proposed scenario in which BHQ interferes with the gating function of the residue Glu309, preventing transported calcium ions from reaching their binding site.[45,46] In addition, the hydrogen bonds formed by the inhibitor lock the relative positions of the transmembrane helices M1 and M4 (or

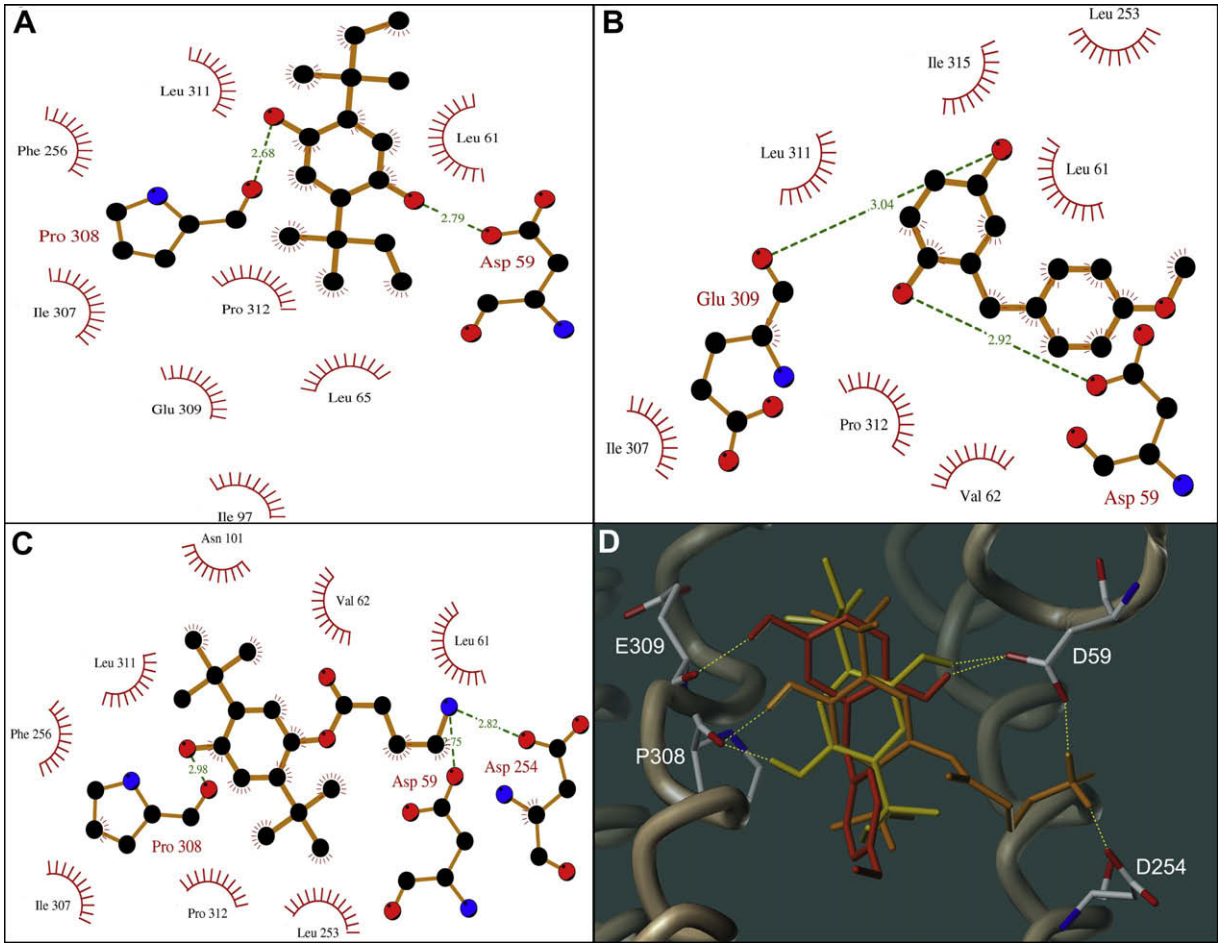


Figure 2. SERCA/inhibitor interactions analyzed by computational docking. Panels A–C: binding profiles of the representative inhibitors **5a** (A), **2c** (B), and **9** (C). Diagrams were created with LIGPLOT.[50] Panel D: docking-predicted binding poses of **5a** (yellow), **2c** (red), and **9** (orange).

M3, if residues 250 and 254 are involved) and prevent their relative movement, which is necessary for the catalytic cycle to continue.

2.4. Conclusion and future directions

In this study, we utilized several synthetic routes for the preparation of hydroquinone-based SERCA inhibitors. We demonstrated that these routes afford access to novel compounds with good inhibitory potencies. In comparison to the preparation of TG analogues, the presented synthetic approaches are straightforward and economical because they involve no more than two steps and require only inexpensive, commercially available starting materials. Future efforts will be directed at a more comprehensive exploitation of the outlined synthetic routes for the refinement and expansion of existing SARs for SERCA inhibition by hydroquinones. Finally, the preparation of inhibitors tethered to a second molecular entity will be explored further.

3. Experimental

3.1. Organic synthesis

Chemistry. All reagents were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were used without further purification. Reactions were carried out under an argon atmosphere. Melting points were measured with a Thomas Hoover melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed using Baker-flex silica gel sheets and detection of spots was made by UV light and/or iodine vapors. Column chromatography was performed using silica gel 70–230-mesh. Proton (500 MHz) and carbon (125.7 MHz) NMR spectra were obtained on a JEOL Eclipse 500 spectrometer (Peabody, MA). Chemical shifts are reported as parts per million (ppm) relative to TMS in $CDCl_3$ or DMSO-d_6. For all compounds not previously characterized in the literature, high resolution mass spectra (HRMS) using either electron impact (EI) or electrospray (ESI) techniques were obtained using the facilities at the Mass Spectrometry Laboratory at the University of Illinois at Urbana.

General procedure for synthesis of monosubstituted hydroquinones (2a–d). Following the procedure of Ozaki et al.,[33] 1,4-cyclohexanedione (1 g, 8.9 mmol), the appropriate aldehyde (8.9 mmol), and LiCl (380 mg, 8.9 mmol) were dissolved in 5 mL of either 1,3-dimethyl-2-imidazolidinone (DMI) or pyridine. The mixture was stirred for 1 h at 160–170 °C or, if pyridine was the solvent, refluxed for 1 h. Then, the reaction mixture was diluted with ethyl ether, washed twice with water, and dried with anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography.

2-Benzylhydroquinone (2a). Solvent: pyridine. Purification by column chromatography (hexanes–ethyl acetate, 3:1) followed by recrystallization from benzene–hexane gave an off-white solid (786 mg, 44%). Mp 105–106.5 °C, literature value: 101–103 °C.[47] ^{1}H NMR (DMSO-d_6): δ 3.78 (s, 2H), 6.42 (m, 2H), 6.60 (m, 1H), 7.12–7.29 (m, 5H), 8.54 (s, 1H, exchangeable with D_2O), and 8.66 (s, 1H, exchangeable with D_2O). ^{13}C NMR (DMSO-d_6): δ 35.8, 113.9, 116.1, 117.3, 126.2, 128.6, 128.7, 129.3, 141.9, 147.9, 150.3.

2-(4-Nitrobenzyl)hydroquinone (2b). Solvent: DMI. Purification by silica gel column chromatography (hexanes–ethyl acetate, 6:4) gave a yellow solid (286 mg, 13%). Mp 152–154 °C. ^{1}H NMR (DMSO-d_6): δ 3.92 (s, 2H), 6.45–6.49 (m, 2H), 6.62 (d, J = 8.7 Hz, 1H), 7.46 (d, J = 8.7 Hz, 2H), 8.14 (d, J = 8.7 Hz, 2H), 8.62 (s, 1H, exchangeable with D_2O), 8.77 (s, 1H, exchangeable with D_2O). ^{13}C NMR (DMSO-d_6): δ 39.76, 114.56, 116.36, 117.51, 123.95, 127.03, 130.32, 146.29, 147.97, 150.41. HRMS (EI): 245.0690 (calcd for $C_{13}H_{11}NO_4$: 245.0688).

2-(4-Methoxybenzyl)hydroquinone (2c). Solvent: DMI. Purification by silica gel column chromatography (hexanes–ethyl acetate, 7:3) yielded a tan solid (734 mg, 36%). Mp 128–130 °C. ^{1}H NMR (DMSO-d_6): δ 3.70 (s, 3H), 3.71 (s, 2H), 6.38–6.42 (m, 2H), 6.59 (d, J = 8.3 Hz, 1H), 6.82 (m, 2H), 7.11 (m, 2H), 8.51 (s, 1H, exchangeable with D_2O), 8.61 (s, 1H, exchangeable with D_2O). ^{13}C NMR (DMSO-d_6): δ 34.92, 55.54, 113.74, 114.18, 116.09, 117.21, 129.10, 130.25, 133.73, 147.81, 150.26, 157.93. HRMS (EI): 230.0942 (calcd for $C_{14}H_{14}O_3$: 230.0943).

2-(2-Furanylmethyl)hydroquinone (2d). Solvent: pyridine. Purification by column chromatography (hexanes–ethyl acetate, 3:1) gave 560 mg (33%) of a pale yellow solid. Mp 57–60 °C.[33] ^{1}H NMR (DMSO-d_6): δ 3.79 (s, 2H), 6.04 (br s, 1H), 6.35–6.46 (m, 3H), 6.60 (d, J = 8 Hz, 1H), 7.51 (s, 1H), 8.58 (s, 1H, exchangeable with D_2O), 8.70 (s, 1H, exchangeable with D_2O). ^{13}C NMR (DMSO-d_6): δ 28.3, 106.7, 110.9, 114.3, 116.1, 116.9, 125.5, 142.0, 147.8, 150.2, 154.7.

General procedure for synthesis of unsymmetrically disubstituted hydroquinones. A mixture of 4-nitrobenzylhydroquinone (200 mg, 0.8 mmol) and the appropriate alcohol (1 mL) was placed in a round bottom flask, cooled in an ice bath, and concentrated sulfuric acid (0.5 mL) was added dropwise with stirring, followed by the addition of glacial acetic acid (1 mL). A brown solid precipitated within 10 min. After 1 h, the reaction was quenched with water and the crude product was taken up in ethyl acetate, washed with water, and dried with sodium sulfate. The organic layer was then concentrated to a crude oil by rotary evaporation.

2-(4-Nitrobenzyl)-5-*tert*-amylhydroquinone (3a). Purified by silica gel column chromatography (hexanes–ethyl acetate, 7:3). The obtained oil was triturated with hexanes to give a yellow powder (40 mg, 15%). Mp 108–112 °C. ^{1}H NMR ($CDCl_3$): δ 0.66 (t, J = 7.8 Hz, 3H), 1.31 (s, 6H), 1.82 (qr, J = 7.8 Hz, 2H), 3.97 (s, 2H), 6.36 (s, 1H), 6.63 (s, 1H), 7.38 (m, 2H), 8.14 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 9.61, 27.67, 29.58, 35.53, 38.10, 114.90, 116.20, 118.56, 123.76, 129.66, 134.64, 146.52, 146.75, 148.18, 148.58. HRMS (ES): 338.1385 (calcd for $C_{18}H_{21}NO_4Na$: 338.1368).

2-(4-Nitrobenzyl)-5-(3-methyl-3-pentyl)hydroquinone (3b). Purified by silica gel column chromatography (hexanes–ethyl acetate, 6:4). The obtained oil was triturated with hexanes to give a yellow solid (70 mg, 26%). Mp 84–87 °C. ^{1}H NMR ($CDCl_3$): δ 0.65 (t, J = 7.4 Hz, 6H), 1.23 (s, 3H), 1.51 (m, 2H), 2.07 (m, 2H), 3.96 (s, 2H), 6.35 (s, 1H), 6.59 (s, 1H), 7.34 (m, 2H), 8.09 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 9.22, 23.59, 32.35, 35.53, 41.93, 117.44, 118.41, 123.64, 123.74, 129.67, 132.87, 146.45, 146.71, 148.23, 148.72. HRMS (ES): 330.1704 (calcd for $C_{19}H_{24}NO_4$ (MH^+): 330.1705).

2,5-Di-*tert*-amylhydroquinone (5a). A mixture of hydroquinone (**4**, 2.0 g, 18 mmol), *tert*-amyl alcohol (3.22 g, 36.5 mmol), and acetic acid (10 mL) was added to a round bottom flask, followed by the dropwise addition of concentrated sulfuric acid (2 mL). The contents of the flask were stirred for 7 h at room temperature. The reaction was then quenched with ice water and the precipitate was collected by suction filtration and washed with water. Purification by recrystallization from benzene yielded a white solid (3.55 g, 78%). Mp 178–180 °C. ^{1}H NMR ($CDCl_3$): δ 0.66 (t, J = 7.8 Hz, 6H), 1.31 (s, 12H), 1.80 (qr, J = 7.8 Hz, 4H), 4.32 (br s, 2H, exchangeable with D_2O), 6.49 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 9.60, 27.61, 33.37, 37.67, 116.90, 132.61, 147.31. HRMS (EI): 250.1932 (calcd for $C_{16}H_{26}O_2$: 250.1933).

2,5-Di-(3-methyl-3-pentyl)hydroquinone (5b). To a mixture of hydroquinone (**4**, 1.0 g, 9 mmol) and 3-methyl-3-pentanol (1.98 g, 19 mmol), sulfuric acid (70% aqueous solution, 15 mL) was added dropwise at room temperature. During the addition, a white precipitate formed. After 1 h, the reaction was quenched with ice water. The precipitate was collected by suction filtration and washed with water. Purification by recrystallization (methanol/water) yielded a white crystalline product (1.46 g, 58 %). Mp

149–150 °C. ^{1}H NMR ($CDCl_3$): δ 0.65 (t, J = 7.5 Hz, 12H), 1.22 (s, 6H), 1.50 (m, 4H), 2.06 (m, 4H), 4.31 (br s, 2H, exchangeable with D_2O), 6.41 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 9.19, 23.43, 32.47, 41.50, 118.00, 130.70, 147.27. HRMS (EI): 278.2246 (calcd for $C_{18}H_{30}O_2$: 278.2246).

2,5-Di-(3-ethyl-3-pentyl)hydroquinone (5c). A mixture of hydroquinone (**4**, 1 g, 9 mmol), 3-ethyl-3-pentanol (2.5 g, 21.8 mmol), and glacial acetic acid (10.0 mL) was added to a round bottom flask. Sulfuric acid (75% aqueous solution, 10 mL) was added dropwise at room temperature while stirring. After 24 h, the reaction was quenched with ice water. The product was collected by suction filtration and washed with water. Purification by recrystallization (methanol/water) yielded a white solid (0.94 g, 34%). Mp 185–187 °C. ^{1}H NMR ($CDCl_3$): δ 0.66 (t, J = 7.3 Hz, 18H), 1.76 (q, J = 7.3 Hz, 12H), 4.28 (br s, 2H, exchangeable with D_2O), 6.42 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 8.42, 26.09, 43.71, 118.40, 130.49, 147.20. HRMS (EI): 306.2561 (calcd for $C_{20}H_{34}O_2$: 306.2559).

2,5-Diallylhydroquinone (6). To a stirred solution of 1,4-bis(allyloxy)benzene[35] (300 mg, 2.0 mmol) in hexane (10 mL), diethyl aluminum chloride (8.0 mL of a 1 M solution in heptane, 8.0 mmol) was added dropwise over 5 min at room temperature.[36] The mixture was stirred for 30 min, chilled in an ice bath, then quenched with diluted aqueous HCl, and extracted with ethyl acetate. The extracts were washed with aqueous HCl, aqueous $NaHCO_3$, and water, and dried over Na_2SO_4. The crude product was concentrated by rotary evaporation. Column chromatography (methylene chloride–hexanes, 98:2) produced the known **6** (97 mg, 31%). ^{1}H NMR ($CDCl_3$): δ 3.42 (m, 4H), 4.60 (br s, 2H), 5.00–5.10 (m, 4H), 5.96 (m, 2H), 6.63 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 30.86, 114.65, 115.79, 125.32, 136.05, 148.30.

Esterification of BHQ (7): preparation of 8a and 8b. To a stirred solution of BHQ (**7**), (1.48 g, 6.66 mmol), $BocNH(CH_2)_3CO_2H$ (451 mg, 2.22 mmol), and DMAP (276 mg, 2.25 mmol) in methylene chloride (7 mL), DCC (2.22 mL of a 1 M solution in methylene chloride; 2.22 mmol) was added at room temperature. The resulting mixture was stirred for 6 h and then filtered. The filtrate was concentrated by rotary evaporation. The crude product was partitioned between ethyl acetate and diluted aqueous HCl. The organic layer was washed with diluted aqueous $NaHCO_3$ and water, and then dried over Na_2SO_4 and concentrated. Purification by preparative TLC (silica gel, methylene chloride–methanol, 99:1) gave 116 mg (12%) of **8b** as a white solid. Mp 146–148 °C. ^{1}H NMR ($CDCl_3$): δ 1.28 (s, 9H), 1.35 (s, 9H), 1.49 (s, 9H) 1.94 (quintet, J = 7.2 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 3.24 (m, 2H) 4.71 (br s, 1H), 5.56 (br s, 1H), 6.67 (s, 1H), 6.78 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 25.12, 28.49, 29.50, 30.24, 32.20, 33.79, 34.23, 40.06, 79.57, 115.20, 122.64, 134.47, 139.19, 141.98, 151.69, 156.26, 172.47. HRMS (ES): 408.2742 (calcd for $C_{23}H_{37}NO_5$ (MH^+): 408.2750).

When the ratio of amino acid, DMAP, and DCC to **7** was changed to 1:1, the major product isolated was the bis-ester **8a**. Purification by column chromatography (methylene chloride/hexanes, 10:1) gave a white solid. Mp 160–162 °C. ^{1}H NMR ($CDCl_3$): δ 1.28 (s, 18 H), 1.43 (s, 18 H), 1.94 (quintet, J = 7.3 Hz, 4H), 2.62 (t, J = 7.3 Hz, 4H), 3.24 (m, 4H), 4.66 (br s, 2H), 6.93 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 25.15, 28.48, 30.14, 32.16, 34.21, 39.95, 79.08, 122.85, 139.59, 146.14, 156.11, 171.86. HRMS (ES): 593.3806 (calcd for $C_{32}H_{53}N_2O_8$ (MH^+): 593.3802).

Deprotection of 8b and preparation of 9. To a stirred solution of **8b** (41.0 mg; 0.1 mmol) in methylene chloride (7 mL), TFA (3 mL) was added dropwise. The solution was stirred for 4.5 h at room temperature. The crude product was concentrated by rotary evaporation. Trituration with benzene gave 24 mg (78%) of a white solid. Mp 191–193 °C. ^{1}H NMR (DMSO-d_6): δ 1.23 (s, 9H), 1.29 (s, 9H), 1.91 (quintet, J = 7.3 Hz, 2H), 2.70 (t, J = 7.3 Hz, 2H), 2.90 (m, 2H), 6.69 (s, 1H), 6.80, (s, 1H), 7.86 (br s, 3H), 9.30 (br s, 1H). ^{13}C NMR (DMSO-d_6): δ 22.74, 29.54, 30.34, 31.32, 33.92, 34.19, 38.58, 114.69, 122.31, 133.76, 138.72 140.83, 153.22, 172.00. HRMS (ES): 308.2223 (calcd for $C_{18}H_{30}NO_3$ (MH^+): 308.2226).

3.2. Determination of inhibitory potencies

The ability of compounds to inhibit the rate of SERCA-catalyzed ATP hydrolysis was evaluated in a spectroscopic assay using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) as described previously.[27] Inhibitory potencies were obtained by measuring the rates of SERCA-catalyzed ATP hydrolysis at ten to twelve different inhibitor concentrations, followed by a three-parameter logistic fit of activity versus inhibitor concentration data.[48] The fit yielded the IC_{50} value, a standard measure for inhibitory potency. All active compounds were subsequently subjected to a second, independent assay that directly monitored the SERCA-catalyzed production of inorganic phosphate. As pointed out previously,[27] the second assay was conducted at only two or three inhibitor concentrations since its purpose was to provide a qualitative confirmation of SERCA inhibition (and rule out inhibition of the enzymes PK and LDH required for the coupled assay) rather than a quantitative measurement of potencies.

3.3. Computational ligand docking

For the prediction of inhibitor binding poses, a previously described protocol was followed.[20,49] Briefly, the three-dimensional structures of synthesized compounds were modeled in Sybyl (Tripos, St. Louis, version 8.0) and their conformational energy minimized by molecular mechanics with the MMFF94s force field in combination with a conjugate gradient and a termination condition of 0.01 kcal/(mol Å). Docking was performed with the program GOLD (Genetic Optimisation for Ligand Docking, Cambridge Crystallographic Data Centre, UK; version 4.1) and the X-ray crystal structure of SERCA (Protein Data Bank entry 2AGV) in a conformation representative of the E_2 state.[29] ChemScore was chosen as the scoring function and the genetic algorithm was executed at the default settings, performing 30 independent repeats under identical conditions for each compound. The docking sphere had a radius of 15 Å centered at the (deleted) C-1 phenyl carbon (atom number 15396) of BHQ in the SERCA/BHQ complex. The descriptor calculation feature of Hermes, a GOLD utility, allowed the identification of ligand and protein moieties involved in hydrogen bonds, close steric contacts, and hydrophobic interactions. Inhibitor/SERCA interactions displayed in Figure 2 were visualized in schematic diagrams created with the program LIGPLOT.[50]

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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.2009.07.075.

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