Published in final edited form as:

Bioorg Med Chem. 2013 July 1; 21(13): 3927–3933. doi:10.1016/j.bmc.2013.04.012.

Structural requirements for inhibitory effects of bisphenols on the activity of the sarco/endoplasmic reticulum calcium ATPase

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Abstract

Bisphenols (BPs) are a class of small organic compounds with widespread industrial applications. Previous studies have identified several BPs that interfere with the activity of the ion-translocating enzyme sarco/endoplasmic reticulum calcium ATPase (SERCA). In order to define the molecular determinants of BP-mediated SERCA inhibition, we conducted enzyme activity assays with rabbit SERCA to determine the inhibitory potencies of 27 commercially available BPs, which were the basis for structure-activity relationships. The most potent BPs inhibited SERCA at low micromolar concentrations and carried at their two phenyl rings multiple non-polar substituents, such as small alkyl groups or halides. Furthermore, the presence of methyl groups or a cyclohexyl group at the central carbon atom connecting the two phenyl moieties correlated with good potencies. For a characterization and visualization of inhibitor/enzyme interactions, molecular docking was performed, which suggested that hydrogen bonding with Asp254 and hydrophobic interactions were the major driving forces for BP binding to SERCA. Calcium imaging studies with a selection of BPs showed that these inhibitors were able to increase intracellular calcium levels in living human cells, a behavior consistent with that of a SERCA inhibitor.

Keywords

calcium homeostasis; fluorescence imaging; bisphenol A; calcium ion transport; computational docking; toxicity; ATPase activity assay

1. Introduction

Bisphenols (BPs) are a class of molecules composed of two hydroxylated phenyl groups that are typically linked by a methylene bridge. Many BPs are widely used as raw materials in industrial processes, such as the production of polycarbonate, polyester, and epoxy resins [1]. Some BPs have endocrine disrupting properties, which makes them a potential hazard to human health. For example, bisphenol A (Scheme 1), the best-known representative from this compound class, has been associated with adverse health effects, particularly in pregnant women [2-4]. Its heavy use for the production of polycarbonate plastics by the food packing industry has led to human exposure through the diet. Bisphenol A is a known endocrine disruptor whose structural resemblance to the synthetic hormone diethylstilbestrol

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allows it to mimic the action of estrogen at the human estrogen receptor. For example, one of many effects of bisphenol A is the alteration of Ca^{2+} handling in female rodent hearts, giving rise to increased field-stimulated Ca^{2+} transients and accelerated Ca^{2+} reuptake rates, ultimately triggering arrhythmias [5]. Another BP, bisphenol AF, has recently been shown to activate the α form of the estrogen receptor, block the β form, while having no effect on the γ form [6]. At the present time, the negative impact of BPs on human health continues to be debated intensively and current safety levels of BPs for humans are being reevaluated [3, 7-9].

Besides the well-established endocrine disrupting effects that typically occur at nanomolar or subnanomolar concentrations and are mediated through estrogen receptors, it has also been shown that several BPs interfere with Ca^{2+} signaling pathways by directly inhibiting the Ca^{2+} -translocating function of the sarco/endoplasmic reticulum calcium ATPase (SERCA) [10-12], a transmembrane enzyme that plays a pivotal role in Ca^{2+} homeostasis. It uses energy gained from ATP hydrolysis to transport Ca^{2+} ions from the cytosol into the endoplasmic or sarcoplasmic reticulum, which serve as Ca^{2+} storage compartments [13-15]. Since the concentration of Ca^{2+} in these reservoirs is orders of magnitude higher than in the cytosol, Ca^{2+} release from these reservoirs can increase cytosolic Ca^{2+} levels rapidly and substantially, thereby triggering a number of physiologically crucial events [16]. Inhibition of SERCA activity results in cessation of loading of Ca^{2+} stores while Ca^{2+} release through ion channels such as ryanodine receptors continues. Permanently elevated cytosolic Ca^{2+} levels are the result, a condition that is lethal to cells since it ultimately triggers apoptosis [17-20].

More than two decades ago, it was shown for the first time that a BP – bis-(2-hydroxy-3-*tert*-butyl-5-methylphenyl)methane – can inhibit SERCA activity by directly binding to the enzyme [10]. A more recent structure-based library screen based on the hydroquinone scaffold identified several BP-like compounds with multiple phenolic fragments that inhibited SERCA activity at concentrations in the low micromolar range [21]. Moreover, two reports demonstrated that the widely used flame retardant tetrabromobisphenol A is a potent SERCA inhibitor [22, 23]. Even though these studies have provided clear evidence for the inhibitory effects of some BPs on SERCA's function, a more comprehensive investigation for BP-mediated SERCA inhibition is currently lacking. In particular, systematic structure-activity relationships (SARs) that correlate molecular structure of a structurally diverse pool of BPs to their SERCA inhibitory potency are not available. As a result, the molecular determinants of bisphenol-mediated SERCA inhibition remain largely elusive at the present time, a fact that has hampered our ability to reliably estimate the potential danger posed by a particular BP to human health.

In the present study, we measured the inhibitory potencies of a series of 27 commercially available BPs using *in vitro* activity assays with rabbit SERCA. The results were the basis for the establishment of SARs that identified the structural requirements for effective SERCA inhibition by BPs. To provide a visualization of relevant enzyme/inhibitor interactions at the molecular level, we conducted computational ligand docking of BPs into the X-ray crystal structure of SERCA. For a small selection of inhibitory BPs, we complemented the enzyme inhibition assays by *in vivo* measurements of their effects on intracellular Ca^{2+} levels in human cells. These experiments were performed by fluorescence imaging and determined the compounds' ability to increase intracellular Ca^{2+} levels, an anticipated and well-documented effect of SERCA inhibitors that are active *in vivo* [22, 24].

2. Materials and Methods

2.1. Materials

Rabbit hind leg tissue for the preparation of microsomes was obtained from Pel-Freez (Rogers, AR). Pyruvate kinase (PK), lactate dehydrogenase (LDH), and Minimum Essential Medium were received from Sigma-Aldrich (St. Louis, MO) whereas all other reagents for enzyme activity assays as well as phosphate-buffered saline were from Fisher Scientific (Pittsburgh, PA). Human skin fibroblasts were obtained from Coriell (Cambden, NJ). Fetal bovine serum, streptomycin, penicillin, and trypsin were from Atlanta Biologicals (Lawrenceville, GA). Fura-2 AM was obtained from Life Technologies (Grand Island, NY). BPs listed in Tables 1-4 were purchased from the following vendors: **1** was from Alfa Esar (Ward Hill, MA), **2-4, 9, 11-14, 16, 18-21, 25**, and **26** were from Sigma-Aldrich, **23** was from SynQuest (Alachua, FL), and **5-8, 15, 22, 24**, and **27** were from TCI (Portland, OR). All compounds were used without further purification.

2.2. ATPase activity inhibition assays

SERCA microsomes from rabbit hind leg tissue were prepared by a standard procedure [13, 25]. The SERCA-catalyzed rate of ATP hydrolysis coupled to the oxidation of NADH by the action of the auxiliary enzymes PK and LDH was measured spectroscopically as described in a previous study [21], using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) operating at a wavelength of 340 nm. Briefly, ATP hydrolysis was initiated in samples placed in the wells of 96-well plates by the addition of 5 mM ATP. Each sample had a volume of 225 μ L and contained SERCA in assay buffer (6.7 μ g/mL protein) composed of 20 mM Tris, 5 mM $MgCl_2$, 100 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM EGTA, 3.3 μ M calcimycin, and 1.5 mM phosphoenolpyruvate at a pH of 7.3. Reaction rates were monitored for several minutes at various inhibitor concentrations and then fit to a three-parameter logistic equation [26]. Inhibitory potencies were expressed in terms of the IC_{50} value, which was the inhibitor concentration that reduced SERCA activity by 50%.

In order to confirm positive results of the coupled assay, a second, independent ATPase activity assay was conducted that was based on the colorimetric reaction between inorganic phosphate and the dye Malachite Green as described before [21, 27]. In a 96-well polystyrene plate, 10 μ L of SERCA solution (100 μ g/mL protein 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 μ M calcimycin, 0.7 mM $CaCl_2$, and 20 mM Trizma (pH 7.5) to give a total volume of 112.5 μ L. SERCA-catalyzed ATP-hydrolysis was initiated by the addition of 112.5 μ L 0.45 mM ATP (total sample volume: 225 μ L) and stopped after well-defined delay times (1 minute intervals up to 10 minutes) by the addition of a solution containing 3 mM Malachite Green, 10 mM sodium molybdate, Triton-X-100 (0.05%), and 0.7 M HCl. The absorbances of the samples were then read with the microplate reader at 630 nm, the wavelength at which the spectrum of the dye/phosphate complex has its maximum. Reaction rates were obtained by linear regression. Unlike the coupled assay described above, this assay was only performed at three inhibitor concentrations to assess whether compounds testing positive in the first assay did so by interfering with the activity of SERCA and not with the auxiliary enzymes PK or LDH.

2.3. Molecular modeling and computational docking

Models of the three-dimensional molecular structures of inhibitors were built in Sybyl-X (version 1.3; Tripos, MO) and their conformational energies were minimized by molecular mechanics using a conjugate gradient method and a termination criterion of 0.01 kcal/(mol $\text{\AA}$). For the minimization, the MMFF94s force field was utilized in conjunction with MMFF94 charges and a distance-dependent dielectric constant of 4, mimicking the

hydrophobic character of the binding site's environment. The X-ray crystal structure of SERCA in the E₂ conformation [28] was obtained from the Protein Databank (PDB ID: 2AGV) and prepared for docking by removing all non-protein components and adding hydrogen atoms. After assignment of partial charges from the Amber library, the positions of the added hydrogen atoms were optimized by molecular mechanics while keeping the positions of the heavy atoms static. For this minimization procedure, the Powell method in conjunction with the Amber7 FF99 Force Field, a distance-dependent dielectric constant of 4, and a convergence criterion of 0.05 kcal/(mol Å) were employed.

Docking was performed with the program GOLD (version 5.0.1; Cambridge Crystallographic Data Centre, Cambridge, UK) [29, 30] using the ChemScore scoring function [31, 32]. Docking runs were executed at the default settings and the docking sphere had a radius of 15 Å centered at the C-1 phenyl carbon (atom number 15395) of the previously deleted inhibitor *tert*-butylhydroquinone (BHQ) in the SERCA/BHQ complex. For each ligand, 30 runs were performed under identical conditions. For the analysis of the docking results, the GOLD utility HERMES was employed, which permitted the identification of hydrogen bonds, close steric contacts, and hydrophobic interactions. Visualization of docking poses was performed with the program MOE (version 2011.10; Chemical Computing Group, Montreal, Canada).

2.4. Calcium uptake inhibition assays in cells

Human skin fibroblasts (GM08333) were grown according to the supplier's guidelines in Minimum Essential Medium complemented with streptomycin/penicillin and 10% fetal bovine serum. Cells were grown on circular glass plates (diameter: 1 cm) placed inside a larger culture dish. Before use in imaging experiments, the glass plates were mounted on a holder and the cells were washed two times with 1 mL of loading buffer composed of 150 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM NaH₂PO₄, 1.8 mM CaCl₂, 10 mM glucose, and 20 mM HEPES (pH 7.2). Cells were loaded with the ratiometric dye Fura-2 AM [33] by adding 10 µL of 200 µM dye (in DMSO) to cells bathed in 1 mL loading buffer. After 40 minutes, external dye was removed in two wash cycles with loading buffer (1 mL each).

To study the effect of a potential inhibitor, the intracellular Ca²⁺ concentration was measured by recording Fura-2 fluorescence (λ_{ex} : 340 and 380 nm; λ_{em} : 510 nm) with an InCyt Im2 imaging system (Intracellular Imaging Inc., Cleveland, OH). Baseline Ca²⁺ levels were recorded for one minute, after which a 20 µL aliquot of a potential inhibitor dissolved in DMSO was layered on top of the cells, giving a total inhibitor concentration of 50 µM. Control experiments were performed with DMSO only to confirm that the observed signal changes were not caused by solvent addition. Processing of the recorded fluorescence traces was done with the software provided by the system manufacturer. Standard curves for the conversion of fluorescence intensity ratios to Ca²⁺ concentration were obtained by measuring standard solutions of known Ca²⁺ concentrations (Life Technologies, Grand Island, NY) as described previously [33].

3. Results and Discussion

3.1. Effect of BP structure on experimentally determined inhibitory potencies

Fig.1 displays inhibition curves obtained for a representative selection of three BPs, showing the effect of chemical structure on inhibitory potency. The potencies expressed as IC₅₀ values in Table 1 show the influence of substituents in the *ortho* position with respect to the hydroxyl groups at the two phenol rings of the bisphenol A scaffold. Bisphenol A (**1**) is a rather weak SERCA inhibitor (IC₅₀ = 140 µM) whose potency is increased by approximately six-fold (IC₅₀ = 24 µM) via the introduction of a single methyl group at each

ring (**2**). Two additional methyl groups, also in the *ortho* position (**3**), cause a further increase in potency by another factor of six ($IC_{50} = 4.1 \mu M$). A very similar effect is observed when the methyl groups in **2** are replaced by three-carbon substituents, such as by allyl (**4**) or isopropyl groups (**5**). A further increase in steric bulk in the *ortho* positions is detrimental to inhibitory potency, as evident from the potencies of the cyclohexyl- (**6**) and phenyl-substituted analogs (**7**), which are only slightly more active than parent compound **1** ($IC_{50} = 94$ and $91 \mu M$, respectively). Tetrachlorinated (**8**) and tetrabrominated (**9**) analogs of **1** display potencies that are similar to that of the tetramethylated bisphenol **3** ($IC_{50} = 4.1$ and $4.7 \mu M$, respectively), thereby reinforcing the notion that small hydrophobic substituents are well-suited for enhancing inhibitory potencies of BPs. In the case of **9**, a commonly used flame retardant, the measured IC_{50} is in good agreement with a previous study that reported pH-dependent inhibitory potencies in the low micromolar range [23]. The introduction of four *ortho* amino groups (**10**) results in a complete loss of activity. Likewise, the exchange of the two hydroxyl groups in the highly active **9** for a small tether terminating in a free hydroxyl group (**11**) or for a tethered epoxide group (**12**) renders the molecule inactive, suggesting that hydroxyl groups directly attached to the ring are a requirement for effective SERCA inhibition.

In order to explore the influence of the chemical structure of the bridge connecting the two phenyl rings, several compounds with differing bridges were tested (Table 2). Whereas the removal of a single methyl group (**13**) in **1** preserves potency, the deletion of both methyl groups results in the loss of activity (**14**, bisphenol F). Interestingly, a conformationally restricted and bulky cyclohexyl bridge (**15**) conveys the highest potency in this series ($IC_{50} = 27 \mu M$), whereas a carbonyl group (**16**) shows little effect and hexafluorination of the two methyl groups (**17**, bisphenol AF) renders the compound inactive.

The four compounds presented in Table 3 are based on a scaffold that places the two hydroxyl groups in direct proximity of the connecting methylene bridge. They differ from each other in nature and position of alkyl or halide substituents at their phenyl rings. In contrast to the BPs in Table 1, the bridge carbon atom connecting the two phenyl rings is not methylated. It should be noted that **19** was the first bisphenol-based compound that was demonstrated to inhibit SERCA [10]. Compound **21**, also known as hexachlorophene, is a commonly used disinfectant. A comparison of **18** and **19** shows that the introduction of *tert*-butyl groups increases potency almost by two orders of magnitude, yielding an inhibitor whose potency rivals those of the most active ones in Table 1 ($IC_{50} = 3.6 \mu M$). The potency of **19** was originally reported as $0.7 \mu M$ [10], which is in reasonable agreement with the present finding, given the differences in experimental conditions and in the source of SERCA used (rat versus rabbit skeletal muscle). The introduction of chlorine groups at the rings is also beneficial for potency, with the hexa-chlorinated **21** being one of the most active inhibitors tested in this study ($IC_{50} = 4.0 \mu M$).

Six other BPs that are not part of a congeneric series were also tested (Table 4). The inactivity of **22** in comparison to its active analog **15** illustrates that hydroxyl groups cannot be exchanged by amino groups. Amino groups in general seem to be unfavorable, as evident from the loss of activity when replacing the four fluorine atoms in the active compound **23** by two amino groups (**27**). Introducing a propionate group at the bridging carbon atom of **8** to yield **26** causes a noticeable drop in potency ($IC_{50} = 25 \mu M$), which may be the result of the molecule having acquired a negative charge. Finally, a comparison of the moderate potency inhibitor **25** ($IC_{50} = 17 \mu M$) with the inactive **14** emphasizes the aforementioned favorable effect of placing moderately sized non-polar substituents – in this case *tert*-butyl groups – at the phenyl rings.

3.2. Interpretation of structure-activity relationships at the molecular level

To date, the only SERCA inhibitors whose binding sites have been unambiguously defined by high-resolution X-ray crystallography are thapsigargin [34], BHQ [28], and cyclopiazonic acid [35, 36], all of which bind to the enzyme in the catalytically important E₂ conformation. In contrast, the only experimental evidence for the location of SERCA's BP binding site stems from a tryptophan fluorescence study which showed that fluorescence quenching induced by binding of compound **9** to SERCA could be reversed by the addition of BHQ, presumably as the result of displacement of **9** by BHQ in a competitive fashion [23]. This interpretation was supported by docking studies that suggested that the binding site of **9** at least partially overlaps with that of the smaller BHQ.

Based on these findings, docking runs with BPs in the present study were centered at the BHQ binding site. Only active compounds were docked and the following discussion focuses on the more potent inhibitors because compounds with high binding affinities are in general more likely to yield reliable docking results. In many but not all cases, good consensus poses were obtained, implying that repeats under identical conditions generated the same results.

Among the *ortho*-substituted BPs (**2-5**, **8**, **9**), a commonly adopted binding pose could be identified (Fig. 2A). In this binding mode, one inhibitor hydroxyl group is engaged in a hydrogen bond with Asp254, a residue located near the entrance of the binding site that has not been implicated in hydrogen bonding with BHQ. Since the BPs are larger in size than BHQ, their binding poses inevitably differs from that of BHQ as they occupy a larger area and protrude somewhat into the opening of the binding site, leaving parts of one of the phenol rings solvent-exposed (Fig. 2B and 2C). As visualized in Figs. 2B-D for **4**, the most potent BP in the series, most non-polar parts of the inhibitor are observed in close proximity to hydrophobic residues lining the binding site, such as Leu61, Val62, Leu65, Ile97, Leu253, Ile307, Pro308, Leu311, and Pro312, suggesting hydrophobic contacts as a major driving force for inhibitor binding. This interpretation is supported by an inspection of the breakdown of the individual ChemScore terms, which predicts hydrophobic interactions to be by far the most important contributor, followed by the single hydrogen bond involving Asp254 (Table S1, Supplementary Materials). Other contributions, such as the loss of conformational entropy upon binding, clash penalties, and unfavorable ligand torsion angles are minor in comparison. This finding is consistent with earlier descriptions of SERCA's BHQ binding site as a predominately hydrophobic pocket buried in the transmembrane domain of the enzyme [25, 28, 37]. It should be noted that the docking-predicted orientation of **9** is similar but not identical to that obtained by Michelangeli and coworkers using a different program [23].

The physical and chemical properties of the binding site and the docking-predicted binding poses are compatible with the experimentally observed trends in potency presented in Table 1. More specifically, the addition of small hydrophobic substituents at the phenol rings enhances affinity, likely through increased hydrophobic interactions. However, due to the finite size of the binding pocket, the space that is available to accommodate such substituents is limited and leads to a decrease in affinity once these groups become too large (**6** and **7**). As far as the potency trends of the inhibitors in Table 2 are concerned, they are also consistent with the picture of a predominately hydrophobic binding pocket of limited size. For instance, the SERCA affinity of compounds **1**, **13**, and **15**, which assume the same binding pose as **2-5**, **8**, and **9**, can be attributed to the non-polar substituents that they carry at their methylene bridge. Compound **16** is unique in that the connecting carbonyl bridge causes the entire molecule to adopt a planar shape, resulting in a binding pose not observed for any of the other inhibitors.

The docking-predicted binding poses of the “original” BP inhibitor **19** and the disinfectant hexachlorophene (**21**) – the most potent representatives in Table 3 – are unique and cannot be superimposed on those of the inhibitors discussed above (Fig. 3). Similar to the other BPs, however, both compounds form hydrogen bonds (**19** with Asp59/Asp254 and **21** with Asp59) and their docking scores are heavily dominated by hydrophobic interactions (Table S1, Supplementary Materials).

3.3. Inhibition of intracellular calcium ion uptake by BPs

In addition to the cell-free ATPase activity assays with rabbit SERCA, a selection of six inhibitors (**6-9**, **20**, **21**) was evaluated in human cells to determine if these compounds trigger the typical response expected for a SERCA inhibitor. For various cell types, it has been observed that upon exposure of the cell to a SERCA inhibitor, the intracellular Ca^{2+} concentration increases transiently within minutes, often followed by a slower decay back to the original concentration [22, 24]. The first phase of the response, the rapid rise of cytosolic Ca^{2+} concentration can be interpreted as a cessation of Ca^{2+} sequestration into the SR while Ca^{2+} release from the SR into the cytosol continues. Whereas this behavior of Ca^{2+} levels is a hallmark of inhibition of SERCA in its native environment, it should be noted that it does not constitute definite proof of its occurrence since the added compound could hypothetically trigger Ca^{2+} release from the SR by activating channels present in the SR membrane.

Human skin fibroblasts, a cell-line known to be sensitive to SERCA inhibitors such as thapsigargin [38], were loaded with the ratiometric dye Fura-2 [33] (Fig. 4A) and then exposed to inhibitors at 50 μM while monitoring the intracellular Ca^{2+} concentration. Fig. 4B shows a representative trace obtained for **8**, which can be described as a quick rise in intracellular Ca^{2+} levels immediately after inhibitor application, followed by a slower decay back to the original concentration. Whereas the initial rise occurred within one minute and was consistently observed for all inhibitors tested, the rate of the subsequent drop was variable and not always as rapid as shown in Fig. 4B. A few cells showed sustained elevated levels throughout the entire duration of the measurements, which lasted up to five minutes. In general, this type of response is typical for cells treated with phenolic and other types of SERCA inhibitors and has been observed in a variety of cell lines [11, 12, 22, 39]. The results presented here show that human skin fibroblasts, which have not been tested previously with BP-based SERCA inhibitors, behave in an identical fashion.

Overall, the results for the six BP-based SERCA inhibitors demonstrate that this compound class affects intracellular Ca^{2+} levels in the same way as other types SERCA inhibitors, which is compatible with inhibition of human SERCA *in vivo*. The results also suggest that BPs readily penetrate the plasma membrane and reach their target in the SR membrane without being deactivated by chemical reactions catalyzed by intracellular enzymes. Consequently, BP-based inhibitors may have the potential for being toxic since it has been established that elevated intracellular Ca^{2+} level cause cells to ultimately undergo apoptosis [40, 41].

Whether a given BP will have a serious impact on health not only depends on its potency, but also on the level of human exposure. The BP that has been investigated most extensively is **1** and the majority of studies agree that typical concentrations of **1** in human blood and urine are found in the low nanomolar range [42], even though some recent reports have questioned the accuracy of these numbers and claim that the exposure levels could be underestimated, particularly in urine [43, 44]. But even at elevated levels, the health hazard posed by the ability of **1** to inhibit SERCA appears insignificant since this BP is a rather weak inhibitor with an IC_{50} value above 100 μM (Table 1). Thus, the main concern regarding **1** remains its interference with estrogen receptors. However, the situation may be

very different for some of the more potent BPs, such as the flame retardant **9**, whose serum levels have been reported as high as 0.3 μ M [23, 45]. At such high concentrations, inhibition of SERCA by **9** could become a concern. Human exposure levels of many other BPs are presently unknown, which underscores the need for more studies to resolve this matter.

3.4. Summary and future directions

This study constitutes the first comprehensive SAR analysis of SERCA inhibition by BPs. With regard to enzyme/inhibitor interactions, nearly all active compounds are engaged in a hydrogen bond with Asp254 and carry moderately sized non-polar substituents, which undergo hydrophobic interactions with the hydrophobic interior of the inhibitor binding site. Some BPs display potencies in the low micromolar range, raising concerns about their safety for humans exposed to BPs at high levels. The SARs established in this study can help guide future searches for alternative BP-based raw materials in industrial processes that are safer than some in use today.

Whereas the ability of certain BPs to inhibit SERCA makes them a potential environmental and health hazard, this property can be exploited to develop new research tools for the study of SERCA's physiological functions. It has also been demonstrated that inactive TG-based SERCA inhibitors tethered to a heptapeptide can be activated by the action of a prostate cancer cell specific protease, PSA, thereby enabling the inhibitor to kill the cancer cell by SERCA inhibition [40, 41]. In principle, the same strategy of conveying specificity to prostate cancer cells should be applicable to other SERCA inhibitor classes as well, including BPs. Having available an arsenal of structurally different SERCA inhibitors could help overcome potential drug resistance developed against one particular inhibitor type, a common challenge encountered in cancer chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from National Center for Research Resources (5P20 RR016481-12) and the National Institute of General Medical Sciences (8P20 GM103436-12 and 1R15GM084431-01). We thank Dr. Patrick Schultheis for making available his fluorescence imaging system and Dr. Robert Kempton for critically reading the manuscript.

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Scheme 1.
Chemical structure of bisphenol A

Figure 1. Representative results of inhibition assays, showing the effects of introducing methyl groups at the bisphenol A scaffold on SERCA activity. ATP hydrolysis rates were measured in coupled assays at varying inhibitor concentrations. ▲: **1** (bisphenol A), ■: **2** (dimethylbisphenol A), ●: **3** (tetramethylbisphenol A). Lines represent fits of the data to a three-parameter logistic equation.

Figure 2.

Docking-predicted binding poses and enzyme/inhibitor interactions. A: Common preferred binding poses assumed by **1** (yellow), **2** (cyan), **3** (grey), **4** (magenta), **5** (green), **8** (black), **9** (pale red), **13** (red), and **15** (orange). B: Detailed view of interactions between **4** and SERCA. BHQ is shown in blue as a reference. The pale blue cylinder with dotted lines represents the hydrogen bond to Asp254. Residues are colored according to the transmembrane helix on which they reside. C: Schematic summary of SERCA/inhibitor interactions for **4** (green dotted line: hydrogen bond; blue shades: ligand and residue exposure). D: Surface of the binding site color-coded according to hydrophobicity, showing the hydrophobic nature of the interior of the BP binding site (green: hydrophobic, purple: hydrophilic).

Figure 3. Docking-predicted binding poses for BPs **19** (A) and **21** (B). The pale blue cylinders with dotted lines represent hydrogen bonds.

Figure 4.

Upper panel: Human skin fibroblast loaded with the ratiometric Ca^{2+} -sensitive dye Fura-2. Lower panel: Representative fluorescence imaging experiment with the inhibitor tetrachlorobisphenol A (**8**). At the time point indicated (arrow), inhibitor dissolved in DMSO (50 μM final concentration) was added to cells bathed in loading buffer. The dotted line represents a control run with DMSO only.

Table 1

Chemical structure and inhibitory potencies of BPs: effects of ring substitution patterns.

#	R ¹	R ²	R ³	IC ₅₀ / μM
1	-H	-H	-OH	140 ± 20
2	-CH ₃	-H	-OH	24 ± 1
3	-CH ₃	-CH ₃	-OH	2.6 ± 0.3
4		-H	-OH	2.2 ± 0.3
5		-H	-OH	3.4 ± 0.4
6		-H	-OH	94 ± 17
7		-H	-OH	91 ± 9
8	-Cl	-Cl	-OH	5.5 ± 1.4
9	-Br	-Br	-OH	4.7 ± 1.1
10	-NH ₂	-NH ₂	-OH	inactive
11	-Br	-Br		inactive
12	-Br	-Br		inactive

Table 2

Chemical structure and inhibitory potencies of BPs: effects of ring substitution patterns: effect of chemical composition of the connecting bridge.

 1 140 ± 20 μ M	 13 90 ± 50 μ M	 14 inactive
 15 27 ± 7 μ M	 16 200 ± 80 μ M	 17 inactive

Table 3

Chemical structure and inhibitory potencies of BPs: compounds with a methylene bridge and hydroxyl groups in its immediate proximity.

#	R ¹	R ²	R ³	IC ₅₀ / μM
18	-H	-CH ₃	-H	300 ± 30
19	- <i>tert</i> -butyl	-CH ₃	-H	3.6 ± 1.2
20	-Cl	-Cl	-H	31 ± 6
21	-Cl	-Cl	-Cl	4.0 ± 1.9

Table 4

Chemical structure and inhibitory potencies of BPs that are not part of a congeneric series.

 22 inactive	 23 $2.8 \pm 1.0 \mu\text{M}$	 24 inactive
 5 $16 \pm 4 \mu\text{M}$	 26 $25 \pm 17 \mu\text{M}$	 27 inactive