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Design, synthesis, and biological evaluation of lipophilically modified bisphenol Z derivatives

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Conflict of Interest

No conflict of interest exists for any of the authors.

Abstract

In the present study, a small library of bisphenol Z (BPZ) derivatives was synthesized and investigated for anti-proliferative effects in cultured breast and glioblastoma cell lines. Synthesized BPZ derivatives varied in molecular size, polarity, and lipophilicity. Of the 8 derivatives tested, compounds **4** and **6**, both of which displayed the highest degree of lipophilicity, were most active at inducing cell death as determined by the XTT assay. Cell membranes were interrogated using trypan blue staining and were shown to remain intact

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during treatments with **4** and **6**. Activation of caspase enzymes (3 and/or 7) was noted to occur following treatment with compound **4**. Polar BPZ derivatives, those with a substituted amine or alcohol, were devoid of any inhibitory or proliferative effects. The remaining derivatives seem to lack sufficient lipophilicity to execute an overt toxic effect. Our results suggest that increasing the lipophilic character of BPZ enhances the cytotoxic effects.

1. Introduction

Bisphenols, including bisphenol A (BPA), are a class of diverse molecules bearing phenolic residues joined by varied linkers. Bisphenol A is the most famous bisphenol derivative as it has been used extensively in industry, primarily in polymerization reactions to form plastics. The synthesis of BPA is relatively straight-forward and can be accomplished by an electrophilic aromatic substitution reaction using acetone and phenol under acid catalysis (Fig. 1) (Dermer, 1983).

Over the past decade, negative effects due to BPA exposure have been reported showing that BPA possess endocrine modulatory effects (de Andrade et al., 2017; Gassman, 2017; Izzotti et al., 2010; Jenkins, Betancourt, Wang, & Lamartiniere, 2012; Lamartiniere, Jenkins, Betancourt, Wang, & Russo, 2011; Lee et al., 2014; Matsumoto, Adachi, & Suzuki, 2005; Rochester & Bolden, 2015). The published data surrounding this effect in exposed populations has led to the withdrawal of BPA from numerous commercial products including plastic water bottles, infant formula bottles, as well as plastic storage containers for fear of BPA leaching into foodstuff. The endocrine modulatory effects of BPA may be a result of the molecule's structural resemblance to estradiol. Research has shown that BPA can act as an estrogen receptor agonist or antagonist and has the capacity to elicit numerous "estrogen-like" effects in cultured cells (Gassman, 2017; Izzotti et al., 2010; Suzuki, Ide, & Ishida, 2001; Washington, Hubert, Jones, & Gray, 2001).

Previous research on BPA and other bisphenol analogs have shown that across all derivatives, a proliferative effect is induced at micromolar concentrations in cell lines which express estrogen receptors (Matsumoto et al., 2005; Mesnage et al., 2017). Importantly, as the concentration of bisphenol derivative was increased, cell viability was shown to decrease. One interesting observation from these studies is that of three bisphenols (BPA, BPF, and BPZ), the more lipophilic the derivative, the greater the cytotoxic effect at higher concentrations (BPF < BPA < BPZ) (Mesnage et al., 2017). The results from this study showed that bisphenol Z (BPZ) displayed the greatest degree of cytotoxicity among the bisphenol analogs tested and may be in part due to its enhanced lipophilic character relative to BPA or BPF (CLogP of 5, 3.6, and 2.8 respectively). This same study demonstrated that the addition of a true estrogen receptor antagonist, ICI 182,780, decreased the proliferative effects of bisphenol analogs. The addition of an antagonist and the removal of proliferative effects strongly indicated that BPZ may be acting as an agonist of estrogen receptors.

In addition to their estrogenic effects, BPA and its derivatives have been shown to modulate PPAR-γ signaling and can also induce caspase-dependent apoptotic pathways in cell culture (Fehlberg, Gregel, Goke, & Goke, 2003; Terasaka, Kadoma, Sakagami, & Fujisawa, 2005). Numerous bisphenol derivatives have been synthesized as BPA substitutes and unfortunately also possess similar endocrine disrupting effects. Simple alterations in the linker between the two phenolic rings is insufficient to prevent hormone receptor activation as seen with bisphenol S and bisphenol F (Rochester & Bolden, 2015). BPZ can be synthesized in a similar manner to BPA by substituting cyclohexanone for acetone (Fig. 2) (Gregor, 2012).

2. Methods and Materials

Given the dichotomy in BPZ activity, this study focused on the design, synthesis and evaluation of both polar and non-polar enhancing modification made to BPZ and their effect on proliferation in breast (MCF-7) and glioblastoma (A-172) cell lines. The present small library focused attention on the 4-position of the cyclohexanone ring used as the phenol linker. Compounds were synthesized in one or two steps with reasonable yields. Synthesized compounds were then screened against the aforementioned cell lines as both culture lines express estrogen receptors and are sensitive to estrogen receptor antagonists (Bardon, Vignon, Derocq, & Rochefort, 1984; He, Liu, Yang, & Yuan, 2015).

Bisphenol Z derivatives were synthesized from phenol and the appropriate cyclohexanone derivative under acidic conditions. Analog **1** was synthesized as shown in Scheme 1a using 4-aminocyclohexanone (1 mmol) dissolved in concentrated HCl followed by the addition of 2.5 mmol phenol and stirred at room temperature overnight. Product was precipitated with ice cold water and filtered. Additional drying in an oven at 100°C removed excess phenol. This synthetic procedure was also used for the preparation of analogs **2**, **5**, **6**, and **8**. For the synthesis of derivatives **4** and **7** (Scheme 1b), analog **1** was dissolved in dry DMF and treated with triethylamine followed by acylation using hexanoyl chloride or palmitoyl chloride at room temperature overnight. Purification of analogs **4** and **7** was done using a 1000 Micron preparative plate with dichloromethane: ethyl acetate (90: 10) as the mobile phase. All compounds were characterized using ^{1}H NMR and high-resolution mass spectrometry. Details on characterization are provided in the supporting information.

The in vitro cytotoxicity of BPZ and synthesized derivatives was measured in A-172 cells and MCF-7 cells using the XTT assay. Briefly, cells were seeded at a concentration of 5,000 cells per well and allowed to equilibrate overnight. The following day, BPZ derivatives were applied in concentrations ranging from 1 to 100 µmol and cells were incubated for 24

hours. The XTT proliferation indicator was added the following day after washing cells and measured per manufacturer instructions on a BioTek Cytation 3 microplate reader.

To assess the mechanism of cytotoxicity for the lead compounds (**4** and **6**), the luminescence-based Caspase-Glo® 3/7 (Promega) assay was conducted in MCF-7 and A-172 cells. Briefly, as per the manufacturer's protocol, cells were plated at a density of 5,000 cells per well and allowed to equilibrate overnight followed by next day treatment with compounds **4** and **6** at 2 or 3 times the calculated IC_{50} values for 12 hours. Camptothecin and cisplatin served as positive controls for the Caspase-Glo® 3/7 assay and provided statistically significant increases in caspase 3/7 activities (data not shown). Luminescence signal as a result of caspase 3 and/or 7 activation was measured on a BioTek Cytation 3 plate reader. Activation of caspase 3 and/or 7 induced by compounds **4** and **6** was expressed in terms of fold change relative to vehicle-treated cells. Statistical significance was determined using GraphPad Prism and a p-value <0.05 represented statistically significant elevation in caspase 3/7 activity (compared to vehicle-treated cells) by the treated compounds.

3. Results and Discussion

Of the analogs tested in the XTT assay, those which appear to have the highest degree of lipophilic character, specifically compounds **4** and **6**, were clearly the most active at inhibiting cell growth in both the A-172 cell line and the MCF-7 cell line. The concentration response curves for all tested derivatives are shown in Figure 3. The IC_{50} values for all tested compounds were calculated and are presented in Table 1 along with their structural modification to the 4-position and the respective CLogP values. Substituents made to the 4-position on the cyclohexane ring of BPZ which were polar or of less lipophilic significance failed to induce any detectable cytotoxic effect or inhibition of growth. Molecules which failed to inhibit growth or induce cell death did not induce proliferation either. We

speculate that the derivatives devoid of activity are excluded from the cells as a consequence of their polarity. Enhanced lipophilic character may contribute to activity by allowing diffusion through membranes followed by an intracellular event which culminates in cell death. Previous studies surrounding bisphenol analogs parallels ours in that as the lipophilicity increases within the bisphenol scaffold, cytotoxic effects increase. The cause of this enhanced cytotoxic effect could point to additional lipophilic interaction with intracellular receptors including the estrogen receptor as a consequence of the substituent coming off the 4-position of the cyclohexane ring. It will be important to determine the nature of the interaction with the estrogen receptors or other receptors of interest.

The observed cell death occurring in A-172 cells and MCF-7 cells is not occurring through a non-specific membrane damaging effect. Trypan blue, a vital dye which is excluded from cells with intact membranes, was used to probe membranes in A-172 and MCF-7 cells after a 6-hour treatment with **4** and **6** at twice the calculated IC_{50} concentration. Relative to acetone-permeabilized controls which took up and retained trypan blue, no significant dye retention was detected in either cell line post-treatment (Fig. 4). Furthermore, preliminary mechanistic studies revealed that the cytotoxicity observed with compound **4** involves the possible activation of caspase-3 and -7. As shown in Table 2, compound **4** treatment led to a statistically significant (9-fold) increase in executioner caspase activity in A-172 cells and a mild yet statistically significant activation of executioner caspase in MCF-7 treated cells. It is possible that the functional deficit of caspase 3 in MCF-7 cells contributed to a much lower signal in the luminescence assay relative to the signal generated in A-172 cells (Jänicke, Sprengart, Wati, & Porter, 1998). Interestingly, our preliminary data did not reveal the activation of caspases-3 or -7 by compound **6.** The lack of significant caspase-3 or -7 activation by compound **6** in this assay does not completely rule out apoptosis as a mechanism of cell death, but may point to an alternative pathway to apoptosis. Studies have

shown that the apoptotic cell death pathway can be achieved despite no net change or an actual reduction in caspase-3 or -7 activation (Peng et al., 2009; Sagar et al., 2014). In lieu of these previous reports we would like to investigate the possible involvement of alternate pathways of cell death in compound **6** treated cells.

We have synthesized a small library of BPZ derivatives with variable lipophilic character. Those with the highest degree of lipophilicity exerted an observable decline in cell proliferation with IC_{50} values in the 30 μmol range while those bearing hydrophilic or minor lipophilic character relative to BPZ were essentially devoid of activity. Additional derivatives are being synthesized which present lipophilicity in a compact manner including an adamantane derivative, planar derivatives including a naphthoyl and piperonyl derivative, and a longer chain fatty acylated derivative using stearoyl chloride. This will help determine if overall lipophilicity or the presentation of the lipophilic group governs activity. One additional aspect to consider is cellular localization after treatment. To determine where BPZ derivatives may accumulate post-treatment, we plan to synthesize a fluorescent derivative using **1** and dansyl chloride to allow for fluorescence microscopy.

It is important to note limitations to our study. The synthetic library overall is comparatively small yet represents a range of molecules bearing extremes in terms of lipophilic character. The synthesis and testing of additional hydrophobic and hydrophilic analogs, as suggested previously, will help to elucidate the overall impact of a bisphenol's lipophilic character on cytotoxic activity. We are in the process of identifying a mechanism to account for the observed cytotoxicity. We have ruled out loss of membrane integrity as a mechanism and are exploring numerous potential causes for cell death. Moving forward, Western blotting for activated caspase enzymes may provide insight for determining a possible mechanism to help explain the observed cell death after treatment with compound

4 or **6**. It will also be important to investigate the role of poly-ADP ribose polymerase (PARP) as a potential mechanism for cytotoxicity.

Figure Legends

Figure 1. Acid-catalyzed synthesis of BPA from phenol and acetone.

Figure 2. Chemical structure of bisphenol Z.

Figure 3. Concentration response curves of A-172 and MCF-7 cell lines following 24-hour incubation with indicated compounds. Results are presented as percent of vehicle control (DMSO). Data points are representative of mean ± SEM of three independent experiments ran in quadruplicate.

Figure 4. Effect of compounds **4** and **6** on membrane integrity as measured by trypan blue dye exclusion. Membranes were still intact following 6-hour incubation with compounds **4** and **6** in the A-172 and MCF-7 cells lines. Data points are representative of mean ± SEM of three independent experiments ran in quadruplicate. Fifteen-minute incubation with ice-cold acetone was used as a positive control. * denotes significance between DMSO and indicated treatment group at $p < 0.05$ (One-Way ANOVA, Dunnett's multiple comparison test).

Scheme 1. Synthesis of aliphatic and acylated bisphenol Z derivatives. (1a) A one-step reaction was used as displayed to synthesize the aliphatic substituted BPZ derivates. (1b) A two-step reaction was used as displayed to synthesize acylated BPZ derivatives via initial synthesis of 4-amino bisphenol Z followed by fatty acid acylation.

Table 1. Bisphenol Z derivatives and respective IC_{50} values. Compounds **1** and **8** did not display activity at 100 μM and were not evaluated using complete response curves. The IC_{50} values represents the mean ± SEM of three independent experiments ran in quadruplicate. CLogP values were calculated using ChemDraw Ultra.

Table 2. Influence of compound **4** and **6** on caspase activation. Compound **4** and **6** were applied to cells at 2 to 3 times the calculated IC_{50} value and incubated for 12 hours. Data represent the fold increase of caspase activation relative to untreated control as determined by

the Caspase®Glo 3/7 assay. Compound **6** failed to influence caspase-3 or -7 activity in both cell lines tested. The values represents the fold increase ± SEM of quadruplicate treatments. Statistical significance was determined using one-way ANOVA and Dunnett's multiple comparison test. A p-value <0.05, denoted with an * represented statistically significant elevation in caspase-3/7 activity compared to vehicle treated cells.

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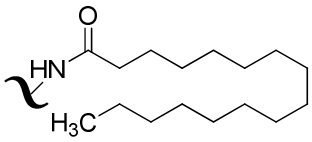
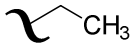
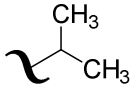
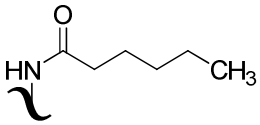
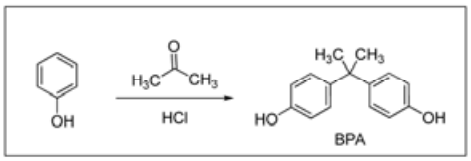
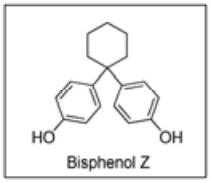
Compound	-R	CLogP	A-172 IC_{50} (µM)	MCF-7 IC_{50} (µM)
BPZ	-	4.9	67 ± 1.5	60 ± 2.0
1	$-NH_2$	2.9	-	-
2	$-CH_3$	5.5	70 ± 2.0	62 ± 1.6
4		9.9	27 ± 0.9	53 ± 2.8
5		6	48 ± 1.8	60 ± 1.9
6		6.4	35 ± 1.1	33 ± 1.7
7		4.7	47 ± 5.9	112 ± 3.6
8	-OH	2.9	-	-

Table 1. Bisphenol Z derivatives and respective IC_{50} values. Compounds **1** and **8** did not display activity at 100 µM and were not evaluated using complete response curves. The IC_{50} values represents the mean ± SEM of three independent experiments ran in quadruplicate. CLogP values were calculated using ChemDraw Ultra.

Treatment	A-172	MCF-7
4	9.34 ± 1.54*	1.23 ± 0.04*
6	-	-

Table 2. Influence of compound **4** and **6** on caspase activation. Compound **4** and **6** were applied to cells at 2 to 3 times the calculated IC_{50} value and incubated for 12 hours. Data represent the fold increase of caspase activation relative to untreated control as determined by the Caspase®Glo 3/7 assay. Compound **6** failed to influence caspase-3 or -7 activity in both cell lines tested. The values represents the fold increase ± SEM of quadruplicate treatments. Statistical significance was determined using one-way ANOVA and Dunnett's multiple comparison test. A p-value <0.05, denoted with an * represented statistically significant elevation in caspase-3/7 activity compared to vehicle treated cells.





Bisphenol Z