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New hydroxystilbenoid derivatives endowed with neuroprotective activity and devoid of interference with estrogen and aryl hydrocarbon receptor-mediated transcription

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ABSTRACT

We have synthesized a series of new (*E*) stilbenoid derivatives containing hydroxy groups at ring positions identical or similar to those of *trans*-resveratrol and bearing one or two bulky electron donating groups *ortho* to 4′-OH and we have evaluated their neuroprotective activity using glutamate-challenged HT22 hippocampal neurons to model oxidative stress-induced neuronal cell death. The most active derivatives, 5-{(*E*)-2-[3,5-bis(1-ethylpropyl)-4-hydroxyphenyl]ethenyl}-1,3-benzenediol (**2**), 5-[(*E*)-2-(3,5-di-*tert*-butyl-4-hydroxyphenylethenyl)]-1,3-benzenediol (**4**) and 5-{(1*E*,3*E*)-4-[3,5-bis(1-ethylpropyl)-4-hydroxyphenyl]-1,3-butadienyl}-1,3-benzenediol (**6**), had EC_{50} values of 30, 45 and 12 nM, respectively, and were ca. 100 to 400-fold more potent than resveratrol. Derivatives **2**, **4** and **6** lacked cytotoxic activity against HT22 cells and estrogen receptor agonist or antagonist activity in estrogen response element-dependent gene expression and in estrogen-dependent proliferation of MCF-7 human breast cancer cells. In addition, they were incapable of interfering with aryl hydrocarbon receptor-mediated xenobiotic response element-dependent gene expression. Derivatives **2**, **4** and **6** might assist in the development of lead candidates against oxidative stress-driven neurodegenerative diseases that will not increase endocrine cancer risk nor affect drug activation and detoxification mechanisms.

1. Introduction

Neuronal cell death from oxidative stress is a strong component of many neurodegenerative diseases.[1] Onset and/or progression of these diseases are primarily attributed to reactive oxygen species (ROS). These form part of the metabolic cycle of oxygen and are continuously generated in low amounts in cells where they participate in cellular signaling.[2] Aberrant upregulation of ROS production can affect cell viability by causing oxidative modification of proteins, DNA chain breaking, lipid peroxidation, mitochondrial dysfunction and cell death.[3] The central nervous system is particularly vulnerable to damage by ROS for many reasons, including high oxygen consumption rates and high concentrations of iron and unsaturated fatty acids as well as low levels of natural antioxidants.[4]

Many natural antioxidants are polyphenols. This category includes hydroxystilbene derivatives and, in particular, *trans*-resveratrol (*trans*-3,5,4′ trihydroxystilbene), one of the most interesting plant-derived polyphenols.[5] The antioxidant activity of resveratrol has been confirmed both in vivo and in vitro and many reports on its promising anti-inflammatory and cancer chemopreventive potential have appeared.[5,6] Resveratrol is also known to behave as mixed estrogen receptor (ER) agonist/antagonist.[7,8] Such ER modulators affect endocrine cancer risk in a tissue-dependent manner as already shown for the selective ER modulator (SERM) tamoxifen.[9] Cancer risk is increased by exogenous hormone mimics as well as endogenous hormones because estrogen induction of cell proliferation is known to increase the probability of random mutation errors.[10] Resveratrol and its derivatives reportedly also interfere with aryl hydrocarbon receptor (AhR)-mediated induction of phases I and II xenobiotic-metabolizing enzymes.[11,12] AhR is a ligand-activated transcription factor that binds a large variety of

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xenobiotics as well as an undisclosed number of endobiotics and regulates adaptation to metabolic and potentially toxic challenges. This occurs through regulation of the expression of a battery of metabolic enzymes that are targeted by the receptor via xenobiotic response elements (XRE) in their gene promoter.[13] In view of the very important roles of ER and AhR in physiology, toxicology and metabolic adaptation, interfering with ER and/or AhR signaling could have unexpected therapeutic implications and/or genotoxic side effects and impact cancer risk.[13,14] The aim of this study, therefore, was to design and synthesize new hydroxystilbene derivatives with strong neuroprotective activity and low ER and AhR agonist/antagonist activities.

The design of the new compounds with respect to neuroprotective activity was based on previous studies relating to the effects of ring substituents on the antioxidant activity of phenolic derivatives. According to these studies, the antioxidant capacity of hydroxystilbene derivatives and phenolic compounds in general is strongly dependent on the O–H bond dissociation enthalpy (BDE). Lowering the BDE of the phenolic O–H bond leads to stabilization of phenoxy radicals and this is very important for the function of antioxidants which is to intercept and react with free radicals at a rate faster than the substrate.[15] It is also known that the introduction of one or two bulky electron donating substituents *ortho* to the OH group leads to compounds having lower BDE and thus to generally better antioxidants.[16,17] The *Z*-isomers of hydroxystilbenes have been found to have BDE's 1.8 kcal/mol greater than those for the *E*-isomers and consequently the latter are better antioxidants.[18] In addition, the bulky substituents are expected to inhibit binding to AhR, since this receptor binds with high affinity rather planar molecules.[19] Moreover, compounds with bulky substituents often display low ER-binding affinity and/or estrogenic activity inspite of fulfilling most of the ER-binding ctiteria.[20,21]

We recently reported that 3′-*tert*-butyl-3,5,4′-trihydroxystilbene (**3**) binds weakly both ER subtypes, ERα and ERβ, and displays non-significant receptor agonist or antagonist activity at concentrations $\leqslant$1 μM.[21] In the present work we have synthesized three sets of stilbenoid derivatives with bulky alkyl substituents at positions *ortho* to 4′-OH (Table 1). The first set (compounds **1**–**4**) contains the resveratrol moiety. In the second set (compounds **5**–**9**) the resveratrol unit has been expanded by introducing an extra double bond between the aromatic rings. Finally, the third set consists of compounds **10** and **11**, in which the hydroxyl groups have been placed in the 4 and 4′ positions in the *E*-stilbene framework. All compounds have been designed to bear bulky electron donating groups, *tert*-butyl or 1-ethylpropyl *ortho* to 4′-OH (except for compound **9**) and the methodology used for their synthesis led exclusively to the desired *E*-isomers. Derivatives **1**–**11** and a selection of synthesized intermediates (**15**–**18**) were screened for effects on ER and AhR-mediated gene transcription using recombinant reporter cell lines and for estrogenic/antiestrogenic activity using endometrial and breast adenocarcinoma cells. In addition, they were tested for neuroprotective activity using HT22 hippocampal neurons. Glutamate-challenged HT22 cells undergo oxidative stress-induced cell death (oxytosis) due to glutamate blockage of cystine/glutamate antiporters leading to inhibition of cystine uptake and depletion of intracellular glutathione.[22] Oxytosis of HT22 cells was shown to faithfully mimic molecular traits of oxidative cytotoxicity in neurodegenerative disorders.[23] Importantly, compounds screened positive in preventing oxytosis of HT22 cells tested positive in reducing stroke-induced brain damage and behavioral defects in rats and rabbits.[24,25]

2. Chemistry

Stilbenoid derivatives **1**–**11** were obtained from the demethylation of the corresponding methoxy-stilbene derivatives **14a–k** synthesized using the Horner–Wadsworth–Emmons reaction. Thus coupling of the respective aldehydes with the appropriate phosphonate in the presence of potassium *tert*-butoxide in DMF generated exclusively the *E*-isomers (no *Z*-isomer could be detected by ^{1}H NMR) (Scheme 1). Cleavage of the methoxy groups was best achieved using MeMgI at 160 °C for 1 h[26]—other conditions, such as BBr_3[27] or 9-iodo-9-BBN[28] led to low yields of isolated material or messy reaction mixtures. Methylmagnesium iodide, however, cleaved only the less hindered methoxy groups, leaving derivative **14k** intact and generating monomethoxy-stilbene derivatives **15** and **16** from **14d** and **14h**, respectively (Scheme 2). The methoxy groups flanked by two bulky *tert*-butyl groups could be successfully cleaved using sodium propylmercaptide[29] in DMF at 100 °C. Although mixtures of compounds were obtained from the demethylation of **14d** and **14k**, increased reaction times and/or addition of further equivalents of NaSPr were not investigated since the mixture could be readily separated and the monomethylated derivatives **17** and **18** could also be evaluated for their antioxidant activity. Demethylation of compound **14h** was not attempted using NaSPr and intermediate **16** was used instead to obtain resveratrol analog **8** (Scheme 3).

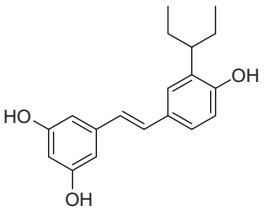
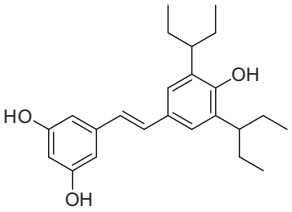
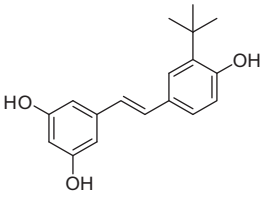
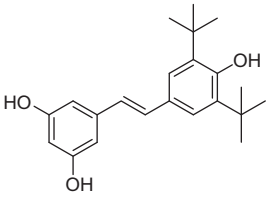
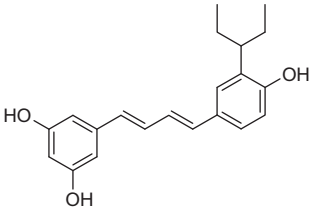
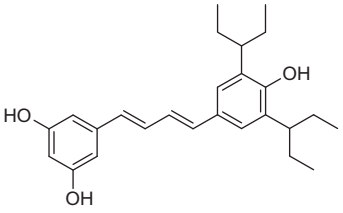
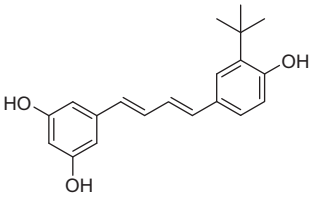
3. Results and discussion

When challenged with glutamate, HT22 cells suffer oxytosis within 24 h as a result of accumulation of ROS due to glutathione depletion.[22] Figure 1 shows a representative assessment of the neuroprotective activity of derivatives **1**–**11** as compared to resveratrol. Evidently, all the derivatives displayed potencies ($EC_{50} \leqslant 3.3$ μM) that are significantly (*t*-test; $p \leqslant 0.05$) higher than resveratrol (Table 1, columns 3 and 4). The synthesized monomethoxy intermediates (**15**–**18**) were also tested for comparison. Intermediate **18** displayed EC_{50} >10 μM and is considered inactive. The rank order of potencies, **6** > **2** ⩾ **4** ⩾ **5** ⩾ **7** ⩾ **8** ⩾ **17** ⩾ **11** ⩾ **16** ⩾ **1** ⩾ **3** ⩾ **15** ⩾ **10** > **9** > res, comprises derivatives with two (**6** > **5** ⩾ **7** ⩾ **8** > **16** > **9** > res) or one alkenic double bond (**2** ⩾ **4** > **17** ⩾ **11** ⩾ **1** ⩾ **3** ⩾ **15** ⩾ **10** > res), the latter comprising 4,4′-dihydroxy derivatives (**11** ⩾ **10** > res) and derivatives with three OH groups (**2** ⩾ **4** > **17** ⩾ **1** ⩾ **3** ⩾ **15** > res). Derivative **6** displayed the highest potency among all compounds tested, while **2** ranked top among derivatives with one double bond. The most active derivatives, defined here as having EC_{50} higher than **6** by a factor $\leqslant$10, were **2**, **4**, **5**–**8**.

The rank order of derivatives with one alkenic double bond shows that the contribution to antioxidant activity of two ethylpropyl and *tert*-butyl substituents *ortho* to 4′-OH is higher than that of one *o*-ethylpropyl and *o*-*tert*-butyl substituent, presumably as a result of higher stabilization of the respective 4′-aryloxyl radical which is generated in the reaction with ROS.[18,30] Alkyl substituents *ortho* to 4′-OH reportedly stabilize the aryloxyl radical via delocalization of the unpaired electron through hyperconjugation with the substituents.[18,30] That **15** was less potent than **4** by a factor of 14 suggests that 4′-OH is requisite for high antioxidant activity. However, **15** was more potent than resveratrol by a factor of 7.5, implying that 3-OH and 5-OH contribute to antioxidant activity independently of 4′-OH. This is in accordance with previous findings that 4′-OH is not the sole determinant of antioxidant activity of resveratrol.[31,32] The contribution of the 3-OH/5-OH pair to antioxidant activity is also reflected in that **17** was less potent than **4** by a factor of 5.8.

The rank order of derivatives with two alkenic double bonds shows again that the contribution of two ethylpropyl substituents to antioxidant activity is higher than that of one such substituent. However, the contribution of two *o*-*tert*-butyl substituents is not in this case higher than that of one such substituent. In fact, **8** was marginally less active than **7**, in accordance with findings that

Table 1
Lipophilicity (*C* log *P*) and neuroprotective potency of the stilbenoids

Compound	*C* log *P*[a]	EC_{50}[b] (nM)	Relative potency[c]	LDH released[d] (% total)
 **1**	5.12	432 ± 126	11	−1.0 ± 0.6
 **2**	7.40	30 ± 5	156	−6.7 ± 3.8
 **3**	4.56	484 ± 188	9.6	−6.0 ± 1.2
 **4**	6.29	45 ± 22	104	−6.7 ± 2.6
 **5**	5.32	79 ± 10	59	−5.0 ± 2.3
 **6**	7.61	12 ± 3	389	−6.0 ± 4.0
 **7**	4.76	91 ± 8	51	−8.7 ± 3.8

(*continued on next page*)

Table 1 (*continued*)

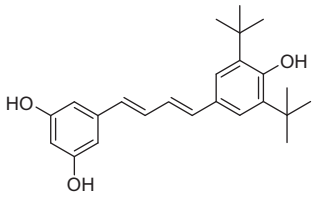
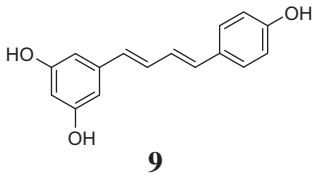
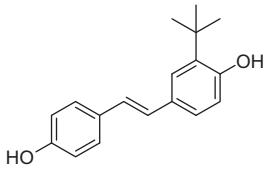
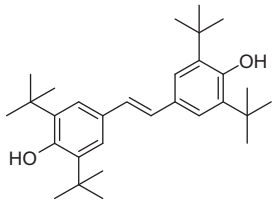
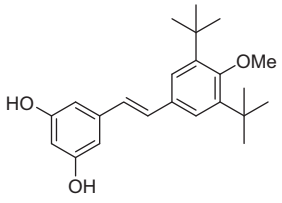
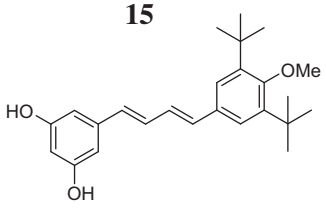
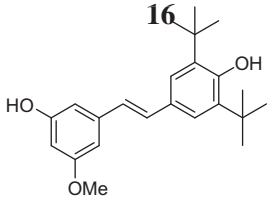
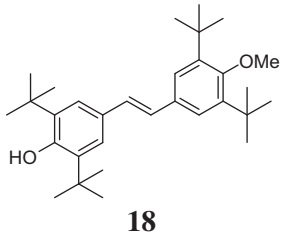
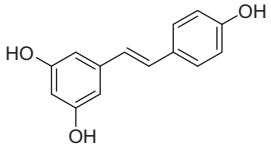
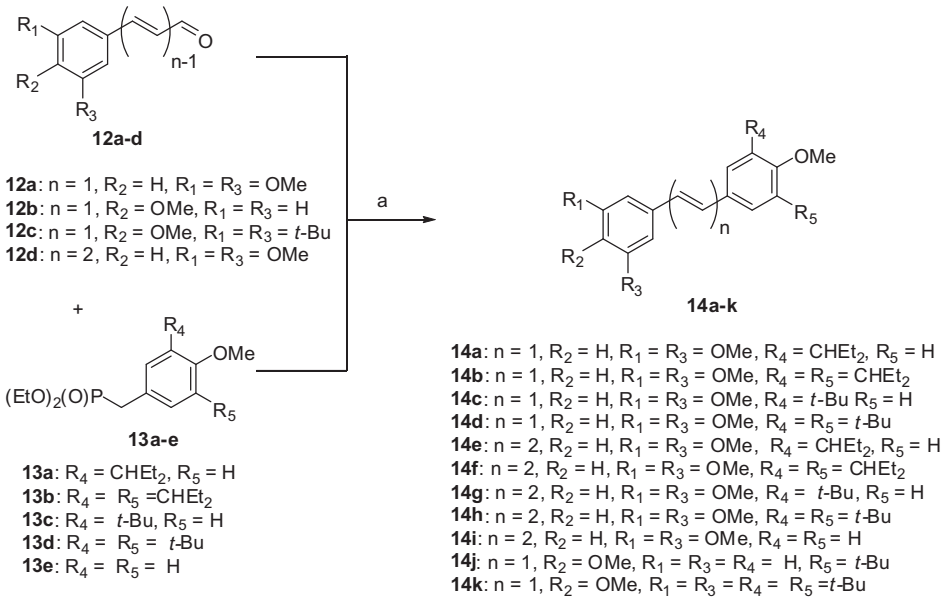
Compound	*C* log *P*[a]	EC_{50}[b] (nM)	Relative potency[c]	LDH released[d] (% total)
 **8**	6.49	119 ± 31	39	−5.3 ± 2.6
 **9**	3.04	3310 ± 383	1.4	8.0 ± 2.6
 **10**	5.23	1060 ± 156	4.4	−5.3 ± 0.9
 **11**	10.40	399 ± 151	12	1.3 ± 0.9
 **15**	7.07	621 ± 143	7.5	nd
 **16**	7.27	410 ± 42	11	nd
 **17**	7.05	258 ± 93	18	nd
 **18**	11.19	>10,000	<1	nd

Table 1 (*continued*)

Compound	*C* log *P*[a]	EC_{50}[b] (nM)	Relative potency[c]	LDH released[d] (% total)
 **Resveratrol**	2.83	4667 ± 200	1.0	4.0 ± 1.7

LDH: lactate dehydrogenase.
nd: not determined.
[a] Calculated using ChemDraw version 10.0.
[b] EC_{50} values are test compound concentrations required to maintain viability of glutamate-challenged HT22 cells to a level equal to 50% of that of not challenged cells. Values are mean ± SEM of at least three independent experiments.
[c] Relative potency was calculated by ($EC_{50\ resveratro}/EC_{50\ compound}$).
[d] LDH released in the cell culture supernatant is a measure of cumulative cell death following incubation with test compounds (1 μM) for 3 days. LDH released in the presence of vehicle alone (innate cytotoxicity) was substracted from the values shown. Data are mean ± SEM of three independent experiments.



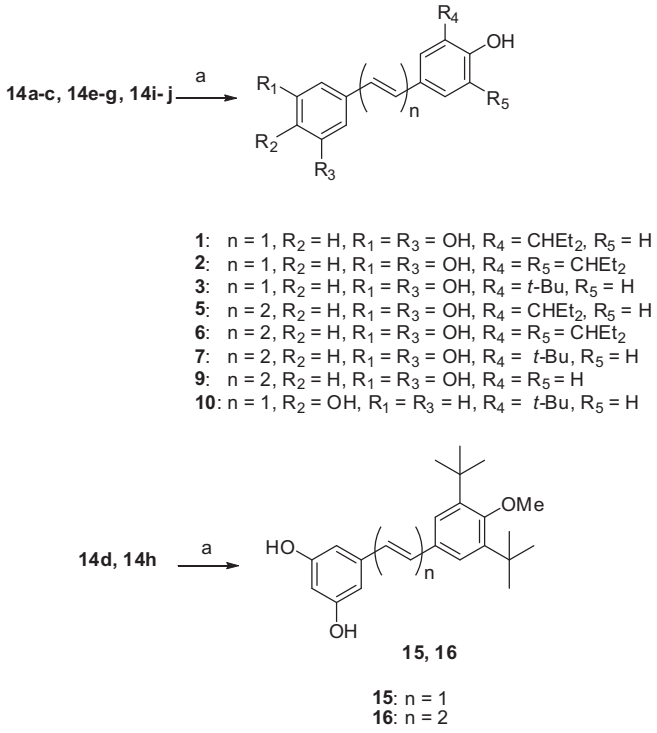
Scheme 1. Reagents and conditions: (a) *t*-BuOK, DMF, rt.

increasing steric crowding around the 4′-OH reduces antioxidant activity.[33] That **16** was 3.4-fold less potent than **8** is in line with 4′-OH being an important determinant of antioxidant activity,[30] while the observation that **16** was eightfold more potent than **9** suggests again that 3-OH and 5-OH in combination with the *tert*-butyl substituents contribute to antioxidant activity independently of 4′-OH. The higher potency of **9** as compared to resveratrol indicates that the second double bond also has a small but significant contribution to antioxidant activity. In summary, the data of Table 1 show that introduction of one ethylpropyl or *tert*-butyl group *ortho* to 4′-OH, especially in combination with a second double bond, increased activity dramatically and that introduction of a second *o*-ethylpropyl group increased activity even further, while this was not the case with introducing a second *o*-*tert*-butyl group in combination with a second double bond.

Table 1 also lists the *C* log *P* values of all the compounds we tested for neuroprotective activity. Derivatives exhibiting relative potency ⩾100, namely **2**, **4** and **6**, can be used to deduce an optimum *C* log *P* range of 6.3–7.6, presumably reflecting a compromise between the role of alkyl substituents in compound solubility and/or permeability through cell membranes and in ROS scavenging activity. This range is higher than *C* log *P* of around 5 which is usually sufficient for most drug applications. This may be rationalized through the central role that mitochondrial damage plays in neuronal cell death.[3] Lipophilic antioxidants are expected to be more effective in protecting neuronal cells from oxidative stress-induced damage since they are more capable of reaching sites of ROS production inside mitochondria.[1,3,34]

We also evaluated the effects of derivatives **1**–**11** and resveratrol on the viability of HT22 cells by measuring lactate dehydrogenase (LDH) release in the supernatant of cell cultures treated with the derivatives for 3 days during which the cells are known to execute nearly three cell cycles. Table 1 (column 5) shows that **9**, **11** and resveratrol had a marginal effect (⩽8%) on cell viability, while the remaining derivatives suppressed innate cell death by about 50%. Similar effects were observed whether compounds were tested at 1 or 10 μM.

We next focused our attention on testing whether the derivatives affect ER-dependent gene expression and/or proliferation and viability of estrogen-dependent breast and endometrial adenocarcinoma cells. Derivatives were tested at concentrations up to 1 μM. Biological effects observed in experiments with cultured



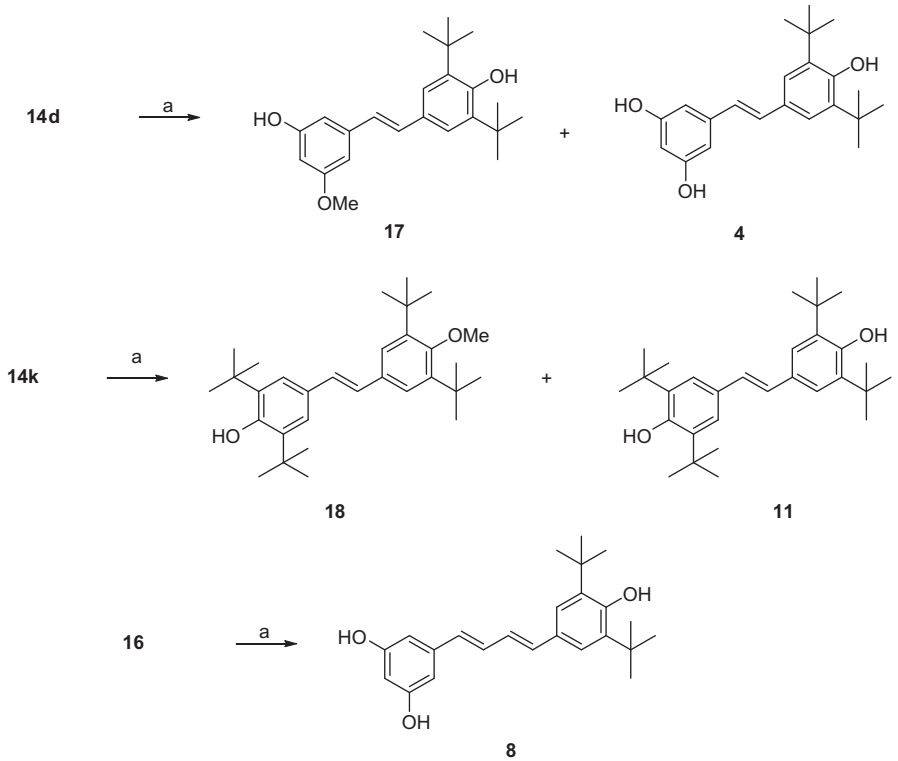
Scheme 2. Reagents and conditions: (a) MeMgI, 160 °C.

cells at concentrations greater than this are probably of little practical interest. Pharmacokinetic studies suggest that, due to extensive metabolism, resveratrol bioavailabilities of 1 μM are practically unattainable even with a daily intake as high as 2 mg per kg of body weight. [5,35]

Initially, we assessed whether **1**–**11** can bind to ERα or ERβ. Successful ER ligands often have two OH groups with an O–O distance considerably higher than that of 17β-estradiol (estradiol).[36,37] Table 2 shows the O–O distance and relative (to estradiol) binding affinity values (relative binding affinity, RBA) of **1**–**11**. The values of resveratrol and the full ER antagonist ICI182,780 (fulvestrant) are also shown for comparison. Evidently, the RBAα and RBAβ of **1**–**11** and resveratrol are fairly modest (RBA <2) as well as comparable. That resveratrol binds ERα and ERβ with affinities much lower than estradiol is in agreement with other reports.[7]

Next we assessed whether **1**–**11** affect estrogen responsive element (ERE)-dependent gene transcription via ERα and ERβ using MCF-7:D5L and HEK:ERβ cells, respectively, as described previously.[38] Treatment of estrogen-free MCF-7:D5L cells with 0.1 nM estradiol resulted in full (~4-fold) induction of the stably transfected luciferase reporter gene.[38] By comparison, **1**, **5**, **7** and **10** behaved as partial agonists, whereas resveratrol and the other derivatives had no effect (Table 3, column 2). Treatment of estradiol-supplemented MCF-7:D5L cells with 1 μM ICI182,780, suppressed hormonal induction of luciferase expression, while none of the derivatives had any effect (Table 3, column 3). However resveratrol stimulated (ca. 40%) induction of ERα-dependent gene expression by estradiol, as already reported by others.[39] Treatment of estrogen-free HEK:ERβ cells with 1 nM estradiol induced the stably transfected luciferase reporter gene by ~5-fold.[38] Resveratrol, **7** and **10** behaved as weak ERβ agonists and **9** as partial agonist; the other derivatives had no effect (Table 3, column 4). Treatment of estradiol-supplemented HEK:ERβ cells with 1 μM ICI182,780 fully suppressed induction of luciferase expression by estradiol, while **9** stimulated the hormonal induction by ca. 70%. However, resveratrol and the other derivatives had no effect (Table 3, column 5). That resveratrol has no effect on estradiol induction of gene expression through ERβ has also been reported by others.[7,39]

In addition, we evaluated the effect of **1**–**11** and resveratrol on the expression of alkaline phosphatase (AlkP) in Ishikawa human



Scheme 3. Reagents and conditions: (a) PrSH, NaH, DMF, 100 °C.

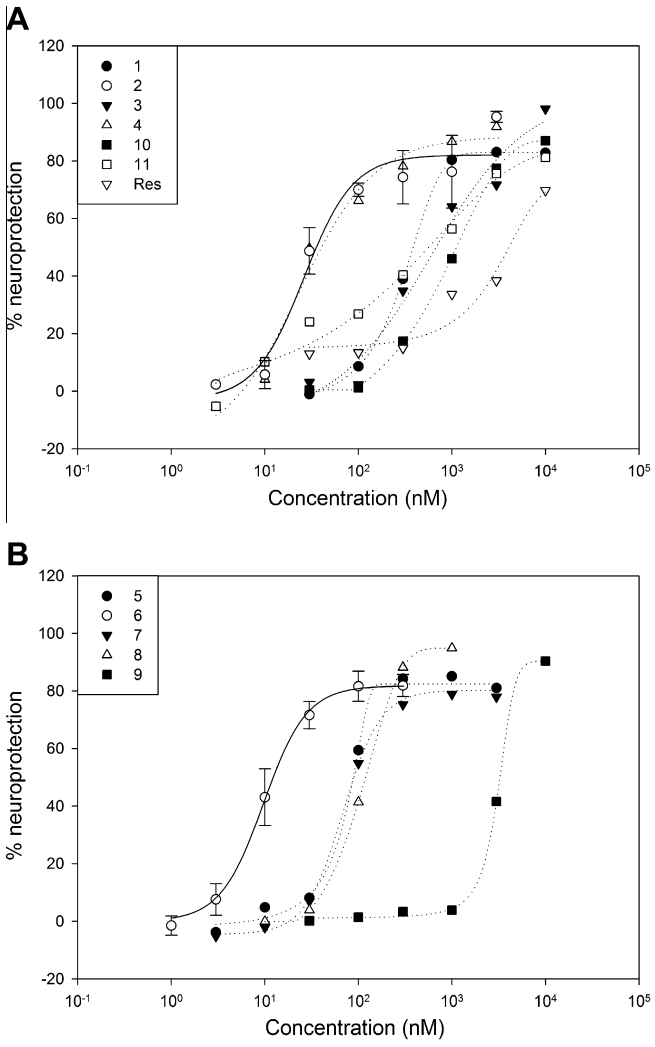


Figure 1. HT22 cells were challenged with 5 mM glutamate in the absence or presence of increasing concentrations of hydroxystilbenoid derivatives or resveratrol (Res) for 24 h and cell viability was assessed using the MTT assay. Data are mean ± SEM of three to seven independent experiments with an intra-assay variation similar to that shown for resveratrol.

uterine adenocarcinoma cells. Treatment of estrogen-free Ishikawa cells with 0.1 nM estradiol resulted in full (~4-fold) induction of AlkP in a manner that was inhibited by 1 μM ICI182,780.[38] By comparison, **5** and **10** behaved as weak agonists, **1** behaved as partial agonist and **9** was cytotoxic (Table 3, column 6). Treatment of estradiol-supplemented Ishikawa cells with 1 μM ICI182,780 fully suppressed estradiol induction of AlkP. However, none of the derivatives was suppressive, while **9** was cytotoxic even in the presence of the hormone (Table 3, column 7). The other derivatives and resveratrol had no discernible effect on Ishikawa cell proliferation and viability, as assessed using the LDH release assay (not shown). We also examined whether **1**–**11** and resveratrol could affect the proliferation of MCF-7 human breast adenocarcinoma cells. These cells proliferate in the presence of estrogen in an ERα-dependent manner. Estradiol is known to fully induce the proliferation of MCF-7 cells at concentrations ⩾0.1 nM and 1 μM ICI172,780 to fully inhibit the hormonal effect.[40] Most of the derivatives, including the topmost neuroprotective **2**, **4** and **6**, could not stimulate cell proliferation or interfere with stimulation of cell proliferation by estradiol nor affect cell viability in the absence or presence of estradiol (not shown). These data are in accordance with **2**, **4** and **6** being unable to interfere with ERα signaling to gene expression in MCF-7:D5L cells at concentrations up to 1 μM (Table 3, columns 2 and 3).

Finally, we tested whether **1**–**11** can interfere with AhR signaling to gene expression using H1L1.1c2 cells.[41] These are AhR-expressing mouse hepatoma cells stably transfected with a luciferase reporter gene regulated by the 5′-flanking region of the CYP1A1 gene promoter. This XRE-dependent promoter is responsive to a vast array of structurally diverse xenobiotics.[19,41,42] We compared test compound effects to those of benzo[*b*]fluoranthene (BbF), an environment contaminant produced by incomplete burning of organic matter (fossil fuel, waste, wood, etc.) that has been detected in the placenta of healthy pregnant women at concentrations of approx 20–60 nM.[43] BbF has been shown to strongly bind AhR and to induce CYP1A1 and other key xenobiotic clearance enzymes in human liver slices, with induction reaching a maximum at concentrations ⩾1 μM.[19] H1L1.1c2 cells responded to 0.01 and 1 μM BbF by inducing luciferase expression by a factor of 12.1 and 87, respectively (Table 4 and data not shown). In contrast, the vast majority of our derivatives and resveratrol failed to significantly induce luciferase expression or to up-regulate the

Table 2
Distal OH pairs and relative ERα- and ERβ-binding affinity and selectivity of the stilbenoids

Compound	O-O distance between[a]		Relative binding affinity (RBA)[b]		
	4′OH and 5OH (Å)	4′OH and 3OH (Å)	RBAα	RBAβ	RBAβ/RBAα
Resveratrol	10.4	10.9	0.29 ± 0.10	0.37 ± 0.07	1.28
1	10.7	11.1	0.32 ± 0.09	0.25 ± 0.04	0.79
2	10.7	11.1	0.21 ± 0.06	0.12 ± 0.01	0.54
3	10.6	11.2	0.29 ± 0.04	0.17 ± 0.02	0.59
4	10.6	11.1	0.16 ± 0.05	0.13 ± 0.04	0.81
5	12.8	13.4	0.41 ± 0.05	0.23 ± 0.06	0.55
6	12.8	13.5	0.26 ± 0.08	0.23 ± 0.03	0.89
7	12.8	13.5	0.25 ± 0.03	0.28 ± 0.05	1.10
8	12.9	13.5	0.23 ± 0.03	0.27 ± 0.06	1.16
9	12.5	13.4	0.23 ± 0.03	0.78 ± 0.12	3.35
10	11.7[c]		0.59 ± 0.09	1.94 ± 0.24	3.28
11	11.9[c]		0.07 ± 0.02	0.04 ± 0.01	0.60
Estradiol	10.4[d]		100	100	1
ICI182,780	10.4[d]		11.2 ± 4.0	2.60 ± 0.46	0.23

[a] Distances were calculated for geometrically optimized structures using MM+ in HyperChem® 7.0.
[b] The RBA values (mean ± SEM of at least three independent experiments) of test compounds for ERα (RBAα) and ERβ (RBAβ) were calculated by [RBA = ($IC_{50\ estradiol}/IC_{50\ compound}$) × 100], where IC_{50} values are estradiol or test compound concentrations capable of inhibiting the binding of the fluorescent estrogen ES2 (1 nM) to ERα and ERβ by 50%. The RBAα and RBAβ of estradiol were set equal to 100.
[c] Refers to the distance between the 4OH and 4′OH groups.
[d] Refers to the distance between the 3OH and the 17βOH groups of estradiol and ICI182, 780.

Table 3
Regulation of the expression of ER-dependent reporter genes by the stilbenoids

Compound	Luciferase expression (MCF-7:D5L cells)		Luciferase expression (HEK:ERβ cells)		Alkaline phosphatase expression (Ishikawa cells)	
	ERα-agonism[a] (% of 0.1 nM E2)	ERα-antagonism[b] (% of 1 μM ICI)	ERβ-agonism[a] (% of 1 nM E2)	ERβ-antagonism[b] (% of 1 μM ICI)	ER-agonism[a] (% of 0.1 nM E2)	ER-antagonism[b] (% of 1 μM ICI)
Resveratrol	ns	na (stimulation)	Weak (27 ± 7)	ns	ns	ns
1	Partial (65 ± 4)	ns	ns	ns	Partial (38 ± 6)	ns
2	ns	ns	ns	ns	ns	ns
3	ns	ns	ns	ns	ns	ns
4	ns	ns	ns	ns	ns	ns
5	Partial (37 ± 6)	ns	ns	ns	Weak (21 ± 5)	ns
6	ns	ns	ns	ns	ns	ns
7	Partial (34 ± 6)	ns	Weak (18 ± 8)	ns	ns	ns
8	ns	ns	ns	ns	ns	ns
9	ns	ns	Partial (66 ± 10)	na (stimulation)	na (toxicity)	na (toxicity)
10	Partial (49 ± 8)	ns	Weak (19 ± 4)	ns	Weak (25 ± 3)	ns
11	ns	ns	ns	ns	ns	ns
Estradiol	100 ± 9	na	100 ± 2	na	100 ± 5	na
ICI182,780	na	100 ± 1	na	100 ± 2	na	100 ± 3

Values are mean ± SEM of three independent experiments. ns, not significant (p >0.05 vs vehicle; ANOVA).
Agonism (or antagonism) through ER was classified as full, partial, or weak depending on whether induction (or suppression of estradiol induction) of gene expression was, respectively, 67–100, 34–66 and ⩽33% of that of 0.1 nM estradiol (or 1 μM ICI182,780)
na: not applicable.
ns: not significant.
[a] Agonism of gene expression (GE) at 1 μM test compound was calculated by $(GE_{compound} - GE_{vehicle}) \times 100/(GE_{E2} - GE_{vehicle})$.
[b] Antagonism of E2 (0.1 nM)-induced GE by 1 μM test compound, was calculated by $(GE_{E2} - GE_{E2+compound}) \times 100/(GE_{E2} - GE_{E2+ICI})$.

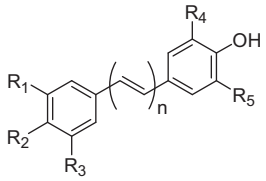
response to 0.01 and 1 μM BbF. The induction mounted by **2** was lower than that at 10 nM BbF by a factor of 6.4 (Table 4).

AhR is known to bind hydrophobic quasi-planar molecules with optimal dimensions of length, width and height equal to 14, 12 and 5 Å, respectively.[19] Table 4 shows that the van der Waals dimensions of our derivatives exceed optimal height (**1**–**4**, **10** and **11**) and/or length (**5**–**9**) for binding to AhR. In contrast, BbF's dimensions are within limits and this is also the case with resveratrol. However, the much more hydrophilic and flexible character of resveratrol compared to BbF may reduce the free energy gain from van der Waals interactions between ligand and receptor and increase the conformational free energy penalty between receptor bound and unbound states,[44] thus limiting affinity of binding to AhR. That resveratrol is unable to induce the XRE-driven luciferase reporter gene of H1L1.1c2 cells at concentrations up to 10 μM has also been reported by others.[42]

4. Conclusions

We have synthesized three sets of (*E*)-hydroxystilbenoid derivatives. In the first set we have retained the resveratrol structure and introduced one or two *tert*-butyl or 1-ethylpropyl substituents *ortho* to 4′-OH. In the second set the resveratrol structure was expanded by an additional double bond and also the above-mentioned substituents were incorporated. For the third set we synthesized two 4,4′-dihydroxystilbenes bearing either one or four *tert*-butyl

Table 4
Stilbenoid substitutions, dimensions and induction of AhR-mediated luciferase expression in H1L1.1c2 cells

Compound						Stilbenoid dimensions [a] (Å)			Luciferase expression (% of 1 μM BbF)	
	n	R1	R2	R3	R4	R5	Length	Width	Height	Level of[b]	Induction of[c]
Resveratrol	1	OH	H	OH	H	H	13.8	8.0	2.5	1.2 ± 0.1	ns
1	1	OH	H	OH	$CH(Et)_2$	H	14.0	8.5	8.5	1.7 ± 0.2	ns
2	1	OH	H	OH	$CH(Et)_2$	$CH(Et)_2$	14.0	10.0	8.5	3.0 ± 0.5*	1.9 ± 0.5
3	1	OH	H	OH	CMe_3	H	14.0	8.0	6.0	1.5 ± 0.1	ns
4	1	OH	H	OH	CMe_3	CMe_3	14.0	11.0	6.0	1.5 ± 0.1	ns
5	2	OH	H	OH	$CH(Et)_2$	H	16.0	9.0	6.5	1.7 ± 0.1	ns
6	2	OH	H	OH	$CH(Et)_2$	$CH(Et)_2$	16.5	12.0	7.2	2.2 ± 0.2	ns
7	2	OH	H	OH	CMe_3	H	16.5	8.3	6.0	1.6 ± 0.1	ns
8	2	OH	H	OH	CMe_3	CMe_3	16.5	10.4	6.1	1.6 ± 0.1	ns
9	2	OH	H	OH	H	H	16.0	7.8	2.5	1.7 ± 0.1	ns
10	1	H	OH	H	CMe_3	H	14.3	8.1	5.9	1.5 ± 0.1	ns
11	1	CMe_3	OH	CMe_3	CMe_3	CMe_3	15.0	10.8	5.9	1.4 ± 0.2	ns
BbF	—	—	—	—	—	—	13.0	9.0	2.6	13.1 ± 2.6[d]*	12.1 ± 1.5[d]

ns: not significant.
[a] Calculated using the programme HyperChem® 7.0.
[b] Values are mean ± SEM of three independent experiments. (*$p \leqslant 0.05$ vs vehicle = 1.1 ± 0.1; ANOVA).
[c] Induction of luciferase gene expression (GE) at 1 μM test compound was calculated as % of that at 1 μM benzo[*b*]fluoranthene (BbF) by $(GE_{compound} - GE_{vehicle}) \times 100/(GE_{BbF} - GE_{vehicle})$. Values are mean ± SEM of three independent experiments.
[d] Values at 10 nM BbF.

groups *ortho* to the two OH groups. These three sets of compounds were evaluated for their biological activity with the following results.

1. Assessment of the neuroprotective activity of these derivatives along with some monomethoxy derivatives using glutamate-challenged HT22 cells revealed that 4′-OH is the most important determinant of antioxidant activity, with 3-OH and 5-OH being of secondary importance; that introduction of one *tert*-butyl and, in particular, one ethylpropyl group *ortho* to 4′-OH, especially in combination with introduction of a second double bond, increases activity relative to resveratrol by a factor of 10–60; and that introduction of a second group of the same type further increases activity by a factor of about 10. However, introduction of a second *tert*-butyl group in combination with a second double bond failed to increase activity. Importantly, none of the derivatives was toxic to HT22 cells.
2. The majority of our derivatives, including the most highly neuroprotective **2**, **4** and **6**, were unable to interfere with ERα and ERβ signaling to gene expression or to significantly affect proliferation and viability of breast (MCF-7) and endometrial adenocarcinoms (Ishikawa) cells.
3. None of the derivatives activated AhR to any substantial extent or interfered with AhR activation by benzo[*b*]fluoranthene, even when the latter was used at concentrations sufficiently low to assimilate its bioavailability following ordinary human exposure.

In the light of these findings it appears that derivatives **2**, **4** and **6** might serve as lead molecules for the development of in vivo neuroprotective antioxidants devoid of endocrine cancer risk due to interference with ER and of deleterious side effects due to interference with AhR-dependent drug activation and/or detoxification mechanisms.

5. Experimental part

5.1. General chemistry methods

All reactions requiring dry or inert conditions were carried out in flame dried equipment under an atmosphere of argon. Solvents were dried under argon by conventional methods. Reactions were monitored by TLC using commercially available Merck Kieselgel 60 F_{254}. After aqueous work-up of reactions mixtures, organic solutions were routinely dried over anhydrous sodium sulfate. Column chromatography was carried out on Kieselgel 60 (particle size 40–63 mm) as supplied by Merck. NMR spectra were recorded in solutions of $CDCl_3$ unless stated otherwise on a Bruker AC 300 spectrometer operating at 300.13 MHz for ^1H and 75.04 MHz for ^{13}C. Chemical shifts are reported with reference to the solvent peak (δ_H 7.27, δ_C (central line of triple) 77.0 for $CDCl_3$). Coupling constants (*J*) are given to the nearest 0.5 Hz. Mass spectra were recorded on a TSQ 7000 Finigan instrument or on a GC–MS Varian Saturn 2000 with a 30 m × 0.25 mm DBS-MS column. For final compounds, purity was assessed as 95% pure or greater unless otherwise noted, by elemental analysis performed at the National Hellenic Research Foundation in Athens using a Perkin Elmer PE2400 II analyzer. Syntheses of the final test compounds are described below, whereas preparation and characterization of intermediates is provided in the online supplement (cf. Supplementary data).

5.2. Synthesis of the stilbene derivatives (14a–k)

A solution of the corresponding phosphonate (**13a–e**) (1.5 equiv) in DMF (5 mL/mmol) was added to a suspension of potassium *tert*-butoxide (2.5 equiv) in DMF (1 mL/mmol) at rt. The mixture was stirred for 10 min before adding a solution of the corresponding aldehyde (**12a–d**) (1 equiv) in DMF (5 mL/mmol). After stirring the reaction mixture at rt for 2 h, water was added and the product was extracted into CH_2Cl_2, the solution dried and evaporated. Compounds **14c**, **14i** and **14j** have already been described.[21]

5.2.1. 4-[(*E*)-2-(3,5-Dimethoxyphenyl)ethenyl]-2-(1-ethylpropyl)-1-methoxybenzene (14a)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14a** as a white solid (1.5 g, 73%). Mp 52–54 °C. ^1H NMR δ 7.31 (1H, dd, *J* 8.5 and 2.0, Ar–H), 7.25 (1H, d, *J* 2.0, Ar–H), 7.05 (1H, d, *J* 16.5, CH=), 6.89 (1H, d, *J* 16.5, CH=), 6.84 (1, d, *J* 8.5, Ar–H), 6.66 (2H, d, *J* 2.0, Ar–H × 2), 6.37 (1H, t, *J* 2.0, Ar–H), 3.83 (6H, s, OMe × 2), 3.82 (3H, s, OMe), 3.10–2.70 (1H, m, C*H*$(CH_2Me)_2$), 1.70–1.50 (4H, m, CH(C*H*$_2$Me)$_2$) and 0.80 (6H, t, *J* 7.5, CH(CH_2*Me*)$_2$); ^{13}C NMR δ 160.9, 157.8, 139.8, 134.3, 129.5, 129.4, 126.1, 125.9, 124.8, 110.6, 104.2, 99.6 55.6, 55.3, 41.0, 27.9 and 12.0. Anal. Calcd for $C_{22}H_{28}O_3$: C, 77.61; H, 8.29. Found: C, 77.70; H, 8.25.

5.2.2. 5-[(*E*)-2-(3,5-Dimethoxyphenyl)ethenyl]-1,3-bis-(1-ethylpropyl)-2-methoxybenzene (14b)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14b** as a clear oil (1.2 g, 80%). ^1H NMR δ 7.15 (2H, s, Ar–H × 2), 7.06 (1H, d, *J* 16.0, CH=), 6.93 (1H, d, *J* 16.0, CH=), 6.68 (2H, d, *J* 2.0, Ar–H × 2), 6.39 (1H, t, *J* 2.0, Ar–H), 3.84 (6H, s, OMe × 2), 3.69 (3H, s, OMe), 2.95–2.75 (2H, m, C*H*$(CH_2Me)_2$ × 2), 1.90–1.40 (8H, m, CH(C*H*$_2$Me)$_2$ × 2) and 0.83 (12H, t, *J* 7.5, CH(CH_2*Me*)$_2$ × 2); ^{13}C NMR δ 160.9, 157.9, 139.7, 138.8, 133.0, 129.7, 127.0, 122.7, 104.3, 99.8, 61.9, 55.3, 40.8, 29.4 and 12.3. Anal. Calcd for $C_{27}H_{38}O_3$: C, 78.98; H, 9.33. Found: C, 79.01; H, 9.46.

5.2.3. 1,3-Di-*tert*-butyl-5-[(*E*)-2-(3,5-dimethoxyphenyl)-ethenyl]-2-methoxybenzene (14d)

Purification of the crude residue by flash column chromatography (100% hexane) afforded stilbene **14d** as a light yellow solid (3.0 g, 88%). Mp 104–106 °C. ^1H NMR δ 7.40 (2H, s, Ar–H × 2), 7.06 (1H, d, *J* 16.0, CH=), 6.92 (1H, d, *J* 16.0, CH=), 6.68 (2H, d, *J* 2.5, Ar–H × 2), 6.34 (1H, t, *J* 2.5, Ar–H), 3.85 (6H, s, OMe × 2), 3.71 (3H, s, OMe), and 1.47 (18H, s, CMe_3); ^{13}C NMR δ 160.9, 159.7, 143.9, 139.7, 131.3, 129.7, 127.1, 124.9, 104.3, 99.7, 64.2, 55.3, 35.7 and 32.0. Anal. Calcd for $C_{25}H_{34}O_3$: C, 78.49; H, 8.96. Found: C, 78.32; H, 8.90.

5.2.4. 4-[(1*E*,3*E*)-4-(3,5-Dimethoxyphenyl)-1,3-butadienyl]-2-(1-ethylpropyl)-1-methoxybenzene (14e)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14e** as a white solid (1.4 g, 74%). Mp 67–70 °C. ^1H NMR δ 7.25–7.20 (2H, m, Ar–H × 2), 6.99–6.75 (3H, m, Ar–H × 3), 6.63 (1H, d, *J* 15.5, CH=), 6.59 (2H, d, *J* 2.0, Ar–H × 2), 6.55 (1H, d, *J* 15.5, CH=), 6.36 (1H, t, *J* 2.0, Ar–H), 3.82 (9H, s, OMe × 3), 3.00–2.57 (1H, m, C*H*$(CH_2Me)_2$), 1.56–1.50 (4H, m, CH(C*H*$_2$Me)$_2$) and 0.79 (6H, t, *J* 7.5, CH(CH_2*Me*)$_2$); ^{13}C NMR δ 160.9, 157.8, 139.7, 134.3, 133.5, 131.2, 130.2, 129.7, 126.6, 125.7, 124.8, 110.7, 104.3, 99.4, 55.5, 55.3, 41.0, 27.9 and 12.3. Anal. Calcd for $C_{24}H_{30}O_3$: C, 78.65; H, 8.25. Found: C, 78.56; H, 8.37.

5.2.5. 5-[(1*E*,3*E*)-4-(3,5-Dimethoxyphenyl)-1,3-butadienyl]-1,3-bis(1-ethylpropyl)-2-methoxybenzene (14f)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14f** as a yellow oil (600 mg, 36%). ^1H NMR δ 7.08 (2H, s, Ar–H × 2), 7.00–6.80 (2H, m, CH= × 2), 6.64 (1H, d, *J* 16.0, CH=), 6.60 (2H, d, *J* 2.5,

Ar–H × 2), 6.59 (1H, d, *J* 15.0, CH=), 6.37 (1H, t, *J* 2.5, Ar–H), 3.83 (6H, s, OMe × 2), 3.68 (3H, s, OMe), 2.95–2.75 (2H, m, C*H*(CH_2Me)$_2$ × 2), 1.80–1.40 (8H, m, CH(C*H*$_2$Me)$_2$ × 2) and 0.83 (12H, t, *J* 7.5, CH(CH_2*Me*)$_2$ × 2); ^{13}C NMR δ 160.9, 157.8, 139.6, 138.8, 133.7, 133.2, 131.7, 130.0, 127.6, 122.5, 104.3, 99.7, 61.9, 55.3, 40.8, 29.4 and 12.3. Anal. Calcd for $C_{29}H_{40}O_3$: C, 79.77; H, 9.23. Found: C, 79.69; H, 9.36.

5.2.6. 2-*tert*-Butyl-4-[(1*E*,3*E*)-4-(3,5-dimethoxyphenyl)-1,3-butadienyl]-1-methoxybenzene (14g)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14g** as a white solid (1.0 g, 54%). Mp 95-98 °C. ^{1}H NMR δ 7.37 (1H, d, *J* 2.0, Ar–H), 7.27 (1H, dd, *J* 8.0 and 2.0, Ar–H), 6.99–6.76 (3H, m, Ar–H and CH= × 2), 6.65 (1H, d, *J* 15.5, CH=), 6.60 (2H, d, *J* 2.0, Ar–H × 2), 6.56 (1H, d, *J* 15.5, CH=), 6.36 (1H, t, *J* 2.0, Ar–H), 3.85 (3H, s, OMe), 3.82 (6H, s, OMe × 3) and 1.40 (9H, s, CMe_3); ^{13}C NMR δ 160.9, 158.5, 139.6, 138.4, 133.5, 131.2, 130.2, 129.4, 126.6, 125.2, 124.9, 111.7, 104.2, 99.7, 55.3, 55.0, 34.8 and 29.6. Anal. Calcd for $C_{23}H_{38}O_3$: C, 78.38; H, 8.01. Found: C, 78.24; H, 8.00.

5.2.7. 1,3-Di-*tert*-butyl-5-[(1*E*,3*E*)-4-(3,5-dimethoxyphenyl)-1,3-butadienyl]-2-methoxybenzene (14h)

Purification of the crude residue by flash column chromatography (hexane/ether 90:10) afforded stilbene **14h** as a white solid (3.2 g, 88%). Mp 117–123 °C. ^{1}H NMR δ 7.35 (2H, s, Ar–H × 2), 7.10–6.80 (2H, m, CH= × 2), 6.67 (1H, d, *J* 15.0, CH=), 6.62 (2H, d, *J* 2.0, Ar–H × 2), 6.48 (1H, d, *J* 15.0, CH=), 6.39 (1H, t, *J* 2.0, Ar–H), 3.84 (6H, s, OMe × 2), 3.72 (3H, s, OMe) and 1.47 (18H, s, CMe_3 × 2); ^{13}C NMR δ 160.8, 159.6, 143.8, 139.5, 133.7, 131.7, 131.5, 130.0, 127.5, 124.7, 104.2, 99.7, 64.2, 55.3, 35.7 and 32.0. Anal. Calcd for $C_{27}H_{36}O_3$: C, 79.37; H, 8.88. Found: C, 79.309; H, 8.86.

5.2.8. 1,3-Di-*tert*-butyl-5-[(*E*)-2-(3,5-di-*tert*-butyl-4-methoxyphenyl)ethenyl]-2-methoxybenzene (14k)

Purification of the crude residue by recrystallisation from MeOH afforded stilbene **14k** as a light yellow amorphous solid (1.25 g, 46%). ^{1}H NMR δ 7.40 (4H, s, Ar–H × 4), 6.95 (2H, s, CH= × 2), 3.72 (6H, s, OMe × 2) and 1.49 (36H, s, CMe_3); ^{13}C NMR δ 159.3, 143.7, 131.9, 127.7, 124.7, 64.2, 35.8 and 32.1. Anal. Calcd for $C_{32}H_{48}O_2$: C, 82.70; H, 10.41. Found: C, 82.91.69; H, 10.55.

5.3. General procedure for the cleavage of the methyl ether

5.3.1. Method A

To a solution of MeMgI [prepared under argon from Mg (20 equiv) and MeI (20 equiv) in Et_2O (1 mL/mmol)] was added dropwise a solution of the methoxy stilbene derivative (**14a–j**) (1 equiv) in Et_2O (5 mL/mmol). The solvent was removed under vacuum and the residue was then heated to 160 °C for 2 h under argon. The cooled reaction mixture was then treated with NH_4Cl (satd solution) and extracted with Et_2O. The combined organic extracts were washed with brine, dried and evaporated.

Compounds **3**, **9** and **10** have already been described.[21]

5.3.1.1. 5-{(*E*)-2-[3-(1-Ethylpropyl)-4-hydroxyphenyl]ethenyl}-1,3-benzenediol (1). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **1** as a yellow amorphous solid (54 mg, 44%). ^{1}H NMR (CD_3OD) δ 8.52 (1H, s, OH), 8.42 (2H, s, OH × 2), 7.31 (1H, d, *J* 2.0, Ar–H), 7.23 (1H, dd, *J* 8.5 and 2.0, Ar–H), 7.02 (1H, d, *J* 16.5, CH=), 6.87 (1H, d, *J* 16.5, CH=), 6.84 (1H, d, *J* 8.5, Ar–H), 6.53 (2H, d, *J* 2.0, Ar–H × 2), 6.25 (1H, t, *J* 2, Ar–H), 3.00–2.75 (1H, m, C*H*(CH_2Me)$_2$), 1.75–1.55 (4H, m, CH(C*H*$_2$Me)$_2$) and 0.80 (6H, t, *J* 7.5, CH(CH_2*Me*)$_2$); ^{13}C NMR (CD_3OD) δ 159.6, 156.8, 141.4, 133.0, 130.2, 129.9, 126.7, 126.5, 125.6, 116.2, 105.7, 102.5, 42.7, 29.1 and 12.5. MS (EI) *m/z*: 298.1 (M)$^+$. Anal. Calcd for $C_{19}H_{22}O_3$: C, 76.48; H, 7.43. Found: C, 76.55; H, 7.38.

5.3.1.2. 5-{(*E*)-2-[3,5-Bis(1-ethylpropyl)-4-hydroxyphenyl]ethenyl}-1,3-benzenediol (2). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **2** as a light yellow solid (420 mg, 47%). Mp 115–120 °C. ^{1}H NMR δ 7.07 (2H, s, Ar–H × 2), 6.99 (1H, d, *J* 16.0, CH=), 6.79 (1H, d, *J* 16.0, CH=), 6.57 (2H, s, Ar–H × 2), 6.24 (1H, s, Ar–H), 2.75–2.50 (2H, m, C*H*(CH_2Me)$_2$ × 2), 2.00–1.45 (8H, m, CH(C*H*$_2$Me)$_2$ × 2) and 0.83 (12H, t, *J* 7.5, CH(CH_2*Me*)$_2$ × 2); ^{13}C NMR δ 156.8, 152.4, 140.7, 130.9, 130.3, 129.2, 124.9, 123.3, 105.8, 101.6, 42.0, 28.4 and 12.1. MS (EI) *m/z*: 368.3 (M)$^+$. Anal. Calcd for $C_{24}H_{32}O_3$: C, 78.22; H, 8.75. Found: C, 78.18; H, 8.76.

5.3.1.3. 5-{(1*E*,3*E*)-4-[3-(1-Ethylpropyl)-4-hydroxyphenyl]-1,3-butadienyl}-1,3-benzenediol (5). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **5** as a yellow amorphous solid (410 mg, 80%). ^{1}H NMR (CD_3OD) δ 7.20–7.00 (2H, m, Ar–H × 2), 6.85–6.70 (3H, m, Ar–H and CH= × 2), 6.56 (1H, d, *J* 15.0, CH=), 6.43 (1H, d, *J* 15.0, CH=), 6.39 (2H, d, *J* 2.0, Ar–H × 2), 6.15 (1H, t, *J* 2.0, Ar–H), 3.00–2.80 (1H, m, C*H*(CH_2Me)$_2$), 1.75–1.50 (4H, m, CH(C*H*$_2$Me)$_2$) and 0.79 (6H, t, *J* 7.5, CH(CH_2*Me*)$_2$); ^{13}C NMR (CD_3OD) δ 159.6, 156.9, 141.2, 134.5, 133.0, 132.2, 130.9, 130.5, 127.3, 127.2, 125.6, 116.2, 105.6, 102.8, 42.7, 29.2 and 12.5. MS (EI) *m/z*: 324.2 (M)$^+$. Anal. Calcd for $C_{21}H_{24}O_3$: C, 77.75; H, 7.46. Found: C, 77.65; H, 7.49.

5.3.1.4. 5-{(1*E*,3*E*)-4-[3,5-Bis(1-ethylpropyl)-4-hydroxyphenyl]-1,3-butadienyl}-1,3-benzenediol (6). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **6** as a light yellow solid (280 mg, 65%). Mp 155–158 °C. ^{1}H NMR (CD_3OD) δ 7.05 (2H, s, Ar–H × 2), 6.95–6.70 (2H, m, CH= × 2), 6.61 (1H, d, *J* 15.0, CH=), 6.48 (1H, d, *J* 15.0, CH=), 6.42 (2H, s, Ar–H × 2), 6.18 (1H, s, Ar–H), 3.00–2.85 (2H, m, C*H*(CH_2Me)$_2$ × 2), 1.75–1.55 (8H, m, CH(C*H*$_2$Me)$_2$ × 2) and 0.83 (12H, t, *J* 6.5, CH(CH_2*Me*)$_2$ × 2); ^{13}C NMR (CD_3OD) δ 159.6, 154.8, 141.2, 134.9, 133.4, 132.2, 130.9, 130.8, 127.1, 123.8, 105.7, 102.8, 42.3, 29.7 and 12.4. MS (EI) *m/z*: 394.3 (M)$^+$. Anal. Calcd for $C_{26}H_{34}O_3$: C, 79.15; H, 8.69. Found: C, 78.94; H, 8.71.

5.3.1.5. 5-[(1*E*,3*E*)-4-(3-*tert* -Butyl-4-hydroxyphenyl)-1,3-butadienyl]-1,3-benzenediol (7). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **7** as a light brown solid (190 mg, 85%). Mp 153–156 °C. ^{1}H NMR (CD_3OD) δ 7.27 (1H, s, Ar–H), 7.15 (1H, d, *J* 8.0, Ar–H), 6.90–6.65 (2H, m, CH= × 2), 6.69 (1H, d, *J* 8.0, Ar–H), 6.57 (1H, d, *J* 15.0, CH=), 6.43 (1H, d, *J* 15.0, CH=), 6.39 (2H, s, Ar–H × 2), 6.15 (1H, s, Ar–H) and 1.40 (9H, s, CMe_3); ^{13}C NMR (CD_3OD) δ 159.6, 157.3, 141.3, 137.2, 134.7, 132.1, 130.9, 129.9, 126.9, 126.3, 125.8, 117.4, 105.7, 102.8, 35.5 and 29.9. MS (EI) *m/z*: 310.2 (M)$^+$. Anal. Calcd for $C_{20}H_{22}O_3$: C, 77.39; H, 7.14. Found: C, 77.36; H, 7.10.

5.3.1.6. 5-{(*E*)-2-[3,5-Di-*tert*-butyl-4-methoxyphenyl]ethenyl}-1,3-benzenediol (15). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **15** as a light brown solid (520 mg, 56%). Mp 160–165 °C. ^{1}H NMR δ 7.38 (2H, s, Ar–H × 2), 7.02 (1H, d, *J* 16.0, CH=), 6.84 (1H, d, *J* 16.0, CH=), 6.58 (2H, d, *J* 2.0, Ar–H × 2), 6.27 (1H, t, *J* 2.0, Ar–H), 3.71 (3H, s, OMe) and 1.47 (18H, s, CMe_3 × 2); ^{13}C NMR δ 159.5, 156.8, 143.9, 140.5, 131.2, 130.2, 126.4, 124.9, 106.0, 101.9, 64.3, 35.7 and 32.0. Anal. Calcd for $C_{23}H_{30}O_3$: C, 77.93; H, 8.53. Found: C, 77.88; H, 8.55.

5.3.1.7. 5-{(1*E*,3*E*)-4-[3,5-Di-*tert*-butyl-4-methoxyphenyl]-1,3-butadienyl}-1,3-benzenediol (16). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **16** as a light yellow amorphous solid (1.0 g, 77%). ^{1}H NMR δ 7.35 (2H, s, Ar–H × 2), 6.95–6.70 (2H, m, CH= × 2), 6.63 (1H, d, *J* 15.0, CH=), 6.51 (1H, d, *J* 15.0, CH=), 6.51 (2H, d, *J* 2.5, Ar–H × 2), 6.25 (1H, t, *J* 2.5, Ar–H), 3.70 (3H, s, OMe) and 1.46 (18H, s, CMe_3 × 2); ^{13}C NMR δ 159.7, 156.8, 143.9, 140.3, 134.0, 131.5, 130.9, 130.4, 127.4, 124.8, 105.8, 102.0, 64.2, 35.7 and 32.0. Anal. Calcd for $C_{25}H_{32}O_3$: C, 78.91; H, 8.48. Found: C, 78.95; H, 8.43.

5.3.2. Method B

A solution of propanethiol (7 equiv) in DMF (50 mL) was added to a suspension of NaH (10 equiv, 60% in oil, previously washed with hexane) in DMF at 25 °C. After stirring the reaction mixture for 30 min. a solution of the corresponding stilbene derivative (**14d**, **14k**, **16**) (1 equiv) in DMF was added and the mixture was warmed to 100 °C. After 20 h, the reaction mixture was allowed to cool down and then water was added followed by 3 M HCl. The product was extracted into Et_2O, the solvent dried and evaporated. Traces of DMF and propanethiol were removed under high vacuum.

5.3.2.1. 2,6-Di-*tert*-butyl-4-[(*E*)-2-(3-hydroxy-5-methoxyphenylethenyl)]phenol (17) and 5-[(*E*)-2-(3,5-di-*tert*-butyl-4-hydroxyphenylethenyl)]-1,3-benzenediol (4). Purification of the crude residue was achieved by flash column chromatography (20% ether in hexane). First to elute was stilbene **17** as a light yellow solid (415 mg, 45%). ^{1}H NMR 7.34 (2H, s, Ar–H × 2), 7.04 (1H, d, *J* 16.0, CH=), 7.85 (1H, d, *J* 16.0, CH=), 6.65 (1H, s, Ar–H), 6.61 (1H, s, Ar–H), 6.32 (1H, s, Ar–H), 5.31 (1H, br, OH), 3.85 (3H, s, OMe), and 1.49 (18H, s, CMe_3 × 2); ^{13}C NMR δ 161.1, 156.7, 153.9, 140.3, 136.1, 130.2, 128.3, 125.4, 123.5, 105.6, 104.7, 100.4, 55.3, 34.3 and 30.2. Anal. Calcd for $C_{23}H_{30}O_3$: C, 77.93; H, 8.53. Found: C, 77.90; H, 8.51.

Second to elute was stilbene derivative **4** as an amorphous light yellow solid (340 mg, 31%). ^{1}H NMR 7.32 (2H, s, Ar–H × 2), 7.02 (1H, d, *J* 16.0, CH=), 6.80 (1H, d, *J* 16.0, CH=), 6.58 (2H, s, Ar–H × 2), 6.27 (1H, s, Ar–H), 4.30–4.00 (3H, br, OH × 3) and 1.48 (18H, s, CMe_3 × 2); ^{13}C NMR δ 156.9, 153.9, 140.6, 136.1, 130.4, 128.3, 125.1, 123.5, 105.8, 101.7, 34.3 and 30.2. MS (EI) *m/z*: 340.2 $(M)^+$. Anal. Calcd for $C_{22}H_{28}O_3$: C, 77.61; H, 8.29. Found: C, 77.72; H, 8.32. In the course of our work, compound **4** was synthesised by another group in an analogous manner.[45]

5.3.2.2. 5-{(1*E*,3*E*)-4-[3,5-Di-*tert* -butyl-4-hydroxyphenyl]-1,3-butadienyl}-1,3-benzenediol (8). Purification of the crude residue by flash column chromatography (hexane/ether 80:20) afforded stilbene **8** as an amorphous light brown solid (550 mg, 66%). ^{1}H NMR δ 7.27 (2H, s, Ar–H × 2), 6.95–6.60 (2H, m, CH= × 2), 6.61 (1H, d, *J* 15.0, CH=), 6.53 (2H, s, Ar–H × 2), 6.42 (1H, d, *J* 15.0, CH=), 6.39 (1H, s, Ar–H), 6.10–5.80 (3H, br, OH × 3) and 1.47 (18H, s, CMe_3 × 2); ^{13}C NMR δ 157.1, 153.8, 140.2, 136.0, 134.2, 130.6, 130.4, 128.6, 126.2, 123.4, 105.7, 102.1, 34.3 and 30.2. MS (EI) *m/z*: 366.2 $(M)^+$. Anal. Calcd for $C_{24}H_{30}O_3$: C, 78.65; H, 8.25. Found: C, 78.36; H, 8.30.

5.3.2.3. 2,6-Di-*tert*-butyl-4-[(*E*)-2-(3,5-di-*tert* -butyl-4-methoxyphenylethenyl)]phenol (18) and 2,6-di-*tert*-butyl-4-[(*E*)-2-(3,5-di-*tert* -butyl-4-hydroxyphenylethenyl)]phenol (11). Purification of the crude residue was achieved by flash column chromatography (gradient 100% hexane to 50% ether in hexane). First to elute was stilbene **18** as a light yellow solid (400 mg, 41%). Mp 168–171 °C. ^{1}H NMR 7.38 (2H, s, Ar–H × 2), 7.33 (2H, s, Ar–H × 2), 6.95–6.85 (2H, m, CH= × 2), 3.21 (3H, s, OMe), 1.49 (18H, s, CMe_3 × 2) and 1.47 (18H, s, CMe_3 × 2); ^{13}C NMR δ 159.0, 153.5, 143.7, 136.0, 132.1, 128.9, 128.0, 126.4, 124.5, 123.2, 64.2, 35.7, 34.4, 32.1 and 30.2. Anal. Calcd for $C_{31}H_{46}O_2$: C, 82.61; H, 10.29. Found: C, 82.60; H, 10.10.

Second to elute was stilbene derivative **11** as a yellow solid (200 mg, 21%). Mp 226–230 °C (Lit.[46] mp 240–241 °C); ^{1}H NMR 7.33 (4H, s, Ar–H × 4), 6.89 (2H, s, CH= × 2) and 1.49 (36H, s, CMe_3 × 2); ^{13}C NMR δ 153.3, 136.0, 129.2, 126.7, 123.0, 34.4 and 30.3. MS (EI) *m/z*: 436.5 $(M)^+$. Anal. Calcd for $C_{30}H_{44}O_2$: C, 82.52; H, 10.16. Found: C, 82.51; H, 10.12.

5.4. Biological evaluation

5.4.1. Cells

HT22 cells were kindly provided by Dr. David Schubert (The Salk Institute). The cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FBS at a confluence not >50%. H1L1.1c2 cells were a generous gift from Dr. Michael S. Denison (University of California, Davis). These cells are a mouse hepatoma (Hepa1c1c7)-derived cells line which has been stably transfected with the XRE-driven firefly luciferase reporter plasmid, pGudLuc1.1.[41] pGudLucl.l contains the 5′-flanking region (−1301 to −819; the region is endowed with four XREs) of the mouse CYP1Al gene directly upstream of the MMTV viral promoter. H1L1.1c2 cells were cultured in DMEM supplemented with 10% FBS and were subcultured using 0.25% trypsin–0.02% EDTA. MCF-7 (human breast adenocarcinoma) cells and Ishikawa (human endometrial adenocarcinoma) cells were purchased from ATCC (American Tissue Culture Collection) and ECACC (European Collection of Cell Cultures), respectively, and were maintained as recommended by the supplier. MCF-7:D5L cells and HEK:ERβ cells were maintained as already reported.[21,47] If not stated otherwise, culture media and fine chemicals administered to the cells were purchased from Sigma–Aldrich, while FBS was from Gibco (Invitrogen). ICI182,780 was purchased from Tocris Bioscience.

5.4.2. Evaluation of the activity of test compounds to prevent oxytosis of glutamate-challenged HT22 hippocampal neurons

Compounds were tested as previously described[48] with minor modifications. In brief, HT22 cells were plated in 96-well flat bottom transparent plates at a density of 4000 cells per well in 100 μM of DMEM (low glucose) containing 2% FBS. 24 h after plating, the cells were challenged with 5 mM glutamate in the absence or presence of increasing concentrations (0.001–10 μM) of test compounds (stock solutions were prepared using tissue culture grade DMSO as vehicle) in fresh medium for 24 h prior to using conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to colored formazan as a means to indirectly assess living cells numbers. The difference in optical density (dOD) at 550 and 690 nm (reference wavelength correcting for background absorbance), determined using a Safire 2 plate reader (Tecan), was taken as a measure of cell number. Direct interference of test compounds with MTT conversion to formazan was excluded using mock cultures deprived of HT22 cells. Interference of test compounds with mitochondrial conversion of MTT to formazan was excluded using the Trypan blue exclusion assay to directly determine the number living cells (see below). No challenged cells served to assess test compound effects on cell proliferation, whereas challenged cells served to assess neuroprotective activity by comparison. Cells exposed only to vehicle (DMSO) or glutamate served as controls. Cell death (CD) in the absence of test compound was calculated by $CD_{Vehicle} = (dOD_{Vehicle} - dOD_{Vehicle+Glutamate}) \times 100/dOD_{Vehicle}$, whereas cell death in its presence was calculated by $CD_{Compound} = (dOD_{Compound} - dOD_{Compound+Glutamate}) \times 100/dOD_{Compound}$. Neuroprotection (%) was calculated by $(CD_{Vehicle} - CD_{Compound}) \times 100/CD_{Vehicle}$.

5.4.3. Cytotoxicity assay

Test compound cytotoxicity was assessed using the LDH Cytotoxicity Detection Kit from Takara Bio Inc. (Japan) according to the manufacturer's instructions. HT22 cells were seeded in a 96-well transparent flat-bottomed tissue culture plates at a density of 1500 cells per well in phenol red-free DMEM supplemented with FBS previously heated at 56 °C for 30 min to inhibit endogenous enzymatic activity. After 24 h, cells were further incubated with 1 or 10 μM test compound for 72 h. Cells incubated in the presence of DMSO (vehicle) only were included to adjust for the innate cytotoxicity of cell culture. Following the 72-h incubation, plates were centrifuged at 400 g for 3 min and 100 μL of the supernatant was transferred to a new 96-well plate and combined with 100 μL of LDH assay mixture. Following incubation at 37 °C for 30 min the activity of LDH was measured at 490 nm and corrected for the absorbance at 690 nm ($OD_{490-690}$), using a Safire 2 plate reader. The remaining cell pellet was lysed by incubation with 100 μL of medium containing 1% Triton X-100 for 150 min at 37 °C and the resulting extracts were treated as described for the supernatant. Cytotoxicity was calculated by $(rLDH_{test} \times 100/tLDH_{test}) - (rLDH_{vehicle} \times 100/tLDH_{vehicle})$, where the latter fraction stands for innate cytotoxicity, rLDH is LDH released in the cell culture supernatant and tLDH is total LDH, that is, the sum of rLDH and LDH in the cell pellet.

For the Trypan blue exclusion assay, cells were suspended in full growth medium and plated in 24-well plates at a density of 625,000 cells per well. After 24 h, cells were treated with test compounds at the concentration of 1 μM and incubated for 1 or 3 days. At the end of the incubation both floating and adherent cells were combined, stained with 0.4% Trypan blue in phosphate buffered saline, and counted using a Neubauer haemocytometer.

5.4.4. Assessment of relative (to estradiol) binding affinity of test compounds for ERα and ERβ

The binding affinities of test compounds relative to that of estradiol (relative binding affinity, RBA) for purified recombinant ERα and ERβ (RBAα and RBAβ) were assessed using a Beacon 2000 Fluorescence Polarization Reader (Invitrogen) as described previously.[40] Briefly, we determined the concentrations of estradiol, ICI182,780, resveratrol and **1–11** that inhibited the binding of the fluorescent estrogen ES2 (Invitrogen) to the isolated recombinant human ERα or ERβ (Invitrogen) by 50% (IC_{50}), and used them to derive the RBA values of Table 2 as described in the legend to the table.

5.4.5. Test compound effects on ER-regulated gene expression

Test compound regulation of ERE-dependent gene expression through ERα and ERβ was assessed using MCF-7:D5L and HEK:ERβ cells, respectively. MCF-7:D5L cells, a clone of MCF-7 cells, that is, stably transfected with the estrogen-responsive reporter plasmid pERE-Gl-Luciferase, have been described.[47] HEK:ERβ cells, a clone of human embryonic kidney (HEK-293) cells, that is, stably transfected with the estrogen-responsive reporter plasmid pERE-tk-Luciferase and an expression plasmid coding for the full-length human ERβ, have also been described.[21] Assessment of test compound regulation of luciferase expression in MCF-7:D5L and HEK:ERβ cells was carried out as already described.[38] Briefly, the cells were plated in 96-well microculture plates at a density of 12,000 cells per well in MEM (MCF-7:D5L cells) or DMEM (HEK:ERβ cells) devoid of phenol-red and supplemented with 1 μg/mL insulin and 5% DCC-FBS, that is, FBS that was treated with 10% dextran-coated charcoal (DCC) to remove endogenous steroids as already described.[49] 72 h after plating, the cells were exposed to increasing concentrations (up to 1 μM) of test compounds in the absence (vehicle was added in this case up to a final concentration ⩽ 0.2%) or presence of estradiol or ICI182,780 for 18 h as indicated, and luciferase expression was assessed using the Steady-Glo Luciferase Assay System (Promega) and a Safire II microplate reader. Controls for full agonism (cells exposed only to estradiol), full antagonism (cells exposed to ICI182,780 as well as estradiol) and non-agonism/antagonism (exposed only to vehicle) served to classify the test compounds that significantly affected luciferase expression as full, partial or weak ERα or ERβ agonists or antagonists as described in the legend to Table 3.

Test compound regulation of alkaline phosphatase (AlkP) expression of Ishikawa cells, was assessed using 96-well microculture plates that were plated with 12,000 cells per well in phenol-red-free MEM supplemented with 1 μg/mL insulin and 5% DCC-FBS. Twenty-four hours after plating, cells were exposed to test compounds in the absence or presence of 0.1 nM estradiol or 1 μM ICI182,780 for 72 h and compound effects on AlkP activity were assessed as already described,[40] with minor modifications. Briefly, the cells were washed with PBS twice, placed at −80 °C for at least 1 h, thawed at room temperature for 5–10 min, and then transferred on ice. Next, 50 μL ice-cold solution containing 5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$ and 1 M diethanolamine (pH 9.8) was added, the cells were warmed to room temperature and yellow-colored *p*-nitrophenol was allowed to accumulate. The color was monitored every 15 min at 405 nm using the Safire II microplate reader until positive controls showed an absorbance (A_{405}) of about 1.2. Cells exposed only to vehicle, ICI182,780 and/or estradiol served as controls, and those of test compounds that significantly affected AlkP expression were classified as agonists or antagonists as described above.

5.4.6. Test compound effects on the proliferation of endocrine cancer cells

Compound effects on the proliferation of MCF-7 cells were assessed as already described,[47] with minor modifications. Briefly, the cells were plated in 96-flat-bottom-well microplates at a density of 8000 cells per well in phenol-red-free MEM supplemented with 1 μg/mL insulin and 5% DCC-FBS. Seventy-two hours after plating, the cells were exposed for 96 h to test compounds in the absence or presence of 0.1 nM estradiol or 1 μM ICI182,780 and relative numbers of viable cells were determined by monitoring MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion to formazan at 550 and 670 nm. The difference in optical density (dOD) at 550 and 690 nm, as determined using a Safire 2 plate reader (Tecan), was taken as a measure of cell number. Cells exposed only to vehicle, ICI182,780 and/or estradiol served as controls. Cell proliferation in the absence or presence of test compounds and/or 0.1 nM estradiol was expressed as % of that in the presence of 0.1 nM estradiol alone. The effect of test compounds on the proliferation of Ishikawa cells was determined as described for MCF-7 cells, except that test compounds were added to the cells 24 h after plating and assayed for 96 h in absence or presence of 0.1 nM estradiol. Effects of test compounds on cell proliferation were expressed as % of that in the presence of 0.1 nM estradiol alone.

5.4.7. Test compound effects on AhR-regulated gene expression

Test compound regulation of XRE-dependent gene expression through AhR was assessed using H1L1.1c2 cells. These cells are known to respond to a large selection of xenobiotics by robustly inducing luciferase activity. In the present study the performance of the cells was validated using benzo[*b*]fluoranthene at 0.01 and 1 μM. Induction of luciferase expression reached maximum at 1 μM, as already reported by others.[19] To assess test compound effects on AhR-regulated gene expression, H1L1.1c2 cells were plated in 96-well flat bottomed microplates at a density of 10^4 cells per well in DMEM supplemented with 10% FBS. After 72 h fresh medium was added followed by test compounds (final

concentration of 1 μM) or test compound combinations with 0.01 or 1 μM benzo[*b*]fluoranthene and incubated for 18 h. Cells were then harvested in Glo Lysis Buffer (Promega) and luciferase activity was measured using a Steady Glo Luciferase Assay system (Promega) in a Safire II microplate reader. The level of luciferase expression of H1L1.1c2 cells at 1 μM benzo[*b*]fluoranthene was set equal to 100. The level of expression in the presence of test compound alone or in combination with 0.01 or 1 μM benzo[*b*]fluoranthene was expressed as percentage of that at 1 μM benzo[*b*]fluoranthene.

5.5. Statistics

The statistical significance of the differences in cell proliferation, viability and gene expression was determined using one-way ANOVA with a Tukey Post Hoc test for multiple comparisons as provided by the SPSS 10.0 statistical package. Differences in neuroprotective activity (EC_{50} values) were analyzed using an independent samples *t*-test. Differences were considered significant for values of $p \leqslant 0.05$.

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Supplementary data

Supplementary data (detailed experimental procedures for the synthesis of intermediate compounds and ^{1}H and ^{13}C NMR spectra of new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.018. These data include MOL files and InChiKeys of the most important compounds described in this article.

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