Studies on the Apoptotic Activity of Natural and Synthetic Retinoids: Discovery of a New Class of Synthetic Terphenyls That Potently Support Cell Growth and Inhibit Apoptosis in Neuronal and HL-60 Cells

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New terphenyl derivatives have been synthesized and tested for their effect on cell survival in serum-free cultures. These compounds protected HL60 cells from death and supported their growth with an activity higher than that of the natural 14-hydroxy-*retro*-retinol. Terphenyls **26** and **28** also possess antiapoptotic activity on neuronal cells, proving them as possible candidates for the treatment of neurodegenerative and ischemic diseases.

Introduction

Apoptotic processes are widespread biochemical events, being involved in, for example, development, differentiation, cell proliferation/homoeostasis, regulation and function of the immune system, and the removal of defective and therefore harmful cells.[1] Thus, dysfunction or deregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer,[2,3] autoimmune diseases, and spreading of viral infections, while neurodegenerative disorders such as Alzheimer and Parkinson diseases, AIDS, and ischemic diseases are caused or enhanced by excessive apoptosis.[4,5] Increasing evidence suggests that inhibition of apoptosis in these severe pathologies may be beneficial as a therapeutic approach to slow disease progression and to improve a patient's prospects.[4] To this end, the discovery of new compounds able to modulate the apoptotic process, both as activators or inhibitors, represents an important challenge in medicinal chemistry.

Retinoids, a class of natural and synthetic compounds structurally related to vitamin A (**1**, Figure 1), are known to modulate cell proliferation, apoptosis, and differentiation, with different effects depending on the cellular context. Thus, *all-trans*-retinoic acid (ATRA, **2**) and its naturally occurring retinoid analogue 9-*cis*-retinoic acid (**3**), are known[6] to be involved in cell differentiation, morphogenesis, proliferation, and antineoplastic processes.

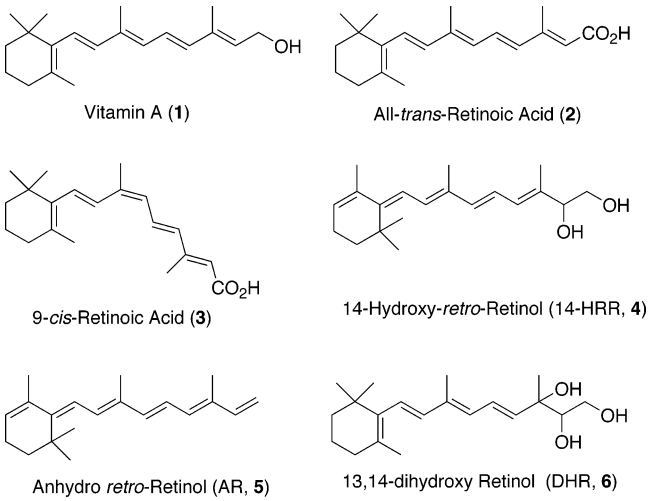

Figure 1. Natural retinoids and *retro*-retinoids.

Two other bioactive metabolites of vitamin A, namely, 14-hydroxy-*retro*-retinol (14-HRR) (**4**) and anhydroretinol (AR) (**5**), are involved in cell proliferation, with growth-supporting properties for normal lymphocyte proliferation being ascribed to **4**, while **5** was discovered to act as a natural antagonist triggering growth arrest and death by apoptosis.[7,8] A mutually reversible relationship exists between these two *retro*-retinoids because one can reverse the effects of the other when given in pharmacological doses.[9−11] Another reported metabolite of vitamin A sharing similar activity as **4** is the 13,-14-dihydroxy retinol (DHR, **6**).[12] Exploitation of this relationship holds much promise for therapeutic control of diseases such as cancer.

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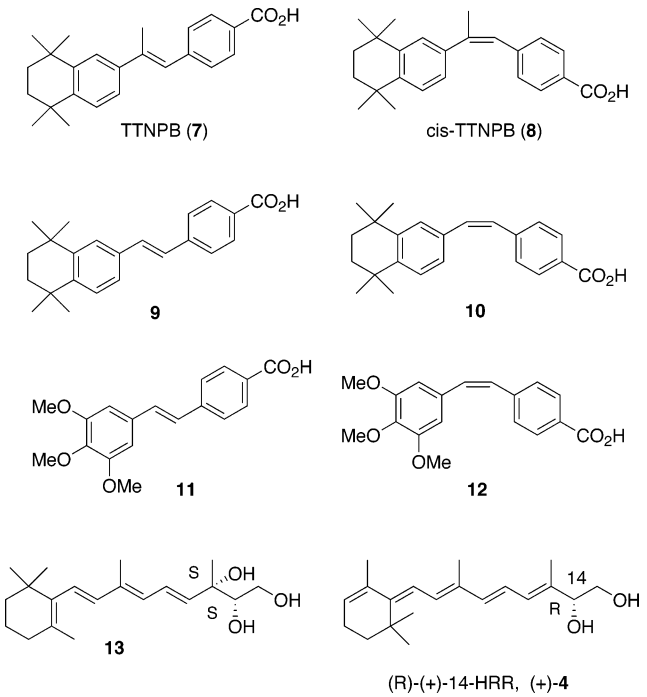


Figure 2. Synthesized retinoids and *retro*-retinoids.

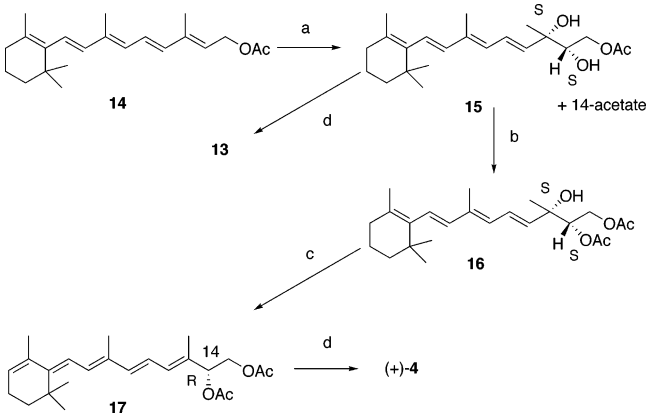
Our continuous interest[13−15] in the field led us to discover that the apoptotic activity of TTNPB (**7**)[16] and *cis*-TTNPB (**8**)[17] (Figure 2) was strictly connected with the properties of the alkenyl portion.[13]

In this research context, we have ascertained that arotinoids such as **9**[18] and the cis derivative **10**[19] are not able to activate apoptosis in HL60 cells. Moreover, **9** is surprisingly able to induce cell growth by preventing apoptosis at 1×10^{-6} M, reproducing to some extent the growth-supporting properties hitherto ascribed only to the natural 14-HRR (**4**) and DHR (**6**).[11,12] We found a similar unexpected activity for **11**,[20] another derivative that we synthesized taking into account the presence of the trimethoxyphenyl moiety in many bioactive molecules but not for its cis stereisomer **12**.[20] To validate our findings, availability of 14-HRR (**4**) was required in order to compare its activity with compounds **9**−**12**. The literature has reported that configuration of natural **4** could not be clearly assigned, but very interestingly, both synthetic enantiomers behaved similarly in biological assays.[8] Thus, we embarked on the synthesis of compound (+)-(**4**) and its hypothetical precursor **13** with (*S*)-configuration at C-14, testing also the biological activity of the intermediates **16** and **17** obtained along the synthetic pathway. Furthermore, we prepared the terphenyl-based derivatives of **11** and **12**, namely, compounds **26**−**28**, considering the phenyl bridge ring as a known bioisosteric substitution of the alkenyl portion in retinoids[17,21] to give conformationally restricted stilbene mimetics.

Chemistry

The natural *retro*-retinoid (+)-(**4**) and its synthetic precursors **16** and **17** have been obtained through the original methodology developed by Corey et al.[22] Compound **13** has been derived from intermediate **15** as outlined in Scheme 1. Standard Wittig reactions between appropriate partners have been used to prepare the benzoic acid derivatives **9**−**12**. A cross-coupling

Scheme 1[a]



[a] (a) $(DHQ)_2PHAL$, $K_3Fe(CN)_6$, K_2CO_3, K_2OsO_4, $CH_3SO_2NH_2$, *t*-BuOH-H_2O; (b) Ac_2O, Et_3N, DMAP, CH_2Cl_2; (c) $SOCl_2$, DiPEA, CH_2Cl_2 , −90 °C; (d) K_2CO_3, BHT, MeOH.

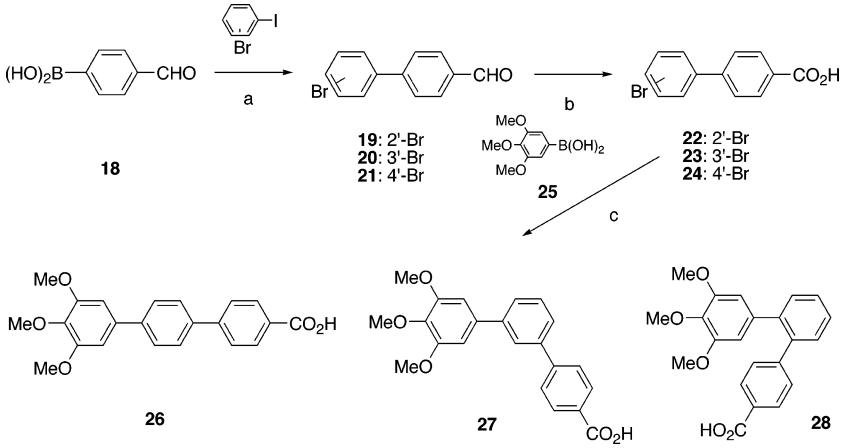
Suzuki reaction between **18** and bromoiodobenzene (ortho, meta, or para isomers) in a mixture of toluene−ethanol in the presence of a catalytic amount of tetrakis-triphenylphosphine palladium with aqueous sodium carbonate solution produced the desired biphenyl aldehydes (**19**−**21**) in good yields. The subsequent oxidation led to the corresponding carboxylic acids (**22**−**24**), which underwent Suzuki cross-coupling with the boronic acid **25** in acetonitrile to give the required **26**−**28** (Scheme 2).

Biological Results and Discussion

In Figure 3a the curves are reporting HL-60 cell growth cultured in complete medium (containing serum) or serum-free medium. When cultured in nonsupplemented serum-free medium, the cell number decreased in 72 h from 200×10^3 to 110×10^3 mL^{-1} and the percentage of apoptotic cells increased from 0% to 43% (Figure 3a and Table 1). Flow cytometry analysis showed a recruitment in the G_0-G_1 phase of the cell cycle (Figure 3b−d), and a sub-G_0-G_1 peak corresponding to apoptotic cells was evident after 48 h of culture (Figure 3c). Morphologic analysis by fluorescence microscopy after staining with acridine orange and ethidium bromide of cells cultured for 72 h in serum-free medium has shown a very low percentage of necrotic cells (less than 10%). The effects of the compounds tested in this study on cell growth and apoptosis are shown in table. Analyses were carried out on the HL60 cell line. In the table are reported the effects of the compounds used at 1 μM. All compounds induced a dose-dependent increase of cell growth and an inhibition of apoptotic cells with a plateau ranging from 0.8 to 1.2 μM; higher concentrations were less effective in supporting cell growth or were cytotoxic (data not shown).

The decrease of living cells (from 200×10^3 to 110×10^3 mL^{-1}) was partially blocked by (+)-**4** at 1 μM to yield 181×10^3 mL^{-1}. (+)-**4** also prevented apoptosis in 9% of cells. At the same concentration, compound **13** was less effective than (+)-**4** in protecting cells from the effects of the serum deprivation, while the diacetate **16** proved to be the most active, being able to partially support cell growth and to inhibit the programmed cell death induced by serum deprivation more efficiently than 14-HRR ($t < 0.01$).

Scheme 2[a]



[a] (a) $Pd(Ph_3P)_4$, aqueous Na_2CO_3, toluene/EtOH; (b) aqueous $KMnO_4$; (c) $Pd(Ph_3P)_4$, aqueous Na_2CO_3, CH_3CN.

Table 1. Effects of Different Compounds on Cell Growth and Apoptosis in HL-60 Cells Cultured for 72 h in Nonsupplemented Serum-Free Medium[a]

compd	no. of living cells/mL ($\times 10^3$)	apoptosis (%)
untreated control	110 ± 4	43 ± 1.56
14-HRR	181 ±6.7 ($p = 0.0003$)	34 ± 1.34 ($p = 0.0002$)
9	155 ± 4.47 ($p = 0.0021$)	36 ± 1.25 ($p = 0.0004$)
10	100 ± 4.33 ($p = 0.07$)	43 ± 1.8 ($p = 0.29$)
11	166 ± 5.3 ($p = 0.0004$)	36 ± 0.9 ($p = 0.028$)
12	100 ± 4.38 ($p = 0.09$)	42 ± 1.65 ($p = 0.44$)
13	149 ± 6 ($p = 0.0035$)	37 ± 1.38 ($p = 0.024$)
15	129 ± 5.5 ($p = 0.062$)	41 ± 1.8 ($p = 0.35$)
16	207 ± 3.57 ($p < 0.0001$)	30 ± 1.16 ($p = 0.0021$)
17	129 ± 5.45 ($p = 0.067$)	40 ± 1.7 ($p = 0.18$)
26	300 ± 15.4 ($p = 0.0002$)	18 ± 6.7 ($p < 0.0001$)
27	350 ± 8.3 ($p < 0.0001$)	17 ± 0.7 ($p < 0.0001$)
28	220 ± 8.2 ($p < 0.0001$)	22 ± 0.9 ($p < 0.0001$)

[a] Data represent the mean ± SEM of five independent experiments. The difference in the value of the number of living cells per milliliter and the % apoptosis between each compound and control was significant ($p < 0.05$) for compounds 14-HRR, **9**, **11**, **13**, **16**, and **26**−**28**. Apoptosis was detected by morphological analysis as described in Materials and Methods for Biological Assays. Compounds were used at 1 μM.

The known antiproliferative[23] and cytodifferentiating[24] properties of **7** in a variety of cancer cell lines and the ability of *cis*-TTNPB (**8**) to induce apoptosis in HL60 cells[13] were in contrast to activities that we found for their desmethyl derivatives. Removal of the methyl group led in some cases, as for the *cis*-stilbenes **10** and **12**, to loss of activity, while trans compounds **9** and **11** were able to slow the rate of cell death and, in part, to support cell growth. This result was quite surprising, since we were unaware of previously reported data for synthetic derivatives of retinoids featuring cell-growth-supporting activity. Taken together, these results further support the idea that both stereochemistry and substitutions at the double bond of stilbene−arotinoids as TTNPB are playing a crucial role in determining the apoptotic or antiapoptotic activity of compounds belonging to this family. Interestingly, potent antiapoptotic activity and cell-growth-supporting activity has been found for the terphenyl-based compounds **26**−**28**, being more active than (+)-**4** and intermediate **16** ($t < 0.01$ both). In particular, compound **27** was the most active, 3-fold more active than the natural *retro*-retinoid (+)-**4**, being able to significantly support cell growth (from 200×10^3 to 350×10^3 cells/mL in 72 h) and to reduce apoptotic HL60 cells from 43% to 17%. Clear evidence of the prevalent role of the terphenyl scaffold rather than the relative positions of the ortho, meta, or para junctions in determining the activity of these compounds came from the data listed in the table.

These very promising results led us to test the most active of these compounds as antiapoptotic agents on neuronal cells. It is apparent that excitoxicity is implicated as a mechanism of cell death in acute neurologic diseases, such as stroke and trauma. The cascade of events initiated by interaction of excessively released glutamate with its receptors may result in either necrosis or apoptosis, depending on the intensity of the original insult.[25,26] Thus, a slow excitotoxic process is likely to induce a delayed neuronal death with apoptotic features in chronic neurodegenerative diseases, such as Alzheimer, Parkinson, and other age-related disorders.[27] Neuronal cell death can be experimentally induced by a variety of neurotoxic agents;[28,29] thus, cultured neurons may undergo apoptosis when treated with 0.15 μM staurosporine for 24 h, with a mechanism involving cytochrome *c* release[30] or excitotoxic necrosis when treated with 30 μM glutamate for 10 min.[31−33] Interestingly, the terphenyl compounds **26** and **28** used at 1 μM were able to prevent staurosporine-induced cell death (Figure 3e), but their protective action was totally ineffective during the glutamate neurotoxic insult. A quantification of the neuroprotective effects cannot be surely made by these methods. However, the increase in cell survival induced by **26** and **28** in different experimentally induced cell death could be attributed to their antiapoptotic action, presumably taking place upstream from mitochondria. In fact, the method used here to quantify cell death is based on the mitochondrial reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a dark-blue formazan product; so it is not suitable for detecting effects occurring downstream of mitochondria, but it is useful for determining the point in the death pathway where agents have their effects.[34] The absorbance detected after staining with MTT is proportional to the number of viable cells, and results obtained using the MTT assay were similar to results obtained with cell count. These results suggest for compounds **26** and **28** a potential neuroprotective action in chronic neurodegenerative diseases. Additionally, neither (+)-**4** nor **16** proved to be active on neuronal cells, thus suggesting a possible

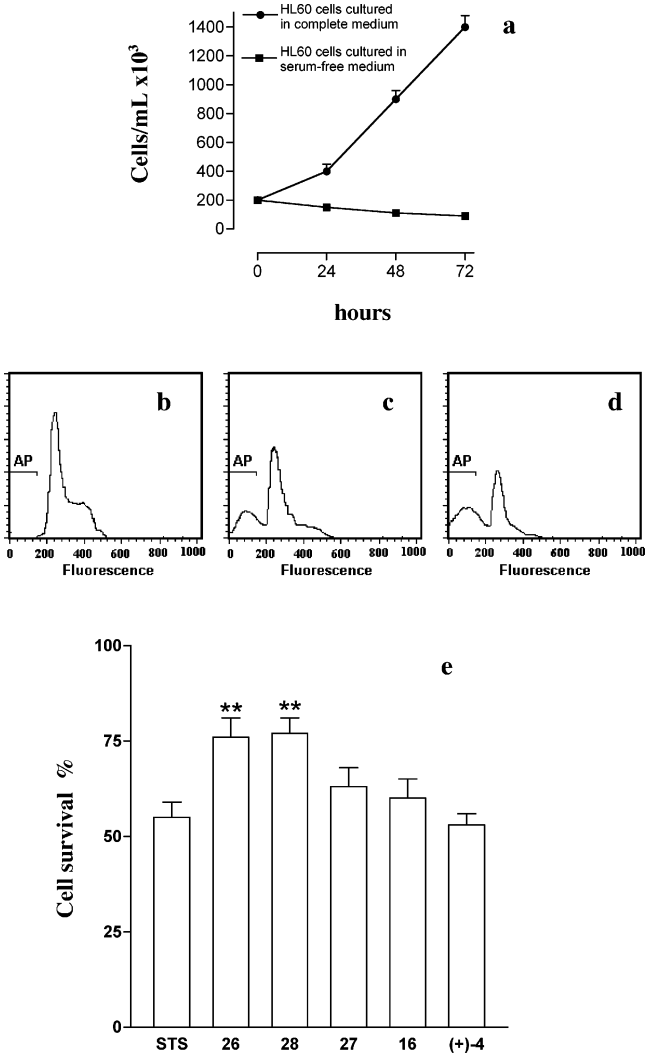


Figure 3. (a) Cell growth of HL-60 cells in complete or serum-free medium, in cells/mL $\times 10^3$. (b−d) Flow cytometry determination of apoptosis. HL60 cells were grown in complete (b) or serum-free medium (c and d): c = evaluation after 48 h of culture; d = evaluation after 72 h of culture. Apoptosis was determined by FACscan flow cytometry assay using the CellQuest software. The hypodiploid sub-GoG1 peak, designed as AP, represents cells undergoing apoptosis. Data reported in parts b−d are representative of four different experiments that have produced similar results. (e) Effects of (+)-**4**, **16**, **26**−**28** (each 1 μM) on cell viability (% survival) of rat primary cortical neurons treated with 0.15 μM staurosporine (STS). Cell viability was evaluated by MTT assay as described in Materials and Methods for Biological Assays. Data are the mean ± SEM of 5−11 experiments in triplicate: (**) $P < 0.01$ significantly different from staurosporine alone, ANOVA followed by Dunnet's test. Effects on % survival without staurosporine were the following (in parentheses is the number of experiments in triplicate): **26**, 91 ± 3% (8); **27**, 101 ± 4% (8); **28**, 106 ± 4 (4).

different mechanism of action or difficulties due to inherent instability or low entrance rate into cells.

Conclusion

Our search for new compounds as apoptosis inducers led us to discover a novel class of potent antiapoptotic agents with even higher cell-growth-supporting activity than the natural *retro*-retinoids. Thus, simple removal of the methyl group located on the double bond of TTNPB (**7**) provided compounds such as **9** and **11** that are able to support growth and to inhibit apoptosis in different cell lines at 1×10^{-6} M. Interestingly, the activity of these compounds is likely related to the trans stereochemistry of the double bond. To the best of our knowledge, synthetic retinoids possessing this biological profile have not been hitherto reported. Moreover, the cell-growth-supporting activity of **16**, together with its relatively greater chemical stability, makes this compound an interesting candidate to substitute the natural 14-HRR (**4**). A noteworthy finding was the disclosure of a new "lead" featuring a terphenyl architecture with a very intriguing biological profile and the right requirements for being considered as a potential drug for treating degenerative diseases. The methoxyphenyl appendages are not comparable to phenolic moieties, characteristic of some known neuroprotective compounds active as ROS (reactive oxygen species) scavengers.[35] Researchers have only recently focused their interest on compounds endowed with antiapoptotic activity as potential drugs useful in degenerative and ischemic diseases. We first describe in this paper that a new class of synthetic terphenyls are able to support cell growth and inhibit apoptosis. Although their effects on survival of neuronal cells are modest, the data reported in this work should be considered as the first step for future studies of structure−activity relationships (SARs) to find other compounds belonging to this class and endowed with more potent antiapoptosis activity. Moreover, considering that these compounds are different from a chemical point of view from other compounds currently studied as antiapoptotic agents (e.g., anti-caspases) it should be interesting to investigate the effects of combination therapies that include our terphenyl compounds. Further studies of this class of natural and synthetic compounds should also provide a better understanding of the mechanism of action of natural bioactive *retro*-retinoids.

Experimental Section

Chemistry. Melting points were obtained with a Kofler apparatus and are uncorrected. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F_{254} Merck plates. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were determined in $CDCl_3$ solution, unless otherwise indicated, with a Bruker AC-200 spectrometer, and peak positions are given in part per million downfield from tetramethylsilane as internal standard. All drying operations were performed over anhydrous sodium sulfate. Column chromatography (medium pressure) was carried out with 60−200 mesh silica gel, using the flash technique. Microanalysis of all new synthesized compounds agreed with calculated values within ±0.4% of the theoretical.

General Procedure for the Synthesis of Stilbene Compounds. To the phosphonium bromide salt (3,4,5-trimethoxybenzyl- or 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphth-2-ylmethyl-, 2 mmol) in anhydrous tetrahydrofuran (15 mL) is added sodium hydride (50% in paraffin, 1.2 equiv), and the mixture is stirred under argon for 30 min. A solution of aldehyde in tetrahydrofuran is added dropwise during 30 min, and stirring is continued for 6 h at room temperature. The suspension is then poured into water and extracted with dichloromethane. The organic phase is washed with brine, dried, and evaporated to give after chromatographic purification a mixture of *cis*/*trans*-stilbenes that is hydrolyzed without separation. To a solution of stilbene esters mixture in methanol (50 mg in 5 mL) is added 10 % LiOH until a slight cloudiness is attained. The mixture is heated to 60−70 °C until completion

of hydrolysis, then cooled, washed once with diethyl ether, acidified, and extracted with diethyl ether. The organic phase is evaporated, and the acids are purified and separated by a chromatographic column.

4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)vinyl]benzoic acid. *trans*-9. White solid. Mp 272−275 °C. Yield 38%. ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 6H), 1.36 (s, 6H), 1.73 (s, 4H), 7.10 (d, J = 16 Hz, 1H), 7.26 (d, J = 16 Hz, 1H), 7.37 (s, 2H), 7.46 (s, 1H), 7.62 (d, J = 8.4 Hz, 2H), 8.11 (d, J = 8.4 Hz, 2H). Anal. ($C_{23}H_{26}O_2$) C, H.

***cis*-10.** Solid. Mp 145−148 °C. Yield 41%. ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 6H), 1.64 (s, 4H), 6.55 (d, J = 11.5 Hz, 1H), 6.66 (d, J = 11.5 Hz, 1H), 7.00 (d, J = 8 Hz, 1H), 7.16 (s, 1H), 7.23 (d, J = 8 Hz, 1H), 7.39 (d, J = 8 Hz, 2H), 7.98 (d, J = 8 Hz, 2H). Anal.($C_{23}H_{26}O_2$) C, H.

4-[2-(3,4,5-Trimethoxyphenyl)vinyl]benzoic Acid. *trans*-11. Solid. Mp 218−221 °C. Yield 35%. ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 3.94 (s, 6H), 6.79 (s, 2H), 7.05 (d, 1H, J = 16.3 Hz), 7.20 (d, 1H, J = 15.9 Hz), 7.61 (d, 2H, J = 8.2 Hz), 8.11 (d, 2H, J = 8.2 Hz). Anal. ($C_{18}H_{18}O_5$) C, H.

***cis*-12.** Solid. Mp 140−143 °C. Yield 33%. ^{1}H NMR ($CDCl_3$) δ 3.67 (s, 6H), 3.85 (s, 3H), 6.46 (s, 2H), 6.59 (d, 1H, J = 12.4 Hz), 6.67 (d, 1H, J = 12.4 Hz), 7.41 (d, 2H, J = 8.1 Hz), 8.01 (d, 2H, J = 8.1 Hz). Anal. ($C_{18}H_{18}O_5$) C, H.

General Procedure for Biphenylcarbaldehyde Synthesis (19−21). To a solution of *o*-, *m*-, or *p*-iodobromobenzene (120 mg, 0.42 mmol) in 1 mL of toluene is added a catalytic amount (3−5% mol) of tetrakis-triphenylphosphine palladium and 0.3 mL of aqueous 2 M Na_2CO_3. A solution of 4-formylbenzeneboronic acid (100 mg, 0.66 mmol) in 1 mL of ethanol is then added, and the mixture is heated to reflux for 3 h in an argon atmosphere. After cooling, the mixture is extracted three times with dichloromethane and the joined organic phases are washed with water and brine, dried, and evaporated under vacuum. The residue is purified by chromatography, giving the bromodiphenylaldehyde derivative.

2′-Bromobiphenyl-4-carbaldehyde (19).[36] Oil. Yield 30%. ^{1}H NMR: δ 7.26−7.41 (m, 3H), 7.60 (d, J = 8.1 Hz, 2H), 7.71 (d, J = 8 Hz, 1H), 7.97 (d, J = 8.3 Hz, 2H), 10.09 (s, 1H).

3′-Bromobiphenyl-4-carbaldehyde (20).[37] Oil. Yield 50%. ^{1}H NMR: δ 7.37, J = 7.7 Hz, 1H), 7.57 (m, 2H), 7.72 (d, J = 9.2 Hz, 2H), 7.77 (m, 1H), 7.96 (d, J = 9 Hz, 2H), 10.07 (s, 1H).

4′-Bromobiphenyl-4-carbaldehyde (21).[38] Solid. Mp 135−140°C. Yield 62%. ^{1}H NMR: δ 7.50 (d, J = 8.8 Hz, 2H), 7.62 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 8 Hz, 2H), 7.96 (d, J = 8.6 Hz, 2H), 10.06 (s, 1H).

General Procedure for Oxidation to Biphenylcarboxylic Acid (22−24). To a gently boiling solution of bromobiphenylcarbaldehyde (**19**−**21**, 100 mg) in sodium carbonate (5 mL) is added potassium permanganate (70 mg in 1 mL of water), and reflux is maintained for 3 h. After cooling, the solution is acidified with concentrated hydrochloric acid and extracted three or more times with diethyl ether. The residue is used in the next reaction with no further purification.

2′-Bromobiphenyl-4-carboxylic Acid (22).[39] Yield 99%. Mp 237−241 °C. ^{1}H NMR (DMSO-d_6): δ 7.40−7.70 (m, 5H), 8.00−8.10 (m, 3H).

3′-Bromobiphenyl-4-carboxylic Acid (23).[39] Yield 99%. Mp 250−253 °C. ^{1}H NMR (DMSO-d_6): δ 7.40−7.85 (m, 6H), 8.11 (d, J = 8.4 Hz, 2H).

4′-Bromobiphenyl-4-carboxylic Acid (24).[39,40] Yield 98%. Mp >300 °C. ^{1}H NMR: δ 7.50 (d, J = 8.8 Hz, 2H), 7.59−7.68 (m, 4H), 8.16 (d, J = 8.6 Hz, 2H).

General Procedure for Terphenyl Synthesis. To a solution of bromobiphenyl acid (**22**−**24**, 100 mg) and 3,4,5-trimethoxybenzene boronic acid (**25**, 77 mg) in 0.4 M aqueous sodium carbonate (5 mL) and acetonitrile (5 mL) is added a catalytic amount (5% mole) of tetrakis-triphenylphosphine palladium, and the mixture is heated to reflux under argon atmosphere for 3 h. The suspension is cooled, filtered, and washed with dichloromethane. The water phase is then acidified and extracted with diethyl ether. Purification of the residue of ether extracts gives the terphenyl product.

3,4,5-Trimethoxy[1,1′;2′,1″]terphenyl-4″-carboxylic Acid (28). Yield 65%. Mp 166−170 °C. ^{1}H NMR (CD_3OD): δ 3.59 (s, 6H), 3.72 (s, 3H), 6.37 (s, 2H), 7.26 (d, J = 8.3 Hz, 2H), 7.45 (m, 4H), 7.91 (d, J = 8.2 Hz, 2H). ^{13}C NMR: δ 56.0, 61.0, 107.3, 127.7, 128.3, 129.9, 130.4, 130.5, 136.2, 137.0, 139.4, 140.6, 147.6, 152.8, 171.7. Anal. ($C_{22}H_{20}O_5$) C, H.

3,4,5-Trimethoxy[1,1′;3′,1″]terphenyl-4″-carboxylic Acid (27). Yield 76%. Mp 198−201 °C. ^{1}H NMR: δ 3.92 (s, 3H), 3.95 (s, 6H), 6.83 (s, 2H), 7.54−7.65 (m, 3H), 7.74−7.78 (m, 3H), 8.23 (d, J = 8.4 Hz, 2H). ^{13}C NMR: δ 56.4, 61.1, 104.7, 126.2, 126.4, 127.2, 127.4, 128.2, 129.4, 130.9, 136.9, 137.9, 140.5, 142.3, 146.5, 153.6, 171.7. Anal. ($C_{22}H_{20}O_5$) C, H.

3,4,5-Trimethoxy[1,1′;4′,1″]terphenyl-4″-carboxylic Acid (26). Yield 72%. Mp 243−247 °C. ^{1}H NMR: δ 3.92 (s, 3H), 3.96 (s, 6H), 6.84 (s, 2H), 7.71 (m, 4H), 7.76 (d, J = 8.4 Hz, 2H), 8.22 (d, J = 8.2 Hz, 2H). ^{13}C NMR: δ 56.3, 61.1, 104.5, 127.1, 127.6, 127.7, 128.0, 130.4, 130.9, 136.4, 138.8, 141.4, 145.9, 153.6, 170.7. Anal. ($C_{22}H_{20}O_5$) C, H.

Materials and Methods for Biological Assays. Cell Culture and Culture Conditions. HL60 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 (Gibco Grand Island, NY) containing 10% FCS (Gibco), 100 U/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), and 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO) in a 5% CO_2 atmosphere at 37 °C. The employed serum-free medium is constituted by RPMI 1640 and ITS+1 liquid media supplement (Sigma). ITS+1 liquid media supplement contains 0.5% delipidated bovine serum albumin, 10 μg/mL insulin, 5.5 μg/mL transferrin, 4.7 μg/mL linoleic acid, and 2 mM L-glutamine (Sigma).

Cytotoxicity Assays. To evaluate the number of live and dead cells, cells were stained with trypan blue and counted on a hemocytometer. Cells that showed trypan blue uptake were interpreted as nonviable.

Morphological Evaluation of Apoptosis and Necrosis. Drugs effects on apoptosis and necrosis were determined morphologically by fluorescence microscopy after labeling with acridine orange and ethidium bromide. Cells (2×10^5) were centrifuged (300 g), and the pellet was resuspended in 25 μL of the dye mixture. An amount of 10 μL of the mixture was placed on a microscope slide, covered with a 22 mm^2 coverslip, and examined in oil immersion with a 100× objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labeling with ethidium bromide.

Determination of Apoptosis by Annexine V. Cells (1×10^6) were washed with PBS and centrifugated at 200*g* for 5 min. The cell pellet was suspended in 100 μL of staining solution containing annexine-V-fluorescein labeling reagent (Annexine-V-Fluos Staining Kit, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at 20 °C. Annexine V positive cells were evaluated by fluorescence microscopy and flow cytometry.

Neuronal Cell Cultures. Cortical neuronal cultures were prepared from 1-day old Sprague Dawley rats, as described by Alho et al.[41] The neurons were plated in 24-well culture vessels (Falcon) coated with 20 μg/mL poly-L-lysine and cultured in modified neurobasal medium (1 mL) by adding 50 mg/mL gentamycin sulfate, 2 % B27, and 500 μM glutamax 500. After 24−48 h, 5 μM cytosin arabinoside (ARA-C) was also added to prevent glial cell replication. The neurotoxic insult was induced on the seventh to ninth day in vitro (7−9 DIV) by adding 0.15 μM staurosporine for 24 h. In another set of experiments, an amount of 500 μL of medium was collected and set aside. The cells were intoxicated by adding 30 μM glutamate for 10 min, and then the medium was removed and replaced with the original one. Drugs to be tested, dissolved in 50 μL of DMSO, were added to the medium 30 min before the intoxication and maintained until the end of

the experiment. An equal amount of DMSO was added to the medium in control wells. The primary effects of the drugs were tested in cultured cells submitted to the same protocol, in the absence of neurotoxins.

MTT Assay. Cell survival was determined either 24 h after staurosporine addition or 30 min after glutamate intoxication. Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to the blue formazan product only by metabolically active mitochondria, and the absorbance is proportional to the number of viable cells. MTT (0.5 mg/mL) was added to the cultures, and the blue color was allowed to develop for 2 h. After aspiration of the medium, 100 μL of 9:1 isopropyl alcohol/HCl was used to solubilize the blue crystals. Samples were read at a test wavelength of 570 nm. The absorbance data are expressed as percentages of control groups.[28]

Statistics. Survival data are given as the mean ± SEM of percent values with respect to control cells. The statistical significance of the differences has been assessed with the analysis of the variance (ANOVA), followed by Dunnet's test (GraphPad software).

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kaufmann, S. H.; Hengartner, M. O. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol*. **2001**, *11*, 526−534.
(2) Zhivotovsky, B.; Orrenius, S. Defects in apoptotic machinery of cancer cells: role in drug resistance. *Semin. Cancer Biol*. **2000**, *13*, 125−134.
(3) Tolomeo, M.; Simoni, D. Drug resistance and apoptosis in cancer treatment: Development of new apoptosis-inducing agents active in drug resistance malignancies. *Curr. Med. Chem.: Anti-Cancer Agents* **2002**, *2*, 387−401.
(4) Haunstetter, A.; Izumo, S. Toward antiapoptosis as a new treatment modality. *Circ. Res.* **2000**, *86*, 371−376.
(5) Friedlander, R. M. Apoptosis and caspases in neurodegenerative diseases. *N. Engl. J. Med.* **2003**, *348*, 1365−75.
(6) *Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action*; Nau, H., Blaner, W. S., Eds.; Springer: Berlin, 1999 and references therein.
(7) Buck, J.; Derguini, F.; Levi, E.; Nakanishi, K.; Hammerling, U. Intracellular signaling by 14-hydroxy-4,14-*retro*-retinol. *Science* **1991**, *254*, 1654−1656.
(8) Derguini, F.; Nakanishi, K.; Hammerling, U.; Buck, J. Intracellular signaling activity of synthetic (14*R*)-, (14*S*)-, and (14*RS*)-14-hydroxy-4,14-*retro*-retinol. *Biochemistry* **1994**, *33*, 623−628.
(9) Chen, Y.; Buck, J.; Derguini, F. Anhydroretinol induces oxidative stress and cell death. *Cancer Res.* **1999**, *59*, 3985−3990.
(10) Vakiani, E.; Buck, J. *Handbook of Experimental Pharmacology*; Springer: New York, 1999; pp 97−115.
(11) O'Connell, M. J.; Chua, R.; Hoyos, B.; Buck, J.; Chen, Y.; Derguini, F. Retro-retinoids in regulated cell growth and death. *J. Exp. Med.* **1996**, *184*, 549−555.
(12) Derguini, F.; Nakanishi, K.; Hammerling, U.; Chua, R.; Eppinger, T.; Levi, E.; Buck, J. 13,14-Dihydroxy-retinol, a new bioactive retinol metabolite. *J. Biol. Chem*. **1995**, *270*, 18875−18880.
(13) Simoni, D.; Roberti, M.; Invidiata, F. P.; Rondanin, R.; Baruchello, R.; Malagutti, C.; Mazzali, A.; Rossi, M.; Grimaudo, S.; Dusonchet, L.; Meli, M.; Raimondi, M. V.; D'Alessandro, N.; Tolomeo, M. Programmed cell death (PCD)-associated with the stilbene motif of arotinoids: Discovery of novel apoptosis inducer agents possessing activity on multidrug resistant tumor cells. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2669−2673.
(14) Simoni, D.; Roberti, R.; Invidiata, F. P.; Rondanin, R.; Baruchello, R.; Malagutti, C.; Mazzali, A.; Rossi, M.; Grimaudo, S.; Capone, F.; Dusonchet, L.; Meli, M.; Landino, M.; D'Alessandro, N.; Tolomeo, M.; Arindam, D.; Lu, S.; Benbrook, D. M. Heterocycle-containing retinoids. Discovery of a novel isoxazole arotinoid possessing potent apoptotic activity in multidrug and drug-induced apoptosis resistant cells. *J. Med. Chem.* **2001**, *44*, 2308−2318.
(15) Simoni, D.; Tolomeo, M. Retinoids, apoptosis and cancer. *Curr. Pharm. Des.* **2001**, *7*, 1823−1837 and references therein.
(16) Loeliger, P.; Bollag, W.; Mayer, H. Arotinoids, a new class of highly active retinoids. *Eur. J. Med. Chem.* **1980**, *15*, 9−15.
(17) Jong, L.; Lehmann, J. M.; Hobbs, P. D.; Harlev, E.; Huffman, J. C.; Pfahl, M.; Dawson, M. I. Conformational effects on retinoid receptor selectivity. 1. Effects of 9-double bond geometry on retinoid X receptor activity. *J. Med. Chem.* **1993**, *32*, 2605−2613.
(18) Kagechika, H.; Himi, T.; Namikawa, K.; Kawachi, E.; Hashimoto, Y.; Shudo, K. Retinobenzoic acids. 3. Structure−activity relationships of retinoidal azobenzene-4-carboxylic acids and stilbene-4-carboxylic acids. *J. Med. Chem.* **1989**, *32*, 1098−1108.
(19) Kagechika, H.; Himi, T.; Kawachi, E.; Shudo, K. Retinobenzoic acids. 4. Conformation of aromatic amides with retinoidal activity. Importance of *trans*-amide structure for the activity. *J. Med. Chem.* **1989**, *32*, 2292−2296.
(20) Osterod, F.; Peters, L.; Kraft, A.; Sano, T.; Morrison, J. J.; Feeder, N.; Holmes, A. B. Luminescent supramolecular assemblies based on hydrogen-bonded complexes of stilbenecarboxylic acids and dithieno[3,2-*b*:2′,3′-*d*]thiophene-2-carboxylic acids with a tris-(imidazoline) base. *J. Mater. Chem.* **2001**, *11*, 1625−1633.
(21) Dawson, M. I.; Jong, L.; Hobbs, P. D.; Xiao, D.; Feng, K.-C.; Chao, W.-R.; Pan, C.; Fontana, J. A.; Zhang, X.-K. 4-[3-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)phenyl]benzoic acid and heterocyclic-bridged analogues are novel retinoic acid receptor subtype and retinoid X receptor α agonists. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1311−1313.
(22) Corey, E. J.; Noe, M. C.; Guzman-Perez, A. Catalytic enantioselective synthesis of (14*R*)-14-hydroxy-4,14-*retro*-retinol from retinyl acetate. *Tetrahedron Lett.* **1995**, *36*, 4171−4174 and references therein.
(23) Schneider, S. M.; Offterdinger, M.; Huber, H.; Grunt, T. W. Activation of retinoic acid receptor α is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells. *Cancer Res*. **2000**, *60*, 5479−5487.
(24) Dawson, M. I.; Hobbs, P. D.; Derdzinski, K.; Chan, R. L.-S.; Gruber, J.; Chao, W.-R.; Smith, S.; Thies, R. W.; Schiff, L. J. Conformationally restricted retinoids. *J. Med. Chem.* **1984**, *27*, 1516−1531.
(25) Nicotera, P.; Bonfoco, E.; Brüne, B. Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. *Adv. Neuroimmunol*. **1995**, *5*, 411−420.
(26) Leker, R. R.; Shohami, E. Cerebral ischemia and trauma—different etiologies yet similar mechanisms: neuroprotective opportunities. *Brain Res. Rev*. **2002**, *39*, 55−73.
(27) Mattson, M. P.; Duan, W.; Pedersen, W. A.; Culmsee, C. Neurodegenerative disorders and ischemic brain diseases. *Apoptosis* **2001**, *6*, 69−81.
(28) Lin, W. W.; Wang, C. W.; Chuang, D. M. Effects of depolarization and NMDA antagonists on the survival of cerebellar granule cells: a pivotal role for protein kinase C isoforms. *J. Neurochem.* **1997**, *68*, 2577−2586.
(29) Loo, D. T.; Rillema, J. R. Measurement of cell death. *Methods Cell Biol*. **1998**, *57*, 251−64.
(30) Ahlemeyer, B.; Klumpp, S.; Krieglstein, J. Release of cytochrome *c* into the extracellular space contributes to neuronal apoptosis induced by staurosporine. *Brain Res*. **2002**, *934*, 107−116.
(31) Cheung, N. S.; Pascoe, C. J.; Giardina, S. F.; John, C. A.; Beart, P. M. Micromolar L-glutamate induces extensive apoptosis in an apoptotic−necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. *Neuropharmacology* **1998**, *37*, 1419−1429.
(32) Gwag, B. J.; Canzoniero, L. M. T.; Sensi, S. L.; Demaro, J. A.; Koh, J. Y.; Goldberg, M. P.; Jacquin, M.; Choi, D. W. Calcium ionophores can induce either apoptosis or necrosis in cultured cortical neurons. *Neuroscience* **1999**, *90*, 1339−1348.
(33) Ankarcrona, M. Glutamate induced cell death: apoptosis or necrosis? *Prog. Brain Res.* **1998**, *116*, 265−272.
(34) Lobner, D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J. Neurosci. Methods* **2000**, *96*, 147−152.
(35) Larget, R.; Lockhart, B.; Pfeiffer, B.; Neudorffer, A.; Fleury, M.-B.; Largeron, M. Synthesis of novel orthoalkylaminophenol derivatives as potent neuroprotective agents in vitro. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2929−2934.
(36) Brown, J. W.; Butcher, J. L.; Byron, D. J.; Gunn, E. S.; Rees, M.; Wilson, R. C. Comparison of the properties of liquid crystals derived from certain lateral halogeno-substituted azomethines. *Mol. Cryst. Liq. Cryst.* **1988**, *159*, 255−266.
(37) Capitosti, G. J.; Guerrero, C. D.; Binkley, D. E., Jr.; Rajesh, C. S.; Modarelli, D. A. Efficient synthesis of porphyrin-containing benzoquinone-terminated, rigid polyphenylene dendrimers. *J. Org. Chem.* **2003**, *68*, 247−261.
(38) van Heerden, P. S.; Bezuidenhoudt, B. C. B.; Ferreira, D. Improved synthesis for the rodenticides, diphenacoum and brodifacoum. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1141−1146.

(39) Byron, D. J.; Gray, G. W.; Wilson, R. C. The synthesis of some substituted biphenyl-4-carboxylic acids, 4-biphenylacetic acids, and 4-aminobiphenyls. *J. Chem. Soc. C* **1966**, 840−845.
(40) Picard, F.; Schulz, T.; Hartmann, R. W. 5-Phenyl substituted 1-methyl-2-pyridones and 4′-substituted biphenyl-4-carboxylic acids. Synthesis and evaluation as inhibitors of steroid-5α-reductase type 1 and 2. *Bioorg. Med. Chem.* **2002**, *10*, 437−448.
(41) Alho, H.; Ferrarese, C.; Vicini, S.; Vaccarino, F. Subset of GABAergic neurons in dissociated cell cultures of neonatal rat cerebral cortex show co-localization with specific modulator peptides. *Brain Res*. **1988**, *39*, 193−204.

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