Cytotoxicity and Cytoprotective Activity of Naphthalenediols in Rat Cortical Neurons

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Some members of the naphthalenediol family have been shown in previous work on PC-12 cells to act as effective antioxidants while being relatively nontoxic. In the present work, we extend that study to examine the effect of naphthalenediols on rat primary cortical neurons exposed to AAPH (2,2′-azobis (2-amidinopropane) hydrochloride), a source of peroxyl radicals. Compounds tested included the acetylated forms of 1,2-naphthalenediol, that is, 1,2-ND, as well as 1,4-ND, 2,3-ND, 1,8-ND, and the known highly potent antioxidant (−)-epigallocatechin gallate (EGCG). In cytoxicity studies, cells were exposed to the compounds for 24 h, leading to observed toxicity in the order of 1,4-ND > 1,2 ND ≫ 2,3-ND ≈ EGCG > 1,8-ND. In cytoprotection studies, the desired compounds were incubated with neurons prior to AAPH exposure, and live cell counts were determined by trypan blue and/or MTT assays. Excellent protection, superior to EGCG, was provided by 2,3-ND and 1,8-ND. Additional studies using glutamate as a stressor showed that 1,8-ND had a significant protective effect at concentrations as low as 500 nM. The results can be understood on the basis of the tendency (or lack thereof) to form the corresponding quinone, which in turn depends on whether or not there is a loss of aromaticity in the ring adjacent to the quinone moiety. Thus, certain members of the family of naphthalenediols are quite cytotoxic, whereas others show promise as neuroprotective antioxidants.

Introduction

Current understanding of the causes of neurodegenerative diseases, such as Alzheimer's disease (AD[1]), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), indicates that there is an oxidative stress component (*1*, *2*). Consequently, dietary antioxidant therapy is being studied as a possible therapeutic approach. A number of studies have examined the protective effects of naturally occurring dietary antioxidants, such as vitamin C and vitamin E, glutathione, carotenoids, flavonoids, polyphenols, and antioxidant enzymes (*3*−*5*). The experimental data on the use of antioxidants in cell cultures and rodent models of neurodegenerative diseases show distinct benefits (*5*), but the clinical and epidemiological outcomes from human trials seem to be less clear-cut.

In contrast, little work has been done on the systematic design of antioxidants targeted toward reducing age-related neurodegenerative disease, but such designer antioxidants are attracting increasing attention now that the principles behind antioxidant design are relatively well understood (*6*−*10*). In a previous work (*11*) using adherent PC-12 adrenal cells, we subjected the cells to oxidative stress in the form of AAPH (2,2′-azobis (2-amidinopropane) hydrochloride), which generates a continuous flux of alkylperoxyl radicals (*12*−*15*). Cytotoxicity studies showed that 1,2-naphthalenediol (1,2-ND) and 1,4-ND were quite toxic and were thus ruled out as potentially useful antioxidants. However, 2,3-ND and especially 1,8-ND showed significant cytoprotection against AAPH, while at the same time showing low inherent toxicity. Thus, further studies were indicated for the latter two species.

We rationalized these experimental results with the help of density functional theory (DFT) by calculating enthalpy changes between the parent naphthalenediol (also called naphthohydroquinone and symbolized NQH_2), the partially oxidized naphthosemiquinone radical ($NQH^{\bullet}$), and the fully oxidized naphthoquinone (NQ) (*11*, *16*). Calculated bond dissociation enthalpy (BDE) values for $NQH_2 \rightarrow NQH^{\bullet}$ (BDE_1) and $NQH^{\bullet} \rightarrow NQ$ (BDE_2) showed that when BDE_2 is low (e.g., <75 kcal mol^{-1}), the formation of the quinone product is observed, whereas when it is high (>85 kcal mol^{-1}), the formation of the quinone product is not observed. Quinone formation is generally accompanied by toxicity in the form of redox cycling and subsequent hydrogen peroxide production or an attack on the electrophilic quinone by cellular nucleophiles such as GSH or cysteine-containing proteins (*17*−*19*). This situation holds for 1,2-ND and 1,4-ND; both redox cycling and thiol depletion mechanisms can contribute to the observed cytotoxicity. However, the loss of aromaticity (in the unsubstituted ring) on quinone formation for 2,3-ND exerts a heavy energy penalty, preventing their

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[1] Abbreviations: AAPH, 2,2′-azobis (2-amidinopropane) hydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGCG, (−)-epigallocatechin gallate; BDE, bond dissociation enthalpy; MLM3, medium-level model 3; 1-2-ND-DA, 1,2-naphthalenediol-diacetate; AD, Alzheimer's disease; ALS, amylotrophic lateral sclerosis; PD, Parkinson's disease; DFT, density functional theory; 1,4-NQH_2, 1,4-naphthohydroquinone; NMDA, *N*-methyl-D-aspartate; NB27 − AO, neurobasal medium supplemented with B-27 minus AO.

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formation. Simple Lewis structures show that no quinone structure can be drawn when starting from 1,8-ND; instead, a triplet diradical state is formed. As a result, the 2,3-NQ and 1,8-NQ products were not formed, and hence, the parent naphthalenediols were much less toxic. They were also shown to be strongly cytoprotective. In the present article, we extend the study of naphthalenediols to primary rat cortical neurons to see whether the conclusions derived from PC-12 cells are more general and whether animal trials should be pursued. For comparison, we include the antioxidant (−)-epigallocatechin gallate (EGCG), the principal polyphenol present in green tea, which is known to be a superior neuroprotective antioxidant (*20*−*24*).

Materials and Methods

Materials. The sources of materials, measures of purity, and so forth are the same as those described previously by Flueraru et al. (*11*). That work also describes the sources from which we obtained 1,2-ND (prepared from its quinone), 1,4-ND, 2,3-ND, 1,8-ND, and EGCG. Glutamate, dimethyl sulfoxide (DMSO), glutamine, glucose, penicillin/streptomycin, and PBS (phosphate buffered saline) were obtained from Sigma Chemical Co. (St. Louis, MO). Neurobasal medium, B-27 serum-free supplement, and B-27 supplement minus AO (antioxidant-free), minimum essential medium (MEM), horse serum, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA).

Cell Culture. We used two methods to prepare the cell culture of cortical neurons; starting either from animals or from frozen cell stock, respectively. In the first, primary cultures of cortical neurons were obtained from 18-day-old pregnant Sprague−Dawley rats (Charles River) following the procedure described previously (*25*). All experiments were performed according to the Guidelines of the Canadian Council on Animal Care and approved by the Carleton University Animal Care Committee. In brief, the rats were sacrificed using CO_2, the pups removed, transferred to ice-cold PBS, and decapitated. Cortices were surgically removed, suspended in 2 mL of plating media consisting of Eagle's minimal essential medium (MEM), supplemented with 10% horse serum, 10% fetal bovine serum, 2 mM glutamine, and 20 mM glucose and mechanically dissociated. The cell suspension was centrifuged at 1100 rpm for 6 min at 20 °C. After removal of the supernatant, the resulting pellets were resuspended in 8−10 mL of plating media. Cell viability was determined by the trypan blue exclusion method. The cells were plated in 35 mm dishes (Corning) previously treated with poly-D-lysine at a concentration of 1.0×10^6 cells/dish. Cultures were maintained in a 5% CO_2 atmosphere at 37 °C. After one week, the medium was replaced with neurobasal medium containing the B-27 supplement. Further media changes occurred bi-weekly.

In the second method, we used cryopreserved rat cerebral cortex neuronal cells (QBM Cell Science, Inc., Ottawa, Canada). The cells were thawed, suspended in neurobasal medium supplemented with B-27 and penicillin (100 U/mL)/streptomycin (100 μg/mL) and seeded in 96-well plates coated with poly-D-lysine at a concentration of 3×10^5 cells/mL. The cells were incubated at 37 °C in a humidified atmosphere (5% CO_2 in air) for 4 h. Then the medium was removed, leaving a small volume to ensure that the cells did not dry out, and a pre-warmed medium was added. For 14 days, 50% of the medium was replaced with fresh medium twice weekly.

Cytotoxicity Test Using MTT Assay. Experiments were performed on days 14−16 of the cell cultures. The medium was removed, and the cells were incubated for an additional 24 h (37 °C, 5% CO_2) in neurobasal medium supplemented with B-27 minus AO (NB27 − AO) (Invitrogen) (*26*). After 24 h, the NB27−AO medium was replaced with fresh medium containing NB27 − AO and the compounds to be tested (in DMSO, final DMSO concentration <0.5%), and the incubation was continued for another 24 h. The neuronal cultures were assessed for viability using the MTT assay. Briefly, the cultures were washed with PBS and incubated with medium and 10 μL of MTT (5 mg/mL) for 2 h. The cells were then lysed in DMSO, and the absorbance values were read at 570 nm with background subtraction at 630 nm. The control consisted of NB27 − AO plus DMSO with no added compound; its absorbance (three measurements) determines cell viability for the control, which was then set at 100%.

Protection against Oxidative Stress Induced by AAPH. For all cytoprotection experiments, the term medium hereafter refers to NB27 − AO. The 96-well microplates prepared in the same way as before (2×10^5 cells/mL) were incubated in medium for 24 h prior to use. Cytoprotection was assessed by the method described previously (*11*). In brief, the cells were incubated with the compounds to be tested (1−150 μM) for 2 h prior to addition of AAPH (1.5 mM). After 24 h of further incubation with AAPH + compound, the cell viability was assessed using MTT (5 mg/mL). At 1.5 mM, the oxidative stress generated by AAPH reduces the cell viability to ca. 36−40% of that of the control (labeled stress on Figures to follow).

Protection against Oxidative Stress Induced by Glutamate. Experiments were performed in dishes on days 14−16 of culture. The maintenance medium was replaced with NB27 − AO. After 24 h of incubation, the medium was discarded and replaced with fresh medium containing the compounds at concentrations ranging from 0.1−100 μM and incubated for 2 h. Glutamate (250 μM) was added to the dishes, and incubation was continued for another 3 h. The cells were washed once with 1 mL of PBS, and new medium was added. Because the cells were in dishes for the glutamate experiments, the MTT assay was inconvenient, and instead, after 24 h, cell viability was assessed by the trypan blue exclusion method. The number of dead cells was counted under the microscope (PIX Cell II) in 8−10 randomly chosen fields in every dish and was expressed as a percentage of the control (0 μM glutamate).

Statistics. Data are expressed as mean ± SEM values, and statistical significance was assessed by one-way ANOVA. Differences were considered to be significant at $p < 0.05$.

Calculation of BDE. The method of calculation of the BDE was previously described (*11*) in which relevant BDE values were described for the naphthalenediols and an EGCG model compound. Briefly, the BDE corresponds to the standard gas-phase enthalpy change at 298 K (ΔH^0_{298}) for ArO-H (g) → ArO• (g) + H• (g). In cases where there are two exchangeable OH groups, BDE_1 refers to the loss of the first (most weakly bound) H atom to form the semiquinone, and BDE_2 to the loss of the second H atom to form the quinone. Starting geometries, which included conformer searching, were obtained with the Spartan 02 builder module (Wave-Function, Inc., Irvine, CA) using the AM1 method; coordinates were then sent to the Gaussian 98 program for subsequent calculations using the lowest-level method (LLM) described by DiLabio et al. (*27*).

Results

Experimental. We first examined the cytotoxicity to primary neuronal cultures of the naphthalenediols, prepared in their diacetylated form, and the reference compound EGCG. The naphthalenediol diacetates cross the cell membrane, and once in the cytosol, the acetate moieties are cleaved by cellular esterases, forming the active diols.

Figure 1 shows the structures for the five compounds tested, where each of the naphthalenediol compounds has been fully acetylated. For each compound, the weakest OH bond is shown as OH(a); after the loss of an H atom and the formation of the corresponding radical, the next weakest OH bond is shown as OH(b). This pattern of reactivity is obvious from a consideration of substituent effects; thus, the center hydroxyl position in pyrogallol is weakest (*8*). Para-substitution by a methyl group will further weaken the central bond, whereas para-substitution

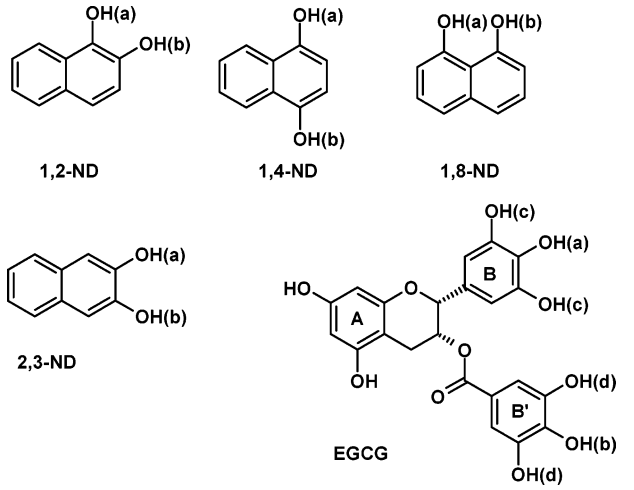


Figure 1. Structural formulas for the five compounds tested, where 1,2-ND = 1,2-naphthalenediol, etc. and EGCG = (−)-epigallocatechin 3-gallate. The weakest OH bond is indicated by OH(a), which corresponds to BDE_1, the next weakest is OH(b), which corresponds to BDE_2, etc.

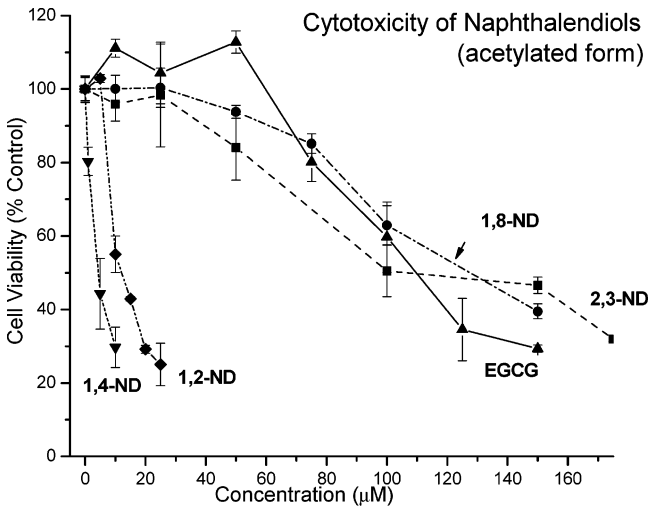


Figure 2. Cell viability of rat cortical neurons (MTT assay) after 24 h of incubation with acetylated naphthalenediols and EGCG.

by an electron-withdrawing carboxyl group will strengthen the bond; the two cases should differ in BDE by ca. 4 kcal/mol.

Figure 2 shows cell viability versus concentration of compound for the five compounds tested, that is, the cytotoxicity. Cell viability is defined here as the percent of live cells (from MTT assay) relative to the control (zero concentration of compound). There was a wide range of cytotoxicity among the compounds tested, ranging from 1,4-ND (most toxic) and 1,2-ND (very toxic) to 2,3-ND ≈ EGCG ≈ 1,8-ND (relatively nontoxic). Note that EGCG showed reproducible enhancement of survival (cell viability above 100%).

Figure 3 shows the cytotoxicity caused by the oxidative stressor AAPH, which generates peroxyl radicals ($ROO^{\bullet}$), at a relatively constant rate. Here, the lethal concentration, 50% level (LC_{50}) is defined as the amount of compound that reduces formazan blue absorbance (MTT assay) to 50% of control (control: no AAPH added). Fitting a quadratic function to the data in Figure 3 gave an LC_{50} of 1.23 ± 0.20 mM, with a correlation coefficient $R^2 = 0.956$.

Because 1,2-ND and 1,4-ND were very cytotoxic, they were not used any further, and our experiments focused on examining the cytoprotective properties of 2,3-ND and 1,8-ND in comparison to that of the well-known antioxidant EGCG. Figure 4

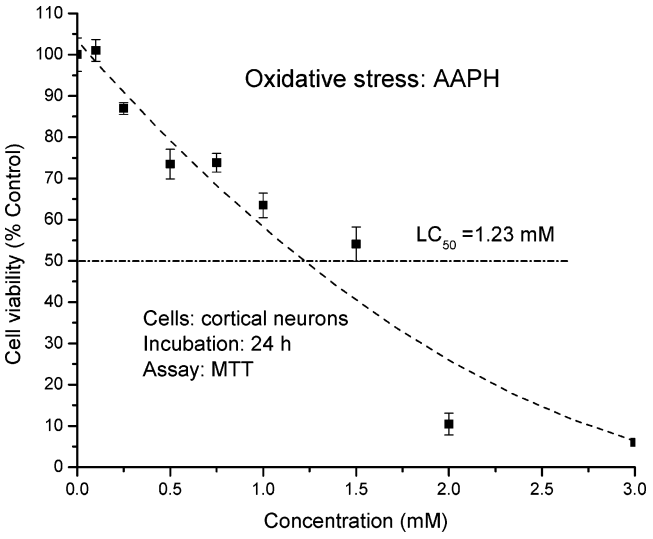


Figure 3. Cell viability (from MTT assay) for primary cortical neurons vs concentration of AAPH. Control: no AAPH added.

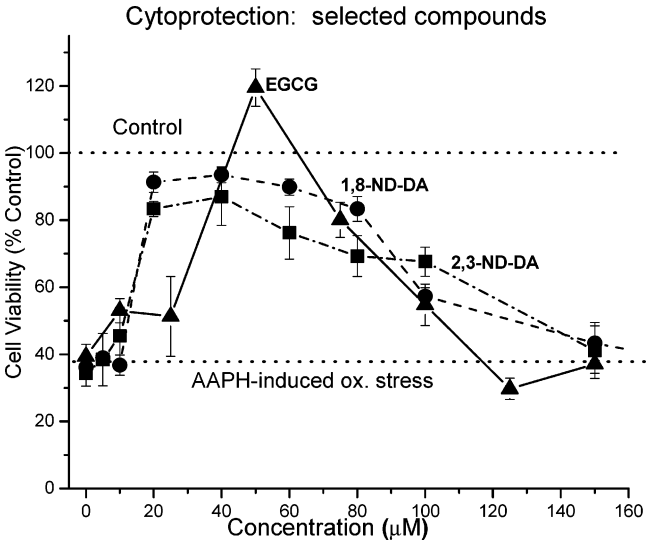


Figure 4. Cytoprotection of cortical neurons by selected naphthalenediols in the presence of 1.5 mM AAPH. Compounds tested: 2,3-ND, 1,8-ND (as diacetates), and EGCG. Protective zone: between dashed lines bounded by 38% viability (AAPH-induced oxidative stress) and 100% (control).

shows the cytoprotection resulting from the pretreatment of the cells for 2 h with 1,8-ND-DA, 2,3-ND-DA, and EGCG prior to adding AAPH. The AAPH stressor reduced the viability from 100% (control) to 38% (stress); therefore, any viability above 38% represents protection of the neurons. The naphthalenediols are protective over the full range tested (10−150 μM), whereas EGCG is only protective up to 120 M. Comparing the 2,3-ND and 1,8-ND, it can be seen that both compounds behave similarly and are strongly protective over the range shown. The cytoprotection curve for EGCG is more sharply peaked, reaching its maximum of 113% at 50 μM, but otherwise showing less protection than the two naphthalenediols.

Figure 5 shows the cytoprotective area (CPA), as defined in our previous work (*11*) and obtained from the data for 1,8- ND. The CPA represents an integral over the viability/concentration plot, with contributions to the CPA occurring when the viability shows a protective effect due to the compound. In the present case, the application of AAPH reduces cell viability to 38%, labeled Stress on Figure 5; this provides the lower boundary for the CPA. Pretreatment with 1,8-ND is protective against

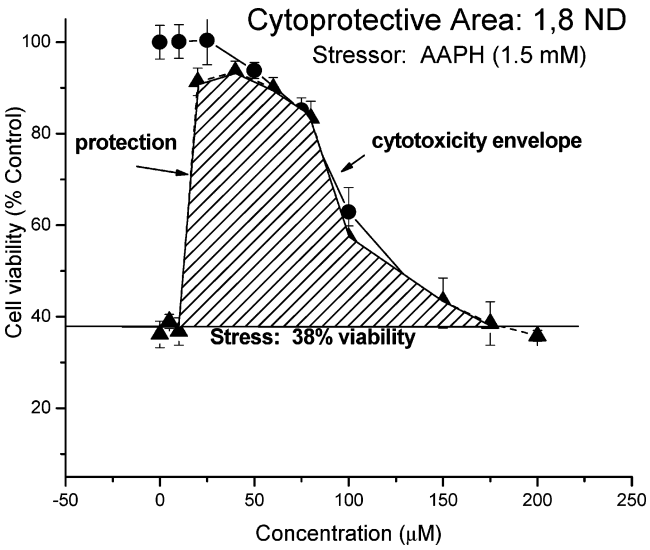


Figure 5. Total cytoprotective area for 1,8-ND (cross-hatched region). Boundaries: cytoprotection experiment (from Figure 4) at left (▲), cytotoxicity envelope (from Figure 2) at top (●), cell viability in presence of AAPH stress at bottom ((—), 38% relative to control).

Table 1. Calculated BDE_1 and BDE_2, the Cytotoxicity LC_{50} for PC12-AC Cells (*11*) and Cortical Neurons (This Work) and the Ratio of the LC_{50}values between the Cell Lines

compd	BDE_1 (kcal/mol)	BDE_2 (kcal/mol)	LC_{50} PC12-AC (μM)	LC_{50} neurons (μM)	ratio
1,4-ND	75	55	15	5	3.00
1,2-ND	70	69	40	12	3.33
2,3-ND	79	84	270	110	2.45
1,8-ND	72	104[a]	270	127	2.12
EGCG	71[b]	74[b]	475	110	4.30

[a] Triplet diradical state. [b] BDE_1 is for the H atom labeled (a) in the parent compound in Figure 1, and BDE_2 is for the H atom labeled (b) in Figure 1.

Table 2. Cytoprotective Efficacy of Antioxidants Against Oxidative Stress Induced by 1.5 mM AAPH[a]

compd	range[b] neurons (μM)	max (% control) neurons	$CPA_{PC12-AC}$ (% control·μM)[c]	$CPA_{neurons}$ (% control·μM)
1,2-ND	2−5	53	100	ca. 75
1,4-ND	3−10	70 at 5 μM[d]	285	50
2,3-ND	5−150	90 at 40 μM	7460	4810
1,8-ND	5−150	93 at 40 μM	9030	5400
EGCG	10−120	110 at 50 μM	17300	3990

[a] Incubation time is 24 h with 2 h of pretreatment with antioxidant. [b] Total protective range; only the region above Stress is counted (see Figure 5). [c] Unit for cytoprotective area. [d] 70 at 5 μM means 70% viability at 5 μM concn of compound.

the oxidative stress at lower doses but eventually becomes even more toxic than 38% viability because of the inherent cytotoxicity of the compound. The upper boundary to the CPA integral is provided by the cytotoxicity envelope (Figure 2). The area of the bounded region (shaded, Figure 5) is the CPA, which provides an integrated comparison of the protection given between different compounds. The protective range is 5−170 μM. The total CPA for 1,8-ND is ca. 5000, in units of % control × μM. The same procedure was repeated for the other compounds, 2,3-ND and EGCG (graphical data not shown). These data are collected in the next section in Table 2.

Another physiological oxidative stressor is the excitory neurotransmitter glutamate. As ischemia/hypoglycemia is known to release large amounts of glutamate, which induces oxidative

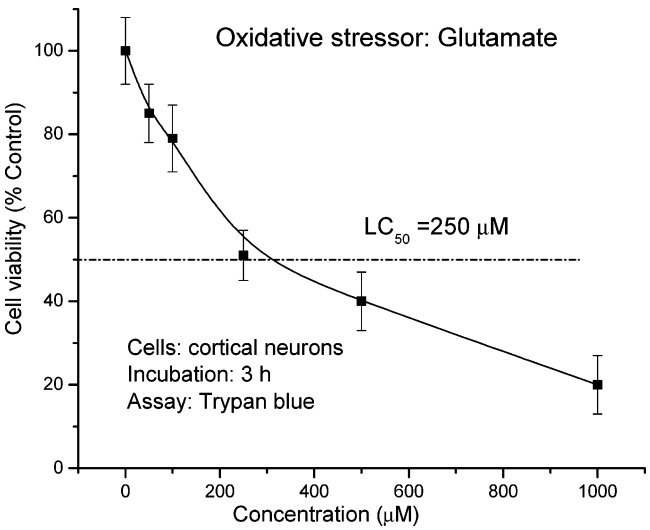


Figure 6. Cytotoxicity of glutamate to cortical neurons (trypan blue assay).

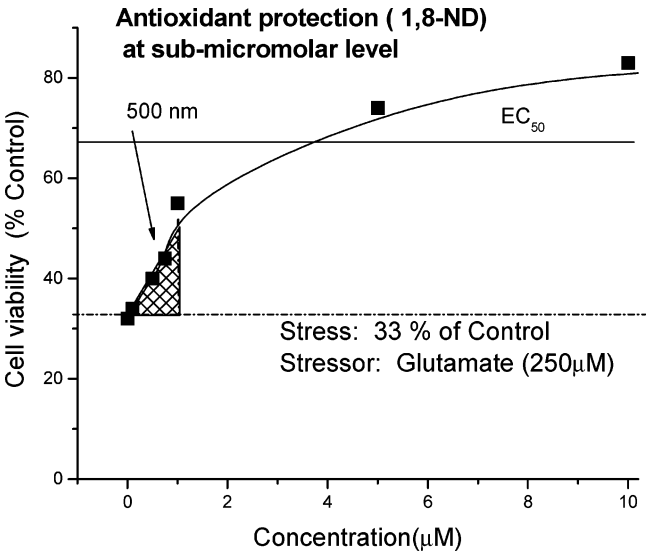


Figure 7. Cytoprotection of cortical neurons by 1,8-ND showing low-dose protection; oxidative stressor is glutamate (250 μM).

stress in neurons (*28*, *29*), this assay would model in vitro what is thought to occur in the intact brain. For these experiments, the cells were subjected to 3 h of exposure to glutamate over the concentration ranges 0.1−1 mM. Cell viability was established using the trypan blue assay after 24 h of incubation of cells in NB27 − AO (see Materials and Methods).

Figure 6 shows that for glutamate, the LC_{50} was about 250 μM under the conditions of our experiment. The LC_{50} values are sensitive to the parameters of the experiment. Relevant parameters are the percentage of supporting glia in the dishes and the initial neuron density, but we did not measure these parameters in the current work. (We used serum-free medium in order to diminish the number of glial cells, which heightens the response of the neurons to oxidative stress.)

Figure 7 shows the cytoprotection resulting when 1,8-ND was added prior to glutamate treatment. A significant ($p < 0.05$) protective effect was observed at a concentration as low as 500 nM. For example, the cell viability was increased from 33 ± 3% SEM (no 1,8-ND present) to 47 ± 4% SEM when 500 nM compound was present. The cell viability goes up rapidly with higher concentration so that the viability at 10 μM is already above 80%. The effective concentration (EC_{50}), which is

halfway between control and stress, that is, 67%, corresponds to 3.7 μM 1,8-ND. Similarly, 2,3-ND at concentrations of 10−50 μM protects against glutamate, but concentrations below 10 μM were ineffective (data not shown).

Theoretical. Theoretical calculations were included to aid in the discussion of the toxicity and protective effects of the compounds studied. Table 1 shows the calculated results for BDE_1 and BDE_2, the cytotoxicity expressed as the LC_{50} values in PC12-AC cells (previous work, ref *11*) and cortical neurons (present work), and the ratio between the LC_{50} values for the two cell types.

Table 2 summarizes the concentration range that is cytoprotective for rat cortical neurons, the concentration that provides the maximum protective effect, the CPA in PC12-AC cells, and the CPA in neurons.

Discussion

Toxicity. Figure 2 shows that for the naphthalenediols tested here, two are relatively toxic (1,2- and 1,4-ND), whereas two are relatively nontoxic (2,3- and 1,8-ND) to rat cortical neurons. The latter cytotoxicity is comparable to that of EGCG, which is generally thought to be neuroprotective (*20*−*24*). In fact, these results are very similar to those found in the adrenal cell line PC12-AC. Thus, Table 1 shows that for the four naphthalenediols the order of cytotoxicity is essentially identical between the two cell lines, as measured by the LC_{50} value for each. Under the conditions of the two experiments, the rat cortical neurons are seen to be more sensitive to the compounds by a factor of approximately 3. It is clear that EGCG differs somewhat from this pattern because it is by far the least toxic of the group in PC12-AC, although it is comparable in toxicity to 2,3-ND and 1,8-ND in cortical neurons.

The location where the toxic action is occurring has not been established in this article; however, some relevant points can be addressed. In ref *11*, we reported the calculated values of log *P*, the octanol−water partition coefficient. Most of the log *P* values were about 2, implying that the naphthalenediols will pass through the cell membrane, and thus, an equilibrium distribution is set up between the extracellular and intracellular regions. In order to act as an antioxidant, the test compounds must be deacetylated first, and this occurs when ester hydrolases are encountered in the intracellular region. Because antioxidant behavior is observed, this proves that the compound must have spent at least some time in the intracellular space. Following deacetylation, it is possible that the compounds were transported back outside the cell where the protective effects were acting. This mode of cytoprotection has been discussed by Brunmark and Cadenas (*18*).

Because the values of log *P* are relatively low, for example, compared to vitamin E or ubiquinone, which are known to exist in the cell membrane, it is unlikely that the naphthalenediols are trapped inside the cell membrane. Thus, the compounds are acting as hydrophilic rather than lipophilic antioxidants. Minimum observable levels of cytoprotective activity would, therefore, be expected to be in the range of ca. 1 μM, as with other hydrophilic antioxidants.

Cytoprotection against AAPH. It is known from the work of Ingold and co-workers (*12*−*14*) that AAPH decomposes with a unimolecular rate constant of 2×10^{-6} s^{-1} into two positively charged carbon radicals. These rapidly add oxygen and may, therefore, be represented as $^{+}AOO^{\bullet}$. This radical cation may be expected to react rapidly with molecular anions, due to the electrostatic attraction (*14*). In the present case, the naphthalenediol parent species have pK_a values as low as 6.4 (for 1,8-ND) and, thus, will be partly in anionic form; the semiquinones have pK_a values of ca. 4.0 and, thus, will be predominantly anionic. Therefore, an important reaction will be $^{+}AOO^{\bullet} + ArO^{-} \rightarrow {}^{+}AOO^{-} + ArO^{\bullet}$, that is, an electron transfer from the anion to the radical cation (written as the charge-separated structure). The main point, however, is to maintain consistency for the oxidative stressor between the different cell lines so that comparisons are possible. This dictated the use of AAPH for most of our experiments.

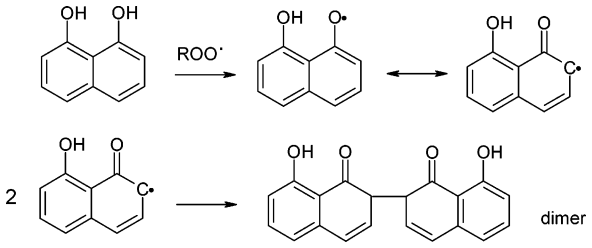
As with cytotoxicity, there are clear trends in the cytoprotective effectiveness that transcend cell lines. Table 2 compares the cytoprotective areas (CPAs) obtained using AAPH as oxidative stressor. Comparing just the naphthalenediols, both 1,2-ND and 1,4-ND have only a small CPA, rendering them useless as antioxidants (their protective window is small). 2,3-ND and 1,8-ND, however, have a CPA of about 2 orders of magnitude greater. EGCG is most effective in PC12-AC cells by a factor of 2; however, in the neurons, both 2,3- and 1,8-ND have higher CPAs than EGCG. Qualitatively, then, the cytoprotective behavior is similar for the two cell lines.

Stoichiometry Reaction Products. In all our experiments using AAPH, the cells were exposed to the (assumed constant) radical flux for 24 h. The question arises as to whether there is sufficient antioxidant so that some will remain over the entire period of incubation. Assuming an average flux of 2×10^{-9} M s^{-1} of radicals (*13*) and 100 μM antioxidant, we can calculate that in $100 \times 10^{-6}/2 \times 10^{-9} = 50\,000$ s = 15 h all of the antioxidant would be used up if the reaction were instantaneous. In fact, as the concentration of ArOH is lowered through the reaction, the reaction rate, k_2[peroxyl$^{\bullet}$][ArOH], must decrease proportionately. This will extend the duration of the protective effect because ArOH is used up at a declining rate. However, the protective effect will not be using the stated concentration but rather by a lesser amount. This point could have been studied further by using different time intervals for incubation, but we have not included such dynamical studies in the present article.

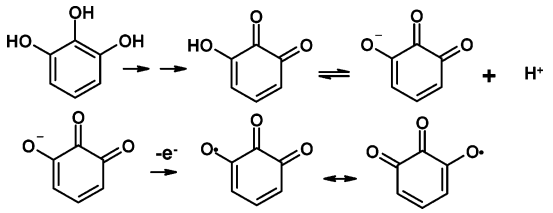
Peroxyl Radical Attack: Significance of BDE (*11*). For naphthalenediols possessing two exchangeable H atoms, BDE_1 describes the tendency to intercept the peroxyl radical, leading to semiquinone formation. Here, the compound is acting as an antioxidant. If the mechanism is H-atom transfer (HAT), then the reaction is $NQH_2 + {}^{+}AOO^{\bullet} \rightarrow NQH^{\bullet} + {}^{+}AOOH$, breaking the chain reaction of lipid peroxidation; therefore, lower values of BDE_1 mean a faster reaction rate with peroxyl radicals and, hence, better radical scavenging. BDE_2 describes the tendency to go from semiquinone to quinone, that is, $NQH^{\bullet} + {}^{+}AOO^{\bullet} \rightarrow NQ + {}^{+}AOOH$. Note that because of the typically low pK_a of semiquinones (ca. 4.0), $NQH^{\bullet}$ actually exists in the deprotonated form $NQ^{\bullet-}$ at physiological pH. The semiquinone radical anion reacts preferentially by electron transfer rather than H-atom transfer (HAT). However, the energetics between electron transfer and HAT are related, and BDEs are still useful as predictors of reactivity: see, for example, the strong correlation between electrochemical reduction potential and BDE (*27*).

Once $NQ^{\bullet-}$ formation occurs, it can easily transfer an electron to oxygen producing superoxide ion, according to $NQ^{\bullet-} + O_2 \rightarrow NQ + O_2^{\bullet-}$. Thus, begins a chain reaction involving redox cycling between semiquinone radical anion and quinone, leading to the formation of superoxide and related species (*17*) or thiol depletion by nucleophilic addition to the electrophilic quinone. Both mechanisms cause cytotoxicity via these prooxidant behaviors (generation of superoxide or removal of antioxidant thiols), and hence, low values of BDE_2 should accelerate

Scheme 1. Termination via Carbon-Centered Radical, Dimer Formation



Scheme 2. Hydroxyquinone Formation, Ionization, and Electron Donation



quinone formation and are to be avoided. Table 2 shows that on the basis of BDE_2 values, the predicted order of cytotoxicity is 1,4-ND > 1,2-ND ≫ 2,3-ND ≫ 1,8-ND. This is essentially the order observed, although the BDE_2 for the formation of 2,3-naphthoquinone is already sufficiently high (84 kcal/mol) that no such quinone is formed; this makes 2,3-ND and 1,8-ND comparably nontoxic.

Experimentally, the quinones of 1,2-ND and 1,4-ND have been observed (*18*, *19*), and thus, their role as prooxidants is well established. For the nonquinone formers 2,3-ND and 1,8-ND, other termination mechanisms can be imagined by analogy to known reaction products, for example, between catechins and peroxyl radicals (*30*, *31*). Scheme 1shows the conversion of an oxygen radical to a carbon radical and subsequent dimerization. Note that dimerization as shown in Scheme 1 leaves additional exchangeable H atoms, thus the molecule retains some antioxidant character after termination, which could show up in the stoichiometry of radical trapping. An alternative termination mechanism is the addition of peroxyl radical at the carbon radical site, forming R′OOR, which also terminates the chain (*30*, *32*).

Why EGCG Is Relatively Nontoxic. EGCG has a relatively low BDE_2 (74 kcal/mol, Table 2) and, hence, is expected to be relatively toxic, contrary to observations (LC_{50} is comparable to 2,3-ND, Table 1). Unlike the naphthalenediols with only two exchangeable H atoms, the EGCG molecule has eight exchangeable H atoms, and a closer examination is required. As shown in Figure 1, the second BDE is actually for the central OH group on the B′ ring. Because the B and B′ rings are not conjugated, they will essentially act independently; thus, in this case, both BDE1 and BDE2 refer to antioxidant reactions and are, therefore, intrinsically different from the case where the loss of the second H atom results in quinone formation and subsequent toxicity (true for 1,2-ND and 1,4-ND). In EGCG, it is BDE3 and BDE4 (not calculated but >74), which become relevant to prooxidant action because the corresponding hydroxyquinones are formed. Here, the recent discussion by Aptula et al. (*32*) on toxicity mechanisms of trihydroxybenzenes is relevant.

The pyrogallol moiety (i.e., 1,2,3-trihydroxybenzene) forms a special type of quinone because it has a third active hydrogen (or electron). Scheme 2 shows the conversion of pyrogallol into 3-hydroxy-1,2-benzoquinone. The hydroxyquinone is very acidic (pK_a 4.3), and hence, the molecule is an anion at pH 7.4. The strongly electron-donating O^- group changes the electronic character of the quinone so that it is no longer strongly electrophilic and, hence, is less subject to nucleophilic attack; this removes the first source of toxicity. The anion in Scheme 1 is then less likely to donate an electron to oxygen because this would leave two electron-withdrawing carbonyl groups and an electron-withdrawing oxygen radical on the aromatic ring; three dipoles oriented in the same direction are repulsive and, hence, are not favored energetically. It is also unlikely to redox cycle because it will be less subject to reduction by cytochrome P450 reductase because of the presence of the negative charge. This removes the three main sources of toxicity; hence, the observation that EGCG, which contains the pyrogallol moiety, is relatively nontoxic.

Comparison of Toxicity and Protection in Immortal versus Primary Neuonal Cells. We expect that the generalizations on cytotoxicity established for the naphthalenediols should hold up for other cell lines as well, although the precise ratios of the LC_{50}'s will depend on the conditions of the experiment, especially the plating density of the cells. Thus we can predict that the order of cytotoxicity in any cell line will be 1,4-ND > 1,2-ND ≫ 2,3-ND ≈ 1,8-ND, with EGCG being generally of toxicity comparable to or less than the least toxic naphthalenediol, 1,8-ND. Similarly, 2,3-ND and 1,8-ND should show significant cytoprotective effects, whereas 1,2-ND and 1,4-ND should not. For such generalizations to hold, *there must be a consistent mechanism underlying the observed toxicity.* As discussed in the introduction, the data are unified by the tendency (or lack thereof) of the naphthalenediols to form naphthoquinones. This tendency explains the data and allows the prediction of toxicity for other naphthalenediols as well.

Cytoprotection against Glutamate. Additional experiments on 1,8-ND using glutamate as the oxidative stressor showed significant protective effects at low (sub-μM) concentrations. In order to explain the very high protection given by high nM concentrations of 1,8-ND, two mechanisms are possible. The most obvious mechanism is that 1,8 ND scavenged the reactive oxygen species created by the glutamate insult. The second possibility is that 1,8 ND antagonized the glutamate receptor-mediated toxicity directly by acting as an antogonist of the glutamate receptors. Neurotoxicity induced by glutamate results from glutamate-activated NMDA receptors. The hyperactivation produces a massive influx of extracellular Ca^{2+} into the cell, producing a cascade of events leading to oxidative stress and superoxide generation (*33*−*38*). We found that this is unlikely because in a few patch clamp recordings, the addition of 1,8 ND had no effect on the ongoing NDMA receptor-driven spontaneous activity present in these cultures (data not shown). This suggests, therefore, that either the low-dose protection (Figure 7) given by 1,8-ND under conditions of glutamate stress is caused by simple antioxidant activity or more subtle effects, for example, cellular signaling, are involved (*39*, *40*).

Conclusions

Certain members of the naphthalenediol family show good activity in scavenging peroxyl radicals, while having toxicity comparable to that in naturally occurring polyphenols. These compounds showed similar behavior in PC-12 cells, and thus, the explanation for their cytotoxicity and cytoprotective activity crosses multiple cell lines. This can be understood from the mechanistic discussion, that is, the tendency to form naphthoquinones is related to the loss of aromaticity (or not) in the quinone moiety. In fact, some of the same arguments have been used to explain cytotoxicity in steroid chemistry, where quinones

are also involved (*41*, *42*). In terms of drug development, it will be of interest to see how well the naphthalenediols penetrate the blood−brain barrier in animal models and look at the toxicity of metabolites.

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