TARGETING NOVEL SYNTHETIC ANTIOXIDANTS TOWARDS REDUCING FREE RADICAL – INDUCED CELL DAMAGE

by

MIHAELA FLUERARU, M.Sc.

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Department of Chemistry

Carleton University

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Abstract

The main objective of the thesis was to develop novel synthetic compounds with antioxidant activity superior to Vitamin E aimed at reducing free radical-induced cell damage. The bond dissociation enthalpy (BDE) for the most active (weakest) OH bond in each molecule was used as predictor of antioxidant activity. After synthesis and testing in chemical solvent, the novel synthetic compounds were tested for cytotoxicity and then for protective effects against oxidative stress in biological systems such as:

a. HL-60 cells used as a model for testing the antioxidant capacity of the novel compounds

b. an adherent clone of rat pheochromocytoma cells (PC12-AC). Oxidative stress was induced by the aqueous-phase peroxyl radical generator AAPH. Four members of the naphthalenediol family were tested, along with the reference compound epigallocatechin gallate (EGCG). The observed cytotoxicity and cytoprotection was explained based on the different electronic structures of the compounds, characterized by the first and second bond dissociation enthalpies and the pKa's for the parent (diol) and semiquinone. The data were combined to create a measure of cytoprotective efficacy for each compound, defined by the CPA (cytoprotective area). The mechanism of cytotoxicity of catechols in PC12-AC cells was also studied. The three catechols tested strongly upregulated glutathione (GSH) synthesis in the first 24 h due to the production of hydrogen peroxide.

c. primary cortical neurons. The study was extended in order to examine the effect of naphthalenediols on primary cortical neurons exposed to glutamate or peroxyl-radical oxidative stress. In cytotoxicity studies, cells were exposed to compounds for 24 h, leading to observed toxicity in the order 1,4-ND > 1,2 ND >> 2,3-ND ≈ EGCG > 1,8-

ND. This order was explained on the basis of the tendency of each compound to form their corresponding quinone, a factor which depends upon the loss of aromaticity in the quinone product. Excellent protection, superior to EGCG, was provided by 2,3- and 1,8-NDs. Additional studies using glutamate as a stressor showed that 1,8-ND prevented neurotoxicity in a concentration-dependent manner, with a significant protective effect observed at concentrations as low as 500 nM.

Dedication

To my husband, Costel and my sons, Andrei and Dragos,

for their love and constant encouragement, support and advice

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Thank you all!

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List of Abbreviations

AAPH – 2,2'-azobis (2-amidinopropane) hydrochloride

ADMET – absorption-distribution-metabolism-excretion-toxicity

AD – Alzheimer's disease

ALS – amyotrophic lateral sclerosis

AM1 – Austin Model 1

$RO^{\bullet}$ – alkoxyl radical

BDE – Bond Dissociation Enthalpy

CPA – Cytoprotective Area

HL-60 – cultured human leukemia cells

DFT – density functional theory

DNA – deoxyribonucleic acid

DPPH – 2,2-diphenyl-1-picrylhydrazyl free radicals

DMSO – dimethyl sulfoxide

DCF – Dichlorofluorescein

H_2DCF-DA – 2'7'-dichlorodihydrofluorescein diacetate

DTNB – 5,5'dithiobis-2-nitrobenzoic acid

EDTA – ethylenediaminetetraacetic acid

EGCG – (-) epigallocatechin gallate

HBSS – Hanks' balanced salt solution

HAT – Hydrogen Atom Transfer

H_2O_2 – hydrogen peroxide

HOCl – hypochlorous acid

$HOO^{\bullet}$ – hydroperoxyl radical

$OH^{\bullet}$ – hydroxyl radical

IP – Ionization Potential

IkB – inhibitor of kappa B

LDBS – locally dense basis sets

LLM – lowest level method

MTT – (3-(3,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide)

NMDA – *N*-methyl-D-aspartic acid

NADH – nicotinamide adenine dinucleotide, reduced form

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

NF-kB – nuclear factor-kappa B

log P – octanol-water partition coefficient

GSSG – oxidized glutathione

PD – Parkinson's disease

$ROO^{\bullet}$ – peroxyl radical

PMA – phorbol 12-myristate 13-acetate

PBS – phosphate buffered saline

ROS – reactive oxygen species

GSH – reduced glutathione

PC12 – rat adrenal pheochromocytoma cells

RPMI 1640 – Roswell Park Memorial Institute medium

SPLET – Sequential Proton Loss Electron Transfer

SET – Single Electron Transfer

$O_2^{\bullet -}$ – superoxide radical

SOD – superoxide dismutase

SAR – structure-activity relationship

α–TOH – α–tocopherol

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Chapter 1

General Introduction

1.1 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are continuously produced in organisms as part of normal aerobic metabolism [1,2] as well as after exposure to environmental stressors such as radiation [3] or cigarette smoke [4]. ROS include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$) [5]. With the exception of H_2O_2 and HOCl, all species contain an unpaired electron in the outer orbital of oxygen. Such free radicals are highly reactive and chemically destructive, reacting with non-radical molecules from which they extract electrons [6]. The non-radical molecule, in turn, becomes a radical itself, leading to a molecular chain reaction which generates more free radicals and leads to an amplification of damage.

ROS are continuously generated through normal aerobic metabolism. Approximately 90% of the oxygen used by the cell is consumed by the mitochondria, a small proportion of which leads to the generation of ROS [7]. Therefore, the rate of mitochondrial ROS formation increases by increasing the metabolic rate [7].

During metabolism, oxygen undergoes a four-electron reduction to produce water in mitochondria, reactions which are catalyzed by cytochrome c oxidase [8,9]. However, a small percentage of the oxygen undergoes one-electron reduction leading to several ROS and hydrogen peroxide according to the following reactions [10]:

$O_2 + e^- \rightarrow O_2^{\bullet -}$	Superoxide radical	(1)
$O_2^{\bullet -} + H^+ \rightleftarrows HO_2^{\bullet}$	Hydroperoxyl radical	(2)
$HO_2^{\bullet} + e^- + H^+ \rightarrow H_2O_2$	Hydrogen peroxide	(3)
$H_2O_2 + e^- \rightarrow OH^{\bullet} + OH^-$	Hydroxyl radical	(4)

Hydrogen peroxide is not a radical but is one of the most studied ROS. It has a relatively long half-life and can readily move through membranes [11]. The dismutation of superoxide radicals, according to the equation:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

is the main source of intracellular hydrogen peroxide. Hydrogen peroxide contributes significantly to oxidative stress. It can undergo Fenton-type reactions with metals such as Fe^{2+} and Cu^+ to produce $OH^\bullet$ radicals [12], according to

$$Fe^{2+} (Cu^+) + H_2O_2 \rightarrow Fe^{3+} (Cu^{2+}) + OH^\bullet + OH^- \quad (6)$$

Hydroxyl radicals are extremely reactive and can damage all major macromolecules of the cell [13,14]. They can also be generated through the Haber-Weiss reaction [15].

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet \quad (7)$$

Other sources of ROS include the peroxisomal β-oxidation of fatty acids, cytochrome P450 reactions, phagocytic cells (respiratory burst), metabolism of xenobiotic compounds, and tissue specific enzymes (e.g. xanthine oxidase).

1.1.1 Lipid peroxidation

An important target of ROS is the biomembrane which contains fatty acids (lipids). The mechanism of lipid peroxidation is a chain reaction which proceeds in three stages [5,16,17]:

Initiation: production of $R^\bullet$: $RH \rightarrow R^\bullet$ (8)

Propagation: $R^\bullet + O_2 \rightarrow ROO^\bullet$ (9)

$ROO^\bullet + RH \rightarrow ROOH + R^\bullet$ (10)

Termination: $ROO^\bullet + ROO^\bullet \rightarrow ROOR + O_2$ (11)

$$2R^{\bullet} \rightarrow R\text{-}R \quad (12)$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \quad (13)$$

The initiation reaction can be nonenzymatic (caused by heat, light, or by a single electron transfer (SET) from a reducing agent such as Fe^{2+} to an acceptor such as ROOH) or an enzyme-catalyzed SET reaction. Once a free radical $R^{\bullet}$ has been generated, then reactions (9) and (10) form a chain reaction. Reaction (9) is very fast, with a rate constant of ca. 10^9 M^{-1} s^{-1} whereas (10) is much slower, typically 10^1 $M^{-1}s^{-1}$ [16]. Termination of the chain reaction takes place with quenching of the propagating free radical through various pathways (11)-(13) [16]. Lipid peroxidation can be lethal to the cell by decreasing the cell membrane fluidity and increasing the "leakiness" of the membrane bilayer to substances such as Ca^{2+} ions that normally cross it through specific channels [5]. The influx of Ca^{2+} activates Ca^{2+}-activated phospholipases which further destroy the cell membrane resulting in cell lysis.

1.2 ANTIOXIDANT DEFENCE SYSTEMS

As a result of a continuous production of ROS and the possibility of lipid peroxidation, cells have evolved potent and complex antioxidant defence systems to protect themselves. The term antioxidant, as defined by Halliwell and Gutteridge [18], is any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate".

There are three kinds of antioxidant systems:

i) antioxidant enzymes: the primary system or preventive antioxidants which limit the initial formation of oxygen centred radicals (peroxyl and alkoxyl radicals).

ii) chemical antioxidants: the secondary scavenging system or chain-breaking antioxidants which interrupt chain reactions propagated by ROS already present (vitamin E, vitamin C, carotenoids, coenzyme Q, glutathione).

iii) repair mechanisms: the third system which includes enzymes for repairing damaged proteins (methionone sulphoxide reductase for repairing oxidised methionine residues), lipids (phospholipase A_2, lecithin cholesterol acyltransferase [5]) and DNA (many enzymes such as endonuclease III, DNA glycosylase) [19].

1.2.1 Antioxidant enzymes

The primary antioxidant system includes enzymes which play important roles in the control of formation and proliferation of ROS resulting from molecular oxygen. Enzymatic defence systems include superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase.

Superoxide dismutase is the principal enzyme of the biological defence against the damage caused by $O_2^{\bullet -}$ [20,21]. SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen, according to reaction (5). The rate of reaction (5) is approximately 10^4 times greater than that of the spontaneous dismutation at physiological pH [1,20,22]. In mammalian tissues, two different superoxide dismutases occur depending on their cellular location and metal ion content: Cu/ZnSOD present in cytoplasm of most cells and MnSOD within the mitochondria [20]. The liver, adrenal gland, kidney, and spleen contain large amounts of SOD.

Catalase is an enzyme (tetrameric hemoprotein) located in peroxisomes of most tissues [5,10]. This enzyme catalyses reduction of H_2O_2 to water, according to the general reaction

$$2\ H_2O_2 \rightarrow 2H_2O + O_2 \quad (14)$$

The activity of catalase varies among the tissues. There is a high level of catalase in liver, kidney and red blood cells and less in brain, heart and skeletal muscle [23]. Catalase shares the function of removing H_2O_2 with another enzyme, glutathione (GSH) peroxidase. At high concentrations, H_2O_2 is removed by catalase and at low concentrations, organic peroxides are removed by GSH peroxidase (utilizing glutathione in the process) [10].

Glutathione (GSH) peroxidase is a selenium dependent enzyme (most forms) which catalyzes the following reactions using glutathione as the reducing substrate:

$$2GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \quad (15)$$

$$2GSH + ROOH \rightarrow GSSG + H_2O + ROH \quad (16)$$

This decreases the oxidative damage produced by lipid peroxidation. There are also two other known GSH peroxidases: selenium independent GSH peroxidase [24] and phospholipid hydroperoxide glutathione peroxidase (an enzyme associated with membranes [25,26]). Both enzymes catalyze only the reduction of lipid hydroperoxides.

Glutathione reductase is a flavoprotein that reduces oxidised glutathione to replenish reduced GSH using NADPH, according to the equation:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (17)$$

The enzyme is localized primarily in the cytosol, also in the membrane-bound organelles and mitochondrial matrix [5]. The ratio GSH/GSSG is used as an indicator of oxidative stress in tissues.

1.2.2 Chemical antioxidants

Chain-breaking antioxidants include vitamin E, vitamin C, glutathione, carotenoids, uric acid and bilirubin. In the next section some of the antioxidants which are relevant for our studies will be discussed.

Vitamin E is the most effective lipid-soluble chain-breaking antioxidant [27]. Vitamin E exists in eight naturally occurring different forms, four tocopherols and four tocotrienols (Figure 1.1) [27]. Each form has its own biological activity.

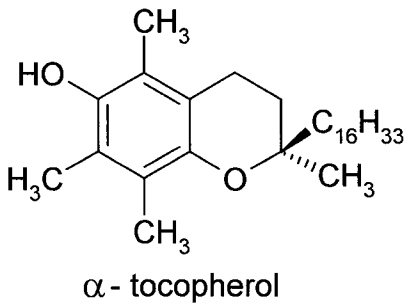


Figure 1.1. The chemical structure of α-tocopherol.

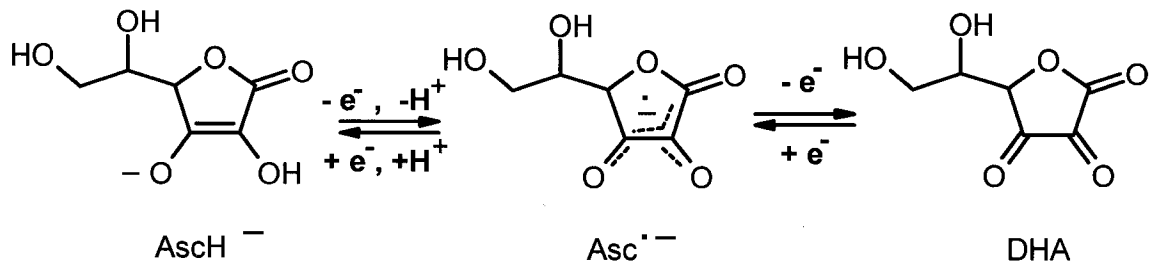
Alpha-tocopherol (or α-TOH) is the most active form of vitamin E in humans. It is also a powerful biological antioxidant [28]. The main role of Vitamin E is to protect against membrane lipid peroxidation by reacting with lipid peroxyl radicals and alkoxyl radicals. α-TOH intercepts peroxyl radicals generated by lipid peroxidation with a rate constant of 10^6 M^{-1} s^{-1} [28], according to the equation:

$$\alpha\text{-TOH} + RO_2^{\bullet} \rightarrow \alpha\text{-TO}^{\bullet} + ROOH \qquad (18)$$

In addition to its antioxidant behaviour, Vitamin E is a membrane-stabilizing agent through its van der Waals interaction with membrane phospholipids, thus preventing the damaging actions of phospholipases [29,30].

Vitamin C is an excellent water-soluble reducing agent. It plays an important role in cellular redox reactions [31,32]. At the pH of 7.4, 99.95% of vitamin C will be present as ascorbate ($AscH^-$) and only 0.05% as ascorbic acid ($AscH_2$) ($pK_1 = 4.17$) [33]. Ascorbate (the negatively charged lactone of ascorbic acid) is found in the cytoplasm of the cells. Ascorbate loses one electron leading to the semidehydroascorbate radical ($AscH^{\bullet}$). This rapidly ionizes to the ascorbyl radical anion ($Asc^{\bullet -}$) which can be further oxidized to give dehydroascorbic acid (DHA), according to Scheme 1.1 [5,33].

SCHEME 1.1



Dehydroascorbic acid is converted back to ascorbic acid by a glutathione-dependent enzyme, dehydroascorbate reductase [34,35], according to the reaction:

$$DHA + 2GSH \rightarrow AscH_2 + GSSG \qquad (19)$$

Ascorbate can react with and scavenge various reactive species such as $O_2^{\bullet -}$, $HO_2^{\bullet}$, $OH^{\bullet}$, and HOCl (in chronic inflammation [5]). Wefers *et al* showed that ascorbate is an efficient scavenger *in vitro* of singlet oxygen [36].

Vitamin C and Vitamin E function synergistically to protect membrane lipids from damage by quenching the free radicals [37-39]. Vitamin C regenerates the reduced form of vitamin E. Ascorbate acts as an electron-transfer agent at the water/membrane interface, where the α–tocopheroxyl radical reacts with ascorbate with the regeneration of α-TOH [37,40], according to the equation:

$$\alpha\text{-TO}^{\bullet} + \text{AscH}^{-} \rightarrow \alpha\text{-TOH} + \text{Asc}^{\bullet -} \quad (20)$$

Glutathione (GSH), a tripeptide composed of L-glutamate, L-cysteine and glycine, protects against free radical damage by providing reducing equivalents for key enzymes such as glutathione-S-transferase (with role in detoxification of xenobiotics) and GSH-peroxidase (antioxidant enzyme) [23]. It is an electron donor via the sulfhydryl group of the cysteine. Its concentration in the cell can be in the millimolar range while most other components generally tend to be in the micromolar range.

Another important role of GSH is that of a free radical scavenger. GSH reacts directly with peroxyl, phenoxyl and semiquinone radicals and is effective in scavenging hydroxyl radicals and singlet oxygen [23]. It is also involved in the regeneration of ascorbate from its oxidized form, dehydroascorbic acid.

Glutathione participates in DNA synthesis and repair, protein synthesis, amino acid transport, enhancement of immune function and enzyme activation [41]. It plays an essential role in the nervous system as a redox modulator of some ionotropic water receptor currents and as a potential neurotransmitter [42].

Natural polyphenols such as flavonoids and catechins are important constituents of fruits, vegetables, nuts, seeds, tea, olive oil and red wine. [43]. These polyphenols inhibit lipid peroxidation and lipoxygenases *in vitro*. Many studies have shown the beneficial effects of tea polyphenolic compounds on quenching free radicals and their role in the prevention and therapy of disease [45,47]. The anti-inflammatory, hepatoprotective, antithrombotic, and antiviral activities of polyphenols are related, in part, to their ability to scavenge a wide range of ROS such as $OH^{\bullet}$, $O_2^{\bullet -}$, $RO^{\bullet}$, and $RO_2^{\bullet}$ [44,45]. They can also chelate metal ions decreasing their metal ion prooxidant effects

[46]. Their antioxidant efficiency will dependent on their bioavailability to cells and tissues and the ability to interact with and penetrate lipid bilayers causing variations in the cell membrane structure and fluidity. The uptake of flavonoids into the cytosol is dependent on cell type [47]. Modification of cholesterol concentrations in cell membranes will influence the incorporation, uniformity of distribution, and the orientation of antioxidants in the lipid bilayers changing in this way their antioxidant efficiency [48]. Cholesterol depletion in erythrocytes causes a significant decrease in the protection offered by antioxidants against oxidative damage induced by ROS.

Many studies have suggested that natural polyphenols exert their biological actions not only through their hydrogen-donating antioxidant capacity but also by interaction with intracellular protein kinase and lipid kinase signalling cascades such as phosphoinositide 3-kinase, Akt/PKB, tyrosine kinases, protein kinase C, and MAP kinases) [49,50,51]. Flavonoids have the ability to bind to the ATP sites on enzymes such as mitochondrial ATPase, protein kinase A, topoisomerase and protein kinase C [50,52,53]. Flavonoids interfere with pathways of intermediary metabolism, and downregulate the expression of adhesion molecules [54].

It was demonstrated that specific flavonoids can suppress the induction of inducible nitric oxide synthase (iNOS – an inflammatory mediator) gene and protein expression, and NO• production by cytokines and endotoxins in mouse macrophage RAW 264.7 cells [49,55,56]. The mechanism was not due to a direct inhibitory effect on enzyme activity, but rather the modulation of cell signalling pathways necessary for NOS gene expression.

In lung cells, low amounts of phenolic antioxidants can regulate redox signalling [57]. They inhibit the inflammatory process by suppressing the activity of nuclear factor-kappa B (NF-kB - an important modulator of inflammatory responses occurring in tubular epithelial cells) either through nuclear factor E2 p45-related factor 2 (Nrf2) induced phase-2 antioxidant enzymes (GSH), inhibitor of kappa B (IkB) pathway or reducing the activity of ROS and p38 mitogen activated protein (MAP) kinase. The antioxidants can also upregulate histone deacetylase (HDAC) activity and /or repair the modified HDAC proteins. Thus, antioxidants as study in this thesis involve scavenging of ROS but in fact they can play an additional role which involve cell signalling.

In all our studies, epigallocatechin gallate (EGCG) was used as a reference compound because of its strong demonstrated antioxidant ability. EGCG is one of the most studied and most active natural antioxidants. It is isolated from green tea and it demonstrates a large spectrum of biological activities, including antioxidant [43], chemopreventive, and chemotherapeutic actions in cellular and animal models of cancer [58]. Many studies have demonstrated the preventive capacity of EGCG on oxidative damage to biomolecules [59,60]. Sugisawa *et al* showed that physiological concentrations of EGCG prevent the effect of ROS-induced chromosomal damage [61].

1.2.3 Pro-oxidant character of antioxidants

In addition to their beneficial health effects due to free radical scavenging ability, the natural antioxidants (Vitamin C, Vitamin E, carotenoids and polyphenols) may exert prooxidant activities at higher doses and under specific conditions [5]. Vitamin C reduces Fe^{3+} to Fe^{2+} which reacts either with O_2, according to reaction (22), or with

hydrogen peroxide resulting in formation of superoxide anions and highly reactive hydroxyl radicals [62,63] according to reaction (23).

$$Fe^{3+} + AscH^- \rightarrow AscH^{\bullet} + Fe^{2+} \quad (21)$$

$$Fe^{2+} + O_2 \rightarrow \quad Fe^{3+} + O_2^{\bullet -} \quad (22)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad (23)$$

In the case of polyphenols (like flavonoids) the prooxidant activity can be explained by a mechanism associated with the formation of quinones [64]. Quinones may either redox cycle or undergo Michael addition (this can lead to protein thiol depletion with associated loss of protein function). The net result of redox cycling is a chain reaction producing superoxide anion ($O_2^{\bullet -}$) and consuming reducing equivalents in the form of NAD(P)H as well as molecular oxygen. In the Chapter 3 and 4 of this thesis, the prooxidant effects of catechols and naphthalenediols were tested.

1.3 OXIDATIVE STRESS AND DISEASES ASSOCIATED WITH INCREASED OXIDATIVE STRESS

Oxidative stress can be defined as an imbalance between the levels of antioxidants and prooxidants in favour of prooxidants. The widely accepted definition of oxidative stress was given by Sies in 1985 [65]: oxidative stress represents "the disturbance in the prooxidant and antioxidant balance in favour of the former, leading to potential damage".

Oxidative stress occurs when the generation of ROS in a system exceeds the ability of the antioxidant defense system to deactivate and eliminate them [65,66,67]. This imbalance can result from a reduction of antioxidant capacity (depletions of dietary antioxidants or mutations in antioxidant enzymes) or from an excess of ROS. The

increase in free radicals in the human body (e.g. due to exposure to toxins and drugs, or activation of phagocytes in chronic inflammatory diseases) is one of the most common causes of oxidative stress and relevant to human diseases [5]. In the case of mild oxidative stress, tissues respond by making more antioxidants in an attempt to restore the balance (e.g. increased SOD synthesis [68], upregulation of GSH [69], etc.).

The accumulation of ROS causes damage to all cellular components (DNA, lipids, proteins, and carbohydrates) [66]. This damage can have additive effects if is not properly repaired resulting in cellular dysfunction and the promotion of various diseases such as cancer [70], cardiovascular disease [71], diabetes [72] neurodegenerative diseases (Parkinson's disease (PD), Alzheimer disease) [5], ischemia-reperfusion injury [73, 74] and aging [75-77].

1.4 THE DESIGN OF SYNTHETIC ANTIOXIDANTS

Oxidative stress can be implicated either in the origin of a disease or in the pathology of the disease. Therefore, an antioxidant treatment should delay or prevent the onset of that disease or can decrease the oxidative damage with therapeutic benefits [78].

Considerable effort has been made in the last ten years to design and develop novel therapeutic antioxidants. The investigation of structure-activity relationships for antioxidants and the absorption-distribution-metabolism-excretion-toxicity (ADMET) studies are of significance in the strategy to design new radical scavenging antioxidants [79].

Many groups have focused on the rational design of new antioxidants. Two types of strategies have been used in designing new antioxidants. The first is to modify the

existing antioxidants to improve their activity (for example, the α-tocopherol analogues, 4-thiaflavans) [80-83]. The second strategy starts with the design of a completely new antioxidant (such as hydroxyphenylurea derivatives, 6-substituted-2,4-dimethyl-3-pyridinols) [84,85].

In 1980, Burton and Ingold proposed a structure-activity relationship (SAR) for α-tocopherol [86,87]. They demonstrated that α-tocopherol is a superb chain-breaking antioxidant by measuring its reactivity toward peroxyl radicals. Their studies demonstrated the steric and stereoelectronic effects that stabilized the phenoxyl radical formed by reaction of α-tocopherol with peroxyl radicals. The overlap between the 2p lone electron pair on the para ether oxygen and the aromatic π system is important in stabilization. This overlap is maximized by contraction of a six-membered heterocyclic ring of α-tocopherol to a five-membered ring (2,3-dihydrobenzofuran) (Figure 1.2) [87]. Thus, new tocopherol analogues with higher antioxidant activity were proposed. One of the most studied is probucol, obtained by substitution of methyls *ortho* to the hydroxyl with *tert*-butyl groups (Figure 1.2) [88,89].

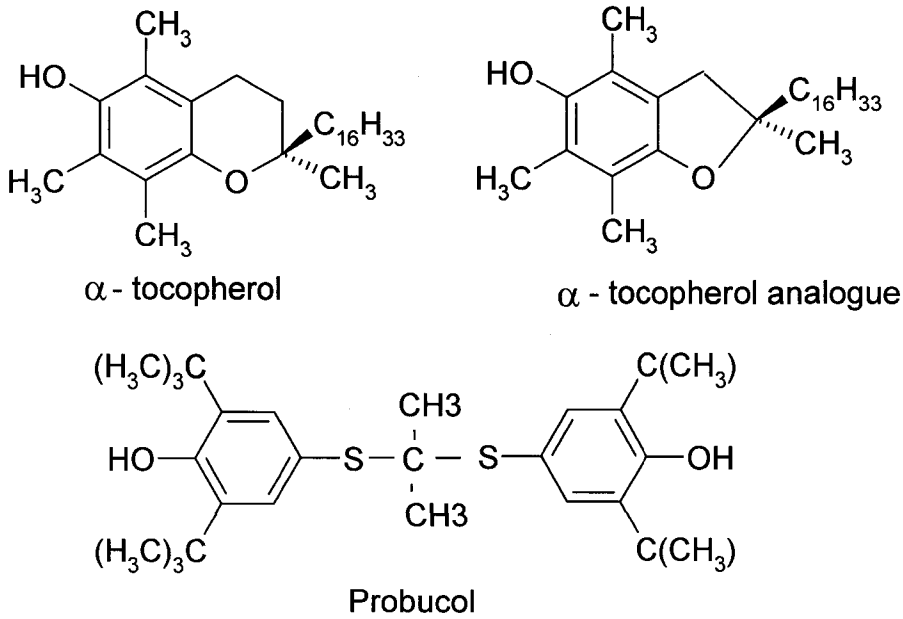


Figure 1.2. Structure of α-tocopherol, an analogue of α-tocopherol and probucol.

It is known that there are three possible mechanisms by which a "chain-breaking antioxidants" can deactivate a free radical. Assuming the attacking radical is a peroxyl radical ($ROO^{\bullet}$) the mechanisms are:

a) Hydrogen Atom Transfer (HAT) – an H-atom is transferred from ArOH to the peroxyl radical, according to the reaction [90]

$$ArOH + ROO^{\bullet} \rightarrow ArO^{\bullet} + ROOH \ (ArO^{\bullet} \text{ must be stable}) \quad (24)$$

b) Single Electron Transfer (SET) – a radical cation ($ArOH^{+}$) is formed which is rapidly deprotonated in solution [90], according to the following reactions:

$$\text{Electron transfer: } RO_2^{\bullet} + ArOH \rightarrow RO_2^{-} + ArOH^{+} \quad (25)$$

$$\text{Deprotonation equilibrium: } ArOH^{+} + H_2O \rightarrow ArO^{\bullet} + H_3O^{+} \quad (26)$$

$$\text{Hydroperoxide formation: } RO_2^{-} + H_3O^{+} \rightarrow ROOH + H_2O \quad (27)$$

The overall reaction is: $RO_2^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet}$ identical to that of the HAT mechanism.

c) Sequential proton loss electron transfer (SPLET) – ArOH loses a proton and forms the anion ArO^- [91,92], according to the equation:

$$ArOH \rightleftarrows ArO^- + H^+ \quad (28)$$

This is followed by Single Electron Transfer and formation of the peroxyl anion, according to the equation:

$$ArO^- + RO_2^{\bullet} \rightarrow ArO^{\bullet} + ROO^- \quad (29)$$

When ROO^- is protonated the product is identical to HAT and SET. All three mechanisms, HAT, SET and SPLET can occur in parallel but one or another is favoured based on rates of reactions. The rate of reaction will strongly correlate with the Bond Dissociation Enthalpy (BDE) of the O-H bond in the case of the HAT mechanism, with the Ionization Potential (IP) for the SET mechanism, and with the pK_a for the SPLET mechanism, which requires the formation of anion. It was suggested that in most reactions of phenolic antioxidants with free radicals, H-atom transfer is predominant [90]. Recent work indicates that the reactions a) and c) are competitive [91].

Therefore, the ability to predict and calculate the BDE of the O-H bond and the pKa of the parent compound could be very useful in designing effective antioxidants.

Chapter 2 of this thesis presents, in detail, a rational strategy for the design of chain-breaking antioxidants. The starting point in the design is an analysis of the structure of Vitamin E.

1.5 HYPOTHESIS

The following hypotheses were tested in the thesis:

1) that BDE can be used to predict the antioxidant reactivity of molecules containing a weak OH bond [90]. Molecules should have a BDE within the "design window" of 68-75 kcal/mol. The weaker the OH bond of the antioxidant, the faster will be the reaction with free radicals (e.g. peroxyl radicals).

2) in the case of synthetic catechols (compounds with two OH groups) a low BDE for the first exchangeable H-atom (BDE_1 is for catechol $\rightarrow$ semiquinone) will favour the antioxidant character. A high BDE for the second exchangeable H-atom (BDE_2 is for semiquinone $\rightarrow$ quinone) makes the formation of the quinone more difficult.

3) naphthalenediols, which are capable of forming the corresponding naphthoquinones, are relatively cytotoxic whereas those which cannot are relatively non-toxic. These compounds show promise as cytoprotective antioxidants.

These hypotheses are based on the following observations: The experimental BDE of vitamin E was found to be 77 kcal mol^{-1} [28]. Since the ascorbate ion has a BDE of 68.5 kcal mol^{-1} [93] this requires that an antioxidant (ArOH) have a BDE higher than this value in order for ascorbate to regenerate the synthetic ArOH. Therefore, it is hypothesized that molecules with a BDE within the "design window" of 68-75 kcal mol^{-1} will react fast with free radicals (DPPH radicals and superoxide produced in cells). The weaker the OH bond of the antioxidant, the faster will be the reaction with free radicals (e.g. peroxyl radicals). (Chapter 2).

Chapter 3 shows that catechols, although they fit in the design window, have a serious flaw in that they tend to form toxic quinones. This led to a redesign of the

molecules so as to increase BDE_2. For this purpose it was chosen the family naphthalenediols.

Chapter 4 shows that the BDE_2 of naphthalenediols is relevant to the formation of the corresponding quinones and this can be used as a predictor of toxicity. The naphthalenediols tested have a BDE_1 within the "design window" of 68-75 kcal mol^{-1}.

1.6 SUMMARY OF AIMS AND OBJECTIVES

The main objective of this work is to create novel synthetic antioxidants which are superior to vitamin E in correlation to its effectiveness in deactivating free radicals generated in living systems, mainly peroxyl radicals ($ROO^{\bullet}$).

The first aim of this thesis is a systematic design of new antioxidants using vitamin E as a starting point in order to develop an optimum antioxidant for use in biological systems. Two generations of molecules were designed and tested: a catechol family and a naphthalenediol family.

The second aim of this thesis is the testing of the novel synthetic compounds for their reactivity in chemical solvent (using the 2,2-diphenyl-1-picrylhydrazyl free radicals - $DPPH^{\cdot}$) and in cultured human leukemia cells (HL-60) for the ability to quench free radicals. I also examined the correlation between calculated BDE values and log k, the second-order rate constant (DPPH assay) as well as the correlation between BDE and IC_{50} (the concentration of antioxidant necessary to suppress 50% of the hydroperoxyl radicals produced).

The third aim of this thesis is the testing of the new antioxidants for cytotoxicity and cytoprotection against oxidative stress induced by various stressors (menadione and

2,2′-azobis (2-amidinopropane) hydrochloride - AAPH) in an adherent clone of rat pheochromocytoma (PC12-AC) cells. The effect of added ascorbate on the cytotoxicity of synthetic catechols and naphthalenediols was examined. Toxicity and protective data were combined to create a measure of the cytoprotective range for each compound. Theoretical calculations were used to see how the toxic or protective effects can be related to molecular structure. The studies of cytotoxicity and cytoprotection of the promising synthetic antioxidants were extended to primary cell culture (neurons) to see if the conclusions derived from PC12-AC cells are more general and whether animal trials should be pursued. The known antioxidant (-)-epigallocatechin gallate (EGCG) from green tea was used for comparison in all studies.

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Chapter 2

Novel synthetic antioxidants as free radicals scavengers: Design strategy and testing with DPPH and HL-60 cell cultures

2.1 INTRODUCTION

Reactive oxygen species (ROS) play major roles in various diseases, in aging and age-related diseases. [1,2]. Depending on their chemical structure, ROS can be very reactive and toxic, leading to oxidative damage of lipids, proteins, and nucleic acids and even death if their production is not well controlled [3-6]. In the case of oxidative stress, when the production of free radicals exceeds the cells defense systems (such as superoxide dismutase, catalase, the glutathione peroxidase system, and the vitamin E/ascorbic acid system), the use of an antioxidant therapy such as synthetic antioxidants (radical scavengers) would be effective [7-9]. Vitamin E is one of the most important chain-breaking, lipid-soluble antioxidant in biological membrane capable of quenching the propagation of free-radical reactions [10,11]. Many other chemical structures have been used to design novel radical scavengers, but the structure of α-tocopherol has often served as a starting point for the design of potent synthetic free-radical scavengers [12]. Over the past several years, considerable progress has been made in understanding what makes a molecule an effective antioxidant and developing methods to design novel and non-toxic antioxidant molecules [13].

In the previous chapter, I have discussed the process of lipid peroxidation by which lipid molecules are converted into lipid hydroperoxides (ROOH). The mechanism of lipid peroxidation involves 3 reactions: initiation, propagation and termination [12]. The control of initiation and propagation reactions is a general approach in designing a synthetic antioxidant.

In this chapter, I am focused on the design of antioxidant molecules that can interrupt the chain reaction of lipid peroxidation, according to the reaction

$$RO_2^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad (1)$$

There are three possible mechanisms (discussed in Chapter 1) for deactivation of peroxyl radicals: Hydrogen Atom Transfer (HAT) correlated with the Bond Dissociation Enthalpy (BDE) of the O-H bond, Single Electron Transfer (SET) [14] correlated with Ionization Potential; and Sequential Proton Loss Electron Transfer (SPLET) correlated with the pK_a [15]. It was suggested that in most reactions of phenolic antioxidants with free radicals, H-atom transfer is predominant [14]. Therefore, prediction and calculation of the BDE of the OH bond could be a very useful tool in the designing effective antioxidants.

One of the best lipid-soluble chain-breaking antioxidants is α–tocopherol (α-TOH), a component of vitamin E which intercepts peroxyl radicals generated in lipid peroxidation with a rate constant of 10^6 M^{-1} s^{-1} [16], according to:

$$\alpha\text{-TOH} + RO_2^{\bullet} \rightarrow \alpha\text{-TO}^{\bullet} + ROOH \quad (2)$$

Then, the α–tocopheroxyl radical reacts with ascorbic acid with the regeneration of α-TOH [17-19], according to:

$$\alpha\text{-TO}^{\bullet} + AscH^{-} \rightarrow \alpha\text{-TOH} + Asc^{\bullet -} \quad (3)$$

The ascorbate radical can then either disproportionate to ascorbate plus dehydroascorbic acid or be reduced back to ascorbic acid by interaction with other reducing species in the cell, such as glutathione or enzyme systems that use NADH and NADPH [20,21]. The BDE for ROO-H is about 88 kcal/mol, so there will be a relatively low barrier for a BDE

of ArO-H less than 88 kcal mol^{-1} (the BDE of α-tocopherol is approximately 77 kcal mol^{-1}). The antioxidant will react faster with the peroxyl radical, preventing reaction with the substrate (lipid molecules - RH). Phenols with a BDE of 88 kcal mol^{-1} will react slowly with $RO_2^{\bullet}$, the reaction being thermoneutral. According to the Hammond postulate [22], the rate of reaction of the substrate with peroxyl radical is correlated with the barrier height of an H-atom transfer from ArOH to $RO_2^{\bullet}$. The reaction of α–tocopherol with peroxyl radicals is exothermic (low barrier) and much faster since the lower the BDE (the weaker the OH bond) the faster will be the reaction with free radicals [14]. Based on additivity values it was predicted that the BDE of α-TOH is –2 (two groups *ortho*-methyl), 0 (*meta*-methyl), 0 (phytyl tail) and -6.0 (*para*-O), giving a predicted BDE of 77 kcal mol^{-1} in agreement with the calculated BDE [14]. Thus Vitamin E, and any other molecules with a lower BDE than 77 kcal mol^{-1}, are effective chain-breaking antioxidants that prevent lipid peroxidation while phenol is not.

In designing compounds as effective antioxidants, the following parameters have to be considered as relevant [23-26]:

1. The compounds must not contain weak C-H bonds because these can form carbon-centered radicals by homolytic cleavage. These radicals react fast with molecular oxygen and become chain-propagating radicals, such as $ROO^{\bullet}$ [27,28].

2. The compounds can have a sulfur, nitrogen or oxygen atom. Sulfur-centered radicals can form $RSOO^{\bullet}$ radicals [5]. However, molecules involving oxygen-centered radicals are more often studied. The oxygen-centered radicals are stable and don't react with oxygen to form $ArOOO^{\bullet}$ because the reaction is endothermic by ca. 20 kcal mol^{-1} [14].

3. The compounds should react fast with peroxyl radicals ($ROO^{\bullet}$). According to equation (1) the $ROO^{\bullet}$ forms by reaction with ArOH a bond in ROO-H with a BDE of 88 kcal mol^{-1} [29]. Therefore, the BDE of the antioxidant should be below this value for a rapid reaction.

4. Our goal is to design an antioxidant superior to vitamin E. Thus, the compounds should have a BDE value lower than 77 kcal mol^{-1}, the experimental BDE of vitamin E [16].

5. Since the ascorbate ion has a BDE of 68.5 kcal mol^{-1} [30] this requires that an antioxidant (ArOH) have a BDE higher than this value in order for ascorbate to regenerate the synthetic ArOH. The useful design window is 68-75 kcal mol^{-1} [31].

6. The radical $ArO^{\bullet}$ formed should not react with lipid, protein or other biological substrates. The radical should react with vitamin C or glutathione in order to regenerate the ArOH [16].

7. The antioxidants should have functional groups that allow them to be transported through cell membranes (for example, by esterifying the OH groups) and increase the lipid solubility (like the phytyl tail in vitamin E) [19].

To predict a BDE value based on group additivity rules, a set of optimized ΔBDEs (BDEs relative to phenol itself) were defined by performing locally dense basis sets (LDBS) calculations [14].

Phenolic antioxidants can contain various functional groups which are electron-donating such as methyl, hydroxy, methoxy, and amino or electron-withdrawing groups such as formyl, acetyl, carboxyl, and ester groups. Table 1 shows the recommended additivity values on the OH BDE in phenolic compounds as calculated by Wright et al. [14].

Table 2.1. Additive effects on the BDE value in phenolic compounds relative to phenol (87 kcal/mol).

Group	*Ortho* substitution	*Meta* substitution	*Para* substitution
NH_2	- 11.5	- 0.2	- 9.4
OCH_3	- 1.4	- 0.6	- 6.1
OH	- 9.2	- 0.4	- 5.9
$CH\text{-}CH_2$	- 4.0	- 0.2	- 4.7
$-C(CH_3)_3$	- 2.7	- 0.6	- 2.5
CH_3	- 2.0	- 0.4	- 2.5
Cl	+ 1	+ 1.2	- 1.4
CN	+ 3.6	+ 2.7	+ 2.2
CHO	+ 8.0	+ 2.2	+ 2.4
COOH	+ 8.1	+ 2.5	+ 2.6

For *ortho* functional groups, both conformational changes and hydrogen bonding must be taken into account. Scheme 2.1 shows the parent catechol and the catechol radical. The OH bond is broken in the parent molecule and the radical is able to rearrange in order to be stable [32]. In the calculation of the BDE of *ortho*-substituted catechols, the electronic effect must be taken into account, considering the H-bond of the parent molecule and the H-bond of the radical. For instance, the calculated ΔBDE of the *ortho*-catechol is (– 5.2 + 4 – 8 = - 9.2):

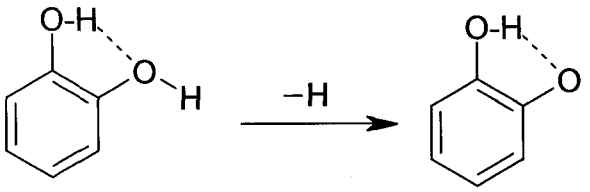
-5.2 kcal/mol – electronic effect

-4 kcal/mol – contribution of hydrogen bond in parent

-8 kcal /mol - contribution of hydrogen bond in radical

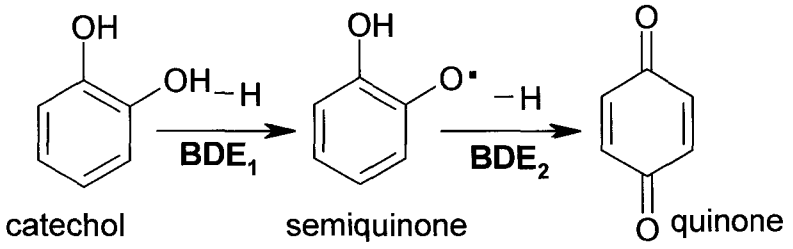
Thus, the BDE of a catechol is 77.9 kcal/mol.

SCHEME 2.1



A catechol donates its first hydrogen to a radical such as ROO forming the semiquinone (the bond dissociation enthalpy of the diol denoted BDE_1). Then, the oxidation of semiquinone leads to a quinone (BDE_2).

SCHEME 2.2



A systematic theoretical investigation of families of antioxidants was started based on knowledge gained in understanding how to predict new and superior target compounds. In this chapter, the design of a series of novel antioxidants with a catechol moiety which have a BDE that falls into or near the design window will be discussed. The objective was to create molecules more effective than a reference compound, vitamin E, as an antioxidant. After the synthesis of promising compounds, I tested them for their capacity to quench free radicals in chemical and biological systems.

The potential antioxidant activity of the new synthetic compounds has been assessed based on two assays: scavenging stable 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH assay) and inhibiting phorbol 12-myristate 13-acetate (PMA)-induced radical formation in cultured human leukemia cells (HL-60).

In the DPPH assay, the antioxidants can react with the stable, purple-coloured free radical DPPH, forming the colourless species $DPPH_2$ by H-atom transfer similar to the reaction with peroxyl radicals [23,32,33]. The weaker the OH bond, the faster the reaction of test samples with DPPH radicals [32].

In order to test the newly designed antioxidants for the capacity to scavenge free radicals in cells, I have chosen the HL-60 cell line as a model. HL-60, a human promyelocytic leukemia cell line first isolated by Collins et al in 1977, is a cell model widely used to evaluate the biological activity of a large number of compounds as potential chemopreventive agents capable of inducing cellular differentiation [34-36]. Some of these reagents cause HL-60 cells to differentiate to granulocyte-like cells, others to monocyte/macrophage-like cells [37]. Dimethyl sulfoxide (DMSO)-differentiated cells, by stimulation with PMA, can generate superoxide radicals at a constant rate [38-40]. Using cytochrome C as a monitor, the inhibitory capacity of test samples on free radicals was determined. Bhimani et al. have used this system for screening of various potential chemopreventive agents for antioxidant activity through their ability to scavenge free radicals [41]. Novel antioxidant compounds, isolated from bamboo leaves, demonstrated superoxide anion scavenging activities stronger than those of ascorbic acid and α-tocopherol [42].

In this chapter, we have tested the hypothesis that the BDE can be used to predict the reactivity of molecules containing a weak OH bond [14]. Molecules with a BDE_1 within the "design window" of 68-75 kcal/mol will react fast with free radicals. The weaker the OH bond of the antioxidant, the faster will be the reaction with free radicals (peroxyl radicals).

2.2 MATERIAL AND METHODS

Materials

RPMI 1640 medium (with L-glutamine and without bicarbonate), Penicillin/Streptomycin solution, Trypan blue solution (0.4%, liquid, sterile-filtered, cell culture tested), DMSO, Hanks' balanced salt solution (HBSS) epigallocatechin gallate (EGCG), Trolox, propyl gallate, resveratrol, PMA, ethanol, sodium bicarbonate, cytochrome c (from horse heart) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DPPH was obtained from Northern Sources, Inc. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). 2-Hydroxymethyl-4-methoxyphenol (H_0), 3-Methyl-4-methoxy-1,2-dihydroxybenzene (H), 4-methoxy-3,5,6-trimethylbenzene-1,2-diol (H1), 2,6,7-trimethyl-2,3-dihydrobenzofuran-4,5-diol (H2), benzo[1,3]dioxole-5,6-diol (H3), and 4-methylbenzo[1,3]dioxole-5,6-diol (H4) were synthesized as described by Hussain et al. [30]. All structures were verified in the laboratory of Tony Durst, University of Ottawa by ^{1}H and ^{13}C NMR, as well as by mass spectrometry.

Cell culture

HL-60, a human promyelocytic leukemia cell line derived by S.J. Collins et al. [35], was purchased from American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, sodium bicarbonate (2g/L) and 1% Penicillin (100 units/mL)/Streptomycin (100 μg/mL) solution. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2 in air) and passed at a density of approximately 1.5 x 10^5 cells/mL twice a week, with a doubling time of approximately 24 h. Their concentration did not exceed 10^6 cells/ml. Cell

numbers and viability were determined by the trypan blue dye (0.1% w/v) exclusion method.

Trypan Blue exclusion method

To an Eppendorf tube was added 250 μL of trypan blue (4% solution), 150 μL PBS and 100 μL solution of cells. After 5 - 10 minutes of incubation, the mixture was vortexed and the cells were counted using a hemocytometer. Living cells appeared colorless, while dead cells stained blue. The proportion of live to dead cells in a population following certain manipulations and treatments was determined using this method. A cell that is metabolically alive has a cell membrane that is active and selects the materials that pass into cytoplasm. The membrane of a dead cell has lost this selective capacity and many materials readily pass into the cell.

HL-60 cell differentiation

HL-60 cells were induced to morphologically differentiate with 1.3% DMSO [37]. The cells in log phase growth (approximately 10^6 cells/mL) were diluted to 200,000 cells/mL and plated in four 175cm^2 flasks (80mL/flask). DMSO was added to the flasks to a final concentration of 1.3%. The effect on viability and proliferation of HL-60 cells was determined every day for seven days of incubation. The rate of superoxide production was measured with the cytochrome c reduction method [36]. Following this treatment, the cells become analogous to neutrophils found in the human immune system and become biological generators of superoxide free radicals by stimulation with PMA.

Cytochrome C reduction assay

HL-60 cells were differentiated in culture medium containing 1,3% DMSO for 5 days. Then the cells were then harvested with centrifugation at 1000 rpm washed twice and resuspended in HBSS. Cells ($1x10^6$ cells/mL) were plated in 96-well microtiter plates [36]. After preincubation of the cells with the new synthetic compounds for 10 min, free radical formation was induced by the addition of PMA (1 μM). Cytochrome C (80 μM) was added in all wells and the plate was incubated at 37 ^{0}C for 30 min. The absorption values were read at 550 nm in a SpectroMax 340^{PC} microplate reader (Molecular Devices Co., Sunnyvale CA USA). The "Blank" is based on the absorbance of cells only in HBSS in which cytochrome C was added. "Controls" were the absorbance of cells treated with PMA in HBSS and cytochrome C.

DPPH: Stopped-flow kinetics assay

The tests were done in the laboratory of Dr. Ingold K. at National Research Council (NRC), Canada. All compounds were dissolved in ether, washed with aqueous sodium dithionite, and then re-isolated prior to the kinetic measurements [31]. A solution of DPPH$^•$ (ca. 5 x 10^{-5} M) was prepared and deoxygenated under nitrogen for 1.5 min prior to mixing with the compounds tested. The concentrations of antioxidants were at least 2 orders of magnitude greater than the concentration of DPPH$^•$.in order to obtain pseudo-first-order rate constants. The DPPH$^•$ solution was mixed 1:1 with (deoxygenated) solutions of antioxidants and the disappearance of DPPH was observed. Stopped-flow was performed using an Applied Photophysics SX 18 MV spectrometer with xenon 150 arc light source. The decay of DPPH$^•$ was monitored at 519 nm in the

presence of a given (large excess) concentration of antioxidant at room temperature. Under these conditions, the decay curve was pseudo-first order, and it was well fitted by a single-exponential function [DPPH] = $[DPPH]_0 \exp(-k_{obs}t)$ + constant. From plots of k_{obs} vs [ArOH] at five concentrations the second-order rate constant was obtained as the slope of the plot.

Calculation of BDE

Starting geometries were obtained with the PC-Spartan [43] builder module using the AM1 (Austin Model 1) method. Cartesian coordinates were then sent to the Gaussian 98 program for all subsequent calculations [44].

For calculation of the BDE, it was used the lowest level method (LLM) described by DiLabio et al. [30]. The AM1 semiempirical method was used to optimize the geometry of parent and radical (BDE_1), or radical and quinone (BDE_2), where T = 298.15 K, P = 1.00 atm., and the AM1 frequencies were scaled by the factor 0.973. At the geometry minimum, a single point calculation was done with (RO)B3LYP/6-311+G-(2d,2p), where RO indicates that, for the radical, the restricted open-shell B3LYP method was used. All electronic energies are then corrected by the thermal contribution to the enthalpy to obtain $H°_{298}$; the standard gas-phase enthalpy at 298 K. To complete the specification of the method, I set the electronic energy of the H-atom to its exact value of -0.50000 hartree, and obtained its enthalpy ($H°_{298}$ = -0.50000 + 5/2RT = -0.49764 hartree).

BDE represents the standard gas-phase enthalpy change at 298 K (ΔH^0_{298}) for reaction,

$$ArOH\ (g) \rightarrow ArO^{\bullet}\ (g) + H^{\bullet}(g) \qquad (4)$$

$$BDE = \sum H^{o}_{298}(products) - \sum H^{o}_{298}(reactants)$$

Statistics

Data are expressed as mean ± SEM values. Competitive inhibition plots are given in Result section, e.g. Figure 2.7. An exponential function gave a good fit through the data (see Figures 2.7-2.12). Using the SEM values, two additional exponentials were fitted through the top and bottom of the error bars. From these an error estimate for the IC_{50} was derived as follows: a line was constructed at 50% of the maximum absorbance and the minimum and maximum curves intersected this line, forming intervals. The error limit of the IC_{50} was taken as half of this interval. Statistical significance was assessed using a one-way ANOVA. Differences were considered significant at $p < 0.05$.

2.3 RESULTS

Figure 2.1 shows the structures of the newly designed compounds and the well – known reference antioxidants such as resveratrol (in red wine), epigallocatechin gallate (in green tea), vitamin E, trolox (solubilized form of vitamin E), and propyl gallate (in food additives). The compounds were tested for H-atom donating ability and antioxidant capacity in cell culture. To predict the order of activity, the theoretical calculations of BDEs were run.

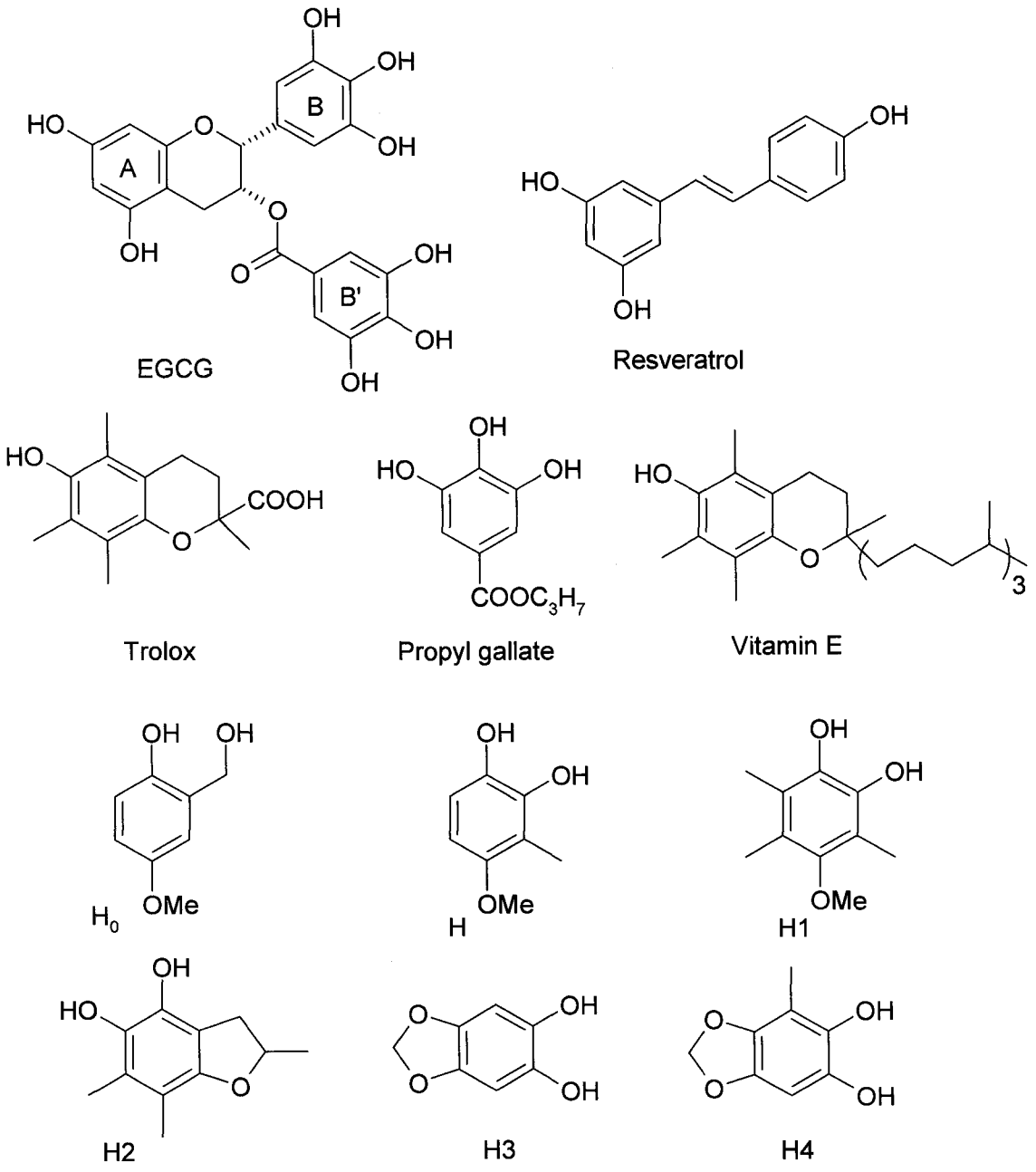


Figure 2.1. Structural formulas for the compounds tested: 2-Hydroxymethyl-4-methoxy-phenol = H_0, new synthetic catechols H, H1, H2, H3, H4 and known reference antioxidants EGCG, propyl gallate, resveratrol, vitamin E, and Trolox (water-soluble vitamin E).

BDE calculations

Table 2.2 shows the BDEs, obtained using the lowest-level method described in Material and Methods, of compounds tested.

Table 2.2. Bond dissociation enthalpy (ΔH°_{298}) for catechols and reference compounds: BDE_1 is for catechol → semiquinone, BDE_2 is for semiquinone → quinone.

Compound	BDE_1[a] (kcal mol^{-1})	BDE_2 (kcal mol^{-1})
α-Tocopherol, Trolox	77[a]	-
Resveratrol	79[a]	-
Propyl gallate	75[a]	74[a]
EGCG	71[a]	74[a]
H_0	79.5[b]	111[b]
H[a]	72.5[b]	75.8[b]
H1	73.6[b]	72.6[b]
H2	68.7[b]	71.7[b]
H3	69[b]	67.5[b]
H4	66.9[b]	67.3[b]

[a]Literature values from ref. [14].

[b]Literature values from ref [31].

DPPH• kinetics results

To determine the H-atom donating ability of the designed catechols and vitamin E, I measured their reactivity toward the DPPH• radical in ethyl acetate. The second-order rate constants, $k_{DPPH.}$ were obtained by following the decay of DPPH• at 519 nm in

the presence of excess antioxidant at various concentrations using a stopped-flow spectrophotometer at room temperature.

Detailed experimental results are showed in Table 2.3 and Figure 2.2, using one of the catechols (H3) as an example. Table 2.3 shows the first order rate constants, k_{exp} , for seven concentrations of H3. The k_{exp} increases as the concentration of H3 rises. The results were plotted and Figure 2.2 shows the concentration of H3 vs. k_{exp}. A very good linear correlation was obtained with the R^2 value being 0.994. The slope, which gives the second order rate constant, had a value of 5037 $M^{-1}s^{-1}$.

Table 2.3. Data obtained from stopped-flow analysis of different concentrations of H3 reacting with DPPH$^{\bullet}$ in ethyl acetate: time of reaction, rate constants of each run (n = 4) and the average of k_{exp}.

Concentration of H3 (M)	Time of reaction (s)	k_{exp} run 1 (s^{-1})	k_{exp} run 2 (s^{-1})	k_{exp} run 3 (s^{-1})	k_{exp} run 4 (s^{-1})	k_{exp} average (s^{-1})
2.49×10^{-4}	5	1.238	1.222	1.246	1.235	1.23533
3.74×10^{-4}	5	1.847	1.863	1.899	1.88	1.87225
5.6×10^{-4}	2	2.623	2.67	2.72	2.627	2.66
8.4×10^{-4}	1	4.338	4.318	4.289	4.335	4.32
1.26×10^{-3}	1	6.143	6.144	6.137	6.11	6.1335
1.89×10^{-3}	1	9.085	9.263	9.222	9.545	9.27875
2.44×10^{-3}	0.5	12.26	12.282	12.522	12.424	12.372

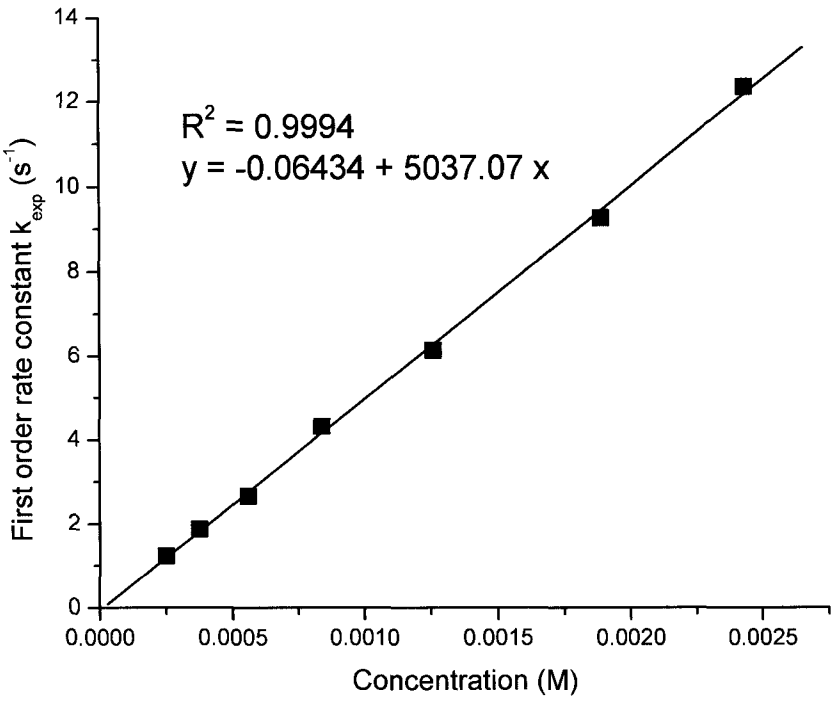


Figure 2.2. Plot of the first order rate constant k_{exp} vs. concentration of H3. The slope of this graph represents the second order rate constant k_{DPPH}.

Table 2.4 shows all the results of the kinetic studies on synthetic catechols and α-tocopherol. Reproducibility in the kinetic runs was excellent with very little deviation in the linear fit of the data, as shown by the high R^2 value. Values of the second order rate constant k_{DPPH} range from 2.9 to 8870, with H3 and H4 being the most reactive (almost 2 orders of magnitude more reactive with DPPH$^{\bullet}$ in ethyl acetate than α-tocopherol). The calculated BDE values are given in Table 2.4.

Table 2.4. Rate constants for DPPH$^{\bullet}$ + compounds in ethyl acetate solvent, R^2 values, calculated BDE_1.

Compound	k (M^{-1} s^{-1}) (EtOAc)	R^2	BDE_1[a] calc. (kcal mol^{-1})
α–tocopherol	160[b]	0.9992[b]	75.0[c]
H_0	2.9[d]	0.9942[d]	79.5[d]
H	200[d]	0.9995[d]	72.5[d]
H1	210	0.9993	73.6
H2	3000	0.9999	68.7
H3	5037	0.9994	69.0[d]
H4	8870	0.9984	66.9[d]

[a] BDE_1 for loss of the second H atom after the first has already been lost.

[b] Literature value from ref [45].

[c] The $C_{16}H_{33}$ (phytyl tail) was replaced by a methyl group in the calculation.

[d] Literature values from ref [31].

Figure 2.3 shows a very good linear correlation between rate constants and calculated BDEs, with a R^2 value of 0.976 for seven points. A lower BDE means a higher rate of scavenging the DPPH radicals.

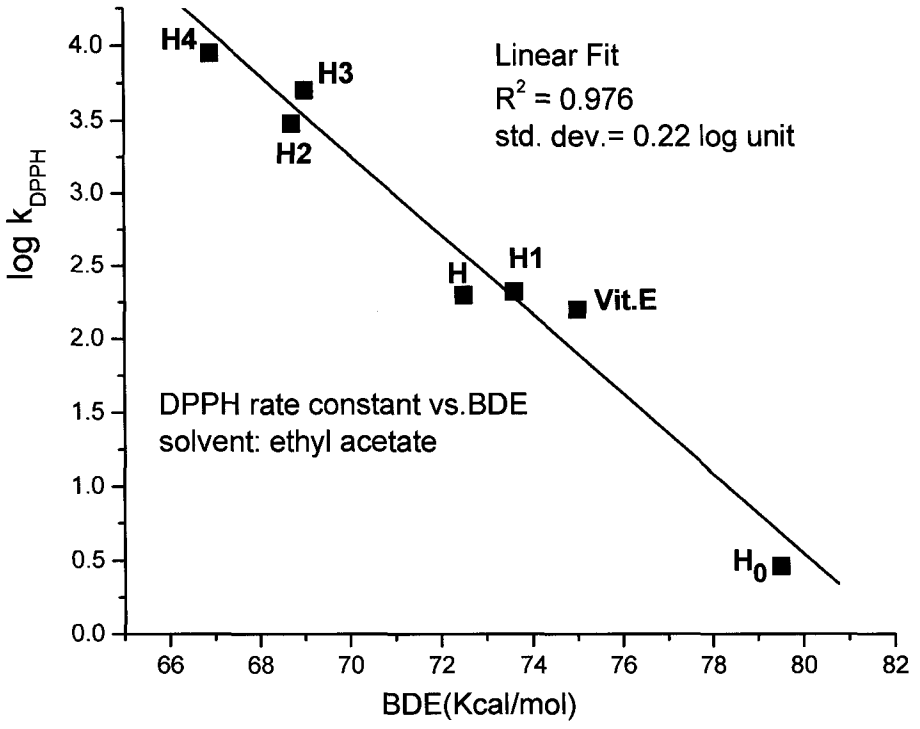


Figure 2.3. Plot of log k_{DPPH} vs. calculated BDEs of H_0, H, H1, H2, H3, H4, and Vitamin E.

Cytochrome C assay with HL-60 cells:

In order to test the ability of antioxidants to quench the ROS produced inside the cells, tests were run on HL-60 cells using the cytochrome C assay. Cells were differentiated and then stimulated with PMA to generate superoxide radicals. The cytochrome C assay is based on the change in absorbance at 550 nm as a result of the change in valence of the heme iron according to the reaction:

Cytochrome C(III) + $O_2^{\bullet -}$ (superoxide anion) → Cytochrome C(II) + O_2 (5)

Orange ↑ colour purple ↑ colour

I first characterized the cells by checking the viability in culture and also the rate of growth over 5 days. I defined cell viability as the ratio of the number of viable cells relative to the total number of cells. The total number and viability of cells were determined by the trypan blue dye exclusion method.

Figure 2.4 shows the concentration of HL-60 cells in culture over the 5 days. I started with approximately 125,000 cells/mL on day one. Cells were doubling exponentially in the linear portion of the log plot. This is between ~ 125,000 –700,000 cells/mL, after which they get crowded and their growth slows. At high concentrations of cells, cell growth declines due to the buildup of metabolites (e.g. lactic acid) in the media that inhibit proper growth. Thus, the cell concentration in culture should be kept below 750,000 cells/mL for a good viability.

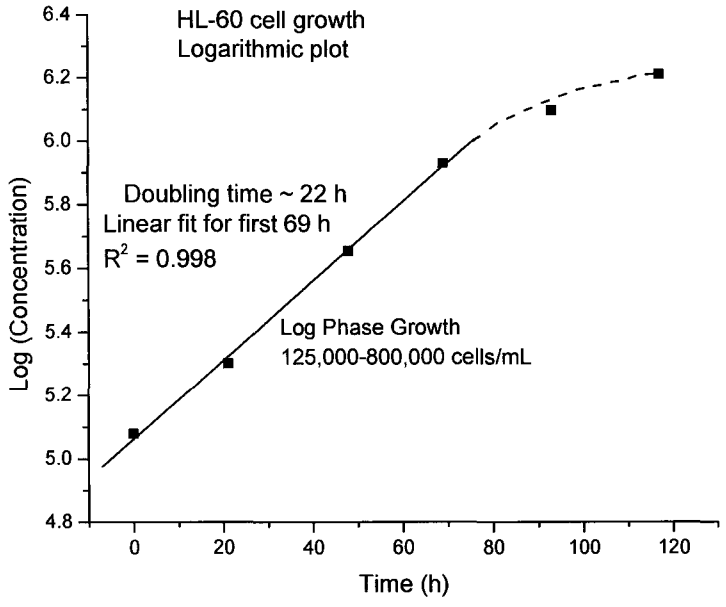


Figure 2.4. Log (concentration) vs time (h). The total number of viable and nonviable cells was determined using the trypan blue method. Dead cells were blue due to the accumulation of the dye in cells.

The conditions of the experiment (such as DMSO treatment, starting concentration of cell, concentration of cytochrome c and so on) were optimized in order to get reproducible results.

The next steps were to induce the differentiation of cells using 1.3% DMSO and determine the optimal time of treatment with the differentiation agent. Cell viability had to be higher than 95 % in order to use them in experiments. Cells at a concentration of ~200,000 cells/mL were incubated in growth medium containing 1.3% DMSO. Cell viability and the total number of cells were checked every 20-24 h over 7 days using the trypan blue assay. HL-60 cells increase their ability to produce superoxide ions after differentiation [44]. In order to test the capacity of the differentiated cells to produce superoxide, the cytochrome C assay was run (data not shown after four, five, six and seven days of differentiation, respectively. I have observed that the cell viability starts to decline after 6 days of differentiation (DMSO is toxic for cells) and the rate of superoxide production reaches a maximum after six days of differentiation.

Figure 2.5 shows the results of growth rate of the cells treated with DMSO compared to controls (no treatment). For the first 24 h, the cell number doubled and after that the cells growth slowed. After six days the cells were no longer dividing.

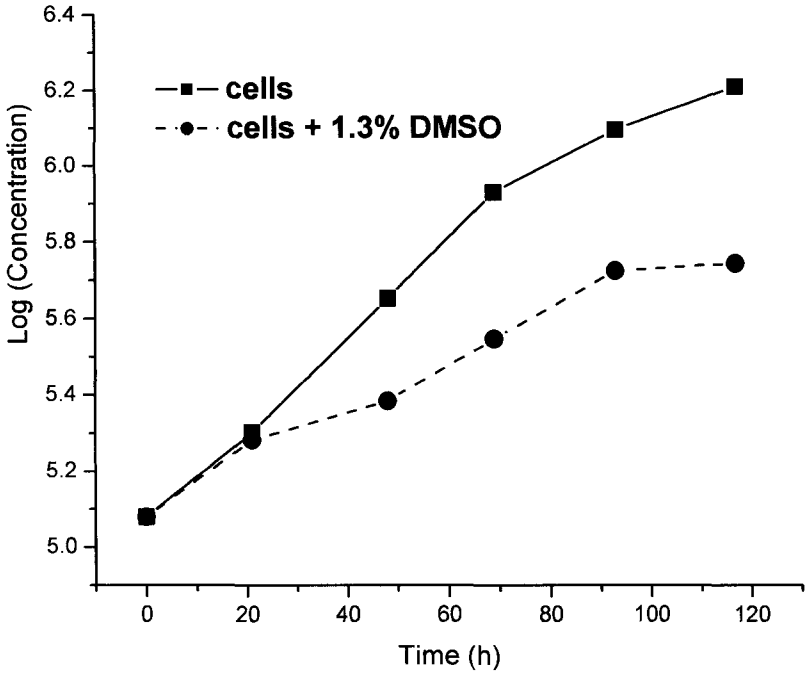


Figure 2.5. Plot of Log (concentration) vs time (h). HL-60 at a density of 200,000 cells/mL were grown in the presence or absence 1.3% DMSO. The concentration of cells was determined using the trypan blue method.

After five days in DMSO, cells were collected by centrifugation, washed twice with HBSS and resuspended in HBSS. Resuspended cells were added to a microplate at 5 different densities. The indicator used in experiments was 80 μM cytochrome C, in the iron (III) state (final concentration in well). Cells were stimulated to produce superoxide with PMA (1 μM). Superoxide converts cytochrome C (III) to cytochrome C (II) [36]. Controls contained 137 μL cells, 75 μL cytochrome C, 4 μL PMA and 4 μL HBSS per well. Figure 2.6 shows the result of this experiment. When all of the 80 μM of

cytochrome C is converted, the absorbance reaches a maximum and then slowly declines due to a back reaction of ferrocytochrome C with O_2 [36].

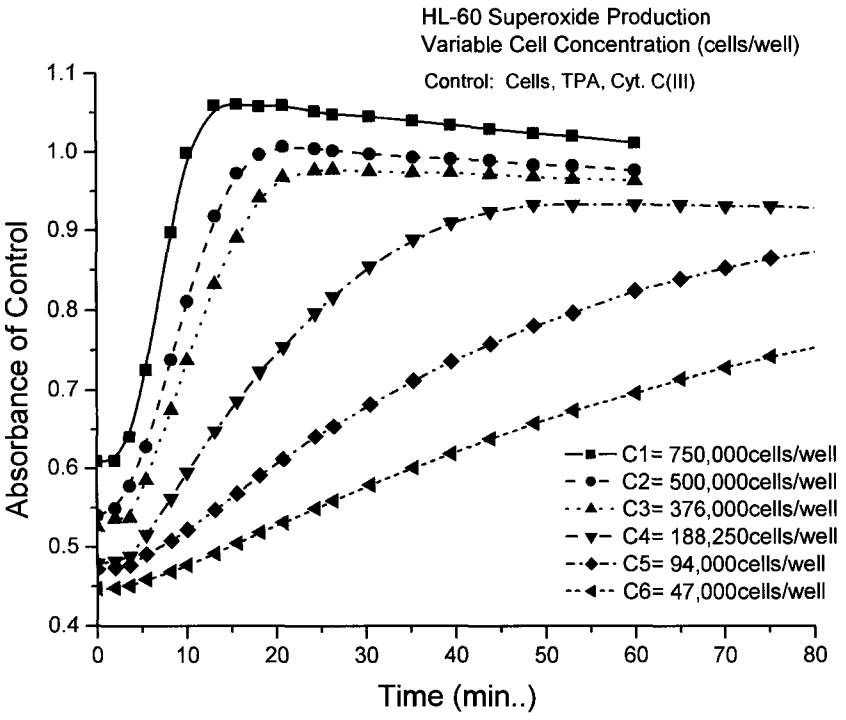


Figure 2.6. Change in cytochrome C absorbance over time for different cell concentrations. The absorbance of a solution containing differentiated cells, 80 μM cytochrome c and 1 μM PMA is recorded at 550 nm in a SpectroMax 340^{PC} microplate reader at 37 °C.

The first two columns of Table 2.5 show five different concentrations of cells used in experiments and the time necessary for them to completely consume 80 μM cytochrome C. Column 3 shows the rate of superoxide production by the cells in each well. From Column 4 of Table 2.5 it is clear that the calculated nmols of superoxide produced per min by 10^6 cells is approximately the same in all wells: (~ 3.00 nmols $O_2^{\bullet -}$/min).

Table 2.5. Rates of $O_2^{\bullet-}$ production by five different concentrations of cells and calculated nmols of $O_2^{\bullet-}$ produced per min by 10^6 cells.

Final Concentration of cells Cells/well (0.22mL)	Time of reaction (min)	Rate of $O_2^{\bullet-}$ production nmols $O_2^{\bullet-}$/min.	nmols of $O_2^{\bullet-}$ produced in 1 min by 10^6 cells
0.94×10^{-5}	112.86	0.29 ± 0.005	3.086 ± 0.05
1.88×10^{-5}	48.7	0.59 ± 0.016	3.15 ± 0.08
3.76×10^{-5}	26.41	1.1827 ± 0.028	3.14 ± 0.07
5×10^{-5}	20	1.44 ± 0.076	2.88 ± 0.15
7.5×10^{-5}	13.13	2.226 ± 0.083	2.968 ± 011

I concluded that the following optimal parameters were necessary for subsequent experiments in order to determine, with reproducible results, the antioxidant activity of the compounds tested:

- The time of incubation with DMSO ~ 5 days
- The viability of the cells > 95%
- The concentration of cytochrome C ~ 80 μM (gives an absorbance of ~ 0.4 OD)
- The density of cells ~220,000 cells/well (each well contains 0.22 mL, so this is a cells concentration of about 1 $x10^6$ cells/mL).

Antioxidants (ArOH) were added to the solution of differentiated cells stimulated with PMA to generate superoxide. According to reaction (5) with no antioxidant present, orange cytochrome C(III) is converted to purple cytochrome C(II), while with antioxidant

present, the production of the purple product is suppressed. Thus, the following mixtures (6 replicates for each) were prepared:

Test: 137 µL Cells, 75 µL cytochrome C(III), 4 µL PMA, 4 µL Compound tested

Standard: 137 µL Cells, 75 µL cytochrome C(III), 4 µL EtOH, 4 µL Compound tested

Control: 137 µL Cells, 75 µL cytochrome C(III), 4 µL PMA, 4 µL HBSS

Blank: 137 µL Cells, 75 µL cytochrome C(III), 4 µL EtOH, 4 µL HBSS

The mixtures were incubated for 40 minutes at 37 °C, in a humidified atmosphere (5% CO_2 in air). Treatments were read on a microplate reader at 550 nm.

Figures 2.7 to 2.14 show the results of these experiments. From these plots, I get the IC_{50}, (i.e. the concentration of antioxidant needed to suppress 50% of the formation of the cytochrome C (II) which represents 50% of the production of free radicals). The lower the IC_{50}, the more potent the antioxidant.

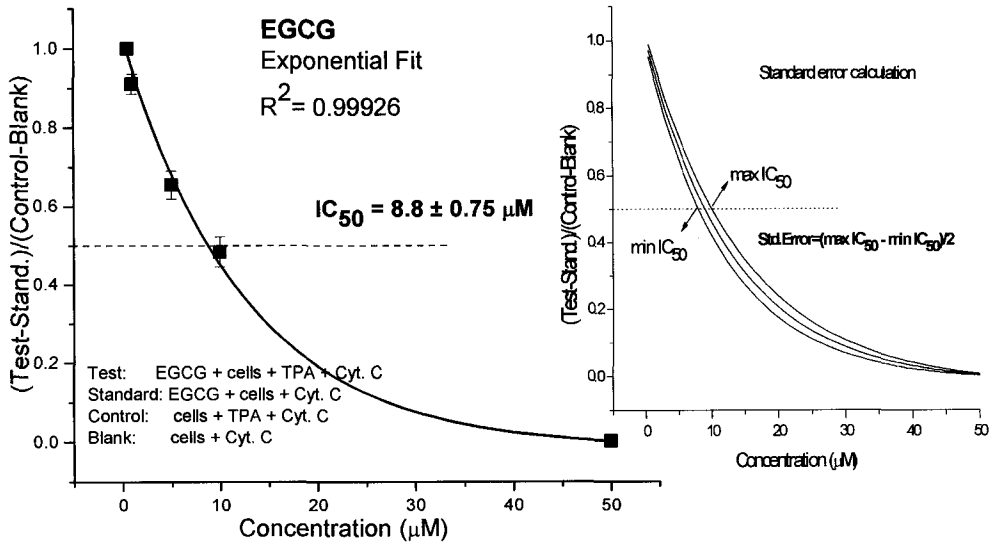


Figure 2.7. Absorbance vs. concentration of EGCG. Differentiated cells, stimulated with PMA, were incubated with 0.5, 1.5, 10, 50 µM EGCG and cytochrome C.

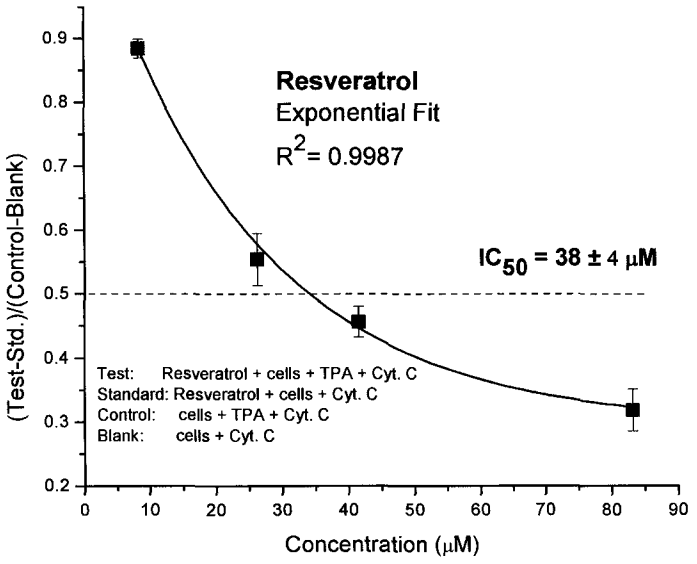


Figure 2.8. Absorbance vs. concentration of resveratrol. Differentiated cells, stimulated with PMA, were incubated with 8.3, 26.2, 41.6, 83.2 μM resveratrol and cytochrome C.

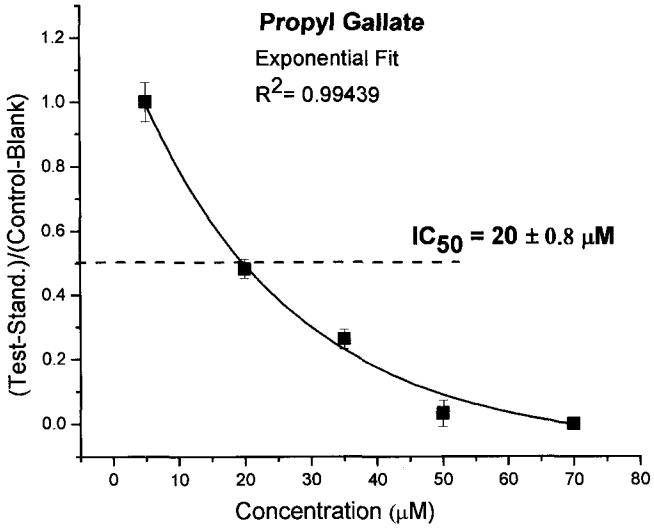


Figure 2.9. Absorbance vs. concentration of propyl gallate. Differentiated cells, stimulated with PMA, were incubated with 5, 20, 35, 50, 70 μM propyl gallate and cytochrome C.

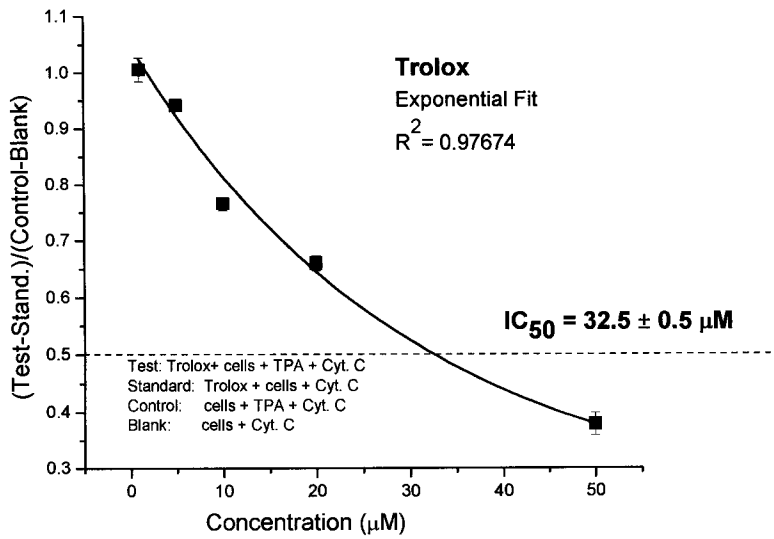


Figure 2.10. Absorbance vs. concentration of Trolox (water-soluble Vit. E). Differentiated cells, stimulated with PMA, were incubated with 1, 5, 10, 20, 50 μM EGCG and cytochrome C.

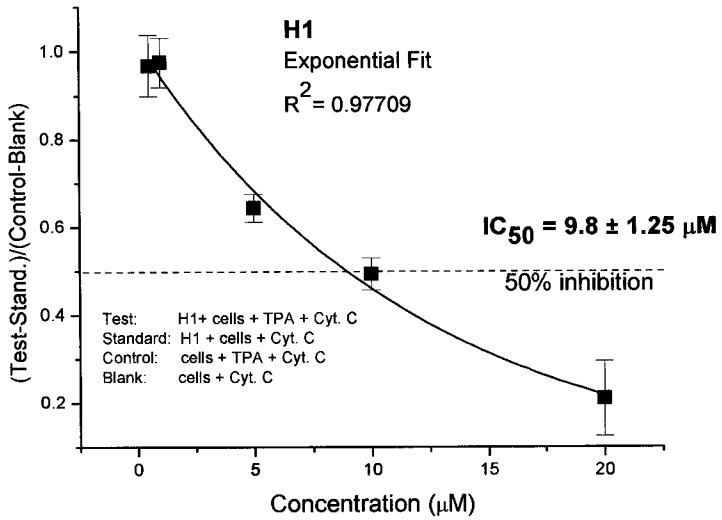


Figure 2.11. Absorbance vs. concentration of H1. Differentiated cells, stimulated with PMA, were incubated with 0.5, 1, 5, 10, 20 μM H1 and cytochrome C.

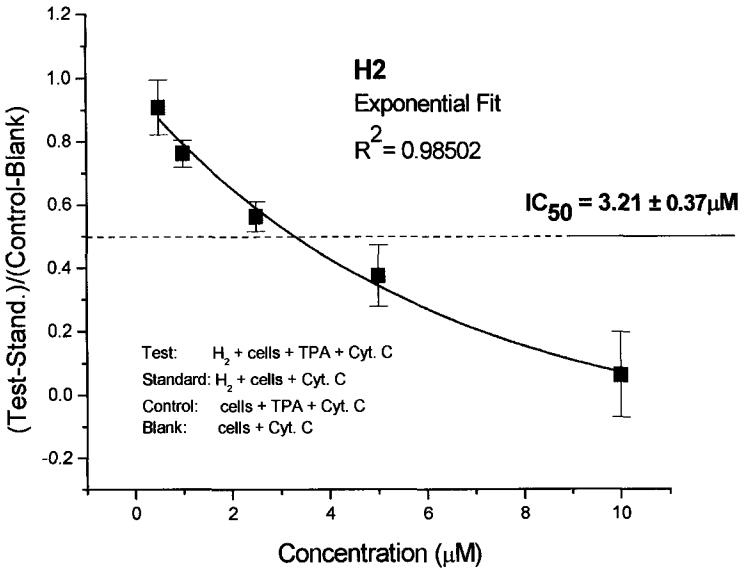


Figure 2.12. Absorbance vs. concentration of H2. Differentiated cells, stimulated with PMA, were incubated with 0.5, 1, 2.5, 5, 10 μM H2 and cytochrome C.

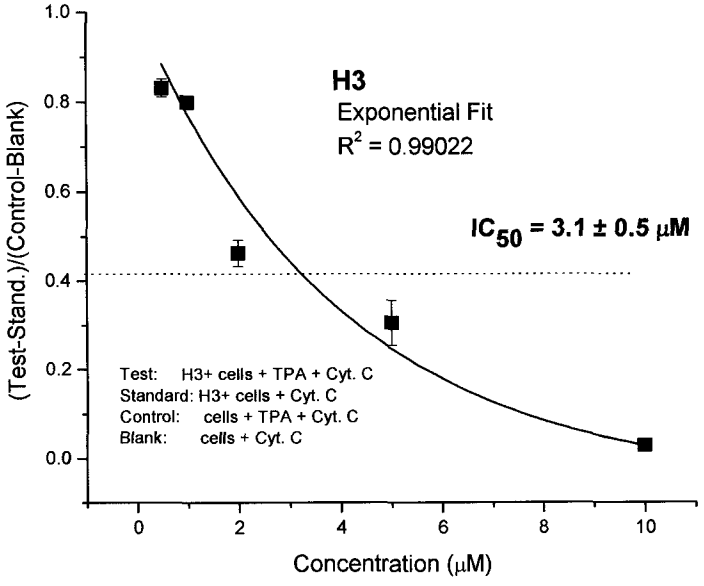


Figure 2.13. Absorbance vs. concentration of H3. Differentiated cells, stimulated with PMA, were incubated with 0.5, 1, 2, 5, 10 μM H3 and cytochrome C.

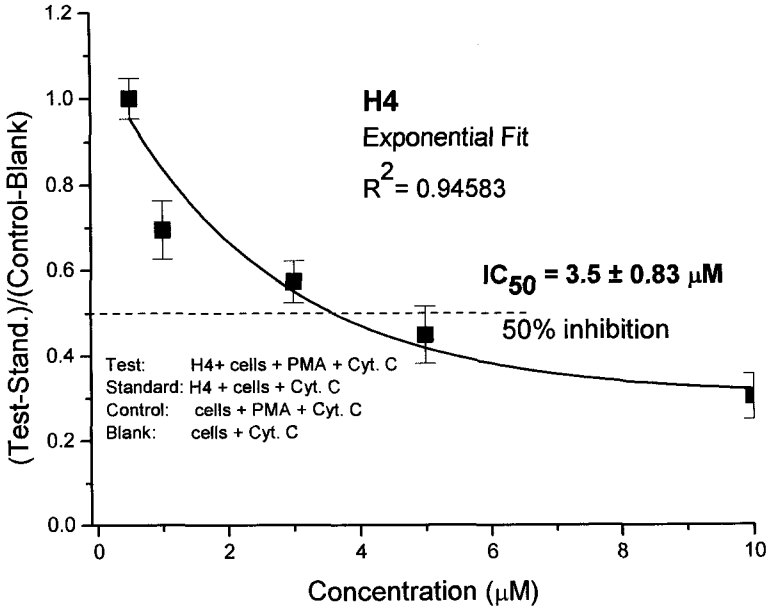


Figure 2.14. Absorbance vs. concentration of H4. Differentiated cells, stimulated with PMA, were incubated with 0.5, 1, 2, 5, 10 μM H4 and cytochrome C.

Figure 2.15 shows a very good linear correlation between IC_{50} and calculated BDEs, with a R^2 of 0.9533 for 8 points. A lower BDE means a lower IC_{50} and therefore a higher rate of scavenging of free radicals.

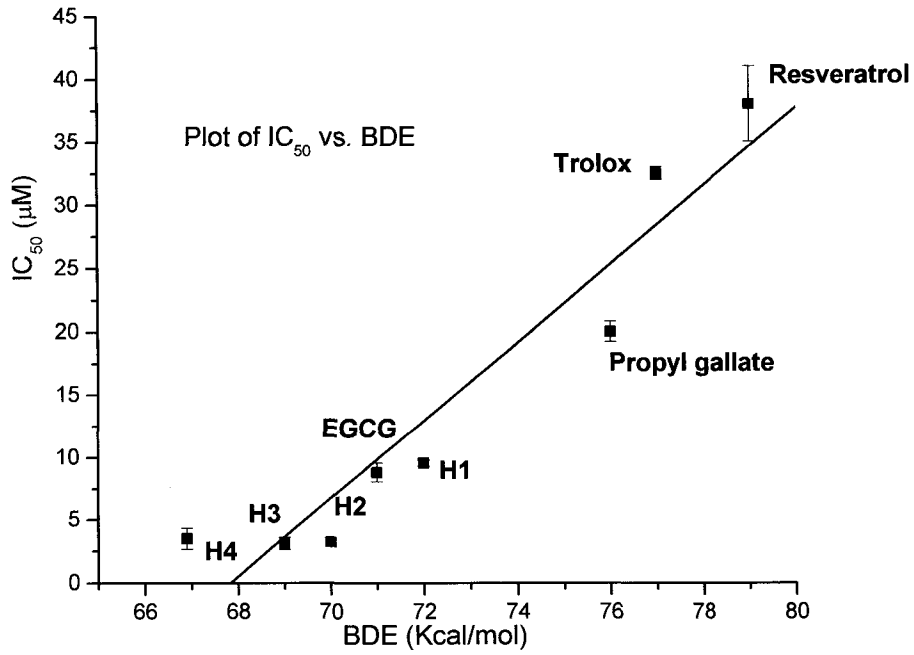


Figure 2.15. Plot of IC_{50} vs. BDE for synthetic compounds H1, H2, H3, H4 and known antioxidant EGCG, propyl gallate, resveratrol, and Trolox.

2.4 DISCUSSION

Design strategy: BDE calculations

In this chapter, we designed and tested molecules with the structure of the catechol, i.e. 1,2-dihydroxybenzene (H_0, H, H1, H2, H3, H4) as well as known reference antioxidants such as EGCG, resveratrol, propyl gallate and Trolox for free radical scavenging ability. Discussions of the parameters which are important in designing molecules with superior antioxidant activity were presented in the Introduction of this chapter.

Thus, the answer to the question "Why were catechols used as the first generation of new antioxidants designed?" is evident. By adding an OH group to the *ortho*-position

of the phenol, the BDE drops from 87.1 kcal mol^{-1} (phenol) to 77.9 kcal mol^{-1} (based on additivity rules as –9.2 kcal mol^{-1}) and thus, the BDE of catechol itself is almost in the design window. By adding other electron-donating functional groups such as methyl, hydroxyl and methoxy to catechols a variety of molecules with a BDE < 77 kcal mol^{-1} (vitamin E) will be created.

We calculated a BDE for phenol of 87.10 kcal mol^{-1}, in good agreement with a current experimental gas-phase value of 87.3 kcal mol^{-1} obtained by Wayner et al. [46]. Catechols (QH_2) donate their first hydrogen to a radical (represented by BDE_1) such as $ROO^{\bullet}$, forming the semiquinone $QH^{\bullet}$ and ROOH, according to:

$$QH_2 + ROO^{\bullet} \rightarrow QH^{\bullet} + ROOH \qquad (6)$$

and breaking the chain reaction of lipid peroxidation. The cellular reducing equivalents, such as ascorbate and glutathione, reduce the semiquinone back to the catechol form. Thus, the effectiveness of QH_2 as an antioxidant is strongly correlated with BDE_1. The semiquinone can lose the second hydrogen (BDE_2) forming the corresponding quinone according to:

$$QH^{\bullet} + O_2 \rightarrow Q + HO_2^{\bullet} \qquad (7)$$

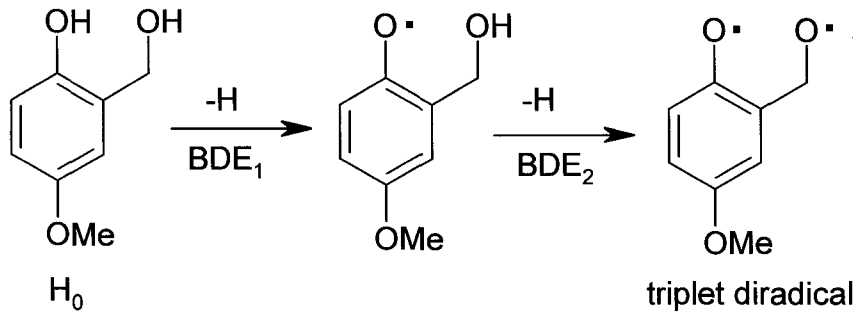
Since the pK_a of the semiquinone tends to be low [47], its conjugate base $Q^{\bullet-}$ donates an electron to oxygen to form superoxide and the quinone Q

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-} \qquad (8)$$

The first compound tested was H_0 (2-hydroxymethyl-4-methoxyphenol), in which the weaker bond is the phenolic OH and the stronger bond is the benzylic OH. Scheme 2.3 shows the formation of phenoxyl radical by abstraction of the first H and then by abstraction of second H, resulting in a triplet diradical formed. We predicted a BDE_1 of

79 kcal mol^{-1} based on additivity rules [87 kcal mol^{-1} (phenol BDE) – 6 kcal mol^{-1} (*p*-methoxy) - 2 kcal mol^{-1} (*o*-methyl)]. The calculated BDE_1 was 79.5 kcal mol^{-1}. A BDE for the second exchangeable OH was calculated to be 111 kcal mol^{-1} and therefore would not participate in any exchange reaction.

SCHEME 2.3



The next antioxidant tested was the catechol H (3-methyl-4-methoxy-1,2-dihydroxybenzene) with a predicted BDE_1 of 71.5 kcal mol^{-1} [87 (phenol BDE_1) – 9 (*ortho*-hydroxy)- 0.5 kcal (*meta*-methyl group) –6 kcal (*para*-methoxy)] = 71.5 kcal mol^{-1}] which is in agreement with observed BDE_1 of 72.5 kcal mol^{-1}. Its BDE_2 of 75.8 kcal mol^{-1} is comparable to the BDE of α-tocopherol.

Catechol H1 (4-methoxy-3,5,6-trimethylbenzene-1,2-diol) has a fully substituted benzene ring. Based on additivity values, it was predicted that the BDE_1 is 71 kcal mol^{-1} [-9 (catechol), -2 (*ortho*-methyl), -1.0 (two *meta*-methyl), and -6.0 (*para*-methoxy)]. We calculated a BDE of 73.6 kcal mol^{-1}. The geometry of catechol H1 (Figure 2.16) could explain this difference.

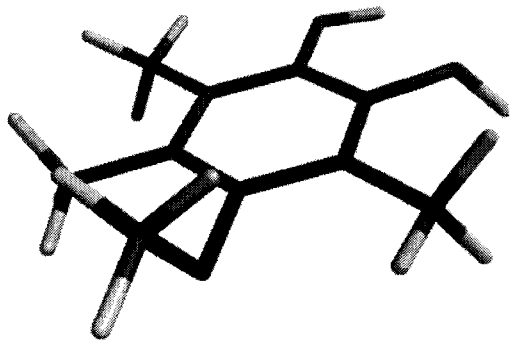


Figure 2.16. Structure of fully substituted catechol H1.

The methoxy group is rotated 90 ^{0} out of the plane because of the interaction with the adjacent methyl groups. This reduces optimal overlap of the methoxy oxygen relative to its normal position, rendering this a substituent that is less likely to donate electrons; the corrected additivity value for this substituent is only -2.6 kcal mol^{-1}, leading to a much better estimate of the effect of this functional group on the BDE. Loss of the second H atom is even faster with a BDE_2 of 72.6 kcal mol^{-1}.

Compound H2 is the bicyclic catechol 2,3-dihydro-2,6,7-trimethyl-4,5-dihydroxybenzofuran, whose structure bears a resemblance to α-tocopherol. This molecule also has a five-membered ring which enhances its planarity [48,49]. The phytyl tail was truncated to a single methyl group. This compound has the very low BDE_1 of 68.7 kcal mol^{-1}, right at the lower limit of our design window. The BDE_2 of 71.7 kcal mol^{-1} is also very low and the compound will be further tested in its acetylated form in order to be protected against autooxidation.

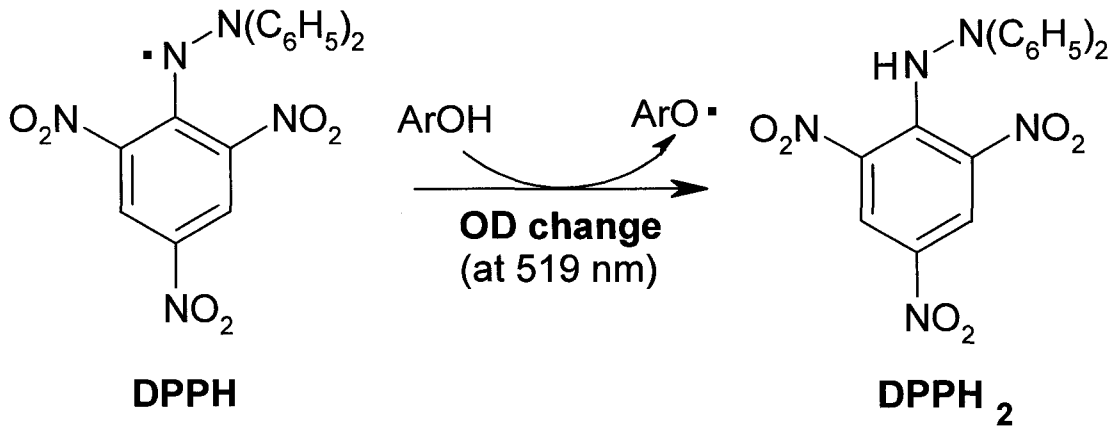
For catechol H3 (benzo[1,3]dioxole-5,6-diol), we calculated a BDE_1 of 69 kcal mol^{-1}. Finally, H4 (4-methylbenzo[1,3]dioxole-5,6-diol) has a BDE_1 slightly below the

"design window" of approximately 67 kcal mol^{-1}. With this low BDE_1, regeneration by ascorbate will be problematic. This will be tested and discussed in the next chapter.

DPPH Kinetics and BDE

The reaction of the phenolic antioxidants (ArOH) with the nitrogen-centered free radical DPPH$^{\bullet}$ is a second order reaction, according to Scheme 2.4:

SCHEME 2.4



The OD change was monitored at 519 nm (due to its odd electron DPPH gives a strong absorption at this wavelength) in presence of large excess of antioxidant at various concentrations. A decrease in absorption over time is seen (the solution starts to decolorize) due to electron pairing in presence of the antioxidant. With the exception of Vitamin E and Trolox, all other compounds are catechols having two exchangeable hydroxyl group H atoms. Therefore, they could react with two molecules of DPPH$^{\bullet}$. It was assumed that it is measured the rate of reaction for abstraction of the H atom from

the weaker phenolic OH. These pseudo-first order rate constants were determined by exponential fit to the decay curves near time zero.

A good correlation between rate constants and calculated BDEs was found (Figure 4). The rate of reaction with the test radical $DPPH^{\bullet}$ increases (hence a better antioxidant) as BDE_1 decreases. The order is H_0 (least reactive with $DPPH^{\bullet}$) < H = vitamin E < H1 < H2 < H3 < H4 (most effective).

Foti et al. [32] also looked at kinetics of reactions with peroxyl radicals and obtained a good linear correlation with BDE values; this confirms that tests with the nitrogen radical $DPPH^{\bullet}$ will mimic behaviour in systems which contain peroxyl radicals $ROO^{\bullet}$. The latter are generated biologically via normal metabolism and are known to play a role in lipid peroxidation.

Correlation between IC_{50} and BDE

The promising compounds (H1, H2, H3, H4) and some reference molecules (resveratrol, propyl gallate, EGCG and Vitamin E) were tested for their antioxidant activity in HL-60 cell culture. Differentiated HL-60 cells when stimulated by PMA produce superoxide radicals at a constant rate. The basis of our assay for superoxide is the reaction (5).

Antioxidant activity is related to BDE_1 (a low BDE_1 gives a more active antioxidant). Based on BDE_1, I predict the following order for antioxidant activity: Resveratrol (least reactive) < Trolox < propylgallate < H1 < EGCG < H2 < H3 ~ H4 (most reactive). According to this order, H2, H3, H4 are the most potent antioxidants. I found a very good correlation between IC_{50} and calculated BDE. The lower the BDE, the

faster the reaction of antioxidant with free radicals. Thus, the IC_{50} of designed catechols H2, H3, H4 were lower than IC_{50} of known resveratrol and EGCG, and more effective in quenching the free radicals produced by cells.

Next, in order to explain the mechanism by which the antioxidants suppress the free radicals produced by cells I describe the sequence of possible reactions and then I calculate the free energy changes in gas phase and in solution for the reactions in which the antioxidant (ArOH or QH_2) is directly involved (Reactions 12, 14, 15, 16):

1. Production of superoxide by cells by stimulation with PMA (superoxide is generated at 1.9 nmol/min/10^6 cells, which agrees with Newburger's data) [40],

$$\text{cells} \rightarrow O_2^{\bullet -}$$

The rate of superoxide production depends on how many days after differentiation the experiment is started and the viability of the cells (has to be higher than 95 %).

2. Reaction of superoxide with indicator, [3,5,50-52].

$$\text{cytochrome C(III)} + O_2^{\bullet -} \rightarrow \text{cytochrome C(II)} + O_2 \qquad (5)$$

3. Back-reaction of oxygen with reduced iron (II) is slow,

$$O_2 + \text{cytochrome C (II)} \rightarrow O_2^{\bullet -} + \text{cytochrome C (III)} \qquad (9)$$

4. Reaction of antioxidant with indicator: electron transfer

Note that depending on the pK_a of the catechols at pH 7.4, there is also the anion form present.

$$ArOH \leftrightarrow ArO^- \qquad (10)$$

$$\text{Thus, } ArO^- + \text{cytochrome C(III)} \rightarrow ArO^{\bullet} + \text{cytochrome C(II)} \qquad (11)$$

5. HAT reaction – Superoxide abstracts H atom from catechol

$$ArOH + O_2^{\bullet -} \rightarrow ArO^{\bullet} + HO_2^{\bullet -} \qquad (12)$$

Reaction 12 does not occur, as it is too endothermic (BDE of $HO_2^{\bullet -}$ is only 17 kcal/mol!).

6. Superoxide is in equilibrium with its conjugate acid $HO_2^{\bullet}$ via the acid-base equilibrium:

$$O_2^{\bullet -} + H^+ \rightarrow HO_2^{\bullet} \text{ (p}K_a \text{ of which is 4.8)} \quad (13)$$

Therefore at pH 7.8 I have 1/1000 of the superoxide concentration present as $HO_2^{\bullet}$. This can react with antioxidant according to the reaction:

7. HAT reaction which generates phenoxyl radical according to:

$$ArOH + HO_2^{\bullet} \rightarrow ArO^{\bullet} + H_2O_2 \quad (14)$$

The pH of the experiment must be known, and also should be approximately invariant over the time of the experiment.

8. Electron transfer from phenoxyl anion to hydroperoxyl radical

$$ArO^- + HO_2^{\bullet} \rightarrow ArO^{\bullet} + HO_2^- \quad (15)$$

9. Electron transfer from phenoxyl anion to oxygen

$$ArO^- + O_2 \rightarrow ArO^{\bullet} + O_2^{\bullet -} \quad (16)$$

10. Dismutation of superoxide ion and hydroperoxyde radical $HO_2^{\bullet}$ [3,5]

$$HO_2^{\bullet} + O_2^- + H^+ \rightarrow HOOH + O_2 \quad (17)$$

11. Dismutation of two superoxide ions [3]:

$$2O_2^- + 2H^+ \rightarrow HOOH + O_2 \quad (18)$$

The same reactions can also be written for any catechol. Knowing the $\Delta G(aq)$ for the catechol, I calculated the equilibrium constant K_{eq} based on the formula:

$$\Delta G(aq) = -RT \ln K_{eq}, \; = -1.364 \log K_{eq} \text{ at } 298 \text{ K}$$

The results are presented in Table 2.6.

Table 2.6. Free energy changes ΔG^0_{298} for various reactions which involve a phenol (ArOH) or a catechol (QH_2) in gas phase and in solution. The constants of reactions equilibrium K_{eq} at 298 K are presented.

Reaction	ΔG^0_{298} (g)[a] kcal mol^{-1}	ΔG^0_{298} (aq)[a] kcal mol^{-1}	K_{eq} at 298 K
$ArOH + HO_2^\bullet \rightarrow ArO^\bullet + H_2O_2$	1.95	1.31	10^{-1}
$QH_2 + HO_2^\bullet \rightarrow QH^\bullet + H_2O_2$	-7.9	-7.8	10^{6}
$ArOH + O_2^{\bullet-} \rightarrow ArO^\bullet + HO_2^{\bullet-}$	23.12	11.16	10^{-8}
$QH_2 + O_2^{\bullet-} \rightarrow QH^\bullet + HO_2^{\bullet-}$	13.3	8.6	10^{-6}
$ArO^- + HO_2^\bullet \rightarrow ArO^\bullet + HO_2^-$	28.26	4.94	10^{-4}
$QH^- + HO_2^\bullet \rightarrow QH^\bullet + HO_2^-$	27.74	-1.45	10
$ArO^- + O_2 \rightarrow ArO^\bullet + O_2^{\bullet-}$	40.78	24.48	10^{-18}
$QH^- + O_2 \rightarrow QH^\bullet + O_2^{\bullet-}$	40.26	18.08	10^{-13}

[a]calculated using MLM3 method

Based on calculated Keq, I concluded that reaction (14) is favoured and explained why the absorbance of cells treated with the test compound is lower than cells with no treatment. The antioxidant scavenges $HO_2^\bullet$ radicals and less cytochrome C(II) is formed. Therefore, I proved that an antioxidant with a lower BDE than the BDE of Vitamin E (77 kcal mol^{-1}) has a higher antioxidant activity since it can more rapidly scavenge free radicals such as $HO_2^\bullet$ or DPPH $^\bullet$.

2.5 CONCLUSION

The Bond Dissociation Enthalpy (BDE) in phenolic antioxidants is an important factor in determining the efficacy of an antioxidant; the weaker the OH bond, the faster will be the reaction with free radicals (peroxyl radicals). A "design window" was defined between 68-75 kcal mol^{-1}, into which the BDE of active bond O-H of the new antioxidants should fit.

In this chapter, I examined a series of target antioxidants containing a weak O-H bond, with a BDE_1 within the "design window" of 68-75 kcal/mol and some outside it, which could be potentially useful for biological purposes. The compounds were then tested for reactivity with the free radical $DPPH^{\bullet}$ and antioxidant activity in HL-60 cell culture. A very good linear correlation was obtained between log k_{DPPH} and the BDE_1 and also between IC_{50} and BDE_1 showing that the BDE can be used to predict reactivity in antioxidants. The toxicity of novel synthetic antioxidants to the cells and their protective effects in cell culture will be discussed in next chapter.

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Chapter 3

Cytotoxicity and cytoprotection of synthetic catechols: On the relationship between structure, toxicity, protection and the effect of added ascorbate

3.1 INTRODUCTION

Catechols (*ortho*-hydroxybenzenes) and hydroquinones (*para*-hydroxybenzenes) are well known in biological systems, often as metabolites of simpler aromatic hydrocarbons such as benzene and being a structural unit in natural polyphenols, as potent antioxidants [1-3]. A wide variety of plants, vegetables and fruits are rich in polyphenols which have been shown to have significant benefits in chronic disease prevention [4-6]. The polyphenols such as quercetin prevent cell death by scavenging oxygen radicals [6-8], protecting against lipid peroxidation [9], and chelating metal ions [10,11]. Due to the presence of two exchangeable hydrogen atoms, the aromatic diols tend to be biologically reactive molecules, capable of exhibiting both anti- and pro-oxidant behaviour. In the previous chapter, novel antioxidants were designed to have a "window" of BDE values of the OH bond between 69-75 kcal/mol. A group of synthetic catechols within this window were synthesized and tested for reactivity with the stable 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH) in organic solvent [12,13]. Then the molecules were tested using differentiated HL-60 cells for their antioxidant capacity in cell culture. The results of cell tests show them to be effective antioxidants. In order to be cytoprotective against different stressors in cells, the compounds should be non-toxic.

Therefore, in the present work I studied their cytotoxicity by using cell cultures, taking into consideration their possible pro-oxidant action. Typically, the pro-oxidant action of catechols and hydroquinones arises following autoxidation, resulting in the formation of *ortho*- and *para*-benzoquinones which are cytotoxic [2,14,15]. Then I tested the potential antioxidants for the protective ability in cells subjected to oxidative stress.

There are two main cytotoxicity mechanisms associated with quinone formation [1,2]. In the first, Michael addition occurs between a nucleophile and the quinone at the ring position β- to the carbonyl group. This can lead to glutathione depletion if the nucleophile is glutathione itself, or more generally to protein thiol depletion with associated loss of protein function. Thiol depletion upsets the redox environment of the cell by causing a change in the GSH/GSSG ratio, and sufficient depletion causes cell death. Another possible reaction of the quinone is the arylation of DNA bases, where the amino groups or ring nitrogen atoms on DNA bases act as the nucleophile. DNA arylation causes transcription errors which can result in tumor-initiating mutations.

The second mechanism of cytotoxicity is redox cycling, a chain reaction resulting in the continuous production of superoxide radical. One likely initiation step occurs between the catechol (QH_2) and its (inevitably present) quinone oxidation product (Q), leading to formation of the semiquinone radical $QH^{\bullet}$ (or its conjugate base $Q^{\bullet-}$, since the pK_a of the semiquinone tends to be low [1]):

$$QH_2 + Q \rightarrow 2\,Q^{\bullet-} + 2H^{+} \qquad (1)$$

The redox cycle begins with the first propagation step:

$$Q^{\bullet-} + O_2 \rightarrow Q + O_2^{\bullet-} \qquad (2)$$

In the second propagation step, Q can undergo a one-electron reduction by the enzyme cytochrome P450 reductase leading to $Q^{\bullet-}$, according to:

$$Q + \text{reducing enzyme} + \text{NAD(P)H cofactor} \rightarrow Q^{\bullet-} + \text{NAD(P)}^{+} \qquad (3)$$

The ascorbate anion, $AscH^{-}$, can play a parallel role by reducing the quinone according to the electron-transfer reaction [16]:

$$Q + AscH^{-} \rightarrow Q^{\bullet-} + AscH^{\bullet} \qquad (4)$$

Alternatively, ascorbate can act by H-atom transfer to give

$$Q + AscH^- \rightarrow QH^\bullet + Asc^{\bullet -} \quad (5)$$

Regardless of whether the reduction occurs via reactions 3, 4, or 5, (or a combination thereof), the net result of the redox cycle is a chain reaction producing superoxide anion ($O_2^{\bullet -}$) and consuming reducing equivalents in the form of NAD(P)H as well as molecular oxygen. The oxygen uptake, measured using a Clark electrode, is often used to monitor the process and to supply information about the extent of redox cycling [2].

Superoxide is a weak oxidant but it is converted by proton transfer to the stronger oxidant, the hydroperoxyl radical, its conjugate acid, according to

$$O_2^{\bullet -} + H^+ \rightleftarrows HO_2^\bullet \quad (6)$$

This factor complicates superoxide-driven chemistry because the pK_a for $HO_2^\bullet$ is only 4.8, so the small percentage of $HO_2^\bullet$ present under physiological conditions may contribute to the oxidizing nature of the free radicals generated during redox cycling, as well as to participate in the dismutation of the above species to produce the strong oxidizing agent (hydrogen peroxide) [17]. Thus redox cycling will generate reactive oxygen species (ROS) in a pH-dependent manner, which is typically measured by oxygen uptake, by an increase in H_2O_2 concentration, or by the presence of lipid peroxidation products (as an indirect measure).

Another possible mechanism which can explain the toxicity of quinone is the autooxidation of QH_2 in the extracellular medium as proposed by Watanabe and Forman [18]. Hydrogen peroxide is the species produced. The quinone reenters the cell and undergoes a two electron reduction to the hydroquinone; a reaction catalyzed by NAD(P)H quinone reductase according to:

$$Q + 2e^- + NAD(P)H \rightarrow QH_2 + NAD(P)^+ \quad (7)$$

The hydroquinone then is transported to the extracellular medium where is autooxidized and more hydrogen peroxide is formed.

Morita et al. [19] explained the *in vitro* cytotoxicity of 4-methylcatechol by the extracellular autooxidation of the catechol leading to formation of the hydrogen peroxide. A complex mechanism of the kinetics of the oxidation of naphthohydroquinones, relevant to the study of catechols, has been given by Munday [20, 21].

As pointed out previously [2] a rise in hydrogen peroxide concentration can usually be countered by the action of the enzyme catalase which consumes hydrogen peroxide, so redox cycling tends to be less cytotoxic than Michael addition. However, if catalase is deactivated, e.g. by azide ion, or overwhelmed by excess H_2O_2, then the cytotoxicity caused by redox cycling can increase significantly.

A rise in the intracellular concentration of hydrogen peroxide is thought to be the cause of a variety of inflammatory diseases. For example, Dopa-quinones, formed by oxidation of L-Dopa, have been shown to generate ROS by redox cycling and are correlated with development of Parkinson's disease [22,23]. The treatment of human fibroblasts with H_2O_2 leads to senescence-like features [24], and deliberate suppression of glutathione peroxidase in knockout mice also leads to accelerated senescence caused by increased concentrations of H_2O_2 [25].

In general, the presence of ascorbate anion ($AscH^-$) would be expected to decrease the toxicity of catechols in a cellular environment due to reduction of the semiquinone, according to:

$$QH^\bullet + AscH^- \rightarrow QH_2 + Asc^{\bullet -} \quad (8)$$

with regeneration of the catechol and formation of the ascorbate radical anion $Asc^{\bullet-}$. This reaction is protective because it prevents quinone formation, and $Asc^{\bullet-}$ is non-toxic due to its spontaneous or enzyme-catalyzed dismutation into $AscH^-$ and dehydroascorbic acid (DHA) which is easily reduced by the cell back to ascorbate.

Protection by ascorbate has been proven in numerous studies: For example, ascorbate needs to be maintained at sufficient levels to prevent oxidative damage in the lens of the eye, which leads to cataract formation [26]. Ascorbate also prevents autoxidation of L-Dopa and its subsequent quinone formation [27]. In endothelial cells, ascorbate reduces the effects of oxidative stress caused by menadione, resulting in the decrease loss of intracellular glutathione [28]. The toxicity of catecholic estrogens was lessened by the presence of reducing agents such as ascorbate and cysteine, again through the reduction of the semiquinone back to the parent catechol [29].

There are also situations where ascorbate can *increase cytotoxicity*. It is well known that ascorbate can act as a prooxidant by the reduction of iron (III) to iron (II) and subsequent Fenton chemistry to produce the highly oxidizing hydroxyl radical [17], although this has been questioned recently as to whether this is relevant to *in vivo* biochemistry [30]. Ascorbic acid increases cytotoxicity-induced by oxidized low-density lipoprotein and thus acts as a prooxidant, although the effect is diminished in the presence of other antioxidants such as Vitamin E [31]. Of more relevance to this paper, ascorbate has been shown to increase the rate of H_2O_2 generation in the autoxidation of some quinones [32]. Thus reaction (4) and/or (5) can accelerate the redox cycling of quinones and increase their toxicity.

In spite of their prooxidant behaviour, catechols demonstrate antioxidant capacity by playing an important role in protection through scavenging free radicals generated during oxidative stress. [2]. Catechols donate their first hydrogen to a radical such as $ROO^{\bullet}$, forming the semiquinone radical and ROOH, according to:

$$QH_2 + ROO^{\bullet} \rightarrow QH^{\bullet} + ROOH \qquad (9)$$

thus breaking the chain reaction of lipid peroxidation. The cellular reducing equivalents, ascorbate and glutathione reduce the semiquinone back to the catechol form.

Here I consider the cytotoxicity of several synthetic catechols and EGCG. H1 is a single-ring structure, 2,3,5-trimethyl-4-methoxylcatechol. H2 is the bicyclic catechol 2,3-dihydro-2,6,7-trimethyl-4,5-dihydroxybenzofuran, whose structure bears a resemblance to α-tocopherol. I also include, in this study, the diacetylated forms, e.g. H2-Diacetate (H2-DA). H4, derived from the naturally occurring sesamol, is a methylated methylenedioxycatechol. These compounds are currently undergoing testing as potential synthetic antioxidants [13] and a study of their cytotoxicity and cytoprotection is thus part of a larger program aimed at creating superior biologically active antioxidants.

Cell viability was studied both with and without added ascorbate. The cell line chosen for cytotoxicity studies was derived from the rat adrenal pheochromocytoma cells (PC12) [33,34], a neuronal cell line that differentiates into preneuronal cells upon treatment with nerve growth factor. In the present work, I used a clone of PC12 [35] denoted PC12-AC which has two advantages: first, the cells are adherent and thus, easy to work with and second, their doubling time is faster than non-adherent PC12s [36,37]. I also used the human promyelocytic leukemia cell line HL-60 [38] to validate the redox

cycling mechanism by an examination of reaction stoichiometry. By treatment with DMSO, the cells become analogous to neutrophils found in the human immune system and become biological generators of superoxide free radicals by stimulation with various agents [39]. Cytotoxicity was measured on PC12-AC by examination of the following experimental parameters: EC_{50} (effective concentration which reduces the number of viable cells by 50%, as measured by the MTT assay; see below), effect of acetylation, effect of added ascorbate, the reduced to oxidized glutathione (GSH/GSSG) ratio, and the H_2O_2 response. H2-DA, H2-DA with ascorbate, and EGCG were tested for protective effects against menadione and the aqueous-phase peroxyl radical generator (2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). Toxicity and protective data will be combined to create a measure of cytoprotective efficacy for each compound. Observed cytotoxicity or protection will be related to calculated molecular properties, such as the bond dissociation enthalpy of the diol (denoted BDE_1) and its semiquinone (BDE_2) and the octanol-water partition coefficient (log P).

3.2 MATERIALS AND METHODS

Materials

H1, H2, and H4 were synthesized as described by Hussain et al. [13]. MTT (3-(3,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide), RPMI 1640 medium (with L-glutamine and without bicarbonate), Penicillin/Streptomycin solution, Trypan blue solution (0.4%, liquid, sterile-filtered, cell culture tested), epigallocatechin gallate (EGCG), dimethyl sulfoxide (DMSO), L-ascorbic acid, Hank's balanced salt solution (HBSS), sodium bicarbonate, cytochrome C (from horse heart), phosphate buffered saline

(PBS), xylenol orange, sorbitol, ferrous sulfate, AAPH and sulfuric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide was obtained from Caledon Laboratories Ltd. (Georgetown, ON, CAN). RPMI 1640, trypsin, horse serum, fetal bovine serum, newborn calf serum, and antibiotic-antimycotic were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Fetal calf serum was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A).

The diacetate forms of compounds (H1-DA, H2-DA and H4-DA) were prepared by reacting the diol with acetic anhydride in the presence of N,N-dimethylaminopyridine and triethylamine at the University of Ottawa. All structures were verified by ^{13}C NMR and mass spectrometry.

Cell culture

PC12-AC cells, an adherent clone of the rat adrenal pheochromocytoma cell line PC12, were a gift from Dr. Steffany Bennett (Biochemistry, Microbiology and Immunology, University of Ottawa). PC12-AC were grown in RPMI 1640 medium supplemented with 5% newborn calf serum, 10% heat-inactivated horse serum, containing 100 U/mL penicillin G sodium salt, 100 μg/mL streptomycin sulfate and 0.025 mg/mL amphotericin B (Invitrogen, Carlsbad, CA, U.S.A.). Cells were maintained at 37 ^0C in a humidified atmosphere of 5% CO_2 in air. Cells were passed at a density of approximately $5x10^5$ cells/mL twice a week with a doubling time of approximately 24 h. Cell numbers and viability were determined by the trypan blue (0.1% w/v) exclusion method.

HL-60 cells, a promyelocytic leukemia cell line (CCL-240) derived by S.J. Collins, et al. [39] was purchased from ATTC. Cells were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, sodium bicarbonate (2g/l) and 1% Penicillin (100units/ml)/Streptomycin (100μg/ml) solution. The cells were maintained at 37 ^{0}C in a humidified atmosphere (5% CO_2 in air) and passed at a density of approximately 1.5 x 10^5 cells/mL twice a week, with a doubling time of approximately 24 h. Their concentration did not exceed 10^6 cells/ml. Cell numbers and viability were determined by the trypan blue dye (0.1% w/v) exclusion method.

Assay of MTT reduction

PC12-AC cells undergoing exponential growth were trypsinized, counted, diluted and seeded in untreated 96-well microplates at a density of 200,000 cells/mL (100 μL/well). Plates were incubated for 24 h (37 ^{0}C, 5% CO_2) to allow adherence. After incubation, the medium was discarded and replaced with fresh medium containing the compounds at concentrations ranging from 1-200 μM (test compounds were dissolved in DMSO; the final concentration of DMSO in the wells was less than 0.5%). Cultures containing test compounds were incubated for another 24 h. The cells were washed once with 100 μL PBS and 100 μL of fresh medium was added, along with 10 μL MTT stock solution (5 mg/mL in PBS). The cells were incubated for another 2 h. MTT is reduced by live cells to a blue, water-insoluble formazan salt [35,40-41]. The medium was removed and the cells were lysed in 50 μL DMSO, which also solubilizes the formazan. After 20 min, the absorption values were read at 570 nm with background subtraction at

630 nm in a SpectroMax 340^{PC} microplate reader (Molecular Devices Co., Sunnyvale CA USA).

Dichlorofluorescein (DCF) assay

The DCF assay is based on the oxidation of the nonfluorescent 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) to the highly fluorescent 2'7'-dichlorofluorescein and used to detect the intracellular peroxides [42].

PC12-AC cells were pre-incubated in 96-well microplates at a density of 400,000 cells/mL for 24 h at 37 °C. The medium was discarded and replaced with HBSS and H2-DA at various concentrations. After 2 hr incubation, 40 μM H_2DCF-DA was added and the incubation continued for another 1.5 h. After the supernatant was removed, the cells were washed and incubated with HBSS and the fluorescence of the cells was measured using a FluoStar Galaxy microplate reader (BMG LABTECH Inc., Durham NC, USA) with an excitation and emission wavelengths of 485-521 nm respectively.

Cytochrome C assay

The cytochrome C assay used to monitor superoxide production was described in Material and Methods, Chapter 2 [43,44]. Briefly, the HL-60 cells (200,000 cells/ml) were treated with 1.3% DMSO to induce granulocyte differentiation. After 5 days, the cells were harvested by centrifugation, washed twice with HBSS and plated in 96-well microplates at a concentration of ca. 500,000 cells/mL. The cells were pre-incubated with test compound (20 μM) for 5 min prior to addition of 75 μL cytochrome C solution in HBSS to a final concentration of 80 μM. Superoxide radical production was observed

by following the colour change from ferrocytochrome C (III) to ferricytochrome C (II), which was monitored at 550 nm using a 96-well microplate reader.

GSH and total protein determination

The assay for glutathione is based on the reaction of reduced glutathione (GSH) with dithionitrobenzene (DTNB) [45]. PC12-AC cells undergoing exponential growth were trypsinized, counted, diluted and seeded in 10 cm dishes at a density of 400,000 cells/mL (8 mL media/dish). Dishes were incubated for 4 h (37 ^{0}C, 5% CO_2) to allow cell adherence. After incubation, the medium was discarded and replaced with fresh medium containing the compounds at concentrations ranging from 1-150 μM (test compounds were dissolved in DMSO; the final concentration of DMSO in the wells was less than 0.5%). The cultures containing test compounds were incubated for another 24 h. The cells were washed once with 5 mL PBS, harvested and centrifuged.

Cells were then lysed in 1:5 (w/v) ice-cold sulfosalicylic acid (previously bubbled with N_2 gas for 15 min), then bubbled with N_2 gas for 10 sec and centrifuged for 1 min at 13,000 *g* in an Eppendorf microcentrifuge. Supernatants were removed to new tubes and immediately neutralized with 9 volumes of 0.5 M potassium phosphate (pH 6.0). Aliquots of neutralized supernatants were used for the measurement of total glutathione equivalents (GSH_{eq} = GSH + 2 GSSG). GSH equivalents were determined by following the rate of reduction of DTNB by GSH at 412 nm and comparing this to a GSH standard curve (0-300 picomoles GSH). The assay medium (100 μL) contained 100 mM sodium phosphate (NaPi) buffer (pH 7.5), 5 mM sodium EDTA, 0.2 mM NADPH, 0.6 mM

DTNB and 3.5 Units/mL glutathione reductase. The reaction was initiated with the addition of either sample or standard (20 μL).

Soluble protein in tissue extracts was measured by the Coomassie blue dye-binding method [46] using the Bio-Rad protein reagent and the micro assay procedure with bovine serum albumin as the standard according to the manufacturer's instructions. Twenty μL of protein sample (pellets suspended in 200 μL potassium phosphate) and 150 μL of Bio-Rad reagent (diluted 1: 4 in water) were added to the well and mixed thoroughly using a microplate mixer. After 10 min incubation at room temperature the absorbance was measured at 595nm.

H_2O_2 (FOX1) Assay

The FOX1 assay will measure all intra- and extracellular peroxides including H_2O_2; i.e. not exclusive to H_2O_2 alone. PC12-AC cells at a concentration of 400,000 cells/mL were plated on 96-well microplates and incubated for 24 h at 37 °C. Medium was removed and replaced with 100 μL HBSS buffer containing different concentrations of the test compounds. Stock solutions of compounds were prepared in DMSO and then diluted with HBSS so that the final concentration of DMSO in each well did not exceed 0.5%. After incubation for 3 h at 37 °C, 10 μL of the supernatant was added to 100 μL of FOX1 reagent [47] (100 mM sorbitol, 125 μM xylenol orange, 250 μM $FeSO_4$, 25 mM H_2SO_4), prepared 30 min in advance. Absorbance at 560 nm was read after 30 min incubation at room temperature. Levels of H_2O_2 in samples were compared to a standard curve of H_2O_2 (0-80 μM).

Protection against oxidative stress

a. Oxidative stress induced by menadione

PC12-AC cells were trypsinized, counted (trypan blue), diluted and then seeded in untreated 96-well microplates at a density of 200,000 cells/mL (100 μL/well). The plates were incubated for 24 h (37 °C, 5% CO_2) to allow adherence. After incubation the medium was discarded and replaced with fresh medium containing the compounds at concentrations ranging from 1-10 μM (test compounds were dissolved in DMSO; final concentration of DMSO in the wells was less than 0.5%). Cultures containing test compounds were incubated for another 24 h. The used medium was then removed and replaced with fresh medium containing menadione (final concentration 50 μM) and the plates were incubated for another 2 hours.

The cell viability was assessed with MTT assay. To each well was added 10 μL of MTT (5 mg/ml) and after 2 h incubation and lysing the cells the absorption values were read at 570 nm with background subtraction at 630 nm in a microplate reader.

b. Oxidative stress induced by AAPH

The 96-well microplates, prepared as previously mentioned (2 x 10^5 cells/mL) were incubated for 24 h prior to use. The used medium was then replaced with fresh medium and the test compounds (in DMSO, diluted in medium) were added to the wells (at final concentrations of 0 - 250 μM) and incubated for 2 h before the addition of AAPH (final concentration 12 mM). After 24 h incubation, the cell viability was assayed using the MTT assay. AAPH has been shown to give carbon-centered radicals which rapidly add oxygen to give hydrophilic peroxyl radicals [41,48]. The term "Stress" is

used to denote the absorbance (% of Control) in the presence of AAPH (12 mM, 24 h incubation).

Calculation of BDE

The method of calculation was presented in the previous chapter (Material and Methods, Chapter 2). Briefly, for calculation of the BDE it was used the lowest-level method (LLM) described by DiLabio et al. [49]. Starting geometries were generally obtained with the Spartan '02 builder module [WaveFunction, Inc., Irvine, CA USA] using AM1; coordinates were then sent to the Gaussian 98 program for all subsequent calculations [50].

Statistics

Data are expressed as mean ± SEM values. Competitive toxicity plots are given in Result section, e.g. Figure 3.2. A B-spline function was fitted through the data (see Figures 3.2--3.6, 3.12). Using the SEM values, two additional curves were fitted through the top and bottom of the error bars. From these an error estimate for the EC_{50} was derived as follows: a line was constructed at 50% of the maximum absorbance and the minimum and maximum curves intersected this line, forming intervals. The error limit of the EC_{50} is half of this interval. An error estimate for the cytoprotective area (CPA) was obtained as follows: a minimum and a maximum area were calculated using the minimum and maximum curves fitted through the top and bottom of the errors bars. The half of the difference between min CPA and max CPA represents the error estimate for

the CPA. Statistical significance was assessed by using one-way ANOVA. Differences were considered significant at $p < 0.05$.

3.3 RESULTS

Figure 3.1 shows structures for the synthetic catechols and their diacetates tested, and the known antioxidant from green tea, EGCG. The catechols were acetylated in order to be protected against extracellular autoxidation. The diacetates cross the PC12-AC cell membrane and are hydrolyzed to the diols by intracellular ester hydrolases.

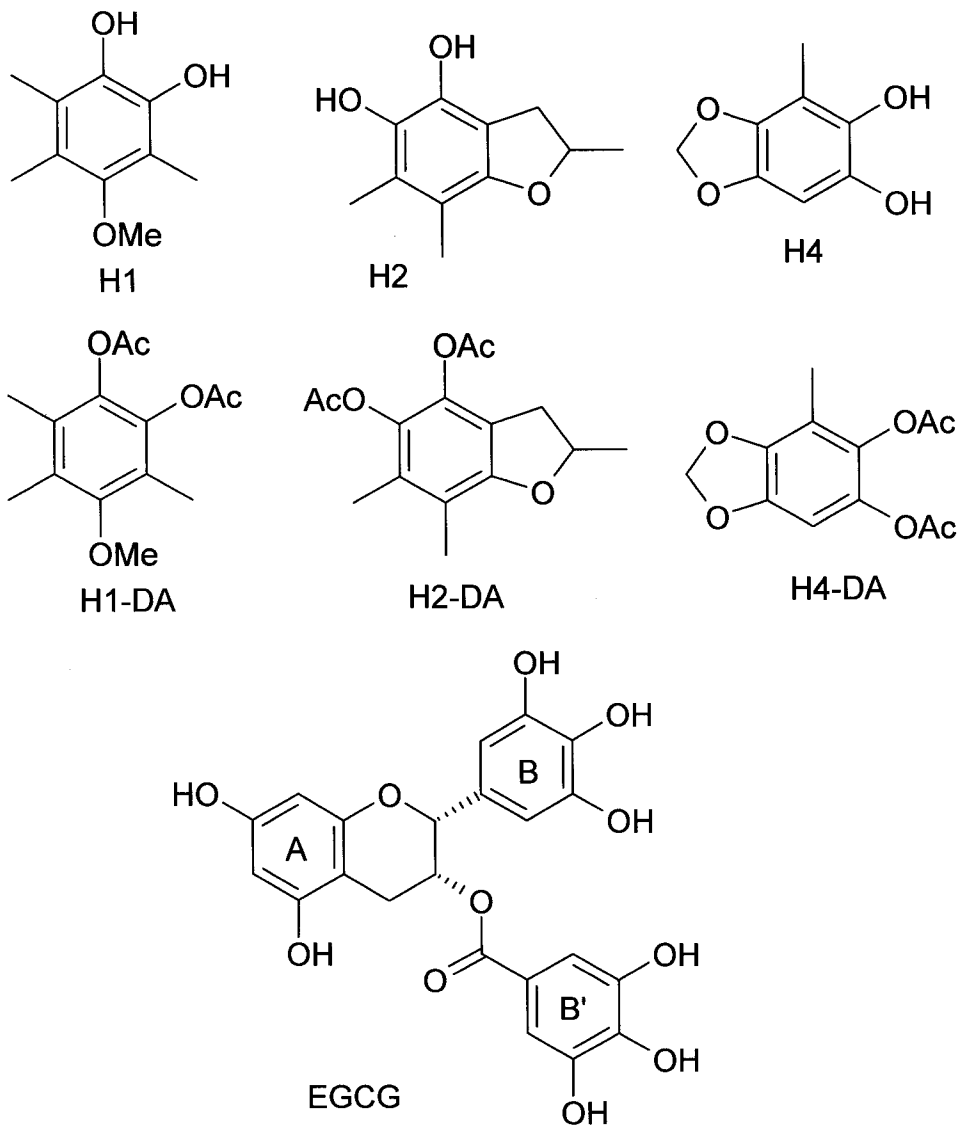


Figure 3.1. Structural formulas for the synthetic catechols H1, H2, and H4 and their diacetates H1-DA, H2-DA, H4-DA and EGCG = (-)-epigallocatechin gallate.

1. BDE and log P calculations

The first two columns of Table 3.1 show the calculated BDE values for the first (BDE_1) and second (BDE_2) homolytic, gas-phase O-H bond dissociation enthalpy. Columns 3 and 4 show the calculated octanol-water partition coefficient (log P) values. Thus for H1, for example, the first dissociation to the semiquinone ($QH_2 \rightarrow QH^{\bullet}$) requires 74 kcal mol^{-1}, whereas the second dissociation to form the quinone ($QH^{\bullet} \rightarrow Q$) requires 73 kcal mol^{-1}. From the Table it is clear that H1, H2 and H4 are similar in that BDE_2 is comparable to BDE_1.

Already from these results one can hypothesize that H1, H2 and H4 should be effective quinone formers. This is true since once the semiquinone radical has been formed they should rapidly autoxidize to form the corresponding quinone and superoxide ions.

Table 3.1. Bond dissociation enthalpy (ΔH°_{298}) and Log P values for catechols and EGCG: BDE_1 is for catechol → semiquinone, BDE_2 is for semiquinone → quinone. QH_2 = parent catechol, Q = quinone derived from the catechol.

Compound	BDE_1 (kcal mol^{-1})	BDE_2 (kcal mol^{-1})	Log P (QH_2)	Log P[b] (Q)
H1[a]	74	73	1.25	0.87
H2[a]	69	72	1.23	1.11
H4[a]	67	67	-0.13	-0.28
EGCG	71	74	2.04	

[a]See Hussain et al. [13].
[b]Log P was calculated using the Villars algorithm in the Spartan '02 program [51].

2. Cytotoxicity and effect of added ascorbate in PC12-AC

Figure 3.2 shows the cytotoxicity vs. concentration for the synthetic catechols (H1, H2, H4) their diacetates (H1-DA, H2-DA, H4-DA) and EGCG, where the dashed line represents the effective concentration (MTT assay) for which the absorbance/cell viability is reduced to 50% of control (only cells, no treatment). There is clearly a wide range of cytotoxicity among the various catechols and their diacetates ranging from H4-DA and H2 (most toxic) to H1-DA (least toxic). Even less cytotoxic is EGCG; an interesting result because this molecule contains the pyrogallol moiety which is known to autoxidize rapidly [52]. The observed order of cytotoxicity is: H2 > H4-DA > H2-DA > H4 > H1 > H1-DA >> EGCG, with EC_{50}'s of 25, 26, 33, 43, 82, 103, >>200 μM, respectively.

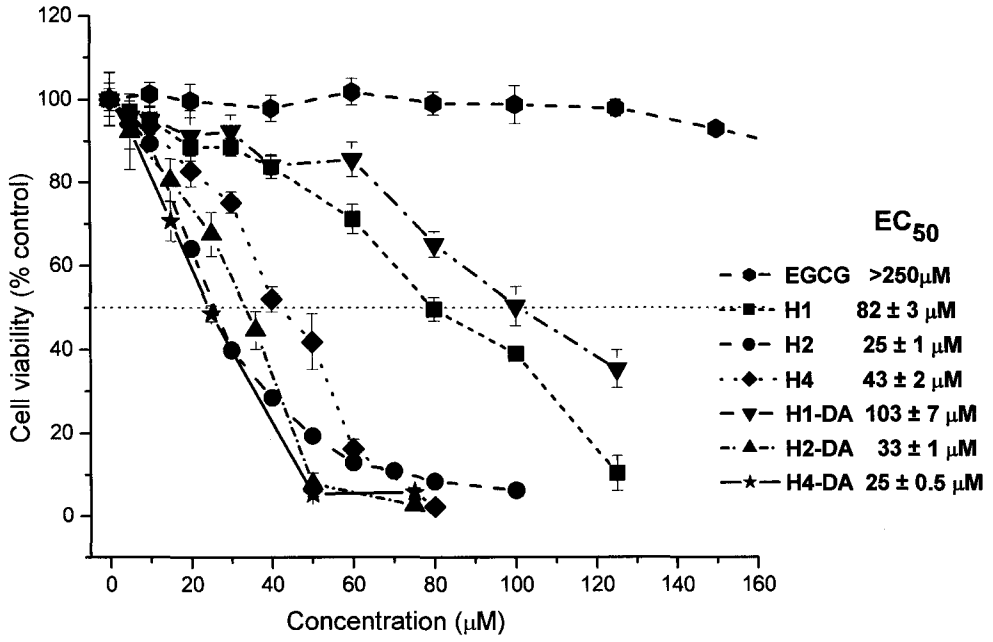


Figure 3.2. Cytotoxicity of three catechols H1, H2, H4 and their diacetates H1-DA, H2-DA, and H4-DA and the known compound EGCG as measured by MTT assay.

Figures 3.3a-3.5b show the number of viable PC12-AC cells, as determined using the MTT assay, for all the compounds in Figure 1 and/or their diacetates, as well as the effect of adding ascorbate at 50 μM. The effective concentration which reduces the number of live cells to 50% of control will be denoted EC_{50} in the discussion to follow.

Figures 3.3a and 3.3b show that the cytotoxicity is somewhat greater for H1 than for its acetylated form H1-DA (EC_{50} values of 82 and 103 μM, respectively). Addition of ascorbate is protective for H1 (82 → 103 μM), but somewhat less so for H1-DA (103 → 110 μM).

Figures 3.4a and 3.4b show that H2 and H2-DA are much more cytotoxic than H1, with an EC_{50} of only 26 μM and 33 μM, respectively. Ascorbate gives a significant protective effect to H2, with an EC_{50} value increasing to 39 ± 0.5 μM in the presence of 50 μM ascorbate, and even higher protection (EC_{50} = 52 ± 5 μM) in the presence of 75 μM ascorbate. For H2-DA the EC_{50} increases from 33 μM to 56 μM when the ascorbate is added.

By contrast, in H4 (Figure 3.5a), ascorbate *increases* the cytotoxicity significantly (43 → 32 μM), making this one of the more cytotoxic combinations of the molecules studied here. H4-DA (Figure 3.5b) has a somewhat different response, showing little difference with or without added ascorbate until the EC_{50} is reached, in which ascorbate is protective beyond the EC_{50}. To try to further understand these different responses to ascorbate, we require information from the hydrogen peroxide and glutathione response curves.

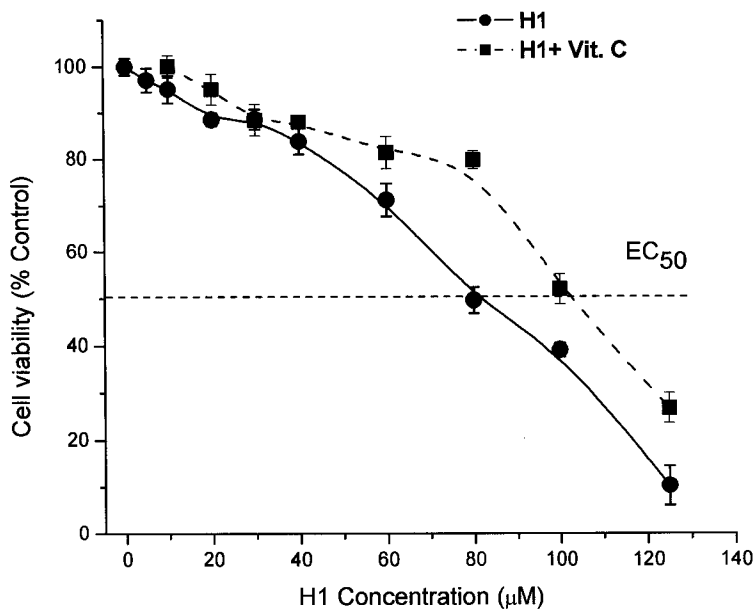


Figure 3.3a. Cytotoxicity response curve for H1, H1 + 50 μM ascorbate, showing % live cells relative to control (MTT assay) vs. conc. of H1.

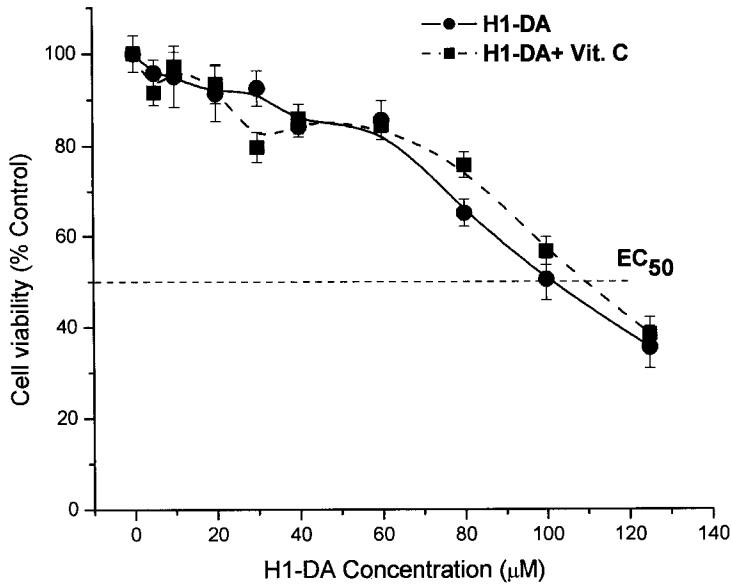


Figure 3.3b. Cytotoxicity response curve for the acetylated compound H1-DA, H1-DA + 50 μM ascorbate.

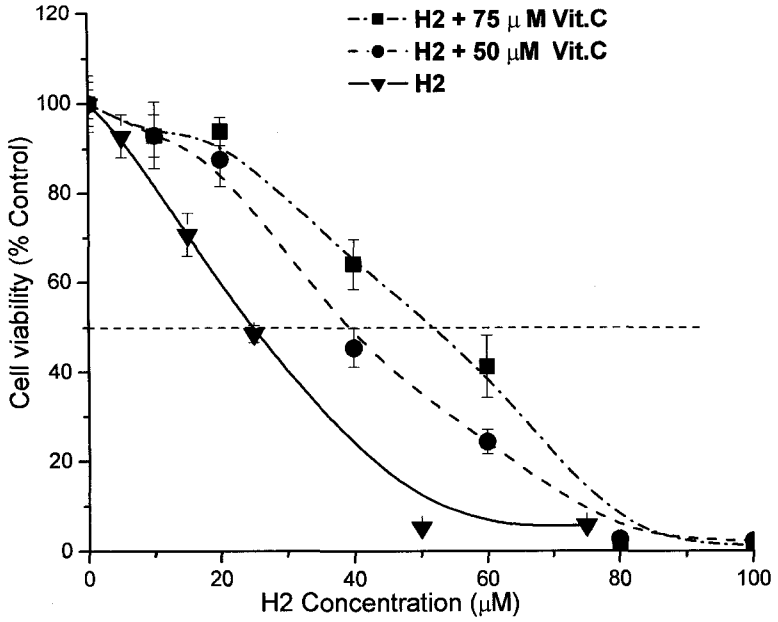


Figure 3.4a. Cytotoxicity response curve for H2, H2 + 50 μM ascorbate and H2 + 75 μM ascorbate.

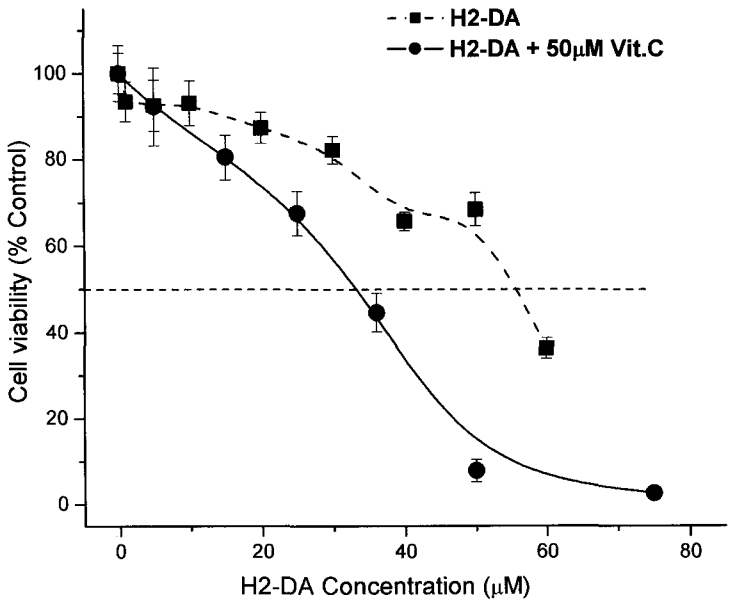


Figure 3.4b. Cytotoxicity response curve for the acetylated compound H2-DA, H2-DA + 50 μM ascorbate.

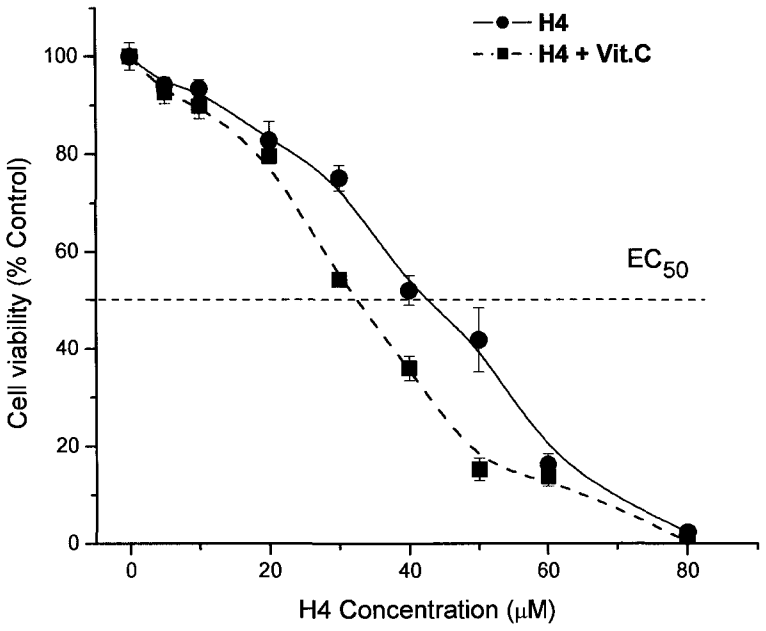


Figure 3.5a. Cytotoxicity response curve for H4, H4 + 50 μM ascorbate.

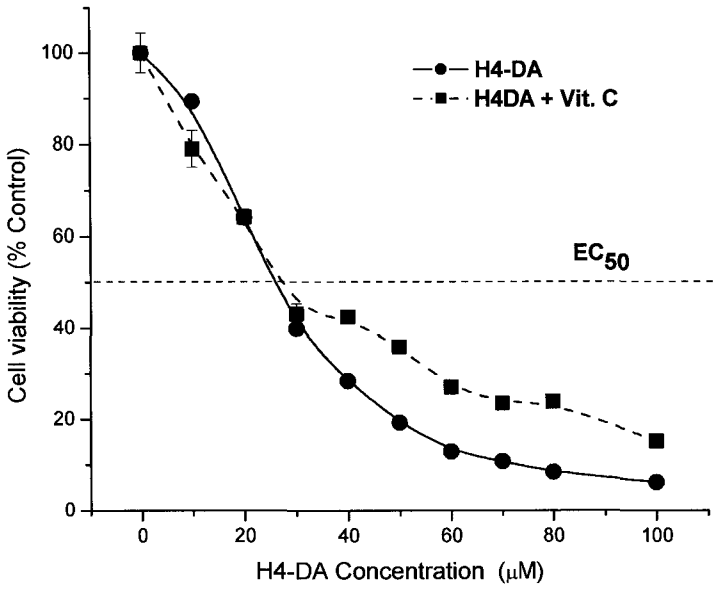


Figure 3.5b. Cytotoxicity response curve for H4-DA, H4-DA + 50 μM ascorbate.

Figure 3.6 shows the number of viable PC12-AC cells (from MTT assay) vs concentration for Vitamin C. Ascorbate anion (since solution pH = 7.4) is very non-toxic (EC_{50} > 200 μM). In this case it is known that ascorbate can dismutate into DHA which will enter the cells easily and then regenerate ascorbate [53] The concentration of 50 μM Vitamin C that is used in the tests with catechols is non-toxic.

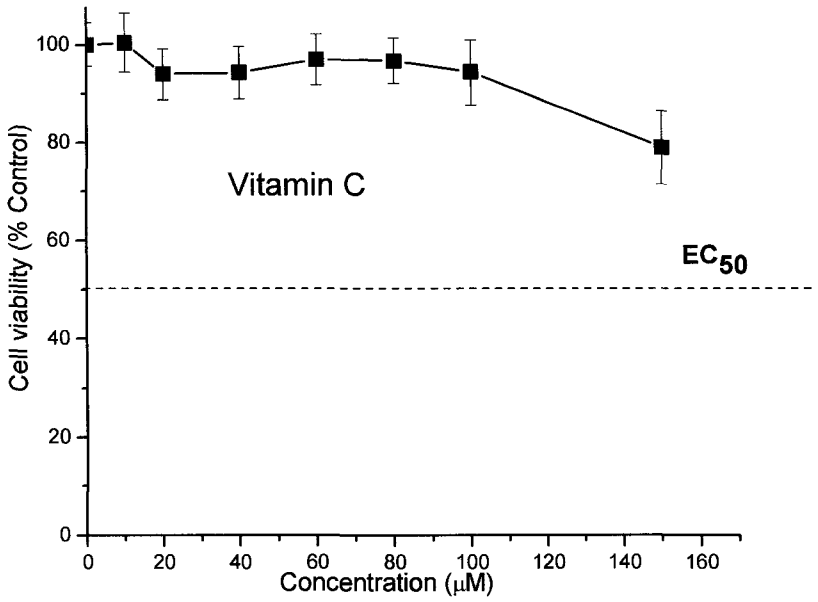


Figure 3.6. Cell viability (MTT assay) for PC12-AC cells vs concentration of Vit. C.

3. Proof of redox cycling

a. H2-DA measurements in HL-60

In an early phase of this work, synthetic catechols were tested for their ability to act as *anti*oxidants, as measured by the scavenging of superoxide radicals generated by HL-60 cells. [54]. Briefly, differentiated HL-60 cells were incubated either with the catechol directly (e.g. H2), or with the diacetate form (H2-DA). Cytochrome C (III) was added, and the cells were stimulated with phorbol acetate (PMA) to produce superoxide (time

zero). The absorbance vs. time plot showed that superoxide was produced and reduced the indicator, causing a rise in absorbance. Antioxidant behavior would cause suppression in the rise of absorbance, i.e. a decreased slope. Alternatively, the HL-60 system can be studied to see whether the molecule in question can act as a *pro-oxidant*, by replacing the PMA in the above assay and stimulating the generation of superoxide. Using H2-DA as an example, hydrolysis inside the cell converts H2-DA into H2, which can then generate superoxide, detected via reduction of cytochrome C (III).

Figure 3.7 shows the results of this experiment. Differentiated HL-60 cells were incubated with H2-DA, which passed through the cell membrane in 10-15 minutes, as shown by the induction period (flat initial slope) in Figure 3.7. The graph is plateauing when the cytochrome C is used up.

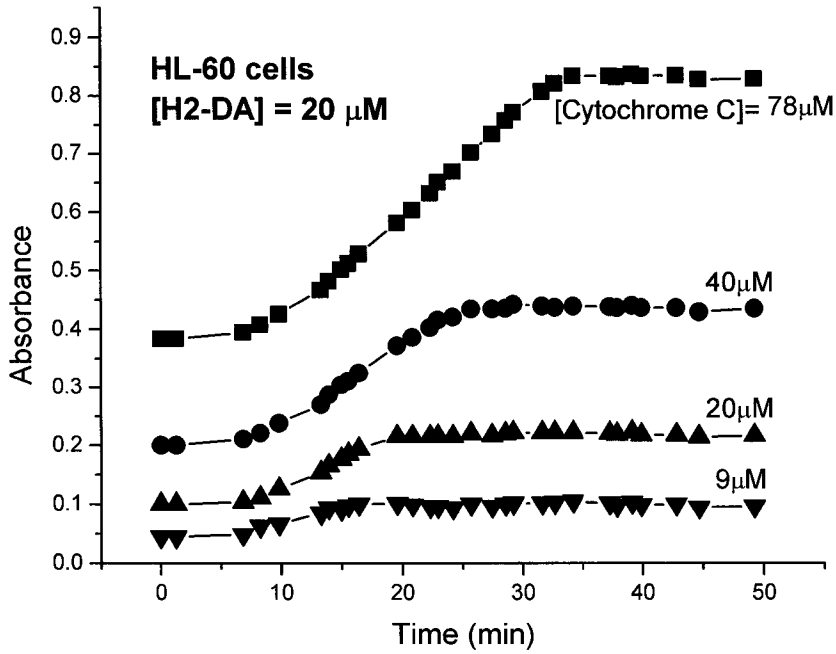


Figure 3.7. H2-DA (20 μM) generates superoxide ion in differentiated HL-60 cells, causing an absorbance change due to reduction of cytochrome C(III).

b. DCF assay on PC12-AC

Another way to monitor the intracellular oxidation mechanism is to measure the extent of intracellular oxidation products (peroxides) using a dichlorofluorescein indicator. First, PC12-AC cells were incubated in HBSS solution with 40 μM DCF-DA for 1.5 h to load the cells. Then H2-DA was incubated with the cells for an additional 2 h. If redox cycling is occurring it should oxidize the DCF and generate a fluorescence signal.

Figure 3.8 shows the result of DCF experiments, for a concentration range of H2-DA of 5 to 150 μM. In this experiment, the cells were washed after the last (H2-DA) incubation and prior to the fluorescence measurement. This removed all excess extracellular DCF-DA and H2-DA, as well as any DCF or H2 which passed from the interior to the exterior of the cell during the incubation period. There is a good dose-response curve up to the maximum concentration of H2-DA used (150 μM); beyond this range the H2-DA toxicity must increase to the point that cells are killed, and the fluorescence response will drop. From Figure 3.8 the maximum fluorescence yield is about 1400 units at 150 μM.

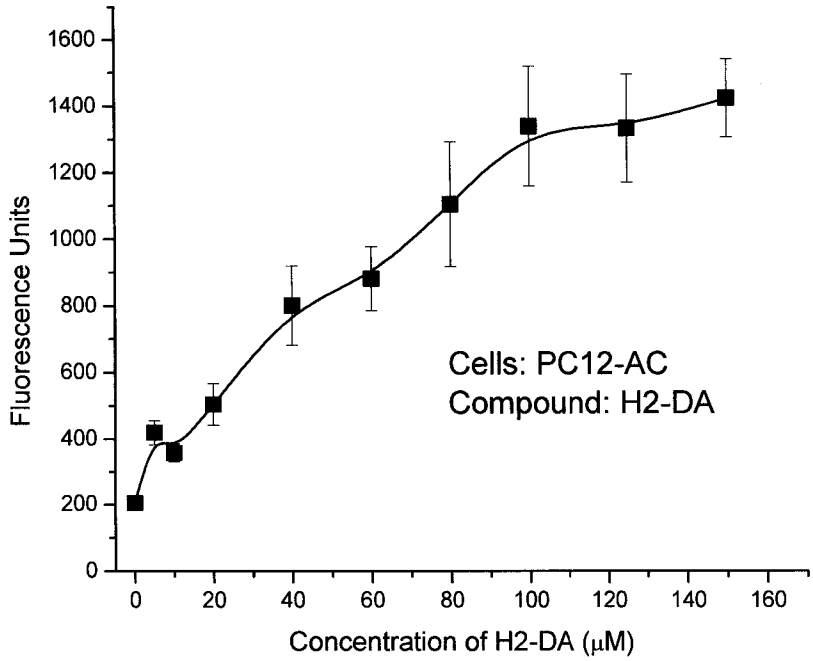


Figure 3.8. Fluorescence vs. concentration of H2-DA. PC-12AC cells (400,000 cells/mL), 24 hours for cell adherence. The cells were treated with H2-DA in HBSS for 2 h, then with 40 μM H2DCF-DA in HBSS, After incubation for 1.5 h, the fluorescence was measured using a Fluogalaxi microplate reader.

4. Hydrogen peroxide response curves

Figures 3.9a-c (the experiments were done in Dr. Wright lab by Chichirau A.) and Figure 3.10 show the amount of H_2O_2 generated as a function of substrate concentration, for H1 and H1-DA, H2-DA, H4 and H4-DA, respectively, along with the effect of added ascorbate at the concentration indicated. For H1, the H_2O_2 response curve (Figure 3.9a) shows an essentially constant upward slope, reaching an H_2O_2 concentration of over 50 μM when H1 is 200 μM, the largest concentration we tested. Even at high concentrations, there was little change in slope, evidence that the cells must be still viable

and undergoing redox cycling. Ascorbate caused a small reduction in H_2O_2 produced, but not until concentrations of H1 above 80 μM. The results for H2-DA (Figure 3.9b) are similar to those for H1. There is a strong rise in H_2O_2 with increasing H2-DA concentration. The H_2O_2 response is somewhat less than for H1, however, reaching a maximum of only 35 μM at a concentration of H1 of 200 μM. A small decrease in H_2O_2 production is seen when ascorbate is added (Figure 3.9b).

The H_2O_2 response to H4 is qualitatively different (Figure 3.9c). There was a much smaller concentration of H_2O_2 reached with a maximum of only ca. 5 μM when the concentration of H4 is 100 μM, followed by a decline. In this case, ascorbate caused an *increase* in production of H_2O_2.

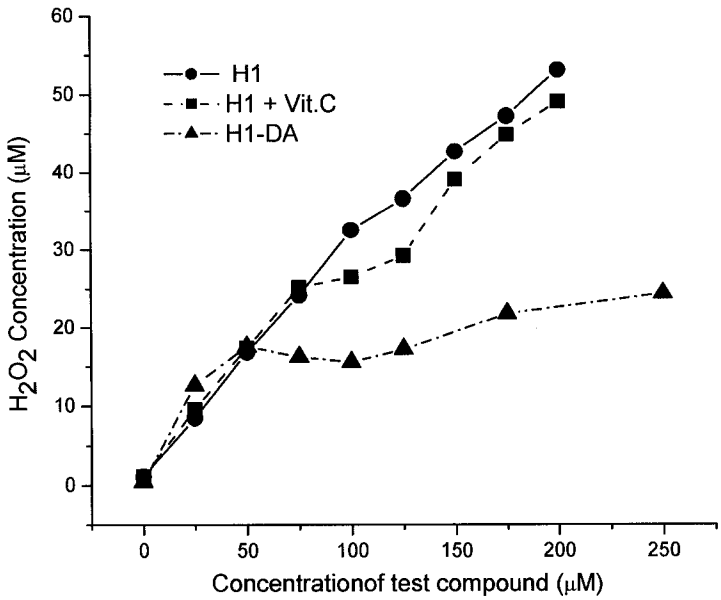


Figure 3.9a. (Extracellular) H_2O_2 concentration vs. concentration of H1, H1 + Ascorbate (50 μM) and H1-DA.

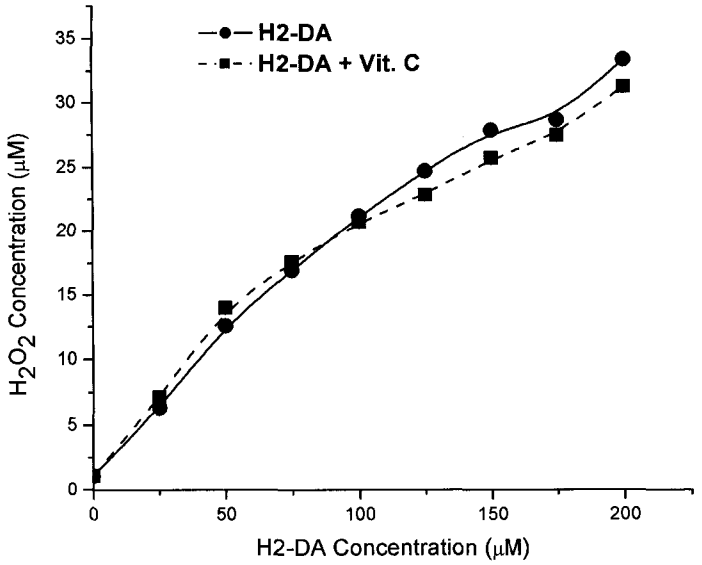


Figure 3.9b. (Extracellular) H_2O_2 concentration vs. concentration of H2-DA, H2-DA + Ascorbate (50 μM).

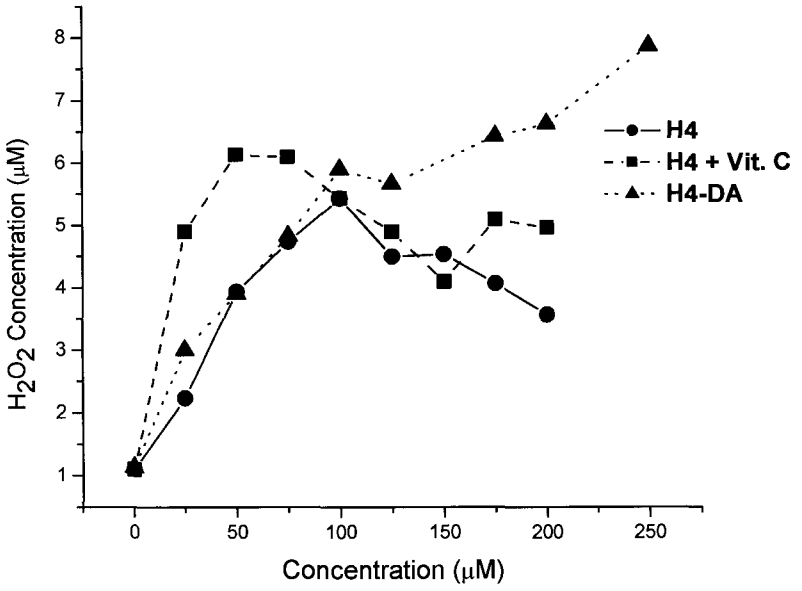


Figure 3.9c. (Extracellular) H_2O_2 concentration vs. concentration of H4, H4 + Ascorbate (50 μM) and H4-DA. More H_2O_2 is produced for [H4] < 100 μM when ascorbate is added.

Figure 3.10 shows the hydrogen peroxide response curves for all three catechols diacetate and EGCG drawn on the same scale, without considering the effects of added ascorbate. In these experiments, the medium was removed, then the compound was added to cells in HBSS and incubated over a variable time period (0-180 min), followed by measurement of H_2O_2 in the supernatant. H1-DA showed a slow but significant rise in time. H2-DA showed a faster rise and H4-DA and EGCG even faster still: in the case of H4-DA did we see a maximum in H_2O_2 concentration, followed by decline. H_2O_2 concentration in the case of EGCG reached a maximum of 20 μM followed by decline and plateau (at 12 μM).

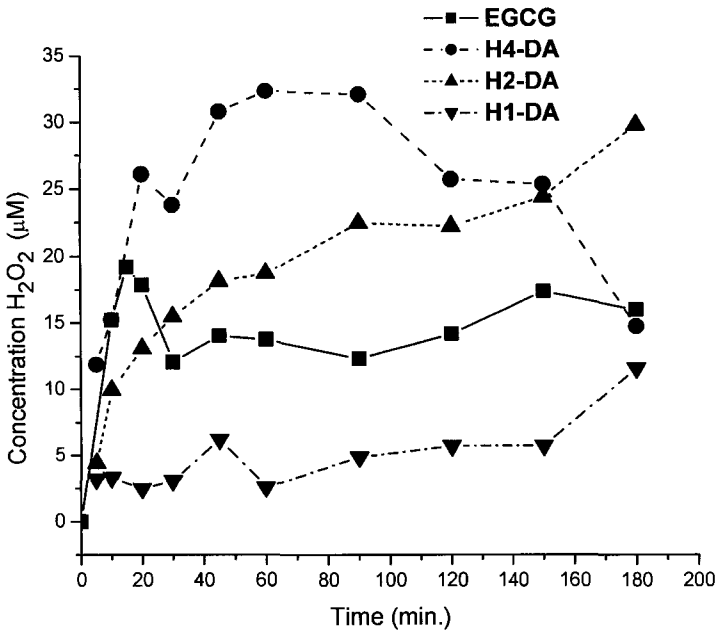


Figure 3.10. H_2O_2 response curves as a function of time (FOX1 assay) for each test compound in its diacetate form. Cytotoxicity can be almost directly related to H_2O_2 production.

5. GSH response curve

The response of GSH to increasing concentrations of substrate is shown in Figure 3.11, for all three diacetates. All GSH results have been normalized to total mg protein. For these experiments which were carried out in medium, it is important to avoid extracellular autoxidation of the catechol, which could be catalyzed by the presence of background superoxide in the RPMI1640 medium [55]. Therefore in these experiments we used only the acetylated forms of the compounds, which were completely unreactive in the GSH assay in the absence of cells (data not shown).

For H1-DA there is a significant rise (ca. factor of two) in pmol GSH /mg protein at the highest concentration of reagent indicated. For all compounds, the statistical error increased dramatically beyond the final concentration (data not shown), due to the decreasing amounts of protein present as a result of cell death. A similar but stronger GSH response is observed for H2-DA. Here the maximum response is almost quadruple that of control, and the slope is much steeper. Thus both redox-cycling compounds are able to cause an increase in GSH concentration in the cell. H4-DA, capable of both thiol depletion and redox cycling, also shows a steep slope and a dramatic increase relative to that of control.

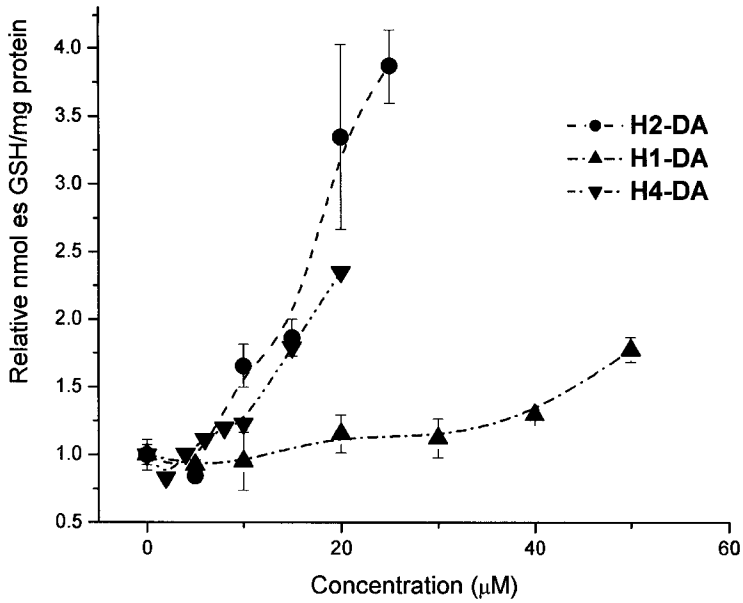


Figure 3.11. GSH response curves as a function of concentration of diacetates H1-DA, H2-DA, and H4-DA. Each plot is normalized to a per milligram protein basis. Again, closely correlated to cytotoxicity and H_2O_2 production.

6. Protection experiments

In order to test our new compounds for the cytoprotective effect in PC12-AC, I have chosen menadione as a classic inducer of oxidative stress. Figure 3.12 shows the cytotoxicity caused by the oxidative stressor menadione. First, the concentration of menadione and cells used in experiments had to be optimized as to obtain about 40 - 50% viability; this corresponds to about 47 μM of menadione (EC_{50}) when the cell concentration is 200,000 cells/mL.

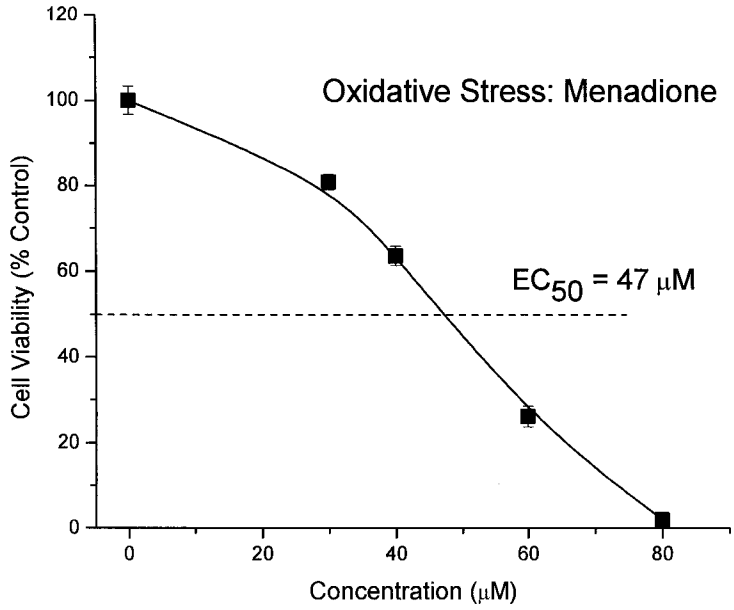


Figure 3.12. Cell viability (MTT assay) for PC12-AC cells vs. concentration of menadione.

Then I looked to see whether the antioxidants or antioxidant/Vitamin C combinations could increase the viability (i.e. protective effect) of the cells stressed with 50 μM menadione (more than 60% of cells were killed). Figure 3.13a shows the effect of H2-DA, H2-DA + ascorbate on viability of PC12-AC cells with and without 50 μM menadione. A synergistic protective response was seen for a combination of 10 μM H2-DA and 50 μM Vitamin C. At 10 μM H2-DA I see a strong protective effect (30% to 60% viability). This increased in the presence of ascorbate (30% to 85% viability, levels similar to that found in controls). Figure 3.13b shows the experiment as before but with concentrations of H2-DA at 20 μM. At this concentration of H2-DA, there was a slight increase in viability of cells stressed with menadione and the only protective effect was

when H2-DA + ascorbate was used, resulting in a two-fold increase in viability. These results suggested that H2-DA has a very narrow protective "window".

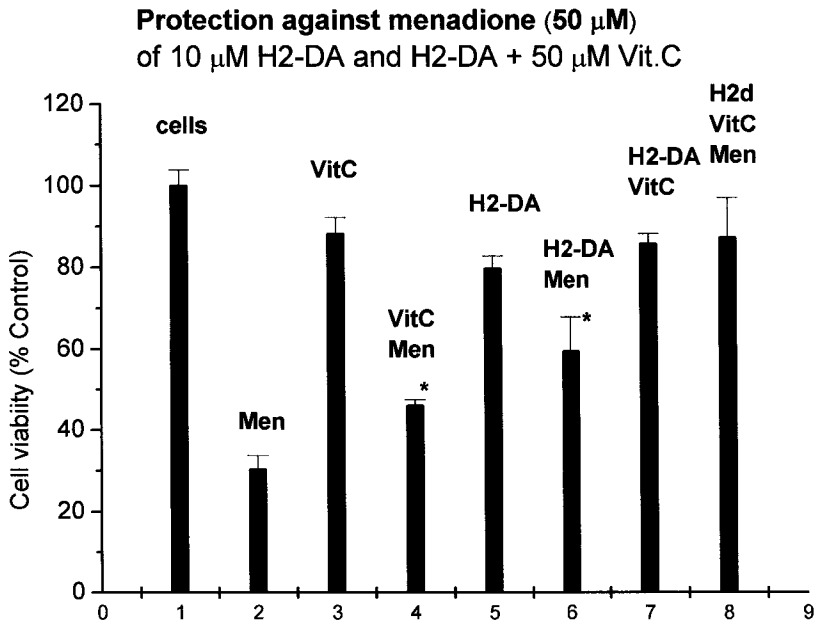


Figure 3.13a. Effects of H2-DA (10 μM) and H2-DA + Vit.C on the viability of menadione (50 μM)-treated PC12-AC cells. Cell viability was assessed by the MTT method as described in Material and Methods. Cells were preincubated with H2-DA for 24 h before treatment with menadione for 2 h. (*) $P < 0.05$ compared with Menadione-treated cells.

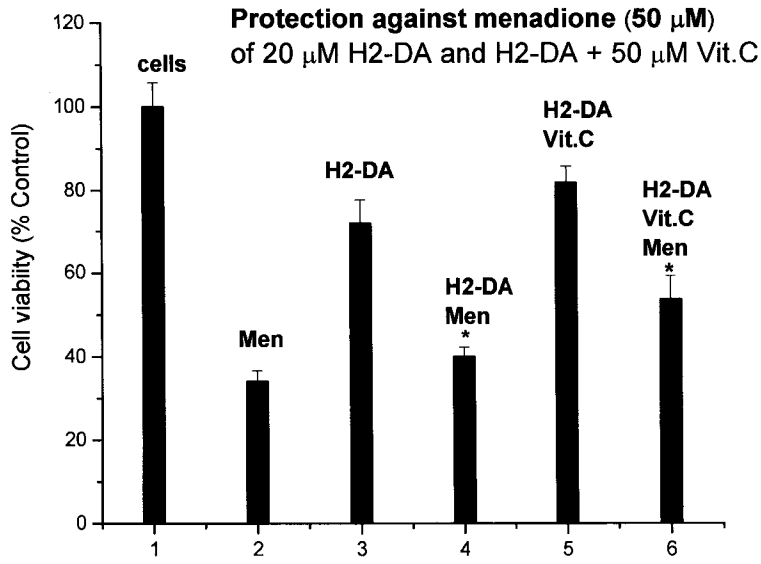


Figure 3.13b. Effects of H2-DA (20 μM) and H2-DA + ascorbate on the viability of menadione (50 μM)-treated PC12-AC cells. Cell viability was assessed by the MTT method as described in Material and Methods. Cells were preincubated with H2-DA for 24 h before treatment with menadione for 2 h. (*) $P < 0.05$ compared with Menadione-treated cells.

Figure 3.14 shows the effect of H2-DA (10 μM) and EGCG, respectively on viability of menadione treated PC12-AC cells. EGCG and H2-DA at the same concentration of 10 μM have almost the same protective effect (the viability is increased from ~ 35 % in cells with menadione to ~ 62 % in cells treated with menadione + compound).

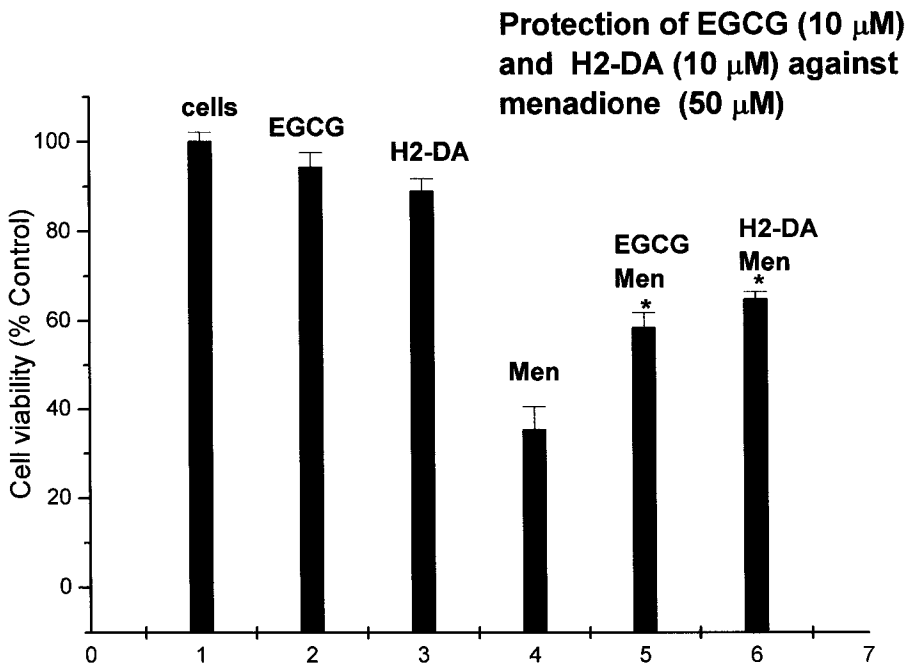


Figure 3.14. Effects of H2-DA and EGCG on the viability of menadione (50 μM) - treated PC12-AC cells. Cell viability was assessed by the MTT method as described in Material and methods. Cells were treated 10 μM H2-DA and EGCG, respectively for 24 h and then for 2 h with menadione. (*) $P < 0.05$ compared with Menadione-treated cells.

When the menadione testing was concluded, it was decided to extend this work to include another oxidative stressor whose mechanism of toxicity is very well known. For this purpose AAPH was chosen, since it is commonly used to induce oxidative stress by generating peroxyl radicals (ROO•) at a relatively constant rate. For these experiments, the cells were subjected to 24 h exposure of AAPH over concentrations ranging from 0 to 25 mM. Cell viability was determined using the MTT assay. Figure 3.15 shows for AAPH, the $EC_{50} = 10.5 \pm 3$mM.

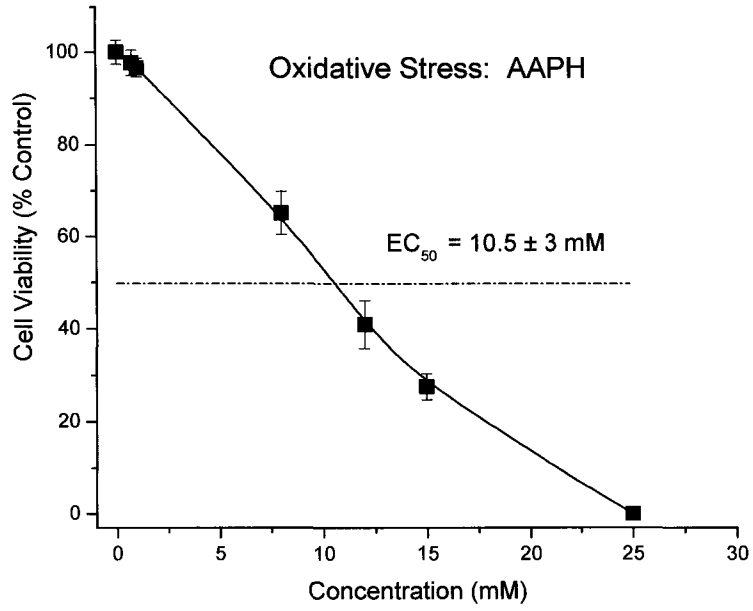


Figure 3.15. Cell viability (MTT assay) for PC12-AC cells vs. concentration of AAPH.

Figure 3.16 shows the cytoprotection of H2-DA and EGCG against oxidative stress caused by AAPH, where the AAPH concentration was 12mM. This was sufficient to reduce cell viability to 40 ± 5 % of Control. The protective range spanned from 40% to 100% of Control. As can be seen in Figure 3.16, the H2-DA has only a slight protective effect until 20 μM, beyond which its became cytotoxic. EGCG is more strongly protective attaining full protection (Control levels) at 50 μM.

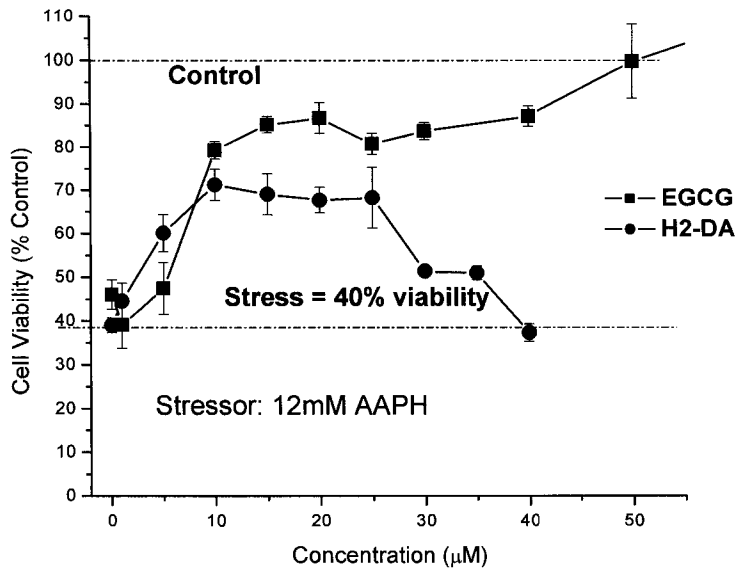


Figure 3.16. Cytoprotection of H2-DA and EGCG against AAPH-induced oxidative stress. PC12-AC cells were pre-incubated with antioxidants for 2 h and then for other 24 h with AAPH (12 mM). The viability was assessed with MTT assay described in Material and Methods.

Figures 3.17 and 3.18 show the relevant data for H2-DA and EGCG, respectively. Oxidative "stress" represented a decrease of cell viability to 40% of Control levels in the presence of AAPH (12 mM, 24 h incubation) and absence of compounds. The left-hand side of the Protection Zone is defined by the cytoprotection experiment (data from Figure 3.16). The cytotoxicity envelope provides the upper curve. The bottom boundary of the protective zone is determined as AAPH "stress". Figure 3.17 shows the H2-DA protective range of 0 to 40 μM, with the maximum peak in protection, 72 ± 3 % viability at a concentration of 15 μM. Figure 3.18 shows the EGCG protective range of 0 to 580 μM, with the maximum peak in protection, 100 ± 5% viability at 60 μM concentration.

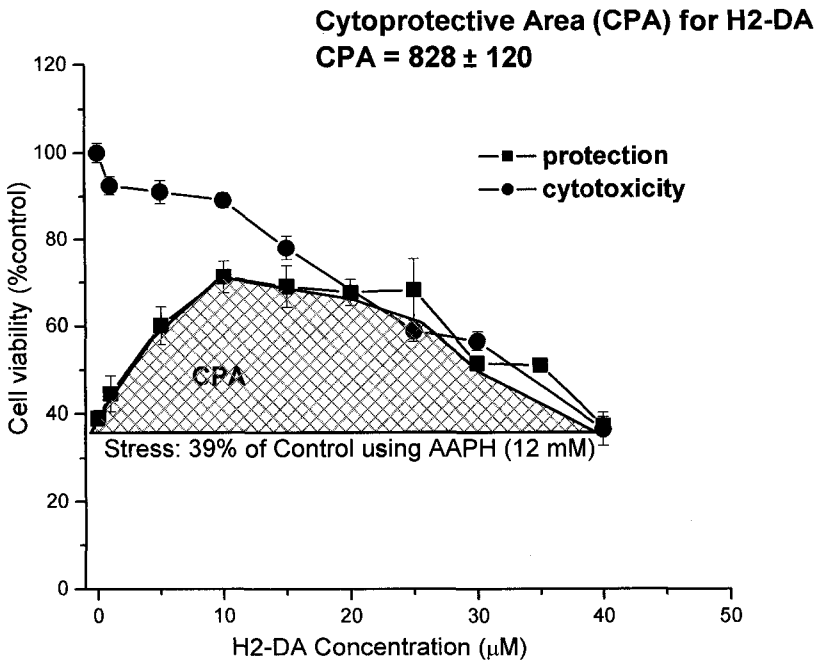


Figure 3.17. Total cytoprotective area (CPA) for H2-DA (the cross-hatched region) showing a narrow range of protection between 0 and 40 μM.

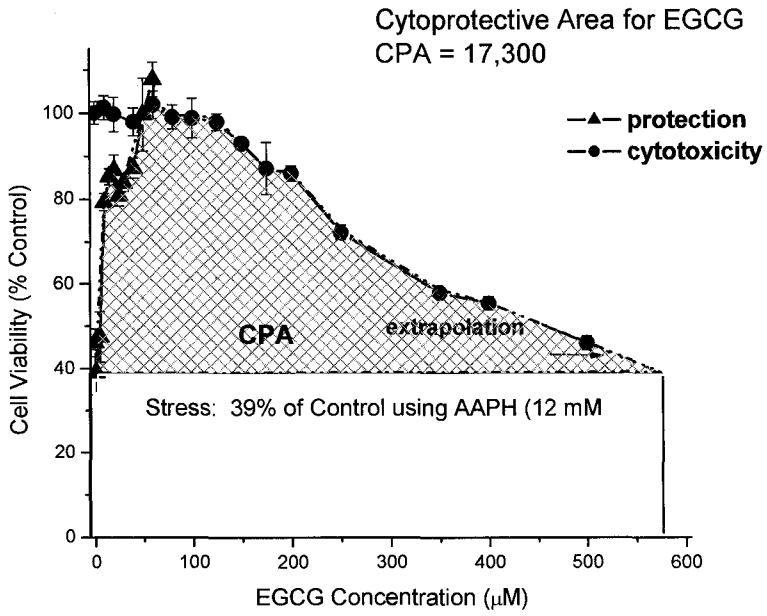


Figure 3.18. Total cytoprotective area for EGCG (cross-hatched region).

3.4 DISCUSSION

1. Predicting reaction mechanism

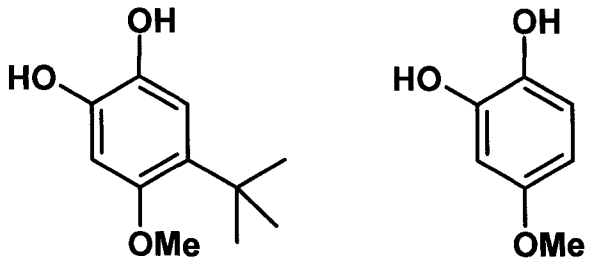
The fully substituted catechols H1 and H2 are unlikely to deplete glutathione via nucleophilic addition to the corresponding quinone, since the electron donors adjacent to the carbonyl positions in the corresponding quinone (methyl or methoxyl) render these positions essentially inactive to nucleophiles (no longer electropositive). The toxicity mechanism of H1 and H2 is expected to be redox cycling, as is known to be the case for 2,3-dimethoxy-1,3-naphthoquinone [56] (see also Watanabe, 2003, for an intra-extracellular variation on this theme) and/or autoxidation of the parent diol [20]. It would therefore be expected that H1 and H2 would show similar variations in H_2O_2 and GSH response but differ from H4, which has an open ring position and could react with thiols as well as undergo redox cycling. (see below).

2. Variations in absolute cytotoxicity and log P

It is of interest to consider variations in absolute cytotoxicity, as well as the difference in cytotoxicity behavior, between a compound and its respective diacetate. A previous comparison of this type was made on a series of catechols and hydroquinones related to butylated hydroxyanisole by Lam and coworkers [57]. These authors examined the cell lines P388 and KB and looked at the effect on cytotoxicity for both acetyl (mono- and di-) substitution, as well as the effect of replacing an OH group by an OCH_3 (methoxy) group. Their most toxic compound was 3-*tert*-butyl-5-methoxy-catechol (their compound 5), with an EC_{50} of 0.09 μg/mL in P-388 cells. Considering only the P-388 data, they found that removing the *tert*-butyl significantly raised the EC_{50} to 1.6 μg/mL

(compound 9). In the case of the catechol, 6-*tert*-butyl-3-methoxy-catechol, mono-acetylation increased the EC_{50} from 0.56 to 2.70 and full acetylation increased it further to 3.70. Similar trends were noted in KB cells.

Based on these data Lam et al. [57] concluded that the presence of a *tert*-butyl group enhanced the cytotoxicity, whereas mono and diacetylation made the compounds less cytotoxic. Figure 19 shows compounds 5 and 9 from their study.



Compound	5	9
Ec_{50} (P388)	0.09	1.60
log P	2.83	1.13

Figure 3.19. Structure of compounds 5 and 9 [57].

We calculated log P for their compounds as described in Table 1 and obtained values of 2.83 and 1.13 for compounds 5 and 9 respectively. This is a very significant difference due to the added *tert*-butyl group in compound 5, which is therefore much more lipid-soluble than compound 9. As shown by Moridani et al. [58] in a QSAR study of cytotoxicity of phenols in hepatocytes, a leading term in the cytotoxicity correlation is log P, i.e. the higher the lipophilicity, the greater the cytotoxicity (and hence the lower the EC_{50}). Although the *tert*-butyl substituent (an electron donor and a sterically bulky group) renders the compound 5 less susceptible to Michael addition, this is clearly a less important factor as compound 5 is far more cytotoxic than compound 9.

Our data show EC_{50}'s increasing in the compound order of H2, H4, H1, EGCG with values of 25, 43, 80, >250 μM, respectively whereas their respective log P values are 1.23, -0.13, 1.25, 2.04, respectively (Table 3.1). Thus the observed order of cytotoxicity of the compounds is H4 > H2 >> H1 >> EGCG, whereas the predicted order using log P values is EGCG >> H1 ≈ H2 > H4; the opposite of observed order. Evidently differences in cytotoxicity arising through different reaction mechanisms far outweigh simple physical variables like lipid solubility.

The lipophilicity of the quinones was found to be similar to that of the parent catechols, to within half a log unit (Table 1). This means that transport into and out of the cell will be similar for both catechols and quinones, i.e. there is no difference between the reduced (catechol) and oxidized (quinone) forms with respect to transport through the cellular membrane.

3. Effect of acetylation or methoxylation on cytotoxicity

Not surprisingly, fully methoxylated compounds were the least toxic in the data set of Lam et al. [57], since no quinone formation is possible, eliminating both thiol depletion and redox cycling mechanisms. Thus, for example, Miller et al. [59] have shown that damage due to catechol oxidation in neurons could be prevented by *O*-methylation (conversion of OH into OCH_3), which reduces the ability to oxidize the catechols. The acetylation data of Lam et al. are not as easy to explain, since nonspecific intracellular ester hydrolases would be expected to quickly catalyze formation of the catechol, such that the only difference between acetylated or non-acetylated compounds would be the presence of an induction period, which is the time required for hydrolysis of

acetyl groups. Evidently this hydrolysis needs to be monitored in each system, since differences between acetylated and non-acetylated molecules are even more pronounced in KB cells. Certainly if an acetyl group is not released then a catechol, for example, is unable to autoxidize and begin a redox cycle, which would drastically decrease its cytotoxicity. The *time involved* in measuring cytotoxicity therefore becomes relevant; if the time scale is too short (e.g. minutes instead of hours) then the delay time for hydrolysis will become a factor and cytotoxicity will be a variable of time of incubation.

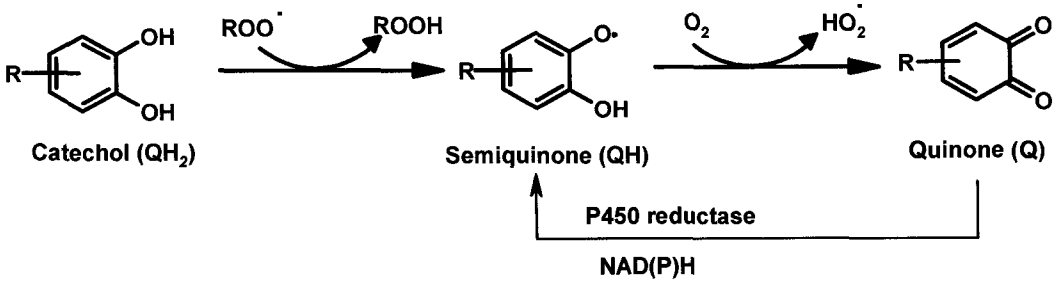
In our experiments, the cytotoxicity of all compounds was determined over a 24 h time period. This long incubation period has the advantage of minimizing any time lags due to hydrolysis by esterases. Thus I would expect the diacetate toxicity to be close to that of the unsubstituted compound. However, I do see significant differences between the compounds and their diacetates. For example, I find H1-DA to be significantly less toxic than H1 (EC_{50} = 100, vs. 80 for H1)(Figure 3.3), whereas the diacetate H4-DA is *more* toxic than H4 (EC_{50} = 26 vs. 43, respectively)(Figure 3.5). This may be a factor of a) the ability of the compound to enter the cell and b) the ability of the compound to form a radical. It goes back to the arguments of protection/toxicity afforded inside/outside of the cell. For example, H4 becomes MORE toxic as it enters the cell and becomes a radical whereas H1 enters the cell and does not (Figure 3.10). In fact, in the case of H1 where the tested concentrations are necessarily high due to the high EC_{50}, I observed formation of a red color in the microplate wells. Since the cytotoxicity tests were carried out in medium, it is probable that this differential toxicity must have come from extracellular autoxidation in the presence of (high concentrations of) H1, probably initiated by superoxide in the medium reacting with catechol, according to $HO_2^{\bullet}$ (from

superoxide) + $QH_2 \rightarrow QH^{\bullet} + H_2O_2$. As a consequence, this would increase the toxicity of H1 relative to H1-DA, as observed. H1 does not generate significant amounts of H_2O_2 and has a high EC_{50}. Whether intracellular or extracellular, it requires much more of this compound to generate the same amount of H_2O_2 as H4. Both diacetylated compounds most likely have an equal chance of entering the cell. However, I have no simple explanation for the higher toxicity of H4-DA than of H4.

4. Reaction mechanism and BDE value

A typical sequence for a catechol undergoing redox reactions and generating superoxide is shown in Scheme 3.1. This scheme shows only the protonated forms of the semiquinone ($QH^{\bullet}$) and of superoxide anion (the hydroperoxyl radical $HO_2^{\bullet}$); whether the protonated or anionic form is present depends on the pH, but does not affect the general mechanistic argument to follow. (Note that pK_a's of semiquinones tend to be rather low at pH 4 to 5, so the dominant form at pH 7.4 is the anion).

SCHEME 3.1



An attacking radical, e.g. peroxyl ($ROO^{\bullet}$) provides the initiation step converting QH_2 into its semiquinone radical, $QH^{\bullet}$. Alternatively, $QH^{\bullet}$ could be produced in a disproportionation reaction between QH_2 and Q (reaction (1)). Note that in the former

case, QH_2 is acting as an antioxidant (deactivates $ROO^{\bullet}$) but in the latter disproportionation QH_2 becomes a pro-oxidant through generation of its semiquinone radical. This illustrates the complexity of catechol/semiquinone/quinone chemistry, where the rates of competing reactions become relevant to the eventual cytotoxicity. In any case once the semiquinone has formed then redox cycling proceeds as shown. The superoxide anion (hydroperoxyl radical) is generated which disproportionates producing H_2O_2 plus molecular oxygen. This is the case with H1, H2 and, to a lesser, extent, H4.

The effectiveness of QH_2 as an antioxidant was strongly correlated with BDE_1. Thus we and others have shown that the rate of reaction with the test radical $DPPH^{\bullet}$ increases (hence a better antioxidant) as BDE_1 decreases; i.e. the weaker the bond in QH_2 the faster the reaction with the free radical $ROO^{\bullet}$ [12,13]. However, the activity as a pro-oxidant must depend both on BDE_1 (related to rate of generation of $QH^{\bullet}$) and BDE_2 (related to rate of production of Q). Thus both BDEs will affect the rate of redox cycling; BDE_1 through generation of the semiquinone, and BDE_2 through generation of the quinone. Kinetic modeling of this type of complex system has been done by Roginsky and coworkers [53] and for a useful summary of the situation we quote from Pattison et al: "whether DOPA and other catechols behave as antioxidants or pro-oxidants is dependent on the nature of the oxidizing radical, the radical flux, and the reactivity of the resulting semiquinone radical. In general, those catechols that give rise to the most stable (least reactive) semiquinones are the most likely to show antioxidant, rather than pro-oxidant behavior" [60].

How does this relate to the cytotoxicity of our catechols? From the above discussion it is clear that a low BDE_1 is protective, whereas a low BDE_2 is destructive to

the cell. In order to obtain a low BDE_1 it is typically needed to load an aromatic ring system with electron donors, e.g. hydroxyl, amino or methoxyl groups or, to a lesser extent, alkyl groups [58]. However, Table 1 shows that BDE_1 values that are similar to BDE_2 values for the catechols H1, H2 and H4 described here (e.g. 74 and 73 for H1), indicative of a reactive semiquinone. When both BDEs are approximately equal, this will favor the pro-oxidant case, since the semiquinone (anion) reaction with oxygen is much faster than the catechol reaction with peroxyl radicals. This is the situation for the catechols H1, H2, and H4, all of which are relatively cytotoxic.

5. Discussion – proof of redox cycling

Figure 3.7 shows the proof that the compounds are redox cycling. Differentiated HL-60 cells were incubated with H2-DA, which passed through the cell membrane in 10 to 15 minutes, as shown by the induction period (flat initial slope) in Figure 3.1. There are then two possible mechanisms which can lead to an increase in absorbance with time. The first is stoichiometric, which begins when the catechol QH_2 in its neutral form being transported back through the cell membrane into the extracellular fluid, where the indicator is present, and is (partially) converted into its anionic form QH^-. This reacts with indicator according to (in abbreviated terminology)

$$QH^- + III \rightarrow QH^\bullet + II \qquad (10)$$

with a rise in absorbance. A second reduction could in principle also occur via the semiquinone radical anion,

$$Q^{\bullet -} + III \rightarrow Q + II \qquad (11)$$

In this simple mechanism a *stoichiometric factor* of two results, i.e. one QH_2 can reduce two molecules of III. Further consideration of the disproportionation possibility leads to a maximum stoichiometric factor of 4; i.e. an initial concentration of H2-DA equal to 20 μM could reduce a maximum amount of cytochrome C (III) equivalent to 80 μM, followed by no further reduction. However, Figure 1 shows that at a concentration of indicator equal to 4 times that of QH_2, a constant absorbance is reached by 35 min showing that *all the indicator was used up*, i.e. the stoichiometric factor must be greater than 4. This result is explained as follows: First, an initiating free radical is generated, probably by disproportionation as in reaction (1), since a small amount of Q is always present along with QH_2. A redox cycle now begins to produce superoxide according to reaction (2) and (3), followed by one-electron reduction of Q back to $Q^{\bullet-}$ to continue the chain, and superoxide ion is exported outside the cell (possibly via its conjugate acid, $HO_2^{\bullet}$). This results in a stoichiometric factor which depends on the redox cycle chain length. Thus Figure 3.7 shows unambiguous proof that a redox cycle is occurring for the catechol H2 inside the HL-60 cells.

Another way to prove the intracellular redox cycling vs. extracellular oxidation mechanism is to measure the extent of intracellular oxidation products using a dichlorofluorescein indicator. The oxidation of the nonfluorescent 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) to the highly fluorescent 2'7'-dichlorofluorescein (DCF) is used to detect the generation of (ROS; previously defined).

First, PC12-AC cells were incubated in HBSS solution with 40 μM DCF-DA for 1.5 h to load the cells. Then H2-DA was incubated with the cells for an additional 2 h. If redox cycling is occurring it should oxidize the DCF and generate a fluorescence signal.

An increasing in fluorescence was observed with increasing of H2-DA concentration. The compound undergoes redox cycling inside the cells, and ROS are produced which oxidize the dye.

6. Effect of ascorbate on toxicity correlates with BDE value

As discussed above, ascorbate is protective (decreases cytotoxicity) in the case of H1 and H2, but increases the cytotoxicity of H4. How can be explained the difference? Assume for simplicity that ascorbate acts by H-atom transfer rather than electron transfer. Reaction (5) shows that ascorbate can increase the rate of the redox cycle by one-electron reduction of Q in parallel with the enzymatic reduction; this must increase the cytotoxicity. On the other hand, ascorbate can reduce the semiquinone according to reaction 8.

Comparing reactions (5) and (8) it is clear that the rate of reduction of the closed-shell quinone Q must be much slower than the reduction of the open-shell radical $QH^{\bullet}$. This statement is consistent with known data from Roginsky and coworkers [16,53], where the rate constant for the quinone reduction is slower by several orders of magnitude than that of semiquinone reduction. Therefore I focused on the first step, the conversion between QH_2 (non-toxic) and $QH^{\bullet}$ (toxic). Now consider the comparative gas-phase BDE values, which I use as a guide to reduction potentials in aqueous solution [49]. The BDE for $AscH^- \rightarrow Asc^{\bullet -} + H$ is 68.5 kcal mol^{-1}. Therefore $AscH^-$ can reduce anything whose radical has a BDE above this, e.g. $QH^{\bullet}$ for H1 and H2, with BDE_1 of 73 and 69 kcal mol^{-1}, respectively. This reaction regenerates the catechol and is protective. On the other hand, for H4, whose BDE_1 lies below that of ascorbate, its semiquinone

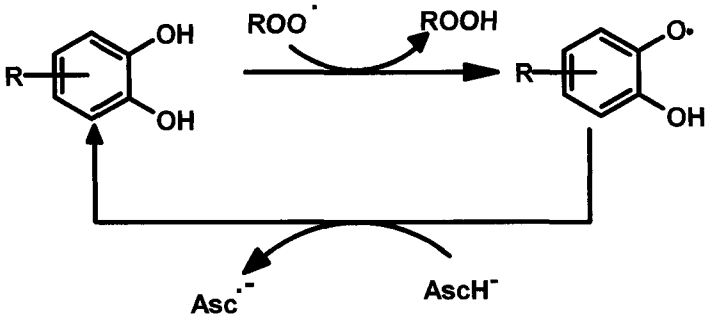
radical is not reduced by ascorbate anion (no protection from ascorbate). But why should a very weak QH_2 bond (< 68.5 kcal mol^{-1}) increase the toxicity of added ascorbate?

One mechanism which provides an answer is that the catechol can then react with ascorbate radical anion according to the *reverse* of reaction 8, i.e.

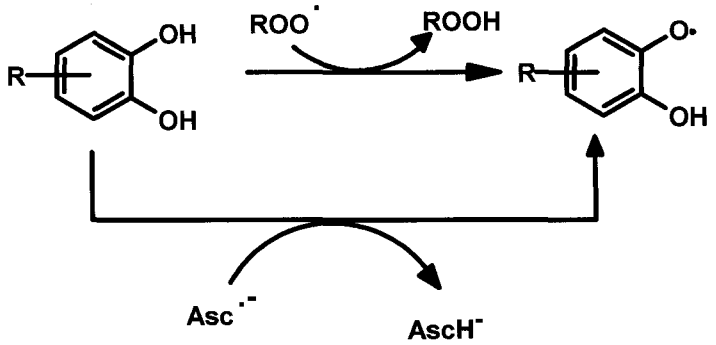
$$QH_2 + Asc^{\bullet -} \rightarrow QH^{\bullet} + AscH^{-} \qquad (12)$$

These two cases are summarized in Scheme 3.2, below. Case 1 is protective, converting semiquinone back to catechol. Case 2 should increase the toxicity, since now the semiquinone is competing with ascorbate radical anion disproportionation (protective, regenerates ascorbate anion) to create more semiquinone (destructive). Thus ascorbate radical anion, which is normally non-toxic, acts as a pro-oxidant through conversion of the catechol into the semiquinone. Creation of a catechol with a BDE_1 even lower than H4 could easily be accomplished, and these low BDE compounds should show strongly increased cytotoxicity in the presence of added ascorbate anion. This binary effect could be beneficial under the right circumstances, such as the targeted delivery of such a compound to a cancer cell [62], combined with ascorbate supplementation.

SCHEME 3.2



Case 1. BDE > 68.5 kcal/mol, ascorbate anion is protective



Case 2. BDE < 68.5 kcal/mol, ascorbate anion is destructive

7. Cytotoxicity correlates with H_2O_2 response curves

As shown in Figure 3.6a, for the pure redox cycling case of H1 the H_2O_2 generation is large and increases almost linearly with concentration of substrate. Ascorbate begins to decrease the production of H_2O_2 at concentrations of H1 greater than ca. 50 μM. This is perfectly mirrored in the cytotoxicity curves for H1 and H1 + ascorbate (Figure 3.2a): Ascorbate becomes protective to H1 at concentrations greater than ca. 50 μM. For H2-DA (Figure 3.6b) there is little effect due to ascorbate and it does not occur until relatively high concentration, so this case is not clear. On the other hand, for H4 at low concentrations, ascorbate increases peroxide production (Figure 3.6c)

and simultaneously increases cytotoxicity (Figure 3.3a). Note also that overall more peroxide is produced for H4-DA than for H4, consistent with its greater cytotoxicity.

In general, for the pure redox cycling of H1 and H2, it appears that the effect of added ascorbate can be determined by examining the amount of H_2O_2 produced. Since it is the redox cycling which is causing toxicity, more H_2O_2 produced means higher cytotoxicity. Conversely, when a diacetate form (H1-DA) leads to reduced peroxide production, this also leads to lower cytotoxicity.

H4 is capable of both redox cycling and thiol depletion. Nevertheless the peroxide response curves give important indicators of cytotoxicity: when peroxide concentrations increase as ascorbate is added, so does cytotoxicity. Thus the more unusual case of ascorbate ion potentiating cytotoxicity can also be predicted from examination of the H_2O_2 response curves.

8. Glutathione response curves

The 24 h GSH response to H1-DA, H2-DA and H4 all show GSH increasing with concentration of substrate (Figure 3.8). Evidently the cell must up-regulate glutathione synthesis in response to catechol treatment on the 24 h time scale of the experiment. In other work [63] it has been shown that this up-regulation does in fact occur in a number of other systems causing oxidative stress which are related to catechols. This phenomenon was discussed in a systematic way by Han et al. [63], who described an increase in GSH in response to catechols, hydroquinones, L-dopa, dopamine and apomorphine. These authors believed that a compound must autoxidize to show this GSH response, e.g. by reaction of semiquinone plus molecular oxygen to produce

superoxide ion. This caused an initial rise in GSSG, which triggered the up-regulation of total glutathione synthesis. Earlier work on quinone-induced oxidative stress showed up-regulation of γ-GCS led to elevated glutathione levels [64]. The same effect was reported later by Ishige et al. [65] and Myhrstad et al. [66] for flavonoids; the latter work explicitly monitored the (rapid) up-regulation of γ-glutamylcysteine synthetase, which preceded the rise in total glutathione levels. Another study using endothelial cells showed that 100 μM hydrogen peroxide was sufficient to cause a doubling in total glutathione levels [67].

The time sequence of measurement of GSH is important, and in our experiments the period of incubation is 24 h. This observation period plays a role because, for example, Dabrosin and Ollinger [68] showed that exposure of hepatocytes to estradiol caused a 25% decrease in GSH at 4 h, whereas the GSH levels at 20 h had nearly doubled.

Considering each compound separately (Figure 3.8), the redox cycler H1-DA shows a steady increase over its range, until the error bars become large near its EC_{50} of ca. 50 μM, as determined using the 10 cm dishes (Note: EC_{50}'s are lower by a factor of two compared to the MTT assay, because the concentration of cells/cm^2 in the GSH assay is much lower than in the MTT assay, which uses microplate wells). The more toxic redox cycler H2-DA shows a very dramatic increase and reaches a value of nearly 4.0 relative to control; the cells are showing an amazingly high up-regulation of GSH in response to the oxidative stress as the EC_{50} is approached.

Compound H4-DA presents an interesting choice between possible thiol depletion and concurrent redox cycling. GSH for H4-DA is evidently still strongly up-regulated (factor of at least three) rather than depleted, in spite of the smaller amount of peroxide generated (Figure 3.7c). Thus based on the assays we chose it is difficult to give a simple

interpretation of the cause of cytotoxicity for these catechols, since the cells are producing H_2O_2 from redox cycling, but attempting to compensate for the increased oxidative stress by the up-regulation of GSH production.

What it was found remarkable is that all three of these compounds, although reacting by different mechanisms, cause an observed 24 h increase in GSH concentration. Clearly the cell is trying to react to a changing redox environment so as to minimize the toxicity.

Literature cited above has shown this to occur in a number of cells and for a number of compounds, and it is speculated that this is a response to oxidative stress. It would be of considerable interest to define the generality and magnitude of this response as a function of cell line and molecular characteristics of the oxidative stressor.

9. Cytoprotection of H2-DA compared with EGCG against oxidative stress induced by menadione or AAPH

In order to test the catechols for their cytoprotection capacity against oxidative stress, using a range of concentrations in which the catechols are non-toxic, I have chosen menadione as a stressor. The cells are first treated with the compounds and then incubated with menadione. Menadione induces cytotoxicity in many cells [69,70]. There are two mechanisms underlying this toxicity: a) menadione produces oxidative stress via redox cycling: menadione is converted to semiquinone radicals by microsomal or mitochondrial enzymes and the radicals generated react with molecular oxygen, thus releasing superoxide anions and subsequent redox cycling and b) the reaction of menadione with soluble thiols (like GSH) or protein thiols results in formation of

quinone-glutathione conjugates or quinone-protein adducts, respectively, resulting in depletion of thiols and a change in the intracellular redox status.

The antioxidant activity and hence cytoprotection is related to BDE_1. A lower BDE_1 means a more active antioxidant and a higher ability to scavenge ROS. In previous chapter it was discussed the antioxidant activity of catechols and EGCG. It was found that the most active in scavenging superoxide radicals is H2-DA. Therefore, I tested H2-DA and also its combination with ascorbate (lower the toxicity of H2-DA) and EGCG for cytoprotective activity against menadione. Based on the BDE_1 (BDE_1 of H2-DA = 69 kcal mol^{-1} < BDE_1 of EGCG = 71 kcal mol^{-1}) it was predicted that H2-DA would be the most protective. For the same concentration tested of 10 μM of the compounds, I obtained a higher protection of H2-DA than EGCG. A synergistic protective effect was seen for a combination of 10 μM H2-DA and 50 μM ascorbate. I suggest that the protective action of catechols against menadione is associated with their superoxide scavenging activity.

Since menadione acts by a wide variety of pharmacological actions that may all contribute to cell death I decided to test the compounds against AAPH. The toxicity of AAPH is due to the peroxyl radical formation.

As shown in Figures 3.17-3.18, the cytoprotection area (CPA) of a compound was defined as an integral of the region where cell viability exceeds that of the cells remaining after AAPH treatment (40% viability) is related both to its protective effect and its toxicity envelope. Basing the protective effect on BDE_1 alone, it was predicted an order of protection which is H2-DA > EGCG. However, the CPA gives the order EGCG (CPA = 17,300) > H2-DA (CPA = 828 ± 120) so BDE_1 is not a useful descriptor of

cytoprotection. Clearly the reason for this is that the cytotoxicity, which provides the upper envelope for the CPA, is far more important in determining the CPA. EGCG is 20 times more protective than H2-DA. The protection of H2-DA, coupled with its high toxicity (its EC_{50} is only 32 μM), suggests that this is a poor compound (low EC_{50}, narrow protective range, low CPA). Based on this, the goal is to design compounds with a low inherent toxicity and a wide protective range.

3.5 CONCLUSION

Based on the above, for any catecholic antioxidant capable of first and second dissociations, a low BDE_1 will favor the antioxidant character, but a low BDE_2 will cause pro-oxidant behavior. This is due to the tendency of the semiquinone to have a low pK_a value and the rapid rate of reaction of its anion $Q^{\bullet -}$ with oxygen to generate superoxide. Thus, making semiquinone formation easy but quinone formation more difficult will be a desirable design. In this way we will lower the toxicity and increase the protection capacity of the antioxidants. In the next chapter it is designed a new class of novel synthetic antioxidants: substituted naphthalenediols. I test them for cytotoxicity and protective effects against oxidative stress in adrenal and primary cortical neuronal cells.

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Chapter 4

Cytotoxicity and cytoprotective activity of naphthalenediols depends on their tendency to form naphthoquinone

4.1 INTRODUCTION

In previous chapters it was discussed the concept of a "design window" for creation of an antioxidant with anti-radical activity superior to vitamin E [1-2]. Molecules which fit into this "window" had bond dissociation enthalpies (BDEs) in the range 69-75 kcal/mol. Using this design window, several catechols were synthesized and tested in organic solvent against the standard free radical $DPPH^{\bullet}$ and also in cell culture using cytochrome C assay. The correlations between calculated BDE values and log k, the second-order rate constant, and between IC_{50} and BDE respectively were excellent [1]. Three catechols and the well-known compound epigallocatechin gallate (EGCG) were then tested in cell culture using PC12-AC cells. The catechols had high cytotoxicity, with EGCG being much less toxic [3]. They also showed a cytoprotective effect against menadione or (2,2'-azobis (2-amidinopropane) hydrochloride (AAPH), but in a very narrow range. Because of their cytotoxicity, the catechols appeared to be a questionable starting point for further antioxidant development. Thus, we started to examine compounds that had a low BDE_1 that will favor the antioxidant character and a high BDE_2 to make more difficult the formation of quinone (redox cycling and Michael addition). Therefore, we designed and tested a new class of synthetic antioxidants, the naphthalenediols which satisfy both criteria.

Naphthoquinones occur naturally in the environment and in biological systems as metabolites of naphthalene, as components involved in mitochondrial respiration, as foodstuffs, as environmental pollutants, and as anti-cancer drugs [4-6]. The cytotoxicity of substituted naphthoquinones was discussed by Miller et al. [7], who examined the series 1,4-NQ, 2-Me-1,4-NQ and 2,3-diMe-1,4-NQ, where Me = methyl and NQ =

naphthoquinone, and found that the order of toxicity was 1,4-NQ ~ 2-Me-1,4-NQ >> 2,3-diMe-1,4-NQ. This order correlated both with the amount of thiol alkylation and its subsequent depletion, and with the amount of redox cycling, so it was not clear which mode caused more toxicity. Later work [8] showed that thiol alkylation via Michael addition is the more important toxic mechanism. Thus, the pure redox cycler 2,3-dimethoxy-1,4-NQ is relatively nontoxic [8] compared to menadione (2-methyl-1,4-NQ), consistent with the fact that the former does not undergo Michael addition whereas the latter does. Other factors considered relevant to cytotoxicity include lipophilicity and the pK_a of the naphthalenediol form [5,9-10].

Naphthoquinones (NQ) can be formed by autoxidation of naphthalenediols (ND, also called naphthohydroquinones). This oxidation was studied with respect to reactivity of the estrogen steroid hormone equilenin [11-12]. Equilenin is metabolized to either 2-hydroxyequilenin or 4-hydroxyequilenin. The latter diol autoxidizes non-enzymatically to the corresponding naphthoquinone. However, the 2-hydroxyequilenin never oxidizes to the corresponding 2,3-naphthoquinone, thus the two naphthoquinones have fundamentally different stability. As early as 1974, Arnold and coworkers [13] and later Scott et al. [14] carried out calculations on ground-state properties of benzo-, naphtho- and anthraquinones. They studied the 1,2-, 1,4-, 1,5- 1,7-, 2,3- and 2,6-NQs and showed that the 1,2- and 1,4-forms were particularly stable due to preservation of a single (fully aromatic) benzene ring adjacent to the quinone ring. The others were much less stable due to the energy penalty associated with loss of aromaticity in the adjacent benzene ring (as with 2,3-naphthoquinone) or when in a completely quinonoid structure (as with 2,6-naphthoquinone) [11].

In addition to being cytotoxic, naphthalenediols and the related catechols can also be cytoprotective, i.e. by acting as antioxidants. Catechols act as an antioxidant by donating its first hydrogen to a radical such as $ROO^{\bullet}$, forming the semiquinone ($QH_2 \rightarrow QH^{\bullet}$) and ROOH, thus breaking the chain reaction of lipid peroxidation [2]. Showing purely antioxidant behavior then requires that cellular reducing equivalents, e.g. ascorbate or glutathione, to reduce the semiquinone back to the diol form. The importance of added antioxidants in reducing toxicity has been shown in many publications, such as in reducing cytotoxicity of catecholic estrogen [15].

If the semiquinone is not reduced, it can act as a pro-oxidant by donating a second hydrogen (or electron, since the semiquinone has a low pK_a), giving $Q^{\bullet -}$, which donates an electron to oxygen to form superoxide and the quinone Q. Toxicity generally arises, as discussed above, from the Q alkylating glutathione or protein thiols via Michael addition or via enzymatic redox cycling between $Q^{\bullet -} \rightarrow Q$, and back [4]. Other mechanisms include extracellular autoxidation [16-17] or some combination of intra/extracellular transport and autoxidation [18].

In this chapter, I tested the hypothesis that naphthalenediols, capable of forming the corresponding naphthoquinone, are relatively cytotoxic, whereas those which cannot are relatively nontoxic. Using PC12-AC cells [19], all compounds were compared for cytotoxicity, including the polyphenolic antioxidant EGCG as a reference compound. Each compound was tested for protective effects against the aqueous-phase peroxyl radical generator AAPH. Toxicity and protective data were combined to create a measure of cytoprotective efficacy for each compound. Theoretical calculations of BDE were utilized to predict the toxic or protective effects of compounds according to

molecular structure. These data are relevant to antioxidant design for biological systems. Finally, I tested the effect of added ascorbate on the naphthalenediols toxicity and the protection of derivatives of 1,8-ND, more water-soluble than 1,8-ND.

4.2 MATERIAL AND METHODS

Materials

2,3-Naphthalenediol and 1,4-naphthalenediol (2,3-ND and 1,4-ND) were obtained from Sigma (St. Louis, MO, USA). Since all commercially available samples of 1,2-ND were found to be of unsatisfactory purity, 1,2-ND was prepared starting from 1,2-naphthoquinone (Sigma) by $NaBH_4$ reduction [20] followed by immediate acetylation (see below) of the crude diol. All syntheses (including the synthesis of 1,2-ND, 1,4-dipropyl-2,3-naphthalenediol (DPND) and its diacetate (DPND-DA), 1,8-ND, 1,8-ND-DA acrylate, 1,8-ND-DA acrylamide and 1,8-ND-diglycerate) were carried by Martin Charron in the Professor Durst group (University of Ottawa). 1,8-ND was prepared by heating 1,8-naphthosultone (Sigma) and KOH together in a stainless steel beaker at 300°C using a Bunsen burner [21] (for an alternative synthesis, see ref. 16). For the synthesis of DPND, commercially available 2,3-naphthalenediol (Sigma) was converted into its diallyl ether by reaction with allyl bromide in the presence of K_2CO_3 in refluxing acetone. This intermediate was dissolved in decalin and heated to 190°C to yield its Claisen rearrangement product. The desired diol DPND was obtained by olefin hydrogenation of the 1,4-diallyl-2,3-naphthalenediol with 30% Pd on activated carbon in a hydrogen atmosphere in methanol solvent. The corresponding diacetates were prepared by reacting the diols with acetic anhydride in the presence of N,N-dimethylaminopyridine

and triethylamine. All structures were verified by ^{1}H and ^{13}C NMR, as well as by mass spectrometry. AAPH, L-ascorbic acid, EGCG, (3-(3,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS), xylenol orange, sorbitol, ferrous sulphate, and sulfuric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide was obtained from Caledon Laboratories Ltd. (Georgetown, ON, CAN). RPMI 1640, trypsin, horse serum, newborn calf serum, and antibiotic-antimyctotic were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

PC12-AC cells, an adherent clone of the rat adrenal pheochromocytoma cell line, were a gift from Dr. Steffany Bennett (Biochemistry, Microbiology and Immunology, University of Ottawa). The cells were thawed and grown in RPMI 1640 medium supplemented with 5% newborn calf serum, 10 % heat-inactivated horse serum, glutamine, bicarbonate, 100 U/mL penicillin G sodium salt, 100 μg/mL streptomycin sulfate and 0.025 mg/mL amphotericin B (Invitrogen). The cells were maintained at 37°C in a humidified atmosphere (5% CO_2 in air) and passed at a density of ca. 5 x 10^5 cells/mL twice a week; doubling time was approximately 24 h. Cell numbers and viability were determined by the trypan blue (0.1% w/v) exclusion method.

Cytotoxicity test using MTT reduction assay

The assay was described in Material and Methods, Chapter 3. Briefly, PC12-AC cells plated at a density of 2 x 10^5 cells/mL (100 μL/well) were incubated for 24 h (37°C,

5% CO_2) to allow adherence and for another 24 h with medium containing test compounds. The incubation was continued for another 2 h with 10 μL MTT stock solution (5 mg/mL in PBS) [22-24]. The absorption values were read at 570 nm with background subtraction at 630 nm in a SpectroMax 340^{PC} microplate reader (Molecular Devices Co., Sunnyvale CA USA). The "Control" is based on the absorbance of cells in medium only, and all treatments are expressed as a percentage of these control values in plots of cell viability.

FOX1 (ferric oxidase 1) Assay

The assay was described in Material and Methods, Chapter 3. Briefly, PC12-AC cells at a concentration of 400,000 cells/mL were incubated for 24 h at 37°C. The medium from the 6 wells was replaced with HBSS and the compound at a final concentration of 100 μM every 15 min for up to 3 h. Afterwards, 10 μL of the supernatant was added to 100 μL of FOX1 reagent [25] (100 mM sorbitol, 125 μM xylenol orange, 250 μM $FeSO_4$, 25 mM H_2SO_4), prepared 30 min in advance. Absorbance at 560 nm was read after 30 min incubation at room temperature. Levels of H_2O_2 in samples were compared to a standard curve of H_2O_2 (0-80 μM).

Protection against oxidative stress

The 96-well microplates prepared in the same way as before (2 x 10^5 cells/mL) were incubated for 24 h prior to use. The used medium was then replaced with fresh medium and the test compounds were added to the wells (at final concentrations of 0-250 μM) and incubated for 2 h before the addition of AAPH (final concentration 12 mM).

After 24 h incubation, cell viability was assayed using the MTT assay. AAPH has been shown to give carbon-centered radicals which rapidly add oxygen to give hydrophilic peroxyl radicals [26-27]. The term "Stress" is used to denote the absorbance (% of Control) in the presence of AAPH (12 mM, 24 h incubation).

Statistics

Data are expressed as mean ± SEM values. In the case of EC_{50} (see Figures 4.2, 4.4, 4.9-4.15) and CPA (see Figures 4.7, 4.8, 4.16-4.18) the same treatment as described in Chapter 3- statistics was applied to calculate the error of the EC_{50} and CPA, respectively. Statistical significance was assessed by using one-way ANOVA. Differences were considered significant at $p < 0.05$.

Calculation of BDE

The method of calculation was presented in the previous chapters. It was used the lowest-level method (LLM) as described by DiLabio et al. [28].

Calculation of log P

The calculation of the octanol-water partition coefficent, Log P, was done using Advanced Chemistry Development Software (ACD Labs, Software Solaris V4.67) available through a structure search on SciFinder Scholar 2004 [29].

4.3 RESULTS

1. Experimental

All of the compounds, with the exception of EGCG, were prepared in their diacetylated form. The purpose of acetylation is to provide protection against extracellular autoxidation, which does not occur in the acetylated form (see also ref. [18]). Figure 4.1 shows structures for the 9 compounds tested.

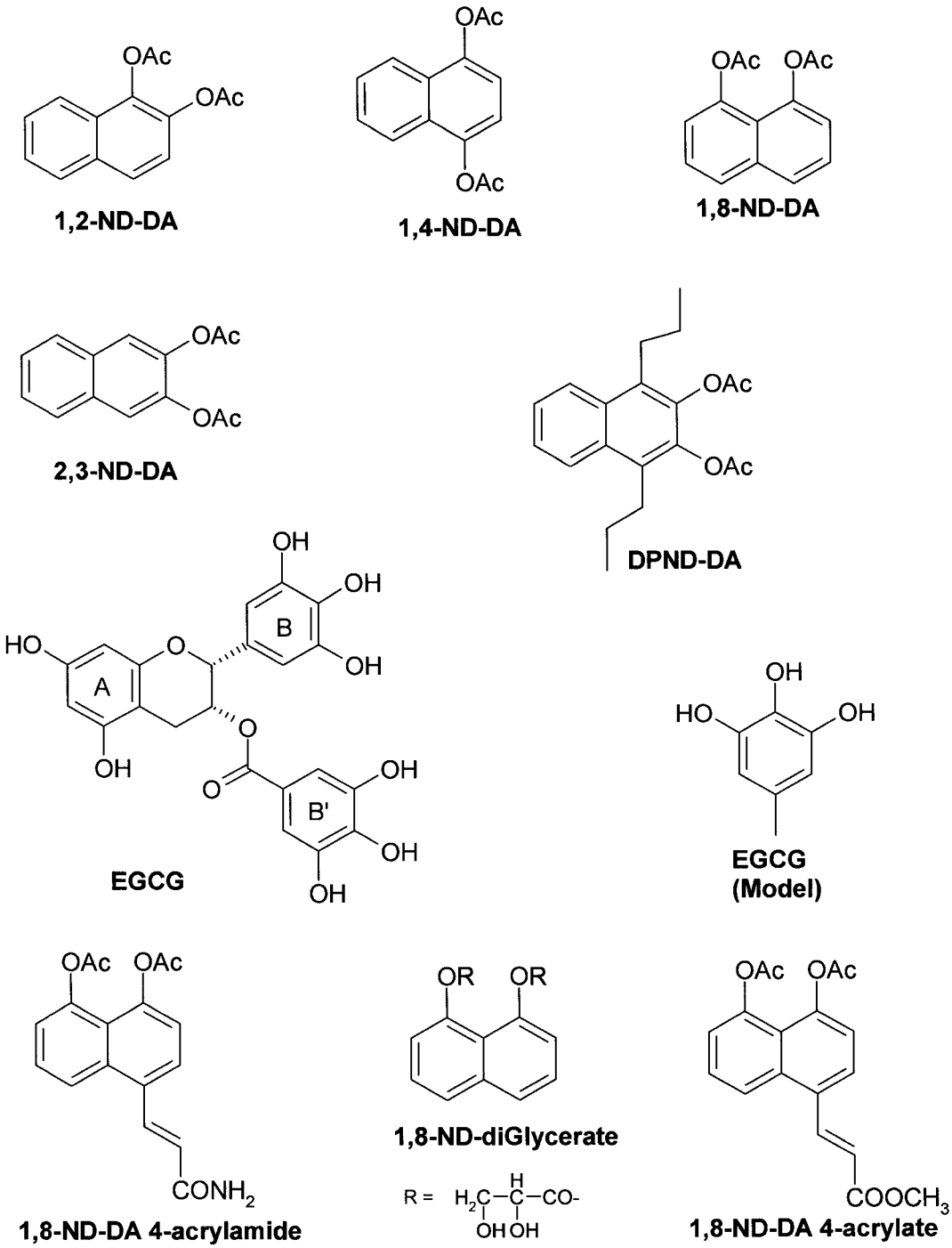


Figure 4.1. Structural formulas for the 9 compounds tested, where 1,2-ND-DA = 1,2-naphthalenediol-diacetate, 1,8-ND-DGly = 1,8-ND-diglycerate and EGCG = (–)-epigallocatechin gallate.

Figure 4.2 shows the cytotoxicity vs. concentration for the 6 compounds, where the dashed line represents the effective concentration (MTT assay) for which the absorbance/cell viability is reduced to 50% of control. There is clearly a wide range of cytotoxicity among the various naphthalenediols, ranging from 1,4-ND (most toxic) to 1,8-ND ≈ 2,3-ND (least toxic). Even less cytotoxic is EGCG, an interesting result since this molecule contains the pyrogallol moiety which is known to autoxidize rapidly [30].

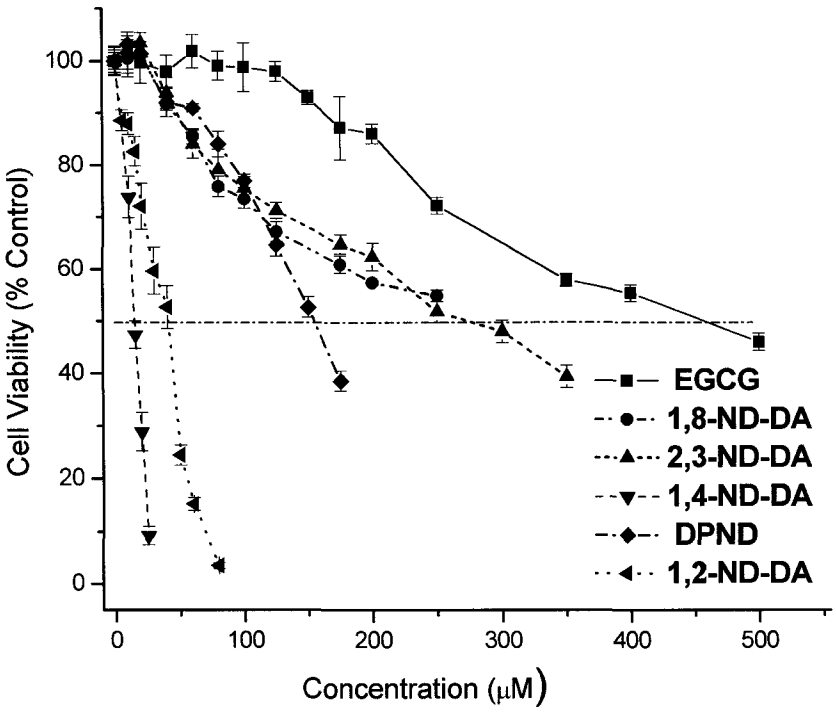


Figure 4.2. Cytotoxicity of the naphthalenediols, initially present as the diacetates, and the reference compound EGCG. Incubation time: 24h.

In order to explain the toxicity of naphthalenediols I measured the amount of H_2O_2 generated. Figure 4.3 shows the amount of H_2O_2 generated as a function of time, for 100 μM concentration of the naphthalenediols. In these experiments, the medium was

removed, then the compound was added to cells in HBSS and incubated over a variable time period, followed by measurement of H_2O_2 in the supernatant. 1,2-ND and 1,4-ND showed a faster rise in time; in the case of 1,4-ND did I see a maximum in H_2O_2 concentration, followed by a rapid decline. Figure 4.3 shows that the amount of H_2O_2 produced in 2 h in the case of 1,8-ND, DPND and 2,3-ND is essentially background (at ca. 1 μM), or even less, and never rises above background even at concentrations of the compounds which are above the EC_{50}.

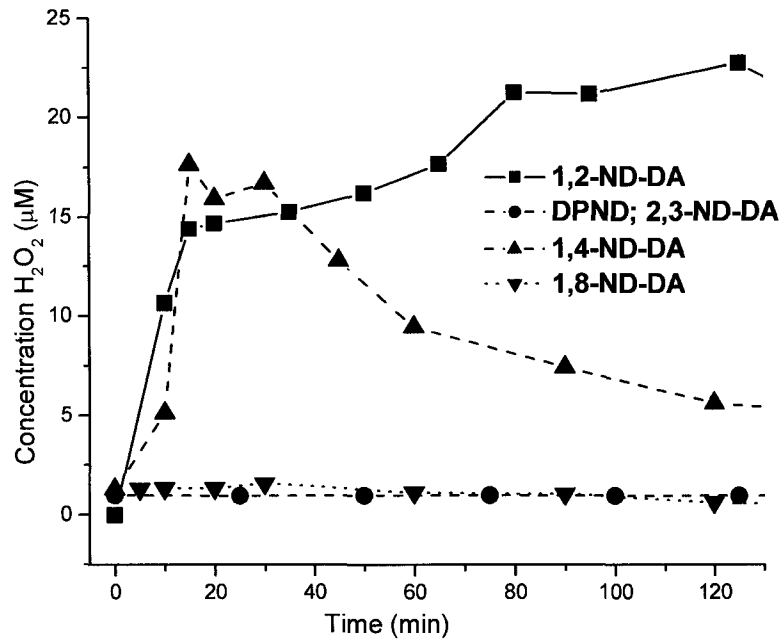


Figure 4.3. H_2O_2 response curves as a function of time (FOX1 assay) for each test compound in its diacetate form.

Figure 4.4 shows the cytotoxicity caused by the oxidative stressor AAPH which generates peroxyl radicals ROO$^{\bullet}$ at a relatively constant rate. Since the MTT assay measures only live (metabolizing) cells, it cannot distinguish between cell death and

inhibition of cell growth. The inhibition process has been demonstrated for resveratrol, catechols [31] and polyphenols [32]. Since the doubling time of 24 h is sufficiently rapid that there can be confusion between these two sources of reduced cell population, I use the term EC_{50} = Effective Concentration which reduces formazan blue absorbance to 50% of control. This is labeled cell viability (expressed as a percentage of Control (no stressor)) in Figure 4.3 and following. For AAPH, the EC_{50} = 10.5 ± 3 mM.

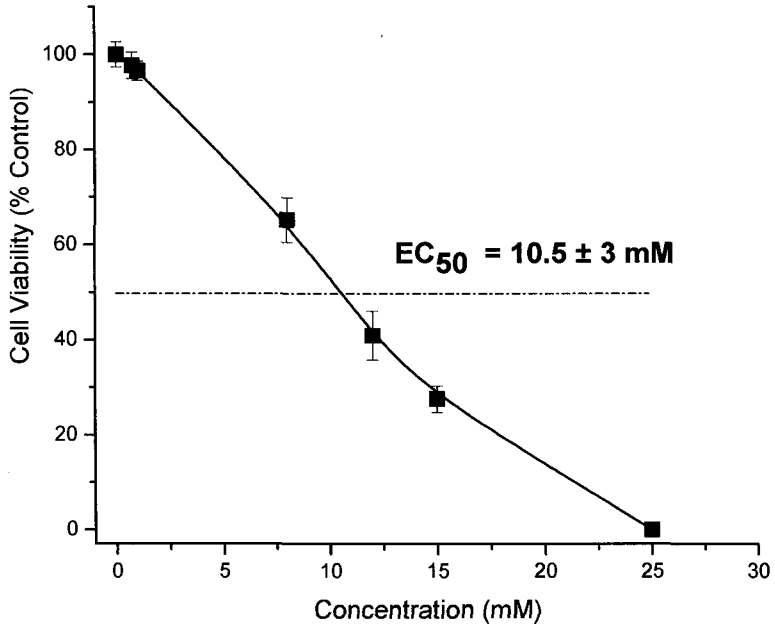


Figure 4.4. Cell viability (MTT assay) for PC12-AC cells vs. concentration of AAPH.

Figure 4.5 shows the cytoprotection of 1,2-ND-DA, 1,4-ND-DA and EGCG against the oxidative stress caused by AAPH, where the AAPH concentration was set at 12 mM. This was sufficient to reduce cell viability to 40 ± 5 % of Control. Since the post-AAPH treated cells had 42% viability, the protective range remaining spans from 42-100% of Controls. The concentration, which inhibits half of the original loss in

viability, is therefore 71%, and an IC_{50} for this particular cell type (PC12-AC), this stressor (AAPH at 12 mM, 24 h), and each compound shown could therefore be defined. However, % viability for compounds can easily cross the IC_{50} line twice (on the way up and on the way down, see Figure 4.6 for 1,8-ND-DA), so the IC_{50} definition was problematic and was not used.

As can be seen in Figure 4.5, the 1,2-ND-DA has only a slight protective effect above "Stress", and then beyond ca. 10 μM its inherent cytotoxicity begins to take its toll on the cells. The 1,4-ND-DA shows a definite protective effect in a range out to 10 μM (no further data were taken beyond this range due to increasing toxicity).

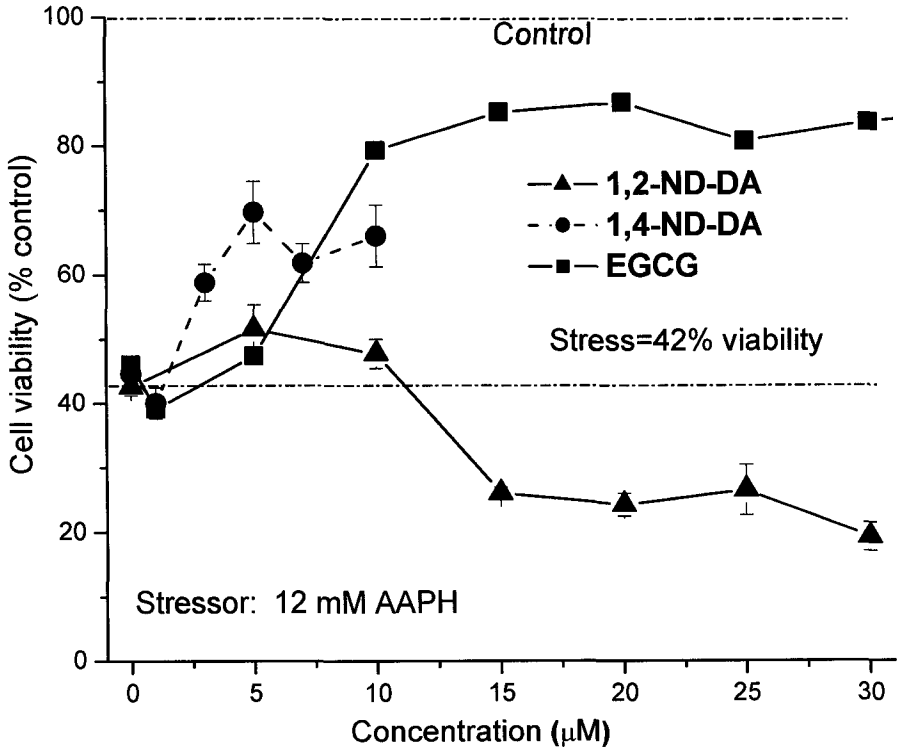


Figure 4.5. Cytoprotection against AAPH-induced oxidative stress for compounds 1,2-ND-DA and 1,4-ND-DA.

Figure 4.6 shows cytoprotection caused by adding 1,8-ND-DA, 2,3-ND-DA and DPND-DA and EGCG to the AAPH-treated cells. The range has now been expanded to 0-60 μM and could have been increased still further since all the compounds continue to be protective in the observed range. 1,8-ND (for simplicity, I omit the DA notation from this point on) was most strongly protective over the range shown, although its maximum was near 20 μM. On the other hand, 2,3-ND showed a more linear dose-response curve. EGCG is more strongly protective and at 50 μM has reached the value of Control (i.e. maximum protection); the slight exceeding of Control is due to experimental error.

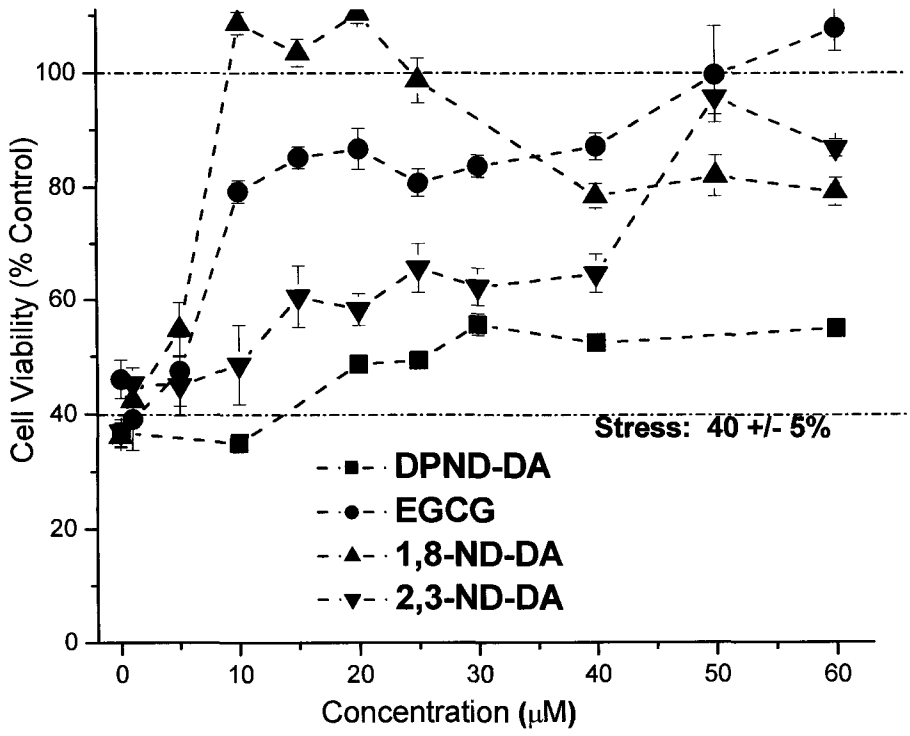


Figure 4.6. Cytoprotection of PC12-AC cells by naphthalenediols with AAPH (12 mM) as a stressor including 2,3-ND, 1,8-ND, DPND (as diacetates), and EGCG.

Figure 4.7 shows the relevant data for EGCG. In the presence of AAPH, but without EGCG, "Stress" was 39% of Control values (12 mM, 24 h incubation). The left-hand side of the Protection Zone is defined by the cytoprotection experiment (data from Figure 4.4 for EGCG, shown as triangles). The cytotoxicity envelope provides the upper curve (filled circles), and since the data were from 0 to 500 μM I extrapolated from 500-580 μM to obtain the final portion (cytotoxicity extrapolation, dotted line). The bottom boundary of the protective zone, or "Stress", is the viability in presence of AAPH (39% in this experiment). Then for EGCG I can characterize the cytoprotection against AAPH-induced stress by (i) its protective range, in this case 0-580 μM, (ii) the maximum peak in protection, in this case 100 ± 5 % viability at 60 μM concentration, or (iii) the "Cytoprotective Area" (CPA), which is the area between the boundaries described above (total cross-hatched area, Figure 4.7).

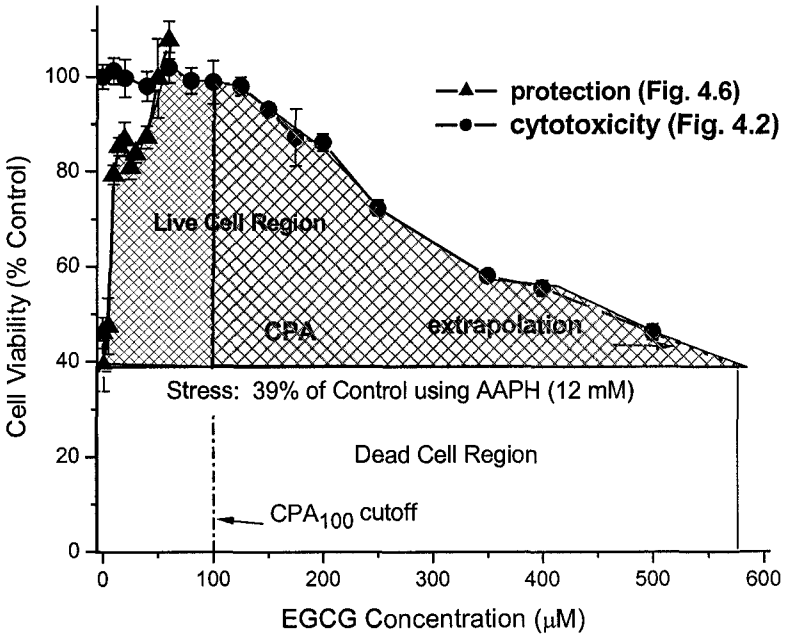


Figure 4.7. Total Cytoprotective area for EGCG (both cross-hatched regions). Boundaries: Cytoprotection experiment (from Figure 4.6) at left (triangles), cytotoxicity envelope (from Figure 4.2) at top (filled circles), extrapolated region 500 - 580 μM (dotted line), viability in presence of AAPH Stress (solid line, 39% Control). CPA_{100}: dark cross-hatched region only.

One assumption I made in constructing Figure 4.7 is that the graph for the cytoprotection experiment (solid triangles), which was only carried out up to 60 μM, will intersect and become coincident with the cytotoxicity data (solid circles). To verify that this was true, I continued the cytoprotection experiment for 2,3-ND out to larger concentrations. Figure 4.8 shows that data for the two experiments do indeed become coincident, to within experimental error, so that an average can be drawn beyond 50 μM (dark solid line) which encloses the cytoprotective area.

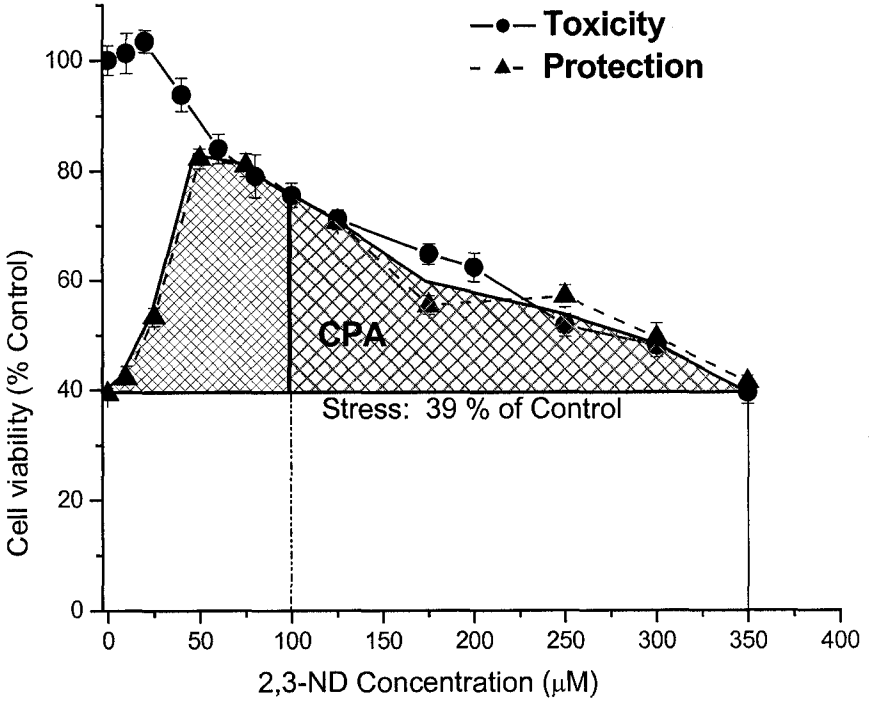


Figure 4.8. Total Cytoprotective area for 2,3-ND (both cross-hatched regions), showing a more extended cytoprotection experiment, with data out to 350 μM. Stressor: AAPH (12mM)

Studies of the effect of ascorbate on cytotoxicity of the NADOLs

Figure 4.9 shows the number of viable PC12-AC cells (from MTT assay) vs concentration for Vitamin C. Ascorbate anion (since solution pH = 7.4) is very non-toxic (EC_{50} > 200 μM). The concentration of 75 μM Vitamin C that is used in the tests with naphthalenediols is non-toxic.

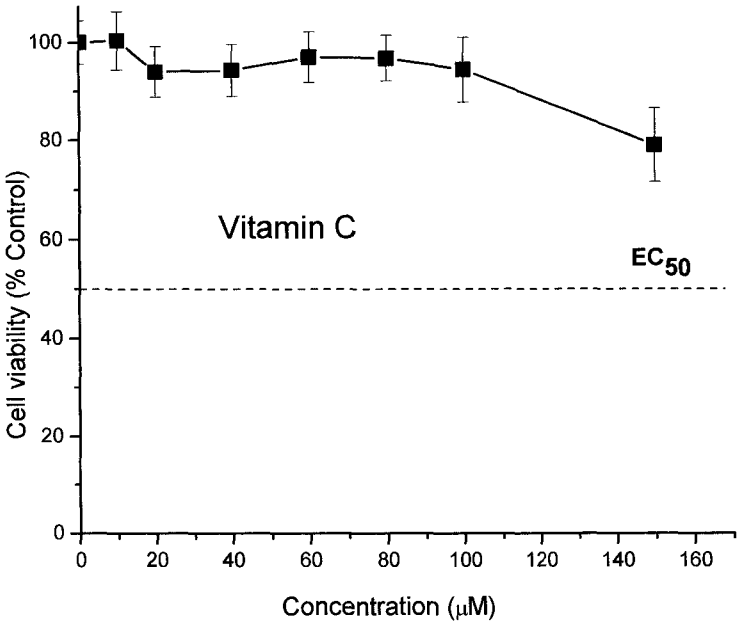


Figure 4.9. Cell viability (MTT assay) for PC12-AC cells vs concentration of Vit. C.

Figures 4.10 - 4.14 show the number of viable PC12-AC cells, as determined using the MTT assay, for some of the compounds in Figure 4.1, as well as the effect of adding ascorbate at 50 μM or 75 μM. The effective concentration which reduces the number of live cells to 50% of control will be denoted EC_{50} in the discussion to follow.

Figure 4.10 shows that 1,8-ND, in the presence and absence of added ascorbate, had essentially the same behavior (EC_{50} value higher than 250 μM). Ascorbate gives a significant protective effect to DPND, with the EC_{50} value increasing to 200 μM in the presence of 50 μM ascorbate (Figure 4.11), and even higher protection (EC_{50} >200 μM) in the presence of 75 μM ascorbate (data not shown). Figure 4.12 shows that the same protective effect of ascorbate is evident for the much less toxic 2,3-ND, where 75 μM ascorbate reduces the toxicity still further.

In contrast, with 1,2-ND (Figure 4.13), ascorbate increases the cytotoxicity significantly (40 → 7.5 μM), making this one of the more cytotoxic combinations of the molecules studied here. 1,4-ND (Figure 4.14) has a somewhat different response, showing little difference with or without added ascorbate (EC_{50} of 15 μM).

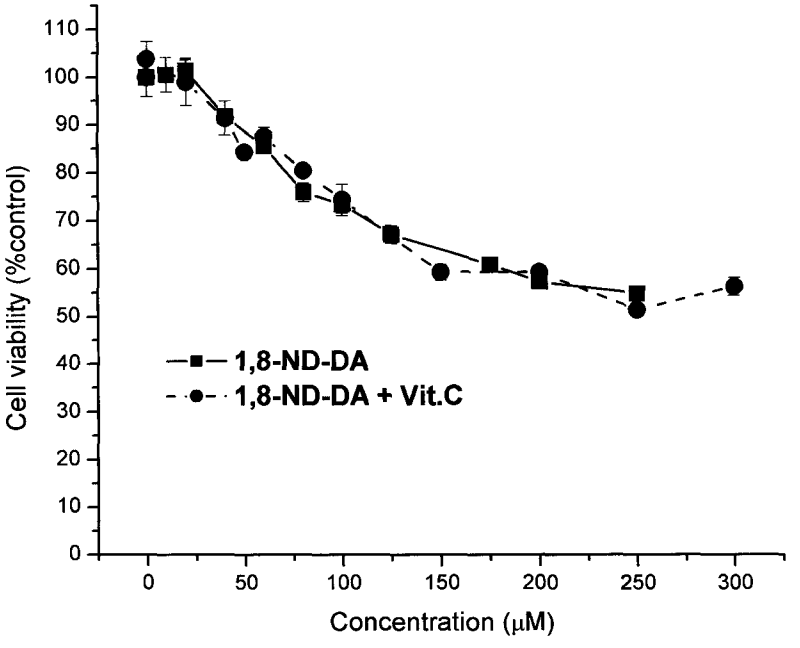


Figure 4.10. Cytotoxicity response curve for 1,8 ND-DA and 1,8 ND-DA with 75 μM ascorbate

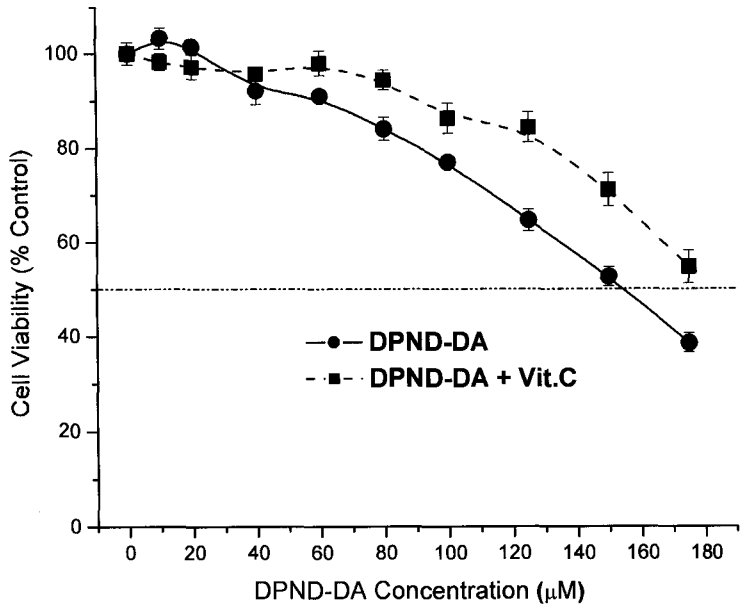


Figure 4.11. Cytotoxicity response curve for DPND-DA and DPND-DA with 50 μM ascorbate.

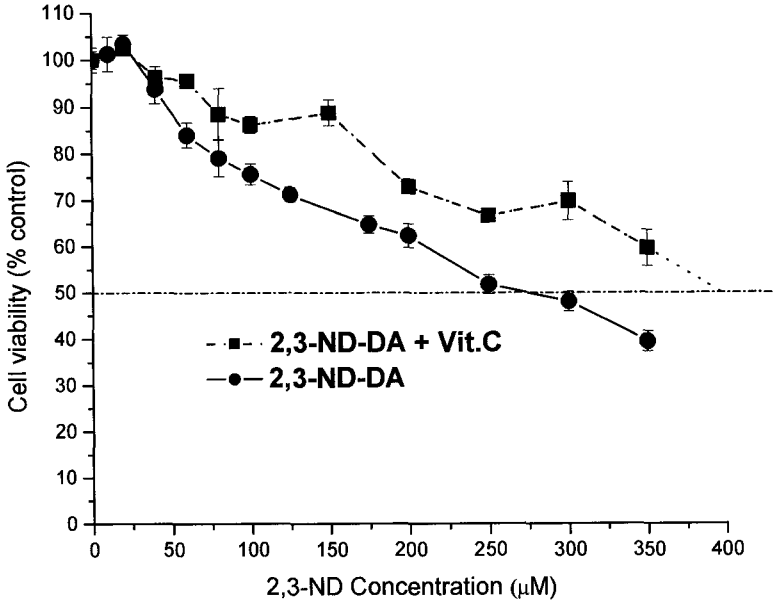


Figure 4.12. Cytotoxicity response curve for 2,3ND-DA and 2,3ND-DA with 75 μM ascorbate.

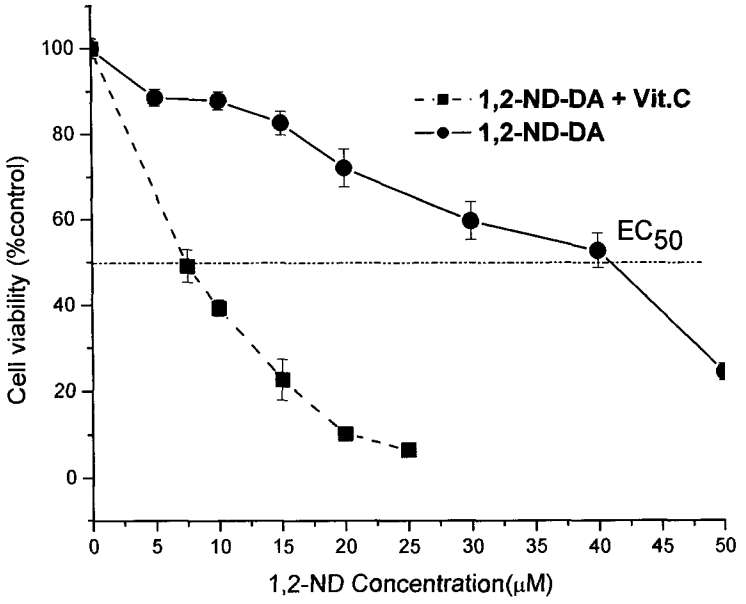


Figure 4.13. Cytotoxicity response curve for 1,2ND-DA and 1,2ND-DA with 50 μM ascorbate.

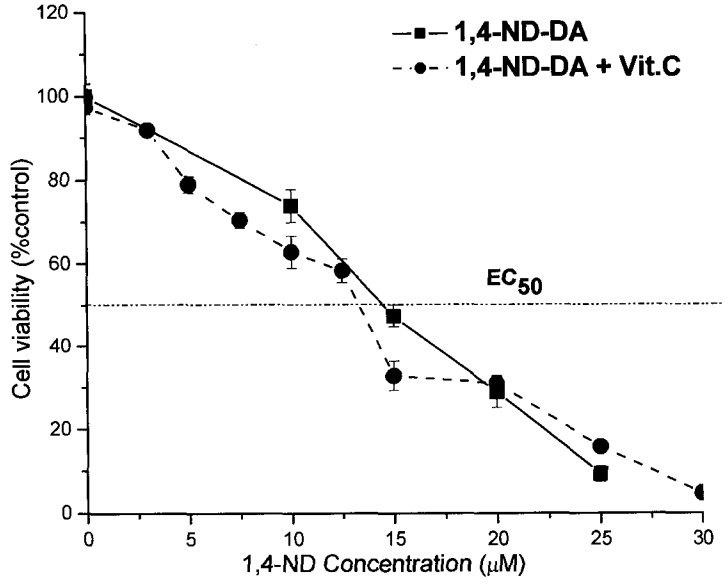


Figure 4.14. Cytotoxicity response curve for 1,4ND-DA and 1,4ND-DA with 50 μM ascorbate.

Toxicity and cytoprotection studies of derivatives of 1,8-ND

1,8-ND-DA showed a very strong protective effect but the compound has a poor solubility in aqueous solutions. We therefore designed and tested three derivatives of 1,8-ND that were more water soluble. 8-ND acrylate and 1,8-ND-DGly have a toxicity similar with 1,8-ND (EC_{50} = 250 μM). Less cytotoxic is 1,8-ND acrylamide with an EC_{50} of 375 μM.

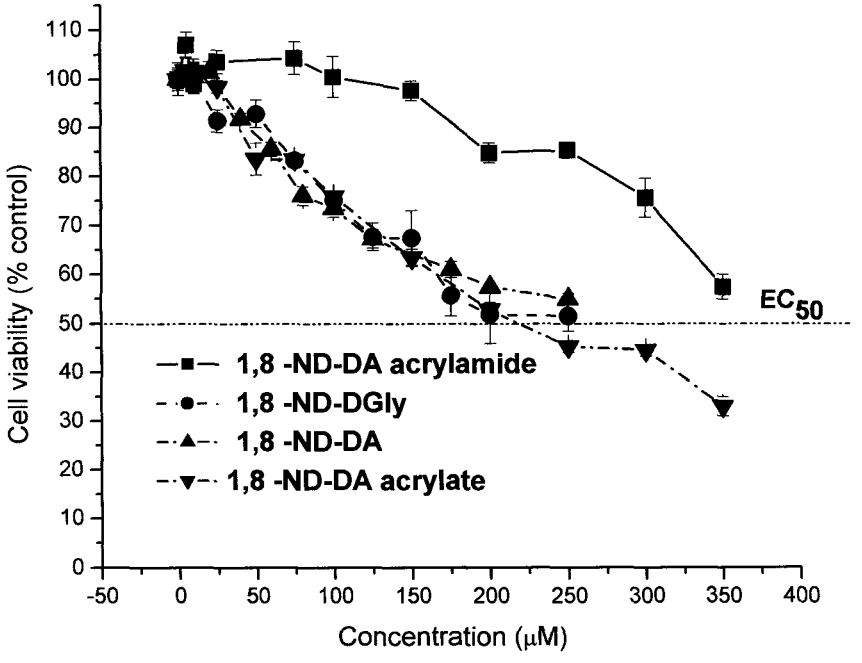


Figure 4.15. Cytotoxicity of the 1,8-ND-DA and substituted 1,8-NDs (1,8-ND-DA acrylamide; 1,8-ND-DA acrylate, 1,8-ND-DGly)

Figures 4.16-4.18 show the relevant cytoprotection data for 1,8-ND derivatives. In the presence of AAPH, but without compound tested, Stress was 40% ± 7 of Control (12 mM, 24 h incubation). For 1,8-ND-DA acrylate and 1,8-ND-DGly the left-hand side

of the Protection Zone is defined by the cytoprotection experiment and the cytotoxicity envelope provides the upper curve. For 1,8-ND-DA acrylamide the cytotoxicity envelope doesn't play any role in defining the cytoprotective area because the protection curve doesn't intersect the cytotoxicity curve (Figure 4.17). The protective range is between 5-300 μM for 1,8-ND-DGly (with maximum protection (75% viability) at 50 μM) and 1,8-ND-DA acrylamide with maximum protection (75% viability) at 60 μM) and 5-260 μM for 1,8-ND-DA acrylate (with maximum protection (80% viability) at 75 μM).

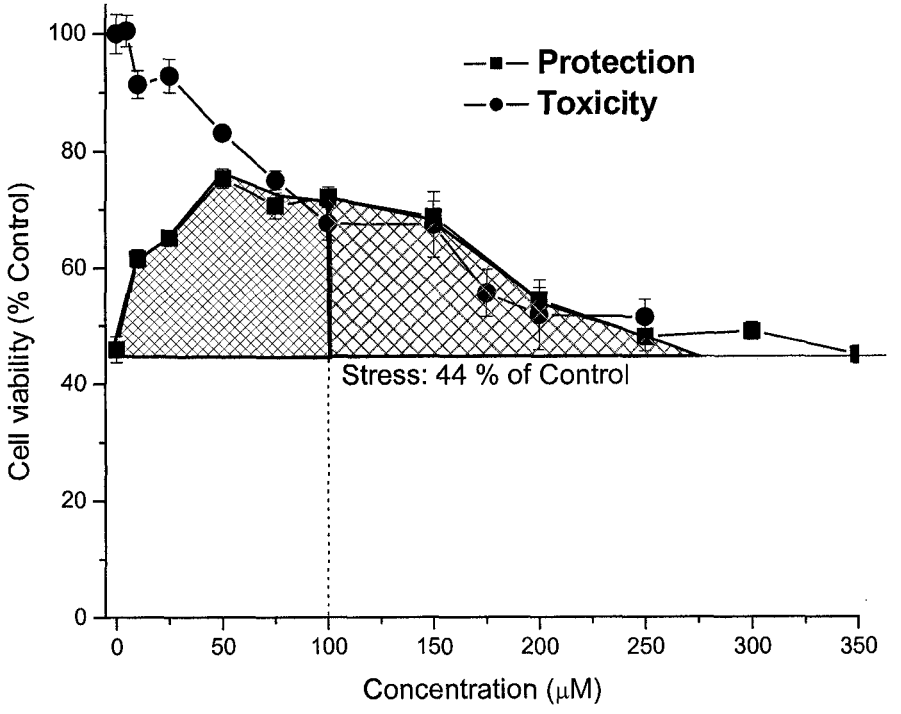


Figure 4.16. Total Cytoprotective area for 1,8-ND-DGly (both cross-hatched regions), showing an extended cytoprotection experiment, with data up to 300 μM. Boundaries: Cytoprotection experiment at left (square), cytotoxicity envelope at top (filled circles), viability in presence of AAPH stress (solid line, 44% relative to Control).

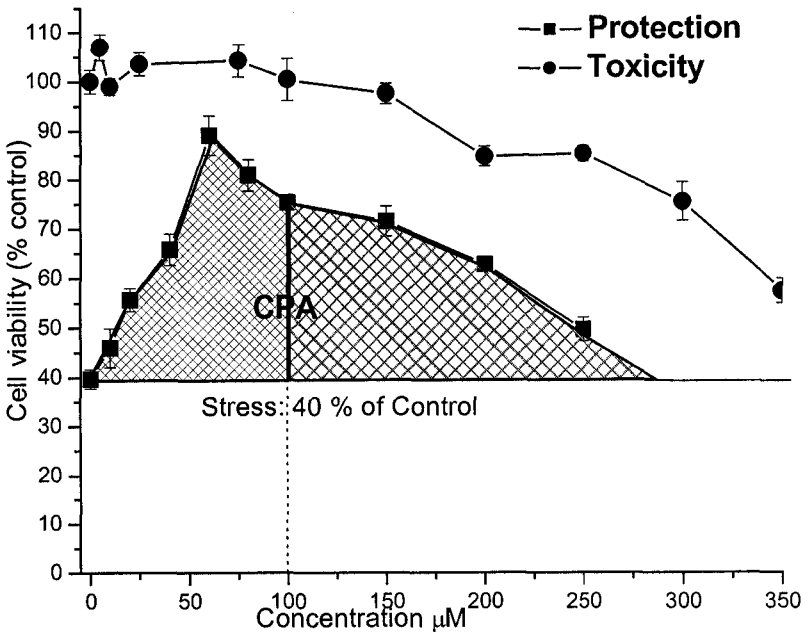


Figure 4.17. Total Cytoprotective area for 1,8-ND-DA-acrylamide (both cross-hatched regions), showing an extended cytoprotection experiment, with data up to 300 μM.

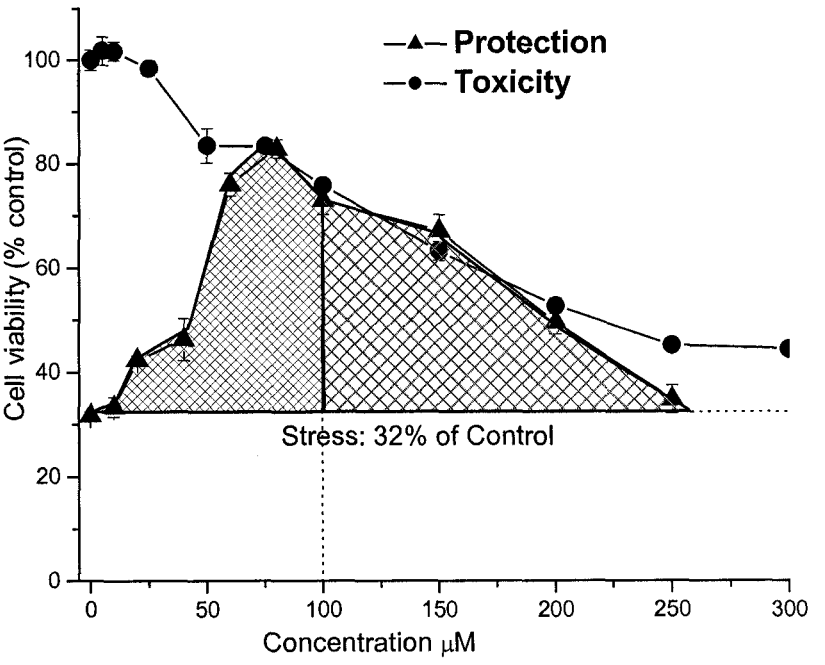


Figure 4.18. Total Cytoprotective area for substituted 1,8-ND-DA acrylate (both cross-hatched regions). Boundaries: Cytoprotection experiment at left (triangles), cytotoxicity envelope at top (filled circles), viability in presence of AAPH stress (solid line, 44% relative to Control)

2. Theoretical

Table 4.1 shows the calculated results for BDE_1 (in generic notation $NQH_2 \rightarrow NQH^{\bullet}$, where NQ = naphthoquinone) and BDE_2 ($NQH^{\bullet} \rightarrow NQ$), the calculated octanol-water partition coefficient LogP, the pK_a of the parent NQH_2, and the cytotoxicity of compounds with and without ascorbate expressed as the EC_{50}.

Table 4.1. Bond dissociation enthalpy (ΔH^{o}_{298}), calculated log P (Log P), pKa , and EC_{50} of naphthalenediols with and without ascorbate, BDE_1 is for naphthalenediol → naphthosemiquinone, BDE_2 is for naphthosemiquinone → naphthoquinone.

Compound	BDE_1 kcal mol^{-1}	BDE_2 kcal mol^{-1}	logP [a]	pK_a (Diol)[a]	EC_{50} (μM)	EC_{50} of the compound tested + ascorbate (μM)
1,4-ND	75	55	1.88	10.3	14 ± 0.6	14 ± 0.5
1,2-ND	70	69	2.11	8.17	40 ± 3	7.5 ± 0.5
DPND[b]	75	86	5.16	9.27	155 ± 4	185 ± 5
2,3-ND	79	84	2.11	9.10	273 ± 20	> 350
1,8-ND	72	104[a]	1.93	6.36	> 250	250
EGCG(M)[c]	71	74	0.75	9.29	----	----
EGCG[d]	71	74	2.04	8.38	456 ± 16	----

[a]Calculated log P, using Advanced Chemistry Development (ACD Labs, Software: Solaris V4.67) in SciFinder Scholar [29].

[b]1,4-dipropyl-2,3-naphthalenediol

[c]EGCG model compound, 4-methyl-1,2,3-trihydroxybenzene

[d] BDE_1 and BDE_2 are based on on calculations for EGCG model compound

4.4 DISCUSSION

Given the data on cytotoxicity (Figure 4.2), the reduction in cell viability due to AAPH (Figure 4.3) and the protection against AAPH-induced cell loss by the various naphthalenediols and EGCG (Figures 4.4-4.5), it is of interest to compare the efficacy of the various compounds on some common basis. "Control" is based on absorbance (MTT assay) of cells plus medium, and "Stress" corresponds to the absorbance of cells plus medium in the presence of AAPH, without compound; this is converted to a percent viability in comparison to Control.

One problem in using these definitions is that the baseline varies in experiments done on different days, due to unavoidable variations in plating density, a parameter which has some effect on the toxic response of the cells. Thus, the cell viability after AAPH incubation for 24 h may range between 40 ± 5% (see Figure 4.5). This makes comparison between experiments on different compounds imperfect. It would be possible to normalize the data by setting the baseline equal to zero in each experiment, but in the interest of simplicity, I have not done that and accept that there is some variation in the "Stress" baseline. This imprecision will not obscure the general validity of the conclusions but could affect the comparison between closely similar compounds, e.g. 2,3-ND and 1,8-ND.

Table 2 shows the result of treating each compound in the same way as 2,3-ND. Clearly the most useful protective index is the Cytoprotective Area (CPA), the integral of the region where cell viability exceeds that of the cells remaining after AAPH treatment. The CPA order is 1,2-ND (least protective) < 1,4-ND << DPND < 2,3-ND ≈ 1,8-ND << EGCG (most protective). This is inversely related to the cytotoxicity (measured by EC_{50},

Table 4.1), which gives 1,4-ND (most toxic) > 1,2-ND > DPND > 2,3-ND ≈ 1,8-ND > EGCG. The only inversion is that 1,4-ND is observed to be more cytotoxic than 1,2-ND but simultaneously offers better protection, although over a very narrow range of concentrations (Fig 2). Similarly, an antioxidant effect has been shown for the related molecule menadiol (2-methyl-1,4-naphthalenediol) [33], which is the reduced form of the known oxidative stressor menadione [2]. Thus, even strongly cytotoxic pro-oxidants can also be cytoprotective antioxidants.

The order of cytotoxicity is also supported by the measurements of hydrogen peroxide. As shown in Figure 4.3, the naphthalenediols were incubated with cells and the H_2O_2 generated was monitored as a function of time (for 2 h). The maximum rate of generation of H_2O_2 was observed with the most toxic compounds which undergo redox cycling: 1,4-ND (EC_{50} = 15 μM), followed by 1,2-ND (EC_{50} = 40 μM). 2,3-ND, DPND and 1,8-ND caused no production of H_2O_2.

Table 4.2. Cytoprotective efficacy of antioxidants against oxidative stress induced by 12 mM AAPH. Incubation time: 24 h, 2 h pretreatment with antioxidant.

Compound	**Range**[a] **(μM)**	**Peak Max. (% Control)**	**CPA (% Control • μM)**[b]	**CPA_{100} (% Control • μM)**[c]
1,2-ND	5-10	53	ca. 100	ca. 100
1,4-ND	3-17	70 @ 5 μM	285 ± 20	285 ± 20
DPND	15-180	56 @ 30 μM	2770 ± 90	1440 ± 60
2,3-ND	1-360	96 @ 50 μM	7225 ± 293	2895 ± 20
1,8-ND	1-350	100 @ 20 μM	9030 ± 200	4600 ± 72
EGCG	5-575	100 @ 60 μM	17,300 ± 1240	4967 ± 159

[a] Total protective range; only the region above "Stress" is counted (see Figure 4.6).
[b] CPA = Cytoprotective Area (total)
[c] CPA_{100} = Cytoprotective Area with cutoff at 100 μM concentration of compound

In general, it is easy to see from Figure 6 that a very cytotoxic compound cannot be very cytoprotective, hence the approximate inverse order of cytotoxicity (EC_{50}) and CPA. This is true regardless of how cytoprotective a compound is at low concentration (triangles, Figure 4.6), the CPA is mostly determined by the cytotoxicity "envelope" (circles, Figure 6). Hence, 1,8-ND and 2,3-ND have too steep a downward slope in their cytotoxicity envelope to be able to compete with EGCG, which has the best CPA by a factor of two. However, this suggests what to consider when cytoprotection is desired and some cytotoxicity can be tolerated. It is more useful (larger increase in CPA) to lower the toxicity of 1,8-ND by appropriate functional group substitutions than it is to try to increase antioxidant activity.

Another possibility for comparing efficacy of compounds is that one may wish to limit the maximum value of concentration to be administered, e.g. 100 μM, 250 μM, etc. In that case, the CPA can be modified so as to introduce a cutoff on the concentration axis. Table 4.2 shows the recalculated area where the CPA is limited to the range 0-100 μM, denoted CPA_{100}. The order stays the same with one exception; 1,8-ND and EGCG are now essentially equivalent, whereas based on the total CPA EGCG was superior by almost a factor of two.

Reaction mechanism: BDE and Redox Potential

A discussion of the parameters which are important in determining the relevant reaction mechanisms would normally begin with the redox potential. In this chapter, I have not measured redox potentials for reduction of the naphthoquinones and the naphthosemiquinones. However, we and others have shown that there is a strong

correlation between gas-phase BDEs (which are easy to calculate) and solution-phase redox potentials, so that weaker O-H bonds correspond to more negative redox potentials. An earlier paper on O-H BDEs showed the BDE (gas) and E^{o} (water) are related by 24 kcal mol^{-1} V^{-1}, with a correlation coefficient of 0.997 [27]. Thus, BDE_2 for 1,4-ND and 2,3-ND (Table 1) differ by 29 kcal mol^{-1}, i.e. by more than a full volt. Next, let us examine some calculated parameters which may explain the observed order, both of cytotoxicity and cytoprotective area.

Figure 4.19 shows the structural changes and BDEs involved in going from diol to quinone. The relevant effects include the presence of hydrogen bonding in parent or semiquinone, interaction of adjacent carbonyl groups, and aromaticity or the lack thereof in the adjacent benzene ring. For catechols, the H-bonding between adjacent OH groups amounts to ca. 4 kcal mol^{-1} [34] whereas for its semiquinone the H-bond is much stronger at 8 kcal mol^{-1}; the effects are similar in the naphthalenediols. We estimate the repulsion due to adjacent carbonyl groups to be 5 kcal mol^{-1} [35]. Now 1,2-ND has a very low BDE_1 = 70 kcal mol^{-1} due to H-bonding in the semiquinone, suggesting an active antioxidant. The corresponding 1,2-NQ has adjacent carbonyl repulsions (destabilizing) but maintains aromaticity in the second ring (stabilizing); the result is a relatively stable quinone leading to a low BDE_2 (69 kcal mol^{-1}), which is similar to BDE_1. The 1,4-ND is missing the H-bond stabilization in the semiquinone and, as a consequence, the BDE_1 rises to 75 kcal mol^{-1}. However, its quinone maintains aromaticity in one ring while losing the adjacent carbonyl repulsions; both effects are stabilizing. As a result, the 1,4-NQ is very stable and BDE_2 has the remarkably low value of 55 kcal mol^{-1}.

The semiquinone of 2,3-ND is stabilized by H-bonding, whereas, in the quinone, the aromaticity in the second ring is lost (no benzene resonance structure can be drawn [10]). The quinone also contains adjacent carbonyl repulsions. The result is a relatively unstable quinone with a high BDE_2 of 84 kcal mol^{-1}. This effect is exaggerated in the dipropyl version DPND, where the electron-donating alkyl groups lower BDE_1 to 75 kcal mol^{-1} but increase BDE_2 to 86 kcal mol^{-1}. Thus DPND should be more cytoprotective and less cytotoxic than 2,3-ND, although the more lipophilic character of DPND (see Table 4.1) could easily outweigh these small BDE differences.

For 1,8-ND, very strong H-bonding in the semiquinone stabilizes it and leads to a low BDE_1 of 72 kcal mol^{-1}. However, in this case no quinone is possible and the lowest energy form is the triplet diradical. Loss of a closed-shell structure requires a very large energy penalty to form the diradical; the result is that BDE_2 is 104 kcal mol^{-1}, so that this high-energy structure will not form under biological conditions (see also ref. 18).

For EGCG, the weakest OH bond is in the B-ring at the center position among the three hydroxyl groups. The *para*-methylpyrogallol subunit provides a close model for BDE calculations, giving the low BDE_1 = 71 kcal mol^{-1}, due to strong H-bonding by the two *ortho*-OH groups which stabilize the semiquinone. The quinone is slightly destabilized by the adjacent carbonyl groups, causing an increase in BDE_2 to 74 kcal mol^{-1}. At this point oxidation usually stops. Figure 7 illustrates this in that the BDE for loss of the third H-atom has increased to 85 kcal mol^{-1}. Since the 2,3-ND has a BDE of 84 kcal mol^{-1}, and is not observed to form, we expect that the quinone form of EGCG (labeled EGCG Quinone in Figure 4.7) will not oxidize to its corresponding radical.

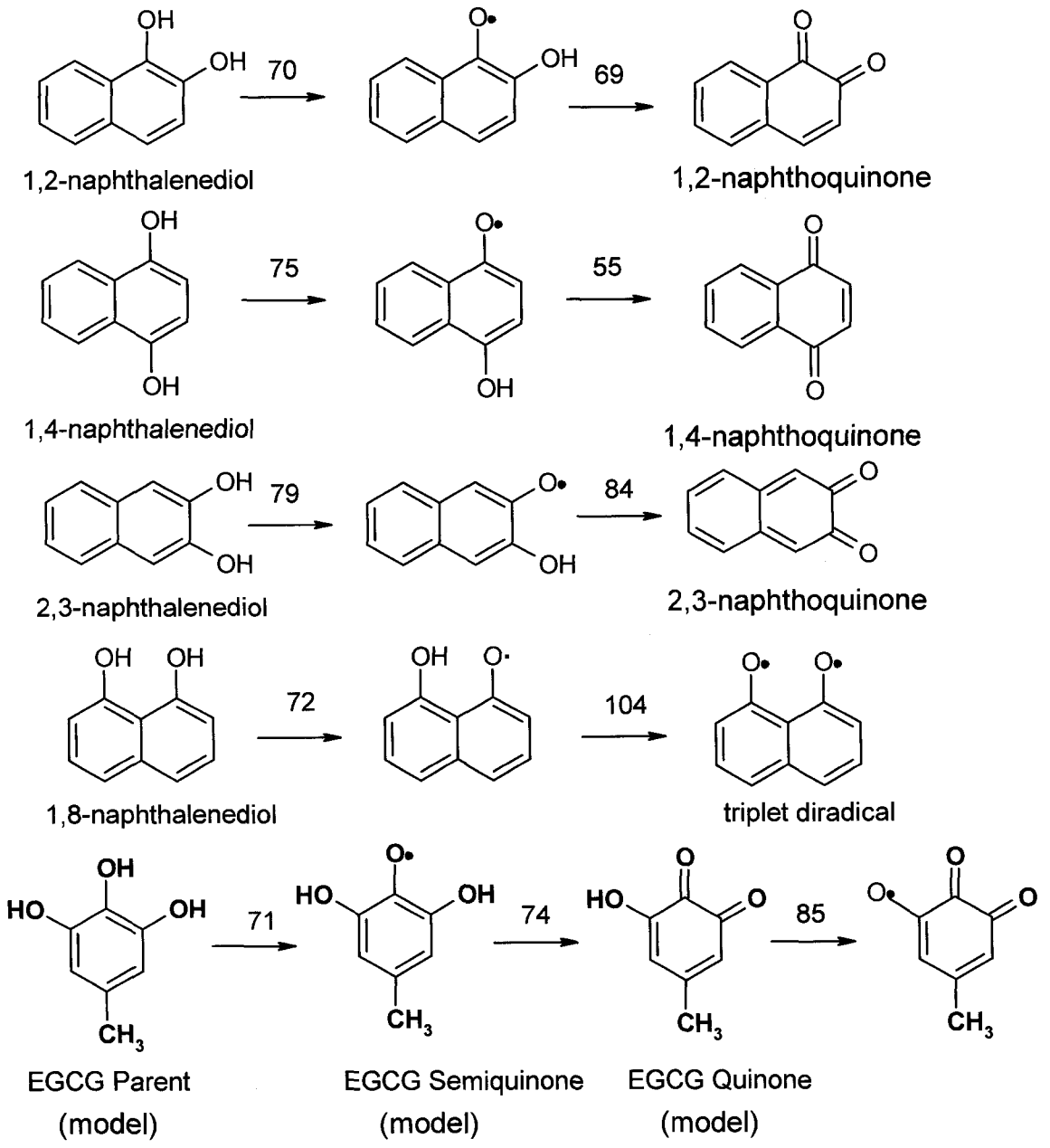


Figure 4.19. Energetics and structural changes for the naphthalenediol → naphthosemiquinone → naphthoquinone. All BDEs are in kcal mol^{-1}.

As discussed above, the antioxidant activity and hence cytoprotection is related to BDE_1, whereas a lower BDE_1 gives a more active antioxidant [1]. However, BDE_2 is related to cytotoxicity since it relates to ease of quinone formation, following which both thiol alkylation or redox cycling can occur. Based on BDE_2, I predicted the following order for cytotoxicity: 1,4-ND > 1,2-ND > EGCG > 2,3-ND > DPND > 1,8-ND. This ordering is correct with one major exception (EGCG) and one minor exception (DPND is more cytotoxic than 2,3-ND). Also, 1,8-ND would be expected to be even less cytotoxic than observed. The discrepancy regarding 2,3-ND vs. DPND probably is related to the fact that DPND is far more lipophilic (Table 4.1), and more lipophilic molecules tend to be more cytotoxic [5].

The result for EGCG is interesting in that Figure 2 shows that it is clearly the least cytotoxic of all molecules tested. It appears that there is a general lack of toxicity in a 1,2,3-trihydroxybenzene moiety which has been oxidized to the 3-hydroxy-1,2-orthoquinone. The reason is that the remaining hydrogen is relatively acidic, with an estimated pK_a of 6.24 [29], so that the anionic form will predominate at pH 7.4. The situation is similar to that in 2-hydroxy-1,4-naphthoquinone, which has been discussed previously [36-37]. This latter compound has been shown not to be a redox cycler *in vitro*, as well as being a poor electrophile. Here the hydroxynaphthoquinone has a very low pK_a of 4.03, so that the anionic form will be present and will retard both Michael addition (ring is no longer electrophilic) and redox cycling (difficult for a reductase enzyme to reduce an already negative ion) [38]. The same arguments apply to EGCG and its model compound. However, toxicity results can sometimes be quite different *in*

vivo, where other reducing enzymes may be present [39] and which can initiate redox cycling.

As shown in Figure 4.7, the cytoprotection area (CPA) is related both to its protective effect and its toxicity envelope. Basing the protective effect on BDE_1 alone, I predict an order of protection which is 1,2-ND > EGCG > 1,8-ND > 1,4-ND = DPND > 2,3-ND. However, the CPA gives the order EGCG > 1,8-ND > 2,3-ND > DPND > 1,4-ND > 1,2-ND so BDE_1 is not a useful descriptor of cytoprotection. Clearly the reason for this is that the cytotoxicity, which provides the upper envelope for the CPA, is far more important in determining the CPA.

Another factor which can be relevant is the pK_a of the parent naphthalenediol. Table 1 shows that 5 out of 6 compounds have pK_a values between 8.2-10.3, so that the parent (protonated) diol is dominant over its anion at physiological pH. However, 1,8-ND has a low pK_a calculated to be 6.36 [29]. This can increase toxicity if the reduction of oxygen is spontaneous according to $NQH^- + O_2 \rightarrow NQH^\bullet + O_2^{\bullet-}$, i.e. the anion becomes a superoxide generator [40]. Finally, even though 1,8-ND and 2,3-ND have only one exchangeable hydrogen atom (or one electron), they still act as a more lipophilic phenol, and phenols have their own inherent cytotoxicity [41].

Effect of added ascorbate on toxicity correlates with BDE value

As discussed in Results section and can be seen in Table 3, ascorbate is protective (decreases cytotoxicity) in the case of 2,3-ND and DPND, but increases the cytotoxicity of 1,2-ND or doesn't have any effect on the cytotoxicity of 1,4-ND and 1,8-ND. How can I explain the difference? It would be expected that by adding ascorbate, the toxicity

of naphthalenediols decreases in a cellular environment due to reduction of semiquinone with the regeneration of the catechol and formation of the ascorbate radical anion Asc•– (reaction 1).

$$QH^{\bullet} + AscH^{-} \rightarrow QH_2 + Asc^{\bullet -} \quad (1)$$

In this way, the quinone formation is prevented and this reaction is protective. The ascorbate radical anion is dismutated spontaneous or enzymatically into $AscH^-$ + DHA [42]. The BDE for $AscH^- \rightarrow Asc^{\bullet -} + H$ is 68.5 kcal mol^{-1}. Therefore $AscH^-$ can reduce anything whose radical has a BDE above this with a reaction rate that is depending on how high the BDE is. In the case of all naphthalenediols, the BDE_1 is higher than 68.5 kcal mol^{-1}, thus ascorbate should have a protective effect by decreasing their toxicity. However, the effect of ascorbate is also strongly correlated with BDE_2.

Ascorbate anion, $AscH^-$, can play a parallel role by reducing the quinone according to the electron-transfer reaction [43].

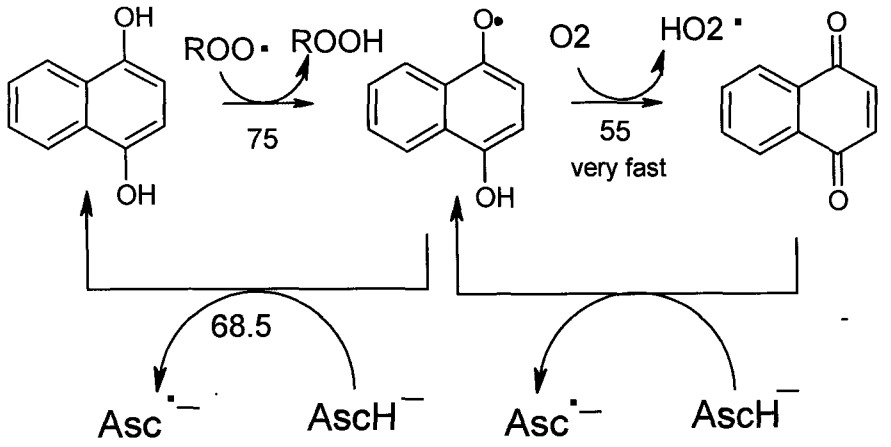
$$Q + AscH^{-} \rightarrow Q^{\bullet -} + AscH^{\bullet} \quad (2)$$

Alternatively, ascorbate can act by H-atom transfer to give

$$Q + AscH^{-} \rightarrow QH^{\bullet} + Asc^{\bullet -} \quad (3)$$

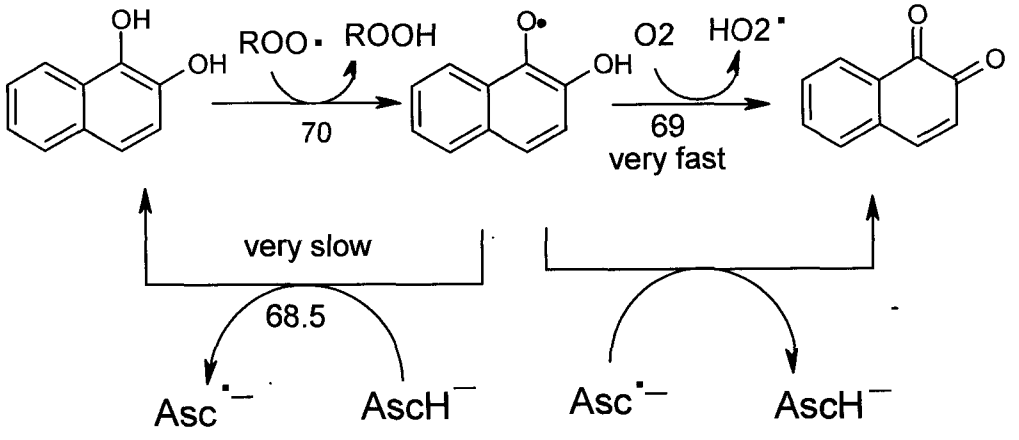
Scheme 4.1 shows the case of 1,4-ND where the QH_2 is regenerated quickly ($BDE_1 >>$ 68.5) but at the same, time the reaction of forming quinone is very fast (BDE_2 is very low; only 55 kcal mol^{-1}). Ascorbate can also reduce the quinone to semiquinone. Overall these two effects are annulled and the ascorbate does not change the cytotoxicity of 1,4-ND ($EC_{50} = 14 \pm 0.6$ μM).

SCHEME 4.1.



Ascorbate lowers the toxicity of 1,2-ND. Scheme 4.2 shows the regeneration of semiquinone by ascorbate; a very slow process. However, the formation of quinone is very fast due to a low BDE_2 leading to a higher toxicity of 1,2-ND when ascorbate is added. Thus ascorbate radical anion, which is normally non-toxic, acts as a pro-oxidant through conversion of the semiquinone into the quinone.

SCHEME 4.2.



In the case of 1,8-ND, 2,3–ND and DPND, the effect of ascorbate on the toxicity depend only of BDE_1 because these naphthalenediols don't form quinones and reaction 2

does not take place. Thus, ascorbate had a protective effect in the case of 2,3-ND and DPND (BDE_1 >> ascorbate BDE of 68.5 kcal mol^{-1}), respectively. $AscH^-$ reduces the semiquinone very fast and the hydroquinone is regenerated by ascorbate. Therefore, the cytotoxicity is decreased. The BDE_1 of 1,8-ND is 70 kcal mol^{-1} (~ 68.5 kcal mol^{-1} with ascorbate), therefore the regeneration of hydroquinone by ascorbate is very slow and will not result in any change in toxicity of 1,8-ND with or without ascorbate.

Cytotoxicity and cytoprotection of substituted 1,8 - naphthalenediol

1,8-ND-DA showed good activity in scavenging peroxyl radicals, while having toxicity comparable to that of naturally occurring polyphenols. This moved us to design and test new substituted 1,8-NDs with a water-solubility higher than 1,8-ND.

The cytotoxicity of the 1,8-ND analogues is the same as 1,8-ND-DA (EC_{50} > 200 μM) with the exception of 1,8-ND acrylamide with an EC_{50} > 350 μM. Table 4.3 shows the result of treating each 1,8-ND analogues in the same way as 1,8-ND-DA. The CPA order for the 1,8-ND derivatives is 1,8-ND DGly (least protective) < 1,8-ND acrylate < 1,8-ND acrylamide < 1,8-ND (most protective). The same order is also obtained for CPA_{100}.

Table 4.3. EC_{50} and cytoprotective efficacy of substituted 1,8-NDs against oxidative stress induced by 12 mM AAPH. Incubation time: 24 h, 2 h pretreatment with antioxidant.

Compound	EC_{50} (μM)	Log P[a]	Range[b] (μM)	Peak Max. (% Control)	CPA (% Control • μM)[c]	CPA_{100} (% Control • μM)[d]
1,8-ND	250	2.2	10-350	100 @ 20 μM	9030 ± 200	4600 ± 20
1,8-ND-DA acrylate	220	2.54	10-300	83 @ 80	6643 ± 460	2790 ± 140
1,8-ND-DA acrylamide	>350	1.62	10-300	89 @ 60	7119 ± 146	2989 ± 113
1,8-ND DGly	240	0.08	10-350	75 @ 50	4700 ± 85	2255 ± 47

[a]Log P was calculated using the Spartan '02 program.
[b]Total protective range; only the region above "Stress" is counted (see Figure 4.14).
[c]CPA = Cytoprotective Area (total)
[d]CPA_{100} = Cytoprotective Area with cutoff at 100 μM concentration of compound

It is very important in evaluating the efficacy of an antioxidant in cells to know the ability of the antioxidant to penetrate into the cells. The more lipophilic a compound, the better it can penetrate lipid membrane. A very interesting case is 1,8-ND-DA acrylamide that showed a very low toxicity (comparable to that of EGCG (>350 μM)) but an unexpected cytoprotection. As can be seen in Figure 4.17 the toxicity envelope does not define the CPA of 1,8-ND-DA acrylamide. There are two mechanisms in order to explain this trend. First, is that the 1,8-ND-DA acrylamide is too water-soluble and does not cross a hydrophobic membrane readily. The calculated Log P for 1,8-ND-DA and 1,8-ND-DA-acrylamide (Table 4.3) are 2.25 and 1.67, respectively. A very significant change in solubility is due to the added *acrylamide* group and 1,8-ND acrylamide is therefore much more water-soluble than 1,8-ND. Thus, its actual concentration inside the cell is lower than expected and explains the lower toxicity as well as the lower protection compared to 1,8-ND. The second mechanism could be explained based on the difference in pK_a of the naphthalenediols. The compounds pass through the PC12-AC cell membrane and are hydrolyzed by intracellular esterase forming the corresponding diols. In the case of 1,8-naphthalenediol, the pK_a is very low (6.3) compared with pK_a of other 1,8-ND derivatives and so its anion form is dominant over its parent (protonated) diol at physiological pH. Therefore, 1,8-naphthalenediol is leaking outside the cell very slowly and in this way it mainly acts inside the cell protecting the cells against intracellular oxidative stress induced by peroxyl radicals.

4.5 CONCLUSION

The results described here suggest some considerations in antioxidant design for compounds with two exchangeable hydrogens. To maximize the cytoprotective area, it is useful to (i) keep BDE_1 relatively low to increase cytoprotection, (ii) keep BDE_2 high to minimize cytotoxicity, (iii) avoid high lipophilicity which may increase toxicity (unless a lipophilic antioxidant is the target), (iv) avoid low pK_a's for the parent compound which can lead to superoxide production, and (v) try to use acidity as an advantage (as in the hydroxyquinone of EGCG). I have made some progress in carrying out such a synthetic program using a variety of substituted naphthalenediols.

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Chapter 5

Cytotoxicity and cytoprotective activity of naphthalenediols in rat cortical neurons

5.1 INTRODUCTION

Current understanding of the causes of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) indicates that there is probably 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 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.

By contrast, little work has been done on the systematic design of antioxidants targeted towards 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 previous chapter using adherent PC-12 adrenal cells, I 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 [11]. 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, symbolized NQH_2), the partially oxidized naphthosemiquinone radical ($NQH^{\bullet}$) and the fully oxidized naphthoquinone (NQ) [11,12]. 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}) formation of the quinone product is observed. Quinone formation is generally accompanied by toxicity in the form of redox cycling and subsequent hydrogen peroxide production, or attack on the electrophilic quinone by cellular nucleophiles such as GSH or cysteine-containing proteins [13-15]. This situation holds for 1,2-ND and 1,4-ND; both redox cycling and thiol depletion mechanisms can contribute to the observed cytotoxicity. On the other hand, loss of aromaticity (in the unsubstituted ring) on quinone formation for 2,3-ND exerts a heavy energy penalty preventing their 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 paper I extend the study of naphthalenediols to primary rat cortical neurons, to see if the conclusions derived from PC-12 cells are more general and to see whether animal trials should be pursued. For comparison, I include the antioxidant (−)-epigallocatechin gallate (EGCG), the principal polyphenol present in green tea, which is known to be a superior neuroprotective antioxidant [16-20].

5.2 MATERIALS AND METHODS

Materials

The sources of materials, measures of purity etc. are the same as those described previously in chapter 4. It also described 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, USA). 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, USA).

Cell culture

I used two methods to prepare the cell culture of cortical neurons; starting either from animals or from frozen cell stock, respectively.

1. Primary cultures of cortical neurons were obtained from eighteen-day-old pregnant Sprague-Dawley rats (Charles River) following the procedure described previously [21]. 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, pups were removed, transferred to ice-cold PBS and decapitated. Cortices were surgically removed, suspended in 2 mL 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 minutes at 20 ^{0}C. After removal of supernatant, the

resulting pellets were resuspended in 8-10 mL 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 $1.0x10^6$ cells/dish. Cultures were maintained in a 5% CO_2 atmosphere at 37 ^{0}C. After one week, the medium was replaced with Neurobasal medium containing B-27 supplement. Further media changes occurred bi-weekly.

2. Cryopreserved rat cerebral cortex neuronal cells were a gift from QBM Cell Science Inc., Ottawa, Canada. The cells were thawed, suspended in Neurobasal medium supplemented with B-27, Penicillin (100 U/mL)/Streptomycin (100 □g/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 ^{0}C in a humidified atmosphere (5% CO_2 in air) for 4 h. Then the medium was removed leaving a small volume to ensure the cells did not dry out and 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 cultures. Medium was removed, and the cells were incubated for an additional 24 h (37 ^{0}C, 5% CO_2) in Neurobasal medium supplemented with B-27 Minus AO (NB27 – AO) (Invitrogen) [22]. 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 MTT (5mg/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. Control consisted of NB27 – AO plus DMSO with no added compound; its absorbance (3 measurements) determines cell viability for Control, set at 100%.

Protection against oxidative stress induced by AAPH

For all cytoprotection experiments the term "medium" refers to NB27 – AO. The 96-well microplates prepared in the same way as before (2 x 10^5 cells/mL) were incubated in medium for 24 h prior to use. The cytoprotection was assessed by the method described previously (Material and Methods - Chapter 3). In brief, the cells were incubated with the compounds tested (1-150 μM) for 2 h prior to addition of AAPH (1.5 mM). After 24 h further incubation with AAPH + compound, the cell viability was assessed using MTT (5mg/mL). At 1.5 mM, the oxidative stress generated by AAPH reduces the cell viability to ca. 36-40% that of 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 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 the incubation was continued for another 3 h. The cells were washed once with 1 mL PBS and new medium was added. After 24 h the 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 Control (0 μM glutamate).

Statistics

Data are expressed as mean ± SEM values. In the case of EC_{50} (see Figure 5.2) and CPA (see Figures 5.5-5.7) the same treatment as described in Chapter 3- statistics was applied in order to calculate the error of the EC_{50} and CPA, respectively. Statistical significance was assessed by using one-way ANOVA. Differences were considered significant at $p < 0.05$.

Calculation of BDE

The method of calculation of the BDE was previously described [chapter 3] 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^{o}_{298}) for ArO-H (g) $\rightarrow$ $ArO^{\bullet}$ (g)+ $H^{\bullet}$ (g). In cases where there are two exchangeable OH groups, BDE_1 refers to loss of the first (most weakly bound) H-atom to form the semiquinone, and BDE_2 to loss of the second H-atom to form the quinone. Starting geometries, which included conformer searching, were obtained with the Spartan '02 builder module (WaveFunction, Inc., Irvine, CA USA) 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 [23].

5.3 RESULTS

Experimental

I first examined the cytotoxicity on primary neuronal cultures of the naphthalenediols prepared in their diacetylated form and well-known compound EGCG. The naphthalenediol diacetates cross the cell membrane and the acetate moieties are cleaved by cellular esterases, forming the diols. Figure 5.1 shows structures for the five compounds tested, where all of the naphthalenediols have been fully acetylated.

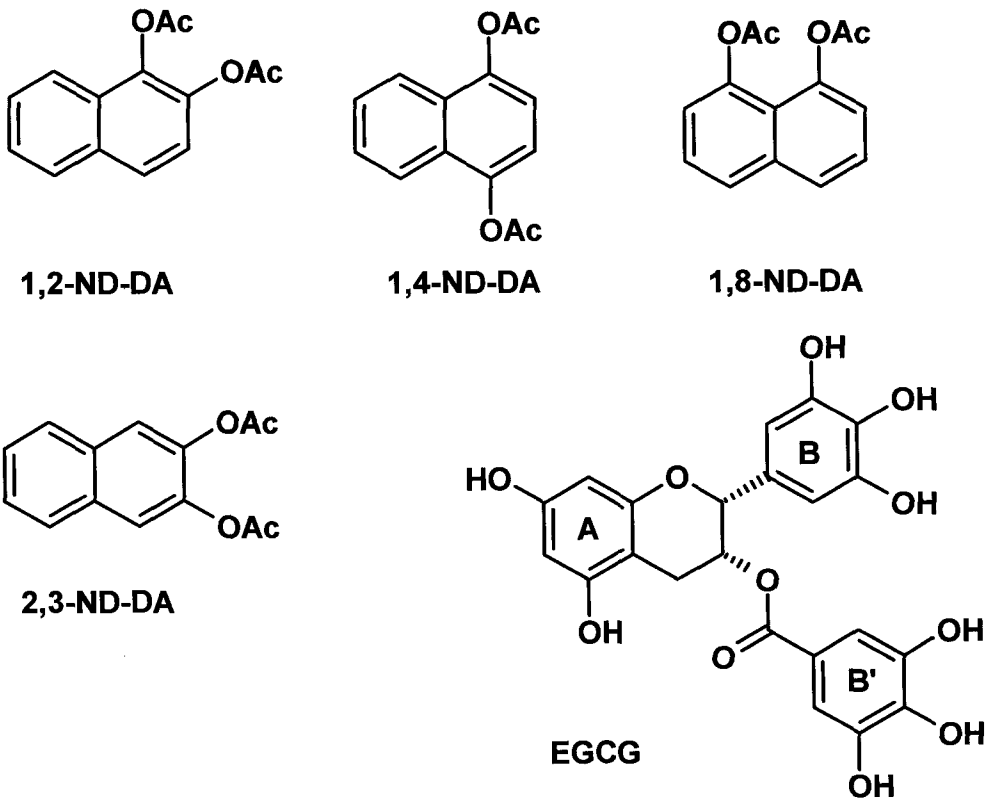


Figure 5.1. Structural formulas for the 5 compounds tested, where 1,2-ND-DA = 1,2-naphthalenediol diacetate, etc. and EGCG = (−)-epigallocatechin gallate.

Figure 5.2 shows the cell viability vs. concentration of compound for the five compounds tested. Cell Viability is defined here as the percent of live cells (from MTT assay) relative to Control (zero concentration of compound). There is clearly a wide range of cytotoxicity among the compounds tested, ranging from 1,4-ND (most toxic) and 1,2-ND (very toxic) >> 2,3-ND ≈ EGCG ≈ 1,8-ND (relatively non-toxic). Note that EGCG showed reproducible enhancement of survival (cell viability above 100%).

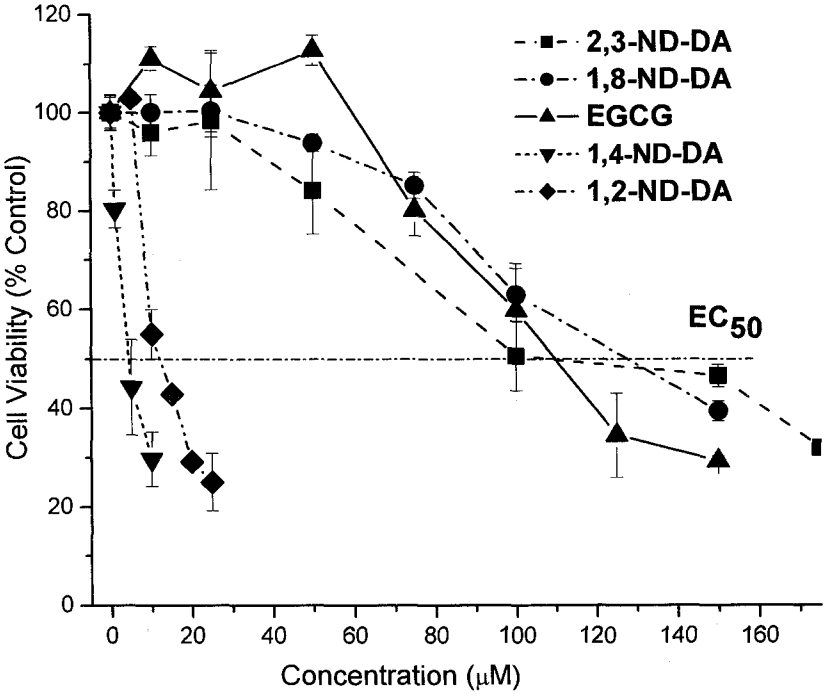


Figure 5.2. Cell viability of rat cortical neurons (MTT assay) after 24 h incubation with naphthalenediols and EGCG. All compounds except EGCG were initially present as diacetates.

Figure 5.3 shows the cytotoxicity caused by the oxidative stressor AAPH, which generates peroxyl radicals ($ROO^{\bullet}$), at a relatively constant rate. Here EC_{50} represents the effective concentration which reduces formazan blue absorbance (MTT assay) to 50% of Control (Control: no AAPH added). Fitting a quadratic function to the data in Figure 5.3 gave an EC_{50} of 1.23 ± 0.20 mM, with a correlation coefficient $R^2 = 0.956$.

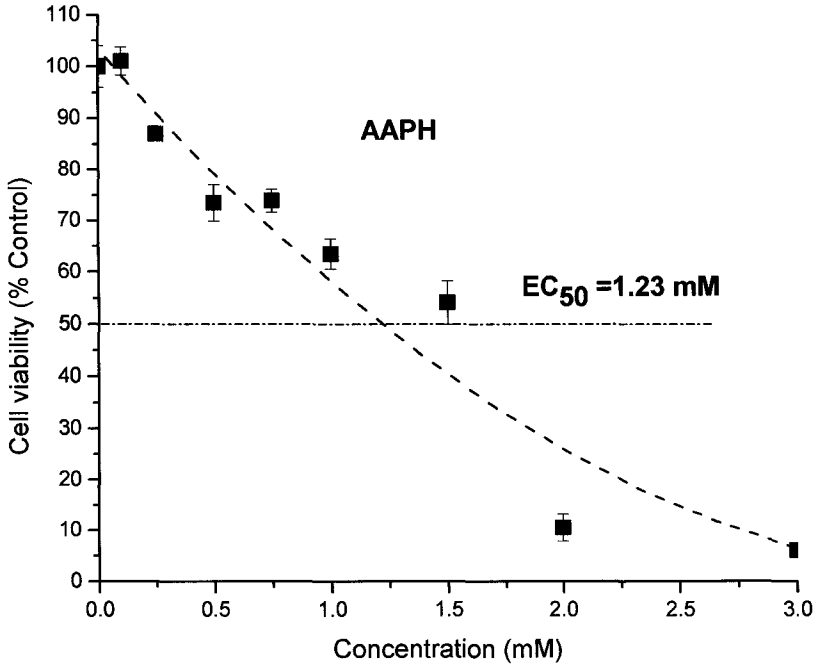


Figure 5.3. Cell viability (MTT assay) for primary cortical neurons vs. concentration of AAPH.

For tests of cytoprotection the relatively toxic 1,2-ND and 1,4-ND were discarded and effort was concentrated on the less-toxic species 2,3-ND, 1,8-ND and the reference compound EGCG. Figure 5.4 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), so any viability above 38% represents protection of the neurons. The naphthalenediols are protective over the full range tested (10-150 μ M), whereas EGCG is only protective up to 120 μ 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.

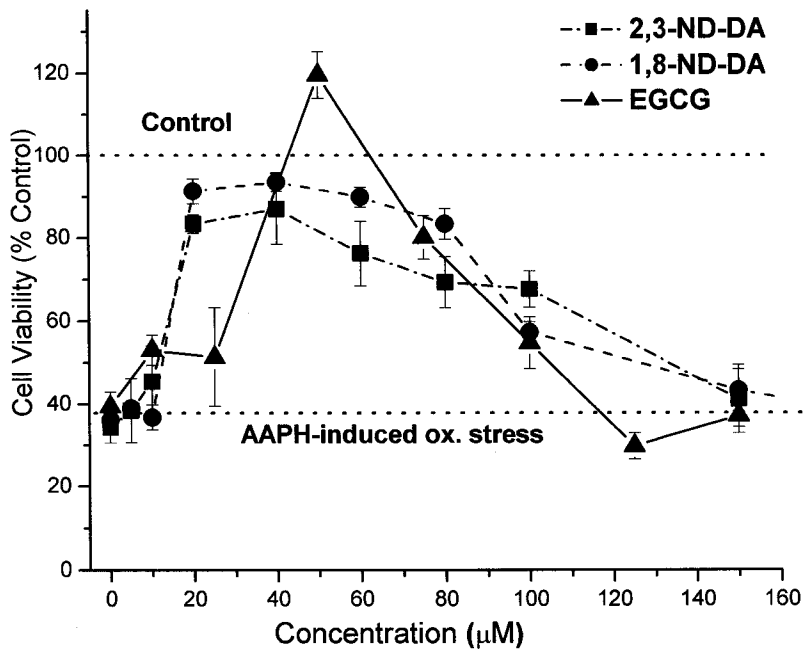


Figure 5.4. Cytoprotection of cortical neurons by selected naphthalenediols, in presence of AAPH (1.5 mM). Compounds tested: 2,3-ND, 1,8-ND (as diacetates) and EGCG. Protective zone: between dashed lines bounded by 38% viability (AAPH-induced stress) and Control.

Figure 5.5 shows the Cytoprotective Area (CPA), as defined in chapter 4 and obtained from data for 1,8- ND. The CPA represents an integral over the viability/concentration plot, with contributions to the CPA occuring when the viability shows a protective effect due to compound. Here "Stress" represents the neuron viability in the presence of the stressor AAPH, applied for 24 h at a concentration of 1.5 mM (i.e. no 1,8 ND present). Figure 5.5 shows that the population of stressed cells has 38 % viability relative to Control; this provides the lower boundary for the CPA integral. The upper boundary is provided by the cytotoxicity envelope (Figure. 5.2). The area of the bounded region (shaded, Figure 5.5) is the CPA, which provides an integrated comparison of the protection given between different compounds. The protective range is 5 - 170 μ M, beyond which the inherent cytoxicity of 1,8-ND outweighs any protective effect (no protection can occur when the cell viability < 38% in this experiment, i.e. the tested compound has not reduced the oxidative stress). The total CPA for 1,8-ND is ca. 5000, in units of % Control x μM. The same procedure was repeated for the other compounds 2,3-ND and EGCG (Figures 5.6-5.7). These data are collected in the next section in Table 5.2.

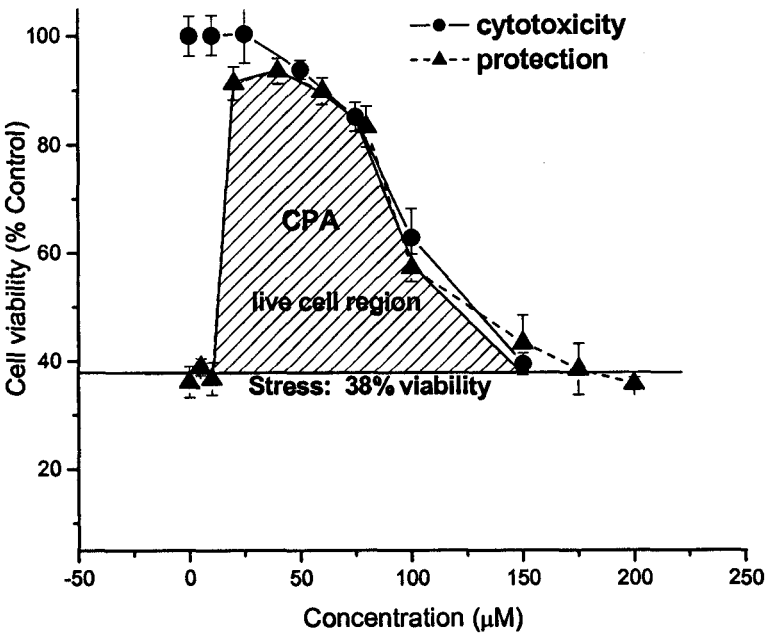


Figure 5.5. Total Cytoprotective Area for 1,8-ND (shaded regions). Boundaries: Cytoprotection experiment (from Figure 5.4) at left (triangles), cytotoxicity envelope (from Figure 5.2) at top (filled circles), viability in presence of AAPH stress (solid line, 38% relative to Control).

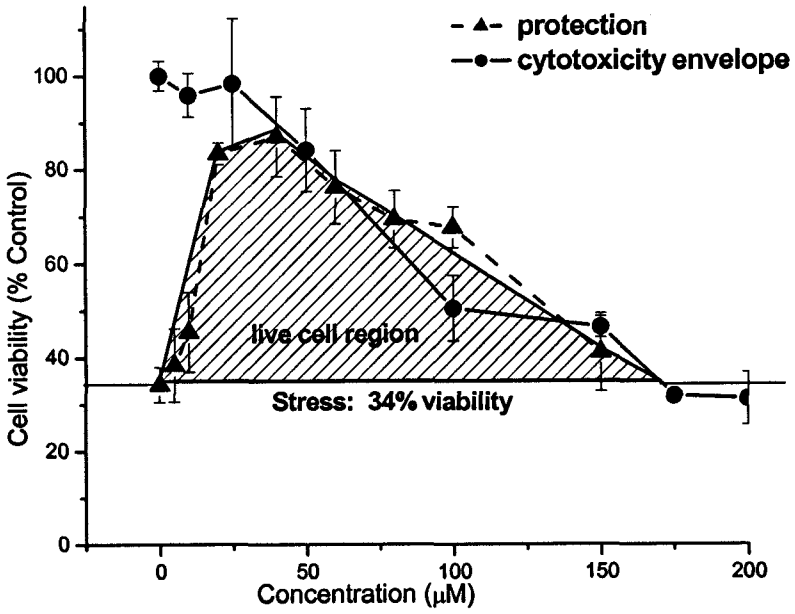


Figure 5.6. Total Cytoprotective Area for 2,3-ND (shaded regions).

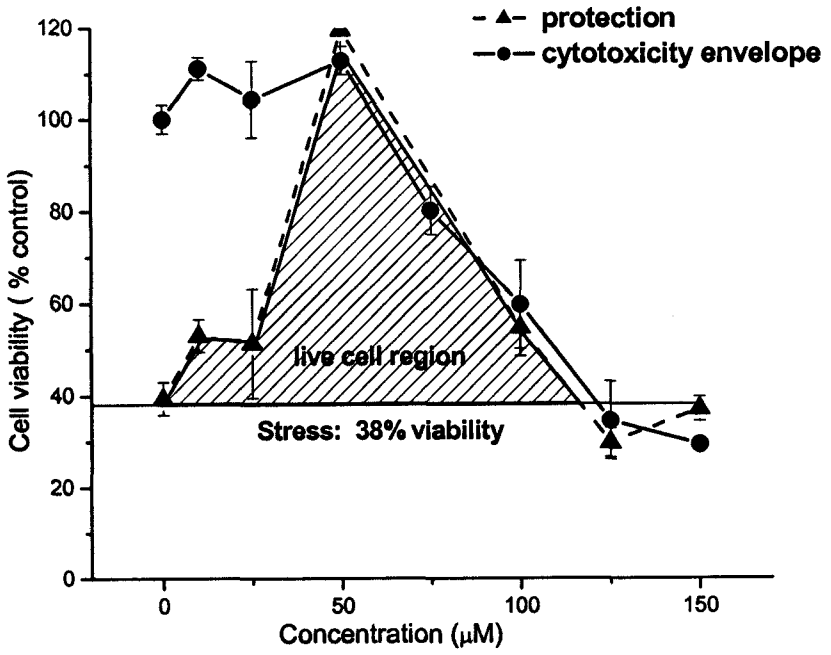


Figure 5.7. Total Cytoprotective Area for EGCG (shaded regions).

When the AAPH testing was concluded, it was decided to extend this work to include another oxidative stressor. For this purpose glutamate, an excitory neurotransmitter was chosen, since it is commonly used to induce oxidative stress in neuronal cell culture work [24,25]. For these experiments the cells were subjected to 3 h exposure of glutamate over the concentration ranges 0.1 – 1 mM. Cell viability was established using the trypan blue assay after 24 h incubation of cells in NB27–AO (see Methods).

Figure 5.8 shows that for glutamate the EC_{50} is about 290 ± 35 μM under the conditions of our experiment. The EC_{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 I did not measure these parameters in the current work. (I used serum-free medium in order to diminish the number of glial cells, which heightens the response of the neurons to oxidative stress.)

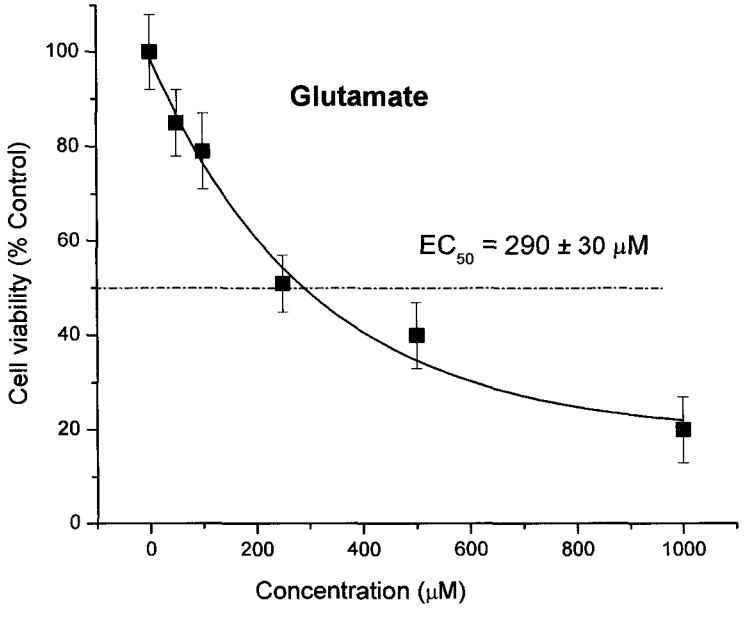


Figure 5.8. Cytotoxicity of glutamate to cortical neurons (trypan blue assay).

Figures 5.9 - 5.11 show images of cultured cortical neurons obtained by (PIXCELL II) inverted microscope after treating the cells with trypan blue. Figure 5.9 shows the cells (14 days old) after 24 h in medium NB27-AO and no treatment (no glutamate, no compound). This is defined as the Control. Two or three dead cells can be seen. The cell viability is very high. Figure 5.10 shows the cells image after 3 h exposure to 250 μM glutamate and 24 h incubation of cells in NB27-AO. The cell viability was approximately 40% (trypan blue assay). Figure 5.11 shows the neurons preincubated with 1,8-ND-DA and then exposed to 250 μM glutamate. The cells were rescued by treatment with the compound and only a few dead cells were counted in the dish.

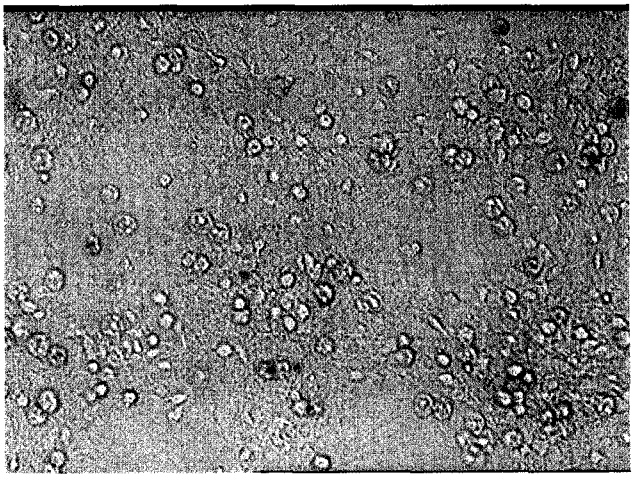


Figure 5.9. Image of primary cortical neurons with no treatment (Control). The cells were stained with trypan blue and counted using an inverted microscope.

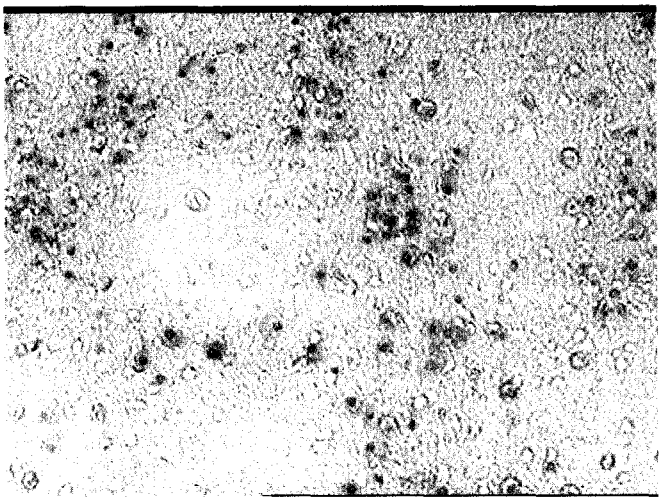


Figure 5.10. Image of primary cortical neurons stressed with 250 μM glutamate. The cells were stained with trypan blue and the dead cells were counted under a PIXCELL II inverted microscope.

Figure 5.11. Image of primary cortical neurons stressed with 250 μM glutamate and protected by 10 μM 1,8-ND-DA treatment. The cells were stained with trypan blue and the dead cells were counted under a PIXCELL II inverted microscope.

Figure 5.12 shows the cytoprotection resulting when 1,8-ND was added prior to glutamate treatment. A significant protective effect was observed at a concentration as low as 500 nM. For example, the cell viability was increased from 33 ± 2% SEM (no 1,8-ND present) to 47 ± 4% SEM when 500 nM compound was present. The cell viability goes up rapidly and monotonically 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, i.e. 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 not protective.

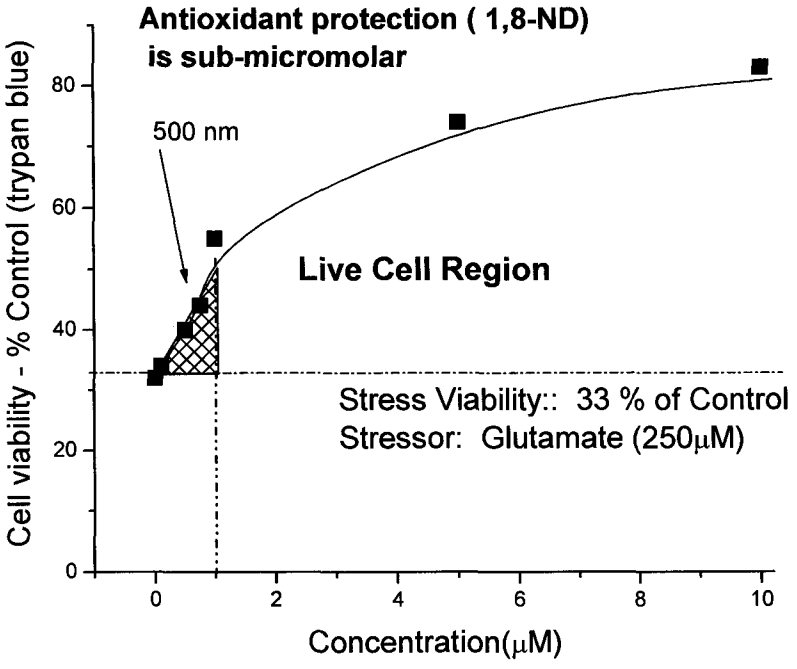


Figure 5.12. Cytoprotection of cortical neurons by 1,8-ND showing low-dose protection; oxidative stressor is glutamate (250 μM).

Theoretical.

Theoretical calculations were included to aid in the discussion of the toxicity and protective effects of the compounds studied. Table 5.1 shows the calculated results for BDE_1 and BDE_2, the cytotoxicity expressed as the EC_{50} in PC12-AC [11] cells and cortical neurons, and the ratio between the EC_{50}'s for the two cell types.

Table 5.1. Calculated BDE_1 and BDE_2, the cytotoxicity EC_{50} for PC12-AC cells [11] and for cortical neurons and the ratio of the EC_{50}'s between the cell lines.

Compound	BDE_1 kcal mol^{-1}	BDE_2 kcal mol^{-1}	EC_{50} (μM) PC12-AC	EC_{50} (μM) Neurons	Ratio
1,4-ND	75	55	14 ± 0.6	4.75 ± 1.2	3.00
1,2-ND	70	69	40 ± 3	12.41 ± 0.8	3.33
2,3-ND	79	84	273 ± 20	118 ± 20	2.45
1,8-ND	72	104[a]	250 ± 20	128 ± 7	2.12
EGCG	71	74	456 ± 16	110 ± 10	4.30

[a]Triplet diradical state

Table 5.2 summarizes the concentration range which is cytoprotective for rat cortical neurons, the concentration which provides the maximum protective effect, the CPA in PC12-AC cells and the CPA in neurons.

Table 5.2. Cytoprotective efficacy of antioxidants against oxidative stress induced by 1.5 mM AAPH. Incubation time is 24 h, 2 h pretreatment with antioxidant.

Compound	Range[a] (μM) Neurons	Max (% Control) Neurons	CPA $_{PC12-AC}$ (% Control • μM)[b]	CPA $_{neurons}$ (% Control • μM)
1,2-ND	2-5	53	100	ca. 75
1,4-ND	3-10	70 @ 5 μM	285 ± 20	50 ± 3
2,3-ND	5-150	90 @ 40 μM	7225 ± 293	4810 ± 170
1,8-ND	5-150	93 @ 40 μM	9030 ± 200	5080 ± 145
EGCG	10-120	110 @ 50 μM	17,300 ± 1240	3990 ± 340

[a] Total protective range; only the region above "Stress" is counted (see Figure 5.5),

[b]Unit for Cytoprotective Area

5.4 DISCUSSION

1. Toxicity

Figure 5.2 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 cytotoxicity of the latter pair is comparable to that of EGCG which is generally thought to be neuroprotective [16-18]. 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 EC_{50} 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 since it 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.

2. Cytoprotection against AAPH

As with cytotoxicity, there are clear trends in the cytoprotective effectiveness which transcend cell lines. Table 5.2 compares the cytoprotective areas (CPA's) 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. 2,3-ND and 1,8-ND, on the other hand, have about 2 orders of magnitude greater CPA. EGCG is most effective in PC12-AC cells by a factor of two,

however in the neurons both 2,3- and 1,8-ND have higher CPA's than EGCG. Qualitatively, then, the cytoprotective behavior is similar for the two cell lines.

3. Peroxyl radical mechanism, effect of BDE

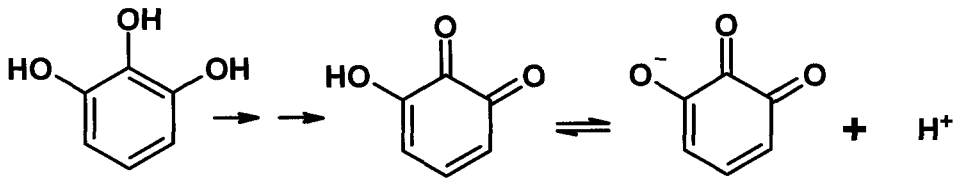
For naphthalenediols possessing two exchangeable H-atoms, BDE_1 describes the tendency for semiquinone formation. Here the compound is acting as an antioxidant, according to $NQH_2 + ROO^{\bullet} \rightarrow NQH^{\bullet} + ROOH$, breaking the chain reaction of lipid peroxidation, so lower values of 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, i.e. $NQH^{\bullet} + ROO^{\bullet} \rightarrow NQ + ROOH$. Note that due to the typically low pK_a of semiquinones (ca. 4.0), $NQH^{\bullet}$ actually exists in the deprotonated form $NQ^{\bullet-}$ at physiological pH, which reacts preferentially by electron transfer rather than H-atom transfer (HAT). However, the energetics are related and HAT is still useful as a predictor of reactivity. Once $NQ^{\bullet-}$ formation occurs, redox cycling between semiquinone radical anion and quinone leads to formation of superoxide and related species [15], or thiol depletion by nucleophilic addition to the electrophilic quinone. Both mechanisms cause cytotoxicity via these prooxidant behaviours (generation of superoxide, or removal or protective thiols) and hence low values of BDE_2 are to be avoided. Table 2 shows that based on BDE_2 values the predicted order of cytotoxicity is 1,4 > 1,2 >> 2,3 >> 1,8-ND. This is essentially the order observed, although the BDE_2 for 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 non-toxic.

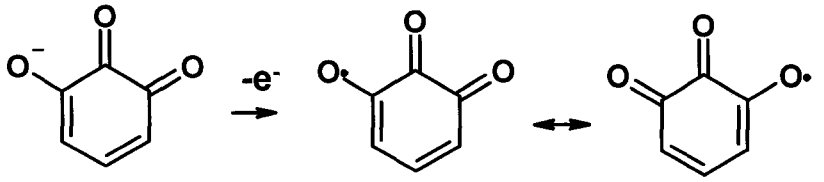
4. EGCG quinone is non-toxic

EGCG has a relatively low BDE_2 (74 kcal/mol, Table 2) and hence is expected to be relatively toxic, contrary to observation. However, the pyrogallol moiety (i.e. 1,2,3-trihydroxybenzene) forms a special type of quinone since it has a third active hydrogen (or electron). Scheme 5.1 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 electrophilic, and hence not subject to nucleophilic attack. This removes the first source of toxicity. The anion in Scheme 5.2 is unlikely to donate an electron to oxygen, since 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 not favored energetically. It is also unlikely to redox cycle since it will not be subject to reduction by cytochrome P450 reductase, due to 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 non-toxic.

SCHEME 5.1



SCHEME 5.2



5. Comparison of toxicity and protection in immortal vs. primary neuronal cells

I 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 EC_{50}'s will depend on the conditions of the experiment, especially the plating density of the cells. Thus I 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 of the naphthalenediols to form naphthoquinones. This tendency explains the data and allows prediction of toxicity for other naphthalenediols as well.

6. Cytoprotection against glutamate

Additional experiments on 1,8-ND using glutamate as oxidative stressor showed significant protective effects at low (sub-μM) concentrations. In order to explain the very high protection given by nM concentrations of 1,8-ND, two mechanisms are possible: First, the ability of the compound to scavenge ROS which, if not deactivated, leads to neuronal cell death, and second, the prevention of apoptosis in cortical neurons exposed to glutamate by blocking *N*-methyl-D-aspartic acid (NMDA) receptors. The mechanism of glutamate neurotoxicity can be divided into acute and delayed toxicity. Delayed neurotoxicity of glutamate is calcium-dependent. Glutamate binds to the NMDA receptor and an influx of extracellular Ca^{2+} leads to a cascade of events leading to oxidative stress. Superoxide generation is then initiated. [25-28].

Using the patch-clamp method we found that the addition of 1,8-ND had no effect on the ongoing NMDA receptor-driven spontaneous activity present in these cultures (data not shown) [29-31]. Therefore, we eliminate blocking of the NMDA receptor as the mechanism of cytoprotection caused by 1,8-ND. That means either the low-dose protection (Fig. 5.7) given by 1,8-ND under conditions of glutamate stress is caused by simple antioxidant activity, or more subtle effects are involved, e.g. cellular signaling [19,20,32,33].

5.5 CONCLUSION

Certain members of the naphthalenediol family show good activity in scavenging peroxyl radicals, while being of 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, i.e. 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 [34,35]. 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 to look at the toxicity of metabolites.

5.6 REFERENCES

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Chapter 6

General Conclusions

The Bond Dissociation Enthalpy (BDE) in phenolic antioxidants is an essential tool in determining the efficacy of a chain-breaking antioxidant. The weaker the O-H bond, the faster will be the reaction with free radicals (peroxyl radicals). A "design window" was defined between 68-75 kcal mol^{-1}, into which the BDE of active bond O-H of the new antioxidants should fit [1].

Novel antioxidants with BDEs values of the O-H bond between 69-75 kcal/mol were designed, synthesized and tested for reactivity with the stable 2,2-diphenyl-1-picrylhydrazyl free radicals (DPPH) in organic solvent and in HL-60 cell culture. A good correlation was found between rates of reaction with DPPH radicals and BDEs and also between IC_{50}'s (concentration of antioxidant that provides 50% inhibition) and BDEs. Both assays can be considered as good models to evaluate the antioxidant capacity of a compound.

Three synthetic catechols H1 (a *p*-methoxycatechol), H2 (a catechol analog of α-tocopherol), and H4 (a dioxymethylene-substituted catechol), their diacetates H1-DA, H2-DA, H4-DA (DA = diacetate) and a well know antioxidant, epigallocatechin gallate (EGCG) were tested for cytotoxicity and protection against oxidative stress in rat pheochromocytoma (PC12-AC) cells, both with and without 50 μM ascorbate. Oxidative stress was induced by menadione and the peroxyl radical generator AAPH. Using assays for H_2O_2 and reduced glutathione (GSH), as well as calculated values of solubility (log P) and bond dissociation enthalpy (BDE), I discussed the cytotoxicity with reference to properties of the parent catechol, the semiquinone and the quinone. We found that the two pure redox-cycling catechols, H1 and H2 and their diacetates, have a strong near-linear dose-dependence in their H_2O_2 response, which correlated well with their

cytotoxicity and with the effect of added ascorbate. For the combined redox cycling-GSH depleting compound H4, less peroxide was formed. In all three compounds, GSH increased relative to control (upregulation of total GSH production). Use of a BDE argument helped us to correlate toxicity both with and without the presence of added ascorbate. In cytoprotection assays, H2-DA showed a narrow zone of protective behavior (5 to 40 μM) compared with EGCG which demonstrated an extended range of protection (5 to 575μM) when cells where stressed with AAPH. In general, the catechols react quickly with radicals but show toxic effects due to redox cycling. This required a redesign of the compounds in order to minimize quinone formation. This led to an investigation of naphthalenediols.

The cytotoxicity and protection against oxidative stress for five members of the naphthalenediol family, as well as the known antioxidant EGCG, was tested. The compounds tested included 1,2-naphthalenediol (1,2-ND), 1,4-ND, 2,3-ND, 1,8-ND and 1,4-dipropyl-2,3-naphthalenediol (DPND). Compounds were tested for their cytotoxicity as well as their antioxidant capacity on an adherent clone of rat pheochromocytoma (PC-12AC). Oxidative stress was induced by the peroxyl radical generator AAPH. The relative order of cytotoxicity (most toxic to least toxic) was found to be 1,4-ND > 1,2-ND > DPND> 2,3-ND > 1,8-ND >> EGCG, with EC_{50}'s of 15, 40, 160, >250, > 250, >> 250 μM, respectively. In spite of their high toxicity, both 1,4-ND and 1,2-ND showed narrow zones of protective behavior whereas DPND, 2,3-ND and 1,8-ND and especially EGCG showed extended ranges of protection. The total protection obtained for the combination of cells/oxidative stressor/protective compounds (PC12-AC/AAPH/naphthalenediols) can be defined, in general, by an integrated measure termed the Cytoprotective Area (CPA).

I related the observed cytotoxicity and CPA to the different electronic structures of the naphthalenediols, characterized theoretically by the first and second bond dissociation enthalpies (BDEs) as well as the pK_a's for the parent (diol) and semiquinone compounds. Since the 2,3- and 1,8-NDs do not form quinones, their cytotoxicity is much lower than for the compounds which do. Thus, selected members of the naphthalenediol family show promise as antioxidants. I also examined the effect of added ascorbate on cytotoxicity of naphthalenediols. I found that 2,3-ND and DPND become less toxic by adding ascorbate, 1,4-ND and 1,8-ND are unaffected by ascorbate and the toxicity of 1,2-ND is increased when the ascorbate is present. The calculated BDEs correlated to their observed toxicity. I also designed and tested derivatives of 1,8-ND for cytotoxicity and cytoprotection. The order of cytoprotection is 1,8-ND-DA > 1,8-ND-DA acrylamide > 1,8-ND glycerate > 1,8-ND-DA ester. Thus certain members of the naphthalenediol family and their derivatives show promise as antioxidants. To test if these conclusions were universal, naphthalenediols were also tested on primary neurons.

The effect of naphthalenediols on rat primary cortical neurons exposed to AAPH. was investigated. Compounds tested included the acetylated forms of 1,2-naphthalenediol, i.e.1,2-ND, as well as 1,4-ND, 2,3-ND, 1,8-ND, and the known highly potent antioxidant EGCG. This set of compounds was the same as those used in the PC12-AC studies. In neuron cytotoxicity 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 based on 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. Therefore these conclusions have been demonstrated in two cell lines, the immortalized cells (PC12-AC) and the primary cells (cortical neurons). I expect that these conclusions will hold for other cell lines, because the underlying chemical mechanism related to quinone formation is universal.

In conclusion, the thesis has demonstrated the following:

1) Weakening the OH bond leads to a faster reaction with free radicals in organic solvents (DPPH radicals) and in HL-60 cells (superoxide radicals and the related hydroperoxyl radicals). This was demonstrated in Chapter 2 for a series of catechols which had BDE_1 in the design window 68-75 kcal mol^{-1}.

2) The designed catechols were tested for cytotoxicity and cytoprotective effects in Chapter 3, using PC12-AC cells. The catechols were found to be quite cytotoxic due to the formation of quinones. This required a redesign of antioxidants to avoid quinone formation, e.g. by creating molecules with a high BDE_2.

3) A series of naphthalenediols were prepared and tested for their cytotoxicity and cytoprotection effects, as described in Chapter 4. The 1,2- and 1,4-NDs were very cytotoxic, due to the formation of the quinone. On the other hand, the 2,3- and 1,8-NDs were relatively non-toxic and, at the same time, cytoprotective due to their high BDE_2

which prevented quinone formation. Measures of efficacy showed that these compounds are comparable to or even superior to the well-known antioxidant EGCG.

4) To test whether the mechanism was universal, experiments were carried out on a primary cell line in Chapter 5. Rat cortical neurons, exposed to the stressors AAPH and glutamate, showed that the 1,8-NDs are highly cytoprotective.

5) Thus the 1,8- and 2,3-ND family of compounds provided a promising starting point for a new type of antioxidant. This could be extended to investigations using higher organisms to test for therapeutic effects when the organism is subjected to oxidative stress.

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PUBLICATION LIST

a). Articles published or accepted in refereed journal

1. **Flueraru, M.**, Willmore, W. G., Poulter, M. O., Durst, T., Charron, M., and Wright, J. S. (2006) Cytotoxicity and cytoprotective activity of naphthalenediols in rat cortical neurons: *Chem. Res. Toxicol.* (in press).
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