Involvement of Semiquinone Radicals in the in Vitro Cytotoxicity of Cigarette Mainstream Smoke

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Free radicals in cigarette smoke have attracted a great deal of attention because they are hypothesized to be responsible in part for several of the pathologies related to smoking. Hydroquinone, catechol, and their methyl-substituted derivatives are abundant in the particulate phase of cigarette smoke, and they are known precursors of semiquinone radicals. In this study, the in vitro cytotoxicity of these dihydroxybenzenes was determined using the neutral red uptake (NRU) assay, and their radical-forming capacity was determined by electron paramagnetic resonance (EPR). All of the dihydroxybenzenes studied were found to generate appreciable amounts of semiquinone radicals when dissolved in the cell culture medium employed in the NRU assay. Hydroquinone exhibited by far the highest capacity to form semiquinone radicals at physiological pH, even though it is not the most cytotoxic dihydroxybenzene. Methyl-substituted dihydroxybenzenes were found to be more cytotoxic than either hydroquinone or catechol. The formation of semiquinone radicals via auto-oxidation of the dihydroxybenzenes was found to be dependent on the reduction potential of the corresponding quinone/semiquinone radical redox couple. The capacity to generate semiquinone radicals was found to be insufficient to explain the variance in the cytotoxicity among the dihydroxybenzenes in our study; consequently, other mechanisms of toxicity must also be involved. The observed interactions between 2,6-dimethylhydroquinone and hydroquinone in the cytotoxicity assay and EPR analysis suggest that care needs to be taken when the bioactivity of cigarette smoke constituents is evaluated, i.e., the effect of the cigarette smoke complex matrix on the activity of the single constituent studied must be taken into consideration.

Introduction

Smoking causes disease (*1*−*5*). Extensive research has been conducted to explain the relationship between individual smoke constituents and smoking-related diseases, but many complex biological processes are involved, and no simple correlations have been found. Numerous constituents of cigarette smoke have been identified as potential agents of biological damage, including tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons, phenolic compounds, and free radicals (*6*−*9*). Free radicals have attracted much attention, because free radicals can cause DNA damage, lipid peroxidation, and protein oxidation (*10*, *11*). Cigarette smoke contains large amounts of free radicals (*8*, *12*) and molecular constituents that readily produce free radicals in aqueous media (*8*, *13*). The involvement of free radicals in cigarette-smoke-related pathologies is plausible, but in-depth studies are needed to delineate their precise effects.

Free radicals are found in both the gas/vapor phase (GVP) and the particulate phase of the cigarette smoke aerosol. To investigate the GVP and particulate-phase radicals independently, the particulate fraction of cigarette smoke is usually separated from the gas/vapor-phase constituents by passing the cigarette smoke through a fiberglass filter called a Cambridge pad (*14*, *15*). Thus, the total particulate matter (TPM) includes all of the smoke condensate collected on the Cambridge filter pad. "Tar" is defined as the TPM less water and nicotine. The gas-phase radicals have been shown to be unstable and very reactive, whereas the radicals in the TPM are much longer lived (*8*, *12*, *16*). The chemical behaviors of the GVP and TPM radicals of cigarette smoke are quite distinct. Experimental evidence suggests that the gas-phase radicals form in a continuous mechanism, whereby nitric oxide (NO) reacts with molecular oxygen in air to form NO_2, and subsequent reactions between NO_2 and unsaturated molecules in cigarette smoke, e.g., isoprene and butadiene, yield alkyl, peroxyl, and alkoxy radicals. Reaction of the peroxyl radicals with NO generates additional NO_2, creating a steady-state cycle (*12*, *17*). The TPM radicals have been tentatively assigned to semiquinone radicals in a polymeric matrix (*8*). The particulate phase also contains constituents, e.g., dihydroxybenzenes, that can generate semiquinone radicals (*18*). Hydroquinone and catechol are well-known examples of smoke constituents that undergo oxidation in air to form semiquinone radicals and ultimately quinones. Further reactions between the semiquinone radicals and molecular oxygen, either in aqueous extracts of cigarette tar (ACT) or in living cells, lead to the creation of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hydroxyl radical (*18*).

Cytotoxicity is regarded as a potential step in several chronic disease processes, including carcinogenesis and emphysema (*19*). This might be especially true for cigarette smoke, where the promoting events might be due to the cytotoxic activity.

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The ultimate cytotoxic constituents of cigarette smoke and their mechanisms of action are poorly understood. Thus far, most research has focused either on the chemistry of cigarette smoke or on the in vitro and in vivo effects of cigarette smoke on biological systems. The lack of a bridge between these two approaches has made it difficult to assess the relationship between cigarette smoke constituents and their deleterious effects in biological systems. It is known, for example, that cigarette smoke contains a substantial amount of dihydroxybenzenes in the TPM (*20*−*22*). In vitro assays have demonstrated that ACT, which contains significant amounts of hydroquinone, catechol, and other mono- and dihydroxybenzenes, can damage DNA (*23*). However, there is no direct evidence to suggest that pure hydroquinone or catechol can induce the same level of free-radical formation and DNA damage as any isolated fraction of ACT.

Even though hydroquinone is the most abundant dihydroxybenzene in cigarette smoke, Blakely et al. (*24*) showed that the yield of particulate-phase radicals is not correlated with the yield of hydroquinone. For a series of cigarettes containing different tobacco blends with variable yields of hydroquinone in their smoke TPM, the amount of free radicals in the TPM remained unchanged. In this case, methylene chloride was used to extract the TPM from a Cambridge pad, was solvent evaporated, and the residue was redissolved in benzene for EPR measurements. Under these experimental conditions, hydroquinone is less likely to undergo auto-oxidation, i.e., oxidation due to exposure to air, which is less favorable in organic solvents. Nevertheless, significant levels of TPM radicals were observed, which calls into question the exact nature of the radicals that could be involved in the biological damage.

Neither the measurements of Pryor et al. (*23*) nor those of Blakley et al. (*24*) are representative of radical formation under physiological conditions or in biological systems. The dihydroxybenzenes can generate semiquinone radicals, quinones, and reactive oxygen species (ROS) in oxygenated physiological media in vitro, for example, in the growth media used in cytotoxicity assays, and in vivo in the epithelial lining fluid of the lungs of smokers. The cytotoxicity of quinols and quinones has been suggested to be due to the concerted action of several processes that include redox cycling; alteration of thiols balance through oxidation or arylation; inhibition of cellular functions; alteration of Ca^{2+} homeostasis; and covalent binding to nucleic acids, proteins, and lipids (*25*). It is difficult to extrapolate in vitro data analysis to an in vivo system, especially if the in vitro experiments were not conducted under conditions close to physiological conditions. Moreover, few data are available on the cytotoxicity of pure dihydroxybenzenes to compare with the toxicity of a complex mixture containing dihydroxybenzenes, such as cigarette smoke TPM.

In this work, we used model systems to evaluate whether the cytotoxicity of several pure dihydroxybenzenes found in the TPM of cigarette smoke is related to their capacity to generate semiquinone radicals under physiological conditions. The cytotoxicity of pure dihydroxybenzenes was determined using the neutral red uptake (NRU) assay, developed by Borenfreund and co-workers (*26*, *27*). This assay has been widely used by the chemical and pharmaceutical industries as an in vitro screening method to determine the basal cytotoxicity of compounds (*28*). The assay is a well-established, reproducible, standardized short-term assay that responds to cytotoxic compounds in a dynamic range of 5 orders of magnitude (*29*). The assay is known to be responsive to both the particulate phase and the gas phase of cigarette smoke (*30*). It can also discriminate between different cigarette tobacco types (*31*) and is recommended by regulatory authorities (*32*).

The TPM from the smoke of 2R4F research cigarettes was collected and analyzed to determine the relative yield of the dihydroxybenzenes. Electron paramagnetic resonance (EPR) was used to quantify the amount of semiquinone radicals formed in DMEM (Dulbecco's Modified Eagle's Medium), the growth medium employed in the NRU assay, both at equimolar concentrations and at concentrations proportional to their yield in the TPM of the 2R4F cigarettes.

Experimental Procedures

Materials. Hydroquinone (HQ), 2-methylhydroquinone (2MHQ), 2,3-dimethylhydroquinone (23DMHQ), 2,6-dimethylhydroquinone (26DMHQ), trimethylhydroquinone (TMHQ), catechol (CAT), 3-methylcatechol (3MCAT), 4-methylcatechol (4MCAT), and 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy (TEMPO) were purchased from Acros Organics (Morris Plains, NJ). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Invitrogen Corporation (Grand Island, NY). All other chemicals were reagent grade.

Cigarette Smoking Procedure. 2R4F cigarettes (low-tar research cigarettes from the University of Kentucky and Health Research Institute) were smoked on a 20-port automatic smoking machine under FTC smoking conditions (35-mL puff volume, 2-s duration, 1 puff/min, with the ventilation holes of the cigarettes left unblocked). For analysis of phenolic constituents, mainstream smoke from 10 cigarettes was trapped on Cambridge fiberglass filters. Four replicate smoke samples were generated per cigarette.

TPM Constituent Analysis. The phenolic constituents in the TPM of cigarette smoke were analyzed by GC/MS. Briefly, the TPM collected on a Cambridge pad was extracted with 10 mL of dichloromethane/methanol (80:20), shaken for several minutes, and centrifuged, and an aliquot of the extract was derivatized with an excess of silylating agent (BSTFA) containing 10% TMCS. The analysis was performed by GC/MS on an Agilent 6890 GC equipped with an Agilent 5973 quadrupole MSD analyzer using selected ion mode for qualitative and quantitative analysis.

Cytotoxicity Assay. Cytotoxicity was determined using the neutral red uptake assay as described by Borenfreund and co-workers (*26*, *27*). Following standard operating procedures (*33*, *34*), 3T3 cells (BALB/c mouse embryo cell line) obtained from ATCC (American Type Culture Collection) were gown in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum and were exposed for 24 h to smoke fractions, or pure constituents were added to the culture medium. For each sample or mixture, four replicate 96-well microtiter plates were used, each with eight test substance concentrations. Each test substance concentration was replicated six times per microtiter plate. After exposure, the medium was replaced by fresh medium containing neutral red dye (25 μg/mL). Following an incubation period of 3 h, the neutral red taken up by viable cells was determined spectrophotometrically. The amount of neutral red taken up is directly proportional to the number of viable cells. The cytotoxic response to the smoke was characterized as the EC_{50} value, i.e., the effective concentration that decreased the number of viable cells by 50% relative to the solvent control. The EC_{50} value is inversely related to the cytotoxicity and was determined by nonlinear least-squares fit of the concentration−response values to the logistic function

$$y = \frac{a}{1 + \left(\frac{x}{b}\right)^c}$$

where x is the concentration, y is the absorbance relative to the untreated control culture, a is a maximum value, and c is the slope at the inflection point. The EC_{50} value is represented by the regression parameter b. The experimental design for testing combinations of compounds was a ray design, as described elsewhere (*35*), using the individual compounds and three mixtures

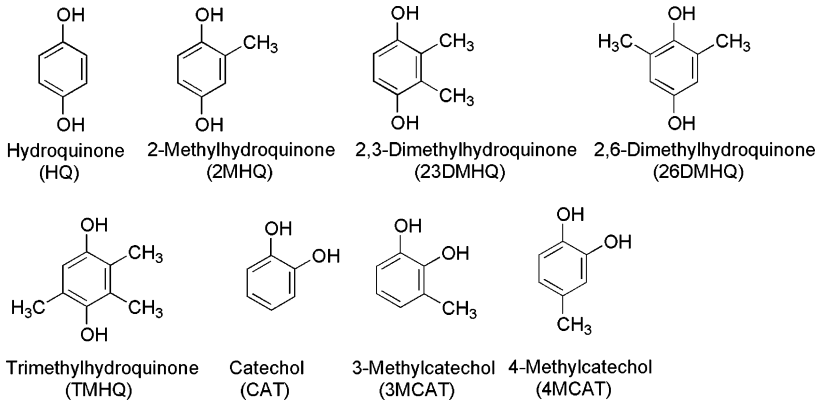

Figure 1. Chemical structures of the dihydroxybenzenes used in this study.

Table 1. Yields of Dihydroxybenzenes in TPM and Their Specific Cytotoxicities Expressed as EC_{50} in the Neutral Red Uptake Assay

dihydroxybenzenes	EC_{50} (mM)[a]	yield in TPM (μg/cig)
HQ	0.021 ± 0.004	34.4 ± 2.0[a]
2MHQ	0.011 ± 0.001	4.02 ± 0.03[b]
23DMHQ	0.015 ± 0.005	1.37 ± 0.07[b]
26DMHQ	0.016 ± 0.002	0.49 ± 0.04[b]
TMHQ	0.026	1.83 ± 0.03[b]
CAT	0.33	45.3 ± 0.5[c]
3MCAT	0.036 ± 0.005	5.3
4MCAT	0.052 ± 0.013	4.4
total		97.1

[a] Mean ± standard deviation, $N = 3$. [b] Mean ± standard deviation, $N = 4$. [c] Mean ± standard deviation, $N = 10$.

with constant combination ratios and serial dilution of any combination. For the estimated response surface modeling, SigmaPlot 8.0 was used with smoothed data.

EPR Analysis. Fresh solutions of dihydroxybenzenes were prepared by dissolving the pure substances in 5 mL of culture medium DMEM, 50 mM pH 7.4 phosphate buffer solution, or other appropriate solution at equimolar concentrations of 1 mM or at concentrations proportional to the yield of the dihydroxybenzenes in TPM. The samples were immediately injected into an AquaX EPR cell (Bruker Biospin Corp., Billerica, MA) mounted in a high-sensitivity EPR cavity. The spectra were recorded at room temperature using a Bruker EMX EPR instrument at X-band. The following EPR parameters were used: microwave power, 3.98 mW; modulation amplitude, 0.2 G; time constant, 20.48 ms; conversion time, 20.48 ms; number of scans, 20. The concentration of free radicals was determined by double integrating the EPR signal using WinEPR software and comparing with known concentrations of TEMPOL free radical. EPR simulation was used to determine the hyperfine splitting constants and confirm the identity of the radical.

The solutions of dihydroxybenzenes were also stored in open-air tubes for 1 or 24 h prior to analysis by EPR to study the effect of aging on semiquinone radical formation. In addition, the interaction of HQ with other dihydroxybenzenes was also studied. Briefly, HQ was mixed with one constituent, and the EPR spectrum was acquired as described above. All experiments were carried out in triplicate, and the results are presented as the mean ± standard deviation.

Results

The TPM of cigarette smoke is a highly complex mixture containing many chemical classes of constituents, including saturated and unsaturated hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, phenols, nitriles, terpenoids, and alkaloids (*14*, *15*). More than 380 phenolic constituents have been reported to be formed in the main stream of cigarette smoke (*7*). Unsubstituted and methyl-substituted dihydroxybenzenes

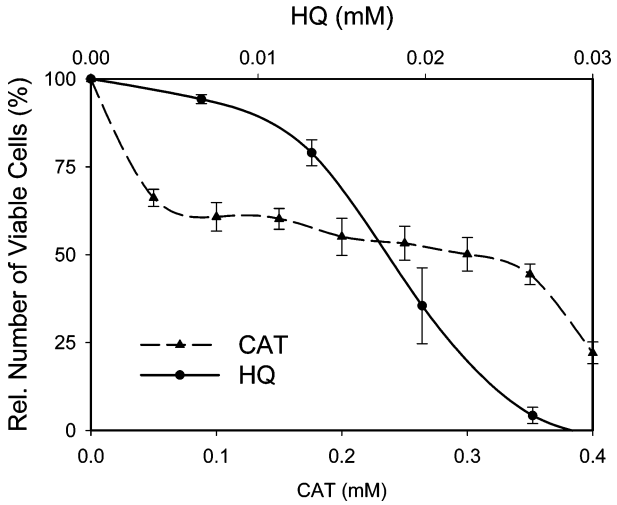

Figure 2. Cytotoxicities of hydroquinone and catechol. Values of the dose−response curves represent the mean and standard deviation of four microtiter plates.

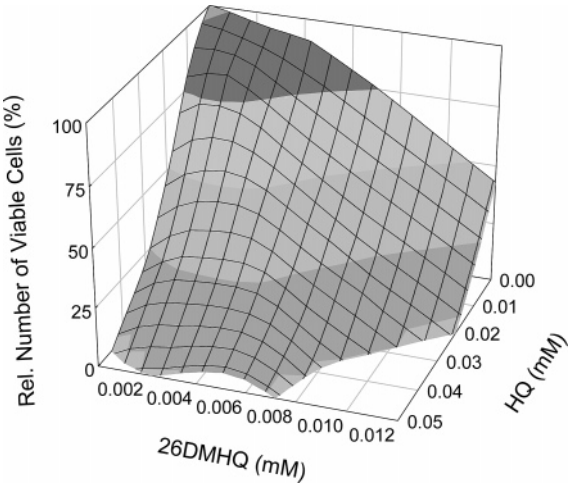

Figure 3. Estimated dose−response surface of hydroquinone, 2,6-dimethylhydroquinone, and three combinations using smoothed data for modeling. The vertical axis represents the relative number of viable cells. The nonlinear contour at 50% represents the EC_{50} values given in the isobologram in Figure 4.

are among the most abundant constituents in TPM. The constituents examined in our study are shown in Figure 1.

TPM Constituent Analysis and Cytotoxicity. As reported in Table 1, CAT and HQ exhibited the highest yields in the TPM of mainstream cigarette smoke, whereas the methyl-substituted HQ and CAT exhibited lower yields. The *para*-dihydroxybenzenes were more cytotoxic than the *ortho*-

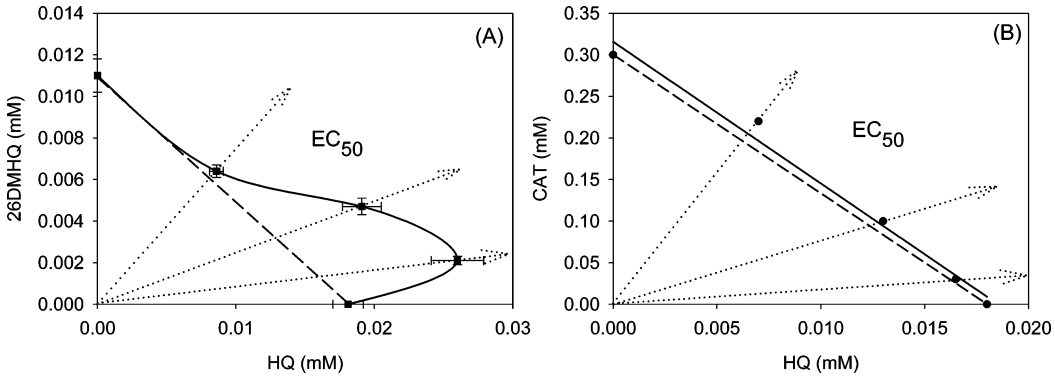

Figure 4. Isobolographic characterization of interaction of hydroquinone with (A) 2,6-dimethylhydroquinone and (B) catechol on cytotoxicity. Data represent EC_{50} values ±95% confidence limits. The three tested mixtures for A consisted of 12/1, 4/1, and 1/1 mixtures of HQ/26DMHQ; those for B consisted of 0.5/1, 0.1/1, and 0.03/1 mixtures of HQ/CAT. The dashed lines indicate the theoretical lines of additivity, whereas the dotted lines indicate the used fixed-ratio rays of the mixtures.

dihydroxybenzenes, and the methyl-substituted dihydroxybenzenes were more cytotoxic than their parent compounds, with the exception of TMHQ. For example, the cytotoxicity of the *para*-dihydroxybenzenes was in the order 2MHQ > 23DMHQ ≈ 26DMHQ > HQ > TMHQ (with a factor of 2.4 between the lowest and the highest EC50 values), and for the *ortho*-dihydroxybenzenes, the order was 3MCAT > 4MCAT > CAT (with a range of a factor of 6.4). Most of the dihydroxybenzenes examined showed similar sigmoidal dose−response curves, as shown for HQ in Figure 2. The exception was CAT, for which the dose−response curve follows a high-order polynomial equation with a long plateau of reduced number of viable cells (i.e., cell cycle arrest during the 24 h of incubation compared to the untreated control) in the middle section (Figure 2). The difference in dose−response curves between catechol and hydroquinone suggests that these two dihydroxybenzenes have different mechanisms of cytoxicity.

To determine whether interactions between the dihydroxybenzenes in the TPM affect their cytotoxicity, we carried out experiments in which HQ was mixed with another dihydroxybenzene and the specific cytotoxicity of the mixtures was determined. For example, the mixtures of 26DMHQ and HQ using constant concentration ratios induced cell viability differently from the theoretical effect of the combination of the pure substances, as seen in the mesh deformation in the 3D graph (Figure 3). The contour of constant 50% viability response in the three-dimensional dose−response curve represents the EC_{50} values plotted in the two-dimensional isobologram (Figure 4A). At low concentrations of 26DMHQ and high concentrations of HQ, the EC_{50} values of two of the three mixtures are higher than the theoretical line for an additive response (dashed line in Figure 4A). This finding indicates a lower cytotoxicity than the sum of the cytotoxicities of the individual compounds and therefore an antagonistic effect of the mixture of 26DMHQ and HQ. By comparison, when HQ was mixed with CAT, the cytotoxicities were found to be additive (the cytotoxicity of the mixture is equal to the sum of the cytotoxicities of the individual compounds), and therefore no interaction was observed, as shown in Figure 4B.

Semiquinone Radical Detection in the Presence of Dihydroxybenzenes. Dihydroxybenzenes are known to undergo auto-oxidation in solution, particularly at alkaline pH (*36*). To gain insight into the mechanism of cytotoxicity of dihydroxybenzenes and to investigate the possible involvement of free radicals, we employed EPR spectroscopy to assay the capacity of several dihydroxybenzenes found in cigarette smoke TPM (Figure 1) to form semiquinone radicals through auto-oxidation in the

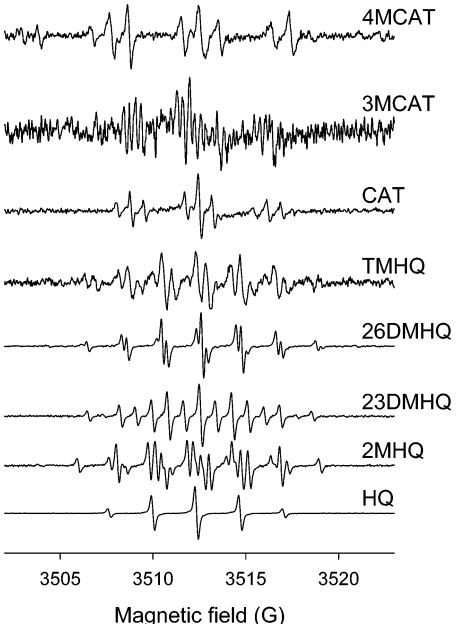

Figure 5. EPR spectra of semiquinone radicals formed in the presence of 1 mM dihydroxybenzenes in DMEM. The spectra were recorded after 1 h of incubation. Refer to the text for experimental conditions.

medium (DMEM) that is commonly employed in the NRU cytotoxicity assay. The pure dihydroxybenzenes were dissolved in aerated aqueous solutions of DMEM at pH 7.4 at 1 mM concentration. The EPR spectra of the corresponding semiquinone radicals were observed as shown in Figure 5. Each EPR spectrum exhibited a pattern of hyperfine couplings characteristic of the particular semiquinone radical, which were identical in each case to those previously reported (*37*). For example, 1 mM HQ in DMEM showed a quintet signal with a 1:4:6:4:1 intensity ratio and a hyperfine splitting constant for the interaction of the impaired electron with four equivalent protons (a_{4H} = 2.37 G), in accordance with the 1,4-benzosemiquinone anion radical (*36*). All other dihydroxybenzenes showed EPR signals and hyperfine splitting constants similar to those reported in the literature (*37*).

The pH and the concentration of the dihydroxybenzene solutions are important factors that affect the ability of the dihydroxybenzenes to auto-oxidize and generate semiquinone radicals. To mimic the actual yield of TPM constituents, we also examined pure dihydroxybenzenes at concentrations proportional to their yield in TPM. These concentrations are summarized in Table 2 along with the actual yields of the dihydroxybenzenes in the TPM of 2R4F cigarettes. When the

Table 2. Concentrations of the Dihydroxybenzenes Used in the EPR Experiments

dihydroxybenzene	yield in TPM (μg/cig)	concentration in EPR assay (μM)
HQ	34.4	743
2MHQ	4.02	67
23DMHQ	1.37	27
26DMHQ	0.49	10
TMHQ	1.83	44
CAT	45.3	819
3MCAT	5.3	85
4MCAT	4.4	71

Table 3. One-Electron Reduction Potentials (mV, 25 °C, pH 7.0) of the Redox Couples ($Q/Q^{-\bullet}$)

	$E(Q/Q^{-\bullet})$[a] at pH 7 (mV)
HQ	78
2MHQ	23
23DMHQ	−74
26DMHQ	−80
TMHQ	−165

[a] Taken from ref *38*.

dihydroxybenzenes were dissolved in either DMEM or phosphate buffer, significant amounts of semiquinone radicals formed in solutions of HQ, 2MHQ, and CAT, but none of the other compounds (Figure 6). The overall yield of semiquinone radicals was slightly higher in DMEM (Figure 6B) than in phosphate buffer (Figure 6A). Semiquinone radical formation from 2MHQ was observed in DMEM, but not in phosphate buffer. HQ produced the highest yield of semiquinone radicals, whereas CAT did not generate a significant amount of radicals, even with a concentration higher than that of HQ. When dissolved in water, DMSO, or ethanol at TPM concentration, only HQ generated semiquinone radicals in significant amounts (not shown).

Because the concentrations used above were different for each dihydroxybenzene and because only HQ, CAT, and 2MHQ generated measurable amounts of their corresponding semiquinone radicals, we also examined the radical formation for solutions of the dihydroxybenzenes at equimolar concentrations (1 mM). As shown in Figure 7, semiquinone radicals were readily detected for all eight dihydroxybenzenes; however, the increase in the amount of radicals did not increase in proportion to the concentration of the parent dihydroxybenzene. In addition, it appears that semiquinone formation is favored when the hydroxyl groups are in the para position (e.g., HQ) compared to the ortho position (e.g., CAT).

The reduction potential is known to be an essential parameter that governs the auto-oxidation of dihydroxybenzene compounds. In Table 3, we summarize the literature data on one-electron reduction potentials of quinone/semiquinone radical redox couples ($Q/Q^{-\bullet}$) for some of the dihydroxybenzenes (*38*). As shown in Figure 8, a linear correlation was found between the structure of the dihydroxybenzene and its reduction potential. In general, methyl substitution decreases the reduction potential of the dihydroxybenzene. Because no reduction potential for $Q/Q^{-\bullet}$ at pH 7 was reported for catechol in the literature, we compared the reduction potential of QH^-/Q^{2-} redox couples for catechol and hydroquinone, 459 and 530 mV, respectively (*39*). Judging from these values, Q^{2-} of HQ appears to be more reducing and should generate semiquinone radicals more easily than Q^{2-} of CAT.

Additional experiments were performed to study the interaction of HQ with other dihydroxybenzenes on semiquinone radical formation. HQ was mixed with each of the other dihydroxybenzenes individually in DMEM at the concentrations reported in Table 2, and the EPR spectra were acquired. In every case, only the EPR signal of 1,4-benzosemiquinone anion radical was observed, except in the presence of CAT, where a very weak signal of 1,2-benzosemiquinone anion radical was also observed (not shown). For example, the formation of semiquinone radicals from HQ in the presence of any of the methyl-substituted 1,4-dihydroxybenzenes decreased after only 10 min, whereas 1 or 24 h after mixing, the same amount of 1,4-benzosemiquinone radical was found (Figure 9). On the other hand, the amount of 1,4-benzosemiquinone radicals increased when HQ was mixed with the methyl-substituted 1,2-dyhydroxybenzenes. These results suggest that the interaction of dihydroxybenzenes with HQ and the formation of semiquinone radicals depend on the structure of the constituent and its concentration.

To elaborate on the interaction of HQ with other dihydroxybenzenes, we followed the effect of each dihydroxybenzene on the capacity of hydroquinone to generate semiquinone radicals, particularly 26DMHQ, which shows an interaction with HQ in the cytotoxicity assay. When 26DMHQ (10 or 100 μM) was added to HQ (740 μM; proportional to the TPM concentration), only 1,4-benzosemiquinone radical was observed, and its yield was decreased at 10 min compared to HQ alone and then appeared to be same within 1 and 24 h, as shown in Figure 10. However, at a higher concentration of 26DMH (500 μM), only the 2,6-dimethyl-1,4-benzosemiquinone radical was observed (Figure 11). Within 24 h, 1,4-benzosemiquinone appeared again (Figure 11), and its yield was almost 2 times higher than in the case of HQ alone (Figure 10). The interaction of CAT with HQ was different from that of 26DMHQ. When CAT was added to HQ at different concentrations, only 1,4-benzosemiqunone radical was observed, and no significant effect on the yield of the radical was observed, except when the concentration of CAT was higher than that of HQ, where an increase in the semiquinone radical yield was observed (Figure 12).

Discussion

The dihydroxybenzenes in the TPM of cigarette smoke are an important class of constituents that can act either as pro-oxidants or as anti-oxidants. Consequently, it is not surprising that the dihydroxybenzenes exhibit significant cytotoxicity. It has long been known that HQ can be oxidized to benzoquinone, a potent hemeatotoxic, genotoxic, and carcinogenic agent (*40*, *41*). Moreover, xenobiotic HQ is known to cause the accumulation of free radicals in the intracellular environment, which can lead to chromosomal aberration and cell damage through oxidative stress (*42*−*44*). HQ and CAT in cigarette smoke TPM have both been shown to block lymphocyte proliferation and inhibit T cell activation in the lung by inhibition of ribonucleotide reductase (binding to tyrosyl radical), the rate-limiting enzyme in DNA synthesis (*45*, *46*). However, the observed differences between the dose−response curves of catechol and hydroquinone in the present study suggest that the underlying cytotoxic processes are different for these two substances. The shape of the dose−response curve for catechol, with its long plateau at intermediate concentrations (0.1−0.3 mM), suggests that CAT is merely cytostatic up to concentrations of 0.3 mM but cytotoxic at higher concentrations.

Our data show that the methyl-substituted dihydroxybenzenes are more cytotoxic than the non-methylated parent compounds, a result that is consistent with previous studies. Moridani et al. (*47*), for example, reported that substituted phenols are more cytotoxic when they are lipophilic and have lower redox

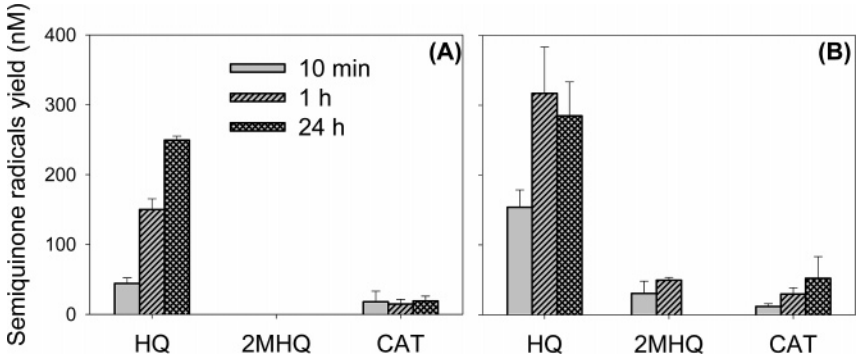

Figure 6. Yield of semiquinone radicals formed during the auto-oxidation of dihydroxybenzenes in (A) 50 mM pH 7.4 potassium phosphate and (B) DMEM. The concentrations of the dihydroxybenzenes used were proportional to their yields in the TPM (see Table 2).

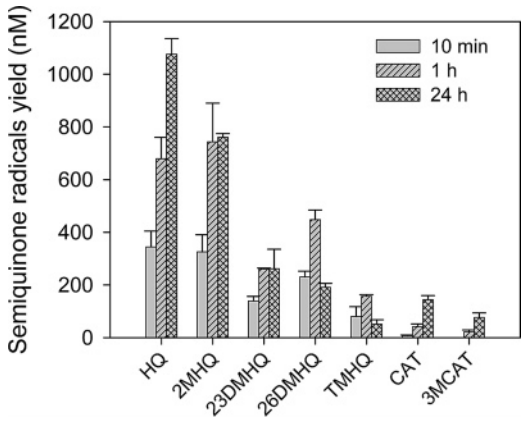

Figure 7. Yield of semiquinone radicals formed during the auto-oxidation of 1 mM dihydroxybenzenes in DMEM.

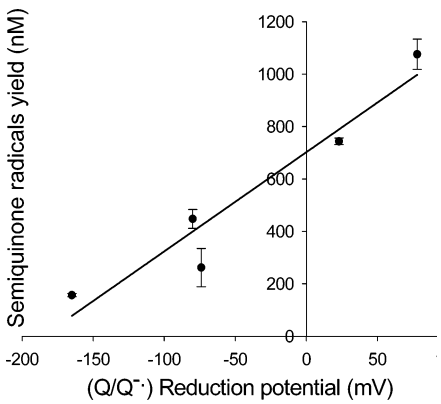

Figure 8. Plot of the yield of semiquinone radicals formed when 1 mM of dihydroxybenzenes was dissolved in DMEM versus the reduction potential of $Q/Q^{-\bullet}$.

potentials, whereas substituted catechols with higher lipophilicity and distribution coefficients, lower degree of ionization, and higher pK_a are more toxic than other catechols (*48*). In a study of quantitative structure−activity relationships (QSARs) of phenols, Smith et al. (*7*) showed that substituted phenols with electron-releasing groups have a greater potential to produce toxic phenoxyl free radicals. However, in our study, whereas mono- and dimethyl substitution had this effect, trimethyl substitution did not.

In certain cases, combinations of dihydroxybenzenes can exhibit cooperative cytotoxic effects, as we have shown for certain concentrations of HQ and 26DMHQ, thus affecting the specific cytotoxicity of the mixture. The apparent cytotoxicity of HQ was reduced (less than additive) by 26DMHQ at low

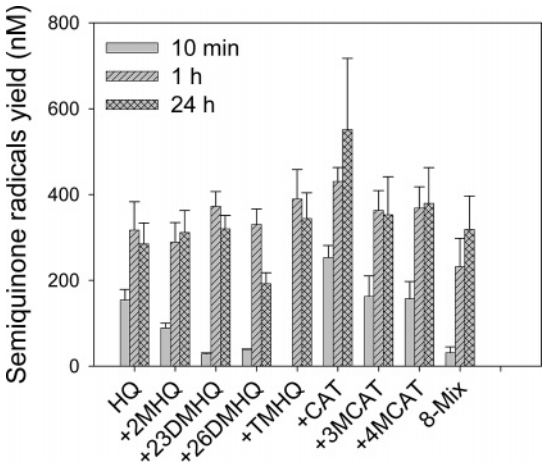

Figure 9. Effect of interactions of hydroquinone with other dihydroxybenzenes in DMEM. The concentrations used were those reported in Table 2.

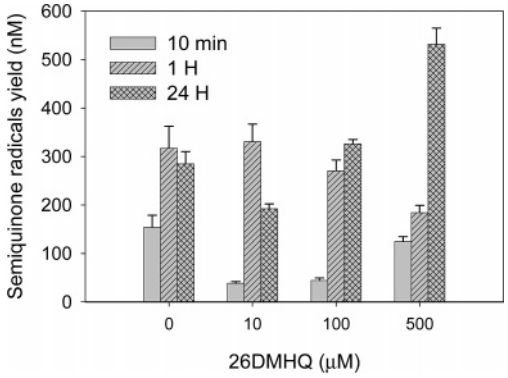

Figure 10. Semiquinone radical yield in the presence of HQ and 26DMHQ at different concentrations in DMEM. The *y* axis represents the total yield of semiquinone radical in solution.

concentrations relative to the EC_{50} value of 26DMHQ. In contrast, higher concentrations of 26DMHQ resulted in a more additive cytotoxicity. This indicates that the two compounds display conflicting actions, probably depending on their relative redox potential and their concentration by as-yet-unidentified mechanisms. However, CAT induced only an additive effect when combined with hydroquinone. The observation that dihydroxybenzenes can interact in the cytotoxicity assay raises the question of whether literature data reporting the biological effects of pure HQ or CAT are representative of their behavior in a complex matrix, as in cigarette smoke TPM.

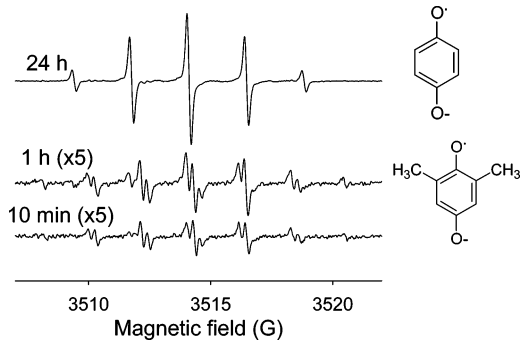

Figure 11. EPR spectra of semiquinone radicals when 500 μM of 26DMHQ was mixed with HQ as function of time.

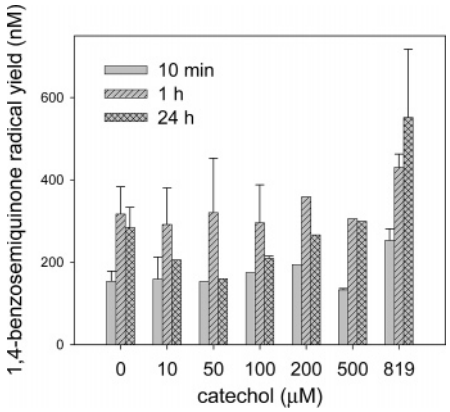

Figure 12. Semiquinone radical yield in the presence of HQ and CAT at different concentrations in DMEM on semiquinone radical yield. The *y* axis represents the total yield of semiquinone radical in the solution.

It is well-known that that semiquinone radical formation from HQ and CAT is favored in alkaline solution (*36*). The results of Pedersen (*36*), as well as our own, show that semiquinone radicals can also be generated at physiological pH. Moreover, we have shown that the dihydroxybenzenes undergo auto-oxidation leading to the formation of semiquinone radicals in cell culture media, such as DMEM.

The reactivity, thermodynamic, and redox properties of quinones, semiquinones, and the parent quinols are of major importance in understanding their biological activity. As we have shown, 2MHQ, 23DMHQ, and 26DMHQ are more cytotoxic than HQ, but at the same concentration, they generate smaller amounts of semiquinone radicals. This suggests that the cytotoxicity of the methyl-substituted dihydroxybenzenes is not solely dependent on radical formation and that other mechanisms should be considered. Smith et al. (*7*) have suggested that the electron-donating character of methyl groups stabilizes the semiquinone radical, thereby prolonging its lifetime and enhancing its potential for biological damage. Thus, the kinetics of semiquinone radical formation can be a determining factor in the cytotoxicity of dihydroxybenzenes. However, for dihydroxybenzenes lacking an electron-donating group, the cytotoxicity can be dominated by other factors, e.g., hydrophobicity (*7*).

A correlation between the cytotoxicity of quinones and their reduction potentials has been previously reported (*49*). It was observed, for example, that the toxicity of natural hydroxyanthraquinones increases at pH 7 with an increase in reduction potential, pointing to an oxidative stress mechanism (*49*). The cytotoxicity and the capacity to form semiquinone radicals vary with the relative position of hydroxyl groups in the dihydroxybenzenes. As we have shown, HQ is more toxic and generates more semiquinone radicals than CAT. It was previously shown that the relative ease of auto-oxidation of dihydroxybenzenes is in the order para > ortho > meta (*39*).

Multiple mechanisms might be involved in the toxicity of dihydroxybenzenes. For example, a study of the structure−toxicity relationships for phenols in isolated rat hepatocytes showed that one or a combination of mechanisms, e.g., mitochondrial uncoupling, phenoxyl radicals, or phenol metabolism to quinine, can contribute to the cytotoxicity of phenols toward hepatocytes (*47*). Two plausible mechanisms by which dihydroxybenzenes can damage cellular macromolecules are (1) the covalent binding of the reactive metabolites (semiquinone radicals and quinones) to essential macromolecules such as protein and DNA and (2) oxidative stress resulting from the formation of ROS via the redox cycling of quinones through semiquinone radical intermediates. Formation of superoxide radicals, hydrogen peroxide, and hydroxyl radicals in ACT has been demonstrated (*23*), suggesting that dihydroxybenzenes are involved in smoking-related oxidative stress.

Because of the large number of constituents found in cigarette smoke TPM, chemical interactions between the constituents or cooperative toxicological effects might affect the cytotoxicity of the TPM. In the NRU cytotoxicity assay, an antagonistic effect was observed when hydroquinone was mixed with 26DMHQ. Similarly, EPR data showed that some dihydroxybenzenes can interact with hydroquinone and alter the yield of semiquinone radicals, whereas other combinations had no effect. A simple relationship, however, was not found between the radical yield and the cytotoxicity of the mixture. There is a paucity of data to indicate whether the critical interactions occur in the extracellular cell growth medium or within the cells. Other factors might influence cytotoxicity and should also be taken into account; for example, the presence of transition metals in the TPM. Iron and copper have been shown to enhance the oxidation of hydroquinone in a concentration-dependent manner (*50*−*52*). It has been shown that, in the presence of NADH, CAT induced more Cu^{2+}-mediated DNA damage than HQ, whereas the reverse relation was observed in the absence of NADH (*53*). The peroxidase-dependent conversion of hydroquinone to benzoquinone has been shown to be stimulated by phenolic constituents such as catechol, resorcinol, and cresol (*54*).

Summary and Conclusions. The dihydroxybenzenes in our study, constituents in the TPM of cigarette smoke, were found to exhibit significant cytotoxicity. The methyl-substituted dihydroxybenzenes were shown to have higher cytotoxicity than the unsubstituted compounds. The dihydroxybenzenes were shown to generate semiquinone radicals in DMEM, the medium used in the NRU cytotoxicity assay. Nevertheless, a correlation between the abundance of semiquinone radicals formed and cytotoxicity was not found. The observed interaction between 2,6-dimethylhydroquinone and hydroquinone in the cytotoxicity assay and EPR analysis demonstrate that the EC_{50} values in binary mixtures of the dihydroxybenzenes cannot, in general, be assumed to be additive. Consequently, the interpretation of the bioactivity of cigarette smoke evaluated by similar methods should consider the possible effects of the complex TPM matrix on the activities of the individual constituents.

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