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Potentiation of DNA adduct formation in HL-60 cells by combinations of benzene metabolites

(32P postlabeling/myeloperoxidase/human leukemogenesis)

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ABSTRACT **Using P_1 nuclease enhanced ^{32}P postlabeling, we investigated DNA adduct formation in HL-60 promyelocytic leukemia cells treated with the benzene metabolites hydroquinone, catechol, and 1,2,4-benzenetriol. Comparison of the slopes of the dose–response curves showed that hydroquinone was 7–9 times more effective than 1,2,4,-benzenetriol and catechol at inducing DNA adducts. Comparison of hydroquinone with catechol showed a good correlation between adduct formation and cytotoxicity. Similar comparisons of hydroquinone and 1,2,4,-benzenetriol suggest that cellular processes in addition to DNA adduct formation contributed to cytotoxicity. In cells treated with the combination of hydroquinone and either catechol or 1,2,4,-benzenetriol, DNA adduct formation was 3–6 times greater than the sum of adduct formation produced by single-agent treatments. Treatment with hydroquinone and 1,2,4,-benzenetriol produced DNA adducts not detected after treatment with either metabolite alone. The synergistic interaction of benzene metabolites in the production of DNA adducts may play an important role in the genotoxic effects of benzene *in vivo*.**

Acute exposure to benzene has a strong toxic effect on the hematopoietic system of laboratory animals (1) and humans (2). Chronic exposure is carcinogenic in rats and mice (3, 4) and leukemogenic in humans (5, 6). To exert its toxic effects, benzene must be metabolized (7–9). The first step of benzene metabolism is the formation of benzene oxide by cytochrome P-450 oxidation followed by either spontaneous conversion to phenol (9) or to 5,6-dihydroxy-1,3-cyclohexadiene by epoxide hydrolase. Phenol is further oxidized to hydroquinone (HQ). Catechol may be obtained by the rearomatization of 5,6-dihydroxy-1,3-cyclohexadiene by dihydrodiol dehydrogenase (10); 1,2,4-Benzenetriol (BT) results from the oxidation of either HQ or catechol. These metabolites accumulate in the bone marrow (7, 11, 12), where they may serve as reducing cosubstrates for peroxidases (12–16). The quinones or semiquinone radicals formed by peroxidase activation can react with both DNA (17–19) and protein (14–16).

Benzene administration has resulted in the formation of DNA adducts *in vivo*. Although the metabolites responsible for this binding have not been identified (20–23), our ^{32}P-postlabeling studies have shown that HQ and *p*-benzoquinone (*p*-BQ) form the same DNA adduct in HL-60 promyelocytic leukemia cells (24). Neither phenol nor HQ is myelotoxic by itself, but in combination they have a myelotoxic effect similar to that of benzene (25). The administration of phenol with either HQ or catechol resulted in a synergistic inhibition of ^{59}Fe uptake by erythroid bone marrow cells in mice (26). Similarly, phenol and HQ have a strong synergistic effect on micronucleus formation in Swiss mice (27) and in human lymphocytes (28). These results suggest that the genotoxic effects of benzene may be due to a synergistic interaction of its metabolites.

In this study, we investigated DNA adduct formation and cytotoxicity in HL-60 cells treated with HQ, catechol, and BT. We also sought to determine whether these benzene metabolites interact synergistically to induce DNA adduct formation.

MATERIALS AND METHODS

Culture Conditions and Cell Treatments. HL-60 cells were grown in 175-cm^2 flasks containing 100 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μg of gentamicin per ml. The cells were maintained and treated at 37°C in a humidified 5% CO_2/95% air atmosphere. HQ, catechol, and BT (Aldrich) were separately dissolved in distilled water and added directly to the culture medium. For the concentration– and time–response studies, cells were exposed to each metabolite at 0–2000 μM for 1–72 hr. In separate experiments, cells were treated with 50–250 μM catechol or 25–100 μM BT as single agents or in combination with 50 μM HQ for 24 hr. Only distilled water was added to the control samples. After treatment, the HL-60 cells were centrifuged at 3000 × *g* for 10 min. The pellets were washed with cold Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free), centrifuged at 3000 × *g* for 10 min, and frozen at −70°C until the DNA was isolated.

Cell Viability. Cell viability was determined by trypan blue dye exclusion. After treatment, an aliquot of cells was mixed with a 0.4% solution of trypan blue (GIBCO) and the cells were counted in a BrightLine Neubauer hemacytometer. The percentage of viable (unstained) cells was estimated by scoring 150–300 cells per sample.

^{32}P Postlabeling. The DNA was isolated (29) and postlabeled by the P_1 nuclease modified ^{32}P method of Reddy and Randerath (30). The postlabeled mixtures were applied to 10 × 10-cm polyethyleneimine (PEI)-cellulose plates (Brinkman, Des Plaines, IL) in aliquots of 5 μl at a distance 2 cm from the bottom and 2 cm from the left edge of the plate. Paper wicks were attached to the top of the PEI plates. After each chromatography, the wicks were removed, and the plates were washed with distilled water and air-dried. The plates were developed overnight in 0.4 M sodium phosphate buffer (pH 6.8) to ≈16 cm onto the wick and then developed in the same direction to the top of the plate with 1.8 M lithium formate/4.5 M urea, pH 3.5. A paper wick was attached to the right edge of each plate, and the plates were developed overnight at a right angle to the previous direction of development in 0.36 M lithium chloride/0.22 M Tris/3.8 M urea, pH 8. The plates were subsequently developed in the same direction with 1.7 M sodium phosphate (pH 6) to 5–6 cm onto

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Abbreviations: HQ, hydroquinone; BT, 1,2,4-benzenetriol; *p*-BQ, *p*-benzoquinone.

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the paper wick. The adducts were located by autoradiography using Kodak XAR-5 film and a DuPont Cronex Lightning Plus intensifying screen.

Calculation of Relative Adduct Levels. The radioactive spots on the PEI plates were scraped into scintillation vials containing 5 ml of scintillation cocktail (Safety Solve; Research Products International) and radioactivity was determined by scintillation counting. Background radioactivity was determined by scraping and counting of areas adjacent to the radioactive spots and was subtracted from the radioactivity of the adducts. The counting efficiency for ^{32}P was 0.76. The relative adduct levels were calculated as described (24). By assuming that 4 μg of DNA was 1.21×10^4 pmol of 2'-deoxyribonucleoside 3'-phosphate and that the specific activity of the [^{32}P]ATP was 9.36×10^6 cpm/pmol, the relative adduct level was calculated as (total cpm in adducts)/11.32×10^{10} cpm.

RESULTS

Treatment with 500 μM HQ produced a single DNA adduct (Fig. 1*A*). The relative adduct level was 7.8 ± 0.8 adducts per 10^7 nucleotides (mean ± SEM). The formation of DNA adducts in HL-60 cells treated with HQ was time- and concentration-dependent (24). No DNA adducts were detected in untreated control samples (data not shown).

Treatment with various concentrations of catechol resulted in the formation of four major DNA adducts of equal intensity (Fig. 1*B*) and similar mobility on the PEI plates. After treatment with 1000 μM catechol for 24 hr, the extent of DNA modification was 1.9 ± 0.6 adducts per 10^7 nucleotides. DNA adduct formation was concentration-dependent up to 1000 μM (Fig. 2*A*) and time-dependent up to 72 hr (Fig. 2*B*). Very little cytotoxicity was observed after treatment intervals up to 8 hr even with 2000 μM catechol; after 24 or 72 hr, the increase in cytotoxicity was concentration- and time-dependent (Fig. 2*C*).

Treatment with 250 μM BT for 8 hr produced a single DNA adduct (Fig. 1*C*). Treatment with 50–250 μM BT for 8 hr produced a linear increase in adduct formation; a plateau was reached at higher concentrations (Fig. 3*A*). DNA adduct formation induced by 250 μM BT was time-dependent up to 8 hr (Fig. 3*B*). BT was more cytotoxic than catechol (Figs. 2*C* and 3*C*). The increase in cytotoxicity of BT was concentration-dependent after 4 hr of treatment and time-dependent at a concentration of 250 μM.

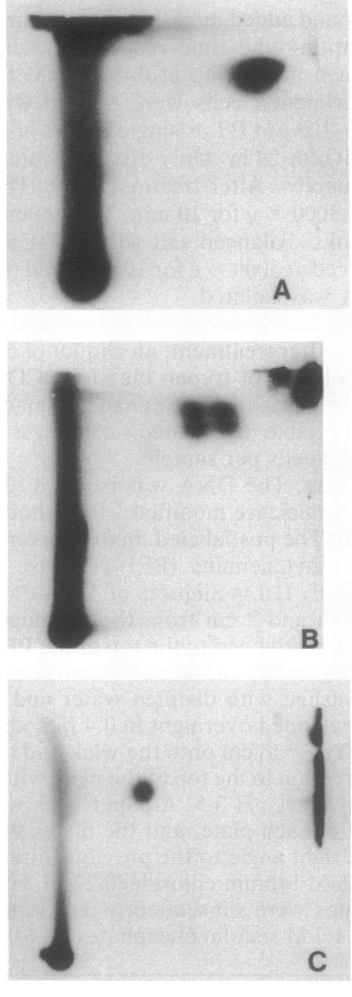

FIG. 1. Autoradiograms of ^{32}P-postlabeled DNA isolated from HL-60 cells treated for 8 hr with 500 μM HQ (*A*), 1000 μM catechol (*B*), or 250 μM BT (*C*). Films were exposed for 2–3 hr at −70°C.

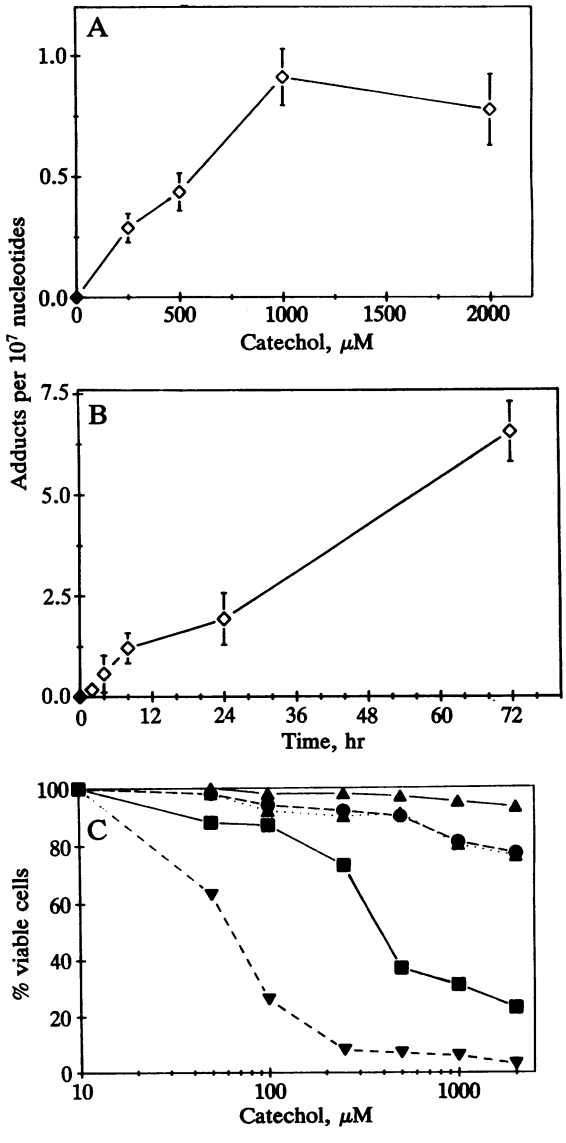

FIG. 2. (*A* and *B*) Induction of DNA adducts in HL-60 cells treated with 0–2000 μM catechol for 24 hr (*A*) and with 1000 μM catechol for 2–72 hr (*B*). (*C*) Percentage of viable cells, determined by trypan blue exclusion, after treatment with various concentrations of catechol for 2 hr (▲–▲), 4 hr (●—●), 8 hr (▲···▲), 24 hr (■–■), or 72 hr (▼- - -▼). Cell treatments were repeated independently at least twice, and each sample was analyzed for DNA adducts one to five times by ^{32}P postlabeling. Error bars show SEM (n = 3–6). For symbols with no error bars, SEM was smaller than the symbol size.

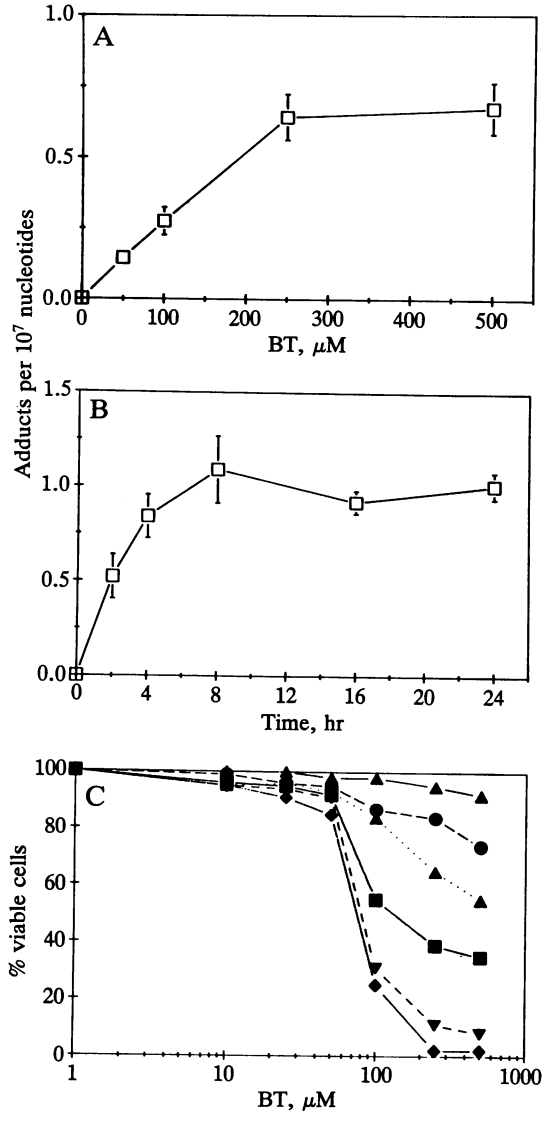

FIG. 3. (*A* and *B*) Induction of DNA adducts in HL-60 cells treated with 0–500 μM BT for 8 hr (*A*) or with 250 μM BT for 2–24 hr (*B*). (*C*) Percentage of viable cells, determined by trypan blue exclusion, after treatment with various concentrations of BT for 1 hr (▲–▲), 2 hr (●—●), 4 hr (▲···▲), 8 hr (■–■), 16 hr (▼- - -▼), or 24 hr (◆–◆). Error bars show SEM (n = 3–10). For symbols with no error bars, SEM was smaller than the symbol size.

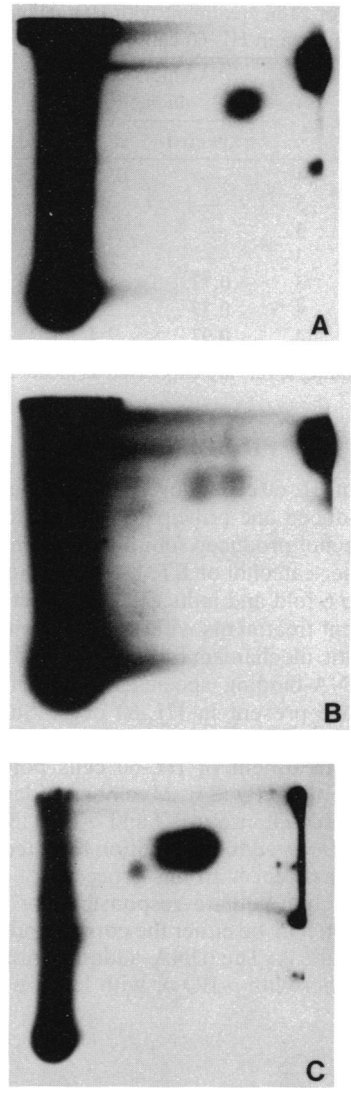

FIG. 4. Autoradiograms of ^{32}P-postlabeled DNA isolated from HL-60 cells treated for 24 hr with 50 μM HQ (*A*), 250 μM catechol (*B*), or 50 μM HQ and 250 μM catechol (*C*). Films were exposed for 3 hr (*A* and *C*) or 6 hr (*B*) at −70°C.

The extents of the DNA modifications induced by the benzene metabolites were compared by analyzing the concentration– and time–response curves for catechol and BT shown in Figs. 2 and 3 and those for HQ from our previous study (24). The slopes of the combined curves were 1.57×10^{-3}, 0.23×10^{-3}, and 0.17×10^{-3} adducts $\times 10^{-7}$ per μM·hr (r = 0.96, 0.61, and 0.97) for HQ, BT, and catechol, respectively. Thus, HQ was ≈7-fold more effective than BT and 9-fold more effective than catechol at inducing DNA adduct formation in HL-60 cells.

The autoradiograms obtained after 24-hr treatment with 50 μM HQ or 250 μM catechol showed barely detectable DNA adduct formation (Fig. 4 *A* and *B*). The extent of DNA modification was 0.77 ± 0.14 and 0.2 ± 0.09 adduct per 10^7 nucleotides, respectively (Table 1). No DNA adducts were detected after treatment with 50 or 100 μM catechol. The autoradiogram obtained after treatment with 50 μM HQ and 250 μM catechol is shown in Fig. 4*C*; individual DNA adducts produced by HQ and catechol were not resolved with the chromatographic conditions used. The combined treatment produced an adduct level of 2.4 ± 0.28 adducts per 10^7 nucleotides; the sum of the individual treatments was 0.97 adduct per 10^7 nucleotides. Thus, the combination treatment increased adduct formation by 2.5-fold. The combination of 50 μM HQ with 50 or 100 μM catechol increased adduct formation by 2.2- and 2.7-fold, respectively (Table 1).

The autoradiograms obtained after 24-hr treatments with 100 μM BT, alone and in combination with 50 μM HQ, are shown in Fig. 5. Four well-resolved DNA adducts were detected after the combination treatment (Fig. 5*B*). The relative distribution of adducts 1, 2, 3, and 4 was 49%, 34%, 14%, and 3%, respectively. The combination of HQ and BT resulted in greater formation of adducts 1 and 3 (Fig. 5*B*) than treatment with either agent alone (Figs. 4*A* and 5*A*). This combination also induced the formation of a new adduct (no. 2) and a minor adduct (no. 4) that were not detected after the single-agent treatments. The relative adduct levels of combined and single-agent treatments are shown in Table 2. Comparison of the observed and expected adduct levels showed that the combination of 50 μM HQ and 100 μM BT increased adduct formation by 5-fold. Similarly, 25 and 50 μM BT in combination with 50 μM HQ increased adduct formation by 5.1- and 6.4-fold, respectively.

Table 1. Effect of 24-hr treatment with HQ and catechol on DNA adduct formation in HL-60 cells

Dose, μM			Adducts per 10^7 nucleotides		
HQ	Catechol	n	Expected	Determined	Potentiation
50	0	4	—	0.77 ± 0.14	—
0	50	3	—	ND	—
0	100	3	—	ND	—
0	250	3	—	0.2 ± 0.09	—
50	50	3	0.77	1.7 ± 0.15	2.2
50	100	4	0.77	2.1 ± 0.22	2.7
50	250	4	0.97	2.4 ± 0.28	2.5

Determined adduct levels are expressed as mean ± SEM. ND, not detectable.

Table 2. Effect of 24-hr treatment with HQ and BT on DNA adduct formation in HL-60 cells

Dose, μM			Adducts per 10^7 nucleotides		
HQ	BT	n	Expected	Determined	Potentiation
50	0	12	—	0.41 ± 0.09	—
0	25	4	—	ND	—
0	50	7	—	0.09 ± 0.02	—
0	100	7	—	0.24 ± 0.02	—
50	25	6	0.41	2.10 ± 0.37	5.1
50	50	7	0.42	2.70 ± 0.47	6.4
50	100	12	0.65	3.24 ± 0.36	5.0

Determined adduct levels are expressed as mean ± SEM. ND, not detectable.

DISCUSSION

These studies in HL-60 cells showed that treatment with either HQ or BT produced one principal DNA adduct, while treatment with catechol produced four adducts. The combination of HQ and either catechol or BT, however, increased adduct formation 3- to 6-fold and induced two adducts not obtained after single-agent treatments with these benzene metabolites.

The enzymatic mechanism for activation of HQ, catechol, and BT to DNA-binding species is probably via cellular myeloperoxidase present in HL-60 cells. Support for this conclusion is based upon our observation that combined H_2O_2 and HQ treatment of HL-60 cells potentiates DNA adduct formation by HQ (24). Myeloperoxidase can oxidize both HQ and catechol into *p*-BQ and *o*-BQ (13–16); the time dependency of DNA adduct formation by catechol and BT in this study is consistent with this process.

The reactive intermediate responsible for cellular DNA adduct formation may be either the corresponding quinone or semiquinone radical. The DNA adducts formed in HL-60 cells treated either with *p*-BQ or with HQ are identical (24).

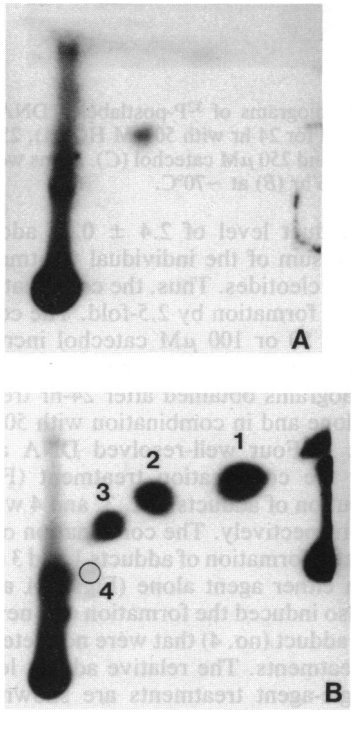

FIG. 5. Autoradiograms of ^{32}P-postlabeled DNA isolated from HL-60 cells treated for 24 hr with 100 μM BT (*A*) or 50 μM HQ and 100 μM BT (*B*). Films were exposed at −70°C for 3 and 1.5 hr, respectively.

However, the adducts formed in purified DNA treated with *p*-BQ do not correspond to those in cells treated with *p*-BQ. These results indicate that the cellular environment influences adduct formation. Further studies are required to determine the nature of the DNA-binding species for the benzene metabolites within cells.

Although it has been shown that benzene administration results in the formation of DNA adducts, it has not been determined which of the metabolites are responsible for this effect (20–23). Our results clearly show that all three benzene metabolites can be activated to form DNA adducts in HL-60 cells. However, HQ is 7-fold more effective than BT and 9-fold more effective than catechol at inducing DNA adduct formation in HL-60 cells. Previous comparisons of HQ with *p*-BQ have shown *p*-BQ to be 20-fold more effective than HQ in the formation of DNA adducts (24). These results in HL-60 cells are similar to those of Reddy *et al.* (31), who showed the formation of DNA adducts in rat zymbal glands in organ culture treated with HQ, catechol, and BT.

Treatment of HL-60 cells with catechol or BT was cytotoxic as measured by trypan blue exclusion. Comparison of catechol and BT treatment showed that BT was ≈12-fold more cytotoxic than catechol. One possible mechanism for this cytotoxicity is the formation of DNA adducts. When formation of DNA adducts and induction of cytotoxicity by catechol and HQ treatment are compared, there is general agreement between these end points. In HL-60 cells, HQ is ≈8-fold more cytotoxic and ≈9-fold more effective at inducing DNA adduct formation than catechol. In contrast, for the same level of cytotoxicity, HQ and catechol induced more adducts than BT. This suggests that mechanisms other than adduct formation contribute to the cytotoxicity of BT. Previous studies have shown that BT effectively induces DNA strand breaks in cells (32), possibly due to the formation of semiquinone radicals, activated oxygen, and hydroxyl radicals (33, 34).

Combination treatment with HQ and catechol increased the relative adduct level in HL-60 cells by 2.2- to 2.7-fold compared with the levels expected from single-agent treatments. The concentrations of catechol that in combination with HQ potentiated DNA adduct formation produced, by themselves, undetectable or very low DNA adduct levels. The DNA adducts formed by the combination of HQ and catechol were not sufficiently resolved to investigate the effects of the combined treatment on the individual adducts.

Similarly, treatment with HQ and BT resulted in a 5- to 6.5-fold increase in DNA modification in HL-60 cells. BT concentrations as low as 25 μM potentiated DNA adduct formation by 50 μM HQ. Comparison of adduct formation induced by HQ combined with either 50 μM catechol or 50 μM BT shows that BT is ≈3 times more effective than catechol. In combination with HQ, BT also induced new DNA adducts (nos. 2 and 4) that were not detected after treatment with HQ or BT alone. The formation of these new

adducts suggests the presence of reactive species that were absent after single-agent treatments. The generation of such reactive species might be due to increased concentrations of reactive oxygen molecules produced during the oxidation of BT (33, 34). These reactive oxygen molecules may activate HQ and BT to reactive species that are different from those formed by myeloperoxidase oxidation and result in the formation of the new DNA adducts observed.

The combination of phenol and HQ did not potentiate adduct formation in a concentration range similar to that used for catechol and BT. Preliminary studies showed that phenol concentrations >1 mM are required to stimulate adduct formation (G.L., unpublished result). This finding suggests that the observed potentiation of biological effects from the combination of phenol with HQ (25–27) may be due to the further metabolism of phenol to HQ and catechol. Our results suggest that the observed potentiation of the biological effects of HQ, catechol, and BT in combination may be due to a synergistic interaction of benzene metabolites in the production of increased levels of DNA adducts.

Several biochemical mechanisms have been proposed to explain the potentiation of DNA adduct formation by HQ combined with catechol or BT. These include alteration of the active sites of the peroxidase enzyme (35, 36) and increased production of the reactive intermediates leading to DNA adduct formation (37–39). Further studies are required to elucidate the mechanisms responsible for the observed increased DNA adduct formation.

The synergistic interaction of metabolites in the production of DNA adducts may have significant biological effects. Human occupational and environmental exposure is often to a complex mixture of these agents. The interaction of individual metabolites from compounds in these mixtures may result in increased DNA adduct formation and potentially increased genotoxic effects from low-level exposure.

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