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# A comparison of the cytogenetic alterations and global DNA hypomethylation induced by the benzene metabolite, hydroquinone, with those induced by melphalan and etoposide

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**Specific cytogenetic alterations and changes in DNA methylation are involved in leukemogenesis. Benzene, an established human leukemogen, is known to induce cytogenetic changes through its active metabolites including hydroquinone (HQ), but the specific alterations have not been fully characterized. Global DNA hypomethylation was reported in a population exposed to benzene, but has not been confirmed *in vitro*. In this study, we examined cytogenetic changes in chromosomes 5, 7, 8, 11 and 21, and global DNA methylation in human TK6 lymphoblastoid cells treated with HQ for 48 h, and compared the HQ-induced alterations with those induced by two well-known leukemogens, melphalan, an alkylating agent, and etoposide, a DNA topoisomerase II inhibitor. We found that rather than inducing cytogenetic alterations distinct from those induced by melphalan and etoposide, HQ induced alterations characteristic of each agent. HQ induced global DNA hypomethylation at a level intermediate to melphalan (no effect) and etoposide (potent effect). These results suggest that HQ may act similar to an alkylating agent and also similar to a DNA topoisomerase II inhibitor in living cells, both of which may be potential mechanisms of benzene toxicity. In addition to cytogenetic changes, global DNA hypomethylation may be another mechanism underlying the leukemogenicity of benzene.**
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## Introduction

Specific cytogenetic alterations are commonly observed in patients with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), and are also characteristic of therapy-related MDS and AML (t-MDS and t-AML).[1,2] For example, loss of whole chromosomes 5 and 7 (−5 and −7) or partial long-arm deletion of these two chromosomes [del(5q) and del(7q)] commonly occurs in t-MDS/AML associated with treatment with alkylating agents, such as melphalan, whereas translocations of chromosomes 11 [t(11;?)] and 21 [t(21;?)] are hallmarks of t-MDS/AML associated with DNA topoisomerase II (topo II) inhibitors, such as etoposide.[1,2] Gain of chromosomes 8 and 21 (+8 and +21) and translocations between 8 and 21 [t(8;21)] are also common in MDS/AML patients.[3–5] These specific cytogenetic alterations are generally thought to be causal in human leukemogenesis.

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As well as cytogenetic aberrations, altered DNA methylation may have an important role in the pathogenesis of AML. DNA methylation, the addition of a methyl group at the C-5 position of cytosine in CpG dinucleotides, is one of the most common epigenetic events in the mammalian genome and has a critical role in controlling gene expression and in maintaining genome stability.[6] Global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes are frequently observed in hematological malignancies.[7]

Altered cytogenetics and DNA methylation are also thought to be potential mechanisms underlying the leukemogenesis associated with benzene. Benzene, an important industrial chemical and universal environmental pollutant, is an established human leukemogen,[8] which causes MDS and AML[9] and probably causes non-Hodgkin's lymphoma.[10] Benzene is known to induce cytogenetic changes through its active metabolites including hydroquinone (HQ), but the characteristic alterations have not been fully characterized.[11] Global DNA hypomethylation was reported in gas station attendants and traffic police officers exposed to low levels of benzene.[12] As the subjects may have been co-exposed to other chemicals, an *in vitro* study with benzene metabolites would help to clarify the association.

In order to shed further light on the mechanisms of benzene-induced leukemogenesis, we examined cytogenetic changes in chromosomes 5, 7, 8, 11 and 21, and global DNA methylation in a human lymphoblastoid cell line, TK6, treated with the benzene metabolite, HQ, for 48 h, and compared the results with those induced by the alkylating agent, melphalan, and the DNA topoisomerase inhibitor, etoposide, both of which are associated with t-AML.

## Materials and methods

### *Cell culture, chemical treatment and cytotoxicity*

The human lymphoblastoid cell line, TK6, was maintained in RPMI 1640 medium (GIBCO, San Diego, CA, USA) containing 10% fetal bovine serum (Omega Scientific, Tarzana, CA, USA) and 1% penicillin and streptomycin (Omega Scientific) at 37 °C in a 5% $CO_2$ moist atmosphere. HQ (⩾99%) was dissolved in sterile 1 × phosphate-buffered saline (PBS) immediately before treatment for all experiments. Melphalan (⩾95%) and etoposide (⩾98%) were dissolved in dimethyl sulfoxide and stored at −20 °C. The final concentration of dimethyl sulfoxide present in cell cultures was 0.1%. The DNA methylation inhibitor, 5-aza-2′-deoxycytidine (⩾97%), was dissolved in sterile 1 × PBS and stored at −20 °C, which served as a positive control for global DNA methylation tests. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). We treated the TK6 cells with HQ (0, 2.5, 5, 10, 15 or 20 μM), melphalan (0, 0.5, 1, 1.5, 2, 4 μM) or etoposide (0, 0.05, 0.1, 0.2, 0.4 μM) for 48 h, which is

equivalent to about three cell cycles, in order to detect stable effects that could be passed on to future generations of cells. Cytotoxicity was measured by the trypan blue exclusion assay.

### Cytogenetic assay

*Metaphase preparation*. Colcemid (0.1 μg/ml, Invitrogen, Carlsbad, CA, USA) was added to each culture 2 h before harvesting in order to obtain a sufficient number of metaphase spreads. After hypotonic treatment (0.075 M KCl) for 30 min at 37 °C, the cells were fixed three times with freshly prepared Carnoy's fixative (methanol/glacial acetic acid = 3:1). The fixed cells were dropped onto glass slides, which were air-dried and stored at −20 °C under a nitrogen atmosphere until use.

*Fluorescence in situ hybridization (FISH)*: A novel FISH assay was designed to simultaneously detect numerical chromosome alterations (NCA) and structural chromosome alterations (SCA) in chromosomes 5, 7, 8 and 21 of the same metaphase spread, in particular leukemia-specific cytogenetic alterations, such as −5, −7, +8, +21, del(5q), del(7q), t(8;21) and t(21;?). Locus-specific probes for chromosome 5 (5p15.2—SpectrumGreen, 5q31—SpectrumOrange; 1 μl) and chromosome 7 (centromere —SpectrumGreen, 7q31—SpectrumOrange; 1 μl), whole chromosome painting probes for chromosome 8 (SpectrumGreen; 1 μl) and chromosome 21 (SpectrumOrange; 1 μl) were mixed with 6 μl hybridization buffer and applied to a 22 × 22 mm area. A whole chromosome painting probe for chromosome 11 was used to detect cytogenetic alterations in chromosome 11, in particular t(11;?), the specific cytogenetic change commonly observed in therapy-related leukemia patients who received etoposide treatments. All the probes and hybridization buffer were purchased from Vysis (Downers Grove, IL, USA). The scoring criteria was previously described in detail.[13]

### Global DNA methylation assay

*Genome DNA isolation*. After the treatment, TK6 cells were spun down and washed once with sterile 1 × PBS. The cells were pelleted and stored at −80 °C until DNA isolation. Genome DNA was isolated using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's protocol. DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The samples were then aliquoted and stored at −80 °C until use.

*Global DNA methylation*. An immunochemistry-based method was set up to determine the global DNA methylation level. Briefly, the black polystyrene 96-well microplate (Corning, catalogue no. 3924, Corning, NY, USA) was coated with 0.01% poly-L-lysine (Sigma-Aldrich) at room temperature for 2 h. The coated microplate was stored at 4 °C for at least 14 days to make it highly hydrophobic. The DNA samples (5 ng/μl) were denatured at 95 °C for 10 min, and then chilled on ice for 10 min. The samples (200 ng/well, six replicates per sample) were loaded and then briefly spun to evenly cover the well bottom. After the wells were dried by incubation at 37 °C overnight with no humidity, they were washed twice with 1 × PBS containing 0.1% Tween-20 and then blocked with 2% bovine serum albumin at 37 °C for 60 min. Mouse anti-5-methylcytosine antibody (CALBIOCHEM, EMD Chemicals, catalogue no. NA81, San Diego, CA, USA) at 1 μg/ml was added, then briefly spun to evenly cover the well bottom. The wells were sealed with parafilm and incubated at 37 °C for 60 min, followed by three washes with 1 × PBS containing 0.1% Tween-20. Secondary antibody conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, catalogue no. 115-095-003, West Grove, PA, USA) at 1.5 μg/ml was added, and the wells were sealed with parafilm and incubated at 37 °C for 60 min, followed again by three washes of 1 × PBS containing 0.1% Tween-20. The plate was read by FL × 800 Fluorescence Microplate Reader (BioTek Instruments, Winooski, VT, USA) using excitation filter 485 ± 20 nm and emission filter 528 ± 20 nm. The fluorescence intensity of one sample was calculated as the average of six replicates, and the methylation level of each dose was calculated as relative percentage of the fluorescence intensity to control. The DNA methylation inhibitor, 5-aza-2′-deoxycytidine, served as positive control.

### Statistical analysis

Poisson regression and linear regression were applied to calculate the *P*-value of the dose–response relationship for the cytogenetic end points and global DNA methylation, respectively.

## Results

### Cytogenetic and global DNA methylation changes induced by melphalan in TK6 cells

We applied FISH to examine the cytogenetic alterations in chromosomes 5, 7, 8, 11 and 21 induced by melphalan in TK6 cells. As shown in Figures 1a and b, melphalan dose-dependently induced −5, −7 and del(5q) cytogenetic alterations characteristic of t-MDS/AML associated with treatment with alkylating agents ($P<0.0001$ for all). Melphalan also increased del(7q), another cytogenetic alteration characteristic of alkylating agents, but the increase was not statistically significant. +8 and +21 were also significantly increased in a dose-dependent manner ($P<0.0001$ for both). As expected, t(11;?) and t(21;?), the cytogenetic hallmarks of DNA topo II inhibitors, were not significantly changed by melphalan treatments. t(8;21), another specific translocation commonly observed in MDS/AML patients, was not detected in the melphalan-treated cultures.

Global DNA methylation level was determined by an immunochemistry-based method. The DNA methylation inhibitor, 5-aza-2′-deoxycytidine, potently decreased the global DNA methylation level in TK6 cells (Figure 1d), validating the assay. As shown in Figure 1c, melphalan did not significantly change the global DNA methylation level in TK6 cells, even at doses producing high cytotoxicity (data not shown).

### Cytogenetic and global DNA methylation changes induced by etoposide in TK6 cells

As expected, etoposide significantly induced t(11;?) and t(21;?), the two specific cytogenetic alterations commonly observed in t-AML associated with prior topo II inhibitor treatment, in a dose-dependent manner ($P<0.0001$ and $P<0.05$, respectively) (Figures 2a and b). Other leukemia-specific changes, including −5, +8, +21, del(5q) and del(7q), were also significantly increased. t(8;21) was not detected at the doses tested.

In contrast to melphalan, etoposide potently induced global DNA hypomethylation in TK6 cells in a dose-dependent manner ($P<0.0001$) (Figure 2c). The average global DNA methylation levels relative to the control at 0.05, 0.1, 0.2 and 0.4 μM etoposide were 76, 60, 47 and 35%, respectively.

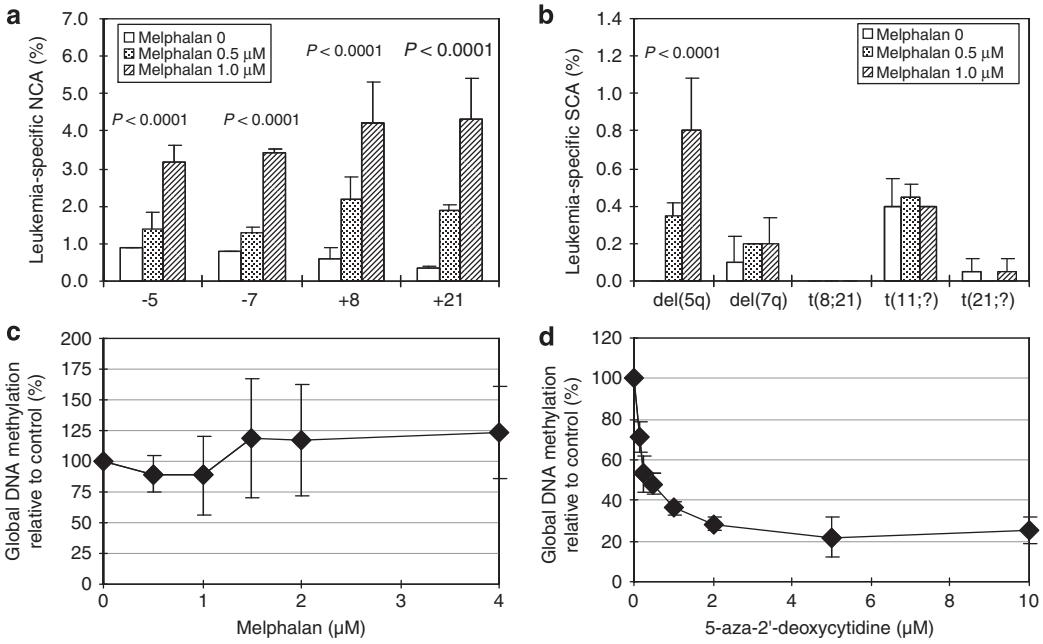


**Figure 1** Leukemia-specific cytogenetic alterations and changes in global DNA methylation in TK6 cells treated with melphalan. (**a**) Leukemia-specific NCA; (**b**) leukemia-specific SCA. Two independent experiments were conducted and 1000 metaphases were scored for each dose in each experiment. Data presented are mean frequency (%), and error bar represents standard deviation. *P* represents the *P*-value of the dose–response test. (**c**) Global DNA methylation in TK6 cells treated with melphalan. Six independent experiments were conducted; (**d**) global DNA methylation in TK6 cells treated with the positive control, 5-aza-2′-deoxycytidine. Three independent experiments were conducted. Data presented are mean global DNA methylation level relative to the control (%), and error bar represents standard deviation.

### *Cytogenetic and global DNA methylation changes induced by HQ in TK6 cells*

As shown in Figures 3a and b, HQ dose-dependently induced del(7q) ($P<0.01$) and t(21;?) ($P<0.05$), typically associated with alkylating agents and topo II inhibitor treatments, respectively. $+8$ and $+21$ were also significantly increased by HQ ($P<0.05$ and $P<0.001$, respectively). A few occurrences of t(8;21) were detected in 10 and 20 μM HQ, compared with none in the untreated control cells, which indicated that HQ treatment might also cause this translocation.

As shown in Figure 3c, HQ moderately induced global DNA hypomethylation in TK6 cells in a dose-dependent manner ($P<0.0001$). The average global DNA methylation levels relative to the control at 2.5, 5, 10, 15 and 20 μM HQ were 86, 83, 72, 66 and 68%, respectively, which were intermediate to melphalan (no effect) and etoposide (potent effect).

## Discussion

AML and MDS are closely related diseases of the bone marrow that arise *de novo* in the general population or following therapy with alkylating agents, topo II inhibitors or ionizing radiation.[1,14] Recent research has shown that the chromosome aberrations and gene mutations detected in therapy-related and *de novo* MDS and AML are very similar, although the frequencies with which they are observed in different subtypes may differ. Hence, therapy-related and *de novo* MDS and AML are considered very similar diseases.[14] At least three cytogenetic subtypes of AML and MDS are commonly observed:[1,2] (a) unbalanced chromosome aberrations, primarily del(5q)/−5 or del(7q)/−7 and +8, following therapy with alkylating agents, such as melphalan; (b) balanced rearrangements, including recurrent balanced translocations (for example, t(11q23;?), t(8;21) and t(15;17)) or inversions (for example, inv(16)), resulting from illegitimate gene recombinations associated with topo II inhibitors, such as etoposide; and (c) normal karyotype. Within these three cytogenetic categories, at least eight different genetic pathways to t-MDS and t-AML have been proposed, defined by the specific chromosome aberrations present in each.[1] The alkylating agents induce t-MDS/AML through pathway I [del(7q)/−7] and pathway II [del(5q)/−5], whereas the topo II inhibitors act through pathway III [t(11q23;?)], pathway IV [t(21q22;?)], pathway V [t(15;17)] and pathway VI [t(11p15;?)]. Patients with a normal karyotype belong to pathway VII, and those with uncharacteristic chromosome aberrations belong to pathway VIII.

Benzene, an important industrial chemical and a ubiquitous environmental pollutant, is an established human leukemogen.[8] Benzene has been shown to be leukemogenic at high doses in the majority of epidemiological studies. Some studies have detected an increased leukemia risk at relatively low levels of exposure. Hayes *et al.*[15] reported the excess leukemia risk at average levels of less than 10 p.p.m. in the large NCI-CAPM cohort study. Glass and co-workers[16] performed a nested case–control study and found that the risk of leukemia was increased at cumulative exposures above 2 p.p.m.-years and with intensity of exposure of highest exposed jobs more than 0.8 p.p.m. We previously observed decreased white blood cell counts in workers exposed to <1 p.p.m. airborne benzene, demonstrating hematotoxic effects of benzene at low occupational exposures.[17] Furthermore, no evidence was found of an exposure threshold below which there was no risk. Occupational exposure to benzene is widely thought to cause leukemias that are similar to t-AML and t-MDS.[18–20] In this study, the benzene metabolite, HQ, induced cytogenetic changes characteristic of

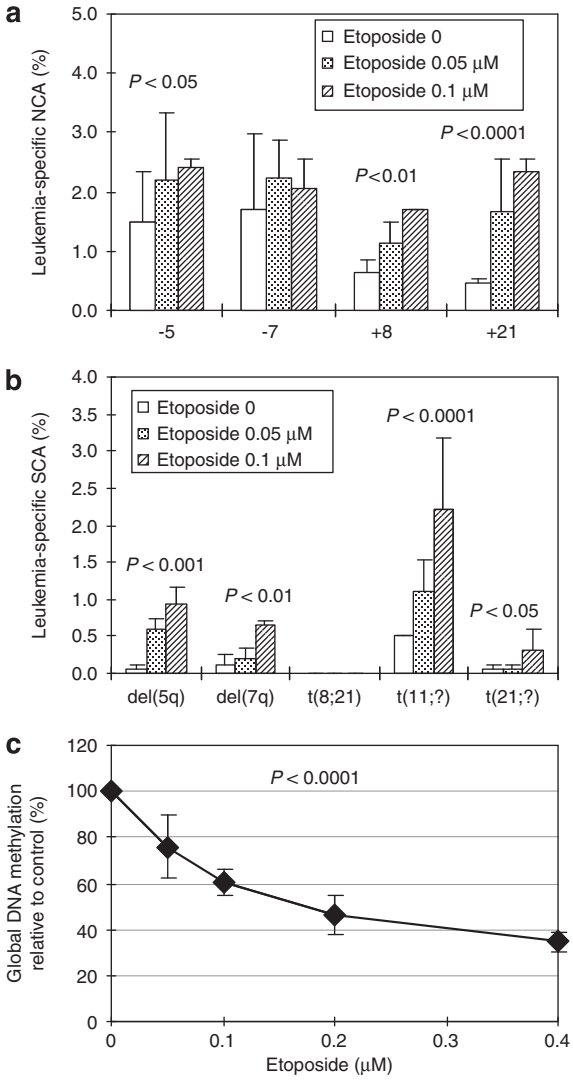


**Figure 2** Leukemia-specific cytogenetic alterations and changes in global DNA methylation in TK6 cells treated with etoposide. (**a**) Leukemia-specific NCA; (**b**) leukemia-specific SCA. Two independent experiments were conducted and 1000 metaphases were scored for each dose in each experiment. Data presented are mean frequency (%), and error bar represents standard deviation. *P* represents the *P*-value of the dose–response test. Data of t(11;?) and t(21;?) were reported previously.[44] (**c**) Global DNA methylation. Four independent experiments were conducted. Data presented are mean global DNA methylation level relative to the control (%). Error bar represents standard deviation. *P* represents the *P*-value of the dose–response test.

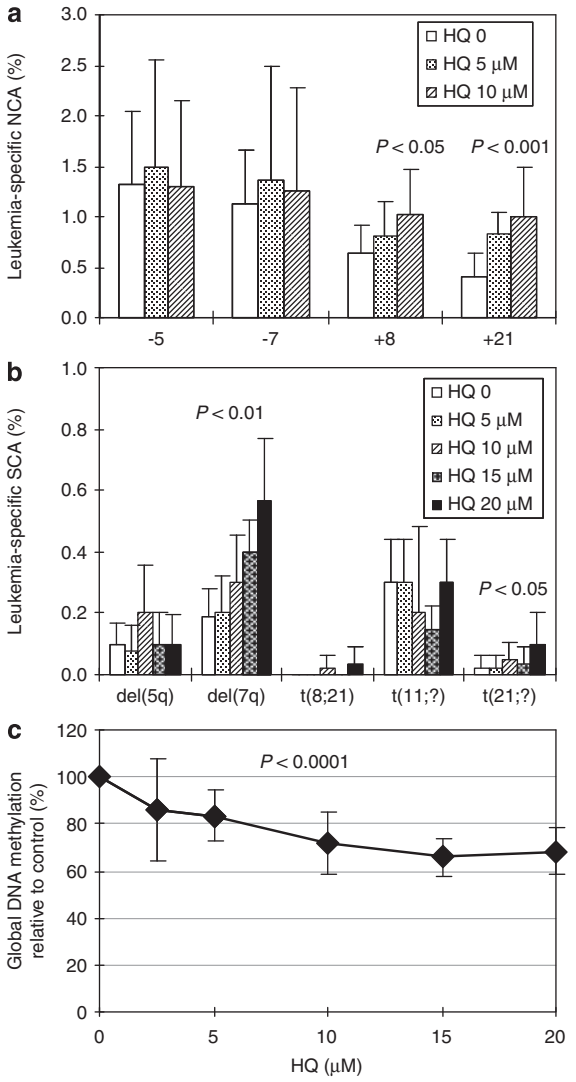


**Figure 3** Leukemia-specific cytogenetic alterations and changes in global DNA methylation in TK6 cells treated with HQ. (**a**) Leukemia-specific NCA; (**b**) leukemia-specific SCA. Five independent experiments were conducted and at least 500 metaphases were scored for each dose in each experiment. Data presented are mean frequency (%), and error bar represents standard deviation. *P* represents the *P*-value of the dose–response test. Data of t(11;?) and t(21;?) were reported previously.[44] (**c**) Global DNA methylation. Seven independent experiments were conducted. Data presented are mean global DNA methylation level relative to the control (%). Error bar represents standard deviation. *P* represents the *P*-value of the dose–response test.

both melphalan and etoposide. HQ at levels between 5 and 20 μM dose-dependently induced del(7q) and t(21;?), cytogenetic alterations characteristic of t-MDS/AML melphalan treatments and etoposide treatment, respectively, in TK6 cells. According to one recent study,[21] the HQ concentration in blood is about 20–120 ng/ml (0.2–1.1 μM) in humans exposed to benzene at 0.19–78.8 mg/m$^3$ (0.1–24.7 p.p.m.). However, HQ accumulates in the bone marrow after benzene exposure.[22,23] In rats exposed to high dose air benzene, the HQ concentration in blood is about 0.2–1.8 μM, which is comparable with the level in exposed humans, whereas in the bone marrow it is about 10–60 μM.[22] Thus, the HQ concentrations in the marrow of highly exposed individuals might reach 10–20 μM. The HQ doses (0, 2.5, 5, 10, 15 and 20 μM) used in this *in vitro* study approximate those in highly exposed workers.

The leukemia-specific cytogenetic changes, +8 and +21, were induced by all three compounds: HQ, melphalan and etoposide. Previously, using the comet assay combined with FISH (comet-FISH), we examined the DNA breakage at chromosome regions 5q31 and 11q23 induced by HQ in TK6 cells, and found that HQ induced DNA breakage at both regions.[24] Together, these studies suggest that the specific cytogenetic alterations induced by HQ overlap with those induced by both melphalan and etoposide. The benzene metabolite, HQ, may act similar to an alkylating agent and also similar to a topo II inhibitor in living cells, both of which may be potential mechanisms of benzene toxicity. This is consistent

with previous studies that HQ can form DNA adducts[25,26] and inhibit topo II activity.[27–29]

Cytogenetic changes are commonly observed in patients with leukemia related to benzene exposure, benzene poisoning patients and 'healthy' workers exposed to benzene.[11] We previously reported that both the characteristic cytogenetic alterations associated with alkylating agents, such as −5, −7, del(5q) and del(7q), and the characteristic changes associated with topo II inhibitors, such as t(21;?), were elevated in workers exposed to benzene.[13,30] Thus, benzene may also induce cytogenetic changes in common with alkylating agents and DNA topo II inhibitors *in vivo*.

An important role for epigenetic changes is also emerging in the development of leukemia. Global DNA hypomethylation is frequently observed in hematological malignancies. Loss of genomic DNA methylation in tumors as compared with their normal-tissue counterparts was one of the first epigenetic changes described in human cancer.[31] In this study, HQ producted an effect on global DNA methylation intermediate to melphalan and etoposide, with no statistically significant induction by melphalan and potent induction by etoposide. The finding that HQ induced global DNA hypomethylation in TK6 cells is consistent with the only report on global DNA methylation in benzene-exposed human subjects,[12] which showed that airborne low-level benzene was associated with global hypomethylation in the peripheral blood DNA of gas station attendants and traffic police officers. Although the subjects may have been co-exposed to other chemicals, the present *in vitro* study with HQ supports an effect of benzene metabolites on global DNA methylation.

Global DNA hypomethylation may have an important role in human leukemogenesis. Currently, there are three mechanisms by which global DNA hypomethylation may contribute to carcinogenesis: (a) generation of chromosomal instability; (b) reactivation of transposable elements; and (c) loss of imprinting. Regarding (a), hypomethylation of DNA can induce chromatin decondensation, centromere and telomere abnormalities and chromosome segregation defects.[32] It has been reported that patients with germline mutations in DNA methyltransferase-3b have numerous chromosome aberrations.[33] Loss of DNA methylation can also favor mitotic recombination, leading to deletions and translocations.[34] Regarding (b), hypomethylation of DNA can reactivate intragenomic endoparasitic DNA,[35] or transposons, which can be transcribed or translocated to other genomic regions, thereby further disrupting the genome. Regarding (c), the loss of methyl groups can affect the expression of imprinted genes. Loss of imprinting of *IGF2* and the tightly linked *H19* locus has been associated with tumorigenesis in a variety of patients.[36,37] Further studies are necessary to determine whether the global DNA hypomethylation induced by HQ acts through one or more of these mechanisms to cause leukemogenesis.

Further studies are also necessary to determine the mechanism by which the global DNA hypomethylation induced by benzene and its metabolites occurs. Several mechanisms have been suggested: (a) DNA damage, including DNA adducts,[38] DNA-strand breaks,[38] oxidative DNA lesions[39] as well as alteration of the topological conformation of DNA,[40] may decrease the substrate efficiency of hemimethylated DNA to accept a methyl group from *S*-adenosylmethionine; (b) changes in expression and/or activity of DNA methyltransferase;[38,41] and (c) alterations in cellular one-carbon metabolism.[42] It is generally accepted that loss of genomic methylation induced by chronic exposure to arsenic occurs mainly through depletion of *S*-adenosylmethionine.[42] However, *S*-adenosylmethionine depletion is not likely the cause of DNA hypomethylation in acute chemical exposure. The benzene metabolite, HQ, can form DNA adducts,[25,26] induce oxidative damage in DNA[43] and inhibit topo II activity.[25,27–29] Each of these factors produces DNA-strand breaks. The DNA adducts, oxidative lesions and strand breaks may decrease the substrate efficiency of hemimethylated DNA to accept methyl group from *S*-adenosylmethionine, thus decrease the genomic methylation level. There is currently no evidence in the literature of alterations in expression and/or activity of DNA methyltransferase associated with HQ.

In conclusion, the benzene metabolite, HQ, may act similar to an alkylating agent and also similar to a topo II inhibitor in living cells, both of which may be potential mechanisms of benzene toxicity. Specific cytogenetic alterations characteristic of both melphalan and etoposide, along with global DNA hypomethylation induced by HQ, may be mechanisms contributing to benzene-induced leukemogenesis.

## Conflict of interest

Dr Smith has received consulting and expert testimony fees from lawyers representing both plaintiffs and defendants in cases involving claims related to exposure to benzene.

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