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Genotoxicity of Stereoisomers of 1,2,3,4-Diepoxybutane in the *gpt* Gene of Chinese Hamster Ovary AS52 Cells

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Abstract Three optical isomers of 1,2,3,4-diepoxybutane, *S*,*S*-, *R*,*R*-, and *meso*-diepoxybutane, are produced by the metabolic processing of carcinogenic 1,3-butadiene. Our previous studies suggested that the observed differences between the biological effects of diepoxybutane optical isomers may be structural in their origin. Therefore, we examined the cytotoxicity and mutation fraction induced by three diepoxybutane stereoisomers in Chinese hamster ovary AS52 cells. All three stereoisomers reduced cell survival and increased *gpt* mutation fraction compared to the control; *S*,*S*-diepoxybutane exhibits the greatest cytotoxic and genotoxic potency, followed by *R*,*R*- and then *meso*-diepoxybutane. These results suggest that the cytotoxic and mutagenic effects of diepoxybutane are mediated by the stereochemical configurations of its isomers.

Keywords 1,2,3,4-Diepoxybutane · Stereoisomers · Genotoxicity · AS52 cells

1,2,3,4-diepoxybutane (DEB) is a carcinogenic metabolite of 1,3-butadiene (BD), an important industrial chemical and environmental contaminant (Morrow 1990) that is also present in tobacco smoke (Hecht 1999). Metabolic activation of BD to DEB involves epoxidation reactions catalyzed by cytochrome P450 monooxygenases (Henderson et al. 1996). The epoxide, 3,4-epoxy-1-butene, may be further oxidized to DEB or hydrolyzed to the corresponding diol, followed by oxidation to 3,4-epoxy-1,2-butanediol (EBD) (Henderson et al. 1996).

All three of the optical isomers of DEB, *S*,*S*, *R*,*R*, and *meso* (Fig. 1), are produced by the metabolic processing of BD. Little evidence exists regarding the effects of stereochemistry of these DEB isoforms to modulate toxicity and mutagenicity. It was reported that *S*,*S*-DEB exhibited the most potent genotoxicity and cytotoxicity (Bianchi and Contin 1962). Recently, we also showed that *S*,*S*-DEB was the most potent mutagen, and mutation specificity and mutant spectra were strongly dependent on DEB stereochemistry in the *supF* gene of pSP189 shuttle vector system (Kim et al. 2007). Meng et al. found that, at the hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) and thymidine kinase (*TK*) loci, all isomers caused increased mutation frequencies. However, there was no significant difference in the types of mutations caused by the three isomers in TK6 lymphoblastoid cells (Meng et al. 2007).

It is not clear, however, whether the three isomers of DEB can induce deletions and other gross structural changes in other types of cells. Therefore, it was of interest to reexamine the effects of stereo-specificity in a sensitive in vitro system for mutation assay. For this reason, we have introduced the Chinese hamster ovary AS52 cell line that carries a single copy of the *E. coli* xanthine-guanine phosphoribosyltransferase (*gpt*) gene, which is functionally expressed using SV40 early promoter and stably integrated into the AS52 cell genome (Tindall et al. 1984). AS52 cells were constructed by transfecting the plasmid vector pSV*gpt* into normal X-linked mammalian *HPRT*-deficient Chinese hamster ovary cells. The AS52 cell line has been demonstrated to be useful with regard to the detection of both deletion and point mutations at *gpt*. The feature of a low spontaneous mutation fraction (MF) and the distinct

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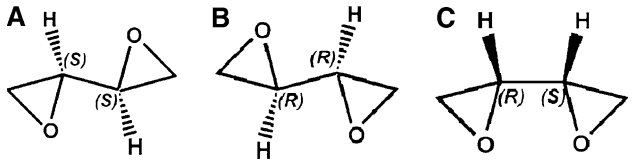

Fig. 1 Chemical structures of DEB stereoisomers: *S*,*S*-DEB (**a**), *R*,*R*-DEB (**b**), and *meso*-DEB (**c**). Each DEB stereoisomer has an optical purity of ≥90% enantiomeric excess over the racemic mixture

advantage of carrying a small easily manipulated mutational target make the AS52 cell line particularly well-suited to quantitative and molecular mutagenesis studies. The *gpt* structural gene consists of 456 base pairs (bp) with no introns. This may be compared to a structural gene size of 654 bp for *HPRT*, which with introns comprises a genomic *HPRT* locus of approximately 35 kb (Tindall et al. 1986). Thus, AS52 cells are sensitive to induced mutagenesis by a variety of clastogens and radiomimetic agents that are often classified as nonmutagens in other assays (Tindall et al. 1986).

In the present work, the toxic and mutagenic potencies of the three stereoisomers of DEB in the *gpt* gene of AS52 cells were examined.

Materials and Methods

Three DEB stereoisomers (*S*,*S*, *R*,*R*, and *meso*, Fig. 1) synthesized as described previously (Park et al. 2005) were kindly prepared by Dr. N. Tretyakova (University of Minnesota). Optically active *S*,*S*- and *R*,*R*-DEB stereoisomers were prepared, starting with dimethyl 2,3-*O*-isopropylidene-L-tartrate and dimethyl 2,3-*O*-isopropylidene-D-tartrate, respectively; *meso*-DEB was prepared from *meso*-erythritol according to the published procedure (Park et al. 2005). All DEB stereoisomers were purified by distillation under atmospheric pressure to give the product as clear oil, bp 138–140°C.

The diastereomeric purity of the resulting DEB stereoisomers was ≥99.5% as determined by ^{1}H and ^{13}C NMR (600 MHz Varian Inova NMR Spectrometer; Palo Alto, CA), and GC–MS (HP5890 series II gas chromatograph; Agilent Technologies, Palo Alto, CA) signal integration and comparison with the corresponding spectra of commercial racemic DEB (97% purity) purchased from Sigma–Aldrich Company (Milwaukee, WI).

Chinese hamster ovary AS52 cells, kindly provided by Dr. Helga Stopper (University of Würzburg, Germany), were grown for 7 days in Ham's F-12 medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum plus mycophenolic acid (MPA) supplements (10 μg/mL MPA, 250 μg/mL xanthine, 22 μg/mL adenine, 11 μg/mL thymidine and 1.2 μg/mL aminopterin) to clear the population of pre-existing *gpt* mutants. The cells were transferred 3 days prior to the experiment to a culture medium enriched with xanthine (11.5 μg/mL), adenine (3 μg/mL) and thymidine (1.2 μg/mL) for recovery. Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C.

Exponentially growing cells (6×10^5 cells/6 well plates) were plated on day 0, and exposed on day 1 to 1, 2, 4, 8 and 16 μM of one DEB stereoisomer (*S*,*S*-, *R*,*R*-, or *meso*-) or racemic DEB, freshly dissolved in Ham's F-12 medium, and incubated for 24 h. Cell viability 24 h after treatment was determined by the trypan blue exclusion assay. Trypsinized cells were pelleted and resuspended in 0.2 mL of Ham's F-12 medium, 0.5 mL of 0.4% trypan blue solution and 0.3 mL of PBS. The untreated cells were used as a control (100% viable), and all values from the experiment were compared to the control. The results are displayed as the percentage of viable cells compared with the untreated control.

Cells were maintained in Ham's F-12 medium for expression of the mutant phenotype after treatment. At the end of incubation for 7 days, 5×10^5 cells from each group were placed in 100 mL of selection medium containing 10 μM 6-thioguanine, and plated at 50,000 cells/10 mL/100 mm dish (10 dishes per group) for determination of mutagenicity. Simultaneously with 6-thioguanine selection, cells were seeded for determination of plating efficiency (200 cells/10 mL/100 mm dish; five dishes per group). Colonies were scored after incubation for 14 days and mutation fraction (MF) was calculated as the number of 6-thioguanine mutants. In one of the experiments, exposure to 8 mM ethyl methansulfonate for 2 h was used as a positive control for mutation induction.

All analyses were replicated three times. Data are presented as the mean ± standard deviation. The two-tailed Student's *t* test was used to evaluate differences in the means between the DEB-treated groups and the untreated control group. Difference with *p* value less than 0.05 and 0.01 were considered statistically significant.

Results and Discussion

When AS52 cells were exposed to all three stereoisomers of DEB for 24 h, ranging from 1 to 16 μM dose, they induced cell death in a dose-dependent manner (Fig. 2). Cell survival in DEB-exposed cells were significantly lower than those in the controls, with the exception of cell cultures exposed to 1 or 2 μM of *R*,*R*- and *meso*-DEB. The relative survival rates for the three isomers of DEB appeared different from each other, with descending order of *meso*-DEB > *R*,*R*-DEB > *S*,*S*-DEB; for example, the

rate of *meso*-DEB was twice that of *S*,*S*-DEB at 8 μM (Fig. 2).

Exposure to all three stereoisomers of DEB caused dose-dependent increases in *gpt* MF compared to the unexposed control (Fig. 3). *S*,*S*-DEB was strongly mutagenic (49.4×10^{-5}) and resulted in a 31-fold increase of the MF over the control MF (1.61×10^{-5}) at a dose of 16 μM. Exposure to 16 μM concentrations of *R*,*R*- and *meso*-DEB increased the MFs 22- and sixfold (34.7 and 9.64×10^{-5}), respectively, as compared with control MF. The MF also increased in a dose dependent manner following racemic DEB treatment (Fig. 3). When cells were treated with 16 μM racemic DEB, the MF (11.9×10^{-5}) was sevenfold higher than that of the unexposed control. While all stereoisomers were mutagenic in the *gpt* gene, the order of potency was *S*,*S* > *R*,*R* > *meso* (Fig. 3). Treatment with *S*,*S*-DEB resulted in two and fivefold increases in MFs at 16 μM, respectively, compared to *R*,*R*- and *meso*-DEB (Fig. 3), confirming that *S*,*S*-DEB was more effective than other stereoisomers in inducing mutations and providing evidence for differing mutagenic potential among the stereoisomers. These findings are consistent with those observed in a recent study (Meng et al. 2007) which evaluated the cytotoxicity and mutagenicity of stereochemical form of DEB in human TK6 lymphoblastoid cells. They reported that all three stereoisomers of 2–6 μM DEB increased *HPRT* and *TK* MFs relative to controls (Meng et al. 2007). The mutagenic responses to the highest concentration of *S*,*S*-DEB appeared to be greater than those for *R*,*R*- and *meso*-DEB, even though the differences were not statistically significant in *HPRT* and *TK* MFs (Meng et al. 2007). As positive control in our study, AS52 cells treated with 8 mM ethyl methansulfonate for 2 h showed 64% survival. The MF under this condition was 79.1×10^{-5}.

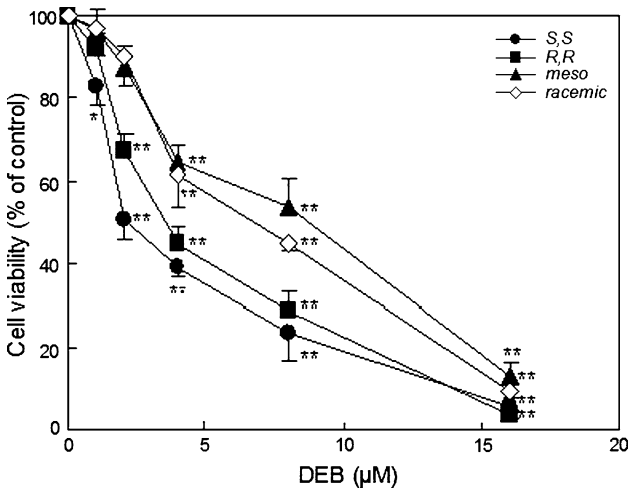

Fig. 2 The percentage of viable AS52 cells after treatment with DEB stereoisomers. Each point is the mean ± SD of three experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$, as compared with untreated control

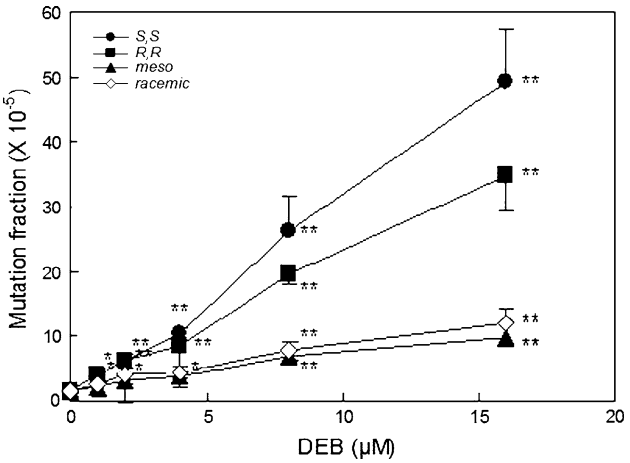

Fig. 3 Dose-dependent relationships between mutagenesis in AS52 cells and exposure to DEB stereoisomers. Each data point is the mean ± SD (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$, as compared with untreated control

Previous studies involving structural characterization of DEB revealed that the ability of DEB to produce DNA-DNA cross-links was strongly dependent on its stereochemistry (Park et al. 2005; Park and Tretyakova 2004). If not repaired, these cross-links blocked DNA replication, transcription, and repair, leading to mutagenic and cytotoxic effects. Based on current information about the structural characterization of DEB, intrastrand DNA-DNA cross-links tend to be mutagenic, whereas interstrand cross-links are cytotoxic (Park et al. 2005). Therefore, our observation that *meso*-DEB is less mutagenic than the *S*,*S* isomer (Fig. 3) is not in accord with that generalization, because only *meso*-DEB induces intrastrand lesions, while the *S*,*S* form induces larger numbers of interstrand lesions (Park et al. 2005). The observed differences in mutagenicity, thus, cannot be explained by known DNA cross-linking specificities of DEB isomers. One likely explanation for these findings is that DNA lesions other than *bis*-N^7G-BD cross-links may play a key role in DEB mutagenicity. For example, recent studies have shown that DEB is capable of forming exocyclic lesions at adenine and guanine nucleobases (Antsypovich et al. 2007; Zhang and Elfarra 2003). These novel lesions are potentially promutagenic because of their inability to form standard Watson–Crick base pairs.

The results of mutagenicity studies in the *HPRT* gene of BD-exposed mice and rats (Meng et al. 2004), and studies of mutation spectra in the *supF* gene (Kim et al. 2007) provide useful data for confirming the stereospecificity in the ability of DEB isomers to induce mutations in the *gpt* gene, presented in this paper. The mutation spectrum analysis identified mutations mainly involving A:T base pairs in both *HPRT* and *supF* genes: A:T to T:A

transversions and G:C to A:T transitions were the base pair substitutions found predominantly and consistently. Moreover, deletions were frequently observed in both the *HPRT* and *supF* genes in the BD- and DEB-treated cells (Kim et al. 2007; Meng et al. 2004). In comparing the nature of the differences in the distribution of mutations between the *HPRT* and *supF* genes, an increased frequency of G:C to C:G transversions was found in the *supF* gene following treatment with *S*,*S*- and *meso*-DEB stereoisomers.

In summary, we have examined the cytotoxicity and mutational fraction induced by individual DEB stereoisomers in the *gpt* gene. Our findings are consistent with stereospecific differences in the mutagenicity of diepoxybutanes. Additional work is in progress to elucidate the observed differences among DEB stereoisomers by molecular analysis.

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