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Mutagenicity of Stereochemical Configurations of 1,3-Butadiene Epoxy Metabolites in Human Cells

Ryan Q. Meng, Linda C. Hackfeld, Richard P. Hodge, Lynne A. Wisse, Diana L. Redetzke, and Vernon E. Walker

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with a Critique by the HEI Health Review Committee

Research Report 150

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI research and analyses to public and private decision makers.

HEI receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or certain research programs. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Health Review Committee are widely disseminated through HEI's Web site (*www.healtheffects.org*), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 150, *Mutagenicity of Stereochemical Configurations of 1,3-Butadiene Epoxy Metabolites in Human Cells*, presents a research project funded by the Health Effects Institute and conducted by Dr. Ryan Q. Meng of Battelle Toxicology Northwest, Richland, Washington, and his colleagues. This research was funded under HEI's Walter A. Rosenblith New Investigator Award Program, which provides support to promising scientists at the early stages of their careers. The report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Health Review Committee's comments on the study.

The Investigators' Report, prepared by Meng and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

The Critique is prepared by members of the Health Review Committee with the assistance of HEI staff; it places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Health Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.

HEI STATEMENT

Synopsis of Research Report 150

Relative Mutagenicity of Stereoisomers of Butadiene Metabolites in Human Cells

BACKGROUND

1,3-butadiene (BD) is used extensively in the chemical industry (e.g., for synthetic rubber production) and is also present in motor vehicle exhaust and cigarette smoke. It is listed by the U.S. Environmental Protection Agency as a mobile-source air toxic and is classified as a human carcinogen when inhaled. Studies in rodents have shown that mice and rats differ in their sensitivity to BD, with mice developing tumors at much lower exposure concentrations than rats. A key research and risk assessment question has been which species is likely to be more relevant to what might happen in humans. Species differences in tumor induction by BD have been attributed to differences in its metabolism. The major metabolites of interest are 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). Studies have shown that these compounds differ in their ability to damage DNA in exposed animals or in cultured cells. In these studies, mostly racemic mixtures that contained all stereoisomers (spatially different forms of the same chemical structure) of each BD metabolite were tested. Limited work with specific stereoisomers has provided some evidence of a possible role of stereochemistry in the metabolism of BD and in the formation of DNA adducts, but none of those studies evaluated the mutagenicity of the individual stereoisomers.

In response to the Walter A. Rosenblith New Investigator Award Request for Applications (RFA 00-2), Dr. Ryan Q. Meng proposed to determine the cytotoxicity and mutagenicity of the stereoisomers of three major butadiene metabolites in the TK6 human lymphoblastoid cell line. The HEI Research Committee recommended the study for funding because they thought that Dr. Meng was a promising investigator and that the proposal was very relevant to the HEI research program on air toxics because it addressed an important question regarding the mutagenicity of BD metabolites.

APPROACH

The study had two major aims: (1) to synthesize the nine stereoisomers of the three main BD metabolites and (2) to evaluate the cytotoxicity and mutagenicity of each stereoisomer of each BD metabolite in cells exposed in vitro. Mutagenicity was evaluated at two genes: the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and thymidine kinase (*TK*) genes. A secondary aim was to characterize the types of mutations induced by BDO_2 stereoisomers at the *HPRT* gene. The synthesis of the stereoisomers was conducted at the University of Texas Medical Branch in Galveston, Texas. The investigators assessed the purity of each compound by evaluating various analytic parameters such as gas chromatography–mass spectrometry and nuclear magnetic resonance spectra, melting point, and optical rotation. Stereoisomers result from the asymmetric bonding of some atoms to a carbon atom, resulting in "left hand" (*S*) and "right hand" (*R*) forms, which differ in their three-dimensional configuration and optical activity but are chemically identical. BDO has two stereoisomers: (2*R*) and (2*S*). BDO_2 has three stereoisomers: (2*R*,3*R*), (2*S*,3*S*), and *meso* (a compound that is not optically active). BDO-diol has four stereoisomers: (2*R*,3*R*), (2*R*,3*S*), (2*S*,3*R*), and (2*S*,3*S*).

The cytotoxicity and mutagenicity of each stereoisomer was evaluated in the TK6 human lymphoblastoid cell line. Cells were exposed in culture to different doses of each stereoisomer for 24 hours. Cytotoxicity, or percent survival, at each dose was calculated as the percentage of cells that form colonies relative to control cells (with the control value set at 100%). Mutations were examined in the *HPRT* and the *TK* genes. Cells with mutations at the *HPRT*

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Ryan Q. Meng of Battelle Toxicology Northwest, Richland, Washington, and colleagues. Research Report 150 contains both the detailed Investigators' Report and a Critique of the study prepared by the Institute's Health Review Committee.

gene grew in a medium containing 6-thioguanine, while cells with mutations at the *TK* gene were grown in the presence of trifluorothymidine; normal cells cannot grow in these media. The frequency of mutations was calculated as the percentage of cells that grew in the presence of the selection agent relative to the percentage that grew in normal medium (without the agent).

In separate experiments, Meng and colleagues determined the spectrum of the mutations at the *HPRT* gene induced by BDO_2 using amplification of the *HPRT* DNA and analysis of the DNA by ultraviolet light. This method primarily detects large deletions.

RESULTS AND INTERPRETATION

The authors successfully synthesized the nine stereoisomers with sufficient yield to conduct the proposed assays. The purity of the stereoisomers was determined to be at least 98% to 99.9%.

A key and novel finding was that the (2*R*,3*S*)-BDO-diol stereoisomer was about 30-fold more cytotoxic and mutagenic than the other three BDO-diol stereoisomers. The dose-related mutagenic responses for the stereoisomers of BDO and BDO_2 were not statistically different. (2*R*,3*S*)-BDO-diol was 5-to-10-fold less mutagenic than any of the BDO_2 stereoisomers, but 10-to-20-fold more mutagenic than the BDO stereoisomers.

The analysis of the types of mutation induced by the stereoisomers of BDO_2 showed a higher frequency of deletions in cells exposed to each stereoisomer of BDO_2 relative to unexposed cells. While appropriately carried out, these experiments did not provide any new insights relating to the mechanism of action of BD. A more detailed analysis of the sizes of the deletions would be helpful to determine whether the deletion spectra of the mutants differ.

In its independent evaluation of the study, the Review Committee thought that the investigators had conducted a well-designed and novel study that combined chemistry and molecular biology to address a relevant research question regarding the mutagenicity of specific stereoisomers of the key BD metabolites. They thought the investigators provided convincing data on the purity of the stereoisomers, determined using a variety of analyses. The study's key finding that one of the four stereoisomers of BDO-diol was responsible for most of the mutagenicity of this metabolite is interesting and could explain some of the possible species differences in the mutagenicity of BD. The study also suggests that the species differences in susceptibility to BD that have been noted in other work are not likely to be related to the stereochemistry of BDO and BDO_2. The caveat to these conclusions is that the normal target cells for BD in rodents may detoxify the different BD metabolites at very different rates, thus leading to steady-state levels of one metabolite or its stereoisomers that are higher than those of others. The results of this study using a cell line and individual stereoisomers provide justification for follow-up studies that consider the kinetics of the formation and distribution of BDO-diol stereoisomers in rodents and humans and that have endpoints relating to both mutagenicity and carcinogenicity.

INVESTIGATORS' REPORT

Mutagenicity of Stereochemical Configurations of 1,3-Butadiene Epoxy Metabolites in Human Cells

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ABSTRACT

The mutagenic and carcinogenic effects of 1,3-butadiene (BD*) are related to its bioactivation to several DNA-reactive metabolites, including 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). Accumulated evidence indicates that stereochemical configurations of BD metabolites may play a role in the mutagenic and carcinogenic action of BD. The objective of this study was to evaluate the cytotoxicity and mutagenicity of each stereoisomer of major BD metabolites in human cells. For this purpose, nine stereochemical forms of BDO, BDO_2, and BDO-diol were synthesized. TK6 cells, a human lymphoblastoid cell line, were exposed to each stereoisomer. Cytotoxicity was measured by comparing cloning efficiencies (CEs) in chemical-exposed cells versus those in control cells. Based on the results of cytotoxicity tests, TK6 cells were exposed to 0, 2, 4, or 6 µM of each form of BDO_2, or to 0, 200, 400, or 600 µM of each form of BDO for 24 hours to determine the mutagenic efficiencies. The exposure concentrations for BDO-diol ranged from 5 to 1000 µM. The mutagenicity was measured by determining, in a lymphocyte cloning assay, the mutant frequencies (M*f*s) in the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and thymidine kinase (*TK*) genes. *HPRT* mutants collected from cells exposed to the three forms of BDO_2 were analyzed by polymerase chain reaction (PCR) to characterize large genetic alterations.

All three stereoisomers of BDO_2 [(2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, and *meso*-BDO_2] caused increased *HPRT* and *TK* M*f*s compared with the concurrent control samples, with *P* values ranged from 0.05 to 0.001. There were no significant differences in cytotoxicity or mutagenicity among the three isomers of BDO_2. Molecular analysis of *HPRT* mutants revealed similar distributions of deletion mutations caused by the three isomers of BDO_2. There were also no statistical differences in mutagenic efficiencies between the two isomers of BDO [(2*R*)-BDO and (2*S*)-BDO] in TK6 cells. These results were consistent with the in vivo finding that there was little difference in the mutagenic efficiencies of (±)-BDO_2 versus *meso*-BDO_2 in rodents. Thus, in terms of mutagenic potency, there was no evidence that stereochemical configurations of BDO and BDO_2 play a significant role in the mutagenicity and carcinogenicity of BD.

The most significant results of this study were the marked differences in cytotoxicity and mutagenicity among the four stereoisomers of BDO-diol [(2*R*,3*R*)-BDO-diol, (2*R*,3*S*)-BDO-diol, (2*S*,3*R*)-BDO-diol, and (2*S*,3*S*)-BDO-diol]. (2*R*,3*S*)-BDO-diol was at least 30-fold more cytotoxic and mutagenic than the other three forms of BDO-diol. This was consistent with the finding that 75% of the adduct N7-(2,3,4-trihydroxybutyl)guanine (THB-Gua) originated from (2*R*,3*S*)-BDO-diol in the lungs of mice exposed to BD. The mutagenic potency of (2*R*,3*S*)-BDO-diol was much closer to that of BDO_2 than previously demonstrated in experiments in which stereochemistry was not considered. The current study demonstrated that the mutagenic potency of (2*R*,3*S*)-BDO-diol was only 5-to-10-fold less than the average equimolar effect of BDO_2 stereoisomers in the *HPRT* and *TK* genes, and was 10-to-20-fold greater than

This Investigators' Report is one part of Health Effects Institute Research Report 150, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Ryan Q. Meng, 902 Battelle Blvd, P.O. Box 999, Richland, WA 99352; email: mengr@battelle.org.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83234701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

the average equimolar effect of BDO stereoisomers in the *HPRT* and *TK* genes. Previous DNA and hemoglobin adduct data demonstrated that BDO-diol is the dominant BD metabolite available to react with macromolecules in vivo after BD exposure (Pérez et al. 1997; Swenberg et al. 2001). Thus, the differences in BD carcinogenesis among rodent species may be significantly accounted for by the stereochemistry-dependent distributions of BDO-diol metabolites and BDO-diol–DNA adducts, and by the mutagenic efficiencies of BDO-diol in mice and rats.

INTRODUCTION

BD, an environmental and workplace air pollutant, is an indirect alkylating agent with the potential to induce DNA damage, mutation, and cancer. Long-term animal studies show that BD is a multisite carcinogen in both mice and rats, and that mice are more sensitive to BD-induced tumorigenicity than rats (Huff et al. 1985; Owen et al. 1987; Melnick et al. 1990). Epidemiologic studies have found some evidence for a causal relationship between BD exposure and the occurrence of leukemia and lymphoma in exposed populations, but the results of these studies have not been conclusive (Acquavella 1996; Himmelstein et al. 1997; Sathiakumar et al. 1998). The species differences in tumorigenicity of BD in rodents and the equivocal epidemiologic human data have complicated the assessment of cancer risk in people occupationally exposed to BD, and have led to mechanistic studies directed at understanding the differences among species in the metabolism and genotoxicity of this agent.

Data from diverse studies suggest that the mutagenic and carcinogenic effects of BD are related to its bioactivation to several DNA-reactive intermediates, including BDO, BDO_2, and BDO-diol. Metabolism studies have shown that blood and tissue levels of BDO and BDO_2 in mice and rats are in accordance with the observed differences between the species in tumors induced by BD (Himmelstein et al. 1994, 1995, 1996; Thornton-Manning et al. 1995, 1996, 1998). In vivo mutagenicity studies have demonstrated that BD causes increased *Hprt* M*f*s in both mice and rats, and the mutagenic response in mice is much greater than that in rats, which is consistent with the observed species differences in BD tumorigenicity in rodents (Meng et al. 1998). BD has been reported to produce increased variant frequencies (VFs) at the *HPRT* locus in the T lymphocytes of workers in a BD manufacturing facility, as measured by the autoradiography assay, and an increase of VFs were associated in a dose-related manner with a specific BD metabolite in urine of workers (Ward et al. 1994). Increased VFs in BD-exposed workers were again observed in two larger biomarker studies in a BD-styrene polymer production plant (Ammenheuser et al. 2001; Ward et al. 2001). In contrast, two BD biomonitoring studies utilizing the lymphocyte cloning assay demonstrated positive trends for increased *HPRT* M*f*s, but the increases were not significant (Hayes et al. 1996; Tates et al. 1996). In an attempt to resolve the conflict in the results of BD exposure studies by using a lymphocyte cloning assay versus an autoradiographic assay, one recent study measured the *HPRT* VFs and M*f*s in the same population (Albertini et al. 2003). In this study, neither form of the *HPRT* mutant lymphocyte assay detected a mutagenic effect of BD exposure at the exposure levels evaluated.

The mutagenic potency and specificity of BD epoxy metabolites were previously evaluated in TK6 cells without regard to the potential effects of stereochemistry. Cochrane and Skopek (1994) found that, at the *HPRT* locus, an induced M*f* value of 5.0×10^{-6} was produced by treatment with 3.5 µM BDO_2, 150 µM BDO, and 450 µM BDO-diol, while at the *TK* locus, a similar increase in M*f*s was produced by treatment with 1.0 µM BDO_2, 100 µM BDO, and 350 µM BDO-diol. Steen and colleagues (1997a,b) observed a significant increase in A:T → T:A transversions in BDO- or BDO_2-induced *HPRT* mutants and a significant increase in genomic deletions in BDO_2-induced *HPRT* mutants in TK6 cells. These results have been extensively used in developing experimental designs and in interpreting data in studies aimed at clarifying the mechanisms of BD mutagenicity and carcinogenicity. However, these reports are problematic because the initial mutagenicity studies of Cochrane and Skopek (1994) were performed with racemic mixtures of BDO and BDO_2 and an uncharacterized mixture of BDO-diol. (A racemic mixture is made up of equal amounts of isomers with opposite configurations at one or more asymmetric centers, for example, (±)-BDO.) Likewise, the mutational spectra studies by Steen and colleagues (1997a,b) were limited to (±)-BDO and (±)-BDO_2. It was assumed that these limited mutagenic potency data for BD epoxy metabolites could be applied in vivo to estimate the relative contributions of BDO, BDO_2, and BDO-diol to the total mutagenicity of BD. On the contrary, accumulating evidence suggests that the dramatic differences between mice and rats in the metabolism and tumorigenicity of BD may involve differences in the levels of BD epoxy metabolites (Koivisto et al. 1997, 1998, 1999, 2001; Krause and Elfarra 1997; Nieusma et al. 1997, 1998).

Thus, the role of stereochemistry in the species-dependent metabolism and toxicity of BD must be considered to clarify the species differences in BD-induced mutagenesis and carcinogenesis. The initial oxidation of BD to BDO creates a stereocenter and influences the stereochemistry

of the subsequent BD metabolism pathways. Stereochemistry affects BD metabolism, formation and persistence of DNA adducts, and cytotoxicity of BD epoxy metabolites (Koivisto et al. 1997, 1998, 1999, 2001; Krause and Elfarra 1997; Nieusma et al. 1997, 1998). For example, Koivisto and colleagues (1997, 1998) showed that 68% of hydroxybutenyl adducts at N7 of guanine (BDO-Gua) in rat liver DNA originated from BDO having *R* stereochemistry, while adducts formed from *S* configurations of BDO dominated in mouse lung and testis (77% and 71%, respectively) after BD inhalation exposure. In lung tissue of mice exposed to 500 ppm of BD for 5 days (6 hours/day), THB-Gua adducts formed from the (2*R*,3*S*) diastereomer of BDO-diol constituted 73% of the adducts derived from BDO-diol (Koivisto et al. 1999). While the mutagenic effects of individual stereoisomers of BD metabolites were not evaluated, cytotoxicity studies demonstrated that (2*R*)-BDO was more toxic than (2*S*)-BDO, and *meso*-BDO_2 was the most toxic of the BDO_2 enantiomers in rat hepatocytes (Nieusma et al. 1997). (A *meso* compound is a nonoptically active member of a set of stereoisomers, at least two of which are optically active.) These findings demonstrated the potential importance of stereochemistry in BD biotransformation, the formation of DNA adducts, and toxicity. They also emphasized the need for studies investigating the relative mutagenic potency of the various stereoisomers of BD epoxy metabolites.

The goals of this study were to assess the mutagenic potency of each stereoisomer of BDO, BDO_2, and BDO-diol in human cells at both *HPRT* and *TK* loci and to characterize and compare the mutational spectra of large-scale genetic alterations caused by the three stereoisomers of BDO_2. Knowledge of the in vivo stereochemical distribution, the formation of DNA adducts, and the relative mutagenic potencies of each stereoisomer is required to define the relative contributions of each stereochemical configuration to the mutagenicity of BD. The stereochemistry factor was considered in previous in vivo mutagenicity studies; however, the evaluation of mutagenic effects was limited to one diastereomer of BDO_2, *meso*-BDO_2 (Walker et al. 2009). It is impractical to evaluate the mutagenic effects of all nine stereoisomers of BD epoxy metabolites in rodents mainly due to the lack of large-scale, cost-effective methods for synthesizing the individual stereoisomers. When rodents are exposed to BD or its metabolites, more than one metabolite or stereoisomer may contribute to the mutagenic response because of the metabolic conversion of the exposure compound to other DNA-reactive intermediates. Conversely, TK6 cells have negligible metabolic capacity, and the observed mutagenic effect of an individual stereoisomer of BD can be attributed solely to the test compound (Shirname-More, 1991; Zhu et al., 2000). Although in vitro studies do not fully mirror the complicated cascade of events leading to BD-induced mutations in vivo, these studies can provide unique information about the mutagenic potency of each BD epoxy stereoisomer, and can be used, along with the data on in vivo stereochemical distributions of BD epoxy metabolites, to improve our understanding of the potential mutagenic mechanisms of BD in laboratory animals and people.

TK6 cells are hemizygous at the *HPRT* locus and heterozygous at the *TK* locus. The measurement of both *HPRT* and *TK* M*f*s resulting from the same exposure provides an exceptional opportunity to estimate the relative contributions of intragene mutational mechanisms and of loss of heterozygosity (LOH) events to the total mutagenicity of BD. Many mutagenesis mechanisms that involve homologous interactions cannot occur at the hemizygous *HPRT* locus. In addition, some multilocus deletions are lethal in the *HPRT* gene, because these gross deletions may span the adjacent genes essential for cell survival. In contrast to the X-linked *HPRT* gene, the autosomal *TK* locus can be used to detect large chromosomal events that can lead to LOH, such as gene conversion, mitotic recombination, and large-scale gene deletions. If a stereoisomer mainly induces LOH events, we expect to find a significantly greater increase in M*f*s at the *TK* locus compared to the *HPRT* locus.

The information on mutagenic potency of each stereoisomer of BDO, BDO_2, and BDO-diol generated from this study, along with the data on in vivo stereochemical distributions of BD epoxy metabolites, can be used to help define the relative contributions of each stereochemical configuration of BD epoxides to the total mutagenicity of BD in mice and rats. Our results offer guidance for the further evaluation of the mutagenicity of various stereochemical configurations of BD epoxy metabolites in vivo. Finally, data generated from this project provide a basis for the development of stereospecific biomarkers for monitoring BD exposure and biological effects in exposed humans.

SPECIFIC AIMS

The objective of this research was to evaluate the cytotoxic and mutagenic potency of each stereoisomer of key BD epoxy metabolites (BDO, BDO_2, and BDO-diol) in human cells. The original aims included synthesis of stereoisomers of BDO, BDO_2, and BDO-diol, measurement of the *HPRT* and *TK* M*f*s in TK6 cells exposed to each isomer of the BD metabolites, and characterization and comparison of the mutational spectra of large-scale genetic alterations caused by the three stereoisomers of BDO_2.

The specific aims were as follows:

1. To synthesize eight stereoisomers of BDO, BDO_2, and BDO-diol. *meso*-BDO_2 was synthesized in a previous HEI-funded study in our laboratory (Walker et al. 2009).
2. To evaluate the cytotoxicity and mutagenicity of each stereoisomer of BDO_2 and to determine the frequencies and types of large-scale changes in the *HPRT* gene in mutant clones obtained from TK6 cells treated with each stereoisomer of BDO_2, compared with control TK6 cells.
3. To evaluate the cytotoxicity and mutagenicity of each stereoisomer of BDO in TK6 cells, as in Aim 2.
4. To evaluate the cytotoxicity and mutagenicity of each stereoisomer of BDO-diol in TK6 cells, as in Aim 2.

During this study, no marked differences in cytotoxicity and mutagenicity among the three stereoisomers of BDO_2 in TK6 cells were found. Preliminary results of molecular analyses of *HPRT* mutants demonstrated similar mutational spectra for each stereoisomer of BDO_2. Therefore, with the approval of the HEI Research Committee, analysis of additional *HPRT* and *TK* mutants was not conducted.

METHODS AND STUDY DESIGN

SPECIFIC AIM 1: SYNTHESIS OF EIGHT STEREOISOMERS OF BDO_2, BDO, AND BDO-DIOL

All solvents and reagents were obtained from Fisher-Acros (Morris Plains, NJ) or Aldrich Chemicals (Milwaukee, WI). Thin layer chromatography (TLC) analyses were carried out on Merck silica gel 60 F_{254} plates (Darmstadt, Germany) and visualized at 254 nm or upon heating with anisaldehyde solution [anisaldehyde, sulfuric acid, acetic acid, ethanol (1:1:10:300)]. Preparative TLC separations were performed on an Analtech (Newark, DE) cyclograph system. Silica gel 60 for column chromatography was purchased from Fisher (Pittsburgh, PA). ^1H (300 MHz) nuclear magnetic resonance (NMR) spectra were obtained on a Varian Mercury Plus 300 MHz (Palo Alto, CA) with a heteronuclear broadband probe, using trimethylsilane as the internal standard. Polarimetry data were obtained with an Optical Activity (Huntingdon, Cambridgeshire, England) AA-5 polarimeter (2.5 × 100 mm cell). The Model AA-5 polarimeter only reads 1 significant figure (± 0.05° accuracy); therefore, though the value for compounds with small rotations (less than 1) is less accurate, the direction (±) of rotation is consistent.

The metabolic pathway and stereostructures of BD metabolites are depicted in Figure 1. Two methods were used and compared to make (2*S*,3*S*)-BDO_2 and (2*R*,3*R*)-BDO_2: method A (Figure 2) and method B (Figure 3).

Synthesis of (2*S*,3*S*)-BDO_2 and (2*R*,3*R*)-BDO_2 — Using Method A

(2S,3S)-O-Isopropylidene-L-threitol-1,4-bis(methanesulfonate) (6a) Methanesulfonyl chloride (CH_3SO_2Cl; 6 mL, 78 mmol) was added dropwise (~1 mL/min) to (2*S*,3*S*)-*O*-isopropylidene-L-threitol (**5a**), shown in Figure 2 (Fluka) (5 g, 31 mmol) in anhydrous pyridine (30 mL) at −10° to −5°C. The mixture was stirred at −5°C overnight. The mixture was then added to ice water, and **6a** (Figure 2) was filtered and dried as a white solid (4.2 g, 43% yield). Analytical data: melting point (MP), 78°–80°C; literature (Lit) MP, 79°–80.5°C (Feit 1964).

(2R,3R)-O-Isopropylidene-D-threitol-1,4-bis(methanesulfonate) (6b) CH_3SO_2Cl (6 mL, 78 mmol) was added dropwise (~1 mL/min) to (2*R*,3*R*)-*O*-isopropylidene-D-threitol (**5b,** Figure 2) (Fluka) (5 g, 31 mmol) in anhydrous pyridine (30 mL) at −10° to −5°C. The mixture was stirred at −5°C overnight. The mixture was then added to ice water, and **6b** (Figure 2) was filtered and dried as a white solid (4.2 g, 43% yield). Analytical data: MP, 79°–80°C; Lit MP 85.5°–86.5°C (two crystal modifications) (Feit 1964).

(2S,3S)-L-Threitol-1,4-bis(methanesulfonate) (7a) Compound **6a** (4.2 g, 13.2 mmol) was suspended in 96% ethanol/water (20 mL). Methanesulfonic acid (Ch_3SO_2OH) (20 µL, 0.31 mmol) was added, and the mixture was refluxed for 8 hours (a clear solution was obtained when the mixture was heated). The mixture was cooled to precipitate **7a** (Figure 2), which was filtered and recrystalized from ethanol (2.5 g, 71.7% yield). Analytical data: MP, 99°–100°C; Lit MP 102°–103°C (Feit 1964).

(2R,3R)-D-Threitol-1,4-bis(methanesulfonate) (7b) Compound **6b** (8.19 g, 25.7 mmol) was suspended in 96% ethanol/water (35 mL). Ch_3SO_2OH (40 µL, 0.62 mmol) was added, and the mixture was refluxed for 8 hours (a clear solution was obtained when the mixture was heated). The mixture was cooled to precipitate **7b** (Figure 2), which was filtered and recrystalized from ethanol (4.48 g, 65.9% yield). Analytical data: MP, 98°–99°C; Lit MP 102°–103°C (Feit 1964).

(2S,3S)-BDO_2 (3a) Potassium hydroxide (KOH) (1.2 g, 21.4 mmol) in water (3 mL) was added dropwise to **7a** (2.5 g, 9.5 mmol) in ether (Et_2O-H_2O) (20 mL). The mixture was stirred at ~20°C for 3 hours with strong agitation (resulting in a biphasic mixture). The layers were then allowed to settle and were separated. The ether layer was washed with

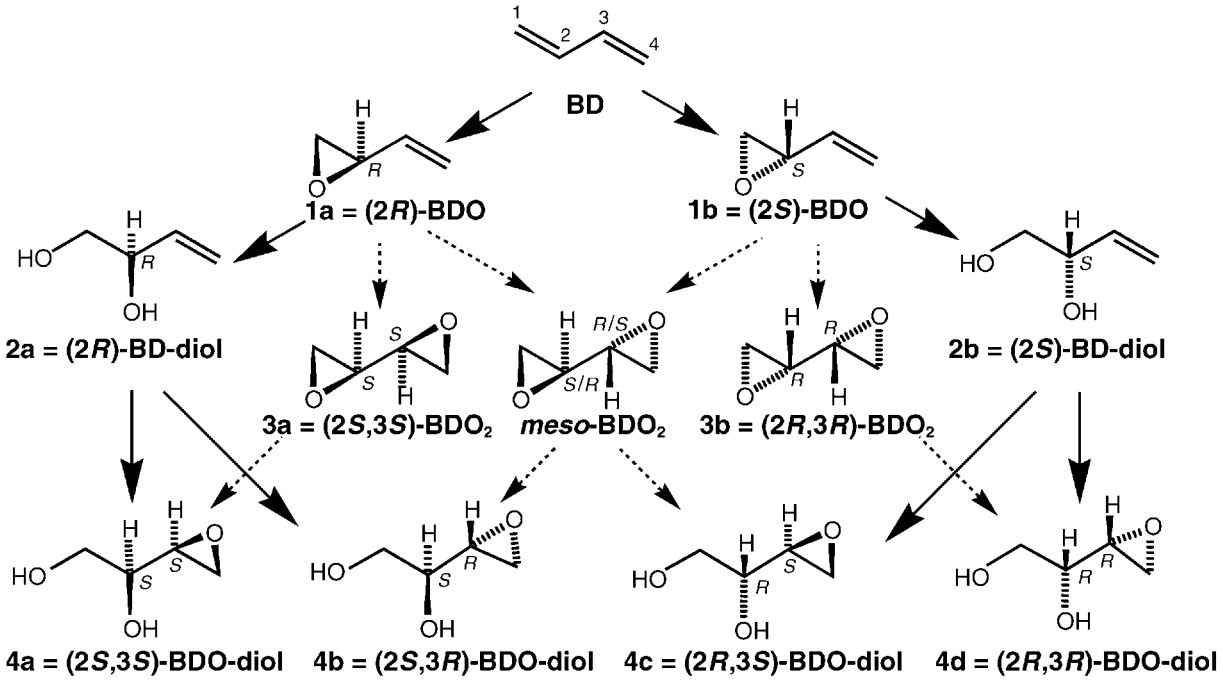

Figure 1. Structures of stereoisomers of major reactive metabolites of BD. Dominant in vivo pathways are indicated with solid arrows.

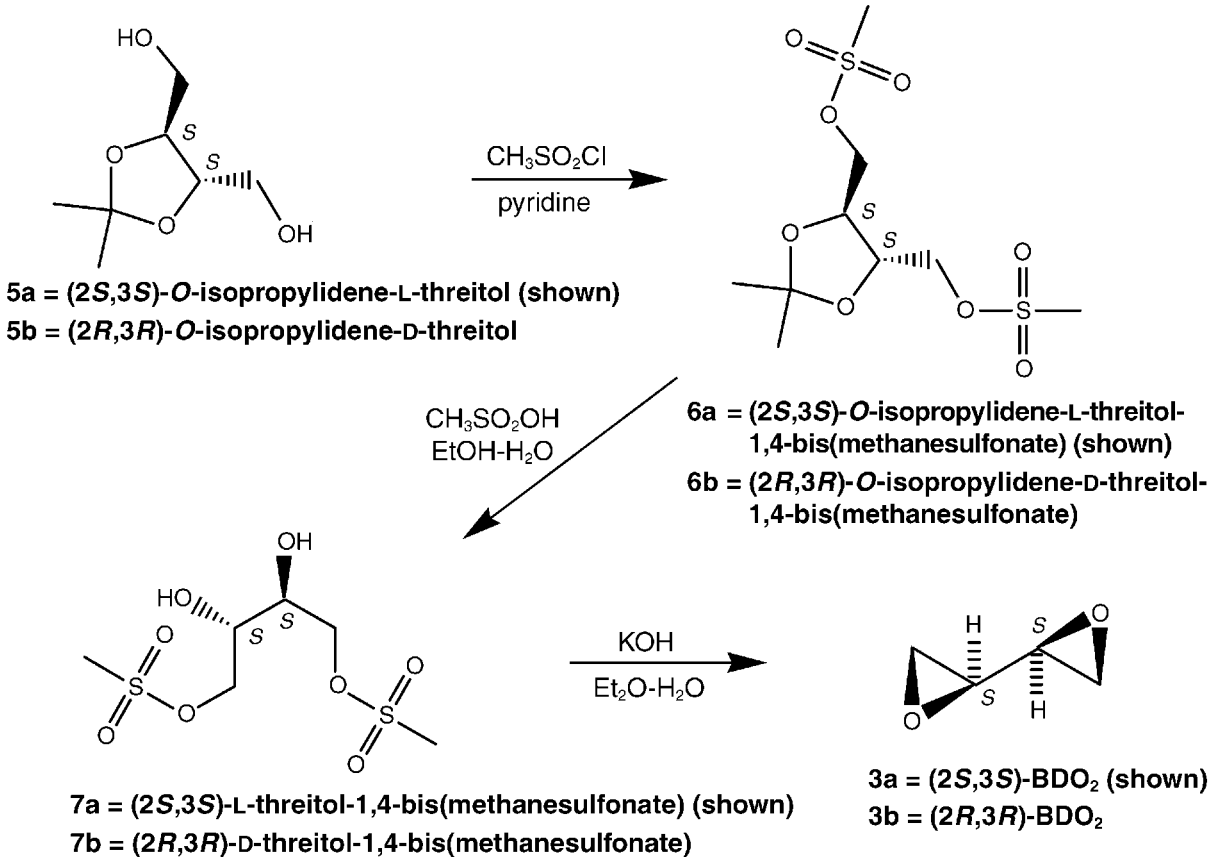

Figure 2. Synthetic scheme for stereoisomers of BDO_2 — Method A. The starting materials were (2*S*,3*S*)-*O*-isopropylidene-L-threitol (**5a**) and (2*R*,3*R*)-*O*-isopropylidene-D-threitol (**5b**).

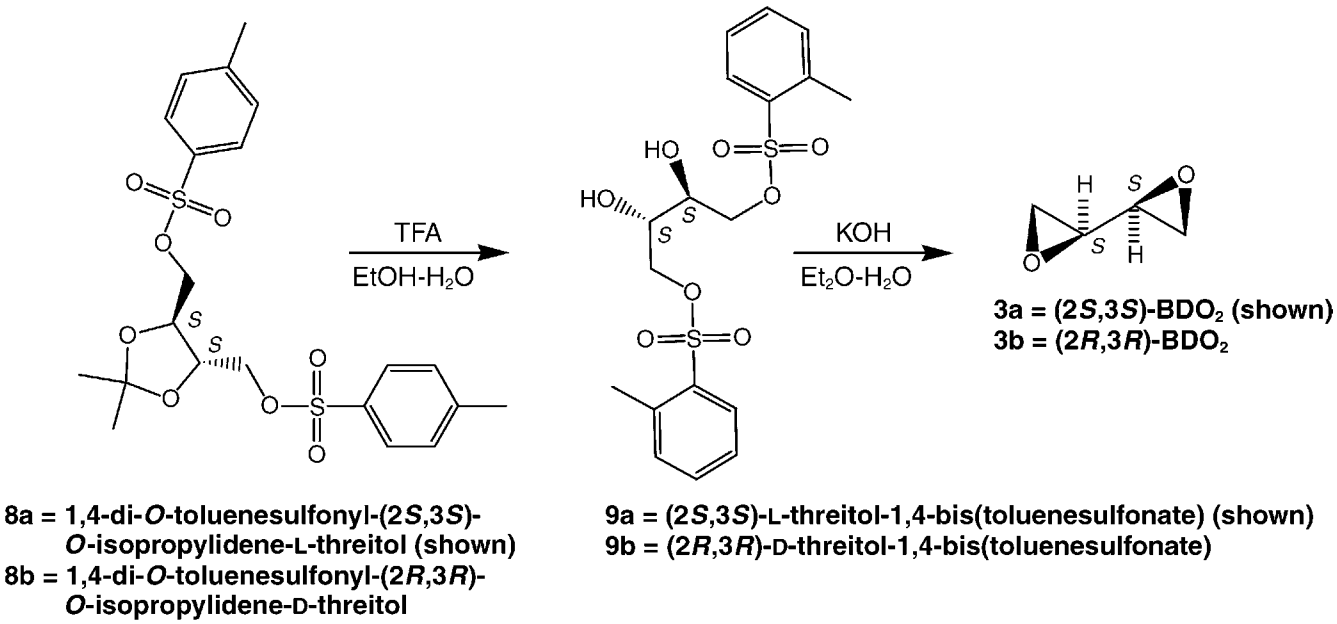

Figure 3. Synthetic scheme for stereoisomers of BDO_2 — Method B. The starting materials were 1,4-di-*O*-toluenesulfonyl-(2*S*,3*S*)-*O*-isopropylidene-L-threitol (**8a**) and 1,4-di-*O*-toluenesulfonyl-(2*R*,3*R*)-*O*-isopropylidene-D-threitol (**8b**).

water, dried over sodium sulfate (Na_2SO_4), and filtered and carefully evaporated to yield **3a** (Figure 2) (360 mg, 44% yield) as a clear oil. Analytical data: Mass spectra positive ion mode (M+1) mass/charge ratio (*m/z*) calculated value, 87.04, observed value 87.03; ^{1}H NMR spectroscopy solvent acetonitrile-d3 (CD_3CN) chemical shift (δ) in ppm: 3.21–3.22 (doublet of doublets [dd], coupling value [*J*] = 1.7 Hz, 2H); 3.28–3.32 (multiplet [m], 4H); optical rotation $[\alpha]_{D}^{23}$ ($^{temp}_{sodium\ D\ line}$) = +17.5° (concentration [c] = 2.0 g/100 mL in the solvent chloroform [$CHCl_3$]); Lit +23.6° (c = 2.0, $CHCl_3$) (Feit 1960).

(2R,3R)-BDO$_2$ (3b) KOH (2 g, 35.6 mmol) in water (3 mL) was added dropwise to **7b** (4.5 g, 17 mmol) in ether (40 mL). The mixture was stirred at ~20°C for 3 hours with strong agitation (resulting in a biphasic mixture). The layers were separated, and the ether layer washed with water, dried over Na_2SO_4, and evaporated to yield **3b** (Figure 2) (680 mg, 46% yield) as an oil. Analytical data: Mass (M+1) *m/z* calculated value, 87.04, observed value, 87.03; ^{1}H NMR (CD_3CN) δ (ppm): 3.21–3.22 (dd, *J* = 1.5 Hz, 2H); 3.28–3.32 (m, 4H); $[\alpha]_D = -18.0°$ (c = 2.0, $CHCl_3$); Lit −23.9° (c = 2.0, $CHCl_3$) (Feit 1960).

Synthesis of (2*S*,3*S*)-BDO_2 and (2*R*,3*R*)-BDO_2 — Using Method B

(2S,3S)-L-Threitol-1,4-bis(toluenesulfonate) (9a) 1,4-di-*O*-toluenesulfonyl-(2*S*,3*S*)-*O*-isopropylidene-L-threitol (**8a**; shown in Figure 3) (10 g, 21.3 mmol) (Aldrich) was suspended in 96% ethanol/water (40 mL). Trifluoroacetic acid (TFA) (500 µL, 6.5 mmol) was added, and the mixture was refluxed for 3 hours (or until the reaction was complete as verified by TLC analysis in 20/80 ethyl acetate/hexane). The solution was cooled to precipitate **9a** (Figure 3), which was filtered and dried to yield 4.89 g (53.7% yield). Analytical data: MP, 68°–71°C; Mass (M+1) *m/z* calculated value, 431.08, observed value, 431.0.

(2R,3R)-D-Threitol-1,4-bis(toluenesulfonate) (9b) 1,4-di-*O*-toluenesulfonyl-(2*R*,3*R*)-*O*-isopropylidene-D-threitol (**8b**) (Figure 3) (10 g, 21.3 mmol) (Aldrich) was suspended in 96% ethanol/water (40 mL). TFA (500 µL, 6.5 mmol) was added, and the mixture was refluxed for 3 hours (or until complete as verified by TLC analysis in 20/80 ethyl acetate/hexane). The solution was cooled to precipitate **9b** (Figure 3), which was filtered to yield 9.1 g (100% yield). Analytical data: MP, 68°–70°C; Mass (M+1) *m/z* calculated value, 431.08, observed value, 431.0.

(2S,3S)-BDO$_2$ (3a) KOH (2.5 g, 40 mmol) in water (1.5 mL) was added dropwise to **9a** (4.89 g, 10 mmol) in ether (30 mL). The mixture was stirred at room temperature for 3 hours (or until complete as verified by TLC analysis in 20/80 ethyl acetate/hexane) with strong agitation (biphasic mixture). The layers were separated and the ether layer washed with water, followed by 5% sodium bicarbonate,

dried over sodium sulfate, and evaporated to yield **3a** (Figure 3) (185 mg, 18.9% yield) as a liquid. Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 3.21–3.22 (dd, J = 1.7 Hz, 2H); 3.28–3.32 (m, 4H); $[\alpha]_D$ = +15.22° (c = 2 in $CHCl_3$); Lit +23.6° (c = 2.0, $CHCl_3$) (Feit 1960).

(2R,3R)-BDO$_2$ (3b) KOH (4.6 g, 82 mmol) in water (2.5 mL) was added dropwise to **9b** (9.1 g, 21.1 mmol) in ether (50 mL). The mixture was stirred at room temperature for 3 hours (or until complete as verified by TLC analysis in 20/80 ethyl acetate/hexane) with strong agitation (biphasic mixture). The layers were separated and the ether layer washed with water, followed by 5% sodium bicarbonate, the organic layer separated, dried over sodium sulfate, and evaporated to yield **3b** (Figure 3) (340 mg, 18.7% yield) as a liquid. Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 3.21–3.22 (dd, J = 1.5, 2H); 3.28–3.32 (m, 4H); $[\alpha]_D$ = −12.42° (c = 2.0, $CHCl_3$); Lit −23.9° (c = 2.0, $CHCl_3$) (Feit 1960).

Synthesis of (2*R*)-BDO and (2*S*)-BDO

1-O-Toluenesulfonyl-3-buten-(2R/2S)-ol (10) Following the method used by Martinelli (2002), Bu_2SnO (dibutyltin oxide) (2.82 g, 11.35 mmol) was added to (2*R*)-BD-diol/(2*S*)-BD-diol [**2**] (10 g, 113.5 mmol) in methylene chloride (CH_2Cl_2) (200 mL) (Figure 4). Toluenesulfonyl chloride (Tos-Cl) (21.6 g, 113.5 mmol) and triethylamine (NEt_3) (16 mL, 113.5 mmol) were added, and the mixture was stirred at room temperature overnight. Water (50 mL) was added, and the layers were separated. The aqueous layer was extracted twice, each time with 50 mL of CH_2Cl_2, and the combined organic layers were washed twice with a 100 mL

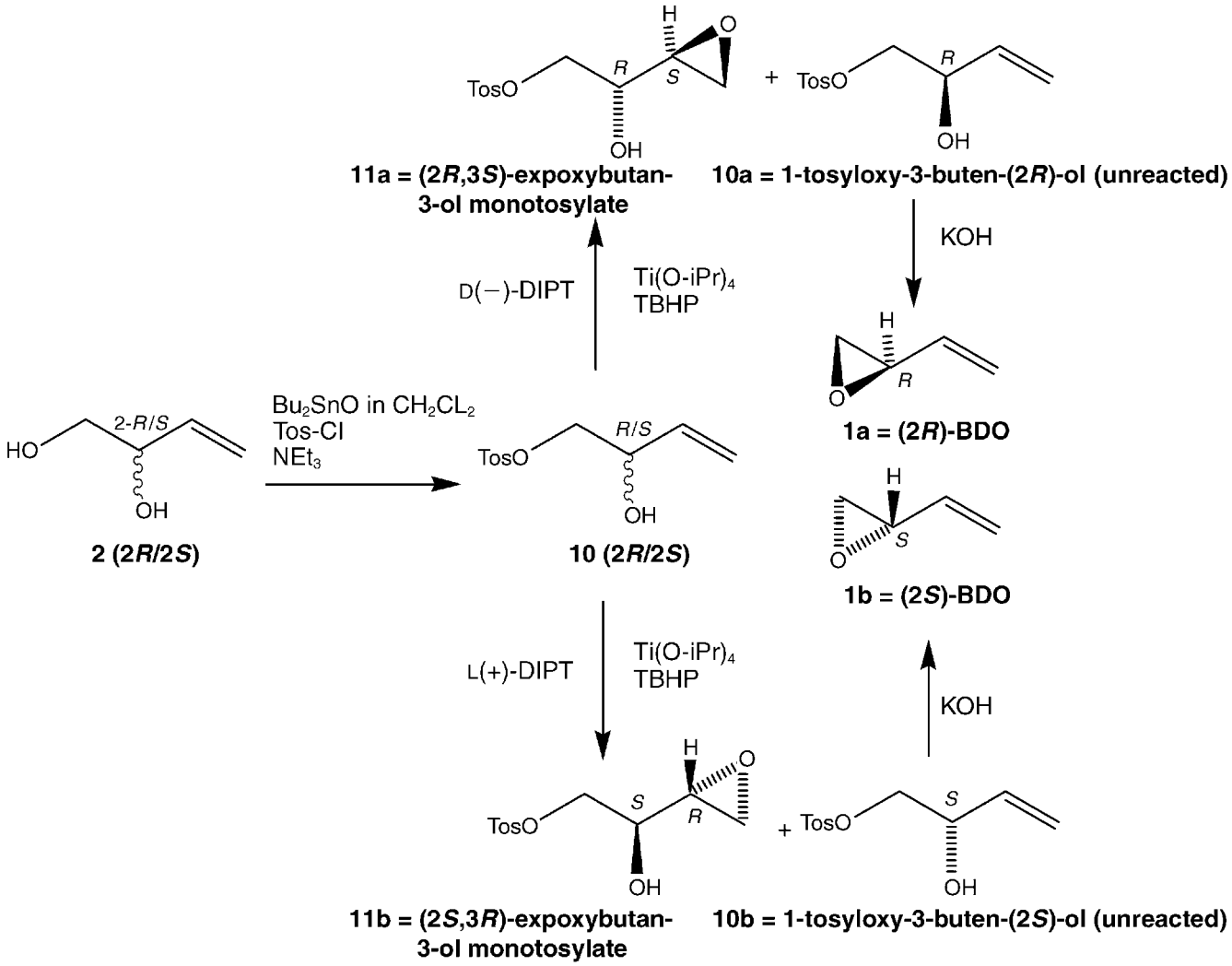

Figure 4. Synthetic scheme for stereoisomers of BDO. The starting materials were (2*R*)-BD-diol (**2a**) and (2*S*)-BD-diol (**2b**).

mixture of water and brine, dried over sodium sulfate, and evaporated to produce **10** (2*R*/2*S*) (Figure 4) as an oil that crystallized upon cooling (26.8 g, 97% yield). Analytical data: ^{1}H NMR ($CDCl_3$) δ (ppm): 2.4 (singlet [s], 3H), 2.5 (s, 1H), 3.87–3.91 (dd, *J* = 7.26 Hz, 1H); 4.01–4.04 (dd, *J* = 3.43 Hz, 1H); 4.35–4.39 (m, 1H), 5.19–5.22 (dd, *J* = 10.49, 1H); 5.32–5.36 (dd, 17.15 Hz, 1H); 5.68–5.77 (m, 1H), 7.32–7.34 (dd, *J* = 8.07 Hz, 2H); 7.76–7.79 (dd, *J* = 8.27 Hz, 2H).

Kinetic Resolution CH_2Cl_2 (50 mL) containing molecular sieves (4A, 4–8 mesh) was cooled to −5°C under argon and D(−) [or L(+)]-diisopropyl-tartrate (DIPT) (430 µL, 2.48 mmol), and titanium isopropoxide ($Ti(O\text{-}iPr)_4$) (615 µL, 2.06 mmol) and *t*-butyl hydroperoxide (TBHP) (1.7 mL, aqueous concentration 5-6 M, 9.29 mmol) were added. The mixture was stirred at −5°C for 4 hours and then cooled to −15°C. Compound **10** (2*R*/2*S*) (5 g, 20.6 mmol) in CH_2Cl_2 (50 mL) was added, and the reaction stirred at −15°C for 36 hours. The reaction was brought to room temperature, filtered through Celite, and evaporated to produce an oil, which was further purified on a silica gel column using 30/70 ethyl acetate/hexane as the eluant. D(−)-DIPT produced the compound 1-tosyloxy-3-buten-(2*R*)-ol (**10a**) (Figure 4) (2.23g, 44.6%) (analytical data: $[\alpha]_D^{23}$ = +3.99° [c = 6.26, methanol (MeOH)]), plus the epoxide (2*R*,3*S*)-epoxybutan-3-ol monotosylate (**11a**) (Figure 4) (800 mg, 15.1%). L(+)-DIPT produced the compound 1-tosyloxy-3-buten-(2*S*)-ol (**10b**) (Figure 4) (2.47 g, 49.4%) (analytical data: $[\alpha]_D^{23}$ = −4.25° [c = 6.0, MeOH]; Lit $[\alpha]_D$ = −3.85° [c = 1.0, MeOH]), plus the epoxide (2*S*,3*R*)-epoxybutan-3-ol monotosylate (**11b**) (Figure 4) (1.12g, 21.2%) (Neagu and Hase 1993).

BDO Resolved **10a** and resolved **10b** (2.9 g, 12 mmol) and powdered KOH (4 g, 71.8 mmol) under N_2 were heated slowly in an oil bath to 100°–110°C (Crawford et al. 1976). The distillates, epoxide (2*R*)-BDO (**1a**), produced from **10a**, and (2*S*)-BDO (**1b**), produced from **10b**, were collected in a −20°C cooled receiver. ^{1}H NMRs of **1a** (2*R*) (Figure 4) and **1b** (2*S*) (Figure 4) were identical. Analytical data: ^{1}H NMR (CD_2Cl_2) δ (ppm): 2.59–2.62 (dd, *J* = 2.7 Hz, 1H); 2.9–2.93 (dd, *J* = 4.2 Hz, 3.9 Hz, 1H); 3.28–3.3 (m, 1H), 5.25–5.3 (m, 1H), 5.48–5.5 (m, 2H). Compound **10a** (2*R*) produced **1a** (2*R*): 212 mg, 25.3% yield. Analytical data: $[\alpha]_D^{23}$ = −0.53° (c = 9.4, CH_2Cl_2). Compound **10b** (2*S*) produced **1b** (2*S*): 292 mg, 36.5% yield. Analytical data: $[\alpha]_D^{23}$ = +0.49° (c = 10.2, CH_2Cl_2); Lit $[\alpha]_D^{23}$ = +0.16° (c = 0.057, CH_3CN) (Neagu and Hase 1993).

Synthesis of (2*R*,3*R*)-BDO-diol, (2*R*,3*S*)-BDO-diol, (2*S*,3*R*)-BDO-diol, and (2*S*,3*S*)-BDO-diol

1-(p-Nitrobenzoyl)-3-buten-2S-ol (12a) and 1-(p-nitrobenzoyl)-3-buten-2R-ol (12b) Bu_2SnO (2.82 g, 11.35 mmol) was added to **2** (2*R*/2*S*) (10 g, 113.5 mmol) in CH_2Cl_2 (200 mL) (Martinelli 2002) (Figure 5). *p*-Nitrobenzoyl chloride (PNB-Cl) (21.1 g, 113.5 mmol) and NEt_3 (16 mL, 113.5 mmol) were added, and the mixture was stirred at room temperature overnight. Water (50 mL) was added and the layers were separated. The aqueous layer was extracted twice, using 50 mL CH_2Cl_2 each time, and the combined organic layers were washed with water and brine (100 mL each), dried over sodium sulfate, and evaporated to produce **12** (2*R*/2*S*) as an oil that crystallized upon cooling. Recrystallization with 1/1 CH_2Cl_2/hexane produced **12a** (2*S*) and **12b** (2*R*) (15.75 g, 58.6% yield). Analytical data: MP, 85°–86°C; ^{1}H NMR ($CDCl_3$) δ (ppm): 2.06 (s, 1H, −OH), 4.35–4.39 (m, 1H, H-1a); 4.45–4.49 (m, 1H, H-1b); 4.55–4.57 (m, 1H, H-2); 5.32 (dd, 1H, *J* = 10.5 Hz, H-4a); 5.47 (dd, 1H, *J* = 17.3 Hz, H-4b); 5.92–6.0 (m, 1H, H-3); 8.22–8.32 [m, 4H, H-*p*-nitrobenzoyl (PNB)] (Claffey and Ruth 1996).

Sharpless Epoxidation CH_2Cl_2 (50 mL) containing molecular sieves (4A, 4–8 mesh) was cooled to −5°C under argon. D(−) [or L(+)] DIPT (430 µL, 2.48 mmol), $Ti(O\text{-}iPr)_4$ (615 µL, 2.06 mmol), and TBHP (1.7 mL, aqueous concentration, 5–6 M, 9.29 mmol) were added. The mixture was stirred at −5°C for 4 hours, and then cooled to −15°C. Compounds **12a** and **12b** (5 g, 20.6 mmol) in CH_2Cl_2 (50 mL) were added, and the reaction was stirred at −15°C for 36 hours. The reaction was brought to room temperature, filtered through Celite, and evaporated to produce an oil that was purified on a silica gel column using 30/70 ethyl acetate/hexane (Gao et al. 1987). D(−)-DIPT produced 1-*O*-(*p*-nitrobenzoyl)-(3*S*),4-epoxybutan-(2*R*)-ol (**13a**) (Figure 5) (2.63 g, 46.8%). Analytical data: ^{1}H NMR ($CDCl_3$) δ (ppm): 2.39 (s, 1H, −OH); 2.84–2.89 (m, 2H, H-4); 3.17–3.18 (m, 1H, H-3); 4.18 (m, 1H, H-2); 4.50 (dd, 1H, *J* = 11.48, 5.84 Hz, H-1a); 4.60 (dd, 1H, *J* = 11.68, 2.92 Hz, H-1b); 8.22–8.31 (m, 4H, H-PNB), plus recovered unreacted **12b** (2*R*) (2.15 g, 43%) (Claffey and Ruth 1996).

L(+)-DIPT produced 1-*O*-(*p*-nitrobenzoyl)-(3*R*),4-epoxybutan-(2*S*)-ol (**13b**) (Figure 5) (2.47 g, 43.9%). Analytical data: ^{1}H NMR ($CDCl_3$) δ (ppm): 2.59 (s, 1H, −OH); 2.81–2.88 (m, 2H, H-4); 3.16–3.17 (m, 1H, H-3); 4.14–4.15 (m, 1H, H-2); 4.48 (dd, 1H, *J* = 11.72, 6.34 Hz, H-1a); 4.57 (dd, 1H, *J* = 11.72, 3.91 Hz, H-1b); 8.19–8.28 (m, 4H, H-PNB), plus recovered unreacted **12a** (2*S*) (1.92 g, 38.4%).

Mitsunobu Reaction Toluene (30 mL) containing triphenylphosphine (PPh_3) (1.2 g, 4.74 mmol), *p*-nitrobenzoic acid (PNB acid) (792 mg, 4.74 mmol), and **13b** (2*S*,3*R*) (1 g, 3.95 mmol) were cooled to −5°C. Diethyl azodicarboxylate (DEAD) (747 µL, 4.74 mmol) was added dropwise over 45 minutes (Mitsunobu and Yamada 1967; Mitsunobu 1981). The mixture was brought to room temperature and stirred for 1 hour. The solvent was evaporated, and the product was

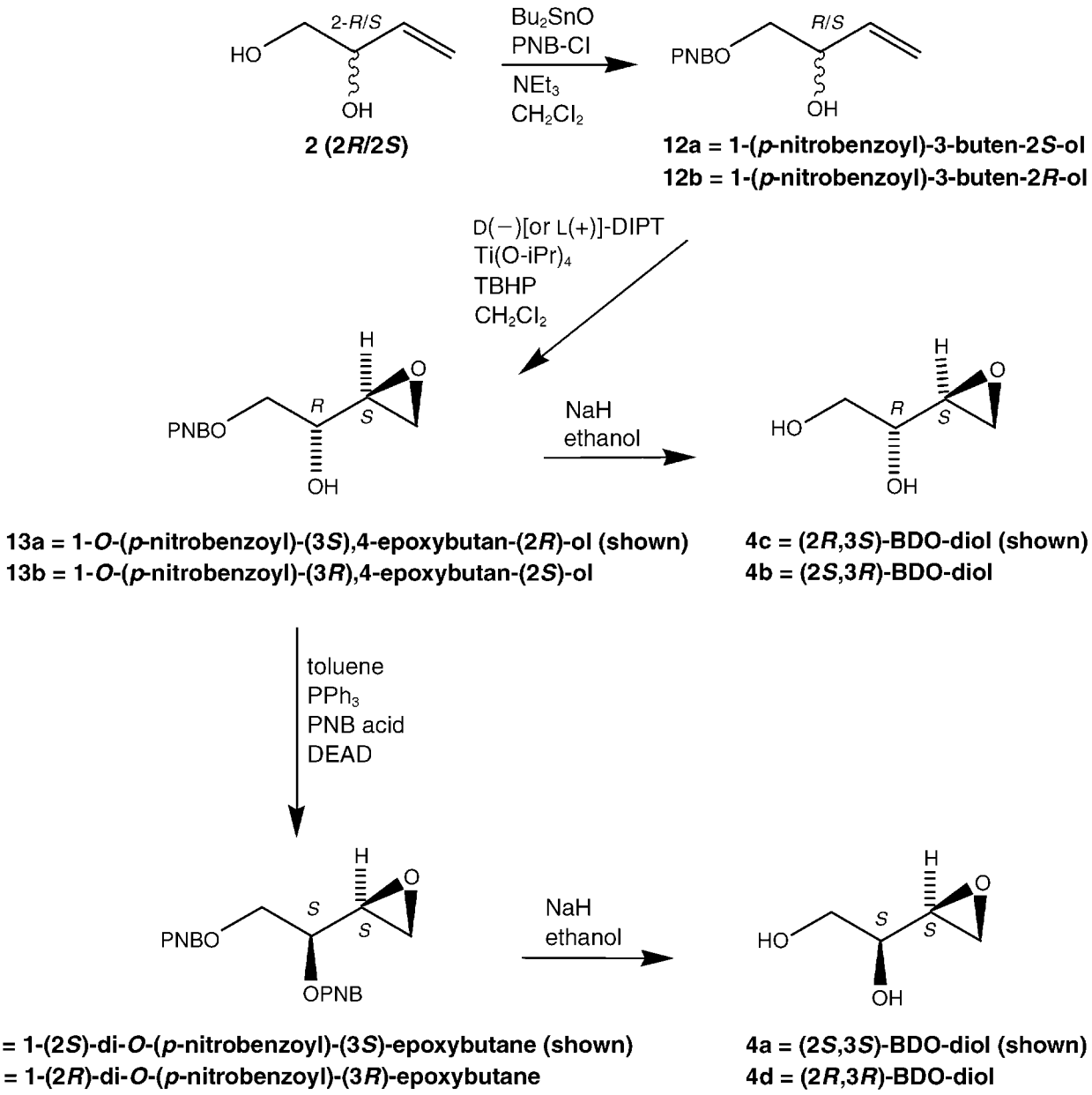

Figure 5. Synthetic scheme for stereoisomers of BDO-diol. The starting materials were (2*R*)-BD-diol (**2a**) and (2*S*)-BD-diol (**2b**).

purified on a silica gel column using 30/70 ethyl acetate/hexane as eluant to produce 1-(2*R*)-di-*O*-(*p*-nitrobenzoyl)-(3*R*)-epoxybutane (**14b**) (Figure 5) (1.33 g, 84% yield). Analytical data: ^{1}H NMR ($CDCl_3$) δ (ppm): 2.74–2.77 (m, 1H, H-4a); 2.87–2.9 (m, 1H, H-4b); 3.33–3.37 (m, 1H, H-3); 4.58 (dd, 1H, J = 11.85, 6.60 Hz, H-1a); 4.74 (dd, 1H, J = 12.0, 3.90 Hz, H-1b); 5.32–5.38 (m, 1H, H-2); 8.05–8.21 (m, 8H, H-PNB) (Claffey and Ruth 1996). Compound **13a** (2*R*,3*S*) (800 mg) produced 1-(2*S*)-di-*O*-(*p*-nitrobenzoyl)-(3*S*)-epoxybutane (**14a**) (Figure 5) (1.04 g, 82.2% yield). Analytical data: ^{1}H NMR ($CDCl_3$) δ (ppm): 2.80–2.83 (m, 1H, H-4a); 2.93–2.97 (m, 1H, H-4b); 3.40–3.42 (m, 1H, H-3); 4.58 (dd, 1H, J = 12.0, 6.60 Hz, H-1a); 4.80 (dd, 1H); 4.80 (dd, 1H, J = 12.0, 3.60 Hz, H-1b); 5.40–5.43 (m, 1H, H-2); 8.13–8.28 (m, 8H, H-PNB).

Deprotection of Alcohols Compounds **13a**, **13b**, **14a**, and **14b** were suspended in anhydrous ethanol (10 mL) and cooled to −5°C. A sodium ethoxide solution (sodium hydride [NaH], 6 mg, in 10 mL anhydrous ethanol) was

added dropwise. The mixture was brought to room temperature and stirred for 30 minutes, at which time completion of the reaction was verified by TLC analysis using 1/1 ethyl acetate/hexane eluant and 1% anisaldehyde detection solution. Amberlite CG-50 type 1 (Rohm and Hass, Philadelphia, PA) (5 g) was added to the mixture, and stirring continued for 15 minutes. The solvent was decanted and the resin was washed three times with ethanol (20 mL each). The decanted ethanol and washes were combined and then evaporated, and the products [(2*S*,3*S*)-BDO-diol (**4a**), (2*S*,3*R*)-BDO-diol (**4b**), (2*R*,3*S*)-BDO-diol (**4c**), and (2*R*,3*R*)-BDO-diol (**4d**)] were purified on a silica gel column using 2/1 ethyl acetate/acetone as the eluant.

Compound **13a** (2*R*,3*S*) (1.83 g, 7.72 mmol) produced **4c** (2*R*,3*S*) (Figure 5), as a liquid (583 mg, 77.5% yield). Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 2.51–2.58 (m, 2H, H-4); 2.78–2.82 (m, 1H, H-3); 2.95 (br [broad] s, 1H, −OH); 3.14 (br s, 1H, −OH); 3.35–3.45 (m, 3H, H-2 and H-1); $[\alpha]_D^{24} = -2.53°$ (c = 1.98, MeOH); Lit $[\alpha]_D = -2.54°$ (c = 1.0, MeOH) (Claffey and Ruth 1996).

Compound **13b** (2*S*,3*R*) (1.27 g, 4.78 mmol) produced **4b** (2*S*,3*R*) (Figure 5), as a liquid (380 mg, 76.4% yield). Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 2.66–2.73 (m, 2H, H-4); 2.93–2.98 (m, 2H, H-3 and −OH); 3.15 (dd, 1H, −OH, J = 4.2 Hz); 3.46–3.64 (m, 3H, H-2 and H-1); $[\alpha]_D^{24} = +3.20°$ (c = 7.81 g/100 mL in MeOH); Lit $[\alpha]_D = +2.49°$ (c = 1.0, MeOH) (Claffey and Ruth 1996).

Compound **14a** (2*S*,3*S*) (1.92 g, 4.80 mmol) produced **4a** (2*S*,3*S*) (Figure 5) as a liquid (325 mg, 65.1% yield). Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 2.58–2.65 (m, 2H, H-4); 2.85–2.89 (m, 1H, H-3); 3.07 (br s, 1H, −OH); 3.25 (br s, 1H, −OH); 3.37–3.56 (m, 3H, H-2 and H-1); $[\alpha]_D^{24} = -1.41°$ (c = 7.1 g/100 mL in MeOH); Lit $[\alpha]_D = -1.32°$ (c = 1, MeOH) (Claffey and Ruth 1996).

Compound **14b** (2*R*,3*R*) (2.36 g, 5.9 mmol) produced **4d** (2*R*,3*R*) (Figure 5), as a liquid (430 mg, 70% yield). Analytical data: ^{1}H NMR (CD_3CN) δ (ppm): 2.59–2.72 (m, 2H, H-4); 2.91–2.96 (m, 1H, H-3); 3.31–3.38 (m, 2H, −OHs); 3.46–3.53 (m, 3H, H-2 and H-1); $[\alpha]_D^{24} = +1.51°$ (c = 6.6 g/100 mL in MeOH); Lit $[\alpha]_D = +1.29°$ (c = 1.0, MeOH) (Claffey and Ruth 1996).

SPECIFIC AIM 2: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, AND *meso*-BDO_2

Chemicals and Cells

TK6 cells were purchased from American Type Culture Collection (Manassas, VA). Reagents for TK6 cell culture, mouse lymphocyte culture, and *HPRT* and *TK* mutant selection were purchased from the following sources: ethyl methanesulfonate (EMS), 6-thioguanine (6-TG), trifluorothymidine (TFT), and HEPES buffer (Fisher Scientific, New York, NY); HL-1 medium, RPMI 1640 medium, fetal bovine serum, L-glutamine, nonessential amino acids, penicillin–streptomycin, and sodium pyruvate (BioWhittaker, Walkersville, MD).

Measurement of *HPRT* and *TK* M*f*s in TK6 Cells Exposed to (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, or *meso*-BDO_2

Cytotoxicity was studied by determining survival of cells treated with a stereoisomer compared with that of control cells. TK6 cells growing at an exponential rate were exposed in triplicate to 0, 2, 4, 6, 8, or 10 μM of each stereoisomer of BDO_2 for 24 hours. Immediately after the end of exposure, the cells were washed and plated at a density of 1 viable cell per well in 96-well, U-bottom microtiter plates in the presence of 4×10^4 lethally irradiated feeder cells. At 8 to 10 days after plating, the clones formed in the 96-well plates were inspected microscopically. Relative cell survival was calculated by comparing CEs in exposed cells versus those in unexposed cells (Meng et al. 2000a).

To evaluate and compare the mutagenic potencies of stereoisomers of BDO_2 in TK6 cells, groups of five cultures were exposed to 0, 2, 4, or 6 μM of each form of BDO_2. In addition, a group of five flasks was exposed to 200 μM of EMS as a positive control. Cell survival assays were not performed for the control cultures; however, live cells were counted by the trypan blue exclusion method at the end of the 24-hour exposure. At these BDO_2 dose levels, an average of at least 30.8 million live cells in each culture could be evaluated for M*f*s. (Live cell counts for each cell culture are listed in the appendix tables.)

TK and *HPRT* M*f*s were determined using the lymphocyte cloning assay either 3 or 7 days after exposure, to allow time for the phenotypic expression of the mutations. The cell cloning assays were performed according to published methods (Cochrane and Skopek 1994). For the cell cloning assays, TK6 cells were grown in suspension in tissue culture flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 12.5 mM HEPES buffer, 4 mM L-glutamine, 100 μM nonessential amino acids, and 100 U/mL penicillin–streptomycin. Cells were subcultured daily at 4×10^5 cells/mL. Prior to exposure, the cells were grown for 2 days in medium containing CHAT (cytidine, 10^{-5} M; hypoxanthine, 2×10^{-4} M; aminopterin, 2×10^{-7} M; and thymidine, 1.75×10^{-5} M) and 1 day in medium containing THC (thymidine, 10^{-5} M; hypoxanthine, 2×10^{-4} M; and cytidine, 10^{-5} M) to reduce the background M*f*s (Liber and Thilly 1982). Two 96-well, U-bottom microtiter plates per sample were seeded with

1 viable cell per well in the presence of 4×10^4 lethally irradiated feeder cells per well to determine CEs. To evaluate the *HPRT* or *TK* M*f*s for each sample, ten 96-well plates were seeded at 4×10^4 cells per well in the presence of 1 µg/mL 6-TG (for *HPRT* mutants) or 1 µg/mL TFT (for *TK* mutants). Plates for *TK* mutation selection were again fed with TFT one week after initial plating. The *HPRT*-negative colonies were scored at 10 days after plating, and the *TK*-negative colonies were scored at 21 to 24 days after plating for both fast-growth and slow-growth *TK* mutants. M*fs* were calculated as the ratio of mean CEs in selection medium to those in nonselection medium (Sussman et al. 1999).

Multiplex-PCR Analysis of *HPRT* Mutant Genomic DNA

To assure isolation of independent mutant clones, 100 individual 10 mL culture flasks were exposed to 4 µM of each BDO_2 stereoisomer, and only one mutant colony from each culture was subcloned in 6-well plates and collected for molecular analysis. For each *HPRT* mutant, exons 1 and 2 were amplified at the 5′ end and exons 7, 8, and 9 were amplified at the 3′ end by genomic PCR. The simultaneous amplification of exons 1, 2, 7, 8, and 9 of human *HPRT* genomic DNA from TK6 cells was achieved using the method of Gibbs (Gibbs et al. 1990). Briefly, 5 µL of a crude cell lysate consisting of 1×10^4 cells was mixed with four pairs of primers in a reaction mixture that had a total volume of 50 µL and contained PCR buffer, 1.5 mM of deoxyribonucleotide triphosphate (dNTP), and 4 units of *Taq* DNA polymerase. The PCR parameters were as follows: 94°C for 5 minutes and then 33 cycles of denaturation (94°C, 1 minute), annealing (59°C, 1 minute), and DNA polymerization (68°C, 2 minutes). Each reaction product (20 µL) was run on 1.4% agarose gel containing ethidium bromide, to check the efficiency of PCR amplification using a transilluminator. For mutants that failed to produce any PCR products, K-*ras* exon 2 was amplified to confirm the quality of the PCR reaction.

SPECIFIC AIM 3: CYTOTOXICITY AND MUTAGENICITY OF (2*R*)-BDO AND (2*S*)-BDO IN TK6 CELLS

Cells and reagents were purchased from the sources described for the BDO_2 study. Exponentially growing TK6 cells were exposed to 0, 200, 400, 600, 800, or 1000 µM of (2*R*)- or (2*S*)-BDO for 24 hours in triplicate for cytotoxicity studies. Immediately after exposure, cells were washed and plated on 96-well plates to determine relative cell survival, calculated by comparing CEs in exposed versus unexposed cells. To determine the mutagenic potency of each stereoisomer of BDO, TK6 cells cultures were exposed in suspension to 0, 200, 400, or 600 µM of each stereoisomer of BDO for 24 hours based upon the cytotoxicity data. Cell survival assay was not performed for these cultures; however, live cells were counted by the trypan blue exclusion method at the end of the 24-hour exposure. With these BDO stereoisomer dose levels, the average number of live cells for M*fs* was at least 40.4 million. Live-cell counts for each group are listed in the appendix tables. The *HPRT* and *TK* M*f*s were measured as described above for BDO_2 stereoisomers (Cochrane and Skopek 1994).

SPECIFIC AIM 4: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO-DIOL, (2*R*,3*S*)-BDO-DIOL, (2*S*,3*R*)-BDO-DIOL, AND (2*S*,3*S*)-BDO-DIOL IN TK6 CELLS

Reagents and TK6 cells were purchased from the sources described for the BDO_2 study. The cytotoxicity of each stereoisomer of BDO-diol was determined by comparing CEs of exposed cells to those of control cells. Triplicate cultures of TK6 cells were exposed to 0, 200, 400, 600, 800, or 1000 µM of each BDO-diol stereoisomer for 24 hours. After exposure, cells were plated on 96-well plates to determine relative cell survival for each culture.

To measure *HPRT* and *TK* M*f*s, exposure concentrations were selected based on results of the cytotoxicity experiments. Five tissue-culture flasks were exposed for 24 hours to 0, 200, 600, or 1000 µM of (2*R*,3*R*)-BDO-diol, (2*S*,3*R*)-BDO-diol, or (2*S*,3*S*)-BDO-diol or to 40, 120, or 200 µM of (*2R,*3*S*)-BDO-diol. A cell survival assay was not performed for these cultures; however, live cells were counted by the trypan blue exclusion method at the end of the 24-hour exposure. For the dose levels of 40 and 120 µM of (*2R,*3*S*)-BDO-diol, the average number of live cells that could be analyzed for M*fs* in each flask was at least 48.6 million. For concentrations of 120 and 200 µM of (2*R,*3*S*)-BDO-diol, an average of only 8.9 and 4.5 million live cells, respectively, could be analyzed for M*fs* in each flask. Live cell counts for each group are listed in the appendix table A.2. The *HPRT* and *TK* M*f*s were measured as described above for BDO_2 stereoisomers (Cochrane and Skopek 1994).

Due to the great cytotoxicity of (2*R,*3*S*)-BDO-diol observed at the dose levels described above (120 and 200 µM), additional cytotoxicity tests of the four BDO-diol stereoisomers were performed at lower concentrations: 5, 10, 20, 30, and 40 µM. Based on the results of these experiments, five flasks were exposed to 0, 5, 10, or 20 µM of each BDO-diol stereoisomer for 24 hours. A cell survival assay was not performed for these cultures; however, live cells were counted by the trypan blue exclusion method at the end of the 24-hour exposure. With these BDO-diol dose levels, the average number of live cells in each group available for

determining M*fs* was at least 171.4 million. Live cell counts for each group are listed in the appendix tables. The *HPRT* and *TK* M*f*s were measured as described above.

STATISTICAL METHODS AND DATA ANALYSIS

The use of five cultures per group to determine the mutagenic potency of BD stereoisomers was based on the historical range of background *HPRT* and *TK* M*f*s in TK6 cells. Results of previous mutagenicity studies of BD metabolites and other agents in this cell line show that using five replicate cultures enabled the detection of a chemically induced mutagenic response as low as two-fold above background (Cochrane and Skopek 1994; Steen et al. 1997a,b; Meng et al. 2000a,b).

Differences in CE and M*f* values between the control and treated cell cultures were tested in a pair-wise format using a *t* test (for parametric data) or the Mann-Whitney test (for nonparametric data; SigmaStat 3.0). To compare stereoisomers of BDO, BDO_2, and BDO-diol, regression models were fit on the data for each stereoisomer across the dose levels. Slope parameters were compared to determine the differences between the fitted curves.

The Fisher exact test was used to assess the difference, in percent of genomic deletions in *HPRT* mutations, between the controls and each BDO_2-treated sample. Differences in percentage of deletion mutations among the cells exposed to the three stereoisomers of BDO_2 were tested with the chi-square test. A P value ≤ 0.05 was considered significant.

RESULTS

SPECIFIC AIM 1: SYNTHESIS OF EIGHT STEREOISOMERS OF BDO_2, BDO, AND BDO-DIOL

The synthesis of eight butadiene epoxide stereoisomers (series **1**, **3**, and **4** in Figure 1) was approached in three phases. Phase I consisted of synthesis of two BDO_2 isomers (series **3**) via readily available, chirally pure 2,3-*O*-isopropylidene-threitols (**5a** or **5b**, Figure 2). Our first attempt (method A, Figure 2) utilized the method of Feit (1964) involving the formation of the bis(methylsulfonyl) esters (**6a** and **6b**) followed by the acid-catalyzed biphasic deprotection of the 2,3-*O*-isopropylidene group to yield **7a** and **7b**. The intramolecular displacement of the bis(methylsulfonyl) esters to form BDO_2 isomers proceeded smoothly under basic biphasic conditions, to produce the desired BDO_2 isomers [**3a** (2*S*,3*S*) and **3b** (2*R*,3*R*)] (Feit 1960). The result of this three-step method was that the overall isomer yield from each starting threitol was approximately 13%.

An alternative two-step method (method B, Figure 3) was also used; it started with the commercially available 1,4-di-*O*-toluenesulfonyl-2,3-*O*-isopropylidene-threitols (**8a** and **8b**). TFA-catalyzed deprotection of the isopropylidene function under biphasic conditions produced **9a** and **9b,** in yields of 53.7% and 100%, respectively. This was followed by formation of the bis(epoxides) (**3a** and **3b**) by displacement of the toluensulfonyl groups, using the same basic biphasic conditions as in method A. **9a** (2*S*,3*S*) produced an 18.9% yield of (2*S*,3*S*)-BDO_2 (**3a**) (10% overall yield), whereas **9b** (2*R*,3*R*) produced an 18.7% yield of (2*R*,3*R*)-BDO_2 (**3b**) (18.7% overall yield). Although method B appeared to be simpler and faster, the starting materials **8a** and **8b** cost more, and the method would not result in an appreciable increase in the efficiency of the reactions to produce **3a** and **3b**; therefore, one method was not preferred over the other. BDO_2 isomers made according to both methods A and B were combined and used in the mutagenicity studies.

Phase II involved synthesis of the two isomers of BDO [**1a** (2*R*) and **1b** (2*S*), Figure 4]. In this procedure, we started from the commercially available racemic butene-1,2-diol and converted it regiospecifically to the 1-*O*-tosyl ester (**10**) with a 97% yield using the method of Martinelli (2002). The asymmetric epoxidation procedure of Sharpless was then used to perform a kinetic resolution of the racemic mixture. The resulting chiral epoxide products (**11a** or **11b**) were separated from unreacted chiral **10** using chiral D(−) or L(+)-DIPT to direct *t*-butyl hydroperoxide attack toward the less sterically hindered face of the resulting chiral complex (Finn and Sharpless 1985**;** Gao et al. 1987). This enabled easy resolution of epoxide products **11a** (2*R*,3*S*) and **11b** (2*S*,3*R*) from the chirally resolved starting materials **10a** (2*R*) and **10b** (2*S*). The latter alkenes were then converted by tosyl displacement under anhydrous, solvent-free basic conditions to produce the epoxide butenes **1a** (2*R*-BDO) and **1b** (2*S*-BDO) upon careful distillation. This two-step procedure (Crawford et al. 1976) resulted in overall yields of 11.3% and 18.0%, for **1a** and **1b**, respectively, for the original racemic butenediol.

For phase III, we originally envisioned that compounds **11a** and **11b** (Figure 4) could be easily converted to their diols **4c** and **4a** (Figure 1). However, owing to the lower yields of the isolated epoxide compounds (15.1% for **11a** and 21.2% for **11b**) in the Sharpless asymmetric epoxidation (most likely due to the lability of the tosylates themselves), we decided to use the more stable **12a** and **12b** (Figure 5) instead, based upon the earlier results of Claffey and Ruth (1996). Again using the method of Martinelli, a

58.6% yield of **12a** and **12b** was produced from **2** (2*R*/2*S*) and used in the Sharpless procedure to resolve the racemic mixture (Finn and Sharpless 1985; Gao et al. 1987; Martinelli 2002). D(−)-DIPT epoxidation of **12a** and **12b** produced a 46.8% yield of product **13a** (2*R*,3*S*) along with 43% unreacted **12b** (2*R*), while L(+)-DIPT epoxidation of **12a** and **12b** produced a 43.9% yield of product **13b** (2*S*,3*R*) along with 38.4% unreacted **12a** (2*S*). Epoxides **13a** (2*R*,3*S*) and **13b** (2*S*,3*R*) were then converted directly to their respective BDO-diol stereoisomers, **4c** (2*R*,3*S*) and **4b** (2*S*,3*R*), by deprotection with sodium ethoxide in ethanol (77.5% and 76.4% yields, respectively). However, a chiral conversion at carbon 2 was required, to provide access to the two remaining diastereomeric diol epoxides, **4a** (2*S*,3*S*) and **4d** (2*R*,3*R*). Thus, compounds **13a** (2*R*,3*S*) and **13b** (2*S*,3*R*) were converted using the Mitsunobu reaction to produce yields of 82.2% and 84%, respectively, of the di-PNB esters **14a** (2*S*,3*S*) and **14b** (2*R*,3*R*). Deprotection of **14a** (2*S*,3*S*) and **14b** (2*R*,3*R*) using sodium ethoxide in ethanol produced 65.1% and 70% yields, respectively, of the required BDO-diol isomers **4a** (2*S*,3*S*) and **4d** (2*R*,3*R*) (Mitsunobu and Yamada 1967; Mitsunobu 1981). Using these methods, we were able to produce all four isomers of BDO-diol, **4a** (2*S*,3*S*), **4b** (2*S*,3*R*), **4c** (2*R*,3*S*) and **4d** (2*R*,3*R*) with overall yields of 14.7%, 19.7%, 21.3%, and 15.1%, respectively, from the original racemic butene-diol **2** (2*R*/2*S*).

SPECIFIC AIM 2: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, AND *meso*-BDO_2

Measurement of *HPRT* and *TK* M*f*s in TK6 Cells Exposed to (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, or *meso*-BDO_2

The cytotoxicity of the three stereoisomers of BDO_2 was evaluated to define an appropriate dose range of BDO_2 exposure for mutagenicity studies. Relative survival was evaluated by comparing CEs in cells exposed to stereoisomers of BDO_2 versus those in unexposed cells (with the control value set at 100%). Cell survival declined with increasing exposure concentration (Figure 6). CEs in most of the exposed cells were lower than those in the control cells, with the exception that cells exposed to 2 µM of *meso*-BDO_2 had an average relative survival rate of 104%. The relative survival rates for the cells exposed to the three isomers of BDO_2 appeared different from each other at the 2 µM exposure level, with survival of cells exposed to *meso*-BDO_2 being the greatest followed by those exposed to (2*R,3R*)-BDO_2 and then those exposed to (2*S*,3*S*)-BDO_2; however, because the sample sizes were small (three cultures/group), the differences were not

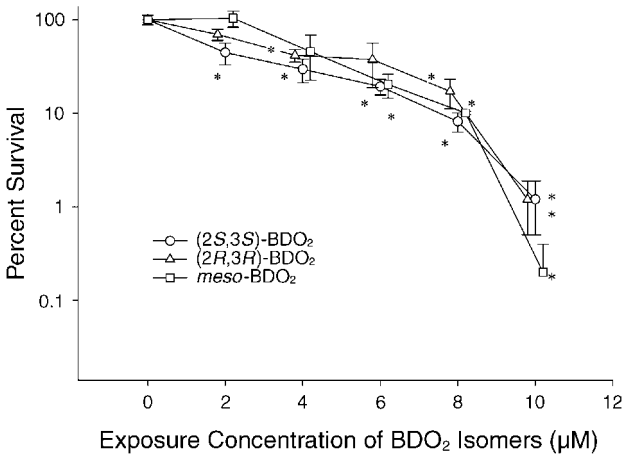

Figure 6. Effects of the exposure concentration of each isomer of BDO_2 on relative survival of TK6 cells. Cultures (n = 3/group) were exposed to 0, 2, 4, 6, 8, or 10 µM of BDO_2 for 24 hours. Relative cell survivals were ratios of CEs in treated versus control samples. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

significant (P = 0.065). At higher exposure concentrations (≥ 4 µM), the relative survival rates for the cells exposed to the three BDO_2 isomers were very similar.

Based on the results of the cytotoxicity experiment, groups of five cultures were exposed to 0, 2, 4, or 6 µM of each stereoisomer of BDO_2 for 24 hours to determine the mutagenic potencies of the BDO_2 stereoisomers. Our previous studies showed that if the relative survival rate is 20% or more (at least 20×10^6 cells) after chemical exposure, there would be sufficient live cells to determine M*f*s with a reasonable coefficient of variation (Meng et al. 2000a,b). Thus, the highest exposure concentration used was 6 µM. To facilitate the comparison with results of previous studies, we chose 4 µM as the middle dose because it had been used by Steen and colleagues (1997a) to evaluate the mutagenic response of the racemic mixture of BDO_2 at the *HPRT* locus in TK6 cells. A lower dose (2 µM) was added to generate a four-point dose-response curve.

The average of *HPRT* M*f*s in unexposed cells was 5.0 ± 2.1×10^{-6} (mean ± SD), which was slightly higher than that reported by Steen and colleagues (1997a) and Cochrane and Skopek (1994), but was consistent with the spontaneous *HPRT* M*f* values observed in this laboratory (Sussman et al. 1999; Meng et al. 2000b). The average value of *HPRT* M*f*s in EMS-exposed cells was 113.0 ± 31.4 × 10^{-6}. All three stereoisomers of BDO_2 caused increased *HPRT* M*f*s at all exposure concentrations, with P values ranging from 0.046 to < 0.001 (Figure 7). The dose-related mutagenic responses caused by the three isomers of BDO_2 were similar,

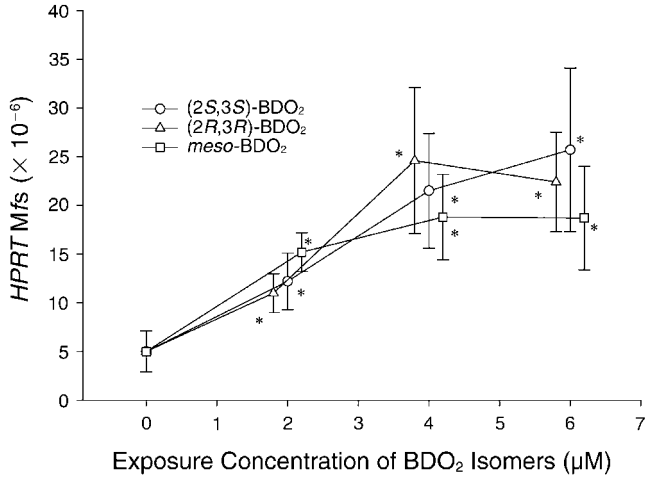

Figure 7. The relationship between the exposure concentration of each isomer of BDO_2 and BDO_2-induced *HPRT* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 2, 4, or 6 μM of BDO_2 for 24 hours. A cell cloning assay was used to measure the *HPRT* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

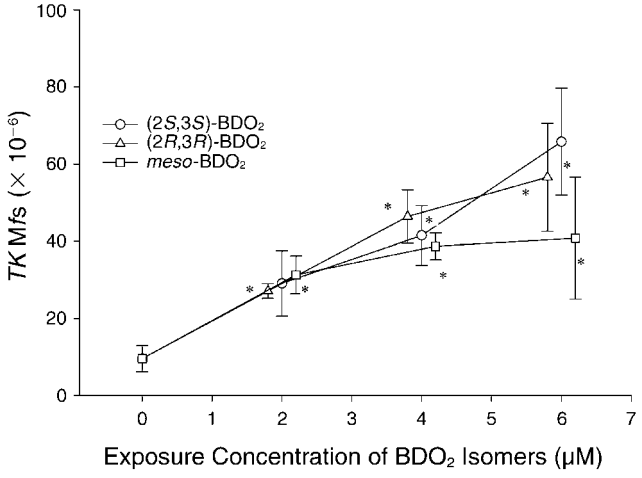

Figure 8. The relationship between the exposure concentration of each isomer of BDO_2 and BDO_2-induced *TK* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 2, 4, or 6 μM of BDO_2 for 24 hours. A cell cloning assay was used to measure the *TK* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

with no significant differences in M*f*s found at any exposure level.

The plates for measuring *TK* M*f*s were scored for both fast- and slow-growth mutants at 21 through 24 days after plating. The average *TK* M*f* value for control cells was 9.6 ± 3.4 × 10^{-6} (mean ± SD), which was similar to the values observed in our previous studies (Meng et al. 2000a, b). The *TK* M*f* value in the EMS-exposed cells was 132.6 ± 39.2 × 10^{-6}. All three forms of BDO_2 induced significantly increased *TK* M*f*s compared to the concurrent controls, with P values ranging from 0.036 to 0.001 except for (2*S*,3*S*)-BDO_2 at 2 μM (Figure 8). At the concentrations of 2 and 4 μM, the mutagenic response induced by the three forms of BDO_2 was essentially the same. The mutagenic response to 6 μM *meso*-BDO_2 appeared to be less than the response to (2*S*,3*S*)-BDO_2 and (2*R*,3*R*)-BDO_2, but in fact (as with the *HPRT* gene) no statistically significant difference in *TK* M*f*s was found at any exposure level across the isomers. The averages for BDO_2-induced *TK* M*f*s (with the background level subtracted) for the three isomers were 19.6, 32.6, and 44.8 × 10^{-6} for the exposure concentrations of 2, 4, and 6 μM, respectively. These values were 2.3-, 2.0-, and 2.8-fold greater, respectively, than those for the *HPRT* M*f*s in the same cultures.

To compare the M*f*s of the three stereoisomers of BDO_2 across the full dose range, an analysis of covariance (ANCOVA) linear model was used to analyze the M*f* data. A $\log_{10}$ transformation was applied to the data to stabilize the variance across the doses. The dose-related mutagenic responses for each of the three stereoisomers of BDO_2 at both the *HPRT* and *TK* loci were not significantly different as shown by an ANCOVA parameter estimate (P values ranging from 0.65 to 0.99).

Multiplex-PCR Analysis of *HPRT* Mutant Genomic DNA

To characterize large-scale changes at the *HPRT* locus in mutant clones obtained from control cells and cells treated with each stereoisomer of BDO_2, *HPRT* mutant clones were transferred to 6-well plates for mutant-cell propagation. Mutant colonies were grown to 5 to 10 million cells and then were collected for DNA extraction and mutant analysis. A total of 274 *HPRT* mutants were analyzed, including 80 spontaneous mutants, 71 mutants from cells exposed to (2*R*,3*R*)-BDO_2, 63 mutants from cells exposed to (2*S*,3*S*)-BDO_2, and 60 mutants from cells exposed to *meso*-BDO_2.

Table 1 presents a summary of the results of the analysis of *HPRT* mutants by genomic PCR. The results showed two general categories of mutations: deletion mutations and other changes. Among deletion mutations, mutants missing exon 1 or exons 1 and 2 were classified as partial 5′ loss, and mutants missing exon 9 or exons 7, 8, and 9 were classified as partial 3′ loss. Mutants missing either exon 2 or exons 7 and 8 were grouped as internal deletions. Mutants that did not produce any PCR products for the *HPRT* gene were classified as complete gene deletions. Mutants with no missing exons were grouped as other genetic changes, which presumably included point mutations, small insertions or

Table 1. Results for Exon-Specific Genomics PCR Analysis of *HPRT* Mutants from TK6 Control Cells and Cells Exposed to BDO_2 Isomers[a]

Types of Mutations	Control	(*R*,*R*)-BDO_2[b,c]	(*S*,*S*)-BDO_2[b,c]	*meso*-BDO_2[b,c]	BDO_2 Isomers Combined[b]
Deletion mutations	23 (28.7)	45 (63.4)	31 (49.2)	28 (46.7)	104 (53.6)
Partial 5′ loss	10 (12.5)	19 (26.8)	15 (23.8)	9 (15.0)	43 (22.1)
Partial 3′ loss	8 (10.0)	11 (15.5)	8 (12.7)	6 (10.0)	25 (12.9)
Internal deletions	2 (2.5)	7 (9.9)	3 (4.8)	6 (10.0)	16 (8.2)
Complete deletions	3 (3.8)	8 (11.3)	5 (7.9)	7 (11.7)	20 (10.3)
Other changes	57 (71.3)	26 (36.6)	32 (50.8)	32 (53.3)	90 (46.4)
Total	80 (100)	71 (100)	63 (100)	60 (100)	194 (100)

[a] Values represent the number of mutations and (in parentheses) the percentage of total mutations.
[b] Significantly different compared to controls, $P < 0.05$, the Fisher exact test.
[c] No significant differences among the cells exposed to the three forms of BDO_2, $P > 0.05$, chi-square test.

deletions, and other small-scale genetic alterations that were not detected by genomic PCR analysis.

The frequencies of deletion mutations as a result of exposure to each stereoisomer of BDO_2 were significantly different from the frequency of spontaneous deletion mutations ($P < 0.05$, the Fisher exact test). There were no significant differences in mutation specificity among the cells exposed to the three stereoisomers of BDO_2 ($P > 0.05$, chi-square test).

SPECIFIC AIM 3: CYTOTOXICITY AND MUTAGENICITY OF (2*R*)-BDO AND (2*S*)-BDO IN TK6 CELLS

The cytotoxicity of the two BDO stereoisomers was evaluated by comparing CEs in BDO-exposed cells with those in unexposed cells (with the control value set at 100%). Cell survival declined with increasing exposure concentration (Figure 9). CEs in BDO-exposed cells were significantly lower than those in the control cells at 600, 800, and 1000 μM, but not at lower doses. The relative survival rates for the cells exposed to one or the other of the two BDO stereoisomers were similar in all the exposure concentrations evaluated.

The mutagenic potencies of (2*R*)-BDO and (2*S*)-BDO were evaluated by determining M*f*s at the *HPRT* and *TK* loci in TK6 cells exposed to 0, 200, 400, or 600 μM of each form of BDO for 24 hours. The average value of *HPRT* M*f*s in unexposed cells was $4.7 \pm 2.7 \times 10^{-6}$ (mean ± SD), which was slightly higher than that reported by Steen and colleagues (1997a,b) and Cochrane and Skopek (1994), but was consistent with the spontaneous *HPRT* M*f* values observed in this laboratory (Sussman et al. 1999; Meng et al. 2000b). (2*R*)-BDO caused increased *HPRT* M*f*s at exposure concentrations of 400 and 600 μM, with *P* values of 0.004 and ≤ 0.001, respectively (Figure 10). Two hundred μM of (2*R*)-BDO induced a 1.9-fold increase of *HPRT* M*f*s, but the increase was not significant ($P = 0.211$). (2*S*)-BDO caused increased *HPRT* M*f*s at all exposure concentrations, with *P* values ranging from 0.013 to < 0.001. Overall dose-related mutagenic responses caused by (2*R*)- or (2*S*)-BDO were similar, with no significant differences in M*f*s found at any exposure level.

TK M*f*s were scored for both fast- and slow-growth mutants at 21 through 24 days after plating. The average

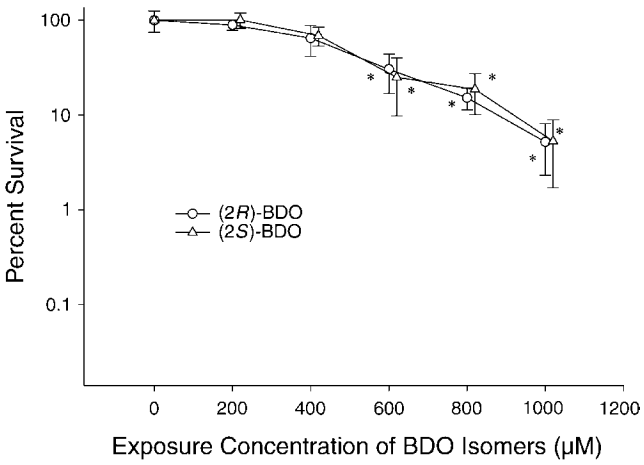

Figure 9. Effects of the exposure concentration of each isomer of BDO on the relative survival of TK6 cells. Cultures ($n = 3$/group) were exposed to 0, 200, 400, 600, 800, or 1000 μM of BDO for 24 hours. Relative cell survivals were ratios of CEs in treated versus control samples determined immediately after exposure. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

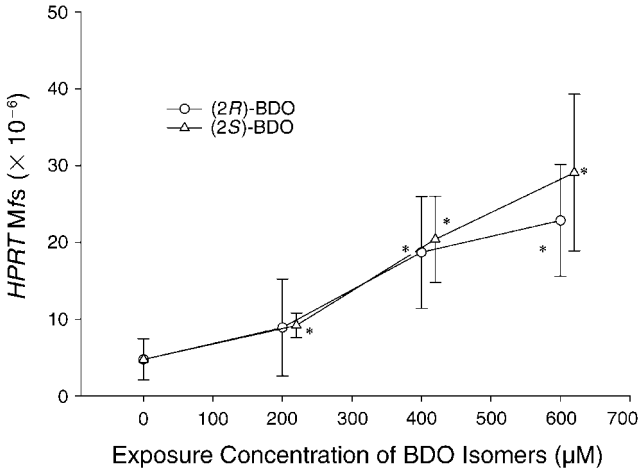

Figure 10. The relationship between the exposure concentration of each isomer of BDO and BDO-induced *HPRT* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 200, 400, or 600 μM of BDO for 24 hours. A cell cloning assay was used to measure the *HPRT* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

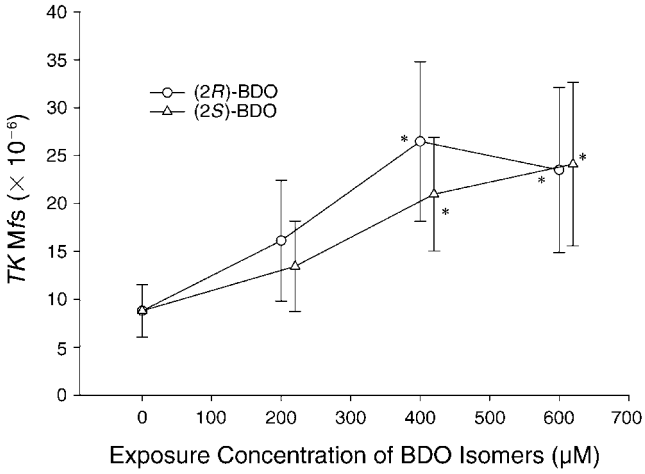

Figure 11. The relationship between the exposure concentration of each isomer of BDO and BDO-induced *TK* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 200, 400, or 600 μM of BDO for 24 hours. A cell cloning assay was used to measure the *TK* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

value of *TK* M*f*s for control cells was 8.8 ± 2.7 × 10^{-6} (mean ± SD), which was similar to the values observed in our previous studies (Meng et al. 2000a,b). Exposure to both (2*R*)-BDO and (2*S*)-BDO induced significantly increased *TK* M*f*s compared to the concurrent controls, with P values ranging from 0.002 to 0.007 at exposure concentrations of 400 and 600 μM (Figure 11). At 200 μM, the mutagenic response induced by (2*R*)- or (2*S*)-BDO was not significant (P values of 0.095 and 0.093, respectively). The dose–response curve for cells exposed to (2*R*)-BDO appeared to reach a plateau after 400 μM. However, no significant differences in *TK* M*f*s between cells exposed to (2*R*)-BDO or (2*S*)-BDO were found at any exposure level. The (2*R*)-BDO-induced *TK* M*f*s (with the background level subtracted) were 7.3, 17.7, and 14.7 × 10^{-6} for the exposure concentrations of 200, 400, and 600 μM, respectively. The (2*S*)-BDO-induced *TK* M*f*s (with the background level subtracted) were 4.7, 12.2, and 15.3 × 10^{-6} for the exposure concentrations of 200, 400, and 600 μM, respectively. These values were similar to those of the *HPRT* M*f*s (4.2, 14.0, and 18.1 × 10^{-6} for (2*R*)-BDO, and 4.5, 15.7, and 24.4 × 10^{-6} for (2*S*)-BDO, for the exposure concentrations of 200, 400, and 600 μM, respectively) measured from the same cultures as described above.

To compare the M*f*s of the two stereoisomers of BDO across the full dose range, an ANCOVA linear model was used after applying a $\log_{10}$ transformation to the data to stabilize the variance across the doses. The dose-related mutagenic responses at both *HPRT* and *TK* loci caused by the two stereoisomers of BDO were not significantly different, as shown by an ANCOVA parameter estimate (P values ranging from 0.34 to 0.45).

SPECIFIC AIM 4: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO-DIOL, (2*R*,3*S*)-BDO-DIOL, (2*S*,3*R*)-BDO-DIOL, AND (2*S*,3*S*)-BDO-DIOL IN TK6 CELLS

Cytotoxic and Mutagenic Responses at High Concentrations

Significant differences in cytotoxicity in TK6 cells were observed among the four stereoisomers of BDO-diol (Figure 12). (2*R*,3*S*)-BDO-diol was markedly more toxic than the other three stereoisomers of BDO-diol, with a relative cell survival of 11.4% at 200 μM, and no cells surviving at higher exposure concentrations. In contrast, (2*R*,3*R*)-BDO-diol was not cytotoxic even at the concentration of 1000 μM. In descending order, the overall cytotoxicity of the four BDO-diol isomers appeared to be (2*R*,3*S*)-BDO-diol, (2*S*,3*R*)-BDO-diol, (2*S*,3*S*)-BDO-diol, and (2*R*,3*R*)-BDO-diol.

Based on the cytotoxicity results for the four BDO-diol isomers, the exposure concentrations for determining *HPRT* and *TK* M*f*s were set at 40, 120, and 200 μM for (2*R*,3*S*)-BDO-diol, and at 200, 600, and 1000 μM for the other three BDO-diol isomers. The *HPRT* M*f* value of 11.3 × 10^{-6} in control cells in this study was slightly higher than the historical range of background *HPRT* M*f*s in this laboratory (3 to 9 × 10^{-6}) (Sussman et al. 1999; Meng et al.

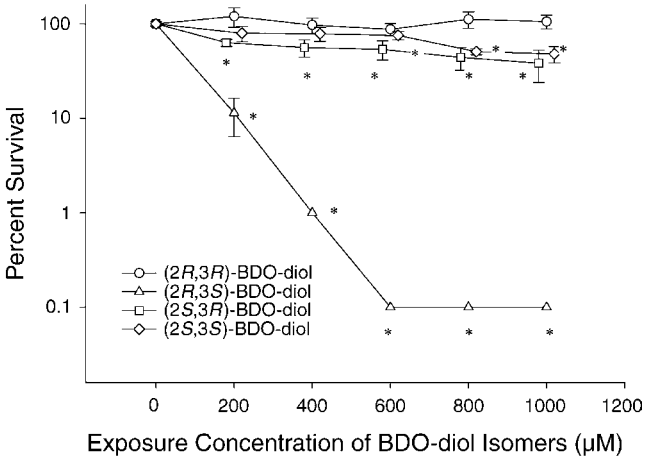

Figure 12. Effects of the exposure concentration of each isomer of BDO-diol on the relative survival of TK6 cells. Cultures (n = 3/group) were exposed to 0, 200, 400, 600, 800, or 1000 μM of BDO-diol for 24 hours. Relative cell survivals were ratios of CEs in treated versus control samples determined immediately after exposure. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

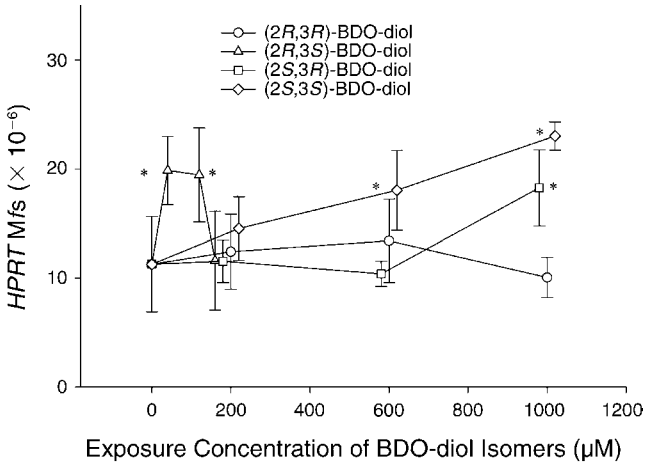

Figure 13. The relationship between the exposure concentration of each isomer of BDO-diol and BDO-diol-induced *HPRT* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 200, 600, or 1000 μM of BDO-diol for 24 hours except for (2*R*,3*S*)-BDO-diol, for which the exposure concentrations were 0, 40, 120, and 200 μM. A cell cloning assay was used to measure the *HPRT* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

2000a,b), but was within the range of background *HPRT* M*f*s reported by other laboratories (Zhu et al. 2000; Tomita-Mitchell et al. 2003). (2*R*,3*S*)-BDO-diol was significantly more mutagenic than the other three BDO-diol isomers, even at the lowest dose tested, causing an increase in *HPRT* M*f*s at 40 μM ($19.9 \pm 3.1 \times 10^{-6}$, $P = 0.007$) (Figure 13). However, at higher exposure concentrations (120 and 200 μM), (2*R*,3*S*)-BDO-diol did not induce any greater mutagenic effects than at 40 μM; this is most likely due to the elevated cytotoxicity of BDO-diol at the higher concentrations. At all exposure concentrations evaluated, (2*R*,3*R*)-BDO-diol did not cause any mutagenic response. (2*S*,3*R*)-BDO-diol induced an increase in *HPRT* M*f*s at 1000 μM ($18.3 \pm 3.5 \times 10^{-6}$, $P = 0.023$), but not at lower concentrations. (2*S*,3*S*)-BDO-diol caused increased *HPRT* M*f*s at both 600 μM ($18.1 \pm 3.7 \times 10^{-6}$, $P = 0.029$) and 1000 μM ($23.0 \pm 1.3 \times 10^{-6}$, $P \leq 0.01$), but not at 200 μM. The order of the overall mutagenic efficiencies of BDO-diol isomers at the *HPRT* locus was as follows: (2*R*,3*S*)-BDO-diol was more mutagenic than (2*S*,3*S*)-BDO-diol, the mutagenicity of which was greater than or equal to that of (2*S*,3*R*)-BDO-diol, which was more mutagenic than (2*R*,3*R*)-BDO-diol.

The mutagenic effects of BDO-diol isomers at the *TK* locus were similar to those at the *HPRT* locus (Figure 14). (2*R*,3*S*)-BDO-diol induced an increase of *TK* M*f*s over the background level ($14.5 \pm 3.1 \times 10^{-6}$) at 40 μM ($30.1 \pm 4.2 \times 10^{-6}$, $P < 0.001$) and at 120 μM ($24.7 \pm 2.6 \times 10^{-6}$, $P < 0.01$), but not at 200 μM ($10.8 \pm 3.8 \times 10^{-6}$, $P = 0.38$) due to high cytotoxicity. At all three exposure concentrations, (2*R*,3*R*)-BDO-diol did not induce any mutagenic effects, while (2*S*,3*R*)-BDO-diol and (2*S*,3*S*)-BDO-diol induced increased *TK* M*f*s only at 1000 μM ($22.6 \pm 1.9 \times 10^{-6}$, $P = 0.023$, and $24.6 \pm 3.2 \times 10^{-6}$, $P = 0.023$, respectively). The order of the overall mutagenic efficiencies of the BDO-diol isomers at the *TK* locus was as follows: (2*R*,3*S*)-BDO-diol

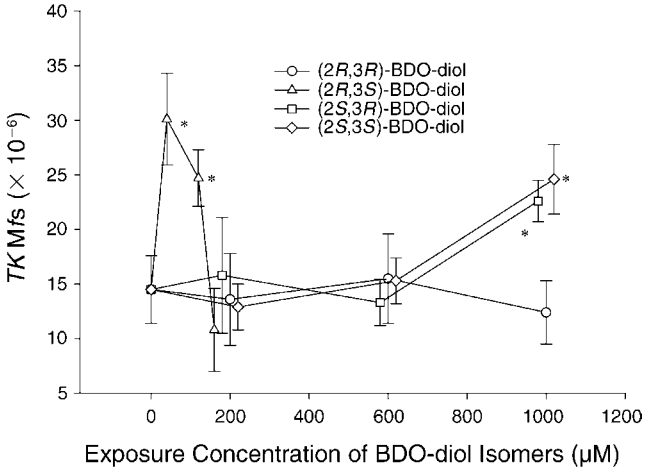

Figure 14. The relationship between exposure concentration of each isomer of BDO-diol and BDO-diol-induced *TK* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 200, 600, or 1000 μM of BDO-diol for 24 hours except for (2*R*,3*S*)-BDO-diol, for which the exposure concentrations were 0, 40, 120, and 200 μM. A cell cloning assay was used to measure the *TK* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

was more mutagenic than (2*S*,3*R*)-BDO-diol, which was equal in mutagenicity to (2*S*,3*S*)-BDO-diol, which was more mutagenic than (2*R*,3*R*)-BDO-diol.

Cytotoxic and Mutagenic Responses at Low Concentrations

The cytotoxicity at concentrations of 5 to 40 μM of each stereoisomer of BDO-diol is plotted in Figure 15. (2*R*,3*S*)-BDO-diol caused statistically significant toxicity at 20, 30, and 40 μM. The other three stereoisomers of BDO-diol did not cause significant cytotoxicity.

The exposure concentrations for determining *HPRT* and *TK* M*f*s at the low-dose range were set at 5, 10, and 20 μM. The *HPRT* M*f* value in control cells was 8.2 ± 3.1 × 10^{-6}. (2*R*,3*S*)-BDO-diol induced significantly higher *HPRT* M*f*s at 20 μM (15.8 ± 1.1 × 10^{-6}, $P < 0.001$), but not at 5 and 10 μM (Figure 16). The *TK* M*f* value in control cells was 12.6 ± 3.3 × 10^{-6}. (2*R*,3*S*)-BDO-diol induced a significant increase in *TK* M*f*s at 20 μM (23.6 ± 2.1 × 10^{-6}, $P < 0.001$), but not at 5 and 10 μM (Figure 17). The other three stereoisomers of BDO-diol did not cause statistically significant increases of *HPRT* or *TK* M*f*s at 5, 10, or 20 μM.

Model Fitting and Regression Analysis of BDO-diol M*f* Data

Comparing trends of *HPRT* and *TK* M*f*s for each BDO-diol stereoisomer across the full range of dose levels proved difficult. The M*f* data did not all follow the same types of patterns within the dose ranges, and therefore no single statistical model type fitted all four treatments well. Model fit is a key assumption in the development of statistical models, and any parameters derived from a model that fits the data poorly are questionable. Since we were more

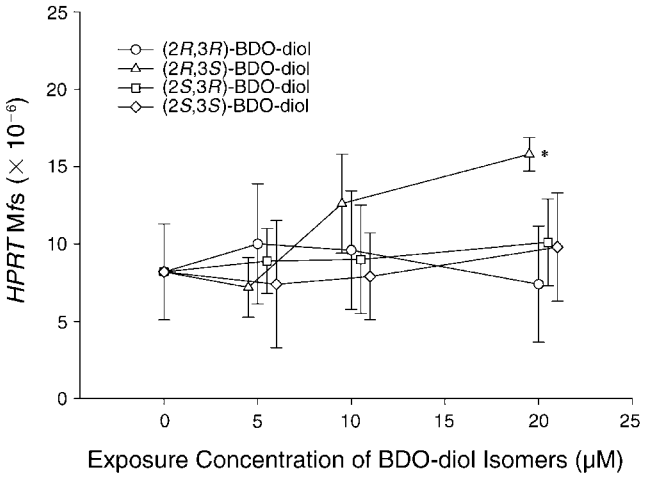

Figure 16. The relationship between the exposure concentration of each isomer of BDO-diol and BDO-diol-induced *HPRT* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 5, 10, or 20 μM of BDO-diol for 24 hours. A cell cloning assay was used to measure the *HPRT* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

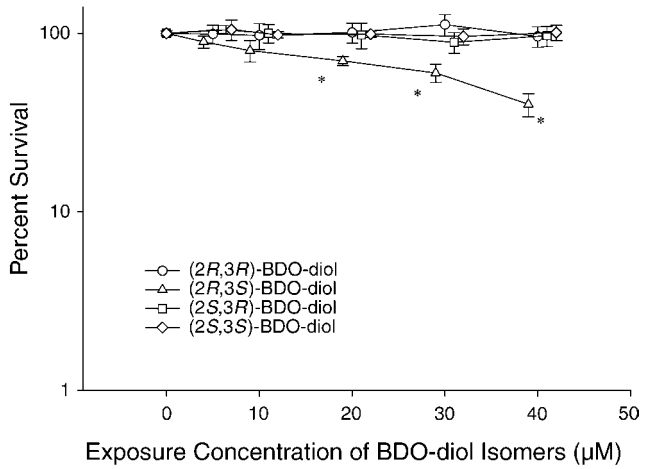

Figure 15. Effects of the exposure concentration of each isomer of BDO-diol on the relative survival of TK6 cells. Cultures (n = 3/group) were exposed to 0, 5, 10, 20, 30, or 40 μM of BDO-diol for 24 hours. Relative cell survivals were ratios of cloning efficiencies in treated versus control samples determined immediately after exposure. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

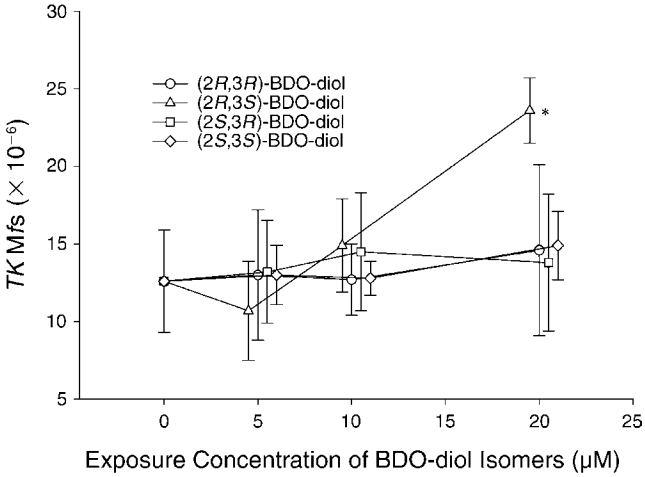

Figure 17. The relationship between the exposure concentration of each isomer of BDO-diol and BDO-diol-induced *TK* M*f*s in TK6 cells. Cultures (n = 5/group) were exposed to 0, 5, 10, or 20 μM of BDO-diol for 24 hours. A cell cloning assay was used to measure the *TK* M*f*s. Error bars show SDs around means. An asterisk (*) indicates a significant change compared to control values. Values for the x-axis are staggered to separate individual symbols.

Table 2. Comparisons of Slopes for *HPRT* and *TK* M*f*s at Doses of 5, 10, and 20 μM of BDO-diol Isomers[a]

Treatment Slopes	Unadjusted *HPRT P* Value	*HPRT* False Discovery Rate[b]	Unadjusted *TK P* Value	*TK* False Discovery Rate[b]
1 vs. 2	0.0002	0.0012	0.0002	0.0006
1 vs. 3	0.1617	0.1940	0.6265	0.7518
1 vs. 4	0.0666	0.0999	0.9780	0.9780
2 vs. 3	0.0150	0.0450	< 0.0001	< 0.0001
2 vs. 4	0.0442	0.0884	0.0003	0.0006
3 vs. 4	0.6519	0.6519	0.6072	0.7518

[a] 1 = slope of (2*R*,3*R*)-BDO-diol, 2 = slope of (2*R*,3*S*)-BDO-diol, 3 = slope of (2*S*,3*R*)-BDO-diol, 4 = slope of (2*S*,3*S*)-BDO-diol.
[b] The expected proportion of tests that are null.

interested in the differences among M*f*s induced by BDO-diol isomers at the low-dose range, we decided to restrict our regression analysis to the low-dose range (5, 10, and 20 μM). At these low-dose levels, there was less variability in distributions, and a linear model fit all treatments well. This provided some insight into how the different treatments were performing at low-dose levels. A *t* test was used to compare each slope parameter of the expression of the *TK* M*f*s versus the doses used, to determine whether the slope parameters were different. (2*R*,3*S*)-BDO-diol appeared to have a significantly different slope from those of treatments (2*R*,3*R*)-BDO-diol, (2*S*,3*R*)-BDO-diol, and (2*S*,3*S*)-BDO-diol for the *TK* M*f*s. This relationship also held for *HPRT* M*f*s, but the differences were borderline significant for a couple of the comparisons, as shown by the *P* values in Table 2.

DISCUSSION

SPECIFIC AIM 1: SYNTHESIS OF EIGHT STEREOISOMERS OF BDO_2, BDO, AND BDO-DIOL

Cochrane and Skopek (1994) evaluated the cytotoxicity and mutagenicity of the three reactive metabolites of BD — BDO, BDO_2, and BDO-diol — in TK6 cells without regard to the potential effects of stereochemistry, and the results were used extensively to explain in vivo mutagenic and carcinogenic effects of BD. Accumulating evidence indicates that stereochemical configurations of BD metabolites may play a role in the mutagenic and carcinogenic action of BD. However, the cytotoxicity and mutagenicity of individual stereoisomers of BD metabolites had not been systematically assessed, mainly due to the lack of availability of these stereoisomers from commercial sources. Therefore, for the current project, it was critical to develop methods for synthesizing each stereoisomer of the reactive metabolites of BD in order to study their toxicity and mutagenicity.

Two methods were used to synthesize (2*R*,3*R*)-BDO_2 and (2*S*,3*S*)-BDO_2. Method B (Figure 3) was relatively fast and simpler compared to method A (Figure 2), but the starting materials (1,4-di-*O*-toluensulfonyl-2,3-*O*-isopropylidene-threitols) (**8a** and **8b**) were more costly. The overall yields for methods A and B were comparable; thus, neither method was superior to the other. In synthesizing the four isomers of BDO-diol, primary mesylates and tosylates were best for production of epoxides by intramolecular displacement, while the Sharpless asymmetric epoxidation proved to be quite useful for the production of stereoisomeric epoxide diols using the more stable **12a** and **12b** (Figure 5). This coupled with the Mitsunobu reaction for conversion of chirality at position 2 allowed access to all four isomers of BDO-diol.

Claffey (2002) developed a method to produce multigram-scale *meso*-BDO_2 for an in vivo inhalation study sponsored by HEI. A portion of the *meso*-BDO_2 was used in the current in vitro study. The remaining eight stereoisomers of BDO, BDO_2, and BDO-diol were synthesized for the current project. Two hundred to 500 mg quantities of eight stereoisomers were synthesized, with the overall yields from all steps ranging from 10% to 21.3%. These overall yields were about 50% of the overall yield for *meso*-BDO_2 synthesized by Claffey (2002); nevertheless, despite the need for multiple steps, the methods used in the current study are efficient for stereo-selective synthesis of BDO, BDO_2, and BDO-diol in quantities suitable for research purposes.

Purity Analysis of Synthesized Chemicals

The compounds synthesized for this study were determined to be at least 98% to 99.9% pure. The purity of the chemicals was estimated based upon a variety of analytical results, because no absolute method exists for determining the actual purity of these compounds. For example, gas chromatography–mass spectrometry can be good for

judging purity; however, it does not take into account any traces of un-ionizable impurities in the sample. A simple melting point for solid samples can rule out the possibility of most impurities if the melting range observed is very narrow, as impurities will both lower the melting point and broaden its melting range. High-performance liquid chromatography can be used with an ionization detector, but water and un-ionizable species would go largely undetected. Water can easily be seen in NMR analysis; however, the percent level is difficult to measure below baseline noise levels (1% to 2%). For the compounds synthesized for this study, using melting points (for solid samples), NMR spectra, mass spectrometry, and optical rotation measurements together safely ruled out the existence of most impurities, especially those that could affect the weight of the samples or skew the results of any biological studies. In the case of optical rotations, since many of these compounds do not carry a significant chromophore, the rotations measured can sometimes be very small, thus leading to larger experimental errors in the readings. However, the optical rotation measurements for mirror image species were of approximately equal and opposite value. Based upon the synthesis and purification methods used in this study, it is not likely that impurities from opposite isomers were present in these samples.

SPECIFIC AIM 2: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, AND *meso*-BDO_2

There were no significant differences in the relative survival of TK6 cells exposed to the three isomers of BDO_2 in the current study. Nieusma and colleagues (1997) observed different cytotoxicities for different BDO_2 isomers in rat hepatocytes exposed to 1000 µM of BDO_2 isomers: From greatest to least, the order of cytotoxicity was *meso*-BDO_2, (2*S*,3*S*)-BDO_2, and (2*R*,3*R*)-BDO_2. The discrepancy is probably related to the marked differences in metabolic capability between TK6 cells and rat hepatocytes (Shirname-More, 1991; Zhu et al., 2000). Rat hepatocytes can convert *meso*-BDO_2 to (2*R*,3*S*)-BDO-diol (the most cytotoxic form of BDO-diol); (2*S*,3*S*)-BDO_2 to (2*S*,3*S*)-BDO-diol; and (2*R*,3*R*)-BDO_2 to (2*R*,3*R*)-BDO-diol (the least cytotoxic BDO-diol) (Figure 1). The order of cytotoxicity of BDO_2 stereoisomers in rat hepatocytes was consistent with the order of cytotoxicity of BDO-diol isomers converted from the respective BDO_2 isomers in TK6 cells.

Each stereoisomer of BDO_2 caused increased *HPRT* and *TK* M*f*s in TK6 cells at all exposure concentrations, compared to the concurrent controls, and the values of BDO_2-induced M*f*s in TK6 cells exposed to racemic mixtures of BDO_2 were comparable to those reported by other investigators (Table 3). These in vitro studies in human cells suggest that there were no significant differences in the mutagenic potency of the three stereoisomers of BDO_2. These findings were consistent with results of another HEI-supported project, where inhalation exposures to equimolar concentrations of *meso*-BDO_2 or (±)-BDO_2 led to similar mutagenic responses at the *Hprt* gene of exposed mice (Walker et al. 2009).

BDO_2-induced *TK* M*f*s were consistently greater than the *HPRT* M*f*s measured from the same cultures at each exposure level. These findings are probably related to the fact that some cells with large deletions at the *HPRT* locus did not survive because essential adjacent genes were missing, and some genetic changes, such as gene conversion, require two copies of the gene and thus could not occur at the *HPRT* locus.

Large genetic changes were evaluated during the molecular analysis of *HPRT* mutants in the current study. BDO_2, a bialkylating agent, is known to form DNA–DNA cross-links and potentially induce gross genetic changes (Park et al. 2004). In an earlier study, Cochrane and Skopek (1994) showed that 54% of *HPRT* mutants from TK6 cells exposed to a racemic mixture of BDO_2 displayed changes in banding patterns when analyzed using the Southern blot method. Steen and colleagues (1997a) reported increased

Table 3. Comparison of Studies of *HPRT* and *TK* M*f*s ($\times 10^{-6}$) Induced in TK6 Cells Exposed for 24 Hours to Isomers of BDO_2

	Cochrane and Skopek 1994 (±)-BDO_2	Steen et al. 1997b (±)-BDO_2	Current Study (*R*,*R*)-BDO_2	Current Study (*S*,*S*)-BDO_2	Current Study *meso*-BDO_2
BDO_2 concentration	3.9 µM	4 µM	4 µM	4 µM	4 µM
Induced *HPRT* M*f*s	~ 9.0	13.8	19.6	16.5	13.8
Induced *TK* M*f*s[a]	~ 20.0	NA[b]	36.8	32.0	29.1

[a] Total includes normal- and slow-growth mutants.
[b] Not available.

frequencies of partial 5′ loss in TK6 cells exposed to a racemic mixture of BDO_2. In the current study, we found increased frequency of deletion mutations as a result of exposure to each stereoisomer of BDO_2, which is consistent with results of earlier studies. These cells show that a large ge-netic change is an important mechanism of the mutagenic action of each isomer of BDO_2. On the other hand, similar distributions of mutation types among cells exposed to the three BDO_2 stereoisomers indicate that stereochemical configurations of BDO_2 did not have a significant impact on the mechanisms for the mutagenic action of BDO_2.

In separate HEI-sponsored studies, the mutagenic potencies of *meso*-BDO_2 and (±)-BDO_2 were evaluated and compared in rodents exposed by inhalation to these compounds (Walker and Meng 2000; Walker et al. 2009). Female $B6C3F_1$ mice and F344 rats were exposed to 2 ppm (±)-BDO_2 for 4 weeks or 2 ppm *meso*-BDO_2 for 3 weeks (6 hours/day, 5 days/week) (exposure was cut short because of insufficient *meso*-BDO_2 for completing the scheduled 4-week exposure). While perfect comparisons of the mutagenic potencies of the stereochemical forms of BDO_2 could not be made because of the differences in exposure durations, the *Hprt* M*f* data were sufficient to support the conclusion that there was little difference in the in vivo mutagenic efficiencies of (±)-BDO_2 versus *meso*-BDO_2 in rodents. Thus, in terms of mutagenic potency, the stereochemistry of these epoxy compounds is not likely to play a significant role in the mutagenicity and carcinogenicity of BD.

SPECIFIC AIM 3: CYTOTOXICITY AND MUTAGENICITY OF (2*R*)-BDO AND (2*S*)-BDO IN TK6 CELLS

The similar relative survival rates of TK6 cells exposed to the two isomers of BDO seem inconsistent with the greater cytotoxicity of (2*R*)-BDO versus (2*S*)-BDO in rat hepatocytes (Nieusma et al. 1997). However, unlike TK6 cells, rat hepatocytes can metabolize BDO to BDO_2, and more BDO_2 was produced from (2*R*)-BDO than from (2*S*)-BDO in rat hepatocytes, which at least partially explains the greater cytotoxicity of (2*R*)-BDO in rats (Nieusma et al. 1997).

The values of (2*R*)-BDO- or (2*S*)-BDO-induced *HPRT* and *TK* M*f*s in TK6 cells exposed to racemic mixtures of BDO were comparable to those reported by other investigators (Cochrane and Skopek 1994; Steen et al. 1997b) (Table 4). Similar *HPRT* and *TK* M*f*s in cells exposed to (2*R*)-BDO or (2*S*)-BDO at each exposure concentration were observed, suggesting that that there were no significant differences in mutagenic potency between the two stereoisomers of BDO.

Given the similar mutagenic potencies of (2*R*)- and (2*S*)-BDO, any effects of the stereochemical configurations of BDO on BD mutagenesis and carcinogenesis are most likely to be caused by the stereochemistry-dependent metabolism of BDO to other DNA-reactive intermediates. (2*R*,3*S*)-BDO-diol, the stereoisomer of BDO-diol most likely to induce *HPRT* and *TK* mutations, is mainly derived from (2*S*)-BDO (Figure 1). In BDO-treated mice and rats, (2*R*)-BDO was predominantly detoxified by glutathione conjugation to form a urinary metabolite, 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene, whereas (2*S*)-BDO was preferentially hydrolyzed to BD-diol, a precursor of reactive BDO-diol metabolites (Richardson et al. 1998). Nieusma and colleagues (1997) showed that, after 30 minutes of incubation with BD, rat liver microsomes produced more (2*R*)-BDO, and mouse liver microsomes produced more (2*S*)-BDO. Furthermore, BDO_2 formation from (2*S*)-BDO by mouse microsomes was significantly greater than BDO_2 formation from (2*R*)-BDO, while in rat microsomes BDO_2 was formed mainly from (2*R*)-BDO. The combined effect of more (2*S*)-BDO generated from BD in mice and the greater production of the highly

Table 4. Comparison of Studies of *HPRT* and *TK* M*f*s ($\times 10^{-6}$) Induced in TK6 Cells Exposed for 24 Hours to Isomers of BDO

	Cochrane and Skopek 1994 (±)-BDO	Steen et al. 1997b (±)-BDO	Current Study (*R*)-BDO	Current Study (*S*)-BDO
BDO concentration	372 µM	400 µM	400 µM	400 µM
Induced *HPRT* M*f*s	~ 12.0	19.7/31.7[a]	14.0	15.7
Induced *TK* M*f*s[b]	~ 17.0	NA[c]	17.7	12.2

[a] Results of two subcultures treated independently.
[b] Total includes normal- and slow-growth mutants.
[c] Not available.

mutagenic BDO_2 from (2*S*)-BDO may contribute to the greater mutagenicity and carcinogenicity of BD in mice.

SPECIFIC AIM 4: CYTOTOXICITY AND MUTAGENICITY OF (2*R*,3*R*)-BDO-DIOL, (2*R*,3*S*)-BDO-DIOL, (2*S*,3*R*)-BDO-DIOL, AND (2*S*,3*S*)-BDO-DIOL IN TK6 CELLS

The role of BDO-diol in BD mutagenicity and carcinogenicity has been significantly underestimated, mainly due to the study of Cochrane and Skopek (1994) that showed that BDO-diol was 200-fold less mutagenic than BDO_2 but that did not take into account the role of stereochemistry. The results of the current study demonstrate that individual stereoisomers of BDO-diol have markedly different mutagenic potencies, and the most potent form of BDO-diol (2*R*,3*S*) has a mutagenic potency much closer to BDO_2 than was previously demonstrated in experiments in which stereochemistry was not considered. The current study demonstrates that the mutagenic potency of (2*R*,3*S*)-BDO-diol is only 5-to-10-fold less than the average equimolar potency of BDO_2 stereoisomers in both *HPRT* and *TK* genes, and is 10- to 20-fold greater than the average equimolar effect of BDO stereoisomers on the *HPRT* and *TK* genes (Table 5).

Emerging evidence demonstrates that BDO-diol may play a significant role in the mutagenic and carcinogenic action of BD. DNA adduct studies showed that the major adduct formed in rodents after exposure to BD was THB-Gua (Tretyakova et al. 1998; Koc et al. 1999; Koivisto et al. 1999; Oe et al. 1999; Boogaard et al. 2001, 2004; Booth et al. 2004). Theoretically, THB-Gua can be formed from either BDO_2 or BDO-diol. Using available DNA adduct and metabolism data for mice and rats, Swenberg and associates were able to calculate that 95% to 98% of the THB-Gua was from BDO-diol (Koc et al. 1999; Swenberg et al. 2001). This finding was confirmed by other investigators using different analysis methods (Koivisto et al. 1999; Boogaard et al. 2001; Koivisto and Peltonen 2001; Boogaard et al. 2004; Booth et al. 2004). Pérez and colleagues (1997) showed that BDO-diol was also the major source of the hemoglobin adduct *N*-(2,3,4-trihydroxybutyl)valine (THB-Val) after rats were exposed to BD. Furthermore, the THB-Val level was substantially greater than that of the BDO-formed hemoglobin adduct (an isomeric mixture of *N*-(1-[hydroxymethyl]-2-propenyl)valine and *N*-(2-hydroxy-3-butenyl)valine [HB-Val]) in both rats and human samples (Pérez et al. 1997). Using an immunoaffinity liquid chromatography–tandem mass spectrometry method, Boysen and colleagues (2004) were able to accurately measure HB-Val, THB-Val, and the BDO_2-specific *N*,*N*-(2,3-dihydroxy-1,4-butadiyl)valine (Pyr-Val) in mice and rats exposed to BD. The ratios of THB-Val to Pyr-Val in mice exposed to 3 or 62.5 ppm BD were 9.3 and 29, respectively, and the corresponding ratios in rats were 87 and 83.

The high ratios of THB-Val to HB-Val and of THB-Val to Pyr-Val in both mice and rats indicate that BDO is primarily metabolized via the BD-diol pathway in both species (Boysen et al. 2004). This was confirmed by the high plasma level of BD-diol in mice and rats exposed to BD by inhalation (Walker et al. 2009). BDO-diol levels in vivo after BD exposure have not been quantified. However, it is reasonable to predict high levels of circulating BDO-diol based on the substantial levels of the adducts THB-Gua and THB-Val, which are derived from BDO-diol, and because BDO-diol is not considered more reactive than BDO_2 toward DNA and protein. The predicted high level of BDO-diol after BD exposure is probably related to the fact that BDO-diol is a poorer substrate for glutathione conjugation or epoxide hydrolysis than is BDO or BDO_2 (Dietze et al. 1993; Nieusma et al. 1998).

The great mutagenic potency of (2*R*,3*S*)-BDO-diol is consistent with the finding that 75% of THB-Gua originated

Table 5. Comparison of Studies of *HPRT* and *TK* M*f*s Induced in TK6 Cells by Isomers of BDO_2, BDO-diol, and BDO[a]

	Cochrane and Skopek 1994 (±)-BDO_2	Steen et al. 1997b (±)-BDO_2	Current Study		
			BDO_2 Isomers[b]	(2*R*,3*S*)-BDO-diol	BDO Isomers[c]
Concentration	3.9 µM	4 µM	4 µM	20 µM	400 µM
Induced *HPRT* M*f*s	9	14	16	8	15
Induced *TK* M*f*s	20	NA[d]	32	11	15

[a] M*f*s are reported as $\times 10^{-6}$; background M*f*s have been subtracted.
[b] Average of (*R*,*R*)-BDO_2, (*S*,*S*)-BDO_2, and *meso*-BDO_2.
[c] Average of (*R*)-BDO and (*S*)-BDO.
[d] Not available.

from (2*R*,3*S*)-BDO-diol in lungs of mice exposed to 500 ppm BD (5 days, 6 hours/day) (Koivisto and Peltonen 2001). (2*R*,3*S*)-BDO-diol is mainly formed from (2*S*)-BDO through (2*S*)-BD-diol (Figure 1). After exposure to 500 ppm of BD, 77% of BDO-Gua adduct in mouse lung tissue was from (2*S*)-BDO, and only 23% was from (2*R*)-BDO, which corresponds to the great amount of (2*R*,3*S*)-BDO-diol adduct formed in mouse lung. In testis, (2*S*)-BDO and (2*R*)-BDO guanine adducts represented 71% and 29% of BDO-Gua adducts, respectively (Koivisto et al. 1998). In contrast, (2*R*)-BDO guanine adduct represented 69% of BDO-Gua adducts in liver of rats exposed to 200 ppm of BD for 5 days (6 hours/day) (Koivisto et al. 1997). Although not in the same tissues, the observed over-representation of (2*S*)-BDO guanine adduct in mice and (2*R*)-BDO guanine adduct in rats probably indicated an excess formation of (2*R*)-BDO in rats and (2*S*)-BDO in mice after BD exposure, implying that more (2*R*,3*S*)-BDO-diol was formed in mice than in rats. These data show that the stereospecific formation of BDO-diol DNA adducts and the different mutagenic potencies of BDO-diol stereoisomers may have a significant impact on differences in mutagenicity and carcinogenicity of BD in mice versus rats.

CONCLUSIONS

The primary objective of this study was to evaluate systematically the mutagenic efficiencies of stereoisomers of major reactive metabolites of BD in human cells. For this purpose, nine stereochemical configurations of BDO, BDO_2, and BDO-diol were synthesized. No significant differences in cytotoxicity or mutagenic efficiencies were found among the three stereoisomers of BDO_2 or the two stereoisomers of BDO. The *HPRT* and *TK* M*f*s induced by stereoisomers of BDO_2 or BDO were comparable to the BDO_2- or BDO-induced *HPRT* and *TK* M*fs* measured in an earlier study of TK6 cells in which stereochemistry was not taken into account (Cochrane and Skopek 1994). Molecular analysis of BDO_2-induced *HPRT* mutants indicated that large-scale genetic alteration was an important mechanism in the mutagenic action of each form of BDO_2, and similar distributions of deletion mutations as a result of exposure to the three BDO_2 stereoisomers indicate that stereochemical configurations of BDO_2 did not have a significant impact on the mechanism of mutagenic action of BDO_2. In separate HEI-sponsored studies, the mutagenic potencies of *meso*-BDO_2 and a racemic mixture of BDO_2 were evaluated and compared in mice and rats exposed by inhalation. The results showed there was little difference in the in vivo mutagenic efficiencies of (±)-BDO_2 versus *meso*-BDO_2 in rodents (Walker and Meng 2000; Walker et al. 2009). Thus, in terms of mutagenic potency, there was no evidence that the stereochemical configurations of BDO and BDO_2 played a significant role in the mutagenicity and carcinogenicity of BD.

The most significant positive results of this study were the marked differences in cytotoxicity and mutagenicity among the four stereoisomers of BDO-diol. (2*R*,3*S*)-BDO-diol was at least 30-fold more cytotoxic and mutagenic than the other three forms of BDO-diol, which was consistent with the finding by Koivisto and Peltonen (2001) that 75% of THB-Gua originated from (2*R*,3*S*)-BDO-diol in lungs of mice exposed to BD. Unlike the 130-fold or 350-fold differences (for *HPRT* and *TK* genes, respectively) in mutagenic potencies between BDO-diol and BDO_2 when stereochemistry was not considered (Cochrane and Skopek 1994), the mutagenic potency of (2*R*,3*S*)-BDO-diol in this study was only 5-to-10-fold less than that of (±)-BDO_2, or the average mutagenic potency of the three individual BDO_2 stereoisomers. In addition, the mutagenic potency of (2*R*,3*S*)-BDO-diol was 10-to-20-fold greater than that of (±)-BDO (Cochrane and Skopek 1994; Steen et al. 1997b).

Mild species differences in DNA adduct levels and mutagenic efficiencies of BDO and BDO_2 are not sufficient to explain the significant species differences in carcinogenicity of BD in mice versus rats. DNA and hemoglobin adduct data demonstrate that BDO-diol is the dominant BD metabolite available to react with macromolecules in vivo after BD exposure (Pérez et al. 1997; Koivisto et al. 1999; Boogaard et al. 2001; Koivisto and Peltonen 2001; Swenberg et al. 2001, 2004; Boogaard et al. 2004; Booth et al. 2004). The species differences in BD-mediated carcinogenesis in rodents may be accounted for in significant portion by the stereochemistry-dependent distributions of BDO-diol metabolites, the BDO-diol-induced DNA adducts, and the mutagenic efficiencies of BDO-diol in mice and rats.

More studies are needed to better understand the significant role of BDO-diol stereochemistry in the mutagenicity and carcinogenicity of BD. First, the mutagenic potency of (2*R*,3*S*)-BDO-diol should be further confirmed in other in vitro systems, using doses ranging from those causing little cytotoxicity to those causing obvious cytotoxicity. Second, in vivo metabolite levels of each stereoisomer of BDO-diol, along with stereochemistry-dependent DNA and hemoglobin adducts of BDO-diol, should be characterized in rodents after inhalation exposure to BD. Finally, the mutagenic efficiencies of BDO-diol stereoisomers should be evaluated and confirmed in rodents. These studies might provide stereospecific biomarkers that can be used to monitor BD exposure and its effects in humans and to improve the assessment of cancer risk related to BD exposure.

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APPENDIX A. Cytotoxicity and Cell Count Results and *HPRT* and *TK* M*f* Data

Table A.1. Summary of Cytotoxicity Assay Results

Stereoisomer and Concentration	Average (%)	Standard Deviation
BDO		
Control, 0 µM	**100.0**	**25.2**
(2*R*)-BDO, 200 µM	89.3	10.7
(2*R*)-BDO, 400 µM	64.8	23.1
(2*R*)-BDO, 600 µM	30.4	13.5
(2*R*)-BDO, 800 µM	15.2	3.9
(2*R*)-BDO, 1000 µM	5.2	2.9
(2*S*)-BDO, 200 µM	101.1	18.7
(2*S*)-BDO, 400 µM	69.1	15.6
(2*S*)-BDO, 600 µM	25.1	15.3
(2*S*)-BDO, 800 µM	18.7	8.6
(2*S*)-BDO, 1000 µM	5.3	3.6
BDO_2		
Control, 0 µM	**100.0**	**11.8**
(2*R*,3*R*)-BDO_2, 2 µM	70.0	9.7
(2*R*,3*R*)-BDO_2, 4 µM	41.8	6.5
(2*R*,3*R*)-BDO_2, 6 µM	37.6	18.7
(2*R*,3*R*)-BDO_2, 8 µM	17.2	6.0
(2*R*,3*R*)-BDO_2, 10 µM	1.2	0.7
(2*S*,3*S*)-BDO_2, 2 µM	44.7	11.6
(2*S*,3*S*)-BDO_2, 4 µM	29.7	8.4
(2*S*,3*S*)-BDO_2, 6 µM	19.4	3.7
(2*S*,3*S*)-BDO_2, 8 µM	8.2	1.9
(2*S*,3*S*)-BDO_2, 10 µM	1.2	0.7
meso-BDO_2, 2 µM	104.0	20.4
meso-BDO_2, 4 µM	45.7	23.0
meso-BDO_2, 6 µM	20.4	5.8
meso-BDO_2, 8 µM	10.1	0.4
meso-BDO_2, 10 µM	0.2	0.2

(*Table continues next page*)

Table A.1 (*Continued*). Summary of Cytotoxicity Assay Results

Stereoisomer and Concentration	Average (%)	Standard Deviation
BDO-diol		
Control, 0 µM	**100.0**	**5.1**
(2*R*,3*R*)-BDO-diol, 200 µM	120.3	28.1
(2*R*,3*R*)-BDO-diol, 400 µM	97.3	18.2
(2*R*,3*R*)-BDO-diol, 600 µM	87.7	13.0
(2*R*,3*R*)-BDO-diol, 800 µM	111.8	22.0
(2*R*,3*R*)-BDO-diol, 1000 µM	105.7	17.4
(2*R*,3*S*)-BDO-diol, 200 µM	11.4	5.0
(2*R*,3*S*)-BDO-diol, 400 µM	1.0	0.0
(2*R*,3*S*)-BDO-diol, 600 µM	0.0	0.0
(2*R*,3*S*)-BDO-diol, 800 µM	0.0	0.0
(2*R*,3*S*)-BDO-diol, 1000 µM	0.0	0.0
(2*S*,3*R*)-BDO-diol, 200 µM	63.2	5.5
(2*S*,3*R*)-BDO-diol, 400 µM	56.2	11.8
(2*S*,3*R*)-BDO-diol, 600 µM	53.8	12.4
(2*S*,3*R*)-BDO-diol, 800 µM	44.3	11.9
(2*S*,3*R*)-BDO-diol, 1000 µM	38.4	14.5
(2*S*,3*S*)-BDO-diol, 200 µM	79.5	14.9
(2*S*,3*S*)-BDO-diol, 400 µM	78.6	13.2
(2*S*,3*S*)-BDO-diol, 600 µM	75.4	7.6
(2*S*,3*S*)-BDO-diol, 800 µM	50.9	3.7
(2*S*,3*S*)-BDO-diol, 1000 µM	48.1	9.6
BDO-diol		
Control, 0 µM	**100.0**	**3.2**
(2*R*,3*R*)-BDO-diol, 5 µM	99.0	12.1
(2*R*,3*R*)-BDO-diol, 10 µM	97.3	16.2
(2*R*,3*R*)-BDO-diol, 20 µM	101.0	13.0
(2*R*,3*R*)-BDO-diol, 30 µM	111.8	15.4
(2*R*,3*R*)-BDO-diol, 40 µM	96.0	12.6
(2*R*,3*S*)-BDO-diol, 5 µM	89.5	6.9
(2*R*,3*S*)-BDO-diol, 10 µM	80.1	11.0
(2*R*,3*S*)-BDO-diol, 20 µM	70.2	4.2
(2*R*,3*S*)-BDO-diol, 30 µM	60.0	7.0
(2*R*,3*S*)-BDO-diol, 40 µM	40.3	6.1
(2*S*,3*R*)-BDO-diol, 5 µM	105.2	5.5
(2*S*,3*R*)-BDO-diol, 10 µM	100.2	11.8
(2*S*,3*R*)-BDO-diol, 20 µM	97.8	16.0
(2*S*,3*R*)-BDO-diol, 30 µM	89.3	11.9
(2*S*,3*R*)-BDO-diol, 40 µM	97.0	12.6
(2*S*,3*S*)-BDO-diol, 5 µM	105.5	13.6
(2*S*,3*S*)-BDO-diol, 10 µM	98.1	3.9
(2*S*,3*S*)-BDO-diol, 20 µM	99.2	4.2
(2*S*,3*S*)-BDO-diol, 30 µM	95.9	9.9
(2*S*,3*S*)-BDO-diol, 40 µM	101.1	10.0

Table A.2. Summary of Cell Count Results at the End of Treatment for the Mutagenicity Studies[a]

Stereoisomer and Concentration	Average ($\times 10^{-6}$)	Standard Deviation
BDO		
Control, 0 µM	**198.7**	**17.7**
(2*R*)-BDO, 200 µM	189.7	26.3
(2*R*)-BDO, 400 µM	134.7	12.2
(2*R*)-BDO, 600 µM	44.9	4.0
(2*S*)-BDO, 200 µM	203.1	11.6
(2*S*)-BDO, 400 µM	160.4	9.9
(2*S*)-BDO, 600 µM	40.4	4.5
BDO_2		
Control, 0 µM	**209.0**	**22.5**
(2*R*,3*R*)-BDO_2, 2 µM	161.0	14.3
(2*R*,3*R*)-BDO_2, 4 µM	65.6	16.2
(2*R*,3*R*)-BDO_2, 6 µM	58.4	8.4
(2*S*,3*S*)-BDO_2, 2 µM	66.2	9.5
(2*S*,3*S*)-BDO_2, 4 µM	31.5	4.3
(2*S*,3*S*)-BDO_2, 6 µM	30.8	3.2
meso-BDO_2, 2 µM	159.2	14.2
meso-BDO_2, 4 µM	78.4	6.7
meso-BDO_2, 6 µM	38.6	2.8
BDO-diol		
Control, 0 µM	**178.9**	**18.1**
(2*R*,3*R*)-BDO-diol, 200 µM	182.4	13.8
(2*R*,3*R*)-BDO-diol, 600 µM	189.3	15.3
(2*R*,3*R*)-BDO-diol, 1000 µM	171.1	9.9
(2*R*,3*S*)-BDO-diol, 40 µM	48.6	5.9
(2*R*,3*S*)-BDO-diol, 120 µM	8.9	1.3
(2*R*,3*S*)-BDO-diol, 200 µM	4.5	1.5
(2*S*,3*R*)-BDO-diol, 200 µM	157.6	12.1
(2*S*,3*R*)-BDO-diol, 600 µM	155.6	9.4
(2*S*,3*R*)-BDO-diol, 1000 µM	147.7	13.5
(2*S*,3*S*)-BDO-diol, 200 µM	146.4	7.8
(2*S*,3*S*)-BDO-diol, 600 µM	137.6	9.6
(2*S*,3*S*)-BDO-diol, 1000 µM	133.8	6.4
BDO-diol		
Control, 0 µM	**201.2**	**21.4**
(2*R*,3*R*)-BDO-diol, 5 µM	178.0	13.8
(2*R*,3*R*)-BDO-diol, 10 µM	189.2	15.1
(2*R*,3*R*)-BDO-diol, 20 µM	209.4	12.4
(2*R*,3*S*)-BDO-diol, 5 µM	207.2	13.8
(2*R*,3*S*)-BDO-diol, 10 µM	213.7	14.8
(2*R*,3*S*)-BDO-diol, 20 µM	195.5	21.1
(2*S*,3*R*)-BDO-diol, 5 µM	220.9	22.2
(2*S*,3*R*)-BDO-diol, 10 µM	196.0	13.9
(2*S*,3*R*)-BDO-diol, 20 µM	210.1	14.6
(2*S*,3*S*)-BDO-diol, 5 µM	171.4	23.1
(2*S*,3*S*)-BDO-diol, 10 µM	189.9	13.7
(2*S*,3*S*)-BDO-diol, 20 µM	199.8	14.5

[a] Cells were counted with trypan blue staining, so only live cells were counted.

Table A.3. Summary of *HPRT* M*f* Data

Stereoisomer and Concentration	Average ($\times 10^{-6}$)	Standard Deviation
BDO		
Control, 0 μM	**4.7**	**2.7**
(2*R*)-BDO, 200 μM	8.9	6.3
(2*R*)-BDO, 400 μM	18.7	7.3
(2*R*)-BDO, 600 μM	22.8	7.3
(2*S*)-BDO, 200 μM	9.2	1.6
(2*S*)-BDO, 400 μM	20.4	5.6
(2*S*)-BDO, 600 μM	29.1	10.2
BDO_2		
Control, 0 μM	**5.0**	**2.1**
(2*R*,3*R*)-BDO_2, 2 μM	11.0	4.5
(2*R*,3*R*)-BDO_2, 4 μM	24.6	16.7
(2*R*,3*R*)-BDO_2, 6 μM	22.4	11.4
(2*S*,3*S*)-BDO_2, 2 μM	12.2	6.5
(2*S*,3*S*)-BDO_2, 4 μM	21.5	13.3
(2*S*,3*S*)-BDO_2, 6 μM	25.7	18.7
meso-BDO_2, 2 μM	15.2	4.4
meso-BDO_2, 4 μM	18.8	9.8
meso-BDO_2, 6 μM	18.7	12.0
BDO-diol		
Control, 0 μM	**11.3**	**4.4**
(2*R*,3*R*)-BDO-diol, 200 μM	12.4	3.5
(2*R*,3*R*)-BDO-diol, 600 μM	13.4	3.8
(2*R*,3*R*)-BDO-diol, 1000 μM	10.1	1.9
(2*R*,3*S*)-BDO-diol, 40 μM	19.9	3.1
(2*R*,3*S*)-BDO-diol, 120 μM	19.5	4.3
(2*R*,3*S*)-BDO-diol, 200 μM	11.6	4.5
(2*S*,3*R*)-BDO-diol, 200 μM	11.6	1.9
(2*S*,3*R*)-BDO-diol, 600 μM	10.4	1.2
(2*S*,3*R*)-BDO-diol, 1000 μM	18.3	3.5
(2*S*,3*S*)-BDO-diol, 200 μM	14.5	2.9
(2*S*,3*S*)-BDO-diol, 600 μM	18.1	3.7
(2*S*,3*S*)-BDO-diol, 1000 μM	23.0	1.3
BDO-diol		
Control, 0 μM	**8.2**	**3.1**
(2*R*,3*R*)-BDO-diol, 5 μM	10.0	3.9
(2*R*,3*R*)-BDO-diol, 10 μM	9.6	3.8
(2*R*,3*R*)-BDO-diol, 20 μM	7.4	3.8
(2*R*,3*S*)-BDO-diol, 5 μM	7.2	1.9
(2*R*,3*S*)-BDO-diol, 10 μM	12.6	3.2
(2*R*,3*S*)-BDO-diol, 20 μM	15.8	1.1
(2*S*,3*R*)-BDO-diol, 5 μM	8.9	2.1
(2*S*,3*R*)-BDO-diol, 10 μM	9.0	3.5
(2*S*,3*R*)-BDO-diol, 20 μM	10.1	2.8
(2*S*,3*S*)-BDO-diol, 5 μM	7.4	4.1
(2*S*,3*S*)-BDO-diol, 10 μM	7.9	2.8
(2*S*,3*S*)-BDO-diol, 20 μM	9.8	3.5

Table A.4. Summary of *TK* M*f* Data

Stereoisomer and Concentration	Average ($\times 10^{-6}$)	Standard Deviation
BDO		
Control, 0 μM	**8.8**	**2.7**
(2*R*)-BDO, 200 μM	16.1	6.3
(2*R*)-BDO, 400 μM	26.5	8.3
(2*R*)-BDO, 600 μM	23.5	8.6
(2*S*)-BDO, 200 μM	13.5	4.7
(2*S*)-BDO, 400 μM	21.0	5.9
(2*S*)-BDO, 600 μM	24.1	8.5
BDO_2		
Control, 0 μM	**9.6**	**3.4**
(2*R*,3*R*)-BDO_2, 2 μM	27.1	4.3
(2*R*,3*R*)-BDO_2, 4 μM	46.4	15.5
(2*R*,3*R*)-BDO_2, 6 μM	56.6	31.3
(2*S*,3*S*)-BDO_2, 2 μM	29.1	19.0
(2*S*,3*S*)-BDO_2, 4 μM	41.6	17.3
(2*S*,3*S*)-BDO_2, 6 μM	65.8	31.1
meso-BDO_2, 2 μM	31.3	10.9
meso-BDO_2, 4 μM	38.7	7.9
meso-BDO_2, 6 μM	40.8	35.3
BDO-diol		
Control, 0 μM	**14.5**	**3.1**
(2*R*,3*R*)-BDO-diol, 200 μM	13.6	4.2
(2*R*,3*R*)-BDO-diol, 600 μM	15.5	4.1
(2*R*,3*R*)-BDO-diol, 1000 μM	12.4	2.9
(2*R*,3*S*)-BDO-diol, 40 μM	30.1	4.2
(2*R*,3*S*)-BDO-diol, 120 μM	24.7	2.6
(2*R*,3*S*)-BDO-diol, 200 μM	10.8	3.8
(2*S*,3*R*)-BDO-diol, 200 μM	15.8	5.3
(2*S*,3*R*)-BDO-diol, 600 μM	13.3	2.1
(2*S*,3*R*)-BDO-diol, 1000 μM	22.6	1.9
(2*S*,3*S*)-BDO-diol, 200 μM	12.9	2.1
(2*S*,3*S*)-BDO-diol, 600 μM	15.3	2.1
(2*S*,3*S*)-BDO-diol, 1000 μM	24.6	3.2
BDO-diol		
Control, 0 μM	**12.6**	**3.3**
(2*R*,3*R*)-BDO-diol, 5 μM	13.0	4.2
(2*R*,3*R*)-BDO-diol, 10 μM	12.7	2.3
(2*R*,3*R*)-BDO-diol, 20 μM	14.6	5.5
(2*R*,3*S*)-BDO-diol, 5 μM	10.7	3.2
(2*R*,3*S*)-BDO-diol, 10 μM	14.9	3.0
(2*R*,3*S*)-BDO-diol, 20 μM	23.6	2.1
(2*S*,3*R*)-BDO-diol, 5 μM	13.2	3.3
(2*S*,3*R*)-BDO-diol, 10 μM	14.5	3.8
(2*S*,3*R*)-BDO-diol, 20 μM	13.8	4.4
(2*S*,3*S*)-BDO-diol, 5 μM	13.0	1.9
(2*S*,3*S*)-BDO-diol, 10 μM	12.8	1.1
(2*S*,3*S*)-BDO-diol, 20 μM	14.9	2.2

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ABBREVIATIONS AND OTHER TERMS

6-TG	6-thioguanine
ANCOVA	analysis of covariance
BD	1,3-butadiene
BD-diol	1,2-dihydroxy-3-butene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
BDO-Gua	total of the two guanine adducts: N7-(1-(hydroxymethyl)-2-propenyl)guanine + N7-(2-hydroxy-3-butenyl)guanine
br	broad
Bu_2SnO	dibutyltin oxide
CE	cloning efficiency
CH_2Cl_2	methylene chloride
CH_3SO_2Cl	methanesulfonyl chloride
CH_3SO_2OH	methanesulfonic acid
CHAT	cytidine, hypoxanthine aminopterin, and thymidine
dd	doublet of doublet
DEAD	diethyl azodicarboxylate
DIPT	diisopropyl tartrate esters
dNTP	deoxyribonucleotide triphosphate
EMS	ethyl methanesulfonate
Et_2O-H_2O	ether
HB-Val	an isomeric mixture of *N*-(1-[hydroxymethyl]-2-propenyl)valine and *N*-(2-hydroxy-3-butenyl)valine
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid
HPRT	human hypoxanthine-guanine phosphoribosyltransferase gene
Hprt	rodent hypoxanthine-guanine phosphoribosyltransferase gene
Hz	Hertz
KOH	potassium hydroxide
Lit	literature
LOH	loss of heterozygosity
m	multiplet
m/z	mass/charge ratio
MeOH	methanol
M*f*	mutant frequency
MP	melting point
Na_2SO_4	sodium sulfate
NaH	sodium hydride
NEt_3	triethylamine
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PNB	*p*-nitrobenzoyl
PNB acid	*p*-nitrobenzoic acid
PNB-Cl	*p*-nitrobenzoyl chloride
PPh_3	triphenylphosphine
Pyr-Val	*N*,*N*-(2,3-dihydroxy-1,4-butadiyl)-valine
s	singlet
TBHP	*t*-butyl hydroperoxide
TFA	trifluoroacetic acid
TFT	trifluorothymidine
THB-Gua	N7-(2,3,4-trihydroxybutyl)guanine
THB-Val	*N*-(2,3,4-trihydroxybutyl)valine
THC	thymidine, hypoxanthine, and cytidine
$Ti(O\text{-}iPr)_4$	titanium isopropoxide
TK	thymidine kinase
TLC	thin layer chromatography
TosCl	toluenesulfonyl chloride
U.S. EPA	U.S. Environmental Protection Agency
VF	variant frequency

ALTERNATE TERMS AND ACRONYMS FOR BUTADIENE AND ITS METABOLITES AND ADDUCTS

BD	α,γ-butadiene biethylene bivinyl buta-1,3-diene divinyl erythrene pyrrolylene vinylethylene *trans*-butadiene
BDO	1,2-epoxybutene (EB) 3,4-epoxy-1-butene (EB) butadiene monoepoxide (BMO or BDO) isomers are referenced as (2*R*)-BDO, (2*S*)-BDO, (±)-BDO

BDO_2	(DEB or DBO_2 are alternate abbreviations for this format) 1,2:3,4-diepoxybutane (DEB) [difference is colon only] butadiene diepoxide (BDE) diepoxybutane (DEB) isomers are referenced as (2*R*,3*R*)-BDO_2, (2*S*,3*S*)-BDO_2, *meso*-BDO_2, (±)-BDO_2
BD-diol	3,4-dihydroxy-1-butene
BDO-diol	3,4-epoxy-1,2-butanediol (EBD) butadiene diol-epoxide butadiene monoepoxide-diol isomers are referenced as (2*S*,3*R*)-BDO-diol, (2*R*,3*S*)-BDO-diol, (2*R*,3*R*)-BDO-diol, (2*S*,3*S*)-BDO-diol
M1	1,2-dihydroxybutyl mercapturic acid (DHB-MA) *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine
M2	1,2-hydroxybutyl mercapturic acid (MHB-MA) 1-(*N*-acetylcysteinyl)-2-hydroxy-butene an isomeric mixture of the regio- and stereoisomers (*R*)/(*S*)-*N*-acetyl-*S*-(1-[hydroxymethyl]-2-propenyl)-L-cysteine + (*R*)/(*S*)-*N*-acetyl-*S*-(2-hydroxy-3-butenyl)-L-cysteine monohydroxy-3-butenyl mercapturic acid (MHB-MA) total 1- and 2-hydroxy-3-butenyl mercapturic acid (MHB-MA)
HB-Val	*N*-(2-hydroxy-3-butenyl)valine total of 1- and 2-monohydroxy-3-butenyl valine
THB-Gua	THB-G

Research Report 150, *Mutagenicity of Stereochemical Configurations of 1,3-Butadiene Epoxy Metabolites in Human Cells*, R.Q. Meng et al.

INTRODUCTION

1,3-butadiene (BD*) is found in motor vehicle exhaust and in cigarette smoke and is widely used in the production of synthetic rubber and thermoplastic resins. BD is one of several pollutants (referred to as mobile-source air toxics) whose levels are not regulated by the National Ambient Air Quality Standards, but that are known or suspected to cause adverse health effects in humans. It is classified by the U.S. Environmental Protection Agency (U.S. EPA) as carcinogenic to humans when inhaled. Studies in rodents have shown that mice and rats differ in their sensitivity to BD, with mice developing tumors at much lower exposure concentrations than rats. A key research and risk assessment question has been which species is likely to be more relevant to what happens in humans. Species differences in tumor induction by BD have been attributed to differences in its metabolism. The major metabolites of interest are 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). Studies conducted to determine the ability of these compounds to damage DNA in vivo as well as in cell cultures in vitro have indicated that these metabolites differ in their genotoxicity. In these studies, mostly racemic mixtures that contained all stereoisomers (spatially different forms of the same chemical structure) of each BD metabolite were tested. Limited work with specific stereoisomers has provided some evidence of a possible role of stereochemistry in the metabolism of BD and in the formation of DNA adducts, but none of those studies evaluated the mutagenicity of the individual stereoisomers.

In response to the Walter A. Rosenblith New Investigator Award Request for Applications (RFA 00-2), Dr. Ryan Q. Meng (then at the Lovelace Respiratory Research Institute) submitted an application to determine the cytotoxicity and mutagenicity of the stereoisomers of three major BD metabolites in the TK6 human lymphoblastoid cell line. The HEI Research Committee recommended the study for funding because they thought that Dr. Meng was a promising investigator and that the proposal was very relevant to the HEI research program on air toxics because it addressed an important question regarding the mutagenicity of BD metabolites. Halfway through the two-year study, Dr. Meng relocated to Battelle Toxicology Northwest, and the work continued in his new laboratory with the approval of the Research Committee.

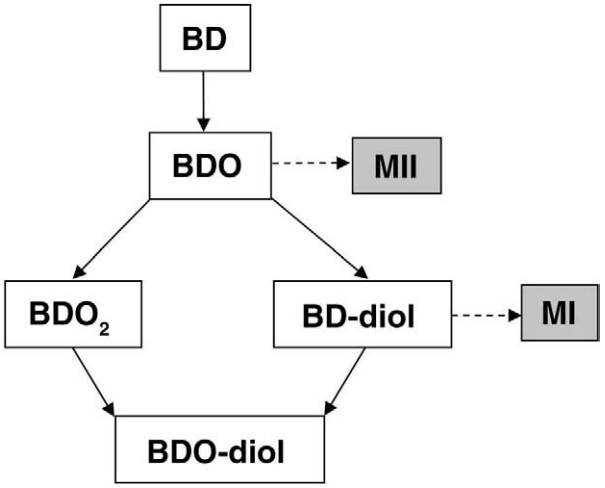
SCIENTIFIC BACKGROUND

The U.S. EPA lists BD as one of the 21 air toxics emitted, at least in part, from mobile sources whose emissions need to be reduced (U.S. EPA 2001) and as one of 8 mobile-source air toxics that, based on their emissions and reported toxicity, pose the greatest health risk (U.S. EPA 2007). In its most recent risk estimate, the U.S. EPA concluded that there is sufficient evidence to consider BD carcinogenic to humans when inhaled (U.S. EPA 2002). In its assessment, the EPA relied primarily on epidemiologic studies on the carcinogenicity of BD in exposed workers, but it also considered evidence from several studies of chronic BD inhalation in rats and mice. Mice have been shown to be more susceptible to BD-induced tumors than rats (Melnick and Huff 1992), and there is uncertainty as to which species is more relevant for human cancer risk assessment. Efforts to understand the basis for this interspecies difference (and its relevance to humans) have focused on the metabolic pathways of BD in the two species and on the ability of the main metabolites of BD to form DNA adducts and induce mutations when animals are exposed to the parent compound. DNA adducts are the product of the reaction of oxygen intermediates with DNA. Mistakes in the repair of these adducts can lead to mutations, which in turn may be associated with tumor development.

A simplified diagram of the metabolism of BD is shown in Critique Figure 1. BD is first metabolized by cytochrome P450 monooxygenase to the reactive monoepoxide BDO. BDO can then be metabolized further to either BDO-diol or the reactive diepoxide BDO_2. BDO-diol can also be formed

The 2-year study, "Mutagenicity of Stereochemical Configurations of 1,3-Butadiene Epoxy Metabolites in Human Cells," by Dr. Meng and colleagues began in November 2002. Total expenditures were $166,226. The draft Investigators' Report from Meng and colleagues was received for review in May 2005. A revised report was received in August 2007. A subsequent version of the report was submitted in March 2008 and accepted for publication in August 2008. The HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and the Review Committee's Critique.

*A list of abbreviations and other terms appears at the end of the Investigators' Report.



Critique Figure 1. Schematic diagram of BD metabolism. MI and MII are glutathione conjugates that are excreted in the urine.

from the oxidation of BDO_2. These metabolic steps are regulated by two different enzymes, cytochrome P450 monooxygenase (which aids in the formation of epoxides) and epoxide hydrolase (which aids in the formation of the alcohols from epoxides). The epoxides BDO, BDO_2, and BDO-diol can (1) react with macromolecules such as DNA or proteins, or (2) be detoxified by glutathione *S*-transferase to a glutathione conjugate, which is excreted in the urine. The chemical structure of these BD metabolites is such that different numbers of stereoisomers of the same molecule exist. Information on the stereochemistry of the BD metabolites can be found in the sidebar.

Several studies have measured the metabolism of BD to better understand the possible basis for the different potency of BD in rats and mice. Significant species differences in the ratio of BDO to BDO_2 in blood were found, with higher levels of BDO_2 in mice than in rats (Himmelstein et al. 1994; Thornton-Manning et al. 1995; Henderson 1996). However, the levels of BDO-diol in mice and rats were not measured. A study of human lymphoblastoid cells exposed in vitro to each of the three epoxy metabolites identified BDO_2 as the most mutagenic and BDO-diol as the least mutagenic (Cochrane and Skopek 1994a), but the relative mutagenicity and carcinogenicity of these metabolites in vivo has not been established.

Some work conducted in the last 10 years, however, has provided some support for a possible role of BDO-diol in the carcinogenicity of BD. For example, Swenberg and colleagues (2001) calculated that the major guanine alkylated DNA adducts found in rodents after exposure to BD are primarily from BDO-diol. Koivisto and Peltonen (2001) showed that the DNA adducts derived from BDO-diol accounted for 98% of the total alkylated adducts in mice exposed to BD. Boogard and colleagues (2004) and Boysen and colleagues (2004) also identified DNA and protein adducts with BDO-diol in both mice and rats. Recent data indicate that BDO-diol is the predominant metabolite in both mice and rats exposed to BD by inhalation (Filser et al. 2007).

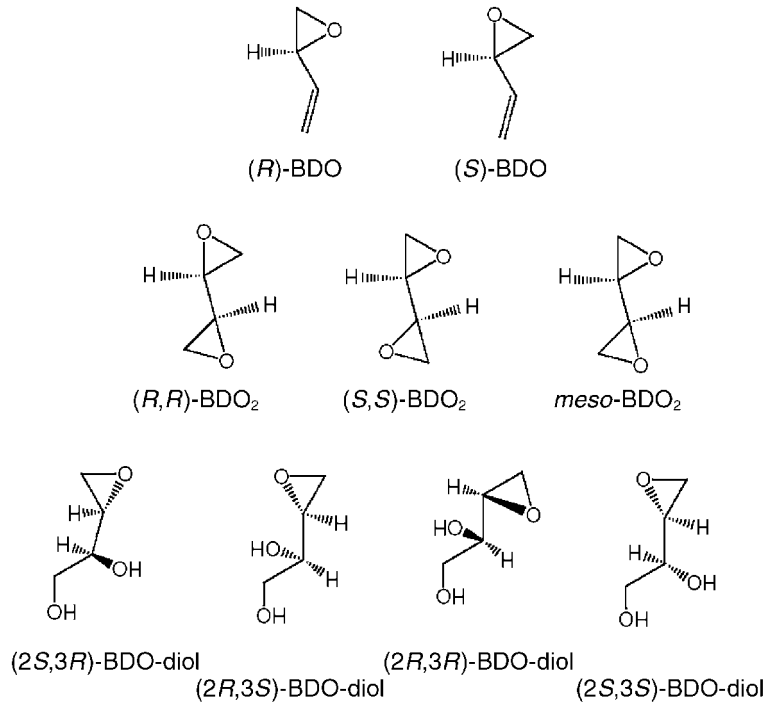
Other work has provided some evidence that the stereochemical configuration of BD metabolites may play a role in the effects of the metabolites. The cytotoxicity of each of the BDO and BDO_2 stereoisomers was examined in freshly isolated rat hepatocytes exposed to BD gas (Nieusma et al. 1997). (2*R*)-BDO showed greater cytotoxicity than (2*S*)-BDO, and *meso*-BDO_2 was more cytotoxic than either (2*R*,3*R*)-BDO_2 or (2*S*,2*S*)-BDO_2. Koivisto and colleagues (1999) reported that 73% of the BDO-diol-derived adducts in mice exposed to BD were derived from (2*R*,3*S*)-BDO-diol.

The most common approach to measure mutations induced by BD or its metabolites has been to identify changes in specific genes. Some studies were conducted in animals exposed by inhalation (Walker and Meng 2000; Cochrane and Skopek 1994b; Tates et al. 1998), and some employed human cells in culture exposed in vitro (Cochrane and Skopek 1994a; Steen et al. 1997). The in vitro studies offer several advantages relative to studies conducted in exposed animals. They are less expensive to conduct and allow the rapid testing of multiple doses and multiple compounds. Depending on the cell line used, in vitro studies also allow the evaluation of the effect of given compounds without interference from possible transformation products, which happens in in vivo studies.

Two commonly used genes that have been evaluated for mutations in response to xenobiotics are hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and thymidine kinase (*TK*). The *HPRT* gene is hemizygous; that is, it is missing one allele (because it is linked to the X chromosome). The *TK* gene is heterozygous; that is, it has two different alleles ($TK^{+/-}$). The TK6 human lymphoblastoid cell line used by Meng and colleagues (and also widely used for mutation assays) was originally isolated as *TK* hemizygote from a male human B lymphoblastoid cell line by Skopek and colleagues (1978). Cells with a normal *HPRT* gene cannot grow in the presence of 6-thioguanine (6-TG), whereas cells with a normal *TK* gene cannot grow in the presence of trifluorothymidine (TFT). Thus, to select the mutant cells after exposure to the target compound, the cells are grown in the presence of 6-TG to identify mutations at the *HPRT* gene; and they are grown in the presence of TFT to identify mutations at the *TK* gene (more details on the *HPRT* assay can be found in Walker and colleagues [2009]). Meng and colleagues applied this approach in their study.

STEREOCHEMISTRY OF BD METABOLITES

In some molecules, the asymmetric bonding of some atoms to a carbon atom results in "left hand" (*S*) and "right hand" (*R*) forms of the molecules, called stereoisomers, which differ in their three-dimensional configurations but are chemically identical (Walker et al. 2009). A *meso*-isomer is a compound that is not optically active. Critique Figure 2 shows the structure of the stereoisomers of the three BD epoxy metabolites.



Critique Figure 2. Stereochemistry of BD. Oxidation of BD to BDO produces one asymmetric center, hence (*R*)-BDO and (*S*)-BDO. Further oxidation converts (*R*)- and (*S*)-BDO to three forms of BDO_2; the designations (*R*,*R*) and (*S*,*S*) — also referred to as (±)-BDO_2 — indicate two symmetric centers; the *meso* form has two asymmetric centers, but their spatial configurations yield overall symmetry within the molecule. Hydration of the three BDO_2 forms, or oxidation of BD-diol, can produce four stereoisomers of BDO-diol. Thus, nine possible stereochemical configurations exist for the three BD epoxides. (Adapted with permission from Nieusma and coworkers [1998].)

Several investigators have also determined the sequence of the DNA of their target genes, to identify the specific types of mutations induced by a given compound. Examples of types of mutations that occur within a gene include point mutations (i.e., single-base substitutions), base insertions or deletions (referred to as frame-shift mutations), DNA sequence reversions, and various types of duplications or deletions of DNA sequences. Gene mutations have varying effects on the gene product, depending on where they occur and whether they alter the function of an essential protein. Recent work by Walker and colleagues (2009) showed that the *HPRT* gene in the spleen of rats exposed to BD had base substitutions, frame-shift mutations, and large deletions.

OVERVIEW OF AIMS AND EXPERIMENTAL APPROACHES

SPECIFIC AIMS

The study had the following specific aims:

1. Synthesize the nine stereoisomers of the three main BD metabolites:
   - BDO_2: (2*R*,3*R*), (2*S*,3*S*), *meso*
   - BDO: (2*R*), (2*S*)
   - BDO-diol: (2*R*,3*R*), (2*R*,3*S*), (2*S*,3*R*), (2*S*,3*S*)
2. Evaluate the cytotoxicity and mutagenicity of each stereoisomer in cells exposed in vitro and determine

the frequency and types of mutations induced by BDO_2 stereoisomers at the *HPRT* and *TK* genes. BDO_2 was presumably chosen because it is the most mutagenic of the three BD metabolites.

STUDY DESIGN AND METHODS

The synthesis of the stereoisomers was conducted at the University of Texas Medical Branch in Galveston, Texas. The investigators assessed the purity of each compound by evaluating various analytic parameters including gas chromatography–mass spectrometry and nuclear magnetic resonance spectra, melting point, and optical rotation.

The cytotoxicity and mutagenicity of each stereoisomer was evaluated in a TK6 human lymphoblastoid cell line. To measure cytotoxicity (i.e., decreased survival), cells were exposed in culture to five different concentrations of each stereoisomer for 24 hours. Subsets of cells from each culture were then grown into colonies for 8 to 10 days, to determine cloning efficiency (CE), reported as the number of cells that form colonies in normal medium relative to the initial number of cells plated, expressed as a percentage. Cytotoxicity, or percent survival, was calculated as CE of exposed cells relative to control cells (with the control value set at 100%). Each concentration was tested in triplicate.

To measure mutagenicity, cells were exposed to three different concentrations of each stereoisomer (selected based on the cytotoxicity results) for 24 hours. Each concentration was tested in five groups of cells. Cytotoxicity was not determined in these experiments, but the number of live cells at the end of the exposure period was determined by counting the cells that excluded trypan blue (a dye that penetrates into dead cells but not live cells.) After exposure, the cells were cultured in growth medium (in the presence of the selection compound) for a period sufficient to allow for the phenotypic expression of the mutation (the period during which the mutation is fixed in the genome). Mutations were examined in the *HPRT* and the *TK* genes. For the *HRPT* assay, the period for phenotypic expression was 7 days; for the *TK* assay, it was 3 days. After the expression period, cells were grown in medium with and without the selection agent (6-TG or TFT) for 10 days (for *HPRT* mutants), or 21 days (for *TK* mutants), to determine CEs (as described above) and mutant frequencies (M*f*s). M*f*s were calculated as the ratio of the mean CEs in the presence of the selection agent to those in medium without the selection agent.

In separate experiments, Meng and colleagues also determined the spectrum of the mutations at the *HPRT* gene induced by BDO_2 (at 4 µM). For these experiments, individual mutant colonies from each culture were grown to a certain cell density; then the cell lysate was mixed with DNA primer for specific exons (sequences that are present in the messenger RNA derived from that gene) of the *HPRT* gene, deoxyribonucleotide triphosphate (dNTP), and DNA polymerase (the enzyme that synthesizes a DNA chain by linking the dNTPs). This method is referred to as multiplex polymerase chain reaction (PCR). Changing the temperature of the solution causes the *HPRT* DNA to be copied several times, thereby yielding sufficient mutant DNA for each exon to be visualized under ultraviolet light after separation on an agarose gel. This method detects primarily large deletions in one or more exons.

DATA ANALYSES

The investigators evaluated differences between the average CE or M*f* values at the various doses of each stereoisomer relative to unexposed cells in pair-wise comparisons. Both parametric and nonparametric analyses were conducted. To compare the mutagenic potency of the stereoisomers of each metabolite, regression models were fit across all dose levels of each stereoisomer and differences between the slopes were assessed.

The numbers of each type of genomic change in each cell culture sample treated with a BDO_2 stereoisomer were compared to those of the controls using a Fisher exact test.

RESULTS

The authors successfully synthesized the nine stereoisomers with sufficient yield to conduct the proposed assays. The purity of the stereoisomers was determined to be at least 98% to 99.9%. The Investigators' Report provides details on the steps involved in the synthesis and purification of the various stereoisomers and data on the yield of the procedures.

The main results of the effects of the stereoisomers of each BD metabolite are summarized below.

TOXICITY AND MUTAGENICITY OF BDO_2 STEREOISOMERS

Cell survival was determined after exposure to 0, 2, 4, 6, 8, and 10 µM of (2*R*,3*S*)-, (2*S*,3*S*)-, and *meso*-BDO_2 isomers. Survival decreased when the dose increased (it was about 20% at 6 µM) for each stereoisomer. Overall, owing to the small sample size, the toxicities of the three isomers did not differ significantly.

Mutagenicity was determined after exposure to 0, 2, 4, and 6 µM of each isomer. The M*f*s increased with the dose and were similar for the three isomers. The value of the M*f*s at the *TK* gene was approximately twofold higher than

that of the M*f*s at the *HPRT* gene. As observed in the cytotoxicity study, cell survival measured in the mutation assay decreased with increasing concentrations (as shown in Appendix A in the Investigators' Report).

TOXICITY AND MUTAGENICITY OF BDO STEREOISOMERS

Cell survival was evaluated at doses of 200, 400, 600, 800, and 1000 µM. The lowest dose that significantly reduced survival was 600 µM (25% survival). Mutagenicity was determined at doses of 200, 400, and 600 µM. (2*S*)-BDO caused a dose-related increase in *HPRT* mutations at all three doses, while (2*R*)-BDO did so only at 400 and 600 µM. Both isomers increased mutations at the *TK* gene at 400 and 600 µM, but not at 200 µM. Overall, dose-related mutagenic responses for (2*R*)-BDO and (2*S*)-BDO were not statistically different.

TOXICITY AND MUTAGENICITY OF BDO-DIOL STEREOISOMERS

The toxicity of the four BDO-diol stereoisomers was determined at 200, 600, and 1000 µM concentrations. The different BDO-diol stereoisomers affected cell survival differently, with the (2*R*,3*S*) stereoisomer being the most toxic; it caused significant cell death even at the lowest dose tested, 200 µM (11% survival). (2*R*,3*R*)-BDO-diol was the least toxic and did not affect survival even at the highest dose (1000 µM); the toxicity of the two other stereoisomers fell in between. Based on the cell toxicity results, mutagenicity was initially determined using doses of 40, 120, and 200 µM for (2*R*,3*S*)-BDO-diol and 200, 600, and 1000 µM for the other isomers.

The BDO-diol stereoisomers had different mutagenic effects. (2*R*,3*S*)-BDO-diol was the most potent and increased the M*f*s at both the *HPRT* and the *TK* genes at the lowest dose tested, 40 µM. However, doses greater than 40 µM (doses of 120 and 200 µM) did not cause mutations.

(2*S*,3*S*)-BDO-diol and (2*S*,3*R*)-BDO-diol increased the M*f*s in both genes only at 1000 µM, while (2*R*,3*R*)-BDO-diol did not show an effect at any of the three doses. The mutagenic effects of the four stereoisomers were similar for the *TK* and the *HPRT* genes.

Because of the steep slope of the toxicity and mutagenicity curves of (2*R*,3*S*)-BDO-diol, the HEI Review Committee was concerned that the compound was killing most of the induced mutants and that the investigators may have underestimated its potency. Therefore, Meng and colleagues repeated the toxicity assays using 5, 10, 20, and 40 µM of all stereoisomers to make a more quantitative assessment of their absolute as well as relative potencies. Cell survival was affected only by (2*R*,3*S*)-BDO-diol: survival was 70% at 20 µM and 40% at 40 µM (as shown in Figure 15 of the Investigators' Report). The mutagenicity assays were repeated using doses of 0, 5, 10, and 20 µM of each BDO-diol stereoisomer. Figures 16 and 17 of the report show the M*f*s at the *HPRT* and *TK* genes, respectively, at these low doses. Only (2*S*,3*S*)-BDO-diol increased the M*f*s at 20 µM. At this dose, the numbers of cells available for cloning after the treatment were similar at all doses for all stereoisomers, as shown in Appendix Table A.2 of the Investigators' Report. No effects were observed at lower concentrations. Meng and colleagues compared the slopes of the M*f*s at the *HPRT* and *TK* genes over the low-dose range (5 to 20 µM) and found that, for the *TK* gene, the slope of the M*f*s for (2*R*,3*S*)-BDO-diol was significantly different from the slopes for the other BDO-diol stereoisomers; the differences between some of the slopes of the M*f*s at the *HPRT* gene were borderline significant.

ANALYSIS OF *HPRT* MUTATIONS INDUCED BY BDO_2

The results showed the presence of gene sequence deletions and other changes such as point mutations and insertions. There was a higher frequency of deletions in cells exposed to each stereoisomer of BDO_2 relative to unexposed cells, but there was no difference among the cells exposed to the three stereoisomers.

REVIEW COMMITTEE EVALUATION

In its independent evaluation of the Investigators' Report, the Review Committee thought that the investigators had conducted a well-designed and novel study that combined chemistry and molecular biology to address a relevant research question regarding the mutagenicity of specific stereoisomers of the key BD metabolites. The Review Committee thought that the investigators provided convincing data on the purity of the stereoisomers, determined using a variety of analyses.

A key finding was that the (2*R*,3*S*)-BDO-diol stereoisomer was about 30-fold more cytotoxic and mutagenic, on an equimolar basis, than the other three BDO-diol stereoisomers. At the same time, the present results support the view that the stereochemistry of BDO and BDO_2 is not likely to play a significant role in the mutagenicity of BD.

(2*R*,3*S*)-BDO-diol was 5-to-10-fold less cytotoxic and mutagenic than any of the BDO_2 stereoisomers. This finding is in contrast with an earlier study of the mutagenicity of BD metabolites in TK6 cells that did not consider stereochemistry (Cochrane and Skopeck 1994a). In that study,

BDO-diol was at least 100-fold less potent than BDO_2. In the current study, (2*R*,3*S*)-BDO-diol was 10-to-20-fold more mutagenic than the BDO stereoisomers.

Although the investigators did not measure cytotoxicity and mutagenicity in the same experiment, they provided data on cell survival (based on the exclusion of a dye) immediately after the end of the exposure in the mutagenicity studies (Appendix A of the Investigators' Report). These data indicate that there should have been a sufficient number of surviving mutants for the determination of M*f*s.

The authors mention that one advantage of using the *TK* cells is their reported lack of metabolic capacity; however, there is no evidence that the cells did not, to some extent, metabolize the various compounds included in this particular study. Thus, the possibility that the ultimate DNA-damaging species could have been derived from the test compound cannot be completely ruled out.

The findings regarding the mutagenicity of (2*R*,3*S*)-BDO-diol are consistent with findings of a study that looked at the stereochemistry of DNA adducts formed in the lung after inhalation exposure of mice to BD (Koivisto and Peltonen 2001). These authors found that the major DNA adduct in the lungs of mice exposed to BD was from (2*R*,3*S*)-BDO-diol. The finding of enhanced mutagenicity in vitro of (2*R*,3*S*)-BDO-diol in the current study suggests that this compound may play a role in BD-induced carcinogenicity, as noted by Meng and colleagues. The authors also suggest that BD mutagenesis and carcinogenesis could in large part be attributable to the stereochemistry-dependent metabolism of BD. The investigators point to evidence, based on what is known about the metabolism of BD (shown in Figure 1 of the Investigators' Report), that (2*R*,3*S*)-BDO-diol is more likely to be derived from (2*S*)-BDO than from (2*R*)-BDO. They cite the work by Nieusma and colleagues (1997) showing that mouse microsomes produced more (2*S*)-BDO than rat microsomes and that the production of BDO_2 from BDO differed in the two species. Specifically, the formation of BDO_2 from (2*S*)-BDO was greater than from (2*R*)-BDO in mice microsomes, whereas the formation of BDO_2 in rats was mainly from (2*R*)-BDO. Meng and colleagues conclude that the combination of greater production of (2*R*,3*S*)-BDO-diol and of BDO_2 in mice, compared with rats, may contribute to the greater carcinogenicity of BD in this species. Although this is a possible scenario, the earlier studies did not measure the production of BDO-diol stereoisomers. More studies that consider the kinetics of the in vivo formation and distribution of BDO-diol isomers are needed to provide insights into the determinants of species susceptibility to BD carcinogenesis.

The analysis by Meng and colleagues of the types of mutations induced by the stereoisomers of BDO_2, while appropriately carried out, did not provide any new insights into the mechanism of action of BD. A more detailed analysis of the sizes of the deletions would be helpful to determine whether the deletion spectra of the mutants differ. A comparison of the mutation spectra of BDO_2 and BDO-diol would also have been informative.

In summary, the study's key finding that one of the four stereoisomers of BDO-diol was responsible for most of the mutagenicity of this metabolite is interesting and could explain some of the possible species differences in the mutagenicity of BD. The study also suggests that the species differences in susceptibility to BD that have been noted in other work are not likely to be related to the stereochemistry of BDO and BDO_2. The caveat to these conclusions is that the normal target cells for BD in rodents may detoxify the different BD metabolites at very different rates, thus leading to steady-state levels of one metabolite or its stereoisomers that are higher than those of others. The results of this study using a cell line and individual stereoisomers provide justification for follow-up studies that consider the kinetics of the formation and distribution of BDO-diol stereoisomers in rodents and humans and that have endpoints relating to both mutagenicity and carcinogenicity.

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