POLYMORPHISMS IN METABOLIC GENES: RELEVANCE FOR HUMAN SUSCEPTIBILITY TO BUTADIENE

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Introduction

The Texas Commission on Environmental Quality (TCEQ) Risk Assessment correctly states that:

> *"Human genetic polymorphisms are likely to affect individual susceptibility to butadiene (BD) and its metabolites. Activation rates in humans exhibit a high degree of variability and appear to span the range of activation rates between mice and rats, ----------" (p.16, lns 22 - 24).*

However, the assessment then goes on to speculate on the effects of these polymorphisms:

> *" ------ so humans may be as sensitive as mice (p.16, ln 24).*

This speculation appears to be based solely on the range of variability of the various enzyme activities, *as measured by in vitro analyses of enzyme kinetics*, and does not take account of the abundant literature that deals with the metabolic and genotoxic consequences of these polymorphisms, as measured in human cells *in vitro* and metabolic and genotoxic biomarkers determined *in vivo.* The relevant literature is reviewed here.

In order to compare humans to mice, and to speculate as to whether any humans can be as sensitive as mice to the gentoxic (and therefore, carcinogenic) effects of BD, it is first important to identify the magnitude of the differences between these species for the metabolic and genotoxic (presumably reflecting carcinogenic) consequences of BD exposures.

Mouse-Human Inter-Species Comparisons

Metabolic endpoints will be considered first, followed by genotoxic consequences.

Metabolism

As regards oxidative metabolism of BD to its most genotoxic metabolites (mediated by P450 enzymes, especially CYP2E1), mice greatly predominate over humans, as measured by the accumulation of 2-hydroxy-3-butanyl-valine (HBVal; 1,2-epoxy3-butene [EB]

derived) and N,N-(2,3-dihydroxy-1,4-butadyl)-valine (*pyr*-Val; 1,2:3,4-diepoxybutane [DEB] derived) hemoglobin adducts in the two species. Comparing mice exposed to 1.0 ppm BD by inhalation for four weeks to humans exposed to an average of 0.8 ppm BD by inhalation for several years showed a *pyr*-Val adduct concentration of between 20 to 30 pmol/g globin (males versus females) in the mice while, for humans, this DEB specific adduct was NOT QUANTIFIABLE (Boysen *et al.* 2007, Albertini *et al.* 2007). Therefore, for this most genotoxic of BD metabolites, the metabolic difference between mice and humans is infinity, using this particular *pyr*-Val assay. Assuming some insensitivity in this assay, an upper bound of the fold-difference between these species for DEB production can be estimated to be greater than 10.

EB production, as determined by HBVal adduct concentrations, was not estimated in the mice exposed to 1.0 ppm BD, but an earlier study exposing mice to 3.0 ppm showed that HBVal adduct concentrations were approximately the same as the *pyr*Val adduct concentrations (Boysen *et al.* 2004). The humans exposed to 0.8 ppm BD showed a HBVal adduct concentration of 2.2 pmol/g globin (Albertini *et al.* 2001, 2003). Therefore, using the *pyr-Val* concentration as a surrogate for the HBVal concentration in mice exposed to 1.0 ppm (estimating ~ 25 pmol/g globin HBVal), the fold-difference in EB production for mice compared to humans can be estimated as also being somewhat greater than 10.

Detoxification of BD metabolites (as opposed to oxidative metabolism) is measured *in vivo* by conjugation and hydrolysis products. The urinary metabolites 1-hydroxy-2-(N-acetylcysteinyl)-3-butene and 2-hydroxy-1-(N-acetylcysteinyl)-3-butene (collectively known as M2 metabolite) are indicators of the percentage of detoxification that occurs using the Glutathione-S-transferase (GST) mediated conjugation pathway. In mice, approximately 77% of BD detoxification occurs via this route (Bechtold *et al.,* 1994). By contrast, in humans, only 1 to 3% of detoxification utilizes this conjugation pathway (Albertini *et al.* 2001, 2003, 2007). Hydrolysis, however, which is mediated by epoxide hydrolase (EH), is greatly enhanced in humans compared to mice. Approximately 97-99% of detoxification occurs by this pathway, as judged by urinary 1,2-dihydroxy-4-(N-acetylcysteinly)-butane (M1 metabolite) concentrations (Albertini *et al.,* 2001, 2003, 2007). In mice, this pathway accounts for approximately 23% of the detoxification (Bechtold *et al.,* 1994). The concentration of the 1,2,3-trihydroxybutyl-valine (THBVal) hemoglobin adduct, which is derived from 1,2-dihydroxy-3,4-epoxybutane (EBD), also reflects hydrolytic detoxification of BD metabolites, and is higher in humans than mice at the various levels of BD exposure (Boysen *et al., 2004,* Albertini *et al.*,2001, 2003).

Mutation Induction

Important inter-species comparisons between mice and humans are those for irreversible genotoxic effects, i.e. gene and chromosome level mutations. Mice show statistically significant increases in *Hprt* gene mutations at an exposure level as low as 1.0 ppm BD for four weeks (where the *pyr*-Val adduct concentrations were also measured, see above) (Meng *et al,* 2007). By contrast, with the exception of a single laboratory studying Texas

BD facilities (Legator *et al.* 1993, Ward *et al.,* 1994, 1996, 1997, 2001, Ma *et al.,*2001, Ammenheuser *et al.* 2001, Abdel-Rahman *et al*. 2001, 2003, 2005), several large multi-institution studies involving many laboratories to study facilities at different locations have failed to find increases in *HPRT* gene mutations at any level of BD exposure (Tates *et al,* 1996, Hayes *et al,*1996, 2000, 2001, Zhang *et al,* 2004, Albertini *et al,* 2001, 2003, 2007).

Reasons for the discordant results between the Texas laboratory and all of the others are not known. The Texas group has employed a variant of the *HPRT* assay (autoradiography) while the others have used a more conventional cloning assay. Results of the two assays, however, while possibly measuring events in different cell sub-populations, should be in the same direction. Furthermore, the Texas laboratory was a collaborator in one of the comprehensive Czech studies (see below), where they too failed to show increases in *HPRT* mutations in exposed workers using autoradiography. Close inspection of the Texas data (different aspects reported in the several references given above) suggest some potential for confounding, as indicated by differences in group mean mutant frequencies in different worker groups having different mean BD exposure levels that were not reflected by within group associations of mutant frequencies with individual exposure levels. Nonetheless, the differences between the Texas results and the others remain to be explained.

An NIH study in China, in which the highest individual BD exposure levels have been reported, not only failed to find an increase in *HPRT* mutations, it failed also to find an increase in Glycophorin A gene mutations (Hayes, 2001, 2003, Zhang *et al.* 2004). As noted, this study was multi-institutional, with the different mutation assays being conducted independently of each other. The two Czech studies were also multi-institutional, and also involved several different laboratories, each conducting its assays independently (Albertini *et al.* 2002, 2003, 2007). Both Czech studies employed extensive external exposure assessments, involving 8 to 10 individual 8 h worker measurements using personal monitors, occurring over a two to four month period prior to the collection of biological samples (to accommodate the time-lag requirements of the different biomarker assays). These studies included rigorous blinding, with the laboratories conducting the assays not knowing sample identities until after they had submitted their complete data sets to the data management facility, which was in a different location managed by different personnel disinterested in the study outcome. As noted above, the autoradiographic assay for *HPRT* mutations was included in the 1st Czech study to help resolve the conflict between the discordant results just described. There were no increases in *HPRT* mutation frequencies related to BD exposure in any of these studies.

Chromosome level mutations (aberrations and or micronuclei) have been found whenever looked for in BD exposed mice (Himmelstein *et al.,* 1997). Similar to the findings for *HPRT* gene mutations, these genotoxic lesions have not been found in humans. A single study that originally reported an increase in mean aberration frequencies (Tates *et al.* 1996, with definitive report by Sram *et al.* 1998), subsequently found the aberration frequencies did not correlate with BD exposure levels, as measured by DNA adduct

levels, and therefore were influenced by other factors (Zhao *et al,* 2001). Numerous other studies have failed to find significantly elevated mean levels of chromosome aberrations in groups of BD exposed humans (Au *et al.,* 1995, Hallberg *et al.,* 1997, Sorsa *et al,* 1994, 1996, Zhao *et al.,* 2001, Hayes *et al.,* 1996, 2000, 2001, Warholm *et al.* 2003, Fustinoni *et al.,* 2004, Albertini *et al.,* 2001, 2003, 2007), although the Sorsa *et al.*1996 paper did report differences according to GST genotypes (see below) and a single poster report did indicate a significant correlation among workers between chromosome aberrations and BD exposure levels, as assessed by surrogate urine metabolite and hemoglobin adduct measurements (Warholm *et al.* 2003). [It is noteworthy that this poster report was not cited by one of the authors, who subsequently reviewed the several chromosome studies in her report of a negative study (Fustinoni *et al.* 2004)]. Increases in chromosome level mutations relative to BD exposure levels were not found in any of the large, multi-institutional studies.

Comparisons of the magnitudes of the differences in *sensitivity* to BD exposure between mice and humans, therefore, range from a factor in excess of 10, for metabolic endpoints, to nearly qualitative differences (magnitude near infinity), for the different genotoxic endpoints. The greatest differences have been observed for production of the DEB metabolite, as assessed by *pyr*-Val hemoglobin adduct concentrations, and for the biomarkers of irreversible genotoxic effects such as gene mutations and chromosome aberrations. It is these latter that most likely serve as indicators of the kinds of genotoxicity that underlies carcinogenesis. These mouse-human inter-species comparisons provide a context for assessing the magnitude of intra-species differences in BD *sensitivity*, as assessed in humans of different metabolic genotypes, as determined by a variety of biomarker responses in several studies.

Human Intra-Species Differences by Genotypes

The several studies of human intra-species differences by genotype have examined one of several of the following metabolic genetic polymorphisms in BD exposed humans:

- CYP2E1: Several genetic polymorphisms for this phase I activating enzyme have been identified in the various studies. The *RsaI* polymorphism encodes for alleles c1/c2 (c2 = high activity). The $G_{-35} \rightarrow T$ polymorphism encodes for a G wild-type and T variant allele, with the latter suggested to be associated with higher transcriptional activity. A 96 bp 5' flanking region repeat polymorphism, and an A→T intron 6 polymorphism have also been studied, both of uncertain biological significance.
- Glutathione-S-transferases (GST): These are phase II enzymes that detoxify by conjugation. Polymorphisms of several GST genes have been studied. These include the wild-type and null alleles for the GST M1 and GST T1 genes, and the Ile→Val exon 5 substitution at residue 104 and the Ala→Val exon 6 substitution at residue 113 for the GST P1 gene. For GST M1 and T1, the null alleles are deficiency alleles: the biological significance of the GST P1 polymorphisms are uncertain. GST phenotypes (activity determined irrespective of genotype) are

determined by various functional tests, such as the administration of chlorzoxazone and analysis of urinary metabolites.

- Microsomal epoxide hydrolysase (EH): This is a phase II enzyme that detoxifies by hydrolysis. Several polymorphisms have been studied. These include 5' flanking region polymorphisms: the -200 linkage group (200 C/T, -259 C/T, -290 T/G), the -600 linkage group (-362 A/G, -613 T/C, -699 T/C), and the independent -399 T/C. These flanking region variants may be associated with increases in gene transcription. The most frequent EH polymorphisms studied are the tyr→his exon 3 substitution at residue 113 and the his→arg exon 4 substitution at residue 139. The 113 his substitution results in a 40% decline in enzyme activity, while the 139 arg substitution produces approximately a 25% increase in activity. These polymorphisms can be combined to give combinations that result in fast, intermediate and low hydroxylation, which constitutes a phenotypic designation for the individual. These combinations are:
    - Fast hydroxylation: tyr/tyr (113) arg/arg (139, tyr/tyr (113); arg.his (139) and try/his (113), arg/arg (139).
    - Intermediate hydroxylation: tyr/tyr (113), his/his (139) and tyr/his (113), arg/his (139)
    - Slow hydroxylation: tyr/his (113), his/his (139); his/his (113), arg/his (139) and his/his (113) and his/his (139).
- Alcohol dehydrogenases (ADH): The ADHs constitute a family of genes that catalyze the oxidation of alcohols and ketones. The ADH 1 gene is not polymorphic; the ADH 2 and 3 genes are. Polymorphisms of both have been identified in BD studies. These are the arg → his substitution at residue 47 in ADH 2 and the Ile → val substitution at residue 349 in ADH3. Both substitutions result in lower enzyme activities compared to wild-type.

Metabolism

A study designed to measure dietary and genetic factors affecting human metabolism of BD in 133 normal human subjects (different races, sexes) exposed to 2.0 ppm BD by inhalation for 20 minutes (administered dose ~ 0.6 ppm h) measured BD uptake and calculated metabolic rates (K_{met}; oxidation) by use of PBPK modeling (Smith *et al.* 2001). CYP2E1 *RsaI* genotypes were determined by DNA analyses and CYP2E1 phenotypes determined by chlorzoxane administration and urine metabolite studies. No significant associations were found between either BD uptake or K_{met} and CYP2E1 genotypes or phenotypes.

Fustinoni *et al.* (2002) examined the relationship between several genetic polymorphisms and both urine M1 metabolite concentrations and THBVal hemoglobin adduct levels in 30 workers exposed to BD at very low levels (range = 0.002 to 0.09 ppm). CYP2E1 *RsaI*, G_{-35}→T and 96 bp repeat polymorphisms, EH113 and 139 polymorphisms, EH hydroxylation phenotypes, GST M1, T1 and P1 (104 and 113) polymorphisms and the ADH3 polymorphism were determined for all subjects. Individuals with either the GST M1 or T1 homozygous null genotype showed significantly higher THBVal adduct

concentrations than did individuals not possessing this genotype, but the differences were small (< 10%). Workers with either the CYP2E1 GT_{-35} or the $T_{-35}T_{-35}$ genotypes had non-significantly lower concentrations of THBVal adducts (<20%) than did workers of the GG genotype. It is of interest that an examination of the influence of hydroxylation phenotypes as indicated by combinations of EH polymorphisms (fast, intermediate, slow hydroxylation) did not show statistically the significant differences in THBVal adduct adduct that might have been predicted from the respective enzyme kinetics conferred by these different genotypes. However, there was only a single individual in the fast hydroxylation category, and the THBVal adduct concentration of that worker was approximately twice the median values for workers in the other two hydroxylation categories (intermediate and slow). Even if this single value is a valid approximation of the median for a group of fast hydroxylators, this would indicate an increase in hydroxylation (detoxification) by only a factor of two in such individuals. The only polymorphism that showed a suggested influence on urinary M1 concentrations was the GST P1 arg→ val substitution at residue 113, where the median urinary concentration of M1 was 73% higher than in workers with the other genotypes at this locus. The difference was not statistically significant, however. Combined analyses that included the GST M1 and T1 genotypes as well as the CYP2E1 G_{-35}→T showed that the THBVal adduct concentrations increased as a function of the number of these polymorphic genotypes that individually increase these adduct concentrations. However, even here, the greatest difference among individual workers with different combined genotypes was a factor of approximately two.

Two relatively small studies examined the influence of GST M1 and T1 polymorphisms on hydrolysis leading to EBD in BD exposed humans. In the study reporting negative results, the THBVal adduct concentrations in 17 BD workers, exposed to a median level of 0.2 ppm BD (range up to ~ 8.0 ppm), showed no relationship with GST M1 or T1 genotype (Begemann *et al.* 2001). In the other, reporting positive findings, N-1-(2,3,4-trihydroxybutyl) adenine (THB A) DNA adduct concentrations and GST M1 and T1 genotypes were analyzed in 15 BD exposed workers (range 0.05 – 8.0 ppm) (Zhao *et al.* 2000, 2001). The mean adduct level was significantly higher in the exposed workers than in a control, unexposed group, and in workers of the M1- (null genotype) compared to the M1+ (non-null genotypes). Fold-differences due to genotypes cannot be determined from these reports, however, because adduct concentrations relative to genotypes are given without corrections for BD exposures levels. (For example, although there is a > 25-fold higher THB A concentration in an individual with the M1-/T1- genotype compared with individuals with the M1+/T1+ genotype, there is only a single worker in the former group, and this worker is an outlier as regards BD exposure, having the highest measured external BD exposure level (8.0 ppm) (Zhao *et al.* 2000). Multiple linear regressions that accounted for BD exposure levels (and other factors) indicated that it was only the M1 genotype that was a significant factor determining adduct concentrations after accounting for BD exposures. It is of note, however, that THB A adduct levels were not positively correlated with chromosome aberration frequencies in this analysis (Zhao *et al.* 2001)

BD metabolic products measured in urine or as hemoglobin adducts have also been measured in three large, multi-institution molecular epidemiological studies, in which

metabolic genotypes were also determined. In an NCI study in China, M1 urine metabolite concentrations and THBVal hemoglobin levels were measured in 41 workers exposed to a median BD concentration of 2.0 ppm (6 h TWA), ranging in the extreme to > 1000 ppm (Hayes *et al.,* 1996, 2000, 2001). Female as well as male workers were included in this study. GST M1 and T1 genotypes were also determined and were reported as having no effect on the metabolic endpoints. There were also no reported differences between the female and male workers.

Two comprehensive molecular epidemiological studies have been conducted in BD exposed workers in The Czech Republic. In the first, including 24 monomer production and 34 polymerization workers, extensive external exposure measurements indicated mean BD exposure levels (8 h TWA) of ~ 0.3 ppm and 0.8 ppms in the monomer and polymerization workers, respectively, with wide ranges in both groups (Albertini *et al.* 2001, 2003). Urinary M1 and M2 metabolites and HBVal and THBVal hemoglobin adducts, as well as CYP2E1 *SaI* and A→T intron 6, EH 113 and 139, GST M1 and T1 and ADH2 and ADH3 genotypes were determined. Genotypes were found to have some significant, but small, influences on the metabolic parameters. Conjugation detoxification, as indicated by the M2/ (M1 + M2) ratio of urinary metabolites, decreased from 0.0143 to 0.009 in workers of the GST M1 null genotype (-/-) compared with workers of the other two genotypes (i.e. +/+ and +/-), a statistically significant difference. A similar decrease in this ratio was seen in workers of the GST T1 null genotype compared to workers with the other two genotypes (from 0.0134 to 0.006), but this difference failed to achieve statistical significance. The M2/(M1 + M2) ratio differences as a function of BD exposure level were seen at all levels of BD exposure. Of note, M2 levels in urine as a function of BD exposure concentrations were also greater in individuals heterozygous for the CYP2E1 intron 6 polymorphism than in individuals homozygous for the wild-type allele, a difference reflected in M2/(M1+M2) ratios as a function of exposure. In these same workers, hemoglobin adducts also showed significant but small, and sometimes inconsistent, associations with polymorphisms of the EH gene. Workers homozygous for the arg139 genotype (high activity allele) had lower HBVal (EB derived) hemoglobin adduct concentrations as a function of BD exposure levels than did individuals of the other two genotypes. However, workers homozygous for the his 139 genotype (low activity allele) also had lower HBVal adduct concentrations, relative to BD exposure levels, than did the heterozygotes (arg139/his139). An unpredicted finding was that workers homozygous for the arg 139 genotype also had significantly lower TBHVal adduct (EBD derived) concentrations as a function of BD exposure levels compared to the other two genotypes, while individuals homozygous his 139 genotype also had lower concentrations of this adduct relative to exposure than did heterozygous workers (arg 139/his 139). Analyses of EH phenotypes (fast, intermediate and slow hydroxylation) based on genotype combinations, as described above, however, showed no influence of hydroxylation status on concentrations of either of the urine metabolites or hemoglobin adducts.

The second Czech molecular epidemiological study included 23 BD exposed female and 30 BD exposed male workers, with some repeats for males that had been included in the first study (Albertini *et al.* 2007). Again, the external exposure assessments were

extensive, revealing mean 8 h TWA BD concentrations of 0.180 and 0.370 ppm for the females and males, respectively. As in the first study, there were wide ranges in the exposure levels. The urinary metabolites and hemoglobin adducts measured in the first study were measured also the second study, with the addition of *pyr*-Val adduct (DEB derived) levels. The same genotypes as assessed in the first study were also measured in the second study. As in the first study, workers with either or both of the GST M1 and T1 null genotypes (-/-) showed lower rates of rise of the urinary M2 metabolite as a function of BD exposure levels. However, in the newer study, it is only the GST T1 null genotype for which this difference is statistically significant. (It was the GST M1 genotype that was significant in the first study). Unlike in the first study, analysis of EH effects using combined genotypes (the different combinations of EH 113 and 139) specifying fast, intermediate and slow hydroxylation, now showed that the rate of rise of urinary M2 production as a function BD exposure was significantly higher in the combinations specifying slow hydroxylation than in the other two combinations, females and males combined. Analyses of HBVal and THBVal hemoglobin adduct concentrations are incomplete for this study at this time, but the important *pyr*-Val adducts were not quantifiable in any of the exposed workers, as indicated above, indicating very low levels of production. This finding was not influenced by any of the metabolic genotypes. Of note, in this study it was found that the females apparently absorbed less BD per unit of exposure than did males, as reflected by lower urinary concentrations of both the M1 and M2 metabolite. However, the M2/(M1+M2) ratio was the same in males and females, reflecting the same relative utilization of the conjugation (producing M2) and hydrolysis (producing M1) detoxification pathways in the two sexes. Conjugation constituted approximately 2 to 4% of detoxification metabolism in this second study, compared to ~ 1% in the first study.

Results of these genotype-metabolism association studies, taken *in toto*, suggest that metabolic genotypes do have some effects on BD metabolism, as reflected by the production of the different metabolites. However, findings of differences related to genotypes are inconsistent. When differences have been shown, they have usually but not always been in the directions predicted by enzyme activities. Furthermore, the differences between genotypes for specific metabolic endpoints have generally been small. Where they could be measured with some confidence, the difference has been by a factor of approximately two – certainly not of the magnitude of the inter-species differences that are seen between mouse and human.

Mutation Induction

The suggestion of metabolic polymorphisms modifying BD's genotoxicity originated from *in vitro* studies. Although such studies are unsatisfactory from a quantitative perspective, as specific metabolites and their exposure concentrations are determined by the experimenter and not the genotype, they can suggest qualitative *susceptibility* differences among individuals. The experimental design used in all of the studies involved incubation of peripheral blood lymphocytes from donors of different genotypes with one of the BD metabolites (EB, EBD or DEB) for a specified time in culture. The most common end-point measured was the sister-chromatid exchange (SCE), which is

not a mutational event *per se*, as it does not change genetic information content. None-the-less, SCEs often reflect the kinds of genotoxic lesions that can result in true mutations at the gene or chromosome level and are, therefore, valid surrogates for such lesions in these studies. Obviously, it is only variability in phase II detoxification metabolism that is assessed by *in* vitro studies as phase I activation metabolism is being bypassed.

Early studies indicated that lymphocytes from human donors homozygous for the GST M1 null allele showed significantly higher percentages of SCEs following short term EB treatments *in vitro* (Uuskula *et al.* 1995). The difference was a 31% increase in SCE's in cells from the null donors. Perhaps because EB is a poor inducer of SCEs *in vitro* (Kligerman *et al.* 2007), most of the *in vitro* studies of BD susceptibility have assessed SCE induction in cells incubated with DEB (Wiencke *et al.* 1995, Pelin *et al.,* 1996, Landi *et al.* 1998, Kligerman *et al,* 1999, 2007). It is of interest that "sensitivity", as reflected by an increase in SCE induction as a function of DEB dose, was associated with the GST T1 genotype; neither the GST M1 (nor GST P1) having an effect. This is in contrast to the findings with EB, noted above. The fold-increase in DEB sensitivity, when it could be determined, ranged from "slight" to a factor of ~2 in GST T1 null cells compared to cells of the other genotypes. The specificity of this sensitivity for DEB, however, might be somewhat open to question as the GST T1 null genotype cells in these studies usually had higher frequencies of *baseline* SCEs, suggesting possibly some mechanisms predisposing to this genotoxic event. GST M1 and T1 polymorphisms were also tested for their ability to confer sensitivity to SCE induction by EBD (Bernardini *et al.* 1996). Neither genotype had an effect.

There are only a few reports of *in vivo* human studies that have suggested genetic sensitivity to mutation induction. Sorsa *et al.* (1996) reanalyzed cytogenetic data from a study of BD exposed workers originally reported as negative (1994, see above), and reported an increase in chromosome aberration and micronuclei frequencies in workers of the GST T1 null, but not the GST M1 genotype. Although statistically significant, the increases were less than a factor of two. There were no genotype effects on SCE induction. Kelsey *et al.* (1995) had earlier reported no differences in SCE induction in workers of the GST T1 genotype exposed to < 2.0 ppm compared to exposed workers of the other two GST T1 genotypes. In a study reported only as a poster, chromosome aberration frequencies were reported to be higher in individuals of the GST M1 and GST T1 null genotypes, and that GST P1 and ADH 3 polymorphisms also had an effect, although there was no increase in frequencies in the overall worker population exposed to BD up to 0.40 ppm (see above) (Warholm *et al.*2003). The fold-increase in the null – individuals was ~ 2.5 or less, where stated.

No other human *in vivo* study has found metabolic genotypes to influence chromosome level mutations in BD exposed workers (references given above). [A study from the Slovak Republic identified a variety of metabolic and DNA-repair polymorphisms and found associations between some and chromosome aberration frequency increases in tire plant workers (Vodika *et al.,* 2004). That study is not considered here because the workers were exposed to a variety of potentially genotoxic xenobiotics in addition to BD]. In fact, the study reported by Zhao *et al.* (2001) actually reported a negative correlation

between chromosome aberration frequencies and the GST null genotype in BD exposed workers. These several negative studies for chromosome aberrations include the large, multi-institution studies (Hayes *et al,* 1996, 2000, 2001, Zhang *et al.* 2004, Albertini *et al.* 2001, 2003, 2007). Among these, the NCI China study assessed aneuploidy as well as structural chromosome aberrations (Hayes *et al.,* 1996, 2000, 2001, Zhang *et al.* 2004). In the Czech studies (Albertini *et al.* 2001, 2003, 2007), in addition to analyses of all genotypes singly, chromosome aberration frequencies relative to BD exposure levels were analyzed as a function of EH fast, intermediate or slow hydroxylation phenotypes. No associations were found (manuscript in preparation).

As for chromosome aberrations, most laboratories have not found associations between genotypes and *HPRT* mutations. The exception again is the Texas group, who reported increases in *HPRT* mutant frequencies associated with BD exposures that correlated with EH genotypes. Three reports describe the associations of different EH polymorphisms, or combinations of polymorphisms, in the same worker cohort, in which the autoradiographic assay had previously assessed mutations (references given above) (Abdel-Rahman *et al.,* 2001, 2003, 2005). In the first analysis, workers who possessed at least one polymorphic EH his 113 allele (genotype his/his, tyr/his) exposed to BD levels of 0.15 ppm or greater, showed an approximately 3-fold in increase mutant frequencies over workers of the tyr/typ genotype (Abdel-Rahman *et al.* 2001). This finding is somewhat unusual in that it suggests that a deficiency allele (his 113) is functioning as a co-dominant, unless some gene dosage effect is operative. There were no effects of GST T1 or M1 polymorphisms, although workers of either the GST T1 or M1 null genotypes, combined with at least a single EH his 113 alleles, showed slightly higher mutant frequencies than did those with his 113 and GST T1 and M1 non-null genotypes. No genotypic effects were observed at BD exposure levels < 0.15 ppm. Somewhat anomalously, however, some controls (without BD exposures), had individual mutant frequency values as high as the "susceptible" BD exposed workers, raising the question of specificity of these findings relative to BD. In the second study, EH exon 4 as well as exon 3 polymorphisms were determined for this same worker group, and mutant frequencies were correlated with EH phenotypes of fast, intermediate and slow (Abdel-Rahman *et al,* 2003). Again, it was found that workers exposed to BD at levels of 0.15 ppm or greater showed mean mutant frequencies that were 3-fold higher for the slow compared to the fast hydroxylators and 2-fold higher for the slow compared to the intermediate hydroxylators. The mutant frequencies by EH hydroxylation status for non-BD exposed individuals are not given in this report. The third study of this series examined the effects of the 5' flanking polymorphisms of EH, again in the same worker cohort (Abdel-Rahman *et al.* 2005). Workers exposed to 0.150 ppm or greater BD and homozygous or heterozygous for the -600 flanking polymorphic variant allele reportedly showed an *HPRT* mutant frequency approximately 3-fold higher than did exposed workers without this variant allele, which was a significant difference. Of interest, workers of these "sensitivity" genotypes not exposed to BD also showed an almost 2-fold increase over the non-exposed worker background mutant frequencies. Workers similarly exposed to BD but homozygous or heterozygous for the -200 flanking polymorphic variant allele showed a similar increase in mutant frequency than did the -600 flanking region variant workers, although, in this case, the increase over workers not

possessing this variant allele was not significant. Mutant frequencies were actually decreased in workers homozygous or heterozygous for EH -399 flanking polymorphism.

In contrast to the findings just described, none of the genotypes, or genotypic combinations, have been found to influence gene mutations frequencies at either the *HPRT* or *GPA* locus in the large multi-institutional studies described above. *HPRT* mutations were assessed by both the conventional cloning assay and by the autoradiographic assay in the 1st Czech study, and EH genotypic effects have been analyzed both by studying gene effects singly and by combining EH genotypes into fast, intermediate and slow hydroxylation status. Including controls, each of these large studies included more subjects than were in the Texas cohort just described. A total of 266 workers have been included in the China and Czech studies, combined, with some repeats between Czech 1 and 2. Multi-racial groups have been investigated. It seems most probable that, if metabolic genotypes really influenced BD induced gene mutations, some evidence would have been found.

Genotypic effects on BD induced true mutations at either the chromosome or gene level have been inconsistently shown, at best, in humans. Most studies have failed to find such effects. Those that have, taken at face value, have reported either chromosome aberrations or gene mutations increased in the range of 2- to 3.5-fold, or frequently less. Again, the *in vivo* gene mutation effects have been reported from a single laboratory, and have not been replicated. However, for estimating a "worst-case scenario", this fold-increase in genotoxic sensitivity might be taken as an upper limit. This can be compared with the upper-limit of a 2-fold increase in sensitivity for sensitive genotypes for metabolic endpoints, as described above. Neither of these, of course, approaches the inter-species differences in sensitivity that have been demonstrated between mice and humans.

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