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2,6-and 3,5-dimethylaniline-induced mutagenesis in Chinese hamster ovary cells expressing human cytochrome P450 1A2 and sulfotransferase

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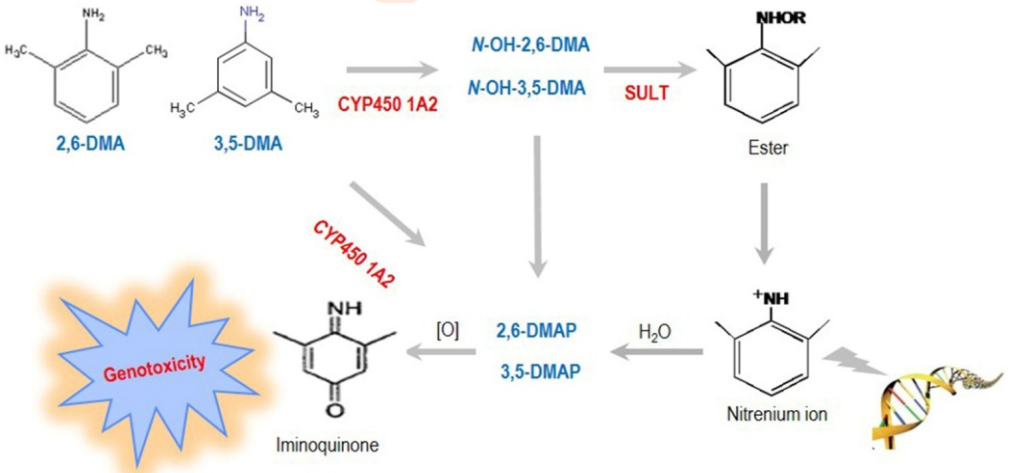
Abstract

Aim: The aim of this study was to test the hypothesis that human cytochrome P450 1A2 (CYP1A2) and sulfotransferase (SULT) contribute to the phase I and II bioactivation of 2,6-dimethylaniline (2,6-DMA) and 3,5-dimethylaniline (3,5-DMA) in affecting the incidence of genotoxicity.

Methodology: 5P3H1 cells carrying cytochrome P450 1A2 (CYP1A2) and SULT cells were treated with various concentrations of 2,6-and 3,5-DMA for 48 hr or their N-hydroxyl and aminophenol metabolites for 1 hr in the absence or presence of 2,6-Dichloro-4-nitrophenol (DCNP). Cell lethality was assayed by trypan blue exclusion and induced mutagenesis of adenine phosphoribosyl transferase (*aprt*) gene was also evaluated.



Results: A significant dose-dependent increase in cytotoxicity and mutant fraction was observed after treatment with 2,6- and 3,5-DMA, and their metabolites; N-hydroxy and aminophenol metabolites are more potent than the parent compounds. Addition of sulfotransferase inhibitor DCNP decreased the cytotoxic and mutagenic effects of 2,6- and 3,5-DMA, and their metabolites in a dose-dependent manner.

Interpretation: This research indicate that 2,6 and 3,5-DMA are mutagenic, and their toxicity in model systems depend on metabolic activation. This activation is mediated by CYP1A2 and SULT enzymes.

Key words: Chinese hamster, Cytochrome, Dimethylaniline, Ovary cells, Sulfotransferase

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Introduction

Humans can be exposed to dimethylaniline (DMA) via various sources like tobacco smoke, some pesticides, anaesthetics and permanent hair dye. The carcinogenic potential of some DMAs has been previously reported, and there is sufficient evidence for the carcinogenicity of 2,6-DMA in experimental animals (Haseman and Hailey, 1997). Moreover, two epidemiologic studies conducted with DMA isomers showed that levels of haemoglobin adducts of 2,6- and 3,5-DMA in peripheral blood samples are significantly associated with an increased risk of bladder cancer in humans (Gan *et al.,* 2004; Tao *et al.,* 2013). However, limited efforts have been made to study the genotoxic effect of 2,6- and 3,5-DMA, and their metabolites. In bacterial reverse mutation assays and *in vitro* chromosomal aberration tests, positive results were for 2,6- and 3,5-DMA with the addition of metabolic activation system (rat liver S9 mix) (Zeiger *et al.,* 1988; Kohara *et al.,* 2018). In previous studies, induction of gene mutations by 2,6- and 3,5-DMA and their metabolites has been reported in the *gpt* of AS52 (Chao *et al.,* 2012) and, *HPRT* and *TK1* genes of TK6 (Chao *et al.,* 2012) cells with human S9.

Metabolism of chemical carcinogens plays a principal role in the aetiology of cancer (Gonzalez andKorzekwa, 1994). A better understanding of the properties of carcinogen metabolizing enzymes will, thus, aid in studies of human risk assessment. Arylamines require metabolic activation, usually in the liver, to be transformed into fully carcinogenic agents (Hecht, 2003). They are oxidized to N-hydroxylamines *in vivo* and react with hemoglobin to form adducts that may persist as long as the hemoglobin adducts remains in circulation (Skipper *et al.,* 2010). Many heterocyclic amines, formed in meat during pyrolysis, are activated by cytochrome P450 1A2 (CYP1A2)-catalyzed N-hydroxylation, including 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (Guengerich, 2006). For xenobiotics such as N-hydroxy arylamines, N-hydroxy heterocyclic amines, and hydroxymethyl polycyclic aromatic hydrocarbons, sulfonation by sulfotransferase (SULT) is a metabolic activation process leading to highly reactive electrophiles that are both mutagenic and carcinogenic (Gamage *et al.,* 2006).

Materials and Methods

Cell cultures: 5P3H1 cells were received as a generous gift from Dr. Gerald N. Wogan (Massachusetts Institute of Technology, Cambridge, MA, USA), derived from CHO cell line AA8, functionally heterozygous at the aprt locus (Wu *et al.,* 2000; Wu *et al.,* 2003). The 5P3H1 cell line carries the mouse CYP1A2 and a human aryl sulfotransferase HAST1 isolated from human liver. Details concerning the construction and characterization of these cell lines has been described previously (Wu *et al.,* 2003; Wu *et al.,* 1997). Prior to each experiment, cells were incubated for 2 days in a medium containing CAAT (10 µM cytidine, 100 µM adenine, 1 µM aminopterin and 17.5 µM thymidine) and 2-5 days in TAC medium (thymidine, adenine and cytidine) to reduce the background *aprt*$^{-}$ mutant fraction (Fujimori *et al.,* 1992). All cells were routinely maintained by monolayer culture in α-minimal essential medium containing L-glutamine supplemented with penicillin (100 unitsml^{-1}), streptomycin (100 µgml^{-1}) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in a humidified atmosphere with 5% CO_2 at 37 ºC. All cell culture reagents were purchased from Lonza (Walkersville, MD).

Cell viability and aprt mutation assay: N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA were synthesized as described previously (Chao *et al.,* 2012). 2,6- and 3,5-DMA were purchased from Sigma Chemical (St Louis, MO, USA) and Acros Organics (Geel, Belgium). 2,6- and 3,5-DMA, and their metabolites dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) was added to exponentially growing cells in 100 mm tissue culture dish containing 0.5×10^6 cells in 10 ml of media. 5P3H1 cells were exposed to 0, 5, 10, 25 and 50 µM of parent compounds for 48 hr or N-hydroxy and aminophenol metabolites for 1 hr in the absence or presence of 0.2 mM 2,6-Dichloro-4-nitrophenol (DCNP, Cole-Parmer, Illinois, USA). A selective SULT inhibitor DCNP was further used to inhibit the sulfation pathway for bioactivation of 2,6- and 3,5-DMA, and their metabolites. In the preliminary experiment, 0.2 mM DCNP itself did not have any cytotoxic and mutagenic effect on the sulfotransferase cells (data not shown).

The control culture was treated with the same volume of DMSO for 48 hr. The final concentration of DMSO to which cells were exposed was less than 0.1%. Test concentrations of 2,6- and 3,5-DMA, and their metabolites were established by MTT assay (data not shown). Following treatment, the cells were rinsed twice with PBS and removed with trypsin. Cells were allowed to recover for 24 hr before determining survival by trypan blue exclusion, and maintained in regular medium for phenotypic expression. At the end of 3 days phenotypic expression, cultures were plated for cloning efficiency and mutation. Total 6×10^5 cells from each group were placed in 100 ml selective medium containing 80 µgm l^{-1} of 8-AA and plated at 600000 cells 10 ml^{-1} 100 mm dish^{-1} (ten replicates) for determining mutagenicity after 14 days. In addition, each mutant was rescreened in selective media in order to confirm the stability of mutant phenotype. Cloning efficiency dishes were seeded with 200 cells 10 ml^{-1} 100 mm dish^{-1} in triplicate and allowed to grow until colonies were visible for 8 days in the absence of selecting agent. Mutation fraction was calculated as ratio of mean cloning efficiency in selective medium to that in non-selective medium. In this study, cells treated with 0.2 µM PhIP (Toronto Research Chemicals, Ontario, Canada) for 48 hr served as a positive control.

Statistical analysis: All experiments were repeated three times. Two-tailed Student's *t*-test (SPSS for Windows, 12.0, SPSS Inc. Chicago, IL, USA) write clearly, and *p* value less than 0.05 were considered significant.

Results and Discussion

2,6-DMA and its hydroxy and aminophenol metabolites were evaluated with 5P3H1 cells to determine their ability to produce cell killing and mutations with/without co-treatment of

sulfotransferase inhibitor DCNP. Hemizygous mutant CHO aprt gene was used as a target locus for mutation assay. *aprt* is constitutively expressed "housekeeping" gene that codes for a purine salvage-pathway enzyme (Bollée *et al.,* 2010). The small size of Chinese hamster *aprt* gene, its convenient distribution of restriction sites, and absence of *aprt* pseudogenes in CHO cells greatly facilitate molecular analysis of *aprt* recombinants (Boll'ee *et al.,* 2010). As shown in Table1, one or forty eight hour treatment of 5P3H1 cells with 2,6-DMA, N-OH-2,6-DMA and 2,6-DMAP resulted in an approximately linear dose-dependent increase in MF at the *aprt* locus, to a maximum for 10 µM of 4.29×10^{-5} for 2,6-DMA or 13.1×10^{-5} for N-OH-2,6-DMA or 16.87×10^{-5} for 2,6-DMAP, respectively, as compared to a spontaneously fraction of 3.34×10^{-5} accompanied with dose-dependent decrease in cell survival (Fig. 1). Cell survival and MF induced by 2,6-DMA and its metabolites were affected by sulfotransferase activity. DCNP is a potent selective inhibitor for human phenol sulfotransferases (Wu *et al.,* 2000). Addition of DCNP caused an increase cell growth of 4- and 6-fold for 50 µM N-OH-2,6-DMA and 2,6-DMAP respectively (Fig. 1), and a decrease in MF by 3.1- and 4.3-fold for 10 µM N-OH-2,6-DMA and 2,6-DMA compared with values obtained without DCNP (Fig. 1).

Sulfonation is an important reaction in the metabolism of numerous xenobiotics, drugs, and endogenous compounds. Sulfonation has been shown to be important in the activation of a range of compounds such as aminoazo dyes, benzidines, heterocyclic amines, hydroxymethyl polycyclic aromatic hydrocarbons, terpenes, b-aminoethyl alcohols, and 2-nitropropane (Gamage *et al.,* 2006). In consistent with our results, these studies have shown that sulfotransferases are very selective in their activation of promutagens(Gamage *et al.,* 2006). 5P3H1 cell line also showed dose-dependent cytotoxicity and *aprt* mutants induced by 3,5-DMA and its hydroxy and aminophenol metabolites (Fig. 2, Table 2). At 10 µM 3,5-DMA, N-OH-3,5-DMA and 3,5-DMAP, induced MFs in the aprt genes were 3.71×10^{-5}, 17.50×10^{-5} and 29.90×10^{-5}, 1.1-, 5.2- and 9-fold higher than background (3.34×10^{-5}) (Table2). Like results of 2,6-DMA and its metabolites, cytotoxicity and MF induced by 3,5-DMA and its metabolites

Table 1: Mutation fraction after treatment with 2,6-DMA for 48 hr or its metabolites for 1 hr in 5P3H1 and AA8 cells

		Mutation fraction ($\times 10^{-5}$)			
Cells	**Groups**	**5 µM**	**10 µM**	**50 µM**	**100 µM**
5P3H1	2,6-DMA	4.42±0.345	4.29±0.317		
	2,6-DMA+DCNP	3.42±0.249*	3.52±0.462*		
	N-OH-2,6-DMA	8.12±1.074	13.10±1.819		
	N-OH-2,6-DMA+DCNP	4.08±0.197*	4.16±0.807*		
	2,6-DMAP	4.65±0.818	16.87±1.610		
	2,6-DMAP+DCNP	3.34±0.609*	3.95±0.577*		
AA8	2,6-DMA			3.11±0.770	3.13±3127
	N-OH-2,6-DMA			3.02±0.643	3.21±0.584
	2,6-DMAP			3.07±0.114	3.16±0.338

Each values is expressed as mean of three replicate±standard deviation. Spontaneous MF was 3.34±0.268 (5P3H1) and 2.90±0.271 (AA8) $\times 10^{-5}$.*$p <$ 0.05 compared to 2, 6-DMA and its metabolites without DCNP (5P3H1)

Table 2: Mutation fraction after treatment with 3,5-DMA for 48 hr or its metabolites for 1 hr in 5P3H1 and AA8 cells

		Mutation fraction ($\times 10^{-5}$)			
Cells	**Groups**	**5 µM**	**10 µM**	**50 µM**	**100 µM**
5P3H1	3,5-DMA	3.65±0.463	3.71±0.238		
	3,5-DMA+DCNP	3.73±0.625	4.18±0.441*		
	N-OH-3,5-DMA	9.06±2.450	17.50±3.092		
	N-OH-3,5-DMA+DCNP	5.29±1.009*	6.18±0.493*		
	3,5-DMAP	15.30±1.310	29.90±4.681		
	3,5-DMAP+DCNP	4.49±0.303*	8.94±0.640*		
AA8	3,5-DMA			3.33±0.521	3.40±0.514
	N-OH-3,5-DMA			3.01±0.362	3.22±0.263
	3,5-DMAP			3.04±0.247	3.41±0.246

Each values is expressed as mean of three replicate±standard deviation. Spontaneous MF was 3.34±0.268 (5P3H1) and 2.90±0.271 (AA8) $\times 10^{-5}$.*$p <$ 0.05 compared to 3,5-DMA and its metabolites without DCNP (5P3H1)

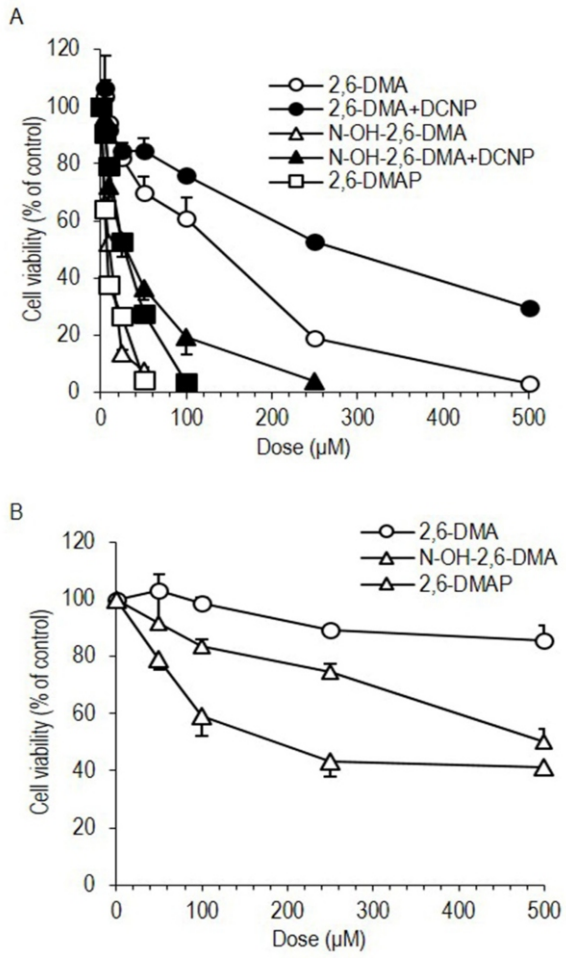

Fig. 1: Dose-dependent of cell survival after treatment with 2,6-DMA for 48 hr or its metabolites for 1 hr in 5P3H1 (A) and Aa8 (B) cells. Survival was determined by trypan blue assay 24 hr after treatment. Data represent mean of three replicates ± S.D.

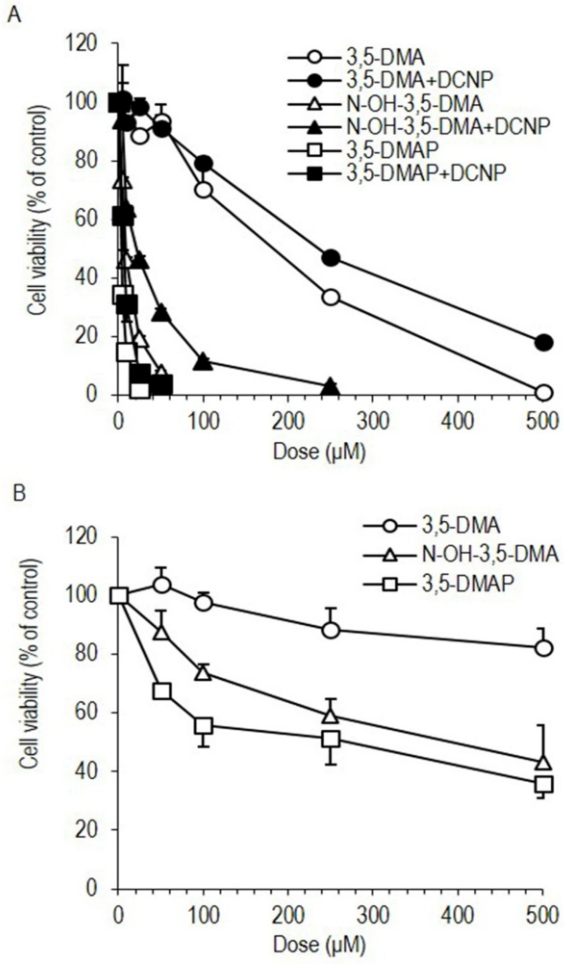

Fig. 2: Dose-dependent of cell survival after treatment with 3,5-DMA for 48 hr or its metabolites for 1 hr in 5P3H1 (A) and AA8 (B) cells. Survival was determined by trypan blue assay 24 hr after treatment. Data represent mean of three replicates ± S.D.

were sharply decreased following co-treatment with DCNP (Fig. 2, Table 2). The mutant factor in the positive control 5P3H1 cells treated with 0.2 μM PhIP for 48 hr was 77.3×10^{-5}.

A number of approaches are available to assess the role of drug and xenobiotic metabolizing enzymes in activation and/or inactivation of chemicals that might act as potential carcinogens (García-Suástegui *et al.,* 2017; Duvai *et al.,* 2016). Heterologous expression of human enzymes in a cell system used for mutagenicity testing has the advantage that metabolites are generated inside the target cell and, thus, enable study of the role of metabolizing enzymes in bioactivation of chemicals (García-Suástegui *et al.,* 2017; Duvai *et al.,* 2016). In this study, we used CHO AA8-derived 5P3H1 cells, which was able to express active CYP1A2 and SULT. With these cells we were able to detect bioactivation of 2,6- and 3,5-DMA. 5P3H1 cell line was more sensitive to the genotoxic effect of these two monocyclic amines and their metabolites relative to parental AA8 line. Exposure to 10 μM of 2,6-DMAP, reduced viability and increased MF in 5P3H1 to 37.5% and 16.87×10^{-5}, respectively, whereas comparable values after treatment with 50 μM of 2,6-DMAP in AA8 were 79.1% and 3.07×10^{-5} (Table 1, Fig. 1). In addition, cell viability and mutation fraction were 14.8% and 29.9×10^{-5} in 5P3H1 cells after a dose of 10 μM 3,5-DMAP, compared to 67.4% and 3.04×10^{-5} after treatment with 50 μM 3,5-DMAP in AA8 cells (Table 2, Fig. 2). It is clear from the data that 2,6- and 3,5-DMA is more genotoxic following oxidative metabolism catalyzed by CYP1A2 and SULT.

In this study, we employed Chinese hamster ovary (CHO)-derived 5P3H1 cell line in which both CYP1A2 and SULT were introduced for determining the role of these enzymes in metabolic activation and genotoxicity of two monocyclic amines, 2,6- and 3,5-DMA. Based on their heterozygosity at the adenine phosphoribosyltransferase (*aprt*) locus, they can be used for

mutational analysis based on selection with 8-azaadenine (8-AA).

The present study indicates that 2,6- and 3,5-DMA investigated appear to have significant genotoxic potential. The evidence also suggests that CYP1A2 and SULT were effective enzymes leading to bioactivation of 2,6- and 3,5-DMA, it strongly enhanced the cytotoxicity and mutagenicity via formation of hydroxyl and aminophenol metabolites of 2,6- and 3,5-DMA.

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