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Research Article

METABOLIC ACTIVATION OF 2,6-DIMETHYLANILINE: MUTATIONAL SPECIFICITY IN THE GPT GENE OF AS52 CELLS

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ABSTRACT

Objective: The purpose of the current work was to characterize the mechanisms of cytotoxicity and mutagenesis of a potential human bladder carcinogen 2,6-dimethylaniline (2,6-DMA).

Methods: Chinese hamster ovary (CHO) AS52 cells were exposed to either human S9 activated 2,6-DMA for 6 h or its *N*-hydroxylamine and aminophenol metabolites for 1 h in serum-free medium. Cell survival was determined by trypan blue exclusion 24 h after treatment, and 6-thioguanine-resistant mutants at the xanthine-guanine phosphoribosyl transferase (*gpt*) gene locus were assessed with doses, of which relative survival is 30% or more. Nested polymerase chain reaction-based deletion analysis was also performed.

Results: AS52 cells exhibited a dose-dependent increase in cytotoxicity and mutant fraction on treatment of 2,6-DMA and its metabolites but show a considerable variation in potency with aminophenol metabolites having the highest potency and parent compound at least; at the highest 2,6-dimethylaminophenol dose (10 μM), the mutant fraction in AS52 cells was 8-fold (13.2×10^{-5}) greater than the spontaneous fraction of 1.62×10^{-5}. Total deletion of the *gpt* gene sequences was found in 1 (4%) spontaneous and 2 (6%) the 6-thioguanine mutants generated by *N*-hydroxy-2,6-DMA.

Conclusions: These findings indicate the mutagenicity of 2,6-DMA at the *gpt* gene, which is mediated through hydroxylamine and aminophenol metabolites, and contribute to the elucidation of mechanisms through which 2,6-DMA may exert its effects *in vivo*.

Keywords: 2,6-Dimethylaniline, Metabolic activation, Genotoxicity, AS52/guanine phosphoribosyl transferase assay.

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INTRODUCTION

2,6-Dimethylaniline (2,6-DMA), a well-known alkylanilines, is used as a chemical intermediate in the manufacture of pesticides, dyestuffs, antioxidants, pharmaceuticals, synthetic resins, fragrances, and other products [1,2]. Exposure to alkylaniline not only has been well documented in cigarette smokers [3] but also may occur from non-smoking-related sources [4]. Smoking is associated with increased rates of bladder cancer [5]. Alkylanilines are believed to be the constituents of tobacco smoke that leads to the development of bladder cancer [6,7]. Several members of alkylanilines including 4-aminobiphenyl, 2-naphthylamine, and benzidine, which are extensive, have been classified and regulated as human bladder carcinogens [8]. Other alkylaniline, including 2,6-DMA, have been implicated as possible human bladder carcinogens [6,9-12].

Genotoxicity of alkylanilines has received very limited investigation to date, and the fragmented available database supports only limited conclusions. Alkylaniline is metabolized to reactive alkylhydroxylamine which can undergo further bioactivation and conjugation reactions [13-15]. These reactions include those catalyzed by cytochrome P450-dependent monooxygenases and other enzymes; the free reactive hydroxylamine may be regenerated from the conjugated form in an acidic environment [13-15]. Possible metabolic pathways leading to the formation of genotoxic DMA damage products of 2,6-DMA are shown in Fig. 1. Recent results described here indicate that alkylaniline scan be potent genotoxins for cultured mammalian cells when activated by exogenous or endogenous Phases I and II xenobiotic-metabolizing enzymes [6,7,10-12,15]. However, there is little information available about mutagenicity of 2,6-DMA and a detailed metabolic profile of 2,6-DMA. Moreover, it is not clear whether 2,6-DMA can induce the deletions and other gross structural changes in other types of cells. Therefore, there is of considerable interest to examine the toxic effects of 2,6-DMA in the reportedly more sensitive *in vitro* system for mutation assay.

For this reason, we have introduced a Chinese hamster ovary (CHO) AS52 cell line that carries a single copy of *Escherichia coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene functionally expressed using SV40 early promoter and stably integrated into the AS52 cell genome [16]. AS52 cells were constructed by transfecting the plasmid vector pSV *gpt* into a normal X-linked mammalian *HPRT*-deficient CHO cells. The AS52 cell line has been demonstrated to be useful with regard to the detection of both deletion and point mutations at *gpt*, and the low spontaneous mutation fraction (MF) and the distinct advantage of carrying a small easily manipulated mutational target make the AS52 cell line particularly suited to quantitative and molecular mutagenesis studies. The *gpt* structural gene is 456 base pairs (bp) with no introns versus a structural gene size of 654 bp for *HPRT*, which with introns comprises a genomic *HPRT* locus of approximately 35 kb [17]. Thus, AS52 cells are sensitive to induced mutagenesis by a variety of clastogens and radiomimetic agents that are often classified as nonmutagens in other assays [17]. The purpose of the present study was to characterize the mutational profile and mechanism induced by 2,6-DMA in the *gpt* gene of AS52cells. We have tested the hypothesis that toxic effects of 2,6-DMA are mediated through its hydroxylamine and/or aminophenol metabolite.

METHODS

Chemicals and reagents

All cell culture reagents were purchased from Lonza (Walkersville, MD). 2,6-DMA, 6-thioguanine (6-TG), dimethyl sulfoxide (DMSO), ethyl

methanesulfonate (EMS), and DL-isocitric acid were purchased from Sigma Chemical Co., St Louis, MO. 3,5-DMA and NADP were obtained from Acros Organics (Geel, Belgium) and Boehringer Mannheim (Indianapolis, IN), respectively.

Cell cultures

CHO AS52 cells, kindly provided by Dr. Gerald N. Wogan (Massachusetts Institute of Technology, Cambridge, MA, USA), were cultured in Ham's F-12 medium supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in a humidified atmosphere with 5% CO_2 at 37°C. Cells were cleansed of preexisting *gpt* mutants by culturing in MPA medium (10 µg/mL MPA, 250 µg/mL xanthine, 22 µg/mL adenine, 11 µg/mL thymidine, and 1.2 µg/mL aminopterin) for 7 days and transferred for 3 days to medium enriched with xanthine (11.5 µg/mL), adenine (3 µg/mL), and thymidine (1.2 µg/mL) for recovery.

2,6-DMA treatment

Cells were placed in 6-well plates at a density of 0.5×10^6 cells per well the day before treatment. Cultures were, just before treatment, washed with phosphate-buffered saline (PBS) and refed with serum-free medium containing human liver S9 mix (Moltox Inc., Boone, NC). Approximately 440 µg S9 protein (16 µL fraction) and 65 µL sterile-filtered core mixture contained 25 mg/mL NADP and 45 mg/mL DL-isocitric acid were added per ml of serum-free medium. Cells were exposed to 25, 50, 100, 250, 500, and 1000 µM of 2,6-DMA dissolved in DMSO for 5 h at 37°C, after which test compounds containing medium were removed and replated with fresh medium supplemented with 10% FBS. The final concentration of DMSO to which cells µL were exposed was <0.1%.

Metabolite treatment

N-hydroxy and aminophenol metabolites of 2,6-DMA were synthesized as described previously [12]. The cells were seeded at 1×10^6 and incubated overnight with the regular medium. Then, medium changed to one without serum and cells were treated in triplicate with 5, 10, 25, 50, 100, and 250 µM of *N*-hydroxy or aminophenol metabolites. After 1 h treatment, the culture was washed with PBS, amended with Ham's F-12 medium containing 10% FBS, and incubated.

Measurement of survival and *gpt* mutations

Cytotoxicity was determined by trypan blue exclusion assay 24 h after treatment, and the cells were maintained for full expression of the mutant phenotype. 7 days after treatment, 5×10^5 cells from each

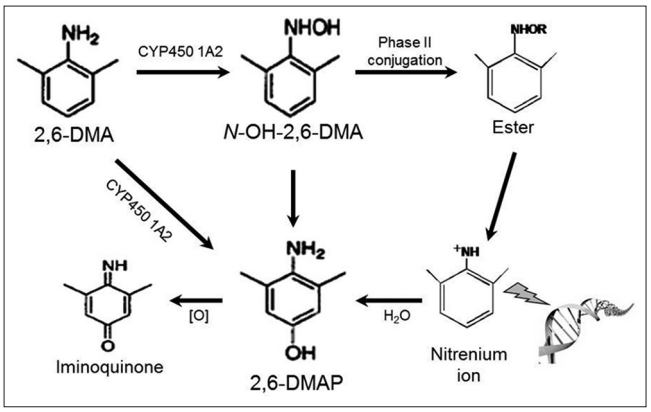

Fig. 1: Schematic representation of the presumed metabolic pathway for 2,6-dimethylaniline, Information was taken from Gan *et al*

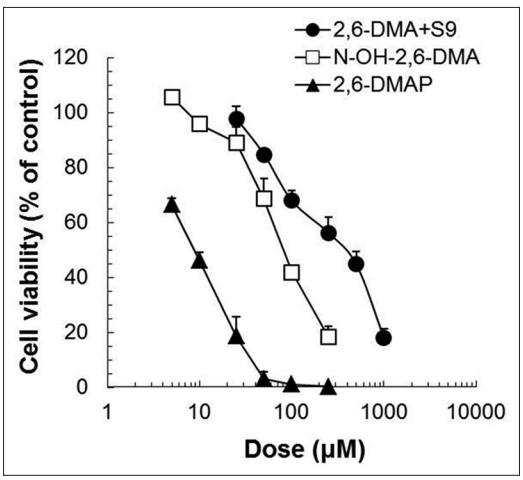

Fig. 2: Dose dependent of cell survival after treatment with 2,6-dimethylaniline, and its *N*-hydroxy and aminophenol metabolites in AS52 cells, Survival was determined by trypan blue assay 24 h after treatment, data represent mean ± standard deviation for three measurements

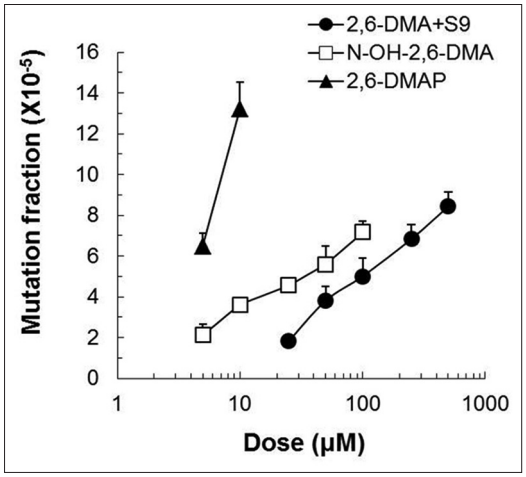

Fig. 3: Induced mutation fraction in AS52 cells treated with 2,6-dimethylaniline, and its *N*-hydroxy and aminophenol metabolites, data represent mean ± standard deviation for three measurements

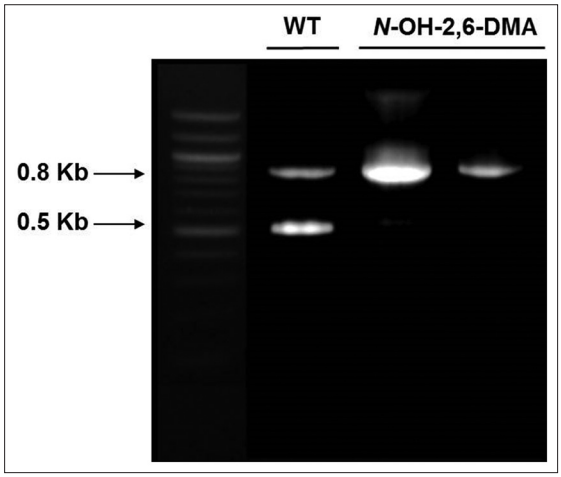

Fig. 4: Nested polymerase chain reaction (PCR) amplication of the *gpt* gene in AS52 cells. Two prominent bands at 0.5 (*gpt* structural gene) and 0.8 kb (rearranged pSV *gpt* gene) are observed in wild-type AS52 cells (WT), mutants generated by *N*-OH-2,6-DMA, rearranged pSV *gpt* band are present, but the PCR product for the structural gene is missing; 1kb ladders are shown in left lane

Table 1: PCR analysis of *gpt* in AS52 Cells after treatment with 2,6-DMA and its metabolites

	Number of mutants (%)			
	Spontaneous	2,6-DMA	*N*-OH-2,6-DMA	2,6-DMAP
No observable alteration[a]	23 (96)	35 (100)	33 (94)	34 (100)
Large deletions/rearrangements[b]	1 (4)	0 (0)	2 (6)	0 (0)
Total of mutants analyzed	24 (100)	35 (100)	35 (100)	34 (100)

[a]No alterations were observable in the migration of the primary PCR products of the structural *gpt* gene on an agarose gel as compared to the AS52 wild-type control, [b]Large deletions or rearrangements resulting in the absence of PCR amplified *gpt* sequences (complete deletions [0.5+0.8 kb] and deletions of the 0.5 kb with normal 0.8 kb product), PCR: Polymerase chain reaction

group was placed in 100 mL of selection medium containing 10 μM 6-TG and plated at 50,000 cells/10 mL/100 mm dish (10 dishes per group) for the determination of mutagenicity. Simultaneously with 6-TG selection, cells were seeded for the determination of plating efficiency (200 cells/10 mL/100 mm dish; 5 dishes per group). Colonies were scored after incubation for 14 days, and mutant fraction was calculated as the number of 6-TG mutants. The MF was calculated by dividing the number of mutants by the number of cells seeded and the plating efficiency. In one of the experiments, exposure to 8 mMEMS for 2 h was used as a positive control for mutation induction. Identified single *gpt* mutant colony was then grown to approximately 2×10^6 mutant cells for molecular analysis.

DNA isolation and polymerase chain reaction (PCR) deletion screening

Identified single *gpt* mutant colony was transferred to 24-well plates for propagating mutant cells. Approximately 2×10^6 mutant cells were collected for molecular analysis. Genomic DNA was extracted from each mutant using a GenElute™ mammalian genomic DNA miniprep kit (Sigma). Amplification of the gDNA was performed in two rounds of nested PCR in PTC-200 DNA Engine Thermal Cycler (Bio-Rad, Hercules, CA). About 1 μg of template DNA was transferred into the first round mix of 10 μL 10× PCR buffer, 2 μL dNTP mix, 0.5 μL taq polymerase, 73.5 μL high-performance liquid chromatography water, 0.2 μL each 25 mM forward (bases - 199 to - 181; 5'-AAGCTTGGACACAAGACAG-3') and reverse (bases 520 to 540; 5'-CCAGAATACTTACTGGAAAC-3') primer and amplified with a PCR profile of 94°C: 1 min, 30 cycles of 94°C: 1 min, 47°C: 1 min, 72°C: 1 min and a final extension of 72°C for 7 min. The product from this reaction was filtered using Centricon 50 concentrator (Amicon, Beverly, MA) and resuspended in 100 μL sterile water to avoid unspecific binding with remaining primers, and 10 μL aliquot was used as template in the second round of PCR using nested primers (bases -23 to -4; 5'-ATAAACAGGCTGGGACACTT-3' and bases 460 to 470; 5'-AGTGCCAGGCGTTGAAAAGA-3'). The PCR conditions were the same in the second round as those in the first round reaction, except annealing temperature (52°C). A quantity of *gpt* gene amplification was analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Survival and MFs

Alkylaniline toxicity is characterized by methemoglobin formation and by hyperplasia and siderosis of the spleen [18]. The compound is nonmutagenic in the standard *Salmonella typhimurium/microsome* test [19]. It is positive, however, in inducing DNA damage in the liver and kidney of rats and in inducing sister chromatid exchanges *in vivo* in mice [20]. Alkylaniline, at a high concentration, induces minimal increases in sister chromatid exchanges *in vitro* [21] and forms DNA adducts [6,11]. Mutagenic activity of a chemical is a function of both the capacity to adduct DNA and the capacity of the cell to respond to the damage. DNA adducts formed from distinct alkylanilines have already been shown to be differentially mutagenic [22].

In the present study, all test compounds reduced the cell survival, whereas they increased the proportion of cells carrying the mutated *gpt* gene in a dose-dependent manner (Figs. 2 and 3). The survival of the test cell lines following 2,6-dimethyaminophenol (2,6-DMAP) treatment was lower than that observed in the corresponding *N*-hydroxy metabolite and parent compound; for instance, cell viability was 3% in AS52 cells 24 h after a dose of 50 μM 2,6-DMAP, compared to 69% and 85% after treatment with *N*-hydroxy-2,6-DMA (*N*-OH-2,6-DMA) and 2,6-DMA, respectively (Fig. 2).

Previous studies showed that, if the relative survival rate is 30% or more after chemical exposure, there will be sufficient live cells to determine mutation responses [23,24]. Thus, based on the results of cytotoxicity experiment, three cultures with those concentration ranges for 2,6-DMA (25–500 μM), *N*-OH-2,6-DMA (5–100 μM), and 2,6-DMAP (5 and 10 μM) were used to determine mutagenic responses (Fig. 3). When cells were treated with 100 μM of 2,6-DMA and *N*-OH-2,6-DMA, MF was 5.01×10^{-5} and 7.17×10^{-5}, 3- and 4.5-fold the spontaneous MF (1.61×10^{-5}), respectively. On the other hand, 5 μM 2,6-DMAP, which is 20 times lower concentration, enhanced the MF from 1.61×10^{-5} to 6.48×10^{-5} (Fig. 3). By comparison, in EMS-treated positive controls, MF at the *gpt* locus was 72.7×10^{-5}.

Our results correlated well with that obtained by previous studies showing both phase I and phase II metabolism are required for genotoxic-specific activation of 2,6-DMA. Nohmi *et al.* reported the presence of the mutagenic *N*-hydroxylated metabolite of 2,4-DMA in an *in vitro* rat liver microsomal system (S9) in which the yield of the mutagenic metabolite increased with increasing S9 content [25]. Beland *et al.* reported the mutagenicity of the *N*-hydroxylated metabolite of 2,6-DMA toward *S. typhimurium* (TA100) [26].

Deletion screening of AS52*gpt* mutants

Thirty-five independent 500 μM 2,6-DMA and 100 μM *N*-OH-2,6-DMA, 34 independent 10 μM 2,6-DMAP, and 24 independent spontaneous 6-TG-resistant mutants in AS52 cells were isolated and analyzed by the nested PCR, to test for the presence or absence of the *gpt* structural gene (0.5 kb PCR product) and the closely linked rearrangement product of this gene (0.8 kb PCR product). Those 6-TG-resistant mutants with both PCR sequences intact are mostly likely to carry point mutations in the *gpt* structural gene. Loss or alteration of one or both bands suggests more complex chromosomal deletions [27] and/or rearrangements involving *gpt* [28,29].

Screening results of the *gpt* gene deletions showed that the percentage of deletion mutations in the spontaneous *gpt* mutants was 4% (1 of 24): That of the 100 μM*N*-OH-2,6-DMA-enhanced mutants was 6% (2 out of 35) (Table 1 and Fig. 4). The mechanisms for the gene deletion are complicated and may involve different pathways such as DNA strand breakage, error in DNA damage repair, and other kinds of DNA damages [30]. Deletion of the *gpt* genein *N*-OH-2,6-DMA-enhanced mutants may be result from DNA damage caused by high cytotoxicity of the chemical.

CONCLUSION

The results reveal that 2,6-DMA is mutagenic and genotoxic in *gpt* gene of AS52 cells when activated by Phases I and II metabolism, in which the aminophenol metabolites were considerably more potent than the corresponding *N*-hydroxylamines. Further study is also needed to investigate the mechanism of genotoxic activity of 2,6-DMA.

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AUTHORS' CONTRIBUTION

All authors had equally contributed the research work.

CONFLICTS OF INTEREST

We declare that there is no conflict of interest.

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