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Research Article

GENOTOXICITY OF N-HYDROXY AND AMINOPHENOL METABOLITES OF 2,6- AND 3,5-DIMETHYLANILINE AT THE HYPOXANTHINEGUANINE PHOSPHORIBOSYLTRANSFERASE LOCUS IN TK6 CELLS

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ABSTRACT

Objective: The objective of this study as to characterize the genotoxicity of reactive metabolites of 2,6-dimethylaniline (2,6-DMA) and 3,5-DMA in the hypoxanthineguanine phosphoribosyltransferase (*HPRT*) gene of human lymphoblastoid TK6 cells.

Methods: Cultures were exposed to *N*-hydroxylamine and aminophenol metabolites of 2,6- and 3,5-DMA for 1 h in serum-free medium. Cell survival 24 h after exposure was determined by trypan blue exclusion. Cells were then subcultured for 7–10 days to allow to phenotypic expression of *HPRT* mutants. After the expression period, cells were plated in the presence of 2 μg/ml 6-thioguanine for the selection of *HPRT* mutants. Plating efficiency was determined and mutant fraction calculated. Electron paramagnetic resonance (EPR) was also used to determine whether 3,5- dimethylaminophenol (DMAP) produced reactive oxygen species (ROS).

Results: All of the metabolites tested were cytotoxic to these cells but exhibited a considerable variation in potency. The aminophenol metabolites of 2,6- and 3,5-DMA were considerably more toxic than the corresponding *N*-hydroxylamines. Furthermore, each metabolite of 3,5-DMA was more toxic than its 2,6-DMA counterpart; *N*-OH-3,5-DMA and 3,5-DMAP were clearly mutagenic at a level of 50 μM. EPR studies showed intracellular oxidative stress induced under 3,5-DMAP treatment.

Conclusions: Our findings suggest that genotoxic responses of 2,6- and 3,5-DMA are mediated through the generation of ROS by hydroxylamine and/or aminophenol metabolites.

Keywords: Dimethylaniline, Metabolites, Genotoxicity, TK6/*HPRT* assay, ROS.

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INTRODUCTION

Alkylanilines are abundantly found environmental chemicals, mainly tobacco smoke and hair dyes. They are considered factors for the development of bladder cancer [1]. Most common alkylanilines in the environment are 2,6-dimethylaniline (2,6-DMA) and 3,5-dimethylaniline ((3,5-DMA). They are metabolized by CYP450s to N-hydroxy-DMA through N-hydroxylation or dimethylaminophenol (DMAP), which can further be metabolized to dimethylquinone imine and then go through redox cycling to generate reactive oxygen species (ROS) [1,2].

Although 2,6-DMA has been shown to be clearly mutagenic for *Salmonella typhimurium* and a rodent nasal cavity carcinogen [3], categorized by IARC as possibly carcinogenic to humans (Group 2B), and it has been shown to form DNA adducts in rats [4], evidence linking exposure to 3,5-DMA, which are structurally closed related to 2,6-DMA, to genotoxicity or carcinogenicity is less extensive. Recently, the mutagenic potential of 3,5-DMA was assessed in the *gpt* gene of AS52 cells and *aprt* gene of AA8 cells [2,5,6]. 3,5-DMA was mutagenic and cytotoxic in AS52 cells when activated by P450-mediated hydroxylation and phase II conjugation, in which the aminophenol metabolites were considerably more potent than the corresponding N-hydroxylamines [2,5], whereas 3,5-DMAP did not caused a statistically significant increase in mutation frequency in AA8 cells [5,6]. In the absence of animal models, *in vitro* studies become particularly important in providing information on the carcinogenic mechanisms of 3,5-DMA. Therefore, all agree that other assays require further study for the understanding of mutagenesis caused by 3,5-DMA and its metabolites. The most promising mutation assays seem to be the hypoxanthine phosphoribosyltransferase (*HPRT*) assay. The *HPRT* gene is on the X chromosome of mammalian cells, and it is used as a model gene to investigate gene mutations in mammalian cell lines [7]. The assay can detect a wide range of chemicals capable of causing DNA damage that leads to gene mutation [7].

In the present study, we have begun testing the hypothesis that their genotoxic effects are mediated through N-hydroxylamine and/or aminophenol metabolites. Target cells used for these experiments include human lymphoblastoid TK6 cells, which have been used extensively in mutagenicity studies. We also used electron paramagnetic resonance (EPR) to test ROS production by aminophenol metabolites of DMA.

METHOD

Chemicals and reagents

Reagents and cell culture materials were purchased from the following sources: Cell culture materials, Lonza (Walkersville, MD); fetal bovine serum, Atlanta Biological; 2,6-DMA, 6-thioguanine (6-TG), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and dimethyl sulfoxide (DMSO), Sigma Chemical Co., St Louis, MO; superoxide dismutase (SOD, 5000 U mg) and beef liver catalase (CAT, 65000 U/mg), Roche Diagnostics (Indianapolis, IN, US); and 3,5-DMA, Acros Organics (Geel, Belgium).

Cell cultures

TK6 cells, provided by Dr. Wogan (Massachusetts Institute of Technology), were maintained in RPMI 1640 medium supplemented with antibiotics and 10% heat-inactivated horse serum (Lonza,

Walkersville, MD). Before each experiment, they were treated with CHAT (10 μM 2´-deoxycytidine, 20 μM hypoxanthine, 0.1 μM aminopterin, and 17.5 μM thymidine) according to a standard protocol to remove pre-existent mutant cells.

Treatments with N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA

N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA were synthesized as described previously [5]. 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 0–250 μM of N-hydroxy or aminophenol metabolites for 1, 2, 4, and 24 h. After treatment, the culture was washed with PBS, amended with RPMI 1640 medium containing 10% horse serum, and incubated.

Measurement of survival and *HPRT* mutations

Cytotoxicity was determined by trypan blue exclusion assay 24 h after treatment. Specifically, conditions were chosen which resulted in 35–50% survival 1 h after exposure because previous studies shown that such conditions optimize statistical estimation of MF [8,9]. TK6 cells were maintained for 7 days to allow phenotypic expression, at which time a total of 24×10^6 cells were placed into ten 96-well microtiter plates at densities of 4×10^4 cells/well in medium containing 2 μg/mL of 6-TG to select *HPRT* mutants, respectively. For plating efficiency, cells from each culture were also plated into 96-well dishes at 1 cell/100 μL/well in the absence of selective agents. After 2 weeks of incubation, colonies were counted and mutation fractions (MFs) were calculated as described by Kim [10]. Identified single *HPRT* mutant colony was then transferred to 24-well plates for propagating mutant cells. Approximately 2×10^6 mutant cells were collected for molecular analysis. The spontaneous MF was estimated from the argon-treated cells for $NO^{\bullet}$ treatment or untreated cells for coculture. Cells treated with 4-NQO (140 ng/mL for 1.5 h) served as positive controls.

EPR spectroscopy

TK6 cells (1×10^7) were gently washed twice with RPMI-serum-free media and then resuspended 400 μL of serum-free media. An aliquot of 400 μL was mixed with DMPO (100 mM), 3,5-DMAP (2 mM), and NADPH (15 mM) and placed into flat cell. Measurements were carried out using 0.3-mm flat cell. All reaction mixtures were air-saturated for the EPR experiments. EPR experiment run by a Broker EMX-300 spectrometer (Bruker, Billerica, MA) operating at modulation frequency 100 kHz; microwave frequency, 9.17 GHz; modulation amplitude, 0.5 G; and receiver gain, 1×10^4.

Statistical analysis

All experiments were repeated 2–4 times. The two-tailed Student's *t*-test (SPSS for Windows, 12.0, SPSS Inc. Chicago, IL, USA) was used for the comparison of test and control groups, and $p<0.05$ and 0.01 were considered to be statistically significant.

RESULTS AND DISCUSSION

Time-course analysis of cell survival

To determine the suppression of cell growth by N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA in human lymphoblastoid cells, TK6 cells were treated with various doses of N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA for 1, 2, 4, or 24 h. As shown in Table 1, N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA exerted cell growth inhibitory effects on TK6 cells in a dose- and time-dependent manner. The growth of the TK6 cells was markedly suppressed following 24 h treatment with the highest concentrations of each metabolite, N-hydroxy-2,6- and 3,5-DMA (250 and 100 μM), and 2,6- and 3,5-DMAP (25 and 25 μM) an 97.1–99.3% reduction as compared with the untreated control cells ($p<0.01$) (Table 1). Aminophenols were far more toxic at low concentration than N-hydroxylamines, and N-hydroxy and aminophenol metabolites of 3.5-DMA were more toxic than those of 2-6-DMA. Cell viability was 0.7% in TK6 cells 24 h after treatment with 25 μM of 3,5-DMAP (Table 1).

Cytotoxicity and mutagenicity of N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA to TK6 cells

The alkylanilines proposed for the study are closely related structurally and would all likely be metabolized to N-hydroylamines *in vivo* [11]. A fraction of the alkylanilines may also be metabolically transformed to DNA reactive quinone imines [12]. Direct genotoxicity as a by-product of N-hydroxylamine or quinone imine formation is the expected carcinogenic mode of action for the chemicals [11,12]. In the present experiments, when TK6 cells were exposed for 1 h to N-hydroxy-2,6 and 3,5-DMA, cell death was induced in a dose-dependent manner (Fig. 1a). N-hydroxy-3,5-DMA was more toxic than N-hydroxy-2,6-DMA; cell viability was 44% in TK6 cells 1 h after a dose of 25 μM of N-hydroxy-3,5-DMA, as compared to 35% after treatment with 100 μM of N-hydroxy-2,6-DMA, respectively (arrows in Fig. 1a). Mutagenicity of N-hydroxy-2,6- and 3,5-DMA in the *HPRT* gene of TK6 cells was also investigated (Fig. 1b). At a dose of 100 μM in N-hydroxy-2,6-DMA and 25 μM in N-hydroxy-3,5-DMA-treated TK6 cells, induced mutation frequencies in the *HRPT* gene were 1.31×10^{-5} ($p<0.05$) and 1.73×10^{-5} ($p<0.05$), 3- and 4-fold higher than background (0.43×10^{-5}), respectively (Fig. 1b).

A dose-dependent decrease in the viability of TK6 cells after treatment with 2,6- and 3,5-DMAP is shown in Fig. 2a. Aminophenol metabolites were more cytotoxic than N-hydroxy metabolites; cell viability in 3,5- and 2,6-DMAP was 39 and 53% after a dose of 10 and 40 μM for

Table 1: Cell survival after treatment with *N*-OH-2,6-DMA, *N*-OH-3,5-DMA, 2,6-DMA, P and 3,5-DMAP in TK6 cells

	Dose (μM)	Survival (%)			
		1 h	2 h	4 h	24 h
N-OH-2,6-DMA	0	100	100	100	100
	50	57.4±3.10**	48.9±2.29**	40.4±2.12**	32.1±1.14**
	100	35.4±1.21**	28.1±1.02**	19.4±1.29**	14.7±2.12**
	250	19.9±1.04**	3.6±0.92**	5.7±0.44**	1.4±0.32**
N-OH-3,5-DMA	0	100	100	100	100
	10	72.5±3.22*	69.3±3.41**	66.8±4.12**	62.3±2.35**
	50	34.6±2.81**	21.3±2.04**	20.0±1.84**	17.9±0.89**
	100	15.7±0.04**	7.9±0.23**	5.2±1.15**	2.9±0.51**
2,6-DMAP	0	100	100	100	100
	5	82.8±3.21*	50.5±2.14**	45.2±2.87**	62.3±4.11**
	10	60.1±3.46**	40.6±1.89**	33.0±1.25**	17.9±0.86**
	25	42.9±2.56**	34.6±3.51**	19.9±2.88**	2.9±0.41**
3,5-DMAP	0	100	100	100	100
	5	55.7±2.14**	53.5±3.14**	38.3±2.21**	36.3±2.10**
	10	35.7±3.12**	29.5±1.12**	14.1±1.37**	19.1±0.98**
	25	13.0±1.57**	7.1±0.91**	2.7±0.12**	0.7±0.04**

Each values is expressed as mean±standard deviation (n=3), *p<0.05 and **p<0.01 compared to vehicle control, DMAP: Dimethylaminophenol

1 h (arrows in Fig. 2a), as compared to 44% and 35% after treatment with 25 μM of N-hydroxy-3,5-DMA and 100 μM of N-hydroxy-2,6-DMA, respectively (Fig. 1a). The MFs induced by exposure to 10 μM in 3,5-DMAP and 40 μM in 2,6-DMAP for 1 h, 9.6 and 7.6×10^{-5} ($p<0.01$), were 13- and 11-fold higher than the background MF, 0.69×10^{-5}, respectively (Fig. 2b). By comparison, in 4-NQO-treated positive controls, MF at the *HPRT* locus was 1.24 and 1.4×10^{-5} (Figs. 1b and 2b).

ROS production by 3,5-DMAP

A growing body of evidence points toward oxygen-free radicals being involved in carcinogenesis and a wide variety of degenerative diseases [13-15]. Oxidative damage may become a threat under conditions of oxidative stress, where defense and repair mechanisms are defective or are overwhelmed by the excessive generation of ROS [16]. Previous studies shown that the mechanism, by which the alkylanilines 2,6-DMA and 3,5-DMA would generate ROS *in vivo*, involves initial oxidative metabolism to an aminophenol, which then can cycle through the corresponding quinone imine structure [5]. In the present study, the possible protective effects of SOD and CAT against the ROS-producing and cytotoxic effects of 3,5-DMAP were investigated by measuring intracellular ROS production and cell viability. As shown in Fig. 3, TK6 cells were exposed to 3,5-DMAP (5, 10, and 25 μM) for 24 h, and intracellular ROS production and cell survival were measured. Cell viability after treatment with 3,5-DMAP decreased by 42% at 5 μM, whereas inhibition of ROS with 500 U SOD and 500 U CAT restored the cell survival to a rate near that of control (81%). The chemical character of the ROS production from the samples treated with 2 mM of 3,5-DMAP as well as its changes caused by cotreatment with SOD and CAT was estimated based on the simulation of EPR spectra. The EPR peak height increased with increases in 3,5-DMAP (Fig. 3b). The addition of 500 U of SOD and CAT resulted in markedly decreases in EPR peak height, suggesting that removal of ROS generated from 3,5-DMAP (Fig. 3b). These data are consistent with the recent demonstration that both hydroxylamine and aminophenol metabolites of both 2,6- and 3,5-DMA were capable of producing ROS intracellularly and that the aminophenols were far more potent [5] and antioxidant enzymes such as N-acetyl cysteine, ascorbate, SOD, CAT, uric acid, and tiron rescue the incidence of 3,5-DMAP-induced mutagenesis in cultured mammalian cells [5,14].

CONCLUSION

The N-hydroxy and aminophenol metabolites of 2,6- and 3,5-DMA can cause both cytotoxicity and mutagenicity in the *HPRT* gene of TK6 cells. Our result also implicates ROS as mediators of 2,6- and 3,5-DMA genotoxicity. Further research will be required to define more specifically the mechanisms through which they act.

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AUTHORS' CONTRIBUTION

All authors had equally contributed to the research work.

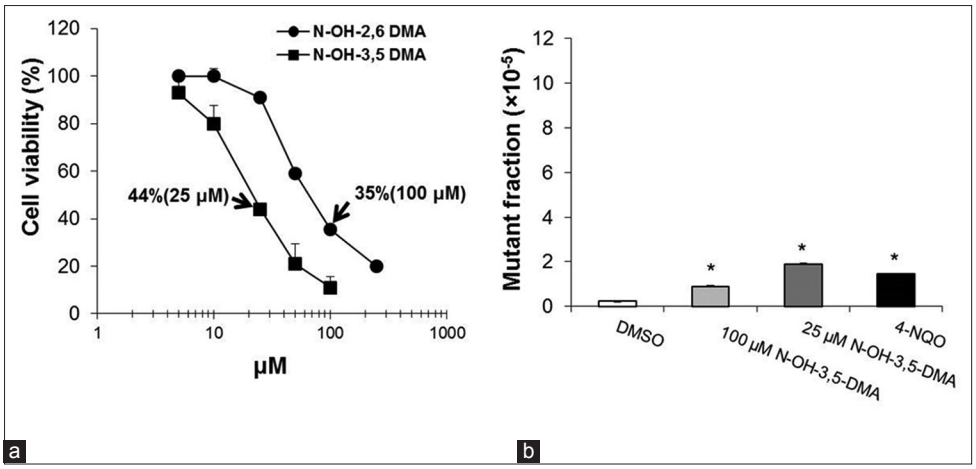

Fig. 1: Cell survival (a) and induced mutation fraction (b) in AS52 cells treated with N-hydroxy metabolites of 2,6-DMA and 3,5-DMA for 1 h, results (a and b) are shown as the mean standard deviation (n=3), *p<0.05 and **p<0.01 compared to vehicle (DMSO) control

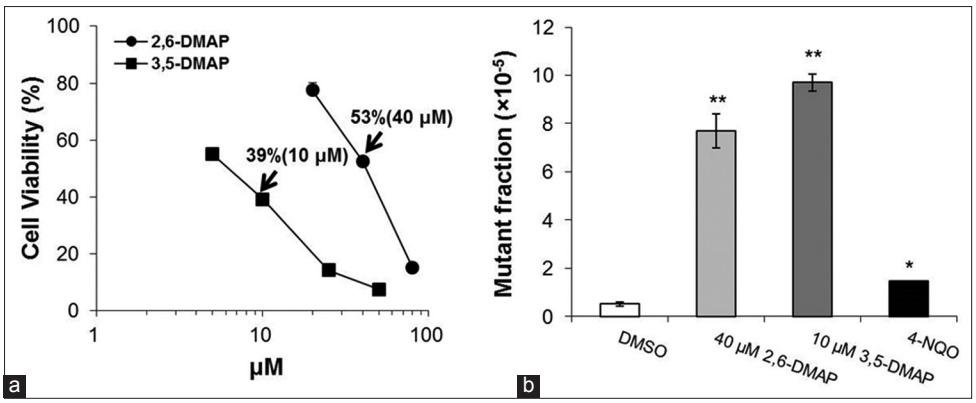

Fig. 2: Cell survival (a) and induced mutation fraction (b) in AS52 cells treated with aminophenol metabolites of 2,6-DMA and 3,5-DMA for 1 h, results (a and b) are shown as the mean ± standard deviation, *p<0.05 and **p<0.01 compared to vehicle (DMSO) control

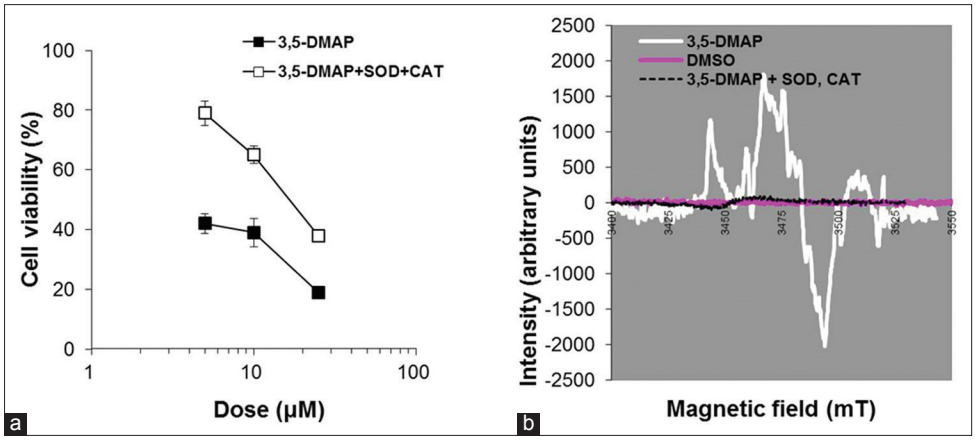

Fig. 3: Survival of TK6 cells (a) and electron paramagnetic resonance spectra (b) after treatment with 3,5-dimethylaminophenol for 24 h in the absence or presence of superoxide dismutase and catalase, results (a and b) are shown as the mean standard deviation (n=3)

CONFLICTS OF INTEREST

We declare that there are no conflicts of interest.

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