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Article

Role of cytochrome P450 1A2 and N-acetyltransferase 2 in 2,6-dimethylaniline induced genotoxicity

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The purpose of the current work was to assess a possible role of cytochrome P450 1A2 (CYP1A2) and N-acetyltransferase 2 (NAT2) in the metabolic activation of 2,6-dimethylaniline (2,6-DMA) and also clarify the function of DNA repair in affecting the ultimate mutagenic potency. Two cell lines, nucleotide excision repair (NER)-deficient 5P3NAT2 and proficient 5P3NAT2R9 both expressing CYP1A2 and NAT2, were treated with 2,6-DMA for 48 h or its metabolites for 1 h. Cell survival determined by trypan blue exclusion and MTT assays, and 8-azaadenine-resistant mutants at the adenine phosphoribosyltransferase (*aprt*) gene locus were evaluated. 5P3NAT2 and 5P3NAT2R9 cells treated with 2,6-DMA and its metabolites showed a dose-dependent increase in cytotoxicity and mutant fraction; N-OH-2,6-DMA and 2,6-DMAP in serum-free α-minimal essential medium (MEM) are more potent than 2,6-DMA in complete MEM. 5P3NAT2 cells was more sensitive to the cytotoxic and mutagenic action than 5P3NAT2R9 cells. H_2DCFH-DA assay showed dose-dependent ROS production under 2,6-DMAP treatment. These findings indicate that the genotoxic effects of 2,6-DMA are mediated by CYP1A2 activation via N-hydroxylation and the subsequent esterification by the phase II conjugation enzyme NAT2, and through the generation of ROS by hydroxylamine and/or aminophenol metabolites. NER status is also an important contributor.

Keywords: N-Acetyltransferase 2. 2,6-Dimethylaniline. Genotoxicity. Cytochrome P450 1A2.

INTRODUCTION

Human is constantly exposed to many chemical carcinogen in their environment. The aromatic amine 2,6-dimethylaniline (2,6-DMA) is used as chemical intermediate in the production of dyes, drugs, pesticides and other products (Marques *et al*., 2002). 2,6-DMA has been identified as a nasal carcinogen with both tumor initiating and promoting properties in rat long-term feeding studies (Haseman, Halley, 1997); high incidence of nasal tumors has been observed in rats fed a diet containing 3000 ppm of 2,6-DMA for 2 years. 2,6-DMA also produces subcutaneous fibromas and fibrosarcomas and increases the incidence of neoplastic nodules in the livers of rats (NTP, 1990). 2,6-DMA is classified as a possible carcinogenic to humans (Category IIB) by the International Agency for Research on Cancer (IARC) (IARC monographs).

2,6-DMA is a recognized risk factor for human bladder cancer, independently of cigarette smoking (Gan, Skipper, 2004; Skipper *et al*., 2003). The Los Angeles study examined hemoglobin adducts of nine alkylanilines, that was previously unstudied in relation to bladder cancer risk, and found three alkylanilines of them, including 2,6-DMA, to be independently and significantly associated with bladder cancer risk among nonsmoking subjects at blood draw (Tao *et al*., 2013). 2,6-DMA is a genotoxic carcinogen (Skipper

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et al., 2010; Skipper *et al*., 2003; Gan *et al*., 2001), which is metabolized to reactive N-hydroxyl-2,6-DMA (N-OH-2,6-DMA) that form covalent adducts with DNA in mouse bladder and liver tissue (Tao *et al*., 2013; Skipper *et al*., 2006). 2,6-Dimethylaminophenol (2,6-DMAP), another primary metabolite of 2,6-DMA, produced by cytochrome P450-catalyzed hydroxylation of the aniline or by nucleophilic attack of H_2O on the appropriate resonance form of the nitrenium ion is oxidized to its quinone imine form (Skipper *et al*., 2003; Gan *et al*., 2001). The electrophilic quinone imine undergoes nucleophilic addition by a DNA base yielding a mutagenic adduct (Skipper *et al*., 2003; Gan *et al*., 2001).

The metabolic activation pathways associated with carcinogenic arylamines, such as 4-aminobiphenyl (ABP) in cigarette smoke, and heterocyclic amines (HCAs), such as 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) in well-cooked meats have been known to involve N-oxidation, catalyzed primarily by cytochrome P4501A2 (CYP1A2), and subsequent N-acetylation of arylanilines and the *O*-acetylation of N-hydroxylated metabolites of arylanilines and heterocyclic amines (HCAs), catalyzed primarily by arylaniline N-acetyltransferase 2 (NAT2) (Hanna, 1996; Hein, 2002). *O*-acetylation catalyzed by NAT2 plays an important role in the metabolic bioactivation and the consequent adduct formation of these carcinogens (Snyderwine, 1999).

In the present study, 2,6-DMA-induced genotoxicity at the hemizygous adenine phosphoribosyltransferase (*aprt*) locus of nucleotide excision repair-deficient 5P3NAT2 and proficient 5P3NAT2R9 cells both expressing CYP1A2 and NAT2 derived from Chinese hamster ovary (CHO) AA8 cell line was analyzed, in an attempt to understand the role of CYP1A2 and NAT2 in the metabolic activation of 2,6-DMA but also clarify the function of DNA repair in affecting the ultimate mutagenic potency. In addition, we identify that ROS has a critical role in causing cell death and mutagenesis in 2,6-DMA-treating cells. The *aprt* gene was chosen because its small size permitted mutations to be easily localized and sequenced (Sodimbaku, Pujari, 2014).

MATERIAL AND METHODS

Cell cultures

Nucleotide excision repair (NER)-deficient 5P3NAT2 and NER-proficient 5P3NAT2R9 CHO cells stably transfected with CYP1A2 and NAT2, kindly provided by Dr. Gerald N. Wogan (Massachusetts Institute of Technology, Cambridge, MA, USA), were used to test the effect of CYP1A2 and NAT2 on 2,6-DMA and its metabolites genotoxicity. The CHO cell line AA8, functionally heterozygous at the *aprt* locus (Thompson, Fong, Brookman, 1980a), was used to derive UV5 cells (Thompson *et al*., 1980b). UV5 cells are defective in the incision step of nucleotide excision repair (Weber *et al*., 1988). Transfection of UV5 cells with CYP1A2 cDNA (Thompson, Wu, Felton, 1991) and NAT2 *N*-acetyltransferase cDNAs resulted in the cell line 5P3NAT2 (Wu *et al*., 1997), which was used to derive the repair-proficient 5P3NAT2R9 cells in the present study. Details concerning the construction and characterization of these cell lines were described previously (Wu, Panteleakos, Felton, 2003). 5P3NAT2 and 5P3NAT2R9 cells were cultured in α-minimal essential medium (MEM) 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 ºC. Cells were cleansed of preexisting *aprt* mutants by culturing in CAAT medium (10 μM cytidine, 100 μM adenine, 1 μM aminopterin and 17.5 μM thymidine) for 2 days and transferred for 2-5 days to medium enriched with thymidine (17.5 μM), adenine (100 μM) and cytidine (10 μM) for recovery. All cell culture reagents were purchased from Lonza (Walkersville, MD).

2,6-DMA and its metabolites treatment

Cells were placed in 100 mm tissue culture dish at a density of 0.5×10^6 cells in 10 mL of media. Cells were exposed to 50, 100, 250, 500 and 1000 μM of 2,6-DMA (Sigma-Aldrich, MO, USA) dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) for 48 h

at 37 °C. N-hydroxy and aminophenol metabolites of 2,6-DMA were synthesized as described previously (Chao *et al*., 2012). The cells were seeded at 1 × 10^6 and incubated overnight with complete MEM containing 10% FBS. Then medium changed to serum-free MEM 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 cultures were washed with PBS, amended with complete MEM, and incubated in complete MEM for additional 24 h prior to determining cell survival. Concentrations and exposure time of 2,6-DMA and 3,5-DMA and their metabolites used for cytotoxicity and mutagenicity experiments were established based on MTT cytotoxicity assays (data not shown) and literature references (Chao *et al*., 2012; Chao *et al*., 2014a; Chao *et al*., 2014b; Erkekoglu *et al*., 2014). Stock solutions of 2,6-DMA and its metabolites (N-hydroxy and aminophenol) were prepared by dissolving the accurately weighed compounds in DMSO to give a final concentration of 100 and 25 mM, respectively. The final concentration of DMSO to which cells µL was exposed was less than 1%.

Measurement of cell viability

To investigate the effect of dose-dependent effects of 2,6-DMA and its metabolites on cell viability, 5P3NAT2 and 5P3NAT2R9 cells were subjected to trypan blue exclusion assay and MTT colormetric method. For trypan blue staining, the cell suspension after treatment was mixed with 0.5% trypan blue solution at a 1:1 ratio. After 1-2 min incubation at room temperature, the mixture was loaded onto one chamber of hemocytometer and squares of the chamber were observed under a light microscope. The viable/ live (clear) and non-viable/dead (blue) cells were counted and the viability was calculated using the formula (number of live cells counted/ total number of cells counted)×100. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 5P3NAT2 and 5P3NAT2R9 cells were plated at 5×10^3 cells/well in 100 µL volume in 96-well plates and were then grown for 24 h in complete MEM medium. 5P3NAT2 and 5P3NAT2R9 cells were exposed to 0-1000 µM of 2,6-DMA for 48 h in complete MEM or its metabolites for 1 h in serum-free MEM. Cells were then incubated with 10 µL of MTT (5 mg/ml) at 37 ºC in the dark for 4 h. The tetrazolium crystals were solubilized by the addition of 100 µL of DMSO at each well. After overnight incubation at 37 ºC, color developed after the reaction was measured at 550 nm using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT, US). The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

aprt mutation assay

Following treatment, 5P3NAT2 and 5P3NAT2R9 cells were rinsed twice with PBS and detached with 0.25% trypsin-EDTA solution. Thereafter, treated cells were maintained in growth medium for at least 7 days to allow full expression of the *aprt* mutant phenotype. Total 6 × 10^5 cells from each group were placed in 100 mL selective medium containing 80 µg/mL of 8-Azaadenine (8-AA, Sigma-Aldrich, MO, USA) and plated at 600000 cells/10 mL/100 mm dish (ten replicates) for determination of mutagenicity at the *aprt* locus after 14 days. In addition, each mutant was rescreened in selective media in order to confirm the stability of the mutant phenotype. Cloning efficiency (CE) dishes were seeded with 200 cells/10 mL/100 mm dish in triplicate and allowed to grow until colonies were visible for 8 days in the absence of selecting agent. Colonies were stained with 0.5% crystal violet in 50% methanol and counted. Mutation fraction (MF) was calculated as the ratio of mean CE in selective medium to that in non-selective medium. In those experiments, cells treated with 0.2 µM PhIP (Toronto Research Chemicals, Ontario, Canada) for 48 h served as a positive control.

Detection of ROS level

Total ROS level in 5P3NAT2 and 5P3NAT2R9 cells was investigated using the fluorogenic probe, 2',7'-dichlorofluorescein-diacetate (H_2DCFDA; Sigma-Aldrich, MO, USA). In brief. cells were seeded at a

density of 2×10^6 cells in a 60 mm culture dish and allowed to attach overnight. The next day, medium was replaced by fresh medium containing 0, 50, 100 and 250 μM of 2,6-DMAP and allowed to incubate for 1 h. Following incubations, the cells were harvested by trypsinization and washed with PBS and resuspended in PBS containing 10 μM H_2DCFH-DA for 30 min at 37 ºC, and the fluorescence intensity in cells was determined by flow cytometry. H_2O_2 was used as a positive control.

Statistical analysis

All the data are expressed as mean ± SD (n = 3). The two-tailed Student's *t*-test using SPSS statistical software (SPSS, ver. 12.0; SPSS Inc., Chicago, IL, US) was used for the comparison of the cell survival, mutation fraction and ROS production between 5P3NAT2 and 5P3NAT2R9 (or AA8) cells. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

There are many factors that can affect the sensitivity of cells to different mutagens and carcinogens. In addition to the DNA damage caused by the reactive intermediates, the capability of the cells to repair DNA damage is also an important factor in the development and evolution of cancer cells (Wu, Panteleakos, Felton, 2003). The 5P3NAT2 and 5P3NAT2R9 cell lines, which both express CYP1A2 and N-acetyltransferase but differ in repair capability, not only offer a sensitive system for evaluating the genotoxicity of a wide range of compounds, but one that can provide further insight into the role of nucleotide excision repair in bulky-adduct mutagenesis (Wu, Panteleakos, Felton, 2003).

In the current study, 5P3NAT2 and 5P3NAT2R9 cells treated with 0, 50, 100, 250, 500 and 1000 μM of 2,6-DMA in complete MEM for 48 h reduced the percentage of trypan blue-negative (viable) cells dose-dependently, and the repair-deficient 5P3NAT2 cells were more sensitive than repair-proficient 5P3NAT2R9 cells (Figure 1). Exposure to 500 and 1000 μM 2,6-DMA for 48 h, reduced viability in 5P3NAT2 to 49 and 35%, respectively, whereas comparable values after treatment with same concentrations of 2,6-DMA for 48 h in 5P3NAT2R9, were 81 and 76%, suggesting that it is important to study the DNA repair mechanisms that play an important part in the stability of adducts affecting the mutational event (Figure 1). Both 5P3NAT2 and 5P3NAT2R9 cells were also treated with 50, 100, 250, 500 or 1000 μM of hydroxyl and aminophenol metabolites of 2,6-DMA in serum-free MEM for 1h. Viability was dramatically decreased in repair-deficient cells expressing CYP1A2 and NAT2, while 5P3NAT2R9, repair-proficient cells, attenuated this cytotoxic effect of N-OH-2,6-DMA and 2,6-DMAP (Figure 1). In addition, a similar trend was observed in cells treated with 2,6-DMA and its metabolites in the MTT assay results (Figure 2). Treating 5P3NAT2 cells with 500 and 1000 μM 2,6-DMA in complete MEM for 48 h decreased the MTT-reducing activity to 53 and 44%, respectively, relative to untreated control cells (Figure 2). N-hydroxy and aminophenol metabolites in serum-free MEM were more cytotoxic than its parent compound in complete MEM, and NER-proficient cells (5P3NAT2R9) exhibited significantly increased survival levels comparable with NER-deficient cells (5P3NAT2), indicating that NER has a distinct role in protecting cells from 2,6-DNA toxicity (Figure 2).

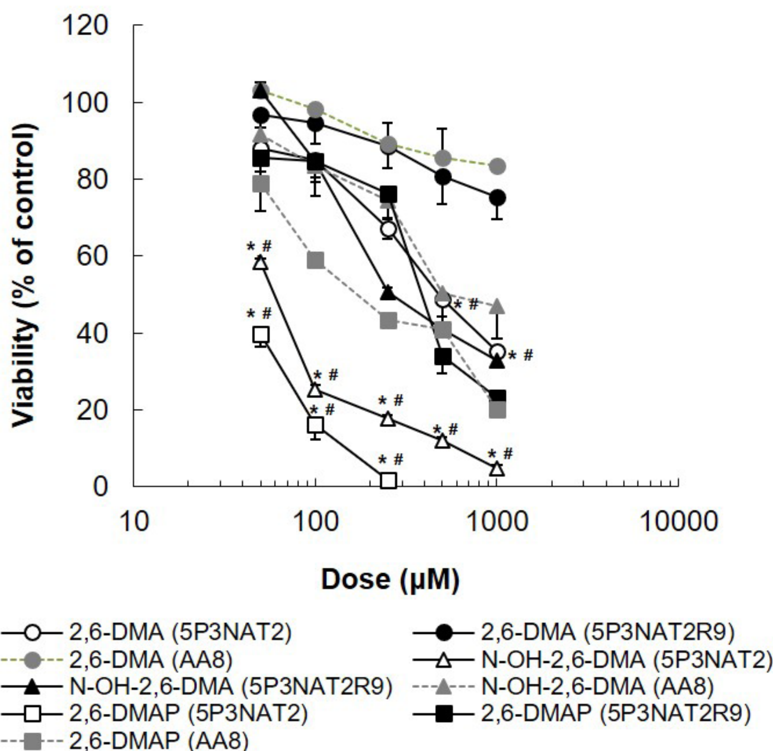

FIGURE 1 - Dose-dependent of cell survival after treatment with 2,6-DMA and its metabolites in 5P3NAT2, 5P3NAT2R9 and AA8 cells. Survival was determined by trypan blue exclusion assay after treatment. Results are presented as a percentage of control cells (mean ± SD, n=3). $^{*}p < 0.05$ compared to 5P3NAT2R9 cells and $^{\#}p < 0.05$ compared to AA8 cells by Student's *t*-test.

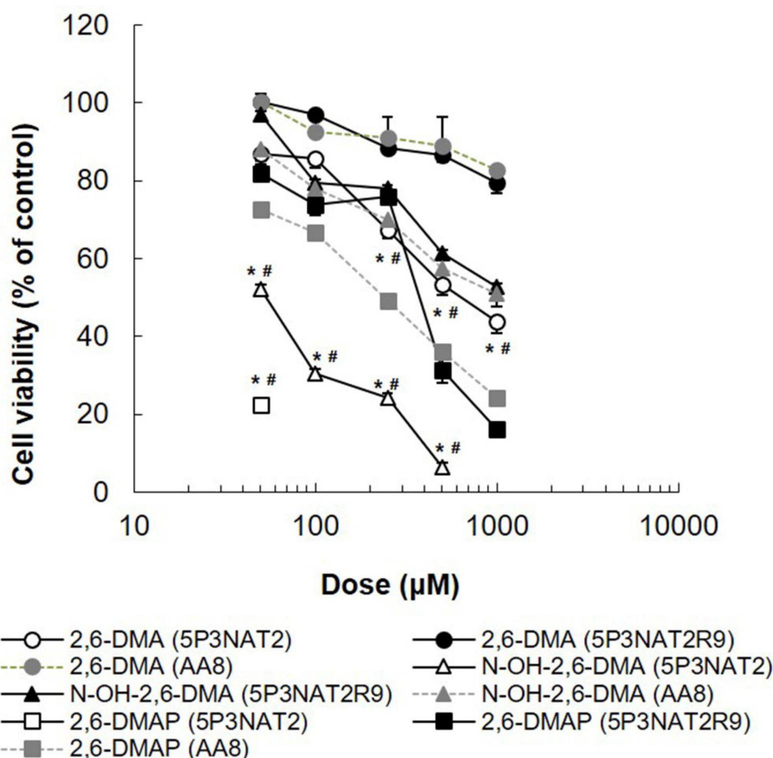

FIGURE 2 - The percentage of viable 5P3NAT2 and 5P3NAT2R9 cells after 2,6-DMA and its metabolites treatment. Cell viability was determined by MTT assay after treatment. Each point is the mean ± SD of three experiments. $^{*}p < 0.05$ compared to 5P3NAT2R9 cells and $^{\#}p < 0.05$ compared to AA8 cells by Student's *t*-test.

The *aprt* locus of CHO cells is well suited for mutation studies. It encodes an enzyme in the nucleotide salvage pathway and is thus nonessential for cell survival in ordinary growth medium. Thus, all classes of mutational events can in principle be expected (Jong, Grosovsky, Glickman, 1988). Advantages of the CHO *aprt* locus for molecular analyses include the availability of hemizygous strains, the apparent absence of pseudogenes and the small kilobases size of the functional CHO gene (Jong, Grosovsky, Glickman, 1988). The *aprt* gene are also more suitable targets for studying large gene deletions in cultured human cells (Meng *et al*., 2000).

The mutagenicity of 2,6-DMA in the *aprt* gene of both 5P3NAT2 and 5P3NAT2R9 cells was investigated (Table I). At a dose of 1000 µM of 2,6-DMA in complete MEM, induced MFs in the *aprt* gene of 5P3NAT2 and 5P3NAT2R9 cells were 27.7×10^5 and 4.8×10^5, 6 and 3fold higher than background (4.4×10^5 and 1.6×10^5), respectively (Table I). N-OH-2,6-DMA and 2,6-DMAP in serum-free MEM were mutagenic to 5P3NAT2 cells co-expressing CYP1A2 and NAT2 even at the lowest concentration level studied (50 µM) (Table I). At 50 µM of N-OH-2,6-DMA and 2,6-DMAP (59 and 40% 5P3NAT2 cell survival, respectively), MF was increased nearly 2- and 4-fold above the level observed in DMSO-treated control cultures (Figure 1; Table I). However, they were very weak mutagenic to repair-proficient 5P3NAT2R9 cells, suggesting repairment of DNA damage caused by N-OH-2,6-DMA and 2,6-DMAP, and NAT2 activity under our experimental condition. The mutagenic effect of 2,6-DMA and N-OH-2,6-DMA was decreased by 4-fold at the highest dose (1000 µM) examined in 5P3NAT2R9 cells (Table I). By comparison, in 0.2 µM PhIP-treated positive controls, MFs at *aprt* locus were 91.9×10^{-5} and 5.9×10^{-5} in 5P3NAT2 and 5P3NAT2R9 cells, respectively. A relatively higher cytotoxic and mutagenic effect of N-OH-2,6-DMA and 2,6-DMAP in serum-free MEM was observed compared to those of 2,6-DMA in complete MEM (Figure 1; Figure 2; Table I), indicating that NAT2 as well as CYP1A2 are strongly involved in bioactivation of 2,6-DMA under our experimental condition.

TABLE I - Mutation fraction after treatment with 2,6-DMA and its metabolites in 5P3NAT2, 5P3NAT2R9 and AA8 cells

Dose (µM)	Mutation fraction ($\times10^{-5}$)					
	0	50	100	250	500	1000
2,6-DMA (5P3NAT2)	4.4±0.14	4.5±1.2	9.6±0.79	12.5±0.63*	24.1±1.93*	27.7±5.99*
2,6-DMA (5P3NAT2R9)	1.6±1.90	1.9±0.43	5.5±0.73	4.9±0.69	3.8±0.17	4.8±0.72
N-OH-2,6-DMA (5P3NAT2)	4.4±0.14	11.9±0.38*#				
N-OH-2,6-DMA (5P3NAT2R9)	1.6±0.29	1.8±0.93	3.1±0.15	3.2±1.09	6.0±0.13	7.7±2.46
2,6-DMAP (5P3NAT2)	4.4±0.14	17.9±4.82*#				
2,6-DMAP (5P3NAT2R9)	1.6±0.29	3.2±0.42	3.6±0.41	3.55±0.41	4.6±0.55	6.5±1.26
2,6-DMA (AA8)	2.9±0.27	3.1±0.77	3.1±0.13			
N-OH-2,6-DMA (AA8)	2.9±0.27	3.0±0.64	3.2±0.58			
2,6-DMAP (AA8)	2.9±0.27	3.1±0.11	3.2±0.34			

Each values is expressed as mean±standard deviation (n = 3). $^*p < 0.05$ compared to 5P3NAT2R9 cells and $^\#p < 0.05$ compared to AA8 cells by Student's *t*-test.

In the present experiments, we used CHO AA8-derived 5P3NAT2 and 5P3NAT2R9 cells, which is able to express active CYP1A2 and NAT2. With these cells we were able to detect bioactivation of 2,6-DMA. Both 5P3NAT2 and 5P3NAT2R9 cell lines were more sensitive to the cytotoxic and mutagenic effects of 2,6-DMA and its metabolites relative to parental AA8 line ($p < 0.05$, Figure 1; Table I). Exposure to 50 µM

of N-OH-2,6-DMA, reduced viability and increased MF in NER-deficient 5P3NAT2 cells to 58.5% and 11.9×10^{-5}, respectively, whereas comparable values after treatment with 50 µM of N-OH-2,6-DMA in NER-deficient AA8 cells, were 91.6% and 3.0×10^{-5} (Table I and Figure 1). In addition, cell viability and mutation fraction were 39.9% and 17.9×10^{-5} in 5P3NAT2 cells after a dose of 50 µM 2,6-DMAP, compared to 79.1% and 3.1×10^{-5} after treatment with 50 µM 2,6-DMAP in AA8 cells (Table I and Figure 1) (Kim, 2019). It is clear from the data that 2,6-DMA become more genotoxic following oxidative metabolism catalyzed by CYP1A2 and NAT2.

In light of our previous findings that 3,5-DMAP could induce ROS generation in TK6 cells (Moon, Kim, 2018b), we were interested to probe if ROS would be generated in 5P3NAT2 and 5P3NAT2R9 cells on the major product of N-hydroxylation of 2,6-DMA, 2,6-DMAP treatment. Both 5P3NAT2 and 5P3NAT2R9 cells were loaded with the ROS probe, H_2DCFDA, and H_2O_2 was included as a positive control. As shown in Figure 3, 2,6-DMAP-induced ROS generation in a dose-dependent manner, as reflected by the increase in fluorescence intensity. The highest amount of ROS was generated after 1 h of exposure to 250 µM 2,6-DMAP, with 60.6 and 21.5 % increases over untreated control in 5P3NAT2 and 5P3NAT2R9 cells, respectively, compared to 26.3% after treatment with 250 µM 2,6-DMAP in AA8 cells (Figure 3). 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 (Chao *et al*., 2012; Chao *et al*., 2014a; Chao *et al*., 2014b; Erkekoglu *et al*., 2014) and antioxidant enzymes such as N-acetyl cysteine, ascorbate, SOD, CAT, uric acid, ascorbic acid and tiron rescue the incidence of 3,5-DMAP-induced mutagenesis in cultured mammalian cells (Chao *et al*., 2012; Chao *et al*., 2014a; Chao *et al*., 2014b; Erkekoglu *et al*., 2014). Oxidative DNA damage is therefore a plausible mechanism of mutagenicity induced by 2,6-DMA (Figure 3). The 5P3NAT2, 5P3NAT2R9 and AA8 cells treated with 100 µM H_2O_2 (positive control) for 6 h resulted in 62.5, 33.1 and 36.4% of ROS production, respectively.

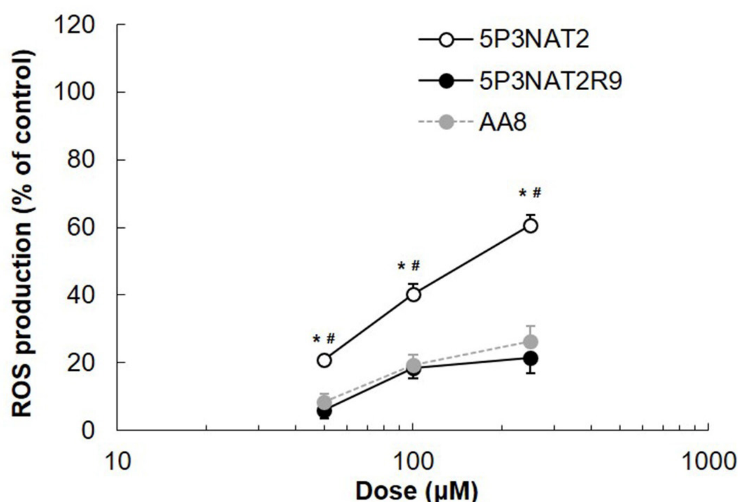

FIGURE 3 – Intracellular ROS level in 5P3NAT2 and 5P3NAT2R9 cells after 2,6-DMAP. The level of ROS in cells treated with 0, 50, 100 and 250 µM 2,6-DMAP for 1 h as measured by flow cytometry. Results are presented as a percentage of control cells (mean ± SD, n=3). $^{*}p < 0.05$ compared to 5P3NAT2R9 cells and $^{\#}p < 0.05$ compared to AA8 cells by Student's *t*-test.

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.* (1983) reported the

presence of the mutagenic *N*-hydroxylated metabolite of 2,6-DMA in an *in vitro* rat liver microsomal system (S9) in which the yield of the mutagenic metabolite increased with increasing S9 content. Beland *et al.* (1997) reported the mutagenicity of the *N*-hydroxylated metabolite of 2,6-DMA towards *Salmonella typhimurium* (TA100). We recently reported that 2,6-DMA is cytotoxic and mutagenic in *gpt* and *HPRT* genes of AS52 (Moon, Kim, 2018a) and TK6 (Moon, Kim, 2018b) cells, respectively, when activated by phase I and phase II metabolism, in which the aminophenol metabolites were considerably more potent than the corresponding N-hydroxylamines.

In summary, we note that 2,6-DMA is potent genotoxic when activated by phase I (CYP1A2) and phase II (NAT2) metabolizing enzymes, and cells deficient in repair of particular DNA adducts or lesions proved more sensitive to the agent causing those lesions than did normally repairing cells. Moreover, our results offer an explanation that ROS generation could be one of the factors leading to an increase in genotoxicity induced by 2,6-DMA. Further investigations are required to examine the mutational spectrum produced by 2,6-DMA for elucidation of the mutational mechanism.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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