FABAD J. Pharm. Sci., 42, 2, 87-93, 2017

RESEARCH ARTICLE

The Mutagenic Potential of 3,5-Dimethylaminophenol in Bacterial and Mammalian Cells

Pınar ERKEKOGLU[*,o], Ming-Wei CHAO[**], Ozge KOSE[*], Suna SABUNCUOGLU[*], Belma KOCER-GUMUSEL[*,o]

The Mutagenic Potential of 3,5-Dimethylaminophenol in Bacterial and Mammalian Cells

SUMMARY

Alkylanilines are ubiquitous environmental chemicals. Most individuals are subject to lifelong exposure to these compounds and this exposure can cause toxic effects in human bladder. It was shown that other than main compounds, their oxidative metabolites (o- or p-phenol derivatives) are cytotoxic. Phenolic metabolite of 3,5-dimethylaniline is 3,5-dimethylaminophenol (3,5-DMAP). It is oxidized to a quinoneimine further metabolite by Phase I enzymes. The aim of this study is to determine whether varying concentrations (2.5, 5, 10, 25, 50, 100 µg/ml) of 3,5-DMAP causes mutagenic effects in bacteria and mammalian cells. To investigate the mutagenicity of 3,5-DMAP in bacteria, "Ames test" was applied. Salmonella typhimurium, TA98 and TA100 strains, were used to determine base-pair and frame shift mutations, respectively (with/without S9 fraction). To determine the mutagenicity of 3,5-DMAP in mammalian cells, "hypoxanthine phosphoribosyltransferase 1 (HPRT) test" was performed on CHO cells. Although 3,5-DMAP caused a dose-dependent increase in mutation frequency, particularly in both TA98 and TA100 strains without S9 fraction, the increases were not significant. Besides, marked increases in the number revertant bacteria were not observed for any concentrations of 3,5-DMAP, with the addition of S9 fraction. 3,5-DMAP did not effect the HPRT mutation frequency in CHO cells. Our results showed that 3,5-DMAP was not mutagenic in CHO cells and different strains of S. typhimurium. As 3,5-DMAP is suggested to be cytotoxic in mammalian cells and not mutagenic in different Salmonella strains, comprehensive studies should be performed to show whether this compound or its derivatives can be used as anti-cancer agents.

Key Words: *3,5-dimethylaminophenol, Ames test, HPRT test, mutagenicity*

Bakteri ve Memeli Hücrelerinde 3,5-Dimetilaminofenolün Motajenik Potansiyeli

ÖZET

Alkilanilinler çevrede yaygın olarak bulunan kimyasal maddelerdir. Birçok kişi bu bileşiklere hayat boyu maruz kalmaktadır ve bu maruziyet insan mesanesinde toksik etkilere neden olabilir. Ana bileşikler dışında, oksidatif metabolitlerinin (o- veya p-fenol türevleri) de sitotoksik olduğu gösterilmiştir. 3,5-dimetilanilinin fenolik metaboliti 3,5-dimetilaminofenoldür (3,5-DMAP) ve Faz I enzimleriyle kinonimin ileri metabolitine okside olur. Bu çalışmanın amacı 3,5-DMAP'ın farklı konsantrasyonlarının (2,5, 5, 10, 25, 50, 100 µg/ml) bakteri ve memeli hücrelerinde motajenik etkilere neden olup olmadığının belirlenmesidir. 3,5-DMAP'ın bakterideki mutajenisitesini araştırmak için "Ames testi" uygulanmıştır. Salmonella typhimurium'un TA98 ve TA100 suşları sırasıyla baz-çifti ve kalıp kayması mutasyonlarını belirlemek için (S9 fraksiyonu ile birlikte veya yokken) kullanılmıştır. 3,5-DMAP'ın memeli hücrelerindeki mutajenitesini belirlemek için CHO hücrelerinde "hipoksantin fosforiboziltransferaz 1 (HPRT) testi" yapılmıştır. 3,5-DMAP her ne kadar TA98 ve TA100 suşlarında S9 fraksiyonu yokken, mutasyon sıklığını artırsa da, bu artışlar anlamlı değildir. Bununla birlikte, 3,5-DMAP'ın herhangi bir konsantrasyonunda S9 fraksiyonu varlığında da revertan bakteri sayısı anlamlı bir şekilde artmamıştır. 3,5-DMAP HPRT mutasyon sıklığını da artırmamıştır. Sonuçlarımız 3,5-DMAP'ın CHO hücrelerinde veya S.typhimurium'un farklı suşlarında mutajenik olmadığını göstermektedir. 3,5-DMAP memeli hücrelerinde sitotoksik olup, farklı Salmonella suşlarında mutajenik olmadığı için, bu bileşik ve türevlerinin anti-kanser ajanlar olarak kullanılıp kullanılmayağının belirlenmesi için kapsamlı çalışmalar yapılmalıdır.

Anahtar Kelimeler: *3,5-dimetilaminofenol, Ames testi, HPRT testi, mutajenite*

Received: 01.03.2017
Revised: 26.03.2017
Accepted: 29.03.2017

[*] Hacettepe University, Faculty of Pharmacy, Department of Toxicology,06100 Ankara, Turkey
[**] Department of BioScience Technology, College of Science, Chung Yuan Christian University, Zhongli district, Taoyuan city, Taiwan 320

[o]Corresponding Author:
Pınar ERKEKOGLU or Belma KOÇER-GÜMÜŞEL,
Tel: +90 312 309 29 58
Fax: +90 312 311 47 77
E-mail: erkekp@yahoo.com, belmagumusel@yahoo.com

INTRODUCTION

Alkylanilines are abundantly found environmental chemicals. The exposure sources are tobacco smoke, colorants (mostly hair dyes), combustion products (motor fuels, fugitive emissions), pharmaceuticals (drugs), and pesticides (Castelao et al. 2001; Skipper et al., 2010). These compounds are widely studied and their biotransformation is well-known, particularly in rodents. They are mainly metabolized to their oxidative metabolites (o- or p-phenol derivatives) by direct ring oxidation in the presence of cytochrome P450s (CYP450s) (Skipper et al., 2010).

3,5-dimethylaniline (3,5-DMA) is an ubiquitous derivative of alkylanilines (Government of Canada 1993). Like other alkylanilines, 3,5-DMA is also metabolized by CYP450s to N-hydroxy-3,5-dimethylaniline (N-OH-3,5-DMA) through N-hydroxylation, which is suggested to be the bioactivation step (Cui et al. 2007). On the other hand, the main metabolite of 3,5-DMA, namely 3,5-dimethylaminophenol (3,5-DMAP), can either be formed by oxidation of 3,5-DMA or as a metabolite of N-OH-3,5-DMA by P450-catalyzed hydroxylation (Chao et al. 2015). 3,5-DMAP can further be metabolized to 3,5-dimethylquinone imine (3,5-DMQI). It was shown that 3,5-DMQI goes through redox cycling and reactive oxygen species (ROS) are the byproducts of this cycle (Chao et al. 2015).

Through ROS production and perhaps through many undiscovered mechanisms, 3,5-DMAP causes cytotoxicity in different Chinese Hamster Ovary (CHO) cells (AS52, *aprt*-transgenic AA8, and *aprt*-transgenic/NER-deficient UV5). It was also shown that 3,5-DMAP caused upregulation of caspase 3 and caspase 8 activities and activation of apoptosis in different types of CHO cells (Chao et al. 2014; Chao et al. 2015; Erkekoglu et al. 2014). Recently, we have shown that N-acetyl cysteine, ascorbic acid and selenocompounds can protect CHO cells from the oxidative damage and cytotoxicity caused by 3,5-DMAP (Chao et al. 2014; Chao et al. 2015; Erkekoglu et al. 2014). Therefore, one of the main cytotoxicity mechanisms of alkylanilines in mammalian cells is the production of ROS and ROS can be encountered by many different antioxidants.

In a recent study, the mutagenicity of 3,5-DMAP was assessed by determination of the induced mutation fraction in the *aprt* gene of AA8 cells. Although at 3,5-DMAP caused an increase in mutation frequency at 25 μM dose, it was not a statistically significant increase vs. control. Besides, this effect was not dose-dependent and was not present when AA8 CHO cells were treated with 3,5-DMAP at 50 μM dose. However, the cytotoxicity of 3,5-DMAP was clearly demonstrated and it was dose-dependent in many types of CHO cells (Chao et al. 2015).

Ames test uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis. These strains are auxotrophic mutants, requiring histidine for growth. The method depends on the test substance's ability to cause mutations that result in a reversion back to a "prototrophic" state, so that the cells can grow on a histidine-free medium. To determine frameshift mutations, strains TA-1537, TA-1538, TA97a or TA98 are used. For point mutations, strains TA-1531, TA100, TA102 or TA104 are used (Ames, 1989; McCann and Ames, 1976; Mortelmans and Zeiger, 2000).

Bacterial gene mutation assay may not detect a small proportion of mammalian specific mutagenic agents. Therefore, at the current time a third assay should be used. The hypoxanthine phosphorybosyl transferase (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. The assay can detect a wide range of chemicals which can cause DNA damage that leads to gene mutation. The test follows a very similar methodology to the thymidine kinase (TK) mouse lymphoma assay (MLA). In HPRT test, mutations which destroy the functionality of the HPRT gene and or/protein, are detected by positive selection using a toxic analogue (6-thioguanine, TG), and HPRT (-) mutants are seen as viable colonies. Unlike bacterial reverse mutation assays, mammalian gene mutation assays respond to a broad spectrum of mutagens, since any mutation resulting in the ablation of gene expression/function produces a HPRT (-) mutant.

Taking into consideration all the available data, this study was performed to determine whether varying concentrations (2.5, 5, 10, 25, 50, 100 μg/ml) of 3,5-DMAP causes mutagenic effects in *S. typhimurium* bacterial strains, by using a renewed version of traditional Ames test. To investigate this effect, *S. typhimurium*, TA98 and TA100 strains, were used to determine base-pair and frame shift mutations, respectively in the presence or absence of S9 fraction.

METHODS AND MATERIALS

Chemicals

N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG), TG, Giemsa stain and DMSO were obtained from Sigma Aldrich (St.Louis, MO, USA). All cell culture chemicals, media and equipment were from Gibco (Grand Island, NY).

Kit

Ames test kit was obtained from Xenometrics (Stilwell, KS).

Synthesis of 3,5-dimethyaminophenol

3,5-DMAP was synthesized according to Chao et al. (2012). Briefly, a mixture of sulfanilic acid, sodium carbonate and water was heated to 60°C and then cooled in an ice bath. Sodium nitrite solution was added and the resulting solution was poured on HCl solution (aqueous, 5.6% w/v) in an ice bath and kept for 15 min. This solution was added to 2,6-dimethylphenol solution (in aqueous NaOH and water) at 5°C. The dark red reaction mixture was stirred well and kept at 25°C for 1 h. Later, it was heated to 60°C and aqueous sodium hydrosulfite (1% w/v) was gradually added till yellow crystals precipitate. After waiting for 15 min at 50°C, the yellow suspension was cooled to 20°C and then filtered. The filtrate was washed with sodium hydrosulfite (1% w/v in water) and dried under vacuum. The final yield of the 3,5-DMAP was found to be 82%.

Ames test

A renewed version of Ames test (384-well plate microfluctuation method) was used throughout the experiments. *S. typhimurium* bacterial strains, TA98 and TA100, were used to determine base-pair and frame shift mutations, respectively. Ames test was performed according to the manufacturer's instructions (Ames MPF™ 98/100, Ames microplate fluctuation). *S. typhimurium* haploid bacteria have particular mutations in the gene encoding an enzyme used to synthesize the amino acid "histidine". The bacteria require histidine to make many proteins, these mutant bacteria will die unless the media in which they are grown contains histidine. The assay involves plating mutant *S. typhimurium* onto media with trace amounts histidine and adding chemicals to be tested for mutagenicity. The number of colonies growing on the plate indicates the number of revertants. In histidine containing medium, bacteria were exposed to six concentrations of a test agent for 90 minutes and a positive and a negative controls were used for generating a dose-response curve. After 90 minutes, the exposure cultures are diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of a 384-well plate. Within two days, cells that have undergone the reversion to histidine prototrophy, will grow into colonies. Bacterial metabolism reduces the pH of the medium, changing the color of that well. The number of wells containing revertant colonies are counted for each dose and compared to a solvent (negative) control. An increase in the number of revertant colonies upon exposure to test chemical relative to the solvent controls indicates that the chemical is mutagenic in the Ames MPF™ 98/100 assay. The mutagenic potential of substances can be assessed directly or in the presence of liver S9 fractions. 2.5, 5, 10, 25, 50, 100 μg/mL concentrations of 3,5-DMAP were used for the exposure. Lyophilized rat liver S9 microsomal fraction, purcased from Xenometrix (Allschwil, Switzerland), was also used for the metabolic activation. Mutagenic potential of 3,5 DMAP was evaluated in the absence and presence of the S9 mix. Positive control chemicals were 2-nitrofluorene (2 μg/mL) and 4-nitroquinoline *N*-oxide (0.1 μg/mL) (for tests without S9) and 2-aminoanthracene (5 μg/mL) (for tests with S9). DMSO (1% v/v) was used as the negative control. The number of positive (yellow) wells out of the total number of wells were counted in triplicate and compared with the negative control. The criteria used to evaluate the Ames results were the fold increase in the number of positive wells over the solvent control baseline and the dose dependency. The fold increase of the revertants relative to the solvent control was determined by dividing the mean number of positive wells at each dose by those in the solvent control at baseline. The solvent control baseline was defined as the mean number of positive wells in the solvent control plus one standard deviation (SD). An increase of ≥2-fold relative to the baseline was classified as being positive and no response ≥2-fold of the baseline as negative.

Hypoxanthine Phosphoribosyltransferase 1 (HPRT) Test

This mutation assay is used to evaluate the potential of 3,5-DMAP to induce mutations at the hgprt locus of CHO cells. Cells deficient in HPRT are selected by resistance to TG (Stock solution: 1 mg/ml in DMSO, stored at 4°C, stable at least for 2 months, used as 10 μg/mL). The HGPRT gene mutation assay was carried out using the following procedure proposed by Johnson (2012), Nestmann et al. (1991) and OECD (1997) with slight modifications. The CHO cells [~1×10^6, in 10 ml F12 medium with 10% fetal bovine serum (FBS)] were trpsinized, seeded on plastic petri dishes and 24 h after cells (~1×10^6) were re-trpsinized, split and again seeded on 10 cm-petri dishes. Cells were later were treated with different concentrations of 3,5-DMAP (0, 2.5, 5, 10, 25, 50 and 100 μg/ml) for 24 h. Then, 200 cells were plated (triplicate per dose) to determine cytotoxicity, and the relative cell survival (RCS) was expressed as a percentage of the negative control. MNNG was selected as positive control (0, 0.5, 1, 1.5 and 2 μg/ml). Negative control was DMSO. Cells were later sub-cultured, re-plated in a 10 cm-petri dish and after a 7-day expression period, 2.0×10^5 cells were exposed

to TG at a final concentration of 10 µg/mL in a 15 ml medium. Cell were incubated and after 7 days, fixed with formal and stained with Giemsa. For each data point, three dishes were used. The mutant frequency is expressed as number of mutant cells per number of surviving cells. A result is classified as positive if there is a concentration-related or reproducible increase in mutant frequency observed. Positive results for the HPRT-mutation assay indicate that the test substance induces gene mutations in the cultured cells used. Test is repeated in three consecutive days and the mean of three assays were given.

Statistical Analysis

The results were expressed as mean±SD for three independent experiments and the differences between the groups were determined by Mann-Whitney test using a Statistical Package for Social Sciences Program (SPSS) version 17.0 for HPRT test. p-values <0.05 were considered as statistically significant.

RESULTS

To evaluate the results, the fold increase of the revertant *S. typhimurium* colonies should be ≥2-fold of the baseline OD and then was considered to be positive. According to our results, none of tested concentrations of the compound caused ≥2-fold increase compared to baseline OD (Figure 1). Significant increases in the number revertant bacteria were not observed for any concentrations of 3,5-DMAP (2.5, 5, 10, 25, 50, 100 µg/ml), with and without the addition of S9 microsomal fraction. Although 3,5-DMAP caused a dose-dependent increase in the mutation frequency, particularly in both TA98 (without S9 mix) and TA100 strains (without S9 mix), the increases were not statistically significant as the baseline mutations were not 2 times higher compared to negative control (as indicated in the test protocol).

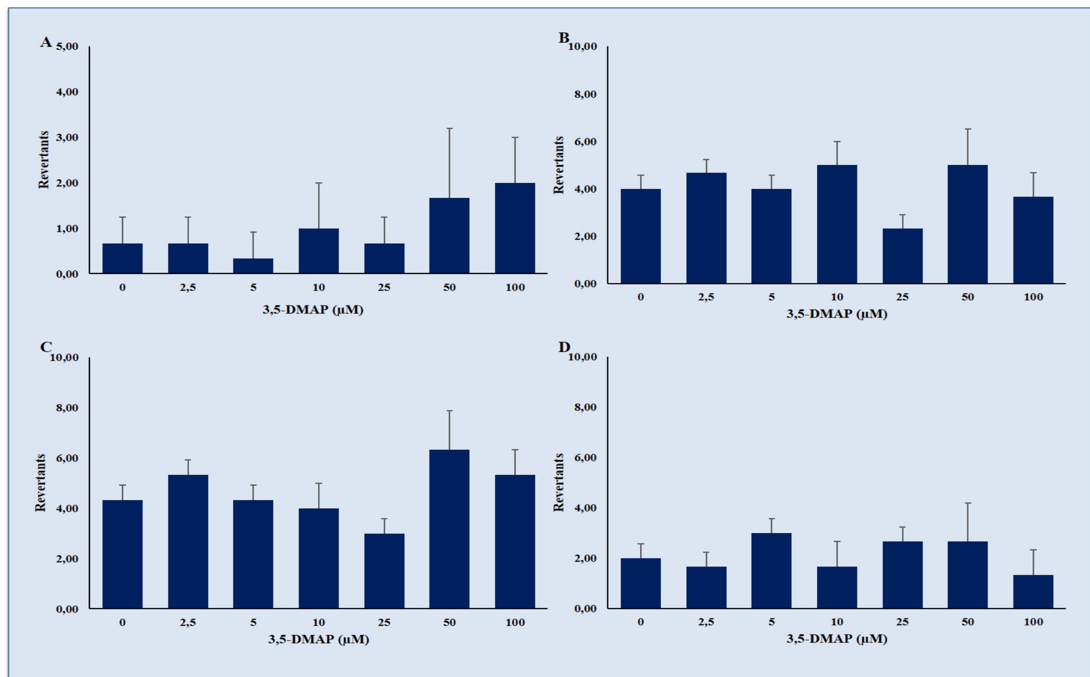

Figure 1. Quantification of Revertant Colonies by Ames Test.

A. Revertant colonies after application of different concentrations of 3,5-DMAP to Salmonella typhimurium TA 98 strain (without S9).

B. Revertant colonies after application of different concentrations of 3,5-DMAP to Salmonella typhimurium TA 98 strain (with S9).

C. Revertant colonies after application of different concentrations of 3,5-DMAP to Salmonella typhimurium TA 100 strain (without S9).

D. Revertant colonies after application of different concentrations of 3,5-DMAP to Salmonella typhimurium TA 100 strain (with S9).

The cell survival in 3,5-DMAP and MNNG applied cells were given in Table 1. These results showed that the HPRT mutation frequency caused by 3,5-DMAP is not statistically significant when compared to MNNG.

Table 1. The cell survival in 3,5-DMAP and MNNG applied cells in HPRT test.

Dose (µg/ml)	RCS (%)	CE (%)	NMC	MF (%)(/10^6)
3,5-DMAP				
0	100	95,71	5	5,22*
2.5	89,71	95,12	5	5,26*
5	83,44	94,22	6	6,37*
10	72,17	93,94	7	7,45*
25	61,46	92,73	8	8,63*
50	49,80	91,70	10	10,91*
100	26,83	85,13	28	32,89*
MNNG				
0	100	10,7	5	46,73
0.5	80,50	55,12	31	56,24
1	27,13	44,22	34	76,89
1.5	15,40	33,94	51	150,27
2	4,62	19,73	101	511,91

RCS: Relative cell survival as a % of the negative control; CE: Cloning efficiency; MNNG: N-Methyl-N′-nitro-N-nitrosoguanidine; NMC: Number of mutant clones; MF (%): Mutant frequency calculated as NMC/CE.

*different than the MNNG applied group ($p<0.05$).

DISCUSSION

Alkylanilines are widely used in industry. Occupational exposure to alkylanilines (i.e. hairdressers, hair saloon workers, chemical manufacturing plant workers) and polycyclic aromatic hydrocarbons (PAHs) are suggested to be cause serious health outcomes, including cancer (Burger et al. 2013; Jiang et al. 2007). Among all the alkylanilines, 3,5-DMA and 2,6-dimethylaniline (2,6-DMA) were particularly suggested to cause alterations in bladder, through different mechanisms (Carreón et al. 2014). The main compounds are generally regarded as weak toxicants; however this may not be valid for their metabolites (Jiang et al. 2007).

3,5-DMA is used in the production of azo dyes, pharmaceuticals, antioxidants, detergents, wood preservatives, textiles, metal complexes and antiozonants (Government of Canada 1993). Studies have suggested that exposure to its main metabolite, namely to 3,5-DMAP, induces an imbalance in cellular antioxidant/oxidant status causing a dose-dependent cytotoxicity in different types of CHO cells (Chao et al. 2012; Chao et al. 2014; Chao et al. 2015; Erkekoglu et al. 2014). Besides, 3,5-DMAP caused apoptosis as evidenced by increases in caspase 3 and caspase 8 activities and by increases in apoptotic cells determined by flow cytometry (Chao et al. 2012; Chao et al. 2014; Chao et al. 2015; Erkekoglu et al. 2014). However, in literature there is no study that show the possible mutagenic effect of 3,5-DMAP on *Salmonella* strains.

The mutagenic potential of the chemicals can be tested on DNA as it is chemically very similar in all biologic systems. Thus, different types bacteria are used as a first step in identifying potential human mutagens and carcinogens before developing cancer. Ames test is a useful method in identification of the classes of mutagenic compounds by using mutant strains of the bacteria *S. typhimurium*. The mutant bacteria are unable to synthesize the amino acid histidine in culture medium. However, it is possible to reverse this mutation with the gene regaining its function. These revertants are able to grow on a medium lacking histidine. If the test chemical is mutagenic to the strain of bacteria, the number of histidine-independent colonies will be significantly grown on those plates compare to the corresponding control plates for that strain of bacteria. The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control (Gold et al., 1992; Johann et al., 2016; McCann and Ames, 1976). Positive test indicates that the chemical is mutagenic and therefore may act as a carcinogen, because cancer is often linked to mutation. The test serves as a quick and convenient assay to estimate the carcinogenic potential of a compound because standard carcinogen assays on mice and rats are time-consuming and expensive. However, false-positives and false-negatives are known as *S. typhimurium* is a prokaryote, therefore it is not a perfect model for humans. Rat liver S9 fraction is sometimes added to the media if the metabolite, not the main compound, is

suspected to cause mutations; however, the differences in the xenobiotic metabolizing enzymes between human and rat can affect outcome of the experiments and the mutagenicity of chemicals being tested (Ames and Gold, 1990; Mortelmans and Zeiger, 2000).

The Ames test was basically developed using agar plates (the plate incorporation technique). However, nowadays a new version of the Ames test is being used and it is known as the "fluctuation method" or "Ames II assay". Fluctuation method is performed entirely in liquid culture by using 96-well or 384-well microplates. The results are comparable to the traditional pour plate method in terms of sensitivity and accuracy. Besides, it requires less test sample, has a simple colorimetric endpoint andi it is preferred because of its robustness (Bridges, 1980). Flückiger-Isler et al. (2004) tested 19 coded chemicals in an international collaborative study for their mutagenic activity by using the fluctuation method (TA98 and TAMix) throughout the experiments. A comparison of the test outcome of the different investigators resulted in an inter-laboratory consistency of 89.5%. The researchers concluded that owing to the high concordance between the two test systems and the low inter-laboratory variability in the results, this method is an effective screening alternative to the standard Ames test, requiring less test material and labor. Kamber et al. (2009) compared the traditional version of Ames test to 384-well plate microfluctuation method. There was 84% agreement between the two procedures in identifying mutagens and non-mutagens, which is equivalent to the intra- and interlaboratory reproducibility of 87% for the traditional test.

In the present work, we used 384-well plate micro fluctuation method as it gives faster and robust results. We did not observe significant increases in 3,5-DMAP-induced mutations in both TA98 and TA100 *S. typhimurium* strains when there was no S9 mix in the media (Figure 1 and Figure 2). Previously, we observed that 3,5-DMAP at 25 µM dose (p>0.05) but not at 50 µM, induced mutation frequency in AA8 CHO cells and ascorbic acid was found to be partially protective against this effect at 25 µM dose but not at 50 µM dose of 3,5-DMAP (Chao et al. 2015).

In HPRT assay, mutant cells are selected by incubation with a purine analogue (i.e. TG) that is toxic to normal cells but not to mutants. The HPRT mutation assay has been already successfully applied to the evaluation of different chemicals. Although there are several studies on the DNA damaging effects of aminophenols, few studies have been conducted to analyze their potential mutagenic effects at gene level. Majeska and Holden (1995) used HPRT assay in CHO and L5178Y tk +/- mouse lymphoma cells to determine the gene mutation frequency caused by aminophenol (PAP), a metabolite of aniline and acetaminophen. In CHO cells, however, there was no increase in TG-resistant cells at dose levels that reduced cell survival to <20%. These results are consistent with published reports on PAP. While the CHO cells were slightly more resistant to the toxic effects of PAP, the dose levels used in the two cell lines did not differ by more than 2-fold. At equivalent survival levels, PAP induced a significant (up to 20% aberrant cells) number of aberrations, primarily complex rearrangements, in both cell lines. The results obtained in this study seems to be in accordance with our results.

In conclusion, this is the first study that shows 3, 5-DMAP was not mutagenic in bacteria (also and mammalian cells) although it is cytotoxic in mammalian cells. Therefore, we can suggest that as 3, 5-DMAP is not mutagenic but cytotoxic, 3, 5-DMAP or its derivatives may have an anticancer effect. However, comprehensive studies are needed to prove this assumption. Furthermore, it should be tested in different mammalian cells, including human bladder cells for its mutagenic potential. Our future aim is to conduct mechanistic *in vitro* and *in vivo* studies that can provide more mechanistic information on the mode of action of 3,5-DMAP.

REFERENCES

Ames BN. What are the major carcinogens in the etiology of human cancer? Environmental pollution, natural carcinogens, and the causes of human cancer: six errors. *Important Adv Oncol* 1989:237-47.

Ames BN, Gold, LS. Chemical carcinogenesis: Too many rodent carcinogens. *Proc Nat Acad Sci USA* 87: 7772–7776, 1990.

Bridges BA. The Fluctuation Test. *Biomed Life Sci* 46: 41–44, 1980.

Burger M, Catto JWF, Dalbagni G, Grossman HB, Herr H, Karakiewicz P, et al. Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol* 63: 234–241, 2013.

Castelao JE, Yuan JM, Skipper PL, Tannenbaum SR, Gago-Dominguez M, Crowder JS, Ross RK, Yu MC. Gender- and smoking-related bladder cancer risk. *J Natl Cancer Inst* 93: 538-545, 2001.

Chao MW, Erkekoglu P, Tseng CY, Ye W, Trudel LJ, Skipper PL, Tannenbaum SR,Wogan GN. Protective effects of ascorbic acid against the genetic and epigenetic alterations induced by 3,5-dimethylaminophenol in AA8 cells. *J Appl Toxicol* 35: 466-477, 2015.

Chao MW, Erkekoglu P, Tseng CY, Ye W, Trudel LJ, Skipper PL, Tannenbaum SR, Wogan

GN. Intracellular generation of ROS by 3,5-dimethylaminophenol: persistence, cellular response, and impact of molecular toxicity. *Toxicol Sci* 141 :300-313, 2014.

Chao MW, Kim MY, Ye W, Ge J, Trudel LJ, Belanger CL, Skipper PL, Engelward BP, Tannenbaum SR, Wogan GN. Genotoxicity of 2,6- and 3,5-dimethylaniline in cultured mammalian cells: the role of reactive oxygen species. *Toxicol Sci* 130: 48-59, 2012.

Cui L, Sun HL, Wishnok JS, Tannenbaum SR, Skipper PL. Identification of adducts formed by reaction of N-acetoxy-3,5-dimethylaniline with DNA. *Chem Res Toxicol* 20: 1730-1736, 2007.

Erkekoglu P, Chao MW, Ye W, Ge J, Trudel LJ, Skipper PL, Kocer-Gumusel B, Engelward BP, Wogan GN, Tannenbaum SR. Cytoplasmic and nuclear toxicity of 3,5-dimethylaminophenol and potential protection by selenocompounds. *Food Chem Toxicol* 72: 98-110, 2014.

Flückiger-Isler S, Baumeister M, Braun K, Gervais V, Hasler-Nguyen N, Reimann R, Van Gompel J, Wunderlich HG, Engelhardt G. Assessment of the performance of the Ames II assay: a collaborative study with 19 coded compounds. *Mutat Res* 558: 181-197, 2004.

Gold LS, Manley NB, Ames BN. Extrapolation of carcinogenicity between species: qualitative and quantitative factors. *Risk Anal* 12: 579-588, 1992.

Government of Canada. 1993. Canadian Environmental Protection Act: Priority Substances List Assessment Report – 3,5´-Dimethylaniline. Environmental Health Center, Health Canada, Ottawa, Ontario, Canada, 10 pp. http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/contaminants/psl1-lsp1/3_5_ dimethylaniline/3_5_ dimethylaniline_e.pdf

Jiang X, Yuan JM, Skipper PL, Tannenbaum SR, Yu MC. Environmental tobacco smoke and bladder cancer risk in never smokers of Los Angeles County. *Cancer Res* 67: 7540-7545, 2007.

Johann S, Seiler TB, Tiso T, Bluhm K, Blank LM, Hollert H. Mechanism-specific and whole-organism ecotoxicity of mono-rhamnolipids. *Sci Total Environ* 548-549:155-163, 2016.

Johnson GE. Mammalian cell HPRT gene mutation assay: test methods. Methods Mol Biol. 2012;817:55-67.

Kamber M, Flückiger-Isler S, Engelhardt G, Jaeckh R, Zeiger E. Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. *Mutagenesis* 24(4):359-366, 2009.

Majeska JB, Holden HE. Genotoxic effects of p-aminophenol in Chinese hamster ovary and mouse lymphoma cells: results of a multiple endpoint test. Environ Mol Mutagen. 1995;26(2):163-70.

McCann J, Ames BN. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals: discussion. *Proc Natl Acad Sci U S A* 73: 950-954, 1976.

Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res* 455: 29-60, 2000.

Nestmann ER, Brillinger RL, Gilman JP, Rudd CJ, Swierenga SH. Recommended protocols based on a survey of current practice in genotoxicity testing laboratories: II. Mutation in Chinese hamster ovary, V79 Chinese hamster lung and L5178Y mouse lymphoma cells. Mutat Res. 1991 Feb;246(2):255-84.

OECD. Organization for Economical Cooperation and Development. Ninth addendum to the OCDE Guidelines for the testing of chemicals vitro Mamm. Cell. Gene Mutat. Test. 1997, 476.

Skipper PL, Kim MY, Sun HL, Wogan GN, Tannenbaum SR. Monocyclic aromatic amines as potential human carcinogens: old is new again. *Carcinogenesis* 31: 50-58, 2010.