Protective Effects of Different Antioxidants against the Molecular Toxicity, Genetic and Epigenetic Alterations induced by 3,5-dimethylaminophenol- A Review of the Recent Work

By Pınar Erkekoglu[*]
Ming-Wei Chao[†]
Belma Koçer-Gümüşel[‡]
Chia-Yi Tseng[+]
Wenjie Ye[•]
Laura J. Trudel[♦]
Paul L. Skipper[°]
Steven R. Tannenbaum[♠]
Gerald N. Wogan[♣]

Extensive human exposure to the monocyclic aromatic amines (MAAs), particularly to 3,5-dimethylaniline (3,5-DMA) has been clearly demonstrated and was significantly associated with bladder cancer. One of the mechanisms underlying their toxicity is suggested to be oxidative stress. 3,5-dimethylaminophenol (3,5-DMAP) is the major metabolite of 3,5-DMA. In mammalian cells, 3,5-DMAP became embedded in the cellular matrix in a form capable of continued redox cycling and induced dose-dependent increase of apoptosis mediated via caspase-3 activation. 3,5-DMAP also caused alterations in the antioxidant enzyme activities, decreases in reduced glutathione levels and increases in lipid peroxidation and protein oxidation. Cellular stress caused by 3,5-DMAP caused both genetic and epigenetic changes, which can eventually lead to cancer. Ascorbic acid and different selenocompounds were found to be protective its toxicity. The protection was observed for both nucleus and cytoplasm, suggesting that the amelioration arises from their antioxidant effects on different subcellular fractions.

Keywords: *alkylaniline, antioxidant, 3,5-dimethylaminophenol, epigenetic alterations*

Introduction

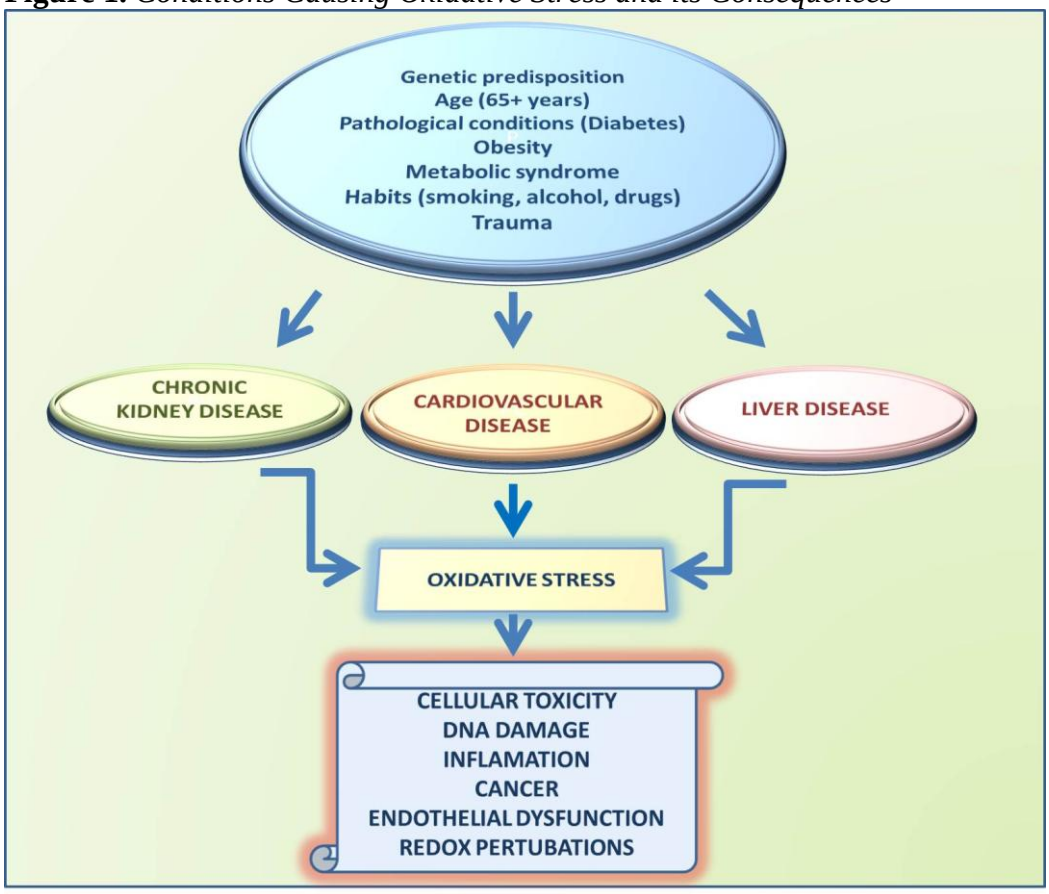
Reactive Oxygen Species

The term "free radical" refers to any molecular species characterized by the presence of one or more unpaired electrons. The "unstable" chemical nature of these radicals gives them the property of being highly reactive to major macromolecules. Thus, this phenomenon can lead to oxidative stress,

[*]Department of Biological Engineering, Massachusetts Institute of Technology, USA and Hacettepe University, Faculty of Pharmacy, Department of Toxicology, Turkey.
[†]Department of Bioscience Technology, Chung Yuan Christian University, Taiwan.
[‡]Hacettepe University, Faculty of Pharmacy, Department of Toxicology, Turkey.
[+]Department of Biomedical Engineering, Chung Yuan Christian University, Taiwan.
[•]Department of Biological Engineering, MIT, USA.
[♦]Department of Biological Engineering, MIT, USA.
[°]Department of Biological Engineering, MIT, USA.
[♠]Department of Biological Engineering, MIT, USA.
[♣]Department of Biological Engineering, MIT, USA.

eventually causing cellular toxicity and genotoxicity (Evans and Halliwell 2001). Conditions causing oxidative stress and its consequences are shown in Figure 1.

Figure 1. *Conditions Causing Oxidative Stress and its Consequences*



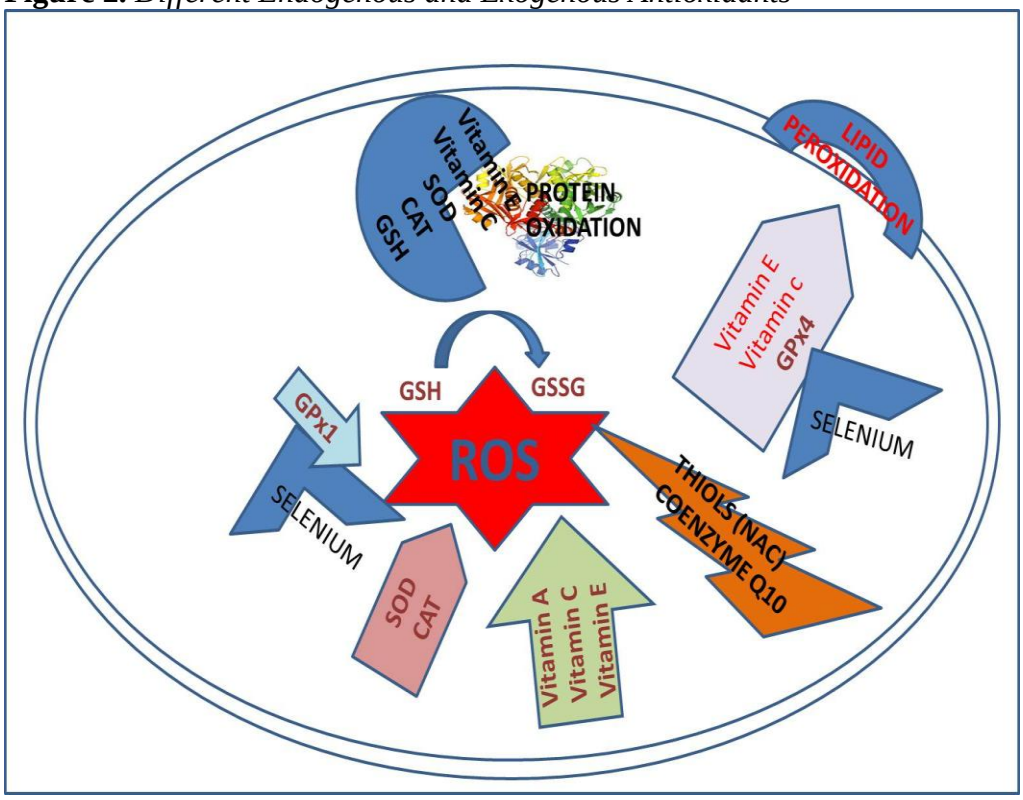
Several potentially damaging species (i.e. reactive oxygen species, ROS) can arise from normal metabolism or from environmental exposure to chemicals (Evans and Halliwell 2001). Several types of ROS contribute to primary or secondary oxidative stress. Hydroxyl radical (•OH) is formed from hydrogen peroxide (H_2O_2) and it is the most detrimental ROS, due to its high interaction with nucleic acids, proteins, and lipids. Superoxide anion ($O_2^{\cdot-}$) is particularly important as the product of the one-electron reduction of dioxygen O_2, which occurs widely in nature (Evans and Halliwell 2001). $O_2^{\cdot-}$ and •OH are recognized as inducers of mutagenesis by direct chemical reaction with DNA. Besides, H_2O_2 can also induce mutagenesis and DNA damage (Maynard et al. 2009).

Antioxidants

The function of antioxidant is to protect the cells from the damage caused by free radicals (Halliwell 2011). If not counteracted by antioxidants, oxidative stress might cause acute damage to vital macromolecules (i.e. DNA, RNA,

proteins, and lipids) (Kern and Kehrer 2005). Low antioxidant levels as well as genetics may contribute to the risk of several types of malignancies (Baliga et al. 2007). Different endogenous and exogenous antioxidants are shown in Figure 2.

Figure 2. *Different Endogenous and Exogenous Antioxidants*



Notes: CAT: catalase; GPx1: glutathione peroxidase 1; GPx4: glutathione peroxidase 4; GSH: reduced glutathione; GSSG: oxidized glutathione; NAC: N-acetyl cysteine; ROS: reactive oxygen species; SOD: superoxide dismutase.

Ascorbic acid (vitamin C, Asc) is a water-soluble dietary antioxidant (Erkekoglu and Baydar 2010). Typically it reacts with •OH (Blokhina et al. 2003). Thus, Asc is able to suppress ROS-induced DNA damage efficiently (Crott and Fenech 1999). Asc also acts with vitamin E, to quench free radicals and prevent LP and protein oxidation (Niki et al. 1995).

The requirement for selenium has been known for several decades. Non-toxic supplementation with either organic or inorganic forms could reduce cancer incidence following exposure to a wide variety of carcinogens (El-Bayoumy and Sinha 2004). Although most of its chemopreventive mechanisms remain unclear, the protective effects of selenium seem to be primarily associated with its presence in the glutathione peroxidases (GPxs), and thioredoxin reductases (TrxRs) (Negro 2008).

N-acetylcysteine (NAC) is an antioxidant that is also used as a nutritional supplement. It is also used in the biosynthesis of glutathione (GSH), which is most abundant cellular thiol. Cellular thiols play important roles in maintaining the cellular redox status (Kerksick and Willoughby 2005).

Cellular Oxidation

If the oxidative stress surpasses the cell's ability of detoxification, intracellular ROS increases and this leads to lipid peroxidation (LP) and/or protein oxidation. LP refers to the oxidative degradation of lipids, which leads to cellular membrane damage (Marnett 1999). LP may lead to protein oxidation. However, protein oxidation can arise independently. Intracellular accumulation of oxidatively modified proteins disrupts the function of cells either by loss of catalytic and structural integrity or by interruption of regulatory pathways. The levels of the oxidized proteins are usually quantitated by measurement of the protein carbonyl content (Stadtman and Levine 2000).

Cellular Antioxidant Enzymes

Intrinsic antioxidant systems, such as enzymatic antioxidants, endogenously produced antioxidants and dietary antioxidants, provide an extensive array of protection that counteracts potentially injurious oxidizing agents (Halliwell and Cross 1994). The enzymatic antioxidants include selenoenzymes, catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione S-transferase (GST). The most important endogenously produced antioxidants are glutathione (GSH) and cellular thiols. Some of the dietary antioxidants are vitamins and trace elements. All together, these antioxidants provide a balance in cellular oxidation state and are protective against the toxic potential of different agents both *in vitro* and *in vivo* (Erkekoglu et al. 2010a, Erkekoglu et al. 2010b).

Genetic and Epigenetic Alterations Caused by Exposure to Environmental Chemicals

After exposure to oxidative chemicals, high levels of ROS may emerge within the cell and this can be followed by damage to DNA. DNA damage may result in disturbances of several physiological processes and may lead to several degenerative diseases, including cancer (Soory 2009).

Multiple lines of evidence from *in vitro* and *in vivo* studies have established that epigenetic modifications caused by exposure to environmental toxicants can induce alterations in gene expression that may be persistent. Thus, the environmentally induced epigenetic changes become increasingly relevant to human health and disease (Liu et al. 2008).

Monocyclic Aromatic Amines

Monocyclic aromatic amines (MAAs) contain a single aromatic ring and at least one amine function to the ring. They are the simplest forms of aromatic amines. Small modifications of the molecular structure greatly affect their mutagenicity (Chung et al. 1997).

These chemicals are abundantly found derivatives of azo dyes that are widely used in textiles, pharmaceuticals, cosmetics, plastics, paper, leather, printing inks, paints, varnishes, lacquers and wood staining (Chung et al. 1997). Cigarette smoking and permanent hair dyes are also high exposure routes to MAAs (Castelao et al. 2001). Moreover, MAAs are also released into

the environment when azo dyes are discharged into the rivers (Chung et al. 1997). Many of the MAAs are toxic, mutagenic and/or carcinogenic to humans and animals (Chung 1983, Reid et al. 1984).

Alkylanilines

Alkylanilines are MAAs that are widely present in the environment, tobacco smoke and hair dyes. They are the leading putative factors responsible for bladder cancer development in smokers and among people who are highly exposed to these dyes (Gago-Dominguez et al. 2001).

2,6-dimethylaniline (2,6-DMA) is an abundant industrial alkylaniline. It is also a metabolite of lidocaine and xylazine. It is an impurity in technical grade pesticide "metalaxyl" and produced as a metabolite to metalaxyl (Bryant et al. 1988, Headley et al. 1996). 2,6-DMA forms hemoglobin adducts and their levels were found to be significantly higher in patients receiving lidocaine vs. control (Bryant et al. 1994). However, 2,6-DMA–hemoglobin adducts are also present in population who are not exposed to lidocaine. This phenomenon suggests that 2,6-DMA exposure can also be attributed to other sources (Bryant et al. 1988). 2,6-DMA was suggested to be involved in human carcinogenesis due to the results of toxicological studies (Bryant et al. 1988).

3,5-dimethylaniline (3,5-DMA) is used as an intermediate in the manufacture of azo dyes. 3,5-DMA does not persist in the environment since, by analogy to aniline, it should readily undergo biological degradation in water and soil, and photolysis in water, air and on soil. The primary metabolite of is 3,5-DMA is 3,5-dimethylaminophenol (3,5-DMAP) (Chao et al. 2012). This metabolite is potentially genotoxic by a mechanism involving nonenzymatic oxidation to the iminoquinone and quinone, which are strongly electrophilic species (Chao et al. 2012, Ye et al. 2012).

Subchronic oral exposure of rats to 3,5-DMA caused hemolysis of erythrocytes, and liver and kidney damage, including massive liver necrosis and necrosis of kidney papilla. Information on the mutagenicity/genotoxicity of 3,5-DMA is limited. Although Zimmer et al. (1980) reported that 3,5-DMA was not mutagenic in various strains (TA98, TA100, TA1537) of *Salmonella typhimurium* (either with or without metabolic activation), Zeiger et al. (1988) reported "weak" mutagenic activity of 3,5-dimethylaniline (94% purity) in *Salmonella typhimurium* in the presence of metabolic activation. The i.p. administration of 3,5-dimethylaniline (100 mg/kg b.w.) to male mice had no effect upon the synthesis of testicular DNA (Seiler 1979).

The results of a non-occupational epidemiologic study, in which exposure to alkylanilines were determined through hemoglobin adduct assays, showed that three of these compounds, namely 2,6-DMA, 3,5-DMA, and 3-ethylaniline (3-EA), were significantly and independently associated with bladder cancer incidence in Los Angeles County (Gan et al. 2004). Besides, exposure assessment based on hemoglobin adduct levels also indicates that tobacco smoke is a distinct source for many alkylanilines and that the levels of 3,5 DMA hemoglobin adducts were increased in women who regularly used permanent hair dyes (Gan et al. 2004).

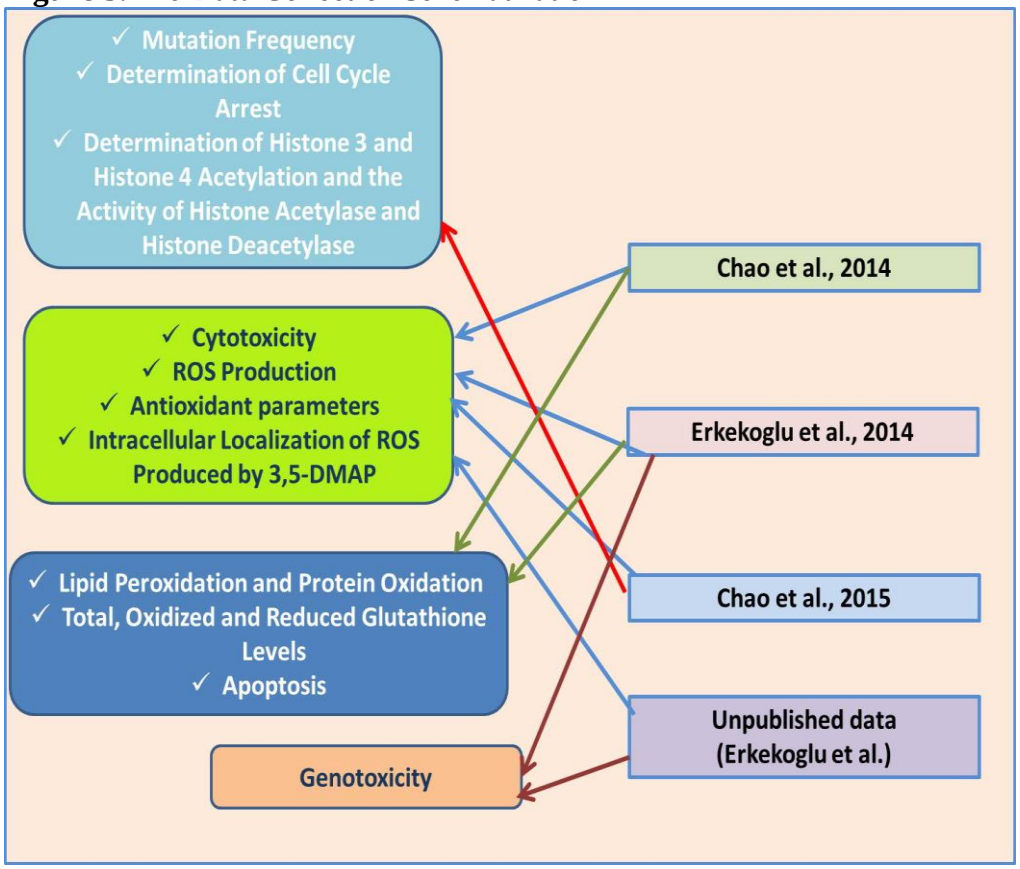
Antioxidants against the Toxicity of 3,5-Dimethylaminophenol

There is considerable interest in using different antioxidant molecules that might prevent cytotoxicity, genotoxicity and cancer. Using antioxidants against the toxic effects of alkylanilines and their metabolites (particularly 3,5-DMAP) has been a research topic for our group in the last years. Recently, we have shown that treatments with ROS scavengers including NAC and selenocompounds can protect AS52 cells from the oxidative damage caused by 3,5-DMAP (Chao et al. 2014, Erkekoglu et al. 2014).

Data Collection

We have used the data obtained from our previous studies (Chao et al. 2014, Erkekoglu et al. 2014, Chao et al. 2015) while explaining the possible toxic effects of 3,5-DMAP and the protective effects of antioxidants against its toxicity. In addition, in this review we have briefly given the unpublished data concerning the toxicity of 3,5-DMAP and antioxidant effects of selenocompounds obtained from primary human bladder cells. The data collection was schematized in Figure 3.

Figure 3. *The Data Collection Schematization*



Results

Molecular Toxicity, Genetic and Epigenetic Alterations Induced by 3,5-Dimethylaminophenol

Cytotoxicity

Previously, we determined the cytotoxic potential of 3,5-DMAP in different cell lines. The parental chemical, 3,5-DMA, as well as the N-OH-3,5-DMA and 3,5-DMAP all caused dose-dependent decreases in cell survival, but with very different potencies: 3,5-DMAP had the highest potency (half maximal inhibitory concentration (IC_{50}):40 μM); N-OH-DMA intermediate (IC_{50}: 250 μM); and 3,5-DMA lowest (IC_{50}: 1500 μM) in nuclear excision repair (NER)-proficient CHO AA8, and NER-deficient UV5 cells. The cell viability of AA8 cells decreased gradually in 7 days after they were exposed to 50 μM of 3,5-DMAP. Only 25% of the cells were viable vs. control on day 3 and 10% of the cells were viable vs. control on the 7^{th} day (Chao et al. 2014). In another study, the IC_{50} of 3,5-DMAP was 25 μM in CHO AS52 cells (Erkekoglu et al. 2014). In human primary bladder cell line, the IC_{50} of 3,5-DMAP was 50 μM and it seems that human cells seem to be more resistant to the cytotoxicity of this compound (unpublished data).

With two different protocols, possible protective effects of Asc against the ROS-producing and cytotoxic effects of 3,5-DMAP were investigated by measuring intracellular ROS production and cell viability. In the first protocol, after AA8 cells were exposed to 3,5-DMAP (5, 10, 25, 50, and 100 μM) for 1 h, intracellular ROS production and cell survival were measured immediately. In the second protocol, after cells were exposed to 3,5-DMAP (5, 10, 25, 50, and 100 μM) for 1 h, cells were incubated for an additional 24 h. The two experiments gave similar results with the increase of both intracellular ROS and decrease of cell viability in a dose-dependent manner. However, concurrent application of 3,5-DMAP and Asc provided complete inhibition of ROS and cytotoxicity in both cell types except for the highest dose (100 μM) of 3,5-DMAP (Chao et al. 2015).

ROS Production

After 1 h exposure to 3,5-DMAP (50 μM), 80% of cells were fluorescent in both AA8 and UV5 cells. In both cases, it appears that ROS production occurs in both nucleus and cytoplasm (Chao et al. 2014). However, in AS52 cells 25 μM 3,5-DMAP treatment caused a significant increase in intracellular ROS generation after 1 h exposure, showing AS52 cells are more sensitive to the toxicity of 3,5-DMAP. On the other hand, both organic (selenomethionine, SM, 10 μM) and inorganic (sodium selenite, SS, 30 nM) selenocompounds were able to decrease ROS production in AS52 cells (Erkekoglu et al. 2014).

Intracellular Localization of ROS Produced by 3,5-DMAP

Intracellular localization of ROS was investigated at 24 h after a 1 h exposure to 3,5-DMAP in both AA8 and UV5 cells by imaging cells with

epifluorescence microscopy. The antioxidant effect of NAC was also determined. At the end of the experiments, responses were the same in AA8 and UV5 cells. After 3,5-DMAP (50 μM) exposure, 80% of cells were fluorescent. However, when cells were treated with 50 μM 3,5-DMAP plus 5 mM NAC or with 5 mM NAC alone, ROS production was undetectable. In both cases, it appears that ROS production occurs in both nucleus and cytoplasm. On the other hand, confocal microscopy revealed that there was evidence of ROS production within the cell nucleus (Chao et al. 2014).

Mutation Frequency

Aprt mutagenicity of 3,5-DMAP with or without Asc was investigated in nucleotide repair-proficient AA8 cells. 25 μM 3,5-DMAP caused almost 2 times increase in the mutation frequency vs. negative control. However, no further induction of *Aprt* mutations was observed with increasing doses of 3,5-DMAP. On the other hand, AA8 cells treated two different doses of 3,5-DMAP (25 μM and 50 μM) along with Asc showed slight dose-dependent decreases in *Aprt* mutagenesis, but with no significant difference was observed when compared to control (Chao et al. 2015).

Determination of Cell Cycle Arrest

3,5-DMAP was found to cause cytotoxicity and modification of histones. We, therefore, examined whether it could affect the progression of AA8 cells through the cell cycle. 3,5-DMAP caused a decrease in the DNA content distribution (at 2 N) and this phenomenon indicated that 3,5-DMAP could affect the cell cycle progression. After 3,5-DMAP (10 and 25 μM) doses exposure, the percentage of cells entering G1 phase raised from 42% to 72%. However, percentage of cells in S and G2/M phase decreased from 40% to 23% and 16% and 3.7%, respectively. These alterations revealed that 3,5-DMAP treatment caused G1 phase arrest. However, when 3,5-DMAP was applied with Asc, the percentage of cells in S and G2/M phases raised from 23% to 44% and 3.7% to 15% ($p<0.01$), respectively (Chao et al. 2015).

Determination of Histone 3 and Histone 4 Acetylation and the Activity of Histone Acetylase and Histone Deacetylase

We wanted to explore whether 3,5-DMAP caused alterations in the Lys of histone 3 (H3) and histone 4 (H4) by changing in the activity of histone-related acetylation enzymes, namely histone acetylase (HAT) and histone deacetylase (HDAC). After 3,5-DMAP treatment (25 μM), H3 and H4 were hyperacetylized (126% and 133%, respectively vs. control), indicating that 3,5-DMAP was able to bind onto the Lys of H3 and H4. On the other hand, when 3,5-DMAP was applied along with Asc, H3 and H4 acetylation levels were similar to that of control. We also observed marked enhancement in HAT activity and marked decreases in HDAC activity after 3,5-DMAP exposure. Meanwhile, HAT and HDAC activities remained at control levels when 3,5-DMAP was applied with Asc (Chao et al. 2015).

Lipid Peroxidation and Protein Oxidation

As an indicator of LP, thiobarbituric acid reactive substance (TBARS) levels showed marked increases after 3,5-DMAP exposure in both cytoplasm and nucleus of AA8 cells. Asc with 3,5-DMAP–treated cells showed significant decreases in TBARS levels in both of the cell fractions when compared to cells treated only with 3,5-DMAP (Chao et al. 2015). The same phenomenon was also observed in UV5 (Chao et al. 2014), AS52 (Erkekoglu et al. 2014) and human bladder cells (unpublished data).

After 3,5-DMAP exposure, protein oxidation in AA8 cells increased significantly in both cytoplasm and nucleus. Asc with 3,5-DMAP exposure provided marked decreases in protein oxidation levels of both of the cellular fractions when compared to 3,5-DMAP treatment alone (Chao et al. 2015). Selenium (SS and SM) was found to protective against the protein oxidation caused by 3,5-DMAP in both AS52 (Erkekoglu et al. 2014) and human bladder cells (unpublished data).

Total, Oxidized and Reduced Glutathione Levels

3,5-DMAP (0 to 100 μM) caused dose-dependent decreases in GSH and increases in GSSG in AA8 and UV5 cells after 1 h (Chao et al., 2014). Total GSH levels showed marked increases in both cytoplasm and in nucleus with 3,5-DMAP exposure vs. control in AA8 cells. Besides, oxidized glutathione (GSSG) levels showed marked increases in both of the cellular fractions after 3,5-DMAP when compared to control. Asc supplementation was found to protective against the toxicity of 3,5-DMAP by decreasing the cytoplasmic and nuclear GSSG and increasing GSH levels. Asc also provided marked increases in [GSH]/[GSSG] redox ratio of both of the cellular fractions (Chao et al. 2015). In both AA8 and UV5 cells, NAC also provided higher levels of GSH and lower levels of GSSG in 3,5-DMAP-treated cells when compared to cells treated with 3,5-DMAP (Chao et al. 2014).

AS52 cells treated with 3,5-DMAP (25 μM) also showed decreased GSH and increased GSSG levels and both organic (SM) and inorganic (SS) selenocompounds provided a higher redox ratio when applied with 3,5-DMAP (Erkekoglu et al. 2014). Interestingly, total thiol levels were not affected by any of the treatment conditions in AA8, UV5 and AS52 cells (Chao et al. 2014, Erkekoglu et al. 2014).

Apoptosis

Apoptotic cells caused by exposure to 3,5-DMAP after 1 h and 24 h were identified by flow cytometric analysis of cells after Annexin V-FITC and PI staining. After 24 h, both AA8 cells and UV5 cells showed a dose-dependent increase in apoptosis (Chao et al. 2014). In accordance, 3,5-DMAP (50 μM) also activated caspase 3 activity. Meanwhile, 3,5-DMAP plus NAC treatment significantly suppressed caspase 3 activity (Chao et al. 2014). On the other hand, 3,5-DMAP (50 μM) also caused increases in caspase 3 and 8 activities in AS52 cells, showing this compound can activate the extrinsic caspase pathway (Erkekoglu et al. 2014).

Genotoxicity

Using a high-throughput Comet assay, we observed that 3,5-DMAP treatment induced genotoxicity in CHO AS52, UV5 and human bladder cells. Selenocompounds (both SS and SM) provided decreases in reduce single-strand breaks caused by 3,5-DMAP (Erkekoglu et al. 2014, Chao et al., 2012, unpublished data).

Antioxidant Parameters

3,5-DMAP caused marked decreases in nuclear and cytoplasmic GPx1 and TrxR activities in both AA and AS52 cells. Both Asc and selenocompounds provided significant increases in the selenoenzyme activities in AA8 and AS52 cells, respectively (Chao et al. 2015, Erkekoglu et al. 2014).

With 3,5-DMAP exposure, both CAT and GR activities decreased in cytoplasmic and nuclear fractions vs. control in both AA8 and AS52 cells. However, Asc or selenocompounds with 3,5-DMAP provided significant increases in both cytoplasmic and nuclear CAT activity compared to 3,5-DMAP treated groups in both of the cells. On the contrary, SOD activity increased in both cytoplasmic and nuclear fractions after exposure to 3,5-DMAP in both AA8 and AS52 cells. Both Asc or selenocompounds with 3,5-DMAP significantly decreased SOD activity in both cytoplasmic and nuclear fractions, compared to 3,5-DMAP treated groups (Chao et al. 2015, Erkekoglu et al. 2014). Only cytoplasmic GST activity decreased in AA8 cells after 3,5-DMAP exposure. When compared to 3,5-DMAP treatment alone, Asc with 3,5-DMAP exposure provided significant increases in GST activity in the both cytoplasm and nucleus. Neither cytoplasmic nor nuclear GST activities were affected from 3,5-DMAP in AS52 cells (Chao et al. 2015, Erkekoglu et al. 2014).

Discussion

If not counteracted by cellular antioxidants, oxidant environmental chemicals can cause cellular toxicity and damage to important biomolecules. Finally, this phenomenon may lead to cell death (Kern and Kehrer 2005). Both low antioxidant status and genetic tendency are contributing factors to the risk of several types of malignancies (Baliga et al. 2007).

Asc is a well-known antioxidant, effective against several ROS-generating agents (Blokhina et al. 2003, Erkekoglu and Baydar 2010c). Selenium is the key component of several antioxidant enzymes. GPxs and TrxRs protect the body from cellular metabolism's endogenous by-products, associated with DNA damage and carcinogenesis (Ganther 1999, Jablonska et al. 2009). Selenium is also protective against the toxic effects of different agents both *in vitro* and *in vivo* (Erkekoglu et al. 2010a, Erkekoglu et al. 2010b). NAC is the precursor of GSH and is effective against the toxicity of several chemical agents (Hernández et al. 2015).

In the last years, we focused on developing strategies to prevent the cytotoxicity, ROS and genotoxicity induced by alkylanilines and their metabolites. We therefore tried to find the right antioxidants and doses with minimal risk or toxicity. Our goal was to search whether cell culture supplementation with right doses that are similar to human plasma concentrations (or plasma concentrations that counterparts after human supplementation doses) can overcome the toxic effects of alkylanilines or not. We applied 3,5-DMAP to AA8, UV5, AS52 and human bladder cells to determine cytotoxicity, ROS generation, genotoxicity (DNA damage and mutagenesis), cell cycle arrest, apoptosis and epigenetic alterations caused by this particular alkylaniline metabolite (Chao et al. 2014, Chao et al. 2015, Erkekoglu et al. 2014, unpublished data). 3,5-DMAP caused cytotoxicity and intracellular ROS production (in both nucleus and cytoplasm) in all of the cell types. 3,5-DMAP caused increases in LP, protein oxidation and GSSG, decreases in reduced GSH and cellular redox ratio, alterations in antioxidant enzyme activities, genotoxicity and apoptosis. Asc and selenocompounds encountered the oxidative stress caused by 3,5-DMAP by decreasing intracellular ROS generation and therefore by preventing cellular oxidation. Besides, Asc with 3,5-DMAP provided a normalized cell cycle and HAT and HDAC activities and decreased the *Aprt* mutation frequency.

Taken together, at least one of the underlying mechanisms of 3,5-DMAP is oxidative stress. This mechanism is observed in many cell types including cancer and primary cell cultures. Right doses of Asc, selenocompounds and NAC seem to prevent high levels of ROS generation and thus, enable a balanced intracellular oxidation state. More studies are needed to discover the other underlying mechanisms of alkylaniline toxicity and its relationship with bladder cancer. Our future aim is to discover the mechanisms underlying the toxicity of different MAAs, particularly 3-EA and 2,6-DMA, and to see whether antioxidant therapy will be beneficial to overcome their toxic effects.

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