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3,5-Dimethyaminophenol is not Mutagenic in Ames Test and HPRT Test and may have Anti-Carcinogenic Potential Against Lung Cancer Cells †

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Abstract: Exposure to 3,5-dimethylaminophenol (3,5-DMAP), the metabolite of the 3-5-dimethylaniline, was shown to cause high levels of oxidative stress in different cells. However, we have shown that this alkylaniline metabolite was non-mutagenic to different strains of Salmonella typhimurium in Ames test and also was found to be not mutagenic to CHO cells in HPRT test. Concerning all the available data, we aimed to observe whether this metabolite may have anti-carcinogenic potential in human non-small cell lung cancer line (A549 cells). 3,5-DMAP caused a dose-dependent increase in cytotoxicity and generation of superoxide (O_2-.) and reactive oxygen species (ROS). 3,5-DMAP did not produce significant cytotoxicity to human lung fibroblasts even at very high concentrations; however showed higher cytotoxic effect on A549 lung cancer cells at the same concentrations. 3,5-DMAP also led to molecular events, like increases in apoptotic markers (i.e., p53, Bad, Bax and cytochrome and decreases anti-apoptotic proteins (Bcl-2). Furthermore, 3,5-DMAP provided significant decreases in cell viability of A549 cells and eventually inhibited growth of A549 cells in an in vivo mouse model. Tumor sections showed that 3,5-DMAP down-regulated c-Myc expression but up-regulated p53 and cytochrome c, all of which might result in tumor growth arrest. In conclusion, our findings demonstrate 3,5-DMAP is not mutagenic to Salmonella typhimurium and CHO cells; toxic to A549 cells and therefore may have anti-cancer properties, the importance of which should be elucidated with further mechanistic studies.

Keywords: 3,5-dimethylaminophenol; alkylaniline; cytotoxicity; apoptosis; Ames test; HGPRT test

1. Introduction

Lung cancer is the primary cause of cancer-related deaths worldwide [1,2]. Active and passive smoking are the two of primary causes of lung cancer [1,2]. Lung cancers are classified as small cell (non-epithelial) or non-small cell carcinomas (epithelial-derived) [3]. Small cell carcinomas are highly malignant; has the ability to metastasize easily and chemotherapy is the choice of treatment [3]. However, treatment of non-small cell cancer primarily involve surgical excision, supplemented by radiation or chemotherapy [4]. Although this treatment method may provide partial or full recovery, it also increases the risk for concurrent diseases [5]. Thus, high efficacy of an anti-cancer drug is the most priority goal in this field. Alkylanilines are classified in the general chemical group "monocyclic aromatic amines" and also under the sub-group of "alkylanilines". These chemicals are present in the environment as well as in cigarette smoke [6,7]. 3,5-dimethyaminophenol (3,5-DMAP) is the main metabolite of 3,5-dimethylaniline (3,5-DMA), which is one of the most abundant alkylanilines in the environment. 3,5-DMA is present in cigarette smoke and is used in the production of different industrial chemicals [6,7]. Recently, we have conducted experiments using Chinese hamster ovary (CHO) cells, revealing an alternative mechanism for cytotoxic and genotoxic effects of 3,5-DMAP [6,7]. Although it was first suggested that the widely accepted toxicity mechanism of phenolic metabolites of the anilines (particularly by 3,5-DMAP) was covalent DNA adduct formation, the production of high intracellular reactive oxygen species (ROS) seems to be the predominant toxicity mechanism of these compounds [6,7].

Due to the results of our previous studies, we can suggest that 3,5-DMAP or its derivatives might have the potential to be used as drug/drug precursor against lung cancer, due to its high cytotoxic potential. Considering all the available data, this study was designed to investigate the mutagenic potential of 3,5-DMAP in bacteria and mammalian cells. Moreover, the anti-cancer effects of 3,5-DMAP on cytotoxicity and apoptosis in lung cancer A549 cells were also determined.

2. Materials and Methods

Ames test and HPRT test: A renewed version of Ames test (384-well plate microfluctuation method, Ames MPF™ 98/100 kit, Ames microplate fluctuation) was used throughout the experiments. Salmonella typhimurium bacterial strains, TA98 and TA100, were used to determine base-pair and frame shift mutations, respectively. A standard HGPRT mutation assay is used to evaluate the potential of 3,5-DMAP to induce mutations at the hgprt locus of CHO cells.

A549 cells were used for cytotoxicity tests. Cells were cultured in Ham's F-12K medium with 10% FBS (at 37 °C, 5% CO_2 and in a humidified atmosphere). A549 cells were treated with 3,5-DMAP in serum-free medium for 1 h followed by additional 24 h for the recovery in culture medium. Cell viability was detected using a commercial MTS assay. Intracellular ROS and superoxide (O_2-.) detection studies were performed by using commercial kits. To determine apoptosis, total cell lysate proteins were extracted and collected after the treatment with 3,5-DMAP. Total protein concentrations of cell lysates were detected by BCA assay. Later, the cell lysates were denatured, loaded onto SDS polyacrylamide gels and thereafter, samples were transferred to PVDF membranes for electrophoresis and primary antibodies of for apoptotic proteins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were probed and visualized. For tumorigenicity testing, nude mice (4-week-old, female) were randomly divided into 4 groups: Group 1: A549 cells were injected for tumor formation as control; Group 2: A549 cells were pretreated with 25 μM of 3,5-DMAP in vitro for 24 h; Group 3: A549 cells were pretreated with 50 μM of 3,5-DMAP in vitro for 24 h; Group 4: A549 cells were pretreated with 50 μM cisplatin in vitro for 24 h. A549 cells (2 × 106) cells were injected in PBS (0.2 mL) per site sc into the dorsal flank of nude mice. The volume of tumor and the weight of nude mice were examined every two days, for 32 days. Growth inhibition (GI), expressed as a percentage of control tumor volumes.

3. Results

In Ames test (Figure 1) and HPRT test (Table 1), 3,5-DMAP was not found to be mutagenic. The cell viability were reduced as the 3,5-DMAP dosage increased, but cytotoxicity was not observed in HLF cells at the doses <25 μM.

Although the mechanism of 3,5-DMAP's lower cytotoxicity towards non-cancerous cells remains unknown, these results mean that 3,5-DMAP can efficiently kill A549 lung cancer cells without causing decreases in the viability of non-cancer, particularly at 25 μM. Therefore, 25 μM of 3,5-DMAP was used as the exposure dose for the further experiments. We also determined that A549 cells had high levels of intracellular ROS and O_2.- following 3,5-DMAP exposure. In a dose dependent manner, apoptotic (Bax, Bad, cytochrome c and p53) protein levels were found to be markedly increased, while anti-apoptotic (Bcl-2) protein expression was decreased after 1 h exposure to 3,5-DMAP. Considering our results, it can be suggested that p53 tumor suppressor protein accumulates immediately and causes cell growth arrest. In nude mice after 32 days, 3,5-DMAP had the ability to inhibit tumor growth. Tumor sections showed that 3,5-DMAP down-regulated c-Myc expression but up-regulated p53 and cytochrome c, all of which might result in tumor growth arrest [8].

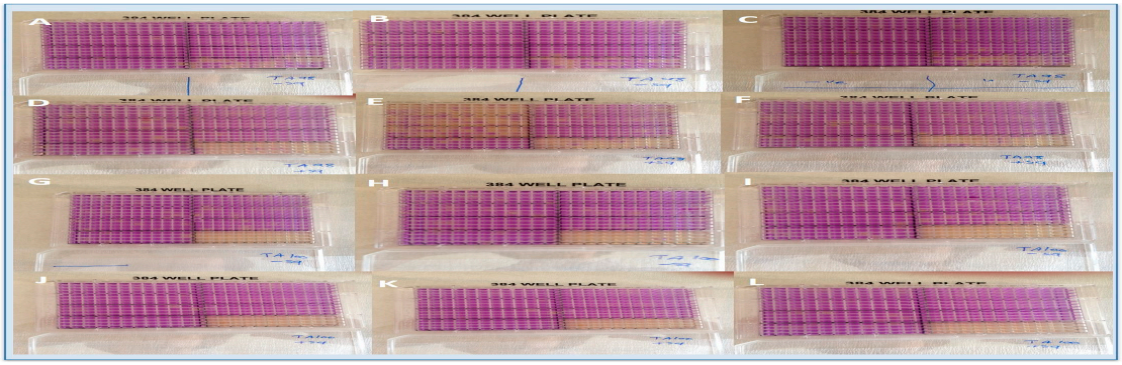

Figure 1. Ames test results for different doses of 3,5-DMAP. In none of the applied doses, 3,5-DMAP was found to be mutagenic.

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

Dose of 3,5-DMAP (μg/mL)	MNNG's MF vs. 3,5-DMAP (-fold)
2.5	196.13 *
5	80.36 *
10	68.71 *
25	59.31 *
50	46.92 *
100	16.88 *

Calculations were made according to the highest dose of MNNG (2 μg/mL) applied to CHO cells. t-test was used for statistical comparison. MF (%): Mutant frequency calculated as NMC/CE. NMC: Number of mutant clones; CE: Cloning efficiency; * different than the MNNG applied group ($p < 0.05$).

4. Discussion

3,5-DMAP causes ROS production in A549 cells. The intracellular oxidation state may be possibly induced by the particular redox cycle of 3,5-DMAP to 3,5-DMQI resulting in cytotoxicity in this particular cancer cell line [6,7]. This phenomenon can further trigger molecular events, which might eventually cause initiation of apoptosis as also suggested previously for other cell lines [6,7]. Moreover, 3,5-DMAP could inhibit tumor growth and reduce the size of tumors in vivo [8]. These findings suggest that 3,5-DMAP or its derivatives might be anti-cancer drug candidates [8]. However, further experiments (particularly on anti-cancer effect—structure relationship) are needed to show the anti-carcinogenic effects of 3,5-DMAP and whether 3,5-DMAP or its analogues are promising agents for the treatment of different types of cancers, particularly for cancer of the lung.

Author Contributions: P.E. and M.-W.C. conceived and designed the experiments; P.E., S.S., Ö.K. and B.K.-G. conducted Ames test; PE conducted HPRT test; C.-Y.T., P.-Y.L., Y.-J.C., and C.-H.L., and Y.-C.C. performed the experiments; P.E., S.S. and B.k.-G. analyzed the data, P.E. and M.-W.C. wrote the paper.

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