Published in final edited form as:

Toxicol Pathol. 2016 August ; 44(6): 835–847. doi:10.1177/0192623316637708.

Molecular Changes in the Nasal Cavity after N,N-Dimethyl-p-toluidine Exposure

June K. Dunnick¹, B. Alex Merrick², Amy Brix³, Daniel L. Morgan⁴, Kevin Gerrish⁵, Yu Wang⁶, Gordon Flake⁶, Julie Foley⁶, and Keith R. Shockley⁷

¹Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

²Biomolecular Screening Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

³Experimental Pathology Laboratories, Inc., Research Triangle Park, North Carolina

⁴NTP Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

⁵Molecular Genomics Core, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

⁶Cellular and Molecular Pathology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

⁷Biostatistics and Computational Biology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

Abstract

N,N-Dimethyl-p-toluidine (DMPT) (Cas No. 99-97-8), an accelerant for methyl methacrylate monomers in medical devices, is a nasal cavity carcinogen in a 2-year cancer study in male and female F344/N rats, with the nasal tumors arising from the transitional cell epithelium. In this study we exposed male F344/N rats for five days to DMPT (0, 1, 6, 20, 60 or 120 mg/kg (oral gavage)) to explore early changes in the nasal cavity after short-term exposure. Lesions occurred in the nasal cavity including hyperplasia of transitional cell epithelium (60 and 120 mg/kg). Nasal tissue was rapidly removed and preserved for subsequent laser capture microdissection and isolation of the transitional cell epithelium (0 and 120 mg/kg) for transcriptomic studies. DMPT transitional cell epithelium gene transcript patterns were characteristic of an anti-oxidative damage response (e.g. *Akr7a3*, *Maff*, *Mgst3*), cell proliferation, and decrease in signals for apoptosis. Amino acid transporters transcripts were upregulated (e. g. *Slc7a11*). The DMPT nasal transcript expression pattern was similar to that found in the rat nasal cavity after formaldehyde exposure with over 1000 transcripts in common. Molecular changes in the nasal cavity after DMPT exposure suggest that oxidative damage is a mechanism for the DMPT toxic and/or carcinogenic effects.

Keywords

N,N-Dimethyl-*p*-toluidine; nasal cavity toxicity; molecular markers

INTRODUCTION

N,N-Dimethyl-*p*-toluidine (DMPT) (Figure 1) is a high production chemical used as an accelerant in methyl methacrylate monomers in medical devices. DMPT exposure is of concern because it can leach out from medical devices and dental materials (Wang *et al.*, 2013). DMPT has other commercial uses and is listed in over 900 patents with the U. S. Patent Office (U. S. Patent and Trademark Office, 2015 (accessed March 2015)). Oral exposure to DMPT, a non-genotoxic chemical, produced liver cancer in rats (at 60 mg/kg) and mice (at 20 and 60 mg/kg) and nasal cavity cancer in rats (at 60 mg/kg) in a 2-year cancer study (National Toxicology Program, 2012). The State of California has listed DMPT as chemical known to cause cancer (Office of Environmental Health Hazard Assessment, 2014) based on the results of the DMPT rodent cancer study (National Toxicology Program, 2012).

After oral administration, DMPT is rapidly absorbed in the rat and is excreted in the urine (Dix *et al.*, 2007). The major DMPT metabolite in the urine is *p*-(*N*-acetylhydroxyamino)hippuric acid. Two lesser metabolites are *N,N*-dimethyl-*p*-toluidine *N*-oxide and *N*-methyl-*p*-toluidine (Kim *et al.*, 2007). After oral administration, DMPT is carcinogenic in the nasal cavity and liver of the rat. The DMPT toxicity and carcinogenic response in the nasal cavity occurred in the transitional epithelium (National Toxicology Program, 2012). The transitional cell epithelium is composed of two cell layers of nonciliated cuboidal or columnar surface cells and basal cells with a scarcity of mucous secretory cells (Hardisty *et al.*, 1999, Harkema *et al.*, 2006). It is found on the lateral walls of the nasal cavity and on turbinate tips. In a series of National Toxicology Program (NTP) cancer studies other chemicals were also found to cause nasal tumors. Often the origin of the nasal cavity tumors in rats is not specified or apparent. Compounds investigated by NTP that cause nasal cavity epithelial tumors include cumene (TR 542; inhalation study), 1,2-dibromo-3-chloropropane (TR 206; inhalation study), 1,2-dibromoethane (TR 210; inhalation study), 2,3-dibromo-1-propanol (TR 400; dermal study), dimethylvinyl chloride (DMVC) (TR 306; oral gavage study), 1,4-dioxane (TR 80; drinking water study), 1,2-epoxybutane (TR 306; inhalation study), naphthalene (TR 410; inhalation study), 2,6-xylydine (TR 278; feed study), and propargyl alcohol (TR 552; inhalation study) (National Toxicology Program, 2014).

The current work describes molecular changes in the rat nasal cavity transitional epithelium after five days of DMPT oral exposure. Rapid dissection was used to capture and preserve a sample of nasal tissue for subsequent laser capture microdissection to obtain transitional cell epithelium tissue for RNA extraction and toxicogenomic evaluation. Methemoglobin was added as an endpoint because DMPT has previously been shown to cause methemoglobinemia in rodents (National Toxicology Program, 2012). This paper expands our knowledge on the use of transcriptomic findings to identify early changes in the nasal

cavity that can elucidate mechanisms or identify early disease biomarkers. While this study focuses on early exposure transcriptomic patterns, other human exposure scenarios may involve longer-term exposure to DMPT. The objective of this study was to evaluate if early molecular changes in the nasal cavity transitional cell epithelium in rats after a 5-day DMPT oral exposure would help to elucidate pathways and mechanisms leading to toxicity.

MATERIALS AND METHODS

Experimental design

N,N-dimethyl-p-toluidine (Sigma-Aldrich, St. Louis, MO, lot #C15Y012; >99% pure) was prepared for oral gavage administration in corn oil (Welch, Holme & Clark Co., Inc. Newark, NJ; Lot# 12-542) to deliver DMPT at doses of 0, 1, 6, 20, 60, or 120 mg/kg/day for five consecutive days to male F344/N rats in a volume of 2.5-mL/kg body weight. The high dose of 120 mg/kg was selected as a five-day maximum tolerated dose based on the findings from a 13-week DMPT study in which the body weights of male F344/N rats at the end of a 125 mg/kg exposure were 12% lower than body weights of controls (National Toxicology Program, 2012). The lower doses (1–60 mg/kg) were selected to give a response over a broad dose range. Male F344/N rats (5 animals/dose level) were obtained from Taconic Laboratory, Germantown, NY. Five male rats were added to the high dose and control groups for frozen nasal tissue collection and RNA extraction. At the start of the study the animals were 5–6 weeks of age. The animals were housed two per cage. Tap water and NTP-2000 diet (Zeigler Brothers, Inc. Gardners, PA) were made available ad libitum.

The care of animals on this study was according to NIH procedures as described in the “The U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals”, available from the Office of Laboratory Animal Welfare, National Institutes of Health, Department of Health and Human Services, RKL1, Suite 360, MSC 7982, 6705 Rockledge Drive, Bethesda, MD 20892-7982 or online at <http://grants.nih.gov/grants/olaw/olaw.htm#pol>. The protocol was approved by the laboratory where the animals were housed and dosed (Alion Science and Technology Animal Care and Use Committee).

Methemoglobin assay

Blood was collected from the retroorbital plexus for methemoglobin determination from the core rats according to the methods of Evelyn and Malloy (Evelyn and Malloy, 1938). Disodium hydrogen phosphate, potassium dihydrogen phosphate, acetic acid, aqueous sodium cyanide and aqueous potassium ferricyanide were purchased from Sigma Chemical Company, St. Louis, MO.

Nasal Cavity Tissue Collection

Animals were euthanized 24 hours after the last dose under CO₂ gas. After decapitation, the skin, mandible and nasal vestibule were removed from the head. Animals not designated for laser capture microdissection (LCM) were infused with 10% NBF through the nasopharyngeal groove and immersed in the fixative for 72 hours. Following fixation, the heads were decalcified in a 3:1 Formical 2000™ (Decal Chemical Corporation, Tallman, NY)/1X PBS (HyClone™ Laboratories Inc., Logan, UT) solution for 60 hours. Following a

rinse in water, the nasal cavities were trimmed to obtain representative sections of Levels 1, 2 and 3. For Level 1, a cut was made directly behind the upper incisor teeth as described in traditional rat nasal cavity trimming (Boorman *et al.*, 1990). Level 2 trimming was modified from the traditional protocol. Instead of cutting directly behind the incisive papilla, the cut was made between the incisive papilla and the first palatal ridge. For Level 3, the tissue trimming was modified to section directly in front of the first molar and not the traditional method of sectioning through the 2nd molar. These modifications were made to obtain improved sections of the respiratory and ethmoid turbinates respectively. Representative sections of the nasal cavity at levels 1, 2 and 3 were routinely processed and paraffin-embedded.

Nasal Cavity Laser Capture Microdissection (LCM)

For the additional control and high dose group animals designated for frozen nasal cavity tissue, transitional epithelium from level 1 was collected by laser capture microdissection (LCM) and RNA extracted. Nasal cavity tissue was collected within 5 minutes to preserve RNA integrity. For LCM studies, the skin, mandible and nasal vestibule were removed from the head. A cut along the sagittal suture at the anterior and posterior ends initiated the splitting of the calvarium. Placing the head on the dorsal surface, a disposable razor blade was placed between the incisors, along the palatine suture of the hard and soft palate. A cut through the hard and soft palate, divided the head into 2 sagittal sections. Each half of the head was placed with the cut surface up in a self-made styrofoam mold. Naso- and maxillo-turbinates were removed, and the epithelium along with submucosal tissue stripped from the inner wall of the calvarium at levels 1 and 2 of the nasal cavity. Tissue strips were placed in a cryomold on dry ice, filled with O.C.T. (Sakura Finetek U.S.A., Inc., Torrance, CA) and stored at -80°C. Tissue strips were collected and frozen within 5 minutes after euthanasia.

The nasal cavity membrane strips were sectioned at 8 µm on the cryostat, with H&E stains performed on every fifth section. Unstained sections were placed on PET foil slides (ACSS, TN) for LCM. The H&E-stained MAP sections were examined microscopically, and the transitional epithelium, identified and marked on the coverslip as a guide for the laser microdissection. Laser microdissection of the transitional epithelium was done on cresyl violet-stained sections.

The presence of transitional epithelium varied from one section to the next with the epithelium often absent from early sections and sometimes depleted from the deeper sections of the frozen block. Transitional epithelium was identified by the cuboidal nature of the epithelium, as opposed to the taller, columnar appearance of the respiratory epithelium. Other features used for identification of transitional epithelium included no or rare ciliated cells and goblet cells, and the usual appearance of 2 cell layers (basal cell and cuboidal cell). Only transitional epithelium on the outer surface of the stripped membranes was labeled and microdissected to facilitate both the labeling and the microdissection process. Transitional epithelium from approximately 40 sections per sample was pooled for the microarray analysis. The amount of RNA obtained for each nasal cavity was 5.7–12.6 ng.

Nasal Cavity RNA Extraction and analysis

After laser capture microdissection of the nasal cavity transitional cell epithelium RNA was extracted from the control and 120 mg/kg groups (according to the NTP protocol for laser capture microdissected samples (<http://www.niehs.nih.gov/research/resources/protocols/extraction/picoscale/index.cfm>)). The RNA was analyzed for quantity and purity by UV analysis using the NanoDrop ND-1000 (NanoDropTechnologies, Wilmington, DE). Samples were concentrated using Microcon filters (Millipore, Billerica, MA). All samples were evaluated for RNA integrity by gel electrophoresis using the Flash Gel RNA cassette system (Lonza, Rockland, ME).

Microarray analysis

Gene expression analysis was conducted using Affymetrix Rat Genome 230 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA). Four nanograms (4 ng) of total RNA was amplified as directed in the WT-Ovation Pico RNA Amplification System protocol, and labeled with biotin following the Encore Biotin Module. Five micrograms (5 µg) of amplified biotin-aRNAs were fragmented and hybridized to each array for 18 hours at 45°C in a rotating hybridization. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip® Hybridization, Wash and Stain Kit and user manual following protocol FS450-0004. Arrays were scanned in an Affymetrix Scanner 3000.

Data normalization

Probe intensity data from all Rat Genome 230 version 2 Affymetrix GeneChip® arrays were read into the R software environment (<http://www.R-project.org>) directly from CEL files using the R/affy package (Gautier *et al.*, 2004). Probe-level data quality was assessed using image reconstruction, histograms of raw signal intensities and hierarchical clustering of samples. Normalization was carried out using the robust multi-array average (RMA) method using all probe intensity data sets together (Irizarry *et al.*, 2003). The RMA method adjusts the background intensities of perfect match (PM) probes, applies a quantile normalization, and calculates final expression measures using the Tukey median polish algorithm. RMA scatterplots were used as an additional quality control measure.

Statistical assessment of differential gene expression

Gene expression between control and treated nasal cavities was made for each probe set using a bootstrap t-test approach. Pairwise tests were conducted while controlling the false discovery rate (FDR) at the 5% level. All statistical calculations were performed in the ORIOGEN software package using 10,000 bootstrap samples (Peddada *et al.*, 2005).

Data were analyzed using the Ingenuity Pathway Analysis database of biologic networks (www.ingenuity.com) and Nextbio (www.nextbio.com), a web-based tool that can be used to compare an experimental gene expression signature to thousands of genomic signatures derived from published microarray data sets in public databases (Kupersmidt *et al.*, 2010). This data mining approach is based on rank-based statistical procedures and is analogous to the Gene Set Enrichment method (Subramanian *et al.*, 2005). NextBio uses a Running Fisher algorithm that can accommodate information from both the query and target sequences, and

can incorporate data of different sizes and filter thresholds, in order to find the most significant signal in a ranked gene expression signature. The resulting correlation scores describe the association between a given gene set and an extensive set of publicly available studies (Kupersmidt *et al.*, 2010).

The upstream regulator analysis presented here is based on known relationships between transcriptional factor molecules and targets stored in the Ingenuity Knowledge Base. For each transcription factor, an overlap p-value is used to compare the overlap between the number of known transcription factor targets and the number of targets found in the DMPT gene list.

Nanostring analysis

Nanostring nCounter is a hybridization-based platform for multiplexing gene expression from total RNA (NanoString®; Seattle, WA; www.nanostring.com). Non-crossreactive probes are custom synthesized and mixed for validation transcripts and normalization (e.g. housekeeping) transcripts. Microarray results were confirmed for nasal epithelium RNA samples with using the nCounter platform by utilizing a Custom CodeSet consisting of 12 DMPT transcripts found to be significantly altered from control after microarray analysis that included: *Spr1a*, *Slc7a11*, *Akr7a3*, *Cyp3a13*, *Maff*, *PscA*, *Krt4*, *Ngfr*, *Nrgn*, *Ernn*, *Ccno*, *GP2*. Seven housekeeping genes (*Gapdh*, *Hprt1*, *LOC100365008*, *Med15*, *Rpl7*, *Spire1* and *ZNF91L*) and a gene that was not significantly increased with DMPT (*Agm*) were used for normalization. For nasal cavity RNA samples in Nanostring analysis, 100 ng of amplified cDNA were obtained utilizing the Nugen™ Ovation Pico WTA System (v2) (Nugen Technologies, Inc., San Carlos, CA). Gene expression was quantified on the nCounter Digital Analyzer™ and raw and normalized counts were generated with nSolver (v2.5)™ software. All data passed nSolver's QA/QC. Data were normalized utilizing the manufacturer's positive and negative control probes, as well as the housekeeping genes. Subsequently, the data were imported into Partek™ Genomic Suite (6.6), quantile normalized, and further inspected for separation of control and DMPT groups.

RESULTS

Survival and Body Weights

There was no effect on survival at any DMPT exposure level in this study (all animals lived until the end of the 5-day exposure). Mean body weights for the 1, 6, 20, 60 and 120 mg/kg exposure groups were 104, 103, 92, 94, and 90% of control mean body weight at the end of the 5-day exposure. Methemoglobin levels were not increased in treated rats (data not shown).

Nasal Tissue Histopathology

Hyperplasia of the nasal cavity transitional epithelium was observed in the 60 and 120 mg/kg dose groups (Table 1 and Figure 2 A&B). Hyperplasia of the transitional epithelium was characterized by an increase in cell layers and an increase in the disorganization of the cells. Control transitional epithelium was typically 1–2 cell layers thick; whereas hyperplastic epithelium was 3 or more layers thick and the cells appeared jumbled and

disorganized, with irregular piling. Only minimal to mild severity hyperplasia of the transitional epithelium was recorded; minimal lesions tended to involve less area than mild lesions. Typically, mild lesions would involve the tips of the nasoturbinates and most of the lateral wall, while minimal lesions might be restricted to the tips of the nasoturbinates, or involve the tips of the nasoturbinates and just a small focal area of the lateral wall. Also, mild lesions tended to be thicker, typically 4 cell layers, whereas the minimal lesions were usually only 3 cell layers thick. None of the lesions had more than 4 or 5 cell layers. The disorganization of the hyperplastic transitional epithelium made determining the exact number of cell layers difficult. The hyperplastic epithelium also contained clear vacuoles within some cells, and occasionally there was a shrunken, eosinophilic cell present within the epithelial layer.

In nasal Level III, olfactory epithelial necrosis was characterized by a layer of proteinaceous substance and cell debris overlying a thin, disorganized layer of olfactory epithelium (Figure 3A–E). The remaining olfactory epithelium was hypocellular, disorganized, vacuolated, and contained individual dead cells, which were characterized by shrunken, eosinophilic round bodies. There were focal areas of attenuated epithelium in affected locations, indicative of epithelial cell loss. The dorsal meatus and immediately adjacent areas were most commonly and severely affected. Severity of olfactory epithelium necrosis was based upon the extent of the lesion, as well as the amount of changes seen in the affected area. Minimal lesions consisted of small areas of dorsal meatus olfactory epithelium characterized by a slight disorganization and vacuolation of the epithelium, which contained decreased numbers of cells within the epithelium. There was a thin layer of proteinaceous and cell debris overlying affected areas. Mild lesions involved a larger area of the dorsal meatus, and the epithelium contained obviously fewer cells. Moderate necrosis involved most of the dorsal meatus and immediately adjacent areas. There was a thick layer of debris overlying the epithelium, which was moderately attenuated in areas. The remaining epithelium was disorganized and vacuolated. The one occurrence of mild olfactory degeneration that was recorded differed from necrosis in that the epithelium appeared normal in thickness and better organized than that in the animals with necrosis. The epithelium contained numerous vacuoles, but lacked evidence of cell death and overlying debris (Figure 3F). One animal exposed to 20 mg/kg DMPT had mild dilation of the vessels in the lamina propria in nasal level III; this was recorded as angiectasis. Angiectasis was not seen in any other animals in this study.

Nasal tissue dissection from frozen specimens

Each of the 10 nasal specimens (5 control and 5 DMPT treated 120 mg/kg specimens) contained both transitional and respiratory epithelium, which varied from one section to the next (Figure 4 A&B). However, enough transitional epithelium was laser microdissected in each of the 10 specimens to allow for sufficient RNA to be extracted from the harvested material. For each specimen the frozen sections were examined blindly, and the epithelium could be correctly identified as either control or treated on the basis of changes seen in the transitional epithelium. These changes in the transitional epithelium of the treated animals included karyomegaly, crowding of nuclei, loss of basal polarity of the nuclei, and sometimes 3 layers of cells indicative of mild hyperplasia.

Nasal tissue significant gene transcripts in the nasal cavity transitional cell epithelium

Principal component analysis across the control and 120 mg/kg samples separated the samples into control and treated groups (Figure 5). Moreover, global gene expression patterns showed a clear difference between treated and control samples. Using a false discovery rate threshold of 0.05, there were 2,561 differentially expressed transcripts (corresponding to 1,730 genes) in the 120 mg/kg group (vs. control). Nanostring analysis of selected transcripts confirmed the direction of the DMPT-induced transcript change (upregulated: *Sprr1a*, *Slc7a11*, *Akr7a3*, *Maff*, *Cyp3a13*, *Psca*, *Krt4*, *Ngfr*; or downregulated: *Nrgn*, *Ermn*, *Ccno*, *GP2*) (Figure 6).

Using Ingenuity Systems Analysis, the differentially expressed transcripts mapped to 1,730 genes, with a total of 994 upregulated genes and 736 downregulated genes (Supplement 1). There were 32 transcripts upregulated > 10 fold, and 38 transcripts downregulated more than 5 fold (Table 2; Supplement 1). Genes predicted to be upstream regulators of the DMPT molecular response were *Myc* (↑ 2.1, *MYCT1*) (Fu *et al.*, 2011), *E2F* (↑ 1.7, *E2F1*; ↑ 2.0, *E2F8*) and Rb pathways (Table 3) (Ma *et al.*, 2013).

DMPT exposure caused upregulation of 35/180 transcripts (e. g. *Akr7a3*, *Maff*, *Mgft3*) in the Nrf2 pathway (Supplement 2), a primary regulator of antioxidant response (Franco *et al.*, 2014, Johnson *et al.*, 2008). Expression of other antioxidant response genes was also increased (e.g. *Srxn1*, *G6pd*, *Prdx4*) (Bensellam *et al.*, 2015).

Transcripts characteristic of epithelial-derived cancers (e. g. *Slc7A11*, *Maff*, *Igfb3*, *Igfb6*) were upregulated more than 1.5 fold (Supplement 3). Cell proliferation signals were also upregulated (Supplement 4). DMPT gene transcript changes were predicted to alter cell death, necrosis, and/or apoptosis processes (e.g. ↓ *Gp2*, *Ccno*) (Supplement 5).

In addition to upregulation of the protein transporter *Slc7a11* (↑ 17.4), other membrane transporters were also upregulated including: *Slc14a1* (↑ 4.6), *Slc4a7* (↑ 3.2), *Slc4A4* (↑ 3.0), *Slc6a9* (↑ 2.9), *Slc6a1* (↑ 2.6), and *Slc1a5* (↑ 2.5) (Supplement 1).

NextBio software was used to compare the DMPT transcript changes with thousands of other microarray datasets accessed by algorithm. NextBio analysis showed the transcriptional changes induced by DMPT were most similar to those in the nasal cavity of adult male F344Cr1BR rat following formaldehyde exposure (Andersen *et al.*, 2010) (Supplement 6). There were over 1000 shared transcripts that were significantly changed by both DMPT and formaldehyde exposure. This included upregulation of *Sprr1a*, *SLC7a11*, *Akr7a3*, *Scin*, *Maff*, *Psca*, and *Cyp3a9*, and down regulation of *Fhit*, *Nrgn*, and *Ace*. The DMPT nasal cavity used for this transcriptomic analysis was transitional epithelium, while the nasal sample in the formaldehyde transcriptomic analysis contained transitional cell epithelium and other portions of the nasal cavity (e. g. respiratory epithelium) (Andersen *et al.*, 2010).

DISCUSSION

After a 5-day DMPT exposure, nasal cavity lesions developed in male F344/N rats, including hyperplasia of the transitional epithelium and degeneration/necrosis of the olfactory epithelium (at 60 and 120 mg/kg). Nasal lesions observed in this 5-day study occurred at the same target site for nasal cancer (transitional cell epithelium) as found in the 2-year DMPT rat study (National Toxicology Program, 2012). In previous studies, after 13 weeks of DMPT exposure, there was treatment-related methemoglobinemia formation (National Toxicology Program, 2012), which occurs by oxidation of the heme moiety (Pallais *et al.*, 2011). Data from the current study support a hypothesis that DMPT nasal toxicity and carcinogenicity effects are the result of oxidative damage (Dunnick *et al.*, 2014, El-Gebali *et al.*, 2013, National Toxicology Program, 2012).

The nasal cavity contains a low level of anti-oxidant associated mucosubstances (Harkema *et al.*, 2006) making it susceptible to DMPT-induced oxidative damage. While 5-days of DMPT exposure was not sufficient for methemoglobin formation, it was sufficient for induction of nasal cavity transitional epithelium toxicity and gene transcript changes. This included activation of an Nrf2 antioxidant response, a process that protects from oxidative stress-induced cell death (Johnson *et al.*, 2008), and provides antioxidant defense and maintenance of cellular redox potential (Franco *et al.*, 2014). Upregulation of transcripts in the Nrf2 pathway included *Mgst3* (microsomal glutathione S-transferase 3), *G6pd* (glucose-6-phosphate dehydrogenase), and *Txnrd1* (thioreductase), whose protein products can function to reduce oxidative stress (Johnson *et al.*, 2008). In addition, *Nqo1* (NAD(P)H dehydrogenase, quinone 1) a major cellular detoxification transcript was upregulated (Nguyen *et al.*, 2009).

Other transcripts induced by DMPT were *Slc7a11* and *Akr7a3* (Katsuoka *et al.*, 2005). *Slc7a11* protein regulates cysteine-glutamate exchange and maintenance of glutathione levels (Huang *et al.*, 2005). *Akr7a3* expression occurs in the liver after aflatoxin treatment of rats, and is a common defense mechanism after free radical generation in several tissue types (Merrick *et al.*, 2012, Merrick *et al.*, 2013).

DMPT-induced transcripts included those associated with promoting and sustaining cell proliferation. This included increased expression of transcripts for *Maff* (a fibrosarcoma oncogene); *Ras* related oncogene (*Rhov*); *Psc* (prostate oncogene); *Casc* (cancer susceptibility candidate 3); and *Krt4* (an epithelial structural protein). Other transcripts upregulated are involved in xenobiotic metabolism (*Cyp3a13*) (Anakk *et al.*, 2003, Liu *et al.*, 2013) or fatty acid transport (*Fabp5*) (Fang *et al.*, 2010). After long-term exposure, the upregulation of *Ngfr* (nerve growth factor receptor) may also enable cell proliferation (Jobling *et al.*, 2015). Upstream regulators of the DMPT-induced nasal transcripts were predicted (based on Ingenuity Analysis) to be those involved in carcinogenesis (e. g. MYC) (Ng *et al.*, 2011).

DMPT increased solute carrier transcripts that code for membrane transporters of various cell nutrients (El-Gebali *et al.*, 2013, Hediger *et al.*, 2013). An increase in the ability to transport nutrients into the cell can provide energy and building blocks for cell proliferation

(Bhutia *et al.*, 2015). Blocking membrane transporters has been proposed as a potential target for cancer therapy (El-Gebali *et al.*, 2013).

DMPT transcript changes created a pattern consistent with changes that could eventually lead to a decrease in cell death and apoptosis including upregulation of: 1. *Scin*, a transcript coding for the scinderin protein, which renders cells resistant to cytotoxic T lymphocyte-associated lysis (Hasmim *et al.*, 2013); 2. *Srxn1* (sulfiredoxin), which codes for an enzyme that catalyzes the reduction of free radicals and prevents apoptosis (Baek *et al.*, 2012); 3. *Igfbp6* whose protein promotes cell survival and inhibits apoptosis (Xu *et al.*, 2009); and 4. *Acaa* whose protein promotes attenuation of the activity of BNIP3 a proapoptotic protein (Cao *et al.*, 2008).

DMPT-induced transcript changes suggest that events leading to apoptosis could eventually be suppressed. This included decreased expression of transcripts for: 1. *Ccno* (cyclin), involved in apoptosis induction (Roig *et al.*, 2009); 2. *Gp2* (glycoprotein 2) coding for a structural protein reported to decrease cell proliferation (Werner *et al.*, 2012); 3. *Fhit* (fragile histidine triad gene transcript) whose protein promotes apoptosis and suppresses cell proliferation (Huang *et al.*, 2014); and 4. *Ace* (angiotensin converting enzyme transcript), an enzyme which converts angiotensin I to the physiologic active peptide angiotensin II, a proapoptotic protein (Wang *et al.*, 2015, Yi *et al.*, 2014). Two other transcripts downregulated were *Ernm* whose protein is an oligodendrocyte-specific protein surrounding myelin axons necessary for action potentials (Brockschneider *et al.*, 2006) and *Nrgn* involved in promoting synaptic transmission (Zhong *et al.*, 2009).

Some of the DMPT-induced transcripts have multiple functions. For example, *Nrgn* (Parsi *et al.*, 2012) and *Bcl2L11* (Kuan *et al.*, 2000, Petrocca *et al.*, 2008) have both a proapoptotic function as well as a tumor suppressor function (Parsi *et al.*, 2012).

The DMPT transcript patterns were similar to those in the rat nasal cavity after formaldehyde exposure (Andersen *et al.*, 2010), a nasal cavity carcinogen in both rats and humans (International Agency for Research on Cancer, 2012). DMPT and formaldehyde both induced transcripts characteristic of an antioxidant response (e. g. *Akr7a3*; *Srxn1*); transcripts in cancer (e.g. *Maff*; *PscA*); and transcripts in cell proliferation and differentiation (e. g. *Sprr1a*). PSCA is usually associated with prostate cancer (Yang *et al.*, 2014) but now appears to also be an important transcript in the nasal cavity. *Sprr1a* (small proline-rich protein 1a) is considered to be an early biomarker of epithelial cell differentiation, and is also upregulated after tobacco smoke exposure (Tesfagzi *et al.*, 1996).

Downregulation of *Fhit* was seen with both formaldehyde and DMPT, and also reported to be downregulated after other carcinogen exposures (Merrick *et al.*, 2012). Additional transcriptomic studies with other nasal carcinogens would add to our knowledge on the use of early molecular makers for identifying toxic and carcinogenic processes in the nasal cavity (e. g. ↑ *Akr7a3*, *Srxn*, *Maff*; *PscA*; ↓ *Fhit*). Development of such biomarkers would help facilitate the use of fewer animals and shorter-term studies for hazard identification.

DMPT transcript changes included upregulation of anti-oxidative stress pathways. Other studies show that oxidative stress can eventually lead to DNA damage (Klaunig *et al.*, 2011)

and promote disease pathways. This is a possible mechanism for DMPT-induced disease (Figure 7). DMPT nasal cavity molecular changes were characteristic of events leading to increased cell proliferation and decreased apoptosis, often considered to be hallmarks of cancer (Hanahan and Weinberg, 2011). Molecular changes in the nasal cavity after DMPT exposure suggest that oxidative damage is a contributory mechanism for the DMPT toxicity and/or carcinogenic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The intramural program of the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC, supported this work. However, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH or the United States government. We gratefully acknowledge technical support from B. Mahler, E.Hou, R. Fannin, O. Lyght, and R. Wilson. The in-life portion of this study was conducted under NIEHS contract NO1-ES-75561 with Alion Science & Technology, Inc. We thank Dr. M. Cesta and Dr. D. Malarkey (NIEHS) for their excellent review of the manuscript.

References

- Anakk S, Kalsotra A, Shen Q, Vu MT, Staudinger JL, Davies PJ, Strobel HW. Genomic characterization and regulation of CYP3a13: role of xenobiotics and nuclear receptors. *FASEB*. 2003; 17:1736–1738.
- Andersen ME, Clewell HJ 3rd, Bermudez E, Dodd DE, Willson GA, Campbell JL, Thomas RS. Formaldehyde: integrating dosimetry, cytotoxicity, and genomics to understand dose-dependent transitions for an endogenous compound. *Toxicol Sc*. 2010; 118:716–731.i. [PubMed: 20884683]
- Baek JY, Han SH, Sung SH, Lee HE, Kim YM, Noh YH, Bae SH, Rhee SG, Chang TS. Sulfiredoxin protein is critical for redox balance and survival of cells exposed to low steady-state levels of H₂O₂. *J Biol Chem*. 2012; 287:81–89. [PubMed: 22086924]
- Bensellam M, Montgomery MK, Luzuriaga J, Chan JY, Laybutt DR. Inhibitor of differentiation proteins protect against oxidative stress by regulating the antioxidant-mitochondrial response in mouse beta cells. *Diabetologia*. 2015; 58:758–770. [PubMed: 25636209]
- Bhutia YD, Babu E, Ramachandran S, Ganapathy V. Amino Acid transporters in cancer and their relevance to “glutamine addiction”: novel targets for the design of a new class of anticancer drugs. *Cancer Res*. 2015; 75:1782–1788. [PubMed: 25855379]
- Boorman, GA., Morgan, KT., Uriah, LC. Nose, Larynx, and Trachea. In: Boorman, GA.Eustis, SL.Elwell, MR.Montgomery, CA., Jr, MacKenzie, WF., editors. *Pathology of the Fischer Rat – Reference and Atlas*. Academic Press; New York: 1990. p. 315-337.
- Brockschneider D, Sabanay H, Riethmacher D, Peles E. Ermin, a myelinating oligodendrocyte-specific protein that regulates cell morphology. *J Neurosci*. 2006; 26:757–762. [PubMed: 16421295]
- Cao W, Liu N, Tang S, Bao L, Shen L, Yuan H, Zhao X, Lu H. Acetyl-Coenzyme A acyltransferase 2 attenuates the apoptotic effects of BNIP3 in two Bhuman cell lines. *Biochim Biophys Acta*. 2008; 1780:873–880.
- Dix KJ, Ghanbari K, Hedtker-Weber BM. Disposition of [14C]N,N-dimethyl-p-toluidine in F344 rats and B6C3F1 mice. *J Toxicol Environ Health A*. 2007; 70:789–798. [PubMed: 17454555]
- Dunnick JK, Brix A, Sanders JM, Travlos GS. N,N-dimethyl-p-toluidine, a component in dental materials, causes hematologic toxic and carcinogenic responses in rodent model systems. *Toxicol Pathol*. 2014; 42:603–615. [PubMed: 23867143]
- El-Gebali S, Bentz S, Hediger MA, Anderle P. Solute carriers (SLCs) in cancer. *Mol Aspects Med*. 2013; 34:719–734. [PubMed: 23506905]

- Evelyn KA, Malloy HT. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. *J Biol Chem.* 1938; 126:655–662.
- Fang LY, Wong TY, Chiang WF, Chen YL. Fatty-acid-binding protein 5 promotes cell proliferation and invasion in oral squamous cell carcinoma. *J Oral Pathol.* 2010; 39:342–348.
- Franco R, Bortner CD, Schmitz I, Cidlowski JA. Glutathione depletion regulates both extrinsic and intrinsic apoptotic signaling cascades independent from multidrug resistance protein 1. *Apoptosis.* 2014; 19:117–134. [PubMed: 24146141]
- Fu S, Guo Y, Chen H, Xu ZM, Qiu GB, Zhong M, Sun KL, Fu WN. MYCT1-TV, a novel MYCT1 transcript, is regulated by c-Myc and may participate in laryngeal carcinogenesis. *PLoS One.* 2011; 6:e25648. [PubMed: 21998677]
- Gautier L, Cope L, Bolstad BM, Irizarry RA. affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics.* 2004; 20:307–315. [PubMed: 14960456]
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–674. [PubMed: 21376230]
- Hardisty JF, Garman RH, Harkema JR, Lomax LG, Morgan KT. Histopathology of nasal olfactory mucosa from selected inhalation toxicity studies conducted with volatile chemicals. *Toxicol Pathol.* 1999; 27:618–627. [PubMed: 10588541]
- Harkema JR, Carey SA, Wagner JG. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. *Toxicol Pathol.* 2006; 34:252–269. [PubMed: 16698724]
- Hasmim M, Badoual C, Vielh P, Drusch F, Marty V, Laplanche A, de Oliveira Diniz M, Roussel H, De Guillebon E, Oudard S, Hans S, Tartour E, Chouaib S. Expression of EPHRIN-A1, SCINDERIN and MHC class I molecules in head and neck cancers and relationship with the prognostic value of intratumoral CD8+ T cells. *BMC Cancer.* 2013; 13:592. [PubMed: 24330498]
- Hediger MA, Clemencon B, Burrier RE, Bruford EA. The ABCs of membrane transporters in health and disease (SLC series): introduction. *Mol Aspects Med.* 2013; 34:95–107. [PubMed: 23506860]
- Huang Q, Liu Z, Xie F, Liu C, Shao F, Zhu CL, Hu S. Fragile histidine triad (FHIT) suppresses proliferation and promotes apoptosis in cholangiocarcinoma cells by blocking PI3K-Akt pathway. *ScientificWorldJournal.* 2014; 2014:179698.
- Huang Y, Dai Z, Barbacioru C, Sadee W. Cystine-glutamate transporter SLC7A11 in cancer chemosensitivity and chemoresistance. *Cancer Res.* 2005; 65:7446–7454. [PubMed: 16103098]
- International Agency for Research on Cancer. IARC Monograph on the evaluation of carcinogenic risks to humans. Chemical agents and related occupations. 2012; 100F:401–435. <http://monographs.iarc.fr/ENG/Monographs/vol100F/index.php>.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics.* 2003; 4:249–264. [PubMed: 12925520]
- Jobling P, Pundavela J, Oliveira SM, Roselli S, Walker MM, Hondermarck H. Nerve-Cancer Cell Cross-talk: A Novel Promoter of Tumor Progression. *Cancer Res.* 2015; 75:1777–1781. [PubMed: 25795709]
- Johnson JA, Johnson DA, Kraft AD, Calkins MJ, Jakel RJ, Vargas MR, Chen PC. The Nrf2-ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration. *Ann N Y Acad Sci.* 2008; 1147:61–69. [PubMed: 19076431]
- Katsuoka F, Motohashi H, Ishii T, Aburatani H, Engel JD, Yamamoto M. Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. *Mol Cell Biol.* 2005; 25:8044–8051. [PubMed: 16135796]
- Kim NC, Ghanbari K, Kracko DA, Weber WM, McDonald JD, Dix KJ. Identification of urinary metabolites of orally administered N,N-dimethyl-p-toluidine in male F344 rats. *J Toxicol Environ Health A.* 2007; 70:781–788. [PubMed: 17454554]
- Klaunig JE, Wang Z, Pu X, Zhou S. Oxidative stress and oxidative damage in chemical carcinogenesis. *Toxicol Appl Pharmacol.* 2011; 254:86–99. [PubMed: 21296097]
- Kuan CY, Roth KA, Flavell RA, Rakic P. Mechanisms of programmed cell death in the developing brain. *Trends Neurosci.* 2000; 23:291–297. [PubMed: 10856938]

- Kupersmidt I, Su QJ, Grewal A, Sundaresh S, Halperin I, Flynn J, Shekar M, Wang H, Park J, Cui W, Wall GD, Wisotzkey R, Alag S, Akhtari S, Ronaghi M. Ontology-based meta-analysis of global collections of high-throughput public data. *PLoS One*. 2010; 5:e13066. [PubMed: 20927376]
- Liu H, Wang LE, Liu Z, Chen WV, Amos CI, Lee JE, Iles MM, Law MH, Barrett JH, Montgomery GW, Taylor JC, MacGregor S, Cust AE, Newton Bishop JA, Hayward NK, Bishop DT, Mann GJ, Affleck P, Wei Q. Association between functional polymorphisms in genes involved in the MAPK signaling pathways and cutaneous melanoma risk. *Carcinogenesis*. 2013; 34:885–892.
- Ma X, Gao Y, Fan Y, Ni D, Zhang Y, Chen W, Zhang P, Song E, Huang Q, Ai Q, Li H, Wang B, Zheng T, Shi T, Zhang X. Overexpression of E2F1 promotes tumor malignancy and correlates with TNM stages in clear cell renal cell carcinoma. *PLoS One*. 2013; 8:e73436. [PubMed: 24023875]
- Merrick BA, Auerbach SS, Stockton PS, Foley JF, Malarkey DE, Sills RC, Irwin RD, Tice RR. Testing an aflatoxin B1 gene signature in rat archival tissues. *Chem Res Toxicol*. 2012; 25:1132–1144. [PubMed: 22545673]
- Merrick BA, Phadke DP, Auerbach SS, Mav D, Stiegelmeier SM, Shah RR, Tice RR. RNA-Seq profiling reveals novel hepatic gene expression pattern in aflatoxin B1 treated rats. *PLoS One*. 2013; 8:e61768. [PubMed: 23630614]
- National Toxicology Program. NTP Technical Report on the toxicology and carcinogenesis studies of N,N-Dimethyl-p-toluidine (CAS NO. 99-97-8) in F344/N rats and B6C3F1/N mice. NTP TR 579. 2012
- National Toxicology Program. NTP long-term study reports and abstracts. 2014. <http://ntp.niehs.nih.gov/?objectid=084801F0-F43F-7B74-0BE549908B5E5C1C>
- Ng SB, Selvarajan V, Huang G, Zhou J, Feldman AL, Law M, Kwong YL, Shimizu N, Kagami Y, Aozasa K, Salto-Tellez M, Chng WJ. Activated oncogenic pathways and therapeutic targets in extranodal nasal-type NK/T cell lymphoma revealed by gene expression profiling. *J Pathol*. 2011; 223:496–510. [PubMed: 21294123]
- Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem*. 2009; 284:13291–13295. [PubMed: 19182219]
- Office of Environmental Health Hazard Assessment, C. Chemical Listed Effective MAY 2, 2014 as known to the State of California to cause cancer: N,N-Dimethyl-p-toluidine. 2014. http://oehha.ca.gov/Prop65/prop65_list/050214list.html
- Pallais JC, Mackool BT, Pitman MB. Case records of the Massachusetts General Hospital: Case 7–2011: a 52-year-old man with upper respiratory symptoms and low oxygen saturation levels. *N Engl J Med*. 2011; 364:957–966. [PubMed: 21388314]
- Parsi S, Soltani BM, Hosseini E, Tousi SE, Mowla SJ. Experimental verification of a predicted intronic microRNA in human NGFR gene with a potential pro-apoptotic function. *PLoS One*. 2012; 7:e35561. [PubMed: 22558167]
- Peddada S, Harris S, Zajd J, Harvey E. ORIOGEN: order restricted inference for ordered gene expression data. *Bioinformatics*. 2005; 21:3933–3934. [PubMed: 16109745]
- Petrocca F, Vecchione A, Croce CM. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. *Cancer Res*. 2008; 68:8191–8194. [PubMed: 18922889]
- Roig MB, Roset R, Ortet L, Balsiger NA, Anfosso A, Cabellos L, Garrido M, Alameda F, Brady HJ, Gil-Gomez G. Identification of a novel cyclin required for the intrinsic apoptosis pathway in lymphoid cells. *Cell Death Differ*. 2009; 16:230–243. [PubMed: 18927588]
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceed Natl Acad Sci*. 2005; 102:15545–15550.
- Tesfaigzi J, Th'ng J, Hotchkiss JA, Harkema JR, Wright PS. A small proline-rich protein, SPRR1, is upregulated early during tobacco smoke-induced squamous metaplasia in rat nasal epithelia. *Am J Respir Cell Mol Biol*. 1996; 14:478–486. [PubMed: 8624253]
- U. S. Patent and Trademark Office. U. S. patent and trademark office Patent Database. 2015. (accessed March 2015) <http://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=>

[%2Fnethtml%2FPTO%2Fsearch-bool.html&r=0&f=S&l=50&TERM1=N%2CN-Dimethyl-p-toluidine&FIELD1=&co1=AND&TERM2=&FIELD2=&d=PG01](#)

- Wang L, Yoon DM, Spicer PP, Henslee AM, Scott DW, Wong ME, Kasper FK, Mikos AG. Characterization of porous polymethylmethacrylate space maintainers for craniofacial reconstruction. *J Biomed Mater Res B Appl Biomater*. 2013; 101:813–825. [PubMed: 23359449]
- Wang X, Yuan B, Dong W, Yang B, Yang Y, Lin X, Gong G. Humid heat exposure induced oxidative stress and apoptosis in cardiomyocytes through the angiotensin II signaling pathway. *Heart Vessels*. 2015; 30:396–405. [PubMed: 24898407]
- Werner L, Paclik D, Fritz C, Reinhold D, Roggenbuck D, Sturm A. Identification of pancreatic glycoprotein 2 as an endogenous immunomodulator of innate and adaptive immune responses. *J Immunol*. 2012; 189:2774–2783. [PubMed: 22891285]
- Xu XF, Guo CY, Liu J, Yang WJ, Xia YJ, Xu L, Yu YC, Wang XP. Gli1 maintains cell survival by up-regulating IGFBP6 and Bcl-2 through promoter regions in parallel manner in pancreatic cancer cells. *J Carcinog*. 2009; 8:13. [PubMed: 19736394]
- Yang X, Guo Z, Liu Y, Si T, Yu H, Li B, Tian W. Prostate stem cell antigen and cancer risk, mechanisms and therapeutic implications. *Expert Rev Anticancer Ther*. 2014; 14:31–37. [PubMed: 24308679]
- Yi L, Li F, Yong Y, Jianting D, Liting Z, Xuansheng H, Fei L, Jiewen L. Upregulation of sestrin-2 expression protects against endothelial toxicity of angiotensin II. *Cell Biol Toxicol*. 2014; 30:147–156. [PubMed: 24838122]
- Zhong L, Cherry T, Bies CE, Florence MA, Gerges NZ. Neurogranin enhances synaptic strength through its interaction with calmodulin. *EMBO J*. 2009; 28:3027–3039. [PubMed: 19713936]

Figure 1.
DMPT structure

Figure 2.

A. Lateral wall region of nasal level I from a young, male F344/N rat exposed to vehicle control corn oil by gavage for 5 days. The transitional epithelium is 1–2 cell layers thick and fairly well organized (arrows). H&E (25×).

B. Lateral wall region of nasal level I from a young, male F344/N rat exposed to 120 mg/kg DMPT by gavage for 5 days. The transitional epithelium is characterized by a thickened, disorganized epithelium (arrows), with occasional vacuoles (arrowheads). H&E (25×).

Figure 3.

A. Normal olfactory epithelium in the dorsal meatus region of nasal level III from a young, male F344/N rat exposed to vehicle control corn oil by gavage for 5 days. The olfactory epithelium is organized and uniform in height (arrows). H&E (12.5×).

B. Higher magnification of figure 1c. The cells are densely packed and uniform in size, and the epithelium is uniform in height. H&E (25×).

C. Dorsal meatus region of nasal level III from a young, male F344/N rat exposed to 120 mg/kg DMPT by gavage for 5 days. Olfactory epithelial necrosis, characterized by irregularity and decreased cellularity of the olfactory epithelium is present (arrows). There is cellular debris on the surface of the epithelium (arrowheads) and in the lumen of the nasal cavity. H&E (12.5×).

D. Olfactory epithelial necrosis in the dorsal meatus region of nasal level III in a young, male F344/N rat exposed to 120 mg/kg DMPT by gavage for 5 days. The olfactory epithelium is decreased in cellularity, vacuolated (arrows) and irregular. H&E (25×).

E. Dorsal meatus region of nasal level III from a young, male F344/N rat exposed to 120 mg/kg DMPT by gavage for 5 days. There is a thick layer of necrotic debris overlying the olfactory epithelium (arrows). The cells of the epithelium are disorganized and many are vacuolated (arrowheads). H&E (25×).

F. Olfactory epithelial degeneration in the dorsal meatus region of nasal level III in a young, male F344/N rat exposed to 120 mg/kg DMPT by gavage for 5 days. The olfactory epithelium is vacuolated (arrows), but appears close to normal in height. H&E (25×).

Figure 4.

A. Frozen nasal section from control rat (40×) showing transitional cell epithelium.

B. Frozen nasal section from treated rat showing transitional cell epithelium (40×), which is thickened by an increase in number and size of the cells.

Figure 5. Principal component analysis. The first three principal components comparing global gene expression profiles of rats exposed to vehicle control and DMPT treatment in the nasal cavity. Samples cluster within group (vehicle or treated), indicating that there are differences in expression between the two treatment conditions.

Figure 6. Nanostring and microarray platform comparison of gene transcript fold change. Bars represent n=10 rats/group.

Figure 7.
Proposed DMPT nasal cavity toxicity pathways

Table 1

Nasal cavity lesions in male rats after five days of DMPT exposure

Mg/kg	0	1	6	20	60	120
Angiectasis	1	0	0	1	0	0
Transitional epithelium hyperplasia	0	0	0	0	4(1.0) ^a	5(1.8)
Olfactory epithelium necrosis	0	0	0	0	0	4(1.75)
Olfactory epithelium degeneration	0	0	0	0	0	1 (2.0)

Five animals per group;

^a severity of lesion

Table 2

DMPT induction of significant transcripts in the nasal cavity transitional cell epithelium

Transcript ^a	Description	Background
Antioxidant response & Nrf2 pathway		
AK7A3 (▲14.8)*	aldo-keto reductase family 7, member A3 (alatoxin aldehyde reductase) (detoxification gene) ;	May contribute to the protection against AFB1-induced hepatotoxicity (Knight <i>et al.</i> , 1999); cellular defense against the toxic/mitogenic effects of the dialdehyde metabolite of AFB1 (Merrick <i>et al.</i> , 2012)
SLC7A11 (▲17)	solute carrier family 7	Increase in transporter caused by oxidative-stress inducing paraquat (Kobayashi <i>et al.</i> , 2012); antioxidant response (Singer <i>et al.</i> , 2015)
SRXN1 (▲7.5)	sulfiredoxin 1	Protein critical for redox balance (Baek <i>et al.</i> , 2012)
MGST3 (▲4.0)	microsomal glutathione S-transferase 3	Utilize reduced glutathione for the cellular defense against oxygen-free radicals (Thameem <i>et al.</i> , 2003)
TXNRD1 (▲3.2)	thioredoxin reductase 1	Helps mediate oxidative stress (Das <i>et al.</i> , 2011)
NQO1 (▲1.7)	NAD(P)H dehydrogenase, quinone 1	Encodes a cytoplasmic 2-electron reductase (Basu <i>et al.</i> , 2011)
Prdx4 (▲1.6)	peroxiredoxin 4	Codes for an antioxidant defense protein
G6PD (▲1.4)	glucose-6-phosphate dehydrogenase	produce NADPH, a key electron donor in the defense against oxidizing agents (Pandolfi <i>et al.</i> , 1995)
Cell proliferation & Cancer		
SCIN (▲14.6)	SCINDERIN	Expressed by tumor cells rendering them resistant to cytotoxic T lymphocyte-mediated lysis (Hasmim <i>et al.</i> , 2013)
MAFF (▲11.4)	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	MAFF in proinflammatory cytokine-mediated control of myometrial gene expression and provide the first link between a small MAF transcription factor and the inflammatory response (Massieh <i>et al.</i> , 2005)
KRT4 (▲8.5)	Keratin 4	Epithelial structural protein (Presland and Dale, 2000)
Fabp5 (▲7.1)	Fatty acid protein	Promotes differentiation and cell proliferation (Dallaglio <i>et al.</i> , 2013; Fang <i>et al.</i> , 2010)
Cyp3a13 (▲11.4)	cytochrome P450, family 3, subfamily a, polypeptide 13	CYP3a13 promoter shows putative binding sites for retinoid X receptor, pregnane X receptor, and estrogen receptor (Anakk <i>et al.</i> , 2003); PPAR alpha pathway (Sahasrabudhe <i>et al.</i> , 2014)
PSCA (▲8.7)	prostate stem cell antigen	Originally identified as a tumor antigen in prostate cancer, but is found in other cancers (Yang <i>et al.</i> , 2014) and in brain (Jensen <i>et al.</i> , 2015); may regulate immune response, cell proliferation, and calcium flows (Yang <i>et al.</i> , 2014) (Jensen <i>et al.</i> , 2015)
IGFBP6 (▲4.6)	insulin-like growth factor binding protein 6	Promotes cell survival and inhibits apoptosis (Xu <i>et al.</i> , 2009)
NGFR (▲3.887)	nerve growth factor receptor	Nerve growth factors important in stem cell development (Cattaneo and McKay, 1990)
RHOV (▲3.1)	ras homolog family member V	Found to be overexpressed in lung cancers (Shepelev and Korobko, 2013)
CASC5 (also known as KNL1) (▲2.5)	cancer susceptibility candidate 5	Kinetic protein required for spindle assembly checkpoint (Izawa and Pines, 2015)
Sprr1 (▲28)	small proline-rich protein 1A	Found in chronic atopic dermatitis skin lesions (Jarzab <i>et al.</i> , 2010); functions as cornified envelope precursors (Fujimoto <i>et al.</i> , 1997); up regulated early after tobacco smoke-induced squamous metaplasia (Tesfaygi <i>et al.</i> , 1996)
FHIT (▼2.1)	fragile histidine triad	↓ liver after aflatoxin exposure (Merrick <i>et al.</i> , 2012); considered a tumor suppressor gene (Waters <i>et al.</i> , 2014)
Control of Apoptosis		
ACAA2 (▲2.387)	acetyl-CoA acyltransferase 2	Attenuates apoptosis by binding to bnip3 (Cao <i>et al.</i> , 2008)
GP2 (▼8.8)	GP2 glycoprotein 2	Membrane protein secreted from intracellular zymogen granules; an autoantigen that triggers autoimmune reaction in Crohn's disease (Pavlidis <i>et al.</i> , 2012)
CCNO (UNG2) (▼7.2)	cyclin	Novel cycle involved in forming complexes with Cdk2 in apoptosis (Roig <i>et al.</i> , 2009)
ERMN (▼6.7)	ermin	Novel marker of myelinating oligodendroglia; plays a role in cytoskeletal rearrangements during the late wrapping and/or compaction phases of myelinogenesis (Brockschneider <i>et al.</i> , 2006)
NRGN (▼5.3)	neurogranin (protein kinase C substrate, RC3)	Human homolog of the neuron-specific rat RC3/neurogranin gene; gene encodes a postsynaptic protein kinase substrate that binds calmodulin in the absence of calcium (Zhong <i>et al.</i> , 2009)
ACE (▼3.2)	angiotensin I converting enzyme	Gene encodes an enzyme involved in catalyzing the conversion of angiotensin I into a physiologically active peptide angiotensin

*
Fold change: DMPT vs. Control^a
(red is an upregulated transcript; blue is a down regulated transcript)

- Anakk, S., Kalsotra, A., Shen, Q., Vu, M.T., Staudinger, J.L., Davies, P.J. and Strobel, H.W. (2003). Genomic characterization and regulation of CYP3A13: role of xenobiotics and nuclear receptors. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 17, 1736–1738.
- Baek, J.Y., Han, S.H., Sung, S.H., Lee, H.E., Kim, Y.M., Noh, Y.H., Bae, S.H., Rhee, S.G. and Chang, T.S. (2012). Sulfiredoxin protein is critical for redox balance and survival of cells exposed to low steady-state levels of H₂O₂. *The Journal of biological chemistry*, 287, 81–89.
- Basu, A., Banerjee, H., Rojas, H., Martinez, S.R., Roy, S., Jia, Z., Lilly, M.B., De Leon, M. and Casiano, C.A. (2011). Differential expression of peroxiredoxins in prostate cancer: consistent upregulation of PRDX3 and PRDX4. *The Prostate*, 71, 755–765.
- Cao, W., Liu, N., Tang, S., Bao, L., Shen, L., Yuan, H., Zhao, X. and Lu, H. (2008). Acetyl-Coenzyme A acyltransferase 2 attenuates the apoptotic effects of BNIP3 in two human cell lines. *Biochimica et biophysica acta*, 1780, 873–880.
- Cattaneo, E. and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature*, 347, 762–765.
- Dallaglio, K., Marconi, A., Truzzi, F., Lotti, R., Palazzo, E., Petrachi, T., Saltari, A., Coppini, M. and Pincelli, C. (2013). E-FABP induces differentiation in normal human keratinocytes and modulates the differentiation process in psoriatic keratinocytes in vitro. *Experimental dermatology*, 22, 255–261.
- Das, S.K., Sharma, N.K., Hasstedt, S.J., Mondal, A.K., Ma, L., Langberg, K.A. and Elbein, S.C. (2011). An integrative genomics approach identifies activation of thioredoxin/thioredoxin reductase-1-mediated oxidative stress defense pathway and inhibition of angiogenesis in obese nondiabetic human subjects. *The Journal of clinical endocrinology and metabolism*, 96, E1308–1313.
- Fang, L.Y., Wong, T.Y., Chiang, W.F. and Chen, Y.L. (2010). Fatty-acid-binding protein 5 promotes cell proliferation and invasion in oral squamous cell carcinoma. *Journal of oral pathology & medicine: official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 39, 342–348.
- Fujimoto, W., Nakanishi, G., Arata, J. and Jetten, A.M. (1997). Differential expression of human cornifin alpha and beta in squamous differentiating epithelial tissues and several skin lesions. *J Invest Dermatol*, 108, 200–204.
- Hasmim, M., Badoual, C., Vielh, P., Drusch, F., Marty, V., Laplanche, A., de Oliveira Diniz, M., Roussel, H., De Guillebon, E., Oudard, S., Hans, S., Tartour, E. and Chouaib, S. (2013). Expression of EPHRIN-A1, SCINDERIN and MHC class I molecules in head and neck cancers and relationship with the prognostic value of intratumoral CD8+ T cells. *BMC cancer*, 13, 592.
- Izawa, D. and Pines, J. (2015). The mitotic checkpoint complex binds a second CDC20 to inhibit active APC/C. *Nature*, 517, 631–634.
- Jarzb, J., Filipowska, B., Zebracka, J., Kowalska, M., Bozek, A., Rachowska, R., Gubala, E., Grzanka, A., Hadas, E. and Jarzb, B. (2010). Locus 1q21 Gene expression changes in atopic dermatitis skin lesions: deregulation of small proline-rich region 1A. *Int Arch Allergy Immunol*, 151, 28–37.
- Jensen, M.M., Arvaniti, M., Mikkelsen, J.D., Michalski, D., Pinborg, L.H., Hartig, W. and Thomsen, M.S. (2015). Prostate stem cell antigen interacts with nicotinic acetylcholine receptors and is affected in Alzheimer's disease. *Neurobiology of aging*.
- Knight, L.P., Primiano, T., Groopman, J.D., Kensler, T.W. and Sutter, T.R. (1999). cDNA cloning, expression and activity of a second human aflatoxin B1-metabolizing member of the aldo-keto reductase superfamily, AKR7A3. *Carcinogenesis*, 20, 1215–1223.
- Kobayashi, S., Kuwata, K., Sugimoto, T., Igarashi, K., Osaki, M., Okada, F., Fujii, J., Bannai, S. and Sato, H. (2012). Enhanced expression of cystine/glutamate transporter in the lung caused by the oxidative-stress-inducing agent paraquat. *Free radical biology & medicine*, 53, 2197–2203.
- Massrieh, W., Derjuga, A., Doualla-Bell, F., Ku, C.Y., Sanborn, B.M. and Blank, V. (2006). Regulation of the MAFF transcription factor by proinflammatory cytokines in myometrial cells. *Biology of reproduction*, 74, 699–705.
- Merrick, B.A., Auerbach, S.S., Stockton, P.S., Foley, J.F., Malarkey, D.E., Sills, R.C., Irwin, R.D. and Tice, R.R. (2012). Testing an aflatoxin B1 gene signature in rat archival tissues. *Chem Res Toxicol*, 25, 1132–1144.
- Pandolfi, P.P., Sonati, F., Rivi, R., Mason, P., Grosveld, F. and Luzzatto, L. (1995). Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *The EMBO journal*, 14, 5209–5215.
- Pavlidis, P., Romanidou, O., Roggenbuck, D., Mytilinaiou, M.G., Al-Sulttan, F., Liaskos, C., Smyk, D.S., Koutsoumpas, A.L., Rigopoulou, E.I., Conrad, K., Forbes, A. and Bogdanos, D.P. (2012). Ileal inflammation may trigger the development of GP2-specific pancreatic autoantibodies in patients with Crohn's disease. *Clinical & developmental immunology*, 2012, 640835.
- Presland, R.B. and Dale, B.A. (2000). Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Critical reviews in oral biology and medicine: an official publication of the American Association of Oral Biologists*, 11, 383–408.
- Roig, M.B., Roset, R., Ortel, L., Balsiger, N.A., Anfosso, A., Cabellos, L., Garrido, M., Alameda, F., Brady, H.J. and Gil-Gomez, G. (2009). Identification of a novel cyclin required for the intrinsic apoptosis pathway in lymphoid cells. *Cell death and differentiation*, 16, 230–243.
- Sahasrabudde, N.A., Huang, T.C., Ahmad, S., Kim, M.S., Yang, Y., Ghosh, B., Leach, S.D., Gowda, H., Somani, B.L., Chaerkady, R. and Pandey, A. (2014). Regulation of PPAR-alpha pathway by Dicer revealed through proteomic analysis. *Journal of proteomics*, 108, 306–315.
- Shepelev, M.V. and Korobko, I.V. (2013). The RHOV gene is overexpressed in human non-small cell lung cancer. *Cancer genetics*, 206, 393–397.
- Singer, E., Judkins, J., Salomonis, N., Matlaf, L., Soteropoulos, P., McAllister, S. and Soroceanu, L. (2015). Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma. *Cell death & disease*, 6, e1601.
- Tesfaigzi, J., Th'ng, J., Hotchkiss, J.A., Harkema, J.R. and Wright, P.S. (1996). A small proline-rich protein, SPRR1, is upregulated early during tobacco smoke-induced squamous metaplasia in rat nasal epithelia. *American journal of respiratory cell and molecular biology*, 14, 478–486.
- Thameem, F., Yang, X., Permana, P.A., Wolford, J.K., Bogardus, C. and Prochazka, M. (2003). Evaluation of the microsomal glutathione S-transferase 3 (MGST3) locus on 1q23 as a Type 2 diabetes susceptibility gene in Pima Indians. *Human genetics*, 113, 353–358.
- Waters, C.E., Saldivar, J.C., Hosseini, S.A. and Huebner, K. (2014). The FHIT gene product: tumor suppressor and genome “caretaker”. *Cellular and molecular life sciences: CMLS*, 71, 4577–4587.
- Xu, X.F., Guo, C.Y., Liu, J., Yang, W.J., Xia, Y.J., Xu, L., Yu, Y.C. and Wang, X.P. (2009). Gli1 maintains cell survival by up-regulating IGFBP6 and Bcl-2 through promoter regions in parallel manner in pancreatic cancer cells. *J Carcinog*, 8, 13.
- Yang, X., Guo, Z., Liu, Y., Si, T., Yu, H., Li, B. and Tian, W. (2014). Prostate stem cell antigen and cancer risk, mechanisms and therapeutic implications. *Expert Rev Anticancer Ther*, 14, 31–37.

Zhong, L., Cherry, T., Bies, C.E., Florence, M.A. and Gerges, N.Z. (2009). Neurogranin enhances synaptic strength through its interaction with calmodulin. *The EMBO journal*, 28, 3027–3039.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3Upstream Transcription Regulators ($|z| > 3$) for DMPT-induced nasal cavity molecular changes^a

Gene	Activation z-score	p-value of overlap	Known Roles
NFE2L2	6.007	7.54E-16	Response to oxidative stress
E2F1	5.696	8.30E-20	Cell cycle; DNA replication
MYC	5.575	3.14E-21	Growth related
TBX2	4.536	1.35E-14	Mesoderm differentiation
FOXO1	4.417	1.14E-06	Insulin signaling; metabolic homeostasis in response to oxidative stress
SREBF1	4.148	1.93E-07	Lipid homeostasis
CCND1	3.891	7.13E-17	Cell cycle
FOXM1	3.814	8.07E-11	Cell cycle; DNA replication; mitosis
SREBF2	3.691	7.76E-14	Lipid homeostasis
E2F2	3.576	3.72E-12	Cell cycle; DNA replication
E2F3	3.400	8.88E-15	Cell cycle; DNA replication
HIF1A	3.311	1.32E-07	Response to hypoxia
MYCN	3.152	1.44E-03	Transcription factor function
RBL1	-3.345	2.12E-06	Cell division; tumor suppressor
RB1	-3.533	1.47E-15	Cell division; tumor suppressor
TCF3	-3.914	2.10E-10	Initiation of neuronal differentiation
CDKN2A	-4.960	2.77E-12	Negative regulator of proliferation

Upstream regulators, Ingenuity pathway analysis (www.ingenuity.com) UniProt knowledgebase (www.uniprot.org)