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RESEARCH ARTICLE

The role of membrane transporters in the absorption of atrazine following nasal exposure

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ABSTRACT

Objective: The purpose of these studies was to investigate the uptake of atrazine across the nasal mucosa to determine whether direct transport to the brain through the olfactory epithelium is likely to occur. These studies were undertaken to provide important new information about the potential for the enhanced neurotoxicity of herbicides following nasal inhalation.

Materials and Methods: Transport of atrazine from aqueous solution and from commercial atrazine-containing herbicide products was assessed using excised nasal mucosal tissues. The permeation rate and the role of membrane transporters in the uptake of atrazine across the nasal mucosa were also investigated. Histological examination of the nasal tissues was conducted to assess the effects of commercial atrazine-containing products on nasal tissue morphology.

Results: Atrazine showed high flux across both nasal respiratory and olfactory tissues, and efflux transporters were found to play an essential role in limiting its uptake at low exposure concentrations. Commercial atrazine-containing herbicide products showed remarkably high transfer across the nasal tissues, and histological evaluation showed significant changes in the morphology of the nasal epithelium following exposure to the herbicide products.

Discussion: Lipophilic herbicides such as atrazine can freely permeate across the nasal mucosa despite the activity of efflux transporters. The adjuvant compounds in commercial herbicide products disrupt the nasal mucosa's epithelial barrier, resulting in even greater atrazine permeation across the tissues. The properties of the herbicide itself and those of the formulated products play crucial roles in the potential for the enhanced neurotoxicity of herbicides following nasal inhalation.

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KEYWORDS
Atrazine; inhalation exposure; olfactory mucosa; nasal absorption; nose-to-brain transport; respiratory mucosa; transporters; BCRP; MRP1

Introduction

Triazine herbicides, such as atrazine, are the most heavily used agricultural chemicals in the United States due to their application flexibility and the ability to combine them with other herbicides (LeBaron et al. 2008; Kwon et al. 2020). Atrazine is used for both pre-emergence and post-emergence control of annual grassy and broadleaf weeds in corn and other crops (Preisler et al. 2020; Bachetti et al. 2021). Atrazine was banned in the European Union in 2005 because of its omnipresence in drinking water (Sass and Colangelo 2006; Montiel-León et al. 2019) however, in the United States, more than 75 million pounds of atrazine continue to be applied annually to corn and sorghum crops, primarily in the Midwest (Cooper et al. 2007). This heavy use occurs despite atrazine being the most reported ground and surface water contaminant in the United States (United States Environmental Protection Agency 2007; Bexfield et al. 2020).

Several studies have shown an association between the inhalation exposure of atrazine and the risk of Parkinson's disease (Shaw 2011; Pantaleon et al. 2021). Atrazine has been reported to be a potential basal ganglia toxicant, and oral and inhalation exposures have been associated with dopaminergic neurotoxicity manifested by decreased striatal dopamine levels (Morgan et al., 1996; Coban and Filipov 2007; Shaw 2011). Curwin et al. showed that atrazine applicators and their families are at increased risk of developing Parkinson's disease (Curwin et al. 2007). Aerial spraying of herbicides can result in spray drift up to hundreds of miles from the treated area, potentially rendering thousands of individuals susceptible to involuntary exposure and subsequent health risks from the millions of pounds of herbicides sprayed annually, and the United States Environmental Protection Agency reports receiving thousands of complaints annually from individuals exposed to spray drift (Van Dijk and Guicherit 1999).

Most studies investigating the adverse health effects of atrazine exposure have focused on oral ingestion, dermal absorption, or acute inhalation exposure through the lung (Silbergeld 1998; Lozier et al. 2013; United States Environmental Protection Agency 2022). Libich et al. reported that for herbicide spray applicators, dermal absorption was the major absorption route being up to 50 times greater than exposure by the inhalation route (Libich et al. 1984; Damalas and Koutroubas 2016), but these

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investigations did not include the evaluation of risk of exposure to the upper respiratory tract following inhalation. Other studies have also reported that the dermal and inhalation routes of exposure are typically the most common routes for agricultural workers' exposure to herbicides (Damalas and Koutroubas 2016). Yet, the likely absorption of atrazine following nasal inhalation and the potential for direct transport of the herbicides and associated additives between the nasal mucosa and the CNS may contribute an unrecognized factor to the increased risk of developing Parkinson's disease in individuals exposed to atrazine.

Transfer of a wide variety of materials, from viruses and bacteria to drug molecules and heavy metals, directly between the nasal cavity and the brain have been reported, and transneuronal, perineuronal, and transcellular pathways have been proposed for transport between these two regions. Reports have described viruses (Tomlinson and Esiri 1983; Van Riel et al. 2015) and a number of heavy metals (Sunderman 2001) being taken up by olfactory neurons in the nasal mucosa with subsequent movement into the brain. The use of the nose-to-brain pathways to deliver proteins, including insulin and insulin-like growth factor, into the brain have also shown that these proteins travel along the perineuronal and perivascular spaces adjacent to the olfactory and trigeminal nerves to reach the olfactory bulb (Thorne et al., 2004; Lochhead and Thorne, 2012; Lochhead et al., 2019). Numerous reports also describe the rapid and enhanced transfer of small, organic drug molecules from the nasal cavity into the brain, along with the inhibition of epithelial membrane transporters, resulting in the modulation of drug transport into the brain. These results show that there is also a transcellular uptake pathway used by molecules able to access the brain *via* the nasal cavity (Illum 2000, 2004; Graff and Pollack 2005; Kandimalla and Donovan 2005; Kandimalla and Donovan 2005; Chemuturi et al. 2006; Dhuria et al. 2010; Lee et al. 2010; Padowski and Pollack 2010; Shingaki et al. 2011; Al-Ghabeish et al. 2015; Iwasaki et al. 2019; Bors et al. 2020).

Membrane influx transporters have been shown to play a role in the transport of chemicals from the olfactory region of the nasal cavity into the CNS by direct nose-to-brain transport (Kandimalla and Donovan 2005; Chemuturi et al. 2006; Lee et al. 2010; Anand et al. 2014; Ponto et al. 2018). Efflux transporters have also been identified in the nasal respiratory and olfactory tissues and have been found to play a role in limiting the uptake of their substrates into the CNS (Graff and Pollack 2005; Kandimalla and Donovan 2005; Padowski and Pollack 2010; Shingaki et al. 2011; Al-Ghabeish et al. 2015; Bors et al. 2020). While there is no information available regarding the interaction of atrazine with influx or efflux transporters in mammals, Bain and Leblanc reported that atrazine did not inhibit MDR1 transporters expressed in the B16/F10 murine melanoma cell line (Bain and Leblanc 1996). An improved understanding of the mechanisms of herbicide absorption across the nasal mucosa, with specific attention to the potential for herbicide transfer to the brain *via* the olfactory system, will provide important new information about the currently unrecognized risks associated with the nasal inhalation of aerosolized herbicides.

Materials and methods

Atrazine, ammonium acetate, sodium azide, Ko143, and eosin were obtained from Sigma Chemical Co (St. Louis, MO). Sunitinib was obtained from LC Laboratories (Woburn, MA). MK571 was purchased from Cayman Chemicals (Ann Arbor, MI). Zosuquidar trihydrochloride was obtained from Tocris Bioscience (Minneapolis, MN). Tissue freezing media (TFM^{TM}) was purchased from Triangle Biomedical Science (Durham, NC). Hematoxylin, xylene, sucrose, glacial acetic acid, and ethanol used for hematoxylin and eosin staining (H&E) for brightfield microscopy were obtained from Research Products International Corp. (Mount Prospect, IL). The CryoJane® Tape-Transfer System for cryosectioning and Snowcoat® slides were obtained from Leica Biosystems Inc. (Buffalo Grove, IL). The two commercial atrazine-containing herbicide products used were Atrazine St. Augustine Weed Killer® from Southern Agricultural Insecticides Inc. (Southern AG®, Boon, NC) and Atrazine 4 L® from WinField United (Arden Hills, MN). Kreb's Ringer's buffer (KRB) salts and acetonitrile were obtained from Fisher Scientific (Chicago, IL).

MRP1 (ABCC1) rabbit anti-human monoclonal antibodies (mAb) and BCRP (ABCG2) rabbit anti-human, mouse, and rat mAb were purchased from Cell Signaling Technology (Danvers, MA) and were used as primary antibodies for immunohistochemistry. The secondary antibody, goat anti-rabbit Alexa Flour® 647, was purchased from Alpha Diagnostic International Inc. (San Antonio, TX). The nuclear stain, DAPI with Vectashield®, was obtained from Vector Laboratories (Burlingame, CA). Dubelcco's phosphate-buffered saline (DPBS) was obtained from Life Technologies Invitrogen (Carlsbad, CA).

Kreb's Ringer buffer

KRB solution was prepared by dissolving 1.67 mM $MgCl_2{\cdot}6H_2O$, 4.56 mM KCl, 119.78 mM NaCl, 1.5 mM $NaH_2PO_4{\cdot}H_2O$, 0.83 mM Na_2HPO_4, 10 mM D-glucose and 15 mM $NaHCO_3$ in 900 mL of deionized water. Carbogen gas (95% O_2 and 5% CO_2) was bubbled through the solution for 15-20 min to lower the pH followed by adding 1.2 mM $CaCl_2{\cdot}2H_2O$. The volume of the solution was completed to 2 liters with deionized water, and the pH was adjusted to 7.4 using HCl or NaOH (1 N).

Nasal tissues

Bovine respiratory and olfactory tissues were obtained from Bud's Custom Meats, Inc. (Riverside, IA). Bovine tissues were selected due to the large surface area of both olfactory and respiratory mucosa available from each animal, and the olfactory mucosa is isolated from the respiratory mucosa, yet is easily accessible following removal of the superior

turbinate. The advantage of using full-thickness, excised nasal mucosa in these studies, compared to cell culture models, lies in the multicellular nature of the bovine tissues providing a more representative model, especially when trans- or peri-neuronal transport pathways may play a role in transmucosal transfer. Olfactory neurons are present in the excised olfactory tissues, and the arrangement relative to their associated sustentacular cells plays a critical role in determining the overall permeability properties of small molecules through this tissue.

Immediately after the animal's death, a longitudinal incision was made along the nasal septum, and the nasal cavity was opened to extract the nasal respiratory and olfactory tissues. The excised tissues were placed in fresh, iced-chilled KRB during transport. To ensure tissue viability for the time course of the transport experiments, the entire experiment was completed within 4 h from the time of tissue collection.

Permeation studies

Excised respiratory and olfactory tissues were mounted between 1 mL NaviCyte™ vertical diffusion cells (Harvard Apparatus, Holliston, MA). The tissues were allowed to equilibrate for 30 min in KRB with carbogen bubbled into the buffer at 3–5 bubbles/min for oxygenation and stirring. The temperature of the diffusion cells was kept at 37°C using a heating manifold (Harvard Apparatus, Holliston, MA) connected to a Lauda™ RM6 circulating water bath (Brinkman Instrument Co. New York City, NY). After equilibration, the donor solution facing the epithelial surface of the tissue segments was removed and replaced with 1 mL of the test solution. The receiver compartment facing the submucosal surface of the tissue segments was replaced with the same volume of fresh KRB. Permeation studies were conducted in the mucosal–submucosal direction, and samples (200 µL) were withdrawn from the receiver chamber every 15 min and replaced by KRB solution for a duration of up to 120 min.

Inhibition of membrane transporters

Atrazine flux was measured in the presence of sodium azide (10 mM) (Kang et al. 2001) sunitinib (30 µM) (Shukla et al. 2009); MK571 (50 µM) (Leier et al. 1994; Lowes et al. 2003); zosuquidar (5 µM) (Choo et al. 2000); verapamil (30 µM) (Wong et al. 2009; Jouan et al. 2016; Perrotton et al. 2007); and Ko143 (1 µM) (Matsson et al. 2009) prepared in KRB. The selected inhibitor concentrations were twice the reported IC_{50} (half maximal inhibitory concentration) value for each inhibitor to ensure maximum inhibition of the transporter's activity. For each of these inhibitors, the tissues were initially equilibrated at 37°C for 30 min with KRB solution containing one of the inhibitors. The donor solutions were replaced with 1 mL of atrazine (93 µM) + inhibitor, and the receiver chamber was replaced with 1 mL of the inhibitor solution. The inhibitors were included in the donor and the receiver solutions for the entire study duration.

Permeation studies with commercial atrazine-containing products

The two commercial products tested for atrazine flux contained different atrazine concentrations (4% w/v atrazine for Atrazine Southern AG® and 42% w/v atrazine for Atrazine 4L®). Both commercial products were diluted with water to the recommended application concentration stated by each manufacturer 0.1% w/v (4.6 mM) atrazine for Atrazine Southern AG® and 21% w/v (974 mM) atrazine for Atrazine 4L®.

Analysis of permeation results

The flux of atrazine across nasal mucosal explants was quantified by evaluating the steady state portion of the cumulative mass transport across the tissue vs. time profile using an application of Fick's Second Law as shown in the following equation (Chemuturi et al. 2005):

$$J = Pe \times (Cd - Cr)$$

$$J(\text{flux}) = \frac{\text{Mass of transported atrazine } (\mu g\ min^{-1})}{\text{Surface area of exposed tissue } (cm^2)} = \text{slope of the cumulative amount of atrazine transported vs.time}$$

$$Pe\ (\text{apparent permeability coefficient of atrazine}) = \frac{J(\mu g/cm^2 min)}{Cd(\mu M)}$$

Cd = Initial atrazine donor concentration (µM)
Cr = atrazine receiver concentration (µM)

Results of the transport studies are reported as the mean of three replicates ± standard deviation. The replicates represent tissue sections obtained from a single animal but collected from different turbinate regions of the nasal respiratory and olfactory mucosa mounted in separate diffusion cells. When comparing the flux across respiratory and olfactory tissues, the flux measured across the respiratory tissues was thickness-normalized by the ratio of the respiratory tissue thickness (0.75 mm) to that of the olfactory tissues (0.4 mm). One-way ANOVA followed by Bonferroni's multiple comparison testing was used to compare permeation results between samples and controls. All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA).

HPLC analysis

Atrazine was measured using reverse phase, high-performance liquid chromatography (HPLC) with an Agilent 1100 system (Santa Clara, CA) equipped with a Zorbax® C18 column (Agilent Technologies Inc., Lexington, MA) and a UV detector set at 226 nm. The mobile phase was acetonitrile including 1 mM ammonium acetate (75:25 v/v) at a flow rate of 1.4 mL/min.

Histologic evaluation

The morphology of the tissue explants exposed to atrazine and commercial atrazine-containing herbicide products was examined using brightfield microscopy. Following a 120 min permeation experiment, the tissues were removed from the NaviCyte™ chambers and fixed using zinc formalin for 48 h. The tissues were processed using sequential dehydration with sucrose solutions of increasing concentrations. Using liquid nitrogen, dehydrated samples were cryo-frozen in Tissue Freezing Media (TFM™). Sections (10 µm) were cut using a Microm® Cryostat II HM505E using a CryoJane system (Microm International, Walldorf, Germany) maintained at −35°C. The tissue sections were stained using hematoxylin and eosin. Finally, the sections were examined using brightfield microscopy with an Olympus BX-61 motorized light microscope (Olympus Microscope and Imagining System Inc., Melville, NY) at 10X magnification.

Immunohistochemical localization of MRP1 and BCRP

For immunohistochemistry (IHC), nasal mucosal explants were prepared using the brightfield microscopy preparation protocol. After tissue fixation and sectioning, the samples were blocked using 5% normal goat serum (NGS) and were exposed to primary antibody (anti-MRP1 or anti-BCRP) raised in rabbits with reactivity against human (Cell Signaling Technology 2024a; 2024b); followed by exposure to a goat, anti-rabbit Alexa Flour® 647 secondary antibody. The homology of the coding sequences for MRP1 (ABCC1) transporter between human (H. sapiens) and cow (B. taurus) was compared using HomoloGene (NCBI) and it was found that the protein sequence is 90.8% homologous between the twospecies (HomoloGene:133779.). For BCRP (ABCG2), the homology of the protein sequence between human (H. sapiens) and cow (B. taurus) is 84.6% (HomoloGene:55852). The tissue sections were also exposed to 0.1% Tween® 20 in DPBS adjusted to pH 7.3 for 5 min before blocking for epitope retrieval. Positive controls were treated by replacing the primary antibody with a blocking solution of normal goat serum (NGS). Vectashield® with DAPI mounting medium was used on all slides for nuclear staining and to prevent photobleaching. The tissue samples were imaged using a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Results

The flux of atrazine across both respiratory and olfactory mucosae increased linearly with increasing atrazine concentrations in the range between 46-140 µM (Figure 1). This range was selected based on the lowest limit of quantification of atrazine in the HPLC assay and the measured solubility of atrazine in KRB (151 µM) (Al Bakri 2014). No saturation of atrazine uptake was observed, and these results indicate that atrazine was readily transported by passive diffusion across both the respiratory and olfactory tissues.

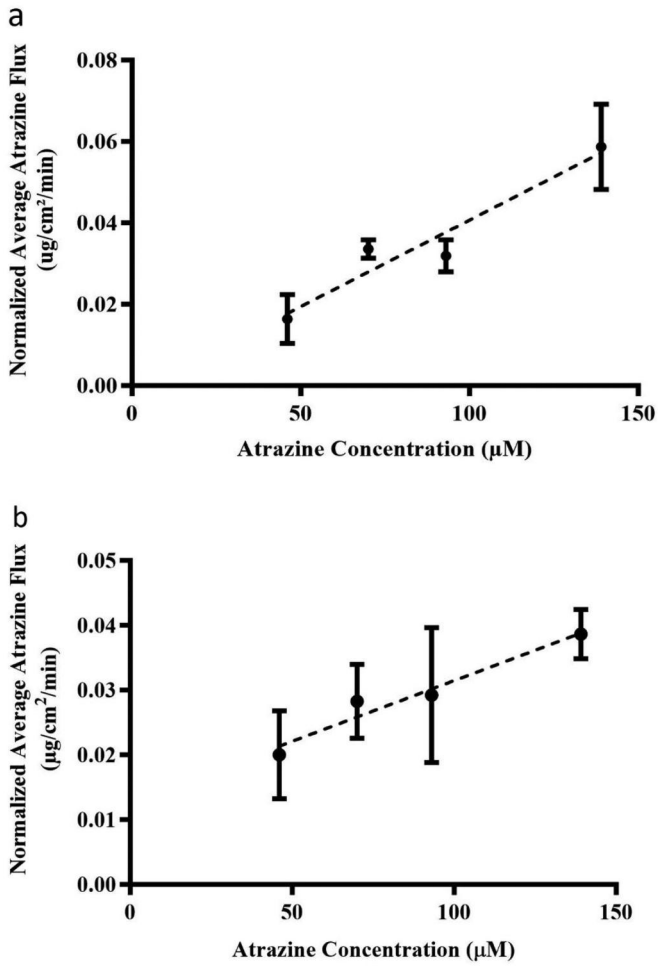

Figure 1. Flux of atrazine across excised bovine nasal (a) respiratory (b) olfactory mucosal tissues mounted in NaviCyte™ vertical diffusion chambers. Flux is presented as the mean of $n = 3 \pm$ standard deviation from separate tissue samples. The respiratory tissue flux values are normalized to the equivalent thickness of the olfactory mucosal sections for comparison.

The permeation of atrazine (93 µM) was also investigated in the presence of the metabolic inhibitor, sodium azide (10 mM). Atrazine uptake was tested at 93 µM, the midpoint between the highest and the lowest concentrations of atrazine measured in the permeation studies. The flux of atrazine incubated with sodium azide was significantly increased across both tissue types (Figure 2), suggesting that atrazine is a substrate for an energy-dependent efflux transport process which reduces net flux across the tissues under non-inhibited conditions.

To identify which membrane efflux transporters may play a role in the efflux of atrazine across the nasal mucosa, studies including the inhibitors sunitinib (non-selective MDR1 (ABCB1) and BCRP (ABCG2) inhibitor) (Shukla et al. 2009; Zhang and Wang 2013), zosuquidar (selective MDR1 (ABCB1) inhibitor), verapamil (MDR1 (ABCB1) and MRP1 (ABCC1) inhibitor) (Perrotton et al. 2007; Wong et al. 2009; Jouan et al. 2016), Ko143 (selective BCRP (ABCG2) inhibitor) (Matsson et al. 2009),and MK571 (selective MRP1 (ABCC1) inhibitor) (Leier et al. 1994; Renes et al. 1999) were performed. Exposing the nasal tissues to Ko143 (selective BCRP inhibitor) or zosuquidar (selective MDR1 inhibitor) did not result in a significant

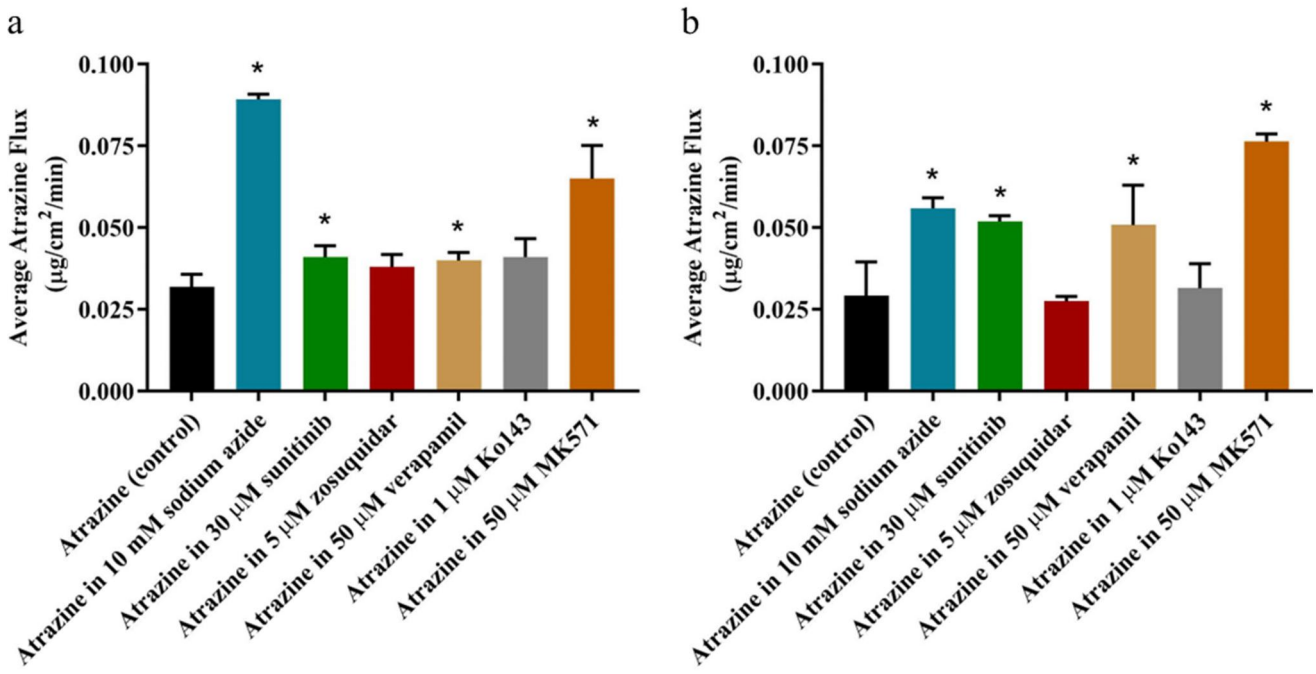

Figure 2. Comparison of atrazine (93 μM) flux across (a) respiratory and (b) olfactory bovine nasal mucosae following exposure to sodium azide (ATPase inhibitor), sunitinib (MDR1 and BCRP inhibitor), zosoquidar (MDR1 inhibitor), verapamil (MDR1 and MRP1 inhibitor), Ko143 (BCRP inhibitor), and MK571 (MRP1 inhibitor). Flux is presented as the average of $n = 3 \pm$ standard deviation from separate tissues. Asterisks indicate a statistically significant difference in flux between atrazine and an atrazine+inhibitor solution (one-way ANOVA followed by bonferroni'smultiple comparison tests). $^*p \leq 0.05$.

increase in atrazine flux (Figure 2). MK571, a selective MRP1 inhibitor, in comparison, resulted in a significant increase in the flux of atrazine across both the nasal respiratory and olfactory tissues. When MDR1 was inhibited along with other efflux transporters by the nonspecific inhibitors verapamil and sunitinib, increased atrazine flux was also observed. Based on these results, atrazine is clearly a substrate for MRP1 efflux in the nasal respiratory and olfactory mucosae. MRP1 is a highly relevant transporter involved in drug distribution, and the inhibition of the MRP1 transporter has been identified to play a critical role in increasing the transfer of several other chemicals and drugs, including vincristine, saquinavir, and grepafloxacin across the blood-brain barrier (BBB), resulting in significant increases in the brain exposure of these molecules (Amai et al. 2000; Park and Sinko 2005; Wang et al. 2010). It is also likely that atrazine has some limited affinity for MDR1 and BCRP, but inhibition of these transporters must be accomplished in combination with other efflux transporters in order to result in notable increases in atrazine flux.

While previous investigations have confirmed the presence of MDR1 protein in the nasal respiratory and olfactory mucosae, the localization of MRP1 and BCRP proteins in these tissues has not been reported. Positive immunoreactivity for MRP1 and BCRP proteins, demonstrated by green fluorescence in antibody-treated tissue sections (Figures 3 and 4), was observed in the epithelial cells of both the bovine respiratory and olfactory mucosae. The green fluorescence appears throughout the epithelial layer and suggests MRP1 and BCRP proteins are located at both the apical and basolateral surfaces of the nasal epithelium for both tissues. The observed distribution of MRP1 and BCRP throughout the nasal mucosal and submucosal regions of the respiratory and olfactory mucosae is different from their previously described localization in the BBB, bronchial epithelium, and gastrointestinal mucosae where BCRP has been reported to be primarily located on the apical surface of the epithelial cells (Aronica et al. 2005; Paturi et al. 2010; Varma et al. 2010) while MRP1 is typically confined in the basolateral region (Bréchot et al. 1998; Soontornmalai et al. 2006; Varma et al. 2010). The continuous distribution of MRP1 and BCRP throughout the apical and basolateral regions of the respiratory and olfactory tissues highlights the unique barrier characteristics of these tissues for limiting the uptake of chemicals from the nasal cavity to the brain (Figure 4). The distribution patterns of MRP1 and BCRP transporters in the mucosal and submucosal regions of the respiratory and olfactory is similar to the reported distribution of MDR1 in the bovine respiratory and olfactory tissues, human nasal tissues and human nasal epithelial cell cultures identified by previous investigators (Wioland et al. 2000; Kandimalla and Donovan 2005; Cho et al. 2011). The broad distribution of the efflux transporters throughout the nasal mucosa suggests they play a significant role in limiting the absorption of potentially deleterious compounds through this highly permeable tissue and also play an important role in determining the distribution of potential toxicants into the brain *via* the olfactory mucosa following inhalation. The submucosal location of efflux transporters may result in the exclusion of highly permeable molecules from entering the systemic circulation *via* the highly perfused nasal mucosa and may also effectively shunt these molecules into nasal mucosal compartments with high metabolic capacities for decomposition prior to transfer to other tissues.

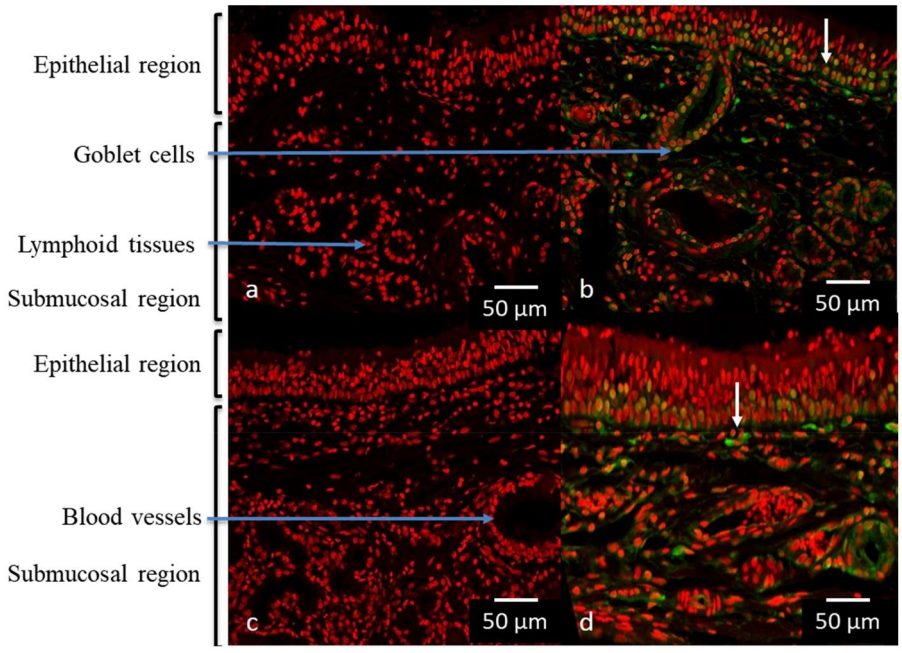

Figure 3. Immunohistochemical staining of MRP1 in bovine respiratory and olfactory tissue sections incubated with either MRP1 monoclonal antibodies or tissue controls not exposed to the primary antibody. The green fluorescence in the epithelial layer shows MRP1 localization, indicated by the white arrows. The red fluorescence represents nuclear staining with DAPI. Magnification is 20X. (a) control respiratory tissue, (b) the epithelial cell layer and submucosal regions of the respiratory mucosa, where the presence of MRP1 is observed by the green coloration in the apical and basolateral surfaces of the epithelial cells; (c) control olfactory tissue; and (d) the epithelial and the submucosal glandular region of the olfactory mucosa, where prominent MRP1 green labeling is observed surrounding the blood vessel in the endothelium and throughout the mucosal and submucosal regions of the olfactory epithelium.

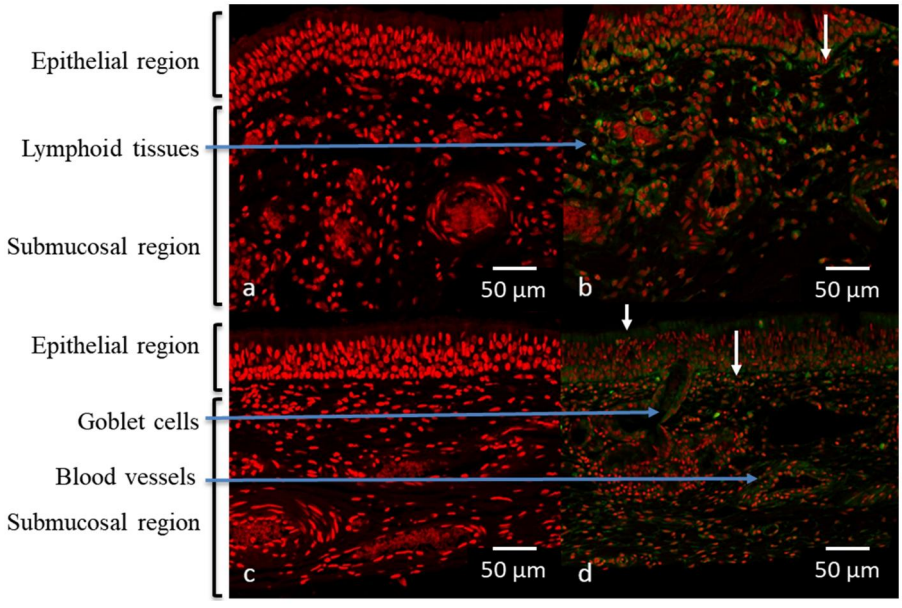

Figure 4. Immunohistochemical staining of BCRP transporters in bovine respiratory and olfactory tissue sections incubated with either BCRP monoclonal antibodies or tissue controls not exposed to the primary antibody. The green fluorescence in the epithelial layer indicates BCRP localization indicated by the white arrows. The red fluorescence represents nuclear staining with DAPI. Magnification is 20X (a) control respiratory tissue; (b) the epithelial cell layer and submucosal regions of the respiratory mucosa, where the presence of BCRP is observed by the green coloration in the apical and basolateral regions of the epithelial cells; (c) control olfactory tissue; and (d) the epithelial and the submucosal glandular region of the olfactory mucosa, where prominent BCRP green labeling is prevalent throughout the apical and basolateral surfaces of the olfactory epithelium.

Studying the flux of atrazine across the nasal mucosal tissues using commercial atrazine-containing products formulated at concentrations directed for application (0.1% = 4.6 mM and 21% = 974 mM for Atrazine Southern AG® and Atrazine 4L®, respectively) across the respiratory and olfactory tissues resulted in remarkably high atrazine transport across the excised mucosal segments due to the high application concentrations of atrazine in these commercial products (Figure 5).

Histologic evaluation of the nasal respiratory and olfactory tissues using brightfield microscopy showed that exposing the nasal tissues to herbicides resulted in complete removal of the epithelial layer from the apical surface of the tissue leaving only a few remaining basal cells. In

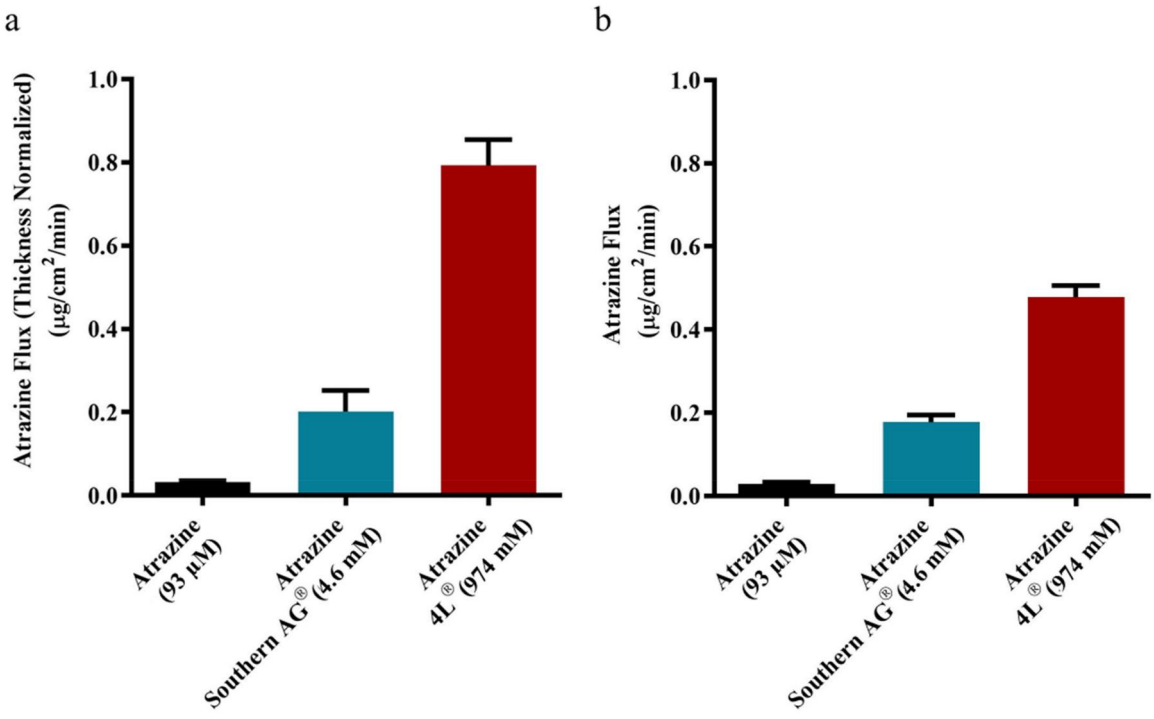

Figure 5. Flux of atrazine dissolved in KRB 0.002% (w/v) (93 µM), atrazine Southern AG® 0.1% (w/v) (4.6 mM), and atrazine 4L® 21% (w/v) (974 mM) used at the recommended application concentrations for the commercial products across bovine nasal (a) respiratory and (b) olfactory tissue. Flux presented as the average of $n = 3 \pm$ standard deviation for separate tissues.

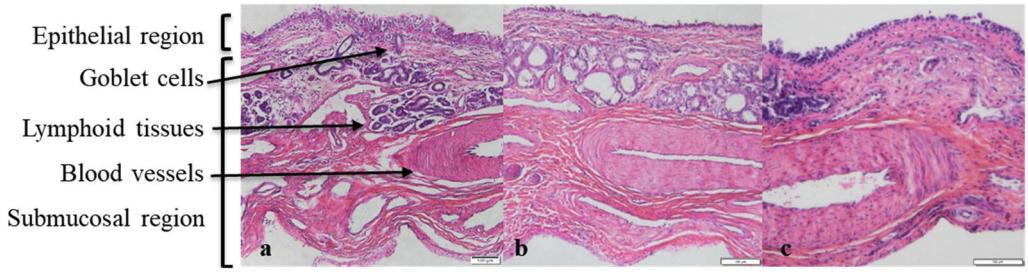

Figure 6. Brightfield microscopic image of H&E stained sections of bovine respiratory tissue (a) tissue exposed to 0.002% (w/v) (93 µM) atrazine solution (2 h); (b) tissue exposed to 0.1% (w/v) (4.6 mM) Atrazine Southern AG® (2 h); and (c) tissue exposed to 21% (w/v) (974 mM) Atrazine 4L® for (2 h). Tissues exposed to commercial atrazine products show significant de-epithelization with scattered presence of basal cells remaining attached to the basement membrane.

comparison, when atrazine was dissolved in KRB and exposed to the nasal tissues under the same conditions, no significant changes in the epithelial layer of the respiratory and the olfactory tissues were observed (Figures 6 and 7). The commercial atrazine-containing products induced alterations in the morphology of the nasal epithelium and likely disrupted the barrier properties of the nasal mucosa. Most commercial atrazine products contain additional compounds, including organic solvents such as xylene, ethylene glycol, ethylbenzene, and 1,2,4 trimethyl benzene, all of which may have additional direct effects on the enhanced permeation and subsequent toxicity of nasally-inhaled atrazine.

Discussion

Humans can be exposed to herbicides through various means, but most characterizations of their toxicity focus on three primary routes of exposure: dermal absorption, oral ingestion, and pulmonary inhalation. Nasal inhalation has been overlooked in most circumstances as a potential route of exposure. These studies demonstrate that atrazine is an excellent substrate for the membrane efflux transporter, MRP1, expressed in the nasal epithelium, which transfers the substrate (atrazine) from the nasal epithelial cells into the nasal lumen/airway secretions. The high passive permeability of atrazine, however, drives net atrazine absorption across the nasal epithelium, and the tissue concentrations achieved appear to be sufficient to saturate MRP1 and other efflux transporters. As a result, a considerable absorption of atrazine still occurs, even in the presence of the population of protective efflux transporters in the nasal cavity.

Brightfield microscopy with hematoxylin and eosin staining showed that low concentrations of atrazine dissolved in aqueous buffer solution did not cause any significant changes in the mucosal epithelium. In contrast, exposure to commercial herbicide products produced extensive changes to the morphology of the excised nasal tissues. These

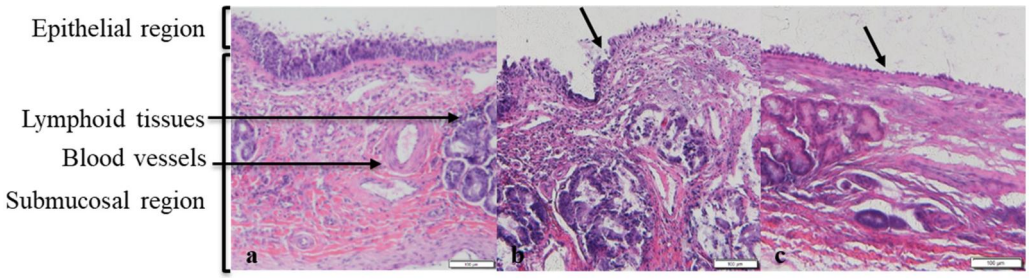

Figure 7. Brightfield microscopic image of H&E stained sections of bovine olfactory tissue (a) tissue exposed to 0.002% (w/v) 93 μM atrazine solution (2 h); (b) tissue exposed to 0.1% (w/v) (4.6 mM) Atrazine Southern AG® (2 h); and (c) tissue exposed to 21% (w/v) (974 mM) Atrazine 4L® for (2 h). Arrows indicate areas where there is removal of the epithelial cells.

changes may be attributed to the high concentration of atrazine (up to 974 mM) and the "inert ingredients" incorporated into these products, which are included to enhance the solubility of atrazine along with other adjuvants such as surfactants and preservatives included to improve formulation properties and permeation across plant cell walls (Cox and Surgan 2006). All of these ingredients can enhance the penetration of atrazine and also contribute to the resulting local and systemic exposures to all the ingredients in atrazine-containing products (Baynes and Riviere 1998; Brand and Mueller 2002). The atrazine concentrations used in the *in vitro* experiments described in this report are far higher than those likely to be experienced following the inhalation of aerosolized atrazine herbicides during typical application procedures due to aerosol dilution in the air and subsequently in the nasal secretions after inhalation. At these lower concentrations, the impact of efflux transporter interactions or saturation, along with confounding effects of other co-inhaled substances should be carefully considered. The concentration of atrazine used in the initial passive transport characterization studies showed transporter interactions for a 93 μM solution, a concentration 10,000 times lower than the application concentration recommended for use of Atrazine 4L®. Since many surfactants retain their interfacial and dissolving properties at extremely low concentrations, this suggests that even after significant dilution of the applied herbicide product, the formulation additives may also continue to exert direct effects on the tissues and cellular mechanisms.

The exact composition of the "other ingredients" in the commercial formulations examined is not publicly available (Tominack 2000; United States Environmental Protection Agency 2017), but reports have described these commercial herbicides to contain a variety of chemicals, such as propylene glycol and formaldehyde, which are known to be toxic above well-described concentrations to humans (US Bureau of Land Management 1989; Cox 2005). Many of the tests conducted to register herbicide products are performed only on the active ingredient and not the entire formulation, while recent investigations have begun to show that commercial atrazine-containing herbicide products are more toxic than the active atrazine ingredient (Cox and Surgan 2006). For instance, Zeljezic et al. reported that commercial atrazine-containing herbicide products increased DNA damage in human lymphocytes, but atrazine alone did not (Zeljezic et al. 2006). The toxic role of formulation additives is also reported for additional herbicides (Song et al. 2012), in particular, polyethyoxylated tallow (POEA), an additive included in glyphosate-containing herbicides, has been shown in several different investigations to play a greater role in the adverse impacts of glyphosate than the herbicide agent alone (Meftaul et al. 2020).

It is also well-known that some formulation aids included in commercial herbicide products, e.g. polyethylene glycol and polyethoxylated surfactants, inhibit the MDR1 efflux transporter (Johnson et al. 2002; Ashiru-Oredope et al. 2011). These inhibitory effects of the formulation additives can result in increased human exposures to agents subject to efflux, including the herbicides themselves, resulting in even greater exposures to these agents than are observed from the pure chemicals in aqueous solution. Inhibition of transporters important in the biodistribution of herbicides, especially efflux transporters that act to limit systemic uptake, can result in significant increases in herbicide exposures, and inhibition resulting from formulation additives rather than the herbicide itself, also need to be considered when evaluating the potential neurotoxicity of herbicides and their commercial products. These results suggest that the evaluation of the potential neurotoxicity of herbicides should include consideration of novel absorption pathways, including those involving the nasal mucosa; the influence of transporters on the uptake, efflux, or clearance of the herbicides; and the impact of additional agents included in commercial product formulations.

Conclusion

While efflux transporters present in the nasal mucosa can play a role in limiting the uptake of atrazine, a substrate of MRP1, through the nasal respiratory and olfactory tissues, at high atrazine concentrations these efflux transporters are saturated and a high net transfer of atrazine across the nasal mucosal tissues occurs. Exposures can be even further increased by the inhibition of important efflux transporters or by epithelial barrier disruption caused by unreported formulation additives included in commercial formulations. The resulting ability of select compounds to enter the CNS *via* the nasal cavity, likely by transfer through the olfactory sustentacular cells or along the olfactory and trigeminal nerve tracts, presents an unrecognized, targeted toxicity for

nasally inhaled substances, including sprayed herbicides and pesticides.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, Wisam Al Bakri, upon reasonable request.

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