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The role of L-type amino acid transporters in the uptake of glyphosate across mammalian epithelial tissues

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HIGHLIGHTS

- Transport properties of glyphosate were investigated using Caco-2 and nasal mucosal tissues.
- Inhibitors were used to investigate the role of LAT transporters in glyphosate permeation in both tissues.
- Glyphosate permeation across both epithelia is mediated by amino acid transporters.

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ABSTRACT

Glyphosate is one of the most commonly used herbicides worldwide due to its broad spectrum of activity and reported low toxicity to humans. Glyphosate has an amino acid-like structure that is highly polar and shows low bioavailability following oral ingestion and low systemic toxicity following intravenous exposures. Spray applications of glyphosate in agricultural or residential settings can result in topical or inhalation exposures to the herbicide. Limited systemic exposure to glyphosate occurs following skin contact, and pulmonary exposure has also been reported to be low. The results of nasal inhalation exposures, however, have not been evaluated. To investigate the mechanisms of glyphosate absorption across epithelial tissues, the permeation of glyphosate across Caco-2 cells, a gastrointestinal epithelium model, was compared with permeation across nasal respiratory and olfactory tissues excised from cows. Saturable glyphosate uptake was seen in all three tissues, indicating the activity of epithelial transporters. The uptake was shown to be ATP and Na^+ independent, and glyphosate permeability could be significantly reduced by the inclusion of competitive amino acids or specific LAT1/LAT2 transporter inhibitors. The pattern of inhibition of glyphosate permeability across Caco-2 and nasal mucosal tissues suggests that LAT1/2 play major roles in the transport of this amino-acid-like herbicide. Enhanced uptake into the epithelial cells at barrier mucosae, including the respiratory and gastrointestinal tracts, may result in more significant local and systemic effects than predicted from glyphosate's passive permeability, and enhanced uptake by the olfactory mucosa may result in further CNS disposition, potentially increasing the risk for brain-related toxicities.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine], an herbicide which has been commercially available since 1974, is commonly used both in large agriculture settings and for residential purposes (Mink et al., 2011). Many commercial products containing glyphosate as the active ingredient have been used worldwide due to their effectiveness and relatively low toxicity to humans and other animals (Jasper et al., 2012). As an herbicide, glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which acts in the biosynthesis of aromatic amino acids and of shikimic acid. The enzyme is not present in mammals, which reduces the likelihood for any direct toxicity for glyphosate in

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humans (Kimmel et al., 2013; Prevot-D'Alvise et al., 2013).

Glyphosate is a broad-spectrum, non-selective, post-emergence herbicide, typically sprayed over the areas being treated. As a result, both inhalation and topical exposures to glyphosate are possible, both for the applicator and for other humans and animals in the area of spray application (Wester et al., 1991; Meza-Joya et al., 2013). Given the frequency of spray applications using glyphosate, nasal inhalation exposure to the spray is extremely likely to occur. While there are no current reports regarding potential glyphosate toxicities following nasal inhalation, studies in cell culture (Slaninova et al., 2009; Koller et al., 2012; Mesnage et al., 2013; Prevot-D'Alvise et al., 2013) have suggested potential toxicities to several human cell types, including those obtained from the buccal, nasal, and lower gastrointestinal tract, and other investigations have demonstrated toxicity to the olfactory systems of several species of fish (Slaninova et al., 2009; Solomon et al., 2013). Although glyphosate itself has been shown to be extremely safe in humans following dermal exposure, toxicities from commercial products have been reported to be induced by the excipients contained in the products, especially the surfactants used to improve the permeability of glyphosate across plant cell walls (Gasnier et al., 2009; Allen and Levy, 2013).

Several investigators have described glyphosate distribution in Sprague Dawley rats following a single oral dose and showed that glyphosate has a limited distribution (1.98%) into the brain (Brewster et al., 1990; Williams et al., 2000). Most of the ingested glyphosate remained associated with the small intestinal tissues, likely being sequestered in the surface epithelial cells. Additional reports, however, continue to suggest a link between glyphosate exposure and a variety of adverse health effects, including Parkinson's and Alzheimer's disease, and recently, even cancer (Kamel et al., 2007; Freire and Koifman, 2012; Allen and Levy, 2013; Guyton et al., 2015).

In plants, glyphosate is reported to be a substrate for several phosphate transport systems (Denis and Delrot, 1993). Since glyphosate is a glycine analog, it is possible that glyphosate may also be a substrate for the glycine-uptake pathways in mammalian cells. A family of transport proteins, glycine transporters (GLYT), which are specific for glycine transport, and the $b^{0,+}$, ASC, asc and L systems of the amino acid transporter family (Kanai and Endou, 2001; Verrey, 2003; Del Amo et al., 2008), are also able to transfer glycine.

Amino acid transporters, including y^+ LAT, $b^{0,+}$, LAT1, and LAT2, are facilitative transporters which act by using a unique mechanism that is described as an “in and out” transfer, where the transporter effluxes one amino acid out of the cell to exchange for another amino acid taken up into the cell at the same time. Given the affinity of several amino-acid-analog drug compounds (L-dopa, gabapentin) for the L system transporters (LAT1, LAT2), the affinity of glyphosate at these transporters was the focus of these investigations. The activity of other, non-LAT transporters was also considered in these studies since there are numerous reports of the overlapping substrate specificities among the amino acid transporters (Del Amo et al., 2008).

If amino acid transport systems are involved in the systemic absorption and distribution of glyphosate, or potentially act to allow the nose-to-brain transfer of glyphosate across the nasal epithelium, the potential for nasal inhalation toxicity may be greater than has been predicted from the previously described pulmonary toxicity profiles (Bradberry et al., 2004). In order to investigate the epithelial transfer properties of glyphosate, two different *in vitro* models were used to evaluate the roles of passive permeability and carrier-mediated uptake. Absorption across the gastrointestinal epithelium was measured using Caco-2 cells, a transformed colonic epithelial cell line frequently used to investigate the gastrointestinal permeability of drug compounds. Excised bovine nasal mucosal tissues were employed to evaluate the uptake of glyphosate by the nasal epithelium and to compare the transport capacities between the nasal respiratory and olfactory mucosae. Previous investigators have shown that LAT1 and LAT2 are expressed in Caco-2 cells, and confirmation of the expression of these transporters throughout the intestinal tract of animals and humans has been reported (Fraga et al., 2005; Terada et al., 2005). The expression of LAT1 and LAT2 gene transcripts in the nasal olfactory and respiratory mucosa of humans, rats and cattle was recently reported by Al Ghabeish et al. (2015) using DNA microarray analysis. Weak expression of LAT1 in cattle mucosal tissues was reported by Zhang (2009) using immunoblotting, and Ferreira and Donovan (2013) more recently reported the confirmation of the expression and localization of LAT2 in cattle nasal mucosa using both PCR and immunohistochemical analyses.

2. Materials and methods

2.1. Reagents

Glyphosate, gabapentin HCl, L-alanine, L-leucine, L-phenylalanine, 2-amino-2-norbornanecarboxylic acid (BCH), tetraethylammonium (TEA) and 2,4-dinitrophenol (2,4-DNP) were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA). HPLC-fluorescence derivatization agent FMOC-Cl and GC-FID derivatization agents, including trifluoroacetic anhydride (TFAA), trifluoroethanol (TFE) and chloroform were also obtained from Sigma–Aldrich Chemical. Dichloromethane and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Krebs Ringer's buffer (KRB) was composed of 1.67 mM $MgCl_2$, 4.56 mM KCl, 119.78 mM NaCl, 1.5 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 10 mM D-glucose, and 15 mM $NaHCO_3$ in 1000 mL deionized water. After bubbling with 95% CO_2/5% O_2, 1.2 mM $CaCl_2$ was added. The pH was adjusted to 7.4 using 1 M HCl. TEA was used in the Na^+ free medium instead of NaCl, and other Na^+ containing compounds were substituted with K^+ salts.

Hank's balanced salt solution (HBSS) was composed of 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 5.55 mM D-glucose, 1.26 mM $CaCl_2$, 0.32 mM $MgCl_2$ and 4.2 mM $NaHCO_3$ in 1000 mL deionized water, and then filtered with a 0.22 μm filter for sterilization. For the Na^+ free HBSS, only NaCl was substituted with TEA (137 mM) while other Na^+ containing compounds were substituted with K^+ salts. All of the chemicals used to prepare the buffer were obtained from Sigma–Aldrich Chemical.

2.2. Glyphosate transport across nasal tissues

2.2.1. Preparation of nasal mucosal tissues

Bovine nasal mucosal tissues were obtained from Bud's Custom Meat (Riverside, IA, USA and Wufeng Meat Corporation, Wuhan, China). The nasal turbinates were retrieved by opening the nasal cavity along the septal midline and removing the turbinate from the lateral wall. The ethmoturbinate, which is covered by olfactory mucosa and the maxilloturbinates, which are covered by respiratory mucosa, were excised carefully and the tissues were immediately transported to the laboratory in ice-cold KRB.

The mucosal tissues were stripped from the underlying cartilage using forceps and returned to the ice-cold KRB prior to mounting onto Navicyte® (Harvard Apparatus, Holliston, MA, USA) vertical diffusion chambers. Once placed into the chambers, the tissues were equilibrated at 37 °C in KRB for 15 min. Carbogen was used to mix, oxygenate, and maintain the buffer pH by bubbling into the donor and receiver chambers at a rate of 3–4 bubbles per second.

2.2.2. Glyphosate transport and inhibition study across nasal tissues

After equilibration, the transepithelial electrical resistance (TEER) of each tissue was measured using an EVOM® volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) to verify the integrity of the tissues; typical TEER values for the respiratory tissues were measured in the range of 165–200 Ω cm^2, and the olfactory tissues were in the range of 100–160 Ω cm^2. Tissues with resistances below 100 Ω cm^2 were regarded as damaged and were discarded. After tissue equilibration and TEER measurement, the buffer solutions in the donor and receiver chambers were replaced with glyphosate-containing solutions (donor) and 1 mL of fresh KRB (receiver).

Transporter inhibition studies were conducted in the presence of several different large amino acid transporter (LAT) inhibitors (BCH, L-alanine, L-leucine, L-phenylalanine) (Del Amo et al., 2008). A 10 mM concentration, 10–100 fold greater than the reported K_i (Grembecka et al., 2000; Gomes and Soares-da-Silva, 2002; Khunweeraphong et al., 2012) was chosen for each inhibitor. 2,4-DNP (10 mM), an ATP-ase inhibitor, was also investigated as a non-specific inhibitor using the same method as for the transport studies, except that the LAT inhibitors or 2,4-DNP were included in the equilibration buffer, in the glyphosate-containing (1200 μM) solutions and in the receiver chamber solution. Samples (200 μL) were collected from the receiver chamber at 15, 30, 45, 60, 75 and 90 min after exposing the tissues to glyphosate. All volumes removed were replaced with the same volume of pre-warmed KRB buffer.

2.3. Glyphosate transport across Caco-2 cells

2.3.1. Cell culture

Caco-2 cells were cultured in Corning® non-treated culture dishes (Diameter: 10 cm, Sigma–Aldrich Chemical, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM), mixed with Ham's F-12 nutrient with a volume ratio of 1:1 (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Sciencell, Carlsbad, CA, USA). After reaching 80–90% confluence, cells were trypsinized and were used for cell passaging; were seeded onto 24-well plates (NEST Biotec Co. Ltd., Wuxi, China) for 3 days with DMEM at a density of 4×10^6 cells cm^{-2}; were seeded onto 96-well plates for MTT assay to check the viability of the cells under test conditions; or were seeded onto 6-well plates for protein extraction for immunoblotting. All of these experiments were conducted on cells between passages 20–45.

2.3.2. Cell cytotoxicity

The Vybrant® MTT cytotoxicity kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the cytotoxicity of glyphosate to Caco-2 cells. Briefly, cells were grown overnight in 96-well plates at a density of 20,000 cells per well. The well medium was replaced with various concentrations (30 μM–1200 μM) of glyphosate in DMEM. Cells were incubated for 4 h at 37 °C prior to adding 20 μL MTT test solution for an additional 2 h incubation at 37 °C. Cell viability was determined by measuring the absorbance at 485 nm of the formazan deriviative produced by the metabolically active cells (ELx 800, BioTek, Winooski, VT, USA).

2.3.3. Immunoblotting

Caco-2 cells were cultured in DMEM at 37 °C on 6-well plates under 95% O_2, 5% CO_2. The cells were scratched down on ice immediately and centrifuged at 3000× g at 4 °C for 1 min, and the supernatants were collected in 1.5 mL vials. Bovine tissues were homogenized using a Biospec® Tissue Tearor (Bartlesville, OK, USA), centrifuged at 3000× g at 4 °C for 1 min and collected into 1.5 mL vials. Protein lysis buffer (30 μL), 10 mM PMSF (phenylmethanesulfonyl fluoride) and Radio-Immunoprecipitation Assay (RIPA) lysis buffer in a ratio of 9:1 (Beyotime®, Shanghai, China) were added to the tube before incubation on ice for 30 min. The vials were centrifuged at 12000× g at 4 °C for 5 min. The supernatant was collected and an aliquot of 50 μg of total protein/sample was resolved in precast gradient Midi gels® (Beyotime, Shanghai, China). A Bio-Rad® system (Hercules, CA, USA) was employed for the gel electrophoresis and proteins were electrotransferred to a Biosharp® (Hefei, China) PVDF membrane (0.45 μm). After the transfer process, the membrane was blocked in Tris-buffered saline (pH 7.6) with 0.05% Tween-20, containing 0.5% non-fat milk for 80 min prior to immunoblotting with a rabbit monoclonal anti-human LAT1 antibody (Cell Signaling®, Danvers, MA, USA) for human LAT1 or a mouse monoclonal anti-human LAT2 antibody (Abcam®, Cambridge, MA, USA). The immunoblotted membranes were visualized using Electro-Chemi-Luminescence reagents (Bio-Rad®, Hercules, CA, USA) and quantified with a Syngene® (Frederick, MD, USA) camera-equipped detection system.

2.3.4. Uptake and inhibition of glyphosate in Caco-2 cells

Unless otherwise indicated, Caco-2 cells were seeded and cultured in DMEM at 37 °C under 95% O_2, 5% CO_2 for 2–3 days. Prior to initiating each uptake experiment, the medium was removed and the cells were rinsed twice with 1 mL phosphate buffered saline (PBS, pH 7.4). Uptake studies were initiated by adding 300 μM glyphosate (or glyphosate plus 10 mM L-alanine, BCH, or L-leucine) in DMEM and then incubated at 37 °C for 10 min. The uptake process was halted by quickly removing the medium and rinsing the cells with ice-cold PBS. Water was added to each well, then the cells were scratched down into a 1.5 mL plastic vial and the supernatant was collected after 10 min ultrasonication followed by a 10 min centrifugation at 18,000× g.

2.3.5. Transport of glyphosate across Caco-2 cells

To better understand the transport mechanisms involved in the uptake/efflux of glyphosate, Caco-2 cells were grown on Millicell®–PCF (Merck KGaA, Darmstadt, Germany) membranes in 24 well plates to investigate the apical-basolateral (A–B) transport of glyphosate. Caco-2 cells were seeded at a density of 50,000 cells per membrane and incubated in DMEM at 37 °C. The monolayer integrity was evaluated using a Millpore TEER device (Millicell®-ERS, Millipore, Billerica, MA, USA). The ΔTEER of the cell monolayer was calculated using the following equation (Eq. (1)):

$$\Delta TEER = (TEER_{Cells} - TEER_{Blank}) \times A \quad (1)$$

$TEER_{Cells}$ – TEER of the well in which cells were cultured.
$TEER_{Blank}$ – TEER of the well without cells.
A – Area of each membrane of the membrane (1.12 cm^2).

Cells were cultured for 21 days until ΔTEER >400 Ω cm^2; the medium was changed every two days during the first week and then changed daily thereafter.

Various inhibitors of amino acid transport were used to evaluate the role of specific transporters in glyphosate transport. For transporters reported to be expressed primarily on the basolateral membrane of Caco-2 cells (LAT1, LAT2) (Fraga et al., 2005), the culture wells were initially equilibrated for 20 min with inhibitor-containing solutions placed in the basolateral-facing culture well. The apical and basolateral media were removed and the apical chamber was replaced with HBSS containing both glyphosate

(30 μM) and one of the inhibitors (10 mM) [L-leucine (LAT1), L-alanine (LAT2), BCH (L system) or L-phenylalanine (L system)]. HBSS containing the inhibitor (10 mM) was placed in the basolateral chamber. Samples (100 μL) were withdrawn from the basolateral chambers at 2, 5, 10, 20, 30, 45, 60, 90 and 120 min, and an equal volume of inhibitor/HBSS solution was replaced into the chamber after each withdrawl.

2.4. Analytical methods

2.4.1. Solid phase extraction (SPE)

Waters Oasis® HLB cartridges were employed for sample preparation. The cartridges were conditioned with 1 mL of methanol followed by 1 mL of water. Samples (200 μL) spiked with gabapentin (4 μg mL^{-1}) as an internal standard were added to the column and eluted with 100 μL water and collected in 10 mL glass tubes (Botero-Coy et al., 2013).

2.4.2. Sample derivatization

2.4.2.1. Gas chromatography. For glyphosate samples obtained from bovine nasal mucosal transport studies, the derivatization procedures were used for quantification by gas chromatographic analysis from a method developed by Lebeck MG et al. at the University of Iowa (personal communication). TFE (400 μL) and TFAA (800 μL) were added to the SPE eluates and the mixtures were heated at 100 °C for 60 min. The tubes were maintained at 60 °C and evaporated under a stream of nitrogen for 40 min. The residue in each tube was reconstituted with 1 mL chloroform, and the samples were injected directly into a GC-FID detection system consisting of an HP 9100 GC equipped with a FID detector using an Agilent HP-1 column (length 30 m, diameter 0.53 mm, film thickness 0.88 μm). The gas flow rate was: H_2 (280 kp), Air (220 kp), Helium (400 kp). A temperature program with a 120 °C hold for 5 min, followed by an increase to 200 °C (5 °C min^{-1}) with a 3 min hold was used. The total run-time for each sample was 24 min, and retention times of 5.7 min for glyphosate and 11.60 min for gabapentin were obtained.

2.4.2.2. HPLC-fluorescence determination. For HPLC measurement, a fluorescent derivative of glyphosate was formed using traditional FMOC procedures. Borate buffer (100 μL, pH 9.1) and 100 μL FMOC-Cl (1 mM in acetonitrile) were added to the cell culture supernatants and kept at room temperature for 2 h. Dichloromethane (400 μL) was added to the mixture and vortexed for 2 min prior to centrifugation for 10 min at 18,000× g. Finally, 100 μL of the supernatant was injected into a HPLC-fluorescence system consisting of a Shimadzu Nexera HPLC system with a RF20A fluorescence detector (excitation wavelength = 260 nm, emission wavelength = 310 nm) and a Thermo C18 column (250 mm × 4.6 mm I.D., 5 μm) was used as described by Bernal et al. (2010).

2.5. Data analysis

The cumulative amount of glyphosate appearing in the basolateral chambers was plotted as a function of time, and the permeability of glyphosate was calculated using a Fick's Second Law treatment. The transepithelial flux in nasal tissues: mucosa to submucosa (M–S), and in Caco-2 cells: apical to basolateral (A–B), J, was calculated from the amount of glyphosate, Q, accumulating in the receiver compartment over time, t, across the exposed surface area, A, as follows (Eq. (2)):

$$J_{M-S \text{ or } A-B} = dQ/dt \cdot (1/A) = P_{app} \cdot C \quad (2)$$

dQ/dt – Slope of the cumulative amount of glyphosate transported vs. time.
A – Exposed area of the nasal mucosa (0.64 cm^2) and Caco-2 cells (1.12 cm^2).
C – Initial donor concentration (mM) of glyphosate.
P_{app} – Apparent permeability coefficient (cm s^{-1}) of glyphosate.

The initial transport rates of glyphosate across the olfactory mucosa in the M-S direction and across the Caco-2 cells in A-B directions were fit to a Michaelis–Menten equation (Eq. (3)):

$$J_{M-S} = J_{max} \cdot C/(K_m + C) \quad (3)$$

J_{M-S} – Measured rate of glyphosate transport across the nasal tissues.
C – Glyphosate concentration.
K_m – Michaelis–Menten constant.

Numerical fitting was performed using a nonlinear least-squares regression analysis. All data and statistical analyses were performed using GraphPad Prism (Version 6.0f; San Diego, CA, USA). Data are presented as mean ± SD. Comparison of mean values for glyphosate flux and glyphosate flux in the presence of an inhibitor were compared individually using the Student's t-test. Between group comparisons were tested using one-way ANOVA.

3. Results

3.1. Transport of glyphosate in bovine nasal respiratory and olfactory tissues

The transport of glyphosate across bovine nasal respiratory or olfactory tissues showed a saturable uptake (Fig. 1), which indicated active transport mechanisms in addition to passive diffusion were involved. Fitting the permeabilty vs. substrate concentration results using a Michaelis-Menten-like approach (Eq. (3)) resulted in K_m values of 100 ± 32 μM in respiratory and 523 ± 68 μM in the olfactory tissues. The J_{max} values were determined to be 128 ± 14 μg min^{-1} cm^{-2} in respiratory and 19 ± 7 μg min^{-1} cm^{-2} in olfactory tissue, respectively. The respiratory mucosa allowed greater amounts of glyphosate to permeate than the olfactory tissues.

3.2. Inhibition of glyphosate transport in nasal tissues

Since the transport studies suggested the activity of a transporter, additional investigations were carried out using competitive amino acids and other general and specific inhibitors to evaluate the potential role of the amino acid transporters in the uptake of glyphosate by the epithelial cells. 2,4-DNP was initially utilized to investigate the role of ATP-dependent transporters. No significant differences between the flux of glyphosate in the presence or absence of 2,4-DNP were observed, suggesting that glyphosate transport was not ATP-dependent. When the amino acid transporter inhibitors L-alanine, L-leucine, L-phenylalanine and BCH were used, glyphosate flux was reduced approximately 30%–50% in olfactory tissues, suggesting that amino acid transporters, especially transporters from the LAT family, may play a role in the uptake of glyphosate across the olfactory epithelium (Fig. 2).

3.3. Glyphosate uptake and transport study in Caco-2 cells

The uptake of glyphosate over a 60–1200 μM concentration

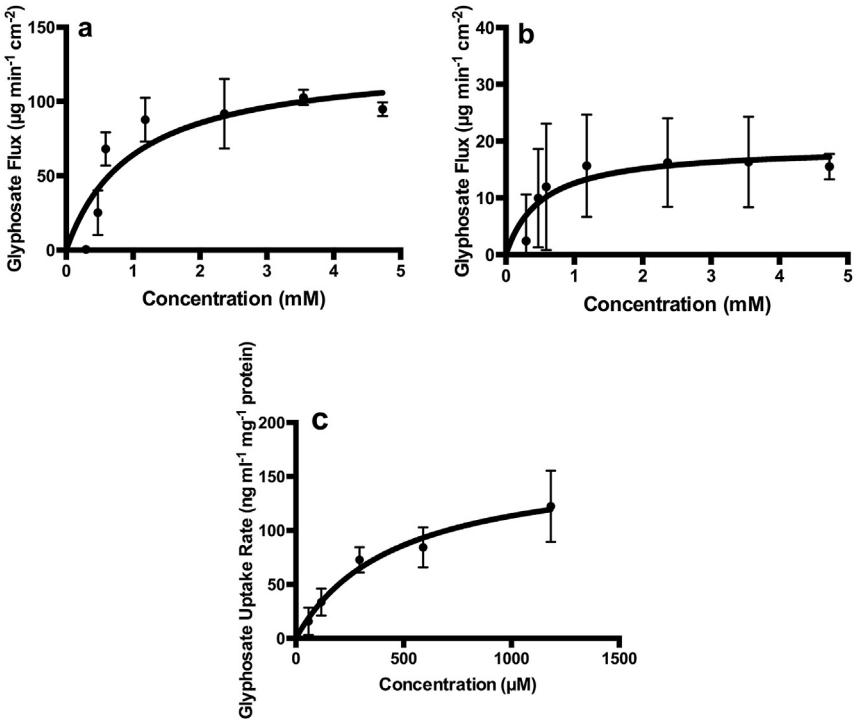

Fig. 1. Flux of glyphosate across nasal respiratory (a), olfactory tissues (b) and uptake rate into Caco-2 cells (c) at various concentrations. Flux and uptake rate presented as mean ± SD (n = 3).

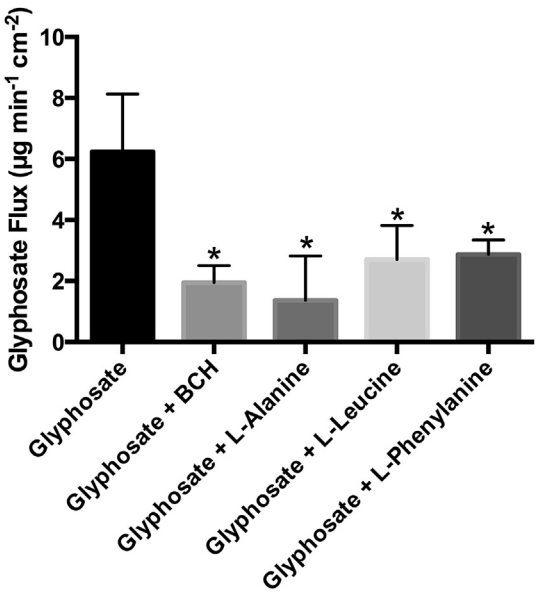

Fig. 2. Reduction of glyphosate (1.2 mM) flux through excised olfactory tissues by different competitive inhibitors (10 mM). * indicates a significant difference compared to glyphosate group ($p < 0.05$, n = 3).

range was also found to be saturable in Caco-2 cells (Fig. 1)(c), with an uptake $K_m = 461 \pm 168$ µM and $J_{max} = 166 \pm 26$ ng mL^{-1} mg^{-1} protein (Eq. 3). As an additional investigation, a Na^+ free medium was substituted in the transport media to investigate the potential activity of the Na^+-dependent amino acid or phosphate transporters, and no significant differences were observed in the glyphosate flux values from either of the two media (Table 1).

When several traditional amino acid transporter inhibitors were included in the media, each was able to significantly decrease the uptake of glyphosate by Caco-2 cells (Fig. 3). In the A-B transport studies, each inhibitor (BCH, L-alanine, L-leucine, L-phenylalnine) was also able to inhibit the flux of glyphosate (Fig. 4).

3.4. Immunoblotting

While previous investigators have reported the expression of LAT1 and LAT2 in the gastrointestinal and nasal mucosae, confirmation of their expression in the tissues utilized in these studies was undertaken using immunoblotting. Fig 5 shows the results and clearly demonstrates the expression of these transporters in the tissues.

3.5. Cell cytotoxicity

The Caco-2 cell viability following a 2 h exposure to glyphosate (30–1200 µM) measured using the MTT assay was essentially 100%, demonstrating that glyphosate was not toxic under the experimental conditions used to evaluate transport properties.

4. Discussion

Glyphosate-based herbicides have been available in the worldwide market for nearly 40 years. Previous toxicity evaluations in humans exposed to glyphosate have shown minimal absorption and toxicity resulting in glyphosate being considered quite safe. Glyphosate absorption and transport across cells and tissues, even in plants, is limited, due to its polarity and low permeability (Mink et al., 2011).

In this study, two different epithelial barriers were employed to further investigate the potential role of carrier-mediated glyphosate absorption and transport. The results showed a concentration-dependence of glyphosate flux in the nasal respiratory and

Table 1
Flux of glyphosate across Caco-2 monolayers in Na^+-included and Na^+free media. Comparisons between media containing the same glyphosate concentrations were made using the Student's t-test. No significant differences were observed between groups (Results are shown as mean ± std dev, n = 3–4).

Glyphosate concentration (μM)	Flux (Na^+ included), (ng min^{-1} cm^2)	Flux (Na^+ free), (ng min^{-1} cm^2)
30	16.47 ± 4.72	10.35 ± 3.23
60	36.03 ± 6.87	32.74 ± 12.60
300	163.18 ± 20.14	150.83 ± 8.78
600	208.27 ± 42.48	184.21 ± 18.53
1200	447.16 ± 128.27	435.24 ± 133.09
3000	867.61 ± 184.35	591.15 ± 111.16

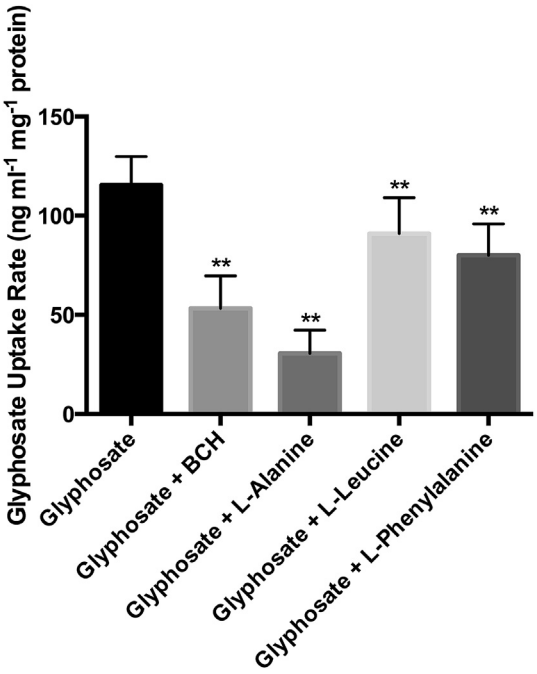

Fig. 3. Inhibition of glyphosate (300 μM) uptake by various competitive inhibitors of amino acid transport (10 mM) in Caco-2 cells. **indicates a significant difference compared to the uninhibited glyphosate uptake group. ($p < 0.01$, n = 4).

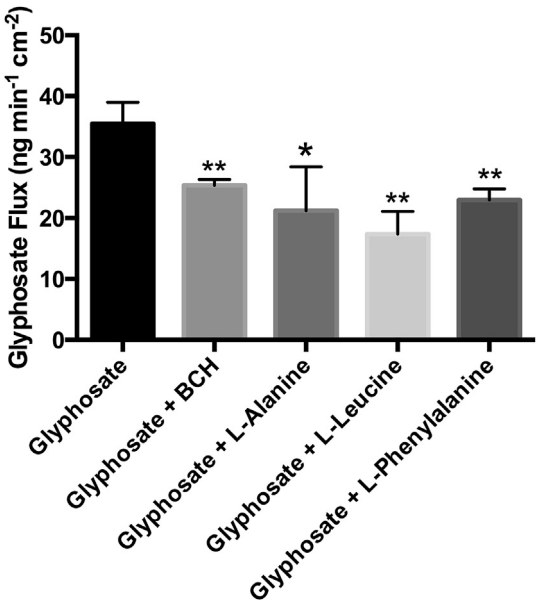

Fig. 4. Inhibition of glyphosate (300 μM) A-B transport by various competitive inhibitors (10 mM) of amino acid transport. *indicates a significant difference compared to glyphosate group, *$p < 0.05$, **$p < 0.01$, n = 4.

olfactory tissues and in Caco-2 cells. Transporter inhibition using non-specific inhibitors showed that the mechanisms involved in glyphosate transport were both ATP and Na^+ independent. This lack of energy and Na^+ independence excludes a significant number of potential transporters from participation in transferring glyphosate. Many amino acid transporters show a dependence on Na^+ to transport effectively, including the y^+L transporters, and many of the glycine and phosphate transporters are also reported to be Na^+ dependent. LAT1 and LAT2, in comparison, are Na^+ independent transporters, so the results obtained using the Na^+ free medium enable us to exclude a significant number of potential amino acid, glycine, and phosphate transporters from consideration when attempting to identify the transporters involved in glyphosate uptake in the nasal and gastrointestinal epithelia.

Glyphosate flux was reduced in the presence of several amino acids and BCH, a specific LAT1/2 amino acid transporter inhibitor, which strongly suggests that the amino acid transporters play a significant role in glyphosate transfer across these epithelial cells. For the 3 amino acids used as competitive inhibitors, glyphosate uptake or flux rates were reduced significantly in the olfactory tissues and in Caco-2 cells, especially with L-alanine, a prototypical substrate for the LAT2 transporter (Figs. 2 and 3). Given that none of the inhibitors was able to completely halt the uptake or transfer of glyphosate into/across the cells, it is likely that glyphosate is a substrate for a number of transport systems present in these epithelial cells, including other Na^+ independent amino acid transporters and potentially transporters in the OCT or OCTN families. Inhibition of any of these transporters enables the activity of the other transporters (either apical or basolateral) to become more prominent.

Previous investigators have assumed that systemic exposure to glyphosate and any resulting toxicities will be minimal due to its high polarity and resulting low passive permeability. Organ-specific toxicities of glyphosate have been reported over recent years (Anadon et al., 2009; Astiz et al., 2009, 2012; Prevot-D'Alvise et al., 2013; Cassault-Meyer et al., 2014), and the current results regarding the affinity of glyphosate for the amino acid transporters suggests that additional toxicities may be exhibited in organs expressing high amino-acid transporter activity. Due to the wide expression of amino acid transporters in a variety of organs, for example, LAT1 is expressed in brain, stomach, liver, intestine, heart, lung and testis and LAT2 is expressed in kidney, intestine, brain, liver, stomach, heart, lung and muscle (Del Amo et al., 2008), these organs are likely sites for increased glyphosate toxicity. A recent publication described the presence of measurable concentrations of glyphosate in the liver, spleen, intestine, kidneys, heart, and muscle tissue of chickens who consumed feed containing glyphosate (Shehata et al., 2014). Each of these organs and tissues is known to possess significant amino acid transporter activity which may explain the observed biodistribution pattern. Another recent publication has

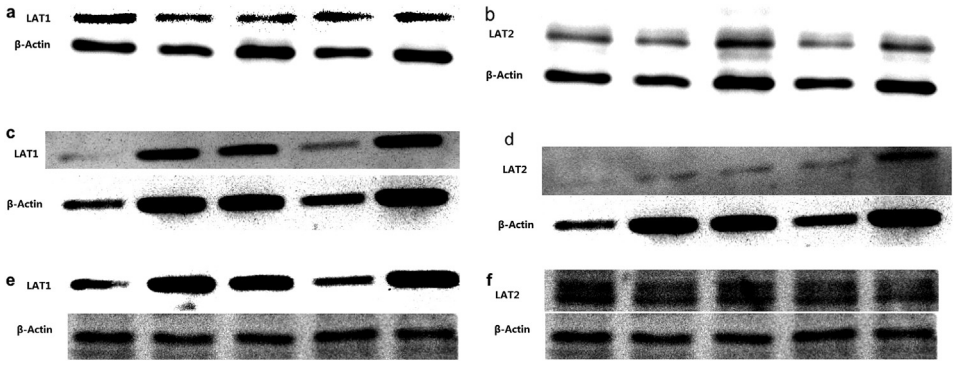

Fig. 5. Immunoblots for LAT1 and LAT2 transporters in Caco-2 cells (a, b), respiratory tissues (c, d), and olfactory tissues (e, f). Each lane contains a tissue sample; β-Actin was blotted as a control.

implicated glyphosate's antibacterial effects on the gut microbiome resulting in a number of subsequent pathologies including celiac disease and potentially a number of neurologic disorders (Samsel and Seneff, 2015). The ability of Caco-2 cells to take up significant quantities of glyphosate supports the observation of Williams et al. (2000), that the intestinal tissues contain high concentrations of glyphosate following oral ingestion. These locally high levels may serve as a continued source of glyphosate able to disturb the microflora patterns in the gut leading to many of the pathologies suggested by Samsel and Seneff (2015). Additional local toxicities, for example the changes in testicular function and sperm morphology reported by Cassault-Meyer et al. (2014), may also be the result of the significant organ concentrations of glyphosate enabled by amino acid transporters present within the tissues.

While inhalation exposure evaluations of glyphosate-containing herbicide sprays have focused on the pulmonary system, the findings reported here regarding the ability of amino acid transporters to transfer glyphosate into and across the nasal respiratory and olfactory tissues suggest that glyphosate inhalation may result in both locally high nasal mucosal tissue concentrations and the potential for the glyphosate associated with the olfactory epithelium to transfer along the nose to brain pathways directly into the CNS (Wu et al., 2008; Lee et al., 2010; Dhuria et al., 2010; Malhotra et al., 2010; Sattler et al., 2011). This additional pathway for glyphosate to enter the brain may result in much higher brain concentrations than previously anticipated based on oral or intravenous exposures, and may also explain the occurrence of reported neurologic toxicities.

Conflicts of interest

The authors have no conflicts of interests with the agencies supporting this research or suppliers and manufacturers of materials described in this report.

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