

## Title page

# **Intranasal co-administration of a diazepam prodrug with a converting enzyme results in rapid absorption of diazepam in rats**

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## Running Title Page

### *Running Title*

Co-administration of prodrug and converting enzyme to rats

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## Abbreviations

AOP, *Aspergillus oryzae* protease; APB, aminopeptidase B; AUC, area under the curve; AVF, avizafone; R, blood to plasma ratio; C, concentration;  $C_{DZP,lim}$ , solution concentration limit for diazepam;  $CL_{int}$ , intrinsic clearance;  $C_{max}$ , maximum concentration;  $C_{nasal,DZP,sol}$ , nasal cavity concentration of diazepam in solution; cLogD, calculated octanol/water partition coefficient at specified pH; DTE, drug targeting efficiency; DZP, diazepam; ee, enantiomeric excess; F, bioavailability;  $f_u$ , fraction unbound; IN, intranasal; IPTG, isopropyl  $\beta$ -D-1-thiogalactopyranoside; IV, intravenous; JM109, bacteria strain;  $k_2$ , cyclization rate constant;  $k_{a,DZP}$ , diazepam absorption rate constant;  $k_{a,ORI}$ , open ring intermediate absorption rate constant;  $k_{cat}$ , catalytic rate constant;  $K_P$ , partition coefficient;  $K_M$ , Michaelis constant;  $\lambda_z$ , terminal rate constant; MDCKII-wt, wild type Madin-Darby canine kidney epithelial cells; NCA, noncompartmental analysis; NBF, neutral buffered formalin; ORI, open ring intermediate;  $P_{app}$ , permeability coefficient; PBPK, physiologically based pharmacokinetic model; PBS, phosphate buffered saline; pQE-30 Xa, vector construct; Q, blood flow rate;  $R^2_{adj}$ , adjusted square correlation coefficient; RMSE, root mean square error; RNPEP, aminopeptidase B gene;  $t_{1/2,z}$ , terminal half-life;  $t_{max}$ , time to peak concentration; V, volume;  $V_{max}$ , maximum velocity

## Section Assignment

special section on Drug Delivery Technologies

## Abstract

Intranasal administration is an attractive route for systemic delivery of small, lipophilic drugs because they are rapidly absorbed through the nasal mucosa into systemic circulation. However, the low solubility of lipophilic drugs often precludes aqueous nasal spray formulations. A unique approach to circumvent solubility issues involves co-administration of a hydrophilic prodrug with an exogenous converting enzyme. This strategy not only addresses poor solubility, but also leads to an increase in the chemical activity gradient driving drug absorption. Herein, we report plasma and brain concentrations in rats following co-administration of a hydrophilic diazepam prodrug, avizafone, with the converting enzyme *human aminopeptidase B*. Single doses of avizafone equivalent to diazepam at 0.500, 1.00, and 1.50 mg/kg were administered intranasally, resulting in  $77.8 \pm 6.0$ ,  $112 \pm 10$ , and  $114 \pm 7$  % bioavailability; maximum plasma concentrations  $71.5 \pm 9.3$ ,  $388 \pm 31$ , and  $355 \pm 187$  ng/mL; and times to peak plasma concentration 5, 8, and 5 min for each dose level respectively. Both diazepam and a transient intermediate were absorbed. Enzyme kinetics incorporated into a physiologically based pharmacokinetic model enabled estimation of the first order absorption rate constants:  $0.0689 \pm 0.0080 \text{ min}^{-1}$  for diazepam and  $0.122 \pm 0.022 \text{ min}^{-1}$  for the intermediate. Our results demonstrate that diazepam, which is practically insoluble, can be delivered intranasally with rapid and complete absorption by co-administering avizafone with aminopeptidase B. Furthermore, even faster rates of absorption might be attained simply by increasing the enzyme concentration, potentially supplanting intravenous diazepam or lorazepam or intramuscular midazolam in the treatment of seizure emergencies.

## Introduction

Status epilepticus and acute repetitive seizures are serious medical emergencies that can cause lasting neurological damage and other life-threatening complications. Prolonged seizure activity associated with these conditions may lead to hypoxia, metabolic acidosis, hyperthermia, aspiration, pulmonary edema, brain injury, and an increase in the likelihood of experiencing additional seizures in the future (Mirski and Varelas, 2008). The risk of injury, medical complications, or death can be mitigated by prompt medical treatment (Pellock, 2007; Sutter et al., 2013). Benzodiazepines are the drugs of choice for treating seizure emergencies. Intravenous (IV) diazepam (DZP), IV lorazepam, or intramuscular midazolam (MDZ) are the preferred first-line rescue therapies (Glauser et al., 2016). However, drug delivery by injection can be problematic, especially when administered by non-medical personnel. Injection is difficult to carry out on obese or emaciated individuals, in the presence of multiple layers of clothing, or in public places. Furthermore, treatment of seizure emergencies can be significantly delayed if the patient must wait for medical personnel or transportation to the emergency room. Therefore, there is a need for outpatient treatments that can be administered immediately at the onset of a seizure by non-medical personnel.

Currently, the only outpatient treatment of a seizure emergencies approved by the United States Food and Drug Administration (FDA) is rectally administered DZP (Diasat<sup>®</sup>). Patients often object to this route of administration despite its effectiveness (Haut et al., 2016). Off-label alternatives to rectal diazepam including intranasal (IN) and buccal MDZ using, respectively, injectable and oral solution formulations (Maglalang et al., 2018). Other routes to deliver benzodiazepines for the treatment of seizure emergencies currently under development include

buccal DZP films (Heller et al., 2018) and IN sprays for DZP or MDZ (Agarwal et al., 2013; Bancke et al., 2013).

Intranasal DZP is an attractive treatment option because administration is easily performed by non-medical personnel, and it has an extended duration of action with low risk of adverse events (Maglalang et al., 2018). However, the poor aqueous solubility of benzodiazepines presents a challenge in formulating them as nasal sprays because they require co-solvents or other additives to improve solubility (Kälviäinen, 2015). These solubilizing excipients can be highly irritating to nasal mucosa. Transient epistaxis, nasal discomfort, nasal congestion, and rhinitis have been reported as side effects with some of these formulations, including those with DZP (Agarwal et al., 2013; Buck, 2013; Sperling et al., 2014). Further, the time to reach the maximum plasma concentration ( $t_{\max}$ ) for IN DZP formulations utilizing solubilizing excipients is relatively slow, ranging from 0.75-1.5 hrs (Agarwal et al., 2013; Ivaturi et al., 2013; Maggio and Pillion, 2013).

One strategy to overcome these challenges is to formulate a nasal DZP spray in an aqueous vehicle employing a water-soluble lysine prodrug of DZP, avizafone (AVF). While prodrug formulations typically rely on endogenous enzymes for *in vivo* biotransformation to the active drug (Jornada et al., 2015), unpublished results from a pilot canine study performed by our group suggested that exogenous enzyme would need to be included in the formulation to achieve the desired prodrug conversion rate and bioavailability. A series of *in vitro* experiments combining water soluble benzodiazepine prodrugs with the fungal converting enzyme *Aspergillus oryzae* protease (AOP) demonstrated that the formulations were capable of producing supersaturated solutions of the active drug, leading to an enhanced permeation rate of drug across cultured cell monolayers (Kapoor and Siegel, 2013; Kapoor et al., 2014; Siegel et al., 2015; Kapoor et al., 2016).

Further *in vitro* studies characterized the conversion kinetics of AVF to DZP (Rautiola et al., 2018), which first involves enzymatically catalyzed release of the lysine and formation of an open ring intermediate (ORI), followed by spontaneous closure of ORI to form DZP (Figure 1). These studies also showed that supersaturated DZP undergoes liquid/liquid phase separation at a critical degree of supersaturation, and resists crystallization over periods of days.

Based on these *in vitro* results, we hypothesized that intranasal administration of prodrug/enzyme formulations should result in rapid and complete *in vivo* absorption of DZP, with rapid distribution into the brain. In the present study, we tested this hypothesis in a rat model, in which we replaced AOP with a human enzyme, aminopeptidase B (APB), and compared the plasma and brain pharmacokinetics of IN AVF/APB with IN AVF and IV DZP. In addition to standard noncompartmental pharmacokinetic analyses, we augmented an established physiologically based pharmacokinetic (PKPB) model to incorporate mechanistic components, including enzyme reaction kinetics, absorption of both ORI and DZP across the nasal mucosa, supersaturation of DZP, and liquid-liquid phase separation of DZP. Finally, we carried out preliminary histological studies to determine whether acute damage would result from the AVF/APB formulations.

## Materials and Methods

### *Experimental design*

The primary aim of this study was to determine whether IN administration of an AVF/APB combination to rats would result in rapid absorption of DZP without damaging the nasal mucosa. In brief, single doses of admixed solutions of AVF and APB equivalent to DZP at either 1.50 mg/kg, 1.00 mg/kg, or 0.500 mg/kg were deposited in the nasal cavities of rats. Plasma samples and brains were collected at time points ranging from 2 to 90 min. DZP concentrations measured in these tissues were compared against concentrations measured in samples collected from rats that were administered a 1.00 mg/kg dose of DZP intravenously (IV). Nasal tissue samples collected from rats sacrificed at the 90 min time point were examined for lesions. Details about the dosing and sample collection schedule are given in the sections that follow and summarized in the supplemental data (Supplemental Table 1S).

### *Animals*

All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Minnesota (protocol 1508-32889A, principal investigator: J.C. Cloyd) and HealthPartners (protocol 14-073, principal investigator: L.R. Hanson). The experiments were conducted at the HealthPartners Animal Care Facility and in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health). Sixty-six male Sprague Dawley rats 8-9 weeks old ( $0.271 \pm 0.023$  kg) from Envigo RMS, Inc. (Indianapolis, IN) were used for this study. Rats were housed under controlled environmental conditions with a standard 12 hr light/dark cycle and free access to food and water.



### *Formulation Components*

S-avizafone dihydrochloride (AVF•2HCl) was synthesized with > 99% purity, 99% ee from 5-chloro-2-methylaminobenzophenone according to the procedure described previously (Kapoor et al., 2016). Recombinant human aminopeptidase B enzyme (APB) was produced by inserting the RNPEP DNA sequence encoding for APB and a His tag into a pQE-30 Xa vector, transforming JM109 *E. coli* with the resultant plasmid, inducing APB expression with IPTG, and purifying the expressed APB by affinity capture chromatography (Ogawa et al., 2014; Ohnishi et al., 2015). Purity of APB was >80% by gel electrophoresis. Phosphate buffered saline (PBS) IN delivery vehicle was prepared at pH 7.4 and concentration 20 mM using sodium phosphate dibasic and sodium phosphate monobasic purchased from Fisher Scientific, Inc. (Hampton, NH: cat# BP332 and BP329), made isotonic with sodium chloride purchased from EMD Millipore (Burlington, MA: cat# 567440), and sterile filtered during formulation. For the IV control arm of the study, a commercial formulation of injectable diazepam USP (5 mg/mL DZP, 40% propylene glycol, 10% alcohol, 5% benzoate-benzoic acid, and 1.5% benzyl alcohol) was purchased from Hospira, Inc. (Rocky Mount, NC: NDC 0409-3213-12, lot 58-257-EV).

### *Enzyme Kinetics*

The Michaelis constant ( $K_M$ ) and maximum reaction velocity ( $V_{max}$ ) for the hydrolysis of AVF by APB in pH 7.4 PBS at 32 °C were determined by fitting the Michaelis-Menten equation to the initial rates of substrate consumption for a series of AVF concentrations. Reactions with 62.5 – 4000  $\mu$ M AVF and 15  $\mu$ g/mL (0.203  $\mu$ M) APB were carried out in an Eppendorf Thermomixer 5436 at 500 rpm. After 1 min, APB was denatured by addition of methanol. Cyclization of ORI to DZP was allowed to progress to completion before measuring UV spectra of the quenched reaction mixtures in a Cary 100 Bio UV/Vis spectrophotometer (Agilent

Technologies, Inc., Santa Clara, CA). The second derivative of the spectrum at 338 nm was used to quantify DZP, and this quantity was taken to be the molar equivalent to the amount of AVF consumed. A curve was fit to the AVF consumption versus initial AVF concentration with  $K_M$  and  $V_{max}$  as fitting parameters using the `nlinfit` function in MATLAB<sup>®</sup> version R2017b by The MathWorks, Inc. (Natick, MA).

### *Dosing*

For IN dosing, rats were anesthetized, placed in the supine position, and cannulated. Rats designated for nasal tissue histology were not cannulated. Solutions of AVF and APB prepared in PBS were admixed to the appropriate concentration for each animal immediately prior to administration. See Table 1 for dose levels. After mixing, the formulation was quickly instilled into the nasal cavity using an Eppendorf pipettor with a gel loading pipette tip inserted to a depth of 14 mm past the nares. A total volume of 30  $\mu$ L was delivered, 15  $\mu$ L into the right nostril followed by 15  $\mu$ L into the left nostril within 0.5 min. There were four IN dose groups: AVF/APB at low, medium, and high doses, and an AVF-only group at the medium dose. These doses were chosen because DZP near 1 mg/kg is commonly used in rat studies and results in plasma concentrations in rats that are clinically relevant.

For IV dosing, a commercial formulation of DZP was injected directly into the femoral vein. A small incision was made in the skin along the mid line of the left hind leg of anesthetized rats that were already cannulated in the right femoral vein. The incision was enlarged by blunt dissection to expose the left femoral vein. A 30-gauge needle was inserted into the vein to deliver a bolus dose of 1 mg/kg (medium dose level, ~54  $\mu$ L) of DZP using a 100  $\mu$ L syringe. Upon

withdrawal of the needle, moderate pressure was applied to the site of injection with a cotton swab for 5-10 seconds to facilitate coagulation and seal the puncture.

### *Sample Collection*

Prior to cannulation for serial blood draws, rats were anesthetized with a 1.0 mL/kg subcutaneous ketamine cocktail containing 43 mg/mL ketamine HCl, 8.6 mg/mL xylazine HCl, and 1.4 mg/mL acepromazine. Anesthesia was maintained throughout surgery, dosing, and sample collection by administering subcutaneous boosters of 50 mg/kg ketamine alternating with boosters of ketamine cocktail. Blood was drawn from the right femoral vein through an PU 3Fr 25cm catheter (Instech Laboratories Inc., Plymouth Meeting, PA: cat# C30PU-RFV1308). Leapfrog sampling was performed, with one cohort of 3 rats sampled at 2, 10, 20, 30, and 60 min and another cohort of 3 rats sampled at 5, 15, 45, 75 and 90 min for each dosing group. Each blood draw was 500  $\mu$ L, with a 500  $\mu$ L saline replacement volume pushed following every other draw. Whole blood was centrifuged at 2000 g for 3 min in BD Microtainer™ Tubes (Becton Dickinson Co., Franklin Lakes, NJ: cat# 365974) containing EDTA to obtain plasma. Additional cohorts of 3 rats each were designated for destructive sampling to obtain 2, 5, 8, and 10 min brain samples for the medium dose IV DZP and medium dose IN AVF/APB groups. At the final time points, blood samples were collected by cardiac puncture. The rats were then euthanized by transcardial perfusion with 120 mL saline. Brains, including the olfactory bulbs, were removed by gross dissection and hemisected sagittally. Brain and plasma samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

### *LC-MS/MS Method*

DZP and AVF were extracted from plasma and brain samples and quantified by LC-MS/MS. For extraction, brain tissue was first combined with two volumes (w/v) of lysis buffer and homogenized. The lysis buffer was composed of 25% methanol in PBS. An internal standard, tolbutamide, was spiked into 50  $\mu$ L aliquots of plasma and 200  $\mu$ L aliquots of brain lysates. The aliquots were then extracted with 2.5 mL ethyl acetate. The supernatant was removed by centrifugation at 2500 rpm for 10 min and evaporated to dryness under nitrogen at 37 °C. The dried extract was resuspended in 150  $\mu$ L of mobile phase for LC–MS/MS analysis.

A Dionex™ UltiMate™ 3000 LC and TSQ Quantum™ Access MAX triple quadrupole mass spectrometer (MS/MS) with electrospray ionization (ESI) manufactured by Thermo Fisher Scientific, Inc. (Waltham, MA) was used for the analysis. Analyte separation was performed at a flow rate of 0.2 mL/min through a Zorbax Eclipse XDB C18 (3.0 x 150 mm x 3.5 $\mu$ m) column (Agilent Technologies, Inc., Santa Clara, CA: cat# 963954-302) with a step gradient mobile phase. Retention times for AVF, DZP, and internal standard were 3.4, 9.3, and 7.6 min, respectively. The mobile phase consisted of acetonitrile (solvent A) and 0.1% acetic acid in water (solvent B). The composition of the mobile phase was held for 2 min at 5:95 A/B, increased to 60:40 A/B for 8 min, and finally decreased to 5:95 A/B for 3 min to condition the column before the next injection. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with a capillary voltage of 3200 V and capillary temperature of 2500 °C. Precursor and product ions for the analytes were detected in positive ion mode with  $m/z$  431  $[MH]^+ \rightarrow 246$  for AVF and 286  $[MH]^+ \rightarrow 193$  for DZP; the internal standard was detected in negative mode with  $m/z$  269  $[M-H]^- \rightarrow 170$ .

The concentration ranges for DZP were 25.0–1600 ng/mL in plasma and 6.25–400 ng/mL in brain lysate.

### *Nasal Tissue Histology*

A separate cohort of rats was utilized to assess potential trauma to the nasal mucosa from the prodrug/enzyme formulation. This cohort did not receive catheters and no blood or brain samples were collected, but the animals did receive anesthesia and were dosed intranasally in the same manner as the others. Three rats at each dose level (vehicle-only, low, medium, and high,  $n = 12$ ) were euthanized 90 min post-dose by perfusion with 120 mL saline followed by 120 mL of 10% neutral buffered formalin (NBF). The heads were removed, fixed in 10% NBF, and submitted to Colorado Histo-Prep (Fort Collins, CO) for histopathological evaluation by a board-certified veterinary pathologist. The samples were decalcified, trimmed, processed, embedded, and sectioned. Five sections of the nasal turbinates from each of the rats were stained with hematoxylin and eosine for microscopic imaging. Slides of the sections were read without knowledge of the dose group. The observed tissue changes were categorized using standardized toxicological pathology criteria (Boorman, 1990; Banks, 1993).

### *Noncompartmental Analysis*

Naïve pooled noncompartmental analysis (NCA) of plasma and brain concentration-time profiles was performed using WinNonlin<sup>®</sup> software version 8.0.0.3176 by Certara USA, Inc. (Princeton, NJ). Maximum concentration ( $C_{\max}$ ) and the  $t_{\max}$  were determined from the average concentrations at nominal time points. The apparent terminal rate constant ( $\lambda_z$ ) was estimated by regression of the log-linear portion of the concentration-time profiles. The apparent terminal half-life ( $t_{1/2,z}$ ) was calculated as  $\ln(2)/\lambda_z$ . Concentrations measured from animals subjected to

destructive and repeated sampling were pooled. Data was treated as sparse and the linear trapezoidal method was used for calculating the area under the curve (AUC) from the time of dosing to 90 min. Intranasal bioavailability (F) was calculated by

$$F = \frac{AUC_{IN,plasma}/Dose_{IN}}{AUC_{IV,plasma}/Dose_{IV}} \times 100 \quad (1)$$

Drug targeting efficiency (DTE) was used to assess direct nose-to-brain transport via the olfactory or trigeminal pathways (Mittal et al., 2014). DTE represents the time average partition ratio between brain and plasma for IN administration versus IV administration. Data from the medium dose level from time of dosing to 90 min was used to calculate DTE from the following equation

$$DTE = \frac{(AUC_{brain}/AUC_{blood})_{IN}}{(AUC_{brain}/AUC_{blood})_{IV}} \times 100 \quad (2)$$

### *PBPK Model*

A physiologically based pharmacokinetic (PBPK) model was used for further analysis. The model's structure and parameters for DZP disposition in rats were originally reported by Igari et al. (1983). Variants of the model have been utilized by other researchers, such as Gueorguieva et al. (2004) and Thompson et al. (2012), to explore aspects of predicting DZP concentration in tissues. We have appended the model with a nasal compartment for IN delivery of AVF and APB as illustrated in Fig. 2.

The conversion rate for AVF to DZP in the nasal cavity is governed by enzyme kinetics and the rate of ORI cyclization. To characterize the enzyme reaction,  $K_M$  and  $V_{max}$  were measured *in vitro* in the formulation vehicle (pH 7.4 PBS) at 32 °C. This temperature is approximately the average temperature of rodent nasal passages (Jackson and Schmidt-Nielsen, 1964). The catalytic rate constant ( $k_{cat}$ ) for enzymatic hydrolysis was then calculated from  $V_{max}$ , i.e.  $k_{cat} = V_{max}/C_{nasal,APB}$

where  $C_{\text{nasal,APB}}$  is the concentration of enzyme in the nasal cavity. Thus, the rate of AVF hydrolysis *in vivo* can be predicted by

$$\frac{dC_{\text{nasal,AVF}}}{dt} = -\frac{k_{\text{cat}}C_{\text{nasal,APB}}C_{\text{nasal,AVF}}}{K_M + C_{\text{nasal,AVF}}} \quad (3)$$

where  $C_{\text{nasal,AVF}}$  is the concentration of AVF in the nasal cavity. ORI, the immediate product of AVF hydrolysis, can accumulate in the nasal cavity and has physicochemical properties such that this species has the potential to passively diffuse across nasal epithelium. Transport of ORI was modeled as unidirectional due to the relatively small volume of the nasal cavity. The volume of the nasal cavity ( $V_{\text{nasal}}$ ) was assumed to be the volume of the deposited formulation, 30  $\mu\text{L}$ . Thus, the rate of change in the concentration of ORI ( $C_{\text{nasal,ORI}}$ ) in the nasal cavity can be expressed as

$$\frac{dC_{\text{nasal,ORI}}}{dt} = \frac{k_{\text{cat}}C_{\text{nasal,APB}}C_{\text{nasal,AVF}}}{K_M + C_{\text{nasal,AVF}}} - (k_2 + k_{\text{a,ORI}})C_{\text{nasal,ORI}} \quad (4)$$

where  $k_2$  is the first order intramolecular cyclization rate constant of ORI and  $k_{\text{a,ORI}}$  is the nasal absorption rate constant of ORI. Similarly, DZP has an associated absorption rate constant ( $k_{\text{a,DZP}}$ ). However, DZP generated in excess of  $C_{\text{DZP,lim}}$  forms an amorphous phase separate from the solution phase. Assuming drug transport only occurs from the solution phase, the rate of change in the concentration of DZP ( $C_{\text{nasal,DZP}}$ ) in the nasal cavity can be expressed as

$$\frac{dC_{\text{nasal,DZP}}}{dt} = k_2C_{\text{nasal,ORI}} - k_{\text{a,DZP}}C_{\text{nasal,DZP,sol}} \quad (5)$$

where  $C_{\text{nasal,DZP,sol}}$  is the concentration of DZP in the solution phase and  $C_{\text{nasal,DZP,sol}} \leq C_{\text{DZP,lim}}$ .

This PBPK model assumes perfusion rate-limited distribution and tissue compartment concentrations that are in instantaneous equilibrium with venous blood flows. A general expression for the rate of change in concentration of DZP in non-eliminating organ or tissue compartments can be written as

$$\frac{dC_{\text{tissue}}}{dt} = \frac{Q_{\text{tissue}}}{V_{\text{tissue}}} \left[ C_{\text{artery}} - \frac{C_{\text{tissue}}}{K_{P,\text{tissue}}} \right] \quad (6)$$

where  $C_{\text{tissue}}$  is the concentration in the specified tissue,  $V_{\text{tissue}}$  is the volume of that tissue,  $Q_{\text{tissue}}$  is the blood flow rate,  $C_{\text{artery}}$  is the concentration in the arterial blood, and  $K_{P,\text{tissue}}$  is the partition coefficient.

DZP is extensively metabolized in the liver and minimally excreted unchanged, so elimination was assumed to be solely by hepatic metabolism. The hepatic extraction ratio for DZP in rats is approximately unity, even for doses as high as 4 mg/kg (Diaz-Garcia et al., 1991). Therefore, the concentration of DZP in the liver was assumed to stay well below  $K_M$  for metabolism by liver enzymes. An expression for the rate of change in the concentration of DZP in the liver can be written as

$$\begin{aligned} \frac{dC_{\text{liver}}}{dt} = \frac{1}{V_{\text{liver}}} \left[ \frac{Q_{\text{stomach}} C_{\text{stomach}}}{K_{P,\text{stomach}}} + \frac{Q_{\text{splanchnic}} C_{\text{splanchnic}}}{K_{P,\text{splanchnic}}} + Q_{\text{liver}} C_{\text{artery}} \right. \\ \left. - \frac{CL_{\text{int}} C_{\text{liver}} (f_u/R)}{K_{P,\text{liver}} R} - \frac{(Q_{\text{stomach}} + Q_{\text{splanchnic}} + Q_{\text{liver}}) C_{\text{liver}}}{K_{P,\text{liver}}} \right] \quad (7) \end{aligned}$$

where  $CL_{\text{int}}$  is intrinsic clearance,  $f_u$  is the fraction unbound in plasma, and  $R$  is the blood to plasma ratio. In rats, the blood to plasma DZP concentration ratio has been reported to be  $R = 1.037 \pm 0.007$  with the fraction unbound in plasma  $f_u = 0.14 \pm 0.003$  (Igari et al., 1983; Igari et al., 1984).

Expressions for the rate of change in the concentration of DZP in the venous and arterial blood compartments are

$$\frac{dC_{\text{vein}}}{dt} = \frac{1}{V_{\text{vein}}} \left[ \sum_i \frac{Q_i C_i}{K_{P,i}} + V_{\text{nasal}} (k_{a,\text{ORI}} C_{\text{nasal,ORI}} + k_{a,\text{DZP}} C_{\text{nasal,DZP,sol}}) - Q_{\text{lungs}} C_{\text{vein}} \right] \quad (8)$$



$$\frac{dC_{\text{artery}}}{dt} = \frac{1}{V_{\text{artery}}} \left[ \frac{Q_{\text{lungs}} C_{\text{lungs}}}{K_{P, \text{lungs}}} - \sum_j Q_j C_{\text{artery}} \right] \quad (9)$$

where  $i$  represents each tissue compartment that has an outflow to the venous pool and  $j$  represents each tissue compartment that has an inflow from the arterial pool. At pH 7.4, the half-life for cyclization of ORI is 1.47 min (Rautiola et al., 2018), so we assume most of the conversion to DZP occurs in the nasal cavity. Absorbed ORI would continue to transform into DZP in the mucosa and in the blood (Upshall et al., 1990). Therefore, systemic exposure to ORI was limited, and any ORI that did reach the circulation was regarded as DZP for the purposes of pharmacokinetic analysis.

The PBPK model, represented by Equations 3-9, was constructed in MATLAB®. Values for the parameters of interest ( $CL_{\text{int}}$ ,  $k_{a, \text{ORI}}$ ,  $k_{a, \text{DZP}}$ , and  $C_{\text{DZP, lim}}$ ) were determined by fitting numerically solved solutions of the differential equations to experimental data using the ode45 function in conjunction with the lsqcurvefit function. Experimental data was input as averages of the DZP concentrations measured at the nominal time points, with data from animals subjected to destructive and repeated sampling pooled. Fitting was performed in two stages. First,  $CL_{\text{int}}$  was determined by fitting the model to DZP concentrations measured in plasma from rats administered IV DZP. Tissue compartment volumes, blood flow rates, and partition coefficients were fixed at the values reported by Gueorguieva et al. (2004) and Thompson et al. (2012). These constants are listed in the supplemental data (Supplemental Table 2S). Then,  $CL_{\text{int}}$  was fixed and *in vivo* values for  $k_{a, \text{ORI}}$ ,  $k_{a, \text{DZP}}$ , and  $C_{\text{DZP, lim}}$  were determined by fitting the model to a matrix of DZP concentrations measured in plasma from rats administered IN AVF/APB at the low, medium, and high dose levels.

### *Statistical Methods*

Naïve pooled noncompartmental analysis was performed using sparse sampling computations (Carter et al., 1995). Concentrations of DZP measured below the limit of quantification were retained in the data set (Jusko, 2012). Squared correlation coefficients adjusted ( $R^2_{\text{adj}}$ ) for the number of points used in the estimation of  $\lambda_z$  were  $>0.7$  for all treatment groups. For AUC, standard error (SE) of the mean estimate was calculated using WinNonlin<sup>®</sup>, which employs equations described by Nedelman and Jia (1998) and unbiased sample covariance estimation described by Holder (2001). The SEs associated with F estimates were calculated by propagating the SEs from the AUCs. Similarly, the SEs of DTEs were calculated by propagating the SEs from the AUCs. Descriptive statistics for the variability in  $t_{\text{max}}$  are not reported because leapfrog serial and destructive sampling data were pooled. The standard deviation (SD) of measured concentrations at  $t_{\text{max}}$  was used to describe the dispersion of  $C_{\text{max}}$ . SEs reported for determined PBPK parameter estimates were obtained from MATLAB<sup>®</sup> by back-calculating from 95% confidence intervals computed using the nlparci function with the Jacobian from the lsqcurvefit function. Root mean square error (RMSE) for the model fits were calculated from the average concentrations at the nominal time points.

## Results

### *Noncompartmental Analysis*

As shown in Fig. 3, DZP generated from IN administration of the AVF/APB formulation appeared rapidly in plasma. Plasma and brain tissue samples were also analyzed for AVF, but concentrations were below the limit of quantification. The short half-life of ORI did not allow for its quantification. The average plasma concentration of DZP at the first measured time point, 2 min, was greater for IN AVF/APB than for IV DZP at an equivalent dose due to rapid distribution following IV dosing (Fig. 3A). Plasma concentrations from IN administration continued to rise, reaching  $C_{\max} = 388 \pm 31$  ng/mL at  $t_{\max} = 8$  min for the medium dose level. Similar  $t_{\max}$  values were observed at each dose level, but  $C_{\max}$  was not proportional to the dose. In fact,  $C_{\max}$  for the high dose was not statistically different from that of the medium dose (Fig. 3B). When AVF was administered without APB, there was a prominent decrease in the rate of DZP absorption. As shown in Table 2,  $C_{\max}$  was nearly fourfold lower without co-administered enzyme.

There was complete absorption of DZP from IN AVF/APB at the medium and high dose levels, as was evident by bioavailabilities of  $112 \pm 10$  and  $114 \pm 7$  % respectively. The AVF-only control group and low dose APB/AVF group had nearly identical bioavailability ( $F = 77.8\%$ ) based on exposure from 0 to 90 min. However, there was a notable difference between these groups at early time points (Fig. 3B). Dose normalized  $AUC_{0-15\min}$  was twice as large for the AVF/APB low dose group compared to the AVF-only control (Table 2).  $AUC_{0-15\min}$  for the IN AVF/APB medium and high dose groups were similar, suggesting saturation of the DZP absorption rate.

NCA results for DZP measured in brain tissue are listed in Table 3. For the IN AVF/APB medium dose group,  $t_{\max}$  was reached at 8 min in both plasma and brain tissue. However, the concentration of DZP measured in plasma at the first time point ( $C_{\text{plasma,DZP}} = 270 \pm 181$  ng/mL  $\pm$ SD at  $t = 2$  min) was already greater than the therapeutic concentration that is expected to lead to seizure threshold elevation in rats,  $C_{\text{plasma,DZP}} \geq 70$  ng/mL (Dhir and Rogawski, 2018). Brain concentrations were not measured at time points between 10 and 60 min, but comparable concentrations were observed at 8 and 10 min ( $415 \pm 32$  and  $404 \pm 35$  ng/g respectively), suggesting the true maxima were in this region. Brain  $C_{\max}$  for IV DZP was recorded at the first time point, 2 min. Rapid transport from blood into brain tissue is normal for small, lipophilic drugs. DTE, a metric commonly used to assess nose-to-brain transport, was less than unity, negating significant transport of DZP through direct nose-to-brain pathways.

### *PBPK Model*

Concentration-time profiles were well described by a PBPK model with the rate of drug input controlled by enzyme kinetics. At 32 °C in pH 7.4 PBS, the Michaelis-Menten enzyme kinetic parameters for APB measured *in vitro* were  $K_M = 370 \pm 63$   $\mu$ M and  $k_{\text{cat}} = 1250 \pm 60$  min<sup>-1</sup>  $\pm$ SE (see Supplemental Fig. 1S). The rate constant,  $k_2 \pm$ SD, for cyclization of ORI, previously measured *in vitro* under the same conditions (Rautiola et al., 2018), was assumed to remain constant at  $0.470 \pm 0.012$  min<sup>-1</sup> *in vivo*. As listed in Table 1, the APB concentrations were scaled with the AVF concentration so that hydrolysis of AVF would be complete at  $t \approx 4$  min, regardless of the dose level. This also means that the predicted mole fraction of the total dose that had been converted to DZP was the same for each dose level at a given time point. For example, approximately 75% of the dose was expected to be converted to DZP at  $t = 5$  min regardless of the dose level.

Fitting the PBPK model to DZP concentrations measured in plasma samples from the IV DZP group resulted in  $CL_{int} = 15.4$  L/min, which was expected since DZP is well known to have a hepatic extraction ratio of approximately one in rats (Rowland et al., 1984) and confirms the assumption that DZP clearance from the liver was flow-limited in this PBPK model. Subsequent fitting of the model to DZP concentrations measured in plasma samples from the IN APB/AVF groups allowed estimation of the parameters governing *in vivo* absorption from the nasal cavity:  $k_{a,ORI} = 0.122 \pm 0.022 \text{ min}^{-1}$ ,  $k_{a,DZP} = 0.0689 \pm 0.0080 \text{ min}^{-1}$ , and  $C_{DZP,lim} = 20.8 \pm 3.1 \text{ mM}$  ( $\pm$ SE). Conversion of AVF to DZP was faster than the combined absorption of ORI and DZP, leading to an accumulation of DZP in the nasal cavity. Since the aqueous equilibrium solubility of DZP is only 130  $\mu\text{M}$ , concentrations entered the supersaturated region. The apparent concentration at which supersaturated DZP began to phase separate from solution in the nasal cavity,  $C_{DZP,lim}$ , was a useful parameter to model the nonlinear absorption kinetics observed at early time points. However, the value of  $C_{DZP,lim}$  should be interpreted within the context of the assumptions that  $V_{nasal}$  was equal to the dose volume and only DZP in the solution phase could be absorbed.

Well-stirred PBPK models predict high initial plasma concentrations with IV administration because the initial volume of distribution is assumed to be the volume of the venous blood compartment. This is illustrated in Fig. 4A where the predicted plasma concentration for the IV DZP group decreases dramatically from the time of dosing as it distributes out of the venous compartment to reach the first measured plasma concentration at  $t = 2$  min. The high concentrations of DZP measured in the brain at the first time point were predicted more accurately with this PBPK distribution behavior compared to a modeling approach which relied on a standard two-compartment model function to drive brain concentrations. In general, concentrations of DZP

in the brain were predicted reasonably well by the PBPK model. For the IN AVF/APB medium dose group, brain concentrations lagged behind plasma concentrations during the first few min. This observation provided additional evidence that transport of DZP through direct nose-to-brain pathways was not significant.

Although there was significant variability in the data, similar average plasma concentrations were observed for the medium and high IN AVF/APB dose groups at early time points (Fig. 5A). This indicated the absorption process was saturable. The nonlinearity was likely caused by phase separation of DZP from solution when  $C_{DZP,lim}$  was reached in the nasal cavity, a phenomenon that has been observed *in vitro* (Rautiola et al., 2018). Phase separation attenuated DZP absorption and produced a shoulder at  $t = 10$  min in the predicted concentration-time profile for the high dose group (Fig. 5A, green curve). The peak preceding the shoulder comes from the absorption of ORI, which contributes to the concentration of DZP observed in plasma. In the first few min after administration, the amount of DZP produced by the reaction is relatively low compared to ORI. Predictions for the relative amount of each reaction species in the nasal cavity are shown in Fig. 5B. Decomposition of the plasma concentration-time profile for the medium IN AVF/APB dose group, shown in Fig. 5C, illustrates how absorption of ORI contributes to the rate at which DZP enters the circulation.

### *Nasal Tissue Histology*

No inflammatory response was observed in the nasal tissue samples. Lesions (changes) found in the samples were few, with severities graded as either minimal or mild. These were located in the anterior portion of the nasal cavity and were consistent with mechanical trauma from insertion of the pipette tip used for dosing. There were some small, focal areas of missing

epithelium on the wall of the nasal septum and/or nasoturbinate, and occasionally the maxilloturbinate. Cellular debris, consisting of small clusters of sloughed epithelial cells and cellular fragments, were found on the wall of the nasal septum or the nasoturbinate and anterior meatus. Other than two instances of minimal focal hemorrhage, the blank vehicle control group was unremarkable. There were no lesions observed in the ethmoid turbinates, olfactory nerves, and olfactory bulbs. The low, medium, and high dose groups (IN AVF/APB equivalent to 0.500, 1.00, and 1.50 mg/kg DZP respectively) did not display a dose response with regard to location, frequency, or severity of the lesions. Some examples of the tissue sections are shown in Fig. 6. A detailed enumeration of the locations and severity of the observations can be found in the supplemental data (Supplemental Table 4S).

## Discussion

Intranasal co-administration of AVF and APB resulted in rapid absorption of DZP with excellent bioavailability, thus demonstrating that a prodrug/enzyme combination in an aqueous vehicle efficiently delivers a hydrophobic drug. PBPK analysis indicated that both DZP and transient ORI were readily absorbed through the nasal mucosa into the systemic circulation. There was no evidence of transport through direct nose-to-brain pathways. Histological analysis of the nasal tissues post-dose did not reveal significant differences between the vehicle-only control group and treatment groups, suggesting that the formulation was well tolerated.

Although aminopeptidases capable of hydrolyzing Lys from AVF to produce DZP exist in the nasal mucosa and blood of both rats and humans (Sarkar, 1992; Hussain et al., 1995; Agu et al., 2009), exogenous APB is necessary to ensure complete and rapid conversion of AVF. In Fig. 3B, the appearance of DZP in plasma at early time points for the IN AVF-only control group suggests that some AVF conversion occurred in the nasal cavity by endogenous enzymes. However, the therapeutic concentration of DZP that is expected to lead to seizure threshold elevation in rats ( $C_{\text{plasma,DZP}} \geq 70 \text{ ng/mL}$ , Dhir and Rogawski, 2018) was not reached until  $t = 10 \text{ min}$  and was not sustained thereafter. AVF also converts to DZP when absorbed into the circulation. Upshall et al. (1990) found the half-life for conversion of AVF in blood was  $0.49 \pm 0.06$ ,  $3.17 \pm 0.09$ , and  $3.85 \pm 0.26 \text{ min}$  for guinea pig, rhesus monkey, and human, respectively (blood and plasma were not significantly different). However, substantial transport of AVF across nasal epithelium is unlikely since it does not readily partition into membranes. Using wild type Madin-Darby canine kidney epithelial cell (MDCKII-wt) monolayers as an *in vitro* model for nasal epithelium to estimate apparent permeability coefficients ( $P_{\text{app}}$ ), we observed negligible



permeation of AVF ( $P_{app} = 1.0\text{--}1.5 \times 10^{-6}$  cm/s) compared to DZP ( $P_{app} = 2.2 \times 10^{-5}$  cm/s) (Kapoor et al., 2014).

IN-administered drugs tend to exhibit variable absorption rates and bioavailabilities when mucociliary clearance to the digestive tract competes with absorption through the nasal mucosa. The high bioavailability calculated for the medium and high IN AVF/APB dose groups suggests that those doses were fully converted and absorbed through the nasal mucosa of the rats (Table 2). The lower bioavailability observed for the low dose IN AVF/APB and AVF-only groups may have been due to a portion of the dose being cleared from the nasal cavity before complete conversion could occur. The first order rate constant for mucociliary clearance is roughly  $0.035 \text{ min}^{-1}$  in healthy adult humans (Gizurason, 2015) and  $0.14 \text{ min}^{-1}$  in Sprague-Dawley rats (Donovan and Zhou, 1995). However, the rate of clearance depends on many factors including dose volume, viscosity, pH, tonicity, component concentrations, and pharmacodynamic effects on the cilia. A separate study to assess the effects of dose level on the beat frequency of cilia may be warranted to reconcile the discrepant bioavailabilities.

From the plasma DZP concentration-time profiles measured at each dose level (Fig. 5A), absorption during the first 15 min displayed nonlinear pharmacokinetics while concentrations at later time points were approximately dose proportional. This behavior was not entirely unexpected since it was known that phase separation of DZP would occur if the accumulation of DZP in the nasal cavity exceeded  $C_{DZP,lim}$ . However, it was surprising that the *in vivo* estimate of  $C_{DZP,lim}$  ( $20.8 \pm 3.1 \text{ mM} \pm \text{SE}$ ), determined by fitting the PBPK model, was nearly 17 times greater than  $C_{DZP,lim}$  measured *in vitro* ( $1.22 \pm 0.03 \text{ mM} \pm \text{SD}$ ) (Rautiola et al., 2018). The *in vivo* estimation is

conspicuously high because  $V_{\text{nasal}}$  was assumed to be equivalent to the dosed formulation volume. However, the total volume would increase when the deposited dose mixes with nasal mucus.

Absorption of DZP directly from phase separated DZP could also cause overestimation of  $C_{\text{DZP,lim}}$ . Transcytosis of the precipitated DZP colloidal particles presents one possible mechanism of transport across the nasal epithelium. Brooking et al. (2001) reported size dependent transcytosis of latex nanoparticles through the nasal mucosa and into the blood of rats. Another potential mechanism invokes an increase in driving force for absorption arising from higher local concentrations of DZP in the diffuse layer of particles that are proximal to cell membranes. Both mechanisms require that DZP precipitate particles be close to cell membranes. Confounding of DZP absorption from solution with absorption from the precipitated phase is exacerbated by the small volume-to-surface area ratio that results when the formulation spreads across the surface of the mucosa, minimizing the distance particles need to travel to reach the cell membranes.

The hydrophobic reaction intermediate, ORI, can accumulate in the nasal cavity as well, so there is potential for phase separation of this species. Since we did not observe precipitate formation at time points where there was expected to be high concentrations of ORI during our *in vitro* experiments (Rautiola et al., 2018), phase separation of ORI was not considered in this PBPK model. However, it was necessary to incorporate absorption of ORI into the model to account for the rapid appearance of DZP in plasma and the shape of the concentration-time profiles. Satisfying Lipinski's rule of five, with  $\text{cLogD} = 1.69$  at pH 7.4 (ChemAxon Ltd., 2016), ORI can partition into membranes and permeate epithelium.  $P_{\text{app}}$  for ORI across MDCKII-wt monolayers was predicted to be  $1.77 \times 10^{-5}$  cm/s using structure activity relationship calculations (Yao and Yang, 2005; Kang, 2017). Since ORI quickly transforms into DZP, absorption of ORI fortuitously

contributes to the rate at which DZP appears in plasma. This contribution is illustrated by the dotted curve in Fig. 5C, but the area under the ORI curve is not representative of exposure. Exposure to ORI was miniscule due to its short half-life.

Exposure to the other reaction species, AVF and APB, was similarly short and most likely only topical. Based on enzyme kinetic calculations, AVF was completely hydrolyzed in the nasal cavity in less than 4 min and increasing the amount of APB could easily speed up its consumption. The residence time of APB in the nasal cavity is controlled by mucociliary clearance and possibly by proteases in the mucus. Once APB or its degradation products reach the digestive tract, they would be digested like any other protein. Histological analysis of the posterior, absorptive nasal tissue sections suggested that the formulation was innocuous. However, the histological findings are incomplete. Collection of tissue samples 90 min post-dose does not provide sufficient time to develop an inflammatory response. Future *in vivo* studies will incorporate later time points to more fully assess acute nasal toxicology.

Approximately 50% of the nasal surface in a rat is olfactory epithelium (Dhuria et al., 2010). Although dosing did not specifically target the olfactory epithelium, rats were in the supine position during treatment. Presumably the formulation spread across the olfactory epithelium, providing ample opportunity for direct nose-to-brain transport via the olfactory bulb. However, there was no evidence that direct nose-to-brain transport was an important pathway for delivery of DZP to the brain, in agreement with the findings of Kaur and Kim (2008). DTE was only 74% (Table 3) and DZP concentrations in plasma preceded brain concentrations (Fig. 4B), indicating absorption into the blood and subsequent advection to the brain was faster than diffusive transport along olfactory or trigeminal nerves.

In conclusion, the pharmacokinetic results presented here demonstrate that IN co-administration of AVF with APB is a viable method to rapidly deliver DZP into the systemic circulation and, subsequently, the brain. Since the highly concentrated formulation does not contain organic solvents, it is expected to be better tolerated and absorb faster than IN formulations of DZP that utilize solubilizing excipients. Administered as a noninvasive nasal spray, IN AVF/APB could be used to quickly terminate seizure emergencies in humans, resulting in reduced emergency department visits and improve the quality of life for patients who experience seizure emergencies. Further progress necessitates development of a device which can store the prodrug and enzyme separately, and then combine them as into sprayed solution at the time of administration.

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## Footnotes

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## Legends for Figures

**Fig. 1. Prodrug/enzyme reaction scheme.** Formation of DZP by enzymatic hydrolysis of AVF with APB progresses through an intermediate, ORI.

**Fig. 2. PBPK model schematic.** An established PBPK model for DZP disposition in rats was modified to include a nasal cavity compartment. ORI and DZP absorb from the nasal cavity into circulation after IN administration of AVF and APB.

**Fig. 3. DZP concentration-time profiles measured in plasma.** Error bars are SD. For IV DZP and IN AVF/APB at the medium dose level (equivalent to DZP at 1.00 mg/kg),  $n = 6$  at 2, 5, and 10 min time points;  $n = 3$  for all other data points. **(A)** IN-administered AVF/APB compared with IV-administered DZP indicated high bioavailability of the IN formulation,  $F = 112 \pm 10\%$  ( $\pm$ SE). The IN-administered AVF/APB formulation has a pharmacokinetic advantage with regards to  $C_{\max}$  and  $AUC_{0-15\min}$  when compared to the AVF-only control. **(B)** Saturation of the absorption process resulted in nearly identical initial rates of absorption for IN-administered AVF/APB at the medium and high dose levels.

**Fig. 4. DZP concentration-time profiles in plasma and brain tissue.** Experimental data (points) and model predictions (lines) at the medium dose level (equivalent to DZP at 1.00 mg/kg). For plasma concentrations,  $n = 6$  at 2, 5, and 10 min time points;  $n = 3$  at all other time points for plasma and brain. Error bars are SD. **(A)** IV-administered DZP. RMSE of the predicted curve was 44 ng/mL for plasma and 246 ng/g for brain. The open purple triangle at the origin is a marker

to highlight the steep initial increase in brain DZP concentration; it is not a measured data point.

(B) IN-administered AVF/APB. Predicted curve RMSE = 66 ng/mL for plasma and 67 ng/g for brain.

**Fig. 5. DZP concentration-time profiles in plasma from IN administration of AVF/APB at each dose level.** Experimental data (points) and model predictions (lines). For medium dose level (equivalent to DZP at 1.00 mg/kg),  $n = 6$  at 2, 5, and 10 min time points;  $n = 3$  for all other data points. Error bars are SD. (A) Saturation of absorption rate observed with the high dose. Predicted curve RMSE = 43 ng/mL for low dose, 66 ng/mL for medium dose, and 61 ng/mL for the high dose. (B) Predicted amounts of each reaction species in the nasal cavity. Solid lines represent the low dose, dashed lines medium dose, and dotted lines high dose level for a 0.250 kg rat. Inset shows expanded view of the first 10 min. (C) Absorption of ORI contributes to the rate of DZP delivery into the systemic circulation.

**Fig. 6. Representative examples of nasal tissue section images.** Minor mechanical trauma from insertion of the dosing pipette tip was observed in some animals. There were no lesions attributed to the drug formulation. (A) Normal tissue. (B) Hemorrhage. (C) Necrosis.

## Tables

**Table 1. IN doses.**

<b>Dose level</b>	<b>Equivalent DZP (mg/kg)</b>	<b>AVF•2HCl (mg/kg)</b>	<b>APB (µg/kg)</b>
high	1.50	2.66	91.4
med	1.00	1.77	60.7
low	0.500	0.885	30.4

**Table 2. Noncompartmental analysis of plasma DZP concentrations.**

<b>Formulation</b>	<b>DZP</b>	<b>AVF</b>	<b>AVF/APB</b>	<b>AVF/APB</b>	<b>AVF/APB</b>
<b>Route</b>	IV	IN	IN	IN	IN
<b>Equiv. DZP dose</b>	1.00	1.00	0.500	1.00	1.50
<b>(mg/kg) [dose level]</b>	[medium]	[medium]	[low]	[medium]	[high]
<b>T<sub>max</sub> (min)</b>	0 <sup>a</sup>	10	5	8	5
<b>C<sub>max</sub> ± SD (ng/mL)</b>	441 <sup>a</sup>	108 ± 30	71.5 ± 9.3	388 ± 31	355 ± 187
<b>t<sub>1/2,z</sub> (min)</b>	32.9	33.4	136 <sup>b</sup>	26.7	37.8
<b>AUC<sub>0-15min</sub></b>					
<b>(min.ng/mL)</b>	1910 ± 70	837 ± 28	793 ± 54	3250 ± 190	3850 ± 270
<b>AUC<sub>0-90min</sub> ± SE</b>					
<b>(min.ng/mL)</b>	5430 ± 190	4230 ± 120	2110 ± 150	6080 ± 510	9280 ± 470
<b>F % ± SE</b>	n/a	77.8 ± 3.5	77.8 ± 6.0	112 ± 10	114 ± 7

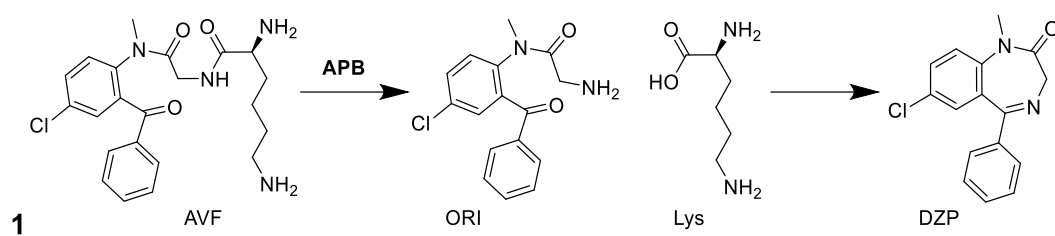
<sup>a</sup> C<sub>max</sub> reported for IV DZP is the concentration extrapolated to t = 0 min (i.e. the initial concentration C<sub>0</sub>) as calculated by WinNonlin<sup>®</sup> NCA analysis. <sup>b</sup> Overestimation of t<sub>1/2,z</sub> due to inherent variability of data below limit of quantification in terminal phase of low dose group.

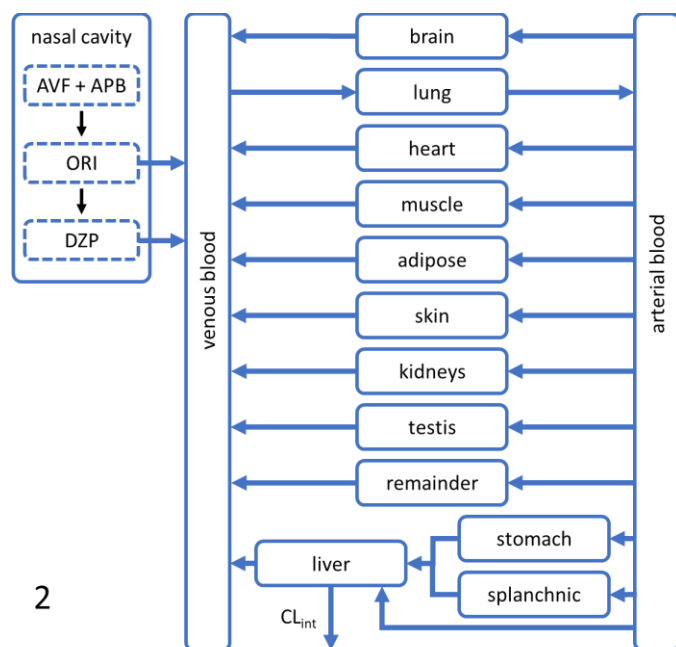


**Table 3. Noncompartmental analysis of brain DZP concentrations.**

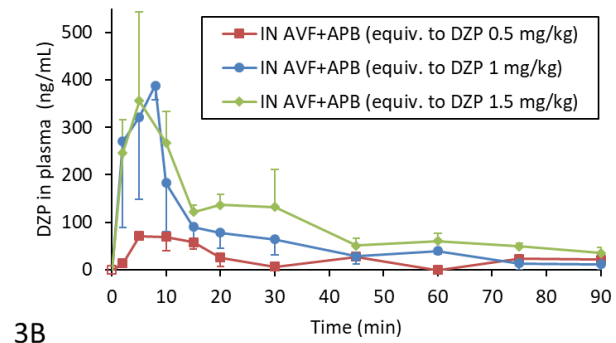
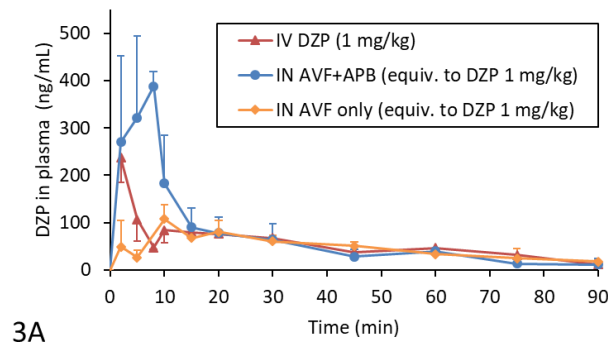
<b>Formulation</b>	<b>Route</b>	<b>Equiv. DZP dose (mg/kg)</b>	<b>t<sub>max</sub> (min)</b>	<b>C<sub>max</sub> ± SD (ng/mL)</b>	<b>AUC<sub>0-90min</sub> ± SE (min.ng/mL)</b>	<b>DTE % ± SE</b>
<b>DZP</b>	IV	1.00	2	938 ± 142	18600 ± 200	n/a
<b>AVF/APB</b>	IN	1.00	8	415 ± 33	15400 ± 500	74.0 ± 7.3

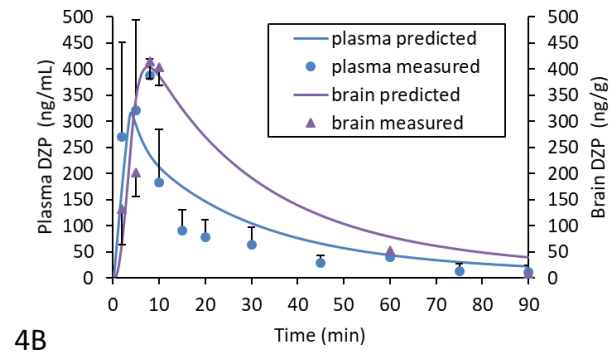
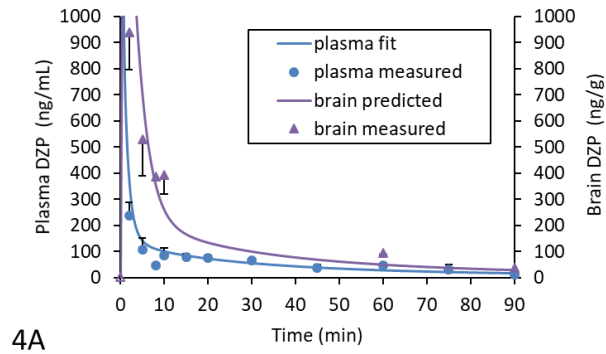
## Figures

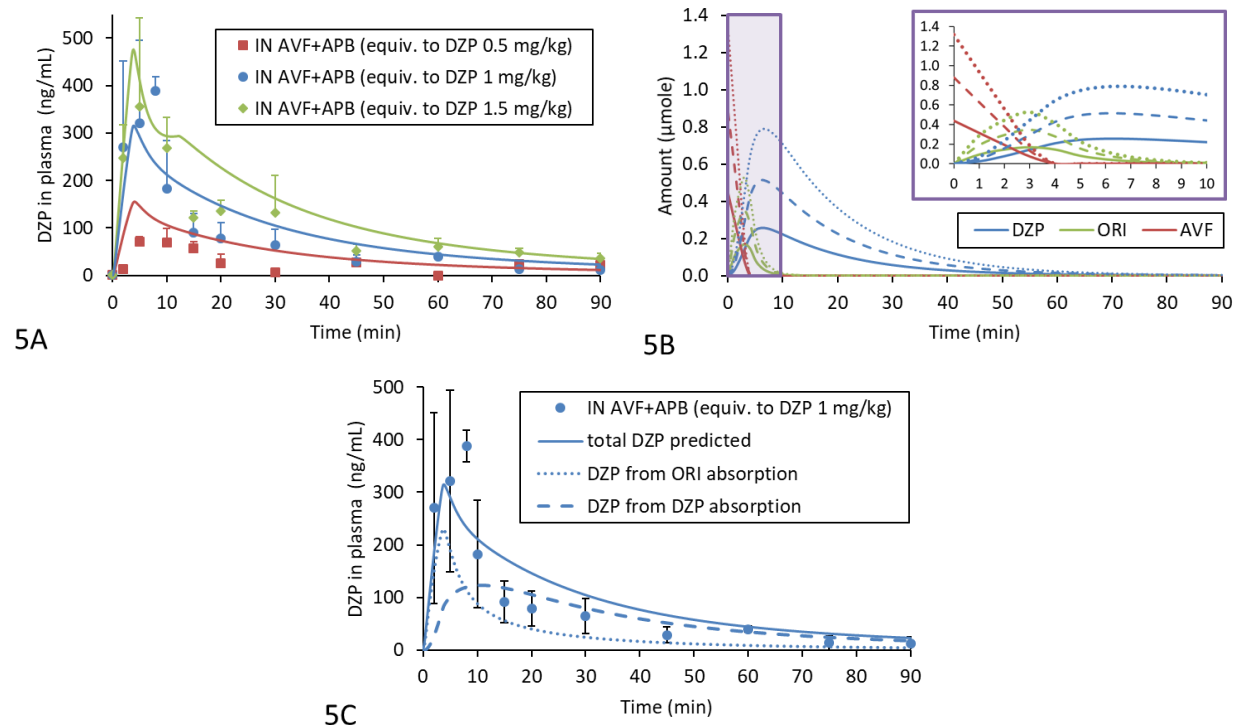


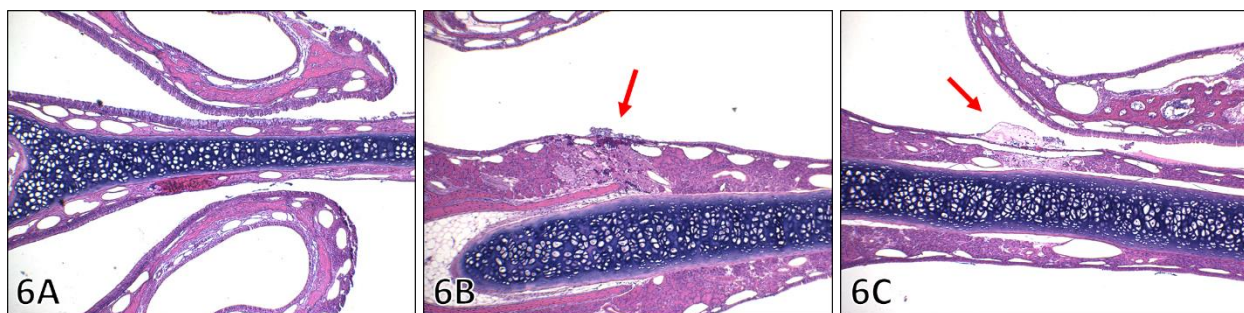


2









**Intranasal co-administration of a diazepam prodrug with a converting enzyme results in rapid absorption of diazepam in rats**

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**SUPPLEMENTAL DATA**

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Diazepam is commonly studied in rats at doses near 1 mg/kg (Schwartz et al., 1965; Igari et al., 1982; Tsang and Wilkinson, 1982; Ableitner et al., 1985; Fenyk-Melody et al., 2004; Gueorguieva et al., 2004a; Gueorguieva et al., 2006; Kaur and Kim, 2008; Musteata et al., 2008). To investigate dose proportionality, doses equivalent to diazepam at 0.50, 1.00, and 1.50 mg/kg were chosen. Since the prodrug has a different molar mass than the active drug, the equivalent prodrug doses were 0.885, 1.77, and 2.66 mg/kg. See Table 1S for dosing and sampling details.

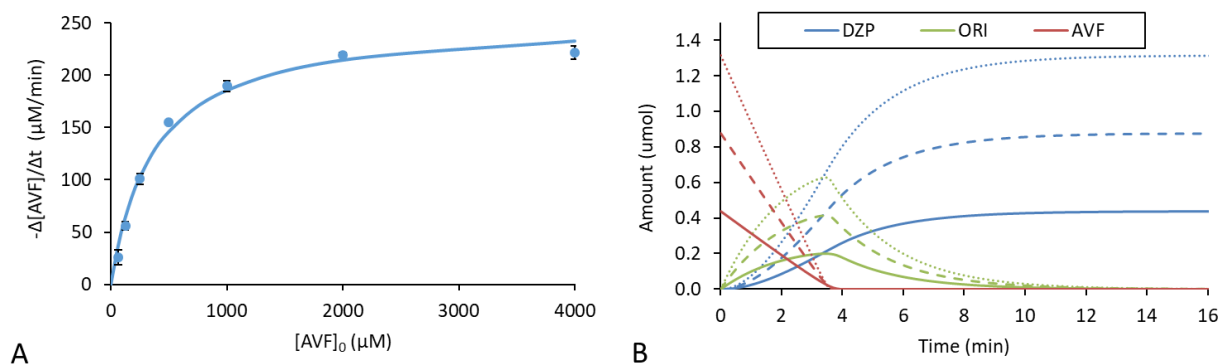
**Table 1S. Study design.** Low dose level is equivalent to diazepam at 0.5 mg/kg, medium dose level is equivalent to diazepam at 1.0 mg/kg, and high dose level is equivalent to diazepam at 1.5 mg/kg. b = brain tissue, h = nasal tissues for histology.

Group	Formulation	Route	Dose level	Number of rats	Blood draw (min)	Tissue collection (min)
1	Vehicle	IN	n/a	3	none	90 <sup>h</sup>
2	AVF	IN	med	3	2, 10, 20, 30, 60	60 <sup>b</sup>
				3	5, 15, 45, 75, 90	90 <sup>b</sup>
3	AVF/APB	IN	low	3	2, 10, 20, 30, 60	60 <sup>b</sup>
				3	5, 15, 45, 75, 90	90 <sup>b</sup>
				3	none	90 <sup>h</sup>
4	AVF/APB	IN	med	3	2, 10, 20, 30, 60	60 <sup>b</sup>
				3	5, 15, 45, 75, 90	90 <sup>b</sup>
				3	none	90 <sup>h</sup>
5	AVF/APB	IN	high	3	2, 10, 20, 30, 60	60 <sup>b</sup>
				3	5, 15, 45, 75, 90	90 <sup>b</sup>
				3	none	90 <sup>h</sup>
6	AVF/APB	IN	med	3	2	2 <sup>b</sup>
				3	5	5 <sup>b</sup>
				3	8	8 <sup>b</sup>
				3	10	10 <sup>b</sup>
7	DZP	IV	med	3	2, 10, 20, 30, 60	60 <sup>b</sup>
				3	5, 15, 45, 75, 90	90 <sup>b</sup>
8	DZP	IV	med	3	2	2 <sup>b</sup>
				3	5	5 <sup>b</sup>
				3	8	8 <sup>b</sup>
				3	10	10 <sup>b</sup>

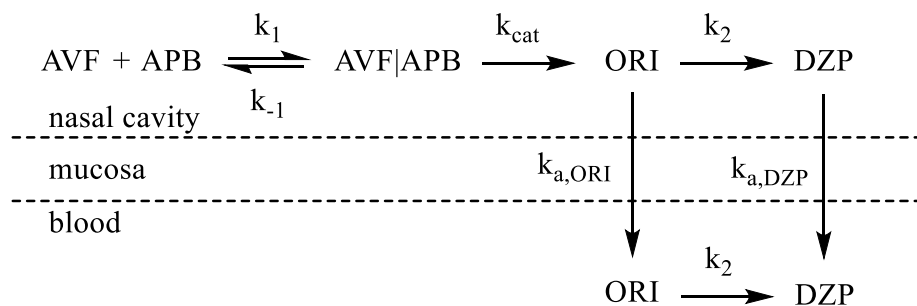
**Table 2S. PBPK model parameters for DZP disposition in rats.** Obtained from Gueorguieva et al. (2004b) and Thompson et al. (2012).

Tissue	Flow Q (mL/min)	Volume V (mL)	Partition coefficient $K_p$
Adipose	2.55	10	21.28
Brain	0.78	1.2	1.67
Heart	4.2	1	5.46
Kidney	16.61	2	5.08
Liver	3.55	11	7.62
Lung	80	1.2	4.91
Muscle	16.25	125	1.88
Skin	7.1	43.8	3.30
Splanchnic	20.25	15	3.67
Stomach	1.9	1.1	5.16
Testes	1.9	2.5	5.13
Remainder	4.91	15.8	17.44
Venous blood	80	13.6	
Arterial blood	80	6.8	

Enzyme kinetic parameters for hydrolysis of AVF by APB and the ring closing rate of ORI measured *in vitro* were used to predict the rate of DZP production *in vivo*. The Michaelis–Menten fit is shown in Figure 1S. As illustrated in Figure 1B, the APB concentrations were scaled with the AVF concentration so that hydrolysis of AVF would be complete at  $t \approx 4$  min, regardless of the dose level. With calculated log partition coefficients (cLogD) of -1.71 at pH 7.4 and -2.85 at pH 6.5 (ChemAxon Ltd., 2016), AVF does not readily partition into membranes. However, ORI and DZP can under passive transcellular transport. Absorption of these species is illustrated in Figure 2S.



**Figure 1S. Predicted time course for the conversion of AVF to DZP by APB.** (A) Michaelis–Menten equation fit to conversion rates measured using 15 μg/mL APB in pH 7.4 PBS at 32 °C,  $n = 3$ ,  $R^2 = 0.988$ ,  $K_M = 370 \pm 63$  μM and  $V_{max} = 254 \pm 13$  μM/min ( $\pm$ SE). (B) Time course prediction for the amounts each reaction species at each dose level based on measured Michaelis–Menten enzyme kinetic parameters,  $K_M$  and  $k_{cat}$ . Solid lines represent the low dose level (0.439 μmol AVF + 0.103 nmol APB), dashed lines medium dose level (0.878 μmol AVF + 0.205 nmol APB), and dotted lines high dose level (1.32 μmol AVF + 0.309 nmol APB) for a 0.250 kg rat. *In vitro* conditions simulated, i.e. no absorption.

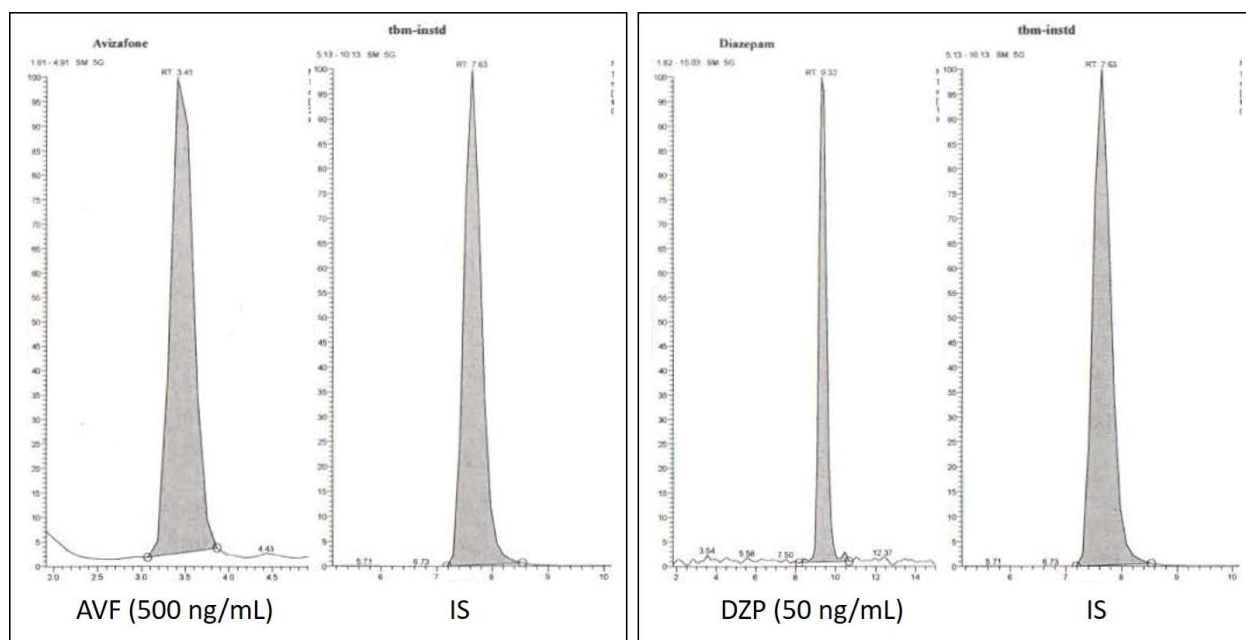


**Figure 2S. Schematic of AVF conversion to DZP.** The rates constants for reversible binding of AVF to the active site of APB are  $k_1$  and  $k_{-1}$ . The catalytic rate constant for conversion of AVF to ORI is  $k_{cat}$ . ORI spontaneously cyclizes to form DZP with cyclization rate constant,  $k_2$ . The values of these reaction rate constants were determined from *in vitro* measurements and their relationship to Michaelis–Menten enzyme kinetic parameters are shown in Equations 1Sa-c.

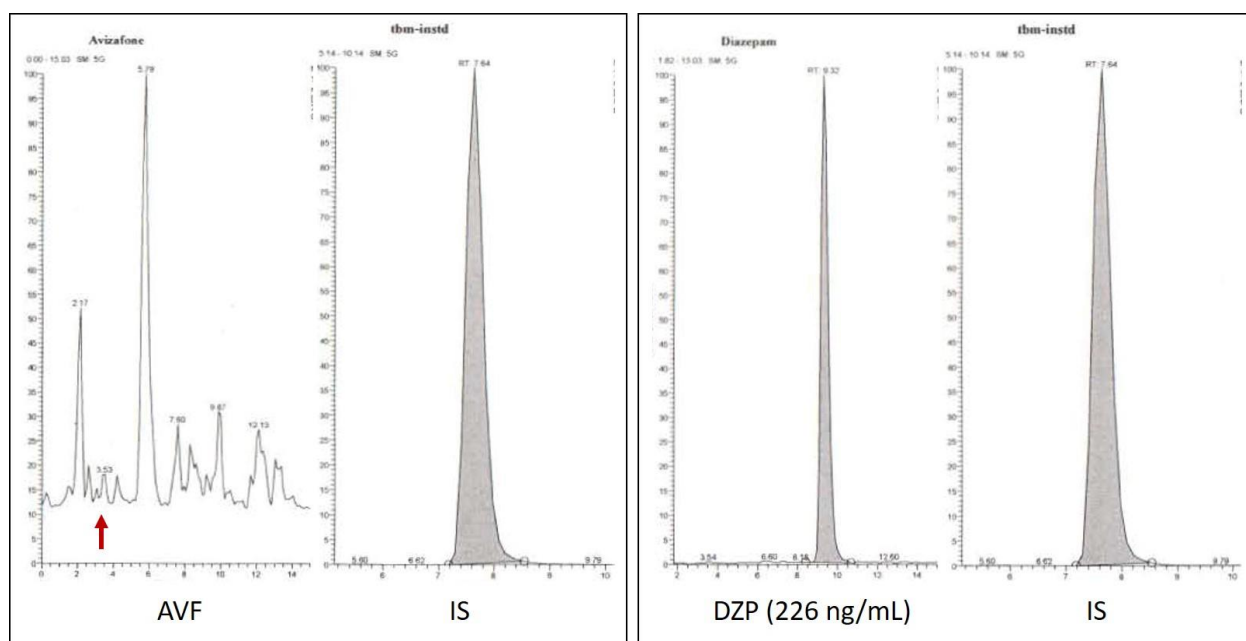
$$K_M = \frac{k_{-1} + k_{cat}}{k_1} = 370 \pm 63 \text{ } \mu\text{M} \quad (1\text{Sa})$$

$$V_{max} = k_{cat}C_{APB} \Rightarrow k_{cat} = 1250 \pm 60 \text{ min}^{-1} \quad (2\text{Sb})$$

$$k_2 = 0.470 \pm 0.024 \text{ min}^{-1} \quad (3\text{Sc})$$



**Figure 3S. Representative LC-MS/MS chromatograms: AVF and DZP standards in plasma.**



**Figure 4S. Representative LC-MS/MS chromatograms: rat #36, IN AVF+APB high dose, 10 min, plasma.** In the AVF chromatogram, the red arrow indicates the location of the AVF retention time. There was no AVF detectable in this sample, only noise.

**Table 3S. Ions monitored by MS/MS.**

Compound	Retention (min)	Precursor ion (m/z)	Product ion (m/z)
AVF	3.4	431 [MH] <sup>+</sup>	246
DZP	9.3	286 [MH] <sup>+</sup> (Cl isotope)	193
Tolbutamide (IS)	7.6	269 [M-H] <sup>-</sup>	170

**Table 4S. Nasal tissue histology incident report.** NSF = nothing significant found.

			Dose Group			
			1	3	4	5
Number of Animals			3	3	3	3
Tissue	Lesion Type	Severity	Number of Incidents Observed			
Turbinate 1	Cellular debris	Minimal		2	1	
	Cellular debris	Mild				1
	Eosinophilic fluid	Moderate			1	
	Epithelium missing	Minimal				3
	Hemorrhage	Minimal		1		
	Necrosis	Minimal		1		
	Necrosis	Mild			1	
	Ulcer	Minimal		1		
	Artifact	Minimal			1	
	NSF		3			
Turbinate 2	Cellular debris	Minimal				1
	Cellular debris	Mild				1
	Degeneration epithelium	Mild				1
	Hemorrhage	Minimal	1			
	Hemorrhage	Mild			1	
	NSF		2	3	2	1
Turbinate 3	Hemorrhage	Minimal	1			
	Hemorrhage	Mild			1	
	NSF		2	3	2	3
Turbinate 4	NSF		3	3	3	3
Turbinate 5	NSF		3	3	3	3

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