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Pharmacokinetics, Metabolism and Partial Biodistribution of 'Pincer Therapeutic' Nitazoxanide in Mice Following Pulmonary Delivery of Inhalable Particles

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***Short Title**: Nitazoxanide Inhalation*

Abstract

Purpose: Nitazoxanide (NTZ) induces autophagy in mammalian cells and also has mycobactericidal activity, displaying a two-pronged therapeutic effect-- on the host as well as the pathogen. The pharmacokinetics and biodistribution of inhaled NTZ were investigated. *Methods*: Particles containing NTZ in a matrix of PLGA were prepared by spray drying. HPLC and LC-MS/MS methods were developed and validated. Particles were administered as inhalations to mice. Drug concentrations in plasma and tissues were estimated at different time points. *Results*: Drug loading (~36%), entrapment efficiency (>90%) and the conversion of NTZ into metabolites in plasma and lung homogenates were assessed satisfactorily by HPLC. NTZ pharmacokinetics and biodistribution following intravenous administration or inhalation were established by LC-MS. NTZ converted in to tizoxanide (99% in 30 min) and other metabolites. Pulmonary delivery of NTZ entrapped in particles increased the half-life of the drug by factors of 3, 12 and 200 in the plasma, lung tissue and alveolar macrophages respectively. *Conclusions:* Targeted delivery and prolonged lung retention along with dose sparing of the kidneys was observed upon pulmonary delivery as compared to intravenous administration.

Keywords: Dry powder inhalation; Tizoxanide; HPLC; LC-MS; one-compartment.

Abbreviations: DPI, Dry Powder Inhalation; ACN, Acetonitrile; TBAHS, Tetra Butyl Ammonium Hydrogen Sulfate; LCMS, Liquid Chromatography Mass Spectrometry; NMR, Nuclear Magnetic Resonance; NTZ, Nitazoxanide; TZX, Tizoxanide; TG, Tizoxanide Glucuronide; SA, Salicylic acid; BA, Benzoic acid.

1. Introduction

The lengthy and arduous multiple-drug regimen for treatment of tuberculosis (TB) is primarily focused towards the objective of killing the pathogen. Host-directed therapies are increasingly being proposed with a view to eliciting host defense responses for the same purpose.[1] Microautophagy or autophagy is a host response that can potentially result in killing of intracellular *Mycobacterium tuberculosis* (Mtb),[2] but equally importantly, has demonstrated pre-clinical efficacy in healing the diseased lung.[3, 4]

Targeted drug delivery to deep lungs by means of a Dry Powder Inhalation (DPI) is an efficient way of maintaining high intracellular drug concentration reducing systemic drug exposure to host-directed or 'pincer' therapeutic agents.[3] The use of biodegradable polymers to prepare inhalable particles also helps in controlling the drug release profile and generating desired pharmacokinetics.[5]

NTZ is a nitrothiazolyl salicylamide having close resemblance to metronidazole. It is a light yellow crystalline powder, practically insoluble in water, slightly soluble in acetone, ethyl acetate, ethanol, and freely soluble in N-methyl 2-pyrrolidone and dimethyl formamide. It is a broad spectrum antiparasitic and antiprotozoal agent.[6, 7] NTZ is a non-competitive inhibitor of pyruvate ferredoxin oxidoreductase (PFOR), an enzyme required for anaerobic energy metabolism in prokaryotes[8] and inhibits the mTORC1 complex in the host cell to induce autophagy.[9] It is effective against clinical isolates of Mtb, however, primarily by changing bacterial membrane potential and disrupting pH homeostasis.[10] In view of the dual action of NTZ, i.e., on both the pathogen and the host, we term this agent as a "pincer therapeutic."[3] NTZ is a prodrug, rapidly hydrolysed non-enzymatically or by plasma esterases with a $t_{½}$ of ~6 min[11] to tizoxanide (TZX) or desacetyl nitazoxanide [(2-hydroxy-N-(5-nitro-2-thiazolyl) benzamide)]. TZX is further glucuronidated to form tizoxanide glucuronide (TG). Therefore, the US FDA recommends the quantification of both NTZ and TZX for pharmacokinetic and bioequivalence studies. NTZ is absorbed from the gastrointestinal tract. One third of the oral dose is excreted in urine and two thirds in faeces. TZX is found in urine, bile and faeces.[11, 12] The half-life of TZX in plasma is approximately 1.5 hrs and it is >99% bound to plasma proteins.[13, 14]

Various pharmacokinetic and biodistribution studies, stability studies and forced degradation studies (photodegradation, hydrolysis, oxidation, acid and alkali induced degradation) on NTZ have been reported,[15] using spectrophotometric,[16] RP-HPLC,[17-19] and LC-MS methods. There is

no report on pharmacokinetics and biodistribution analysis of NTZ in mice. Since the mouse is an animal model of choice in translation of drug discovery and delivery system leads in the area of TB, the present report describes analytical methods and results for pharmacokinetic and biopharmaceutic analysis of NTZ and its metabolites.

Material and methods

Materials

Nitazoxanide (NTZ, CAS-55981-09-4, purity 98%) was purchased from Hangzhou Terphenyl LCD Co., Ltd, Hangzhou, China. The primary metabolite/degradation product of NTZ, i.e., tizoxanide (TZX) was synthesized by refluxing NTZ with 1M HCL. TZX glucuronide (TG) sodium salt was purchased from (CAS 221287-83-8) Santa Cruz Biotechnology, USA. Salicylic acid (SA) and Benzoic acid (BA) was purchased from Merck, Mumbai India. HPLC grade acetonitrile (ACN), analytical grade tetrabutylammonium hydrogen sulfate (TBAHS) and ammonium acetate were purchased from Spectrochem Pvt. Ltd., Mumbai, India. All other reagents and chemicals were obtained from Merck, Mumbai, India. LC-MS grade acetonitrile and methanol were purchased from Merck Millipore. Milli Q water was obtained from a Milli Q water system from Merck Millipore.

Methods

Synthesis and structure confirmation of TZX

TZX was prepared by acidic hydrolysis of NTZ. NTZ (250mg) was taken in a 250 ml round bottom flask and refluxed with 100 ml of 1M HCl at 100°C for 24 hr. The product was filtered and re-crystallized three times with chloroform. The structure of TZX was confirmed (Table S1) by UV-visible spectroscopy (UV-1700 Pharma Spec, Shimadzu), Mass spectroscopy [(JMS-T100LC, Accu TOF (DARTMS)] and ^{1}H and ^{13}C NMR spectroscopy (Bruker DRX-300; and JEOL AL 300 FT-NMR).

HPLC method development and validation

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of dual piston reciprocating pumps (LC-10 AT Vp and LC-20 AT) with a system controller (SCL-10A Vp) and PDA detector (SPD-M10A Vp). Data collection and analysis were performed using Class VP software. A

LiChrospher 100 C18 column (RP-18e, 5µm, 250mm×4mm, Merck) and ACN: 0.005M TBAHS in triple distilled water (TDW) in the ratio of 50:50 was used as a mobile phase at a flow rate of 1ml/min to achieve separation at room temperature. The mobile phase was filtered through a 0.22µm membrane filter. Detection was carried out using a photodiode array (PDA) detector at 240 nm.

Standard solutions of NTZ and TZX were prepared by dissolving accurately weighed 10 mg of NTZ or TZX in 100 ml of ACN. The RP-HPLC method was validated as per International Conference on Harmonization (ICH) guidelines[20] in terms of linearity, accuracy, intra-day and inter-day precision and robustness of method. Limit of detection (LOD) and Limit of quantitation (LOQ) for both NTZ and TZX was calculated from the values of standard deviation of response (SD) and slopes (S) of the calibration curves.

Estimation of NTZ and TZX in bulk and formulation

Particles of volume-mean diameter 3.8±0.3µm, aerodynamic diameter 2.2±0.2µm and NTZ content of 36.3±1.4% w/w in a matrix of *poly*(lactic-co-glycolic acid) (PLGA) were prepared by spray drying.[3] Briefly, both the drug and polymer were dissolved in acetone and spray dried using inlet temperature 65ºC, feed rate 2ml/min, air flow rate 108 Nl/min. Drug loading and entrapment efficiency was calculated by estimating drug by HPLC after dissolving the particles in dichloromethane and extracting the drug using methanol as reported earlier.[3]

NTZ degradation kinetics in plasma and lung homogenate ex vivo

Rat blood plasma and lung homogenate were used to study the metabolic profile of NTZ. NTZ converts to TZX non-enzymatically. Thus, the source of the biomatrix is unlikely to contribute to the rate of NTZ hydrolysis. However, NTZ is also metabolized by blood plasma esterases. To our knowledge, there are no significant differences between activities of esterases present in rat and mouse blood plasma, although both species differ significantly from humans.[21] Since normal rat plasma is easily obtained in abundance without killing the animal, we chose to employ this resource as a surrogate.Blood (5 ml) was withdrawn from the tail vein and collected in Vacutainer® tubes coated with heparin. The blood sample was centrifuged at 1000 rpm for 15 min at 4°C to obtain plasma. Aliquots (100 µl) of plasma and lung homogenate were spiked with 4µg/ml NTZ for different time periods- 5min, 15min, 30min, 1hr, 1.5hr, 2hr, 3hr, 6hr, 24hr, 48hr and 72 hr. After incubation, 900µl of ACN was added to precipitate proteins and extract the drug

and metabolites. The sample was vortex-mixed for 10min and centrifuged at 10,000rpm for 15min at 4°C. The supernatant was separated in a clean glass tube and vacuum dried. The sample was reconstituted in 1 ml of ACN and injected for chromatographic analysis using HPLC. Blank plasma and lung homogenate was also processed as above and the ACN extract injected to check interference. The peak area was measured for calculation of NTZ and TZX concentrations at different time points, with respect to the calibration curve. Degradation kinetics was fitted to determine the decay rate constant.

LC-MS method development and identification of metabolites

The LC-MS system consisted of a Waters Milford USA (quaternary pump -600) equipped with Waters 717 auto sampler and Waters in line AF degasser. The analytes were chromatographically separated on a Waters Symmetry-Shield C18 (5µm, 4.6 × 150 mm) column, using acetonitrile: 10mM ammonium acetate (pH 3), (80:20, v/v) as the mobile phase. All separations were performed in binary mode at a flow rate of 0.7 ml/min and the injection volume was kept at 20 µl. Rinsing mode was set to before and after injection aspiration to ensure no carry over effect using acetonitrile: water (50:50) as a rinsing solution.

Bioanalysis of analytes was performed using API 3200 mass spectrometer (Applied Biosystems, Canada) coupled with an electro spray ionization (ESI) source using negative multiple reaction monitoring (MRM) mode. Topiramate was used as internal standard (IS). The optimized mass parameters for identification of major and minor metabolites is given in Table 1. Spectra of blank ACN as well as blank plasma and tissue homogenate were recorded to identify any interferences in detection due to biological sample.

Table 1: LC-MS parameters for identification and quantitation of NTZ and its metabolites

Mass parameters	
Source Parameters	**Values**
LC-MS/MS System	API 3200
Ion source	ESI
Ion spray voltage	-4500
Polarity (mode)	Negative
GS-1	40
GS-2	45
Curtain gas	20

CAD gas		3
Temp.		250˚C
Splitter (Yes/No)		No
Probe	X-axis	0.5 cm
	Y-axis	0.5 cm

Compound Parameters

Analyte	**MRM(Q1/Q3)***	**DP***	**CE***	**CXP***	**EP***	**Molecular Weight**	**Dwell (msec)**
Topiramate	338.16/77.96	-90	-70	-1	-10	339.36	200
Nitazoxanide	306.1/186.1	-21	-18	-1	-10	307.28	200
Tizoxanide	263.8/217	-30	-21	-1	-10	265.24	200
Tizoxanide glucuronide	439.9/264	-29	-24	-1	-10	463.02	200
Salicylic acid	136.9/93.10	-32	-25	-1	-10	138.12	200
Benzoic acid	121.1/76.9	-29	-18	-1	-10	122.12	200

Abbreviation: MRM (Q1/Q3) (Multiple Reaction Monitoring (Precursor ion/ Product ion); DP (Declustering potential); CE (Collision Energy); CXP (Collision cell exit potential); EP (Entrance potential).

Pharmacokinetics and biodistribution in mice

NTZ pharmacokinetics and biodistribution were investigated with the approval of the IAEC (approval number IAEC/2014/149/DATED 03.12.2014). Healthy male Swiss mice aged 8-10 weeks and weighing 22-25g were used. Animals were bred and housed ethically in the National Laboratory Animal Facility located in our Institution in conformity with the US NIH's Guide for the Care and Use of Laboratory Animals.

NTZ was dissolved in DMSO and diluted with PBS. NTZ solution was filter sterilized and 806 µg/kg was administered intravenously to mice through the tail vein. For delivering inhalations, an in-house nose only apparatus was used.[22] This apparatus is constructed of a 15 ml centrifuge tube with a hole just large enough to accommodate the nares of the test animal, and another hole in the apex that allows flexible tubing to be admitted into the interior of the tube. When a rubber bulb at the other end of the tubing is actuated, turbulent air enters the tube and aerosolizes powder placed in the cap of the tube. Powder weighing approximately 12.5 mg was placed in the apparatus. Mice were manually restrained with nostrils inserted in the delivery port. The powder

was fluidized with turbulent air admitted by squeezing a rubber bulb once per second for 30 seconds. Under these conditions, the inhaled dose of NTZ was 18.42 ± 1.36 µg / mouse or 750µg/Kg.[3]

Following intravenous and inhalation administration, sets of four animals were anaesthetized and blood samples were withdrawn from hepatic portal vein for complete exsanguination at 4 min, 8 min, 12 min, 16 min, 30 min, 1h, 3h, 6h, 12h, 24h, and 48h. Bronchio-alveolar lavage (BAL) was conducted to recover lung macrophages and viscera were collected and weighed fresh. NTZ, TZX, TG, BA and SA concentrations was determined by LC-MS following extraction of drug from plasma, BAL cell lysate and organ homogenate samples with acetonitrile. A one-compartment PK model was fitted to data on concentrations in tissue homogenates and blood plasma, using PK Solver as an add-in to Microsoft Excel® and PK parameters was calculated.

Results and Discussion

HPLC method development and validation

The HPLC method was suitable for detection and quantitation of NTZ and TZX in formulations and ex vivo samples. HPLC conditions were optimized to obtain good peak shapes, baseline resolution and purity indices of ≥0.9999. The method was linear in the concentration range of 1-20µg/ml with a correlation coefficient of 0.99. The values of LOD and LOQ were 5.49 ng/mL and 16.62 ng/mL for NTZ, and 5.43ng/ml and 16.45 ng/ml for TZX. Accuracy of analyte recovery was ~ 99% (Table S2). To determine the intra-day and inter-day precision of the method, % CV was calculated and was within acceptable range as shown in Table S3. The robustness of the method was checked by deliberately changing the mobile phase composition and flow rates. Changes in retention time, peak shape, tailing and effect on separation were observed and are shown in Table S4. Using the analytical HPLC method, it was established that spray drying yielded particles with a practical drug content of 36.36 ± 1.39 % and the process had an entrapment efficiency of 90.9 ± 3.48 %.

NTZ degradation kinetics in plasma and lung homogenate ex vivo

Almost all NTZ (99%) spiked into plasma samples was observed to be metabolized to TZX within 30 minutes. TZX itself is not a stable metabolite, as indicated by time-dependent reduction in TZX peak area in plasma and lung homogenate samples. NTZ converted in to TZX

very rapidly and TZX decayed exponentially with the values of exponential decay rate constant of 24.79 h^{-1} and 82.42 h^{-1} in plasma and lung homogenate respectively (Figure 1).

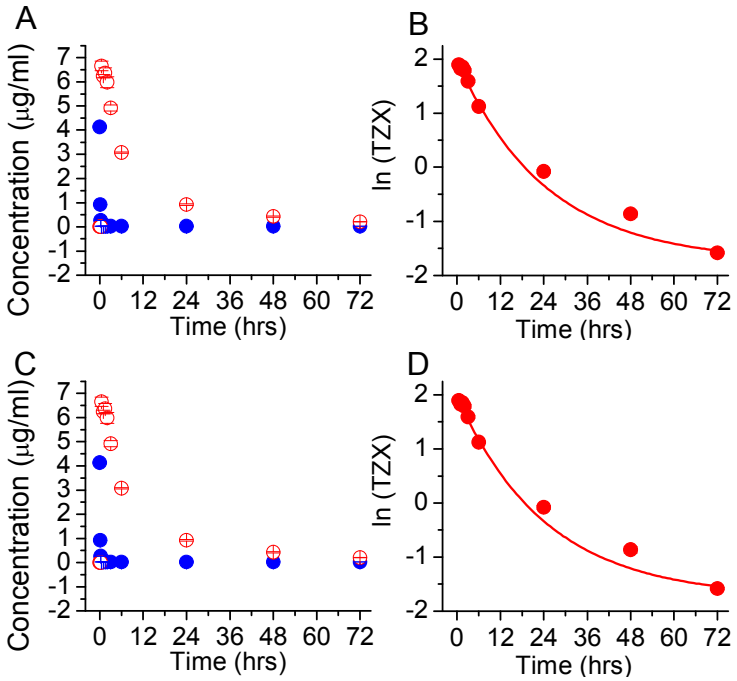

Figure 1: Degradation of NTZ (*filled symbols)* in blood plasma (*A*) and lung homogenate (*C*) was rapid while TZX (*open circles*) decayed exponentially at 37°C. Mean ± SD of three replicate experiments is shown. The exponential decay rate constant of TZX in calculated by fitting the natural log of TZX concentrations to time was 24.79 h^{-1}(R^2= 0.993) in plasma (*B*), while in lung homogenate (*D*), the decay constant was obtained from fitting natural log of TZX concentrations to time ($y = A1e^{(-k/t)} + y_0$) was 82.42 h^{-1} (R^2= 0.987).

LC-MS method development and identification of metabolites

The sensitivity of the HPLC method was not sufficient for biopharmaceutics, so an LC-MS method was developed for identification and quantitation of NTZ, TZX and minor metabolites. The retention times of topiramate (IS), NTZ, TZX, Tizoxanide glucuronide (TG), Salicylic acid (SA), and Benzoic acid (BA) in LC-MS were 2.51, 2.78, 2.77, 2.09, 2.27 and 2.51 min respectively. The respective chromatograms are shown in Figure 2. The metabolites of NTZ are summarized in Figure 3. Calibration curves were plotted over 0.97-1000ng/ml concentration ranges for each analyte in every organ used for determining drug concentration. The value of the correlation coefficient was 0.99 in each case. The value of LOQ and presence of metabolites is shown in Table 2. For determining pharmacokinetics and biodistribution, the sum total of NTZ and TZX was considered.

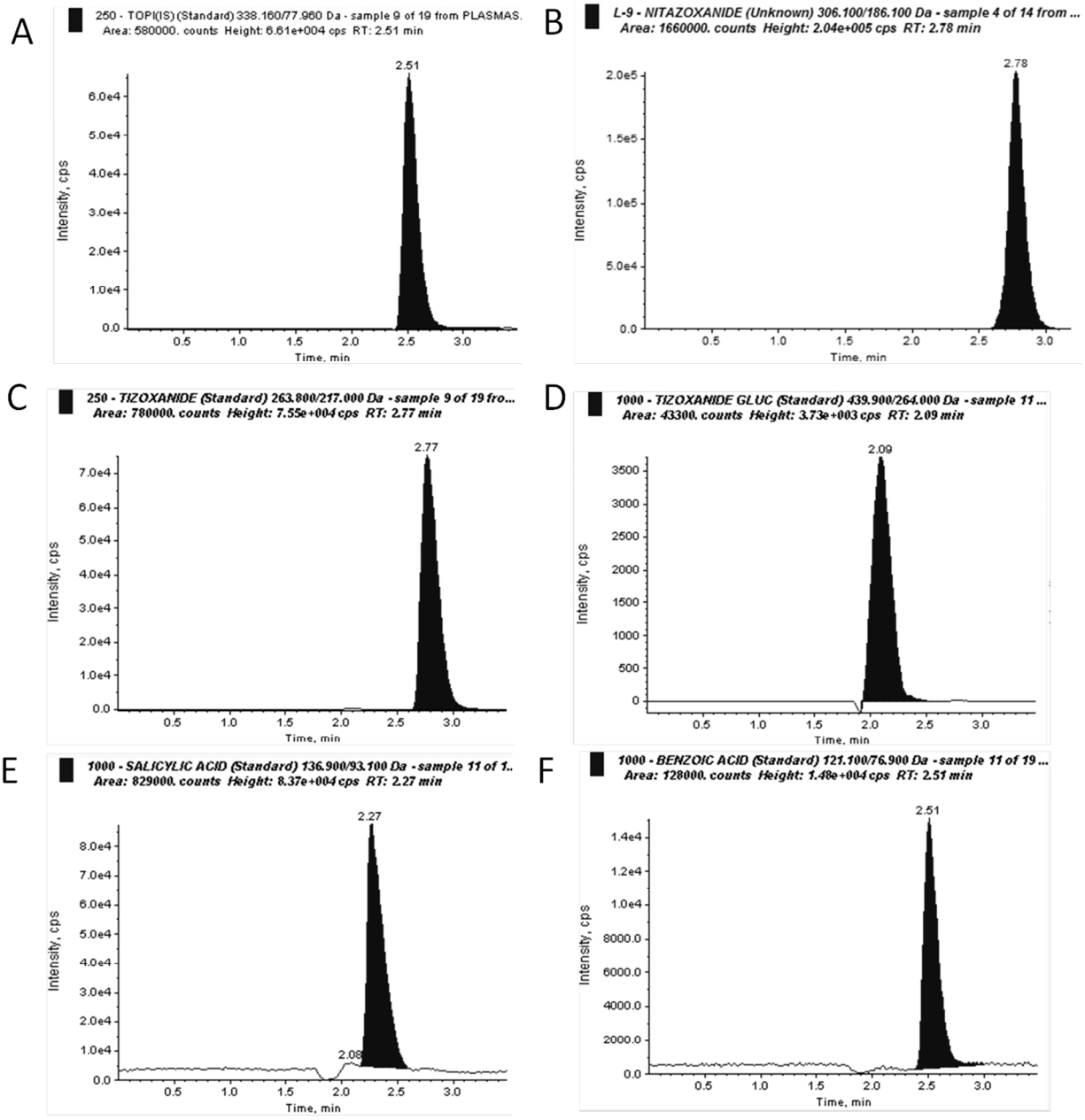

Figure 2: LC-MS chromatograms of *(A)* Topiramate, *(B)* NTZ, *(C)* TZX, *(D)* TZX-glucuronide, *(E)* Salicylic acid, and *(F)* Benzoic acid.

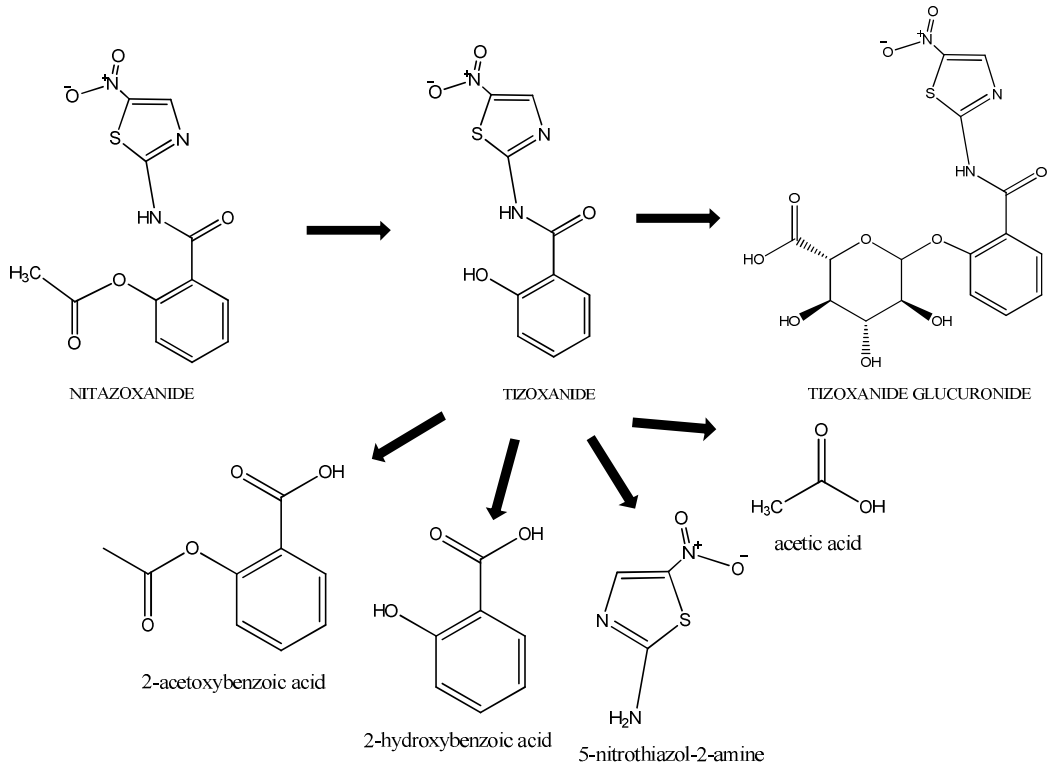

Figure 3: Structure and metabolic conversion of NTZ into its metabolites

Table 2: Parent compound and metabolites that were either "found" or "not found" in plasma, cells or tissue homogenates at 72 hours after IV or inhalation dosing, and their limits of quantitation

Intravenous					
Organs/ LOQ (ng/ml)	**NTZ**	**TZX**	**TG**	**SA**	**BA**
Plasma	Not found	Found (0.97)	Found (0.97)	Found (7.85)	Not found
Lung	Not found	Found (0.97)	Found (0.97)	Not found	Not found
BAL cells	Not found	Found (0.97)	Not found	Not found	Not found
Liver	Not found	Found (0.97)	Not found	Found (7.85)	Not found
Spleen	Not found	Found (0.97)	Found (0.97)	Not found	Found (31.2)
Kidney	Not found	Found (0.97)	Found (0.97)	Found (15.6)	Found (31.2)
Heart	Not found	Found (0.97)	Found (3.9)	Not found	Not found
Inhalation					
Organs/ LOQ (ng/ml)	**NTZ**	**TZX**	**TG**	**SA**	**BA**
Plasma	Not found	Found (0.97)	Found (0.97)	Found (7.85)	Not found
Lung	Found (0.97)	Found (0.97)	Found (0.97)	Found (15.6)	Not found
BAL cells	Found (0.97)	Found (0.97)	Found (0.97)	Found (7.85)	Found (3.9)
Liver	Not found	Found (0.97)	Found (1.95)	Found (7.85)	Not found
Spleen	Not found	Found (0.97)	Found (0.97)	Found (15.6)	Found (31.2)

Kidney	Not found	Found (0.97)	Found (0.97)	Found (15.6)	Found (31.2)
Heart	Not found	Found (0.97)	Found (3.9)	Found (7.85)	Found (15.6)

Pharmacokinetic parameters were calculated by applying non compartmental, one compartment and two compartment models. Based on the value of R^2 and AIC, a one compartment model was best fitted to data and PK parameters were calculated (Table 3). The concentration-time graphs highlight the differences in concentrations over time as shown in Figure 4. NTZ was retained in the lungs and alveolar macrophages following inhalation and intracellular drug concentration were maintained for up to 48 h. This observation suggested that efficacy studies should examine an alternate-day dosing regimen.[23] NTZ was not detected in plasma after i.v. dosing in mice despite the reported $t_{½}$ of 6 min in humans following per-oral administration.[11] TZX was detected up to 6 h. TZX glucuronide and minor metabolites benzoic acid and salicylic acid were detected at later time points but their amount was not taken into consideration for calculation due to variability. NTZ and TZX were both found in BAL cells, plasma and lungs after inhalation dosing. TZX was detected for up to 48 h demonstrating that NTZ particles were able to maintain drug concentrations for a prolonged period of time. PK parameters indicated that the $t_{½}$ of NTZ increased from 0.68 h to 2.28 h in plasma, 0.19 h to 11.6 h in lungs and 0.10 h to 21.94 h in alveolar macrophages. Clearance was reduced from 7.81 to 0.04 (μg/kg) /(ng/ml)/h following intravenous and inhalation dosing.

During peer-review, it was pointed out that relatively higher concentrations were observed in the heart, liver and spleen following pulmonary delivery as compared to IV injection. A possible explanation for these observations may be that particles deposited in the lungs traffic to these organs. Since the particles are fairly large (volume-mean diameter 3.8±0.3μm), it is extremely unlikely that they would enter blood circulation by crossing epithelial barriers. It is far more likely that macrophages on the lung and airway surface pick up deposited particles and move to the bloodstream and then to visceral organs. This speculation is supported by the observations that macrophage-rich organs (liver, spleen) also show high drug concentrations, whereas the kidneys (which macrophages infiltrate only in disease or upon injury) show low concentrations.

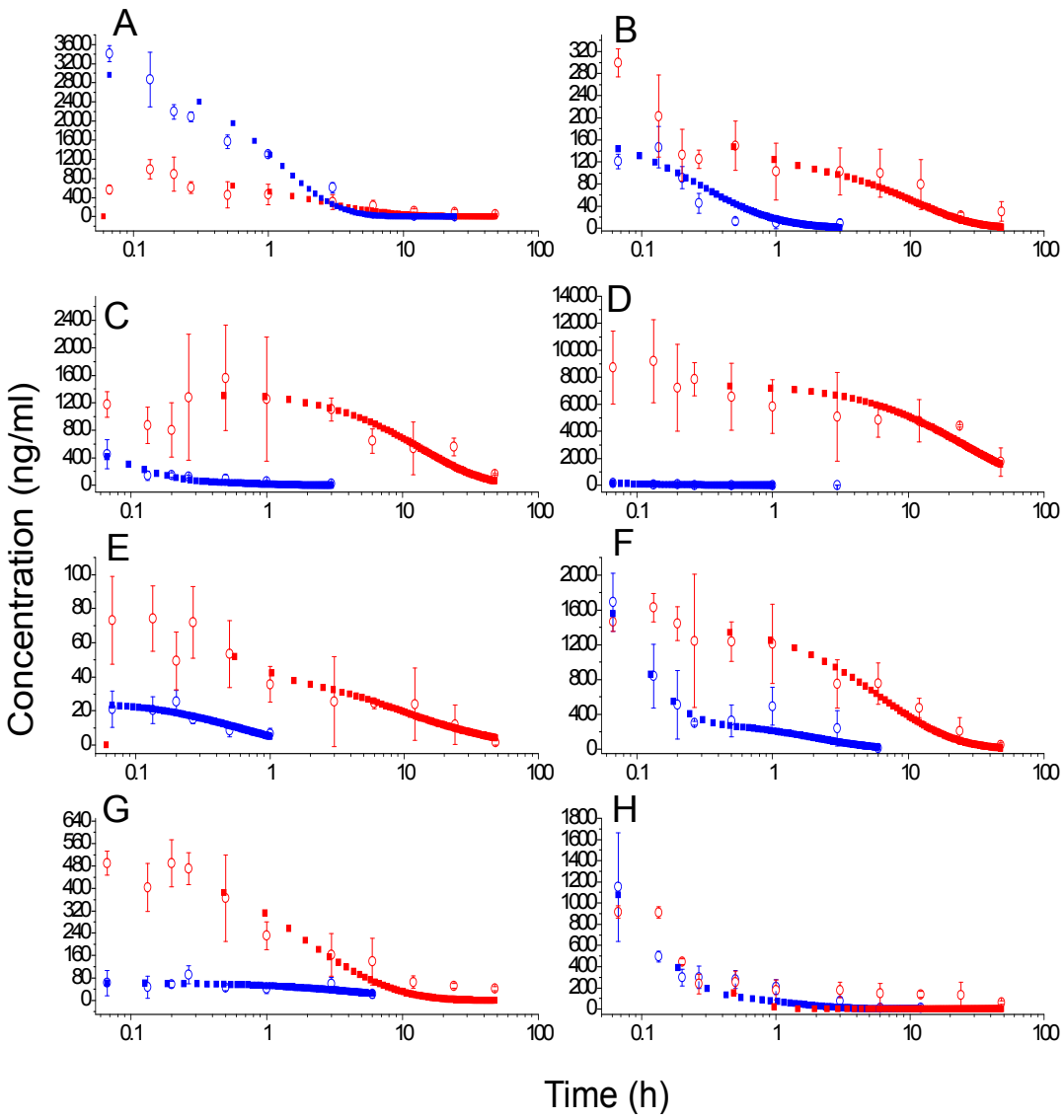

Figure 4: Pharmacokinetics and partial bio distribution of NTZ + TZX. Drug concentrations time graph resulting from IV injection (*blue empty circles*) or inhaled (*red empty circles*) in mice (N=3 per time point). *(A)* Plasma, *(B)* heart, *(C)* lungs, *(D)* cell lysate, *(E)* BAL fluid, *(F)* liver, *(G)* spleen *(H)* kidney. Data are shown as means ± standard deviation. Respective predicted concentrations obtained after one compartment modelling are shown by scatter points. Data from three animals per time-point ± SD is plotted.

Table 3: Pharmacokinetic parameters calculated using a one-compartment model to data from three animals per time-point

Intravenous										
Parameter/ Organs	**C_0 ng/ml**	**k_a 1/h**	**k_{10} 1/h**	**$t_{1/2ka}$ h**	**$t_{1/2}$ h**	**V (µg/kg) /(ng/ml)**	**CL (µg/kg) /(ng/ml)/h**	**AUC $_{0-t}$ ng/ml*h**	**AUMC ng/ml*h^2**	**MRT h**
Plasma	3206.79±407.67	-	1.14±0.4	-	0.68±0.22	0.26±0.03	0.28±0.05	3007.3±620.1	3139.9±1666.4	0.98±0.32
Cell lysate	235.15±58.32	-	6.81±0.4	-	0.10±0.01	3.60±1.04	24.51±7.18	34.6±9.0	5.1±1.4	0.15±0.01
Lungs	874.26±650.09	-	8.81±6.3	-	0.19±0.23	1.63±1.55	7.81±3.31	114.9±42.5	38.6±53.8	0.27±0.33
Heart	188.43±6.62	-	4.11±0.7	-	0.17±0.03	4.28±0.15	17.59±3.15	46.8±8.7	11.9±4.2	0.25±0.04
Liver	3401.19±2097.8	-	9.26±7.7	-	0.54±0.85	0.39±0.37	1.72±1.17	952.9±1066.8	1617.45±2758.7	0.78±1.23
Spleen	527.55±421.55	-	5.18±4.3	-	0.14±0.12	1.41±0.32	6.10±2.44	55.5±54.1	31.3±26.9	0.20±0.17
Kidney	2080.22±1368.5	-	7.82±5.3	-	0.18±0.20	0.71±0.73	2.92±0.29	277.6±28.1	76.9±91.6	0.26±0.29
BAL Fluid	26.13±7.28	-	1.61±0.3	-	0.44±0.07	32.57±9.4	51.03±7.95	16.03±2.2	10.0±1.4	0.63±0.10
Inhalation										
Parameter/ Organs	**A ng/ml**	**k_a 1/h**	**k_{10} 1/h**	**$t_{1/2ka}$ h**	**$t_{1/2k10}$ h**	**V/F (µg/kg) /(ng/ml)**	**CL/F (µg/kg) /(ng/ml)/h**	**AUC $_{0-t}$ ng/ml*h**	**AUMC ng/ml*h^2**	**MRT h**
Plasma	814.54±138.20	182.22±10.33	0.48±0.4	0.00±0.0	2.28±1.73	1.01±0.19	0.45±0.33	2454.6±147.94	10483.81±11698.25	3.29±2.49
Cell lysate	7484.62±921.47	164.99±116.22	0.04±0.0	0.01±0.0	21.94±10.17	0.11±0.01	0.00±0.00	172595.6±56698.6	8346942.8±5904206.9	31.66±14.6
Lungs	1377.95±442.56	113.27±181.34	0.07±0.0	0.06±0.1	11.16±3.59	0.64±0.25	0.04±0.01	19455.9±3378.9	331950.4±103510.1	16.18±5.1
Heart	209.55±38.88	273.32±17.07	0.84±1.3	0.00±0.0	5.27±4.41	3.94±0.70	2.78±4.13	1435.0±1220.7	16342.53±16120.5	7.61±6.37
Liver	1443.34±143.15	233.59±70.80	0.15±0.1	0.00±0.0	5.84±3.37	0.56±0.05	0.08±0.04	11584.5±5709.8	118849.2±119003.7	8.44±4.86
Spleen	483.39±36.31	271.53±24.34	0.48±0.4	0.00±0.0	2.23±1.76	1.68±0.13	0.78±0.55	1491.9±1067.8	6601.7±8339.1	3.22±2.53
Kidney	1304.39±130.90	1739.7±2079.8	4.46±0.8	0.00±0.0	0.16±0.03	0.63±0.07	2.76±0.24	293.4±25.3	68.5±18.5	0.23±0.04
BAL fluid	78.39±27.67	168.78±100.4	0.83±1.3	0.01±0.0	5.13±5.11	11.14±3.1	6.67±9.15	401.0±344.8	5600.9±7398.2	7.40±7.37

Where **C_0, A:** Drug concentration at time zero, **k_a**: First order absorption rate constant, **k_{10}**: Elimination rate constant (first-order) from the central (1) compartment,
$t_{1/2ka}$: Absorption half-life, **$t_{1/2}$:** Half-life of drug in plasma, **$t_{1/2k10}$:** Elimination half-life, **V:** Volume of distribution, **CL:** Total body clearance, **AUC_{0-t}:** Area under the curve from time zero to t, **AUMC:** Total area under the first moment curve, **MRT:** Mean residence time, **F:** Fraction of administered dose which reaches the systemic circulation unchanged.

Conclusion

The high payload DPI formulation of NTZ, intended to target alveolar macrophages following aerosol delivery to the lungs, maintained a high intracellular drug concentration for a longer duration than intravenous injection. The HPLC method developed and validated was sufficient for the purpose of determining drug content in the formulation, to estimate drug loading and entrapment efficiency. For determining drug and metabolite concentrations in animal tissue, a more sensitive LC-MS method was required. Using this method, it was established that NTZ is converted into TZX which then gives rise to TG, SA and BA as terminal metabolites. NTZ particles served the purpose of deep lung targeting and increasing the half-life of drug as evident from secondary PK parameters.

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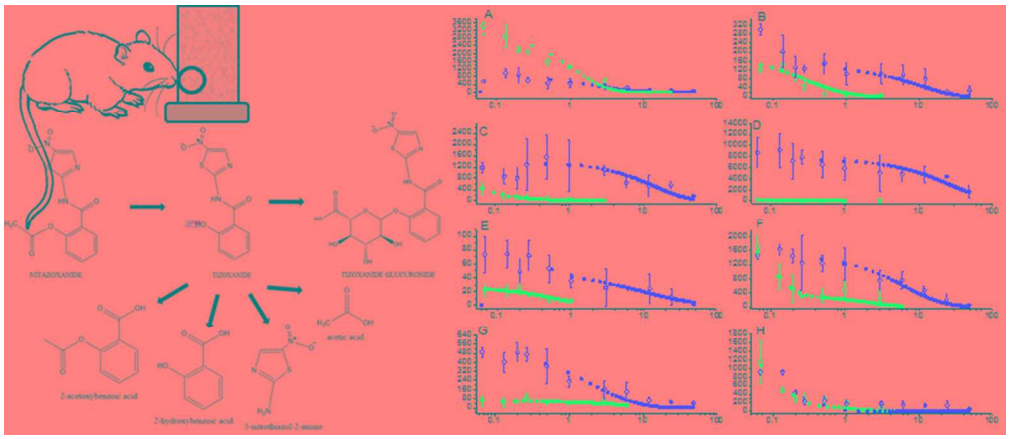

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