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Evaluation of gender differences in the pharmacokinetics of oral zileuton nanocrystalline formulation using a rat model

Chandra Mohan Reddy Muthumula [a], Sangeeta Khare [a], Rajan Jog [b,1], Bhagya Wickramaratne [a], Angela Lee [a], Sushanta Chakder [c], Diane J. Burgess [b], Kuppan Gokulan [a,*]

[a] Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR 72079, United States of America
[b] Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, United States of America
[c] Center for Drug Evaluation and Research, US Food and Drug Administration, White Oak Campus, Silver Spring, MD 20993, United States of America

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ABSTRACT

Zileuton is a leukotriene inhibitor used to treat asthma. As a BCS class II drug it exhibits challenges with solubility which likely impact its absorption. As patient gender significantly impacts the pharmacokinetics of many drugs, this study aimed to investigate potential gender-based pharmacokinetic differences after oral zileuton administration in rats. Male and female Sprague Dawley rats received single oral gavage doses of pure zileuton as an active pharmaceutical ingredient (30 mg/kg body weight (bw)), physical mixture (PM; at 30 mg/kg bw of the formulation contains zileuton, kollidon VA64 fine, dowfax2A1 and trehalose), and nanocrystalline formulation of zileuton (NfZ; at 30 mg/kg bw of the formulation). Plasma, tissue, and urine concentrations were quantified using high performance liquid chromatography (HPLC). Noncompartmental pharmacokinetic analysis showed higher zileuton levels in the plasma of female versus male rats across all evaluated forms of zileuton (API, PM, and NfZ). Female rats demonstrated higher peak plasma concentrations (C_{max}) and increased area under the plasma concentration-time curve (AUC) relative to males, regardless of formulation. These findings reveal substantial gender disparities in the pharmacokinetics of zileuton in the rat model. This study emphasizes the critical need to evaluate gender differences during preclinical drug development to enable gender-based precision dosing strategies for equivalent efficacy/safety outcomes in male and female patients. Additional studies are warranted to investigate underlying mechanisms of such pharmacokinetic gender divergences.

1. Introduction

Pharmacokinetics (PK) of a drug reveals how the body interacts with the drug over its entire duration within the body, based on the absorption, distribution, metabolism, and elimination (ADME) of the drug. Understanding these factors provides insights into bioavailability, allows optimization of dosing, and minimizes toxicity for therapeutic efficacy and safety (Li et al., 2019). PK considers both drug-specific factors such as solubility and permeability, as well as physiological variables like age, gender, and genetics (Ladumor et al., 2019; Maharaj and Edginton, 2014; Vinarov et al., 2021; Yanamadala et al., 2023). By elucidating the complete time-course of a drug's journey throughout the body, PK is considered a foundational science for drug development and therapeutic optimization (Gallo, 2010; Yu and Wilson, 2010).

Asthma is a major chronic inflammatory disorder of the airways affecting over 300 million individuals globally (Rogliani et al., 2020; Menzies-Gow et al., 2020; Johnson and Harker, 2017). It is characterized by reversible airflow limitation and bronchospasm. Asthma's onset and progression is majorly induced by environmental triggers such as allergens, airborne pollens, and dust mites (Johnson and Harker, 2017; Baldacci et al., 2015; Gautier and Charpin, 2017). The key symptoms of asthma include increased mucus secretion, bronchial hyperresponsiveness, wheezing, chest tightness and pain, coughing, and shortness of breath (Holgate et al., 2015; Porsbjerg et al., 2023).

Zileuton works as an asthma medication through its action as a 5-lipoxygenase inhibitor, preventing the formation of inflammatory leukotrienes (Rossi et al., 2010a; Holgate et al., 1996). Leukotrienes are proinflammatory mediators that contribute to the pathogenesis of asthma.

* Corresponding author.
E-mail address: Kuppan.gokulan@fda.hhs.gov (K. Gokulan).
[1] Present address: U.S. Food and Drug Administration, Office of Generic Drugs, Center for Drug Evaluation and Research, Silver Spring, MD 20993, USA.

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The approved adult dosage of zileuton tablets is 2400 mg daily in divided doses (Rossi et al., 2010b). Adverse effects include liver enzyme elevations, sleep disorders and behavioral changes. Importantly, zileuton is categorized as a biopharmaceutics classification system (BCS) class II drug. BCS class II compounds have low solubility but high permeability (Papich and Martinez, 2015). The poor aqueous solubility of zileuton might lead to low oral bioavailability, which poses challenges for effective drug delivery. To overcome solubility-limited absorption, nanocrystal drug formulations strategies can be utilized (Bertoni et al., 2020; Gigliobianco et al., 2018; Junyaprasert and Morakul, 2015; Yanamadala, 2023).

There is an extensive evidence that gender plays a significant role as a risk factor for asthma, in addition to environmental contributors. During childhood, asthma prevalence is higher in boys. However, after puberty there is a shift and asthma become more common in women (Fuseini and Newcomb, 2017; Zein and Erzurum, 2015; Chowdhury et al., 2021; Almqvist et al., 2008). Risk of developing asthma is nearly twice in women than men. Recent data from Center of Disease Control and Prevention (CDC) states that approximately 25 million people are currently suffering from asthma in US; approximately 59% of these are estimated to be females and 41% are males (*Most Recent National Asthma Data*, 2023; Pate et al., 2021). Further studies in mice have shown females have greater susceptibility to airway inflammation, implying that gender may contribute to the onset and progression of asthma (Yung et al., 2018).

Given the clear evidence that gender significantly influences asthma susceptibility, severity, and developmental patterns, it is critical to consider potential gender differences when evaluating asthma therapies. Gender disparities likely extend to the pharmacokinetics of asthma medications like zileuton, but these factors are often overlooked. PK parameters (ADME) of drugs can be affected by gender-based differences such as body composition, hormone levels, expression of biotransformation enzymes and renal/hepatic blood flow (Soldin and Mattison, 2009; Zucker and Prendergast, 2020; Franconi and Campesi, 2014). Females tend to have slower gastric emptying times and differences in gastrointestinal mobility that influence bioavailability (Islam et al., 2017). There are also disparities in clearance mechanisms; for example, females exhibit lower glomerular filtration rates and decreased clearance of P-glycoprotein substrates such as digoxin (Anderson, 2008). Given zileuton's metabolism by CYP enzymes and potential for liver toxicity, it is especially important to elucidate any gender differences in PK (Lu et al., 2003). By avoiding gender-specific factors, the safety and efficacy of a drug may be compromised in certain patient groups.

The current research investigated gender-differences in PK parameters in a rat model following oral administration of different forms of zileuton (zileuton, a physical mixture of zileuton (Kollidon VA64 fine, Dowfax 2A1 and Trehalose), and a nanocrystalline formulation of zileuton (NfZ)). In addition, the impact of particle size of zileuton (Papich and Martinez, 2015) vs the zileuton nanocrystal formulation was evaluated for gender-specific PK differences. The outcome of the present research provides essential information and aids in understanding of how gender may influence the pharmacokinetic profile and disposition of zileuton. Overall, this research provides a preliminary insight to optimize safety, efficacy, and therapeutic outcomes of a model drug for the treatment of asthma by characterizing gender differences in drug pharmacokinetics.

1.1. Materials

Crystalline zileuton (>99% purity, irregularly shaped coarse particles with a heterogeneous size distribution will hereafter simply be referred to as “API” throughout this paper) was procured from Beijing Mesochem Technology Co. Ltd., China. KollidonVA (vinyl acetate) 64 fine was obtained from BASF Pharmaceutical Products (Ludwigshafen, Germany), Dowfax 2A1 was obtained from Dow Chemical Company (Midland, MI, USA) and Trehalose from Gattefose (Lyon, France). HPLC grade solvents were purchased from Fisher Scientific (Waltham, MA, USA). Gelatin capsules was purchased from Torpac, (Fairfield, New Jersey, USA), and polyurethan dosing tubes (#9 capsules, 85 mm long, sterile) were obtained from Instech Laboratories, Inc. (Plymouth Meeting, PA, USA).

2. Methods

The animal study was conducted with the approval of National center for Toxicological research (NCTR) Institute Animal Care Use Committee (IACUC) and protocol number is E0759021. The three forms of zileuton used in this study were: 1) API; 2) physical mixture (PM) of API and excipients (PM contains mixture of API, 0.5% *w/v* Kollidon VA64 fine, 0.05% w/v of Dowfax 2A1, 1:1% *w*/w of drug: trehalose); and 3) nanocrystalline formulation of zileuton (NfZ). The NfZ formulation was prepared using wet media milling and spray drying. All three forms were characterized using dynamic light scattering (DLS) and scanning electron microscopy (Zhao et al., 2018).

2.1. Rationale for selection of API

Zileuton was selected as a model BCS class II drug due to its poor aqueous solubility. It is expected that there will be a significant improvement of solubility and concurrent bioavailability of such low solubility drugs via a nanocrystal formulation strategy. Additionally, nanocrystalline drugs would require low amounts of drug that probably could decrease the side effects compared to parent drug (coarse particles). Drug selection and formulation development was previously described in detail (Khare et al., 2023).

2.2. Preparation of nanocrystalline formulation

Selection of poorly soluble drug substance and formulation development were performed in collaboration with Center for Drug Evaluation and Research (Reinecker et al., 1993) and the research team at University of Connecticut. zileuton (BCS class II drug) was selected as a model drug for the preparation of a nanocrystal formulation at the University of Connecticut, Storrs, Connecticut. The risk assessment was conducted at National Center for Toxicological Research (NCTR) using rat model. The detailed method development and method validation via design of experiments (DoEs) of wet media milling and spray drying of spray dried nanocrystalline zileuton formulations and solid-state analysis were presented previously (Jog and Burgess, 2019).

2.3. Wet media milling

Briefly, nanocrystalline zileuton suspensions were manufactured using wet media milling. Zileuton (1% *w/v*) was suspended in distilled water containing stabilizers – KollidonVA64 (0.75% w/v) and Dowfax 2A1 (0.05% w/v). The prepared suspension was stirred for 30 min for complete wetting of the drug by the stabilizer solution. The microsuspension (100 mL) was milled using a wet media mill (Microcer® Netzsch, bead diameter: 0.05 mm ZetaBeads® nano (yttrium-stabilized, high purity zirconium oxide powder)) at a milling speed of 1200 rpm, pump speed of 92 rpm, and milling time of 18 min. The temperature of the nanosuspension was maintained at 2–8 °C using two cooling bath recirculators (one attached to the mill and the other to the suspension recirculation chambers).

2.4. Spray drying

Briefly, solid dry powder of nanocrystalline zileuton was prepared via spray drying. The nanosuspension was spray-dried using a B-290 spray dryer (Buchi Labortechhnik AG). The spray dryer was equilibrated using distilled water. Once the spray dryer was equilibrated, distilled water was replaced with the nanosuspension formulation. The

nanosuspension formulation was spray dried at an inlet temperature of 118 °C, an aspirator rate of 90%, and a feed flow rate of 10%, using trehalose as an excipient [drug:sugar (1:1%*w*/w)] to prevent nanocrystal aggregation. Spray-dried powder [nanocrystalline formulation of zileuton (NfZ)] was collected from the collection chamber and immediately evaluated for particle size.

2.5. Characterization of nanocrystalline formulation

2.5.1. Particle size analysis via scanning electron microscopy

SEM was used to analyze the surface morphology of the drug particles in this study. The SEM images were obtained using a Zeiss Merlin Field Emission Gus Scanning Electron Microscope (Zeiss Merlin FESEM) with an electron high tension voltage of 3.0 kV. In this method, a small amount of the samples (API, NfZ, and the physical mixture of API and excipients (PM)) were mounted on a conductive tape and sputter coated with a thin layer of metal to enhance conductivity. The samples were then loaded into the SEM and bombarded with a beam of electrons. The magnification of the images was adjusted to generate high-resolution images of the zileuton samples under study. SEM images were used to analyze size, shape, and surface morphology of the zileuton samples (API, NfZ, and PM).

2.5.2. Particle size analysis via dynamic light scattering (DLS)

The hydrodynamic diameter, polydispersity index and zeta potential of NfZ via dynamic light scattering (DLS) was determined using continuously monitored phase-analysis light scattering. The samples were prepared by homogeneously dispersing the NfZ in ethanol. The measurements were performed using an Anton Paar Litesizer 500.

2.5.3. HPLC Chromatographic condition

HPLC analysis was performed using an Agilent Technology 1260 infinity hyphenated with mass spectrophotometer (Agilent Technology infinity lab LC/MSD) with an auto sampler system. Chromatographic separation and identification of zileuton was performed using a C18 reverse-phase column (100 mm × 4.6 mm, 5 µm particle size, Discovery C18 from Waters) and a UV detector set at 230 nm using spectral diode array system. The mobile phase consisted of a mixture of ammonium acetate buffer (pH adjusted to 7.5) and 50% methanol at compositions of 10% and 90%, respectively and was freshly prepared every time. The mobile phase flow rate was set at 1 mL/min. The sample injection volume was set to 20 µL and injected into the column using an automatic sample loader.

2.6. Study design

2.6.1. Experimental design

The animal study was approved by the Institute Animal Care Use Committee (IACUC) at the National Center for Toxicological Research (NCTR), Jefferson, Arkansas. The strategy for the life stage animal experimental groups and three test materials-exposure is provided in the experimental Scheme (Fig. 1). Sprague Dawley rats were selected because they share many anatomical and physiological features with humans and are a close animal model to non-human primates (Weber et al., 2019). Ten-week-old male and female Sprague Dawley rats (approximately 150-g body weight) were obtained from Charles River Co. (Wilmington, MA). NfZ, zileuton API and PM were orally administrated to both male and female Sprague Dawley rats (n = 3 in each group) at a dose of 30 mg/kg bw daily for 15 consecutive days. One control group (n = 4) did not receive any drug. Blood, plasma, and urine samples from this control group were used as a biological matrix to spike zileuton and generate calibration curves. Thirty (30) mg of NfZ had approximately 7.2 mg of API, which is 4.2 times less drug load when compared to the API treated animals. Similarly, 30 mg of PM had approximately 7.2 mg of API and remaining constituents are excipients (approximately 22.8 mg). In humans, zileuton is orally taken after meals to increase the drug absorption, hence, rats were also orally gavaged after feeding. All the test materials were filled in 9 h clear gelatin capsules (Torpac, Fairfield, New Jersey 07004, USA) under sterilized conditions according to the weight of an individual rat to mimic drug treatment in patients. The capsule was orally administered using flexible oral gavage syringes (Polyurethane dosing tubes #9 capsules, 85 mm long, sterile, Instech Laboratories, Inc. 450 South Gravers Road, Plymouth Meeting, PA 19462 USA). Animals were housed individually in a metabolic cage to collect urine and blood samples at various time points. Plasma samples were collected at 0, 1, 2, 4, 6, and 24 h after the first dose on day 1 to evaluate the pharmacokinetics of zileuton, and urine samples were collected over a 24-h period after the first dose on day 1. Animals were not sacrificed during these sample collection periods. Following the completion of the 15-day daily dosing regimen, animals were sacrificed, and the ileum tissue was dissected out to evaluate zileuton levels in the ileum tissue of the API, NfZ, and PM treatment groups.

2.6.2. Preparation of zileuton standards for HPLC analysis

Stock solutions of zileuton API were prepared in methanol at a concentration of 4 mg/mL. The control samples (plasma, urine and ileal tissue homogenate) were spiked with zileuton stock solution to obtain concentration ranges from 125 to 16,000 ng/mL and underwent identical extraction procedures with some modifications as described previously (Prakash et al., 2014). The calibration curves were constructed

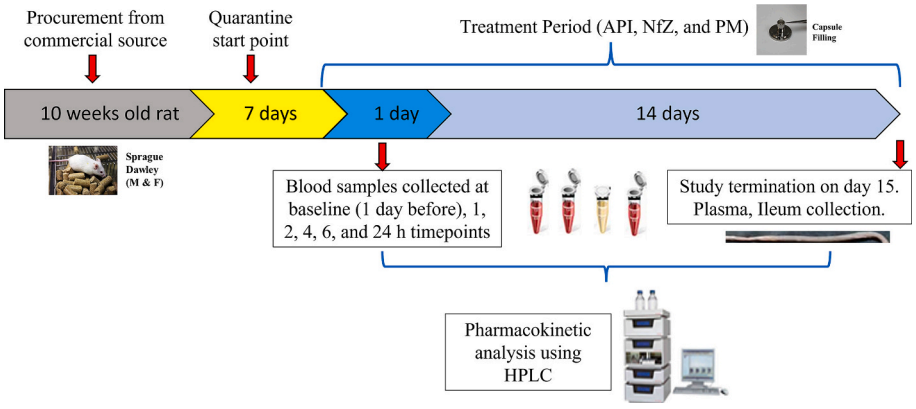

Fig. 1. Schematic representation of the zileuton's Pharmacokinetic study design in Sprague Dawley rats. Rats were treated with API, PM, and NfZ drug for 15 days. Blood samples for PK were collected at various time points (within 24 h) as indicated in the figure for PK studies.

for each biological matrix by plotting peak area of zileuton versus the spiked concentration of standards. Good linearity was observed across the concentration ranges (R2 = 0.9995 for plasma standards, R2 = 0.9995 for urine standards, and R2 = 0.9979 for ileal tissue homogenate standards). Percent recovery of zileuton in the biological matrices were observed to be 91.88% for plasma, 94.78% for urine, and 95.45% for ileum. Based on the recovery analysis the data was readjusted and plotted the graphs. Plasma samples of female rats treated with API was diluted to two-fold to fall within the standard curve. While plotting the graph dilation factor was included in the calculation.

2.6.3. Extraction of zileuton from rat biological matrices

Zileuton was extracted from plasma and urine using the method described by Prakash et al. (Prakash et al., 2014). Intestinal ileal tissue was homogenized as described by Gokulan et al., with some modifications (Gokulan et al., 2018) as described below.

Extraction of zileuton from plasma

Plasma samples were mixed with methyl tert-butyl ether (MTBE) at a 1:5 ratio (by volume) and vortexed for 10 mins at setting 4 (Daigger Vortex Genie 2, Hanover Park, IL). The samples were centrifuged at 3000 rpm for 10 mins (Eppendorf Centrifuge 5425, Hanover Park, IL). After centrifugation, the supernatant MTBE layer from the samples were removed and evaporated in vacufuge at 45 °C. The obtained residue was reconstituted with 300 µL of mobile phase, filtered through 0.22 µm syringe filters into HPLC vials for analysis. The concentration of Zileuton was expressed as ng/mL of plasma. All concentration values of the assay above LOD (125 ng/mL) were reported in this study.

2.6.3.1. Extraction of zileuton from urine. Urine samples were mixed with equal volumes of MTBE. The samples were vortexed for 10 mins at setting 4 (Daigger Vortex Genie 2, Hanover Park, IL) to allow extraction of zileuton into the organic layer. The MTBE layer containing extracted zileuton was collected and dried under vacuum at 45 °C for 30–40 min to evaporate any residual solvent. The obtained residue was reconstituted with 300 µL of mobile phase, filtered through 0.22 µm syringe filters into HPLC vials for analysis.

2.6.3.2. Extraction of zileuton from ileum tissue. Ileum tissue was minced using hand scalpel by adding cell lysis buffer and transferred to M-tube (at a concentration of 100 mg tissue/1 mL of lysis buffer) (Miltenyi Biotec Inc., Auburn, CA). Tissue homogenization was carried out using the gentleMACS dissociator (Miltenyi Biotec Inc., Auburn, CA) at setting: Protein_0.1.01. The homogenate was centrifuged at 750 rpm at 4 °C for 10 min. The supernatant was collected and further centrifuged at 15,000 rpm at 4 °C for 15 min. The final supernatant was collected, dried overnight under vacuum at 45 °C and resuspended in 1 mL MTBE. After centrifugation at 7000 rpm for 2 min, the MTBE layer was collected and dried under vacuum at 45 °C. The obtained residue was reconstituted with 300 µL of mobile phase, filtered through 0.22 µm syringe filter into HPLC vials for analysis.

2.7. Data analysis

Pharmacokinetic analysis of the plasma concentration-time profiles was performed using non-compartmental modeling in PKSolver 2.0, an add-in program for Microsoft Excel (Zhang et al., 2010). The linear trapezoidal method was applied for the calculations after extravascular administration of zileuton. Time to reach maximum plasma concentration (T_{max}) and maximum observed plasma concentration (C_{max}) were obtained directly from the observed data. Additional pharmacokinetic parameters calculated included: The terminal elimination rate constant (Lambda_z), ($t_{1/2}$), area under the plasma concentration-time curve from time zero to last observed concentration ($AUC_{0\text{-}t}$), area under the moment curve from time zero to infinity ($AUMC_{(0\text{-}inf\_obs)}$), mean residence time extrapolated to infinity ($MRT_{0\text{-}inf\_obs}$), apparent volume of distribution during the terminal phase after extravascular administration (Vz/F_obs), apparent plasma clearance (Cl/F_obs), and several others as estimated by the software algorithms. A time delay/lag time for absorption was set to 0 h for the extravascular routes of administration. Interpretation of pharmacokinetic data was based entirely on the computational outputs from PKSolver.

For statistical comparison of pharmacokinetic parameters between male and female treatment groups, an unpaired two-sample *t*-test was performed at a 5% significance level ($P \leq 0.05$). The t-test was used to assess whether significant differences existed between the means of the male and female data sets based on calculating the variance across all animals in each group ($n = 3$ males and n = 3 females for each formulation). *P*-values below the 0.05 threshold were considered statistically significant.

3. Results

3.1. Characterization of zileuton particles using SEM analysis

The SEM images of API, PM, and NfZ provided distinct differences in their surface morphological characteristics. The API crystals exhibited rough surfaces with irregular edges with varying particle sizes (Fig. 2a). The PM showed a heterogeneous mixture of API and excipients with varying sizes and shapes (Fig. 2b). In contrast, the NfZ displayed uniform and spherical-shaped crystals with smooth surfaces (Fig. 2c & d). Overall, NfZ particles have uniform surface morphology compared to the API crystals. The heterogeneous surface morphology of PM indicates that the two components (API and excipients) did not completely integrate into a single phase. These findings have important implications in drug development, as the uniform particle size and surface morphology of nanosized drugs may enhance their bioavailability.

3.2. Characterization of zileuton particles using DLS

DLS is best suited for analyzing sub-micron particles in the nanometer size range. API and PM particles exhibit very slow Brownian motion which is difficult to accurately measure by light scattering. Additionally, due to gravity, micron-sized particles settle rapidly. This impacts measurement accuracy as DLS requires the particles to exhibit free Brownian motion without disturbance over the measurement time. Hence, we analyzed the size of nanocrystal formulation but not the API and physical mixture.

The particle size distribution of the zileuton NfZ formulation was analyzed via DLS. The particle size of the NfZ formulation was found to be 571.1 nm. The size distribution of the particles was also examined by calculating the percentage polydispersity index (PDI). The NfZ had a PDI of 21.3%, indicating a narrower size distribution. The zeta potential of the NfZ was observed to be −14.5 mV, indicating a stable colloidal system. These results demonstrate that NfZ has good physicochemical characteristics for effective drug delivery. The smaller particle size and narrow size distribution suggest good uniformity and homogeneity. Hence, the stable zeta potential and a narrower size distribution suggests that NfZ will have a reduced tendency to aggregate.

3.3. Analysis of zileuton levels in plasma

Plasma concentration-time profiles of zileuton were determined at 0, 1, 2, 4, 6, and 24 h in male and female Sprague Dawley rats following oral administration of API, NfZ, and PM, as shown in Fig. 3. The corresponding PK parameters were calculated using PKSolver are presented in Table 1.

Plasma concentration time profiles showed higher levels of zileuton in female rats compared to male rats following oral administration of API, PM and NfZ ($P < 0.05$). By 24 h, plasma levels were undetectable in both genders for all administered forms of zileuton. Time to reach maximal plasma concentration (T_{max}) differed between male and female

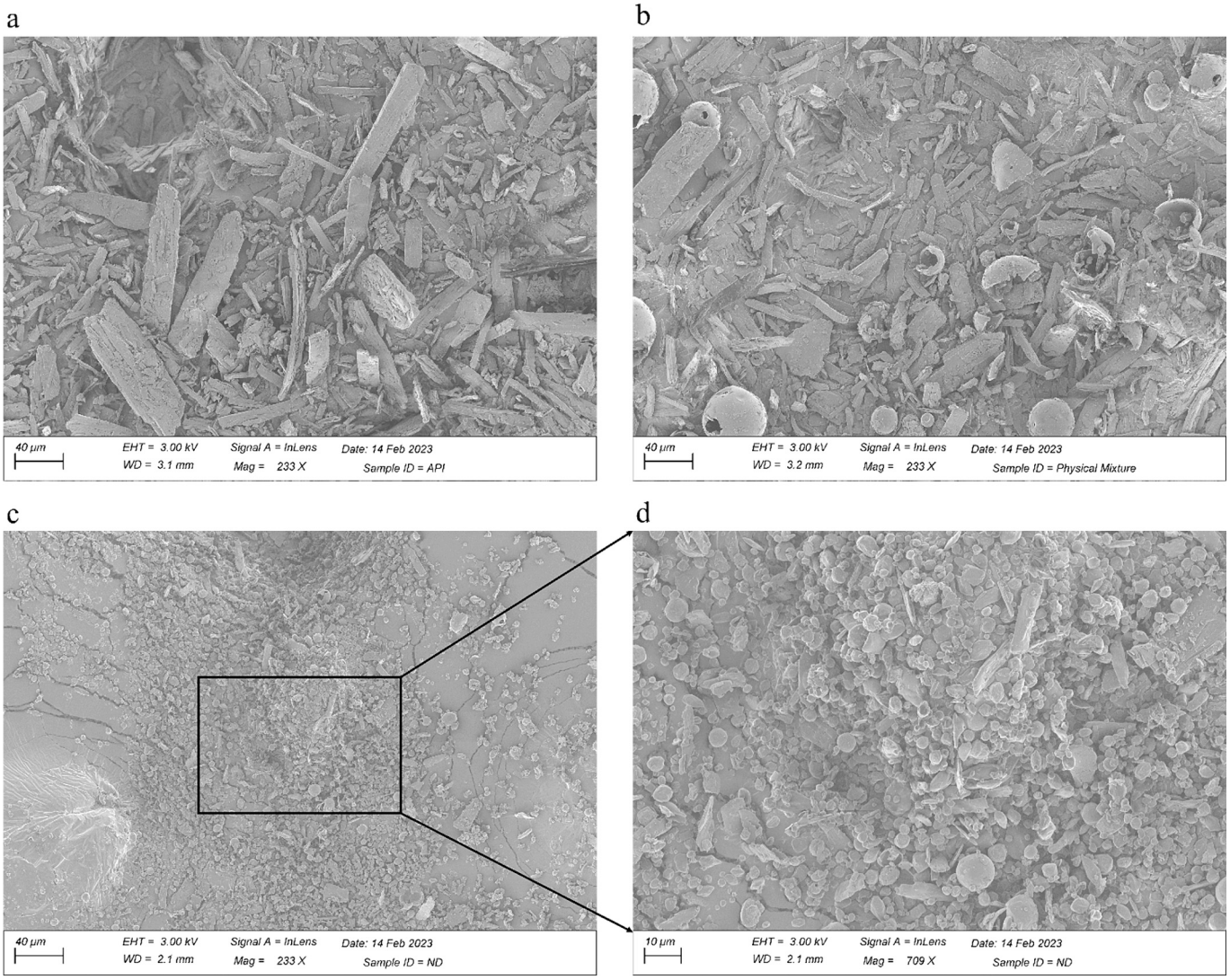

Fig. 2. SEM images of zileuton's API (a), PM (b), and NfZ (c & d).

rats depending on zileuton formulation administered. For the groups treated with API, T_{max} was attained quicker in males (at 1 h) versus females (at 2 h). In contrast, for the NfZ and PM groups, female rats achieved maximal levels sooner (at 1 h) than males (at 2 h). Peak concentration (C_{max}) was observed to be 1.5–3 times higher in female rats compared to male rats following oral administration of API, PM, and NfZ.

Zileuton levels in female rats were evidenced by 2–4 fold greater area under the plasma concentration-time curve from time 0 to infinity ($AUC_{0\text{-}inf}$) metrics ($P < 0.05$ for API, NfZ, and PM). Shorter terminal elimination half-life ($t_{1/2}$) values were observed in male rats compared to female rats for the forms of zileuton (API, and NfZ) tested ($P < 0.05$). Larger apparent volume of distribution during the terminal elimination phase (Vz/F) ($P < 0.05$) and faster apparent total clearance of drug from plasma (CL/F) ($P < 0.05$) was observed in male compared to female rats across the API, PM and NfZ formulations tested.

3.4. Analysis of zileuton levels in Ileum

In this study we have evaluated the concentrations of zileuton in the ileal tissue extract of male and female Sprague Dawley rats following 15 days of daily treatment with API, NfZ, and PM. Animals were sacrificed after 15th day treatment and ileum tissue was collected to evaluate zileuton levels. Our findings demonstrate that male rats had higher zileuton concentrations ($P < 0.05$) in the ileum compared to female rats following oral administration of API, whereas female rats showed higher zileuton concentrations ($P < 0.05$) in the ileum following oral administration of PM. No significant differences were observed in zileuton levels in the ileum between male and female rats following oral administration of NfZ (Fig. 4). However, it is important to consider that the difference in zileuton levels between male and female rats administered with NfZ is relatively small.

3.5. Analysis of zileuton levels in urine

The levels of zileuton in the urine (24 h samples) of male and female Sprague Dawley rats were measured following oral administration of API, NfZ, and PM. No significant differences based on gender were detected within any treatment groups at the $p > 0.05$ level. Both male and female subjects displayed comparable urinary zileuton levels across all groups tested including API, NfZ and PM (Fig. 5). Overall, this analysis indicates total urinary elimination was not impacted by gender after oral administration of zileuton in the selected preclinical rat models.

4. Discussion

This pharmacokinetic study in rats provides critical insights into

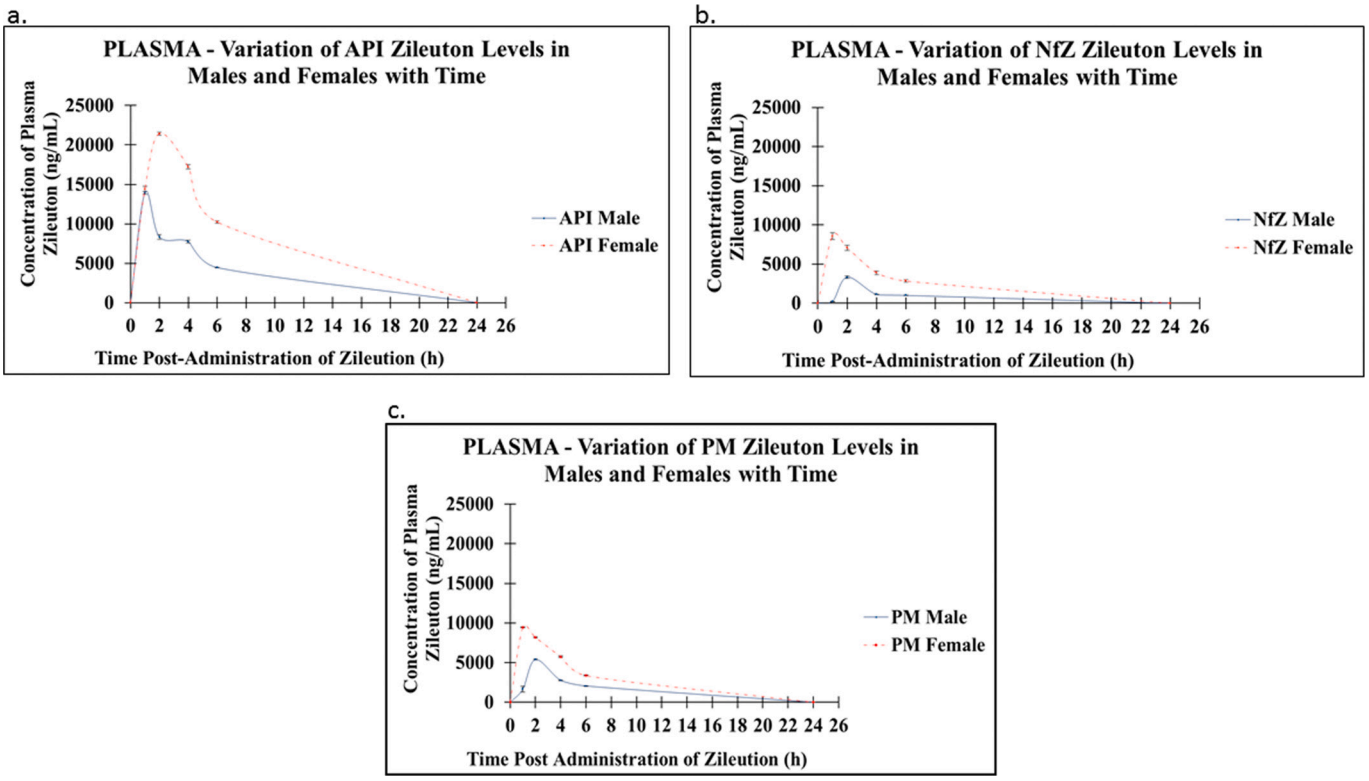

Fig. 3. (a-d): Mean plasma concentrations (ng/mL) versus time (h) curves (females represented in dotted lines and males represented in solid lines) for zileuton following oral administration of API, NfZ, and PM at a dose of 30 mg/kg bw (n = 3). d) Standard curve was generated using biological matrices (control rat plasma samples).

Table 1
Pharmacokinetic parameters for zileuton following oral administration of API (30 mg/kg bw), NfZ (30 mg contains 7.2 mg of API), and PM (30 mg contains 7.2 mg of API) at a dose of 30 mg/kg bw (n = 3).

		API		NfZ		PM	
Parameter	Unit	Male	Female	Male	Female	Male	Female
Lambda_z	1/h	0.2 ± 0	0.19 ± 0	0.3 ± 0	0.23 ± 0	0.24 ± 0	0.21 ± 0.01
$t_{1/2}$	h	3.49 ± 0.02	3.75 ± 0.05	2.32 ± 0.01	3.01 ± 0	2.89 ± 0.03	3.3 ± 0.17
T_{max}	h	1 ± 0	2 ± 0	2 ± 0	1 ± 0	2 ± 0	1 ± 0
C_{max}	ng/mL	13,918.21 ± 175.72	21,420.25 ± 189.18	3259.44 ± 161.54	8594.17 ± 425.59	5375.46 ± 27.2	9441.49 ± 66.79
T_{lag}	h	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
C_{last_obs}/C_{max}		0.32 ± 0.01	0.48 ± 0.01	0.3 ± 0	0.33 ± 0	0.38 ± 0	0.36 ± 0
$AUC_{0\text{-}t}$	ng/mL*h	46,416.87 ± 810.28	91,245.94 ± 807.18	8327.3 ± 400.3	29,826.36 ± 1468.04	17,330.4 ± 403.72	36,605.84 ± 353.84
$AUC_{0\text{-}inf\_obs}$	ng/mL*h	68,854.33 ± 907.34	146,487.43 ± 2081.77	11,621.45 ± 545.76	42,164.38 ± 2054.66	25,917.11 ± 441.46	52,663.07 ± 1400.15
$AUC_{0\text{-}t/0\text{-}inf\_obs}$		0.67 ± 0	0.62 ± 0	0.72 ± 0	0.71 ± 0	0.67 ± 0.01	0.7 ± 0.01
$AUMC_{0\text{-}inf\_obs}$	ng/mL*h^2	375,331.86 ± 3971.54	907,975.7 ± 21,991.14	55,551.04 ± 2518.36	205,576.75 ± 9840.42	139,663.97 ± 2449.3	273,102.51 ± 16,298.54
$MRT_{0\text{-}inf\_obs}$	h	5.45 ± 0.04	6.2 ± 0.07	4.78 ± 0.01	4.88 ± 0.01	5.39 ± 0.09	5.18 ± 0.17
Vz/F_obs	(mg)/(ng/mL)	0.0022 ± 0	0.0011 ± 0	0.0021 ± 0.0001	0.0008 ± 0	0.0012 ± 0	0.0007 ± 0
Cl/F_obs	(mg)/(ng/mL)/h	0.0004 ± 0	0.0002 ± 0	0.0006 ± 0	0.0002 ± 0	0.0003 ± 0	0.0001 ± 0

potential gender differences in the plasma exposure profiles of an asthma drug following oral administration. Key finding was that female rats demonstrated higher plasma zileuton concentrations compared to males across all the three forms of zileuton tested (API, PM and NfZ). The observed differences in the plasma zileuton levels between male and female rats suggest that gender-specific factors may influence the pharmacokinetics of the drug. These factors could be related to differences in absorption, distribution, metabolism, and/or excretion process between males and females. However, further studies are required to determine the specific mechanisms contributing to the different oral exposure profiles observed in this study. These findings have important clinical implications for optimization of zileuton therapy based on the gender.

The observed gender differences in plasma zileuton concentrations between male and female rats could be influenced by the solubility and dissolution properties of the various formulations tested in this study. As reported by Khare et al. (Khare et al., 2023), the solubility and dissolution rate of zileuton can vary significantly depending on the formulation. The in vitro dissolution profile data showed that only 8% of the API dissolved in 24 h, while 95% of the spray- dried nanocrystal

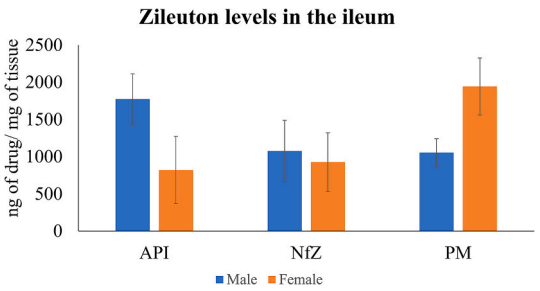

Fig. 4. A comparison of zileuton levels in the ileum following oral administration of API, NfZ, and PM in male and female Sprague Dawley rats at a dose of 30 mg/kg bw. Each bar represents the mean ± SD of three observations (n = 3).

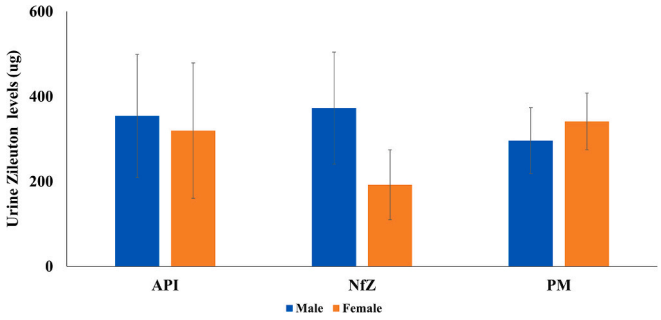

Fig. 5. A comparison of zileuton levels in the urine following oral administration of API, NfZ, and PM in male and female Sprague Dawley rats at 24 h post an oral dose of 30 mg/kg bw. Each bar represents the mean ± SD of three observations (n = 3).

formulation (NfZ) dissolved within 4 h, and the nanosuspension formulation dissolved 98% within 1.5 h. These findings suggest that the improved solubility and faster dissolution rate of the NfZ formulation could potentially lead to enhanced oral exposure compared to the API. While we did not directly evaluate the relative bioavailability of zileuton from the NfZ and PM formulations compared to the API, our plasma concentration data suggests that there may be differences in the oral exposure profiles among these formulations. Further studies evaluating the relative bioavailability of these formulations is required to better understand the relationship between improved solubility and the in vivo pharmacokinetic behavior of zileuton.

Gender differences were evidenced in both the peak concentration (C_{max}) and Area under the curve (AUC) metrics. Females demonstrated 1.5–3 fold and 2–4 fold higher C_{max} and AUC values respectively, versus males for the API, PM, and NfZ. This again indicates increased circulating levels of zileuton in female compared to male rats, after equivalent oral delivery. The fact that gender differences persisted across zileuton forms implies gender-specific factors, rather than solely formulation properties, drive the pharmacokinetic disparities. However, further studies are required to confirm mechanisms causing the gender skew towards heightened oral plasma zileuton levels uniquely in female rats.

Additionally, the time to reach maximal plasma concentrations (T_{max}) was found to be influenced by both gender and formulation properties. Our data confirms the existence of gender based differences in the absorption rate of zileuton. However, the relationship between these gender differences and the specific formulation properties remains unclear. Further investigations are required to elucidate the underlying physiological or physicochemical factors contributing to the observed differences in the absorption kinetics between male and female rats for each formulations tested (API, PM, and NfZ). Such studies would provide a better understanding of how gender and formulation properties interact to influence the absorption of zileuton.

There are several potential explanations for the observed gender divergences in zileuton pharmacokinetics. Firstly, sex hormones such as estrogen may alter gastric emptying, gut motility, and drug transporter expression in the intestine, impacting oral absorption (Matos et al., 2016; Chen et al., 1995; Hogan et al., 2009; Yang et al., 2017; Grimsrud et al., 2015). Females exhibit slower gastric emptying, which can prolong drug dissolution and increase the bioavailability of compounds like zileuton with solubility-limited absorption (Datz et al., 1987; Koch, 2010; Stillhart et al., 2020; Rangaraj et al., 2022). Moreover, expression of influx transporters like OATP1A2 is approximately double in women than men that facilitate uptake of drugs like zileuton across the gut epithelium (Kalliokoski and Niemi, 2009; Taniguchi et al., 2020; Bose et al., 2012; Franke et al., 2009; van de Steeg et al., 2013).

The substantially higher plasma concentrations of zileuton observed in female rats align with human studies showing increased bioavailability of various drugs tested in women (Ciccone and Holdcroft, 1999; Schwartz, 2003). Reduced first-pass metabolism in females (due to lower CYP expression and activity) likely contributes to this effect for zileuton and other drugs. Studies have demonstrated that males exhibit higher expression CYP enzymes, including CYP1A2, which metabolizes zileuton (Ou-Yang et al., 2000; Finnström et al., 2002; Khare et al., 2022). Therefore, males likely have enhanced first-pass metabolism and faster clearance of the drug leading to lower plasma levels.

Gender differences in body composition and physiology may also contribute to the differences in pharmacokinetics (Tamargo et al., 2017; Beierle et al., 1999). Females tend to have higher body fat percentages, altering drug distribution into tissues (Chen et al., 2007). Plasma protein binding may also factor into gender disparities in zileuton clearance and volume of distribution we observed, since only unbound drug is available for elimination and tissue uptake (Gandhi et al., 2004). Human studies have shown lipophilic drugs exhibit larger volumes of distribution in women (Rosano et al., 2015). Zileuton is a lipophilic drug, hence increased adiposity in females may enhance distribution and retention.

Several studies have shown pharmacokinetic evidence of reduced clearance in female rats (Li et al., 2021; Arora et al., 2021; Zhou et al., 2002; Czerniak, 2001). Gender differences in clearance mechanisms are complex and may depend on the formulation (Soldin et al., 2011). Lower female glomerular filtration rates would be expected to reduce renal elimination across all forms (Fenton et al., 2018). However, factors such as CYP-mediated metabolism, plasma protein binding, active tubular secretion, and reabsorption can also modulate renal excretion in a gender and formulation-specific manner (Palleria et al., 2013; Zhao et al., 2021; Smith and Waters, 2018; Hu et al., 2020). However, Analysis of urinary zileuton levels over 24 h did not show significant differences between male and female rats across the API, NfZ or PM treatment groups. Unlike common patterns of reduced renal clearance in human females, the rat models in this study showed consistent 24-h zileuton levels in urine between genders. While factors such as glomerular filtration, tubular reabsorption, protein binding, and enzymatic metabolism can modulate clinical urinary drug excretion in a gender-dependent manner, such trends were not significantly manifested in this rat study. Given the lack of statistical divergence in renal clearance outcomes between male and female subjects across groups, gender does not appear to markedly impact urinary levels of zileuton in the rat model.

Interestingly, we observed gender based and formulation based differences in zileuton concentrations in the ileal tissue. This suggests gender as well as formulation factors may impact zileuton accumulation at the site of absorption in the small intestine. Overall, the data implies that gender and formulations factors may influence zileuton uptake and retention in the ileum. Further studies are needed to clarify these aspects.

Overall, our findings clearly demonstrate gender is a major determinant of zileuton pharmacokinetics that should be considered in dose optimization. Consistent with guidelines recommending evaluation of sex differences during drug development (Evaluation of Gender

Differences in Clinical Investigations, 1998), our study provides a model for preclinical characterization of gender effects on pharmacokinetics. Due to higher plasma levels at equivalent dosing, female asthma patients may have increased risks of zileuton side effects such as liver toxicity (Watkins et al., 2007). Our results suggest, dose reduction may be feasible in women while still maintaining efficacy, thus improving the benefit-risk balance. In contrast, male patients may require higher zileuton doses to achieve comparable therapeutic outcomes and avoid suboptimal treatment. Gender-based pharmacokinetic monitoring and personalized dosing are warranted to ensure safe and effective zileuton therapy in men and women.

Several limitations should be considered when interpreting the study findings. Firstly, rats exhibit physiological and metabolic differences compared to humans that may limit translatability of the results (Radermacher and Haouzi, 2013; Indorf et al., 2021). For instance, rats utilize CYP2C11 instead of human CYP2C9 to metabolize zileuton and other drugs which could cause species-specific pharmacokinetic variability (Wang et al., 2015; Wójcikowski et al., 2013). However, qualitative gender trends are likely conserved across species. Additionally, pure zileuton API was used rather than commercial tablets containing excipients. Excipient effects on GI dissolution, permeability, and bioavailability may further influence gender pharmacokinetic differences in patients (Mai et al., 2019). Nevertheless, evaluating the pharmacokinetics of the pure API is useful for isolating the inherent contributions of gender and identifying formulations for optimization.

Studies are ongoing to further investigate the mechanisms driving gender divergent zileuton pharmacokinetics. The pharmacokinetic modeling using compartmental and non-compartmental approaches could provide greater insight into absorption, distribution, metabolism, and elimination processes (Foster and Vicini, 2022). Additional research on tissue distribution would help characterize gender differences in drug uptake across organs (Foster and Vicini, 2022). Integrated analysis of pharmacokinetic and pharmacodynamic relationships are also warranted to fully understand efficacy and safety in males versus females.

5. Conclusions

This study clearly demonstrates substantial gender disparities in pharmacokinetics of zileuton that necessitate consideration in dose optimization to achieve equivalent efficacy and safety in male and female patients. These results highlight the fundamental importance of evaluating gender differences in pharmacokinetics during preclinical drug development to guide gender-personalized therapy. By elucidating gender divergences in drug disposition and leveraging them to inform dosing strategies, this research exemplifies the value of personalized, precision medicine approaches for optimal treatment across patient demographics.

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CRediT authorship contribution statement

Chandra Mohan Reddy Muthumula: Writing – original draft. **Sangeeta Khare:** Writing – review & editing, Conceptualization. **Rajan Jog:** Methodology, Formal analysis. **Bhagya Wickramaratne:** Methodology. **Angela Lee:** Methodology. **Sushanta Chakder:** Writing – review & editing. **Diane J. Burgess:** Writing – review & editing, Conceptualization. **Kuppan Gokulan:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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