Revefenacin Absorption, Metabolism, and Excretion in Healthy Subjects and Pharmacological Activity of Its Major Metabolite

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ABSTRACT
Revefenacin inhalation solution is an anticholinergic indicated for the maintenance treatment of patients with chronic obstructive pulmonary disease. Mass balance, pharmacokinetics, and metabolism of revefenacin were evaluated after intravenous and oral administration of [14C]-revefenacin in healthy subjects. Pharmacological activity of the major metabolite revefenacin was also assessed. Adult males (n = 9) received 20 μg intravenously of approximately 1 μCi [14C]-revefenacin and/or a single 200-μg oral solution of approximately 10 μCi [14C]-revefenacin. Mean recovery of radioactive material was 81.4% after intravenous administration (54.4% in feces; 27.1% in urine) and 92.7% after oral dosing (88.0% in feces, 4.7% in urine). Mean absolute bioavailability of oral revefenacin was low (2.8%). Intact revefenacin accounted for approximately 52.1% and 13.1% of the total radioactivity in plasma after intravenous and oral administration, respectively. Two main circulating metabolites were observed in plasma. After an intravenous dose, a hydrolysis product, THRX-195518 (M2), was observed that circulated in plasma at 14.3% of total radioactivity. After an oral dose, both THRX-195518 and THRX-697795 (M10, N-dealkylation and reduction of the parent compound) were observed at 12.5% of total circulating radioactivity. THRX-195518 was the major metabolite excreted in feces and comprised 18.8% and 9.4% of the administered intravenous and oral dose, respectively. The major metabolic pathway for revefenacin was hydrolysis to THRX-195518. In vitro pharmacological evaluation of THRX-195518 indicated that it had a 10-fold lower binding affinity for the M2 receptor relative to revefenacin. Receptor occupancy analysis suggested that THRX-195518 has minimal contribution to systemic pharmacology relative to revefenacin after inhaled administration.

SIGNIFICANCE STATEMENT
The major metabolic pathway for revefenacin was hydrolysis to the metabolite THRX-195518 (M2), and both revefenacin and THRX-195518 underwent hepatic-biliary and fecal elimination after oral or intravenous administration with negligible renal excretion. Pharmacological evaluation of THRX-195518 indicated that it had a 10-fold lower binding affinity for the M2 muscarinic receptor relative to revefenacin and that THRX-195518 has minimal contribution to systemic pharmacology after inhaled administration.

Introduction
Revefenacin (YUPELRI) is a novel once-daily, lung-selective, muscarinic antagonist developed and approved as a nebulized inhalation solution for the maintenance treatment of patients with chronic obstructive pulmonary disease (COPD). As a potent and selective antagonist of human M1–M3 muscarinic receptors with kinetic selectivity for the M3 over the M2 subtype (Hegde et al., 2018), revefenacin demonstrates bronchoselectivity with limited systemic effects after nebulized delivery. In phase 2 studies of patients with moderate to severe COPD, single or multiple doses of nebulized revefenacin, 22–700 μg once daily led to a rapid onset of effect and sustained bronchodilation for more than 24 hours with no clinically significant antimuscarinic adverse events (AEs), changes in laboratory variables, or prolongation of the QTc interval (Pudi et al., 2017; Quinn et al., 2018; Borin et al., 2020). In subsequent phase 3 studies, once-daily revefenacin, 88 and 175 μg (approved dose), was well tolerated and demonstrated clinically significant improvements from baseline in bronchodilation (measured using forced expiratory volume in 1 second) and in several respiratory health outcomes following up to 52 weeks of treatment (Donohue et al., 2019a,b; Ferguson et al., 2019).

In clinical pharmacokinetic (PK) studies evaluating single and repeated revefenacin dosing in patients with COPD, plasma concentrations of

ABBREVIATIONS: AE, adverse event; AMS, accelerator mass spectrometry; AUC, area under the curve; AUC(t-last), AUC-time curve up to time t, where t is the last time point with concentrations above the lower limit of quantitation; BCRP, breast cancer resistance protein; B.P, fraction of total radioactivity in whole blood relative to plasma; COPD, chronic obstructive pulmonary disease; FRO, fractional receptor occupancy; hCES, human carboxylesterase; [3H]NMS, [N-methyl-3H]scopolamine methyl chloride; HPLC, high-performance LC; LC, liquid chromatography; PK, pharmacokinetic; TEAE, treatment-emergent AE.
revefenacin and its major metabolite THRX-195518 (M2) were low after inhaled administration and declined rapidly in a biexponential manner, resulting in limited plasma accumulation after repeated administration (Pudi et al., 2017; Quinn et al., 2018). Studies in patients with COPD also suggested THRX-195518 was a major metabolite because plasma exposure to the metabolite was approximately 4-fold higher than that of revefenacin (Quinn et al., 2018). No significant renal excretion of either compound was observed (Quinn et al., 2018).

This absorption, metabolism, and excretion study was conducted to evaluate the absolute bioavailability, mass balance, PK, and metabolic fate of revefenacin after intravenous and oral administration of 14C-labeled revefenacin ([14C]-revefenacin) in healthy subjects. Conducting radiolabeled absorption, distribution, metabolism, and excretion studies via the intended clinical route of administration is challenging for respiratory products that are administered via nebulization owing to numerous factors, including the inability to accurately quantify the delivered dose. Therefore, an intravenous microtainer was used in the current study as a surrogate for the portion of the inhaled revefenacin that reaches the systemic circulation via the lungs. An oral arm was also included in the study as a surrogate to understand the absorption and metabolism of any revefenacin that may be swallowed after inhaled administration. In vitro pharmacological characterization of the major revefenacin metabolite was also conducted, and its contribution to the overall systemic pharmacology relative to revefenacin was assessed with a receptor occupancy modeling approach.

Materials and Methods

Materials. [N-methyl-1H]scopolamine methyl chloride ([1H]NMS) (specific activity 82 Ci mmol\(^{-1}\)) was obtained from GE Healthcare (Piscataway, NJ). Revefenacin, THRX-195518, THRX-600975, THRX-177860, THRX-193305, and THRX-697795 were prepared at Theravance Biopharma US, Inc. (South San Francisco, CA). [14C]-revefenacin was synthesized at Vitrax (Placentia, CA).

Study Design and Treatments. This was an open-label, fixed-sequence, two-period study (NCT02772159) in nine healthy, nonsmoking males aged 19–55 years. For eligibility, the subjects had to have a body mass index of 18–32 kg/m\(^2\), inclusive, and weigh at least 55 kg.

Subjects received one (n = 3 receiving intravenous only; n = 3 receiving oral only) or both (n = 3 receiving both intravenous and oral) of the following treatments in a fasted state with a single 20-mg oral solution of approximately 1.7 mg carbon) was added as a carbon carrier. Process standards (oxalic acid II and sodium benzoate) were placed in separate sample tubes containing prebaked copper oxide powder. All samples, standards, and controls were dried under vacuum. The glass sample tube containing the dried sample and copper oxide was placed into a larger quartz glass combustion tube with a drawn point, which was heat-sealed under vacuum and then heated at 900°C for 2 hours in a furnace. After combustion, the tube was allowed to cool to ambient temperature. CO\(_2\) was produced in the sealed tube by the oxidation of experiments and controls during the heating process, and then CO\(_2\) was cryogenically transferred and sealed into a glass tube containing Ti\(_2\)H\(_2\) and Zn, with cobalt powder as the catalyst. Samples were heated for 4 hours at 500°C and then for 6 hours at 550°C, reducing CO\(_2\) to solid carbon. Carbon was pressed into aluminum cathodes, which were placed in the ion source of a 5 MV tandem pelletron. The ion source created a beam of negative ions by directing a Cs\(^+\) ion beam onto the graphite in each cathode in turn and sputtering the surface. The resultant secondary beam that was extracted from the ion source contained 12C, 13C, and 14C ions. The 14C content of each sample was calculated based on the 14C:12C ratio and the total carbon content. Carbon content was measured using a CHN analyzer (2400 Series 2; Perkin Elmer), or generic values were used. When appropriate, predose samples from the same subject were graphitized and analyzed to determine background 14C:12C ratios.

Metabolite Profiling in Plasma, Urine, and Feces. Plasma, urine, and feces samples from the study period 1 and period 2 were assayed for total 14C content using AMS at Xceleron. For metabolite profiling, plasma samples were pooled across individual subjects and time points (0–72 hours) according to the Hamilton area under the curve (AUC) pooling approach (Hamilton et al., 1981). Urine samples were pooled individually for the 0–4, 4–8, 8–12, and 12–24 collection intervals and then for each 24-hour interval up to 168 hours and subsequently pooled across subjects. Fecal samples were pooled individually and then across subjects for the 0–216 hours interval. LC-UV (UV)AMS analyses were conducted using HPLC/UV with offline 14C detection by AMS at Xceleron. For the LC-UV/AMS analysis, cross-subjects pooled plasma 300 μl (period 1) and 500 μl (period 2) were each protein precipitated separately with three volumes of acetonitrile/methanol (1:1, v/v) containing 2 mM ammonium acetate, vortex mixed, supernatant separated, and evaporated to dryness. The residue was then reconstituted into 200 μl of ammonium acetate (50 mM) in water. A portion of the reconstitution solution was then injected on the HPLC column, and fractions were collected for subsequent AMS analysis.
For urine samples of each study period 1 and 2, a 1/500th proportion was taken from each collection period from the 0–4 hours collection interval to the 144–168 hours collection intervals. After each individual subject pool was prepared, a cross-subject pool was prepared by taking a 1/10th portion (period 1) and 1/5th (period 2) of each subject pool and combining them together for each period. A portion of the reconstitution solution was used for AMS fractionation.

For fecal samples of each subject from study period 1 and 2, a 1/200th proportion of the feces homogenate sample aliquot was taken from each period to make an individual subject pool for 0–216 hours. After each subject pool was prepared, a cross-subject pool was made using one-fifth portion of each subject pool. Cross-subjects pooled fecal homogenate 300 mg (period 1 and 2), each was extracted with three volumes of dimethylacetamide, vortex mixed and sonicated, and centrifuged, and then the supernatant was separated and diluted with ammonium acetate (50 mM) in water. A portion of the diluted solution was used for AMS fractionation.

Plasma, urine, and feces metabolite profiling samples were spiked with reference standards [revefenacin; THRX-600075 (carbamate hydrolysis-revefenacin-M1); THRX-195518 (amide hydrolysis-revefenacin); THRX-177860 (N-dealkylation and oxidation-revefenacin-M8); THRX-193305 (hydrolysis and N-dealkylation-revefenacin-M9); THRX-697795 (N-dealkylation and reduction-revefenacin-M10)] and then fractionated by HPLC. Metabolite peaks were separated on a Phenomenex, Phenyl-Hexyl Luna column (4.6 × 250 mm, 5 μm) at a temperature set at 50°C. Mobile phase A consisted of 50 mM ammonium acetate in water, pH 7, and mobile phase B consisted of acetonitrile:methanol (1:1, v/v). The flow rate was 1 ml/min, and peaks were separated using a stepwise gradient from 5% to 95% mobile phase B over 39 minutes (5%–30% B from 0 to 8 minutes, 30%–50% B from 8 to 9 minutes, 50%–60% B from 9 to 31 minutes, 60%–95% B from 31 to 35 minutes, held at 95% B for 5 minutes, 95%–5% B from 39 to 40 minutes held at 5% B for 5 minutes before column re-equilibration at 5% B). The eluent was collected as a series of fractions at 12-second intervals across each run. Fractions were analyzed either individually or pooled and then analyzed for 14C content by AMS as described earlier. UV chromatograms with the spiked reference standards were overlaid with the radio-chromatograms based on the AMS detection of fractions and fraction pools to confirm the identity of revefenacin and its metabolites in each matrix.

**Metabolite Identification.** Retention times of the metabolite peaks from plasma, urine, and fecal metabolite profiling samples were compared with the metabolites observed in a dog 14C absorption, distribution, metabolism, and excretion study (data on file). The structures of the metabolites were determined from the dog study using HPLC-UV–mass spectrometry.
Thermo Finnigan LTQ-Orbitrap (ThermoFisher Corporation) in a full scan mode (m/z 150–900) at a resolution setting of 30,000. Capillary temperature was set at 275°C, and the source potential was 5000 V. Other potentials were optimized based upon ionization and fragmentation of the parent compound. UV absorption spectra were obtained by an in-line photodiode array detector. Metabolite peaks were separated on a Phenomenex, Phenyl-Hexyl Luna column (4.6 × 250 mm, 5 μm) at a temperature set at 50°C. Mobile phase A consisted of 50 mM ammonium acetate in water, pH 7, and mobile phase B consisted of acetonitrile: methanol (1:1, v/v). The flow rate was 1 ml/min, and peaks were separated using a stepwise gradient from 5% to 95% mobile phase B over 39 minutes (5%–30% B from 0 to 8 minutes, 30%–50% B from 8 to 9 minutes, 50%–60% B from 9 to 31 minutes, 60%–95% B from 31 to 35 minutes, held at 95% B for 5 minutes, 95%–5% B from 39 to 40 minutes held at 5% B for 5 minutes before column re-equilibration at 5% B). Proposed structures were compared with available authentic standards THRX-609075, THRX-195518, THRX-177860, THRX-193305, and THRX-697795.

Pharmacokinetic Evaluations. Noncompartmental PK parameters were calculated from the total radioactivity of [14C]-revefenacin–derived concentration equivalent-time data in whole blood and plasma as well as plasma revefenacin and THRX-195518 concentration-time data (Phoenix WinNonLin Version 6.3; Certara USA, Inc., Princeton, NJ). Urinary excretion and renal clearance were estimated from individual total radioactivity concentration equivalents in urine or urine revefenacin and THRX-195518 concentrations and volumes using SAS, Version 9.3 (SAS, Institute Inc., Cary, NC). Fecal excretion was estimated from individual total radioactivity concentration equivalents in feces and fecal homogenate weights. Mass balance was calculated individually as the sum of the percent of total radioactivity recovered in urine and feces. The absolute bioavailability of revefenacin was assessed using plasma concentration data from subjects who completed both dosing periods.

Radioligand Binding Studies at hM1, hM2, hM3, hM4, and hM5. Studies were conducted using Chinese hamster ovary–K1 cell membrane fractions stably expressing human recombinant M1, M2, M3, M4, or M5 muscarinic acetylcholine receptors. Assays were conducted with 1 nM [3H]NMS in a 10-mM HEPES buffer containing 100 mM NaCl, 10 mM MgCl2, and 0.025% bovine serum albumin, pH 7.4, at 37°C. Nonspecific binding was defined in the presence of 10 μM atropine.

Revefenacin and THRX-195518 Receptor Occupancy. Receptor occupancy at the M3 receptor for revefenacin and THRX-195518 after nebulized inhalation dosing was calculated using a fractional receptor occupancy (FRO) model. The FRO for revefenacin and THRX-195518 was calculated using the unbound plasma concentration (in nanomolar) of revefenacin and THRX-195518 at each time point determined in a multiple-dose clinical trial at steady state (Quinn et al., 2018). FRO for revefenacin and THRX-195518 was calculated using the equations:

\[ \text{FRO} = \frac{\text{unbound revefenacin(m) \times (1 + \text{unbound THRX-195518/K}_{\text{unbound}}} \times \text{unbound THRX-195518(m))}}{\text{unbound THRX-195518(m) + (K}_{\text{unbound}} \times \text{unbound THRX-195518/K}_{\text{unbound}} \times \text{unbound THRX-195518(m))}} \]

Total combined M3 receptor occupancy for the contribution of revefenacin and THRX-195518 was then determined using the sum of the respective fractional receptor occupancies.

Safety Assessments. Safety was evaluated by physical examination, vital sign measurement, 12-lead electrocardiogram, clinical laboratory tests, treatment-emergent adverse events (TEAEs), and concomitant medication monitoring.

Results

Study Participants. A total of nine male subjects were enrolled in the study: Three received only the 20-μg intravenous infusion, three received only the 200-μg oral solution, and three received both treatments. All subjects completed dosing and the required assessments. Demographic and baseline characteristics were similar between treatments [mean age 31.8 years, range 22.0–46.0 years; n = 6 (66.7%) White, n = 3 (33.3%) Black or African American; mean body weight 80.0 kg ± 15.9 S.D.; mean body mass index 25.3 ± 4.08 (S.D.).]

Mass Balance and Excretion. A mean (S.D.) of 81.4 (6.0)% of drug-related radioactive material was recovered after intravenous administration of revefenacin, with 54.4 (3.8)% excreted in feces and 27.1...
(4.0)% excreted in urine. After oral administration, 92.7 (2.2)% of the total dose was recovered, with 88.0 (2.9)% excreted in feces and 4.7 (1.5)% excreted in urine. Figure 1, A and B show the mean cumulative percentage of radioactivity excreted in urine and feces and the combined total excretion over time.

**Total Radioactivity in Whole Blood and Plasma.** After intravenous and oral administration of [14C]-revefenacin, mean total radioactivity plots showed similar profiles for whole blood and plasma (Fig. 2, A and B). Intravenous administration led to a multiphasic, relatively parallel postpeak decline in mean total blood and plasma concentration equivalents up to 72 hours postdose. Total radioactivity remained measurable in the plasma until 312 hours after infusion; however, blood concentrations were unable to be reliably quantified at these later time points. Maximal blood and plasma concentrations were achieved after 30 minutes and 2 hours after oral dosing, respectively.

**Total Radioactivity, Revefenacin, and THRX-195518 Plasma Concentration-Time Profiles.** After intravenous administration, maximal mean plasma concentrations of total radioactivity and revefenacin were achieved 30 minutes after initiation of the infusion (Fig. 2C). There was a postpeak multiphasic, parallel decline in plasma concentrations up to 36 hours after infusion. After 48 hours, almost all radioactive revefenacin was undetectable. Plasma THRX-195518 was detectable in most subjects 45 minutes after starting the infusion and remained measurable in the plasma to 36 hours after infusion. After 48 hours, almost all radioactive radioactivity AUC0–t last (1.26 and 1.64 ng eq*h/ml), and Cmax (0.11 and 0.14 ng eq/ml) were similar in whole blood and plasma, respectively. Mean B:P AUC and Cmax ratios were approximately 0.9, indicating equal partitioning between red blood cells and plasma.

**Pharmacokinetics of Revefenacin and THRX-195518 in Plasma.** A summary of PK parameters of Revefenacin and THRX-195518 is shown in Supplemental Table 1. After intravenous administration, AUC and Cmax ratios of revefenacin to total radioactivity in plasma (mean [S.D.]: 0.56 [0.26] and 0.73 [0.35], respectively) showed that the majority of radioactivity was associated with intact revefenacin (Fig. 2C). By contrast, ratios of 0.16 [0.03] and 0.20 [0.04], respectively, after oral administration, indicated that total radioactivity was primarily associated with revefenacin metabolites rather than the parent revefenacin (Fig. 2D). Mean [S.D.] absolute bioavailability of oral revefenacin was low (2.8 [1.2%]) based on within-subject comparison (n = 3).

Plasma concentrations of THRX-195518 were generally very low (<0.02 ng/ml), especially after oral administration of revefenacin, when PK parameters could not be determined (Fig. 2C). After intravenous administration, mean [S.D.] AUC0–last and Cmax, THRX-195518 to revefenacin ratios were 0.08 [0.06] and 0.03 [0.01], respectively.

**Metabolite Profiling in Plasma.** Unchanged revefenacin was identified as the primary species circulating in human plasma after intravenous and oral administration. The percentages of unchanged revefenacin and revefenacin metabolites identified in pooled plasma after intravenous and oral administration are shown in Table 2. The radio-chromatogram based on AMS detection and overlaid with the UV chromatogram containing known revefenacin and metabolite reference

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intravenous Dose</th>
<th>Oral Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0–last (ng eq h/ml)</td>
<td>1.32 (0.79) 1.09 (0.69, 2.86)</td>
<td>1.22 (0.72) 1.26 (0.24, 2.18)</td>
</tr>
<tr>
<td>AUCint (ng eq h/ml)</td>
<td>1.11 (0.28) 1.18 (0.77, 1.48)</td>
<td>1.14 (0.66) 1.01 (0.30, 1.81)</td>
</tr>
<tr>
<td>Cmax (ng eq/ml)</td>
<td>1.50 (2.13) 0.70 (0.50, 5.84)</td>
<td>0.11 (0.06) 0.110 (0.03, 0.17)</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.45 (0.11) 0.52 (0.25, 0.52)</td>
<td>2.84 (1.16) 2.52 (2.00, 5.00)</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>25.29 (8.33) 25.82 (12.63, 35.40)</td>
<td>25.59 (20.31) 15.31 (10.34, 60.15)</td>
</tr>
<tr>
<td>B:P AUC ratio</td>
<td>— — — — —</td>
<td>0.85 (0.36) 0.95 (0.12, 1.09)</td>
</tr>
<tr>
<td>B:P Cmax ratio</td>
<td>— — — — —</td>
<td>0.65 (0.29) 0.73 (0.07, 0.83)</td>
</tr>
</tbody>
</table>

AUC0–last: AUC-time curve from time zero to infinity; max, maximum; min, minimum; ng eq, ng equivalent revefenacin; Tmax, time to attain Cmax.
TABLE 2
Percentage of revefenacin and its metabolites identified in pooled plasma after a single intravenous dose of 20 μg [14C]-revefenacin (n = 6) and an oral dose of 200 μg [14C]-revefenacin (n = 6)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intravenous (%)</th>
<th>Oral (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revefenacin</td>
<td>52.1</td>
<td>13.1</td>
</tr>
<tr>
<td>THRX-195518 (M2)</td>
<td>14.3</td>
<td>12.5</td>
</tr>
<tr>
<td>THRX-697795 (M10)</td>
<td>4.79</td>
<td>12.5</td>
</tr>
<tr>
<td>THRX-193305 (M9)</td>
<td>2.81</td>
<td>2.93</td>
</tr>
<tr>
<td>THRX-609075 (M1)</td>
<td>1.66</td>
<td>3.63</td>
</tr>
<tr>
<td>THRX-177860 (M8)</td>
<td>1.58</td>
<td>3.69</td>
</tr>
</tbody>
</table>

TABLE 3
Percentage of revefenacin and its metabolites identified in pooled feces and urine after a single intravenous dose of 20 μg [14C]-revefenacin (n = 6) and an oral dose of 200 μg [14C]-revefenacin (n = 6)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Feces (%)</th>
<th>Urine (%)</th>
<th>Administered Dose (%)</th>
<th>Oral (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revefenacin</td>
<td>15.4</td>
<td>11.8</td>
<td>66.8</td>
<td>0.32</td>
</tr>
<tr>
<td>THRX-195518 (M2)</td>
<td>18.8</td>
<td>2.19</td>
<td>9.38</td>
<td>0.18</td>
</tr>
<tr>
<td>THRX-697795 (M10)</td>
<td>1.03</td>
<td>&lt;1</td>
<td>1.36</td>
<td>0.98</td>
</tr>
<tr>
<td>THRX-193305 (M9)</td>
<td>&lt;0.10</td>
<td>&lt;1</td>
<td>0.38</td>
<td>0.69</td>
</tr>
<tr>
<td>THRX-609075 (M1)</td>
<td>0.98</td>
<td>1.76</td>
<td>0.31</td>
<td>0.19</td>
</tr>
<tr>
<td>THRX-177860 (M8)</td>
<td>&lt;0.10</td>
<td>2.84</td>
<td>&lt;0.10</td>
<td>0.73</td>
</tr>
</tbody>
</table>
The relatively lower recovery of total radioactivity after administration of microtracer doses has been previously observed for other inhaled therapeutics and may reflect slow tissue redistribution, longer elimination, and/or logistical challenges with complete recovery of the dose after microtracer administration and AMS analysis (Harrell et al., 2013, 2019).

The poor oral absorption of revefenacin demonstrated in this study may be partially attributed to the susceptibility of revefenacin to efflux by both P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210598Orig1s000ClinPharmR.pdf). Given the low inhaled dose of revefenacin (175 μg), it is unlikely that the portion of that dose that ends up being swallowed would saturate efflux transport in the intestine, and thus P-gp and BCRP may be an important mechanism limiting absorption of revefenacin. Expression of both P-gp and BCRP transporters has also been reported in human lung tissue and human lung-derived cells (Berg et al., 2014, 2018; Fallon et al., 2018). Although the relative abundance of these efflux transporters in the lung is believed to be lower than that observed in other tissues, including the intestine, their functional role with respect to lung disposition remains to be fully elucidated. Studies in rat and multidrug resistance 1a/1b knockout mouse isolated lung models have suggested that a prototypical P-gp substrate (rhodamine-123) exhibits greater lung absorption under conditions of transporter knockout relative to wild-type controls, suggesting that P-gp may play some role in lung disposition (Al-Jayyoussi et al., 2013). The relative impact of P-gp or BCRP on the lung disposition and efficacy of revefenacin remains to be investigated.

The excretion profile of revefenacin was consistent with elimination via the hepatic-biliary and fecal route. Revefenacin and the primary metabolite THRX-195518 were subject to low recovery in urine after intravenous and oral administration, suggesting that renal clearance of the drug is a minor elimination route. This finding is consistent with the excretion profile from prior PK studies of inhaled revefenacin in patients with moderate to severe COPD, in which no significant renal excretion was observed (Quinn et al., 2018).

Comparison of the whole blood and plasma total radioactivity concentration equivalents showed that [14C]-revefenacin–related radioactivity after intravenous or oral administration was predominantly associated with plasma during the early absorption phase and then gradually shifted toward red blood cells. The total radioactivity B:P AUC ratio after intravenous or oral administration averaged >0.9, suggesting that [14C]-revefenacin–related total radioactivity was partitioned almost equally between red blood cells and plasma.

After intravenous administration, most plasma radioactivity was associated with intact revefenacin with a smaller contribution from metabolites (plasma revefenacin to total radioactivity mean AUC and Cmax ratios of approximately 0.6 and 0.7, respectively). After oral administration, plasma revefenacin to plasma total radioactivity mean AUC and Cmax ratios were approximately 0.2, which is suggestive of extensive first-pass metabolism.

**TABLE 4**

Binding affinities of revefenacin and THRX-195518 at M₁, M₂, M₃, M₄, and M₅ mAChRs

<table>
<thead>
<tr>
<th></th>
<th>hM₁</th>
<th>hM₂</th>
<th>hM₃</th>
<th>hM₄</th>
<th>hM₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revefenacin, Kᵦ, nM ± S.D.</td>
<td>0.4 ± 0.03</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>6.7 ± 2.8</td>
</tr>
<tr>
<td>THRX-195518, Kᵦ, nM ± S/D.</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>17.0 ± 2</td>
</tr>
</tbody>
</table>

Kᵦ, inhibition binding constant; mAChR, muscarinic acetylcholine receptor.

*Hegde et al. (2018).*
Unchanged revefenacin accounted for 52% and 13% of total circulating radioactivity in plasma after intravenous and oral administration, respectively. The major metabolic pathway after intravenous and oral administration was hydrolysis of revefenacin to THRX-195518, although additional pathways, including N-dealkylation, generated five further metabolites. Two main circulating metabolites were observed in plasma: THRX-195518 accounted for 14.3% of the total radioactivity after intravenous administration, and THRX-195518 (hydrolysis) and THRX-697795 (N-dealkylation and reduction of revefenacin) each comprised 12.5% of total radioactivity after the oral dose. THRX-195518 exceeded the 10% threshold for circulating metabolites as outlined in the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and Food and Drug Administration Safety Testing for Drug Metabolites guidance documents after intravenous administration, which is used as a surrogate for the inhaled route of administration (Food and Drug Administration, 2010; https://www.fda.gov/media/72279/download). However, adequate animal exposure in the toxicology program was demonstrated for THRX-195518 during the course of development of revefenacin (data on file), and thus no additional studies were conducted. Revefenacin was excreted unchanged in pooled feces at 27.2% and 66.8% of the administered dose after intravenous and oral administration, respectively. THRX-195518 was the only metabolite excreted in feces at dose after intravenous and oral administration, respectively. THRX-195518 unchanged in pooled feces at 27.2% and 66.8% of the administered dose for either dosing route.

The hydrolysis of revefenacin to THRX-195518 represents the major metabolic pathway and raises questions with regard to the mechanism of hydrolysis and the potential for drug-drug interactions. A number of specific and nonspecific hydrolyses have been implicated in the hydrolysis of drugs in various organs, including liver and intestine, and in blood and plasma (Fukami and Yokoi, 2012). Human carboxylesterases (hCES1 and hCES2) have been relatively well studied and are known to catalyze the hydrolysis of amides to the corresponding carboxylic acid (Di, 2019). Revefenacin exhibits significant conversion to THRX-195518 in human hepatocytes (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210598Orig1s000PharmR.pdf), suggesting that hCES1 (high expression in human liver) may play a role in its conversion in the liver. Studies to evaluate the potential of hCES1 to catalyze formation of THRX-195518 have not been conducted to date. However, the general lack of reported clinically significant drug-drug interactions for substrates of hCES or other hydrolase enzymes (Di, 2019) and low contribution of oxidative CYP-based metabolism for revefenacin (<5% contribution of M8, M9, and M10 excreted after intravenous administration) indicate that the specific enzymes responsible for metabolism of revefenacin are unlikely to result in clinically significant drug-drug interactions.

The pharmacological properties of THRX-195518 showed moderate binding affinity for the five human muscarinic acetylcholine receptors. By comparison, revefenacin exhibited high affinity for all five muscarinic receptor subtypes (Hegde et al., 2018). Interestingly, the PK profile of THRX-195518 after nebulized administration in patients with COPD differs from that observed in healthy volunteers. Plasma exposure to THRX-195518 in patients with COPD is approximately 4-fold higher than that of revefenacin (Quinn et al., 2018). The higher exposure to THRX-195518 in patients with COPD raises the question of whether it may contribute to any systemic pharmacological effects despite its 10-fold lower potency at the M3 receptor. It should be noted that the prescribing information for revefenacin (YUPELRI, 2018) describes a range of potency for THRX-195518 that indicates it is 3-fold to 10-fold less potent than parent revefenacin. However, the potency for revefenacin was underestimated in a pilot study that suggested only a 3-fold difference in potency due to an inappropriately short incubation time (1 hour) compared with the longer incubation times (16 hours) used in the definitive studies (Hegde et al., 2018). A slowly dissociating ligand, such as revefenacin, requires longer incubation periods to achieve equilibrium with the target, and thus the 10-fold difference in potency between THRX-195518 and revefenacin at the M3 receptor remains the most robust assessment of relative potency and was used in the fractional occupancy analysis. Unbound clinical exposure of both revefenacin and THRX-195518 was used in the fractional receptor occupancy model to assess the likely contribution of each species to systemic antimuscarinic effects. This analysis demonstrated that the THRX-195518 M3 occupancy at the therapeutic dose of revefenacin (175 μg) was negligible and did not exceed 10% at any time point postdose. These results imply that any systemic antimuscarinic effects observed after dosing of revefenacin primarily result from exposure to revefenacin as opposed to the THRX-195518 active metabolite. Overall, the calculated levels of combined M3 receptor occupancy for revefenacin and THRX-195518 at the therapeutic dose level were low, transient, and not expected to result in clinically meaningful systemic antimuscarinic pharmacodynamic effects consistent with the reported AE profile for revefenacin.

Conclusions

Revefenacin had low absolute bioavailability (<3%) after oral administration. The major metabolic pathway was hydrolysis to THRX-195518, and both revefenacin and THRX-195518 underwent
intravenous or oral doses of revefenacin were generally well tolerated. Single intravenous or oral doses of revefenacin were generally well tolerated.

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Authorship Contributions
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Contributed new reagents or analytic tools: Bourdet, Hegde, Colson, Borin.
Performed data analysis: Bourdet, Hegde, Borin.
Wrote or contributed to the writing of the manuscript: Bourdet, Yeola, Hegde, Colson, Barnes, Borin.

References

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Supplementary Materials

Liquid chromatography with Tandem Mass Spectrometric (LC/MS/MS) Bioanalysis Materials and Methods

Human plasma samples were treated with dipotassium (K₂) ethylenediaminetetraacetic acid (EDTA) anticoagulant, and aliquots of urine samples were treated with 0.1% (v/v) TWEEN 20. Treated plasma and urine samples were stored at −70°C. Revefenacin and THRX-195518 were isolated from K₂ EDTA plasma samples (100 μL) and urine samples (20 μL) using a 96-well strong cation exchange solid phase extraction procedure. The extracts are evaporated to dryness, reconstituted, and aliquots of the extracts were analyzed by turbo ion spray LC/MS/MS in the positive ion mode. The assay was validated in human plasma with a calibration range of 0.500–250 pg/mL for revefenacin and 5.00–2500 pg/mL for THRX-195518. For urine, the calibration range was 0.1–100 ng/mL for both revefenacin and THRX-195518. The liquid chromatography/mass spectrometry system consisted of two LC-10AD pumps (Shimadzu Scientific Instruments, Inc.), an SCL-10A pump controller (Shimadzu Scientific Instruments, Inc.), a LEAP CTC PAL autosampler (Leap Technologies), an HALO C18 (50 x 2.1 mm, 2.7 μm, MAC-MOD) at 50°C with an in-line frit (0.5 μm x 2/3 mm, Thermo Scientific Keystone), and an API 6500 mass spectrometer (AB SCIEX) using Analyst (v 1.6.2) software for plasma and AB SCIEX API 5000 mass spectrometer using Analyst (v 1.4.2) for urine. Mobile phase solvent A was 1000:1 water/1 M ammonium bicarbonate adjusted to pH 9. Mobile phase solvent B was methanol. HPLC column for plasma was Mac-Mod Halo C18 column (2.1 x 50 mm; 2.7 μm particle size) with a 0.5 μm x 2/3 mm in-line frit, column temperature of 50°C. HPLC column for urine was Phenomenex Gemini C6 Phenyl, 5-μm particle size, 50 x 2.0 mm. The initial flow rate was 300 μL/min for both plasma and urine samples.
TABLE S1 Mean (SD) pharmacokinetic parameters for revefenacin in plasma following a single intravenous dose of 20 μg (≈1.0 μCi) [14C]-revefenacin and an oral dose of 200 μg (≈10.0 μCi) [14C]-revefenacin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intravenous (Mean (SD))</th>
<th>Oral (Mean (SD))</th>
<th>n</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-last} (ng*h/mL)</td>
<td>0.727 (0.339)</td>
<td>0.142 (0.0734)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>AUC_{inf} (ng*h/mL)</td>
<td>0.605 (0.173)</td>
<td>0.145 (0.0826)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>0.0280 (0.0118)</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>C_{max} (ng/mL)</td>
<td>0.944 (0.555)</td>
<td>0.0241 (0.0128)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>T_{max} (h)^a</td>
<td>0.45 (0.25, 0.82)</td>
<td>2.52 (0.58, 5.00)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>39.7 (10.6)</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
<td>-</td>
<td>2090 (1530)</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>V_{SS} (L)</td>
<td>218 (59.2)</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>V_{Z}/F (L)</td>
<td>-</td>
<td>12800 (7290)</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>11.16 (3.39)</td>
<td>5.04 (2.56)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>AUC ratio^b</td>
<td>0.564 (0.259)</td>
<td>1.161 (0.0251)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C_{max} ratio^c</td>
<td>0.729 (0.347)</td>
<td>0.199 (0.0398)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ae_{0-336} (μg)</td>
<td>1.39 (0.613)</td>
<td>0.231 (0.232)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CLr (L/h)</td>
<td>3.01 (1.79)</td>
<td>2.24 (0.839)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Fe (%)</td>
<td>6.17 (2.71)</td>
<td>0.110 (0.111)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

^aT_{max} is presented as median (minimum, maximum).

^bAUC ratio = AUC (plasma)/AUC (total radioactivity in plasma), where AUC was based on latest common T_{last}.

^cC_{max} ratio = C_{max} (plasma)/C_{max} (total radioactivity in plasma).

Ae_{0-336}, mean cumulative amount of unchanged revefenacin excreted in urine over 336 h after intravenous infusion; AUC, area under the curve; AUC_{0-last}, area under the plasma concentration-time curve up to time t, where t is the last point with concentrations above the lower limit of quantitation; AUC_{inf}, area under the plasma concentration-time curve from time zero to infinity; CL, apparent total radioactivity or plasma total clearance after intravenous administration (following [14C]-revefenacin intravenous dose only); CL/F, apparent total radioactivity or plasma clearance after extravascular administration (following [14C]-revefenacin oral dose only); CLr, renal clearance; C_{max}, peak plasma concentration; F, absolute bioavailability; Fe, feces; SD, standard deviation; t_{1/2}, terminal elimination.
half-life; $T_{\text{last}}$, time to $C_{\text{last}}$; $T_{\text{max}}$, time to attain $C_{\text{max}}$; $V_{\text{ss}}$, steady-state volume of distribution of total radioactivity or plasma (following $[^{14}\text{C}]$-revafenacin intravenous dose only); $V_{z}/F$, apparent volume of distribution of total radioactivity or plasma during the terminal elimination phase after extravascular administration (following $[^{14}\text{C}]$-revafenacin oral dose only).
**Supplementary Fig. S1.** UV chromatogram (242 nm) generated from a 75 µL injection of reconstituted extract with revefenacin, THRX-609075 (M1), THRX-195518 (M2), THRX-177860 (M8), and THRX-193305 (M9) at approximately 1 µg on column overlaid against a $^{14}$C radio-chromatogram based on the AMS detection of fractions and fraction pools generated from the injection of the processed 0-72 h AUC cross-subject Period 2 plasma pool.

AUC, area under the curve; AMS, accelerator mass spectrometry; min, minutes; REV, revefenacin; UV, ultraviolet.
**Supplementary Fig. S2.** UV chromatogram (242 nm) generated from a 50 µL injection of feces homogenate supernatant diluted 1:10 with Mobile Phase A and spiked with THRX-609075 (M1), THRX-195518 (M2), and revefenacin at approximately 1 µg on column overlaid against a ¹⁴C radio-chromatogram based on the AMS detection of fractions and fraction pools generated from the injection of the 0-216 h cross-subject Period 1 feces pool.

AUC, area under the curve; AMS, accelerator mass spectrometry; min, minutes; REV, revefenacin; UV, ultraviolet.
Supplementary Fig. S3. UV chromatogram (242 nm) generated from a 50 µL injection of
THRX-609075 (M1), THRX-195518 (M2), THRX-177860 (M8), THRX-193305 (M9), and revefenacin
at approximately 1 µg on column overlaid against a ^14C radio-chromatogram based on the AMS
detection of fractions and fraction pools generated from the injection of the 0-168 h cross-
subject Period 2 urine pool.

AUC, area under the curve; AMS, accelerator mass spectrometry; min, minutes; REV, revefenacin; UV, ultraviolet.
Supplementary Fig. S4. UV chromatogram (242 nm) generated from a 10 µL injection of THRX-697795 (M10) at approximately 1 µg on a column overlaid against a $^{14}$C radiochromatogram based on the AMS detection of fractions and fraction pools generated from the injection of the 0-72 h cross-subject Period 2 plasma pool.

AUC, area under the curve; AMS, accelerator mass spectrometry; min, minutes; REV, revefenacin; UV, ultraviolet.