



# Plasma and Intrapulmonary Concentrations of ETX2514 and Sulbactam following Intravenous Administration of ETX2514SUL to Healthy Adult Subjects

Keith A. Rodvold,<sup>a</sup> Mark H. Gotfried,<sup>a,b</sup> Robin D. Isaacs,<sup>c</sup> John P. O'Donnell,<sup>c</sup> Emily Stone<sup>c</sup>

<sup>a</sup>University of Illinois at Chicago, Chicago, Illinois, USA

<sup>b</sup>Pulmonary Associates, PA, Phoenix, Arizona, USA

<sup>c</sup>Entasis Therapeutics, Inc., Waltham, Massachusetts, USA

**ABSTRACT** ETX2514 is a novel  $\beta$ -lactamase inhibitor that broadly inhibits Ambler class A, C, and D  $\beta$ -lactamases. ETX2514 combined with sulbactam (SUL) *in vitro* restores sulbactam activity against *Acinetobacter baumannii*. ETX2514-sulbactam (ETX2514SUL) is under development for the treatment of *A. baumannii* infections. The objective of this study was to determine and compare plasma, epithelial lining fluid (ELF), and alveolar macrophage (AM) concentrations following intravenous (i.v.) ETX2514 and sulbactam. Plasma, ELF, and AM concentrations of ETX2514 and sulbactam were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in 30 healthy adult subjects following repeated dosing (ETX2514 [1 g] and sulbactam [1 g] every 6 h [q6h], as a 3-h i.v. infusion, for a total of 3 doses). A bronchoalveolar lavage (BAL) was performed once in each subject at either 1, 2.5, 3.25, 4, or 6 h after the start of the last infusion. Penetration ratios were calculated from area under the concentration-time curve from 0 to 6 h ( $AUC_{0-6}$ ) values for total plasma and ELF using mean and median concentrations at the BAL fluid sampling times. Respective ELF  $AUC_{0-6}$  values, based on mean and median concentrations, were 40.1 and 39.4 mg · h/liter for ETX2514 and 34.7 and 34.5 mg · h/liter for sulbactam. Respective penetration ratios of ELF to total/unbound plasma concentrations, based on mean and median  $AUC_{0-6}$  values, of ETX2514 were 0.37/0.41 and 0.36/0.40, whereas these same ratio values were 0.50/0.81 and 0.50/0.80 for sulbactam. ETX2514 and sulbactam concentrations in AM were measurable and fairly constant throughout the dosing interval (median values of 1.31 and 1.01 mg/liter, respectively). These data support further study of ETX2514SUL for the treatment of pneumonia caused by multidrug-resistant *A. baumannii*. (This study has been registered at ClinicalTrials.gov under identifier NCT03303924.)

**KEYWORDS** ETX2514, sulbactam, ETX2514SUL, pharmacokinetics, epithelial lining fluid, alveolar macrophages

**A** *Acinetobacter baumannii* is a Gram-negative pathogen that causes serious infections which are associated with high morbidity and mortality (1–3). Isolates of *A. baumannii* are associated with high rates of multidrug-resistance (e.g., ~63% in the United States), and many are considered extensively drug resistant (XDR) (3–6). The emerging prevalence of multidrug-resistant (MDR) and XDR *A. baumannii* limits the choice of antimicrobial agents to treat these infections (7, 8). The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have recognized *A. baumannii* as a significant global threat and placed it at the top of priority lists as an urgent unmet medical need requiring new antibiotic treatment options (9, 10).

Sulbactam is a  $\beta$ -lactam agent which is a  $\beta$ -lactamase inhibitor but also has intrinsic activity against *A. baumannii* (11, 12). Although sulbactam is available as a standalone product in a small number of countries (e.g., as Combactam in Germany), the vast

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Address correspondence to Keith A. Rodvold, [kar@uic.edu](mailto:kar@uic.edu).

**TABLE 1** Characteristics of 30 healthy adult subjects enrolled in the study<sup>a</sup>

Sampling time (h)	No. and sex of patients <sup>b</sup>	Age (yr)	Height (cm)	Weight (kg)	eCL <sub>CR</sub> <sup>c</sup> (ml/min)	Total cell count in BAL fluid (cells/mm <sup>3</sup> )	Macrophages (%)
All subjects	18 M, 12 F	42 ± 11	173 ± 9	79.7 ± 11.8	110 ± 22	145 ± 159	81 ± 10
1	3 M, 3 F	46 ± 12	168 ± 5	71.6 ± 7.0	93 ± 23	114 ± 61	82 ± 6
2.5	3 M, 3 F	45 ± 6	174 ± 8	82.2 ± 10.0	115 ± 15	137 ± 87	82 ± 17
3.25	5 M, 1 F	33 ± 10	176 ± 8	84.3 ± 11.8	111 ± 15	141 ± 57	86 ± 6
4	3 M, 3 F	46 ± 13	170 ± 12	76.6 ± 14.7	103 ± 17	267 ± 368 <sup>d</sup>	78 ± 10
6	4 M, 2 F	37 ± 12	176 ± 8	80.0 ± 12.6	122 ± 31	72 ± 54	75 ± 10

<sup>a</sup>Data are expressed as means ± SDs except for the data on sex.

<sup>b</sup>M, male; F, female.

<sup>c</sup>eCL<sub>CR</sub>, estimated creatinine clearance based on Cockcroft-Gault equation (31).

<sup>d</sup>One subject at this sampling time had an extremely high total cell count of 900 cells/mm<sup>3</sup>.

majority of human uses is in combination with  $\beta$ -lactams (e.g., ampicillin-sulbactam, Unasyn). Unfortunately, *A. baumannii* resistance to sulbactam, generally mediated by  $\beta$ -lactamases, is now widespread among resistant isolates; the expression of class D  $\beta$ -lactamase is nearly ubiquitous, but class A and/or extended-spectrum class C coexpression is also common (12–15). Therefore, the restoration of  $\beta$ -lactam activity against *A. baumannii* would require a  $\beta$ -lactamase inhibitor capable of broadly inhibiting class A, C, and D  $\beta$ -lactamases. Currently, there are no  $\beta$ -lactamase inhibitors available which provide broad coverage of class D  $\beta$ -lactamases (16, 17).

ETX2514 is a potent broad inhibitor of Ambler class A, C, and D  $\beta$ -lactamases. ETX2514 has no significant intrinsic activity against *A. baumannii* (13, 18, 19). The addition of ETX2514 to sulbactam *in vitro* restores the activity of sulbactam, such that the MIC<sub>90</sub> from a collection of globally diverse contemporary *A. baumannii* clinical isolates ( $n = 2,177$ ) decreases from >32 mg/liter in the absence of ETX2514 to 2 mg/liter in the presence of ETX2514 (held constant at 4 mg/liter) (20). The combination of ETX2514-sulbactam (ETX2514SUL) is being developed to treat infections caused by MDR *A. baumannii* infections. The U.S. Food and Drug Administration (FDA) has granted the qualified infectious disease product (QIDP) designation and fast track status to ETX2514SUL for the treatment of hospital-acquired and ventilator-acquired bacterial pneumonia and bloodstream infections due to *A. baumannii*.

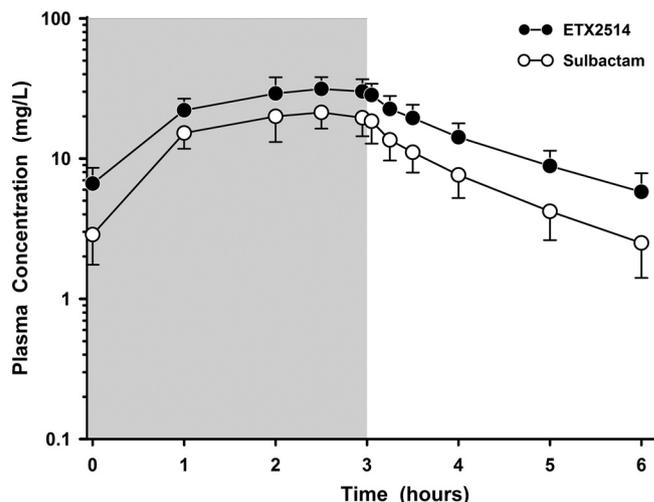
The objectives of this study were to (i) determine and compare plasma, epithelial lining fluid (ELF), and alveolar macrophage (AM) concentrations of ETX2514 and sulbactam after ETX2514 1 g was given concurrently with sulbactam 1 g every 6 h infused intravenously (i.v.) over 3 h for a total of three consecutive doses, (ii) characterize the plasma pharmacokinetics of ETX2514 and sulbactam before, during, and after the third i.v. infusion, and (iii) assess the safety and tolerability of concurrent administration of ETX2514 1.0 g and sulbactam 1.0 g for three consecutive i.v. doses in healthy adult subjects (ClinicalTrials registration no. NCT03303924).

(This work was presented in part at the American Society for Microbiology Microbe conference, Atlanta, GA, June 2018.)

## RESULTS

Forty adult male and female subjects signed informed consent forms and were screened. Ten subjects did not meet the inclusion criteria or had an exclusion criterion. Thirty subjects participated in the study, completed the required procedures, and were included in the safety and pharmacokinetic analysis. The demographic and laboratory characteristics of these subjects are listed in Table 1.

The concurrent administration of ETX2514 and sulbactam as a 3-h i.v. infusion was generally well tolerated, and no serious adverse events were reported during this study. Four subjects (13.3%) experienced a total of five treatment-emergent adverse events (TEAEs) over the course of the study. The observed TEAEs included constipation ( $n = 1$ ), i.v. infusion site pain ( $n = 2$ ), eye contusion ( $n = 1$ ), and hepatic enzyme elevation ( $n = 1$ ). All TEAEs were considered mild ( $n = 3$ ) or moderate ( $n = 2$ ) in severity. One



**FIG 1** Mean ( $\pm$  SD) plasma concentration versus time profiles of ETX2514 and sulbactam with the third dose of ETX2514SUL (1 g ETX2514, 1 g sulbactam) administered as a 3-h i.v. infusion every 6 h. Shaded region represents the 3-h infusion period. The y axis is log scale.

TEAE (infusion site pain) was considered related to the study drug administration. All TEAEs resolved during the study.

The plasma concentration-time profiles of ETX2514 and sulbactam before and following the third dose of ETX2514SUL are displayed in Fig. 1. The times of the observed maximum plasma concentration ( $T_{max}$ ) were similar for ETX2514 and sulbactam, with arithmetic means (ranges) of 2.62 (2.0 to 3.05) and 2.56 (2.0 to 3.05) h, respectively. The means and standard deviation (SDs) of the minimum plasma concentrations ( $C_{min}$ ) of ETX2514 at the 6-h sampling time after the first, second, and third infusions were  $5.71 \pm 1.77$ ,  $6.63 \pm 1.97$ , and  $5.79 \pm 2.08$  mg/liter, respectively. The means and SDs for the  $C_{min}$  values of sulbactam at the 6-h sampling time after the first, second, and third infusions were  $2.43 \pm 1.02$ ,  $2.87 \pm 1.12$ , and  $2.50 \pm 1.08$  mg/liter, respectively. The plasma pharmacokinetic parameters of ETX2514 and sulbactam associated with the third i.v. dose of ETX2514SUL are shown in Table 2.

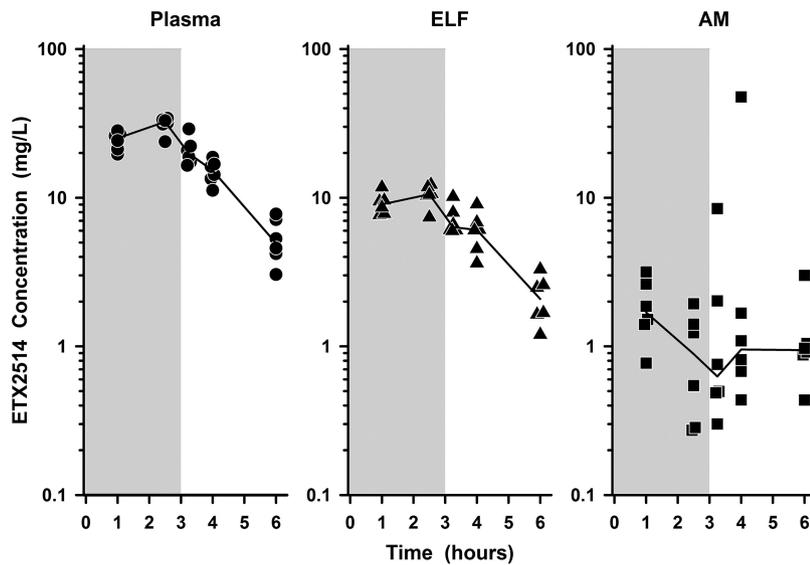
The individual concentrations of ETX2514 and sulbactam after the third dose in plasma (total), ELF, and AM at the five bronchopulmonary sampling times are displayed in Fig. 2 and 3, respectively. One subject displayed a higher AM concentration for ETX2514 (47.60 mg/liter) and sulbactam (29.65 mg/liter) at the 4-h sampling time, because the bronchoalveolar lavage (BAL) fluid was cloudy and the red cell count in BAL fluid was an extremely high value ( $11,025/mm^3$ ). These values were not included in the reporting of mean ( $\pm$  SD) concentrations of ETX2514 and sulbactam at the bronchopulmonary sampling times in Tables 3 and 4, respectively. Figure 4A and B illustrates the mean ( $\pm$  SD) concentration values of ETX2514 and sulbactam, respectively.

The mean ( $\pm$  SD) ratios of ELF or AM to the simultaneous unbound plasma concentrations of ETX2514 and sulbactam are reported in Tables 3 and 4. The unbound fractions of ETX2514 and sulbactam in plasma have been previously reported as 0.90 and 0.62, respectively (21 and data on file, Entasis Therapeutics, Inc., correspondence 5

**TABLE 2** Pharmacokinetic parameters of ETX2514 and sulbactam in plasma following the third i.v. dose of ETX2514SUL<sup>a</sup>

Drug	$C_{max}$ (mg/liter)	$C_{min}$ (mg/liter)	$AUC_{0-6}$ (mg · h/liter)	$t_{1/2}$ (h)	$V_{ss}$ (liters)	CL (liters/h)
ETX2514	$33.41 \pm 8.99$	$5.79 \pm 2.08$	$109.05 \pm 23.44$	$1.40 \pm 0.18$	$16.7 \pm 3.0$	$9.56 \pm 1.92$
Sulbactam	$23.10 \pm 7.61$	$2.50 \pm 1.09$	$67.89 \pm 16.69$	$1.12 \pm 0.14$	$20.7 \pm 4.3$	$15.56 \pm 3.63$

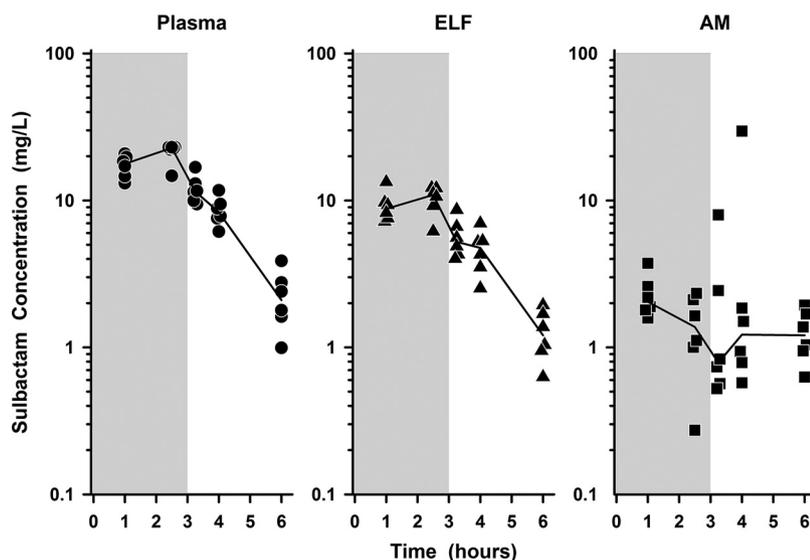
<sup>a</sup>Data (means  $\pm$  SDs) from 30 subjects per parameter estimate.



**FIG 2** Individual concentrations of ETX2514 in plasma, epithelial lining fluid (ELF), and alveolar macrophages (AM) at the BAL fluid sampling times after the third dose of ETX2514SUL (1 g ETX2514, 1 g sulbactam) administered as a 3-h i.v. infusion every 6 h. Lines represent the median concentrations. Shaded regions represent the 3-h infusion period. The y axes are log scale.

January 2018 [study report PC2514-2016-0024]). The individual ratios of ELF and AM to simultaneous unbound plasma concentrations for ETX2514 during the 6-h dosing interval ranged from 0.24 to 0.60 and 0.01 to 1.10, respectively. The individual ratios of ELF and AM to the simultaneous unbound plasma concentration for sulbactam were 0.43 to 1.69 and 0.01 to 4.52, respectively. In comparison, the same ratios of ELF and AM to the simultaneous total plasma concentration for ETX2514 ranged from 0.22 to 0.54 and 0.01 to 0.99, respectively. For sulbactam, ELF and AM ratios to simultaneous total plasma were from 0.27 to 1.05 and 0.01 to 2.80, respectively.

The values of area under the concentration-time curves from 0 to 6 h ( $AUC_{0-6}$ ) based on mean and median ELF concentrations of ETX2514 at the bronchopulmonary



**FIG 3** Individual concentrations of sulbactam in plasma, epithelial lining fluid (ELF), and alveolar macrophages (AM) at the BAL fluid sampling times after the third dose of ETX2514SUL (1 g ETX2514, 1 g sulbactam) administered as a 3-h i.v. infusion every 6 h. Lines represent the median concentrations. Shaded regions represents the 3-h infusion period. The y axes are log scale.

**TABLE 3** ETX2514 concentrations and ratios of ELF or AM to unbound plasma concentration at BAL fluid sampling times<sup>a</sup>

Sampling time (h) <sup>b</sup>	ETX2514 concentration (mg/liter) in:			Ratio of ETX2514 <sup>c</sup>	
	Plasma (total)	ELF	AM	ELF to plasma (unbound)	AM to plasma (unbound)
1	24.33 ± 3.35	9.14 ± 1.51	1.89 ± 0.87	0.42 ± 0.04	0.09 ± 0.04
2.5	31.23 ± 3.80	10.47 ± 1.70	0.95 ± 0.68	0.37 ± 0.02	0.04 ± 0.03
3.25	20.78 ± 4.54	7.14 ± 1.66	2.09 ± 3.18	0.38 ± 0.04	0.12 ± 0.21
4	15.10 ± 2.68	6.03 ± 1.88	0.94 ± 0.47 <sup>d</sup>	0.45 ± 0.11	0.07 ± 0.03 <sup>d</sup>
6	5.33 ± 1.80	2.15 ± 0.78	1.21 ± 0.91	0.46 ± 0.11	0.32 ± 0.38

<sup>a</sup>Data are expressed as means ± SDs.<sup>b</sup>Six subjects per sampling period.<sup>c</sup>The unbound fraction of ETX2514 in plasma was assumed to be 0.90.<sup>d</sup>Value reflects the mean ± SD from 5 samples.

sampling times were 40.1 and 39.4 mg · h/liter, respectively. The ratios of ELF to total plasma ETX2514 concentrations based on the mean and median AUC<sub>0-6</sub> values were 0.37 and 0.36, respectively. The ratios of ELF to unbound plasma ETX2514 concentrations based on the mean and median AUC<sub>0-6</sub> values were 0.41 and 0.40, respectively. The AUC<sub>0-6</sub> values based on mean and median AM concentrations of ETX2514 were not determined, since these AM concentrations were relatively low (approximately 1 to 2 mg/liter) and remained at a fairly constant value throughout the 6-h dosing interval (Fig. 2 and 4A).

The AUC<sub>0-6</sub> values based on mean and median ELF concentrations of sulbactam were 34.7 and 34.5 mg · h/liter, respectively. The ratio values of ELF to total plasma sulbactam concentrations based on the mean and median AUC<sub>0-6</sub> values were both 0.5. The ratios of ELF to unbound plasma sulbactam concentrations based on the mean and median AUC<sub>0-6</sub> values were 0.81 and 0.80, respectively. The AUC<sub>0-6</sub> values based on mean and median AM concentrations of sulbactam were not determined, since these AM concentrations were relatively low (approximately 1 to 2 mg/liter) and remained at a fairly constant value throughout the 6-h dosing interval (Fig. 3 and 4B).

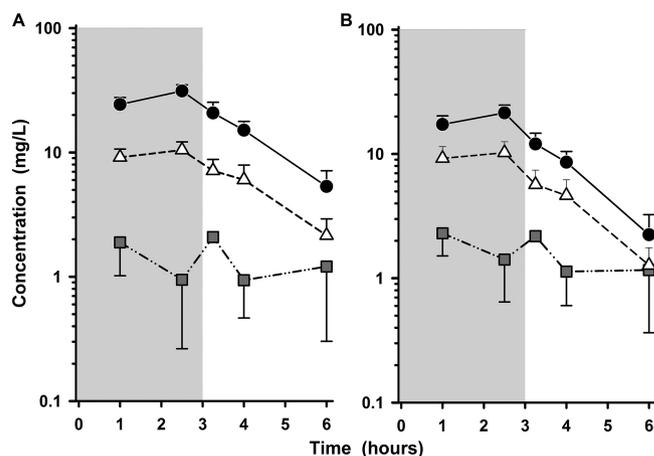
## DISCUSSION

The observed steady-state plasma concentration-time profiles and pharmacokinetic parameters of ETX2514 and sulbactam in our study (Table 2) were similar to those in previous pharmacokinetic studies as single agents and as ETX2514SUL in healthy adult subjects (22). Our mean penetration ratio of 55% for ELF to total plasma sulbactam concentrations at the 4-h sampling time was similar to the 61% (30-min after 30-min infusion of sulbactam 1 g) in eight patients with bacterial respiratory tract infections (23). Serum and ELF concentrations of sulbactam in patients were significantly higher and demonstrated greater variability compared to the values observed in our healthy subjects. Felton and colleagues showed a similar trend in serum and ELF concentrations of piperacillin in critically ill patients compared to those in healthy subjects (24).

**TABLE 4** Sulbactam concentrations and ratios of ELF or AM to unbound plasma concentration at BAL fluid sampling times<sup>a</sup>

Sampling time (h) <sup>b</sup>	Sulbactam concentration (mg/liter) in:			Ratio of sulbactam <sup>c</sup>	
	Plasma (total)	ELF	AM	ELF to plasma (unbound)	AM to plasma (unbound)
1	17.28 ± 2.97	9.22 ± 2.25	2.30 ± 0.79	0.81 ± 0.14	0.21 ± 0.09
2.5	21.42 ± 3.31	10.25 ± 2.29	1.41 ± 0.77	0.73 ± 0.16	0.11 ± 0.07
3.25	12.03 ± 2.66	5.67 ± 1.74	2.18 ± 2.94	0.72 ± 0.14	0.26 ± 0.34
4	8.58 ± 1.90	4.64 ± 1.56	1.31 ± 0.53 <sup>d</sup>	0.83 ± 0.25	0.21 ± 0.11 <sup>d</sup>
6	2.24 ± 1.01	1.27 ± 0.49	1.16 ± 0.80	0.94 ± 0.42	1.22 ± 1.63

<sup>a</sup>Data are expressed as means ± SDs.<sup>b</sup>Six subjects per sampling period.<sup>c</sup>The unbound fraction of sulbactam in plasma was assumed to be 0.62.<sup>d</sup>Value reflects the mean ± SD from 5 samples.



**FIG 4** Mean ( $\pm$  SD) plasma (black circles), epithelial lining fluid (ELF) (white triangles), and alveolar macrophages (AM) (gray squares) concentrations of ETX2514 (A) and sulbactam (B) at the BAL fluid sampling times after the third dose of ETX2514SUL (1 g ETX2514, 1 g sulbactam) administered as a 3-h i.v. infusion every 6 h. Shaded regions represent the 3-h infusion period. The y axes are log scale.

Intravenous infusion of ETX2514 given concurrently with i.v. sulbactam, administered every 6 h as a 3-h infusion for three consecutive doses to reach steady state, achieved similar time courses and patterns of concentrations for both agents in plasma, ELF, and AM. The magnitude of total plasma concentrations of each agent was higher than those observed in ELF. The intrapulmonary penetrations of ETX2514 and sulbactam, based on  $AUC_{0-6}$  values of mean ELF and total plasma concentrations at the BAL fluid sampling times, were 38% and 50%, respectively. If unbound plasma concentrations of ETX2514 and sulbactam are considered, these values were 41% and 81%, respectively. These results lend support to exploring the combination of ETX2514 and sulbactam (ETX2514SUL) as a potential antimicrobial agent for the treatment of lower respiratory tract bacterial infections caused by susceptible extracellular pathogens such as MDR *A. baumannii*.

The percent time that unbound plasma concentrations remained above the MIC ( $\%fT_{>MIC}$ ) during the dosing interval has been the pharmacokinetic-pharmacodynamic parameter that best correlates with antibacterial activity for sulbactam (25). Yokoyama and colleagues have reported the respective target values for  $\%fT_{>MIC}$  of sulbactam for a bacterial static effect and 1- $\log_{10}$  and 2- $\log_{10}$  reductions in the number of CFU of *A. baumannii* from baseline as 21.0%, 32.9%, and 43.6% in the thigh murine infection model and 20.4%, 24.5%, and 29.3% in the lung murine infection model (25). Although contemporary isolates of *A. baumannii* have lost susceptibility to sulbactam treatment alone, Barnes and colleagues recently demonstrated that combining ETX2514 (4 mg/liter) can effectively restore the susceptibility of sulbactam against highly drug-resistant *A. baumannii* isolates (26). The respective  $MIC_{50}$  and  $MIC_{90}$  values of 8 and 32 mg/liter for sulbactam were lowered to 1 and 2 mg/liter for the combination of ETX2514SUL. Time-dependent activity of the ETX2514SUL combination has recently been demonstrated against MDR *A. baumannii* in murine infection models where a  $>1\text{-}\log_{10}$  reduction in CFU from baseline was achieved when sulbactam concentrations were above the "potentiated" MIC (MIC of sulbactam in the presence of 4 mg/liter ETX2514) for 50% of the dosing interval (13). Combining the pharmacodynamics target values and *in vitro* MIC data with our observed plasma and ELF concentrations in healthy subjects provides support for the i.v. dosage regimen of ETX2514 1 g concurrently with sulbactam 1 g every 6 h, as a 3-h infusion, for the treatment of pneumonia caused by susceptible strains of MDR *A. baumannii*. On the basis of an initial population pharmacokinetic analysis of sulbactam plasma concentrations in targeted patients, unbound concentrations in the ELF are anticipated to exceed the proposed breakpoint of 4 mg/liter for 96.4%  $\pm$  6.7% (mean  $\pm$  SD) of the dosing interval using ETX2514 1 g with

sulbactam 1 g, administered every 6 h as a 3-h i.v. infusion (data on file, Entasis Therapeutics, Inc., correspondence 8 August 2018).

Concentrations of ETX2514 and sulbactam were detected in alveolar macrophages. The median concentrations tended to remain constant over the 6-h sampling period at between 1 and 2 mg/liter for both agents. Although  $\beta$ -lactam antibiotics are considered to have poor cellular uptake, measured AM concentrations have been observed in older single-dose studies of oral  $\beta$ -lactams cefuroxime and amoxicillin (27, 28). Potential explanations for our observed AM concentrations are that they may reflect the higher dose of ETX2514 and sulbactam (1 g), the administration of three doses, the lower quantification level of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay, and extracellular cross contamination. Previous studies have observed AM concentrations of the  $\beta$ -lactamase inhibitors clavulanate, vaborbactam (RPX7009), and relebactam (MK-7655) at similar magnitudes as ETX2514 in this study (28–30).

In summary, ETX2514SUL was generally safe and well tolerated at a dosing regimen of ETX2514 1 g and sulbactam 1 g administered every 6 h as a 3-h i.v. infusion for a total of three doses. The concentrations in plasma and ELF of each agent displayed similar time courses and patterns. The magnitude of ETX2514 concentrations in plasma and ELF was greater than for sulbactam throughout the 6-h dosing interval. Intrapulmonary concentrations of ETX2514 and sulbactam were also detected in AM, and median values remained fairly constant (approximately 1 to 2 mg/liter) throughout the 6-h dosing interval. The results from this study support further considerations of ETX2514SUL as a potential agent for the treatment of lower respiratory tract bacterial infections caused by MDR *A. baumannii*.

## MATERIALS AND METHODS

**Study design.** This was a phase 1, multiple-dose open-label pharmacokinetic study in healthy adult male and female subjects. The study was reviewed and approved by the Quorum Review Institutional Review Board in Seattle, WA. All participants provided written informed consent prior to enrollment. This study was conducted according to the U.S. Code of Federal Regulations, the ethical principles set forth in the Declaration of Helsinki, and the ICH guidelines for good clinical practice. Study enrollment and procedures were conducted at Pulmonary Associates PA (Phoenix, Arizona).

A screening evaluation was performed within 28 days of the initial dosing to determine the eligibility for enrollment in the study. If clinical laboratory tests (hematology, serum chemistry, and urinalysis) at screening were performed more than 3 days before confinement, these clinical laboratory tests were repeated during an additional screening visit (within 3 days prior to confinement) to ensure there were no changes in parameters. Subject eligibility was reviewed to ensure the subjects remained eligible for the study since the initial screening, and a review of concomitant medications and adverse events was conducted. Eligible subjects were admitted on day 1 and remained confined in the study center until the completion of all scheduled procedures, including the collection of blood at the final sampling time, the completion of bronchoscopy procedures, and safety evaluations after the third i.v. doses of ETX2514 and sulbactam. Subjects were discharged from the study center on day 2 and returned on days 4, 6 ( $\pm 1$  day), and 13 ( $\pm 2$  days) (e.g., at 2, 4, and 11 days after receiving the last dose of the study drug, respectively) for safety evaluations.

**Study population.** Male and female subjects were required to fulfill the following inclusion criteria to be eligible for participation in the study: (i) age between 18 and 55 years at the time of screening; (ii) body mass index  $\geq 18$  kg/m<sup>2</sup> and  $\leq 32$  kg/m<sup>2</sup>; (iii) body weight between 55 and 100 kg; (iv) be in general good health without clinically significant medical history as judged by the principal investigator; (v) clinical laboratory values within the normal limits or determined as not clinically significant by the principal investigator; (vi) nonsmoking defined as no tobacco or nicotine-containing product use for a minimum of 6 months before the time of screening; (vii) female subjects of child-bearing potential were required to have a negative serum pregnancy test at screening and a negative urine pregnancy test on day 1 before drug administration and were required to use two approved methods of birth control from the time of screening and until 30 days following the last administration of study drug; (viii) male subjects agreed to be sexually abstinent or use two approved methods of contraception when engaging in sexual activity from the time of the screening visit until 90 days following the last administration of study drug and to not donate sperm during same time period; and (ix) female subjects who were postmenopausal (defined as 12 months spontaneous amenorrhea) with serum follicle-stimulating hormone (FSH) levels  $\geq 40$  mIU/ml or who had undergone one of the protocol-defined and documented sterilization procedures at least 6 months prior to screening.

Subjects were excluded from participation if any of the following criteria were met: (i) history of any moderate or severe hypersensitivity or allergic reaction to any  $\beta$ -lactam antimicrobial (e.g., penicillin, cephalosporin, sulbactam, or carbapenem); (ii) history of allergic or other serious adverse reactions to lidocaine or amide anesthetic agents; (iii) use of probenecid within 30 days before confinement; (iv) use of any prescription medication (with the exception of hormonal contraceptives or hormone replacement

therapy for females) within 14 days prior to confinement; and (v) use of medication (except for acetaminophen, which was allowed up to 3 days before confinement), multivitamins, and vitamin C were allowed up to 7 days before confinement (day 1), and all other medication (including over-the-counter medication, health supplements, and herbal remedies such as St. John's Wort extract) must have been stopped at least 14 days prior to confinement, unless agreed as not clinically relevant by the principal investigator.

Exclusion criteria also included a history or presence of (i) significant oncologic, cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrine, immunologic, dermatologic, neurological, or psychiatric disease; (ii) seizures, head injury, or meningitis (e.g., epilepsy); (iii) bleeding disorders; (iv) surgery within the past 3 months prior to screening determined by the investigator to be clinically relevant; (v) any acute illness including clinically significant infection within 30 days prior to screening; and (vi) any other condition or prior therapy, which, in the opinion of the principal investigator, would make the subject unsuitable for this study. Subjects were excluded if a test result for HIV, hepatitis B, or hepatitis C was positive or for laboratory tests (chemistry, hematology, and urinalysis) at screening or prior to confinement that were considered clinically significant or if the following test values were reported: white blood cell count,  $<3,000/\text{mm}^3$ ; hemoglobin,  $<11 \text{ g/dl}$ ; absolute neutrophil count,  $<1,200/\text{mm}^3$ ; or platelet count,  $<120,000/\text{mm}^3$ . Female subjects could not be pregnant or currently lactating. Subjects must not have had a history or presence of alcohol or drug abuse within the 2 years prior to screening, excessive intake of alcohol in the last 6 months prior to screening, or a positive alcohol breath test or urine drug screen test at screening or confinement. Subjects were excluded if estimated creatinine clearance ( $[\text{eCL}_{\text{CR}}]$  Cockcroft-Gault method [31]) was  $<60 \text{ ml/min}$ , there was a clinically significant pulmonary disease or any other disease that prevented a subject from undergoing bronchoscopy with BAL, and for spirometry results showing a median forced expiratory volume in 1 s ( $\text{FEV}_1$ ) of  $<80\%$  of predicted. Subjects who had an unwillingness or inability to comply with the study protocol for any other reason, had used caffeine- or xanthine-containing products or consumed Seville oranges (sour) or grapefruit or grapefruit juice within 48 h prior to study drug dosing, and had engaged in strenuous activity within 96 h of confinement (day 1) and until the time of discharge were excluded. Subjects who had difficulty in donating blood, had given a blood or plasma donation (i.e.,  $>500 \text{ ml}$ ) or had significant blood loss within 60 days prior to screening, or had participated in another investigational drug/device study or been treated with an investigational drug within 30 days or five half-lives (whichever is longer) prior to screening were excluded.

**Drug administration.** Subjects were administered ETX2514 1 g given concurrently with sulbactam 1 g i.v. every 6 h for a total of three doses. Three doses of ETX2514SUL were administered to achieve steady-state concentrations of each agent. ETX2514 and sulbactam were simultaneously administered via a peripheral i.v. catheter, using programmed infusion pumps, over a duration of 3 h. ETX2514 and sulbactam were supplied by Entasis Therapeutics, Inc. (Waltham, MA).

**Blood sample collection.** Blood samples for determining plasma ETX2514 and sulbactam concentrations were collected within 5 min before the administration of the infusion and at 1.0, 2.0, 2.5, 2.95, 3.05, 3.25, 3.5, 4.0, 5.0, and 6.0 h after start of the third dose. Blood samples for determining plasma ETX2514 and sulbactam concentrations were also collected within 15 min before the administration of the first infusion and within 5 min of the second infusion.

**Bronchoscopy and BAL.** Each subject had undergone one standardized bronchoscopy with BAL in the outpatient bronchoscopy suite at one of the five bronchoscopy time points at 1.0, 2.5, 3.25, 4.0, or 6.0 h after start of the infusion of the third doses of ETX2514 and sulbactam (6 subjects per BAL fluid sampling time point). Blood and BAL fluid samples for determining urea concentrations were obtained at each BAL fluid sampling time. Further details concerning bronchoscopy and BAL procedures and the handling, processing, and storage of samples have been previously described (32–34).

**Determination of plasma concentrations of ETX2514 and sulbactam.** The concentrations of ETX2514 and sulbactam in plasma were determined by a validated liquid chromatography with tandem mass spectrometry (LC-MS/MS) assay operated in the negative ion mode (method number M8351072B) performed at Covance (Salt Lake City, UT). A total of 390 plasma samples were assayed for ETX2514 and sulbactam concentrations between 16 October 2017 and 30 October 2017 (Covance Bioanalytical report 8371374). A total of 47 samples were selected for incurred sample assay reproducibility (ISR) testing, and the calculated assay variability values met acceptance criteria with at least two-thirds (rounded up) of the repeat results and original results falling within 20% of the mean of the two values. For all analytical runs, fortified calibration standards and quality controls (QCs) met acceptance criteria of within  $\pm 15\%$  of the nominal concentration ( $\pm 20\%$  at the lower limit of quantification [LLOQ]).

In brief, the method used protein precipitation (acetonitrile used as the solvent) to isolate ETX2514, sulbactam, and the internal standards (ETX2514-13C2-15N2, [AstraZeneca, lot no. AZ135725 14-015] and sulbactam sodium-D2 [Entasis Therapeutics, lot no. 15601]). The blank control matrix was made up of human plasma (BioreclamationIVT) diluted 1:1 with SigmaFast (reconstituted protease inhibitor cocktail tablet [product S8820; Sigma-Aldrich, St. Louis, MO] in 10 ml of water) for calibration standards and QC samples. After extraction, diluted plasma samples were centrifuged, and 100  $\mu\text{l}$  of supernatant was transferred to 96-well plates. Samples were diluted further with 400  $\mu\text{l}$  of water, and 5  $\mu\text{l}$  of sample was injected into the LC-MS/MS system for analysis.

The standard curves were linear for ETX2514 ( $r^2 \geq 0.997$ ) and sulbactam ( $r^2 \geq 0.996$ ) over the concentration ranges of 5.0 to 5,000 ng/ml. The respective precision and accuracy for ETX2514 QC samples were 6.2% and 4.7% at 15 ng/ml, 6.0% and 4.0% at 175 ng/ml, 4.2% and 0.5% at 2,000 ng/ml, and 6.0% and 2.0% at 4,000 ng/ml. The respective precision and accuracy for sulbactam QC samples were 6.3% and 1.3% at 15 ng/ml, 4.4% and  $-1.7\%$  at 175 ng/ml, 3.7% and  $-7.0\%$  at 2,000 ng/ml, and 7.0%

and  $-6.5\%$  at  $4,000$  ng/ml. The LLOQ for both drugs was  $5$  ng/ml. Reported concentrations were multiplied by a factor of  $2$  to account for the  $1:1$  dilution of plasma samples with SigmaFast protease cocktail solution.

**Determination of BAL fluid and cell pellet concentrations of ETX2514 and sulbactam.** The determinations of ETX2514 and sulbactam in BAL fluid supernatants and reconstituted cell pellet suspensions were assayed with LC-MS/MS using a Turbo ion spray interface operated in negative ion mode at Keystone Bioanalytical (North Wales, PA) (report number 171001). A total of  $30$  BAL fluid and  $30$  cell pellet samples were assayed for ETX2514 and sulbactam concentrations between  $16$  October  $2017$  and  $30$  October  $2017$ . A total of  $13$  samples for both BAL fluid and cell pellets ( $43.3\%$ ) were selected for ISR testing, and the calculated assay variability values were within  $20\%$ .

In brief, the method used protein precipitation (acetonitrile used as the solvent) to isolate ETX2514, sulbactam, and the internal standards (ETX2514-13C2-15N2 [AstraZeneca, lot no. AZ135725 14-015] and sulbactam sodium-D2 [Entasis Therapeutics, lot no. 1S601]) for both matrices. The blank control matrix used for calibration standards and QC samples was human BAL fluid (BioreclamationVT). After extraction, BAL fluid samples were centrifuged, and  $50$   $\mu\text{l}$  of supernatant was transferred to plastic injection vial with  $150$   $\mu\text{l}$  of Nanopure water. A  $10$ -to  $20$ - $\mu\text{l}$  sample was injected into the LC-MS/MS system for analysis. For the cell pellet samples, the cells were resuspended with  $1$  ml of Nanopure water, vortexed, and carried through three freeze-thaw cycles. After the third cycle, macrophage samples were extracted by the same procedure as for BAL fluid.

The standard curves were linear for ETX2514 ( $r^2 \geq 0.997$ ) and sulbactam ( $r^2 \geq 0.995$ ) over the concentration ranges of  $2.0$  to  $1,000$  ng/ml. The respective precision and accuracy for ETX2514 QC samples were  $15.53\%$  and  $3.27\%$  at  $6$  ng/ml,  $2.89\%$  and  $-3.96\%$  at  $250$  ng/ml, and  $4.86\%$  and  $-2.49\%$  at  $750$  ng/ml. The respective precision and accuracy for sulbactam QC samples were  $9.93\%$  and  $10.27\%$  at  $6$  ng/ml,  $1.90\%$  and  $-2.58\%$  at  $250$  ng/ml, and  $3.62\%$  and  $-2.45\%$  at  $750$  ng/ml. The LLOQ for both drugs and matrices was  $2$  ng/ml.

**Determination of urea concentrations.** The concentrations of urea in plasma and BAL fluid supernatants were determined using LC-MS/MS at Keystone Bioanalytical (North Wales, PA) (report numbers 171101 and 171011, respectively). A total of  $30$  human plasma and  $30$  BAL fluid samples were assayed for urea concentrations between  $1$  November  $2017$  and  $6$  November  $2017$ . A total of  $10$  samples for both plasma and BAL fluid ( $33.3\%$ ) were selected for ISR testing, and the calculated assay variability values were within  $20\%$ .

The calibration range of the urea assay for plasma was linear ( $r^2 > 0.997$ ) over a range of concentrations from  $100$  to  $3,000$   $\mu\text{g}/\text{ml}$ . The respective precision and accuracy of urea QC samples in plasma were  $4.75\%$  and  $0.90\%$  at  $300$   $\mu\text{g}/\text{ml}$ ,  $1.57\%$  and  $0.84\%$  at  $1,000$   $\mu\text{g}/\text{ml}$ , and  $2.95\%$  and  $-2.97\%$  at  $2,250$   $\mu\text{g}/\text{ml}$ . The LLOQ for urea in human plasma was  $100$   $\mu\text{g}/\text{ml}$ .

The calibration range of the urea assay for BAL fluid was linear ( $r^2 > 0.998$ ) for the range of concentrations from  $0.2$  to  $10$   $\mu\text{g}/\text{ml}$ . The respective precision and accuracy for urea QC samples in BAL fluid were  $2.73\%$  and  $3.67\%$  at  $0.6$   $\mu\text{g}/\text{ml}$ ,  $1.42\%$  and  $-3.87\%$  at  $3.0$   $\mu\text{g}/\text{ml}$ ,  $0.95\%$  and  $-4.72\%$  at  $7.5$   $\mu\text{g}/\text{ml}$ , and  $0.88\%$  and  $-4.42\%$  at  $30$   $\mu\text{g}/\text{ml}$ . The LLOQ of urea in human BAL fluid was  $0.2$   $\mu\text{g}/\text{ml}$ .

**Pharmacokinetic analysis.** Plasma pharmacokinetic parameters for ETX2514 and sulbactam were determined by noncompartmental analysis using Phoenix WinNonlin (version 7.0) software (Pharsight Corp., Cary, NC). Following the administration of the third dose of study drug, the maximum plasma concentration ( $C_{\text{max}}$ ) and the minimum plasma concentration ( $C_{\text{min}}$ ) were read directly from the observed plasma concentration-time profiles of ETX2514 and sulbactam. The plasma concentration at  $6$  h after the third (last) dose was considered the  $C_{\text{min}}$ . The area under the concentration-time curve (AUC) for the last dose was determined for the  $6$ -h dosing interval ( $\text{AUC}_{0-6}$ ) and calculated with the linear-log trapezoidal method. The elimination half-life ( $t_{1/2}$ ) for each agent was calculated by dividing the terminal elimination rate constant ( $\lambda_z$ ) by  $\ln 2$ , where  $\lambda_z$  was determined by nonlinear least-squares regression. The systemic clearance (CL) ( $\text{CL} = \text{dose}/\text{AUC}_{0-6}$ ) and volume of distribution at steady state ( $V_{\text{ss}}$ ) ( $V_{\text{ss}} = \text{MRT}_{\text{inf}} \times \text{CL}$ , where  $\text{MRT}_{\text{inf}}$  is the mean residence time extrapolated to infinity) were calculated for i.v. infusion and steady-state data.

**Calculation of ELF volume and antibiotic concentrations in ELF and AM.** The calculations of the ELF volume and the drug concentrations in ELF and AM were performed with BAL fluid supernatants and cell pellets from pooled aspirates recovered from the 2nd, 3rd, and 4th instillations (35). The concentration of ETX2514 or sulbactam in ELF ( $C_{\text{ELF}}$ ) was determined as follows:  $C_{\text{ELF}} = C_{\text{BAL}} \times (V_{\text{BAL}}/V_{\text{ELF}})$ , where  $C_{\text{BAL}}$  is the concentration of ETX2514 or sulbactam measured in BAL fluid,  $V_{\text{BAL}}$  is the volume of aspirated BAL fluid, and  $V_{\text{ELF}}$  is the volume of ELF sampled by BAL.  $V_{\text{ELF}}$  is derived from the following:  $V_{\text{ELF}} = V_{\text{BAL}} \times (\text{urea}_{\text{BAL}}/\text{urea}_{\text{plasma}})$ , where  $\text{urea}_{\text{BAL}}$  is the concentration of urea in BAL fluid and  $\text{urea}_{\text{plasma}}$  is the concentration of urea in plasma.

The concentration of ETX2514 or sulbactam in AM ( $C_{\text{AM}}$ ) was determined as follows:  $C_{\text{AM}} = C_{\text{pellet}} \text{ suspension}/V_{\text{AM}}$ , where  $C_{\text{pellet}}$  is the amount of ETX2514 or sulbactam measured in the  $1$ -ml cell suspension, and  $V_{\text{AM}}$  is the volume of alveolar cells in the  $1$ -ml cell suspension.  $V_{\text{AM}}$  was determined by multiplying the cell count in BAL fluid by the mean macrophage cell volume of  $2.42$   $\mu\text{l}/10^6$  cells (36, 37). The concentration of ETX2514 or sulbactam measured in AM was derived from  $C_{\text{AM}}$  by adjusting for the percentage of macrophages in AM, as determined by a differential cell count of the BAL fluid.

The ratios of the ELF and AM concentrations to the simultaneous plasma concentrations were calculated for each subject and summarized per group at each sampling time. The mean and median concentrations of ETX2514 and sulbactam in BAL fluid obtained at the different BAL fluid sampling times (e.g.,  $1$ ,  $2.5$ ,  $3.25$ ,  $4$ , and  $6$  h) were used to estimate the  $\text{AUC}_{0-6}$  for ETX2514 and sulbactam in plasma, ELF, and AM. The concentration at the final sampling time ( $6$  h) also served as the time zero value for

determining the  $AUC_{0-6}$  value of each matrix. The linear-log trapezoidal method was used for determining  $AUC_{0-6}$  values using Phoenix WinNonlin (version 7.0) software. The ratios of the  $AUC_{0-6}$  of ELF or AM to the  $AUC_{0-6}$  of plasma (total and unbound) were calculated. Plasma protein binding analyses were not conducted as part of this study. The values used for the unbound fraction of ETX2514 and sulbactam in plasma were 0.90 and 0.62, respectively (21 and data on file, Entasis Therapeutics, Inc., correspondence 5 January 2018 [study report PC2514-2016-0024]). The measured concentrations in ELF and AM were assumed to represent unbound concentrations, since only unbound plasma fractions are considered to penetrate the lung compartments.

**Laboratory and safety assessment.** Safety was assessed throughout the study by adverse event monitoring, clinical laboratory tests (serum chemistry, hematology, and urinalysis), electrocardiogram (ECG), physical examination, and vital signs monitoring. Subjects who received at least 1 dose of either ETX2514 or sulbactam were included in the safety analysis. Safety data were summarized by treatment group, and the incidence of adverse events was presented by system organ class, the relationship to the study medication, and severity. The descriptive statistics of clinical laboratory, vital sign, and ECG results and the change from the baseline, as well as a summary of clinically notable values, were assessed.

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R.D.I., J.P.O., and E.S. are current employees of Entasis Therapeutics, Inc.

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