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Host-Mediated Bioactivation of Pyrazinamide: Implications for Efficacy, Resistance, and Therapeutic Alternatives

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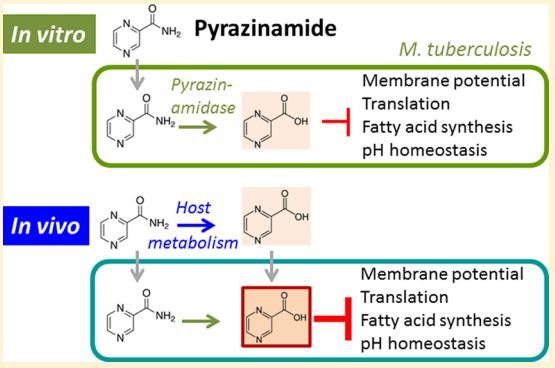
Supporting Information

ABSTRACT: Pyrazinamide has played a critical role in shortening therapy against drug-sensitive, drug-resistant, active, and latent tuberculosis (TB). Despite widespread recognition of its therapeutic importance, the sterilizing properties of this 60-year-old drug remain an enigma given its rather poor activity in vitro. Here we revisit long-standing paradigms and offer pharmacokinetic explanations for the apparent disconnect between in vitro activity and clinical impact. We show substantial host-mediated conversion of prodrug pyrazinamide (PZA) to the active form, pyrazinoic acid (POA), in TB patients and in animal models. We demonstrate favorable penetration of this pool of circulating POA from plasma into lung tissue and granulomas, where the pathogen resides. In standardized growth inhibition experiments, we show that POA exhibits superior in vitro potency compared to PZA, indicating that the vascular supply of host-derived POA may contribute to the in vivo efficacy of PZA, thereby reducing the apparent discrepancy between in vitro and in vivo activity. However, the results also raise the possibility that subinhibitory concentrations of POA generated by the host could fuel the emergence of resistance to both PZA and POA. In contrast to widespread expectations, we demonstrate good oral bioavailability and exposure in preclinical species in pharmacokinetic studies of oral POA. Baseline exposure of oral POA can be further increased by the xanthine oxidase inhibitor and approved gout drug allopurinol. These promising results pave the way for clinical investigations of oral POA as a therapeutic alternative or an add-on to overcome PZA resistance and salvage this essential TB drug.



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Pyrazinamide (PZA) plays a complex but crucial role in modern tuberculosis (TB) chemotherapy. It was officially

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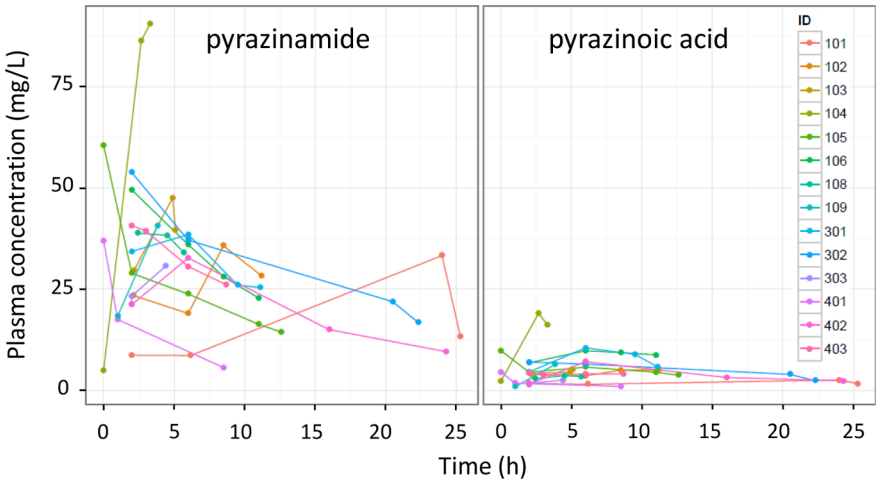

Figure 1. Concentration−time profile of PZA and POA in plasma following oral administration of 1500 mg of PZA to 14 TB patients. Each subject contributed three to five plasma samples from predose to 25 h after drug administration.

adopted as part of the first line regimen in the 1980s after it was found to reduce the duration of therapy from 9 to 6 months when added to a regime of isoniazid and rifampicin for the first 2 months.[1−4] PZA is also part of most regimens used against multi-drug-resistant (MDR)-TB, where it plays an important role given the limited therapeutic options of these patients.[5] Finally, PZA has shown synergies with several drugs or drug candidates currently in clinical development for TB, such as bedaquiline, clofazimine, and PA-824.[6−9] Thus, PZA has a unique therapeutic potential across many TB patient populations.

Despite widespread recognition of its therapeutic importance, the sterilizing and synergistic properties of PZA remain an enigma.[10] This knowledge gap stems from a very unusual in vitro−in vivo disconnect: the discovery of PZA activity in a murine model of TB rather than a culture of replicating bacteria.[11] Unlike all other TB drugs, PZA exhibits poor in vitro activity. Minimum inhibitory concentrations (MICs) vary widely depending on the pH, inoculum size, and other assay conditions.[12,13] The clinical susceptibility breakpoint above which therapy fails is around 100 μg/mL (as determined by the BACTEC MGIT 960 method with an adjusted pH of 5.9),[14] whereas "normal" peak plasma concentrations are 20−60 μg/mL 1 to 2 h postdose.[15] Despite PZA being one of the oldest anti-TB drugs, we have failed to uncover the biological mechanisms underlying this pharmacokinetic−pharmacodynamic conundrum.

PZA is a biologically inactive prodrug that undergoes activation by a bacterial deamidase to generate pyrazinoic acid (POA), a weak carboxylic acid. That POA is the major active metabolite has been demonstrated by exposing *Mycobacterium tuberculosis* cultures to extracellular POA and measuring growth inhibition at pH 5.5 to 6.0 against laboratory strains or various panels of clinical isolates. A surprisingly wide range of minimum inhibitory concentrations (MIC) has been reported for POA, from 8- to 16-fold less potent than PZA[16] to slightly more active than PZA[17,18] and up to 8-fold more potent than PZA.[12] The reported MICs of PZA itself vary between 4 and 400 μg/mL at acidic pH.[12,13] Under the current model, bioactivation primarily occurs inside *M. tuberculosis* bacilli and is catalyzed by the *pncA*-encoded nicotinamidase.[19,20] Accordingly, a large proportion of *M. tuberculosis* PZA-resistant strains have *pncA* mutations that reduce or abolish POA production.[21]

On the basis of these findings, it was concluded that PZA undergoes intramycobacterial activation to POA and targets a subpopulation of nonreplicating or slowly replicating bacilli that reside in an acidified niche. These observations have motivated a series of studies focusing on the mechanism(s) of action of PZA and POA against *M. tuberculosis* and have identified a diverse range of potential targets, including fatty acid synthesis,[22] trans-translation,[18] membrane potential and integrity,[23] pantothenate biosynthesis,[24,25] and the host immune response[26,27] (N. Ammerman, unpublished). Collectively these studies indicate that PZA acts through a multiplicity of targets and mechanisms, many of which require biotransformation into POA.

Because POA is active in vitro, it must accumulate in *M. tuberculosis* bacilli when provided exogenously, as shown by Zhang and colleagues.[28] Thus, the direct administration of POA to overcome *pncA*-mediated resistance, particularly in MDR/XDR patients, appears to be a sensible therapeutic strategy. However, oral administration of POA is not currently considered, mostly based on the assumption that it would likely exhibit poor oral bioavailability and tissue distribution given its physicochemical properties. This expectation also stems from a 1967 anecdotal report mentioning the lack of POA efficacy (administered at 2 to 5% in food) in a mouse model of acute intravenous infection.[29]

Here we have first characterized the pharmacokinetics of PZA in TB patients and in animal species commonly used to model TB. Our first objective was to establish doses that reproduce human exposure of PZA and POA in preclinical species using population pharmacokinetic approaches. In line with early but largely forgotten observations in healthy volunteers and TB patients,[30,31] we have measured a substantial host-mediated conversion of the prodrug (PZA) to the active form (POA) and have systematically characterized and quantified this conversion in a panel of preclinical species. In addition, we have shown that this pool of circulating POA generated by the host effectively distributes into lung tissue and pulmonary lesions, suggesting that host-derived POA contributes to the puzzling efficacy of PZA. Our second objective was to investigate the oral bioavailability and exposure of POA in various animal models. The results pave the way for preclinical and clinical investigations of oral POA as a potential therapeutic alternative to overcome *pncA*-mediated resistance to PZA.

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Table 1. Parameter Estimates of the Pharmacokinetic Model for PZA and POA Metabolite Across Animal Species and Strains, with Parameters are Reported as Typical Values[a] and Standard Errors (in Parentheses)[b]

	TB patients[c]	marmosets	rabbits	guinea pigs	CD1 mice	C3HeB/FeJ mice	C3HeB/FeJ infected mice (steady state)	BALB/c mice
median weight (kg)	57.8	0.426	2.87	0.235	0.023	0.035	0.040	0.023
CL/F (L/h/kg)	0.028 (7)	0.23 (27)	0.75 (6)	0.75 (2)	0.75 (3)	0.62 (13)	0.38 (3)	0.47 (5)
V/F (L/kg)	0.49 (8)	1.1 (25)	3.72 (61)	2.54 (2)	1.28 (6)	1.02 (15)	1.85 (2)	0.78 (4)
k_a (h^{-1})	0.72 (18)	0.81 (7)	4.8 (2)	7.7 (NA)	15 (11)	4.47 (21)	220 (1)	18 (8)
K_{el} (h^{-1})	0.057	0.21	0.20	0.29	0.58	0.61	0.21	0.60
CL_m/F_m (L/h/kg)	0.032 (1)	1.1 (19)	0.24 (6)	2.14 (1)	2.35 (4)	1.04 (17)	2.18 (6)	1.15 (6)
V_m/F_m (L/kg)	2.27 (0.22)	4.3 (21)	0.73 (11)	2.71 (1)	0.71 (9)	0.75 (14)	0.47 (13)	0.16 (1)
PZA proportional error (CV %)	37 (20)	62 (22)	42 (8)	79 (15)	38 (15)	50 (10)	47 (13)	35 (16)
POA proportional error (CV %)	47 (17)	66 (10)	64 (10)	54 (17)	45 (9)	87 (10)	40 (11)	66 (6)

[a]CL/F, oral clearance; V/F, oral volume of distribution; k_a, absorption rate constant; CL_m/F_m and V_m/F_m, oral clearance and volume of distribution of the POA metabolite, respectively. [b]The mouse sample sizes were too small to estimate SE. [c]For consistency, clinical parameters are normalized to the median body weight; note that non-normalized values were used in the model.

RESULTS

Conversion of PZA to POA in Tuberculosis Patients. In a clinical research study of PZA pharmacokinetics, we measured the plasma concentrations of PZA and POA in 14 active TB patients scheduled to receive elective lung surgery to treat their disease. Blood samples were collected predose and at four to five time points from 3 to 24 h following a single standard dose of 1500 mg PZA. Although the time points were flexible due to uncertainty surrounding surgical timing, the exact time postdose was accurately recorded in all cases. The absorption of PZA was delayed in several subjects, possibly due to comorbidities for which they received medication that might have interfered with TB drug absorption or related to sedation and anesthesia prior to lung resection. We found substantial levels of POA in all plasma samples across subjects (Figure 1). PZA and POA exposures were estimated from the pharmacokinetic model simultaneously fitted to the PZA and POA data. A one-compartment model both for the parent compound and metabolite provided an adequate fit and enabled an estimation of the PZA and POA pharmacokinetic (PK) parameters summarized in Table 1. Using this model, we integrated individual predicted concentrations over 24 h to assess the daily exposure of PZA and POA. On the basis of these estimates, the median POA/PZA exposure ratio was 19% (range 17−32%) over a 24 h period for this population. The total plasma clearance of PZA was 1.7 L/h or approximately half the values reported in other patient populations.[31,32] This again may be related to anesthesia which was administered 2 to 3 h prior to lung resection and/or to interactions with non-TB drugs which a number of the subjects received to treat various comorbidities. The total plasma clearance of POA was similar at 1.9L/h.

Circulating POA Penetrates Lung Tissue and Pulmonary Lesions. The presence of a circulating pool of POA raised two immediate questions: (1) Does pulmonary *M. tuberculosis* affect the conversion of PZA to POA measured in the bloodstream? (2) Does POA found in plasma distribute into lung and pulmonary lesions? The chemical structure and physicochemical properties of POA, a weak carboxylic acid more hydrophilic than PZA (calculated log *D* at pH 7.4 of −3.28 to −4.43 for POA and −0.27 to −0.37 for PZA) would argue for moderate to poor tissue distribution by passive diffusion. To answer these questions, we resorted to the rabbit model of *M. bovis* infection because (1) *M. bovis* fails to effectively convert PZA to POA due to a defective *pncA* gene[21] and (2) *M. bovis* generates humanlike TB pathology.[33,34]

PZA was administered to uninfected rabbits and to rabbits infected with either *M. tuberculosis* or *M. bovis*. In naïve rabbits, we found higher POA/PZA ratios than those measured in humans, with the clearance of PZA and POA being higher and lower than in TB patients, respectively (Figure 2A and Table 1). We measured the same POA/PZA ratio in plasma regardless of infection status and infecting agent, i.e. the conversion of PZA to POA measured in plasma was independent of infection with *M. tuberculosis* (Figure 2B). This ruled out the possibility that POA produced by *M. tuberculosis* bacilli within infected lesions is released from the tissue and subsequently recirculated into the central compartment, indicating that bioactivation is host-mediated. The concentration ratio of POA to PZA increased over time. However, the POA/PZA ratio remained constant across the range of doses studied (Figure 2B). To determine whether circulating POA produced by the host reaches the site of infection and is therefore available to inhibit the growth of *M. tuberculosis* inside lesions, we measured POA concentrations in the lung and cellular lesions of *M. bovis*-infected rabbits, relative to plasma, 2, 6 and 24 h post PZA dosing. POA lung-to-plasma and granuloma-to-plasma ratios in these rabbits were approximately 0.3 and 0.2 at 2 h and 0.6 and 0.3 at 6 h postdosing. In comparison, PZA lung-to-plasma and lesion-to-plasma ratios were approximately 0.3 and 0.1 at 2 h and 0.4 and 0.2 at 6 h, respectively (Table 2). Both PZA and POA concentrations were below the limit of quantification at 24 h.

Thus, POA distributes from plasma to tissue/lesions at least as effectively as PZA, indicating that POA molecules circulating in plasma and extracellular space have the potential to reach tubercle bacilli contained within a lung granulomatous lesion. This observation prompted us to compare the MIC of PZA and POA under standardized conditions to resolve conflicting numbers reported in the literature.[12,16,17] We measured the MIC of both agents against the two laboratory strains H37Ra (which has been used for numerous mechanistic studies with PZA and POA) and H37Rv in liquid and on solid media, using turbidometry and colony formation as read-outs of growth rather than indirect measurements relying on fluorometric or other growth reporters because these might be influenced by the drug's mechanism of action. Growth curves of *M. tuberculosis* H37Ra and H37Rv were established at pH 5.5,

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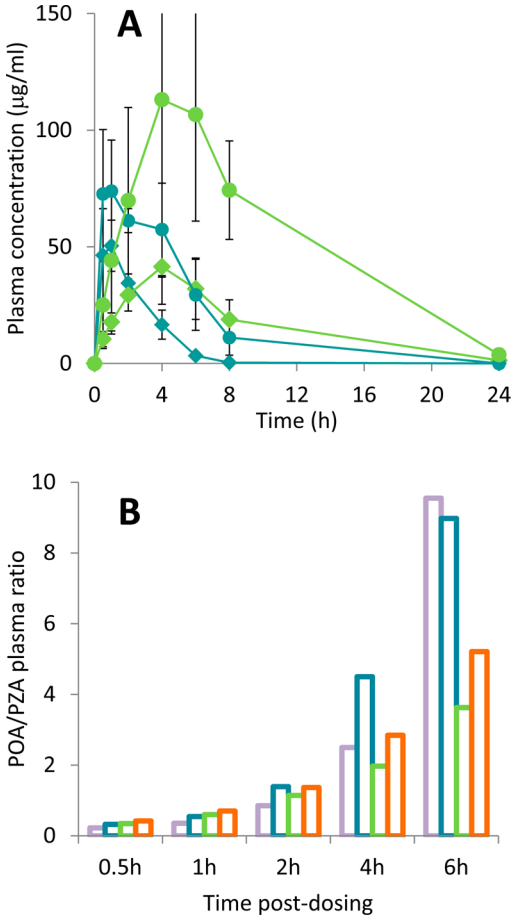

Figure 2. (A) Concentration–time profile of PZA and POA following the oral administration of 150 and 300 mg/kg to uninfected rabbits. Blue diamonds, PZA 150 mg/kg; green diamonds, POA from PZA dosed at 150 mg/kg; blue circles, PZA 300 mg/kg; green circles, POA from PZA dosed at 300 mg/kg. (B) POA/PZA concentration ratio in plasma at sequential time points following oral dosing of PZA to naïve, *M. tuberculosis*-infected (12 weeks postinfection), and *M. bovis*-infected (4 weeks postinfection) rabbits. Purple bars, uninfected rabbits dosed with 150 mg/kg PZA; blue bars, *M. bovis* AF2122 infected rabbits dosed with 125 mg/kg PZA; green bars, uninfected rabbits dosed with 300 mg/kg PZA; orange bars, *M. tuberculosis* HN878 infected rabbits dosed with 250 mg/kg PZA.

Table 2. Lung-to-Plasma and Granuloma-to-Plasma Concentration Ratio of PZA and POA Following Oral Administration of a Single PZA Dose to *M. bovis*-Infected New Zealand White Rabbits[a]

	$PZA_{granuloma}$	PZA_{lung}	$POA_{granuloma}$	POA_{lung}
concentration ratio to plasma 2 h postdose	0.07	0.25	0.24	0.32
concentration ratio to plasma 6 h postdose	0.15	0.38	0.32	0.56

[a]Uninvolved lung pieces and individual granulomas were dissected, weighed, and homogenized prior to PZA and POA quantitation.

5.8, and 6.5 in the presence of an increasing concentration of PZA or POA. The pH was carefully monitored throughout the entire experiment. Both 50% and 90% growth inhibition were recorded for each agent at each pH in liquid media and on agar plates (Supporting Information Table S1 and Figure S2). POA was consistently more potent than PZA in the liquid medium at each pH tested, in agreement with the results reported by Cynamon and colleagues[12] who observed that POA was more potent than PZA against the vast majority of 30 clinical isolates they tested. While POA was similarly more active than PZA against both H37Ra and H37Rv, the latter strain appeared to be less susceptible to PZA than H37Ra across the pH range (Table S1 and Figure S2). On agar plates, POA was reproducibly twice as potent as PZA.

Collectively, the results of the PK experiments conducted in uninfected and infected rabbits indicate a high POA/PZA exposure ratio, independent of mycobacterial infection. They also demonstrate the favorable tissue distribution of POA from the bloodstream into lung tissue and granulomas, indicating that host-derived POA reaches bacilli present in pulmonary lesions.

Pharmacokinetics and Bioconversion of PZA to POA in Mice, Guinea Pigs, and Marmosets. Given the much higher POA/PZA ratio in rabbits than in humans, we asked whether other animal species used to evaluate the efficacy of TB drug regimens would exhibit a humanlike host-mediated activation of PZA. Because the C3HeB/FeJ mouse model of TB infection is increasingly used for the preclinical evaluation of drug efficacy, we first established the plasma concentration–time profiles of PZA and PZA-derived POA in naïve C3HeB/FeJ mice following a single oral dose of 150 mg/kg PZA, the human equivalent dose (Figure 3A). To examine the effect of Mtb infection, mouse strain, and multiple dosing on the pharmacokinetics of PZA and host-mediated conversion to POA, we also measured the plasma concentrations of PZA and POA in uninfected CD-1 mice and in *M. tuberculosis*-infected C3HeB/FeJ and BALB/c mice, following the administration of a single dose as well as at steady state during PZA treatment in C3HeB/FeJ mice (Figure S1). Table 1 summarizes the PK parameter estimates for the 3 naïve mouse strains as well as the infected C3HeB/FeJ mice. The exposure of PZA and conversion to POA was consistent regardless of mouse strain, infection status, and repeated dosing. Although the half-life of PZA was shorter in mice than in humans and rabbits (Figure 3A and Table 1), the kinetics and extent of conversion of PZA to POA in plasma were similar to those observed in humans (refer to Figure 1). As seen in rabbits, the POA to PZA exposure ratios were similar in infected and uninfected mice (Table 3). In conclusion, the relative exposure of PZA and POA is similar in mice and humans.

Next we investigated the conversion of PZA to POA in naïve guinea pigs, another model of TB infection and disease used for the evaluation of drug combinations.[35] Guinea pigs convert PZA to POA to the same extent as mice and humans, but PZA is eliminated faster in guinea pigs than in humans, as is the case in mice (Figure 3B and Table 1).

Finally, we measured the plasma concentrations of PZA and POA in uninfected marmosets, an emerging nonhuman primate model increasingly used to study TB vaccine and drug efficacy, following a single oral dose of 125 mg/kg. In this species, the pharmacokinetic profile of PZA was closest to that observed in TB patients, and the conversion of PZA to POA was similar or slightly lower than in humans (Figure 3C). The exposure of PZA and POA and the corresponding ratios across species and in TB patients are summarized in Table 3. Together, the results show that substantial concentrations of POA are generated not only in humans but in all major animal species used to study TB.

Oral Pharmacokinetics of POA in Preclinical Species. Given the favorable penetration of POA from plasma into

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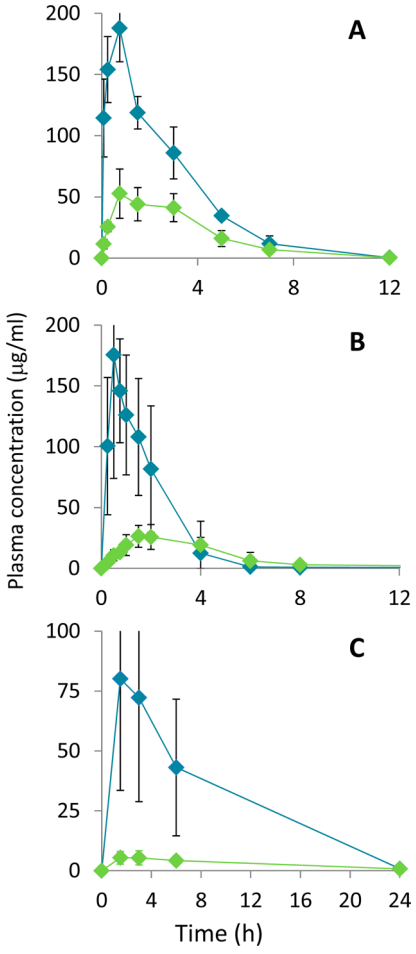

Figure 3. (A) Concentration−time profile of PZA and POA metabolite following oral administration of (A) a single 150 mg/kg PZA dose to naïve C3HeB/FeJ mice, (B) a single 300 mg/kg PZA dose to naïve guinea pigs, and (C) a single 125 mg/kg PZA dose to naïve marmosets. Blue diamonds, plasma concentrations of PZA; green diamonds, plasma concentrations of POA metabolite.

pulmonary tissues and the superior potency of POA compared to PZA in vitro, we then asked whether direct oral administration of POA to mice, guinea pigs, and rabbits would provide sufficient bioavailability to support efficacy studies. A single oral dose of POA was administered to CD-1 and BALB/c mice (150 mg/kg), guinea pigs (300 mg/kg[6]), and NZW rabbits (200 mg/kg) (Figure 4). In the absence of prior knowledge regarding the PK of POA in preclinical species, we arbitrarily selected POA doses identical to human-equivalent PZA doses for mice and guinea pigs and a lower dose in rabbits since we knew that POA clearance was significantly lower in this species (Table 1). In mice, the compound was rapidly absorbed to reach peak concentrations similar to those of PZA, but elimination was fast, with plasma concentrations decreasing rapidly within 3 h postdose. In guinea pigs, absorption was again excellent and clearance was slower than in mice, with a concentration−time profile suggesting enterohepatic cycling (Figure 4). Animals exhibited a rebound during the elimination phase, between 2 and 6 h postdose. Interanimal variability was seen in the timing of the rebound, but the increase was observed in each individual animal (n = 4, Figure S3A). This rebound was also observed, though to a lesser extent, in naïve CD-1 mice (not shown). Two different POA formulations were

Table 3. Average PZA and POA Exposure Values (AUC) and Exposure Ratios across Species Following a Single Oral Dose of PZA or POA[a]

	naïve CD1 mice	naïve C3HeB/FeJ mice	infected C3HeB/FeJ mice	naïve BalbC mice	infected BalbC mice	naïve guinea pigs	naïve rabbits	infected rabbits[b]	naïve marmosets	Korean subjects
PZA dose (mg/kg)	150	150	150		150	300	300	250	125	22−30
AUC_{PZA} (μg·h/ml)	210 (23)	580 (280)	425 (101)		388 (35)	342 (183)	457 (95)	296 (61)	743 (471)	617−738
$Cmax_{PZA}$ (μg/mL)	109 (11)	190 (24)	89 (20)		163 (38)	179 (100)	82 (17)	90 (35)	80 (47)	43/59[c]
$AUC_{POA\ met}$ (μg·h/ml)	59 (3)	223 (32)	76 (16)		131 (13)	130 (98)	1288 (486)	504 (139)	72 (32)	116−212
$Cmax_{POA\ met}$ (μg/mL)	28 (8)	60 (15)	16 (5)		43 (10)	29 (13)	123 (68)	111 (27)	5.6 (2.8)	7/34
AUC_{POA}/AUC_{PZA} ratio	0.29 (0.05)	0.38 (0.04)	0.18 (0.02)		0.38 (0.02)	0.36 (0.07)	3.12 (1.93)	1.70 (0.21)	0.11 (0.03)	0.19
POA dose (mg/kg)	150			150		300	200			
AUC_{POA} (μg·h/ml)	136 (65)			287 (93)		449 (265)	2,013 (159)			
$Cmax_{POA}$ (μg/mL)	102 (32)			155 (33)		165 (74)	244 (51)			
human-equivalent PZA dose (mg/kg)	n/a	n/a	160		175	400	300	n/d	100	n/a

[a]The human-equivalent PZA doses were calculated to achieve a target AUC of 450 mg·h/L. [b]AUC, area under the curve; POA met, POA metabolite derived from dosing of PZA. [c]Single dose/steady state. AUC ratios were calculated using $AUC_{[0-24]}$ obtained from noncompartmental analysis, except for the exposure ratios of infected rabbits for which $AUC_{[0-6]}$ was used. Standard deviations are in parentheses.

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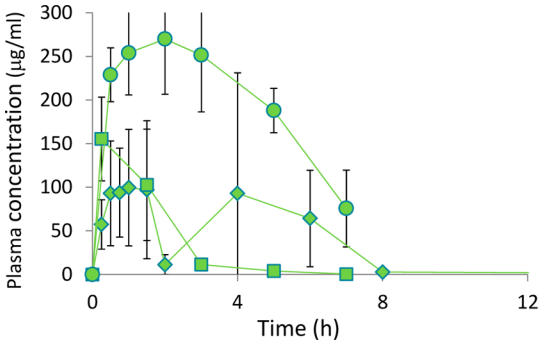

Figure 4. Concentration−time profile of POA following oral administration of a single POA dose to naïve BALB/c mice (150 mg/kg, squares), guinea pigs (300 mg/kg, diamonds), and NZW rabbits (200 mg/kg of Na-POA, circles). At the last time point of 24 h, all plasma concentrations were below the limit of detection.

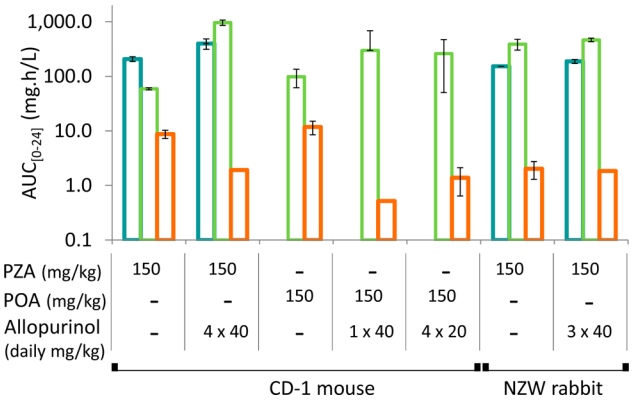

Figure 5. Effect of xanthine oxidase inhibition by varying allopurinol predosing schedules on the metabolism of PZA and POA in CD-1 mice and NZW rabbits. Blue bars, $AUC_{[0-24]}$ of PZA; green bars, $AUC_{[0-24]}$ of POA; orange bars, $AUC_{[0-24]}$ of 5-hydroxy-POA.

tested in rabbits given the poor solubility of POA in aqueous media: 40% sucrose in water providing a homogeneous suspension and a sodium salt in pH-adjusted buffer providing a clear solution. The overall exposure was very high and comparable following the administration of both preparations, significantly higher than for PZA at the same dose, but absorption of the sodium salt was faster as expected because the compound was fully solubilized (Figure S3B). The respective AUC in each species is shown in Table 3. In contrast to widespread expectations, our results thus indicate that POA is orally bioavailable in the three species tested.

In TB patients receiving a standard daily dose of 1500 mg PZA, the mean AUC is around 380 to 625 mg·h/L,[32,36,37] and one of the top predictors of a poor outcome is a PZA $AUC_{[0-24]}$ lower than 363 mg·h/L.[38] Thus, we chose a target AUC of 450 mg·h/L to calculate human-equivalent dose of PZA for each animal species/strain (Table 3).

Species-Specific Metabolism of POA in Mice and Rabbits. POA is primarily converted by the liver xanthine oxidase to 5-hydroxypyrazinoate.[39,40] Given the drastic difference in POA pharmacokinetics and clearance between mice and rabbits, we suspected the species-specific metabolism of POA, whereby POA is more rapidly and extensively metabolized in mice than in rabbits. We thus set out to test the hypothesis that pretreatment with an inhibitor of xanthine oxidase could boost the exposure of POA, whether produced following the administration of PZA or orally dosed as POA, in mice while having a less pronounced effect in rabbits. Allopurinol is a common inhibitor of xanthine oxidase used primarily to treat hyperuricemia, including chronic gout.[41] We designed a series of pharmacokinetic studies where CD-1 mice received 1 or 4 daily predoses of allopurinol at 20 or 40 mg/kg and rabbits received 3 daily predoses of 40 mg/kg. The doses and schedules were selected on the basis of published studies of xanthine oxidase inhibition in mice.[42,43] PZA or POA was administered orally 30 to 60 min after the (last) dose of allopurinol, and serial blood samples were collected from 0 to 24 h postdose to quantify PZA, POA, and 5-hydroxy-POA, the product of POA conversion by xanthine oxidase.[31] When PZA was administered, the effect of allopurinol pretreatment (4 daily doses of 40 mg/kg) on POA exposure was drastic, with a 16-fold increase in exposure (measured as $AUC_{[0-24]}$), whereas the $AUC_{[0-24]}$ of PZA increased only around 2-fold (Figure 5 and S4). When POA was administered orally following 4 daily predoses of 40 mg/kg allopurinol, we measured a 12-fold increase in POA exposure. When allopurinol pretreatment was reduced to either 4 doses of 20 mg/kg or 1 dose of 40 mg/kg, we observed a modest increase in POA AUC of 2- to 3-fold and a concomitant decrease in 5-hydroxy-POA exposure (Figure 5). Thus, allopurinol pretreatment increases the exposure of POA whether it is derived from PZA or administered orally, and the effect increases as the allopurinol dose or dose number increases. Predosing of allopurinol in rabbits (3 doses of 40 mg/kg) followed by the oral administration of PZA 150 mg/kg had no effect on POA exposure (Figure 5). The average AUC values of allopurinol and its active metabolite oxypurinol in rabbit plasma were 8.0 and 123.7 mg·h/L (Figure S5C), respectively, or at least twice as high as the exposure in humans following a high dose of 300 mg;[44] (http://www.rxlist.com/aloprim-drug/clinical-pharmacology.htm), ruling out the possibility that the rabbits received a subtherapeutic dose of allopurinol. The results indicate that POA is metabolized by the xanthine oxidase in mice but not in rabbits, thus explaining the unique pharmacokinetics and high exposure of POA in rabbits, whether it is derived from PZA or dosed orally.

DISCUSSION

It is generally assumed that the bioactivation of PZA to POA taking place inside *M. tuberculosis* bacilli is the primary source of PZA-derived activity.[28] The fact that most clinical resistance to PZA is conferred by mutations in the mycobacterial pyrazinamidase (encoded by the *pncA* gene) is often used to support this notion.[21] However, when phenotypic resistance data, pyrazinamidase activity, and the mutation in *pncA* are directly compared, there appear to be substantial disconnects with as many as 20% of PZA-resistant strains retaining pyrazinamidase activity.[45] Indeed, at least some of the genotypic polymorphisms identified in *pncA* have been shown to be simply markers of phylogeny.[46,47] Very few of the known polymorphisms in *pncA* have been shown biochemically to compromise pyrazinamidase activity or alter the hydrolysis rate of PZA. PZA susceptibility is not routinely tested in many endemic regions and is hampered by technical difficulties and reproducibility issues.[48] For this reason and given the lack of a clear association between PZA resistance and a poor clinical outcome, the WHO treatment guidelines recommend including PZA regardless of the susceptibility status.[49] Thus, a significant proportion of patients with PZA-resistant isolates are treated with PZA-containing regimens.

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Our studies show that there is a substantial host-mediated conversion of PZA to POA in TB patients and in all major preclinical models of experimental chemotherapy. Systematic pharmacokinetic studies across species revealed significant concentrations of POA in plasma following the oral administration of human-equivalent doses of PZA. The systemic concentration−time profile of the POA metabolite was similar in infected and naïve animals, indicating that the host-mediated conversion of PZA to POA accounts for most of the POA found in plasma. Earlier studies in healthy volunteers showed that following a single oral dose of 1500 mg, PZA was converted to at least four metabolites, including 5-hydroxyl-PZA, pyrazinuric acid, POA, and 5-hydroxyl-POA.[31,50] Our quantification data of PZA and POA in the plasma of active TB patients showed a POA/PZA exposure ratio of approximately 0.2, in agreement with these early studies.

Could host-derived POA contribute to the observed efficacy and surprising sterilizing activity of PZA? A 1996 study by Cynamon and colleagues supports this hypothesis: when the activity of PZA was evaluated in a mouse model against clinical isolates of *M. tuberculosis* with varying susceptibility to PZA (32 to >2000 μg/mL), there was an inconsistent correlation between the PZA MIC measured in vitro and the reduction of bacterial burden in mouse lungs.[51] Indeed, PZA was efficacious against one isolate that was fully resistant in vitro. POA in vitro susceptibility of this isolate was measured in a subsequent study and appeared to be a better predictor of efficacy in mice than PZA MIC.[12] This PZA-resistant isolate had retained partial susceptibility to POA, thus remaining sensitive to in vivo killing by host-derived POA. Depending on the resistance mutation (in *pncA* versus the POA target), other isolates may have been differentially susceptible to POA in comparison to PZA, thus explaining the general lack of correlation between PZA MIC and PZA-mediated killing in vivo. Clinically, the contribution of host-derived POA to PZA efficacy has not been detected thus far for several reasons. In addition to the lack of systematic PZA susceptibility testing, the combinatorial nature of TB therapy has made it difficult to establish a link between PZA resistance and clinical outcomes.[52] Recent small-scale studies suggest that resistance to PZA in MDR-TB is associated with poor cure rates,[5,53] but this finding awaits confirmation in powered prospective trials.

When we tested the activity of POA and PZA in vitro at various pH and inocula, we found that POA was 2- to 4-fold more potent than PZA against *M. tuberculosis* in vitro, in agreement with the work of Cynamon et al., who showed superior potency of POA over PZA in a panel of 30 clinical isolates.[12] Thus, POA may exert substantial activity at therapeutically achieved concentrations, particularly against bacterial subpopulations that are most susceptible, such as those present in acidic microenvironments. Accounting for the production of significant amounts of this potent metabolite by the host may reduce the apparent discrepancy between the in vitro potency and in vivo efficacy of PZA in preclinical and clinical studies. The graphical abstract illustrates the disconnect between the activity of PZA measured in vitro, where bioactivation takes place exclusively inside *M. tuberculosis* bacilli, and the in vivo situation where substantial amounts of POA are generated by the host (Figure S6).

Another critical finding is the observation that POA produced by the host effectively distributes into lung tissue and tuberculous lesions, with tissue-to-plasma ratios ranging between 0.2 and 0.5 in animals infected with *M. bovis* (a *pncA*-deficient mycobacterial species). Similar studies in infected C3HeB/FeJ mice dosed with PZA showed a favorable distribution of PZA-derived POA into necrotic lesions with $AUC_{tissue}/AUC_{plasma}$ ranging between 0.5 for uninvolved lung and 1.2 for large caseous granulomas (Anne Lenaerts, unpublished). This has two pharmacological implications: (1) If POA can be delivered either orally or via another route, then a reasonable distribution into lung and lesions can be expected. (2) Because the POA-to-PZA concentration ratio is clearly lower than 1 in tissue, spatial and temporal windows of subinhibitory POA concentrations could be present in the lungs. What are the potential consequences of sustained subtherapeutic POA concentrations? The present work raises the possibility that in these patients host-derived POA exerts residual activity against selected pockets of bacteria, dampening the effect of *pncA*-mediated resistance to PZA. However, the mutation of *pncA* being the most common mechanism of clinical resistance suggests that POA generated by the host is not sufficient to prevent the selective outgrowth of *pncA* mutants. Indeed, the average POA-to-PZA ratio is around 0.2 in human plasma. Though this might be partially compensated for by the higher potency of POA, at least in vitro (this work and ref 12), it is possible that POA made by the host does not achieve/maintain therapeutic concentrations in humans. This is a critical observation because it suggests that the use of PZA against PZA-resistant TB caused by *pncA* mutations (as practiced in most settings in the absence of susceptibility data) could fuel the development of further resistance to POA due to subinhibitory concentrations of the active metabolite in plasma and tissues. *pncA* mutants exposed to inadequate concentrations of host-generated POA within lesions could provide an ideal background for the further selection of resistance mutations in POA targets. POA resistance has recently been associated with mutations in *panD*, the aspartate decarboxylase gene in the pantothenate biosynthetic pathway, although the underlying mechanism awaits further investigations.[24,25] The emergence of POA resistance could be tested in animal models given the good oral bioavailability of POA in mice, guinea pigs, and rabbits.

The kinetics of bioconversion of PZA to POA are similar in mice, guinea pigs, nonhuman primates, and TB patients, where POA and PZA have parallel profiles with POA concentrations lower than PZA concentrations at all times and POA/PZA exposure ratios ranging between 0.1 and 0.4. It must be noted that a noncompartmental analysis (NCA) approach was used to calculate PZA and POA exposures in each animal species. This approach has intrinsic limitations because in some of the PKs the sampling designs were sparse and also varied across animals, with outbred species being more prone to design-related NCA error. Although POA is eliminated rapidly in humans, NHP, guinea pigs, and mice, rabbits exhibit distinct kinetics of POA metabolism, leading to higher exposure to POA than to PZA in plasma and lung tissue over a 24 h period. This finding is in agreement with early reports of high pyrazinamidase activity in rabbit compared to that in mouse liver homogenates.[54] In addition, rabbit liver homogenates were reported in 1953 "to be relatively deficient in xanthine oxidase".[55] Early PK studies demonstrated increased exposure of POA when allopurinol was administered prior to POA in dogs or prior to PZA in human volunteers.[39,56] In agreement with these observations, the coadministration of allopurinol with PZA or POA in mice and rabbits confirmed that the conversion of POA to 5-hydroxy-POA by xanthine oxidase significantly contributes to POA

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elimination in mice but not in rabbits. Such high POA exposure presents a unique opportunity to study the efficacy of exogenous POA, i.e., POA not made inside the bacillus but provided via blood supply and tissue distribution, against TB in vivo. On the basis of the POA/PZA exposure ratio measured in rabbits (Figure 2), the oral administration of human-equivalent doses of PZA to rabbits infected with a *pncA M. tuberculosis* mutant is expected to deliver efficacy results comparable to those of PZA if indeed exogenous POA can inhibit or kill *M. tuberculosis* bacilli in vivo.

Direct dosing of POA has been proposed as an alternative to PZA in order to overcome drug resistance mediated by mutations in *pncA*.[57] Because of the suspected poor oral bioavailability of POA, pulmonary delivery was suggested as a development strategy. Indeed, POA is more polar than PZA, is largely ionized at physiological pH, and is therefore expected to exhibit poor passive diffusion and poor permeability through biological membranes. However, the dissolution kinetics and absorption of weak acids along the gastrointestinal tract are functions of multiple factors (including the formulation composition, buffering capacity, ionic strength, and pK_a of the drug) and hence are difficult to predict. Given the promising POA oral bioavailability shown here in mice, rabbits, and guinea pigs, consideration should be given to the preclinical evaluation of oral POA.

We have demonstrated that POA resulting from host metabolism in humans and in major animal species distributes into lung and lesion tissues, thus reaching the site of infection. In addition, POA is orally bioavailable in preclinical models, albeit with a short half-life which can be significantly extended by preadministration of the xanthine oxidase inhibitor allopurinol. Our results provide a series of tools to measure the efficacy of POA and characterize resistance development. If confirmed, POA as an add-on or as a substitute for PZA could be a successful therapeutic strategy to circumvent *pncA*-mediated resistance and prevent the emergence of resistance to POA/PZA through mutations in POA targets. Co-dosing of a xanthine oxidase inhibitor such as allopurinol could also be considered to reduce clearance and boost exposure of POA while decreasing the exposure of 5-OH-POA, which is shown to be primarily responsible for PZA-induced hepatotoxicity.[58] Formulation, tolerability, and pharmacokinetic/pharmacodynamic studies are needed to support phase I–II trials of POA and potentially salvage one of the most sterilizing anti-TB drugs.

METHODS

Human Subjects. Adults with pulmonary MDR-TB scheduled for elective lung resection surgery were asked to participate in the study "Pharmacokinetics of Standard First and Second Line anti-TB Drugs in the Lung and Lesions of Subjects Elected for Resection Surgery" (www.ClinicalTrials.gov, no. NCT00816426). The institutional review boards of the National Institute of Allergy and Infectious Diseases; National Institutes of Health, Bethesda, Maryland, USA; and hospitals Pusan National University Hospital, Pusan, Republic of Korea (ROK); Asan Medical Center, Seoul, ROK; and National Medical Center, Seoul, ROK all approved the study. The procedures followed were in accordance with the ethical standards of the Helsinki Declaration. Consenting subjects were randomized to receive three first-line and two second-line anti-TB drugs including 1500 mg of PZA concomitantly at one of five predetermined times 2 to 24 h prior to the anticipated pulmonary vessel ligation during surgery. Subjects were followed for 1 month postsurgery for drug-related adverse effects. All patients were under treatment for multi-drug-resistant TB and had at least one prior episode of TB treatment (median episodes 1.5, range 1 to 8). The median age was 45 (range 23 to 59), nine were male and all were Asian. The weight range was 49.8 to 84.0 kg, with a median of 57.8 kg. Mean weights (±SD) were 65 ± 12 and 53 ± 3 kg for males and females, respectively. Table S2 summarizes patient demographics.

Animals and Ethics Assurance. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, with approval from the Committee on the Ethics of Animal Experiments of the National Institute of Allergy and Infectious Diseases (*Callithrix jacchus* or common marmosets and *M. bovis*-infected New Zealand White (NZW) rabbits), the Institutional Animal Care and Use Committee of the New Jersey Medical School, Newark, NJ (NZW rabbits and CD-1 mice), Colorado State University's Institutional Animal Care and Use Committee (C3HeB/FeJ mice), and the Institutional Animal Care and Use Committee of the Johns Hopkins University (C3HeB/FeJ mice and guinea pigs). All animals were maintained under specific pathogen-free conditions and fed water and chow ad libitum, and all efforts were made to minimize suffering or discomfort. Studies in *M. tuberculosis*- and *M. bovis*-infected animals were performed in biosafety level 3 facilities approved for the containment of *M. tuberculosis*.

Pharmacokinetic Studies. *Clinical Study.* Blood was collected in a Li-heparinized tube for plasma preparation just prior to drug administration, 2 and 6 h postdose, at the time of central line placement, and at the time of vessel ligation. Two or 6 h samples that overlapped in time with the surgery samples were not collected twice. Samples were collected in 6 mL Na-heparin tubes and inverted 8 to 10 times. They were kept at room temperature for no more than 30 min prior to centrifugation at 1000*g* units for 10 min. Plasma was recovered and kept frozen at −80 °C until assayed. PZA and/or POA were quantified by HPLC coupled to tandem mass spectrometry (LC/MS-MS).

Rabbits. Groups of four female New Zealand white naïve rabbits (uninfected) received a single dose of PZA (150 or 300 mg/kg as indicated) or POA (200 mg/kg). In selected studies, allopurinol (in 0.5% carboxymethyl cellulose) was predosed daily on 3 consecutive days at 40 mg/kg and immediately prior to PZA dosing on the day of the PK study (day 3). PZA was prepared in 40% sucrose/10% PEG400, and POA was formulated either in 40% sucrose (delivering a cloudy suspension) or in water adjusted to pH 7.0 with NaOH (delivering a clear solution). These two formulations were used to verify that exposure remained unchanged whether POA was delivered as a solution or suspension. Blood samples were collected in heparinized tubes predose and at 0.5, 1, 2, 4, 6, 7–8, and 24 h postdosing. Specific pathogen-free, individually housed female NZW rabbits, weighing 2.2 to 2.6 kg, were used for aerosol infection by *M. tuberculosis* HN878 or *M. bovis* AF2122, as previously described.[59,60] The two strains were selected because they are known to generate a representative range of humanlike lesions in infected rabbits. Briefly, rabbits were exposed to *M. tuberculosis*- or *M. bovis*-containing aerosol using a nose-only delivery system. Three hours postinfection, three rabbits were euthanized, and serial dilutions of the lung homogenates were cultured on Middlebrook 7H11 agar plates

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to enumerate the number of bacterial-colony-forming units (CFUs) implanted in the lungs. The infection was allowed to progress for 4 weeks (*M. bovis*) or 12 to 16 weeks (*M. tuberculosis*). Groups of three or four infected rabbits received a single dose of PZA (125 or 250 mg/kg as indicated) prepared in 40% sucrose/10% PEG400 or in a pediatric suspension.[60] Blood samples from naïve and infected rabbits were collected in EDTA-coated or lithium heparin tubes predosed and at 0.5, 1, 2, 4, and 6 h postdosing. Samples were inverted 8 to 10 times and kept on ice for no more than 10 min prior to centrifugation at 2000*g*−3000*g* units for 10 min to recover plasma and quantify PZA and/or POA by LC/MS-MS.

Mice. Groups of three CD-1 (outbred) and BALB/c (inbred) female mice 4 to 6 weeks of age, weighing 20−22 g, received a single dose of 150 mg/kg PZA or POA prepared in 40% sucrose/10% PEG400. In selected PK studies, allopurinol (in 0.5% carboxymethyl cellulose) was predosed orally 30 to 60 min prior to PZA or POA administration, according to the regimens and doses specified. Blood samples were collected in plasma separator tubes containing lithium heparin (Becton, Dickinson and Co.) predosed and 15 min, 30 min, 1 h, 1.5 h, 3 h, 5 h, 7−8 h, and 24 h postdose. Samples were kept on ice for 30 to 60 min prior to centrifugation at 10 000 RCF for 2 min and then kept frozen at −80 °C until assayed for PZA and/or POA concentration by LC/MS-MS. Groups of three to four 6-week-old uninfected inbred female C3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, ME) received a single 150 mg/kg dose of PZA. The drug was prepared in water warmed to 70 °C to facilitate dissolution. (The stability of PZA at this temperature was verified by LC/MS-MS.) Blood samples were collected predose and at serial time points from 5 min to 12 h postdose. For the PK study in *M. tuberculosis*-infected mice, 6- to 8-week-old C3HeB/FeJ mice were infected with strain Erdman (TMCC107) as previously described[61] by low-dose aerosol infection using an inoculum of 1.5×10^6 CFU/mL to achieve a deposition of 25 to 75 CFU in the lungs. At 8 weeks postinfection, 35 mice received a single dose of 150 mg/kg PZA via oral gavage. Blood was collected in lithium heparin tubes from groups of five mice at 5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 3 h, 5 h, and 8 h postdose. Samples from naïve CD-1, naïve C3HeB/FeJ, and infected C3HeB/FeJ mice were kept at room temperature for 15 to 30 min prior to centrifugation at 400*g* to 800*g* units and kept frozen at −80 °C until assayed.

Guinea Pigs. Female outbred Hartley guinea pigs (250−300 g) with jugular vein vascular catheters were purchased from Charles River Laboratories (Wilmington, MA). Separate groups of four catheterized guinea pigs each were given a single dose of PZA or POA at 300 mg/kg. Doses were prepared in water in a final volume of 0.5 mL and delivered in the posterior oropharynx by an automatic pipet with a disposable tip.[62] Blood (∼0.4 mL) was drawn serially from guinea pigs through the intravenous catheter at the following time points after antibiotic dosing: 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, and 24 h. Blood was collected in lithium heparin tubes (BD Microtainer). Samples were kept at room temperature for 30 to 45 min prior to centrifugation at 2000*g* units for 5 min and kept frozen at −80 °C until analyzed for POA or PZA concentration determination by LC/MS-MS.

Marmosets. Uninfected marmosets (2−6 years of age, weight range 375 to 480 g) received a single dose of 125 mg/kg PZA prepared in ORA-PLUS/ORA-SWEET/grape flavoring compounded 48:48:4% (Paddock Laboratories, Minneapolis, MN). Blood samples were collected in lithium heparin blood collection tubes predose and at 1.5, 3, 6 and 24 h postdose. They were inverted 8 to 10 times and kept at room temperature for no more than 20 min prior to centrifugation at 2000*g*−3000*g* for 10 min, and then plasma supernatants were transferred to cryovials and flash frozen on dry ice. Frozen samples were kept at −80 °C until assayed.

Sample Processing and Analytical Method. Individual lung granulomas and pieces of uninvolved lung tissue were weighed and homogenized in approximately but accurately recorded 5 volumes of phosphate-buffered saline (PBS). Homogenization was achieved using a FastPrep-24 instrument (MP Biomedicals) and 1.4 mm zirconium oxide beads (Precellys). Proteins were precipitated by adding 9 volumes of 1:1 acetonitrile/methanol containing 0.5 μg/mL of pyrazinamide-^{15}N-D3 or pyrazinecarboxylic acid-D3 (Toronto Research Chemicals, Inc.) as internal standards to 1 volume of plasma or a homogenized tissue sample. The mixtures were vortex mixed for 5 min and centrifuged at 4000 rpm for 5 min. The supernatant was then transferred for LC/MS-MS analysis. PZA and POA standards were obtained from Acros and Sigma-Aldrich, respectively. LC/MS-MS analysis was performed with an Agilent 1260 system coupled to an AB Sciex 4000 Q-trap mass spectrometer (positive-mode electrospray ionization) and an Agilent SB-C8 column, 4.6 × 75 mm^2, 3.5 μm, with the column temperature fixed at 24 °C. Mobile phase A was 0.1% formic acid in 100% H_2O, and mobile phase B was 0.1% formic acid in 100% acetonitrile. Injection volumes were routinely 2 μL. -The mass-selective detector was set to MRM (multiple reaction monitoring) mode using positive polarity ionization, monitoring for the ions of interest (m/z 124.0/81.1 for PZA; m/z 125.2/81.1 for POA) and the internal standards (m/z 128.2/84.1 for PZA-N15-d^3 and POA-d^3). The lower limit of quantification was 0.1 μg/mL for PZA and 0.5 μg/mL for POA. Predicted *c* log *D* values of PZA and POA were calculated using the ACD/Laboratories Percepta Platform - PhysChem Module (ChemSpider resources).

Pharmacokinetic Modeling. All PZA and POA pharmacokinetic data were modeled using a compartmental approach and nonlinear mixed effect methodology as implemented in the NONMEM software (version 7.2). This approach was adopted because it allowed the simultaneous modeling of both parent and metabolite data and was able to deal with the different PK study designs used in different species, which would not have been possible with a noncompartmental modeling strategy. Concentration values below the lower limit of quantification were excluded from the pharmacokinetic evaluation. For all species, a model for PZA was developed first, metabolite POA data were then added, and finally the PZA and POA data were analyzed simultaneously. The basic model structure was a one-compartment disposition model. The final model was parametrized using the oral clearance (CL/F), the oral volume of distribution (V/F), the clearance of the metabolite corrected for the fraction of metabolized drug (CL_m/F_m), and the volume of the metabolite corrected for the fraction of metabolized drug (V_m/F_m). The individual parameters were assumed to be log-normally distributed, and the residual variability was described with the proportional error model. The model building process was guided by the likelihood ratio test, diagnostic plots, and internal model validation techniques, including visual and numerical predictive checks. Given the small group sizes, variability estimates could not be determined and thus empirical Bayesian estimates were not used. Final models and

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model parameters were used to simulate concentration time profiles and AUC integration for PZA and POA at several dose levels and infer the human-equivalent dose based on AUC.

In Vitro Growth Inhibitory Assay. *M. tuberculosis* strains H37Rv and H37Ra (ATCC 25177) were used to measure growth inhibition in the presence of increasing doses of PZA and POA using a standardized turbidometric assay. The choice of strains is based on the observation that extensive PZA and POA mechanism of action studies have been conducted in both H37Rv and H37Ra,[17,18,23,28,63,64] with no notable discrepancies with regard to potency and factors influencing potency. In addition, the *pncA* DNA sequence is 100% conserved in H37Ra (MRA_2058) and H37Rv (Rv2043c). *M. tuberculosis* was grown in Middlebrook 7H9 liquid medium (BD Difco) supplemented with 10% albumin-dextrose-catalase (ADC), 0.5% glycerol, and 0.05% Tween-80 (Sigma) in T25 culture flasks at 37 °C at 80 rpm. PZA and POA (pyrazinecarboxylic acid, Sigma-Aldrich) were freshly dissolved in 90% DMSO at a concentration of 500 mM and sterilized using a 0.2 μm PTFE membrane filter (Acrodisc PALL). Serial 2-fold dilutions of the compounds were prepared in either standard 7H9 broth or 7H9 broth adjusted to pH 5.5, 5.8, or 6.5 with HCl and filter sterilized in order to achieve a range of final concentrations from 0 to 492 μg/mL. The media were inoculated with mid-log-phase *M. tuberculosis* H37Rv or H37Ra cultures at a starting $OD_{600\ nm}$ of 0.01 to 0.05, corresponding to a density of 5×10^5 to 2×10^6CFU/mL (no inoculum effect could be detected up to 2×10^6 CFU/mL). Each set of assays included a drug-free control at the same pH. These were then incubated at 37 °C and 200 rpm, and the turbidity was measured after 7 days (Ultrospec 10 cell density meter, Amersham Biosciences). Assays were performed as three independent replicates, and the results of the best two are shown, with each data point showing the average and standard deviation of three turbidity measurements.

To determine the susceptibility of *M. tuberculosis* to PZA and POA on solid media, 10^4 CFU from mid-log-phase *M. tuberculosis* H37Rv or H37Ra cultures was plated onto 7H10 agar plates supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) with either PZA or POA (concentrations in the range of 0.2−8 mM) in replicates. Drug-free controls were included in each assay set. The plates were incubated at 37 °C and screened for growth inhibition after 4 weeks as compared to the drug-free plates. The "agar MIC" reported here is the concentration of PZA/POA which inhibits colony formation after 4 weeks.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/id500028m.

Minimum inhibitory concentrations of PZA and POA in liquid and on solid media against *M. tuberculosis* H37Ra and H37Rv.

Demographics of the 14 Korean subjects.

Concentration−time profile of PZA and POA metabolites following oral administration of 150 mg/kg PZA.

Growth inhibition of *M. tuberculosis* H37Ra (A, B and E, F) and H37Rv (C, D and G, H) by PZA (A−D) and POA (E−H) at various pH values.

Individual concentration−time profiles of POA following oral administration of 300 mg/kg POA to naïve guinea pigs.

Effect of allopurinol predoses on the plasma concentration−time profiles.

Proposed model to explain the apparent disconnect between in vitro potency and the unique sterilizing activity of PZA in vivo.

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Author Contributions

LEV, SE, ML, TSS, JSC, WK and CEB coordinated the clinical pharmacokinetic studies in Korean subjects; LEV and DW conducted infected rabbit PK studies; RS analyzed and modeled all PK data; MDZ and BP quantified drug contents in plasma and tissue samples; SMI, EL, AL, JPL and EN coordinated and conducted mouse PK experiments; POB performed naïve rabbit PK experiments; PK and NKD coordinated and conducted guinea pig PK experiments; PG performed MIC experiments; CEB is the principal investigator for the clinical research protocol; CEB, RS and VD designed the experiments. VD, LEV and CEB wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PZA, pyrazinamide; POA, pyrazinoic acid; CFU, colony-forming units; LC/MS-MS, high-pressure liquid chromatography coupled to tandem mass spectrometry; AUC, area under the concentration−time curve

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