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Review

Impact of the host environment on the antitubercular action of pyrazinamide

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ABSTRACT

Pyrazinamide remains the only drug in the tuberculosis pharmacopeia to drastically shorten first-line therapy from nine to six months. Due to its unparalleled ability to sterilize non-replicating bacilli and reduce relapse rates, PZA is expected to be irreplaceable in future therapies against tuberculosis. While the molecular target of PZA is unclear, recent pharmacokinetic studies using small animal models and patient samples have highlighted the importance of host metabolism and immune responses in PZA efficacy. Delineating which host factors are important for PZA action will be integral to the design of next-generation therapies to shorten current TB drug regimens as well as to overcome treatment limitations in some patients. In this review, we discuss evidence for influence of the host environment on PZA activity, targets for PZA mechanism of action, recent studies in PZA pharmacokinetics, PZA antagonism and synergy with other first-line anti-TB drugs, and implications for future research.

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is responsible for an estimated 1.6 million deaths each year [1]. As the case fatality rate for untreated TB is approximately 70%, the discovery and integration of combinatorial drug therapy in clinical practice marked a major milestone in the fight against TB. While first-line treatment of drug-susceptible TB achieves cure rates of up to 95%, success rates involving second-line treatments for drug resistant TB are on the order of 50% [1,2]. Thus, a comprehensive understanding of the factors that underlie treatment success and failure are paramount in advancing toward global eradication of TB.

Pyrazinamide (PZA) is an irreplaceable drug used for treatment of both drug-susceptible and multidrug-resistant TB infections. Discovery efforts that led to identification of PZA were inspired by Vital Chorine's research on nicotinamide (vitamin B3) as a treatment for leprosy [3]. Chorine and others [4] found that treatment of *M. leprae* infected rats and mice with high concentrations of nicotinamide prolonged their survival. Chorine translated these findings to TB infected guinea pigs and demonstrated that subcutaneous administration of nicotinamide similarly delayed disease progression.

Eager to contribute other antitubercular drugs to the growing TB pharmacopeia, Lederle Laboratories of American Cynamid [5] and Merck Laboratories [6] developed numerous pyrazine analogues of nicotinamide with the hope that they would show increased potency compared to the parent compound. Contrary to standard practices for drug development and validation, these pyrazine analogues were exclusively screened in TB infected mice without prior in vitro susceptibility testing. Both laboratories found that the most active pyrazine analogue against *M. tuberculosis* was PZA. Testing in animals proved to be fortuitous as PZA showed potent activity in vivo paradoxical to inactivity of the drug on pure cultures of *M. tuberculosis* at near neutral pH. Future experimentation by McDermott and Thompsett would show that the culture medium needed to be mildly acidic (pH of 5.8) for PZA to inhibit growth of *M. tuberculosis* [7]. Subsequent studies revealed enhanced PZA activity in the presence of alkaline pH [7], low temperature [8], nutrient limitation [9], and hypoxia [10]. Further testing of experimentally infected mice and guinea pigs by Malone [11], Dessau [12] and colleagues showed that PZA treatment resulted in decreased lung pathology and showed superior antitubercular activity compared to *para*-aminosalicylic acid and nicotinamide. Clinical evaluation of PZA treatment in forty-three TB infected patients at Summit Park Sanatorium commenced from 1949 until 1951 [13]. Yeager, Monroe, and Dessau noted improvement of symptoms and disposition in patients treated with PZA, even those with infections that were resistant to streptomycin. However, rapid resistance to

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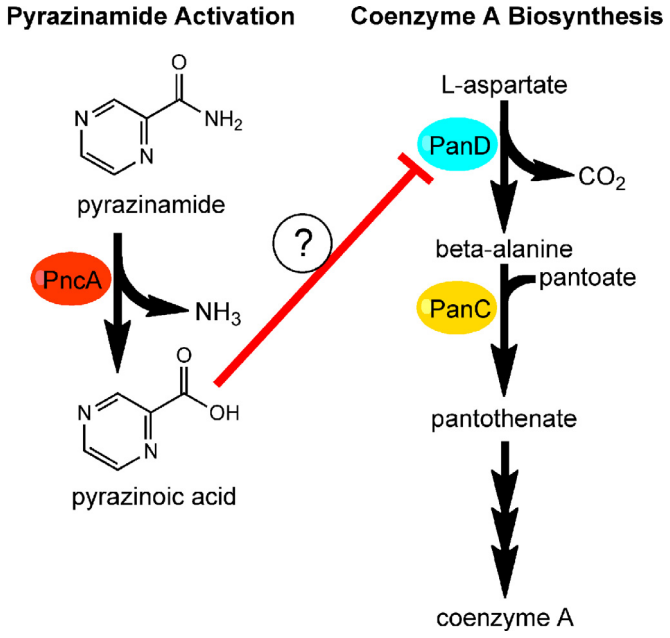

Fig. 1. Working mode for pyrazinamide action. Pyrazinamide is activated by PncA to pyrazinoic acid (left). Pyrazinoic acid impairs coenzyme A metabolism possibly through interaction with PanD. Intermediates of coenzyme A synthesis antagonize pyrazinamide and pyrazinoic acid activity.

PZA was observed in patients, particularly those with large cavitary lesions. Favorable outcomes from PZA treatment led to the rapid inclusion of PZA in second-line therapy for drug resistant TB.

PZA was considered in the combinatorial first-line therapy after reports of synergistic activity between PZA and isoniazid (INH) in experimentally infected mice [14] and TB patients [15]. The addition of PZA to first-line therapy dramatically reduced relapse rates and enabled treatment shortening from nine to six months [16]. PZA has remained a cornerstone of TB therapy over the last 50 years and is projected to be a mainstay in future TB regimens. It is fortunate that PZA was first evaluated in infected animals as its inactivity in standard culture medium would have likely excluded this drug for further testing. The unique efficacy of PZA in vivo has also provided researchers with evidence of the importance of the host environment in antimicrobial drug action.

While the majority of basic science research surrounding PZA has focused on molecular and biochemical aspects of its antitubercular activity, recent studies have highlighted the indispensable role of the host environment in the sterilizing action of PZA in vivo [17–21]. Defining which aspects of the host response enhance PZA activity will provide key insight for development of host-directed therapies as adjunctive treatments to current antitubercular drugs. In this review, we focus on proposed modes of action for PZA, recent pharmacokinetic studies using host cells, patient samples and small animal models, PZA antagonism and synergy by other first-line antitubercular drugs, and implications for future TB drug research.

2. Proposed molecular targets for PZA

PZA is a pro-drug that is hydrolyzed to its active form, pyrazinoic acid (POA), by the *M. tuberculosis* intracellular pyrazinamidase/nicotinamidase (PncA, Fig. 1) [22]. This enzyme, encoded by *pncA*, is involved in the salvage pathway for the production of nicotinamide adenine dinucleotide (NAD^+), a critical cofactor required for hundreds of biological reactions [23]. Since *M. tuberculosis* can also synthesize NAD^+ by the de novo pathway, the NAD^+ salvage pathway is non-essential for fitness in vivo [24,25]. Thus, loss-of-function mutations in *pncA* represent the primary mechanism of PZA resistance in clinical isolates. Diverse *pncA* mutations, including single and multi-nucleotide polymorphisms and indels, have been reported in PZA resistant clinical isolates along the entire 561 base pair gene length [26,27]. Recently, Yadon et al. [28] created a comprehensive library of PncA polymorphisms through saturating mutagenesis and identified over 300 substitutions, some of which were previously reported in clinical isolates, that conferred PZA resistance in vitro and in vivo. Interestingly, more PZA resistant substitutions were observed in the in vivo infection model, suggesting that there are unique selective pressures mediated by the host that are not captured by in vitro experimentation [28]. In addition, many non-synonymous substitutions that did not elicit PZA resistance were also observed. Biochemical and bioinformatic analysis indicated that most PZA resistance-conferring substitutions led to impaired PncA structure and stability or disrupted active site catalytic triad and iron coordinating residues [28]. While mutation of *pncA* is the primary molecular mechanism of PZA resistance, as much as 30% of PZA resistant clinical isolates encode wild type *pncA* and retain full PncA activity [29,30]. PZA resistant isolates with functional PncA indicate that there are other as yet undescribed resistance mechanisms.

Over the last twenty years, several mechanisms of action for PZA have been proposed [31–36]. Some models, such as POA-mediated acidification of the *M. tuberculosis* cytoplasm, inhibition of fatty acid synthase I (FAS-I), and inhibition of ribosome rescue have been challenged by multiple research groups [31,37–39]. Recently, numerous studies have shown that a component of the coenzyme A (CoA) biosynthetic pathway may be a molecular target of POA (Fig. 1) [40–45]. Analysis of spontaneous PZA and POA resistant isolates with functional PncA revealed missense mutations in *panD* that encodes L-aspartate-α-decarboxylase, a rate-limiting step in the CoA biosynthetic pathway [42,44]. In the CoA pathway, L-aspartate undergoes decarboxylation by PanD to form β-alanine (Fig. 1) [46]. PanC then catalyzes ligation β-alanine with pantoate to form pantothenate that is further modified to ultimately yield CoA (Fig. 1), an essential cofactor for numerous metabolic pathways [47]. While *M. tuberculosis* PanD shows sequence and structural similarity to other members of the PanD family, it has a unique 13 amino acid C-terminal extension [48]. The C-terminal extension appears to be a hot spot for POA resistance mutations [45]. In addition to isolation of *panD* mutant strains in vitro, Gopal et al. recovered POA resistant *panD* mutant strains (82% of POA resistant strains) from the lungs of mice that were infected with *M. tuberculosis* and treated with POA [41]. Mice infected with a *panD* missense mutant strain had bacterial lung burdens comparable to those infected with wild-type *M. tuberculosis* [41], indicating that *panD* mutant strains maintain fitness in vivo.

In support of PanD as a target of POA, Gopal et al. showed binding of POA to PanD ($K_D = 6.1\ \mu M \pm 0.88\ \mu M$), which was abrogated with resistant variants [40]. Treatment of wild-type *M. bovis* BCG with POA was also found to decrease CoA abundance after 24–48 h of exposure [42], and a mutant strain disrupted in the acyl-CoA ligase FadD2 showed heightened susceptibility to POA [49]. CoA depletion appears to be specific for POA as the structural analogues, nicotinic acid and benzoic acid, failed to significantly change CoA levels [40]. Multiple studies demonstrate that POA-mediated inhibition of *M. tuberculosis* growth can be antagonized by precursors of the CoA biosynthetic pathway (Fig. 1), such as β-alanine, pantothenate and pantetheine [42–44]. These data are consistent with a model in which POA is able to bind PanD and ultimately inhibit production of CoA. However, it is important to note that the pantothenate auxotrophic strain *M. tuberculosis* $mc^2$7000 ($\Delta panCD$) remains susceptible to PZA when cultivated with sub-antagonistic concentrations of panthetheine, indicating that there must be other factors in the CoA biosynthetic pathway involved in PZA-mediated

growth inhibition [43]. While data supporting PanD as a target of POA are intriguing, *panD* missense mutations identified in the laboratory have yet to reported in PZA resistant clinical isolates [50]. Additional in vitro, in vivo and clinical studies are essential to better understand the association between CoA metabolism and the sterilizing activity of PZA in TB therapy.

3. PZA and the host environment

While the majority of efforts toward dissecting PZA action have focused on its direct interaction with *M. tuberculosis*, recent studies have also explored host related aspects of PZA activity [51–54]. The current understanding of TB indicates that the disease presents as a spectrum of responses to infection rather than a simple binary distribution of pathology in the host [55]. Recent studies have shown multiple lesions types within a single host that vary in cell composition, microenvironment, metabolic activity, and bacterial burden [56]. Differences in microenvironments govern the support and suppression of *M. tuberculosis* subpopulations [57] as well as drug penetration, activity, and availability [17]. Ultimately, a few granulomas may dictate treatment responses and disease outcome. Understanding drug activity in different cell and lesion types is essential to optimize drug combinations, dosing, and timing.

Drug distribution and efficacy studies involving PZA have been conducted in macrophages, small animal models, patient sera and lung tissues collected from surgical resections. PZA is most efficacious during the first two months of therapy and it is generally assumed to target non- or slowly-replicating bacilli residing in acidic compartments such as the macrophage phagosome [58,59]. Studies with cultured macrophages have shown that PZA accumulates intracellularly within 3 h of exposure independent of the cellular metabolic state [60], suggesting that the drug can enter cells through passive diffusion. Despite this observation, PZA and POA antitubercular activity in macrophages range from no inhibition [61] to bacteriostatic [62] to sterilizing [63]. These disparate results stem from use of different experimental designs, including source and activation state of macrophages, drug concentration, and time of drug exposure. It is also important to note that culture conditions, such as the inclusion of fetal bovine serum [64] and carbon dioxide [65], can be antagonistic for the antitubercular activity of PZA and POA.

Several therapeutic drug monitoring studies have been conducted to assess the concentration of circulating PZA and POA in plasma and the impact of disease status on drug absorption and clearance. Clinical data indicate that the susceptibility breakpoint at which PZA therapy fails is 100 μg/mL [66], while PZA peak plasma concentrations are 20–60 μg/mL at 2 h post-dose in most patients [67]. Plasma clearance of PZA ranges from 1.7 L/h to 3.42 L/h, whereas the rate for POA is approximately 1.9 L/h [21,68]. There also appears to be slow and fast absorbers of PZA [68]. PZA pharmacokinetic studies in children with pulmonary TB demonstrate a slower rate of absorption and clearance compared to adults when dosage is based on body weight (mg/kg) [69]. Amendments to the WHO guidelines for drug dosage in children recommended increased concentrations of antitubercular drugs and the PZA range was changed to 30–40 mg/kg [70]. The few studies that have conducted therapeutic drug monitoring in children using the revised WHO guidelines report that target plasma PZA concentrations were typically achieved [66–68].

Absorption and clearance of PZA in the context of TB-HIV co-infection is less clear. Some studies report that co-infection with HIV reduces PZA plasma concentrations [69,71,72]; while others state that no significant differences are observed between TB only and co-infected patients [73,74]. Factors that may influence disparate drug concentrations include nutritional status [75], access to antiretroviral therapy, and state of immunosuppression. Few studies have examined disease outcome with respect to low PZA and POA concentrations in the plasma. Three studies within the last eight years have shown an association between low serum concentrations of PZA and poor disease outcome [76–78]. For example, Chideya et al. [76] reported that patients with inadequate PZA levels in serum (adjusted for HIV status and $CD4^+$ *T* cell counts) were at three times greater risk for poor outcome compared to patients with normal concentrations of PZA. However, data collected by Park et al. [79] indicated that low serum concentrations of isoniazid rather than PZA had a slight association with TB recurrence and drug resistance.

While activation of PZA to POA is typically attributed to hydrolysis by *M. tuberculosis*, recent evidence indicates that TB-independent host-mediated activation may be relevant for drug action [21]. Following oral administration of PZA to uninfected mice, guinea pigs, rabbits and primates, a substantial level of POA could be detected in plasma [21]. Further, POA was found to penetrate and accumulate within the lung and pulmonary lesions of rabbits infected with *M. bovis* lacking PncA activity [21]. While PZA shows no activity against most other PncA deficient mycobacteria [53], additional studies are required to discern whether host mediated activation of PZA can be exploited to enhance exposure of tubercle bacilli to POA.

Small animal models have been informative in pharmacokinetic and pharmacodynamic studies involving antituberculosis drugs [80,81]. Murine models of TB infection have been extensively used in assessing TB drugs due to the availability of reagents, tractability of host genetics, cost-effectiveness, and ability to achieve statistical power [82–84]. The majority of classical murine models display one disease state in the TB spectrum [85,86]. BALB/C and C57/BL6 mice represent immunocompetent hosts that experience a prolonged chronic infection [87,88]. BALB/C and C57/BL6 mice develop aerobic and diffuse lesions that fail to progress to an advanced state, which is atypical in human TB infection [89,90]. The inability of BALB/C and C57/BL6 mice to capture the full gamut of granuloma heterogeneity has led researchers to explore other small animal models for TB drug studies. One such model, C3HeB/FeJ mice (also referred to as the Kramnik model), forms necrotic, hypoxic, liquefying granulomas followed by occasional cavitation in response to *M. tuberculosis* challenge due to a mutation in the *Ipr1* (intracellular pathogen resistance) isoform of the *Ifi75* (interferon-inducible-75) gene [91]. Drug efficacy studies pioneered by Anne Lenaerts and colleagues using the Kramnik mouse model showed a refractory response to PZA monotherapy at 7–8 weeks post *M. tuberculosis* infection [52]. Extension of the post-infection time course by six weeks prior to PZA monotherapy resulted in the separation of two distinct response groups within Kramnik mice; one group showed bactericidal effects similar to BALB/C control mice and the other group displayed little to no reduction in bacterial burden [92]. The lungs from Kramnik mice from the bactericidal group contained a relatively homogenous population of lesions comparable to BALB/C mice, while the other group developed a majority of necrotic, caseous lesions [92] despite having similar PZA concentration profiles in plasma and lesions [20]. PZA and POA were found to accumulate to within cellular and necrotic lesions by 0.5–1 h post-dose [17]. However, a higher concentration of PZA was distributed throughout lesions compared to POA. PZA penetration into lesions was also observed in TB infected rabbits [19]. Granulomas isolated from patients undergoing lung resection for drug refractory TB also showed PZA and POA distribution throughout the cellular cuff and caseum [93]. Combined, PZA and POA spatial distribution data in animals and human subjects suggest that PZA is able to penetrate into critical lesion compartments where recalcitrant populations of bacilli are thought to reside.

Given the importance of the host environment in PZA action, assessment of PZA within host compartments is imperative. The

pH of liquefied caseum in lesions from the PZA unresponsive Kramnik mice was near neutral (pH 7.4), which provides an intriguing explanation for PZA inactivity [17,92]. The pH of caseum in lesions from TB infected guinea pigs was also a circumneutral (pH 7.2) environment and is thought to be the basis for inadequate PZA treatment response in this model [20]. Data involving use of the in vitro hollow-fiber model of TB suggested PZA sterilizing activity may be primarily directed against extracellular bacteria found within acidic fluid lining the alveolar epithelium [94]. While acidic pH is not a strict requirement for PZA activity, acidic conditions may be important to drive PZA susceptibility in acellular environments that present limited host-induced stresses. Further supports for this concept comes from the observation that PZA promotes dramatic reduction of *M. tuberculosis* burden in immune competent mice, such as BALB/C and C57BL/6, which have cellular restricted bacilli [87,88]. *M. tuberculosis* restricted to cellular compartments of the host experience multiple immune mediated stresses irrespective of low pH. These conditions are likely responsible for enhanced PZA activity in vivo. In fact, multiplexed in situ imaging of cytokine and immune effector transcripts in TB infected Kramnik mice showed that cellular granulomas displayed networks, like *CD68-iNOS* (inducible nitric oxide synthase) and *IFNG* (gamma interferon), associated with a robust response against *M. tuberculosis* [95]. This is in stark contrast to enriched transcript signatures found in necrotic granulomas, such as *IL10* and *FOXP3*, which are associated with immunosuppression [95]. Additional studies using the Kramnik model should consider mapping immune transcripts in multiple granulomas that are responsive and refractory to PZA treatment.

In addition to Kramnik mice, TB infected athymic nude mice showed similar PZA inactivity after 7–8 weeks of treatment [53]. While the basis for this drug inactivity has not been elucidated, it is consistent with the need for macrophage activation and sufficient phagosomal acidification for PZA to be efficacious against intracellular *M. tuberculosis* [62]. It has also been demonstrated that PZA can dampen expression of pro-inflammatory cytokines and chemokines [54]. Some studies have suggested that PZA potentiation by the host may be related to drug upregulation of the autophagy pathway [96]. Multiple studies have reported the occurrence of dysregulated autophagy during diseases that cause altered immune states [97–100] While many outstanding questions remain, these studies indicate that the appropriate cell-mediated responses are critical for optimal PZA efficacy, and both local and systemic immune modulation are highly impactful for PZA efficacy.

It was recently hypothesized that some antituberculosis drugs fail to diffuse through lesions due to caseum macromolecule binding. There was no discernable amount of PZA binding to caseum in contrast to another TB drug, bedaquiline [101]. Caseum binding provides a rationale as to why PZA can distribute throughout lesions as opposed to drugs like bedaquiline [17], which are retained in the cellular regions surrounding the caseum. Collectively, these exciting data suggest that lung pathology heterogeneity and lesion microenvironment have a dramatic influence on PZA efficacy and are important considerations for future TB drug discovery and design [102].

4. Other considerations

Another intriguing aspect of PZA is its paradoxical relationship to another first-line TB pro-drug, isoniazid (INH). Although PZA and INH are structurally similar and appear to target different metabolic populations of *M. tuberculosis*, INH has been shown to antagonize PZA action in vivo in a dose dependent manner [103–105]. It is well established that INH is activated via the *M. tuberculosis* catalase-peroxidase KatG to form an isonicotinoyl radical [106–110]. This radical reacts with NAD^+ to produce an INH–NAD adduct that is a potent inhibitor of the enoyl-ACP reductase (InhA) involved in mycolic acid biosynthesis [111,112]. It is possible that INH accelerates metabolism and clearance of PZA. Alternatively, INH may more directly interfere with PZA action at the site of the bacilli. Pharmacokinetic studies measuring the concentrations and distribution of PZA and POA within lesions isolated from mice co-treated with PZA and INH may shed light on the mechanism behind this antagonism.

Interestingly, the addition of INH to combination therapy with rifampicin (RIF) and PZA has an ameliorating affect in reducing hepatotoxicity in uncomplicated active TB cases [113]. This observation is in stark contrast to the severe hepatotoxicity and occasional fatalities noted in individuals treated with a combination of RIF and PZA for latent TB infection [113–116]. Hepatotoxicity is thought to occur due to the accumulation of toxic, reactive drug metabolites [117] or drug-metabolite adducts within the liver [118–121]. Drug-metabolites may impair critical cellular functions [122,123]. For example, POA is hydroxylated by the host xanthine oxidase to form 5-hydroxypyrazinoic acid (5-OH-PA) [21,124], which was shown to be cytotoxic in Hep-G2 cells [125] and has been correlated with hepatotoxicity in rats [117,125] and TB patients [126]. Co-administration with the amidase inhibitor, bis-*p*-nitrophenyl phosphate, reduced or eliminated PZA induced hepatotoxicity in *M. tuberculosis* infected rats [125]. This observation suggests that limiting host-mediated conversion of PZA to POA and subsequent formation of 5-OH-PA may suppress deleterious side-effects of PZA. Future studies examining INH, RIF, and PZA mono- and combination treatment in TB infection animal models should consider measuring the accumulation of PZA drug metabolites in the liver to explore important drug-drug interactions.

5. Outstanding questions and conclusion

Since its introduction into clinical use, PZA has become part of the bedrock of TB drug regimens. Early screening of pyrazine analogues in small animal models revealed that the host environment is an integral factor PZA efficacy. Recent studies show this drugs works optimally in conjunction with an antitubercular cell-mediated immune response and the granuloma microenvironment may help to drive PZA activity. Evidence provided by Anne Lenaerts and colleagues [17] show that PZA fails to sterilize *M. tuberculosis* in caseous granulomas with neutral pH. However, it is unclear if pH is the sole host stress that is required for drug activity or if other host responses may similarly potentiate PZA. It is possible that low pH may be required for PZA efficacy in the caseum but other host factors, including oxidative/nitrosative stress and nutrient limitation, may be sufficient at the periphery of the granuloma. In order to appreciate the diversity of host stresses with respect to spatial and temporal considerations, future studies should employ a combination of multiplexed in situ imaging of immune mRNAs and proteomics to map the landscapes of various granulomas after PZA treatment. Furthermore, studies should seek to characterize PZA metabolites in the caseum throughout the spectrum of TB pathology. Recently, an elegant study reported by Eric Rubin and colleagues [127] characterized the host proteome of multiple lesion types. This study showed that the centers of the granulomas contained antimicrobial peptides, reactive oxygen species, and pro-inflammatory signals, while the surrounding tissues had anti-inflammatory associated markers. Important host factors mediating PZA activity in vivo may also be identified through the use of mutant mouse strains with defined knockdowns of antimicrobial peptides and factors for cell-mediated immunity. Delineating which factors drive PZA action in vivo may reveal novel therapeutic approaches to further decrease the length of the TB drug regimen as well as illuminate the basis for PZA failure in some patients.

6. Search strategy and selection criteria

Data for this review were identified by searches of PubMed, MEDLINE, and references from relevant articles using the search terms “pyrazinamide”, “tuberculosis”, “host immune system”, “mode of action”, “pharmacokinetics”, and “mouse models.” Articles between 1945–2019 were included.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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