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PYRAZINAMIDE/PYRAZINOIC ACID RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*: RECENT FINDINGS AND IMPLICATIONS FOR IMPROVING THE TREATMENT OF TUBERCULOSIS

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Abstract. Pyrazinamide (PZA) is unique in that it is a component of the first line therapy for drug sensitive tuberculosis and in most current and experimental treatments also for multi drug resistant tuberculosis. Furthermore, PZA has been shown to help to ensure lasting cure and prevent relapse in shorter multi drug regimens. PZA is a prodrug. *Mycobacterial tuberculosis* (MTB) PncA enzyme activates the anti-mycobacterial prodrug PZA by transforming it into pyrazinoic acid (POA). The majority of clinical PZA resistant isolates contain mutations within the *pncA* gene and therefore remain sensitive to POA as they no longer activate PZA. Resistance to the active compound POA requires an alternative resistance mechanism and *in vitro* selected spontaneous MTB POA resistant mutants typically acquire a range of mutations in *panD* or mutations in one of a series of genes most of which are associated with the regulation of the bacterial stringent response. Clinically isolated PZA resistant MTB strains resistant to PZA and POA with mutations in any of these genes are unusual. Thus, it is likely the stringent response is critical for MTB *in vivo* and a damaged stringent response results in at least a reduction in fitness. Various lead compounds that disrupt the MTB stringent response have been identified that might form the basis for drugs with activity against latent mycobacteria with the potential to shorten tuberculosis treatment. Here we discuss the role of latency in the lifecycle of MTB and possible links to the activity PZA with a focus on potential new targets and drugs.

Key words: *Mycobacterium tuberculosis, drug resistance, pyrazinamide, pyrazinoic acid, latent tuberculosis.*

УСТОЙЧИВОСТЬ *MYCOBACTERIUM TUBERCULOSIS* К ПИРАЗИНАМИДУ/ПИРАЗИНОЕВОЙ КИСЛОТЕ: НОВЫЕ СВЕДЕНИЯ И ИХ ЗНАЧЕНИЕ ДЛЯ ПОВЫШЕНИЯ ЭФФЕКТИВНОСТИ ЛЕЧЕНИЯ ТУБЕРКУЛЕЗА

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Резюме. Пиразинамид (PZA) уникален тем, что является противотуберкулезным препаратом первого ряда как при лечении лекарственно-чувствительного туберкулеза, так и компонентом современных курсов лечения мультирезистентного туберкулеза. Также было показано, что PZA помогает обеспечить длительное лече-

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ние и предотвратить рецидив в более коротких схемах приема нескольких лекарств. Пиразинамид является неактивным пролекарством и фермент PncA *Mycobacterium tuberculosis* превращает его в активную форму — пиразиноевую кислоту (POA). Большинство клинических PZA-резистентных штаммов содержат мутации внутри гена *pncA* и поэтому остаются восприимчивыми к POA, поскольку не активируют PZA. Устойчивость к активному соединению POA требует альтернативного механизма резистентности, и полученные *in vitro* POA-резистентные спонтанные мутанты MTB имеют ряд мутаций в гене *panD* или в серии генов, большинство из которых связаны с регуляцией строгого ответа бактерий. Клинические штаммы MTB, устойчивые к PZA и POA с мутациями в любом из этих генов, являются нетипичными. Таким образом, вероятно, строгий ответ имеет важное значение для MTB в условиях *in vivo*, а нарушенный ответ приводит к снижению жизнеспособности микроорганизма. Были идентифицированы различные лекарственные соединения-прототипы, нарушающие строгий ответ MTB, которые могут стать основой для препаратов с активностью против латентных форм микобактерий с целью сокращения сроков противотуберкулезного лечения. В данном обзоре мы обсуждаем роль латентного периода в жизненном цикле MTB и возможные связи с активностью PZA с особым вниманием к потенциально новым мишенями и препаратам.

Ключевые слова: *Mycobacterium tuberculosis, лекарственная устойчивость, пиразинамид, пиразиноевая кислота, латентный туберкулез.*

Latency and the activity of (PZA) POA

The critical importance of bacterial latency on the epidemiology and treatment of tuberculosis is widely accepted [11]. Lethal infectious diseases as well as transient infections that result in protective immunity require a continuous supply of naive hosts to be maintained in a population. *Mycobacterium tuberculosis* (MTB) adopts a distinct strategy establishing active infections in only a small proportion of individuals and latent infection in the majority of the infected population. Latent TB infections may spontaneously clear, reactivate or die with the host. The continuing long-term success of *M. tuberculosis* is thus largely due to its ability to (undetected) spread in a population by establishing large numbers of slowly progressing incipient or dormant infections [14]. Subclinical MTB infections have the potential to transform into new transmittable active infections, predominantly in vulnerable populations, maintaining the epidemic in a human population over a long period [25, 37]. To establish a long-term latent infection requires the infecting mycobacteria to respond effectively to stress and to have the capacity to enter a dormant/latent phenotype (variously termed; latent, fat lazy, viable non-culturable, persister). The transition to these phenotypes thus appears to be critical for the long term success of MTB and we will argue here is closely linked to transmission dynamics, treatment outcomes, and probably also the emergence of drug resistant clones.

PZA is a pro drug which can be modified by the mycobacterial enzyme PncA to form the active compound POA. Recent reports demonstrate that resistance to pyrazinoic acid, a drug primarily active against stressed/dormant MTB *in vitro* can be caused by disrupting the stress responses resulting in the a failure to express the sensitive phenotype. As regulation of bacterial stress responses in this pathogen resulting in a dormant/latent phenotype is essential for the spread and survival of the MTB species, this form of resistance comes with a cost. And it is thus logical to investigate the disrupted stress responses seen in PZA and POA resistant strains, and use these data to identify potential targets for new drugs.

A further complication is neither PZA nor POA show any activity in routine culture, an effect is only seen when cultured bacteria are subjected to environmental stress. Typically an acidic growth medium is used for sensitivity testing, but a wide range of other stresses, that result in a switch to a latent/dormant phenotype, have a similar effect [30, 46]. In order to identify the target of POA multiple groups have generated POA resistant mutants *in vitro* and identified mutations in a range of genes, for example: *panD, clpC1*, and *gpsI* [26, 45, 55, 66, 67]. Recent evidence suggests *panD* is the primary target of POA [5, 26] therefore, other inhibitors of the pantothenate synthetase pathway [13] would be expected to have similar activity to PZA/POA against MTB under stressed conditions.

Apart from mutations in the likely primary target of POA *panD*, many of the mutations observed in spontaneous *in vitro* POA resistant mutants are the result of a damaged ability to enter the stressed state in which the activity of POA is inhibitory for bacterial growth [5, 26].

Disrupting the stringent response

Based on the range of genes identified in *in vitro* POA resistant spontaneous mutants the stringent response appears to play a key role in susceptibility to POA. The bacterial stringent response is a specific and very rapid cascade response to a change in environment. In *E. coli* the stringent response has been shown to be induced within seconds and is initiated by the accumulation of the so called stringent response alarmone (p)ppGpp [6]. This

response was detected 20 minutes after *M. tuberculosis* log-phase cultures were transferred into nutrient free buffer and (p)ppGpp declined to a new steady state by 90 to 120 min [57]. Interestingly, an enzyme (Gps1) involved in the metabolism of (p)ppGpp was recently suggested as a new target for POA after it was observed in 4 clinical PZA resistant isolates with wildtype *pncA*, *panD* and *clpC1* [45]. When this gene *(gps1)* was mutated in a sensitive strain the PZA MIC was increased. An altered enzymatic activity of mutated Gps1 in the presence and absence of POA was also demonstrated. Based on the activity of the mutant gene in the presence of POA the authors suggested Gps1 as yet another target of POA [45]. This may be correct but the role of *gps1* in the initiation of the stringent response suggests that absence of the wild type *gps1* may disrupt the regulation of entry into a fully POA susceptible phenotype [5].

Because of its importance for the regulation of latency and virulence in multiple species, the bacterial stringent response has already received attention as a potential drug target. A compound (relacin) structurally similar to the alarmone (p)ppGpp has been shown to disrupt the bacterial stringent response [64]. Relacin and related compounds are of interest also against MTB [7] but to our knowledge have not been investigated in detail.

In most bacteria the Clp protease complex is a non-essential ATP-dependent protease that regulates the response to various stresses. The Clp protease complex is composed of two heptameric sections ClpP1 and ClpP2 which are involved in substrate unfolding and breakdown into short peptides. In *M. tuberculosis* the ClpP1 ClpP2 complex is active when bound to either hexameric ClpX or ClpC1. In *M. tuberculosis* ClpX and ClpC1 are both essential and involved in substrate recognition and specificity [36, 49]. Along with *panD* point mutations in *clpC1* are among the most frequently reported mutations in *in vitro* selected *M. tuberculosis* POA resistant mutants [26, 66, 67].

It has been proposed that *clpC1* is involved in the stringent response by regulating CarD levels, a probable substrate of ClpP1P2 [48]. In *E. coli* DksA is a key regulator of the stringent response. Despite having little structural similarity mycobacterial CarD can functionally complement an *E. coli* DksA deficient mutant. Thus both DksA/CarD can work as general transcription factors when combined with

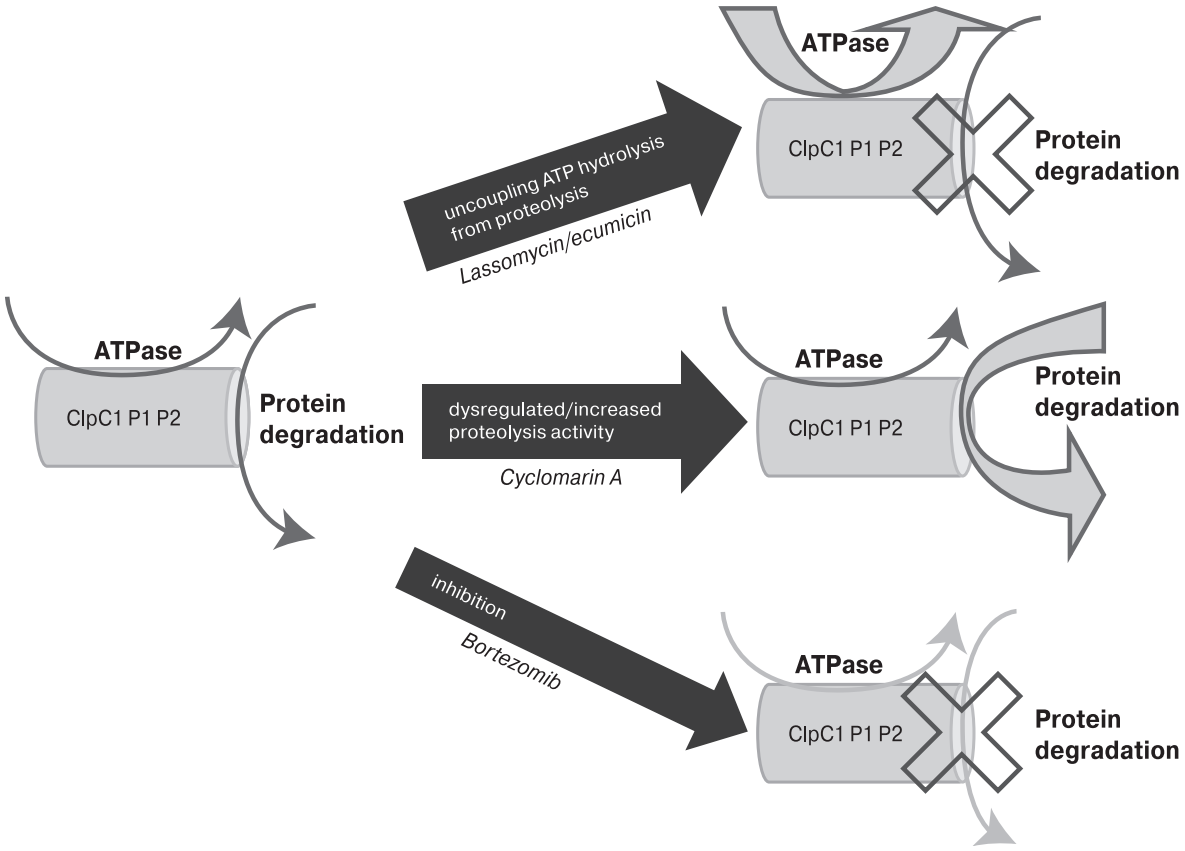

Figure. Overview of the different mechanisms of compounds known to disrupt the activity of the ClpP1P2 complex in *M. tuberculosis*

Proposed mechanisms are indicated in the straight arrows and compounds identified with this mechanism in italic below. ATPase and Protein degradation activity level is illustrated by the thickness of the curved arrows (left hand side normal activity), an X indicates inhibition

the stringent response alarmone (p)ppGpp to activate the stringent response [57]. It is therefore possible that the POA resistance associated mutations seen in *clpC1* are the result of a disrupted stringent response [5] due to dysregulation of the control of *carD* levels in the mycobacterial cell.

The absence of the Clp protease complex in eukaryotic cells and its requirement for normal growth of *M. tuberculosis* make this complex an interesting drug target [49]. The potential of this target is further supported by the fact that although *clpC1* mutants are resistant to both PZA and POA *in vitro*, *clpC1* mutants have, to our knowledge, never been observed in PZA resistant clinical isolates. Multiple lead compounds with activity against this protease complex with different mechanisms of action have been described [36] illustrated in Fig. and briefly described here: Cyclomarin A is a naturally occurring cyclic peptide isolated from a marine *Streptomyces* spp. which is active against mycobacteria with reportedly good specificity [53]. Cyclomarin A appears to act by binding to ClpC1 and dysregulating the proteolysis activity pf the MTB ClpC1P1P2 complex resulting in uncontrolled protein degradation [60]. Two other cyclic and looped peptides, lassomycin and ecumicin found after screening libraries of extracts obtained from actinomycetes, have also been reported to disrupt the activity of ClpC1P1P2 activity but by a different mechanism, uncoupling the ATPase activity from the proteolysis activity [20, 22]. These are far from being fully developed drugs but lassomycin demonstrated good specificity with activity against mycobacterial ClpC1 but none of the other bacterial ClpC homologs or eukaryotic proteases screened [22]. Finally, bortezomib is a compound which disrupts the MTB ClpC1P1P2 proteolytic catalytic sites [42]. Unfortunately, as this compound is used as a proteasome inhibitor approved by the U.S. FDA for the treatment of human multiple myeloma [1] it lacks (myco)bacterial specificity. However, recent work on derivatives of bortezomib, by the group who identified the potential of this compound, demonstrates scope for improving its specificity [43].

Trans-translation and PZA?

RpsA is a component of trans-translation, a rescue mechanism for stalled ribosomes. Although the role of *rpsA* mutations in PZA resistance is disputed, association studies of larger collections of clinical isolates does suggest some involvement [63]. RspA does not appear to be a target of PZA/POA, as recent work using laboratory mutants did not show any effect of PZA (POA) on trans-translation or the expression of RpsA [17], but RspA does seem to play a role in the susceptibility to PZA/POA. It has also been shown that overexpression of RpsA increases the PZA MIC [54]. Trans-translation is also closely linked to the stringent response and the available data supports that it is probable that disturbances in the balance of these interacting pathways, may disrupt entry into a latent, pyrazinamide-susceptible phenotype [5].

Inhibitors of trans-translation have been identified and at least one (KKL-35) was shown to be active against MTB under both aerobic and anoxic conditions [3], although others have questioned KKL-35's mode of action [10].

Based on this idea above we have suggested RpsA and trans-translation do not have a direct role in the mode of action of pyrazinamide but mutations in *rpsA* reduce the efficiency of a switch to a pyrazinamide-susceptible phenotype [5]. Interestingly, in 2018 a mutation in another gene lprG was associated with PZA resistance in 4 POA-resistant laboratory mutants [55]. The authors speculated this mutation probably results in a state of higher metabolism during *in vitro* culture that antagonizes PZA/POA activity *in vitro*, a suggestion that appears to be analogous with our speculation on the role of *rpsA* mutations [5].

Novel strategies to target PZA resistant *pncA* mutants?

As a clear majority of clinically PZA resistant isolates are resistant due to mutations in *pncA* and do not transform PZA into its active form POA [40] might it be possible to reverse this resistance? Although it has been frequently argued that the loss of the *pncA* gene has little or no cost this may not be the case [32]. NAD^+ is produced in *M. tuberculosis* by either the de novo or the salvage pathway. PncA is part of the NAD^+ salvage pathway. *M. bovis* PncA has dramatically reduced activity and as a result *M. bovis* is resistant to PZA and has a negative result in the niacin test [56]. The *pncA* gene in *M. bovis* has a single mutation (His57Asp) and does appear to retain some activity as *M. bovis* strains in which the de novo NAD^+ pathway is also deleted remain viable but are killed if starved of nicotinamide [62]. This indicates that either a (partially) functional de novo or active salvage pathway for NAD^+ is essential for the viability of the MTB complex. In support of this interpretation detailed work on these pathways suggests when the do novo pathway cannot function due to prolonged starvation recycling of NAD^+ by the salvage pathway prevents cell death [62]. This implies that the total loss of PncA activity, probable in a large proportion of PZA resistant *pncA* mutants as a result of AA substitutions frame shifts or even *pncA* gene deletion, will have a lethal cost under extended starvation.

Based on the argument above, either exposing the cost of a total loss of the salvage pathway or conversion of PZA to POA by another (host) pathway would be expected to restore sensitivity to PZA

in MTB PZA resistant mutants lacking any PncA activity. Therefore, inhibition of the *de novo* NAD^+ pathway should be lethal for PZA resistant strains with no PncA activity making this pathway a potential target for a large proportion of M(X)DR-TB isolates as suggested by Vilcheze et al. in 2010 [62].

Secondly, PZA is not only converted into the active form (POA) by bacterial PncA but also in the infected patient's liver by microsomal deamidase. POA is then further metabolized by human xanthine oxidase. It has been shown that compounds inhibiting human xanthine oxidase activity, such as allopurinol, result in increased the levels of circulating pyrazinoic acid [44, 61] but it does not appear to be known if this increase in circulating host-derived pyrazinoic acid would restore pyrazinamide activity against infecting bacteria with *pncA* mutations.

Preventing the accumulation of PZA resistance

The lack of PZA activity in routine culture and an incomplete understanding of the mechanism of action have added to the complexity of optimising the use of this compound in patients. The diversity of resistance mutations in *pncA* in PZA resistant clinical isolates suggests ongoing selection of PZA resistance in most settings [4]. This may be a particular problem for MDR-TB patients who are not detected as infected with resistant TB at diagnosis and receive first line therapy which is in fact likely to be monotherapy with PZA supplemented with ineffective drugs [65]. PZA is usually given only for the first two months of standard TB therapy because clinical trials conducted by the British Medical Research Council in the 1960s and 1970s did not detect any benefit of PZA beyond 2 months [19]. However, an effect of PZA beyond 2 months was seen in second line regimens in a murine model [2] and treatment of MDR-TB frequently includes longer periods of PZA exposure [59]. RIF and INH are very effective at clearing replicating bacilli in an infection whereas PZA is known to be most active against difficult to eradicate non-replicating mycobacteria therefore, PZA given at the end of therapy to eradicate any remaining bacteria instead of exposing the large numbers of bacilli at the start of therapy appears logical. Furthermore delaying the use of PZA in this way should reduce the chance of inadvertent monotherapy with PZA for yet to be identified M(X)DR-TB patients starting TB therapy [4]. To our knowledge, the utility of PZA at the end of therapy vs at the beginning of therapy has not been tested.

Recent insights into the pharmacological mechanism underlying PZA's unique clinical efficacy and modelling suggest a potential benefit of PZA beyond the first 2 months in some patients [9]. This combined with knowledge on the mechanism of action of PZA, which has become much clearer by the efforts of different groups in the past few years, should provide a basis for trials to explore the more optimal use of this drug in multidrug regimens as well as how to time and dose new drugs with related mechanisms of action.

The regulation of latency and the success of MTB strains

Latency is critical for the success of MTB in the population and in an individual patient. It is often stated that the infectious dose of TB is low possibly as low a single viable cell [52]. This may be true but caution is warranted as routine culture often misses > 90% of viable cells [18]. Also, the observation that infections with double MIRU-VNTR bands are mixed infections that can be transmitted between patients [33] provides circumstantial evidence that new infections are often the result of larger numbers of mycobacterial cells that preserve some of the genetic diversity present in the source case. This preserved diversity within the infecting bacterial population may ultimately be useful to identify more details of transmission dynamics [8, 33, 39]. Even more interestingly, it was recently observed that patients with a higher proportion of latent bacteria in their sputum appeared more likely to transmit the disease to close contacts [16]. At first sight this may appear to be a paradox but establishing a new infection is a critical step in the life cycle of *M. tuberculosis* and expressing a phenotype able to remain viable in the environment and initiate a new infection without provoking a lethal immune response from the host could well be an essential ability for continued success. It has long been recognised that microscopically the cells in patient's sputum appear different to those in culture, being slightly more elongated and with more apparent internal structure when stained with ZN or auramine O. These elongated cells with structure were studied in detail by Garton et al. (2008) [21] and termed "fat lazy bacteria". Presumably these bacteria are the subpopulation of cells that are transmitting in the study of Datta et al. (2017) [16]. Downregulation of growth in a large proportion of the mycobacteria in an infection thus appears to be normal and is probably important for efficient transmission.

The critical importance of persistence on the natural life cycle of MTB raises the possibility that different linages may have adapted their propensity to enter or exit latency as a survival strategy. The presence of bacilli simultaneously in different states in an active infection is assumed to be one of the primary reasons why the curative treatment of tuberculosis is so ineffective and requires at least 6 months to eliminate all the latent cells, even

Table. Potential drug targets and compounds of interest likely to be active against stressed mycobacteria based on our current understanding of PZA activity

Target/mechanism of action	Compounds of interest	Notes	Literature
Alarmone (p)ppGpp analogue	Relacin	Disrupt the stringent response shown to be active against MTB	[64] [7] [58]
Disruption of clpCP1P2 complex activity	Cyclomarin A Bortezomib Lassomycin Ecumicin	Disrupt the regulation of clpC targeted protein degradation to disrupt the stress response *(see Fig.)*	[53] [42] [22] [36]
Reversion of PZA resistance in *pncA* mutants	Alopurinol + PZA	Inhibit host degradation of POA to increase levels of host derived POA, restoring sensitivity in *pncA* mutants	[61] [44]
	unidentified inhibitor of the *de novo* NAD^+ pathway + PZA	Inhibition of the NAD^+ *de novo* pathway. Loss of PncA activity is predicted to make the *de novo* pathway essential	[62]
Avoiding the selection of *de novo* PZA resistance	PZA dosed differently/ given at the end of therapy	The diversity of *pncA* mutations suggests ongoing selection of resistance in some settings	[4] [9]
Disrution of trans-translation	KKL-35	Trans-translation has been shown to be essential for MTB and the activity of of KKL-35 agains MTB demonstrated but the mechanism of action of KKL-35 is disputed	[3] [10]
Alternative inhibitors of pantothenate synthesis	Unknown (sulfamoyl analogues)	Based on the ideas presented here would be expected to have similar activity as PZA	[13]

though an active tuberculosis infection is probably effectively "cured" within the first few weeks [27]. The majority of drugs used to treat tuberculosis appear to be at best only partially effective at eliminating latent bacteria. Two notable exceptions may be pyrazinamide (PZA) and high dose rifampicin but even these compounds probably do not rapidly eliminate all persistent MTB [28, 29]. Here the discussion focusses on the bacteria but this is certainly also in part due to the location of many of these less active mycobacterial cells in tissue or granulomas where the concentration of antimicrobials is suboptimal as a result of limited penetration [23], a situation that may also help amplify resistance. Furthermore, as an active MTB infection does not usually result in protective immunity [51] the immune system of even a "virtually cured" patient may be unable to eliminate even a few reactivating MTB cells that escaped the treatment.

Differences in the propensity of strains to enter a latent phenotype may result in some strains being more likely to rapidly breakdown into active disease with others being more likely to establish latent infections. An association with specific clades with treatment failure and drug resistance is established [24, 41] but the explanation for this association remains controversial [12, 35]. Although representatives of virtually all genotypes of *M. tuberculosis* have independently acquired multi-drug resistance by similar mechanisms it does appear that in many settings similar genotypes are more often associated with an MDR genotype than other genotypes. This may be chance, but as these differences between lineages are consistent between different geographical areas and because first line treatment of tuberculosis is highly standardised throughout the world, it is probable that certain genotypes are more able to develop resistance or are more likely to maintain their ability to spread and cause disease after having acquired resistance. There is tantalising evidence that variation in the initial bactericidal effect of antibiotic exposure plays a role in the development of resistance in *M. tuberculosis*: it has recently been observed that when different genotypes of *M. tuberculosis* are initially exposed to rifampicin the rate of killing and initial response differs [31, 34]. The increased ability to resist exposure to an antibiotic in the absence of a specific resistance mechanism (mutation) by a proportion of the bacterial cells in a population is termed persistence. It is likely these effects are linked to differing proportions of metabolically active cells in a nutrient rich environment for different genotypes [50], termed Class 1 persistence [38], possibly a result of differences in the "magic spot" setting of the stringent response [15].

Conclusions

In this paper we discuss the insights that mutations seen in in vitro PZA/POA resistant strains have provided regarding the formation of a latent/dormant phenotype of TB. The increasing amount of data on POA resistance mechanisms many of which appear to disrupt the formation of these phenotypes, provides an opportunity to determine the clinical relevance of blocking this phenotype switching and research compounds capable of specifically blocking the activity of the relevant enzymes.

In the discussion above we present a series of arguments that suggests the precise regulation of latency in MTB is of critical importance for the disease process, the development of resistance, as well as the epidemiology of tuberculosis. If the argument that PZA attacks latent cells is accepted this explains the value of PZA in (shortening) tuberculosis treatment regimens. We further suggest that it is likely the regulation of latency is disrupted in many unsuccessful POA resistant mutants, seen in culture but rarely in clinical isolates, and compounds that disrupt related targets (Table) would be expected to also help shorten treatment duration and prevent relapse if developed into drugs.

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Список литературы/References

1. Adams J., Kauffman M. Development of the proteasome inhibitor Velcade™ (Bortezomib). *Cancer Invest., 2004, vol. 22, no. 2, pp. 304–311. doi: 10.1081/CNV-120030218*
2. Ahmad Z., Tyagi S., Minkowski A., Peloquin C.A., Grosset J.H., Nuermberger E.L. Contribution of moxifloxacin or levofloxacin in second-line regimens with or without continuation of pyrazinamide in murine tuberculosis. *Am. J. Respir. Crit. Care Med., 2013, vol. 188, no. 1, pp. 97–102. doi: 10.1164/rccm.201212-2328OC*
3. Alumasa J.N., Manzanillo P.S., Peterson N.D., Lundrigan T., Baughn A.D., Cox J.S., Keiler K.C. Ribosome rescue inhibitors kill actively growing and nonreplicating persister Mycobacterium tuberculosis cells. *ACS Inf. Dis., 2017, vol. 3, no. 9, pp. 634–644. doi: 10.1021/acsinfecdis.7b00028*
4. Anthony R.M., Cynamon M., Hoffner S., Werngren J., den Hertog A.L., van Soolingen D. Protecting pyrazinamide, a priority for improving outcomes in multidrug-resistant tuberculosis treatment. *Antimicrob. Agents. Chemother., 2017, vol. 61, no. 6: e00258-17. doi: 10.1128/AAC.00258-17*
5. Anthony R.M., den Hertog A.L., van Soolingen D. 'Happy the man, who, studying nature's laws, Thro'known effects can trace the secret cause.' Do we have enough pieces to solve the pyrazinamide puzzle? *J. Antimicrob. Chemother., 2018, vol. 73, no. 7, pp. 1750–1754. doi: 10.1093/jac/dky060*
6. Arenz S., Abdelshahid M., Sohmen D., Payoe R., Starosta A.L., Berninghausen O., Vasili Hauryliuk V., Beckmann R., Wilson D.N. The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Res., 2016, vol. 44, no. 13, pp. 6471–6481. doi: 10.1093/nar/gkw470*
7. Bag S., Das B., Dasgupta S., Bhadra R.K. Mutational analysis of the (p) ppGpp synthetase activity of the Rel enzyme of Mycobacterium tuberculosis. *Arch. Microbiol., 2014, vol. 196, no. 8, pp. 575–588. doi: 10.1007/s00203-014-0996-9*
8. Black P.A., De Vos M., Louw G.E., Van der Merwe R.G., Dippenaar A., Streicher E.M., Abdallah A.M., Sampson S.L., Victor T.C., Dolby T., Simpson, J.A., van Helden, P.D., Warren, R.M., Pain A. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in Mycobacterium tuberculosis isolates. *BMC genomics, 2015, vol. 16, no. 1: 857. doi: 10.1186/s12864-015-2067-2*
9. Blanc L., Sarathy J.P., Cabrera N.A., O'Brien P., Dias-Freedman I., Mina M., Sacchettini J., Savic R.M., Gengenbacher M., Podell B.K., Prideaux B., Ioerger T., Dick T., Dartois V. Impact of immunopathology on the antituberculous activity of pyrazinamide. *J. Exp. Med., 2018, vol. 215, no. 8: 1975. doi: 10.1084/jem.20180518*
10. Brunel R., Descours G., Durieux I., Doublet P., Jarraud S., Charpentier X. KKL-35 exhibits potent antibiotic activity against Legionella species independently of trans-translation inhibition. *Antimicrob. Agents. Chemother., 2017, AAC-01459. doi: 10.1128/AAC.01459-17*
11. Caño-Muñiz S., Anthony R., Niemann S., Alffenaar J.W.C. New approaches and therapeutic options for Mycobacterium tuberculosis in a dormant state. *Clin. Microbiol. Rev., 2018, vol. 31, no. 1: e00060-17. doi: 10.1128/CMR.00060-17*
12. Carey A.F., Rock J.M., Krieger I.V., Chase M.R., Fernandez-Suarez M., Gagneux S., Sacchettini, J.C., Ioerger, T.R., Fortune S.M. TnSeq of Mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PLoS Pathog., 2018, vol. 14, no. 3, e1006939. doi: 10.1371/journal.ppat.1006939*
13. Ciulli A., Scott D.E., Ando M., Reyes F., Saldanha S.A., Tuck K.L., Chirgadze, D.Y., Blundell, T.L., Abell C. Inhibition of Mycobacterium tuberculosis pantothenate synthetase by analogues of the reaction intermediate. *ChemBioChem, 2008, vol. 9, no. 16, pp. 2606–2611. doi: 10.1002/cbic.200800437*
14. Cobelens F., Kik S., Esmail H., Cirillo D.M., Lienhardt C., Matteelli A. From latent to patent: rethinking prediction of tuberculosis. *The Lancet Respiratory Medicine, 2017, vol. 5, no. 4, pp. 243–244. doi: 10.1016/S2213-2600(16)30419-2*

15. Connolly L.E., Cox J.S. CarD tricks and magic spots: mechanisms of stringent control in mycobacteria. *Cell Host Microbe, 2009, vol. 6, no. 1, pp. 1–2. doi: 10.1016/j.chom.2009.07.001*
16. Datta S., Sherman J.M., Tovar M.A., Bravard M.A., Valencia T., Montoya R., Quino W., D'Arcy N., Ramos E.S., Gilman R.H., Evans C.A. Sputum microscopy with fluorescein diacetate predicts tuberculosis infectiousness. *J. Infect. Dis., 2017, vol. 216, no. 5, pp. 514–524. doi: 10.1093/infdis/jix229*
17. Dillon N.A., Peterson N.D., Feaga H.A., Keiler K.C., Baughn A.D. Anti-tubercular activity of pyrazinamide is independent of trans-translation and RpsA. *Scientific Reports, 2017, vol. 7, no. 1: 6135. doi: 10.1038/s41598-017-06415-5*
18. Dillon N.A., Peterson N.D., Rosen B.C., Baughn A.D. Pantothenate and pantetheine antagonize the antitubercular activity of pyrazinamide. *Antimicrob. Agents Chemother., 2014, vol. 58, pp. 7258–7263. doi: 10.1128/AAC.04028-14*
19. East African/British Medical Research Councils. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis: second report. *Lancet, 1973, vol. 1, pp. 1331–1339. doi: 10.1016/S0140-6736(74)91411-1*
20. Gao W., Kim J.Y., Anderson J.R., Akopian T., Hong S., Jin Y.Y., Kandror O., Kim J.-W., Lee I.-A., Lee S.-W., McAlpine J.B., Mulugeta S., Sunoqrot S., Wang Y., Yang S.H., Yoon T-M., Goldberg A.L., Pauli G.F., Syh J.-W., Franzblau S.G., Cho S. The cyclic peptide ecumicin targeting ClpC1 is active against Mycobacterium tuberculosis *in vivo. Antimicrob. Agents. Chemother., 2014, vol. 59, no. 2, pp. 880–889. doi: 10.1128/AAC.04054-14*
21. Garton N.J., Waddell S.J., Sherratt A.L., Lee S.M., Smith R.J., Senner C., Hinds J., Rajakumar K., Adegbola R.A., Besra G.S., Butcher P.D. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med., 2008, vol. 5: e75. doi: 10.1371/journal.pmed.0050075*
22. Gavrish E., Sit C.S., Cao S., Kandror O., Spoering A., Peoples A., Ling L., Fetterman A., Hughes D., Bissell A., Torrey H., Akopian T., Mueller A., Epstein S., Goldberg A., Clardy J., Lewis K. Lassomycin, a ribosomally synthesized cyclic peptide, kills Mycobacterium tuberculosis by targeting the ATP-dependent protease ClpC1P1P2. *Chem. Biol., 2014, vol. 21, no. 4, pp. 509–518. doi: 10.1016/j.chembiol.2014.01.014*
23. Gillespie S.H. Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective. *Antimicrob. Agents. Chemother., 2002, vol. 46, no. 2, pp. 267–274. doi: 10.1128/AAC.46.2.267-274.2002*
24. Glynn J.R., Whiteley J., Bifani P.J., Kremer K., van Soolingen D. Worldwide occurrence of Beijing/W strains of Mycobacterium tuberculosis: a systematic review. *Emerg. Infect. Dis., 2002, vol. 8, no. 8: 843. doi: 10.3201/eid0808.020002*
25. Gomez J.E., McKinney J.D. M. tuberculosis persistence, latency, and drug tolerance. *Tuberculosis, 2004, vol. 84, no. 1, pp. 29–44. doi: 10.1016/j.tube.2003.08.003*
26. Gopal P., Tasneen R., Yee M., Lanoix J.-P., Sarathy J., Rasic G., Li L., Dartois V., Nuermberger E., Dick T. In vivo-selected pyrazinoic acid-resistant Mycobacterium tuberculosis strains harbor missense mutations in the aspartate decarboxylase PanD and the unfoldase ClpC1. *ACS Infect. Dis., 2017, vol. 3, pp. 492–501. doi: 10.1021/acsinfecdis.7b00017*
27. Honeyborne I., McHugh T.D., Phillips P.P., Bannoo S., Bateson A., Carroll N., Perrin F.M., Ronacher K., Wright L., Van Helden P.D., Walzl G. Molecular bacterial load assay, a culture-free biomarker for rapid and accurate quantification of sputum Mycobacterium tuberculosis bacillary load during treatment. *J. Clin. Microbiol., 2011, vol. 49, no. 11, pp. 3905–3911. doi: 10.1128/JCM.00547-11*
28. Hu Y., Coates A.R., Mitchison D.A. Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant Mycobacterium tuberculosis. *Int. J. Tuberc. Lung Dis., 2006, vol. 10, no. 3, pp. 317–322.*
29. Hu Y., Mangan J.A., Dhillon J., Sole K.M., Mitchison D.A., Butcher P.D., Coates A.R. Detection of mRNA transcripts and active transcription in persistent Mycobacterium tuberculosis induced by exposure to rifampin or pyrazinamide. *J. Bacteriol., 2000, vol. 182, no. 22, pp. 6358–6365. doi: 10.1128/JB.182.22.6358-6365.2000*
30. Hertog A.L. den, Menting S., Pfeltz R., Warns M., Siddiqi S.H., Anthony R.M. PZA is active against Mycobacterium tuberculosis cultures at neutral pH with reduced temperature. *Antimicrob. Agents. Chemother., 2016, vol. 60, no. 8, pp. 4956–4960. doi: 10.1128/AAC.00654-16.*
31. Hertog A.L. den, Menting S., van Soolingen D., Anthony R.M. Mycobacterium tuberculosis Beijing genotype resistance to transient rifampin exposure. *Emerg. Infect. Dis., 2014, 20(11), 1932. doi: 10.3201/eid2011.130560*
32. Hertog A.L. den, Sengstake S., Anthony R.M. Pyrazinamide resistance in Mycobacterium tuberculosis fails to bite? *Pathog. Dis., 2015, vol. 73, no. 6, doi: 10.1093/femspd/ftv037*
33. Jajou R., Kamst M., van Hunen R., de Zwaan C.C., Mulder A., Supply P., Anthony R., van der Hoek W., van Soolingen D. The occurrence and nature of double alleles in VNTR patterns of more than 8,000 Mycobacterium tuberculosis complex isolates in The Netherlands. *J. Clin. Microbiol., 2017, vol. 56, iss. 2: e00761-17. doi: 10.1128/JCM.00761-17*
34. Keijzer J. de, Mulder A., de Ru A.H., van Soolingen D., van Veelen P.A. Parallel reaction monitoring of clinical Mycobacterium tuberculosis lineages reveals pre-existent markers of rifampicin tolerance in the emerging Beijing lineage. *J. Proteomics, 2017, vol. 150, pp. 9–17. doi: 10.1016/j.jprot.2016.08.022*
35. Lan N.T. N., Lien H.T. K., Tung L.B., Borgdorff M.W., Kremer K., Van Soolingen D. Mycobacterium tuberculosis Beijing genotype and risk for treatment failure and relapse, Vietnam. *Emerg. Infect. Dis., 2003, vol. 9, no. 12: 1633. doi: 10.3201/eid0912.030169*
36. Lee H., Suh J.W. Anti-tuberculosis lead molecules from natural products targeting Mycobacterium tuberculosis ClpC1. *J. Ind. Microbiol. Biotechnol., 2016, vol. 43, no. 2–3, pp. 205–212. doi: 10.1007/s10295-015-1709-3*
37. Lillebaek T., Dirksen A., Baess I., Strunge B., Thomsen V.Ø., Andersen Å.B. Molecular evidence of endogenous reactivation of Mycobacterium tuberculosis after 33 years of latent infection. *J. Infect. Dis., 2002, vol. 185, pp. 401–404. doi: 10.1086/338342*
38. Lupoli T.J., Vaubourgeix J., Burns-Huang K., Gold B. Targeting the proteostasis network for mycobacterial drug discovery. *ACS Inf. Dis., 2018, vol. 4, no. 4, pp. 478–498. doi: 10.1021/acsinfecdis.7b00231*
39. Meehan C.J., Moris P., Kohl T.A., Pecerska J., Akter S., Merker M., Utpatel C., Beckert P., Gehre F., Lempens P., Stadler T. The relationship between transmission time and clustering methods in Mycobacterium tuberculosis epidemiology. *bioRxiv, 2018, 302232. doi: 10.1101/302232*

40. Miotto P., Cabibbe A.M., Feuerriegel S., Casali N., Drobniewski F., Rodionova Y., Bakonyte D., Stakenas P., Pimkina E., Augustynowicz-Kopeć. E., Degano M. Mycobacterium tuberculosis pyrazinamide resistance determinants: a multicenter study. *MBio, 2014, vol. 5, no. 5: e01819-14. doi: 10.1128/mBio.01819-14*
41. Mokrousov I., Jiao W.W., Sun G.Z., Liu J.W., Valcheva V., Li M., Li M., Narvskaya O., Shen A.D. Evolution of drug resistance in different sublineages of Mycobacterium tuberculosis Beijing genotype. *Antimicrob. Agents. Chemother., 2006, vol. 50, no. 8, pp. 2820–2823. doi: 10.1128/AAC.00324-06*
42. Moreira W., Ngan G.J.Y., Low J. L, Poulsen A., Chia B.C.S., Ang M.J.Y., Yap A., Fulwood J., Lakshmanan U., Lim J., Khoo A.Y.T., Flotow H., Hill J., Raju R.M., Rubin E.J., Dick T. Target mechanism-based whole-cell screening identifies bortezomib as an inhibitor of caseinolytic protease in mycobacteria. *mBio, 2015, vol. 6, no. 3: e00253-15. doi: 10.1128/mBio.00253-15*
43. Moreira W., Santhanakrishnan S., Dymock B.W., Dick T. Bortezomib warhead-switch confers dual activity against mycobacterial caseinolytic protease and proteasome and selectivity against human proteasome. *Front. Microbiol., 2017, vol. 8: 746. doi: 10.3389/fmicb.2017.00746*
44. Naftalin C.M., Verma R., Gurumurthy M., Lu Q., Zimmerman M., Yeo B.C.M., Tan K.H., Lin W., Yu B., Dartois V., Paton N.I. Co-administration of allopurinol to increase anti-mycobacterial efficacy of pyrazinamide: evaluation in a whole-blood bactericidal activity model. *Antimicrob. Agents. Chemother., 2017, vol. 61, no. 10: e00482-17. doi: 10.1128/AAC.00482-17*
45. Njire M., Wang N., Wang B., Tan Y., Cai X., Liu Y., Mugweru J., Guo J., Hameed H.A., Tan S., Liu J. Pyrazinoic acid inhibits a bifunctional enzyme in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother., 2017, vol. 61, no. 7: e00070-17. doi: 10.1128/AAC.00070-17*
46. Peterson N.D., Rosen B.C., Dillon N.A. Baughn A.D. Uncoupling environmental pH and intrabacterial acidification from pyrazinamide susceptibility in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother., 2015, vol. 59, pp. 7320–7326. doi: 10.1128/AAC.00967-15*
47. Primm T.P., Andersen S.J., Mizrahi V., Avarbock D., Rubin H., Barry C.E. The stringent response of Mycobacterium tuberculosis is required for long-term survival. *J. Bacteriol., 2000, vol. 182, no. 17, pp. 4889–4898. doi: 10.1128/JB.182.17.4889-4898.2000*
48. Raju R.M., Jedrychowski M.P., Wei J.R., Pinkham J.T., Park A.S., O'Brien K., Rehren G., Schnappinger D., Gygi, S.P., Rubin E.J. Post-translational regulation via Clp protease is critical for survival of Mycobacterium tuberculosis. *PLoS Pathog., 2014, vol. 10, no. 3: e1003994. doi: 10.1371/journal.ppat.1003994*
49. Raju R.M., Unnikrishnan M., Rubin D.H., Krishnamoorthy V., Kandror O., Akopian T.N., Rubin E.J. Mycobacterium tuberculosis ClpP1 and ClpP2 function together in protein degradation and are required for viability *in vitro* and during infection. *PLoS Pathog., 2012, vol. 8, no. 2: e1002511. doi: 10.1371/journal.ppat.1002511*
50. Reed M.B., Gagneux S., DeRiemer K., Small P.M., Barry C.E. The W-Beijing lineage of Mycobacterium tuberculosis overproduces triglycerides and has the DosR dormancy regulon constitutively upregulated. *J. Bacteriol., 2007, vol. 189, no. 7, pp. 2583–2589. doi: 10.1128/JB.01670-06*
51. Rie A. van, Warren R., Richardson M., Victor T.C., Gie R.P., Enarson D.A., Beyers N., van Helden P.D. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *N. Eng. J. Med., 1999, vol. 341, no. 16, pp. 1174–1179. doi: 10.1056/NEJM199910143411602*
52. Russell D.G., Barry C.E., Flynn J.L. Tuberculosis: what we don't know can, and does, hurt us. *Science, 2010, vol. 328, no. 5980, pp. 852–856. doi: 10.1126/science.1184784*
53. Schmitt E.K., Riwanto M., Sambandamurthy V., Roggo S., Miault C., Zwingelstein C., Krastel P., Noble C., Beer D., Rao S.P., Au M. The natural product cyclomarin kills Mycobacterium tuberculosis by targeting the ClpC1 subunit of the caseinolytic protease. *Angewandte Chemie, 2011, vol. 123, no. 26, pp. 6011–6013. doi: 10.1002/ange.201101740*
54. Shi W., Chen J., Zhang S., Zhang W., Zhang Y. Identification of novel mutations in LprG (rv1411c), rv0521, rv3630, rv0010c, ppsC, cyp128 associated with pyrazinoic acid/pyrazinamide resistance in Mycobacterium tuberculosis. *Antimicrob. Agents. Chemother., 2018, vol. 62, no. 7: e00430-18. doi: 10.1128/AAC.00430-18*
55. Shi W., Zhang X., Jiang X., Yuan H., Lee, J.S., Barry C.E., Wang H., Zhang W., Zhang, Y.Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. *Science, 2011, vol. 333, pp. 1630–1632. doi: 10.1126/science.1208813*
56. Sreevatsan S., Pan X., Zhang Y., Kreiswirth B.N., Musser J.M. Mutations associated with pyrazinamide resistance in *pncA* of Mycobacterium tuberculosis complex organisms. *Antimicrob. Agents. Chemother., 1997, vol. 41, no. 3, pp. 636–640.*
57. Stallings C.L., Stephanou N.C., Chu L., Hochschild A., Nickels B.E., Glickman M.S. CarD is an essential regulator of rRNA transcription required for Mycobacterium tuberculosis persistence. *Cell, 2009, vol. 138, no. 1, pp. 146–159. doi: 10.1016/j.cell.2009.04.041*
58. Syal K., Flentie K., Bhardwaj N., Maiti K., Jayaraman N., Stallings C.L., Chatterji D. Synthetic (p) ppGpp analogue: inhibitor of stringent response in mycobacteria. *Antimicrob. Agents. Chemother., 2017, vol. 61, iss. 6: e00443-17. doi: 10.1128/AAC.00443-17*
59. Van Deun A., Salim H., Kumar Das A.P., Bastian I., Portaels F. Results of a standardised regimen for multidrug-resistant tuberculosis in Bangladesh. *Int. J. Tuberc. Lung Dis., 2004, vol. 8, no. 5, pp. 560–567.*
60. Vasudevan D., Rao S.P., Noble C.G. Structural basis of mycobacterial inhibition by cyclomarin A. *J. Biol. Chem., 2013, vol. 288, no. 43: 30883-91. doi: 10.1074/jbc.M113.493767*
61. Via L.E., Savic R., Weiner D.M., Zimmerman M.D., Prideaux B., Irwin S.M., Lyon E., O'Brien P., Gopal P., Eum S., Lee M. Host-mediated bioactivation of pyrazinamide: implications for efficacy, resistance, and therapeutic alternatives. *ACS Inf. Dis., 2015, vol. 1, no. 5, pp. 203–214. doi: 10.1021/id500028m*
62. Vilchèze C., Weinrick B., Wong K.W., Chen B., Jacobs, Jr W.R. NAD^+ auxotrophy is bacteriocidal for the tubercle bacilli. *Mol. Microbiol., 2010, vol. 76, no. 2, pp. 365–377. doi: 10.1111/j.1365-2958.2010.07099.x*
63. Werngren J., Alm E., Mansjö M. Non-*pncA* Gene-mutated but pyrazinamide-resistant Mycobacterium tuberculosis: why is that? *J. Clin. Microbiol., 2017, vol. 55, pp. 1920-1927. doi: 10.1128/JCM.02532-16*
64. Wexselblatt E., Oppenheimer-Shaanan Y., Kaspy I., London N., Schueler-Furman O., Yavin E., Glaser G., Katzhendler J., Ben-Yehuda S. Relacin, a novel antibacterial agent targeting the stringent response. *PLoS Pathog., 2012, vol. 8, no. 9: e1002925. doi: 10.1371/journal.ppat.1002925*

65. Wollenberg K.R., Desjardins C.A., Zalutskaya A., Slodovnikova V., Oler A.J., Quiñones Abeel T., Chapman S.B., Tartakovsky M., Gabrielian A., Hoffner S., Skrahin A., Birren B.W., Rosenthal A., Skrahina A., Earl A.M. Whole-genome sequencing of Mycobacterium tuberculosis provides insight into the evolution and genetic composition of drug-resistant tuberculosis in Belarus. *J. Clin. Microbiol., 2017, vol. 55, pp. 457–469. doi: 10.1128/JCM.02116-16*
66. Yee M., Gopal P., Dick T. Missense mutations in the unfoldase ClpC1 of the caseinolytic protease complex are associated with pyrazinamide resistance in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother., 2017, vol. 61: e02342-16. doi: 10.1128/AAC.02342-16*
67. Zhang S., Chen J., Shi W., Cui P., Zhang J., Cho S., Zhang W., Zhang Y. Mutation in clpC1 encoding an ATP-dependent ATPase involved in protein degradation is associated with pyrazinamide resistance in Mycobacterium tuberculosis. *Emerg. Microbes Infect., 2017, vol. 6: e8. doi: 10.1038/emi.2017.1*

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