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Salicylanilide pyrazinoates inhibit *in vitro* multidrug-resistant *Mycobacterium tuberculosis* strains, atypical mycobacteria and isocitrate lyase

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ABSTRACT

The development of antimicrobial agents represents an up-to-date topic. This study investigated *in vitro* antimycobacterial activity, mycobacterial isocitrate lyase inhibition and cytotoxicity of salicylanilide pyrazinoates. They may be considered being mutual prodrugs of both antimycobacterial active salicylanilides and pyrazinoic acid (POA), an active metabolite of pyrazinamide, in which these esters are likely hydrolysed without presence of pyrazinamidase/nicotinamidase. Minimum inhibitory concentrations (MICs) of the esters were within the range 0.5–8 μmol/l for *Mycobacterium tuberculosis* and 1–32 μmol/l for nontuberculous mycobacteria (*Mycobacterium avium*, *Mycobacterium kansasii*). All esters showed a weak inhibition (8–17%) of isocitrate lyase at the concentration of 10 μmol/l. The most active pyrazinoates showed MICs for multidrug-resistant tuberculosis strains in the range of 0.125–2 μmol/l and no cross-resistance with clinically used drugs, thus being the most *in vitro* efficacious salicylanilide esters with 4-chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate superiority (MICs ⩽ 0.25 μmol/l). This promising activity is likely due to an additive or synergistic effect of released POA and salicylanilides. Selectivity indexes for the most active salicylanilide pyrazinoates ranged up to 64, making some derivatives being attractive candidates for the next research; 4-bromo-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate showed the most convenient toxicity profile.

1. Introduction

Tuberculosis (TB), a contagious infectious disease caused by *Mycobacterium tuberculosis* complex, represents one of the global health threats. Although the treatment has brought a considerable amelioration, TB is still the most fatal infectious disease with many negative consequences (Ducati et al., 2006). A standard therapy of new TB patients is based on a six months regimen consisted of two month taking of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) and then four month of INH and RIF (WHO, 2010). In this scheme, PZA affects primarily mycobacterial subpopulations with low metabolic activity due to acidic and hypoxia environment. It represents a pivotal component of the killing of a subset of bacteria unaffected by other drugs. Its inclusion reduces the therapy course up to a half (Dover and Coxon, 2011). Treatment of latent infection is a key component of TB control programmes; PZA may be included in some regimens for the latent TB eradication (Lobue and Menzies, 2010). The various infections caused by nontuberculous (atypical) mycobacteria with a problematic susceptibility to established antimicrobial agents bring a need of novel drugs. Compounds targeting both tuberculous and atypical mycobacteria may be particularly beneficial (Cook, 2010).

Unfortunately, the global incidence of drug-resistant TB is especially alarming and evoking a serious challenge for the effective TB control. Multidrug-resistant tuberculosis (MDR-TB) was defined as the infection that is resistant at least to INH and RIF and the graver extensively drug-resistant TB (XDR-TB) consists in MDR in combination with both resistance to any fluoroquinolone and at least one second-line injectable drug (kanamycin, amikacin, capreomycin) (Caminero, 2008). For the therapy of MDR-TB, WHO recommends administration of PZA in the intensive phase of the treatment (WHO, 2011). PZA may be useful as an adjunct for the treatment of MDR- and XDR-TB for the entire treatment duration, while many MDR- and XDR-TB retain susceptible (Caminero et al., 2010).

While PZA is the only anti-TB drug now available that kills dormant organisms more effectively than those that are actively metabolizing, Mitchison and Fourie (2010) suggested that the

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future development of anti-tuberculosis drugs should be, inter alia, targeted to this essential molecule and its derivatives.

PZA is active only against *M. tuberculosis* complex organisms [*M. tuberculosis* (*Mtb.*), *Mycobacterium africanum* and *Mycobacterium microti*, but not *Mycobacterium bovis*] (Zhang and Mitchison, 2003). Despite the wide use, the mechanism of action has not been fully elucidated for a long time; it was considered being rather non-specific without a clear target.

PZA as a prodrug enters *M. tuberculosis* cell probably by passive diffusion and it is hydrolyzed intracellularly into its active form, pyrazinoic acid (pyrazine-2-carboxylic acid; POA), by nicotinamidase/pyrazinamidase (PZase). This enzyme encoded by *pncA* gene converts nicotinamide into nicotinic acid, primarily. Its various defective mutations are thought to be the main reason for PZA-resistance (Jureen et al., 2008; Zhang and Mitchison, 2003; Zhang et al., 2008). A "classical" mechanism of action involving POA protonization, deprotonization and migration, was described by Zhang and Mitchison (2003) and Zhang et al. (2003a). In this way, POA has been proposed to collapse the proton gradient, disrupt membrane potential and transport functions, acidify cytoplasm and thereby influence vital function. POA also decreases respiratory synthesis of ATP and its intracellular level (Lu et al., 2011).

Fatty acid synthase I (FAS I) has been suggested as a PZA target, but a subsequent study ended negatively (Boshoff et al., 2002). By contrast, some newer studies have found PZA, POA and its simple esters diminishing FAS I function (Ngo et al., 2007; Zimhony et al., 2007); recently, it has been revealed that PZA inhibits binding of NADPH competitively, whereas POA showed a greater affinity for FAS I and a different binding site (Sayahi et al., 2011). The PZA hydrolysis to POA is not required for FAS I inhibition. Interestingly, alkyl pyrazinoates have been shown to be inhibitors of FAS I, likely on the same binding site (Sayahi et al., 2012). A previously unrecognized target of POA was identified in 2011: the ribosomal protein S1 (RpsA) involved in protein translation and the ribosome-sparing process of trans-translation. This mechanism could explain PZA activity against non-replicating mycobacteria (Shi et al., 2011).

PZA-resistant *M. tuberculosis* strains possess loss of PZase activity mostly due to mutations of *pncA* (rarely mutations in promoter or an undefined regulatory gene have been discussed), which is conventionally considered being a major reason of the resistance (Jureen et al., 2008; Zhang and Mitchison, 2003; Zhang and Yew, 2009). PZA-resistance in some strains with an optimal PZase activity can be explained by the variation of POA efflux rate due to mutations altering the efficiency of the POA efflux pump (Zimic et al., 2012) and also the changes in RpsA protein represent another resistance mechanism (Shi et al., 2011).

Nontuberculous mycobacteria share mostly natural PZA-resistance. In *Mycobacterium kansasii*, it results from the reduced PZase activity. Additionally, there exists a weak POA efflux mechanism (Sun and Zhang, 1999). The natural PZA-resistance in other atypical mycobacteria such as *Mycobacterium smegmatis* and *Mycobacterium avium* consists most likely in a highly active POA efflux which abolishes its accumulation within cells at an acidic pH; their PZase is fully functional (Sun and Zhang, 1999). A lack or a lowering of ATP-dependent PZA uptake was proposed being an additional factor participating in lower PZA susceptibility at some atypical mycobacteria as well as at *M. tuberculosis* with acquired PZA-resistance (Raynaud et al., 1999). That is why PZA is not usually used in the treatment of infections caused by nontuberculous mycobacteria.

The observation that PZA-resistant *M. tuberculosis* retains susceptibility to POA has led to the development of its esters. While ionized POA does not penetrate through mycobacterial cell wall well, its derivatives may prevent this obstacle and may circumvent PZase deficient *M. tuberculosis* and nontuberculous strains (Sayahi et al., 2012; Zhang and Mitchison, 2003). Esters have been found to have a greater *in vitro* antimycobacterial activity than POA, generally assumed that it is a consequence of increased lipophilicity and that esters, after non-enzymatic or rather enzymatic hydrolysis, share identical mechanism of action. Despite *in vitro* improved antimycobacterial activity of POA, efficacy studies in mice have failed, presumably due to instability of the POA esters *in vivo* (Zhang and Mitchison, 2003).

In contrast, propyl-pyrazinoate, unlike PZA or POA, is active at neutral pH indicating that POA esters are not only POA prodrugs, but they likely have intrinsic antimycobacterial activity interacting with FAS I, without necessary previous hydrolysis. Similarly, POA esters are active against POA-resistant *M. smegmatis* and *M. avium*, where the resistance is not caused due to defective PZase (Sayahi et al., 2012).

Pyrazinoic acid esters, which are mostly active towards extended spectrum of mycobacterial species as well as with improved efficacy for TB strains including those with acquired PZA-resistance, have been reported (Bergmann et al., 1996; Cynamon et al., 1992; Cynamon et al., 1995; Seitz et al., 2002; Speirs et al., 1995; Yamamoto et al., 1995); however, *M. avium* avoid uniform susceptibility to POA esters (Cynamon et al., 1992; Speirs et al., 1995). POA esters with higher linear alcohols were designed to increase the hydrolytic stability in serum and mycobacterial cell barriers penetration. These highly lipophilic esters showed significantly a greater activity than POA or PZA against *M. tuberculosis* and they were more resistant to plasma and liver hydrolysis than short chain esters, positively correlating with increased lipophilicity. The authors concluded that more hydrolysis-resistant esters appear convenient as POA prodrugs, overcoming the limitations of previously described esters (Simões et al., 2009). On the other side, ester of POA with protected L-serine avoided any activity against *M. tuberculosis* (Pinheiro et al., 2007).

In agreement with these results, the investigation of prodrugs has been expanded during the last decades. The prodrug design offers the improvement of drug candidate undesired properties, typically in means of chemical instability, poor solubility, pharmacokinetics, efficacy or side effects. Esters and amides are the most common prodrug strategies used to improve the lipophilicity. Carboxylates are converted to the parent compounds by ubiquitous esterases (Huttunen and Rautio, 2011) or spontaneously. Interestingly, the intrinsic antimicrobial activity of the POA counterpart alcohol or phenol may be a further advantage, while it might result in a synergistic action of the mutual prodrug. That is why we selected antimicrobially active salicylanilides (2-hydroxy-*N*-phenylbenzamides) for the esterification of POA in this study.

Salicylanilides have just revealed many pharmacological activities; some members of this group are established in human or veterinary medicine. Importantly, they are investigated for their activity against bacteria including mycobacteria, fungi and protozoa (Fomovska et al., 2012; Garner et al., 2011; Krátký and Vinšová, 2011; Krátký et al., 2012b; Lee et al., 2013). The exact mechanism of salicylanilides action is not still fully elucidated; the brief summarization of particular effects on bacterial cells is outlined in our review (Krátký and Vinšová, 2011). Recently, a moderate inhibition of mycobacterial methionine aminopeptidase and isocitrate lyase was reported (Krátký et al., 2012b; Krátký et al., 2013), as well as others like transglycosylase (Cheng et al., 2010) or "resurrected" disruption of the membrane proton gradient (Lee et al., 2013). For salicylanilides as phenolic compounds, increased lipophilicity and subsequent better passing through biomembranes and decreased cytotoxicity represent the main reasons for their esterification. However, it is still not fully elucidates, if salicylanilide esters act only as prodrugs releasing parent salicylanilide and acid, or if they may interact with target sites as original entities (Krátký and Vinšová, 2011).