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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).

Salicylanilide pyrazine-2-carboxylates (pyrazinoates) were demonstrated having a significant activity towards Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* and filamentous fungi in micromolar range (Krátký et al., 2012a). Six salicylanilide pyrazinoates were reported inhibiting *M. tuberculosis* $H_{37}Rv$ within a range of 0.5–2 μmol/l (Krátký et al., 2012b).

Here we present the antimycobacterial activity (including MDR-TB) of eighteen salicylanilide pyrazinoates as well as their impact on mycobacterial isocitrate lyase function and toxicity study.

2. Materials and methods

2.1. Chemistry

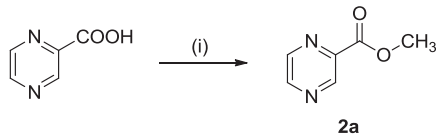
Salicylanilide pyrazinoates **1** [2-(phenylcarbamoyl)phenyl pyrazine-2-carboxylates] were synthesized previously by our group (Scheme 1). Their straightforward synthesis mediated by *N*,*N*′-dicyclohexylcarbodiimide (DCC), physical and spectral characteristics as well as antifungal and antibacterial activity were published by Krátký et al. (2012a). Their synthetic plan, general structure and substitution patterns are depicted in Scheme 1.

Methyl pyrazine-2-carboxylate (methyl pyrazinoate; **2a**) was synthesized *via* direct esterification (Scheme 2) with a yield of 86%. Its structure was confirmed by ^{1}H and ^{13}C NMR spectra, IR: 1722 (C=O ester).

4-Chlorophenyl pyrazine-2-carboxylate **2b** was obtained either similarly to salicylanilide pyrazinoates **1** by the reaction *via* DCC in *N*,*N*-dimethylformamide (DMF) with an addition of a catalytic amount of 4-dimethylaminopyridine (DMAP; Method A) or *via in situ* generated pyrazinoyl chloride (Method B; Scheme 3). After the esterification mediated by carbodiimide, the resulted urea **2c** was isolated as a by-product in 53% yield. This rearrangement and product has been described previously by Pinheiro et al. (2007). Yields of **2b**: 34% (Method A) or 59% (Method B). The structures of ester and urea were confirmed by ^{1}H and ^{13}C NMR spectra. IR for **2b**: 1732 (C=O ester) and for **2c**: 3271 (N—H) and 1702 (C=O amide).

All of the reagents and solvents were purchased from Sigma–Aldrich (Darmstadt, Germany) or Penta Chemicals (Prague, Czech Republic), and they were used as received. Reactions and the purity of the products were monitored by thin layer chromatography with a toluene/ethyl-acetate 4:1 9:1 mixture as eluent; plates were coated with 0.2 mm Merck 60 F254 silica gel and were visualised by UV irradiation (254 nm). Infrared spectra (ATR) were recorded on FT-IR spectrometer Nicolet 6700 FT-IR in the range of 400–4000 cm^{-1}. The NMR spectra were measured in $CDCl_3$ at ambient temperature on a Varian V NMR S500 instrument (500 MHz for ^{1}H and 125 MHz for ^{13}C; Varian Comp. Palo Alto, CA, USA).

The calculated log*P* values (Clog*P*), that are the logarithms of the partition coefficients for octan-1-ol/water, were determined using the program CS ChemOffice Ultra version 12.0 (CambridgeSoft, Cambridge, MA, USA).



Scheme 2. Synthesis of methyl pyrazine-2-carboxylate **2a** (reagents and conditions: (i) MeOH, catalytic amount of H_2SO_4, reflux, 3 h).

2.2. In vitro antimycobacterial susceptibility determination

Salicylanilide pyrazinoates were evaluated for their *in vitro* antimycobacterial activity against *M. tuberculosis* 331/88 ($H_{37}Rv$; dilution of this strain was 10^{-3}), *M. avium* 330/88 (resistant to INH, RIF, ofloxacin (OFX) and ethambutol (EMB); dilution 10^{-5}) and two strains of *M. kansasii*: 235/80 (dilution 10^{-4}) and clinically isolated strain 6509/96 (dilution 10^{-5}). The description of the used method can be found in (Krátký et al., 2013). The following concentrations were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μmol/l. MIC (reported in μmol/l) was the lowest concentration at which the complete inhibition of mycobacterial growth occurred. The front-line anti-tuberculosis drugs isoniazid (INH), pyrazinamide (PZA) and *p*-aminosalicylic acid (PAS) as a structural similar second-line drug and pyrazine-2-carboxylic acid (POA) as a synthetic precursor of salicylanilide pyrazinoates **1** were chosen as the reference compounds.

Six compounds with the lowest MIC (⩽1 μmol/l) against *M. tuberculosis* 331/88 were evaluated against MDR-TB and XDR-TB strains under the same conditions and concentrations using six *M. tuberculosis* strains (dilution 10^{-3}) with different resistance patterns: 7357/1998, 234/2005, 53/2009, Praha 1, Praha 131 (XDR-TB), and 9449/2006. All strains are resistant to INH, rifamycines (RIF and rifabutine) and streptomycin (STM).

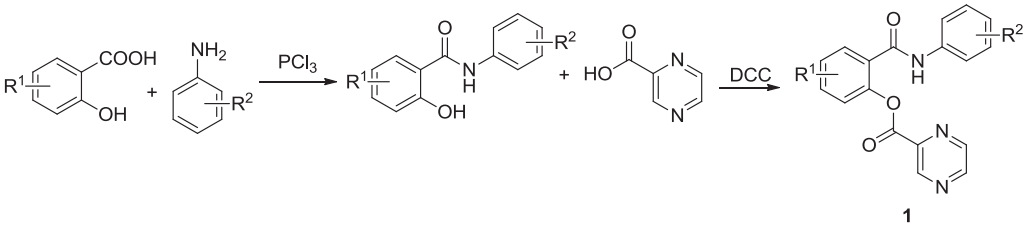
2.3. Isocitrate lyase inhibition assay (ICL1)

The description of the used method can be found in (Krátký et al., 2013). The isocitrate lyase activity was assayed according to the protocol reported by Dixon and Kornberg (1959) *via* glyoxylate phenyl hydrazone formation; the concentration of investigated compounds was 10 μmol/l. Isoniazid was employed as a negative control (inhibition 0%), and 3-nitropropionic acid (3-NP) served as a positive control.

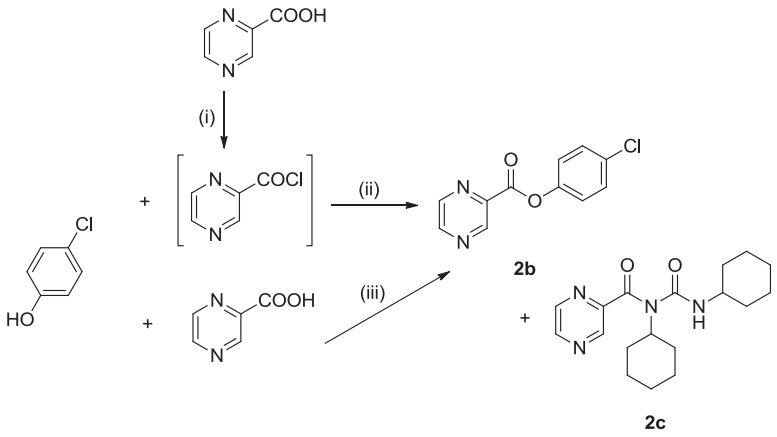
3. Results and discussion

3.1. Antimycobacterial activity

Salicylanilide pyrazinoates **1** inhibited the growth of four mycobacterial strains with MICs from 0.5 up to 16 μmol/l (Table 1). Four esters (**1e**, **1o**, **1p**, **1r**) are fully comparable to INH against *M. tuberculosis*; all pyrazinoates showed the better efficacy against INH-resistant strains of *M. avium* and *M. kansasii* 235/80 and most

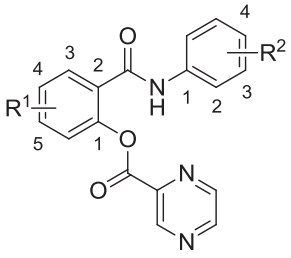


Scheme 1. Synthesis of salicylanilide pyrazinoates **1** (R^1 of esters: 4-Cl, 4-Br, 5-Cl; R^2 = 3-Cl, 4-Cl, 3,4-diCl, 3-Br, 4-Br, 3-F, 4-F, 3-CF_3, 4-CF_3).



Scheme 3. Two ways of 4-chlorophenyl pyrazine-2-carboxylate **2b** synthesis (reagents and conditions: (i) $SOCl_2$, DCM, reflux, 2 h; (ii) Et_3N (3 eq.), mixture of DCM and THF (1:1), room temperature, 3 h; (iii) DCC (1.2 eq.), DMAP (0.1 eq.), DMF, −20 °C to +4 °C, 24 h).

Table 1
Antimycobacterial activity of the pyrazinoates **1**.



	R^1	R^2	MIC (μmol/l)									
			M. tuberculosis 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
1a	4-Cl	3-Cl	2	4	8	8	**2**	4	4	**2**	**2**	4
1b	5-Cl	3-Cl	2	2	4	8	**2**	4	4	4	4	8
1c	4-Cl	4-Cl	2	2	4	8	**2**	**2**	4	**1**	**2**	4
1d	5-Cl	4-Cl	2	4	**2**	**2**	**2**	**2**	4	**2**	**2**	**2**
1e	4-Cl	3,4-diCl	**1***	**1***	4	16	2	4	4	**1**	**2**	**2**
1f	5-Cl	3,4-diCl	2	2	8	16	4	4	8	**2**	4	4
1g	4-Cl	3-Br	**1***	2*	8	16	4	4	4	**1**	**2**	4
1h	5-Cl	3-Br	2	8	8	16	4	4	4	**2**	4	4
1i	4-Cl	4-Br	2	2	8	8	**2**	**2**	4	**2**	**2**	4
1j	5-Cl	4-Br	2	2	4	4	4	4	4	**2**	**2**	4
1k	4-Cl	3-F	2	4	8	16	**2**	4	8	4	8	8
1l	5-Cl	3-F	2	8	8	16	4	4	8	4	4	8
1m	4-Cl	4-F	4	8	8	16	4	8	8	4	8	8
1n	5-Cl	4-F	2	8	**2**	4	**2**	4	4	**2**	4	8
1o	4-Cl	4-CF_3	**0.5***	**1***	**2**	4	**2**	**2**	**2**	**1**	**2**	**2**
1p	5-Cl	4-CF_3	**1***	**1***	4	8	**1**	**1**	**2**	**1**	**1**	**1**
1q	4-Cl	3-CF_3	**1***	2*	8	32	8	8	8	4	8	8
1r	4-Br	4-CF_3	**0.5***	**1***	**2**	4	**2**	**2**	4	**1**	**1**	**2**
INH			0.5–1	0.5–1	>250	>250	>250	>250	>250	2	4	4–8
PAS			62.5	62.5	32	125	125	1000	>1000	32	125	500
POA			250–1000	>1000	>1000	>1000	250–1000	>1000	>1000	250–1000	1000	1000

INH: isoniazid; PAS: *p*-aminosalicylic acid; POA: pyrazinoic (pyrazine-2-carboxylic) acid.
One or two best MIC(s) for each strain are given in bold.
* These MIC values were taken from Krátký et al. (2012b).

of esters additionally for *M. kansasii* clinical isolate. MIC values of PAS were many times higher (⩾32 μmol/l). Pyrazinoic acid alone showed a weak intrinsic activity particularly against *M. tuberculosis* and *M. kansasii* (MICs ⩾ 250 μmol/l). Since this assay was realized at the conditions not convenient for a standard PZA susceptibility testing, PZA inhibited mycobacteria at the concentrations of 125 μmol/l or higher, but lower than POA.

Salicylanilide derivatives bearing 4-trifluoromethyl moiety ($R^2 = CF_3$) expressed the highest activity: **1o** and **1r** against *M. tuberculosis* (0.5–1 μmol/l) and **1p** for *M. kansasii* (1–2 μmol/l). For *M. avium*, 4-chloroaniline derivative **1d** was evaluated possessing the lowest MIC of 2 μmol/l. Besides 4-CF_3, also 3-CF_3 (**1q**), 3,4-dichloro (**1e**) or 3-/4-bromine (mainly **1g**) substituents (as R^2) produced improved activity against *M. tuberculosis*. Contrarily, especially derivatives of fluoroanilines brought the least benefit (e.g. **1m**). For tuberculous strain, 4-chloroesters (R^1 = 4-Cl) showed mostly a better activity than 5-chloroesters (R^1 = 5-Cl) – e.g. for pairs **1e** and **1f** or **1g** and **1h**, similarly for *M. kansasii* with pairs

1m vs. **1n** and **1o** vs. **1p** being an exception, but for *M. avium* is this relationship in the opposite way (**1c** vs. **1d**, **1i** vs. **1j**, **1m** vs. **1n**).

In comparison to other salicylanilide esters with aromatic organic acids, pyrazinoates **1** showed approximately similar MIC values as benzoates (Krátký et al., 2012d), but they are significantly more active than benzenesulfonates (Krátký et al., 2012c) against *Mtb.* 331/88 and atypical mycobacteria.

The most active pyrazinoates **1e**, **1g**, **1o**–**1r** (i.e. whose MIC value after 14 days of incubation against *M. tuberculosis* was ⩽1 μmol/l) were evaluated against one XDR- and five MDR-TB strains. Table 2 overviews the results.

All evaluated pyrazinoates revealed the excellent antimycobacterial activity against six drug-resistant strains within the concentration range of 0.125–2 μmol/l. All of drug-resistant strains exhibited a similar susceptibility despite their resistance pattern including XDR strain and, interestingly, salicylanilide pyrazinoates **1** affected their growth at even lower concentrations (up to eight times) than for drug-sensitive *Mtb.* 331/88 ($H_{37}Rv$). These findings indicate no cross-resistance to clinically used drugs (INH, rifamycines, EMB, STM, OFX, clofazimine, aminoglycosides), qualifying presented esters as potential agents for combating drug-resistant TB.

The lowest MICs were found for 4-chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate **1o** (⩽0.25 μmol/l) followed by its 5-chloro isomer **1p** and by ester **1r** with 4-chlorine replaced by 4-bromine. In comparison to previously reported salicylanilide esters, pyrazinoates displayed lower MICs against drug-resistant TB strains than esters with *N*-acetyl-L-phenylalanine (Krátký et al., 2010), benzoic acid (Krátký et al., 2012d), 4-(trifluoromethyl)benzoic acid (Krátký et al., 2013) and aliphatic salicylanilide carbamates (Férriz et al., 2010).

Although salicylanilide pyrazinoates **1** possess the lowest lipophilicity from all esters evaluated against MDR-TB (for **1e** see illustratively Table 3), pyrazinoates exhibited significantly the highest *in vitro* activity towards MDR- and XDR-TB strains with MICs from 0.125 μmol/l and 4-chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate **1o** represents the most *in vitro* active salicylanilide derivative with MIC values of 0.125–0.25 μmol/l.

As expected, salicylanilide pyrazinoates **1** affected also significantly the growth of nontuberculous mycobacteria naturally resistant to PZA. In our assay, hydrophilic POA displayed certain, but just mild MIC values towards *M. kansasii* (⩾250 μmol/l). In accordance with previous reports (Bergmann et al., 1996; Cynamon et al., 1992; Cynamon et al., 1995; Seitz et al., 2002; Speirs et al., 1995; Yamamoto et al., 1995), we proposed that the salicylanilide pyrazinoates are hydrolyzed at least partly *in vivo* into parent POA and salicylanilide without any presence of PZase activity, thus bypassing the main reason of the natural PZA-resistance in *M. kansasii* as well as the acquired resistance of tuberculosis strains. The hydrolysis may be specific by mycobacterial esterase enzymes or spontaneous. Esters **1** also effectively inhibit *M. avium*, which resistance is conferred by alternative mechanism(s) different from deficient PZase.

Salicylanilide pyrazinoates may be considered being prodrug forms of either salicylanilides or pyrazinoic acid, i.e. mutual prodrugs with respect to significant antimycobacterial activity of both individual components. However, newer study indicates the possibility of intrinsic antimycobacterial activity of POA esters, not only being considered as POA prodrugs with indispensable hydrolysis before exhibiting any pharmacological action (Sayahi et al., 2012). It may also explain the activity of POA esters against PZA-resistant mycobacterial strains with efficient PZase.

While aliphatic POA esters with shorter alcohols failed *in vitro* in animal studies, probably due to rapid hydrolysis in plasma (Zhang and Mitchison, 2003), the presented more lipophilic aromatic POA esters should be more stable against hydrolysis than simple aliphatic ones, similarly as it was described for POA esters with linear higher alcohols. However, the stability during the transport phase is only one of the requirements, while convenient prodrug must also be efficiently activated within the mycobacteria (Simões et al., 2009).

Furthermore, we presume that the excellent *in vitro* activity is caused by the molecular additive or synergistic effect of both active moieties of esters **1**, salicylanilide and pyrazinoic acid, in which the esters are probably hydrolyzed at least in part within mycobacterial cells.

For the evaluation of antimycobacterial activity of individual components, pyrazinoic acid and salicylanilides and to verify or disprove the hypothesis, that the excellent activity is conferred only by the presence of pyrazinoyl moiety, we performed two experiments. First, we synthesized two simple POA esters with aliphatic alcohol, methanol (methyl pyrazinoate **2a**) and 4-chlorophenol, which is an analogue of the most active salicylanilides without phenylcarbamoyl group (4-chlorphenyl pyrazinoate **2b**). Additionally, we evaluated the antimycobacterial activity of urea by-product **2c** (Table 4). If the antimycobacterial activity will depend only on POA or PZA scaffold, these derivatives should exhibit a significant antimycobacterial activity like salicylanilide esters **1**. However, both esters as well as urea displayed a low activity or inactivity with MICs ⩾ 250 μmol/l. 4-Chlorophenyl derivative **2b** showed the highest activity among them, but still poor when compared to esters **1**. It is obvious that the excellent *in vitro* antimycobacterial activity is connected only with salicylanilide pyrazinoates (MICs ⩾ 0.125 μmol/l).

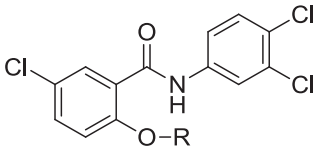
Second, we evaluated the antimycobacterial activity of four equimolar mixtures of different parent salicylanilides with free POA (Table 4). For *M. avium*, the mixtures displayed mostly the

Table 2
MIC of salicylanilide pyrazinoates **1** towards MDR- and XDR-TB.

	MIC (μmol/l)													
	R^1	R^2	*Mtb.* 7357/1998		*Mtb.* 9449/2006		*Mtb.* 53/2009		*Mtb.* 234/2005		*Mtb.* Praha 1		*Mtb.* Praha 131	
			14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d	14 d	21 d
1e	4-Cl	3,4-diCl	**0.125**	**0.25**	**0.5**	**0.5**	**0.5**	1	**0.125**	**0.125**	**0.25**	**0.5**	**0.5**	1
1g	4-Cl	3-Br	**0.5**	1	1	1	**0.5**	1	**0.25**	**0.5**	**0.125**	1	**0.25**	1
1o	4-Cl	4-CF_3	**0.25**	**0.25**	**0.125**	**0.25**	**0.125**	**0.25**	**0.25**	**0.25**	**0.125**	**0.125**	**0.125**	**0.125**
1p	5-Cl	4-CF_3	**0.5**	**0.5**	**0.125**	**0.25**	**0.125**	**0.5**	**0.5**	**0.5**	**0.125**	**0.25**	**0.125**	**0.125**
1q	4-Cl	3-CF_3	**0.5**	1	1	2	1	2	1	2	1	1	1	2
1r	4-Br	4-CF_3	**0.125**	**0.25**	**0.5**	**0.5**	**0.5**	1	**0.25**	**0.5**	**0.125**	**0.25**	**0.25**	**0.5**

MIC values lower than 1 μmol/l are given in bold.
MDR-TB strains: 234/2005 and 7357/1998 both resistant to INH, RIF, rifabutine, streptomycin, ethambutol and ofloxacin; 53/2009 resistant to INH, RIF, rifabutine, streptomycin, ethambutol; Praha 1 resistant to INH, RIF, rifabutine, streptomycin, ethambutol and clofazimine; 9449/2006 resistant to INH, RIF, rifabutine and streptomycin. XDR-TB strain: Praha 131 resistant to INH, RIF, rifabutine, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin.

Table 3
Comparison of Clog*P* and MIC values of various salicylanilide derivatives.



R	Clog*P*	MIC(s) for *Mtb.* $H_{37}Rv$ (μmol/l)	MICs for MDR- and XDR-TB (μmol/l)
H (parent salicylanilide)[a]	4.12	4–8	NT
Benzoyl (benzoate)[b]	6.0	1	0.25–1
4-(Trifluoromethyl)benzoyl [4-(trifluoromethyl)benzoate][c]	6.92	1	1–4
Benzenesulfonyl (benzenesulfonate)[d]	5.6	1–2	0.5–1
Pyrazinoyl (pyrazinoate) **1e**	3.75	1	0.125–1
(*S*)-2-acetamido-3-phenylpropanoyl (ester with *N*-acetyl-L-phenylalanine)[e]	5.12	0.5–1	1–4
Hexylcarbamoyl (*N*-hexylcarbamate)[f]	6.08	0.5	0.5–1
POA (its anion POA^-)	−0.66 (−0.77)	⩾250	NT

NT: not tested.
[a] MIC values were taken from Krátký et al. (2012b).
[b] MIC values were taken from Krátký et al. (2012d).
[c] MIC values were taken from Krátký et al. (2013).
[d] MIC values for *Mtb.* H37Rv were taken from Krátký et al. (2012c).
[e] MIC values were taken from Krátký et al. (2010).
[f] MIC values were taken from Férriz et al. (2010).

Table 4
MICs of simple pyrazinoic acid derivatives **2a–c** and the comparison of the activity of selected parent salicylanilides, their pyrazinoates **1** and equimolar mixtures of salicylanilides and POA.

	M. tbc. 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96		
	14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
Ester **1e**	1	1	4	16	2	4	4	1	2	2
Parent salicylanilide	4	8	32	32	4	8	8	8	8	8
Mixture SAL + POA (1:1)	4	4	16	16	16	16	32	16	32	32
Ester **1i**	2	2	8	8	2	2	4	2	2	4
Parent salicylanilide	4	4	16	16	4	8	8	4	8	8
Mixture SAL + POA (1:1)	2	4	8	16	8	8	16	8	8	16
Ester **1n**	2	8	2	4	2	4	4	2	4	8
Parent salicylanilide	16	16	32	32	8	16	16	8	16	16
Mixture SAL + POA (1:1)	8	16	32	32	16	32	32	32	32	32
Ester **1p**	1	1	4	8	1	1	2	1	1	1
Parent salicylanilide	4	4	8	8	2	2	4	2	2	4
Mixture SAL + POA (1:1)	1	2	4	8	2	4	4	2	4	4
2a	>500	>500	>500	>500	>500	>500	>500	500	500	500
2b	250	250	500	>500	250	500	500	250	250	500
2c	500	500	500	>500	500	>500	>500	500	500	500

SAL: corresponding salicylanilide; POA: pyrazine-2-carboxylic acid.

activity comparable to salicylanilides indicating no general significant improvement resulted from the addition of POA to the salicylanilides. For *M. tuberculosis*, the mixture exhibited somewhat lower MIC values than parent salicylanilides, especially for 4-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide, a synthetic precursor of **1p**, thus probably reflecting the mild intrinsic activity of POA. Surprisingly and in spite of the MICs of POA for these strains, the mixtures showed a weaker activity against *M. kansasii* than parent salicylanilides. In sum, the esters **1** remain the most active molecular entities for all mycobacterial strains. Presented facts support the hypothesis that salicylanilide esters with POA **1** enter the mycobacterial cells as original molecules at least in part and subsequently, they are likely hydrolyzed *in vitro* intracellularly. The excellent antimycobacterial activity is connected with the esters **1**, not with only a mechanistic mixture of both individual components added into the testing medium. This temporary transport form seems to be essential for the excellent antimycobacterial action.

The esterification of salicylanilides by pyrazine-2-carboxylic acid leads to the esters that are a bit more hydrophilic than parent salicylanilides (Table 3). However, a lot of highly effective antituberculosis drugs (typically PZA, INH or aminoglycosides) are classified as hydrophilic despite the highly lipophilic mycobacterial cell wall. From the point of view of POA, esters **1** are strongly more lipophilic than free acid, thus attractively enhancing its non-specific transport into mycobacterial cells. This effect overcoming poor penetration through mycobacterial cell wall may be one explanation ("non-specific") of the improved antimycobacterial activity of salicylanilide pyrazinoates **1**.

Salicylanilides and POA share some aspects of mechanism of action: they disrupt membrane potential and proton motive force *via* proton shuttling; they interfere with energy metabolism thus leading to the energy depletion or they acidify cytoplasm (de Carvalho et al., 2011; Krátký and Vinšová, 2011; Lee et al., 2013; Lu et al., 2011; Zhang et al., 2003a). *M. tuberculosis* showed a poor ability to maintain membrane proton motive force. The disruption of

membrane functions and potential has been also referred as a non-specific class effect of weak acids for *M. tuberculosis*. Local acidic pH also enhances intracellular accumulation of POA (Zhang et al., 2003b). Previously, an *in vivo* synergy between PZA and weak acids acetylsalicylic acid or ibuprofen has been demonstrated. The authors hypothesized that this effect is more likely caused by the inducing of low metabolic state by these weak acids, thus increasing efficacy of PZA (Byrne et al., 2007). Salicylanilides as phenols belong to weak acids, although less strong than carboxylic ones. That is reason why these possible mechanisms may be also kept in the mind as possible reasons of improved antimycobacterial activity of salicylanilide esters with POA.

It has been described that the PZA activity especially against old-culture *M. tuberculosis* is enhanced by the presence of some weak acids like benzoic acid or propyl 4-hydroxybenzoate, probably with an additive effect. Similar, but stronger and likely synergistic effect has been found for energy inhibitors including uncoupling agents (Wade and Zhang, 2006). Salicylanilides as both acidic and protonophore compounds may also increase PZA/POA activity in this way.

The synergy or dual action of POA as PZA active metabolite and salicylanilides may be especially beneficial for the targeting of persistent, non-replicating mycobacteria, while it has been demonstrated that proton motive force is necessary for the maintaining ATP homeostasis and viability of this mycobacterial subpopulation (Rao et al., 2008).

3.2. Mycobacterial isocitrate lyase inhibition

Salicylanilide esters including six pyrazinoates and other salicylanilide-scaffold based derivatives have been described to be mostly mild inhibitors of mycobacterial isocitrate lyase, one of two glyoxylate-shunt-pathway enzymes, at the concentrations of 10 and 100 μmol/l. Some of them have been comparable or superior to 3-nitropropionic acid, a known inhibitor (Krátký and Vinšová, 2012; Krátký et al., 2012b; Krátký et al., 2013). Isocitrate lyase represents an attractive drug target especially for combating persistent mycobacteria and it is believed that the introduction of its inhibitors into clinical practise may lead to the shortening of TB treatment course. Especially compounds with dual activity against actively growing and non-replicating mycobacterial subpopulations should be more beneficial. Advantageously, the glyoxylate shunt does not operate in humans (Krátký and Vinšová, 2012; Krátký et al., 2012b; Muñoz-Elías and McKinney, 2005; Smith et al., 2004).

Based on these facts, we evaluated salicylanilide pyrazinoates for the mycobacterial ICL inhibition (Table 5). The esters exhibited consistently a mild enzymatic inhibition within the range of 8–17% at the concentration of 10 μmol/l. We considered this concentration being more convenient than previously applied concentration of 100 μmol/l; the main reason consists in the limited solubility of salicylanilide esters in testing medium.

Only two esters demonstrated ⩾15% inhibition of ICL enzyme inhibition rate (**1f** and **1q**) with 4-chloro-2-[(3,4-dichlorophenyl)carbamoyl]phenyl pyrazine-2-carboxylate **1f** superiority (17%). None of pyrazinoates was comparable to 3-nitropropionic acid, a known standard inhibitor, at 10 μmol/l (25%). At the concentration of 100 μmol/l, for which the inhibition rates were published previously (Krátký et al., 2012b), only 4-trifluoromethyl derivative **1o** was almost as active as a standard (59% vs. 67%). However, as pointed, the activity could be a little confusing; two esters (**1g**, **1q**) caused even a lower ICL inhibition at 100 μmol/l when compared to 10 μmol/l concentration and for three esters (**1e**, **1p** and **1r**) the ten-fold increased concentration produced only about twice higher activity.

Table 5
ICL inhibition activity of salicylanilide pyrazinoates **1.**

	R^1	R^2	% ICL inhibition at 10 μmol/l (±standard deviation)	% ICL inhibition at 100 μmol/l (±standard deviation)
1a	4-Cl	3-Cl	14 ± 2.87	NT
1b	5-Cl	3-Cl	10 ± 4.25	NT
1c	4-Cl	4-Cl	13 ± 2.85	NT
1d	5-Cl	4-Cl	10 ± 2.43	NT
1e*	4-Cl	3,4-diCl	9 ± 1.3	17.5 ± 2.5
1f	5-Cl	3,4-diCl	**17 ± 3.84**	NT
1g*	4-Cl	3-Br	14 ± 2.4	13 ± 2
1h	5-Cl	3-Br	14 ± 1.65	NT
1i	4-Cl	4-Br	14 ± 3.65	NT
1j	5-Cl	4-Br	9 ± 4.20	NT
1k	4-Cl	3-F	14 ± 3.18	NT
1l	5-Cl	3-F	10 ± 2.06	NT
1m	4-Cl	4-F	11 ± 2.53	NT
1n	5-Cl	4-F	10 ± 2.88	NT
1o*	4-Cl	4-CF_3	8 ± 0.6	**59 ± 5.8**
1p*	5-Cl	4-CF_3	10 ± 0.9	23 ± 2
1q*	4-Cl	3-CF_3	15 ± 3.6	14.5 ± 1.5
1r*	4-Br	4-CF_3	9 ± 2.3	19 ± 2
3-NP			25 ± 4.1	67 ± 2.7
INH			0	NT

INH: isoniazid; 3-NP: 3-nitropropionic acid; NT: not tested.
The best inhibition rate value is given in bold.
* Data for **1e**, **1g**, **1o**–**1r** were taken from Krátký et al. (2012b).

When focused on structure–activity relationship, the derivatives of 3-substituted aniline showed a higher potency than those derived from 4-substituted anilines (e.g. markedly for the pairs **1h** vs. **1j** or **1o** vs. **1q**). In general, salicylanilides related to 5-chlorosalicylic acid affected isocitrate lyase function more strongly than molecules derived from 4-chlorosalicylic acid (**1a** vs. **1b**, **1c** vs. **1d**, **1i** vs. **1j**, **1k** vs. **1l**).

Similarly to other salicylanilide esters, salicylanilide pyrazinoates **1** act as modest ICL inhibitors. This property represents a certain benefit in addition to their excellent MIC values against growing mycobacteria, but it should not be overestimated with respect to determined inhibition rates. As expected, there is not a clear relationship of *in vitro* MICs and ICL inhibition. Here reported MIC values were obtained for actively growing mycobacteria, while ICL inhibition has displayed the potential against persistent or non-growing mycobacterial subpopulations. Inhibitors of mycobacterial isocitrate lyase may have a potential in a long-term use in the treatment of TB, not in an acute phase of the infection.

3.3. Cytotoxicity

Salicylanilides as phenolic compounds have demonstrated some cytotoxicity (Krátký and Vinšová, 2011; Zhu et al., 2011). That is why we evaluated cellular toxicity of the six salicylanilide pyrazinoates with superior antimycobacterial activity. The cytotoxicity is expressed as IC_{50}, *i.e.*, concentration which decreases the viability of the cells to 50% from the maximal viability; these values were taken from Ref. (Krátký et al., 2012b). The esters have been reported sharing IC_{50} for Hep G2 cells within the range of 1.66–8.02 μmol/l (Table 6), thus showing significantly less cytotoxicity than parent salicylanilides, probably due to the masking of phenolic group. Free pyrazinoic acid exhibited many times higher IC_{50} value of 2240.0 μmol/l. Based on this finding, the cytotoxicity properties of esters **1** may be predominantly attributed to the salicylanilide core.

4-Bromo-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate **1r**, followed by 2-[(3-bromophenyl)carbamoyl]-4-chlorophenyl pyrazine-2-carboxylate **1g**, expressed the highest IC_{50} of 8.02 and 7.04 μmol/l, respectively; on the other side,

Table 6
Cytotoxicity and selectivity indexes of selected antimycobacterial pyrazinoates **1**.

	R^1	R^2	IC_{50} (μmol/l) Hep G2	SI for *Mtb.* 331/88	SI for MDR-TB strains	SI for XDR-TB strain
1e	4-Cl	3,4-diCl	3.16	3.16	3.16–25.28	3.16–6.32
1g	4-Cl	3-Br	7.04	3.52–7.04	7.04–56.34	7.04–28.16
1o	4-Cl	4-CF_3	1.66	1.66–3.32	6.64–13.28	13.28
1p	5-Cl	4-CF_3	3.68	3.68	7.36–29.44	29.44
1q	4-Cl	3-CF_3	6.28	3.14–6.28	3.14–12.56	3.14–6.28
1r	4-Br	4-CF_3	8.02	8.02–16.04	8.02–64.16	16.04–32.08

IC50 values of salicylanilide pyrazinoates were taken from Krátký et al. (2012b). SI = IC50/MIC100.

4-chloro-2-{[4-(trifluoromethyl)phenyl]carbamoyl}phenyl pyrazine-2-carboxylate **1o** showed the lowest value of 1.66 μmol/l.

Based on IC_{50} and MIC values, we calculated selectivity indexes (SI) for drug-sensitive and drug-resistant *M. tuberculosis* strains. Selectivity indexes as ratio IC_{50}/MIC_{100} ranges from 1.66 up to 16.04 for *M. tuberculosis* 331/88, from 3.14 to 64.16 for MDR strains and from 3.14 up to 32.08 for XDR-TB. Due to a better efficacy of salicylanilide pyrazinoates **1** against drug-resistant mycobacteria, SI are more convenient for them. SI values higher than 10 indicate rather acceptable toxicity (based on the analogy of the therapeutic index) and selectivity for *M. tuberculosis*. The most favourable SI values for all tuberculosis strains exhibited ester **1r**, making it the most attractive drug-candidate from salicylanilide pyrazinoates, especially in a combination with its very low MIC values. Two other esters with the best activity against drug-resistant strain **1o** and **1p**, also derivatives of 4-(trifluoromethyl)aniline, showed satisfied SI values only for drug-resistant strains, whereas **1r** remains exclusively borderline sufficiently selective for drug-sensitive strain.

Similarly, one study reported highly lipophilic pyrazinoic acid esters with SI exceeding 10 (Simões et al., 2009).

Among salicylanilide esters with aromatic organic acids, pyrazinoates showed predominantly more alleviated cytotoxicity than salicylanilide benzoates, benzenesulfonates and 4-(trifluoromethyl)benzoates (Krátký et al., 2012b; Krátký et al., 2012d; Krátký et al., 2013), although with values also in micromolar range. Moreover, since pyrazinoates share superior antimycobacterial potency especially against MDR-TB, the combination of lower toxicity and enhanced activity favours them from the point of view of propitious selectivity indexes. Thus, salicylanilide pyrazinoates seem to be the most promising antimycobacterial salicylanilide esters derived from aromatic acids.

Interestingly, Appleton et al. (2010) described similar effect: the esterification of cytotoxic scaffold with an intrinsic antimycobacterial activity by pyrazinoic acid led to the decreased MIC and, concomitantly, cytotoxicity, thus both improving SI.

4. Conclusions

In this study, we investigated salicylanilide pyrazinoates as potential antimycobacterial agents. These compounds inhibit drug-sensitive as well as drug-resistant strains of *M. tuberculosis*, being also efficacious for nontuberculous mycobacteria naturally resistant to pyrazinamide (*M. avium*, *M. kansasii*). Salicylanilide pyrazinoates exhibited the lowest MIC values against MDR- and XDR-TB from all previously reported salicylanilide esters. The salicylanilide esterification by POA also resulted in derivatives with significantly improved activity and, concomitantly, decreased toxicity making these derivatives attractive. We suggest the hydrolysis of esters as mutual prodrugs in biological systems and then additive/synergistic action of released salicylanilide and pyrazinoic acid, which share some cellular effects. These esters may be as well considered being the prodrugs of POA with sharply increased lipophilicity when compared to free acid, which means facilitating of passing through cell wall and biomembranes. Being POA esters, they are likely activated within mycobacterial cells with dispensable presence of PZase, which means that they bypass the main mechanism of natural and acquired PZA-resistance in atypical and tuberculous mycobacteria, respectively. This hypothesis is supported by the fact that salicylanilide pyrazinoates inhibit *M. kansasii* with deficient PZase. However, there is a possibility of the action and biological activity of unhydrolyzed esters as molecular entities. Aromatic esters of phenols are hydrolytically more stable than esters derived from aliphatic alcohols, especially against spontaneous hydrolysis; additionally, previously it was described that more lipophilic POA esters are hydrolyzed slower by serum and liver esterases than those with higher hydrophilicity. In summary, salicylanilide pyrazinoates seem to be successful and promising mutual modification of both pyrazinoic acid and salicylanilides.

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