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SHORT COMMUNICATION

Synthesis and evaluation of a pyrazinoic acid prodrug in *Mycobacterium tuberculosis*

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Abstract Tuberculosis (TB) is a disease caused mainly by infection of *Mycobacterium tuberculosis* affecting more than ten million people around the world. Despite TB can be treated, the rise of MDR-TB and XDR-TB cases put the disease in a worrying status. As pyrazinamide-resistant strains exhibit low or none pyrazinamidase activity, it is proposed that the active form of pyrazinamide (PZA) is pyrazinoic acid (POA), although this acid has poor penetration in mycobacteria. In this work, we present a convenient one-pot synthesis of 2-chloroethyl pyrazinoate, and its activity in *M. tuberculosis* $H_{37}Rv$ (ATCC27294) in MIC assay using the MABA technique. The obtained MIC of the compound was 3.96 g/mL, and discussion about the activity profile of some previously evaluated pyrazinoates is also performed.

1. Introduction

Tuberculosis (TB) is a disease caused by the infection with members of *Mycobacterium tuberculosis* complex, affecting more than ten million people worldwide (Barry et al., 1998). The increase in HIV infection cases was the most important factor in the growth in TB prevalence rate. Nowadays, the disease is getting more worrying status since resistant cases are rising every day. Despite this, TB resistant cases can be classified in multidrug resistant TB (MDR-TB, when the resistance to first-line agents is detected, including resistance to isoniazid or rifampin) and extensively drug-resistant TB (XDR-TB, when second-line agent resistance is detected) (WHO, 2012; Zumla et al., 2013).

Although there is an increase in MDR-TB and XDR-TB cases, the development of new therapeutic options to TB is stagnant. Since the discovery of rifampin, in 1965, no other important specific antimycobacterial drug has been introduced in therapeutics against susceptible TB. However, bedaquiline (a diarylquinoline) was recently introduced in the treatment of MDR-TB (Sirturo, 2013). Nevertheless, several research groups worldwide are involved in obtaining new chemical enti-

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ties (NCE) for TB therapy, some in clinical trials (Working Group on New TB Drugs, 2013; Zumla et al., 2013).

Mycobacteria are slow-growing bacilli that possess an external cell wall composed by highly lipophilic fatty acids, the mycolic acids (Barry et al., 1998; Brennan and Nikaido, 1995; Zumla et al., 2013). Because of this unusual characteristic, antimycobacterial agents might have good penetration through this barrier. In fact, this is one of the most important roles to be fulfilled by antimycobacterial agents. Many of the anti-TB compounds have difficulties to cross the mycobacterial cell wall (Liu et al., 1996).

Pyrazinamide (PZA) was discovered during investigations of analogs of nicotinamide (Kushner et al., 1952). It is considered a bioisostere of nicotinamide. PZA is valuable in TB control, because it becomes active in acid conditions and may kill bacteria inside the granuloma, while other drugs lose their activity under these conditions, but some mycobacterial strains are naturally resistant to PZA. It is proposed that PZA interfere with the energetics and functions of the membrane (Zhang et al., 2003). However, the entire mechanism of action of PZA is unknown. It was verified that the conversion of PZA to pyrazinoic acid (POA) (Fig. 1) could play the main role in its action, because PZA-resistant strains do not express the enzyme responsible for the conversion to POA (named as pyrazinamidase, PZAse) (Bergmann et al., 1996; Cynamon et al., 1992, 1995; Konno et al., 1967). POA itself is poorly active in antimycobacterial tests, while it cannot pass through mycobacterial cell walls. This inactivity is due to its low lipophilicity and highly ionized state in physiological pHs. Thus, the prodrug approach to direct POA inside mycobacteria is suitable to increase its activity, including into PZA-resistant mycobacterial strains, since it can change its physicochemical characteristics (Cynamon et al., 1992, 1995).

Cynamon et al. (1992, 1995) synthesized a series of POA esters and derivatives as prodrugs, exhibiting good *in vitro* antimycobacterial activity against several mycobacterial strains, including the PZA-resistant strain of *M. tuberculosis* ATCC 35828. In an earlier work (Bergmann et al., 1996), they proposed a classical quantitative structure–activity relationship (QSAR) model ($N = 47$, $r^2 = 0.56$, $s = 0.54$; and, $N = 34$, $r^2 = 0.57$, $s = 0.54$) to those derivatives and several mycobacterial strains using the following independent variables: Hammet's σ, n-octanol/water partition coefficient (log P), the time of ester hydrolysis in serum (log *t*), and Charlton's steric substituent constant (). More recently, our group presented a multivariate QSAR model of these compounds ($N = 32$, $r^2 = 0.68$, $q^2 = 0.59$, LOF = 0.25, LSE = 0.19, outliers = 0), obtaining the Balaban index *J*, C log P, the stretching energy contribution ($E_{stretch}$), the van der Waals molecular surface area (S_{vdW}) and the dipole moment (μ) as important descriptors (Fernandes et al., 2010). The validated model showed that lipophilicity and molecular size are the most important factors that determine the activity of these esters.

Figure 1 Activation of PZA into POA.

Although pyrazinoate esters are important alternatives to be considered in resistant TB strains, it did not achieve the therapeutics yet. Clinical studies were not performed with it, and economical aspects should be considered as a factor in its indifference. Considering these, the aim of this study is to present a convenient one-pot synthesis of a POA prodrug, and also its antimycobacterial activity against *M. tuberculosis* H37Rv (ATCC 27294).

2. Material and methods

Chemical compounds were commercially acquired in adequate purity and used without previous treatment. Spectrometric analysis in infrared (IR) was performed in Bomem FTIR Michelson series equipment, between wave numbers 4000 and 400 cm^{-1}, using NaCl plates as support. NMR analyses were performed in Bruker DPX-300 equipment, operating at 300 for ^1H and 75 MHz for ^{13}C. Chemical shifts (δ = ppm) were measured using tetramethylsilane (TMS) as reference. Elemental analysis was performed in Perkin–Elmer CHN 2400 equipment.

2.1. Synthetic procedure

In approximately 20 mL of 2-chloroethanol, 5 mmol (0.620 g) of POA was added under vigorous stirring. The solution was kept in reflux, when 5.5 mmol (0.650 g – 10% excess) of thionyl chloride was added. The solution was maintained under heating and stirring for 3 h. The reaction mixture was then evaporated, and the remaining oily liquid was dried under vacuum. After this, 10 mL of diethyl ether was added, and the organic solution was washed with 15 mL of saturated $NaHCO_3$ and brine. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. The reaction was monitored by TLC, using chloroform:methanol (8:2) as eluent. The product was obtained as a yellowish oily liquid. Yield: 0.84 g (90%). IR (NaCl): ν_{max} 1737.38 cm^{-1} (C═O). ^1H NMR: (300 MHz, $CDCl_3$, TMS, δ = ppm) 9.34 (s, ArH7, 1H); 8.87 (s, ArH4, 1H); 8.82 (s, ArH5, 1H); 4.75 (t, OCH_2, $J = 5.7$ Hz, 2H); 3.95 (t, CH_2Cl, $J = 5.7$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$, TMS, δ = ppm) 163.54 (C1); 147.96 (C5); 146.44 (C7); 144.60 (C4); 143.05 (C2); 65.48 (OCH_2); 41.18 (CH_2Cl). Elemental analysis calculated: C = 45.0%; H = 3.8%; N = 15.0%; Cl = 19.0%. Found: C = 44.8%; H = 3.7%; N = 14.6%; Cl = 18.9%.

2.2. Determination of antimycobacterial activity

The microplate Alamar blue assay (MABA) was used to measure the minimal inhibitory concentration (MIC) for the tested compounds (minimum concentration necessary to inhibit 90% growth of *M. tuberculosis* $H_{37}Rv$ ATCC 27294) (Franzblau et al., 1998). In a sterile 96-well microplate was added 200 μL of distilled water in each well of the outer-perimeter, to avoid water evaporation during incubation. The test compounds (POA, 2-chloroethyl pyrazinoate and ciprofloxacin) were diluted in DMSO to obtain solutions, and thereafter, were diluted in Middlebrook 7H9 to obtain variable concentrations of the compounds, with starting concentration of 250 g/mL. The *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) strain was cultivated in 7H9 broth at 37 °C until reaching the turbidity

equivalent to McFarland 1 scale. The culture was diluted 25 times, and then 100 μL of bacterial suspension was inoculated in each well containing the compound solutions. The microplates were sealed with parafilm and incubated at 37 °C for 6 days, when Alamar Blue solution was added to the control wells containing the mycobacterial strain. The plates were reincubated for 24 h, when the reading was performed. The blue color in the wells was defined as negative bacterial growth, while the pink color development was defined as positive growth. The microplates showing wells with violet color were reincubated for 24 h, and if color change to pink was detected, the growth was considered positive. If the color was maintained, the growth is negative.

2.3. Log P calculation

The logP of POA and of the esters 2-chloroethyl, allyl, *n*-propyl, isobutyl, tetradecyl and hexadecyl pyrazinoates were calculated using the freeware software Marvin 5.5 (Chemaxon, 2011). The structures were built up in the program prior to the calculation in its neutral form. The logP value was calculated using the methodology described by Viswanadhan et al. (1989). These calculations are fragment-based and are implemented in the software. The program MarvinSketch allows the user to weigh the calculation, extended by the methods of Klopman et al. (1994) and PhysProp database (Syracuse Research Corporation, 1994), being 1 for all of them. The stipulated Na^+, K^+ and Cl^- concentrations used in the calculations were 0.1 M.

3. Results and discussion

Among the alternatives explored to achieve new antimycobacterial agents is the prodrug approach. Prodrugs are transport forms that can solve several problems regarding well-known drugs (Chung et al., 2008). POA prodrugs can be considered an alternative to obtain antimycobacterial molecules. Despite POA being considered the active form of PZA, it cannot cross the hydrophobic cell wall of mycobacteria, due to its high hydrophilic and ionizable characteristics. Cynamon et al., 1992; proposed the esterification of POA to obtain more lipophilic compounds of the active agent, able to cross the cell wall. Several compounds exhibiting activity were synthesized, 5-chloropyrazinoates and 5-methylpyrazinoates being the most active analogs (Cynamon et al., 1992, 1995). Other groups also evaluated some POA esters, with good activity results (Seitz et al., 2002).

Cynamon et al. (1992) presented the synthesis of POA esters through the alcoholysis reaction of pyrazinoyl chloride in two steps. The pyrazinoyl chloride was prepared using excess of thionyl chloride (15 mL to 30 mmol of POA), isolated, and then the acyl chloride was used in the reaction with the corresponding alcohol in dichloromethane with the presence of pyridine as base to neutralize the HCl formed *in situ*. Using this procedure, the authors obtained 74% yield to the pyrazinoyl chloride preparation, and variable yields ranging from 24% to 79% to the ester syntheses. Specifically to the synthesis of 2-chloroethyl pyrazinoate, the total obtained yield was 42%. In a following work (Cynamon et al., 1995), the authors reported again the pyrazinoate syntheses by the same methodology, obtaining yields of 70–80% to the pyrazinoyl chloride, and in the alcoholysis step, the yields were 60–90%. After two steps, the effective yields were around 50%.

In a latter work, Simões et al. (2009) reported the synthesis of higher chain pyrazinoates than that previously reported by Cynamon and colleagues. The synthetic procedure was similar to earlier reports, however the pyrazinoyl chloride was obtained using thionyl chloride as solvent (25 mL to 26.5 mmol of POA), and the alcoholysis step was done using triethylamine as base. The final yields to the preparation of the esters were 42–46%.

In addition to this synthetic route, there are in the literature different procedures to obtain the pyrazinoate esters. Seitz et al. (2002) reported the esterification of POA by the reaction with dicyclohexylcarbodiimide (DCC) in the presence of *N*,*N*-dimethylaminopyridine (DMAP) as catalyst. This method is widely used in ester synthesis, but the yields are frequently variable and it gives byproducts, such as dicyclohexylurea (DCU) and *N*-acylureas, very hard to remove (March, 1992). In a previous work, our group presented the synthesis of POA esters by the reaction between alkyl halides and carboxylates, through nucleophilic substitution (Fernandes and Felli, 2009). With this method, were obtained two pyrazinoate esters, the ethyl and *n*-hexyl pyrazinoates, with 62–76% yields, higher than that presented by Cynamon et al. (1992), but with the advantage to be done in a one-pot reaction. The carboxylates were generated *in situ* using DBU or triethylamine.

In the present work, is reported the synthesis of POA esters in a one-pot step, through the reaction with thionyl chloride and the corresponding alcohol, without any additional base (Fig. 2). This is possible because the pyrazine nitrogen can act as a base to quench the formed HCl, dispensing the use of triethylamine or pyridine. In fact, the pyrazine ring can act as pyridine, since they have the same characteristic.

It was observed that POA exhibit good solubility in 2-chloroethanol. This finding became possible using this reagent as the solvent of the reaction, improving the yield. Moreover, the 2-chloroethanol can easily be removed by evaporation due its low boiling point. The yield obtained with this methodology (90%) is higher than that obtained by Cynamon et al. (1992, 1995). The characterization data of the product show that the desired 2-chloroethyl pyrazinoate was obtained and isolated with good purity. Thus, the results indicate the present methodology is more adequate to synthesize pyrazinoate esters.

The results obtained in the MIC assay showed a significant increase in the POA activity after esterification. The obtained MIC value (3.96 g/mL) is comparable to the MIC of ciprofloxacin, a second-line agent with high activity, and used in the treatment of MDR-TB and other mycobacteriosis (Table 1) and better than PZA (reported MIC 50–100 g/mL). This increase was predicted, however there were no MIC data of this compound against *M. tuberculosis* H37Rv.

Kushner et al. (1952) evaluated the activity of thiopyrazinoates and verified that some of these compounds, mainly the isopropyl-thiopyrazinoate, showed antimycobacterial activity.

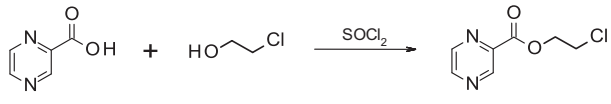

Figure 2 Preparation of 2-chloroethyl pyrazinoate.

Table 1 MICs results for POA, ciprofloxacin and 2-chloroethyl pyrazinoate using the Alamar Blue (MABA) technique.

Compounds	MIC (g/mL)
POA	15.62
Ciprofloxacin	3.96
2-Chloroethyl pyrazinoate	3.96

However, the activity was attributed to the generation of ethylmercaptane, and not to POA. The first report that POA esters could present antimycobacterial activity was done by Solomons and Spoerri (1953), in a work searching for local anesthetic activity of POA and 2,3-pyrazinedioic acid.

It is noted that PZA present better activity *in vitro* in acidic pH (Cynamon et al., 1992; Seitz et al., 2002). It is verified that the activity increases as pH decreases, being the optimum activity reached when the medium pH is approximately 5.8 (Cynamon et al., 1992, 1995).

In the MIC assay performed in this work, we obtained activity of 3.96 g/mL to the 2-chloroethyl pyrazinoate in pH 6.6 against *M. tuberculosis* H37Rv. It is possible to infer that 2-chloroethyl pyrazinoate may show better activity in pH 5.8, and comparing with other compounds reported in the literature, it is considered a highly active compound (Fernandes et al., 2010). It is observed a 2 to 8-fold increase in the activity of POA esters when the pH is lowered from 6.6 to 5.8 (Cynamon et al., 1992). Thus, it can be estimated the activity of 2-chloroethyl pyrazinoate can be even lower.

In order to evaluate the potential biological activity of non-tested compounds, some pyrazinoates previously synthesized by us [18] had their activity predicted by our previously published QSAR model (Fernandes et al., 2010). The predicted pMIC value to 2-chloroethyl pyrazinoate was 2.32, but in a different protocol from that presented now. In this work, this compound had its activity evaluated against the strain *M. tuberculosis* H37Rv ATCC 27294, using the Alamar Blue (MABA) methodology [20], and its experimental pMIC was 1.67 (equivalent to 3.96 g/ml).

It is reported in the literature a MIC value of 6.25 g/ml to 2-chloroethyl pyrazinoate in pH 5.8 against *M. bovis* ATCC 27289, and to *M. tuberculosis* BUR (Cynamon et al., 1992). It is important to highlight the *M. bovis* is considered naturally resistant to PZA. Thus, the 2-chloroethyl pyrazinoate can be considered active in some resistant strains of mycobacteria. In this previous report, the authors did not perform the MIC assay of this compound in *M. tuberculosis* H37Rv. The compounds that exhibited better activity were the allyl and the *n*-propyl pyrazinoates, with MIC values lower than 3.25 g/mL (Fig. 3). In the latter work, the authors obtained MIC values lower than 2 g/mL to four compounds, isobutyl, *n*-decyl, *n*-pentadecyl and benzyl pyrazinoates (Cynamon et al., 1995).

More recently, Simões et al. (2009) presented a work reporting the synthesis, antimycobacterial activity and enzymatic stability of POA esters and the equivalent amides. The authors observed that the amides were very stable, even in *M. smegmatis* homogenates, concluding that they will not be activated inside mycobacteria. MIC assays corroborate with this finding, showing MIC values higher than 800 g/mL to these amides. However, the long-chain esters of POA (dodecyl, tetradecyl and hexadecyl esters) showed MIC values ranging from 10 to 40 g/mL (Fig. 3).

Analyzing these previous works, it is possible verify a strong positive relationship of the side chain of the esters (and also its lipophilicity) with the antimycobacterial activity. The higher the side chain, the higher is the lipophilicity and higher the antimycobacterial activity. Regarding this, in counterpart, it is possible to verify that lower chain derivatives show similar activity than the higher chain derivatives (Table 2). This can be explained by the excessive lipophilicity,

Table 2 Log P values for the selected compounds.

Compounds	log P	MIC (g/mL)
POA	−0.42	15.62
2-Chloroethyl pyrazinoate	0.39	3.96
Allyl pyrazinoate	0.46	3.25[a]
n-Propyl pyrazinoate	0.61	3.25[a]
Isobutyl pyrazinoate	0.97	2[b]
n-Tetradecyl pyrazinoate	5.50	10[c]
n-Hexadecyl pyrazinoate	6.39	40[c]

[a] Obtained from Ref. Franzblau et al. (1998).
[b] Obtained from Ref. Klopman et al. (1994).
[c] Obtained from Ref. Seitz et al., (2002).

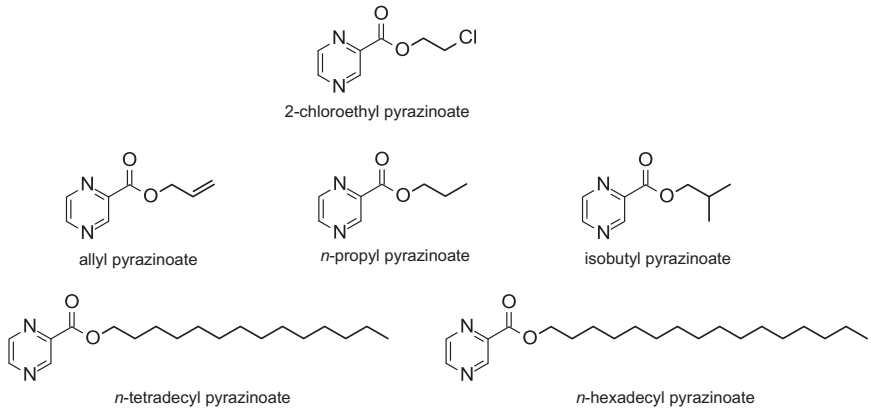

Figure 3 POA esters.

which reduces the water solubility, becoming more difficult the diffusion through the bacterial cell wall, and compromising the usefulness of these compounds as future drugs. Thus, molecules with low logP and adequate MIC should be considered as more promising. Considering this, the 2-chloroethyl pyrazinoate can be defined as a promising compound, since its logP is lower than derivatives with similar activity.

It is concluded that the 2-chloroethyl pyrazinoate can be synthesized by an efficient one-pot procedure, with high yield and adequate purity. Moreover, it can be considered a promising alternative as prodrug with activity in PZA resistant strains.

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