Esters of pyrazinoic acid are active against pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and other naturally resistant mycobacteria *in vitro* and *ex vivo* within macrophages.

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Running Title: New POA derivatives active in PZA resistant mycobacteria

ABSTRACT

Pyrazinamide (PZA) is active against major *Mycobacterium tuberculosis* species (*M. tuberculosis, M. africanum,* and *M. microti*), but not against *M. bovis and M. avium.* The latter two are mycobacteria species involved in human and cattle tuberculosis and in HIV co-infections, respectively. PZA is a first-line agent for the treatment of human tuberculosis and requires activation by a mycobacterial pyrazinamidase to form the active metabolite pyrazinoic acid (POA). As a result of this mechanism, resistance to PZA as often found in tuberculosis patients is caused by point mutations in pyrazinamidase.

In previous work, we have shown that POA esters and amides synthesized in our laboratory were stable in plasma. Although the amides did not present significant activity, the esters were active against sensitive mycobacteria at concentrations 5-to-10 fold lower than those of PZA. Here, we report that these POA derivatives possess antibacterial efficacy *in vitro* and *ex vivo* against several species and strains of *Mycobacterium* with natural or acquired resistance to PZA, including *M. bovis* and *M. avium*. Our results indicate that the resistance was probably overcome by cleavage of the prodrugs into POA and a long-chain alcohol. Although it is not possible to rule out that the esters may have intrinsic activity *per se*, we bring evidence here that long-chain fatty alcohols possess a significant anti-mycobacterial effect against PZA-resistant species and strains and are not mere inactive promoieties. These findings may lead to candidate dual-drugs having enhanced activity against both PZA-susceptible and PZA-resistant isolates and being suitable for clinical development.

INTRODUCTION

Mycobacterium tuberculosis is a particularly successful pathogen which latently infects about 2 billion people, almost one third of the world population (1). Each year, there are about 8 million new cases of tuberculosis (TB) and 1.3 million deaths worldwide. Fifty million people have already been infected with drug-resistant TB (1). This situation arose from a) the spread of HIV worldwide, a virus that weakens the immune system allowing the reactivation of latent drug-susceptible or drug-resistant *M. tuberculosis* strains, and b) non-compliance, often due to toxic side-effects during the long lasting treatment.

Pyrazinamide (PZA) is an important frontline anti-TB drug and plays a key role in shortening TB therapy from 9–12 months to the current 6 months (2). The ability of PZA to shorten TB therapy is due to its activity against a population of non-growing, persistent tubercle bacilli residing in an acidic pH phagosomal environment that are not killed by other anti-TB drugs (3-5). Furthermore, studies have shown that a regimen of PZA combined with rifampicin for 2 months is as effective to prevent TB in HIV carriers as a 6-month isoniazid treatment (6).

Cynamon group postulated in 1992 that pyrazinamide was converted to pyrazinoic acid (POA) within the bacteria lumen and POA was the active antimycobacterial agent (7). Indeed PZA is a prodrug whose activation requires hydrolysis by the pyrazinamidase enzyme encoded by the *pncA* gene of *M. tuberculosis* (2, 3). The unique susceptibility of *M. tuberculosis* to PZA is at least partly due to a inefficient efflux mechanism that allows POA to be increasingly accumulated in the acidic interior of *M. tuberculosis* (2).

While acquired resistance to PZA in *M. tuberculosis* occurs mainly by

mutations in the *pncA* gene (8), the mechanism of natural PZA resistance in other mycobacteria is more complex. Like *M. tuberculosis, M. bovis* strains including BCG are naturally resistant to PZA due to a single point mutation (C⊙G) at nucleotide position 169 in *pncA* (9). In contrast, naturally PZA-resistant *M. smegmatis* and other bacteria such as *E. coli* have a highly active POA efflux mechanism that prevents accumulation of POA even at acidic pH (10). *M. avium* has high pyrazinamidase activity, and its natural resistance to PZA is probably due to the POA efflux mechanism (11).

If PZA is a prodrug then it would be possible to develop alternative prodrugs that deliver POA intracellularly. This hypothesis was successfully developed by the work of Cynamon *et al* in a series of papers dedicated to explore the potential of such esters as anti-tuberculosis agents (7, 12, 13). The advantage of these compounds seems evident; because esterases are abundant in mycobacteria, prodrugs can be easily activated *in situ*. Moreover resistant strains will be hardly selected, as the mechanism would no longer depend on a single gene point mutation. Esterases also exist in human plasma, therefore these esters may be hydrolyzed before reaching their bacterial target, their active moiety (POA) being too polar for effective permeation into mycobacteria (12-14).

In order to improve the stability of the POA esters we designed and synthesized several long-chain esters, showing that esters with long alkoxy chains are particularly resistant towards hydrolysis in the plasma (14). This fact suggested that it might be possible to identify some POA ester prodrugs with increased resistance toward plasmatic hydrolysis. Here, we demonstrate that such POA ester prodrugs are active against PZA-resistant *M. tuberculosis* isolates, as well as against naturally PZA-resistant species of mycobacteria such as *M. bovis* and *M. avium*. Our results

indicate that their resistance was overcome by two factors, the release of POA by enzymes other than pyrazinamidase, and the intrinsic anti-mycobacterial effect of long-chain alkanols.

The new compounds were shown to possess *in vitro* and *ex vivo* activities within macrophages infected either with sensitive strains of *M. tuberculosis* or *M. avium* even at concentrations 10-to-100-fold lower than PZA.

MATERIALS AND METHODS

Materials

Balanced salt solution, phosphate-buffered saline, DMEM medium, and L-glutamine were purchased from Invitrogen. Sodium dodecyl sulfate (SDS), Triton X-100, pyrazinamide (PZA), pyrazinoic acid (POA), *n*-dodecanol (12-ol), *n*-tetradecanol (14-ol), *n*-hexadecanol (16-ol), *n*-butanol (4-ol) and trypan blue were purchased from Sigma Aldrich Quimica SA. Middlebrook 7H10 agar was purchased from Difco. The materials and equipment used for antibiotic susceptibility tests of *M. tuberculosis* with the BACTEC MGIT 960 PZA kit system were purchased from Becton Dickinson and prepared according to the recommendations of the manufacturer. Microwell tissue culture plates were purchased from Nunc. Pyrazinamide (PZA) and the esters of POA, namely E-12 (*n*-dodecyl pyrazinoate), E-14 (*n*-tetradecyl pyrazinoate), and E-16 (*n*-hexadecyl pyrazinoate,) were synthesized as described previously (14). They were then prepared in stock solutions of 8 mg/ml in dimethyl sulfoxide (DMSO), and diluted in Middlebrook 7H9 medium containing ADC (albumin, dextrose, catalase-Difco, but no oleic acid as it is toxic for mycobacteria at acidic pH) (15), and acidified to pH = 5.9 (14).

Bacterial strains

M. tuberculosis H37Rv (ATCC 27294), a PZA-susceptible strain, served as the positive control; five clinical strains with acquired resistance to PZA (MDR-TB) and maintained in the Unit of Bacteriology, Health Institute of Lisbon, Lisbon, Portugal, were used as *M. tuberculosis* PZA-resistant strains (16). Two out of the five clinical strains were selected for their susceptibility to POA. *M. bovis* isolated from infected cattle was provided by the National Laboratory for Veterinary Research, Lisbon; *M. bovis* BCG (CIP 105050) was purchased from the Pasteur Institute, Pasteur Collection, Paris, and the two *M. avium* strains, DZMC 44156 and DZMC 44157, were from the German Collection (17). *M. smegmatis* ATCC607 variant mc2 155 was used for the production of the homogenate.

Activation of the prodrugs in *M. avium* and BCG homogenates.

M. avium homogenate and BCG homogenate were prepared using the technique described for *M. smegmatis* (14, 18). Incubation of the homogenate with prodrug was performed at 37ºC in a total volume of 1500 µl. Concentration of substrate in incubation media was 1.2 $x10^{-4}$ M and protein concentration was 0.12 mg/ml. Dilutions were performed using PBS. At predetermined time points, aliquots of 150 µl were taken into vials containing 300 µl of acetonitrile and 300 µl of a 1% $ZnCl_2$ aqueous solution. The suspension was then agitated in a vortex and centrifuged for 5 minutes. The supernatant was injected in HPLC as in (14) for quantification of the prodrug and POA**.**

Determination of the Minimum Inhibitory Concentrations (MIC)

The MIC of PZA, POA and the long-chain ester prodrugs of POA (E-12, E-14 and E-16), as well as the corresponding long-chain fatty alcohols used as promoieties (12-ol, 14-ol and 16-ol), were determined individually by two methods, the broth dilution method available on our laboratory (19), and the BACTEC MGIT 960 PZA kit provided by the National Health Institute, as described previously (16). Briefly, five MDR-TB isolates and the susceptible *M. tuberculosis* used as reference were tested for susceptibility to PZA by the BACTEC MGIT 960 PZA kit at a reduced pH of 5.9 and a 100 µg/ml concentration of PZA (Becton Dickinson) according to the manufacturer's manual. We retested these five MDR-TB strains for PZA susceptibility by using the BACTEC MGIT 960 PZA with 100-, 300-, and 900 µg/ml concentrations of PZA. In parallel, we used the dilution method, screening drug concentrations ranging from 5 up to 1000 µg/ml for the same *M. tuberculosis* strains tested by the BACTEC system, for *M. bovis*, *M. bovis* BCG and *M. avium*. The acidified Middlebrook 7H9 medium (pH 5.9) supplemented with ADC (Difco) (14) and an adjusted concentration of mycobacteria (approximately 10^5 colony-forming units). Each agent or prodrug screened in serial dilutions, was incubated for 10 days at 37 ºC. For the long-chain alkanols, the pH was adjusted to 7 as usual for compounds other than POA prodrugs. The Minimum Inhibitory Concentration (MIC) was the minimum drug concentration where no visible turbidity was observed. The assay was extended until day 30. A test tube without drug was used as control. The MIC values obtained were similar in the two methods.

Macrophage cell line J774A.1.

The J774 macrophage cell line was kindly provided by Gareth Griffiths, EMBL, Heidelberg, and has been maintained as described previously (17).

Macrophage infection and *ex vivo* intracellular killing activity.

Bacterial cultures in the exponential growth phase were pelleted, washed twice in PBS pH 7.4 and re-suspended in DMEM medium to a final optical density (OD_{600}) of 0.1 corresponding to 10^7 colony-forming micro-organisms per ml. Clumps of bacteria were removed by ultrasonic treatment of bacteria suspensions in a ultrasonic water bath for 15 min followed by low-speed centrifugation at 120 *g* for 2 min. Single cell suspension was verified by light microscopy. J774 cells were seeded onto 24 well tissue culture plates at a density of 0.5 x 10^5 cells per well and were incubated for 2 days until 80% confluence and infected with bacteria at a multiplicity of infection 10:1. After 3 hours of bacteria internalization by macrophages, at 37ºC and 5% CO_2, remaining extracellular bacteria were removed by intensive washing with PBS. The prodrugs were then added to the medium and incubated during 7 days. Every second day, the medium was replaced with fresh medium containing the test compound.

At the end of the bacterial internalization by macrophages (3 hours) and after one, three, five and seven days post-infection, macrophages were washed with PBS and disrupted with 1% IGEPAL CA-630 (Sigma-Aldrich) solution in water, a nonionic, non-denaturing detergent that disrupts eukaryotic cells but does not affects mycobacteria viability, with the goal being to assess the colony-forming microorganisms of viable intracellular bacteria.

Serial dilutions of the macrophage culture lysate were prepared in water and platted in Middlebrook 7H10 agar medium supplemented with OADC (Difco). After about 3 weeks of incubation at 37ºC, colonies were counted. The proportion of surviving mycobacteria in samples treated with the compounds was compared to controls of infected cells at the same time point treated with the same dilution of the dilution solvent (mock control) and was taken as a measure of the *in vivo* activity of

the screened compounds. The assays were done in triplicate and from independent experiments.

Cytotoxicity assay.

J774A.1 cells were incubated with the compounds at concentrations of 20 µg/ml for esters E-12, E-14 and E-16, and at 5 and 10 µg/ml for the *n*-alkanols 12-ol, 14-ol and 16-ol for 5 days. Culture media with the compounds was replenished every second day during the course of the experiment. DMSO, at the same proportions as in the tested compounds, was used as control. The highest concentration of DMSO used was 1%. Puromycin (Sigma-Aldrich) was used as a positive control for cell death. Cell viability was determined after 5 days of treatment using alamarBlue® (Molecular Probes) following the manufacturer's indications. Briefly, 10% (v/v) of alamarBlue reagent was added to each well and incubated for 4 h at 37 ºC and 5% CO_2. Fluorescence was measured at an excitation of 570 nm and emission of 595 nm in a TecanM200 plate spectrophotometer. Viability was calculated as percentage fluorescence intensity relative to the untreated controls. Test results were obtained from at least four independent experiments, each performed in triplicate.

Statistical analysis.

All values are reported as means ± SD of 3 independent experiments. The statistical significance of the differences observed in bacterial loads was analyzed by the Student's *t*-test. Differences were considered significant at $P<0.05$ (*) or $P<0.01$(**).

RESULTS

Hydrolysis of the esters

Figure 1 shows the structures of the esters and alcohols used in this study and also the hydrolysis reaction to POA and the corresponding alcohol. Incubation of the esters with mycobacterial homogenate (BCG or *M. avium*) gives rise to POA and the corresponding alcohol. As can see from Figure 1 disappearance of the ester corresponds quantitatively to the formation of POA.

Effect of PZA, POA and POA esters on PZA-susceptible and resistant mycobacteria *in vitro*.

The calculated Minimum Inhibitory Concentrations (MIC) of PZA, POA and POA esters (PAEs) are summarized in Table 1 for a pH of the medium adjusted to 5.9. As expected, the results for PZA are in agreement with the literature with a MIC of 10-20 µg/ml when using pH 5.5 (20) and 100 µg/ml at a pH of 5.9 (16, 21). By using exactly the same conditions all three POA esters displayed a 5-fold lower MIC than PZA or POA *in vitro* against the *M. tuberculosis* susceptible strain.

When PZA-resistant strains of *M. tuberculosis*, or natural resistant species such as *M. avium* and *M. bovis*, including BCG, were used to screen the new agents, the results were as depicted in Table 1: while PZA has a MIC higher than 1000 µg/ml for all strains and species tested, confirming their resistance, the esters E-12 and E-14 (obtained from 12-ol and 14-ol, respectively) showed MICs of 10-40 µg/ml indicating their efficacy against PZA-resistant mycobacteria. For E-16 (*n*-hexadecyl pyrazinoate), the MIC was slightly higher than for E-12 and E-14 but still two orders of magnitude lower than those of PZA against resistant mycobacteria.

The results also indicate that POA, with a MIC similar to PZA in sensitive strains, is active against PZA resistant mycobacteria except *M. avium*. Indeed the differences observed between *M. tuberculosis* H37Rv and the *M. tuberculosis* PZA-resistant strain with sensitivity to POA indicates that the resistance on these *M. tuberculosis* clinical strains is due to a mutation in pyrazinamidase as confirmed by DNA sequencing of the *pncA* gene (16). For all *M. avium* strains tested, a resistance against PZA and indeed POA was detected with MIC higher than 1000 µg/ml. However all POA esters (E-12 to E-16) were shown to possess antimycobacterial activity against *M. avium* with MIC ranging from 10 to 40 µg/ml.

Antibacterial effects of the pyrazinoate esters on susceptible and resistant mycobacteria in infected macrophages

We first determined the minimal inhibitory concentration of each PAEs against an intracellular *M. tuberculosis* susceptible strain. For each compound we selected a range of concentrations above and below the *in vitro* MIC, ranging from 10 to 200 µg/ml. Figure 2A shows the antimycobacterial effects on the susceptible strain within J774 macrophages. The percentage of intracellular survival is represented at days 0, 1, 3, 5 and 7. The culture medium with the drug was added 3 hours post-bacterial internalization by macrophages and was replaced every second day. For PZA and POA, the *ex vivo* active concentration was 100 µg/ml while for the PAEs E-12, E-14 and E-16 was 20 µg/ml, confirming an *ex vivo* MIC similar to that obtained *in vitro*. Lower concentrations of these compounds were ineffective in infected macrophages. On day one post-infection all the compounds were effective against *M. tuberculosis*. E-12 and E-14, at a concentration fivefold lower than that of PZA or POA, presented approximately a 50% higher antimycobacterial activity compared

with PZA- and POA (Fig. 2A). This activity was maintained throughout the experiment. At day seven post infection the activity of the esters was significantly more effective against intracellular mycobacteria than PZA or POA (Fig. 2A).

Since ester E-12 had higher activity against intracellular susceptible *M. tuberculosis* plus a MIC of 10-20 µg/ml against two strains of *M. avium*, we selected this compound to determine its activity against intracellular *M. avium*. Figure 2B shows how its activity evolved during the days following infection, thus emphasizing its effects on intracellular survival rate as compared with PZA and POA. As the MIC determined *in vitro* for *M. avium* DZMC 44156 was 20 µg/ml at pH 5.9 we chose to test three concentrations below the MIC in infected macrophages. The concentration found to be effective was 10 µg/ml with 50% decrease of intracellular *M. avium* from day 3 post-infection relatively to PZA or POA treated cells (Fig. 2B).

Mycobacterium avium is naturally resistant to most antibiotics including PZA. Unlike *M. tuberculosis* whose PZA resistance is due to mutations in the *pncA* gene, *M. avium* has high PZase activity. *M. avium* natural resistance to PZA is most likely due to a POA efflux mechanism, but this mechanism has not been fully proven so far (11). The MICs in Table 1 clearly suggest that *M. avium* is resistant to POA in contrast to the other tested mycobacteria. This led us to investigate the effect of these POA esters against the two *M. avium* reference strains. Incubation of esters with a *M. avium* homogenate gives rise to quantitative hydrolysis of the POA esters to POA (Fig. 1). Because POA did not present activity when tested against *M. avium* (table 1), we decided to test the liberated long-chain alcohols as these are likely candidates to account for antimycobacterial activity. We therefore examined alcohols with alkyl chain lengths from C4 to C16 and determined their MICs against different mycobacteria strains including *M. avium*, as depicted in Table 2.

As expected, all long-chain alcohols displayed an antimycobacterial activity slightly higher than POA esters, with MICs ranging from 5 to 40 µg/ml and efficacy increasing with chain length.

Interestingly Kushner et al (22) obtained relevant antimycobacterial activity with ethyl thiopyrazinoate and also found that ethanethiol the potential liberated thiol presented very good activity (even higher than the ester) when tested alone, shifting the focus of their research from the pyrazine nucleus to thiol moietie.

Antibacterial effect of the long-chain alcohols against intracellular mycobacteria

We next tested the effect of long-chain alkanols on the intracellular survival of PZA resistant mycobacteria. Effects of the long-chain alcohols on intracellular survival of *M. tuberculosis* in macrophages were determined first. The greatest effects on bacterial killing were observed after 5 or more days of incubation. The results in Figure 3 show that the 12-carbon alcohol was the most effective at killing the bacteria at 5 days post-infection. Although the 12-carbon alcohol was usually less effective on most strains in broth culture than its homologs, inside macrophages the 12-ol was very effective. The PZA-resistant strain of *M. tuberculosis* demonstrated comparable sensitivities to 100 µg/ml POA and to 12-ol at 10 µg/ml. No differences were observed between the *M. tuberculosis* reference strain H37Rv relative to the PZA resistant strain for all compounds with the exception of PZA (Fig 3B left panel compared to Fig 2A, at day seven post-infection).

In this context, *n*-dodecanol again proved to be the most active *n*-alkanol screened. We then compared the effect of 12-ol with the corresponding POA ester E-12 on the intracellular killing of *M. tuberculosis* PZA resistant strain and/or *M. avium* DZMC44156, seven days post-infection. The results (Fig. 3B right panel) show that

comparable effects were caused by 10 µg/ml E-12 and 5 µg/ml 12-ol, namely 50% difference in survival when comparing to PZA- or POA-treated infected cells in *M.avium* infection. As a reminder, one molecule of E-12 liberates one molecule of dodecanol and one of POA and thus 10 µg of E-12 contain approximately 5 µg of the corresponding alcohol. However for *M. tuberculosis* the results in Figure 3B (left panel) show a slight higher activity of the ester relatively to 12-ol or POA alone.

Cytotoxicity evaluation of the new prodrugs and alkanols to J774 cells

To confirm that the decrease in CFUs after treatment with the esters and long-chain alkanols were not due to a potential cytotoxicity against J774 cells and therefore caused by the loss of infected macrophages in the culture, the alamarBlue test was used as described in Methods. The alamarBlue reagent allows quantification of cell viability by measuring their metabolic functions. We verified that after 5 days of exposure, concentrations of prodrugs or *n*-alkanols equal or below their MICs resulted in no reduction of macrophages viability in non infected control host cells (Fig. 4). Cytotoxicity was tested in parallel over infected J774 cells for seven days but no effects were detected even at concentrations 10-fold higher than the MIC (not shown).

DISCUSSION

The present study has shown that POA esters are more effective in clearing intracellular mycobacteria within J774 macrophages than PZA or POA (Figs.2A and B). It was found that 100 µg/ml was an appropriate concentration to distinguish between susceptible strains (used as controls) and PZA-resistant *M. tuberculosis*. Moreover, MICs higher than 1000 µg/ml were found for all naturally or acquired

resistant mycobacteria to PZA.

The POA esters, when tested against mycobacteria with natural or acquired resistance, were shown to be active with MICs ranging from 5 to 80 µg/ml. This was true for five drug resistant *M. tuberculosis* isolates and for *M. bovis*, including BCG. These are all mycobacteria whose PZA resistance mechanism is related to mutations in the *pncA* gene together with poor efflux pump mechanisms characteristic of this group of microorganisms.

It is intriguing to note that with *M. avium*, a naturally PZA resistant species, both strains tested were highly susceptible to all esters but resistant to POA. Cynamon, , also reported that POA esters when obtained with similar alkanols (10-ol and 15-ol) presented activity against *M. avium* (12).

Since tested POA did not show activity against *M. avium* (table 1), the activity presented by pyrazinoate esters could be due to increased accumulation of POA inside the cells, intrinsic activity of the ester molecules or intrinsic activity of the alcohol moiety.

Regarding the first hypothesis It is possible that POA is not active against *M. avium* because it cannot penetrate inside the bacteria and thus the ester furnishes a way of delivering POA inside the cell, however it is likely that *M. avium* possess an effective POA efflux mechanism (11). If so, POA should also be expelled from the mycobacteria after being formed by hydrolysis making the accumulation hypothesis less likely (unless of course the rate of entrance supplants the rate of efflux).

So two possibilities remain: either the esters present antimycobacterial activity *per se* or the alkanols have activity against *M. avium*. Since pyrazinoic acid esters are easily hydrolysed to the corresponding alcohols in *M. avium* homogenate

(Fig 1), we favour the long-chain alcohols as the most likely agents accounting for the observed antimycobacterial activity. Indeed it can be seen from Fig. 3 B that the activity of the alkanol is comparable to the activity of the ester. This is not surprising as the activity of the other moiety (POA) against *M. avium*, is very low. It is known that Tween 80, a detergent used to disperse clumps of mycobacteria in liquid media cultures, can kill mycobacteria at acidic pH. Tween 80 releases oleic acid (23) and its structure has some similarities with long-chain alkanols. Both have a long hydrocarbon chain with a polar group capable of hydrogen bonding. Moreover it is well known that alcohols can easily be oxidized *in vivo* to carboxylic acids (24, 25). Indeed the lethal effects of oleic acid and other fatty acids were reported against mycobacteria (26, 27). Other reports point to the lethal effect on *M. tuberculosis* of extracts of some South African plants enriched with palmitic, oleic and linoleic acid and used to treat tuberculosis (28). More recently the antimycobacterial activities of C6 to C13 *n*-alkanols were examined against *M. smegmatis and M. tuberculosis* H37Rv, the best antimycobacterial activity being found with 10-ol (29), very similar to our 12-ol for which we obtained the highest activity. The alcohols 12-ol, 14-ol and 16-ol have the respective log P values of 4.2, 5.2 and 7.2 (as calculated by ALOGPS) (30). It seems possible that 14-ol and 16-ol, which are poorly soluble in water, do not dissolve satisfactorily in the test medium, explaining why better results are observed with alkanols of lower log P (4.2).

For *M. tuberculosis* our results indicate that their resistance was overcome by the release of POA and by the anti-mycobacterial effect of the long-chain alcohol released following POA ester hydrolysis (Fig. 3B, left panel) while for *M. avium* it is more likely that only the 12-ol contributed for the antimycobacterial effect (Fig.3B, right panel). In a context of emergency with MDR-TB increasing the world over,

these preliminary results should provide a potential therapeutic alternative when other treatments have failed. This approach applied to other active moieties may lead to candidate dual-drugs with enhanced activity against both PZA-susceptible and PZA-resistant isolates suitable for clinical development.

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FIGURE LEGENDS

Figure 1. Hydrolysis reaction of pyrazinoate esters to POA and the corresponding alcohol and structure of the compounds studied: concentration of ester E-12 and POA during the hydrolysis of E-12 in *M. bovis* BCG or *M.avium* homogenates.

Figure 2. *Ex vivo* antibacterial effects of POA esters on mycobacteria. The ordinate represents the percentage remaining intracellular survival measured by colony-forming units/ml along seven days post-infection. (A) Effects of E-12, E-14 and E-16 on intracellular *M. tuberculosis* compared with PZA and POA. (B) Effect of E12 on *M. avium* within J774 macrophages. PZA and POA were used to confirm the natural resistance of *M. avium* to these compounds. The statistical significance of the differences observed in bacterial loads was analyzed by the Student's *t*-test. Differences were considered significant at $P<0.05$ (*) or $P<0.01$ (**).

Figure 3. *Ex vivo* antibacterial effects of long-chain alkanols and corresponding E-12 ester on PZA-resistant mycobacteria. (A) Effects on intracellular *M. tuberculosis* of long-chain alkanols. (B) Effects on *M. tuberculosis and on M. avium* of 12-ol compared with PZA and POA (100 µg/ml), and corresponding PZA ester E-12. The statistical significance of the differences observed in bacterial loads was analyzed by the Student's *t*-test. Differences were considered significant at $P<0.05$ (*) or $P<0.01$ (**).

520 **Figure 4.** Cytotoxicity of PZA esters and the corresponding long-chain alkanols on
521 host macrophages. The viability of macrophages is expressed on percentage of
522 survival relative to control macrophages.

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TABLE 1. Minimal Inhibitory Concentrations of POA esters, POA and PZA *in vitro.* (data in µg/ml) E-12 (*n*-dodecyl pyrazinoate), E-14 (*n*-tetradecyl pyrazinoate), E-16 (*n*-hexadecyl pyrazinoate), PZA (pyrazinamide), POA (pyrazinoic acid).

Compound	*M. bovis BCG*	*M. bovis*	*M. avium 44156*	*M. avium 44157*	*M. tb H37Rv*	*M. tb PZAR 1*	*M. tb PZAR 2*
E-12	20	40	20	10	20	40	20
E-14	20	40	20	10	20	40	20
E-16	>40	>40	40	40	20	>40	>40
PZA	>1000	>1000	>1000	>1000	100	>1000	>1000
POA	100	100	>1000	>1000	100	100	100

M. tb H37Rv is the reference sensitive strain. All other species and strains are resistant to PZA

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566 **TABLE 2.** Minimal inhibitory concentrations of the *n*-alkanols *in vitro* (data in
567 µg/ml). 4-ol (butanol) was used to estimate the effect of a short-chain alcohol.

Compound	*M. bovis BCG*	*M. avium 44156*	*M. avium 44157*	*M. tuberculosis H37Rv*
4-ol	>80	>80	>80	10
12-ol	10	40	40	5
14-ol	5	20	20	5
16-ol	5	20	20	5
Kanamycin	0.5	10	10	1

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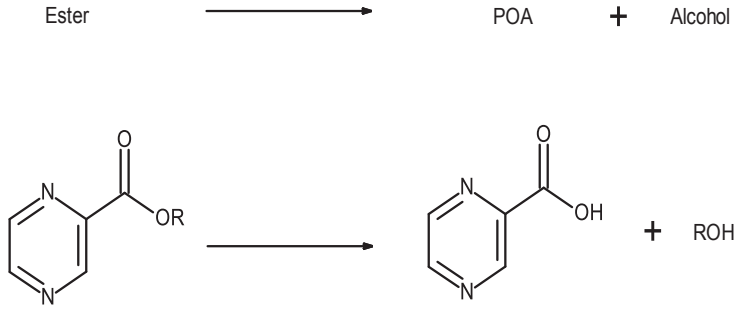
Figure 1 Pires et al.2015



R	Ester	Alcohol
n-Butyl	E-4	4-ol
n-Dodecyl	E-12	12-ol
n-Tetradecyl	E-14	14-ol
n-Hexadecyl	E-16	16-ol

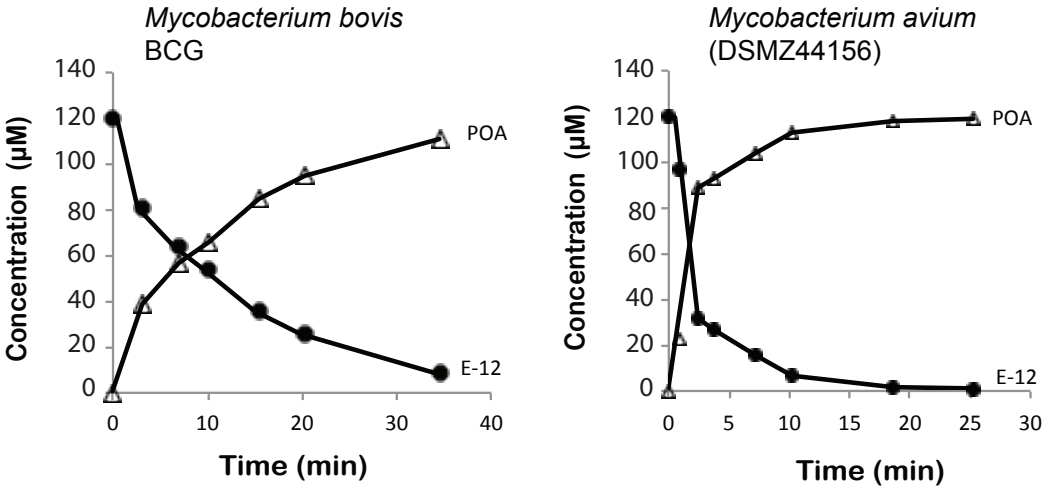


Figure 2 Pires et al.2015

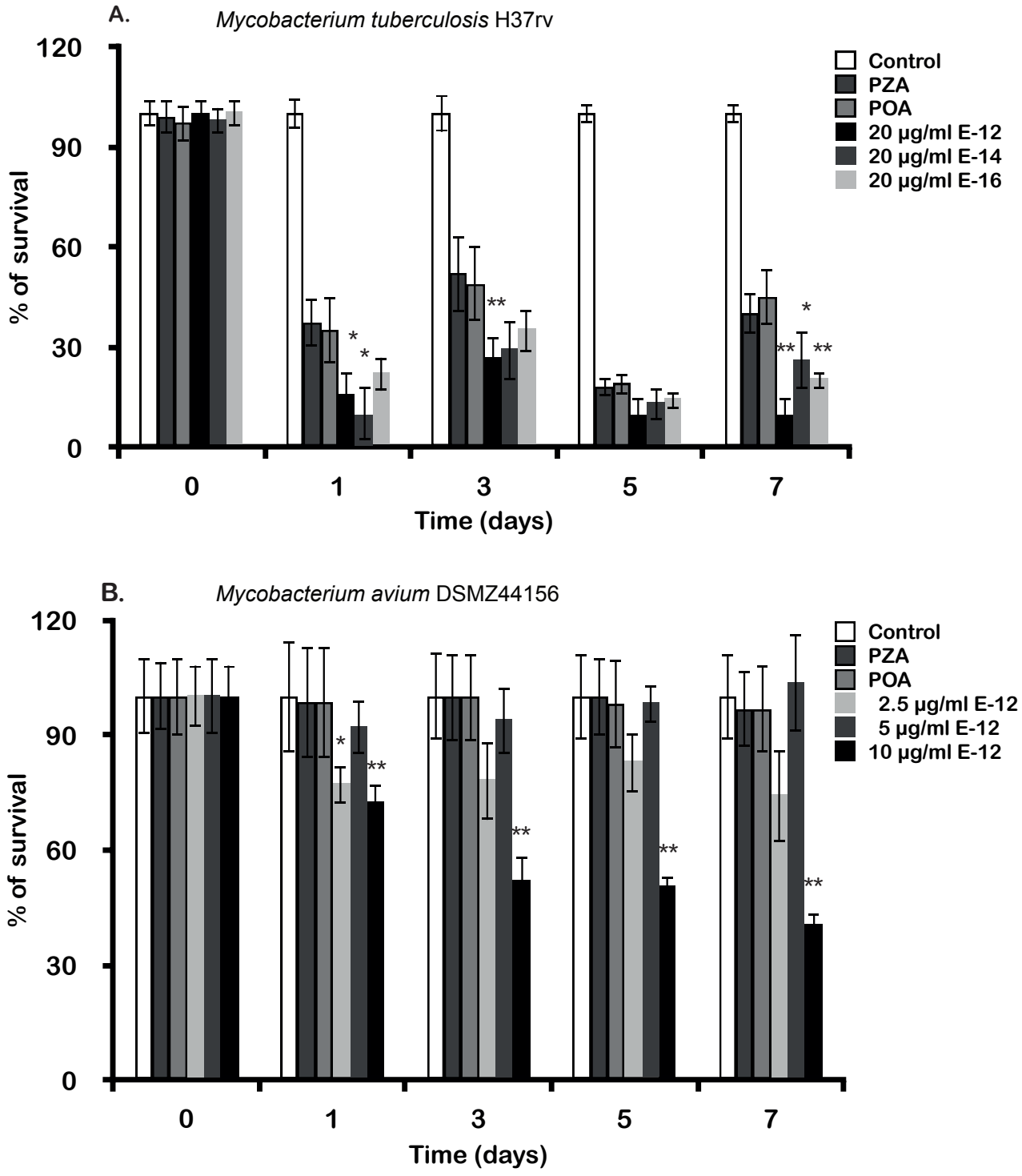


Figure 3 Pires et al 2015

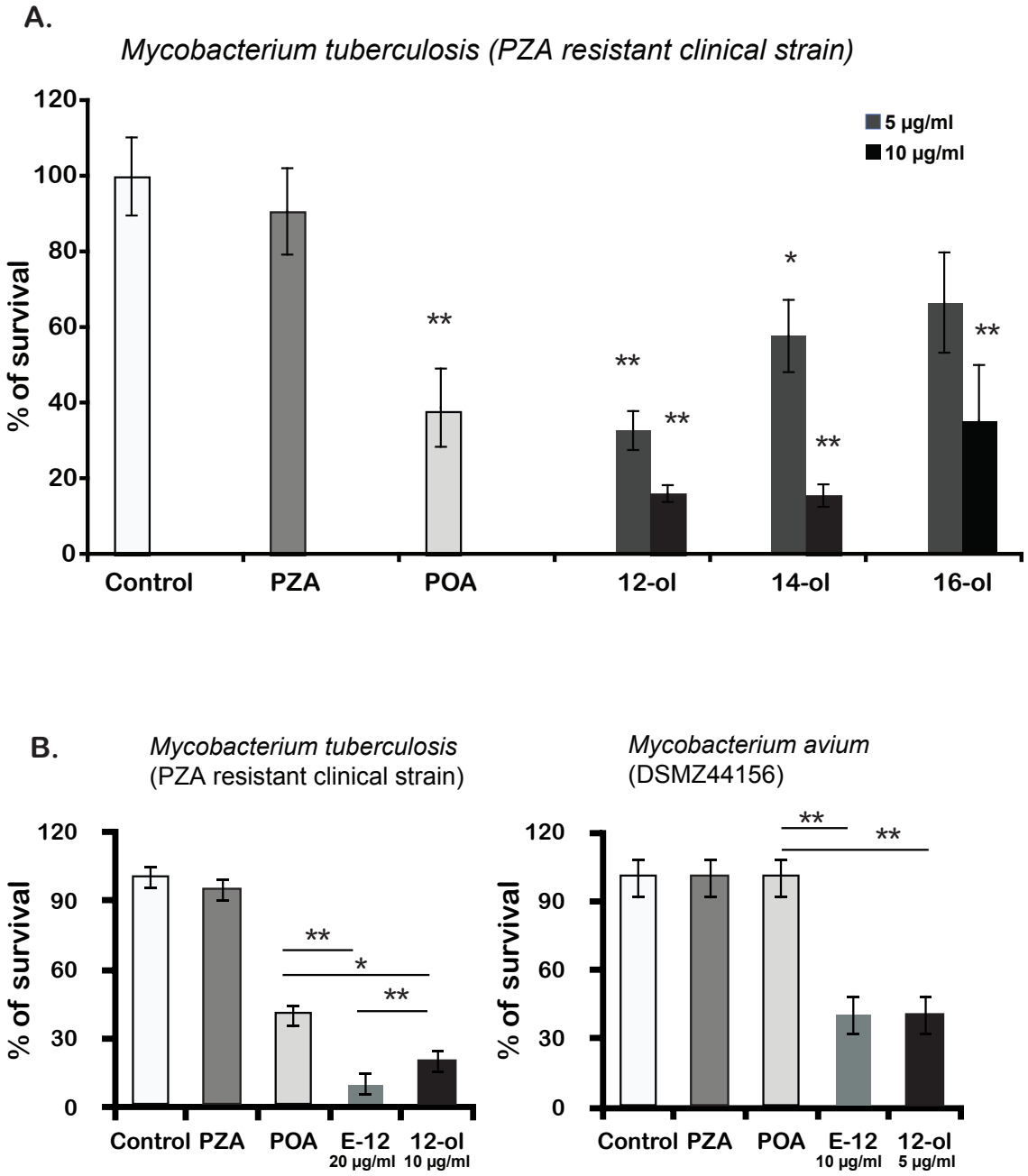


Figure 4 Pires et al, 2015

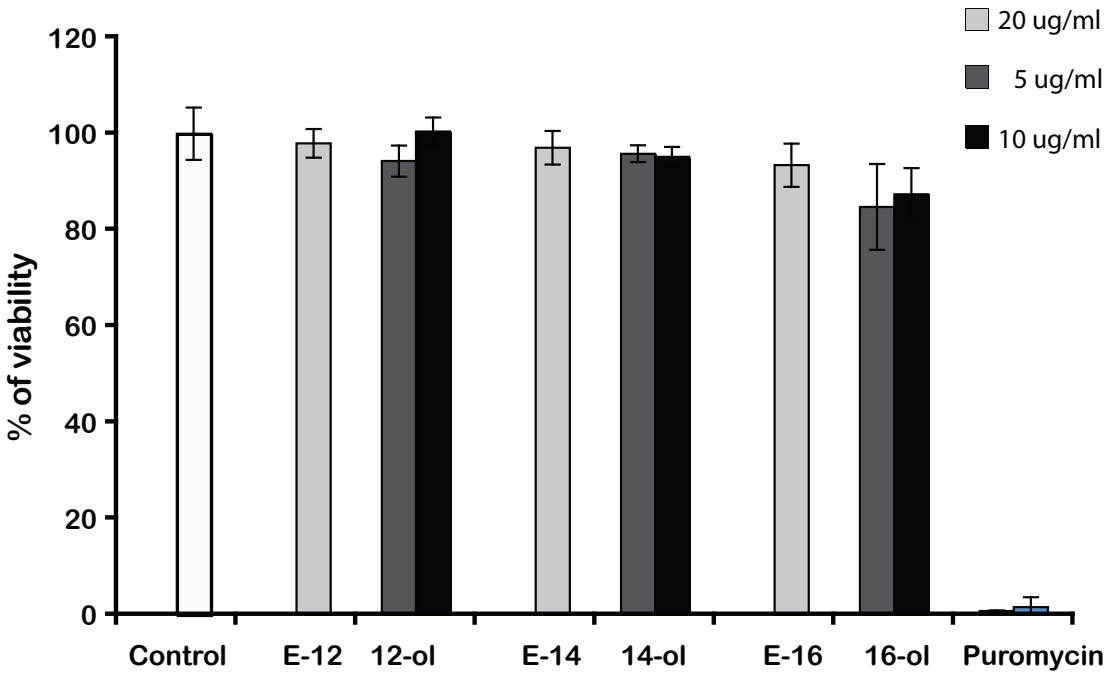


TABLE 1. Minimal Inhibitory Concentrations of POA esters, POA and PZA *in vitro.* (data in µg/ml) E-12 (*n*-dodecyl pyrazinoate), E-14 (*n*-tetradecyl pyrazinoate), E-16 (*n*-hexadecyl pyrazinoate), PZA (pyrazinamide), POA (pyrazinoic acid).

Compound	*M. bovis BCG*	*M. bovis*	*M. avium 44156*	*M. avium 44157*	*M. tb H37Rv*	*M. tb PZAR1*	*M. tb PZAR2*
E-12	20	40	20	10	20	40	20
E-14	20	40	20	10	20	40	20
E-16	>40	>40	40	40	20	>40	>40
PZA	>1000	>1000	>1000	>1000	100	>1000	>1000
POA	100	100	>1000	>1000	100	100	100

M. tb H37Rv is the reference sensitive strain. All other species and strains are resistant to PZA

TABLE 2. Minimal inhibitory concentrations of the *n*-alkanols *in vitro* (data in µg/ml). 4-ol (butanol) was used to estimate the effect of a short-chain alcohol.

Compound	*M. bovis BCG*	*M. avium 44156*	*M. avium 44157*	*M. tuberculosis H37Rv*
4-ol	>80	>80	>80	10
12-ol	10	40	40	5
14-ol	5	20	20	5
16-ol	5	20	20	5
Kanamycin	0.5	10	10	1