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Development of a method to investigate the hydrolysis of xenobiotic esters by a *Mycobacterium smegmatis* homogenate

Emilia Valente [a], Marta Filipa Simões [a], Bernard Testa [b], Luis Constantino [a,*]

[a] iMed.UL, Faculty of Pharmacy, University of Lisbon, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal
[b] Department of Pharmacy, University Hospital Centre (CHUV), Rue du Bugnon, CH-1011 Lausanne, Switzerland

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ABSTRACT

One of the main problems in combating tuberculosis is caused by a poor penetration of drugs into the mycobacterial cells. A prodrug approach via activation inside mycobacterial cells is a possible strategy to overcome this hurdle and achieve efficient drug uptake. Esters are attractive candidates for such a strategy and we and others communicated previously the activity of esters of weak organic acids against mycobacteria. However very little is known about ester hydrolysis by mycobacteria and no biological model is available to study the activation of prodrugs by these microorganisms. To begin filling this gap, we have embarked in a project to develop an *in vitro* method to study prodrug activation by mycobacteria using *Mycobacterium smegmatis* homogenates. Model ester substrates were ethyl nicotinate and ethyl benzoate whose hydrolysis was monitored and characterized kinetically. Our studies showed that in *M. smegmatis* most esterase activity is associated with the soluble fraction (cytosol) and is preserved by storage at 5 °C or at room temperature for one hour, or by storage at −80 °C up to one year. In the range of homogenate concentrations studied (5–80% in buffer), k_{obs} varied linearly with homogenate concentration for both substrates. We also found that the homogenates showed Michaelis–Menten kinetics behavior with both prodrugs. Since ethyl benzoate is a good substrate for the mycobacterial esterases, this compound can be used to standardize the esterasic activity of homogenates, allowing results of incubations of prodrugs with homogenates from different batches to be readily compared.

1. Introduction

Tuberculosis is the second most deadly infectious disease the world over (Frieden et al., 2003). According to the World Health Organization (WHO) in 2008 one third of the world's population was infected with *Mycobacterium tuberculosis* and there were 9.4 million new cases of tuberculosis and 1.8 million deaths (WHO, 2009). As new weapons to fight this situation are urgently needed, a viable contribution can come from the development of new agents from already available drugs. The prodrug approach via mycobacterial activation is a possible alternative especially considering the problematic penetration of drugs into mycobacterial cells. One of the first-line drugs used in tuberculosis treatment, pyrazinamide, is in fact a prodrug. This compound has a good penetration into mycobacteria but must be activated by an amidase (pyrazinamidase) in order to be transformed in the active form, pyrazinoic acid (POA) (Zhang and Mitchison, 2003). This active form of the drug is not directly used in patients presumably due to its poor gastrointestinal absorption and significant serum protein binding (Konno et al., 1967). Unfortunately mutations in the *M. tuberculosis* gene *pncA* abolish pyrazinamidase activity and were shown to be responsible for pyrazinamide resistance (Cheng et al., 2000; Zhang and Mitchison, 2003). Since pyrazinamide is a prodrug of pyrazinoic acid, other prodrugs of this compound could have activity against *M. tuberculosis* if they penetrate the mycobacterial cell and are properly activated. Cynamon and co-workers proposed for the first time the use of esters of pyrazinoic acid and observed that some of these compounds exhibit *in vitro* activity against *M. tuberculosis* (Cynamon and Klemens, 1992; Cynamon et al., 1995). In addition, we observed in a previous work that lipophilic ester prodrugs of pyrazinoic acid displayed increased *in vitro* and *ex vivo* activity when compared with pyrazinamide and pyrazinoic acid (Simões et al., 2009). It was also shown that these lipophilic esters were easily activated to pyrazinoic acid by contact with a crude mycobacterial homogenate while the corresponding amide prodrugs did not present activity and were not activated by the same homogenate (Simões et al., 2009). This prompted us to investigate in more detail the hydrolysis of esters by mycobacteria, as the same approach could be used to develop other prodrugs targeted to tuberculosis and other mycobacterial diseases. Since very little is known about ester hydrolysis by mycobacteria and no model is available to study the activation of prodrugs by these microorganisms we decided to develop, standardize and characterize a

* Corresponding author.
E-mail address: constant@ff.ul.pt (L. Constantino).

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Mycobacterium smegmatis homogenate of the type we used to probe the activation of pyrazinoates, in order to allow kinetic results of incubations of drugs with homogenates from different batches to be readily compared. We think a *M. smegmatis homogenate* is suitable to be used in medicinal chemistry research, as this microorganism is non-pathogenic, grows rapidly and does not pose biohazard problems. In addition an ample literature exists on *M. smegmatis* and comparison between *M. smegmatis* and *M. tuberculosis* (Shiloh and DiGiuseppe Champion, 2010; Tyagi and Sharma, 2002; Reyrata and Kahnb, 2001; Gupta and Chatterji, 2005). We choose as model substrates ethyl benzoate (EB) and ethyl nicotinate (EN) as there are many studies on the chemical and enzymatic hydrolysis of these compounds (Nielsen and Bundgaard 1987; Durrer et al., 1992) and also because it was observed that weak organic acids and esters of these compounds demonstrate activity against mycobacteria (Gu et al., 2008; Zhang et al., 2003). In order to develop this model we studied: (a) the preparation and conservation of the homogenate, (b) the activity of the homogenate fractions, (c) the variation of *pseudo*-first order hydrolysis constants with the concentration of the homogenate and (d) the variation of hydrolysis initial rate with substrate concentration.

2. Materials and methods

All starting materials were commercially available research grade chemicals and were used without further purification. All solvents were dried and distilled prior to use. The HPLC system for the quantification of the prodrugs and the correspondent organic acid consisted in a Merck-Hitachi L-7100 pump, a Merck-Hitachi L-7400 UV detector, a Merck-Hitachi L-7500 integrator and a 5 μm Merck RP-8 24 cm column. The eluant consisted in 45% acetonitrile in 0.05 M KH_2PO_4 buffer with 0.1% (v/v) H_3PO_4. The flow rate was 1 ml min^{-1} and the wavelength was set at 260 nm for the determination of nicotinic acid/ethyl nicotinate and 230 nm for benzoic acid/ethyl benzoate.

2.1. Mycobacterial studies

A culture of *M. smegmatis* ATCC607 variant mc2 155 incubated at 37 °C with Middlebrook 7H9 OADC supplemented broth (with oleic acid, albumin, dextrose and catalase) was used for the production of the mycobacterial homogenate. A crude whole homogenate was prepared from a culture of exponentially growing bacteria with an $O.D._{600nm} = 0.8–1.0$. Mycobacteria were harvested by centrifugation at T = 4 °C for 10 min, washed and re-suspended in pH = 7.4 phosphate buffer saline PBS (25 ml for each 750 ml of the initial growing broth). The bacterial homogenate was prepared with a CV375 600 W 20 KHz Vibracell Ultrasonic processor (Sonics and Materials Inc.) and a CV17 ultra-sound probe (Sonics and Materials Inc.) using a sequence of 5 cycles of 2 min each. The homogenate was afterwards divided in 1 ml portions and kept at −80 °C till use. Protein concentration was accessed at 595 nm using the Bradford method.

Experiments regarding homogenate stability and fractionation were performed with homogenate batch *Ms*1 (0.38 mg protein ml^{-1}), remaining experiments were performed with batch *Ms*2 (1.4 mg protein ml^{-1}).

2.2. Activation of the prodrugs in M. smegmatis homogenates

The esterase activity of the mycobacterial homogenate was tested using EB and EN 5×10^{-4} M as substrates, (or at 10^{-5} to 5×10^{-3} M in the kinetics experiments). The prodrugs were added in acetonitrile solution and final acetonitrile concentration in the incubations medium was 2%. The homogenate was diluted with phosphate buffer, total concentration $C_t = 0.05$ M, ionic strength I = 0.15 M, pH = 7.4 and incubated at T = 37 °C with agitation. At several time points, aliquots of 50 μl were taken into vials containing 450 μl of a 1:1 solution of 1% zinc sulfate and acetonitrile, agitated in a vortex and centrifuged for 10 min at 15,000 rpm. The supernatant was then injected in HPLC for quantification of the prodrug and the corresponding organic acid. All quantifications were performed using calibration curves for the substrates and products. The results reported with no standard deviation were the mean of two experiments; the other results were obtained from triplicate experiments. Each single value of value of k_{obs} used to calculate the average and standard deviation was obtained using incubations with 6–10 time points extending trough at least two 3 half-lives. For each time point duplicate aliquots were analysed.

2.3. Whole homogenate separation in different fractions

A sample of *M. smegmatis* whole homogenate was separated into 4 portions. One portion was used as crude whole homogenate and the others were subjected to centrifugation at different speeds at 4 °C in order to obtain different fractions. After supernatant separation the same buffer volume was added to the remaining pellet and this preparation was homogenized with a vortex. Centrifugations were performed at 2500 *g* for 10 min, 12,500 *g* for 10 min or 100,000 *g* for one hour. Each fraction was incubated with EB in order to determine the remaining esterasic activity.

2.4. Homogenate activity at different pHs

The esterase activity of the mycobacterial homogenate was tested using EB 5×10^{-4} M as substrate. The prodrugs were added in acetonitrile solution and final acetonitrile concentration in the incubation medium was 2%. Mycobacterial homogenate was diluted to 0.14 mg ml^{-1} using phosphate buffer with the desired pH (total concentration $C_t = 0.05$ M, ionic strength I = 0.15 M). The initial pH of the medium ranged from 5.5 to 8.0 and was controlled routinely in the beginning and at the end of incubations.

3. Results and discussion

A first screen in the method of preparation of the mycobacterial homogenate was done by comparing homogenates prepared by the French press method (2 cycles) with homogenates prepared by sonication. The two methods applied to the same culture gave rise to crude homogenates of comparable esterasic activity when corrected for protein concentration. However, the French press method was abandoned at an early stage because it is time consuming, leads to more diluted homogenates and also uses equipment not as common as a sonication probe. All the results in this paper were obtained with homogenates prepared by sonication using 5 cycles of 2 min each. An increased number of cycles did not improve final homogenate protein concentration.

3.1. Homogenate separation in different fractions

Different fractions of *M. smegmatis* whole homogenate were prepared as described in Materials and methods and the *pseudo*-first order rate constants evaluated for each fraction with EB. The results are summarized in Table 1.

It was observed that hydrolysis of EB occurred in all fractions. Esterasic activity was always higher in the supernatant fractions but was also detectable in all resuspended pellet fractions. The activity present in the supernatant fractions is most likely due to cytosolic enzymes since it is not removed even by a centrifugation at 100,000 *g*. Since the centrifugation at 100,000 *g* removes approximately the same activity that is removed by a simple centrifugation at 2500 *g* or a centrifugation at 12,500 *g* most of the activity presented by the resuspended pellets was already present in membrane and cell debris fraction. The fact that significant esterasic activity was present in the