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Activation of benzoate model prodrugs by mycobacteria. Comparison with mammalian plasma and liver hydrolysis

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A B S T R A C T

Due to difficulties in drug penetration in *M. tuberculosis*, a prodrug approach based on mycobacterial activation appears as a promising strategy to increase the delivery of antitubercular drugs to the target microorganisms. Esters have been successful used by us and others to deliver drugs to mycobacteria, however because very little is known about the metabolic hydrolysis of esters by mycobacteria in connection with prodrug activation, we decided to study the process further. For that we selected a series of 13 benzoates with different chain lengths and ramifications in the alkoxy side as model prodrugs and examined their hydrolysis by a mycobacterial homogenate, comparing the results with those obtained parallelly in human plasma and in total rat liver homogenate. In all biological media, the benzoates with a linear alkyl group showed a parabolic dependence between log(*k*) and logP (or the number of carbons of the linear alkyl chain) that reached a maximal value for the *n*-butyl chain. Considering linear correlations for the total number of compounds between log(*k*) and chosen descriptors, for mycobacterial esterases, pKa of the leaving alcohol (pKa_{LG}) seem to be the most important descriptor. Plasma esterases seem to be quite sensitive to the Taft polarity parameter σ* and also to pKa_{LG} and less sensitive to steric effects. Liver esterases seem to be more sensitive to the Taft steric descriptor E_S^S. Lipophilicity correlates weakly with log (*k*) in all the 3 media, however, is more important when one looks for mycobacterial activation selectivity in relation to plasma metabolism or in relation to liver homogenate metabolism. The importance of lipophilicity increases further when biparametric expressions are considered. We showed that it is easy to activate a wide variety of benzoate esters using a mycobacterial homogenate. The data also suggest that with careful design is possible to obtain tuberculostatic prodrug esters sensitive to mycobacterial hydrolases while reasonably resistant to plasma and liver hydrolysis. One important observation is that mycobacterial hydrolysis is less affected by bulky substituents than liver homogenate or plasma hydrolysis. tert-Butyl is probably the substituent in the alkoxy side that seems more adequate to resist simultaneously plasma and liver metabolism, while allowing activation by mycobacterial esterases. Hexyl is also a good option for the medicinal chemist if a linear alkoxy chain is needed.

1. Introduction

In the third decade of the 21st century, tuberculosis remains a significant cause of illness and death worldwide. *Mycobacterium tuberculosis*, the organism responsible for this disease, is intrinsically resistant to many antibiotics, which is traditionally attributed in part to the unusual structure of its cell wall. This slow-growing organism possesses an external cell wall composed mainly of a covalently attached complex of peptidoglycan, arabinogalactan and highly lipophilic fatty acids (the mycolic acids) (Batt et al., 2020; Dulberger et al., 2020). In addition, *M. tuberculosis* has an outer layer composed of an array of glycolipids, lipoglycans and lipids associated with the cell wall. Due to its high lipophilicity and tight packing, this structure severely hinders the penetration of xenobiotics; as a result, many potential antimycobacterial agents fail to cross the barrier (Batt et al., 2020; Chiaradia et al., 2017; Gygli et al., 2017).

The prodrug approach via mycobacterial activation offers a possible solution to increase drug penetration. In a previous work, our group observed that lipophilic pyrazinoic ester prodrugs displayed increased *in vitro* and *ex vivo* activity when compared with pyrazinamide and pyrazinoic acid (Simões et al., 2009; Pires et al., 2015). Also, it was observed that esters of other organic acids, namely benzoates, were active against *Mycobacterium tuberculosis* (Gu et al., 2008), inciting us to study in more detail the activation of ester prodrugs by mycobacteria as very little information was available for the rational design of prodrugs to be activated by mycobacteria. Esters activated by mycobacterial esterases are attractive prodrug candidates to use in mycobacterial diseases as pyrazinoic acid and other antimycobacterial agents can be transformed easily into ester prodrugs. This approach has been used by us and others (Simões et al., 2009; Pires et al., 2015; Cynamon et al., 1995; Bergmann et al., 1996) and has at least two main advantages: (i) Esters are not ionized at physiological pH and can permeate more easily

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than ionized molecules, (ii) Since esters will be activated by esterases and mycobacteria have a large array of esterases (Gygli et al., 2017), resistance due to lack of inactivation is not likely to occur.

In previous work, we developed a method to investigate the hydrolysis of xenobiotic esters by a *Mycobacterium smegmatis* homogenate as a model to study *M. tuberculosis* activation of prodrug esters (Valente et al., 2011). This method was validated using two ester model prodrugs, ethyl benzoate and ethyl nicotinate. The present study reports the results on the hydrolysis of 13 benzoates using the same mycobacterial homogenate (My). In order to compare mammalian and mycobacterial hydrolysis, the stability of these esters was also investigated in two media containing mammalian esterases, namely human plasma (Pl) and total rat liver homogenate (Lv). The comparison of the hydrolysis in the three media aimed identifying the structural features leading to a relative resistance towards mammalian hydrolysis in conjunction with a fair susceptibility towards mycobacterial activation. Deriving structure-metabolism relationships from these data proved promising. With this work we hope to contribute for the development of new therapeutic approaches using suitable prodrugs activated by mycobacterial esterases.

2. Materials and methods

2.1. Ester prodrugs synthesis

Cyclopropylmethyl benzoate (BMecPr, **3**), 2-Methoxyethyl benzoate (BEtOMe, **4**), Isopropyl benzoate (BiPr, **6**), *sec*-Butyl benzoate (B*s*Bu, **8**), *tert*-Butyl benzoate (B*t*Bu, **9**) and Octyl benzoate (BOc, **11**) were synthesized according to the following general method. Characterization of the products can be found in the supplementary material.

General method. A solution of the appropriate acyl chloride (1.2 mmole *per* mmole of alcohol) in dichloromethane was added dropwise to a solution of corresponding alcohol and triethylamine (1 mmole *per* mmole of acid chloride) in dichloromethane at 0°C. When the reaction was complete (as assessed by TLC using hexane:ethyl acetate, 5:1 to 1:1, or ethyl acetate as eluent) the reaction mixture was filtered and the filtrate washed successively with 10 mL of distilled water and with 15 mL of saturated sodium bicarbonate solution. The dichloromethane solution was subsequently dried, and the solvent evaporated. The residue was purified by column chromatography (silica gel 60) using hexane: ethyl acetate, 5:1 to 1:1, or ethyl acetate as eluent.

2.2. HPLC system

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 eluent consisted in 45% acetonitrile in 0.05M KH_2PO_4 buffer with 0.1% (v/v) H_3PO_4. The flow rate was 1 mL min^{-1}. The wavelength was set at 230 nm.

2.3. Phosphate buffer

The chemical stability of some model esters - ethyl benzoate, propyl benzoate and phenyl benzoate - was evaluated in phosphate buffered solution, total concentration $C_t = 0.05$M, ionic strength I = 0.15M, pH = 7.4 and incubated at T = 37°C with agitation.

2.4. Mycobacterial homogenate preparation and use

A crude whole mycobacterial homogenate (My) was prepared according to (Valente et al., 2011). Briefly a culture of exponentially growing *M. smegmatis* ATCC607 variant mc2 155 with an $O.D._{600nm}$ of 0.8 – 1.0 was harvested by centrifugation at T = 4°C for 10 minutes, 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 using an ultra-sound probe with a sequence of 5 cycles of 2 minutes each. The homogenate was afterwards divided in 1 mL portions and kept at -80°C till use. Total protein concentration was 1.4 mg mL^{-1}.

2.5. Mammalian media

Preparation of human plasma (Pl). Human blood was recovered in sodium heparinate, the supernatant separated after centrifugation was divided in 1mL portions and refrigerated at T = -20°C till use. Total protein concentration was 19.5 mg mL^{-1}.

A total rat liver homogenate (Lv) was prepared from the livers of 3 rats (7 months old, weight about 400 grams) according to (Constantino et al., 1999). The livers (total weight 32.2 grams) were mixed with 100 mL of isotonic phosphate buffer – PBS (about 3 mL per gram of liver weight), homogenized in a "Potter – Elvehjem" equipment and finally centrifuged at 700g for 10 minutes. The supernatant was separated in 1 mL portions and kept at T = -80°C until use. Total protein concentration was 20.0 mg mL^{-1}. In the stability studies, the total rat liver homogenate was diluted to 1% with the phosphate buffered solution described above and incubated at T = 37°C under agitation.

2.6. Conditions of incubations and preparation of samples

The initial substrate concentration was 5×10^{-4} M in all stability assays. All incubations were carried out at pH 7.4 and 37°C under agitation using the phosphate buffer described above as diluting agent. The levels of dilution of the mycobacterial homogenate (20%), human plasma (80%) and total rat liver homogenate (1%) in the incubates were chosen following preliminary assays to ensure *pseudo*-first order kinetics in the hydrolysis of benzoates. Acetonitrile (2%) was used in all studies to ensure adequate solubilization of the substrates. The benzoates were added from 10^{-1}M acetonitrile stock solutions.

After incubation, aliquots of 50 μL were taken into vials containing 450 μL of a 1:1 solution of 1% zinc sulfate and acetonitrile, mixed in a vortex and centrifuged for 10 minutes at 15,000 rpm. The supernatant was then injected into the HPLC and analyzed for quantification of benzoic acid and remaining benzoate. All quantifications were performed using calibration curves for the substrates and products. The reaction rates were measured by the disappearance of the parent compound from the reactions media, which in all cases matched the concurrent appearance of benzoic acid. This correspondence proved that the disappearance of the ester was due exclusively to its hydrolysis.

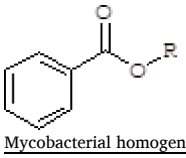
3. Results and discussion

3.1. Hydrolysis of benzoates in a phosphate buffer and in biological media

The esterasic activity of the mycobacterial homogenate (My) was studied using 13 benzoates as substrates. The same reactions was also studied in parallel in media containing mammalian esterases, namely human plasma (Pl) and a total rat liver homogenate (Lv). All three biological media used in this assay presented esterasic activity towards all substrates. The reactions obeyed *pseudo*-first order hydrolysis and liberate quantitatively the free acid from the model prodrug in all cases studied. The rate constants and corresponding half-lives are reported in Table 1. All the pseudo first order rate constants are represented in this paper as *(k)*. The ratio between (*k*) rate constants and the pseudo-first order rate constants for methylbenzoate $(k)_{Me}$ in the same bio-media. The rate is represented as (m) and its purpose is to obtain a ratio that could allow for the effect of different esterase concentrations in each of the three bio-media used.

To evaluate whether chemical hydrolysis significantly contribute to the overall rate of hydrolysis in the various biological media studied, the *pseudo*-first order rate constants of hydrolysis of ethyl, propyl and phenyl benzoates (**2, 5** and **12**, respectively) were determined under conditions

Table 1
Pseudo-first order rate constants ((*k*)/h^{-1}) in the three bio-media studied, and the ratio between the same pseudo first order rate constants and the *pseudo*-first order rate constants for methylbenzoate in the same bio-media (*m*). the purpose of (m) is to obtain a ratio that could allow for the effect of different esterase concentrations in each biomedia: 20% mycobacterial homogenate (My), 80% human plasma (Pl), and 1% total rat liver homogenate (Lv). (ND - not determined).



Compound and Number	R	Mycobacterial homogenate (My)		Plasma (Pl)		Liver Homogenate (Lv)	
		(*k*)	(*m*)	(*k*)	(*m*)	(*k*)	(*m*)
BMe (**1**)	-CH_3	1.64 ±0.11	1,00	0.81 ±0.13	1,00	18.5 ±0.61	1,00
BEt (**2**)	-CH_2CH_3	3.53 ±0.21	2,15	0.48 ±0.04	0,59	24.6 ±1.94	1,33
BmecPr (**3**)	-$CH_2CH(CH_2CH_2)$	17.9 ±4.71	10,91	3.77 ±0.15	4,65	57.5 ±5.24	3,11
BEtOMe (**4**)	-$CH_2CH_2OCH_3$	7.16 ±0.53	4,37	14.4 ±1.00	17,78	29.3 ±1.45	1,58
BPr (**5**)	-$(CH_2)_2CH_3$	14.1 ±3.5	8,60	2.15 ±0.17	2,65	47.8 ±3.66	2,58
B*i*Pr (**6**)	-$CH(CH_3)_2$	2.22 ±0.14	1,35	0.25 ±0.098	0,31	30.1 ±0.84	1,63
BBu (**7**)	-$(CH_2)_3CH_3$	14.9 ±0.33	9,09	3.68 ±0.16	4,54	63.1 ±3.27	3,41
B*s*Bu (**8**)	-$CH(CH_3)CH_2CH_3$	4.16±0.48	2,54	0.09 ±0.002	0,11	23.6 ±2.06	1,28
B*t*Bu (**9**)	-$C(CH_3)_3$	1.58 ±0.23	0,96	0.03 ±0.002	0,04	4.15 ±1.21	0,22
BHe (**10**)	-$(CH_2)_5CH_3$	7.66 ±0.34	4,67	0.53 ±0.08	0,65	19.8 ±3.33	1,07
BOc (**11**)	-$(CH_2)_7CH_3$	1.45 ±0.18	0,88	0.75 ±0.04	0,93	ND	ND
BPh (**12**)	-C_6H_5	103 ±7.3	62,80	81.5 ±17.0	100,6	70.6 ±15.0	3,82
BBz (**13**)	-$CH_2C_6H_5$	24.9 ±3.4	15,18	7.58 ±0.40	9,36	22.7 ±2.60	1,23

of pH, buffer and temperature identical to those in the biological media. The ratios between the *pseudo*-first order rate constants in the phosphate buffer and in the biological media are shown in Table 2. As expected, hydrolysis was always much faster in the biological media than in buffer alone, demonstrating that esterases were the main driver for the reactions of hydrolysis observed in the biological media. Also, the (*k*) values for chemical hydrolysis remained below the standard deviations observed with the biological media. For practical purposes, it follows that no correction for chemical hydrolysis was necessary for the *pseudo*-first order rate constants obtained in the biological media.

3.2. Effect of the n-alkyl chain on the hydrolysis of benzoates in the three biological media

The *n*-alkyl benzoate esters (including the methyl ester) contain some valuable information on the structure-metabolism relations of these compounds. It was indeed observed that, in the three biological media, the rate constants of hydrolysis increased from the methyl to *n*-butyl ester (**7**), and decreased beyond this point (Fig. 1). A similar effect was described by others for non-aromatic carboxylate esters incubated in human plasma (Buchwald and Bodor, 2002).

An interesting point to mention in Fig. 1 is the fact that the curves show comparable shapes with a maximum at 4 carbons but the mycobacterial enzymes are much more sensitive towards the hydrolysis of butyl esters than plasma or liver esterases. The same effect can be extended to a lesser degree to chains of three and five atoms. These differences appear promising when examining the non-*n*-alkyl benzoates (shown below).

The drop in the rate from Me to Et in the plasma hydrolysis of benzoates is consistent with values reported by Nielsen and Bundgaard (Nielsen and Bundgaard, 1987). A drop in the (*k*) values for longer *n*-alkyl moieties seen here was reported for other types of esters. Durrer and colleges found trends comparable to ours in the hydrolysis of nicotinates both in plasma and hog liver carboxyesterase (Durrer et al., 1991).

Table 2
Pseudo-first order rate constants ((*k*)/h^{-1}) and half-lives ($t_{1/2}$/h) of hydrolysis of ethyl, propyl and phenyl benzoates at T = 37°C in a phosphate buffer of pH = 7.4, total concentration C_t = 0.05M and ionic strength I = 0.15 M containing 2% acetonitrile. Initial substrate concentration $C_i = 5 \times 10^{-4}$ M. Also shown are the ratios between the *pseudo*-first order rate constants in the phosphate buffer and in the three biological media.

Compound	10^3 x (*k*)	$t_{1/2}$	Ratio A [a)]	Ratio B [b)]	Ratio C [c)]
BEt (**2**)	10.5±1.7	67±10	336	46	2340
BPr (**5**)	97.4±12	7.1±0.9	145	22	491
BPh (**12**)	252±30	2.8±0.3	409	323	280

a) Ratio A = $k_{(My)}/k_{(Buffer)}$
b) Ratio B = $k_{(Pl)}/k_{(Buffer)}$
c) Ratio C = $k_{(Lv)}/k_{(Buffer)}$

3.3. Effect of branched and functionalized esterifying moieties on the hydrolysis of benzoates in the three biological media

As expected, esters with branched alkyl chains have in general much lower *pseudo*-first order rate constants than their linear isomers. The hydrolysis of the *sec*-butyl ester (**8**) was ca. 3-4 times slower in mycobacterial homogenate and liver homogenate (and ca. 40 times slower in plasma), when compared with that of the *n*-butyl ester (**7**). The hydrolysis of the *tert*-butyl ester (**9**) was almost 9-fold slower in mycobacterial homogenate, 15 times in liver homogenate and ca 120 times slower in plasma. In contrast, the rate difference between the cyclopropylmethyl and the *n*-butyl esters (**3** and **7**, respectively) was not significant in all the three media. Furthermore, the phenyl and benzyl benzoates (**12** and **13**, respectively) showed that an aromatic moiety may markedly increase the sensitivity of benzoates towards mycobacterial and plasma esterases (but not markedly towards rat liver esterases). As expected, this effect was high for the phenyl group (the leaving group having a pKa = 9.9), but the effect of the benzyl group in mycobacterial and plasma hydrolysis is interesting as the pKa of benzyl alcohol is quite different from the pKa of phenol (table 3).

The introduction of the methoxyl group as a substituent in the alkoxy chain raised the *pseudo*-first order hydrolysis constant as can be seen by comparing the values obtained with methoxyethyl benzoate **(4)** and ethyl benzoate **(2)**. This fact was especially relevant within plasma hydrolysis where methoxyethyl benzoate was degraded *ca.* 30 times faster than ethyl benzoate and *ca.* 4 times faster than butyl benzoate. Other authors had already observed this effect in the hydrolysis of nicotinates, either in plasma or in liver preparations (Durrer et al., 1992; Testa and Krämer, 2007; Testa and Mayer, 2006).

In addition to characterization of hydrolysis of esters in distinct media containing esterases (mycobacterial homogenate versus mammalian plasma and mammalian liver homogenate), the other main objective of this work was searching for substituents that would be as

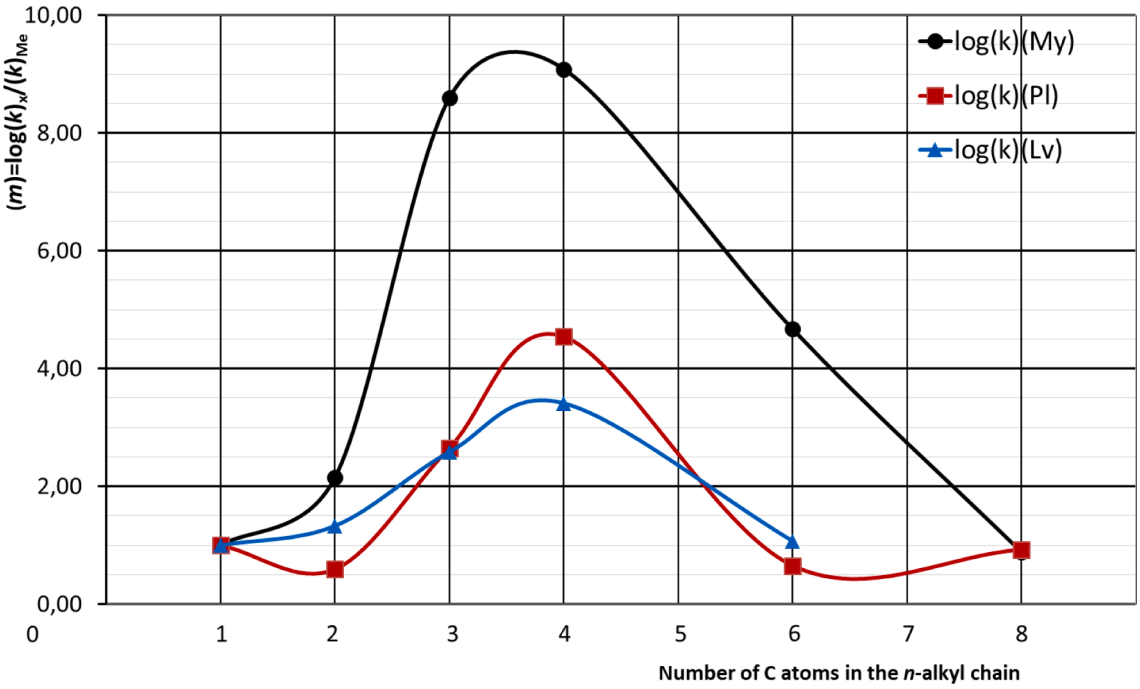


Fig. 1. *Pseudo*-first order rate constants of hydrolysis of the *n*-alkyl benzoates, in mycobacterial homogenate (My) human plasma (Pl) and rat liver homogenate (Lv) normalized in relation to that rate of hydrolysis of methylbenzoate (Me) in the same bio-media (m)=$\log(k)_x/(k)_{Me}$

Table 3
Descriptors values used in the hydrolysis correlations of benzoates esters.

Compound	R	$ES_{(corr)}$ [a]	σ* [b]	pKa [c]	log P [c]
BMe (**1**)	$-CH_3$	0	0	15.73	1.85
BEt (**2**)	$-CH_2CH_3$	-0.07	-0.1	15.76	2.54
BmecPr (**3**)	$-CH_2CH(CH_2CH_2)$	-	0.01	15.76	2.70
BEtOMe (**4**)	$-CH_2CH_2OCH_3$	-0.77	0.24	14.97	1.71
BPr (**5**)	$-(CH_2)_2CH_3$	-0.36	-0.12	15.76	2.92
BiPr (**6**)	$-CH(CH_3)_2$	-1.08	-0.19	17.60	2.72
BBu (**7**)	$-(CH_2)_3CH_3$	-0.39	-0.13	15.76	3.40
B2Bu (**8**)	$-CH(CH_3)CH_2CH_3$	-1.74	-0.21	17.60	3.20
B*t*Bu (**9**)	$-C(CH_3)_3$	-2.46	-0.3	19.20	3.39
BHe (**10**)	$-(CH_2)_5CH_3$	-0.30	-0.25	15.76	4.51
BOc (**11**)	$-(CH_2)_7CH_3$	-0.33	0.0	15.76	5.23
BPh (**12**)	$-C_6H_5$	-0,23	0.60	9.90	3.38
BBz (**13**)	$-CH_2C_6H_5$	-0.69	0.22	15.04	3.79

a) Taft steric parameter (Hansch, C.; Leo, 1979) corrected by considering the value of the methyl group as being equal to zero.
b) Taft polarity parameter (Hansch, C.; Leo, 1979).
c) pKa of the leaving alcohol or phenol and logP values were taken from E. Valente (Valente, 2009) and were calculated respectively using SPARC and ALogPS2.1 software.

resistant as possible to mammalian esterases, while allowing easy prodrug activation by mycobacteria. To unravel the semi-quantitative effects of substituents on hydrolysis in the mycobacterial homogenate when compared to human plasma and a rat liver homogenate, each (*k*) was normalized by dividing it by the (k) obtained for methyl benzoate for each biomedia (Table 1) and obtained the dimensionless (*m*). The higher the ratio (*m*) the more the substitution increases the hydrolysis over methylbenzoate in the bio-media under consideration. Using the (m) values of Table 1 we than calculated the ratio between (m) for the same compound in mycobacterial homogenate over plasma and on mycobacterial homogenate over liver homogenate and called it $(M)_{My/Pl}$ and $(M)_{My/Li}$. The results are represented in Fig. 2.

In this figure the bars quantify the relative effects of the esterifying moiety of benzoates on increasing their hydrolysis in the mycobacterial homogenate compared to the mammalian media. The higher the bar reach, the more the substituent facilitates hydrolysis in the mycobacterial homogenate compared to plasma (A) or liver homogenate (B). In other words, these (*M*) values can be taken as an index of hydrolytic selectivity in comparing the mycobacterial homogenate with mammalian preparations. The higher the index the more the compound is stable in relation to the mammalian esterases and the easier it is activated by the mycobacterial esterases. To begin with the bars in Fig. 2A and classifying the six benzoates with the greatest tendency towards mycobacterial versus plasma selectivity, one gets B*t*Bu (**9**) ≈ B*s*Bu (**8**)> BHe (**10**) > B*i*Pr (**6**) ≈ BEt (**2**) > BPr (**5**). This sequence can be understood as meaning that steric hindrance at the alpha-carbon of the esterifying moiety is a major factor in resisting plasma hydrolysis while simultaneously favoring mycobacterial hydrolysis.

A partly different sequence emerges when examining the ranking of the bars in Fig. 2B using similar criteria, namely BPh (**12**) > BBz (**13**) > B*t*Bu (**9**) ≈ BHe (**10**) > BmecPr (**3**) > BPr (**5**). Here aromatic moieties clearly play a major role in favoring mycobacterial esterases hydrolysis over liver esterases, but steric hindrance may also be important.

3.4. QSAR studies of the hydrolysis of benzoates in the biological media

Based on the results described above, an attempt was made to gain better insights into prevailing structure-metabolism relations using a few parameters and descriptors. Some classical parameters were used to describe relevant physicochemical features of the esterifying moieties. As steric parameters, we considered the Taft steric parameter ES and Charton's steric parameter based on van der Waals radii (Charton, 1975, 1976, Taft, 1952a,b). Because a cross-correlation between the two descriptors was seen before (Hansch and Leo, 1979), only the Taft steric parameter was used here. The ES values were corrected to become E_S^c by considering the value of the methyl group as being equal to zero. The Taft σ* parameter of the esterifying moieties was used to express the polarity of this part of the substrate molecules (Durrer et al., 1991; Taft, 1952a,b). A related parameter was the pKa of the leaving alcohol or phenol, a stereo-electronic parameter usually correlated with the relative ease of ester hydrolysis (Bundgaard, 1992). The parameters mentioned so far concern only the alkoxy portion of the ester molecule.

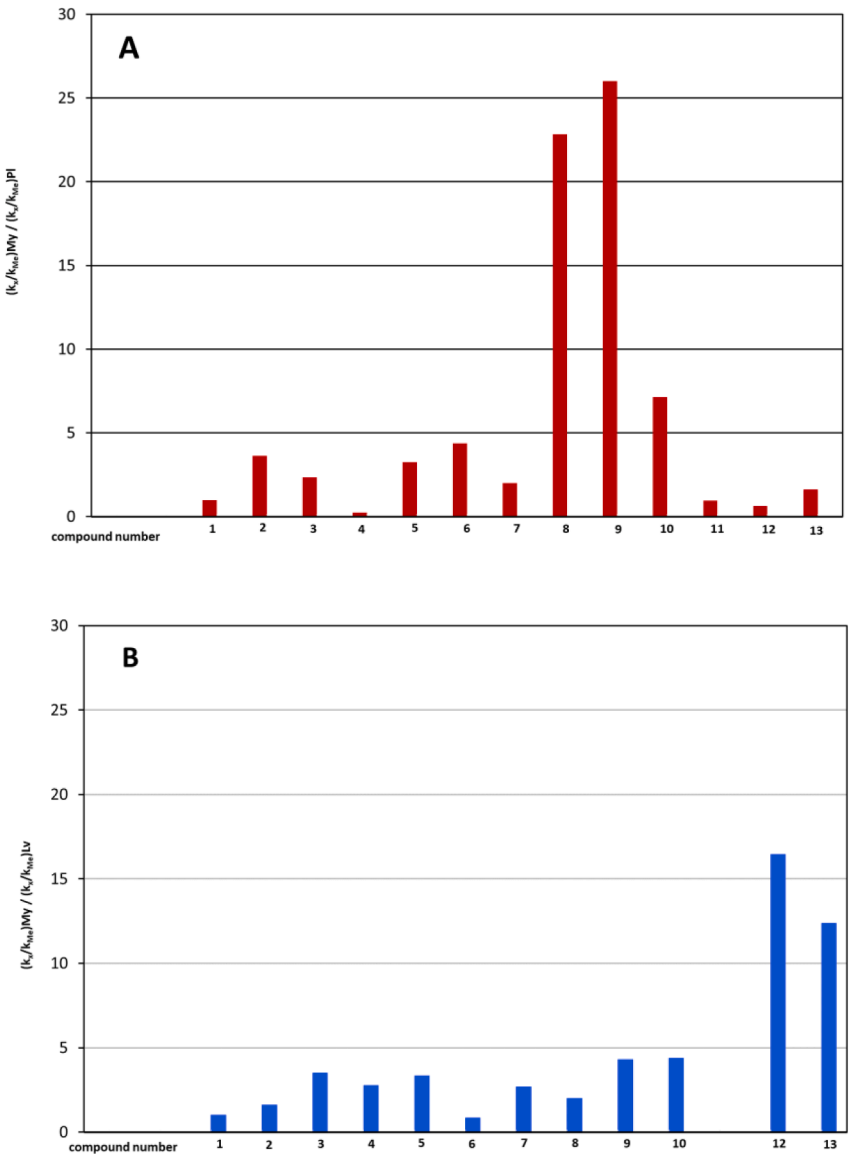


Fig. 2. Plots showing the differential influence of esterifying groups on the hydrolysis of benzoates in: **(A)** Mycobacterial homogenate (My) relative to human plasma (Pl) or, **(B)** Mycobacterial homogenate relative to a rat liver homogenate (Lv). The graphs represent the ratio (*M*) between the normalized (k) values obtained in mycobacterial homogenate (table 1) and the normalized (*k*) values (*m*) obtained in the other bio-media under comparison (Pl or Lv). The higher the bar the more the substituent facilitates activation by the mycobacterial homogenate while increases the odds of survival in the mammalian media.

A very useful parameter describing the complete molecule of the substrates is their lipophilicity as expressed by log P, the log of their octanol/water partition coefficient (Kirchmair et al., 2015). The values of the different descriptors were taken as much as possible from the same source.

The first step in these QSAR analyzes was to investigate autocorrelation among the descriptors chosen to describe the physicochemical properties of the esters under study. This can be achieved by obtaining a correlation matrix. The correlation matrix is useful in determining which independent variables are likely to help explain the variation of dependent variables. The dependent variables considered here are the *pseudo*-first order rate constants of the prodrugs in the biomedia, evaluated as $\log(k)_{My}$, $\log(k)_{Pl}$ and $\log(k)_{Lv}$ and also the selectivity of the prodrugs mycobacterial activation towards the metabolism, both in human plasma and total rat liver homogenate, S_1=log $((k)_{My}/(k)_{Pl})$ and S_2=$\log((k)_{My}/(k)_{Lv})$, respectively (calculated in Table 4). To simplify the discussion, we use the following definitions of correlations: 0-0.09 no correlation, 0.1-0.39 weak, 0.4-0.69, moderate, 0.69-1 Strong. Obtained r values are always reported for reader's own inference about the significance of the correlations.

We look for correlations between dependent variables and the independent ones, whereas no correlation should exist among the selected independent variables. In the present case, the obtained correlation matrix (Table 5) led us to observe that in our compounds the pKa of the alcohol leaving group, had a strong negative correlation (-0.78) with the Taft electronic descriptor σ* for the alkoxyl portion of the ester prodrug.

Also, it is possible to observe that both Taft descriptors, σ* (electronic) and E_S^c (steric hindrance) are related to each other with a moderate correlation (0.44) (see Table 5). Therefore, regressions using these combinations of descriptors face auto-collinearity and give redundant information. As a last note, σ* has also a weak negative correlation with the lipophilicity descriptor log P (-0.18). Considering the dependent variables, a strong positive correlation (+0.71) between $\log(k)_{My}$ and $\log(k)_{Lv}$, and a strong positive correlation (+0.81) between $\log(k)_{My}$ and $\log(k)_{Pl}$ are observed (Table 5). $\log(k)_{Lv}$ also shows a strong positive correlation (0.73) with $\log(k)_{Pl}$ (Table 5).

It is also worth noting that the correlation between selectivity of the prodrugs mycobacterial activation towards metabolism in total rat liver *S*2ss=log$((k)_{My}/(k)$Lv) and the selectivity of the prodrugs mycobacterial activation towards metabolism in plasma S_1=$\log((k)_{My}/(k)_{Pl})$ is weak and negative (-0.19). Furthermore, it is also possible to observe also that S_1, has a strong negative correlation with $\log(k)_{Pl}$ (about -0.82), a moderate negative correlation with $\log(k)_{Lv}$ (-0.54) and a weak negative correlation with log $(k)_{My}$ (about -0.34) (Table 5). S_2, shows a strong

Table 4
Selectivity values measuring the differential influence of esterifying groups on the hydrolysis of different benzoates in a mycobacterial homogenate (My) relative to human plasma (Pl) or in (My) relative to a rat liver homogenate (Lv). S_1 is calculated as $\log((k)_{My}/(k)_{Pl})$ and S_2 as $\log((k)_{My}/(k)_{Lv})$. Due to differences in the dilutions of the biomaterials used, both S_1 and S_2 represent a rank of the compounds in relations to the easiness of hydrolysis should not be viewed as absolute values.

Compound and Number	R	S_1 $\log((k)_{My}/(k)_{Pl})$	S_2 $\log((k)_{My}/(k)_{Pl})$
BMe (**1**)	$-CH_3$	0,306	-1,052
BEt (**2**)	$-CH_2CH_3$	0,867	-0,843
BmecPr (**3**)	$-CH_2CH(CH_2CH_2)$	0,677	-0,507
BEtOMe (**4**)	$-CH_2CH_2OCH_3$	-0,303	-0,612
BPr (**5**)	$-(CH_2)_2CH_3$	0,817	-0,530
B*i*Pr (**6**)	$-CH(CH_3)_2$	0,948	-1,132
BBu (**7**)	$-(CH_2)_3CH_3$	0,607	-0,627
B*s*Bu (**8**)	$-CH(CH_3)CH_2CH_3$	1,665	-0,754
B*t*Bu (**9**)	$-C(CH_3)_3$	1,722	-0,419
BHe (**10**)	$-(CH_2)_5CH_3$	1,160	-0,412
BOc (**11**)	$-(CH_2)_7CH_3$	0,286	ND
BPh (**12**)	$-C_6H_5$	0,102	0,164
BBz (**13**)	$-CH_2C_6H_5$	0,517	0,040

positive correlation with $\log(k)_{My}$ (about +0.80), a moderate positive correlation with $\log(k)_{PL}$ (0.56) and a weak positive correlation with $\log(k)_{Lv}$ (0.14).

Regarding correlations between dependent and independent variables, $\log(k)_{My}$ has a high negative correlation with pKa_{LG} (-0.76), moderate positive correlations with σ* (0.55) and with E_S^c (0.42) and does not correlate with log P (0.019) (Table 5). $\text{Log}(k)_{Lv}$ shows a high positive correlation with E_S^c (+0.70), a moderate negative correlation with pKa_{LG} (-0.65), a moderate positive correlation with σ* (+0.50) and no correlation with logP (-0.092) (Table 5).

Regarding stability in human plasma, $\log(k)_{Pl}$, it is possible to observe a strong negative correlation with pKa_{LG} (-0.89), a high positive correlation with σ* (+0.78), a moderate positive correlation with E_S^c (+0.67) and a weak negative correlation with log P (-0.13). It seems that within the chosen descriptors, regarding hydrolysis by mycobacterial and plasma esterases, pKa_{LG} is the most important descriptor, whereas for rat liver homogenate hydrolysis the Taft steric descriptor E_S^c seems to be the most important descriptor. An important observation is the fact that mycobacterial hydrolysis is less affected by steric factors than plasma or liver hydrolysis.

Other dependent variables included in Table 5 are indexes of hydrolytic selectivity of the prodrugs i.e, the mycobacterial activation selectivity towards plasma S_1 and the mycobacterial activation selectivity towards liver homogenate S_2, evaluated as $\log((k)_{My}/(k)_{Pl})$, and S_2=$\log((k)_{My}/(k)_{Lv})$, respectively.

Regarding S_1, it is possible to observe a moderate to strong positive correlation to pKa_{LG} (+0.69), a weak correlation with log P (+0.23) and moderate to strong negative correlations with σ* (-0.72) and to the Taft descriptor E_S^c (-0.68). When S_2 is considered, it is possible to observe moderate positive correlations with log P (+0.56) and σ* (+0.40) and moderate negative correlations with pKa_{LG} (-0.59). Only a weak correlation is observed with the steric descriptor E_S^c (+0.12).

LogP becomes more important regarding the hydrolytic selectivity of mycobacterial homogenate, in relation to the plasma or rat liver homogenate, than when the log(*k*) of the hydrolysis was considered alone for each media, as both the hydrolytic selectivity of mycobacterial homogenate in relation to plasma (S_1), and in relation to rat liver homogenate (S_2) have positive weak and moderate correlations with logP, whereas when the log(*k*) of the hydrolysis was considered alone for each media, no relevant correlation with lipophilicity was observed.

Since pKa correlates with σ*(-0.78) and ES (-0.75), and ES correlates with σ* (0.44) different regression expressions have been tried for the dependent variables leaving aside those combinations. Table 6 summarizes the results of simple and multiple correlations and the regression expressions obtained based on Table 5 correlation results.

The best correlations were observed for the prodrugs stability rate constant in human plasma, log $(k)_{Pl}$, considering either the pKa_{LG} or the Taft electronic descriptor σ* - Expressions 4 and 3; a better correlation was observed in this kinetics when logP is also associated to pKa_{LG} – Expression 9.

Considering any of the selectivity expressions S_1 or S_2 (Table 6), we were able to conclude that log P becomes more important when biparametric correlations are considered, ameliorating the correspondent correlation coefficient r^2. For this reason, log P should be a descriptor to be considered in the benzoate prodrugs selection to mycobacteria. From expression 10 and 11, we can see that mycobacterial selectivity towards plasma (evaluated as S_1) and mycobacterial activation selectivity towards liver homogenate (evaluated as S_2), correlates simultaneously with logP and pKa_{LG}, and also that S_2 correlates simultaneously with logP and σ* (expression 12).

4. Conclusion

Esters activated by mycobacterial esterases are attractive prodrug candidates to use in mycobacterial diseases if the compounds can survive mammalian esterases and be activated by the mycobacterial enzymes. We have studied the hydrolysis of a series of benzoates using a mycobacterial homogenate, a rat liver homogenate and human plasma. The first media was used to assess the easiness of activation of the prodrugs while the other two media were used to evaluate the ability of the prodrugs to survive in contact with mammalian esterases. These biohydrolysis are much faster than the chemical hydrolysis observed in the same buffer used on the biological media. The studies performed led us to conclude that, the mycobacterial homogenate presented hydrolytic activity against all the benzoates in our study and that the hydrolytic activity can be useful for the activation of other esters, namely, prodrug esters. As expected, the esterasic activity of the mycobacterial homogenate towards the benzoates depends on the alkoxy portion of the

Table 5
Correlation matrix for inter-correlation of molecular descriptors and their correlation with the prodrugs *pseudo*-first order rate constants (for the activation in mycobacterial homogenate and for the stability in human plasma and total rat liver homogenate and also with the selectivity of the drugs activation towards their stability in the other biological media).

	$\log(k)_{My}$	$\log(k)_{Lv}$	$S_{2\ (My/Lv)}$	$\log(k)_{Pl}$	$S_{1\ (My/Pl)}$	logP	E_S^c	σ*	pKa_{LG}
$\log(k)_{My}$	1								
$\log(k)_{Lv}$	0.71	1							
$S_{2\ (My/Lv)}$	0.80	0.14	1						
$\log(k)_{Pl}$	0.81	0.73	0.56	1					
$S_{1(My/Pl)}$	-0.34	-0.54	-0.19	-0.82	1				
logP	0.019	-0.092	0.56	-0.13	0.23	1			
E_S^c	0.42	0.70	0.12	0.67	-0.68	-0.00025	1		
σ*	0.55	0.50	0.40	0.78	-0.72	-0.18	0.44	1	
pKa_{LG}	-0.76	-0.65	-0.59	-0.89	0.69	-0.020	-0.75	-0.78	1

Table 6
Statistically significant QSAR models for modeling the activation, stability, and selectivity of benzoate esters prodrugs with regression expressions and parameters.

Dp Var	Exp	logP	E_S^c	σ*	pKa_{LG}	Intercept	n	s	r^2	F
$\log(k)_{My}$	1	-	-	1.21±0.56	-	0.82±0.13	13	0.48	0.308	4.9
$\log(k)_{Pl}$	2	-	0.83±0.29	-	-	0.63±0.29	12	0.74	0.454	8.3
$\log(k)_{Pl}$	3	-	-	2.9±0.70	-	0.08±0.17	13	0.60	0.610	17
$\log(k)_{Pl}$	4	-	-	-	-0.38±0.06	6.2±0.96	13	0.44	0.787	41
$\log(k)_{Lv}$	5	-	0.29±0.10	-	-	1.6±0.10	11	0.25	0.495	8.8
S_1	6	-	-0.52±0.18	-	-	0.37±0.18	12	0.46	0.460	8.5
S_1	7	-	-	-1.7±0.48	-	0.74±0.11	13	0.41	0.519	12
S_2	8	0.28±0.13	-	-	-	-1.4±0.40	12	0.33	0.318	4.7
$\log(k)_{Pl}$	9	-0.14±0.13	-	-	-0.39±0.06	6.6±1.0	13	0.44	0.809	21
S_1	10	0.14±0.13	-	-	0.19±0.06	-2.7±1.0	13	0.43	0.534	5.7
S_2	11	0.27±0.10	-	-	-0.10±0.03	0.21±0.63	12	0.25	0.648	8.3
S_2	12	0.34±0.10	-	0.82±0.32	-	-1.6±0.32	12	0.27	0.610	7.0

n – number of compounds; s – regression standard deviation

molecules.

Regarding the benzoates with linear alkoxy portion, a parabolic dependence between log (k) and log P (or number of carbons of the linear alkoxy chain) was observed in the three media (mycobacterial homogenate, plasma and rat liver homogenate). It was also shown that the pseudo-first order rate constant for the hydrolysis has a maximum value when the alkoxy carbon number is four. The form of the mycobacterial curve is closer to the liver homogenate curve than to the plasma curve, however the mycobacterial enzymes are much more sensitive towards the hydrolysis of butyl esters than plasma or liver esterases. The same effect can be extended to a smaller degree to chains of three and five atoms and can probably be used in the design of a prodrug.

The maximum of activity in the hydrolysis of linear chains with 4 carbons is in accordance with previous results described for the hydrolysis of non-aromatic carboxyl esters in plasma (Buchwald and Bodor, 2002). What was not known was that the curve is much more pronounced in mycobacterial homogenate than in plasma or liver.

The differences between hydrolysis in the mycobacterial homogenate and hydrolysis in the two other bio-media increased when all benzoates studied are considered. The pKa_{LG} of the leaving alcohol became the most important descriptor in relation to mycobacterial esterasic activity. Plasma esterases appear to be quite sensitive to the pKa_{LG} but also to the Taft polarity parameter σ*. Rat liver esterases seem to be more sensitive to the Taft steric descriptor E_S^c. Interestingly, the fact that mycobacterial hydrolysis is less sensitive to steric effects can probably be used in the advantage of prodrug design in tuberculosis. Indeed bulky groups like t-butyl or sec butyl can be used to help survive plasma and liver hydrolysis and can still be easily activated by mycobacterial esterases. These findings can probably be explained by the type of esterases contained in mycobacteria, needed to the metabolism of the complex lipids of this microorganism. *M. tuberculosis* expresses more than 250 enzymes related to ester/lipid metabolism. In contrast, only about 50 enzymes are involved in the ester/lipid metabolism in E. coli (Cole et al., 1998; Camus et al., 2002). The extraordinary diversity of lipids synthesized by Mtb is directly related with the unusual level of complexity regarding the fate of fatty acids, as they are substrates of all the different lipid biosynthetic pathways (Gago et al., 2018).

Although, lipophilicity correlates weakly with *k* in all the three media, is important when seeking a prodrug that it is resistant to mammalian esterases while is still able to be activated by mycobacterial enzymes. Lipophilicity becomes more important to the prodrugs mycobacterial activation selectivity S_1 and S_2, mainly when biparametric expressions are considered (Table 6). S_1 ($\log((k)_{My}/(k)_{Pl})$), correlates with logP and pKa_{LG} (equation 10). S_2 ($\log((k)_{My}/(k)_{Lv})$) correlates also with logP and pKa_{LG} (equation 11), but also with logP and σ* (equation 12).

Our results show is possible to easily activate a wide variety of benzoates esters by the mycobacterial homogenate. Our data also suggest that it is possible to identify structural features leading to a relative resistance towards mammalian hydrolysis in conjunction with a fair susceptibility towards mycobacterial activation. Some groups are particularly suited to enhance stability in plasma, while maintaining a good rate of activation by mycobacterial esterases (t-butyl > sec-butyl > hexyl). Other groups maintain a good activation by mycobacterial esterases, while being more resistant to liver esterases (phenyl > benzyl >. t-butyl > hexyl). t-Butyl is probably the group that seems more adequate to resist simultaneously plasma and liver metabolism, while allowing activation by mycobacterial esterases. Hexyl is also a good option for the medicinal chemist if a linear alkoxy chain is needed.

CRediT authorship contribution statement

Emília Valente: Writing – original draft, Investigation, Formal analysis. **Bernard Testa:** Conceptualization, Methodology, Writing – review & editing. **Luís Constantino:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Supplementary materials

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