Enhancement of the antituberculosis activity of weak acids by inhibitors of energy metabolism but not by anaerobiosis suggests that weak acids act differently from the front-line tuberculosis drug pyrazinamide

Peihua Gu,¹ Luis Constantino² and Ying Zhang¹

Correspondence

Ying Zhang

yzhang@jhsph.edu

¹Department of Molecular Microbiology & Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA

²Faculty of Pharmacy, University of Lisbon, Av Prof Gama Pinto 1649-019, Lisbon, Portugal

Mycobacterium tuberculosis is uniquely susceptible to weak acids compared with other mycobacteria or bacteria. The antituberculosis activity of the front-line drug pyrazinamide (PZA), a weak acid (pyrazinoic acid) precursor, can be enhanced by inhibitors of energy metabolism and anaerobiosis. Here, we investigated the effect of inhibitors of energy metabolism and anaerobiosis on weak acid activity against *M. tuberculosis* in general. The susceptibility of *M. tuberculosis* to benzoic acid (BA) esters and amides was determined alone and in the presence of inhibitors of energy metabolism such as *N,N'*-dicyclohexylcarbodiimide (DCCD) and azide and also under anaerobic conditions in the form of MIC and drug exposure followed by colony count. Some BA esters such as propyl hydroxybenzoic acid and 4-dodecyloxybenzoic acid had significant activity whereas amides of BA had no activity. As for PZA, inhibitors of energy metabolism DCCD and azide enhanced the antituberculosis activity of weak acids under normal atmospheric oxygen tension. However, unlike PZA, weak acids did not show antituberculosis activity and the inhibitors of energy metabolism did not enhance the weak acid activity under anaerobic conditions. The enhancement of weak acid activity by inhibitors of energy metabolism for *M. tuberculosis* was not seen in other bacterial species such as *Helicobacter pylori*. These results suggest that while the antituberculosis activity of weak acids can be enhanced by inhibitors of energy metabolism as for PZA, weak acids act differently from PZA in that they were inactive against *M. tuberculosis* under anaerobic conditions. The significance of these findings is discussed in the context of the unique physiology of *M. tuberculosis* and the development of new tuberculosis drugs.

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INTRODUCTION

Tuberculosis (TB) is a leading infectious cause of morbidity and mortality worldwide, especially in developing countries (Raviglione, 2003). Drug-resistant TB has become an increasing public health concern in recent years and poses a significant threat to the control of the disease (Raviglione, 2003). There is currently a great deal of interest in developing new drugs that are not only active against drug-resistant TB but also shorten the therapy (Zhang, 2005). Improved understanding of the frontline TB drug pyrazinamide (PZA), a paradoxical drug that has poor *in vitro* activity but high *in vivo* activity and shortens

TB therapy from 9–12 months to 6 months, may lead to design of new drugs that can shorten TB therapy (Zhang & Mitchison, 2003).

During our study of the mode of action of PZA, we have found that *Mycobacterium tuberculosis* seems to be uniquely susceptible to the weak acid pyrazinoic acid (POA), the active form of PZA, whereas other mycobacteria such as *Mycobacterium smegmatis* or bacteria such as *Escherichia coli* are naturally resistant to POA (Schaller *et al.*, 2002; Sun & Zhang, 1999a; Zhang *et al.*, 1999, 2003b). Subsequently, we have demonstrated that *M. tuberculosis* is more susceptible to weak acids in general compared with other mycobacterial or bacterial species (Zhang *et al.*, 2003b), presumably due to deficient efflux (Sun & Zhang, 1999a; Zhang *et al.*, 1999) and poor ability to maintain membrane energetics (Zhang *et al.*, 2003b). In addition, we have shown that the susceptibility of *M. tuberculosis* to PZA, the weak acid POA precursor, can be

Abbreviations: ASP, aspirin; BA, benzoic acid; DBA, 4-dodecyloxybenzoic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHB, propyl 4-hydroxybenzoate; POA, pyrazinoic acid; PZA, pyrazinamide; SA, salicylic acid; TB, tuberculosis.

enhanced by anaerobiosis (Wade & Zhang, 2004) and by energy inhibitors such as *N,N'*-dicyclohexylcarbodiimide (DCCD), azide and rotenone, which inhibit proton-ATPase, cytochrome *c* oxidase and NADH dehydrogenase, respectively (Wade & Zhang, 2006; Zhang *et al.*, 2003a). However, it is not clear whether anaerobiosis and inhibitors of energy metabolism can also enhance the activity of other weak acids or their precursors against *M. tuberculosis*. In this study, we looked at the effects of inhibitors of energy metabolism on the activity of weak acids and their precursors against *M. tuberculosis* under normal atmospheric and anaerobic conditions.

METHODS

Reagents and chemicals. DCCD and sodium azide were obtained from Sigma-Aldrich. Sodium azide was dissolved in deionized water at stock concentrations of 10 mM and filter-sterilized. DCCD was dissolved in 95 % ethanol at a stock concentration of 200 mM.

Weak acid derivatives (Table 1) 1a, 1b, 1c, 2a, 2b, 2c, 2e and 2k were obtained from Sigma-Aldrich. Amides 1d, 1e, 1f and 1g were synthesized by adding benzoyl chloride to a solution of the parent amine in 10 % sodium hydroxide under reflux followed by extraction with dichloromethane. Compounds 1d and 1e were recrystallized from ethanol, and compounds 1f and 1g were purified by column chromatography using ethyl acetate:hexane 5:2 as eluant. Esters 2d, 2f, 2g, 2h, 2i and 2j were

Table 1. BA derivatives and their activity on *M. tuberculosis* at pH 5.5

An exponential-phase *M. tuberculosis* H37Ra culture washed and resuspended in 7H9 medium at pH 5.5 was incubated with weak acids at 100 and 500 $\mu\text{g ml}^{-1}$ for 3 days before the MTT viability measurement. The percentage of growth inhibition was calculated by $[1 - (\text{MTT absorbance values of weak acid treated samples}) / \text{MTT absorbance value of control}] \times 100$. A negative percentage value indicates slight growth stimulation rather than growth inhibition with some weak acids at a low concentration of 100 $\mu\text{g ml}^{-1}$. The experiment was repeated at least three times and representative data are shown here.

Compound	R1	R2	Name	Growth inhibition (%)	
				100 µg ml ⁻¹	500 µg ml ⁻¹
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			Benzoic acid amide		
1a	H	H	Benzamide	5.03	0.63
1b	H	CH ₃	<i>N</i> -Methylbenzamide	13.21	91.19
1c	H	CH ₂ CH ₃	<i>N</i> -Ethylbenzamide	−1.26	3.77
1d	H	CH ₂ (CH ₂) ₂ CH ₃	<i>N</i> -Butylbenzamide	12.58	10.06
1e	H	<div> </div>	<i>N</i> -Cyclopropylbenzamide	6.29	6.29
1f	CH ₃	CH ₃	<i>N,N</i> -Dimethylbenzamide	0.00	5.03
1g	CH ₂ CH ₃	CH ₂ CH ₃	<i>N,N</i> -Diethylbenzamide	−6.29	5.03
1h	CH ₃	<div> </div>	<i>N</i> -Methyl- <i>N</i> -cyclopropylbenzamide	13.84	5.03
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			Benzoic acid ester		
2a	CH ₃		Methylbenzoate	15.09	45.91
2b	CH ₂ CH ₃		Ethylbenzoate	10.69	61.64
2c	CH ₂ CH ₂ CH ₃		Propylbenzoate	38.99	88.68
2d	CH(CH ₃) ₂		Isopropylbenzoate	−24.53	56.60
2e	CH ₂ (CH ₂) ₂ CH ₃		Butylbenzoate	28.30	77.99
2f	CH(CH ₃) ₃		<i>tert</i> -Butylbenzoate	−8.18	4.40
2g	CH ₂ (CH ₂) ₄ CH ₃		Hexylbenzoate	38.36	76.10
2h	CH ₂ (CH ₂) ₆ CH ₃		Octylbenzoate	20.75	60.38
2i	CH ₂ (CH ₂) ₈ CH ₃		Decylbenzoate	88.05	95.60
2j	CH ₂ (CH ₂) ₁₀ CH ₃		Dodecylbenzoate	22.01	5.03
2k	C ₆ H ₅		Phenylbenzoate	78.62	76.73

synthesized by the reaction of benzoyl chloride with the corresponding alcohol. Briefly, benzoyl chloride was added dropwise to a solution of the desired alcohol in diethyl ether in the presence of excess triethylamine. After 2 h water was added and the organic layer was washed with 5 % HCl, and then with saturated aqueous sodium bicarbonate solution. The organic layer was dried and the solvent was removed under vacuum. The compounds were purified by silica-gel column chromatography using ethyl acetate:hexane 5:2 as eluant. Amide was synthesized as previously described (Constantino *et al.*, 1992). The weak acid precursors were dissolved in DMSO at appropriate concentrations.

Bacterial growth and weak acid susceptibility. *M. tuberculosis* strain H37Ra was grown in 7H9 liquid medium (Difco) supplemented with 0.05 % Tween 80 and 10 % BSA-dextrose-catalase (ADC) enrichment (Difco) at 37 °C for approximately 2 weeks with occasional agitation. *Helicobacter pylori* was grown in Brucella broth containing 5 % horse serum under microaerophilic conditions for 4–7 days. The bacterial susceptibility to weak acids was determined by the viability dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) method (Sun & Zhang, 1999b), MIC, or by c.f.u. counts following weak acid exposure for 3–5 days. The principle of the MTT assay is that the yellow tetrazolium salt MTT is cleaved to purple-blue formazan by the succinate-tetrazolium reductase enzyme present in live cells. The purple-blue formazan was then dissolved in DMSO and the A_{570} was measured in a microplate reader. The MTT assay was used as an initial screen to rapidly identify weak acids that have antimycobacterial activity. We have previously validated the correlation between MTT absorbance readings and c.f.u. result for fresh cultures (Sun & Zhang, 1999b).

Effect of DCCD and azide on weak acid activity. Three- to four-week-old *M. tuberculosis* H37Ra cultures were prepared as described above, harvested by centrifugation, washed in one volume of PBS (pH 7.0) and resuspended in 7H9 medium at acid pH 5.5. Bacilli were treated with DCCD (0.1–1 mM), sodium azide (0.5–1 mM) or appropriate concentrations of weak acids, or DCCD or sodium azide in combination with weak acids for 3 days, when the cells were washed with PBS buffer and the c.f.u. for each sample was determined by serial dilution and plating on 7H11 agar supplemented with ADC enrichment.

Effect of anaerobic conditions on weak acid activity. Three-week-old *M. tuberculosis* H37Ra bacilli were exposed to weak acids benzoic acid (BA), salicylic acid (SA) and propyl 4-hydroxybenzoic acid (PHB), and inhibitors of energy metabolism azide and DCCD, alone and in combination, under anaerobic conditions and normoxia (20 % oxygen) as a control using procedures as previously described (Wade *et al.*, 2004). Briefly, a BBL GasPak 100 anaerobic system (Becton Dickinson Microbiology Systems) was used for anaerobic conditions with a BBL GasPak Plus anaerobic system envelope with palladium catalyst. A methylene blue indicator strip was used to verify the anaerobic conditions. After incubation for 7 days in 7H9 acid pH 5.5, the cells were harvested by centrifugation, washed with PBS (pH 7.0), serially diluted and plated in triplicate on 7H11 agar plates supplemented with ADC. Plates were incubated at 37 °C for 4 weeks, when the c.f.u. was determined.

Statistical treatment. Pairwise comparison of the c.f.u. data for statistical significance was performed using Student's *t*-test.

RESULTS AND DISCUSSION

Activity of weak acid precursors on *M. tuberculosis*

We have previously shown that weak acids such as BA are quite active against *M. tuberculosis* especially at acid pH

(Zhang *et al.*, 2003b). We were interested in using weak acid precursors to check whether they had better lipophilicity and antimycobacterial activity than the weak acids. To determine the antimycobacterial activity of the weak acid precursors and the ability of *M. tuberculosis* to break them down, a series of 19 BA precursors (esters and amides) was synthesized (see Methods for details). *M. tuberculosis* H37Ra bacilli were seeded in acidic pH 5.5 7H9 broth and incubated with these BA precursors for 3 days. The MTT viability assay was then done. Prodrugs of BA may have the advantage of a more favourable bioavailability or increased penetration into mycobacteria. The studied compounds differ mainly in lipophilicity and susceptibility to hydrolysis (chemical functionality and steric hindrance). Table 1 shows the susceptibility of *M. tuberculosis* to 19 esters and amides of BA. Two BA derivatives, phenylbenzoate (compound 2k) and decylbenzoate (compound 2i), had significant activity against *M. tuberculosis* whereas other BA derivatives had much less activity (Table 1). We further determined the MIC values for the two active weak acid esters phenylbenzoate and decylbenzoate, which were found to be 80 and 160 $\mu\text{g ml}^{-1}$, respectively, at acid pH 5.5 for *M. tuberculosis*. Esters are hydrolysed by esterases to their corresponding carboxylic acids (Testa, 2004), and the different activities observed can be due to different rates of hydrolysis and different permeation of the compounds into mycobacteria. However, it is clear from Table 1 that *M. tuberculosis* is not susceptible to the amides under study. This can be attributed to the lack of hydrolysis of the amide compounds due to absence of appropriate amidases to break down the amides to release active weak acids.

Fig. 1 shows the susceptibility of *M. tuberculosis* to BA esters with linear alkyl groups as a function of log P of the

Fig. 1. Susceptibility of *M. tuberculosis* to the BA esters with linear alkyl groups as a function of log P of the compounds. Log P calculation was performed by the ChemProp program included in ChemOffice 5.5. DO 100 and DO 500 indicate weak acid dose at 100 and 500 $\mu\text{g ml}^{-1}$, respectively.

compounds. Log P refers to the log of the octanol/water partition coefficient of a compound (here a weak acid), and is a useful parameter in quantitative structure–activity relationships in drug design. In general, the susceptibility of *M. tuberculosis* to weak acids increased with the log P value of the compounds, reaching a maximum with 2i (log P of 5.7). This finding is consistent with the previous observation that the lipophilicity of the compounds seems to fit well with the log P values of fluorescently labelled lipophilic probes (Christensen *et al.*, 1999). A significant drop in activity is noted from 2i to 2j; however, this compound is approximately 10 times less soluble in water than the already poorly soluble 2i, and the lack of solubility of the compound 2j (dodecylbenzoate) in the media could explain the drop in activity. It should be noted that the lipophilicity of the compound per se does not explain all the results, but this parameter is probably important for facilitating the entry of the prodrugs into the mycobacteria. However, once inside, the prodrug must give rise to BA. Chemical hydrolysis of the esters is dependent on steric hindrance on the alkyl portion of the esters and on the pK_a of the alcohol (Robinson & Matheson, 1969). In the ester series, *tert*-butyl and isopropyl groups were included to assess the importance of steric factors on the susceptibility of *M. tuberculosis* H37Ra to weak acid esters. *tert*-Butylbenzoate has a log P very close to that of butylbenzoate (2.73 vs 2.89), and isopropylbenzoate has a log P close to that of propylbenzoate (2.57 vs 2.44); however, their activity is significantly lower. This can be explained by steric factors as the bulkier *tert*-butyl and isopropyl groups, and poor hydrolysis of the esters. Further animal studies are needed to investigate the hydrolysis, toxicity and *in vivo* activity of the highly active weak acid precursors such as PHB and compound 2i.

Enhancement of antituberculosis activity of weak acids by inhibitors of energy metabolism

Since we have shown previously that inhibitors of energy metabolism such as azide (inhibitor of cytochrome c oxidase) and DCCD (inhibitor of F_1F_0 -ATPase) could enhance PZA activity (Zhang *et al.*, 2003a; Wade & Zhang, 2004, 2006), we tested whether the enhancement effect of inhibitors of energy metabolism is specific to PZA (a weak acid precursor) by examining the effect of inhibitors of energy metabolism on the antituberculosis activity of other weak acids, including the most active BA derivative 2i. Interestingly, DCCD and azide also enhanced the anti-mycobacterial activity of a range of weak acids, including BA, SA and aspirin (ASP), with DCCD having a much higher enhancement effect than azide (Table 2). BA (100 $\mu\text{g ml}^{-1}$) alone showed a 10-fold reduction in c.f.u. compared with control and addition of azide had a slight enhancement effect. However, DCCD plus BA caused about a 100-fold decrease in c.f.u. compared with BA or DCCD alone ($P < 0.05$). SA at 100 $\mu\text{g ml}^{-1}$ showed a threefold drop in c.f.u. compared with the control, and addition of azide led to a further reduction of 10-fold c.f.u.

Table 2. Effect of inhibitors of energy metabolism on weak acid activity for *M. tuberculosis*

The c.f.u. values, in $\log_{10}$ scale, represent the difference between the c.f.u. counts for the drug-free control (6.2 $\log_{10}$ c.f.u. ml^{-1}) and drug-treated groups.

Drug treatment	Mean $\log_{10}(\text{c.f.u. ml}^{-1}) \pm \text{SD}$
0.1 mM DCCD	1.47 $\pm$ 1.39
1 mM sodium azide	0.90 $\pm$ 0
100 $\mu\text{g BA ml}^{-1}$	1.20 $\pm$ 0.45
100 $\mu\text{g BA ml}^{-1}$ + DCCD	3.40 $\pm$ 2.94
100 $\mu\text{g BA ml}^{-1}$ + sodium azide	1.90 $\pm$ 1.21
100 $\mu\text{g SA ml}^{-1}$	0.53 $\pm$ 0.09
100 $\mu\text{g SA ml}^{-1}$ + DCCD	2.90 $\pm$ 3.45
100 $\mu\text{g SA ml}^{-1}$ + sodium azide	1.98 $\pm$ 1.69
100 $\mu\text{g ASP ml}^{-1}$	0.60 $\pm$ 0.21
100 $\mu\text{g ASP ml}^{-1}$ + DCCD	2.40 $\pm$ 2.05
100 $\mu\text{g ASP ml}^{-1}$ + sodium azide	2.08 $\pm$ 1.69

($P < 0.05$). DCCD plus SA caused an over 20-fold reduction in c.f.u. compared with DCCD alone ($P < 0.05$). ASP at 100 $\mu\text{g ml}^{-1}$ had similar activity against *M. tuberculosis* to SA and addition of azide and DCCD caused enhancement of its activity ($P < 0.05$) (Table 2).

Other BA esters such as PHB (MIC=25 $\mu\text{g ml}^{-1}$ at pH 5.5) and 4-dodecylbenzoic acid (DBA) (MIC=11 $\mu\text{g ml}^{-1}$ at pH 5.5) (Zhang *et al.*, 2003b) were quite active against *M. tuberculosis* (Table 3). At 100 $\mu\text{g ml}^{-1}$ and 200 $\mu\text{g ml}^{-1}$, PHB alone produced a 5-fold and 100-fold reduction in c.f.u., respectively. However, addition of azide enhanced the activity of PHB by 36-fold at 100 $\mu\text{g ml}^{-1}$ and resulted in no c.f.u. when PHB (200 $\mu\text{g ml}^{-1}$) was in combination with azide ($P < 0.05$) (Table 3). By far the most dramatic enhancement effect was seen when DCCD was added to

Table 3. Effect of DCCD and azide on the activity of the weak acids PHB and DBA for *M. tuberculosis*

The c.f.u. values, in $\log_{10}$ scale, represent the difference between the c.f.u. counts for the drug-free control (6.48 $\log_{10}$ c.f.u. ml^{-1}) and drug-treated groups.

Drug treatment	Mean $\log_{10}(\text{c.f.u. ml}^{-1}) \pm \text{SD}$
0.5 mM DCCD	2.11 $\pm$ 1.24
1 mM sodium azide	0.48 $\pm$ 0
100 $\mu\text{g PHB ml}^{-1}$	0.70 $\pm$ 0.24
100 $\mu\text{g PHB ml}^{-1}$ + DCCD	6.48 $\pm$ 0*
100 $\mu\text{g PHB ml}^{-1}$ + sodium azide	2.26 $\pm$ 1.24
200 $\mu\text{g PHB ml}^{-1}$	2.18 $\pm$ 0.58
200 $\mu\text{g PHB ml}^{-1}$ + DCCD	6.48 $\pm$ 0*
200 $\mu\text{g PHB ml}^{-1}$ + sodium azide	6.48 $\pm$ 0*
100 $\mu\text{g DBA ml}^{-1}$	2.84 $\pm$ 1.64
100 $\mu\text{g DBA ml}^{-1}$ + DCCD	4.48 $\pm$ 2.76
100 $\mu\text{g DBA ml}^{-1}$ + sodium azide	3.66 $\pm$ 2.94

*The c.f.u. value is zero.

100 $\mu\text{g PHB ml}^{-1}$, which led to no c.f.u. (Table 3). DBA was more active than other weak acids and at 100 $\mu\text{g ml}^{-1}$ caused a 693-fold reduction in c.f.u. compared with control, and addition of DCCD and azide led to a further reduction of 43-fold and 65-fold in c.f.u., respectively ($P < 0.05$). The above data strongly suggest that the weak acid activity against *M. tuberculosis* can be enhanced by inhibitors of energy metabolism such as DCCD and to a lesser extent by azide, with the exception of DBA, where azide was slightly more effective than DCCD in enhancing its activity (Table 3). These findings indicate that, in *M. tuberculosis*, inhibitors of energy metabolism enhanced not only the activity of PZA (Zhang *et al.*, 2003a) but also the antituberculosis activity of weak acids in general.

Inhibitors of energy metabolism do not generally enhance the activity of weak acids against other bacteria

To determine whether the enhancement of weak acid activity by inhibitors of energy metabolism is unique to *M. tuberculosis*, we tested the effect of inhibitors of energy metabolism on the weak acid activity against *H. pylori*. *H. pylori* was exposed to weak acids POA, SA, ASP and PHB at acid pH 5.0 for 4–7 days in the presence or absence of DCCD, azide and rotenone. The results showed that the weak acids alone had relatively weak activity against *H. pylori* at high concentrations (4 mM) (Table 4). However, inhibitors of energy metabolism had no statistically significant effect on enhancing the activity of weak acids, compared with weak acid alone or inhibitors of energy metabolism alone (Table 4). This is in contrast to the 2–3 orders of magnitude difference in c.f.u. caused by inhibitors of energy metabolism on weak acid activity for *M. tuberculosis* (Tables 2 and 3). These findings suggest that inhibitors of energy metabolism preferentially increase the activity of weak acids against *M. tuberculosis* and are consistent with our previous observation that *M. tuberculosis* has poor ability to maintain its proton motive force (membrane potential and ΔpH) (Zhang *et al.*, 2003b). It is conceivable that new drugs or treatment strategies may be designed for improved treatment of TB based on the unique susceptibility of *M. tuberculosis* to weak acids and the enhancement of weak acid activity by inhibitors of energy metabolism.

Effect of anaerobiosis on the antimycobacterial activity of weak acids

We previously showed that anaerobic conditions could enhance the activity of PZA (Wade & Zhang, 2004). Since PZA is a precursor of the weak acid POA, we tested whether anaerobic conditions could also enhance the activity of other weak acids and whether the enhancement of weak acid activity mediated by inhibitors of energy metabolism under normal atmospheric conditions (20 % oxygen) as shown above is also true under anaerobic conditions. Three-week-old *M. tuberculosis* bacilli were exposed to weak acids BA, SA and PHB in the presence and absence of azide and DCCD under anaerobic conditions and normoxia (20 % oxygen) at pH 5.5 for 7 days, when the c.f.u. was determined. Rifampicin was included as a control. As shown in Table 5, inhibitors of energy metabolism significantly increased the antituberculous activity of weak acids at normoxia (Table 5). Azide slightly enhanced the activity of SA as shown by a 10-fold reduction in c.f.u. compared with SA or azide alone ($P < 0.05$) (Table 5). As shown above, DCCD had the greatest effect on enhancement of the weak acid BA, SA and PHB activity at normoxia ($P < 0.05$). However, in contrast to PZA, whose activity is enhanced by anaerobiosis (Wade & Zhang, 2004), weak acids did not have significant activity against *M. tuberculosis* and inhibitors of energy metabolism did not significantly enhance weak acid activity against *M. tuberculosis* under anaerobic conditions, producing overall less than a 10-fold difference in c.f.u. compared with the control (Table 5). This finding is consistent with the observation that inhibitors of energy metabolism, while enhancing the activity of PZA under aerobic conditions, failed to do so under anaerobic conditions (Wade & Zhang, 2004). In addition, *M. tuberculosis* was found to be generally less susceptible to inhibitors of energy metabolism under anaerobic conditions compared with normoxia (Table 5). These findings suggest that the antimycobacterial activity of the weak acids and inhibitors of energy metabolism themselves at least partly rely on the presence of oxygen.

The finding that inhibitors of energy metabolism enhance the antituberculosis activity of weak acids suggests a synergy between them in depleting the energy of tubercle bacilli. Weak acids serve as proton carriers and bring protons inside the tubercle bacilli, cause anion accumulation at acid pH

Table 4. Effect of inhibitors of energy metabolism on weak acid activity for *H. pylori*

The c.f.u. values, in $\log_{10}$ scale [mean $\log_{10}(\text{c.f.u. ml}^{-1}) \pm \text{SD}$], represent the difference between the c.f.u. counts for the drug-free control ($6.30 \log_{10} \text{c.f.u. ml}^{-1}$) and drug-treated groups.

<i>H. pylori</i> acid pH 5.0	POA	SA	ASP
4 mM weak acid	-0.07 ± 0.24	0.48 ± 1.24	0.30 ± 0
1 mM DCCD	-0.18 ± 0.24	-0.18 ± 0.24	-0.18 ± 0.24
4 mM weak acid + 1 mM DCCD	0.30 ± 0	1.03 ± 1.38	0.91 ± 1.29
1 mM sodium azide	0.48 ± 0.06	0.48 ± 0.06	0.48 ± 0.06
4 mM weak acid + 1 mM sodium azide	-1.22 ± 0.40	0.84 ± 1.44	0.80 ± 1.82

Table 5. Effect of oxygen on weak acid activity for *M. tuberculosis* with or without inhibitors of energy metabolism

The c.f.u. values, in log₁₀ scale [mean log₁₀(c.f.u. ml⁻¹) ± SD], represent the difference between the c.f.u. counts for drug-free controls (6.5 log₁₀c.f.u. ml⁻¹ for normoxia and 6.0 log₁₀c.f.u. ml⁻¹ for anaerobiosis) and drug-treated groups.

Drug treatment	Normoxia	Anaerobiosis
4 µg RIF ml ⁻¹	3.21 ± 3.51	3.00 ± 0
0.1 mM DCCD	2.48 ± 0	0.12 ± 0.18
0.5 mM sodium azide	0.60 ± 0.94	0.40 ± 0.30
100 µg BA ml ⁻¹	1.00 ± 0	0.27 ± 0.24
BA + sodium azide	0.92 ± 0.94	0.63 ± 0.24
BA + DCCD	4.96 ± 4.24	0.52 ± 0
100 µg SA ml ⁻¹	3.36 ± 3.24	0.40 ± 0
SA + sodium azide	4.11 ± 3.68	1.40 ± 0.76
SA + DCCD	4.96 ± 4.24	0.88 ± 0.19
100 µg PHB ml ⁻¹	1.36 ± 1.24	0.30 ± 0
PHB + sodium azide	1.51 ± 1.94	0.57 ± 0.24
PHB + DCCD	6.5*	0.44 ± 0.24

*The c.f.u. value is zero.

and disrupt the membrane potential (Zhang *et al.*, 2003b). The lower membrane potential caused by weak acids can be further compromised by DCCD and azide, which inhibit the production of membrane potential. This can account for the synergy between weak acids and inhibitors of energy metabolism. This explanation is also consistent with our recent finding that weak acids also enhance PZA activity (Wade & Zhang, 2006). That the inhibitors of energy metabolism enhance the activity of weak acids only in *M. tuberculosis* but not in other bacterial species is likely related to a deficiency in efflux mechanism (Zhang *et al.*, 1999) and inefficient maintenance of membrane energetics in *M. tuberculosis* as previously demonstrated (Zhang *et al.*, 2003b). However, it is worth noting that under anaerobic conditions weak acids or inhibitors of energy metabolism did not show significant activity against *M. tuberculosis* and that inhibitors of energy metabolism had no apparent enhancement effect on weak acids (Table 5). In this respect, weak acids are quite different from PZA, a POA precursor, which is more active against *M. tuberculosis* under anaerobic conditions (Wade & Zhang, 2004). The difference in PZA activity versus weak acid activity under anaerobic conditions implies that despite the common enhancement of anti-mycobacterial activity by inhibitors of energy metabolism for both PZA and weak acids, PZA works quite distinctly from a general weak acid effect. Further studies are needed to address the role of efflux and energy maintenance in the unique susceptibility of *M. tuberculosis* to weak acids and how PZA acts differently from weak acids in general.

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