In vitro synergistic interactions of oleanolic acid in combination with isoniazid, rifampicin or ethambutol against *Mycobacterium tuberculosis*

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Reports have shown that oleanolic acid (OA), a triterpenoid, exists widely in food, medicinal herbs and other plants, and that it has antimycobacterial activity against the *Mycobacterium tuberculosis* strain H37Rv (ATCC 27294). In this study it was found that OA had antimycobacterial properties against eight clinical isolates of *M. tuberculosis* and that the MICs of OA against drug-sensitive and drug-resistant isolates were 50–100 and 100–200 μg ml^{-1}, respectively. The combination of OA with isoniazid (INH), rifampicin (RMP) or ethambutol (EMB) showed favourable synergistic antimycobacterial effects against six drug-resistant strains, with fractional inhibitory concentration indices of 0.121–0.347, 0.113–0.168 and 0.093–0.266, respectively. The combination treatments of OA/INH, OA/RMP and OA/EMB displayed either a synergistic interaction or did not show any interaction against two drug-sensitive strains. No antagonism resulting from the OA/INH, OA/RMP or OA/EMB combination was observed for any of the strains tested. OA exhibited a relatively low cytotoxicity in Vero cells. These results indicate that OA may serve as a promising lead compound for future antimycobacterial drug development.

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INTRODUCTION

Tuberculosis (TB) remains a major public health problem worldwide. It is estimated that approximately 8 million new TB cases cause an estimated 2 million deaths annually (Bloom & Murray, 1992). The 13th Annual Tuberculosis Report from the World Health Organization showed that there were an estimated 9.27 million new cases of TB worldwide in 2007 (WHO, 2009). The resurgence of TB caused by *Mycobacterium tuberculosis* is associated with the emergence of the human immunodeficiency virus and the rapid spread of multidrug-resistant (MDR) TB strains [defined as resistance to isoniazid (INH) and rifampicin (RMP); WHO (2000)]. Infections related to MDR TB are more difficult to treat and have limited the efficacy of TB control programmes (Kremer & Besra, 2002). Drug combinations are the key to achieving high cure rates in the treatment of TB (Dye, 2009). The failure of treatment with first-line drug regimens requires the use of second-line drugs; however, some circulating strains are resistant to these second-line drugs as well, and these extensively drug-resistant strains are very disturbing (Koenig, 2008). The development of new antibiotics or novel combination therapeutic strategies is therefore necessary.

Plants and other natural materials may prove to be valuable sources of useful new antimycobacterials (Newton *et al.*, 2000). Oleanolic acid (OA) (3β-hydroxy-olea-12-en-28-oic-acid) is a triterpenoid compound that exists widely in the human diet, medicinal herbs and various other plants either in free form or bound to glycosides (Somova *et al.*, 2003). OA has been shown to have hepatoprotective (Liu,

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Abbreviations: EMB, ethambutol; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; INH, isoniazid; MDR, multidrug resistant; OA, oleanolic acid; OADC, oleic acid, albumin, dextrose and catalase; RMP, rifampicin; TB, tuberculosis.

1995), anti-inflammatory (Ovesna *et al.*, 2004), anti-allergic (Banno *et al.*, 2004), anti-ulcer (Ovesna *et al.*, 2004) and antiparasitic properties, as well as antiviral properties against human immunodeficiency virus (Ovesna *et al.*, 2004), and antimicrobial properties and potential antitubercular properties against *M. tuberculosis* (Gu *et al.*, 2004). The antitubercular properties of OA against clinical isolates of *M. tuberculosis* are still unknown. The aim of this study was to evaluate the *in vitro* antimycobacterial activity of OA used alone, and its synergistic interactions in combination with the first-line drugs INH, RMP and ethambutol (EMB) against clinical isolates of *M. tuberculosis*.

METHODS

Reagents and antibiotics. OA was purchased from the National Institute of the Control of Pharmaceutical and Biological Products, Beijing, PR China. It was dissolved in DMSO (Sigma) at a concentration of 5 g l^{-1} under sterile conditions and stored at –70 °C until use. The antibiotics INH, RMP and EMB were purchased from Sigma. Stock solutions of INH and EMB were prepared in deionized water, and RMP was prepared in DMSO. When drugs were used, the final concentration of DMSO in the culture medium was 0.5 % (v/v) (De Logu *et al.*, 2002).

Micro-organisms. *M. tuberculosis* strain H37Rv (ATCC 27294) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products. This *M. tuberculosis* strain is sensitive to three first-line anti-TB drugs: INH, RMP and EMB. Eight clinical isolates of *M. tuberculosis* (CRY1–CRY8) were obtained from patients at the Tuberculosis Hospital in Changchun, PR China. Organisms were cultured in Middlebrook 7H10 medium (Difco Laboratories) supplemented with oleic acid, albumin, dextrose and catalase (OADC) (Becton Dickinson) for 10 days at 37 °C.

Determination of antimycobacterial susceptibility *in vitro*. The antimycobacterial activities of OA and INH, RMP and EMB against the above *M. tuberculosis* strains were tested by an agar dilution method in 7H11 agar (Difco) as described by Gu *et al.* (2004). The strains were subcultured in 7H9 broth (Difco) with OADC and 0.04 % Tween 80, and allowed to grow at 37 °C for 2 weeks. The bacteria were harvested by centrifugation (2000 ***g***, 10 min), suspended in saline (0.15 M NaCl) with 0.04 % Tween 80, and sonicated in a bath-type sonicator to disrupt the clumps. The inoculum was adjusted to 3×10^6 c.f.u. ml^{-1} by comparison with a McFarland no. 1 turbidity standard, and 100 µl bacterial suspension containing approximately 3×10^5 c.f.u. was spotted onto 7H11 agar in 24-multiwell plates containing test drugs. After cultivation at 37 °C for 14 days, MICs were read as minimum concentrations of drugs completely inhibiting visible growth of the organisms.

Activities of drug combinations. The effects of combinations of OA with INH, RMP and EMB were determined in 7H11 agar as described by De Logu *et al.* (2005). A total of 10 µl of the appropriate dilution of antimicrobial agents was dissolved in 1 ml 7H11 agar with OADC to obtain final concentrations of the two drugs that ranged from eight dilutions below the MIC to 2 × MIC using twofold or threefold dilutions. Interpretation of the data was achieved by calculating the fractional inhibitory concentration index (FICI) as follows: $FICI = (MIC_{a\ combination}/MIC_{a\ alone}) + (MIC_{b\ combination}/MIC_{b\ alone})$. The FICI was used to interpret the test results as follows: FICI $\leqslant 0.5$, synergy; FICI 0.5–4, no interaction; FICI >4.0, antagonism (De Logu *et al.*, 2002). Employing the chequerboard technique, the lowest concentration of each agent that inhibited the organisms was plotted as an isobologram, and the effect of a drug combination was considered synergistic when the MIC for each drug was reduced to one-quarter of the original MIC so that the sum of the FICI was less than or equal to 0.5 (Stratton & Cooksey, 1991). In addition, the combination of INH and RMP was tested against the sensitive strain CRY6 and the resistant strain CRY3.

Time–kill assay. A time–kill assay was conducted using a modification of a previous method (Kent *et al.*, 1992). One strain, CRY3, was selected for further investigation. The MICs for the strain were determined in 7H9 broth containing 0.05 % Tween 80 (pH 6.9) with an inoculum of approximately 5×10^6 c.f.u. ml^{-1}. Tubes of broth were prepared containing each antibiotic alone at a concentration equivalent to the MIC or combinations of two of the test antibiotics at concentrations equivalent to the MIC of each one. A control tube without antibiotics was included with each series. Each tube (final volume 5 ml) was inoculated with 50 µl of a 3-day-old broth culture to give an initial inoculum of approximately 5×10^5 c.f.u. ml^{-1}. The tubes were incubated in air at 35 °C, and 100 µl aliquots were withdrawn at the start of the study and at daily intervals thereafter for 7 days. Tenfold dilutions of the aliquots were made in peptone water and 20 µl each dilution was spread onto 7H11 agar plates containing 10 % OADC supplement. Colonies were counted with the aid of a hand lens after incubation for 7 days. Synergy was defined as a 100-fold or greater increase in killing by the combination at 7 days compared with killing produced by the most active single drug; similarly, antagonism was defined as a 100-fold or greater decrease in killing by the combination at 7 days compared with that produced by the most active drug alone.

Cytotoxic activity. The cytotoxicity of OA was evaluated against Vero cell lines obtained from the American Type Culture Collection. Cells were cultured at 37 °C in 5 % CO_2 in Dulbecco's modified Eagle's medium (Sigma Diagnostics), supplemented with 10 % fetal calf serum, 2 mM glutamine and 1 % penicillin/streptomycin solution. Cells were resuspended in growth medium at a final concentration of 6×10^4 cells per well, placed in a 96-well culture microplate (200 µl per well) and incubated with various concentrations of OA for 48 h. Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The IC_{10} value was calculated, representing the concentration of the compound that inhibited 10 % of cell growth.

RESULTS

Antimycobacterial susceptibility *in vitro* and cytotoxicity

The chemical formula of OA is shown in Fig. 1. The MICs of OA, INH, RMP and EMB were determined according to a standard twofold agar dilution method as described in Methods. The antimycobacterial susceptibility results showed that clinical isolates CRY1, CRY2 and CRY4 were resistant to EMB, CRY5 and CRY7 were resistant to INH, CRY3 was resistant to both INH and RMP, and CRY6 and CRY8 were sensitive to INH, RMP and EMB (Table 1). The MIC of OA against the standard strain of *M. tuberculosis* (H37Rv, ATCC 27294), which is sensitive to the three anti-TB drugs (RMP, INH and EMB), was 100 µg ml^{-1}. The MICs of OA against the drug-sensitive isolates CRY6 and CRY8 were 50 and 100 µg ml^{-1}, respectively. The MICs of

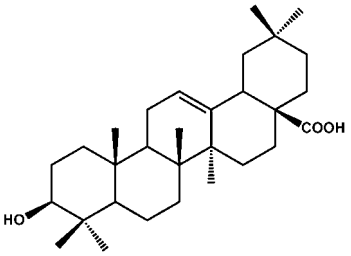

Fig. 1. Chemical formula of OA.

OA against the six drug-resistant *M. tuberculosis* clinical isolates ranged from 100 to 200 μg ml^{-1}. These results indicated that OA has potential antimycobacterial properties. DMSO was used as an appropriate solvent control and the highest concentration of DMSO used (0.5 %, v/v) did not inhibit the growth of bacteria. In terms of cytotoxicity, OA showed an IC_{10} value of 43 μg ml^{-1} in Vero cells.

Combination testing

The results of the combination of OA with INH, RMP or EMB against *M. tuberculosis* H37Rv ATCC 27294 and clinical isolates are listed in Table 1. For the six drug-resistant strains tested, the combination of OA with INH, RMP or EMB showed favourable synergistic antimycobacterial effects. Upon addition of subinhibitory concentrations of OA to these first-line agents, the MICs of the latter decreases ranged from 4-fold to 16-fold, 8-fold to 16-fold and 4-fold to 16-fold for INH (FICI 0.121–0.347), RMP (FICI 0.113–0.168) and EMB (FICI 0.093–0.266), respectively. The MICs of OA against the six drug-resistant strains tested were significantly decreased, ranging from 4-fold to 32-fold, 16-fold to 128-fold and from 16-fold to 128-fold, in combination with INH, RMP and EMB, respectively. For each combination of antimycobacterials,

Table 1. Combination testing of OA plus antituberculous drugs against *M. tuberculosis* isolates

Drug combination	Strain	Status of strains	Individual MICs (μg ml^{-1})	Combination MICs (μg ml^{-1})	Combined FICIs	Conclusion
OA/INH	CRY1	EMB^R	100/0.09	12.5/0.02	0.347	Sy
	CRY2	EMB^R	100/0.09	25/0.006	0.317	Sy
	CRY3	MDR	200/1.56	6.25/0.19	0.153	Sy
	CRY4	EMB^R	200/0.09	12.5/0.01	0.174	Sy
	CRY5	INH^R	200/1.56	12.5/0.09	0.121	Sy
	CRY6	INH^S, RMP^S, EMB^S	50/0.04	25/0.006	0.650	N
	CRY7	INH^R	200/3.12	12.5/0.19	0.124	Sy
	CRY8	INH^S, RMP^S, EMB^S	100/0.09	25/0.01	0.361	Sy
	H37Rv	INH^S, RMP^S, EMB^S	100/0.09	100/0.006	1.067	N
OA/RMP	CRY1	EMB^R	100/0.39	3.12/0.04	0.134	Sy
	CRY2	EMB^R	100/0.19	6.25/0.02	0.168	Sy
	CRY3	MDR	200/6.25	12.5/0.39	0.125	Sy
	CRY4	EMB^R	200/0.19	1.56/0.02	0.113	Sy
	CRY5	INH^R	200/0.39	12.5/0.02	0.114	Sy
	CRY6	INH^S, RMP^S, EMB^S	50/0.09	1.56/0.02	0.253	Sy
	CRY7	INH^R	200/0.78	1.56/0.09	0.123	Sy
	CRY8	INH^S, RMP^S, EMB^S	100/0.09	3.12/0.02	0.253	Sy
	H37Rv	INH^S, RMP^S, EMB^S	100/0.19	3.12/0.09	0.505	N
OA/EMB	CRY1	EMB^R	100/25	3.12/3.12	0.156	Sy
	CRY2	EMB^R	100/12.5	3.12/0.78	0.093	Sy
	CRY3	MDR	200/3.12	3.12/0.78	0.266	Sy
	CRY4	EMB^R	200/25	12.5/1.56	0.125	Sy
	CRY5	INH^R	200/3.12	3.12/0.78	0.266	Sy
	CRY6	INH^S, RMP^S, EMB^S	50/3.12	3.12/1.56	0.562	N
	CRY7	INH^R	200/3.12	1.56/0.78	0.258	Sy
	CRY8	INH^S, RMP^S, EMB^S	100/3.12	3.12/0.78	0.281	Sy
	H37Rv	INH^S, RMP^S, EMB^S	100/3.12	3.12/1.56	0.531	N
INH/RMP	CRY3	MDR	1.56/6.25	0.78/3.12	1.0	N
	CRY6	INH^S, RMP^S, EMB^S	0.04/0.09	0.005/0.01	0.236	Sy

N, No interaction; R, resistant; S, sensitive; Sy, synergistic.

the MICs of OA against all strains were decreased below 25 μg ml^{-1}, except for the MIC of OA/INH against H37Rv. For the drug-sensitive strains tested, including H37Rv ATCC 27294, the OA/INH, OA/RMP and OA/EMB combination treatments displayed either synergistic effects or did not show any interaction. Noticeably, no antagonism resulting from the combination of OA with INH, RMP or EMB was observed for any of the strains tested. The synergistic effects of OA/INH, OA/RMP and OA/EMB against the various strains were visualized with individual fractional inhibitory concentrations (FICs) plotted as isobolograms. Examples of synergistic results for the OA/INH, OA/RMP and OA/EMB combinations against CRY3 are shown in Fig. 2.

Time–kill assay

Further time–kill studies were conducted using OA, INH, RMP and EMB against one chosen clinical MDR isolate, *M. tuberculosis* CRY3. For this strain, time–kill curves verified synergism for the OA/INH, OA/RMP and OA/EMB combinations (Fig. 3). After 7 days of incubation, the combinations of OA/INH, OA/RMP and OA/EMB were bactericidal, yielding a >3 $\log_{10}$ c.f.u. ml^{-1} decrease compared with the more active agents INH, RMP and EMB alone, respectively.

DISCUSSION

We found that OA not only significantly decreased the MICs of INH, RMP and EMB, but also that the MICs of OA itself were significantly reduced by INH, RMP and EMB. In particular, for the OA/RMP and OA/EMB combinations, the MICs of OA against all strains tested were decreased to below 12.5 μg ml^{-1}. EMB is used in many regimens due to its activity against both extracellular and intracellular bacilli. It inhibits the development of resistant *M. tuberculosis*; however, EMB is reported to have ocular toxicity and toxicity to retinal ganglion cells *in vitro* and *in vivo* (Yoon *et al.*, 2000). In addition, owing to the central role of INH and RMP in the treatment of TB, it is important to identify the synergistic properties of OA with INH and RMP. To the best of our knowledge, we have described for the first time the antimicrobial properties of OA in combination with other agents against *M. tuberculosis* strains. In clinical practice, combined treatment of INH and RMP is used. In the present study, the FICI values of the combination of INH and RMP against the sensitive strain CRY6 and the resistant strain CRY3 were 1.0 and 0.236, respectively. This result showed that the OA/INH, OA/RMP and OA/EMB combinations were more favourable than the INH/RMP combination against the resistant strain.

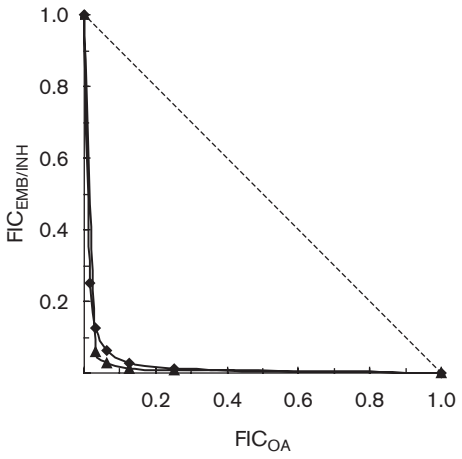

Fig. 2. Synergistic activity of OA and INH (▲) or EMB (◆) against clinical *M. tuberculosis* CRY3. Values are expressed as individual FICs (the FIC for each combination). The dotted line shows the theoretical plot for an additive effect.

Our results showed that OA is relatively non-toxic, which was consistent with the previous study (Liu, 1995). Recently, it has been shown that OA can be modified at the C-3 position to form cinnamate-based esters; modification of the parent structure of OA to form *p*-coumarate, the analogous ferulate ester, resulted in high antimycobacterial activity (Tanachatchairatana *et al.*, 2008). This ability to modify OA may allow the creation of new compounds with more effective derivatives and less toxicity.

It has been shown that plant extracts of *Buddleja saligna* and *Leysera gnaphaloides* exhibit significant antimycobacterial properties, which appear to be primarily associated with the presence of pentacyclic triterpenoids, OA and ursolic acid (Bamuamba *et al.*, 2008). The ubiquity of OA makes the study of its antimycobacterial properties more attractive and practical than that of other compounds. It has been suggested that the mechanism of action of OA depends on the lipophilicity of the compound, which allows rapid penetration across the lipid-rich mycobacterial cell wall (Caldwell *et al.*, 2000). In addition, Deng *et al.* (2000) indicated that the mechanism of antimicrobial action of OA and its derivatives is based on the inhibition of DNA polymerase. These possible mechanisms of antimicrobial action of OA are significantly different from that of INH, RMP and EMB, which may explain the synergistic effects of OA/INH, OA/RMP and OA/EMB. In addition, we found that the combinations of OA/INH, OA/RMP and OA/EMB showed more favourable synergistic antimycobacterial effects against drug-resistant strains than against drug-sensitive ones, and we suggest that OA may be an antibiotic resistance inhibitor. Interestingly, INH did not decrease the MICs of OA to the same levels observed for combinations of OA with RMP and EMB, indicating that there may be different synergistic mechanisms operating in the OA/INH, OA/RMP and OA/EMB combinations.

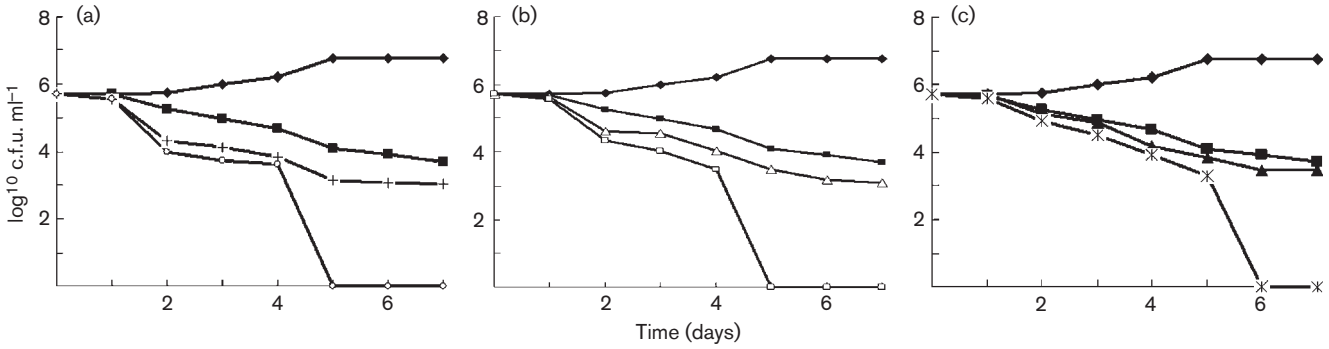

Fig. 3. Time–kill curves for *M. tuberculosis* CRY3 in the presence of individual and combinations of antimicrobials: (a) INH, (b) RMP and (c) EMB. ◆, Control; ■, 200 μg OA ml^{-1}; +, 1.56 μg INH ml^{-1}; ○, 200 μg OA ml^{-1} and 1.56 μg INH ml^{-1}; △, 6.25 μg RMP ml^{-1}; □, 200 μg OA ml^{-1} and 6.25 μg RMP ml^{-1}; ▲, 3.12 μg EMB ml^{-1}; ∗, 200 μg OA ml^{-1} and 3.12 μg EMB ml^{-1}.

In conclusion, our study showed that OA has potent antimycobacterial properties against drug-sensitive and drug-resistant *M. tuberculosis*, and that OA shows favourable synergistic activity with the first-line drugs INH, RMP and EMB against clinical *M. tuberculosis* isolates, especially drug-resistant strains. Our results are preliminary but are of sufficient interest to warrant further study, and indicate that OA is worthy of further investigation as a template for the development of novel antimycobacterial compounds.

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