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Cholesteroid nature of free mycolic acids from *M. tuberculosis*

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Abstract

Mycolic acids (MAs) are a major component of the cell walls of *Mycobacterium tuberculosis* and related organisms. These α-alkyl β-hydroxy long fatty acids have been the subject of numerous studies for their immunological properties. We previously reported that an interaction between cholesterol and mycolic acids could be responsible for the low accuracy in the serodiagnosis of TB when using free mycolic acid in an ELISA assay. The aim of this work was to investigate if this interaction could be due to a similarity in the structural properties between mycolic acids and cholesterol. The investigation revealed that patient sera cross-reacted with mycolic acids and cholesterol in an ELISA experiment suggesting that both molecules may present related functionality in a similar structural orientation. This relation was further supported by the interaction of mycolic acids with Amphotericin B (AmB), a known binding agent to ergosterol and cholesterol. Using a resonant mirror biosensor, we observed that AmB recognised both cholesterol and mycolic acids. In addition, a specific attraction was observed between mycolic acid and cholesterol by the accumulation of cholesterol from liposomes in suspension onto immobilized mycolic acids containing liposomes, detected with a biosensor technique. Combined, these results suggest that mycolic acids can assume a three-dimensional conformation similar to a sterol. This requires that mycolic acid exposes its hydroxyl group and assumes rigidity in its chain structure to generate a hydrophobic surface topology matching that of cholesterol. A particular folded conformation would be required for this, of which a few different types have already been proven to exist in monolayers of mycolic acids.

Keywords: Mycolic acids; Cholesterol; Lipid conformation; Amphotericin B; Resonant mirror biosensor

1. Introduction

Tuberculosis is one the most life-threatening infectious diseases. Its re-emergence has brought new interest in finding novel methods for its treatment and diagnosis, especially in developing countries where the population is most burdened by this pandemic and where the connection between TB and HIV/AIDS infection is particularly worrying. In fact, TB is the major cause of death in HIV/AIDS co-infected individuals (Uma Devi et al., 2003).

In the search for new surrogate markers for the diagnosis of tuberculosis, antibodies to cord factor (trehalose-6,6′-dimycolate, TDM) have attracted much attention. Although having a hydrophobic nature, TDM is a very immunogenic and biologically active substance present in the mycobacteria and a few related genera. TDM is a glycolipid consisting of trehalose, which is identical for all cord factors, and two of a set of mycolic acids (MAs) (**1**, Fig. 1), which differ within and among species and genera (Ryll et al., 2001). For example, *Mycobac-*

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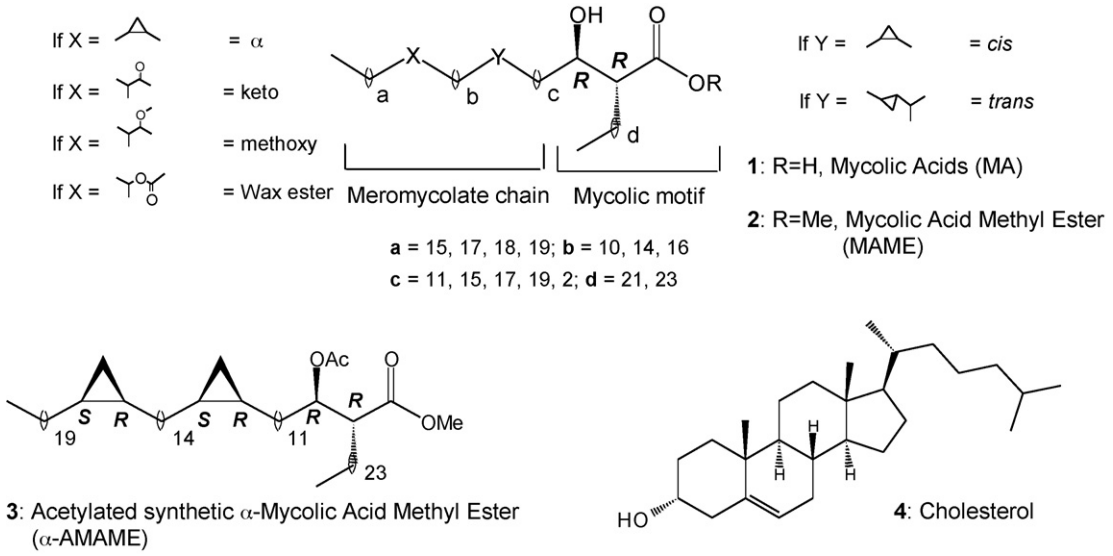


Fig. 1. Molecules under investigation for their similarity in function.

terium tuberculosis contains α-, methoxy- and keto-mycolic acids subclasses, whereas *Mycobacterium avium* contains wax ester—instead of methoxymycolic acids (Dobson et al., 1985). Fujiwara et al. (1999) identified the antigenic epitope in cord factor molecules to be the mycolic acids by proving that antibodies against TDM from TB patients could distinguish between mycolic acids subclasses.

We have reported the use of free mycolic acids as antigens for the serodiagnosis of tuberculosis; they have considerable potential because HIV–TB co-infected patients maintain high antibody levels to mycolic acids, despite the severity of the immune deficiency measured by the fall in CD4 T cell count (Schleicher et al., 2002). However, in an ELISA assay, the use of free mycolic acids was not adequate for serodiagnosis of tuberculosis (accuracy = 57%). An association between mycolic acids and cholesterol was hypothesised as a possible reason for this low accuracy (Schleicher et al., 2002). Cholesterol may be non-specifically attracted to mycolic acids by means of hydrophobic Van der Waals type of binding, or by a more specific interaction such as a hydrogen bond, arising from conformational features present in the two molecules.

Recently, free mycolic acids have been demonstrated to be able to adopt a folded conformation to give a hydrophobic surface. In particular, the "W" conformation has the alkyl chains folded to give four parallel arms, while in the "Z" conformation three folded arms provide the three-dimensional, curved hydrophobic surface (Villeneuve et al., 2007; Sekanka et al., 2007). The existence of these conformations has been suggested by analyses of Langmuir monolayers consisting of free mycolic acids over a range of temperatures (Villeneuve et al., 2005, 2007). In an extended form, the shape and structure of a mycolic acid appears very different to that of cholesterol and does not suggest particularly strong interactions between these molecules. However, the folded conformations of mycolic acids could be imagined to assume a shape similar to that of cholesterol.

The current study was designed to investigate the possibility that the low accuracy measured for a serodiagnostic ELISA assay based on the use of mycolic acid as antigen could be the result of the folding of free mycolic acids to resemble a "cholesteroid" shape. In order to investigate this, we have used an approach similar to that employed by Prendergast et al. (1998) to suggest molecular mimicry between microbial and self-structures in autoimmune diseases. The basic assumption of this approach is that the specific molecular recognition of two substances by an established binding agent indicates resemblance in the three-dimensional structure of the two compounds. Therefore we further investigated the interaction of mycolic acids and cholesterol with sera from human TB patients, using the ELISA technique described by Schleicher et al. (2002). Secondly, using a resonant mirror biosensor (Cush et al., 1993; Buckle et al., 1993; Athanassopoulou et al., 1999), we compared the interaction of these two lipids with Amphotericin B (AmB), a known cholesterol binding molecule (Baginski et al., 2002, 2005). Thirdly, the attraction between cholesterol and mycolic acids was studied, also using the resonant mirror biosensor.

2. Materials and methods

2.1. General procedures

^{1}H, ^{13}C and COSY nuclear magnetic resonance (NMR) were recorded on a Bruker advance DRX-500 spectrometer or Bruker AC-300 spectrometer with chemical shifts in ppm. Analytical thin layer chromatography (TLC) was performed on Alugram SIL G/UV, layer: 0.2 mm silica gel 60 with fluorescent indicator UV_{254} plates. The plates were viewed under ultraviolet light (254 and 366 nm). Flash column chromatography was performed using Merck silica gel. FT-IR was obtained with a

PerkinElmer BX-1 instrument of 2.00 cm^{-1} resolution fitted with a MIR source, an internal LiT aO_3 detector and FT-IR Spectrum Software version 5.1. Optical rotations were measured on a PerkinElmer model 341 polarimeter. For mycolic acids analyses HPLC was performed using a Merck Hitachi Chromatograph fitted with a Phenomenex Luna 5 μm C18 column and a Merck Hitachi L-4500 Diode Array detector. For AmB derivative purification a Waters high performance liquid chromatography (HPLC) system was used with a Waters 610 fluid unit, Waters 600 controller and a Waters 996 Photodiode Array detector. A Waters C18 reverse phase column (150 mm × 4.6 mm) was used for optimization of methods and a Phenomenex Luna C-18 reverse phase column (250 mm × 10 mm, 10 μm) was used to collect the product. Electrospray mass spectrometry performed on a Micromass Micro Triple quadrupole mass spectrometer in full scan mode, range 500–2000 amu, was used to characterise the product. Sample infusion was directly into the electron spray source by means of a Hamilton syringe pump at 10 μl/min.

2.2. Preparation of methyl mycolic acids isolated from *M. tuberculosis* (H37Rv)

A mixture of mycolic acids (**1**) was isolated from the cell wall of the virulent Erdman strain of *M. tuberculosis* as described by Goodrum et al. (2001). HPLC and NMR spectra were in agreement with the data reported in literatures (Steck et al., 1978; Watanabe et al., 2001).

To form the methyl esters, mycolic acids (**1**, 100 mg, ∼0.1 mmol) were dissolved in a mixture of toluene:methanol (5:1, 18 ml), trimethylsilyldiazomethane (TDM, 2 M solution, 0.2 ml, 0.4 mmol) added, followed by a further 4 additions of TDM (0.1 ml, 0.2 mmol) every 45 min. The mixture was stirred for 72 h, and then quenched by evaporation. The residue was dissolved in dichloromethane (15 ml) and water (10 ml) was added. The two layers were separated and the water layer extracted with dichloromethane (2× 10 ml). The combined organic layers were dried and the solvent evaporated to give the desired compound (98 mg, ∼97%). The NMR spectra of the compounds obtained corresponded to those reported in the literatures for methyl esters of mycolic acids (MAME, **2**) (Watanabe et al., 2001; Al Dulayymi et al., 2003, 2007; Koza and Baird, 2007).

The acetylated synthetic alpha-mycolic acid methyl ester (α-AMAME, **3**) was kindly provided by Dr. J. Al Dulayymi, University of Bangor, UK (Al Dulayymi et al., 2003, 2005). Cholesterol (**4**), isoniazid, AmB, $NaBH_4$, terephthalaldehyde and phosphatidylcholine were purchased from Fluka or Sigma–Aldrich. All organic solvents were purchased from Merck while the inorganic materials were from Sigma.

2.3. Synthesis of Amphotericin B derivative

2.3.1. Preparation of *N′-(4-formylbenzylidene)isonicotinohydrazide* (*6*)

Isoniazid (**5**, 0.5 g, 3.65 mmol) was added in portions over an hour to a solution of terephthalaldehyde (0.49 g, 3.65 mmol) in ethanol (50 ml) and stirred at room temperature (RT) overnight. The precipitate that formed was removed by filtration and the mother liquor was concentrated to give a crude product (0.15 g) that was purified by flash column chromatography on silica gel in ethyl acetate:methanol:ethanol containing 1% triethylamine (90:9:1, v/v) to give pure *N′*-(4-formylbenzylidene) isonicotinohydrazide (**6**, 0.12 g, 14% yield).

Molecular weight 253.2599 g mol^{-1}. R_f 0.57 (ethyl acetate:methanol:ethanol with 1% Et_3N (v/v) (90:9:1); mp 219–220 °C; IR ν_{max} 3465, 3197 (NH), 1698 (CHO) cm^{-1}. ^1H NMR (300 MHz, $(CD_3)_2$ SO) δ 12.25 (1H, s, NH), 10.06 (1H, s, 1a), 8.80 (2H, d, J = 5.4 Hz, 10a), 8.55 (1H, s, 6a), 8.01 (2H, d, J = 9 Hz, 3a), 7.97 (2H, d, J = 9 Hz, 4a), 7.84 (2H, d, J = 5.4 Hz, 9a); ^{13}C NMR (300 MHz, $(CD_3)_2$ SO) δ 193.1, 162.3, 150.7, 148, 140.7, 139.9, 137.4, 130.3, 128.1, 121.9 ppm; HR–MS (EI) calculated for $C_{14}H_{10}O_2N_3$ [M^+] 253.08513 g mol^{-1} found 253.08389 g mol^{-1}, *m/z* (EI) 122 (74), 106 (1 0 0), 79 (10), 78 (47), 51 (22).

2.3.2. Preparation of *N-(4-((2-isonicotinoylhydrazono)methyl)benzyl)-AmB (7, AmB derivative)*

In the next step, the aldehyde (**6**, 0.01 g, 0.04 mmol) and Amphotericin B (0.043 g, 0.05 mmol) were stirred at RT in 2 ml dimethylsulfoxide in the dark for an hour. The derivative that formed was reduced *in situ* with $NaBH_4$ (0.001 g, 0.04 mmol) overnight and purified by RP-HPLC (methanol:H_2O a gradient from 20% to 100% methanol). The column eluent was monitored at 300 and 407 nm and AmB derivative collected and concentrated.

Molecular weight 1159.35 g mol^{-1} (calculated), melting point not determined due to decomposition of compound at about 40 °C, $[\alpha]_D^{20}$ (*c* 2.0, DMF), IR ν_{max} 3391, (NH, OH's), 1564 (double bonds) cm^{-1}. ^1H NMR (500 MHz, $(CD_3)_2$ SO) δ 12.06 (1H, s, H–NH), 8.79 (2H, d, J = 3.3 Hz, H-10a), 8.46 (1H, s, H-6a), 7.83 (2H, d, J = 4.4 Hz, H-6a), 7.51 (2H, d, J = 7.9 Hz, H-4a), 7.7 (2H, d, J = 7.9 Hz, H-3a), 6.45–6.07 (m, 12H, olefinic), 5.95 (1H, dd, J = 13, 5.3 Hz, H-20), 5.43 (1H, dd, J = 11.3, 12 Hz, H-33), 5.21 (1H, m, H-37), 4.48 (1H, broad-s, H-1′), 4.4 (1H, m, H-19), 4.24 (1H, m, H-11), 4.23 (1H, t, H-17), 4.06 (1H, m, H-3), 3.99 (1H, dt, H-15), 3.89 and 3.67 (2H, s, H-1a), 3.73 (1H, d, H-2′), 3.54–3.46 (HOD plus 3H), 3.2 (1H, m, H-5′), 3.09 (2H, m, H-4′, H-35), 2.82 (1H, m, H-3′), 2.28 (1H, s, H-34), 2.16 (3H, m, H-2, H-18), 1.88–1.05 (14H, m, CH_2, CH), 1.87 (1H, t, J = 6.7 Hz, H-16), 1.16 (3H, d, J = 5.5 Hz, CH_3), 1.11 (3H, d, J = 5.6 Hz, CH_3), 1.04 (3H, d, J = 5.6 Hz, CH_3), 0.91 (3H, d, J = 6.5 Hz, CH_3), ^{13}C NMR (300 MHz, $(CD_3)_2$ SO) δ 174.4, 170.6, 161.54, 150.3, 149.1, 143.6, 140.51, 133.9, 136.8, 133.7–128.5, 131.9, 127.2, 121.5, 97.2, 77.1, 73.8, 74.5, 74.4, 69.8, 73.6, 69.4, 68.8, 66.2, 65.4, 65.2, 63, 57.1, 44.7, 49.5–44.3, 42.3, 42, 35.1, 29, 18.5, 12.1 ppm.

2.4. ELISA

The human sera used in the ELISA experiments were from two sources: the first was from a pulmonary TB positive collection that was made in 1994 by the MRC Clinical and Biomedical TB Research Unit at King George V Hospital, Dur-

ban, KwaZulu-Natal and donated by Dr. P.B. Fourie. The second source was from a collection made in the year 2000 from patients for another study by Schleicher et al. (2002). From the latter, five serum samples were selected from patients who were determined to be negative for both tuberculosis and HIV (TB^-). These TB negative patients were hospitalised for various reasons other than TB or AIDS.

Mycolic acids (MA, **1**), acetylated synthetic α-mycolic acid methyl ester (α-AMAME, **3**) and cholesterol were used at final concentrations of 60 μg/ml. To prepare the coating solutions, the antigens were heated in PBS buffer for 20 min at 85 °C. The hot solutions were sonicated and kept at 85 °C during loading into the ELISA plates at 3 μg antigen per well. The assay with sera was done according to Schleicher et al. (2002). The results obtained were analysed for statistical differences using the Student's *t*-test.

2.5. Preparation of liposomes

Empty liposomes were prepared consisting of only phosphatidyl choline (PC), whereas the other liposomes consisted of PC in some ratio to the lipid under investigation. Mycolic acids (MA, **1**) liposomes and other chemically prepared mycolic acid liposomes contained MA or MAME (**2**) or α-AMAME (**3**) (7.8 mol%; mycolic acid: PC = 1 mg: 9 mg), whereas the cholesterol (Chol, **4**) liposomes contained Chol (50 mol%, Chol:PC = 3 mg:6 mg). The appropriate lipids were initially dissolved in chloroform in an amber glass vial and vortexed to ensure mixing. The samples were then dried at 80 °C under a stream of N_2 and sonicated in 2 ml saline for 5 min at room temperature. Sonication was done using a Branson sonifier (model B30, Branson Sonicpower Co. USA) with 30% duty cycles and output of 5. Subsequently the liposomes were divided into 200 μl aliquots, freeze-dried and stored at −70 °C until required for use. Before use, the liposomes were reconstituted with 2 ml of phosphate buffered saline (PBS/AE) pH 7.4 containing EDTA (1 mM), sodium azide (0.025%, m/v), heated at 80 °C for 15 min and then sonified as above. The final liposome concentration came to 500 μg/ml. The liposome suspensions were analysed for their mycolic acid content by HPLC according to Goodrum et al. (2001). Recovery of mycolic acids ranged between 70% and 110% of the expected values.

2.6. Measurements of interaction between mycolic acids, cholesterol and Amphotericin B derivatives on an IAsys affinity biosensor

The binding interactions among Amphotericin B, cholesterol, mycolic acids and acetylated synthetic α-mycolic acid methyl ester were measured by means of an IAsys resonant mirror biosensor (IAsys Affinity Sensors, Bar Hill, Cambridge, UK) according to the activity sequence outlined in Fig. 2. IAsys software was used to set the device at a data-sampling interval of 0.4 s, temperature of 25 °C and stirring rate of 75% for all experiments on the biosensor. The cells of the cuvette were rinsed with ethanol (95%) 3 times prior to use, followed by extensive washing with PBS/AE. A 60-μl volume of PBS/AE was

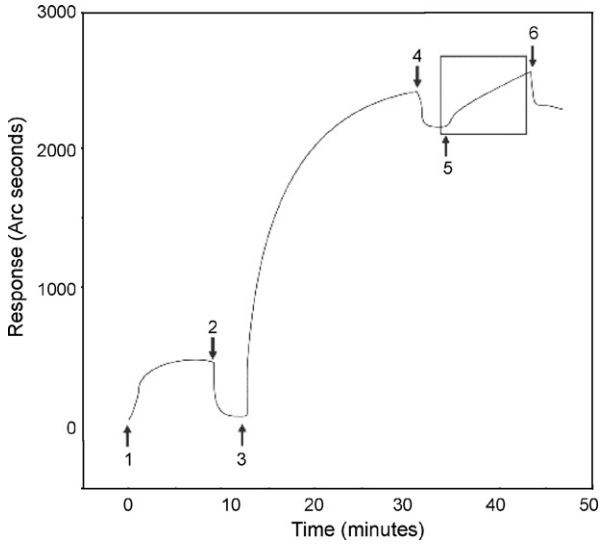
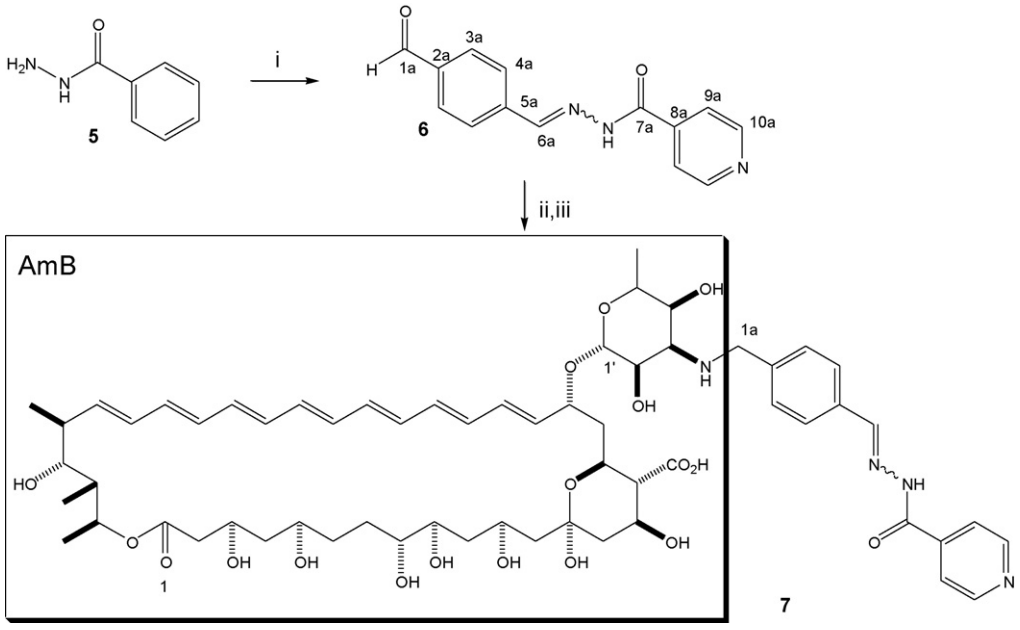


Fig. 2. Biosensor activity sequence to obtain sensorgrams of the binding of cholesterol from cholesterol-containing liposomes onto various immobilized lipid-containing liposomes. 1: activation of surface with CPC, 2: PBS/AE wash step, 3: addition of liposomes for coating, 4: PBS/AE wash step, 5: addition of the test solution, 6: PBS/AE wash step. The cholesterol binding sensorgram appears in the rectangle.

pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 μl of cetyl-pyridinium chloride (CPC, 0.02 mg/ml in PBS/AE) for 10 min. This was followed by washing 5 times with 60 μl PBS/AE and then substituting with 25 μl PBS/AE for a new baseline before immobilization of mycolic acids (or cholesterol, or a synthetic α-AMAME, or PC only) containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed 5 times with 60 μl PBS/AE. The cuvette cell content was substituted with 25 μl of PBS/AE and allowed to equilibrate for 5–10 min to achieve a stable baseline. An addition of either 25 μl liposomes under investigation or 25 μl of a solution of AmB (1×10^{-4} M) or the AmB derivative (**7**, Scheme 1, 1×10^{-4} M) in PBS/AE was made. Direct interaction between the immobilized cholesterol/mycolic acids and the dissolved Amphotericin B compounds or lipids contained in the suspended liposomes was monitored for 5–10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE. Finally, regeneration of the cuvette was effected by 5 times washing with 50 μl 95% ethanol for 1 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The surface was then finally treated for 5 times with 50 μl potassium hydroxide (12.5 M) for 2 min, followed by 7 times washing with 70 μl PBS/AE for 1 min. The results obtained were analysed for statistical differences using the Student's *t*-test.

2.7. Measurements of direct interaction between mycolic acids and cholesterol

After immobilization of either the mixture of natural mycolic acids (**1**), their methyl esters (**2**), or protected acetylated synthetic α-mycolic acid methyl ester (**3**) containing liposomes using the same method as described above and illustrated in



Scheme 1. Preparation of an AmB derivative: (i) terephthalaldehyde, (ii) AmB and (iii) $NaBH_4$.

Fig. 2, 25 μl cholesterol-containing liposomes were added. Direct interaction between the immobilized mycolic acids and cholesterol was monitored for 10 min, after which the cuvette was washed 3 times with 60 μl PBS/AE and regenerated as before.

3. Results and discussion

3.1. Interactions of patient sera to mycolic acids and cholesterol

It is known that all humans have anti-cholesterol antibodies (ACHA), which have been proven to be very specific for their interaction with the sterol. ACHA recognise selectively 3β-hydroxy-sterols in a stereospecific manner, but they cannot distinguish between enantiomers (Geva et al., 2001; Bíró et al., 2007). Concurrently, tuberculosis patients have been shown to produce antibodies against mycolic acids (Schleicher et al., 2002). Pan et al. (1999) reported that even small changes in the structure of the mycolic acids present in TDM are important for their antigenicity. In particular, among the three subclasses present in *M. tuberculosis*, methoxymycolic acids were shown to be the most antigenic. For this reason, we expected TB negative patient sera to react to cholesterol, but not to mycolic acids.

TB positive and TB negative sera were tested in ELISA for antibody binding to natural mycolic acid and cholesterol. To verify the specificity of the interaction between sera and mycolic acids, a synthetic α-mycolic acid (**3**), with the carboxylic acid protected as methyl ester and the hydroxyl group as acetyl ester, was used as negative control. This compound was chosen because it lacks a hydrogen donor at either the α-carboxylic acid, the β-hydroxyl group or in the meromycolate chain, which diminishes the possibility of hydrogen bonds with antibodies without alleviating the hydrophobic nature of the molecule that can be expected to be the major force of non-specific binding from serum components. Moreover, the protection of the polar groups in the mycolic motif also discourages any folded arrangement with the merochain (Villeneuve et al., 2005, 2007). Therefore, this change in the three-dimensional structure may be hypothesised to preclude specific interactions with antibodies against mycolic acids or cholesterol.

Coating of the wells with the lipid antigens was confirmed under the microscope as visible fatty deposits adsorbed on the polystyrene. Eleven TB positive patient sera were randomly chosen from the 1994 collection and five TB and HIV negative patients were randomly selected from the 2000 collection. For the TB negative controls, it was deemed important to exclude HIV positive individuals, for whom false negative TB diagnosis is known to occur at high frequency with the currently available TB diagnostics (Uma Devi et al., 2003). The sera were diluted 1:20, which was found to be the highest dilution where significant binding to the antigens on the plate could still be observed with most sera.

As expected there was a tendency for TB positive patient antibodies to bind more strongly to mycolic acid than those of the TB negative patients (average absorbance value 0.76 compared to 0.37 respectively, Fig. 3, $P < 0.1$). The selectivity of binding with MAs was confirmed by the negligible antibody activity of the same patients to the synthetic alpha-mycolic acid in both groups of sera (Fig. 3, $P < 0.0001$). Both TB positive and TB negative sera also recognised cholesterol as an antigen. In either TB positive or TB negative patients, the difference in ELISA signal between mycolic acid and cholesterol was not significant (Fig. 3, $P > 0.25$). Although TB negative patients should not present antibodies against *M. tuberculosis* mycolic acids, the results obtained show that their sera recognise in a similar fash-

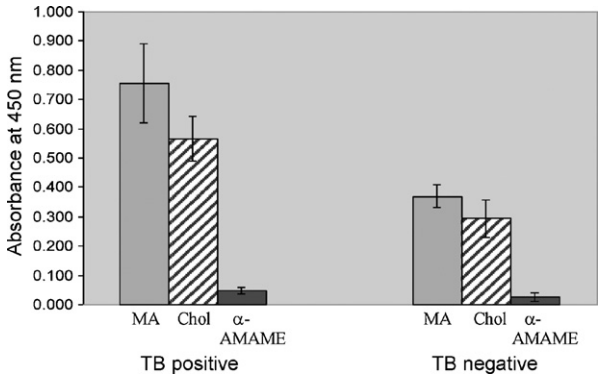


Fig. 3. Normalised ELISA results of TB positive ($n = 11$) and TB negative sera ($n = 5$) against natural mycolic acids (1) (MA, grey), cholesterol (4) (Chol, hatched) and an acetylated, synthetic α-mycolic acid methyl ester (3) (α-AMAME, black). The error bars indicate the standard error of the mean.

ion either cholesterol or mycolic acids. Such congruence of the titres of both antibody specificities could suggest cross-reactivity of recognition of cholesterol and mycolic acid by antibodies.

3.2. AmB cross-reactivity to mycolic acids and cholesterol

To further probe the possibility of resemblance in the nature of mycolic acid and cholesterol to bind to a ligand, an alternative experiment was designed using Amphotericin B (AmB) as a cholesterol binding agent. AmB is a macrolide antibiotic that exerts its antifungal activity by binding to ergosterol and cholesterol (Baginski et al., 2002). The major interaction between the macrolide and the sterol is based on the formation of a hydrogen bond between the amine or hydroxyl groups of the mycosamine moiety of AmB and the hydroxyl group of ergosterol (Herve et al., 1989; Matsumori et al., 2005). Other sterols, in particular cholesterol, are also recognised and bound by AmB, albeit with one order of magnitude weaker affinity. This is due to the more rigid hydrophobic sterol surface of ergosterol (which has two extra double bonds and a methyl group compared to that of cholesterol), making the Van der Waals interaction with the hydrophobic part of AmB stronger (Readio and Bittman, 1982; Baginski et al., 2002). The difference in binding affinity of AmB to ergosterol and cholesterol proves the specificity of the interaction between AmB and its sterol ligands. According to the approach of Prendergast et al. (1998), the specific molecular recognition of two substances by an established binding agent of one of them indicates a resemblance in the three-dimensional structure of the two compounds. For this reason, AmB was selected to assess its binding activity to cholesterol and to mycolic acid using resonant mirror biosensor technology. This technique was preferred to ELISA because it does not need a fluorescent tag on the binding agent that may affect the structure and interaction properties of AmB (Cush et al., 1993). Moreover using this technique it is possible to use liposomes which better mimic the biological environment within which these lipids are presented (MacKenzie et al., 1997).

In order to immobilize the liposomes onto the surface of the biosensor, a new procedure was used. This is based on the activation of the cuvette cell surface with cetyl-pyridinium chloride, a

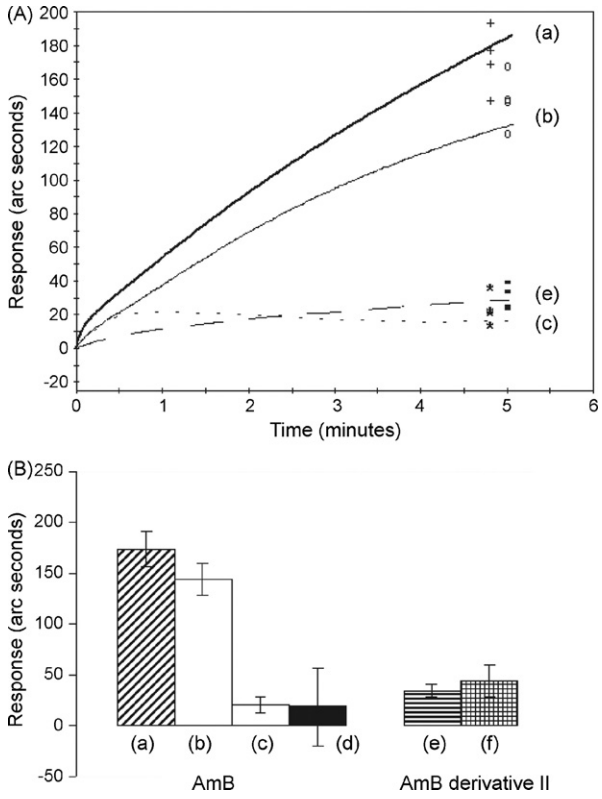


Fig. 4. Resonant mirror biosensor binding curves (A) and binding capacity (B) of AmB or AmB derivative (7) on immobilized lipid antigens. Binding of AmB on (a) mycolic acids (1) (thick line, +, hatched bar); (b) cholesterol (thin line, o, white bar); (c) synthetic protected α-MA (3) (dotted line, *, grey bar) or (d) PC liposomes (black bar). Binding of AmB derivative (7) on immobilized (e) cholesterol liposomes (dashed line, -, horizontal stripes bar) or (f) mycolic acid (1) containing liposomes (grid bar); $n = 5$ for each set. Immobilization of lipid ligate containing liposomes was monitored to achieve binding of at least 2000 arc seconds before contacting with AmB or AmB derivative (7) solutions.

frequently employed cationic, amphipathic compound that gives glazed and metal surfaces a hydrophobic character. Hydrophobic lipid antigen-containing liposomes can then adhere to the surface (see Fig. 2). Following the coating of the cuvette cells with either cholesterol-containing or empty liposomes, consisting only of phosphatidylcholine, the biosensor registered a direct accumulation of AmB on cholesterol liposomes, while it did not show any binding to empty liposomes (Fig. 4). Remarkably, a binding sensorgram comparable to that of immobilized cholesterol was obtained when AmB was allowed to interact with immobilized mycolic acids.

The binding between the AmB macrolide and cholesterol could be abrogated by covalent modification of the single amino group on the AmB macrolide by linking it to isoniazid to give Amphotericin B-derivative (**7**). The mycolic acid binding was similarly abrogated by the covalent modification of the AmB macrolide amino group. This modification to the amino group of the AmB molecule destroyed the latter's ability to bind to both cholesterol and the mycolic acid, showing that the attraction of AmB for both these lipids is determined by the fine structure of the ligand–receptor pair and possibly that this requires a specific hydrogen bond between the binding partners. Such an analysis would be in agreement with the literature, confirming the impor-

tance of the hydrogen bond for a stable interaction (Baginski et al., 2002). Matsumori et al. (2005) demonstrated that AmB's binding to sterols could be hindered when its amino-sugar moiety was forced in a conformation that prevented hydrogen bonding with its ligate. This suggests that the interaction between AmB and mycolic acids could also be based on the formation of a hydrogen bond with the amino-sugar moiety of AmB. It is noteworthy that AmB did not bind to the synthetic protected alpha-mycolic acid (**3**) immobilized on the sensor surface (Fig. 4), thus supporting the specificity of this interaction in terms of the requirement for both Van der Waal's and hydrogen bonding in the recognition event. These results imply that mycolic acid and cholesterol share structural features that are similarly recognised by AmB and that may provide the basis for the cross-reactivity observed with the TB patient antibodies.

3.3. Interaction between mycolic acids and cholesterol

In order to determine whether the presumed structural relatedness between mycolic acid and cholesterol would also lead to their interaction directly, the interaction between mycolic acids and cholesterol liposomes was analysed using the biosensor technique.

Following coating of the cuvette surface with either mycolic acid (**1**), mycolic acid methyl ester (**2**) or synthetic acetylated α-mycolic acid methyl ester (**3**), the cuvettes were exposed to cholesterol-containing test liposomes and the sensorgrams for the binding of cholesterol onto the different surfaces were recorded (Fig. 5). A mixture of methyl esters of natural mycolic acids (MAME), still containing hydrogen binding groups (β-hydroxyl and the oxygenated groups in the meromycolate chain), was also used as a negative control for this test. The experiments described earlier compared the interactions of mycolic acids and cholesterol with binding agents known to be able to differentiate between small conformational changes. The present experiment measured the attraction between two lipids and therefore it was important to verify that this is not just due to Van der Waals interactions between two hydrophobic molecules but also to a more specific conformational match between cholesterol and

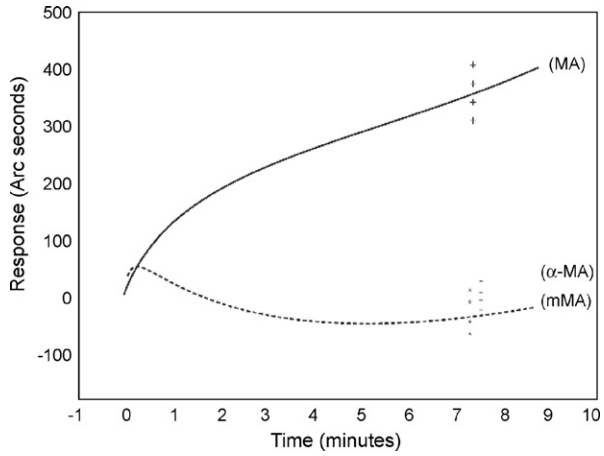


Fig. 5. Resonant mirror biosensor binding curves for the attraction of cholesterol to immobilized mycolic acids (1) (MA, thick line/+), their methyl esters (2) (mMA, dashed line/*) or synthetic protected alpha-mycolic acid (3) (α-MA, thin line/-). Each line represents a typical curve of five repeats with the end points of each indicated after 7.5 min of contact.

mycolic acid. The formation of a hydrogen bond between the carboxylic and the β-hydroxyl of mycolic acid is particularly favoured for the natural erythro configuration of 2*R*,3*R* 2-alkyl-3-hydroxy acids and has been shown to have a stabilising effect on the alignment of the alkyl chains, affecting the physical properties of these acids (Durand et al., 1979a,b). Therefore, the use of methyl esters of mycolic acids as a negative control is particularly important, as this modification will destroy the specific hydrogen bond in the mycolic motif that is expected to destabilise the natural conformations of the alkyl chains to assume a sterol fold, similar to that assumed by the steroid-precursor, the linear 2,3-oxidosqualene, before its conversion into lanosterol in cholesterol biosynthesis (Van Tamelen, 1982).

Fig. 5 shows that the mycolic acid liposomes-coated cuvette surface accumulated cholesterol from the solution while the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**) were unable to do so (no significant difference between binding of cholesterol to (**2**) and (**3**), $P > 0.1$, but highly significant difference of cholesterol bind-

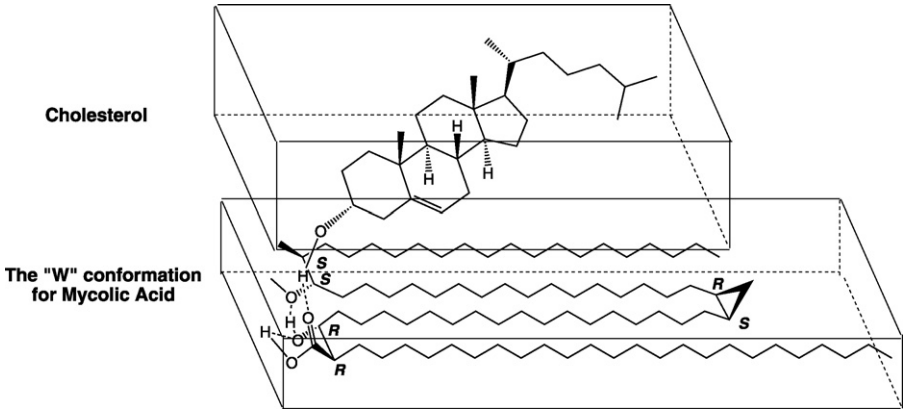


Fig. 6. A pictorial representation of a hypothesised mechanism of interaction between a "W"-folded methoxy MA (Villeneuve et al., 2005, 2007) and cholesterol. The absolute stereochemistry of the cyclopropane and the methoxy group are not completely clarified. Here for simplicity the S,R stereochemistry has been used for the cyclopropyl group and the SS for the methoxy-group. RR is known to be the absolute configuration of the mycolic motif. The "W" fold is drawn here in a flat plane, whereas in practice the fold shown in earlier models had a cylindrical three-dimensional structure (Villeneuve et al., 2005, 2007).

ing to mycolic acid or either the methyl esters of mycolic acid (**2**) and the synthetic, acetylated alpha-mycolic acid methyl ester (**3**), $P < 0.001$). These results demonstrate a pronounced attraction between cholesterol and mycolic acid that is determined by the presence of a free carboxylic acid group and the degree of structural fit by a particular conformation that free mycolic acids assume, which can be eliminated by formation of a simple methyl ester of the acid. In Fig. 6 a graphical representation is attempted of a mycolic acid, folded to assume a cholesteroid nature. A more accurate representation can be expected from virtual models based on Langmuir–Blodgett analysis of synthetic subclasses of mycolic acids interacting with cholesterol. The current advances in the stereo-controlled chemical synthesis of mycolic acids may make this possible in the near future.

4. Conclusion

The following observations support the hypothesis of a similarity in nature between the mycolic acids of *M. tuberculosis* and cholesterol that can affect the accuracy of serodiagnostic assays based on antibodies to mycolic acids as surrogate markers of active TB: (i) both mycolic acid and cholesterol are recognised by AmB and human TB sera, (ii) both human TB sera and AmB are able to distinguish between natural occurring mycolic acid (**1**) and a closely related chemically synthetic structure, i.e. acetylated alpha-mycolic acid methyl ester (**3**), (iii) TB negative patient sera containing antibodies to cholesterol (Bíró et al., 2007) bind to mycolic acid comparatively well, but not to the acetylated alpha-mycolic acid methyl ester (**3**) and (iv) the association of AmB to both cholesterol and mycolic acid could be disrupted by covalent modification of the AmB that prevented the formation of hydrogen bonds but not Van der Waals association. Likewise, modification of mycolic acid, aiming to disfavour its arranging into the "W" or any other folded conformation and preclude hydrogen bonding with ligate, abrogated its interaction with patient sera, AmB and cholesterol. The specific attraction between mycolic acid and cholesterol in liposomes probably depends on a particular conformation that free MAs assume in the phospholipid bilayer of liposomes, and this will probably also apply to biological membranes. The existence of a condensed conformation for MAs has already been proposed by several groups, based on Langmuir studies. Here we present the first evidence that these folded conformations could possibly exist in biological conditions to determine the antigenicity of mycolic acids.

The implications of such a discovery and of the relatedness between cholesterol and mycolic acids may be significant for several reasons.

First it could confirm the explanation of the poor results obtained with ELISA testing the usefulness of antibodies to mycolic acids as surrogate markers for tuberculosis (Schleicher et al., 2002).

Second, it impacts on the observation that mycolic acids, intraperitoneally or intratracheally administered as liposomes in mice, resulted in a pathogen-associated type of innate immune response that mainly affects macrophages, converting them into cholesterol-rich foam cells (Korf et al., 2005). Moreover, when administered intratracheally, mycolic acid could also prevent experimentally induced asthma in mice (Korf et al., 2006). The ability of mycolic acid to attract cholesterol to the macrophage may be critical for these phenomena.

Third, cholesterol has been shown to play a role in the entry and survival of *M. tuberculosis* in the host macrophage (Gatfield and Pieters, 2000; de Chastellier and Thilo, 2006). The cholesteroid nature of at least some mycolic acids may imply their active participation in this manifestation of virulence.

The different subclasses of mycolic acids from *M. tuberculosis* are currently under investigation to learn if the cholesteroid nature of mycolic acid is due only to the mycolic motif or to the other functional groups in the meromycolate chain. Finally, an improved understanding of the nature of the mycolate coat of *M. tuberculosis* could lead to the development of improved diagnostics and treatments of tuberculosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemphyslip.2008.01.004.

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