Cholesteroid Nature of Free Mycolic Acids from *M. tuberculosis*.

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Abstract

Mycolic acids 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. The observation that patient's sera cross-reacted with mycolic acids and cholesterol suggested the possibility of a functional relationship between these apparently very different molecules. 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 AmB recognised both cholesterol and mycolic acids. In addition, a specific attraction was observed between these two lipids, established by the accumulation of cholesterol liposomes on immobilised mycolic acids, detected with a biosensor technique. Combined, these results suggest that mycolic acids can assume a three dimensional configuration similar to sterols. This requires that mycolic acid exposes its hydroxyl group and assumes rigidity in its chain structure to match that of cholesterol and could be generated through a "W" conformation which has 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 (**1**, **Fig 1**), which differ within and among species and genera (Ryll *et al.* 2001). For example, *M. tuberculosis* contains α-, methoxy- and keto-mycolic acids subclasses, whereas *Mycobacterium avium* contains wax ester- instead of methoxy-mycolic acids (Barry III *et al.* 1998). 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 "W" conformation with the alkyl chains folded to give four parallel arms (Villeneuve *et al.* 2007; Sekanka *et al.* 2007). The existence of this conformation has been suggested by

analyses of Langmuir monolayers consisting of free mycolic acids over a range of temperatures (Villeneuve *et al.* 2005; Villeneuve *et al.* 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 "W" conformation of mycolic acids could be imagined to be much closer to the shape of cholesterol. The current study was designed to investigate the possibility that this folding of free mycolic acids could lead to a "cholesteroid" shape which would either allow a strong attraction between these two lipids or lead to similar biological effects. In order to investigate this, we have used approaches similar to those employed to suggest molecular mimicry between microbial and self structures in autoimmune diseases. These are based on the assumption that the specific molecular recognition of two substances by an established binding agent indicates resemblance in the three-dimensional structure of the two compounds (eg. Prendergast *et al.* 1998). Therefore we further investigated the interaction with mycolic acids and cholesterol of sera from human TB patients, using the ELISA technique described by Schleicher *et al.* (2002), and compared the interaction of these two lipids with Amphotericin B (AmB), a known cholesterol-binding molecule (Baginski *et al.* 2002; Baginski *et al*. 2005). Finally, the attraction between cholesterol and mycolic acids was studied. These interactions were examined using a resonant mirror biosensor (Cush *et al.* 1993, Buckle *et al.* 1993, Athanassopoulo *et al.* 1999).

2. Material 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 nm and 366 nm). Flash column chromatography was performed using Merck silica gel. FT-IR was obtained with a Perkin Elmer BX-1 instrument of 2.00 cm^{-1} resolution fitted with a MIR source, an internal LiT aO3 detector and FT-IR Spectrum Software version 5.1. Optical rotations were measured on a Perkin Elmer model 341 polarimeter. For mycolic acids analyses HPLC was performed using a Merck Hitachi Chromatograph fitted with a Phenomenex Luna 5µ 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 C-18 reverse phase column (150 x 4.6 mm) was used for optimization of methods and a Phenomenex Luna C-18 reverse phase column (250 x 10 mm, 10 μ) was used to collect the product. Electrospray mass spectrometry performed on a Micromass Micro Triple quadrupole mass spectrometer in full scan mode, range 500 to 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 ul/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 literature (Steck *et al.* 1978; Watanabe *et al.* 2001).

To form the methyl esters, mycolic acids (**2**, 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 minutes. The mixture was stirred for 72 hr, 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 x 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 literature for methyl mycolic acids (Watanabe *et al.* 2001; Al Dulayymi *et al.* 2003; Al Dulayymi *et al.* 2007; Koza *et al.* 2007).

The synthetic protected alpha mycolic acid (**3**) was kindly provided by Al Dulayymi (Al Dulayymi *et al.* 2003; Al Dulayymi *et al.* 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 (compounds) salts were from Sigma.

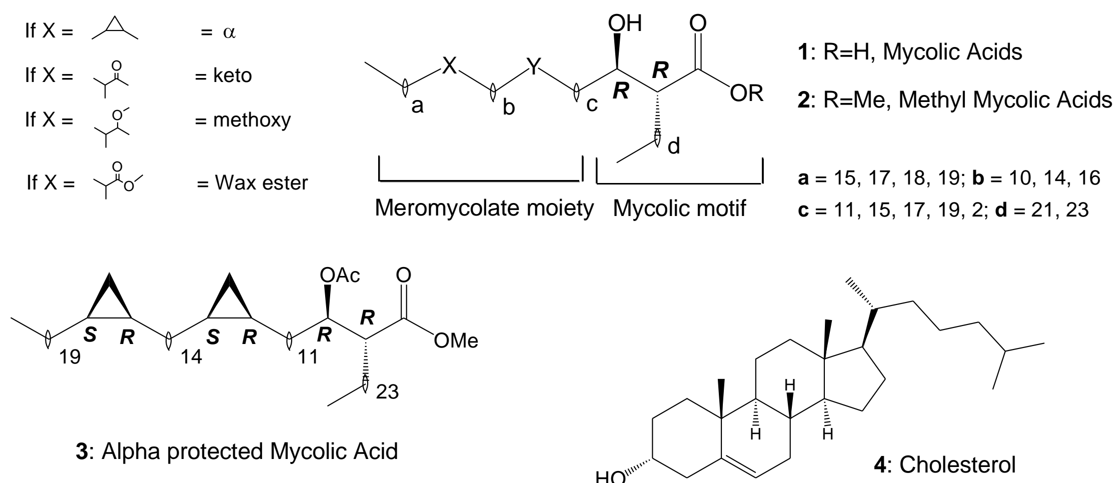
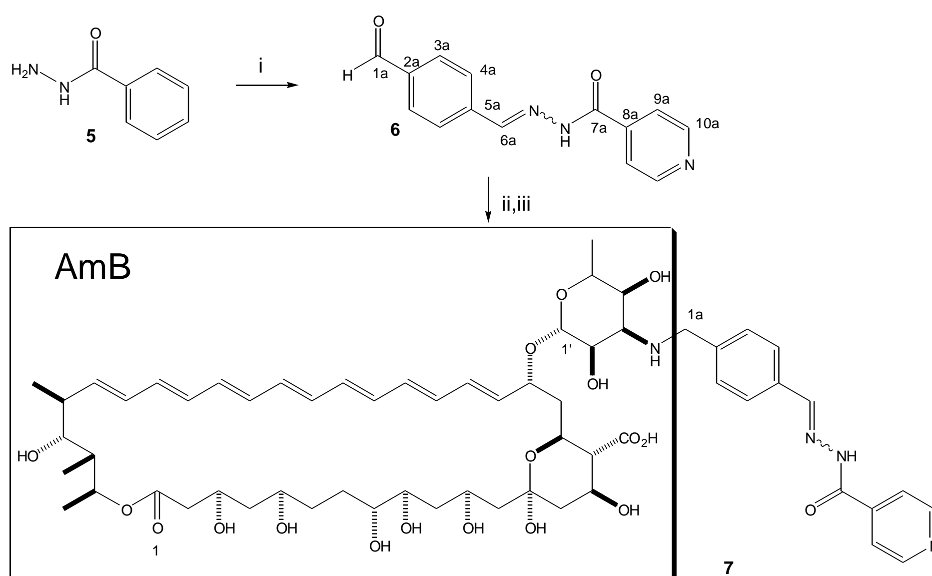


Fig. 1: Molecules under investigation for their similarity in function

2.3. Synthesis of Amphotericin B derivative



Scheme 1: Preparation of an AmB derivative: i- terephthalaldehyde, ii-AmB, iii-NaBH$_4$

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. 219-220ºC; IR ν_{max} 3465, 3197 (NH), 1698 (CHO) cm^{-1}. ^{1}H 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 (100), 79 (10), 78 (47), 51 (22).

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) was 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}, melting point not determined due to decomposition of compound at about 40 ˚C, $[\alpha]^{20}_D$ +328 (*c* 2.0, DMF), IR ν_{max} 3391, (NH, OH ‘s), 1564 (double bonds) cm^{-1}. ^{1}H 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, Durban, KwaZulu-Natal and donated by Dr PB 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 (**1**), synthetic protected α-mycolic acid (**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 minutes 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 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 synthetic protected mycolic acid (**3**) liposomes contained MA: PC (1 mg: 9 mg), whereas the cholesterol (Chol, **4**) liposomes consisted of 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 minutes 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 minutes and then sonified as above. The final liposome concentration came to 500 µg/ml. The liposome suspensions were analysed for their mycolic acids 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

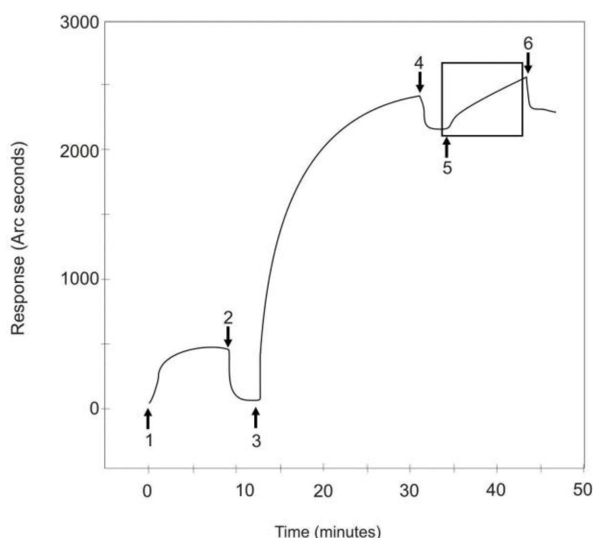


Fig. 2: Biosensor activity sequence to obtain sensorgrams of the binding of cholesterol from cholesterol containing liposomes onto various immobilized lipid-containing liposomes. 1 - Activation of surface with CPC, 2 - PBS/AE wash step, 3 - Addition of liposomes for coating, 4 - PBS/AE wash step, 5 -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

The binding interactions among Amphotericin B, cholesterol, mycolic acids and protected α-MA 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 ^{0}C and stirring rate of 75 % for all experiments on the biosensor. The cells of the cuvette were rinsed with ethanol (95 %) three times prior to use, followed by extensive washing with PBS/AE. A 60 µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 minute. 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 minutes. This was followed by washing five 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 protected MA, or PC only) containing liposomes to the surface for 20 minutes. 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 minutes to achieve a stable baseline. An addition of either 25 µl liposomes under investigation or 25 µl of a solution of AmB (1 x 10^{-4} M) or the AmB derivative (**7 Scheme 1**, 1 x 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 minutes, 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 one minute, followed by 7 times washing with 70 µl PBS/AE for 1 minute. The surface was then finally treated for 5 times with 50 µl potassium hydroxide (12.5 M) for 2 minutes, followed by 7 times washing with 70 µl PBS/AE for 1 minute. The results obtained were analysed for statistical differences using the student 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 synthetic α-mycolic acid (3) containing liposomes using the same method as described above and illustrated in Fig. 2, 25 µl cholesterol-containing liposomes were added. Direct interaction between the immobilized mycolic acids and cholesterol was monitored for 10 minutes, 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 can not 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) proved 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 proven 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 MA 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 the "W" structural arrangement (Villeneuve *et al.* 2005; Villeneuve *et al*. 2007). Therefore, this change in the three dimensional structure may be hypothesised to preclude specific interactions with antibodies against MAs 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.

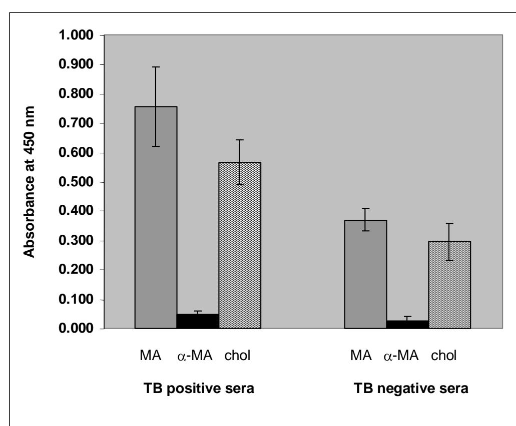


Fig. 3. Normalised ELISA results of TB positive (n = 11) and TB negative sera (n = 5) against natural mycolic acids (1) (MA, grey), cholesterol (4) (Chol, hatched) and a protected, synthetic α-mycolic acid (3) (α-MA, black). The error bars indicate the standard error of the mean.

As expected there was a tendency for TB positive patient antibodies to bind more strongly to MA 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**, $P < 0.0001$). Strikingly, 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 MA 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 fashion either cholesterol or mycolic acids. Such congruence of the titres of both antibody specificities is most easily explained by assuming cross-reactivity for recognition of cholesterol and MA.

3.2. AmB cross-reactivity to mycolic acids and cholesterol

To further assess the proposed resemblance in the nature of mycolic acid and cholesterol, an alternative experiment was designed using Amphotericin B (AmB) as a cholesterol specific ligand. AmB is a macrolide antibiotic that exerts its antifungal activity by binding to ergosterol (Baginski *et al.* 2002). The major interaction between the macrolide and the sterol is based on the formation of a hydrogen bond between the amino group of AmB and the hydroxyl group of ergosterol (Herve *et al.* 1989). 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 tail

chain of ergosterol (which has an extra double bond and methyl group compared to that of cholesterol), making the Van der Waals interaction with the hydrophobic part of AmB stronger (Readio *et al.* 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. For this reason, AmB was selected to assess its binding activity to cholesterol and to MA 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).

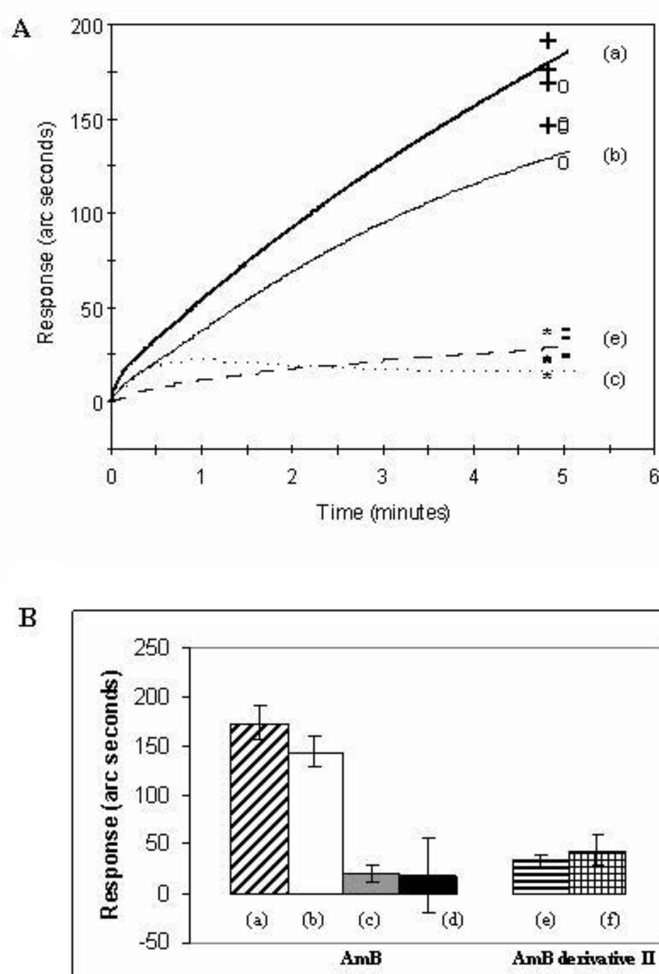


Fig. 4. Resonant mirror biosensor binding curves (Fig. 4A) and binding capacity (Fig. 4B) of AmB or AmB derivative (7) on immobilized lipid antigens. Binding of AmB on (a) mycolic acids (1) (thick line, +, hatched bar); (b) cholesterol (thin line, o, white bar); (c) synthetic protected α-MA (3) (dotted 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.

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 frequently employed cationic, amphipatic 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**). Apparently, phosphatidylcholine, which does not have a hydroxyl group to display, cannot interact with AmB sufficiently to register any significant degree of binding. Moreover, 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**). This modification to the amino group of the AmB molecule destroyed the latter's ability to bind to cholesterol, showing that the attraction of AmB for a lipid 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 importance of the hydrogen bond for a stable interaction (Baginski *et al.* 2002).

Remarkably, a binding sensorgram comparable to that of immobilized cholesterol was obtained when AmB was allowed to interact with immobilized mycolic acids. The mycolic acid binding was similarly abrogated by the covalent modification of the AmB macrolide amino group. This suggests that the interaction between AmB and mycolic acids could also be based on the formation of a hydrogen bond with the AmB amino group

AmB did not bind to the synthetic protected alpha-mycolic acid (**3**) immobilised 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 MA 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 MA and cholesterol would also lead to their interacting directly, the interaction between mycolic acids and cholesterol liposomes was analysed using the biosensor technique.

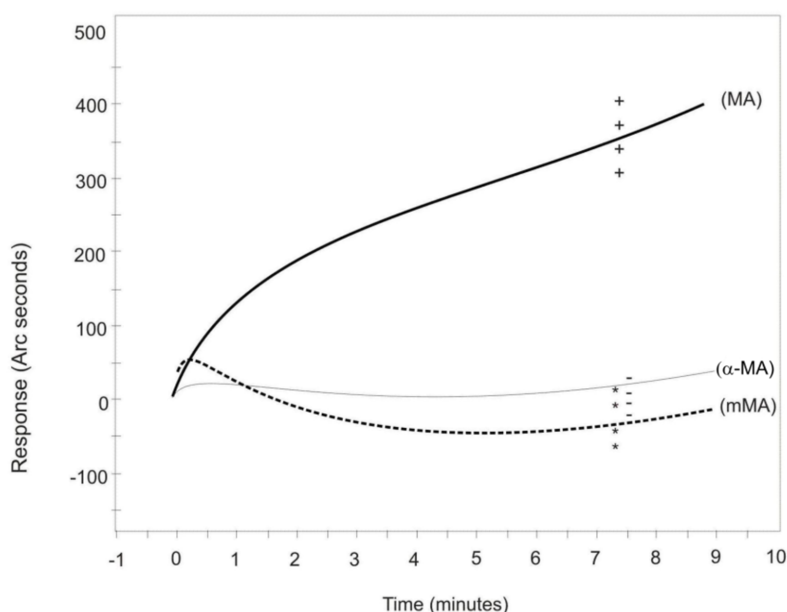


Fig. 5 Resonant mirror biosensor binding curves for the attraction of cholesterol to immobilized mycolic acids (1) (MA, thick line / +), their methyl esters (2) (mMA, dashed line / *) or synthetic protected alpha-mycolic acid (3) (α-MA, thin line / -). Each line represents a typical curve of five repeats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

Following coating of the cuvette surface with either MA (**1**), mMA (**2**) or synthetic protected α-mycolic acid (**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 (mMA), 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 MA. 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 *2R,3R* 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, Durand *et al.* 1979b). Therefore, the use of methyl mycolic acids as a negative control is particularly important in this experiment because this modification will destroy this specific hydrogen bond and therefore can be expected to destabilise the formation of the "W" conformation.

Fig. 5 shows that the MA-liposomes coated cuvette surface accumulated cholesterol from the solution while the methyl esters of MA (**2**) and the synthetic, protected alpha MA (**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 binding to either MA or protected α-MA, $P < 0.001$).

These results demonstrate a pronounced attraction between cholesterol and MA that is determined by the presence of a carboxylic acid group, and the degree of structural fit and particular conformation that free mycolic acids assume and that can be eliminated by formation of a simple methyl ester of the acid (**Fig. 6**).

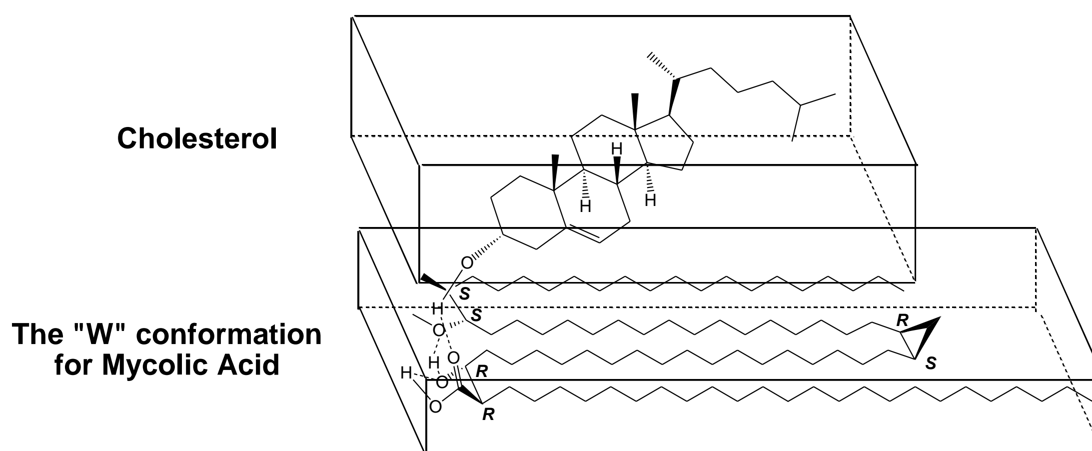


Fig. 6: The hypothesised mechanism of interaction between "W"-folded MA (Villeneuve *et al.* 2007) and cholesterol. The absolute stereochemistry of the cyclopropane and the methoxy group are not completely clarified. Here, for simplicity of explanation, the S,R stereochemistry has been used for the cyclopropyl group and the SS for the methoxy-group. The RR stereochemistry is known to be that of the mycolic motif.

4.Conclusion

Observing that both human TB sera and AmB are able to distinguish natural occurring MA (**1**) from a closely related chemically synthetic structure such as protected alpha-MA (**3**), and that both MA and cholesterol are recognised by AmB, led to the hypothesis of a similarity in nature between the mycobacterial fatty acids and the sterol. This was further supported by analysing the specific interaction of MA with cholesterol.

The association of both AmB and cholesterol to mycolic acid was shown to depend on the formation of hydrogen bonds that also affect the structural rigidity of the molecules. The attraction between MA and cholesterol in liposomes was shown to be specific, probably depending on a particular conformation that free mycolic acids assume in the phospholipid bilayer of liposomes, and this will probably also apply to biological membranes.

The existence of a condensed conformation for mycolic acids has already been proposed by several groups, based on Langmuir studies. Here we present the first evidence that this conformation 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. 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). In addition, it impacts on the observation that mycolic acids, intraperitoneally or intratracheally administered as liposomes in mice, act as a pathogen associated molecular pattern of innate immunity that affect mainly the macrophages and convert them into cholesterol-rich foam cells (Korf *et al.* 2005). When administered intratracheally, MA 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 the conversion of macrophages into foam-like cells with the ability to influence the manifestation of asthma in mice. Finally, cholesterol has been shown to play an important role in the entry and survival of *M. tuberculosis* in the host macrophage (Gatfield, *et al.* 2000, de Chastellier, *et al.* 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.

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