RESEARCH LETTER

Antibacterial activity of long-chain fatty alcohols against mycobacteria

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Abstract

Tuberculosis is caused by the bacterium *Mycobacterium tuberculosis* and results in innumerable deaths across the world. The emergence of multidrug-resistant and extremely drug-resistant tuberculosis strains and its coinfection with HIV has made tuberculosis more difficult to treat. Therefore, new antimycobacterial agent(s) for both therapy and disinfection are urgently required. In this context the present study describes the antibacterial property of long-chain fatty alcohols against mycobacteria. The antimycobacterial activities of alcohols with chain length ranging from C_5 to C_{13} were examined against *Mycobacterium smegmatis* mc^2155 and *M. tuberculosis* $H_{37}R_v$. The best activity was found with one with a C_{10} chain length. This bactericidal activity can partly be attributed to its ability to damage the robust and complex cell envelope of *Mycobacteria*. Moreover, our study reveals the ability of decanol to attenuate biofilm formation by *M. smegmatis*. This knowledge can be used to develop new therapeutics and disinfectants against mycobacteria.

Introduction

Tuberculosis is caused by *Mycobacterium tuberculosis* and results in innumerable deaths worldwide. *Mycobacterium tuberculosis* is highly infectious and is able to establish persistent infection in individuals even with a healthy immune system and thus has infected more than one-third of the world's population. The emergence of multi-drug-resistant (MDR) and extremely drug-resistant tuberculosis strains (XDR) and its coinfection with HIV has made tuberculosis more difficult to treat (Global tuberculosis control, full report, 2011). The search for antimycobacterial agents for both therapy and disinfection is therefore now of major research interest across the world. In this regard, research describing new anti-mycobcaterial agents from natural and synthetic sources is being reported with different target sites and mode of actions (Koyama *et al.*, 2010; Kakwani *et al.*, 2011; Lougheed *et al.*, 2011).

Different types of alcohols, their derivatives and some lipophilic or amphiphilic compounds are known to exhibit antimycobacterial activity (Júnior *et al.*, 2009; Rugutt & Rugutt, 2011; Falkinham *et al.*, 2012). Among the different class of alcohols, aliphatic alcohols are the most widespread compounds occurring naturally in plants and foods. There have been a number of reports on the antibacterial activities of long-chain fatty alcohols (Lansford *et al.*, 1960; Sheu & Freese, 1973; Mates, 1974; Kato *et al.*, 1978; Kato & Shibasaki, 1980; Kubo *et al.*, 1993a, b; Tanaka *et al.*, 2002; Kabelitz *et al.*, 2003; Togashi *et al.*, 2007). These studies showed that the antimicrobial activity is influenced by the number of carbon atoms present in the alkanol chain and can be modulated by the presence of an effective head group that alters its polarity. The presence of double or triple bonds and their position can also play a crucial role in determining their antimicrobial activities (Ravel *et al.*, 1955; Lansford *et al.*, 1960; Sheu & Freese, 1973; Mates, 1974; Kabelitz *et al.*, 2003). In addition, the type of organism and its cell-wall composition also influence the anti-microbial activity of a particular agent. However, no antimyobacterial activity of these long-chain fatty alcohols has previously been reported.

In this context, in the present study we have investigated the effect of long-chain fatty alcohols against

Mycobacterium smegmatis mc^{2}155 and *M. tuberculosis* $H_{37}R_v$. Our study revealed that fatty alcohols with carbon number 7–10 are capable of exhibiting considerable antimycobacterial activity. Among them, 1-decanol (C_{10}) showed highest activity against both *M. smegmatis* and *M. tuberculosis*. In addition, the current study also shows that the presence of a terminal double bond in a fatty alcohol potentiates its antimycobacterial activity. This antimycobacterial activity of the alcohols was found to be partly, if not exclusively, due to damage to the cellular envelope. The ability of 1-decanol and 9-decene-1-ol to attenuate biofilm formation by *M. smegmatis* was also investigated.

Material and methods

Chemicals, strains and media

All the alkanes, alkanols and alkene-1-ol used in this study were purchased from Sigma-Aldrich (St Louis, MO). *Mycobacterium smegmatis* mc^{2}155 (ATCC 700084) and *M. tuberculosis* $H_{37}R_v$ (ATCC 25618) used in this study were a kind gift from Prof. Sujoy Dasgupta, Bose Institute, Kolkata, India, and Prof. N. K. Pal, IPGMER, Kolkata, India. Middlebrook 7H9 broth base supplemented with glycerol and bovine serum albumin was used for cultivation of *M. smegmatis* and Kirchner's broth supplemented with antibiotic cocktail Polymyxin B, Amphotericin B, Carbenicillin, Trimethoprim was used for cultivation of *M. tuberculosis*. The medium contains phenol red as a pH indicator that turns yellow from pink upon growth of the *M. tuberculosis*.

Agar diffusion assay

Preliminary assessment for antimycobacterial activity of long-chain fatty alcohols was done by agar diffusion method as described previously (Bauer *et al.*, 1966). Briefly, paper discs of 4 mm in diameter soaked with 3 μL of each alcohol were placed on agar plates overlaid with soft agar (0.6%) that was inoculated with *M. smegmatis* mc^{2}155. Plates were incubated for 48 h at 37 °C. The extent of inhibition was measured by the diameter of the zone of inhibition created around the disc.

Determination of minimum inhibitory concentration by broth dilution shaking (BDS)

The BDS method was performed as described previously (Charles, 1974). Briefly the compound to be tested was dissolved at a concentration of 8 mg mL^{-1} in 70% dimethyl sulfoxide and was further diluted twofold in each consecutive test tube in either Middlebrook 7H9 broth (Difco, Detroit, MI) for *M. smegmatis* mc^{2}155 or in Kirchner's broth for *M. tuberculosis* $H_{37}R_v$. An aliquot (10 μL) of an overnight culture of either *M. smegmatis* mc^2 155 or *M. tuberculosis* $H_{37}R_v$ (ca. 1×10^5 CFU mL^{-1}) were added to each tube. Each culture was incubated at 37 °C for 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which there was no visible growth of the bacteria after 48 h of incubation. *Mycobacterium tuberculosis* growth in Kirchner's medium is indicated by the change in colour from pink to yellow due to pH change of the medium by acid produced during growth of *M. tuberculosis*. The minimum concentration of agent at which no colour change of the growth medium was observed was designated as the MIC.

Atomic force microscopy (AFM)

Mycobacterium smegmatis mc^{2}155 cells were grown to log phase and either treated with 0.8 mM of decanol for 2 h or left untreated. Cells were smeared on a glass cover slip, dried in air for 30 min and examined under AFM (Veeco, Singapore). All images were obtained with a scan speed of 1.0 Hz and a resolution of 512 × 512 pixels.

Dual staining for assessment of loss of membrane integrity

Mycobacterium smegmatis culture was grown for 14–16 h at 37 °C and the culture was either treated with 0.8 mM of decanol for 2 h or left untreated. Cells were harvested, washed with phosphate-buffered saline (PBS) and treated with 4 μg mL^{-1} acridine orange for 15 min. Thereafter, cells were washed with PBS and treated with 4 μg mL^{-1} ethidium bromide. Cells were viewed under a fluorescence microscope.

Assay for biofilm formation

Each well of a six-well polysterene Petri dish of 9.6 cm^2 was poured with 2 mL of Middlebrook 7H9 medium. Each well was inoculated with *M. smegmatis* mc^{2}155 culture grown for 48 h (10^6 CFU mL^{-1}) and incubated at 37 °C for 4 days to allow biofilm formation. Thereafter, planktonic cells were pipetted out carefully and the adhered biofilm was stained with 4 μg mL^{-1} of acridine orange in PBS for 15 min and viewed under a fluorescence microscope.

For crystal violet (CV) assay, 200 μL of a saturated culture of *M. smegmatis* was added to each well of a 96-well plate and incubated at 37 °C 48 h. Thereafter, culture broths from the wells were discarded. Wells were washed

with mQ water and to each well, 200 μL of 0.4% CV was added. CV was allowed to adsorb to the biofilm components for 15 min at room temperature. Next, each well was washed with mQ water to remove any unadsorbed CV from the wells. Then, 33% acetic acid was added to dissolve the CV adsorbed to the biofilm and the amount was measured by determining its absorbance in a microplate reader at 630 nm (Molecular Devices, Sunnyvale, CA).

Results and discussion

1-Alkanols with carbon number C_7–C_{10} show considerable antimycobacterial activity

Long-chain fatty alcohols are long known to exhibit antimicrobial activity. To test the activity of long-chain fatty alcohols against mycobacteria, primarily the antimycobacterial activity of alcohols containing 5–13 carbons in their chain were assessed by the disc diffusion method in an agar plate against *M. smegmatis* as described. The radius of zone of inhibition increased almost linearly with the number of carbon atoms in the chain from 1-hexanol to 1-decanol (Fig. 1a). Alkanols with more than 10 carbon atoms showed a drastic reduction in activity (Fig. 1a). In contrast, long-chain hydrocarbons starting from *n*-hexane to *n*-decane showed no inhibitory action against *M. smegmatis*.

Alcohols with a different number of carbon molecules starting from pentanol to tridecanol show not only a wide range of molecular weight but also a variable degree of polarity. The ability to diffuse in the agar plate depends strongly on their polarity, viscosity and other physical properties, and thus can influence its antimicrobial activity in a plate assay. To overcome solubility and diffusion problems of different alcohols with the agar diffusion method the alcohols were solubilized in a universal solvent such as DMSO (70%) and subjected to determination of MIC by the BDS method. Table 1 summarizes the antimicrobial activities of long-chain fatty alcohols on *M. smegmatis* mc^2155 and *M. tuberculosis* $H_{37}R_v$ as determined by the BDS method. Consistent with the agar diffusion method, the results from BDS showed that fatty alcohols with carbon number 7–10 possess considerable antimycobacterial activity, and among which decanol is the most potent candidate with an MIC of 0.4 mM (Table 1). In addition, results from BDS also showed no or very little antimycobacterial activity for long-chain fatty alcohols with an aliphatic carbon chain containing fewer than seven and more than 11 carbon atoms.

To establish the relationship between the lipophilicity of the alkanols and their antimycobacterial activity we plotted MIC values against the corresponding log *P* (water/octanol partition coefficient) value (Table 1).

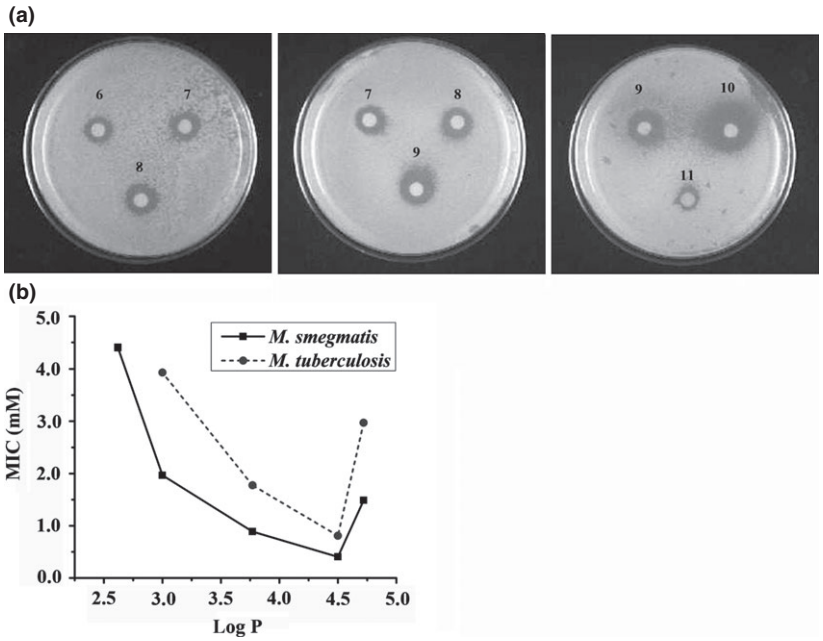

Fig. 1. Antimycobacterial activity of long-chain fatty alcohols. (a) Agar diffusion assay. Paper discs soaked with 3 μL of alcohols (containing an equal amount of alcohol by weight) with different chain length were placed on *Mycobacterium smegmatis* lawn made on Middlebrook 7H9 agar plate. The number given near each disc denotes the number of carbon atoms in the 1-alkanols tested. Antimycobacterial activity was assessed by the radius of zone of inhibition created around the disc. (b) Plot of MIC vs. lipophilicity. MIC values calculated in mM from Table 1 were plotted against log *P* values of each alcohol.

Table 1. MIC of long-chain alcohols against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* by the BDS method

Alcohol	log $P_{o/w}$	MIC (mM) against *M. smegmatis* mc^{2}155	MIC (mM) against *M. tuberculosis* $H_{37}R_v$
1-Hexanol	1.82	ND	ND
1-Heptanol	2.62*	4.40	ND
1-Octanol	3.0	1.96	3.93
1-Nonanol	3.77	0.88	1.77
1-Decanol	4.5	0.40	0.80
1-Undecanol	4.72	1.48	2.97
1-Dodecanol	5.13	ND	ND
1-Tridecanol	5.4	ND	ND

ND, not detected at the concentration tested.
*Value obtained from the Pubchem compound database (NCBI). All other log *P* values were obtained from the manufacturer's website (Sigma-Aldrich).

The result revealed a marked reduction of MIC for alkanols with an increase in lipophilicity up to decanol. A further increase in carbon number resulted in a very sharp increase in MIC (Fig. 1b), with no toxicity with 1-dodecanol and 1-tridecanol. It appears that these alcohols with high lipophilicity should be taken up preferentially by the membrane, but possibly due to their poor partition coefficient from water to the membrane ($P_{M/W}$) (De Bont Jan, 1998) they failed to reach higher membrane concentration, thereby resulting in low toxicity. On the other hand, smaller alkanols did not show higher toxicity as expected from their high $P_{M/W}$. The reason of this disparity may lie in the fact that partitioning into the membrane does not depend solely on the value of the $P_{M/W}$ coefficient, but also on the cell-wall composition of the organism. In our case it could be the unique cell-wall composition of the mycobacteria that did not allow smaller alkanols to accumulate in the membrane at a toxic concentration. Therefore, both the partition coefficient of the alkanol between water and membrane and the cell-wall composition of a particular organism will determine the extent of accumulation of the agent in the membrane and thus determine toxicity.

Unsaturation in the alkanol chain modulates antimycobacterial activity

Naturally available alcohols often occur with unsaturations at different positions of the alkanol chains. To verify if unsaturation has any influence on antimycobacterial activity, we used decanol as it showed maximum activity against mycobacteria and compared its activity with its alkene and alkene-1-ol counterparts, i.e. 1-decene and 9-decene-1-ol. The results showed that 9-decene-1-ol has greater activity than decanol and 1-decene has no activity against both *M. smegmatis* and *M. tuberculosis* (Table 2). These data are also true for other alcohols with moderate antimycobacterial activity; for example, hexene-1-ol exhibits greater activity than hexanol and hexene shows no activity (Table 2). These results suggest that a long-chain aliphatic hydrocarbon and a hydrocarbon with only a terminal double bond were completely inactive against mycobacteria. However, the presence of a terminal double bond along with a hydroxyl group provided greater activity against mycobacteria. This result indicates that hydrophobicity of an agent is not a sufficient condition for exhibiting antimycobacterial activity but that it requires a hydrophobic–hydrophilic balance. With both the hydroxyl group and terminal unsaturation, the hydrophobic–hydrophilic balance is better than the respective alkanol and thus shows greater activity.

The extremely low permeability of the mycobacterial cell wall is known to be a major factor that contributes towards its intrinsic resistance to several disinfectants and chemotherapeutics. Hydrophilic agents diffuse poorly through the mycobacterial cell wall because the mycobacterial porin is inefficient in allowing the passage of hydrophilic solutes and also because they exist at low concentration. Again, the lipophilic compounds are slowed by the complex fatty acid and unique glyoclipid content of the wall and by the lipid bilayer (Jarlier & Nikaido, 1994). Thus, it can be expected that a compound with perfect amphiphilic balance will be effective in inserting itself into such a cell-wall structure.

Decanol causes damage to cell envelope structure

Previous studies have shown that long-chain fatty alcohols exert their antimicrobial activity by nonspecifically damaging the cellular envelope and thus perturbing the ion homeostasis across the membrane (Ingram, 1976; Sikkema *et al.*, 1995; Togashi *et al.*, 2007). In our case the alcohol treatment may also cause damage to the mycobacterial cell envelop. To test this hypothesis, the loss of *M. smegmatis* membrane integrity upon alcohol treatment was assessed by dual staining with acridine

Table 2. Comparison of MIC values of alkene-1-ol with its respective alkanols against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*

Alcohol	MIC (mM) against *M. smegmatis* mc^{2}155	MIC (mM) against *M. tuberculosis* $H_{37}R_v$
1-Hexanol	ND	ND
5-Hexene-1-ol	1.28	2.32
1-Decanol	0.4	0.8
9-Decene-1-ol	0.20	0.40

ND, not detected at the concentration tested.

orange and ethidium bromide. The assay is based on the principle that neutral dyes such as acridine orange can enter passively into both live cells with an intact membrane and dead cells with a damaged membrane, whereas charged dyes such as ethidium bromide are unable to diffuse through the intact cell membrane and thus can enter only cells that have lost membrane functionality. Our result showed a large number of cells stained with ethidium bromide when a log phase culture was treated with 0.8 mM (twofold higher than the MIC) decanol for 2 and 4 h and viewed under a fluorescence microscope. The total microbial population either dead or alive was stained with acridine orange and only the dead cells with damaged membrane were stained with ethidium bromide. Orange cells in the merged picture indicate the number of dead cells in the total population. From Fig. 2a it is evident that the number of dead cells with a damaged membrane increased in the population with the time of alcohol treatment. Therefore, this result suggests a considerable loss of membrane integrity of *M. smegmatis* on alcohol treatment. To further confirm this result, we have also performed AFM analysis of *M. smegmatis* treated with alcohol. While the untreated cells exhibit a smooth envelope structure, disruption of cellular envelope at several different locations (indicated by arrowhead) was observed for cells treated with 1-decanol (Fig. 2b). Moreover, in untreated samples most of the fields were occupied with mycobacteria that adhered and clustered together. This is a normal tendency of biofilm-forming bacteria such as mycobacteria. On treatment with alcohol, most of the bacteria lose their cell shape and morphology and as a consequence remain unattached and occur mostly as single cells. Thus, the growth inhibitory activity of decanol can be attributed partly, if not exclusively, to its ability to damage the cellular envelope. Perhaps this damage is a result of the well-known event of accumulation of alkanols in the membrane thus affecting the general membrane functions.

Decanol and 9-decene-1-ol inhibits biofilm formation by *M. smegmatis*

Biofilm formation in many cases is important for bacterial virulence and survival (Parsek & Singh, 2003). So a successful attenuation of biofilm formation can be of wide interest for the management of disease progression and elimination of the pathogen. An intact cellular envelope and its hydrophobicity helps in cell to cell adhesion and thus promotes biofilm formation in microorganisms such as mycobacteria. Thus, any damage to the cell envelope may hinder its ability to adhere to each other and subsequently inhibits biofilm formation. In this context we have assessed the ability of long-chain fatty alcohols in biofilm formation by performing CV assay and acridine orange staining of the biofilm. Interestingly, our result showed that decanol concentrations of 0.1 and 0.2 mM, far lower than its MIC (0.4 mM), were able to attenuate biofilm formation (Fig. 3a). Furthermore, the quantitative CV assay also revealed that 9-decene-1-ol concentrations of 0.05 and 0.1 mM, again lower than its MIC (0.2 mM), were able to attenuate biofilm formation considerably (Fig. 3b). The same concentration of the alcohols tested had no effect on planktonic growth as measured by $OD_{600\ nm}$. These results clearly suggest that a sublethal

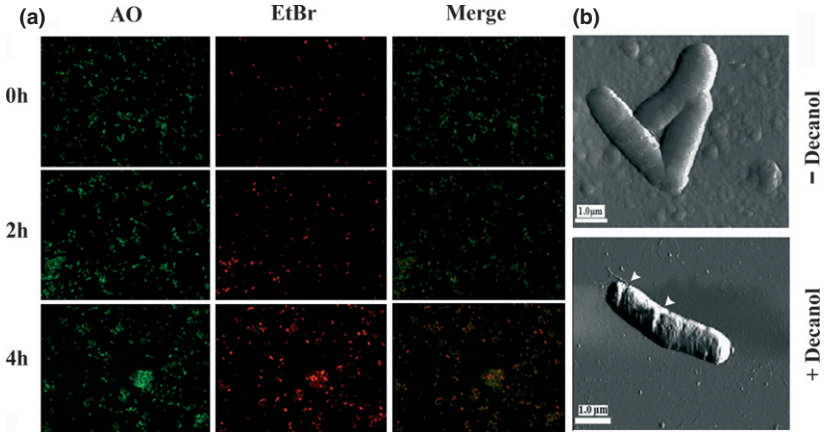

Fig. 2. Alcohols damage the cell envelop of *Mycobacterium smegmatis*. *Mycobacterium smegmatis* cells were grown to log phase and treated with 0.8 mM decanol for 2 h. (a) Cells were stained with ethidium bromide and acridine orange and viewed under the fluorescence microscope. The figure is representative of images obtained from 20 different fields for each group and from three independent experiments. (b) AFM image of cells untreated (above) and treated with decanol (below). The figure is representative of images obtained from 15 different fields for each group and from three independent experiments.

dose of both 1-decanol and 9-decene-1-ol is able to attenuate biofilm formation *in vitro*. This inhibition may result from the ability of these agents to damage the cellular envelope and thus in turn perturb the cell to cell adhesion, which is a key factor in biofilm formation. Exploring new agents that can attenuate biofilm formation and insight into the mechanism involved may shed light into therapeutic strategies for infections with microbes such as mycobacteria whose pathogenic potential strongly depends on successful biofilm formation within the host.

Surface active agents such as surfactants and other membrane-damaging compounds are drawing significant attention in the field of antimicrobial chemotherapy.

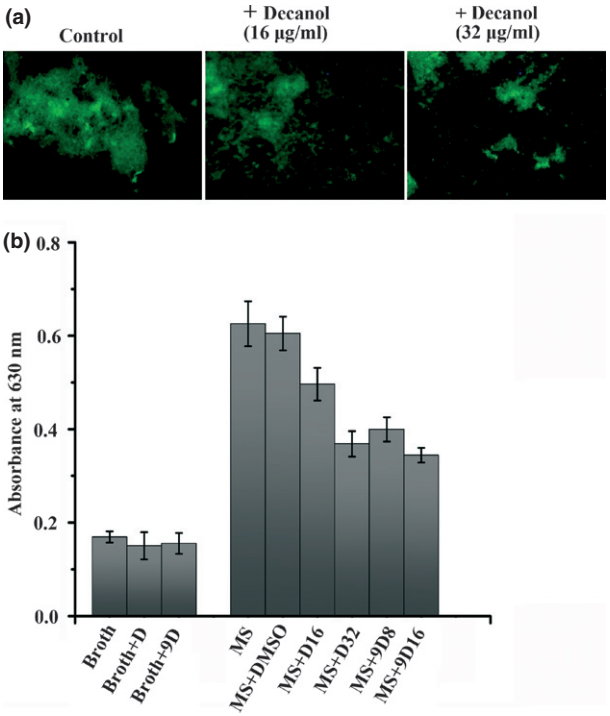

Fig. 3. Decanol attenuates biofilm formation of *Mycobacterium smegmatis*. (a) Micrograph of *M. smegmatis* biofilm. *Mycobacterium smegmatis* was grown in Middlebrook 7H9 medium either containing only DMSO (Control) or decanol ((0.1 and 0.2 mM)) in six-well polystyrene plates for 4 days at 37 °C. Biofilms formed were stained with acridine orange and viewed under a fluorescence microscope. The figure is representative of images obtained from 15 different fields for each group and from three independent experiments. (b) CV assay. In 96-well microtitre plates overnight cultures of *M. smegmatis* in Middlebrook 7H9 broth was added in four sets at 200 µL. One set was left untreated (MS) and two other sets were respectively treated with 0.1 and 0.2 mM 1-decanol (MS + D16 and MS + D32) or with 0.05 and 0.1 mM 9-decene-1-ol (MS + 9D8 and MS + 9D16). Another set was treated with vehicle control, i.e. DMSO (MS + DMSO). Wells with only media (Broth), media with drugs (Broth + D and Broth + 9D) served as controls for abiotic factors. Five replicates of each experimental set were maintained.

Drugs such as daptomycin clofazimine derivatives that are known to disrupt membrane integrity are already being used either clinically or are at the final stage of drug development (Adams *et al.*, 1999; Pogliano *et al.*, 2012). Membrane active agents generally have multiple target sites and diverse modes of action against the organism, reducing the chance of mutation at the target site (Andries *et al.*, 2005; Koul *et al.*, 2008). Besides perturbation in membrane integrity, these agents also interfere with some important membrane functions such as cell–cell adhesion or substrate attachment that are key factors in events like biofilm formation (Hurdle *et al.*, 2011). Thus, membrane active agents at sublethal dose are often found to inhibit biofilm formation and thus reduce infection. Consistent with this idea, we have shown here the inhibitory effect of both the alcohols tested against biofilm formation by *M. smegmatis*. Given its toxicity to mammalian cells and its broad spectrum of target sites, exploring selective membrane active agents may provide a platform for future drug designs.

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