Direct Growth Inhibitory Effect of Platelet Activating Factor C-16 and its Structural Analogues on Mycobacteria

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Abstract

*Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis, is one of the leading causes of human deaths due to a single infectious agent. *M. tuberculosis* infection of the host initiates a local inflammatory response, resulting in the production of a range of inflammatory factors at the site of infection. These inflammatory factors may come in direct contact with *M. tuberculosis* and immune cells to activate different signalling pathways. One such factor produced in excess during inflammation is a phospholipid compound, Platelet Activating Factor C-16 (PAF C-16). In this study, PAF C-16 was shown to have a direct inhibitory effect on the growth of *Mycobacterium bovis BCG* (*M. bovis BCG*) and *Mycobacterium smegmatis* (*M. smegmatis*) in a dose and time-dependent manner. Use of a range of PAF C-16 structural analogues, including the precursor form Lyso-PAF, revealed that small modifications in the structure of PAF C-16 did not alter its mycobacterial growth inhibitory properties. Subsequent experiments suggested that the attachment of aliphatic carbon tail via ether bond to the glycerol backbone of PAF C-16 was likely to play a vital role in its growth inhibition ability against mycobacteria. Fluorescence microscopy and flow cytometry using Propidium iodide (PI) indicated that PAF C-16 treatment had a damaging effect on the cell membrane of *M. bovis BCG* and *M. smegmatis*. Furthermore, the growth inhibitory effect of PAF C-16 was partially mitigated by treatment with membrane-stabilizing agents, α-tocopherol and Tween-80, which further suggests that the growth inhibitory effect of PAF C-16 was mediated through bacterial cell membrane damage.
Introduction

Tuberculosis (TB) is an infectious disease, caused by the acid-fast bacillus, *Mycobacterium tuberculosis* (*M.tb*). According to the World Health Organization (WHO), TB is one of the leading causes of human mortality, resulting in more than 1 million human deaths each year. Approximately 10.4 million new cases of TB were reported worldwide in the year 2016 (WHO, TB report 2017). It is estimated that about one-third of the world’s population (1.7 billion people) is latently infected with *M.tb*, a condition with no active disease symptoms due to containment of the pathogen by the host immune system (Houben and Dodd, 2016). This latent infection with *M.tb* provides a huge reservoir for reactivation into active TB and its spread across the globe. TB continues to be a major health problem due to the non-availability of an effective vaccine. The only preventive vaccine against TB is BCG, which is almost 100 years old and not effective in controlling the spread of *M.tb* infections in economically active adult humans, which comprises the major population of TB patients in developing countries (Lahey and von Reyn, 2016; Aronson *et al*., 2004; Colditz *et al*., 1994; Sepulveda *et al*., 1992). New challenges, such as cases of HIV and *M.tb* co-infection (Daley *et al*., 1992), multidrug-resistant tuberculosis (MDR-TB) (Espinal *et al*., 2000), and extensively drug resistant tuberculosis (XDR-TB) (Liu *et al*., 2011; Jain and Mondal, 2008) have compounded the severity of the problem and therefore, novel therapeutic interventions are required to control TB.

Infection of a host by *M.tb* activates the host’s immune system resulting in a localized inflammatory response (Giacomini *et al*., 2001; Cooper and Flynn, 1995). This causes an increase in blood flow and changes in vascular permeability, resulting in the leakage of several proteinaceous and non-proteinaceous factors and the migration of cellular components from the blood to the site of infection (Clark *et al*., 2007; Sherwood and Toliver-Kinsky 2003). In addition to the plasma-derived factors, complement proteins such as C1q and lipid compounds such as PAF C-16 and Lyso-PAF are also synthesized by inflammatory cells such as macrophages at the site of infection (Kaul and Loos 1995; Camussi *et al*., 1987). These factors are likely to come in contact with the pathogens and different immune cells and thus may modulate the outcome of the infection.

PAF C-16 or PAF-acether, a membrane-derived phospholipid is chemically known as 1-O-alky-2-acetyl-*sn*-glycero-3-phosphocholine. PAF C-16 is normally present in low amounts in human serum (approximately 127pg/ml), however, its concentration increases by
PAF C-16 inhibits mycobacterial growth

6 fold during allergic reactions (Vadas et al., 2013). PAF C-16 is produced by a range of cells, including platelets, neutrophils, macrophages, endothelial and mast cells (Gardiner et al., 1999; Biffl et al., 1996; Leaver et al., 1990; Bussolino et al., 1986; Schleimer et al., 1986; Alam et al., 1983). PAF C-16 is synthesized by two distinct pathways; the remodelling pathway and the de novo synthesis pathway (Prescott et al., 1990). The remodelling pathway is the major PAF C-16 synthesis pathway used by activated inflammatory cells and involves the modification of membrane ether-linked phospholipids by the enzymes PLA$_2$ and acetyl coenzyme A acetyltransferase in a two-step process to produce PAF C-16 (Shindou et al., 2007).

PAF C-16 binds to specific transmembrane G-protein coupled receptors, known as PAF receptor (PAFR), on the plasma membrane of target cells (Ishii et al., 2002; Honda et al., 2002). Binding of PAF C-16 to PAFR results in the activation of different signal transduction mechanisms such as phosphatidylinositol-calcium second messenger system and the activation of different kinases including protein tyrosine kinase, mitogen-activated protein kinases and protein kinase C pathways (Honda et al., 2002).

PAF C-16 is endowed with diverse biological activities including its well-known ability to cause platelet aggregation (Chignard et al., 1979). PAF C-16 also has important roles in inflammatory and allergic responses (Henderson et al., 2000). PAF C-16 induces apoptosis in neuronal cells and epidermal cells (Brewer et al., 2002; Barber et al., 1998), causes the production of reactive oxygen and nitrogen species by macrophages (Borges et al., 2017; Aliberti et al., 1999; Hartung et al., 1983), and plays an important role in angiogenesis (Seo et al., 2004). Unregulated production of PAF C-16 has also been shown to be involved in a number of diseases including multiple sclerosis (Hostettler et al., 2002), thrombosis (Mueller et al., 1995), myocardial infarctions (Montrucchio et al., 2000), rheumatoid arthritis (Hilliquin et al., 1995), bronchial asthma (Cuss et al., 1986), acute pancreatitis and inflammatory bowel disease (Rosam et al., 1986). PAFR antagonists are compounds that bind to PAFR with high affinity and have been shown to successfully inhibit certain pathological effects such as asthma, cardiac and circulatory disorders that are driven by PAF C-16 (Singh et al., 2013).

There is limited information available on the direct antimicrobial activity of PAF C-16. PAF C-16 was shown to inhibit the growth of Gram-positive bacteria in cultures, but not Gram-negative bacteria (Steel et al., 2002). Furthermore, the direct anti-mycobacterial role of
PAF C-16 has not been investigated. In this study, exogenous PAF C-16 and its structural analogues were examined for their direct effect on mycobacterial growth using *M. bovis* BCG (Pasteur 1173P2) and *M. smegmatis* (mc² 155) as models for *M. tb*. We report that PAF C-16 and a number of PAF C-16 structural analogues inhibited the growth of both *M. bovis* BCG and *M. smegmatis*. The active portion of PAF C-16 responsible for mycobacterial growth inhibition and the underlying mechanisms of growth inhibition by PAF C-16 were also investigated.

**Material and Methods**

**Chemicals**

PAF C-16 (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine), Lyso-PAF C-16 (1-O-hexadecyl-2-hydroxy-sn-glyceryl-3-phosphorylcholine), PAF C-18 (1-O-octadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine), Hexanolamino PAF C-16 (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphoryl (N,N,N-trimethyl) hexanolamine), 2-O-methyl PAF C-16 (1-O-hexadecyl-2-O-methyl-sn-glyceryl-3-phosphorylcholine), Pyrrolidino PAF C-16 (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphoryl-N-methyl-pyrrolidinium ethanol) and Miltefosine (1-hexadecylphosphorylcholine) were obtained from Cayman Chemical Company, USA. 1-O-hexadecyl-sn-glycerol (Bachem) was obtained from Cambridge Biosciences UK, Palmitic acid (Hexadecanoic acid), Vitamin E [(±)-α-Tocopherol] and Phosphocholine chloride calcium salt tetrahydrate were purchased from Sigma-Aldrich Company, USA and Hexadecyl lactate was purchased from Santa Cruz Biotechnology, USA. All other chemicals used in the experiments were of analytical grade.

Stock solutions of phospholipids and fatty acids were prepared in ethanol (10mg/ml). Miltefosine was dissolved in PBS (10mg/ml), Hexadecyl lactate in DMSO (10mg/ml) and Phosphocholine chloride calcium salt tetrahydrate in water (10mg/ml). Appropriate solvent controls were always included in all the experiments involving PAF C-16, its structural analogues or any other chemical compound used with bacteria.

**Mycobacterial Strains and growth conditions**

Liquid cultures of *M. smegmatis* (mc² 155) were grown in Luria-Bertani (LB) broth (Lennox; Sigma Aldrich) containing 50μg/ml carbenicillin (Fisher Chemical), 0.2% (v/v) glycerol and 0.2% (v/v) tween-80 in a shaking incubator at 37°C until the O.D₆₀₀nm reached 0.8-0.9. The number of *M. smegmatis* colony forming units (CFUs) per microliter was determined by
plating different dilutions of the bacterial stock on LB agar plates in triplicates and counting the number of CFUs after incubation at 37°C for 72h.

Liquid cultures of *M. bovis* BCG (Pasteur 1173P2) were grown in Middlebrook 7H9 broth (Sigma Aldrich), supplemented with 10% (v/v) albumin dextrose catalase (ADC) and 0.2% (v/v) tween-80. ADC was prepared by dissolving 5g of bovine albumin fraction V (Fisher Chemical), 2g dextrose (Fisher Chemical), 0.85g sodium chloride (Fisher Chemical) and 4mg catalase from bovine liver (Sigma Aldrich) in 100ml of sterile water and the solution was passed through a 0.22µm filter. The growth cultures were kept in a shaking incubator at 37°C for 2-3 weeks until the O.D$_{(600nm)}$ reached 0.8-0.9. The number of CFUs per microliter for stock *M.bovis* BCG was determined by plating different dilutions of the bacterial stock on 7H10 plates (BD Biosciences) in triplicates and counting the number of CFUs after incubation at 37°C for 2-3 weeks.

**Direct growth inhibition assays**

Appropriately diluted stock *M.bovis* BCG and *M.smegmatis* (2.5x10$^4$) in suspensions of 1ml 7H9 or LB broth respectively were exposed to a range of concentrations of PAF C-16 or PAF C-16 analogues (10-100µg/ml) for 2h at 37°C with mixing every 15 min. Appropriate solvent controls for the test chemicals were also included in all the experiments. After incubation, 200µl of bacterial suspensions from test compound treated and solvent control tubes were seeded on agar plates in triplicate and viable colony counting methods were used to detect the direct growth inhibitory effects of PAF C-16 and its analogues. *M.smegmatis* was seeded on LB agar plates and incubated for 72h while 7H10 plates were used for *M.bovis* BCG and incubated for 3-4 weeks at 37°C after which the CFUs were enumerated by the naked eye.

The above described protocol was also used to investigate *in vitro* direct growth inhibitory effect of Palmitic acid (10-100µg/ml), Phosphocholine chloride calcium salt tetrahydrate (10-100µg/ml), 1-O-hexadecyl-sn-glycerol (10-100µg/ml), Miltefosine (10-100µg/ml) and Hexadecyl lactate (10-100µg/ml) in order to localize the biologically active portion of PAF C-16 contributing to the growth inhibition.

Additional experiments to determine the effect of incubation time on the growth inhibitory concentration of PAF C-16 against *M.bovis* BCG and *M.smegmatis* were performed in a similar manner. However, lower concentrations of PAF C-16 (1-25µg/ml) were used and the incubation periods with bacteria were increased to 6h, 12h and 24h.
Fluorescence microscopy and flow cytometry to detect damage to the mycobacterial cell membrane

Propidium Iodide (PI) (BioLegend, San Diego, USA), a nucleic acid binding fluorescent dye, was used to detect damage to the mycobacterial cell membrane by using fluorescence microscopy and flow cytometry. 1ml of M.bovis BCG or M.smegmatis (O.D(600nm)=0.9) from fresh culture were washed with PBS via centrifugation (5,000rpm, 10min), resuspended in 1ml of broth culture media, and then incubated with 100µg PAF C-16 for 2h at 37°C with mixing every 15min. Ethanol treated (10µl/ml for 2h) and heat killed (100°C for 10min) bacterial samples were used as solvent control and positive control respectively. Next, PAF C-16 treated test and untreated control bacterial samples were washed twice with PBS (5000rpm for 10min) and then stained with PI (1µg/ml) for 20min at room temperature in dark. The excess dye was removed by washing with PBS.

For fluorescence microscopy, the bacterial pellets were resuspended in 100µl of PBS, and 5µl of bacteria from each condition was examined at 400X magnification using a Leica DM4000® fluorescence microscope. For flow cytometry the bacterial pellets were resuspended in 250µl of PBS and 25,000 events were acquired using ACEA NovoCyte® Flow Cytometer; the acquired data was analysed by NovoExpress® software. To determine the percentage of PI stained bacteria, first the solvent control (10µl ethanol/ml of bacterial suspension) condition stained with PI was analysed on a density plot by applying gates such that most of the bacterial population was negative for PI. The same gates were then applied on the density plots for heat-killed (100°C for 10min) positive control and 100µg/ml PAF C-16 treated test conditions.

Effect of α-tocopherol and tween-80 on PAF C-16 induced growth inhibition

α-tocopherol and tween-80 were used to determine if they can mitigate the PAF C-16 induced bacterial growth inhibition as shown previously (Li et al., 2011; Kaghan, 1989). M.smegmatis (2.5x10⁴) and M.bovis BCG (2.5x10⁴) was resuspended in 1ml broth medium and treated with either 100µg/ml α-tocopherol or 1% v/v tween-80 for 1h at 37°C. After 1h, PAF C-16 (100µg/ml bacterial suspension) was added to the samples and incubated at 37°C for 2h with mixing at after every 15min. Solvent (20µl ethanol/ml bacterial suspension), α-tocopherol (100µg/ml bacterial suspension), tween-80 (1% v/v bacterial suspension) and PAF C-16 (100µg/ml bacterial suspension) treated M.smegmatis or M.bovis BCG were included as controls. After incubation, 200µl of bacterial suspension from the test and control tubes was
seeded on agar plates in triplicate and the CFUs were counted using viable colony counting method for each condition.

**Statistical Analysis**

All the experiments were repeated 3-6 times. The data for growth inhibition assays were expressed as mean ± standard error of means (S.E.M), and the solvent treated samples were considered 100% bacterial survival. Statistical analysis was performed using GraphPad Prism® software (Version 5.01) to determine the level of significance (p-value) by applying non-parametric multiple comparison Kruskal-Wallis test on ranks and individual data-sets were compared through post hoc Dunn’s multiple comparison test. For comparison of two particular datasets, non-parametric Mann Whitney test was used. A p-value of less than or equal to 0.05 (p≤0.05) was considered to be significant. On the graphs p≤0.05 was denoted by *, p≤0.01 was denoted by ** and p≤0.001 was denoted by ***.

**Results**

**PAF C-16 inhibits M.smegmatis and M.bovis BCG growth in a dose-dependent manner**

Treatment of *M.smegmatis* and *M.bovis* BCG with PAF C-16 (10µg, 25µg, 50µg and 100µg/ml) for 2h resulted in a dose-dependent growth inhibition, as evident from the decrease in the number of surviving CFUs when compared with PAF C-16 solvent control (10µl ethanol/ml bacterial culture) (Figure 1A & 1B). PAF C-16 treatment of *M.smegmatis* and *M.bovis* BCG at the lower range of concentrations (10µg/ml and 25µg/ml) showed 15-40% reduction in the number of CFUs. The number of *M.smegmatis* CFUs after treatment with 50µg/ml PAF C-16 on an average decreased by 65%, whereas, 100µg/ml PAF C-16 treatment caused 97% reduction (Figure 1A). Similar results were obtained for *M.bovis* BCG where 50µg/ml and 100µg/ml PAF C-16 treatment on an average reduced the number of surviving CFUs by 66% and 88%, respectively, when compared to the number of CFUs from the solvent control (Figure 1B). Although a trend of *M.smegmatis* and *M.bovis* BCG growth inhibition was seen in all the experiments using the above range of PAF C-16 concentrations, only PAF C-16 at the concentration of 50µg/ml (p≤0.01) and 100µg/ml (p≤0.001) reached statistical significance using stringent non-parametric Dunn’s multiple comparison test.
PAF C-16 inhibits *M. smegmatis* and *M. bovis* BCG growth in a time-dependent manner

To assess the effect of PAF C-16 exposure time on the growth inhibition of *M. smegmatis* and *M. bovis* BCG, bacteria were treated for extended durations of 6, 12 and 24h with PAF C-16 (1µg-25µg/ml) (Figure 2A & 2B). It was observed that in addition to concentration, the growth inhibition of both *M. smegmatis* and *M. bovis* BCG by PAF C-16 was also dependent on the treatment duration. PAF C-16 treatment at 5µg/ml or at higher concentrations was effective in inhibiting the growth of both *M. smegmatis* and *M. bovis* BCG by ≥50% at all three time points. At 6h, PAF C-16 treatment at a concentration of 5µg/ml caused a reduction of 55% in the number *M. smegmatis* CFUs when compared to the solvent control. The PAF C-16 induced *M. smegmatis* growth inhibition at 5µg/ml increased to ≥95% as evident from the decrease in CFUs when the treatment times were increased to 12h and 24h (Figure 2A). Similar results were obtained with *M. bovis* BCG. At 6h, PAF C-16 at a concentration of 5µg/ml reduced the number of *M. bovis* BCG CFUs by 51% which increased to 85% after 12h treatment and finally reached 96% after 24h (Figure 2B). PAF C-16 at the concentration of 2.5µg/ml after 6h treatment on an average caused 14% and 12% reduction in the number of *M. smegmatis* and *M. bovis* BCG CFUs respectively, which increased to ~29% after 24h treatment. Furthermore, 1µg/ml PAF C-16 showed negligible growth inhibition at all three time points tested against both *M. smegmatis* and *M. bovis* BCG.

Subsequent experiments with PAF C-16 and its structural analogues were performed at concentrations ranging from 10µg-100µg/ml with treatment duration of 2h. Since we established the growth inhibition of *M. smegmatis* and *M. bovis* BCG at the lowest concentration of 5µg/ml PAF C-16 at increased treatment durations.

**PAF C-16 analogues show a similar growth inhibitory effect against *M. smegmatis* and *M. bovis* BCG**

Different PAF C-16 structural analogues, including the naturally occurring precursor form Lyso-PAF C-16, PAF C-18 and Hexanolamino PAF C-16 as well as synthetic analogues such as 2-O-methyl PAF C-16 and Pyrrolidino PAF C-16 were tested against both *M. smegmatis* and *M. bovis* BCG in order to assess the impact of small modifications of the structure of PAF C-16 on the *in vitro* bacterial growth inhibition potential (Figure 3). PAF C-16 structural analogues used were selected such that each analogue represented a change in different functional group when compared to PAF C-16. These PAF C-16 structural analogues were able to inhibit the growth of both *M. smegmatis* and *M. bovis* BCG in a dose dependent
PAF C-16 inhibits mycobacterial growth

manner, when tested at concentrations ranging from 10-100µg/ml (Figure 4A & 4B). Significant levels of *M. smegmatis* and *M. bovis* BCG growth inhibition (*p*≤0.01-*p*≤0.001) were observed at 50µg/ml (≤50% reduction in CFUs) and 100µg/ml (≤90% reduction in CFUs) for all PAF C-16 analogues tested. Furthermore, the mycobacterial growth inhibition potency of these PAF C-16 structural analogues was comparable to PAF C-16. This showed that small changes in the structure of PAF C-16 did not affect its direct growth inhibitory potential against mycobacteria.

**Structural dissection of PAF C-16 to localize the antimicrobial active portion**

To localize the biologically active portion of PAF C-16 that contributed to its growth inhibitory properties against mycobacteria, compounds with structures similar to different portions of PAF C-16 (Figure 5) were investigated for their direct mycobacterial growth inhibitory potential using *M. smegmatis* as a model. Palmitic acid (hexadecanoic acid) having a linear 16-carbon atoms chain similar to the carbon tail of PAF C-16 in the number of carbon atoms, was tested to determine the role of the carbon chain in the growth inhibition activity of PAF C-16. However, Palmitic acid (10-100µg/ml) did not show any inhibitory effect on *M. smegmatis* growth, in fact, it showed a slight growth enhancing effect. Phosphocholine chloride calcium tetrahydrate salt with a structure resembling the phosphocholine head region of PAF C-16 also did not show any direct inhibitory effect on the growth of *M. smegmatis* (Figure 6). Finally, 1-O-hexadecyl sn-glycerol, a compound with a 16-carbon atoms tail attached via ether bond to a glycerol backbone as in PAF C-16, showed direct dose-dependent growth inhibition of *M. smegmatis* following 2h treatment (Figure 6). This suggested that the attachment of a carbon tail through an ether linkage may be important for the antimycobacterial characteristics of PAF C-16. Further experiments with other compounds such as miltefosine and hexadecyl lactate, each having a 16-carbon atoms tail attached via ester linkage to a phosphate and lactyl group respectively, also showed growth inhibition of *M. smegmatis* (Figure 6).

**PAF C-16 causes damage to the mycobacterial cell membrane**

Fluorescence microscopy and flow cytometry were performed to assess the damaging effect of PAF C-16 on the cell membrane integrity of mycobacteria. The cell membrane integrity was determined by using a nucleic acid binding fluorescent dye, Propidium Iodide (PI). PI only enters the bacteria when the cell membrane is damaged while bacteria with intact cell membranes are impermeable to this dye (Cox *et al.*, 2000).
The qualitative analysis of *M. smegmatis* and *M. bovis* BCG by fluorescence microscopy following treatment with 100μg/ml PAF C-16 for 2h revealed loss of membrane integrity, as determined by positive staining with PI (Figure 7C & 7F). No PI staining was observed in the solvent control (10μl ethanol/ml of bacterial suspension), suggesting that the bacterial membrane was intact (Figure 7A & 7D). Heat-killed (100°C for 10min) *M. smegmatis* and *M. bovis* BCG were also included as a positive control and majority of the bacteria were stained positively with PI (Figure 7B & 7E). These results from fluorescence microscopy suggested that PAF C-16 had a damaging effect on the cell membranes of both *M. smegmatis* and *M. bovis* BCG that can lead to the growth inhibition of these bacteria.

To further quantify the percentage of injured/dead bacteria, flow cytometry was performed using *M. smegmatis* and *M. bovis* BCG treated with 100μg/ml PAF C-16 for 2h. It was observed that ~50% of *M. smegmatis* were stained with PI following treatment with PAF C-16, whereas, only 2% of *M. smegmatis* were stained with PI in the solvent control and 90% staining was observed for the positive control heat-killed *M. smegmatis* (Figure 8A, 8B & 8C). Similar results were obtained with *M. bovis* BCG; 100μg/ml PAF C-16 treatment resulted in ~40% of bacteria to be stained with PI as compared to 2% PI positive staining in solvent control and 68% staining in heat-killed *M. bovis* BCG (Figure 8D, 8E & 8F). The histogram overlays (Figure 8G & 8H) showed a more than 4-fold increase in the mean channel fluorescence (MeanX) for PAF C-16 treated *M. smegmatis* (MeanX=318) and *M. bovis* BCG (MeanX=135) when compared to the solvent only controls for *M. smegmatis* (MeanX=74) and *M. bovis* BCG (MeanX=27), respectively. These flow cytometry results reaffirmed the notion that PAF C-16 induced growth inhibition in *M. smegmatis* and *M. bovis* BCG was through damage to their cell membrane.

**α-tocopherol and tween-80 prevent the growth inhibitory effect of PAF C-16 on mycobacteria**

Since PAF C-16 showed damaging effects on both *M. smegmatis* and *M. bovis* BCG cell membranes, compounds such as α-tocopherol and tween-80 were used to assess whether they can mitigate the growth inhibitory effect of PAF C-16, and thus, have protective effects on mycobacteria. Both α-tocopherol and tween-80 have previously been shown to reduce the anti-bacterial activity of hydrophobic compounds such as fatty acids, phospholipids and antibiotics like rifampicin (Nielsen *et al.*, 2016; Li *et al.*, 2011; Kaghlan, 1989). The addition of either α-tocopherol (100μg/ml) or tween-80 (1% v/v) to *M. smegmatis* or *M. bovis* BCG
suspension for 1h prior to PAF C-16 (100µg/ml) treatment partially mitigated the inhibitory effect of PAF C-16 on the growth of bacteria as indicated by the increase in number of surviving CFUs on the agar plates when compared with the number of CFUs from bacteria treated with 100µg/ml PAF C-16 only (Figure 9A, 9B & 9C). Furthermore, both α-tocopherol and tween-80 did not show any direct effect on the growth of either *M.smegmatis* or *M.bovis* BCG on their own.

**Discussion**

PAF C-16 belongs to a class of single-chained ether linked lipids and is produced by a variety of immune cells including monocytes, macrophages (Elstad *et al*., 1988) and neutrophils (Lynch *et al*., 1979). The production of PAF C-16 is enhanced several fold at the site of infection, where it is available to interact with invading pathogens (Huseyinov *et al*., 1999). However, there is limited information regarding the direct effects of this pro-inflammatory phospholipid on the growth of bacterial pathogens both *in vivo* and *in vitro*. A previous study showed that PAF C-16 can directly inhibit the growth of Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) *in vitro*, without affecting the growth of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Steel *et al*., 2002). In the current study, we have shown, for the first time, that PAF C-16 and its structural analogues can directly inhibit the growth of mycobacteria using *M.smegmatis* and *M.bovis* BCG as models, in a dose and time-dependent manner *in vitro*.

We first investigated the effect of PAF C-16 on the growth of *M.smegmatis* and *M.bovis* BCG at various concentrations (10-100µg/ml) and for a shorter treatment duration (2h), which revealed a decrease in the surviving bacterial CFUs in a dose-dependent manner, suggesting that PAF C-16 had a direct growth inhibitory effect *in vitro*. Since PAF C-16 at higher concentrations can have certain adverse side effects on the host (Stafforini *et al*., 2003), we further investigated the effect of PAF C-16 on *M.smegmatis* and *M.bovis* BCG growth at lower concentrations (1-25µg/ml) and at increased treatment durations of 6, 12 and 24h. We observed that this compound was effective at a concentration as low as 5µg/ml after 6h of treatment. In order to get an insight into the structure-function relationship of PAF C-16, different structural analogues were assessed *in vitro* for their direct growth inhibitory potential against *M.smegmatis* and *M.bovis* BCG. PAF C-16 analogues have minor but subtle structural modifications such as variations in functional groups or carbon tail length (Prescott *et al*., 1990; Tence *et al*., 1981). Previous studies with PAF C-16 analogues in mammalian
PAF C-16 inhibits mycobacterial growth

systems have shown that small structural modifications in the structure can alter the biological activity of PAF C-16 to varying degrees (Stewart and Grigoriadis, 1991; Rose et al., 1990; O’Flaherty et al., 1987; Shigenobu et al., 1985). These PAF C-16 analogues have not been investigated for their direct effect on the growth of mycobacteria.

Lyso-PAF C-16, the precursor form of naturally produced PAF C-16, has a hydroxyl group at position sn-2 instead of an acetyl group and is considered to be biologically inactive (Montrucchio et al., 2000; Aliberti et al., 1999; Pendino et al., 1993). In our experiments, however, Lyso-PAF was effective in inhibiting the growth of both M. smegmatis and M. bovis BCG at the levels similar to PAF C-16. Since Lyso-PAF is the inactive precursor form and does not possess most of the side effects associated with PAF C-16, this compound may have therapeutic potential and needs further investigation for its anti-M.tb properties. Similar mycobacterial growth inhibition results were obtained with other PAF C-16 structural analogues including PAF C-18 (18 carbon atoms chain at sn-1), Hexanolamino PAF C-16 (additional 4-carbon atoms chain attached to terminal amino group), 2-O-methyl PAF (methyl group sn-2) and Pyrrolidino PAF (5-member lactam ring attached to the phosphate group) indicating that these small modifications had insignificant effects on the bacterial growth inhibition potential.

Structurally, PAF C-16 is composed of a glycerol backbone with a single chained aliphatic carbon tail attached via ether bond at position sn-1, an acetyl group at position sn-2 and a phosphocholine group attached at sn-3 position (Venable 1993; Prescott et al., 1990). Different compounds with structures similar to the phosphocholine head and the aliphatic carbon tail of PAF C-16 were tested to identify the structurally active portion of PAF C-16, which might be involved in the mycobacterial growth inhibition using M. smegmatis as a model. It seems that the aliphatic carbon chain linked with oxygen bond to the rest of the molecule is essential for the anti-mycobacterial activity. This is supported by our results which showed that compounds containing such structure (1-O-hexadecyl-sn-glycerol, miltefosine and hexadecyl lactate) possessed anti-mycobacterial activity, whereas palmitic acid although containing aliphatic carbon chain, but lacking oxygen bond, showed no such growth inhibitory activity. To the best of our knowledge, there is currently no information about the inhibitory effect of these compounds on mycobacterial growth and our results show a novel activity for these compounds. Miltefosine has previously been shown to kill Leishmania (Machado et al., 2010; Jha et al., 1999), pathogenic bacteria such as Streptococcus pneumoniae (Llull et al., 2007) and fungi such as Aspergillus fumigatus and
PAF C-16 inhibits mycobacterial growth

*Candida* species (Biswas *et al.*, 2013; Widmer *et al.*, 2006). Similarly, hexadecyl lactate has also been shown to be safe in humans and is used as an additive in food, medicines and personal care products (Zhang *et al.*, 2010; Clary *et al.*, 1998).

Most of the biological activities of PAF C-16 in mammalian systems are carried out by its binding to specific G-protein coupled receptors known as PAFR (Honda *et al.*, 2002; Ishii *et al.*, 2002). Currently, it is not known if any PAF C-16 receptor exists on bacteria. However, certain activities attributed to PAF C-16 are independent of its receptor binding, such as its incorporation into biological membranes, which can affect the molecular organization of membrane lipids, and hence membrane functions (Sawyer and Andersen, 1989). In addition, very limited research has been done to establish the mechanism through which PAF C-16 can inhibit the growth of prokaryotic organisms. Exogenous PAF C-16 affects the cell membrane in Gram-positive bacteria by causing the dysfunction of potassium ion (K\(^+\)) transport and leads to the bacterial death but it is ineffective against Gram-negative bacteria (Steel *et al.*, 2002). In this study, fluorescence microscopy and flow cytometry analysis to determine the effect of PAF C-16 on the cell membrane of *M. smegmatis* and *M. bovis* BCG revealed positive PI staining, which occurred due to the entry of the dye in these bacteria, indicating damage to the bacterial cell membrane. Furthermore, prior treatment of the bacteria with \(\alpha\)-tocopherol, which is a well-known anti-oxidant and membrane stabilizer (Urano *et al.*, 1992; Kaghan, 1989), partially mitigated the PAF C-16 induced bacterial growth inhibition. However, ascorbic acid, another compound with anti-oxidant properties, failed to prevent PAF C-16 induced growth inhibition of *M. smegmatis* (data not included), suggesting that the protective role of \(\alpha\)-tocopherol is through its membrane stabilizing mechanism. Similarly, another membrane stabilizing agent tween-80 (Li *et al.*, 2011), also mitigated the inhibitory effect of PAF C-16 on the growth of *M. smegmatis*. This hypothesis is further supported by a previous study in which tween-80 was shown to reduce the activity of antimicrobial compounds such as essential oils, hydrophobic antibiotics like rifampicin and bile salts (Nielsen *et al.*, 2016; Li *et al.*, 2011). Thus, it is evident that PAF C-16 mediated growth inhibition of *M. smegmatis* and *M. bovis* BCG is via cell membrane damage, as membrane stabilizing compounds successfully prevented the PAF C-16 induced growth inhibition of mycobacteria. However, these membrane stabilizing compounds also have the ability to form micelles in aqueous solutions due to their amphipathic nature (Aizawa, 2009) and there is a possibility that the increased
survival of mycobacteria when treated with both α-tocopherol/tween-80 and PAF C-16 might be due to the sequestration of PAF C-16 in these micelles.

In summary, PAF C-16 inhibits the growth of both \textit{M.smegmatis} and \textit{M.bovis BCG} in a dose and time-dependent manner. The growth inhibitory effect PAF C-16 seems to be through damage to the mycobacterial cell membrane. The presence of an ether bond at position \textit{sn}-1 along with a 16 atom carbon chain seems to be important in conferring the bacterial growth inhibition potential to PAF C-16. Small structural modifications of PAF C-16 do not affect growth inhibitory potential against these mycobacteria. Since these PAF C-16 analogues may lack most of the biological side effects associated with PAF C-16, they have the potential to be used as anti-TB drugs. Miltefosine, a compound structurally related to PAF C-16 investigated in this study, is currently used for treating Leishmaniasis in humans and has been shown to be well tolerated at 100mg orally per day (Soto \textit{et al}., 2004; Sundar \textit{et al}., 2002). Furthermore, these compounds can be delivered via inhaler at a higher dose directly to the disease site as in the case of pulmonary TB. In this respect, one of the anti-TB drugs, capreomycin, has already been tested in a phase I clinical trial in healthy subjects. In this single-dose escalation study, the maximum dose delivered was 300mg by handheld inhaler into the lung with minimum side effects (Dharmadhikari \textit{et al}., 2013). It is worthwhile to note that differences exist between \textit{M.tb} and \textit{M. bovis BCG} (as well as \textit{M. smegmatis}) with respect to the composition of cell wall components. Therefore, these PAF C-16 analogues need further investigation both \textit{in vitro} and \textit{in vivo} using \textit{M.tb}.

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PAF C-16 inhibits mycobacterial growth

References

Aizawa H. Morphology of polysorbate 80 (Tween 80) micelles in aqueous 1, 4-dioxane solutions. *Journal of Applied Crystallography* (2009) **42**:592-6.

Alam I, Smith J, Silver MJ. Human and rabbit platelets form platelet-activating factor in response to calcium ionophore. *Thromb Res* (1983) **30**:71-9.

Aliberti JC, Machado FS, Gazzinelli RT, Teixeira MM, Silva JS. Platelet-activating factor induces nitric oxide synthesis in Trypanosoma cruzi-infected macrophages and mediates resistance to parasite infection in mice. *Infect Immun* (1999) **67**:2810-4.

Aronson NE, Santosham M, Comstock GW, Howard RS, Moulton LH, Rhoades ER, et al. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: a 60-year follow-up study. *JAMA* (2004) **291**:2086-91.

Barber LA, Spandau DF, Rathman SC, Murphy RC, Johnson CA, Kelley SW, et al. Expression of the platelet-activating factor receptor results in enhanced ultraviolet B radiation-induced apoptosis in a human epidermal cell line. *J Biol Chem* (1998) **273**:18891-7.

Biffl WL, Moore EE, Moore FA, Barnett CC,Jr, Silliman CC, Peterson VM. Interleukin-6 stimulates neutrophil production of platelet-activating factor. *J Leukoc Biol* (1996) **59**:569-74.

Biswas C, Sorrell TC, Djordjevic JT, Zuo X, Jolliffe KA, Chen SC. In vitro activity of miltefosine as a single agent and in combination with voriconazole or posaconazole against uncommon filamentous fungal pathogens. *J Antimicrob Chemother* (2013) **68**:2842-6.

Borges AF, Morato CI, Gomes RS, Dorta ML, de Oliveira, Milton Adriano Pelli, Ribeiro-Dias F. Platelet-activating factor increases reactive oxygen species-mediated microbicidal activity of human macrophages infected with Leishmania (Viannia) braziliensis. *Pathogens and disease* (2017) **75**: doi.org/10.1093/femspd/ftx082

Brewer C, Bonin F, Bullock P, Nault M, Morin J, Imbeault S, et al. Platelet activating factor-induced apoptosis is inhibited by ectopic expression of the platelet activating factor G-protein coupled receptor. *J Neurochem* (2002) **82**:1502-11.

Bussolino F, Brexviero F, Tetta C, Aglietta M, Sanavio F, Mantovani A, et al. Interleukin 1 stimulates platelet activating factor production in cultured human endothelial cells. *Pharmacol Res Commun* (1986) **18**:133-7.

Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *J Exp Med* (1987) **166**:1390-404.

Chignard M, Le Couedic J, Vargaftig B, Benvenisie J. Platelet-Activating Factor (PAF-Acether) Secretion from Platelets: Effect of Aggregating Agents. *Br J Haematol* (1980) **46**:455-64.

Chignard M, Le Couedic J, Tence M, Vargaftig B, Benveniste J. The role of platelet-activating factor in platelet aggregation. *Nature* (1979) **279**:799-800.
PAF C-16 inhibits mycobacterial growth

Clark PR, Manes TD, Pober JS, Kluger MS. Increased ICAM-1 expression causes endothelial cell leakiness, cytoskeletal reorganization and junctional alterations. *J Invest Dermatol* (2007) **127**:762-74.

Clary J, Feron V, Van Velthuijsen J. Safety assessment of lactate esters. *Regulatory toxicology and pharmacology* (1998) **27**:88-97.

Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis: meta-analysis of the published literature. *JAMA* (1994) **271**:698-702.

Cooper AM, Flynn JL. The protective immune response to *Mycobacterium tuberculosis*. *Curr Opin Immunol* (1995) **7**:512-6.

Cox S, Mann C, Markham J, Bell H, Gustafson J, Warmington J, et al. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* (2000) **88**:170-5.

Cuss F, Dixon CS, Barnes P. Effects of inhaled platelet activating factor on pulmonary function and bronchial responsiveness in man. *The Lancet* (1986) **328**:189-92.

Daley CL, Small PM, Schecter GF, Schoolnik GK, McAdam RA, Jacobs Jr WR, et al. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus: An analysis using restriction-fragment—Length polymorphisms. *N Engl J Med* (1992) **326**:231-5.

Demopoulos CA, Pinckard RN, Hanahan DJ. Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J Biol Chem* (1979) **254**:9355-8.

Dharmadhikari AS, Kabadi M, Gerety B, Hickey AJ, Fourie PB, Nardell E. Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: a new approach to therapy of drug-resistant tuberculosis. *Antimicrob Agents Chemother* (2013) **57**:2613-9 doi: 10.1128/AAC.02346-12 [doi].

Elstad MR, Prescott SM, McIntyre TM, Zimmerman GA. Synthesis and release of platelet-activating factor by stimulated human mononuclear phagocytes. *J Immunol* (1988) **140**:1618-24.

Espinal MA, Kim SJ, Suarez PG, Kam KM, Khomenko AG, Migliori GB, et al. Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. *JAMA* (2000) **283**:2537-45.

Gardiner C, Harrison P, Chavda N, MacKie I, Machin S. Platelet activation responses in vitro to human mast cell activation. *Br J Haematol* (1999) **106**:208-15.

Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, et al. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J Immunol* (2001) **166**:7033-41.

Hanahan DJ, Demopoulos CA, Liehr J, Pinckard RN. Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. *J Biol Chem* (1980) **255**:5514-6.
PAF C-16 inhibits mycobacterial growth

Hartung H, Parnham MJ, Winkelmann J, Englberger W, Hadding U. Platelet activating factor (PAF) induces the oxidative burst in macrophages. *Int J Immunopharmacol* (1983) **5**:115-21.

Henderson WR, Jr, Lu J, Poole KM, Dietsch GN, Chi EY. Recombinant human platelet-activating factor-acetylhydrolase inhibits airway inflammation and hyperreactivity in mouse asthma model. *J Immunol* (2000) **164**:3360-7.

Hilliquin P, Guinot P, Chermat-Izard V, Puechal X, Menkes CJ. Treatment of rheumatoid arthritis with platelet activating factor antagonist BN 50730. *J Rheumatol* (1995) **22**:1651-4.

Honda Z, Ishii S, Shimizu T. Platelet-activating factor receptor. *The Journal of Biochemistry* (2002) **131**:773-9.

Hostettler ME, Knapp PE, Carlson SL. Platelet-activating factor induces cell death in cultured astrocytes and oligodendrocytes: Involvement of caspase-3. *Glia* (2002) **38**:228-39.

Houben RM, Dodd PJ. The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. *PLoS medicine* (2016) **13**:e1002152.

Huseyinov A, Kutukculer N, Aydogdu S, Caglayan S, Coker I, Goksen D, et al. Increased gastric production of platelet-activating factor, leukotriene-B4, and tumor necrosis factor-α in children with Helicobacter pylori infection. *Dig Dis Sci* (1999) **44**:675-9.

Ishii S, Nagase T, Shimizu T. Platelet-activating factor receptor. *Prostaglandins Other Lipid Mediat* (2002) **68**:599-609.

Jain A, Mondal R. Extensively drug-resistant tuberculosis: current challenges and threats. *Pathogens and Disease* (2008) **53**:145-50.

Jha T, Sundar S, Thakur C, Bachmann P, Karbwang J, Fischer C, et al. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *N Engl J Med* (1999) **341**:1795-800.

Kagan VE. Tocopherol stabilizes membrane against phospholipase A, free fatty acids, and lysophospholipids. *Ann N Y Acad Sci* (1989) **570**:121-35.

Kaul M, Loos M. Collagen-like complement component C1q is a membrane protein of human monocyte-derived macrophages that mediates endocytosis. *J Immunol* (1995) **155**:5795-802.

Lahey T, von Reyn CF. Mycobacterium bovis BCG and New Vaccines for the Prevention of Tuberculosis. *Microbiol Spectr* (2016) **4**: doi:10.1128/microbiolspec.TNMI7-0003-2016. 38. Leaver H, Qu J, Smith G, Howie A, Ross W, Yap P. Endotoxin releases platelet-activating factor from human monocytes in vitro. *Immunopharmacology* (1990) **20**:105-13.

Li J, Zhang L, Du M, Han X, Yi H, Guo C, et al. Effect of tween series on growth and cis-9, trans-11 conjugated linoleic acid production of Lactobacillus acidophilus F0221 in the presence of bile salts. *International journal of molecular sciences* (2011) **12**:9138-54.

Liu CH, Li L, Chen Z, Wang Q, Hu YL, Zhu B, et al. Characteristics and treatment outcomes of patients with MDR and XDR tuberculosis in a TB referral hospital in Beijing: a 13-year experience. *PloS one* (2011) **6**: doi.org/10.1371/journal.pone.0019399.
PAF C-16 inhibits mycobacterial growth

Llull D, Rivas L, Garcia E. In vitro bactericidal activity of the antiprotozoal drug miltefosine against Streptococcus pneumoniae and other pathogenic streptococci. *Antimicrob Agents Chemother* (2007) **51**:1844-8.

Lynch JM, Lotner GZ, Betz SJ, Henson PM. The release of a platelet-activating factor by stimulated rabbit neutrophils. *J Immunol* (1979) **123**:1219-26.

Machado PR, Ampuero J, Guimarães LH, Villasboas L, Rocha AT, Schriefer A, et al. Miltefosine in the treatment of cutaneous leishmaniasis caused by Leishmania braziliensis in Brazil: a randomized and controlled trial. *PLOS Neglected tropical diseases* (2010) **4**: doi.org/10.1371/journal.pntd.0000912.

Merendino N, Dwinell MB, Variki N, Eckmann L, Kagnoff MF. Human intestinal epithelial cells express receptors for platelet-activating factor. *Am J Physiol* (1999) **277**:G810-8.

Montrucchio G, Alloatti G, Camussi G. Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol Rev* (2000) **80**:1669-99.

Mueller HW, Haught CA, McNatt JM, Cui K, Gaskell SJ, Johnston DA, et al. Measurement of platelet-activating factor in a canine model of coronary thrombosis and in endarterectomy samples from patients with advanced coronary artery disease. *Circ Res* (1995) **77**:54-63.

Nielsen CK, Kjems J, Mygind T, Snabe T, Meyer RL. Effects of Tween 80 on growth and biofilm formation in laboratory media. *Frontiers in microbiology* (2016) **7**: doi.org/10.3389/fmicb.2016.01878

O'Flaherty JT, Redman J, Schmitt JD, Ellis JM, Surles JR, Marx MH, et al. 1-0-alkyl-2-N-methylcarbamyl-glycerophosphocholine: A biologically potent, non-metabolizable analog of platelet-activating factor. *Biochem Biophys Res Commun* (1987) **147**:18-24.

Pendino KJ, Gardner CR, Laskin JD, Laskin DL. Induction of functionally active platelet-activating factor receptors in rat alveolar macrophages. *J Biol Chem* (1993) **268**:19165-8.

Prescott SM, Zimmerman GA, McIntyre TM. Platelet-activating factor. *J Biol Chem* (1990) **265**:17381-4.

Rosam A, Wallace JL, Whittle BJ. Potent ulcerogenic actions of platelet-activating factor on the stomach. *Nature* (1986) **319**:54-6.

Rose JK, Debs RA, Philip R, Ruis NM, Valone FH. Selective activation of human monocytes by the platelet-activating factor analog 1-O-hexadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine. *J Immunol* (1990) **144**:3513-7.

Rouis M, Nigon F, Chapman MJ. Platelet activating factor is a potent stimulant of the production of active oxygen species by human monocyte-derived macrophages. *Biochem Biophys Res Commun* (1988) **156**:1293-301.

Ryan SD, Harris CS, Carswell CL, Baenziger JE, Bennett SA. Heterogeneity in the sn-1 carbon chain of platelet-activating factor glycerophospholipids determines pro- or anti-apoptotic signaling in primary neurons. *J Lipid Res* (2008) **49**:2250-8.

Sawyer DB, Andersen OS. Platelet-activating factor is a general membrane perturbant. *Biochimica et Biophysica Acta (BBA)-Biomembranes* (1989) **987**:129-32.
Schleimer RP, MacGlashan Jr DW, Peters SP, Pinckard RN, Adkinson Jr NF, Lichtenstein LM. Characterization of Inflammatory Mediator Release from Purified Human Lung Mast Cells 1–3. Am Rev Respir Dis (1986) 133:614-7.

Seo KH, Ko H, Choi JH, Jung HH, Chun YH, Choi I, et al. Essential role for platelet-activating factor-induced NF-κB activation in macrophage-derived angiogenesis. Eur J Immunol (2004) 34:2129-37.

Sepulveda R, Parcha C, Sorensen R. Case-control study of the efficacy of BCG immunization against pulmonary tuberculosis in young adults in Santiago, Chile. Tubercle Lung Dis (1992) 73:372-7.

Sherwood ER, Toliver-Kinsky T. Mechanisms of the inflammatory response. Best Practice & Research Clinical Anaesthesiology (2004) 18:385-405.

Shigenobu K, Masuda Y, Tanaka Y, Kasuya T. Platelet activating factor analogues: lack of correlation between their activities to produce hypotension and endothelium-mediated vasodilation. J Pharmacobiodyn (1985) 8:128-33.

Shindou H, Hishikawa D, Nakanishi H, Harayama T, Ishii S, Taguchi R, et al. A single enzyme catalyzes both platelet-activating factor production and membrane biogenesis of inflammatory cells. Cloning and characterization of acetyl-CoA:LYSO-PAF acetyltransferase. J Biol Chem (2007) 282:6532-9.

Singh P, Singh IN, Mondal SC, Singh L, Garg VK. Platelet-activating factor (PAF)-antagonists of natural origin. Fitoterapia (2013) 84:180-201.

Soto J, Arana B, Toledo J, Rizzo N, Vega J, Diaz A, et al. Miltefosine for new world cutaneous leishmaniasis. Clinical infectious diseases (2004) 38:1266-72.66. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Rev Clin Lab Sci (2003) 40:643-72.

Steel H, Cockeran R, Anderson R. Platelet-activating factor and lyso-PAF possess direct antimicrobial properties in vitro. APMIS (2002) 110:158-64.

Stewart AG, Grigoriadis G. Structure-activity relationships for platelet-activating factor (PAF) and analogues reveal differences between PAF receptors on platelets and macrophages. J Lipid Mediat (1991) 4:299-308.

Stoddart NR, Roudebush WE, Fleming SD. Exogenous platelet-activating factor stimulates cell proliferation in mouse pre-implantation embryos prior to the fourth cell cycle and shows isoform-specific stimulatory effects. Zygote (2001) 9:261-8.

Sundar S, Jha T, Thakur C, Engel J, Sindermann H, Fischer C, et al. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med (2002) 347:1739-46.

Tence M, Coeffier E, Heymans F, Polonsky J, Godfrroid J, Benveniste J. Structural analogs of platelet-activating factor (PAF-acether). Biochimie (1981) 63:723-7.

Urano S, Inomori Y, Sugawara T, Kato Y, Kitahara M, Hasegawa Y, et al. Vitamin E: inhibition of retinol-induced hemolysis and membrane-stabilizing behavior. J Biol Chem (1992) 267:18365-70.
PAF C-16 inhibits mycobacterial growth

Vadas P, Perelman B, Liss G. Platelet-activating factor, histamine, and tryptase levels in human anaphylaxis. *J Allergy Clin Immunol* (2013) **131**:144-9.

Venable ME, Zimmerman GA, McIntyre TM, Prescott SM. Platelet-activating factor: a phospholipid autacoid with diverse actions. *J Lipid Res* (1993) **34**:691-702.

Widmer F, Wright LC, Obando D, Handke R, Ganendren R, Ellis DH, et al. Hexadecylphosphocholine (miltefosine) has broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis. *Antimicrob Agents Chemother* (2006) **50**:414-21.

World Health Organization. "Global tuberculosis report 2017". In: *Global tuberculosis report 2017*. (2017).

Zhang J, Liu M, Jin H, Deng L, Xing J, Dong A. In vitro enhancement of lactate esters on the percutaneous penetration of drugs with different lipophilicity. *Aaps Pharmscitech* (2010) **11**:894-903.
Figure legends

Figure 1: Effect of PAF C-16 treatment on *M. smegmatis* and *M. bovis* BCG survival in vitro. Number of surviving colony forming units of *M. smegmatis* (A) and *M. bovis* BCG (B) after 2 hours of treatment with the indicated concentrations of PAF C-16. Data is expressed in terms of percent survival where solvent control is taken as 100% survival and different PAF C-16 treated test conditions are compared to it. Each bar represents the average of six individual experiments performed in triplicate and the error bars show the standard error of means (±SEM). Statistically significant differences from the solvent control by Kruskal-Wallis test with post hoc Dunn's multiple comparison test are indicated ** *p* ≤ 0.01, *** *p* ≤ 0.001.

Figure 2: Effect of PAF C-16 on *M. smegmatis* and *M. bovis* BCG growth inhibition at increased incubation durations. Number of surviving colony forming units of *M. smegmatis* (A) and *M. bovis* BCG (B) after 6, 12, 24 hours of treatment with indicated concentrations of PAF C-16. Data is expressed in terms of percent survival where solvent control is taken as 100% survival. Each data point represents the average of four individual experiments performed in triplicate and the error bars show the standard error of means (±SEM).

Figure 3: Chemical structures of PAF C-16 and different PAF C-16 structural analogues. Each analogue has a small modification in the structure as compared to naturally occurring PAF C-16 which is highlighted in the red circles.

Figure 4: Comparison of the levels of growth inhibition by different PAF C-16 analogues and PAF C-16 for *M. smegmatis* and *M. bovis* BCG. Number of surviving colony forming units of *M. smegmatis* (A) and *M. bovis* BCG (B) after 2 hours treatment with indicated concentrations of different PAF C-16 analogues and PAF C-16. The data is expressed as percentage survival where solvent control is taken as 100% survival. Each data point represents the average of three individual experiments performed in triplicate and the error bars show the standard error of means (±SEM).

Figure 5: Chemical structures of Palmitic acid, Phosphocholine chloride calcium tetrahydrate, 1-O-Hexadecyl-sn-glycerol, Miltefosine and Hexadecyl lactate.

Figure 6: Effect of Palmitic acid, Phosphocholine chloride calcium tetrahydrate, 1-O-hexadecyl-sn-glycerol, Miltefosine and Hexadecyl lactate on *M. smegmatis* survival. The data is expressed as percentage where solvent control is taken as 100% survival. Each data point represents the average of three individual experiments performed in triplicate and the error bars show the standard error of means (±SEM).

Figure 7: Fluorescence microscopy to detect the effect of PAF C-16 treatment on *M. smegmatis* and *M. bovis* BCG cell membrane integrity. *M. smegmatis* (A-C) and *M. bovis* BCG (D-F) treated with PAF C-16 (100µg/ml) or solvent control (10µl/ml ethanol) for 2 hours and heat killed (100°C, 10min) as a positive control were stained with the nucleic acid binding dye propidium iodide and imaged at 400X magnification in bright field and CY3 (PI) channels. Panels show a merger of the two channels. A and D are solvent treated negative control, B and E are heat treated positive control, C and F are PAF C-16 treated test.
Figure 8: Flow cytometric analysis to determine cell membrane integrity of PAF C-16 treated *M*. *smegmatis* and *M*. *bovis* BCG. Flowcytometry density plots for *M*. *smegmatis* (A-C) and *M*. *bovis* BCG (D-F) treated with PAF C-16 or solvent control for 2 hours and heat killed positive control after staining with propidium iodide. Density plots A and D are solvent treated (10µl/ml ethanol) negative control, density plots B and E are heat treated (100°C, 10min) positive control, density plots C and F are PAF C-16 treated (100µg/ml) test condition. Each plot shows percentage of unstained and PI stained bacteria for different conditions. The difference in fluorescence intensity for PAF C-16 treated test, solvent treated negative control and heat treated positive control are shown using frequency histogram overlay for *M*. *smegmatis* (G) and *M*. *bovis* BCG (H). The blue colour histograms represent PAF C-16 treated bacteria, red colour is for solvent treated negative control and green colour represents heat treated positive control.

Figure 9: α-Tocopherol and Tween-80 partially mitigate the growth inhibitory effect of PAF C-16 against *M*. *smegmatis* and *M*. *bovis* BCG. Number of surviving colony forming units of *M*. *smegmatis* (A and B) and *M*. *bovis* BCG (C) after treatment with the indicated concentrations of α-tocopherol/tween-80 and PAF C-16. The data is expressed as percentage survival where solvent control is taken as 100% survival. Each bar represents the average of three individual experiments performed in triplicate and the error bars show the standard error of means (±SEM). The *p*-value was calculated by applying Mann Whitney test on the given two data sets and was found to be significant *(*p*≤0.05).
PAF C-16 inhibits mycobacterial growth

Figure 1

(A) Percent M. smegmatis survival

(B) Percent M. bovis BCG survival

(n=6)
PAF C-16 inhibits mycobacterial growth

Figure 2

(A) Percent *M. smegmatis* survival
- Green line: 6 hours
- Blue line: 12 hours
- Red line: 24 hours

(B) Percent *M. bovis BCG* survival
- Green line: 6 hours
- Blue line: 12 hours
- Red line: 24 hours

 Yên (n=4)
PAF C-16 inhibits mycobacterial growth

Figure 3

PAF C-16
(1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine)

Lyso PAF C-16
(1-O-hexadecyl-sn-glyceryl-3-phosphorylcholine)

PAF C-18
(1-O-octadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine)

Hexanolamino PAF C-16
(1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphoryl(N,N,N-trimethyl)hexanolamine)

2-O-methyl PAF C-16
(1-O-hexadecyl-2-O-methyl-sn-glyceryl-3-phosphorylcholine)

Pyrrolidino PAF C-16
(1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphoryl-N-methyl-pyrrolidinium ethanol)
PAF C-16 inhibits mycobacterial growth

Figure 4

(A) Percent *M. smegmatis* survival vs. PAF C-16/analogue concentration (µg/ml) for different compounds: Lyso PAF, PAF C-18, 2-O-methyl PAF, Hexanolamino PAF, Pyroliddino PAF, and PAF C-16. (n=3)

(B) Percent *M. bovis* BCG survival vs. PAF C-16/analogue concentration (µg/ml) for different compounds: Lyso PAF, PAF C-18, 2-O-methyl PAF, Hexanolamino PAF, Pyroliddino PAF, and PAF C-16. (n=3)
Figure 5

**Palmitic acid**

![Palmitic acid structure](image)

**Phosphocholine chloride calcium salt tetrahydrate**

![Phosphocholine structure](image)

**1-O-Hexadecyl-sn-glycerol**

![1-O-Hexadecyl-sn-glycerol structure](image)

**Miltefosine**

![Miltefosine structure](image)

**Hexadecyl lactate**

![Hexadecyl lactate structure](image)
Figure 6

![Graph showing percent M. smegmatis survival against compound concentration (µg/ml).](image)

Figure 7

- **M. smegmatis** (Solvent Control)
- **M. smegmatis** (Heat treated)
- **M. smegmatis** (PAF C-16 treated)

- **M. bovis DCG** (Solvent Control)
- **M. bovis DCG** (Heat treated)
- **M. bovis DCG** (PAF C-16 treated)
Figure 8

Flow cytometry with *M. smegmatis*

Flow cytometry with *M. bovis* BCG

Frequency Histograms for *M. smegmatis* and *M. bovis* BCG

PAF C-16 inhibits mycobacterial growth
Figure 9

(A) PAF C-16 inhibits mycobacterial growth

(B) PAF C-16 inhibits mycobacterial growth

(C) PAF C-16 inhibits mycobacterial growth