Dissecting the mechanism of intracellular *Mycobacterium smegmatis* growth inhibition by Platelet activating factor C-16

Muhammad Suleman Riaz\(^1\), Anuvinder Kaur\(^1\), Suha Nadim Shwayat\(^1\), Shahriar Behboudi\(^3\), Uday Kishore\(^1\), Ansar Ahmed Pathan\(^1\)*

\(^1\)College of Health and Life Sciences, Division of Biosciences, Brunel University London, Uxbridge, UB8 3PH, United Kingdom

\(^2\)Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan.

\(^3\)The Pirbright Institute, Ash Road, Pirbright, Surrey, GU24 0NF, United Kingdom

\(^4\)Faculty of Health and Medical Sciences, School of Veterinary Medicine, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom.

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**Running Title:** PAF C-16 inhibits intracellular *M. smegmatis* growth

*Correspondence:* Dr Ansar Ahmed Pathan (E-mail: Ansar.Pathan@brunel.ac.uk).
Abstract

*Mycobacterium tuberculosis* (*M.t.b*) infection results in approximately 1.3 million human deaths each year. *M.t.b* resides primarily inside macrophages, and maintains persistent infection. In response to infection and inflammation, platelet activating factor C-16 (PAF C-16), a phospholipid compound, is released by various cells including neutrophils and monocytes. We have recently shown that PAF C-16 can directly inhibit the growth of two representative non-pathogenic mycobacteria, *Mycobacterium bovis BCG* and *Mycobacterium smegmatis* (*M. smegmatis*), by damaging the bacterial cell membrane. Here, we have examined the effect of PAF C-16 on *M. smegmatis* residing within macrophages, and identified mechanisms involved in their growth inhibitory function. Our results demonstrated that exogenous PAF C-16 inhibited the growth of *M. smegmatis* inside phagocytic cells of monocytic cell line, THP1; this effect was partially blocked by PAF receptor antagonists, suggesting the involvement of PAF receptor-mediated signalling pathways. Arachidonic acid, a downstream metabolite of PAF C-16 signalling pathway, directly inhibited the growth of *M. smegmatis in vitro*. Moreover, the inhibition of phospholipase C and phospholipase A2 activities, involved in PAF C-16 signalling pathway, increased survival of intracellular *M. smegmatis*. Interestingly, we also observed that inhibition of inducible nitric oxide synthase (iNOS) enzyme and antibody-mediated neutralization of TNF-α partially mitigated the intracellular growth inhibitory effect of PAF C-16. Use of a number of PAF C-16 structural analogues, including Lyso-PAF, 2-O-methyl PAF, PAF C-18 and Hexanolamino PAF, revealed that the presence of acetyl group (CH$_3$CO) at sn-2 position of the glycerol backbone of PAF is important for the intracellular growth inhibition activity against *M. smegmatis*. Taken together, these results suggest that exogenous PAF C-16 treatment inhibits intracellular *M. smegmatis* growth, at least partially, in a nitric oxide and TNF-α dependent manner.
Introduction

*Mycobacterium tuberculosis* (*M. tb*) belongs to the acid-fast group of bacteria and causes an infectious disease in humans, known as Tuberculosis (TB), which mostly affects the respiratory system. Approximately 1.3 million people died as a result of *M. tb* infections in 2017, which is the highest number of human mortalities caused by any single bacterial pathogen (World Health Organization, 2018). Nearly 10 million new cases of *M. tb* infection were reported worldwide during 2017, the majority of infected population belongs to developing countries such as India, Indonesia, China, the Philippines, Pakistan and Nigeria (World Health Organization, 2018). It is estimated that about one-third of the world’s population are latently infected with *M. tb* (Flynn and Chan, 2001). The latently infected individuals show no evidence of active disease due to the containment of the pathogen by the host immune system. Over the course of time, this latent *M. tb* infection can reactivate into active disease, and thus, provides a vast reservoir for the spread of *M. tb* infection.

New challenges such as HIV and *M. tb* co-infections in TB patients, multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tb* have also emerged recently. During HIV and *M. tb* co-infection, the virus infects the CD4+ T cells, which are the most important immune cells involved in controlling *M. tb* infection (Daley et al., 1992). The HIV infection, thus, not only predisposes the patients to new *M. tb* infections, but also increases the chances of reactivation of latent TB due to host’s immunocompromised status. The emergence of MDR and XDR strains of *M. tb* is a global challenge in treating TB patients as the patients fail to respond to multiple anti-TB drugs, and hence, can act as a reservoir for the spread of drug-resistant *M. tb* strains (Liu et al., 2011; Ormerod, 2005; Espinal et al., 2000). The current vaccine against TB consists of attenuated *Mycobacterium bovis* strain, Bacillus Calmette-Guérin (BCG), which is almost 100 years old with variable efficacy, and is not effective in the adult population (Lahey and von Reyn, 2016; Aronson et al., 2004; Colditz et al., 1994; Sepulveda et al., 1992). Therefore, to control the global menace of TB, novel interventions are required on the therapeutic and preventive fronts.

*M. tb* infection of the host evokes localized inflammation in the lungs, resulting in the migration of different immune cells and the leakage of plasma proteins and non-proteinaceous factors at the site of infection due to changes in vascular permeability (Amaral et al., 2016; Sherwood and Toliver-Kinsky, 2004). In addition, phospholipids, such as PAF C-16 and proteins such as C1q, are synthesized by the host’s immune cells, which are present at the site of infection (Kaul and Loos, 1995; Camussi et al., 1987). These host factors are likely to come in direct contact with the bacterial pathogens and immune cells, and thus, may modulate the outcome of the infection. The effects of the majority of these host factors on *M. tb* growth, intracellular as well as extracellular, are either poorly understood or completely unknown.

Platelet activating factor (PAF) is a phospholipid compound that is involved in a number of important biological processes in mammals, including platelet aggregation (Chesney et al., 1982), inflammation and allergy (Henderson et al., 2000). Chemically, PAF is 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine (Demopoulos et al., 1979). The most common form of naturally produced PAF in humans contains a 16-carbon chain attached at the sn-1 position via ether linkage, and is known as PAF C-16 (Clay et al., 1984). PAF C-16 is normally present in picogram per milliliter concentrations in human serum; however, its production is increased during inflammation, allergic reactions and in newly diagnosed TB patients, particularly in cavitary form of pulmonary TB (Vadas et al., 2013; Kaminskaia and Aladysheva, 1995). Moreover, neutrophils from TB patients, when stimulated with BCG *in vitro*, produced 3 times more PAF C-16 than control (Kaminskaia and Aladysheva, 1995). A variety of cell types including platelets (Alam et al., 1983), monocytes/macrophages (Yagnik, 2014; Leaver et al., 1984), and macrophages (Yagnik, 2014; Leaver et al., 1984).
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1990), neutrophils (Biffl *et al.*, 1996), endothelial cells (Bussolino *et al.*, 1986) and mast cells (Schleimer *et al.*, 1986) are able to produce PAF C-16 upon stimulation. PAF C-16 binds to a specific G-protein coupled receptor, PAF receptor (PAFR), on the target cells (Ishii *et al.*, 2002). PAF-PAFR engagement activates downstream signalling pathways, including the activation of phospholipases (PLC and PLA₂), kinases such as protein tyrosine kinase and protein kinase C as well as the production of cytokines such as TNF-α, IL-1α and prostaglandins (Honda *et al.*, 2002; Ishii and Shimizu, 2000).

It has been shown that PAF C-16 possess direct growth inhibition activity against mycobacteria (*M. smegmatis* and *M. bovis* BCG) (Riaz *et al.*, 2018) and a number of Gram-positive bacteria (Steel *et al.*, 2002) by causing damage to the cell membrane. Exogenous PAF C-16 has also been shown to inhibit the growth of intracellular pathogenic protozoans such as Leishmania and Trypanosoma inside human and mouse macrophages by causing the production of reactive oxygen and nitrogen species (Borges *et al.*, 2017; Lonardoni *et al.*, 2000; Aliberti *et al.*, 1999). Similarly, administration of exogenous PAF C-16 in mice, infected with lethal doses of *Candida albicans*, reduced the number of pathogens and improved the survival via production of NO and TNF-α in serum (Kim *et al.*, 2008; Im *et al.*, 1997).

In the current study, we examined PAF C-16 and its structural analogues in vitro for their effect on the growth of *M. smegmatis* (as a model for *M. tb*) inside human monocytes derived THP-1 cells. Furthermore, the underlying mechanisms of PAF C-16 induced growth inhibition of intracellular *M. smegmatis* were also investigated.

**Experimental procedures**

**Chemicals**

PAF C-16 (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine) and different PAF C-16 structural analogues, including Lyso-PAF (1-O-hexadecyl-2- hydroxy-sn-glyceryl-3-phosphorylcholine), PAF C-18 (1-O-octadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine), Hexanolamino PAF (1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphoryl (N,N,N-trimethyl) hexanolamine), 2-O-methyl PAF (1-O-hexadecyl-2-O-methyl-sn-glyceryl-3-phosphorylcholine), as shown in Figure 1, along with ABT-491 (1-(N,N-Dimethylcarbamoyl)-4-ethyl-3-(3-fluoro-4-((1H-2-methylimidazo[4,5-c]pyridin-1-yl)methyl)benzoyl)-indole, HCl), U-73122 (1-[6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) and arachidonic acid were purchased from Cayman Chemical Company, USA. WEB-2086 (3-[4-(2-Chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-2-yl]-1-(4-morpholinyl)-1-propanone), benzenesulfonamide and aminoguanidine hemisulfate were purchased from Sigma-Aldrich Company USA. All the chemicals used in different experiments were of analytical grade.

Stock solutions of PAF C-16, PAF C-16 structural analogues, WEB-2086, U-73122 and arachidonic acid were prepared in ethanol according to the manufacturer’s protocoll. ABT-491 and aminoguanidine hemisulfate stock solutions were prepared using distilled water; while the stock solution of benzenesulfonamide was prepared in methanol. Appropriate solvent controls were included in all the experiments involving PAF C-16, its structural analogues or any other chemical compound used.

**Culturing of *M. smegmatis***

*M. smegmatis* (mc² 155) were grown in Luria-Bertani (LB) broth (Lennox; Sigma Aldrich) containing 50µg/ml carbenicillin (Fisher Scientific, UK) 0.15% (v/v) glycerol (Fisher Scientific, UK) and 0.10% (v/v) Tween-80 (Fisher Scientific, UK) in a shaking incubator at
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37°C until the O.D$_{(600\text{nm})}$ reached 0.8-0.9. The number of *M. smegmatis* colony forming units (CFUs) per µl 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 72 hours.

The LB agar plates for growing *M. smegmatis* colonies were prepared by dissolving tryptone (10 g) (Fisher Scientific, UK), yeast extract (5 g) (Fisher Scientific, UK), sodium chloride (0.5 g) (Fisher Scientific, UK) and agar (15 g) (Fisher Scientific, UK) in 1 litre distilled water and autoclaving the media at 121°C for 15 minutes. About 15-20 ml of melted LB agar was poured in each plate (petri dish) under sterile conditions and the plates were allowed to solidify for 90-120 minutes before plating *M. smegmatis*.

**THP-1 cell culture**

THP-1 cells, (a human monocytic leukemic cell line; (ATCC® TIB-202™), used as the model phagocytic cells, were grown in complete RPMI (cRPMI) medium at 37°C using an incubator with 5% CO$_2$ supply. The cRPMI medium was prepared by adding 10% v/v fetal bovine serum (FBS) (HyClone, UK), 1 mM sodium pyruvate (Sigma Aldrich, UK), 2 mM L-glutamine (Sigma Aldrich, UK) and PenStrep (100U/mL Penicillium and 100µg/mL Streptomycin) (Sigma Aldrich, UK) to RPMI-1640 (Sigma Aldrich, UK). Cells were fed every 3 days by removing half of the culture medium and replacing it with fresh cRPMI; the cell density was kept at about 0.5x10$^6$-0.75x10$^6$/ml.

**Intracellular growth inhibition assay for *M. smegmatis* using THP-1 cells**

Intracellular bacterial growth inhibition assays were performed to investigate the effect of different test compounds, including PAF C-16 and PAF structure analogues, on the growth of phagocytosed *M. smegmatis* inside THP-1 cells. THP-1 cells, grown at a density of 0.5-0.75x10$^6$/ml, were washed twice with plain RPMI medium and adjusted to ~0.25x10$^6$ cells/ml in cRPMI without antibiotics. One ml of this cell suspension was added to individual eppendorf tubes. Approximately 1.25x10$^6$ *M. smegmatis* (bacteria to THP1 ratio; 5:1) was then added to each tube. These tubes were incubated at 37°C in a CO$_2$ incubator for 2 hours with intermittent shaking to allow phagocytosis of bacteria.

Pan anti-mouse IgG coated magnetic Dynabeads (Thermo Fisher Scientific, UK), bound to mouse pan anti-human MHC class I (HLA A, B & C) antibody W6/32 (BioLegend, USA), were prepared to remove non-phagocytosed *M. smegmatis*. For each sample, 10$^6$ Dynabeads were incubated with 1µg of W6/32 for 90 minutes on ice to allow the binding of the beads with W6/32 antibody. Later, the beads were washed twice with plain RPMI by applying a Dynal® magnet (Thermo Fisher Scientific, UK) and this reagent (Dynabeads-W6/32) was resuspended in 25µl of RPMI. The ‘Dynabeads-W6/32’ were then added to the previously prepared tube, containing THP-1 cells with *M. smegmatis* (beads to cells ratio; 4:1). The eppendorf tubes were then placed on ice for 45 minutes with intermittent shaking to allow the binding of W6/32 component of ‘Dynabeads-W6/32’ with MHC Class I molecules present on THP-1 cells. After incubation, extracellular *M. smegmatis* was removed by applying a Dynal® magnet and washing the cells twice with plain RPMI. This was followed by resuspending the cells in 1ml cRPMI without antibiotic. For each experiment, a solvent control and test compounds treated samples were included and the tubes were incubated at 37°C in a CO$_2$ incubator for another 24 hours. After incubation, Dynal® magnet was applied to the eppendorf tubes. The ‘Dynabeads-W6/32’attached to THP-1 cells migrated to the side of eppendorf tube. The supernatant was removed and stored in 15ml falcon tubes to collect any extracellular bacteria released from dying cells. The THP-1 cells left in the eppendorf tubes were lysed by adding 1ml of 1% w/v saponin solution in water (Fisher Scientific, UK) and vortexing the mixture for 15 minutes.
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The cell lysate for each condition was transferred to the respective 15ml falcons already containing 1ml of bacterial supernatant, collected earlier. The contents of each falcon tube were mixed by vortexing for 10 seconds and serial dilutions (10<sup>1</sup>, 10<sup>2</sup> and 10<sup>3</sup>) were prepared in sterile PBS.

Finally, the bacterial suspensions, at dilutions of 10<sup>2</sup> and 10<sup>3</sup>, were used for plating. 200µl of bacterial suspension was plated for each experimental condition in triplicates using LB-agar plates. Plates were incubated at 37°C for 72 hours, after which the number of bacterial CFUs were counted. A comparison of CFUs between test compound treated plates and the solvent control was done to determine the effect of the test compound on the growth of intracellular *M. smegmatis* inside THP-1 cells.

**Assessing the effects of PAF receptor antagonists on PAF C-16 induced intracellular *M. smegmatis* growth inhibition**

Two PAF receptor (PAFR) antagonists, ABT-491 and WEB-2086, were used to examine their effects on PAF C-16 induced intracellular *M. smegmatis* growth inhibition. The assays were carried out according to the intracellular growth inhibition assay as described before, with a difference that *M. smegmatis* infected THP-1 cells were initially treated with PAFR antagonists (ABT-491 or WEB-2086, 2µg/ml each) for 1 hour before adding PAF C-16 (1µg/ml) to the cell culture, the samples were incubated further for 24 hours at 37°C in a CO<sub>2</sub> incubator. Solvent control along with an additional control comprising of *M. smegmatis* infected THP-1 cells treated with PAFR antagonists only, were also included in the experimental design.

**Assessing the effects chemical inhibitors of PLC, PLA<sub>2</sub> and iNOS and anti-TNF-α antibody on PAF C-16 induced intracellular *M. smegmatis* growth inhibition**

Assays using inhibitors of PLC (U-73122), cPLA<sub>2</sub> (benzenesulfonamide) and iNOS (aminoguanidine hemisulfate) were also performed similar to the intracellular growth inhibition assay described before. The only difference was in the treatment step where *M. smegmatis* infected THP-1 cells were first treated with U-73122 (2μM) (Macmillan and McCarron, 2010), benzenesulfonamide (56nM) (Farooqui et al., 2006) or aminoguanidine hemisulfate (1mM) (Nascimento et al., 2002) for 1 hour. Subsequently, PAF C-16 (1µg/ml) was added and the THP-1 cells containing phagocytosed *M. smegmatis* were further incubated for 24 hours at 37°C in a CO<sub>2</sub> incubator. An additional control comprising of *M. smegmatis* infected THP-1 cells treated with U-73122 (2µM), benzenesulfonamide (56nM) or aminoguanidine hemisulfate (1mM), was also included in the respective experimental designs.

Anti-TNF-α neutralizing antibody (BD Biosciences, USA) was also used to investigate the role of TNF-α in PAF C-16 induced growth inhibition of intracellular *M. smegmatis*, according to the intracellular growth assay described earlier. *M. smegmatis* infected THP-1 cells were treated with 10µg/ml of mouse anti-human TNF-α antibody, an isotype antibody control (10µg/ml mouse IgG + 1µg/ml PAF C-16) or ethanol (PAF C-16 solvent) for 1 hour prior to treatment with 1µg/ml PAF C-16 and the cells were further incubated for another 24 hours before cell lysis and plating.

**Direct growth inhibition assay**

This assay was carried out as described previously (Riaz et al., 2018), to investigate the direct effect of arachidonic acid on *M. smegmatis* growth. Briefly, the diluted stock of *M. smegmatis* (2.5x10<sup>4</sup>) in suspensions of 1ml LB broth was exposed to a range of concentrations of arachidonic acid for 2 hours at 37°C with mixing every 15 minutes. Appropriate solvent control (10µl/ml) for the test compound was also included. After incubation, 200µl of bacterial
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Suspensions from test compound treated and solvent control tubes were seeded on LB agar plates in triplicate and the plates were incubated at 37°C for 72 hours. Colony counting method was used to detect the direct growth inhibitory effects of test compounds.

**Statistical analysis**

At least three independent experiments were performed. The data were presented as box plots of medians (25th-75th percentile), and the solvent treated samples (solvent control) were considered 100% bacterial survival. Statistical analysis was performed using GraphPad Prism® software (Version 5.01) to determine the level of significance (*p*-value). For intracellular growth inhibition assays, *p*-value was determined by applying non-parametric multiple comparison Kruskal-Wallis test on ranks and any two datasets were compared using Dunn’s post hoc 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 by ** and *p*≤0.001 by ***.

**Results**

**PAF C-16 suppresses *M. smegmatis* growth inside THP-1 cells**

Intracellular growth inhibition assays were performed by treating *M. smegmatis* infected THP-1 cells with PAF C-16 at concentrations ranging between 0.001µg-10µg/ml (1.90nM-19.09µM) for 24 hours and the resulting growth inhibitory effect was determined by counting the number of surviving CFUs on LB agar plates. PAF C-16, at a concentration of 1µg/ml (1.90µM), on average inhibited the growth of intracellular *M. smegmatis* by 49% (n=4, independent experiments), compared with the solvent control (10µl ethanol/ml cell suspension) (*p*≤0.01), (Figure 2). Treatment of *M. smegmatis* infected THP-1 cells with PAF C-16 at concentrations higher or lower than 1µg/ml (0.001µg (1.90nM), 0.01µg (19.09nM), 0.1µg (190.9nM), 5µg (9.5µM) and 10µg/ml (19.09µM)) for 24 hours showed no significant inhibitory effect on the growth of intracellular *M. smegmatis* (Figure 2).

**Acetyl group at sn-2 position is essential for the inhibitory effect of PAF C-16 on *M. smegmatis* growth inside THP-1 cells**

To assess the impact of small modifications in the structure of PAF C-16 on its intracellular *M. smegmatis* growth inhibition potential, different PAF C-16 structural analogues, including its precursor form Lyso-PAF, 2-O-methyl PAF, PAF C-18 and Hexanolamino PAF (Figure 1) were tested. The results showed that structural analogues lacking an acetyl group at sn-2 position such as Lyso-PAF and 2-O-methyl PAF were unable to inhibit the growth of intracellular *M. smegmatis* at concentrations range of 1µg, 5µg and 10µg/ml (Figure 3A and 3B). Both PAF C-18 and Hexanolamino PAF having acetyl group at sn-2 position were able to inhibit the growth of intracellular *M. smegmatis* at concentrations range of 1µg, 5µg and 10µg/ml (Figure 3C and 3D) and their intracellular *M. smegmatis* growth inhibition ability was similar to PAF C-16 at 1µg/ml (~50% reduction in CFUs).

**PAF receptor antagonists partially mitigated PAF C-16 induced intracellular *M. smegmatis* growth inhibition**

Two structurally distinct PAFR antagonists, ABT-491 and WEB-2086, were used to investigate their effect on PAF C-16 induced growth inhibition of *M. smegmatis*. Prior treatment of *M. smegmatis* infected THP-1 cells with ABT-491 or WEB-2086 (Test-1 and Test-2, Figure 4) at a concentration of 2µg/ml partially mitigated the inhibitory effect of PAF C-16 on the growth of intracellular *M. smegmatis* as indicated by an increase in the number CFUs (20-24.5%) compared to 1µg/ml PAF C-16 treated only condition (n=4, independent...
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experiments) (Figure 4A, 4B). This difference was significant (*p*=0.02, Mann-Whitney test) for both PAFR antagonists. In the absence of PAF C-16, ABT-491 as well as WEB-2086, showed no effect on the survival of intracellular *M. smegmatis* on their own (Figure 4A, 4B).

**PLC inhibitor (U-73122) partially overcomes PAF C-16 induced intracellular *M. smegmatis* growth inhibition**

PAF C-16 binding to its receptor causes the activation of phospholipase C (PLC). PLC inhibitor, U-73122, was therefore, used to investigate the role of PLC in PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. It was observed that U-73122 at a concentration of 2µM was effective in partially mitigating the inhibitory effect PAF C-16 on the growth of intracellular *M. smegmatis*. Treatment of *M. smegmatis* infected THP-1 cells with U-73122 along with 1µg/ml PAF C-16 resulted in ~25.2% increase in the number of surviving *M. smegmatis* CFUs when compared to 1µg/ml PAF C-16 only treated condition (Figure 5); this difference was significant (*p*=0.02, Mann Whitney test).

**PLA₂ inhibitor (Benzenesulfonamide) partially overcomes PAF C-16 induced intracellular *M. smegmatis* growth inhibition**

PAF C-16 also causes the activation of cytosolic phospholipase A₂ (cPLA₂), which leads to the intracellular production of arachidonic acid (AA) and lysophosphatidylcholine. Benzenesulfonamide, a cPLA₂ inhibitor, was used to investigate the role of cPLA₂ in PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 cells were treated with benzenesulfonamide at a concentration of 56nM along with 1µg/ml PAF C-16 for 24 hours. Benzenesulfonamide partially mitigated the inhibitory effect PAF C-16 on the growth of intracellular *M. smegmatis* as indicated by an increase in the number of surviving *M. smegmatis* CFUs. On average, this compound increased the number of *M. smegmatis* CFUs by ~30.1% when compared to the number of CFUs from 1µg/ml PAF C-16 only treated condition (Figure 6), and this difference was significant (*p*=0.02, Mann Whitney test).

**Arachidonic acid (AA) directly inhibits *M. smegmatis* growth in vitro**

Arachidonic acid (AA) is produced by activated cPLA₂ from intracellular phospholipids during PAF C-16 signalling pathway. This compound was investigated *in vitro* for its direct effect on the growth of *M. smegmatis* and the results showed dose-dependent growth inhibition of *M. smegmatis* in all the three independent experiments. AA at the concentrations of 5µg/ml and 2.5µg/ml caused a reduction of 99% and 86.4%, respectively in the number of surviving *M. smegmatis* CFUs as compared to solvent control (Figure 7); these results were found to be significant (*p*≤0.001 and *p*≤0.01 respectively) using Dunn’s multiple comparison test. Similarly, other intracellular molecules, Phosphoinositol bisphosphate (PIP₂) and Diacylglycerol (DAG) produced during PAF C-16 signalling were also tested *in vitro* at concentrations of 5µg, 10µg, 25µg, 50µg and 100µg/ml, however, they showed no direct inhibitory effect on *M. smegmatis* growth (supplementary Figure 3 & Figure 4).

**Nitric oxide synthase inhibitor (aminoguanidine hemisulfate) partially overcomes PAF C-16 induced growth inhibition of intracellular *M. smegmatis***

Treatment of *M. smegmatis* infected THP-1 cells with iNOS inhibitor, aminoguanidine hemisulfate (1mM), partially mitigated PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. On an average, prior treatment of THP-1 cells having phagocytosed *M. smegmatis* with 1mM AG increased the number of surviving *M. smegmatis* CFUs by 23.4% when compared to 1µg/ml PAF C-16 only (Figure 8) and this difference was significant (*p*=0.02, Mann-Whitney test).
Anti-TNF-α antibody partially overcomes PAF C-16 induced intracellular \textit{M. smegmatis} growth inhibition

To investigate the role of TNF-α on PAF C-16 induced intracellular growth inhibition, \textit{M. smegmatis} infected THP-1 cells were incubated with 10µg/ml mouse anti-human TNF-α monoclonal antibody (neutralizing antibody) and 1µg/ml PAF C-16 for 24 hours. This antibody partially mitigated the PAF C-16 induced growth inhibition of intracellular \textit{M. smegmatis}, as indicated by 17% and 18% increase in the number of surviving CFUs as compared to the isotype control antibody (10µg/ml mouse IgG + 1µg/ml PAF C-16) and 1µg/ml PAF C-16 only treated conditions respectively (Figure 9) ($p=0.02$, Mann-Whitney test). Furthermore, neutralizing antibodies specific to IL-6 and IL-10 showed no effect on the intracellular growth inhibitory effect of PAF C-16 (supplementary Figure 1 and Figure 2).

Discussion

PAF C-16 is a pro-inflammatory phospholipid; its production increases during inflammation (Gawaz \textit{et al}., 2005) and bacterial infections including \textit{M.tb} infection (Huseyinov \textit{et al}., 1999, Kaminskaia and Aladysheva, 1995). In this study, the effect of exogenous PAF C-16 was investigated \textit{in vitro} on the growth of intracellular mycobacteria. Phagocytic THP-1 cells infected with \textit{M. smegmatis} were used as a model to investigate the intracellular growth inhibitory effect of PAF C-16. THP-1 cells are human leukaemia derived monocytic cells that mimic blood-derived monocytes and are a valuable tool for research because of their homogenous genetic background (Chanput \textit{et al}., 2014). THP-1 cells have previously been used as an \textit{in vitro} model to study intracellular mycobacteria (Iona \textit{et al}., 2012; Fontán \textit{et al}., 2008; Rohan \textit{et al}., 2008; Rajavelu and Das, 2007). THP-1 cells are usually treated with phorbol 12-myristate 13-acetate (PMA) in order to differentiate them into macrophages (Riendeau and Kornfeld, 2003). PMA treatment of THP-1 cells causes the rearrangement of macrophage-specific kinome that leads to the activation of pro-inflammatory genes, such as IL-1β, IL-8 and TNF-α, making these cells biased towards a more pro-inflammatory phenotype (Richter \textit{et al}., 2016). Furthermore, IL-1β has been shown to be a potent stimulator of PAF C-16 synthesis in monocyte cell line U-937 (Vlachogianni \textit{et al}., 2013). In addition, PAF C-16 is known to modulate the ratio of GHS/GSSG and also modulates the redox status of monocytes (Verouti \textit{et al}., 2011). Therefore, in our study, untreated THP-1 cells were used to provide a microenvironment in which the specific effect of PAF C-16 on the growth of intracellular \textit{M. smegmatis} could be examined.

PAF C-16 can activate monocytes/macrophages by binding to PAFR (Simon \textit{et al}., 1994) and stimulate the production of inflammatory mediators such as TNF-α, IL-1α and β, reactive oxygen species (ROS) and reactive nitrogen species (Muehlmann \textit{et al}., 2012; Poubelle \textit{et al}., 1991; Bonavida \textit{et al}., 1989; Hartung \textit{et al}., 1983), and thus may play a protective role during infection. We have recently shown that PAF C-16 and its analogues have direct inhibitory effect on the growth of mycobacteria (Riaz \textit{et al}., 2018). Therefore, we investigated the effects of these compounds on mycobacterial survival inside phagocytic cells, as these cells have the ability to control mycobacterial infection when they are in an activated state (Leemans \textit{et al}., 2005). We hypothesised that PAF C-16 and its structurally related compounds may activate macrophages to enhance its intracellular killing ability. In this study, PAF C-16 at an optimal dose of 1µg/ml was shown to inhibit \textit{M. smegmatis} growth inside THP-1 cells, whereas any deviation (increase or decrease) from this optimal concentration was ineffective. This observation suggests that PAF C-16 acts in a concentration-specific manner. This is consistent with the previous study demonstrating that the treatment of human macrophages with higher concentration ($10^{-8}$ M) of PAF C-16 showed reduced phagocytosis of \textit{Leishmania braziliensis} as compared to cells treated with the lower concentrations ($10^{-8}$ and $10^{-10}$ M) (Borges \textit{et al}.,
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2017). Similarly, human monocytes showed maximum production of ROIs when stimulated with PAF C-16 at 2x10^6 M; increasing the concentration of PAF C-16 (5x10^6 M) led to a decrease in ROIs production (Pustynnikov *et al*., 1991). The fact that PAF C-16 at higher concentrations (5μg and 10μg/ml) showed less intracellular growth inhibition than 1μg/ml in our study, may be due to higher level of PAFR down regulation at these concentrations, as a result of the interaction of PAF C-16 with PAFR. This explanation is supported by the observation made by Choa *et al*., who showed that PAF C-16 binding to PAFR results in down regulation of PAFRs in a dose dependent manner on the surface of Kuffer cells of rat (Chao *et al*., 1990).

A number of previous studies have shown that exogenous PAF C-16 can inhibit the growth of intracellular pathogens, including *Leishmania donovani* (Lonardoni *et al*., 2000), *Leishmania braziliensis* (Borges *et al*., 2017), *Trypanosoma cruzi* (Aliberti *et al*., 1999) and *Candida albicans* (Kim *et al*., 2008; Im *et al*., 1997) both *in vivo* as well as *in vitro*. PAF C-16 induced growth inhibition of these intracellular pathogens was shown to be associated with an enhanced production of reactive oxygen intermediate species (ROIs), reactive nitrogen intermediate species (RNIs) and TNF-α. There is limited information regarding the effect of PAF C-16 on the growth of intracellular mycobacteria. One study investigating the role of endogenous PAF C-16 during *M.tb* infection in mice found that there was no significant difference in mortality between PAFR deficient (*PAFR^−/−*) and wild-type control mice when infected with *M.tb* and similar *M.tb* loads were observed in the lungs and liver (Weijer *et al*., 2003). However, to the best of our knowledge, there is currently no information about the effect of exogenous PAF C-16 or its structural analogues on the growth inhibition of intracellular mycobacteria.

The biological activity of PAF C-16 in eukaryotic cells can be affected by small modifications in its structure (Stewart and Grigoriadis, 1991; Rose *et al*., 1990; O’Flaherty *et al*., 1987; Shigenobu *et al*., 1985). Therefore, the effect of small changes in the structure of PAF C-16 was also investigated on the growth inhibition of intracellular *M. smegmatis* using different PAF C-16 structure analogues. Lyso-PAF is the precursor form of PAF C-16 that contains a hydroxyl group in place of the acetyl group at sn-2 position of the glycerol backbone. The enzyme platelet-activating factor acetylhydrolase (PAF-AH) tightly regulates the level of active PAF C-16 in the body and converts excess PAF C-16 into Lyso-PAF (McIntyre *et al*., 2009). Due to the inability of Lyso-PAF to perform most of the biological functions associated with PAF C-16, this precursor analogue is mostly used as a control in experiments performed with PAF C-16 (Montrucchio *et al*., 2000). We observed that exogenous Lyso-PAF failed to inhibit the growth of intracellular *M. smegmatis*, suggesting that the acetyl group at position sn-2 was important for the intracellular growth inhibitory effect of PAF C-16. Another synthetic PAF C-16 analogue, 2-O-methyl PAF (the acetyl group of PAF C-16 at position sn-2 is replaced by a methyl group), was also unable to inhibit the growth of intracellular *M. smegmatis*. Structural analogues with changes in functional group at sn-2 position have previously been shown to lack different PAF C-16 associated activities such as aggregation of platelets (McManus *et al*., 1981), NO production from endothelial cells (Kikuchi *et al*., 2008), excitation of synaptic transmission in neuronal cells (Clark *et al*., 1992), and bronchial hyper-responsiveness (Cuss *et al*., 1986).

PAF C-18, a naturally occurring PAF C-16 analogue, was also used to investigate the effect of increase in the number of carbon atoms in the aliphatic carbon tail on the intracellular *M. smegmatis* growth inhibition. PAF C-18 has two additional carbon atoms in the aliphatic carbon tail attached at position sn-1 as compared to PAF C-16, was also able to inhibit the growth of intracellular *M. smegmatis*. Previous studies have shown that PAF C-18 is less potent in
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inducing platelet aggregation but is as potent as PAF C-16 in activating guinea pig macrophages (Stewart and Grigoriadis, 1991).

Hexanolamino PAF differs from PAF C-16 in the position of the terminal amino group, which is linked by an additional 4-carbon atoms chain to the phosphate group. This compound also inhibited the growth of intracellular *M. smegmatis*. Hexanolamino PAF has previously been shown to act as both PAF C-16 antagonist and agonist. Hexanolamino PAF can inhibit PAF C-16 stimulated production of ROS by human macrophage (Rouis et al., 1988) and act as a partial PAF C-16 agonist in guinea pig macrophages (Stewart and Grigoriadis, 1991).

To investigate the role of PAF C-16 signalling through PAFR during intracellular growth inhibition of *M. smegmatis*, PAFR antagonists were used. PAFR antagonists are compounds that can bind to PAFR and reduce PAF C-16 activity by blocking PAFR (Singh et al., 2013). Prior treatment with PAFR antagonists, ABT-491 or WEB-2086, increased the growth of intracellular *M. smegmatis* as compared to the PAF C-16 only treated condition, suggesting *M. smegmatis* intracellular growth inhibition was partly mediated through PAFR. It has also been suggested that PAF C-16 can perform certain biological activities via pathways independent of PAFR signalling (Dyer et al., 2010). PAFR antagonist, ABT-491, has been shown to be highly effective in suppressing PAF C-16 induced platelet degranulation and PAF C-16 mediated pathological conditions such as inflammation, hypotension and other lethal effects in rat and guinea pig models (Albert et al., 1997). Similarly, WEB-2086 is also a known potent PAFR antagonist, which has been shown to inhibit PAF C-16 induced activities such as platelet aggregation, hypotension and vascular permeability in rats (Clavijo et al., 2001).

Binding of PAF C-16 to PAFR results in the activation of intracellular signalling components. Therefore, we investigated the role of PAF C-16 intracellular signalling pathway components such as PLC, cPLA<sub>2</sub> and different second messengers in PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. Binding of PAF C-16 to PAFR on the target cell results in the activation of a membrane bound enzyme, PLC, through the associated G-proteins (Shukla, 1992). Treatment of *M. smegmatis* infected THP-1 cells with PLC inhibitor, U-73122, increased the number of surviving CFUs by 25.2%. During PAF C-16 signalling pathway, the activated PLC causes the production of second messengers, Diacylglycerol (DAG) and Inositol triphosphate (IP<sub>3</sub>) (Ishii and Shimizu, 2000). Since these second messengers are produced inside the cell and may come in contact with the intracellular *M. smegmatis*, we investigated PIP<sub>2</sub> and DAG for their direct inhibitory effect on the growth of *M. smegmatis*. However, both these compounds did not show any direct inhibitory effect on the growth of *M. smegmatis* when tested at a range of concentrations (5µg, 10µg, 25µg, 50µg and 100µg/ml) (Supplementary Figure 3 & Figure 4), suggesting they are not directly involved in the intracellular growth inhibition activity of *M. smegmatis*.

PAF C-16 binding to PAFR also causes the activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) due to increased levels of intracellular Ca<sup>2+</sup> (Ishii and Shimizu, 2000). Treatment of *M. smegmatis* infected THP-1 cells with cPLA<sub>2</sub> inhibitor, Benzenesulfonamide, increased the surviving *M. smegmatis* CFUs by 30% when compared to the *M. smegmatis* infected THP-1 cells treated with PAF C-16 alone. Activated cPLA<sub>2</sub> causes the production of arachidonic acid (AA) from phospholipids inside the cell (Nakashima et al., 1989). In our study, AA at a concentration as low as 2.5µg/ml (8.21µM) significantly reduced the number of surviving *M. smegmatis* CFUs in vitro. This compound has previously been shown to inhibit the growth of *Staphylococcus aureus*, *Bacillus licheniformis* and *Streptococcus pyogenes* (Zheng et al., 2005; Raychowdhury et al., 1985). AA has also been shown to be present in resting islet cells of Langerhans at a concentration of 15uM/2000 cells, which increases to 10-fold after stimulation.
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with carbacol (Ramanadham *et al.*, 2001). In normal human serum the AA is present at a concentration of 500nM/ml (Chilton *et al.*, 1996), whereas, in lungs the AA concentration reported is 1–5μg/ml (Manku *et al.*, 1983). Therefore, AA is available to interact with the both intra and extra-cellular pathogens. Collectively, these results appear to suggest that PAF C-16 signalling via PAFR leads to the production of an intermediate intracellular signalling molecule, AA, which possesses direct growth inhibition capacity and might be involved in the growth inhibition of intracellular *M. smegmatis*. Furthermore, activation of cPLA$_2$ leads to the production of Lyso-PAF from the membrane lipids inside the cell that can be converted into PAF C-16, resulting in its higher intracellular concentration. It is also known that PAF C-16 can activate its own synthesis in human monocytes (Valone *et al.*, 1991). This intracellular PAF C-16 can bind to its nuclear receptors and elicit various signalling pathways resulting in the expression of proinflammatory genes such as iNOS and COX-2 (Zhu *et al.*, 2006).

Nitric oxide (NO) and RNIs such as peroxynitrite have a damaging effect on mycobacteria (Jamaati *et al.*, 2017; Long *et al.*, 2005). The role of RNIs in PAF C-16 induced intracellular growth inhibition of *M. smegmatis* was investigated by using a potent iNOS inhibitor, Aminoguanidine hemisulfate (AG), which increased the survival of intracellular *M. smegmatis* by 23.4% as compared to PAF C-16 only treated condition. PAFC-16 has previously been shown to cause the production of NO and RNIs in murine cells including monocytes, macrophages and Kupffer cells through activation of iNOS enzyme (Mustafa *et al.*, 1996; Szabo *et al.*, 1993). NO and related nitrogen compounds have been shown to inhibit the growth of *M.tb* inside mouse macrophages (Bose *et al.*, 1999). Furthermore, it has been shown that PAF C-16 treatment of *T.cruzi* infected mouse macrophages results in the inhibition of intracellular parasite due to PAF C-16 induced NO production (Aliberti *et al.*, 1999).

We also observed that TNF-α neutralizing antibody (anti-TNF-α mAb) partially mitigated the growth inhibitory effect of PAF C-16 on intracellular *M. smegmatis*. PAF C-16 has previously been shown to stimulate macrophages to produce TNF-α (Ruis *et al.*, 1991; Dubois *et al.*, 1989). The protective role of TNF-α during *M.tb* infection is well established (Mohan *et al.*, 2001; Bean *et al.*, 1999; Keane *et al.*, 1997; Flynn *et al.*, 1995). Previous studies have shown that TNF-α is involved in the induction of NO by activation of iNOS genes in macrophages, which causes the elimination of intracellular pathogens such as *L.major* (Fonseca *et al.*, 2003) and *T.cruzi* (Silva *et al.*, 1995).

In conclusion, this study shows that exogenous PAF C-16 can inhibit the intracellular growth of *M. smegmatis* involving PAFR receptor signalling pathways, which is at least partially mediated in a Nitric oxide and TNF-α dependent manner. The underlying mechanism arising from this study is summarized in the Figure 10.

**Author Contributions Statement:** AAP and MSR contributed conception and design of the study. The work was supervised by AAP. MSR wrote the manuscript with contributions from AAP, UK and SB. MSR did the experiments with contribution from AK and SNS. MSR, AAP and AK analysed the data. All the researcher reviewed the final version of the draft approved for submission.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the content of this article.
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**Figure legends**

**Figure 1:** Chemical structures of PAF C-16 and its various analogues tested against *M. smegmatis*. The changes in structure for different analogues are highlighted in red circles.

**Figure 2:** Effect of PAF C-16 treatment on the growth of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 cell were treated for 24 hours either with solvent control (10µl ethanol/ml) or indicated concentrations of PAF C-16, before lysis and plating. The data shown as box plots represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. The data is expressed in terms of percentage, where solvent control is considered as 100% survival. Level of significance was calculated by applying multiple comparison non-parametric Kruskal-Wallis test on ranks ($p=0.0012$) and any two data sets were compared using post hoc Dunn’s multiple comparison, where PAF C-16 (1µg/ml) vs solvent control was found to be significant, $**p \leq 0.01$.

**Figure 3:** Effect of PAF C-16 structural analogues on the growth of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 cells were treated for 24 hours either with solvent control (10µl ethanol/ml), Lyso-PAF (Graph A), 2-O-methyl PAF (Graph B), PAF C-18 (Graph C) or Hexanolamino PAF (Graph D) at indicated concentrations, before lysis and plating. The data is expressed in terms of percentage, where solvent control is considered as 100% survival. The data shown as box plots represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant differences between tests samples (PAF analogues) v solvent controls were determined using Kruskal-Wallis test and any two data sets were compared using post hoc Dunn's multiple comparison ($**p \leq 0.01$ and $*p \leq 0.05$).

**Figure 4:** Effect of PAFR antagonists (ABT-491 and WEB-2086) on PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 samples were treated for 24 hours either with solvent control (10µl ethanol/ml), ABT-491 (2µg/ml), PAF C-16 (1µg/ml) and a combination of ABT-491(2µg/ml) and PAF C-16 (1µg/ml) (Test-1) (Graph A) or WEB-2086(2µg/ml) and a combination of WEB-2086 (2µg/ml) and PAF C-16 (1µg/ml) (Test-2) (Graph B), before lysis and plating. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different treated conditions are compared to it. The data shown as box plots represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant (*$p=0.02$) differences were found in case of both PAFR antagonists with PAF C-16 only treated samples using Mann-Whitney test.

**Figure 5:** Effect of PLC inhibitor U-73122 on PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 samples were treated for 24 hours either with solvent control (10µl ethanol/ml), U-73122 (2µM), PAF C-16 (1µg/ml) or a combination of U-73122 (2µM) and PAF C-16 (1µg/ml) (Test), before lysis and plating. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different treated conditions are compared to it. The data represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant (*$p=0.02$) difference was found for Test v 1µg/ml PAF C-16 using Mann-Whitney test.

**Figure 6:** Effect of cPLA$_2$ inhibitor Benzenesulfonamide on PAF C-16 induced growth inhibition of intracellular *M. smegmatis*. *M. smegmatis* infected THP-1 samples were treated for 24 hours either with solvent control (10µl ethanol/ml), Benzenesulfonamide (BS, 56nM), PAF C-16 (1µg/ml) or a combination of BS (56nM) and PAF C-16 (1µg/ml) (Test), before
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Lysis and plating. The data is expressed in terms of percentage, where solvent control is taken as 100% survival and different treated conditions are compared to it. The data represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant (*p* = 0.02) difference was found for Test v 1µg/ml PAF C-16 only treated condition using Mann-Whitney test.

**Figure 7: Direct effect of Arachidonic acid on *M. smegmatis* growth in vitro.** *M. smegmatis* samples were directly treated either with solvent control (10µl ethanol/ml) or indicated concentrations of arachidonic acid for 2 hours before plating. Data is expressed in percentage where solvent control is taken as 100% survival and different arachidonic acid treated conditions are compared to it. The data represent median, interquartile and minimal and maximal values for three individual experiments performed in triplicates. Statistically significant differences between arachidonic acid treated and solvent control samples were estimated using Kruskal-Wallis test (*p* = 0.004) and any two sets of data were compared using post hoc Dunn's multiple comparison. ** *p* ≤0.01 and *** *p* ≤0.001.

**Figure 8: Effect of Aminoguanidine hemisulfate treatment on PAF C-16 induced intracellular *M. smegmatis* growth inhibition.** *M. smegmatis* infected THP-1 were treated for 24 hours either with solvent control (10µl ethanol/ml), Aminoguanidine hemisulfate (AG, 1mM), PAF C-16 (1µg/ml) or a combination of AG (1mM) and PAF C-16 (1µg/ml) (Test), before lysis and plating. The data is expressed in terms of percentage, where solvent control is considered as 100% survival and different treatment conditions are compared to it. The data represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant (*p* = 0.02) difference was found for Test v 1µg/ml PAF C-16 using Mann-Whitney test.

**Figure 9: Effect of anti-TNF-α neutralizing antibody on PAF C-16 induced intracellular *M. smegmatis* growth inhibition.** *M. smegmatis* infected THP-1 cells were treated for 24 hours either with solvent control (10µl ethanol/ml), anti-TNFα antibody (10µg/ml), PAF C-16 (1µg/ml), a combination of isotype control antibody (10µg/ml) with PAF C-16 (1µg/ml) and anti-TNFα antibody (10µg/ml) with PAF C-16 (1µg/ml) (Test), before lysis and plating. The data is expressed in terms of percentage, where solvent control is considered as 100% survival and different treatment conditions are compared to it. The data represent median, interquartile and minimal and maximal values for four individual experiments performed in triplicates. Statistically significant (*p* = 0.02) differences were found for ‘Test’ v 1µg/ml PAF C-16 and ‘Test’ v Isotype control condition using Mann-Whitney test.

**Figure 10: Proposed mechanism for PAF C-16 induced growth inhibition of intracellular *M. smegmatis*.** Binding of PAF C-16 to its receptor PAFR on the target cell activates phosphatidylinositol specific phospholipase C (PI-PLC) enzyme which causes the production of second messengers IP$_3$ and DAG inside the cell. The second messenger IP$_3$ then causes the mobilization of intracellular Ca$^{++}$. DAG along with Ca$^{++}$ further leads to the activation of phosphokinase C (PKC) enzyme that upregulates the production of reactive nitrogen intermediates and TNF-α, which can inhibit the growth of intracellular *M. smegmatis*. In addition, the elevated level of intracellular Ca$^{++}$ also causes the activation of cytosolic phospholipase A$_2$ (cPLA$_2$) enzyme that hydrolyses phospholipids and results in the production of arachidonic acid inside the cell. This arachidonic acid has also the potential to inhibit the growth of *M. smegmatis*. 
PAF C-16 inhibits intracellular *M. smegmatis* growth

Figure 1.
PAF C-16 inhibits intracellular *M. smegmatis* growth

**Figure 2.**
PAF C-16 inhibits intracellular *M. smegmatis* growth

Figure 3.
PAF C-16 inhibits intracellular *M. smegmatis* growth

**Figure 4.**

- (A) Graph showing percent *M. smegmatis* survival across different treatments: Solvent Control, ABT, PAF C-16, Test 1. (n=4)
- (B) Graph showing percent *M. smegmatis* survival across different treatments: Solvent Control, WEB-2086, PAF C-16, Test 2. (n=4)
PAF C-16 inhibits intracellular *M. smegmatis* growth

Figure 5.
PAF C-16 inhibits intracellular *M. smegmatis* growth

Figure 6.
PAF C-16 inhibits intracellular *M. smegmatis* growth

Figure 7.
Figure 8.

PAF C-16 inhibits intracellular *M. smegmatis* growth
Figure 9.

PAF C-16 inhibits intracellular *M. smegmatis* growth
Figure 10.