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# *cis*-2 and *trans*-2-eicosenoic Fatty Acids Inhibit *Mycobacterium tuberculosis* Virulence Factor Protein Tyrosine Phosphatase B

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**Abstract:** The present study aims to investigate the potential inhibitory effect of eicosenoic fatty acids on protein tyrosine phosphatase B of *Mycobacterium tuberculosis* (PtpB). PtpB is recognized to play a vital role in *Mycobacterium tuberculosis* (Mtb) successful latent infection. It prevents the fusion even between phagocytosed mycobacteria with lysosomes so that the bacteria escape from degradation. We have over-expressed recombinant Mtb *PtpB* within *Escherichia coli* BL21(DE3), and further, we have used the protein for inhibition assay with *cis*-2 and *trans*-eicosenoic fatty acids. It is revealed that at a concentration of 16 µM, *cis*-2- and *trans*-2-eicosenoic fatty acids can inhibit PtpB by 63.72% and 74.67%, respectively. Docking analysis has confirmed strong interactions of PtpB with *cis*-2 and *trans*-2-eicosenoic fatty acids, with the binding energy of -60.40 and -61.60 kcal/mol, respectively. These findings underline both fatty acids' high potential to be further investigated to discover drugs against latent tuberculosis infection.

**Keywords:** Eicosenoic fatty acid, latent tuberculosis infection, protein tyrosine phosphatase B, molecular docking.

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## INTRODUCTION

The World Health Organization estimates that Mtb infects one-fourth of the world population in a latent fashion (1,2). Although not all infections develop into active tuberculosis cases, the number of people with tuberculosis (TB) is still very high. There are 10 million new TB cases with a death rate of 1.2 million registered annually (1).

At the onset of *M. tuberculosis* infection, macrophages -the host cells that act as the first line of the defense system- engulf the bacteria in a process called phagocytosis. Phagocytosis results in membrane-enclosed structures called phagosomes. Phagosomes undergo a series of maturation processes by fusion with early endosomes, late endosomes, and lysosomes. This maturation process includes several stages to form early,

intermediate, and, finally, mature phagolysosomes (3). The presence of specific marker proteins characterizes each stage. The mature form of phagolysosome is a membrane-enclosed structure with an acidic lumen (pH 4.5) containing an array of lysosome-derived proteases/hydrolases and reactive oxygen species (ROS). They collectively serve as host weapons to degrade invading bacterial cells (4).

Together with monocytes and dendritic cells, macrophages constitute the mononuclear phagocyte system (MPS). The hallmark of this system is the recognition and elimination of pathogens (5). Mtb and related species, however, are equipped with the ability to evade the hostile environment of macrophage cells. Mtb does this by secreting different types of molecules that can interfere and prevent phagosome fusion with lysosomes, which are otherwise lethal for the Mtb bacteria. They include mycobacterial lipid and glycolipid such as trehalose-6,6'-dimycolate (TDM) and lipoarabinomannan (LAM), respectively. TDM has been shown to facilitate Mtb survival by decreasing phagosomal acidification and phagolysosomal fusion in murine macrophage (6). LAM was reported to inhibit early phagosomal markers (7). The mycobacterial may also secrete proteins or enzymes, such as protein tyrosine phosphatase A (PtpA), protein tyrosine phosphatase B (PtpB), secretory acid phosphatase M, zinc-dependent metalloprotease 1, lipoamide dehydrogenase C, serine/threonine protein kinase G (PknG), and PEPGRS62 protein (5,8). These proteins have been demonstrated to play important roles in the Mtb survival from macrophage elimination. Once the bacteria escaped from the degradation process, they enter a latent infection phase (9). This so-called latent tuberculosis infection (LTBI) can last for years and even decades before resurrecting into active TB (10). Therefore, understanding the interactions between the Mtb pathogen and host macrophages is essential to overcome tuberculosis.

PtpA and PtpB are among the proteins secreted by Mtb, and both of them play a role in facilitating the persistence of mycobacterial infection (11). Deletion of PtpA and PtpB reduces Mtb survival within macrophages and dramatically reduces the Mtb bacillary load in the lung of chronical guinea pigs (12,13). Researchers proposed PtpA to interfere with the formation of the acidic lysosome by blocking the V-type proton pump responsible for the lysosome's acidification (14,15). The void of acidification eventually switches the lysosomal hydrolases into an inactive form, a condition that favors Mtb's survival (16). Likewise, PtpB protein phosphatase is also known to promote mycobacterial survival from host degradation. A recent report suggested that PtpB reduces the expression of proinflammatory cytokines as well as the apoptosis of macrophages (17). It also limits macrophages' bactericidal responses by inhibiting NF-κB and MAPK signaling pathways (18). Therefore, these phosphatases constitute a high potential to be research targets due to their essential role in Mtb infection.

It has recently been shown that *cis*-2 and *trans*-2-eicosenoic fatty acids can inhibit PtpA (19). A few reports suggest that PtpA and PtpB have common inhibitors, although the inhibitory effect may vary (20). In this context, the present study aims to reveal whether *cis*-2 and *trans*-2-eicosenoic fatty acids can also inhibit PtpB.

## MATERIALS AND METHODS

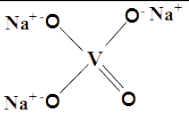
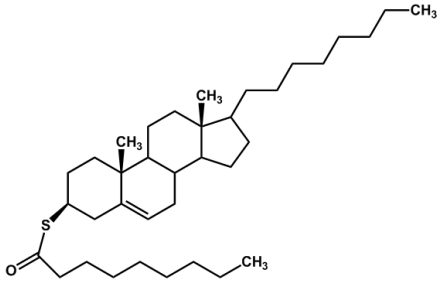
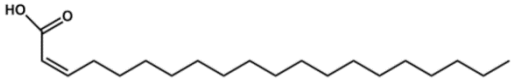
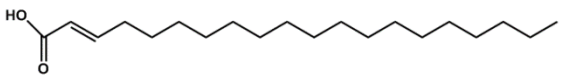
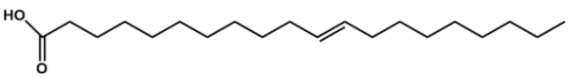
### Materials

Recombinant plasmid pET-30a bearing *M. Tuberculosis PtpB* is maintained in *Escherichia coli* strain XL1-Blue. The strain BL21(DE3) of *E. Coli* was used for expression. Bacterial growth media was standard lysogeny broth (LB), which consisted of 0.5% yeast extract (BD), 1% Tryptone (Bio Basic), 1% NaCl (Merck), 2% bacto agar (Difco) for plated culture, and 25 µg/mL kanamycin (Bioworld). The inducer of gene expression was Isopropyl 1-thio-β-D-galactopyranoside (IPTG), supplied by Thermo Scientific, whereas the substrate for PtpB was *para*-nitrophenyl phosphate (pNPP, Sigma). Materials for SDS PAGE were acrylamide and bis-acrylamide (Bio Basic), sodium dodecyl sulfate (Bio Basic), tetramethylethylenediamine (Bio Basic), ammonium persulfate (Bio Basic), glycine (Bio Basic), beta-mercaptoethanol (Sigma), bromophenol blue, and Coomassie Brilliant Blue (Sigma), dithiothreitol (Sigma) and protein ladder (Thermo Scientific). Sample degradation by protease was prevented by the addition of Phenylmethylsulfonyl fluoride (PMSF, Sigma). Guanidine hydrochloride (GuHCl) was purchased from MP BioScience.

Inhibitor *cis*-2- and *trans*-2-eicosenoic fatty acids were purchased from Larodan AB. *Trans*-11-eicosenoic acid and sodium orthovanadate were purchased from Sigma. S-((3S,10R,13R)-10,13-dimethyl-17-octyl-2,3,4,7,8,9,10,11,12,13,14,15, 16,17-tetradecahydro- 1H-cyclopenta[a] phenanthren-3-yl) nonanethioate or DTP was purchased from Interbioscreen. The inhibitors structures are shown in Table 1.

DMSO (MP BioScience) was used as the solvent for the substrate in the presence of imidazole (Thermo Scientific). Graphics were plotted in Graphpad Prism 7. Docking analysis was performed by Discovery Studio (Accelrys, San Diego, CA, USA).

**Table 1:** Compounds tested their inhibitory effect on PtpB.

| No | Compound | Structure/formula | Note |
|---|---|---|---|
| 1 | Sodium orthovanadate |  | general inhibitor for phosphatases as a control (21) |
| 2 | DTP* |  | proposed to inhibit PtpB (22) |
| 3 | *cis*-2-eicosenoic acid |  | PtpA inhibitor (19) |
| 4 | *trans*-2-eicosenoic acid |  | PtpA inhibitor (19) |
| 5 | *trans*-11-eicosenoic acid |  | a related fatty acid with double bond position lies apart from carboxyl group |

*) DTP: S-((3S,10R,13R)-10,13-dimethyl-17-octyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl) nonanethioate

**Methods**

*Expression and Production of PtpB*

The recombinant pET30a plasmid bearing the Mtb *PtpB* gene was used to transform *Escherichia coli* BL21(DE3). Expression of PtpB under the control of T7 promotor within the pET30a vector was induced by adding 0.5 mM IPTG to the culture of transformant *E. coli* (23) in its early log phase, i.e., when the culture reached to $OD_{600}$ of **"**0.3. Incubation of *E. coli* culture was prolonged at 37 °C with a shaking speed of 250 rpm and harvested after 4 hours of induction. The high PtpB expression leads to the formation of an inclusion body (IB), which is further subjected to the recovery process described below.

*Recovery of PtpB* and activity assay

Recovery of PtpB from inactive IB was made re-solubilization and dialysis against refolding buffer, as described elsewhere (24). Briefly, IB was washed three times with wash buffer (50 mM Tris pH 8; 0.1 mM NaCl; 0.1 mM EDTA, 5% glycerol; 0.1 mM DTT, and 5% Triton X-100). In each washing step, IB suspension was sonicated and recovered by centrifugation at 14.000 rpm at 4 °C for 10 minutes. No Triton X-100 was added in the second and third washing steps. The washed IB was further dissolved in the same buffer containing 6 M GuHCl and shaken vigorously (250 rpm) until a clear lysate was observed. The denatured protein was transferred into a dialysis tube (Carolina Biological Supply Company) with a cutoff between 12,000 to 14,000 Daltons. The dialysis was undertaken against refolding buffer (20 mM Tris pH 8; 5 mM EDTA; 5 mM DTT; 50 mM NaCl, 20% glycerol, and 0.32 M GuHCl) overnight. On the next day, the refolding buffer was exchanged, and dialysis further proceeded for two hours. The buffer exchange and dialysis cycle were subsequently repeated for two times.

A time-course curve that shows the activity of recombinant PtpB was created by plotting the absorbance of *para*-nitrophenol over time. pNP is the product of *para*-nitrophenyl phosphate hydrolysis by PtpB. The reaction was carried out in a 96 well-plate containing 40 µL of 100 mM imidazole, 20 µL of 50 mM pNPP substrate, 135 µL mili-Q water, and 5 µL of recovered PtpB in a total volume of 200 µL. The reaction was allowed to proceed within the spectrophotometer chamber (Multiskan Go, Thermo Scientific). The spectrophotometer was set to automatically record the absorbance at 410 nm in five minutes intervals for two hours.

*Inhibition of PtpB with cis-2 and* trans*-2-eicosenoic fatty acids*

We have performed an inhibition assay of PtpB with a procedure adapted from Mascarello et al. (20). The reaction mixture contained 100 ng PtpB (in phosphate buffer, pH 8.0), 20 mM imidazole, 20 mM pNPP substrate, 16 µM inhibitor (Table 1), and Milli-Q water to complete the reaction volume to 200 µL in a 96-well plate. The reaction was carried out at 37 °C. After 15 minutes, the reaction was stopped

by the addition of 80 µL of 0.25 M NaOH, and the absorbance was recorded at 410 nm immediately.
PtpB activity was calculated by the following equation:

$$A = \frac{Vol \times \Delta A\,410}{\epsilon \times time}$$

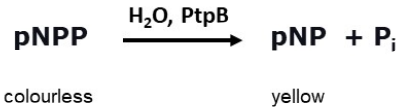
Where
A : activity (Unit)
Vol : reaction mixture volume (µL)
$\Delta A_{410}$ : sample absorption – blank absorption, measured at 410 nm
ε : molar extinction of pNP (1.78 x $10^4$ $M^{-1}.cm^{-1}$)
t : measurement time (minutes)

*Molecular Docking*
In order to describe the interaction between PtpB with the inhibitor molecules, we have performed docking analysis by using the software Discovery Studio (Biovia, Accelrys, San Diego USA). The protein structure of PtpB was retrieved from RCSB Protein Data Bank with accession number 1YWF.

## RESULTS AND DISCUSSION

The required material for the inhibition assay was prepared by over-expressing the Mtb *PtpB* gene in *E. Coli* BL21(DE3). We found that the protein exists as an IB (Figure 1A). Despite initially being inactive protein, expression as IB offered a high protein level that can be recovered by an established procedure to gain protein with sufficient purity for inhibition assays. As shown in Figure 1B, re-solubilization with 6 M GuHCl followed by dialysis effectively removes the excessive salt. The application of GuHCl gave a better result compared to previous work with 8 M urea (24). The observation might come from the relatively efficient solubilization of the IB by GuHCl compared to urea (25).

The re-solubilized protein was active, as shown by its ability to cleave pNPP substrate into para-nitrophenol and phosphate.




The absorbance of the reaction mixture was recorded at 410 nm every five minutes for two hours. The data was plotted in a time-course curve (Figure 1C). The curve showed good linearity within the first 45 minutes. We employed this reaction to test whether prospective inhibitors (Table 1) can decrease PtpB activity. The inhibition mixture containing PtpB, substrate pNPP, and inhibitor was allowed to react, and the absorbance at 410 nm was recorded after 15 minutes of reaction.

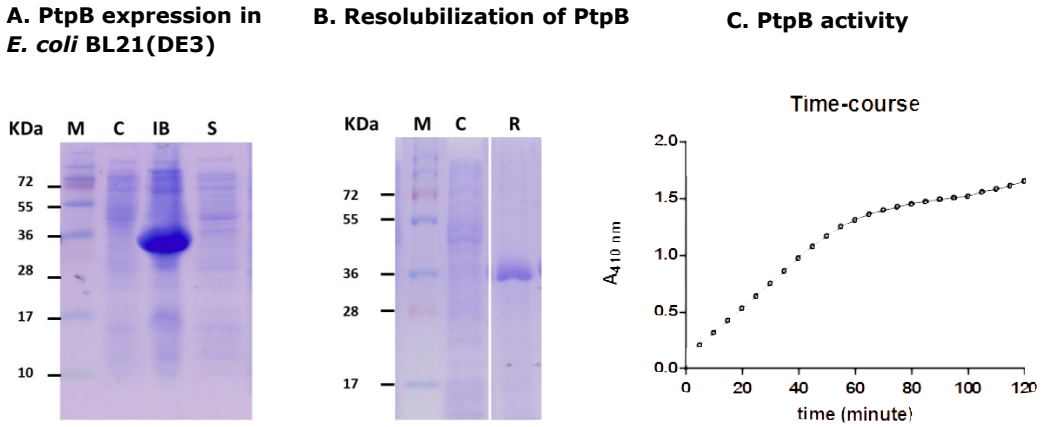



**Figure 1:** Expression of PtpB in *E. coli* BL21(DE3).

A. Expression of PtpB in *E. coli* BL21(DE3) under IPTG induction. M: protein ladder; C: untransformed *E. coli* extract; IB: inclusion body found in the insoluble fraction; S: soluble fraction or supernatant.
B. Solubilization of PtpB with Guanidine hydrochloride and its recovery in refolding buffer. M: protein ladder; C: lysate from untransformed *E. coli*; R: recovered PtpB. Proteins were separated by 12.5% SDS-PAGE.
C. Activity of recombinant PtpB was measured over time. PtpB hydrolyzed pNPP substrate to release the yellow color of *para*-nitrophenol. The absorbance was measured at 410 nm at 5-minute intervals for 120 minutes.

We tested the inhibitory effect of *cis*-2 and *trans*-2 eicosenoic fatty acids on the activity of PtpB. In this preliminary work, we employed a single dose of inhibitors of 16 µM that represent a low dose (26). Figure 2 shows the activity of PtpB is reduced by *cis*-2 and *trans*-2-eicosenoic fatty acids to 36.28% and 25.33, respectively. In other words, *cis*-2 and *trans*-2-eicosenoic fatty acids inhibit PtpB by

63.72% and 74.67%, respectively. The inhibition of PtpB by *trans*-2-eicosenoic fatty acid was in line with the effect of sodium orthovanadate, a general inhibitor for phosphatases (27). In an *in silico* study, Dhanjal and co-workers suggested that the compound DTP is a potential inhibitor for PtpB (22). We also showed for the first time that DTP inhibits PtpB with a slightly less effect than *cis*-2 and *trans*-2-eicosenoic acids. *Trans*-11-eicosenoic fatty acid, a related fatty acid whose double bond lies apart from the carboxyl group, only slightly inhibited PtpB activity (Figure 2). It showed that the double bond position that is close to the carboxy group is crucial for *cis*-2- and *trans*-2-eicosenoic to inhibit PtpB.

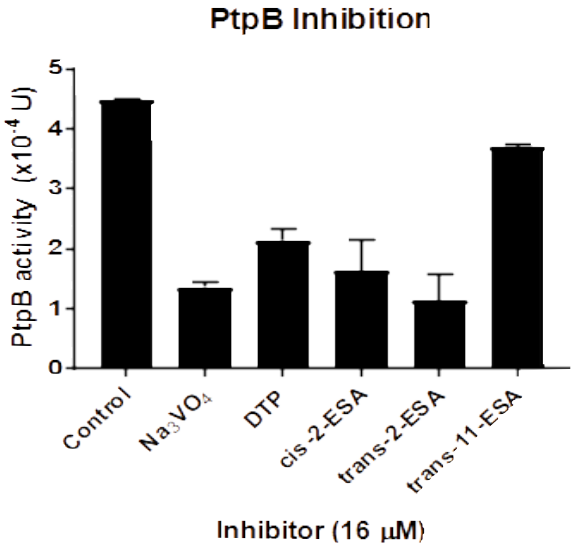
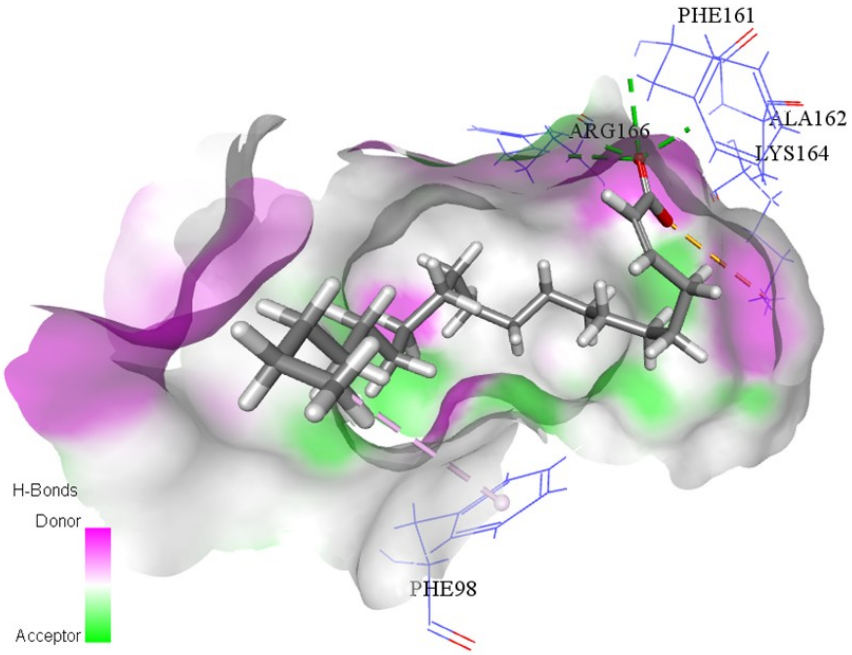



**Figure 2:** The activity of PtpB in the presence of several inhibitors.

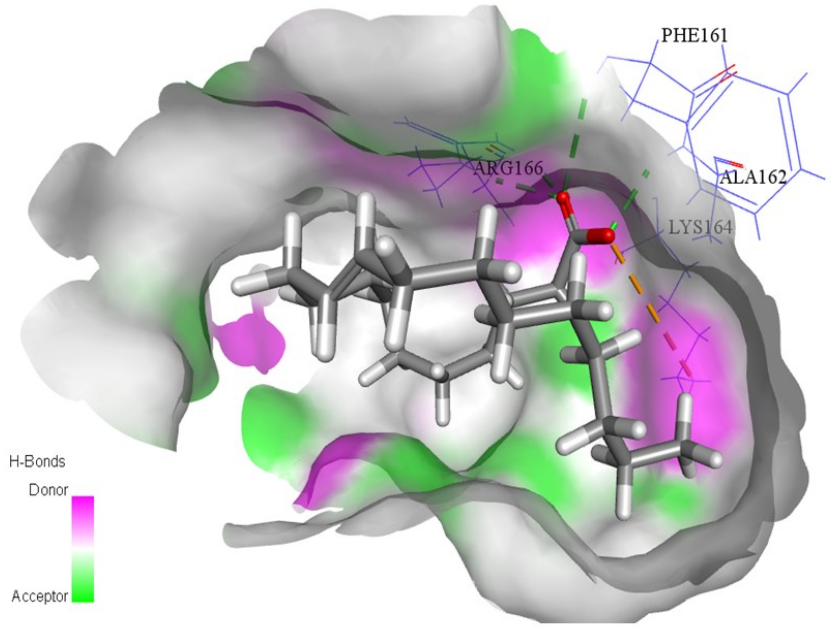
PtpB activity in the presence of several inhibitors at a concentration of 16 µM. Control: PtpB without inhibitor. Inhibition by sodium orthovanadate ($Na_3VO_4$), DTP, *cis*-2-eicosenoic fatty acid, *trans*-2-fatty acid, and *trans*-11-fatty acid correspond to 69.44%, 52.22%, 63.72%, 74.67% and 17.53%, respectively, with regard to control. All data are averages of two measurements. ESA: eicosenoic fatty acid.

To reveal the interaction between those inhibitors with PtpB, we performed a docking experiment. Figure 3A-B shows that both *cis*-2 and *trans*-2-eicosenoic acids fit the active site of the PtpB structure with the binding energy of -60.40 and -61.60 kcal/mol, consecutively. These values are closed to the cDocker energy of native phosphate ligand of -81.47 kcal/mol; Table 2). This shows that both *cis*-2 and *trans*-2-eicosenoic fatty acids have stable interaction with PtpB. The strong interactions are supported by the presence of three and four hydrogen bonds between *cis*-2 and *trans*-2-eicosenoic fatty acids, respectively, with critical amino acids of PtpB. Besides, both hydrophobic inhibitors are assumed to have a pi stacking ($\pi$-$\pi$ stacking) interaction with the aromatic ring of $Phe_{98}$ residue. A weaker interaction of PtpB is observed with *trans*-11-eicosenoic fatty acid. Although *trans*-11-eicosenoic fatty acid forms three hydrogen bonds and an attractive charge interaction with PtpB, the lack of pi stacking interaction seems to be responsible for the weaker interaction (summarized in Table 2). Based on the binding energy and the presence of hydrogen bonds between *cis*-2 and *trans*-2-eicosenoic fatty acids with PtpB, it can be concluded that both fatty acids have high inhibition activity against the virulence factor of Mtb. This finding is also in agreement with the *in vitro* inhibition assay described in Figure 2.

**A. Complex of PtpB and *cis*-2-eicosenoic acid**




**C. Complex of PtpB and *trans*-11-eicosenoic acid**



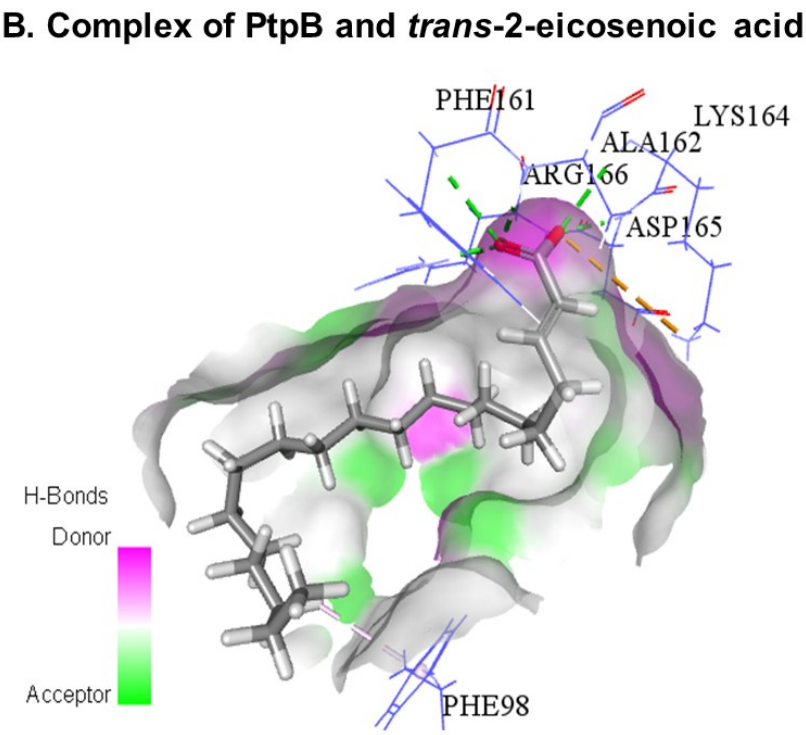



**Figure 3:** Docking of PtpB with selected inhibitors.

The strong interaction of PtpB with *cis*-2 and *trans*-2 eicosenoic fatty acids is supported by three potential hydrogen bonds of the protein through residues $Phe_{161}$, $Ala_{162}$, and $Arg_{166}$. The interaction is also strengthened by the presence of attractive charge interaction with the positively charged residue $Lys_{164}$, which is also observed with *trans*-11-eicosenoic fatty acid (A-C). An additional hydrogen bond between PtpB with *trans*-2-eicosenoic acid via $Asp_{165}$ (B) confirmed the more potent inhibitory effect of *trans*-2-eicosenoic acid than its *cis*-2 isomer (A). In contrast to the *cis*-2 and *trans*-2-eicosenoic fatty acids, a Pi stacking (π-π stacking) interaction is absent in the docking of PtpB with *trans*-11-eicosenoic fatty acid (C).

**Table 2:** Docking parameters of PtpB with several inhibitors.

| Compound | cDocker Energy (kcal/mol) | Interaction |
|---|---|---|
| *cis*-2-eicosenoic acid | -60,3957 | Hydrogen bonds: $Phe_{161}$, $Arg_{166}$, $Ala_{162}$<br>Pi stacking (π- π stacking): $Phe_{98}$<br>Attractive Charge: $Lys_{164}$ |
| *trans*-2-eicosenoic acid | -61,6195 | Hydrogen bonds: $Phe_{161}$, $Arg_{166}$, $Ala_{162}$, $Asp_{165}$<br>Pi stacking (π- π stacking): $Phe_{98}$<br>Attractive Charge: $Lys_{164}$ |
| *trans*-11-eicosenoic acid | -55,4227 | Hydrogen bonds: $Phe_{161}$, $Arg_{166}$, $Ala_{162}$<br>Attractive Charge: $Lys_{164}$ |
| co-crystalized ligand $PO_4^{2-}$ | -81,4651 | Hydrogen bonds: $Phe_{161}$, $Ala_{162}$, $Lys_{164}$, $Asp_{165}$, $His_{94}$, $Pro_{81}$<br>Pi stacking (π- π stacking): $Phe_{98}$, $Tyr_{125}$, $Leu_{227}$, $Leu_{101}$, $Val_{231}$<br>Attractive Charge: $Met_{126}$ |

Although the primary host substrate of PtpB is primarily unknown, accumulated evidence emphasizes the crucial role of PtpB in preventing the host degradative pathway (28). Hence, targeting PtpB to prevent the onset of latent tuberculosis infection has lead to the screening of many potential inhibitors (29). Various types of compounds, either naturally occurring or modified compounds, have been reported to inhibit PtpB. A series of chalcones derivatives, for example, has

been reported to inhibit PtpB at low micromolar concentration, i.e., with $IC_{50}$ < 30 μM (27). Further development has expanded the inhibition at submicromolar concentration, such as reported by Liu and coworkers who tested polypropionate derivatives isolated from deep-see fungus to inhibit PtpB. They revealed that the compounds have $IC_{50}$ in the range of 5.1 to 12 μM (30). Here we demonstrated that *cis*-2 and *trans*-2-eicosenoic fatty acids strongly inhibit PtpB. This finding could be helpful for the development of drugs to prevent latent tuberculosis infection. Targeting PtpB offers an advantage since the protein is secreted into the macrophage's cytoplasm. There is no requirement that the inhibitor has to overcome the thick hydrophobic envelope of mycobacteria (14). Moreover, it is also worth considering alternative application strategies; for instance, using the potential inhibitors in combination with first-line TB drugs such as rifampicin and isoniazid as PtpB inhibitors were reported to enhance mycobacterial elimination (11,31). However, before further exploration, the selectivity of inhibition among human phosphatases might also be deemed necessary.

## CONCLUSION

The present study shows that both *cis*-2 and *trans*-2-eicosenoic fatty are able to inhibit Mtb PtpB. This is also the first report that long fatty acids can inhibit PtpB. The inhibitory potential was also supported by docking analysis. Further study can be directed to investigate whether these compounds can also inhibit macrophage cells or animal model infection by Mtb.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

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