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What is This?

# Assay Development for Identifying Inhibitors of the Mycobacterial FadD32 Activity

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#### Abstract

FadD32, a fatty acyl-AMP ligase (FAAL32) involved in the biosynthesis of mycolic acids, major and specific lipid components of the mycobacterial cell envelope, is essential for the survival of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The protein catalyzes the conversion of fatty acid to acyl-adenylate (acyl-AMP) in the presence of adenosine triphosphate and is conserved in all the mycobacterial species sequenced so far, thus representing a promising target for the development of novel antituberculous drugs. Here, we describe the optimization of the protein purification procedure and the development of a high-throughput screening assay for FadD32 activity. This spectrophotometric assay measuring the release of inorganic phosphate was optimized using the *Mycobacterium smegmatis* FadD32 as a surrogate enzyme. We describe the use of  $T_m$  (melting temperature) shift assay, which measures the modulation of FadD32 thermal stability, as a tool for the identification of potential ligands and for validation of compounds as inhibitors. Screening of a selected library of compounds led to the identification of five novel classes of inhibitors.

#### **Keywords**

mycobacteria, fatty acyl-AMP ligase, FAAL, assay adaptation, screening of inhibitors

#### Introduction

Tuberculosis (TB) remains the leading cause of mortality due to a single infectious agent and, in conjunction with the spread of human immunodeficiency virus (HIV) infection, is today among the worldwide health threats. Emergence of "MDR" (multidrug-resistant) and "XDR" (extensively drug-resistant) *Mycobacterium tuberculosis* (*Mtub*) strains is partly responsible for the treatment failure and for the recrudescence of this disease. The development of new antibiotics effective against resistant strains is one of the major priorities of worldwide programs of the World Health Organization<sup>1</sup> to fight against TB.<sup>2</sup>

Mycobacteria produce a remarkable collection of lipophilic molecules ranging from simple short-chain fatty acids to the very complex long-chain mycolic acids.<sup>3,4</sup> Among the unique lipids of *Mtub*, being the main constituent of the outer membrane, mycolic acids play a critical role in both the architecture and permeability of the mycobacterial cell envelope.<sup>5,6</sup> The biosynthesis of these exceptionally long ( $C_{70}$ – $C_{90}$ ), alpha-branched, beta-hydroxylated fatty acids is essential for the survival of *Mtub* and provides thereby opportunities to target numerous enzymes of this metabolic pathway. The synthesis of mycobacterial lipids <sup>1</sup>Institut de Pharmacologie et de Biologie Structurale (IPBS), Centre National de la Recherche Scientifique (CNRS), Département "Mécanismes Moléculaires des Infections Mycobactériennes," Toulouse, France

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Mamadou Daffé and Hedia Marrakchi, Institut de Pharmacologie et de Biologie Structurale (IPBS), Centre National de la Recherche Scientifique (CNRS), Département "Mécanismes Moléculaires des Infections Mycobactériennes," Université de Toulouse, Université Paul Sabatier, 205 route de Narbonne, BP 64182, F-31077, Toulouse, France. Email: mamadou.daffe@ipbs.fr and hedia.marrakchi@ipbs.fr requires the combined action of fatty acid synthases (FASs) and polyketide synthases (PKSs). The mycolic acid synthesis involves both FAS-I (for de novo synthesis) and FAS-II (for elongation), as well as the action of Pks13 to achieve the final condensation between two activated fatty acids (**Fig. 1A**). A specific subclass of the FadD (fatty acid activating) family of enzymes establishes the crosstalk between FASs and PKSs by providing the activated fatty acyl adenylates to their cognate PKSs.<sup>4</sup>

The Mtub genome contains 34 fadD genes encoding for these FadD proteins grouped in two subclasses: fatty acyl-CoA ligases (FACLs) involved in lipid degradation and fatty acyl-AMP ligases (FAALs) dedicated to lipid biosynthesis.7 The FAALs activate and transfer fatty acids to PKSs for further chain extension to provide most of the highly specialized lipids of *Mtub*. Many of the *Mtub* FadD proteins represent potential drug targets due to their confirmed essentiality or requirement for virulence.8 Several FAAL functions have been characterized and shown to activate carboxylic acid substrates and load them onto cognate PKS proteins. For instance, FAAL22, which loads onto Pks15/1, was demonstrated to be essential for the synthesis of the virulence-conferring phenolic glycolipids (PGLs),<sup>9</sup> whose synthesis also involves FAAL29, which loads onto the polyketide synthases PpsA-E.<sup>10</sup> Similarly, FAAL26, which loads onto PpsA-E, and FAAL28, which loads onto Mas, are involved in the biosynthesis of the phthiocerol and mycocerosate, respectively, of the phthiocerol dimycocerosates (DIMs) virulence factor. Likewise, FAAL23, which loads onto Pks2, is implicated in the synthesis of sulfolipids, whereas FAAL33 (also called MbtM), which loads onto MbtL, is involved in mycobactin synthesis.

One of the most extensively studied FAALs, probably because of its confirmed essentiality in mycobacteria, is FAAL32, more known as and thereafter named FadD32, required for mycolic acid synthesis.<sup>11</sup> FadD32 acts in concert with Pks13 and activates the very long meromycolic acid  $(C_{50}-C_{60})$  prior to its condensation with a  $C_{24}-C_{26}$  fatty acid,<sup>12</sup> which itself is activated by the AccD4-containing acyl-CoA carboxylase ACCase, to yield, upon reduction, mycolic acids (Fig. 1A).<sup>11,13,14</sup> The operon fadD32-pks13-accD4, present in all the mycobacterial species and Mtub strains sequenced so far, was proved to be essential for the viability of mycobacteria.<sup>11,13</sup> Conditional expression of *fadD32* has confirmed its essentiality for the growth of Mtub,15,16 Mycobacterium smegmatis (Msmeg),<sup>11</sup> and Mycobacterium abscessus,<sup>17</sup> whereas knockdown studies have established that the fadD32 operon is a vulnerable target.<sup>18</sup> Accordingly, FadD32 represents a particularly attractive drug target.

Substrate analogues as inhibitors of FadD32 have been characterized using a radiometric assay coupled with thinlayer chromatography (TLC).<sup>19</sup> However, limited throughput of this assay is not suitable for screening large libraries of compounds to find novel inhibitors. We describe, in the present study, the development and the optimization of a nonradiometric assay for FadD32 activity using the *Msmeg* FadD32 orthologue as a surrogate enzyme source. The adapted assay was used to screen a selected library, which led to the identification of five novel classes of inhibitors.

#### **Materials and Methods**

#### Cloning of the fadD32 Orthologous Genes

The *fadD32* genes were PCR-amplified from total DNA of *Msmeg mc*<sup>2</sup>155 and *Corynebacterium glutamicum* (*Cglu*) 13032 strain using the high-fidelity Pfu DNA polymerase (Fermentas, Vilnius, Lithuania) with the following specific primers (Sigma-Aldrich, St. Louis, MO):

*Msmeg* fadD32 5' (NdeI) GCACATATGATGCCGTT CCACAATCCG

*Msmeg* fadD32 3' (NdeI) GCACATATGATGGCGTT GTTGGTCAGTC

*Cglu* fadD32 5′ (NdeI) GCGTCGGCGCATATGAGAG GGGTTCACATGGA

*Cglu* fadD323' (BamHI) ACGGATCCCTGCGGTGTCT GCAAAGA

The PCR fragments were ligated into the pET15b expression vector (Novagen/Merck, Darmstadt, Germany) digested with appropriate restriction enzymes (Fermentas) and dephosphorylated to avoid recircularization (CIAP; Fermentas). Calcium-competent *Escherichia coli* cells (DH5 $\alpha$ ; Invitrogen, Paisley, UK) were transformed with ligation products, and carbenicillin-resistant (50 µg/mL carbenicillin; Sigma-Aldrich) clones were selected. DNA sequencing (MilleGen, Toulouse, France) of the cloned fragments after plasmid preparation (Qiagen, Valencia, CA) confirmed the correctness of the sequences.

#### Protein Expression and Purification

Competent E. coli BL21 Star (DE3) One Shot (Invitrogen) were transformed with the pET15b-fadD32 plasmid,<sup>19</sup> and expression of FadD32 proteins was performed using autoinducible medium as described previously by Studier.20 Briefly, a starter culture grown overnight in LB medium was diluted in auto-induction medium.<sup>20</sup> Cells were grown for 72 h at 20 °C and collected by centrifugation at 4 °C for 15 min. The pellet was then resuspended in lysis buffer consisting of 50 mM HEPES (pH 7.5), 10% glycerol (v/v), 30 mM imidazole, 500 mM NaCl, 0.75 mg/mL lysozyme, and 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF; Sigma-Aldrich) and frozen overnight at -80 °C. The frozen bacterial pellets were thawed at room temperature (RT) and disrupted by sonication (four intermittent pulses of 30 s) on a VibraCell (Fisher Bioblock Scientific, Illkirch, France) and centrifuged at 20 000 g for 30 min at 4 °C. The clarified lysate was loaded onto an HisTrap HP (1-mL) affinity column (GE



**Figure 1.** The FadD32 activity, purification, and assay adaptation. (**A**) Role of FadD32 in the biosynthesis of mycolic acids. The synthesis of mycolic acids involves the intervention of fatty acid synthases (FAS): FAS-I for de novo synthesis and FAS-II for elongation of fatty acids (FAs). The condensation (by Pks13) between two activated fatty acids produces, after reduction (by the ketoacyl reductase CmrA), the mature mycolic ester with the characteristic mycolic motif (alpha-branch, beta-hydroxyl). Both condensation substrates are activated: The acyl-CoA ( $C_{24}-C_{26}$ ) is carboxylated by the acyl-CoA carboxylase complex (AccD4-containing complex), yielding the alpha-branch ( $R_2$ ) of mycolic acids. The very long-chain fatty acid (meromycolic acid,  $C_{50}-C_{60}$ ) is activated as acyl-AMP by FadD32 (FAAL32), at the origin of the meromycolic chain ( $R_1$ ) of mycolic acids.  $R_1$  and  $R_2$  represent long hydrocarbon chains, and X is an unknown acceptor. (**B**) A schematic representation of FAAL reaction catalyzed by FadD32 and detection of inorganic phosphate (Pi) using the Pyrophosphatase-coupled reaction. PPi is pyrophosphate. (**C**) Purity of the different FadD32 orthologues after affinity and gel filtration chromatography purification. Protein fractions were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (**D**) FAAL activity of FadD32 orthologues: *Mycobacterium tuberculosis* (dotted line), *Corynebacterium glutamicum* (solid line), *Mycobacterium smegmatis* (dashed line), and inset. Assays were conducted in Tris-HCI (pH 8) using 200  $\mu$ M lauric acid ( $C_{12}$ ) and 2 mM adenosine triphosphate (ATP) as substrates. Serial dilutions of enzymes were performed in Tris-HCI (pH 8). Means and standard errors of the mean (SEM) of four replicates are shown. Lines were obtained by linear regression, and data are representative of at least three independent experiments.

Healthcare, Little Chalfont, Buckinghamshire, UK), and FadD32 protein was eluted using 150 mM imidazole in 50 mM HEPES (pH 7.5), 500 mM NaCl, and 0.2 mM AEBSF. The fractions containing FadD32 protein were pooled and concentrated using Vivaspin 2 (10 000 Da molecular weight [MW] cutoff; Sartorius, Goettingen, Germany) and loaded onto a gel filtration column (HiLoad 16/60 Superdex 200; GE Healthcare) preequilibrated with 50 mM HEPES (pH 7.5), 500 mM NaCl, and 0.2 mM AEBSF. The purified proteins (as checked on sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] followed by a Coomassie blue staining) were then concentrated and stored at –20 °C in 50% glycerol.

#### Radiometric-TLC Enzyme Assays

The formation of the acyl-AMP product by FadD32 from fatty acid and adenosine triphosphate (ATP) in the presence of Mg<sup>2+</sup> was monitored by radiolabeled-TLC (radio-TLC), as described previously.<sup>7,19</sup> Briefly, the reaction mixtures were performed as described for spectrophotometric assays, in 30 µL in glass microtubes, and incubated for 80 min at RT. Reactions were then stopped by adding 5% final acetic acid. The reaction mixture was then spotted in duplicates on silica gel TLC plates (G60; Macherey-Nagel, Düren, Germany), with the radiolabeled fatty acid substrate being subsequently separated from the acyl-AMP product on the basis of their differential  $R_{f}$  (frontal ratio). Radiolabeled products were resolved in butan-1-ol/acetic acid/water (80:25:40, v/v/v) at RT and visualized by exposing the TLC to a Fujifilm (Tokyo, Japan) imaging plate prior to phosphorimager detection (Typhoon Trio; GE Healthcare). The percentage of product was calculated after quantitation of both fatty acid and acyl-AMP using the ImageQuant software version 5.1 (Molecular Dynamics, Sunnyvale, CA).

#### Spectrophotometric Enzyme Assays

FadD32 enzyme catalyzes the formation of acyl-AMP using fatty acid and ATP as substrates (Fig. 1B). Pyrophosphate (PPi) released during this reaction was hydrolyzed using a Pyrophosphatase coupled reaction, and the resulting inorganic phosphate (Pi) was measured using a colorimetric assay. Addition of Malachite Green reagent to the reaction mix resulted in the formation of phosphomolybdate complex with an increase in absorbance at 630 nm  $(A_{630})$  (Fig. 1B). All assays were performed in quadruplicates in 384-well polystyrene microplates (Greiner Bio One, Courtaboeuf, France), using the PiColorLock Gold kit (Innova Biosciences, Cambridge, UK). Reactions were conducted at RT, in 30 µL of assay mix containing 50 mM HEPES (pH 7.5), 8 mM MgCl<sub>2</sub>, 0.01% Brij-58, 2 mM ATP, 20 µM lauric acid, 1 mM DTT (1,4-dithiothreitol), and 2 mU/mL Pyrophosphatase (Sigma-Aldrich). Reactions were initiated by the addition of 15 µL FadD32 enzyme diluted in 50 mM HEPES (pH 7.5) to 15 µL of 2× assay mix. After 80 min, reactions were stopped by adding 30 µL of cold reaction buffer and 15 µL of Malachite Green. The  $A_{630}$  was read after 5 min of incubation at RT in a µQuant counter (Bio-Tek, Winooski, VT). A reaction without enzyme (for protein dose curve experiments) or without substrate (for  $K_m$  and  $V_{max}$  determinations) was performed in each experiment and used as blank. To calculate the concentration of Pi, a calibration curve using known concentrations of Pi varying from 10 to 80 µM was performed in each experiment according to the manufacturer's recommendations.

For apparent kinetic parameters  $(K_{\rm m}, V_{\rm max}, \text{ and } k_{\rm cat})$ determination, the initial velocity was measured (incubation time of 40 min) as a function of the studied substrate concentration at saturating concentrations of the nonvaried substrate. The kinetic parameters were determined when initial velocities were measured at a 90-min incubation time and found identical to those calculated at 40 min. The saturation curve was fit by nonlinear regression analysis using the following GraphPad Prism 4.02 (GraphPad Software, La Jolla, CA) equation:  $V_i = V_{max} * [S]/(K_m + [S])$ , where  $V_{max}$  is the maximal velocity, [S] is the substrate concentration, and  $K_{\rm m}$  is the Michaelis-Menten constant. Kinetic parameters for fatty acids were determined at fixed concentrations of ATP (2 mM) and by varying concentrations of the fatty acid  $(3.1-200 \ \mu M)$ , whereas kinetic constants for ATP were determined at fixed concentrations of lauric acid  $(C_{12})$  (200 µM) and by varying concentrations of ATP (0.05-4 mM) (Suppl. Fig. S1). The catalytic constant  $(k_{m})$ reflects the maximum rate of product formation and was calculated by dividing  $V_{\rm max}$  by the total enzyme concentration  $k_{\text{cat}} = V_{\text{max}} / [\text{E}_{\text{T}}]$ . The  $[\text{E}_{\text{T}}]$  was 1.2 pmol in the 30-µL assay reaction, and  $V_{\text{max}}$  was inferred from the Michaelis-Menten equation (described above and in **Suppl. Fig. S1**).

For inhibition studies, concentrations of ATP and lauric acid were adjusted to be above or around the  $K_{\rm m}$  (0.25 mM ATP and 20  $\mu$ M lauric acid). The substrate analogue alkyladenylate AMPC12 (chemically synthesized as described previously<sup>19</sup>) was first diluted in DMSO, and the substrate mixture was applied to increasing concentrations of the compound. The reaction was started by adding the enzyme. Data were expressed as percentage of inhibition. Curve fitting and EC<sub>50</sub> calculations were performed using sigmoidal dose response as the equation model (GraphPad Prism 4.02 software).

#### Thermostability of FadD32

The melting point  $(T_m)$  of FadD32 was measured by differential scanning fluorimetry (DSF) and was used both to characterize the enzyme thermal stability under various conditions (buffer, pH) and to identify ligands or inhibitors.<sup>21</sup> A mix of enzyme (4  $\mu$ M), SYPRO orange (5×) (Invitrogen), buffer (100 mM), and NaCl (500 mM) was exposed to a temperature gradient from 25 to 80 °C with a 0.3 °C increment. All measurements were performed in triplicates in 96-well plates (Biorad, Marnes-la-Coquette, France). The thermal transition was monitored using an RTQPCR CFX96 Real-Time System (Biorad). T was given by the inflexion point of the curve RFU (relative fluorescence unit) = f(T). Before fluorimetry experiments, the FadD32 samples were soaked in the presence of substrate analogue (AMPC12) or in buffer at a final concentration of 40 µM. For the DSF experiments, the His-tag of the affinity-purified FadD32 was removed by thrombin cleavage (Novagen). The protein solution (after 5-fold dilution to reduce the imidazole concentration down to 30 mM) was submitted to a 3-h cleavage reaction at RT with 0.28 U/mL of thrombin. The cleaved protein was then reloaded onto a HisTrap HP (1-mL) affinity column to eliminate the noncleaved fraction. The flow-through protein-containing fractions were then pooled, concentrated, and purified by size exclusion chromatography onto a Superdex 200 Prep Grade column (GE Healthcare). Samples used for DSF experiments were stored at -80 °C without glycerol.

#### Biochemical High-Throughput Screening

The above-mentioned biochemical assay to measure FadD32 activity was adapted for high-throughput screening (HTS). All the chemicals and biochemicals were procured from Sigma-Aldrich unless otherwise stated. The *Msmeg*FadD32 enzyme was prepared as described above. The 30-µL reactions containing 50 mM HEPES buffer (pH 7.7), 0.25 mM ATP, 8 mM MgCl, 0.001% Brij-35P, 1 mM DTT, 0.1 U/mL Pyrophosphatase, 20 nM MsmegFadD32 enzyme, and 20 µM lauric acid were carried out in 384-well microplates (Corning Costar; Corning Life Sciences, Tewksbury, MA) at room temperature (25 °C) for 100 min. Then, 30 µL of modified Baykov's Malachite Green reagent<sup>22</sup> was added and mixed for color development. Assay additions and mixing were done using the Apricot PP-384-M Liquid Handling System (Apricot Designs, Covina, CA). The plates were further incubated at 25 °C for 20 min. At the end of this incubation time, the plates were read at a 635-nm wavelength in a Spectramax 384 Plus microplate reader (Molecular Devices, Sunnyvale, CA). The single-point primary screening and the 10-point concentration response (CR) studies were performed using this assay. The inhibition was calculated against the following reaction controls: MIN (0% reaction control) = 1  $\mu$ L of 0.5 M EDTA (16.7 mM final) and MAX (100% reaction control) = 1  $\mu$ L of 100% DMSO (3.3% final). Since the enzymatic mechanism process for the two substrates is yet unknown, the concentrations of ATP and lauric acid were adjusted to be above or around their respective  $K_m$  to select moderate to weak inhibitors of both substrates.

In the primary screening, 10 177 compounds were screened in singlicates at 20  $\mu$ M. These compounds were randomly selected from a large, chemically diverse library of the AstraZeneca Corporate Compound collection to check the feasibility of HTS. This collection was made by acquiring chemically diverse sets of compounds from various sources as well as by adding compounds synthesized during various drug projects within AstraZeneca.

#### Promiscuity Test

High protein effect (promiscuity) assays were done to ascertain high protein (*MsmegFadD32*) effect on the compound CR. Basically, the above-mentioned high-throughput biochemical assay was done for a given set of compounds simultaneously at  $1 \times (20 \text{ nM})$  and  $5 \times (100 \text{ nM})$  enzyme concentrations and with a 10-point CR similar to the assay described before, except that the reaction time was reduced to 20 min to maintain the reaction linearity with increased enzyme concentration.<sup>23</sup>

## Thermofluor (T<sub>m</sub> Shift) Assay

The Thermofluor assay was performed as described before<sup>24</sup> to ascertain binding of these compounds to MsmegFadD32 enzyme. Thermal stability measurements were recorded in 96-well PCR plates covered with optical film (Bio-Rad, Hercules, CA), using a real-time PCR thermal cycler (iQ5 from Bio-Rad). The environmentally sensitive dye, SYPRO Orange (Invitrogen), was used to monitor protein unfolding. Thermal unfolding was monitored at 0.5 °C intervals from 25 °C to 95 °C. The assay was performed at the optimal reaction buffer in a 50-µL volume (50 mM HEPES [pH 7.7], 8 mM MgCl., 6.25× SYPRO Orange, and 0.15 mg/mL MsmegFadD32). The final DMSO concentration was maintained at 4% (v/v). Under these conditions, ATP, nonhydrolysable ATP analogue adenosine 5'-[y-thio] triphosphate (ATP-y-S) (Sigma-Aldrich) and lauric acid were tested for binding to MsmegFadD32 at  $1 \times$  and  $5 \times$ concentrations of their respective  $K_{\rm m}$  values. All 32 compounds selected after hit evaluation were tested in this assay for binding to MsmegFadD32 at three concentrations (12.5, 25, and 50 µM) (Suppl. Table S1A). The 16 "nonpromiscuous" compounds were assessed for the competition with ATP-γ-S (**Suppl. Table S1B**).

#### **Results and Discussion**

## Enhancement of the FadD32 Purification Yield

Previously, we successfully expressed and purified the *Mtub*FadD32 (*Mtub*FadD32), yet with a relatively low protein yield (0.7 mg/L of culture).<sup>19</sup> To improve the

MtubFadD32 expression and/or solubility, we reasoned that expression host strains reported to allow stabilization of mRNA levels of recombinant proteins should allow improved protein expression levels and stability, whereas auto-induction media should slow down the expression rate while decreasing handling of cultures. Accordingly, the MtubFadD32 protein was expressed by culturing Star One Shot (Invitrogen) BL21(DE3)/pET15b-MtubfadD32 in autoinduction media for 72 h at 20 °C. Using these culture conditions, the amount of MtubFadD32 protein recovered after purification using the reported protocols was more than 7 mg/L of culture, which represented at least a 10-fold increase in the protein yield compared with conditions where protein expression was performed in LB medium (5-h induction with 0.5 mM isopropyl-β-d-thiogalactopyranoside [IPTG] at 25 °C).<sup>19</sup> The protein obtained after the two-step purification was homogeneous (Suppl. Fig. S2).

#### FadD32 Assay Development

The *Mtub*FadD32 activity was previously characterized using a radiometric assay coupled to thin-layer chromatography (radio-TLC). The search for FadD32 inhibitors through screening of chemical libraries requires the design of suitable readouts compatible with low volumes and robotics. The FadD32 protein displays FAAL activity in vitro, catalyzing the formation of an acyl-adenylate (acyl-AMP) from a fatty acid and ATP (in the presence of Mg<sup>2+</sup>), with the release of PPi (**Fig. 1B**). We decided to couple the FadD32 reaction to inorganic Pyrophosphatase (iPPase), which cleaves the PPi into Pi, the latter being measurable with inorganic phosphate-interacting reagents such as Malachite Green, followed by spectrophotometric detection at 630 nm, as demonstrated for Pi-generating enzymes.<sup>22</sup>

The initial assays of FadD32 activity using the Pyrophosphatase-coupled reaction were performed in 384well plates in conditions similar to those applied in the radio-TLC assay previously reported,19 except for the [1-<sup>14</sup>C]C<sub>12</sub> fatty acid substrate, which was replaced by cold lauric acid  $(C_{12})$  and the addition of Pyrophosphatase in the reaction mixture. The FadD32 reaction was followed at 630 nm, and a Pi standard curve allowed quantitation of the Pi released, thus enabling the pyrophosphate or acyl-AMP concentrations formed during the reaction to be deduced. To test the linearity of the product formation in these conditions, a protein dose-response curve (with increasing concentrations) of MtubFadD32 in the reaction was performed. For protein concentrations up to 600 nM in the assay, the increase in  $A_{630}$  remained low, and at least 1.2  $\mu$ M was needed to detect FAAL activity (Fig. 1D, dotted line).

Among the various parameters to be optimized to adapt the FadD32 assay to HTS are (1) the protein concentration, optimally in the nanomolar range, and (2) the substrate's (fatty acid) chain length and concentration, which should be kept to a minimum given its hydrophobic nature and low solubility.

The relatively high concentrations of *Mtub*FadD32 added in the reaction seemed not compatible with the adaptation of the assay to HTS. We therefore considered using FadD32 orthologues as surrogates to develop an HTS assay, knowing that the use of the *M. smegmatis* orthologue in the mycobacterial PimB protein biochemical and structural studies has been shown to be valuable.<sup>25</sup>

The FadD32 proteins being highly conserved in all bacteria (grouped in the mycolic acid-producing Corynebacteriales suborder), two FadD32 orthologues from Msmeg and Cglu—sharing, respectively, 75% and 40% sequence identity with the MtubFadD32-were selected as surrogate candidates for comparative biochemical and inhibition studies. The respective genes were cloned in pET15b expression vectors with an N-terminal 6xHistidine tag for production in *E. coli* strains. The three FadD32 proteins were expressed and purified (Fig. 1C) according to improved protocols of expression and purification mentioned above (see Materials and Methods). The protein expression rates and purification yields obtained for MsmegFadD32 and CgluFadD32 were significantly higher than those of MtubFadD32 (Table 1). Indeed, the calculated volumes of culture required for performing a significant number of assays clearly distinguished the *Msmeg*FadD32 and CgluFadD32 from the Mtub protein and indicated that the former orthologues are by far more convenient for HTS purposes than the *Mtub* protein (**Table 1**).

When the thermal stability of the three FadD32 orthologues was investigated through determination of the melting temperature ( $T_{\rm m}$ ), *Mtub*FadD32 was the least stable protein, with a  $T_{\rm m}$  of 37 °C, whereas *Msmeg*FadD32 and *Cglu*FadD32 were much more thermally stable in our experimental conditions, with a T of 46 °C and 59 °C, respectively.

The activities of the *Mtub*FadD32, *Msmeg*FadD32, and CgluFadD32 proteins were then measured in parallel in a protein dose-response experiment, and their abilities to release Pi in the FadD32-Pyrophosphatase coupled assay were compared (Fig. 1D). A linear relationship between velocity and enzyme concentration was observed throughout the enzyme concentrations tested. The specific activity of CgluFadD32 was only twice that of MtubFadD32 (Table 1). In sharp contrast, the dose-response curve for the MsmegFadD32 enzyme showed a linear response down to a few nanomolar of protein in the assay (Fig. 1D, inset). Indeed, the specific activity of MsmegFadD32 was approximately 75 times higher than that of the MtubFadD32 enzyme (Table 1). The CgluFadD32 displayed high thermal stability and purification yield, yet its low specific activity is not suitable for performing inhibition and HTS assays. Furthermore, when combining the purification yield, the amount of protein required in the assay, and enzyme activity for the three FadD32 orthologues (Table 1), the Msmeg protein displayed the lowest volume of culture necessary to perform a 100-microplate (384-well plates) assay, thus permitting the achievement of an HTS campaign with

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FadD32 Protein	Identity, %	Purification Yield, mg/L	Specific Activity (200 µM Lauric Acid), nmol·min <sup>-1</sup> ·mg <sup>-1</sup>	Volume of Culture for a 100-Microplate Assay, L
M. tuberculosis	100	7	32 ± 2	14
M. smegmatis	75	45	2443 ± 99	0.08
C. glutamicum	40	50	71 ± 2	I

Table 1. Properties of the Selected FadD32 Orthologues from Mycobacterium Species and Corynebacterium glutamicum

Identity scores with the *M. tuberculosis* protein were computed from protein sequence alignment performed using the BLAST program (NCBI, Bethesda, MD). The protein purification yields are calculated per liter of culture expressing the protein of interest. The specific activity of each FadD32 was deduced from the linear regression protein dose-response curve (**Figure 2B**) (protein ranging from 1200 to 300 nM for *Mtub*FadD32 and *Cglu*FadD32 and from 100 to 3.125 nM for *Msmeg*FadD32). The volume of culture required for performing a 100-microplate assay (384-well plates) was calculated based on addition of 1200 nM of *Mtub*FadD32, 40 nM of *Msmeg*FadD32, and 600 nM of *Cglu*FadD32 protein per reaction.

a unique batch of protein. The higher sequence identity of *Msmeg*FadD32 with the *Mtub* enzyme, combined with its favorable biophysical properties and high specific activity, made the *Msmeg*FadD32 enzyme the most promising surrogate for conducting the assay development and optimization toward the HTS of inhibitors.

# Assay Optimization Using FadD32 from M. smegmatis

To gain insight into the optimal assay conditions (i.e., reaction volume and composition), several parameters were investigated using *Msmeg*FadD32. A reaction volume of 30  $\mu$ L was used to reduce the amount of protein in the assay while keeping the most favorable optical performance. To keep the benefit of using larger volumes at the reading step, which allowed reducing reading errors due to meniscus curvature and shorter path length,<sup>26</sup> the reaction mixture was diluted 1:1 (v/v) postincubation with basal buffer before taking a readout. Indeed, higher ratios of signal/background (S/B of 4.2 vs. 2.6) and signal/noise (S/N of 97 vs. 23) compared with no buffer dilution were achieved (**Suppl. Fig. S3A**).

The critical parameters affecting the enzyme activity were the reaction buffer and pH. These were first investigated by inspecting the protein thermal stability using DSF, a widely used technique for screening optimal conditions for biological macromolecules.<sup>21</sup> When thermal stability of the MsmegFadD32 protein was inspected at different buffer and pH conditions (from 5–8), a higher  $T_{\rm m}$  was observed at neutral pH, indicating an increased stability of the protein at this pH (Fig. 2A). Investigation of FAAL activity of *Msmeg*FadD32 under the same conditions revealed that the use of acidic buffer (pH 5) in the reaction mixture did not allow detection of FAAL activity, even at a higher protein concentration in the assay (Fig. 2B). The highest protein activity was observed at neutral pH using HEPES buffer, with the pH 7.5 being the most suitable for further enzymatic assays (Fig. 2B, insert), in agreement with the thermal stability data.

A time course experiment of *Msmeg*FadD32 activity was performed in the presence of three enzyme concentrations to determine the time window for reaction linearity. The

reaction rate was linear between 40 and 120 min, ensuring initial velocity  $(V_0)$  conditions at all enzyme concentrations tested (**Fig. 2C**).

The low solubility of the fatty acids in the reaction mixture was a critical issue for the availability of the substrate in the FAAL reaction. Accordingly, the effects of detergents on the kinetic parameters of *Msmeg*FadD32 (for lauric acid) were examined in the absence or presence of different agents. The presence of detergent in the assay significantly improved the reaction rate, with Brij (58 and 35) exhibiting the most pronounced effect (**Suppl. Fig. S3B**).

Since most of the library compounds are solubilized in DMSO, the effect of DMSO on *Msmeg*FadD32 activity was investigated, and concentrations up to 7.5% did not appear to affect the activity of the enzyme (data not shown).

# Enzymatic Characterization of M. smegmatis FadD32

The choice of the fatty acid substrate and determination of kinetic parameters for both substrates (ATP and fatty acid) of FadD32 are critical for the determination of the conditions to set for inhibition and screening experiments. The apparent  $K_{m}$ ,  $V_{max}$ , and  $k_{cat}$  were determined for ATP and for fatty acids of various chain lengths (**Table 2**). The myristic acid ( $C_{14}$ ) displayed the highest catalytic efficacy  $(k_{cat}/K_m)$ , in agreement with the FadD32 substrate preference for long-chain fatty acids.<sup>19</sup> For further inhibition and HTS experiments, lauric acid  $(C_{12})$  was chosen since it combines the advantage of having a relatively short aliphatic chain with a high catalytic efficacy. In addition, the  $K_{\rm m}$  value for  $C_{12}$  permits including a concentration of the substrate in the assay close to the K (20) µM), which would allow the detection of all types (competitive, uncompetitive, and noncompetitive) of inhibitors with a sufficient absorbance change. The lower  $K_{m}$  of the C<sub>14</sub> could be a disadvantage if one is looking for competitive inhibitors, and the assay would require several-fold the  $K_m$  of the substrate to generate sufficient signal. The  $K_m$  of ATP measured in the assay conditions was 0.08 mM.

We evaluated the correlation between the release of Pi using the PiColorLock Gold (Innova Biosciences)



**Figure 2.** Assay optimizations using the FadD32 from *Mycobacterium smegmatis*. (**A**) Melting temperature  $(T_m)$  measurements of *Msmeg*FadD32 as a function of pH. The differential scanning fluorimetry measurements for the protein at the indicated pH are displayed. (**B**) Optimization of buffer and pH:Assays were conducted during 80 min in different buffers using 100 nM of the *Msmeg*FadD32 enzyme. Inset: Fatty acyl-AMP ligase (FAAL) activity of *Msmeg*FadD32 at different pH. Serial dilutions of enzymes were performed in the indicated HEPES buffer (dashed line: pH 6.5; dotted line: pH 7.5; solid line: pH 7.0) using 200  $\mu$ M lauric acid and 2 mM adenosine triphosphate (ATP) as substrates. Means and standard errors of the mean (SEM) of four replicates are shown. Lines were obtained by linear regression. (**C**) Time course of *Msmeg*FadD32 activity. Assays were conducted at room temperature in HEPES pH 7.5 using 20  $\mu$ M lauric acid and 0.2 mM ATP using 80 nM (solid line), 40 nM (dashed line), or 20 nM (dotted line) of enzyme.

 Table 2. Apparent Kinetic Parameters of MsmegFadD32

Substrate	<i>K</i> <sub>m</sub> , μM	V <sub>max</sub> , pmol∙min <sup>-1</sup>	$k_{\rm cat}$ , min <sup>-1</sup>	$k_{\rm cat}/K_{\rm m}$ , min <sup>-1</sup> $\mu$ M <sup>-1</sup>	
ATP	76.0 ± 6.7	16.8 ± 0.4	$14.0 \pm 0.3$	0.18	
C10	259.0 ± 57.9	19 ± 2.8	16.1 ± 2.4	0.06	
C12	25.9 ± 1.6	13.5 ± 0.3	11.2 ± 0.2	0.43	
CI4	5.2 ± 0.5	5.0 ± 0.1	4.2 ± 0.1	0.81	

Initial velocities of the FadD32 reaction were assayed spectrophotometrically using the coupled assay. Reactions were conducted at room temperature during 40 min in HEPES pH 7.5 as described in Materials and Methods. The steady-state apparent parameters  $K_m$  (Michaelis constant),  $V_{max}$  (maximum velocity),  $k_{cat}$  (catalytic constant), and  $k_{cat}/K_m$  (catalytic efficacy) were determined using nonlinear regression to fit the data to the Michaelis-Menten equation. ATP, adenosine triphosphate.

detection and the formation of acyl-AMP determined by radio-TLC (**Fig. 3**). In the same conditions (except for the radiolabeled substrate), a protein dose-response curve experiment was performed, and the product formation was analyzed with either the radio-TLC assay (**Fig. 3A**) or the Pyrophosphatase-coupled FadD32 assay. The good correlation of the amount of product formed using both assays validated the PiColorLock as a reliable and quantitative assay for measuring FadD32 activity (**Fig. 3B**). Thus, the optimization of the assay conditions and its adaptation to HTS afforded a robust and time-saving assay, which also represents a powerful tool for studying the biochemical properties of this FAAL and others (determination of enzyme kinetic parameters, inhibition studies) and makes the assay an attractive option for the identification of FAAL inhibitors.

# Binding of a Substrate Analogue and Inhibition of FadD32 Activity

The shift in the thermal stability of *Msmeg*FadD32 in the presence of a substrate analogue (alkyl-adenylate, AMPC12), previously reported to inhibit the *Mtub*FadD32 enzyme activity,<sup>19</sup> was investigated using  $T_m$  measurements. The addition of AMPC12 to the protein induced a significant (6 °C) thermal shift ( $\Delta T_m$ ) compared with the protein alone at pH 7.5 (**Fig. 4A**). This shift indicated that the inhibitor binds to the protein, and this interaction very likely stabilizes *Msmeg*FadD32. The effect of AMPC12 on the protein activity was tested and, expectedly, a dose-dependent inhibition of the enzyme activity was observed in the presence of increasing concentrations of the alkyl-AMP (**Fig. 4A**), with a calculated EC<sub>50</sub> (half-maximal inhibitory concentration) of 1.4  $\mu$ M.

As the use of the  $T_{\rm m}$  shift appeared to be an interesting tool for evaluating the potential binding properties of compounds, as well documented in the literature,<sup>21</sup> it was thus employed in the screening of the limited library in the *Msmeg*FadD32 HTS.

#### Screening Cascade and Compound Progression

The biochemical assay was validated for HTS by testing its reproducibility. An elaborate assay validation was performed before the throughput screening to assess the dayto-day assay variability (data not shown). A set of 200 compounds was screened on different occasions, and the calculated Z' value<sup>27</sup> was 0.7 (data not shown), thus allowing initiation of the screening campaign. For the primary screening, 10 177 compounds were screened in singlicates (at 20  $\mu$ M) and, based on the 33% inhibition cutoff and stringent statistical criteria (hits outside mean + 2.5 interquartile range [IQR]\*), 412 potential inhibitors were selected (Suppl. Fig. S4). These compounds were tested for the 10-point (100 µM down to 0.2 µM) CR assay, and those showing CR and EC  $_{50}$  values less than 100  $\mu M$  were progressed further. Of 412 compounds, 200 were shortlisted: 98 compounds with good CRs (with Hill slopes between 0.5 and 2.0) and 102 with acceptable CRs (with a relaxed criterion for Hill slopes between 0.5 and 3). These 200 CR-confirmed hits were then classified into chemical clusters. Only 32 of these compounds were further selected based on rigorous hit evaluation criteria,



**Figure 3.** Fatty acyl-AMP ligase (FAAL) activity of *Msmeg*FadD32 after assay optimizations. (**A**) Radio-thin-layer chromatography (TLC) analysis of the [1-<sup>14</sup>C]C12-AMP synthesized using increasing concentrations of the *Msmeg*FadD32 enzyme. (**B**) A protein dose-response curve of *Msmeg*FadD32 showing the formation of enzyme product using either inorganic phosphate (Pi) released in the PiColorLock-based assay (Innova Biosciences, Cambridge, UK) (open squares) or acyl-AMP formed in the radio-TLC assay (black triangles). Assays were performed at room temperature during 80 min in HEPES pH 7.5 using 40 µM lauric acid and 2 mM adenosine triphosphate (ATP) as substrates. Means and standard errors of the mean (SEM) of four replicates (PiColorLock assay) or duplicates (radio-TLC) are shown. Lines were obtained by linear regression, and data are representative of three independent experiments.

such as chemical properties, ligand efficiency (cutoff value = 0.3), chemical tractability, and other druglike properties.<sup>†</sup> The 32 compounds were then tested in

<sup>\*</sup>Compound inhibition data sets follow "normal (Gaussian) distribution," wherein the spread of the middle-half of the data is called *interquartile range* or IQR. Statistically, this spread is  $4\sigma/3$ , where  $\sigma$  is the standard deviation. Statistically significant inhibition data points usually reside beyond the Median + 2 \* IQR range as "outliers." These outliers deserve attention as potential inhibitors.†If the compound is identified as a hit, it should satisfy the following hit evaluation criteria: (1) *chemical nature:* should have drug-like properties with adequate lipophilicity, ligand efficiency, and so on; (2) *chemical tractability:* should have multiple modification points to be able to make several analogues to form a chemical series in order to explore the most desirable derivative; and (3) *pharmacophores:* should *not* have known toxic motifs.



**Figure 4.** Inhibition of *Msmeg*FadD32 by the substrate analogue AMPC12 and screening for inhibitors of its activity. (**A**) Inhibition curve of *Msmeg*FadD32 activity by AMPC12. The chemical structure of adenosine 5'-alkylphosphate (alkyl-AMP) is displayed; R = C12 for AMPC12. Increasing concentrations of the compound were added to the assay, and FadD32 activity was measured in HEPES pH 7.5 in the presence of 20 µM lauric acid (C<sub>12</sub>) and 0.2 mM adenosine triphosphate (ATP) as substrates. Reactions were conducted at room temperature during 80 min and were done in quadruplicates. (**B**) Chemical classes, EC<sub>50</sub>, and T<sub>m</sub> shift measurements of the five selected compounds. <sup>\$</sup>Competes with ATP- $\gamma$ -S in a dose-dependent manner for binding to the enzyme. For details, refer to **Supplementary Table S1B**.

parallel in the promiscuity test (high protein effect) and the Thermofluor ( $T_m$  shift) assays.

In the promiscuity test, CR studies were done simultaneously at 1× and 5× protein concentrations to ascertain an undesirable protein effect or nonspecific inhibition. A compound is defined as "promiscuous" (i.e., exhibits undesirable protein effect) when it shows a 2-fold or more increase in its EC<sub>50</sub> value at higher (5× or higher) protein concentrations. The 2-fold shift criterion for determining the promiscuity was used since, for nonpromiscuous compounds, even a large change in enzyme concentration (2×–10×) is not expected to lead to changes in the EC<sub>50</sub> as the inhibitor's concentration is in far more excess than that of the protein, and unless the inhibitor binds nonspecifically, the  $EC_{50}$ s remain within a 2-fold change.<sup>23,28,29</sup> Sixteen compounds were found to pass this test (i.e., these compounds did not show a high protein effect) (**Suppl. Fig. S4**).

The Thermofluor assay was done for the same 32 compounds to ascertain a significant dose-dependent shift in MsmegFadD32  $T_{\rm m}$  as a result of binding of the compounds to the enzyme. Five of these compounds showed dose-dependent binding to the enzyme (Suppl. Table S1A) and did not show a nonspecific high protein effect and thus were "nonpromiscuous" in the promiscuity test (data not shown). Together, these data suggest that these compounds bind to the enzyme specifically. To substantiate these results, competition studies were done, where binding of all 16 nonpromiscuous compounds to MsmegFadD32 was checked in the presence of ATP-y-S in the Thermofluor assay. Convincingly enough, the same five compounds indicated dose-dependent binding, preferentially to the free form of the enzyme (with  $\Delta T_m 1 > \Delta T_m 2$ ), suggesting that they are competitive inhibitors of *Msmeg*FadD32 and that they competed with the ATP analogue for binding to the enzyme (Suppl. Table S1B). Binding of any of these compounds to the enzyme in the presence of the second substrate, lauric acid, however, could not be demonstrated because compounds did not show significant  $T_{\rm m}$  shifts in the presence of C12. The latter alone or in the presence of ATP- $\gamma$ -S did not show significant T shifts upon binding to *MsmegFadD32* (data not shown). It is not uncommon that some substrates do not show significant Tshifts upon binding to enzymes (in-house data) and that their binding to the enzyme can be shown with other biophysical assays such as 1D nuclear magnetic resonance spectroscopy.<sup>30</sup> These five hits represent five chemical classes as shown in Figure 4B, thus exhibiting (1) dose-dependent enzyme inhibition, (2) no protein effect (nonpromiscuous inhibitors), and (3) binding to *Msmeg*FadD32 in the Thermofluor assay.

Thus, this screening campaign demonstrated that the essential FadD32 enzyme is a "druggable" target. This HTS was performed on the *M. smegmatis* FadD32 protein using a 10 000-compound limited library and has validated the optimized HTS assay, leading to the selection of five compounds as inhibitors of this enzyme with a high hit rate (4%). These are true inhibitors, as shown by binding as well as competition studies. This proof of concept being made, the perspective of this work will be to apply the same strategy using an HTS campaign with an extended library to select more compounds with potentially lower EC<sub>50</sub>. The selected molecules will be the starting point for testing the corresponding chemical classes through "backscreening" on the M. tuberculosis enzyme. The efficacy of the most potent molecules will be tested on *M. tuberculosis* growth and checked on sensitive and resistant isolates. The application of high-throughput chemical screening for the discovery of small molecules that could inhibit FadD32 activity has significant potential in the development of novel anti-TB drugs.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### **Supplemental Material**

Suppl. Fig. S1: Michaelis-Menten curves for the determination of the appatent K<sub>m</sub> and V<sub>max</sub> for lauric acid (C<sub>12</sub>) and ATP. Data were obtained using the PiColorLock-based assay.
Reactions were conducted at RT during 40 min in HEPES pH7.5 buffer using increasing concentration of lauric acid with 2mM ATP (A) or increasing concentration of ATP with 200 μM of lauric acid (B). The K<sub>m</sub> and V<sub>max</sub> were determined using non-linear regression to fit

the data to the Michaelis-Menten equation.



**Suppl. Fig. S2:** Optimization of FadD32 expression and purification. Purification of *Mtub*FadD32 following either expression in BL21(DE3)/purification in Tris pH8 (**A**) or expression in *E.coli* BL21 Star<sup>TM</sup> (DE3) One Shot/purification in HEPES pH7.5 (**B**). Upper panels display fractions from the first step affinity chromatography and lower panels show fractions from second-step S-200 gel filtration chromatography, as analyzed by 12% SDS-PAGE.



Suppl. Fig. S3: Assay optimizations using the FadD32 from *M. smegmatis*. (A) Optimization of the reaction volume. Assays were conducted during 100 min at RT in Tris-HCl pH8 using 2 mM ATP, 200 $\mu$ M lauric acid in the absence (grey) or in the presence of 400 nM enzyme (black). The reaction volumes were either 20  $\mu$ L of substrate mixture/20  $\mu$ L of enzyme (20:20) or 15  $\mu$ L substrate mix/15  $\mu$ L enzyme with addition of 30  $\mu$ L of cold buffer (4°C) after incubation (15:15:30). (B) Effect of detergents on *Msmeg*FadD32 activity: assays were conducted at room temperature during 80 min in HEPES buffer at pH7.5 using 40 nM enzyme and 25  $\mu$ M lauric acid (C<sub>12</sub>) in the absence or in the presence of Brij<sup>®</sup>58 (0.01%), Brij<sup>®</sup>35P (0.01%) or NP-40 (0.1%). Means and standard errors of the mean (SEM) of four replicates are shown and data are representative of at least three independent experiments.



**Suppl. Fig. S4: Screening for** *Msmeg***FadD32 inhibitors.** Chart showing the cascade for the screening of a library of 10,177 compounds and the strategy for "Hit" selection and evaluation leading to selection of five compounds, non-promiscuous and binders.



Suppl. Table 1A:  $T_{\rm m}$  shift data for compounds binding to the *M. smegmatis* FadD32. Five compounds were checked for binding to *Msmeg*FadD32 at three concentrations, namely, 12.5, 25 and 50  $\mu$ M. These compounds showed dose-dependent  $T_{\rm m}$  shifts.

T <sub>m E</sub> =	41.5	+/- 0.12	(°C)
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Conc. (µM)	<b>Δ</b> T <sub>m</sub> ( <sup>0</sup> C)							
$\downarrow$	1	2	3	4	5			
12.5	0.1	0.2	0.2	0.5	-0.2			
25	0.5	0.2	0.7	0.7	0.3			
50	0.8	0.5	0.8	0.7	0.9			

Suppl. Table 1B:  $T_{\rm m}$  shift data for compounds binding to the *M. smegmatis* FadD32 enzyme in presence of  $\gamma$ -S-ATP. Binding of the five compounds to the *Msmeg*FadD32 enzyme was checked in presence of 0.2 mM and 1 mM ATP- $\gamma$ -S. These compounds were found to compete with the ATP analog for binding to the enzyme. Compounds at 50  $\mu$ M concentration were mixed with enzyme (E) alone, E+1x ATP- $\gamma$ -S and E+5x ATP- $\gamma$ -S in the reaction. From the  $T_{\rm m}$  Shift data, values of  $\Delta T_{\rm m}1$  and  $\Delta T_{\rm m}2$  were calculated for each compound (I) at 1X and 5X ATP- $\gamma$ -S concentrations. (where,  $\Delta T_{\rm m}1=T_{\rm mEI}$  -  $T_{\rm mE}$  and  $\Delta T_{\rm m}2=$  $T_{\rm mEIS}$  -  $T_{\rm mES}$  and  $T_{\rm mE}$ ,  $T_{\rm mEI}$ ,  $T_{\rm mEIS}$  were the  $T_{\rm m}$  values of free enzyme (E), E+S, E+I and E+S+I respectively; S being ATP- $\gamma$ -S).

	T <sub>m</sub> (°C)						
	E	E+1X S	E+5X S				
	T <sub>mE</sub>	T <sub>mES</sub>	T <sub>mES</sub>				
(AVG)	41.38	45.08	47.14		$\Delta T_m$ (°C)		
	I+E	I+(E+1x S)	I+(E+5x S)	I+E	I+(E+1x S)	I+(E+5x S)	
Compound (I)	T <sub>mEl</sub>	T <sub>mEIS</sub>	T <sub>mEIS</sub>	$\Delta T_m 1$	$\Delta T_m 2$	$\Delta T_m 2$	Result
1	42.23	45.32	47.12	0.85	0.24	-0.02	$\Delta T_m 1 > \Delta T_m 2$
2	41.90	45.13	47.18	0.52	0.05	0.04	$\Delta T_m 1 > \Delta T_m 2$
3	42.60	45.32	47.05	1.22	0.24	-0.09	$\Delta T_m 1 > \Delta T_m 2$
4	42.33	45.31	47.19	0.95	0.23	0.05	$\Delta T_m 1 > \Delta T_m 2$
5	42.60	45.48	47.42	1.22	0.40	0.28	$\Delta T_m 1 > \Delta T_m 2$