BBA - General Subjects 1867 (2023) 130444

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Small molecule inhibitors of the fosfomycin resistance enzyme FosM from *Mycobacterium abscessus*

Skye Chiasson [a], Tatum Smith [a], Landon Bello [a], Nishad Thamban Chandrika [b], Keith D. Green [b], Sylvie Garneau-Tsodikova [b], Matthew K. Thompson [a,*]

[a] Department of Chemistry & Biochemistry, The University of Alabama, 250 Hackberry Lane, Tuscaloosa, AL 35487, United States of America
[b] Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone St., Lexington, KY 40536, United States of America

ARTICLE INFO

Keywords:
Antimicrobial resistance
Fosfomycin
ForM
Mycobacterium abscessus

ABSTRACT

Fosfomycin is a safe broad-spectrum antibiotic that has not achieved widespread use because of the emergence of fosfomycin-modifying enzymes.

Inhibition of fosfomycin-modifying enzymes could be used to help combat pathogens like *Mycobacterium abscessus*.

Our previous work identified several inhibitors for the enzyme FosB from *Staphylococcus aureus*.

We have tested those same compounds for inhibition of FosM, the fosfomycin-modifying enzyme from *M. abscessus*.

The work described here will be used as the basis for more detailed studies into the inhibition of FosM.

Fosfomycin (Fig. S1) is a broad-spectrum antibiotic that exhibits antibacterial activity in both Gram-positive and Gram-negative bacterial species by inhibiting cell wall biosynthesis *via* irreversible inactivation of the enzyme UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase (MurA). [1] Originally characterized in 1969, fosfomycin was approved by the US Food and Drug Administration in 1996 for treatment of uncomplicated cystitis. [2] It exhibits low toxicity in humans and is typically given as a single 3-g oral dose, [3] which allows it to achieve concentrations in the body high enough to inhibit the growth of most pathogens. However, fosfomycin has not been able to achieve broad use due to the emergence of fosfomycin resistance enzymes.

Currently, there are three well-characterized classes of fosfomycin resistance enzymes: FosA, FosB, and FosX/M (Fig. S1), all of which confer resistance by catalyzing a nucleophilic addition reaction that opens the epoxide ring of fosfomycin and destroys the bactericidal properties of the antibiotic. FosA is a Mn^{2+}- and K^{+}-dependent glutathione transferase found in Gram-negative bacteria like *Pseudomonas aeruginosa*; [4] FosB enzymes are Mn^{2+}-dependent bacillithiol or L-cysteine transferases found in Gram-positive bacteria such as *Staphylococcus aureus*; [5] FosX enzymes are Mn^{2+}-dependent epoxide hydrolases that are found in both Gram-positive and Gram-negative species. [6] Recently, a FosX-type epoxide hydrolase, FosM, was characterized in *Mycobacterium abscessus* subsp. *bolletii*, representing the first fosfomycin-modifying enzyme characterized from any *Mycobacterium* species. [7]

M. abscessus is a complex of non-tuberculosis mycobacteria (NTM) comprising three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*. [8] Among NTM species, *M. abscessus* has been shown to be one of the most likely to cause a wide range of infections, including pulmonary and skin infections. [9] *M. abscessus* subsp. *bolletii* is associated with soft tissue infections in patients with chronic pulmonary diseases like cystic fibrosis. [10] *M. abscessus* poses a particularly significant threat due to its resistance to most currently available antibiotics, including fosfomycin. [11]

After we previously identified several potential small-molecule inhibitors of FosB, [12] we chose to investigate the effect of those compounds on FosM. We used ^{31}P NMR to determine if each compound successfully inhibited the activity of FosM *in vitro* (Fig. S2). Detailed experimental methods are outlined in the Supporting Information, and the full chemical names and structures of each compound can be found in Table S1. ^{31}P NMR is useful for qualitatively determining changes in FosM activity, but it cannot be used to obtain true Michaelis-Menten kinetic parameters. The reason is because many successive NMR scans are required before the peaks can be observed in the spectra due to peak averaging. This has been reported in several of our previous experiments [13] and appears to correlate to the slight curve in the early time points of our resulting data (Fig. S2) where each trace appears to curve instead

* Corresponding author at: Department of Chemistry & Biochemistry, The University of Alabama, Box 870336, Tuscaloosa, AL 35487, United States of America.
E-mail address: mthompson10@ua.edu (M.K. Thompson).

https://doi.org/10.1016/j.bbagen.2023.130444
Received 20 July 2023; Received in revised form 9 August 2023; Accepted 10 August 2023
Available online 12 August 2023

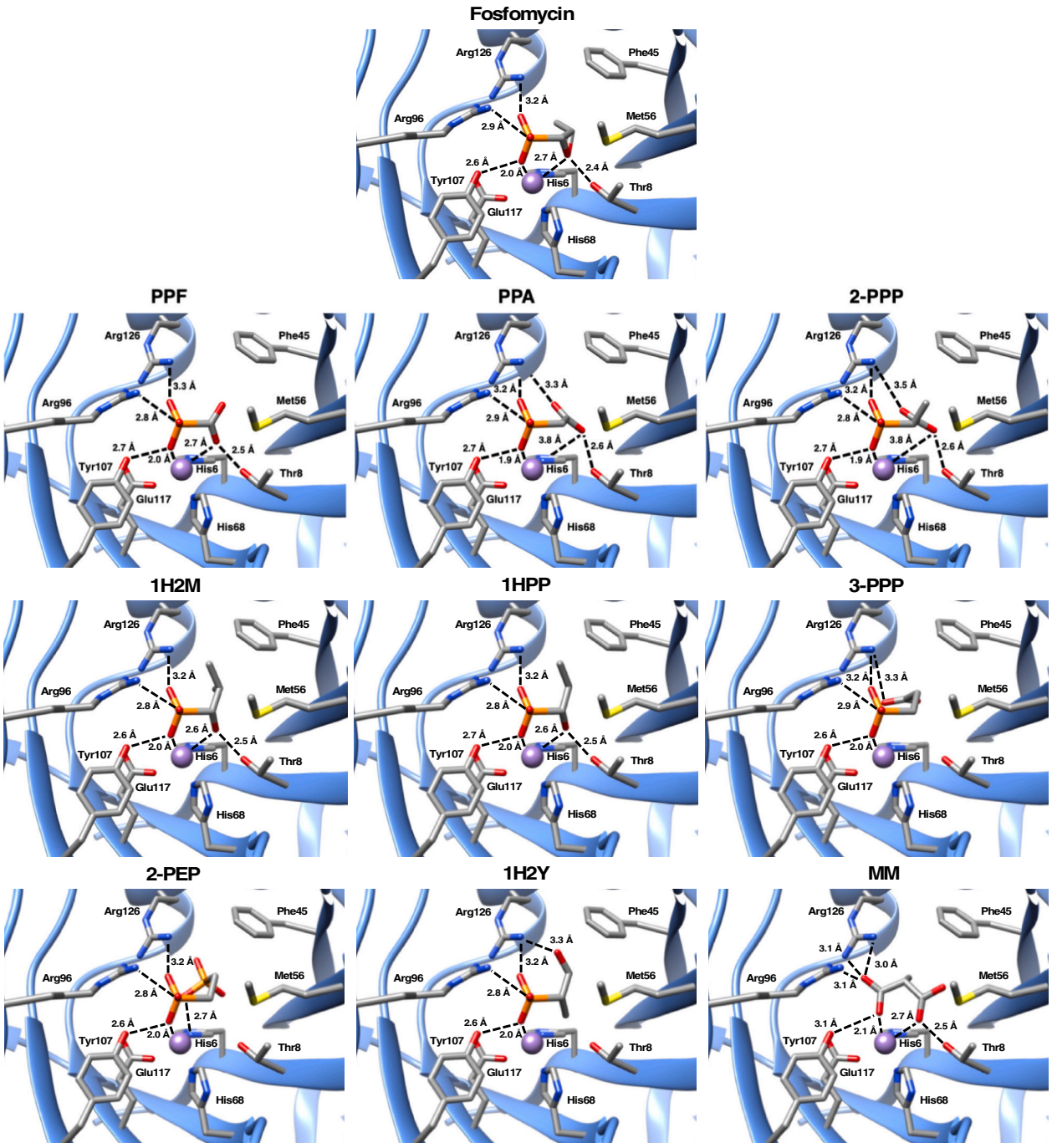

Fig. 1. Glide docking output of each of the compounds in our working library. Each compound has been docked into our FosM AlphaFold model. Important interactions with the metal and active site residues are shown as dashed lines.

of the first region being linear as would be expected. Nevertheless, the ^{31}P NMR technique allowed us to directly compare each inhibitor to the uninhibited enzyme. The results are summarized in Fig. S2.

The apparent k_{cat} of uninhibited FosM was 3.9 s^{-1}. This differs from our previously reported value of 15.1 s^{-1} and can most likely be attributed to the preparation of FosM for kinetic testing. As with all the fosfomycin resistance enzymes characterized thus far, [4–7,14] FosM typically co-purifies with Zn^{2+} in the active site, which is known to inhibit the enzyme. [7] We then remove the co-purified Zn^{2+} and replace it with Mn^{2+} to activate FosM. Because of this, every protein prep had slight variations in activity. It is possible that the Zn^{2+} was not fully removed for this protein preparation, thereby lowering the apparent k_{cat} of the enzyme. However, since we were only testing to determine which compounds successfully inhibited FosM, this lowered activity did not affect our comparison given we used the same stock of enzyme for all experiments.

Of the compounds evaluated, phosphonoformate (PPF), phosphonoacetate (PPA), and 2-phosphonopropionic acid (2-PPP) inhibited FosM at much lower concentrations than the others, indicating they are stronger inhibitors. At concentrations of 500 μM, PPF, PPA, and 2-PPP reduced the apparent k_{cat} of FosM to 1.1 s^{-1}, 2.3 s^{-1}, and 3.2 s^{-1}, respectively. For comparison, these three compounds were also the best low concentration inhibitors of FosB from *S. aureus*. [12,15] The remaining compounds required concentrations of 5 mM to observe any appreciable inhibition of FosM using the ^{31}P NMR technique. At 5 mM, 1-hydroxypropylphosphonic acid (1HPP) and (1-hydroxy-2-

methylpropyl)phosphonic acid (1H2M) reduced the apparent k_{cat} of FosM to 3.3 s^{-1} and 3.4 s^{-1}, respectively. Similar concentrations of these compounds were required to inhibit FosB. [12] The most interesting result from our experiments was the inhibition of FosM by 3-phosphonopropionic acid (3-PPP) and methylmalonate (MM). At 6 mM, 3-PPP had no effect on FosB, and MM had no effect on FosB at concentrations as high as 10 mM. [12] Here, MM and 3-PPP both demonstrated inhibition of FosM, reducing the apparent k_{cat} to 1.7 s^{-1} and 2.4 s^{-1}, respectively. (1-Hydroxypropan-2-yl)phosphonic acid (1H2Y) and 2-phosphonoethylphosphonic acid (2-PEP) were tested at concentrations up to 5 mM with no observable effect on FosM activity. These results indicate subtle differences in the active site and binding affinity for the compounds between FosB and FosM.

Thus far, all attempts to crystallize FosM have resulted in crystals that contain unresolvable translational non-crystallographic symmetry (tNCS). Therefore, to better understand why 3-PPP and MM inhibited FosM but not FosB, we generated a structural model of FosM using AlphaFold. [16] The model was generated as described in the Supporting Information. We then performed molecular docking of our compounds to the FosM structural model using the Schrodinger Program suite to generate Glide scores for comparison to FosB. The resulting Glide screening and docking scores, which are calculated based on predicted binding affinity to the active site, are provided in Table S1. A more negative Glide score is an indication of stronger binding. Differences between the docking and Glide scores reflects state penalties applied by the Glide program. Comparison of the Glide scores first indicated that fosfomycin itself has weaker binding to FosM than FosB (−9.61 compared to −10.73, respectively). This suggested a greater propensity for fosfomycin to be displaced from the active site of FosM in the presence of an inhibitor. Interestingly, the compounds which provided the best inhibition of FosM relative to concentration (PPF, PPA, and 2-PPP) returned weaker scores (−7.90, −7.99, and − 7.98, respectively), whereas the compounds which showed the weakest inhibition (1H2M and 1HPP) had the strongest scores (−9.87 and − 9.43, respectively). For comparison, PPF, PPA, 2-PPP, 1H2M, and 1HPP had Glide scores of −10.73, −10.11, −10.11, −10.42, and − 10.61, respectively, with FosB. [12,15] Finally, 3-PPP and MM returned Glide scores of −9.29 and − 7.63, respectively, in FosM, whereas they had Glide scores of −8.08 and − 7.73 in FosB. [12]

Observation of the docked structures (Fig. 1) indicates that all the phosphonate containing compounds have the phosphonate group positioned in the active site of FosM in the same orientation as fosfomycin. The same residues, Arg96, Tyr107, and Arg126, all form the same hydrogen bonding interactions with each compound. Of the three most effective inhibitors (PPF, PPA, and 2-PPP), all form a bidentate metal coordination geometry like fosfomycin. As the carboxylate oxygen of each compound moves further away from the metal, there is a correlation with reduced inhibitor potency. The carboxylate oxygen-metal distances for PPF, PPA, and 2-PPP are 2.7, 3.8, and 3.8 Å, respectively. This is the same trend observed for these three compounds with FosB. [12,15] We also note the potential interaction between the carboxylate oxygen of each compound and Thr8 of FosM. FosB has a L-cysteine residue at this position. The weak inhibitors that returned the best Glide scores, 1H2M and 1HPP, also form bidentate metal coordination and interaction with Thr8. However, both have an alcohol for the second metal coordinating ligand rather than a carboxylic acid. Formation of the alcohol group during inactivation of the fosfomycin epoxide has been implicated in product release. [14] This may explain why they have better Glide scores but are poor inhibitors. MM also forms bidentate metal coordination with hydrogen bonding to Arg96, Tyr107, and Arg126. In addition, we can see that MM forms a hydrogen bond interaction with Thr8. Once again, Thr8 is replaced with an L-cysteine residue in FosB and may contribute to the differences observed for inhibition. Finally, 3-PPP, 2-PEP, and 1H2Y do not form bidentate metal coordination. Consequently, 2-PEP and 1H2Y were the least effective inhibitors. 3-PPP, however, may gain stability from additional hydrogen bond formation between its carboxylic oxygen and Arg126.

The analysis presented here will be used to determine which FosM inhibitors we will pursue as lead compounds for future combinatorial administration with fosfomycin. Our goal is to inhibit the FosM enzyme and lower the minimum inhibitory concentration (MIC) of fosfomycin in *M. abscessus*. Given their effect on FosM activity relative to concentration PPF, PPA, and 2-PPP are the obvious choices. MM is interesting and also worth investigating for FosM, especially considering it showed no effect on the activity of FosB in our previous work. [12] We will continue to perform full Michaelis-Menten kinetic analyses to determine K_i values for each inhibitor and perform cell-based assays to determine if the compounds can act synergistically with fosfomycin. We hope these preliminary results will aid future structure-based drug design efforts to combat fosfomycin resistance.

Funding

This work was supported by The University of Alabama startup funds, the Cystic Fibrosis Foundation Grant #THOMPS2010, and National Institutes of Health grant #1R15GM148956.

CRediT authorship contribution statement

Skye Chiasson: Methodology, Investigation, Data curation, Writing – original draft. **Tatum Smith:** Validation, Investigation. **Landon Bello:** Validation, Investigation. **Nishad Thamban Chandrika:** Methodology, Formal analysis, Writing – review & editing. **Keith D. Green:** Methodology, Investigation, Writing – review & editing. **Sylvie Garneau-Tsodikova:** Writing – review & editing, Supervision, Project administration. **Matthew K. Thompson:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

There are no conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgements

M. K. T. would like to acknowledge the generosity of the Richard N. Armstrong family and Vanderbilt University Department of Biochemistry for initial laboratory equipment along with plasmids and reagents specific to this project. S. C. would like to acknowledge the Department of Education GAANN Grant #P200A150329.

Appendix A. Supplementary data

Supplementary material: The Supporting Information includes detailed materials and methods, a general mechanism of fosfomycin resistance enzymes, a summary figure of kinetic parameters, and a table of the full chemical names and structures of the compounds tested herein.

References

[1] F.M. Kahan, J.S. Kahan, P.J. Cassidy, H. Kropp, The mechanism of action of fosfomycin (phosphonomycin), Ann. N. Y. Acad. Sci. 235 (1974) 364–386.
[2] M.E. Falagas, A.C. Kastoris, D.E. Karageorgopoulos, P.I. Rafailidis, Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies, Int. J. Antimicrob. Agents 34 (2009) 111–120.
[3] S.S. Patel, J.A. Balfour, H.M. Bryson, Fosfomycin tromethamine. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy as a single-dose oral treatment for acute uncomplicated lower urinary tract infections, Drugs 53 (1997) 637–656.

[4] C.L. Rife, R.E. Pharris, M.E. Newcomer, R.N. Armstrong, Crystal structure of a genomically encoded fosfomycin resistance protein (FosA) at 1.19 a resolution by MAD phasing off the L-III edge of Tl(+), J. Am. Chem. Soc. 124 (2002) 11001–11003.
[5] M.K. Thompson, M.E. Keithly, M.C. Goodman, N.D. Hammer, P.D. Cook, K. L. Jagessar, J. Harp, E.P. Skaar, R.N. Armstrong, Structure and function of the genomically encoded fosfomycin resistance enzyme, FosB, from *Staphylococcus aureus*, Biochemistry 53 (2014) 755–765.
[6] K.L. Fillgrove, S. Pakhomova, M.R. Schaab, M.E. Newcomer, R.N. Armstrong, Structure and mechanism of the genomically encoded fosfomycin resistance protein, FosX, from *Listeria monocytogenes*, Biochemistry 46 (2007) 8110–8120.
[7] S. Travis, M.R. Shay, S. Manabe, N.C. Gilbert, P.A. Frantom, M.K. Thompson, Characterization of the genomically encoded fosfomycin resistance enzyme from *Mycobacterium abscessus*, MedChemComm 10 (2019) 1948–1957.
[8] E. Tortoli, T.A. Kohl, B.A. Brown-Elliott, A. Trovato, S.C. Leão, M.J. Garcia, S. Vasireddy, C.Y. Turenne, D.E. Griffith, J.V. Philley, R. Baldan, S. Campana, L. Cariani, C. Colombo, G. Taccetti, A. Teri, S. Niemann, R.J. Wallace Jr., D. M. Cirillo, Emended description of *Mycobacterium abscessus, Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* and designation of *Mycobacterium abscessus* subsp. *massiliense* comb. nov, Int. J. Syst. Evol. Microbiol. 66 (2016) 4471–4479.
[9] M.D. Johansen, J.-L. Herrmann, L. Kremer, Non-tuberculous mycobacteria and the rise of *Mycobacterium abscessus*, Nat. Rev. Microbiol. 18 (2020) 392–407.
[10] I. Sermet-Gaudelus, M. Le Bourgeois, C. Pierre-Audigier, C. Offredo, D. Guillemot, S. Halley, C. Akoua-Koffi, V. Vincent, V. Sivadon-Tardy, A. Ferroni, P. Berche, P. Scheinmann, G. Lenoir, J.L. Gaillard, *Mycobacterium abscessus* and children with cystic fibrosis, Emerg. Infect. Dis. 9 (2003) 1587–1591.
[11] R. Nessar, E. Cambau, J.M. Reyrat, A. Murray, B. Gicquel, *Mycobacterium abscessus*: a new antibiotic nightmare, J. Antimicrob. Chemother. 67 (2012) 810–818.
[12] S. Travis, K.D. Green, N. Thamban Chandrika, A.H. Pang, P.A. Frantom, O. V. Tsodikov, S. Garneau-Tsodikova, M.K. Thompson, Identification and analysis of small molecule inhibitors of FosB from *Staphylococcus aureus*, RSC Med. Chem. 14 (2023) 947–956.
[13] V. Wiltsie, S. Travis, M.R. Shay, Z. Simmons, P. Frantom, M.K. Thompson, Structural and functional characterization of fosfomycin resistance conferred by FosB from *Enterococcus faecium*, Protein Sci. 31 (2022) 580–590.
[14] M.K. Thompson, M.E. Keithly, J. Harp, P.D. Cook, K.L. Jagessar, G.A. Sulikowski, R.N. Armstrong, Structural and chemical aspects of resistance to the antibiotic fosfomycin conferred by FosB from Bacillus cereus, Biochemistry 52 (2013) 7350–7362.
[15] S. Travis, K.D. Green, N.C. Gilbert, O.V. Tsodikov, S. Garneau-Tsodikova, M. K. Thompson, Inhibition of fosfomycin resistance protein FosB from Gram-positive pathogens by phosphonoformate, Biochemistry 62 (2023) 109–117.
[16] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S.A. A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Highly accurate protein structure prediction with AlphaFold, Nature 596 (2021) 583–589.