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Inhibition of *Mycobacterium tuberculosis* InhA by 3-nitropropanoic acid

**SHORT TITLE:** Inhibition of InhA by 3NP

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/prot.26268 © 2021 Wiley Periodicals, Inc.

Received: Jul 25, 2021; Revised: Oct 05, 2021; Accepted: Oct 12, 2021

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Abstract

3-Nitropropanoic acid (3NP), a bioactive fungal natural product, was previously demonstrated to inhibit growth of Mycobacterium tuberculosis. Here we demonstrate that 3NP inhibits the 2-trans-enoyl-acyl carrier protein reductase (InhA) from Mycobacterium tuberculosis with an IC50 value of 71 μM, and present the crystal structure of the ternary InhA-NAD+-3NP complex. The complex contains the InhA substrate-binding loop in an ordered, open conformation with Tyr158, a catalytically important residue whose orientation defines different InhA substrate/inhibitor complex conformations, in the “out” position. 3NP occupies a hydrophobic binding site adjacent to the NAD+ cofactor and close to that utilized by the diphenyl ether triclosan, but binds predominantly via electrostatic and water-mediated hydrogen-bonding interactions with the protein backbone and NAD+ cofactor. The identified mode of 3NP binding provides opportunities to improve inhibitory activity towards InhA.

Keywords
3-nitropropanoic acid; bioactive compound; InhA; antituberculosis; Mycobacterium tuberculosis

1 INTRODUCTION

Mycobacterium tuberculosis is the causative pathogen of tuberculosis (TB) an infectious disease listed among the top 10 causes of mortality worldwide. Globally, approximately 1.4 million people died from TB-associated illness in the year 2019 (WHO), increasing TB antibiotic
resistance makes studies of new, potent inhibitors of known and potential anti-TB drug targets of great interest.

The NADH-dependent enoyl-acyl carrier protein reductase from *M. tuberculosis*, InhA, is the target of isoniazid, one of the main anti-TB drugs, and contributes to the biosynthesis of essential components of the mycobacterial cell walls through involvement in the type II fatty acid biosynthetic pathway. As isoniazid requires prior activation for InhA inhibition, and resistance is most commonly associated with disruption of the activation mechanism, inhibitors acting directly upon InhA are expected to circumvent isoniazid resistance and so represent attractive potential routes to anti-TB drug development. Structures of InhA in complex with various potent inhibitors have been reported in the PDB.¹

One advantage of the great biodiversity in Thailand is the profusion of natural products, some of which are candidate antimycobacterial agents.² One of these is 3-nitropropanoic acid (3NP, also known as 3-nitropropionic acid). 3NP has been extracted from an endophytic fungus, *Phomopsis* sp. usia5, isolated from plant hosts located in Thailand.² 3NP is a small compound (C₃H₅NO₄, molecular weight 119.08 g/mol (Figure S1)), and exhibits minimum inhibitory concentration (MIC) values of 50.0 µg/mL and 12.5 µg/mL against *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv, respectively.³ A derivative of 3NP, methyl 4-nitrobutyrate, had MIC values of 12.5 and 25.0 µg/mL against H37Ra and H37Rv strains, respectively.³

The toxicity of 3NP towards respiratory succinate dehydrogenase (SDH) is well known.⁴ However, 3NP has also been identified as an inhibitor of *M. tuberculosis* isocitrate lyase (ICL), with the crystal structure of the 3NP-ICL complex (PDB entry 6C4A) revealing the nitro group to act as a masked electrophile and form a covalent bond with Cys191.⁵ Given this demonstrated inhibition of targets beyond SDH, we were anxious to investigate whether the activity of 3NP against *M. tuberculosis* might extend to additional enzymes, specifically the isoniazid target InhA.

In this work inhibition of InhA by 3NP was evaluated, and the mode of binding of 3NP to InhA was identified by X-ray crystallography. These data will support further exploration and optimization of structures based upon 3NP, with the aim of designing new derivatives that reduce toxicity to humans, while retaining InhA inhibitory, and antimycobacterial, activity.

2 MATERIALS AND METHODS
2.1 Expression, purification and crystallization

Recombinant expression and purification of InhA from *M. tuberculosis* and inhibition assays, were carried out as previously described.6 3NP and NADH were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). CocrySTALLization of InhA with 3NP was performed at 293 K using the microbatch method with final concentrations of 5.0 mg/ml InhA, 50 mM NADH, and 10 mM 3NP and a precipiant solution containing 5 % (w/v) ethanol, 5 % (w/v) MPD, 200 mM sodium chloride and 100 mM Tris-HCl pH 8.5. Crystals were obtained within three days of incubation.

2.2 Data collection and processing

Crystals of the InhA-NAD⁺-3NP ternary complex were soaked in a cryoprotectant containing precipitant solution (see above) supplemented with 20% (v/v) glycerol and quickly vitrified in a nitrogen gas stream at 100 K. X-ray diffraction data from InhA-NAD⁺-3NP crystals were collected using a Mar165 CCD detector system (Rayonix, L.L.C., Evanston, IL, USA) at macromolecular crystallography beamline BL7.2W of the Synchrotron Light Research Institute (SLRI), Thailand, at a wavelength of 1.24 Å. Diffraction data were indexed and integrated using iMOSFLM7 and subsequently scaled using the program Scala from the CCP4 suite.8

2.3 Structure determination and refinement

The structure of InhA-NAD⁺-3NP was solved by molecular replacement using Phaser9 with PDB code 6R9W as the search model. A model of 3NP was generated using a structure data file obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov) as input to the eLBOW program10 and modelled into difference density present in the initial structure. The structure was then refined using iterative cycles of crystallographic model rebuilding with WinCoot11 and refinement with REFMAC512. The geometry of the refined structure was validated by MolProbity.13 Statistics for X-ray diffraction data collection and refinement of the InhA-NAD⁺-3NP ternary complex structure are presented in Table 1. Atomic coordinates and structure factors have been submitted to the PDB with accession 7E48. All the structures and electron density maps were generated and displayed with PyMOL.14
TABLE 1 Crystallographic data collection and refinement statistics

Values for the outer shell are given in parentheses.

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3 RESULTS AND DISCUSSION

3.1 Inhibition of InhA activity

As previously reported, 3NP exhibited measurable MIC values against both the attenuated H37Ra and virulent H37Rv *M. tuberculosis* strains. To establish whether 3NP might be acting on InhA, as well as the established *M. tuberculosis* target ICL, IC$_{50}$ values for InhA inhibition by 3NP
were determined in comparison with the phenolic diphenyl ether triclosan, an established InhA inhibitor.\textsuperscript{1} Plots of relative inhibition versus the log of inhibitor concentration are shown in Figure S2. The IC\textsubscript{50} value ($71.2 \pm 2.79 \, \mu M$) shows 3NP to display lower potency than triclosan ($5.32 \pm 0.30 \, \mu M$). However, the micromolar IC\textsubscript{50} values exhibited by 3NP are comparable to those observed for some other classes of InhA inhibitors such as arylamides and oxopyrrolidine carboxamides.\textsuperscript{1}

3.2 Overall structure of InhA-NAD\textsuperscript{+}-3NP

Crystals of the InhA-NAD\textsuperscript{+}-3NP complex belonged to a trigonal space group ($P3_2$) with unit-cell parameters, $a = b = 96.39, \, c = 139.50 \, \AA$, and contained four molecules per asymmetric unit. Positive electron density evident at early stages of refinement allowed 3NP to be placed and modelled, a difference electron density map calculated after removal of ligand from the final refined structure is shown in Figure 1A. The real-space correlation coefficient (RSCC) for bound 3NP was calculated by the PDB as $\geq 0.91$ for all chains, giving further confidence in the correct assignment of bound ligand. The asymmetric unit contains four molecules of the InhA-NAD\textsuperscript{+}-3NP ternary complex, with each InhA chain containing 267 amino acid residues (modelled as a continuous polypeptide spanning InhA residues Gly3 to Leu269 without gaps), along with one molecule of glycerol and 369 water molecules. The average atomic temperature factor ($B$-factor) for the ternary InhA-NAD\textsuperscript{+}-3NP complex is 37.53 Å\textsuperscript{2}, and the average $B$-factor for the bound inhibitor, 3NP, is 57.23 Å\textsuperscript{2}. The overall structure of bound 3NP has a chairlike conformation in which the cofactor is buried in the binding site, as illustrated in Figure 1B.

3.3 Interactions of 3NP in the InhA binding pocket

Figure 1C illustrates the interactions of the 3NP inhibitor with adjacent amino acids and the NAD\textsuperscript{+} cofactor via hydrogen bonds and hydrophobic interactions. Two water molecules (W103 and W193), whose presence is confirmed by an omit difference density map contoured at 4.5σ (Figure 1C, green), are involved in mediating these interactions. The 3NP binding site is located in the substrate binding pocket of the InhA catalytic center, and thus involves residues from the InhA substrate-binding loop (SBL, residues Met199 and Ile202, see below). The 3NP carboxylate group is hydrogen bonded to the NAD\textsuperscript{+} ribose hydroxyl (distances to the two carboxylate oxygen atoms 2.9 Å and 3.5 Å, respectively). One carboxylate oxygen atom is positioned to form an
electrostatic interaction with the nitrogen atom of the pyridine base of NAD⁺, at a distance of 4.0 Å. The 3NP nitro group is stabilized by water-mediated hydrogen bonds (via W103, distance 3.8 Å) to the amide and carbonyl groups of Met98 (distances of 3.6 Å and 3.7 Å, respectively). W193 forms hydrogen bonds with the carboxylate group of 3NP and the nicotinamide group of NAD⁺, with distances of 4.1 Å and 2.7 Å, respectively. In various InhA complexes, two conformations, termed “in” and “out”, of Tyr158 have been identified.¹ In our InhA crystal structure, Tyr158 in all four molecules, A, B, C and D, of the asymmetric unit adopts the “out” conformation, thus rotating away from the nicotinamide moiety of NAD⁺ and pointing towards the ordered SBL (Figure 1C). The “Tyr158-out” conformation avoids a steric clash with one carboxylate oxygen of 3NP while retaining the interaction of the inhibitor with the nicotinamide of NAD⁺ via a water-mediated hydrogen bond (Figure 1C) between the phenolic oxygen of Tyr158 and W193 (distance 2.7 Å).

3.4 Orientation of the InhA substrate-binding loop and comparison with other InhA complex structures

InhA contains a substrate-binding loop (SBL, residues Ile194 - Gly208) located on the outer surface of the structure. It is proposed that movement of the InhA SBL can influence accommodation of the C16 fatty acyl substrate (trans-2-hexadecenoyl-(N-acetyl-cysteamine)-thioester; THT; PDB entry 1BVR) and of some inhibitors, for example triclosan (PDB entry 1P45) and the related diphenyl ether PT70 (PDB entry 2X23). Previous structures of InhA:inhibitor complexes feature different conformations of the SBL: the triclosan complex (PDB entry 1P45) has an SBL conformation that is classified as a “wide open” state, also observed in PDB entries 6R9W (complex with inhibitor compound 7) and 1BVR (complex with C16 fatty acyl substrate THT); structure 2X23 (diphenyl ether PT70 complex) has a “closed” SBL conformation, whereas the SBL of 2NSD (arylamide inhibitor complex) has a conformation intermediate between these two (Figure S3A).

In the 3NP complex structure (PDB entry 7E48), the InhA SBL (Ile194 - Gly208) is relatively well defined, with an average B-factor of 43.30 Å², and forms an α-helix that covers the entrance to the active site. To compare orientations of the SBL, the structure of the InhA-NAD⁺-3NP complex was superimposed upon structures of representative InhA complexes differing in their SBL conformations (PDB entries 6R9W, 1P45, 1BVR, 2NSD and 2X23, see above). Average
root-mean-square deviation (RMSD) values between the 267 equivalent Cα atoms of 7E48 and these structures were 0.65, 0.71, 0.73, 0.79 and 1.14 Å, respectively. This comparison reveals that in the InhA-NAD⁺-3NP complex the SBL conformation is oriented in a “slightly open” state, between those of the SBLs in 1P45 and 2NSD (Figure S3A). This is consistent with the observation of an intra-loop hydrogen bond between the carbonyl group of Gly205 and the amide group of Gly208, and an inter-loop hydrogen bond between the carbonyl group of Leu207 and the amide group of Ile105 (in loop B) as shown in Figure S3B.

3.5 Comparison between the InhA binding sites of 3NP, the C16 substrate analogue THT and other inhibitors

Comparison of our 3NP-inhibitor-bound structure with various InhA complexes was undertaken to investigate the relationship of the 3NP binding site and mode to those of previously described compounds. The structure of InhA-NAD⁺-3NP was superimposed upon various InhA complex structures (PDB entries 6SQ5 and 6SQB from a study of fragment based InhA inhibitors; 4BQP and 4BQR from a study of methyl-thiazole inhibitors; and 6R9W, 1P45 and 1BVR (see above)). R.m.s.d. values for the superpositions of these structures upon the InhA-NAD⁺-3NP complex were 0.21, 0.22, 0.54, 0.54, 0.65, 0.71 and 0.73 Å, respectively, between the equivalent 267 Cα atoms, revealing a high similarity of the overall folds of these structures.

Structural comparison of the InhA-NAD⁺-3NP ternary complex structure with the complex of InhA bound to the C16 substrate analogue THT (PDB entry 1BVR) indicates that the binding region of 3NP partially overlaps with that of the C16 fatty acyl substrate (Figure 2A). In particular, the nitrogen atom of the inhibitor 3NP and the sulfur atom of the THT thioester bond occupy the same location, while 3NP cannot replicate interactions made by the more extensive THT aliphatic tail. Comparison of 3NP binding with that of triclosan is complicated by the presence in PDB entry 1P45 of two molecules of the InhA inhibitor triclosan in two distinct binding sites. The binding mode of 3NP overlaps with the first triclosan site (Figure 2B), with one carboxylate oxygen of 3NP located in the same region (within ~1.6 Å) as the ether oxygen atom linking the two triclosan phenolic rings. In contrast, our previously studied InhA inhibitor compound 7 (PDB entry 6R9W, Figure 2C) occupies the second of the triclosan binding sites evident in structure 1P45, i.e. that more distant from the NAD⁺ cofactor binding site. Comparison of the 3NP complex structure with
the structure of compound 7 bound to InhA shows that the 3NP nitro group is close to the position of the benzimidazole group of compound 7 (~1.5 Å) but there is otherwise little overlap in binding.

The complexes of InhA with THT, triclosan and compound 7 all feature Tyr158 in the “in” position, contrasting with the “out” orientation observed in the current complex structure. Hence, we also superimposed our InhA complex structure on those of the methyl thiazole compounds 7 and 11 (PDB entries 4BQP and 4BQR, respectively, Figure 2D) which feature Tyr158 in the “out” orientation. The carbon backbone and two carboxylate oxygens of 3NP overlay in the approximate plane of the terminal thiazole rings of 7 and 11, with one oxygen roughly overlapping with the methyl-thiazole methyl group and the other close to the position of the thiazole ring nitrogen atom.

Lastly, the carboxylate groups of two inhibitory fragments: fragment 1 (which contains a prop-2-enoic acid group (PDB entry 6SQ5)) and fragment 4 (3-(3-chlorophenyl) propanoic acid (PDB entry 6SQB)) identified in a study applying fragment-based methods to InhA inhibitor discovery, orient their carboxylate groups in the same region as, and providing partial overlap with, that of 3NP (Figure 2E). Binding of these fragments, both of which are larger than 3NP, however positions Tyr158 in the “in” orientation.
Figure 1 (A) A difference electron density map generated from the final structure with ligand removal and contoured at 4.5$\sigma$. (B) The overall structure of InhA-NAD$^+$-3NP complex (PDB entry 7E48). A ribbon representation is depicted in salmon. The substrate-binding loop is labelled. (C) The structural components of InhA-NAD$^+$-3NP complex (PDB entry 7E48, salmon). The labelled amino acid residues are depicted as ball-and-stick models with carbon atoms in the colour noted above, sulphur atoms in yellow and oxygen atoms in red. The 3NP inhibitor (salmon) and NAD$^+$ (limegreen) are shown as stick models with nitrogen in blue and oxygen in red. Black dash lines are presented for hydrogen bonds formation in the crystal structure of InhA complex with their distances given in Å. Water-mediated hydrogen bonds which stabilized both 3NP and NAD$^+$ are shown. A difference electron density map was generated from the final structure with water removal and contoured at 4.5$\sigma$. 
Figure 2 (A) The superposed structures of InhA-NAD⁺-3NP (PDB entry 7E48, salmon) and InhA-NAD⁺-THT (PDB entry 1BVR, magenta). (B) The superposed structures of InhA-NAD⁺-3NP (PDB entry 7E48, salmon) and InhA-NAD⁺-triclosan (PDB entry 1P45, orange). (C) The superposed structures of InhA-NAD⁺-3NP (PDB entry 7E48, salmon) and InhA-NAD⁺-Cpd7 (PDB entry 6R9W, cyan). (D) The superposed structures of InhA-NAD⁺-3NP (PDB entry 7E48, salmon), InhA-NAD⁺-7 (PDB entry 4BQP, lightpink) and InhA-NAD⁺-11 (PDB entry 4BQR, purple). (E) The superposed structures of InhA-NAD⁺-3NP (PDB entry 7E48, salmon) and InhA-NAD⁺-fragment 1 (PDB entry 6SQ5, lemon) and InhA-NAD⁺-fragment 4 (PDB entry 6SQB, sand). Substrate-binding loop is depicted in ribbon representation. The labelled Tyr158 residues are represented as ball-and-stick models with carbon in the colors noted and oxygen in red. The 3NP inhibitor (salmon) and NAD⁺ (limegreen) are shown as stick models with nitrogen in blue and oxygen in red. Other inhibitors are shown as stick model with carbon in the colors noted, sulfur in yellow, nitrogen in blue, oxygen in red, chloride in green and fluoride in light cyan.

4 CONCLUSIONS

Here, we show 3-nitropropanoic acid (3NP) to be a micromolar inhibitor of InhA and report the crystal structure of the InhA-NAD⁺-3NP ternary complex, revealing the interactions between protein and inhibitor. The complex features an ordered open state of the InhA SBL, defined by intra-loop and inter-loop hydrogen bond formation¹⁵ and has Tyr158 in the “out” conformation as seen in some other inhibitor complexes (PDB entries 4BQP, 4BQR, 4QXM, 4UVG, 5G0U and 5JFO). 3NP binds InhA via direct contacts and water-mediated hydrogen bonds with both active site amino acids and the NAD⁺ cofactor. 3NP orients in the InhA active site with its nitro group overlapping the position of the thioester bond of the fatty acyl substrate analogue THT, and its carbon backbone overlapping the thiazole rings of the methyl thiazole inhibitors 7 and 11 (PDB entries 4BQP and 4BQR, respectively). While there is some overlap with the first (i.e. more proximal to the NAD⁺ cofactor binding site) of the two triclosan binding sites identified in earlier studies (PDB entry 1P45), binding of 3NP as a smaller and more polar molecule is dominated by hydrogen-bonding and electrostatic contacts rather than hydrophobic interactions as observed for triclosan.
The IC50 value of 3NP (71.2 μM) is considerably less potent than that of triclosan (5.32 μM), and its well characterized mitochondrial toxicity precludes therapeutic use. However, elucidation of the mode of 3NP binding to InhA is of value in identifying starting points for potential antituberculosis drugs owing to the ready availability of 3NP from natural and commercial sources, the possibility of developing derivatives that may reduce toxicity and improve InhA inhibitory activity, and the possibility that the combination of improved activity against InhA with retention of ICL inhibition might result in an agent with multitargeting activity against *M. tuberculosis*. Our data indicate that 3NP binding to InhA predominantly involves interactions with NAD+ cofactor or, via intermediary water molecules, the backbone amide and carbonyl groups of active site amino acids. The lack of involvement of specific amino acid side chains in 3NP binding suggests that compounds exploiting these interactions might show reduced susceptibility to emergence of resistance by mutation of the InhA active site. These data indicate that further exploration and compound optimization, for example derivatization of 3NP at the 2- or 3-positions, may be warranted to exploit these findings in potential antituberculosis agents.

DECLARATION OF COMPETING INTEREST

All authors declare that they do not have any financial competing interests which occurred to effect the research work reported in this paper.

ACKNOWLEDGEMENTS

This research was granted by the Thailand Research Fund (Grant No. RSA5980057), RGJ Advanced Programme (Grant No. RAP60K0009), the Thailand Graduate Institute of Science and Technology (TGIST) (Grant No. SCA-CO-2560-4375TH) and Center of Excellence for Innovation in Chemistry (PERCH-CIC). JS acknowledges funding from the BristolBridge antimicrobial resistance network (EPSRC EP/M027546/1). We acknowledge Miss Narumol Mothong, SLRI staff for her technical assistance at the beamline. PP thanks Ubon Ratchathani for facilitating this research.

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Chayanin Hanwarinroj: Protein expression and purification, Characterization, Crystallization

Bongkochawan Pakamwong: Biological assay

Potjanee Srimanote: Protein expression and purification, Characterization

Nitima Suttipanta: Protein expression and purification, Characterization

Sanya Sureram: Characterization.

Khomson Suttisintong: Writing - revise & editing

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Auradee Punkvang: Writing - revise & editing.

James Spencer: Writing - revise & editing.

Prasat Kittakoop: Characterization, Writing - revise & editing.

Pornpan Pungpo: Conceptualization, Supervision, Resources, Project administration, Funding acquisition, Writing - revise & editing.

DATA ACCESS STATEMENT

Atomic coordinates and structure factors for the InhA-NAD⁺-3NP ternary complex have been submitted to the PDB (www.rcsb.org/pdb) with accession 7E48. Other data are provided in Supplementary Information.

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