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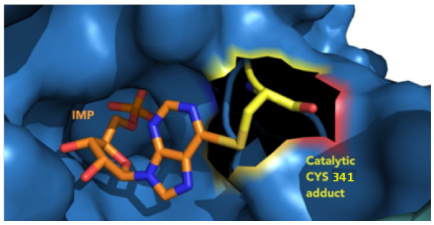

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Covalent Inactivation of *Mycobacterium thermoresistibile* inosine-5′-monophosphate dehydrogenase (IMPDH)

Ana Trapero[a,‡], Angela Pacitto[b,‡], Daniel Shiu-Hin Chan[a], Chris Abell[a], Tom L. Blundell[b,*], David B. Ascher[b,c,*], Anthony G. Coyne[a*]

[a]*Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom.*
[b]*Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, United Kingdom.*
[c]*Department of Biochemistry and Molecular Biology, Bio21 Institute, University of Melbourne, 30 Flemington Road, Parkville, Victoria 3052, Australia.*
*Corresponding authors at: Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom (AGC).
E-mail addresses: david.ascher@unimelb.edu.au (DBA), tom@cryst.bioc.cam.ac.uk (TLB), agc40@cam.ac.uk (AGC).
‡ These authors contributed equally.

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ABSTRACT

Inosine-5′-monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme involved in nucleotide biosynthesis. Because of its critical role in purine biosynthesis, IMPDH is a drug design target for immunosuppressive, anticancer, antiviral and antimicrobial chemotherapy. In this study, we use mass spectrometry and X-ray crystallography to show that the inhibitor 6-Cl-purine ribotide forms a covalent adduct with the Cys-341 residue of *Mycobacterium thermoresistibile* IMPDH.

Inosine-5′-monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205), a crucial enzyme required for *de novo* synthesis of guanine nucleotides, is considered an important drug target for immunosuppressive,[1] cancer,[2,3] antiviral[4] and antimicrobial chemotherapy.[5]

IMPDH is the enzyme that catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with the concomitant reduction of the cofactor nicotinamide adenine dinucleotide (NAD^+) to NADH. The reaction involves the initial attack of an active site cysteine 331 (numbering in human IMPDH) on the 2-position of IMP, followed by hydride transfer from the E-IMP intermediate to NAD^+. The resulting covalent enzyme intermediate E-XMP* is hydrolysed to XMP, regenerating the free enzyme.[6]

IMPDH has been isolated from 11 different sources and several X-ray crystal structures of IMPDH, including the *Mycobacterium thermoresistibile* (*Mth*) IMPDH protein,[7, 8, 9] have recently been determined in complex with substrate, product and cofactors along with a number of new inhibitors. IMPDH is a homotetramer, with each subunit composed of

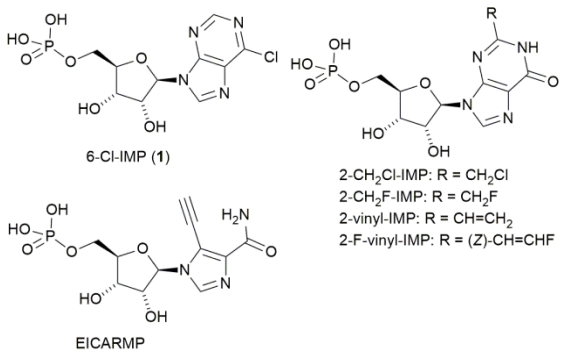


Fig. 1. Structures of selected covalent inhibitors of IMPDH.

two domains, the larger catalytic domain and a smaller domain containing tandem cystathione β-synthase (CBS) motifs.[10] Deletion of the CBS domain does not impact enzymatic properties or tetramer formation, but it does improve crystallization and solubility.

The existing IMPDH inhibitors have been divided into three major groups with respect to the binding position and

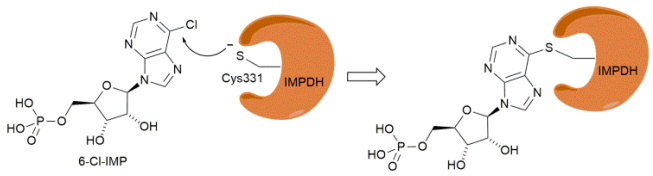


Fig. 2. Mechanism of human IMPDH type II inactivation by 6-Cl-purine ribotide.

the competitive relationship with IMP and NAD^+.[11] One group comprises ligands that target the binding pocket of the natural substrate, IMP. The other group of ligands includes those that target the site of the cofactor, NAD^+/NADH.[6] Moreover, it has been suggested that a third ligand binds to a putative allosteric site far from the IMP and NAD^+ pockets.

Covalent inhibitors contain specific functional groups, designed to react with a corresponding site in the target, typically an amino acid side chain.[12] Analogs of IMP have been designed to react with Cys-331 (numbering in human IMPDH) of the enzyme to form covalent bonds, including 6-Cl-purine ribotide,[13, 14] 2-CH_2Cl-IMP,[15] 2-CH_2F-IMP,[15] 2-vinyl-IMP,[15, 16] 2-F-vinyl-IMP[17] and EICARMP[18] (Fig. 1).

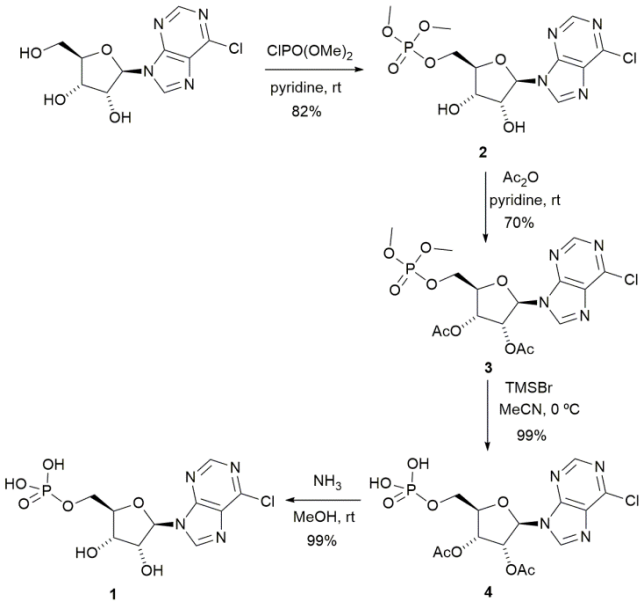
Covalent inhibitors have been shown to be useful in identifying amino acid residues that are important for substrate binding and catalysis.[19] It is particularly interesting to develop an inhibitor, which is also a substrate analog as in the case of 6-Cl-purine ribotide, since its primary reactive site would be in the active site of the enzyme.

6-Cl-purine ribotide has previously been reported to irreversibly inactivate IMPDH from *Aerobacter aerogenes*.[20] Modification of the catalytic cysteine has been verified,[14] and an X-ray crystal structure at a 2.9 Å resolution of human IMPDH type II in complex with 6-Cl-purine ribotide and the NAD analogue selenazole-4-carboxamide adenine dinucleotide has previously been reported.[13] The X-ray crystal structure of 6-Cl-purine ribotide with human IMPDH type II shows that the 6-chloro-substituted purine base is dehalogenated and forms a covalent bond at C-6 position on the purine ring with the thiol group of Cys-331 (Fig. 2).

In this paper, we report on the novel synthesis of 6-Cl-purine ribotide and the use of mass spectrometry and X-ray crystallography analysis of *Mth* IMPDH modified by the covalent inhibitor 6-Cl-purine ribotide. This study shows that 6-Cl-purine ribotide can irreversible bind to a Cys-331 located at the IMP binding site of the enzyme offering the possibility to develop selective covalent inhibitors.

A synthesis of 6-Cl-purine ribotide has been previously described in the literature,[21, 22] however the reproducibility of of the reported methods proved challenging. It was decided to develop an alternative route to this compound.

The covalent IMPDH inhibitor 6-Cl-purine ribotide (**1**) was synthesized from 6-chloropurine riboside according to the reaction sequence described in Scheme 1. In the first step, the primary hydroxyl group of the 6-chloropurine riboside was phosphorylated using dimethylchlorophosphate in pyridine at room temperature to obtain the phosphate



Scheme 1. Synthesis of 6-Cl-purine ribotide (**1**).

dimethyl ester **2** in 82% yield. The next step to directly obtain 6-Cl-purine ribotide was to remove the methyl phosphate protecting groups of compound **2** but attempts with TMSBr in MeCN failed. A likely depurination reaction took place during isolation of 6-Cl-purine ribotide as evidenced by LCMS and ^{1}H-NMR. It was anticipated that protection of the 3,4-diol **2** with acid resistant and electron attracting protecting groups should retard glycosidic bond cleavage during removal of the methyl phosphate protecting groups with TMSBr.[23] Acetyl groups were chosen for protection, as they can be conveniently removed under mild alkaline conditions. Protection of the 3,4-hydroxyl groups of **2** by acetyl groups using acetic anhydride and pyridine gave the diacetate **3** in 70% yield (Scheme 1).

The next steps in the synthetic path from compound **3** was the removal of the methyl esters and acetyl groups. Removal of the methyl esters was carried out in a quantitative yield with an excess of TMSBr in MeCN to give **4**, which was obtained by evaporation of the volatiles and was sufficiently pure, as evidenced by ^{1}H-NMR and LCMS, to be used for the next step without purification. This showed that protection of the 3,4-hydroxyl functions by acetyl groups suppressed depurination during removal of the methyl phosphate protecting groups with TMSBr.

The final step of the synthesis requires the removal of the acetyl groups in compound **4**. This was accomplished by treatment with a solution of ammonia in methanol to give the desired covalent inhibitor 6-Cl-purine ribotide (**1**) in an overall yield of 56% over the four steps (Scheme 1).

Mth IMPDH, which shares 85% sequence identity with *Mycobacterium tuberculosis* (*Mtb*) IMPDH and is 100% identical in the active site, was chosen for the mass spectroscopy and structural studies because it gave higher protein expression yields and better diffracting crystals than the *Mtb* orthologue. The deletion of the CBS domains facilitated crystallization of enzymes from several other organisms,[24] therefore a variant of *Mth* IMPDH with a GG linker replacing the two CBS domains was constructed,

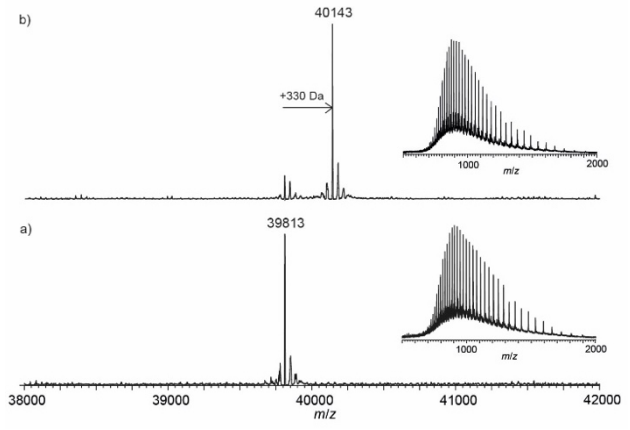


Fig. 3. Deconvoluted ESI-MS spectra for a) *Mth* IMPDH ΔCBS and b) *Mth* IMPDH ΔCBS treated with **1**, showing addition of 330 Da after addition of **1**. Insets show mass spectra before deconvolution.

this will be referred to as *Mth* IMPDH ΔCBS. We recently reported the development of potent *Mth* IMPDH ΔCBS inhibitors using fragment-based screening and structure-based design techniques.[7]

Mass spectroscopy is an excellent technique to confirm the formation of a covalent enzyme-inhibitor adduct.[25] The verification of the covalent nature of the interaction between *Mth* IMPDH ΔCBS and **1**, was examined using electrospray ionization-mass spectrometry (ESI-MS) under denaturating conditions (Fig. 3).

In the absence of **1**, *Mth* IMPDH ΔCBS displayed a *m/z* signal at 39813 Da (Fig. 3a), consistent with the expected mass of the construct (39812 Da). The treatment of *Mth* IMPDH ΔCBS with an excess of the covalent inhibitor **1** caused a 330 Da shift in the *m/z* signal to 40143 Da (Fig. 3b). This result is consistent with the covalent attachment of 6-Cl-purine ribotide (**1**) (366 Da) to the enzyme with the elimination of HCl (36 Da). The presence of just a single shifted peak indicates that the covalent modification has occurred on only one residue.

The X-ray crystal structure of *Mth* IMPDH ΔCBS with the inhibitor 6-Cl-purine ribotide (**1**) was solved at a resolution of 1.6 Å (PDB ID: 6MJY). The electron density showed 6-Cl-purine ribotide covalently bound in the IMP binding pocket of *Mth* IMPDH ΔCBS. In addition to the covalent bond to a nearby cysteine residue (C341), 6-Cl-purine ribotide made a strong network of primarily polar and hydrogen bonds to the protein (Fig. 4a).

The analysis of the binding pocket revealed a strong hotspot binding[26] potential region that was occupied by the 6-Cl-purine ribotide (Fig. 4b), suggesting that this site could be target from a drug discovery approach. A second hotspot binding potential region, which corresponded to the NADH binding site, was observed towards the entrance to the pocket. When the comparison of the binding orientation of 6-Cl-purine ribotide in the *Mth* IMPDH ΔCBS structure to previous structure with human IMPDH enzyme (PDB IDs: 1B3O,[13] 1JCN and 1NFB) revealed that due to differences in the binding pockets, 6-Cl-purine ribotide bound in a different orientation (Fig. 4c). This opens up potential for the development of novel inhibitors that can target mycobacterial enzymes.

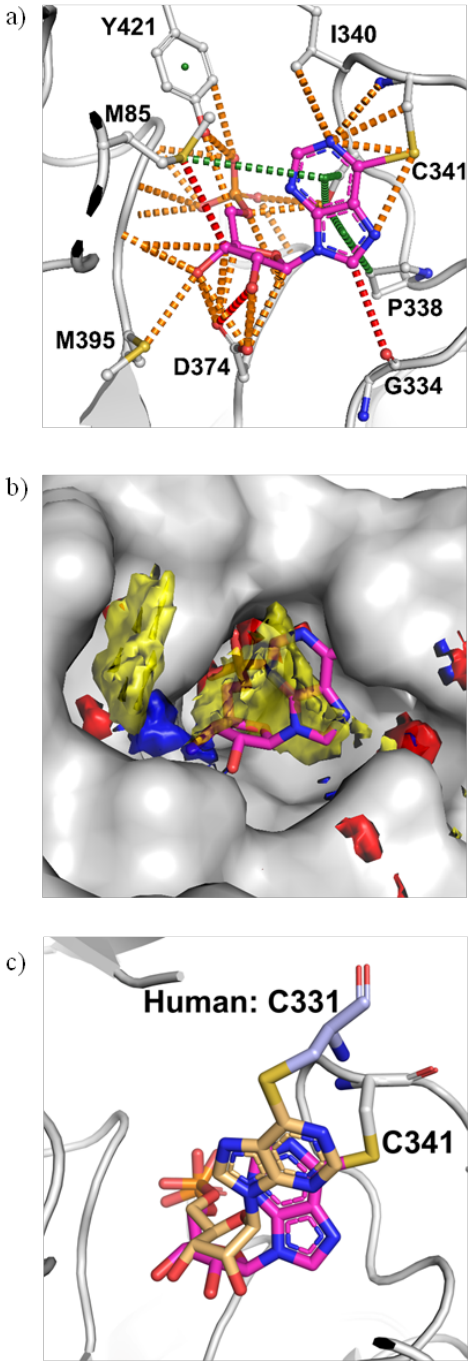


Fig. 4. (a) The X-ray crystal structures of *Mth* IMPDH ΔCBS (grey ribbon) with 6-Cl-purine ribotide (magenta sticks). (A) The interactions made by 6-Cl-purine ribotide (**1**) in the X-ray crystal structures of the complexes with *Mth* IMPDH ΔCBS (grey) were calculated by Arpeggio,[27] with ring and pi interactions shown in green, amide–amide in blue, hydrogen bonds in red, and polar interactions in orange. Residue numbering is of the mycobacterium tuberculosis sequence. (b) The hotspot[26] binding potential of *Mth* IMPDH ΔCBS Calculated hydrogen donor regions are coloured in blue, hydrogen acceptor regions in red and apolar regions in yellow. The additional binding potential towards to entrance to the pocket is where NADH binds. (c) Comparison of the binding orientation of 6-Cl-purine ribotide in *Mth* IMPDH ΔCBS to the crystal structure of human type II IMPDH (PDB ID: 1NFB).

In conclusion, this study reports on the synthesis of the irreversible inhibitor 6-Cl-purine ribotide and the covalent engagement of the Cys-341 residue of *Mth* IMPDH ΔCBS enzyme and the inhibitor. This covalent interaction was confirmed by mass spectrometry and X-ray crystallography studies. The mass spectrometry results indicated a shift of 330 Da upon treatment of Mth IMPDH ΔCBS with an excess of inhibitor 6-Cl-purine ribotide, consistent with the covalent attachment of the inhibitor to the enzyme. In addition, an X-ray crystal structure of 6-Cl-purine ribotide bound to *Mth* IMPDH ΔCBS showed that the 6-chloro-subtituted purine base was dehalogenated and forms a covalent bond at C-6 position on the purine ring with the thiol group of Cys341 in a novel conformation in comparison to previous covalent inhibitors. The information from the X-ray crystal structure offers the possibility for the development of selective covalent inhibitors of IMPDH from mycobacteria.

Declaration of Competing Interest

The authors declare no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at XXXXXX. The data include Experimental details, characterization, and additional supportive data.

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