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Oxanosine monophosphate is a covalent inhibitor of inosine 5'-monophosphate dehydrogenase

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Abstract

Reactive nitrogen species (RNS) are produced during infection and inflammation and the effects of these agents on proteins, DNA and lipids are well recognized. In contrast, the effects of RNS damaged metabolites are less appreciated. 5-Amino-3-β-(D-ribofuranosyl)-3 H-imidazo-[4,5-d][1,3]oxazine-7-one (oxanosine) and its nucleotides are products of guanosine nitrosation. Here we demonstrate that oxanosine monophosphate (OxMP) is a potent reversible competitive inhibitor of IMPDH. The value of K_i varies from 50 to 340 nM among IMPDHs from five different organisms. UV spectroscopy and x-ray crystallography indicate that OxMP forms a ring-opened covalent adduct with the active site Cys (E-OxMP^{*}). Unlike the covalent intermediate of the normal catalytic reaction, E-OxMP^{*} does not hydrolyze, but instead re-cyclizes to OxMP. IMPDH inhibitors block proliferation and can induce apoptosis, so the inhibition of IMPDH by OxMP presents another potential mechanism for RNS toxicity.

Graphical Abstract

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Supporting Information Available

Procedures to obtain spectra of E-OxMP^{*} using two methods, one table with molecular weight and extinction coefficient of IMPDHs, one table of kinetic parameters and ten figures showing ¹H-NMR and LC-MS spectrum of OxMP, kinetic and LC-MS characterization of OxMP inhibition, spectra of E-OxMP^{*} and contribution of two E-OxMP^{*} complex. This material (18 pages) is available free of charge via the Internet at <https://pubs.acs.org/oxmp-si.pdf>.

Introduction

Reactive nitrogen species (RNS) are produced during infection and inflammation and the effects of these agents on proteins, DNA and lipids are well recognized¹. In contrast, the effects of RNS damaged metabolites are less appreciated. 5-Amino-3-β-(D-ribofuranosyl)-3 H-imidazo-[4,5-d][1,3]oxazine-7-one (oxanosine) and oxanosine nucleotides are produced in the reaction of guanosine and guanosine nucleotides with RNS (Scheme 1A)², and oxanosine is a potential biomarker for nitrosation³. Oxanosine was originally isolated as an antibiotic from *Streptomyces capreolus* MG265-CF3^{4, 5}, and it also displays antitumor^{6, 7} activity. Both activities require phosphorylation to oxanosine monophosphate (OxMP)^{7, 8}. Therefore oxanosine and its nucleotides are examples of toxic damaged metabolites⁹.

OxMP was previously reported to be a modest inhibitor of inosine 5'-monophosphate dehydrogenase (IMPDH)⁷. This enzyme catalyzes the first and rate-limiting step in the *de novo* guanosine nucleotide biosynthesis pathway, the oxidation of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP) with the reduction of NAD⁺. The inhibition of IMPDH decreases the guanosine nucleotide pool, which blocks cell proliferation and leads to apoptosis¹⁰. IMPDH is a clinically validated target for immunosuppressive, anticancer and antiviral drugs^{11, 12} and a potential target for antimicrobial drugs¹³. The IMPDH mechanism involves two different chemical reactions, hydride transfer and hydrolysis (Scheme 1B)¹¹. The catalytic Cys attacks C2 of IMP and a hydride is transferred to NAD⁺, forming the covalent intermediate E-XMP*. NADH is released, and a mobile flap docks in the vacant dinucleotide site, forming the closed conformation required for hydrolysis of E-XMP*.

OxMP would be expected to be a competitive inhibitor of IMPDH given its similarity to IMP and the competitive inhibitor GMP. Surprisingly, OxMP was previously reported to be a mixed inhibitor with respect to IMP⁷. More recently, oxanosine was shown to be substrate of adenosine deaminase³. In this reaction, water attacks the 6-position, resulting in C6-O1 ring-opening to produce 1-β-(D-ribofuranosyl)-5-ureido-1 H-imidazole-4-carboxylic acid (Ur). Oxanosine can also react with thiol and amine nucleophiles to form analogous ring opened products³. Such reactions are believed to cause DNA-protein cross-links that are responsible for RNS-induced DNA damage¹⁴. We hypothesized that IMPDH might likewise catalyze this ring-opening reaction if the catalytic Cys attacked the 6 position of OxMP, resulting in a thioester enzyme adduct (E-UrMP*) (Scheme 1C). A similar reaction at C6 is observed when IMPDH is inactivated by 6-Cl-purine ribose monophosphate¹⁵. Alternatively,

the catalytic Cys might attack C2 of OxMP as observed in the normal catalytic cycle with IMP (Scheme 1D). In this case, C2-O6 ring opening would result in formation of a carbamimidothioate adduct (E-OxMP*), with the potential to hydrolyze to UrMP.

Here we report the kinetic and structural characterization of the inhibition of IMPDH by OxMP, which reveals the reversible formation of the carbamimidothioate adduct E-OxMP*. X-ray crystal structures of the OxMP complex suggests that the ring-opened inhibitor occupies the site of the catalytic water, preventing hydrolysis to UrMP.

Experimental procedures

Materials

IMP disodium salt was purchased from MP Biochemical (Solon, OH). GMP was purchased from Sigma-Aldrich (St. Louis, MO). NAD⁺ free acid was purchased from Roche. TCEP and DTT was purchased from GoldBio (St. Louis, MO). NaNO₂, Tris, glycerol, EDTA, KCl, triethylamine, methanol were purchased from Thermo Fisher Scientific.

Protein expression and purification

All IMPDHs were expressed and purified as previously described¹⁶. Briefly, BL21 *guaB* cells containing the appropriate expression plasmid were grown in LB medium with corresponding antibiotic (kanamycin and/or ampicillin) at 37 °C. When the OD₆₀₀ reached 0.6 to 0.8, the culture was induced with 0.5 mM IPTG and then grown at 25 °C overnight. The cell paste was collected by centrifugation at 4 °C for 20 min at 5,000 × g in a Beckman JLA10.5 rotor. Cells were then resuspended in phosphate buffer (50 mM K₂HPO₄, pH 8.0, 500 mM KCl, 5 mM imidazole, 0.1 mM TCEP, 10% glycerol) and sonicated on ice. After centrifugation at 9,000 × g for 1 h in a Beckman JA21 rotor, the supernatant was applied to Ni-NTA Sepharose beads (GE Healthcare). IMPDH was eluted with 250 mM imidazole and dialyzed in Tris buffer (50 mM Tris, pH 8.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, 10% glycerol). Purified fractions containing the IMPDH were characterized by SDS gel electrophoresis, concentrated to 100 μM, and flash-frozen in a dry ice/acetone cooling bath (final yields 3-8 mg/g of cell paste). Enzymes were stored at -80 °C. Enzyme concentrations were determined by measuring A₂₈₀ using the dialysis buffer as a blank.

Synthesis of OxMP

OxMP was synthesized from GMP, adapting the procedure to synthesize oxanosine^{2, 17}. GMP (80 mg) and NaNO₂ (140 mg) were added in 20 mL sodium acetate buffer (200 mM, pH = 3.7) (Scheme 2). The reaction mixture was shaken at 37 °C for 18 h and then filtered through a 0.2 μm filter. GMP was completely consumed after 18 h. OxMP was separated and purified by preparative RP-HPLC. The purified product was treated with a Dowex 50WX8-400 column to remove triethylammonium salt. The final product was confirmed by ¹H-NMR and LC-MS. The peak of OxMP appears at 246 nm and 287 nm in analytical RP-HPLC. The yield of the reaction is 20%. UV: λ_{max} 246 nm and 287 nm (ε₂₄₆ = 11,200 M⁻¹cm⁻¹, ε₂₈₇ = 7,400 M⁻¹cm⁻¹). ¹H-NMR (400 MHz, D₂O at 25 °C): δ (ppm) 8.30 (s, 1H, H-2), 7.93 (s, 2H, H-N), 5.72 (dd, 2H, H-2'), 4.56 (m, 1H, H-3'), 4.23 (m, 1H, H-4'), 4.17 (m, 1H, H-5'), 3.94 (m, 2H, H-6') (Figures S1 and S2).

RP-HPLC Analysis and Preparations

Preparative RP-HPLC was performed on a Waters HPLC system using a XBridge Prep C18 OBD column (19 mm × 250 mm, 5 μm). The mobile phase was a mixture of 100 mM triethylammonium bicarbonate buffer, pH = 7.0, and 0.05% formic acid (Buffer A) and 0.05% formic acid in methanol (Buffer B). The percentage of Buffer B was increased from 2% to 20% over 20 min in a linear gradient. The flow rate was 12 mL min⁻¹. Analytical RP-HPLC was performed in Varian Prostar HPLC system using a SiliaChrom dt C18 column (4.6 mm × 250 mm, 5 μm) using a linear gradient from 2% to 11% Buffer B over 9 min, maintaining 11% Buffer B for 6 min. The flow rate was 1.0 mL min⁻¹. UV spectra was obtained with Varian Prostar 325 UV-Vis detector and monitored by Galaxie Workstation.

LC-MS Analysis

Liquid chromatography mass spectrometry (LC-MS) was performed on a Waters Acquity UPLC system coupled to a photodiode array detector and Micromass ZQ4000 single quadrupole mass spectrometer, using Acquity UPLC BEH C18 columns (2.1 mm × 50 mm, 1.7 μm). The mobile phase was a mixture of water, pH = 7.0, and 0.07% formic acid (Buffer A) and acetonitrile and 0.1% formic acid (Buffer B). The percentage of Buffer B was maintained at 1% for 4 min and then increased from 1% to 95% over 2 min in a linear gradient. The flow rate was 0.330 mL min⁻¹. The peak of OxMP appears at 246 nm and 287 nm in the UPLC and is well separated from other nucleotides.

Steady-state Kinetics

IMPDH steady-state kinetics assays were performed in assay buffer (50 mM Tris-HCl, pH 8, 100 mM KCl and 1 mM DTT) with 20 nM enzyme at 25 °C. The production of NADH was monitored by changes in absorbance at 340 nm ($\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) on a Shimadzu UV-2600 UV-Vis spectrophotometer. Steady-state parameters were determined by collecting initial velocity data at varying concentrations of IMP (5 μM to 1,000 μM) or NAD⁺ (5 μM to 10,000 μM). All reactions were carried out in a total of 1 mL volume in 1 cm pathlength quartz cuvettes. Due to NAD⁺ substrate inhibition¹⁸, kinetic constants with respect to NAD⁺ were first obtained by varying NAD⁺ concentrations at fixed saturating concentrations of IMP. The values of $K_{m(\text{NAD}^+)}$ and $K_{ii(\text{NAD}^+)}$ were determined by fitting the initial to the equation describing uncompetitive substrate inhibition:

$$V_{max} = \frac{k_{cat}[E]}{1 + \frac{K_m}{[NAD^+]} + \frac{[NAD^+]}{K_{ii}}} \quad (1)$$

The value of $K_{m(\text{IMP})}$ was determined similarly by measuring initial velocities at varying IMP concentrations and fixed optimal concentrations of NAD⁺. The data were fit to the Michaelis-Menten equation:

$$v = \frac{V_{max}[\text{IMP}]}{K_m + [\text{IMP}]} \quad (2)$$

SigmaPlot (SPSS, Inc) was used for data analysis.

Inhibition Mechanism

The inhibition of GMP and OxMP was determined by measuring the initial velocity at varying concentrations of GMP (0 μ M to 1,200 μ M) or OxMP (0 μ M to 1.2 μ M) at fixed optimal concentrations of NAD⁺ (500 μ M for human IMPDH2, *Ta*IMPDH, *Cj*IMPDH and *Cj*IMPDH, 2,000 μ M for *Ba*IMPDH) and varying concentrations of IMP (5 μ M to 800 μ M). Initial velocity data best fit a competitive inhibition model (Equation 3) when compared with mixed and non-competitive inhibition models, as judged by Akaike's information criteria.

$$v = \frac{v_0[IMP]}{K_m \left(1 + \frac{[OxMP]}{K_{is}} \right) + [IMP]} \quad (3)$$

where v_0 is the initial velocity in the absence of inhibitor, $[S]$ is the concentration of IMP, $[I]$ is the inhibitor concentration, K_m is the Michaelis constant for IMP, and K_{is} is the slope inhibition constant, respectively. SigmaPlot (SPSS, Inc) was used for data analysis.

Jump Dilution Experiment

The reversibility of inhibition was determined by measuring the recovery of activity after the rapid dilution of the enzyme-inhibitor complex. IMPDH (4 μ M for *Ba*IMPDH and 2 μ M for *Cj*IMPDH) was pre-incubated with 10 μ M OxMP for 20 min. Then the E-I complex was diluted 100-fold into assay buffer containing IMP (160 μ M for *Ba*IMPDH and 230 μ M for *Cj*IMPDH) and NAD⁺ (2 mM for *Ba*IMPDH and 1 mM for *Cj*IMPDH) to initiate the reaction. In control reactions, IMPDH (4 μ M for *Ba*IMPDH and 2 μ M for *Cj*IMPDH) was pre-incubated alone and diluted into assay buffer containing 0 μ M, 10 μ M (as pre-incubation) and 0.1 μ M (the final diluted concentration) OxMP. The rate constant for the recovery of activity, k_{off} , was determined by equation 4¹⁹.

$$P = v_s t + \frac{(v_i - v_s) \left(1 - e^{-k_{off} t} \right)}{k_{off}} \quad (4)$$

where $[P]$ is the concentration of NADH, v_s is the steady-state velocity, v_i is the initial velocity immediately after dilution, k_{obs} is a rate constant for conversion between conditions of v_i and v_s .

Active-site Titration

Active-site concentrations of *Ba*IMPDH and *Cj*IMPDH were determined using titration with the nanomolar inhibitor 2-(2-chloro-5-(3-(2-(3-(prop-1-en-2-yl)phenyl)propan-2-yl)ureido)phenoxy)acetic acid²⁰. Initial velocity data at varying concentrations (0.001 μ M to 1,000 μ M) against approximately 0.05 μ M enzyme were fit into Morrison equation²¹ for tight-binding inhibition model.

$$v = v_0 \frac{([E] - [I] + K_i^*) + \sqrt{([E] - [I] - K_i^*)^2 + 4[E]K_i^*}}{2} \quad (5)$$

Enzyme (4.7 μM active site concentration) was then titrated with OxMP (1-10 μM). The data were fit by linear regression. SigmaPlot (SPSS, Inc) was used for data analysis. Similar values of K_i^* were obtained using R to fit Equation 5 and using Dynafit.

Product Determination

Varying concentrations of OxMP (0-10 μM) were incubated with 5 μM enzyme in 500 μL for 5 min. Free small molecules were separated from enzyme by filtering through a cellulose membrane (nominal molecular weight limit 30-kDa; Amicon (New York, NY)) by centrifuging at 14,000xg for 5 minutes. Samples were analyzed by LC-MS. OxMP was quantified by integration of absorbance at 287 nm. The remaining concentrated enzyme solution (60 μL) was diluted back to 440 μL with assay buffer containing IMP (1 mM for *Bal*IMPDH, 500 μM for *Cj*IMPDH, respectively) and NAD^+ (1 mM) and the reaction was monitored by UV. The inactive enzyme inhibitor complexes were quantified by relative activity.

Difference spectrum of the enzyme inhibitor reaction.

Enzyme (20 μM *Bal*IMPDH, 13 μM *Cj*IMPDH) was placed in one chamber of a split cuvette and OxMP (20 μM for *Bal*IMPDH, 13 μM for *Cj*IMPDH) was placed in the other. The unmixed spectrum was recorded every 5 minutes for 30 min. No changes were observed during this preincubation. The chambers were mixed and the spectrum was recorded again within 2 minutes of mixing. The spectrum was recorded every 5 minutes over the following 30 minutes. Enzyme or OxMP was mixed with assay buffer separately as controls. Spectrum of assay buffer was subtracted from all the spectra. Difference spectra of enzyme and OxMP before and after mixing were determined. Spectrum of the enzyme inhibitor complex was obtained by subtracting the spectrum of the enzyme from that of the enzyme-inhibitor complex.

Pre-steady state kinetics

The reactions of OxMP with *Cj*IMPDH and *Bal*IMPDH were investigated at 25 °C using Applied Photophysics SX17MV stopped flow spectrophotometer. Syringe A contained 10 μM IMPDH. Syringe B contained 0, 5, 10 μM OxMP. After pre-incubation, the contents of both syringes were mixed in 1:1 ratio. The reaction progress was monitored by recording absorbance at 287 nm and 321 nm in separate experiments. The extinction coefficients (ϵ) of OxMP were determined to be 7400 $\text{M}^{-1} \text{cm}^{-1}$ at 287 nm and 0 $\text{M}^{-1} \text{cm}^{-1}$ at 321 nm. Each data trace contained 16,000 points over 305 s and was an average of 3 separate injections. Data sets with enzyme alone were subtracted as background. Dynafit²² was used for data analysis. Progress curves were fit into 1) one-step model to form E-OxMP* (Scheme 3A) and 2) two-step model involving initial formation of E•OxMP followed by formation of reversible E-OxMP (Scheme 3B). The ϵ values of E•OxMP and E-OxMP* adduct were fit by Dynafit simultaneously. The values of k_{-1} for one-step model and k_{-2} for two-step model

were assigned to the value of k_{obs} determined in the jump dilution experiments. Kinetic parameters were derived by Dynafit and $K_{\text{is}}^{\text{app}}$ was calculated using equation 6 and 7 (for one-step model and two-step model, respectively).

$$K_{\text{is}}^{\text{app}} = \frac{k_{-1}}{k_1} \quad (6)$$

$$K_{\text{is}}^{\text{app}} = \frac{k_{-1}k_{-2}}{k_1(k_{-2} + k_2)} \quad (7)$$

Protein production and crystallization

*Ba*IMPDH L (the CBS domain E92-R220 deleted and replaced with GG), and *Cj*IMPDH S (CBS domain V92-T195 deleted and replaced with G)²³ were expressed and purified according to the Center for Structural Genomics of Infectious Diseases (CSGID) standard protocol²⁴. The proteins contained an N-terminal His₆-tag plus Tobacco Etching Virus (TEV) protease cut site. Briefly, a culture of enriched M9 medium was grown at 37 °C with a 180 rpm shaking. At $OD_{600} \approx 0.9$, the culture was cooled down to 4 °C for a half hour and supplied with 0.5 mM (final) IPTG. Protein expression was induced overnight at 18 °C. After a centrifugation, the cell pellet was suspended in a 35 ml lysis buffer containing 500 mM KCl, 5 % (v/v) glycerol, 50 mM HEPES pH 8.0, 20 mM imidazole and 10 mM β -mercaptoethanol per liter culture, treated with lysozyme (1 mg mL⁻¹) and sonication²⁵. The enzymes were purified using nickel(II) affinity chromatography (IMAC) using 5 mL HisTrap HP column (GE Healthcare Life Sciences, Piscataway, NJ) on AKTA express (GE Healthcare). Following TEV cleavage reaction, *Ba*IMPDH L was further purified by the second IMAC using the same 5 mL HisTrap HP column. The protein without the His-tag was collected from the flow-through fractions. Since the TEV cleavage reaction for *Cj*IMPDH S shown to be incomplete previously, the purification proceeded with size exclusion chromatography using Superdex 200 16/60 (GE Healthcare Life Sciences) after the first IMAC without the TEV cleavage reaction. The buffer solutions used in the purification included KCl in place of NaCl since most IMPDHs require K⁺ to be most active. Purified fractions containing the IMPDH were characterized by SDS gel electrophoresis, concentrated to 20 mg mL⁻¹, and flash-frozen in liquid nitrogen. The final yields for *Ba*IMPDH L and *Cj*IMPDH S were 3.8 and 4.0 (mg/g cell paste) respectively.

Crystallization experiments were performed using the sitting-drop, vapor-diffusion method in 96-well CrystalQuick plates (Greiner Bio-One, Monroe, NC) with the liquid dispenser Mosquito (LabTech, Cambridge, MA). For co-crystallization trials, ligands were used at a 10-20 fold molar excess over protein concentration. For each condition, 0.4 μ L of protein solution containing protein, IMP and inhibitor and 0.4 μ L crystallization formulation were mixed and the mixture was equilibrated against a 135 μ L reservoir. The suites of index (Hampton Research, Aliso Viejo, CA), JBScreen Wizard (Jena Bioscience, Jena, Thuringia,

Germany) and four MCSG crystallization screens (MCSG1-4, Anatrache Inc, Maumee, OH) were used and conditions yielding diffraction quality crystals typically appeared within 2–7 days. The complex *Ba*IMPDPH L·OxMP crystallized to a primitive tetragonal crystal P4 from the condition containing 0.1 M magnesium chloride, 0.1 M MES, pH 6.5 (by sodium hydroxide), and 30 % (volume/volume) PEG 400 which belongs to the condition of MCSG4 E6. *Cj*IMPDPH S·OxMP crystallized in a primitive monoclinic crystal P2₁ from the crystallization condition of MCSG4 D10 which contains 0.1 M lithium sulfate, 0.1 M MES, pH 6.0 (by sodium hydroxide), and 35 % (volume/volume) 2-methyl-2,4-pentanediol (MPD). Both complexes crystallized at 16°C.

Data Collection

All the X-ray diffraction experiments were performed at the Structural Biology Center 19-ID beamline at the Advanced Photon Source, Argonne National Laboratory²⁶. For the *Ba*IMPDPH L·OxMP crystals, prior to flash-cooling in liquid nitrogen, the crystals were cryoprotected in a solution containing the crystallization mother liquor plus 15% ethylene glycol. The *Cj*IMPDPH S·OxMP crystals did not require additional cryoprotectant. The crystals were mounted on Litholoops (Molecular Dimensions, Apopka, FL). All data were collected at 100 K on a Pilatus3 × 6M detector using SBCCOLLECT with an x-ray energy near 12.66 keV. HKL3000 suite²⁷ was used to process the diffraction images and to scale to final data sets for structure determination and refinement.

Structure solution and refinement

All crystal structures reported here were determined by the molecular replacement (MR) method using Molrep²⁸ and remlac²⁹ as a part of HKL3000 suite. The structure of chain A of the complex *Ba*IMPDPH L·P32 (4MYX), after removing IMP and the inhibitor molecule P32, was used as a search model for *Ba*IMPDPH L·OxMP structure. Similarly, the structure of chain A of the *Cj*IMPDPH S·P176 complex (5URQ), after removing the inhibitor P176 and IMP molecules, was used for *Cj*IMPDPH S·OxMP structure. In each of resulting MR structures, the presence of the well-defined extra electron density was apparent in the IMP site from the initial electron density map (F_0) calculated without any ligand molecule. The subsequent iterative steps of alternating manual adjustments and computational refinements were done using Coot³⁰ and Phenix³¹ until the convergence was achieved with an optimal stereochemistry for each structure. All structures were checked with PROCHECK³² and Molprobit³³ and Ramachandran plot and validated with PDB validation server.

PDB accession codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) and the accession codes are 6MGU and 6MGR for *Ba*IMPDPH L·OxMP and *Cj*IMPDPH S·OxMP, respectively.

Results and Discussion

Enzyme targets and their steady-state parameters

We chose to examine the inhibition of human IMPDH2 and IMPDHs from two protozoal pathogens, *Trichomonas foetus* (*Tf*) and *Cryptosporidium parvum* (*Cp*), and two bacterial

pathogens, *Campylobacter jejuni* (*Cj*) and *Bacillus anthracis* (*Ba*). These enzymes are good candidates for elucidating the mechanism of OxMP inhibition because they have well characterized kinetics and crystal structures. The steady-state kinetic parameters for all five enzymes were in good agreement with previously reported values (Table 1)^{25, 34, 35}. We also determined the values of K_{is} for GMP in order to compare with OxMP. As expected, GMP was a competitive inhibitor with respect to IMP for all five enzymes. The values of K_{is} varied from 8 μ M (*Cj*IMPDH) to 115 μ M (*Ba*IMPDH) and were in good agreement with previous reports (Table 1)^{36, 37}.

OxMP is a potent competitive inhibitor of IMPDH

We found that OxMP was a potent competitive inhibitor for all five IMPDHs versus IMP (Table 1; Figure S3). None of the IMPDHs displayed time dependent inhibition in the reaction progress curves or when enzyme and inhibitor were preincubated for 15 min prior to addition of substrates. The values of K_i varied from 51 nM (*Cj*IMPDH) to 340 nM (*Ba*IMPDH). OxMP affinity generally correlated with GMP affinity, although OxMP was more potent than GMP by factors of 140- to 350-fold. These findings conflict with a previous report of mixed inhibition ($K_i = 1\text{--}5\text{ }\mu$ M)⁷ using partially purified rat IMPDH. This discrepancy may result from the presence of a contaminating enzyme in the earlier experiment.

The dissociation of OxMP is slow

*Ba*IMPDH and *Cj*IMPDH were selected for further examination since these two enzymes had the most different affinities for OxMP. We verified that OxMP was a reversible inhibitor using a jump dilution experiment, where enzyme-inhibitor complexes were pre-formed then diluted into assay buffer containing IMP and NAD⁺. Activity recovered slowly over 5-10 min for both *Ba*IMPDH and *Cj*IMPDH (Figure 1). No lag was observed in the absence of inhibitor. The values of the dissociation rate constant k_{off} were determined to be 0.0019 s⁻¹ for *Ba*IMPDH and 0.0009 s⁻¹ for *Cj*IMPDH by fitting the progress curves to a single exponential (Figure 1). Interestingly, the similar values of k_{off} suggest that difference in the OxMP affinities of *Cj*IMPDH and *Ba*IMPDH derives primarily from k_{on} ($1.4 \times 10^4\text{ M}^{-1}\text{s}^{-1}$ for *Cj*IMPDH and $5.7 \times 10^3\text{ M}^{-1}\text{s}^{-1}$ for *Ba*IMPDH, assuming a one-step binding mechanism).

OxMP is not hydrolyzed by IMPDH

We hypothesized that IMPDH might catalyze the hydrolysis of OxMP to UrMP (Scheme 1C, D). *Ba*IMPDH and *Cj*IMPDH were incubated with different ratios of OxMP for 5 min. The unbound small molecules were isolated by filtration and characterized by LC-MS using conditions that separate OxMP from other nucleotides, including XMP, which has same mass (Figure S4 and S5). Only OxMP was observed. The stoichiometry of inhibition was determined by adding IMP and NAD⁺ to the enzyme samples (Figure S6). Using the initial rates, the stoichiometry of inhibition was 1 OxMP/active site (Figure 2). All of the OxMP could be accounted for in the enzyme-bound and free fractions, further confirming that no hydrolysis had occurred (Figure 2). In addition, no free UrMP was detected when enzyme and OxMP were incubated in a 1:1 ratio for 4 h.

OxMP forms a ring-opened covalent enzyme adduct

We reasoned that ring opening might be observed by monitoring absorbance since OxMP has an absorbance peak at 287 nm that is absent in UrMP³. We used a split cuvet to detect changes in the spectrum of OxMP after mixing with *Cj*IMPDPH and *Bal*IMPDPH (Figure 3 and S7). In both cases, the difference spectra between mixed and pre-mixed samples displayed troughs at approximately 250 nm and 280 nm and peaks at approximately 220 nm. Assuming that the spectrum of the enzyme is unchanged, the new inhibitor species is characterized by a spectrum with peaks at 262 and 311 nm. Since no UrMP could be detected (see above), the change in absorbance was most likely due to the formation of a covalent ring-opened adduct with the catalytic Cys (Scheme 1C,D).

We monitored the kinetics of adduct formation by measuring the change in absorbance at 287 (A_{287} , the peak of OxMP absorbance) and 321 nm (A_{321} , the maximum difference in absorbance of OxMP and adduct) in a stopped flow spectrophotometer (Figure 4A-D). We used Dynafit²² to globally fit both the A_{287} and A_{321} data sets for each enzyme to a one step binding mechanism (Scheme 3A). The values of k_{-1} were fixed to the values of k_{off} determined in the jump dilution experiment and the values of k_1 were determined in the global fit. The values for the extinction coefficients for E-OxMP* were initially assigned to the values determined in the split cuvette experiment, but were allowed to optimize during the fit.

For the reaction of *Bal*IMPDPH, the progress curves for both wavelengths were well described by a one-step mechanism with reversible formation of a covalent adduct (Figure 4A and Scheme 3A). The final extinction coefficients for E-OxMP* were 5160 M⁻¹ cm⁻¹ at 287 nm and 3560 M⁻¹ cm⁻¹ at 321 nm, in good agreement with the measured values, and the calculated value of K_i was 330 nM, in excellent agreement with that measured in the steady state experiment (340 nM). The absence of a detectable noncovalent E•OxMP complex was not surprising-assuming the dissociation constant is similar to that of GMP ($K_i = 110 \mu\text{M}$), little E•OxMP would be present under these experimental conditions ($[E] = 5 \mu\text{M}$ and $[OxMP] = 2\text{-}10 \mu\text{M}$).

The reaction progress curves for *Cj*IMPDPH reaction could also be described by a one step reaction, although the fits were not as good as those for the *Bal*IMPDPH reaction (Figure S10). The extinction coefficients were similar to those obtained for the *Bal*IMPDPH adduct, 5360 M⁻¹ cm⁻¹ at 287 nm and 4280 M⁻¹ cm⁻¹ at 321 nm. However, the calculated value of K_i was 6.7 nM, which was not consistent with the value measured in the steady state experiment (51 nM). In contrast to the *Bal*IMPDPH reaction, the noncovalent complex would be significantly populated during the *Cj*IMPDPH reaction if the dissociation constant for E•OxMP was similar to that of E•GMP ($K_i = 8 \mu\text{M}$). Therefore we considered a two step binding reaction (Scheme 3B, Figure 4C,D). The values of the extinction coefficients were fixed to the values determined in the *Bal*IMPDPH experiment. The progress curves were better described by the two step mechanism, although only an upper limit could be obtained for the value of k_{-2} ($< 0.029 \text{ s}^{-1}$). We calculated the value of k_{-2} using the values of k_{-1} and k_2 determined in the preliminary fit and the value of k_{off} measured in the jump dilution

experiment ($k_{-2} = \frac{k_{-1} + k_2}{k_{-1}} \times k_{off} = 0.0012 \text{ s}^{-1}$). Fixing the value of k_2 to 0.0012 s^{-1} , we

repeated the fit to yield the values shown in Scheme 3B (fit shown in Figure 4B). The calculated value of K_i was 39 nM, which is in good agreement with that measured in the steady-state experiment (51 nM).

Slow binding inhibitors usually display deviations from classical competitive inhibition, yet the steady state experiments clearly indicated that OxMP was a competitive inhibitor. We simulated the inhibition of *Ba*IMPDPH by OxMP using the previously determined microscopic rate constants for the kinetic mechanism¹⁶ and kinetic parameters determined in the current manuscript (Scheme 3A). The time dependent portion of the progress curve is essentially complete by 30 sec (Figure S11), which is within the dead time for our steady state kinetic experiments. The simulated initial rates clearly fit a competitive inhibition mechanism and the simulated K_i (320 nM) is in good agreement with the experimental value. Moreover, the simulated data did not fit a mixed inhibition model. The simulated data was also consistent with the observed jump dilution experiment. Thus all of the experiments are internally consistent.

Structures of the OxMP complexes of *Ba*IMPDPH and *Cj*IMPDPH

To establish if the covalent adduct is E-UrMP* or E-OxMP* (Scheme 1C,D), we determined the x-ray crystal structure of the OxMP complexes of *Ba*IMPDPH and *Cj*IMPDPH variants lacking the CBS subdomain (*Ba*IMPDPH L and *Cj*IMPDPH S, respectively). Deletion of the subdomain has no effect on catalytic activity²³. The statistics for crystallographic data processing, structure determination and refinement are given in Table 2. For the structure of the *Ba*IMPDPH L•OxMP complex, the asymmetric unit contains two protein chains, A and B, from two different tetramers. Each tetramer structure with a square planar symmetry is formed by four of A chains or four of B chains in the crystal. The structure was refined to 1.54 Å resolution and the final model for both chains consists of residues M1-S486. The two subunits are nearly identical with RMSD of 0.21 Å. The N-terminal portion includes three vector-derived residues, Ser, Asn, Ala directly preceding M1 when the His₆-tag is removed and S(-2)N(-1)A0 in chain A and N(-1)A0 in chain B are visible in the structure. The electron density for residues GG that were introduced in place of the CBS domains (resulting in a S91G92G220 motif) is well defined in both chains. A portion of the catalytic flap (*B. anthracis* residues 380-420) is disordered with 13 amino acids missing in both chains (residues 399-412). The final model also includes one OxMP molecule and one potassium ion per protein chain (Figure 5A), two ethylene glycols, six PEG molecules (two different lengths), and 237 water molecules.

The 1.97 Å structure of the complex of *Cj*IMPDPH S with OxMP is in the monoclinic space group P21 and the asymmetric unit consists of one tetramer with chains A, B, C, and D. The four subunits also have nearly identical conformations (RMSD 0.13 Å). The His₆-tag is not visible for each of all four chains but all protein chains contain residues M1-K481 along with one vector-derived alanine residue at the N-terminus. The other two vector-derived residues Ser-Asn are missing in all four chains. The junction region where the CBS domains (92-195) were removed is also not visible (Chain A: 90, 91, 198; Chain B: 90, 91, 198-200;

Chain C: 90, 91, 198, 199, Chain D: 90, 91, 198, 199). The S92 residue created after the removal of the CBS domains is also not defined well in all chains. Portions of the catalytic flap are disordered in the structure (Chains A and B: 393-395, 402-405, Chain C: 402-404 and Chain D: 393-396, 402-406). The final model of the *Cj*IMPDPH S·OxMP complex includes one molecule of OxMP and one potassium ion per protein chain (Figure 5B) and 10 chlorides, 15 sulfates, and 439 water molecules.

Structure of the ring-opened covalent enzyme adduct

OxMP occupies the IMP site and makes similar interactions in both enzymes (Figure 5A,B). Hydrogen bonds are observed between N7 of OxMP and the peptide nitrogen of Met391, O11 of OxMP and the Gly392 peptide nitrogen, O1 of OxMP and the thiol of Cys308, and N10 of OxMP and the hydroxyl of Thr310. The sugar moiety is in the C3'-endo conformation and both O2' and O3' form hydrogen bonds with Asp341. The phosphate moiety forms hydrogen bonds with Ser306, Gly343, Gly364, Ser365 and Tyr388. The oxanine ring is opened at O1-C2 bond (the distance between O1 and C2 is 2.71 Å in *Bal*IMPDPH L·OxMP and 2.70 Å in *Cj*IMPDPH S·OxMP). The thiol of Cys308 is 2.03 Å and 2.21 Å from C2 in both *Bal*IMPDPH L and *Cj*IMPDPH S (*Bal*IMPDPH numbering, Figure 5A,B), respectively, indicating the formation of an E-OxMP* adduct (Scheme 1D, Figure 5C,D). The C2-SG bonds are slightly longer than the ideal of 1.82 Å, perhaps due to the close proximity of the OxMP carboxylate. We performed six independent refinements (4 *Cj*IMPDPH and 2 *Bal*IMPDPH), and all of the electron density maps are most consistent with the E-OxMP* tautomer shown in Scheme 1D. The Cys S and OxMP NH₂, C2 and N3 are located in a single plane. All of the C-N distances are between the length of a conventional single bond (1.47 Å) and double bond (1.25 Å), indicating considerable resonance. The distance between C4 and N3 is the longest (1.38 Å), while the distances between C2 and N3 and C2 and N are 1.27 and 1.33 Å, respectively. We estimated that no more than 15% of an alternative tautomer or adduct can be present.

Fifteen structures of *Bal*IMPDPH and its CBS domain deleted variants (*Bal*IMPDPH L and *Bal*IMPDPH S) are currently available in the Protein Data Bank, including complexes that mimic several steps in the catalytic cycle: apoenzyme (4MJM)²³, E•IMP (3USB), E•XMP (3TSD) and E•P_i (phosphate ion-bound form, 3TSB), where the flap is found in the closed conformation²⁵. With the exception of the flap and the loop that contains Cys308, the protein conformation of *Bal*IMPDPH L·OxMP is nearly identical to those found in these previously reported complexes (Figure 5E). In contrast, the mononucleotide conformations are different in each complex. Neither IMP nor XMP form covalent adducts with Cys308. The purine ring of XMP is rotated slightly away from Cys308 relative to IMP (O4'-C1'-N9-C4 torsion angles are -143 ° and -132 ° for XMP and IMP, respectively), while the ring-opened base of OxMP is rotated 39 ° toward Cys308 (O4'-C1'-N9-C4 torsion angle = -93°) (Figure 5F). Cys308 also moves relative to its position in the IMP and XMP complexes. Curiously, Cys308 is found in the same position as in the E•P_i complex.

In contrast, little difference is observed in the conformations of IMP and OxMP and the position of the catalytic Cys in *Cj*IMPDPH (O4'-C1'-N9-C4 torsion angles are -93 ° and -99 ° for IMP and OxMP, respectively; Figure 5G). These observations suggest that the

active site of *Cj*IMPDH is poised to react with OxMP, which may account for the higher affinity of OxMP for this enzyme.

IMPDHs employ different conformations for each step of the catalytic cycle, an open conformation for hydride transfer and a closed conformation for hydrolysis of E-XMP*¹¹. Although the flap is disordered in both OxMP complexes, the last visible residues clearly indicate the flap is in an open conformation (Figure 5H). Moreover, at least two different open conformations are observed in *Cj*IMPDH S·OxMP. The failure of the E-OxMP* to hydrolyze might derive from the inability to form the closed conformation. Superposition of the *Ba*IMPDH L·OxMP structure with that of the closed conformation found in *Ba*IMPDH·P_i (3TSB) suggests that the E-OxMP* adduct does not prevent formation of the closed conformation (Figure 5I). However, superposition with the structure of the transition state analog complex with *T. foetus* IMPDH (1PVN)³⁸ shows that N10 of E-OxMP* is in the same position as the putative catalytic water (Figure 5I). While N10 prevents the attack of water, the close proximity of O1 and C2 indicates that cyclization can readily occur to regenerate OxMP.

Conclusions

Oxanosine and its nucleotides are RNS-damaged metabolites. The experiments described above demonstrate that OxMP is a potent competitive inhibitor of IMPDH. An O1-C2 ring-opened covalent enzyme adduct is formed with the catalytic Cys, but does not hydrolyze. X-ray crystal structures suggest that the inhibitor blocks access of the catalytic water, while the O1 is poised for recyclization. IMPDH inhibitors block proliferation, leading to apoptosis¹⁰, so the inhibition of IMPDH by OxMP serves as another mechanism for the RNS toxicity.

OxMP can form directly by the nitrosation of GMP, by phosphorylation of oxanosine, or possibly via further metabolism of other oxanosine nucleotides and oligonucleotides. Given the ubiquitous nature of RNS, it seems likely that OxMP is processed by yet to be discovered metabolite repair enzymes, as is observed for other toxic nucleotides such as 8-oxo-GTP⁹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used:

***Ba*IMPDH** IMPDH from *B. anthracis*

CjIMPDH	IMPDH from <i>C. jejuni</i>
GMP	guanosine 5'-monophosphate
IMP	inosine 5'-monophosphate
IMPDH	inosine 5'-monophosphate dehydrogenase
OxMP	oxanosine monophosphate
P_i	phosphate ion
RNS	reactive nitrogen species
UrMP	1-β-(D-ribofuranosyl)-5-ureido-1 H-imidazole-4-carboxylic acid monophosphate
XMP	xanthosine 5'-monophosphate

References

- (1). Martínez MC, and Andriantsitohaina R (2009) Reactive Nitrogen Species: Molecular Mechanisms and Potential Significance in Health and Disease. *Antioxidants & Redox Signaling* 11, 669–702. [PubMed: 19014277]
- (2). Suzuki T, Yamaoka R, Nishi M, Ide H, and Makino K (1996) Isolation and Characterization of a Novel Product, 2'-Deoxyoxanosine, from 2'-Deoxyguanosine, Oligodeoxynucleotide, and Calf Thymus DNA Treated by Nitrous Acid and Nitric Oxide. *Journal of the American Chemical Society* 118, 2515–2516.
- (3). Majumdar P, Wu H, Tipton P, and Glaser R (2005) Oxanosine Is a Substrate of Adenosine Deaminase. Implications for the Quest for a Toxicological Marker for Nitrosation Activity. *Chemical Research in Toxicology* 18, 1830–1841. [PubMed: 16359173]
- (4). Shimada N, Yagisawa N, Naganawa H, Takita T, Hamada M, Takeuchi T, and Umezawa H (1981) Oxanosine, a Novel Nucleoside from Actinomycetes. *The Journal of Antibiotics* 34, 1216–1218. [PubMed: 7328060]
- (5). Nakamura H, Agisawa N, Imada N, Takita T, Umezawa H, and Iitaka Y (1981) The X-Ray Structure Determination Of Oxanosine. *The Journal Of Antibiotics* 34, 1219–1221. [PubMed: 7328061]
- (6). Itoh O, Kuroiwa S, Atsumi S, Umezawa K, Takeuchi T, and Hori M (1989) Induction by the Guanosine Analogue Oxanosine of Reversion toward the Normal Phenotype of K-ras-transformed Rat Kidney Cells. *Cancer Research* 49, 996–1000. [PubMed: 2643467]
- (7). Uehara Y, Hasegawa M, Hori M, and Umezawa H (1985) Increased Sensitivity to Oxanosine, a Novel Nucleoside Antibiotic, of Rat Kidney Cells upon Expression of the Integrated Viral src Gene. *Cancer Research* 45, 5230–5234. [PubMed: 2413989]
- (8). Yagisawa N, Shimada N, Takita T, Ishizuka M, Takeuchi T, and Umezawa H (1982) Mode Of Action Of Oxanosine, A Novel Nucleoside Antibiotic. *The Journal Of Antibiotics* 35, 755–759. [PubMed: 6288649]
- (9). Linster CL, Van Schaftingen E, and Hanson AD (2013) Metabolite damage and its repair or pre-emption. *Nature Chemical Biology* 9, 72. [PubMed: 23334546]
- (10). Khan M, Dhanwani R, Patro IK, Rao PVL, and Parida MM (2011) Cellular IMPDH enzyme activity is a potential target for the inhibition of Chikungunya virus replication and virus induced apoptosis in cultured mammalian cells. *Antiviral Research* 89, 1–8. [PubMed: 21070810]
- (11). Hedstrom L (2009) IMP Dehydrogenase: Structure, Mechanism, and Inhibition. *Chemical Reviews* 109, 2903–2928. [PubMed: 19480389]

- (12). Cuny GD, Suebsuwong C, and Ray SS (2017) Inosine-5'-monophosphate dehydrogenase (IMPDH) inhibitors: a patent and scientific literature review (2002-2016). *Expert Opinion on Therapeutic Patents* 27, 677–690. [PubMed: 28074661]
- (13). Hedstrom L, Liechti G, Goldberg JB, and Gollapalli DR (2011) The Antibiotic Potential of Prokaryotic IMP Dehydrogenase Inhibitors. *Current Medicinal Chemistry* 18, 1909–1918. [PubMed: 21517780]
- (14). Chen H-JC, Chiu W-L, Lin W-P, and Yang S-S (2008) Investigation of DNA-Protein Cross-Link Formation Between Lysozyme and Oxanine By Mass Spectrometry. *ChemBioChem* 9, 1074–1081. [PubMed: 18351683]
- (15). Colby TD, Vanderveen K, Strickler MD, Markham GD, and Goldstein BM (1999) Crystal structure of human type II inosine monophosphate dehydrogenase: Implications for ligand binding and drug design. *Proceedings of the National Academy of Sciences* 96, 3531–3536.
- (16). Wei Y, Kuzmi P, Yu R, Modi G, and Hedstrom L (2016) Inhibition of Inosine-5'-Monophosphate Dehydrogenase from *Bacillus Anthracis*: Mechanism Revealed by Pre-Steady-State Kinetics. *Biochemistry* 55, 5279–5288. [PubMed: 27541177]
- (17). Suzuki T (2000) Formation of 2'-deoxyoxanosine From 2'-deoxyguanosine and Nitrous Acid: Mechanism and Intermediates. *Nucleic Acids Research* 28, 544–551. [PubMed: 10606654]
- (18). Kerr KM, and Hedstrom L (1997) The Roles of Conserved Carboxylate Residues in IMP Dehydrogenase and Identification of a Transition State Analog. *Biochemistry* 36, 13365–13373. [PubMed: 9341229]
- (19). Copeland RA, Basavapathruni A, Moyer M, and Scott MP (2011) Impact of enzyme concentration and residence time on apparent activity recovery in jump dilution analysis. *Analytical Biochemistry* 416, 206–210. [PubMed: 21669181]
- (20). Gollapalli D, and Hedstrom L (2018) Manuscript in preparation.
- (21). Morrison JF (1969) Kinetics of the Reversible Inhibition of Enzyme-Catalysed Reactions By Tight-Binding Inhibitors. *Biochimica et Biophysica Acta (BBA) - Enzymology* 185, 269–286.
- (22). Kuzmi P (1996) Program DYNAFIT for the Analysis of Enzyme Kinetic Data: Application to HIV Proteinase. *Analytical Biochemistry* 237, 260–273. [PubMed: 8660575]
- (23). Makowska-Grzyska M, Kim Y, Maltseva N, Osipiuk J, Gu M, Zhang M, Mandapati K, Gollapalli DR, Gorla SK, Hedstrom L, and Joachimiak A (2015) A Novel Cofactor-Binding Mode in Bacterial IMP Dehydrogenases Explains Inhibitor Selectivity. *Journal of Biological Chemistry* 290, 5893–5911. [PubMed: 25572472]
- (24). Kim Y, Babnigg G, Jedrzejczak R, Eschenfeldt WH, Li H, Maltseva N, Hatzos-Skintges C, Gu M, Makowska-Grzyska M, Wu R, An H, Chhor G, and Joachimiak A (2011) High-Throughput Protein Purification and Quality Assessment for Crystallization. *Methods* 55, 12–28. [PubMed: 21907284]
- (25). Makowska-Grzyska M, Kim Y, Wu R, Wilton R, Gollapalli DR, Wang XK, Zhang R, Jedrzejczak R, Mack JC, Maltseva N, Mulligan R, Binkowski TA, Gornicki P, Kuhn ML, Anderson WF, Hedstrom L, and Joachimiak A (2012) *Bacillus Anthracis* inosine 5'-Monophosphate Dehydrogenase in Action: The First Bacterial Series of Structures of Phosphate Ion-, Substrate-, and Product-Bound Complexes. *Biochemistry* 51, 6148–6163. [PubMed: 22788966]
- (26). Rosenbaum G, Alkire RW, Evans G, Rotella FJ, Lazarski K, Zhang R-G, Ginell SL, Duke N, Naday I, Lazarz J, Molitsky MJ, Keefe L, Gonczy J, Rock L, Sanishvili R, Walsh MA, Westbrook E, and Joachimiak A (2005) The Structural Biology Center 19id Undulator Beamline: Facility Specifications and Protein Crystallographic Results. *Journal of Synchrotron Radiation* 13, 30–45. [PubMed: 16371706]
- (27). Minor W, Cymborowski M, Otwinowski Z, and Chruszcz M (2006) Hkl-3000: the Integration of Data Reduction and Structure Solution - From Diffraction Images To an Initial Model in Minutes. *Acta Crystallographica Section D Biological Crystallography* 62, 859–866. [PubMed: 16855301]
- (28). Vagin A, and Teplyakov A (1997) Molrep: an Automated Program for Molecular Replacement. *Journal of Applied Crystallography* 30, 1022–1025.
- (29). Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, and Vagin AA (2011) Refmac5 for the Refinement of Macromolecular Crystal Structures. *Acta Crystallographica Section D Biological Crystallography* 67, 355–367. [PubMed: 21460454]

- (30). Emsley P, Lohkamp B, Scott WG, and Cowtan K (2010) Features and Development Ofcoot. *Acta Crystallographica Section D Biological Crystallography* 66, 486–501. [PubMed: 20383002]
- (31). Adams PD, Afonine PV, Bunkóczi G, Chen VB, Echols N, Headd JJ, Hung L-W, Jain S, Kapral GJ, Kunstleve RWG, McCoy AJ, Moriarty NW, Oeffner RD, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, and Zwart PH (2011) The Phenix Software for Automated Determination of Macromolecular Structures. *Methods* 55, 94–106. [PubMed: 21821126]
- (32). Laskowski RA, MacArthur MW, Moss DS, and Thornton JM (1993) Procheck: a Program To Check the Stereochemical Quality of Protein Structures. *Journal of Applied Crystallography* 26, 283–291.
- (33). Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB, Snoeyink J, Richardson JS, and Richardson DC (2007) Molprobity: All-Atom Contacts and Structure Validation for Proteins and Nucleic Acids. *Nucleic Acids Research* 35, W375–W383. [PubMed: 17452350]
- (34). Digits JA, and Hedstrom L (1999) Species-Specific Inhibition of Inosine 5'-Monophosphate Dehydrogenase by Mycophenolic Acid. *Biochemistry* 38, 15388–15397. [PubMed: 10563825]
- (35). Riera TV, Wang W, Josephine HR, and Hedstrom L (2008) A Kinetic Alignment of Orthologous Inosine-5'-Monophosphate Dehydrogenases. *Biochemistry* 47, 8689–8696. [PubMed: 18642884]
- (36). Umejiego NN, Li C, Riera T, Hedstrom L, and Striepen B (2004) *Cryptosporidium Parvum* IMP Dehydrogenase. *Journal of Biological Chemistry* 279, 40320–40327. [PubMed: 15269207]
- (37). Verham R, Meek TD, Hedstrom L, and Wang CC (1987) Purification, Characterization, and Kinetic Analysis of Inosine 5'-Monophosphate Dehydrogenase of *Tritrichomonas Foetus*. *Molecular & Biochemical Parasitology* 24, 1–12. [PubMed: 2886911]
- (38). Gan L, Seyedsayamdost MR, Shuto S, Matsuda A, Petsko GA, and Hedstrom L (2003) The Immunosuppressive Agent Mizoribine Monophosphate Forms a Transition State Analogue Complex with Inosine Monophosphate Dehydrogenase. *Biochemistry* 42, 857–863. [PubMed: 12549902]

Figure 1: Activity recovery of IMPDH-OxMP complexes upon dilution. Progress curves of (A) *Ba*IMPDH and (B) *Cj*IMPDH when incubated with 10 μM OxMP and diluted 100 times in assay buffer (blue dots). Progress curves with no OxMP (black dots), 0.1 μM OxMP (green dots) and 10 μM OxMP (red dots) are shown for reference. Data were fit to Equation 4 (solid line).

Figure 2:

Stoichiometry of inhibition. (A) Scheme of the OxMP titration experiment. OxMP was incubated with enzyme in various ratios for 5 min. Free OxMP was removed using a 30 kDa cut-off filter. The enzyme was diluted into assay buffer containing IMP and NAD⁺ to determine activity. LC-MS was used to determine the concentration of free OxMP. **(B)** Stoichiometry and reversibility of OxMP inhibition for *Cj*IMPDPH. Relative activity of each inhibitor-preincubated enzymatic assay were shown. A lag was observed when OxMP concentration was approximately equal or greater than the enzyme concentration (Figure S5). The initial relative activity (closed circles) and final relative activity (open circles) are shown. Concentrations of free (pink) and bound (purple) OxMP are shown. Initial concentrations of OxMP are shown as grey bar.

Figure 3:

Spectrum of the complex between *BaIMPDH* and OxMP. (A) Scheme of the split cuvette experiment. Enzyme is depicted in orange and OxMP is depicted in pink. E-OxMP* adduct is depicted in orange and purple. (B) *BaIMPDH* (20 μM) was placed in one chamber of a split cuvette and OxMP (20 μM) was placed in the other. The initial spectrum was recorded (light blue), then the chambers were mixed and the spectrum was recorded again within 2 minutes of mixing (dark blue). No additional changes were noted over the following 30 minutes. The difference spectrum of enzyme and OxMP before and after mixing is shown in grey. The spectrum of enzyme is shown in orange and the spectrum of OxMP is shown in green. The spectrum of the assay buffer is subtracted in all traces. (C) Spectrum of E-

OxMP* adduct determined from the difference of the spectra of enzyme alone and enzyme mixed with OxMP.

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Figure 4:

OxMP reaction progress curves. Stopped-flow transient kinetic data of OxMP modification with (A,B) *Ba*IMPDPH (C,D) *Cj*IMPDPH monitored at $\lambda = 287$ nm (A,C) and $\lambda = 321$ nm (B,D). In each set of experiments, 5 μ M IMPDPH reacts with varying concentrations of OxMP (2 μ M, closed red circles), (5 μ M, closed blue triangles) and (10 μ M, closed green rectangles). Data sets with enzyme alone were subtracted as background. The solid curves display the global fits to a one-step (*Ba*IMPDPH, Scheme 3A) or a two-step (*Cj*IMPDPH, Scheme 3B) binding mechanism.

Figure 5:

Structures of E-OxMP complex. (A) Overlay of the two chains of *Ba*IMPDPH L•OxMP structure (tan and blue) showing the position of OxMP (tan and blue, respectively). Residues within 5Å of OxMP are shown. Hydrogen bonds are depicted by blue lines. The potassium ion is depicted as purple ball. (B) Overlay of four monomers of the *Cj*IMPDPH S•OxMP structure showing the position of OxMP. (C-D) Electron density showing the ring-opened E-OxMP* in (C) *Ba*IMPDPH L•OxMP (D) *Cj*IMPDPH S•OxMP. (E) Overlay of the apo *Ba*IMPDPH (4MJM, blue) and the *Ba*IMPDPH complexes of IMP (3USB, green), XMP (3TSD, pink), OxMP (tan) and P_i (3TSB, orange). (F) Overlay of the *Ba*IMPDPH complexes of IMP (3USB, green), XMP (3TSD, pink) and OxMP (tan) complexes showing the position of IMP, XMP and OxMP. The torsion angle of O4'-C1'-N9-C4 is depicted as arcs. (G) Overlay of the *Cj*IMPDPH complexes with IMP (4MZ1, green) and OxMP (tan) complexes showing position of IMP and OxMP (green and tan sticks, respectively). (H) Flap conformations in different complexes of IMPDPH. Overlay of one monomer of *Ba*IMPDPH• P_i (3TSB) with ordered closed flap (blue), two chains of the *Ba*IMPDPH L•OxMP structure (tan and orange), four monomers of the *Cj*IMPDPH S•OxMP (green, forest, pink and purple). (I) Overlay of *Ba*IMPDPH L•OxMP (green), *Cj*IMPDPH S•OxMP (tan), *Ba*IMPDPH• P_i (blue) and *T. foetus* structure of the transition state analog MZP (1PVN, pink)³⁸. The catalytic water is depicted by red sphere. Hydrogen bonds are depicted in blue.

Scheme 1: IMPDH and OxMP.

(A) OxMP and GMP structure. (B) Mechanism of IMPDH reaction. The covalent intermediate E-XMP* is highlighted in the frame. R5P = ribose-5'-monophosphate. (C) Proposed mechanism of inactivation of IMPDH by OxMP with nucleophilic Cys attacking C6. (D) Proposed mechanism of inactivation of IMPDH by OxMP with nucleophilic Cys attacking C2.

Scheme 2:
OxMP synthesis.

A. *Ba* **B. *Cj*** **Scheme 3:**

Kinetic models of the inhibition of IMPDH by OxMP. (A) One-step model for *Ba*IMPDH reaction with kinetic parameters determined by Dynafit²². (B) Two-step model for *Cj*IMPDH reaction with kinetic parameters determined by Dynafit.

Table 1:

Steady-state kinetics parameters and inhibition mechanism of IMPDHs

<i>Source</i>	K_m, IMP (μM)	K_m, NAD (μM)	$K_{ii, \text{NAD}}$ (mM)	k_{cat} (s^{-1})	$K_{is, \text{GMP}}^a$ (μM)	$K_{is, \text{OxMP}}^a$ (nM)	$K_{is, \text{GMP}} / K_{is, \text{OxMP}}$
<i>Human II</i>	3.3 ± 0.3	7.0 ± 1.7	0.49 ± 0.12	0.22 ± 0.01	37 ± 4	130 ± 20	280
<i>T. foetus</i>	6.3 ± 0.4	80 ± 3	5.4 ± 0.3	0.70 ± 0.01	24 ± 2	170 ± 20	140
<i>C. parvum</i>	10 ± 0.7	45 ± 3	6.5 ± 0.5	3.2 ± 0.4	21 ± 1	90 ± 10	240
<i>C. jejuni</i>	12 ± 0.3	66 ± 5	4.1 ± 0.3	2.2 ± 0.1	8.0 ± 0.9	51 ± 6	160
<i>B. anthracis</i>	83 ± 5	700 ± 40	8.1 ± 0.5	3.0 ± 0.1	115 ± 7	340 ± 50	340

^aversus IMP, competitive, no time dependency; rapid reversible unless noted.

Table 2.

Crystal and Data Collection and Refinement Statistics.

	<i>Ba</i> IMPDPH L•OxMP	<i>Cj</i> IMPDPH S•OxMP
Data collection		
Space group	P4	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å),	86.41, 86.41, 90.98	69.34, 119.59, 119.31
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90, 90	90, 89.99, 90
Protein molecules/ASU	2	4
Wavelength (Å)	0.97924	0.97924
Resolution * (Å)	1.54 (1.57-1.54) *	1.97 (2.00-1.97)
Unique reflections	97222 (4080)	135052 (5009)
<i>R</i> _{merge} [†]	0.066 (0.881)	0.131(0.876)
<i>⟨I⟩</i> / <i>⟨σI⟩</i>	20.6 (0.7)	17.0(1.0)
Completeness (%)	98.3 (82.6)	98.2 (86.7)
Redundancy	4.6 (2.4)	5.7 (4.1)
CC1/2 **	0.400	0.591
Refinement		
Resolution (Å)	1.54 (1.56-1.54)	1.97 (1.99-1.97)
Reflections: work/test set	92294 / 4781	127920/ 6506
<i>R</i> _{work} (<i>R</i> _{free} [‡])	0.165 (0.198)	0.173 (0.202)
No. of atoms: protein/ligands [§] /water	5191 / 96 / 236	10954 / 220 / 428
Mean B factor (Å ²): protein/ligand/water; ligands	41.9 / 48.8 / 42.3 OXP, K ⁺ , ethylene glycol, PEG: 39.5, 40.3, 50.4, 61.2	61.1 / 91.1 / 56.0 OXP, K ⁺ , sulfate, Cl ⁻ , MPD: 52.9, 76.4, 129.8, 95.2, 100.2
Bond lengths (Å)	0.006	0.007
Bond angles (°)	1.006	0.960
Ramachandran plot: most favored/outliers, (%)	97.4 / 0.00	97.7 / 0.00
PDB code	6MGU	6MGR

ASU, Asymmetric Unit.

* Values in parentheses correspond to the highest-resolution shell.

$$^{\dagger}R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

where $I_i(hkl)$ is the intensity for the i th measurement of an equivalent reflection with indices h , k , and l .

$$^{\ddagger}R = \frac{\sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}|}{\sum_{hkl} F_{\text{obs}}}$$

where F_{obs} and F_{calc} are observed and calculated structure factors, respectively. R_{free} is calculated analogously for the test reflections, which were randomly selected and excluded from the refinement.

$§$

Ligands include all atoms excluding protein and water atoms.

$**$

CC1/2 values are for the highest resolution shells.