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Inhibition of Guanosine Monophosphate Synthetase by the Substrate Enantiomer L-XMP

Nicholas B. Struntz^{a,b}, Tianshun Hu^{a,b}, Brian R. White^a, Margaret E. Olson^a, and Daniel A. Harki^a

^a Department of Medicinal Chemistry University of Minnesota 717 Delaware Street S.E., Minneapolis, MN 55414 (USA)

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Studies with mirror-image L -enantiomer nucleosides and nucleotides have revealed relaxed enantioselectivities of several cellular kinases and viral polymerases.^[1,2] This feature of enzyme-ligand molecular recognition has been exploited in the design of efficacious antiviral L -nucleoside drugs, which have lowered host cell toxicity.^[3-6] For example, lamivudine (2',3'-dideoxy-3'-thiacytidine, 3TC), an L -nucleoside drug, exploits the relaxed enantioselectivity of HIV reverse transcriptase to inhibit viral replication.^[7] Conversely, the enantioselectivities of the majority of nucleotide biosynthesis enzymes have not been characterized. The depletion of cellular nucleotide pools has been shown to result in antiproliferative, antibacterial, and immunosuppressive effects.^[8-11]

GMP Synthetase (GMPS), an enzyme in *de novo* nucleotide biosynthesis, catalyzes the amination of xanthosine 5'-monophosphate (XMP) to guanosine 5'-monophosphate (GMP) in the presence of glutamine (the amine source) and ATP.^[10,12] GMPS possesses two active sites that are separated by approximately 30 Å, suggesting that GMPS undergoes a significant conformational change during catalysis.^[13,14] In the amidotransferase active site, a glutamine residue is hydrolyzed to liberate ammonia, which subsequently functions as the nucleophile in the amination of XMP (Equation 1).^[15] In the synthetase active site, the 2-carbonyl of XMP is adenylated with ATP (Equation 2) to activate the aromatic ring for subsequent aminolysis (Equation 3).^[10,12,13,16] Formation of adenylyl-XMP is believed to trigger glutamine hydrolysis in the amidotransferase active site.^[12]

Fax: (612) 626-6318 daharki@umn.edu.

^bThese authors contributed equally to this work.

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A crystal structure of *E. coli* GMPS has been solved that reveals a large solvent-accessible synthetase pocket with considerable surface area.^[13] Several base-modified D-XMP analogues have been shown to function as substrates for GMPS and be converted to their amine derivatives,^[17] and non-hydrolyzable adenylyl-XMP analogues have been synthesized.^[18] Based on this structural information and our interest in characterizing for the first time the enantioselectivity of GMPS, we hypothesized that L-XMP, the enantiomer of native ligand D-XMP, could target GMPS and modulate enzymatic activity. We hypothesized that L-XMP could incorporate into the synthetase active site and inhibit enzyme function, or less likely, L-XMP could function as a substrate for GMPS and undergo aminolysis to yield L-GMP. In either case, enzymatic synthesis of D-GMP would be affected, either by direct enzyme inhibition or by the activity of a suicide substrate. Given the central importance of GMPS in eukaryote and prokaryote biochemistry, we examined the enantioselectivity of the enzyme.

Preceding this work, a synthesis of L-XMP (**6**), the enantiomer of natural ligand D-XMP, had not been reported. Our synthesis of L-XMP (**6**) started from L-arabinose, which was elaborated to 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl- β -L-ribofuranoside (**1**) by reported methods (Scheme 1).^[19] A Vorbrüggen coupling with trimethylsilyl protected xanthine **2** gave a separable mixture protected L-xanthosine isomers **3** (*N*⁹ isomer) and **4** (*N*⁷ isomer, not shown).^[20, 21] Deprotection of the benzoyl protecting groups of **3** using ammonia afforded L-xanthosine (**5**). Selective phosphorylation of the 5'-OH of **5** utilizing phosphorous oxychloride gave L-XMP (**6**).^[22]

E. coli GMPS was overexpressed and purified (Figure S1) and an HPLC-based assay was developed to quantitate enzymatic reaction products. GMPS was incubated with test substrates and NH₄OAc (ammonia source), and the reaction was terminated at various time points by addition of EDTA. GMPS protein was then removed by a molecular weight spin-column (30 kDa), and enzymatic reaction products were analyzed by reverse-phase HPLC. Surprisingly, we found that incubation of L-XMP and GMPS yielded a new peak of identical retention time as D-GMP (Figure 1). Characterization of this new peak (MS analysis, Figure S3) confirmed that L-XMP was converted to L-GMP by GMPS, demonstrating that turn over of the opposite enantiomer substrate was possible.

Biochemical characterization of the kinetics of L-XMP conversion to L-GMP by GMPS, as well as D-XMP conversion to D-GMP, was measured by fitting the individual GMP/CMP (cytosine 5'-monophosphate, an external standard) ratios from each sample into the slope-intercept equation from the calibration plot. Initial velocity measurements of GMP production as a function of time were measured at a variety of substrate (XMP) concentrations, and at fixed saturating concentrations of the non-varied substrates ATP and NH₄OAc (Figure 2A). Fitting these data to the Michaelis-Menten equation and analysis by non-linear regression allowed measurement of kinetic parameters (Table 1).

Analysis of D-XMP revealed an apparent K_m of 35.3 μM , which was similar to previously reported K_m values for *E. coli* GMPS (29 μM and 166 μM).^[23, 24] The turnover number (k_{cat}) was found to be $4.8 \times 10^{-2} \text{ s}^{-1}$, which was comparable to a previous report of $9.4 \times 10^{-2} \text{ s}^{-1}$.^[24] Analysis of L-XMP revealed an apparent K_m of 316.7 μM , which is ~10-fold higher than the natural enantiomer. Surprisingly, the k_{cat} was measured at $3.2 \times 10^{-6} \text{ s}^{-1}$, which is a 15,000-fold difference in turnover number compared to D-XMP. The specific activity (k_{cat}/K_m) of L-XMP decreased 140,000-fold from the natural enantiomer ($1.4 \times 10^{-3} \mu\text{M}^{-1} \text{ s}^{-1}$ for D-XMP versus $1.0 \times 10^{-8} \mu\text{M}^{-1} \text{ s}^{-1}$ for L-XMP). These results suggest that L-XMP may also inhibit GMPS.

To confirm the values derived from the HPLC assay, a known continuous UV spectrophotometric assay was also employed.^[23,25] This assay monitors a reduction in 290 nm absorbance resulting from conversion of XMP ($\epsilon_{290} = 4800 \text{ M}^{-1} \text{ cm}^{-1}$) to GMP ($\epsilon_{290} = 3300 \text{ M}^{-1} \text{ cm}^{-1}$). UV-based kinetic data was calculated analogously to the HPLC-derived data (Figure 2B). Analysis of both D-XMP and L-XMP revealed nearly identical results to the HPLC assay (for L-XMP: $k_{\text{cat}}/K_m = 9.4 \times 10^{-9} \text{ } \mu\text{M}^{-1} \text{ s}^{-1}$ (UV) versus $1.0 \times 10^{-8} \text{ } \mu\text{M}^{-1} \text{ s}^{-1}$ (HPLC); Table 1).

Although L-XMP conversion to L-GMP by GMPS was demonstrated, the weak affinity of L-XMP for GMPS, coupled with its slow turnover number, suggested possible enzyme inhibition by this ligand. To probe for GMPS inhibition, we performed enzymatic activity experiments with our xanthosine-based molecules to demonstrate reduction of GMPS-mediated amination of D-XMP. Addition of a fixed concentration of inhibitor to varying D-XMP concentrations, followed by analysis by UV-Vis and fitting to the competitive inhibition equation (or uncompetitive for decoyinine towards XMP),^[10] yielded the K_i data shown in Table 2. Evaluation of the known GMPS uncompetitive inhibitor decoyinine revealed a $K_i = 54.1 \text{ } \mu\text{M}$, which was similar to a previous report ($26 \text{ } \mu\text{M}$).^[26] Mizoribine (bredinin trade name), a known immunosuppressive drug and competitive GMPS inhibitor, was found to be more potent in our hands ($K_i = 1.8 \text{ } \mu\text{M}$), and this activity is similar to reports of the same compound against GMPS isolated from rat Walker sarcoma cells ($K_i = 10 \text{ } \mu\text{M}$).^[11] L-XMP (**6**) inhibition results were quite interesting, revealing that L-XMP is almost 7-fold more potent than decoyinine inhibition against *E. coli* GMPS ($K_i = 7.5 \text{ } \mu\text{M}$). Both D-xanthosine and L-xanthosine nucleosides were also tested and neither molecule inhibited GMPS, suggesting that 5'-monophosphorylation is required for inhibition. Mizoribine does not require phosphorylation for GMPS inhibition. Our results suggest that L-XMP can inhibit GMPS enzymatic activity with potency similar to or slightly better than other known inhibitors.^[9, 26-28]

To understand the molecular interactions of D-XMP and L-XMP within the GMPS active site, energy minimized three-dimensional conformations of the biochemical reaction intermediates (adenyl-D-XMP and adenyl-L-XMP; Figure S4B) were docked (Surflex-dock in the SYBYL software suite) into the crystal structure of *E. coli* GMPS (PDB 1GPM).^[13] The molecule of AMP observed in the x-ray crystal structure was extracted and re-docked into GMPS with a calculated similarity of 0.908 (1.0 is the theoretical maximum), showing reliability in docking accuracy (Figure S4A). Several stabilizing molecular interactions were observed between adenyl-D-XMP and GMPS, such as hydrogen bonds between Lys856 and the xanthine nucleobase; Arg875, Arg765, and Glu768 to ribose alcohols; and Asn761 to the phosphate of D-XMP (Figure 3A). The considerable size of the GMPS synthetase pocket readily accommodated the docking of adenyl-L-XMP; however, the L-ribose sugar occupied a substantially different position within the synthetase domain (Figure 3B). No longer present were many of the key molecular interactions between the nucleobase and ribose alcohols as evident by a decreased in consensus score, which is an estimate of the overall ligand binding affinity (CScore = 7.68 for D-XMP versus 6.16 for L-XMP). One compensating molecular interaction was observed for adenyl-LXMP, which was a hydrogen bond between Asn761 to a ribose alcohol. The conformation of the L-ribose sugar in adenyl-L-XMP also forces C^2 of the nucleobase to be positioned approximately $2.0 \text{ } \text{\AA}$ away from the region in space occupied by natural adenyl-D-XMP ligand. This perturbation to nucleobase conformation may deter aminolysis of the adenylated unnatural monophosphate, thereby slowing enzyme turnover. Additionally, the loss of key hydrogen-bonding interactions may also contribute to the loss in enzyme efficiency. Nonetheless, our observation of the synthesis of L-GMP from L-XMP implies the large size of the synthetase pocket must allow some movement of adenyl-L-XMP to obtain the correct conformation for amination.

In conclusion, the synthesis of L-GMP from L-XMP provides new insight into the substrate promiscuity of GMPS. GMPS was also inhibited by L-XMP at low micromolar levels, which is comparable to other known inhibitors. These results provide new insight into GMPS-ligand interactions that will be useful for future inhibitor designs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Synthesis of L-XMP: Reagents and conditions: (i) TMSOTf, DCE, reflux, 70% (for **3**), 21% (for *N*⁷-isomer **4**, not shown); (ii) NH₃, MeOH, 55 °C (sealed tube), 93%; (iii) POCl₃, PO(OMe)₃, Proton-Sponge; aq. TEAB, 19%.

Figure 1.
HPLC analysis of L-XMP conversion to L-GMP by GMPS.

Figure 2. Initial velocities versus substrate plots for D-XMP and L-XMP as measured by A) HPLC analysis and B) UV-Vis analysis.

Figure 3. Docking of A) adenylyl-D-XMP and B) adenylyl-L-XMP intermediates into the *E. coli* GMPS synthetase binding pocket. Key hydrogen-bonding interactions between the adenylylated ligands and GMPS are marked with dashed lines. The 2-position of the xanthine nucleobase is marked (2.0 Å conformational shift).

Table 1

Kinetic parameters of GMPS enzymatic activity in the presence of enantiomeric substrates *D*-XMP and *L*-XMP.^[a]

Substrate	k_{cat} (s ⁻¹)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ (μM ⁻¹ s ⁻¹)
<i>D</i> -XMP ^[b]	$4.8 (\pm 0.3) \times 10^{-2}$	35.3 ± 8.5	1.4×10^{-3}
<i>D</i> -XMP ^[c]	$4.3 (\pm 0.7) \times 10^{-2}$	24.9 ± 6.6	1.7×10^{-3}
<i>L</i> -XMP ^[b]	$3.2 (\pm 0.3) \times 10^{-6}$	316.7 ± 55.6	1.0×10^{-8}
<i>L</i> -XMP ^[c]	$3.1 (\pm 0.7) \times 10^{-6}$	329.9 ± 104.9	9.4×10^{-9}

^[a] Performed in triplicate. Values shown are the average $\pm$ S.D.

^[b] HPLC analysis.

^[c] UV-Vis analysis.

Table 2GMPS inhibition data of known inhibitors and xanthosine analogues.^[a]

Inhibitor	K_i (μM)
Decoyinine	54.1 ± 14.5
Mizoribine	1.8 ± 0.7
D-Xanthosine	> 1500
L-Xanthosine	> 500
L-XMP	7.5 ± 1.8

^[a] Performed in triplicate. Values shown are the average $\pm$ S.D.