Crystal Structure of Avian Aminomidazole-4-carboxamide Ribonucleotide Transformylase in Complex with a Novel Non-folate Inhibitor Identified by Virtual Ligand Screening*

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Aminimidazole-4-carboxamide ribonucleotide transformylase (AICAR Tfase), one of the two folate-dependent enzymes in the de novo purine biosynthesis pathway, is a promising target for anti-neoplastic chemotherapy. Although classic antifolates, such as methotrexate, have been developed as anticancer agents, their general toxicity and drug resistance are major issues associated with their clinical use and future development. Identification of inhibitors with novel scaffolds could be an attractive alternative. We present here the crystal structure of avian AICAR Tfase complexed with the first non-folate based inhibitor identified through virtual ligand screening of the National Cancer Institute Diversity Set. The inhibitor 326203-A (2-[5-hydroxy-3-methyl-1-(2-methyl-4-sulfophenyl)-1H-pyrazol-4-ylazo]-4-sulfo-benzoic acid) displayed competitive inhibition against the natural cofactor, 10-formyl-tetrahydrofolate, with a Ki of 7.1 μM. The crystal structure of AICAR Tfase with 326203-A at 1.8 Å resolution revealed a unique binding mode compared with antifolate inhibitors. The inhibitor also accessed an additional binding pocket that is not occupied by antifolates. The sulfonate group of 326203-A appears to form the dominant interaction of the inhibitor with the proposed oxanion hole through interaction with a helix dipole and Lys267. An aromatic interaction with Phe318 also likely contributes to favorable binding. Based on these structural insights, several inhibitors with improved potency were subsequently identified in the National Cancer Institute Compound Library and the Available Chemical Directory by similarity search and molecular modeling methods. These results provide further support for our combined virtual ligand screening rational design approach for the discovery of novel, non-folate-based inhibitors of AICAR Tfase.

The biological significance of the folate cofactor stems from the key role that folates play as one-carbon carriers in several important metabolic pathways, including de novo purine and thymidylate synthesis. The recognition of the critical role of reduced folates for the synthesis of DNA precursors led to the discovery of folate analogues as anti-metabolite agents. Antifolates typically interfere with the binding of the natural cofactor to key enzymes in these biosynthetic pathways, such as thymidylate synthase, dihydrofolate reductase, glycinamide ribonucleotide transformylase (GAR Tfase), and AICAR Tfase. The first generation of antifolates, aminopterin and methotrexate, heralded the era of anti-metabolite cancer chemotherapy (1). Subsequent design and development of folate analogues has led to new generations of antifolates, some currently under clinical evaluation. For example, pemetrexed (ALIMTA) can bind multiple targets, such as thymidylate synthase, dihydrofolate reductase, GAR Tfase, and AICAR Tfase, and is one of the most promising of these current antifolates. Pemetrexed exhibits antitumor activity in several types of solid tumors (2), and its phase III clinical trial has recently been approved by the Food and Drug Administration.

Nevertheless, general toxicity issues associated with antifolates have posed major challenges for folate analogue development. Folic acid and its cellular derivatives function mainly in their fully reduced form and are involved in many cellular metabolic pathways, such as cell proliferation and amino acid metabolism. Because at least 18 folate-dependent enzymes are involved in 1-carbon transfer reactions, as well as transformation of folate cofactors themselves, in human cells, antifolate agents often inhibit multiple pathways, resulting in adverse cytotoxic side effects. One such example is methotrexate, whose general toxicity stems from the inhibition of several folate-dependent enzymes involved in methionine biosynthesis, thymidylate synthesis, and de novo purine biosynthesis (3). Lomotrexol is a selective inhibitor to GAR Tfase (nt) in de novo purine biosynthesis, but can also inhibit AICAR Tfase at higher concentrations (4). Although lometrexol has exhibited promising anti-neoplastic activity, it was suspended from further clinical evaluation because of folate depletion after administration of the drug (4). However, more recent studies have indicated that supplementation with folate can reduce its toxicity (5).

Currently, substantial efforts have been focused on specific tailoring of folate analogues to individual enzymatic targets to achieve greater selectivity, as well as increased potency. Nevertheless, only a limited number of antifolates for cancer chemotherapy have been approved by the Food and Drug Administration for clinical applications, including methotrexate and

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The atomic coordinates and structure factors (code 1thz) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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§ The abbreviations used are: GAR Tfase, glycinamide ribonucleotide transformylase; 10-f-THF, 10-formyl-tetrahydrofolate; AICAR, aminimidazole-4-carboxamide ribonucleotide; AICAR Tfase, AICAR transformylase; IMPCH, IMP cyclohydrolase; BME, β-mercaptopetanol; MAI, multisubstrate adduct inhibitor; rmsd, root mean square deviation; ATIC, AICAR Tfase/IMPCH.

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raltitrexed, a thymidylate synthase inhibitor for treatment of
colon cancer in Europe (3). Therefore, inhibitors with novel
scaffolds that differ from traditional antifolates are attractive
because they are unlikely to produce the same undesired side
effects. Moreover, such classes of inhibitors would not neces-
sarily possess a glutamate or multiple glutamates as in tradi-
tional antifolates and would eliminate the requirement for
active transport, a frequent source of antifolate drug resist-
ance. Nevertheless, the added folate requirement in tumor cells
leads to up-regulation of these folate transporters and in-
creased accumulation of folates and antifolates in tumor cells.
Thus, bypassing of the active transport system and reliance on
passive diffusion raises its own challenges for selective uptake
into tumor cells to obtain an efficacious chemotherapeutic
agent.

In this study, a virtual ligand screening approach via dock-
ing was undertaken to search for inhibitors of AICAR Tfase
with novel scaffolds. AICAR Tfase/IMPCH (ATIC), which cat-
alyzes the last two steps in the de novo purine biosynthesis
pathway, is a promising target for the development of thera-
peutic intervention in various types of cancer (6). The de novo
purine biosynthesis pathway consists of 10 enzymatic reac-
tions that sequentially convert 5-phosphoribosyl-1-pyrophosphate
to IMP. The homodimeric ATIC is a bifunctional enzyme com-
posed of two distinct active sites. The high resolution crystal
structure of ATIC (7) revealed that the C-terminal domain
(residues 200–593) contains the AICAR Tfase activity, which
formyl transfer from the cofactor 10-f-THF to AICAR
producing 5-formyl-AICAR (Fig. 1) in the penultimate step of
purine biosynthesis. The N-terminal IMPCH domain (residue
1–199) is responsible for the final cyclohydrolase ring closure
step that converts 5-formyl-AICAR to IMP (Fig. 1). The IMPCH
active site and catalytic mechanism have been elucidated by
the structure of ATIC complexed with the IMPCH domain
inhibitor, xanthosine 5′-monophosphate (8, 9). Crystal struc-
tures of ATIC with the substrate AICAR (8) and two sulfonyl-
containing folate inhibitors, BW1540 and BW2315, have illu-
minated the cofactor-binding site (10). The crystal structure of
a multisubstrate adduct inhibitor β-DADF (MAI), mimicking
both the substrate AICAR and the cofactor when complexed
with ATIC, unambiguously demonstrated that the AICAR
Tfase active site was located at the dimer interface and iden-
tified putative key catalytic residues (11). Furthermore, the
complexed structures revealed that AICAR Tfase possesses an
unusually large active site, with unoccupied binding pockets
and cavities even when both AICAR and cofactor are bound.
Thus, the structural information provides invaluable guide-

lines for the rational design of novel inhibitor scaffolds. ATIC
has no structural or sequence similarity to any other folate-de-
pendent enzymes in the de novo biosynthesis pathways. In
addition, no clinically useful inhibitors for ATIC have been
developed to date, which underscores the opportunity to pursue
structure-based inhibitor design targeting of ATIC.

Molecular docking and de novo design are widely used in
the discovery of enzyme inhibitors with the desired selectivity
and potency profiles. Virtual screening of the structural database
of known and commercially available small molecules can rapidly
generate lead compounds with complementary shapes and
favorable hydrogen bonding, electrostatic and hydrophobic in-
teractions within the active site of the target (12). Such docking
studies also enable discovery of novel ligands with chemical
scaffolds that are dissimilar to those already tested. Here, we
describe the outcome of the molecular docking strategy for
the discovery of novel inhibitors of ATIC, where crystal structures
of ATIC provided the necessary entry points for the docking
and rational design of inhibitors.

Compounds from the National Cancer Institute Diversity Set
were docked into the three-dimensional structure of human
ATIC using AutoDock. The screening led to the identification
of 44 new compounds with distinct chemical structures, compared
with traditional antifolates, that inhibited ATIC (13). In vitro
inhibition assays confirmed that 8 of the 16 compounds that
had good solubility in water surprisingly inhibited AICAR Tfase
activity at the micromolar level. To establish the struc-
tural basis for their specificity and affinity, we determined the
crystal structure of ATIC with one of the most potent inhibitors
identified from the screening effort.

Thus, we report here the discovery of a new class of inhibi-
tors that bind to ATIC with a binding mode that differs from
the traditional folate-based inhibitors. The crystal structure of
ATIC complexed with one of the novel inhibitors, 326203-A, a
commercially available dye named Acid Yellow 54, at 1.8 Å
resolution (Fig. 2) has revealed the key enzyme-inhibitor inter-
actions that now provide a valuable structural template for
further improvement via rational design.

**EXPERIMENTAL PROCEDURES**

**Materials**—LB and agar were obtained from Invitrogen. All common
buffers and reagents were purchased from Sigma-Aldrich. The inhibitor
candidates were obtained from the National Cancer Institute Open
Chemical Repository (Bethesda, MD) and the Sigma-Aldrich Library of
Rare Chemicals.

**Protein Expression and Purification**—The plasmid pET28a, encoding the
N-terminal hexahistidine-tagged human ATIC (kindly provided by
Dr. G. Peter Beardsley, Yale University), was transformed into Esche-
richia coli BL21.DEC3 cells (Novagen) by heat shock. The E. coli
transformants were grown in 2YT medium (Invitrogen) at 37 °C to an
optical density of 0.6. The cells were harvested by centrifugation and
were resuspended in 100 ml of solution of 100 mM sodium phosphate,
100 mM KCl, 150 mM NaCl, 5 mM imidazole, and 5 mM β-mercaptoethanol
(BME) (Buffer A). The resultant mixture was clarified by centrifugation
at 20,000 × g at 4 °C for 50 min. The supernatant was incubated with nickel
affinity beads (Qiagen) overnight before transferring to a 2.5 × 10 cm Econo
column (Bio-Rad) and further washing with 10 column volumes of Buffer A.
The protein was eluted with a 10–250 mM imidazole gradient. Fractions
containing protein were analyzed by SDS-PAGE, pooled, and further
purified on a Superdex 200 HR column (Amersham Biosciences) equil-
ibrated with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM KCl, 5 mM
BME, with or without 5 mM EDTA. The peak fractions were again analyzed
by SDS-PAGE and pooled.

The plasmid pET28a, encoding the avian ATIC cDNA with an N-
terminal hexahistidine tag (a kind gift from Dr. Stephen J. Benkovic),
was transformed into E. coli BL21.DEC3 cells (Novagen, Inc.) for over-
expression. Avian ATIC was purified in the same manner as human
ATIC (10).

**FIG. 1. Reactions and structure of the natural cofactor.** Formyl
transfer and IMP cyclohydrolase reactions catalyzed by ATIC. The
structure of the natural cofactor (10-f-THF) is depicted in the black box.
Virtual Ligand Screening by AutoDock—The human AICAR Tfase holo-template was extracted from its crystal complex with AICAR and BW1540 (Protein Data Bank code 1p4r) (10). All crystallographic water molecules and active site ligands were removed. The protonation states of His residues were assigned as follows: HD1 on His267, His385, His469, His584, and His592; HE2 on His213, His290, His293, His453, His470, and His473. Non-polar hydrogens were merged with heavy atoms, and Kollman charges (14) were assigned. The active site in the homodimer that had lower average B values (2 monomers/asymmetric unit) was chosen as the docking site. A 60 × 50 × 66 three-dimensional energy grid with 0.375 Å spacing was calculated for each of the following atom types: carbon, aromatic carbon, nitrogen, oxygen, sulfur, hydrogen, fluoride, chlorine, bromine, iodine, iron, phosphorus, and e (electrostatic) using Autogrid3 (15). The docking screenings were carried out with AutoDock3.0.5 (15), and the jobs were distributed to the Scripps Atlas SGI Origin 2000 cluster, the NBCR Meteor, and the UCSD KeckII linux cluster. The National Cancer Institute Diversity Set (dtp.nci.nih.gov/branches/dsch/diversity_explanation.html) (1990 compounds with a rich structural and pharmacophore diversity) was chosen as the compound library. All-atom Gasteiger charges were added, and non-polar hydrogens were merged with their connecting heavy atoms (16). The simulation parameters were: trials of 100, population size of 150, random starting position and conformation, translation step of 0.5 Å, rotation step of 35°, elitism of 1, mutation rate of 0.02, cross-over rate of 0.8, local search rate of 0.06, and 10 million energy evaluations. Final docked conformations were clustered using a tolerance of 1.5 Å root mean square deviation (rmsd). The top compounds were picked based on the higher binding energies.

Preparation of 10-f-THF—10-f-THF was prepared by a modified procedure of Rowe (17) and Black et al. (18). 10 mg of (6R,S)-5-f-THF (Schircks Laboratories, Jona, Switzerland) was dissolved in 500 μl of 3.5 M BME, and 12 μl HCl was then added to adjust the pH to 1–2. This solution was flushed with nitrogen and stored at 4 °C for 3 days. Yellow precipitate of (6R)-5,10-methenyl-THF formed and was harvested by centrifugation at 11,000 rpm for 10 min. The precipitates were resuspended in 5 ml of 0.1 M Tris-HCl, pH 8.0, 0.1 M BME. The conversion of (6R,S)-5, 10-methenyl-THF to 10-f-THF was monitored by the decrease of the UV absorbance at 356 nm (A356) and was usually complete after 3 h. The concentration of 10-f-THF was determined by A298 using the extinction coefficient of 9.54 × 105 cm⁻¹ M⁻¹ (18).

AICAR Tfase Inhibition Assay—The human ATIC enzyme was used for the inhibition assay. In an initial screen to determine approximate Ki values, 1 mg of each compound was dissolved in 100 μl of Me2SO and diluted to 1 mM using the assay buffer (32.5 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM BME) as described previously (19–21). The assay buffer was flushed with nitrogen to minimize oxidation of 10-f-THF. The Me2SO used in this assay did not have any inhibitory effect on the activity of ATIC. 25 nM of human ATIC, 10 μM inhibitors, and different concentrations of cofactor 10-f-THF were mixed in the assay buffer to a volume of 150 μl and incubated for 2 min. The reaction was initiated by adding 50 μM of substrate AICAR (total volume, 300 μl). Using a SpectraMax Plus384 microplate reader, the reaction was monitored at 298 nm by measuring the increase in absorbance corresponding to the formation of THF. The THF was generated in the transfer reaction of the formyl group from cofactor to AICAR to produce 5-formyl-AICAR. IC50 values were measured at 25 nM ATIC, 50 μM AICAR, and 8.5 μM 10-f-THF.
The $K_i$ was obtained by generating plots of $1/V_i$ versus $1/[S]$ at several levels of inhibitor concentration, where the slopes of these plots are given by Equation 1.

$$\text{slope} = \frac{K_m}{V_{\max}}[I] + \frac{K_m}{V_{\max}}$$  

(Eq. 1)

The apparent $K_i$ was obtained from the plot of these slopes versus $[I]$, which generated a straight line with a $y$-intercept of $K_m/V_{\max}$ and a $x$-intercept of $-K_i$.

### Crystallization and Data Collection

Because avian ATIC protein crystallizes more readily than human ATIC, apo avian ATIC was used for crystallization experiments. Apo avian ATIC crystals were grown at 22 °C, as described previously (7), via the sitting drop vapor diffusion method by mixing equal volume of avian ATIC (10 mg/ml) with a reservoir solution consisting 18% (w/v) polyethylene glycol 8000, 0.2 M imidazole, pH 7.2, and 5 mM dithiothreitol. Crystals of ATIC/326203-A complex were obtained by soaking a native avian ATIC crystal for 6 h with 10 mM 326203-A in mother liquor (20% polyethylene glycol 8000, 0.2 M imidazole pH 7.2, and 5 mM dithiothreitol). Ethylene glycol at 20% was used as cryo-protectant. The data were collected on an ADSC 3 CCD detector at Beamline 11-1 at Stanford Synchrotron Radiation Laboratory. The data were processed to 1.8 Å using DENZO-Scalepack (23). The crystal is isomorphous with the apo avian ATIC structure previously reported (7) and was indexed in monoclinic space group $P2_1$, with cell dimensions of $a = 64.8$ Å, $b = 105.3$ Å, $c = 102.4$ Å, $\beta = 108.1^\circ$. The Matthews' coefficient ($V_m = 2.6$ Å$^3$/Da) (24) indicated one dimer/asymmetric unit (solvent content of 51.9%).

### Structure Determination and Refinement

The structure of ATIC with 326203-A was determined to 1.8 Å resolution by molecular replacement with AMoRe (CCP4 program suite) (25) using the apo avian ATIC coordinates (Protein Data Bank code 1g8m) (7) as the search model. The initial molecular replacement solution after rigid body refinement yielded a correlation coefficient of 53.4% and an $R_{	ext{four}}$ of 36.1% for data between 15 and 4 Å. The bound inhibitor was identified using the initial difference $F_o - F_c$ and $2F_o - F_c$ electron density maps. Subsequent refinement was performed with CNS (26) using simulated annealing, conjugated gradient minimization, and individual $B$ value refinement with intervening rounds of manual building using the graphic software O (27). A bulk solvent correction was applied throughout the entire refinement. Two-fold noncrystallographic symmetry restraints were applied for the initial five rounds of refinement and then released completely for the final rounds of refinement between the two monomers. The final model has $R_{	ext{cryst}}$ and $R_{	ext{free}}$ values of 21.3 and 24.0%, respectively (see Table I). Because the electron density was weaker in one of the active sites in the dimer, most of the structural analysis presented here was carried out on the active site with the better ordered inhibitor.

The model was analyzed with Procheck (28) and WHATCHECK (29). The program MS (30) was used to calculate buried surface area using a 1.4 Å probe. Hydrogen bonds and van der Waals' interactions were identified with LIGPLOT (31) and CONTACTSYM (32). Calculations of rmsd on the complexed and apo avian ATICs were carried out on the active site with the better ordered inhibitor.

### RESULTS AND DISCUSSION

#### Kinetic Analysis of AICAR Tfase and 326203-A

The $K_i$ value was determined using the reciprocal plot of AICAR Tfase activity. The concentration of inhibitor used was 0 μM (open circles), 5 μM (filled diamonds), 10 μM (filled rectangles), 20 μM (filled circles), or 25 μM (filled triangles). The $x$ axis is 1/[I], the reciprocal of 10-f-THF concentration (μM$^{-1}$); the $y$ axis is 1/V, (min/mMμg). Inhibition is replotted in A against 326203-A concentration [I], giving a $K_i$ of 7.1 μM.

#### CG- Docking

AutoDock (15) was then used to screen 1990 compound from the National Cancer Institute Diversity Set with diverse pharmacophores. Energy scoring was calculated on the sum of the electrostatic and van der Waals' interaction energy between the ligand and the enzyme. Based on the calculated binding energy, AutoDock retrieved 44 best energy-scoring compounds that have scaffolds that differ substantially from known antifolates. We tested 16 of these compounds that had good solubility in water for their ability to inhibit the formyl transfer activity. The compound now serves as a promising lead for future generations of novel inhibitors for ATIC.
**Inhibitor Binding**—The initial $F_o - F_c$ electron density map of the inhibitor was well defined (Fig. 2C). Almost every atom of the inhibitor can be distinguished in the electron density map, except for the anticipated Cr$^{3+}$. No Cr$^{3+}$ chelation is observed, which differs from the chemical structure of the inhibitor provided by the National Cancer Institute (Fig. 2A). In the National Cancer Institute chemical structure, Cr$^{3+}$ is chelated by the carboxylate oxygen (O22) (labeled in Fig. 4B), the hydroxyl oxygen from the pyrazole ring (O17), and the nitrogen from the diazoo moiety (N-19), as well as a hydroxide ligand. The chelation of the free ligand with the Cr$^{3+}$ creates a rigid and planar inhibitor structure. It is possible that the 5 mM EDTA present in the protein solution reacted with the Cr$^{3+}$ complex and removed the Cr$^{3+}$ ion from compound 326203-A. Nevertheless, without Cr$^{3+}$, the bound ligand in the crystal complex remains rigid and planar, with the pyrazole ring rotated about 30° from the plane of ring A. It is plausible that a proton replaced the Cr$^{3+}$, acting as a chelation site to complex the three lone pairs of electrons on oxygens O-17, O-22, and on nitrogen N-19 to form an intramolecular hydrogen bond network (Fig. 2B).

The bound ligand is located at the ATIC dimeric interface around the AICAR Tfsase active site (8, 11), as anticipated. However, the ligand surprisingly only occupies part of the natural cofactor (10-f-THF)-binding cleft and does not enter the AICAR-binding subsite (Fig. 5A). This structural result corresponds well with the kinetic data, which show that the ligand is a competitive inhibitor only of the cofactor (Fig. 3), but appears to be a noncompetitive inhibitor of the substrate AICAR (not data shown). The ligand consists of three parts: two benzene sulfonate ring-binding regions (A and B) at each end of the ligand and the middle pyrazole moiety (Fig. 2C), 28% (124 Å$^2$) of the total surface area (437 Å$^2$) of the ligand is exposed to the solvent. The corresponding buried surface areas are 77% for the benzene sulfonate moiety of ring A and 71% for the pyrazole ring component, whereas ring B is slightly more exposed to solvent (64% buried surface area).

The 326203-A ligand represents a new class of inhibitors with a novel scaffold that is dissimilar to folates. Consequently, this inhibitor adopts a binding mode that differs substantially from any ATIC-specific inhibitor, such as a MA with avian ATIC (11), and two folate analogues (BW2315 and BW1540) in complex with human ATIC (10). Thus, in retrospect, it is not surprising to find a novel mode of binding that engages different active site residues. The substantial number of new hydrogen bonding and hydrophobic interactions (Fig. 4B) must then account for the observed binding potency of the inhibitor. All of the interactions are from residues in the biological dimer; no crystal contacts that might lead to artificial binding were observed. A relatively "conserved" interaction was noted that arises from binding of the sulfonate moiety of ring A in 326203-A to the N terminus of helix 17 (residues 451–469) and suggests that the helix dipole stabilizes the binding of the negatively-charged sulfonate. Furthermore, this proposed oxanion hole, which is composed of the main-chain amides from the N terminus of helix 17, has previously been proposed to dominate the location and orientation of two sulfonate anti-folate inhibitors (BW2315 and BW1540) in the active site (10). Similarly, a key hydrophilic interaction between Lys$^{267}$ and the benzoyl sulfonate moiety of 326203-A is conserved with the antifolates. Here, O-32 and O-34 of the sulfonate form hydrogen bonds with the side chain of Lys$^{267}$, with distances of 2.9 and 3.3 Å, respectively. The positively charged Lys$^{267}$ has been suggested to stabilize the oxanion transition state of the formyl transfer reaction (8, 11). In this complex, the polar interactions involving Lys$^{267}$, as well as with the proposed binding (8, 10, 11), ligand soaking experiments were performed. Because avian ATIC originally crystallized more readily than human ATIC, soaking experiments were performed on the apo avian ATIC crystals. Avian and human ATIC have 83% sequence identity, and their structures are highly conserved (9). The crystals soaked in the solution of compound 326203-A were indexed in monoclinic space group $P2_1$ (see "Experimental Procedures"). The structure was determined at 1.8 Å resolution by molecular replacement using the apo avian ATIC structure (Protein Data Bank code 1g8m) (7) as the search model. The final model of the complex included residues 4–593 for both monomers of the dimer, two 326203-A ligands in the AICAR Tfsase active sites, two potassium ions, and 748 water molecules (Table 1). Superposition of monomer A onto monomer B gave an rmsd of 0.45 Å for the main chain and 0.78 Å for all atoms. The corresponding values were similar for individual superposition of the IMPCH domain (0.45 and 0.80 Å), and the AICAR Tfsase domain (0.45 and 0.79 Å). The ATIC structures in complex and in the apo enzyme are essentially the same (rmsd of 0.61 and 0.55 Å for the main chains of monomers A and B). The binding of the ligand to the AICAR Tfsase active site does not perturb the overall structure, but differences between the apo and the liganded enzyme structures are observed in one of the IMPCH domains with an rmsd of 0.95 Å (0.38 Å for the other subunit) because of an endogenously bound nucleotide (xanthosine 5’-monophosphate) in one of subunits in the apo avian ATIC structure (7). The equivalent comparisons for the individual AICAR Tfsase domains are 0.29 and 0.30 Å.

### Table 1

**Data collection and refinement statistics**

| Parameter                                      | Value               |
|------------------------------------------------|---------------------|
| Space group                                    | $P2_1$              |
| Unit cell $a$ (Å)                               | 64.8                 |
| $b$ (Å)                                        | 105.3               |
| $c$ (Å)                                        | 102.4               |
| $β$ (°)                                        | 108.1               |
| Number of molecules asymmetric unit            | 2                   |
| Resolution (Å)                                 | 45–1.80 (1.85–1.80) |
| Unique reflections                             | 115,650 (8413)      |
| Completeness (%)                               | 91.4 (92.1)         |
|Multiplicity                                    | 3.3 (3.2)           |
| Average $\langle U \rangle$ (Å$^2$)             | 11.9 (4.6)          |
| $R_{cryst}$ (%)                                | 10.1 (31.2)         |
| Data cutoff                                    | $F_o > 0 \sigma$    |
| No. of reflections (test set)                  | 113,750 (2836)      |
| Protein atoms                                  | 9022                |
| Water molecules                                | 745                 |
| Inhibitor atoms                                | 66                  |
| Average protein (mol1) B value (Å$^2$)         | 25.0                |
| Average protein (mol2) B value (Å$^2$)         | 28.4                |
| Inhibitor (mol1) B value (Å$^2$)               | 36.6                |
| Inhibitor (mol2) B value (Å$^2$)               | 41.6                |
| Average solvent B value (Å$^2$)                | 33.8                |
| rmsd from ideal                                | 0.008               |
| Bond length (Å)                                | 1.40                |
| Bond angle (deg)                               |                     |
| $R_{cryst}$ (%)                                | 21.4 (24.6)         |
| $R_{free}$ (%)                                 | 24.0 (28.8)         |
| Ramachandran plot (%)                          | Most favored 93.1   |
| Additionally allowed                           | 6.7                 |
| Generously allowed                             | 0.2                 |

*a* Number in parentheses correspond to the highest resolution shell.  
*b* $R_{cryst} = \sum |F_o| - |F_c| / \sum |F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes.  
*c* $R_{free}$ is the same as $R_{cryst}$ but for 2.5% of the data randomly omitted from refinement.
oxyanion hole, both appear to be the key features that confer selectivity and probably a substantial portion of the binding energy of this compound.

The proposed catalytic His268 does not form a direct hydrogen bond with this ligand, but instead a water molecule W327 mediates this interaction via a relatively long hydrogen bond (3.6 Å). O-33 of the ligand hydrogen bonds with main chain amide of Arg452 from the opposite subunit. No direct interactions are made between active site residues and the ring A benzoate moiety. Nevertheless, a well-organized water shell forms around the carboxylate oxygens to create an extensive hydrogen bonding network that assists in the interaction be-

**Fig. 4. AICAR Tase-326203-A interactions.** A, residues in the AICAR Tase active site interact with the inhibitor. The side chains of the protein and the inhibitor are depicted in ball-and-stick representations. The protein and inhibitor are colored as in Fig. 2C. The side chains of the protein are colored in gray. Asn490 has a different conformation in the apo human enzyme and is colored in green. B, schematic drawing of AICAR Tase interactions with 326203-A, generated by LIGPLOT (31). Residues forming van der Waals’ interactions are indicated by an arc with radiating spokes toward the ligand atom they contact; those participating in the hydrogen bonding are shown in ball-and-stick representations. Hydrogen bonds are illustrated as dotted lines with the donor-acceptor distance given in Å. Water molecules are colored in cyan, and carbon atoms are in black; other atom types are colored according to Fig. 2C.
between the main-chain amide of Ala<sup>567</sup> in the opposing subunit of the dimer with the ligand carboxylate oxygens O<sub>26</sub> and O<sub>22</sub> (Fig. 4B). Otherwise, the remaining interactions are via hydrophobic packing and π stacking of the aromatic rings. The benzene ring of Phe<sup>316</sup> forms parallel π stacking with ring A with a ring-to-ring distance of 3.5 Å, whereas Pro<sup>544</sup> stacks against the opposing face of ring A (Fig. 4A).

The primary interaction between the pyrazole ring and the enzyme involves the side chain of Asn<sup>490</sup> and N-12 of the inhibitor with a hydrogen bonding distance of 2.7 Å. Phe<sup>316</sup>, apart from π stacking with ring A, also provides a hydrophobic environment for the pyrazole ring by forming van der Waals’ interactions with C-14 and C-15 of the ligand. The sulfonate of ring B is anchored atop the N terminus of α-helix 19 (residues...
484–498), and its negative charge also appears to be stabilized by the dipole interaction of the helix. A hydrophilic interaction of the sulfonate of ring B is made between the side chain of Glu487 and O-10. O-8 and O-9 form electrostatic interactions with the side chains of Lys294 and Glu487 through two water molecules W400 and W401. Ala486 and Val338 provide a favorable hydrophobic environment above the ring B (Fig. 4A).

AutoDock predicted several different binding clusters for 326203 with similar docking energy ranging between −12.4 and −14.4 kcal/mol. However, there is no dominant binding cluster and no preferred conformation over the others (13). Nevertheless, the interaction between Lys294 and the sulfonate of ring A is conserved both in the crystal structure and in some of the predicted binding configurations. The successful prediction of this important polar interaction is probably due to the dominant role of this sulfonate in enzyme-inhibitor binding, as suggested for the two sulfonyl groups in the two antifolate-ATIC complex structures (10). Indeed, docking identified three other novel sulfonate-containing inhibitors; ongoing crystallization studies revealed superimposable benzene sulfonate positions; these form dominant interactions with Lys294. The consensus orientations of these inhibitors were, again, successfully reproduced by docking. 2 What is most likely and consistent with all of the docking, enzymatic and structural data, is the domination of the sulfonate interaction with the oxyanion hole that minimizes the contributions of the other interactions affecting the binding mode. The predicted number of clusters with a similar binding energy is consistent with that observation. However, in almost 50% of the binding modes, the sulfonate occupies the same relative location. Different placement of the other sulfonate interactions; these form dominant interactions with Lys294. The consensus orientations of these inhibitors were, again, successfully reproduced by docking. 2 What is most likely and consistent with all of the docking, enzymatic and structural data, is the domination of the sulfonate interaction with the oxyanion hole that minimizes the contributions of the other interactions affecting the binding mode. The predicted number of clusters with a similar binding energy is consistent with that observation. However, in almost 50% of the binding modes, the sulfonate occupies the same relative location. Different placement of the rest of 326203 could partly arise from the interactions mediated by ordered water molecules, which were not included in the docking of the ligand, or might be due to electrostatic and π–π interactions that play a more important role in the binding than accounted for in the energy scoring function (13).

Comparison with Folate Analogues—Superimposition of inhibitor 326203-A onto MAI, BW1540 and BW2315 (Fig. 5B) reveals that the only overlapping moiety is the benzene ring A with the benzoyl moieties of the ATIC-specific inhibitors. Although π stacking of aromatic rings is predominantly involved in all of these interactions, minor differences are apparent. Ring A rotates about 50° with respect to the benzoyl ring of MAI that enables Phe331 to form a perpendicular π stacking with the benzoyl ring of MAI, but parallel π stacking with ring A of 326203-A (Fig. 4A). The benzoyl rings of BW2315 and BW1540 also align in a perpendicular orientation compared to ring A where a small rotation of Phe331 of the human enzyme, the equivalent residue to avian Phe331, enables a similar perpendicular π stacking. Another form of π stacking involves the imidazole ring of substrate AICAR with the benzoyl rings of the antifolates BW2315 and BW1540 (10), which assists in the orientation of these inhibitors in the vicinity of one of the key catalytic residues, Lys294, in the human enzyme. Although AICAR is not present in the complex structure of 326203-A and ATIC, Phe331 provides a similar function.

As discussed above, the sulfonate moiety of ring A in 326203-A is anchored in a position similar to that of the sulfonyl groups of BW1540 and BW2315, which are adjacent to the N terminus of helix 17. Therefore, the helix dipole and the oxyanion hole in the N terminus of the helix seem to play the same role of stabilizing the negatively-charged sulfonate and directing the binding of 326203-A, as proposed for sulfonyl antifolates (10). Another common feature of binding for these four ATIC-specific inhibitors is the conserved residue, Lys294.

Lysine is essential for the catalytic formyl transfer reaction, as shown by mutagenesis studies (39) and various crystal structures (8, 11) and appears to be one of the key elements for high affinity binding of BW1540 and BW2315 (10). Slight differences of the attached benzene ring systems appeared such that their polar functional groups adjacent to the benzene rings (the amide carbonyl oxygen of MAI, sulfonyl for BW1540 and BW2315, sulfonate for 326203-A) occupy similar positions (Fig. 5B) and are within electrostatic interactions range of the Lys294 (the equivalent of Lys296 in human enzyme).

Otherwise, 326203-A is accommodated in shallow hydrophobic pockets (Fig. 5A) that are not occupied by MAI, BW2315 and BW1540. Most of these residues, Phe331, Met332, Val338, and Ala486 (Fig. 4A), are not utilized in folate binding, except for Phe331. For the ATIC-specific inhibitors, most of their surface area is buried (77% for MAI, 78% for BW1540, and 86% for BW2315), which consequently leads to more extensive interactions with the enzyme than 326203-A, which explains their higher binding affinities. Nevertheless, this novel binding mode of 326203-A utilizes new binding pockets and additional active site residues that can be further exploited to improve future generations of folate and nonfolate-based inhibitors.

Structural Differences upon Binding—One challenge for automated docking arises from the difficulty in accounting for the degrees of freedom of the enzyme in the inhibitor-enzyme interactions. For example, in E. coli GAR Tfase, isomerism of the folate-binding loop occurs at different pH values and upon ligand induced binding (40–45). Similarly, in the human enzyme, the substrate-binding loop is pH-dependent and affected by ligand binding (45). However, ATIC provides a relatively rigid protein scaffold that is optimal for docking studies. Examination of known AICAR Tfase complex structures reveals that the protein undergoes minimal conformational changes in the protein main chain, whereas some side chain flipping occurs upon inhibitor binding. In the ATIC complex structures with MAI, BW1540, and BW2315, upon AICAR binding, Arg208 rotates about 50° into the AICAR-binding site to interact with one of the phosphate oxygens of AICAR. Because no AICAR is present in the 326203-A crystal structure, this arginine residue points away from the active site and hydrogen bonds with Ser235 at a distance of 3.5 Å. The side chain of Asn490 adopts a different conformation compared with the apo enzyme by rotating about 80° toward the ligand, acting as a hydrogen bond donor to the nitrogen atom (N-12) in the pyrazole ring (Fig. 4A).

In human ATIC complexes with BW2315 and BW1540, the side chain of Asn341 rotates more than 90° (relative to the MAI complex structure) toward the AICAR-binding pocket to hydrogen bond with one of the sulfonyl oxygens, as well as the 5-amino moiety of AICAR. This conformational change appears to be induced by antifolate binding (10). Although this same movement occurs for Asn341 in one of the active sites of 326203-A avian complexes, it is not involved in a direct interaction with the ligand because of its considerable distance from the ligand sulfonate (about 5 Å). Arg542 in the same active site moves about 4 Å toward the AICAR-binding site to hydrogen bond with Asn342.

Similar Structure Search for Improved Binding Affinity—Although the affinity of 326203-A to ATIC is modest (μM), the compound is intriguing because it possesses an unexplored scaffold, different from conventional antifolates. Furthermore, the ATIC-326203 complex structure assists in identifying interactions that have not been observed in previous ATIC complex structures. To optimize 326203-A as a lead compound for future inhibitor design via chemical synthesis, a further similarity search in Available Chemical Directory or National Cancer Institute Compound Library was performed. Compounds

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2 L. Xu, C. Li, A. J. Olson, and I. A. Wilson, unpublished result.
with similar shape and higher complementarity to the active site of ATIC should exhibit higher binding affinity to the enzyme, which could then serve as a better lead. Visual examination of ligand binding reveals that ring A and the central pyrazole ring are mostly buried in the active site and presumably provide most of the binding specificity (Fig. 5). In addition, the molecular mass of the inhibitor already exceeds 500; therefore, ring B was eliminated from the subsequent substructure design. Furthermore, the planar and rigid structure of 326203-A suggested formation of an intramolecular hydrogen bond network (Fig. 2B); it is conceivable that heterocyclic structures such as quinoline or indole, which partially mimic this ring system, could preserve a similar set of interactions with ATIC.

Substructure searches of the Available Chemical Directory with a quinoline or an indole ring as templates were performed and followed by visual inspection that lead to seven candidate compounds. In addition, a similarity search of the National Cancer Institute Compound Library yielded 10 compounds. AutoDock was then used to score the binding of these compounds to the active site of ATIC, which was followed by in vitro inhibition assays on the top-scoring candidates with good solubility. The most potent of these compounds, SALOR2, has an IC50 of 1.4 μM, which is about eight times more potent than 326203-A (Table II). Although our initial substructure and similarity searches were relatively crude, we believe that a more systematic substructure search, combined with rational design, will lead to more potent inhibitors containing these novel scaffolds.

**Implications in Future Lead Discovery**—326203-A represents a completely new chemical scaffold that differs from known folate analogues and possesses a novel binding mode. It has already served as a good structural template for the rational design and optimization of 326203-A as a lead compound. Initial exploitations of small scale similar chemical structure searches in the National Cancer Institute Compound Library and Available Chemical Directory have generated compounds with increased binding affinity. Inhibitor 326203-A and its

| Compound | Database | Label | % lowest energy cluster | $E_{\text{binding}}$ (Kcal/mol) | IC50 (μM) |
|----------|----------|-------|-------------------------|-------------------------------|----------|
| ![Chemical Structure](image1) | NCI | 326203-A | 4 | -11.9 | 11.6 ± 0.1 |
| ![Chemical Structure](image2) | NCI | 47729-M | 14 | -12.8 | 3.3 ± 0.7 |
| ![Chemical Structure](image3) | NCI | 324571-C | 10 | -12.1 | 41.6 ± 1.8 |
| ![Chemical Structure](image4) | NCI | 324572-F | 15 | -12.5 | 11.6 ± 0.9 |
| ![Chemical Structure](image5) | ACD | Acid Yellow | 11 | -12.5 | 20.1 ± 0.7 |
| ![Chemical Structure](image6) | ACD | SALOR2 | 6 | -11.2 | 1.4 ± 0.3 |

* a Chemical structure is provided by National Cancer Institute compound library. From the 326203-A complex structure, no Cr3+ electron density was visible (see Fig. 2).

* b NCI, National Cancer Institute Compound Library.
Another structural feature of the ATIC 326203-A complex structure that could be exploited further is the aromatic π stacking interaction of Phe316 with the sulfabenzoic ring that assists in anchoring the sulfonate group of 326203-A in the vicinity of Lys267. Frequently, optimization of specific hydrophobic interactions has been attributed to enhanced inhibitor binding affinity. For example, aromatic π stacking formed between the pyrimidine ring of dUMP and benzoquinazoline ring of antifolate, BW1843U89, has been suggested to be the main inhibition mechanism in thymidylate synthase (46) by blocking the key interaction between dUMP and the catalytically essential cysteine residue. The increased affinity of the BW1843U89 over its predecessor is attributed to the enhanced aromatic properties of the inhibitor. Similarly, comparison of the more potent compound SALOR2 and inhibitor 326203-A revealed SALOR2 possesses a naphtho-thiophene ring and an indole ring. Hence, the enhanced aromatic properties and rigidity of compound SALOR2 over inhibitor 326203-A may also be responsible for the improved binding potency. Therefore, one future direction is to generate different fused heterocyclic rings present in the ATIC-326203 complex should also be maintained, especially the interaction with Lys267. A charged hydrogen bond can contribute up to 4.7 kcal/mol of binding energy, which is equivalent to a 3000-fold increase in affinity (47).

The active site of AICAR Tfase has been well characterized (8, 11), which provides an excellent platform for iterative cycles of structure-based inhibitor design. Traditional folate analogues are developed based on the natural folate cofactor scaffold. The structural similarities of these folate analogues pose a great challenge for improving their binding specificity to the target folate-dependent enzymes. It is certainly likely that binding specificity and potency profile could be improved by exploring the surprisingly large binding pockets and cavities of AICAR Tfase that are not occupied by substrate or cofactor. Binding of inhibitor 326203-A leaves the AICAR-binding site essentially empty. In addition, the benzene ring B of the inhibitor extends toward the other subunit, where it occupies a binding site not used by folates that has not been explored previously in folate analogue design.

Although direct hydrophobic and electrostatic interactions involving the active site residues are important for attaining the binding potency of an inhibitor, hydrogen bonds with active site water molecules also need to be taken into account. A well organized water shell (water molecules W695, W696, and W697) is seen in the AICAR-binding pocket (Fig. 5A) mediated through main chain interactions, including Gly73Ile299 and Asn280. These water molecules have been observed in most of the apo ATIC and complex structures, except for ATIC-BW2315 complex, where the water molecules W695 and W696 are absent. Although these water molecules do not seem to be involved in the catalytic process, they assist in anchoring the substrate AICAR by mediating hydrogen bonding between AICAR and enzyme (8). They appear to act as the integral part of the protein structure in the absence of substrate or ligand. Displacement of the active site water molecules has been recognized as one important design strategy to gain additional binding efficiency (33). The appropriate selection of functional groups that provide favorable steric and polar interactions could potentially replace the water molecules and enable analogous interactions directly with the protein.

In conclusion, this proof of concept study not only revealed the structural basis for the binding specificity and affinity of this novel inhibitor of ATIC but also offers an attractive platform for future lead discovery and subsequent structure-based lead optimization efforts for ATIC.

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