Selective Inhibition of Bacterial Tryptophanyl-tRNA Synthetases by Indolmycin Is Mechanism-based*

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Indolmycin is a natural tryptophan analog that competes with tryptophan for binding to tryptophanyl-tRNA synthetase (TrpRS) enzymes. Bacterial and eukaryotic cytosolic TrpRSs have comparable affinities for tryptophan (K_m ~ 2 μM), and yet only bacterial TrpRSs are inhibited by indolmycin. Despite the similarity between these ligands, Bacillus steaethermophilus (Bs)TrpRS preferentially binds indolmycin ~1500-fold more tightly than its tryptophan substrate. Kinetic characterization and crystallographic analysis of BsTrpRS allowed us to probe novel aspects of indolmycin inhibitory action. Previous work had revealed that long range coupling to residues within an allosteric region called the D1 switch of BsTrpRS positions the Mg\(^{2+}\) ion in a manner that allows it to assist in transition state stabilization. The Mg\(^{2+}\) ion in the inhibited complex forms significantly closer contacts with non-bridging oxygen atoms from each phosphate group of ATP and three water molecules than occur in the (presumably catalytically competent) pre-transition state (preTS) crystal structures. We propose that this altered coordination stabilizes a ground state Mg\(^{2+}\)-ATP configuration, accounting for the high affinity inhibition of BsTrpRS by indolmycin. Conversely, both the ATP configuration and Mg\(^{2+}\) coordination in the human cytosolic (Hc)TrpRS preTS structure differ greatly from the BsTrpRS preTS structure. The effect of these differences is that catalysis occurs via a different transition state stabilization mechanism in HcTrpRS with a yet-to-be determined role for Mg\(^{2+}\). Modeling indolmycin into the tryptophan binding site points to steric hindrance and an inability to retain the interactions used for tryptophan substrate recognition as causes for the 1000-fold weaker indolmycin affinity to HcTrpRS.

The accumulation of resistance in pathogenic organisms over time and with prolonged drug use necessitates the continued development of new anti-infective therapeutics. Such developments can include modifications to current drugs that are active against exploited targets while counteracting current resistance mechanisms or novel compounds targeted against underexploited targets. One group of enzyme targets that has been validated but remains underexploited is the class of aminoacyl-tRNA synthetases (aaRSs). Aminoacyl-tRNA synthetases maintain the fidelity of the genetic code by ensuring the charging of tRNA with its cognate amino acid via the following two-step reaction.

\[
\text{aaRS} + \text{ATP} + \text{aa} \rightarrow \text{aaRS}-\text{aa-AMP} + \text{PPi}
\]

\text{REACTION 1}

\[
\text{aaRS}-\text{aa-AMP} + \text{tRNA} \rightarrow \text{aaRS} + \text{aa-tRNA} + \text{AMP}
\]

\text{REACTION 2}

All aaRS enzymes bind ATP and activate a specific amino acid by catalyzing the formation of an aminoacyl 5'-adenylate (aa-AMP) during the first step. This is followed by transfer of the activated amino acid to the 3'-end of the correct tRNA. Structural and mechanistic differences among the different aaRS enzymes as well as orthologs of individual synthetases make it possible to selectively modulate the activity of specific synthetases, e.g. prokaryotic over eukaryotic TrpRS (1). This makes the aaRS enzymes attractive targets for novel anti-infective therapeutics.

Any compounds intended for clinical use must be much less inhibitory against the eukaryotic orthologs of its intended target. Naturally occurring aminoacyl-tRNA synthetase inhibitors include indolmycin (TrpRS), gramicidin (LeuRS), mupirocin (IleRS), and ochratoxin A (PheRS) (1–4). Of these, mupirocin displays the required selectivity for prokaryotic over eukaryotic IleRS and has been developed for the treatment of infections in humans (5).

Indolmycin produced by Streptomyces griseus displays selective inhibition for prokaryotic TrpRS (9 nm; Escherichia coli) over eukaryotic TrpRS (4 nm; Bos taurus) (6). Problems with off-target effects on tryptophan metabolism have prevented its clinical use (7). However, if we could understand the molecular basis for the observed inhibition and selectivity we could exploit this information for the rational design of antibiotics targeted against TrpRS on pathogens.

Structurally, tryptophan and indolmycin are quite similar with a heterocyclic indole moiety at the root of each ligand (Fig.

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† The atomic coordinates and structure factors (code SDK4) have been deposited in the Protein Data Bank (http://wwwpdb.org/).

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2 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase; Bs, B. steaethermophilus; Hc, human cytoplasmic; LTN, tryptophanamidine; IND, indolmycin; PreTS, pre-transition state; Bt, B. taurus; TEV, tobacco etch virus; OXA, methylamino-substituted oxazolinone ring.
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![Tryptophan and Indolmycin](image)

**Tryptophan**  
**Indolmycin**

**FIGURE 1. Functional equivalences of tryptophan and indolmycin.** Indolmycin differs from tryptophan in three key ways: 1) the incorporation of C α constituents into an oxazolinone ring, 2) a methylamino group extending from the oxazolinone ring, and 3) replacement of a hydrogen on C β with a hydrophobic methyl group.

1. Indolmycin differs from tryptophan in the following ways. (i) The carbon that is functionally equivalent to C α is substituted with a methyl group. (ii) The carbonyl carbon is part of an oxazolinone ring. (iii) The hydroxyl and amine groups of tryptophan are replaced by the nitrogen and oxygen atoms of the oxazolinone ring, respectively. (iv) The —NH–CH3 moiety attached to the oxazolinone ring does not have functionally equivalent atoms in tryptophan.

BsTrpRS is one of the most extensively characterized TrpRS enzymes. Mechanistically a Mg2+ ion is linked to what appears to be a dissociative transition state for tryptophan activation (8–10). During catalysis, the Mg2+ ion helps compensate for the increased negative charge that develops on the PPi leaving group, resulting from breaking the αP–O–βP bond. The Mg2+ ion must move to be catalytically competent, but no protein–metal interactions have been observed in any of the BsTrpRS crystal structures determined. Instead, a remote allosteric location, the D1 switch, must undergo significant conformational change to promote the Mg2+ ion to a catalytically competent position. The metal moves closer to the PPi, leaving group, whose charge is further stabilized in the transition state by the KMSKS loop. ATP binding is required for the conformational switching between the open and closed states that allows for catalysis. ATP-dependent induced fit closing of the active site brings ATP ~4 Å closer to tryptophan in a predominantly translational movement mediated by relative movement of the catalytic and anticodon-binding domains.

In the absence of ATP, tryptophan binding is promoted by hydrophobic van der Waals interactions, π–π interactions with Phe5, and a hydrogen bond between the indole nitrogen and Asp132 of the specificity helix. When both substrates bind, the tryptophan substrate undergoes a rotational movement that brings the indole ring deeper into the binding pocket and results in more stabilizing interactions between tryptophan and active site residues. This change is facilitated by the inward movement of the specificity helix that is not observed when only tryptophan is bound.

ATP-dependent induced fit rearrangement of the active site facilitates proper ATP positioning in BsTrpRS, and molecular dynamics simulations demonstrate that tryptophan is required to achieve the requisite movement of the αP in H₄TrpRS (11, 12). Even a modest substitution of tryptophanamide in place of tryptophan prevents the repositioning of ATP. These findings support the idea that H₄TrpRS is intrinsically better at discriminating between tryptophan and its structural analogs than is BsTrpRS.

H₄TrpRS uses different structural elements for substrate recognition than its prokaryotic orthologs (13). Such elements include an extended N terminus with a β1–β2 hairpin structure shown to have a role in ATP binding as well as the amino acid activation reaction in H₄TrpRS (14). In contrast to BsTrpRS, it is tryptophan binding that leads to induced fit rearrangement of the active site in H₄TrpRS. There are a greater number of binding determinants for tryptophan recognition as eight direct and water-mediated hydrogen bonds with polar side chains stabilize tryptophan in the active site. It has been proposed that amino acid activation proceeds via an associative transition state in H₄TrpRS with an unclear role of Mg2+ in the catalytic transition state (11). However, comparison of the pre-transition (Protein Data Bank code 2QUI) and product states (Protein Data Bank code 2QUI) shows that, as with BsTrpRS, the αP of ATP must move 5.3 Å to be in a position for nucleophilic attack by tryptophan.

Despite mechanistic and structural differences, BsTrpRS and H₄TrpRS have comparable tryptophan binding affinities. However, these inherent differences between prokaryotic and eukaryotic TrpRS enzymes promote the binding of indolmycin to prokaryotic TrpRSs ~1500-fold while protecting eukaryotic TrpRSs from such inhibition by a comparable amount. Determining the structure of BsTrpRS bound by Mg2+·ATP and indolmycin allowed us to probe the structural basis for indolmycin inhibition and selectivity. Specifically, we examined this structure along with the catalytically relevant structures of BsTrpRS and H₄TrpRS deposited in the Protein Data Bank to answer the following questions. 1) What are the structural consequences of binding indolmycin? 2) Why is indolmycin a tight inhibitor of prokaryotic TrpRS? 3) Why is indolmycin not an inhibitor of eukaryotic cytosolic TrpRSs?

**Experimental Procedures**

**Construction of pet28-His-BsTrpRS Vector**—The full-length BsTrpRS sequence was PCR-amplified from a pet11 construct made previously in the laboratory. PCR primers contained restriction sites for BamHI and HindIII. The resultant PCR product was digested with BamHI and HindIII. A three-way ligation among the PCR product (BamHI/HindIII), double-stranded oligo encoding for the TEV site (NdeI/BamHI), and pet28b (NdeI/HindIII) yielded an expression vector for His-TEV-BsTrpRS.

**Expression and Purification of His-BsTrpRS**—BsTrpRS was expressed by autoinduction with BL21(DE3)pLysS cells at 37 °C (15). The cells were pelleted at 4500 rpm for 30 min, resuspended in lysis buffer, and frozen at −20 °C. Upon thawing, cells were sonicated and centrifuged (16,000 rpm, 4 °C, 1 h). His-BsTrpRS was captured from the lysate on nickel-nitrilotriacetic acid resin and eluted with 0.3 M imidazole. Purified protein was cleaved overnight with TEV while dialyzing out the imidazole. The cleaved protein mixture was passed back over a nickel-nitrilotriacetic acid column to capture both uncleaved protein and His-TEV protease.

**Active Site Titration**—Active sites were titrated by following the loss of ATP to determine the fraction of molecules compre-
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\[ F(t) = (a_1 + b_1 T) \times p_1 + (a_2 + b_2 T) \times p_2 \quad \text{(Eq. 2)} \]

where \(a_1\) and \(a_2\) are adjustable parameters representing intercepts, \(b_1\) and \(b_2\) are the slopes of the linear dependences of the initial and final states, and \(T\) is the Kelvin temperature. The pipeline consists of the following three parts.

Part A is reading the data from high throughput, 384-well, real time PCR files and transforming them into a matrix consisting of four columns: (i) number of the well from which temperature-dependent readings were taken, (ii) an index representing the protein variant, and finally the data, (iii) temperature and (iv) fluorescence readings.

Part B is fitting the thermoﬂuor data to a thermodynamic model (Equations 3 and 4).

\[ F = F_0 + \frac{a_1 + b_1 T + (a_2 + b_2 T) e^{-\Delta G(T)/RT}}{1 + e^{-\Delta G(T)/RT}} \]

\[ = F_0 + (a_2 + b_2 T) \frac{(a_1 - a_2) + (b_1 - b_2) T}{1 + e^{-\Delta G(T)/RT}} \quad \text{(Eq. 3)} \]

where \(\Delta G\) is the Gibbs energy difference between the two states and \(e^{-\Delta G(T)/RT}\) is the Boltzmann factor that determines the state probabilities \(p_1\) and \(p_2\).

\[ \Delta G = \Delta H(T_m) + \Delta c_p \times (T - T_m) - T \Delta S(T_m) + c_p \ln \left(\frac{T}{T_m}\right) \quad \text{(Eq. 4)} \]

where \(\Delta H\) and \(\Delta S\) are the enthalpy and entropy changes between the states, \(c_p\) is the heat capacity at temperature \(T\), and \(\Delta c_p\) is the heat capacity change between the two states at the melting temperature \(T_m\).

Part C is independent determination of \(T_m\) assuming that the state probabilities \(p_1\) and \(p_2\) can be estimated from distances between the intersection of the melting curve with vertical lines connecting the extrapolated linear final and initial slopes. Data initially worked up using both methods B and C agreed closely, and the analysis reported here follows C.

Crystalization, Data Collection, and Structure Determination—Crystals of seleno-Met-substituted BsTrpRS in complex with ATP, Mg^{2+}, and indolmycin were grown by vapor diffusion against a reservoir of 1.4 M potassium citrate and 0.1 M Hepes, pH 7.4. Crystals were cryoprotected in Fomblin-Y and passed in a nitrogen airstream before plunging into liquid nitrogen. Data were collected remotely at Southeast Regional Collaborative Access Team (Beamline ID-22) using inverse beam geometry at 0.979 Å to obtain experimental phases from the Bijvoet differences and processed with XDS (20). PHENIX (21) and COOT (22) were used for phase determination, to interpret the map, and to iteratively refine the final structure (Protein Data Bank code 5DK4).

Results

BsTrpRS Binds Indolmycin ~1500× More Tightly than Tryptophan—Indolmycin is a competitive inhibitor of Bacillus stearothermophilus and other bacterial TrpRS enzymes that competes with tryptophan for binding to the active site of the enzyme. By conducting Michaelis-Menten experiments at

\[^3\text{V. Weinreb and G. Weinreb, unpublished data.}\]
increasing tryptophan concentrations in the presence of different indolmycin concentrations and fitting all 64 data points simultaneously to Equation 1, we were able to determine $K_{\text{cat,tryptophan}}$ (3 μM) and $K_{\text{cat,indolmycin}}$ (2 nM) (Table 1). As these experiments were carried out under exchange conditions (23), we determined the standard free energy, $\Delta G^0 = -R\ln K$, at 310 K for tryptophan and indolmycin binding to be 7.8 and 12.3 kcal/mol, respectively. This translates to a free energy difference of 4.5 kcal/mol between the affinities of the catalytic and inhibited complexes for the indole-containing ligand. To determine what factors account for the observed difference in binding free energy, we determined the structure of the BsTrpRS-ATP-indolmycin ternary complex and then conducted differential scanning fluorimetric experiments in the presence of various ligands.

**Indolmycin and ATP Form a Ternary Complex with BsTrpRS—**
Extensive crystallization studies conducted on BsTrpRS have revealed three distinct conformational states: an open conformation (ligand-free, tryptophan, low ATP (12, 24)), a closed pre-transition state (high ATP, ATP + tryptophanamide (9, 12)), and a closed product conformation (Trp-5'-AMP (25, 26)).

A previously unpublished structure of BsTrpRS bound to Mg$^{2+}$-ATP and indolmycin was never deposited (27). Nevertheless, that structure was the first example of a series of subsequently solved structures that have been described as “pre-transition state” (PreTS) structures (Protein Data Bank codes 1MAU and 1M83 (12)). In these structures, the initial ATP binding site in the small domain composed of the N-terminal α-helix and the anticodon-binding domain closes on the remainder of the Rossmann fold, bringing the nucleotide α-phosphate from 6.7 Å away to within van der Waals contact distance of the tryptophan carboxyl oxygen (12).

The new structure presented here is at higher resolution (1.9 Å) and, the experimental phases greatly enhanced the quality of electron density maps (Table 2 and Fig. 2). Details of the new structure, such as the orientation of the ribose and the metal position, are quite similar to those observed in deposited PreTS structures 1MAU and 1M83. Detailed differences that appear functionally relevant are discussed below.

**Indolmycin Induces New Contacts with Active Site Side Chains—**
Indolmycin makes contacts with the side chains of His$^{43}$, Asp$^{132}$, and Gln$^{147}$ as well as two water molecules (Fig. 3A). The interaction between O62 of Asp$^{132}$ in the specificity helix and the nitrogen atom of the indole ring is observed when tryptophan (3.1 Å), tryptophanamide (3.0 Å), or indolmycin (2.9 Å) is bound. The addition of the oxazolinone group to the ligand allows for stabilizing interactions with His$^{43}$ and Gln$^{147}$ with functionalities on either side of the ring, which have the effect of fixing the rotation of the ring (Fig. 3B). N61 of His$^{43}$ can donate and/or accept a hydrogen bond from N2 (methylamino group) of indolmycin. In addition to these hydrogen bonds, His$^{43}$ can form a salt bridge with O62 of Asp$^{132}$ (2.8 Å). The amide group of Gln$^{147}$ forms two hydrogen bonds, one with the carbonyl oxygen of the oxazolinone ring (3.0 Å) and another with O62 of Asp$^{146}$ (3.0 Å). O61 of Asp$^{132}$ makes a highly conserved hydrogen bond with the 2'-OH group of ATP (2.7 Å). These side chain interactions in the conserved GEDQ motif link the indolmycin and ATP binding sites while reinforcing the linkage between opposite sides of the indole-binding pocket.

Structurally, indolmycin binding also prevents the Tyr$^{125}$ rotamer switch that occurs upon the enzyme going from its open to closed conformation (Fig. 4, A and B). During the catalytic cycle, Tyr$^{125}$ changes hydrogen-bonding partners from His$^{150}$ (2.7 Å) in the open conformation to the α-amino group (2.4 Å) of the tryptophan substrate in the closed PreTS. The tryptophanyl-adenylate intermediate is stabilized by two polar contacts with the hydroxyl group of Tyr$^{125}$ (Protein Data Bank code116K). The inhibited state maintains the side chain interaction between Tyr$^{125}$ and His$^{150}$ (2.8 Å) observed in the open state.

**Structural Modifications Induced by Indolmycin to the Mg$^{2+}$-ATP Configuration—**
Superposition of the inhibited structure onto the closed PreTS structure (Protein Data Bank code 1MAU) gives a root mean square deviation of 0.28 Å for 323 Ca pairs. For comparison, the two closed PreTS structures,
1MAU and 1M83, have a root mean square deviation 0.17 Å for 328 Cα pairs. The greatest structural difference between the PreTS and inhibited states occurs around Glu103–Ala120 with a root mean square deviation of 0.63 Å for these 18 residues. This mobile loop, which contains Gln107 and Lys111, is more open by 0.5 Å in the inhibited structure as measured from the PO to the α-carbons of residues Gln107, Lys111, and Lys115. The carbonyl oxygen of the Gln107 side chain accepts a hydrogen bond from the water molecule coordinated to the Mg2+ ion (2.8 Å) and another from Ne2 of Gln147 (3.0 Å) in the pre-transition state. Steric clashing with the constrained Tyr125 rotamer prevents Gln107 from switching rotamers in the inhibited state. As such, the interaction with Gln147 is not observed. Instead, the side chain of Gln107 accepts a hydrogen bond at Oe1 from a water molecule (3.0 Å) and donates a hydrogen bond at Ne2 to another water molecule (3.3 Å).

In the pre-transition state, the Nζ atom of Lys111 forms a salt bridge with the Oγ1 atom of ATP (2.9 Å), which also forms a strong electrostatic interaction with the catalytic Mg2+ ion (2.4 Å). This interaction presumably is important for stabilizing the developing charge on the PP, leaving group released after tryptophan activation. Additionally, in the pre-transition state, Lys111 is in position to act as a hydrogen bond donor to the one water molecule coordinated to the Mg2+ ion (Fig. 5). The subtle opening of this loop in the inhibited state weakens the salt bridge between the Lys111 Nζ atom and the Oγ1 atom of ATP (3.4 Å) from that observed in the preTS. Now the closest interactions are with three water molecules (2.6, 2.8, and 2.8 Å), none of which are coordinated directly to the catalytic Mg2+ ion so they are not shown in Fig. 5.

Replacement of tryptophan (amide) with indolmycin in the active site alters the coordination and placement of the Mg2+ ion used during the activation step of the aminoacylation reaction. Presumably because its orientation is fixed by the hydrogen bonding network described above, the oxazolinone forms hydrogen bonds with two water molecules. Introduction of these two water molecules is associated with the movement of the Mg2+ ion into a hexavalent coordination that closely resembles stable configurations generated in quantum mechanical simulations of Mg2+·ATP.

As is also true in the PreTS structure, the Mg2+ ion in the inhibited BsTrpRS structure coordinates with a non-bridging oxygen from each phosphate group and three water molecules (Figs. 3B and 4B), two of which are further stabilized by the presence of indolmycin. In addition to the three electrostatic interactions with ATP, only one water molecule was seen to

4 S. Liu, unpublished data.
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**FIGURE 4. Comparison of ligand-free, preTS, and inhibited BsTrpRS structures.** A, compared with the apo form (pink; Protein Data Bank code 1D2R), the fully occupied preTS structure (black; Protein Data Bank code 1MAU) assumes a closed conformation. The Cα of Tyr125 is shifted inward by 2.4 Å, and the side chain is flipped −45° (measured from OH-Ca-OH). A Mg²⁺ ion (black sphere) forms electrostatic interactions with ATP and one water molecule (salmon sphere). B, binding of indolmycin and ATP causes similar shifts in the backbone (gray) as the enzyme adopts a closed conformation. However, due to the addition of the methylenedioxy-substituted oxazolone ring, this movement to the closed conformation is not accompanied by a rotamer change of Tyr125 in the inhibited structure. C, consequently, Gln107 is constrained and is rotated 106° around Cβ away from the specificity helix in the inhibited state compared with the pre-transition state. Finally, the Mg²⁺ ion (green sphere) is shifted toward the γ-P0, and has hexavalent coordination to ATP and three water molecules (red spheres) as compared with the preTS structure.

coordinate with the Mg²⁺ ion in the PreTS structure (Protein Data Bank code 1MAU) (Figs. 4A and 5). The side chain residues that accept and donate hydrogen bonds to this water molecule differ between these two states (Fig. 5). Due to the different Gln¹⁰⁷ rotamer and slight opening of the mobile loop around Lys¹¹¹, these two residues no longer interact with this water molecule in the inhibited state.

The Mg²⁺ ion is closer and more central to the triphosphate moiety in the inhibited structure than in the PreTS structure (Fig. 4C) and makes equivalent interactions with the phosphate oxygen atoms. The significance of the differences in metal position is evident from several measurements. (i) Metal to oxygen distances are significantly shorter (0.2 Å; p = 0.002) in the inhibited complexes. (ii) Movement of the divalent metal ion into closer contact with the ATP phosphate oxygen atoms is associated with a subtle but statistically significant opening of the active site crevice among the N-terminal helix of the second crossover connection (the GADQ motif), the KMSKS signature, and the mobile loop containing Lys¹¹¹.

**FIGURE 5. Differential BsTrpRS side chain interactions with the water molecules electrostatically coordinated with Mg²⁺ in the PreTS and inhibited structures.** Gln⁹, Gln¹⁰⁷, and Lys¹¹¹ are in position to accept (Gln¹⁰⁷) or donate (Gln⁹ and Lys¹¹¹) hydrogen bonds (black dashed lines) to the water molecule (salmon sphere) coordinated with Mg²⁺ (black sphere) in the PreTS (black sticks). As indolmycin binding leads to opening of the mobile loop containing Gln¹⁰⁷ and Lys¹¹¹, these residues are too far (red dashed lines) to form stabilizing interactions with the equivalent water molecule (red sphere) in the inhibited complex (gray sticks). The three water molecules (red spheres) electrostatically coordinated to Mg²⁺ (green sphere) in the inhibited complex are stabilized by interactions (cyan dashed lines) with side chain atoms of residues Gln⁹, Asp¹⁴⁶, Gln¹⁴⁷, and Lys¹⁹².

Ligand-induced Stability Changes Imply Cooperative Sources of High Indolmycin Affinity—The nature of the ligands within the active site has a significant effect on the conformation and thermal stability of an enzyme. For small perturbations, the fractional change in melting temperature (ΔTm/Tm) induced by ligand binding is proportional to the free energy change in stability, the proportionality constant being the enthalpy change (ΔH) (28). We use this implicit relationship to assess the stabilizing or destabilizing effects of various ligands on the BsTrpRS enzyme. Binding of ATP, tryptophan, or tryptophanamide stabilizes the thermal transition of molten globule formation by 3, 7, and 7%, respectively (Table 3). Indolmycin enhances the thermal stability of BsTrpRS by 20%, increasing Tm by 13.5 °C. The enhanced affinity for indolmycin over tryptophan results in a shift by 8 °C to higher temperature in the thermal transition due to molten globule formation in the presence of indolmycin compared with tryptophan.

The linkage between protein stability and ligand binding (29–31) implies that we can attribute differences in stability changes to binding affinity. Two of the stabilizing interactions formed between indolmycin and Mg²⁺-coordinated water molecules are associated with a change in the metal position relative to the ATP phosphate oxygen atoms. A key implication of the structural observations in Figs. 3A and 4C is that binding of indolmycin to BsTrpRS should be potentiated by the presence of Mg²⁺-ATP.
The presence/position of Mg$^{2+}$ in the active site is strictly dependent on ATP because the protein makes no contacts with the metal. As no direct ATP-indolmycin interactions are observed, we therefore expected that ATP would enhance the thermal stability of the BsTrpRS-indolmycin complex by a larger amount in the presence compared with the absence of Mg$^{2+}$. Additionally, we did not expect Mg$^{2+}$ to contribute to thermal stability in the absence of ATP. As expected, the differential scanning fluorimetry measurements show that the BsTrpRS in complex with indolmycin and Mg$^{2+}$-ATP has a 27% increase in melting temperature compared with ligand-free enzyme with Mg$^{2+}$-ATP contributing an additional 5 °C of thermal stability on top of the 13.5 °C provided by indolmycin binding. By contrast, binding of indolmycin, indolmycin + Mg$^{2+}$, or indolmycin + ATP all elicit far smaller changes of ~20% in thermal stability, demonstrating that both Mg$^{2+}$ and ATP are required to confer additional thermal stability to the BsTrpRS-indolmycin (IND) complex.

The conclusion that the metal is essential to the enhanced affinity of indolmycin to the pre-transition state complex can also be derived using the three-dimensional thermodynamic cycle of contributions to stability from ATP, the methylamino-substituted oxazoline ring (OXA), and the presence/absence of Mg$^{2+}$ (Fig. 6). Differences in binding and thermal stability between tryptophanamide and indolmycin were attributed to the methylamino-substituted oxazoline ring as this is the major structural difference between these two ligands.

Stabilizing and destabilizing interactions are distinguished by positive and negative non-additivity, respectively. If thermal stability were unaffected by interactions between the ligands, then we expect the effects of binding multiple ligands, e.g. ATP and LTN, to be additive and the effects of binding one ligand not to be affected by the presence of a second ligand, thus giving Equations 5 and 6.

$$\Delta T_m(\text{ATP}) + \Delta T_m(\text{LTN}) = \Delta T_m(\text{ATP + LTN})$$

(Eq. 5)

$$\Delta T_m(\text{LTN}) = T_m(\text{LTN}) - T_m(\text{LF}) = T_m(\text{ATP + LTN}) - T_m(\text{ATP})$$

(Eq. 6)

An interaction between the ligands would introduce a term, $\Delta T_{\text{m, int}}$, to describe the non-additivity (32), giving Equation 7.

$$\Delta T_m(\text{ATP}) + \Delta T_m(\text{LTN}) + \Delta T_{\text{m, int}} = \Delta T_m(\text{ATP + LTN})$$

(Eq. 7)

In the absence of Mg$^{2+}$, there is no significant ATP-OXA interaction, and binding either IND or LTN reduces the effect of ATP on $T_m$ by ~1.5 °C. The ATP-LTN and ATP-IND interactions are both destabilizing; i.e. the doubly liganded complexes melt at lower temperatures. In contrast, addition of Mg$^{2+}$ stabilizes the interactions of ATP with LTN and IND to varying degrees. In the case of LTN-ATP, addition of Mg$^{2+}$ compensates for the destabilizing ATP-LTN interaction such that the interaction is no longer significant. The metal compensates for the ~1.3 °C destabilizing ATP-LTN interaction and allows for an additional stabilizing interaction of 2.6 °C. Thus, the $\Delta T_m$ (ATP-OXA) interaction is comparable with that of the $\Delta T_m$ (ATP-IND) interaction. The crystal structure suggests that the stabilizing effect of Mg$^{2+}$ on the interaction...
between ATP and indolmycin is mediated through the oxazolinone ring, whose orientation is, in turn, stabilized by hydrogen bonds to His43 and Gln147 as discussed above.

**Discussion**

An array of crystal structures of both BsTrpRS and H₄-TrpRS provide snapshots of the enzymes along their catalytic paths and demonstrate the conformational changes that result from binding of various ligands (12, 13, 24). From these structures, it is evident that H₄-TrpRS uses a greater number of binding determinants for tryptophan recognition and that binding of tryptophan causes an induced fit rearrangement of the active site in H₄-TrpRS but not BsTrpRS. Here we discuss possible structural and mechanistic reasons for the tight binding of indolmycin to BsTrpRS and the inability of indolmycin to inhibit eukaryotic TrpRSs.

**Why Is Indolmycin a High Affinity Inhibitor of Bacterial TrpRS?**—There are no drastic global changes between structure 1MAU and the inhibited BsTrpRS structure (Protein Data Bank code 5DK4). We propose that subtle, mechanistically relevant differences in the active site metal coordination account for the ability of indolmycin to inhibit BsTrpRS as tightly as it does. We observe stronger Mg²⁺-ATP and weaker BsTrpRS-ATP interactions (Fig. 6) as well as altered Mg²⁺ coordination and placement in the inhibited state (indolmycin + Mg²⁺-ATP; Figs. 3B and 4) compared with the pre-transition state (tryptophanamide + Mg²⁺-ATP) structure. We attribute these differences to the replacement of tryptophanamide with indolmycin that varies mainly at the methylamino-substituted oxazolinone ring of indolmycin. Interactions among His43, Gln147, and indolmycin restrict the oxazolinone ring orientation, thereby reducing the entropy of the α-carbon mimic in the inhibited complex compared with the pre-transition state complex. This unfavorable entropy change is compensated by the enthalpy from additional hydrogen bonds formed among the Mg²⁺-coordinated water molecules and the oxazolinone nitrogen and carbonyl oxygen atoms as well as the interaction with His43.

These hydrogen bonds stabilize the water molecules that are also tightly coordinated to the catalytic Mg²⁺ ion. Functional groups of the α-carbon atoms of tryptophan and tryptophanamide can adopt alternative conformations that are similar in energy, none of which allow for completion of the Mg²⁺ coordination sphere. We conclude from these observations that completion of that coordination sphere allows the metal to form significantly tighter interactions with all three phosphate oxygen atoms and hence that indolmycin stabilizes a ground state Mg²⁺-ATP configuration, opposing the tendency of the PreTS state to promote the metal to a high energy state that assists in transition state stabilization.

Furthermore, the oxazolinone ring of indolmycin, stabilized by hydrogen bonds with His43 and Gln147, prevents the rotamer switch of Tyr125 in the specificity helix that is part of the structural transition from the open to the closed state. To avoid a steric clash with the constrained Tyr125 residue, Gln107 likewise does not switch rotamers in the presence of indolmycin. Gln107 is part of a highly mobile loop that shows a subtle but significant opening in the inhibited state compared with the pre-transition state. This opening results in the weakening of ATP-BsTrpRS interactions, specifically those between Lys111 and the γ-phosphate group.

In the catalytically competent PreTS configuration, coordination by lysine residues of the phosphate oxygen atoms promotes the metal to an activated, less stable state with weaker interactions to the three phosphate oxygen atoms and prevents the Mg²⁺ ion from assuming a lower energy position with stronger contacts to ATP. The positively charged Nε atom of Lys111 competes with the Mg²⁺ ion for stabilization of a negatively charged oxygen atom (Oγ) of the γ-phosphate group. In the PreTS state, the Mg²⁺-Oγ and Lys111-Oγ distances are 2.4 and 2.9 Å, respectively.

Substitution by indolmycin for tryptophanamide simultaneously weakens the Lys111-Oγ (3.4 Å) interaction and strengthens that between that oxygen atom and the Mg²⁺ ion (2.2 Å). Additionally, the 0.4-Å shift in Mg²⁺ placement along with the opening of the mobile loop around Lys111 allows for tight, hexavalent Mg²⁺ coordination accompanied by stronger, more nearly equivalent interactions between Mg²⁺ and the three ATP phosphate groups.

**Mutation of His43 Results in Indolmycin Resistance**—When both tryptophanamide and ATP are bound, the His43 side chain switches from one rotamer to another in the transition from open to closed PreTS state and back again in the closed product state. This rotamer switch does not occur when the active site is bound to AMP, PPᵢ, and tryptophan (33). In the PreTS (Mg²⁺-ATP + LTN) and inhibited (Mg²⁺-ATP + IND) states, Nε2 of His43 interacts with Oδ2 of Asp132. In all other observed states, Nε2 of His43 forms an interaction with the carbonyl oxygen of Tyr125. His43 also contributes to indolmycin binding via Nδ1. This reorientation of His43 appears to be correlated with the succession of ligands most similar to the putative catalytic reaction path, and it may thus also be functional.

Several groups have identified mutations that confer high level indolmycin resistance (34–36). One of the mutant sites, His13, is of direct interest in the context of the present inhibited structure, which furnishes a semiquantitative explanation for the mutational effects at position 43. We have implicated His43 in a hydrogen bond network that requires hydrogen bonds to both indole nitrogen atoms that stabilize the orientation of the plane of the oxazolinone ring of indolmycin. Fixing the orientation of the ring consequently allows formation of a full hexacoordinated environment for the catalytic Mg²⁺ ion, which we have shown accounts for the additional stabilization of the indolmycin-Mg²⁺-ATP complex.

Indolmycin inhibition of the *B. stearothermophilus* TrpRS H43N mutant is weaker by 3.5 kcal/mol than that of the native enzyme (37). Depending on the stabilization energy provided by these two hydrogen bonds and their coupling, we would predict that an H43N mutant would lose 2–4 kcal/mol binding energy compared with wild-type enzyme. All rotamers of an asparagine substitution at position 43 would result in the loss of two of the three hydrogen bonds observed in the network among indolmycin, His43, and Asp132. Similarly, the homologous H48Q mutation in *Streptomyces coelicolor* similarly appears incapable of forming both hydrogen bonds we observe for His43 (36).
Mechanism-based Selectivity of Indolmycin Inhibition

Modeling Reveals Why Indolmycin Is a Weak Inhibitor of Eukaryotic Cytosolic TrpRS Enzymes—The selectivity ratio of indolmycin for cytosolic B. taurus (Bt)TrpRS versus BsTrpRS is 10⁶-fold in favor of BsTrpRS binding. This selectivity factor far surpasses those of most therapeutic drugs, including trimethoprim (selectivity ratio rat/Toxoplasma gondii dihydrofolate reductase, 49) and metoprolol (selectivity ratio β₂/β₁-adrenergic receptor, 6.0), which treat toxoplasmosis and cardiovascular disease, respectively (38, 39). This dramatic selectivity arises by enhancing indolmycin binding recognition by BsTrpRS as we have just shown while reducing eukaryotic selectivity arises by enhancing indolmycin binding recognition by BsTrpRS enzymes evade inhibition by indolmycin (13, 14, 40, 41).

Whereas BsTrpRS uses an induced fit mechanism for ATP binding, H₄TrpRS uses induced fit for tryptophan binding. Binding of tryptophan to BsTrpRS is stabilized by one hydrogen bond between the indole nitrogen of tryptophan and Oδ2 of Asp¹³² and π-π interactions with Phe⁵. Meanwhile, H₄TrpRS makes seven direct and water-mediated contacts to its tryptophan substrate. The determinants for tryptophan binding to H₄TrpRS include Glu¹⁹⁹, which has a direct and water-mediated interaction with the α-amino group of tryptophan (Fig. 7A). Modeling of indolmycin into the amino acid binding site introduces steric clashes between indolmycin and Glu¹⁹⁹ (Fig. 7B). Furthermore, Glu¹⁹⁹ cannot adopt an alternative rotamer conformation without introducing additional clashes between Glu¹⁹⁹ and Thr¹⁹⁶, Trp²⁰₃, or Phe²⁰⁶. Besides clashing with Glu¹⁹⁹, this indolmycin conformer would not form any of the hydrogen bonds observed when tryptophan is bound aside from the bifurcated hydrogen bond with the indole nitrogen, Tyr¹⁵⁹, and Gln¹⁹⁴. These interactions are preserved because indolmycin was modeled into the active site by overlaying its indole moiety with that of tryptophan (Protein Data Bank code 2QUH).

Indolmycin can be modeled into the active site of H₄TrpRS by rotating the oxazolinone ring away from Glu¹⁹⁹ to an orientation perpendicular to the indole moiety (Fig. 7B). Although this indolmycin conformer does not clash with active site residues, it does disrupt the hydrogen bonding pattern used by the cytosolic enzyme to identify tryptophan as the bound substrate. The α-amino group of tryptophan, which is protonated at physiological pH, is recognized by Glu¹⁹⁹, Gln¹⁹⁴, and Gln³¹³ (Fig. 7A). Each of these residues acts a hydrogen bond acceptor, and the negatively charged carboxylate of Glu¹⁹⁹ forms additional electrostatic interactions with the amino group. According to our model, the side chain amide group of Gln²⁸⁴ would act as a hydrogen bond donor for the cyclic oxygen atom in indolmycin that is in the equivalent position of the α-amino group (Fig. 7B). Although Glu¹⁹⁹ cannot form a salt bridge with indolmycin, it can instead share a bifurcated hydrogen bond from the methy lamino group nitrogen with Gln³¹³. This nitrogen can accept a hydrogen bond from the hydroxyl group of Tyr¹⁵⁹.

Finally, Lys²⁰⁰ cannot form a salt bridge with indolmycin as it does with the tryptophan carboxylate. This electrostatic interaction is also missing when tryptophanamide is bound in place of tryptophan and appears to be critical for progression from the pre-transition state to transition state as this is the only interaction used for tryptophan substrate recognition and binding that cannot form when tryptophanamide occupies the active site. Indolmycin differs from tryptophan by a greater degree than does tryptophanamide. The inability of indolmycin to fully retain the tryptophan-H₄TrpRS side chain interactions, including the salt bridge with Lys²⁰⁰, allows H₄TrpRS to discriminate between tryptophan and the inhibitor. For these reasons, a stable H₄TrpRS-ATP-indolmycin complex is ~1000 less likely to form than that of H₄TrpRS-ATP-tryptophan. Contrastingly, BsTrpRS, which has a 10³-fold higher affinity for indolmycin over tryptophan, is ~1500 more likely to form a stable, inhibited complex than a catalytically competent tryptophan-bound complex.
Mechanism-based Selectivity of Indolmycin Inhibition

In this work, we determined the structural basis for high affinity inhibition of BsTrpRS by indolmycin. The simultaneous binding of indolmycin and Mg\(^{2+}\) -ATP results in (i) movement of the Tyr\(^{125}\) and Gln\(^{107}\) side chains, (ii) opening of the mobile loop containing Lys\(^{111}\), (iii) displacement of the Mg\(^{2+}\) ion by 0.4 Å, (iv) hexavalent metal coordination, (v) stronger, nearly equivalent electrostatic interactions of Mg\(^{2+}\) with an oxygen from each phosphate group of ATP, and (vi) weaker coordination of phosphate group oxygen atoms by active site lysine residues. These changes are reinforced by the hydrogen bonding of phosphate group oxygen atoms by active site lysine residues. These changes are reinforced by the hydrogen bonding of phosphate group oxygen atoms by active site lysine residues thereby significantly increasing affinity by preventing activation of the metal required for use in amino acid activation.

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References

1. Werner, R. G., Thorpe, L. F., Reuter, W., and Nierhaus, K. H. (1976) Indolmycin inhibits prokaryotic tryptophanyl-tRNA ligase. Eur. J. Biochem. 68, 1–3
2. Ogilvie, A., Wiebauer, K., and Kersten, W. (1975) Inhibition of leucyltransfer ribonucleic acid synthetase. Biochim. Biophys. Acta 152, 511–515
3. Nakama, T., Nureki, O., and Yokoyama, S. (2001) Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. J. Biol. Chem. 276, 47387–47393
4. Konrad, I., and, R. (1977) Inhibition of phenylalanine tRNA synthetase from Bacillus subtilis by ochratoxin A. FEBS Lett. 83, 341–347
5. Sutherland, R., Boon, R. J., Griffin, K. E., Masters, P. J., Slocombe, B., and White, A. R. (1985) Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob. Agents Chemother. 29, 495–498
6. Kanamaru, T., (2001) In vitro and in vivo antibacterial activities of TAK-083, an agent for treatment of Helicobacter pylori infection. Antimicrob. Agents Chemother. 45, 2465–2469
7. Werner, R. G., and Reuter, W. (1979) Interaction of indolmycin in the metabolism of tryptophan in rat liver. Arzneimittelforschung 29, 59 – 63
8. Weinreb, V., Li, L., Campbell, C. L., Kaguni, L. S., and Carter, C. W., Jr. (2009) Mg\(^{2+}\)-assisted catalysis by B. stearothermophilus TrpRS is promoted by allosteric effects. Structure 17, 952–964
9. Retailleau, P., Weinreb, V., Hu, M., and Carter, C. W., Jr. (2007) Crystal structure of tryptophanyl-tRNA synthetase complexed with adenosine-5’-triphosphate: evidence for distributed use of catalytic binding energy in amino acid activation by class I aminoacyl-tRNA synthetases. J. Mol. Biol. 369, 108–128
10. Weinreb, V., Li, L., and Carter, C. W., Jr. (2012) A master switch couples Mg\(^{2+}\)-assisted catalysis to domain motion in B. stearothermophilus tryptophanyl-tRNA Synthetase. Structure 20, 128–138
11. Zhou, M., Dong, X., Shen, N., Zhong, C., and Ding, J. (2010) Crystal structures of Saccharomyces cerevisiae tryptophanyl-tRNA synthetase: new insights into the mechanism of tryptophan activation and implications for anti-fungal drug design. Nucleic Acids Res. 38, 3399–3413
12. Retailleau, P., Vonrhein, C., Bricogne, G., Roversi, P., Ilyin, V., and Carter, C. W., Jr. (2003) Interconversion of ATP binding and conformational free energies by tryptophanyl-tRNA synthetase: structures of ATP bound to open and closed, pre-transition-state conformations. J. Mol. Biol. 325, 39–63
13. Shen, N., Zhou, M., Yu, Y., Dong, X., and Ding, J. (2008) Catalytic mechanism of the tryptophan activation reaction revealed by crystal structures of human tryptophanyl-tRNA synthetase in different enzymatic states. Nucleic Acids Res. 36, 1288–1299
14. Yang, X. L., McRee, D. E., and Schimmel, P. (2007) Functional and crystal structure analysis of active site adaptations of a potent anti-angiogenic human tRNA synthetase. Structure 15, 793–805
15. Studier, F. W. (2005) Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. 41, 207–234
16. Fersht, A. R., Ashford, J. S., Brouton, C. J., Jakes, R., Koch, G. H., and Hartley, B. S. (1975) Active site titration and aminoacyl adenylate binding stoichiometry of aminoacyl-tRNA synthetases. Biochemistry 14, 1–4
17. Franklyn, C. S., First, E. A., Perona, J. J., and Hou, Y. M. (2008) Methods for kinetic and thermodynamic analysis of aminoacyl-tRNA synthetases. Methods 44, 100–118
18. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to Image: 25 years of image analysis. Nat. Methods 9, 671–675
19. SAS Institute (2013) JMP: The Statistical Discovery Software, SAS Institute, Cary, NC
20. Kabsch, W. (2010) XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132
21. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, S. J., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221
22. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
23. Cleland, W. W., and Northrop, D. B. (1999) Energetics of substrate binding, catalysis, and product release. Methods Enzymol. 308, 3–27
24. Ilyin, V. A., Temple, B., Hu, M., Li, G., Yin, Y., Vachette, P., and Carter, C. W., Jr. (2000) 2.9 Å crystal structure of ligand-free tryptophanyl-tRNA synthetase: domain movements fragment the adenine nucleotide binding site. Protein Sci. 9, 218–231
25. Doublié, S. (1993) 2.9 Å Crystal Structure of Bacillus steatorrhophilus Tryptophanyl-tRNA Synthetase Complexed to its Adenylate. Tryptophanyl-5’-AMP. Ph.D. thesis, University of North Carolina at Chapel Hill
26. Retailleau, P., Yin, Y., Hu, M., Roach, J., Bricogne, G., Vonrhein, C., Roversi, P., Blanc, E., Sweet, R. M., and Carter, C. W., Jr. (2001) High resolution experimental phases for tryptophanyl-tRNA synthetase (TrpRS) complexed with tryptophanyl-5’-AMP. Acta Crystallogr. D Biol. Crystallogr. 57, 1595–1608
27. Yin, Y. (1995) Crystallographic Study of Bacillus steatorrhophilus tryptophanyl-tRNA synthetase in its catalytic reaction. Ph.D. thesis, University of North Carolina at Chapel Hill
28. Calvin, M., Hermans, J., and Schraga, H. A. (1959) Effect of deuterium on the strength of hydrogen bonds. J. Am. Chem. Soc. 81, 5046–5050
29. Matulis, D., Kranz, I. K., Salemmeh, F. R., and Todd, M. J. (2005) Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using thermofluor. Biochemistry 44, 5258–5266
30. Niesen, F. H., Berglund, H., and Vedadi, M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat. Protoc. 2, 2212–2221
31. Weber, P. C., Wendoloski, J. J., Pantoliano, M. W., and Salemme, F. R. (1992) Crystallographic and thermodynamic comparison of natural and synthetic ligands bound to streptavidin. J. Am. Chem. Soc. 114, 3197–3200
32. Jencks, W. P. (1981) On the attribution and additivity of binding energies. J. Mol. Biol. 152, 213–221
33. Ilyin, V. A., Temple, B., Hu, M., Li, G., Yin, Y., Vachette, P., and Carter, C. W., Jr. (2001) High resolution experimental phases for tryptophanyl-tRNA synthetase (TrpRS) complexed with tryptophanyl-5’-AMP. Acta Crystallogr. D Biol. Crystallogr. 57, 1595–1608
34. Jancs, W. P. (1981) On the attribution and additivity of binding energies. J. Mol. Biol. 152, 213–221
in molecular simulations. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 1790–1795
34. Kitabatake, M., Ali, K., Demain, A., Sakamoto, K., Yokoyama, S., and Söll, D. (2002) Indolmycin resistance of *Streptomyces coelicolor* A3(2) by induced expression of one of its two tryptophanyl-tRNA synthetases. *J. Biol. Chem.* **277**, 23882–23887
35. Hurdle, J. G., O’Neill, A. J., and Chopra, I. (2004) Anti-staphylococcal activity of indolmycin, a potential topical agent for control of staphylococcal infections. *J. Antimicrob. Chemother.* **54**, 549–552
36. Vecchione, J. J., and Sello, J. K. (2009) A novel tryptophanyl-tRNA synthetase gene confers high-level resistance to indolmycin. *Antimicrob. Agents Chemother.* **53**, 3972–3980
37. Ali, K. S. (2002) *Bacillus stearothermophilus* Tryptophanyl-tRNA Synthetase: Mutations Leading to Indolmycin Resistance. Ph.D. thesis, Yale University

38. Gangjee, A., Vasudevan, A., Queener, S. F., and Kisliuk, R. L. (1996) 2,4-Diamino-5-deaza-6-substituted pyrido[2,3-d]pyrimidine antifolates as potent and selective nonclassical inhibitors of dihydrofolate reductases. *J. Med. Chem.* **39**, 1438–1446
39. Smith, C., and Teitler, M. (1999) β-Blocker selectivity at cloned human β1- and β2-adrenergic receptors. *Cardiovasc. Drugs Ther.* **13**, 123–126
40. Yang, X. L., Otero, F. J., Skene, R. J., McRee, D. E., Schimmel, P., and Ribas de Pouplana, L. (2003) Crystal structures that suggest late development of genetic code components for differentiating aromatic side chains. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 15376–15380
41. Yu, Y., Liu, Y., Shen, N., Xu, X., Xu, F., Jia, J., Jin, Y., Arnold, E., and Ding, J. (2004) Crystal structure of human tryptophanyl-tRNA synthetase catalytic fragment: insights into substrate recognition, tRNA binding, and angiogenesis activity. *J. Biol. Chem.* **279**, 8378–8388