An Antifungal Agent Inhibits an Aminoacyl-tRNA Synthetase by Trapping tRNA in the Editing Site

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Aminoacyl–transfer RNA (tRNA) synthetases, which catalyze the attachment of the correct amino acid to its corresponding tRNA during translation of the genetic code, are proven antimicrobial drug targets. We show that the broad-spectrum antifungal 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690), in development for the treatment of onychomycosis, inhibits yeast cytoplasmic leucyl-tRNA synthetase by formation of a stable $tRNA^{Leu}$-AN2690 adduct in the editing site of the enzyme. Adduct formation is mediated through the boron atom of AN2690 and the 2′- and 3′-oxygen atoms of tRNA's 3′-terminal adenosine. The trapping of enzyme-bound $tRNA^{Leu}$ in the editing site prevents catalytic turnover, thus inhibiting synthesis of leucyl-$tRNA^{Leu}$ and consequentially blocking protein synthesis. This result establishes the editing site as a bona fide target for aminoacyl-tRNA synthetase inhibitors.

Aminoacyl-tRNA synthetases (AARSs) perform a pivotal role in translating the genetic code by catalyzing the attachment of the correct amino acid to its cognate tRNA (*1*). The aminoacylation reaction occurs in two steps: the formation of an enzyme-bound aminoacyl-adenylate, followed by transfer of this activated amino acid to either the 2′- or 3′-hydroxy group on the 3′-terminal adenosine of tRNA. The accuracy of the tRNA aminoacylation reaction is critical to ensuring the fidelity of the genetic code (*2*). To achieve this accuracy, many AARS enzymes possess a proofreading (editing) mechanism that hydrolyzes tRNAs aminoacylated with the incorrect amino acid (*3*). Leucyl-tRNA synthetase (LeuRS) is a proofreading AARS, which possesses distinct synthetic (aminoacylation) and editing active sites separated by more than 30 Å (*4*, *5*). We show that 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690) inhibits LeuRS by trapping $tRNA^{Leu}$ in the editing active site.

AN2690 is a member of a new class of broad-spectrum antifungals (table S1), the benzoxaboroles, which have an unusual chemical attribute: a boron atom (*6*). We isolated spontaneous and ethyl-methanesulfonate (EMS)–induced AN2690-resistant mutants in the yeast *Saccharomyces cerevisiae* (*7*). These genetically dominant mutants were 32- to 512-fold more resistant to AN2690 than the parental *S. cerevisiae* strain (table S2), and their resistance mutations were found to lie in the *CDC60* gene, which encodes the cytoplasmic LeuRS (Cdc60p). Furthermore, all AN2690-resistant mutations mapped to the editing domain (Fig. 1A and table S2) and all but two, $Cys^{326} \rightarrow Arg^{326}$ (C326R) and $Cys^{326} \rightarrow Phe^{326}$ (C326F) (*8*), to the two highly conserved regions that form the editing active site of LeuRS (*9*). Four mutations lie in the threonine-rich region, a locus known in bacterial LeuRS homologs to be involved in binding and hydrolyzing mischarged tRNAs (*9*–*12*). Seven of the nine mutants exhibited an editing defect based on their sensitivity to the structurally related noncognate amino acid norvaline (fig. S1). These results suggest that the editing pocket of Cdc60p is the binding site for AN2690.

To delineate its mode of action, we investigated the effect of AN2690 on the ability of LeuRS to hydrolyze mischarged $tRNA^{Leu}$. Addition of AN2690 to the posttransfer editing assay inhibited the hydrolysis of Ile-$tRNA^{Leu}$ in a dose-dependent manner (Fig. 1B). In addition, we found that AN2690 inhibited tRNA aminoacylation (fig. S2A), and, as would be expected for a LeuRS inhibitor, it blocked protein synthesis in vivo (fig. S2B). Initial aminoacylation experiments also revealed that AN2690 required the presence of tRNA for effective inhibition of aminoacylation activity. Kinetic analysis of aminoacylation inhibition showed that AN2690 acted as a noncompetitive inhibitor with respect to both adenosine triphosphate (ATP) and leucine (fig. S3, A and B). Analysis of the noncompetitive nature of AN2690 revealed that the inhibition constant (K_i) decreased on increasing AN2690's incubation time with tRNA and Cdc60p, before initiating the aminoacylation reaction with ATP. When enzyme and tRNA were incubated with AN2690 for 2 min, the K_i was 31.4 ± 2.8 (SEM) μM, whereas after a 20-min incubation the K_i decreased to 1.85 ± 0.1 μM (fig. S3). To better understand this process, we measured inhibition of aminoacylation as a function of incubation time and AN2690 concentration (Fig. 2A). We found a direct linear relationship between the observed rates of inactivation (k_{obs}) and AN2690 concentrations, with no apparent plateau even at the highest concentration tested (fig. S4). From these data, we deduced a rate of inactivation of the enzyme ($k_{inactivation}$) of 0.66 ± 0.10 min^{-1}

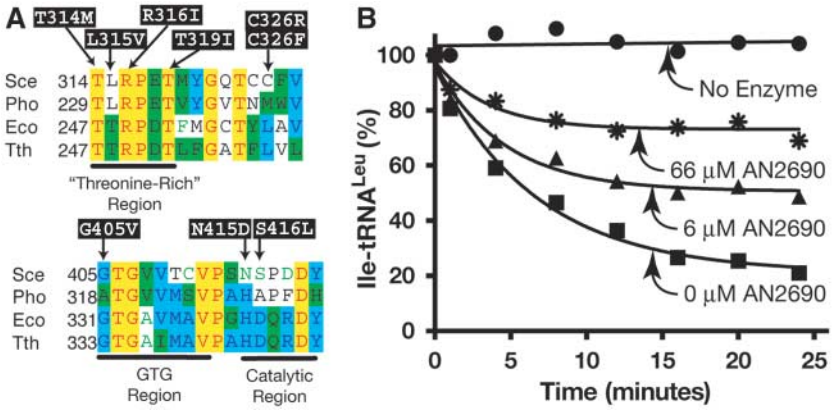

Fig. 1. (**A**) *S. cerevisiae* AN2690 resistance mutations in the editing active site of Cdc60p (*8*). Alignment of the conserved regions of the LeuRS editing domains from *S. cerevisiae* (Sce) from CAA97865, *Pyrococcus horikoshii* (Pho) from O58698, *Escherichia coli* (Eco) from AAC73743, and *T. thermophilus* (Tth) from BAD69984. The amino acid substitutions that confer resistance in *S. cerevisiae* to AN2690 are in black (table S2). (**B**) AN2690 inhibits posttransfer editing. Deacylation of total brewer's yeast tRNA mischarged with isoleucine, no enzyme control (circles), enzyme control (squares), Cdc60p treated with 6 μM AN2690 (triangles), and enzyme treated with 66 μM AN2690

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(fig. S5). Measurement of the rate of recovery demonstrated that AN2690's inactivation of Cdc60p was reversible, albeit with a very slow reactivation rate of $1.64 \times 10^{-4} \pm 0.15 \times 10^{-4}$ min^{-1} (Fig. 2B), which corresponds to a half-life of 424 min. These kinetic properties are consistent with AN2690 being characterized as a slow-tight-binding inhibitor.

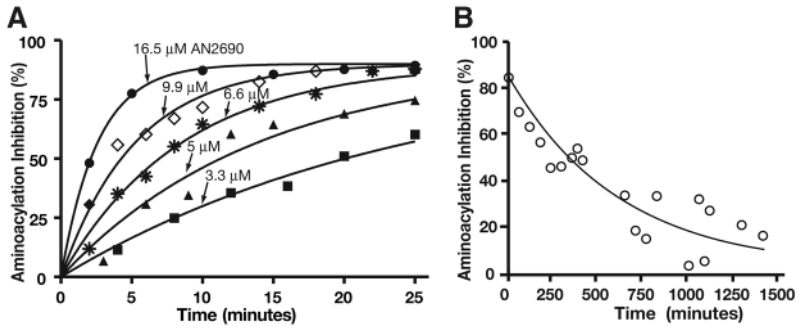


Fig. 2. (**A**) The rate of Cdc60p inactivation by AN2690. The percentage of inhibition from different concentrations [3.3 μM (squares), 5 μM (triangles), 6.6 μM (asterisk), 9.9 μM (open diamonds), and 16.5 μM (circles)] of AN2690 were plotted versus the time of incubation. All reactions were performed in triplicate, and the mean values were plotted. (**B**) The recovery of Cdc60p activity from AN2690 inhibition. All reactions were performed in triplicate, and the mean values were plotted. The data were fitted to a first-order exponential decay.

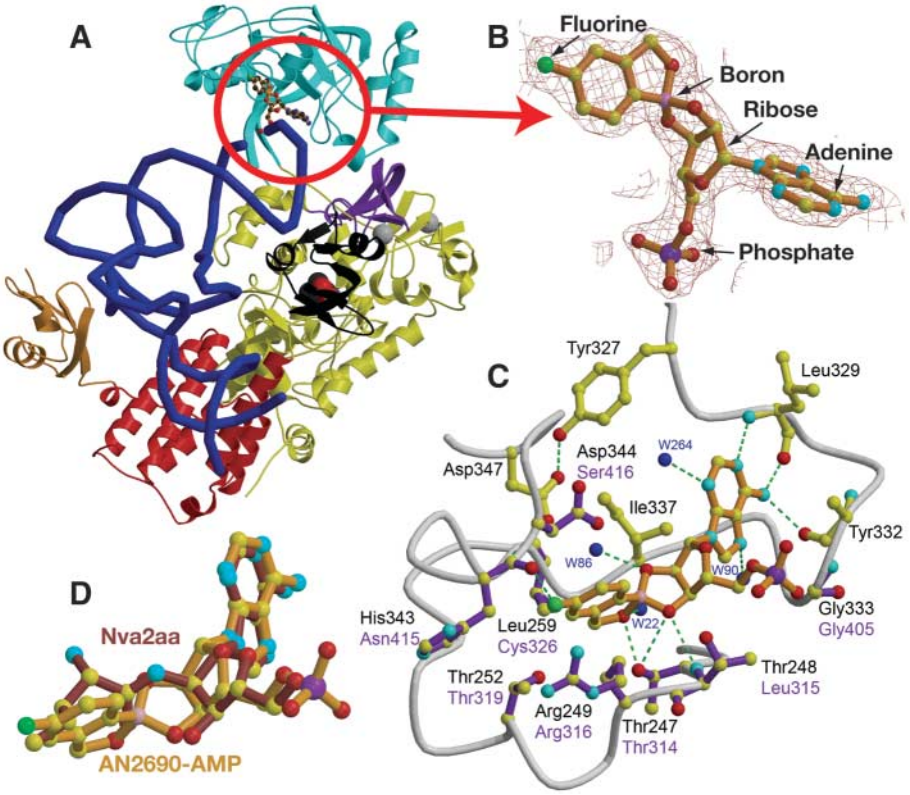


Fig. 3. AN2690 forms an adduct with the terminal adenosine (A76) of $tRNA^{Leu}$ in the editing active site of LeuRS. (**A**) Overall structure of the complex of *T. thermophilus* LeuRS with $tRNA^{Leu}$ and AN2690, showing the adenosine-AN2690 adduct (ball-and-stick model, ringed in red) in the editing site and leucine (space-filling model) in the synthetic site. The editing domain is cyan; the catalytic domain, yellow; Zn-1 domain, purple; the leucyl-specific insertion domain, black; the anticodon-binding domain, red; the C-terminal domain, gold; zinc atoms, gray spheres; and tRNA, blue tube. (**B**) Unbiased difference map (1.85 Å resolution) for the AMP-AN2690 adduct in the editing site. (**C**) Diagram showing water molecules (dark blue spheres) and hydrogen bonds (green dotted lines) between editing site residues of LeuRS and the AMP-AN2690 adduct (orange). Amino acid residues that are mutated in the *S. cerevisiae* AN2690-resistant mutants are labeled and colored in purple (table S2). The atoms are colored accordingly: boron, mauve; fluorine, green; oxygen, red; nitrogen, light blue; carbon, yellow; and phosphate, purple. (**D**)

Equilibrium dialysis demonstrated that adenosine-containing ribonucleotides were essential for measurable binding of AN2690 to Cdc60p (fig. S6A). To determine more precisely the mode of action of AN2690, we obtained a 3.5 Å crystallographic structure of *Thermus thermophilus* LeuRS complexed with $tRNA^{Leu}$ and AN2690 (Fig. 3A). Significant positive difference density found in the editing site (fig. S7) was interpreted as a tRNA-AN2690 adduct with the boron from the oxaborole ring bound to the 2′,3′-hydroxy groups on the 3′-terminal adenosine (A76). A 1.85 Å resolution structure of an adenosine monophosphate (AMP)–AN2690 adduct bound in the editing site of LeuRS (Fig. 3B) confirmed this tetrahedral spiroborate structure, whose configuration is stabilized by two hydrogen bonds to the conserved threonine-rich peptide and a water molecule (Fig. 3C). These results show that AMP can act as a surrogate for the 3′-terminal adenosine of $tRNA^{Leu}$, as in our direct binding assay, and that AN2690 occupies the noncognate amino acid–binding pocket in the editing site. The adenosine and the 2′-hydroxy group of the AMP-AN2690 adduct can be exactly overlaid on the analogous groups of a posttransfer editing substrate analog, 2′-(L-norvalyl) amino-2′-deoxyadenosine (Nva2aa), bound in the editing site (*9*). However, the planar benzoxaborole only partially overlaps with the noncognate amino acid (Fig. 3D). In particular, AN2690 has no equivalent of the amino acid amino group, and the absence of any associated strong interactions with the enzyme probably explains why the compound has very low affinity for the editing site in the absence of AMP or tRNA.

Because benzoxaboroles can bind to the *cis*-diols of sugars (*13*, *14*), we tested the requirement for adenosine's 2′- and 3′-hydroxy groups in AN2690 binding to Cdc60p and found that both hydroxy groups were required (fig. S6C). This implies that AN2690 can only form an

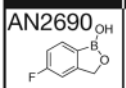
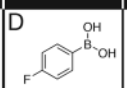
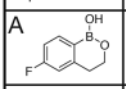
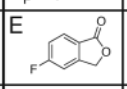
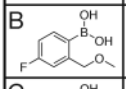
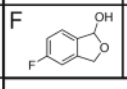
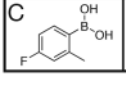
Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
AN2690 	2.1	D 	>100
A 	96	E 	>100
B 	>100	F 	>100
C 	>100		

Fig. 4. Boron and the oxaborole ring are required for inhibition of aminoacylation. All reactions were performed in triplicate, and the mean

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adduct with nonaminoacylated tRNA: The free 3′ end of the bound tRNA must sample the editing site before aminoacylation for AN2690 to inhibit LeuRS (fig. S8). This is the configuration observed in the *T. thermophilus* LeuRS-tRNA^{Leu} crystal structure (*15*).

The requirement for boron and the oxaborole ring in aminoacylation inhibition was tested by using various AN2690 analogs (Fig. 4). Expanding the oxaborole ring to form a benzoxaborin, compound A, adversely affected its ability to inhibit the aminoacylation reaction (Fig. 4). Because the ring-opened boronic acids are known also to bind to tRNA (*16*), we tested compounds B, C, and D. Each was found to be inactive, further demonstrating the importance of the oxaborole ring (Fig. 4). Lastly, the compounds E and F, where boron is replaced with a sp^2 or sp^3 carbon, were inactive, showing that boron cannot simply be replaced by carbon (Fig. 4).

This work demonstrates that AN2690 is a highly specific inhibitor of LeuRS and that the boron atom in the oxaborole ring is essential for AN2690's unique mechanism of inhibition. With appropriate compounds, the oxaborole tRNA trapping (OBORT) mechanism could be used to inhibit other AARS enzymes that perform posttransfer editing. Therefore, the incorporation of a boron atom into rationally designed enzyme inhibitors represents a promising approach to the discovery of new classes of therapeutic agents.

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7. Materials and methods are available on *Science* Online.
8. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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17. GraphPad, GraphPad Software, Incorporated.

18. We thank M. Kully and J. Khan at NAEJA Pharmaceuticals, Incorporated, for their contribution to the minimal inhibitory concentrations in table S1. Part of this work was funded by an NIH grant awarded to J.J.P. (R01 DE16835). The coordinates for LeuRSTT:tRNA:AN2690 and LeuRSTT:AMP:AN2690 have been deposited in the Protein Data Bank as 2V0G and 2V0C. S.J.B. and L.S. are co-founders and members of the board of Anacor Pharmaceuticals, Incorporated. S.C. and S.A.M. have received honoraria and/or consulting fees from Anacor Pharmaceuticals, Incorporated.

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Materials and Methods
Figs. S1 to S9
Tables S1 to S3
References

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