Chapter 2

Creating Synthetic “Blanks” in the Genetic Code Using A Novel Class of Aminoacyl Adenylate Mimics as tRNA Synthetase Inhibitors

Abstract

Efforts to reengineer the protein synthesis apparatus should expand our fundamental understanding of the central macromolecular synthesis machinery inside all cells. The UAG stop codon has often been referred to as a "blank" in the genetic code because it can be suppressed by an appropriate tRNA. We would like to eliminate the activity of specifically chosen aminoacyl tRNA synthetase (aaRS), creating our own blanks in the codes. We generated various high-affinity inhibitors of the synthetases—aminoacyl sulfamide to create synthetic blanks in our translation extracts. The gaps were filled with chemically aminoacylated orthogonal tRNAs. This unnatural strategy enables peptides and proteins to be constructed containing a single novel residue at a specific location. Biocytin, a biotin derivative of lysine, can be incorporated into globin, efficiently enriched by using aminoacyl sulfamide to inhibit natural aminoacylated tRNA and adding orthogonal tRNA coupled with biocytin.

Introduction

The protein synthesis machinery translates genetic information into folded, functional polypeptides, but is constrained by the universal genetic code to using the 20 naturally occurring amino acids as building blocks. Each of these 20 amino acids is escorted into the machine by transfer RNA that recognizes a specific codon in a message RNA template (Figure 1). Rewriting genetic code requires reassigning the codons to correspond to new amino acids.

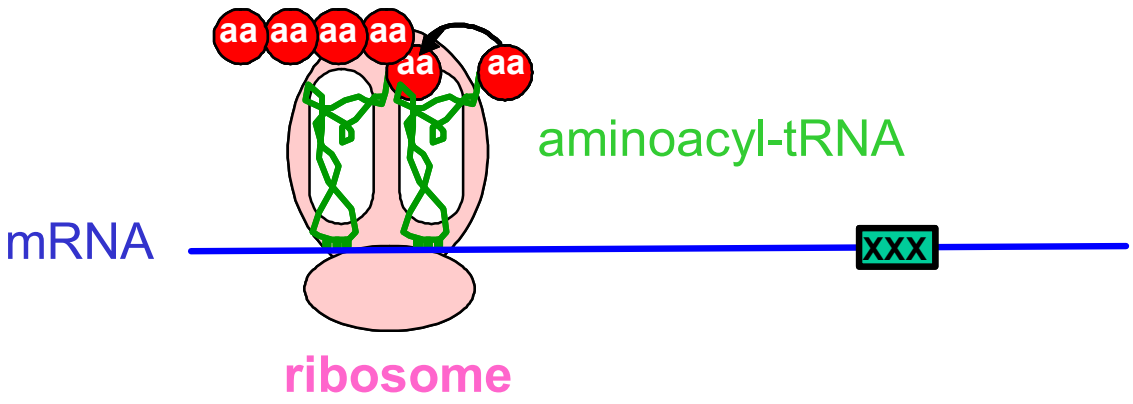

Figure 1. The protein synthesis machinery - ribosome.

The incorporation of unnatural amino acids into peptides and proteins has been pursued intensively over a couple of decades. Several unnatural amino acids have been incorporated into dipeptides through the use of chemically misacylated tRNAs in the

Hecht lab[1]. The UAG stop codon has often been referred to as a "blank" in the genetic code because it can be suppressed by an appropriate suppressor tRNA. Schultz and coworkers demonstrated the insertion of unnatural amino acids into proteins by nonsense UAG suppression[2,3]. While the original description of the method utilized an *E. coli* translation extract, the technique has been applied to eukaryotic translation extracts such as rabbit reticulocyte lysate[4-7] or *in vivo* in *Xenopus* oocytes[8-10]. This unnatural strategy enables peptides and proteins to be constructed containing a single novel residue at a specific location, amenable to the insertion of various entities into proteins including: 1) affinity tags, 2) spectroscopic probes, and 3) various amino acid derivatives for physical organic and mechanistic studies of protein function.

We would like to create our own blanks in the code by eliminating the activity of specific aaRS as we choose. All aaRSs act by using ATP to create an activated form of an amino acid – a mixed carbon-phosphorus anhydride termed an aminoacyl adenylate (Figure 2). Aminoacyl sulfamides are stable structural mimics of aminoacyl adenylates and generally have submicromolar K_i values for their corresponding synthetases[11]. We generated various high-affinity inhibitors of the synthetases to create synthetic blanks in our translation extracts. Aminoacyl tRNA synthetase inhibitors are combined with translation extracts and chemically aminoacylated orthogonal tRNAs to reprogram specific codons as desired. The genetic code will be determined by the chemically acylated tRNAs used, rather than by the natural aminoacyl-tRNA synthetases, the gatekeepers of the genetic code.

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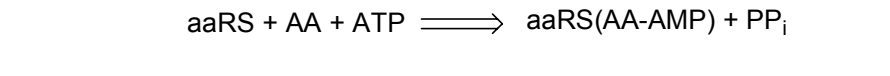

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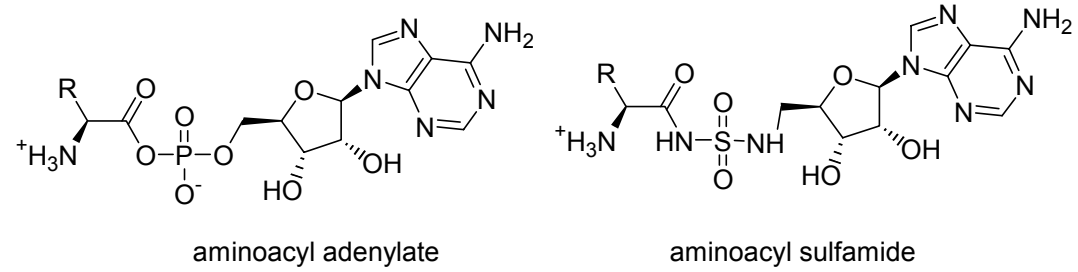


Figure 2. (A) Aminoacyl tRNA synthetases (aaRSs) specifically catalyze the aminoacylation of tRNA in a two step reaction. (B) The tRNA synthetases typically catalyze the formation of tightly bound aminoacyl-adenylates in their first reaction step. The analogues of these reaction intermediates (aminoacyl sulfamides) have been synthesized and evaluated for the ability to inhibit aminoacylation.

Application of non-sense and sense-codon suppression in protein selection has been examined in our group[12,13]. First, we reported that biocytin, a biotin derivative of lysine, can be inserted into an mRNA-protein fusion molecule through amber stop codon suppression. We also demonstrated that templates containing the codon corresponding to the biocytin tRNA (a UAG stop codon) can be enriched by iterative cycles of selection against a streptavidin agarose matrix. The universal genetic code links the 20 naturally occurring amino acids to the 61 sense codons. We have developed a selection methodology to investigate whether the unnatural amino acid biocytin could be incorporated into an mRNA display library at sense codons. In these experiments we probed a single randomized NNN codon with a library of 16 orthogonal, biocytin-acylated tRNAs. *In vitro* selection for efficient incorporation of the unnatural amino acid resulted in templates containing the GUA codon at the randomized position. This sense suppression occurs via Watson-Crick pairing with similar efficiency to UAG-mediated nonsense suppression. These experiments suggest that sense codon suppression is a viable means to expand the chemical and functional diversity of the genetic code. We envision that these aaRS inhibitors should facilitate the construction of novel proteins and selection of unnatural amino acids incorporated by sense codon suppression using the mRNA display approach (Figure 3).

mRNA display provides a strategy to make up to 10^{13} unique peptides that fuse with their own genes via a covalent linkage. Compared to other *in vitro* selection methodologies, such as phage-display[14,15] and surface-display[16] libraries, mRNA display[17,18] libraries have several unique features, including the incorporation of unnatural residues, the chemical derivatization of libraries, and the feasibility of mutagenic PCR and DNA recombination.

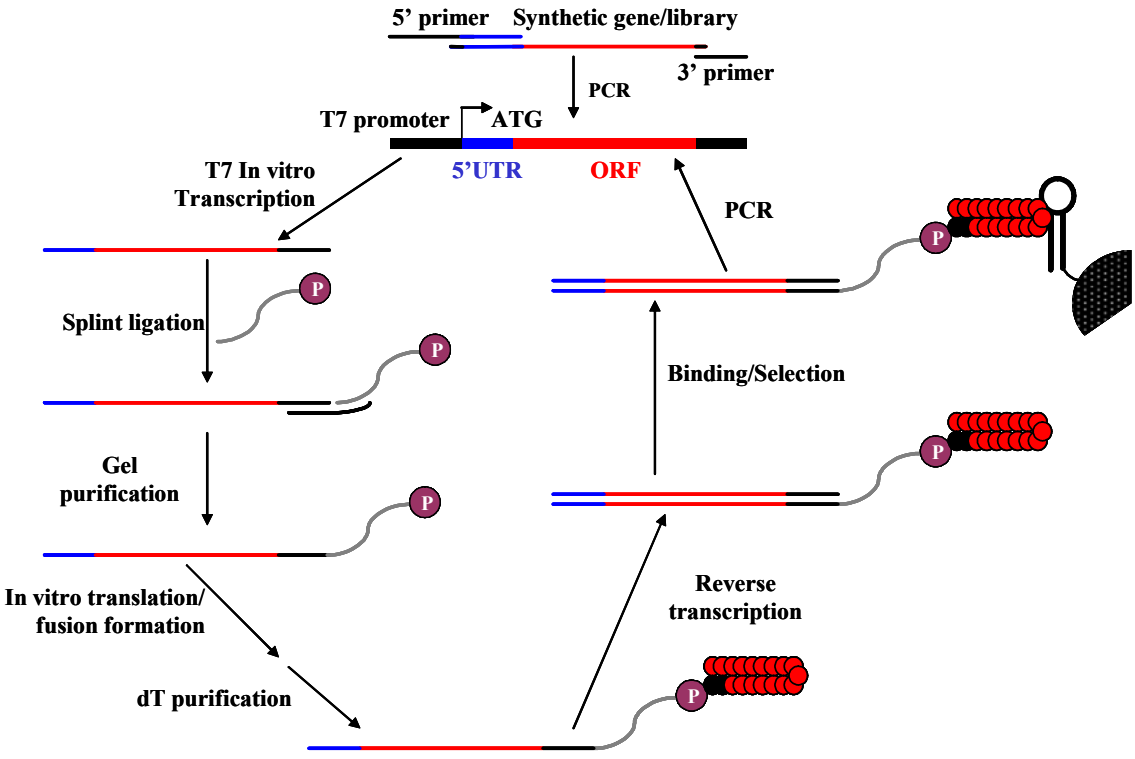


Figure 3. Selection cycle via mRNA display[17].

In affinity selection with mRNA display library, the synthetic DNA library is transcribed to make mRNA, which is modified with a 3'-puromycin. The engineered mRNA template is translated to prepare mRNA-peptide fusion. After a cDNA•mRNA duplex is generated by reverse transcription, the fusion library is subjected to affinity selection against targets of interest. The enriched library is amplified by PCR for the next cycle of selection.

Biocytin can be detected by ECL Plus Western Blotting Detection, and it binds to streptavidin (protein) coupled with Horseradish Peroxidase (HRP enzyme) which catalyzes a chemoluminence reaction.

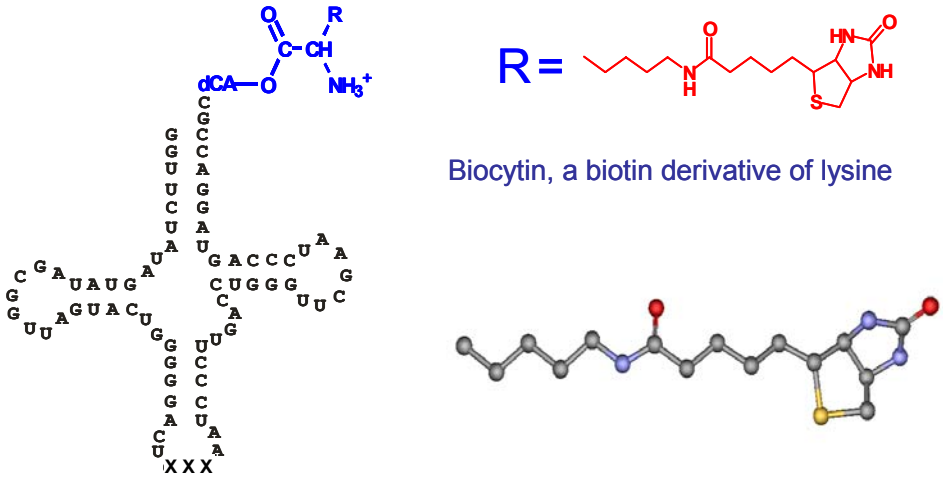
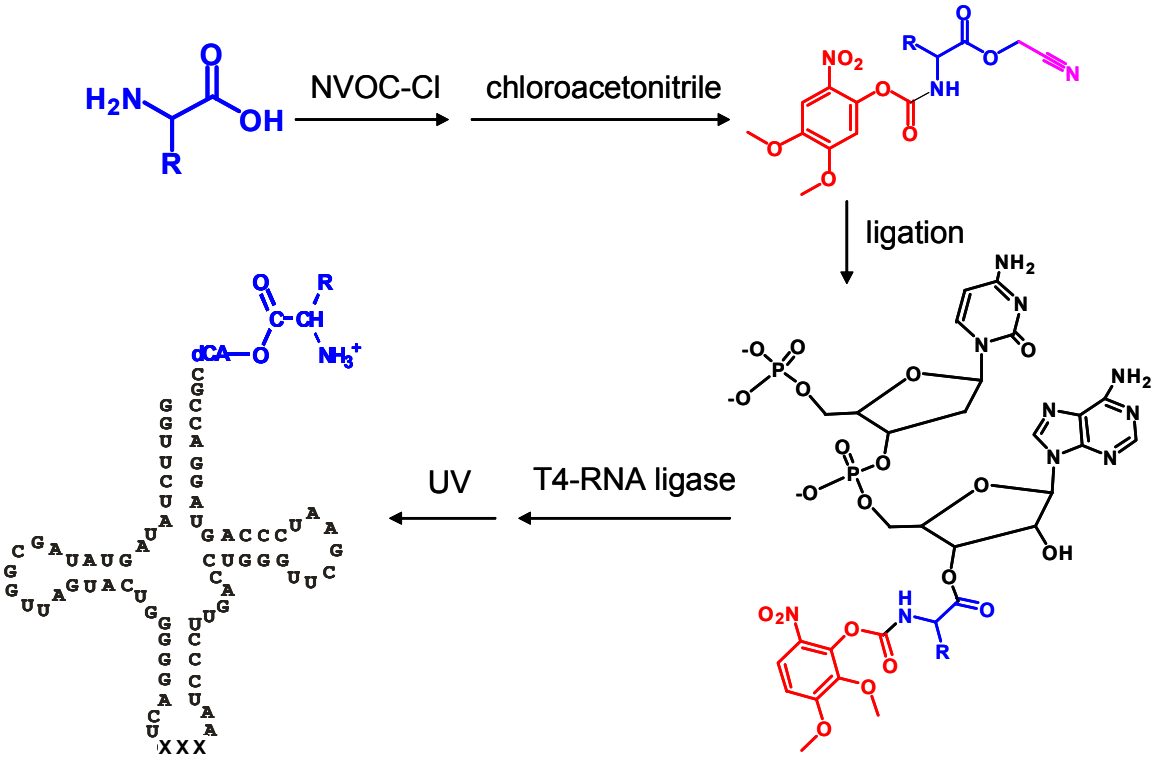


Figure 4. Biocytin charged suppressor tRNA and biocytin structure.

All the tRNAs we used are made from the pUC19-based plasmid harboring the gene for THG73, which was mutated at the tRNA anticodon position by Quikchange (Stratagene). To synthesize chemically aminoacylated tRNA with unnatural amino acid residue, the amino group of amino acid was protected by NVOC group first and the

carboxylic group was activated by acetonitrile. The activated amino acid was then coupled to dinucleotide pdCpA. Using T4-RNA ligase, dinucleotide pdCpA coupled with unnatural amino acid was ligated to the engineered truncated 73-nucleotide tRNA to form intact charged suppressor tRNA. Right before applying it to the translation system, NVOC protecting group was deprotected using UV irradiation (Scheme 1).



Scheme 1. Preparation of amino acylated THG73 tRNAs.

Since various high-affinity inhibitors of the synthetases suppress the formation of natural amino acylated tRNA and create synthetic blanks in our translation extracts, the selection rounds will be shortened for the discovery of novel ligands with functionalities beyond those provided by the 20 naturally occurring amino acids.

Materials and Methods

General Information. ^{1}H and ^{13}C NMR spectra were recorded on a UNITY INOVA instrument (Varian, Inc.) operating at 500 MHz using D_2O or DMSO-*d6* as the solvent. ^{1}H NMR data are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet; dd, doublet of doublets. High-resolution mass spectra (FAB) were recorded on a JMS-600H double-focusing, high-resolution, magnetic sector mass spectrometer (Mass Spectrometry Laboratory, Division of Chemistry and Chemical Engineering, California Institute of Technology). Column chromatography was carried out on silica gel (40-63 μm, EM Science). All reagents were of highest available commercial quality and were used without further purification. Triphenolphosphine, phthalimide, 2',3'-O-isopropylideneadenosine, hydrazine, benzyl alcohol, diethyl azodicarboxylate, chlorosulfonyl isocyanate, Pd 10% on activated carbon wet Degussa type, carbonyldiimidazole and diazabicycloundecene were purchased from Aldrich. Sodium azide was purchased from Sigma Chemical Co. Boc-Ala-OH, Boc-Val-OH, Boc-Leu-OH, Boc-ILe-OH, Boc-Phe-OH, Boc-Trp-OH, Boc-Tyr(2-Br-Z)-OH, Boc-Met-OH, Boc-Pro-OH and Boc-Gly-OH were purchased from Novabiochem.

Rabbit reticulocyte lysate was purchased from Novagen. Rabbit globin mRNA was obtained from Sigma Chemical Co. Ras mRNA was prepared by using two DNA primers complementary to the 5'- and 3'- ends of the coding region for H-Ras (pProEX HTb

vector, a kind gift from Dafna Bar-Sagi)[19] to amplify the gene using PCR. mRNA was produced by T7 runoff transcription[20] of the H-Ras DNA in the presence of RNAsecure (Ambion) followed by gel purification via denaturing urea-PAGE and 'crush and soak' RNA isolation. L-[^{35}S]methionine (1,175 Ci/mmol) was purchased from NEN Life Science Products. GF/A glass microfiber filters were from Whatman. Scintillation counting was carried out using a Beckman LS-6500 liquid scintillation counter.

General Procedure for Preparation of Aminoacyl-Sulfamides. 5'-Amino-2', 3**'**-O-isopropylidene -5'-deoxyadenosine was prepared from commercially available 2',3'-O-isopropylideneadenosine via the procedure disclosed previously[21]. It was then converted to 5'-deoxy-2', 3**'**-O-isopropylideneadenosine-5'-N-[(phenylmethoxy) carbonyl]-sulfamide by treatment with N-carbobenzyloxysulfamoyl chloride and a base such as triethylamine in dichloromethane at 0 °C to ambient temperature. CBZ protected sulfamide was reduced with Pd 10% on activated carbon wet Degussa type and hydrogen gas in EtOH/MeOH to afford 5'-deoxy -2', 3**'**-O-isopropylideneadenosine-5'-N-sulfamide, which was further reacted with BOC protected amino acids catalyzed by carbonyldiimidazole and base diazabicycloundecene in acetonitrile. The obtained 5'-deoxy-2',3**'**-O-isopropylideneadenosine-5'-N-(N-tertbutoxycarbonyl-aminoacyl) sulfamides were fully deprotected according to standard procedures and purified by trituration[22].

IC_{50} determination. Translation reactions containing [^{35}S]Met were mixed in batch on ice and added in aliquots to microcentrifuge tubes containing an appropriate amount of aminoacyl-sulfamide (2.5 μL of different concentrations). Typically, a 25 μl translation mixture consisted of 1.0 μL of 2.5 M KCl, 0.5 μL of 25 mM MgOAc, 2.0 μL of 12.5X translation mixture without methionine, (25 mM dithiothreitol (DTT), 250 mM HEPES (pH 7.6), 100 mM creatine phosphate, and 312.5 μM of 19 amino acids, except methionine) (Novagen), nuclease-free water, 2.0 μL (6.1 μCi) of [^{35}S]Met (1175 Ci/mmol), 10 μL of Red Nova nuclease-treated lysate (Novagen), and 5 μL of 0.05 μg/μL globin mRNA (Sigma). Inhibitor, lysate preparation (including all components except template), and globin mRNA were mixed simultaneously and incubated at 30 ºC for 60 min. Biocytin-tRNAs were added for translation restoration test. Then 2 μL of each reaction was combined with 8 μL of tricine loading buffer (80 mM Tris-Cl (pH 6.8), 200 mM DTT, 24% (v/v) glycerol, 8% sodium dodecyl sulfate (SDS), and 0.02 % (w/v) Coomassie blue G-250), heated to 90 ºC for 5 min, and applied entirely to a 4% stacking portion of a 16% tricine-SDS-polyacrylamide gel containing 20% (v/v) glycerol (30 mA for 1h, 30 min)[23]. Gels were fixed in 10% acetic acid (v/v) and 50% (v/v) methanol, dried, exposed overnight on a PhosphorImager screen, and analyzed using a Storm PhosphorImager (Molecular Dynamics). Inhibition of translation to a variety mRNA templates was tested in a similar condition.

Preparation of biocytin-acylated tRNAs. The pUC19-based plasmid harboring the gene for THG73 was mutated at the tRNA anticodon position by Quikchange (Stratagene). Resulting clones were verified by DNA sequencing before synthesizing individual tRNAs by *in vitro* transcription with T7 polymerase from *Fok I*-linearized plasmids. Transcribed tRNAs were then gel-purified and desalted by ethanol precipitation. Purified tRNAs were ligated to NVOC protected biocytin-dCA with T4 RNA ligase (New England Biolabs). Reaction mixtures were extracted in an equal volume of phenol:$CHCl_3$:isoamyl alcohol (25:24:1, pH 5.2, Fisherbiotech), and precipitated with 3.0 volumes of cold ethanol (-20 °C). After drying, the pellets were resuspended in 1.0 mM sodium acetate, pH 5.2, and adjusted to 4.0 mg/mL and 1.0 mg/mL for each biocytin-acylated tRNA. Biocytin-tRNA concentrations were determined by UV absorbance at 260 nm. Before adding to translation reactions, biocytin-tRNAs were deprotected by a xenon lamp outfitted with a 315 nm cut-off filter for 5 min to remove the NVOC group.

Restoration of Translation Probed with Streptavidin-capture of Biocytin-containing Globin. A portion of streptavidin-agarose (0.75 mL) [Pierce, 50% slurry (v/v)] was washed 3 times with a buffer (1X PBS containing 0.1% Triton X-100) and resuspended in 0.75 mL of buffer. To 100 μL of this suspension, 10 μL of the translation

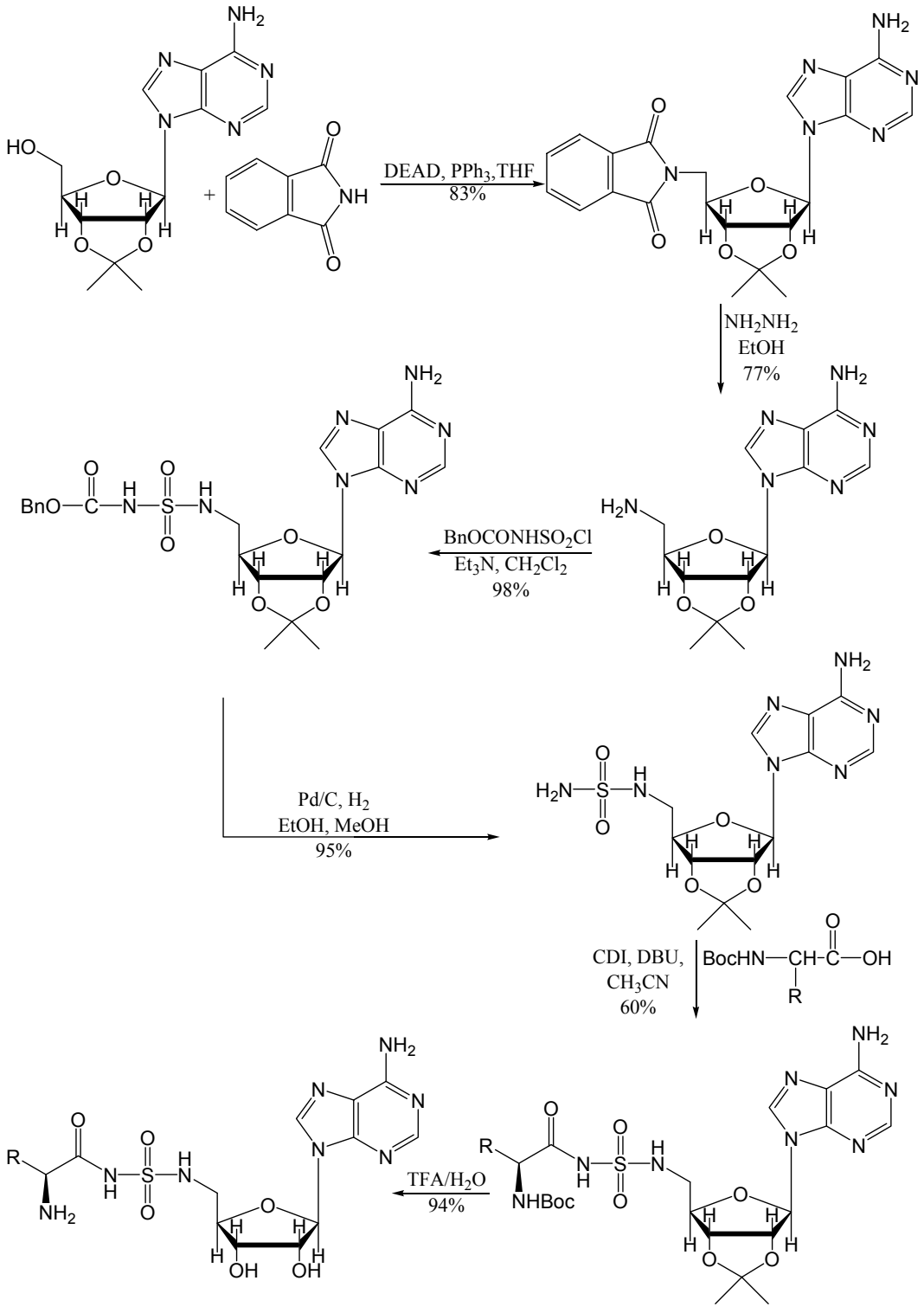
reaction and up to 0.7 mL of buffer were added. The samples were rotated at 4 ºC for 1.5 h and washed with buffer until the cpm of [^{35}S]Met were <500 in the wash. Immobilized [^{35}S]Met-biocytin-containing-globin was eluted by adding tricine loading buffer (80 mM Tris-Cl (pH 6.8), 200 mM DTT, 24% (v/v) glycerol, 8% sodium dodecyl sulfate (SDS), and 0.02 % (w/v) Coomassie blue G-250) (50 μL for each sample) directly to the streptavidin-agarose beads with heating at 90 ºC for 10 min. The amount of immobilized [^{35}S]Met-biocytin containing globin was determined by scintillation counting of the eluent (Figure 10).

Restoration of Translation Probed by Western Blot Analysis. A portion of each translation reaction (2 μL) was combined with 8 μL of tricine loading buffer, heated to 90 ºC for 5 min, and applied entirely to a 4% stacking portion of a 15% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose membrane in 1X transfer buffer w/ 10% MeOH (10X transfer buffer: Glycine 290g, Tris base 58g to 2L H_2O) using Miniprotean II wet transfer apparatus (Bio-Rad). The nitrocellulose membrane was blocked w/ 1% BSA in 1X PBS +0.1% Tween-20 for 1h and probed with Streptavidin-HRP conjugate (1:2000) for 1h. The membrane was washed four times with 30ml 1X PBS-T and visualized using ECL plus Western Blotting Detection System (Amersham Biosciences). Luminescence was then detected using Hyperfilm^{TM} (Amersham) with various exposure times.

Results

We generated various high-affinity inhibitors of the tRNA synthetases—aminoacyl sulfamides to create synthetic blanks in our translation extracts. We demonstrated that we can create and reprogram synthetic "blanks" in the genetic code. Aminoacyl tRNA synthetase inhibitors were combined with translation extracts and chemically aminoacylated orthogonal tRNAs to reprogram specific codons as desired.

A series of aminoacyl sulfamides were synthesized starting from 2', 3'-O-isopropylidene adenosine[21,22] (Scheme 2). 5'-hydroxy to amino group transformation was achieved by reacting with phthalimide followed by reducation using hydrazine. The first step reaction was catalyzed by Diethyl azodicarboxylate $EtO_2CN{=}NCO_2Et$ and triphenylphosphine. 5' free amino group reacted with N-Benzyloxycarbonylsulfamoyl chloride to afford protected aminoacylsulfamide. Deprotection using Pd catalyzed hydrogenation gave adenosine-5'-N-sulfamide which was coupled with Boc-protected amino acids catalyzed by CDI (carbonyldiimidazole) and DBU (diazabicycloundecene). After Boc protecting group was deprotected using TFA condition, the residue was triturated with anhydrous ether to give aminoacyl sulfamide as a white solid. There are additional procedures according to different amino acids with more complex side chains.



Scheme 2. Synthesis of aminoacyl sulfamides.

Addition of micromolar concentrations of synthetically constructed sulfamides allows us to specifically block the action of one or more synthetases as we choose. Inhibition is general to a variety of templates we tested, such as globin, Ubiquitin, and Ras mRNA templates (Figure 5). Clearly the potency of different aminoacyl sulfamides to inhibit protein translation varies according to the amino acid bearing and the mRNA template tested.

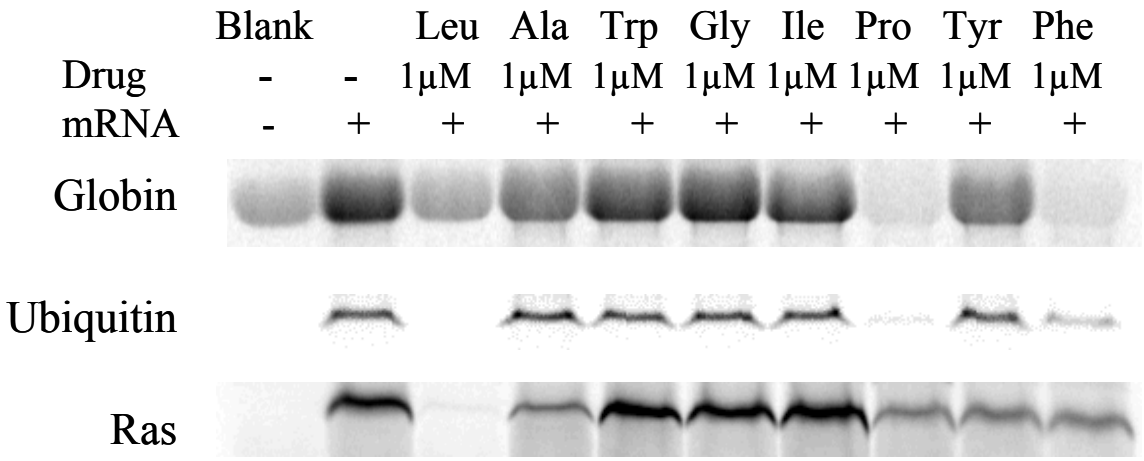
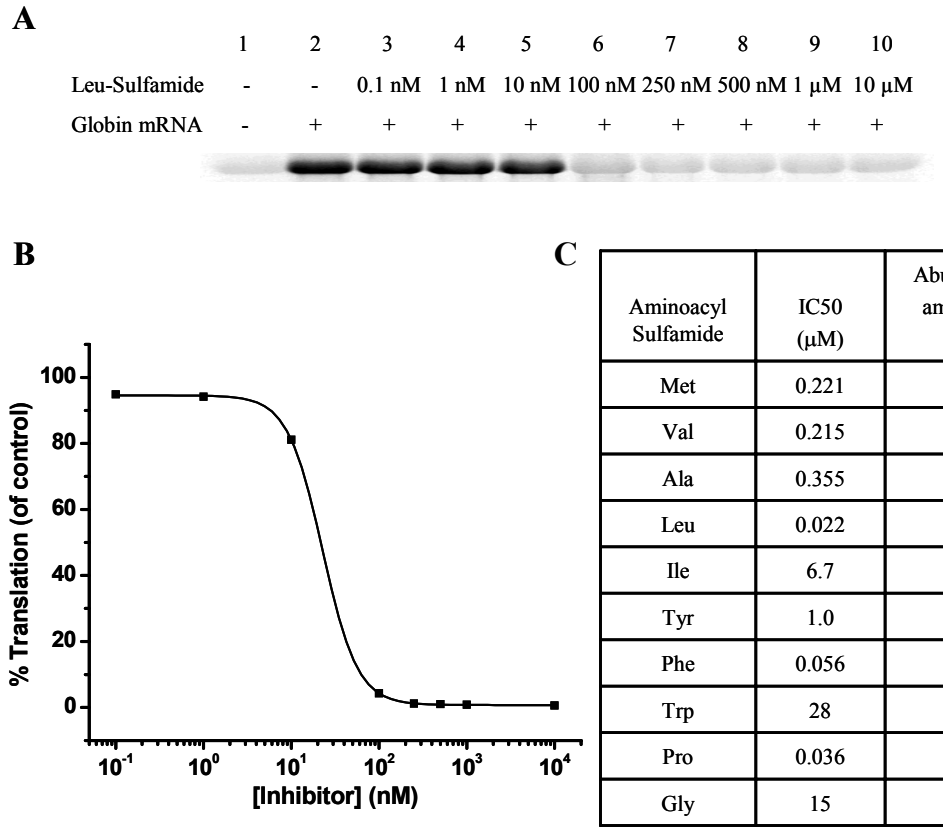


Figure 5. Inhibition of protein translation by aminoacyl-sulfamide. Tricine-SDS-PAGE analysis of [^{35}S]Met-protein translation reactions in the presence of 1 μM of aminoacyl-sulfamides.

We then measured the activity of each compound in a high dynamic-range IC_{50} potency assay using the rabbit reticulocyte protein synthesis system.[24] The various aminoacyl sulfamides inhibit globin translation with IC_{50} in the range of 22 nM to 28 μM (Figure 6). There appears to be a general correlation between the potency of the drugs and the amino acid abundance for a particular template. For example, Leu-sulfamide is a very potent inhibitor (IC_{50} 22 nM), whereas Ile-sulfamide is a poor inhibitor (IC_{50} 6.7 μM) for globin translation, and there are more positions coded for Leu (17/18) than Ile (3/1) in globin template. Presumably, the larger abundance of an amino acid in the template, the easier to inhibit the translation by shutting down the aminoacyl tRNA synthetase activity. All inhibitors are specific to corresponding aminoacyl tRNA synthetases due to the structural similarity to their corresponding aminoacyl adenylates.

A key issue is whether we can restore translation of globin by supplying aminoacyl tRNAs orthogonal to the codon blank created by the inhibitor. We tested the restoration of globin translation by biocytin-tRNAs coding for valine (Figure 7). We used an orthogonal tRNA charged with biocytin to eliminate it as a substrate for the cognate aminoacyl-tRNA synthetase and provide an easy handle for probing.



C

Aminoacyl Sulfamide	IC50 (μM)	Abundance of amino acids (α/β)
Met	0.221	1/2
Val	0.215	10/18
Ala	0.355	13/15
Leu	0.022	17/18
Ile	6.7	3/1
Tyr	1.0	3/3
Phe	0.056	8/8
Trp	28	1/2
Pro	0.036	7/4
Gly	15	9/11

Figure 6. Inhibition of globin translation by aminoacyl sulfamide. (A) Tricine-SDS-PAGE analysis of [^{35}S]Met-globin translation reactions in the presence of Leu-Sulfamide: Lane 1, no template; lane 2, globin alone; lanes 3-10, concentrations of aminoacyl sulfamide from 0.1 nM to 10 μM. (B) Percent globin translation relative to the no drug control for Leu-Sulfamide from gel analysis of (A). (C) IC_{50} values for various aminoacyl sulfamides.

The total globin translation level with the Val-sulfamide inhibitor and biocytin-tRNAs coding for valine is shown in Figure 7. At 1 μM of val-sulfamide, we were able to reduce the translation level to ~10% (lane 3). In the presence of biocytin-tRNAs, globin translation was restored to about 40% relative to no drug translation, depending on which biocytin-tRNAs were added (lane 4-6).

Biocytin-tRNA-CAC	-	-	-	4 μg	-	-
Biocytin-tRNA-GAC	-	-	-	-	4 μg	-
Biocytin-tRNA-AAC	-	-	-	-	-	4 μg
Val-Sulfamide	-	-	1 μM	1 μM	1 μM	1 μM
Globin mRNA	-	+	+	+	+	+

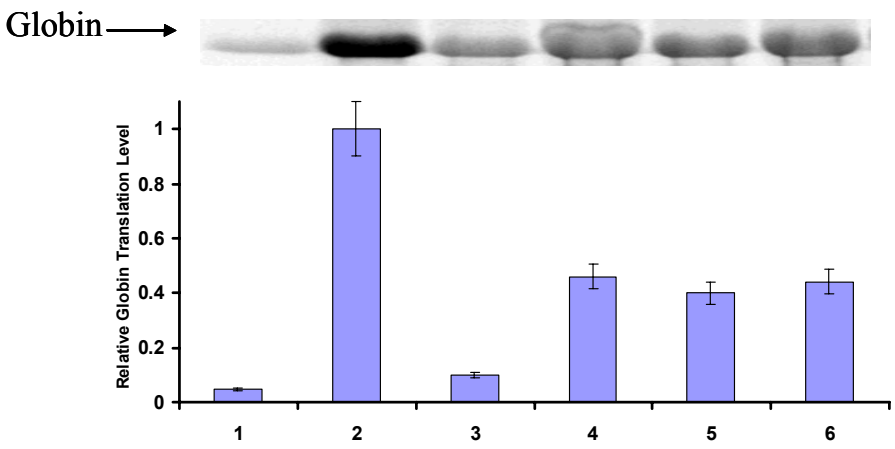


Figure 7. Globin translation restoration by biocytin-tRNA THG73 variant containing anticodon CAC, AAC or GAC in the presence of Val-sulfamide inhibitor: Lane 1, no template; lane 2, globin alone; lane 3, 1μM of val-sulfamide; lanes 4 - 6, biocytin-tRNA-CAC, GAC or AAC was added individually.

More important is the relative ratio of biocytin incorporation to total translation level. Here, specific biocytin incorporation was probed by Western blot (Figure 8). In α-globin,

there are 10 valine codons, all are GUG (anticodon CAC); in β-globin, there are 18 valine codons, 12 of them are GUG, and 2 GUC (anticodon GAC) and 4 GUU (anticodon AAC), no GUA. The globin bands may represent heterogeneous populations of biocytin incorporation and the more abundant codon sites (lane 4, CAC) are over-represented than less abundant codons (lane 6 GAC, lane 7 AAC). Also when we use biocytin–tRNA with stop codon UAG (lane 5, anticodon CUA), there is no significant biocytin incorporation, and this demonstrates that the Val sulfamide inhibitor shows specificity toward the corresponding Val synthetase due to the structural similarity.

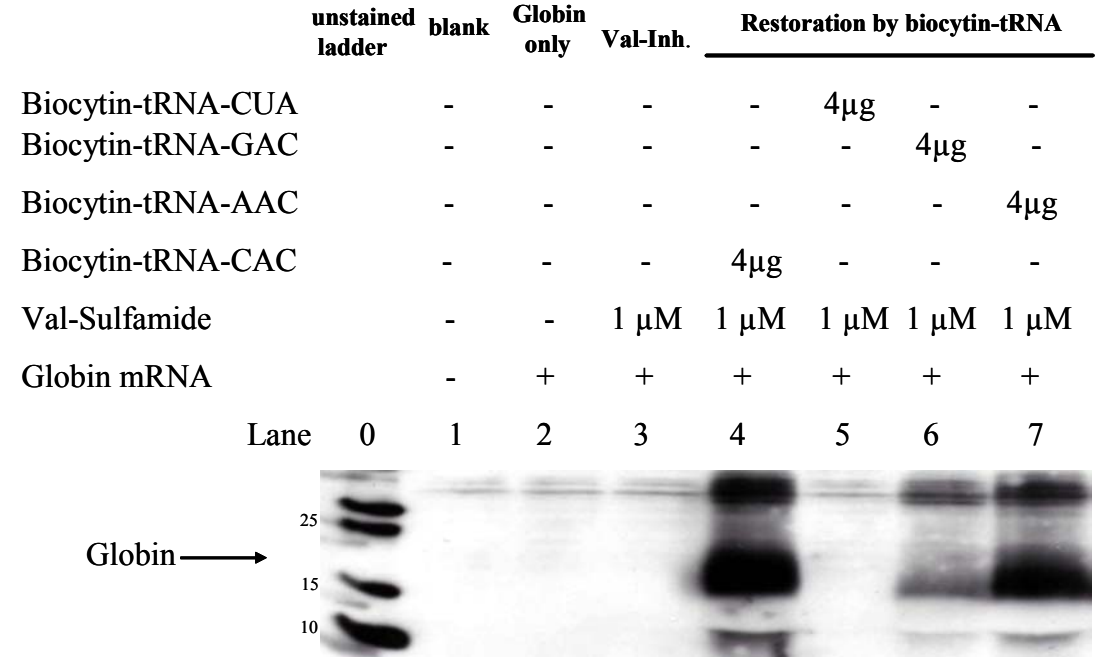


Figure 8. Globin Translation Restoration probed with Western blot. Lane 0, precision protein standard unstained; lane 1, no template; lane 2, globin alone; lane 3, 1μM of val-sulfamide; lane 4, biocytin-tRNA-CAC (Valine codon GUG); lane 5, biocytin-tRNA-CUA (stop codon UAG); lane 6, biocytin-tRNA-GAC (Valine codon GUC); lane 7, biocytin-tRNA- AAC (Valine codon GUU) was added individually.

We further compared biocytin incorporation level in the presence and absence of Val-sulfamide. As shown in Figure 9, biocytin incorporation level in the presence of the inhibitor (lane 6 and 7) is higher than that in the absence of the inhibitor (lane 4 and 5). This suggests that the presence of the inhibitor provides enhancement of unnatural amino acids incorporation.

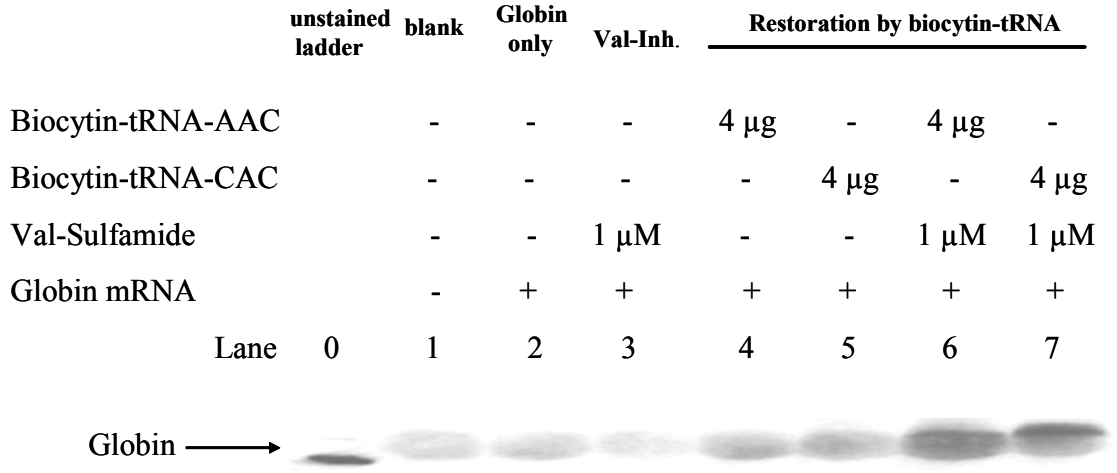


Figure 9. Globin Translation Restoration probed with Western blot in the presence of inhibitor and in the absence of inhibitor. Lane 0, precision protein standard unstained; lane 1, no template; lane 2, globin alone; lane 3, 1 μM of Val-sulfamide; lane 4, biocytin-tRNA-AAC was added without the inhibitor; lane 5, biocytin-tRNA-CAC was added without the inhibitor: lane 6, biocytin-tRNA-AAC in the presence of 1 μM of Val-sulfamide; lane 7, biocytin-tRNA-CAC in the presence of 1 μM of Val-sulfamide.

We also tested the restoration of globin translation by biocytin-tRNAs coding for valine probed with Streptavidin-capture of biocytin-containing globin. [^{35}S]Met-biocytin-containing-globin was immobilized to streptavidin-agarose beads and then eluted by adding tricine loading buffer directly to the streptavidin-agarose beads with heating at 90 ºC for 10 min. The amount of immobilized [^{35}S]Met-biocytin containing globin was determined by scintillation counting of the eluent (Figure 10). This also demonstrated that in the presence of biocytin-tRNAs, globin translation was restored to about 35-40% relative to no drug translation.

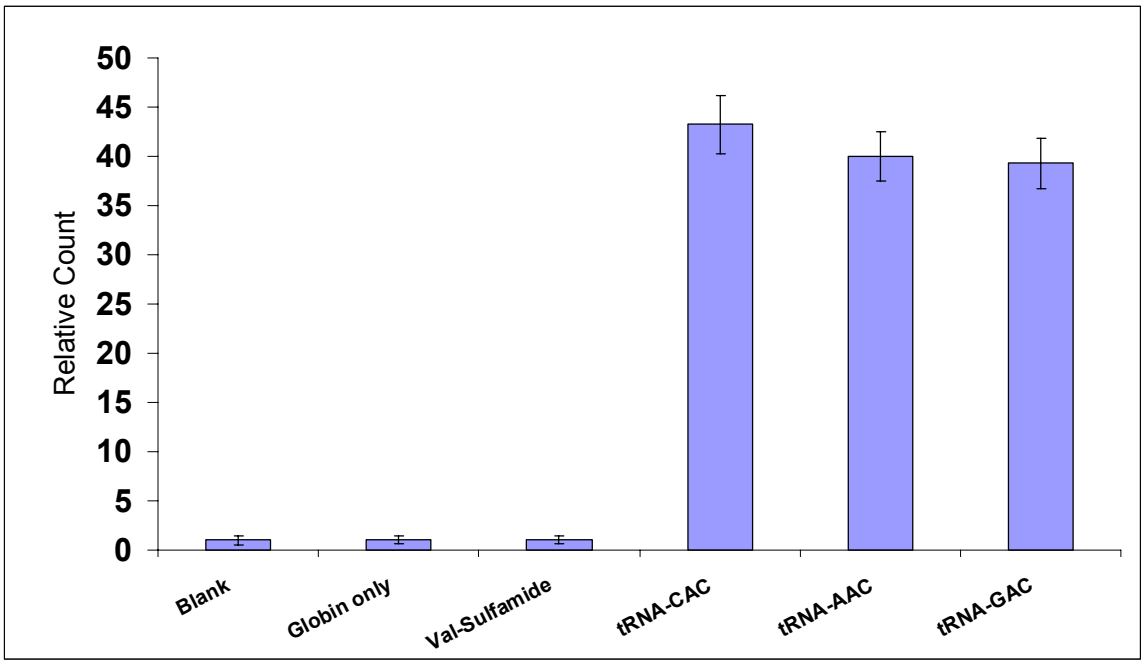


Figure 10. Restoration of translation probed with streptavidin-capture of biocytin-containing globin.

Discussions

tRNA synthetase inhibitors enhance In Vitro suppression

In this work, we prepared a series of aminoacyl-adenylate analogues and evaluated their potency for inhibition of protein translation. Further, we characterized the ability of aminoacyl sulfamides to facilitate incorporation of an unnatural amino acid, biocytin, into globin protein. This approach provides a facile means to rewrite the universal genetic code at will, without extensively re-engineering cells. Therefore, this work provides an experimental means to pursue fundamental questions relating to the construction, functional diversity, and organization of the genetic code (Figure 11).

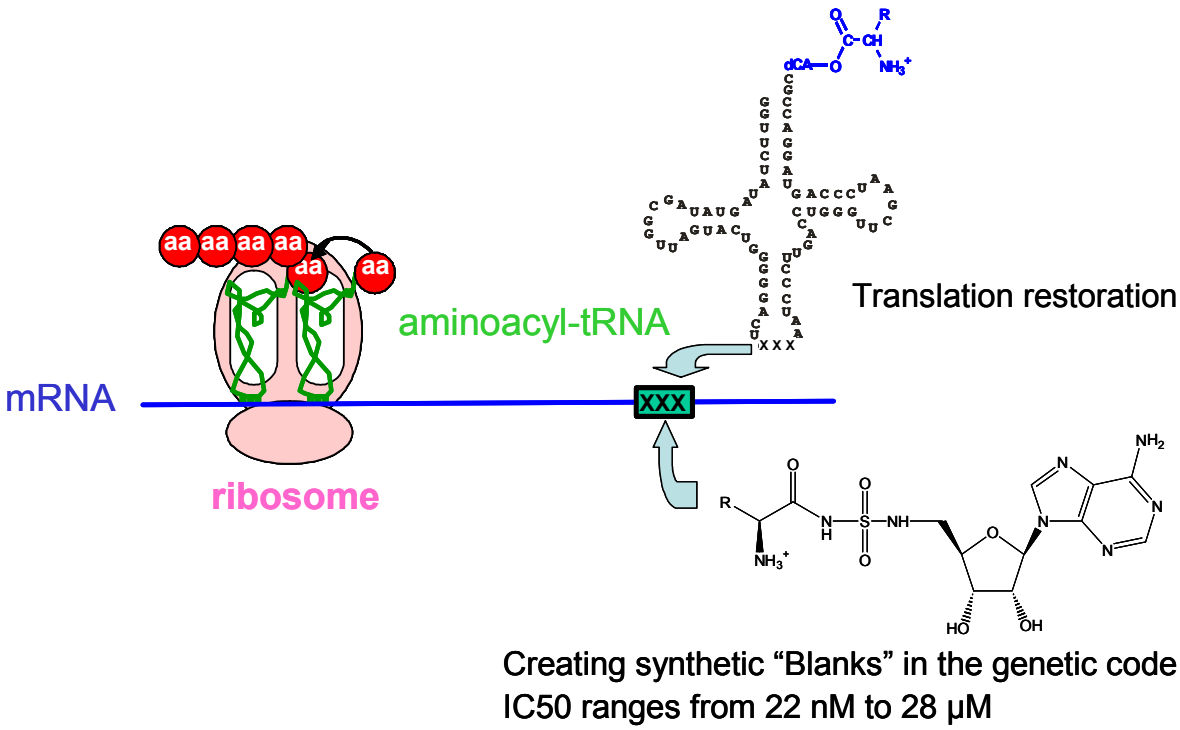


Figure 11. Enhance *In Vitro* suppression by tRNA synthetase inhibitors.

Uniting mRNA display, In Vitro Suppression and tRNA synthetase inhibitors

The incorporation of unnatural amino acid into selectable, amplifiable peptide and protein libraries expands the chemical diversity of such libraries, thus considerably facilitating the process of obtaining ligands with improved properties (affinity, specificity, and function), particularly against therapeutically interesting targets. Here we also demonstrated that sense codon suppression was enhanced by tRNA synthetase inhibitors. To apply this unnatural strategy to selection cycle via mRNA display library, we can facilitate the enrichment of desired unnatural amino acid residue and speed up the selection process (Figure 12).

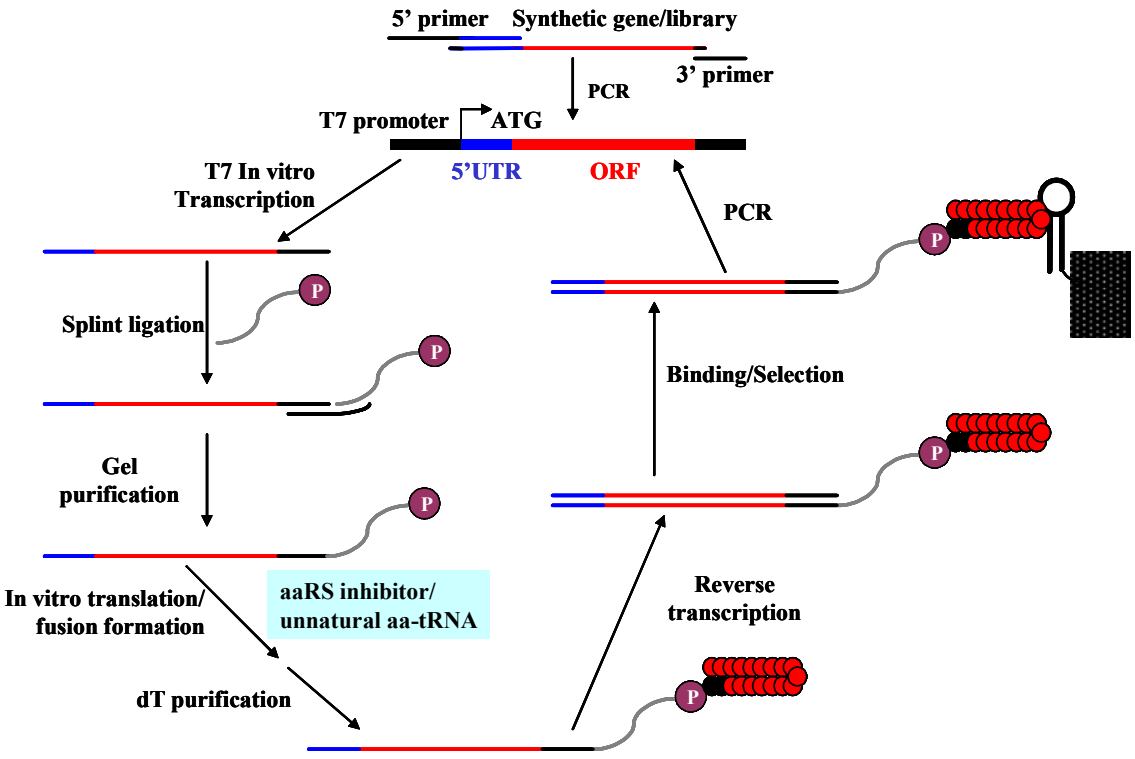


Figure 12. Selection cycle via mRNA display enhanced by tRNA synthetase inhibitor.

Conclusions

We generated various high-affinity inhibitors of the tRNA synthetases—aminoacyl sulfamides to create synthetic blanks in our translation extracts. Aminoacyl tRNA synthetase inhibitors were combined with translation extracts and protein translation was shown to be inhibited. We then measured the activity of each compound in a high dynamic-range IC_{50} potency assay using the rabbit reticulocyte protein synthesis system. The various aminoacyl sulfamides inhibit globin translation with IC_{50} in the range of 22 nM to 28 μM.

We further demonstrated the restoration of globin translation by biocytin-tRNAs coding for valine in the presence of Val-sulfamide inhibitor. Here, specific biocytin incorporation was probed by Western blot, and biocytin incorporation level in the presence of inhibitor is higher than that in the absence of inhibitor. This suggests that the presence of inhibitor provides enhancement of unnatural amino acids incorporation.

In this work, we demonstrated that we can create and reprogram synthetic "blanks" in the genetic code. Most importantly, we can facilitate the enrichment of desired unnatural amino acid residue and speed up the selection process when we unit mRNA display, *in vitro* Suppression and tRNA synthetase inhibitors.

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