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Genetic Code Expansion using Aminoacylated Orthogonal tRNAs in Conjunction with Aminoacyl Sulfamides

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Abstract: ***Re-engineering the protein synthesis apparatus is a powerful approach to expand our understanding of the central macromolecular synthesis machinery present in all cells. In the current study, we showed that the high-affinity inhibitor—aminoacyl sulfamide significantly inhibited the activity of specific aminoacyl tRNA synthetase (aaRS), thereby creating synthetic gaps in the genetic code that can be filled with chemically aminoacylated orthogonal tRNAs. We further demonstrated the restoration of globin translation and enhancement of unnatural amino acids incorporation by biocytin-tRNAs coding for valine in the presence of Val-sulfamide. Taken together, this study demonstrates that we can create and reprogram synthetic "gaps" in the genetic code and provides an experimental means to pursue fundamental questions relating to the construction and expansion of the genetic code to facilitate the enrichment of desired unnatural amino acid residues.***

Index Terms**: *Aminoacyl sulfamids, Aminoacylated orthogonal tRNAs, Genetic code expansion.***

I. INTRODUCTION

In all living organisms, aminoacyl tRNA synthetases (aaRSs)–the gatekeepers of the genetic code play a key role in protein biosynthesis by catalyzing the esterification of amino acids to cognate tRNAs in a two-step process (Figure 1A). Recent studies further illustrated the connection of new domains of tRNA synthetases to new functions [1]. As proof of principle, the evolutionary addition of amino acid editing domain CP1 to the class I enzyme enhances fidelity of the synthetic aminoacylation reaction for tRNA synthetases [2]. Furthermore, aminoacyl-tRNA synthetases have drawn interest as potential targets for the development of new antibiotics due to the differences between the active sites of the bacterial and eukaryotic enzymes [3]. In light of the versatile and modular functions of orthogonal tRNA/ aaRS pair, the protein translation machinery has been evolutionary optimized due to role of tRNA orthogonality [4].

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Enhanced understanding of the expanding roles of tRNA synthetases in disease, together with the increasing knowledge of genetic difference among different kingdoms of life, have provided new opportunities for expanding genetic code [5]. Incorporation of unnatural amino acids into peptides and proteins has been extensively employed for mechanistic studies of protein function. The suppression technique, using either chemically misacylated tRNAs or unnatural amino acids incorporation by nonsense stop codon suppression [6-13]has been applied for sense codon silencing both *in vitro* [14-18] and *in vivo* [19-21]. Since tRNA synthetases could directly link to the tumorigenesis as a result of their non-canonical functions in angiogenesis, immune responses and signal transduction pathways [22], tRNA synthetase inhibitors is an ideal model system for developing new anticancer agents leading to potential application in regulating oncoprotein expression and modification for anti-cancer therapeutics.

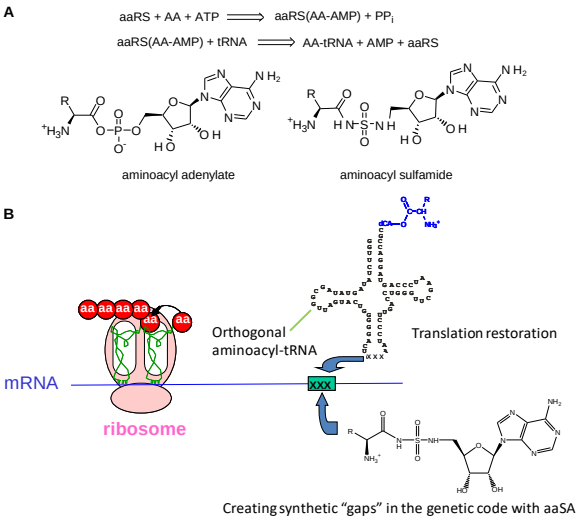

Figure 1. (A) Aminoacylation of tRNA is catalyzed by aaRSs in a two-step reaction. (B) "Inhibition and restoration" suppression approach.

All aaRSs act by using ATP to generate an activated form of an amino acid–a mixed carbon-phosphorus anhydride termed aminoacyl adenylate (aaAMP). Aminoacyl sulfamides (aaSAs) are stable structural mimics of aaAMPs and generally have sub-micromolar K_i values for their corresponding synthetases [23,24].

Importantly, since the reacylation of the residue-specific endogenous tRNA is inhibited by aaSA, the genetic code could be manipulated by chemically acylated orthogonal tRNAs, rather than by the endogenous aaRSs (Figure 1B) [25,26]. In addition to non-sense and sense codon suppression in protein selection [15,16], we envision that these aaRS inhibitors may facilitate unnatural amino acid incorporation by sense codon suppression for novel protein construction. To enhance the efficiency of sense codon suppression and to effectively compete with endogenous tRNAs, we designed an *in situ* "inhibition and restoration" strategy. We first eliminated the activity of specific aaRSs using aaRS inhibitors–aminoacyl sulfamides since aaRSs are pivotal in determining how the genetic code is interpreted into amino acids. Once we created sense codon gaps in the genetic code, we reprogrammed specific codons as desired by supplying chemically aminoacylated orthogonal tRNAs in combination with translation extracts. In this study, we demonstrated the use of aminoacylated orthogonal tRNAs in conjunction with aminoacyl sulfamides to incorporate unnatural amino acid residues to expand genetic code.

II. Experimental

A. General information

^{1}H and ^{13}C NMR spectra were recorded on an Oxford Activated Shield NMR instrument (Varian, Inc.) operating at 400 MHz for ^{1}H, and 100 MHz for ^{13}C using D_2O or DMSO-*d6* as the solvent. High-resolution mass spectroscopic data were acquired on an Agilent 6220 ESI-TOF with electro spray ionization (ESI) mode (Mass Spectrometry Laboratory, University of Florida). 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. Triphenylphosphine, phthalimide, 2',3'-O-isopropylideneadenosine, hydrazine, benzyl alcohol, diethyl azodicarboxylate, chlorosulfonyl isocyanate, Pd 10% on activated carbon, 1,1'-carbonyldiimidazole (CDI) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) 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(tBu)-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 cDNA was PCR amplified 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). mRNA was produced by T7 runoff transcription 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 [27]. 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.

B. 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 [28]. It was converted to 5'-deoxy-2', 3'-O-isopropylideneadenosine-5'-N-[(phenylmethoxy)carbonyl]-sulfamide treated with N-carbobenzyloxysulfamoyl chloride and a base such as triethylamine in dichloromethane at 0 °C to ambient room temperature. Cbz-protected sulfamide was deprotected by using Pd 10% on activated carbon and hydrogen gas in EtOH to afford 5'- deoxy-2',3'-O -isopropylideneadenosine -5'-N-sulfamide, which was further reacted with Boc-protected amino acids activated by CDI and DBU in dry acetonitrile. The obtained 5'-deoxy-2',3'-O-isopropylideneadenosine-5'-N-(N-tertbutoxycarbonyl-aminoacyl) sulfamides purified by column chromatography were fully deprotected according to standard procedures and purified by trituration [29].

C. 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 uL of each reaction was combined with 8 uL 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 1.5 h) [10]. 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 of mRNA templates was tested in a similar condition (Figure 2A).

D. Restoration of translation probed by Western blot analysis.

An aliquot 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.

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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™ (Amersham) with various exposure times.

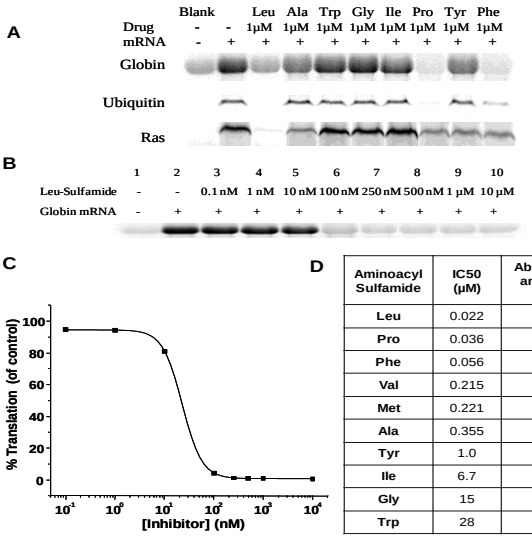
E. Restoration of translation probed with streptavidin-capture of biocytin-containing globin.

A portion of streptavidin-agarose (0.75 mL) [Pierce, 50% slurry (v/v)] was washed 3 times with buffer (1X PBS containing 0.1% Triton X-100) and resuspended in 0.75 mL 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.

III. Results & Discussion

A. Inhibition of protein translation by aminoacyl sulfamides

We designed and synthesized a series of aaSAs [28,29] and demonstrated that the addition of micromolar concentrations of synthetically constructed sulfamide specifically blocked the synthetase activity. First, we evaluated their ability to inhibit protein synthesis in a rabbit reticulocyte protein synthesis system. Inhibition was observed, irrespective of the mRNA templates tested, including globin, Ras, and ubiquitin (Figure 2A). We next measured the activity of each compound in a high dynamic-range IC_{50} potency assay [27,30]. The various aminoacyl sulfamides inhibited globin translation with IC_{50} in the range of 22 nM to 28 µM (Figure 2B-D). The potency of the drugs appeared to correlate with the amino acid abundance for a particular template. For example, Leu-sulfamide and Ile-sulfamide showed distinct inhibition of globin translation, with IC_{50} at 22 nM and 6.7 µM respectively. It is conceivable that the abundance of an amino acid in the template may be a factor for specific inhibition of translation by shutting down the aaRS activity.



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

Figure 2. (A) Inhibition of protein translation by aminoacyl sulfamides. (B) Tricine-SDS-PAGE analysis of [35S]Met-globin translation reactions in the presence of Leu-sulfamide as an example: Lane 1, no template; lane 2, globin alone; lanes 3-10, varying concentrations of aminoacyl sulfamide from 0.1 nM to 10 µM. (C) Percent globin translation relative to the no drug control for Leu-sulfamide from gel analysis of (B). (D) IC50 values for various aminoacyl sulfamides.

B. Restoration of protein translation by exogenous orthogonal aminoacyl tRNAs

Several crystal structures provided mechanistic insights and illustrated the structural basis of inhibition by Val-sulfamide [31,32]. A key issue here is whether we can restore protein translation by supplying aminoacyl tRNAs orthogonal to the codon gaps created by the inhibitors. To examine this possibility, we assayed the restoration of globin translation by using an orthogonal valine-coding tRNA THG73 that is charged with biocytin for easy probing (Figure 3). The total globin translation level with or without the Val-sulfamide inhibitor and/or orthogonal biocytin-tRNAs coding for valine is shown in Figure 3. There are four sets of codons coded for valine: GUG, GUC, GUU and GUA. All 10 valine codons in α-globin are GUG (anticodon CAC); among 18 valine codons in β-globin, there are 12 GUG, 2 GUC (anticodon GAC), 4 GUU (anticodon AAC), and no GUA. At 1 µM of Val-sulfamide, we were able to reduce the translation level to ~10% (lane 3). In the presence of biocytin-tRNAs and Val-sulfamide, globin translation was restored to about 40% relative to the no drug control, with only subtle differences among the biocytin-tRNAs added (lane 4-6).

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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	-	+	+	+	+	+

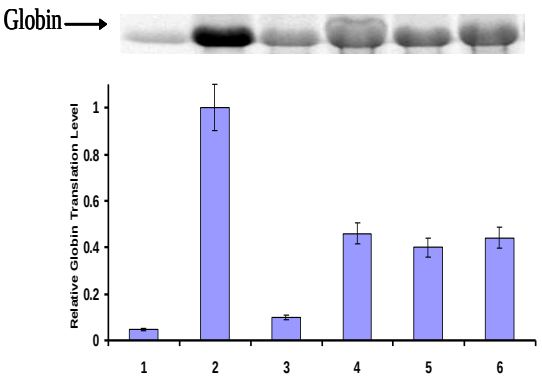

Figure 3. Restoration of globin translation by biocytin-tRNA THG73 variant containing anticodon CAC, GAC or AAC in the presence of Val-sulfamide inhibitor: Lane 1, no template; lane 2, globin alone; lane 3, 1 µM of Val-sulfamide; lanes 4-6, biocytin-tRNA-CAC, GAC or AAC was added individually.

The observed globin in Figure 3 likely corresponded to heterogeneous populations with biocytin incorporation. To evaluate the restoration directly from biocytin-tRNA, an important parameter is the relative ratio of biocytin incorporation vs. total translation level. Here, specific biocytin incorporation was probed by Western blotting (Figure 4A). A positive correlation was found between the more abundant codon sites (lane 4, CAC) and the less abundant codons (lane 6 GAC, lane 7 AAC). Moreover, when we used a biocytin–tRNA with the stop codon UAG (lane 5, anticodon CUA), no significant biocytin incorporation was observed, demonstrating that the Val sulfamide inhibitor specifically inhibited the corresponding Val synthetase due to structural similarity. Next, when comparing biocytin incorporation level in the presence and absence of Val-sulfamide, elevated biocytin incorporation level was observed in the presence of the inhibitor (lane 6 is higher than lane 4 and lane 7 is higher than lane 5, respectively. Figure 4B), showing that a stimulatory role of the inhibitor in unnatural amino acids incorporation.

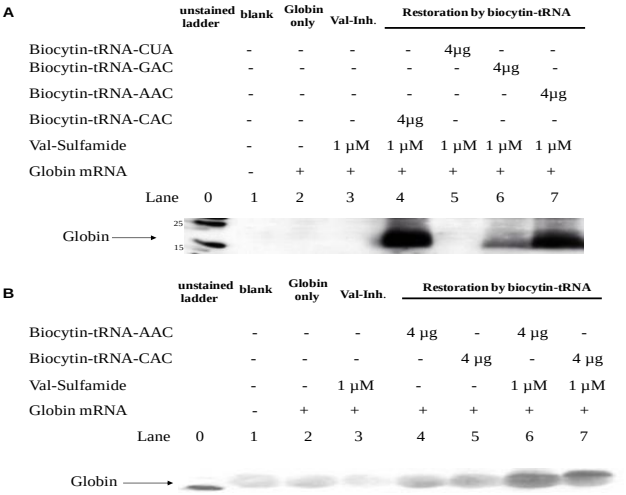

Figure 4. (A) Western blot analysis of restoration of globin translation. Lane 0, precision protein standard unstained; lane 1, no template; lane 2, globin alone; lane 3, 1 µM of val-sulfamide; lane 4, biocytin-tRNA-CAC (Valine codon GUG); lane 5, biocytin-tRNA-CUA (stop codon UAG); lane 6, biocytin-tRNA-GAC (Valine codon GUC); lane 7, biocytin-tRNA-AAC (Valine codon GUU) was added individually. (B) Western blot analysis of restoration of globin translation in the presence and 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 and 6, biocytin-tRNA-AAC was added in the absence and presence of 1 µM of Val-sulfamide; lane 5 and 7, biocytin-tRNA-CAC was added in the absence and 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. The amount of immobilized [^{35}S]Met-biocytin containing globin was determined by scintillation counting of the eluent (Figure 5). This also demonstrated that in the presence of biocytin-tRNAs, globin translation was restored to about 40% relative to no drug control that is consistent with specific biocytin incorporation probed by Western blot (Figure 3).

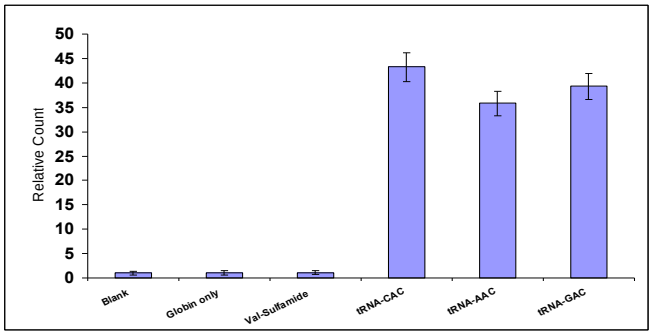

Figure 5. Restoration of translation probed with streptavidin-capture of biocytin-containing globin.

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IV. CONCLUSION

In summary, we prepared a series of aaSAs analogous to aminoacyl-adenylates. We evaluated their potency for inhibition of protein translation. In particular, we demonstrated the restoration of globin translation and enhancement of unnatural amino acids incorporation by biocytin-tRNAs coding for valine in the presence of Val-sulfamide. Collectively, our observations demonstrate that we can create and reprogram synthetic “gaps” in the genetic code. This approach provides a facile means to manipulate the universal genetic code at will, without extensively re-engineering cells. Therefore, this work illustrates an experimental means to pursue fundamental questions in relation to the construction, functional diversity, and organization of the genetic code. Furthermore, our approach allows more powerful construction of peptide and protein libraries containing much more expanded repertoire of unnatural amino acids.

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