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Effects of Conformational Change on the Angiogenic Activity of Threonyl-tRNA Synthetase

Defense presented by: Khadar Abdi

Performed at the University of Vermont in College of Arts and Science

In partial fulfillment of the requirements for College Honors
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List of Abbreviations

$A_{280}$-Absorbance at 280nM

aaRS-Aminoacyl-tRNA synthetases

B-ME- β-mercaptoethanol

BN- Borrelidin

EBM-Endothelial Basal Media

EGM-Endothelial Growth Media

*E. Coli- Escherichia coli*

HUVECs- Human umbilical vascular endothelial cells

PBS-Phosphate buffered saline

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TARS-Threonyl-tRNA synthetase

VEGF-Vascular Endothelial Growth Factor
Abstract

Aminoacyl-tRNA synthetases (aaRs) have been known for their importance in protein synthesis. However, the Francklyn/Lounsbury lab discovered that some aaRs, like tyrosyl-tRNA synthetase and tryptophanyl-tRNA synthetase are linked to angiogenesis. Recently, the lab discovered that threonyl-tRNA synthetase (TARS) was an angiogenic factor that is linked to ovarian cancer. Studies have shown that a derivative of macrolide inhibitor Borrelidin, known as BC194, inhibits TARS angiogenic and functional activity and altering TARS conformation. Sites of BC19 were found and used to generate mutated version of human TARS. Q566W and H388A TARS mutations were investigated to see whether induced conformational change or loss of aminoacyl-transferase activity is the key to inhibit angiogenic activity respectively. Q566W and H388A plasmids were generated using site-directed mutagenesis. The plasmids were expressed in Escherichia coli cells and purified using sonication, protamine sulfate salting, His6tag affinity chromatography and Hydroxyapatite chromatography. Purified mutants were found to have a molecular weight of 83kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Following purification, TARS enzymes were applied to the in vitro tube assay using human umbilical vein endothelial cells to test angiogenic activity. Q566W promoted a similar number of pieces and branches as wild type TARS, while H388A had the greatest amount tube pieces, branches, and branching length. The study didn’t find a difference in angiogenic activity between the wild type and mutant TARS. However, the study shows that TARS has dose-dependent angiogenic activity and may link to α5β3 integrin migration signaling. More studies on angiogenic activity using integrin inhibitor dose response in vitro tube assay under TARS are needed to verify this mechanism.
I. Introduction

The primary function of aminoacyl-tRNA synthetases (aaRS) is to transfer a specific amino acid to an uncharged tRNA containing the corresponding anticodon, which is necessary for protein synthesis (Ibba & Soll, 2000). AaRSs perform this mechanism in a two-step process: 1) Adding an adenosine monophosphate (AMP) to the amino acid with adenosine triphosphate 2) Exchange of AMP with tRNA\textsuperscript{AA} (with AA meaning the specific amino acid).

Interestingly, studies have linked some human diseases to mutation in aaRS. For instance, mutant forms of tyrosyl-tRNA synthetase (YARS) were found to be linked to Charcot-Marie-Tooth, a peripheral neuropathy disorder causing axonal degeneration starting at upper and lower extremities and spreads toward the body (Jordanova et al., 2006). But more surprising was the idea that aaRSs are connected with angiogenesis (Keisuke Wakasugi & Schimmel, 1999).

Angiogenesis, development of new blood vessels from pre-existing ones, is an important part of the developing tumor microenvironment (Cassavaugh & Lounsbury, 2011). Tumor microenvironments are necessary for survival, growth, and proliferation of cancer cells, as seen in ovarian cancer (Wellman et al., 2014). The normal process of angiogenesis includes endothelial cell migration that consists of cellular movement involving rearrangement of actin filaments resulting in stretching the endothelial cells into a leading tip (Michaelis, 2014).

Endothelial cell migration during angiogenesis occurs by Vascular Endothelial Growth Factor receptor 2 (VEGFR2) signaling mechanism (Lamalice, Le Boeuf, & Huot, 2007). First, VEGFR2 activates cdc42, a Rho-GTPase protein, that stimulates the formation of actin stress fibers that breakthrough the cell in the direction of VEGF gradient. Phosphoinositide 3-kinase signaling then stimulates the stretch of the endothelial cell, while attachment of the endothelial cell’s focal adhesion occurs. The focal adhesion’s attached stress fibers then contracts due to
stimulation of the Rho kinase pathway. Finally, integrin-focal adhesion kinase inside-out signaling occurs to release focal adhesions from the fibronectin extracellular matrix. This pathway is dynamic as the VEGFR2 interacts with α5β3 integrin receptor, another receptor that stimulates endothelial cell migration through regulating VEGFR2 signaling and stimulating assembly of focal adhesion by activating focal adhesion kinase.

Experiments carried out on Class 1 aaRSs, with a monomeric structure, such as YARS and tryptophanyl-tRNA synthetase have shown that these enzymes have angiogenic and angiostatic properties affecting VEGF migration pathway (K. Wakasugi, 2002). The phenomenon of regulation of angiogenesis in both enzymes was found to occur through proteolysis of YARS and WARS, which a portion of the enzyme acts as a signal molecule to activate/inhibit endothelial cell migration through inhibition of ERK (K. Wakasugi, 2002; Keisuke Wakasugi & Schimmel, 1999). These experiments opened the possibility that aaRs enzymes may have important roles in angiogenesis that are outside of their housekeeping function. This led to investigation on bacterial threonyl-tRNA synthetase (TARS) inhibitor, borrelidin (BN), as a potential antiangiogenic agent as it was discovered to suppress metastasis in mouse models (Funahashi et al., 1999).

BN was isolated from *Streptomyces* species and was identified to bind to bacterial TARS in *Escherichia coli* (*E. coli*) cells (Olano et al., 2004; Paetz & Nass, 1973). Studies on BN resistant hamster ovary cells showed that BN also targeted eukaryotic cells, which suggest that angiogenesis occurs through TARS (Gerken & Arfin, 1984). However, BN is unable to examine physiological activity as its cytotoxic in normal epithelial cells. Variants of BN varying C17 side chain, such as BC194, were developed to reduce the cytotoxic effect (Wilkinson et al., 2006).
The Lounsbury/Francklyn lab became intrigued about the antiangiogenic effect of BN after recently observing secretion of TARS in endothelial through VEGF signaling in ovarian cancer (Tamara F. Williams, Adam C. Mirando, Barrie Wilkinson, Christopher S. Francklyn, & Karen M. Lounsbury, 2013). Recently, *in vitro* angiogenic assays studies showed that extracellular TARS increases the amount of blood vessel branching. This finding led to the theory that TARS acts as an extracellular signal to stimulate endothelial cell migration.

Recent crystallization studies on TARS bound to BC194 and BN have shown to interact hydrophobic interaction in the catalytic site of TARS and inducing an open conformation (Adam C. Mirando et al., 2015). Theories from these studies suggest that the conformational change of TARS is the key to the inhibition of angiogenic activity. However, it’s not fully understood whether inducing conformation of TARS or inhibiting the amino-transfer reaction is effective way to reduce angiogenesis.

Current studies turn towards generating BN resistant TARS mutants to compare level of angiogenic activity (Adam C. Mirando et al., 2015). Most mutant TARS that have the same open-lock conformation but still present catalytic activity can be formed by changing an amino acid within the binding pocket to a bulky amino acid, like tryptophan. Examining the clef structure of TARS showed that Q566 site is a potential area of inducing the open conformation without losing any function of the enzyme (Fang et al., 2015).

At the same time, studies looked at generating BN resistant TARS that had loss of aminoacyl-transfer activity. Kinetic analysis had showed that H309 on bacteria TARS (H388 in human) had low level of aminoacyl transfer after converting site to alanine (Minajigi & Francklyn, 2008). This possible target as H388 is also an amino acid that is known to bind to both BN and BC194 (Figure 1).
In this study, mutants Q566W and H388A wild type human TARS were analyzed to see any changes in angiogenesis activity. These mutants were generated from *E. coli* to see whether inducing conformational change or inhibition of TARS catalytic activity reduces angiogenic activity respectively. To investigate whether the mutation site affected the angiogenic activity, tube assay formation was used (DeCicco-Skinner et al., 2014). The technique mimics the formation of blood vessels by utilizing human umbilical vascular endothelial cells (HUVECs). The cells grow under negative control media, positive control media, and low concentration of

**Figure 1.** Crystal structure identified amino acids within TARS that bind to the angiogenic inhibitor BC194. Interaction shown by dotted lines. From Adam C. Mirando et al., 2015.
WT, Q566W, and H388A TARS enzymes, and measures the number of tubes formed by using the plugin Angiogenesis Analyzer on ImageJ.

The mutants in this study were successfully purified and tested in the angiogenesis assay. Thus far, Q566W mutant didn’t change the level of tube production while H388A had high levels of activity. This shows support for the idea that neither changing conformation nor removing catalytic function reduces angiogenetic activity. However, the study found that TARS angiogenic activity is dose-dependent, as TARS had low level of activity at 200 nM than 100nM. This influences the comparison tube assay results to have no difference of activity. This is similar to studies on dose response angiogenic activity on integrin inhibitors. Future studies on Q566W and H388A TARS mutants as well as integrin inhibitor dose response under 100nM TARS should be investigated using in vitro tube formation assay.
II. Materials and Methods

Generating mutant forms of TARS:

N-terminal His$_6$-tagged human wildtype TARS sequence was purified using the QIAprep$^\text{®}$ spin miniprep kit (Qiagen). Plasmid DNA concentration was recorded at an absorbance at 260nm provided by UVM DNA Analysis facility’s Nanodrop.

Purified TARS plasmid was mutated at the desired site using the QuickChange II Mutagenesis kit provided by Agilent Technologies. The mutant DNA plasmid was inserted into XL-10 competent blue cells (Agilent) and JM109 cells (Promega). These cells were selected in the presence of Kanamycin and Chloramphenicol. Bacteria colonies were harvested for mutant DNA strains using QIAprep$^\text{®}$ spin miniprep kit (Qiagen). Purified mutant genes were verified using the University of Vermont’s (UVM) DNA sequencing facility. Validated mutated genes were inserted into *E. coli*. Rosetta$^{\text{TM}}$ 2(DE3) pLac cells for expression and purification. Mutants Q566W and H388A were developed from this method.

Purification of TARS:

Purification of wild type and mutant versions of human TARS were performed by techniques described in Mirando et al. 2015. Transformed *E. coli* cultures, containing TARS plasmids with recombinant His$_6$-tag gene, were grown in LB containing 10 ug/ml Kanamycin and 10 ug/ml Chloramphenicol and were shaken at 37 °C until cell density reached 0.6 at 600nM. Expression of TARS was induced with 5mM isopropyl-1-thio-β-D-galactoside at 18 °C overnight.

After bacterial expression, the *E. coli* culture was subjected to centrifugation at 7000 rpm for 10 min at 4 °C. The *E. coli* cell pellet was resuspended in sonication buffer (cold 20 mM KH$_2$PO$_4$, 100 mM KCl, 35 mM Imidazole, 5 mM β-mercaptoethanol (B-ME)) and lysed by
sonication. The sonicate was spun at 11,000 rpm for 30 minutes. The lysate was saturated to 0.1% Protamine Sulfate to precipitate DNA plasmid, followed by centrifugation using the condition described above.

TARS supernatant was loaded onto a 5 mL HisTrap™ FF column (GE Healthcare) equilibrated with the sonication buffer and run at a flow rate of 0.75 mL/min. HisTrap column was washed using the same sonication buffer and eluted by an imidazole gradient of 35-250 mM through 20 column volumes. Fractions yielding high peak absorbance at 280nm (A₂₈₀) was run on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with GelCode™ Blue (Thermo Scientific) to detect presence of TARS. Pooled fractions containing TARS were dialyzed in buffer A (50 mM KH₂PO₄ pH: 6.8, 5% glycerol and 5 mM B-ME).

The dialyzed TARS was further purified in a 23 mL CHT-Tricorn Hydroxyapatite column using the buffer A as the equilibration and wash solution. Once washed, the hydroxyapatite column was eluted using pH gradient from buffer A to buffer B (500 mM KH₂PO₄ pH: 8, 5% glycerol and 5 mM B-ME). Fractions yielding high A₂₈₀ detecting for TARS using SDS-PAGE. Purified TARS were dialyzed and stored in storage buffer C (20 mM HEPES pH:8, 100 mM KCl, 5 mM B-ME 40% glycerol). Concentration of TARS in storage solution was recorded at A₂₈₀ provided by UVM DNA Analysis facility’s Nanodrop. TARS samples were verified with Western blot stained in 1:500 TARS anti-mouse in 3% bovine serum albumin (BSA), 0.1% Azide, and Tris-buffered saline and Tween 20/Triton X-100 (TBST) (Abnova).

**Tube Assay Formation Technique:**
The Tube Assay was performed as described by Williams et al., 2015. Cultured HUVECs, between passages 3-7, were trypsinized and aliquoted onto a 48-well plate, coated with Matrigel\textsuperscript{TM} (Basement Membrane Matrix Growth Factor Reduced; BD Biosciences) at a 5 x 10\textsuperscript{5} cells/ml concentration in Endothelial basal media (EBM media). Cells were incubated in the following treatments: EBM media (negative control), EGM media with 10% fetal bovine serum (positive control), EBM media with 0.2 uM wild type TARS, 0.2 uM Q566W TARS, and 0.2 uM H388A TARS. Cells were incubated for 4-6 hours, washed twice with phosphate buffered saline (PBS) and fixed with 10% Formalin for 30 min. HUVECs were then washed again with PBS, followed by incubation in 1:150 Oregon green 488 Phallodin in 3% BSA/TBST (Molecular Probes), then washed again with PBS. These cells were imaged using an inverted fluorescence microscope (Olympus IX70) provided by UVM’s microscope imaging center. The number of Endothelial branches, pieces, and branching length were quantified using the Angiogenesis Analyzer plugin on Image J software. For dose-response experiments, a similar Tube Formation Assay was done using TARS concentrations ranging from 10 nM to 500 nM to view effects of fibronectin binding to Matrigel.

**Statistical Analysis**

All experimental values were presented as means ± SEM and analyzed by one-way ANOVA and Turkey Post-hoc test using GraphPad Prism V7. Significance was defined at P < 0.05.
III. Results

Expression Mutant TARS Plasmids

Mutant TARS plasmids purified from the mutagenesis included Q566W and H388A. For example, the H388A plasmid was inserted onto XL-10 gold compacted cells and JM109 cells for selection and was sequenced to verify the mutation site. The image of the H388A sequence can be seen in Figure 2.

The bases highlighted represents the three-letter codon for amino acid 388. The wildtype amino acid encodes for histidine while the mutant plasmid encodes for alanine. The sequence data verifies correct base change and proper selection of *E. coli* cells based on the codon table. This insures that the H388A mutant was successful generated. A similar procedure was done for the Q566W mutant.
Purified TARS enzymes

Wild type TARS, Q566W, and H388A were purified from *E. coli* cells by saturating sample with protamine sulfate, followed by His6Tag and HA chromatography. SDS-PAGE results verified the presence of protein with a ~83kDa molecular weight, the molecular weight of TARS subunits (Figure 3). Western blot of concentrated protein further verified the presence of TARS (Figure 4).

Figure 3. An 8% SDS-PAGE Gel stained with GelCode™ Blue (Thermo Scientific) presenting purified TARS wild type and mutants showing the correct molecular weight (~83kDa).
Both data verify the purification of TARS. However, the Western blot demonstrates that earlier purification of wild-type TARS contained contaminants that shared similar characteristic of TARS.

**Tube Assay Formation:**

Previous data suggested that a point mutation at Q566W would result in decreased tube formation compared to wildtype TARS (Adam Christopher Mirando, 2015). To confirm this, Q566W was tested against both positive, and negative controls, as well as wildtype TARS (Figure 5).
Figure 5. Representative images of HUVECs grown on Matrigel™ and stained with 1:150 Phalloidin Oregon green. Image A: Treated with EBM media (negative control), Image B: Treated with 2% EGM media (positive control), Image C: Treated with EBM media and 200nM TARS, Image D: Treated with EBM media and 200nM Q566W, and Image E: Treated with EBM media and 200nM H388A.
Although the tube assay results showed a visual trend, neither Q566W nor wildtype TARS had a significant quantitative difference in the number of tubes relative to the negative control (Figure 6). A possible explanation of this results comes from TARS having low angiogenic activity compared to earlier studies performing tube assays (Tamara F. Williams et al., 2013). In addition, the negative control groups had higher baseline tubes than in previous assays. More trials should be performed to reduce random error of low angiogenic activity in TARS. As discussed below, experiments that studied the dose-dependent effects of TARS also provide an explanation for the variable results (Figure 8 and 9).
The H388 site was also investigated, as this site is known to interact with Borrelidin and tRNA\textsuperscript{Thr} (Figure 1). It was found that the H388A mutation had the most number of tubes compared to the treatments, making it seem that removal of catalytic site of stimulates TARS induced angiogenesis (Figure 7). However, this stems from the wildtype TARS not show any tube
formation. It should be noted that only one trial was performed comparing the H388A mutants. More trials of H388A against wild type TARS should be done before a conclusion can be made. Like the results with the Q566W mutant, the dose-dependent effects of TARS need to be considered in evaluating these results as will be described below.

Figure 7. (A,B,C) Quantification of HUVECs Tube Assay in response EBM Media, EGM media, wild type TARS, Q566W, and H388A TARS. Treatments were measured using Image J\textsuperscript{TM} Angiogenesis Analyzer and statistics were recorded in Graphpad V7. (A) Comparing the sum number of tube pieces formed for each treatment. (B) Comparing the sum number of branches formed for each treatment. (C) Comparing the branch length of tubes for each treatment.
Dose response of TARS in tube assay:

In the experiments described above, 200 nM wild-type TARS was used for stimulating tubes because that concentration proved to be effective for other investigators using previous TARS protein preparations. However, the current results showed variable or even inhibitory effects of TARS on tube formation of HUVECs using that dose. There was a concern that the different TARS preparations may exhibit different effects on the tube assay or that TARS at high concentrations may inhibit tube formation. To address this question, a dose response of TARS in the Tube Assay was performed at concentrations from 10 nM to 500 nM. The results revealed that TARS induced tube formation at concentrations up to 100 nM but higher concentrations led to a reduced effect, with loss of cell contact at 500 nM (Figure 8). As a result, tubes formed through the earlier experiments resulted in some tubes falling out of the Matrigel due to the inhibitory effect. This property of TARS led to a low amount of tubes forming in response to higher concentrations of both wild type and mutant TARS.

Figure 8 (a & b) HUVECs dose response bell curve data for wild type TARS. HUVECs were exposed to various concentration TARS ranging from 10nM to 500nM and were assayed using in vitro endothelial tube assay. HUVECs were fixed with 10% formalin and stained in 1:150 Oregon green Phalloidin. Tubes were measured in Image J analysis and plotted on Graphpad V7.
Similar experiments were performed with mutants Q566W and H388A. A dose response with mutant Q566W revealed the lowest angiogenic activity occurred around 200 nM; while H388A inhibitory effects occurred around 50 nM (Figure 9). The results demonstrated that mutant Q566W was at peak concentration in earlier studies to perform inhibitory effects, compared to H388A mutant. This must had influenced the high angiogenic activity found in Figure 6.

Figure 9 (a,b,c,d) HUVEC Dose Response bell curve data for mutant TARS. HUVECs were exposed various concentration of mutant TARS ranging from 10nM to 500nM were assayed using in vitro endothelial tube assay. Graphs above represents mutants Q566W (a&b) and H388A (c&d) dose response curve respectively.
IV. Discussion

Investigation of Q566W site

Prior studies have proposed that Q566W mutant was shown to decrease angiogenic activity due to mimicking BC194 bound TARS open conformation disrupting protein-protein interaction (Adam Christopher Mirando, 2015). Based on ANOVA, the in vitro Tube Assay results oppose this idea because mutant Q566W does not show significant change of angiogenic activity compared to the wildtype TARS enzyme. However, only a few trials have been done comparing mutants to wild type TARS, and there are questions regarding the concentrations of TARS preparations, thus more tests are needed to confirm these results.

Inhibition of TARS amino-transferring activity causes increase angiogenic activity

Purified H388A was generated to observe whether inhibition of TARS amino-transfer activity affects the level of angiogenic activity. This study has found that H388A has increased the angiogenic activity level of wildtype TARS and seemed to be more potent in the dose response assay. It is clear from these studies that the TARS concentrations that elicit optimal activity need to be carefully titrated. Future investigation should perform dose response tube assays before comparing to wildtype TARS.

Concentration of purified TARS affects HUVECs affinity to fibronectin

During the tube assay experiments, TARS also exhibited signs of excess migration with HUVECs that led to loss of cell contact. These trends are seen in both wild type and mutant derivatives of TARS indicating that there is a concentration threshold of TARS to use for HUVECs tube assay before tubes fall off. This may be a result of TARS having an effect of fibronectin formation as fibronectin is necessary to bind to Matrigel well. The removal of fibronectin would result in HUVECs being removed as well as the tubes formed. Based on the
curve, it seems that a concentration of around 100nM for wildtype TARS is the peak of observing tube formation. Studies on dose response curve on Matrigel binding to fibronectin reveal that most angiogenic activators, like VEGF, stimulates migration of HUVECs resulting in falling off the wells when washing. Investigation of the fibronectin binding with Matrigel is a potential gateway of understanding TARS migration phenomenon as fibronectin is found to bind to integrin receptors causing formation of actin filament stress fibers and attachment of focal adhesion to matrix (Danen, Sonneveld, Brakebusch, Fässler, & Sonnenberg, 2002). Future study using the fibronectin inhibitor (RGDS) in the tube assay is underway, as a comparison between wildtype dose response and RGDS could verify this idea (Reynolds et al., 2009).

Troubleshooting in vitro endothelial tube assay technique

Growth of Tubes Formation

A key issue in the tube assay experiments was timing. Although earlier studies mentioned HUVECs tube formation peak occurs around 6 hrs, the experiments performed had variations of peak formation between 4 hrs to 8 hrs (T. F. Williams, A. C. Mirando, B. Wilkinson, C. S. Francklyn, & K. M. Lounsbury, 2013). In some cases, the tubes were under development in EGM media when being fixed. To prevent under developed tubes in future studies, monitoring both positive and negative control of HUVECs tube formation every 3 hrs is recommended.

Verifying correct TARS concentration

Another issue in the tube assay experiments was the TARS concentration. Seen in the dose response, TARS concentration does play a role in angiogenic response. In earlier purifications, concentrations of purified wildtype TARS, Q566W, and H388A were recorded and diluted down to 20uM concentration to be used for tube assays. However, testing diluted version concentration using similar nanodrop technique revealed higher level of concentration than
expected. This was resolved prior to performing dose response experiments. Future experiments should nanodrop diluted TARS samples to ensure correct concentration as this could possibly influence the angiogenic response.

**Research significance in ovarian cancer treatment**

The goal of the project was to investigate how TARS activity stimulates angiogenesis in ovarian cancer. As discussed earlier in the study, angiogenesis is necessary component to cancer cell environment as its used to create an “escape route” for tumor cells to travel to different tissues and provide nutrition for the cells to live. Research in oncology has come to understand this method and begin developing antiangiogenic therapies. Developing known knockout enzyme, such as Q566W, could impact on anti-angiogenetic researcher as this would show mechanistically how TARS induces angiogenesis and how inhibitors such as BC194 and possibly RGDS inhibits activity. This would lead to development of better detection of severity of ovarian cancer compared to low detection molecules like Mucin-16 (Vaughan et al., 2011). Also, development of inhibitors such as BC194 and RGDS can make a major impact as a future targeted therapy against ovarian cancer cells (Vaughan et al., 2011).

**Conclusions**

The angiogenic activity of TARS mutants Q566W and H388A has been successfully studied. Q566W does not show any difference in angiogenic activity with wild type TARS, while H388A shows an increase in activity. As mentioned, only a few trials were performed in this study, so further trials of *in vitro* tube assay should be done. Future studies should continue investigate mutants as studies have shown promise of inhibition of angiogenic activity (Egri, 2015)
V. References


https://www.nature.com/articles/srep13160#supplementary-information


