

Targeting a Regulatory Element in Human Thymidylate Synthase mRNA

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Thymidylate synthase (TS) is a key enzyme in the biosynthesis of thymidine. The use of TS inhibitors in cancer chemotherapy suffers from resistance development in tumors through up-regulation of TS expression. Autoregulatory translation control has been implicated with TS overexpression. TS binding at its own mRNA, which leads to sequestration of the start codon, is abolished when the enzyme forms an inhibitor complex, thereby relieving translation suppression. We have used the protein-binding site from the TS mRNA in the context of a bicistronic

expression system to validate targeting the regulatory motif with stabilizing ligands that prevent ribosomal initiation. Stabilization of the RNA by mutations, which were studied as surrogates of ligand binding, suppresses translation of the TS protein. Compounds that stabilize the TS-binding RNA motif and thereby inhibit ribosomal initiation might be used in combination with existing TS enzyme-targeting drugs to overcome resistance development during chemotherapy.

Introduction

The enzyme thymidylate synthase (TS) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to produce 2'-deoxythymidine-5'-monophosphate (dTMP) which is further phosphorylated to the triphosphate dTTP, an essential building block for cellular DNA synthesis.^[1] TS is an attractive target for cancer chemotherapy since it provides the only de novo source for dTTP.^[2] Inhibitors of TS are important drugs for cancer therapy, including 5-fluorouracil (5-FU), which was one of the earliest anticancer agents and is still used in the treatment of colorectal cancer.^[3] 5-FU is metabolized to the active TS inhibitor 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP). FdUMP has a dual mechanism of action. It is incorporated into cellular nucleic acids and also directly inhibits TS through the formation of a covalent ternary complex with the methyl donor cofactor *N*⁵-*N*¹⁰-methylene-tetrahydrofolate (mTHF).^[2]


A major drawback of the clinical use of 5-FU is the development of resistance against the drug in tumors through, among other more complex mechanisms,^[4] upregulation of TS expression.^[2] TS overexpression emerging during 5FU chemotherapy has been implicated with the autoregulatory mechanism of translation control for the enzyme. In the absence of a substrate ligand, TS associates at two independent binding sites within its own mRNA and thereby represses translation.^[5] Complex formation with the cognate substrate dUMP or the inhibitor FdUMP abolishes mRNA binding of TS.^[6] The presence of FdUMP during chemotherapy with 5-FU thus leads to increased levels of TS expression despite inactivation of the enzyme, which ultimately results in emergence of tumor resistance. Whereas such feedback regulation of translation is common in bacteria, the TS system represents the first known example of translational autoregulation in humans.^[7] It has been demonstrated that full translational repression is achieved through TS binding at both mRNA sites.^[5b] One of the

TS-binding sequences (site 2) is located in an extended stretch of 200 nucleotides within the mRNA coding region. The site 1 is predicted to fold into a stem-loop structure that spans the translation initiation site (Figure 1).^[5a] It has been suggested that protein binding to the regulatory mRNA site 1 motif stabilizes the hairpin loop which sequesters the start codon, making it unavailable for ribosomal recognition.

We propose that small-molecule ligands of the TS mRNA might bind and stabilize the site 1 hairpin independent of TS binding and suppress translation sufficiently to overcome 5-FU dependent overexpression of the enzyme. Such hairpin-stabilizing ligands might overcome resistance development during 5-FU chemotherapy. Previously, it has been reported that promiscuous nucleic acid binders including aminoglycosides and a bis-benzimidazole dye used for DNA staining (Hoechst 33258) interact with RNA constructs resembling the upper part of the TS site 1 hairpin.^[8] Here, we have used the authentic regulatory element from the TS mRNA to validate the approach of targeting the hairpin motif with stabilizing ligands. As a proof of concept, we aimed to investigate if interaction energies typically provided by the binding of a small-molecule ligand would provide sufficient stabilization of the hairpin RNA to impact translation initiation. We have constructed a reporter system carrying mutations within the TS site 1 motif as a surro-

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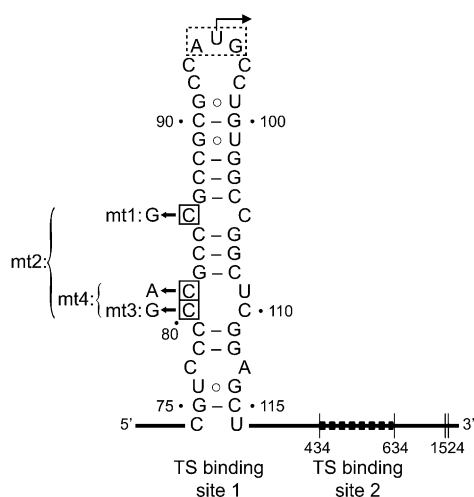


Figure 1. Secondary structure of the human TS mRNA which contains two binding sites for the enzyme. Site 1 is predicted to adopt a stem-loop structure that contains several base mismatches and a hairpin sequestering the AUG initiation codon.^[5a] Site 2 is located within the reading frame.^[5b] Base changes are shown for four stabilized mutant sequences (mt1–mt4) which are used in this work. Numbering is according to the *Homo sapiens* sequence, record NM \cong 001071 of the NCBI Nucleotide Database.^[16]

gate for stabilizing ligands to assess the potential energetic contribution required for translation suppression through sequestration of the start codon. A small exploratory set of compounds was tested for their impact on reporter expression under the control of the TS regulatory motif.

Results and Discussion

The TS site 1 regulatory motif confers TS-dependent repression of reporter translation

To test the function of the TS site 1 motif as a protein-binding hairpin that confers TS-dependent regulation of translation we inserted the site 1 motif upstream of a reporter gene to be used in a coupled *in vitro* transcription-translation (IVT) assay. A bicistronic system was constructed in which a firefly luciferase reporter is translated cap-driven under the control of the TS site 1 motif followed by a *Renilla* luciferase reporter initiated at a hepatitis C virus (HCV) internal ribosome entry site (IRES; Figure 2A).^[9] Bicistronic expression constructs provide an internal control of translation integrity and allow the exclusion of false-positive inhibitors which affect the ribosome independently of the presence of the TS site 1 regulatory motif. In addition, the use of bicistronic transcripts obviates the need for determining absolute mRNA concentrations or screening for polymerase inhibition in the IVT assay.

The proper function of the TS site 1 regulatory motif in the context of the bicistronic reporter constructs was demonstrated by titration of human TS protein in the IVT assay (Figure 2B). Addition of the enzyme suppressed reporter expression in a dose-dependent fashion and at 25 μ M concentration completely inhibited translation in the construct carrying the TS site 1 motif, thus reproducing the effect shown for TS pro-

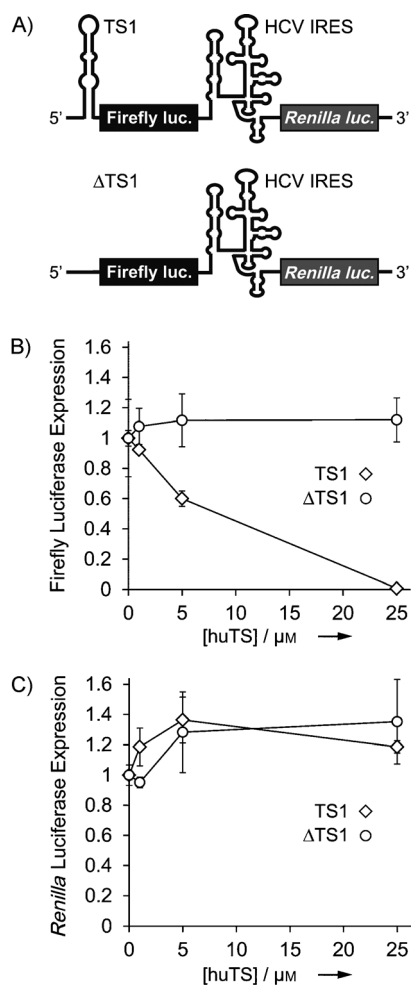


Figure 2. Reporter translation under the control of the TS site 1 regulatory element. A) Bicistronic reporter constructs used for experiments in a coupled *in vitro* transcription-translation assay (IVT). In the TS1 construct a cap-driven firefly reporter gene under the control of the TS site 1 motif is followed by an HCV IRES-driven *Renilla* luciferase reporter. The Δ TS1 construct lacks the site 1 motif upstream of the firefly reporter. B) Impact of huTS protein addition on cap-driven firefly luciferase reporter expression from the TS1 and Δ TS1 constructs. C) Impact of huTS protein addition on IRES-driven *Renilla* luciferase reporter expression from the same two constructs. Data are averages of three replicates with error bars showing $\pm 1\sigma$.

tein acting on its authentic mRNA.^[7] In the absence of the TS site 1 hairpin (Δ TS1), cap-driven translation was not affected by the TS protein (Figure 2B) and neither was the IRES-driven process in any of the bicistronic constructs (Figure 2C). Compared to the translation of a cap-driven luciferase from the Δ TS1 construct, reporter expression under the TS regulatory motif was about 40% lower (Figure 3A). We assumed that the reduced translation activity was due to TS protein present in the reticulocyte lysate (full cell extract) which was used in the IVT assay. After immunodepletion of TS protein from the lysate, the expression of cap-driven reporter under the TS site 1 motif was restored to the level of the Δ TS1 construct (Figure 3B). This demonstrates that the secondary structure of the wild-type TS site 1 hairpin by itself does not provide sufficient stabilization to prevent the ribosome accessing the initiation site.

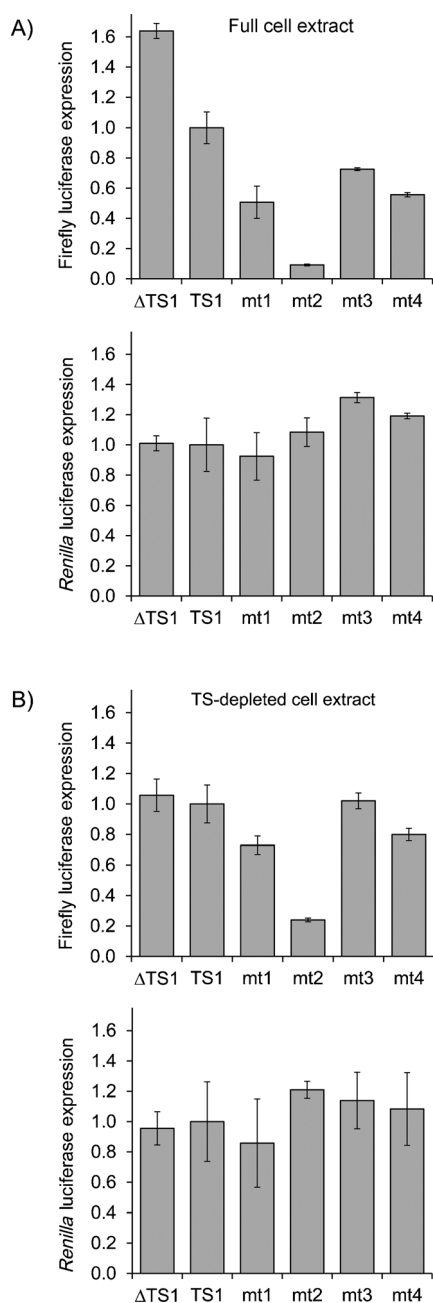


Figure 3. Impact of stabilizing mutations on reporter translation under the control of the TS site 1 regulatory element. A) Expression levels of the cap-driven firefly and IRES-driven *Renilla* luciferase reporters in bicistronic constructs containing wild-type (TS1) and mutant (mt1–mt4) TS site 1 regulatory elements in the presence of full reticulocyte extract. B) Reporter expression from the same two constructs in cell extract that was depleted of residual TS protein. Mutations in mt1–mt4 are shown in Figure 1. The ΔTS1 construct is included as a control. Note that expression data has been normalized to the level of translation from the TS1 construct (= 100%) for the separate experiments shown in panels A and B. Absolute expression from the ΔTS1 construct does not change between experiments with full extract (A) and TS-depleted extract (B) while expression levels from TS1 and mutants rise uniformly in TS-depleted extract. Data are averages of three replicates with error bars showing $\pm 1\sigma$.

Binding of TS protein to the RNA is required to efficiently sequester the start codon for translation suppression.

Stabilization of the TS site 1 regulatory motif suppresses mRNA translation

We propose to exploit the site 1 regulatory motif in the TS mRNA as a target for small molecule translation inhibitors that stabilize a hairpin motif in which the initiation codon is sequestered. The feasibility of this approach was tested by investigation of stabilizing mutations in the stem part of the site 1 motif and their impact on translation (Figure 1). In the absence of selective ligands, we planned to use stabilizing mutations as surrogates to correlate stabilization energy with the extent of translation suppression in the IVT assay. We assumed that translation inhibition in stabilized mutant hairpins would result from an overall increased stability of the TS protein–RNA hairpin complexes. However, this holds true only if protein binding is not adversely affected by the mutations. Therefore, we selected three positions for mutations upstream from the initiation codon since preliminary crosslinking studies and truncated TS mutants examined in the IVT assay indicated that the TS protein binds to the site 1 motif downstream from the start site.

Testing in the IVT assay of four mutant constructs (mt1–mt4), which introduce stabilizing base changes in the TS site 1 structure, showed that each additional Watson–Crick base pair formed led to further repression of reporter translation under control of the site 1 element (Figure 3A). Reduction of reporter expression was a specific consequence of the stabilizing mutations in the TS site 1 motif as indicated by the relatively unaffected levels of IRES-driven translation from the bicistronic constructs. Whereas in vitro translation in cell lysate that was depleted of residual TS protein partially restored reporter expression of the stabilized mutant constructs (Figure 3B), only the singly mutated mt3 reached an expression level comparable to the wild-type sequence. The other three mutants maintained relatively suppressed translation that was correlated to the stabilization introduced by the base exchanges. Marginal stabilization, corresponding to one additional stacked base pair as in mt1, of the wild-type TS site 1 hairpin was sufficient to reduce translation efficiency, even in the absence of TS protein binding. In conjunction with our finding that the TS site 1 motif by itself does not provide sufficient stabilization to prevent translation initiation, this suggests that the wild-type hairpin is only just labile enough to allow free read-through by the ribosome whereas binding with TS protein affords a stable complex that efficiently inhibits translation.

Stabilization energies introduced by the mutations, which were estimated from RNA folding predictions for the TS site 1 motif, suggest a correlation to reporter expression in full cell extract (see the Supporting Information). As expected, TS site 1 mutants with higher stability led to lower translation efficiency, presumably through requiring more energy for the ribosome to dissociate the TS complex and disrupt the site 1 motif RNA secondary structure during initiation of protein synthesis. In agreement with this hypothesis was the observation that the wild-type TS site 1, which recruits TS protein present in the reticulocyte lysate, reduced translation efficiency compared to the ΔTS1 construct. The correlation of energy stabilization and

reporter expression in TS-depleted extract confirmed the notion of the TS site 1 hairpin by itself as a marginally stable roadblock to ribosomal initiation. Whereas an additional contribution of $\sim 3\text{--}4\text{ kcal mol}^{-1}$ of free energy to the RNA secondary structure, as in the mutant mt3, did not yet reduce translation, stabilization beyond this level reduced reporter expression.

From the correlation of secondary structure stability and translation efficiency, it was estimated that a contribution of $\sim 8\text{ kcal mol}^{-1}$ of stabilization free energy was sufficient to reduce translation by $\sim 50\%$. This suggests that a small-molecule ligand engaging in just two to three intermolecular hydrogen bonds to the RNA target will induce inhibition of TS translation upon binding to the site 1 regulatory element. Thus, compounds selectively binding to the TS mRNA might stabilize the site 1 hairpin independent of protein binding and suppress TS synthesis sufficiently to overcome 5-FU dependent overexpression of the enzyme.

Exploratory screen for compounds that affect translation under control of the TS site 1 motif

To discover compounds that selectively impact protein expression under the control of the TS site 1 regulatory element, we performed a screen of a small exploratory compound set emerging from our efforts to design and synthesize molecules biased for RNA binding. Compounds were tested in the IVT assay using the bicistronic reporter construct which carried the wild-type TS site 1 motif. Testing was performed initially at two compound concentrations of 10 and 100 μM . In the primary screen, we did not discover ligands that selectively suppressed expression of the firefly luciferase under the control of the TS site 1 regulatory element. However, two compounds, derivatives of diamino-propionic acid (**1**) and dihydropyrimidine (**2**), were identified that increased reporter translation (Figure 4). Subsequent titration in the IVT assay revealed that both molecules selectively stimulated TS1-specific translation in a dose-dependent fashion while not affecting reporter expression in the ΔTS1 construct (Figure 4A).

When TS-depleted lysate was used in the IVT assay, compounds **1** and **2** did not affect reporter expression, suggesting that these molecules may ablate the TS protein's ability to repress translation by binding to the site 1 motif. We hypothesized that the compounds perhaps target the cosubstrate-

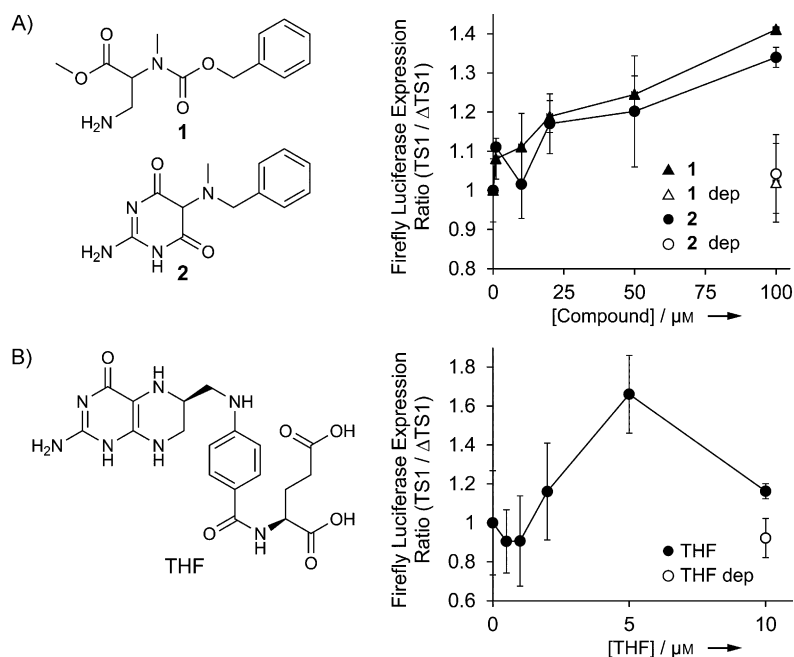


Figure 4. Compounds that increase translation of luciferase reporter under control of the TS site 1 regulatory element in an IVT assay using full reticulocyte extract. A) Derivatives of diamino-propionic acid (**1**) and dihydropyrimidine (**2**). B) Tetrahydrofolic acid (THF), which is structurally related to the TS enzyme cosubstrate N^5 - N^{10} -methylene-tetrahydrofolic acid (mTHF). Graphs show firefly luciferase expression under control of the TS site 1 motif normalized to reporter expression from a construct that lacks the TS site 1 motif (see Figure 2A). Open symbols show reporter expression using TS-depleted extract ("dep") at the maximum concentrations tested for compounds **1**, **2** and THF. Data are averages of three replicates with error bars showing $\pm 1\sigma$.

binding site of the enzyme, which is known to exert allosteric control over RNA binding of the TS protein.^[2] As a control, we investigated the impact of tetrahydrofolic acid (THF) whose pteridine moiety shows structural similarity to the heterocyclic scaffold in the dihydropyrimidine **2** (Figure 4B). THF is a precursor of the TS enzyme cosubstrate mTHF and a ligand of the cosubstrate-binding site. We found that THF, like compounds **1** and **2**, increased TS1-specific translation albeit with better potency. At 5 μM concentration, THF led to increased reporter expression in the TS1-controlled construct comparable to the level of ΔTS1 mRNA, thus confirming that ligand binding to the enzyme cosubstrate site ablates translational repression conferred by TS binding to the site 1 motif.

The hypothesis that compounds **1** and **2** may directly interact with the cosubstrate-binding site of TS was further explored by testing both molecules for their impact on the protein's enzymatic activity. Competition with the cognate cosubstrate mTHF would lead to inhibition of the enzyme. Addition of **1** or **2** to a TS in vitro activity assay showed that both molecules suppressed enzymatic function in a dose-dependent fashion (Figure 5). The finding of direct enzyme inhibition along with the ability to ablate TS-mediated repression of translation under the control of the site 1 motif suggest that **1** and **2** function as ligands of the TS cosubstrate-binding site and might provide starting points for the development of more potent TS inhibitors. However, similar to 5FU, such inhibitors will still suffer from resistance development through ablation of enzyme binding to its mRNA and relieving translation suppression.

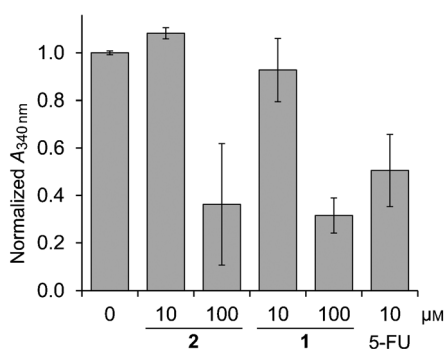


Figure 5. Inhibition of TS enzymatic activity by compounds **1** and **2**, which likely are competitors of the mTHF cosubstrate, and the active-site substrate analogue 5-FU as a control. Data are averages of three replicates with error bars showing $\pm 1\sigma$.

Discovery of peptides that bind to the TS site 1 motif and suppress translation

Because screening of the small exploratory set of compounds did not yield a stabilizing ligand for the TS site 1 motif, we decided to explore short arginine-rich peptides as potential binders that have a propensity for RNA interaction.^[10] Peptides for binding testing were designed following two criteria. Firstly, the length of peptides was limited to six amino acids to avoid problems with solubility and permeability in future cell-based testing. Secondly, we included sequences that contained dyads of arginines around pairs of neutral polar residues (Figure 6). The selection and distribution of arginines was inspired by observations from a high-resolution crystal structure of the TS enzyme in which a cluster of tightly bound phosphate ions is found around two spatially close pairs of arginines at the surface of the protein.^[11] It has been suggested that the phosphate cluster indicates the location of an RNA-binding site in TS. Based on these considerations we initially explored hexapeptides of the general sequence RRXXRR (X = any amino acid) as potential ligands. A 33-mer RNA construct representing the TS site 1 motif was used to analyze the interaction of peptides by native polyacrylamide gel electrophoresis (PAGE). Peptides containing tyrosine or tryptophan pairs (**3** and **6**) showed non-specific binding, and in the case of **6** led to aggregation. Two peptides (**4** and **5**) which had arginine dyads separated by leucine or serine pairs formed specific complexes with the RNA (Figure 6A). Titration of **4** and **5** revealed dose-dependent binding of the peptides to the TS site 1 motif with affinities of $\leq 50 \mu\text{M}$ (Figure 6B).

To assess whether peptide binding would occur to the TS site 1 target as part of reporter mRNA, we tested the impact of **4**, **5** and, as a control, **6** in the IVT assay. Reporter expression from constructs TS1 and Δ TS1 was measured in the presence of $200 \mu\text{M}$ peptide (Figure 7). Strong general translation inhibition occurred in the presence of peptide **6**, consistent with the observation of RNA aggregation in the PAGE analysis (Figure 6A).

The serine-containing peptide **5**, which formed a defined complex with TS site 1 RNA (Figure 6B), inhibited translation in a nonspecific fashion, reducing expression of reporter both in

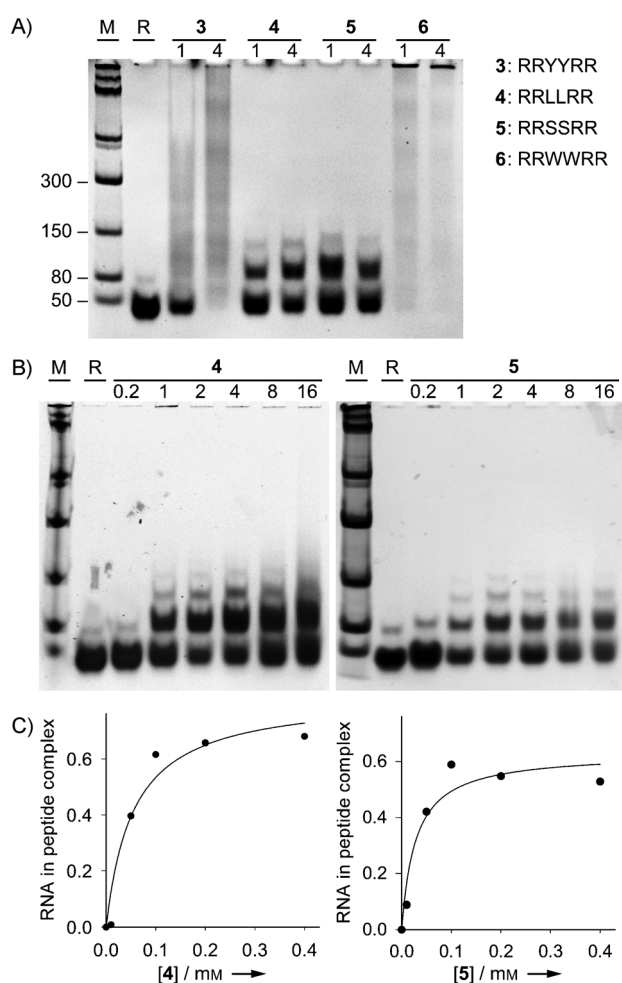


Figure 6. Identification of arginine dyad peptides that bind the TS site 1 regulatory element. A) Native PAGE assay of four hexapeptides (**3–6**) interacting with a 33-mer TS site 1 RNA construct (residues C80–G112, Figure 1). “M” indicates a single-stranded RNA marker. “R” indicates the free RNA. Peptides were tested at stoichiometric ratios of 1:1 and 4:1 peptide/RNA. B) Dose-response titration of peptides **4** and **5** in the PAGE assay. Numbers indicate the stoichiometric ratio of peptide/RNA. C) Quantification of peptide–RNA complex formation in gels shown in panel B. For both peptides the binding affinity is $K_D \leq 50 \mu\text{M}$.

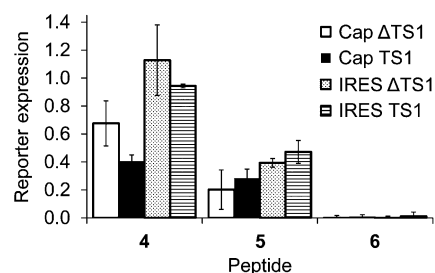


Figure 7. Impact of hexapeptides **4–6** on reporter translation from bicistronic constructs TS1 and Δ TS1. Peptides ($200 \mu\text{M}$) were added to the IVT assay. Reporter expression was normalized to levels measured in the absence of peptides. Peptide **6** induces aggregation and likely precipitation of RNA (see Figure 6A), resulting in general translation inhibition. Data are averages of three replicates with error bars showing $\pm 1\sigma$.

the presence and absence of the TS site 1 motif as well as affecting the IRES-driven process. In contrast, the leucine-con-

taining peptide **4** showed selective stronger (60%) inhibition of cap-initiated translation under the control of the TS site 1 motif. Reporter expression from the IRES element was not diminished in the presence of **4**, demonstrating that the leucine-containing peptide does not promiscuously affect the translational machinery.

Comparison of binding behavior (Figure 6) and inhibition potential (Figure 7) of hexapeptides **3–6** suggests that target-selective ligands might be developed by optimization of the lead peptide **4**. Sequence variation of residues separating the arginine dyads and modification of the number as well as nature of basic amino acids will be used in future studies to explore peptide inhibitors derived from **4**.

Conclusions

We have demonstrated that the TS site 1 hairpin constitutes an autonomous regulatory RNA motif that can be transplanted to reporter systems for mechanistic studies and the development of screening assays. The secondary structure of the wild-type motif by itself provides only a marginally stable roadblock to ribosomal initiation, whereas binding of the TS protein reduces translation initiation by sequestration of the start codon. Concluding from mutational studies, we determined that stabilization of the RNA secondary structure by the binding of a small-molecule ligand may be a viable approach to suppress translation.

Discovery of a peptide (**4**) that forms a defined complex with the TS site 1 RNA and inhibits translation under the control of this regulatory motif provides validation of the TS mRNA as a target for translation inhibitors that stabilize a hairpin motif in which the initiation codon is sequestered. The finding that among ligands that bind the TS site 1 (peptides **4** and **5**) selective inhibition of translation under control of the site 1 RNA motif can be achieved in a sequence-specific fashion (peptide **4**) suggests that both target affinity and selectivity might be improved in future studies by modification of the lead inhibitor **4**.

Reduction of TS expression levels might be beneficial to counter overexpression of the enzyme during cancer therapy with TS-inhibitor drugs which ablate binding of the protein to its mRNA. A combination therapy approach of a TS enzyme inhibitor and a site 1 RNA-targeting ligand might overcome emergence of tumor resistance.

Experimental Section

TS protein expression and purification: A DNA construct coding for a codon optimized C-terminal His₆-tagged human TS (huTS) was purchased from GenScript (Piscataway, NJ) and inserted into the pET22b+ plasmid (Invitrogen), which was transformed into Neb5 α competent cells. His₆-tagged huTS protein was expressed and purified as described previously,^[12] with the following modifications. The enzyme was expressed in T7 Express *E. coli* (Neb) and purified on a Ni²⁺-affinity column. An additional wash step utilizing ten column volumes of 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) buffer was performed prior to elution to remove any bacterial TS from the solution. The huTS

was eluted on a linear gradient of 40–100 mM imidazole. Fractions were pooled, concentrated, and dialyzed into Tris buffer (20 mM, pH 8) containing DTT (1 mM).

Construction of the bicistronic reporter constructs: Cloning of bicistronic reporter constructs (Figure 2A) was performed as outlined in the Supporting Information.

In vitro translation assay and luciferase detection: The in vitro transcription-translation assay (IVT) was conducted using the TNT Quick coupled reticulocyte lysate system (Promega). The TNT Quick system was chosen because it works efficiently with templates that lack an optimal Kozak sequence such as the TS1 construct which had to contain the native TS site 1 sequence upstream of the initiation codon. IVT reactions were run in 7.5 μ L volume according to the manufacturer's recommendations. In brief, each reaction (7.5 μ L) contained plasmid DNA (100 ng μ L⁻¹, 1.5 μ L), H₂O or compound solution (1.5 μ L), and reaction buffer containing reticulocyte lysate (5 μ L), SP6 polymerase, and RNase inhibitor. Incubation was at 30 °C.

Detection of firefly and *Renilla* luciferase levels was done using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's recommendations. In brief, 5 μ L of the reaction mixture was mixed with Dual-Glo luciferase substrate (25 μ L) on a 96-well plate. After incubation for 60 s at RT the firefly luminescence was measured on a SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA). Then, Dual-Glo Stop & Glo substrate (25 μ L) was added to each well, incubated for 60 s, and the luminescence of the *Renilla* luciferase was quantified.

IVT TS enzyme titration: Previously frozen TS enzyme was diluted in buffer to a fivefold concentrated sample (i.e., 5, 25, and 125 μ M), and 1.5 μ L was added to the transcription-translation reaction in order to achieve a final concentration of 1, 5, or 25 μ M. No other conditions were modified.

IVT Compound screening: Aqueous compound stock (1.5 μ L) was added to the assay mixture to a final concentration of 10 or 100 μ M, respectively. Controls were H₂O or 1% DMSO.

TS immunodepletion: To deplete the TNT extract of cellular TS, mouse antibody (MAb-TS(106) at 200 μ g mL⁻¹; Thermo Fisher) was mixed with the extract at a 1:250 dilution. This mixture was gently agitated at 4 °C for 45 min, at which point red agarose-conjugated goat-anti-mouse antibody was mixed (1:250 v/v) with the antibody/reticulocyte lysate mixture and allowed to gently agitate for 30 min at 4 °C. The agarose-conjugated complex was removed from the mixture by centrifugation (1000g, 1 min).

TS enzyme assay: The TS enzyme activity assay was conducted as described in the literature.^[13] In brief, the enzymatic conversion of mTHF to dihydrofolate results in an increased absorbance at 340 nm. The reaction conditions were Tris buffer (50 mM, pH 7.0) containing KCl (125 mM), dUMP (5 μ M), and mTHF (5 μ M). TS protein was added to a final concentration of 5 μ M. Enzymatic reactions were carried out at 37 °C for 15 min and quenched by the addition of HCl (0.36 N). Absorbance was measured on a Shimadzu UV-2401PC spectrophotometer.

Compound synthesis: Methyl 3-amino-2-(*N*-benzyloxycarbonyl-*N*-methylamino)propanoate (**1**) was synthesized in five steps from benzyloxycarbonyl-protected L-asparagine following published procedures.^[14] ¹H NMR ([D₆]DMSO, 400 MHz): δ = 8.30 (brs, 2H), 7.35–7.29 (m, 5H), 5.10–5.03 (m, 2H) 4.73 (t, *J* = 6.89 Hz, 1H), 3.63 (s, 3H) 3.32–3.26 (m, 2H), 2.91 ppm (s, 3H); ¹³C NMR ([D₆]DMSO, 400 MHz): δ = 169.74, 156.68, 137.17, 129.09, 128.41, 128.10, 67.55,

59.22, 53.15, 38.11, 34.85 ppm; MS (ESI, positive-ion mode): m/z calcd for $C_{13}H_{18}N_2O_4$: 266.13 $[M+H]^+$; found: 267.09.

2-Amino-5-(*N*-benzyl-*N*-methylamino)pyrimidine-4,6-(1*H*,5*H*)-dione (**2**) was obtained from condensation of 2-(*N*-benzyl-*N*-methylamino)malonate and guanidine following established procedures.^[15] 1H NMR (CD_3OD , 400 MHz): δ = 7.57–7.35 (m, 5H), 4.65–4.57 (d, J = 11.73 Hz, 1H), 4.20–4.12 (m, 1H), 3.96–3.79 (m, 1H), 2.92 ppm (s, 3H); ^{13}C NMR (CD_3OD , 400 MHz): δ = 167.02, 157.35, 151.66, 131.22, 130.18, 129.78, 129.23, 129.06, 60.06, 54.77, 52.36, 42.84 ppm; HRMS (ESI-TOF): m/z calcd for $C_{12}H_{14}N_4O_2$: 246.1117 $[M+H]^+$; found: 247.1190, Delta (ppm) –0.19.

Peptide RNA binding gel shift assay: RNA representing the TS site 1 sequence (nt C80–G112, Figure 1) was obtained as HPLC-purified oligonucleotide from IDT (Coralville, IA). Peptides were purchased from Genscript and dissolved in water to a final concentration of 5 mM. Individual reaction mixtures consisted of 2× reaction buffer [12% glycerol, Tris (25 mM, pH 7.0), DTT (5 mM), $MgCl_2$ (2 mM); 5 μ L], RNA (50 μ M) and an appropriate aliquot of the desired peptide, brought to a final volume of 10 μ L with distilled H_2O . Mixtures were allowed to incubate at RT for 5 min, then placed on ice for another 5 min.

Native polyacrylamide gels (6%) were prepared using 40% acrylamide-bisacrylamide solution (1.5 mL, 19:1), 10× MOPS buffer [1 mL, 200 mM, pH 7.0, NaOAc (80 mM)] diluted with distilled H_2O (7.3 mL), and polymerized by addition of TEMED (20 μ L) and 10% ammonium persulfate (0.2 mL). The gel was pre-run in 1× MOPS running buffer [20 mM, pH 7.0, NaOAc (8 mM)] at 4°C for 30 min at 100 V, then loaded with the reaction mixtures and allowed to run for another 40 min. Visualization of RNA was achieved using SYBR Gold nucleic acid gel stain (Invitrogen) according to the manufacturer's protocol.

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