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Phosphorothioate analogs of m^{7}GTP are enzymatically stable inhibitors of cap-dependent translation

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ABSTRACT

We report synthesis and properties of a pair of new potent inhibitors of translation, namely two diastereomers of 7-methylguanosine 5′-(1-thiotriphosphate). These new analogs of mRNA 5′cap (referred to as m^{7}GTPαS (D1) and (D2)) are recognized by translational factor eIF4E with high affinity and are not susceptible to hydrolysis by Decapping Scavenger pyrophosphatase (DcpS). The more potent of diastereomers, m^{7}GTPαS (D1), inhibited cap-dependent translation in rabbit reticulocyte lysate ~8-fold and ~15-fold more efficiently than m^{7}GTP and m^{7}GpppG, respectively. Both analogs were also significantly more stable in RRL than unmodified ones.

A characteristic feature of all eukaryotic mRNAs is a cap structure, m^{7}Gppp(Np)$_n$, consisting of 7-methylguanosine linked to the first nucleotide of mRNA chain by 5′,5′-triphosphate bridge. This structure is involved in numerous events of mRNA metabolism, including intracellular transport, translation and turnover.[1] During the initiation of translation, the cap is specifically recognized by eukaryotic Initiation Factor 4E (eIF4E), which participates in the recruitment of ribosomes to mRNA.[2] eIF4E plays a crucial role in translational control of gene expression. It is known that eIF4E is overexpressed in many types of tumor cells and elevated eIF4E levels selectively increase the translation of mRNAs important in malignant transformation and metastasis.[3] Studies demonstrated that targeting eIF4E may inhibit the growth of tumor cells and induce apoptosis.[4,5] An antisense oligonucleotide-based antitumor therapy directed against eIF4E is currently under clinical trials.[6]

Synthetic cap analogs, such as 7-methylguanosine 5′-polyphosphates (m^{7}Gp$_n$), dinucleotides of m^{7}Gp$_n$N structure and their various derivatives, were shown to inhibit cap-dependent translation in vitro by competing with mRNA for eIF4E.[7,8] From this fact, an idea of employing cap analogs in therapy, as specific inhibitors that might counteract elevated eIF4E levels in tumor cells, originated. Further studies revealed that the ability of an individual analog to inhibit translation is qualitatively correlated with its binding affinity for eIF4E.[9] Two general conclusions can be drawn from these works. First, the binding affinity for eIF4E and the resulting inhibitory potency is increasing with the length of polyphosphate bridge (e.g., m^{7}GTP is ~1.7-fold and ~88-fold more potent inhibitor than m^{7}GDP and m^{7}GMP, respectively). Second, mononucleotides are better inhibitors than corresponding dinucleotides with the same length of polyphosphate bridge.[8,9]

This work is a part of our project aimed at designing new, highly specific eIF4E inhibitors. Recently, we synthesized several phosphorothioate analogs of m^{7}GpppG that bind tightly to eIF4E.[10] Both diastereomers of the analog modified at the position closest to m^{7}Guo (abbreviated as m^{7}Gp$_S$ppG (D1) and m^{7}Gp$_S$ppG (D2)) were resistant to Decapping Scavenger pyrophosphatase (DcpS), which is responsible for degradation of such compounds in living cells.[11] Both of them proved also to be stronger inhibitors of in vitro translation than m^{7}GpppG. Since it is known, that m^{7}GTP is a better inhibitor than m^{7}GpppG, in this work we synthesized and studied a diastereomeric pair of m^{7}GTP analogs modified with phosphorothioate moiety at the corresponding position (m^{7}GTPαS, Fig. 1).

The chemical synthesis of m^{7}GTPαS was accomplished by 5′-thiophosphorylation of 7-methylguanosine in (MeO)$_3$PO in the presence of 2,6-lutidine,[12] followed by addition of tributylammonium pyrophosphate and subsequent hydrolysis of the trimeta-

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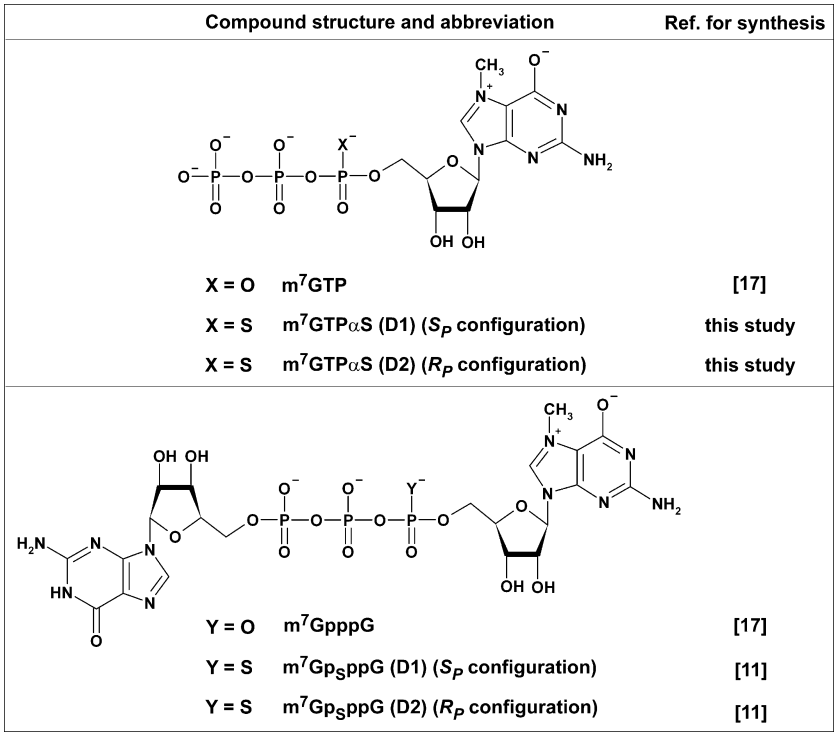
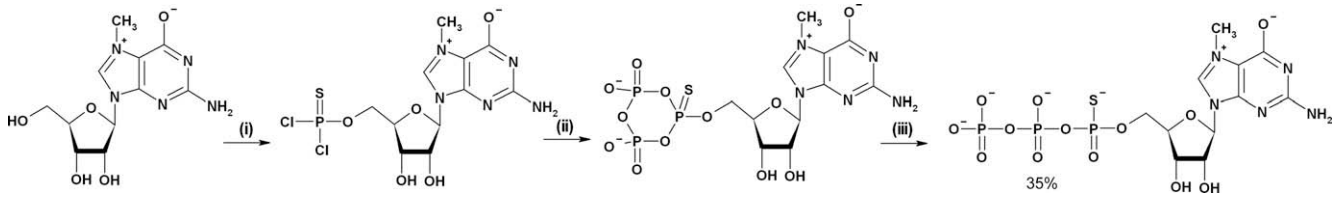


Figure 1. Structures and abbreviations of compounds used in this study. Due to the presence of P-stereogenic centers, two diastereomers of phosphorothioate cap analogs ($m^7GTP\alpha S$ and m^7Gp_SppG) exist. (See above-mentioned references for further information.)



Scheme 1. Synthesis of 7-methylguanosine 5′-(1-thiotriphosphate). Conditions: (i) $PSCl_3$, 2,6-lutidine, $(MeO)_3PO$, 0 °C (ii) 0.5 M tributylammonium pyrophosphate in DMF (iii) TEAB.

Table 1
Summary of biophysical and biological characteristics of the studied inhibitors of translation

Cap analog	K_{AS}[a] cap-eIF4E (μM^{-1})	ΔG° (kcal/mol)	Susceptibility to hDcpS	IC_{50}[b] in RRL conditions A (μM)	IC_{50}[b] in RRL conditions B (μM)	Reported IC_{50} values
m^7GpppG	9.4 ± 0.4[c]	−9.35 ± 0.02[c]	Hydrolyzed	8.3 ± 0.2	N.D.[d] (<20)	17.1 ± 1.0[c]
m^7Gp_SppG (D1)	30.8 ± 0.5[c]	−10.04 ± 0.01[c]	Resistant[c]	1.1 ± 0.1	1.2 ± 0.3	4.1 ± 0.2[c]
m^7Gp_SppG (D2)	10.0 ± 0.2[c]	−9.39 ± 0.01[c]	Resistant[c]	3.8 ± 0.6	2.1 ± 0.6	12.1 ± 3.2[c]
m^7GTP	124 ± 6	−10.85 ± 0.03	Hydrolyzed	4.5 ± 0.9	8.0 ± 1.5	4.39 ± 0.89[e]
$m^7GTP\alpha S$ (D1)	371 ± 19	−11.49 ± 0.03	Resistant	0.56 ± 0.08	0.56 ± 0.02	—
$m^7GTP\alpha S$ (D2)	190 ± 7	−11.10 ± 0.02	Resistant	1.44 ± 0.2	1.3 ± 0.3	—

[a] Determined by fluorescence quenching titration.[15]
[b] Cap analog concentration at which cap-dependent translation is inhibited by 50%; values are means of $n = 2–7$ exp, except m^7GpppG, for which $n = 22$.
[c] Data from Ref. 10.
[d] The value could not be determined since the experimental data did not fit to theoretical curve.
[e] Data from Ref. 8.

phosphate-like intermediate (Scheme 1, complete synthetic details and spectroscopic analysis are available online in Supplementary data).

Besides the desired product, the after-reaction mixture contained 7-methylganosine 5′-thiophosphate (m^7GMPS) and minor amounts of 7-methylguanosine 5′-(1-thiopentaphosphate) which

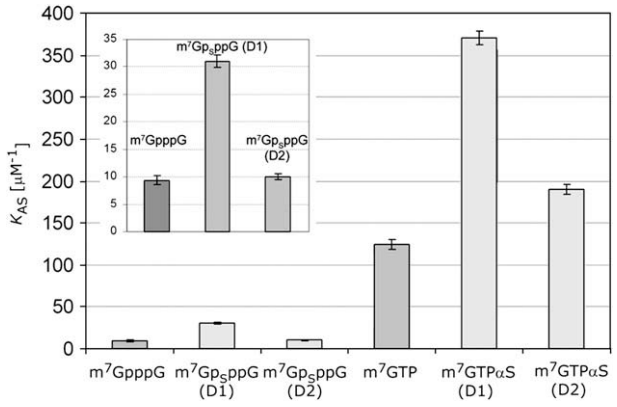


Figure 2. Comparison of the association constants for complexes of eIF4E with $m^7GTP\alpha S$, m^7Gp_SppG diastereomers and their parent analogs. Fluorescence titration measurements were carried out in 50 mM HEPES/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, 1 mM DTT at 20.0 ± 0.2 °C.

probably resulted from the attack of pyrophosphate on the cyclic intermediate. The Sephadex ion-exchange purification afforded $m^7GTP\alpha S$ diastereomeric mixture with 35% isolated yield. We encountered difficulties in preparative separation of diastereomers by RP HPLC, however, it was successfully accomplished with triethylammonium acetate as a mobile phase. The diastereomers, referred to as D1 and D2, according to their elution order during separation, were subsequently converted to sodium salts on Dowex resin. Their structures and homogeneities were confirmed by MS ESI(-), ^1H and ^{31}P NMR.[13] The absolute configurations of D1 and D2 were assigned as S_P and R_P, respectively, on the basis of H8 protons shifts in ^1H NMR,[13] as previously shown for similar compounds.[14]

The binding affinities of $m^7GTP\alpha S$ diastereomers for murine eIF4E were determined by fluorescence quenching titration.[15] The equilibrium association constants (K_{AS}) and free binding energies (ΔG°) of these analogs are shown in Table 1, together with the same data for m^7GTP, m^7GpppG and both diastereomers of m^7Gp_SppG. The most important finding is that the phosphorothioate moiety stabilizes also the binding of mononucleotides to eIF4E. For both isomers of $m^7GTP\alpha S$ the K_{AS} values are higher than that for the parent analog. The eIF4E ability to bind a cap analog is strongly dependent on its absolute configuration around P-stereogenic center: D1 isomer (S_P) shows more than 3-fold greater affinity to eIF4E than m^7GTP, whereas the affinity of D2 is only ~1.5-fold higher. We observed similar structure-affinity dependence previously, in case of m^7Gp_SppG diastereomers (Fig. 2).[10] This indicates that in case of both mono- and dinucleotides, an oxygen-to-sulfur substitution in the proS_P position is energetically favorable by $\Delta\Delta G^\circ$ ~−0.7 kcal/mol, whereas in proR_P it is less significant, not exceeding −0.25 kcal/mol.

The susceptibility to hydrolysis by human DcpS was tested with an HPLC-based assay (Fig. 3).[10] Cap analogs were subjected to enzymatic digestion by DcpS in conditions leading to complete degradation of m^7GpppG (positive control) within less than 10 min. We found that in analogous conditions m^7GTP is also hydrolyzed by DcpS, however, notably slower than m^7GpppG. On the contrary, both isomers of $m^7GTP\alpha S$ remained completely undigested by DcpS. Hence, the susceptibility to DcpS can be ordered as follows: $m^7GpppG > m^7GTP \gg m^7GTP\alpha S$ (D1 and D2).

The ability of cap analogs to inhibit cap-dependent translation was tested in a micrococcal nuclease treated rabbit reticulocyte lysate. In previously reported assays, the system was programmed with natural rabbit β-globin mRNA and the protein synthesis was determined by measuring the incorporation of [^3H] leucine.[8,9] In our study, an in vitro transcribed, ARCA-capped, β-globin 5′ UTR containing mRNA encoding firefly luciferase was used to allow determination of protein synthesis by luminometry. To verify the influence of this alteration on the reliability of the assay and to make our results comparable with the previous findings, in addition to m^7GpppG, we also included m^7GTP, m^7Gp_SppG (D1) and (D2) as reference compounds. The IC_{50} values for these compounds, obtained from our experiments, were different but in good correlation with those reported previously (see Table 1).

As expected, both $m^7GTP\alpha S$ isomers were more potent inhibitors of cap-dependent translation than m^7GTP. Although there were some variations between individual experiments, the general order from the most to the least potent inhibitor was always the same, that is, $m^7GTP\alpha S$ (D1) > m^7Gp_SppG (D1) ≈ $m^7GTP\alpha S$ (D2) > m^7Gp_SppG (D2) > m^7GTP > m^7GpppG. Representative experimental data for selected analogs are shown in Figure 4A. Analogously as for dinucleotides, $m^7GTP\alpha S$ (D1) was ~3-fold more inhibitory than its D2 counterpart, which is in agreement with its higher binding affinity for eIF4E from fluorescent titration measurements.

Strikingly, we also noticed that the inhibitory potency of unmodified cap analogs is diminished if they are preincubated in RRL before addition of mRNA to the system (See Supplementary Fig. S5). We performed another series of experiments, in which appropriate cap analog was added to RRL not together with mRNA (Fig. 4A), but 60 min prior to addition of mRNA (Fig. 4B). We observed that 60 min preincubation in RRL completely destroys m^7GpppG inhibitory properties up to 20 μM concentrations and also increases IC_{50} value of m^7GTP. The preincubation of either phosphorothioate cap analog did not alter their IC_{50} significantly, which suggests that they are much more stable in RRL.

To summarize our results, the new pair of m^7GTP analogs represents potent and enzymatically stable inhibitors of translation. The new assay conditions were developed to allow measurement of protein synthesis in RRL by luminometry and to assess stability of cap analogs in this cell-free system. Although the determined IC_{50} values differed from the reported ones, they correlated with them and with the binding affinity of individual analogs for eIF4E. The differences may result from combination of several reasons. First, the translation rate depends on many factors including the type of mRNA being translated, the pH, the ionic strength, the mRNA concentration, and the particular batch of reticulocyte lysate that is used.[16] Furthermore, by luminometry only full-length, properly folded luciferase is detected, whereas measurement of radioactively labeled amino acid incorporation may also include truncated or misfolded proteins, which would result in virtually higher IC_{50} values.

The stability of analogs in RRL, estimated indirectly via preincubation experiment, appears to correlate with their susceptibility to human DcpS. However, we have no evidence that DcpS is actually present in RRL, thus it may as well be an effect of other enzymatic activity. Still, we consider DcpS-resistance particularly important in the context of potential in vivo applications, since this decapping enzyme is responsible for degradation of compounds of this type in living cells.[11]

Recently, Graff et al. reported potent antitumor effects of antisense oligonucleotides targeting eIF4E encoding mRNA, thereby providing the first in vivo evidence that cancers may be more susceptible to eIF4E inhibition than normal tissues.[6] However, the ability of cap analogs to inhibit translation has not yet been demonstrated in vivo, even in cultured cells. One of the reasons may be that the analogs developed to date do not have sufficient stability in cellular environment. This thesis is supported by our results from preincubation experiments. We hope that the phosphorothioate analogs of m^7GTP offer a promising new solution to this problem.

Due to their stability, such analogs may be also useful in studying interrelations between cap-dependent and independent

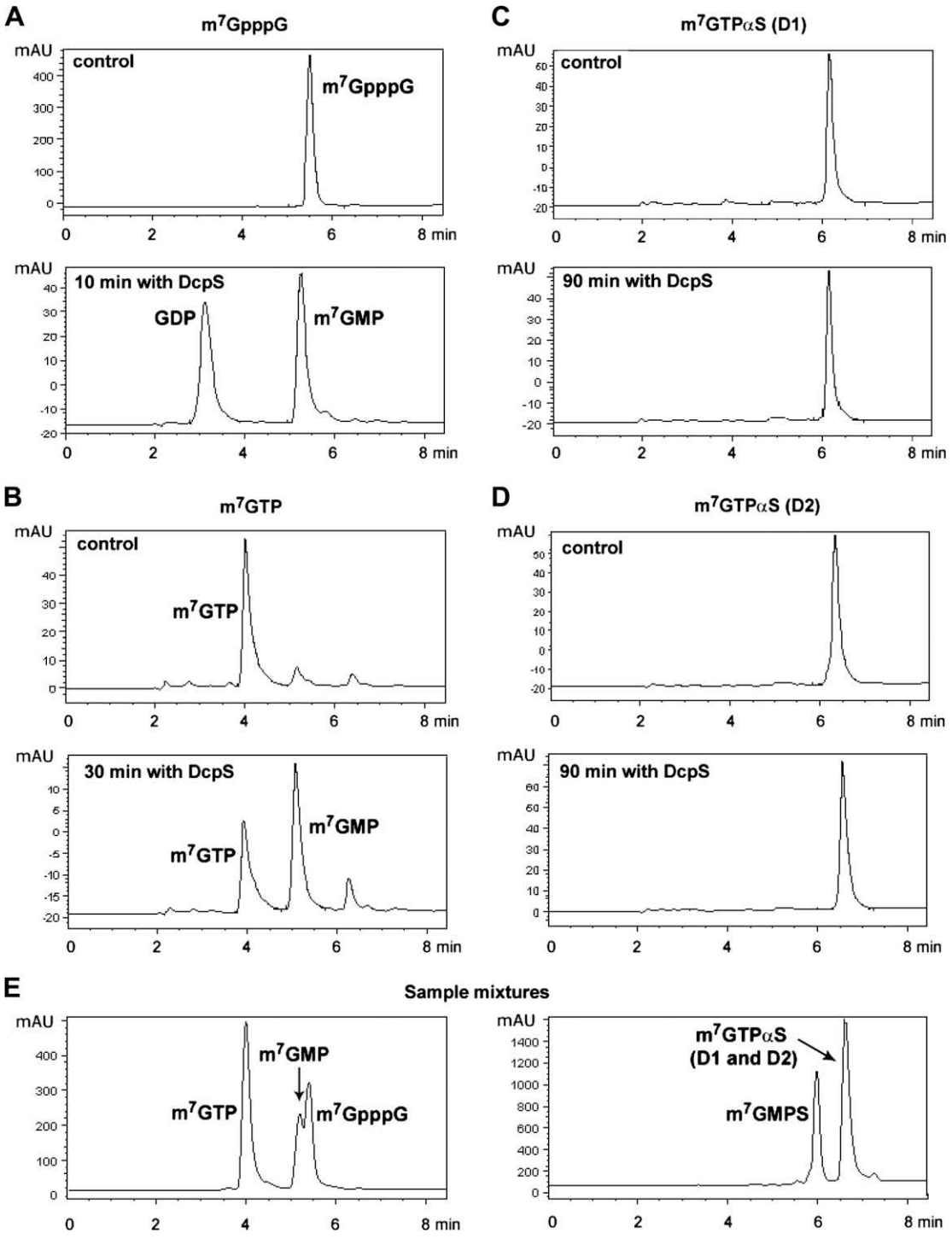


Figure 3. Example HPLC profiles from human DcpS susceptibility assay. Under the applied conditions (see Supplementary data) m^7GpppG was hydrolyzed by hDcpS in less than 10 min (A). m^7GTP was also hydrolyzed by hDcpS, but noticeably slower than m^7GpppG (B). m^7GTPαS (D1) and (D2) remained undigested by DcpS even after 90 min reaction time (C and D). E represents sample mixtures of substrates and expected degradation products—m^7GMP and m^7GMPS, respectively.

processes (e.g., IRES mediated translation) in various cellular and cell-free systems. Finally, they may be exploited as ligands for affinity isolation and purification of cap-binding proteins with decapping activity. In conclusion, we hope that these new compounds will prove useful for verifying the concept of exploiting cap analogs as translational inhibitors in living cells, helpful in designing new potential therapeutics as well as expand the spectrum of cap analog tools for studying cap-dependent processes.

Acknowledgements

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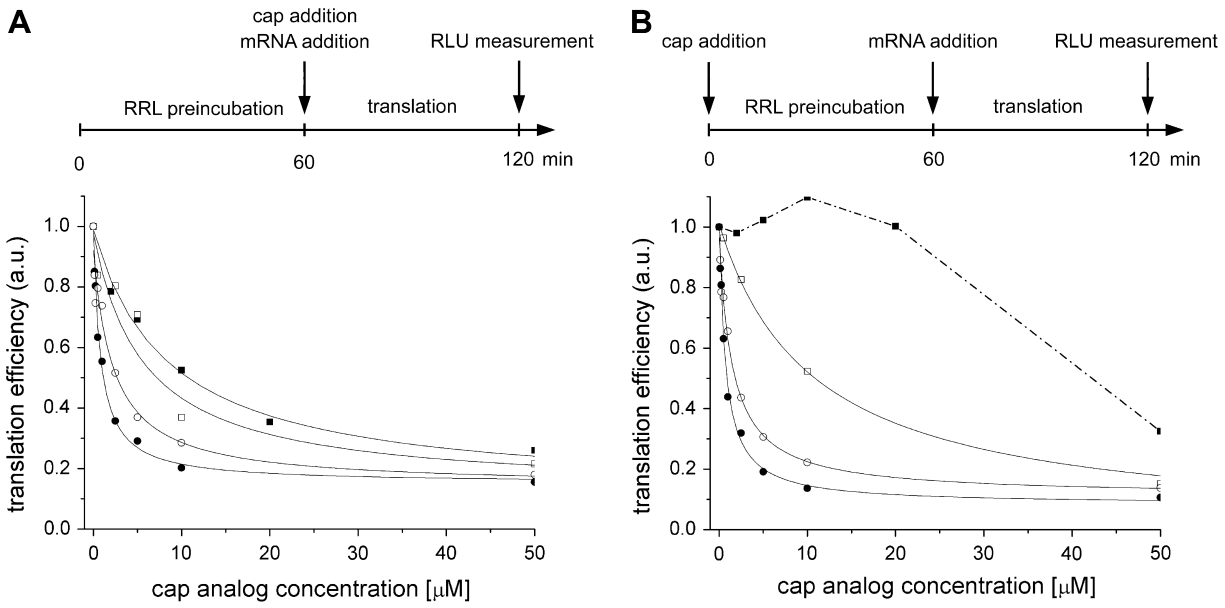

Figure 4. Inhibition of luciferase encoding mRNA translation in rabbit reticulocyte lysate by m^7GpppG (■), m^7GTP (□), m^7GTPαS (D1) (●) or m^7GTPαS (D2) (○). In experiment A the cap analog and mRNA were added to RRL at the same time point. In experiment B the cap analog was preincubated in RRL for 60 min prior to addition of mRNA. In both experiments the luciferase activity was detected by luminometry 60 min after addition of mRNA. It was observed that the 60 min preincubation in RRL completely destroyed inhibitory properties of m^7GpppG up to 20 μM concentrations, and increased IC_{50} value of m^7GTP, but did not alter IC_{50} of phosphorothioate cap analogs significantly.

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Supplementary data

Complete synthetic details. Details of DcpS susceptibility assay and inhibition of cap-dependent translation assay are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.053.

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13. m^7GTPαS (D1): ^{1}H NMR δ (ppm): 9.36 (1H, s, H8G); 6.08 (1H, d, H1′); 4.74 (1H, ~t, H2′); 4.63 (1H, ~t, H3′); 4.43 (1H, dd, H4′); 4.36 (2H, m, H5′, H5″); 4.10 (3H, s, CH_3). ^{31}P NMR δ (ppm): 44.97 (1P, d, Pα); −8.46 (1P, d, Pγ); −22.92 (1P, dd, Pβ). m^7GTPαS (D2) ^{1}H: 9.31 (1H, s, H8G); 6.09 (1H, s, H1′); 4.74 (1H, ~t, H2′); 4.57 (1H, ~t, H3′); 4.45 (1H, m, H4′); 4.43 (1H, m, H5′); 4.33 (1H, m, H5″,); 4.12 (3H, s, CH_3). ^{31}P: 44.05 (1P, d, Pα); −10.11 (1P, d, Pγ); −23.21 (1P, dd, Pβ). All spectra were recorded in D_2O at 25 °C. MS ESI (−) *m/z* ($M-H^-$): 552.01. Calcd for $C_{11}H_{17}N_5O_{13}P_3S$: 551.98.
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17. The synthesis was accomplished accordingly to the strategy described in Refs. 9a,b.