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Synthesis and biological validation of N^7-(4-chlorophenoxyethyl) substituted dinucleotide cap analogs for mRNA translation

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ABSTRACT

Design, synthesis and biological validation of dinucleotide cap analogs, N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) are reported. The effect of N^7-(4-chlorophenoxyethyl) substitution on cap analogs has been evaluated with respect to its in vitro transcription by using T7 RNA polymerase capping efficiency, and translational activity. The gel shift assay indicates that the new cap analogs (**5a**, **5b**) showed 77% and 76% capping efficiency respectively, whereas the standard cap analog, m^{7}G(5′)ppp(5′)G has a capping efficiency of 63%. The capping efficiency experiment clearly demonstrates that the N^7-modified analogs are good substrate for T7 RNA polymerase. It is noteworthy that the mRNA poly(A) capped with N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) was translated ~1.64-fold more efficiently, while compound (**5a**) was translated ~0.72-fold less efficiently than mRNA capped with standard cap analog. The observed low translation activity for (**5a**) could be due to stability in the form of dinucleotide cap analogs. Based on the substrate compatability of the N^7 modification in dinucleotide form, these new analogs may be used for structure function studies as well as protein production.

1. Introduction

M^{7}G-capped mRNA plays an important role in eukaryotic mRNA metabolism, such as mRNA processing, splicing, localization, nuclear transport, translation, and mRNA degradation pathways.[1–8] The unique cap molecular structure is necessary for the capped mRNA to bind the protein eukaryotic initiation factor eIF4E vital for gene express and cell metabolism. The cap binding protein possesses undisputed role in initiation of mRNA translation and subsequent protein synthesis. Specifically the cap binding protein eIF4E has become favorable target for anti-cancer studies. The recent findings on anti-cancer drug agents have focused on molecular design to increase the binding affinity of potential drug candidates to the protein eIF4E.[9–14] Potential drug candidates serve as inhibitors for the capped mRNA in cancer cell to bind with eIF4E, which results in interrupting abnormal cancerous cell proliferation and inducing apoptosis.[14] The capped mRNA, m^{7}GpppN contains a positive charge resulting from N^7-methyl substitution, which delocalizes over aromatic bicyclic rings of guanosine moiety. The positive charge plays an important role in stabilizing the cap structure, and thus, m^{7}G-capped mRNA results in increase in binding affinity of m^{7}G-capped mRNA to cap binding protein compared with G-capped mRNA without positive charge. The N^7-alkylation 5′-GMP derivatives displayed stronger binding affinity to eIF4E than m^{7}GMP, and served as competitive inhibitors of capped mRNA translation.[15,16] The N^7 modified 5′-GMP derivatives are utilized as effective inhibitors for the binding and interaction between m^{7}G-capped mRNA and cap binding protein. N^7-Substituted phenoxyethyl guanosine 5′-monophosphate derivatives have shown higher binding affinity for the protein eIF4E. In particular, 4-chloro/bromophenoxyethyl analogues exhibited IC_{50} values in nanomolar range, which is over 200-folds increase in binding affinity to protein eIF4E compared with m^7-GMP.[14] These results and our continuous interest to identify novel modified cap analogs exhibiting higher translational efficiency and longer half-life for mRNA,[17–19] promted us to synthesize N^7-(4-chlorophenoxyethyl) substituted dinucleotide form of cap analog and study its biological application. Herein, we report the first example for the synthesis of dinucleotide form of cap analog containing N^7-(4-chlorophenoxyethyl) moiety such as, N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) and their biological evaluation was compared with the standard cap analog.

2. Experimental

2.1. General

All commercial reagents and solvents are used as such without further purification. Guanosine-5′-diphosphate sodium salt and

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4-chlorophenyl-2-bromoethyl ether were purchased from Sigma–Aldrich. ^{1}H NMR spectra were obtained in D_2O on a Bruker 400 MHz and ^{31}P NMR were acquired on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). ESI mass spectra were recorded on Applied Biosystems/Scies MDX API 150 model and MALDI-TOF spectra were obtained on Applied Biosystems Voyager DE-PRO model. The crude reaction mixture was purified by using GE ÄKTA Purifier 900 (GE Healthcare, Piscataway, NJ, USA) with diethylaminoethyl (DEAE) Sepharose column, and purity of the fractions was determined by Waters Alliance 2695 HPLC with 2996 PDA detector (Waters Corporation, Milford, MA, USA) using Dionex DNAPac PA200 (4 x 250 mm) column with elution solvent system of ammonium phosphate mono basic (pH 2.8, pH 3.7).

Gel shift assay for the capping efficiency was conducted by utilizing MAXIscript T7 kit (Life Technologies Corporation), while IVT reactions were performed with linearized AmbLuc poly(A) DNA template with the use of MEGAcript™ kit (Life Technologies Corporation). Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Life Technologies Corporation) and associated vendor's protocol. Luciferase signals were detected using FLUO star Omega multi-mode microplate reader as per manufacturer's protocol (BMG Labtech).

2.2. Chemistry

2.2.1. Synthesis of Guanosine 5′-monophosphate (2a)

Synthesis of Guanosine 5′-monophosphate (**2a**) was accomplished by following the reported literature monophosphorylation[20] of Guanosine (**1a**) involving phosphorous oxychloride.[21]

2.2.2. Synthesis of N^7-(4-chlorophenoxyethyl)-guanosine 5′-monophosphate (3a)

To a mixture of guanosine-5′-monophosphate (**2a**) (0.463 g, 1 mmol, 1 equiv) in DMSO (4 mL) at 25 °C was added 4-chlorophenyl-2-bromoethyl ether (0.944 g, 4 equiv). The reaction mixture was stirred for 48 h at 55 °C, and was quenched by addition of water (50 mL). Aqueous layer was adjusted to pH 5.5 by 2.5 M NaOH, and washed with DCM (20 mL). The crude mixture was purified by FPLC (DEAE Sepharose FF media) by elution of linear gradient of 0–1 M TEAB, and the pure fractions with the product were pooled, evaporated, and dried to give N^7-(4-chlorophenoxyethyl) guanosine-5′-monophosphate TEA salt (**3a**) (0.192 g, 27.5%).

2.2.3. Synthesis of guanosine 5′-diphosphate imidazolide (Im-GDP) (4)

Imidazolide derivative of guanosine 5′-diphosphate (**4**) was synthesized from commercially available sodium salt of 5′-GDP by following reported procedure elsewhere. [22]

2.2.4. Synthesis of N^7-(4-chlorophenoxyethyl) G(5′)ppp(5′)G (5a)

To a mixture of N^7-(4-chlorophenoxyethyl) GMP (**3a**) (0.070 g, 0.1 mmol, 1 equiv) and GDP-imidazole (**4**) (0.048 g, 0.8 equiv) in DMF (2 mL) at 25 °C was added $ZnCl_2$ (0.068 g, 5 equiv). The reaction mixture was stirred for 6 h at 25 °C, and was quenched by addition of a solution of EDTA (0.054 g) in water (10 mL). Aqueous layer was adjusted pH 5.5 by 2.5 M NaOH, and washed with DCM (20 mL). The crude mixture was purified by FPLC (DEAE Sepharose FF media) by elution of linear gradient of 0–1 M TEAB, and the pure fractions with the product were pooled, evaporated, and dried to give of N^7-(4-chlorophenoxyethyl) G[5′]PPP[5′]G TEA salt . The TEA salt was dissolved into 2 mL of nuclease free water, and the resulting solution was poured into a solution of sodium perchlorate (0.040 g) in acetone (10 mL) and the resulting solid was separated by centrifuge, and was repeated above precipitate procedure once. The solid was washed with acetone (10 mL). The resulting white solid was dried by vacuum to give pure N^7-(4-chlorophenoxyethyl) G[5′]PPP[5′]G sodium salt (**5a**) (0.045 g, 45%). ^{1}H NMR (D_2O, 400 MHz): δ 7.92 (1H, s), 7.03 (2H, d, J = 8.8 Hz), 6.65 (2H, d, J = 8.8 Hz), 5.82 (1H, d, J = 3.2 Hz), 5.68 (1H, d, J = 6.0 Hz), 4.58 (2H, t, J = 5.6 Hz), 4.44 (1H, t, J = 4.0 Hz), 4.39–4.31 (4H, m), 4.29 (2H, m), 4.22 (2H, m), . 4.17 (4H, m), ^{31}P NMR (D_2O, 162 MHz): δ −11.5 (2P, d, J = 9.4 Hz), δ −23.1 (1P, t, J = 17.8 Hz). MS (m/z) 941 [M−H].

2.2.5. Synthesis of 3′-O-methylguanosine 5′-monophosphate (2b)

Synthesis of 3′-*O*-methylguanosine 5′-monophosphate (**2b**) was accomplished by following literature reported monophosphorylation[20] of 3′-*O*-methylguanosine (**1b**) involving phosphorous oxychloride (**1**).[21]

2.2.6. Synthesis of N^7-(4-chlorophenoxyethyl)-3′-O-methylguanosine 5′-monophosphate (3b)

To a stirred solution of 3′-*O*-methylguanosine 5′-monophosphate TEA salt (**2b**) (0.477 g, 1 mmol) in DMSO (4 mL) was added 4-chlorophenyl-2-bromoethyl ether (0.944 g, 4 mmol). The resulting reaction mixture was heated at 55 °C for 48 h, and quenched by addition of water (50 mL). Aqueous layer was adjusted to pH 5.5 using 2.5 M NaOH, and washed with DCM (20 mL). The crude mixture was purified by FPLC (DEAE Sepharose FF media) by elution of linear gradient of 0–1 M TEAB. The fractions containing pure product were pooled, evaporated, and dried to afford N^7-(4-chlorophenoxyethyl)-3′-*O*-methylguanosine 5′-monophosphate as triethylammonium salt (**3b**) (0.391 g, 55%). ^{1}H NMR (D_2O, 400 MHz): 7.10 (2H, m), 6.78 (2H, m), 5.9 (1H, m), 4.9 (2H, s), 4.35–4.45 (2H, m), 4.1–4.2 (1H, m), 3.9–4.1 (2H, m), 3.39 (3H, s), 3.11 (4H, q, J = 7.2 Hz), 1.19 (6H, t, J = 7.2 Hz). ^{31}P NMR (D_2O, 162 MHz): δ 0.57 (1P, s). MS (m/z) 530 [M−H].

2.2.7. Synthesis of N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (5b)

To a stirred solution of N^7-(4-chlorophenoxyethyl)-3′-*O*-methyl 5′-GMP, TEA salt (**3b**) (0.072 g, 0.1 mmol) and Im-GDP (**4**) (0.048 g, 0.08 mmol) in 2 mL of dry DMF, zinc chloride (0.068 mg, 0.5 mmol) was added under nitrogen atmosphere. The resulting reaction mixture was stirred for 72 h at 4 °C, and was quenched by addition of a solution of EDTA (0.054 g) in water (10 mL). Aqueous layer was adjusted to pH 5.5 using 2.5 M NaOH, and washed with DCM (20 mL). The crude mixture was purified by FPLC (DEAE Sepharose FF media) by elution of linear gradient of 0–1 M TEAB. The fractions containing pure product were pooled, evaporated, and dried to afford N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G as triethylammonium salt. The TEA salt was dissolved in nuclease free water (2 mL) and poured into a solution of sodium perchlorate (40 mg) in acetone (10 mL. The resulting solid was separated by centrifugation. The ion exchange procedure was repeated one more time. The solid thus obtained was washed with acetone (10 mL) and dried under vacuum to afford pure N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) as sodium salt (0.080 mg, 79%). ^{1}H NMR (D_2O, 400 MHz): δ 7.91 (1H, s), 6.99 (2H, dd, J = 6.8, 2.0 Hz), 6.62 (2H, dd, J = 6.8, 2.0 Hz), 5.79 (1H, d, J = 4 Hz), 5.67 (1H, d, J = 6.0 Hz), 4.58 (2H, t, J = 5.2 Hz), 4.41 (1H, t, J = 4.0 Hz), 4.39–4.25 (4H, m), 4.25–4.2 (1H, br), 4.20–4.10 (3H, br), 4.02 (1H, t, J = 5.2 Hz), 3.39 (3H, s). ^{31}P NMR (D_2O, 162 MHz): δ −11.5 (2P, m), δ −23.1 (1P, t, J = 19.4 Hz). MS (m/z) 955 [M−H].

2.3. Biology

2.3.1. Gel shift capping assay

The capping efficiency of modified cap analogue N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) was compared with the standard cap m^{7}G(5′)ppp(5′)G. The pTri β-actin template was used in an in vitro transcription reaction omitting pyrimidine nucleotides which results in the termination of transcription after the first 7 coded all purine nucleotides. Syntheses of the capped and uncapped oligoribonucleotides were performed by using the MAXIscript™ kit (Life Technologies Corporation) following manufacturer's protocol. Typically, 20 μl of the transcription reaction mixture contained the following final concentrations of components: linearized pTri β actin vector template, 25 ng/μl (0.5 μg total); ATP, 2 mM; GTP, 0.4 mM; standard cap or modified cap (**5a**, **5b**), 1.6 mM each in separate reaction; reaction buffer, 1X; T7 RNA Polymerase-Plus™, 20 units/μl; and (α-^{32}P) ATP, 800 (Ci/mmol). In the control reaction, no cap analog was added. The transcription reactions were incubated at 37 °C for 2 h. After that 10 μl of the reaction mixtures were applied to a 20% dPAGE gel. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare).

2.4. Transcription reaction

T7 RNA polymerase transcription was performed by using the MEGAscript™ kit (Life Technologies Corporation). All transcription reactions were performed in a 20 μl final volume at the following final concentrations of components: linearized AmbLuc poly(A) DNA, (1.0 μg total); 1X reaction buffer; ATP, UTP, and CTP, 7.5 mM each; and 50 units/μl of T7 RNA polymerase. Additionally, GTP was present at 7.5 mM in the no-cap control reaction; and reactions with cap analog included GTP at 1.5 mM and the cap analog (standard cap or modified cap **5a**, **5b**) at 6.0 mM. The transcription reactions were incubated at 37 °C for 2 h. In order to hydrolyze the remaining plasmid DNA, 1 μl of turbo DNase was added and the reaction mixture was further incubated at 37 °C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Life Technologies Corporation) as per manufacturer's protocol. The transcription yield of N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) was comparable with the standard cap.

2.5. Luciferase assay

The translational efficiency of dinucleotide cap N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) was determined by transfecting 5′-capped mRNA generated from transcription reaction into Hela cells. Cells were plated in 96-well plate one day before transfection with 8000 cells in 100 μl 10% FBS DMEM each well. A complex of 5′-capped RNA was prepared by mixing 100 ng (2 μl) of RNA, and 0.2 μl Lipofectamine 2000 transfection reagent by following manufacturer's protocol (Life Technologies Corporation, Catalog number 11668027), and incubated for 20 min at room temperature. The resulting complex was added onto cultured cells. The transfected plates were incubated at 37 °C. Cells were harvested and lysed at 4, 8, 16, 24, 32, and 40 h post-transfection. Luciferase production was measured with Dual-Light® Luciferase & β-Galactosidase Reporter Gene Assay System (Life Technologies Corporation, Catalog number T1003). Luciferase signals were detected using FLUO star Omega multi-mode microplate reader (BMG Labtech). The fold induction of luciferase protein data was normalized to the no cap, mRNA poly(A) transfection results (control reaction).

3. Results and discussions

All cellular eukaryotic mRNAs have at their 5′ end a unique structure (Fig. 1), known as a 'cap' which consists of a 7-methylguanosine linked via a 5′,5′-triphosphate bridge to the first transcribed nucleotide (m7GpppN, where N = G, A, C or U). The cap plays a crucial role in several cellular processes such as it promotes pre-mRNA splicing, enables transport of RNA from the nucleus to the cytoplasm, protects mRNA from degradation by 5′ exonucleases and is required for efficient translation.[1–8] During the initiation step of translation, the cap is specifically recognized by eIF4E (eukaryotic initiation factor 4E), the smallest subunit of a larger complex, eIF4F, which also consists of a RNA helicase, eIF4A, and a scaffolding protein, eIF4G.[23]

The prevailing method for generating capped mRNA using in vitro transcription employs a preformed dinucleotide of the form m^{7}G(5′)ppp(5′)G (standard mCAP) in excess over regular G in the transcription reaction, so that it is incorporated as the initial base in preference to regular G.[1-4] However, the drawback of standard mCAP analog is that the 3′ OH of either the G or m^{7}G can serve as the initiating nucleophile for transcriptional elongation leading to the synthesis of two isomeric RNAs of either forward or reverse form in approximately equal proportions depending upon the ionic conditions of the transcription reaction. The reverse form of capped mRNAs that is, G[5′]pppm^{7}G[pN]^n will not be recognized during the translation process, with only forward orientated sequences that is, m^{7}G[5′]pppG[pN]^n being translated.[24] The finding that the synthesis of two 'anti-reverse cap analogs' (ARCAs) such as m$_2^{7,3'\text{-}O}$G[5′]ppp[5′]G and m^7·3′dG[5′]ppp[5′]G are also exclusively incorporated only in the forward orientation because of modifications at the 3′ position of the N^7-methylguanosine ribose.[19,25,26] The biological activity of RNAs capped with N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-m$^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) were compared with the standard m^{7}G(5′)ppp(5′)G cap analog with respect to incorporation efficiency, in vitro transcription yield, and translational activity.

The reaction pathway leading to the formation of desired cap analogues **5a** and **5b** for the biological testing is depicted in Scheme 1. Generally preparation of N^7-alkyl GMP involves reaction between sodium salt of GMP and alkyl halide in organic solvents such as DMSO and DMF.[16] Alkylation of sodium salt of N^7-GMP invariably results in very low yields (~2%) of N^7-alkyl GMP.[14] We presumed that the low yield of N^7-alkylation 5′-GMP results from insoluble nature of nucleotide sodium salt in common organic solvents. It is to be noted that the presence of hydrophobic counter ion present in the nucleotide helps to solubilize in the organic medium.[7] Therefore, we tried the reaction using triethyl

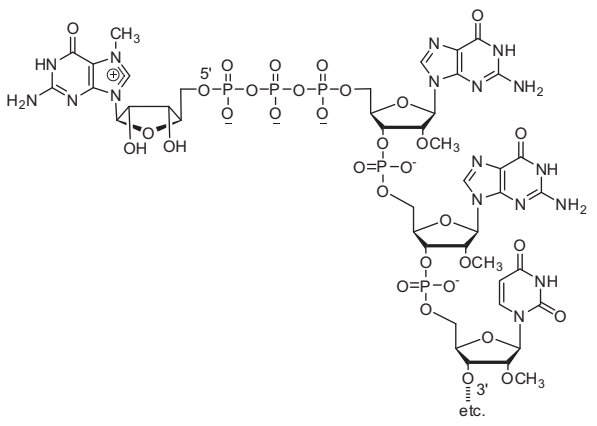
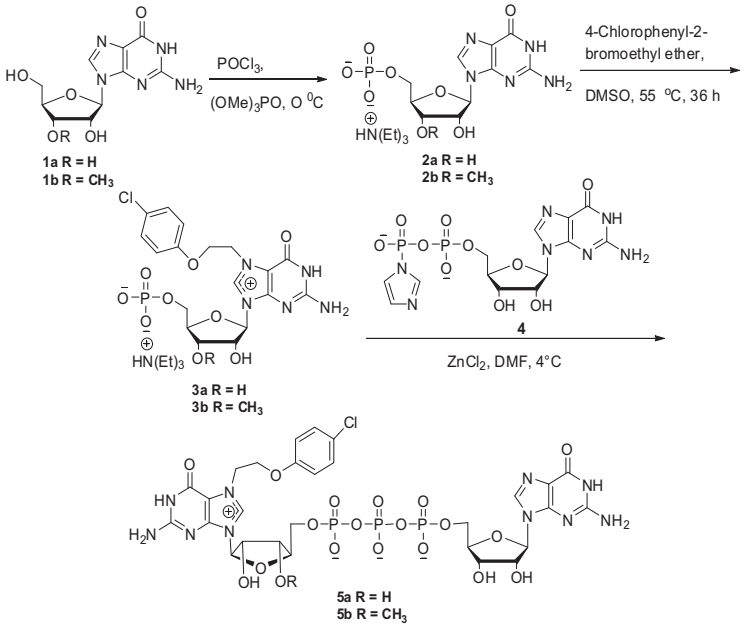


Figure 1. The chemical structure of the 5′ terminus of a capped mRNA.



Scheme 1. Synthesis of N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) cap analogues.

ammonium salt of guanosine 5′-monophosphate **2a** and 3′-*O*-methylguanosine 5′-monophosphate **2b**. Reactions of **2a** and **2b** with 4-chlorophenyl-2-bromoethyl ether in DMSO led to significantly improved yield of the corresponding N^7-(4-chlorophenoxyethyl) substituted GMP **3a** and **3b** in 28% and 59% yields, respectively. Finally, the coupling reaction of N^7-(4-chlorophenoxyethyl) substituted GMP **3a** and **3b** with Im-GDP (**4**) in the presence of anhydrous zinc chloride as a catalyst using DMF as a solvent afforded the corresponding cap analogues **5a** and **5b** in 45% and 79% yields, respectively.

Next, the capping efficiency of the modified cap analogs (**5a**, **5b**) with respect to standard cap analog during transcription was measured by gel shift assay (Fig. 2). pTri β actin vector was used to generate transcripts of 6 nucleotides in length. For gel shift assay, the reactions were performed in the presence of (α-^{32}P) ATP to internally label the transcript. Gel shift assay (Fig. 2) revealed 63% capping efficiency for standard cap analogue and 77%, 76% capping efficiency for the modified cap analogues (**5a**, **5b**). As can be seen from Figure 2, N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) and N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) capped mRNA migrates slower than the standard capped mRNA. This is essentially due to additionally increased molecular weight and bulky size of modified dinucleotide cap analogs (**5a**, **5b**). Observed facile incorporation of triphosphates and higher capping efficiency in the case of N^7-(4-chlorophenoxyethyl)-substituted cap analogues (**5a**, **5b**) over standard m^7G(5′)ppp(5′)G cap analogue might be due to increased activation of ribose 3′-hydroxyl group by bulky N^7-(4-chlorophenoxyethyl) substitution over N^7-methyl and 3′-*O*-methyl substitution on guanosine.

The ability of modified cap analogs (**5a**, **5b**) to be incorporated into 1.83 Kb mRNA was explored using AmbLuc poly(A) supercoiled plasmid (Life Technologies Corporation). Linearized plasmid, generated from the above plasmid by digestion with Blp 1 enzyme, was used as a template for in vitro transcription. The reaction was carried out in the presence of either modified cap analogs (**5a**, **5b**), or standard cap analog or no cap followed by the purification of the transcribed mRNAs using the MEGAclear™ Kit. The transcripts yield with T7 RNA polymerase indicates that modified cap analogs (**5a**, **5b**) is a good substrate similar to standard cap analog (Fig. 3).

The translational efficiency of modified cap analogs (**5a**, **5b**) was determined with respect to standard cap analog by transfecting capped mRNA that was transcribed from AmbLuc poly(A) vector with T7 RNA polymerase into HeLa cells. Cells were harvested

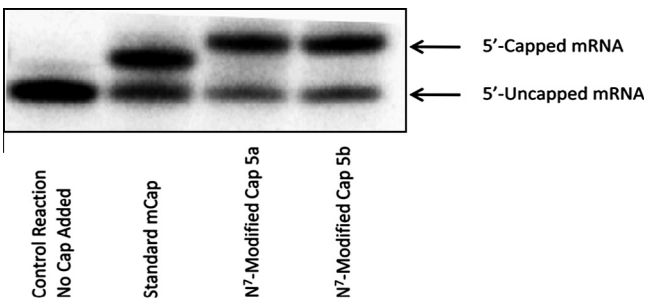


Figure 2. The capping efficiency of standard m^7G(5′)ppp(5′)G and N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**), N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) cap analogues by gel shift assay.

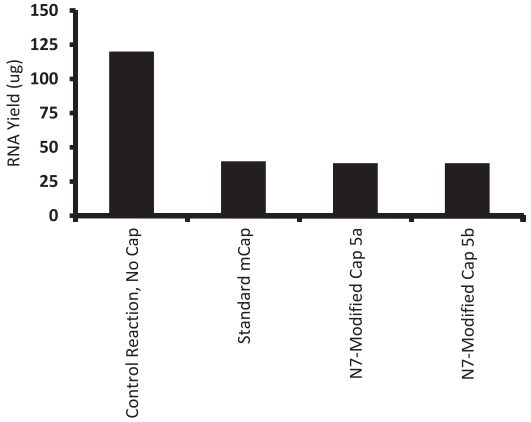


Figure 3. T7 RNA polymerase transcription reaction yields (quantified at A260 nm by using Nanodrop) involving standard m^7G(5′)ppp(5′)G cap and N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**), N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}$G(5′)ppp(5′)G (**5b**) cap analogues.

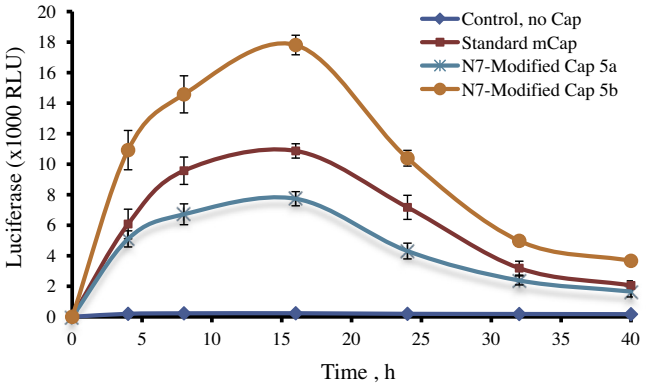


Figure 4. Translation efficiency of 5′-capped mRNA poly(A) from standard $m^7G(5')ppp(5')G$ cap and N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**), N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}G(5')ppp(5')G$ (**5b**) cap analogues. The standard mCAP, and modified cap (**5a, 5b**), 5′-capped in vitro transcribed poly(A) tailed luciferase RNA(100 ng) was transfected into HeLa cells. The fold induction of luciferase protein data was normalized to the control reaction that is, no cap, mRNA poly(A) transfection results.

and lysed at different time points of post-transfection and the protein production was measured in terms of luciferase activity. Luciferase activity data was normalized to the control reaction wherein uncapped mRNA was used. After 16 h of post-transfection, the luciferase activity of mRNA containing N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}G(5')ppp(5')G$ cap analog (**5b**) was almost ~1.64-fold higher than that of mRNA containing standard $m^7G(5')ppp(5')G$ cap analog. However, the N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**) dinucleotide form of the cap analog showed only ~0.72-fold than the mRNA capped with standard cap analog. The observed low translation activity for (**5a**) could be due to stability in the form of dinucleotide cap analogs. Both the longer life of modified cap analog (**5b**) capped transcripts and the absence of nonfunctional (reverse-capped) capped transcripts during translation could be responsible for the higher translational efficiency of modified cap analog (**5b**) capped mRNA (Fig. 4). The outcome of the effect of N^7-substituted phenoxyethyl guanosine in the form of dinucleotide form is quiet different from its N^7-substituted than phenoxyethyl guanosine 5′-monophosphate form. The N^7-substituted phenoxyethyl guanosine dinucleotide form (**5a**) and (**5b**) are substrate for RNA polymerase and recognized by eIF4E during translation process. The resulting translation efficiency is slightly lower for (**5a**), could be due to stability of the capped mRNA complex.

4. Conclusion

In conclusion, the synthesis of novel cap analogs, N^7-(4-chlorophenoxyethyl)-G(5′)ppp(5′)G (**5a**), N^7-(4-chlorophenoxyethyl)-$m^{3'\text{-}O}G(5')ppp(5')G$ (**5b**), that carry N^7 guanosine modification is reported. The new cap analogue (**5b**) generates exclusively forward-oriented capped mRNA during in vitro transcription. The new modified dinucleotide cap analogs (**5a**, **5b**) were shown to be a substrate for T7 RNA polymerase. Modified analog (**5b**) shows ~1.64-fold higher translational activity compared to standard cap analog that could be attributed to the presence of exclusive forward capped transcripts, strong binding affinity to the cap binding proteins and its increased cellular stability. Considering the biological compatibility of N^7 modified cap analogs (**5a**, **5b**) for both transcription and translational processes one can expect its application in protein production, anti-cancer and gene therapy.

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