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ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 880–884
Bioorganic & Medicinal Chemistry Letters

Synthesis and biological evaluation of trimethyl-substituted cap analogs

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Received 3 December 2007; revised 17 December 2007; accepted 19 December 2007
Available online 4 January 2008

Abstract—The N^7-methyl guanosine cap located on the 5′-terminus of mRNAs is important for a number of biochemical processes. A new dinucleoside triphosphate cap analog was synthesized with methyl groups on the N^7 of both guanine moieties, as well as the 3′-OH of one of the ribose moieties ($m_2^{7,3'O}$G[5′]ppp[5′]m^7G). The function of this trimethylated cap analog was compared with those of three other, less-methylated cap analogs: one omitting the ribose methylation (m^7G[5′]ppp[5′]m^7G), one omitting the N^7 methylation linked to the unmodified ribose ($m_2^{7,3'O}$G[5′]ppp[5′]G), and the standard cap analog, m^7G[5′]ppp[5′]G. These cap modifications were assayed with respect to their effects on capping efficiency, yield of RNAs during in vitro transcription, and the translational activity of these RNAs upon transfection into HeLa cells. The translational activity was monitored by measuring the luciferase activity of a luciferase-fusion protein produced from the in vitro synthesized RNAs. The RNA capped with the trimethylated analog ($m_2^{7,3'O}$G[5′]ppp[5′]m^7G) was translated the most efficiently, with ~2.6-fold more activity than the conventional cap (m^7G[5′]ppp[5′]G). The other two variants were also more efficient, generating, ~2.2 times (for the $m_2^{7,3'O}$G[5′]ppp[5′]G analog) and, ~1.6 times (for the m^7G[5′]ppp[5′]m^7G analog) more luciferase function than the conventional cap.

The nucleotide structure found at the 5′ terminus of many eukaryotic messenger RNAs, consisting of a 7-methylguanosine, connected, via a triphosphate bridge, to the 5′-end of the first transcribed nucleotide, resulting in m^7G(5′) ppp(5′)N, where N is any nucleotide. The 5′-terminal 'cap' structure, m^7G(5′)ppp(5′)N, that is present in most eukaryotic mRNAs, serves several functions. It facilitates their function in protein synthesis at the level of initiation.[1] The presence of the N^7-methyl group on the terminal guanosine promotes the formation of stable initiation complexes between mRNA and ribosomal 40S subunits.[2,3] The mRNA cap also plays an important role in processing, it protects the mRNAs from degradation by exonucleases and enables transport of RNAs from the nucleus to the cytoplasm. The prevailing method for generating capped mRNA using in vitro transcription employs a preformed dinucleotide of the form m^7G[5′]ppp[5′]G (mCAP) in excess over regular G in the transcription reaction, so that it is incorporated as the initial base in preference to regular G.[4–9] However, the drawback of this mCAP analog is that the 3′ OH of either the G or m^7G 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, i.e., G[5′]pppm^{7}G[pN]^n will not be recognized during the translation process, with only forward oriented sequences i.e. m^7G[5′]pppG[pN]^n being translated.[10] The recent finding that the synthesis of two 'anti-reverse cap analogs' (ARCAs) such as $m_2^{7,3'-O}$G[5′]ppp[5′]G and m^7,3′dG[5′]ppp[5′]G is also exclusively incorporated only in the forward orientation because of modifications at the 3′ position of the N^7-methylguanosine ribose.[11,12] In a subsequent study, it was also reported that chemical modification at either the 2′ or 3′ position of N^7-methylguanosine, the cap incorporated solely in the forward orientation, even though the 2′ OH group does not participate in the phosphodiester linkage.[13] As the N^7-methylguanosine cap is involved in diverse molecular events as stated above, another natural cap structure, the $m_3^{2,2,7}$G[5′]ppp[5′]G trimethylated cap, found on small nuclear RNAs is required for shuttling these RNAs between nucleus and cytoplasm, indicating the potentially powerful roles additional methyl groups

Keywords: Capping efficiency; In vitro transcription; Translation efficiency; Trimethylated cap analog; Luciferase activity; HeLa cells.
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doi:10.1016/j.bmcl.2007.12.049

could play.[14,15] In continuation of our efforts[16] to test the potential beneficial effects additional chemical modifications could play, we have synthesized a trimethylated cap analog, $m_2^{7,3'O}G[5']ppp[5']m^7G$, and studied it with other methylated cap variants incorporated into in vitro synthesized RNA. The variants we created were $m_2^{7,3'O}G[5']ppp[5']m^7G$ **7**, $m_2^{7,3'O}G[5']ppp[5']G$ **9**, and $m^7G[5']ppp[5']m^7G$ **10**. The biological activity of RNAs capped with these and $m^7G[5']ppp[5']G$ was studied with respect to incorporation efficiency, in vitro transcription yield, and translational activity. These three variants all hold an advantage over the conventional cap analog. Compound **10**, $m^7G[5']ppp[5']m^7G$, presents an identical cap structure to be recognized during translation regardless of its orientation. The cap variant $m_2^{7,3'O}G[5']ppp[5']G$ **9** is the anti-reverse cap analog (ARCA) currently supplied by Applied Biosystems. The 'anti-reverse' designation in ARCA refers to a modification at the 3′ position of the ribose attached to the methylated guanine where the OH group is replaced with an OCH_3 group. Because of this substitution, the RNA polymerase can only initiate transcription with the remaining 3′-hydroxyl group, thus forcing ARCA incorporation in the forward orientation. The compound **7**, $m_2^{7,3'O}G[5']ppp[5']m^7G$, is a new version of ARCA, in which one more methyl group is added at the N^7 position of the second guanine. As a result, unlike transcripts synthesized with conventional cap analog, 100% of the transcripts synthesized with our anti-reverse compounds **7** and **9** at the 5′ end should be translatable leading to a strong increase in translational yield.

The reaction pathway leading to the formation of desired $m_2^{7,3'O}G[5']ppp[5']m^7G$ **7** is shown in Scheme 1. The starting compound 3′-*O*-methylguanosine **1** was prepared as previously described.[17] Treatment of 3′-*O*-methylguanosine with $POCl_3$ in the presence of trialkyl phosphate afforded the corresponding 3′-*O*-methyl GMP **2**.[18] The imidazolide reaction of 3′-*O*-methyl GMP **2** with imidazole, triphenyl phosphine, and aldrithiol afforded the corresponding imidazolide salt **3** with 76% yield.[19] The phosphorylation reaction of 3′-*O*-methyl ImGMP with $(Et_3NH)_3PO_4$ in the presence of $ZnCl_2$ as the catalyst afforded the corresponding 3′-*O*-methyl GDP **4** with 65% yield.[19] The desired $m_2^{7,3'O}GDP$ **5** to make $m_2^{7,3'O}G[5']ppp[5']m^7G$ **7** was obtained by the methylation of 3′-*O*-methyl GDP **4** using dimethyl sulfate as the methylating agent with 78% yield. It is noteworthy that the methylation reaction is highly regioselective affording single m^7 methylated product, $m_2^{7,3'O}GDP$ **5**.[20] Treatment of $m_2^{7,3'O}GDP$ **5** with m^7ImGMP **6** in the presence of $ZnCl_2$ as the catalyst furnished the final $m_2^{7,3'O}G[5']ppp[5']m^7G$ **7** with 61% yield.[21,22]

The preparation of $m_2^{7,3'O}G[5']ppp[5']G$ **9** is depicted in Scheme 2. The coupling reaction of $m_2^{7,3'O}GDP$ **5** with ImGMP **8** took place in the presence of $ZnCl_2$ as the catalyst to form compound **9** with 70% yield.[21] The structure of compounds **7** and **9** was confirmed by ^1H and ^{31}P NMR and mass data.

In order to determine the capping efficiency of the methylated cap variants, compounds **7**, **9**, and **10** and conventional mCAP, were next tested in an in vitro transcription system. The template was a pTri β actin vector from the MAXIscript® Kit (Ambion, Inc.) and the products were analyzed by a gel shift assay.[23] Under the reaction conditions, of the four NTPs, only ATP and GTP were used, while CTP and UTP were omitted from the transcription reaction. Due to this omission only the six nucleotides at the 5′ end were transcribed by T7 RNA polymerase, producing a transcript of a short enough length to distinguish whether the cap or regular G had been incorporated. During the transcription reaction, GTP was included along with compounds **7**, **9**, **10** or conventional mCAP in presence of (α-^{32}P) ATP, and the control reaction was performed without any cap analog. The resulting transcription products (6-mer RNAs) were analyzed by 20% denaturing polyacrylamide/8 M urea gel electrophoresis. The outcome of the gel shift assay, shown in Figure 1, indicates that the capped RNAs migrate slower than uncapped RNA, allowing determination of the relative incorporation of cap versus unmodified G. All reactions were performed in triplicate and the capping efficiency was determined by quantitating the intensities of capped versus uncapped RNA using phosphorimagery. From these gel shift assays we calculate that conventional cap $m^7G[5']ppp[5']G$ has a capping efficiency of 61%, while two-way cap variant, compound **10**, has a 56% capping efficiency, the ARCA, compound **9**, has 52% capping efficiency, and compound **7**, which has a N^7 methyl on both guanine of the cap structure and a 3′-OCH_3 substitute, has a 51% capping efficiency. The capping efficiency experiment clearly indicates that the methylated cap variants were substrate for T7 RNA polymerase, although the capping efficiency of compounds **7** and **9** were slightly lower compared to the conventional mCAP. This could be because of the modification and the polymerase can still fit the dinucleotide in either orientation, but since only one led to a productive complex, the aborted complexes decrease yield or at least the speed of production.

To study the ability of these methylated cap variants to stimulate translation, all four compounds were used in a transcription reaction to generate a luciferase message.[24] The supercoiled plasmid, AmbLuc Poly(A) (Ambion, Inc.), used as directed, creates a luciferase mRNA with a 60-base poly(A) tail. This in vitro transcription was performed with compounds **7**, **9**, **10**, and conventional mCAP, along with a control reaction which contained no cap. The transcript containing 5′-capped and -uncapped mRNAs was purified by using the MEGAclear™ Kit (Ambion, Inc.). Transcripts produced with T7 RNA polymerase using compounds **7**, **9**, and **10** were of the predicted length (1.85 kb) and indistinguishable in size and homogeneity from those produced with either conventional mCAP or no cap analog. Analysis of each transcribed mRNA with poly(A) tail was performed on RNA chips in the Agilent 2100 Bioanalyzer, which revealed that no mRNAs were degraded and all were of similar size (data not shown). The Yield of the T7 RNA polymerase transcription reaction with compounds **7**, **9**, and **10**, conventional mCAP, and no cap analog is shown in Figure 2. All methylated