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Bioorganic & Medicinal Chemistry Letters 15 (2005) 2177–2180
Bioorganic & Medicinal Chemistry Letters

Synthesis and evaluation of potential inhibitors of eIF4E cap binding to 7-methyl GTP

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Received 9 August 2004; revised 25 January 2005; accepted 27 January 2005
Available online 18 March 2005

Abstract—Cap-dependent translation is initiated by the binding of eIF4E to capped mRNA (m^7GpppN). We have prepared a small library of 7-methyl guanosine nucleoside and nucleotide analogs and evaluated their ability to inhibit eIF4E binding to 7-methyl GTP with a competitive eIF4E binding immunoassay. 5′-*H*-Phosphonate derivatives in which the 2′- and 3′-riboside hydroxyls were tethered together by an isopropylidene group were shown to be a new class of inhibitors of eIF4E binding to capped mRNA.

In eukaryotes, eukaryotic initiation factor 4E (eIF4E) plays a key role in initiation of the translation process by recognition of the 5′ terminal mRNA cap structure, 7-methyl G(5′)ppp(5′)X (X = any nucleoside). A strong interaction between the 5′-cap of mRNAs and eIF4E is required for initiation of translation.[1] Formation of the eIF4E—cap mRNA complex is considered to be rate limiting for translation initiation under most circumstances[2] and its up-regulation is associated with cell growth, tumorgenicity and inhibition of apoptosis.[3,4] Recently, inhibition of cap-dependent translation by the ectopic expression of the eIF4E repressor protein, 4E binding protein 1 (4E-BP1), was shown to reduce breast cancer tumorgenicity and resistance to apoptosis.[4] These findings have renewed interest in the development of pharmacological tools and assays capable of modulating and monitoring translation initiation in general and eIF4E binding to capped RNA in particular.[5,6] In this report, we describe the synthesis and evaluation of the ability of a small library of 7-methyl G nucleoside and nucleotide analogs to inhibit eIF4E binding to 7-methyl GTP with an immunoassay.

A library of 29 members was assembled. The triphosphates **1**–**3** and nucleosides **7**–**9** were obtained from commercial sources. The monophosphates **10**–**13** were prepared from guanosine monophosphate as previously described.[7] The synthesis of the m^7G derivatives **14**–**29** is summarized in Scheme 1. Guanosine was first treated with dimethoxy propane and toluene sulfonic acid (TsOH) to yield 2′,3′-isopropylidene guanosine, **16**. Next, **16** was either alkylated with the appropriate alkyl or aryl halide to yield compounds, **17**–**19**, or treated with diphenylphosphite and pyridine to afford the protected *H*-phosphonate of guanosine, **20**. Compounds **21**–**24** were obtained from alkylation of **20** by a procedure similar to that employed to synthesize compounds **17**–**19**. Since removal of the isopropylidene from compounds **21**–**24** resulted in significant depurination, compound **14** was obtained by deprotection of compound **20** Dowex 50W resin, followed by alkylation of compound **13** with benzyl bromide. The amino acid phosphoramidates of compound **20** were prepared by oxidation with timethylsilyl chloride (TMSCl) and I_2, followed by the addition of the carbomethoxy amino acid. The carbomethoxy group was removed by treatment with sodium hydroxide, yielding compounds **25**, **26**, **28**, and **29**.

Unlike previous assays[6–8] that have monitored indirectly eIF4E antagonism in the presence of accessory proteins, we have successfully developed a high throughput procedure for the direct measurement of the inhibition of eIF4E binding capped mRNA in tissue extracts.[9] Typically, eIF4E from pooled cell extracts[10] was co-incubated with variable concentrations of the potential inhibitors in the presence of 7-methyl-GTP agarose

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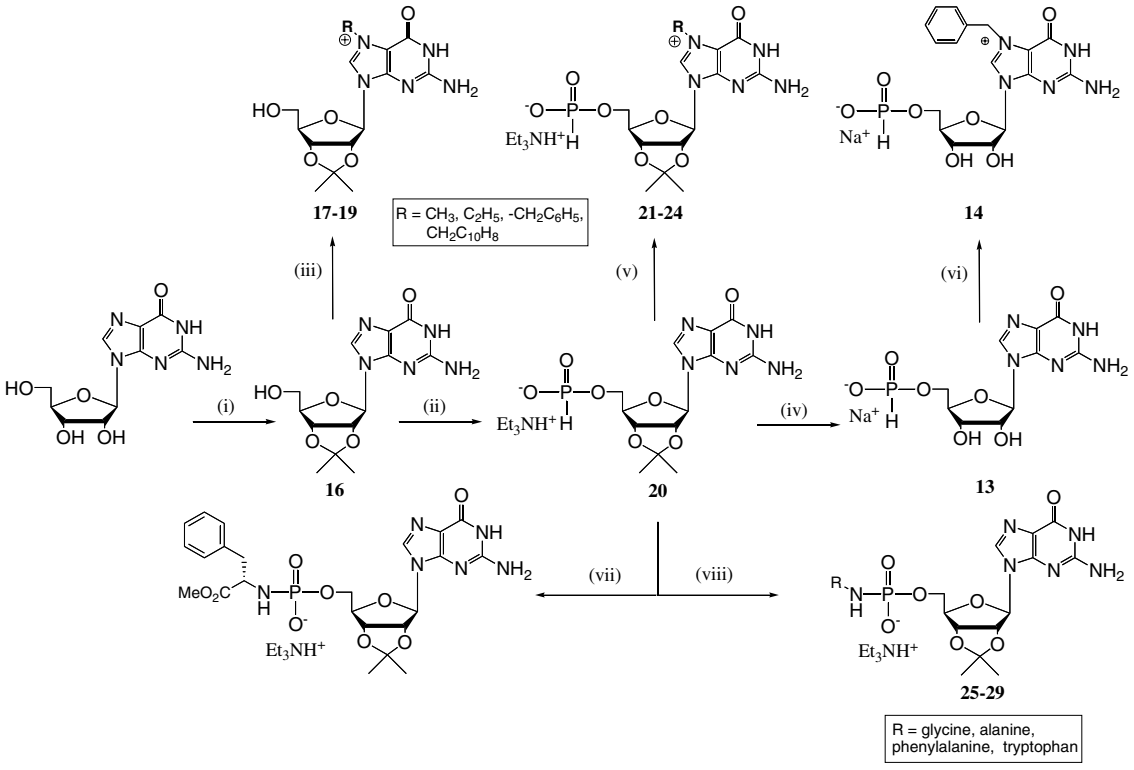
doi:10.1016/j.bmcl.2005.01.080



Scheme 1. Reagents and conditions: (i) Guanosine·xH$_2$O, 2,2-dimethoxy propane, TsOH, acetone, 63%; (ii) diphenylphosphite, Py, rt, 6 h; H_2O, Et_3N, 0.5 h; silica column, $CHCl_3$–MeOH–H_2O (5:2:0.25) + 1% NH_4OH, 44%; (iii) RI, R = CH_3, C_2H_5, $C_6H_5CH_2$, $C_{10}H_8CH_2Br$–DMF, rt, 18–24 h; (iv) 20, Dowex 50W × 4 resign, H_2O, 8 h, rt, silica column, $CHCl_3$–MeOH–H_2O (5:2:0.25) + 1% NH_4OH, 58%; (v) RI, R = CH_3, C_2H_5, or $C_6H_5CH_2Br$–DMF, rt, 7–24 h; (vi) $C_6H_5CH_2Br$, DMF, rt, 48 h; 0.5 mL DMSO, rt, 24 h, 15%; (vii) (a) TMSCl, Py, I_2, Et_3N, (AA)-OMe·HCl, argon, rt, 20–24 h; (viii) (vii); 1 N NaOH, rt, 2 h.

beads. After thorough washing to remove unbound proteins, bound eIF4E was assayed by eluting the beads with 7-methyl-GTP followed by spot immunoblot analysis (Fig. 1a). As can be seen for the representative inhibitor compound **24**, the optical densities determined by densitometry were plotted and the IC_{50} values extracted (Fig. 1b, Table 1).

The IC_{50} for 7-methyl GTP (**1**) was similar to the previously reported K_i value for inhibition of in vitro translation (5 vs 4.39 μM)[8] (Table 1). While loss of the 7-methyl group reduced the potency by greater than 80-fold, no loss of potency was observed for the 7-methyl 2′-deoxy GTP. This is consistent with the placement of the 7-methyl guanosine cation in a deep hydrophobic pocket composed of three tryptophans, while the 2′- and 3′-hydroxyl groups are solvent exposed. None of the nucleosides, compounds **4**–**9**, exhibited binding affinity for eIF4E, establishing that effective binding by a 7-methyl GTP analog is likely dependent on having at least one 5′-phosphate group. Indeed, binding could be recovered when the 7-methyl G analog, **9** (IC_{50} = >400 μM), was converted to the respective monophosphate, **10** (IC_{50} = 50 μM). Increasing the steric bulk at N-7 by substitution with either ethyl, allyl, or benzyl groups reduced inhibitor potency from 2- to 4-fold. This is in contrast to K_i values previously reporting a 3.5-fold increase in potency for

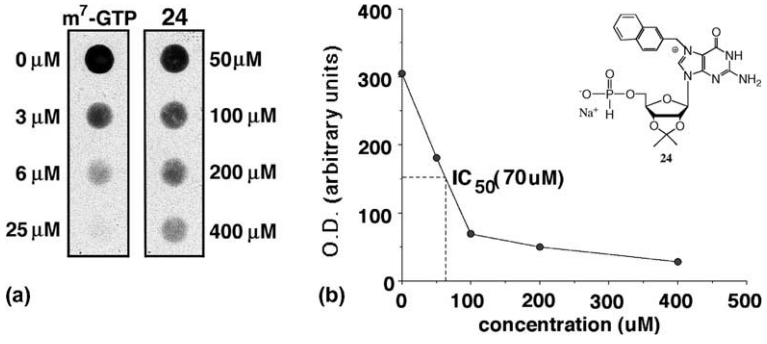
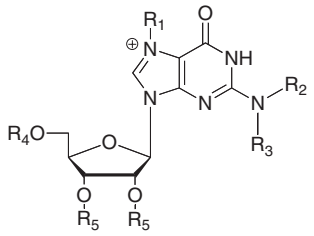


Figure 1. eIF4E binding assay for **24**.[9]

Table 1. Inhibition data for 7-methyl guanosine nucleoside and nucleotide analogs[a]



No.	R_1	R_2	R_3	R_4	R_5	IC_{50}[b] (μM)
1	$-CH_3$	–H	–H	$(-P(=O)(-O^-Na^+)_2)_3$	–H	5
2	—	–H	–H	$(-P(=O)(-O^-Na^+)_2)_3$	–H	>400
3	$-CH_3$ (2′-deoxy)	–H	–H	$(-P(=O)(-O^-Na^+)_2)_3$	–H	4
4	—	$-CH_3$	–H	–H	–H	>400
5	—	$-CH_3$	$-CH_3$	–H	–H	>400
6	—	–H	*p*-CH_3–PhSH–	–H	–H	>400
7[c]	$-CH_3$	–H	–H	–H	–H	>400
8[d]	$-CH_3$	–H	–H	–H	–H	>400
9	$-CH_3$	–H	–H	–H	–H	>400
10	$-CH_3$	–H	–H	$-P(=O)(O^-Na^+)_2$	–H	50
11	$-C_2H_5$	–H	–H	$-P(=O)(O^-Na^+)_2$	–H	100
12	$-CH_2(CH=CH)$	–H	–H	$-P(=O)(O^-Na^+)_2$	–H	220
13	$-CH_2C_6H_5$	–H	–H	$-P(=O)(O^-Na^+)_2$	–H	220
14	—	–H	–H	$-P(H)(=O)(O^-Na^+)$	–H	130
15	$-CH_2C_6H_5$	–H	–H	$-P(H)(=O)(O^-Na^+)$	–H	200
16	—	–H	–H	–H	$-C(CH_3)_2$	>400
17	$-CH_3$	–H	–H	–H	$-C(CH_3)_2$	>400
18	$-C_2H_5$	–H	–H	–H	$-C(CH_3)_2$	>400
19	$-CH_2C_6H_5$	–H	–H	–H	$-C(CH_3)_2$	>400
20	—	–H	–H	$-P(H)(=O)(O^-Et_3NH^+)$	$-C(CH_3)_2$	180
21	$-CH_3$	–H	–H	$-P(H)(=O)(O^-Na^+)$	$-C(CH_3)_2$	55
22	$-C_2H_5$	–H	–H	$-P(H)(=O)(O^-Na^+)$	$-C(CH_3)_2$	45
23	$-CH_2C_6H_5$	–H	–H	$-P(H)(=O)(O^-Na^+)$	$-C(CH_3)_2$	85
24	$-CH_2C_{10}H_8$	–H	–H	$-P(H)(=O)(O^-Na^+)$	$-C(CH_3)_2$	70
25	—	–H	–H	$-P(=O)(O^-Na^+)NHCH_2C(=O)O^-Na^+$	$-C(CH_3)_2$	>400
26	—	–H	–H	$-P(=O)(O^-Na^+)NH(CH_2)_2C(=O)O^-Na^+$	$-C(CH_3)_2$	400
27	—	–H	–H	$-P(=O)(O^-Na^+)NHCH(CH_2C_6H_5\text{-}(C(=O)OCH_3$	$-C(CH_3)_2$	>400
28	—	–H	–H	$-P(=O)(O^-Na^+)NHCH(CH_2\text{-}3\text{-indolyl.})C(=O)O^-Na^+$	$-C(CH_3)_2$	>400
29	—	–H	–H	$-P(=O)(O^-Na^+)NHCH_2(4\text{-benzoyl})C(=O)O^-Na^+$	$-C(CH_3)_2$	400

[a] All compounds were characterized by ^1H, ^{13}C, and ^{31}P NMR and HRMS.
[b] The IC_{50} (in μM) is the concentration necessary to inhibit 7-Me-GTP binding to eIF4E by 50%. Percent variance was within 20%.
[c] 7-Methylinosine.
[d] 7-Methyl-6-thioguanosine.

7-benzyl GMP relative to 7-methyl GMP when assayed as inhibitors of in vitro translation. This discrepancy may stem from differences in the conformation of eIF4E bound to 7-methyl GTP resin and when actively engaged in translation.[11]

Since nucleoside 5′-monophosphates are not stable in vivo and are not cell permeable, we prepared *H*-phosphonate analogs, **14** and **15**, as potential phosphate mimics.[12] No significant difference in the binding affinity of 7-benzyl GMP (Table 1, **13**) an 7-benzyl G *H*-phosphonate (**15**) was observed, thus confirming the ability of this substitution to replace 5′-monophosphate. Surprisingly, the *H*-phosphonate of guanosine (Table 1, **14**) exhibited nearly 2-fold greater potency than 7-benzyl G *H*-phosphonate (Table 1, **15**). Since the 2′- and 3′-hydroxyl groups of 7-methyl GTP do not interact with eIF4E, we chose to examine the role of the ribose ring conformation on the binding of potentially cell permeable *H*-phosphonates by constraining the ring with an isopropylidene group. While this modification had little effect on the activity of the *H*-phosphonate of guanosine (Table 2, **14** vs. **20**), the potency of the isopropylidene **23** was enhanced over 2-fold when compared to that for the unconstrained *H*-phosphonate, **15**. Replacement of the 7-benzyl group with either methyl or ethyl moieties further enhanced inhibitory potency by approximately 2-fold. Surprisingly, replacement of 7-benzyl with 7-naphthyl modestly enhanced binding affinity. The requirement for incorporating the *H*-phosphonate group was demonstrated by observing little or no activity for the corresponding nucleosides. Because phosphoramidates of nucleosides have been shown to be stable intracellularly, we proposed that they might serve as potential di- and tri-phosphate mimics, since they are dianions under physiological conditions. Nevertheless, none of the phosphoramidates (**25**–**29**) of compound **20** exhibited inhibitory activity.

In conclusion, a new assay for direct binding to eIF4E was developed and used to screen a small library of **29** 7-methyl guanosine nucleoside and nucleotide analogs. Constrained 5′-*H*-phosphonate derivatives were shown to be a new class of potential cell permeable inhibitors of eIF4E binding to cap mRNA and thus regulators of cap-dependent translation. Results of a cell based translational screen are underway and will be reported in due course.

Acknowledgements

This work was partially supported by a grant from NIH-NCI (U01-CA91220).

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9. *Experimental detail of the cap binding assay*: Up to **6** compounds were tested in triplicate in each run and compared to the reference compound, **7** methyl GTP, to assess their relative activity. Cell lysate generated from 3T3/4E cells using a freeze–thaw lysis method was prepared in advance and aliquots stored at −70 °C. Each sample required 20 μL of packed 7-methyl GTP agarose beads (Amersham Biosciences) pre-equilibrated with freeze–thaw lysis buffer. Beads were placed into micro-centrifuge tubes and 7-methyl GTP was added to form a set of standards. Test compounds were added in parallel to other sets of tubes. Freeze–thaw lysis buffer was added to the tubes to bring the volume in each tube to 150 μL. At the start of the binding step 50 μL of 3T3/4E cell lysate at a concentration of 3 mg/mL was added to each tube, which was placed in a horizontal rotating platform to mix for 2 h at 4 °C. Following the binding step, tubes were centrifuged to form a pellet, which was washed with 0.5 mL of freeze–thaw lysis buffer and centrifuged. The wash step was repeated once. Bound eIF4E was eluted from the beads by the addition of 250 μL of elution buffer (150 mM KCl, 25 mM Tris pH 7.5, and 100 μM 7-methyl GTP). The beads were centrifuged to form a pellet and 200 μL of each eluate was removed and spotted onto a nitrocellulose membrane using a vacuum dot blotting manifold. The membrane was blocked for 1 h with 5% NFD milk in TTBS buffer and probed with mouse anti-eIF4E (1:500, B D Biosciences) for 1 h. The blot was washed three times for 5 min each and then incubated with HRP conjugated Goat anti mouse IgG (1:2000, Sigma Chemical) for 1 h, rewashed and signal was visualized on film using ECL reagents (Amersham Biosciences). Densitometric analysis was performed by scanning and quantitating spot densities with Molecular Analyst software (Bio-Rad). Values were expressed in arbitrary optical density (OD) units.
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