Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 14, pp. 1647-1652, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00295-8

DECAPITATION OF A 5' CAPPED RNA BY AN ANTISENSE COPPER COMPLEX CONJUGATE

Brenda F. Baker,* Kanda Ramasamy,[a] and John Kiely[b]

Isis Pharmaceuticals, Inc., 2292 Faraday Ave., Carlsbad, CA 92008

Abstract: This report presents the synthesis and 5' cap reactivity of an antisense oligodeoxynucleotide equipped with two equivalents of an analog of N-(2-mercaptopropionyl)glycine (MPG). MPG is a peptide derivative which coordinates to copper(II) in solution as a monomeric species via the thiol, amide, and carboxylate groups. Decapitation of a complementary 5' capped RNA was observed for the oligonucleotide-ligand conjugate coordinated to copper. Copyright © 1996 Elsevier Science Ltd

Introduction

Cellular and viral messenger RNAs synthesized by RNA polymerase II have a unique chemical structure at their 5' termini[1] that is required in varying degrees for splicing and maturation of the transcript in the nucleus,[2] transport of the transcript from the nucleus to the cytoplasm,[3] transcript stability,[4] and the initiation of translation of the transcript to the encoded protein.[5] The 5' cap of a messenger RNA is a N7 methylated guanosine residue that is connected to the 5' terminus of the mRNA via a triphosphate linkage.[1b] Due to the function of the 5' cap in mRNA metabolism and its inherent chemical properties, we are exploring it as a target for antisense directed chemistries.[6,7] Antisense oligonucleotides equipped with moieties that react upon the triphosphate linkage or the methylated guanosine residue may yield an effective means to incapacitate a specific transcript and thus prevent protein expression.

N-(2-mercaptopropionyl)-glycine (MPG) is a peptide derivative[8] that coordinates to copper(II) in solution as a monomeric species via the thiol, amide, and carboxylate groups (Figure 1A).[9] Preliminary experiments showed that the MPG:Cu(II) complex acts as a RNA decapitating agent free in solution.[10] Although the complex demonstrated relatively low reactivity in comparison to OP:Cu(II),[7] other attributes[9] including ease of analog synthesis supported further evaluation of this type of complex.

In this initial study we evaluated an antisense oligonucleotide equipped with two equivalents of an analog of MPG, N-(2-mercaptoacetyl)glutamate, for its reaction upon the 5' cap structure of a complementary RNA (Figure 1B).

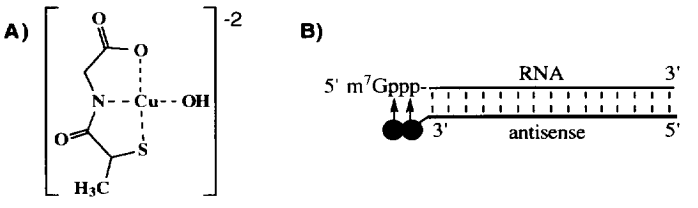

Figure 1. (A) MPG:Cu(II) (B) Antisense decapitation reaction.

[a]Current Address: ICN, 3300 Highland Ave., Costa Mesa, CA 92626.
[b]Current Address: Houghton Pharmaceuticals, 350 General Atomics Ct., San Diego, 92121.

Materials and Methods

Synthesis of α-Methyl-ε-(t-butyl)-$N_α$-(chloroacetyl)glutamate (**1**). α-Methyl-ε-(t-butyl)glutamate-HCl (0.5 g, 2 mmol, Bachem Bioscience Inc.) and triethylamine (0.6 mL, 4 mmol) were dissolved into 25 mL of dichloromethane and then added dropwise to a 15 mL solution of chloroacetyl chloride (0.2 mL, 2.2 mmol) in dichloromethane at 0 °C. The reaction was monitored by TLC (silica, EtOAc). The reaction was quenched with 50% saturated sodium bicarbonate upon completion. Product was recovered as a syrup from the dried dichloromethane layer. ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s, tBOC), 2.05 (1H, m, $COCH_2CH_2$), 2.20 (1H, m, $COCH_2CH_2$), 2.32 (2H, m, $COCH_2CH_2$), 3.78 (3H, s, OCH_3), 4.06 (2H, s, CH_2Cl), 4.61 (1H, m, CH), 7.28 (1H, s, NH).

Synthesis of α-Methyl-ε-(t-butyl)-N-(S-benzoyl-2-mercaptoacetyl)glutamate (**2**). Compound **1** was solubilized into 5 mL of THF and added dropwise to a solution of thiobenzoic acid (0.26 mL, 2 mmol) and TEA (0.6 mL, 4 mmol) in THF (0 °C). The reaction mixture was stirred for 6 h and then warmed to room temperature. 50 mL of diethyl ether was added and the solution was extracted three times with 50% saturated sodium bicarbonate. Crude product was recovered from the dry ether layer and purified by flash chromatography through silica using EtOAc:Hexane (1:3) as the solvent. ^{1}H NMR ($CDCl_3$) δ 1.41 (9H, s, tBOC), 1.96 (1H, m, $COCH_2CH_2$), 2.14 (1H, m, $COCH_2CH_2$), 2.30 (2H, m, $COCH_2CH_2$), 3.70 (3H, s, OCH_3), 3.85 (2H, s, CH_2S), 4.61 (1H, m, CH), 7.18 (1H, d, NH), 7.48 (2H, t, ArH), 7.60 (1H, d, ArH), 8.02 (2H, d, ArH).

Synthesis of α-Methyl-N-(S-benzoyl-2-mercaptoacetyl)glutamate (**3**). Compound **2** was dissolved into 10 mL of dichloromethane, purged with argon, and cooled to 0 °C. Trifluoroacetic acid (0.5 mL) was added dropwise to the solution. The reaction mixture was then stirred for 16 h at room temperature. Solvent and TFA were removed by rotovap. A white solid material was recovered from the syrup upon storage of the material overnight at -20 °C followed by trituration with EtOAc:Hexane (1:1). The compound was recrystallized from dichloromethane. Overall yield = 60% (H-Glu(OtBu)-OMe). TLC R_f = 0.65, EtOAc:Hex:EtOH (2.5:2.5:2.0); mp 105-106 °C; FAB (+) = 339; ^{1}H NMR (CD_3OD) δ 2.02 (1H, m, $COCH_2CH_2$), 2.22 (1H, m, $COCH_2CH_2$), 2.42 (2H, t, $COCH_2$-CH_2), 3.75 (3H, s, OCH_3), 4.32 (1H, m, CH), 7.55 (2H, m, ArH), 7.65 (1H, m, ArH), 8.02 (2H, d, ArH), 8.64 (1H, d, NH).

Synthesis of the N2 Modified Guanine Phosphoramidite. The N2-modified guanine phosphoramidite, 3'-O-[(N,N-diisopropylamino)(ß-cyanoethoxy)phosphanyl]-5'-O-(4,4'-dimethoxy-trityl)-6-O-di-phenylcarbamoyl-N_2-(3-trifluoroacetamidopropyl)-9-(2'-deoxy-ß-D-erythro-pentofuranosyl)-guanosine, was synthesized from the key intermediate 2-chloro-9-(2'-deoxy-ß-D-erythro-pento-furanosyl)-inosine by methods described previously for analogous amidites.[11]

Synthesis of Oligonucleotides. Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems 380B DNA synthesizer) by standard phosphoramidite chemistry. Commercially available phosphoramidites and 5' phosphorylating agents were purchased from Glen Research.

Synthesis of Oligodeoxynucleotide-MAG Conjugate. Compound **3** was activated for oligonucleotide conjugation chemistry as a N-hydroxysuccinimide ester by premixing the free acid **3** (100 mM, DMF) with EDC (200 mM, H_2O:DMF at 1:1) and NHS-sulfonate (200 mM, H_2O:DMF at 1:1) for 25 min at r.t. 10 μL of the activated acid mixture was then added to 10 μL of the amine modified oligonucleotide precursor (15 nmol) in 200 mM NaBicarbonate (pH 9.2). After 5 h at rt the conjugation reaction was complete as shown by PAGE analysis. The oligonucleotide-**3** conjugate product was separated from the free reagents and buffer utilizing a NAP25 column (Pharmacia). Conjugated ligand groups were then deprotected by incubation of the oligonucleotide conjugate in 50 mM NaOH for 12 h at 55 °C. Excess sodium hydroxide was removed using a NAP25 column and the deprotected oligonucleotide conjugate (**Antisense 1-G***) was recovered as dried material following speedvac.

Antisense Oligodeoxynucleotide Composition Analysis. The compositions of the unmodified oligodeoxynucleotide, **Antisense 1-G**; the amine modified oligodeoxynucleotide, **Antisense 1-G'** (where G' represents N2-(propylamine)-guanosine; and the oligodeoxynucleotide conjugate, **Antisense 1-G*** were determined by reverse phase HPLC analysis of the released nucleosides following complete nuclease digestion of 5 nmole of each oligomer.[12] Commercially available nucleosides (Sigma) and the modified nucleoside, N2-(propylamine)guanosine (K.R. Ramasamy), were used as standards.

*Metallation of **Antisense 1-G**.* 1 μL of 1M NaOH was added to 5 μL of a 1 mM aqueous solution of the oligonucleotide-ligand conjugate. 1.5 μL of 10 mM $CuSO_4$ was then mixed into the solution. After 2 min at rt an additional microliter of 6M NaOH was added. Five minutes later 15 μL of cold EtOH (-20 °C) was added which resulted in the formation of a precipitate(s). The precipitated material was collected by microcentrifugation, washed with 80% cold EtOH and then dried by speedvac. The oligonucleotide-ligand conjugate was resolubilized from the dried ethanol precipitated material with 10 μL of buffer (20 mM Hepes, 200 mM KCl, pH 7.4) and separated from the remaining insoluble material, presumably copper hydroxide, by microcentrifugation. 90% of the oligonucleotide-ligand conjugate was recovered by this procedure, as determined by UV spectral analysis.

Synthesis of RNA Substrates. The 5' capped 3' [^{32}P]-radiolabeled RNA substrate, m^{7}Gppp-GAGCUCCUC-UGCUACUCAGA^{32}pCp 3', was synthesized by enzymatic ligation[13] of the chemically synthesized 3' radiolabeled oligoribonucleotide, 5' pUCCUCUGCUACUCAGA-^{32}pCp 3', to the enzymatically synthesized 5' capped tetramer, 5' m^{7}GpppGAGC 3', using T4 RNA Ligase (Pharmacia). The 16mer was radiolabeled by ligation of cytidine 3',5'-[5'-^{32}P] bisphosphate (ICN) to the 3' terminus of the oligomer with T4 RNA ligase (Pharmacia).[13] The 3' radiolabeled product was PAGE purified prior to ligation to the 5' capped tetramer. The 5' capped tetramer was synthesized from a single stranded DNA template[14] using T7 RNA polymerase (Ambion). The 5' capped tetramer was purified by anion exchange chromatography.

The 5' uncapped 3' [^{32}P]-radiolabeled RNA, 5' HO-GAGCUCCUCUGCUACUCAGA^{32}pCp 3' was synthesized by ligation of cytidine 3',5'-[5'-^{32}P] bisphosphate to the 3' terminus of the chemically synthesized oligoribonucleotide, 5' GAGCUCCUCUGCUACUCAGA 3', using T4 RNA Ligase (Pharmacia). The 5' phosphorylated RNA, 5' p-GAGCUCCUCUGCUACUCAGA^{32}pCp 3', was enzymatically synthesized from the radiolabeled 5' HO-RNA using T4 polynucleotide kinase (Boehringer Mannhein).

The compositions of the 3' radiolabeled RNAs (5' capped and uncapped) were verified by base hydrolysis and RNase (T1 and Cl3) cleavage patterns.

Antisense 1G*:Cu(II) 5' Cap Cleavage Analysis. [^{32}P]-radiolabeled RNA was mixed with each oligonucleotide/reagent (10 μM/20 μM) in 10 mM Hepes (pH 7.4), 150 mM KCl, and 10 mM NaCl. Samples were preincubated at 50 °C for 5 min and cooled to room temperature. This step facilitated antisense hybridization to the RNA substrate whose sequence allowed for formation of a competing RNA hairpin/pseudoknot structure. Aliquots for the zero time point were removed and stored at -20 °C. Reaction mixes were incubated at 37 °C. Samples were analyzed by PAGE under denaturing conditions.

Results and Discussion

A post-conjugation strategy[15] was selected as the means to synthesize the oligonucleotide equipped with the MPG analog. A 20 nt antisense oligodeoxynucleotide which binds to the 5' terminal region of the cytokine induced ICAM-1 transcript[16] was functionalized at the 3' terminus with two N2-propylamine deoxyguanosine residues[11] for this purpose (5' TCTGAGTAGCAGAGGAGCTC**G'G'T** 3', where the RNA binding region is underlined and G' represents N2-(propylamine)-guanosine). Utilization of purine residues allowed for a means to span the double helix of the RNA:DNA duplex, as well as stabilize the placement of the attached reactive moiety through intrastrand base stacking.[17]

MPG was functionalized, for conjugation to the N2-propylamine groups, with a complementary carboxylic acid by replacement of the glycine portion with glutamate. The one chiral center of MPG was removed by replacement of the 2-mercaptopropionyl portion with a 2-mercaptoacetyl to give N-(2-mercaptoacetyl)glutamate (MAG). The ligation groups of MAG were protected during synthesis such that conjugation to the oligonucleotide occurred only through the gamma carboxyl group (Figure 2).

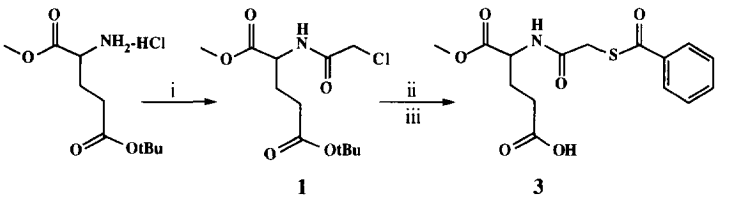

Figure 2. Synthetic scheme for the MPG analog, N-(2-mercapto-acetyl)glutamate (MAG), designed for conjugation chemistry.
(i) chloroacetyl chloride/CH_2Cl_2
(ii) thiobenzoic acid/TEA/THF
(iii) trifluoroacetic acid/CH_2Cl_2

The MAG precursor, **3**, was conjugated to the amine functionalized oligonucleotide via the N-hydroxysulfosuccinimide ester derivative. The ligating groups of the conjugated MAG moiety were deprotected by moderate base treatment (50 mM NaOH, 12 h at 55 °C). Conjugation chemistry was verified by PAGE

analysis, and by HPLC analysis of the released nucleosides following complete nuclease digestion of the oligonucleotide amine precursor (**Antisense 1-G'**) in comparison to the oligonucleotide-MAG conjugate (**Antisense 1-G***).[12]

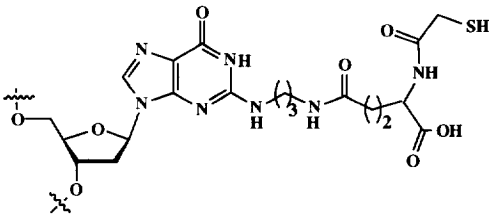

Figure 3. MAG modified residue, **G***.

Copper was coordinated to the conjugate by a method similar to that employed for MPG.[9a] The 5'-cap reactivity of the **Antisense 1-G*:**Cu(II) complex was determined using the complementary 5' capped 3' radiolabeled 20 nt oligoribonucleotide as substrate, *i.e.* corresponding to nt 1-20 of the hICAM-1 transcript.[16b] Antisense oligonucleotides were preannealed to the RNA substrate at 50 °C for 5 min in 10 mM Hepes (pH 7.4), 150 mM KCl, and 10 mM NaCl. Reaction mixtures were then incubated at 37 °C.

Analysis of the reaction mixtures by gel electrophoresis (Figure 4) showed a single RNA band (20%) with faster mobility after 120 h for the metallated antisense oligonucleotide conjugate, **1-G*:**Cu(II), lane 4. In this experiment the reactivity of the unmodified oligonucleotide, **Antisense 1-G**, in the presence of MPG:Cu(II) was utilized as a control. No reaction was observed in this control reaction, lane 2. RNA markers showed that the gel mobility of the RNA band generated in the **Antisense 1-G*:**Cu(II) reaction was similar to an uncapped RNA equivalent, lane 6.

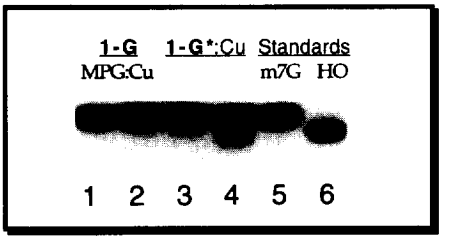

Figure 4. Decapitation of 5' capped RNA by **Antisense 1-G*:**Cu(II). Autoradiogram of acrylamide gel (20%, 1:20 crosslinkage, 40% urea). Lanes 1 and 2: m^7Gppp-RNA-^{32}pCp + 10 μM Antisense 1-G + 20 μM MPG:Cu(II) at t=0 and 120 h, respectively. Lanes 3 and 4: m^7Gppp-RNA-^{32}pCp + 10 μM Antisense 1-G*:Cu(II) at t=0 and 120 h. Lane 5: m^7Gppp-RNA-^{32}pCp. Lane 6: HO-RNA-^{32}pCp.

Additional experiments showed that uncapped RNAs (5' p-RNA and 5' HO-RNA) were not substrates for the antisense conjugate copper complex (96 h, 37 °C), which further demonstrated the 5' cap substrate specificity (*vs.* the RNA backbone)[18] of the reaction. Finally, no reaction was observed upon the 5'-capped RNA substrate by the unmodified oligonucleotide, **Antisense 1-G**, prepared through the metallation procedure.

These studies demonstrate that an antisense oligonucleotide equipped with an analog of the copper chelating agent, N-(2-mercaptopropionyl)glycine, reacts specifically upon the 5' cap structure of a target RNA in solution. Future research will focus on increasing the rate of the reaction via changes in the linker length and composition to optimize placement and positioning of the metal complex(es), and the metal ligands to optimize the stability and reactivity of the metal center for intracellular conditions.

Acknowledgements. ISIS Pharmaceuticals is grateful to the National Institutes of Health for support of this research through a Small Business Innovative Research Award (R44 AI30333-02A1). The authors also thank Maryann Zounes for the synthesis of the oligonucleotides, Todd Burckin for assistance in RNA analysis, and Len Cummins and Boyd Conklin for assistance in composition analysis of the conjugate.

References and Notes

1. (a) Banjeree, A. K. *Microbiol. Rev.* **1980**, 44, 175. (b) Shatkin, A. J. *Cell* **1976**, 9, 645. (c) Cory, S.; Adams, J. M. *J. Mol. Biol.* **1975**, 99, 519.

2. (a) Ohno, M.; Sakamoto, H.; Shimura, Y. *Proc. Natl. Acad. Sci. USA* **1987**, 84, 5187. (b) Edery, I.; Sonenberg, N. *Proc. Natl. Acad. Sci. USA* **1985**, 82, 7590. (c) Konarska, M. M.; Padgett, R. A.; Sharp, P. A. *Cell* **1984**, 38, 731.

3. (a) Hamm, J.; Mattaj, I. W. *Cell* **1990**, 63, 109. (b) Ohno, M.; Kataoka, N.; Shimura, Y. *Nucl.Acids Res.* **1990**, 18, 6989.

4. (a) Murthy, K. G. K.; Park, P.; Manley, J. L. *Nuc. Acids Res.* **1991**, 19, 2685. (b) Furuichi, Y.; LaFiandra, A.; Shatkin, A. J. *Nature* **1977**, 266, 235.

5. (a) Thach, R. E. *Cell* **1992**, 68, 177. (b) Kozak, M. *J. Biol. Chem.* **1991**, 266, 19867. (c) Hershey, J. W. B. *Ann. Rev. Biochem.* **1991**, 60, 717. (d) Rhoads, R. E. *Prog. Mol. Cell. Biol.* **1985**, 9, 104.

6. Baker, B. F. In *Antisense Research and Applications*; Crooke, S. T., LeBleu, B., Eds.; CRC Press: Boca Raton, 1993; pp 37-53.

7. Baker, B. F. *J. Am. Chem. Soc.* **1993**, 115, 3378.

8. Sóvagó, I. In *Biocoordination Chemistry: Coordination Equilibria in Biologically Active Systems*; Burger, K., Ed.; Ellis Horwood: New York, 1990; pp 168-171.

9. (a) MPG preferentially binds (or stabilizes) copper in one oxidation state, Cu(II); and shows a slow rate of dissociation from the divalent ion at pH 7.0. Sugiura, Y.; Hirayama, Y.; Tanaka, H.; Ishizu, K. *J. Am. Chem. Soc.* **1975**, 97, 5577. (b) MPG has a greater affinity for copper over other more abundant divalent cations, *e.g.* two orders of magnitude greater than zinc. Sugiura, Y.; Hirayama, Y. *Inorg. Chem.* **1976**, 15, 679.

10. A 5' capped internally radiolabeled RNA hybridized to its DNA complement was utilized to determine the 5' cap reactivity of MPG:Cu(II).[7] After 48 h at 37 °C a single RNA product, similar in gel mobility to the OP:Cu product, was observed in the amount of 56% at 1.5 mM MPG:Cu(II) and 25% at 0.15 mM MPG:Cu(II). The lower reactivity of MPG:Cu(II) relative to OP:Cu(II) is attributed to the net negative charge of the MPG:Cu(II) complex and the lack of "open" coordination sites for the triphosphate substrate.

11. (a) Ramasamy, K. S.; Bakir, F.; Baker, B. F.; Cook, P. D. *J. Heter. Chem.* **1993**, 30, 1373. (b) Ramasamy, K. S.; Zounes, M.; Gonzalez, C.; Freier, S. M.; Lesnik, E. A.; Cummins, L. L.; Griffey, R. H.; Monia, B. P.; Cook, P. D. *Tetrahedron Lett.* **1994**, 35, 215.

12. Kawasaki, A. M.; Casper, M. D.; Freier, S. M.; Lesnik, E. A.; Zounes, M. C.; Cummins, L. L.; Gonzalez, C.; Cook, P. D. *J. Med. Chem.* **1993**, 36, 831.

13. Romaniuk, P. J.; Uhlenbeck, O. C. *Methods Enzymol.* **1983**, 100, 52.

14. (a) Milligan, J. F.; Uhlenbeck, O. *Methods Enzymol.* **1989**, 180, 51. (b) Yisraeli, J.K.; Melton, D.A. *Methods Enzymol.* **1989**, 180, 49.

15. Goodchild, J. *Bioconj. Chem.* **1990**, 1, 165.

16. (a) Bennett, C. F.; Crooke, S. T. In *Advances in Pharmacology*; August, J. T., Anders, M. W., Murad, F., Coyle, J. T., Eds.; Academic Press: San Diego, 1994: Vol. 28, pp 1-43. (b) Ledebur, H. C.; Parks, T. P. *J. Biol. Chem.* **1995**, 270, 933.

17. Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984; pp 132-140.

18. Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, 116, 5981.

(*Received in USA* 30 *April* 1996; *accepted* 14 *June* 1996)