Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 10, pp. 1225-1230, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00201-1

PHOSPHOROTHIOATE OLIGONUCLEOTIDE METABOLISM: CHARACTERIZATION OF THE "N+"-MER BY CE AND HPLC-ES/MS

Lendell L. Cummins,* Michael Winniman, and Hans J. Gaus

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

Abstract: Unique phosphorothioate oligonucleotide metabolites of ISIS 2302 were isolated from pig kidney and characterized by capillary electrophoresis (CE) and high pressure liquid chromatography-electrospray mass spectrometry (HPLC-ES/MS) analysis. Fractionation by anion exchange HPLC followed by CE and HPLC-ES/MS analysis enabled facile identification of many metabolites, including a unique metabolite that has a slower migration time than intact ISIS 2302. The appearance of this slower migrating "N+" metabolite in CE analysis was correlated to three masses observed during HPLC-ES/MS analysis. These masses are consistent with the addition of a ribonucleotide or a phosphorothioate deoxyribonucleotide to intact ISIS 2302.

Introduction

In the past few years, oligonucleotides have become the focus of intense research regarding their use as antisense therapeutic agents. Much of the research has been directed towards the development of phosphate backbone analogs that provide enhanced metabolic stability relative to the naturally occuring phosphodiester species. Phosphorothioate oligonucleotides have emerged as promising analogs, as they have shown promise as therapeutic agents in a variety of studies.[1-8] Successful development of phosphorothioate oligonucleotides as therapeutics requires an in-depth understanding of their metabolism. Previous studies of phosphorothioate metabolism characterized the observed metabolites by HPLC, slab gel electrophoresis, or capillary gel electrophoresis.[9-13] The predominant metabolites observed in these studies were consistent with exonuclease degradation. It was expected that exonuclease activity would be a major metabolic pathway, but other unanticipated metabolites with an electrophoretic mobility slower than the intact oligonucleotide (N+mers) were also observed.[9,14] Given their slower mobility and spacing, it was hypothesized that these metabolites were the result of the addition of nucleotides to the intact oligonucleotide. It is also possible that alkylation of the phosphorothioate linkage would result in a net reduction of charge and have a similar effect upon mobility. Electrospray mass spectrometry (ES/MS) methods have been used to characterize oligonucleotides.[15-20] Recently, we have developed methods to further characterize phosphorothioate metabolites using HPLC-ES/MS.[21] In this report, we extend our mass spectrometry studies to the characterization of the n+ mer metabolites.

Animal Studies: Animal studies were carried out by H. T. I. Bioservices, Inc., Ramona, CA and conducted entirely under the FDA Good Laboratory Practice Regulations (21 CFR Part 58). Female Yucatan minipigs (60-80 kg) were dosed subcutaneously with ISIS 2302 at 300 mg/day for nine days. Organs were removed after the final dose and stored at -80 °C.

Oligonucleotide and metabolite extraction and purification: One pig kidney was homogenized using a commercial blender in 250 mL of 4 M guanidinium thiocyanate, 0.5% NP-40, 50 mM tris-HCl pH 9.0,

100 mM NaCl and 250 mL of phenol:chloroform (1:1). The aqueous layer was removed, and the phenol:chloroform layer was re-extracted with 250 mL of 15% NH_4OH. The aqueous layers were combined, extracted with 250 mL chloroform, and precipitated with 3 volumes of ethanol. The precipitated material was dissolved in H_2O and filtered through a 0.2 mm filter prior to HPLC purification. Anion exchange HPLC fractionation was accomplished using a BioCad HPLC system and a Poros II HQ/M 25.4 mm x 100 mm column (Perseptive Biosystems). Phosphorothioate oligonucleotide metabolites were separated using a linear gradient from 0 M NaBr, 10 mM tris-HCl, pH 9.0 to 1.5 M NaBr, 10 mM tris-HCl, pH 9.0 over 40 column volumes at a flow rate of 20 mL/min. Fractions containing phosphorothioate oligonucleotide and metabolites were collected (~45 mL each) and brought to ~ 0.1 M triethylammonium acetate (TEAA), pH 7.0 by the addition of 5 mL of 1 M TEAA. Each fraction was then desalted using a Poros II R/H 4.6 mm x 100 mm column (Perseptive Biosystems). The column was equilibrated with 0.1 M TEAA pH 7.0, and each anion exchange fraction was loaded manually using a 50 mL superloop (Pharmacia). After loading, the column was washed with H_2O until the conductivity trace showed no further decrease. The column was then washed with 1 mM triethylamine (TEA) until the pH trace stabilized (between pH 10 and 11). Bound phosphorothioate oligonucleotide and metabolites were then eluted with 20% acetonitrile. Eluted material was evaporated to dryness and resuspended in H_2O.

Capillary electrophoresis analysis: Desalted metabolite fractions were analyzed using a Beckman P/ACE 5010 capillary electrophoresis instrument. Electrophoresis was carried out using a ssDNA-R kit (Beckman Instruments). Capillaries were cut to 27 cm total length (20 cm to detector). Analyses were carried out with an applied voltage of 15 kV at 30 °C.

HPLC-ES/MS: Mass spectrometry was carried out essentially as described.[21] An HP 5989 quadrupole mass spectrometer with an extended mass range (2600 *m/z*) was employed in the negative ionization mode as described[21] for all studies. Electrospray was achieved using a HP 59987A source with a pneumatic nebulizer and a 70 psi flow of nitrogen gas. The curtain gas mixture consisted of 10 l/min nitrogen and 2 L/min oxygen. A step size of 0.1 amu over the mass range *m/z* 500 to 1200 was used during acquisition. Molecular masses were determined using the HP Chemstation analysis package. Manual evaluation was performed by comparing a table of calculated *m/z* values corresponding to expected metabolites with the peaks present in a given spectrum. Peaks closely matching the expected metabolite peaks were manually selected and deconvoluted using the manufacturer's mass deconvolution program.

Results and Discussion

Previous studies have shown the utility of HPLC-ES/MS for analysis of phosphorothioate oligonucleotides and their metabolites.[21] These studies demonstrated that ~15 metabolites could be detected and identified from a single spectrum. Analysis of such spectra, however, can be tedious, since many of the metabolite charge states overlap substantially.

ISIS 2302 and its metabolites were extracted from Yucatan minipig kidneys, and further purified by anion exchange chromatography. Because of their high affinity for strong anion exchange matrices, phosphorothioate oligonucleotides and metabolites are well separated from the endogenous proteins and nucleotides remaining after phenol:chloroform extraction and ethanol precipitation (data not shown).

In order to simplify mass spectrometry studies, fractions of the anion exchange separation were collected and further desalted as described in the experimental section. CE analyses of anion exchange

fractions 1, 2, 3, and 4 are shown in Figure 1. Fraction 1 contains metabolites estimated to be primarily from 13 to 17 nucleotides in length. Fraction 2 contains intact ISIS 2302 and shorter metabolites down to ~17 nucleotides in length. Fractions 3 and 4 show essentially the same metabolic profile as fraction 2, but a new metabolite species is also observed. This species, the "n+" metabolite, is first observed in fraction 3, and is more abundant in fraction 4.

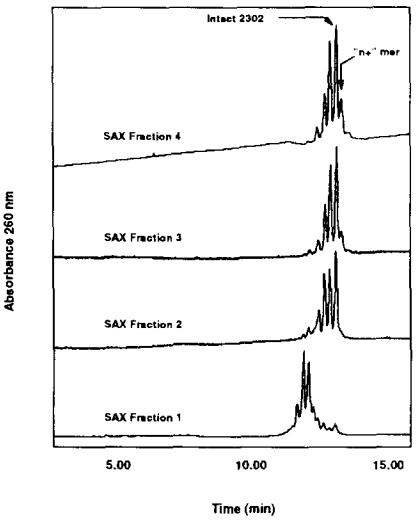

Figure 1. CGE analysis of SAX fractions

Anion exchange fractions 1, 2, 3, and 4 were analyzed by HPLC-ES/MS. The use of reversed phase HPLC prior to mass spectrometry resulted in a substantial reduction of the sodium and potassium adducts observed during mass spectrometry. The HPLC-ES/MS conditions utilized in this study are the same as previously reported.[21] The spectra of these fractions are shown in Figure 2, and the observed metabolite masses are shown in Table 1. Analysis of the spectrum for fraction 1 indicates that the masses observed are consistent with metabolites 13, 14, 15, 16, and 17 nucleotides in length. These masses suggest that the metabolites are generated by the action of either 3'- or 5'-exonucleases, or a combination of 3'- and 5'-exonucleases. Analysis of the spectrum for fraction 3 indicates that masses consistent with intact ISIS 2302 and its metabolites are present. These masses are consistent with exonuclease degradation. These correlate with the size of metabolites observed by CE analysis (Figure 1). The absence of shorter metabolites from the spectrum of fraction 2 is observed by comparing the 505 and 1114 *m/z* regions. In the spectrum for fraction 1, the -4 species for metabolite T (a 14-mer) is present, but examination of these same two regions in the spectrum for fraction 2 shows this species to be absent. Mass spectrometry analysis of fractions 3 and 4 shows a new set of peaks (X, Y, and Z) in the 1111 to 1118 *m/z* region with a charge state of -6 (Figure 2). Five charge states (-6 to -10) were found for these new peaks (Figure 2). Five charge states were also observed for metabolite T which shares a common *m/z* species at 1111, but these charge states were from -4 to -8 (Figure 2). Deconvolution of

Table 1: Oligonucleotide sequence, calculated mass and observed mass of SAX fractions 1-4 by HPLC-ES/MS. Numbers in brackets indicate the standard deviation.

Oligonucleotide	calculated mass	fraction 1: mass found	fraction 2: mass found	fraction 3: mass found	fraction 4: mass found
A: gcc caa gct ggc atc cgt ca	6369.24	-	6369.59 (3.1)	6369.73 (2.1)	6369.73 (2.8)
B: gcc caa gct ggc atc cgt c	6039.97	-	6038.17 (2.6)	6037.94 (2.8)	6038.59 (2.7)
C: gcc caa gct ggc atc cgt	5734.72	-	5733.91 (2.1)	5734.18 (2.1)	5734.13 (2.6)
D: gcc caa gct ggc atc cg	5414.45	-	5413.87 (1.5)	5413.62 (2.1)	5413.72 (2.5)
E: gcc caa gct ggc atc c and/or cc caa gct ggc atc cg	5069.18	5069.16 (2.8)	5068.55 (2.8)	-	-
F: gcc caa gct ggc atc and/or c caa gct ggc atc cg	4763.93	4762.56 (2.5)	-	-	-
G: gcc caa gct ggc at and/or caa gct ggc atc cg	4458.68	4456.10 (2.4)	-	-	-
M: cc caa gct ggc atc cgt ca	6022.59	-	6021.91 (3.1)	-	-
N: c caa gct ggc atc cgt ca	5718.72	-	5717.81 (1.8)	-	-
O: caa gct ggc atc cgt ca	5413.47	-	5413.87 (1.5)	5413.62 (2.1)	5413.72 (2.5)
P: aa gct ggc atc cgt ca	5108.22	5108.45 (2.6)	-	-	-
Q: a gct ggc atc cgt ca and/or caa gct ggc atc cgt and/or aa gct ggc atc cgt c	4778.94	4776.68 (1.7)	-	-	-
R: gct ggc atc cgt ca and/or a gct ggc atc cgt c	4449.67	4450.96 (2.1)	-	-	-
S: cc caa gct ggc atc cgt c	5693.56	-	5692.66 (1.1)	-	-
T: c caa gct ggc atc cgt c and/or cc caa gct ggc atc cgt	5389.44		5388.59 (1.9)	-	-
U: caa gct ggc atc cgt c and/or c caa gct ggc atc cgt	5084.19	5082. 23 (1.4)	-	-	-
V: cc caa gct ggc atc c	4723.90	4723.06 (2.2)	-	-	-
W: aa gct ggc atc cgt	4473.69	4471.61 (3.1)	-	-	-
X: c caa gct ggc atc c and/or cc caa gct ggc atc	4418.65	4418.85 (2.6)	-	-	-
I: gcc caa gct ggc atc cgt ca+c or gcc caa gct ggc atc cgt ca+t	6674.43	-	-	6673.47 (3.1)	6673.03 (3.1)
K: gcc caa gct ggc atc cgt ca+a	6698.45	-	-	6697.14 (2.5)	6697.09 (3.2)
L: gcc caa gct ggc atc cgt ca+g	6714.45	-	-	6713.27 (3.2)	6713.79 (3.1)

the spectrum generated for fractions 3 and 4 shows the presence of three metabolites with masses higher than that of ISIS 2302. The mass differences determined for these peaks are +303.3, +327.36, and +344.06 daltons greater than the mass for ISIS 2302 (mass differences were calculated by subtracting the observed mass for ISIS 2302 from the observed mass for species I, K, and L in anion exchange fraction 4, as shown in Table 1). These are consistent with the addition of phosphodiester cytosine or uridine, adenosine, or guanosine respectively. These masses are also consistent with the addition of the phosphorothioate analog of deoxycytidine, deoxyadenosine, or deoxyguanosine. The abundance of these three species is greater in the spectrum for fraction 4 than in the spectrum for fraction 3, in agreement with the increase in abundance of the "n+" peak observed in the CE analyses (Figure 1).

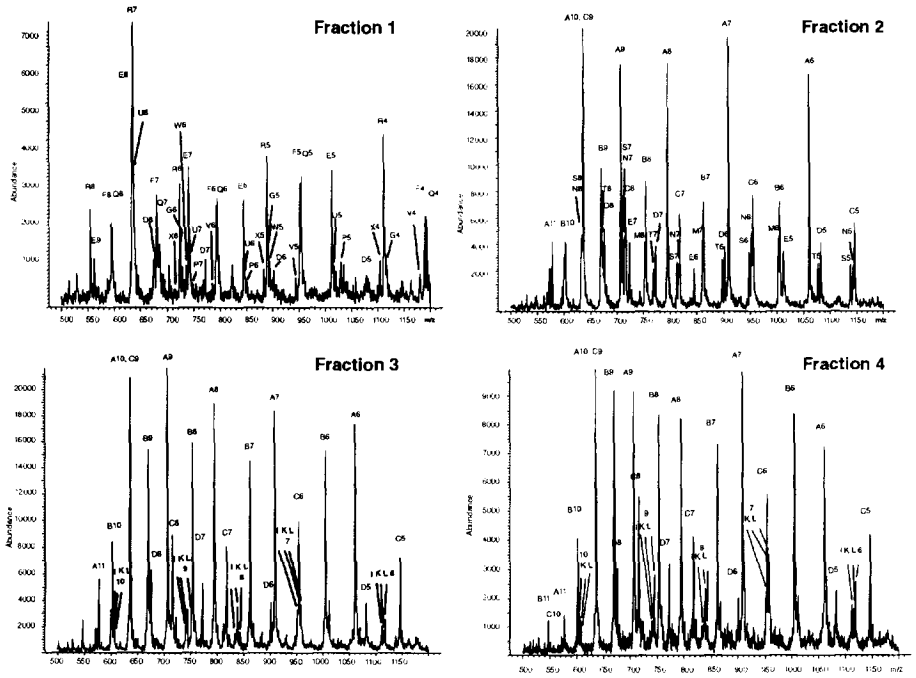

Figure 2. HPLC-ES/MS analysis of SAX fractions 1-4.

The results presented here confirm our previous observations that the major degradation products of phosphorothioate oligonucleotide metabolism are consistent with either 3'- and/or 5'-exonuclease activity. In addition to these degradation pathways, these results suggest that slower migrating species observed by CE is the result of the addition of a nucleotide to intact phosphorothioate oligonucleotide. Capillary electrophoresis data show that only one species was present, but mass spectrometry studies indicated three distinct metabolites. Our results are consistent with the hypothesis that the slower electrophoretic mobility was due to the addition of a nucleotide.[9,14] The finding of multiple nucleotides provides additional clues to understanding this unique metabolic pathway of phosphorothioate oligonucleotides.

References

1. Agrawal, S.; Goodchild, J.; Civeira, M. P.; Thornton, A. H.; Sarin, P. S. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7079.

2. Azad, R. F.; Driver, V. B.; Tanaka, K.; Crooke, R. M. *Antimicrobial Agents Chemotherapy* **1993**, *37*, 1945.

3. Bennett, C. F. In *Antisense Research and Applications*; Crooke, S. T. and Lebleu, B., Eds.; CRC Press: Boca Raton, FL, 1993; pp 547-562.

4. Bennett, C. F. In *Delivery Strategies for Antisense Oligonucleotide Therapeutics*; Aktar, S., Ed.; CRC Press: Boca Raton, FL, 1995; pp 223-232.

5. Cowsert, L. In *Antisense Research and Applications*; Crooke, S. T. and Lebleu, B., Eds.; CRC Press: Boca Raton, FL, 1993; pp 521-533.

6. Cowsert, L. M.; Fox, M. C.; Zon, G.; Mirabelli, C. K. *Antimicrobial Agents Chemotherapy* **1993**, *37*, 171.

7. Monia, B. P.; Johnston, J. F.; Ecker, D. J.; Zounes, M. A.; Lima, W. F.; Freier, S. M. *Journal of Biological Chemistry* **1992**, *267*, 19954.

8. Dean, N. M.; McKay, R. *Proceedings of the National Academy of Sciences, USA* **1994**, *91*, 11762.

9) Agrawal, S.; Temsamani, J.; Tang, J. Y. *USA Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7595.

10. Crooke, S. T.; Graham, M. J.; Zuckerman, J. E.; Brooks, D.; Conklin, B. S.; Cummins, L. L.; Grieg, M. J.; Guinosso, C. J.; Kornbrust, D.; Manoharan, M.; Sasmor, H. M.; Schleich, T.; Tivel, K. L.; Griffey, R. G. *J. Pharmcol. Exp. Ther.* **1996**, *277*, 923.

11. Cossum, P. A.; Sasmor, H.; Dellinger, D.; Truong, L.; Cummins, L.; Owens, S. R.; Markham, P. M.; Shea, J. P.; Crooke, S. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 1181.

12. Leeds, J. M.; Graham, M. J.; Truong, L.; Cummins, L. L. *Anal. Biochem.* **1996**, *235*, 36.

13. Sands, H.; Gorey-Feret, L. J.; Cocuzza, A. J.; Hobbs, F. W.; Chidester, D.; Trainor, G. L. *Mol. Pharmacol.* **1994**, *45*, 932.

14. Crooke, R. M.; Graham, M. J.; Cooke, M. E.; Crooke, S. T. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 462.

15. Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882.

16. Limbach, P. A.; Crain, P. F.; McCloskey, J. A. *Current Opinion in Biotechnology* **1995**, *6*, 1 (author's copy).

17. Potier, N.; Van Dorsselaer, A.; Corider, Y.; Roch, O.; Bischoff, R. *Nucleic Acids Res.* **1994**, *22*, 3895.

18. Bothner, B.; Chatman, K.; Sarkisian, M.; Siuzdak, G. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2863.

19. Bleicher, K.; Bayer, E. *Chromatographia* **1994**, *39*, 405.

20. Greig, M.; Griffey, R. H. *Rapid Commun. Mass. Spectrom.* **1995**, *9*, 97.

21. Gaus, H. J.; Owens, S. R.; Winniman, M.; Cooper, S.; Cummins, L. L. *Anal. Chem.* **1997**, 69, 313.

(*Received in USA* 23 *December* 1996; *accepted* 7 *April* 1997)