ELSEVIER

International Journal of Pharmaceutics 133 (1996) 257–263

international journal of pharmaceutics

Rapid communication

Stability of antisense oligonucleotides during incubation with a mixture of isolated lysosomal enzymes

Andrew J. Hudson[a], Wayne Lee[a], Jayne Porter[a], Jehngir Akhtar[a], Ruth Duncan[b], Saghir Akhtar[a,*]

[a]*Pharmaceutical Sciences Institute, Department of Pharmaceutical and Biological Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK*

[b]*Centre for Polymer Therapeutics, The School of Pharmacy, University of London, London, UK*

Received 20 October 1995; revised 8 November 1995; accepted 14 November 1995

Abstract

Cellular uptake of antisense oligonucleotides occurs predominantly via the endocytic route but the subsequent intracellular trafficking and metabolic fate is still poorly understood. We have examined the stability of 20-mer phosphodiester (D-oligo) and phosphorothioate (S-oligo) oligonucleotides ([^{32}P]-radiolabelled either internally and at the 3′- or 5′- end) during exposure to a mixture of isolated rat liver lysosomal enzymes (Tritosomes). Whereas the oligonucleotides remained stable in aqueous buffer at pH 5.4 for at least 48 h, the D-oligos (at 10 nM) were degraded relatively rapidly ($t_{50\%} = 30–40$ min) by Tritosomes and at a rate similar to S-oligos ($t_{50\%} = 50$ min). Degradation of both was verified using reverse-phase HPLC and in this case for D- and S-oligos (2 μM) the $t_{50\%}$ was 90 min and 150 min, respectively. Enzymatic digestion by lysosomal nucleases, and not simple acid-catalysed hydrolysis, accounts for the rapid degradation of oligonucleotides.

Keywords: Antisense; Oligonucleotide stability; Lysosomes; Tritosomes; Intracellular fate; Phosphorothioate

1. Introduction

Antisense oligonucleotides have emerged as novel therapeutic agents for the sequence-specific inhibition of gene expression (Stein and Cheng, 1993; Wagner, 1994) and are currently undergoing clinical evaluation for the treatment of viral infections in AIDS patients and both chronic and acute myelogenous leukaemias (Agrawal and Akhtar, 1995). However, despite this fact little is still known regarding their intracellular trafficking and intracellular fate (for review see Akhtar, 1995). Recent reports suggest that unmodified antisense oligonucleotides and their polyanionic analogues enter mammalian cells by some form(s) of active endocytosis (Akhtar and Juliano, 1992; Stein et

* Corresponding author. Tel: +44-121-3593611, ext. 4766; Fax: +44-121-3590733; E-mail: S.AKHTAR@ASTON.AC.UK.

SSDI 0378-5173(95)04390-V

al., 1993; Beltinger et al., 1995), but the exact mechanism of uptake still remains unclear. It has been proposed that endocytic uptake of phosphodiester and phosphorothioate oligonucleotides by certain cells may be mediated by a 'receptor' or binding protein (Loke et al., 1989; Yakubov et al., 1989; Hawley and Gibson, 1992), whereas in other cell types uptake may occur by fluid-phase endocytosis or non-specific adsorptive endocytosis (Shoji et al., 1991; Stein et al., 1993; Wu-Pong et al., 1994; Krieg, 1995; Beltinger et al., 1995). All endocytosed oligonucleotides will localize first within the endosomal compartment and from there may transfer into secondary lysosomes. Intact oligonucleotides must escape from these acidic compartments if they are to become bioavailable, and it is unlikely that they can leave by passive diffusion (Akhtar et al., 1991a).

The subcellular distribution of fluorescently labelled oligonucelotides suggest that they remain localized within these vesicular compartments for a considerable time (Shoji et al., 1991; Wu-Pong et al., 1994; Krieg, 1995), but still very little is known about their stability within endosomal or lysosomal vesicles. Both compartments have an acidic interior (pH 5–6) and the lysosome also contains a battery of hydrolases including ribonuclease, desoxyribonuclease, acid phosphatase, phosphodiesterase and pyrophosphatase which together may be responsible for the degradation of oligonucleotides entering the lysosomes via autophagy.

We have studied the stability profiles of 20-mer polyanionic phosphodiester and phosphorothioate oligodeoxynucelotides during their incubation in acid pH and also in the presence of lysosomal enzymes isolated from rat liver (Tritosomes (Trouet, 1974)). The use of 5′-end [^{32}P]-radiolabelling alone is considered undesirable for monitoring stability because oligonucleotides are prone to dephosphorylation and/or rapid degradation by exonucleases. Hence, here stability was monitored by reverse-phase HPLC of non-radiolabelled product, or with the aid of both internal radiolabels, and 5′-end and 3′-end labelling for autoradiographic detection on PAGE gels.

2. Materials and methods

2.1. Oligonucleotide synthesis

Antisense phosphodiester and phosphorothioate oligodeoxynucleotides were synthesised on an automated DNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry (0.2 μM scale). The 20-mer all phosphodiester (D-oligo) and an all phosphorothioate (S-oligo) sequence complementary to the 3′-splice site of the *tat* gene in HIV-RNA (5′ ACA CCC AAT TCT GAA AAT GG 3′) were used in this study.

2.2. Radiolabelling of oligonucleotides

2.2.1. 5′-end and 3′-end labelling

The oligonucleotides were labelled at the 5′-end with [^{32}P]-labelled ATP (Amersham, UK) using bacteriophage T4 polynucleotide kinase (Gibco, UK) in 100 mM Tris pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA at 37°C for 30 min. Alternatively, oligonucleotides were labelled at the 3′-end with [^{32}P]-labelled ddATP (Amersham, UK) using terminal transferase (Gibco, UK) at 37°C for 60 min as described by the manufacturer.

2.2.2. Internal labelling

Internally labelled D-oligos were synthesised using Klenow fragment of *Escherichia coli* DNA polymerase 1 (Sigma, UK) as described by Akhtar et al. (1991b) except that a 25-mer template DNA oligonucleotide (5′ TGA GTG TCC AGC TTC TTA CCA GCA T 3′) and a 13-mer primer DNA oligonucleotide (5′ GG TAA GAA GCT GG 3′) designed to form a duplex seven nucleotides in from the 5′-end of the template were used in this case. The resultant 20-mer internally labelled oligodeoxynucleotide contained three labelled adenine residues; 5′ GGT AAG AAG CTG GAC ACT CA 3′ (shown as bold letters). The position of the radiolabelled adenines within the 20-mer sequence were confirmed with DNase footprinting (data not shown). The radiolabelled oligonucleotides were then purified by native 15% polyacrylamide gel electrophoresis (PAGE). The excised

bands were eluted in water and were concentrated in a Savant DNA Speed Vac. The concentration of the oligonucleotide solution was determined by UV absorption at 260 nm assuming that a value of 1 OD was equivalent to 29.4 μg for the 20-mer sequence (Sambrook et al., 1989). Oligonucleotides were resuspended in sterile water for use in stability studies

2.3. Preparation of the mixture of rat liver lysosomes (Tritosomes)

Tritosomes were prepared according to the method of Trouet (1974). As lysosomes and mitochondria have approximately the same buoyant density they are difficult to separate by density gradient centrifugation. Administration of the detergent Triton WR-1339 intraperitoneally (i.p.) is followed by its endocytosis and subsequent accumulation in liver lysosomes. The concomitant decrease in lysosome density allows these vesicles, so-called 'Tritosomes', to be efficiently separated from other sub-cellular organelles, producing a very concentrated preparation of lysosomal enzymes. Briefly, rats were injected i.p. with Triton WR-1339 (1 ml/100 g body weight) and sacrificed 4 days later after overnight fasting. The liver was homogenised in ice-cold sucrose (0.25 M, 5 ml/g of liver), and the homogenate was then centrifuged at 1650 rev./min for 10 min at 4°C. The supernatant was removed and kept, and the pellet re-suspended in cold sucrose (0.25 M) then re-centrifuged at 1400 rev./min for 10 min. The supernatants were pooled and centrifuged at 17 000 rev./min for 10 min, the pellet resuspended and then centrifuged again at 17 000 rev./min for 10 min. The supernatant was discarded, and the pellet re-suspended in cold sucrose 45% (w/v) to a volume of 30 ml. This was divided into three 9-ml aliquots, and placed in a 25-ml centrifuge tube. Onto this were carefully layered 8 ml of a 34.5% (w/v) sucrose solution, and finally 4 ml of 14.3% (w/v) sucrose were layered on top. The density gradient was centrifuged for 2 h at 17 500 rev./min, and then 1-ml aliquots of Tritosomes recovered from the interface between the 34.5% and 14.3% sucrose layers.

2.4. Degradation studies

The desired concentration of radiolabelled (10 nM) or unlabelled oligonucleotide (2 μM) was incubated with 100 μl of either the Tritosome preparation (approximately 1 mg protein/ml) in citrate buffer (0.2 M) at pH 5.4 or snake venom phosphodiesterase 1 (20 units/ml; Pharmacia, UK). The latter enzyme was used as a positive control for the rapid degradation of oligonucleotides. Samples (10-μl aliquots) were removed at timed intervals, incubated with denaturing loading buffers to quench reactions and the degradation products analysed by either PAGE as described above or by HPLC according to a method described previously (Becker et al., 1985; Applied Biosystems DNA Synthesiser User Bulletin Issue No. 13, 1987)

3. Results and discussion

The aim of this study was to elucidate the intracellular fate of oligonucleotides by assessing their stability under conditions they would encounter within endosomal and lysosomal compartments of the cell. Rat liver Tritosomes have be used for many years to provide a concentrated source of lysosomal hydrolases (Trouet, 1974) and have proven a useful tool for evaluation, and further design, of peptidyl spacers used to control the intracellular release of anticancer agents from polymer-drug conjugates designed for lysosomotropic delivery (Duncan et al., 1980, 1984; Duncan, 1992). Not only has it been shown that the rate of proteolytic cleavage of such substrates by Tritosomes in vitro is similar to that seen in vivo (Subr et al,, 1992; Seymour et al., 1994), but first clinical evaluation of polymer-anthracycline conjugates shows good correlation of both pharmacokinetic and pharmacological data observed in man with the preclinical in vivo data (Duncan et al., 1995). Thus, there is good justification to suppose that the rates of degradation observed here reflect the intracellular situation.

Degradation of 5'end-[^{32}P]-radiolabelled D-oligo by Tritosomes is shown in Fig. 1. The D-oligo degraded relatively rapidly, the time

taken for 50% degradation ($t_{50\%}$) being 30 min for the phosphodiester oligodeoxynucleotide. A semi-log plot of the degradation profile (see inset to Fig. 1) was linear suggesting that degradation proceeded via first-order or pseudo-first-order kinetics. Interestingly, both the 5′-end and 3′-end [^{32}P]-radiolabelled D-oligos exhibited similar kinetics ($t_{50\%} = 30$ min), and furthermore, both the 5′- and 3′-end-labelled oligonucleotides exhibited kinetics approaching those obtained with internally [^{32}P]-radiolabelled D-oligo ($t_{50\%} = 40$ min) suggesting a minor involvement of enzymes that may remove end labels from D-oligos. Removal of end labels may be facilitated by either dephosphorylating enzymes such as phosphatases for the 5′-end [^{32}P]-radiolabelled oligonucleotides or by 3′-end exonucleases in the case of 3′-end labelled oligonucleotides. The 5′-end [^{32}P]-radiolabelled S-oligo was significantly more stable ($t_{50\%} = 50$ min) than the corresponding D-oligo ($t_{50\%} = 30$ min) in Tritosomes. The kinetic data are summarized in Table 1. A phosphodiesterase enzyme derived from the snake venom was used as a positive control for these studies and in its presence both

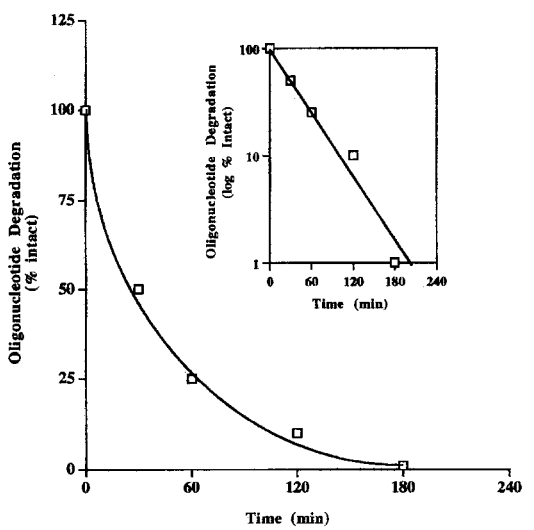

Fig. 1. The degradation-time profile of 20-mer 5′-end [^{32}P]-radiolabelled D-oligo in the presence of Tritosomes. The amount of intact oligonucleotide remaining (expressed as a percentage) was determined by scanning laser densitometry of PAGE autoradiographs. Inset shows a semi-log plot of the data from the main Figure.

phosphodiester and phosphorothioate oligonucleotides degraded instantly as would be predicted ($t_{50\%} = < 1$ min). It is noteworthy that radiolabelled D-oligos were stable at pH 5.5 for the entire study period (2 days) suggesting that nuclease activity, and not the acidic pH, is responsible for degradation.

To investigate further the degradation of D- and S-oligos by Tritosomes, stability of unlabelled oligonucleotides was examined by reverse-phase HPLC. However, lower sensitivity of the UV-detection used for HPLC necessitated the use of higher oligonucleotide concentration in the incubation mixture (see Table 1). The HPLC data confirmed that D-oligos were degraded more rapidly by Tritosomes ($t_{50\%} = 90$ min) compared with S-oligo ($t_{50\%} = 150$ min). However, the slower rates of degradation observed at these higher (2 μM) concentrations may indicate enzyme saturation. Further studies are undoubtedly required to identify exactly which enzymes are responsible for degradation and also define the effect of substrate concentration.

Comparison of the degradation rates seen during exposure of oligonucleotides to Tritosomes (Table 1) with those reported earlier during incubation with whole cytosolic extracts (Akhtar et al., 1991b) suggests that lysosomal degradation is much more rapid. Interestingly, a 32-mer hammerhead ribozyme (an RNA oligonucleotide motif that exhibits catalytic activity), also thought to enter cells by endocytic pathways (Akhtar et al., 1995) also exhibited rapid degradation in Tritosomes (data not shown). These findings suggest that intracellularly the lysosomal compartment represents the major site of degradation of both RNA and DNA oligonucleotides. This notion is further supported by evidence from a recent study examining efflux kinetics of D-oligos from cultured HL60 cells which suggests that DNA oligonucleotide degradation can occur within vesicular compartments leading to exocytosis of degradation products (Tonkinson and Stein, 1994).

Intracellular trafficking of oligonucleotides is a complicated process which certainly requires further study if we are to understand clearly the key issues relating to their bioavailability. Ultimate

Table 1
Degradation of antisense oligonucleotides in the presence of enzymes or at acid pH

Oligonucleotide name	Length, type and sequence	Label and analysis method	Degradation medium	Degradation rate, $t^{a}_{50\%}$
20D-TAT	20 mer Phosphodiester 5′ ACA CCC AAT TCT GAA AAT GG 3′	5′ end [^{32}P]-radiolabelled; 20% PAGE/7 M urea	Tritosomes	30 min
		3′ end [^{32}P]-radiolabelled, 20% PAGE/7 M urea	Tritosomes	30 min
		Internally [^{32}P]-radiolabelled using Klenow, 20% PAGE/7 M urea	Tritosomes	40 min
		Unlabelled, HPLC[b]	Tritosomes	90 min
		5′ end [^{32}P]-radiolabelled; 20% PAGE/7 M urea	S100 Cytosolic fraction	60–90 min[c]
		Internally [^{32}P]-radiolabelled using Klenow, 20% PAGE/7 M urea	Phosphodiesterase I	<1 min
		5′ end [^{32}P]-radiolabelled; 20% PAGE/7 M urea	Citrate buffer, pH 5.5	>48 h
20S-TAT	20 mer Phosphorothioate 5′ ACA CCC AAT TCT GAA AAT GG 3′	5′ end [^{32}P]-radiolabelled; 20% PAGE/7 M urea	Tritosomes	50 min
		Unlabelled, HPLC[b]	Tritosomes	150 min

[a]Estimated from plots of % intact oligomer remaining as a function of time. Data were obtained from either scanning densitometric analysis of PAGE gel autoradiographs or determined from peak heights of HPLC traces. Data are the mean of at least two separate experiments.
[b]The use of a higher oligonucleotide concentration was necessary for detection by HPLC (2 μM) compared with radiolabelled oligomers (10 nM).
[c]Data from Akhtar et al. (1991b).

pharmacological activity will be governed by a variety of factors, including premature loss by exocytosis, degradation within lysosomes, difficulty in transferring into the cytoplasm and ultimately ability to interact with their target. The relative contributions of these parameters will determine the true bioavailability, but this study highlights the importance of the hydrolytic capacity of lysosomal enzymes, and suggests the need to develop delivery systems that will either circumvent the lysosome or protect an oligonucleotide against degradation during its residence there.

Acknowledgements

We would like to thank Alysa Hume (Keele University) for her technical assistance in the preparation of Tritosomes. BBSRC and Cruachem are thanked for a CASE studentship to A.H. This research programme is funded in part by grants to S.A. from the Cancer Research Campaign, MRC AIDS Directed Programme and the Nuffield Foundation.

References

Agrawal, S. and Akhtar, S., Advances in antisense efficacy and delivery. *Trends Biotechnol.*, 13 (1995) 197–199.

Akhtar, S., *Delivery Strategies for Antisense Oligonucleotide Therapeutics*. CRC Press, Boca Raton, USA, 1995.

Akhtar, S. and Juliano, R.L., Cellular uptake and intracellular fate of antisense oligonucleotides. *Trends Cell Biol.*, 2 (1992) 139–144.

Akhtar, S., Basu, S., Wickstrom, E. and Juliano, R.L., Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic Acids Res.*, 19 (1991a) 5551–5559.

Akhtar, S., Kole, R. and Juliano, R.L., Stability of antisense DNA oligodeoxynucleotide analogs in cellular extracts and sera. *Life Sci.*, 49 (1991b) 1793–1801.

Akhtar, S., James, H. and Gibson, I., Molecular DIY with hairpins and hammerheads. *Nat. Med.*, 1 (1995) 300–302.

Applied Biosystems DNA Synthesiser User Bulletin Issue No. 13, *The Evaluation and Purification of Synthetic Oligonucleotides*, April 1987, Applied Biosystems (Perkin Elmer), Warrington, UK, pp. 28–39.

Becker, C.R., Efcavitch, J.W., Heiner, C.R. and Kaiser, N.F., Use of a reverse phase column for the HPLC purification of synthetic oligonucleotides. *J. Chromatogr.*, 326 (1985) 293–299.

Beltinger, C., Saragovi, H.U., Smith, R.M., LeSauter, L., Shah, L., DeDionisio, L., Christensen, L., Raibie, A., Jarett, L. and Gewirtz, A.M., Binding, uptake and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. *J. Clin. Invest.*, 95 (1995) 1814–1823.

Duncan, R., Drug polymer conjugates — potential for improved chemotherapy. *Anti-Cancer Drugs*, 3 (1992) 175–210.

Duncan, R., Lloyd, J.B. and Kopecek, J., Degradation of side chains of N-(2-hydroxypropyl)methacrylamide copolymers by lysosomal enzymes. *Biochim. Biophys. Res. Commun.*, 94 (1980) 284–290.

Duncan, R., Cable, H.C., Lloyd, J.B., Rejmanova, P. and Kopecek, J., Polymers containing enzymatically degradable bonds, 7. Design of oligopeptide side chains in poly N-(2-hydroxypropyl)methacrylamide copolymers to promote efficient degradation by lysosomal enzymes. *Makromol. Chem.*, 184 (1984) 1997–2008.

Duncan, R. Vasey, P.A., Kaye, S.B. and Cassidy, J., Development of PK1, an HPMA copolymer doxorubicin conjugate. *EORTC Early Drug Development Meeting*, Corfu, June 1995.

Hawley, P. and Gibson, I. The detection of oligodeoxynucleotide molecules following uptake into mammalian cells. *Antisense Res. Dev.*, 2 (1992) 119–127.

Krieg, A., Uptake and localization of phosphodiester and chimeric oligodeoxynucleotides in normal and leukemic primary cells. In Akhtar, S. (Ed.), *Delivery Strategies for Antisense Oligonucleotide therapeutics*, CRC Press, Inc., Boca Raton, USA, 1995, pp. 177–190.

Loke, S.L., Stein C., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S. and Neckers, L.M., Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA*, 86 (1989) 3474–3478.

Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Vol 1–3, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, pp. 11.28, E5.

Seymour, L.W., Ulbrich K., Styger, P.S., Brereton, M., Subr V., Strohalm, J. and Duncan, R., Tumor tropism and anticancer efficacy of polymer-based doxorubicin prodrugs in the treatment of subcutaneous murine B16F10 melanoma. *Br. J. Cancer*, 70 (1994) 636–641.

Shoji, Y., Akhtar, S., Periasamy, A., Herman, B. and Juliano, R.L., Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res.*, 19 (1991) 5543–5550.

Stein, C.A. and Cheng, Y.-C., Antisense oligonucleotides as therapeutic agents — Is the bullet really magical? *Science*, 261 (1993) 1004–1012.

Stein, C.A., Tonkinson, J.L., Zhang, L.-M., Yakubov, L., Gervasoni, J., Taub, R. and Rotenberg, S.A., Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. *Biochemistry*, 32 (1993) 4855–4861.

Subr, V., Strohalm, J., Ulbrich, K. and Duncan, R., Polymers containing enzymatically degradable bonds. 12. Effect of

spacer structure on the rate of release of daunomycin and adriamycin from poly[n-(2-hydroxypropyl)-methacrylamide] copolymer drug carriers in vitro and antitumor-activity measured in vivo. *J. Controlled Release*, 18 (1992) 123–132.

Tonkinson, J.L. and Stein, C.A., Patterns of intracellular compartmentalization, trafficking and acidification of 5'-fluorescein phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells. *Nucleic Acids Res.*, 22 (1994) 4268–4275.

Trouet, A. Isolation of modified liver lysosomes. In Fleischer, S. and Packer, L. (Eds.), *Methods in Enzymology*, Vol. XXXI, Academic Press, New York, 1974, pp. 323–329.

Wagner, R., Gene Inhibition using antisense oligonucleotides. *Nature*, 372 (1994) 333–335.

Wu-Pong, S., Weiss, T.L. and Hunt, C.A., Antisense c-myc oligonucleotide cellular uptake and activity. *Antisense Res. Dev.*, 4 (1994) 155–163.

Yakubov, L.A., Deeva, E.A, Zarytova, V.F., Ivanova, E.M., Ryte, A.S., Yurchenko, L.V. and Vlassov, V.V., Mechanism of oligonucleotide uptake by cells: involvement of receptors? *Proc. Natl. Acad. Sci. USA*, 86 (1989) 6454–6458.