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International Journal of Pharmaceutics 152 (1997) 121–125

international journal of pharmaceutics

Rapid communication

Effect of ultrasound on the stability of oligodeoxynucleotides in vitro

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Received 3 January 1997; accepted 7 January 1997

Abstract

In order for oligodeoxynucleotides (ODNs) to be viable candidates for phonophoresis (i.e. ultrasound-enhanced delivery), they must remain stable on exposure to therapeutic ultrasonic waves. We have examined the stability of radiolabelled ODNs as a function of their chemistry (phosphodiester (PO) and phosphorothioate (PS)) and chain length (7-mer and 20-mer) in aqueous solutions at three different pH values (1, 2 and 7) when subjected to ultrasound at an intensity of 1.5 W cm^{-2} for 30 min. Whereas all ODNs remained stable at pH 1, pH 2 or pH 7 in the absence of ultrasound, significant degradation was observed at pH 1 upon ultrasound treatment. The sensitivity of ultrasound-enhanced ODN degradation at this pH, starting with the most rapidly degradable species first, was 7-mer PO > 20-mer PO > 7-mer PS > 20-mer PS. Interestingly, ODN stability was unaffected by exposure to an equivalent heat-alone application (pH 1 at 44°C) thus indicating that the mechanical, rather than heating, effects of ultrasound are responsible for the observed ODN degradation. © 1997 Elsevier Science B.V.

Keywords: Degradation; Antisense; Oligonucleotide; Phonophoresis; Phosphorothioate; Phosphodiester; Ultrasound

1. Introduction

Oligodeoxynucleotides (ODNs) are short chains of nucleotides (typically 6–100 units long) which resemble single-stranded DNA or RNA. In recent years, these agents. as either antisense DNA or RNA ribozyme sequences, have been proposed as important new classes of pharmaceuticals which can be used to directly inhibit gene expression (Weintraub, 1990; Szymkowski, 1996; Akhtar and Agrawal, 1997). Such a capability promises beneficial pharmaceutical applications because of the possibility of down-regulating the expression

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PII S0378-5173(97)04915-6

of specific gene-products which induce disease states such as cancer (Narayanan and Akhtar, 1996). However, the in vivo delivery of oligonucleotides represents a novel and exciting pharmaceutical challenge (Akhtar, 1995) and various routes of administration have been investigated for these molecules, including transdermal delivery (Oldenburg et al., 1995; Brand and Iversen, 1996).

Phonophoresis is defined as the use of ultrasound to enhance percutaneous drug delivery (Meidan et al., 1995). In principle, phonophoresis would be a valuable technique for promoting ODN delivery across the skin. Alternatively, it can be used to enhance degradation and drug release from biodegradable polymeric delivery devices (Kost et al., 1989) and such systems have recently been proposed for oligonucleotide delivery (Lewis et al., 1995; Hudson et al., 1996a; Akhtar and Lewis, 1997). However, currently, there are no data available on the chemical stability of ODNs on exposure to ultrasonic fields. Such data would be important if ODNs were eventually delivered by phonophoresis. The aim of this study was to determine the effect of ultrasound on the in vitro degradation of four candidate ODNs, of varying chemistry and chain length, in aqueous solutions at three different pH.

2. Methods

2.1. Oligonucleotide synthesis and radiolabelling

Antisense phosphodiester (PO) and phosphorothioate (PS) ODNs were synthesised on an automated DNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry (0.2 mM scale). The 7-mer sequence complementary to the AUG initiation codon of human c-*myc* oncogene exon 2 (3′ TAC GGG G 5′) and a 20-mer 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. ODNs were radiolabelled at the 5′-end with [^{32}P]-γ 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. The radiolabelled ODNs thus produced were then purified by 20% polyacrylamide gel electrophoresis as described previously (Akhtar et al., 1991)

2.2. Degradation studies

Five μl of the ODN under study and 95 μl of the appropriate buffer (pH 1, 2 or 7) were aliquoted into an Eppendorf tube and vortexed to ensure adequate mixing. A pH 1 buffer was prepared by mixing 25 ml of 0.2 M potassium chloride and 25 ml of 0.2 M hydrochloric acid. A pH 2 buffer was prepared by mixing 25 ml of 0.2 M potassium chloride with 2.8 ml of 0.1 M hydrochloric acid. A pH 7 buffer was prepared from 50 ml of 0.1 M potassium dihydrogen phosphate and 29.1 ml of 0.1 M sodium hydroxide. The ODN-buffer solution was then exposed to either ultrasound (see below), heat-alone (44°C in an incubator) or room temperature for 30 min. At successive time intervals during the exposure treatment (0, 5, 10, 20 and 30 min), the Eppendorf tube was removed and a 5-μl aliquot of ODN solution was placed into another labelled Eppendorf tube prefilled with 20 μl of stop mixture [7 M urea in tris borate buffer]. Each such Eppendorf tube containing a time-point sample was spun on a bench centrifuge and subsequently stored at −20°C pending analysis by 20% PAGE and subsequent autoradiography (Akhtar et al., 1991).

2.3. Ultrasound application and analysis of heating effects

Ultrasound exposure was mediated by the small transducer of an ultrasound generator (Therasonic no. 50, EMS Greenham Ltd.) which was fixed in position through the central axis of a Franz donor cell by a specially designed teflon collar (Meidan, 1996). The donor compartment was clamped in an inverted position so that the transducer was at the base of the assembly, pointing upwards towards the narrow end of the donor compartment at the top. The donor cell was entirely filled with coupling gel (Henleys Medical Supplies Ltd). The Eppendorf tube containing the

ODN solution was positioned so that its conical base was totally immersed within the coupling gel but the upper cylindrical part protruded above the gel level. Each ODN solution was sonicated for 30 min with a 1.1 MHz beam, exhibiting an intensity of 1.5 W cm^{-2}. A separate temperature profile study was performed in order to determine the ultrasound effect on the temperature of the ODN solution in the Eppendorf tube. An Eppendorf tube was filled with 100 μl sterile double-distilled water and then placed in the sonication system described above. Then, 1.1 MHz ultrasound was applied for 30 min, at an intensity of 1.5 W cm^{-2}. The tube was removed at specific time intervals (0, 5, 10, 20 and 30 min) so that the water temperature could be recorded with a thermocouple (Digitron Instruments, 3202 K). The experiments were performed in triplicate.

3. Results and discussion

Studies in the absence of ultrasound for all ODNs (at pH 1, 2 and 7) and after sonication of ODNs with an ultrasound intensity of 1.5 W cm^{-2} for 30 min at pH 2 or pH 7 demonstrated that the radiolabeled 7-mer and 20-mer ODNs of both PO and PS backbone chemistries remained stable as no degradation products were evident following densitometry of 20% PAGE autoradiographs. Over longer time periods, ODN degradation in the absence of ultrasound was observed at acidic pH. The PO sequences degraded within 2–3 h at pH 1 and after around 16 h at pH 2 (data not shown).

Accelerated degradation of ODNs in the presence of ultrasound was, however, visible at pH 1. Fig. 1A, B illustrates the results of the sonication studies performed at pH 1 for PO and PS ODNs. It can be seen from Fig. 1A that progressive degradation of both the 7-mer PO and 20-mer PO sequences occurred in the presence of ultrasound. However, the rate of degradation was dependent on ODN chain length with the shorter 7-mer degrading more rapidly under these conditions (with an estimated time for 50% degradation ($t_{50\%}$) of only 7 min). For the 7-mer PO sequence a faster migrating band was visible almost instantaneously ($t = 0$) and complete degradation of the intact ODN occurred within 20 min. The band appearing at $t = 0$ may represent a rapid degradation product or possibly an alternative conformation assumed by this ODN. This requires further study. In contrast, the 20-mer PO sequence retained its original length and remained stable for a slightly longer period (with an estimated $t_{50\%}$ of 11 min) and was completely degraded within the 30-min ultrasound exposure period.

The extent and rate of ultrasound-mediated degradation was also dependent on the backbone chemistry of the ODN. Fig. 1B shows that the 7-mer PS ODN appears more stable (with an estimated $t_{50\%}$ of 15 min) than the corresponding PO ODN. The disappearance of full-length product and the concomitant appearance of smaller length products was clearly visible at the 20 min and 30 min sample for the 7-mer PS ODN. However, the 20-mer PS ODN appeared to be the

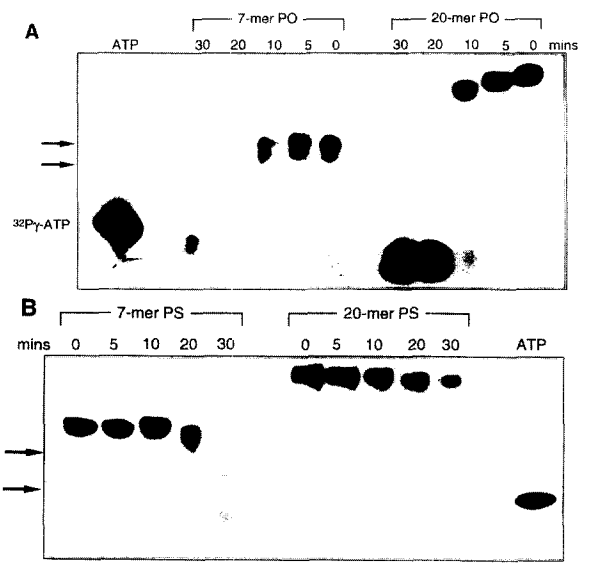

Fig. 1. The stability of oligonucleotides in aqueous solution at pH 1 when subjected to 1.1 MHz ultrasound beam of intensity 1.5 W cm^{-2} over a 30-min time period. (A) PO chemistry; (B) PS chemistry. Representative autoradiographs of 20% denaturing PAGE gels are shown for both the 7-mer and 20-mer in each ODN chemisty as a function of ultrasound exposure time in min. Migration of the 'free' monomeric ^{32}P-γ-ATP radiolabel as a control is also shown. The migration of the full length 7-mer is faster than the 20-mer for each chemistry. Arrows in the panels indicate degradation products.

most stable of all ODNs studied with only modest degradation being evident even after 30 min of ultrasound application.

The pattern of degradation indicates that both the 7-mer PO and PS ODNs clearly undergo chain shortening under the influence of ultrasound suggesting that intramolecular cleavage at the phosphodiester internucleoside linkage is occurring (see Fig. 1). This is not so clear with the longer 20-mer sequences of both chemical backbones, where visualisation of chain shortened products may have been complicated by the accelerated loss of the 5′-end radiolabel following ultrasound treatment. Furthermore, it can be surmised that each PS sequence was more stable than the comparable PO ODN. Interestingly, these ultrasound-mediated degradation results for PO and PS chemistries mirrored the trends observed upon nuclease digestion in cellular and biological fluid assays (Akhtar et al., 1991; Hudson et al., 1996b).

Since sonication raised the sample temperature to 44°C within 20 min (Fig. 2A), the possibility existed that ultrasonic heating was responsible for the observed ODN degradation. In order to test this hypothesis, the 7-mer PO ODN, the most sensitive to ultrasound degradation in the above studies, was incubated at 44°C for 30 min in pH 1. It can be seen from Fig. 2B that this ODN remained stable under these conditions and indicates that ultrasound-induced degradation of ODNs at pH 1 must have been produced by the mechanical effects (e.g. hydrodynamic shear stresses or cavitation effects) of the beam rather than the thermal effects alone. Similar results have been observed in double-stranded (ds) DNA. For example, Hill et al. (1972) demonstrated that a 3-min application of 1 MHz ultrasound at intensities as low as 0.4 W cm^{-2} degraded dsDNA in vitro. This was thought to be due to stable cavitation as the process was influenced by the availability of dissolved gas in solution. Other studies with ultrasound suggest that the nucleotide chains in dsDNA are being stretched to breaking point by the hydrodynamic shear fields developing secondarily to stable cavitation (for a review see Williams, 1983). It is likely that similar forces were exerted on the single-stranded ODNs

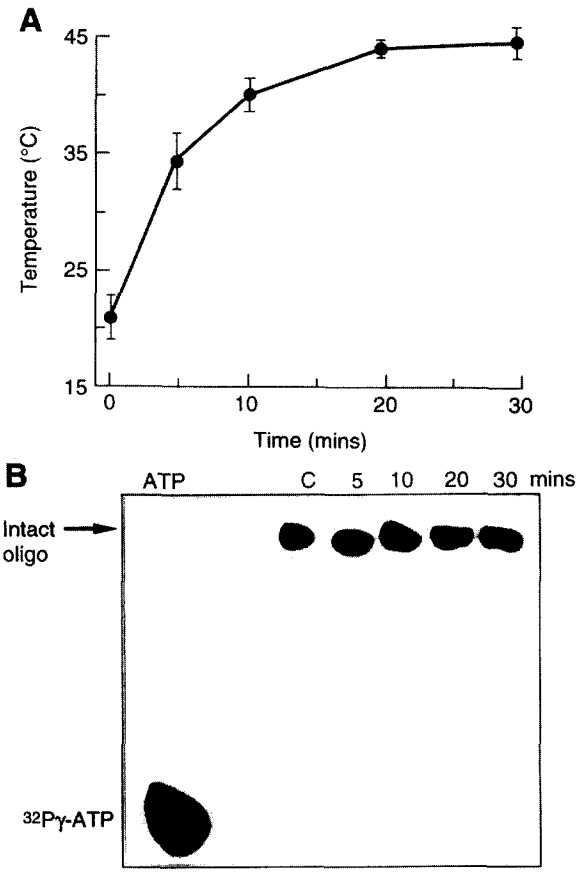

Fig. 2. (A) The rise in sample temperature over time following exposure to 1.1 MHz ultrasound beam of intensity 1.5 W cm^{-2}. (B) The lack of degradation of 7-mer PO ODN when incubated for up to 30 min at 44°C in aqueous buffer at pH 1. Band (C) control ODN not exposed to ultrasound. All other times and conditions are as per Fig. 1.

used in the present study such that there was sufficient cumulative disruption to degrade this ODN. However, one might expect mechanical effects to impart greater degradation in longer chain length ODNs, the converse of that observed in this study. A possible explanation for this may be that longer oligonucleotide sequences assumed a more stable secondary structure in solution resulting from Watson:Crick (WC) and non-WC intramolecular associations which can not always be predicted from currently available computer algorithms (Coulson et al., 1996). Clearly, further work is needed in order to elucidate the exact nature of these sonochemical-induced changes.

In conclusion, a 30-min ultrasound exposure at an intensity of 1.5 W cm^{-2} does not degrade any of the four ODN sequences examined in buffer solutions of either pH 2 or pH 7. This indicates that phonophoresis of ODNs would not be associated with stability problems under normal applications. However, when the ODNs were sonicated in aqueous solutions at pH 1, all of the sequences exhibited degradation but the extent and rate of degradation was dependent on ODN chemistry and chain length. Furthermore, this degradation was not simply mediated by the heating effects associated with ultrasound.

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