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Sequence and chemistry requirements for a novel aptameric oligonucleotide inhibitor of EGF receptor tyrosine kinase activity

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Abstract

We have previously identified a phosphorothioate oligonucleotide (PS-ODN) that inhibited epidermal growth factor receptor tyrosine kinase (TK) activity both in cell fractions and in intact A431 cells. Since ODN-based TK inhibitors may have anti-cancer applications and may also help understand the non-antisense mediated effects of PS-ODNs, we have further studied the sequence and chemistry requirements of the parent PS-ODN (sequence: 5′-GGA GGG TCG CAT CGC-3′) as a sequence-dependent TK inhibitor. Sequence deletion and substitution studies revealed that the 5′-terminal GGA GGG hexamer sequence in the parent compound was essential for anti-TK activity in A431 cells. Site-specific substitution of any G with a T in this 5′-terminal motif within the parent compound caused a significant loss in anti-TK activity. The fully PS-modified hexameric motif alone exhibited equipotent activity as the parent 15-mer whereas phosphodiester (PO) or 2′-*O*-methyl-modified versions of this motif had significantly reduced anti-TK activity. Further, T substitutions within the two 5′-terminal G residues of the hexameric PS-ODN to produce a sequence, TTA GGG, representing the telomeric repeats in human chromosomes, also did not exhibit a significant anti-TK activity. Multiple repeats of the active hexameric motif in PS-ODNs resulted in more potent inhibitors of TK activity than the parent ODN. These results suggested that PS-ODNs, but not PO or 2′-*O*-methyl modified ODNs, containing the GGA GGG motif can exert potent anti-TK activity which may be desirable in some anti-tumor applications. Additionally, the presence of this previously unidentified motif in antisense PS-ODN constructs may contribute to their biological effects *in vitro* and *in vivo* and should be accounted for in the design of the PS-modified antisense ODNs. © 2002 Published by Elsevier Science Inc.

Keywords: Phosphorothioate; Aptamer; Receptor tyrosine kinase inhibitor; Antisense oligodeoxynucleotide

1. Introduction

Antisense oligonucleotides (ODNs) are sequence selective nucleotide sequences that can bind to hybridization accessible sites within a target mRNA to inhibit gene expression [1–3]. An efficacious antisense ODN will result in a decrease in the levels of the target gene product (protein) and if the chemistry of ODN supports RNase H activity, a concomitant decrease in mRNA levels will also be observed due to this enzyme selectively hydrolyzing the mRNA in the antisense ODN:mRNA heteroduplex [2].

Phosphorothioate (PS) ODNs support RNase H activity and are the most widely used antisense ODN chemistry to date in both clinical trials studies and in cell culture experiments [1,4]. Indeed the first antisense ODN to be marketed as a drug for the treatment of human cytomegalovirus-induced retinitis is a PS-ODN (Vitravene, ISIS Pharmaceuticals, Carlsbad) [2]. Although antisense PS-ODNs can exert sequence specific antisense effects, they can also exert non-antisense mediated biological effects. Furthermore since these biological effects often can also be sequence-specific, they are sometimes mistakenly

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Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TK, tyrosine kinase; ODN, oligodeoxynucleotide; PS, phosphorothioate; PO, phosphodiester.

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interpreted as demonstrative of an antisense mechanism of action [1–3,4]. It is now known that some G-rich ODNs, termed "aptamers," can exert biological effects by interacting directly with cellular proteins. A positive example of an aptameric ODN is one that inhibits thrombin activity and is being developed for therapeutic use in vascular diseases [5]. This prototype aptamer of thrombin, selected from a large combinatorial library of oligonucleotides, has a unique double G-tetrad structure capable of inhibiting thrombin at nanomolar concentrations through binding to a specific region within thrombin exosite I [5–7].

In a project designed to develop antisense ODNs for the down regulation of epidermal growth factor receptor (EGFR) in the potential treatment of brain cancer, we serendipitously discovered an aptameric ODN that inhibited EGFR activity. A 15-mer PS-ODN complementary (antisense) to the 5′ AUG initiation codon of the human EGFR inhibited cell growth and altered the cell morphology of A431 epidermoid cancer cells in a non-antisense mechanism of action [8]. Preliminary mechanistic studies suggested that A431 cell growth and morphology changes were resulting from an inhibition of the EGF receptor tyrosine kinase activity as no changes in receptor protein or mRNA levels were observed by Western and Northern Blotting analyses [8]. In this study, we have further examined the sequence and chemistry requirements of this aptamer in its role to inhibit EGFR tyrosine kinase activity and report on its non-specificity in also inhibiting insulin receptor tyrosine kinase activity.

2. Methods

2.1. ODN synthesis and cell culture

Aptamer ODNs were synthesized as PS, phosphodiester (PO) or 2′-*O*-methyl modified chemistries and 5′-end [^{32}P]-radiolabeled as described previously [8–10].

A431 and U87-MG cells from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) were maintained at 37°, in a 5% CO_2 atmosphere, in DMEM containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2 or 1 mM glutamine, respectively (all from Life Technologies Inc.). The L6 skeletal muscle cells were provided by Luck (AstraZeneca), and were originally obtained from ECACC. A sub clone was isolated in the laboratory of Luck on their basis to fuse and form myotubes in 0.5% serum. These cells were frozen at Aston University, and aliquots thawed for the experiments described in this paper. L6 cells were cultured in DMEM/5% FCS as described previously [11]. To induce differentiation, confluent cells were left overnight in DMEM with the serum reduced to 0.5%.

For morphological studies, cells were seeded in 24-well plates at 2.5×10^4–5.0×10^4 cells per well, ODNs were added in culture medium (containing 10% FCS or serum-free) 24 hr after seeding, when the cells were approximately 50% confluent. For cell growth assays, cells were trypsinized 24 hr after aptamer or Tyrphostin A25 addition and the number of viable cells was counted in triplicate wells. The morphology of cells was examined by light microscopy 24 hr after addition of aptameric ODNs and representative replicate wells were photographed.

2.2. Preparation of solubilized EGF receptors

A431 cells were homogenized in 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 2 mM EDTA, 20 μg/mL soybean trypsin inhibitor, 0.1 mM PMSF and the membranes spun down at 10,000 *g* for 20 min. A crude solubilized extract was made by agitating membranes (2.5 mg protein/mL) in 50 mM Tris, 1% Triton X-100, 20% glycerol, 0.1% BSA, 0.05% sodium azide for 15 min at 4° and then centrifuging at 15,000 *g* for 10 min. This extract was aliquoted and stored at −20°.

2.3. Assay of EGFR tyrosine kinase activity

EGFR tyrosine kinase activity was determined using the BIOTRAK EGF-receptor tyrosine kinase enzyme assay kit according to the manufacturer's instructions (Amersham Life Sciences, UK). Briefly, 10 μL of solubilized membranes (prepared as above) were mixed with an equal volume of substrate peptide (a model poly(tyr–glu) peptide specific for EGFR) and 5 μL of 1.2 mM ATP containing 0.05 μCi γ-[^{32}P]-ATP. These were stimulated with 4 μg/mL of EGF (final concentration) in the presence of oligonucleotides for 30 min, in a total volume of 33 μL. Labeled peptide was separated from unreacted γ-[^{32}P]-ATP by binding to phosphocellulose filter paper and quantified by scintillation counting.

2.4. Glucose uptake as an indirect assay for insulin receptor tyrosine kinase activity

Uptake of [^{3}H]-2-deoxyglucose was measured as described elsewhere [12]. In brief, cells grown in 24-well plates were washed with glucose-free Krebs and then incubated for 10 min in glucose-free Krebs supplemented with 0.1 mM [^{3}H]-2-deoxyglucose (0.5 μCi per well) at room temperature. This medium was then discarded, the cells were washed twice in ice-cold Krebs and solubilized with 0.5 mL of 1 M NaOH. Radioactivity was measured by scintillation counting.

2.5. In vivo studies in nude mice xenografts

The hexameric active motif, APT6, was administered intra-tumorally as a single injection (100 μL) at a dose of 16 mg/kg into NCR nude mice (six mice per group) bearing established A431 cell xenografts. Mice were obtained from the National Cancer Institute (USA). Tumor

volumes were calculated from two dimensional caliper measurements using the formula $(a^2 \times b)/2$ where a is the smaller diameter and b is the larger diameter. Tumor volumes were recorded on day 3, 7, 10 and 14 following administration. Data were compared with untreated and those mice receiving only an equivalent volume of saline. UKCCR Guidelines [13] were followed throughout for the vivo studies.

3. Results and discussion

In our earlier study we showed that the parent PS-ODN (5′-GGA GGG TCG CAT CGC-3′) inhibited EGFR autophosphorylation in intact A431 cells. The underlying mechanism for inhibition of EGF receptor autophosphorylation was thought to be a direct inhibition of receptor kinase activity by the PS-ODN rather than interference with receptor-ligand interactions or even receptor dimerization [8]. Since an *in vitro* receptor kinase assay using solubilized EGF receptors showed that the parent aptamer inhibited the enzyme in a dose-dependent manner and was the likely cause of the reduced autophosphorylation, we have used this assay again in this study to examine the sequence and chemistry requirements of this aptamer. Since the aptamer also altered cell morphology, we have additionally examined the changes in morphology of A431 cells in response to treatment of various sequences and chemistries of ODNs.

3.1. Elucidation of the EGFR tyrosine kinase inhibitory sequence motif by mutation and deletion studies

Table 1 summarizes the changes in cell morphology and receptor tyrosine kinase activity for various doses of aptameric ODNs housing substitutions/mutations or deletions investigated in this study. Similar to our previous findings, the parent aptamer had a marked effect on A431 cell morphology (see also Fig. 1) and on the tyrosine kinase activity resulting in about 65% inhibition (Table 1). An altered colony morphology resulting from the aggregation of cells into tight clusters was observed for the parent compound but not in the scrambled control sequence (Fig. 1). This indicated that the order of the nucleotides

Table 1
The effect of aptamer sequence and chemistry on EGF receptor tyrosine kinase and A431 cell morphology (mean ± SD; $n = 3$)

ODN name	Sequence (5′–3′) and chemistry[a]	Concentration (μM)	Effect on cell morphology[b]	% Tyrosine kinase activity[c]
Parent	GGA GGG TCG CAT CGC	0	−	100 ± 3
		0.01	+	n.d
		0.1	++	n.d
		1.0	+++	n.d
		2.5	++++	35 ± 1
Scrambled	CGC ACG TGG ACT GGC	2.5	−	98 ± 2
G1A	**A**GA GGG TCG CAT CGC	2.5	−	n.d
G1C	**C**GA GGG TCG CAT CGC	2.5	−	n.d
G1T	**T**GA GGG TCG CAT CGC	2.5	−	81 ± 4
G2T	G**T**A GGG TCG CAT CGC	2.5	−	80 ± 7
G3T	GGA **T**GG TCG CAT CGC	2.5	−	82 ± 1
G4T	GGA G**T**G TCG CAT CGC	2.5	−	96 ± 6
G5T	GGA GG**T** TCG CAT CGC	2.5	+	62 ± 5
APT6	GGA GGG	0.01	+	105 ± 5
		0.1	++	95 ± 6
		1.0	+++	57 ± 5
		2.5	++++	37 ± 9
TEL6	**TT**A GGG	2.5	−	84 ± 4
APT6-PO	GGA GGG (PO)	2.5	−	85 ± 6
APT6-2OM	GGA GGG (2′-*O*-methyl)	2.5	+	72 ± 5
2.5 × APT6	GGA GGG GGA GGG GGA	0.01	++	n.d
		1.0	++++	n.d
		2.5	+++++	15 ± 2
3 × APT6	GGA GGG GGA GGG GGA GGG	2.5	+++++	11 ± 1
G_4	GGG G	2.5	+++	23 ± 2
G_6	GGG GGG	2.5	+++++	14 ± 2

Some nucleotides are shown in bold to highlight substitutions in parent aptamer. n.d. refers to "not determined".

[a] All phosphorothioate internucleoside chemistry unless stated; PO refers to all phosphodiester modified internucleoside linkages and 2′-*O*-methyl as having 2′-*O*-methyl modifications on the sugar residues.

[b] The effect on A431 cell morphology was scored from +, least effect; to a maximum effect with ++++ and relates visually to those shown in Fig. 1 for the corresponding concentrations of parent aptamer. A score of +++++ is assigned when the morphology effect was even greater than observed for parent motif as in the case of repeat motifs. A score of − indicates minimal or no effect on cell morphology.

[c] Relative to activity obtained with the control untreated A431 cells which was taken as 100%.

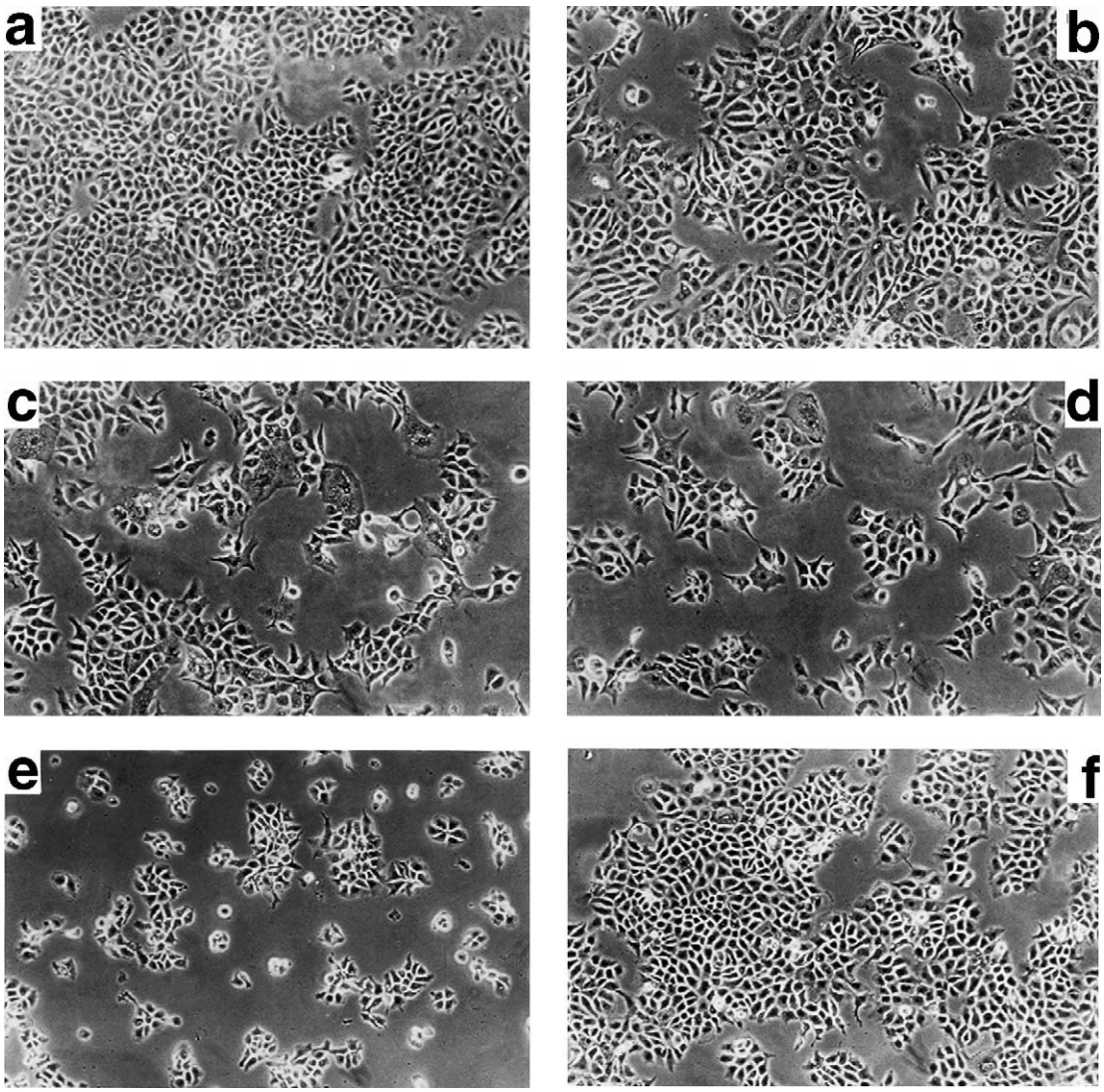

Fig. 1. Effect of parent aptamer on A431 cell morphology. Panel a represents untreated control cells. Panels b–e represent cells treated with 0.01, 0.1, 1 and 2.5 μM of parent aptamer, respectively. Panel f represents cells treated with 2.5 μM scrambled ODN (see Table 1 for sequences). In all cases cells were photographed at a 100× magnification following treatment with ODNs for 24 hr. Bar = 15 μM.

in the sequence rather than base composition *per se* was important for this aptameric effect.

We next began to elucidate which sequence motifs were critical for the observed effects on cell morphology and receptor tyrosine kinase activity. Based on the assumption that G-rich regions may be involved, we focused on the 5′-terminus of the parent compound that comprised the GGA GGG string of nucleotides. Sequential substitution of the G residues within this 5′-terminus with a T resulted in a near abolition of the anti-tyrosine kinase activity associated with the parent sequence (see Table 1). The aptamer-induced effect on A431 cell morphology was also removed upon sequential substitution of G with T (Table 1). Similarly there was no effect on morphology when A431 cells were treated with sequences where the first 5′-G residue was substituted with either an A or C nucleotide (Table 1). These data suggested that G residues were important for activity. Substitution of the first four G residues with T residues at the 5′-terminus resulted in aptamers that at best only modestly inhibited tyrosine kinase (less than 20% inhibitory activity). However, substitution of the fifth G residue with a T nucleotide in the parent sequence still exhibited about a 40% inhibition in TK activity. These findings suggested that all the first four G residues were essential for activity of the parent ODN and that the fifth G in the sequence was the least important as some inhibitory activity (~25–30%) was still present upon its substitution.

These studies involving mutations of G to T also suggested that the TK inhibitory activity was probably due to a sequence motif within the 5′-terminus of the parent compound. Splitting of the 15-mer parent PS-ODN into two molecules, a 6-mer 5′-GGA GGG-3′ (5′-terminal motif; APT6) and the 9-mer 5′-TCG CAT CGC-3′ (3′-motif) confirmed that the activity was a direct result of the 5′-terminal sequence. Indeed the 5′-terminal APT6 motif alone (i.e., the effective deletion of the 3′-terminus)

resulted in the same level of TK inhibition as the parent compound (see Table 1) confirming that this was the active motif in the parent compound. It also had a marked effect on cell morphology at a similar level to the parent compound (Table 1; data not shown) providing further supportive evidence for this being the active motif.

Further attempts to shorten this motif significantly reduced the inhibitory action. For example, splitting of the hexamer to two trimer sequences 5′-GGA-3′ or GGG failed to markedly alter the morphology of A431 cells upon treatment with either sequence individually (data not shown). Furthermore, substitution of the two terminal G residues with T as in the sequence 5′-TTA GGG-3′, which resembles the telomeric tandem repeat sequence found at the ends of human chromosomes (e.g. [14] and references therein), also failed to inhibit tyrosine kinase activity (Table 1). These studies collectively suggested that the active motif in the parent PS-ODN aptamer was the 5′-GGA GGG-3′ sequence found at its 5′-terminus. The hexameric motif identified by the present study appears to be novel and is distinct from the aptameric sequences previously reported as having anti-tyrosine kinase activity. For example, studies by Bergan *et al*. [15,16] suggested that ODN sequences housing GGC motifs were important for the inhibition of p210 bcr-abl tyrosine kinase autophosphorylation. This motif was not present in our parent aptamer and thus not contributing to the activity of the sequences studied here. However, it was present at the 3′-terminus of the scrambled control ODN (Table 1) and yet failed to exert any effect on the cell morphology or EGFR tyrosine kinase activity (Table 1). This finding further suggests that sequence context and/or the number of repeating motifs in a given sequence may be additional determinants for aptamer activity. Indeed sequence context is important for stimulation of immune response with CpG containing ODNs [17–19]. In another study, a 28-mer poly-C phosphorothioate ODN was effective in inhibiting EGFR tyrosine kinase activity [20] suggesting that several different motifs may be important for aptameric action at the level of receptor signaling. The effective concentration of the aptamers reported in the present study are similar (in the low micromolar range (∼1–5 μM)) to those reported previously by Bergan *et al*. [15,16], Krieg *et al*. [21] and Rockwell *et al*. [20]. However, the parent aptamer was about 10-fold more sensitive in the present study than previously reported by Coulson *et al*. [8]. A likely explanation is the different source and sensitivity of the A431 cells used in each study. Those used by Coulson *et al*. [8] were from an unidentified primary source and with an unknown age/passage number. They also appear less sensitive than the present A431 cells (obtained from ECACC) with respect to their effects with Tyrphostin A25 [22], a small molecular weight preferential inhibitor of EGF receptor tyrosine kinase, and further emphasizes the need to use cells from the same source and with defined characteristics for comparative studies (for a comparison of the effects of aptamer and Tyrphostin A24 on A431 tumor cell growth, see below).

3.2. The effect of aptamer ODN chemistry on tyrosine kinase activity

To investigate if the inhibition of EGFR tyrosine kinase activity by the hexamer aptameric motif was dependent on ODN chemistry, we synthesized PO and 2′-*O*-methyl modified versions of the motif. Table 1 shows that full modification of the aptamer to either of the new chemistries significantly reduces its inhibitory effect on tyrosine kinase activity. Approximately 15% inhibition with PO chemistry and only about 25–30% inhibition of tyrosine kinase activity were observed with the 2′-*O*-methyl modified APT6 aptamer. Neither of these chemical modifications had any marked effect on cell morphology (Table 1 and Fig. 2). This is in contrast to the PS-modified APT6 which inhibited tyrosine kinase activity by about 65–70% and significantly altered cell morphology (Table 1 and Fig. 1). This confirmed that the aptamer required an all phosphorothioate chemistry to exert its potent effect on cell morphology and EGF receptor kinase activity.

3.3. PS-ODNs with multiple repeats of the active motif enhance aptamer activity

As both the parent aptamer and the hexameric active motif only inhibited receptor tyrosine kinase activity by about 65–70% at the maximal doses studied, we investigated whether more potent aptamers could be produced by having sequences with multiple repeats of the active motif. Table 1 shows that by having 2.5 repeats (5′-GGA GGG GGA GGG GGA), coded as 2.5 × APT6, or three repeats ([5′-GGA GGG]$_3$) in a single new sequence improved the observed effect on EGF receptor kinase activity with almost 85–90% inhibition being observed with these aptamers. The more enhanced effect on cell morphology with 2.5 × APT6 is shown in Fig. 2 and can be compared with that observed for the parent aptamer in Fig. 1. For example 2.5 × APT6 produces markedly greater rounding of cells (Fig. 2d) compared with the same dose of aptamer in Fig. 1e. From a therapeutic viewpoint, the potential use of repeat motif ODNs as tyrosine kinase inhibitors has to be assessed in the light of the likely increased potential for such ODNs to interact with proteins involved in many other biological pathways. For example G-rich ODNs, especially those forming G-quadraplex or higher structures, are known to bind and/or interfere with the functions of DNA polymerases and transcription factors (e.g., see references [23,24]). Indeed in Table 1, we show that both GGGG and GGG GGG phosphorothioate-modified sequences markedly affect A431 cell morphology and inhibit TK activity. Hence, these results suggest that some of the biological effects of G-quadraplex forming ODNs may be mediated by inhibition of receptor tyrosine kinase

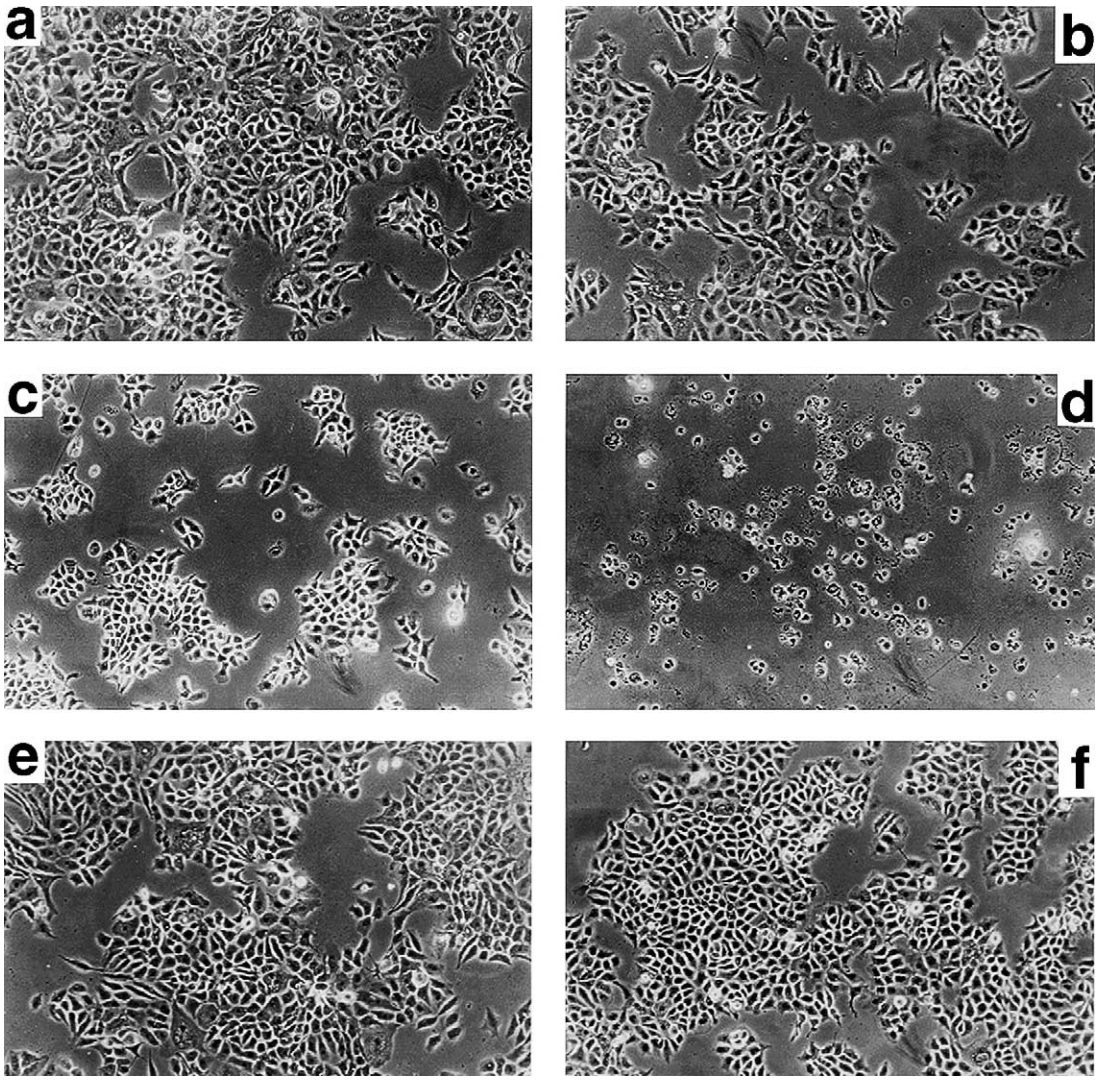

Fig. 2. Effect of 2.5 × APT6, an aptamer housing 2.5 multiple repeats of the active sequence, and aptamer chemistry on A431 cell morphology. Panel a represents untreated control cells. Panels b–d represent cells treated with 0.01, 1 and 2.5 μM of 2.5 × APT6, respectively. Panel e and f represent cells treated with 2.5 μM of 2′-*O*-methyl modified and all PO chemistry of APT6 (see Table 1 for sequences). In all cases cells were photographed at a 100× magnification following treatment with ODNs for 24 hr. Bar = 15 μM.

activity and further confirms that several different ODN motifs can modulate TK activity (see also references [15,16,20,21]).

3.4. Aptamer is a non-specific inhibitor of receptor tyrosine kinase activity

Previously we have shown that the anti-TK activity of the parent aptamer sequence was not due to impairment of EGF ligand–receptor binding or interference with EGFR receptor dimerization [8]. In this study, we have further examined the mechanism of action by investigating its specificity of action. The purpose of this study was to simply determine if a receptor tyrosine kinase other than EGFR could be inhibited by the aptamer ODN. In this preliminary investigation, we examined the effects of the parent aptamer on insulin receptor tyrosine kinase activity using an indirect assay that was already available in our group. This assay involved examining insulin stimulated glucose uptake in cultured L6 muscle cells, a process that required insulin receptor tyrosine phosphorylation.

Table 2 indicates that aptamer action is not specific to EGFR tyrosine kinase but also affects insulin receptor activity. Insulin (1 μM) caused a significant increase in glucose uptake compared to unstimulated cells and was

Table 2
The effect of aptamer on insulin stimulated glucose uptake in L6 muscle cells (mean ± SD; $n = 3$)

Treatment	Glucose uptake (%)
Insulin alone (1 μM)	101 ± 7
Insulin (1 μM) + 2.5 μM scrambled ODN	93 ± 6
Insulin (1 μM) + 2.5 μM APT6	57 ± 5

taken as control (i.e., 100% stimulation). In the presence of 2.5 μM scrambled control ODN, there was no significant change in this value (93 ± 6% stimulation). However, in the presence of 2.5 μM parent aptamer, insulin-stimulated uptake was significantly reduced (57 ± 5%). Thus, the aptamer appeared to inhibit insulin receptor-TK activity as assessed by this indirect assay without affecting differentiation of L6 cells. Since stimulation of glucose uptake by insulin depends on insulin tyrosine phosphorylation, this result is consistent with the aptamer inhibiting the insulin receptor tyrosine kinase.

Taken together the mechanistic studies reported here and those previously [8] suggest that inhibition of receptor tyrosine kinase activity is not specific to EGFR, is not due to inhibition of ligand binding, and is not by a direct inhibition of receptor homodimerization during receptor phosphorylation. As to what other receptor kinases are affected and whether heterodimerization between related growth factor receptors e.g., EGFR and HER2 is affected requires further study to help explain exactly how the aptamer is functioning.

3.5. Anti-tumor effects of the hexameric PS-ODN aptamer in vitro and in nude mice xenografts

Receptor tyrosine kinases play an important role in the initiation and development of cancers and inhibitors appear useful as anti-cancer agents [25,26]. In an attempt to investigate whether the hexameric ODN aptamer motif was an effective anti-tumor agent, we first examined its effects on cell growth in A431 cells in culture and also performed a preliminary *in vivo* study in nude mice xenografts. Fig. 3 shows the effects of APT6 and Tyrphostin A25, a positive control that is a preferential inhibitor of EGF receptor tyrosine kinase [27], on A431 tumor cell growth *in vitro*. Fig. 3a confirms that Tyrphostin A25 is an effective, dose-dependent inhibitor of the growth of the EGFR-overexpressing A431 cell line with an IC_{50} of approximately 3.5 μM which is similar to that reported previously [28,29]. In contrast, Tyrphostin A25 had little effect on the growth of the U87-MG glioma cell line that has largely lost its EGFR expression [8] and its dependence on the growth-promoting effects of EGF [30]. This further suggested that Tyrphostin A25 was mediating its effects by preferentially interfering with EGF receptor signaling. Fig. 3b shows that the APT6 also had a dose-dependent inhibitory effect on the growth of A431 cells in culture in a similar manner to the positive control. For example at a dose of 2.5 μM, A431 cell growth was reduced to about 68 % of the control with the aptamer treatment compared to 62% with Tyrphostin A25 treatment (see Fig. 3). These data suggested that dose-dependent inhibition of tyrosine kinase activity by APT6 (see Table 1) was proportional to its effects on tumor cell growth *in vitro* (see Fig. 3b). These encouraging anti-tumor results *in vitro* led to the testing of this aptamer *in vivo*.

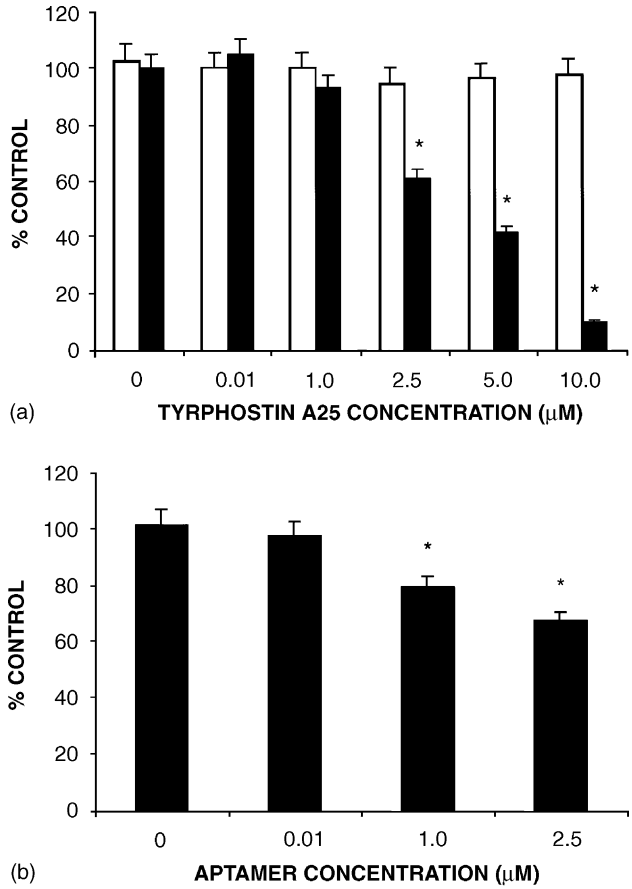

Fig. 3. (a) Effects of Tyrphostin A25 on cell growth in A431 (solid bars) and U87-MG (open bars) cell lines. Cells were incubated with increasing concentrations (0.1–10 μM) of Tyrphostin A25 for 24 hr and then cell numbers determined by trypan blue exclusion assay. (b) The effect of APT6 on growth of A431 cells in culture as a function of aptamer concentration. Cells were incubated with increasing concentrations (0.01–2.5 μM) of APT6 for 24 hr and then cell numbers determined as above. All data are expressed as percentage of control (untreated) cell number. Bars represent SD where $n = 3$. Asterisk (∗) denotes a significant reduction ($P < 0.05$) from the untreated control.

For the *in vivo* study, cultured A431 epidermoid cancer cells were injected sub-cutaneously in nude mice to establish xenografts. Once the mice exhibited palpable tumors, a single intra-tumoral dose (16 mg/kg) of the 6-mer APT6 aptamer was administered. The 6-mer was selected for this study based on the *in vitro* results above but also because of its lower molecular weight (compared to the 15-mer parent), which may facilitate its delivery to tumor cells *in vivo*. After 14 days, relative tumor volumes were compared for untreated, saline treated and aptamer treated mice (see Fig. 4). There was a general, but not statistically significant, trend to lower tumor volumes with saline treated animals compared to untreated controls. The reason for this trend is unclear but may be related to damage following needle injection that could somewhat impair tumor growth. The explanation for this effect requires further study. Although, there was no difference in the relative tumor volume between mice receiving aptamer

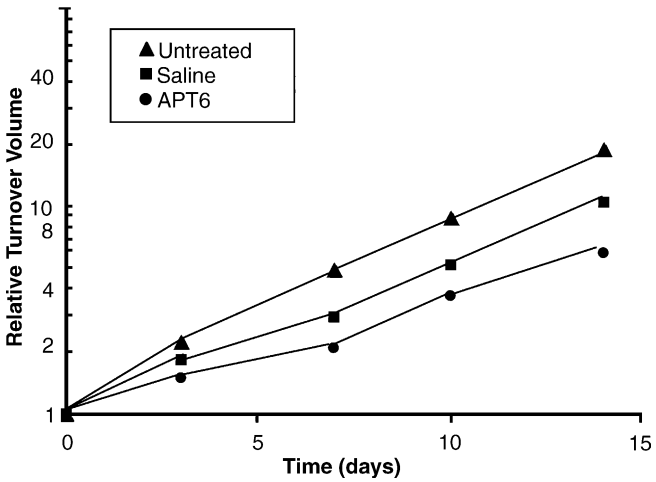

Fig. 4. The effect of APT6 on A431 tumor growth in nude mice. The mean relative tumour volume is given for each group of six mice. A statistically significant difference between data for untreated and APT6 treated mice was observed ($P > 0.01$) but not between untreated and saline or even saline and APT6 treated. SDs are omitted for clarity of the figure.

or saline, there was a modest but statistically significant anti-tumor effect with the 6-mer active motif ($P < 0.01$) compared to the untreated controls. This suggested that such aptameric inhibitors of EGFR kinase activity may have a role in inhibiting tumor growth *in vivo* and are worthy of further study. A more thorough study including a full dose-response and examination of the specificity of effect in animal models would further assess the full potential of APT6 efficacy *in vivo*. Further work is planned with the more active repeat motif sequences and with more frequent dosing regimens. There is also the possibility that a single dose administration of ODN may not be sufficient to maintain an effective dose in the tumors for the duration of this study. Indeed we have recently shown that a direct intra-tumoral, single-dose administration of a fluorescently-labeled antisense ODN resulted in only about 20% of the dose remaining 6-hr post-injection [2]. The subsequent use of this ODN entrapped within a sustained-release polymer microsphere formulation significantly improved residence time of the nucleic acid within the tumor [2]. In another study, we have shown that polymer formulations can also improve the cellular biodistribution as well as increase the residence time of nucleic acids in the brain of rats when administered site-specifically in the neostriatum [31]. In addition, the localized intra-tumoral administration of aptameric ODNs as polymer implants, of even those aptamers that are non-specific inhibitors of receptor TK activity, should limit their activity to the site of administration (e.g., solid tumors) and thereby minimize unwanted effects in normal tissue. Thus the use of polymeric sustained release formulations may not only improve the pharmacokinetic and pharmacodynamics of ODNs but may further help localize the activity of aptameric ODNs to target tissues and as such they are being actively studied in the laboratory [2,31,32].

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References

[1] Hughes MD, Hussain M, Nawaz Q, Sayyed P, Akhtar S. Cellular delivery of antisense oligonucleotides and ribozymes. Drug Discov Today 2001;6(6):303–15.
[2] Akhtar S, Hughes MD, Khan A, Double J, Hussain M, Nawaz Q, Bibby M, Sayyed P. The delivery of antisense therapeutics. Adv Drug Deliv Rev 2000;44(1):3–21.
[3] Sohail M, Southern EM. Selecting optimal antisense reagents. Adv Drug Deliv Rev 2000;44:23–34.
[4] Akhtar S, Agrawal S. In vivo studies with antisense oligonucleotides. Trends Pharmacol Sci 1997;18:12–8.
[5] Tsiang M, Gibbs CS, Griffin LC, Dunn KE, Leung LL. Selection of a suppressor mutation that restores affinity of an oligonucleotide inhibitor for thrombin using in vitro genetics. J Biol Chem 1995;270:19370–6.
[6] Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature 1992;355:564–6.
[7] Griffin LC, Tidmarsh GF, Bock LC, Toole JJ, Leung LLK. In vivo anticoagulant properties of a novel nucleotide-based thrombin inhibitor and demonstration of regional anticoagulation in extracorporeal circuits. Blood 1993;81:3271–6.
[8] Coulson JM, Poyner D, Chantry A, Irwin WJ, Akhtar S. A non-antisense sequence-selective effect of a phosphorothioate oligonucleotide targeted to EGFR in A431 cells. Mol Pharmacol 1996;50(2):314–25.
[9] Fell P, Reynolds M, Usman N, Akhtar S. Cellular uptake properties of 2′-amino/2′-*O*-methyl-modified hammerhead ribozyme targeted to the epidermal growth factor receptor mRNA. Antisense Nucleic Acid Drug Dev 1997;7:319–26.
[10] Wan MSK, Fell PL, Akhtar S. Synthetic 2′-*O*-methyl-modified hammerhead ribozymes targeted to the RNA component of telomerase as sequence-specific inhibitors of telomerase activity. Antisense Nucleic Acid Drug Dev 1998;8:309–17.
[11] Poyner D, Andrew DP, Brown D, Bose C, Hanley MR. Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat L6 myocytes. Br J Pharmacol 1992;105:441–7.
[12] Klip A, Li G, Logan WJ. Induction of sugar uptake response to insulin by serum depletion in fusing L6 myoblasts. Am J Physiol 1984;247:E291–6.
[13] UKCCR Research Guidelines for the welfare of animals in experimental neoplasia. Br J Cancer 1998;77:1–10.
[14] Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989;49:521–9.
[15] Bergan R, Connell Y, Fahmy B, Kyle E, Neckers L. Aptameric inhibition of $p210^{bcr\text{-}abl}$ tyrosine kinase autophosphorylation by oligodeoxynucleotides of defined sequence and backbone structure. Nucleic Acids Res 1994;22:2150–4.
[16] Bergan R, Kyle E, Connell Y, Neckers L. Inhibition of protein-tyrosine kinase activity in intact cells by the aptameric action of oligodeoxynucleotides. Antisense Res Dev 1995;5:33–8.
[17] Krieg AM. Immune effects and mechanisms of action of CpG motifs. Vaccine 2000;19:618–22.
[18] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. Nature 1995;374:546–9.

[19] Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J Immunol 1992;148:4072–6.
[20] Rockwell P, O'Connor WJ, King K, Goldstein NI, Zhang LM, Stein CA. Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides. Proc Natl Acad Sci USA 1997;94:6523–8.
[21] Krieg AM, Matson S, Kunrong C, Fisher E, Koretzky GA, Koland JG. Identification of an oligodeoxynucleotide sequence motif that specifically inhibits phosphorylation by protein tyrosine kinases. Antisense Nucleic Acid Drug Dev 1997;7:115–23.
[22] Fell P. Cellular uptake and biological activity of synthetic hammerhead ribozymes. Ph.D. Thesis, Aston University, Birmingham, UK, 1999.
[23] Higgins KA, Perez JR, Coleman TA, Dorshkind K, McCommas WA, Sarmiento UM, Rosen CA, Narayanan R. Proc Natl Acad Sci USA 1993;90:9901–5.
[24] Stein CA, Cheng YC. Antisense oligonucleotides as therapeutic agents: is the bullet really magical? Science 1993;261:1004–12.
[25] Kolibaba KS, Drucker BJ. Protein tyrosine kinases and cancer. Biochem Biophys Acta 1997;1333:F217–48.
[26] Noonberg SB, Benz CB. Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily. Drugs 2000;59:753–67.
[27] Sion-Vardy N, Vardy D, Rodeck U, Kari C, Levin RM, Malkowicz SB. Antiproliferative effects of tyrosine kinase inhibitors (Tyrphostins) on human bladder and renal carcinoma cells. J Surg Res 1995;59:675–80.
[28] Gazit A, Yaish P, Gilon C, Levitzki A. Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem 1989;10:2344–52.
[29] Lyall RM, Zilberstein A, Gazit A, Gilon C, Levitzki A, Schlessinger J. Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. J Biol Chem 1989;264(24):14503–9.
[30] Hoi-Sang U, Espiritu OD, Kelley PY, Klauber MR, Hatton JD. The role of the epidermal growth factor receptor in human gliomas: II. The control of glial process extension and the expression of glial fibrillary acidic protein. J Neurosurg 1995;82:847–57.
[31] Khan A, Sommer W, Fuxe K, Akhtar S. Site-specific administration of antisense oligonucleotides using biodegradable polymer microspheres provides sustained delivery and improved subcellular biodistribution in the neostriatum of the rat brain. J Drug Target 2000;8(5):319–34.
[32] Hussain M, Beale G, Hughes MD, Akhtar S. Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release P(LA-GA) microsphere formulations for potential combination therapy in cancer. Int J Pharm 2001;234:129–38.