Inhibition of gene expression by anti-sense C-5 propyne oligonucleotides detected by a reporter enzyme

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Using a reporter plasmid containing the luciferase gene under the control of the insulin-like growth factor 1 (IGF-1) promoter region [including its 5′ untranslated region (UTR)], we demonstrate that a 17-mer oligophosphorothioate containing C-5 propyne pyrimidines is able to inhibit luciferase gene expression in the nanomolar concentration range when the anti-sense oligonucleotide is targeted either to a coding sequence in the luciferase gene or to the 5′ UTR of the gene for IGF-1. Inhibition was obtained independently of whether the plasmid and the anti-sense oligonucleotide were co-transfected or transfected separately into hepatocarcinoma cells. However, the efficiency of inhibition by the anti-sense oligonucleotides was 10-fold greater in the first case. The unmodified oligophosphorothioate targeted to the 5′ UTR of IGF-1 did not inhibit luciferase gene expression at a 100-fold higher concentration unless its length was increased from 17 to 21 nt, in which case an inhibition of gene expression was obtained and an IC_{50} of 200 nM was observed.

Key words: cationic lipid, insulin-like growth factor 1, luciferase, phosphorothioate.

INTRODUCTION

Synthetic oligonucleotides can be used to control gene expression selectively by one of several strategies [1]. In the anti-sense strategy, the oligonucleotide is targeted to a complementary sequence in a messenger RNA and leads to an inhibition of translation of the targeted gene [1]. In the anti-gene strategy, the oligonucleotide binds to the DNA duplex via triple-helix formation and inhibits gene transcription [2].

Although anti-sense oligophosphorothioates have been used extensively as gene inhibitors in cellular systems and are used at present in clinical trials (reviewed in [3]), some unexpected effects of oligophosphorothioates, unrelated to their intended anti-sense activity, have been described: non-sequence-specific inhibition of gene expression, inhibition of cellular adhesion, induction of immune response and protein trapping (reviewed in [4]). These non-specific effects could be observed at high concentration and with long sequences of oligophosphorothioates.

Short oligophosphorothioates containing C-5 propyne pyrimidines might represent a good alternative to oligophosphorothioates. Phosphorothioate oligonucleotides containing C-5-propynyl-2′-deoxyuridine (pdU) and C-5-propynyl-2′-deoxycytidine (pdC) have been shown to be potent and specific anti-sense inhibitors of protein expression such as simian virus 40 (SV40) TAg [5–7], p34(cdc2) kinase [8], cyclin B1 [8], p27kipl [9,10], HIV *env* [11], HIV *rev* [12], tumour necrosis factor α [13], *Escherichia coli* β-galactosidase [5] and firefly (*Photinus pyralis*) luciferase [14,15] in various cellular systems. In most of these studies, C-5 propyne oligophosphorothioates were introduced together with their plasmid targets in cells by nuclear microinjection to compensate for their poor cellular uptake. A new cationic cytofectin has been successfully used with C-5 propyne oligonucleotides to inhibit gene expression [8,10,14,15]. More recently, phosphorothioates containing C-5 propyne were shown to exhibit non-specific effects when delivered to leukaemia cells by means of permeabilization with streptolysin O [16].

Insulin-like growth factor 1 (IGF-1) is a highly conserved 70-residue circulating peptide that is expressed in a wide variety of tissues in adult animals [17]; it is a key regulator of cell growth in normal cells including fibroblasts, keratinocytes and haemopoietic cells. IGF-1 and its receptor, IGF-1-R, also seem to have a major role in the transformation and tumorigenesis of various cells [18] such as glioblastoma [19,20], prostatic carcinoma [21], melanoma [22], breast carcinoma [23] and hepatocarcinoma cells [24]. IGF-1 or IGF-1-R synthesis can be inhibited by using an anti-sense RNA strategy targeted to the IGF-1 or IGF-1-R mRNA species in glioblastoma and hepatocarcinoma cells. In these cell lines, inhibition of IGF-1 or IGF-1-R expression leads to the inhibition of tumorigenesis *in vivo* [19,20,24]. Anti-sense oligophosphorothioates targeted to the IGF-1-R mRNA have been used to demonstrate the important role of IGF-1-R in cellular proliferation, transformation and tumorigenesis (reviewed in [25]).

In the present study we designed anti-sense oligonucleotides directed to the 5′ untranslated region (UTR) of the IGF-1 gene to inhibit IGF-1 synthesis in rat hepatocarcinoma cell lines. A transient transfection assay with two reporter genes, the *P. pyralis* and the *Renilla* luciferases, was used to monitor rapidly the effect of anti-sense oligonucleotides targeted to the 5′ UTR of IGF-1. We show that an anti-sense oligonucleotide 17 nt long containing C-5 propyne pyrimidines and phosphorothioate linkages is a more potent inhibitor ($IC_{50} \approx 5$ nM) than the oligophosphorothioate lacking the propynyl groups, which did not exhibit any activity up to 500 nM. When the length of the

Abbreviations used: IGF-1, insulin-like growth factor 1; IGF-1-R, insulin-like growth factor 1 receptor; SV40, simian virus 40; T_m, melting temperature; UTR, untranslated region.

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unsubstituted anti-sense oligophosphorothioate was increased from 17 to 21 nt, a sequence-specific inhibition of luciferase expression was observed with an IC_{50} of 200 nM. Our results demonstrate the potency of anti-sense C-5-propyne-containing oligophosphorothioates compared with oligophosphorothioates to target IGF-1 mRNA and show that anti-sense strategies against IGF-1 could be used in hepatocarcinoma therapy.

MATERIALS AND METHODS

Oligonucleotides and UV absorption spectroscopy

All oligonucleotides used in this study were purchased from Eurogentec (Seraing, Belgium). They were purified either by denaturing PAGE or by gel filtration on Sephadex G-25 mini-columns (Boehringer Mannheim) and precipitation with ethanol. The quality of all oligonucleotides including the C-5-propyne-containing oligophosphorothioates was checked by PAGE and UV shadowing with a chromatography paper containing fluorescent indicator that was irradiated at 254 nm (TLC plastic sheets silica gel 60 F254; Merck). Oligonucleotide concentrations were determined by measuring A_{260} with the use of the molar absorption coefficients calculated as described in [26]. The absorbances of the C-5-propyne-containing oligophosphorothioates were also measured at 260 nm, with molar absorption coefficients of 5000 $M^{-1} \cdot cm^{-1}$ for 5-(1-propynyl)uracil and of 3200 $M^{-1} \cdot cm^{-1}$ for 5-(1-propynyl)cytosine (http://lomand.glenres.com/ProductFiles/Technical/Extinctions.html). Melting experiments with anti-sense oligonucleotides were performed as described previously [27].

Plasmids

The pIGF1630/luc and the p1711b/luc plasmids, kindly supplied by Peter Rotwein (St. Louis, MO, U.S.A.), contained the firefly (*P. pyralis*) luciferase gene (*luc*) under the control of the promoter regions (including the 5′ UTR) of the human and rat IGF-1 genes respectively. The pIGF1630/luc plasmid was obtained by subcloning the 5′-flanking region of the human IGF-1 gene in the plasmid pOLuc [28]; it contained the promoter sequence (from −1630 to +1) and the 5′ UTR (from +1 to +322) of the human IGF-1 gene ([29], GenBank accession no. M77496). The p1711b/luc plasmid contained the promoter sequence (from −1711 to +1) and the 5′ UTR (from +1 to +328) of the rat IGF-1 gene ([30,31], GenBank accession no. J02743) and was subcloned in pGL2-Basic contruct (Promega). The pGL2-Control (Promega) contained the firefly luciferase gene (*luc*) under the control of the SV40 promoter and enhancer sequences.

The pRL family of *Renilla* luciferase control vectors (Promega) were used to monitor transfection efficiency. The pRL-CMV contained the cytomegalovirus (CMV) immediate-early enhancer/promoter region controlling the transcription of the sea pansy (*Renilla reniformis*) luciferase gene (*Rluc*). The pRL-TK plasmid contained the herpes simplex virus thymidine kinase (HSV-TK) promoter region upstream of *Rluc*. All plasmids used in the transfection experiments were first propagated in JM109 bacterial strain (Promega), purified by Qiagen Maxipreps kits (Qiagen) and then precipitated with ethanol.

Cells and transfections

The LFCL2A rat hepatocarcinoma cell line was used for the co-transfection of anti-sense oligonucleotides and reporter vectors. This cell line was established from a hepatocellular carcinoma induced in the Commentry rat by 4-dimethylaminoazobenzene [32] and expressed IGF-1 [24]. Cells were maintained in minimal essential medium containing 10% (v/v) newborn-calf serum (heat-inactivated for 30 min at 60 °C).

Most transfections (plasmids and oligonucleotides) performed in this study were conducted with TFX50 reagent (Promega), which is a mixture of a synthetic cationic lipid molecule [*N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis(2-hydroxyethyl)-2,3,-dioleoyl-oxy-1,4-butanediammonium iodide] and L-dioleoylphosphatidyl-ethanolamine. Lipofectin reagent (Bethesda Research Laboratories), DOTAP (Boehringer Mannheim) and polyethyleneimine (PEI MW800, a gift from Dr. J. P. Behr [33]) were also used to transfect DNA plasmids in LFCL2A cells. DNA and lipids were combined in accordance with the recommendation of the suppliers. No centrifugation of cells was performed, to help the binding of the DNA–cytofectin precipitates to cell membranes.

A typical transfection experiment with TFX50 reagent was performed as follows. Various quantities of oligonucleotides were mixed with 6 μg of firefly luciferase-containing plasmid DNA and 0.1 μg of *Renilla* luciferase-containing plasmid DNA in a total volume of 1.5 ml (medium without serum). TFX50 reagent (final concentration 18 μM) was then added to the mixture to obtain a cationic lipid-to-DNA ratio of 3:1. The mixture was split into triplicates and then added to LFCL2A cells (2×10^5 cells per well spread in 12-well plates, 500 μl per well). After 4 h of incubation at 37 °C, the medium was diluted by adding minimal essential medium (500 μl) containing 10% (v/v) serum. Cells were then incubated overnight before harvesting 24 h after transfection.

P. pyralis and *Renilla* luciferase assays

Both luciferase activities (*P. pyralis* and *Renilla*) were measured in cell extracts by using the dual-luciferase assay kit (Promega). In brief, transfected LFCL2A cells were lysed passively with lysis buffer (250 μl per well; Promega) for 30 min. Protein concentrations of cell extracts were determined by using the Bradford reagent (the protein assay kit from Bio-Rad). Equal quantities of proteins were then analysed sequentially for the expression of both luciferase genes with a Microlite TLX1 dual-injector luminometer (Dynatech). First, substrates for the *P. pyralis* luciferase (ATP, beetle luciferin and CoA) were automatically injected into the sample. The light generated by the firefly luciferase was measured for 10 s and is given in relative light units. After 10 s, a quencher of the *P. pyralis* luciferase and substrates for the *Renilla* luciferase (coelenterazine) were injected with the second injector and light was recorded for 10 s. Light emission was then due only to the *Renilla* luciferase. The second luciferase (*Rluc*) gene expression provided an internal control value to which expression of the experimental firefly reporter gene could be normalized. The luciferase activity shown on figures (in arbitrary units) corresponds to the ratio of the relative light units detected for the *P. pyralis* luciferase and the *Renilla* luciferase. All transfection experiments were done in triplicate and repeated at least twice.

RESULTS AND DISCUSSION

Design of anti-sense oligonucleotides

The IGF-1 gene from several species has been characterized (reviewed in [34]). The IGF-1 genes in rats and humans contain six exons. Multiple transcription initiation sites define two leader exons (exon 1 and 2 with two different promoters, P1 and P2) that encode different 5′ UTRs and distinct putative IGF-1 signal peptides [35]. In rats, IGF-1 mRNA species containing the 5′ UTR of exon 1 represent the predominant IGF-1 mRNA species

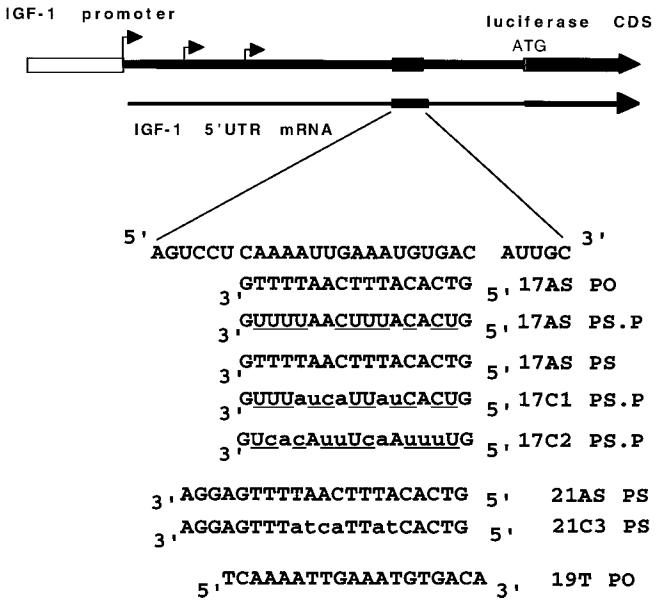

Figure 1 Sequences of the anti-sense oligonucleotides used in this study

The anti-sense oligonucleotides (17AS PO, 17AS PS.P, 17AS PS and 21AS PS) are targeted to a sequence located in the 5′ UTR of the rat and human IGF-1 gene (see Figure 2). The promoter P1 and the 5′ UTR sequences of the IGF-1 gene have been subcloned upstream of a firefly luciferase gene in pOluc or pGL2-basic vectors (see the Materials and methods section). PO, oligophosphodiesters; PS, oligophosphorothioates; PS.P, oligophosphorothioates containing C-5 propyne pyrimidines. The C-5 propyne uracils and C-5 propyne cytosines are underlined. The control oligonucleotides 17C1 PS.P and 21C3 PS are the six-base mismatch counterparts of 17AS PS.P and 21AS PS. Mismatches are noted in lower-case letters. The 17C2 PS.P has the sequence of 17AS PS.P but in the reverse orientation, which corresponds to a ten-base mismatch oligonucleotide compared with the 17AS PS.P. The sequence of the 19T PO used in melting experiments is also indicated.

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HUMAN     AATCTCACTGTCACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
RAT       AATCTCACTGTCGCTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATCGAAATAAAGTCC
MOUSE     AATCTCACTGTCACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
GOAT      AATCTCACTGTCACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
SHEEP     AATCTCACTGTCACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
PORCINE   AATCTCACTGT-ACTGCTAAATTCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
CHICKEN   AATCTCACTGTCACTGCTAAAATCAGAGCAGATAGAGCCTGCGCAATGGAA-TAAAGTCC
XENOPUS   AATCTCACTGTCACTGCTAAAATCAGAGCAGATAGT--CTACGCAATGGAA-CAAAGTCC
          ********** ******** ************   ** ****** ***  *******

HUMAN     TCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCTCTGGATTTCTTTTTGCT
RAT       TCAAAATTGAAATGTGACTTTGCTCT-AACATCTCCCATCTCTCTGGATTTCTTTTTGCC
MOUSE     TCAAAATTGAAATGTGACATTGCTCT-AACATCTCCCATCTCTCTGGATTTCTTTTTCGC
GOAT      TCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGATTTCTTTTTGCC
SHEEP     TCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGATTTCTTTTTGCC
PORCINE   TCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGATTTCTTTTTGCC
CHICKEN   TCAATATTGAAATGTGACATTGCTCTCAACATCTCACATCTCTCTGGATTTCTTTTTTC-
XENOPUS   TCTATATTGAAATGTGACATTGCTCTGAACATCTCCA------CTGGATTCCTTTT----
          ** * ************** ******* ********        ******* *****

HUMAN     -TCATTATTCCTGCTAACCAATTCATTTTCAGACTTTGTACTTC--AGAAGCAATGGGAA
RAT       -TCATTATTCCTGCCCACCAATTCATTTCCAGACTTTGTACTTC--AGAAGCGATGGGGA
MOUSE     CTCATTATCCCTGCCCACCAATTCATTTCCAGACTTTGTACT--TCAGAAGCGATGGGGA
GOAT      -TCATTATTCCTGCTAACCAATTCATTTCCAGACTTTGCACTTC--AGAAGCAATGGGAA
SHEEP     -TCATTATTCCTGCTAACCAATTCATTTCCAGACTTTGCACTTC--AGAAGCAATGGGAA
PORCINE   -TCATTATTCCTGCTAACCAATTCATTTCCAGACTTTGCACTTC--AGAAGCAATGGGAA
CHICKEN   -TCATCATTACTGCTAACAAATTCATTTCCAGACTTTGCACTTTAAGAAGCAATGGAAA
XENOPUS   ---ATTATCACAGCTGACCATTCCATTCCCAGTCTCTGCTCTCCTCAGAAGCCATGGAAA
             ** **  * **  ** * * ****  *** ** **  **    ****** ****  *

HUMAN     AAATCAGCAGTCTTCCAACC-CAATTATTTAAGTGCTGCTTTTG-TGATTTCTTGAAGGT
RAT       AAATCAGCAGTCTTCCAACT-CAATTATTTAAGATCTGCCTCTG-TGACTTCTTGAAGGT
MOUSE     AAATCAGCAGCCTTTCAACTTCAATTATTAAAGATCTGCCTCTGGTGACTTCTTGAAGAT
GOAT      AAATCAGCAGTCTTCCAACC-CAATTATTTAAGTGCTGCTTTTG-TGATTTCTTGAAGGT
SHEEP     AAATCAGCAGTCTTCCAACC-CAATTATTTAAGTGCTGCTTTTG-TGATTTCTTGAAGGT
PORCINE   AAATCAGCAGTCTTCCAACC-CAATTATTTAAGTGCTGCTTTTG-TGATTTCTTGAAGGT
CHICKEN   AAATCAACAGTCTTTCAACA-CAATTAGTTAAGTGCTGCTTTTG-TGATTTCTTGAAGGT
XENOPUS   CAAACAACAATCTTTCAACA-CAATTATTTAAGTGCTACTTTTG-TGATATCTTAAAGCT
           ** ** **  *** ****  ****** * ***  ** * * ** ***  **** *** *
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Figure 2 Sequence similarities of the 5′ UTRs of the IGF-1 mRNAs between various species

The sequence of the 5′ UTR of human IGF-1 was compared with sequences in the GenBank database by using the FASTA program. Similar sequences were then aligned by using the CLUSTALW program. Only a part of the alignment is shown. Completely identical nucleotides between species are indicated by asterisks below the sequences [accession numbers are given in parentheses after the locus names: HUMGFIAB1 (M12659, M77496), RATGFIL1 (M15647, J02743), W10072 (W10072), GOTIGFI1 (D26116), OAIGFIEX1 (X17229), SSIGF1E1 (X52388, X17491), CHKIGFIEX1 (M74176), XELIGFIA (M29857) respectively]. The target sequence for the anti-sense oligonucleotide is indicated in bold.

in all tissues [31,36,37]. The major promoter (P1) located in the 5′-flanking region of exon 1 activates transcription over a relatively dispersed region of exon 1, leading to IGF-1 mRNA species with heterogeneous 5′ ends [29,31], because the promoter lacks proximal transcriptional control elements such as TATAAA and CAAT boxes [31]. The transcription initiation sites cover approx. 100 bp (represented with arrows in Figure 1).

Anti-sense oligonucleotides could be targeted upstream or downstream of the translation initiation codon of mRNA species. Although it was initially thought that the AUG region of mRNA species was more accessible to anti-sense hydridization, numerous anti-sense effects were described with oligonucleotides targeted to the 5′ UTR or to the non-coding 3′ end of mRNA species, which might be accessible regions. It was shown previously that an unmodified 15 nt anti-sense oligodeoxynucleotide complementary to the 5′ human IGF-1 mRNA sequence, including the translation start site, inhibited IGF-1 protein synthesis and DNA synthesis of human embryonic lung fibroblasts [38]. To our knowledge, no anti-sense oligonucleotides have previously been targeted to the 5′ UTR of IGF-1 mRNA.

We chose to target anti-sense oligonucleotides to a common sequence of the 5′ UTR region of the rat and human IGF-1 mRNA species transcribed from the major promoter P1 of the IGF-1 gene. As shown by the sequence similarity analysis performed with the FASTA program in Genbank, the sequence of the 5′ UTR of the IGF-1 mRNA is well conserved among various animal species (Figure 2). Almost 95% nucleotide identity was observed. The target sequence of the anti-sense oligonucleotides is present in all rat and human IGF-1 mRNA species synthesized from the multiple transcription start sites (see Figures 1 and 2).

In the culture medium of LFCL2A rat hepatocarcinoma cells, unmodified oligonucleotides were degraded rapidly by nucleases (results not shown); we therefore chose to study nuclease-resistant anti-sense oligonucleotides such as C-5 propyne pyrimidine-containing oligophosphorothioates. The role of oligonucleotide length and target mismatches has been previously investigated for C-5-propyne-containing oligophosphorothioates [15]. The length (17 nt) of the anti-sense oligonucleotides (17AS PO, 17AS PS and 17AS PS.P) was chosen to be long enough to have a unique target sequence in all human and rodent genes of GenBank [39] and short enough to limit potential non-anti-sense effects. In fact the lack of sequence specificity of anti-sense oligophosphorothioates tends to increase with their length [40]. It was shown that a 21 nt C-5-propyne-containing anti-sense oligophosphorothioate exhibited non-sequence-specific effects [16]. A shorter oligophosphorothioate, such as a 17-mer, should exhibit no or fewer non-specific effects. This could be confirmed by designing the appropriate control oligonucleotides with the same length. Two different 17-base control oligonucleotides were designed by permuting the nucleotide sequence of the anti-sense oligonucleotide and conserving the base composition. The 17C1 PS.P and 17C2 PS.P oligonucleotides exhibited six-base and ten-base mismatches when aligned against the corresponding 17AS PS.P (Figure 1). Their complementary sequences are absent from the rat and human gene databases (Genbank).

Table 1 Comparison of various cytofectin agents in transfecting pGL2-control and pRL-TK vectors in LF(CL2A) hepatocarcinoma cells

pGL2-control (5 μg) and pRL-TK (0.16 μg) were mixed in 1.5 ml of minimal essential medium with the cytofectins (final concentrations: TFX50, 18 μM; polyethyleneimine, 0.12 mM; DOTAP and lipofectin, 20 μg/ml) in accordance with the manufacturers' recommendations and were added to cells (500 μl per triplicate). After 4 h of incubation at 37 °C, cell medium was diluted by the addition of 500 μl of serum-containing medium per well. Relative light unit (RLU) values, determined as indicated in the Materials and methods section, were subtracted from the RLU obtained with mock-transfected cells and normalized for protein content. Cell backgrounds were 6 RLU/μg of protein for *P. pyralis* luciferase expression and 58 RLU/μg of protein for *Renilla* luciferase expression.

Cytofectin reagent	*P. pyralis* luciferase activity (RLU/μg)	*Renilla* luciferase activity (RLU/μg)
TFX50	12883 ± 2014	280 ± 44
Polyethyleneimine	3524 ± 783	201 ± 36
DOTAP	7799 ± 790	272 ± 39
Lipofectin	343 ± 292	71 ± 8

Choice of cationic lipid to transfect plasmids in LFCL2A cells

It is well known that DNA transfection efficiency can vary greatly from one cell type to another one and depends on the cytofectins used. We compared the transfection efficiencies of four commercial cytofectins (TFX50, DOTAP, lipofectin and polyethyleneimine) in LFCL2A rat hepatocarcinoma cells. The DNA (pGL2-control, pRL-TK) and cytofectins were mixed as recommended by the suppliers and were applied to cells for 4 h at 37 °C. Cell culture medium was then diluted with serum-containing medium. Luciferase activities in cell extracts were determined after 24 h expression as described in the Materials and methods section. As shown in Table 1, the TFX50 reagent gave better transfection efficiencies in LFCL2A cells than did the polyethyleneimine, DOTAP and lipofectin reagents. Therefore TFX50 was used for all further transfections of DNA plasmids and oligonucleotides in the LFCL2A cell line.

Anti-sense activity of C-5-propyne-containing phosphorothioate oligonucleotides

Anti-sense oligonucleotides were co-transfected with the reporter plasmid p1711b/luc containing the promoter and the 5′ UTR region of the rat IGF-1 gene with the use of TFX50 cytofectin (Figure 3). Only the sequence-specific anti-sense oligonucleotide 17AS PS.P induced a concentration-dependent inhibition of luciferase expression with an IC_{50} of approx. 5 nM (Figure 3). No inhibition was observed with 17AS PS.P at 1 nM (results not shown). The control oligomer, 17C1 PS.P, did not inhibit luciferase expression but it slightly increased its expression, even at the lowest concentration used in our study (10 nM). This was also observed with the second control oligonucleotide 17C2 PS.P (results not shown) and the phosphorothioate 17AS PS, which did not contain C-5 propyne derivatives (Figure 3). Therefore these observations were probably due to a non-sequence-specific effect of the phosphorothioate backbones.

Similar anti-sense effects with 17AS PS.P were obtained with the plasmid containing the human 5′ UTR IGF (p1640IGF/luc) (results not shown). No inhibition of luciferase gene expression occurred with the anti-sense oligonucleotide 17AS PS.P, when the control plasmid pGL2 containing the SV40 promoter was used for transfection instead of the plasmids containing the 5′ UTR region of the IGF-1 (results not shown). This result emphasizes the sequence specificity of the anti-sense oligonucleotide.

We also compared the effect of the C-5-propyne-containing anti-IGF-1 oligophosphorothioate (17AS PS.P) with a C-5-propyne-containing anti-sense oligophosphorothioate targeted to the coding region of *P. pyralis* luciferase (GenBank accession no. M15077). This C-5-propyne-containing anti-luciferase 15-mer oligonucleotide, 5′-dUUUGGCGUCUUCCAU-3′, has been previously used in the nanomolar range (IC_{50} between 1 and

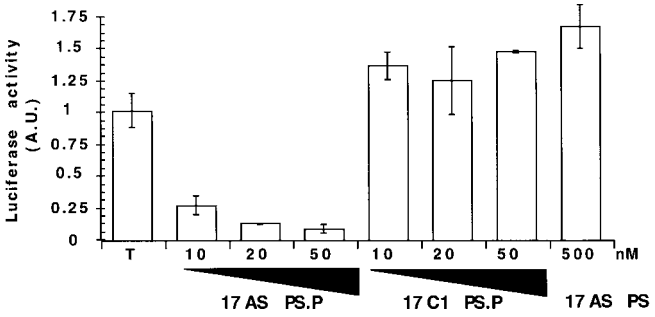

Figure 3 Anti-sense-specific inhibition of luciferase enzymic activity by anti-sense oligonucleotides targeted to a sequence located in the 5′ UTR of the IGF-1 gene (see Figures 1 and 2)

Dose–response inhibition of *P. pyralis* luciferase in LFCL2A cells with the C-5-propyne-containing oligophosphorothioate 17AS PS.P and the six-base mismatch 17C1 PS.P. A range of oligonucleotide concentrations (10, 20 and 50 nM) were delivered to the LFCL2A cells together with reporter vectors (p1711b/luc and pRL-TK; see the Materials and methods section) with 18 μM TFX50 reagent. The oligophosphorothioate 17AS PS was used at 500 nM. Both luciferase activities were measured 24 h later and ratios of *P. pyralis* luciferase to *Renilla* luciferase were calculated and normalized to the expression of the cells that did not receive oligonucleotides (T). Error bars were obtained from triplicates (mean ± S.D.). Abbreviation: A.U., arbitrary units.

Table 2 Melting temperatures for the dissociation of anti-sense oligonucleotides from a DNA target (19T PO).

The sequences of oligonucleotides are shown in Figure 1. T_m values were obtained with a concentration of 1 μM for both the anti-sense oligonucleotide and the target in a 10 mM sodium cacodylate buffer, pH 7, containing 1 mM $MgCl_2$ in presence of 10 mM KCl or 100 mM KCl. T_m values are given at ± 1 °C.

Oligonucleotide	T_m (°C) 10 mM KCl	100 mM KCl
17AS PO	50.5	54
17AS PS	40.5	43
17AS PS.P	54.5	58
17C1 PS.P	23.5	29

Table 3 Inhibition of luciferase expression by 21AS PS, a 21 nt oligophosphorothioate targeted to the 5′ UTR region of the IGF-1 gene

The sequences of oligonucleotides are shown in Figure 1. Transfection experiments were performed as indicated in Figure 3 with a range of oligonucleotide concentrations (50, 100 and 200 nM). 21C3 PS is a six-base mismatch control oligophosphorothioate. The percentage of luciferase activity was obtained by calculating the ratios of *P. pyralis* luciferase to *Renilla* luciferase and normalization to the expression of the cells that did not receive oligonucleotides. Results are given as means ± S.D.

Oligonucleotide	Concentration (nM)	Luciferase activity (%)
None	0	100 ± 7.3
21AS PS	50	94.3 ± 7.8
21AS PS	100	75.2 ± 8.8
21AS PS	200	35.9 ± 12.5
21C3 PS	50	88 ± 9
21C3 PS	100	92.8 ± 13.7
21C3 PS	200	110 ± 1.4

2 nM) to inhibit luciferase gene expression in cells that stably expressed the luciferase protein [14,15]. In our transient transfection assay, the anti-luciferase oligonucleotide inhibited the expression of luciferase with the same IC_{50} as the 17AS PS.P ($IC_{50} \approx 5$ nM) (results not shown).

None of the oligonucleotides used in this study could bind to DNA via triple-helix formation and therefore none of them could interfere with plasmid transcription in all transfection experiments. The anti-sense 17AS PS.P is designed to interact with its complementary sequence present in the mRNA transcribed from the plasmid. Although no known protein has been described to bind to the target sequence of the anti-sense oligonucleotide 17AS PS.P, we could not completely exclude the potential recruitment of *trans*-acting proteins regulating the IGF-1 gene and not the thymidine kinase promoter by the anti-sense oligonucleotide. Nevertheless, we showed that control oligonucleotides such as the reverse sequence of 17-mer (17C1) and a mutated oligonucleotide (17C2) could not inhibit the expression of *P. pyralis* luciferase, strongly suggesting that the inhibitory effect of 17AS PS.P is due to an anti-sense mechanism involving oligonucleotide sequence-specific binding and RNAse H activity.

C-5 propyne substitution stabilized phosphorothioate binding

As shown in Figure 3, a high concentration (500 nM) of 17AS PS, the phosphorothioate counterpart of 17AS PS.P, failed to inhibit luciferase expression. Thermal denaturation experiments were performed with anti-(IGF-1) oligonucleotides and a complementary DNA target (19T PO) (see sequences in Figure 1). The duplex formed with the 17AS PO oligophosphodiester had a melting temperature (T_m) of 50.5 °C (Table 2). Changing the phosphodiester backbone to a phosphorothioate backbone decreased the T_m values of the duplex by 10 °C (T_m of 17AS PS is 40.5 °C), in agreement with previous results [41]. The C-5 propyne substitution in all pyrimidines of 17AS PS increased the stability of the duplex by 14.5 °C (T_m of 17AS PS.P is 54.5 °C). The resulting C-5 propyne oligophosphorothioate, 17AS PS.P, bound to the 19T PO with greater affinity than the 17AS PO oligophosphodiester (Table 2). These melting experiments showed that the C-5-propyne modification stabilized the binding of phosphorothioates to the DNA target, probably by increasing the stacking interactions of the bases [13,42]. Therefore the C-5-propyne modification should stabilize the binding of phosphorothioates to the IGF-1 RNA target. We performed melting experiments with a DNA target instead of using a complementary RNA target because it has been shown recently that propynyl-modified pyrimidine substitution in oligonucleotides led to a net stabilization of duplex formation when binding to either DNA or RNA sense strands [13].

Not only the stability of the oligonucleotide–mRNA duplex but also other parameters such as duplex dissociation rates or trapping of oligonucleotides by proteins in cells can have a crucial role in the efficiency of an anti-sense oligonucleotide. For example, it was recently demonstrated that unmodified oligophosphorothioates prebound to their target mRNA introduced by cytoplasmic or nuclear microinjection could rapidly dissociate in cells before cleavage by RNase H [6,7]. Under the same conditions, a C-5 propyne oligophosphorothioate did not dissociate and could induce cleavage by RNase H [7]. The same phenomenon might occur with the 17-mer anti-sense oligophosphorothioate targeted to IGF-1. It might either dissociate too quickly from its target RNA before cleavage by RNase H or bind to proteins that prevent it from hybridizing to the mRNA target sequence.

Increasing the length of the oligophosphorothiate increases its anti-sense effect

Table 3 shows that a longer oligophosphorothioate, 21AS PS, could inhibit luciferase gene expression in a dose-dependent manner. Nevertheless, this 21 nt anti-sense oligonucleotide was less efficient than 17AS PS.P (the IC_{50} of 21AS PS was approx. 200 nM). Increasing the length of the phosphorothioate increased the binding affinity of the anti-sense oligonucleotide to its target and it also probably increased the half-life of the oligonucleotide/mRNA duplex in cells. Because a long oligophosphorothioate can also have non-specific effects, we designed appropriate controls such as mutated oligonucleotides to demonstrate clearly the specificity of the observed effect. The control oligonucleotide with six base mismatches, 21C3 PS, did not inhibit luciferase synthesis, suggesting a sequence-specific mechanism for the anti-sense phosphorothioate.

Two separate transfections for anti-sense oligonucleotides and plasmids

Next, we investigated whether C-5 propyne anti-sense oligonucleotides that had entered cells via the endocytosis of oligonucleotide–TFX50 complexes could find their target mRNA species (the 5′ UTR of the IGF-1 mRNA), when the corresponding plasmids were separately introduced in cells via transfection. Two successive transfections were performed with

Table 4 Luciferase activity was inhibited when C-5-propyne oligophosphorothioate and reporter plasmids were separately transfected

Plasmids (pIGF1630/luc and pRL-TK) were transfected with TFX50 reagent in LFCL2A cells 6 h after the anti-sense oligonucleotides. Final oligonucleotide concentrations were 50 nM. The percentage of luciferase activity was calculated as described in Table 3. Results are given as means ± S.D.

Oligonucleotide	Luciferase activity (%)
None	100 ± 0.2
17AS PS.P	53.5 ± 2
17C1 PS.P	93 ± 1.8
17C2 PS.P	107 ± 6.1
17AS PS	90.8 ± 1.8
17AS PO	107.5 ± 1.5

the same cytofectin reagent, TFX50. The first was done with the anti-sense oligonucleotide, followed 6 h later by the reporter plasmids (pIGF1630/luc and pRL-TK). The anti-sense oligonucleotide 17AS PS.P was able to inhibit luciferase synthesis. A study with a concentration range of 5–50 nM demonstrated that slight inhibitions (20 % and 30 %) were observed with low concentrations of 17AS PS.P (5 and 10 nM) (results not shown). The luciferase mRNA translation is inhibited by 50 % with 50 nM of the 17AS PS.P (Table 4). In this case, the anti-sense oligonucleotide was less efficient (approx. 10 % as efficient) than when it was co-transfected with the reporter plasmids. The oligophosphorothioate 17AS PS and the oligophosphodiester 17AS PO showed no inhibitory activity (Table 4).

Conclusion

In an attempt to assay the capability of various anti-sense oligonucleotides to inhibit IGF-1 gene expression in cells, we developed a cellular system employing the transfection of a reporter plasmid containing the luciferase gene under the control of the rat or human IGF-1 promoter (including their 5′ UTR region). We have used this reporter gene strategy to facilitate oligonucleotide uptake, to optimize the choice of anti-sense oligonucleotide targeted to IGF-1 mRNA and to measure rapidly the protein synthesis controlled by the IGF-1 promoter. Reporter genes such as luciferase and alkaline phosphatase were previously used to determine the most efficient anti-sense or triplex-forming oligonucleotides in cells [43–48]. This recent method, based on the measurement of two different luciferases in the same tube, could be used for the rapid screening of many different anti-sense sequences targeted to a specific mRNA. Here we targeted a region of the 5′ UTR region of the mRNA, which is common to the rat and human IGF-1 gene, by using several anti-sense oligonucleotide constructs. Co-transfection experiments of reporter plasmids and oligonucleotides were performed in rat hepatocarcinoma cells with the use of various cationic lipids. Good transfection efficiencies have been obtained in these cells with the cationic lipid TFX50.

We demonstrated that a phosphorothioate oligonucleotide containing C-5 propynyl pyrimidines was able to inhibit gene expression in the nanomolar concentration range, although an unmodified phosphorothioate oligonucleotide did not inhibit gene expression at a 100-fold higher concentration. Propynyl modification strongly increased the binding affinity of anti-sense oligonucleotides for their targets, as confirmed by the T_m measurements. Oligophosphorothioates substituted with C-5 propynyl pyrimidines will be used in further studies to inhibit the expression of the endogenous IGF-1 gene in rat hepatocarcinoma cells. In agreement with previously published results [5–15], this paper demonstrates that C-5 propyne oligophosphorothioates are able to inhibit gene expression efficiently in cells in a sequence-specific manner, with little or no non-sequence-specific binding of the phosphorothioates.

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