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PAPER

Synthesis and biological activity of phosphonoacetate- and thiophosphonoacetate-modified 2′-*O*-methyl oligoribonucleotides†

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Chimeric 2′-*O*-methyl oligoribonucleotides (2′-OMe ORNs) containing internucleotide linkages which were modified with phosphonoacetate (PACE) or thiophosphonoacetate (thioPACE) were prepared by solid-phase synthesis. The modified 2′-OMe ORNs contained a central phosphate or phosphorothioate sequence with up to 4 PACE or thioPACE modifications, respectively, at either end of the ORN in a "gapmer" motif. Both PACE and thioPACE 2′-OMe ORNs formed stable duplexes with complementary RNA. The majority of these duplexes had higher thermal melting temperatures than an unmodified RNA:RNA duplex. The modified 2′-OMe ORNs were effective passenger strands with complementary, unmodified siRNAs, for inducing siRNA activity in a dual luciferase assay in the presence of a lipid transfecting agent. As single strands, thioPACE 2′-OMe ORNs were efficiently taken up by HeLa cells in the absence of a lipid transfecting agent. Furthermore, thioPACE modifications greatly improved the potency of a 2′-OMe phosphorothioate ORN as an inhibitor of microRNA-122 in Huh7 cells, without lipid transfection.

Introduction

The discovery of cellular regulation and defense mechanisms which are controlled by RNAs[1,2] provides a new, potentially important opportunity to develop novel therapies based on targeted gene silencing. This type of gene silencing can be carried out with short sequences of synthetic RNA which augment cellular functions through a variety of mechanisms, for example directing the cleavage of a mRNA in the RNA-induced silencing complex, or regulating gene expression through binding to and sequestering microRNAs. However, unmodified RNA is extremely susceptible to serum nucleases[3] and is rapidly cleared from the plasma by the kidneys. Thus, unmodified RNA has a short half-life *in vivo*[4] and this makes the application of unmodified RNA as an *in vivo* therapeutic problematic. The ability to deliver RNA-based therapeutics to their cellular targets has also been a significant obstacle in the development of these compounds for some time. As single strands or duplexes, unmodified RNAs cannot penetrate the cell membrane to any great extent because of their size and large negative charge. The use of cationic transfection lipids or techniques such as electroporation can ensure uptake of short RNAs into cultured cells *in vitro*; however, these methods are not practical for *in vivo* applications.

Numerous strategies have been applied in attempts to improve the biophysical properties of RNAs for use *in vivo*. Phosphorothioate backbones,[5] locked nucleic acids (LNAs),[6] methylboranes[7] and 2′-*O*-(2-methoxyethyl)[8] are common chemical modifications which provide increased nuclease resistance, whereas viral delivery vectors[9] or conjugation to cell-penetrating entities[10–12] can enhance cellular uptake. Oligoribonucleotides with a 2′-*O*-methyl modification (2′-OMe ORNs) are known to be nuclease resistant[13] and increase the stability of a duplex which is formed with complementary RNA.[14] 2′-OMe ORNs also do not induce activation of RNaseH.[15] Because these properties are desirable for applications in biological systems, 2′-OMe ORNs have been used to inhibit siRNA and miRNA function.[16] As 2′-OMe ORNs do not have the free 2′-hydroxyl group of RNAs, solid-phase synthesis and incorporation of modifications into 2′-OMe ORNs are also less problematic than with unmodified RNA. Therefore, this combination of features provides a convenient platform to assess the effects of other chemical modifications of ORNs on interactions with RNA.

Recent work has identified phosphonoacetate (PACE) and thiophosphonoacetate (thioPACE) as oligodeoxynucleotide (ODN) modifications with significant potential for biological activity.[17] A PACE or thioPACE modification consists of an acetate group in place of a non-bridging oxygen in an internucleotide phosphate linkage. A thioPACE modification is an analogous substitution in a phosphorothioate linkage (Fig. 1, R = H). In a previous study, ODNs modified with PACE or thioPACE were shown to be nuclease resistant, perhaps as a result of the presence of the

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† Electronic supplementary information (ESI) available: Reagents and techniques, mass, UV, ^{1}H NMR, ^{13}C and ^{31}P NMR spectra for compounds **2a–d**, mass and ^{31}P NMR spectra and HPLC chromatograms for ORNs **4–7**, **9–13**, cell viability data. See DOI: 10.1039/c1ob06614e

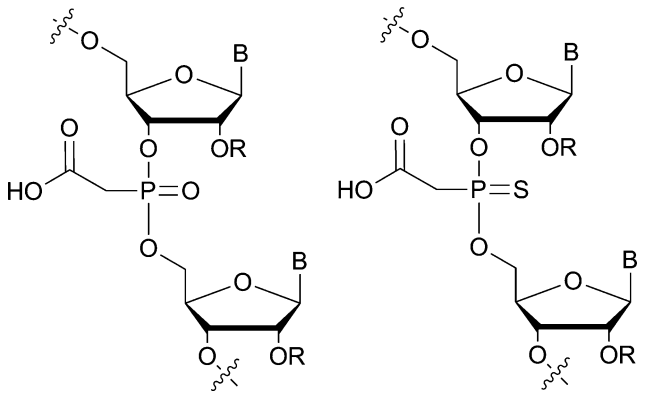


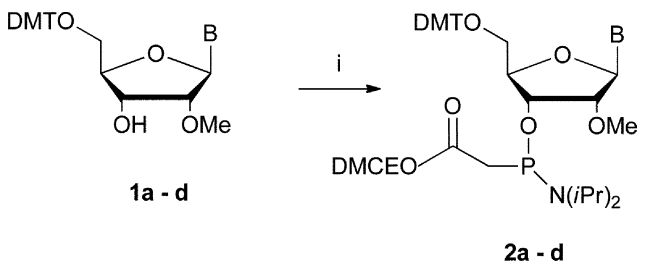
Fig. 1 General structure of phosphonoacetate (left) and thiophosphonoacetate (right) as oligodeoxynucleotide modifications; B = uridine (U), adenine (A), guanine (G) and cytosine (C).

phosphorous–carbon bond in the internucleotide linkage. These ODNs were also shown to activate RNase H1 *in vitro*.[17] To further investigate the biological potential of PACE and thioPACE, we inserted these modifications into 2′-OMe ORNs (Fig. 1, R = OMe) and evaluated their effects on RNA binding, cell uptake, siRNA activity and microRNA inhibition. Herein, we describe the synthesis of 2′-OMe PACE phosphinoamidites, PACE and thioPACE-modified 2′-OMe ORNs and the results of various biological assays which were performed with the modified ORNs.

Results and discussion

Chemical synthesis

Protected 2′-OMe ribonucleoside phosphinoamidites were prepared by using a strategy which was modified from the method used to synthesise the deoxy analogues (Scheme 1).[17] Acetic acid [bis(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl (DMCE) ester was prepared by a Reformatsky reaction with bis(*N*,*N*-diisopropylamino) chlorophosphine, elemental zinc and 1,1-dimethylcyanoethyl bromoacetate, to give acetic acid [bis(*N*,*N*-diisopropylamino)phosphino]-DMCE ester.



Scheme 1 Synthesis of 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1-dimethylcyanoethyl ester-5′-*O*-(4,4-dimethoxytrityl) ribonucleosides. (i) Acetic acid [bis(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethyl ester, 4,5-dicyanoimidazole, dichloromethane. **2a** 84% yield; **2b** 79% yield; **2c** 76% yield; **2d** 95% yield. B: **a** = A^{Bz}, **b** = C^{Ac}, **c** = G^{iBu}, **d** = U. DMCE = 1,1-dimethyl-2-cyanoethyl, DMT = 4,4′-dimethoxytrityl.

The 2′-*O*-methyl-5′-*O*-(4,4′-dimethoxytrityl) ribonucleosides with appropriate heterocyclic base protection (**1a–d**) were then treated with excess acetic acid [bis(*N*,*N*-diisopropylamino)phosphino]-DMCE ester in the presence of a catalytic activator. In this case, 4,5-dicyanoimidazole (4,5-DCI) was the most efficient activator, with reaction times between 4–7 h. Phosphinoamidites **2a–d** were obtained in yields of 76–95% after flash column chromatography on silica gel. The silica gel was neutralised with 1% triethylamine in hexane prior to use.

Solid-phase synthesis of phosphonoacetate and thiophosphonoacetate 2′-OMe ORNs

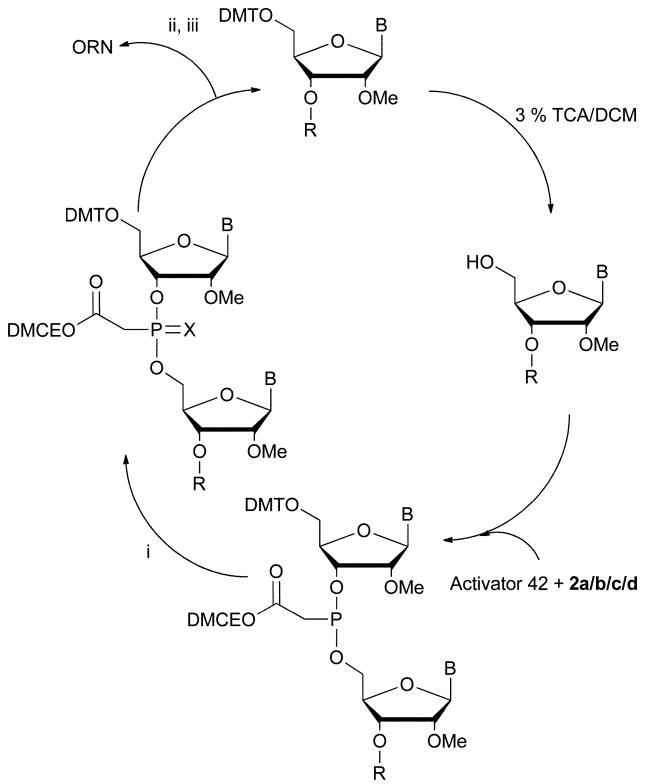
The standard procedure for oligonucleotide synthesis using the phosphoroamidite method[18] was adapted to accommodate the 2′-OMe phosphinoamidites. Of the four activators tested for ORN synthesis, 5-(ethylthio)-1*H*-tetrazole, 4,5-DCI, 5-(bis-3.5-trifluoromethylphenyl)-1*H*-tetrazole (also known as Activator 42)[19] and tetrazole, Activator 42 gave the highest coupling yields and fewest side products, as assessed by HPLC. Although Activator 42 has been shown to be an efficient activator for the synthesis of DNA,[19] this result was surprising in light of the size of the modified phosphinoamidite and the activator, relative to the corresponding components in standard DNA synthesis. With Activator 42, the optimum wait time for the coupling reaction was determined to be 16 min. Longer wait times resulted in more unidentified side products, which were probably due to the increased acidity of Activator 42 (pK_a 3.4) relative to the other activators tested (tetrazole pK_a 4.9,[20] 5-(ethylthio)-1*H*-tetrazole pK_a 4.3,[21] 4,5-DCI pK_a 5.2[22]). Both detritylation and degradation of P(III) internucleotide linkages are known to occur with the more acidic activators. The coupling time was less than half of that used in the synthesis of the analogous PACE-modified ODNs with tetrazole as the activator.

To make the chimeric gapmer sequences **4–7** (Table 1), which contained both PACE and phosphate internucleotide linkages, coupling times of 6 min were used with Activator 42 for the 2′-OMe phosphoramidites. Because no significant issues were identified in coupling a 2′-OMe phosphoramidite with the PACE-modified oligonucleotide during ORN synthesis with Activator 42, it was not necessary to change from Activator 42 to another activator when coupling the 2′-OMe phosphoramidites. ThioPACE/phosphorothioate ORNs **9–12** (Table 1) were synthesised by using the same method as for the PACE/phosphate ORNs, except that a sulfurizing reagent (0.2 M phenylacetyl disulfide in

Table 1 Sequences and modification patterns of PACE or thioPACE 2′-*O*-methyl ORNs **3–12**

ORN	Sequence
Phosphate/PACE ORNs	
3	GAUUAUGUCCGGUUAUGUAUU
4	G*AUUAUGUCCGGUUAUGUAU*U
5	G*A*UUAUGUCCGGUUAUGUA*U*
6	G*A*U*UAUGUCCGGUUAUGU*A*U*U
7	G*A*U*U*AUGUCCGGUUAUG*U*A*U*U
Phosphorothioate/thioPACE ORNs	
8	GAUUAUGUCCGGUUAUGUAUU
9	G†AUUAUGUCCGGUUAUGUAU†U
10	G†A†UUAUGUCCGGUUAUGUA†U†U
11	G†A†U†UAUGUCCGGUUAUGU†A†U†U
12	G†A†U†U†AUGUCCGGUUAUG†U†A†U†U

* = PACE; † = thioPACE; sequences are given in 5′ → 3′ direction.



Scheme 2 Synthesis of PACE- and thioPACE-modified 2′-*O*-methyl-ORNs. (i) For X = O, 0.02 M I_2/THF/pyridine/H_2O; for X = S, 0.2 M phenylacetyl disulfide, acetonitrile/*N*-methylimidazole 9 : 1. (ii) 1.5% DBU/acetonitrile, 1 h. (iii) 40% aqueous $MeNH_2$, 55 °C, 15 min. B: **a** = A^{Bz}, **b** = C^{Ac}, **c** = G^{iBu}, **d** = U. CPG = controlled-pore glass, DMCE = 1,1-dimethyl-2-cyanoethyl, DMT = 4,4′-dimethoxytrityl, TCA = trichloroacetic acid.

acetonitrile containing 10% by volume *N*-methylimidazole) was used instead of the oxidiser (Scheme 2).

The ORNs were characterised by ^{31}P NMR spectroscopy and mass spectrometry (see the ESI, S13–28†). The resonances which corresponded to internucleotide PACE groups were between 26–29 ppm, and the signals for the thioPACE groups were between 94–95 ppm in the ^{31}P NMR spectra. Each set of peaks integrated correctly for the expected ORNs. Ions corresponding to decarboxylation during the analysis were evident in the mass spectrum of each of the modified ORNs.

Duplex formation and thermal denaturation experiments

All of the PACE and thioPACE ORNs formed stable duplexes with complementary RNA. The T_m of a control, unmodified RNA:RNA duplex was 64.0 °C (Fig. 2). As expected, a duplex consisting of 2′-OMe ORN **3** with complementary RNA had a higher T_m of 71.3 °C. The T_m of the duplex formed from RNA and ORN **4**, which had one PACE modification at each end, was 0.9 °C higher (72.2 °C) than the ORN **3**:RNA duplex. The introduction of more PACE modifications resulted in a linear decrease of 1 °C per modification, from 72.2 °C for the ORN **4**:RNA duplex to 70.2 °C for the ORN **5**:RNA duplex, 68.2 °C for the ORN **6**:RNA duplex and 66.2 °C for the ORN **7**:RNA duplex. In the thioPACE series, no increase in T_m relative to the control ORN **8**:RNA duplex was recorded for the duplex which consisted of ORN **9** and

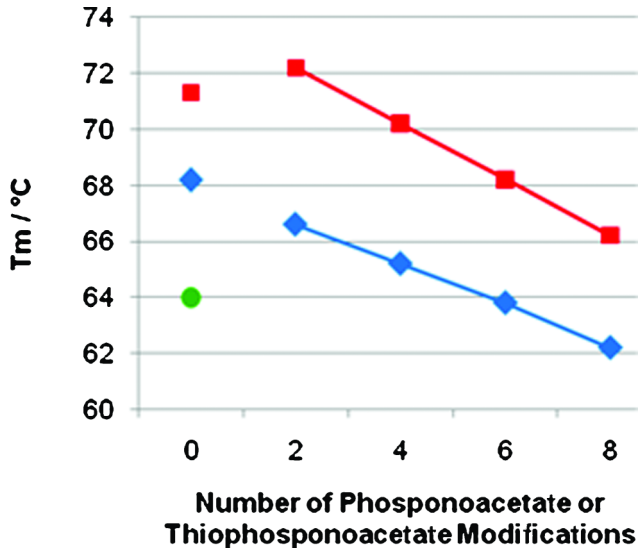


Fig. 2 Melting temperature (T_m) of phosphonoacetate- and thiophosponoacetate-modified oligoribonucleotides with complementary RNA. Melting temperatures were measured at 1 μM duplex in buffer which contained 0.1 M NaCl and 10 mM Na_2HPO_4. ● Unmodified RNA:RNA control; ■ RNA:phosphonoacetate ORNs **3–7**; ◆ RNA:thiophosphonoacetate ORNs **8–12**.

unmodified RNA. The T_m of a duplex of 2′-OMe phosporothioate ORN **8** with complementary, unmodified RNA was 68.2 °C. The introduction of thioPACE modifications resulted in an average reduction in T_m of 0.75 °C per modification from 66.6 °C for the ORN **9**:RNA duplex to 65.2 °C for the ORN **10**:RNA duplex, 63.8 °C for the ORN **11**:RNA duplex and 62.2 °C for the ORN **12**:RNA duplex. This was slightly more than the 0.5–0.7 °C which would be expected for a standard phosphorothioate modification.[5]

Biological activity of phosphonoacetate- and thiophosphonoacetate-modified ORNs

To investigate the effect of ORNs modified with PACE and thioPACE in biological systems, ORNs **3–12** were applied as passenger strands for targeted siRNA silencing of a Firefly Luciferase gene in a dual Firefly Luciferase/Renilla Luciferase (FLuc/RLuc) assay. The modified ORNs were annealed with complementary, unmodified RNA guide strands and transfected into HeLa cells containing a plasmid stably expressing both FLuc and RLuc genes. As shown in Fig. 3, duplexes of PACE ORNs **4–7** with siRNA down-regulated expression of FLuc relative to RLuc in the presence of cationic lipids by between 55 and 65% at 100 nM concentration, and by 30–45% at 10 nM concentration. The PACE ORNs **4–7** were not as effective at silencing FLuc as 2′-OMe ORN **3** at either concentration. ThioPACE ORNs:siRNA duplexes **9–12** were more effective in this assay than the corresponding PACE ORNs, and were approximately as potent as the control ORN **8**:RNA duplex at 100 nM concentration.

In a manner similar to the control ORN **8**:siRNA duplexes, thioPACE ORN:siRNA duplexes reduced cell viability by approximately 40% at 100 nM, but did not cause significant toxicity at 10 nM. PACE ORNs were well tolerated at both concentrations (see Fig. S29–30 in the ESI†). With both sets of ORNs there was little correlation between the number of PACE or thioPACE modifications in the ORNs and silencing activity.

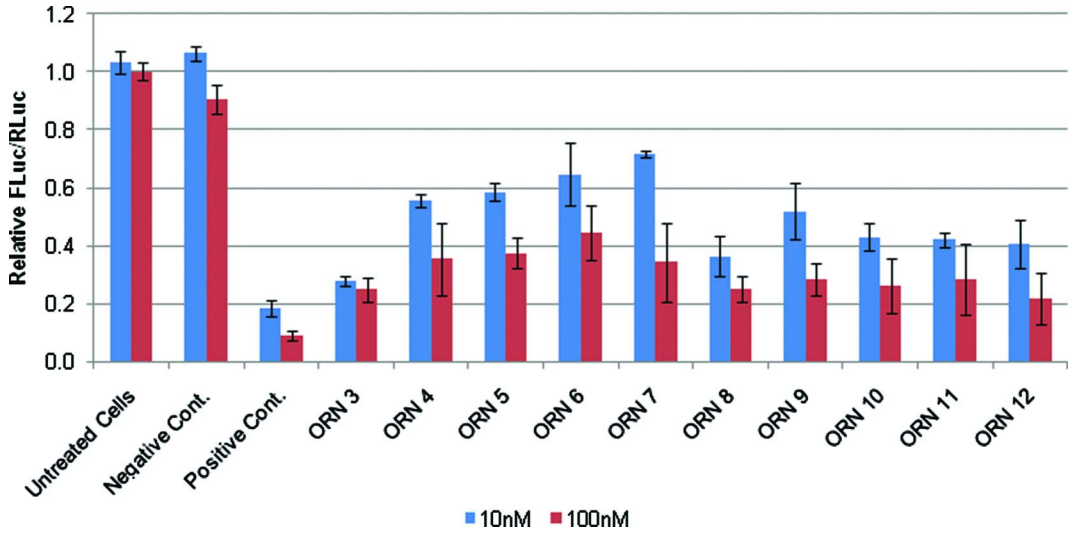


Fig. 3 siRNA activity of phosphonoacetate- and thiophosphonoacetate-modified ORNs as duplexes with siRNA in HeLa cells with lipid transfection as measured by down-regulation of firefly *versus* Renilla luciferase in a DualGlo luciferase assay. Positive control was AntiLuc siRNA1 (Dharmacon, Lafayette, CO), negative control was Silencer Negative Control 1 (Ambion, Foster City, CA). The concentrations indicated for untreated cells denote that the untreated cell experiments were run on the same plate as the cells that were treated with ORNs at the corresponding concentration.

To test if PACE or thioPACE modifications could affect uptake of ORNs by cells, Fluorescence-activated Cell Sorting (FACS) was used to assess the extent to which duplexes which consisted of PACE or thioPACE ORNs with complementary, fluorescently-labelled RNA strands were taken up into cells. At 2 μM, these modified ORNs were taken up by HeLa cells in the absence of lipids, but with a low efficiency. For example fluorescence was detected in 15% of cells which were treated with a duplex of ORN **6**:RNA (Fig. 4, center) and 10% of cells which were treated with a duplex of ORN **11**:RNA (Fig. 4, right) after 24 h.

This finding correlated well with the dual luciferase assay when the assay was performed in the absence of a lipid transfecting reagent. Both PACE and thioPACE ORNs as duplexes with siRNA were able to elicit some down-regulation of FLuc *versus* RLuc, but less than that produced with the lipid transfecting reagent. At 1 μM, the relative knockdown of FLuc was between 12 and 34% for the PACE series and 13–27% for the thioPACE series (Fig. 5). As for the experiments with lipids for transfection, the silencing activity did not seem to be dependent on the number of PACE or thioPACE modifications in the ORNs. Although some of the ORNs, for example ORNs **4** and **5**, did appear to perform better than the same positive control duplex used in experiments with lipids, the difference in down-regulation was not significant at this concentration.

The results of the FACS and dual luciferase experiments suggested that the PACE and thioPACE modifications did improve cell uptake of the siRNA duplex to a limited extent. Therefore, we tested the uptake of the modified ORNs as single strands. As single strands and in the absence of lipids, the uptake of thioPACE ORNs into HeLa cells was extremely efficient. For example, fluorescently-labelled ORN **11**, which contained six thioPACE modifications, was taken up by 90% of cells after 24 h incubation at 1 μM concentration (Fig. 6, center). At concentrations above 5 μM, almost 100% of cells carried fluorescently-labelled ORN within 1 h (Fig. 6, right). Under these conditions, fluorescence microscopy showed that the distribution of ORNs inside the cells appeared to be punctate throughout the cytoplasm, which suggested possible localisation of the ORNs in endosomes (Fig. 7). The cell nuclei,

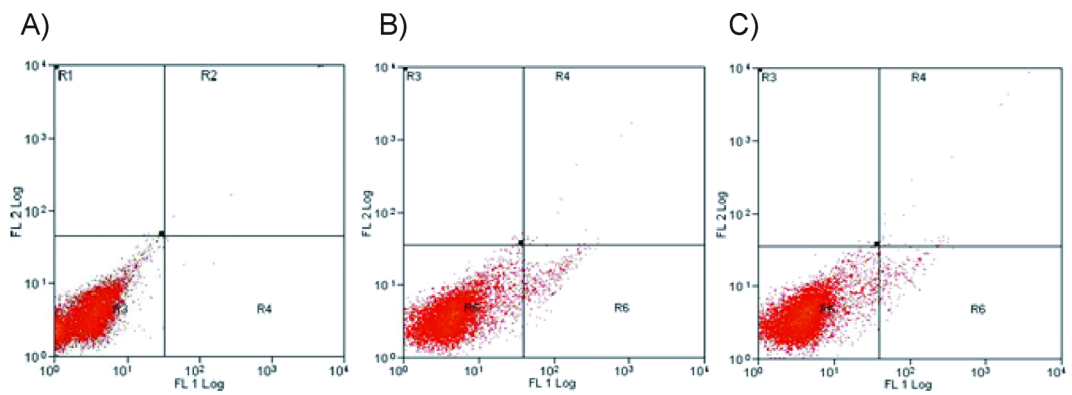


Fig. 4 Fluorescence-activated cell sorting scatter plots of untreated HeLa cells (left) and HeLa cells which were incubated with duplexes of fluorescently-labelled siRNA with ORN **6** (center) or ORN **11** (right). Percentage uptake was calculated as number of cells shifted to the right of the fluorimeter gate (set at FL-1 Log = 10) for samples *versus* control.

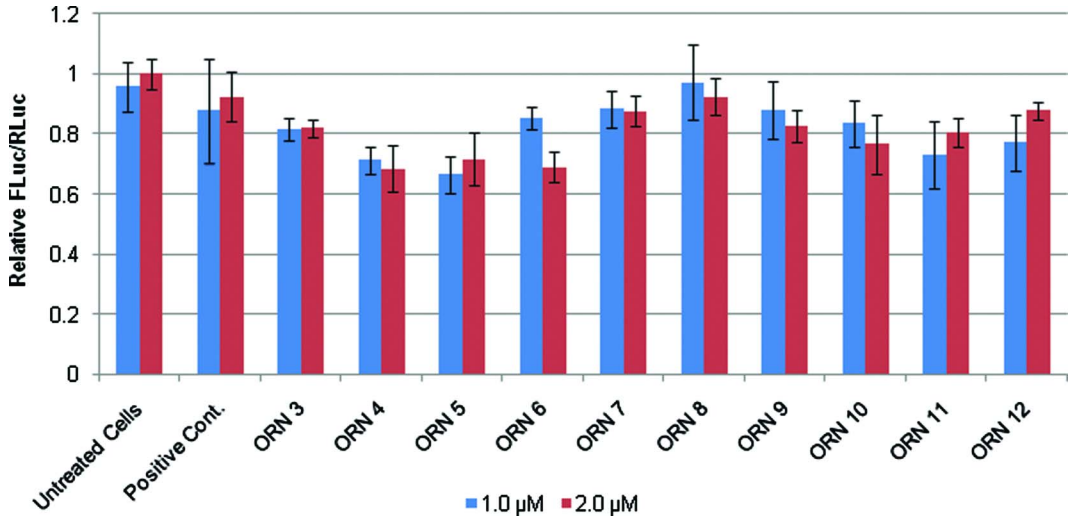


Fig. 5 siRNA activity of phosphonoacetate- and thiophosphonoacetate-modified ORNs as duplexes with siRNA in HeLa cells without lipid transfection as measured by down-regulation of Firefly *versus* Renilla luciferase in a DualGlo luciferase assay. Positive control was AntiLuc siRNA1, the same duplex used in experiments with lipids. The concentrations indicated for Untreated Cells denote that the untreated cell experiments were run on the same plate as the cells that were treated with ORNs at the corresponding concentration.

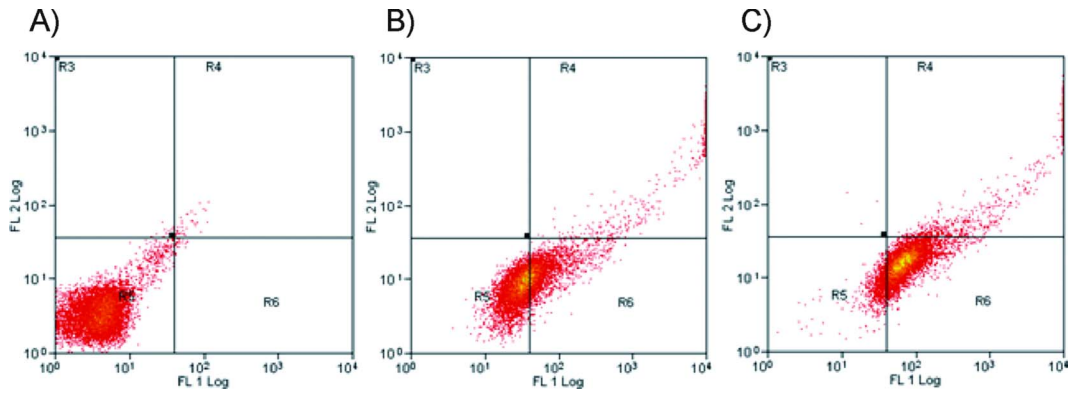


Fig. 6 Fluorescence-activated cell sorting scatter plots of untreated HeLa cells (left) and HeLa cells incubated with fluorescently-labelled ORN **11** at 1 μM for 24 h (center) and 5 μM for 1 h (right). Percentage uptake was calculated as number of cells shifted to the right of the fluorimeter gate (set at FL-1 Log = 10) for samples *versus* control.

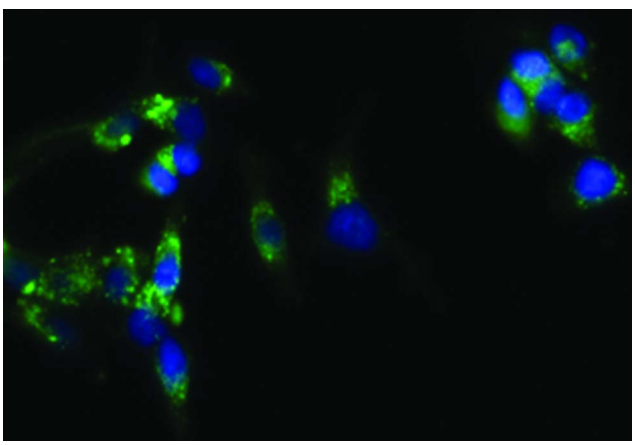


Fig. 7 Fluorescence microscopy image of HeLa cells incubated with fluorescently-labelled ORN **11** at 1 μM in the absence of a lipid transfecting agent. DAPI was used to stain the nuclei.

which were stained with DAPI, appeared free of fluorescently-labelled ORNs. This distribution was similar for cells treated with duplexes of PACE and thioPACE with RNA, which indicated that the mode of uptake for single strands and duplexes might be similar.

Although apparently encapsulated in endosomes, the results of these FACS experiments demonstrated that cell uptake of single-stranded thioPACE ORNs was much more efficient than for duplexes that contained the same ORN. These findings indicated that investigating the use of thioPACE in a system such as microRNA (miRNA) inhibition, in which only a single strand rather than a duplex is required to elicit a regulatory response, would be worthwhile. We tested a thioPACE ORN as an inhibitor of microRNA-122 (miR-122) in a steric block approach, without the use of cationic lipids for transfection. MicroRNA-122 is a liver-specific microRNA which has been implicated in lipid metabolism[23] and Hepatitis C viral replication,[24] and it is known to be expressed at high levels in human Huh7 hepatocarcinoma cells.[25,26]

To test the efficacy of a thioPACE ORN for miRNA inhibition, a miRNA-122 recognition site was cloned into the 3′-UTR of the RLuc reporter gene of a plasmid which contained both FLuc and RLuc genes[27] and transfected into Huh7 cells.

With the expression of RLuc under the control of endogenous miR-122, the cells were then incubated in the absence of a lipid transfection reagent with 2′-OMe thioPACE ORN 13 (5′-A†C†A†A†ACACCAUUGUCACACUCCA-3′; † = thioPACE, the remaining internucleotide linkages were phosphorothioate). ORN 13 was designed to bind and sequester miR-122 to block the miR-122-controlled down-regulation of RLuc expression. The relative expression of the two luciferase genes was then monitored for up-regulation of RLuc *versus* FLuc. The modifications were placed at the 5′-end of ORN 13 because binding strength in the 5′-seed region of miRNAs influences the activity and specificity of miRNA inhibitors.[28] Therefore, the effects of the thioPACE modifications on miRNA inhibition could be directly assessed through comparison with a 2′-OMe phosphorothioate control ORN 14 (5′-ACAAACACCAUUGUCACACUCCA-3′), without the thioPACE modifications affecting binding in the seed region. ORN 14 was shown to inhibit miR-122 in Huh7 cells in the absence of transfection agents[27] and had the same sequence and the same construct at the 3′-end as ORN 13.

As with ORNs **9–12**, ORN **13** formed a duplex with complementary RNA with a slightly reduced T_m, relative to control ORN **14**. The T_m of the ORN **14**:RNA duplex was 78.8 °C, whereas the ORN **13**:RNA duplex had a T_m of 76.4 °C. Compared with control ORN **14**, ORN **13** was significantly more potent as a miR-122 inhibitor. As expected, in the absence of a lipid transfecting reagent, control ORN **14** was able to inhibit miR-122 at sub-micromolar concentrations and up-regulate RLuc *versus* FLuc (Fig. 8). The dose–response curve shows that ORN **13** consistently up-regulated RLuc by a magnitude of approximately two times greater than ORN **14**. Inhibition was detected as little as 24 h after 0.5 μM ORN transfection and was maintained for 72 h, as demonstrated by a continuous increase in RLuc/FLuc ratio with ORN **13**. The inhibitory effect of ORN **13** was maintained for slightly less time than for control ORN **14**, which maintained inhibition for at least 96 h in cell culture.[27] The target binding affinity of 2′-OMe phosphorothioate RNA with modifications such as LNAs is known to be positively correlated with the potency of miR-122 inhibition.[29] However, binding strength does not always correlate with higher anti-miR activity in cells[28] and *in vivo*.[30] Given that the duplex T_m for ORN **13** with complementary RNA was lower than the control, these findings and other studies suggest that other factors, as well as the binding affinity for the target sequence, determine inhibitory activity. Davis *et al.* have also demonstrated that the activity of various 2′-modified RNA inhibitors of miRNA increased relative to a 2′-OMe phosphorothioate RNA as a function of the location of the modification within the oligonucleotide sequence, as well as binding affinity for miRNA targets.[29] This paves the way for future studies of PACE and thioPACE, perhaps involving structure–activity relationships relative to the location of the modifications.

Fluorescence microscopy showed that initial uptake of thioPACE ORNs into HeLa cells may be predominantly through an endosomal pathway. The mechanism of cellular transport of oligonucleotides has been the subject of much debate for a number of years. Some theories suggest oligonucleotides are accepted into cells following receptor-mediated endocytosis.[31] Interestingly, miRNAs have also been found in endosomal compartments.[32] One possibility is that the increase in miRNA inhibition with ORN **13** relative to ORN **14** is a result of improved uptake through

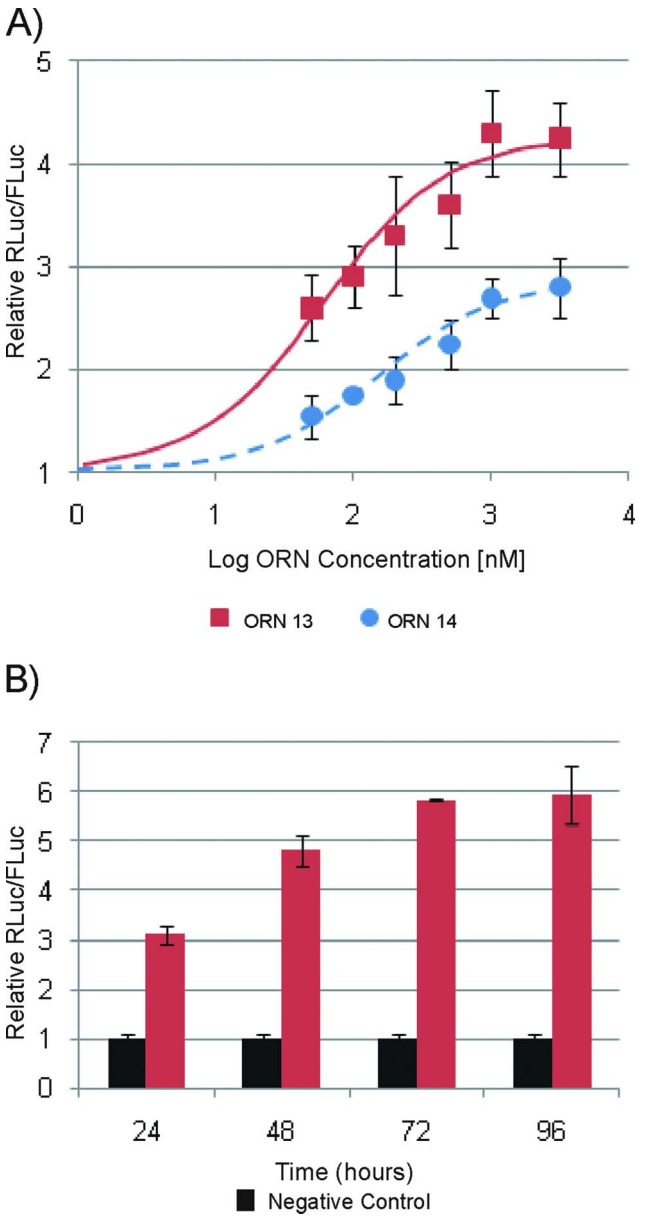


Fig. 8 Dose-response of miR-122 inhibition in Huh7 cells by ORNs **13** and **14** (top) and time dependence of miR-122 inhibition by 0.5 μM ORN **13** (bottom) as measured by a miR-122 sensor luciferase assay. Experiments were performed without lipid transfection. For dose–response, Huh7 cells expressing endogenous miR-122 were treated with ORNs at 0, 50, 100, 200, 500, 1000 and 3000 nM.

endocytosis of the thioPACE ORN in Huh7 cells, which leads to ORN **13** finding miR-122 within endosomal compartments.

Another possible pathway for the penetration of oligonucleotides into cells is through interaction with divalent metal ions. Divalent metal ions are known to change the conformation of single-stranded phosphorothioate ODNs in a sequence- and cation-dependent manner,[33] and also when ODNs are associated with anionic liposomes.[34] Uptake of ODNs into cells also increases in the presence of divalent metal ions, possibly through direct interactions with the phospholipid bilayer.[35] Thus, one could speculate that the presence of the carboxylate group in thioPACE provides an available binding site for a divalent metal ion which

then facilitates uptake by the ORN through interaction with the phospholipid membrane.

Conclusions

In summary, 2′-OMe PACE phosphinoamidites were synthesised and incorporated into chimeric 2′-OMe ORNs as PACE or thioPACE modifications. These ORNs formed stable duplexes with complementary RNA, and the majority of these duplexes had higher thermal melting temperatures than an unmodified RNA:RNA control duplex. ORNs with thioPACE modifications were more effective as passenger strands for siRNA than PACE ORNs in the presence of a lipid transfecting agent, and both sets of ORNs also showed some ability to penetrate unaided into HeLa cells to elicit siRNA activity. The number of modifications in the ORNs did not appear to be correlated with silencing activity in either case. Unassisted cellular uptake was notably more efficient with thioPACE ORNs as single strands. In the absence of a transfecting agent, a 2′-OMe ORN which contained 4 thioPACE modifications was found to significantly improve the potency of miRNA-122 inhibition when compared with a control 2′-OMe phosphorothioate RNA. These results suggest that further investigation of the cell penetrating properties and potential therapeutic value of thioPACE 2′-OMe ORNs is warranted.

Experimental

Chemical synthesis

General procedure for preparation of 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1,1-dimethylcyanoethyl ester-5′-*O*-(4,4′-dimethoxytrityl) ribonucleosides: Protected 2′-*O*-methyl-5′-*O*-(4,4-dimethoxytrityl) ribonucleosides (**1a–d**) (8.0 mmol) were dissolved in anhydrous dichloromethane under an argon atmosphere. Acetic acid [bis(*N*,*N*-diisopropylamino)phosphino]-1,1-dimethylcyanoethyl ester (4.5 g, 12.0 mmol) and 4,5-DCI (0.76 g, 6.4 mmol) were added and the solution stirred at ambient temperature until starting material was consumed, as assessed by tlc and ^{31}P NMR spectroscopy. The reaction solvent was evaporated under reduced pressure, then the foam or oily residue was re-dissolved in a minimum amount of ethyl acetate and purified by flash column chromatography on silica gel. Before loading the crude product onto the column, the silica was first neutralised with 1 column volume of 1% triethylamine in hexane, washed with 2 column volumes of hexane, and then washed with 2 column volumes of ethyl acetate. Recrystallisation was performed by dissolving the purified, foam-like products in a minimum volume of anhydrous dichloromethane and adding this solution dropwise to vigorously stirred anhydrous hexane. The products were then isolated by filtration.

Compound 2a: 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1,1-dimethylcyanoethyl ester-5′-*O*-(4,4-dimethoxytrityl)-*N*-6-benzoyl adenosine. Reaction time was 6 h. Yield: 6.4 g (6.7 mmol, 84%). ^{31}P NMR (121 MHz, CD_3CN) δ 125.4, 122.3 (diastereomers). Found ESI$^+$: *m*/*z* 958.5 (M + H)$^+$, 980.5 (M + Na)$^+$, 996.5 (M + K)$^+$, $[C_{52}H_{60}N_7O_9P + H]^+$ requires 958.4

Compound 2b: 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1,1-dimethylcyanoethyl ester-5′-*O*-(4,4-dimethoxytrityl)-*N*-4-acetyl cytidine. Reaction time was 4 h. Yield 5.5 g (6.3 mmol, 79%). ^{31}P NMR (121 MHz, CD_3CN) δ 125.4, 124.9 (diastereomers). Found ESI$^+$: *m*/*z* 872.4 (M + H)$^+$, 894.3 (M + Na)$^+$, 910.3 (M + K)$^+$, $[C_{46}H_{58}N_5O_{10}P + H]^+$ requires 872.4

Compound 2c: 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1,1-dimethylcyanoethyl ester-5′-*O*-(4,4-dimethoxytrityl)-*N*-2-isobutyryl guanosine. Reaction time was 4 h. Yield 5.7 g (6.1 mmol, 76%). ^{31}P NMR (121 MHz, CD_3CN) δ 126.2, 122.0 (diastereomers). Found ESI$^+$: *m*/*z* 940.4 (M + H)$^+$, 962.3 (M + Na)$^+$, 978.3 (M + K)$^+$, $[C_{49}H_{62}N_7O_{10}P + H]^+$ requires 940.4

Compound 2d: 2′-*O*-methyl-3′-*O*-(diisopropylamino)phosphino acetic acid-1,1-dimethylcyanoethyl ester-5′-*O*-(4,4-dimethoxytrityl) uridine. Reaction time was 7 h. Yield: 6.3 g (7.6 mmol, 95%). ^{31}P NMR (121 MHz, CD_3CN) δ 125.7, 123.4 (diastereomers). Found ESI$^+$: *m*/*z* 831.4 (M + H)$^+$, 853.3 (M + Na)$^+$, 869.4 (M + K)$^+$, $[C_{44}H_{55}N_4O_{10}P + H]^+$ requires 831.4

Solid-phase oligoribonucleotide synthesis

ORNs were synthesised on an Applied Biosystems 394 automated DNA synthesiser on a controlled-pore glass (CPG) solid support. Deblocking of the 5′-*O*-DMT protecting group prior to each coupling was achieved with 3% trichloroacetic acid in dichloromethane. Modified 2′-*O*-methyl-3′-*O*-phosphinoamidite ribonucleotides **2a–d** were dissolved in anhydrous acetonitrile at 0.1 M concentration and delivered to the column with 0.25 M Activator 42. Coupling time was 16 min. Oxidation was performed prior to the capping step with 0.02 M I_2 in THF/pyridine/H_2O and a 3 min wait time. Failure sequences were capped with a combination of anhydrous tetrahydrofuran/pyridine/acetic anhydride (cap mix A) and 6.5% dimethylaminopyridine in anhydrous tetrahydrofuran (cap mix B).

After completion of the solid-phase synthesis, the dimethylcyanoethyl protecting group was cleaved from the ORNs by treating the solid support beads with a solution of 1.5% 1,8-diazabicycloundec-7-ene (DBU) in acetonitrile for 60 min. ORNs were cleaved from the solid support and the heterocyclic bases deprotected by treating the beads with aqueous methylamine (40% methylamine in water) at 55 °C for 15 min. After cooling, the CPG was removed by filtration and the remaining solution of the ORN was evaporated to dryness.

Crude PACE ORNs were dissolved in 0.5 ml water/acetonitrile (4 : 1) and applied to a semi-preparative RP-HPLC column. Using the 5′-*O*-DMT group for hydrophobicity, failure sequences were separated from the full length products. The appropriate fractions of products were combined and evaporated to dryness. After drying, the residues were treated with 80% acetic acid for 90 min to remove the 5′-*O*-DMT protecting group. After evaporating the acetic acid, the ORNs were dissolved in 0.5 mL 0.05 M triethylammonium bicarbonate, re-applied to the HPLC column and purified using the same mobile phase gradients.

ThioPACE ORNs were synthesised and purified using the same method as for PACE ORNs, except that the oxidizing solution was replaced with a sulfurizing reagent (0.2 M phenylacetyl disulfide in anhydrous acetonitrile with 10% by volume of *N*-methylimidazole). The sulfurizing reagent was prepared and left

to stand for at least 12 h before use. Sulfurizing wait time was 3 min and the post-synthesis cleavage and deprotection procedures remained unchanged.

Fluorescent Labelling of ORNs

Modified ORNs were labelled at the 5′-end using fluorescein. Fluorescein phosphoramidite was dissolved at 0.1 M in anhydrous acetonitrile and coupled for 15 min using Activator 42. The remaining cycle was then performed as described for unlabelled ORNs.

HeLa cell luciferase assay

The stable HeLa cell line contains modified versions of both *Renilla reniformis* (sea pansy) luciferase (RLuc), and *Photinus pyralis* (firefly) luciferase (FLuc) and was designed by adopting the psi-CHECK II vector (Promega, Sunnyvale, CA). HeLa cells were maintained at 37 °C/5% CO_2 in Eagle's Minimum Essential Media (EMEM) with Earle's Balanced Salt Solution which contained 20% Fetal Bovine Serum (FBS), supplemented with 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 1.0 nM non-essential amino acids, 0.3 μg mL^{-1} Puromyicin, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin.

Approximately 1×10^4 cells in 100 μL of supplemented EMEM/20% FBS without antibiotics were plated per well in 96-well plates. After 24 h cells were incubated for a further 24 h at 37 °C/5% CO_2 with 100 μl OptiMEM which contained the desired concentration of ORN duplex. For experiments with lipids, the ORN duplexes were treated with 0.2 μL per well of Dharmafect1 (ThermoFisher, Lafayette, CO) prior to transfection, according to the manufacturer's protocol for adherent cell lines. After incubation, the medium containing the ORNs was substituted with 50 μL of 1 × PBS and the expression of luciferase was measured using the DualGlo Luciferase Assay (Promega, Sunnyvale, CA) according to the manufacturer's protocol. Luminescence was measured for 400 milliseconds per well using a DTX 880 Multimode Detector (Beckman Coulter, Miami, FL). All experiments were carried out in triplicate.

MicroRNA-122 sensor luciferase assay in Huh7 cells

The plasmid construct psi-1-122, containing a miR-122 recognition site which was cloned into the 3′-UTR of the Renilla luciferase gene of double luciferase plasmid psi-Check II (Promega), is described elsewhere.[27] Huh7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and penicillin/streptomycin antibiotics (full media) at 37 °C/5% CO_2. Twenty-four hours before transfection, 2×10^4 cells in 100 μL of DMEM/10% FBS without antibiotics were plated per well in 96-well white plates with a clear base. Twenty-four hours after plating, the cells were transfected overnight with 100 ng psi-1-122 and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, in a final transfection volume of 150 μL per well.

After 24 h, the cells were washed once with 150 μL PBS and incubated for 4 h at 37 °C/5% CO_2 in 100 μL Opti-MEM which contained the ORNs at the desired concentration. After incubation, the ORN-containing Opti-MEM was replaced with 150 μL full media per well. Unless stated otherwise, after a further 48 h the full media was replaced with 75 μL PBS and the expression of luciferase was measured using the DualGlo Luciferase Assay (Promega) according to the manufacturer's protocol, except that the luciferase substrate incubations were for 10 min in the dark. Luminescence was measured for 5 s per well in an Orion microplate luminometer (Berthold Detection System GmBH, Pforzheim, Germany). All experiments were carried out in triplicate.

Curve fitting was carried out using a dose–response stimulation equation (log[agonist] *vs.* response) and a bottom constraint of 1 (which corresponds to the basal relative RLuc/Fluc signal for the negative control cells). Since it is not possible to plot "log 0" values that correspond to negative control cells (0 nM oligonucleotides), we have used a value close to zero, 0.001 (log0.001 = −3, not shown in graph), instead. This modification did not alter the statistical analysis or the curve fitting.

Fluorescence-activated cell sorting

Twenty-four hours before transfection, approximately 150,000 HeLa cells per well were seeded in a 24-well plate in 500 μl FBS containing media (DMEM). The transfection was carried out at 0.05–10 μM of the fluorescently-labelled ORNs in OptiMEM for 24 h. After removal of growth media, the cells were washed with PBS 3 times, treated with trypsin and profiled on a MoFlo High Speed Sorter (DakoCytomation, Fort Collins, CO) with a 488 nm argon laser.

Fluorescence microscopy

Epifluorescence microscopy was used to visualise fluorescently-labelled ORNs in cells. HeLa cells were grown on a cover glass placed in a 6-well cell culture plate which contained DMEM media. After 24 h the media was removed and the cells were treated with fluorescently-labelled ORN at 3 μM concentration in serum reduced OptiMEM media without antibiotics. After a further 24 h, the slides were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich), washed 3 times with 1 × PBS buffer and stained with ProLong Gold antifade reagents which contained the blue-fluorescent nuclear counterstain DAPI (Invitrogen). Microscopy was performed at 40× resolution on a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY). Images were captured using Nikon NIS Element software.

Cell viability

Cell viability was monitored by fluorescence changes in the cells by using the redox indicator dye resazurin (AlamarBlue, Invitrogen). HeLa cells were incubated as per the HeLa luciferase assay with ORNs at 1 μM concentration and 15 μl of AlamarBlue reagent per 100 μl of media for 1 h. Fluorescence was measured using a Safire² microplate reader (Tecan, Männedorf, Switerland) with XFLUOR software (XFLIOR4SAFIREII version V4.62n) with the following parameters: fluorescence excitation wavelength 540–570 nm (peak at 570 nm); emission wavelength 580–610 nm (peak emission 585 nm). All experiments were carried out in triplicate.

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Notes and references

1 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806–811.
2 R. C. Lee, R. L. Feinbaum and V. Ambros, *Cell*, 1993, **75**, 843–854.
3 K. A. Whitehead, R. Langer and D. G. Anderson, *Nat. Rev. Drug Discovery*, 2009, **8**, 129–138.
4 J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H. P. Vornlocher, *Nature*, 2004, **432**, 173–178.
5 C. A. Stein, C. Subasinghe, K. Shinozuka and J. S. Cohen, *Nucleic Acids Res.*, 1988, **16**, 3209–3221.
6 M. Frieden, H. F. Hansen and T. Koch, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 1041–1043.
7 H. Krishna and M. H. Caruthers, *J. Am. Chem. Soc.*, 2011, **133**, 9844–9854.
8 K.-H. Altmann, N. M. Dean, D. Fabbro, S. M. Freier, T. Geiger, R. Haner, D. Husken, P. Martin, B. P. Monia, M. Muller, F. Natt, P. Nicklin, J. Philips, U. Picks, H. Sasmor and H. E. Moser, *Chimia*, 1996, **50**, 168–176.
9 C. Kimchi-Sarfaty, S. Brittain, S. Garfield, N. J. Caplen, Q. Tang and M. M. Gottesman, *Hum. Gene Ther.*, 2005, **16**, 1110–1115.
10 F. Marlin, P. Simon, T. Saison-Behmoaras and C. Giovannangeli, *ChemBioChem*, 2010, **11**, 1493–1500.
11 C. Wolfrum, S. Shi, K. N. Jayaprakash, M. Jayaraman, G. Wang, R. K. Pandey, K. G. Rajeev, T. Nakayama, K. Charrise, E. M. Ndungo, T. Zimmermann, V. Koteliansky, M. Manoharan and M. Stoffel, *Nat. Biotechnol.*, 2007, **25**, 1149–1157.
12 T. Endoh and T. Ohtsuki, *Adv. Drug Delivery Rev.*, 2009, **61**, 704–709.
13 H. Inoue, Y. Hayase, A. Imura, S. Iwai, K. Miura and E. Ohtsuka, *Nucleic Acids Res.*, 1987, **15**, 6131–6148.
14 M. Majlessi, N. C. Nelson and M. M. Becker, *Nucleic Acids Res.*, 1998, **26**, 2224–2229.
15 H. Inoue, Y. Hayase, S. Iwai and E. Ohtsuka, *FEBS Lett.*, 1987, **215**, 327–330.
16 G. Meister, M. Landthaler, Y. Dorsett and T. Tuschl, *RNA*, 2004, **10**, 544–550.
17 D. J. Dellinger, D. M. Sheehan, N. K. Christensen, J. G. Lindberg and M. H. Caruthers, *J. Am. Chem. Soc.*, 2003, **125**, 940–950.
18 S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.*, 1981, **22**, 1859–1862.
19 A. Wolter and M. Leuck, *Activators for Oligonucleotide and Phosphoramidite Synthesis*. Sigma-Aldrich Co. [7897758]. 2011. Missouri, USA. 2006.
20 R. Welz and S. Müller, *Tetrahedron Lett.*, 2002, **43**, 795–797.
21 F. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, D. Tracz, C. Workman, D. Sweedler, C. Gonzalez, S. Scaringe and N. Usman, *Nucleic Acids Res.*, 1995, **23**, 2677–2684.
22 C. Vargeese, J. Carter, J. Yegge, S. Krivjansky, A. Settle, E. Kropp, K. Peterson and W. Pieken, *Nucleic Acids Res.*, 1998, **26**, 1046–1050.
23 M. Lagos-Quintana, R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel and T. Tuschl, *Curr. Biol.*, 2002, **12**, 735–739.
24 R. E. Lanford, E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen and H. Orum, *Science*, 2010, **327**, 198–201.
25 H. Xu, J. H. He, Z. D. Xiao, Q. Q. Zhang, Y. Q. Chen, H. Zhou and L. H. Qu, *Hepatology*, 2010, **52**, 1431–1442.
26 C. L. Jopling, M. Yi, A. M. Lancaster, S. M. Lemon and P. Sarnow, *Science*, 2005, **309**, 1577–1581.
27 A. G. Torres, R. N. Threlfall and M. J. Gait, *Artificial DNA: PNA & XNA*, 2011, **2**, 2–8.
28 S. Davis, B. Lollo, S. Freier and C. Esau, *Nucleic Acids Res.*, 2006, **34**, 2294–2304.
29 M. M. Fabani and M. J. Gait, *RNA*, 2008, **14**, 336–346.
30 S. Davis, S. Propp, S. M. Freier, L. E. Jones, M. J. Serra, G. Kinberger, B. Bhat, E. E. Swayze, C. F. Bennett and C. Esau, *Nucleic Acids Res.*, 2009, **37**, 70–77.
31 X. Ming, *Expert Opin. Drug Delivery*, 2011, **8**, 435–449.
32 D. Gibbings and O. Voinnet, *Trends Cell Biol.*, 2010, **20**, 491–501.
33 S. D. Patil and D. G. Rhodes, *Nucleic Acids Res.*, 2000, **28**, 4125–4129.
34 S. D. Patil and D. G. Rhodes, *Nucleic Acids Res.*, 2000, **28**, 2439–2445.
35 S. Wu-Pong, *Biochem. Mol. Biol. Int.*, 1996, **39**, 511–519.

