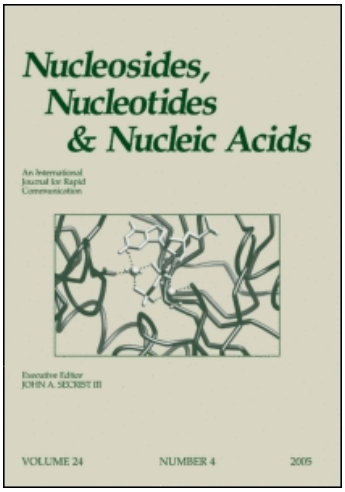

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SECOND GENERATION ANTISENSE OLIGONUCLEOTIDES - INHIBITION OF PKC-α AND *c-RAF* KINASE EXPRESSION BY CHIMERIC OLIGONUCLEOTIDES INCORPORATING 6'-SUBSTITUTED CARBOCYCLIC NUCLEOSIDES AND 2'-O-ETHYLENE GLYCOL SUBSTITUTED RIBONUCLEOSIDES

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Abstract: 6'-substituted carbocyclic deoxyribonucleosides and 2'-O-ethylene glycol substituted ribonucleosides have been evaluated as building blocks for antisense oligonucleotides. Within the former class 6'-*hydroxy* substituted building blocks in combination with internucleoside phosphorothioate linkages have the potential to enhance antisense activity. 2'-O-ethylene glycol substituted ribonucleosides generally allow for the construction of potent antisense oligonucleotides with reduced phosphorothioate content, but differences exist in their effects on biological activity in cell culture in spite of virtually identical effects on RNA-binding affinity. Activity enhancement was most pronounced for a 2'-O-methoxyethyl substituent.

Introduction

The chemical modification of oligonucleotides represents a key element for the successful implementation of antisense-based drug design strategies.[1] Due to their pronounced susceptibility to nucleolytic degradation natural oligonucleotides (i. e. short pieces of DNA or RNA based on an unmodified phosphodiester backbone) do not yield potent antisense effects under physiological conditions and changes in oligonucleotide structure are a fundamental prerequisite in order to generate antisense inhibitors of sufficient metabolic stability.[1] In addition, structural alterations of antisense oligonucleotides can lead to enhanced RNA-binding affinity and possibly more favorable pharmacokinetic and pharmacodynamic properties *in vivo*.

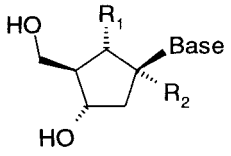
The issue of nuclease resistance has been successfully addressed in *first generation* antisense oligonucleotides by the replacement of one of the non-

bridging oxygen atoms of the phosphodiester group by sulfur;[1] such phosphorothioate based antisense oligonucleotides in several cases have been shown to be potent inhibitors of protein expression *in vitro*[2] as well as *in vivo*[2d,3] (i. e. in experimental animal models) and several of these analogs are currently undergoing phase I or even phase II clinical trials. However, even though they certainly have to be considered as serious drug candidates, phosphorothioates are not free of disadvantages. In addition to exhibiting sub-optimal RNA-binding properties, one of the most critical problems associated with this class of compounds is their pronounced tendency for unspecific protein binding, which can elicit biological effects that are not related to an antisense mechanism of action.[4] As a consequence, extensive efforts have been made to identify other types of modified oligonucleotides that would display improved RNA-binding affinity, but would not, or at least not entirely, depend on a phosphorothioate backbone for metabolic stability.[5] Alternatively, phoshorothioate related toxicities might also be diminished or even completely eliminated, if additional modifications would allow for a profound improvement in potency of these compounds.[2b,6]

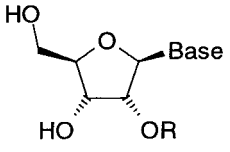
In this paper we now want to report on the *in vitro* biological activity (i. e. activity in cell culture) of sugar modified antisense oligonucleotides incorporating either 6'-substituted carbocyclic nucleosides **I** or a novel class of modified ribonucleosides **II**, containing ethylene glycol based 2'-substituents (*Figure 1*).

The synthesis of these modified nucleosides and nucleoside analogs has been previously described and the biophysical properties of the corresponding oligonucleotides have been evaluated.[7,8] In particular, it was demonstrated that ethylene gycol based 2'-substituents *(Fig. 1*, R = $(CH_2CHR'O)_nOR''$ lead to significantly enhanced RNA-binding affinity as well as superior nuclease resistance.[8] In contrast to simple 2'-O-alkyl groups these novel substituents are not plagued by counterbalancing effects on nuclease resistance and RNA-binding affinity, i. e. in spite of their size, which forms the basis for their protective effect against nuclease degradation, they promote RNA-binding to the same extent as a 2'-methoxy group at least up to the level of a triethylene glycol chain attached to the 2'-oxygen. In comparison, 2'-O-alkyl groups of similar length do not significantly increase or even decrease RNA-binding affinity of the corresponding oligonucleotides.[9]

Chimeric oligonucleotides incorporating 2'-O-methoxyethyl substituted ribonucleotide units (*Fig. 1*, R = $CH_2CH_2OCH_3$), in combination either with phosphorothioate or with natural phosphodiester linkages, have been shown to exhibit improved biological activity over the corresponding parent



I



R = $(CH_2CHR'O)_nR''$

II

Figure 1

2'-deoxyphosphorothioates in cell culture and even in *in vivo* anti-tumor experiments.[10] We have now extended these studies to other ethylene glycol based 2'-substituents and in this paper we compare the effects of different members of this class on the *in vitro* biological activity of *c-raf* kinase inhibitor **ISIS 9826** (*vide infra*).

As fully modified oligonucleotides incorporating either modified building blocks **I** or **II** upon binding to complementary RNA do not elicit RNAse H-catalyzed RNA cleavage, chimeric structures ("gapmers")[11] had to be employed in order to assess the effects of these modifications on antisense activity. This involved incorporation of modified building blocks **I** and **II** into the terminal regions ("wings") of two different sequences, which as 2'-deoxyphosphorothioates are potent inhibitors of PKC-α (**ISIS 6633**) and *c-raf* kinase (**ISIS 9826**) mRNA expression, respectively, in cell culture. The central part of these modified oligonucleotides ("gap", "RNAse H window") retains its 2'-deoxy-phosphorothioate structure in order to ensure RNase H mediated cleavage of bound target RNA, while the modified building blocks are connected either by natural phosphodiester linkages or by phosphorothioate groups (*Figure 2*). The latter format allows the effect of a particular modification on antisense activity to be assessed more or less independently of (nuclease) stability issues, while the former design also critically tests the ability of the modification to protect the oligonucleotide from nucleolytic degradation.

Results and Discussion

The effects of 6'-substituted carbocyclic nucleosides on the antisense activity of **ISIS 6633**, as measured by the ability of modified oligonucleotides to reduce PKC-α mRNA levels, are summarized in *Table 1*.

ISIS 9826-analogs

5'-**txtxcxtxcs**GsCsCsGsCsTsCsCs**txcxcxtxcx**C-3'

ISIS 6633-analogs

5'-**txtxcxtxcs**GsCsTsGsGsTsGsAsGs**txtxtxcx**A-3'

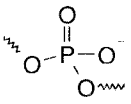
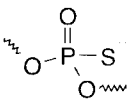
o =  s = 

Figure 2: General scheme for the biological evaluation of modified oligonucleotides. The parent 2'-deoxyphosphorothioates **ISIS 9826** and **ISIS 6633** are potent antisense inhibitors of *c-raf* kinase and PKC-α mRNA synthesis, respectively. **t, c** are modified analogs of thymidine and 5-methyl-2'-deoxycytidine, respectively. x = o or s.

Table 1: Inhibition of PKC-α mRNA expression in A549 cells by chimeric analogs of **ISIS 6633** incorporating 6'-substituted carbocyclic nucleotide units.[a, b]

		Full PS (x = s)[b]		PO-wings (x = o)[b]	
R_1[a]	R_2[a]	T_m (°C)[c]	IC_{50} (nM)[d]	T_m (°C)[c]	IC_{50} (nM)[d]
2'-Deoxy (**ISIS 6633**)		51.5	200	n. d.	n. d.
OH	H	55.7	250	60.9	> 500
CH_3	H	52.0	200	56.6	>> 500
CH_2OH	H	50.8	250	57.8	>> 500
$CH_3O(CH_2CH_2O)_2$	H	39.6	> 1000	44.6	n. d.
-CH_2-	H	43.1	> 1000	46.7	> 1000

[a]*Cf. Fig. 1,* **I**. [b]*Cf. Fig. 2.* [c]T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+. For further experimental details *cf.* ref. 12. [d]Cells were treated with oligonucleotides in the presence of 10 μg/ml of lipofectin for 4h at 37°. Following treatment, medium was removed and replaced with normal cell culture medium. PKC-α mRNA was determined by Northern blot analysis 24 h after initiation of oligonucleotide treatment by means of a random primed ^{32}P-labeled full-length cDNA probe.

For those analogs that are based on a full phosphorothioate backbone (x = s) modifications that do not adversely affect RNA-binding affinity do not lead to any significant change in antisense activity as compared to **ISIS 6633**. In contrast, and not too surprisingly, the incorporation of modified building blocks resulting in substantially decreased RNA-binding affinity completely abolishes biological activity at oligonucleotide concentrations < 1μM. It is worth pointing out that for bicyclo[3.1.0]hexane based nucleoside analogs (*Table 1*, R_1, R_2 = -CH_2-, x = s) this profound reduction in biological potency is associated with a decrease in T_m of less than 1°/modification (*vide infra*).

Significantly reduced antisense activity is observed for all oligonucleotides with a mixed phosphodiester/phosphorothioate backbone (x = o), even for those modifications which lead to enhanced RNA-binding affinity (compared to **ISIS 6633**).

This finding indicates that those 6'-substituents that do not compromise or even enhance RNA-binding affinity do not protect adjacent phosphodiester groups from nucleolytic cleavage to an extent that would allow for significant antisense activity in a cellular environment (*cf.* also ref. 7). In contrast, the loss in biological activity for the chimeric **ISIS 6633** analog incorporating bicyclic nucleotide units in combination with unmodified phosphodiester linkages (*Table 1*; R_1, R_2 = -CH_2-; x = o) is more likely related to insufficient RNA-binding affinity, as phosphodiester based oligodeoxyribonucleotides incorporating this type of modified building block in independent experiments were found to be at least as nuclease resistant as the corresponding phosphorothioates. Not considering the effect of the modification on RNAse H cleavage efficiency a decrease in T_m of only 5° *vs.* **ISIS 6633** thus results in a remarkable loss in inhibitory activity. In combination with the data obtained for the corresponding full phosphorothioate (*Table 1*, R_1,R_2 = -CH_2-, x = s) this might be taken as an indication that in order for a particular type of modification to be truly useful for antisense purposes it should not reduce RNA-binding affinity to a significantly larger extent than the incorporation of phosphorothioate linkages.

Based on these data it seems clear that none of the 6'-modified carbocyclic nucleoside analogs shown in *Table 1* is an appropriate tool for the design of potent antisense oligonucleotides with a reduced number of phosphorothioate linkages. On the other hand, some of these analogs can be incorporated into **ISIS 6633** without apparent loss in biological activity in cell culture. It is not clear at this point whether the presence of certain types of carbocyclic nucleotide units in phosphorothioate based antisense oligonucleotides could lead to

changes in the overall properties of the parent 2'-deoxyphosphorothioates (e. g. with respect to pharmacokinetic or toxicity profile) that would result in improved efficacy *in vivo*.

6'-Hydroxy substituted carbocyclic nucleosides have also been incorporated into **ISIS 9826**, which results in a significant (~ 5-fold) improvement in antisense activity (IC_{50} = 75 nM *vs.* 350 nM for **ISIS 9826**) (*Fig. 3*). The increase in biological potency of **ISIS 9826** associated with the incorporation of 6'-hydroxy substituted carbocyclic nucleosides (full phosphorothioate backbone) is even more pronounced than observed for the corresponding 2'-methoxy modified analog (IC_{50} = 175 nM), in spite of the slightly higher RNA-binding affinity of the latter (*cf. Fig. 3*).

A comparison of the activity data obtained for 6'-hydroxy substituted (phosphorothioate based) analogs of **ISIS 6633** and **ISIS 9826** clearly indicates that the effects of a particular modification on antisense activity can differ significantly for different base sequences and/or RNA target sites. The investigation of a single sequence may thus not conclusively reveal the true potential of chemical modifications for the design of improved antisense inhibitors. In this conjunction, 6'-hydroxy substituted carbocyclic nucleosides in combination with internucleoside phosphorothioate linkages may represent an interesting tool for the design of more potent antisense oligonucleotides than 2'-deoxyphosphorothioates.

ISIS 9826 has also served as a host oligonucleotide to establish the effects of different types 2'-O-oligoethylene glycol substituents (*Fig. 1*) on antisense activity (within the limitations related to the sequence dependence of such effects as pointed out above) (*Table 2*).

As has been reported previously for other antisense inhibitors of signal transduction, the incorporation of 2'-O-methoxyethyl substituted ribonucleotide units into **ISIS 9826** (*Table 2*, $R = CH_2CH_2OCH_3$) leads to significantly enhanced antisense activity. The fact that the same biological potency is observed for 2'-O-methoxyethyl substituted analogs that are based on a full phosphorothioate or a mixed phosphodiester/phosphorothioate backbone, respectively, also corroborates previous findings that this modification enables the construction of antisense oligonucleotides with improved potency and a reduced phosphorothioate content. Other ethylene glycol based 2'-O-substituents also allow for a reduction in the number of phosphorothioate linkages in **ISIS 9826** without loss in antisense activity, but not in every case do they lead to significantly *improved* activity (*cf. Table 1*, $R = (CH_2CH_2O)_3CH_3$ and

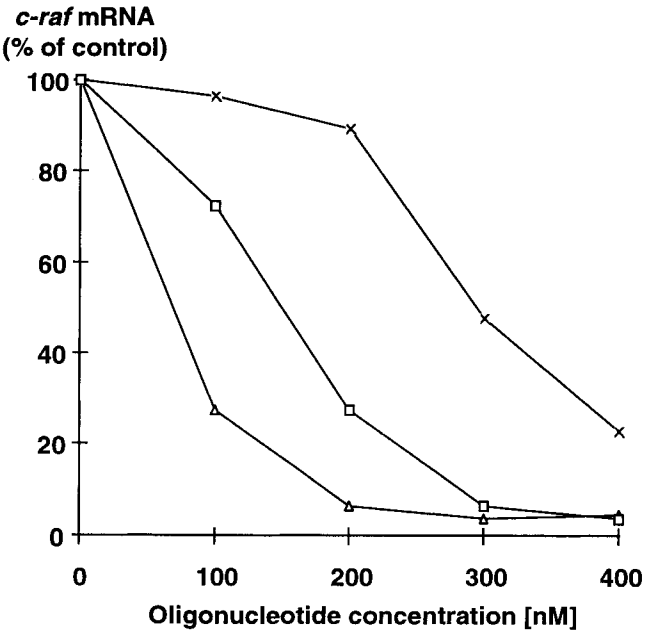


Figure 3: Inhibition of *c-raf* kinase mRNA expression in T24 cells by **ISIS 9826** (-x-) and chimeric analogs incorporating 6'-hydroxy substituted carbocyclic thymidine and 2'-deoxycytidine (-Δ-) and 2'-O-methyl uridine and cytidine (-□-) (*cf. Fig. 2*, x = s). For experimental details *cf. Table 1.* T_m values of the duplexes with complementary RNA are 68.6° for **ISIS 9826**, 72.4° for the 6'-hydroxy analog, and 76.8° for the 2'-O-methyl analog.

R = CH_2CH_2OH *vs.* R = $CH_2CH_2OCH_3$). In view of the very similar RNA-binding affinities of the corresponding oligonucleotides (and presumably very similar nuclease resistance[8]) this finding is rather surprising and (among other possibilities) might be related to differences in RNAse H cleavage efficiency for differently modified chimeric oligonucleotides of the same sequence or to differences in uptake efficiency, i. e. differences in the interactions of these modified oligonucleotides with uptake promoting cationic lipids. Regarding the former possibility it should be noted that *in vitro* RNAse H cleavage experiments performed on modified analogs of **CGP69846A** (**ISIS 5132**) have indicated a less than 1.5-fold difference in the intitial cleavage rate by *E. choli* RNAse H between modified DNA/RNA duplexes incorporating either 2'-methoxytriethoxy (*Fig. 1*, R = $(CH_2CH_2O)_3CH_3$) or 2'-O-methoxyethyl modified chimeric DNA strands.[13] On the other hand, data pertaining to the relative activities of these

Table 2: Inhibition of *c-raf* kinase mRNA expression in T24 cells by chimeric analogs of **ISIS 9826** incorporating 2'-O-ethylene glycol substituted ribonucleotide units.[a, b, c]

	Full PS (x = s)[b]		PO-wings (x = o)[b]	
R[a]	T_m (°C)[c]	IC_{50} (nM)[d]	T_m (°C)[c]	IC_{50} (nM)[d]
2'-Deoxy (**ISIS 9826**)	68.6	350	-	-
CH_3	76.8	150	n. d.	n. d.
C_3H_7	n. d.	200	74.8	200
$CH_2CH_2OCH_3$	n. d.	40	84.9	40
$(CH_2CH_2O)_3CH_3$	78.2.	150	84.3	350
(R)-$CH_2CH(CH_3)OCH_3$	78.1	50	82.6	70
CH_2CH_2OH	n. d.	n.d.	82.7	320
$CH_2CH_2OCH_3$/5-propynyl[d]	85.9	100	92.4	125

[a]*Cf. Fig. 1,* **II**. [b]*Cf. Fig. 2.* [c]T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+. For experimental details *cf.* ref.12. [d]For experimental details *cf. Table 1.* [d]Modified nucleosides are 2'-O-methoxyethyl-5-propynyl-uridine and 2'-O-methoxy-ethyl-5-propynyl-cytidine, respectively.

latter analogs *in vitro* and *in vivo* do indicate that differences in cell culture activity are *not* reflected in different anti-tumor activities *in vivo*. At least for a 2'-methoxytriethoxy substituent reduced uptake efficiency (including unfavorable effects on intracellular localization) may thus at least in part account for the differences in *in vitro* activity between 2'-methoxytriethoxy and 2'-O-methoxyethyl modified **ISIS 9826** as indicated in *Table 1.* Finally, it should be noted that the incorporation of 2'-O-methoxyethyl 5-propynyl pyrimidine nucleosides into **ISIS 9826** does not result in any improvement in antisense activity over the 2'-O-methoxyethyl T/5-MeC analog, despite a further increase in RNA-binding affinity. It is not clear why the latter oligonucleotide apparently is a slightly more potent inhibitor of *c-raf* kinase mRNA expression than its base modified analog, as 5-propynyl modified oligonucleotides in several cases have been demonstrated to be significantly more potent antisense inhibitors of protein expression than the corresponding parent 2'-deoxyphosphorothioates.[2b,6] This finding again emphasizes that the effect of a particular modification on antisense activity (in this case the 5-propynyl modification) may depend on

oligonucleotide sequence and also on the overall structure of the oligonucleotide, i. e. the presence of additional other modifications.

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