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Second Generation of Antisense Oligonucleotides: From Nuclease Resistance to Biological Efficacy in Animals

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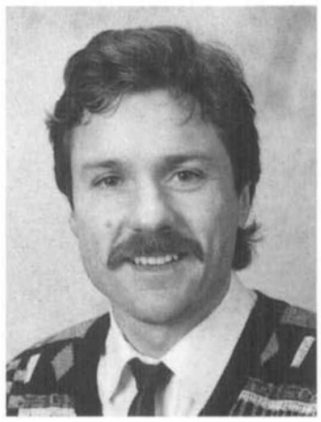
Dedicated to Prof. *Albert Eschenmoser* on the occasion of his 70th birthday

Abstracts. From efforts to improve the biophysical properties of antisense oligonucleotides by incorporating backbone- or sugar-modified nucleoside analogs, 2'-*O*-methoxyethyl ribonucleosides **8b** were identified as building blocks for a second generation of antisense oligonucleotides. Compounds containing these modifications were demonstrated to combine the benefit of a high binding affinity to the RNA complement with a large increase in nuclease resistance, allowing the use of regular phosphodiester linkages. Chimeric oligonucleotides with 2'-*O*-methoxyethyl ribonucleosides, **8b**, in the wings and a central DNA-phosphorothioate window were shown to efficiently downregulate C-'raf' kinase and PKC-α *messenger*-RNA in tumor cell lines resulting in a profound inhibition of cell proliferation. The same compounds were able to effectively reduce the growth of tumors in animal models at low concentrations indicating the potential utility of these second generation antisense oligonucleotides for therapeutic applications.



Heinz E. Moser, born 1957 in Uttigen/BE, studied chemistry at the department of natural sciences of the ETH in Zürich, where he graduated in 1980. He joined Prof. A. *Eschenmoser*'s group working on the synthesis and properties of di- and tri-peptamidinium salts, a new class of peptide-analog compounds. After receiving his Ph.D. in 1985, he moved to the California Institute of Technology in Pasadena/CA where he worked under Prof. *P.B. Dervan*'s supervision on the sequence-specific recognition of double-stranded DNA by triplex-forming oligonucleotides. In 1987 he joined the Central Research Laboratories of *Ciba-Geigy* in Basel, where he initiated research activities in the area of nucleic-acid chemistry, especially the antisense field, and currently holds the position as head of nucleic-acid chemistry. His scientific contributions were honored with the *Ciba* fellow award (1994) and the *Ruzicka* Prize (1995).

Introduction

Zamecnik and *Stephenson* [1] were the first authors to propose synthetic oligonucleotide analogs as a new class of potential therapeutics which could be rationally designed and should be able to specifically inhibit the synthesis of chosen target proteins. This strategy – known today as 'antisense strategy' – is in principle overwhelmingly simple and offers the advantage of a general applicability as outlined below.

The coded building plan of every protein is stored as double-helical DNA which, upon requirement of a particular protein, is transcribed into single-stranded messenger RNA (mRNA) as intermediate carrier of information. After processing and maturation, the mRNA is translocated from the cell nucleus to the cytoplasm where it serves as template for protein synthesis by ribosomes (generally known as 'central dogma of molecular biology', *Fig. 1a*). Due to the ability of single-stranded nucleic acids to form double helices according to the rules of *Watson-Crick* base pairing [2] (*Fig. 1b*), it is straightforward to design a unique oligonucleotide which – in principle – should only bind to the complementary site of a single mRNA by forming a local duplex structure and thereby inhibit the synthesis of the corresponding protein.

The antisense strategy was not only proposed by scientists but also nature learned how to make use of this elegant principle. An increasing variety of gene products are being discovered that are regulated by antisense RNA molecules expressed intracellularly [3][4]. Similar approaches might become feasible in the future for therapeutic applications in connection with gene therapy.

For synthetic oligonucleotides, the antisense strategy is convincing from a mechanistic point of view. However, a few hurdles have to be overcome before this class of compounds will become suitable as therapeutics (*Fig. 2*). Natural DNA or RNA oligonucleotides are rapidly degraded under physiological conditions by a variety of nucleases that primarily hydrolyze the phosphodiester of the internucleosidic backbone. As a consequence, oligonucleotides have to be stabilized in order to possess a reasonable half-life *in vivo*. Even though oligodeoxyribonucleotides (DNA) bind quite well to complementary RNA targets, RNA/RNA duplexes of the same sequence are usually much more stable [5]. Therefore, it should be possible to increase the RNA binding affinity of oligonucleotides by the incorporation of well-chosen modifications to improve the overall biological efficacy. The achievement of these two issues, stability and affinity, will still not be sufficient to observe biological activity, if the compounds are not able to penetrate cells and reach the cytoplasma or cell nuclei where they should

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become effective. As these molecules are reasonably big (molecular weight between 4000 and 8000 Da) and highly charged, cellular uptake is considered to be a critical issue. Inside the cell the oligonucleotide has to be able to bind its target site on the mRNA. The secondary and tertiary structures of RNA greatly influence its accessibility [6] and only 5% or less of an entire mRNA are estimated to be potential target sites for antisense compounds. Due to limited abilities to predict secondary and tertiary structures of RNA in a reliable way, oligonucleotides which bind accessible sites on RNA have so far been identified by screening a large number of compounds. On the longer run, experimental methods for the identification of accessible binding sites on structured RNA may generally become available [7]. A variety of studies indicated that binding of a modified oligonucleotide to a particular mRNA does usually not guarantee the biological potency [8], unless the target site is of crucial importance like *e.g.* the initiation codon. High antisense activities are observed, if the oligonucleotide will initiate the cleavage of the bound mRNA leading to rapid destruction of the encoded building plan for protein synthesis. Cleavage of mixed DNA/RNA hybrids is accomplished within the cell by ubiquitous nuclease(s) RNase H which naturally serve(s) the purpose to remove RNA primers during DNA replication. This enzyme, however, is highly sensitive to structural changes within the antisense (DNA) strand and only a few modifications as phosphorothioates **1** (*Fig. 3*, [9]) or phosphorodithioates [10] are known to be accepted as substrates to induce cleavage of the bound RNA strand. Antisense oligonucleotides which fulfill all these criteria still have to possess a reasonable pharmacokinetic behavior in organisms; metabolic degradation or clearance from the body should not be too fast and the overall bioavailability should be substantial. Finally, the technical feasibility to synthesize and purify such molecules has to be improved in order to guarantee acceptable therapeutic costs for this class of potential drugs.

When we became involved in this research field, we first focused our activities on improving nuclease resistance and RNA binding affinity of oligonucleotides by chemical modifications. We considered those two issues as the most important hurdles to be overcome in an initial phase because these properties are not only a requirement for functional antisense molecules but also a prerequisite to address questions like *e.g.* target site accessibility within cells or pharmacokinetic behavior in animals.

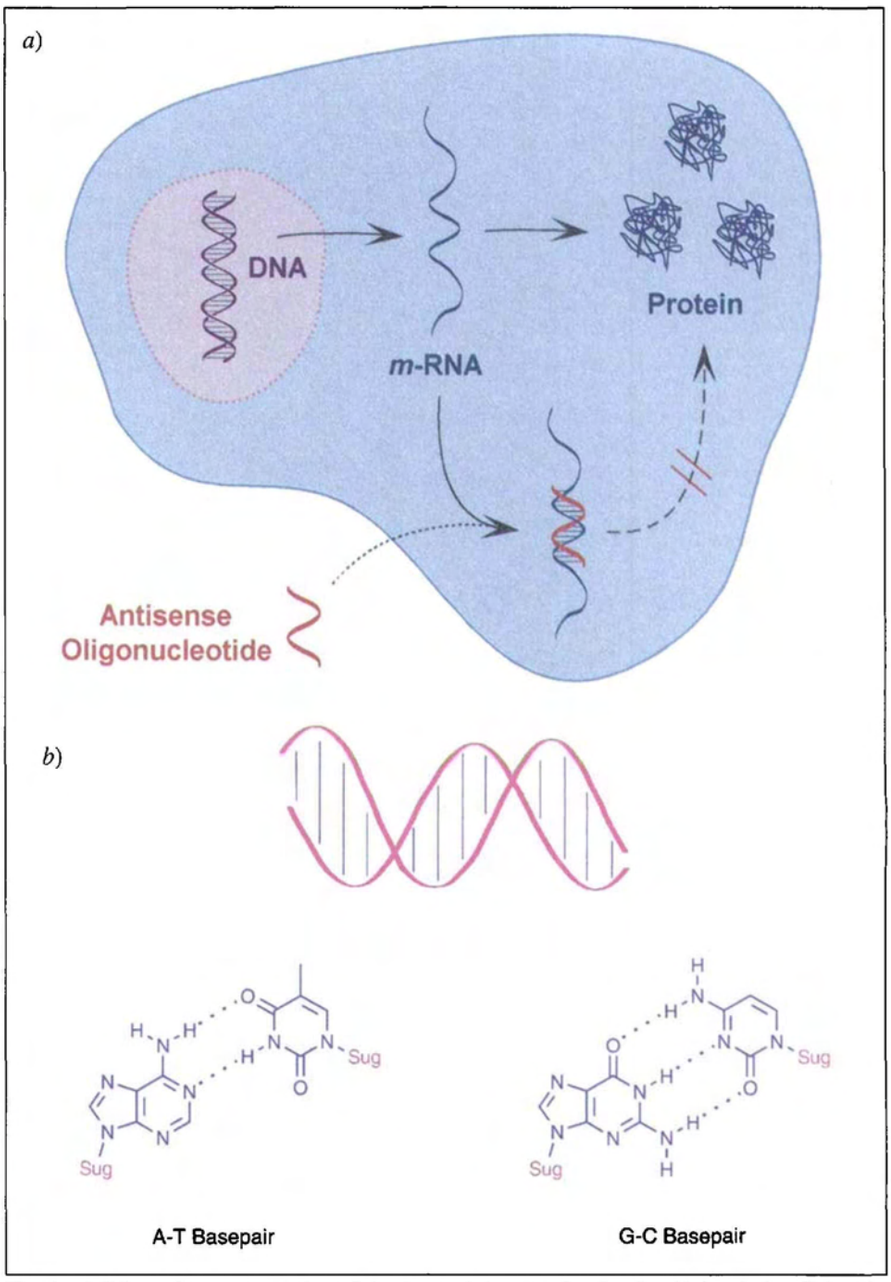


Fig. 1. a) *Schematic representation of the 'central dogma of molecular biology' visualizing the strategy by which antisense molecules interfere in the translational process and* b) *specific* Watson-Crick *hydrogen bonding of pyrimidine and purine bases in nucleic acids as prerequisite of duplex formation*

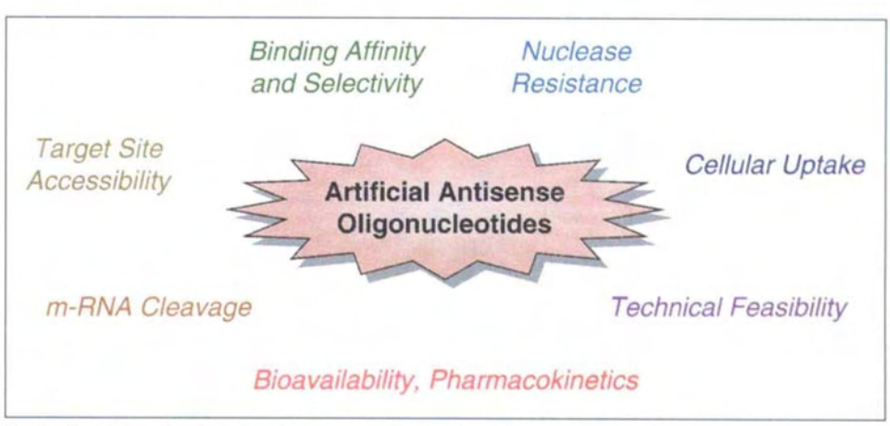


Fig. 2. *Key issues for functional antisense oligonucleotides*

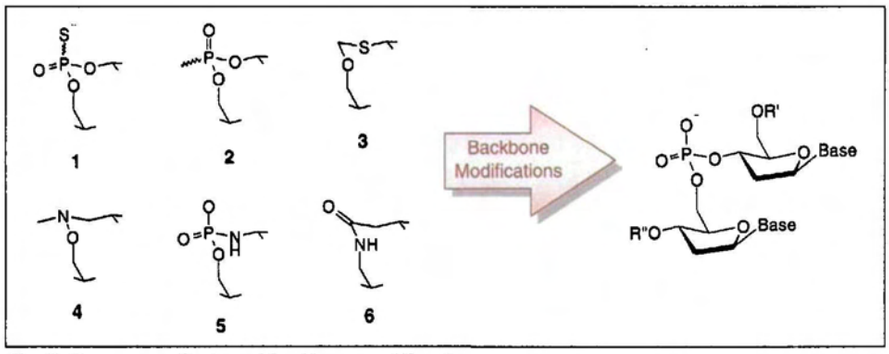


Fig. 3. *Structures of selected backbone modifications*

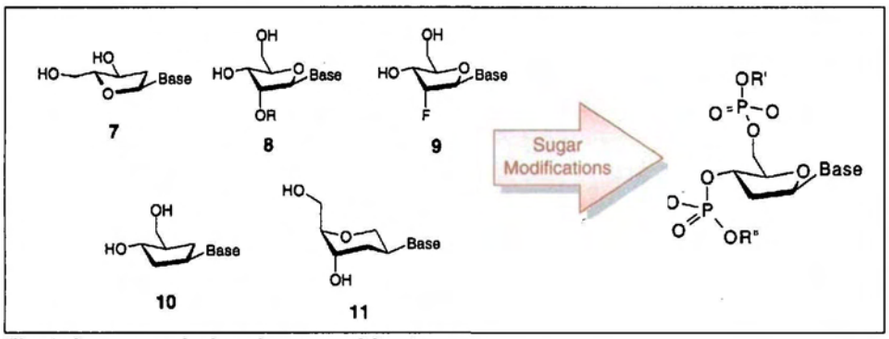


Fig. 4. *Structures of selected sugar modifications*

Table. *Average RNA Hybridization and Stability Data of 2'-O-Alkyl Ribonucleosides.* The average T_m/mod. values were assembled from 1–3 different sequences as described [35] (**A**: 5'-TTTTtCTCTCTCTCT; **B**: 5'-**ttttt**c^m**t**c^m**t**c^m**t**c^m**t**c^{m}T (c^m: C(5)-methyl derivatives); **C**: 5'-CTCGTAC**ttttt**CCGGTCC; **D**: 5'-GCG**ttttttttttt**GCG). If not stated otherwise, the values given are based on sequences **A**, **C**, and **D**. The half-life time was determined for the sum of n and $(n-1)$ species of 5'-TCCAGGTGTCCG**tttt**C. The melting curves were determined under the following conditions: 100 mM Na^+, 10 mM phosphate pH 7.0, 0.1 mM EDTA at 4 μM strand concentration and heating at 0.5°/min. The ΔT_m/mod. rather than the $\Delta\Delta G^\circ_{37}$/mod. values (as calculated by mathematical curve fitting) from the same or similar sequences were chosen as more reliable data to generally judge the influence on the binding affinity of individual modifications for fully modified oligonucleotides containing pyrimidine-nucleoside analogs. For a detailed discussion on this topic see references [35][56]. For the oligonucleotides examined, the following guideline can be applied as a rule of thumb: a change of the melting temperature by 3–5° corresponds roughly to a tenfold change in binding affinity.

	R	ΔT_m / mod. [°]	rel. stability [c])
8a		+1.0	8
8b		+1.1	24
8c		+1.1	>48
8d		−0.3	16
8e		+1.6[a])	-
8f		+1.5[b])	-
8g		+1.5[b])	-

[a]) Value determined from sequences **A** and **B**. [b]) Value based on a single modification incorporated in **A**. [c]) The factor of increased stability in 10% heat-denatured calf-fetal serum was established as compared to the wild-type DNA oligonucleotide.

A possible part for incorporating structural alterations in DNA or RNA is the phosphodiester backbone (*Fig. 3*, [11–13]). Such chemical modifications should allow to increase the nuclease resistance, and backbone analogs are known since the late sixties, especially phosphorothioates **1** [9][14][15] and methylphosphonates **2** [16] as the two most prominent representatives. Even though these derivatives are quite resistant towards degradation by nucleases, their RNA binding affinity is generally lower as compared to oligodeoxyribonucleotides. Part of this observation is due to the fact that the synthetic methods used are not stereospecific. Therefore, roughly a 1:1 mixture is produced of both possible diastereoisomers for each modified backbone linkage. To overcome this drawback, a few achiral backbone replacements were identified more recently, giving not only rise to stability towards nucleases but also exhibiting an improved binding behavior towards RNA. The most promising candidates are thioformacetals **3** [17], *N*-methylhydroxylamines **4** [18], phosphoramidates **5** [19], and amides **6** [20][21], the latter resulting from our internal efforts to optimize backbone modifications [22]. Despite the promising properties of such derivatives, they are usually more difficult to prepare as they have to be incorporated as dimeric building blocks. Nevertheless, they offer the oportunity to dramatically change the biophysical properties of oligonucleotides (*e.g.* charge reduction) and thereby might affect the pharmacokinetic behavior. Peptide Nucleic Acids (PNA) can be considered as a special type of backbone modifications with the additional replacement of the sugar moiety. These derivatives possess interesting properties but are still awaiting demonstration of their utility as therapeutic agents [23].

The improvement of oligonucleotide properties by modifying the base moieties is limited due to the involvement in *Watson-Crick* hydrogen bonding with the complementary strand. Base modifications should not interfere with this property for specific recognition but, nevertheless, some interesting derivatives emerged over the past few years [24–27]. Even though some of these analogs are able to increase the RNA binding affinity of corresponding oligonucleotides to a remarkable extent, usually the nuclease resistance is not sufficient and has to be further improved by concomitant modification of the backbone [28].

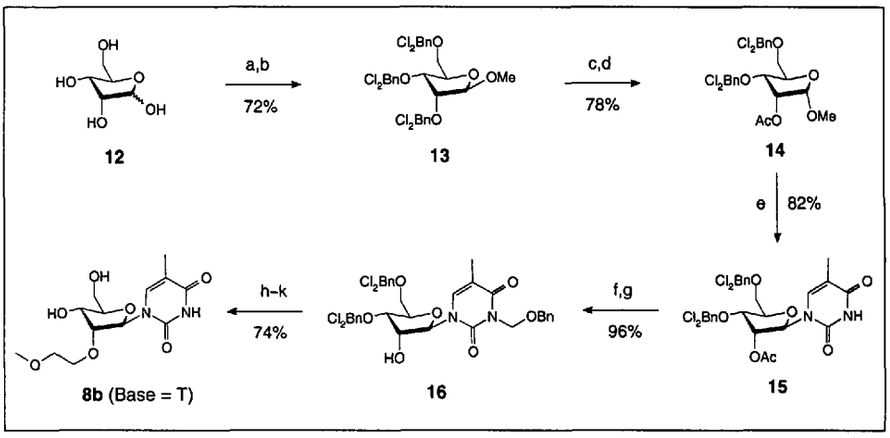
Among a whole variety of oligonucleotides containing sugar-modified nucleosides (*Fig. 4*, for reviews, see [14][22][29]

[30]), especially the following derivatives were investigated in some detail: α-anomeric nucleosides **7** [31][32], 2'-*O*-alkylated ribonucleosides **8** [33–36], 2'-fluoro derivatives **9** [37], carbocyclic deoxyribonucleotides **10** [38–40], and more recently the six-membered derivatives **11** [41][42].

Based on the experimental fact that RNA/RNA duplexes are usually more stable than the corresponding RNA/DNA hybrids of the same sequence [5][43], we chose to prepare ribonucleoside analogs rather than 2'-deoxyribonucleoside derivatives. In support of this working hypothesis, it has been demonstrated that 2'-*O*-methyl ribonucleosides (**8a**) [33][35] or the 2'-fluoro analogs (**9**) [37], both carrying electronegative substituents in the 2'-α-position like ribonucleosides, greatly contribute to an increased RNA binding affinity of corresponding oligonucleotides. However, the increase in nuclease resistance caused by these modifications is insufficient to obtain biologically active antisense molecules, unless the stability is further improved by additionally modifying the phosphodiester backbone. This drawback could be overcome by increasing the size of the 2'-*O*-substituent; unfortunately, the increase of the 2'-*O*-alkyl side chain also gave rise to a decrease of the RNA binding affinity [35]. With this background, it was surprising to find that 2'-*O*-alkoxyalkyl substituents – in particular 2'-*O*-methoxyethyl (**8b**), 2'-*O*-[methyltri(oxyethyl)] (**8c**), and other groups related to ethylene glycol (**8e-g**) – retain or even slightly surpass the high RNA binding affinity of 2'-*O*-methyl ribonucleosides (**8a**), despite the larger substituents in 2'-position (*Table*) [44].

The general preparation of this class of nucleoside derivatives is exemplified with the synthesis of the 2'-*O*-methoxyethyl ribothymidine (**8b**) (*Scheme*, [44]). Starting from D-ribose (**12**), the anomeric hydroxy function was first methylated, followed by dichlorobenzylation of the three remaining hydroxy groups to give crystalline **13** in good yield. Treatment with $SnCl_4$ in CH_2Cl_2 at 0° allowed the specific removal of the 2-*O*-protecting group which was acylated to give crystalline **14** as glycosyl donor. *Lewis*-acid-catalyzed reaction with bissilylated thymine exclusively gave the β-ribonucleoside in good yield. The 2'-*O*-acetyl group was cleaved with base to give **16** which served as general intermediate to introduce a variety of 2'-*O*-substituents by alkylation after base protection. Methoxyethylation proceeded smoothly to give the fully protected ribonucleoside derivative that was efficiently deprotected in three consecutive steps to give **8b**. The 2'-*O*-modified nucleoside analogs were 5'-*O*-dimethoxy-tritylated, base-protected where required, and converted to the phosphoramidites to allow automated oligonucleotide synthesis [44].

Scheme. *Synthesis of 2'-O-Methoxyethyl Ribothymidine* **8b**



a) MeOH, conc. H_2SO_4 (cat.); 5°; 16 h (95%). *b*) 4 equiv. Cl_2BnCl, 4 equiv. powdered KOH, DMSO; 20°, 24 h (76%). *c*) 1.2 equiv. $SnCl_4$, CH_2Cl_2; 0°, 30 h (99%). *d*) Ac_2O, DMAP (cat.), pyridine; 20°, 17 h (79%). *e*) 1.3 equiv. bissilylated thymine, 1.2 equiv. TMS-triflate, MeCN; 70°, 20 h. *f*) 1.1 equiv. $BnOCH_2Cl$, DBU, MeCN; 20°, 24 h (98%). *g*) NaOMe, MeOH; 20°, 4 h (98%). *h*) 1.5 equiv. $CH_3OCH_2CH_2Cl$, 2 equiv. powdered KOH, DMSO; 20°, 26 h (87%). *i*) Pd/C (5%), 1 atm H_2, THF; 20°, 2 h. *j*) NaOMe, MeOH; 20°, 4 h (93%, 2 steps). *k*) Pd/C (5%), 1 atm H_2, MgO, MeOH; 20°, 4 h (92%).

The rationale for the beneficial behavior of ethyleneglycol-like substituents in 2'-position can be hypothesized twofold (*Table*): first, by additional solvation of the alkoxy substituent in water. This influence on the duplex stability is difficult to predict, nevertheless binding results of carbocyclic nucleoside analogs suggest that a 2'-methoxytriethoxy substituent has a rather negative influence on the RNA duplex formation as compared to the corresponding 2'-deoxy analog [22][45]. On the other hand, the second O-atom in the 2'-chain of the ethylene-glycol type ribonucleosides **8b**, **c**, and **8e–g** is responsible for a structural preorganization of the side chain which – due to the *gauche* effect – preferably adopts a conformation with a torsion angle 2'-O–CH_2–CH_2–O of either +60° or –60°. For the 2'-*O*-methoxyethyl derivative **8b**, both conformations are well accommodated in the minor groove of the formed hybrid without causing any steric constraints. In addition, model building indicated that additional side groups at the second C-atom of the 2'-substituent would fit extremely well, irrespective of the new stereocenter formed, as was experimentally proven with derivatives **8f, g** (*Table*). This hypothesis is even further supported with the ribonucleoside analog **8d** that prefers, due to the anomeric effect, a different and unfavorable conformation of the side chain resulting in a slightly destabilizing contribution for the duplex formation. As expected, the increasing size of the 2'-chain (**8c**) adds to the nucleolytic stability without negatively influencing the binding properties to complementary RNA. We chose with **8b** the simplest of these promising derivatives to evaluate their influence on the efficacy of functional antisense molecules in cell cultures as well as in animals.

A collaborative project was initiated a few years ago to identify active antisense molecules for the downregulation of C-'raf' kinase [46]. The family of 'raf' genes encodes serine-threonine-specific protein kinases that play key roles in signaling processes. Evidence exists supporting the importance of 'raf' kinases in the development and maintenance of certain human malignancies and the downregulation of C-'raf' kinase was viewed as a potential possibility to stop cell proliferation. From initial screening efforts of 20-mer phosphorothioates, one particular compound (**17**, CGP-69846A / ISIS-5132) was identified which was shown to specifically inhibit the synthesis of C-'raf' kinase by a RNase H-dependent mechanism resulting in potent antiproliferative effects, both in cell cultures and *in vivo* [46] (*Fig. 5*). Due to known side effects of phosphorothioates at usually much higher concentrations (>1–5 μM), presumably based on non-specific protein binding [15][47][48], we put a lot of emphasis on the concept validation of this particular compound. Therefore, a series of compounds (**18–24**) was prepared, containing 1–7 mismatches in the center of the fully matched antisense oligonucleotide **17**. As shown in *Fig. 5*, an increasing number of mismatches not only lowered, as expected, the RNA binding affinity (melting temperature) but also re-

a)

Compound	Sequence	Tm (°C)	IC_{50} (nM)
17	5'-TCCCGCCTGTGACATGCATT-3'	62	90
18	5'-TCCCGCCTGCGACATGCATT-3'	56	300
19	5'-TCCCGCCTGCTACATGCATT-3'	47	500
20	5'-TCCCGCCTACTACATGCATT-3'	42	1000
21	5'-TCCCGCCTACTTCATGCATT-3'	36	--
22	5'-TCCCGCCCACTTCATGCATT-3'	27	--
23	5'-TCCCGCCCACTTGATGCATT-3'	--	--
24	5'-TCCCGCGCACTTGATGCATT-3'	--	--

b)

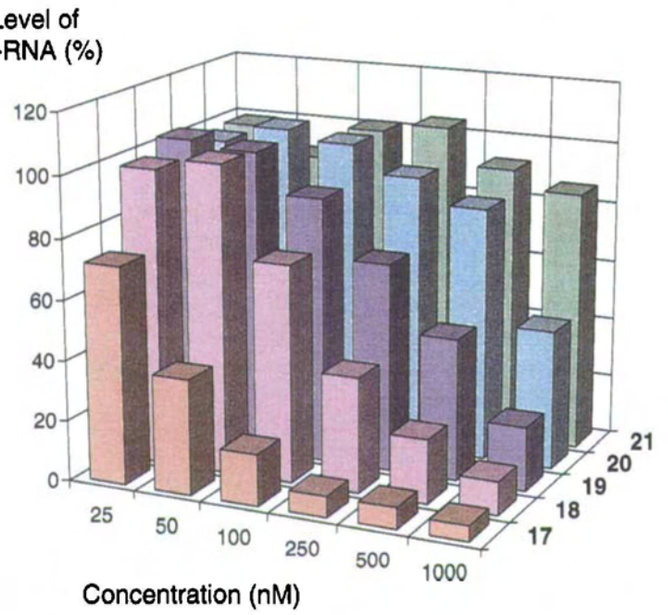


Fig. 5. *Influence of the match antisense oligonucleotide 17 and its control compounds 18–24 containing 1–7 mismatches on the level of C-raf mRNA.* T24 cells (human bladder-carcinoma cell line) were grown on 10-cm-plates at a density of 50–75% confluency, washed with phosphate-buffered saline (prewarmed to 37°); Opti-MEM containing 10 μM DOTMA:DOPE was then added to each plate (5 ml/plate). Antisense oligonucleotides were added from 200 μM stock solutions to each plate and incubated at 37° for 4 h. Following this treatment, the solution was removed and replaced with normal cell-culture media. After 20 h incubation at 37°, the mRNA levels were determined by Northern blots as described [46].

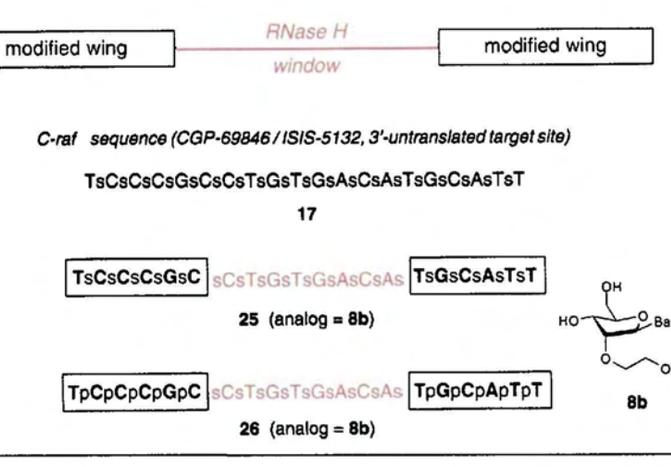


Fig. 6

duced the ability to downregulate the C-'raf'-kinase message, thereby demonstrating the specificity of **17**. The same observation was made with these compounds in animal experiments: whereas the fully matched phosphorothioate **17** was able to efficiently inhibit the growth of human tumors in mice *(vide infra)*, the mismatched controls clearly revealed less activity with an increasing number of mismatches up to 4. This particular oligonucleotide (**21**) showed no activity and behaved like a placebo or a phosphorothioate oligonucleotide with unrelated sequence (data not shown). Based on statistical considerations, the limited expression of the human genome, and the restricted accessibility of mRNA's, an oligonucleotide-containing 1–3 mismatches with the ability to efficiently bind any other message is quite unlikely to occur.

To further increase the therapeutic window of this phosphorothioate antisense oligonucleotide **17**, we intended on one hand to increase the binding affinity for the mRNA by incorporation of modified nucleoside derivatives **8b** without destroy-

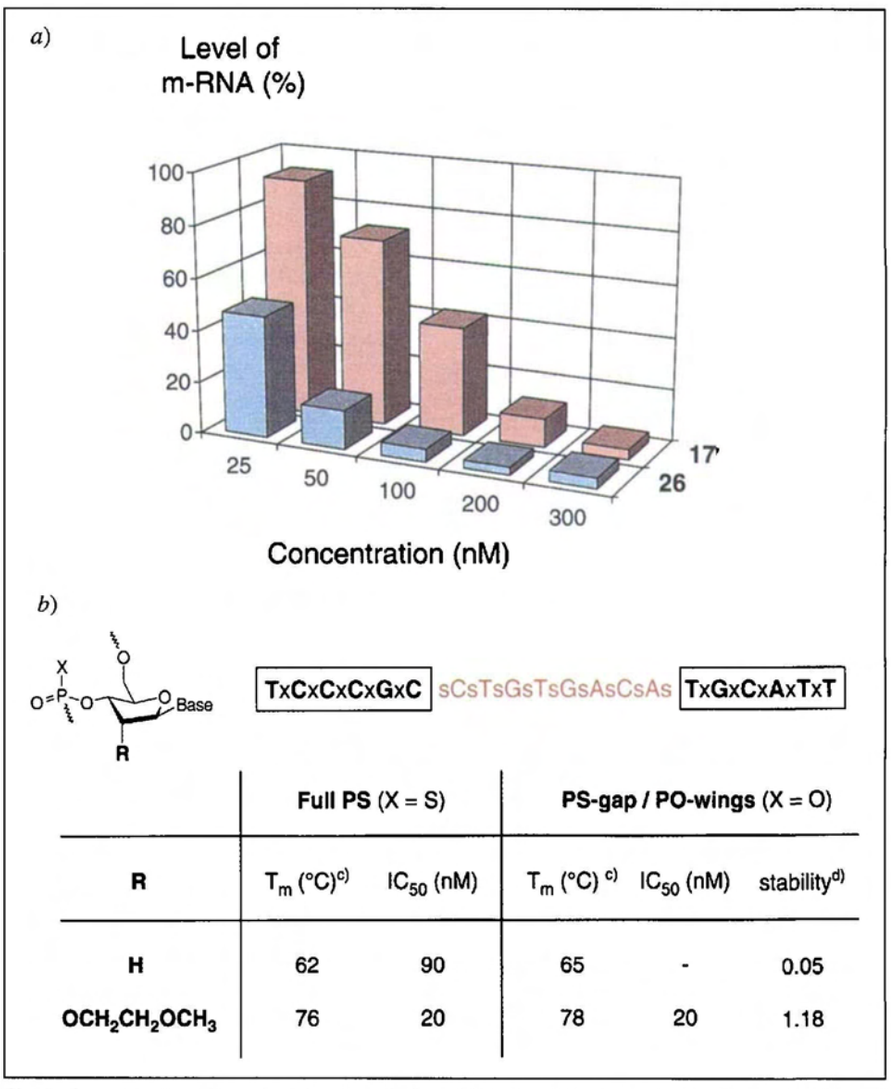
Fig. 6. *Chimeric antisense oligonucleotides with modified wings and a central RNase H window consisting of DNA phosphorothioates.* Oligonucleotide syntheses were performed on an Applied Biosystems 380B automated DNA synthesizer using Pharmacia primer support [57]. The following modifications were made to the standard synthesis protocol for oligodeoxyribonucleotides: *i*) the modified phosphoramidites were coevaporated three times from benzene and dried over night in high vaccum, *ii*) the coupling times were doubled *iii*) anhydrous oxidation was achieved with 0.3M *t*-BuOOH in CH_2Cl_2, and *iv*) extended sulfurization was realized with *Stec*'s reagent in acetonitrile/pyridine 3:1; twice 2 min [58]. On research scale (1.5 μmoles), the crude oligonucleotides contained on average 85% full-length material as judged by capillary gel electrophoresis and MALDI-TOF MS [59]. The large-scale synthesis (280 μmol) was run on a *Milligen 8800* DNA synthesizer with the same reagents as described above. Prolonged coupling times were used (20 min for modified building blocks and 8 min for DNA phosphoramidites) with the following equiv. of modified amidites: 5.5 (T), 6 (5-MeC and A), and 7 (G). After deprotection (30% aq. ammonia, 55°, 16 h), the crude material was purified DMT-on by HPLC (RPC-18, 12 μ *Merck Lichrosphere 300*, 5 x 25 cm; A: 50 mM TEAA pH 7, B: A in 70% acetonitrile. Elution: 15% B for 5 min, then linear gradient from 15–45% B in 60 min. Flow: 100 ml/min; 12500 OD_{260}/run). Pure fractions were pooled, concentrated by ultrafiltration and deprotected in 80% aq. acetic acid for 30 min. After neutralization with ammonia, ultrafiltration was used to desalt, exchange the counterion to sodium and deionize the oligonucleotide. Usually 26% material was obtained with the correct mass and a purity similar to the research-scale syntheses.

ing the ability of RNase H-mediated cleavage of the target complement. For this purpose we applied the gap-mer strategy as outlined in *Fig. 6* [8]. On the other hand, we wanted to reduce the amount of phosphorothioate linkages in oligonucleotides, as some of the sequence-independent side effects are presumably based on non-specific binding to certain proteins. It was demonstrated with nucleic-acid-processing enzymes that a reduction of the sulfur content can have a dramatic effect on such non-specific interactions [49]. We, therefore, exchanged six nucleosides at each end of **17** by the corresponding ribonucleoside analog **8b**, using both, phosphorothioate (**25**) and phosphodiester linkages (**26**). This leaves in the middle a DNA-phosphorothioate window of eight nucleotides that was demonstrated to be sufficiently big for inducing efficient cleavage of the bound RNA by RNase H [8]. In general, the direct comparison of such related compounds (*e.g.* **25** and **26**) allows an excellent assessment of the influence on stability by a particular modification in cell culture.

In the chimeric oligonucleotides examined, the 2'-*O*-modified ribonucleoside **8b** contributed to an increased affinity for the complementary RNA target (*Fig. 7*). Even the phosphodiester-linked nucleoside analogs revealed a remarkable stability (**26**) which – in combination with the increased RNA binding affinity – resulted in an improvement of the biological activity in cell culture. Even after 60 h readout, **26** shows a roughly fourfold higher potency compared with **17**, indicating a remarkable extent of nuclease resistance (data not shown). This particular sugar modification represents to our knowledge a unique case as the beneficial properties allow the preparation of oligonucleotides with increased biological potency with a simultaneous reduction of phosphorothioate linkages that should lead to an increased therapeutic window of such compounds.

Similar chimeric oligonucleotides containing phosphodiester-linked 2'-*O*-methyl ribonucleosides (**8a**) in the wings were shown to possess a clearly reduced potency as compared to the parent phosphorothioate compounds, most likely due to an insufficient nuclease stability in cellular assays ($IC_{50} > 500$ nM, data not shown). As expected, the biological efficacy could easily be restored by stabilizing the backbone linkages in the wings with phosphorothioates.

As mentioned before, **17** was shown to specifically inhibit tumor growth in nude mice in a dose-dependent manner (*Fig. 8a*).



	Full PS (X = S)		PS-gap / PO-wings (X = O)		
R	T_m (°C)[c]	IC_{50} (nM)	T_m (°C)[c]	IC_{50} (nM)	stability[d]
H	62	90	65	-	0.05
$OCH_2CH_2OCH_3$	76	20	78	20	1.18

Fig. 7. *Concentration-dependent downregulation of C-'raf' mRNA by antisense oligonucleotides **17** and **25/26*** (for details, see *Fig. 5*). [c]) Melting temperatures were determined as described in the Table. [d]) Nuclease stability was experimentally determined as follows: Oligonucleotides were purified by gel electrophoresis and desalted using Poly Pak cartridges (*Glen Research*, Sterling, VA). After 5'-labeling with γ^{32}P-ATP and T4 polynucleotide kinase, the samples were heated to 95° for 2 min to inactivate the kinase prior to performing the SVP assay. The oligonucleotides (0.1 μM) were incubated in 50 mM Tris-HCl, pH 8.5, 72 mM $CaCl_2$, and 14 mM $MgCl_2$ with SVP (0.005 U/ml, USB Cleveland, OH) in a final volume of 50 μl. 5 μl aliquots were removed at different timepoints, added to an equal volume of 80% formamide containing bromophenol blue and xylene cyanol as dyes, and then heated for 2 min to 95°. These treated aliquots were stored at –20° until analysis by denaturing polyacrylamide electrophoresis. Quantitation was performed on a Molecular Dynamics Phosphor-Imager. The number given represents the half-life of the oligonucleotide indicated divided by the half-life of the parent compound **17**.

Assuming that the known pharmacokinetic behavior of phosphorothioates [9] [50][51] would be influenced by partially modified building blocks and reduction of 'sticky' phosphorothioate linkages, the increase of potency observed in cell cultures could not be directly extrapolated to the *in vivo* potency. Therefore, we were pleased to observe for the chimeric oligonucleotide **26** a slight increase of potency in these animal studies (*Fig. 8b*).

For the validation of this strategy to use antisense oligonucleotides with 2'-*O*-methoxyethyl ribonucleosides in the wings and a DNA phosphorothioate window we wanted to apply it to a different target. Protein kinases C were identified as a family of serine/threonine kinases involved in mediating intracellular responses to a variety of growth factors, hormones, and neurotransmitters. In analogy to C-'raf' kinase, PKC's are believed to play a crucial role in cell proliferation. As a consequence, inhibitors of individual isozymes of PKC would be of considerable value, both as potential therapeutic agents and as

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powerful research tools to examine the function played by PKC isozymes in regulating cellular and biochemical processes. With this background in mind, a collaborative program was initiated to specifically inhibit the synthesis of PKC-α by antisense oligonucleotides. One 20-mer phosphorothioate oligonucleotide (**27**, CGP-64128A/ISIS-3521; *Fig. 9a*) was identified that was not only shown to specifically suppress the formation of PKC-α without affecting the other isozymes [52],but which was also able to efficiently inhibit the growth of various human tumors in mice [53]. By the end of last year an IND (Investigational New Drug) application was filed in the US and this compound is currently under clinical investigation. We used exactly the same approach as outlined above for C-'raf' kinase as target and prepared the chimeric oligonucleotide **28** (CGP-75182A) using phosphodiester-linked 2'-*O*-methoxyethyl ribonucleosides **8b** in the wings. In a cell-culture-based assay (A549 cells), **28** was shown to be sixfold more active than the parent compound **27** (data not shown, [53]) with a IC_{50} of roughly 20 nM. Both of these compounds were examined for their potency to inhibit tumor growth (human colon cancer, Colo 205) in nude mice (*Fig. 9*). The DNA phosphorothioate inhibited tumor growth of Colo 205 cells, even though the efficiency was reduced as compared to the same experiment with A549 cells. The chimeric oligonucleotide **28** with

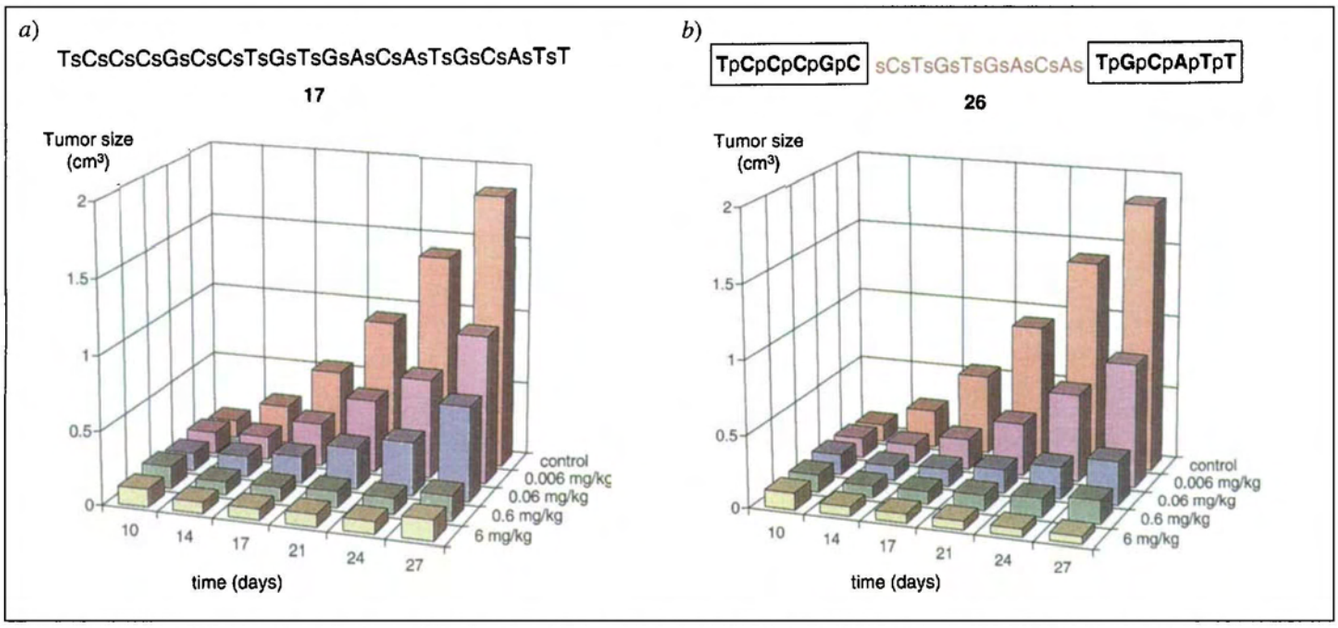

Fig. 8. *Antitumor activity of antisense oligonucleotides. a)* ***17*** *(CGP-69846A / ISIS-5132) and b)* its chimeric analog **26** (CGP-69845A) in female Balb/c nude mice. Previously grown tumor fragments (human A549 lung adenocarcinoma, ~25 mg) were implanted subcutaneously. Oligonucleotide treatment was initiated after the tumor reached a mean volume of 100 mm³. The oligonucleotides were daily administered intravenously at the indicated doses in saline; each experimental value is averaged from at least five animals [46]. The control represents placebo treatment, but sequence-unrelated or mismatched oligonucleotides (*e.g.* **21–24**) were also shown to have no effect on tumor growth at the highest dose examined.

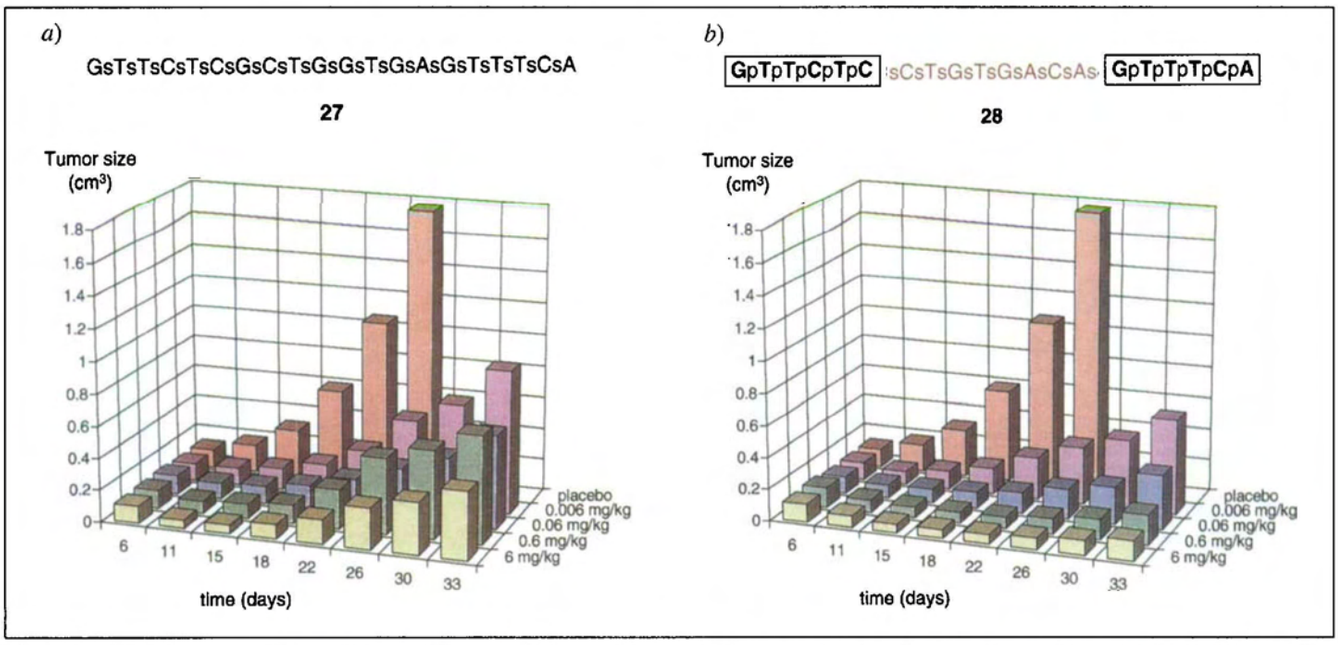

Fig. 9. *Antitumor activity of antisense oligonucleotides. a)* **27** (CGP-64128A / ISIS-3521) and *b)* its chimeric analog **28** (CGP-75182A) in female Balb/c nude mice. The experiments were performed as described in *Fig. 8*, the only difference being the cancer cell line used (human colon cancer Colo 205 instead of A549).

the same sequence was much more potent in inhibiting tumor growth. At a daily administered dosis of 0.06 mg/kg (*Fig. 9b*, blue bars), this compound was roughly as active as the DNA phosphorothioate **27** at a 100-fold higher concentration (*Fig. 9a*, yellow bars). This additional example demonstrates the potential utility of the sugar-modified ribonucleosides **8b** in antisense oligonucleotides, and studies to further improve the potency are ongoing.

In combination with the efficacy studies in mice, it was of particular interest to understand the influence of these modifications (and sulfur reduction) on the pharmacokinetic behavior. Therefore, one representative oligonucleotide of each class (**17** and **26**) was labeled with tritium at C(8) of the purine bases as described [54]. The behavior of phosphorothioate **17** turned out to be as expected (*Fig. 10a*): rapid, biphasic clearance from the blood with kidney, liver, and spleen as high affinity tissue. Reasonable concentrations were also observed in skin, bone, heart, lung, and tumor whereas the oligonucleotide level remained low in brain and fat throughout the experiment. Interestingly, the chimeric oligonucleotide **26** behaved quite differently (*Fig. 10b*). This compound was cleared from the body much more rapidly as demonstrated by the high levels in urine and kidney. On the other hand the affinity towards liver and spleen is clearly reduced and reasonable concentrations are still found in skin, bone, heart, lung, and tumor, especially in the initial timeframe. In light of these findings it is even more remarkable that both chimeric compounds, **26** and **28**, are superior in terms of biological potency in animals as compared to the parent phosphorothioates **17** and **27**.

As mentioned above, the reduction of phosphorothioate linkages from 19 (**17**) to 9 (**26**) should be beneficial regarding non-specific side effects known for fully backbone-modified DNA phosphorothioates [49]. In particular complement activation and hemodynamic changes were observed after rapid intravenous injections in monkeys [55], requiring a slow infusion of phosphorothioates for therapeutic applications. Based on this observation, we decided to examine the influence of oligonucleotides on the clotting time of human plasma (*Fig. 11*). According to our expectations, the effect of the modified oligonucleotide **26** on the observed clotting time was much less pronounced, indicating the beneficial properties of such chimeric oligonucleotides on non-specific side effects.

Based on the results given above, we believe that chimeric oligonucleotides with phosphodiester-linked 2'-*O*-methoxyethyl ribonucleosides **8b** in the wings and DNA-phosphorothioate windows perform better as antisense compounds compared to their parent DNA phosphorothioates. These molecules belong to a second generation of antisense-oligonucleotide analogs with the potential of increased efficacy *in vivo* combined with fewer side effects that should finally result in an increase of the therapeutic window.

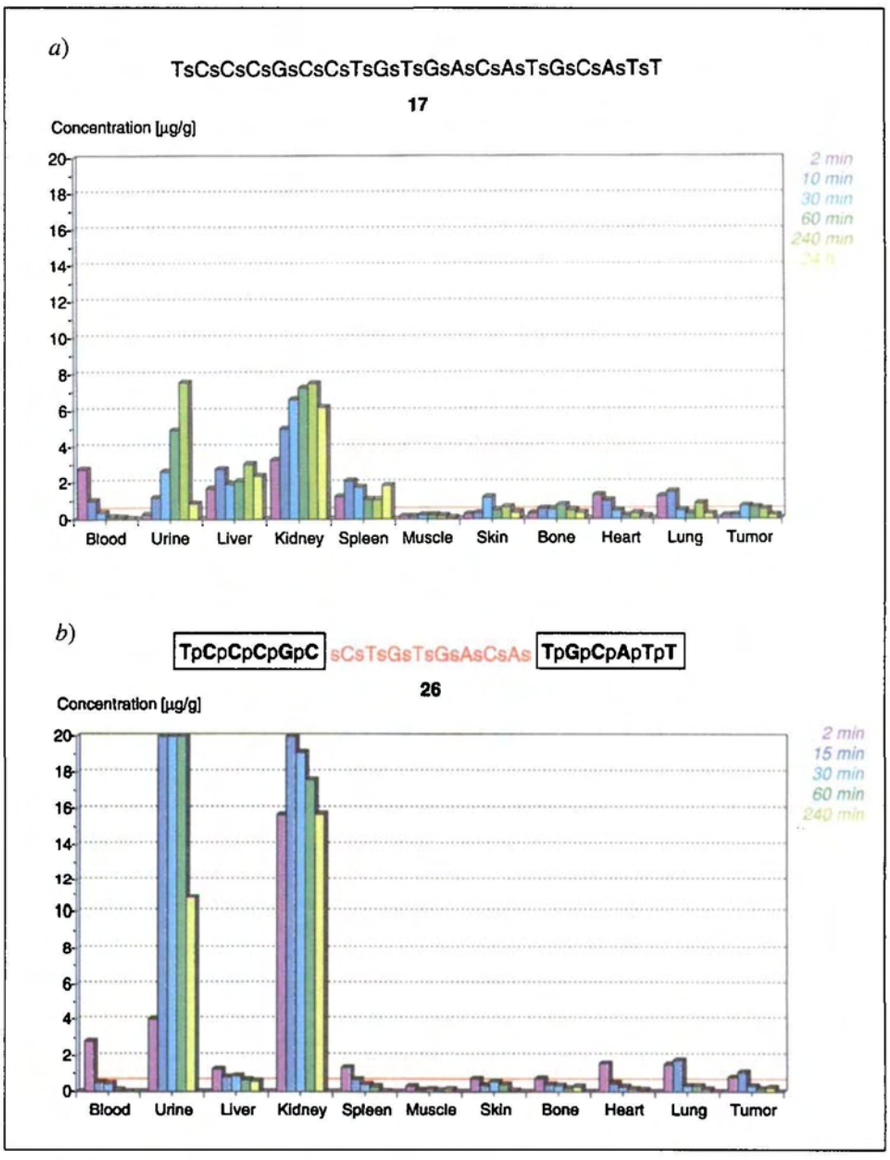

Fig. 10. *Pharmacokinetic behavior of oligonucleotides 17 and 26.* Female nu:nu Balb/c mice (20–25g) were fed *ad libitum* with a standard laboratory diet and kept under controlled conditions (12 h light cycle; 20°). Tumor-bearing mice were generated by subcutaneous administration of 1.0×10^6 A549 cells in 200 µl of *Hanks'* balanced salt solution to the right flank of the mice. They were allowed to develop for 7–10 d and only those mice with palpable tumors (0.5–1.5 cm) were selected for pharmacokinetic studies. Oligonucleotides *a*) **17** or *b*) **26** in 50 µl 0.9% saline, containing 0.25 µCi of tracer [^{3}H]oligonucleotide per animal, were administered intravenously to conscious tumor-bearing mice by tail vein injection, at doses of 0.6 mg/kg. Animals were kept in solid bottomed cages with free access to food and water for up to 24 h throughout the experimental period. Animals were sacrificed by sodium-pentabarbitone overdose at the times indicated after dosing. Organs of interest (blood, urine, liver, kidney, spleen, heart/thymus, lung, muscle, skin, bone, fat, brain, and tumor) were collected and their [^{3}H]-content determined. The red line in the graphs indicates a concentration of 0.6 mg/kg.

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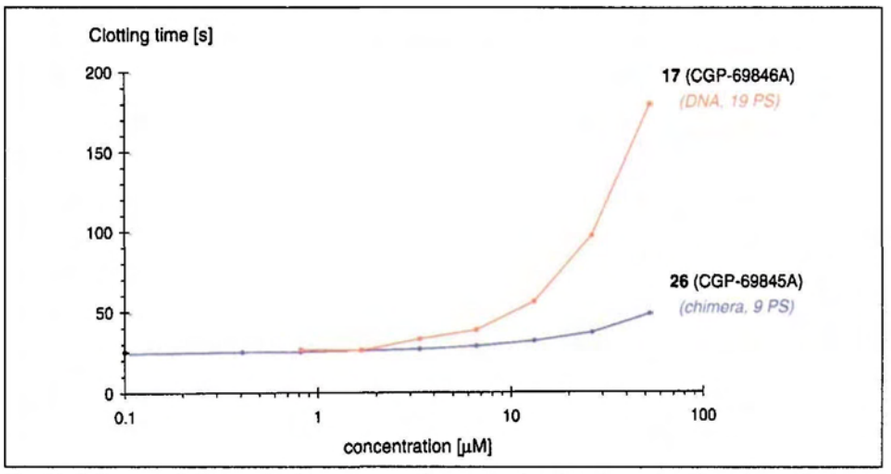

Fig. 11. *Influence of oligonucleotide concentration on clotting time determined by using an Activated Partial Thromboplastin Time (APTT) method – an* in vitro *diagnostic test designed to identify deficiencies in the intrinsic coagulation pathway.* Briefly, 50 µl of human plasma containing a known concentration of oligonucleotide **17** (red) or **26** (blue) was activated by incubation with 50 µl of bovine-cephalin reagent before coagulation was initiated by the addition of $CaCl_2$ (50 µl, 20 mM). The time taken for coagulation to occur was measured using an *Instrumentation Laboratory ACL 300R* coagulometer. The concentrations of **17** (CGP-69846A) and **26** (CGP-69845A) required to double the clotting time were 12.1 and >53 µM, respectively.

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