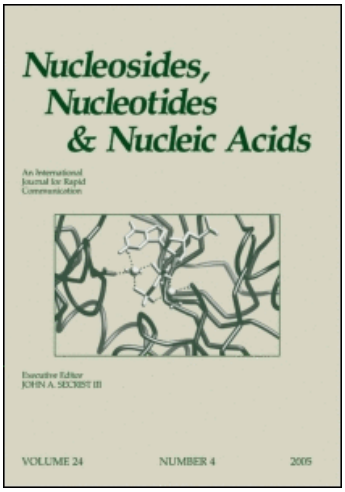

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PRECLINICAL PROFILING OF MODIFIED OLIGONUCLEOTIDES: ANTICOAGULATION AND PHARMACOKINETIC PROPERTIES

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Abstract: Backbone, sugar and pendant-group modifications were shown to influence the anticoagulant properties of a 20-mer oligonucleotide in human plasma *in vitro*. The pharmacokinetics, tissue-distribution and metabolism of a chimeric oligonucleotide (CGP 69845A), which had reduced anticoagulation properties, were compared with the analogous phosphorothioate oligodeoxynucleotide (CGP 69846A) in a tumour-bearing mouse model.

INTRODUCTION

The ability of antisense oligonucleotides to hybridise with target mRNA and cause protein-specific translation arrest[1,2] has stimulated interest in their therapeutic potential[3], notably for tumour[4,5,6] and viral[7] indications. The discovery of an antisense oligonucleotide, which is able to knock-down target mRNA, initiates a series of preclinical studies which characterise its potency, specificity, mechanism of action, pharmacokinetics, metabolism and safety profile. Potential routes of administration and oligonucleotide formulation are further considerations. Preclinical studies in monkeys have shown that phosphorothioate oligodeoxynucleotides can elicit acute haemodynamic side-effects (anticoagulation and complement activation) once threshold plasma levels are exceeded. These side-effects are not dose-limiting, but can be dose rate-limiting. These compounds are therefore administered by controlled intravenous infusions in clinical trials where treatment is aimed at systemic conditions. Nucleotide modifications which are able enhance the activity of oligonucleotides and reduce their haemodynamic side-effect profile are candidates for second-generation clinical compounds. In the present study, the

influence of several nucleotide modifications on the *in vitro* anticoagulant properties of oligonucleotides is reported. Furthermore, the *in vivo* pharmacokinetics, tissue distribution and metabolism were characterised for the most promising compounds (*Fig. 1*).

MATERIALS AND METHODS

Activated partial thromboplastin time (APTT) is an *in vitro* diagnostic test designed to identify insufficiencies in the intrinsic coagulation pathway. It is sensitive to deficiencies or impairment of prekallikrein, high molecular weight kininogen, factors-V, VIII, IX, X, XI, XII and prothrombin. APTT can also be increased in severe hypo- or dys-fibrinogenaemia. The concentration of compound required to double the clotting time in the APTT assay was used as an anticoagulation index for several oligonucleotides containing modified nucleotides. Fresh human blood was anticoagulated with 10% v/v tri-sodium citrate (3.8 g/100 ml). Anticoagulated blood was centrifuged at 1300 x g for 20 min and the supernatant collected as platelet-free plasma. Two plasma pools were prepared, each comprising the combined plasma of three individuals. Plasma was treated with known concentrations of the test oligonucleotide and its influence on the clotting time was assessed. APTT was determined by activating plasma (50 µl) by incubation with bovine cephalin reagent (50 µl, 5 min, 37°C) before initiating coagulation with the addition of calcium chloride solution (50 µl, 20 mM). The coagulation time was determined using an Instrumentation Laboratory ACL 300R coagulometer. The concentration of oligonucleotide required to double APTT was determined in duplicate in each plasma pool.

Intravenous pharmacokinetic studies were performed in tumour-bearing mice using tritium labelled CGP 69846A and CGP 69845A. These oligonucleotides were radiolabelled by tritium exchange at the C_8-position of purine bases according to the method of Graham *et al.*[8] and purified by strong anion exchange high-performance liquid chromatography. Tumour-bearing mice were generated by subcutaneous inoculation of female nu:nu Balb/c mice with 1.0 x 10^6 A549 cells. Tumours were allowed to develop for 7-10 days and only those mice with palpable tumours were selected for pharmacokinetic studies. Animals were allowed free access to food and water throughout the experimental period. [^{3}H]CGP 69846A and [^{3}H]CGP 69845A (0.5 µCi, 0.6 mg/kg) were administered by tail vein injection. At defined time points animals were killed by sodium pentabarbitone overdose, tissues of interest (blood, urine, liver, kidney,

spleen, heart/thymus, lung, muscle, skin, bone, fat, brain and tumour) were collected and their [^{3}H]-content determined by tissue-oxidation (Canberra-Packard 306) followed by liquid scintillation counting (Beckman LS6500). The metabolic fate of the oligonucleotides was assessed by the analysis of kidney and liver extracts using capillary gel electrophoresis as described previously.[9,10] Briefly, A549 tumour-bearing mice were intravenously dosed with 6.0 mg/kg of oligonucleotide and the animals were killed by sodium pentabarbitone overdose at 1440 min. The kidney and liver were collected. Parent oligonucleotide and its metabolites were extracted from selected tissues as follows: (i) sample digestion with proteinase K, (ii) phenol:chloroform extraction, (iii) ammonium hydroxide treatment, (iv) SAX-SPE, (v) RP-SPE and (vi) membrane de-salting. Samples were analysed by capillary gel electrophoresis using UV_{260nm} detection (Beckman PACE 5010).

RESULTS AND DISCUSSION

Nucleotide modifications were incorporated into a model sequence - TCC CGC CTG TGA CAT GCA TT - complementary to the 3'-untranslated region of C-*raf*-1 kinase. The parent phosphorothioate oligodeoxynucleotide (CGP 69846A, **1**) was a moderately potent anticoagulant which doubled APTT at 12.1 μM. The incorporation of modified nucleotides into this sequence resulted in oligonucleotides with a range of anticoagulation potencies. Base modification had relatively little effect. The phosphorothioate oligodeoxynucleotide in which all of the seven cytidine bases were replaced with 5-methyl cytidine (**5**) or 5-propyne cytidine (**6**) doubled APTT at 9.1 μM and 12.3 μM, respectively. The influence of 2'-modifications were substituent-dependent. The addition of a 2'-fluoro substituent increased the anticoagulation properties (**2**; APTT doubled at 7.0 μM). Conversely, 2'-O-alkyl substituents decreased anticoagulant effects; 2'-methoxy (**3**) and 2'-propoxy (**4**) doubled APTT at 14.4 μM and 22.2 μM, respectively. The greatest diminution of anticoagulation properties was achieved through replacement of phosphorothioate inter-nucleoside linkages with the natural phosphodiester group. The phosphodiester 2'-deoxy (**8**), 2'-methoxy (**9**) and 2'propoxy (**10**) oligonucleotides did not double APTT at concentrations up to 31.8 μM. Anticoagulation properties of CGP 69846A were also attenuated by conjugation of cholesterol at the 5-terminus (**7**; APTT doubled at 18.7 μM). Chimeric oligonucleotides (*Fig. 1*), which comprised a 2'-deoxy phosphorothioate 'RNase H window' sandwiched between two 2'-modified 'wings' (the so called 'gapmer strategy'), were also assessed. The incorporation of 2'-

CGP 69846A
5'-TsCsCsCsGsCsCsTsGsTsGsAsCsAsTsGsCsAsTsT-3'

CGP 69845A
5'-**t**o**c**o**c**o**c**o**g**o**c**sCsTsGsTsGsAsCsAs**t**o**g**o**c**o**a**o**t**o**t**-3'

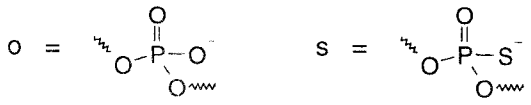

Figure 1: Structures of CGP 69846A and chimeric oligonucleotide analogues CGP 71849A and CGP 69845A, **t,c,a** and **g** are 2'-methoxyethoxy modified analogues of thymidine and 5-methyl-cytidine, adenine and guanine, respectively.

methoxyethoxy phosphorothioate wings (**12**) reduced anticoagulant effects; APTT was doubled at 16.6 μM compared to 12.1 μM for the uniform 2'-deoxy phosphorothioate compound. Interestingly, incorporation of 2'-methoxyethoxy phosphodiester wings (**14**; CGP 69845A), reduced the anticoagulation properties at least 4.5-fold compared to the uniform 2'-deoxy phosphorothioate compound (*Table 1 and Fig. 2*).

The phosphorothioate oligodeoxynucleotide (**1**; CGP 69846A) can specifically knock-down C-*raf*-1 mRNA *in vitro* (IC_{50} = 90 nM) and has potent *in vivo* anti-tumour activity in the A549 human tumour xenograft nude mouse model (50% inhibition of tumour growth between 0.006 and 0.06 mg/kg).[11] In addition to having reduced anticoagulant properties *in vitro*, CGP 69845A is more active *in vitro* (IC_{50} = 20 nM) and its antitumour activity is at least comparable with CGP 69846A (50% inhibition of tumour growth at 0.006 mg/kg) in the same models.[11] CGP 69846A and CGP 69845A were selected for pharmacokinetic and metabolic profiling.

The blood kinetics for [^{3}H]CGP 69846A and [^{3}H]CGP 69845A were indistinguishable following intravenous administration of a 0.6 mg/kg dose to A549 tumour-bearing mice (*Fig. 3*). They were rapidly cleared from the blood; the circulating radiolabel decreased to 30% by 2 min and to 1% by 60 min. Tissue distribution, however, was dependent on the nature of the oligonucleotide (*Fig. 3*). The tissue distribution of CGP 69846A was similar to that reported for

TABLE 1: Effect of nucleotide modifications on the *in vitro* anticoagulation properties of oligonucleotides in human plasma. **1** = CGP 69846A and **14** = CGP 69845A. 5-MeC = 5-methyl cytosine, 5-propnylC = 5-propyne cytosine, F = fluoro, MoE = methoxyethoxy, OMe = methoxy, OPr = propoxy, PO = phosphodiester, PS = phosphorothioate. † 2'-sugar modification. ‡ 2'-sugar modification and 5-MeC.

#	Modification				Concentration doubling APTT
	Backbone	**Sugar**	**Base**	**Pendant**	**(μM)**
Uniform					
1	PS	—	—	—	12.1
2	PS	uniform 2'-F	—	—	7.0
3	PS	uniform 2'-OMe	—	—	14.4
4	PS	uniform 2'-OPr	—	—	22.2
5	PS	—	5-MeC	—	9.1
6	PS	—	5-propnylC	—	12.3
7	PS	—	—	5'-cholesterol	18.7
8	PO	—	—	—	>31.8
9	PO	uniform 2'-OMe	—	—	>31.8
10	PO	uniform 2'-OPr	—	—	>31.8
Chimeric					
11	PS†-PS-PS†	2'-F	—	—	8.0
12	PS‡-PS-PS‡	2'-MoE	5-MeC	—	16.6
13	PO†-PS-PO†	2'-OPr	—	—	18.4
14	PO‡-PS-PO‡	2'-MoE	5-MeC	—	≥53.0

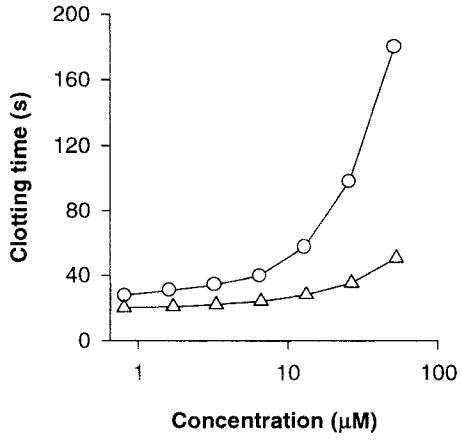

Figure 2: Influence of oligonucleotide concentration on clotting time of human plasma in an activated partial thromboplastin time assay. O = CGP 69846A and Δ = CGP 69845A.

other phosphorothioate oligonucleotides.[10] It was distributed amongst high (*e.g.* kidney, liver, spleen), low (*e.g.* lung, skeletal muscle) and negligible (*e.g.* brain) accumulating tissues. CGP 69845A had a much lower hepatic accumulation whilst its renal uptake was increased. In addition, a much greater proportion of the administered radiolabel was excreted in the urine for [^{3}H]CGP 69845A than for [^{3}H]CGP 69846A. Interestingly, the tumour-association of CGP69845A was higher than for CGP 69846A at 60 min.

The metabolic profiles for CGP 69846A and CGP 69845A were compared in kidney and liver at 1440 min after a 6.0 mg/kg dose using capillary gel electrophoretic analysis (*Fig. 4*). Intact CGP 69846A (N) was still present and represented 9.0±1.9% and 15.3±2.1% of the total oligonucleotide pool in the kidney and liver, respectively. A series of chain-shortened metabolites (N_{-n}; N_{-1} to N_{-10} in kidney and N_{-1} to N_{-9} in liver) which migrated more rapidly than the parent compound were also detected. The kidney contained a greater proportion of higher-order metabolites than the liver. In addition, a metabolite which migrated more slowly than the parent compound (N_{+x}) was observed but not defined. A less pronounced metabolite pattern was observed for CGP 69845A. Intact CGP 69845A was still present and accounted for 47.0±4.9% and 80.7±4.6% of the total oligonucleotide pool in the kidney and liver, respectively.

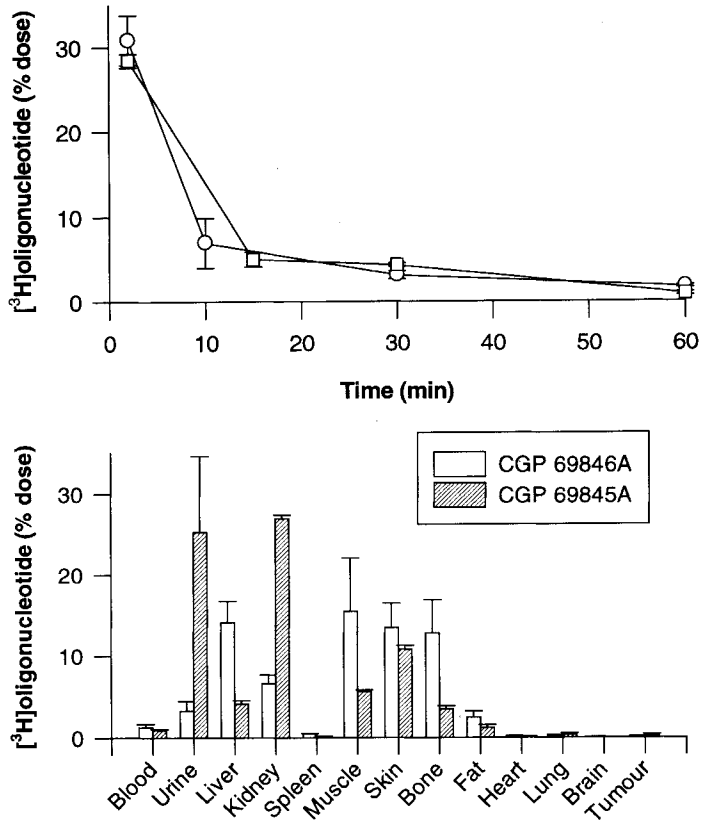

Figure 3: Blood kinetic and tissue distribution for [^{3}H]CGP 69846A and [^{3}H]CGP 69845A in A549 tumour-bearing mice. O = CGP 69846A and □ = CGP 69845A.

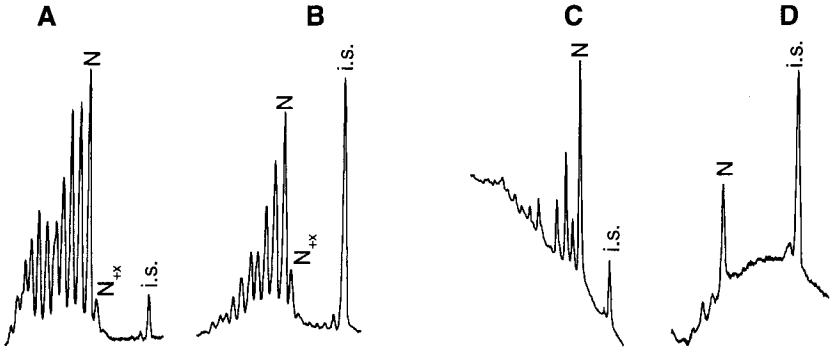

Figure 4: Metabolite profile for CGP 69846A and CGP 69845A in mice. **A** = CGP 69846A - kidney, **B** = CGP 69846A - liver, **C** = CGP 69845A - kidney and **D** = CGP 69845A - liver. N = parent compound, N_{+x} = slower migrating species and i.s. = internal standard.

Some chain-shortened metabolites were also detected; N_{-1}, N_{-2}, N_{-3} and small quantities of higher-order metabolites were detected in the kidney, N_{-1} and N_{-2} were present in the liver. Metabolites which migrated more slowly than CGP 69845A (*i.e.* N_{+x} species) were not observed.

The incorporation of phosphodiester, 2'-methoxyethoxy modified into an oligonucleotide using the 'gapmer strategy' has produced a compound (CGP 69845A) with significantly lower *in vitro* anticoagulation properties when compared to the uniform phosphorothioate oligodeoxynucleotide. It was also metabolically stable in tissues *in vivo*. Despite an altered tissue distribution and high initial urinary clearance, CGP 69845A has potent antitumour activity in the human A549 tumour-bearing mouse model.[11] This second generation antisense oligonucleotide analogue which offers increased efficacy *in vivo* combined with fewer side-effects could potentially expand the therapeutic window for this class of therapeutic agents.

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