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Co-administration of Polyanions with a Phosphorothioate Oligodeoxynucleotide (CGP 69846A): A Role for the Scavenger Receptor in its *In Vivo* Disposition

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ABSTRACT. The effects of co-administering polyanions on the pharmacokinetics of a 20-mer phosphorothioate oligodeoxynucleotide (CGP 69846A), and the role of scavenger receptors in its *in vivo* disposition, have been investigated. Following i.v. administration, CGP 69846A was rapidly cleared from the plasma and distributed amongst high (e.g. kidney, liver, spleen), low (e.g. skeletal muscle) and negligible (e.g. brain) accumulating tissues. In addition it was shown that: 1) dextran sulphate co-administration has a dose-dependent effect on the disposition of CGP 69846A; 2) CGP 69846A undergoes renal filtration and renal accumulation largely results from tubular reabsorption; 3) cross-inhibition studies are consistent with CGP 69846A being recognized by scavenger receptors *in vitro* and *in vivo*; and 4) the scavenger receptor may be an important determinant for the *in vivo* disposition of CGP 69846A in mice. These studies contribute toward an increased understanding of the mechanism underlying the pharmacokinetic behaviour of phosphorothioate oligodeoxynucleotides. BIOCHEM PHARMACOL **56**;4:509–516, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Antisense; c-*raf* kinase; ISIS 5132; oligonucleotide

The pharmacokinetics, metabolism and elimination of phosphorothioate oligodeoxynucleotides *in vivo* are well characterised [1–8]. They are well absorbed after parenteral administration (intramuscular, subcutaneous, intraperitoneal or intradermal injections) and are rapidly cleared from plasma and broadly distributed to most peripheral tissues with certain tissues accumulating a high proportion of the dose (e.g. kidney, liver and, to a lesser extent, spleen). Uptake into cells *in vivo* has been demonstrated by a number of laboratories using a variety of methods. For instance, the intracellular localisation of phosphorothioate oligodeoxynucleotides has been unequivocally demonstrated in cells lining the renal proximal convoluted tubules and nonparenchymal hepatic cells using autoradiography [9, 10] and immunohistochemistry [11]. Metabolism occurs in plasma predominantly through 3′-exonuclease-mediated degradation and more slowly in tissues by 3′-alone, 5′-alone and combined 3′- and 5′-exonuclease-mediated cleavage [8, 12, 13]. Tissue associated phosphorothioate oligodeoxynucleotides are eliminated slowly [3, 8, 14] with metabolism being the primary clearance mechanism [15].

The mechanisms responsible for the accumulation of phosphorothioate oligodeoxynucleotides by specific cell types and their broad distribution to peripheral tissues have received little attention and are poorly understood. This was addressed by investigating the possibility that short, single stranded phosphorothioate oligodeoxynucleotides are recognized by scavenger receptors and that this interaction is a key determinant of their pharmacokinetic behaviour *in vivo*. For these studies, we performed cross-inhibition studies between a model phosphorothioate oligodeoxynucleotide (CGP 69846A) and a series of polyanions with differential binding to scavenger receptors.

MATERIALS AND METHODS

Oligonucleotides

CGP 69846A (TsCsCsCsGsCsCsTsGsTsGsAsCsAsTsGsCs AsTsT where s = phosphorothioate; Lot number NGMP-0746–5132; otherwise known as ISIS 5132), was provided by Dr. Brett Monia of ISIS Pharmaceuticals; the active substance constituted a single peak by capillary gel electrophoresis and the molecular weight by electrospray mass spectrometry (6343.7 ± 1.3 Da) was consistent with the calculated value for this phosphorothioate sequence (6344.6 Da).

[^{3}H]Labeling of CGP 69846A

CGP 69846A was tritiated using the method described by Graham *et al.* [16] to produce 5′-TsCsCsCs[3H]GsCsCs Ts[3H]Gs[3H]G[3H]sAsCs[3H]AsTs[3H]GsCs[3H]AsTsT-3′. The reaction product was shown to have a radiochemical purity greater than 97% and a specific activity of 320 Ci/mol.

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[^{3}H]CGP 69846A was thermally stable below 95° in line with a specific trituration at the C8-position of purine bases.

Polyanions

Dextran sulphate (molecular weight = 8000 Da), chondroitin sulphate, fucoidan, polycytidylic acid and polyinosinic acid were purchased from Sigma Chemical Company.

Culture of J774 Cell Line

The J774 murine macrophage-like cell line [17] was obtained from ECACC. Cells were routinely cultured in vented 75 cm^2 flasks (Costar); they were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Culture-medium, comprising Dulbecco's Modified Eagle's Medium supplemented with 4.5 g/L of D-glucose, 4 mM L-glutamine, 1% nonessential amino acids, sodium pyruvate and 10% fetal bovine serum, was replaced daily. At 75% confluence, cells were passaged by trypsinization (0.25% trypsin, 0.02% EDTA in PBS (without calcium and magnesium) for 2 min at 37°) with a 1:5 split ratio. For uptake experiments, cells were trypsinized and seeded in 24-well plates at a viable cell density of 250,000 cells per well in 1 mL of culture-medium. After 24 h, the culture-medium was aspirated and the cells washed (1 × 1 mL × 1 min at 37°) with Hank's balanced salt solution buffered to pH 7.4 with *N*-[2-hydroxyethyl]-piperazine-*N*′-2-ethanesulphonic acid. The washed cells were incubated with 5 μM [^{3}H]CGP 69846A in Hank's balanced salt solution at 37° for 15 min. At the end of the incubation period the cells were washed (1 × 1 mL × 1 min with Hank's balanced salt solution at 37°) and the cells harvested by shaking with 0.1% Triton X-100 (aq). The [^{3}H]content of solubilized cells was determined by liquid scintillation counting (Beckman LS6500). Uptake experiments were performed for [^{3}H]CGP 69846A alone or in the presence of a 1:1, 1:10 or 1:100 mass ratio of dextran sulphate, chondroitin sulphate or fucoidan.

Animals

Male Wistar rats (240–270 g) and female Balb/c mice (20 g) were fed *ad lib*. with a standard laboratory diet (animals and husbandry supplies purchased from Bantin and Kingman) and kept under controlled conditions (12 hr light cycle; 20°).

Intravenous Administration Studies to Rats

Animals were immobilized through light sedation induced by a 40-μL i.m. injection of fentanyl in combination with fluanisone (0.315 mg/mL and 10 mg/mL, Hypnorm, Janssen Pharmaceuticals Ltd.). [^{3}H]CGP 69846A (0.6 mg/kg, 1.0 μCi in 200 μL of 0.9% saline) was administered i.v. by tail vein injection. For co-administration studies, the [^{3}H]CGP 69846A dose was formulated with a known concentration of competitor polyanion. Aliquots of blood (200 μL) were withdrawn from the contralateral tail vein at 5, 15, 30, 60, 120, 240 and 360 min after dosing. The rats were housed in metabolism cages (North Kent Plastics). Animals were killed by sodium pentobarbitone overdose (Expiral, Sanofi Animal Welfare) after 360 min. Tissues of interest (blood, urine, liver, kidney, spleen, heart, lung, skeletal muscle, skin, bone, fat and brain) were immediately collected and their [^{3}H]content determined.

Intravenous Administration Studies to Mice

[^{3}H]CGP 69846A (0.6 mg/kg, 0.25 μCi in 100 μL of 0.9% saline) was administered i.v. by tail vein injection. For co-administration studies, the [^{3}H]CGP 69846A dose was formulated with a known concentration of competitor polyanion. The mice were housed in plastic cages with free access to food and water. Animals were killed by sodium pentobarbitone overdose (Expiral, Sanofi Animal Welfare) after 10 min. Selected tissues (liver, spleen, kidney and skeletal muscle) were immediately collected and their [^{3}H]content determined.

Autoradiography on Mouse Kidney

[^{3}H]CGP 69846A (10 μCi, 6 mg/kg) was administered i.v. to female Balb/c mice (20 g). The animals were sacrificed by asphyxiation in carbon dioxide after 2 min or 60 min. Their kidneys were immediately removed, fixed for 24 hr in 10% neutral buffered formalin, embedded, sectioned at 3 μm, mounted on glass slides and then de-waxed. K5 nuclear emulsion was applied to each sample prior to exposure at 4° in light-free conditions. Autoradiographs were developed after 8 weeks exposure using D19 developer (4 min; Kodak) and fixed with 30% sodium thiosulphate (8 min). Samples were counter-stained with haemotoxylin and eosin using standard protocols.

[^{3}H]-Quantitation

The [^{3}H]content of blood, urine and preweighed tissue samples was determined using a tissue-oxidizer (Canberra-Packard 306) followed by liquid scintillation counting (Beckman LS6500). The oxidizer efficiency ranged from 90% to 96%. The [^{3}H]content of each sample was adjusted for total tissue weight and expressed as a percentage of the dose administered or as the equivalent concentration of CGP 69846A (μg/mL or μg/g). For calculations of tissue content, muscle, skin, bone and fat were assumed to be 41%, 16%, 10% and 10% of total body weight, respectively.

Statistical Analysis

Statistical significance was determined using a non-paired Student's *t*-test assuming equal variance.

TABLE 1. Dose-dependent effect of dextran sulphate on the tissue distribution of [^{3}H]CGP 69846A after 360 min in rats

Tissues	0 mg/kg (control group)	0.6 mg/kg	6 mg/kg	60 mg/kg
Blood	0.9 ± 0.4	0.9 ± 0.20	1.5 ± 0.2	1.6 ± 0.1
Urine	4.8 ± 1.5	6.2 ± 1.1	9.6 ± 0.5*	12.3 ± 1.5*
Liver	38.4 ± 0.6	42.6 ± 7.6	21.9 ± 1.9†	12.7 ± 1.1‡
Kidney	16.0 ± 0.3	11.1 ± 2.6	6.3 ± 0.5‡	4.1 ± 0.6‡
Spleen	1.4 ± 0.2	1.2 ± 0.4	1.2 ± 0.05	0.6 ± 0.1*
Muscle	5.5 ± 0.7	7.4 ± 0.6	11.6 ± 1.0†	14.5 ± 1.6†
Skin	4.6 ± 0.4	6.0 ± 0.5*	8.5 ± 0.8†	13.8 ± 1.2‡
Bone	2.3 ± 0.5	5.1 ± 0.7*	12.4 ± 5.9	6.7 ± 0.4†
Fat	3.9 ± 0.7	4.1 ± 1.0	19.6 ± 8.0	11.1 ± 1.3†
Heart	0.07 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Lung	0.16 ± 0.06	0.11 ± 0.02	0.26 ± 0.04	0.32 ± 0.04
Brain	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00

Data are presented as means ± SEM, N = 3–4.
*, † and ‡ represent statistical significance at 0.05, 0.01 and 0.001, respectively.

RESULTS

Effect of Dextran Sulphate on the Disposition of CGP 69846A in Rats

[^{3}H]CGP 69846A showed a characteristic pharmacokinetic and tissue distribution profile for a phosphorothioate oligodeoxynucleotide (Table 1). Following bolus i.v. administration it was rapidly cleared from the blood and distributed amongst high (e.g. kidney, liver, spleen), low (e.g. lung, skeletal muscle, skin) and negligible (e.g. brain, fat) accumulating tissues. Co-administration of dextran sulphate with CGP 69846A had little effect on its blood kinetics (Fig. 1) but a significant and dose-dependent effect on tissue distribution (Table 1). Increasing doses of dextran sulphate (oligonucleotide:polyanion mass ratios 1:0 [control], 1:1, 1:10 to 1:100) caused a progressive redistribution of [^{3}H]CGP 69846A from high affinity tissues to the well-perfused low-affinity tissues. For instance, the hepatic uptake of [^{3}H]CGP 69846A (0.6 mg/kg) was reduced from 38.4 ± 0.6% to 12.7 ± 1.1% at 360 min upon co-administered with 60 mg/kg of dextran sulphate. This was matched by an increased accumulation by skeletal muscle from 5.5 ± 0.7% to 14.5 ± 1.6%. Similarly, 60 mg/kg of dextran sulphate caused a 3.9-fold reduction in the renal accumulation of [^{3}H]CGP 69846A and increased its excretion in urine by 2.6-fold.

Autoradiographic Distribution of [^{3}H]CGP 69846A in Kidney

Temporal autoradiographic studies provided direct evidence for the glomerular filtration of [^{3}H]CGP 69846A (Fig. 2). After 2 min, radioactivity was associated with the glomerulus but concentrated in the Bowman's capsule and the lumen of proximal tubules. At a later time point, 60 min, radioactivity was only associated with the cells lining the proximal tubules. These observations are consistent with glomerular filtration followed by reabsorption into the proximal tubules.

Effect of Polyanions on the Uptake of CGP 69846A by J774 Cells

The cellular-association of [^{3}H]CGP 69846A by J774 cells was significantly reduced by dextran sulphate and fucoidan in a dose-dependent fashion (Fig. 3A, B). In contrast, chondroitin sulphate did not affect the cellular association over the same mass ratio range (Fig. 3C).

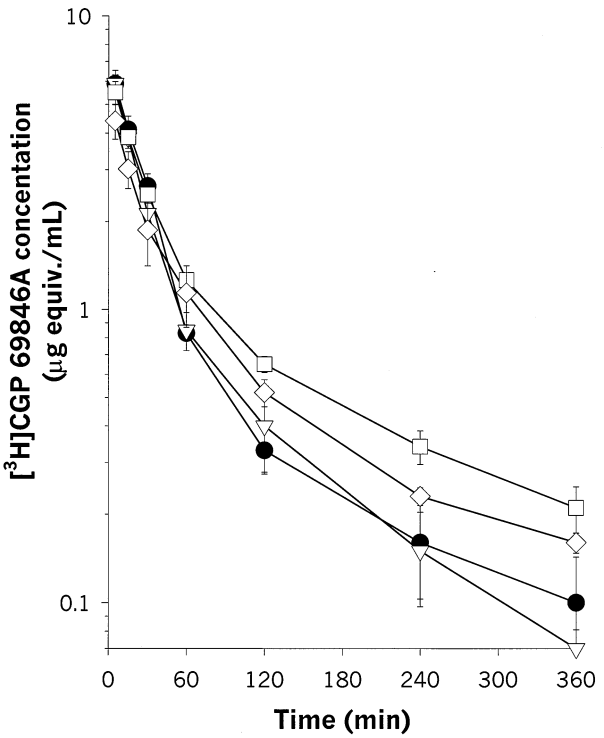

FIG. 1. Dose-dependent effect of dextran sulphate on the plasma kinetics of [^{3}H]CGP 69846A; 0.6 mg/kg of [^{3}H]CGP 69846A co-dosed with 0 mg/kg (closed circle = control), 0.6 mg/kg (open triangle), 6.0 mg/kg (open square) or 60.0 mg/kg (open diamond) dextran sulphate, respectively. Data are presented as mean ± SEM, N = 6.

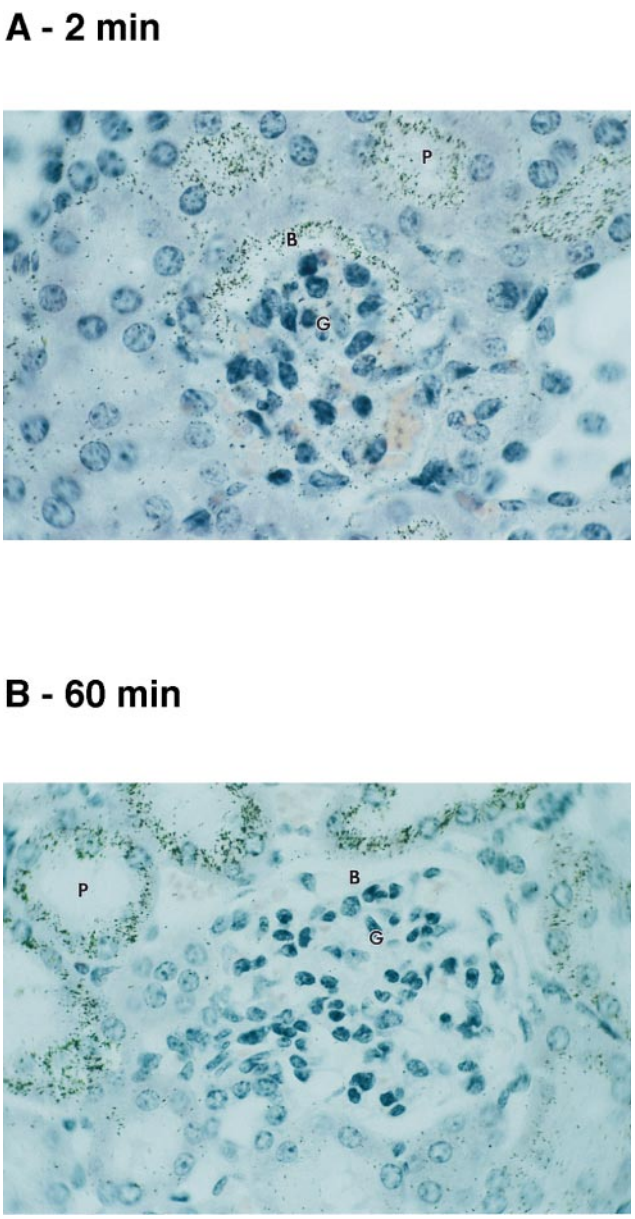

FIG. 2. Autoradiography of [^{3}H]CGP 69846A in mouse kidney. [^{3}H]CGP 69846A (10 μCi, 6 mg/kg) was administered i.v. to groups of four female Balb/c mice which were sacrificed at 2 min (A) and 60 min (B), processed for autoradiography and visualized using a Leitz Orthoplan microscope. Photographs are annotated as follows: G = glomerulus, B = Bowman's capsule, P = proximal tubule.

Effect of Polyanions on the Distribution of CGP 69846A in Mice

The pharmacokinetics and tissue distribution of 0.6 mg/kg of [^{3}H]CGP 69846A were qualitatively similar in mice and rats and was similarly affected by the co-administration of 60 mg/kg of dextran sulphate (data not shown). The effect of polyanions, having differential abilities to block the scavenger receptors, on [^{3}H]CGP 69846A accumulation by selected high- (liver, spleen, kidney; Fig. 4A-C) and low- (skeletal muscle; Fig. 4D) affinity tissues was examined. Known substrates for the scavenger receptor (dextran sulphate, fucoidan and polyinosinic acid) significantly reduced hepatic and splenic uptake and simultaneously increased the accumulation in skeletal muscle. In contrast, polyanions which are not substrates for scavenger receptors (chondroitin sulphate and polycytidylic acid) did not alter the distribution between these tissues. Although the kidney was a high-affinity tissue, there was no consistent pattern between renal accumulation and the nature of the co-administered polyanion.

DISCUSSION

The disposition, metabolism and elimination of phosphorothioate oligodeoxynucleotides *in vivo* have been reported previously [1–8]. Nevertheless, the mechanisms responsible for their distinctive pharmacokinetic profile are poorly understood. We have addressed this using a model 20-mer phosphorothioate oligodeoxynucleotide, CGP 69846A, complementary to the 3′-untranslated region of human c-*raf*-1 kinase. It specifically knocks-down c-*raf*-1 *in vitro* [18] and has potent *in vivo* antitumour activity in the human tumour xenograft nude mouse model through an antisense mechanism [18, 19]. It is currently undergoing clinical evaluation for the treatment of solid tumours. We have previously reported a dose-dependent tissue distribution and nonlinear pharmacokinetic profile for this compound in rats [8]. As the administered dose increased from 0.06 mg/kg to 60 mg/kg, the proportion of dose distributed to the high accumulating tissues (e.g. kidney, liver and spleen) decreased significantly. At the same time, plasma clearance was reduced and distribution to well perfused low accumulating (e.g. skeletal muscle) tissues increased. These data suggested a saturable uptake of CGP 69846A by high accumulating tissues. Similar dose-dependent disposition is observed in mice (Phillips, JA, unpublished observations). A saturable cellular uptake mechanism, rather than saturable binding to tissue matrix, is the most likely explanation since autoradiography studies in mice show the majority of [^{3}H]CGP 69846A-derived radioactivity in kidney and liver to be localized within cells. Indeed, uptake via receptor-mediated membrane transport could explain the ability of high accumulating tissues to concentrate CGP 69846A to levels many times higher than those present in blood. In addition to these specific mechanisms, the shuttling of phosphorothioate oligodeoxynucleotides between proteins, by ligand exchange along an affinity gradient, may account for the bulk distribution to peripheral tissues (Crooke ST, *et al.*, manuscript submitted).

In the present study, our understanding of the dose-dependent disposition for phosphorothioate oligodeoxynucleotides has been extended by showing that dextran sulphate, a sulphated polysaccharide, had dose-dependent effects on the tissue distribution of CGP 69846A. The dose-dependent reduction of CGP 69846A uptake by high accumulating tissues (kidney, liver and spleen) was associated with a redistribution to lower accumulating tissues such as skeletal muscle. The effects of co-administered

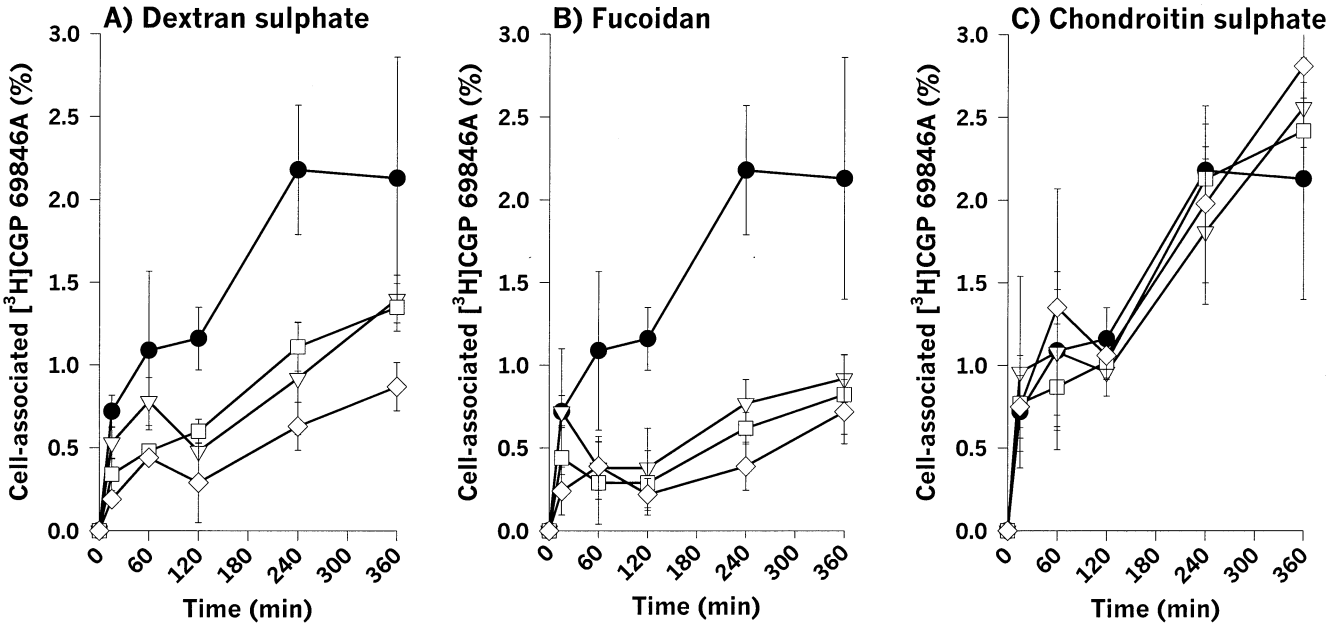

FIG. 3. Effect of polyanions on the association [^{3}H]CGP 69846A to J774 cells. Cellular association of [^{3}H]CGP 69846A to J774 cells in the absence (filled circles, control) or presence of a 1:1 (open triangle), 1:10 (open square) or 1:100 (open diamond) mass ratio of polyanion. Data are presented as mean ± SEM, N = 6.

dextran sulphate suggest that phosphorothioate oligodeoxynucleotides may be handled *in vivo* by mechanisms shared with other polyanions. Moreover, the dose-dependent reduction in renal accumulation was matched by an increase in urinary elimination. This inverse relationship between renal accumulation and urinary excretion of CGP 69846A upon dextran sulphate administration, suggests that both are filtered by the glomerulus and dextran sulphate is able to block the tubular reabsorption of CGP 69846A. This indirect evidence for filtration and reabsorption of CGP 69846A is supported by autoradiography studies in mice which show i.v. administered [^{3}H]CGP 69846A to be present in the Bowman's capsule and the lumen of the renal tubules at 2 min then localized within cells lining the proximal tubules at later time points.

Scavenger receptors [20] are involved in the cellular recognition and uptake of several polyanions such as acetylated low density lipoprotein, dextran sulphate, fucoidan, maleylated-albumins, polyinosinic acid, but not others such as acetylated albumin, chondroitin sulphate, methylated albumin, and polycytidylic acid [21]. Polynucleotide recognition was first described by Pearson *et al.* [22] who showed that binding to the type I bovine scavenger receptor was dependent upon the formation of base-quartet-stabilized four-stranded helices (G-tetrads). Although this rule can be used to explain why polyguanylic acid and polyinosinic acid, but not polycytidylic acid and polyadenylic acid, are ligands for the receptor, it cannot account for all subsequent findings. For instance, scavenger receptors are involved in the hepatic extraction of double-stranded plasmid DNA in mice and rats [23, 24] and renal accumulation of a partially phosphorothioated oligonucleotide [25]. In the latter case, however, the oligonucleotide (5′-AoAoGoCoToAoAoCoGoToToGoAoGoGoGoGsCsAsT-3′, where o = phosphodiester and s = phosphorothioate) contains a string of four contiguous guanines and G-tetrad formation cannot be ruled out. Thus, competition studies between CGP 69846A (which is not able to form G-tetrads) and a range of polyanions were used to determine if single-stranded phosphorothioate oligodeoxynucleotides are recognized by scavenger receptors. The uptake of CGP 69846A by the J774 murine macrophage-like cell line [17], known to express scavenger receptors [26], was significantly reduced by dextran sulphate and fucoidan but not chondroitin sulphate. This cross-inhibition profile was consistent with binding to scavenger receptors. Because CGP 69846A cannot form G-tetrads and does not have a complex secondary structure, this work demonstrates that a short single stranded phosphorothioate oligodeoxynucleotide can bind scavenger receptors *in vitro* and that receptor-blockade reduces its cellular association.

The role of scavenger receptors in *in vivo* disposition of CGP 69846A in mice was assessed by the effect of polyanions on the accumulation of CGP 69846A by selected tissues. For these studies, a 10 min end point was chosen to ensure minimal metabolism of the oligonucleotide and to reduce the theoretical complications arising from differential pharmacokinetics and metabolism of the co-administered polyanions. The inhibition of hepatic and splenic uptake by dextran sulphate, polyinosinic acid and fucoidan but not polycytidylic acid or chondroitin sulphate suggests class A type I/II scavenger receptor-mediated uptake by these tissues [27, 28, 29]. Indeed, the dextran sulphate reduced hepatic uptake by 70%, suggesting it to be the predominant uptake mechanism. This reduction in hepatic and splenic uptake by scavenger receptor ligands was

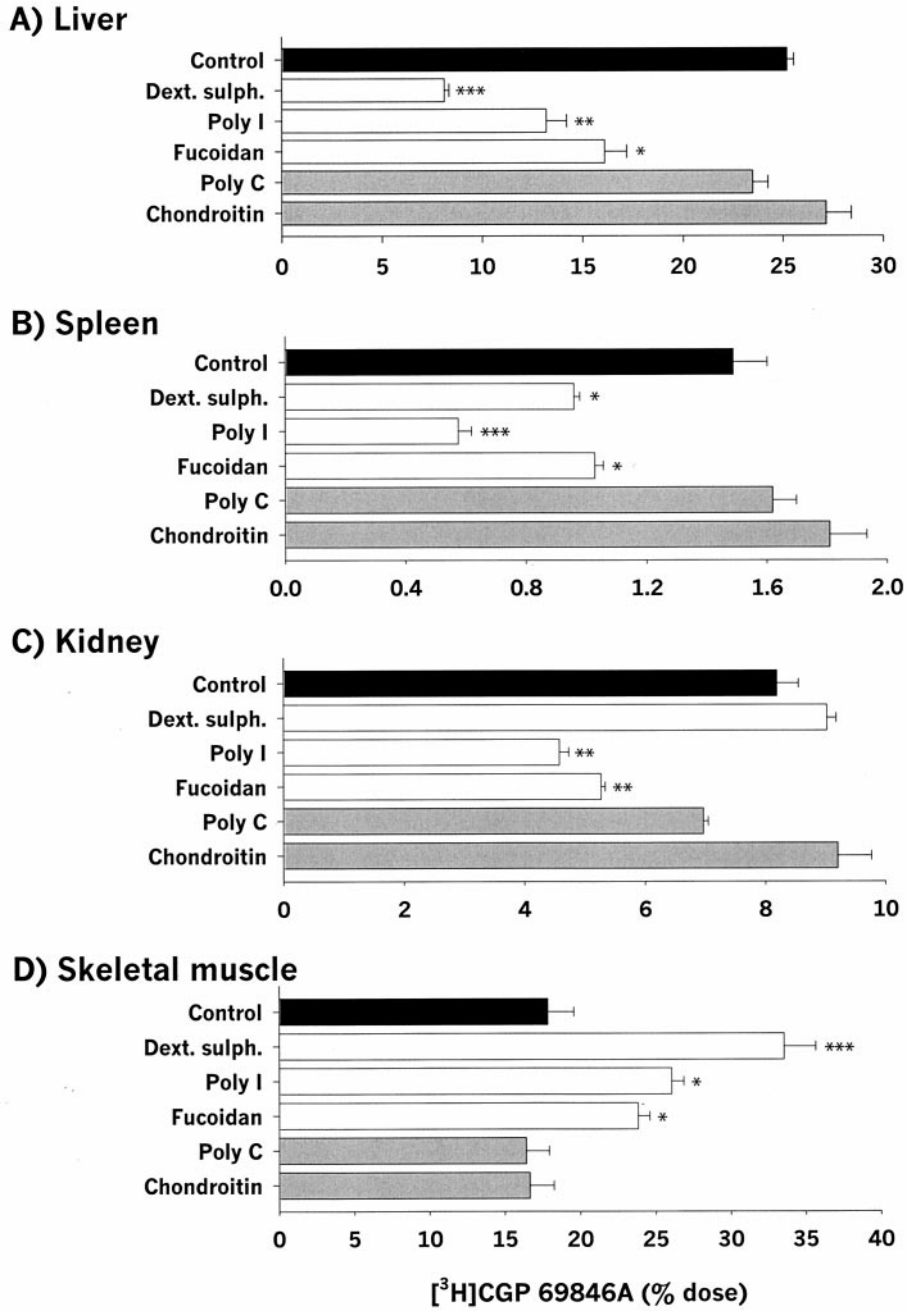

FIG. 4. Effect of polyanions on the accumulation of [^{3}H]CGP 69846A by liver (A), spleen (B), kidney (C) and skeletal muscle (D) after 10 min in mice. Black bar = control, grey bar = polyanions known not to bind scavenger receptors. Data are presented as mean ± SEM, N = 5–6; *, ** and * represent statistical significance at 0.05, 0.01 and 0.001, respectively.**

matched by a concomitant increase in the accumulation by skeletal muscle. Polyanions which are not recognized by the scavenger receptor had no effect on the uptake by skeletal muscle. Although scavenger receptor-mediated processes have been implicated in the renal uptake of oligonucleotides in isolated kidney perfusions [25], this was not confirmed in our cross-inhibition studies. This apparent discrepancy probably results from the 10-min end point, at which time renal accumulation represents a composite value of the radioactivity in tissue and that in the renal filtrate. Urinary excretion would therefore effectively mask the scavenger receptor-mediated tissue uptake and prevent meaningful data interpretation. Similar cross-inhibition experiments performed at time points after the distribution-phase may show scavenger receptor involvement in renal uptake. In conclusion, CGP 69846A was recognized by scavenger receptors in certain tissues *in vivo* and receptor-blockade caused a redistribution from high accumulating organs to lower accumulating peripheral tissues.

The involvement of scavengers receptors, particularly on endothelial cells in the liver, in the *in vivo* fate of another phosphorothioate oligonucleotide has recently been reported [30]. Their observations confirm our data and suggest this mechanism to be generic, rather than sequence specific, for single stranded phosphorothioate oligodeoxynucleotides.

The present study has shown that: 1) dextran sulphate co-administration has a dose-dependent effect on the dis-

position of CGP 69846A; 2) CGP 69846A undergoes renal filtration and renal accumulation largely results from tubular reabsorption; 3) CGP 69846A was recognized by scavenger receptors *in vitro* and *in vivo*; and 4) the scavenger receptor was an important determinant for the *in vivo* disposition of CGP 69846A in mice. It also raises some interesting questions. Firstly, does the intracellular fate of oligonucleotides differ between cells bearing scavenger receptors and those without? Moreover, does this result in an altered sensitivity toward antisense phosphorothioate oligodeoxynucleotides? Secondly, how does the co-administration of scavenger receptor ligands influence the activity oligonucleotides in efficacy models? Thirdly, what would be the pharmacokinetic profile of oligonucleotides in class A type I/II knockout mice? Finally, do other chemically modified oligonucleotides differentially bind scavenger receptors and does this influence their tissue distribution (e.g. cholesterol-conjugated phosphorothioate oligodeoxy-nucleotides and phosphodiester, 2′-methoxyethoxy modified compounds which show very high and low hepatic uptake, respectively)? These questions are currently being explored.

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