Pharmaceutical Research, Vol. 15, No. 4, 1998

Pulmonary Bioavailability of a Phosphorothioate Oligonucleotide (CGP 64128A): Comparison with Other Delivery Routes

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Received September 16, 1997; accepted November 20, 1997

Purpose. Phosphorothioate antisense oligodeoxynucleotides are promising therapeutic candidates. When given systemically in clinical trials they are administered via slow intravenous infusion to avoid their putative plasma concentration-dependent haemodynamic side-effects. In this study, we have evaluated alternative parenteral and non-parenteral administration routes which have the potential to enhance the therapeutic and commercial potential of these agents.
Methods. The delivery of CGP 64128A by intravenous, subcutaneous, intra-peritoneal, oral and intra-tracheal (pulmonary) routes was investigated in rats using radiolabelled compound and supported by more specific capillary gel electrophoretic analyses.
Results. Intravenously administered CGP 64128A exhibited the rapid blood clearance and distinctive tissue distribution which are typical for phosphorothioate oligodeoxynucleotides. Subcutaneous and intra-peritoneal administration resulted in significant bioavailabilities (30.9% and 28.1% over 360 min, respectively) and reduced peak plasma levels when compared with intravenous dosing. Administration via the gastro-intestinal tract gave negligible bioavailability (<2%). Intra-tracheal administration resulted in significant but dose-dependent bioavailabilities of 3.2, 16.5 and 39.8% at 0.06, 0.6 and 6.0 mg/kg, respectively.
Conclusions. Significant bioavailabilities of CGP 64128A were achieved following subcutaneous, intra-peritoneal and intra-tracheal administration. Pulmonary delivery represents a promising mode of non-parenteral dosing for antisense oligonucleotides. The dose-dependent increase in pulmonary bioavailability suggests that low doses may be retained in the lungs for local effects whereas higher doses may be suitable for the treatment of a broader spectrum of systemic diseases.

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ABBREVIATIONS: AUC, area under the curve; Cl, clearance; C_{max}, maximum concentration; Da, Dalton; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; μg equiv/g, microgram equivalents per gram of tissue; μg equiv/ml, microgram equivalents per millilitre of blood; mRNA, messenger ribonucleic acid; N, parent oligonucleotide peak in capillary gel electrophoresis; N_{-n}, peaks migrating more rapidly than N in capillary gel electrophoresis; N_{+x}, peaks migrating more slowly than N in capillary gel electrophoresis; RP-SPE, reverse-phase solid-phase extraction; SAX-SPE, strong anion-exchange solid-phase extraction; $t_{1/2\alpha}$, half-life for distribution; $t_{1/2\beta}$, half-life for elimination; T_{max}, time after dosing to maximum concentration; Tris, tris(hydroxymethyl)methylamine and V_d = volume of distribution.

KEY WORDS: administration; antisense; bioavailability; gastrointestinal; intra-peritoneal; intra-tracheal; ISIS 3521; oligonucleotide; oral; pharmacokinetics; subcutaneous.

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. CGP 64128A, a 20-mer phosphorothioate oligonucleotide complementary to the 3′-untranslated region of human protein kinase C-alpha (PKC-α), can specifically knock-down PKC-α and exhibits potent anti-tumour activity in the human tumour xenograft model through an antisense mechanism (6). It has pharmacokinetic properties and a metabolism profile in rodents which are typical for a phosphorothioate oligonucleotide (8). It is rapidly cleared from the blood and has a distinctive distribution amongst high (*e.g.* kidney, liver, spleen), low (*e.g.* lung, skeletal muscle, skin) and negligible (*e.g.* brain) accumulating tissues from which it is slowly eliminated.

Phosphorothioate oligonucleotides are currently undergoing clinical trials. For systemic conditions they are administered by controlled intravenous infusion in order to maintain plasma concentrations below threshold levels above which haemodynamic side-effects have been observed in preclinical studies in monkeys (9,10). This mode of administration is not ideal and the identification of alternative dose routes is a key issue if phosphorothioates are to realise their full therapeutic and commercial potential. Parenteral dosing is invasive, is associated with pain and usually requires the involvement of a healthcare professional. In turn, this may result in poor patient compliance and high treatment costs for chronic therapy. Reliance on parenteral administration could, therefore, restrict the use of oligonucleotides to highly motivated patients or niche diseases which cannot be treated by conventional therapy. A non-parenteral administration route, which is effective and acceptable to patients, would greatly enhance their therapeutic scope. Oral administration is the most familiar route for patients, however, the gastrointestinal tract has a hostile luminal environment and a competent epithelial barrier which together efficiently retard the absorption of macromolecules. The pulmonary tract offers a less hostile environment and an epithelium with a much greater absorptive capacity for macromolecules (11,12).

In this paper, the pharmacokinetics and bioavailability of CGP 64128A following administration by parenteral (intravenous, i.v.; intra-peritoneal, i.p.; subcutaneous, s.c.) and non-parenteral (gastrointestinal, pulmonary) routes are compared. The advantages, opportunities and limitations for each route are discussed.

MATERIALS AND METHODS

Chemicals

CGP 64128A (GsTsTsCsTsCsGsCsTsGsGsTsGsAsGsTsTsTsCsA, where s = phosphorothioate; Lot number NGMP-0723-3521; otherwise known as ISIS 3521), was provided by Dr. Nicholas Dean of ISIS Pharmaceuticals (Carlsbad, CA) and its identity was confirmed by electrospray mass spectrometry (calculated M_r = 6430.59 Da and measured M_r = 6430.73 Da).

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Poly-T_{27} phosphorothioate oligonucleotide, used as an internal standard for capillary gel electrophoretic analysis, was also provided by ISIS Pharmaceuticals. Hybrid-BDBB-α-interferon was provided by Novartis, Basel, Switzerland.

[^{3}H]Labeling of CGP 64128A

CGP69846A was tritiated using the method described by Graham *et al.* (13) to produce 5′-$^{[3H]}$GsTsTsCsTsCs$^{[3H]}$GsCsTs$^{[3H]}$Gs $^{[3H]}$GsTs$^{[3H]}$Gs$^{[3H]}$As$^{[3H]}$GsTsTsTsC$^{[3H]}$A-3′. The reaction product was shown to have a radiochemical purity greater than 98% by strong anion-exchange high-performance liquid chromatography and a specific activity of 320 Ci/mol. 10 μCi aliquots were stored at −70°C. [^{3}H]CGP 64128A was thermally stable below 95°C in line with a specific tritiation at the C8-position of purine bases (data not shown).

CGP 64128A Administration to Rats

Male Wistar rats (240–270 g) were fed *ad libitum* with a standard laboratory diet (animals and husbandry supplies purchased from Bantin and Kingman, Hull, UK) and kept under controlled conditions (12 h light cycle; 20°C) prior to experimentation.

Anesthesia

A light anesthesia was induced by 20 μl intra-muscular injection of fentanyl (Hypnorn; Janssen Pharmaceuticals Ltd., Oxford, UK) to immobilise the animals. This was followed by an i.v. injection of sodium pentabarbitone (3 mg/100 g; May and Baker Ltd., Dagenham, UK) diluted two-fold with 150 mM NaCl (saline). The anesthesia was maintained for a 360 min period by additional i.v. injection of sodium pentabarbitone as required.

Administration of Dose

CGP 64128A in saline, containing 1.0 μCi of [^{3}H]CGP 64128A per animal, was administered to rats by a variety of routes (see below) at a dose of 0.06, 0.6 or 6.0 mg/kg. The rats were maintained horizontal on a heated stage throughout the sampling period. *Intravenous*—CGP 64128A (0.6 mg/kg) was administered in 100 μl of saline by tail vein injection to anesthetised rats. *Subcutaneous*—CGP 64128A (0.6 mg/kg) was administered in 10 μl of saline by s.c. injection to the left of the flank of anesthetised rats. *Intraperitoneal*—CGP 64128A (0.6 mg/kg) was administered in 100 μl of saline by direct injection through the abdominal wall to anesthetised rats. *Gastrointestinal* (GI)—CGP 64128A (6.0 mg/kg) was administered in 100 μl of saline by gavage, intra-duodenal (i.d.) or intra-ileal (i.i.) injection. Gavage dosing introduced the dose directly into the stomach of non-anesthetised rats. Intra-duodenal and intra-ileal administration was performed after laporotomy to allow access to the GI tract. The dose was introduced into the lumen of the proximal side of the intestinal region. *Intra-tracheal* (i.t.)—Intra-tracheal administration was used as a model of pulmonary delivery. The trachea was exposed during the cannulation of the carotid artery. The rat's head was inclined slightly (30°) in order to minimise nasal drainage of the dose. CGP 64128A (0.06, 0.6 or 6.0 mg/kg) in 10 μl of saline was injected directly into the trachea between two cartilage rings just below the thyroid gland. The dosing was performed by forceful expulsion on an intake of breath using a 25G needle and 100 μl Hamilton syringe. Using this method, the administration of Evans Blue dye stained the mid/deep regions of the lungs suggesting effective pulmonary delivery (data not shown).

Cannulation, Blood Sampling and Organ Collection

At defined time intervals between 0 and 360 min, 200 μl aliquots of blood were taken from a cannulated carotid artery. At each time point the volume of blood withdrawn from the animal was replaced with an equal volume of heparinised saline (10 IU in 200 μl saline). After the final time point, the animal was sacrificed by a lethal i.p. injection of sodium pentabarbitone (Sagatal, Rhône Mérieux). Organs of interest were immediately excised then representative portions collected and weighed. Total urine was collected directly from the bladder.

Sample Analysis

[^{3}H]-quantification

The [^{3}H]-content of blood, urine and pre-weighed tissue samples was determined using a tissue-oxidiser (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 equiv/ml or μg equiv/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.

Extraction and Analysis of Plasma and Tissue Samples

Plasma samples were extracted as described by Leeds *et al.* (14). Tissue samples (150 mg) were homogenized in Tris buffer pH8.0 (20 mM Tris-HCl, 20 mM EDTA, 0.1 M NaCl, 0.5% NP 40) containing 200 nM of poly-T_{27} as an internal standard. The homogenate was incubated overnight at 37°C together with proteinase K (final concentration 1.5 mg/ml; Boehringer, UK) and extracted twice with phenol/chloroform (volume ratio 2:1). The aqueous phases were concentrated using a vacuum drier (MAXI dry plus, Heto, Denmark), treated overnight with 500 μl of 30% ammonium hydroxide at 55°C and concentrated once again. The samples were loaded onto a pre-equilibrated strong anion-exchange solid-phase extraction cartridge (SAX-SPE; Accubond 100 mg, J+W Scientific, USA) in loading-buffer (10 mM Tris-HCl, pH 9.0, 0.5 M KCl, 20% acetonitrile), washed with loading-buffer and eluted with elution-buffer (10 mM Tris-HCl, pH 9.0, 0.5 M KCl, 1.0 M NaBr, 30% acetonitrile). The SAX-SPE eluate was loaded onto a pre-equilibrated RP-SPE cartridge (C_{18} end-capped Isolute 100 mg; International Sorbent Technology, USA), washed with Nanopure water and eluted with 20% aqueous acetonitrile. The RP-SPE eluate was concentrated using a vacuum drier and reconstituted to 20 μl with Nanopure water. The sample was further desalted by placing it on a 0.025 μm membrane filter (Type VS, Millipore, UK) floating on Nanopure water for 60 min. Desalted plasma and tissue samples were analysed by capillary gel electrophoresis (Beckman P/ACE 5010, Beckman, UK) using a Beckman DNA capillary (Cat. No. 477477), 20

cm effective length containing a dextran polymer solution (40% $dextran_{500,000}$, 100 mM Bis-Tris borate, 8.3 M urea) with 100 mM Bis-Tris Borate running buffer. Separations were performed at 40°C with an applied voltage of 30 kV and UV_{260nm} detection. These conditions provided baseline resolution of a phosphorothioate T_{7-25} ladder and CGP 64128A from its N_{-n} metabolites (data not shown). A 1:1 ratio for the extraction and analysis of CGP 64128A and a T_{27} phosphorothioate oligodeoxynucleotide was achieved and concentrations were calculated by comparison with T_{27} as an internal standard.

Integrity of the Pulmonary Tract

Histology of Trachea and Lung

Male Wistar rats (four per group) were dosed with (i) i.t. saline vehicle, (ii) i.t. CGP 64128A at 6.0 mg/kg, or (iii) i.v. CGP 64128A at 6.0 mg/kg. The animals were sacrificed by a lethal i.p. injection of sodium pentabarbitone and exsanguinated by cardiac puncture. Respiratory tract tissues were immediately excised. Tracheae were removed and fixed in 10% neutral buffered formalin (NBF) for 48 h. The lungs were gently inflated with 10% NBF via the residual trachea and then immersed in 10% NBF fixative for 48 h. Tissues were processed, sectioned and stained with haematoxylin and eosin according to accepted standard protocols. Stained sections were examined for evidence of local toxicity (minor pathological changes) by an independent pathologist using light microscopy.

Permeability Marker Studies

The influence of CGP 64128A on the functional integrity of the pulmonary tract was assessed using a permeability marker. In separate experiments, rats were dosed intra-tracheally with hybrid-BDBB-α-interferon (1.3×10^7 IU/kg) and simultaneously with (i) i.t. saline vehicle, (ii) i.t. CGP 64128A at 6.0 mg/kg or (iii) i.v. CGP 64128A at 6.0 mg/kg. Plasma levels of intact hybrid-BDBB-α-interferon were assayed using a dual monoclonal antibody 'sandwich' solid-phase enzyme linked immuno assay (ANAWA, Zurich, Switzerland).

Calculation of Bioavailability

Bioavailability (BAV) values were calculated by comparing the area under the curve over 360 min ($AUC_{0-360\ min}$) compared to that for an i.v. dose of 0.6 mg/kg using Equation 1. Calculated values were confirmed by quantitation of the radioactivity in the tissues excluding the administration site.

$$\%\ BAV = (AUC_{dose\ route}/AUC_{i.v.})*(Dose_{i.v.}/Dose_{dose\ route})*100 \quad (1)$$

RESULTS

Tritium did not back-exchange from [^{3}H]CGP 64128A at temperatures less than 95°C and the radiolabel was considered to be physically stable under *in vivo* experimental conditions. Samples were analysed by complementary techniques; tritium quantitation which is rapid and convenient and capillary gel electrophoresis which provides additional information about the metabolic status of the oligonucleotide. In agreement with previous studies (15,16), a good correlation between the oligonucleotide concentration determined by tritium quantitation and capillary gel electrophoretic analysis was observed.

Parenteral Administration

The blood kinetics and tissue concentrations of radiolabel following [^{3}H]CGP 64128A administration were dose route-dependent. Three parenteral routes were investigated—i.v., s.c. and i.p.. Intravenous administration resulted in a bi-phasic blood profile (Fig. 1A) comprising a rapid distribution phase (0–240 min; $t_{1/2\alpha}$ = 19.6 min), followed by a prolonged elimination phase (240 min onwards; $t_{1/2\beta}$ = 693 min) in which circulating levels of radiolabel were less than 1.0% of the administered dose. Total blood clearance (Cl) was 1.57 ml/min/kg with a volume of distribution (V_d) of 391 ml (or 1564 ml/kg, approximately 2-fold greater than the total body water volume of the rat). Although CGP 64128A was metabolised during the initial phase, its distribution to tissues was the primary mechanism for clearance from the vascular compartment. The radiolabel was distributed amongst high (*e.g.* kidney, liver and to a lesser extent spleen), low (*e.g.* lung, skeletal muscle, skin) and negligible (*e.g.* brain, eye) accumulating tissues (Fig. 1B). Intact CGP 64128A was detected in plasma after extraction and capillary gel electrophoretic analysis at 10 and 120, but not 360 or 1440 min and represented 51.8% and 35.4% of the total oligonucleotide pool at 10 and 120 min, respectively (Fig. 2A–D). CGP 64128A was detected in liver and kidney extracts at all time

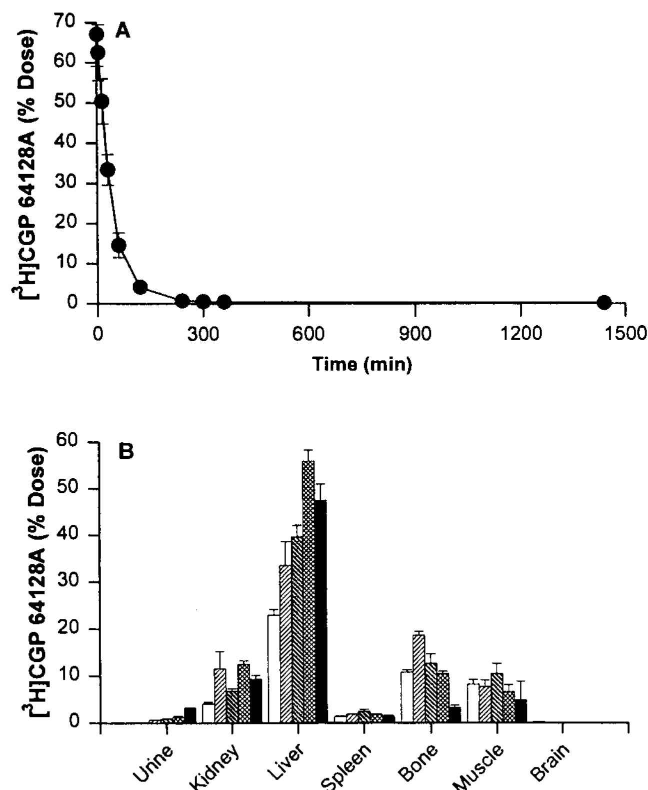

Fig. 1. Intravenous administration of [^{3}H]CGP 64128A (0.6 mg/kg). (**A**) Blood kinetics of [^{3}H]CGP 64128A-derived radioactivity and (**B**) its tissue distribution at 5 (white), 60 (white hatch), 120 (grey hatch), 360 (cross hatch) and 1440 (black) min. Data are presented as mean±sem (n = 6).

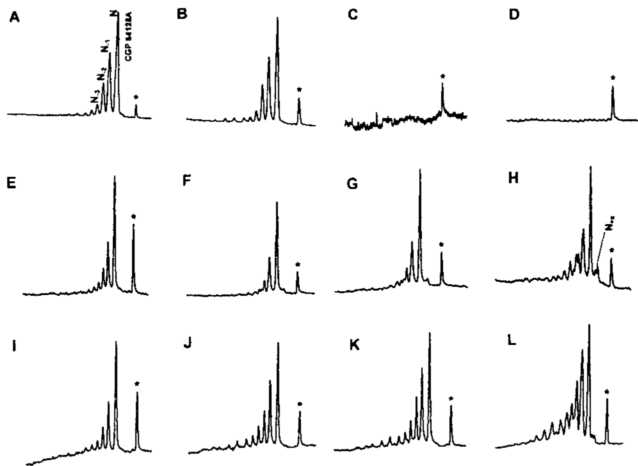

Fig. 2. Capillary gel electrophoresis of plasma, kidney and liver extracts following intravenous administration of CGP 64128A (6.0 mg/kg) to rats. Plasma: A = 10 min, B = 120 min, C = 360 min and D = 1440 min; Liver: E = 10 min, F = 120 min, G = 360 min and H = 1440 min; Kidney: I = 10 min, J = 120 min, K = 360 min and L = 1440 min. N_{+1} and N_{+2} represent slower-migrating metabolites and * denotes T_{27} internal standard.

points (Fig. 2E–H and I–L, respectively). In the kidney, intact CGP 64128A represented 51.8%, 35.4%, 27.7%, and 35.7% at 10, 120, 360 and 1440 min, respectively. Intact compound in the liver represented at 54.5%, 48.7%, 49.0% and 29.6% at the same time points. In addition to the parent molecule (N), chain-shortened metabolites (N_{-n}) and peaks which migrated more slowly than CGP 64128A (N_{+x}) were also observed (Fig. 2H and L).

Subcutaneous dosing (Fig. 3) resulted in a rapid rise in blood levels, achieving a peak concentration 30 min after administration, 12-fold lower than the i.v. C_{max}, before declining rapidly. The bioavailability compared to i.v. administration was 30.9% (Table 1). Binding of [^{3}H]CGP64128A to tissues at the site of administration is the most likely explanation for the incomplete s.c. bioavailability. After i.p. dosing of [^{3}H]CGP 64128A (Fig. 3), a 15 min lag-time was observed prior to its appearance in the systemic circulation. Blood levels gradually increased, achieving their maximum concentration between 60 and 180 min. Blood levels decreased at a slower rate than for either i.v. or s.c. administration. At sampling times greater than 240 min, blood levels remained at least two-fold higher than after i.v. or s.c. administration. Bioavailability from the i.p. route was 28.1% by comparison to i.v. administration (Table 1). Interestingly, the bioavailability based on the percentage of dose associated with tissues at 360 min was 59.4%. The reason for this discrepancy is unclear, however, it may indicate that intra-peritoneally administered CGP 64128A can access tissues without entering the systemic circulation.

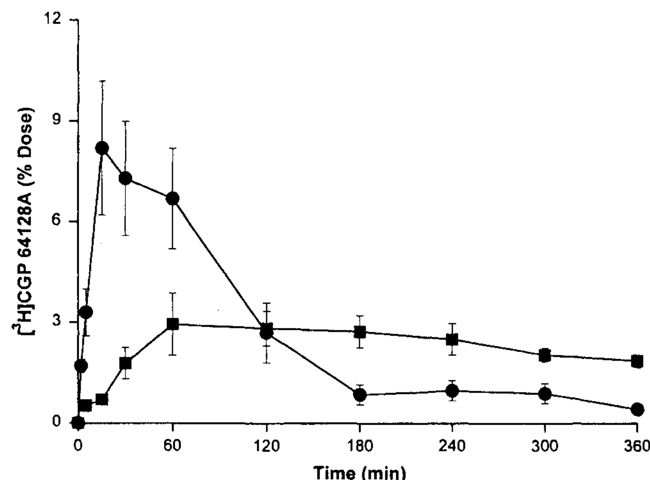

Fig. 3. Subcutaneous and intra-peritoneal administration of [^{3}H]CGP 64128A (0.6 mg/kg). Blood kinetics of [^{3}H]CGP 64128A-derived radioactivity following subcutaneous (●) or intra-peritoneal (■) administration. Data are presented as mean ± sem (n = 6).

Table 1. Comparative Bioavailabilities for Parenteral and Non-parenteral Routes Measured by Comparison with Intravenous Blood Levels or [^{3}H]-content of Tissues

Administration route	Dose [mg/kg]	Bioavailability by comparison to intravenous over 360 min [%]	Bioavailability based on tissue accumulation at 360 min [%]
Parenteral			
Intravenous	0.6	100.0	95.9
Subcutaneous	0.6	30.9	24.9
Intra-peritoneal	0.6	28.1	59.4
Non-parenteral			
Oral gavage	6.0	0.3	0.1
Intra-duodenal	6.0	0.7	0.9
Intra-ileal	6.0	0.1	2.4
Intra-tracheal	0.06	3.2	2.7
	0.6	16.5	21.1
	6.0	39.8	23.0

Note: Data are presented as mean ± sem (n = 6).

Non-parenteral Administration

Absorption from two non-parenteral administration routes—gastrointestinal and pulmonary—was also investigated. [^{3}H]CGP 64128A was administered to the GI tract by gavage or direct injection into duodenum and ileum. The bioavailability from the gastrointestinal tract, by comparison with i.v. administration, was less than 1% in all cases. Based on blood levels (Fig. 4A), direct intra-duodenal administration resulted in the highest bioavailability of 0.7%, whereas dosing by gavage resulted in only 0.3% being absorbed. The low bioavailability from the gastrointestinal tract by comparison with i.v. dosing was confirmed by radiochemical determinations in tissues after 360 min (duodenal = 0.9%, ileal = 2.4%; gavage = 0.1%) with the small quantity of radiolabel which was absorbed being principally distributed to the liver and kidney. Metabolism of CGP 64128A in the GI tract and visualisation of CGP64128A-derived oligonucleotides in tissues was assessed by capillary gel electrophoresis after its administration to an isolated duodenal segment. High concentrations of oligonucleotides remained in the luminal contents and the duodenal wall after 360 min (Fig. 4C). CGP 64128A was appreciably degraded in the GI tract; chain-shortened metabolites down to N_{-6} were detected with the parent compound representing 50.4 ± 8.0% and 48.8 ±

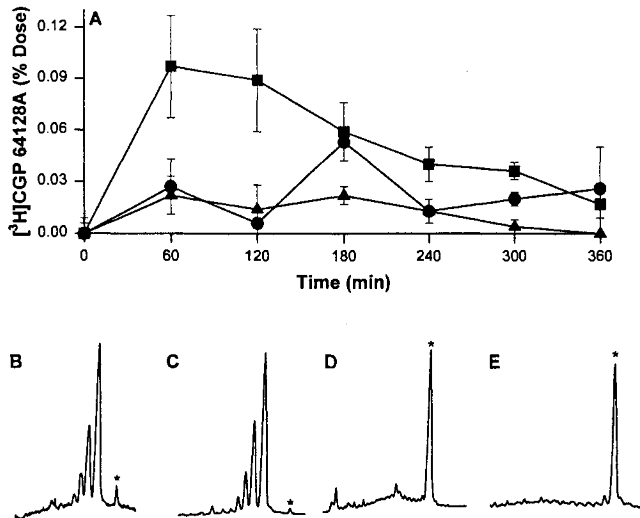

Fig. 4. Gastrointestinal absorption of [^{3}H]CGP 64128A (6.0 mg/kg). **A** Blood kinetics of [^{3}H]CGP 64128A-derived radioactivity following oral gavage (●), intra-duodenal (■) or intra-ileal (▲) administration. Data are presented as mean ± sem (n = 6). **B–E** represent capillary gel electrophoretic analyses of extracts from luminal contents (**B**), mucosal wall (**C**), liver (**D**) and kidney (**E**), * denotes T_{27} internal standard.

2.9% of the total oligonucleotide pool in the duodenal lumen and mucosa, respectively. Trace concentrations of oligonucleotides—possibly parent compound and high-order metabolites—were observed in the liver but not the kidney (Fig. 4D and E).

Intra-tracheal administration was used as a model for pulmonary delivery. The systemic bioavailability of [^{3}H]CGP 64128A following i.t. administration was significant and dose-dependent (Fig. 5A); 3.2, 16.5 and 39.8% at 0.06, 0.6 and 6.0 mg/kg, respectively by comparison with an i.v. dose at 0.6 mg/kg (Table 1). These values represent an under-estimation since absorption did not reach completion after 360 min. After the 6.0 mg/kg intra-tracheal dose of [^{3}H]CGP 64128A, there was a lag-time of approximately 60 min before radiolabel appeared in the systemic circulation. It achieved maximal concentration between 180 and 240 min then decreased at a slower rate than for i.v. administration. Pulmonary absorption was incomplete and the percentage radiolabel remaining in the lung tissue at 360 min was inversely related to the administered dose. Moreover, CGP 64128A was only slowly metabolised in the pulmonary tract (Fig. 5B); 95% of a 6.0 mg/kg dose extracted from lungs at the end of the experiment was intact parent compound. The relative distribution of radiolabel amongst tissues was comparable to that after i.v. administration. Furthermore, kidney and liver extracts showed significant levels of intact CGP 64128A and a typical spectrum of metabolites (principally chain-shortened metabolites (N_{-n}) with some evidence of chain-extended (N_{+n}) species; Fig 5C and D).

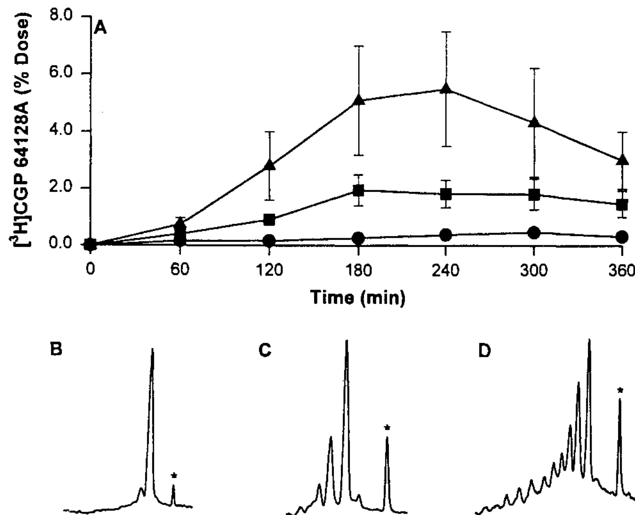

Fig. 5. Pulmonary absorption of [^{3}H]CGP 64128A. **A** Blood kinetics of [^{3}H]CGP 64128A-derived radioactivity following intra-tracheal administration at 0.06 (●), 0.6 (■) or 6.0 mg/kg (▼) administration. Data are presented as mean ± sem (= 6). **B–D** represent capillary gel electrophoretic analyses of extracts from lungs (**B**), liver (**C**) and kidney (**D**) after a 6 mg/kg dose, * denotes T_{27} internal standard.

The effects of CGP 64128A on the morphological and functional integrity of the respiratory tract were assessed at the highest dose. Tracheal and lung sections from animals dosed intra-tracheally with saline showed normal morphology indicating that the intra-tracheal dosing procedure did not cause any physical disruption. Moreover, no pathological changes were observed in the trachea and lung in response to either tracheal or i.v. administration of CGP 64128A over the time course of these experiments (Fig. 6A–F). Similarly, CGP 64128A had no effect on the functional integrity of the respiratory tract since the pulmonary absorption of a permeability marker was not increased by the simultaneous i.v. or intra-tracheal administration of CGP 64128A (Fig. 7).

Over the time-course of these experiments, and even after dosing to the gastrointestinal tract, the dose was not excreted in faeces. Urinary elimination was less than 2% in all cases.

DISCUSSION

There is compelling evidence that intravenously administered phosphorothioate antisense oligodeoxynucleotides have antisense effects in human tumour xenograft murine models (5,6,17). Evidently, these compounds are able to overcome a range of biological hurdles which are presented *in vivo* — they resist nuclease degradation, have appropriate pharmacokinetics, localise to target tissues, penetrate cells and are available in sufficient quantities to hybridise with target mRNA and cause protein-specific knock-down. From a clinical perspective, the pharmacokinetic hurdles relating to the administration and absorption of antisense oligonucleotides are amongst the most important and yet have received very little attention to date.

The rate of dose input is a key factor determining the acute tolerance of phosphorothioate oligodeoxynucleotides. Preclinical studies in monkeys have shown that they can elicit haemodynamic (anticoagulation, complement activation) and cardiovascular (hypotension) side-effects once plasma levels exceed a 'threshold' concentration (9,10). Extrapolation of clinically relevant doses to initial plasma concentrations suggest that bolus i.v. administration of phosphorothioate oligodeoxynucleotides could precipitate haemodynamic side-effects in human subjects. This is managed in ongoing clinical programmes by local administration or slow i.v. infusions. Local administration has great potential for dose-retention and there-

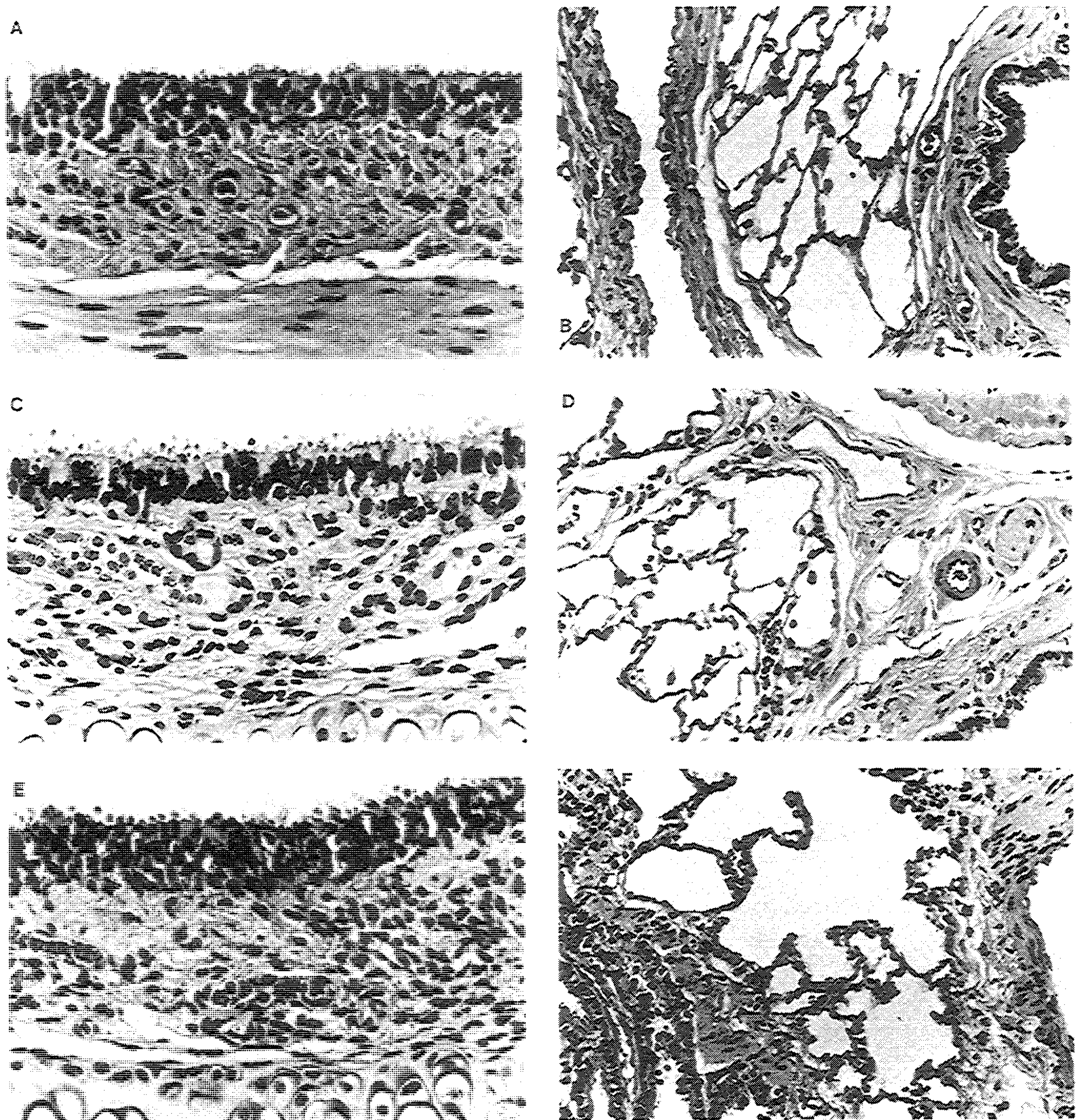

Fig. 6. Effect of CGP 64128A on the morphological appearance of the pulmonary tract. Haemotoxylin and eosin staining of tracheal and lung sections following the administration of saline intra-tracheally (A and B), 6.0 mg/kg CGP 64128A intravenously (C and D) or 6.0 mg/kg CGP 64128A intra-tracheally (E and F). Representative pictures from n = 4 are shown.

fore reduction of systemic side-effects by minimising circulating plasma levels and distribution to non-target tissues. It is, however, only applicable to a limited number of diseases (*e.g.* intra-ocular administration of antisense oligonucleotides directed against CMV-induced retinitis). Slow i.v. infusion results in low but sustained levels; whilst this circumvents the acute haemodynamic side-effects, additional inconvenience to patients and healthcare professionals is incurred. Alternative delivery strategies which (i) reduce haemodynamic and cardiovascular side-effects, (ii) do not incur dose route-specific toxicologies, (iii) are more clinically acceptable and (iv) are broadly applicable, could greatly enhance the therapeutic and commercial potential for this class of compounds. In the present study, we have compared the blood kinetics and tissue distribution of [^{3}H]CGP 64128A following i.v., i.p., s.c., oral and intratracheal administration.

Intravenously administered CGP 64128A showed typical blood kinetics and tissue distribution for a phosphorothioate

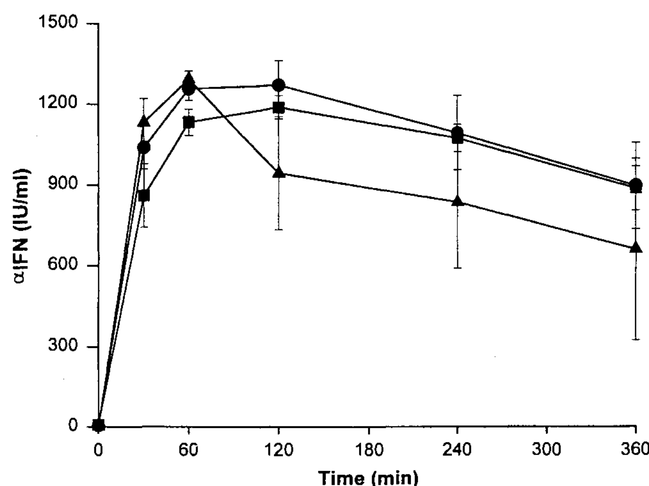

Fig. 7. Effect of CGP 64128A on the pulmonary absorption of permeability marker compounds. Blood levels of marker compound hybrid (BDBB)α-interferon following intra-tracheal dosing and simultaneous administration with saline (●), 6.0 mg/kg CGP 64128A intravenously (▲) or 6.0 mg/kg CGP 64128A intra-tracheally (■). Data are presented as mean ± sem (n = 6).

oligodeoxynucleotide (15,16,18–25). The blood kinetics of were bi-phasic and it was distributed amongst high (*e.g.* kidney, liver, spleen), low (*e.g.* lung, skeletal muscle, skin) and negligible (*e.g.* brain, fat) accumulating tissues. The metabolism profile was characteristic for a phosphorothioate oligodeoxynucleotide; chain shortened metabolites (N_{-n}) via 3′, 5′ and mixed 3′+5′-exonuclease-mediated degradation, metabolic adducts (N_{+x}) and more subtle metabolites, probably arising through metabolic oxidation of the phosphorothioate linkage, are observed (8). Subcutaneous and i.p. administration of CGP 64128A resulted in appreciable bioavailabilities (~30–50% at a dose of 0.6 mg/kg over 360 min), reduced peak plasma levels when compared to i.v. administration, and efficacy in the human tumour xenograft models (unpublished observations, M. Müller, Novartis, Switzerland). The s.c. profile was similar to that reported previously for GEM-91 (23) and CGP 69846A (16). Whilst each obviates the need for administration by slow i.v. infusion, and thereby avoid haemodynamic side-effects, they are parenteral routes and as such are associated with lower patient acceptability. Non-parenteral routes were therefore considered in more detail.

Non-parenteral administration, particularly oral dosing, combines clinical convenience with patient acceptability. The implicit requirement for efficient transepithelial transport, however, presents a formidable challenge for antisense oligonucleotides. The oral bioavailability of CGP 64128A in fasted rats was negligible. Less than 1% of [^{3}H]CGP 64128A was absorbed following administration to the gastrointestinal tract by gavage or direct introduction into isolated duodenal or ileal segments. Even this poor bioavailability probably represents an overestimation of the actual oral bioavailability since partial degradation of the [^{3}H]CGP 64128A in the gastrointestinal tract would generate smaller, and potentially more absorbable, radioactive fragments. The small amount of absorbed radioactivity was distributed to the liver and kidney and probably represented partially intact oligonucleotides since radioactive mononucleotides are accumulated in the spleen (22). There are many possible explanations for the poor systemic availability after oral administration, including (i) precipitation in the acidic gastrointestinal environment, (ii) binding to luminal contents, (iii) metabolic instability in the lumen or at the absorptive epithelium, (iv) binding to the mucosal surface, (v) low permeability of the gastrointestinal epithelium or (vi) first-pass hepatic clearance. CGP 64128A may precipitate at gastric pH but would not under the mildly acidic conditions of the small intestine. Precipitation, therefore, is not considered to be the principle absorption-limiting factor in these experiments since the oral bioavailability was equally poor after dosing by gavage and direct instillation into the small intestine. The rats were fasted, making binding to luminal contents an unlikely explanation. Since CGP 64128A was only partially degraded in the GI tract (duodenal segment), metabolic instability is also unlikely to account for its poor absorption. The large proportion of CGP 64128A which becomes associated with the GI mucosa may contribute to its poor absorption since bound compound is unavailable for absorption. The physicochemical properties of phosphorothioate oligodeoxynucleotides—high molecular weight, multiple negative charges and hydrophilic (Log $D_{(octanol/water)}$ ~ −3.5) —predict that the gastrointestinal epithelium should present a significant physical barrier which is probably responsible for limiting their absorption. This is supported by the very low apparent permeability coefficient (P_{app}) of 2.0×10^{-8} cm/s for the transport of [^{3}H]CGP 64128A across the Caco-2 monolayers—a model of the human gastrointestinal epithelium (26–29)—which predicts an oral absorption <<1% according to the correlation described by Artursson and Karlsson (30; R. Fox and P. L. Nicklin, *unpublished observations*). First-pass hepatic extraction may further reduce the systemic availability after oral dosing. In summary, these data suggest that the oral route is not feasible for phosphorothioate oligodeoxynucleotides. The situation is apparently more promising for metabolically stable oligonucleotides. Agrawal *et al.* reported the oral bioavailability of a hybrid oligonucleotide (5′-CUCUCGCACCCATCTCTCTCCUUCU-3′, a uniform phosphorothioate backbone with additional 2-O-methyl modification on underscored bases) to be 25.9±4.7% in rats based on the percentage of the orally administered radiolabel dose associated with tissues other than the gastrointestinal tract (31). Closer examination of these data, by comparison with the dose-corrected tissue levels for the same compound following i.v. administration (32), suggest an oral bioavailability of ~1% to be more realistic and comparable to similarly stabilised oligonucleotides (J. A. Phillips, *manuscript in preparation*). Because of clear discrepancies between different workers and its potential importance to the field, we are currently defining the absolute oral bioavailability for several chemically modified oligonucleotides in a variety of animal species.

Intra-tracheal administration was the most promising non-parenteral delivery route giving a significant and dose-dependent systemic bioavailability of CGP 64128A. Relatively high pulmonary bioavailabilities have been reported previously for peptides (12) and the present work shows that this is equally true for antisense oligonucleotides. After intra-tracheal dosing, [^{3}H]CGP 64128A displayed absorption-limiting kinetics where blood levels increased slowly to a peak 20-fold lower than after i.v. administration and were maintained over extended time periods. The systemic availability was confirmed by the detection of intact CGP 64128A and a typical array of metabolites in kidney and liver using capillary gel electrophoresis. Possible

reasons for the dose-dependent bioavailability include, (i) a dose related toxicity which increased the permeability of the pulmonary epithelium and (ii) saturation of absorption-limiting tissue-binding at higher doses. Experiments with permeability markers and histological examination of the pulmonary tissues showed that the highest dose of CGP 64128A had no apparent effect on the functional or morphological integrity of tracheal or lung epithelia supporting the latter hypothesis. Indeed, the dose-dependent bioavailability parallels that seen for the s.c. administration of CGP 69846A which was also attributed to a saturation of absorption-limiting local tissue-binding at higher doses (16). This dose-dependence provides two delivery opportunities; low doses of oligonucleotide are retained within the lung whereas higher doses achieve significant systemic bioavailability. This presents the opportunity of treating local respiratory diseases with low pulmonary doses, thereby minimising the exposure of non-target tissues, or of treating systemic conditions with higher doses and circumventing the haemodynamic effects of phosphorothioate oligodeoxynucleotides.

An example of *in vivo* efficacy following the pulmonary administration of an aerosolised phosphorothioate oligodeoxynucleotide has recently been described by Nyce and Metzger (33). Phosphorothioate oligodeoxynucleotides were used to specifically down-regulate adenosine A1 receptors in a dust mite-conditioned rabbit model resulting in reduced hyper-responsiveness in antigen or adenosine challenged animals. Antisense-mediated down-regulation of Bradykinin B2 receptor expression was also achieved with different antisense sequences. This provides a clear demonstration that some molecular targets relevant to respiratory disease can be modulated through antisense-mediated down-regulation of gene expression. These encouraging observations make the present work timely in that the delivery, metabolism, bioavailability and acute effects of phosphorothioate oligonucleotides in the lungs have been characterised. A number of important questions have also been raised, for instance (i) is multiple dosing of phosphorothioate oligodeoxynucleotides to the lungs tolerated?, (ii) which cell types, tissue matrices or extracellular components accumulate oligonucleotides in the lungs *in vivo*? and (iii) what is the pulmonary bioavailability of other chemically modified oligonucleotides and especially those having reduced protein-binding? These issues are currently being addressed in our laboratories.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Paul Skelton-Stroud for his histopathological reading of lung sections. Drs. Hans Gaus and Mark Graham are also thanked for their efforts in developing the necessary capillary electrophoretic analytical methods.

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