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Distribution of a 20-Mer Phosphorothioate Oligonucleotide, CGP69846A (ISIS 5132), into Airway Leukocytes and Epithelial Cells Following Intratracheal Delivery to Brown-Norway Rats

Henry Danahay,[2] June Giddings,[1] Robin A. Christian,[1] Heinz E. Moser,[1] and Judy A. Phillips[1]

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Purpose. To evaluate the pulmonary distribution of CGP69846A (ISIS 5132), a phosphorothioate oligonucleotide, following intra-tracheal (i.t.) instillation into Brown-Norway rats.

Methods. The pharmacokinetic profile of [^{3}H]-CGP69846A was investigated following i.t. instillation into both naïve and inflamed airways of Brown-Norway rats. The cellular distribution was determined using autoradiography, immunohistochemistry and flow cytometry/fluorescence microscopy, in inflamed airways.

Results. CGP69846A displayed a dose-dependent lung retention following i.t. administration which was unaffected by local inflammation. Autoradiography and immunohistochemistry showed distribution to alveolar macrophages, eosinophils, bronchial and tracheal epithelium and alveolar cells. Studies with [FITC]-CGP69846A demonstrated a preferential association of oligonucleotide with leukocytes in bronchial lavage fluid of: macrophages > eosinophils = neutrophils >> lymphocytes.

Conclusions. The dose-dependency of lung retention together with cell-specific uptake suggests that the lung can be used as a local target for antisense molecules with potentially minimal systemic effects. Furthermore, the preferential targeting of macrophages and the airway epithelium by oligonucleotides may represent rational cellular targets for antisense therapeutics.

KEY WORDS: antisense; Brown-Norway rat; oligodeoxynucleotide; pulmonary delivery; ISIS 2105; pharmacokinetics; airway inflammation.

INTRODUCTION

Antisense oligonucleotide technology has spawned numerous compounds with targets in a wide variety of disease states, notably for tumor, viral and inflammatory indications. A number of these compounds have now reached the clinic and are showing therapeutic promise (reviewed in references 1–3). One compound, fomivirsen (ISIS 2922) has recently been licensed for the treatment of CMV-induced retinitis in AIDS patients. Antisense oligodeoxynucleotides (ODN) can work through a number of potential mechanisms involving hybridization with mRNA leading to an enzyme (RNase H) catalysed degradation of the target message. A major limitation to the widespread use of antisense therapy in some diseases is the difficulty of targeting the specific tissue and cell types. Local administration would enable high concentrations of ODN to be reached at the target tissue whilst minimizing any potential for systemic toxicity (4–6). Furthermore, knowledge of the cell specific uptake and intra-cellular localization of these compounds would enable rational target selection.

When administered systemically phosphorothioate (PS) ODNs are rapidly cleared from the circulation (7,8). The clearance reflects uptake of up to 80% of the administered dose by liver and kidney. The fraction of the dose accumulated in the lung is low (7,8) therefore reducing the opportunity for pulmonary targeting. The lung is an ideal site for local delivery as it can be accessed non-invasively and relatively specifically (4,5). Furthermore, lung surfactant is zwitterionic and at physiological pH is cationic thereby potentially facilitating oligonucleotide uptake into pulmonary tissue (5). It was recently demonstrated that an inhaled oligonucleotide reduced the expression of a target protein (PA_1-receptor), with reported functional consequences, in a rabbit model of asthma (9).

In this paper, the organ and cellular distribution of a 20-mer phosphorothioate oligonucleotide, CGP69846A (ISIS 5132), targeted against the 3′-untranslated region of human *c-raf*-1 kinase mRNA (10) is described following intra-tracheal delivery into the airways of Brown-Norway (BN) rats. The BN rat was chosen because of the ability to sensitise to antigen and its widespread use in models of allergen-induced airway inflammation. CGP69846A was used only as a model ODN to assess a class specific behaviour of these compounds. In studying the cellular distribution within the lung, an eosinophilic inflammation was induced before dosing to provide an environment containing raised numbers of inflammatory cells. An understanding of the cellular distribution of ODNs within the lung could identify specific cell populations, in which antisense targets would have optimal chances of success in treating pulmonary disease.

MATERIALS AND METHODS

Chemicals

CGP69846A (5′-TsCsCsCsGsCsCsTsGsTsGsAsCsAsTs-GsCsAsTsT where s = PS; otherwise known as ISIS 5132) and ISIS 2105 (5′-TsTsGsCsTsTsCsCsAsTsCsTsTsCsCsTsCs GsTsC) were used in these studies. ISIS 2105, [FITC]-CGP69846A and antibody 2E1 were kindly provided by Dr. M. Butler, ISIS Pharmaceuticals (Carlsbad, C.A.). CGP69846A was tritiated using the method described by Graham et al. (11).

Animals, Sensitization, and Antigen Challenge

BN rats were used throughout this study because of their ability to mount an eosinophilic response in the lungs. Male, BN rats (250–300 g) (Harlan Olac, Oxon) were fed *ad lib.* (Harlan Diet 9607) under controlled conditions (12 h light cycle;

[1] Novartis Horsham Research Centre, Horsham, West Sussex RH12 5AB, U.K.

[2] To whom correspondence should be addressed. (e-mail: henry.danahay@pharma.novartis.com)

ABBREVIATIONS: ARG, autoradiography; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IHC, immunohistochemistry; mRNA, messenger ribonucleic acid; ODN, oligonucleotide; PS, phosphorothioate; TRITC, tetramethylrhodamine-isothiocyanate.

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20°C). Animals used for antigen challenge experiments were sensitized on day 1 with ovalbumin (OA, 0.2 μg s.c. Sigma, U.K.) and pertussis vaccine (1:4 i.p.) followed with identical OA boosters (0.2 μg s.c.) on days 15 and 22. Animals were then challenged with antigen or vehicle on days 28–30. An eosinophilic airway inflammation was induced by challenging sensitized animals with nebulized OA (0.32% w/v in 0.9% saline, in a whole body cloud chamber for 60 min).

Oligonucleotide Dosing

Oligonucleotides were administered to either naïve (no pretreatment) or eosinophilic (dosed 48 hours after OA challenge) airways of BN rats. Intratracheal (i.t.) instillation of CGP69846A (200 μl 0.9% saline) or vehicle was performed using a micro-sprayer (Penncentury, P.A.), placed into the trachea through the mouth, whilst the animals were under short-acting anaesthesia (halothane/N_2O) (Merial Animal Health, U.K.).

Organ Distribution Studies

[^{3}H]-CGP69846A (0.5 μCi/animal; • 0.006 mg) was used as a tracer in doses of CGP69846A (0.06–6.0 mgkg^{-1} i.t.). The distribution of CGP69846A was studied in naïve animals and also in OA-sensitized/challenged animals. After dosing 200 μl blood samples were taken from the tail vein at regular intervals up to 24 hours. At 24 hours after dosing, animals were overdosed with pentobarbitone sodium (100 mg i.p. Euthatal, Merial Animal Health, U.K.) and were exsanguinated. A plastic cannula was fixed into the top of the trachea and lungs were lavaged (3 × 4 mL 0.9% saline, 0.1% K^+ EDTA, Sigma, U.K.). Trachea and lungs were then removed, together with samples (200–500 mg) of liver, kidney, spleen, bone marrow, stomach, duodenum, ileum and ceacum. Bone marrow was obtained by lavaging the left femur with 10 mL lavage solution and collecting the cellular pellet (2000*g* 10 min). Cytospins were prepared from bronchoalveolar lavage (BAL) fluid and were stained with Diff-Quick (Sigma, U.K.) for differential cell counting. The BAL cell pellet was separated from the BAL fluid (centrifugation at 2000*g* 10 min) and samples of both collected. The whole lung, in 300 mg portions and the samples of other organs and BAL components were then oxidized using a Packard 307 Sample Oxidizer and counted on a Beckman LSC-6500. Values are expressed as the percentage of the dose administered. Blood volume was assumed to be 18 mL.

Pulmonary Metabolism of CGP69846A

Samples of lung taken from BN rats previously dosed with CGP69846A (6.0 mgkg^{-1}) were extracted as described by Leeds *et al.* (12). Samples were analyzed by capillary gel electrophoresis (Beckman P/ACE MDQ, Beckman, U.K.) using a Beckman DNA capillary (# 477477), 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.

Autoradiography

[^{3}H]-CGP69846A (7 μCi/animal i.t. • 0.08 mg) was dosed to either saline or OA challenged rats. Animals were overdosed with pentobarbitone sodium at either 15 or 360 min after dosing and exsanguinated. Lungs were removed and then inflated with neutral buffered formalin (4.5 mL; 10%) instilled into the trachea through a plastic cannula. Samples were processed as previously described (8) and sections exposed for 4 weeks.

Immunohistochemistry

CGP69846A and ISIS2105 were both used for these studies as the primary antibody directed against the oligonucleotides, 2E1/AS, was raised against the ISIS2105 sequence (13) and the extent of cross reactivity with CGP69846A was unknown. Animals previously exposed to OA were dosed with oligonucleotide (0.06–6.0 mgkg^{-1} i.t.) and were killed as previously described 120 min later. Animals either underwent BAL, or lungs were removed, inflated and fixed with neutral buffered formalin and processed essentially as described by Butler *et al.* (13).

[FITC]-CGP69846A Studies

Animals previously exposed to OA were dosed with [FITC]-CGP69846A (0.06–6.0 mgkg^{-1} i.t.) and were killed, as previously described, 120 min later. Animals then underwent BAL or lungs were removed and inflated with 50-50 TBS-Cryo-M-bed. Frozen sections were immunostained for macrophages (ED1), eosinophils (Major Basic Protein) and B-lymphocytes (Pan B), using a TRITC labeled second stage antibody and were examined for the cellular location of oligonucleotide using the FITC filter, and for identification of the cells using the rhodamine filter. BAL samples were washed once with lavage buffer to minimize contamination with free unbound compound, and cytospins were prepared. [FITC]-CGP69846A association with BAL leukocytes was also analyzed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, C.A.). Cytofluorometric analysis was performed using laser excitation at 488nm and the mean fluorescence intensity per 30,000 cells was determined. Background non-specific fluorescence was assessed in the saline dosed group.

RESULTS

Organ Distribution Studies

Naïve Rats

[^{3}H]-CGP69846A was absorbed into the systemic circulation displaying a dose-dependent C_{max} and T_{max}, following inhaled delivery to naïve BN rats (Fig. 1). At 6.0 mgkg^{-1}, the levels of CGP69846A peaked at 2 h after dosing in contrast to 0.6 mgkg^{-1} which peaked at 6 h and 0.06 mgkg^{-1} which peaked at between 6 and 12 h. There was also a dose-dependent reduction in lung retention with 25% of the administered dose retained within the lung at 24 h in the 6 mgkg^{-1} group compared with 38% and 52% with the 0.6 and 0.06 mgkg^{-1} groups respectively. This pattern of dose-retention was also evident in the BAL pellet (Table 1) with 1.5% of the dose present at 0.06 mgkg^{-1} down to 0.4% at 6.0 mgkg^{-1}. There was also a dose-dependent increase in accumulation in the liver, kidney, spleen, and bone marrow (Table 1). Levels in the alimentary canal were variable although they appeared to be higher in the 6.0 mgkg^{-1} treated groups in each of the gut tissues except the ceacum. Metabolism of CGP69846A (6.0 mgkg^{-1}), through nuclease-mediated degradation, was evident by 24 h after dosing

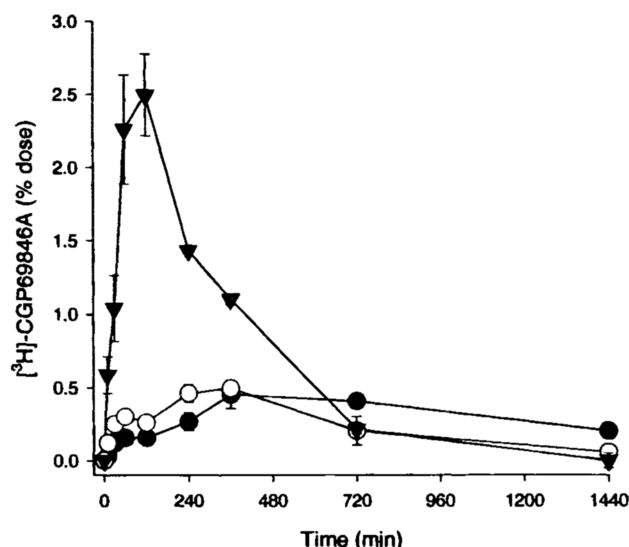

Fig. 1. Blood levels of [^{3}H]-CGP6946A 0.06 [-●-], 0.6 [-○-] and 6.0 mgkg^{-1} [-▼-] following inhaled delivery to naive BN rats. Results expressed as % administered dose (mean ± SEM, n = 6).

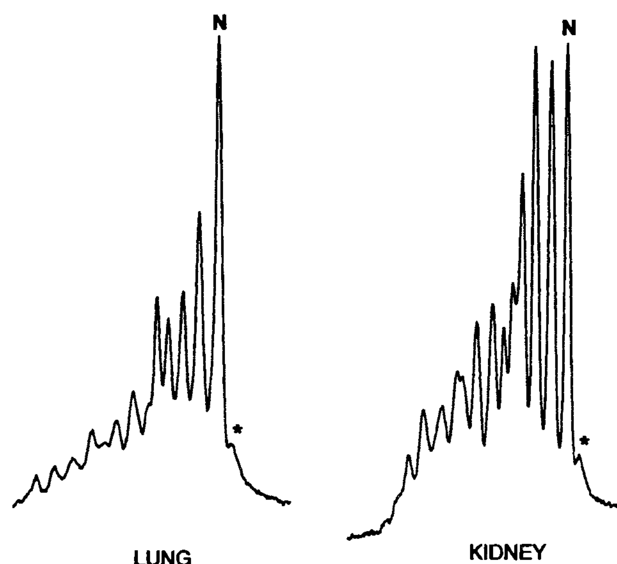

Fig. 2. Sample traces (CE) showing metabolism of CGP69846A (6.0 mgkg^{-1}) in lung and kidney 24 h after inhaled delivery to naive BN rats. N = parent ODN, * = N_{+1}.

in kidney and to a lesser degree in lung (Fig. 2). In the lung, approximately 30% of the oligonucleotide was present as parent molecule compared to 11% in the kidney. In addition to parent molecule (N), chain shortened metabolites (N_{-n}) and peaks that migrated more slowly than CGP69846A (N_{+x}) were observed in both tissues.

Sensitized Rats

Inhaled OA induced a lung eosinophilia in sensitized animals (Fig. 3A). Administration of CGP69846A into the OA-sensitized airways of either saline or OA-challenged BN rats produced a similar pattern of blood levels (Fig. 3B) and tissue distribution (Table 2) to that observed in naïve animals. However, although there were no significant differences in systemic exposure in terms of blood levels between the saline and OA-challenged groups, differences were observed in organ distribution. Whilst lung dose retention was similar, accumulation in the peripheral organs was reduced following CGP69846A administration to OA-challenged animals in liver ($P < 0.001$), spleen ($P < 0.0002$), kidney ($P < 0.002$), BALF ($P < 0.05$) and bone marrow ($P < 0.02$) but not gut tissues.

Pulmonary Distribution in Eosinophilic Airways

Autoradiography

The pulmonary distribution of [^{3}H]-CGP69846A (7 μCi, i.t.) into the airways of OA sensitized/challenged BN rats was time-dependent and ultimately cell type specific. At 15 min after dosing, radioactivity was localized to the areas surrounding the larger airways although not uniformly distributed (data not shown). This activity was predominantly associated to alveolar macrophages (Fig. 4i), with some uptake in eosinophils (Fig.4ii) and tracheal (Fig. 4iii) and alveolar epithelial cells (Fig. 4iv). There was no evidence of uptake into lymphoid tissue (Fig. 4v). Activity in macrophages was localized to the cytoplasm and not the nucleus, whilst some activity was concentrated in the nucleus of epithelial cells. The intra-cellular localization in the eosinophils was difficult to determine due to much lower activity. By 6 h after dosing, activity was more uniformly distributed throughout all levels of the airways and was primarily localized to the alveolar macrophages (Fig. 4iv). At this time, there was no evidence of activity in eosinophils (Fig. 4iv) and only limited sites of alveolar cell uptake.

Table 1. Tissue Distribution of [^{3}H]-CGP69846A (0.06–6.0 mgkg^{-1}) Following Inhaled Delivery to Naive BN Rats

Dose	Lung	Trachea	Liver	Spleen	Kidney	BALF	BALP	BM	Stom	Duod	Ileu	Caec
0.06	52.20	0.43	1.08	0.03	1.62	0.80	1.47	0.10	0.18	0.03	0.38	2.09
mgkg^{-1}	(1.56)	(0.05)	(0.06)	(0.00)	(0.17)	(0.21)	(0.23)	(0.02)	(0.05)	(0.00)	(0.07)	(0.45)
0.6	38.44	0.36	4.57	0.63	5.29	1.66	0.97	0.31	0.16	0.07	0.42	3.78
mgkg^{-1}	(2.54)	(0.04)	(0.50)	(0.53)	(1.48)	(0.17)	(0.09)	(0.02)	(0.03)	(0.01)	(0.05)	(0.32)
6.0	25.51	0.34	19.85	0.40	13.30	1.85	0.39	1.00	0.47	0.17	0.92	2.89
mgkg^{-1}	(0.81)	(0.02)	(0.33)	(0.01)	(0.38)	(0.06)	(0.04)	(0.06)	(0.06)	(0.02)	(0.06)	(0.22)

Note: Results expressed as % administered dose (±SEM) (n = 6). BALF, bronchoalveolar lavage fluid; BALP, cell pellet from BALF; BM, bone marrow; Stom, stomach; Duod, duodenum; Ileu, ileum; Caec, caecum.

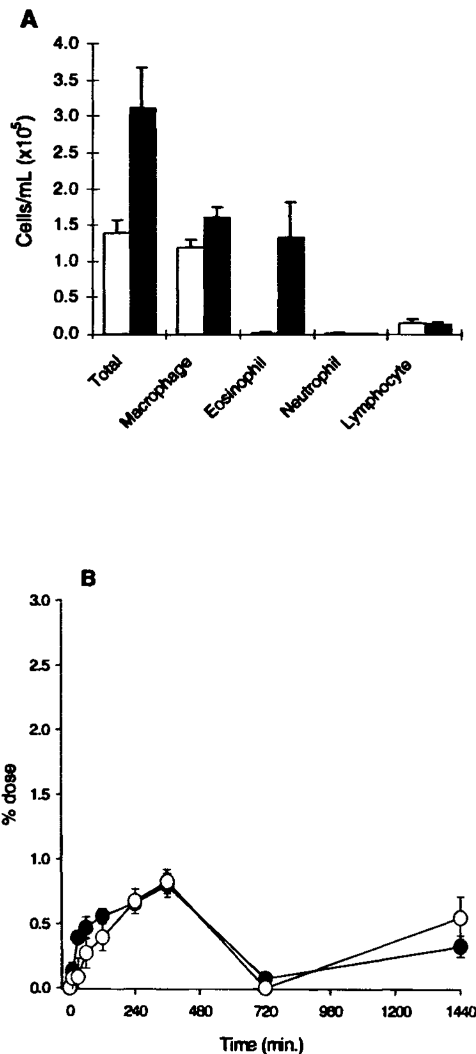

Fig. 3. A. Cellular composition of BAL fluid 72 h after saline (open bars) or OA (filled bars) challenge (24 h after dosing with [^{3}H]-CGP69846A, 0.6 mgkg^{-1}). B. Blood levels of [^{3}H]-CGP69846A (0.6 mgkg^{-1}) following inhaled delivery to sensitised and challenged (saline [-○-]/OA [-●-]) BN rats. Results expressed as % administered dose (mean ± SEM, n = 5).

Immunohistochemistry

At 2 h after dosing, CGP69846A was detected in macrophages and the alveolar epithelium at all of the studied doses (Fig. 5i, shows 0.6 mg kg^{-1}). Macrophage staining again appeared to be cytoplasmic. Staining was however only noted in macrophages with CGP69846A, whereas staining was more pronounced and widespread with ISIS 2105. In common with CGP69846A, the alveolar macrophage was a major site of uptake of ISIS 2105 (Fig. 5ii) where again distribution appeared to be cytoplasmic. There was also association of this oligonucleotide with eosinophils and alveolar cells (Fig. 5ii) and both tracheal (Fig. 5iii) and bronchial epithelium (Fig. 5iv) where it was located in the cytoplasm and nucleus.

Fluorescence Microscopy

[FITC]-CGP69846A (0.6–6.0 mgkg^{-1}) was detected in alveolar macrophages (ED1 positive TRITC cells) and eosinophils (major basic protein positive TRITC cells) (Fig. 6) at 2 h after dosing. None of the cells staining positive for Pan B showed association with [FITC]-CGP69846A (Fig. 6).

[FITC]-CGP69846A associated with leukocytes in a dose-dependent manner following i.t. administration (Fig. 7A) as measured using laser excitation at 488 nm. It is important to note that the dose dependent uptake demonstrated with [FITC]-CGP69846A is expressed in absolute units in contrast to the kinetics studies where lung retention was expressed as % dose. Macrophages were the major associating inflammatory cell, with less neutrophil and eosinophil association and limited lymphocyte fluorescence. Cytospin preparations of these samples (Fig. 7B) demonstrated that the fluorescence was not surface bound but was predominantly cytoplasmic, indicating that the ODN had been internalized by the leukocytes.

DISCUSSION

Antisense technology theoretically opens up numerous therapeutic opportunities, some of which are now being explored in animal models of disease and clinical trials (1–3). There are however limitations to this approach and target selection is likely to play a pivotal role in the success of an ODN based therapy. Cellular uptake and internalization, rapid clearance and non-specific ODN-effects in addition to manufacturing costs, are the major obstacles to be overcome with antisense therapies and this has lead to increased interest in local delivery (4–6,9,15). Recently, interest has focused on utilizing the lung for antisense approaches both as a disease target and as a non-parenteral route of administration for achieving systemic availability (4,5,9,14). This study examines the potential for utilizing localized airway delivery for antisense therapeutics by determining the pulmonary distribution of PS ODNs after intratracheal administration.

Table 2. Tissue Distribution of [^{3}H]-CGP69846A (0.6 mgkg^{-1}) Following Inhaled Delivery to Sensitized and Challenged (Saline/OA) BN Rats

	Lung	Trachea	Liver	Spleen	Kidney	BALF	BALP	BM	Stom	Duod	Ileu	Caec
Saline	41.09	0.33	6.13	0.13	7.80	2.05	0.64	0.33	0.34	0.11	0.64	4.58
	(3.09)	(0.04)	(0.82)	(0.02)	(0.89)	(0.23)	(0.08)	(0.04)	(0.02)	(0.03)	(0.06)	(0.46)
OA	43.39	0.24	1.58*	0.04*	2.58*	1.35*	0.82	0.15*	0.30	0.05	0.38	4.55
	(3.40)	(0.04)	(0.30)	(0.00)	(0.53)	(0.20)	(0.17)	(0.05)	(0.18)	(0.01)	(0.08)	(1.03)

Note: Results expressed as % administered dose (±SEM).

* Indicates significant difference between groups using unpaired students t-test ($P < 0.05$; n = 5).

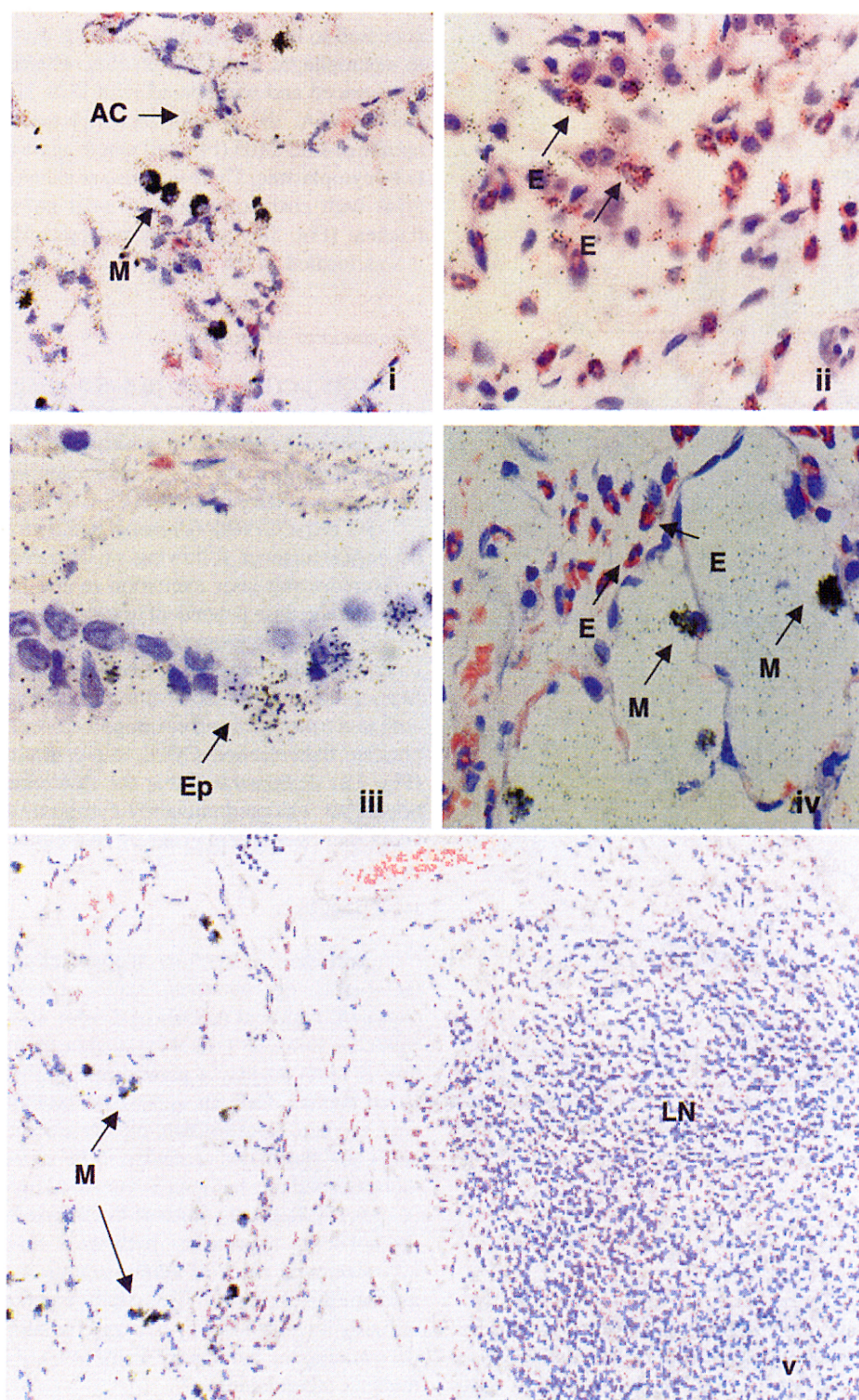

Fig. 4. Autoradiographic sections of airways from Brown-Norway rats following dosing with [^{3}H]-CGP69846A (7 μCi/animal). Evidence of association with alveolar macrophages (i, ×340), alveolar cells (i), eosinophils (ii, ×550) and tracheal epithilium (iii, ×865) at 15 min after dosing. By 360 min, there was no association with eosinophils although positive macrophages were apparent (iv). There was no evidence of uptake into lymphoid tissue (v). [AC = alveolar cell, M = macrophage, E = eosinophil, Ep = epithelium, LN = lymph node].

CGP69846A was absorbed systemically in a dose-dependent manner following intra-tracheal administration into the lungs of BN rats, accumulating in the liver, kidney and spleen as previously described for this compound after intravenous administration (8). From Table 1, it is evident that a proportion of the dose is unaccounted for which may be present in tissues not specifically examined although it is more likely that this fraction has been excreted (8). In parallel with systemic accumulation, there was a dose-dependent reduction in lung retention of the administered dose, suggesting the existence of a saturable

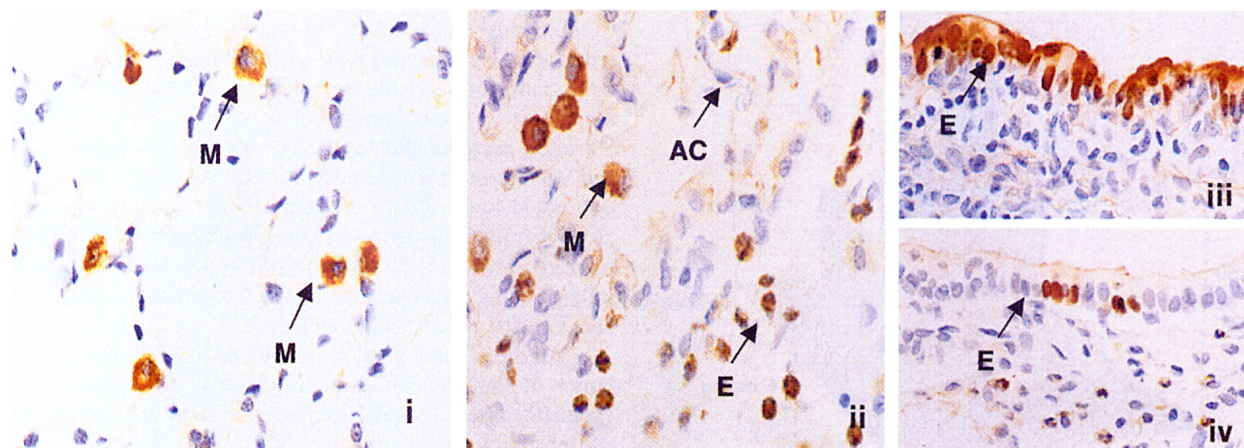

Fig. 5. Immunostaining of (i) alveolar macrophages in lung tissue from BN rats (×550) 120min following dosing with CGP69846A (0.6 mgkg^{-1} i.t.). Using ISIS 2105 (0.6 mgkg^{-1} i.t.) immunostaining is also evident in (ii) alveolar macrophages, eosinophils and alveolar cells (×430), (iii) tracheal epithelial cells (×680) and (iv) bronchial epithelial cells (×430). [AC = alveolar cell, M = macrophage, E = eosinophil, Ep = epithelium].

uptake site in the airways. Saturable uptake was evident in inflammatory cells recovered in BAL fluid in both the [^{3}H]- and [FITC]-labeled ODN studies. Other saturable binding sites may also exist within the lung. A similar saturable uptake mechanism has also been described in rats following systemic dosing with a profile suggestive of scavenger receptors (15). Scavenger receptors have been identified on a number of tissues relevant to potential ODN uptake in the airways such as: alveolar macrophages (16), airway epithelium (17), fibroblasts (18), endothelium (19) and smooth muscle (18).

The more rapid absorption into the circulation at 6.0 mgkg^{-1} may be as a consequence of increased deposition of the aerosol in the alveolar regions. However, the microsprayer delivery device used in these studies produces particles with a mass median diameter of 5–10 μm (Penncentury, product information) which would be expected to impact predominantly in the small bronchi with limited alveolar deposition. Systemic absorbance from the lung would occur primarily from the alveolar regions (4). Limited alveolar deposition would therefore support the concept of a saturable uptake/retention site.

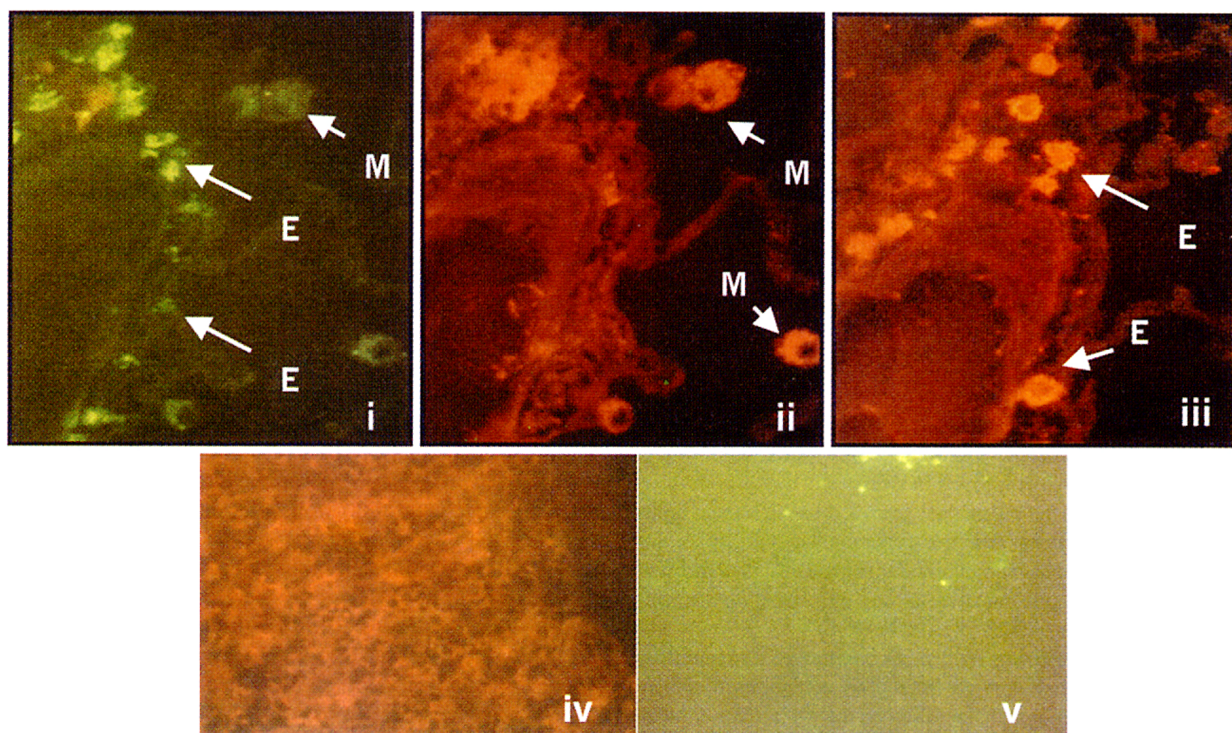

Fig. 6. Sequential sections of lung tissue illustrating: (i) the distribution of [FITC]-CGP69846A (6 mgkg^{-1}, i.t.), and the identity of the leukocytes present using a TRITC double staining technique (ii and iii). Panels ii and iii stain for ED1 (macrophages) and Major Basic Protein (eosinophils) respectively. Pan B positive lymphoid tissue is shown in panel iv, with no evidence of oligonucleotide staining (v). [M = macrophage, E = eosinophil].

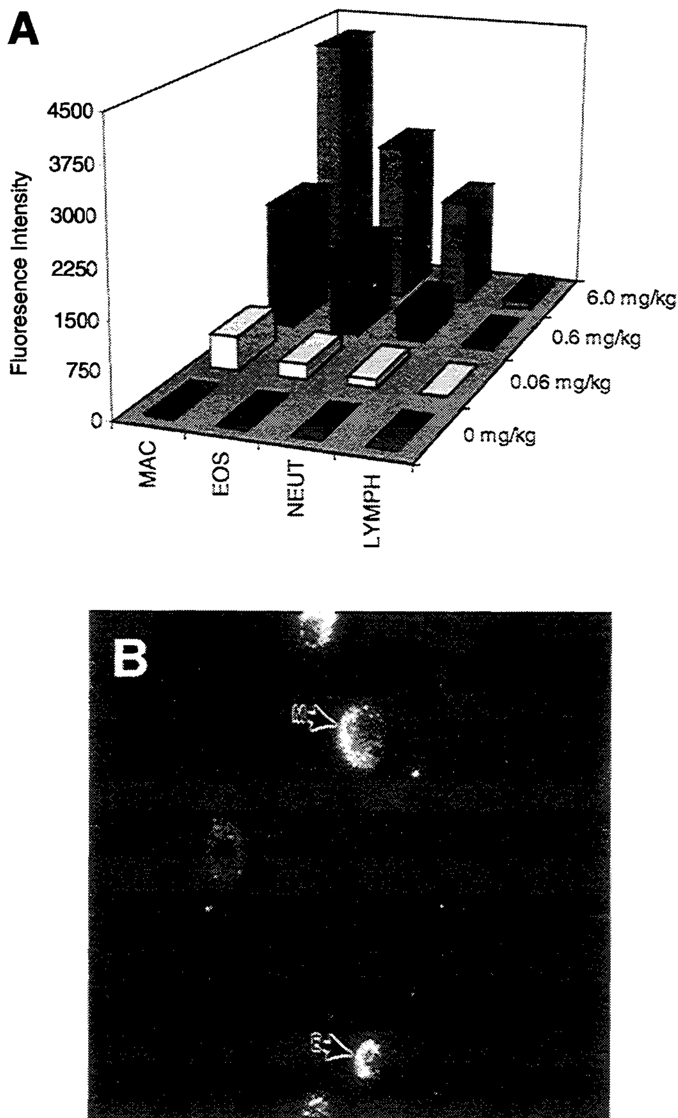

Fig. 7. A. [FITC]-CGP69846A (0.06-6.0 $mgkg^{-1}$ i.t.) association with BALF leukocytes removed from OA-sensitised and challenged BN rats. Rats were dosed *in vivo* with ODN, 48 h after inhaled OA challenge. Animals were lavaged 2 h after ODN dosing. Control was dosed with saline. Results are expressed as Mean Fluoresence Intensity after laser excitation at 488 nm (n = 2 per group). B. Cytospin preparation of BALF from BN rat dosed with [FITC]-CGP69846A (6.0 $mgkg^{-1}$ i.t.).

Although not specifically examined in this study it has been previously demonstrated that a 20-mer phosphorothioate, CGP64128A, at doses employed in this study, did not compromise the integrity of the pulmonary tract following local administration (14) indicating that the systemic availability is not simply due to an increased epithelial permeability. These observations suggest that systemic availability of inhaled oligonucleotides can be minimized by dose selection.

Previously sensitizing BN rats to ovalbumin and administering an antigen-challenge to induce an eosinophilic airway inflammation had no significant effect on the systemic availability of inhaled CGP69846A. There was however a small, although statistically significant, reduction in the systemic accumulation of the compound. That the lung retention of CGP69846A is unaffected, but that the systemic accumulation reduced when delivered into the inflamed airway, implies that more compound is being removed from the body. The reason for this is as yet unknown but may be as a consequence of enhanced excretion due to the systemic response to anaphylaxis and will require more detailed studies. An alternative explanation could involve the trafficing of inflammatory cells to the airways from these peripheral sites, potentially relocating internalized compound. In conclusion, the presence of an acutely inflamed lung does not have a marked effect on the pharmacokinetics of the inhaled oligonucleotide.

All three of the methods employed to determine the distribution of oligonucleotides in the airways (ARG, immunohistochemistry (IHC) and fluorescence studies) produced a consensus picture of specific cellular uptake. ISIS 2105 was used for the IHC studies because of the greater sensitivity of the antibody, 2E1/AS, to this compound. Only at 15 min after dosing is there limited non-specific binding to mucus and tissue matrix. At all times studied, there is cell specific uptake which appears to consolidate predominantly in macrophages and to a lesser extent in eosinophils. Uptake of antisense molecules by macrophages and monocytes has been previously demonstrated in vitro in both primary cells and cell lines (20–23). Furthermore, activity of oligonucleotides has also been demonstrated in this cell type *in vitro* (20,21,23). Uptake does not however automatically suggest activity since a number of workers have noted that ODNs remain in the cytoplasm in apparent granular structures and activity is only observed upon the co-administration of cationic lipid (20,22) or chemical modification of the ODN (21,23). This apparent 'trapping' of ODNs in endosomal vesicles is a barrier that may also be present *in vivo*, since in this study, both CGP69846A and ISIS 2105 were detected only in the cytoplasm of macrophages. Fluorescence microscopy and laser excitation flow cytometry of BALF leukocytes confirmed a selective uptake of [FITC]-CGP69846A by macrophages; the order of uptake in BALF leukocytes being: macrophages > eosinophils = neutrophils >> lymphocytes. Fluorescence microscopy confirmed that the compound had been internalized by BAL leukocytes and was not bound to the cell membrane. In common with the ARG and immunostaining, [FITC]-CGP69846A was located in the cytoplasm and not the nucleus of macrophages.

To our knowledge, ODN uptake by eosinophils has not been previously described. ARG, IHC and fluorescence studies all confirm this observation in BN rats which demonstrate eosinophilia. Using ARG, uptake appeared to be transient as it was only observed at 15 min after dosing and not by 6 hours. IHC with CGP69846A and ISIS 2105 and fluorescence studies confirmed eosinophilic uptake at 2 hours. Uptake was also noted in tracheal and bronchial epithelium using ARG and IHC, which appeared to be both cytoplasmic and nuclear. The nuclear staining in the epithelium may be a consequence of a differential uptake process in this cell type compared with leukocytes or may be as a result of the higher local concentration the ODN would present at the surface of the lumen.

This study is unique in employing three unrelated techniques to demonstrate cell specific uptake of PS ODNs in the lung. We have also attempted to relate this to whole organ systemic pharmacokinetics following local administration. All three techniques showed 20-mer PS-ODN uptake into a variety of pulmonary cells with most uptake detected in the cytoplasm

of alveolar macrophages. Uptake has also been demonstrated in eosinophils, alveolar cells and tracheal and bronchial epithelial cells. Combined with the kinetic and metabolic behaviour of these compounds, these cell types would therefore appear to be logical targets for an antisense approach to a relevant disease of the airways. It will be of interest to compare the distribution and subsequently the functional activity, of suitably designed sequences, of a variety of oligonucleotide chemistries within the airways.

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