Brief/Technical Note

Development of an *In Vivo* Ovine Dry Powder Inhalation Model for the Evaluation of Conventional and Controlled Release Microparticles

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INTRODUCTION

The local treatment of disease states such as asthma and chronic obstructive pulmonary disease has suggested the potential for controlled release formulations designed to treat circadian attacks or have improved residence for the treatment of respiratory infections. While the relationship between the physico-chemical properties of particles within the respiratory size range and their aerosolisation efficiency is still not fully understood, the fate of deposited particles requires detailed investigation prior to use in humans.

Clearly, with such complex systems, the use of *in vivo* animal models to assess the feasibility of drug delivery and the fate of medicaments in the respiratory tract following inhalation is crucial to the process of drug development. In general, small animals (mice, rats and guinea pigs) are commonly used to test inhalation formulations. Such models are useful for evaluating toxicology, since population size requirements make the use of larger animals prohibitive.

Sheep have previously been chosen as a good model for lung injury (1). Furthermore, because of the similarity of the lung size, anatomy and pathophysiological changes to humans, sheep have been extensively used to study the pathophysiology of asthma, chronic obstructive pulmonary disease cystic fibrosis and allergic rhinitis (2–4).

Previous studies by this group have focussed on the development and evaluation of model controlled release inhalation dry powders (5,6). Although *in vitro* diffusion models have successfully characterised these systems (in comparison with conventional pharmacopoeia methodologies), a critical *in vivo* model is required. The aim of this work was to develop an *in vivo* inhalation model using sheep and to compare the pharmacokinetics of a controlled release formulation with a regular dry powder formulation. A dry powder system containing disodium cromoglycate (DSCG) as

the active ingredient with polyvinyl alcohol (PVA) as a release modifier was used as a model system and has been characterised extensively elsewhere (5).

MATERIAL AND METHODS

Test Formulations

Microparticles of DSCG alone or with 90% *w/w* PVA were prepared and characterised as described previously (5,6). Briefly, DSCG was spray dried under optimised conditions to produce a yield of microparticles with desired characteristics suitable for pulmonary inhalation. Particle sizing, imaging, aerosolisation performance and *in vitro* release studies were the key assessments performed. Although previous studies focussed on a series of PVA concentrations, for this study, the two extremes were chosen: DSCG with 0% PVA and DSCG with 90% *w/w*. They will be referred to as DSCG and DSCG/PVA formulations, respectively.

Animal Study

Ethics approval was obtained prior to animal handling from the Royal North Shore Hospital, University of Technology Sydney Animal Care and Ethics Committee (no. 0702-011A). Merino first-cross female sheep (*Ovis aries*), 44.2–51.4 kg weight, were kept indoors at all times after arrival for at least 2 weeks in temperature-, humidity- and light-cycle-controlled rooms. They were fed standard compressed pellets for sheep in the morning and hay or chaff in the evening.

A total of four animals were used in a cross-over design for this study. Jugular access lines were surgically implanted into two sheep at a time in order to withdraw blood samples for plasma analysis. The two sheep under experiment were kept in their metabolic crates in the same room for 1–2 weeks prior to dosing, for recovery and acclimation. On the day of the experiment, after overnight fasting, animals were placed in a linen sling (designed to keep them upright) in the metabolic crates prior to general anaesthesia. Anaesthesia was induced by slow intravenous propofol (Diprivan® 1%, AstraZeneca, UK; all doses between 180 and 200 mg) until

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effect was reached. Sheep were kept upright during the course of each experiment to maintain normal ventilation/perfusion ratios throughout the lung and were intubated with an endotracheal (ET) tube for pulmonary drug administration. Immediately after intubation, the ET tube was quickly fitted to a powder dispersing device previously developed in-house (7). During an end-expiratory pause, a dose of either DSCG or DSCG/PVA was fired using a bolus of medical grade air (BOC, Sydney, Australia) at 2 atm for 3 s. This produces 2 L of atmospheric pressure air sufficient to deliver the dose and suitable for the size of the sheep's lungs. Previous laboratory experiments for estimated powder loss into the device and the ET tube ($n=3$) for each formulation were the bases to calculate the total powder dose used for each formulation. A net of 25 mg DSCG was delivered in case of the DSCG formulation and 17.5 mg of DSCG in case of the DSCG/PVA formulation. After dose administration (≤ 5 min), the animal was allowed to recover from anaesthesia, and the ET tube was removed. The device and ET tube were rinsed with deionised water for mass balance assessment. Animals were fed their usual day's meals after 5 h of the experiment. In addition, for comparison, an intravenous (i.v.) bolus dose (20 mg DSCG in sterile water for injection) was delivered under similar conditions. Blood samples (4 mL) were collected in heparinised glass tubes at 0, 5, 10, 15 and 30 min and 1, 2, 3, 4, 6, 8 and 24 h for the i.v. and DSCG formulations. In addition, samples were taken at 2, 3, 4 and 6 days after administration for the DSCG/PVA formulation. All blood samples were immediately centrifuged, and plasma was harvested and stored at -20°C pending analysis. A washout period of 30 days was allowed between experiments, and a zero time point was collected prior to the start of each study.

Analytical Methodology

The concentration of DSCG in plasma samples was determined by high-performance liquid chromatography (HPLC) using the method described by Kato *et al.* (8), with modifications in the extraction process. Solid phase extraction (SPE) using Oasis® HLB 3 cc (Waters Corporation, Massachusetts, USA) cartridges was used instead of the liquid-liquid extraction described in the original method and with modification to the SPE of DSCG used by Ozoux *et al.* (9). Plasma (1 mL) was diluted with 5% ammonia solution (2 mL) and vortexed. Diluted samples were loaded into the cartridges, which were equilibrated with methanol (1 mL) followed by water (1 mL). Cartridges were then washed with 0.1 M ammonium acetate solution (2 mL, Aldrich, USA) followed by 2 mL of methanol in 0.1 M ammonium acetate mixture (15:85% *v/v*). DSCG was then eluted by methanol (1 mL) and collected into small glass vials and concentrated under a stream of nitrogen gas to 150 μL final volumes. Samples were then loaded into small volume inserts (150 μL glass Inserts, Phenomenex, Australia) for the HPLC assay. A Waters HPLC system was used [Waters 600 controller, 600 pump, 486 tuneable absorbance detector, column oven TCM controller and heater (set at 40°C) and 717+ auto-sampler, Waters Australia, Sydney] using a HPLC column (Luna 5 μm NH2 100A, 250×4.60 mm, Phenomenex, Australia) with a security guard cartridge system (cartridges NH2 4×3.0 mm, Phenomenex, Australia). The mobile phase was composed of KH_2PO_4 solution (4.5 g in 350 mL

water) and acetonitrile 35:65% *v/v*, adjusted to $\text{pH}=3.2$ by H_3PO_4 (Sigma Chemical, USA). A flow rate of 2 mL min^{-1} was used, and DSCG absorbance was recorded at a wavelength of $\lambda=240$. All solvents used throughout were supplied by Biolab (Australia) and were analytical or HPLC grade. Water used was purified by reverse osmosis (Milli-Q, Sydney, Australia). Each plasma sample was analysed twice.

The plasma concentration of DSCG was calculated using calibration curves of peak area *vs.* standards' concentrations in sheep plasma. Freshly prepared DSCG standards were used to spike the zero time sheep plasma for the construction of the calibration curves prior to each set of plasma analysis. Linearity over the range $5\text{--}500 \text{ ng mL}^{-1}$ ($r^2=0.994$) was observed. Percentage recovery was $107.8 \pm 7.1\%$ and $97.5 \pm 9.9\%$ at 500 and 50 ng mL^{-1} , respectively. The retention time for DSCG was 5.88 ± 0.42 min, and the lower limit of detection was 0.4 ng mL^{-1} . Intra-day assay coefficient of variation (CV) was 2.7% ($n=4$) and 1.62% ($n=5$) for 500 and 50 ng mL^{-1} , respectively. Inter-day assay CV was 4.24% ($n=7$) and 5.57% ($n=6$) for 500 and 50 ng mL^{-1} , respectively.

For pharmacokinetic analysis, the area under the DSCG plasma concentration-time curve was calculated by the trapezoidal rule up to the last collection point (AUC_{0-t}). Area under the curve to infinity ($\text{AUC}_{0-\infty}$) was calculated as $\text{AUC}_{0-t} + C_t/k_{el}$, where C_t is the concentration at the time of last collection point and k_{el} is the elimination rate constant, calculated from the slope of the terminal portion of the log-concentration-time profile. The peak plasma concentration (C_{max}) and time to the peak concentration (T_{max}) were obtained by observation.

RESULTS AND DISCUSSION

General Observations

A novel method for the delivery of dry powder to the sheep's lung by pulmonary inhalation was studied. The dry powder disperser used in this study, previously developed and evaluated (7), was successful in dispersing the DSCG dry powder dose to the lung of the sheep via ET tube. Preliminary *ex vivo* studies evaluating the disperser-ET tube assembly indicated that a 2-atm air pressure and a 3-s time interval was the optimum combination of parameters efficient to deliver the powder dose from the disperser with a tolerable effective volume of air [approximated as 2 L of uncompressed air, measured by manual breathing bags of 2.0 L (GE Healthcare, Australia), data not shown]. Another aspect of this design was animal orientation. Keeping the animal in the upright position during the dose administration process was a design meant to help mimic the gravitational effect on the inhalation dose without modifying ventilation or perfusion in the lungs from normal. Generally, this method was feasible and allowed delivery of the DSCG formulations into the sheep's lung with minimum invasion or distress.

Pharmacokinetic Analysis

Figure 1 shows the plasma concentration-time profile of DSCG and DSCG/PVA dry powder formulations after delivery to sheep using the disperser-ET tube assembly. It can be seen that a very rapid peak plasma concentration was achieved within a T_{max} of 5–10 min (7.5 ± 2.5 min) post-

Fig. 1. a DSCG plasma concentration-time profile for DSCG and DSCG/PVA formulations administered by inhalation to sheep ($n=4$). b The first 2 h of the concentration-time profile graph

inhalation for both the DSCG and DSCG/PVA formulations. It is worth mentioning that the reported $T_{\max}$ for inhaled DSCG in human studies was from 10–15 min (10) to 30 min (8), a similar order to that reported here for the sheep model.

It is an interesting observation that such a fast peak concentration with the formulation containing 90% w/w PVA was observed, since it would be expected to have a delayed absorption from the lungs due to the effect of the release modifier. However, previous *in vitro* studies, using a diffusion model, suggested that there is an initial burst release of the DSCG from the PVA-based dry powder formulation, with approximately 40% being released within the first 60 min (in comparison to 100% for DSCG alone) (6). Although a simplified *in vitro* model, analysis of this previously reported data suggested that the release profile followed a classical Higuchi diffusion model for homogeneous matrices (11,12), where initial diffusion would be rapid due to the higher drug/sink concentration gradient. Furthermore, comparison of the DSCG and DSCG/PVA drug-plasma concentration time profiles suggested different release mechanisms *in vivo*. It may be considered that such a high concentration of PVA in the formulation is an extreme case; however, it is interesting to note that a study of these systems, using conventional *in vitro* dissolution methods, failed to detect any differences in the

release profiles when compared to plain DSCG formulation (6). However, such observations are expected to some extent, since previous studies by Yamamoto *et al.* had shown that the incorporation of 1% PVA into a liquid inhalation formulation affected the absorption of the 5(6)-carboxyfluorescein from the lungs in a murine model (13).

DSCG Alone

Previous studies by Moss *et al.* (10) indicated that urinary excretion of DSCG had decreased to negligible proportions after 8 hours post administration in humans, and the authors suggested that no further absorption from the lungs was taking place. Interestingly, similar urinary excretion rates of DSCG in rats, rabbits, monkeys and humans was also observed by Moss *et al.*, which suggested that lung clearance mechanism of DSCG in humans would follow the same pattern as found in mammalian species (14).

The data for microparticles containing only DSCG reported here showed similar behaviour with a rapid decrease in plasma concentration due to clearance and excretion into the urine and bile. The fraction that reached the systemic circulation has a clearance of approximately 1 L h⁻¹ [calculated from the i.v. average data of the four sheep ($\pm$ SD): $AUC_{0-\infty} = 20.05 \pm 0.1 \mu\text{g h mL}^{-1}$, clearance = $1.01 \pm 0.2 \text{ L h}^{-1}$, volume of distribution = $26.7 \pm 4 \text{ L}$ and $t_{1/2} = 18.4 \pm 3 \text{ h}$]. DSCG concentrations in this study remained detectable after 24 h; however, the detection limit is 100-fold smaller than in previously reported studies (10).

DSCG/PVA Controlled Release System

As seen Fig. 1, the DSCG plasma concentration from the DSCG/PVA microparticles did not decline as fast as the PVA free formulation. In general, the DSCG concentration remained at an approximately constant level for up to 2 days before it started to decline. In addition, DSCG was still within the limit of detection after 6 days. Table I shows the pharmacokinetic parameters calculated from both the DSCG and DSCG/PVA controlled release formulation ($AUC_{0-\infty}$, apparent $t_{1/2}$ and F %). Since a value of only 8% bioavailability for inhaled DSCG from DPIs was reported in humans (15) and since the percentage excreted in faeces would be attributed to bile excretion more than GI absorption (14), it comes as no surprise that the percent bioavailability for DSCG microparticles was $2.58\% \pm 0.31$ (SD). In addition, it is expected that the polar nature of the DSCG molecule and the defence clearance mechanisms (mucociliary clearance and alveolar macrophages)

Table I. Pharmacokinetic Parameters for DSCG Administered by Inhalation in Sheep

Pharmacokinetic parameter	DSCG	DSCG/PVA
$C_{\max}$ ($\mu\text{g mL}^{-1}$)	0.240 ± 0.009	0.191 ± 0.019
Apparent half-life (h)	4.28 ± 0.58	62 ± 8 *
$AUC_{0-\infty}$ ($\mu\text{g h mL}^{-1}$)	0.509 ± 0.064	10.5 ± 1.3 *
F (%)	2.58 ± 0.31	52.5 ± 6.6 *

Data are presented as mean ($\pm$ SD)

* $p < 0.05$, t test significant statistical difference

of the airways may have contributed to the rapid clearance of the bulk of the drug from the lungs before being subject to absorption. In comparison, it is hypothesised that the presence of PVA in the microparticles effectively slowed the release of DSCG over the duration of the experiment, acting as a depot for the drug. In addition, the gel-like nature of these wetted microparticles may promote air interface adhesion and reduce mucociliary removal and phagocytosis. In general, a ~25-fold increase in the bioavailability of DSCG was observed in the controlled release formulation.

CONCLUSION

Previous studies by the investigators have suggested that the release of DSCG from co-spray-dried microparticles is through initial wetting of the particles resulting in both dissolution of the solid drug molecules and gelling of the hydrophilic polymer matrix. This is then followed by the diffusion of the drug through this swelled layer rather than dissolution of the polymer. As part of an ongoing study, we have developed an ovine *in vivo* model to further evaluate the delivery and release of these complex systems. Preliminary studies have shown that the use of an *in vivo* model is capable of assessing the fate of inhaled dry powder medicaments intended for respiratory therapy, with preliminary studies using DSCG, being similar to that obtained in humans. Furthermore, the model has shown that it is possible to evaluate controlled release formulations that to date have only limited toxicology and thus require an animal model. Further studies are needed to accurately investigate the exact pattern by which these particles are deposited in the lungs, the mechanism by which the drug is released from the particles, cellular uptake and absorption as well as the clearance from the lung.

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REFERENCES

1. Murakami K, Bjertnaes LJ, Schmalstieg FC, McGuire R, Cox RA, Hawkins HK, *et al.* A novel animal model of sepsis after acute lung injury in sheep. *Crit Care Med.* 2002;30:2083–90.
2. Abraham WM. Modeling of asthma, COPD and cystic fibrosis in sheep. *Pulm Pharmacol Ther.* 2008;21:743–54.
3. Begin R, Rola-Pleszczynski M, Sirois P, Masse S, Nadeau D, Bureau MA. Sequential analysis of the bronchoalveolar milieu in conscious sheep. *J Appl Physiol.* 1981;50:665–71.
4. Bischof RJ, Snibson K, Shaw R, Meeusen ENT. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy.* 2003;33:367–75.
5. Salama R, Hoe S, Chan H-K, Traini D, Young PM. Preparation and characterisation of controlled release co-spray dried drug-polymer microparticles for inhalation 1: influence of polymer concentration on physical and *in vitro* characteristics. *Eur J Pharm Biopharm.* 2008;69:486–95.
6. Salama RO, Traini D, Chan H-K, Young PM. Preparation and characterisation of controlled release co-spray dried drug-polymer microparticles for inhalation 2: Evaluation of *in vitro* release profiling methodologies for controlled release respiratory aerosols. *Eur J Pharm Biopharm.* 2008;70:145–52.
7. Tang P, Fletcher DF, Chan HK, Raper JA. Simple and cost-effective powder disperser for aerosol particle size measurement. *Powder Technol.* 2008;187:27–36.
8. Kato Y, Muraki K, Fujitaka M, Sakura N, Ueda K. Plasma concentrations of disodium cromoglycate after various inhalation methods in healthy subjects. *Br J Clin Pharmacol.* 1999;48:154–7.
9. Ozoux ML, Girault J, Malgouyat JM, Pasquier O. Determination of sodium cromoglycate in human plasma by liquid chromatography–mass spectrometry in the turbo ion spray mode. *J Chromatogr B Biomed Sci Appl.* 2001;765:179–85.
10. Moss GF, Jones KM, Ritchie JT, Cox JSG. Plasma levels and urinary excretion of disodium cromoglycate after inhalation by human volunteers. *Toxicol Appl Pharmacol.* 1971;20:147–56.
11. Higuchi T. *J Soc Cosmet Chem.* 1960;11:85–97.
12. Higuchi T. Rate of release of medicaments from ointment bases containing drugs in suspension. *J Pharm Sci.* 1961;50:874–5.
13. Yamamoto A, Yamada K, Muramatsu H, Nishinaka A, Okumura S, Okada N, *et al.* Control of pulmonary absorption of water-soluble compounds by various viscous vehicles. *Int J Pharm.* 2004;282:141–9.
14. Moss GF, Ritchie JT. Absorption and clearance of disodium cromoglycate from lung in rat, rabbit, and monkey. *Toxicol Appl Pharmacol.* 1970;17:699–707.
15. Martindale: the complete drug reference. London: The Pharmaceutical Press; 2008.