CHAPTER 7

Dry powder inhalation of hemin to induce heme oxygenase expression in the lung

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Eur J Pharm Biopharm. 2007 Nov;67(3):667-75. Epub 2007 Mar 31

ABSTRACT

The purpose of this study was to formulate hemin as a powder for inhalation and to show proof of concept of heme oxygenase 1 (HO-1) expression in the lungs of mice by inhalation of hemin. Hemin was spray dried from a neutralized sodium hydroxide solution. The particle size distribution of the powder was between 1-5 µm. Dispersion from the Twincer dry powder inhaler showed a fine particle fraction (<5 µm) of 36%. A specially designed aerosol box based on the Twincer®-inhaler was used for a proof of concept study of HO-1 induction by inhalation of hemin in mice. The aerosol in the exposure chamber of the aerosol box remained aerosolized up to 5 minutes. A rhodamin B containing aerosol was used to show that the aerosol box gave deposition over the entire lung indicating the suitability of the model. Additionally, inhalation of hemin showed a dose dependent increase in HO-1 protein expression in the lungs. In conclusion, hemin was successfully formulated as a powder for inhalation and the inhalation model allowed controlled HO-1 expression in the lungs of mice. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

INTRODUCTION

The heme oxygenase (HO) metabolic pathway has recently received an explosion of research interest due to its potent physiological and cytoprotective properties, e.g. (1-3). The inducible (HO-1) and the constitutively expressed (HO-2 and HO-3) forms of HO catalyze the oxidation of heme to biliverdin, carbon monoxide and iron (4). Subsequently, biliverdin is rapidly converted to bilirubin which is a potent endogenous antioxidant (5). All three downstream products (biliverdin/bilirubin, CO and Fe/ferritin) participate in the cellular defense. HO-1 has an integral role in the response to oxidative stress and inflammation and is induced by numerous oxidative and inflammatory stimuli (6).

Recently, it was shown that HO-1 expression in lung tissue and macrophages obtained by bronchoalveolar lavage of patients with chronic obstructive pulmonary disease (COPD) is decreased compared with healthy controls (7, 8). COPD is a respiratory disease characterized by a chronic inflammatory response in the lungs, which is primarily caused by the toxic effects of smoking. It is tempting to hypothesize that in COPD patients an imbalance between systemic reactive oxygen species and relative antioxidant systems exists, which is possibly caused by insufficient upregulation of HO-1 (9). One of the substances that induces HO-1 is hemin (ferriprotoporphyrin IX), the oxidized form of heme (10, 11). Induction of the heme oxygenase pathway by inhalation of natural substrates, such as hemin, could therefore be of great benefit in diseases like COPD (12, 13). So far, inhalation of hemin in humans has been performed only once as a control to show that the HO-1 pathway is involved in asthma (13). In that study, nebulization of 2 ml of 10^{-4} M hemin resulted in increased levels of exhaled CO, indicating that the HO-1 pathway can be induced by inhalation of hemin. However, no direct evidence of HO-1 induction was given.

In the clinical setting, nebulization is characterized by a low deposition efficiency (typically 2-27%), and needs electricity or pressurized air for the aerosol to be created. In contrast, dry powder inhalation is characterized by higher deposition efficiency (up to 60%) and the patient delivers the driving force for the aerosol to be created (14-17). Moreover, nebulization is accompanied by patient immobility, whereas dry powder inhalers are easy to carry and use. Furthermore, dry powder inhalation is likely to result in increased patient compliance, especially in chronic use.

The aim of this study was to formulate hemin as a powder for inhalation and to show a proof of concept for induction of heme oxygenase 1 expression in the lungs of mice by inhalation of hemin. To obtain the proof of concept we administered hemin as a powder for inhalation to mice by using a specially designed aerosol box, which is based on the Twincer® dry powder inhaler (18).

MATERIALS AND METHODS

Materials

Hemin (51280) and rhodamin B (83689) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Inulin, with a degree of polymerization of 23, was a generous gift of Sensus (Breda, The Netherlands). Sodium hydroxide (NaOH), hydrochloric acid (HCl) and ammonia (NH_4) were of analytical grade and purchased from commercial suppliers. Demineralized water was used throughout the experiments.

Methods

Quantitative determination of hemin

Sample concentrations for determination of solubility and drug load were spectrophotometrically analyzed in phosphate buffered saline (PBS) with the Ultraspec 4052TDS apparatus at 385 nm (LKB, Zoetermeer, the Netherlands).

Solubility

In order to measure the maximum solubility in different alkaline solutions, 7 solutions with a pH between 11.0 and 14.0 were prepared in steps of 0.5 pH units. To these solutions excess hemin was added under constant stirring. After 30 minutes, the solutions were centrifuged at 10,000 rpm for 10 minutes. The supernatant was pipetted and diluted with a phosphate buffered solution and measured spectrophotometrically.

Powder production by spray drying

Due to an excess of sodium hydroxide in the solution to increase dissolution of hemin, the pH of that solution remains high. If such a solution is spray dried, the resultant powder causes an alkaline pH shift upon reconstitution. To avoid alkaline pH changes upon reconstitution of the spray dried powder, the pH needs adjustments during or before spray drying. Two methods were used for pH adjustment. In the first method a

sodium hydroxide solution (0.7% w/v) with pH 13.5 was immediately before spray drying adjusted to pH 7.6 with hydrochloric acid. The second method consisted of spray drying an ammonia solution (1.2% w/v) with a pH of 13.5. During spray drying, ammonia evaporates and is not present in the dried powder any longer.
Four powders were spray dried. The first powder was prepared by dissolving hemin in a sodium hydroxide solution (0.7% w/v) at a pH of 13.5 to obtain a total concentration of 4.5% w/v. After pH adjustment the solution was filtered through a 1.2 µm filter. Hemin was not removed by filtration from the solution. After spray drying, this powder thus contained sodium chloride and hemin. The hemin drug load was determined to be 63% w/w.
The second powder was produced by dissolving hemin in an ammonia solution (1.2% w/v) with a pH of 13.5. After spray drying, this powder thus only contained pure hemin. This powder was used as a control in the dissolution experiments.
Finally, spray dried controls containing rhodamin B or inulin were prepared by dissolving the respective controls in a sodium hydroxide solution followed by pH adjustment before spray drying. Rhodamin B served as a control for the *in vivo* deposition characteristics and thus the suitability of the aerosol box; inulin served as a control for the local effect of powder inhalation with sodium chloride.
Spray drying was performed with a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) equipped with a two-fluid nozzle (0.5 mm). The solutions were sprayed at a fluid flow rate of 3.2 ml/min and an atomizing air flow rate of 800 l/h. The settings of the aspirator (14) and heater (14) resulted in temperatures at the inlet and outlet of 180 °C and 120 °C, respectively.
The obtained powders were stored in a vacuum desiccator at room temperature.

Dissolution
Dissolution experiments were carried out using an USP dissolution apparatus I (Rowa Techniek B.V., Leiderdorp, The Netherlands) at 37 °C and 100 rpm in 1000 mL phosphate buffered solution with 10 mg hemin, corrected for drug load. Samples from the dissolution vessel were spectrophotometrically analyzed at 385 nm.

Laser diffraction, scanning electron microscopy and cascade impactor analysis

Particle size distributions of the spray dried samples were measured with a Helos Compact model KA laser diffraction apparatus (100 mm lens, Fraunhofer theory) equipped with a RODOS dispersing system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) operated at a dispersion pressure of 5 bar. All measurements were performed in triplicate. The particle size distribution in the exposure chamber was measured by placing the complete aerosol box in the laser beam. To avoid scattering by the Perspex walls, the light was directed through two opposing openings.

Scanning electron micrographs (SEM) were recorded with a JEOL JSM 6301-F Microscope (JEOL, Japan). The powder was dispersed on top of double-sided sticky carbon tape on metal disks and coated with 150 nm of gold/palladium in a Balzers 120B sputtering device (Balzers UNION, Liechtenstein).

The aerodynamic particle size distribution was measured with cascade impactor analysis using a multi-stage liquid impinger (Erweka, Heusenstamm, Germany) with a Twincer®-inhaler (18) attached to the induction port. A solenoid valve was used in combination with a timer to control the flow (corresponding with 4 kPa pressure difference) through the inhaler and the cascade impactor for the duration of 3 seconds. The stages of the cascade impactor were filled with 20 mL phosphate buffered saline (PBS). A total amount of ~100 mg was delivered in 10 separate doses. The concentration of the drug on the different stages was measured by UV absorbance at 385 nm; deposition was subsequently calculated as the percentage of the metered dose. The fine particle fraction (< 5 µm at the flow rate corresponding with 4 kPa across the Twincer, FPF) was calculated by interpolation of the cumulative mass plot versus effective cut-off diameter. The FPF is expressed as a percentage of the metered dose. All cascade impactor experiments were carried out in triplicate.

Aerosol box for passive pulmonary delivery of powders for inhalation

As a proof of concept of HO-1 induction by inhalation of hemin a new device was developed to allow passive, pulmonary dry powder delivery to small laboratory animals. The device, hereafter called aerosol box (figure 1), is actually an accessory for the Twincer®, an effective dry powder inhaler which delivers high dosed formulations in highly constant particle

size distributions (fine particle fraction of 40-50%) independent of the pressure drop between 1 and 4 kPa (18). The efficient aerosol generation at low pressure drops makes the Twincer® most suitable for the aerosol box where the operator manually generates the air flow that disperses the powder. The device consists of the Twincer®, a cylinder with piston to provide a pressure drop across the inhaler and transfer of the aerosol to last part of the device, the exposure chamber in which a fan is built. Mice are attached to the exposure chamber. In total, up to twelve mice can be attached to the exposure chamber.
About 15 mg of powder, equivalent to 9.45 mg active ingredient (hemin, inulin or rhodamin B) and 5.55 mg sodium chloride, was dispersed in the aerosol box. In this study, one dose is defined as 5 minutes exposure to an aerosol that has been generated from 15 mg of drug formulation.

Animals
Female A/J mice (aged 8-10 weeks) were obtained from Harlan (Zeist, the Netherlands) and were held at the Central Animal Facility of the Groningen University. The experiments were approved by the Committee on Animal Experimentation of the University Medical Center Groningen and were performed under strict governmental law, adhering to the Principles of Laboratory Animal Care.

Experimental setup
The mice used in this study were divided into 6 groups. Group 1 consisted of one mouse that was not exposed to any aerosol and served as a control. Groups 2, 3, 4 (n=2 for each group) were exposed to 2, 4 and 6 doses of spray dried hemin, respectively. Group 5 (n=2) was exposed to 6 doses of spray dried inulin and served as control for sodium chloride that was present in the spray dried powders. Inulin, a naturally occurring oligosaccharide was chosen because oligosaccharides contribute marginally to the osmotic pressure due to their high molecular weight. Group 6 (n=2) was exposed to 6 doses of spray dried rhodamin. Group 6 was used to investigate the deposition of dispersed powder in the lungs of mice. Deposition of rhodamin was visualized in 4 µm sections of frozen lung tissue by fluorescent microscopy.

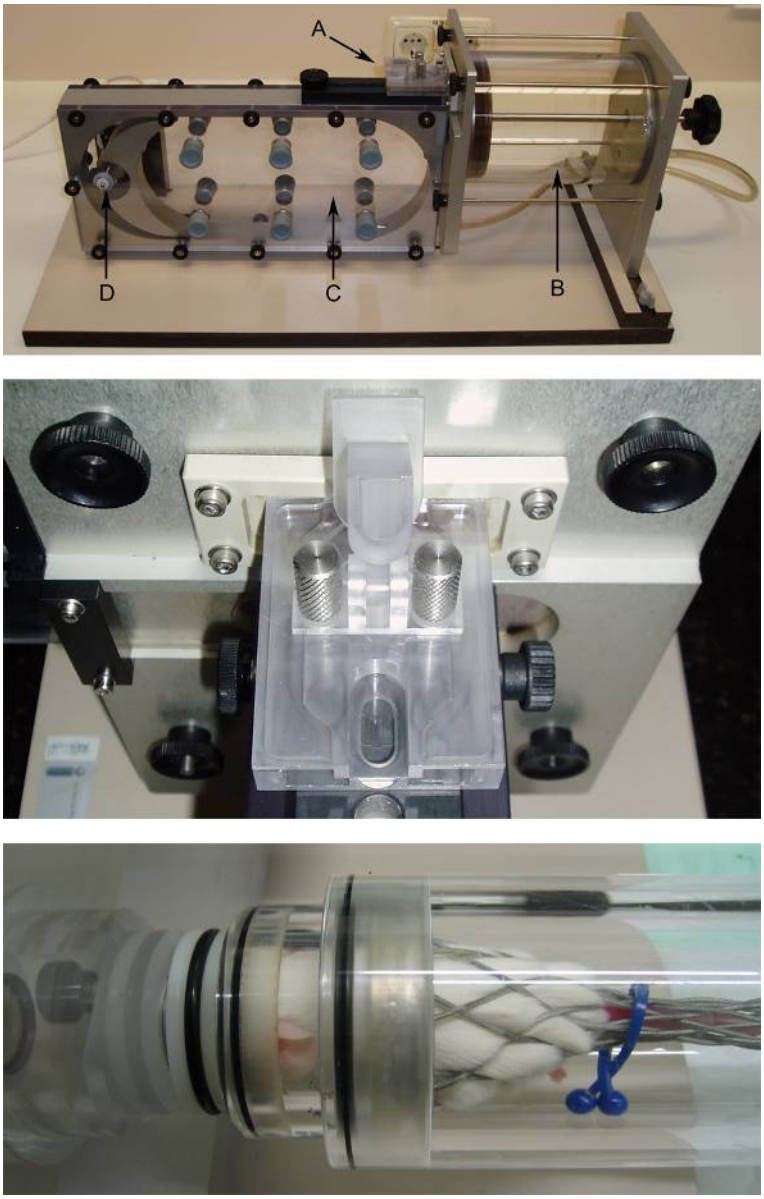

Figure 1. (Top) The aerosol box, a utility for the Twincer® (A). By pulling the piston in the cylinder (B) an aerosol is created and can be transferred to the exposure chamber (C) to which 12 small laboratory animals can be fitted. A fan (D) creates a laminar airflow in the exposure chamber to minimize sedimentation effects. **(Middle)** Detail of the Twincer® attached to the aerosol box. **(Bottom)** Detail of mouse attached to exposure chamber.

Western Blot

Protein samples were made from whole lung tissue using a sonicator (Bandelin sonopuls HD2070, Berlin, Germany). The proteins were separated on molecular weight using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane. The membrane was blocked overnight in 5% low fat milk and the proteins were detected with anti-heme oxygenase-1 (Stressgen, Victoria, Canada) followed by a peroxidase labeled goat-anti-rabbit antibody (DakoCytomation, Heverlee, Belgium). As a protein loading control the membrane was stripped using an 25mM Glycine-HCl buffer containing 1% SDS (pH:2) and stained for ß-actin (Abcam loading control, Cambridge, UK) followed by a peroxidase labeled goat-anti-rabbit antibody (Dako). The bands of interest were visualized using enhanced chemiluminescence.

Band-sizes of each group were quantified using Matlab 6.5.1 (The MathWorks Inc, Natick, USA) and the Dipimage toolbox (Quantitative Imaging Group, Faculty of Applies Sciences, Delft University of Technology, the Netherlands) (19, 20). In short, the grey-scale images were made binary with the Isodata threshold algorithm which calculates the best threshold value out of the grey value histogram of the image (21). Data are given as the ratio between the number of pixels of the HO-1 band of interest divided by the number of pixels of the corresponding ß-actin band.

Histology

HO-1 protein expression was demonstrated in 3 µm sections of paraffin embedded lung tissue with antibodies against HO-1 (Stressgen 896, San Diego, CA, United States). Presence of leukocytes was demonstrated in 4 µm sections of frozen lung tissue with monoclonal antibodies anti-CD45 (Pharmingen).

RESULTS

Formulation. A series of experiments was carried out to measure the solubility of hemin at different pH levels. Hemin is practically insoluble in water (at pH ~7) and organic solvents (22). However, under alkaline conditions hemin is soluble. In alkaline solutions, hemin (FePP-Cl) reacts with excess hydroxide to form hematin (FePP-OH). Hematin acts as an

intermediate in a polymerization reaction which results in a oxy-bridged dimer and probably other polymers (22).

$FePP\text{-}Cl + OH^- \rightarrow FePP\text{-}OH + Cl^-$
$2FePP\text{-}OH \rightarrow (FePP)_2O + H_2O$

The solubility of hemin (figure 2; solid line, primary y-axis) reached a maximum at pH 13.5. At pH>13.5 a large variation in solubility was observed which indicates the occurrence of degradation reactions, such as hydrolysis, at high pH values.

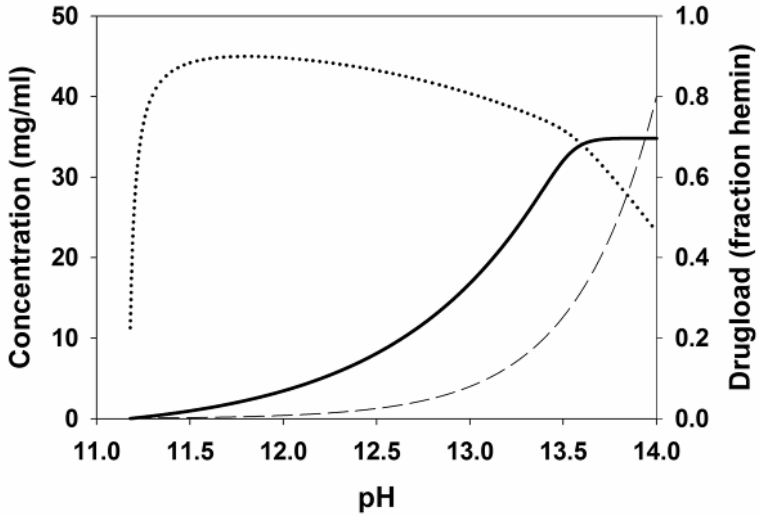

Figure 2. Solubility of hemin (solid line, primary y-axis) as a function of pH and calculated content of hemin in spray dried powder (dotted line, secondary y-axis) as a function of pH. The calculated concentration of NaOH versus pH is represented by the dashed line (primary y-axis).(n=3)

Since the pH of the sodium hydroxide solution determines the amount of hemin that can be dissolved, the sodium hydroxide concentration is the main parameter that determines the efficiency of the spray drying process. The higher the sodium hydroxide concentration, the higher the hemin concentration in the solution. On the other hand, it should be realized that an increase in the sodium hydroxide concentration requires increased amounts of hydrochloric acid for neutralization. This effect will lead to a shift towards higher sodium chloride fractions in the powder,

which of course reduces process efficiency in terms of drug load. Finding the most efficient process condition is a matter of balancing between the solubility of hemin, which is positively affected by the sodium hydroxide concentration, and the hemin drug load in the powder, which is negatively affected by the sodium hydroxide concentration. These effects are shown in figure 2 (dotted line, secondary y-axis).

With increasing pH the drug load of hemin in the spray dried product initially rose up to a maximum of 90% at a pH of 11.8. At this pH the total concentration of hemin and NaOH was only 2.5 mg/ml. Spray drying of a solution with such a low concentration does not result in a high yield. Therefore the pH was increased to 13.5, which resulted in a solution with a total concentration of 45 mg/ml and a drug load of 63% after spray drying as was confirmed by chemical analysis. The spray dried hemin powder consisted of highly irregular particles (figure 3), which were in a size range that corresponded with laser diffraction (table 1).

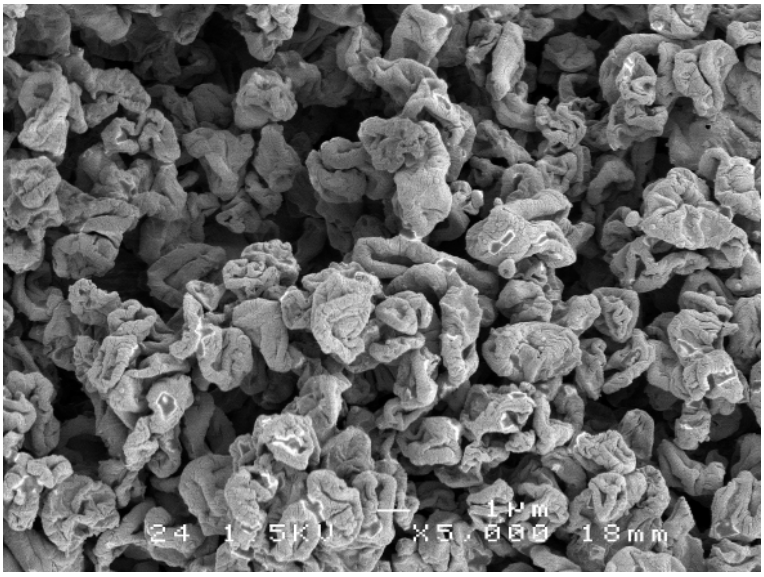

Figure 3. Scanning electron micrograph of spray dried hemin at a magnification of 5000x.

Table 1. particle size obtained from RODOS dispersion (5 bar) at 10, 50 and 90% cumulative undersize of the spray dried powders. All powders were spray dried from a sodium chloride solution, except pure spray dried hemin, which was spray dried from ammonia.

Formulation	x_{10} (µm)	x_{50} (µm)	x_{90} (µm)	Span*
Spray dried hemin	1.01	2.14	3.67	1.25
Spray dried hemin from ammonia	0.95	2.06	3.50	1.24
Spray dried inulin	0.92	2.29	4.66	1.64
Spray dried rhodamin B	0.87	2.02	3.42	1.26

* Span = $(x_{90}-x_{10})/x_{50}$

Spray dried hemin from a neutralized sodium chloride solution displayed a faster dissolution profile compared to spray dried hemin from NH_3 as shown in figure 4. After 2 minutes 50% of spray dried hemin was dissolved, compared to 20 minutes for spray dried hemin from NH_3, while the powders had approximately the same particle size (Table 1).

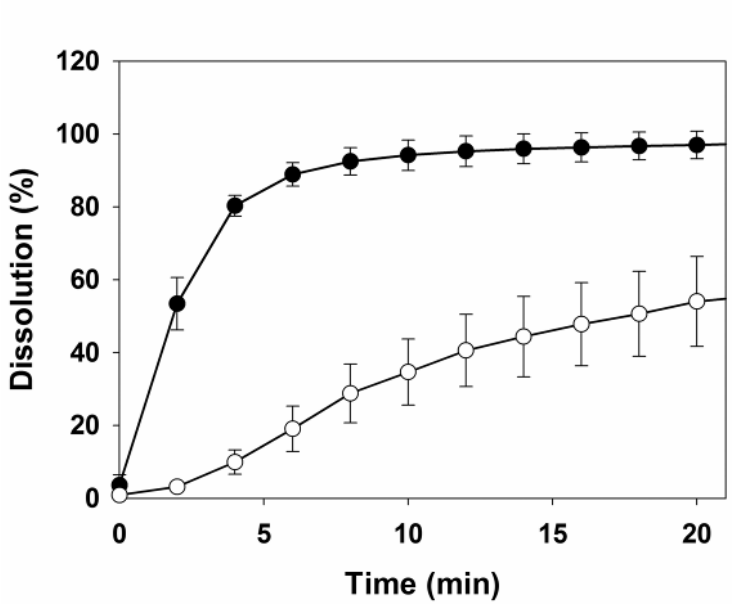

Figure 4. Dissolution rate of spray dried hemin in the presence of NaCl (●) and pure spray dried hemin from ammonia (○). (n=3)

Complete dissolution of the powder spray dried from the NH_3 solution took 110 minutes. The powder with the fastest dissolution rate was used to perform further experiments (aerosol behavior and proof of concept).
In table 1 the particle size distributions of the spray dried powders are shown. The particle size distributions are comparable, only spray dried inulin had a somewhat wider size distribution.
Cascade impactor analysis (figure 5) showed a delivered dose from the Twincer at 4 kPa of approximately 77% with a fine particle fraction (<5 µm) of 36% of the metered dose.

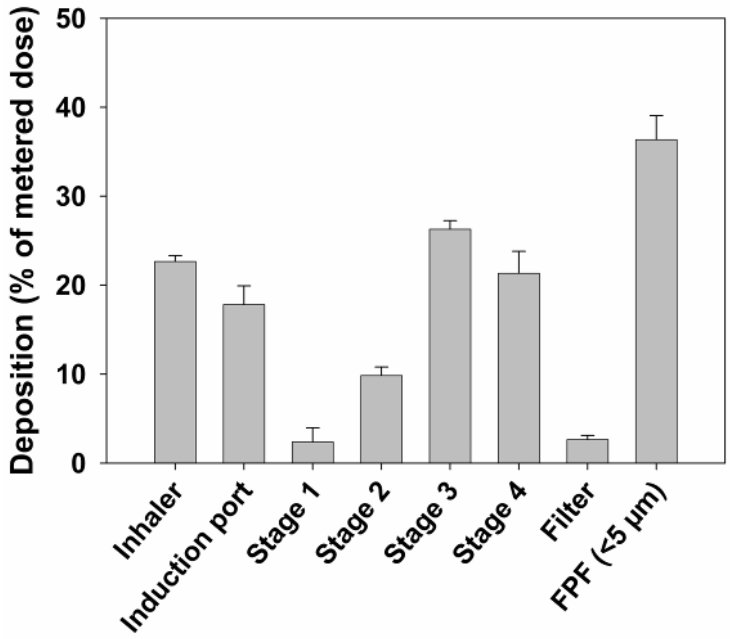

Figure 5. Cascade impactor analysis of spray dried hemin with NaCl after dispersion by the Twincer® at 4 kPa. Fine particle fraction (FPF) represents particles <6.8 µm. Error bars represent highest and lowest value.

Validation of aerosol box.
After dispersion in the aerosol box (figure 6B), most particles (<90%) were between 1 and 5 µm. In the aerosol box the size distribution moved towards finer particles in time, indicating sedimentation. Larger agglomerates (5-10 µm) sediment fast and such agglomerates are no longer present after 1 minute. The change in particle size distribution will not substantially affect the inhalable fraction in the inhaled air however,

since the volume fraction of such agglomerates in the aerosol is small. Sedimentation was confirmed by a decline in the optical concentration (i.e. amount of laser light that is blocked by the aerosol). After 5 minutes the optical concentration was 0.0%, whereas the starting concentration was 1.54% on average. The amount of aerosol in the exposure chamber after 5 minutes was therefore negligible due to sedimentation. Therefore, we exposed the mice for 5 minutes per dose to the aerosol because a longer period would have no additional effect.

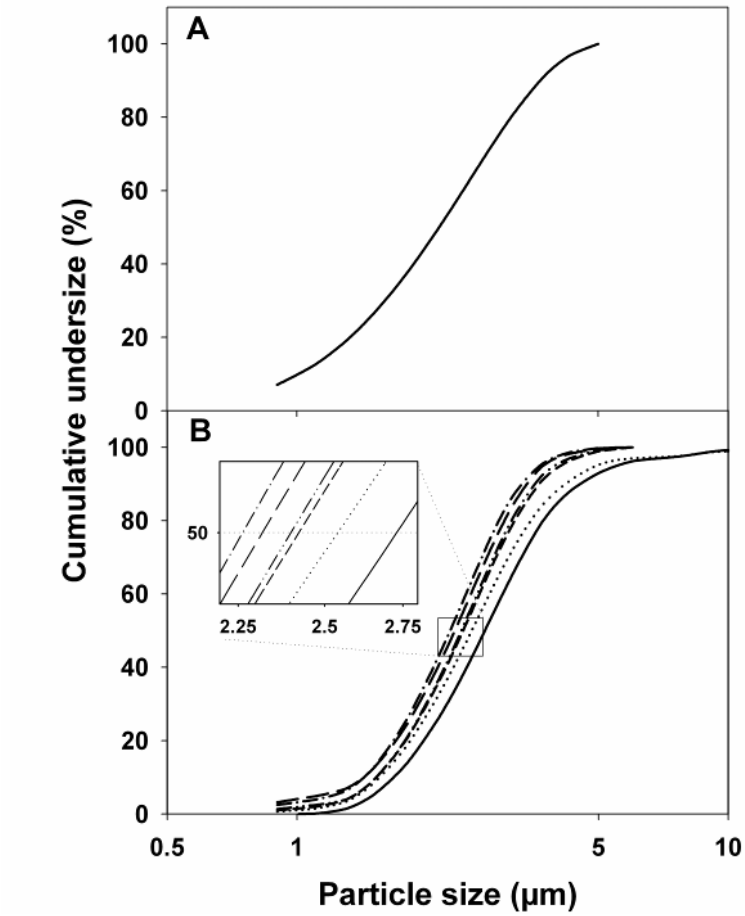

Figure 6. (A) Geometrical particle size distributions of spray dried hemin as measured by RODOS dispersion at 5 bar and (**B**) in the exposure chamber of the aerosol box immediately (———), 1 minute (·····), 2 minutes (— — —), 3 minutes (— ·· —), 4 minutes (—— ——) and 5 minutes (— · — ·) after dispersion by the Twincer®. (Fig.4A: n=3; Fig.4B n=4)

To obtain an indication of the amount of drug that a mouse actually inhales, an *in vitro* measurement was performed by withdrawing 20 ml/min of air during 5 minutes from the exposure chamber. It was shown that this volume contained about 0.02% of each dose, an amount that consequently can be assumed as the administered dose. The assumption of 20 ml/min was on the low side, since respiratory minute volumes of mice of 45 – 50 ml/min have been described (23, 24).
In vivo deposition of inhaled powder in the lungs from the aerosol box was demonstrated by exposing mice to rhodamin B. Rhodamin B had a similar particle size distribution as spray dried hemin (Table 1). By using fluorescent microscopy, rhodamin deposition on the epithelial lining of the airways was demonstrated (figure 7). Rhodamin deposition was detected from the trachea until the large airways.

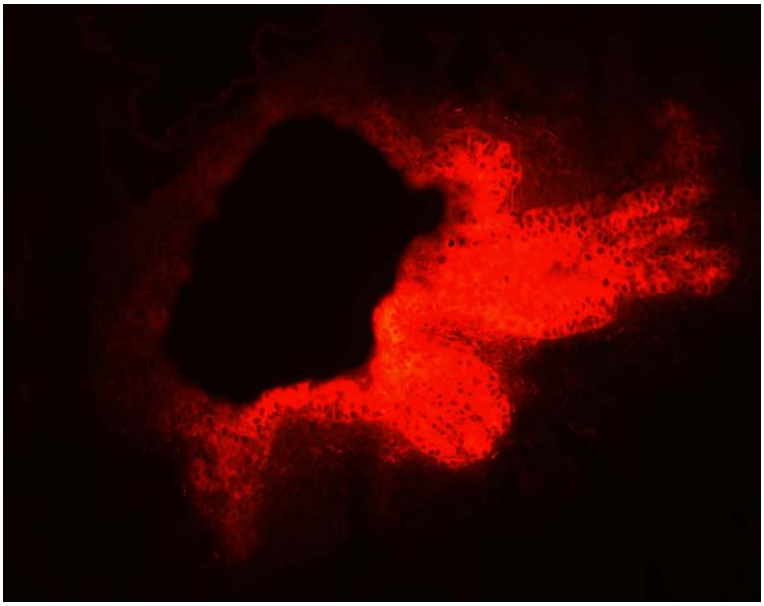

Figure 7. Example of rhodamin fluorescence in lung tissue. The fluorescent signal (red) of rhodamine in lung tissue is shown to be present in the epithelial lining of the airways. Magnification: 20x

Proof of concept
Different doses of spray dried hemin were administered to achieve HO-1 induction in lung tissue. Figure 8 shows a clear relationship between the dose of spray dried hemin and the level of HO-1 protein expression in lung

homogenate. Furthermore, inhalation of inert powder (spray dried inulin) containing sodium chloride did not result in increased expression of HO-1. HO-1 protein expression was found to be particularly increased in alveolar macrophages (figure 9). The airway epithelium stains faintly and shows no differences after hemin inhalation. The HO-1 expression as shown with HO-1-staining in the spray dried inulin exposed mice was comparable to control.

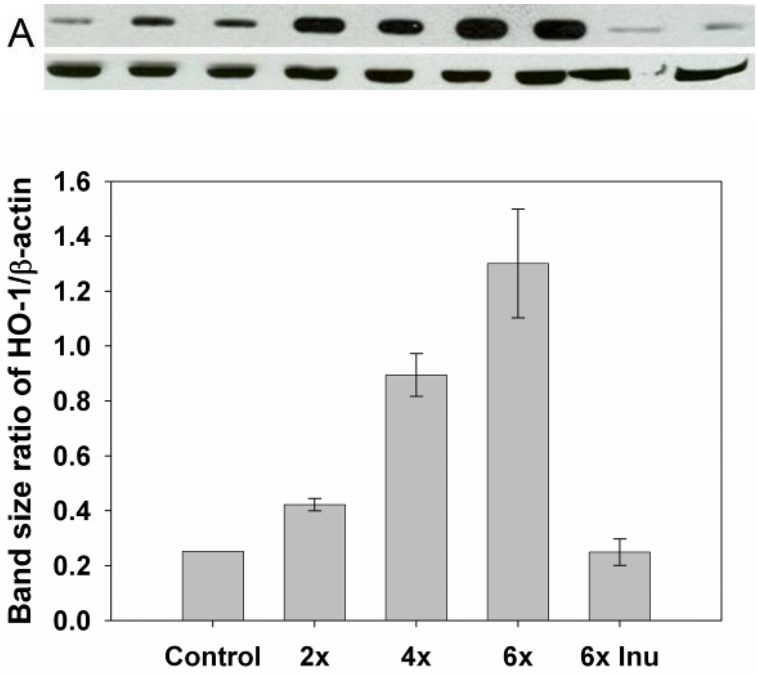

Figure 8. (A) HO-1 protein expression. Protein bands for HO-1 (top) and ß-actin (loading control, bottom) detected by Western blot analysis are shown; each band represents 1 mouse. From left to right: one control mouse that was not exposed to aerosol, 2 mice that were exposed to 2, 4 or 6 doses (2x, 4x and 6x) of spray dried hemin during 10, 20 or 30 minutes, respectively, and finally 2 mice that were exposed to 6 doses spray dried inulin (6x inu) during 30 minutes. **(B)** Ratio of the HO-1 and □-actin band size as obtained with Western blot for the different groups. Error bars indicate highest and lowest values.

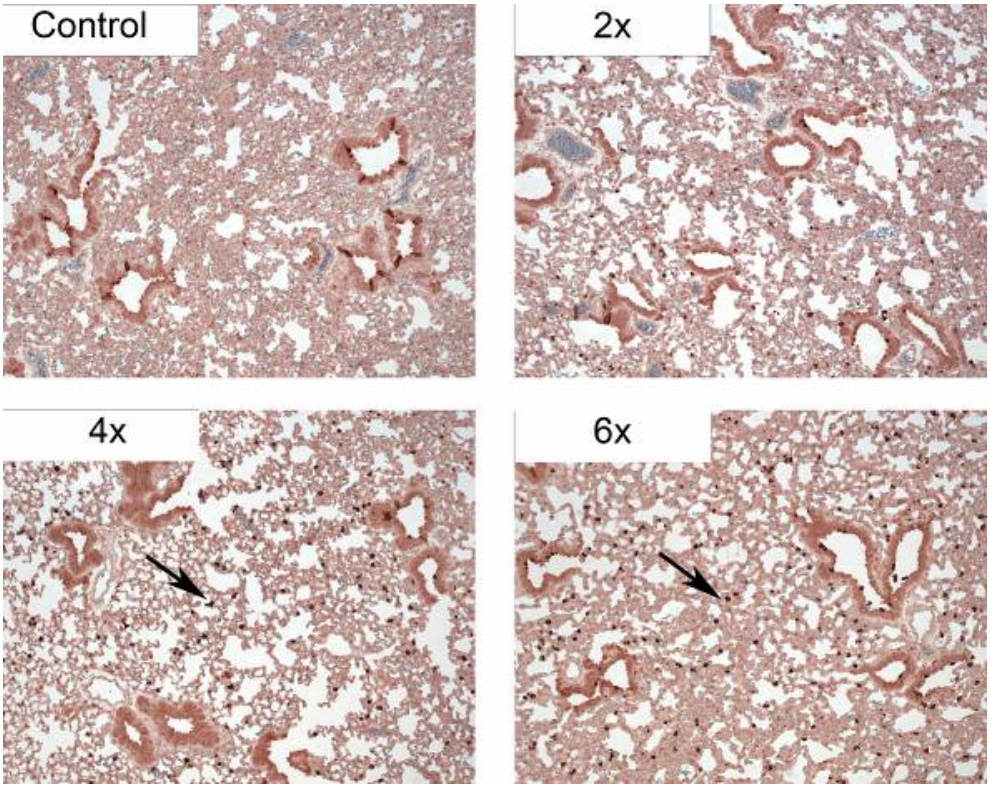

Figure 9. A representative picture of the HO-1 expression (dark red) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown (25x magnification). Particularly, alveolar macrophages (indicated with an arrow) show an increased HO-1 expression after hemin inhalation. Magnification: 25x

The inflammatory side effects of spray dried hemin inhalation were studied by histological staining of leukocytes in lung tissue. Visual observation showed increased numbers of leukocytes surrounding the airways after inhalation of 4 and 6 doses of spray dried hemin, while in the control mouse and after inhalation of 2 doses of spray dried hemin this was not visible (figure 10).

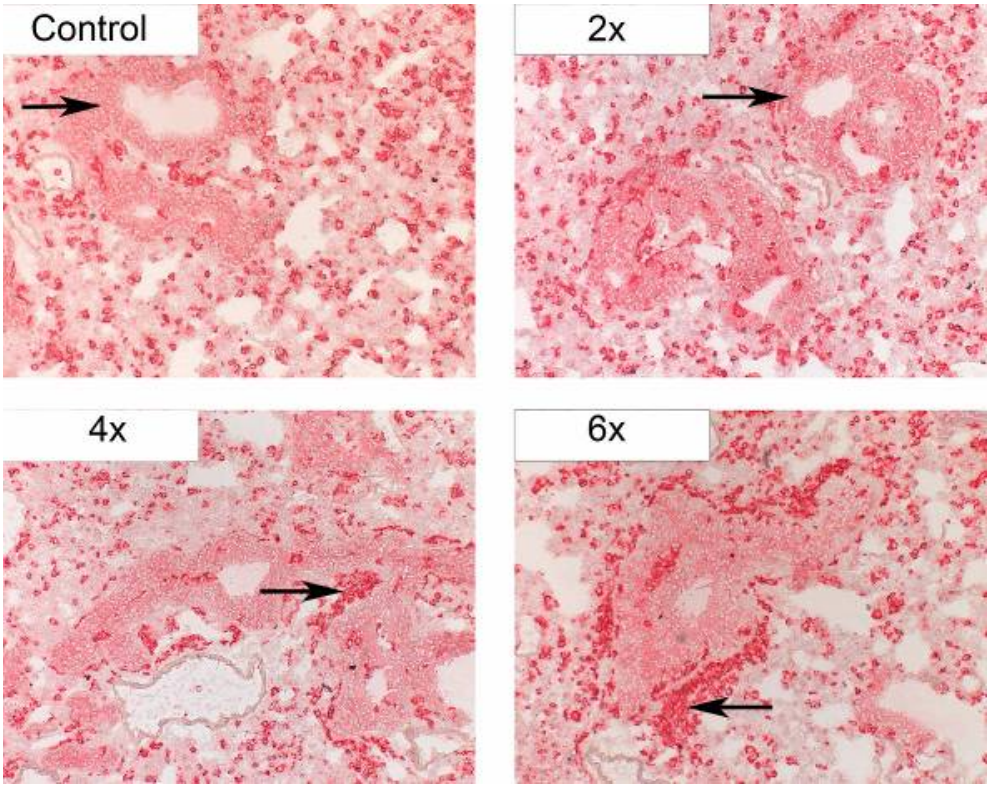

Figure 10. Leukocytes expressed in lung tissue. A representative picture of the leukocyte expression (CD45 positive cells) in lung tissue of the control and after inhalation of 2, 4 and 6 doses of spray dried hemin is shown. Arrows in top pictures indicate airways; arrows in bottom pictures indicate areas with increased numbers of leukocytes surrounding the airways. Magnification: 50x

DISCUSSION

Hemin, as shown in this study, can be formulated as a powder for inhalation by spray drying. Furthermore, spray dried hemin resulted in dose-dependent HO-1 induction after nose-only exposure of mice to the aerosol, showing that dry powder inhalation of hemin is possible and effective in inducing the HO-1 metabolic pathway.

Spray drying of hemin was performed from two solutions that had initial high pH values. An advantage of spray drying an ammonia solution that contains hemin is that ammonia evaporates during spray drying, resulting in pure hemin powder. In contrast, spray drying of the neutralized sodium hydroxide solution results in a powder that contains 37% w/w sodium

chloride and 63% w/w hemin. However, the latter solution results in a powder that dissolves much more rapidly than the pure spray dried powder. This is attributed to the presence of sodium chloride in the spray dried powder which improves wetting and increases the surface over which dissolution occurs. The technique may be beneficial for other poorly soluble compounds too, especially for those compounds that have a pH-dependent solubility.

Spray drying has become one of the standard techniques for the preparation of inhalation powders (25, 26). One of the advantages of spray drying over conventional techniques such as milling is the improved control over the particle size. From both the sodium hydroxide and ammonia solution the obtained powders were in the appropriate size range for inhalation, as measured with laser diffraction and cascade impactor analysis.

Clearly, the sodium chloride in the formulation did not increase HO-1 expression as was shown by the similarity in band size of HO-1 expression of the inulin control, after 6 doses, and the control that did not receive any aerosol.

The suitability of the Twincer-based aerosol box for pulmonary administration in freely breathing mice was demonstrated in this study. The *in vitro* laser diffraction measurements showed that an aerosol consisting of particles suitable for inhalation is present in the aerosol box for at least 5 minutes. Moreover, the in vivo study with rhodamin B showed that the aerosol reached all parts of the lung. It should however be realized that the individual mouse receives only a small fraction, about 0.02%, of the total dose in the Twincer. The major limitation in this setup is the relative small volume that is inhaled by mice.

One study exists where inhalation of hemin in human subjects has been investigated (13). However, the dose in our study is much higher than the dose used in the human study. The nebulized dose in the human study was 2 ml of 10^{-4} M hemin, which is equivalent to 1.6 μg/kg for a person of 80 kg. The lowest dose in our study is estimated to be 171 μg/kg, which corresponds to at least 100 times the dose used by Horvath *et al.* (13).

Since there was no information available on the dose-effect relationship of hemin we exposed mice to different doses. The dose escalation in our study was thus 171, 342 and 523 μg/kg. The relatively high doses that the mice inhaled led to concerns about the toxicity of hemin since it has been reported that hemin possesses oxidative properties especially at high

doses which is due to excess iron (27). Ambiguous effects have been ascribed to hemin (28). In moderate amounts and bound to proteins it is an essential element in various biological processes (10, 29). Large amounts of hemin can be toxic by mediating oxidative stress and inflammation, which is mainly due to release of iron (27).
The higher doses indeed led to infiltration of leukocytes into the airways. This inflammatory cell recruitment could be the result of increased production of inflammatory mediators by epithelial cells in response to toxic effects of the increasing dose of spray dried hemin. This possibility is in agreement with other *in vitro* and *in vivo* studies showing increased adhesion molecule expression and tissue infiltration of leukocytes after hemin administration to vascular cells (30, 31). On the other hand, we found that 171 µg/kg hemin did not result in increased numbers of inflammatory cells. However, inhalation of this dose of spray dried hemin was sufficient for a clear increase in HO-1 expression in lung tissue, indicating that this was a safe and effective dose of spray dried hemin.
To conclude, we presented a novel preparation of a hemin formulation by spray drying and showed proof of concept of the hemin formulation and a new pulmonary administration technique. The substance, hemin, induced the HO-1 expression in a dose dependent manner. Future studies investigating the utility of inhaled hemin in treating disease states are warranted.

ACKNOWLEDGEMENTS

The authors wish to thank the research workshop of the Faculty of Medicine for constructing the aerosol box, Paul Hagedoorn for his assistance with the aerosol box measurements, Pieter Klok for his assistance with the animal experiments, Marie Geerlings for the immunohistologic stainings, and Prof Wim Timens for aiding with evaluation of the histology.

REFERENCES

1. L.E. Otterbein, F.H. Bach, J. Alam, M. Soares, H. Tao Lu, M. Wysk, R.J. Davis, R.A. Flavell and A.M. Choi, Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway, Nat. Med., 4 (2000) 422-8.
2. L.E. Otterbein and A.M. Choi, Heme oxygenase: colors of defense against cellular stress, Am. J. Physiol. Lung Cell. Mol. Physiol., 6 (2000) L1029-37.
3. D. Willis, A.R. Moore, R. Frederick and D.A. Willoughby, Heme oxygenase: a novel target for the modulation of the inflammatory response, Nat. Med., 1 (1996) 87-90.
4. E.P. Carter, C. Garat and M. Imamura, Continual emerging roles of HO-1: protection against airway inflammation, Am. J. Physiol. Lung Cell. Mol. Physiol., 1 (2004) L24-5.
5. R. Stocker, Y. Yamamoto, A.F. McDonagh, A.N. Glazer and B.N. Ames, Bilirubin is an antioxidant of possible physiological importance, Science, 4792 (1987) 1043-6.
6. A.M. Choi and J. Alam, Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury, Am. J. Respir. Cell. Mol. Biol., 1 (1996) 9-19.
7. P. Maestrelli, C. Paska, M. Saetta, G. Turato, Y. Nowicki, S. Monti, B. Formichi, M. Miniati and L.M. Fabbri, Decreased haem oxygenase-1 and increased inducible nitric oxide synthase in the lung of severe COPD patients, Eur. Respir. J., 6 (2003) 971-6.
8. D.J. Slebos, H.A. Kerstjens, S.R. Rutgers, H.F. Kauffman, A.M. Choi and D.S. Postma, Haem oxygenase-1 expression is diminished in alveolar macrophages of patients with COPD, Eur. Respir. J., 4 (2004) 652-3; author reply 653.
9. J.E. Repine, A. Bast and I. Lankhorst, Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group, Am. J. Respir. Crit. Care Med., 2 Pt 1 (1997) 341-57.
10. A. Almolki, C. Taille, G.F. Martin, P.J. Jose, C. Zedda, M. Conti, J. Megret, D. Henin, M. Aubier and J. Boczkowski, Heme oxygenase attenuates allergen-induced airway inflammation and hyperreactivity in guinea pigs, Am. J. Physiol. Lung Cell. Mol. Physiol., 1 (2004) L26-34.
11. Z.A. Khan, Y.P. Barbin, M. Cukiernik, P.C. Adams and S. Chakrabarti, Heme-oxygenase-mediated iron accumulation in the liver, Can. J. Physiol. Pharmacol., 7 (2004) 448-56.

12. P.J. Barnes and T.T. Hansel, Prospects for new drugs for chronic obstructive pulmonary disease, The Lancet, 9438 (2004) 985.
13. I. Horvath, L.E. Donnelly, A. Kiss, P. Paredi, S.A. Kharitonov and P.J. Barnes, Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress, Thorax, 8 (1998) 668-72.
14. S.P. Duddu, S.A. Sisk, Y.H. Walter, T.E. Tarara, K.R. Trimble, A.R. Clark, M.A. Eldon, R.C. Elton, M. Pickford, P.H. Hirst, S.P. Newman and J.G. Weers, Improved lung delivery from a passive dry powder inhaler using an Engineered PulmoSphere powder, Pharm. Res., 5 (2002) 689-95.
15. C. Dunbar, G. Scheuch, K. Sommerer, M. DeLong, A. Verma and R. Batycky, In vitro and in vivo dose delivery characteristics of large porous particles for inhalation, Int. J. Pharm., 1-2 (2002) 179-89.
16. A.H. De Boer, P.P.H. Le Brun, H.G. van der Woude, P. Hagedoorn, H.G. Heijerman and H.W. Frijlink, Dry powder inhalation of antibiotics in cystic fibrosis therapy, part 1: development of a powder formulation with colistin sulfate for a special test inhaler with an air classifier as de-agglomeration principle, Eur. J. Pharm. Biopharm., 1 (2002) 17-24.
17. P.P.H. Le Brun, A.H. De Boer, G.P. Mannes, D.M. De Fraiture, R.W. Brimicombe, D.J. Touw, A.A. Vinks, H.W. Frijlink and H.G. Heijerman, Dry powder inhalation of antibiotics in cystic fibrosis therapy: part 2. Inhalation of a novel colistin dry powder formulation: a feasibility study in healthy volunteers and patients, Eur. J. Pharm. Biopharm., 1 (2002) 25-32.
18. A.H. De Boer, P. Hagedoorn, E.M. Westerman, P.P. Le Brun, H.G. Heijerman and H.W. Frijlink, Design and in vitro performance testing of multiple air classifier technology in a new disposable inhaler concept (Twincer((R))) for high powder doses, Eur. J. Pharm. Sci., 3 (2006) 171-8.
19. http://www.ph.tn.tudelft.nl/DIPlib/, (accessed 01/02/2007)
20. Y.S. Wu, H.W. Frijlink, L.J. van Vliet, I. Stokroos and K. van der Voort Maarschalk, Location-dependent analysis of porosity and pore direction in tablets, Pharm. Res., 8 (2005) 1399-405.
21. T.W. Ridler and S. Calvard, Picture tresholding using an iterative selection method, IEEE Trans. Syst. Man Cybern, (1978) 630-632.
22. J. Bauer and J. Fornnarino, High-performance liquid chromatographic analysis of "available" hemin in hematin solutions, J. Chromatogr., (1984) 378-82.

23. P.C. Withers, Metabolic, respiratory and haematological adjustments of the little pocket mouse to circadian torpor cycles, Respir Physiol, 3 (1977) 295-307.
24. G.A. Fairchild, Measurement of respiratory volume for virus retention studies in mice, Appl Microbiol, 5 (1972) 812-8.
25. N.R. Rabbani and P.C. Seville, The influence of formulation components on the aerosolisation properties of spray-dried powders, J. Control. Release, 1 (2005) 130-40.
26. G.S. Zijlstra, W.L.J. Hinrichs, A.H. De Boer and H.W. Frijlink, The role of particle engineering in relation to formulation and de-agglomeration principle in the development of a dry powder formulation for inhalation of cetrorelix, Eur. J. Pharm. Biopharm., 2 (2004) 139-149.
27. L. Goldstein, Z.P. Teng, E. Zeserson, M. Patel and R.F. Regan, Hemin induces an iron-dependent, oxidative injury to human neuron-like cells, J. Neurosci. Res., 1 (2003) 113-21.
28. F.A. Wagener, H.D. Volk, D. Willis, N.G. Abraham, M.P. Soares, G.J. Adema and C.G. Figdor, Different faces of the heme-heme oxygenase system in inflammation, Pharmacol. Rev., 3 (2003) 551-71.
29. B.O. Attuwaybi, R.A. Kozar, S.D. Moore-Olufemi, N. Sato, H.T. Hassoun, N.W. Weisbrodt and F.A. Moore, Heme oxygenase-1 induction by hemin protects against gut ischemia/reperfusion injury, J. Surg. Res., 1 (2004) 53-7.
30. Wagener, E. Feldman, T. de Witte and N.G. Abraham, Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells, Proc. Soc. Exp. Biol. Med., 3 (1997) 456-63.
31. F.A. Wagener, A. Eggert, O.C. Boerman, W.J. Oyen, A. Verhofstad, N.G. Abraham, G. Adema, Y. van Kooyk, T. de Witte and C.G. Figdor, Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase, Blood, 6 (2001) 1802-11.