Inhaled Agonists of Soluble Guanylate Cyclase Induce Selective Pulmonary Vasodilation

Oleg V. Evgenov^{1*}, Daniel S. Kohane^{2,3*}, Kenneth D. Bloch¹, Johannes-Peter Stasch⁴, Gian P. Volpato¹, Evangelia Bellas³, Natalia V. Evgenov⁵, Emmanuel S. Buys¹, Mark J. Gnoth⁴, Amanda R. Graveline¹, Rong Liu¹, Dean R. Hess⁶, Robert Langer³, and Warren M. Zapol¹

¹Department of Anesthesia and Critical Care and Anesthesia Center for Critical Care Research, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; ²Department of Anesthesiology and Laboratory for Biomaterials and Drug Delivery, Children's Hospital, Boston, Massachusetts; ³Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts; ⁴Pharma Research Center, Bayer HealthCare, Wuppertal, Germany; ⁵Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and ⁶Department of Respiratory Care, Massachusetts General Hospital and Department of Anesthesia, Harvard Medical School, Boston, Massachusetts

Rationale: Nitric oxide-independent agonists of soluble guanylate cyclase (sGC) have been developed.

Objectives: We tested whether inhalation of novel dry-powder micro-particle formulations containing sGC stimulators (BAY 41-2272, BAY 41-8543) or an sGC activator (BAY 58-2667) would produce selective pulmonary vasodilation in lambs with acute pulmonary hypertension. We also evaluated the combined administration of BAY 41-8543 microparticles and inhaled nitric oxide (iNO). Finally, we examined whether inhaling BAY 58-2667 microparticles would produce pulmonary vasodilation when the response to iNO is impaired.

Methods: In awake, spontaneously breathing lambs instrumented with vascular catheters and a tracheostomy tube, U-46619 was infused intravenously to increase mean pulmonary arterial pressure to 35 mm Hg.

Measurements and Main Results: Inhalation of microparticles composed of either BAY 41-2272, BAY 41-8543, or BAY 58-2667 and excipients (dipalmitoylphosphatidylcholine, albumin, lactose) produced dose-dependent pulmonary vasodilation and increased transpulmonary cGMP release without significant effect on mean arterial pressure. Inhalation of microparticles containing BAY 41-8543 or BAY 58-2667 increased systemic arterial oxygenation. The magnitude and duration of pulmonary vasodilation induced by iNO were augmented after inhaling BAY 41-8543 microparticles. Intravenous administration of 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), which oxidizes the prosthetic heme group of sGC, markedly reduced the pulmonary vasodilator effect of iNO. In contrast, pulmonary vasodilation and transpulmonary cGMP release induced by inhaling BAY 58-2667 microparticles were greatly enhanced after treatment with ODQ.

Conclusions: Inhalation of microparticles containing agonists of sGC may provide an effective novel treatment for patients with pulmonary hypertension, particularly when responsiveness to iNO is impaired by oxidation of sGC.

Keywords: pulmonary hypertension; soluble guanylate cyclase; BAY 41-2272; BAY 41-8543; BAY 58-2667

(Received in original form July 29, 2007; accepted in final form September 13, 2007)

*These authors contributed equally to this article.

Supported in part by the National Institutes of Health (grants HL42397 to W.M.Z., HL70896 to K.D.B., and EB-00351 to R.L.) and the Research Council of Norway (grant 161151/V40 to O.V.E.).

Correspondence and requests for reprints should be addressed to Oleg V. Evgenov, M.D., Ph.D., Department of Anesthesia and Critical Care, Massachusetts General Hospital, 55 Fruit Street, Gray/Bigelow 444, Boston, MA 02114. E-mail: evgenov@etherdome.mgh.harvard.edu; oevgenov@partners.org

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 176, pp 1138-1145, 2007

Originally Published in Press as DOI: 10.1164/rccm.200707-1121OC on September 13, 2007
Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary hypertension is a heterogeneous group of life-threatening disorders with limited therapeutic options and poor outcome.

What This Study Adds to the Field

Inhalation of microparticles containing agonists of sGC may provide an effective novel treatment for patients with pulmonary hypertension, particularly when responsiveness to iNO is impaired by oxidation of sGC.

Pulmonary hypertension (PH) is a heterogeneous group of life-threatening disorders usually characterized by excessive pulmonary vasoconstriction. Progression of PH over time is associated with remodeling of the pulmonary vascular wall, localized thrombosis, and, when severe, right heart hypertrophy and failure (reviewed in Reference 1). Although there have been significant advances in the treatment of PH, including the clinical introduction of prostacyclin analogs, endothelin receptor antagonists, phosphodiesterase (PDE) inhibitors, and their combinations (reviewed in Reference 2), major unmet needs for additional therapeutic modalities remain.

In the lung, nitric oxide (NO) is synthesized from L-arginine by a family of NO synthases. NO activates soluble guanylate cyclase (sGC), which converts guanosine 5'-triphosphate (GTP) to cGMP, leading to vasodilation and inhibition of platelet aggregation and vascular smooth cell proliferation (reviewed in Reference 3). Two novel drug classes that modulate the sGC-cGMP signal transduction in an NO-independent manner have been developed: sGC stimulators can enhance the sensitivity of reduced sGC to low levels of NO, whereas sGC activators can increase sGC enzyme activity even when the enzyme is oxidized and unresponsive to NO (reviewed in Reference 4). We reported that, in sheep, intravenous administration of the sGC stimulator BAY 41-2272 reversed acute PH induced by infusion of the thromboxane analog U-46619, but had limited pulmonary vascular selectivity (5). Similar pulmonary vasodilator effects of intravenous BAY 41-2272 were found in fetal and newborn lambs after partial ligation of the ductus arteriosus (a model of persistent PH of the newborn) (6). Furthermore, in rodent models of hypoxia- or monocrotaline-induced PH, oral treatment or intramuscular injections of BAY 41-2272 or oral administration of the sGC activator BAY 58-2667 attenuated the increase in right ventricular

systolic pressure, right ventricular hypertrophy, and structural remodeling of the lung's vasculature (7, 8).

Treatment of PH with systemically administered vasodilators including NO-releasing drugs, prostacyclin analogs, PDE inhibitors, as well as agonists of sGC can be associated with potentially catastrophic systemic hypotension and impairment of pulmonary gas exchange (9). In contrast, targeted drug delivery to the lungs via inhalation can result in a rapid onset of action, high local bioavailability, and low metabolism, potentially avoiding or reducing systemic side effects (reviewed in Reference 10). In this study, we tested the hypothesis that inhalation of novel, biodegradable, dry-powder microparticles containing sGC stimulators BAY 41-2272 (3-[4-amino-5-cyclopropylpyrimidine-2-yl]-1-[2-fluorobenzyl]-1H-pyrazolo[3,4-*b*]pyridine) and BAY 41-8543 (3-[4,6-diamino-5-morpholine-pyrimidine-2-yl]-1-[2-fluorobenzyl]-1H-pyrazolo[3,4-*b*]pyridine), or an sGC activator, BAY 58-2667 (4-[(4-carboxybutyl){2-[(4-phenylethylbenzyl)oxy]phenylethyl}amino)methyl] benzoic acid), would provide selective pulmonary vasodilation in an ovine model of PH. We also examined whether the combined administration of an inhaled sGC stimulator and inhaled NO (iNO) gas would produce greater pulmonary vasodilation than either intervention alone. A final goal was to test whether inhalation of an sGC activator would produce pulmonary vasodilation during a state of impaired responsiveness to iNO after oxidation of sGC by 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ). Some of the results of this study have been previously reported in the form of an abstract (11).

METHODS

This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council and was approved by the institutional subcommittee on research animal care. See the online supplement for additional details on methods.

Preparation and *in Vitro* Characterization of Microparticles

To produce blank microparticles, a solution of dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Alabaster, AL), bovine serum albumin, and α -lactose monohydrate (Sigma-Aldrich, St. Louis, MO) (60:20:20, wt/wt/wt) was spray-dried (190 mini spray dryer; Büchi, Postfach, Switzerland). For microparticles containing BAY 41-2272 (5% of the particles by weight), BAY 41-8543 (5% of the particles by weight), or BAY 58-2667 (0.1, 1, or 2.5% of the particles by weight), the test compounds were added to the final solution of DPPC, albumin, and lactose (DAL). BAY 41-2272, BAY 41-8543, and BAY 58-2667 were provided by Bayer HealthCare AG (Wuppertal, Germany).

Microparticles were imaged with a JSM 6060 scanning electron microscope (JEOL, Peabody, MA). The deposition of microparticles was evaluated *in vitro* with a cascade impactor (1-ACFM nonviable particle-sizing sampler; Andersen Instruments, Smyrna, GA) (12). Bulk mass density was estimated by measuring the volume of 50 mg of microparticles.

Instrumentation and Hemodynamic Measurements

Thirty-five lambs (16–35 kg) were anesthetized with ketamine (15 mg · kg⁻¹, intramuscularly) and propofol (0.1–0.2 mg · kg⁻¹ · min⁻¹, intravenously) and instrumented with a 7.5-Fr pulmonary artery thermal dilution catheter (Edwards Lifesciences, Irvine, CA) inserted via the left jugular vein, a polyvinylchloride catheter (inner diameter, 1.5 mm) in the left common carotid artery, and an 8.0-mm cuffed tracheostomy tube (SIMS Portex, Keene, NH), as described previously (5, 13). The animals were allowed at least 2 hours for recovery from anesthesia. Mean arterial pressure (MAP), mean pulmonary arterial pressure (PAP), and central venous pressure were recorded continuously (PowerLab 8SP; ADInstruments, Colorado Springs, CO). Cardiac output (SAT2; Edwards Lifesciences) and pulmonary capillary wedge pressure were measured at 15-minute time intervals. Cardiac index, pulmonary vascular resistance index (PVRI), systemic vascular resistance index (SVRI), stroke volume index,

right ventricle stroke work index (RVSWI), and left ventricle stroke work index were calculated on the basis of standard equations (13).

Experimental Protocols

During the study, the lambs were awake, breathed spontaneously at an inspired oxygen fraction (F_IO₂) of 0.7 via a ventilator (model 7200; Puritan Bennett, Pleasanton, CA), and received an intravenous infusion of lactated Ringer's solution (8 ml · kg⁻¹ · h⁻¹). After baseline measurements had been performed, U-46619 (Cayman Chemical, Ann Arbor, MI) was infused intravenously (1–2 µg · kg⁻¹ · min⁻¹) to increase the mean PAP to approximately 35 mm Hg (5, 13). The effects of each new and subsequent intervention were tested after a 30-minute period of stable PH.

Four lambs received 10-minute inhalations of nebulized ethanol (8 ml) delivered via an oxygen-powered nebulizer (PARI LC Star; PARI Respiratory Equipment, Monterey, CA) connected directly to the tracheostomy tube. This was followed by inhalations of nebulized BAY 41-2272 (0.1, 0.3, and 1 mg · kg⁻¹) dissolved in ethanol (8 ml). The time intervals between each drug treatment were at least 1 hour.

Three animals inhaled blank DAL microparticles (100 mg) delivered into the trachea in synchrony with inspiration. In experiments with DAL microparticles containing BAY 41-2272, BAY 41-8543, or BAY 58-2667, all doses refer to the amount of the active compound inhaled.

Twelve animals received microparticles composed of DAL and BAY 41-2272 or BAY 41-8543 (0.05, 0.1, and 0.15 mg · kg⁻¹) inhaled in random order with 2-hour time intervals between each dose (*n* = 6 lambs per group). In an additional six lambs, iNO (10 ppm) was first administered for 10 minutes, as described previously (13). Thirty minutes later, DAL/BAY 41-8543 microparticles (0.1 mg · kg⁻¹) were inhaled. Fifteen minutes after inhalation of these microparticles, a second dose of iNO (10 ppm) was administered for 10 minutes. Two hours later, a continuous intravenous infusion of the PDE inhibitor zaprinast (1,4-dihydro-5-[2-propoxyphenyl]-7H-1,2,3-triazolo[4,5-*d*]pyrimidine-7-one; BIOMOL, Plymouth Meeting, PA) was started at 1 mg · kg⁻¹ · hour⁻¹. Thirty minutes later, the infusion rate of U-46619 was increased by two- to threefold to maintain the mean PAP at approximately 35 mm Hg. Thereafter, inhalation of DAL/BAY 41-8543 microparticles (0.1 mg · kg⁻¹) was repeated.

Five lambs received inhalations of DAL/BAY 58-2667 microparticles (0.001, 0.01, and 0.1 mg · kg⁻¹) administered in random order with 2-hour time intervals between each dose. In another group of five lambs, iNO (40 ppm) was administered for 10 minutes followed, 30 minutes later, by inhalation of DAL/BAY 58-2667 microparticles (0.01 mg · kg⁻¹). Three hours later, the sGC inhibitor ODQ (Cayman Chemical) was injected intravenously (1 mg · kg⁻¹), and a second dose of iNO (40 ppm) was administered for 10 minutes. Thirty minutes later, ODQ (1 mg · kg⁻¹) was injected again, and the animals received a second inhalation of DAL/BAY 58-2667 microparticles (0.01 mg · kg⁻¹).

Analysis of Blood Gases, Transpulmonary cGMP Release, and Drug Concentrations

Blood samples were simultaneously obtained from the carotid and pulmonary arteries, and analyzed for pH, P_O₂, PCO₂, oxygen saturation, and hemoglobin concentration. Venous admixture (Q_s/Q_t) was calculated according to standard equations. Arterial and mixed venous plasma concentrations of cGMP were determined by radioimmunoassay (BT-340; Biomedical Technologies, Stoughton, MA). The quantity of cGMP released by the lung during each treatment was calculated as the product of cardiac output times the difference between the arterial and mixed venous plasma cGMP concentrations (5, 13). Arterial plasma concentrations of BAY 41-2272 and BAY 41-8543 were measured by high-performance liquid chromatography (2300HTLC; Cohesive Technologies, Franklin, MA) and tandem mass spectrometry (API 3000; PE Sciex, Concord, ON, Canada) (14).

Data Analysis

Data are expressed as means ± SEM. The half-time of the reversal of pulmonary vasodilation (T_{1/2}) after termination of NO inhalation was determined as previously described (5, 13). Treatment effects were tested by repeated measures analysis of variance followed by a Holm-Sidak post hoc test for comparisons with baseline PH or a Student-Newman-Keuls post hoc test for comparisons between treatments

(SigmaStat 3.0; Systat Software, Richmond, CA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inhalation of Aerosolized BAY 41-2272

Inhaled administration of aerosolized BAY 41-2272 by a conventional nebulizer produced, at higher doses, balanced pulmonary and systemic vasodilation as reflected by decreased mean PAP, PVRI, MAP, and SVRI ($P < 0.05$) with unchanged PVRI/SVRI, $\text{PaO}_2/\text{FiO}_2$, and Qs/Qt (see Table E1 of the online supplement). Pulmonary vasodilation lasted for 30 to 40 minutes (see Figure E1). Of note, by the end of the nebulization period a significant amount of BAY 41-2272 was dispersed into the ambient air, as well as precipitated on the inner surface of the nebulizer and respiratory circuit. Inhalation of the solvent (ethanol) alone had no significant effects on hemodynamics, $\text{PaO}_2/\text{FiO}_2$, or Qs/Qt (see Table E1).

In Vitro Characterization of Microparticles

Figure 1 shows a representative scanning electron micrograph of spray-dried DAL/BAY 41-8543 microparticles ranging from 2 to 6 μm in diameter. There was a morphologic continuum ranging from irregularly shaped particles to spheroids. There were no significant differences in respirable fraction (ranging from 53 to 63%), mass median aerodynamic diameter (ranging from 4.5 to 5.2 μm), and particle size geometric standard deviation (approximately 1.7) between blank DAL microparticles and microparticles encapsulating any of the three test compounds (see Figure E2). The bulk mass density of all microparticles studied was less than 0.04 $\text{g} \cdot \text{cm}^{-3}$.

Inhalation of Blank DAL Microparticles

Inhalation of blank DAL microparticles (without test compounds) in lambs with acute PH produced no changes in pulmonary and systemic hemodynamics (see Figure E3) or gas exchange (data not shown).

Figure 1. Representative scanning electron micrograph of dipalmitoylphosphatidylcholine/albumin/lactose-based microparticles encapsulating BAY 41-8543. Scale bar: 5 μm .

Inhalation of DAL/BAY 41-2272 and DAL/BAY 41-8543 Microparticles

As shown in Figure 2 (and see Table E2), inhalation of DAL/BAY 41-2272 or DAL/BAY 41-8543 microparticles produced dose-dependent reductions of mean PAP and PVRI ($P < 0.05$). The maximal level of pulmonary vasodilation was attained within 10 to 15 minutes after inhaling each dose of microparticles, and this effect lasted for at least 1 hour ($P < 0.05$). The pulmonary vasodilator effect of inhaled DAL/BAY 41-8543 was greater than that of inhaled DAL/BAY 41-2272 ($P < 0.05$). There were no significant changes in MAP after inhalation of either compound at the doses we tested; however, inhalation of DAL/BAY 41-8543 increased cardiac index and reduced SVRI ($P < 0.05$). Nevertheless, the magnitude of pulmonary vasodilation was much greater than that of systemic vasodilation, as reflected by decreased PVRI/SVRI at all dose levels ($P < 0.05$). In addition, inhaled DAL/BAY 41-8543 reduced RVSWI ($P < 0.05$). Other hemodynamic variables including pulmonary capillary wedge pressure, central venous pressure, heart rate, stroke volume index, and left ventricle stroke work index remained unchanged after inhaling either compound. At 0.1 and 0.15 $\text{mg} \cdot \text{kg}^{-1}$, inhaled DAL/BAY 41-8543 microparticles increased $\text{PaO}_2/\text{FiO}_2$ ($P < 0.05$), whereas Qs/Qt tended to decrease ($P = 0.09$). Inhaled DAL/BAY 41-8543 also markedly increased the rate of transpulmonary cGMP release into the circulation, as measured 15 minutes after administration ($P < 0.05$). The arterial plasma levels of BAY 41-2272 and BAY 41-8543 peaked 15 minutes after inhalation.

Combination of Inhaled DAL/BAY 41-8543 Microparticles with iNO or Zaprinast

As shown in Figure 3 (and see Table E3), iNO caused selective pulmonary vasodilation and increased $\text{PaO}_2/\text{FiO}_2$ ($P < 0.05$). After iNO was discontinued, mean PAP rapidly returned to baseline PH level, with a $T_{1/2} < 1$ minute. Inhaling DAL/BAY 41-8543 microparticles in this experimental group produced effects similar to those described above. When iNO was administered after inhalation of DAL/BAY 41-8543 microparticles, the reductions in mean PAP, PVRI, PVRI/SVRI, and RVSWI were all markedly greater than those occurring after either intervention alone ($P < 0.05$). In addition, the duration of pulmonary vasodilation after iNO was discontinued, as reflected by $T_{1/2}$, was prolonged about fivefold ($P < 0.05$). Furthermore, concurrent administration of zaprinast enhanced and prolonged the pulmonary vasodilator effect of inhaled DAL/BAY 41-8543 microparticles ($P < 0.05$). Combinations of inhaled DAL/BAY 41-8543 microparticles with iNO or zaprinast also increased $\text{PaO}_2/\text{FiO}_2$ ($P < 0.05$).

Inhalation of DAL/BAY 58-2667 Microparticles

As depicted in Figure 4 (and see Table E4), inhaled DAL/BAY 58-2667 microparticles potently reduced mean PAP, PVRI, PVRI/SVRI, and RVSWI in a dose-dependent manner ($P < 0.05$). The pulmonary vasodilation that occurred after inhaling DAL/BAY 58-2667 microparticles at doses of 0.01 and 0.1 $\text{mg} \cdot \text{kg}^{-1}$ lasted nearly 2 hours ($P < 0.05$). The largest dose also reduced MAP, central venous pressure, and SVRI and increased heart rate ($P < 0.05$). Furthermore, inhaling DAL/BAY 58-2667 microparticles increased transpulmonary cGMP release and arterial oxygenation ($P < 0.05$), whereas Qs/Qt tended to decrease ($P = 0.14$).

Inhalation of DAL/BAY 58-2667 Microparticles When Responsiveness to iNO Is Impaired

As shown in Figure 5 (and see Table E5), an intravenous injection of ODQ markedly reduced the pulmonary vasodilation

Figure 2. (A) Hemodynamic changes after inhalation of DAL/BAY 41-2272 or DAL/BAY 41-8543 microparticles in lambs with U-46619-induced acute pulmonary hypertension (PH). (B) Time course of changes in mean pulmonary arterial pressure after inhaling either type of microparticle at 0.15 mg $\cdot$ kg $^{-1}$. (C) Transpulmonary cGMP release rate after inhalation of DAL/BAY 41-8543 microparticles. Data represent means $\pm$ SEM ($n = 6$ per group); * $P < 0.05$ versus baseline PH; † $P < 0.05$ versus DAL/BAY 41-2272. MAP = mean arterial pressure; PAP = mean pulmonary arterial pressure; PVRI = pulmonary vascular resistance index; SVRI = systemic vascular resistance index.

induced by iNO and abolished its ability to increase $\text{PaO}_2/\text{FiO}_2$ and decrease $\dot{Q}_s/\dot{Q}_T$ ($P < 0.05$). In contrast, the reductions of mean PAP, PVRI, and RVSWI and the increase in transpulmonary cGMP release rate caused by inhaled DAL/BAY 58-2667 microparticles were each greatly enhanced after ODO administration ($P < 0.05$). The ability of DAL/BAY 58-2667 microparticles to enhance $\text{PaO}_2/\text{FiO}_2$ and reduce $\dot{Q}_s/\dot{Q}_T$ was not blocked by ODO ($P < 0.05$). Of note, DAL/BAY 58-2667 microparticles also caused marked systemic vasodilation when inhaled after ODO injection ($P < 0.05$).

DISCUSSION

In this study, we developed and evaluated novel inhaled selective pulmonary vasodilators composed of sGC agonists (BAY 41-2272, BAY 41-8543, and BAY 58-2667) encapsulated into dry-powder, lipid/protein/sugar-based microparticles. In an awake lamb model of acute PH, inhalation of these microparticles produced dose-dependent pulmonary vasodilation associated with increased transpulmonary cGMP release into the circulation and enhanced arterial oxygenation. Moreover, we found that inhaling microparticles containing BAY 41-8543 augmented the magnitude and duration of pulmonary vasodilation induced by iNO. In turn, the pulmonary vasodilator effect of BAY 41-8543 was enhanced by concurrent pharmacologic inhibition of the cGMP-metabolizing PDEs. We also demonstrated that after oxidation of the prosthetic heme group of sGC by ODO, the pulmonary vasodilator effect of iNO was greatly diminished, whereas inhaled BAY 58-2667 microparticles became even more effective in producing pulmonary vasodilation and increasing transpulmonary cGMP release.

Inhaled administration of pulmonary vasodilators represents an attractive alternative to oral or parenteral drug therapy, because inhaled drugs are directly delivered to the desired site of action, potentially increasing pulmonary selectivity and diminishing systemic side effects such as systemic vasodilation (reviewed in References 10 and 15). We first examined the effects of aerosolized BAY 41-2272, dissolved in ethanol and delivered by a conventional nebulizer. Of note, inhaling ethanol alone had no adverse effects on hemodynamics or pulmonary gas exchange, a finding that was in agreement with a previous investigation demonstrating no acute toxicity of nebulized ethanol on rat lungs (16). Inhalation of aerosolized BAY 41-2272 produced balanced pulmonary and systemic vasodilation without impairing systemic oxygenation or increasing venous admixture. However, to achieve a similar magnitude of the pulmonary vasodilator response, 20-fold higher doses of aerosolized BAY 41-2272 were required as compared with the intravenous doses we infused in a previous investigation (5). The latter observation is not surprising because commonly used nebulizers deliver only 8 to 20% of a drug into the lungs (9).

Because intrapulmonary delivery of BAY 41-2272 via nebulization required large drug doses and produced neither selective nor long-lasting pulmonary vasodilation, we employed an alternative approach. We used spray-dried microparticles composed of the major constituent of pulmonary surfactant, DPPC, as well as albumin and lactose (12, 17), to provide a vehicle for inhalation delivery of sGC agonists. A similar formulation of DPPC, albumin, and lactose (all are natural, biodegradable, and biocompatible materials) was reported to be suitable as a carrier for inhalation delivery of albuterol or estradiol in terms of particle

Figure 3. (A) Changes in mean pulmonary arterial pressure (PAP) and pulmonary vascular resistance index (PVRI) after administration of inhaled nitric oxide (iNO, 10 ppm), DAL/BAY 41-8543 microparticles ($0.1 \text{ mg} \cdot \text{kg}^{-1}$), or combinations of DAL/BAY 41-8543 microparticles with iNO or intravenous zaprinast ($1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) in lambs with U-46619-induced acute pulmonary hypertension (PH). (B) The half-time of reversal of pulmonary vasodilation ($T_{1/2}$) after discontinuation of iNO administered alone or after inhalation of DAL/BAY 41-8543 microparticles. (C) Time course of changes in PAP after inhaling DAL/BAY 41-8543 microparticles alone or in combination with intravenous zaprinast. Data represent means $\pm$ SEM ($n = 6$); * $P < 0.05$ versus baseline PH; † $P < 0.05$ versus iNO; ‡ $P < 0.05$ versus DAL/BAY 41-8543.

density, size, and drug release rate (12). Aerosol particles of 1 to $5 \mu\text{m}$ in mass median aerodynamic diameter are usually used for maximal deep-lung penetration; larger particles tend to deposit in the upper airways, and smaller ones are exhaled (10, 18). The DAL-based microparticles we used had a low mass density, an acceptable mass median aerodynamic diameter (approximately $5 \mu\text{m}$), and a high respirable fraction (approximately 60%), as measured *in vitro* with an Andersen cascade impactor. In agreement with a previous investigation of DAL-based microparticles demonstrating neither pulmonary nor systemic toxicity in guinea pigs (17), inhalation of blank DAL microparticles produced no changes in either hemodynamics or gas exchange in our control animals.

Inhaling BAY 41-2272, BAY 41-8543, or BAY 58-2667 encapsulated in DAL microparticles produced dose-dependent pulmonary vasodilation in lambs with acute PH. This effect was accompanied by an increased rate of transpulmonary cGMP release into the circulation, consistent with the known ability of these compounds to directly activate lung sGC (19–21). The maximal level of pulmonary vasodilation was attained between 10 and 15 minutes after inhaling drug-containing microparticles (concurrent with peak arterial plasma drug concentrations), and pulmonary vasodilation persisted for at least 1 hour when both sGC stimulators were used and for almost 2 hours after inhalation of DAL/BAY 58-2667. The duration of pulmonary vasodilation induced by inhaling sGC agonists was longer than that produced by the inhaled prostacyclin analog iloprost (9), which has been approved by the U.S. Food and Drug Administration for the treatment of patients with chronic PH. Although a reduction in SVRI was noted after inhaling BAY 41-8543 and BAY 58-2667, there was no significant systemic arterial hypotension (except at the highest dose of BAY 58-2667 we used). Importantly, PVRI/SVRI decreased at all dose levels for all three test compounds, indicating selective pulmonary vasodilation. In

addition, BAY 41-8543 and BAY 58-2667 reduced the workload of the right ventricle, as evidenced by a decreased RVSWI. We found that, on an equimolar basis, the magnitude of pulmonary vasodilation after inhalation of BAY 41-8543 was greater than that produced by inhaling BAY 41-2272. Moreover, BAY 58-2667 was the most potent pulmonary vasodilator, producing this effect at an inhaled dose of $1 \mu\text{g} \cdot \text{kg}^{-1}$. The latter findings are in agreement with *in vitro* studies demonstrating that the vasodilator potencies of BAY 41-8543 and BAY 58-2667 are about 3- and 160-fold greater, respectively, than that of BAY 41-2272 (20, 21). Another important observation was that none of the sGC agonists we studied had adverse effects on pulmonary gas exchange: inhaling DAL-based microparticles containing either BAY 41-8543 or BAY 58-2667 increased the systemic arterial oxygen tension and tended to decrease venous admixture. Our model was not specifically designed to mimic the pulmonary vascular abnormalities seen in patients with acute lung injury. However, we would speculate that in patients with hypoxia due to major mismatching of ventilation and perfusion, inhalation of sGC agonists might augment systemic arterial oxygenation by selectively dilating vessels in well-ventilated lung regions, similar to the effects of iNO (22).

Combination therapies are being actively investigated for the management of PH (1, 2). Therefore, we evaluated the effects of combined administration of the inhaled sGC stimulator BAY 41-8543 with iNO or zaprinast. In agreement with previous studies, iNO produced selective pulmonary vasodilation, but this effect disappeared immediately after ceasing its administration (5, 13). However, when iNO was delivered after inhalation of DAL/BAY 41-8543 microparticles, pulmonary vasodilation and a corresponding reduction in RVSWI were markedly augmented as compared with either intervention given alone. Importantly, the duration of the pulmonary vasodilator response after discontinuation of iNO breathing was prolonged about fivefold. These

Figure 4. (A) Hemodynamic changes after inhalation of DAL/BAY 58-2667 microparticles in lambs with U-46619-induced acute pulmonary hypertension (PH). (B) Time course of changes in mean pulmonary arterial pressure (PAP) after inhaling three different doses of DAL/BAY 58-2667 microparticles. (C) Transpulmonary cGMP release rate after inhalation of DAL/BAY 58-2667 microparticles. Data represent means $\pm$ SEM ($n = 5$); * $P < 0.05$ versus baseline PH. See Figure 2 for definition of abbreviations.

observations support previous findings in ovine models of PH that sGC stimulators can augment the therapeutic efficacy of iNO, most likely by increasing the sensitivity of the enzyme (5, 6). In clinical practice, combined administration of an sGC stimulator with iNO might result in an increased proportion of patients with PH responding to therapy with low concentrations of iNO. In addition, prolongation of the pulmonary vasodilator effect of iNO by an sGC stimulator may facilitate chronic therapy by intermittent inhalations of NO gas. Another important observation was that concurrent intravenous infusion of zaprinast enhanced and prolonged the pulmonary vasodilator effect of inhaled BAY 41-8543. Zaprinast inhibits several cGMP-metabolizing PDEs including PDE5, an isoform that is highly expressed in the pulmonary vasculature (23). Our observation that zaprinast augments the effects of BAY 41-8543 in lambs with PH is supported by an *in vitro* study demonstrating that the PDE5 inhibitor sildenafil potentiates the relaxant effects of the sGC stimulator BAY 41-2272 in isolated rabbit aortic rings (24). Because progression of PH is associated with pulmonary vascular remodeling, combined stimulation of sGC and inhibition of PDE5 may be beneficial to produce antiproliferative effects, as shown in isolated human pulmonary artery smooth muscle cells (25). Taken together, our findings suggest that combining inhaled stimulators of sGC with iNO and/or PDE inhibitors may enhance their therapeutic efficacy.

A significant number of patients with acute or chronic PH have a minimal response to vasodilators including iNO and PDE inhibitors (26, 27). It is conceivable that in some of these patients, sGC may have become oxidized, rendering it resistant to activation

by endogenous and/or exogenous NO. In this study, we intravenously injected ODQ, which irreversibly oxidizes the ferrous form of sGC to the ferric form (28), to produce a state of impaired responsiveness to iNO. Correspondingly, administration of ODQ markedly reduced the ability of iNO to produce pulmonary vasodilation, improve arterial oxygenation, and decrease Q_s/Q_t . One of our most striking findings was that under such conditions the pulmonary vasodilation induced by inhalation of DAL/BAY 58-2667 microparticles was greatly augmented, and this effect was associated with an additional increase in transpulmonary cGMP release. These observations are in agreement with investigations demonstrating that BAY 58-2667 activates oxidized, NO-unresponsive sGC (21, 29). Importantly, DAL/BAY 58-2667 microparticles significantly enhanced arterial oxygenation and reduced Q_s/Q_t , and these effects were preserved despite prior treatment with ODQ. Our findings underscore the concept of using activators of sGC in the therapy of PH and related conditions, particularly in disease states characterized by NO resistance due to sGC oxidation (4).

From a clinical point of view, inhaling sGC agonists encapsulated into dry-powder microparticles is an attractive means of selective intrapulmonary drug delivery. Compared with nebulization, inhalation of dry-powder microparticles is faster and more efficient because of the much higher respirable fraction and minimal extrapulmonary drug losses. Moreover, inhaled encapsulated sGC agonists are likely to avoid most of the side effects associated with systemically administered vasodilators. Optimization of the controlled-release technology by varying the excipients,

Figure 5. Changes in mean pulmonary arterial pressure (PAP), pulmonary vascular resistance index (PVRI), and transpulmonary cGMP release after administration of inhaled nitric oxide (iNO, 40 ppm) or DAL/BAY 58-2667 microparticles ($0.01 \text{ mg} \cdot \text{kg}^{-1}$) before and after intravenous injection of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) ($1 \text{ mg} \cdot \text{kg}^{-1}$) in lambs with U-46619-induced acute pulmonary hypertension (PH). Data represent means $\pm$ SEM ($n = 5$); * $P < 0.05$ versus baseline PH; † $P < 0.05$ versus iNO; ‡ $P < 0.05$ versus DAL/BAY 58-2667.

particle type and morphology, and manufacturing process could further increase the pulmonary deposition and bioavailability of sGC agonists and extend the duration of therapeutic effect from a single application (17, 18). These factors may potentially improve patient compliance and overall treatment outcomes.

In conclusion, encapsulation of sGC agonists into lipid/protein/sugar-based microparticles for inhalational delivery represents a promising novel approach from both pharmaceutical and biological perspectives. Inhalation of such microparticles may provide an effective treatment modality for patients with PH, particularly when responsiveness to iNO is impaired. Further development and preclinical testing, including assessment of the long-term efficacy and safety of microparticles containing agonists of sGC, are warranted.

Conflict of Interest Statement: O.V.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.D.B. has served on the Scientific Advisory Board of Ikaria/INO Therapeutics LLC and research in his laboratory has been supported by a sponsored research agreement between Massachusetts General Hospital and Ikaria/INO Therapeutics LLC. J.-P.S. is a full-time employee of Bayer HealthCare and a coinventor in several patent applications on sGC agonists. G.P.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.V.E. does not have a financial relationship with a commercial entity that has an interest in the subject of

this manuscript. E.S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.G. is a full-time employee of Bayer HealthCare. A.R.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.R.H. is a consultant for Respiroics and PARI and his research has been supported by grants from INO Therapeutics. R.L. is a coinventor in several patents on formulations of aerosol microparticles. W.M.Z. receives royalties from patents on inhaled NO owned by Massachusetts General Hospital and licensed to Ikaria/INO Therapeutics LLC; he is chairman of the Scientific Advisory Board of INO Therapeutics LLC.

References

- McLaughlin VV, McGoon MD. Pulmonary arterial hypertension. *Circulation* 2006;114:1417–1431.
- Hooper MM, Rubin LJ. Update in pulmonary hypertension 2005. *Am J Respir Crit Care Med* 2006;173:499–505.
- Hampel V, Herget J. Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol Rev* 2000;80:1337–1372.
- Evgenov OV, Pacher P, Schmidt PM, Hasko G, Schmidt HH, Stasch JP. NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. *Nat Rev Drug Discov* 2006;5:755–768.
- Evgenov OV, Ichinose F, Evgenov NV, Gnoth MJ, Falkowski GE, Chang Y, Bloch KD, Zapol WM. Soluble guanylate cyclase activator reverses acute pulmonary hypertension and augments the pulmonary vasodilator response to inhaled nitric oxide in awake lambs. *Circulation* 2004;110:2253–2259.
- Deruelle P, Grover TR, Abman SH. Pulmonary vascular effects of nitric oxide–cGMP augmentation in a model of chronic pulmonary hypertension in fetal and neonatal sheep. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L798–L806.
- Deruelle P, Balasubramaniam V, Kunig AM, Seedorf GJ, Markham NE, Abman SH. BAY 41-2272, a direct activator of soluble guanylate cyclase, reduces right ventricular hypertrophy and prevents pulmonary vascular remodeling during chronic hypoxia in neonatal rats. *Biol Neonate* 2006;90:135–144.
- Dumitrescu R, Weissmann N, Ghofrani HA, Dony E, Beuerlein K, Schmidt H, Stasch JP, Gnoth MJ, Seeger W, Grimminger F, et al. Activation of soluble guanylate cyclase reverses experimental pulmonary hypertension and vascular remodeling. *Circulation* 2006;113:286–295.
- Lowson SM. Inhaled alternatives to nitric oxide. *Crit Care Med* 2005;33:S188–S195.
- Paton JS, Byron PR. Inhaling medicines: delivering drugs to the body through the lungs. *Nat Rev Drug Discov* 2007;6:67–74.
- Evgenov OV, Petersen B, Bellas E, Evgenov NV, Stasch JP, Hess DR, Ichinose F, Bloch KD, Langer R, Kohane DS, et al. Inhalation of microparticles containing stimulators of soluble guanylate cyclase produces potent pulmonary vasodilation [abstract]. *Proc Am Thorac Soc* 2006;3:A686.
- Vanbever R, Mintzes JD, Wang J, Nice J, Chen D, Batycky R, Langer R, Edwards DA. Formulation and physical characterization of large porous particles for inhalation. *Pharm Res* 1999;16:1735–1742.
- Evgenov OV, Busch CJ, Evgenov NV, Liu R, Petersen B, Falkowski GE, Petho B, Vas A, Bloch KD, Zapol WM, et al. Inhibition of phosphodiesterase 1 augments the pulmonary vasodilator response to inhaled nitric oxide in awake lambs with acute pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L723–L729.
- Schuhmacher J, Zimmer D, Tesche F, Pickard V. Matrix effects during analysis of plasma samples by electrospray and atmospheric pressure chemical ionization mass spectrometry: practical approaches to their elimination. *Rapid Commun Mass Spectrom* 2003;17:1950–1957.
- Gessler T, Schmehl T, Olschewski H, Grimminger F, Seeger W. Aerosolized vasodilators in pulmonary hypertension. *J Aerosol Med* 2002;15:117–122.
- Choi WS, Murthy GG, Edwards DA, Langer R, Klivanov AM. Inhalation delivery of proteins from ethanol suspensions. *Proc Natl Acad Sci USA* 2001;98:11103–11107.
- Ben-Jebria A, Chen D, Eskew ML, Vanbever R, Langer R, Edwards DA. Large porous particles for sustained protection from carbachol-induced bronchoconstriction in guinea pigs. *Pharm Res* 1999;16:555–561.
- Edwards DA, Ben-Jebria A, Langer R. Recent advances in pulmonary drug delivery using large, porous inhaled particles. *J Appl Physiol* 1998;85:379–385.
- Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A, Gerzer R, Minuth T, Perzborn E, Pleiss U, et al. NO-independent

- regulatory site on soluble guanylate cyclase. *Nature* 2001;410:212–215.
20. Stasch JP, Alonso-Alija C, Apeler H, Dembowski K, Feurer A, Minuth T, Perzborn E, Schramm M, Straub A. Pharmacological actions of a novel NO-independent guanylyl cyclase stimulator, BAY 41-8543: *in vitro* studies. *Br J Pharmacol* 2002;135:333–343.
21. Stasch JP, Schmidt P, Alonso-Alija C, Apeler H, Dembowski K, Haerter M, Heil M, Minuth T, Perzborn E, Pleiss U, *et al.* NO- and haem-independent activation of soluble guanylyl cyclase: molecular basis and cardiovascular implications of a new pharmacological principle. *Br J Pharmacol* 2002;136:773–783.
22. Rossaint R, Falke KJ, Lopez F, Slama K, Pison U, Zapol WM. Inhaled nitric oxide for the adult respiratory distress syndrome. *N Engl J Med* 1993;328:399–405.
23. Cohen AH, Hanson K, Morris K, Fouty B, McMurty IF, Clarke W, Rodman DM. Inhibition of cyclic 3',5'-guanosine monophosphate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. *J Clin Invest* 1996;97:172–179.
24. Priviero FB, Baracat JS, Teixeira CE, Claudino MA, De Nucci G, Antunes E. Mechanisms underlying relaxation of rabbit aorta by BAY 41-2272, a nitric oxide-independent soluble guanylate cyclase activator. *Clin Exp Pharmacol Physiol* 2005;32:728–734.
25. Wharton J, Strange JW, Moller GM, Growcott EJ, Ren X, Franklyn AP, Phillips SC, Wilkins MR. Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med* 2005;172:105–113.
26. Ichinose F, Roberts JD Jr, Zapol WM. Inhaled nitric oxide: a selective pulmonary vasodilator: current uses and therapeutic potential. *Circulation* 2004;109:3106–3111.
27. Chockalingam A, Gnanavelu G, Venkatesan S, Elangovan S, Jagannathan V, Subramaniam T, Alagesan R, Dorairajan S. Efficacy and optimal dose of sildenafil in primary pulmonary hypertension. *Int J Cardiol* 2005;99:91–95.
28. Schrammel A, Behrends S, Schmidt K, Koesling D, Mayer B. Characterization of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol Pharmacol* 1996;50:1–5.
29. Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, Arun Kumar HS, Meurer S, Deile M, Taye A, Knorr A, Lapp H, *et al.* Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 2006;116:2552–2561.