Ambrisentan and Tadalafil Synergistically Relax Endothelin-Induced Contraction of Rat Pulmonary Arteries

Faquan Liang, Suya Yang, Lina Yao, Luiz Belardinelli, John Shryock

Abstract—Endothelin receptor antagonists and phosphodiesterase type 5 inhibitors are used to treat pulmonary arterial hypertension. We tested the hypothesis that a selective endothelin type A receptor antagonist (ambrisentan) and a phosphodiesterase type 5 inhibitor (tadalafil) may act synergistically to relax endothelin-constricted pulmonary arteries. Rat isolated intrapulmonary arterial rings contracted with 8 nmol/L endothelin-1 were relaxed by 10 nmol/L ambrisentan and 30 nmol/L tadalafil alone by 26±3% and 21±1%, respectively, whereas both drugs in combination acted synergistically to relax arterial rings by 83±6%. The nonselective endothelin type A and B receptor antagonists bosentan (100 nmol/L) and macitentan (30 nmol/L) alone relaxed endothelin-contracted rings by 30±5% and 24±3%, respectively. Combinations of 30 nmol/L tadalafil with 100 nmol/L bosentan or 30 nmol/L macitentan relaxed endothelin-contracted rings by 53±5% or 46±7%, respectively; these values are similar to the calculated sums of the individual effects of these compounds. Denudation of endothelium from pulmonary arterial rings abolished the vasodilator response to 30 nmol/L tadalafil and the synergistic vasorelaxant effect of tadalafil with ambrisentan. In the presence of 1 μmol/L BQ-788, a selective endothelin type B receptor antagonist, the vasorelaxant effects of 10 nmol/L ambrisentan and 30 nmol/L tadalafil were additive but not synergistic. These data can be interpreted to suggest that ambrisentan and tadalafil synergistically inhibit endothelin-1-induced constriction of rat intrapulmonary arteries and that endothelin type B receptors in endothelium are necessary to enable a synergistic vasorelaxant effect of the drug combination. (Hypertension. 2012;59:705-711.)

Key Words: endothelin-1 ■ endothelin receptor antagonist ■ phosphodiesterase inhibitor ■ pulmonary artery ■ pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a chronic and progressive disease characterized by elevation of mean pulmonary arterial pressure and pulmonary vascular resistance, which may lead to right ventricular failure and death. Patients with PAH have a median survival time of 2.8 years if untreated. Current pharmacotherapies include prostacyclin analogues, endothelin (ET) receptor antagonists, and phosphodiesterase 5 (PDE5) inhibitors.

ET-1 is a potent vasoconstrictor that binds to 2 distinct G protein–coupled receptor subtypes, the ET type A (ET_{A}) and ET type B (ET_{B}) receptors. In the vasculature, smooth muscle cells express mainly the ET_{A} subtype of receptors, which contribute to vascular constriction and remodeling, whereas the ET_{B} subtype of receptors is predominantly expressed in endothelial cells. ET_{A} receptors mediate clearance of ET-1 and the release of nitric oxide and ET_{B} receptor-mediated vasodilation. Plasma ET-1 levels are elevated in PAH patients and strongly correlate with the severity of this disease. ET receptor antagonists have been approved for treatment of PAH. These include the nonselective or dual ET_{A}/ET_{B} receptor antagonist bosentan, and the selective ET_{A} receptor antagonist ambrisentan. Ambrisentan has K_{i} values of 1 nmol/L for ET_{A} and 195 nmol/L for ET_{B} receptor antagonism, resulting in an ~200-fold selectivity for the ET_{A} receptor. Both bosentan and ambrisentan block ET_{A} receptor-dependent vascular smooth muscle contraction, and this is thought to contribute to their effectiveness in treatment of PAH. However, bosentan but not ambrisentan also blocks endothelial cell ET_{B} receptors and may thereby reduce ET-1-mediated endothelial formation of nitric oxide and ET_{B} receptor-mediated clearance of ET-1.

PDEs degrade the intracellular second messengers cAMP and cGMP, both of which promote the relaxation of vascular smooth muscle. PDE5 is a cGMP-selective enzyme and one of the principal PDEs in the pulmonary vasculature. In patients with chronic PAH, PDE5 levels are increased in lung tissue, promoting the degradation of cGMP and favoring smooth muscle contraction. The PDE5 inhibitors sildenafil and tadalafil have been approved for treatment of patients with PAH.
cGMP in smooth muscle cells and enhance nitric oxide-mediated vasodilatation of the vasculature.\textsuperscript{15}

The use of combinations of drugs with different mechanisms of action is an evolving strategy for treatment of advanced PAH.\textsuperscript{16} Results from a recent clinical study of ambrisentan in combination with tadalafil indicated that the drug combination was safe and well tolerated and that no pharmacokinetic and safety interactions were observed.\textsuperscript{17} In the present study, we tested the hypotheses that the combination of ambrisentan and tadalafil may be more effective than either drug alone to reduce an ET-1 induced increase of pulmonary vascular constriction and that the synergistic effects of these drugs depend on the presence of both ETA and ET\textsubscript{B} receptors. The effects of ambrisentan and tadalafil alone and in combination to relax ET-contracted rat isolated pulmonary arteries were determined. For comparison, the effects and in combination to relax ET-contracted rat isolated pulmonary arteries were determined. (For details of methodology, please see the online-only Data Supplement.)

Materials and Methods

Materials
Human ET-1 (catalog No. 88-1-10B) and BQ-788 (catalog No. 88-2-55B) were purchased from American Peptide Company, Inc (Sunnyvale, CA). Ambrisentan was synthesized by Gilead Sciences, Inc (Foster City, CA). Tadalafil (cat No. SRP008000) and bosentan (cat No. SRP02325b) were purchased from Sequoia Research Products (Pangbourne, United Kingdom). Macitentan was purchased from Sai Advantium Pharma Ltd (Hyderabad, India).

Animals
Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). The use of animals was approved by the Animal Care and Use Committee of Gilead Sciences, Inc. Rats were housed in climate-controlled conditions with a 12-hour light and dark cycle and had free access to chow and water.

Pulmonary Artery Preparation and Isometric Tension Measurement
Male Sprague-Dawley rats (300–325 g) were anesthetized, their chests were opened, and intact right and left lungs were excised. Under a stereomicroscope, intrapulmonary arteries at sizes ranging from 100 to 500 \textmu m diameter were dissected and then cut into rings. Each ring was mounted to a pair of thin pins or wires in a myograph system, preincubated with selected drugs alone and in combination for 10 minutes, and then treated with 8 nmol/L ET-1 for 30 seconds. Rings were homogenized for measurement of cGMP content. (For details, please see the online-only Data Supplement.)

Measurement of cGMP
Isolated rat intrapulmonary arterial rings were mounted to a myograph system, preincubated with selected drugs alone and in combination for 10 minutes, and then treated with 8 nmol/L ET-1 for 30 seconds. Rings were homogenized for measurement of cGMP content. (For details, please see the online-only Data Supplement.)

Western Blot Analysis
To determine the effects of drugs on ET-1-induced phosphorylation (ie, activation) of myosin light-chain kinase (MLCK), isolated rat intrapulmonary artery rings were mounted to the myograph system and preincubated with selected drugs for 10 minutes. ET-1 was then added to each chamber for an additional 10 minutes. Vascular rings were homogenized for Western blot analysis with an anti-myosin light chain-2 (anti-MLC2) antibody or an anti-phospho-MLC2 (Thr18/Ser19) antibody. (For details, please see the online-only Data Supplement.)

Statistics
Data were expressed as mean±SEM and analyzed using 2-way ANOVA with Bonferroni post hoc tests or 1-way ANOVA with Newman-Keuls tests. All statistical analyses were performed using GraphPad Prism (version 5.0, GraphPad Software, Inc, La Jolla, CA). Differences among values were considered significant at \( P<0.05 \).

Results

Effects of the ET Receptor Antagonists Ambrisentan, Bosentan, and Macitentan, Individually and in Combination With Tadalafil, to Attenuate ET-1-Induced Contraction of Rat Pulmonary Arterial Rings

ET-1 contracted rat intrapulmonary arterial rings in a concentration-dependent manner with an EC\textsubscript{90} value of 7.4 nmol/L (Figure 1A). Therefore, in subsequent experiments, an ET-1 concentration of 8 nmol/L was used to elicit a submaximal contraction of rat intrapulmonary arterial rings that would be suitable for assay of the responses to ET receptor antagonists. The contractile response to 8 nmol/L ET-1 was stable and sustained for at least 60 minutes (Figure 1A).

To investigate synergistic effects of combinations of ET receptor antagonists and the PDES inhibitor tadalafil, we selected a low concentration of each compound to cause a modest inhibition of the ET-1-induced contraction by 20% to 30%. As shown in Figure 1B, 10 nmol/L ambrisentan, 30 nmol/L tadalafil, 100 nmol/L bosentan, and 30 nmol/L macitentan individually relaxed ET-1-constricted rat intact intrapulmonary arterial rings by 26±3%, 21±1%, 30±5%, and 24±3%, respectively. The same drug concentrations were used in all subsequent experiments. The combination of ambrisentan and tadalafil relaxed intrapulmonary arterial rings by 83±6% (\( P<0.01 \) versus ambrisentan or tadalafil alone), more than the calculated sum of individual effects of each drug (Figure 1B, dotted line), suggesting that ambrisentan and tadalafil synergistically inhibited ET-1-induced constriction of intrapulmonary arteries. In the absence and presence of tadalafil, the IC\textsubscript{50} values of ambrisentan were 18±4 and 3±2 nmol/L (\( n=5, P<0.05 \)), respectively, indicating that tadalafil increased the potency of ambrisentan to relax ET-1-constricted rat intrapulmonary arteries. Combinations of tadalafil with either bosentan or macitentan relaxed the ET-1-dependent contractions of arterial rings by 53±5% or 46±7%, respectively (\( P<0.05 \) versus bosentan, macitentan, or tadalafil alone or the combination of ambrisentan with tadalafil) (Figure 1B). The effects of tadalafil in combinations with either bosentan or macitentan were therefore additive but not synergistic.

The Synergistic Effect of Ambrisentan and Tadalafil Is Dependent on the Presence of Endothelium

In endothelium-denuded pulmonary arterial rings, tadalafil failed to inhibit contraction induced by ET-1, whereas ambrisentan, bosentan, and macitentan reduced ET-1-induced
contractile force by 23\% ± 3\%, 19\% ± 2\%, and 22\% ± 2\%, respectively (Figure 1C). A combination of tadalafil and ambrisentan was not more effective than ambrisentan alone and was not more effective than the combination of tadalafil and bosentan or macitentan in denuded rings (Figure 1C). These data suggest that the presence of endothelium is necessary to enable a response to tadalafil and is required for the synergistic effect of ambrisentan with tadalafil to relax pulmonary arteries constricted with ET-1.

The ET\textsubscript{B} Receptor in Endothelium Is Involved in the Synergistic Effect of Ambrisentan and Tadalafil

Although ambrisentan, bosentan, and macitentan are all antagonists of the smooth muscle ET\textsubscript{A} receptor, the latter 2 drugs may also block endothelial ET\textsubscript{B} receptor-dependent nitric oxide production.\textsuperscript{7,8} It is thus possible that the presence of synergism between tadalafil and ambrisentan, but not between tadalafil and either bosentan or macitentan, can be explained by the absence (ambrisentan) or presence (bosentan, macitentan) of an ET\textsubscript{B} receptor blocking effect. To test this hypothesis, intrapulmonary arterial rings were preincubated with 1 \mu mol/L BQ-788, a selective ET\textsubscript{B} receptor antagonist,\textsuperscript{19} for 10 minutes and then contracted with ET-1. In the continued presence of both agents, responses to tadalafil in combination with ambrisentan, bosentan, or macitentan were measured. As shown in Figure 2, in the presence of BQ-788, the vasorelaxant effect of ambrisentan combined with tadalafil was significantly reduced from 82\% ± 3\% to 57\% ± 6\% (n = 5; \( P < 0.05 \) versus the combination of ambrisentan with tadalafil). In contrast, the presence of BQ-788 did not have an effect on the relaxation induced by a combination of

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**Figure 1.** Effects of ambrisentan (AMB), bosentan (BOS), and macitentan (MAC) individually and in combination with tadalafil (TAD) to attenuate endothelin-1 (ET-1)-induced contraction of rat intrapulmonary arterial rings. A, ET-1 concentration-response curve for contraction (n = 4 experiments) and the time course of the response to 8 nmol/L ET-1 from a representative experiment. B, The combination of AMB and TAD produced a synergistic effect to relax 8 nmol/L ET-1-constricted rings, whereas the effects of TAD in combination with either BOS or MAC were additive (n = 6 experiments). Horizontal dotted lines represent the calculated sum of the individual effects of AMB and TAD. *\( P < 0.01 \) vs each compound alone, BOS + TAD, or MAC + TAD; #\( P < 0.01 \) vs BOS, MAC, or TAD. C, Denudation of endothelium from intrapulmonary arterial rings caused loss of vasorelaxant effect of TAD and synergistic or additive effect of TAD with AMB, BOS, or MAC to inhibit ET-1-induced vasoconstriction (n = 4 experiments). Concentrations of AMB, BOS, MAC, and TAD were 10, 100, 30, and 30 nmol/L, respectively. The three bars for TAD in B and C are the same pooled data.

**Figure 2.** Blockade of endothelin type B (ET\textsubscript{B}) receptor by BQ-788 led to loss of the synergistic effect of ambrisentan (AMB) and tadalafil (TAD) to relax ET-1-induced contraction of intrapulmonary arterial rings. Concentrations of ET-1, AMB, bosentan (BOS), macitentan (MAC), TAD, and BQ-788 were 8, 10, 100, 30, and 1000 nmol/L, respectively. *\( P < 0.05 \) vs AMB + TAD, n = 5 experiments.
tadalafil with either bosentan or macitentan. In the absence and presence of BQ-788, 30 nmol/L tadalafil relaxed ET-1 constricted rat intrapulmonary arteries by 23% and 11% (n=4, P<0.05), respectively, suggesting that blockade of ETB receptors reduces tadalafil-dependent vasorelaxation. These results indicate that ETB receptors on endothelial cells contribute to the effect of tadalafil in combination with ambrisentan (but not bosentan or macitentan) to relax ET-1–constricted pulmonary arteries.

In addition, we tested the effect of varying concentrations (1–1000 nmol/L) of IRL-1620, a selective ET B receptor agonist, on rat isolated intrapulmonary arterial rings. IRL-1620 did not induce intrapulmonary artery contraction, suggesting that ETB receptors do not contribute to ET-1–mediated vasoconstriction in our intrapulmonary artery preparations.

Effects of ET Receptor Antagonists, Tadalafil, and 1.-NAME on cGMP Production in Rat Isolated Intrapulmonary Arterial Rings

Activation of the ETB receptor in endothelium leads to production of nitric oxide, which in turn stimulates soluble guanylate cyclase to increase cGMP synthesis in vascular smooth muscle.7,8,20 In isolated pulmonary arterial rings, ET-1 caused a rapid increase of cGMP that reached a peak at 30 seconds (Figure 3A); therefore, in all subsequent experiments, rings were exposed to ET-1 for 30 seconds before being frozen for measurement of cGMP. Preincubation of rings with the nitric oxide synthase inhibitor L-NAME (100 μmol/L) or denudation of endothelium by rubbing with a steel wire completely eliminated ET-1-stimulated cGMP synthesis (Figure 3B and 3C). The content of cGMP in pulmonary arterial rings was not increased by ambrisentan, macitentan, or BQ-788 alone, whereas tadalafil increased cGMP content by 2-fold (Figure 3D). Both macitentan and BQ-788, but not ambrisentan, significantly inhibited cGMP production stimulated by ET-1, whereas tadalafil potentiated ET-stimulated cGMP production (Figure 3D). Both macitentan and BQ-788 also blocked the effect of tadalafil to potentiate ET-1-stimulated cGMP production in pulmonary arterial rings (Figure 3D), consistent with their known effects to block ETB receptors. These data suggest that ETB receptors in endothelium mediate the effect of ET-1 to stimulate nitric oxide (NO) production and a subsequent increase of cGMP in rat pulmonary arteries.

Effects of Ambrisentan and Tadalafil Individually and in Combination to Attenuate ET-1-Stimulated MLCK Phosphorylation in Rat Isolated Intrapulmonary Artery Preparations

Ca2+/calmodulin-dependent MLCK regulates contraction of smooth muscle cells. Phosphorylation of MLCK leads to

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kinase activation and phosphorylation of its substrate, MLC2. Phosphorylation of MLC2 is correlated with myosin ATPase activity and smooth muscle contraction. In smooth muscle, MLC2 is phosphorylated at threonine 18 (Thr18) and serine 19 (Ser19) by MLCK in a Ca²⁺/calmodulin-dependent manner. In our experiments, the activity of MLCK was determined using an anti-phospho-MLC2 (Thr18/Ser19) antibody that detects endogenous levels of MLC2 in smooth muscle cells only when dually phosphorylated at Thr18 and Ser19 by MLCK. As shown in Figure 4, ambrisentan and tadalafil each individually inhibited ET-1-stimulated MLCK activity in rat isolated pulmonary arterial rings by 34% and 30%, respectively. Treatment of rings with a combination of ambrisentan and tadalafil resulted in inhibition of ET-1-induced MLCK activity by 89%, which is significantly greater than calculated sum (ie, 64%) of the individual inhibitory effects of the 2 compounds. These data suggest that inhibitions of MLCK caused by both ambrisentan and tadalafil (by 2 different mechanisms) can explain their synergistic effect to relax rat pulmonary arterial ring preparations.

**Discussion**

The results in this study demonstrate that the ETA receptor antagonist ambrisentan and the PDE5 inhibitor tadalafil act synergistically to relax ET-1-constricted rat pulmonary artery preparations, and that the endothelial ETB receptor is required for this synergism. Blockade of the ETB receptor or denudation of the endothelium resulted in the loss of synergism between vasorelaxant effects of ambrisentan and tadalafil. The selective ETB receptor antagonist BQ-788, the nitric oxide synthase inhibitor L-NAME, and denudation of endothelium each abolished the effect of ET-1 to stimulate cGMP synthesis in rat pulmonary artery rings (Figure 3). These data are supported by previous findings that stimulation of ETB receptors leads to an increase in production of NO by endothelium, which in turn stimulates vascular smooth muscle guanylate cyclase activity, production of cGMP, and the activity of cGMP-dependent protein kinase, leading to vasorelaxation. Inhibition of vascular smooth muscle PDE5 by tadalafil would be expected to enhance ETB receptor-mediated cGMP accumulation and subsequent smooth muscle relaxation.

The nonselective ETA and ETB receptor antagonists bosentan and macitentan did not act synergistically with tadalafil to attenuate an ET-1-stimulated constriction of rat intrapulmonary arterial rings (Figure 1), which is consistent with the hypothesis tested in this study that a selective ETA receptor antagonist but not a nonselective ETA and ETB receptor antagonist would act synergistically with a PDE5 inhibitor. ETB receptor block would prevent ET-1-induced endothelial NO and cGMP formation that appear to be a prerequisite for a vasorelaxant response to tadalafil, which acts to prevent or slow the degradation of cGMP in vascular smooth muscle. It has been reported that activation of the small number of ETB receptors present on vascular smooth muscle cells may contribute to the vasoconstrictor effect of ET-1. However, selective ETB receptor antagonism increases peripheral vascular resistance in healthy subjects. Genetic and pharmacological disruptions of ETB receptors increase arterial blood pressure in mice, and selective ETB receptor antagonism induces hypertension in the hamster. ETB receptor-deficient rats also develop severe salt-sensitive hypertension. Monocrotaline- and hypoxia-induced pulmonary hypertension in ETB receptor-deficient rats is accelerated and exaggerated, suggesting that the ETB receptor has a role to inhibit the progression of PAH development. These findings suggest that the balance between vasodilator and vasoconstrictor effects of ETB Receptor activation favors vasodilation. Our data are in agreement with these findings.

The proposed mechanisms by which ET receptor antagonists and tadalafil relax ET-1-induced smooth muscle contraction are illustrated in Figure 5. Activation of ETB receptors in vascular smooth muscle increases intracellular calcium, MLC phosphorylation, and smooth muscle contraction via calcium/calmodulin-dependent MLCK. Ambrisentan, bosentan, and macitentan block ET receptor antagonists in smooth muscle cells and thereby inhibit ET-1-induced vasoconstriction. ET-1 stimulates formation of NO through activation of ETB receptors on endothelial cells. Tadalafil inhibits PDE5 and degradation of cGMP and thereby enhances endothelial ETB receptor-mediated formation of NO, which acts to stimulate smooth muscle cGMP. Cyclic GMP subsequently activates cGMP-dependent protein kinase, which acts to inhibit calcium release from intracellular stores and thereby decrease MLCK activity. The selectivity of ambrisentan for the ETA receptor leaves intact the effect of ET-1 to activate the endothelial ETB receptor. However, bosentan and macitentan block ETB receptor-dependent NO-mediated vasorelaxation and thus lack synergism with tadalafil. Therefore, the combination of ambrisentan and tadalafil, but not the combinations of bosentan or macitentan with tadalafil, has a synergistic effect to relax ET-1-constricted pulmonary arteries.
**Study Limitations**

The experimental models used in this study were chosen to provide a mechanistic proof-of-concept for a synergistic vasorelaxant effect of a PDE5 inhibitor and a selective ETA receptor antagonist. These models are not sufficient to demonstrate the applicability of the concept to treatment of PAH or any other disease. It was assumed in this study that ET-1 plays an important role as a vasoconstrictor of pulmonary arteries and contributes to the pathology of PAH. Additional studies of animal models of PAH are therefore needed to validate the pathological role of ET-1 and to determine the relevance of the findings in this study to the treatment of PAH.

**Perspectives**

Because there is a high rate of monotherapy failure in treatment of PAH, targeting multiple pathways with combination drug therapy may lead to better outcomes. The results from this study suggest that the combination of ambrisentan (a selective ETA receptor antagonist) and tadalafil (a PDE5 inhibitor) may be superior to the use of each drug alone for reduction of ET-induced pulmonary artery contraction in experimental PAH models and PAH patients. The biochemical mechanisms of this combination therapy, including a critical role for endothelial ETA receptors in the synergism of tadalafil with ambrisentan, are delineated. The clinical utility of the combination of ambrisentan and tadalafil is currently under investigation in several clinical trials, and these results in combination with our findings will hopefully provide new therapeutic options for the treatment of PAH.

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**Disclosures**

Drs Liang, Yang, Belardinelli, and Shryock are employees of Gilead Sciences, Inc.

**References**


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AMBRISENTAN AND TADALAFIL SYNERGISTICALLY RELAX ENDOTHELIN-INDUCED CONTRACTION OF RAT PULMONARY ARTERIES

Faquan Liang, Suya Yang, Lina Yao, Luiz Belardinelli and John Shryock

Gilead Sciences, Inc., Palo Alto, CA 94304

Correspondence to: Faquan Liang, Ph. D., Research Scientist. Gilead Sciences, Inc., 1651 Page Mill Road, Palo Alto, CA 94304. Phone: 650-384-8417; Fax: 650-475-0391; Email: faquan.liang@gilead.com
Expanded Methods

Pulmonary artery preparation and isometric tension measurement
Male Sprague–Dawley rats (300–325 g) were anesthetized by intraperitoneal injection of a mixture of 60 mg/kg ketamine plus 8 mg/kg xylazine. After anesthesia was complete, the chest was opened and intact right and left lungs were excised. Under a stereomicroscope, intrapulmonary arteries at sizes ranging from 100 to 500 μm diameter were dissected, and the surrounding tissues were removed using ocular dissection scissors. The intrapulmonary arteries were then cut into rings. In selected experiments wherein denudation of pulmonary artery endothelium was desired, a steel wire was used to rub the lumen of the ring(s). Each ring was mounted to a pair of thin pins or wires in a DMT myograph chamber [Myograph 610M System; Danish MyoTechnologies (DMT), Aarhus, Denmark] to measure isometric force. Each chamber was filled with 8 mL of oxygenated Krebs–Henseleit solution containing 115 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.5 mmol/L KHPO4, 25 mmol/L NaHCO3, 2.5 mmol/L CaCl2, and 10 mmol/L glucose. Isometric force was recorded by a force transducer connected to a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO). The intrapulmonary arterial rings were stretched to a resting force of 3 mN. After a 60-min equilibration period, the rings were contracted with a submaximal concentration (8 nmol/L) of ET-1. The effects of bath additions of ET receptor antagonists (10 nmol/L ambrisentan, 100 nmol/L bosentan, 30 nmol/L macitentan) and 30 nmol/L tadalafil alone and in combination were determined in the continued presence of ET-1. The effect of drug to relax a constricted vessel was expressed as percentage reduction of ET-1-induced isometric force.

Measurement of cyclic guanosine monophosphate (cGMP)
Isolated rat intrapulmonary arterial rings were mounted to a DMT myograph system, preincubated with 10 nmol/L ambrisentan, 30 nmol/L macitentan, 1 μmol/L BQ-788 and 30 nmol/L tadalafil alone and in combination for 10 min, then treated with 8 nmol/L ET-1 for 30 sec. Pulmonary artery tissues were flash frozen in liquid nitrogen and stored at -80ºC. Frozen tissues were weighted, homogenized in 10 volumes of 0.1 mol/L HCl (e.g., 0.1 g of tissue in 1 ml of 0.1 mol/L HCl), and centrifuged at ≥600 × g. Pelleted material was discarded and supernatants were assayed for cGMP content using a cGMP complete EIA kit from Enzo Life Sciences (Plymouth Meeting, PA).

Western blot analysis
To determine the effects of drugs on ET-1-induced phosphorylation (i.e., activation) of myosin light-chain kinase (MLCK), isolated rat intrapulmonary artery rings were mounted to the DMT myograph system and preincubated with either 10 nmol/L ambrisentan, 30 nmol/L tadalafil, or ambrisentan plus tadalafil for 10 min. ET-1 (8 nmol/L) was then added to each chamber for an additional 10 min. Vascular rings were removed from the myograph and quickly immersed for 1 h in pre-cooled acetone containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol (DTT), then washed at least three times with acetone containing 10 mmol/L DTT to remove TCA. The vessels were then air-dried and stored at minus 80ºC until use. The dried vessels were homogenized in RIPA buffer (catalog# 89900, Thermo Scientific) containing protease and phosphatase inhibitors (catalog# 78442, Pierce) and 1 mmol/L PMSF (Sigma). Five μg of homogenized protein from each sample was electrophoresed on
12% Bis-Tris SDS gels (Invitrogen), transferred onto nitrocellulose membranes and subjected to immunoblotting with an anti-myosin light-chain 2 (MLC2) antibody (1:1000 dilution, CAT#3672, Cell Signaling Technology) or an anti-phospho-MLC2 (Thr18/Ser19) antibody (1:1000 dilution, catalog# 3674, Cell Signaling Technology). The membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (1:2000, catalog# 7074, Cell Signaling Technology) and developed in Chemiluminescent Substrate (Cat#34095, Pierce). Luminescent intensity was detected and quantified using a Luminescent Image Analyzer (Fuji LAS-3000 Imaging System). ET-1 incubation time of 10 min was selected based on the results of a time course where 8 nmol/L ET-1 induced a maximal MLC2 phosphorylation from 5 min to 15 min (data not shown).