Safety and Ergogenic Properties of Combined Aminophylline and Ambrisentan in Hypoxia

Thies Schroeder[1], Claude A. Piantadosi[2], Michael J. Natoli[2], Julie Autmizguine[3],
Michael Cohen-Wolkowieczs[4], Karyn L. Hamilton [5], Christopher Bell[5], Jelena Klawitter [6],
Uwe Christians[6], David C. Irwin[7] and Robert J. Noveck[2]

We hypothesized that concomitant pharmacological inhibition of the endothelin and adenosine pathway is safe and improves exercise performance in hypoxic humans, via a mechanism that does not involve augmentation of blood oxygenation. To test this hypothesis, we established safety and drug interactions for aminophylline (500 mg) plus ambrisentan (5 mg) in normoxic volunteers. Subsequently, a placebo-controlled study was employed to test the combination in healthy resting and exercising volunteers at simulated altitude (4,267 m). No serious adverse events occurred. Drug interaction was minimal or absent. Aminophylline alleviated hypoxia-induced headaches. Aminophylline, ambrisentan, and their combination all significantly ($P < 0.05$ vs. placebo) improved submaximal hypoxic exercise performance (19.5, 20.6, and 19.1% >placebo). Single-dose ambrisentan increased blood oxygenation in resting, hypoxic subjects. We conclude that combined aminophylline and ambrisentan offer promise to safely increase exercise capacity in hypoxemic humans without relying on increasing blood oxygen availability.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Hypoxemia reduces exercise capacity and causes serious health problems. It is often assumed that reduced oxygen concentration in the systemic blood directly translates into reduced oxygen bioavailability to tissue. However, hypoxemia also causes pulmonary vasoconstriction, concealed hypotension, and precapillary vasoconstriction, each obstructing oxygen delivery to tissue. As an alternative to oxygen supplementation, hypoxemia might thus be alleviated by reversing microvascular disorder with pleiotropic drug treatment.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ We investigated the safety and efficacy to combine the adenosine and endothelin blockers aminophylline and ambrisentan in healthy resting and exercising humans under hypobaric hypoxia.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ We demonstrate the safety of combined aminophylline and ambrisentan in hypoxemic resting and exercising volunteers, and potential to alleviate exercise decrement without supplementing oxygen.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ This pharmacological concept could improve treatment and outcome of hypoxemia when oxygen supplementation is insufficient or unfeasible.

When exposed to high altitude, most humans experience decreased physiological function (such as declines in endurance exercise capacity) and face increased risk for health problems, including acute mountain sickness (AMS), and/or pulmonary or cerebral edema.[1,2] Similar to exercise-induced hypoxemia at sea level, diminished blood oxygenation is often thought to limit endurance at high altitude.[3] However, since even at 4,500 m residual central venous oxygen saturation still approximates 60% in resting and 40% in exercising subjects, the performance-limiting role of the blood oxygen concentration is potentially overestimated.[4] On the other hand, systemic hypoxemia causes a battery of microvascular disorders that represent a barrier to oxygen delivery to tissues, including excessive release of the peptide hormone endothelin-1 (ET-1), which causes hypoxic pulmonary hypertension,[5] and capillary occlusion in the skeletal musculature.[6] Hypoxemia also triggers global vasodilation, causing relative hypotension that is compensated by increased cardiac output.[7,8] Pharmaceutical targeting can alleviate the hypoxia-induced decline of exercise endurance in rats. Specifically, endothelin receptor A antagonists (ETRA), combined with either

Table 1 Adverse events

Study	Type of AE	Percent and (absolute) no. of subjects affected by treatment				
		Placebo	Aminophylline	Ambrisentan	Combination	Mean
#1	Headaches	N/A	11.1 (2)	27.8 (5)	11.1 (2)	16.7 (3)
	Cramping, extremities	N/A	11.1 (2)	16.7 (3)	16.7 (3)	14.8 (3)
	Tremors/shakiness	N/A	22.2 (4)	0	11.1 (2)	11.1 (2)
	Urinary frequency	N/A	5.6 (1)	5.6 (1)	22.2 (4)	11.1 (2)
	Dizzy/lightheaded	N/A	11.1 (2)	0	11.1 (2)	7.4 (1)
	Nausea, vomiting	N/A	16.7 (3)	0	5.6 (1)	7.4 (1)
	Other AEs that occurred only in Study #1 (listed below)	N/A	(4)	(1)	(10)	(5)
	Number of subjects	N/A	18	18	18	
#2 Period 1	Nausea, vomiting	57.1 (4)	87.5 (7)	62.5 (5)	42.9 (3)	62.5 (5)
	Headache	28.6 (2)	62.5 (5)	37.5 (3)	71.4 (5)	50.0 (4)
	Dizzy/ lightheaded	42.9 (3)	75.0 (6)	37.5 (3)	42.9 (3)	49.6 (4)
	Fatigue	28.6 (2)	37.5 (3)	25.0 (2)	28.6 (2)	29.9 (2)
	Tremors/ shakiness	0	12.5 (1)	0	0	3.1 (0)
	Urinary frequency	0	12.5 (1)	0	0	3.1 (0)
	Number of subjects	7	8	8	7	
#2 Period 2	Nausea, vomiting	42.9 (3)	12.5 (1)	0	28.6 (2)	21.0 (2)
	Headache	28.6 (2)	37.5 (3)	0	14.3 (1)	20.1 (2)
	Dizzy/ lightheaded	14.3 (1)	25 (2)	14.3 (1)	14.3 (1)	17.0 (1)
	Stomach ache, stomach cramping	0	0	0	14.3 (1)	3.6 (0)
	Paresthesia (buttock, fingers/toes)	0	12.5 (1)	0	0	3.1 (0)
	Number of subjects	7	8	7	7	

The following adverse events (Aes) only occurred in Study #1 and in less than 8% of the subjects (averaged in all treatment groups): palpitations and tachycardia, sweaty/clammy hands and feet, feeling different and increased energy, anxiety and restlessness, facial flushing and hot flashes, hiccups, nasal congestion, contact dermatitis, erythema at injection site. Fatigue did not occur in Study #1 and in Study #2 Period 2.

adenosine receptor antagonists (ARA) or hypertensive sympathomimetics, improve exercise performance of rats at altitude.[9,10] Importantly, this performance-restoring effect did not involve any augmentation of blood oxygen concentrations.

We hypothesized that the combination of the ARA aminophylline and the ETRA ambrisentan (Letairis, Gilead, Foster City, CA) is well tolerated in resting and exercising humans at simulated high altitude (>4,000 m) and that the combination improves exercise performance without augmenting blood oxygenation. We also tested whether the treatment would interfere with early AMS in human subjects.

RESULTS
Study population
For Study 1, 71 (100%) subjects were screened. Forty (56.3%) qualified and 31 (43.7%) did not meet inclusion criteria (**Supplemental Table 1**), withdrew consent, or failed in follow-up. Twenty-two (31.06%) were admitted, four (5.6%) withdrew consent, and 18 (25.4%) completed the study. For Study 2, 91

(100%) subjects consented, of which 58 (63.7%) did not meet inclusion criteria, 30 (33%) were enrolled, and 27 (29.7%) completed the study. Demographics are summarized in **Supplemental Table 2**.

Adverse events (AEs) and hepatic safety
In Study 1, 70 transient AEs were reported in nine (50%) subjects; mostly in Period 1 (91%) and after aminophylline (38%) or ambrisentan (47%, **Table 1**). No serious adverse events (SAE) occurred. Most frequent AEs were headache, leg cramping, tremor, and increased urinary frequency. Extremity cramping, tachycardia, and facial flushing occurred only during Study 1. Glutamate-oxaloacetate-transaminase (AST) and glutamate-pyruvate-transaminase (ALT) levels were raised over normal in one subject on Day 4 and six who had received sequence B, but were normalized by Day 22. Highest AST and ALT levels were 80 and 162 IU/L, which was less than thrice the upper normal limit (40 and 55 IU/L, respectively), our boundary of clinical significance. In Study 2, most frequent AEs were nausea, headaches,
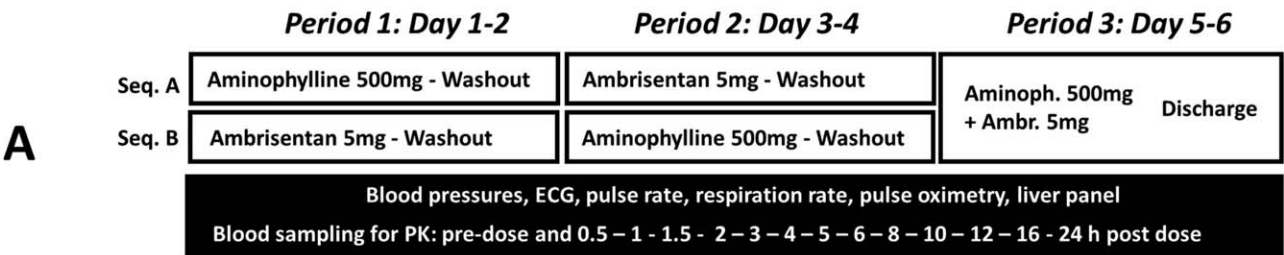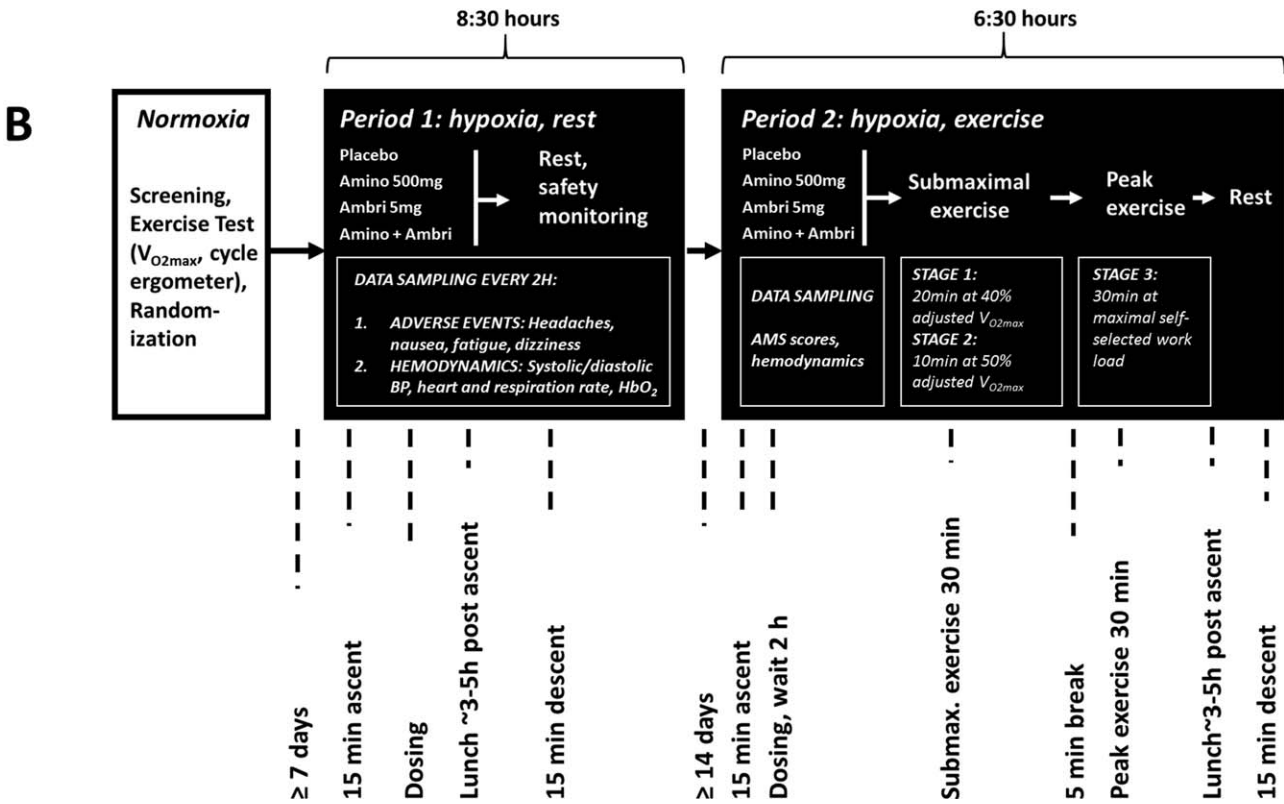
Figure 1 Diagram of treatment interventions

and dizziness during Period 1 (**Figure 1b**), and headaches and nausea in Period 2 (**Table 1**). When comparing the most common AEs (headaches, cramping, tremors, urinary frequency, dizziness, nausea, tachycardia, and fatigue) between study periods, there was a significant decrease from Period 1 to Period 2 in the aminophylline group (paired t-test, $P < 0.05$). Two subjects voluntarily revoked consent due to intolerable side effects. Their symptoms resolved after supplementation of oxygen, descent, and provision of fluid and Tylenol. One subject was discontinued by the investigator. Mildly elevated hepatic parameters were found in four subjects: Subject 25 had significantly elevated AST at screening that was resolved on follow-up. Subject 33 (combination) had elevated bilirubin after Period 2. Subject 49 (aminophylline)

had elevated ALT after Period 1 (both resolved at follow-up). Subject 81 (ambrisentan) had elevated bilirubin after Period 2 (resolved on follow-up).

Pharmacokinetics and analysis of bioequivalence

Pharmacokinetic analyses were done during Study 1 only. Eighteen subjects were analyzed after single drugs and 17 after combination. Pharmacokinetic (PK) data and interaction analyses are listed in **Supplemental Tables 3 and 4**. The 90% confidence limits for both theophylline and ambrisentan after combined dosing were within the 80% and 125% boundaries for the area under the curve (AUC) from zero to infinity (AUC_{0-inf}) after aminophylline single dosing. The mean time of peak concentration

(T_{max}) for theophylline was earlier when administered with ambrisentan (1.94 h) than alone (2.42 h). T_{max} for ambrisentan was earlier when administered with aminophylline (1.71 h) than alone (1.9 h).

Hemodynamic results

In Study 1, systolic blood pressure (BP) increased significantly after aminophylline and combination and returned to baseline at 8 h. Average systolic BP post-aminophylline was significantly higher than post-ambrisentan (**Figure 2c–e**). Diastolic BP was elevated after aminophylline and combination, but returned to baseline in both groups 10 h postdosing. Ambrisentan produced diminished diastolic BP 12 h postdosing (**Figure 2f–h**). Heart rates increased significantly 6 h after both single drugs. Combination produced significantly elevated heart rates at 6–24 h (**Figure 2i–k**). Ambrisentan significantly increased breathing rates 10–24 h postdose (**Figure 2l–n**) and reduced hemoglobin oxygen saturation (SaO_2) 6–10 h postdosing (**Figure 2o–q**). QT intervals did not change in and between any group (**Figure 2r–t**). In Study 2, systolic BP after combination was significantly lower 1 h postexercise and 1 h postdescent than preascent (**Figure 3c**). No differences were found between groups (**Figure 3c**). Diastolic BP decreased after combination treatment 2 h postascent and 2 h postexercise, compared with time 0. There were no differences between treatment groups. Heart rates increased significantly in all groups during exercise, compared with preascent and with time of dosing. After exercise, heart rates remained elevated, compared to time 0, in all groups except ambrisentan. Average heart rates did not differ between groups during exercise. Top heart rates seen under maximum exercise conditions were significantly lower than maximum heart rates during (normoxic) maximum oxygen uptake capacity (VO_2max) screening (paired t-test, $P < 0.0005$). SaO_2 declined in all treatment groups postascent to altitude (**Figure 3j**) until 2 h postascent. SaO_2 in the combination group 7 h postdosing was higher than placebo. Comparing averaged SaO_2 values between groups, blood oxygen was higher in ambrisentan-treated than in placebo subjects (**Figure 3k**). SaO_2 declined further during exercise in all groups (**Figure 3l**). Breathing rates did not change during the resting phases of Period 2, except with combination treatment, where rates increased 2 h postdosing and postexercise, compared to time 0 (**Figure 3o**). After normalization to the time of dosing, ambrisentan produced significantly lower breathing rates postexercise than both placebo and combination (**Figure 3o**).

Hypoxic exercise tolerance

During screening, mean $VO_2max \pm$ standard deviation (range) was 49.1 ± 6.4 (41.8–64.1) ml/kg/min. Average maximum heart rate (HR_{max}) was 182.9 ± 11.8 (157.0–205.0) bpm. Mean power during exercise screening was 241.0 ± 41.9 (145.0–302.2) watt (W). Average work rates during screening were 272.1 ± 40.7 W (placebo), 231.2 ± 43.0 W (aminophylline), 234.4 ± 32.3 W (ambrisentan), and 228.6 ± 44.4 W (combination). Mean group work rates during exercise episodes are listed in **Table 2**. Individual power levels did not differ between groups during any of the stages. After normalization to individual work rate during

screening, all treatment groups performed significantly better than placebo during Stage 1 and Stage 2, and both aminophylline and combination-treated subjects performed better than placebo during Stage 3 (**Figure 5c**). Aminophylline, ambrisentan, and combination improved hypoxic work performance by 16.9%, 19.2%, and 19.0% (Stage 1), 19.5%, 20.6%, and 19.1% (Stage 2), and 23.8%, 18.8%, and 23.0% (Stage 3), compared to placebo, and using normalized data for comparison. There was no difference in exercise performance between subjects treated with aminophylline, ambrisentan, and the combination. Performance significantly increased between Stages 1 and 2 in all groups, but not between Stages 2 and 3 (repeated measures analysis of variance (ANOVA), $P < 0.05$).

AMS (Figure 4a–e)

Cumulative scores were significantly elevated 8 h after placebo dosing and 4 h after aminophylline. Headaches increased significantly during Period 1 after placebo (6 h and 8 h, vs. zero), and after ambrisentan and combination (8 h vs. baseline). Aminophylline-dosed subjects had significantly less severe headaches than placebo-treated subjects at 8 h. During Period 2, severity of headaches did not differ between groups. Fatigue did not change significantly during both periods. Aminophylline produced dizziness and nausea 4 h postdosing that resolved 2 h later.

DISCUSSION

Combined treatment with aminophylline and ambrisentan was tolerated relatively well by healthy volunteers, with no SAE. Drug interaction was minimal or absent. The combination improved hypoxic exercise performance without inducing a change in SaO_2.

Alveolar hypoxia causes hypoxic pulmonary vasoconstriction (HPV) as part of the autonomous response of blood flow to ventilation.[11] However, extended systemic hypoxemia triggers excessive release of the powerful vasoconstrictor ET-1 by endothelial cells, which exacerbates pulmonary vasoconstriction.[5,12] ET-1 binds to two main receptors, ETR-A and ETR-B, that are distributed throughout the body, and although ETR-B can mediate vasodilation, ET-1 causes net vasoconstriction.[13,14] Constitutive release of ET-1 contributes to the ubiquitous basic vascular tone, whereas acute release antagonizes excessive vasodilation during exercise hyperemia.[15] Altitude-induced reduction of peak performance can be partially reversed by blockade of ET-1 signaling,[16] also supported by our findings. ET-1 acts predominantly on vascular elements near the end of the arterial tree, including pericytes, thus reducing oxygen transport to the parenchymatic skeletal muscle and other organs.[6,17] Combined with augmented perfusion pressure, ET-1 inhibition strongly enhances tissue oxygenation under hypoxemia.[9] In addition to ET-1 dependent vasoconstriction, hypoxemia also triggers vasodilation of (extrapulmonary) resistance arteries.[18] The resulting hypotension is promptly antagonized by increased cardiac output.[7] Hypoxic vasodilation is part of the normal hyperemic reaction of skeletal muscle to exercise-induced hypoxia and is partially mediated by adenosine.[19] Inhibition of adenosine signaling may thus stabilize arterial perfusion pressure under hypoxemia.[20] Thus, adenosine

Study 1

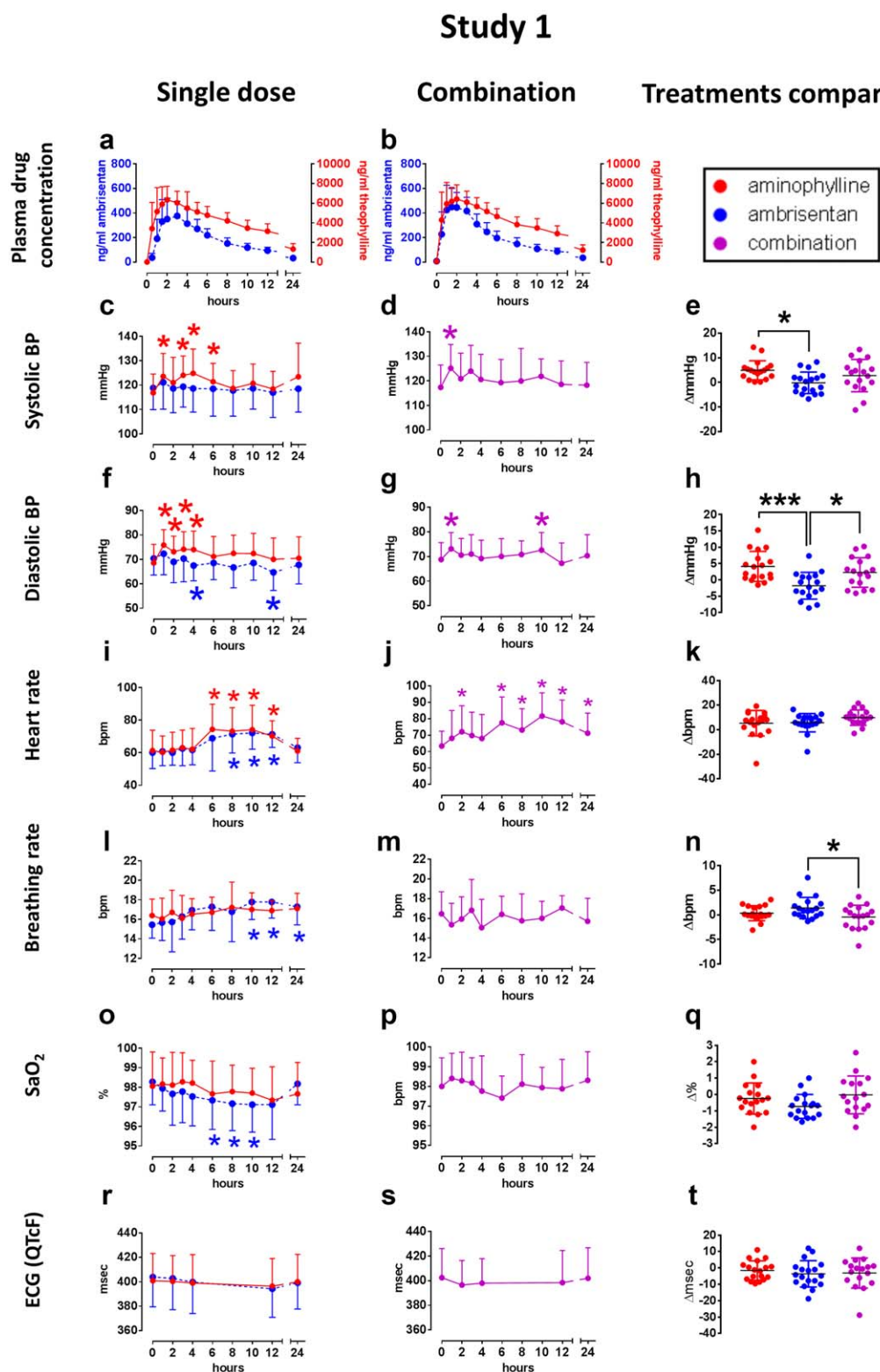**Single dose** **Combination** **Treatments compared**

Figure 2 Hemo- and pharmacodynamic changes during Study 1. Asterisks indicate significant differences to time of dosing and the color of the asterisk indicates the type of treatment (paired ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). **a,b**: theophylline and ambrisentan plasma concentrations, alone and in combination. **c–e**: systolic BP. **f–h**: diastolic BP. **i–k**: heart rate. **l–n**: breathing rate. **o–q**: SaO$_2$. **r–t**: time intervals between q and t spikes during EKGs.
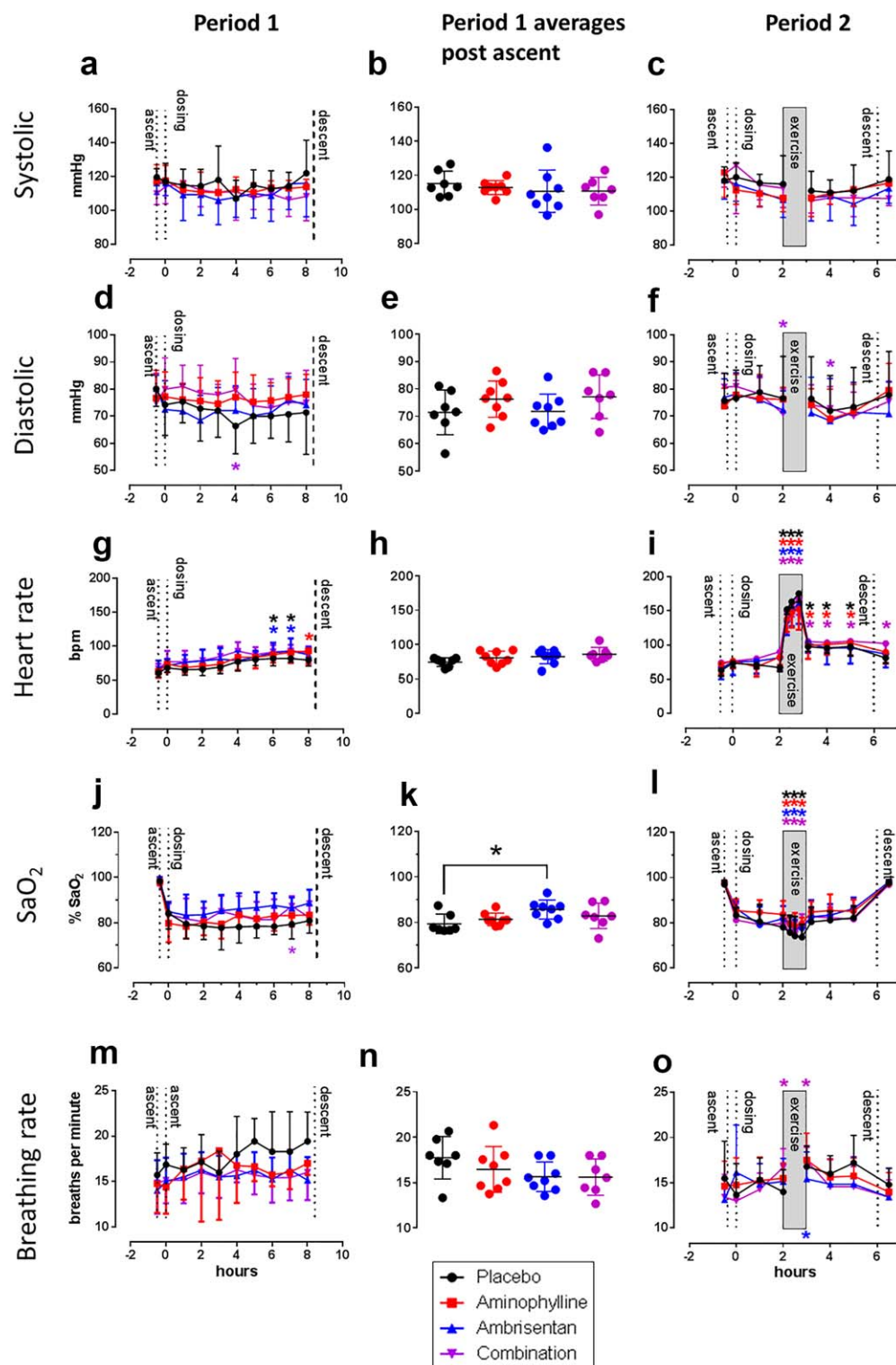
Study 2

Figure 3 Hemodynamic changes during hypoxia (Study 2): Column 1 and 3: parameter changes during Period 1 and 2. Column 2: averages during Period 1, excluding pre-ascent data. Asterisks above data points indicate significant changes compared to the time of dosing and the color of the asterisk indicates the type of treatment (paired ANOVA, $*p < 0.05$). Asterisks below data points indicate significant difference to placebo group (unpaired ANOVA). **a–c**: systolic BP, **d–f**: diastolic BP, **g–i**: heart rate, **j–l**: hemoglobin oxygen saturation (SaO_2), **m–o**: breathing rate.

Table 2 Work rates during exercise episodes

Exercise episode		Placebo	Aminophylline	Ambrisentan	Combination
		Work rate in watt ± standard deviation			
Screening		272.1 ± 40.7	231.2 ± 43.0	234.4 ± 32.3	228.6 ± 44.4
Study 2 Period 2	Stage 1	73.9 ± 7.1	57.6 ± 13.2	62.1 ± 10.1	59.3 ± 17.1
Study 2 Period 2	Stage 2	91.1 ± 6.2	76.8 ± 15.1	78.6 ± 37.1	79.0 ± 36.5
Study 2 Period 2	Stage 3	88.6 ± 27.6	83.3 ± 40.7	73.3 ± 37.1	79.0 ± 36.5

inhibition might synergize with ETRAs to improve capillary conductance under hypoxemia. Indeed, adenosine blockade increases hypoxic endurance in rats, if combined with an ETRA.[10] This concept contrasts with other treatments that increase oxygen levels in the blood.[21]

Ambrisentan was the ETRA of choice because of its low hepatotoxicity[22] and high likelihood of compatibility with theophylline.[10] Hepatic enzyme elevations in Study 1 were likely not caused by ambrisentan, since the elevation was stable 10 days after treatment, bilirubin was unchanged, and no other hepatotoxicities occurred. All cases with elevated liver enzymes in Study 2 resolved quickly and were spread across treatment groups. This argues against drug treatment as a cause.

In addition to its adenosine receptor antagonism, aminophylline (theophylline) is also an unspecific phosphodiesterase (PDE) inhibitor.[23] However, PDE inhibition is only at half maximum when plasma theophylline reaches 100 μmol/l (18,016.4 ng/ml).[24] Because maximum plasma concentration (C_{max}) for theophylline in our study approximated 7,000 ng/ml (38.9 μmol theophylline), with only one value exceeding 10,000 ng/ml, PDE inhibition was probably negligible in this study.

Comparison of T_{max} of the drug plasma profiles did not fully rule out mutual drug interaction; however, an AUC comparison suggested it was minimal or absent. This is consistent with the different elimination routes of the drugs.[23,25]

Increased blood pressures after aminophylline in Study 1 are probably caused by adenosine blockade.[26] These effects were lost in hypoxic individuals, likely because of peripheral vasodilation. Elevated heart rates in Study 1 were probably unrelated to the study drugs, but elevations in Study 2 / Period 2 may have been caused by environmental hypoxia.[7] Peak heart rates at hypoxia never exceeded those seen during VO$_2$max testing. Average SaO$_2$ during hypoxia was appropriate for the chosen altitude and activity level.[27,28] The effect of ET-1 blockade on SaO$_2$ in both studies could reflect changes in pulmonary ventilation-perfusion matching.[29] Importantly, no such effect was seen with the drug combination. An important safety concern was the interaction of theophylline, which AEs include hypokalemia, hypotension, nausea, and headaches[23] with ambrisentan. Dizziness, nausea, and headaches are lead symptoms of AMS, and their increase in Study 2, as seen in **Table 1**, is likely caused by simulated altitude. The aminophylline=associated spike in nausea and dizziness 4 h postdosing could be, in part, due to potentiation of the drug by hypoxia; notably, however, there was no significant difference to placebo treatment. Theophylline reportedly reduces some AMS

symptoms,[30] but longer observation periods are required for conclusive information.

Our data suggest that all three treatments offer a reversal of hypoxemic exercise fatigue by 18–24%, even though the placebo group showed the (nonsignificantly) highest average performance of all groups during VO$_2$max screening. In laboratory rats we found that the combination of aminophylline and ambrisentan significantly improved exercise performance (unpublished data) compared to both single drugs during submaximal exercise burden, whereas under heavy hypoxic exercise, the synergistic advantage of the combination vanished, although it was still efficacious vs. vehicle control. This could mean that the exercise burden in Study 2 might have been too high to detect synergistic advantages of the combination. A dedicated efficacy study in humans will produce more precise information about the potential synergism between aminophylline and ambrisentan, and how this combination compares with other related treatments, such as acetazolamide, dexamethasone, and sildenafil.

One important limitation of the studies described in the current article pertains to the laboratory setting. Our initial studies were performed in laboratory-based, simulated high altitude.[21,31–33] While a simulated high altitude can never fully capture all of the stressors associated with mountain activity, this study provides close safety monitoring of human subjects during the first-time administration of combined aminophylline and ambrisentan in hypoxia.

In summary, simultaneous inhibition of the adenosine and endothelin pathway is feasible and potentially efficacious to safely improve exercise performance in hypoxemic humans. This might represent a novel treatment option of hypoxemic pathologies that does not rely on increasing oxygen concentration in the blood.

METHODS
Clinical study protocol overview
Two studies were conducted to test the safety and drug interaction of combined aminophylline and ambrisentan in healthy, normoxic subjects, followed by testing the safety and efficacy of this combination in resting and exercising hypoxic, hypobaric subjects. Both studies were conducted at Duke University Medical Center under the US Food and Drug Administration (FDA) investigational new drug program (IND). All clinical procedures followed the principles of the Helsinki Declaration of 1975 (revised 1983) and were preapproved by the Duke University Institutional Review Board (IRB). All subjects consented in writing after full explanation of study procedures. ClinicalTrials.gov IDs were NCT01530464 and NCT01794078.

Study 1 was a phase I, three period, two sequence, open-label, randomized crossover design. Periods 1 and 2 included oral single-dose
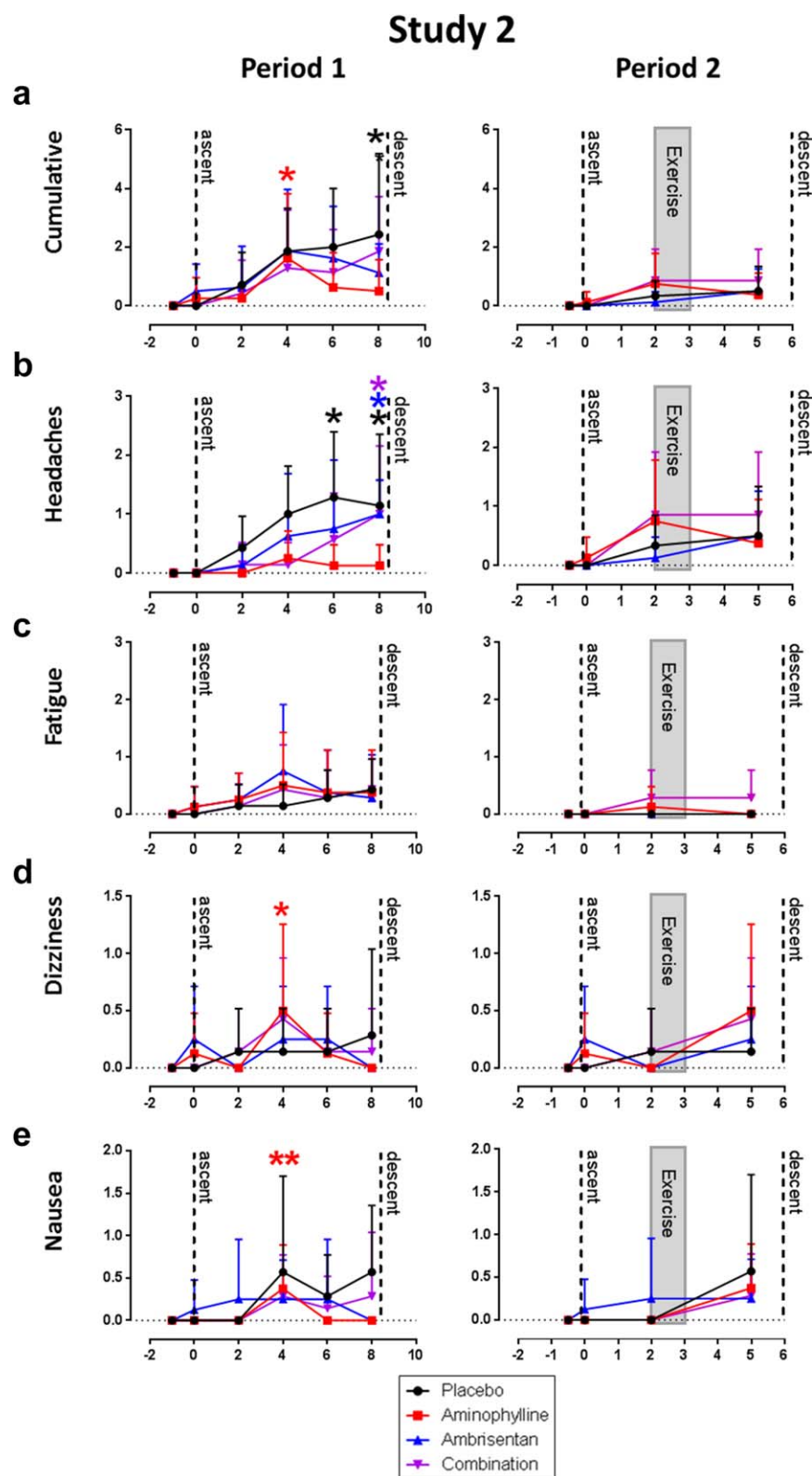
Figure 4 AMS during Study 2 Period 1 and 2. An asterisk indicates a significant change compared to the time of dosing and the color of the asterisk indicates the type of treatment (ANOVA on paired values, *$P < 0.05$, **$P < 0.01$). **a:** Average cumulative score, aminophylline at 4 h and placebo at 8 h significantly higher than baseline. Average headaches **(b)**, fatigue **(c)**, dizziness **(d)**, nausea **(e)**.
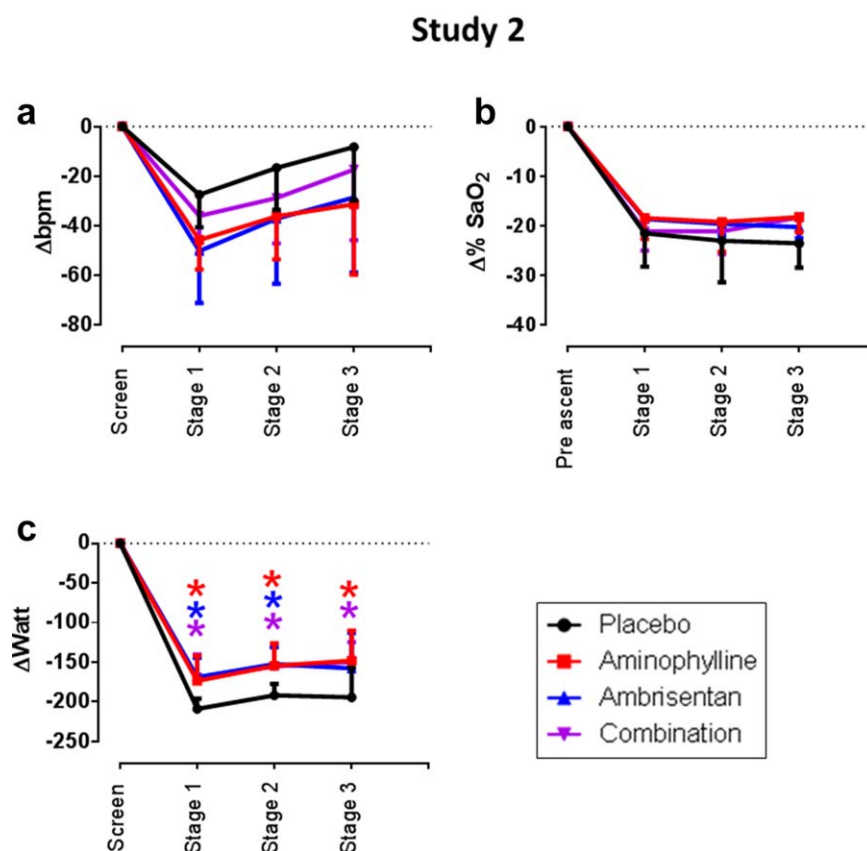
Study 2

Figure 5 Hypoxic exercise results. **a:** Average maximum heart rates normalized to the individual max. heart rate during screening. **b:** Average SaO_2 normalized to individual SaO_2 before ascent to hypoxia. **c:** Average power achieved during Stages 1–3 of hypoxic exercise, normalized to the power achieved during VO_{2max} screen testing. Asterisks indicate significant difference of a treatment to placebo and the color of the asterisk indicate the type of treatment (ANOVA, $*P < 0.05$).

aminophylline (500 mg) or ambrisentan (5 mg), followed by a 48-h washout. Period 3 included simultaneous ingestion of both drugs. Subjects were confined in the Duke Early Phase Clinical Research Unit (DEPRU) throughout study procedures. Recruitment goals were to enroll 24 to complete at least 16 subjects, following a common design strategy for crossover safety studies.[34] Primary outcome measures were safety and PK alterations. Safety parameters were 1) AEs, 2) vital signs (respiration rate, pulse, systolic and diastolic pressure), 3) blood chemistry, and 4) hepatic safety (AST, ALT, total bilirubin) on Days 2, 4, and 6. Screening involved medical history, physical examination, vital signs, electrocardiograph (ECG), and blood and urine testing. Admission criteria (**Supplemental Table 1**) were designed to ensure mental and physical ability to participate, to preclude subjects who use drugs or abuse alcohol, to ensure abstinence from substances that may alter blood levels of the drugs, and, because ambrisentan is a suspected teratogen, to exclude pregnancy or fatherhood during the study.[35] Randomization to sequences A (aminophylline-ambrisentan-combination) or B (ambrisentan-aminophylline-combination) occurred on study Day 1 (**Figure 1a**). Blood for PK analysis was sampled predose, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, and 24 h postdose. Subjects had a follow-up visit 3–7 days after discharge, for physical examination, vital signs, and assessment of unresolved events (**Figure 1a**). Plasma drug analysis was done at iC42 Integrated Solutions (Aurora, CO; details in Methods Supplement). PK parameters were computed using noncompartmental methods within WinNonLin Phoenix v. 6.3 (Pharsight, Sunnyvale CA; see Methods Supplement). Bioequivalence was analyzed from log (ln)-transformed plasma–time concentrations of each drug alone and in combination. Nondifference was assumed when 90% confidence intervals of C_{max} and AUC_{0-inf} after combined dosing were within 80–125% of C_{max} and AUC_{0-inf} after single drug.[36]

Study 2 was a phase I, two-period, randomized, placebo-controlled, double-blinded, parallel study to test the safety of combined aminophylline and ambrisentan in resting and exercising subjects under simulated high altitude, their efficacy to reverse altitude-induced performance loss, and interference with early AMS symptoms. Subjects were screened for medical history and health, pregnancy, drug/alcohol abuse, and adherence to admission criteria (**Supplemental Table 1**), followed by a test for maximum oxygen uptake on a cycle ergometer (see Methods Supplement). Subjects had to meet a VO2max of at least 42 ml/kg/min, or were admitted at the discretion of the investigator if slightly below the cutoff. They were then randomized to receive either placebo, 500 mg aminophylline, 5 mg ambrisentan, or combination of the latter two, with treatments remaining the same throughout both periods (**Figure 1b**). Drugs and placebo were deidentified by over-encapsulation. On study days, continued eligibility was verified, followed by safety labs, urine tests for pregnancy and drugs of abuse, alcohol breathalyzer test, physical examination, ECG, and vital signs assessment. During Period 1 (hypoxia–rest), subjects ascended to an equivalent of 4,267 m within ~15 min. Drugs were ingested on arrival and subjects remained in the chamber for ~8 h and were offered a small lunch after ~4 h. AEs were assessed nonsystematically, initiated by study attendants or subject, and systematically, using a customized AMS test,[37] involving hourly severity review of headaches, fatigue, dizziness, and nausea, on a scale from 0 to 3. Subjects were then descended and discharged (**Figure 1b**). Subjects entered Period 2 (hypoxia–exercise), ≥14 days after Period 1, to avoid acclimatization. Subjects ascended to simulated 4,267 m and ingested study medication on arrival. AEs and AMS criteria were assessed as before. Approximately 2 h after dosing, subjects cycled on an ergometer for 20 min at a burden of 40% of the calculated maximal exercise

capacity at this altitude, based on VO_2max at screening.[38] This was followed by cycling for 10 min at 50% of altitude-adjusted maximal exercise capacity and, after a break of ~5 min, of 30 min cycling at maximum capacity (Methods Supplement). Six hours after ascent, subjects were descended and discharged. Hepatic safety labs were done 2–4 days after each period and follow-up labs after 7 ± 3 days, if applicable, and repeated if necessary. The recruitment goal was ~10 subjects to finish all procedures, which was based on similar studies in the field that commonly have sample sizes between 5 and 10 per group.[39–42]

Statistics were done with GraphPad Prism (GraphPad Software, La Jolla, CA). Paired ANOVA with Bonferroni correction served to analyze changes from baseline. Cross-sectional comparisons between treatment groups were investigated with nonpaired ANOVA with Bonferroni correction.

SUPPLEMENTARY MATERIAL is linked to the online version of the article at http://www.cpt-journal.com

CONFLICT OF INTEREST/DISCLOSURE

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

T.S. and R.J.N wrote the artciel; T.S., C.A.P., M.J.N., K.L.H., C.B., D.C.I., and R.J.N. designed the research; T.S., C.A.P., M.J.N., and R.J.N. performed the research; T.S., M.J.N., J.A., M.C.W., J.K., U.C., and R.J.N. analyzed the data.

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