JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page

Pharmacological characterization of a novel 5-hydroxybenzothiazolone (5-HOB) derived β_2-adrenoceptor agonist with functional selectivity for anabolic effects on skeletal muscle resulting in a wider cardiovascular safety window in preclinical studies

Magdalena Koziczak-Holbro, Dean F. Rigel, Bérengère Dumotier, David A. Sykes, Jeffrey Tsao, Ngoc-Hong Nguyen, Julian Bösch, Marie Jourdain, Ludivine Flotte, Yuichiro Adachi, Michael Kiffe, Moïse Azria, Robin A. Fairhurst, Steven J. Charlton, Brian P. Richardson, Estelle Lach-Trifilieff, David J. Glass, Thomas Ullrich, Shinji Hatakeyama

Affiliation: Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland (M.K-H., B.D., N-H.L-N., J.B., M.J., L.F., M.K., M.A., R.A.F., B.P.R., E.L-T., T.U., S.H.), East Hanover NJ, USA (D.F.R.), Horsham, UK (D.A.S, S.J.C.), Cambridge MA, USA (J.T., Y.A., D.J.G.).

The current affiliations of the authors who left Novartis Institutes for BioMedical Research after this work:

School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK (D.A.S., S.J.C.)

MilliporeSigma, Boston MA, USA (J.T.)

RWA Pharma Ltd., Bath, U.K. (B.P.R.)

Rigel BioPharma Consulting LLC, Berkeley Heights, NJ (D.F.R.)

Running Title Page

Running title: 5-HOB, skeletal muscle selective β_2-agonist

Corresponding author: Magdalena Koziczak-Holbro (magdalena.koziczak@novartis.com, Phone: +41795367720), Shinji Hatakeyama (shinji.hatakeyama@novartis.com, Phone: +41799585947), Novartis Institutes for BioMedical Research, Novartis Pharma AG, CH-4056 Basel, Switzerland

Number of text pages: 35

Number of tables: 5

Number of figures: 7

Number of references: 50

Abstract 249 words

Introduction 638 words

Discussion 1453 words

Abbreviations:

AR	Adrenoceptor
5-HOB	5-hydroxybenzothiazolone derivative

A recommended section: Drug Discovery and Translational Medicine

Abstract

The anabolic effects of β_2-adrenoceptor (β_2-AR) agonists on skeletal muscle have been demonstrated in various species. However, the clinical use of β_2-AR agonists for skeletal muscle wasting conditions has been limited by their undesired cardiovascular effects. Here, we describe the preclinical pharmacological profile of a novel 5-hydroxybenzothiazolone (5-HOB) derived β_2-AR agonist in comparison to formoterol as a representative β_2-AR agonist which has been well characterized. *In vitro*, 5-HOB has nanomolar affinity for the human β_2-AR and selectivity over the β_1-AR and β_3-AR. 5-HOB also shows potent agonistic activity at the β_2-AR in primary skeletal muscle myotubes and induces hypertrophy of skeletal muscle myotubes. When compared to formoterol, 5-HOB demonstrates comparable full-agonist activity on cAMP production in skeletal muscle cells and skeletal muscle tissue derived membranes. In contrast, a greatly reduced intrinsic activity was determined in cardiomyocytes and cell membranes prepared from the rat heart. In addition, 5-HOB shows weak effects on chronotropy, inotropy and vascular relaxation when compared to formoterol. *In vivo*, 5-HOB significantly increases hind limb muscle weight in rats with attenuated effects on heart weight and ejection fraction, unlike formoterol. Furthermore, changes in cardiovascular parameters after bolus subcutaneous treatment in rats and rhesus monkeys are significantly lower with 5-HOB when compared to formoterol. In conclusion, the pharmacological profile of 5-HOB indicates superior tissue selectivity compared to the conventional β_2-AR agonist formoterol in preclinical studies, and supports the notion that such tissue-selective agonists should be investigated for the safe treatment of muscle wasting conditions without cardiovascular limiting effects.

Introduction

Skeletal muscle atrophy is a common debilitating co-morbidity currently without any approved treatment options. In humans, skeletal muscle atrophy occurs under various physiological and disease conditions, such as: following an injury resulting in immobilization, critical illness, burns, cancer, congestive heart failure, liver disease, chronic obstructive pulmonary disease, chronic kidney disease, acquired immune deficiency syndrome, and diabetes. The reduction in strength and endurance associated with the involuntary loss of muscle mass results in functional limitations, loss of independence, a reduced quality of life, increased disability, and increased mortality (Dudgeon et al 2006, Lynch et al 2007, Thomas 2007).

β-adrenoceptors (ARs) are a subfamily of G-protein-coupled receptors that are activated by the endogenous catecholamines, adrenaline and noradrenaline. They regulate diverse physiological functions, from heart pacemaker activity, myocardial contractility, vascular and bronchial smooth muscle tone, to glucose and lipid metabolism (Zheng et al 2005). β-AR can be classified into three distinct subtypes, β_1, β_2 and β_3 which share 65-70% homology between these sub-groups. β_1-ARs are the main subtype in the heart while β_2-ARs are prominently found in smooth and skeletal muscles and β_3-ARs in white and brown adipose tissues, gallbladder and urinary bladder (Sarsero and Molenaar 1995, Kim et al 1991, Ursino et al 2009). Synthetic β_2-AR agonists were initially developed to facilitate bronchodilation to relieve asthma and chronic obstructive pulmonary disease (Solis-Cohen 1990). However, several studies have shown efficacy of β_2-AR agonists in addressing skeletal muscle wasting conditions in animal models such as cancer cachexia and sarcopenia (Busquets et al 2004, Ryall et al 2007) and also in human clinical trials in orthopaedic patients, cancer cachexia, and muscular dystrophy patients (Martineau et al 1992, Maltin et al 1993, Kissel et al 2001, Skura et al 2008, Fowler et al 2008, Greig et al 2014). In animals, β_2-AR agonists can prevent skeletal muscle atrophy (e.g. injury) and elicit hypertrophy, which is associated with improvements in strength in both fast- and slow-twitch muscles (Burniston et al 2007, Ryall et al 2006, Ryall et al 2008). The increase in muscle mass results from an increase in protein synthesis and a decrease

in protein degradation, mediated via β_2-AR coupling to $G\alpha_s$ followed by adenylate cyclase induction, and a consequent increase in intracellular cAMP concentration. In addition, the $G\beta\gamma$ subunits are thought to activate the PI3K-Akt pathway (Lynch and Ryall 2008, Ryall et al 2010).

Besides the induction of skeletal muscle hypertrophy and the inhibition of atrophy, β-AR stimulation also serves as a powerful way to increase cardiac output in response to stress or exercise. As a consequence, the clinical use of β_2-AR agonists has been limited by their cardiovascular effects, for example, tachycardia and palpitation (Inamizu et al 1984, Löfdahl and Svedmyr 1989). Moreover, sustained β-AR stimulation can promote pathological cardiac remodeling such as cardiomyocyte hypertrophy and apoptosis, and ultimately contribute to heart failure. As shown in studies using heart-specific overexpression of β_1-AR and β-AR subtypes knockout animal models, β_1-AR plays a predominant role in regulation of contractilty and pathological remodeling effects (Engelhardt et al 1999, Rohrer et al 1996, Chruscinski et al 1999), β-AR-induced positive contractile responses in human heart are also predominantly mediated by β_1-AR stimulation but β_2-AR causes a significant positive chronotropic and inotropic effect (Levine and Leenen 1989, Brodde 1991).

Here, we describe the preclinical pharmacological profile of a novel 5-hydroxybenzothiazolone (5-HOB) derived, β-AR subtype and tissue selective β_2-AR agonist, (*R*)-7-(2-(1-(4-butoxyphenyl)-2-methylpropan-2-ylamino)-1-hydroxyethyl)-5-hydroxybenzo[*d*]thiazol-2(3*H*)-one. Our data demonstrate that 5-HOB has strong anabolic and functional skeletal muscle effects at doses associated with reduced cardiovascular effects in animal models, indicating a substantially improved safety margin compared to the β_2-AR agonist formoterol when administered systemically. To understand the unique efficacy profile of 5-HOB a series of *in vitro* studies was carried out, including binding affinity measurements and β_2-AR kinetics, receptor selectivity and cellular activity, as well as functional studies with isolated organs, and *in vivo* studies.

Materials and Methods

Compounds

(*R*)-7-(2-(1-(4-butoxyphenyl)-2-methylpropan-2-ylamino)-1-hydroxyethyl)-5-hydroxybenzo[*d*]thiazol-2(3*H*)-one (5-HOB; Figure 1A) was synthesized at Novartis Pharma AG (Basel, Switzerland) according to the synthetic method descibed in WO 2014132205. Formoterol hemifumarate was purchased from Tocris Bioscience (catalog number 1448, Bristol, UK) and AK Scientific (catalog number 67361; Mountain View, CA, USA). Doses of formoterol are quoted as the non-salt (molecular weight ratio = 1.169). 2-Hydroxypropyl β-cyclodextrin was purchased from Sigma-Aldrich (catalog number 332593; St. Louis, MO, USA).

Cell Culture Techniques

Chinese hamster ovary (CHO) cells stably expressing recombinant human β_1- and β_2-AR with matched expression levels were made in-house and maintained as described before (Battram et al 2006). Human Embryonic Kidney 293 (HEK293) cells expressing human β_3-AR were maintained at Eurofins Panlabs Taiwan Ltd. according to standard conditions. β_3-AR expression levels in HEK293 were comparable to the β_1-AR and β_2-AR receptors levels in the CHO cells (Bmax values 0.55, 0.334, 0.356 pmol/mg protein, respectively)

Skeletal muscle cells: Human primary fetal skeletal muscle cells (skMC, catalog number CC-2561 female; Lonza, Basel, Switzerland) were maintained and differentiated for 5 days as described previously (Lach-Trifilieff et al., 2014). To enhance differentiation the medium was supplemented with 1 μM SB431542 (Sigma-Aldrich St. Louis, Missouri, USA). Primary myoblasts were isolated from the quadriceps of Wistar Han rats at the age of 2 to 3 days, mixed gender (neonatal), from the gastrocnemius of adult male beagle dogs, and from the gastrocnemius of adult female rhesus monkeys. Briefly, for digestion muscle pieces were incubated in a dissociation medium (DMEM/F12; 6 mM NaHCO3; 1x ITSX; 1% PS) containing Collagenase IA (600 U/ml, Sigma-Aldrich) and Hyaluronidase I-S (600 U/ml,

Sigma-Aldrich) for 10 min at 37°C. Supernatants were discarded and tissue pieces were incubated and agitated in the dissociation medium containing Dispase II (2.5 mg/ml, Roche, Mannheim, Germany) for further 15 min at 37°C. The supernatant was collected and strained through a 100 µm mesh filter into ice-cold stop medium (DMEM/F12; 10% FBS; 1% ITSX; 1% PS), centrifuged at 130 g at 4°C for 5 min. Pellets containing cells were resuspended in the same medium and preplated in order to separate fibroblasts from muscle cells. After 1 h the supernatant containing muscle cells was collected and centrifuged at 130 g at 4°C for 5 min. Cells were resuspended in the stop medium and plated in 384 well plates coated with Matrigel (Falcon, Bedford, MA, USA) or collagen (Sigma-Aldrich). On the following day this medium was exchanged for one containing reduced serum content (4% FBS), two days later cells were transfered to a differentiation medium (DMEM/F12; 2% HS; 1% ITSX; 1% PS) for 5 days.

Cardiomyocytes: Human iPS-derived cardiomyocytes (catalog number CMC-100-110-001, female, Fujifilm Cellular Dynamics, Inc., Madison, WI, USA) were plated on fibronectin (Sigma-Aldrich) and maintained according to the supplier's protocol. Primary neonatal rat ventricular cardiomyocytes (NRVM) were prepared from 2 to 3 day-old pups of Wistar Han rats. Briefly, hearts were quickly excised, the atria were cut off, then the ventricles were minced and digested at 4°C overnight in calcium-free HEPES-buffered Hanks' solution, pH 7.4, plus trypsin (100 units in 10 ml). Soybean Trypsin Inhibitor (2 mg/ml in HBSS, Worthington, Lakewood, USA) was added and further digestion was performed at 37°C for 45 min by collagenase II (300 Units/ml in HBSS, Worthington).The isolated cells were centrifuged at 80 g, room temperature (RT) for 5 min. The cell pellet was resuspended in preplating medium containing DMEM/F12; 4.5 mM $NaHCO_3$; 1x Primocin ; 5% FCS; 5% HS and preplated for 1 h to reduce the contribution of non-myocardial cells. For the cAMP assay, cardiomyocytes from the supernatant were transferred into 384 well plates coated with collagen in medium containing DMEM/F12; 4.5 mM $NaHCO_3$; 1x Primocin; 2% FCS; 2% HS; 1% ITSX; 10 µM BrdU. After 24 h cells were transfered to a starvation medium without FCS and HS, and cAMP assay was performed on the following day.

Binding kinetics

CHO cells expressing the human β_2-AR were used for cell membrane preparation for radioligand binding studies. Cell membranes were prepared and competition binding assays were performed as described previously (Sykes et al 2012). To obtain affinity estimates for unlabeled 5-HOB, [^{3}H]-DHA competition binding experiments were performed. [^{3}H]-DHA (Perkin Elmer, Waltham, MA) was used at a concentration of approx. 0.6 nM such that the calculated total binding never exceeded more than 10% of total ligand concentration added, therefore avoiding ligand depletion. [^{3}H]-DHA was incubated in the presence of the indicated concentration of unlabeled 5-HOB and CHO-cell membranes expressing the β_2-AR at 37°C in assay binding buffer in 96-deep well plates with gentle agitation for 2.5 h to ensure equilibrium was reached. The kinetic parameters of unlabeled 5-HOB were assessed using the methodology of Sykes and Charlton 2010. All experiments were analyzed by non-linear regression using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a four parameter logistic equation. IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff, 1973. [^{3}H]-DHA association data was fitted as follows to a global fitting model using GraphPad Prism 5.0 to simultaneously calculate k_{on} and k_{off}. Association and dissociation rates for unlabeled antagonists were calculated using the equations described by Motulsky and Mahan, 1984. The % occupancy was calculated by estimating the concentration of each compound producing a cAMP response equivalent to 40% of the isoprenaline control in the cAMP assay (% control [EC_{40}] i.e. identical system response levels). k_{obs} curves were then simulated using the kinetic parameters, k_{off} and k_{on} at the concentration of compound producing a response equivalent to the % control [EC_{40}]. From this type of analysis it was possible to estimate the % of receptors occupied by each agonist (Sykes et al 2010).

In Vitro cell-based assays

cAMP assay:

Increases in cAMP levels were determined using HTRF technology (cAMP dynamic 2 bulk HTRF-Assay, Cisbio, Codolet, France) in 384-well plate format according to the manufacturer's protocol. Cells were stimulated with β_2-agonists for 30 min in presence of 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and then lysed by using Cisbio reagents. The measurement was performed with the Molecular Devices SpectraMax Paradigm (Molecular Devices LLC, Sunnyvale, CA, USA). Results were calculated from the 665 nm/620 nm ratio, and data were expressed in percentage of the DMSO stimulated control. The functional β_3-AR assay was performed at Eurofins Panlabs Taiwan Ltd.

Myotube hypertrophy assay:

Human skMC were differentiated to myotubes in 12-well pates coated with Matrigel and stimulated with compounds for 48 h. Immunostaining with anti-myosin heavy chain antibody (catalog numer 05-833, Upstate Biotechnology, Lake Placid, NY, USA) was performed and myotubes diameters were determined as described previously (Trendelenburg AU et al 2009). Shown images were taken with Cellomics CellInsight HCS Reader with 10x objective (ThermoFisher Scientific, Paisley, France).

Animals

Male Wistar Han or Sprague Dawley rats were purchased from Charles River Laboratories (Germany or USA), acclimatised to the facility for 7 days, housed in groups of 2 to 3 animals at 25 °C with a 12:12 h light-dark cycle, and fed a standard laboratory diet. Food and water were provided *ad libitum*. Female rhesus monkeys were housed in a colony at Novartis Pharma AG. Besides following the Guide for the Care and Use of Laboratory Animals, the experiments described here were performed according to the regulations effective in the Canton of Basel-City, Switzerland, as well as East Hanover (NJ) and Cambridge (MA), USA.

In Vitro tissue membrane assays

Procedures for the preparation of cell membrane fragments for assessment of β-ARs activity have been described previously (MacEwan et al 1996, Beitzel et al 2007). The gastrocnemius muscle and the heart were collected from 3 to 4 month-old male Wistar rats. The frozen tissues were pulverized in a Cryopress (Microtec Co., Ltd, Chiba, Japan) and homogenized in a buffer containing 20 mM HEPES, 0.25 M sucrose, 1 mM EGTA pH 7.0 and a protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). The homogenate was centrifuged at 1,000 g for 10 min, and the supernatants were filtered through a polyamide mesh (pore size 250 μm) into an ultracentrifuge tube. The filtered supernatants were further cleared by centrifugation at 10,000 g for 15 min, and the supernatant containing cell membrane fragments were prepared by repeated ultracentrifugation at 100,000 g for 30 min, to isolate the sarcolemma membrane. Enrichment in membrane fraction was validated by WB (Supplemental Figure 1). After this step, the cell membrane pellets were resuspended in a buffer containing 10 mM HEPES, 0.1 mM EDTA pH 7.4 and a protease inhibitor cocktail. The cell membrane suspensions and compounds (diluted in a buffer containing 30 mM HEPES pH 7.4, 20 mM phosphocreatine, 20 units/mL creatine phosphokinase, 20 μM GTP, 400 μM ATP, 10 mM $MgCl_2$, 300 μM IBMX, 200 μM Rolipram) were mixed with a ratio of 1:1 and incubated at room temperature for 30 min. Sequentially, HTRF cAMP CisBio reagents were added, followed by measurement and analysis (performed as described above).

In Vitro isolated organ assays

Chronotropy in rabbit sino-atrial node: the right atrium was separated from the rest of the heart (collected from New Zealand white female rabbits from Charles River Laboratories, Sulzfeld, Germany). The preparations were mounted in a tissue bath and kept at 37°C for at least 1 h stabilization. The stabilization phase lasted at least 60 min during the period that the sinoatrial (SA) pacemaker activity should stay stable for at least 20 min. Action potentials were intracellularly recorded with a standard glass microelectrode filled with 3 M KCl, connected to a high input impedance-neutralizing microelectrode

amplifier VF-180 (Bio-Logic Science Instruments, Seyssinet-Pariset, France). The action potentials were displayed on a digital oscilloscope HM-407 (Rohde & Schwarz (HAMEG Instruments), Munich, Germany), analyzed by means of high resolution data acquisition system Notocord software hem 4.2 (Notocord Systems, Le Pecq, France). Inotropy assay in guinea pig left atria: the assay was conducted at Eurofins Panlabs (Taipei, Taiwan). Left atria were isolated from Dunkin Hartley derived male or female guinea pigs with body weights of 600 g, and the inotropy response as measured by isometric changes in gram were evaluated according to the method described elsewhere (Grodzińska and Gryglewski 1971). Vascular relaxation in rat aortic ring: The thoracic aorta was isolated from Sprague Dawley rats, and cut into 2 to 3 mm long rings. The rings were mounted on stainless steel hooks and suspended in organ glass chambers (Radnoti, Covina, CA, USA). Tension development was measured by isometric force transducers 159901A (Radnoti) connected to a data acquisition system, PowerLab (ADInstruments, Oxford, UK). Cumulative dose-response curves to formoterol and 5-HOB were obtained. Absolute tension of all points was analyzed, and the relaxant response to each concentration of compound was expressed as the percentage decrease from pre-contraction induced by 100 nM phenylephrine.

Effects on hind limb muscle and heart in Wistar rats

Compound adnimistration:

Formoterol and 5-HOB were subcutaneously administered to rats using Alzet osmotic minipumps (the model 2ML4 with pumping rate of 2.5 μL/hr, Charles River Laboratories, Sulzfeld, Germany) for 4 weeks. Formoterol was dissolved in 0.9% NaCl to achieve doses at 0.003, 0.01 and 0.03 mg/kg/day, and 5-HOB was dissolved in 20% Cremophor EL and ethanol mixed with a ratio of 1:2 in 0.9% NaCl to achieve doses at 0.01, 0.03 and 0.1 mg/kg/day. The dose range was selected to achieve a similar range of plasma compound concentrations at steady state.

Evoked force:

After treatment with the compounds for 4 weeks, the sciatic nerve was attached to a nerve stimulating electrode (Harvard Apparatus, Holliston, MA, USA) and the Achilles tendon with a piece of calcaneus was attached with a polyethylene thread (Spiderwire 16/1135590; 0.30 mm/110 m; Pure Fishing, Hattersheim Germany) to a force displacement transducer (Model FT10; Grass Technologies, West Warwick, RI, USA) under anesthesia. The knee and ankle joints were immobilized with a clamp, and the stimulating electrode was connected to an electrical stimulator (ADInstruments). The force of contraction was acquired with PowerLab system and analyzed using LabChartPro (ADInstruments).

Dissection and data analysis:

The tibialis anterior, gastrocnemius and soleus muscles were dissected and weighed. The brain weight was also measured for normalization of heart weights (Sellers et al 2007). Body weight was expressed as % change from day 0 as the start of treatment (initial body weight). Muscle weight was normalized to initial body weight and then expressed as % change from the vehicle control group. The organ weight was normalized to brain weight and then expressed as % change from the control group.

Effects on calf muscle mass and ejection fraction in Wistar rats

Compound adnimistration:

Formoterol and 5-HOB were administered subcutaneously to rats once daily for 4 weeks and then the treatment was discontinued for 5 weeks. Formoterol was dissolved in 0.9% NaCl, and 5-HOB was dissolved in 50% PEG200 in distilled water.

MRI measurement and data analysis:

Magnetic resonance imaging (MRI) measurement was performed in the rats under anesthesia with isoflurane at a concentration of 1 to 1.3%, using a Bruker Avance 7 T / 30 cm wide-bore instrument

(Bruker BioSpin, Billerica, MA, USA) equipped with a 12-cm inner-diameter (i.d.) actively shielded gradient insert. Two electrocardiography (EKG) electrodes 3M Red Dots (3M, St. Paul, MN, USA) were attached. Respiration, body temperature and EKG were monitored throughout the experiment using the physiology monitoring system by SA Instruments, Inc. Cardiac images were acquired in the standard cardiac views which are aligned along the principle axes of the organ. The segmentation and length measurements were performed for both diastole and systole. Left-ventricular volume was calculated by integrating the blood pool volume using a truncated cone model, and ejection fraction was also calculated. Muscle volume was measured using an imaging method that separates water and fat signals (Tsao and Jiang 2013). The 3D water-only image was reconstructed in Matlab (Mathworks). Lower leg muscle volume was calculated using in-house generated software.

Effects on heart rate and mean arterial pressure in Wistar rats

Surgical procedure:

Rats were surgically instrumented with a chronically indwelling femoral arterial catheter to allow direct measurement of arterial blood pressure and repeated blood sampling, and with a catheter terminating subcutaneously to allow subcutaneous administration of vehicle or compounds.

A femoral artery was isolated and a catheter inserted. The catheter was tunneled subcutaneously and exteriorized in the mid-dorsal abdominal region. The catheter exited through a subcutaneously anchored skin button/tether/swivel system which allowed the animal to move unrestrained in a specialized plexiglass wire-bottom cage.

For implanting the s.c. catheter, a narrow tunnel was created by inserting a Teflon catheter/needle subcutaneously from the skin button incision site to the mid-scapular region. The tip of the catheter/needle was pushed through the mid-scapular skin and the needle removed, leaving the Teflon catheter intact and still protruding through the skin. The hub of the Teflon catheter was cut off. The Tygon s.c. catheter was inserted into the Teflon catheter such that the tip of the s.c. catheter extended

slightly beyond the tip of the Teflon catheter. The Tygon catheter and tubing were clamped at the exit site from the skin. The Tygon catheter was anchored at the skin button incision site. The Tygon and Teflon tubings were unclamped and the Teflon catheter was removed through the skin puncture wound. The mid-scapular skin was then lifted to retract the tip of the Tygon catheter s.c. The Tygon catheter was exteriorized through the spring tether along with the arterial catheter.

Compound adnimistration and data acquisition:

Formoterol was dissolved in 0.9% NaCl, and 5-HOB was dissolved in 0.27% 2-hydroxypropyl β-cyclodextrin in saline. The compound or vehicle was administered subcutaneously via the implanted catheter over 40 to 45 sec followed by an air flush over 10 to 15 sec. The arterial catheter was attached to a pre-calibrated blood pressure transducer, Statham P23 (AMETEK Power Instruments, Rochester, NY, USA) for continuously monitoring arterial pressure. The pulsatile blood pressure signals were conditioned and amplified with Gould pre-amplifiers and further processed with a Modular Instruments, Inc. digital data acquisition system. For an individual rat, mean arterial pressure and heart rate were continuously derived from all beats over consecutive 15-sec intervals.

Effects on heart rate in rhesus monkeys

Formoterol and 5-HOB were administered subcutaneously to rhesus monkeys at the age of 6 to 8 years-old with the body weight around 5 to 7 kg. Formoterol was dissolved in 0.9% NaCl, and 5-HOB was dissolved in 2.5% Pluronic F-127 in 0.9% NaCl. The monkeys were restrained on a chair up to 4 h after subcutaneous administration, and then returned to their pens. Heart rate, potassium and glucose were measured. Heart rate was measured by Surgivet V3304 device (Smiths Medical, Adliswil, Switzerland). Blood samples were collected at 0, 0.08, 0.25, 0.5, 1, 2 and 4 h on the chair, and blood glucose was measured with a glucometer (Accu-Check Aviva Nano; Roche Diagnostics, Rotkreuz, Switzerland). Potassium concentration was measured by an automated blood biochemistry analyzer (NOVA CRT8A; Laboratory Systeme Flükiger, Menziken, Switzerland).

Compound concentration monitoring

In some experiments, compound concentrations in plasma were measured to assess the relationship between concentration and response. Plasma protein was precipitated by mixing with acetonitrile and removed by centrifugation. The remaining solvent in the supernatant was evaporated, dissolved, and injected into a LC-MS/MS system, AB SCIEX QTRAP 5500 (AB Sciex, Baden, Switzerland) for analysis.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism; comparison of 2 groups with unpaired t-test, multiple comparisons with Bonferroni's test or Dunnett's test following one-way ANOVA, or Holm-Sidak's test or Sidak's test following 2-way ANOVA as indicated in each figure or table legend. Differences were considered to be significant when the probability value was < 0.05. Concentration-response data were evaluated by sigmoid curve fitting using XLfit (IDBS) yielding EC_{50} values (concentrations causing half-maximal effects) and E_{max} (maximal effects).

JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Binding affinity and kinetic characteristics

The affinity of 5-HOB and formoterol for the β_2-AR was assessed in a [^{3}H]-DHA radio-ligand competition binding assays using membranes derived from CHO cells expressing the human β_2-AR (Table 1). Both agonists showed high binding affinity for the β_2-AR in the nanomolar range, with 5-HOB exhibiting approximately a 10-fold higher binding affinity than formoterol (Table 1). We also measured binding kinetic parameters of both agonists using the competition-association binding method first described by Motulsky and Mahan, 1984. 5-HOB showed a longer residence time at the human β_2-AR, with a slower dissociation half-life when compared to formoterol. The mean kinetic k_{on} and k_{off} values determined for 5-HOB were 7.43 x 10^8 $M^{-1}min^{-1}$ and 0.733 min^{-1} respectively, producing a kinetically derived dissociation constant K_d (k_{off}/ k_{on}) value of 1.08 ± 0.14 nM which is in excellent agreement with the equilibrium K_i value obtained from competition binding experiments. Displacement of [^{3}H]-DHA from the β_2-AR by β_2 agonists was determined in the presence of 1 mM GTP to uncouple the receptor from downstream G proteins and therefore these affinity measurements are reflective of a low affinity form of the receptor (Kent et al 1980). 5-HOB had higher binding affinity to the low affinity form of the receptor compared to formoterol.

In vitro functional activity of 5-HOB in cellular systems and membrane extracts

The functional activity and receptor selectivity of 5-HOB were evaluated by the measurement of cAMP production in CHO or HEK293 cells stably expressing a comparable level of each of the three human β-AR subtypes: β_1, β_2, and β_3 (Table 2). 5-HOB showed a potent agonistic response at the human β_2-AR (EC_{50} = 3.5 nM) and high selectivity over the other subtypes β_1-AR and β_3-AR (EC_{50} = 793 nM and 925 nM, respectively). In comparison to formoterol, 5-HOB showed a similar selectivity profile on all three human β-AR subtypes but with an overall 10-fold lower potency. Importantly, a clear difference in the maximum effect (E_{max}) was observed between the two agonists on the human β_1-AR, 40 ± 6.8% for 5-

HOB compared to 84 ± 6.5% for formoterol ($P < 0.0001$ by t-test), which is considered to be advantageous for 5-HOB in terms of cardiac safety, as the β_1-AR is the main subtype expressed in the heart.

Based upon the above β_2-AR functional activity and binding data we performed a modelling simulation of receptor occupancy at the EC_{40} of cAMP response. The simulation revealed that 5-HOB requires a much higher degree of β_2-AR receptor occupancy (70.4%), relative to formoterol (1.8%), to produce an equivalent effect (Table 2), indicating a lower intrinsic efficacy of 5-HOB.

In addition, functional cAMP assays were conducted using the relevant primary human, rat, dog and monkey cells with endogenous expression of β_1- and β_2-ARs. 5-HOB was shown to be highly potent and efficacious at inducing cAMP production in skeletal muscle myotubes, in a similar manner to formoterol (Table 3). However, 5-HOB was less efficacious (appearing as a partial agonist) than formoterol at inducing cAMP production in iPS-derived human cardiomyocytes and rat cardiomyocytes when compared to the effect on skeletal muscle myotubes. The EC_{50} values were comparable among species, indicating that cross-species differences seem to be minimal in terms of cAMP responses in these cell types.

The induction of cAMP was also evaluated in cell membranes isolated from rat skeletal muscle and heart tissue (Table 4, Figure1B). The effect of 5-HOB on cAMP induction was quite prominent in rat skeletal muscle membranes, but much weaker in rat heart membranes. When compared to formoterol, 5-HOB demonstrated comparable maximal efficacy on skeletal muscle membranes and a significantly reduced efficacy on heart membranes (P=0.005 by t-test), clearly demonstrating the tissue selective characteristics of 5-HOB. In conclusion, all *in vitro* functional studies point to a potent effect of 5-HOB on skeletal muscle comparable to that of formoterol, and an intrinsically weaker effect of 5-HOB on the cardiac tissue (membranes or cells) including in comparison to formoterol.

In vitro effect on skeletal muscle hypertrophy

The functional activity of 5-HOB was additionally tested in an *in vitro* human primary skeletal muscle hypertrophy assay (Figure 2). 5-HOB induced a significant increase in skeletal myotube diameter, an effect that was blocked by the β_2-AR antagonist ICI-118,551, but not by the selective β_1-AR antagonist CGP20712. These data indicate that skeletal muscle hypertrophy is specifially induced by agonistic activity of 5-HOB on the β_2-AR.

In vitro functional activity of 5-HOB in isolated organs

To assess tissue selectivity, the effects of 5-HOB on the cardiovascular system have been evaluated in *in vitro* isolated-organ assays: rabbit sinoatrial node for measuring chronotropic effects (Figure 3), guinea pig left atria for measuring inotropic effects (Table 5), and rat aortic ring for measuring vascular relaxation effects (Table 5). Formoterol was shown to be a potent inducer of sinoatrial node pacemaker activity (Figure 3B; +11% at 5 nM and +45% at 150 nM), while 5-HOB showed a weak effect on chronotropy up to a concentration of 150 nM (Figure 3C; +6.5% from baseline). No direct effects of 5-HOB were noted on inotropy at concentrations of up to 10 μM, while formoterol induced atrial contractility with an EC_{50} of 17 nM. In an aortic ring relaxation assay, formoterol showed a potent effect with an EC_{50} of 0.8 nM, but 5-HOB showed a weaker effect with an EC_{50} of 31 nM. Overall, the effect of 5-HOB on chronotropy, inotropy and vascular relaxation were observed only at the highest concentrations, and were negligible in the concentration range producing skeletal muscle changes described below.

In vivo 5-HOB effects on skeletal muscle and on heart in Wistar rats

The effect of 5-HOB on skeletal muscle and heart weight was evaluated in Wistar rats with subcutaneously implanted osmotic mini-pumps, in comparison to the effect of formoterol. Formoterol significantly increased both skeletal muscle and heart weight to a similar extent: 13% and 15% at 0.003 mg/kg (both $P < 0.05$), 20% and 17% at 0.01 mg/kg (both $P < 0.05$), and 27% and 21% at 0.03 mg/kg

(both $P < 0.05$), for the weight of pooled hind limb skeletal muscle and heart in comparison to the vehicle control, respectively (Figure 4A, Supplemental Table 1). The delta in weight changes between skeletal muscle and heart were not statistically significant at all doses for formoterol ($P > 0.05$, Supplemental Table 1). In contrast, 5-HOB showed a selective increase for skeletal muscle weight over heart weight: 11% and 6.9% at 0.01 mg/kg ($P < 0.05$, $P = 0.2324$), 17% and 9.3% at 0.03 mg/kg ($P < 0.05$, $P = 0.0939$), and 26% and 12% at 0.1 mg/kg (both $P < 0.05$), for the weight of pooled hind limb skeletal muscle and heart in comparison to the vehicle control, respectively (Figure 4B, Supplemental Table 1). It should be noted that the weight changes in the heart at the two lower doses were not statistically significant from the vehicle control (Figure 4B). The delta in weight changes between skeletal muscle and heart were not statistically different at lower doses and became statistically significant at 0.1 mg/kg (Supplemental Table 1). A significant increase in evoked force was also observed in parallel to skeletal muscle hypertrophy for each formoterol and 5-HOB (tested at 0.01 mg/kg, Figure 4C), and the increase in evoked force between formoterol and 5-HOB was not statistically significant ($P = 0.8086$ by t-test). The doses of β_2-AR agonists showed comparable increase in plasma exposure across the three doses (Supplemental Table 1). Therefore, the relationship between plasma concentration and skeletal muscle effects were similar for both formoterol and 5-HOB.

To gain further insights into the effect of formoterol and 5-HOB on skeletal muscle mass and ejection fraction as a measure of cardiac function, longitudinal monitoring with MRI was conducted. Once daily subcutaneous treatment for 4 weeks followed by washout for 5 weeks allowed an assessment of duration of action and reversibility. The effect of 5-HOB on skeletal muscle mass was equivalent to the effect with formoterol at the same dose level (0.03 mg/kg qd), and this hypertrophy effect was maintained for 5 weeks after cessation of treatment, when compared to the vehicle treated groups (Figure 5A). In the formoterol- and 5-HOB-treated groups, the increases in skeletal muscle mass when compared to the vehicle control were statistically significant at 2, 4, 6, 9 weeks ($P < 0.05$; Supplemental Table 2). There was no statistical difference on the increases in skeletal muscle mass between formoterol and 5-HOB ($P >$

0.05; Supplemental Table 2). In contrast to the effect on muscle volume, there was a clear difference in ejection fraction between formoterol and 5-HOB. During the 4 week treatment period, formoterol significantly reduced ejection fraction when compared to the vehicle control ($P < 0.05$ at 2 and 4 weeks), whereas 5-HOB did not ($P > 0.05$ at all time points) (Figure 5B, Supplemental Table 2). The change in ejection fraction between formoterol and 5-HOB was statistically significant at 2 weeks ($P < 0.0001$) and became not significantlly different after 4 weeks ($P > 0.05$ at 4, 6 and 9 weeks) (Supplemental Table 2). During the washout period, the effect of formoterol on ejection fraction was found to be reversible, and returned close to baseline.

Cardiovascular effects in Wistar rats and rhesus monkeys

In order to evaluate if 5-HOB would show attenuated acute cardiovascular effects *in vivo* after single subcutaneous treatment, as indicated by the cellular and isolated organ assays, we monitored heart rate in Wistar rats and rhesus monkeys. In the rat, formoterol induced a maximum heart rate increase of approximately 150 bpm at the 3 highest doses tested (0.01 to 0.1 mg/kg), and a nearly maximal effect at the lowest dose (0.003 mg/kg, C_{max} = 2.2 nM) within 5 min of dosing, as shown in Figure 6A. In contrast, when compared to the vehicle control, the effect of 5-HOB on heart rate was apparent only at the highest tested dose (0.3 mg/kg, C_{max} = 18 nM) with an increased heart rate of +58 bpm (a 14% increase from the baseline) and showed almost no effect at the lower doses (Figure 6B). Thus, formoterol is more potent than 5-HOB in inducing a positive chronotropic effect, as reflected by the relative shift in the dose-response relationships (Figure 6C). The effect of formoterol was statistically significant at all doses, and the effect of 5-HOB was statistically significant only at 0.3 mg/kg (Supplemental Table 3). Unlike the changes in heart rate, formoterol dose-dependently decreased mean arterial pressure by a maximum of approximately 30 mmHg whereas the effect of 5-HOB on mean arterial pressure was again apparent only at 0.3 mg/kg (Figure 6D). The effect of formoterol was statistically significant above 0.01 mg/kg and the effect of 5-HOB was statistically significant only at 0.3 mg/kg (Supplemental Table 3). Nevertheless,

formoterol was still 1.5-2 orders of magnitude more potent than 5-HOB in eliciting a vasodepressor response.

In rhesus monkeys, a single subcutaneous dose of formoterol increased heart rate by +60 bpm at 5 min post dosing (+32% from pre-dose baseline) at 0.01 mg/kg with plasma concentration of 7.7 nM (Figure 7A). In contrast, a single subcutaneous dose of 5-HOB showed a heart rate change of +17 bpm at 30 min post dosing (+9.2% from pre-dose baseline), which is in the range of the variability observed in the control groups after being returned to their pens, at 0.03 mg/kg with plasma concentration of 4.4 nM (Figure 7A). There was no obvious heart rate change observed with 5-HOB dosed at 0.01 mg/kg. The effect on heart rate with 5-HOB was clearly lower than that of formoterol when plasma concentrations to heart rate response relationships are compared, as illustrated in Figure 7B. Blood glucose and serum potassium levels were also evaluated as well described β_2-AR mediated pharmacodynamic markers (Supplemental Figure 3): formoterol dosed at 0.01 mg/kg induced hyperglycemia between +40% to +43% compared to pre-dose baseline, while 5-HOB dosed at 0.03 mg/kg increased blood glucose by around 13%, which is in the range of variation observed in the control groups after they were returned to their pens, when compared to the pre-dose baseline levels. There was no obvious change in blood glucose levels observed with 5-HOB dosed at 0.01 mg/kg. The hyperglycemic effect of 5-HOB was clearly lower than that of formoterol. Moreover, the degree of hypokalemic effects showed a similar trend as observed with the blood glucose changes, and 5-HOB clearly showed a lesser effect on serum potassium levels (Supplemental Figure 3). Overall, 5-HOB has a favorable profile in terms of cardiac and metabolic effects when compared to formoterol, i.e. on heart rate, blood glucose and serum potassium changes.

Discussion

In the present study, we have investigated the preclinical pharmacological profile of 5-HOB, a novel β_2-AR agonist with a potent anabolic effect on skeletal muscle but with markedly attenuated cardiovascular effects, which was aimed for the treatment of skeletal muscle wasting conditions, where cardiovascular effects associated with this class of drugs would be undesireable. To benchmark the profile, 5-HOB has been compared to formoterol as a representative and well characterized β_2-AR agonist. Before this study was performed, it had not been previously demonstrated that one could achieve relative tissue selectivity with a β_2-AR agonist.

In a radioligand binding assay, 5-HOB demonstrated high affinity for the human β_2-AR. The β_2-AR binding affinity translated into observed *in vitro* efficacy, exemplified by the rise in intracellular cAMP in which 5-HOB had high potency in the nanomolar range on the β_2-AR and displayed selectivity over β_1-AR and β_3-AR. The receptor selectivity profile of 5-HOB against the β-AR subtypes is comparable to that of formoterol, but with lower intrinsic efficacy on the β_1-AR, which is advantageous for 5-HOB in terms of cardiac safety as the β_1-AR is the main subtype expressed in the heart (Zheng et al 2005) and implicated in pathological remodeling effects (Engelhardt et al 1999, Morisco et al 2001). Additionally, studies in β-AR subtype KO mice imply that the β_1-AR plays a predominant role in catecholamine-mediated regulation of heart rate and myocardial contractility (Rohrer et al 1999; Chruscinski et al 1999). However, the β_2-AR also contributes to these cardiovascular effects (Molenaar et al 2000). This suggests that β_2-AR selective agonists could offer an advantage for a reduced cardiovascular side-effect profile. Therefore, lower efficacy on the β_1-AR for 5-HOB, when compared with formoterol, might contribute to the compound's favorable cardiac profile. The relevance of β_3/ β_2 selectivity is currently less clear, although it has been shown that activation of β_3-AR in the ventricle may cause negative inotropic effects in the heart (Gauthier et al 2000, Angelone T et al 2008). The main roles described for the β_3-AR are in lipolysis and bladder relaxation, i.e. tissues where this receptor sub-type is predominant (Philipson 2002, Yamaguchi 2002). Our studies have also investigated β_2-AR-agonist-mediated stimulation in systems

with an endogenous distribution of receptors: in primary cells and in cell membranes prepared from rat tissues. In a functional cAMP assay using primary skeletal muscle cells, 5-HOB was shown to be a highly efficacious agonist, while in cardiomyocytes 5-HOB behaved as a partial agonist with a reduced intrinsic efficacy versus formoterol. Consistent with the result obtained with skeletal muscle myotubes and cardiomyocytes, both 5-HOB and formoterol appeared to be equally efficacious agonists in skeletal muscles membranes, while 5-HOB was less efficacious compared to formoterol in heart membranes. Similar differences between the two agonists were also observed in the assays using isolated organs for evaluating the effect on the cardiovascular system. Thus, 5-HOB showed only a weak effect on pacemaker activity and on aortic ring relaxation, and no effect on atrial contractility. In contrast, formoterol exerted potent effects on chronotropy, inotropy and vascular relaxation, as would be expected from a conventional β_2-AR agonist.

In our studies both β_2-AR agonists, 5-HOB and formoterol, displayed comparable anabolic action on skeletal muscle *in vitro* and *in vivo*. In addition, we demonstrated that the 5-HOB-induced skeletal muscle hypertrophy is β_2-AR mediated. *In vitro*, 5-HOB promoted significant hypertrophy in human skeletal muscle myotubes and this effect was blocked by the β_2-AR antagonist ICI-118,551, but not by the β_1-AR selective antagonist CGP20712. This is in line with what has previously been shown *in vivo* by others: the β_2-AR agonist (clenbuterol) mediated skeletal muscle hypertrophy can be blocked by selective β_2-AR antagonists, or by genetic deletion of the β_2-AR (Choo et al 1992; Hinkle et al 2002). Also in our *in vitro* assay formoterol- and clenbutrol-induced hypertrophy were blocked by ICI-118,551 (Supplemental Figure 2). In line with our *in vitro* hypertrophy data, in rat studies both 5-HOB and formoterol were capable of inducing comparable strong skeletal muscle hypertrophy as well as increasing muscle function measured by evoked force at an equivalent plasma concentration. However, 5-HOB caused lower increase in heart weight compared to skeletal muscle weight while formoterol produced nearly equivalent increases in both heart and skeletal muscle weight. This selective action of 5-HOB on skeletal muscle could be further demonstrated by the lack of an effect on cardiac function, as measured by ejection

fraction, in comparison to formoterol which significantly decreased ejection fraction during the treatment period. It seems clear that the increased heart rate was associated with the decreased cardiac function in the formoterol treatment group.

When acute cardiovascular effects were evaluated, formoterol at a dose of 0.003 mg/kg, with a C_{max} of 2.2 nM, was sufficient to elicit almost the maximum heart rate increase of approximately 150 bpm (approx. 35-40% increase) in the rat. In contrast, although the highest doses of 5-HOB achieved approximately 10-fold higher C_{max} value (18 nM at 0.3 mg/kg), heart rate was increased by only 58 bpm. The effect of 5-HOB on mean arterial pressure was also less compared to formoterol. Overall, 5-HOB was about 1.5 to 2 orders of magnitude less potent than formoterol in eliciting cardiovascular responses. These differences in heart rate responses between formoterol and 5-HOB were also well reproduced in a similar plasma concentration range in the rhesus monkey. These *in vivo* findings clearly reflect the results obtained with isolated organs for inducing cardiovascular effects, implying that 5-HOB is indeed a partial agonist, not only in the cardiovascular system, but also in other organs such as the liver based upon the effect on glucose levels (Supplemental Figure 3). The lack of effect on cardiac function as well as the attenuated acute cardiovascular responses with 5-HOB provide a clear advantage over conventional β_2-AR agonists, such as formoterol, which apparently do not exhibit such tissue selectivity. What is particularly encouraging were the effects of 5-HOB on rhesus monkeys. In these animals, formoterol had a significant effect on heart rate even at the lowest dose, inducing a 60 bpm increase; in contrast, 5-HOB did not cause a statistically significant effect, even at therapeutic levels.

As discussed above, the attenuated effect of 5-HOB on heart rate could be partially explained by its receptor selectivity for the β_2-over the β_1-AR, detected in *in vitro* functional studies. However, other factors may contribute to the greater effect of 5-HOB on skeletal muscles when compared to effects on the heart. The differential responses between tissues may also be related to differences in absolute β_2-AR numbers, as well as differences in β_2-AR/G protein/AC ratios between the different tissue types. A drug that is a partial agonist in one tissue could also be a full agonist in a different tissue wherein either

receptor density or receptor-to-response coupling efficiency is relatively high. Supporting this notion, it has been shown for the partial agonist salmeterol that the maximal agonist-mediated stimulation of cAMP production can be increased through the elevation of total levels of β_2-AR (McDonnell et al 1998). Additionally, other studies have demonstrated that enhanced expression of AC enables greater maximal cAMP generation following receptor activation (MacEwan et al 1996). Our data clearly demonstrate that 5-HOB shows the characteristics of a partial agonist: in cAMP functional assays, it displayed partial efficacy in both cardiomyocytes and membranes isolated from the heart. On the other hand, we detected higher β_2-AR expression at the mRNA level in skeletal muscle compared to heart tissue in the rat, and in cells isolated from these organs (Supplement Figure 4). Thus, in skeletal muscle tissue, a lower intrinsic efficacy agonist such as 5-HOB may be able to provoke a maximal functional response because the receptor density is relatively high and, or, the receptor reserve is relatively large. In contrast, in the heart with a lower level of receptor expression a lower intrinsic activity compound, such as 5-HOB, would have minimal efficacy. Interestingly, such tissue selectivity has been described previously for adenosine A_1 receptor agonists. Low intrinsic efficacy adenosine A_1 receptor agonists can reduce lipolysis at concentrations that do not cause effects on heart rate due to a greater receptor reserve in adipose tissue compared with cardiac tissue (Fatholahi et al 2006; Wu et al 2001).

In summary, the preclinical data reported in this study show that 5-HOB is a potent, selective β_2-AR agonist that is effective in promoting skeletal muscle growth. Furthermore, 5-HOB displays tissue selectivity and reduced cardiovascular effects, when compared to the well-described representative β_2-AR agonist formoterol in preclinical studies. Hence, these data suggest that 5-HOB may provide a new valuable treatment option for muscle atrophy conditions. Clinical studies will determine whether 5-HOB has the potential for reduced cardiac side effects at therapeutic doses in humans compared with other conventional β_2-AR agonists, such as formoterol.

Acknowledgements

We thank Ralf Endres, Daniel Lehmann for bioanalytics support, and Michael S. Hansen and Yun Jiang for imaging support, and Wei Chen, Fumin Fu, Michael Beil, Jing Liu and Marie-Claude Del Grosso for cardiovascular profiling support, and Antonia Rosenstiel for cAMP assay support.

Autorship Contributions

Participated in research design: Koziczak-Holbro, Rigel, Dumotier, Sykes, Tsao, Adachi, Kiffe, Azria, Charlton, Richardson, Lach-Trifilieff, Glass, Hatakeyama

Conducted experiments: Koziczak-Holbro, Rigel, Dumotier, Sykes, Tsao, Nguyen, Bösch, Jourdain, Flotte, Adachi, Kiffe, Azria, Hatakeyama

Contributed new reagents or analytic tools: Fairhurst, Ullrich

Performed data analysis: Koziczak-Holbro, Rigel, Dumotier, Sykes, Tsao, Nguyen, Bösch, Jourdain, Flotte, Adachi, Kiffe, Azria, Hatakeyama

Wrote or contributed to the writing of the manuscript: Koziczak-Holbro, Rigel, Dumotier, Sykes, Tsao, Adachi, Kiffe, Fairhurst, Charlton, Lach-Trifilieff, Glass, Ullrich, Hatakeyama

References

Angelone T, Filice E, Quintieri AM, Imbrogno S, Recchia A, Pulerà E, Mannarino C, Pellegrino D, Cerra MC (2008) β_3-adrenoceptors modulate left ventricular relaxation in the rat heart via the NO-cGMP-PKG pathway. *Acta Physiol (Oxf)* **193**:229-39

Battram C, Charlton SJ, Cuenoud B, Dowling MR, Fairhurst RA, Farr D, Fozard JR, Leighton-Davies JR, Lewis CA, McEvoy L, et al. (2006) In vitro and in vivo pharmacological characterization of 5-[(R)-2-(5,6-diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1H-quinolin-2-one (indacaterol), a novel inhaled β_2-adrenoceptor agonist with a 24-h duration of action. *J Pharmacol Exp Ther* **317**: 762-770.

Beitzel F, Sillence MN, Lynch GS (2007) β-Adrenoceptor signaling in regenerating skeletal muscle after β-agonist administration. *Am J Physiol Endocrinol Metab* **293**: E932-40.

Brodde OE (1991) β_1- and β_2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. *Pharmacol Rev* **43**, 203-242

Burniston JG, McLean L, Beynon RJ, Goldspink DF (2007) Anabolic effects of a non-myotoxic dose of the β_2-adrenergic receptor agonist clenbuterol on rat plantaris muscle. *Muscle Nerve* **35**: 217-23.

Busquets S, Figueras MT, Fuster G, Almendro V, Moore-Carrasco R, Ametller E, Argilés JM, López-Soriano FJ (2004) Anticachectic effects of formoterol: a drug for potential treatment of muscle wasting. *Cancer Res* **64**: 6725-31.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099-108.

Choo JJ, Horan MA, Little RA, Rothwell NJ (1992) Anabolic effects of clenbuterol on skeletal muscle are mediated by β_2-adrenoceptor activation. *Am J Physiol* **263**: E50-6.

Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK (1999) Targeted disruption of the β_2-adrenergic receptor gene. *J Biol Chem* **274**: 16694-700.

Dudgeon WD, Phillips KD, Carson JA, Brewer RB, Durstine JL, Hand GA (2006) Counteracting muscle wasting in HIV-infected individuals. *HIV Med* **7**: 299-310.

Engelhardt S, Hein L, Wiesmann F, Lohse MJ (1999) Progressive hypertrophy and heart failure in β1-adrenergic receptor transgenic mice. *Proc Natl Acad Sci USA* **96**: 7059-64.

Fatholahi M, Xiang Y, Wu Y, Li Y, Wu L, Dhalla AK, Belardinelli L, Shryock JC (2006) A novel partial agonist of the A(1)-adenosine receptor and evidence of receptor homogeneity in adipocytes. *J Pharmacol Exp Ther* **317**: 676-84.

Fowler EG, Graves MC, Wetzel GT, Spencer MJ (2008) Pilot trial of albuterol in Duchenne and Becker muscular dystrophy. *Neurology* **62**: 1006-8.

Gauthier C, Leblais V, Moniotte S, Langin D, Balligand JL (2000) The negative inotropic action of catecholamines: role of β_3-adrenoceptors. *Can J Physiol Pharmacol* **78**: 681-90.

Greig CA, Johns N, Gray C, MacDonald A, Stephens NA, Skipworth RJ, Fallon M, Wall L, Fox GM, Fearon KC (2014) Phase I/II trial of formoterol fumarate combined with megestrol acetate in cachectic patients with advanced malignancy. *Support Care Cancer* **22**: 1269-75.

Grodzińska L and Gryglewski R (1971) Action of β-adrenolytics on the isolated guinea-pig atria. *Arch Int Pharmacodyn Ther* **191**: 133-41.

Hinkle RT, Hodge KM, Cody DB, Sheldon RJ, Kobilka BK, Isfort RJ (2002) Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the β_2-adrenergic receptor. *Muscle Nerve* **25**: 729-34.

Inamizu T, Ikuta T, Tanabe M, Nishimoto Y (1984) Evaluation of a new bronchodilator, Formoterol, using biochemical parameters. *Rinsho Kenkyu* **61**: 251-60.

Kent RS, De Lean A, Lefkowitz RJ (1980) A quantitative analysis of β-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol Pharmacol* **17**: 14-23.

Kim YS, Sainz RD, Molenaar P, Summers RJ (1991) Characterization of β_1- and β_2-adrenoceptors in rat skeletal muscles. *Biochem Pharmacol* **42**: 1783-9.

Kissel JT, McDermott MP, Mendell JR, King WM, Pandya S, Griggs RC, Tawil R (2001) Randomized, double-blind, placebo-controlled trial of albuterol in facioscapulohumeral dystrophy. *Neurology* **57**: 1434-40.

Lach-Trifilieff E, Minetti GC, Sheppard K, Ibebunjo C, Feige JN, Hartmann S, Brachat S, Rivet H, Koelbing C, Morvan F, Hatakeyama S, Glass DJ (2014) An antibody blocking activin type II receptors induces strong skeletal muscle hypertrophy and protects from atrophy. *Mol Cell Biol* **34**: 606-18.

Levine MA and Leenen FH (1989) Role of β_1-receptors and vagal tone in cardiac inotropic and chronotropic responses to a β_2-agonist in humans. *Circulation* **79**: 107-15.

Löfdahl CG, Svedmyr N (1989) Formoterol fumarate, a new β_2-adrenoceptor agonist. Acute studies of selectivity and duration of effect after inhaled and oral administration. *Allergy* **44**: 264-71.

Lynch GS, Schertzer JD, Ryall JG (2007) Therapeutic approaches for muscle wasting disorders. *Pharmacol Ther* **113**: 461-87.

Lynch GS and Ryall JG (2008) Role of β-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. *Physiol Rev* **88**: 729-67.

Maltin CA, Delday MI, Watson JS, Heys SD, Nevison IM, Ritchie IK, Gibson PH (1993) Clenbuterol, a β-adrenoceptor agonist, increases relative muscle strength in orthopaedic patients. *Clin Sci* **84**: 651-4.

Martineau L, Horan MA, Rothwell NJ, Little RA (1992) Salbutamol, a β_2-adrenoceptor agonist, increases skeletal muscle strength in young men. *Clin Sci* **83**: 615-21.

McDonnell J, Latif ML, Rees ES, Bevan NJ, Hill SJ (1998) Influence of receptor number on the stimulation by salmeterol of gene transcription in CHO-K1 cells transfected with the human β_2-adrenoceptor. *Br J Pharmacol* **125**: 717-726.

MacEwan DJ, Kim GD, Milligan G (1996) Agonist regulation of adenylate cyclase activity in neuroblastoma x glioma hybrid NG108-15 cells transfected to co-express adenylate cyclase type II and the β_2-adrenoceptor. Evidence that adenylate cyclase is the limiting component for receptor-mediated stimulation of adenylate cyclase activity. *Biochem J* **318**: 1033-9.

Molenaar P, Bartel S, Cochrane A, Vetter D, Jalali H, Pohlner P, Burrell K, Karczewski P, Krause EG, Kaumann A (2000) Both β_2- and β_1-adrenergic receptors mediate hastened relaxation and phosphorylation of phospholamban and troponin I in ventricular myocardium of Fallot infants, consistent with selective coupling of β_2-adrenergic receptors to G(s)-protein. *Circulation* **102**: 1814-21.

Morisco C, Zebrowski DC, Vatner DE, Vatner SF, Sadoshima J (2001) β-adrenergic cardiac hypertrophy is mediated primarily by the β1-subtype in the rat heart. *J Mol Cell Cardio* **33**: 561-73.

Motulsky HJ and Mahan LC (1984) The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol* **25**: 1-9.

Philipson LH (2002) β-agonists and metabolism. *J Allergy Clin Immunol* **110** (6 Suppl): S313-7.

Rohrer DK, Chruscinski A, Schauble EH, Bernstein D, Kobilka BK (1999) Cardiovascular and metabolic alterations in mice lacking both β_1- and β_2-adrenergic receptors. *J Biol Chem* **274**: 16701-8.

Ryall JG, Sillence MN, Lynch GS (2006) Systemic administration of β_2-adrenoceptor agonists, formoterol and salmeterol, elicit skeletal muscle hypertrophy in rats at micromolar doses. *Br J Pharmacol* **147**: 587-95.

Ryall JG, Schertzer JD, Lynch GS (2007) Attenuation of age-related muscle wasting and weakness in rats after formoterol treatment: therapeutic implications for sarcopenia. *J Gerontol A Biol Sci Med Sci* **62**: 813-23.

Ryall JG, Schertzer JD, Alabakis TM, Gehrig SM, Plant DR, Lynch GS (2008) Intramuscular β_2-agonist administration enhances early regeneration and functional repair in rat skeletal muscle after myotoxic injury. *J Appl Physiol* **105**: 165-72.

Ryall JG, Church JE, Lynch GS (2010) Novel role for ß-adrenergic signalling in skeletal muscle growth, development and regeneration. *Clin Exp Pharmacol Physiol* **37**: 397-401.

Sarsero D and Molenaar P (1995) Effects of chronic infusion of (-)-isoprenaline on rat cardiac muscarinic (M2)-cholinoceptors and β_1- and β_2-adrenoceptors. *J Auton Pharmacol* 15: 239-55.

Sellers RS, Morton D, Michael B, Roome N, Johnson JK, Yano BL, Perry R, Schafer K (2007) Society of Toxicologic Pathology position paper: organ weight recommendations for toxicology studies. *Toxicol Pathol* **35**: 751-5.

Skura CL, Fowler EG, Wetzel GT, Graves M, Spencer MJ (2008) Albuterol increases lean body mass in ambulatory boys with Duchenne or Becker muscular dystrophy. *Neurology* **70**: 137-43.

Solis-Cohen S (1990) The use of adrenal substance in the treatment of asthma. *J Asthma* **27**: 401-6.

Sykes DA and Charlton SJ (2012) Slow receptor dissociation is not a key factor in the duration of action of inhaled long-acting β_2-adrenoceptor agonists. *British Journal of Pharmacology* **165**: 2672-83.

Sykes DA, Dowling MR, Charlton SJ (2010) Measuring receptor target coverage: a radioligand competition binding protocol for assessing the association and dissociation rates of unlabeled compounds. *Current protocols in Pharmacology*, Unit 9.14.

Thomas DR (2007) Loss of skeletal muscle mass in aging: examining the relationship of starvation, sarcopenia and cachexia. *Clin Nutr* **26**: 389-99.

Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ (2009) Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am J Physiol Cell Physiol* **296**: 1258-70.

Tsao J and Jiang Y (2013) Hierarchical IDEAL: fast, robust, and multiresolution separation of multiple chemical species from multiple echo times. *Magn Reson Med* **70**: 155-9.

Ursino MG, Vasina V, Raschi E, Crema F, De Ponti F (2009) The β_3-adrenoceptor as a therapeutic target: current perspectives. *Pharmacol Res* **59**: 221-34.

Wu L, Belardinelli L, Zablocki JA, Palle V, Shryock JC (2001) A partial agonist of the A(1)-adenosine receptor selectively slows AV conduction in guinea pig hearts. *Am J Physiol Heart Circ Physiol* **280**: H334-43

Yamaguchi O (2002) β_3-adrenoceptors in human detrusor muscle. *Urology* **59** (Suppl 5A): 25–29

Zheng M, Zhu W, Han Q, Xiao RP (2005) Emerging concepts and therapeutic implications of β-adrenergic receptor subtype signaling. *Pharmacol Ther* **108**: 257-68.

Footnotes

Financial support for this work was provided by Novartis Institutes for BioMedical Research, Novartis Pharma AG.

Co-author Moïse Azria passed away before the manuscript was written

Legends for Figures

Figure 1

(A) Chemical structure of 5-HOB: (*R*)-7-(2-(1-(4-butoxyphenyl)-2-methylpropan-2-ylamino)-1-hydroxyethyl)-5-hydroxybenzo[*d*]thiazol-2(3*H*)-one. (B) Effect of formoterol and 5-HOB on cAMP production in membranes isolated from Wistar rat gastrocnemius muscle and from the heart. Percent of cAMP responses were determined relative to the E_{max} of formoterol in skeletal muscle membranes. Data shown are means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ on efficacy of 5-HOB versus formoterol in heart membranes and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ in skeletal muscle membrane (T-test, unpaired).

Figure 2

Hypertrophy in human primary skeletal muscle myotubes. (A) Differentiated myotubes treated with DMSO control, formoterol (1µM) or 5-HOB (1µM) for 48 h were stained with anti-myosin heavy chain (MyHC). Shown are representative pictures. (B) *In vitro* differentiated human skeletal muscle myotubes were treated with 5-HOB (1µM) in the absence or presence of a β_1-antagonist (CGP20712A, 1 µM) or a β_2-antagonist (ICI-118'551, 1µM) for 48 h and changes in myotubes diameter were measured. Data are expressed as % increase compared to the DMSO control treatment (as 100%). The values are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ increase versus DMSO control (one-way ANOVA, followed by Bonferroni's test).

Figure 3

Effects of formoterol and 5-HOB on the beating rate of rabbit sino-atrial (SA) node. (A) values are means ± SEM (n=5-6) and % changes from baseline values. (B) superimposed SA Action Potentials exposed to Formoterol (mean of n=5). (C) superimposed SA Action Potential exposed to 5-HOB (mean of n=6).

Figure 4

Dose-response effects of formoterol (A) and 5-HOB (B) on the weight of hind limb muscle and heart in Wistar rats. Effects of formoterol and 5-HOB on evoked force of hind limb muscle (C). Compounds were administered with subcutaneously implanted alzet minipump for 4 weeks. Values are means ± SEM or mean (n=5-6), $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control (Dunnett's test following one-way ANOVA).

Figure 5

Time course changes of hind limb muscle mass (A) and ejection fraction (B) in Wistar rats. Changes in hind limb muscle and ejection fraction were evaluated by MRI during treatment with formoterol and 5-HOB once daily subcutaneously at 0.03 mg/kg for 4 weeks and during washout for 5 weeks. Values are means ± SEM (n=6-8), $^{**}P < 0.01$ versus vehicle control (Holm-Sidak test following 2-way repeated measurement ANOVA).

Figure 6

Heart rate changes in Wistar rats after single subcutaneous treatment with formoterol (A) or 5-HOB (B). Dose response (0 to 10 min average) plots of formoterol and 5-HOB for heart rate (C) and mean arterial pressure (D). Values are expressed as means (A, B) or means ± SEM (C, D) (n=3-4) of changes from pre-dose baseline values. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control (Dunnett's test following one-way ANOVA).

Figure 7

Heart rate changes in rhesus monkeys after single subcutaneous treatment with formoterol or 5-HOB (A). Heart rate was measured with a Surgivet V3304 device under restrained condition. Relationships of plasma concentration (nM) to heart rate response (bpm) (B). Values are expressed as means ± SEM (n=6) of absolute values.

Tables

TABLE 1

Binding and kinetic parameters of 5-HOB and formoterol for the human β_2-AR

The values are expressed as mean ± SEM of at least three independent experiments.

	5-HOB	Formoterol
Equilibrium K_i (nM)	1.62 ± 0.55	12.9 ± 4.3
k_{on} ($M^{-1}min^{-1}$)	7.43 ± 2.42 x 10^8	1.78 ± 0.21 x 10^8
k_{off} (min^{-1})	0.73 ± 0.13	3.0 ± 0.4
Kinetic K_d (nM)	1.08 ± 0.14	16.80 ± 0.04
$t_{1/2}$ of dissociation (sec)	57	14

TABLE 2

Functional activity and selectivity of 5-HOB and formoterol against the different human β-AR subtypes

β_1-AR and β_2-AR were expressed in CHO cells; β_3-AR was expressed in HEK293 cells. E_{max} values were determined relative to isoprenaline, a reference non-selective β-AR agonist. The values are expressed as mean ± SEM of two to four independent experiments.

cAMP response	5-HOB		Formoterol	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
β_1-AR	793 ± 92	40 ± 1.7	67 ± 3.8	84 ± 1.6
β_2-AR	3.5 ± 0.3	93 ± 0.7	0.3 ± 0.03	97 ± 1.0
β_3-AR	925 ± 11	97 ± 0.7	97 ± 1.4	113 ± 7
β_2-AR occupancy at % control [EC_{40}]	70.4		1.8	

JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 3

Functional activity of 5-HOB and formoterol in primary cells with endogenous expression of β-ARs

E_{max} values were determined relative to formoterol, where formoterol was defined as 100%. The values are expressed as mean ± SEM of at least four independent experiments, each performed with 7 concentrations of compounds (10-fold dilutions) in triplicates.

cAMP response		5-HOB		Formoterol	
		EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
Skeletal muscle myotubes	Rat	1.9 ± 0.4	104 ± 0.8	0.1 ± 0.02	100 ± 0.0
	Dog	2.1 ± 0.2	89 ± 0.8	0.6 ± 0.03	100 ± 0.0
	Monkey	5.1 ± 0.8	90 ± 3.7	1.5 ± 0.37	100 ± 0.0
	Human	1.7 ± 0.1	96 ± 0.3	0.3 ± 0.01	100 ± 0.0
Cardiomyocytes	Rat	4.4 ± 0.5	72 ± 0.3	1.6 ± 0.13	100 ± 0.0
	Human iPS-derived	3.4 ± 0.2	84 ± 2.5	0.5 ± 0.03	100 ± 0.0

TABLE 4

Functional activity of 5-HOB and formoterol in cell membranes isolated from rat tissues

% cAMP responses was determined relative to the E_{max} of formoterol in skeletal muscle membranes. The EC_{50} and E_{max} values ± SEM of experiments from three independent membrane preparations, each performed with 7 concentrations of compounds (10-fold dilutions) in triplicates.

cAMP response	5-HOB		Formoterol	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
Skeletal muscle	80 ± 17	92 ± 1	6 ± 0.3	100 ± 0
Heart	361 ± 179	14 ± 2	62 ± 8	53 ± 4

TABLE 5

Effects of 5-HOB and formoterol on inotropy and vascular relaxation

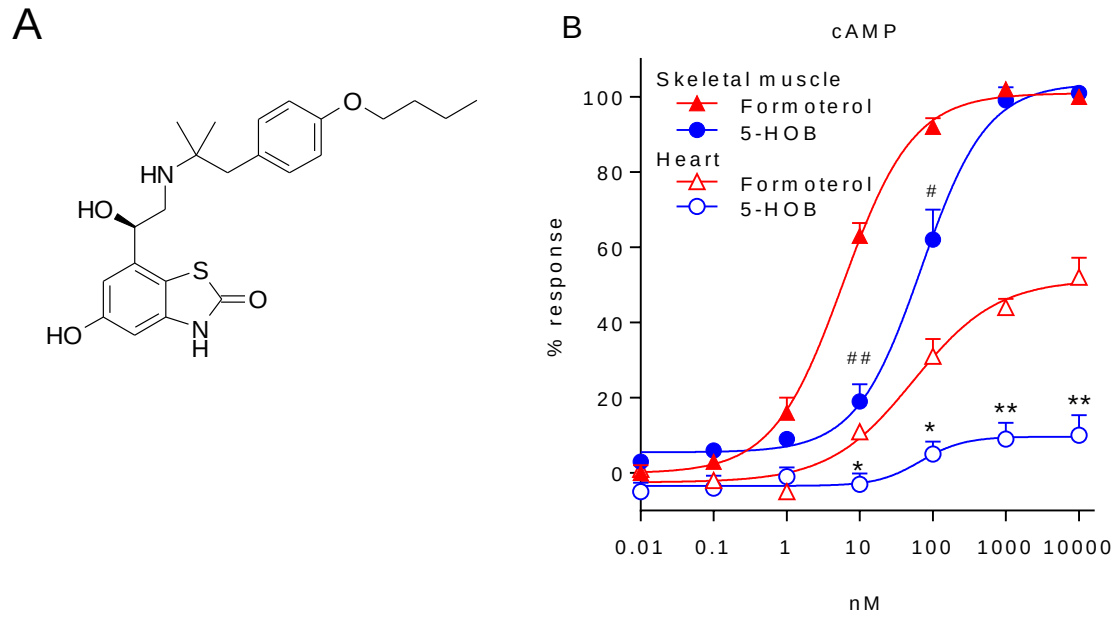
Left atrium was isolated from Dunkin Hartley guinea pigs and aortic ring was isolated from Sprague Dawley rats. The values are expressed as means of n=2 for left atrium and n=7 for aortic ring.

EC_{50} (nM)	Formoterol	5-HOB
Inotropy	17	>10,000
Vascular relaxation	0.8	31

JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

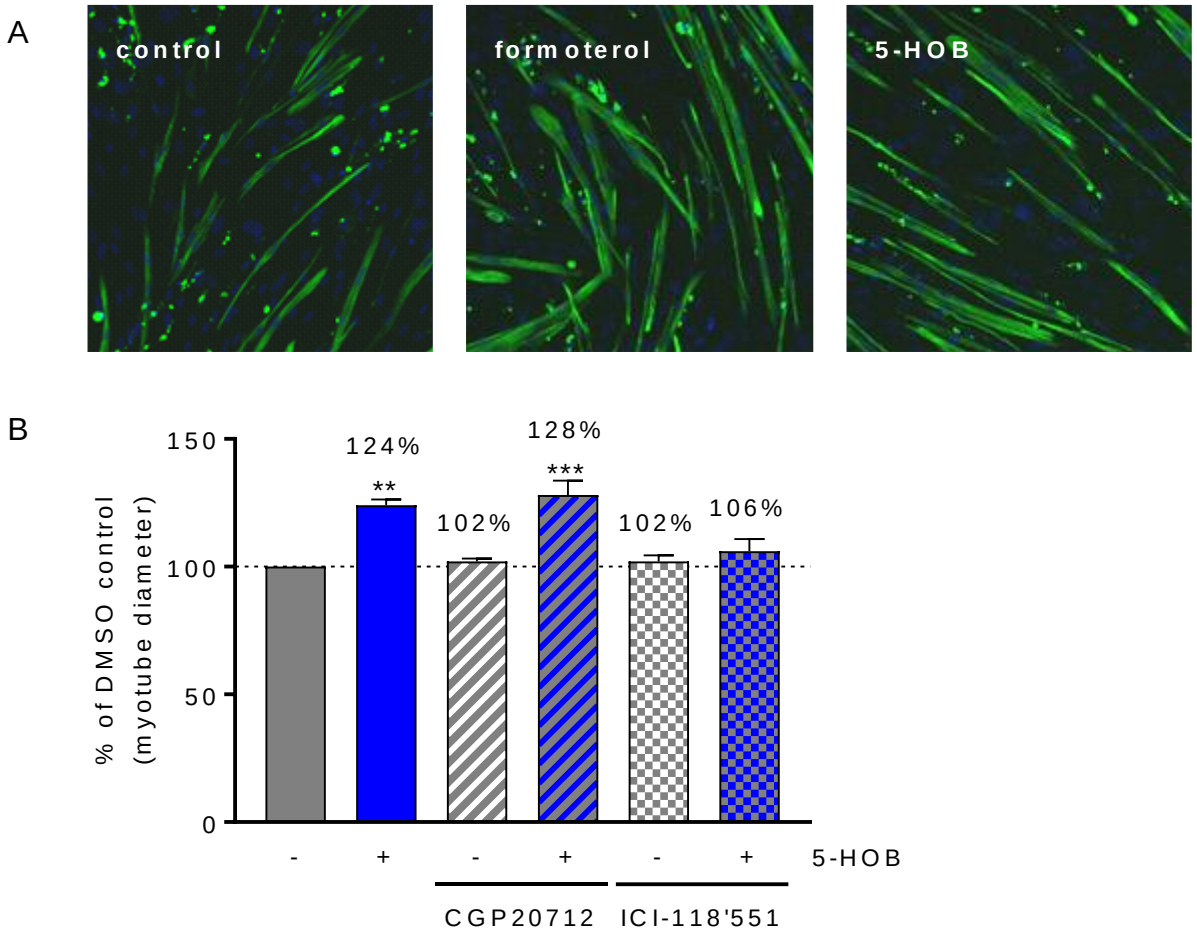
Figures

Figure 1



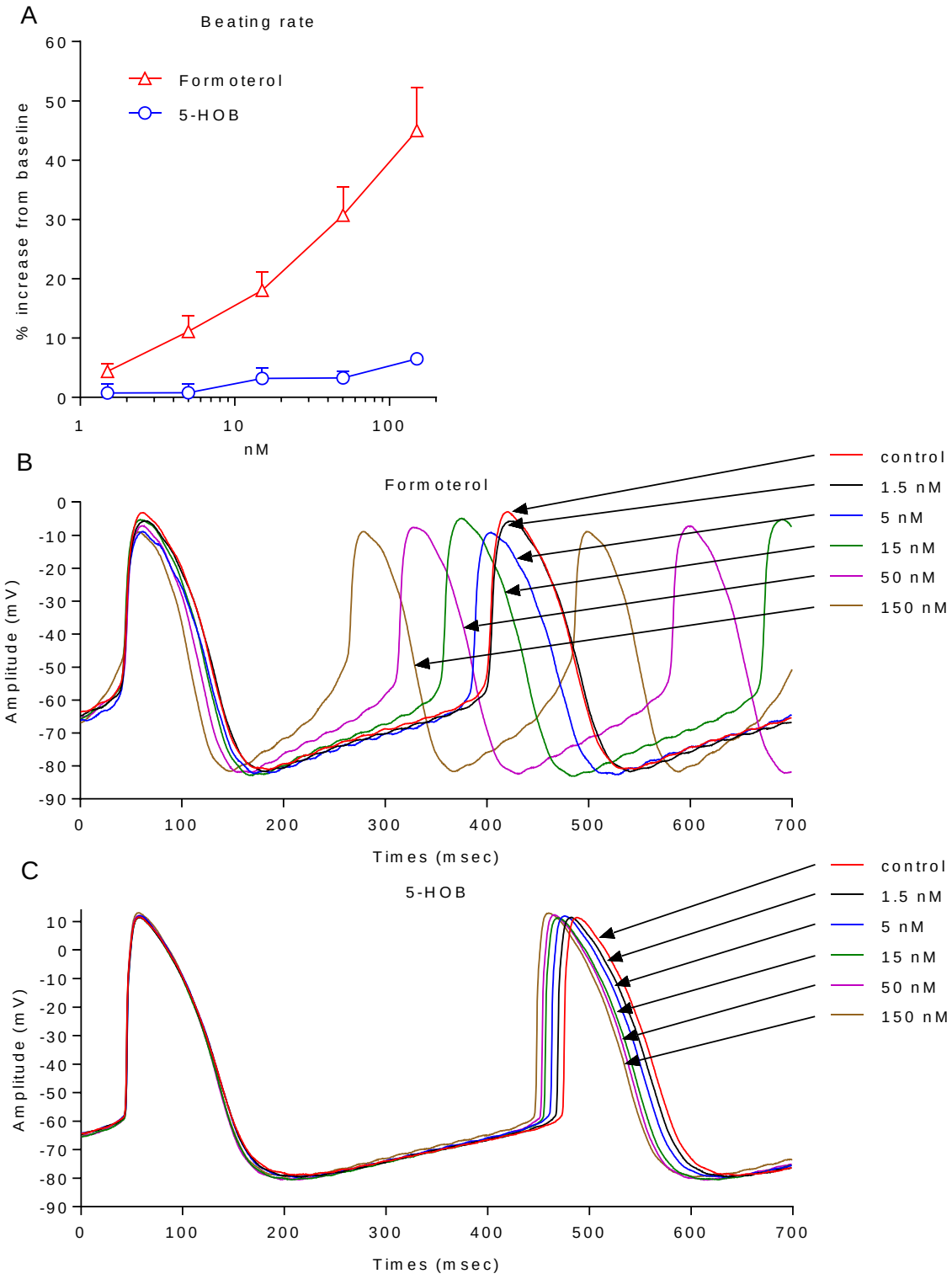
JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



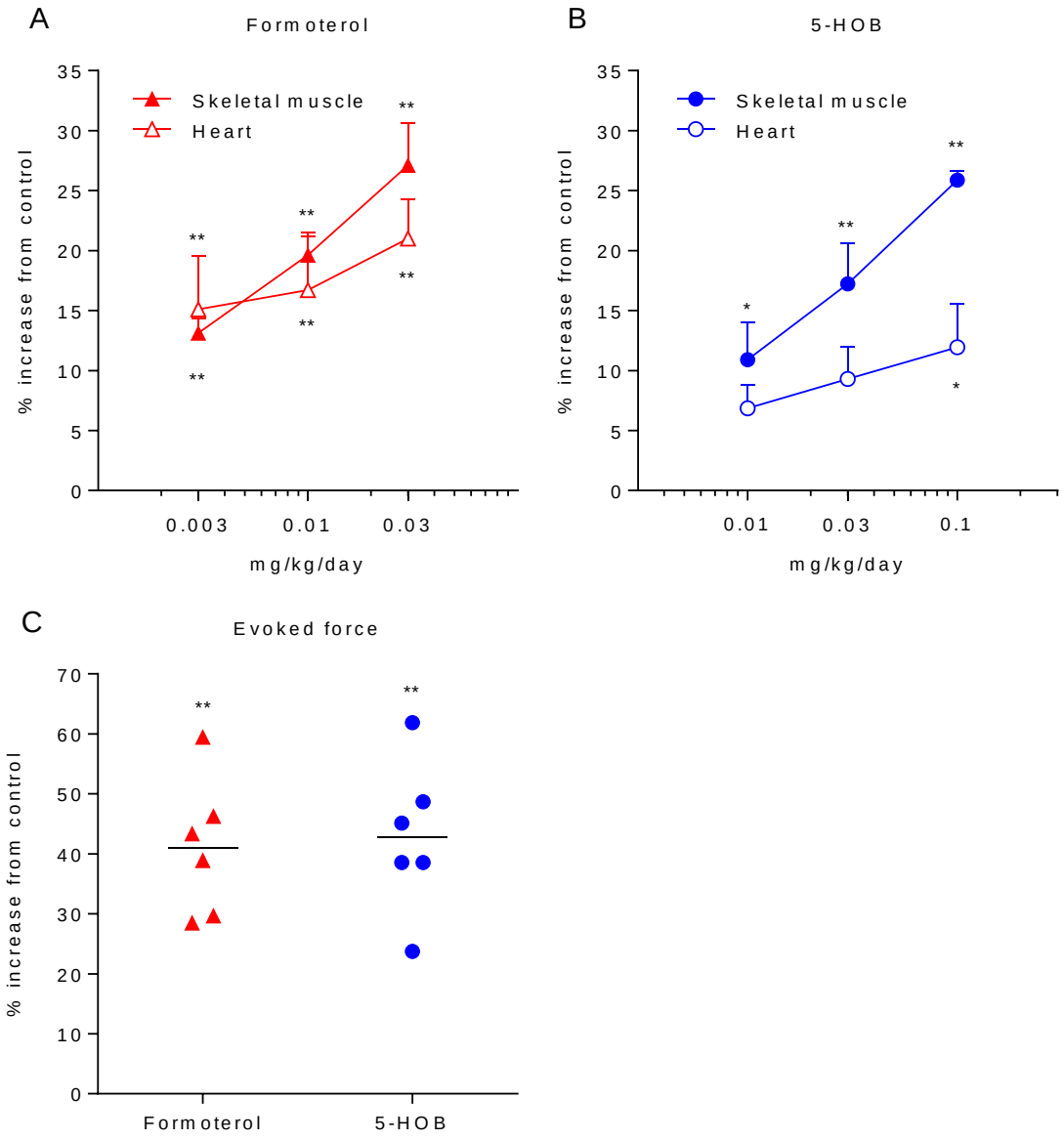
JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



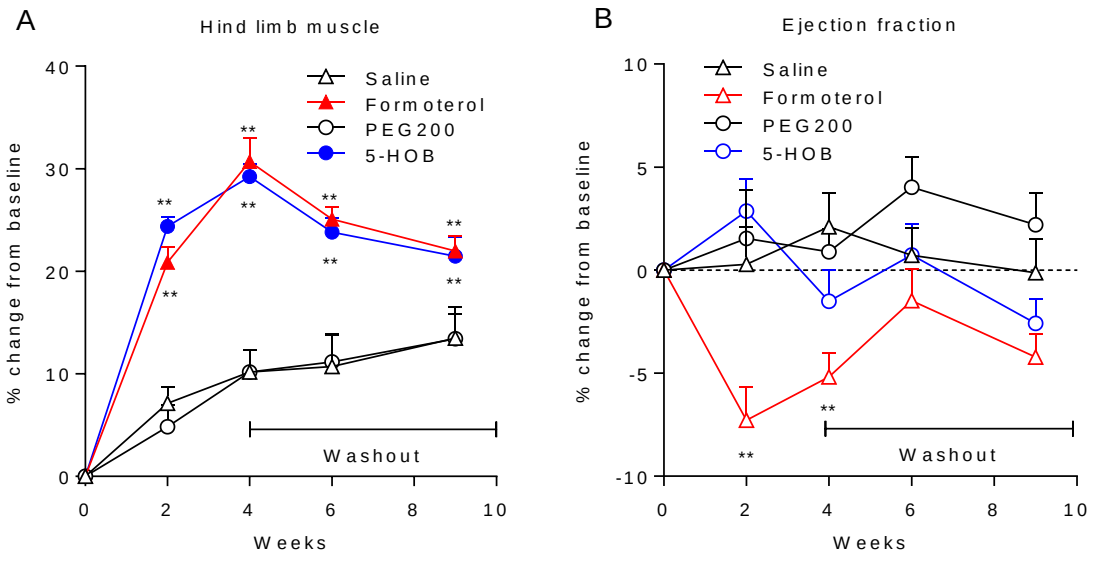
JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



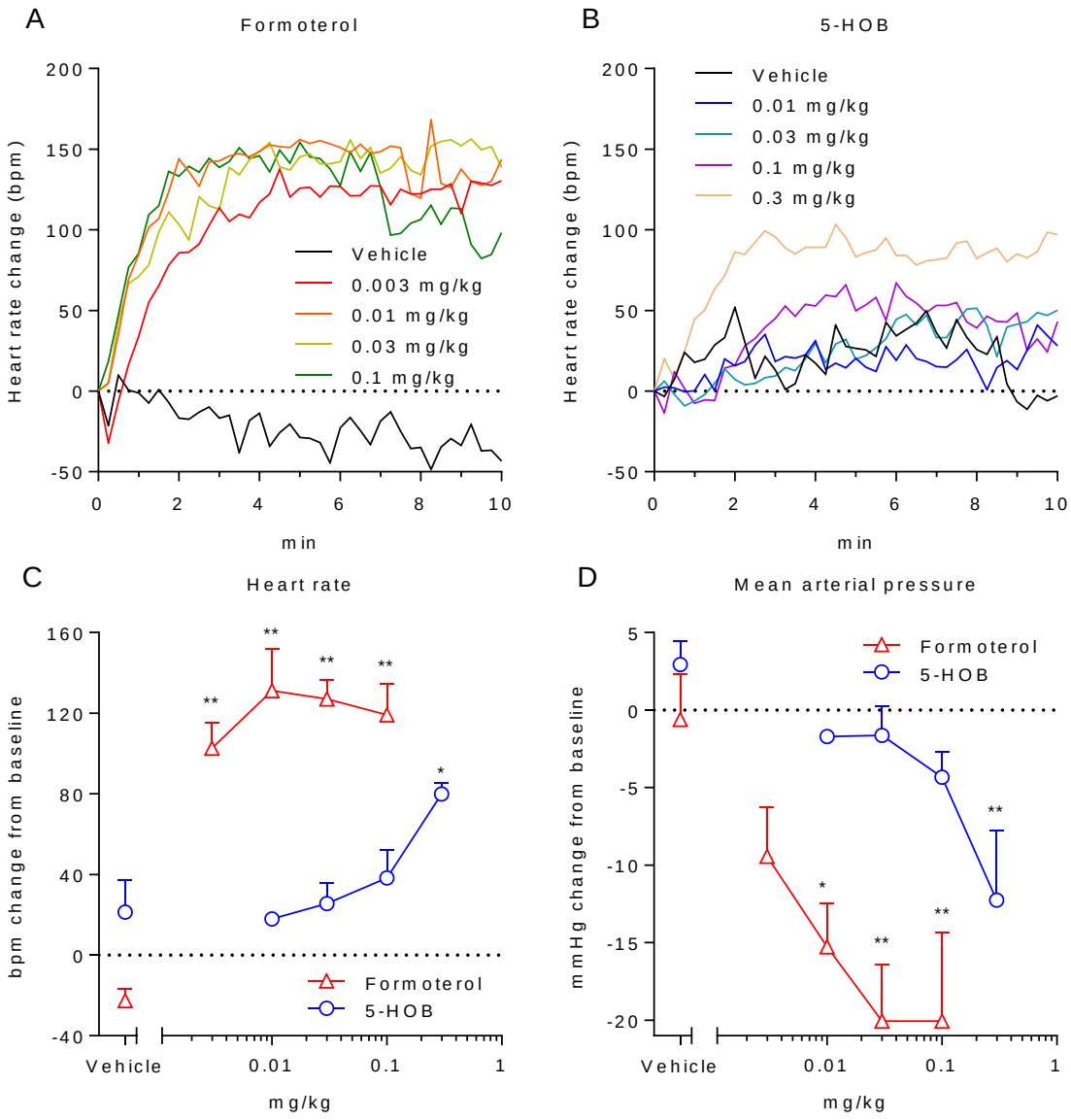
JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



JPET Fast Forward. Published on February 28, 2019 as DOI: 10.1124/jpet.118.255307
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7