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1. Title Page

Identification of a Human Whole Blood-Based Endothelial Cell Impedance Assay for Assessing Clinical TRPV4 Target Engagement Ex Vivo

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2. Running Title Page

a) Running Title *(≤60 characters including spacing)***:** Cellular impedance assay to predict human TRPV4 inhibition

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Significance Statement word count: 80

Introduction word count: 735

Discussion word count: 1306

d) Non-Standard Abbreviations:

DMSO	Dimethyl sulfoxide
EC_{50}	Concentration required to elicit 50% activation
EC_{95}	Concentration required to elicit 95% activation
E_{max}	Maximum response achievable
FLIPR	Fluorometric imaging plate reader
FTIH	First time in human
GSK	GlaxoSmithKline
GSK1016790	TRPV4 channel activator
GSK2798745	TRPV4 channel blocker
HF	Heart failure

HLMVEC	Human Lung Microvascular Endothelial Cells
HUVEC	Human umbilical vein endothelial cell
IC_{50}	Concentration required to inhibit response by 50%
IV	Intravenous
LW/BW	Lung wet weight to body weight ratio
NCI	Normalized cell index
PD	Pharmacodynamic
PET	Positron emission tomography
PK	Pharmacokinetic
SD	Standard deviation
SEM	Standard error of the mean
TRPV4	Transient receptor potential vanilloid 4

e) Recommended section assignment:

Drug Discovery and Translational Medicine

f) Conflict of interest:

All authors worked for and owned shares in GSK during the study.

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3. Abstract

TRPV4 channels expressed on pulmonary endothelial cells are activated by elevated pulmonary vascular pressure, resulting in endothelial shape change, pulmonary barrier disruption and edema. As such, TRPV4 blocker GSK2798745 was recently investigated in Phase I/IIa trials to reduce pulmonary edema caused by heart failure (HF). In the absence of a suitable TRPV4 target engagement biomarker, we hypothesized that an ex vivo assay could be utilized to predict pharmacological activity at the intended site of action (endothelial cells) of subjects. In this assay, the ability of GSK2798745 to block TRPV4 agonist GSK1016790-induced impendence reduction in human umbilical vein endothelial cells (HUVECs) in the presence of human whole blood was assessed. Blood from healthy volunteers drawn 1-12 h after single or repeated dose of GSK2798745 (5 mg) inhibited GSK1016790-induced impedance reduction by ≥85%. Similarly, blood samples from 16 HF subjects dosed with GSK2798745 (2.4 mg) inhibited GSK1016790-induced HUVEC impedance reduction by ≥58% 1-24 h after single dosing and ≥78% 1-24 h after 7 days of repeated dosing. No inhibition was detected using blood from placebo subjects. Using matched GSK2798745 plasma levels, a pharmacokinetic/pharmacodynamic (PK/PD) relationship was calculated as 2.9 nM IC_{50}, consistent with the 6.5 nM IC_{50} of GSK2798745 obtained from a rat in vivo PK/PD model of pulmonary edema after correcting for rat-to-human differences. These results indicate circulating levels of GSK2798745 in the recently completed Phase I/IIa trials were sufficient to block TRPV4 in lung vascular endothelial cells to a large extent, supporting this dosing regimen for assessing efficacy in HF.

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4. Significance Statement

In the absence of a suitable target engagement biomarker, we developed an ex vivo assay to predict the pharmacological activity of the TRPV4 blocker GSK2798745 in healthy volunteers and HF subjects from Phase I/IIa trials. The potency values from the ex vivo assay were consistent with those predicted from a rat in vivo PK/PD model of pulmonary edema, strongly suggest circulating levels of GSK2798745 were sufficient to robustly block TRPV4, supporting use of GSK2798745 for assessing efficacy in HF.

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5. Introduction

Transient Receptor Potential Vanilloid 4 (TRPV4) plays a significant role in a range of physiological systems. This non-selective, calcium (Ca^{2+})-permeant, ion channel exhibits polymodal activation, functioning as a thermosensor (Gao, Wu and O'Neil, 2003), osmosensor (Liedtke et al., 2000; Strotmann et al., 2000; Alessandri-Haver et al., 2003; Mizuno et al., 2003) and mechanosensor (Liedtke et al., 2003), and is also activated by endogenous and exogenous chemicals (Watanabe et al., 2003; Nilius et al., 2004; Vriens et al., 2004; Thorneloe et al., 2008).

Considerable research has focused on the prominent expression of TRPV4 in pulmonary vascular endothelial cells which, together with alveolar epithelial cells and the basement membrane separating the two cell types, constitute the alveolar-capillary barrier. In endothelial cells, TRPV4 activation elicits enhanced Ca^{2+} entry resulting in reorganization of actin cytoskeleton, microtubule disassembly, and disruption of cell-cell and cell-matrix adhesion (Thodeti et al., 2009; Goswami et al., 2010; Phuong et al., 2017). This is evident in both cultured cells and isolated intact lungs, where pharmacologic activators of TRPV4 induce endothelial detachment and increase lung barrier permeability (Alvarez et al., 2006; Willette et al., 2008). Similarly, consistent with a mechanosensor role for TRPV4, elevated pulmonary vascular pressure increases permeability in isolated, perfused lungs, and this response is attenuated in TRPV4 knockout mice and by pharmacologic TRPV4 blockade (Jian et al., 2008; Yin et al., 2008; Thorneloe et al., 2012). In vivo, pharmacologic activation of TRPV4 induces vascular leakage and tissue hemorrhage in the lung (Willette et al., 2008). Furthermore, TRPV4 blockade inhibits pulmonary edema formation in both acute and chronic animal models of heart failure (HF), suggesting a significant role for TRPV4 in the pathogenesis of pulmonary edema associated with HF in preclinical species (Thorneloe et al., 2012). Consistent with these findings, TRPV4

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expression is elevated in the pulmonary vasculature of lungs from humans with HF (Thorneloe et al., 2012).

Based on the above, GSK2798745, a selective, small molecule blocker of TRPV4 channels, was recently investigated as a medicine to reduce pulmonary edema caused by HF, a clinical syndrome of dyspnea, impaired exercise tolerance, frequent hospitalization and high mortality (Goyal et al., 2019; Stewart et al., 2020).

While performing such clinical studies, it is important to confirm that the small molecule therapeutic interacts robustly with its intended protein target, a measure referred to as target engagement (Morgan et al., 2012; Simon, Niphakis and Cravatt, 2013). Indeed, appropriate measures of target engagement are pivotal for contextualizing drug efficacy. For instance, if a drug elicits full target engagement but lacks clinical efficacy, then the mechanism of the drug was properly tested, and the original clinical hypothesis is appropriately refuted. Conversely, without a suitable target engagement measure, it can be extremely difficult to interpret the cause of a negative therapeutic effect (Wagner, 2008).

The preferred method for confirming target engagement is via in vivo pharmacokinetic/pharmacodynamic (PK/PD) studies using positron emission tomography (PET) or radiolabeled ligands (Morgan et al., 2012). Alternatively, a proximal circulating biomarker could also serve as a useful measure of target engagement (Krishna et al., 2008). Unfortunately, efforts to develop a suitable PET or radiolabeled TRPV4 ligand for in vivo PK/PD studies have been unsuccessful to date, and identification of an appropriate circulating biomarker proximal to TRPV4 function also remains elusive.

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In the absence of a suitable target engagement tool, the aim of the present study was to identify and utilize a translational ex vivo whole blood assay designed to predict levels of TRPV4 blockade at the appropriate site of action, vascular endothelial cells, of individual clinical subjects. A cellular impedance assay utilizing human umbilical vein endothelial cells (HUVECs) was selected considering the relationship between TRPV4 channel activation, cytoskeletal and related morphological changes in endothelial cells, pulmonary barrier disruption and pulmonary edema. Indeed, HUVECs have been utilized previously in impedance-based assays to assess drug-induced vascular leakage (Kustermann et al., 2014), and TRPV4 channel activation has been shown to reduce HUVEC impedance (Thorneloe et al., 2012).

Results from the herein described ex vivo whole blood assay were consistent with pharmacological activity predictions obtained from a rat in vivo PK/PD model of pulmonary edema and together suggest that circulating levels of GSK2798745 in the recently completed Phase I/IIa trials were sufficient to adequately assess clinical efficacy in HF subjects. Additionally, such an ex vivo paradigm may offer a more compelling tool to estimate in vivo target engagement than the combined measurement of simple PK projected onto in vitro activity.

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6. Materials and Methods

Rat in vivo PK/PD

All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council, 2011), the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and the GlaxoSmithKline (GSK) Policy on the Care, Welfare and Treatment of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at GSK. Male Sprague Dawley (SD) rats were obtained from Charles Rivers Laboratories Inc. (Raleigh, NC) and held in rooms environmentally controlled for temperature, humidity, and lighting (12 hour light/dark cycle, lights on 06:00 am) standard for the vivarium. Rats were maintained on a normal rodent diet (Lab Diet 5001, Purina Mills, LLC, St. Louis, MO, USA) and filtered tap water *ad libitum* and allowed to acclimate for at least four days prior to use.

An established in vivo pharmacokinetic/pharmacodynamic (PK/PD) model of TRPV4-mediated pulmonary edema (Thorneloe et al., 2012) was used to evaluate the effects of TRPV4 blocker GSK2798745 (Brooks et al., 2019; Goyal et al., 2019) on lung wet weight (normalized to body weight; LW/BW) in SD rats (10-12 week old) treated with TRPV4 agonist (Willette et al., 2008). Prior to agonist administration (10 min intravenous [i.v.] infusion with the TRPV4 agonist GSK1016790 at 10 µg/kg/min), rats were pre-treated with vehicle (3% DMSO, 20% Cavitron, pH= 4.5; n=10) or GSK2798745 (n=9) via an unblinded, randomized regimen consisting of an i.v. bolus loading dose (0, 5, 14, or 43 µg/kg) followed by a continuous 60 min i.v. infusion (0, 16, 50, or 150 ng/kg/min) to provide steady-state plasma exposure. Following GSK1016790 infusion, lungs were excised and weighed and blood was obtained for GSK2798745 plasma concentration determination. Lungs were also harvested from a separate control group of age-

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matched Sham and naïve rats (n=13) for determination of basal LW/BW ratios. Lastly, plasma concentrations of GSK2798745 were correlated with LW/BW ratios to approximate an IC_{50}. In absence of a direct receptor occupancy test, the IC_{50} obtained from the rat PK/PD experiment reflects the potency that can be employed to predict target (TRPV4) inhibition for a corresponding GSK2798745 concentration.

Pharmacokinetics (PK) Measurement and Model Prediction

As described in Goyal et al., 2019, single and repeat doses of GSK2798745 were administered to healthy volunteers and subjects with stable HF, and blood samples were collected over time to assess the clinical PK. TRPV4 channel inhibition cannot be measured directly in humans. However, the potency estimate (IC_{50}) for GSK2798745 from the rat PK/PD experiment described in the preceding section can be utilized to characterize the in vivo TRPV4 channel inhibition for any observed plasma concentration utilizing the relationship listed in equation 1. Consequently, the % TRPV4 inhibition was characterized for every individual at various time points utilizing their available GSK2798745 plasma concentration at that time point.

Equation 1: $\% \ TRPV4 \ inhibition = 100 * Concentration / (Concentration + IC_{50})$

IC_{50} is the half maximal potency estimate obtained from the rat in vivo PK/PD study and corrected for interspecies difference in protein binding as well as differences in IC_{50} potency between the rat and human TRPV4 channel from a cellular fluorometric imaging plate reader (FLIPR) assay (Thorneloe et al., 2012). Plasma protein binding of GSK2798745 was 91.8% in rat and 87% in human, and the IC_{50} potency of GSK2798745 was 1.3 nM at rat TRPV4 and 2.0 nM at human TRPV4.

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The channel blockade estimated based on clinical exposure data with this approach could be then compared with estimates from the other approach mentioned in this publication (i.e., HUVEC impedance assay).

Human Blood Samples

Heparinized (sodium heparin 50 units/mL) human blood samples were collected from donors at GSK (King of Prussia, PA) and used according to GSK's human biological sample management policy and Standard Operation Procedures. Dimethyl sulfoxide (DMSO) vehicle or varying concentrations of GSK2798745 were added to the blood (final DMSO concentration = 0.1%). The blood was either used on the same day (fresh) or frozen at -80°C for later (≥2 days) use (termed "previously frozen"). In addition, as part of the first-time-in-human (FTIH) clinical evaluation of GSK2798745 (NCT02119260), single and repeat doses were administered to healthy volunteers and subjects with stable HF, and blood samples were collected over time to assess the clinical PK and impedance assay activity of GSK2798745. The detailed safety and PK results of the study are a separate publication (Goyal et al., 2019). Heparinized blood samples drawn from healthy volunteers dosed with placebo or 5 mg GSK2798745, and from HF subjects dosed with placebo or 2.4 mg GSK2798745 (dose lowered due to interim pre-clinical toxicity findings; Goyal et al., 2019), were shipped on dry ice from clinical centers in the United Kingdom and stored at -80°C until being tested. The collection of blood samples complied with the Declaration of Helsinki 2008 and ICH Good Clinical Practice guidelines, and full written informed consent was obtained from all participants. Blood samples from heathy volunteers and the first 6 HF subjects were blinded until after the HUVEC experiment data were analyzed.

Human Umbilical Vein Endothelial Cells (HUVECs)

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HUVECs (single donor catalog #C2517A and pooled donors catalog #C2519A, sex unknown) were purchased from Lonza (Allendale, NJ) and cultured in a 95% humidified incubator at 37^{o}C and 5% CO_2 in plates using complete EBM-2 medium obtained by mixing 500 mL EBM-2 (catalog #CC-3156, Lonza, Allendale, NJ) with a bottle of EGM-2 SingleQuots (catalog #CC-4176, Lonza, Allendale, NJ). Both single donor and pooled HUVECs at passage 2 were frozen in small vials (500,000 cells/mL) in the complete EBM-2 medium+10% DMSO and stored in a liquid nitrogen tank. Frozen HUVECs were cultured in T-75 culture plates and medium was changed every 2-3 days. HUVECs were sub-cultured at 70~80% confluence and used up to passage 7 for the impedance assay. Single donor HUVECs were used for testing blood drawn from donors and healthy volunteers, whereas pooled donor HUVECs were utilized for testing blood collected from HF subjects considering HUVECs from the single donor lot were no longer available. In the presence of previously frozen blood, the EC_{50} potencies of GSK1016790 between single and pooled donor HUVECs were marginally but not significantly different (224±51 nM [n=3] and 462±71 nM [n=9], respectively; P=0.10, unpaired *t*-test).

HUVEC Impedance and Cell Index measurement

Impedance between electrodes in individual wells of an E-plate 96 (ACEA Biosciences; San Diego, CA) containing a HUVEC monolayer was measured as "Cell Index" (Kustermann et al., 2014) using an xCELLigence Real-Time Cell Analysis Single Plate Instrument (RTCA SP; ACEA Biosciences). The impedance between the electrodes in an individual well depends on the HUVECs that are attached to the electrodes, and the impedance reduction can occur due to the HUVEC contraction and detachment induced by TRPV4 activation (Thorneloe et al., 2012). HUVECs were seeded at a density of ~8,000 cells/well in 160 µL complete EBM-2 medium and cultured for ~17-26 hours before testing. The E-plate 96 was placed on the RTCA SP reader

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inside a 37°C and 5% CO_2 incubator for impedance recording. Impedance was recorded every 20 min during culture, and every 1 min during experiments except when blood or drug was added in a biological safety cabinet at room temperature (~22°C). Human blood was pre-warmed to 37°C for 10 min before being added to the E-plate 96.

TRPV4 agonist GSK1016790 and TRPV4 blocker GSK2798745 were dissolved in DMSO, respectively, to make 10 mM stock solutions. Aliquots of the stock solutions were stored at -20°C.

TRPV4 Agonist Assay Using Donor Blood

Using fresh and previously frozen blood from healthy donors, concentration-responses to the TRPV4 agonist GSK1016790 on HUVEC impedance were generated to determine the EC_{95} concentration for each fresh and previously frozen sample. In these experiments, 140 µL (of 160 µL total) culture medium was removed from each well of the E-plate and 170 µL blood per well was added. HUVECs in the E-plate 96 were incubated with the blood for ~20 min and then stimulated with various concentrations of GSK1016790 (10 µL at 20X final concentration in medium). Final volume/well was 200 µL, with 85% blood and 15% medium. The content of each well was mixed gently (enough to mix solution contents without disrupting the HUVEC cell monolayer) 3-4 times following GSK1016790 addition using a multi-channel pipette. GSK1016790 concentrations and impedance reduction were correlated to determine EC_{95}.

TRPV4 Antagonist Assay Using Donor Blood

To determine the GSK2798745 concentration-dependent inhibition of GSK1016790-induced HUVEC impedance reduction in donor blood, a similar procedure was followed except that various concentrations of GSK2798745 or vehicle control (DMSO; 0.1% final concentration)

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were added to the blood prior to GSK1016790 (~EC_{95}) addition. HUVECs in the E-plate 96 were incubated with the blood including the DMSO or GSK2798745 for ~15-40 min (enough time for the impedance to reach steady-state) and then stimulated with TRPV4 agonist at the corresponding ~EC_{95} determined earlier for the particular donor and treatment (i.e., fresh or previously frozen blood).

Ex Vivo Assessment of TRPV4 Inhibition Using Blood From Clinical Subjects

The ex vivo inhibitory effects of blood samples from clinical subjects orally dosed with placebo or GSK2798745 on TRPV4 agonist GSK1016790-induced HUVEC impedance reduction was determined using a procedure similar to that described above. The clinical blood samples were pre-warmed to 37°C for 10 min and then incubated with the E-plate 96 containing HUVECs for 30-40 min prior to GSK1016790 (~EC_{95}) addition. Clinical blood samples from each subject collected before the first oral dosing were used as the negative control (0% inhibition of GSK1016790-induced HUVEC impedance reduction). For 4 healthy volunteers, blood samples were not collected before dosing and blood samples collected from donors at GSK were used as the negative control. Blood from the same subject (except for the 4 healthy volunteers mentioned above) with added GSK2798745 (120-300 nM) was used as the positive control (100% inhibition of GSK1016790-induced HUVEC impedance reduction; based on results from the previously described experiment). The inhibitory effects of the clinical blood samples on TRPV4 agonist GSK1016790-induced HUVEC impedance reduction were quantified as the percentage of the maximal inhibition by the positive control. Due to limited amount of clinical blood samples, we were not able to determine GSK1016790 potency for individual subjects. Rather, the mean ~EC_{95} of GSK1016790 determined using GSK donor blood (unpublished data, 1 μM

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for single donor HUVECs or 1.6 μM for pooled HUVECs) was utilized to induce HUVEC impedance reduction.

Data Analysis

The HUVEC impedance (termed "Cell Index") response to GSK1016790 was determined using an *in vitro* TRPV4 agonist assay. Time-dependent measures of Cell Index, recorded in each well after the addition of DMSO or GSK1016790, were normalized to the Cell Index measured in respective wells (containing untreated fresh or previously frozen human blood) immediately prior to addition (time=0) to obtain the Normalized Cell Index (NCI; RTCA Software version 2.0, ACEA Biosciences). An average NCI (n=4 replicating wells) was then determined for the DMSO control and the various concentrations of GSK1016790 over time. The maximal response to GSK1016790 was identified (time point when maximal NCI reduction was achieved, ~25 minutes after addition of GSK1016790) and the concentration-dependent NCI reduction of HUVECs caused by GSK1016790-induced TRPV4 activation was calculated using the formula: NCI reduction = average NCI of DMSO controls (n=4 wells) – individual NCI of wells treated with GSK1016790 at each concentration. NCI reduction was averaged per concentration group (n=4 wells/group) and correlated with log-transformed concentrations of GSK1016790. Using non-linear regression (GraphPad Prism 5 or 7, La Jolla, CA; log(agonist) vs. response-variable slope), the EC_{50} was determined for fresh and previously frozen blood. The EC_{95} was also determined for individual donors (in fresh and previously frozen blood) for respective evaluation in the TRPV4 antagonist assay.

An *in vitro* TRPV4 antagonist assay was utilized to evaluate the effects of GSK2798745 on GSK1016790-induced NCI reduction. To begin, the average NCI was determined for wells (n=4) containing fresh or previously frozen human blood with added DMSO or various concentrations

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of GSK2798745. Maximal NCI reduction was determined by stimulating wells containing DMSO treated, fresh or previously frozen blood with GSK1016790 (~EC_{95}). Similarly, wells containing fresh or previously frozen blood treated with increasing concentrations of GSK2798745 were stimulated with GSK1016790 (~EC_{95}) and reduction in NCI was recorded. The % inhibition of NCI reduction by GSK2798745 [% Inhibition of NCI Reduction = 100 x (1 - NCI reduction with GSK2798745 pre-treatment / maximal NCI reduction)] was determined and correlated with log-transformed concentrations of GSK2798745. Nonlinear regression curve fitting was used to calculate respective IC_{50}s (GraphPad Prism 5 or 7).

To estimate target (TRPV4) inhibition in clinical samples, blood collected from healthy volunteers or HF subjects at various times after oral administration of GSK2798745 or placebo was evaluated in the TRPV4 antagonist assay (as described above) to determine the ability of circulating concentrations of GSK2798745 or placebo to inhibit GSK1016790-induced NCI reduction. The % Inhibition of NCI Reduction was calculated over 12-24 hours on Day 1, Day 7 and Day 14 of dosing and compared with the predicted TRPV4 channel blockade (described previously using Equation 1). Additionally, the % Inhibition of NCI Reduction for all blood samples from GSK2798745-treated HF subjects was correlated with the clinical PK for corresponding subjects and times, and an IC_{50} was generated using nonlinear regression curve fitting (GraphPad Prism 7).

Averaged results are presented as mean ± SEM. Concentration responses in fresh and previously frozen blood were compared using a two-tailed, unpaired t-test, where $P<0.05$ was considered significant.

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For the rat PK/PD study, LW/BW ratios from control (n=13) and GSK2798745 treated rats (n=3 per group) were compared to vehicle (n=10) using a one-way ANOVA with Dunnett's multiple comparisons post hoc test. In all cases, significance was considered $P<0.05$. Steady-state plasma concentrations of GSK2798745 were log-transformed and plotted with respective LW/BW ratios. The IC_{50} value was estimated with an unconstrained four parameter dose-response model which included data from the vehicle and control groups. Statistical comparisons were performed using GraphPad Prism 7 and nonlinear regression curve fitting was performed with SAS® 9.4.

7. Results

Rat in vivo PK/PD

Activation of TRPV4 in the rat, via a 10 min i.v. infusion of the selective TRPV4 agonist GSK1016790, increased the LW/BW ratio by 51% (from 4.36 in control animals to 6.58 in vehicle-treated animals; Fig 1A), consistent with formation of profound lung edema. TRPV4 channel blocker GSK2798745 dose-dependently inhibited the formation of pulmonary edema in this animal model, with complete inhibition seen at the highest dose tested where LW/BW was held to control levels (LW/BW = 4.21; Fig. 1A). The steady-state total rat plasma GSK2798745 concentration and LW/BW relationship yielded an estimated IC_{50} of 3.1 ng/mL (6.7 nM; Fig. 1B) with 95% confidence intervals from 0.79 to 12.0 ng/mL. This rat IC_{50} value equated to a human equivalent IC_{50} of 3.0 ng/mL (6.5 nM) with 95% confidence intervals from 0.77 to 11.6 ng/mL after correcting for rat-to-human species differences in TRPV4 cellular IC_{50} potency and plasma protein binding.

Rat PK/PD Model Prediction of Human Target Engagement

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The safety and PK of single and repeat doses of GSK2798745 ranging from 0.25 to 12.5 mg was recently evaluated in a FTIH study (Goyal et al., 2019). The healthy volunteer cohort (Cohort 3) of the FTIH study was administered 5 mg GSK2798745 once daily with repeat dosing for up to 14 days. Enrolled subjects with stable HF (Cohorts 4 and 5) were administered 2.4 mg of GSK2798745 once daily for up to 7 days.

Predicted TRPV4 channel blockade based on GSK2798745 concentrations measured in the subjects in these cohorts after a single and once-daily repeat dose regimen is presented in Fig. 2A and 2B. An IC_{50} of 3.0 ng/mL was utilized in estimating the channel inhibitions as described in Equation 1. This value was derived from the rat PK/PD study data and corrected for protein binding differences between human and rat species. Corrections were also made for IC_{50} potency differences between species based on cell-based assays. The plots demonstrate high and sustained levels of target engagement with the once daily regimen at the doses evaluated in clinic. The predicted maximal channel inhibition on Day 1 and Day 14 in healthy volunteers with the 5 mg once daily regimen reaches above 85% and 90%, respectively, as seen in Fig. 2A. The lower 2.4 mg once daily regimen evaluated in the HF subjects is also predicted to provide high channel inhibition (>80%) on repeat dosing at peak drug exposure (Fig. 2B). The decline in drug exposure over the dosing interval retains channel blockade to >60% at trough.

HUVEC Impedance

The ability of GSK2798745 to block TRPV4 agonist GSK1016790-induced impendence reduction in HUVECs, a surrogate for pulmonary endothelial cells, in the presence of human whole blood was assessed.

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Fig. 3A showed the time course of Normalized Cell Index (NCI) of HUVEC treated with DMSO or GSK1016790 (0.03-1 μM). GSK1016790 concentration-dependently induced NCI reduction compared with DMSO control. Pre-treatment of HUVEC with GSK2798745 (2.55-25.5 nM) concentration-dependently decreased GSK1016790-induced NCI reduction compared with DMSO pre-treatment (Fig. 3B). As shown Fig. 3C and D, TRPV4 agonist GSK1016790 in previously frozen human blood induced HUVEC impedance reduction with a half maximal activation concentration (EC_{50}) of 224±51 nM (n=3) compared with that of 98±10 nM (n=3) in fresh blood from the same donors (P>0.05). With equivalent stimulation of HUVECs using GSK1016790 (~EC_{95}), pre-treatment with GSK2798745 in previously frozen blood inhibited GSK1016790-induced HUVEC impedance reduction with a half maximal inhibition concentration (IC_{50}) of 6.2±1.1 nM (n=3; Fig. 3F) compared with that of 6.5±1.4 nM (n=3; Fig. 3E) in fresh blood (P>0.05). These results demonstrate that freezing blood prior to impedance assessment did not alter the inhibitory potency of GSK2798745, supporting the use of frozen clinical blood samples in the HUVEC impedance assay.

The above assay was utilized to assess the inhibitory effect of previously frozen blood from clinical trial subjects on TRPV4 agonist (GSK1016790)-induced HUVEC impedance reduction. Blood from 3 healthy volunteers drawn 1-12 h after 5 mg GSK2798745 repeat daily dosing (Day 1 or Day 14) inhibited GSK1016790-induced HUVEC impedance reduction by ≥85% (Fig. 4A), consistent with the predictions based on the rat PK/PD model (Fig. 2A). As expected, blood from healthy volunteers dosed with placebo (Day 1 and Day 14 combined) elicited no inhibitory effect (Fig. 4A). The averaged results of 16 HF subjects dosed with 2.4 mg GSK2798745 (Day 1 or Day 7) and 4 HF subjects dosed with placebo (Day 1 and Day 7 data combined) are summarized in Fig. 4B. On average, blood samples from HF subjects dosed with 2.4 mg GSK2798745

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inhibited GSK1016790-induced HUVEC impedance reduction by ≥58% at 1-24 h after single dosing (Day 1), and by ≥78% at 1-24 h after receiving the seventh daily dose during repeated dosing (Day 7), consistent with expectations based on the rat PK/PD model (Fig. 2B). Blood samples from HF subjects dosed with placebo had no inhibitory effect.

Inhibition of GSK1016790-induced HUVEC impedance reduction (i.e., pharmacodynamics [PD]) was plotted against the corresponding plasma GSK2798745 concentration (PK) for all tested blood samples from the HF subjects dosed with 2.4 mg GSK2798745 to establish a PK/PD relation (Fig. 5). Curve fitting yielded an IC_{50} of 1.3 ng/mL (2.9 nM) with 95% confidence intervals from 1.1 to 1.4 ng/mL, similar to the 3.0 ng/mL IC_{50} (95% confidence intervals from 0.77 to 11.6 ng/mL) of GSK2798745 predicted from the rat in vivo PK/PD model of pulmonary edema after correcting for rat-to-human species differences in TRPV4 cellular IC_{50} potency and plasma protein binding.

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8. Discussion *(≤1500 words)*

GSK2798745 was recently investigated as a novel medicine to reduce pulmonary edema caused by HF, but the test agent lacks a suitable biomarker to confirm appropriate target engagement for clinical efficacy. In the present study, we established a translational ex vivo whole blood assay designed to predict levels of TRPV4 blockade at the intended site of action, vascular endothelial cells, of clinical subjects with stable HF. Using this assay, blood samples from HF subjects dosed with 2.4 mg GSK2798745, a dose selected based on emerging clinical exposure data and to maintain exposure within adequate safety margins based on data from pre-clinical studies, inhibited GSK1016790-induced HUVEC impedance reduction by ≥78% at 1-24 h after 7 days of repeated daily dosing. These values were consistent with target engagement predictions obtained from a rat in vivo PK/PD model of pulmonary edema, and together strongly suggest the circulating levels of GSK2798745 were sufficient to robustly block TRPV4 in lung vascular endothelial cells, supporting this dosing regimen for assessing clinical efficacy in HF subjects.

Growing evidence indicate that the level of target occupation necessary for therapeutic effect is ~60-90% for antagonists of G-protein-coupled receptors, neurotransmitter transporters and ion channels (Grimwood and Hartig, 2009). In the absence of a suitable technique for directly measuring TRPV4 target occupancy, our first step was to perform mechanism-based PK/PD modeling to predict levels of GSK2798745 necessary to exhibit clinical efficacy. Indeed, in some instances, a PK/PD model/readout is preferred as receptor occupancy does not always directly predict drug inhibitory activity (Rosario et al., 2008). A previously described TRPV4 agonist-mediated rat model of pulmonary edema (Thorneloe et al., 2012) was chosen for this purpose due to its simplicity, reproducibility, throughput and ability to infuse test compound to steady-state. In this model, GSK2798745 dose-dependently inhibited the TRPV4 agonist-induced

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increase in LW/BW to the level observed in control animals with a potency of 6.7 nM, in-line with the IC_{50} potency determined in a rat TRPV4 cell-based FLIPR assay (1-2 nM). This inhibitory potency is predicted to translate to a high and sustained level of TRPV4 channel blockade in both the healthy volunteer (5 mg GSK2798745 once daily with repeat dosing for 14 days) and HF (2.4 mg GS2798745 once daily for 7 days) cohorts after both single and once-daily repeat dose regimens. Importantly, in the HF cohort in which the efficacy of GSK2798745 on lung edema will be assessed, repeat 2.4 mg daily dosing resulted in predicted TRPV4 inhibition of >80% at peak drug exposure and remained at >60% at trough, suggesting adequate target engagement for further clinical assessment in HF subjects.

Although in vivo PK/PD models offer many advantages over in vitro models, such as enabling a more integrated perspective on the relationship between drug dose, exposure and response, one limitation of utilizing a pre-clinical species is the requirement for cross-species conversions. As such, we sought a secondary, in vitro, PK/PD tool, preferably one employing cells expressing endogenous human TRPV4, to confirm the rat in vivo PK/PD results.

A HUVEC impedance assay was selected as a secondary, ex vivo, PK/PD tool. Importantly, TRPV4 agonist-mediated reduction in HUVEC impedance remained in the presence of whole blood, supporting its potential use as an ex vivo target engagement assay utilizing whole blood from clinical subjects.

In addition to HUVECs, the suitability of human lung microvascular endothelial cells (HLMVECs) for the TRPV4 impedance assay were assessed considering HLMVECs constitute the alveolar barrier, but HUVECs were selected as they can be used up to passage 7 whereas HLMVECs can only be used up to passage 4 (Xu, unpublished observation). Also, TRPV4

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blockers were found to have similar potencies between these two cell types (Xu, unpublished observation), and therefore further substantiated using HUVECs as a surrogate of HLMVECs.

As utilization of frozen blood over fresh blood offers greater flexibility in sample shipments and experimental timings, an initial goal was to determine whether previously frozen blood could be utilized without altering the HUVEC impedance assay performance. Whereas the potency of response to TRPV4 agonist was marginally (2.3-fold) rightward-shifted in previously frozen blood (not statistically significant), using equivalent stimulation (~EC_{95}) with TRPV4 agonist, the inhibitory potency of GSK2798745 was not altered by freezing prior to impedance assay assessment, supporting the use of frozen clinical blood samples.

When adding GSK2798745 to human blood, the inhibitory potency of GSK2798745 in the impedance assay (IC_{50}s of 6.5 nM and 6.2 nM in fresh and frozen blood, respectively) was comparable to that predicted from the rat PK/PD model (6.5 nM). These potency values were also similar to the 2.9 nM IC_{50} obtained from HUVEC impedance assay when impedance reduction was plotted against the corresponding plasma GSK2798745 concentration for all tested blood samples from HF subjects. Together, these correlations provide further confidence in the translatability of the HUVEC impedance assay to the clinic.

Blood from healthy volunteers and HF subjects dosed with GSK2798745 (5 mg and 2.4 mg, respectively) robustly inhibited TRPV4 agonist-mediated HUVEC impedance. Day 7 samples were selected to assess the steady-state PD effect as steady-state PK was attained in approximately 4 to 6 days with the once daily dosing regimen (Goyal et al., 2019). As expected, the impedance reduction tracked with the PK (Goyal et al., 2019), with the maximum PD effect occurring within 2 h of the first or repeat daily dose. Importantly, at steady-state in the HF

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cohort, 2.4 mg daily dosing resulted in ≥78% attenuation of the GSK1016790-induced HUVEC impedance reduction over the entire 24 h assessment period, further supporting this dosing regimen for clinical evaluation of GSK2798745 in HF patients.

A notable limitation of this study was the use of TRPV4 agonist-based PK/PD models/assays to predict the exposure of GSK2798745 necessary to mitigate a non-agonist, disease-mediated clinical readout. Indeed, the i.v. dose of TRPV4 agonist GSK1016790 utilized in the rat PK/PD model of lung edema resulted in >50% increase in lung weight and marked cardiovascular collapse within 10 min, and it is anticipated that this level of TRPV4 activation is not attained in a disease state such as HF. Similarly, the ~EC_{95} concentration of GSK1016790 employed in the HUVEC impedance assay detaches the majority of HUVECs from the assay plates and therefore also likely activates TRPV4 more than in human HF. It is also important to note that GSK2798745 inhibits both small molecule agonist- and hypotonicity-induced activation of TRPV4 with IC_{50}s ranging from 1.6 – 2 nM (Brooks et al., 2019 and unpublished observations), suggesting that GSK2798745 inhibits TRPV4 with similar potencies regardless of the mode of activation. As such, the level of GSK2798745 exposure required to elicit a PD effect in this study might be an overestimate of the amount needed in a disease state such as HF where TRPV4 activation is presumably less robust. Another important limitation to this study is that the target engagement predicted from the in vivo and ex vivo PK/PD models is useful only for diseases in which the vascular endothelial cell is the site of pharmacological action. Indeed, GSK2798745 levels could differ in target cells/tissues for other potential TRPV4-related diseases, requiring an appropriate adjustment of GSK2798745 dose.

In summary, the current study describes the identification of an HUVEC impedance assay in which blood from HF subjects dosed with GSK2798745 (2.4 mg daily) were utilized to predict

TRPV4 target engagement at the intended site of action (endothelial cells) of clinical subjects with HF. Results from this ex vivo whole blood assay were consistent with target engagement predictions obtained from a rat in vivo PK/PD model of pulmonary edema and together suggest that circulating levels of GSK2798745 in the recently completed Phase I/IIa trials were sufficient to block TRPV4 in pulmonary vascular endothelial cells to a large extent, supporting this dosing regimen for assessing clinical efficacy in HF subjects. This ex vivo impedance assay represents an approach to estimate target engagement for suitable targets when more traditional methods have failed.

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9. Acknowledgement

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10. Authorship Contributions

Participated in research design: Xu, Goyal, Roethke, James, Thorneloe, Sprecher and Behm

Conducted experiments: Xu and James

Contributed new reagents or analytic tools: Patterson, Stoy and Goodman

Performed data analysis: Xu, Goyal, Costell, Roethke, James and Behm

Wrote or contributed to the writing of the manuscript: Xu, Goyal, Costell and Behm

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12. Footnotes

This work received no external funding.

All authors worked for and owned shares in GSK during the study.

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13. Figure Legends

Figure 1. GSK2798745 dose-dependent inhibition of TRPV4 agonist-mediated lung edema in rats. A: Compared to controls, administration of the TRPV4 agonist GSK1016790 (10 μg/kg/min i.v. for 10 min) increased the lung wet weight/body weight ratio (LW/BW), a measure of lung edema, in vehicle treated rats by 51%. Pre-treatment for 60 min with a low, middle, or high dose of the TRPV4 inhibitor GSK2798745 (16, 50, or 150 ng/kg/min i.v., respectively) dose-dependently inhibited the development of lung edema by 37%, 79%, and 107%, respectively, compared to vehicle. Data are presented as mean ± SEM as well as individual data points, and n/group are indicated within the bars. *** $p<0.001$, * $p<0.05$ vs. vehicle, one-way ANOVA with Dunnett's multiple comparison post-test. B: Steady-state plasma concentrations of GSK2798745 were log-transformed and plotted with respective LW/BW ratios from rats treated with a low, middle, or high dose of GSK2798745. Using an unconstrained four parameter dose-response model (SAS® 9.4) which included data from the vehicle and control groups, the half maximal inhibition concentration (IC_{50}) was approximated at 3.1 ng/mL (6.7 nM) with 95% confidence intervals (CI) from 0.79 to 12.0 ng/mL and a Hill slope of -1.84. The upper limit was 6.57 (95% CI from 5.77 to 7.36) and the lower limit was 4.32 (95% CI from 3.66 to 4.99).

Figure 2. Predicted TRPV4 channel blockade from rat PK/PD model. A: Predicted TRPV4 channel blockade for healthy volunteers dosed 5 mg once daily GSK2798745 on Day 1 and Day 14. B: Predicted TRPV4 channel blockade for HF patients dosed 2.4 mg once daily GSK2798745 on Day 1 and Day 7.

Figure 3. Effects of TRPV4 antagonist GSK2798745 on TRPV4 agonist GSK1016790-induced HUVEC impedance reduction. A: Example time course of Cell Index normalized to the value prior to GSK1016790 addition (Normalized Cell Index; NCI). GSK1016790 concentration-

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dependently induced NCI reduction compared with DMSO control (open circles). B: Similar to A, except HUVECs were pre-treated with DMSO or GSK2798745 (2.55-25.5 nM) and GSK1016790 concentration was 0.5 µM (~EC_{95}). Open squares were negative control where HUVECs were pre-treated with DMSO and stimulated with DMSO instead of GSK1016790. C: Concentration-dependent NCI reduction of HUVECs caused by GSK1016790-induced TRPV4 activation (results obtained using fresh blood from 3 donors). The mean half maximal activation concentration (EC_{50}) of GSK1016790 was 98 ± 10 nM. D: Results obtained using previously frozen blood from the same 3 donors, EC_{50}=224 ± 51 nM. E: Concentration-dependent inhibition of GSK1016790-induced HUVEC impedance reduction by GSK2798745 with a mean half maximal inhibition concentration (IC_{50}) of 6.5 ± 1.4 nM (n=3) in fresh blood. F: Results obtained using the previously frozen blood from the same 3 donors, IC_{50}=6.2 ± 1.1 nM (n=3). All data are expressed as Mean ± SEM.

Figure 4. % Inhibition of GSK1016790-induced HUVEC impedance reduction by pretreatment of HUVECs with clinical blood samples containing GSK2798745. A: % Inhibition of GSK1016790-induced HUVEC impedance reduction obtained using blood drawn from healthy volunteers at various time points after being dosed with placebo (2 subjects) or 5 mg GSK2798745 (3 subjects). B: Results obtained using blood drawn from HF patients at various time points after being dosed with placebo (3-4 subjects) or 2.4 mg GSK2798745 (5-16 subjects). All data are expressed as Mean ± SEM.

Figure 5. Inhibition of TRPV4 agonist-induced HUVEC impedance reduction by blood drawn from HF subjects dosed with GSK2798745. % Inhibition of GSK1016790-induced HUVEC impedance reduction for all tested blood samples from HF patient dosed with 2.4 mg once daily GSK2798745 was plotted against the plasma concentration of GSK2798745 determined for the

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corresponding patient and time. Using three-parameter concentration-response curve fitting, GSK2798745 exhibited a half maximal inhibition concentration (IC_{50}) of 1.3 ng/mL (2.9 nM).

Figure 1

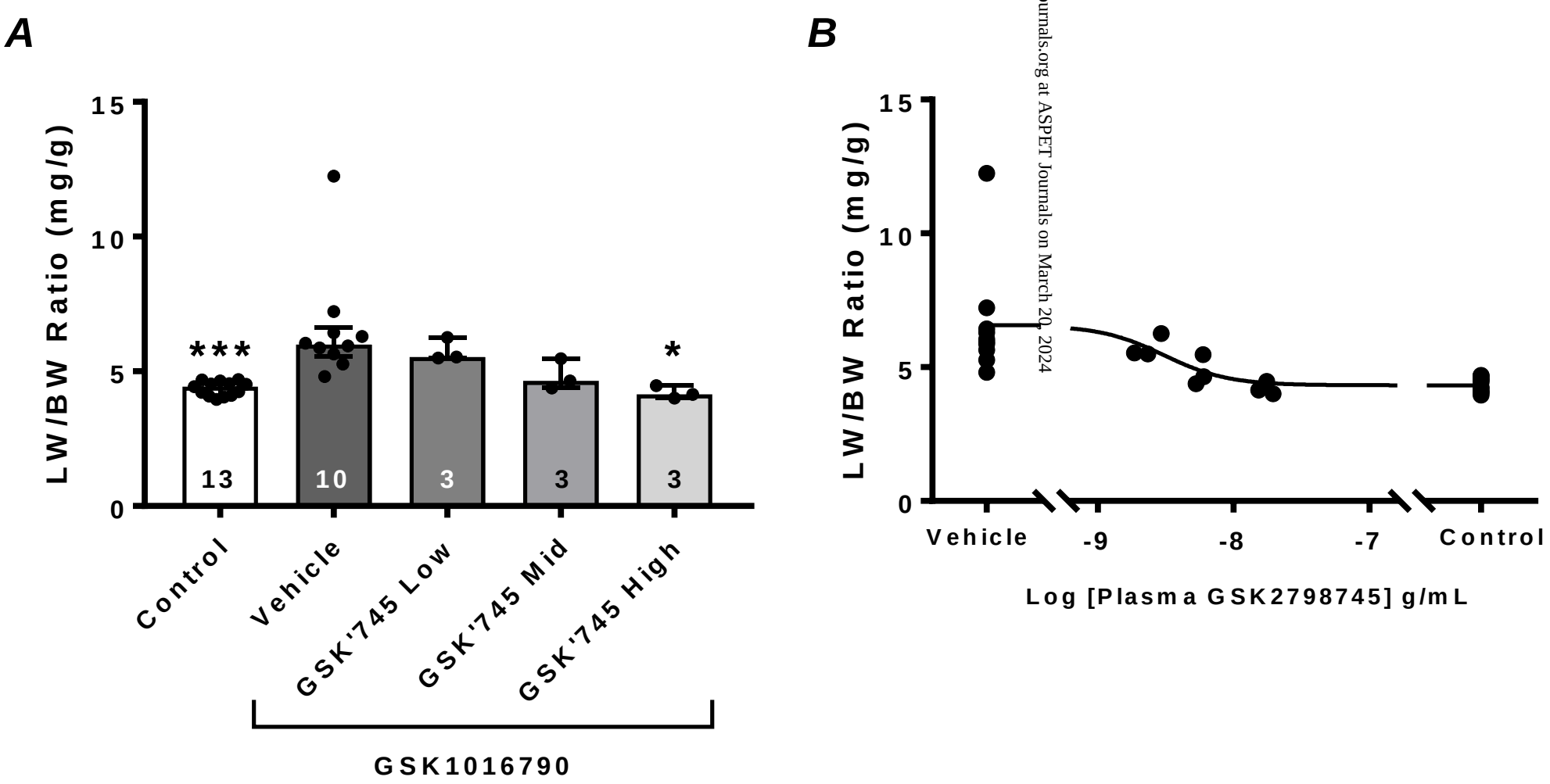
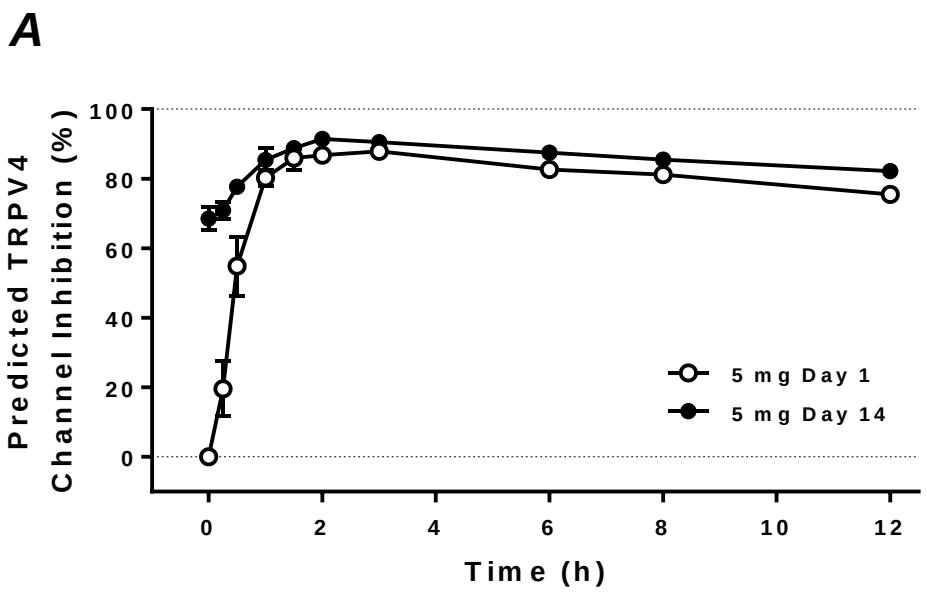


Figure 2



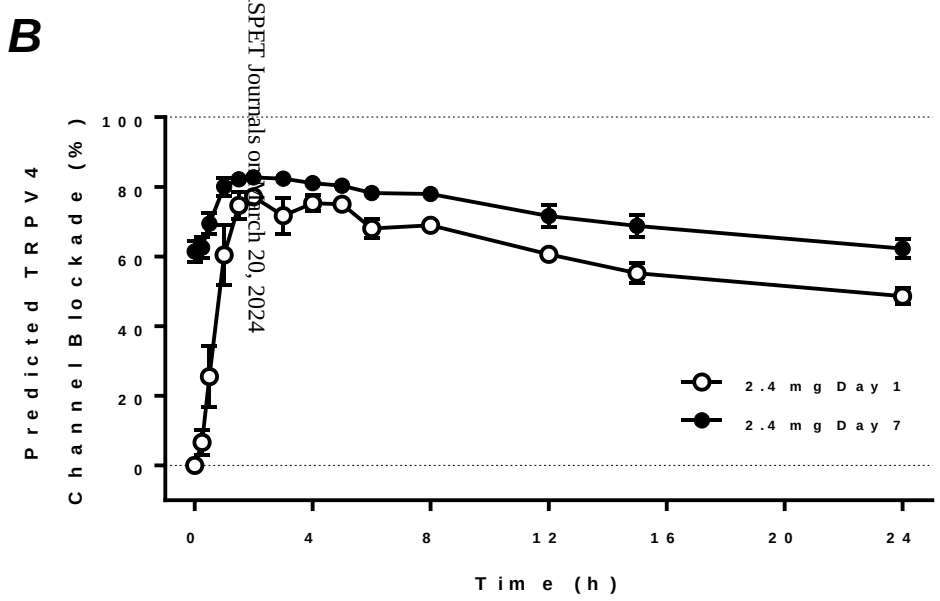
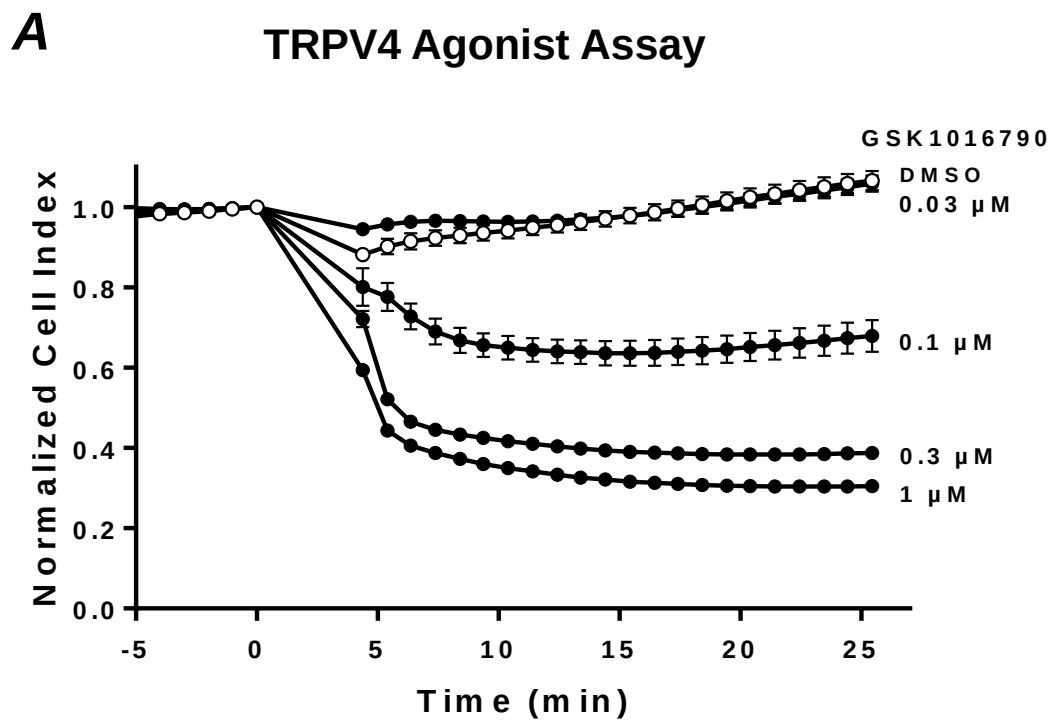
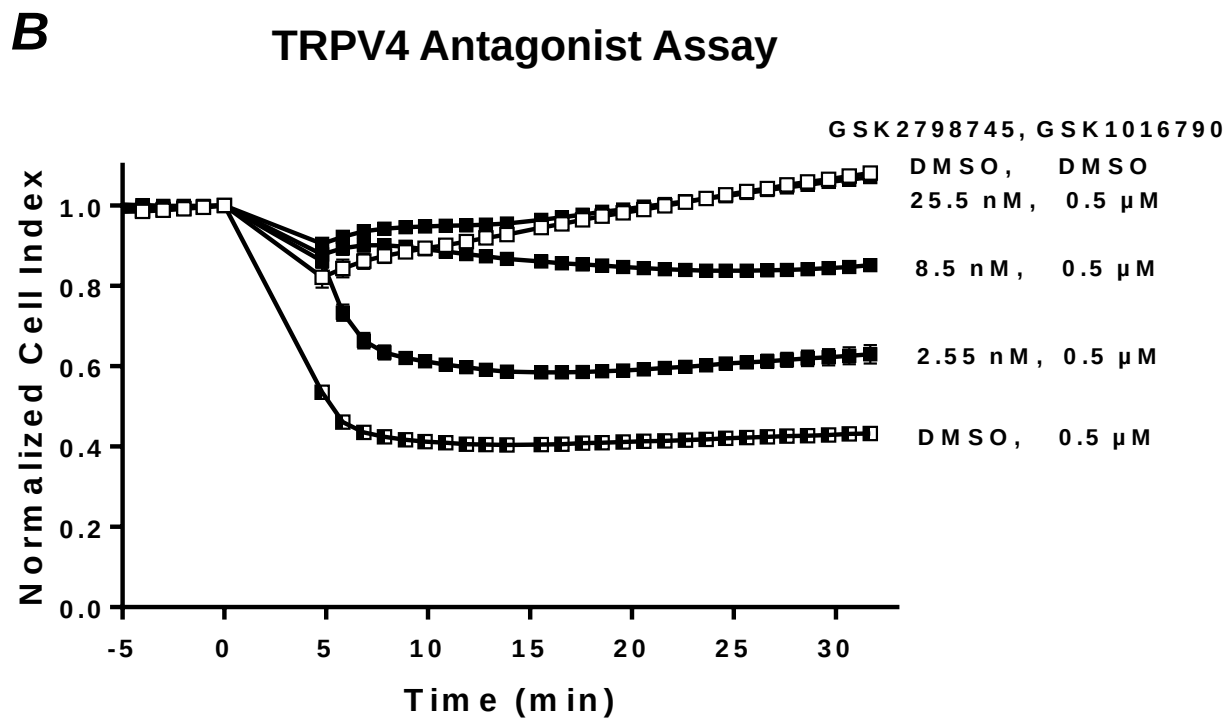
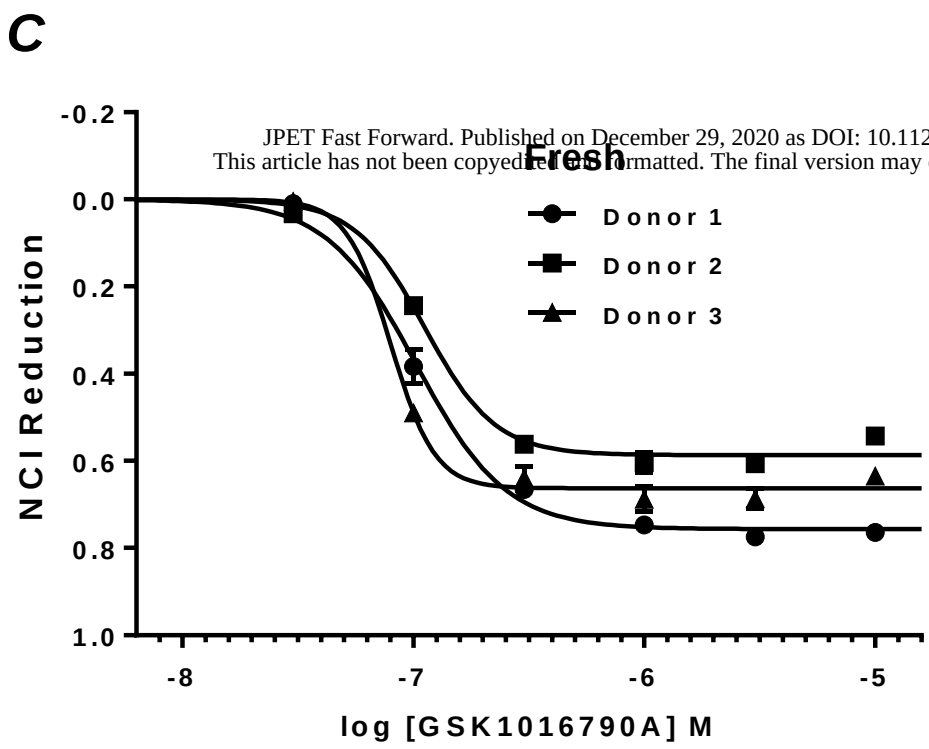
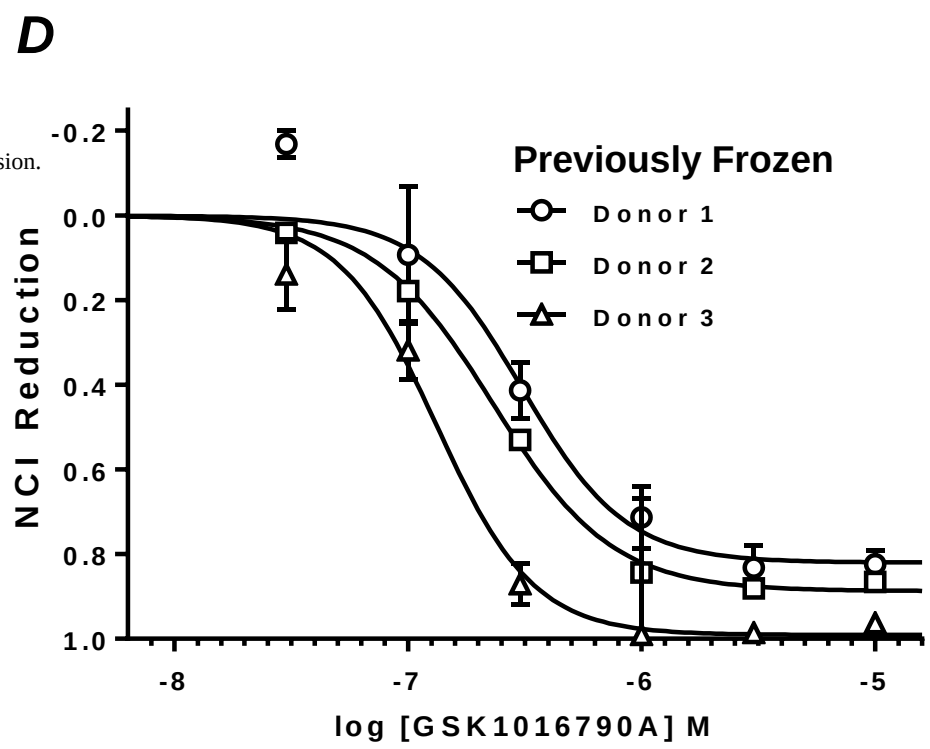


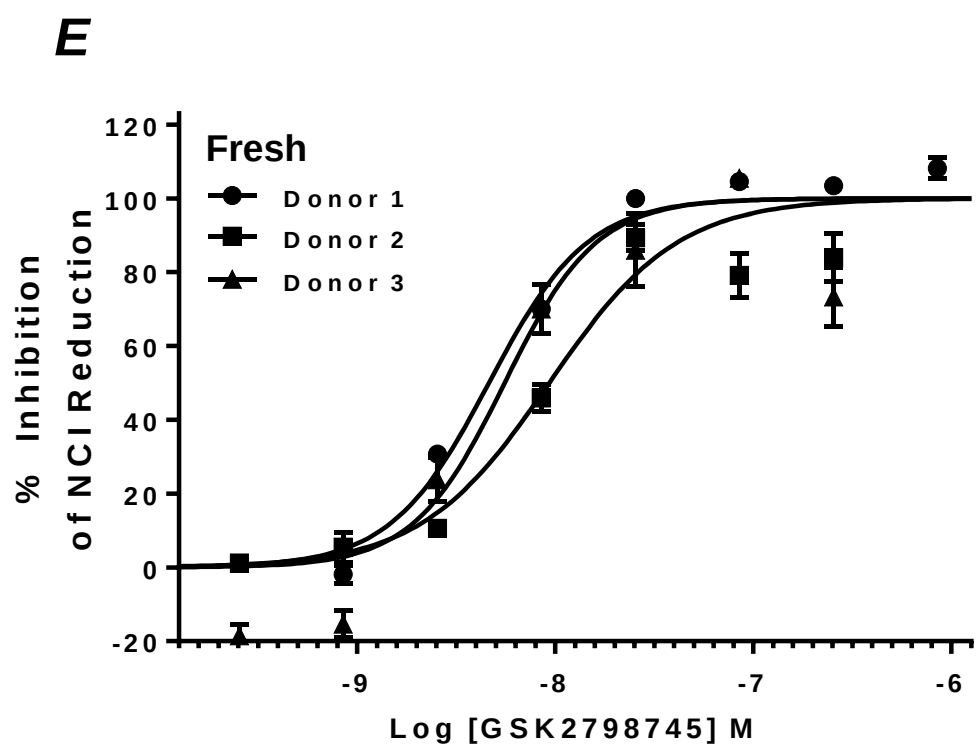
Figure 3











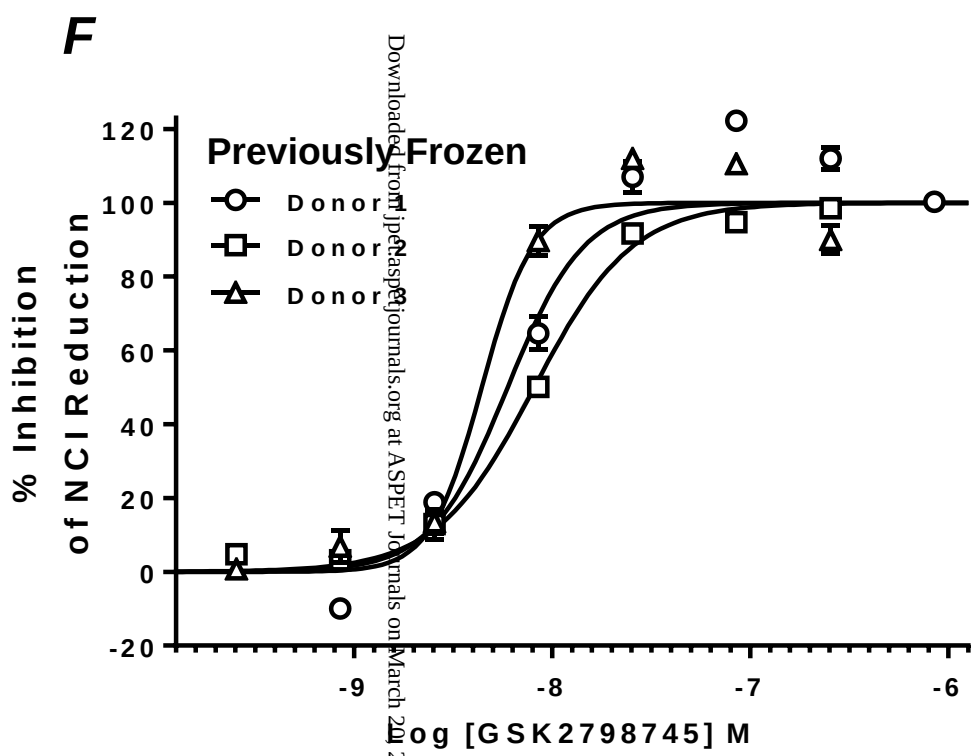
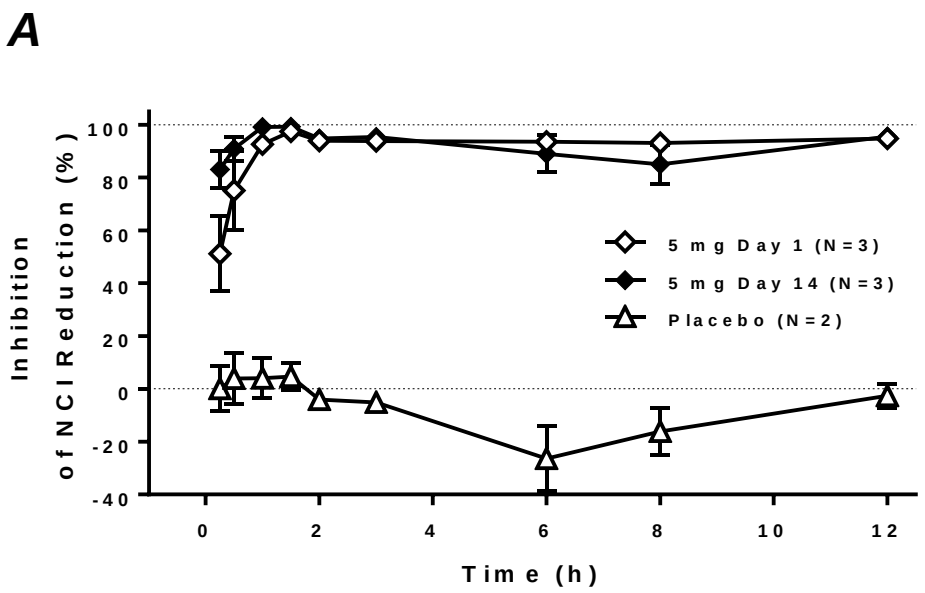


Figure 4



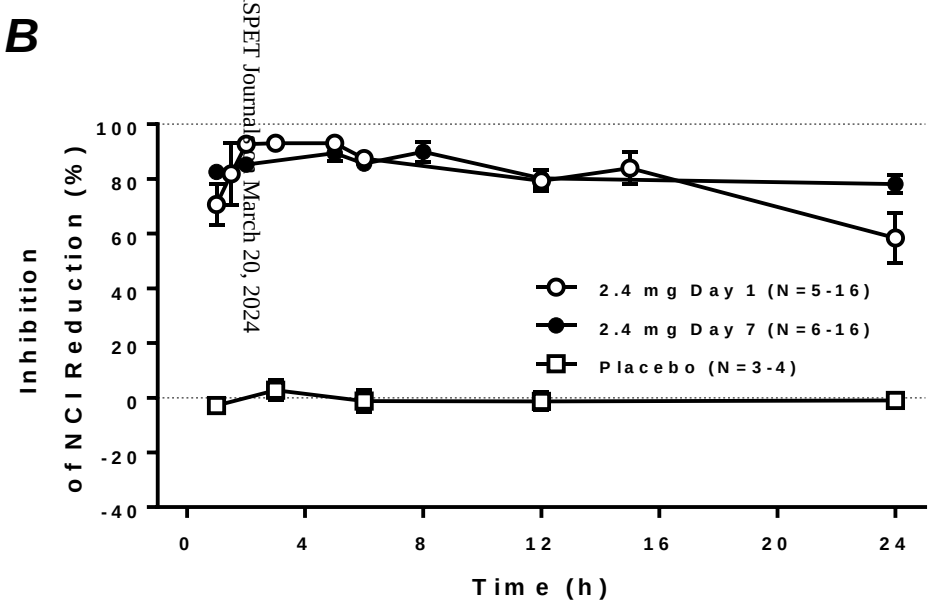


Figure 5