

Characterizing Pharmacokinetic-Pharmacodynamic Relationships and Efficacy of PI3K $\delta$ -inhibitors in Respiratory Models of TH2 and TH1 Inflammation

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List of abbreviations

AUC – area under the curve

C<sub>max</sub> - maximum (or peak) plasma concentration

CND – central nervous system

COPD - chronic obstructive pulmonary disease

IgG - Immunoglobulin G

IgM - Immunoglobulin M

MDSC – myeloid-derived suppressor cells

NKA –neurokinin A

NKB – neurokinin B

PD –pharmacodynamic

PI3K- phosphatidylinositol 3-kinases

PK – pharmacokinetic

TH1 Type 1 T helper cell

TH2 Type 2 T helper cell

ToT – Time on target

T<sub>max</sub> – time to maximum plasma concentration

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## Abstract

We leveraged a clinical PK/PD/efficacy relationship established with an oral PI3K $\delta$  inhibitor (Idelalisib) in a nasal allergen challenge study to determine if a comparable PK/PD/efficacy relationship with PI3K $\delta$  inhibitors was observed in preclinical respiratory models of TH2 and TH1 inflammation. Results from an *in vitro* rat blood basophil (CD63) activation assay were used as a PD biomarker. IC<sub>50</sub> values for PI3K $\delta$  inhibitors, MSD-496486311, MSD-126796721, Idelalisib and Duvelisib were 1.2, 4.8, 0.8 and 0.5  $\mu$ M. In the Ovalbumin Brown Norway TH2 pulmonary inflammation model, all PI3K $\delta$  inhibitors produced a dose dependent inhibition of BAL eosinophils (maximum effect between 80-99%). In a follow-up experiment designed to investigate PK attributes (C<sub>max</sub>, AUC, Time on target (ToT)) that govern PI3K $\delta$  efficacy, MSD-496486311(3 mg/kg, QD and 100 mg/kg QD) produced 16% and 93% inhibition of eosinophils, while doses (20 QD-, 10 BID- and 3 TID -mg/kg) produced 54 to 66 % inhibition. Our profiling suggests that impact of PI3K $\delta$  inhibitors on eosinophils is supported by a PK target with a ToT over the course of treatment close to the PD IC<sub>50</sub> rather than strictly driven by AUC, C<sub>max</sub>, or C<sub>min</sub> coverage. Additional studies in an *A. alternata* rat model, a sheep *Ascaris*-sensitive sheep model and a TH1 driven rat ozone exposure model did not challenge our hypothesis, suggesting that an IC<sub>50</sub> level of TE sustained for 24 hours is required to produce efficacy in these traditional models. We conclude that the PK/PD observations in our animal models appear to align with clinical results associated with a TH2 airway disease.

## Introduction

The use of animal models in drug discovery has historically been integral to the development of novel therapies. Unfortunately, in many disease areas the translatability of animal data into the clinical arena has been and continues to be challenging, with notable disconnects between the apparent effectiveness of various pharmacological agents across species, including man (McGonigle and Ruggeri 2014; de Caestecker et al., 2015; Gidyk et al., 2015; McNamee et al., 2015). This is particularly true in heterogeneous inflammatory diseases with complex multifaceted pathophysiology such as severe asthma or COPD (Grainge et al., 2016; Han et al., 2016; Powell, 2016; Vanfleteren et al., 2016). As an example, in the early 1990s, a very exciting emerging area of asthma research was directed at understanding the physiology, pathology and potential therapeutic potential relating to tachykinins, a family of neuropeptides. These include substance P, neurokinin A (NKA) and neurokinin B (NKB) that mediate inflammation, airway constriction and cough in the lungs through their respective receptors (NK1, NK2 and NK3) (Chapman et al, 1998; Ramalho et al., 2011). Many pharmaceutical companies invested significant resources into developing tachykinergic antagonists. Unfortunately, the respiratory animal models, over-predicted the actions of tachykinin antagonists compared to what was seen in patients with respiratory disease (Boot et al., 2006). With that being said, NK1 antagonists with different pharmacokinetic properties such as Aprepitant (i.e. CNS penetrating capabilities) have successfully entered the human clinical market for chemotherapy mediated nausea and vomiting, (Singh et al., 2016). As a result of pipeline attrition of new drug candidates, pharmaceutical companies are utilizing several strategies, including a better anchoring of animal to human genetics and more extensive evaluation of pharmacokinetic (PK)–pharmacodynamic (PD)-efficacy relationships, to enhance the probability of successfully advancing novel compounds from the laboratory to human patients.

Phosphatidylinositol 3-kinases (PI3Ks) are enzymes (lipid kinases) that mainly function by phosphorylating phosphoinositides on the D3 position in the inositol ring (Fruman 2004; Ghigo et al., 2010). Based on structure and function, there are three classes of PI3Ks, designated, I, II and III. Class I is further partitioned into Ia and Ib subtypes (Hawkins and Stephens, 2014). Phosphoinositide 3-kinase delta (PI3K $\delta$ ) is one

of four heterodimeric Class I PI3K isoforms that has engendered considerable interest as a drug target due to its central role in signaling cascading leading to initiation of inflammation responses (Ghigo et al., 2010; Barnes et al., 2013). The PI3K $\delta$  isoform is primarily expressed in leukocytes and utilizes lipid phosphorylation to regulate signaling pathways responsible for coordinating a broad range of cellular activities, including cell survival, proliferation, differentiation, and trafficking. That said, these drugs have been found to well tolerated in animals and man (Perry et al., 2018). PI3K $\delta$  also impacts tumor surveillance and genetic deletion or pharmacologic antagonism has been demonstrated to suppress tumor growth in a broad range of solid tumors in murine syngeneic models (Stark et al., 2015). PI3K $\delta$  oncological actions may, in part, be mediated by direct regulation of immune suppressor cells, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs); attenuating their suppressive function, and thus enabling a more effective T cell mediated anti-tumor response. Clinical proof of concept for selective PI3K $\delta$  inhibition has been established in several hematologic malignancies (Sanford et al., 2015). Idelalisib (Zydelig; formerly Cal-101) recently achieved regulatory approval for treatment in follicular lymphoma and chronic lymphocytic leukemia and in combination with Rituxin in indolent non-Hodgkin lymphoma.

Pulmonary pharmacologists have been considering PI3K pathways as an anti-inflammatory target for diseases such as allergic rhinitis (Horak et al., 2016), asthma (Marwick et al., 2010; Lee et al., 2016), idiopathic pulmonary fibrosis (Yan, et al., 2014; Kral et al., 2016; Campa et al., 2018), cystic fibrosis (Marwick et al., 2010) acute lung injury (Ma et al., 2015) and COPD (Barnes, 2013; Horiguchi et al., 2015; Hsu et al., 2015). A small proof of biology study with Idelalisib in patients with allergic rhinitis, a predominately TH2 inflammatory driven upper airway disease, was conducted by Horak et al., (2016). These investigators demonstrated that PI3K $\delta$  inhibition with Idelalisib significantly diminished total nasal symptom scores, nasal airflow, nasal secretion weights and congestion. Noteworthy was the employment of an *ex vivo* whole blood basophil (CD63) activation assay in the study, which confirms that the PI3K pathway was effectively engaged throughout the observation/measurement period in their study. Amassing similar data in preclinical respiratory models may begin to help bridge the translatability between animal results and clinical outcomes. To this end, in the current

study we describe the potency and selectivity of four PI3K $\delta$  inhibitors (MSD-496486311, MSD-126796721, Idelalisib and Duvelisib), in *in vitro* enzyme and cellular assays. In addition these compounds were profiled in *in vivo* studies of traditional allergic TH2 (Ovalbumin sensitized and whole body challenged rat; *Altenaria alternata* intratracheal instilled rat; *Ascaris*-sensitive sheep) and TH1 (rat ozone whole body exposure) models, with the goal of relating CD63 potency, PK and efficacy.

## Materials and Methods

### Biochemical Assay

The *in vitro* inhibitory potencies of MSD-496486311, MSD-126796721, Idelalisib and Duvelisib across PI3K  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  isoforms were measured by an Homogeneous Time-Resolved Fluorescence energy transfer (HTRF) assay that was developed and further optimized from a kit produced by Upstate (Millipore catalog # 33-047). This procedure utilizes a pre-formed HTRF complex between four binding partners: 1) biotinylated PIP3, 2) GST tagged pleckstrin homology (PH) domain, 3) Europium labeled anti-GST monoclonal antibody, and 4) Streptavidin-Allophycocyanin (APC). The native PIP3 produced by PI3K activity displaces biotin-PIP3 from the PH domain, resulting in the dissociation of the HTRF complex and a decrease in the fluorescence signal. This loss of signal was nonlinear with respect to both increasing product and time. A correction was derived from a PIP3 standard curve run in a separate assay plate. All data were calculated using the ratio of acceptor (APC) to donor (Europium) fluorescence in each well of the assay plate. The percent inhibition for each compound concentration was calculated as follows: % inhibition =  $100 \times (\text{fluorescence ratio} - \text{CtrlB}) / (\text{CtrlA} - \text{CtrlB})$  where CtrlA= PI3Kinase reaction + known reference inhibitor and CtrlB=PI3K + DMSO. An IC<sub>50</sub> was then calculated fitting the % inhibition data to the equation: %inhibition =  $\text{min} + (\text{Max} - \text{min}) / (1 + ([\text{inhibitor}] / \text{IC}_{50})^n)$  where min is the % inhibition with inhibitor, max was the signal in DMSO control, and n was the Hill slope.

### **Cell-based Assay**

The cell-based potencies of MSD-496486311, MSD-126796721, Idelalisib and Duvelisib were measured by the ability to inhibit downstream effectors of PI3K $\delta$  as assessed by analysis of the effects of the compound on the phosphorylation status of Akt Serine 473 in the Ramos lymphoma-derived B cell line. The Ramos cell line expresses cell surface IgM and responds to IgM crosslinking by activating PI3K $\delta$ -dependent signaling. Treatment of Ramos cells with PI3K $\delta$  inhibitors robustly suppresses the anti-IgM stimulated PI3K $\delta$ -dependent phosphorylation of Akt S473. The cellular selectivity of PI3K $\delta$  inhibitors over other Class I PI3K isoforms was determined by measuring the inhibition of Akt phosphorylation in cell-based assays selective for PI3K $\alpha$  (heregulin-stimulated SK-BR-3 cells), PI3K $\beta$  (PTEN-null MDA-MB-468 cells), or PI3K $\gamma$  (C5a-stimulated RAW 264.7 cells).

### **In vitro Whole Blood Assays**

#### ***Anti-CD79b Induced pAKT in Human Whole Blood***

Human whole blood was incubated with PI3K $\delta$  inhibitors for 30 min and stimulated with anti-CD79B for 10 min at 37 °C. Samples were stained with anti-CD45, anti-CD20, and anti-pAKT, mixed, and incubated at 4 °C for 20 min. Sample red blood cells were lysed for 15 min at room temperature and were fixed with 2% formaldehyde for 10 min followed by incubation in FACs read buffer. CD20+ cells were gated

#### ***Anti-IgE-Mediated CD63 in Rat Whole Blood***

Whole blood withdrawn from male Brown Norway rats (225-275g) was incubated with PI3K $\delta$  inhibitors for 30 mins and stimulated with anti-IgE for 20 min at 37°C. Reaction was stopped by placing at 4°C for 5 min. Samples were stained with anti-CD45-FITC, anti-HAR-PE and anti-CD63-APC into blood, mixed and incubated at 4 °C for 30 min. Sample red blood cells were lysed for 15 min at room temperature and were fixed with 2% formaldehyde for 10 min followed by incubation in FACs read buffer. CD45+HARP+ cells were gated for CD63 analysis.

## **Ex vivo Whole Blood Assay**

### ***Anti-IgE-Mediated CD63 in Rat Whole Blood***

Male Brown Norway rats were dosed with vehicle, control compound or one of four PI3K $\delta$  inhibitors. Whole blood was collected at Day 0 to establish baseline control and then dosed at Day 1. Whole blood was then sampled at 1, 4, 8, and 24 hr time points. Withdrawn blood was stimulated with anti-IgE for 20 min at 37 °C. Samples were stained with (anti-CD45-FITC, anti-HAR-PE and anti-CD63-APC) into blood, mix and incubated at 4 °C for 30 min. Sample red blood cells were lysed for 15 min at room temperature and were fixed with 2% formaldehyde for 10 min followed by incubation in FACs read buffer. CD45+HARP+ cells were gated for CD63 analysis.

### **Concanavalin A-stimulated histamine release in sheep whole blood**

Sheep whole blood plasma was removed and remaining fraction diluted with buffered saline and incubated with PI3K $\delta$  inhibitors for 30 min and stimulated with 10 ug/ml of Concanavalin A for 30 min at 37 °C. Histamine released into supernatants was measured by ELISA.

## **Animal Care and Use**

These studies were performed in accordance to the guidelines of the Institute for Laboratory Animal Research (ILAR). All studies were part of an institutional animal care and use committee (IACUC)-approved protocol and animals were housed in an AAALAC international accredited research facility. Since all key experimental readouts were terminal with the exception of studies conducted in sheep, rodent study groups were used once only and were euthanized at the end of each study.

## **Respiratory Rat Models of TH2 and TH1 Inflammation**

Ovalbumin sensitized and challenged rat model studies were conducted by Charles River Laboratories (Montreal, QC) as per as per our protocol. To examine allergic-mediated lung inflammatory responses, Brown Norway rats (225–275 g) were actively sensitized to ovalbumin over a 14 day regimen. On day 1, animals were administered 20  $\mu$ g of ovalbumin (i.p.) and 8 mg/kg of aluminum hydroxide (i.p.) in 1 ml



of physiological saline. Seven days later, an i.p. booster containing ovalbumin (20  $\mu$ g) and aluminum hydroxide (8 mg/kg) was administered. Non-sensitized rats received 2 doses of aluminum hydroxide (8 mg/kg) administered on day 1 and day 7. Pulmonary BAL inflammation (total and differential cell counts) was assessed following ovalbumin sensitization and challenge on day 14 using method described in detail previously (Celly et al., 2006). In the first study, we profiled the dose response characteristics of several PI3 $\delta$  inhibitors, MSD-496486311 (0.3 – 30 mg/kg), MSD-126796721 (10 – 100 mg/kg), Idelalisib (10 – 100 mg/kg), Duvelisib (0.3 – 30 mg/kg) and the glucocorticoid agonist dexamethasone (0.003 – 3.0 mg/kg) for 2 days. These drugs were given orally 1 hour before ovalbumin exposure on Day 14 and BAL cellular content was examined 48 hours after Ovalbumin challenge. The second experiment was designed to investigate pharmacokinetic attributes (C<sub>max</sub>, AUC, ToT) that drive PI3K $\delta$  efficacy in this model. We conducted a “split dosing experiment” which modulated C<sub>max</sub> and AUC across treatment groups and allowed investigations into the PK/PD relationship. Specifically, we administered MSD-126796721 orally at 20 mg/kg QD 1 hour before Ovalbumin challenge to one group of animals, then “splitting” the dose to 10 mg/kg, BID and 3 mg/kg, TID to separate groups of rats. Additionally, we tested 3 mg/kg and 100 mg/kg (QD) to round out a full dose response. Note that this dosing regimen was levied for 2 days and BAL samples were collected 48 hours post ovalbumin exposure. Separate PK studies in Brown Norway rats were conducted as described in the “Pharmacokinetics in Rats” section.

The rat *A. alternata* model has been described previously (Gil et al., 2014). Briefly, male Brown Norway rats (225–275 g) were purchased from Charles River Laboratories (Kingston, NY). In the first experiment, rats were intratracheally challenged with *A. alternata* extracts (Greer Labs, Lenoir, NC) in 100  $\mu$ l sterile phosphate buffered saline (PBS), using a Penn-Century microsyringe device (Penn-Century, Philadelphia, PA). MSD-126796721 (10, 30 and 100 mg/kg, QD) was administered 1 hour before instillation of *A. alternata*. Twenty four hours after *A. alternata* challenge BAL fluid was analyzed for total inflammatory cells, neutrophils and eosinophils population (Celly et al., 2006). The dose response characteristics of MSD-126796721 were compared to an equivalent experiment in Ovalbumin sensitized and challenged Brown Norway Rat model.

Ozone studies were conducted by Pneumolabs (London, UK) as per our design. Male Brown Norway Rats (200-275 g, Charles Rivers UK Ltd) were exposed to air from a primary cylinder which passed through a Sander ozoniser (S500 MG) and into a 5 L glass reservoir, where it was mixed with the air from a second inlet cylinder. The ozone output (3 ppm) was monitored using Gastec ozone detector tubes and the generated ozone was passed through an whole body exposure chamber (30 x 30 x 45 cm) at a rate of 2 L/min. For these experiments, rats were exposed to ozone for 180 min, with 4 animals sharing a chamber at a time. Rats were given MSD-496486311 at doses of 10 (QD), 30 (QD), 15 (BID) 100 (QD) 50 (BID) and 300 (QD) mg/kg. MSD-496486311 or air was given 1 hour prior to ozone exposure. Twenty-four hours after ozone provocation, the animals were euthanized by an overdose of pentobarbital and BAL fluid was collected. The BAL fluid collection was performed as previously described (Celly et al., 2006). BAL fluid was analyzed for total inflammatory cells and neutrophils populations using a XT-2000iV analyzer (Sysmex).

### **Pharmacokinetics in Rats.**

Male Brown Norway Rats were dosed orally by gavage. MSD-496486311 was dosed at 10, 30, 100, and 300 mg/kg QD. MSD-126796721 was dosed at 20 mg/kg QD. Whole blood was withdrawn immediately prior to dosing and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after drug administration. Plasma concentrations were determined using liquid chromatography–tandem mass spectrometry (LC–MS/MS) instruments and validated bioanalytical methods. Pharmacokinetic parameters, including the peak plasma concentration (C<sub>max</sub>), the time to C<sub>max</sub> (T<sub>max</sub>), and the area under curve (AUC), were calculated by using Watson LIMS software (version 7.2, Thermo Electron Corporation, Philadelphia, PA). Based on those pharmacokinetic data, plasma concentration–time profiles resulting from BID (twice a day) and TID (three times a day) dosing at the levels used in the efficacy experiments were predicted by using Phoenix WinNonlin (Version 6.3, Pharsight Corp, Mountain View, CA, USA).

### Sheep Ascaris Model

Sheep with a naturally occurring allergic response to *Ascaris suum* were utilized in this study. These animals have a stable and reproducible increase in pulmonary resistance (RL) following inhaled challenge with *Ascaris suum*. Pulmonary resistance following compound treatment is then compared to this historical RL response to assess the effects of the compound. On the day of antigen challenge, baseline values of pulmonary resistance (RL) were obtained and then three sheep were treated with a 2.0 mg/kg intravenous bolus of Idelalisib followed by a 1.92 mg/kg infusion delivered over 9 h (via the external jugular vein). The intravenous infusion was initiated 1 h prior to the challenge of the sheep airways with aerosolized extracts of the antigen (*Ascaris suum* extract). Plasma concentration was maintained at ~4.0  $\mu\text{M}$  for the duration of the experiment. Measurements of RL were obtained 5-10 min after injection, to ensure there was no adverse effect of the drug itself on RL. Then 1 hour after the start of the infusion, the sheep were challenged with *Ascaris suum* antigen. Measurements of RL were obtained immediately after challenge, hourly from 1-6 h after challenge, and on the half-hour from 6 1/2-8 h after challenge. Measurements of RL were also obtained 24 h after challenge followed by the 24 h post challenge carbachol concentration response curve to determine the post challenge PC400. Detailed methods for this procedure can be found in the Supplementary Materials of Moy et al. (2013).

### PK/PD Modeling

An Emax PK/PD model describing anti-IgE Basophil degranulation CD63 expression response was established using data from *in vitro* and *ex vivo* rat whole blood assays. For our model,  $(E = E_0 - I_{\text{max}} \times C / (IC_{50} + C))$ , where E represents CD63 response, C compound plasma concentration,  $E_0$  CD63 stimulation in the absence of compound treatment,  $I_{\text{max}}$  the maximum inhibitory effect,  $IC_{50}$ , concentration required to achieve 50% of  $I_{\text{max}}$  *in vitro*.

## Results

### Characterization of novel PI3K $\delta$ -selective small molecule inhibitors in human Biochemical Assays

We identified potent, selective inhibitors of PI3K $\delta$  during the course of our internal research efforts. Additional compounds for the described studies were selected from commercial vendors, based on publically disclosed knowledge of the target. MSD-496486311, MSD-126796721, Idelalisib and Duvelisib selectively inhibited PI3K $\delta$  enzymatic activity (compared to PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\gamma$ ) with IC<sub>50</sub> values of 1.3 nM, 5.7 nM, 2.2 nM, and 0.17 nM, respectively (Table 1). MSD-496486311 and MSD-126796721 were also highly selective against a broad panel of >300 additional kinases. For example, at 10  $\mu$ M, MSD-496486311 and MSD-126796721 did not display significant inhibition (>50%) of any of the kinases tested (data not shown).

### Characterization of Novel PI3K $\delta$ -Selective Small Molecule Inhibitors in Human Cell Assays

PI3K $\delta$  is the primary PI3K isoform that mediates B cell receptor signaling. Antigen receptor induced AKT phosphorylation and induced CD69 expression serve as surrogate markers for PI3K $\delta$  mediated cellular activity. MSD-496486311, MSD-126796721, Idelalisib and Duvelisib inhibit B cell receptor signaling in Ramos B cells in a concentration-dependent manner with IC<sub>50</sub> values of 20 nM, 58 nM, 6.5 nM, and 0.4 nM, (Table 2), respectively. These values correlated with their respective enzymatic potency (Table 1). The cellular selectivity of PI3K $\delta$  inhibitors over other PI3K isoforms was determined by measuring the inhibition of AKT phosphorylation in cell-based assays selective for PI3K $\alpha$  (heregulin-stimulated SK-BR-3), PI3K $\beta$  (PTEN-null MDA-MB-468 cells) or PI3K $\gamma$  (C5a-stimulated RAW 264.7 cells). Consequently, MSD-496486311, MSD-126796721, Idelalisib and Duvelisib are effective and selective PI3K $\delta$  inhibitors in both enzymatic and cellular contexts and can serve as pharmacological tools to establish a PK/PD relationship in preclinical respiratory models of TH2 and TH1 inflammation.

## **PI3K $\delta$ Inhibitors Attenuate B cell and Basophil Derived Pharmacodynamic Biomarkers in Both Rat and Human Whole Blood.**

The importance of monitoring relevant biomarkers to improve the efficiency of drug development is increasingly recognized. We identified and validated two PD biomarkers (CD63 in rat whole blood and anti-CD79b induced pAKT modulation in human whole blood) in this study. MSD-496486311, MSD-126796721, Idelalisib and Duvelisib inhibited anti-CD79b induced pAKT in human whole blood with IC<sub>50</sub> values of 42 nM, 1,534 nM, 90 nM, and 112 nM respectively (Table 3), which are consistent with potencies observed in the Ramos cell-based assay. To establish a relationship between plasma exposure in preclinical models and putative translation to humans, PI3K $\delta$  inhibitors were assessed for their target coverage by evaluating basophil degranulation CD63 biomarker in rat. This very same approach was used in the clinical study by Horak et al., (2016). Anti-IgE was used to induce basophil degranulation. We then measured surface CD63 up-regulation on basophils. MSD-496486311, MSD-126796721, Idelalisib and Duvelisib blocked completely in a concentration-dependent manner with IC<sub>50</sub> values (*ex vivo*) of 300 nM, 1,200 nM, 490 nM, and 96 nM, respectively (Table 3). The *in vitro* IC<sub>50</sub> values are also shown in Table 3.

### **PK/PD Modeling**

A direct relationship was observed when plasma concentrations were correlated to the PD effect. These time-course and dose-response data and the estimated PK/PD parameters led to the establishment of an Emax mathematical model linking plasma concentrations to CD63 basophil degranulation inhibition over time, in which a correlation factor was calculated between predicted versus experimentally measured CD63 inhibition. Thus, a quantitative relationship between dosing regimen, exposure, and PD effects of PI3K $\delta$  was identified based on the correlation of *ex vivo* PD effects potency, which allowed for the extrapolation of a fitting mathematical PK/PD model.

### **Respiratory Rat Models of TH2 and TH1 Inflammation**

In sensitized rats, ovalbumin exposure on day 14 produced a consistent increase in the number of BAL eosinophils ranging from  $124 \pm 15$  to  $160 \pm 21 \times 10^3$  cells/ml. All

PI3K $\delta$  inhibitors tested inhibited allergic induced eosinophilic inflammation (Figure 1). The maximum achievable efficacy of the PI3K $\delta$  inhibitors was similar to the positive comparator dexamethasone (3 mg/kg). Efficacy and simulated PK results from the MSD-126796721 split dosing study are displayed in Figure 2. The 3 mg/kg QD and the 100 mg/kg QD dose levels produced 16% and 93% inhibition of BAL eosinophils respectively, while the 20 mg/kg QD, 10 mg/kg BID and the 3 mg/kg TID doses produced equivalent effects ranging from 54 to 66 % BAL inflammation (Figure 2 and Table 4). Figure 2 also displays the effect of PI3K $\delta$  inhibition on TH2 BAL cytokine secretion, which mimicked these drugs actions on BAL inflammation. The data shown in Table 4 supports that the PK target is consistent with time on target (ToT) rather than strictly C<sub>max</sub>, or C<sub>min</sub> driven. Modeling data relating time above the *in vitro* CD63 IC<sub>50</sub> assay and *in vivo* efficacy of the PI3K inhibitors, support the ToT as being an important driver of efficacy in the Ovalbumin Sensitized and Challenged Model (Figure 3).

MSD-126796721(10 – 100 mg/kg) produced a dose dependent attenuation of the increases in eosinophils produced by intratracheal instillation of *A. alternata* (Figure 4). In the allergic rat models, our aim was to determine the impact of PI3K $\delta$  inhibition on TH2 inflammation. Thus, we focused our attention on eosinophils as a primary endpoint. However, it is noteworthy to point out that PI3K $\delta$  efficacy was observed on total cell counts and other BAL immune cell types (data not shown). Similarly, MSD-126796721(10 – 100 mg/kg) produced dose related inhibition of an eosinophilic pulmonary response in the BAL of Brown Norway rats sensitized and challenged with Ovalbumin. While the degree of eosinophilic inflammation was greater in the *A. alternata* phenotype compared to the Ovalbumin model, the normalized (percent inhibition) BAL cellular response produced by PI3K $\delta$  inhibition was comparable across the two studies (Figures 2 and 4). Additionally, the terminal plasma exposure of MSD-126796721(10, 30 and 100 mg/kg) was equivalent across the *A. alternata* (0.052, 0.155 and 0.428  $\mu$ M) and Ovalbumin (0.066, 0.259 and 0.600  $\mu$ M) models.

Similar to studies conducted in the Ovalbumin Sensitized and Challenged Model, we performed a split dosing experiment in the rat ozone model. Vehicle control animals challenged with ozone displayed a significant increase in the number of neutrophils and total cells in the BAL (Figure 5). MSD-496486311(10, 30, 100 and 300 mg/kg, QD, PO)

elicited a dose related attenuation in the ozone pulmonary inflammatory response. The split doses (i.e. 15 mg/kg BID and 50 mg/kg BID) produced comparable effects on BAL neutrophils and total cells. The PK profile of MSD-496486311 shown in Figure 5 and Table 5. Results in Table 5 indicate that the efficacy in this model is not driven by C<sub>max</sub> or AUC but is more likely driven by ToT above the *in vitro* CD63 IC<sub>50</sub> value.

### Ascaris-Sensitive Sheep Model

We evaluated the ability of clinical benchmark PI3K $\delta$  inhibitor Idelalisib to attenuate airway resistance induced by aerosolized ascaris to the lungs. Idelalisib was dosed by intravenous infusion and achieved a 5  $\mu$ M steady state for study duration (i.e. 9 hrs). This level of exposure represented an approximate 2.5 fold over the *in vitro* sheep washed whole blood basophil histamine IC<sub>50</sub> value of 0.638  $\mu$ M (2.0  $\mu$ M adjusted for sheep PPB). At the single dose tested, Idelalisib significantly attenuated EAR and LAR by 40  $\pm$  5 and 78  $\pm$  8 percent respectively (Figure 6). The airway hyper-responsiveness was fully blocked by Idelalisib.

### Discussion

Respiratory diseases such as asthma and COPD continue to be associated with substantial morbidity and mortality with a significant portion of patients being poorly controlled by current standard of care (Grainge et al., 2016; Han et al., 2016; Powell, 2016). Novel treatments, particularly for COPD, have been difficult to advance to the market place (Barnes et al., 2013), in part because promising pharmacological results from animal studies have not always translated into effective human efficacy (Wendler and Wehling, 2010). A better understanding of target engagement in historical models, and whenever possible linking those results to PK/PD observations in the clinic, may help offset drug pipeline failures (Danhof et al., 2008). In the current study, we leveraged published clinical data from Horak et al., (2016) which indicated Idelalisib (a selective PI3K $\delta$  inhibitor) provided meaningful benefit to allergic rhinitis patients in a seven-day grass pollen Vienna Challenge Chamber crossover study. In addition to efficacy endpoints, the study employed an *ex vivo* whole blood basophil activation assay as a surrogate PI3K $\delta$  target engagement PD marker. An Idelalisib dose level of 100 mg/kg

BID produced a plasma exposure concentration of 792.7 ng/ml (3 hrs after the first dose) which corresponded to a 59% (value minus control responses) suppression of *ex vivo* basophil activation. Consequently, we sought to understand the relationship between attenuation of whole blood basophil activation, plasma exposure and respiratory anti-inflammatory effects of PI3K $\delta$  inhibitors in preclinical pulmonary animal models.

Evaluation of PI3K $\delta$  inhibitors in human whole blood enables human dose projections. The panel of PI3K $\delta$  inhibitors was tested systematically across individual human donors for their ability to inhibit basophil activation in response to anti-IgE. In addition, PI3K $\delta$  inhibitors were found to display similar potencies in an alternative human whole blood assay measuring signaling via measurement of pAKT. Collectively, application of these PD biomarkers across species not only validated their biological relevance, but allowed us to determine the exposure-effect relationship and the correlation between PI3K $\delta$  inhibition and efficacy in preclinical respiratory models of TH2 and TH1 inflammation.

In the current *in vivo* experiments we both recapitulate and expand upon literature reports indicating that PI3K $\delta$  inhibitors can effectively attenuate both TH2 and TH1 evoked inflammatory responses in the lungs (Chen et al 2004; Doukas et al., 2008; Finan and Thomas, 2004; Takeda et al., 2010). Our rodent studies were conducted in the Brown Norway rat, as this strain is genetically TH2-predisposed and produces robust and consistent pulmonary inflammatory responses after allergic sensitization and challenge (Hylkema et al., 2002). Moreover, TH1 respiratory inflammatory responses can also be evoked in Brown Norway rats by various stimuli, such as ozone, LPS and cigarette smoke (Tsukagoshi et al., 1995; Hisada et al, 1999; Bassett et al., 2005). Using the same strain eliminates confounding interpretation of our results as we associate efficacy to PK and to the *in vitro* basophil activation levels. Based on the clinical observations by Horak et al., (2016) we speculated that maintaining PI3K $\delta$  target engagement or “time on target” (ToT) at our basophil CD63 IC<sub>50</sub> value would elicit significant efficacy in the models tested. To evaluate the effectiveness of a ToT above a threshold concentration set by the CD63 IC<sub>50</sub>, we conducted a split dosing study in the Brown Norway (BN) rat ovalbumin lung allergen challenge model with MSD-126796721 at 20 mg/kg QD, 10 mg/kg BID, 3 mg/kg TID, which modulated C<sub>max</sub> and



AUC across these treatment groups. This study allowed investigations into the exposure-response relationship and the concentration-time profiles were used to compute AUC, extract other PK parameters ( $C_{max}$ ,  $T_{oT}$ ,  $C_{min}$ ), and assess their relationships with percent inhibition of eosinophils. An Emax-model, as illustrated in Figure 4A, provides a good representation of the AUC-PD relationship with multiple PI3K $\delta$  inhibitors. The use of Emax-models is limited to cases where the dose range covers the entire spectrum of the response; otherwise they are unreliable to make predictions outside the linear portion of the curve. Indeed, our initial exploration of the PK-PD relationship showed that AUC provides reasonable exposure response projections through an Emax-model. Evaluation of the PD response against other PK parameters revealed that  $T_{oT}$  provides the most robust estimate. It provides a simple linear relationship that correlates well (see Figure 4B) with the response and does not have the stringent dose range requirements imposed by the Emax-model. Recognizing that AUC and  $T_{oT}$  are not completely independent parameters, our data strongly supports previous findings regarding the use of  $T_{oT}$  to make reasonable dose-response projections. The impact of PI3K $\delta$  inhibition on BAL TH2 cytokines such as IL-5 and IL-13 paralleled the effects on BAL cells in the Brown Norway rat Ovalbumin model. Interesting, at the equal doses, PI3K $\delta$  inhibition (MSD-126796721) in the *A. alternata* model (on a Brown Norway strain) displayed a qualitatively equivalent anti-inflammatory action to those in the Ovalbumin model suggesting a similar PK/PD relationship. Additional support for the  $T_{oT}$  hypothesis is suggested in a second species by a small allergic sheep study, where intravenous infusion of Compound C, at 2.5 fold above an *in vitro* whole blood basophil histamine  $IC_{50}$  value attenuated early and late airway hyper-responsiveness (EAR, LAR). Nonetheless, additional sheep experiments would be needed to fully support this position. Taken together, the data in our allergic models are in alignment with the allergic rhinitis PI3K $\delta$  inhibitor proof of biology study by Horak et al., (2016) in regard to the degree of target engagement needed to produce respiratory anti-inflammatory responses, which is not surprising, given that allergic rhinitis and allergic lung allergen challenge preclinical models share a common pathophysiology, (i.e. Th2-type inflammation).

Th2 cytokines, including IL-4, IL-5, IL-9 and IL-13, have long been recognized as having a key function in the pathogenesis of atopic asthma and IgE-mediated

eosinophilic inflammation of the lungs (Romagnani et al., 2002; Fischer et al., 2007). Moreover, Th1 pathway mediators including IL-1b, IL-8 and IL-17 and lung neutrophilia are also believed to play a key role in certain severe asthmatic populations (Wenzel et al., 1999; Douwes et al., 2002) as well contributor to the underlying inflammation in COPD (Caramori et al., 2014; Barnes, 2015;). In the current experiments we used whole body ozone exposure to increase total cell and neutrophil influx into the BAF of Brown Norway rats. We conducted a split dose experiment similar to the experimental designed mentioned above for the Ovalbumin model and observed that 24h coverage of MSD-496486311(duration to the ozone experiment) at the basophil IC<sub>50</sub> value resulted in maximum BAL anti-inflammatory efficacy between 69 and 84% (Table 4). On the other hand, the relationships between AUC, Cmax and efficacy were not as clear in the ozone experiment.

Combination treatments such as Trelegy Ellipta®, Combivent®, Advair®, Symbicort®, Dulera® are becoming more common place for the treatment of respiratory diseases (Calzetta et al.,2019). For example, administration of triple therapy by adding a LAMA to ICS/LABA combination provides significant clinical benefit to asthmatic and COPD patients over ICS/LABA alone. While combination treatments offer the possibility of improving a desired efficacy it must be pointed out that the emergence of greater unwanted side effects may become more evident. An good understanding of PK/PD relationships may offset over dosing and minimize the liability of combination treatments. While we did not currently study the impact of co-administration of PI3K  $\delta$ -inhibitors with other respiratory drugs we believe that target engagement experiments (such as presented here) can be the basis of future explorations. From our point of view, this can be expanded to potential novel respiratory targets such as NOX4, and Nrf2 (Zhao et al., 2015; Zhou et al., 2016; Cui et al., 2018).

In summary, we studied the anti-inflammatory actions of four PI3K $\delta$  inhibitors in animal models of Th1 and Th2 inflammation. We conclude that the PK/PD observations in our animal models appear to align with clinical results associated with a Th2 airway disease, allergic rhinitis.

## Authorship Contributions

**Participated in research design:** McLeod, Shah, Cabal, Cicmil, Gervais, Bass, Alves, Houshyar

**Conducted experiments:** Gil, Chen, Woodhouse, Dorosh, Geda, Mehta, Baltus, Caniga Yu,

**Contributed new reagents or analytic tools:** Katz, Methot

**Performed data analysis:** Yu, Shah, McLeod, Shah, Gil, Chen, Cabal

**Wrote or contributed to the writing of the manuscript:** McLeod, Shah, Cabal, Caniga

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Table 1: Biochemical potency of compounds across human class I PI3K isoforms

<i>Isoform</i>	Intrinsic potency IC <sub>50</sub> (nM)			
	<i>MSD-496486311</i>	<i>MSD-126796721</i>	<i>Idelalisib</i>	<i>Duvelisib</i>
PI3K $\delta$	1.3	5.7	2.2	0.17
PI3K $\alpha$	190	525	1,134	96
PI3K $\beta$	1,650	385	409	6
PI3K $\gamma$	4,800	3,755	208	1.8

Table 2: Cell-based potency of compounds across human class I PI3K isoforms

Cell-based potency IC <sub>50</sub> (nM)				
<i>Isoform</i>	<i>MSD-496486311</i>	<i>MSD-126796721</i>	<i>Idelalisib</i>	<i>Duvelisib</i>
PI3K $\delta^a$	20	58	6.5	0.4
PI3K $\alpha^b$	>12,500	3,906	4755	3891
PI3K $\beta^c$	>12,500	4,413	327	27
PI3K $\gamma^d$	7,018	4,139	487	17

<sup>a</sup>Ser473-pAKT phosphorylation in anti-IgM-stimulated Ramos lymphoma-derived B cells.

<sup>b</sup>Ser473-pAKT phosphorylation in heregulin-stimulated SK-BR-3 cells.

<sup>c</sup>Ser473-pAKT phosphorylation in PTEN-null MDA-MB-468 cells.

<sup>d</sup>Ser473-pAKT phosphorylation in C5a-stimulated RAW 264.7 cells.



Table 3: Potency of compounds in human or rat whole blood.

Whole blood potency IC <sub>50</sub> (nM)				
<i>Biomarker</i>	<i>MSD-496486311</i>	<i>MSD-126796721</i>	<i>Idelalisib</i>	<i>Duvelisib</i>
Human pAKT ( <i>in vitro</i> )	42	1,534	90	112
Rat CD63 ( <i>in vitro</i> )	1,200	4,800	800	500
Rat CD63 ( <i>ex vivo</i> )	300	1,200	490	96

Table 4: MSD-126796721 split dosing experiment in the Brown Norway (BN) rat OVA allergic lung inflammation model. The data are indicative of a “time on target” PK target.

<b>Dose (mg/kg)</b>	<b>C<sub>max</sub> (μM)</b>	<b>AUC<sub>0-48h</sub> (μM•h)</b>	<b>C<sub>trough</sub> (μM)</b>	<b>ToT (&gt;rat CD63 IC<sub>50</sub> 48hr)</b>	<b>BAL Eosinophils (% inhibition)</b>	<b>BAL Neutrophils (% inhibition)</b>	<b>BAL Totals (% inhibition)</b>
20 (QD)	38	230	0.18	21 h	66	57	77
10 (BID)	20	230	0.34	32 h	67	72	84
3 (TID)	6	103	0.4	26 h	54	59	59
3 (QD)	6	34	0.03	8 h	16	17	17
100 (QD)	192	115	0.9	42 h	93	96	>100

C<sub>max</sub> and AUC values from BID and TID studies were predicted from the simulation using Phoenix WinNonlin software. C<sub>max</sub> and AUC values from 3, 20, and 100 mg/kg QD studies were measured from a separate PK study.

Table 5: MSD-496486311 split dosing experiment in the Ozone inflammation model. The data are indicative of a “time on target” PK target.

<b>Dose (mg/kg)</b>	<b>ToT (0.3 <math>\mu</math>M IC<sub>50</sub>)</b>	<b>AUC (<math>\mu</math>M•h)</b>	<b>Cmax (<math>\mu</math>M)</b>	<b>Efficacy neutrophils (% inhibition)</b>
10 (QD)	18 hr	37	13	12
30 (QD)	18 hr	106	34	38
15 (BID)	24 hr	114	21	69
100 (QD)	24 hr	353	113	70
50 (BID)	24 hr	372	56	73
300 (QD)	24 hr	1270	158	84

Cmax and AUC values from BID and TID studies were predicted from the simulation using Phoenix WinNonlin software. Cmax and AUC values from 30 mg/kg QD studies were measured from a separate PK study.

## Legends for Figures

Figure 1: PI3K $\delta$  inhibition attenuates allergic-mediated pulmonary inflammation in the Ovalbumin sensitized and challenged Brown Norway rat. Displayed are the actions of MSD-496486311(Panel A), MSD-126796721(Panel B), Idelalisib (Panel C) and Duvelisib (Panel D) on eosinophils 48 hr post antigen challenge. Each bar represents the MEAN  $\pm$  SEM (n=8 per treatment group). \*p<0.05 compared to vehicle control; \*\*p<0.05 vs. OVA control group. As a comparator, the effects of dexamethasone on antigen mediated inflammation is shown in Panel E.

Figure 2: Efficacy of PI3K $\delta$  inhibition in a “split dosing” paradigm. Panel A displays the simulated plasma exposures of MSD-126796721 after different dosing paradigms based on a two-compartment model relative to the drug’s in vitro IC<sub>50</sub> basophil CD63 value. Shown in Panels B and C are the inhibitory actions of MSD-126796721(3 mg/kg QD, 20 mg/kg QD, 10 mg/kg QD and 3 mg/kg TID) on inflammatory TH2 BAL cytokines. Each bar represents the MEAN  $\pm$  SEM (n=8 per treatment group). \*p<0.05 compared to vehicle control; \*\*p<0.05 vs. OVA control group. As a positive comparator the effects of dexamethasone (3 mg/kg QD) is also displayed in the figure.

Figure 3: Correlation graph: Efficacy of pulmonary eosinophil influx to AUC (Panel A) and efficacy of pulmonary eosinophil to time on target (ToT; Panel B).

Figure 4: Comparative anti-inflammatory effect PI3K $\delta$  inhibition in the Ovalbumin sensitized–challenged and A. alternate Brown Norway rat models. The dose response inhibitory actions of MSD-126796721 on allergic-mediated pulmonary eosinophilic responses 48 hr post antigen challenge in the Ovalbumin sensitized–challenged (Panel A) and the A. alternate (Panel B) models are depicted. Each bar represents the MEAN  $\pm$  SEM (n=8 per treatment group). \*p<0.05 compared to vehicle control; \*\*p<0.05 vs. OVA control or A. alternate group. Values in parentheses are mean percent change values from the challenged response in the control group.

Figure 5: Efficacy of PI3K $\delta$  inhibition in a “split dosing” paradigm. Panel A displays the simulated plasma exposures of MSD-496486311 after different dosing paradigms based on a two-compartment model relative to the drug’s *in vitro* IC<sub>50</sub> basophil CD63 value. Shown in Panels B and C are the inhibitory actions of MSD-496486311 (10 mg/kg QD, 30 mg/kg QD, 15 mg/kg BID, 100 mg/kg QD, 50 mg/kg BID and 300 mg/kg QD) on TH1 driven BAL inflammation. Each bar represents the MEAN  $\pm$  SEM (n=8 per treatment group). \*p<0.05 compared to vehicle control; \*\*p<0.05 vs. OVA control group.

Figure 6: Idelalisib (steady state intravenous administration @ 5  $\mu$ M) improved lung function in the allergic sheep model. The upper insert demonstrates that Idelalisib reversed carbachol airway hyper-responsiveness. The lower insert depicts the PK profile relative to *in vitro* wash blood basophil IC<sub>50</sub>. Each point/bar represents the MEAN  $\pm$  SEM (n=3 sheep per treatment group). A statistically significant (p<0.05) effect on EAR, LAR and AHR compared to vehicle control was observed.

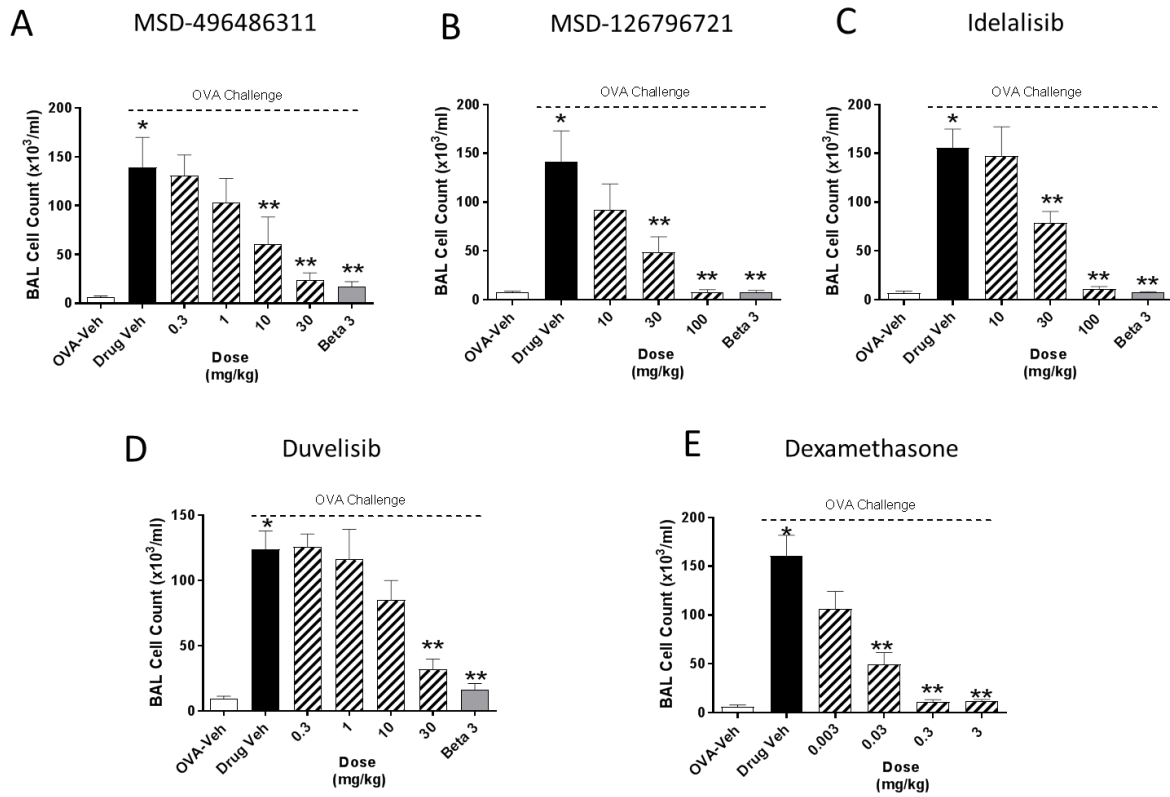
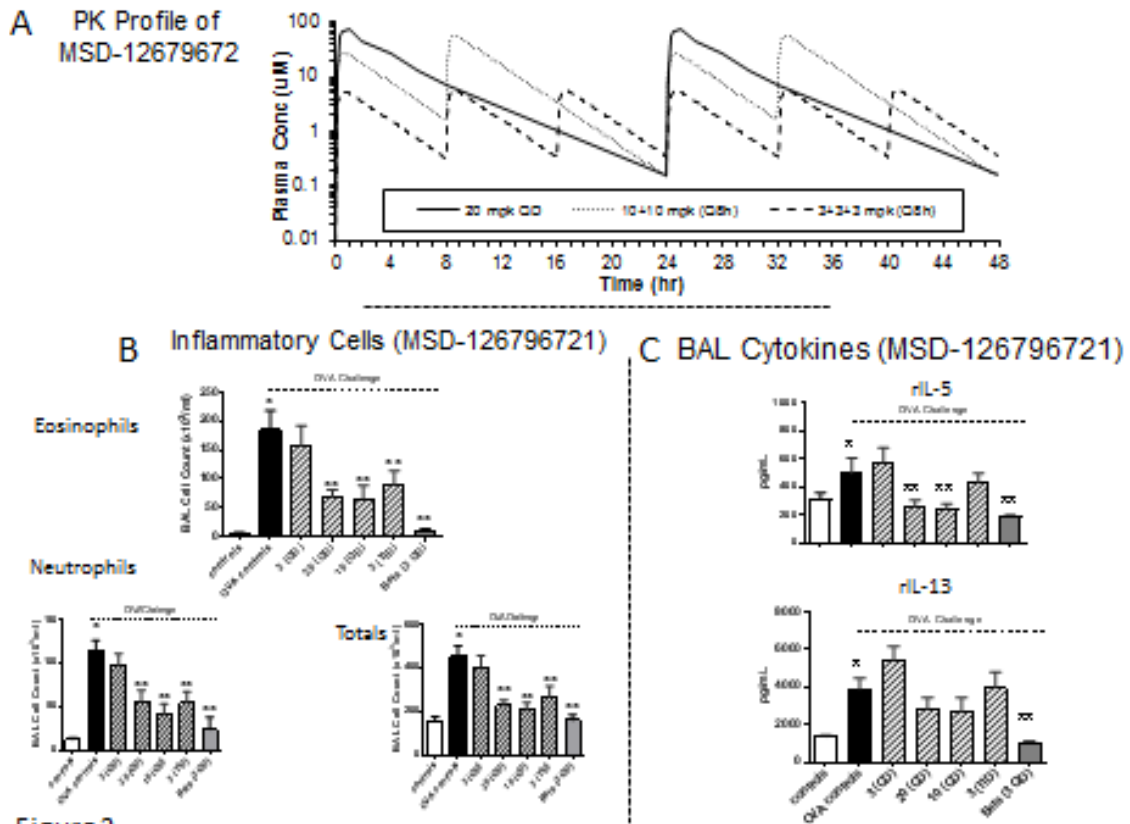


Figure 1



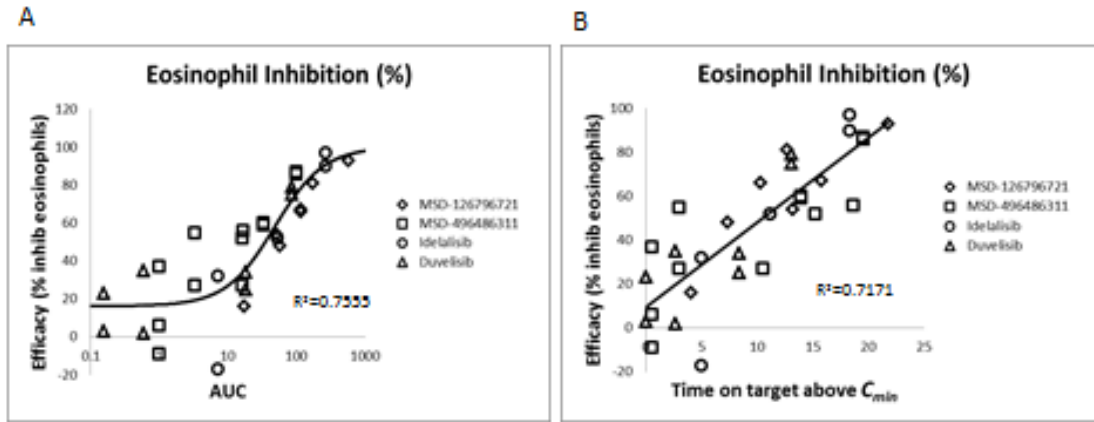


Figure3



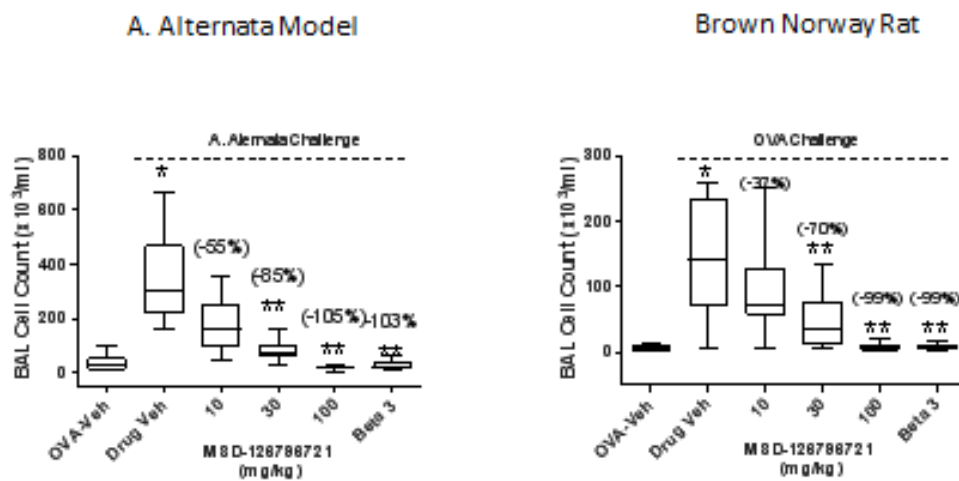
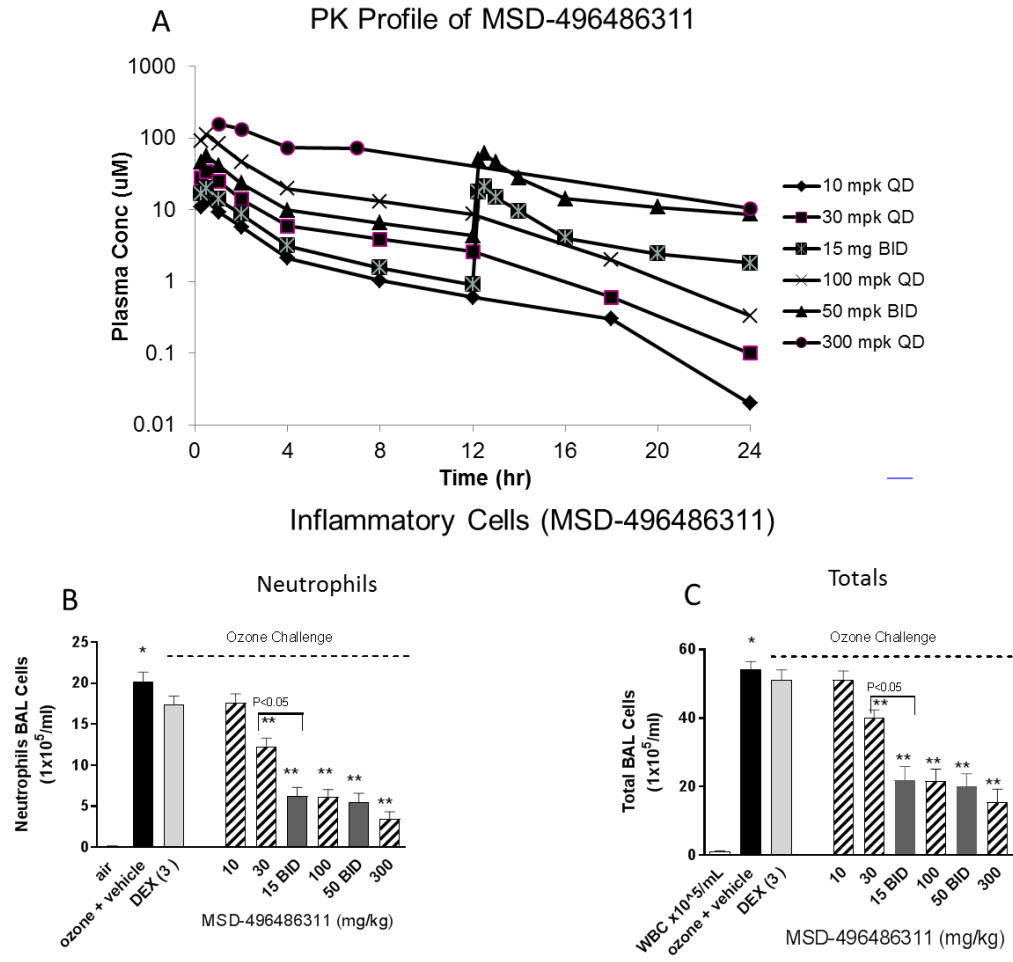


Figure 4



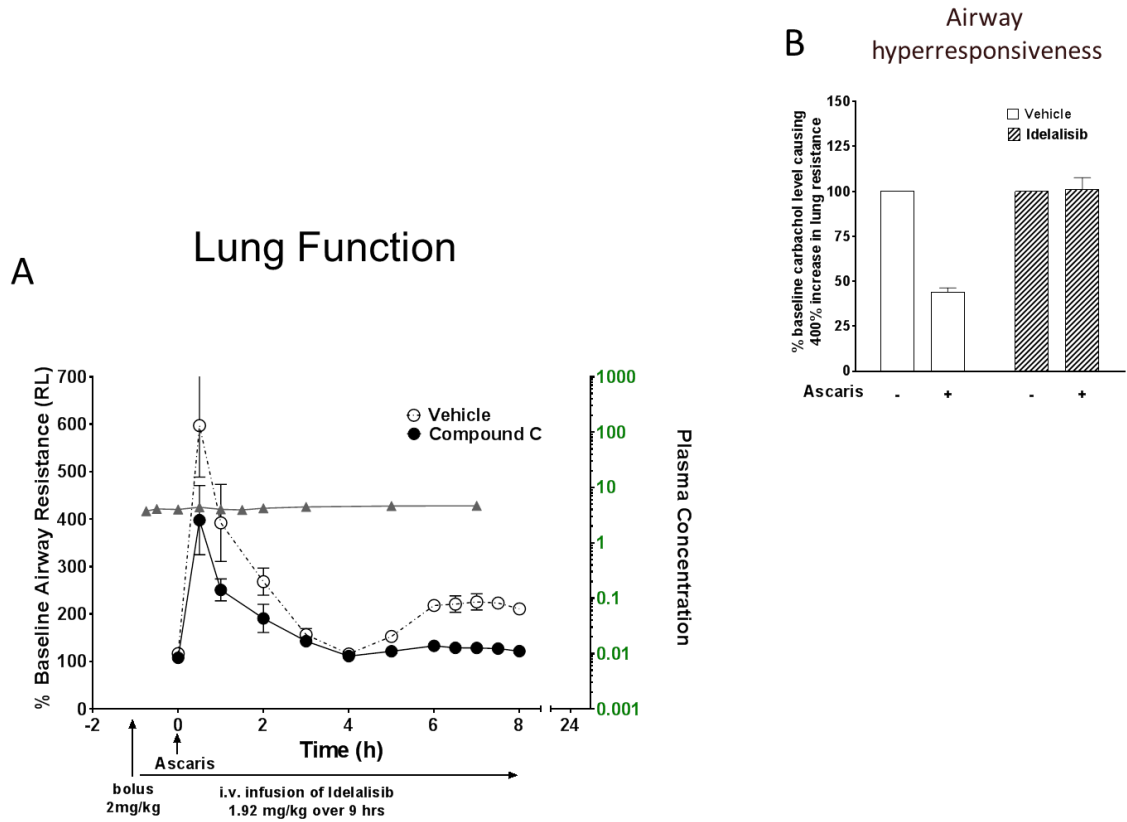


Figure 6