

# Effect of Phosphatidylinositol-3-Kinase Gamma (PI3K $\gamma$ ) Inhibitor CAY10505 in Nicotine and Monocrotaline induced Pulmonary Vascular Endothelium Dysfunction

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## Abstract

This study has been designed to investigate the role of phosphatidylinositol-3-kinase gamma (PI3K $\gamma$ ) in Mono Cro Taline (MCT)- and nicotine-induced endothelial dysfunction in pulmonary arteries. MCT (60 mg/kg, s.c. once in 4 weeks) and nicotine (2 mg/kg, i.p. regularly) were administered in respective groups to produce pulmonary vascular endothelium dysfunction. Vascular endothelial dysfunction was assessed in terms of attenuation of acetylcholine-induced endothelium-dependent relaxation, sodium nitroprusside-induced endothelium-independent vasorelaxation (isolated pulmonary artery ring preparation), decrease in serum nitrate/nitrite level, mRNA expression of eNOS (rtPCR) and increased oxidative stress (superoxide anions and TBARS level), mean arterial blood pressure and right ventricular hypertrophy. PI3K $\gamma$  inhibitor CAY10505 (0.6 mg/kg, p.o) [(5-[5-4-fluorophenyl]-2-furanyl]-2, 4-thiazolidinedione) and atorvastatin (30 mg/kg, p.o, positive control) treatment for 7 days (21<sup>st</sup>-28<sup>th</sup> day) significantly improved, mean arterial blood pressure and right ventricular hypertrophy in both nicotine and Mono Cro Taline-induced pulmonary vascular endothelial dysfunction. Therefore, it may be concluded that CAY10505, a specific inhibitor of PI3K $\gamma$  up regulates eNOS expression and nitric oxide production to improve Mono Cro Taline and nicotine-induced pulmonary vascular endothelial dysfunction.

## Introduction

The pulmonary vascular endothelium is a quiescent genetically stable cell line. It maintains vascular tone, homeostasis, leukocyte trafficking, transduction of luminal signals to abluminal vascular tissues, production of growth factors, cellular signaling and barrier functions [1]. Pulmonary vascular endothelial dysfunction is a pathological condition characterized by an imbalance of vasodilators and vasoconstrictors, coagulants and anticoagulants, prothrombotic and antithrombotic factors. It is a prognosis and initiating marker of Pulmonary Hypertension (PH) [2,3]. Cigarette smoking and Nicotine affects cardiovascular function and could contribute to cardiovascular diseases. Nicotine is also associated with the vascular endothelial dysfunction that leads to secondary complications of various organs [4].

Monocrotaline is an 11-membered macrocyclic pyrrolizidine alkaloid. Monocrotaline has several advantages as a model of pulmonary vascular endothelium dysfunction and hypertension [5,6]. A single subcutaneous administration of MCT leads to progressive pulmonary vascular endothelium dysfunction and hypertension with striking vascular lesions without apparent cytotoxicity or destruction of alveolar capillaries [7,8]. Lesions resulting from MCT administration include medial hypertrophy and extracellular matrix secretions in pulmonary arteries. MCT is converted into reactive metabolite in the liver and is transported to red blood cells where it causes endothelial injury and is further specifically reported to cause pulmonary vascular endothelium dysfunction [9,10]. One of the many causes of pulmonary hypertension is the lack of production or response to nitric oxide. Both monocrotaline and nicotine are reported to induce pulmonary vascular endothelium dysfunction by attenuation of eNOS; however, the exact mechanism is not elucidated.

Phosphoinositide-3-kinase (PI3K) is a ubiquitously expressed enzyme family that regulates cell functions, aging and malignant transformation. Members of the PI3K family are grouped into 3 classes (classes I, II, and III). The member of the class Ib PI3Ks i.e. PI3K $\gamma$  after binding to Rac has been reported to have a crucial role in the triggering of Reactive Oxygen Species (ROS) production [11]. Oxidative stress and inflammation are the main characteristic features of nicotine and monocrotaline-induced pulmonary endothelial dysfunction [12]. Furthermore, PI3K $\gamma$  is also involved in Rac induced hypertension and inflammatory response [11]. Rac attenuates eNOS expression and decreases nitric oxide that causes vasoconstriction [13]. Pharmacological inhibition

of PI3K $\gamma$  has been reported to increase nitric oxide level and consequently improve DOCA salt-induced endothelial dysfunction in aortas [14]. Furthermore, PI3K $\gamma$  is a key mediator in Angiotensin-II-induced L-type calcium channel activation and calcium influx. Inhibition of PI3K $\gamma$  has also been explored as a potential target in inflammation [15]. Thus the present study is designed to investigate the effect of PI3K $\gamma$  specific inhibitor CAY10505 in monocrotaline and nicotine-induced pulmonary vascular endothelium dysfunction.

## Materials and Methods

### Experimental Animals

The study was carried in male Wistar rats weighing 180-240 g. All the experiments were conducted in approval with Institutional Animal Ethics Committee (IAEC) and were carried out in accordance with the guidelines of CPCSEA for the care and use for laboratory animals. The animals were housed at 12 hour light and 12-hour dark cycle.

### Drugs and Chemicals

CAY10505 was procured from Cayman Chemical Company, Ann Arbor, Michigan, USA and Monocrotaline was procured from sigma Aldrich Ltd, St Louis, MO, USA. Atorvastatin was obtained from Ind-swift laboratories, Dera Bassi, India. All other chemicals and reagents used were of analytical grade.

## Experimental Protocol (Each group n=6)

### Group I Normal Control

#### Group II

(Monocrotaline control): MCT (60 mg/kg; s.c) was administered by a single injection at the 1<sup>st</sup> day of 4 weeks period.

#### Group III

(MCT + CAY10505 [(5-[5-(4-fluorophenyl)-2-furanyl]-2,4-thiazolidinedione]): MCT (60 mg/kg; s.c) administered rats were treated with CAY10505 (0.6 mg/kg; p.o) from 21<sup>st</sup> to 28<sup>th</sup> day.

#### Group IV

(MCT + Atorvastatin): MCT (60 mg/kg; s.c) administered rats were treated with Atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> to 28<sup>th</sup> day.

#### Group V

(Nicotine Control): Nicotine (2 mg/kg; i.p) was administered daily over the 4 week treatment period.

#### Group VI

(Nicotine + CAY10505): Nicotine (2 mg/Kg; i.p) administered rats were treated with CAY10505 (0.6 mg/kg; p.o) from 21<sup>st</sup> to 28<sup>th</sup> day.

#### Group VII

(Nicotine + Atorvastatin): Nicotine (2 mg/kg; i.p) administered rats were treated with Atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> to 28<sup>th</sup> day.

## Wire Myograph Assessment of Pulmonary Artery Vascular Endothelial Dysfunction

The rats were sacrificed by cervical dislocation, followed by decapitation. Pulmonary artery was removed, cut into a ring of 4-5 mm in length and mounted on the Myograph (Radnoti, US) containing the krebs-Henseleit solution and maintained at 37°C. The preparation was allowed to equilibrate for 90 min under 1.5 g tension. The isometric contractions were recorded on the computer with the help of data acquisition system (PowerLab 4/30 Ad Instrument). The pulmonary artery ring preparation was primed with 80mM KCl to check its functional integrity and to improve its contractility. Endothelium-dependent relaxations to graded doses of acetylcholine (10<sup>8</sup> to 10<sup>4</sup> M) were recorded in phenylephrine (3×10<sup>6</sup> M) precontracted isolated pulmonary artery ring preparation. Similarly, endothelium independent relaxation to sodium nitropruside (10<sup>6</sup> to 10<sup>4</sup> M) in phenylephrine (3×10<sup>6</sup> M) precontracted isolated aortic ring preparation was recorded [16].

### Estimation of Serum Nitrite/Nitrate Concentration

A total of 400  $\mu$ l of carbonate buffer (pH 9.0) was added to 100  $\mu$ l of serum sample followed by the addition of small amount (0.15 g) of copper-cadmium alloy. The tubes were incubated at room temperature for 1 h to reduce nitrate to nitrite. The reaction was stopped by adding 100  $\mu$ l of 0.35 M sodium hydroxide. Following this, 400  $\mu$ l of zinc sulfate solution (120 mM) was added to deproteinate the serum samples. The samples were allowed to stand for 10 min and then centrifuged at 4,000g for 10 min. Griess reagent (250  $\mu$ l of 1.0% sulfanilamide and 250  $\mu$ l of 0.1%N-naphthylethylenediamine) was added to aliquots (500  $\mu$ l) of clear supernatant and serum nitrite/nitrate was measured spectrophotometrically at 545 nm. The standard curve of sodium nitrite (1-40 $\mu$ M) was plotted to calculate concentration of serum nitrite [17].

### Assessment of Oxidative Stress

The oxidative stress was assessed by estimating serum Thiobarbituric Acid Reactive Substances (TBARS) and pulmonary artery superoxide anion generation.

### Estimation of TBARS

A total of 1 ml of 20% trichloroacetic acid was added to 100  $\mu$ l of serum and 1% Thio Barbituric Acid (TBA) reagent (1.0 ml), which were mixed and incubated at 100°C for 30 min. After cooling on ice, samples were centrifuged at 1,000g for 20 min. Serum concentration of TBARS was measured spectrophotometrically at 532 nm. A standard curve using 1, 1, 3, 3-tetramethoxypropane (1-10  $\mu$ M) was plotted to calculate the concentration of TBARS [17].

### Estimation of Superoxide Anion

Pulmonary artery was cut into transverse rings of 6 mm in length and placed in 5 ml of Krebs-Henseleit solution containing 100 $\mu$ M of Nitro Blue Tetrazolium (NBT) and incubated at 37°C for 1.5 h. The NBT reduction was stopped by adding 5 ml of 0.5 N HCl. The rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% Sodium Dodecoyl Sulphate (SDS) in water containing 40 mg/l of Diethylene Triamine Pentaacetic Acid (DTPA). The mixture was centrifuged at 20,000g for 20 min and the resultant pellets were

re suspended in 1.5 ml of pyridine and kept at 80°C for 1.5 h to extract formazan. The mixture was centrifuged at 10,000g for 10 min and the absorbance of formazan was determined spectrophotometrically at 540 nm. The amount of reduced NBT was calculated using the following formula:

$$\text{Amount of reduced NBT} = (A \times V) / (T \times Wt \times e \times l)$$

Where A is absorbance, V is volume of solution (1.5 ml), T is time for which pulmonary artery rings were incubated with NBT (90 min), Wt is blotted wet weight of pulmonary artery rings, e is extinction coefficient (0.72 l mM<sup>-1</sup>mm<sup>-1</sup>), and l is length of light path (10 mm). The result was expressed as reduced NBT in picomoles per min per mg of wet tissue [17].

### Measurement of Right Ventricular Hypertrophy

The heart was dissected, and the ratio of the right ventricular free wall weight divided by the length of the tibia was calculated as an index of right ventricular hypertrophy which is unaffected by changes in body weight or left ventricular mass [18].

The ratio was calculated by using formula

$$\text{Ratio} = (\text{Right ventricular}) / (\text{Left ventricular Septum})$$

### Mean arterial Blood Pressure

The rat blood pressure was measured by tail cuff method (NIBP-BIOPAC MP100, USA). Rat tail was heated with heater with exact time of 3 minutes after that tail was inserted into the cuff, pressure was applied and mean arterial blood pressure is recorded on BIOPAC [16].

### Estimation of mRNA level of eNOS

The expression of mRNA for endothelial Nitric Oxide Synthase (eNOS) was assessed by using reverse transcriptase polymerase chain reaction. Isolation of RNA was done with XNAPS RNA Flex spin Kit (Renege) and for the formation of tracer Kit (Renege) was used. Twenty-four PCR cycles of GAPDH and thirty PCR cycles of eNOS were performed by using one half of the reverse transcription mixture. The sense and antisense primers of eNOS and primers of GAPDH were used.

Primers for Enos: (5'-TCCAGAAACACAGACAGTGCA-3' and 5'-CAGGAAGTAAGTGAGAGC-3' respectively) and for GAPDH (5'-TCCCTCAAGATTGTGACGAA-3' and 5'-AGATCCACAACGGATACATT-3' respectively) were used.

The PCR products so obtained were analyzed on ethidium bromide stained agarose gel on Gel Electrophoresis apparatus (Biorad). The eNOS and GAPDH products were quantified using image (Gel Doc EZ image, Bio-Rad) and amount of eNOS was normalized with respect to amount of GAPDH product [16].

### Statistical Analysis

All values are expressed as mean ± S.D. Data for isolated pulmonary artery ring preparation was statistically analyzed using one way ANOVA followed by Newman Keuls test. The data for nitrite, Superoxide anions, TBARS, MABP and RVH were statistically analyzed using one way ANOVA followed by Turkey's multiple comparison tests. P value ≤ 0.05 was considered to be statistically significant.

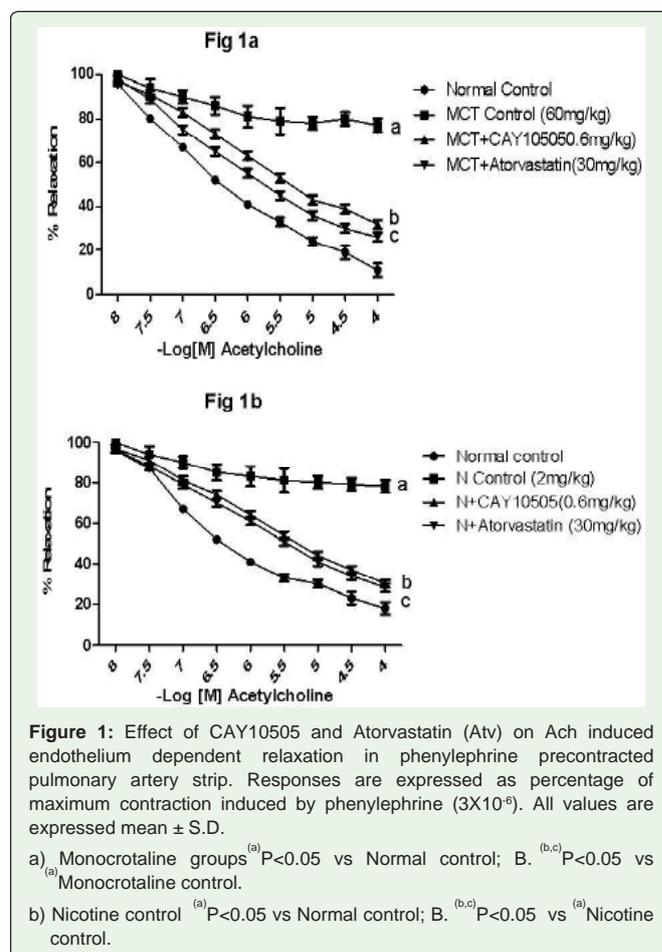
## Results

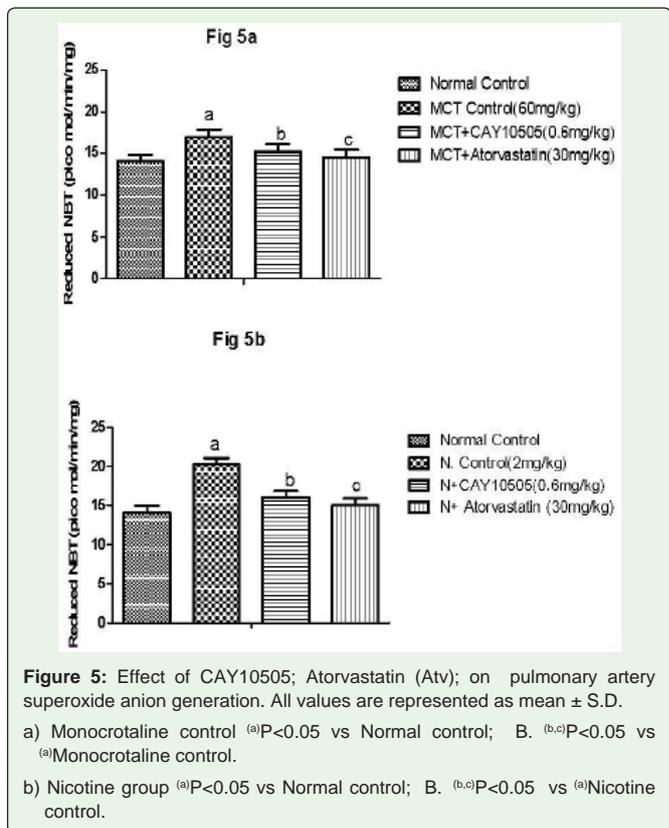
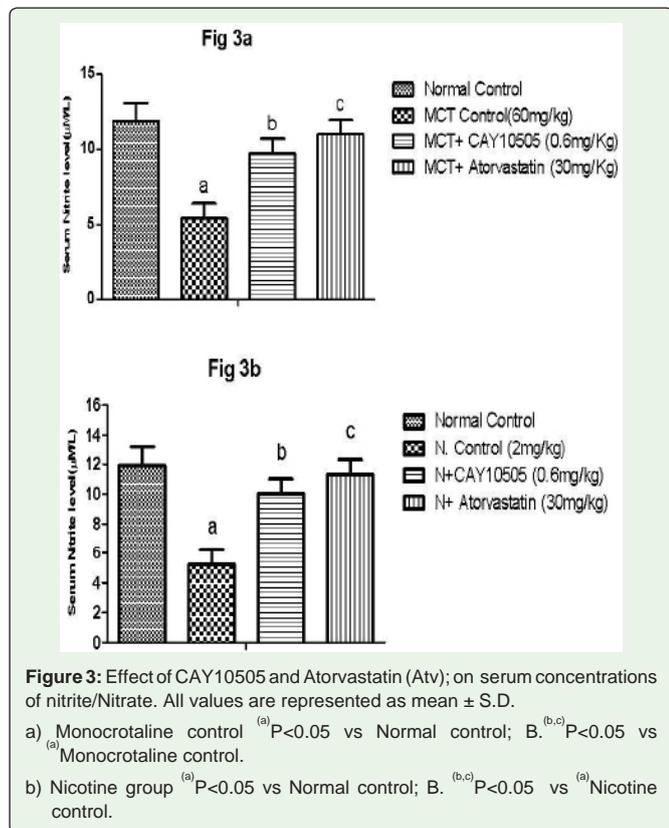
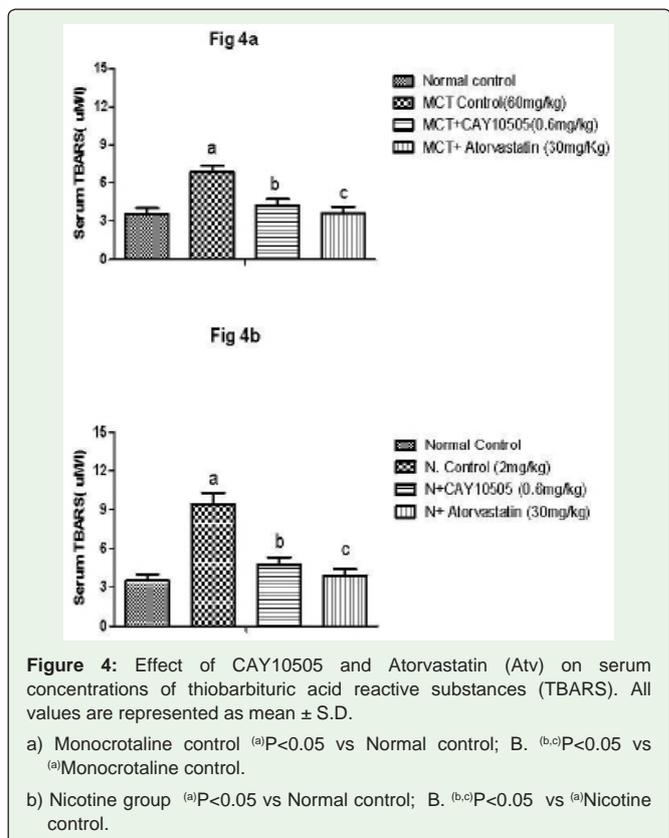
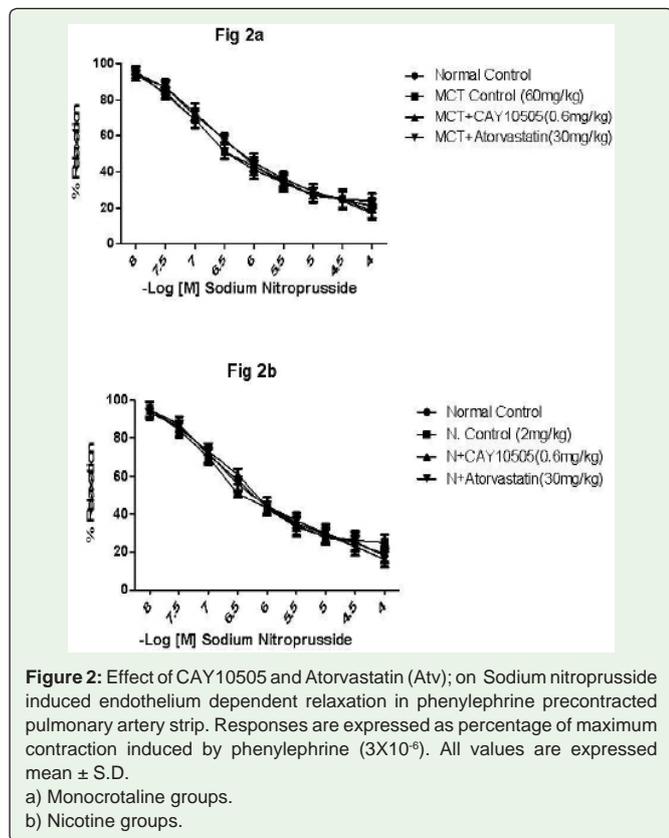
### Effect of Pharmacological Interventions on Endothelium Dependent and Independent Relaxation

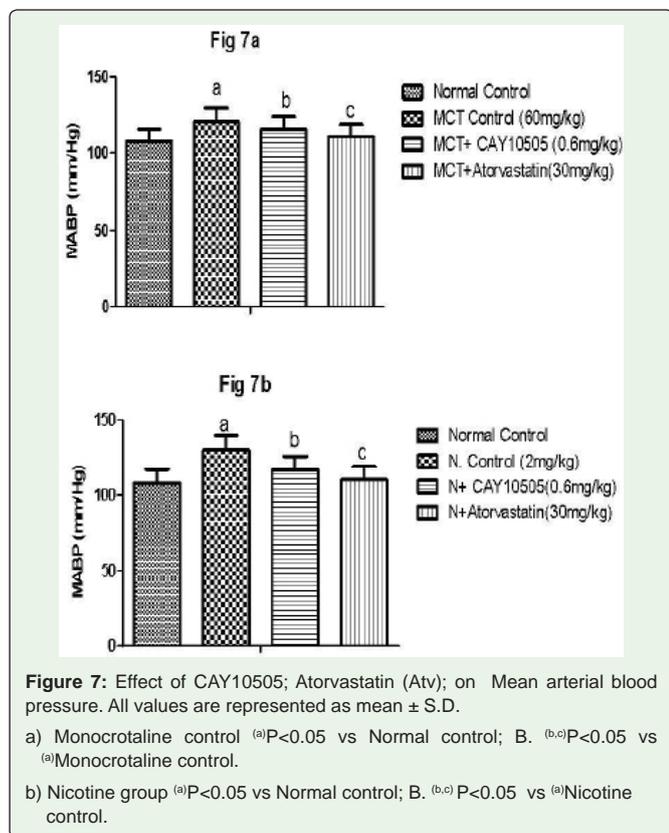
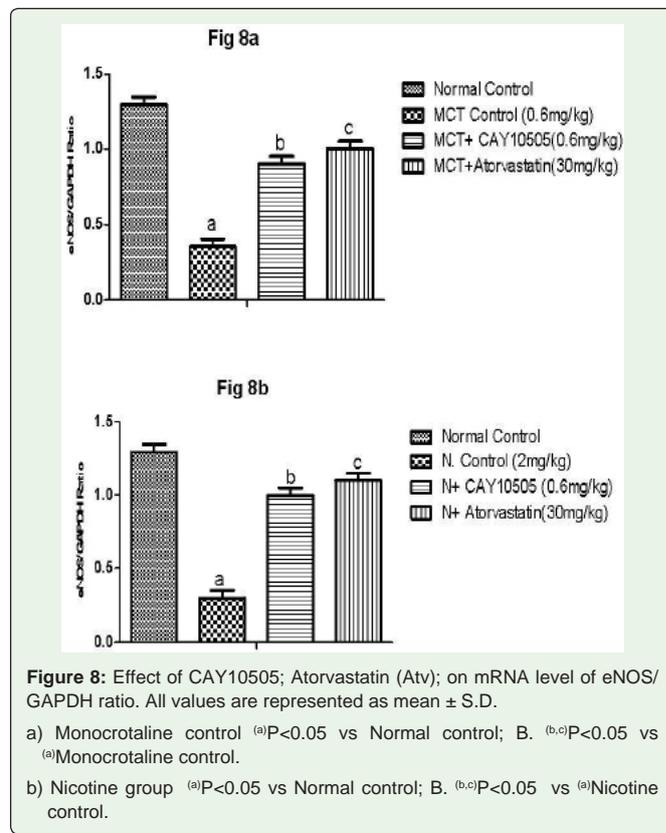
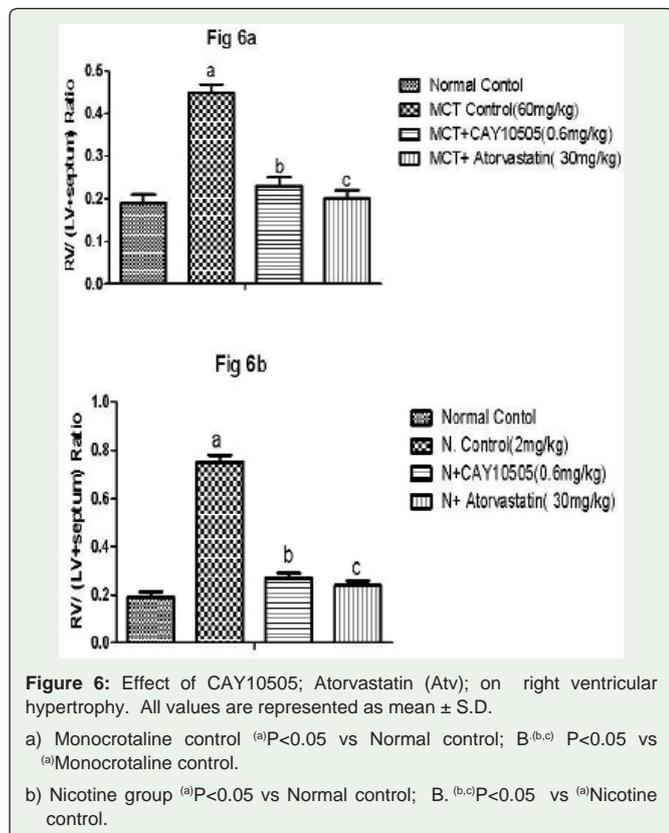
Acetylcholine (Ach) (Figure 1a, Figure 1b) and Sodium Nitro Prusside (SNP) (Figure 2a, Figure 2b) were noted to produce endothelium dependent and independent relaxation, respectively in phenylephrine (3 X 10<sup>6</sup> M) precontracted isolated normal rat pulmonary artery ring preparation in a dose dependent manner. In pulmonary vascular endothelial dysfunction, ach induced endothelium dependent relaxation was noted to be significantly attenuated CAY10505 (0.6 mg/kg; p.o.) from 21<sup>st</sup> day to 28<sup>th</sup> day and atorvastatin (30 mg/kg; p.o.) from 21<sup>st</sup> day to 28<sup>th</sup> day. Significantly improved ach induced endothelium dependent relaxation in pulmonary artery rings.

### Effect of Pharmacological Interventions on Serum Nitrite/ Nitrate Concentrations

The serum nitrite/nitrate concentrations were noted to be reduced in pulmonary vascular endothelial dysfunction when compared with normal control group. However treatment with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day significantly increases serum nitrite/nitrate concentrations MCT and nicotine administered groups (Figure 3a, Figure 3b).







**Effect of Pharmacological Interventions on Serum TBARS**

The increase in serum TBARS concentration was noted in pulmonary vascular endothelial dysfunction induced groups when compared with normal control group. However treatment with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day significantly decreases serum TBARS as compared to MCT and Nicotine control groups (Figure 4a, Figure 4b).

**Effect of Pharmacological Interventions on Pulmonary Artery Superoxide Anions Generation**

The increase in pulmonary artery superoxide anion generation was noted in pulmonary vascular endothelial dysfunction induced groups when compared with normal control group. However treatment with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day significantly attenuated in comparison to MCT and Nicotine control groups. (Figure 5a, Figure 5b).

**Effect of Pharmacological Interventions on Right Ventricular Hypertrophy**

Monocrotaline and Nicotine administration increases right ventricular hypertrophy as compared to the normal control group. Treatment with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day in rats produced marked reduction in the right ventricular hypertrophy (Figure 6a, Figure 6b).

**Effect of Pharmacological Interventions on Mean Arterial Blood Pressure (MABP)**

Monocrotaline and nicotine Administration increases mean

arterial Blood Pressure (MABP) in rats within 4 weeks as compared to normal control group. Treatment with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day in rats produced marked reduction on MABP (Figure 7a, Figure 7b).

### Effect of Pharmacological Interventions on mRNA level of eNOS

Monocrotaline and Nicotine administration decreases mRNA level of eNOS as compared to the normal control group. The treatments with CAY10505 (0.6 mg/kg; p.o) and atorvastatin (30 mg/kg; p.o) from 21<sup>st</sup> day to 28<sup>th</sup> day in rats produced marked increase in the mRNA level of eNOS (Figure 8a, Figure 8b).

### Discussion

Pulmonary circulation is at maximum risk as a consequence of inhalation of nicotine as cigarette smoke directly into the lungs. Chronic nicotine deteriorates the single cell layer barrier of pulmonary vascular endothelium that further leads to pulmonary vascular endothelial dysfunction and pulmonary hypertension. Thus intervention of vascular endothelial dysfunction may provide potential therapeutic interventions in further propagation of its adverse effects [19]. The adverse effects are likely a major contributing factor for the generation of oxidative stress [4]. Dysfunction of pulmonary vascular endothelium attenuates activity and expression of eNOS and consequently reduces release of NO [5,6]. In present study nicotine administration also produce pulmonary vascular endothelial dysfunction assessed in terms of decrease in mRNA expression of eNOS, serum concentrations of nitrate/nitrite, attenuation of acetylcholine induced endothelium dependent relaxation and increase in mean arterial blood pressure and oxidative stress.

MCT-induced pulmonary vascular endothelial dysfunction is a well established standard model characterized by a decrease in endothelium-dependent relaxation, oxidative stress, proliferative pulmonary vasculitis, pulmonary hypertension and right ventricular hypertrophy [9,10]. Monocrotaline is an 11-membered macrocyclic pyrrolizidine alkaloid. MCT is converted into reactive metabolite in the liver and is transported to red blood cells where it causes endothelial injury and is further specifically reported to cause pulmonary vascular endothelial dysfunction [7,8]. Both Monocrotaline and nicotine have been reported to reduce endothelium-dependent vasorelaxation and nitric oxide bioavailability however exact molecular mechanism has not yet elucidated.

In the present study cumulative dose response curves were recorded for Ach-induced endothelium dependent relaxation in isolated pulmonary artery ring preparation. Further, sodium nitroprusside induced endothelium-independent vasorelaxation has been used as the control in this study, to investigate the effect of endothelium independent vascular reactivity. The earlier results suggest that NO is, in fact, the most important endothelium-derived mediator involved in Ach-induced relaxation of rat pulmonary artery rings. Endogenous formation of NO is unstable and gets converted to nitrate/nitrite and estimation of serum nitrite/nitrate has been used as an indirect measure of NO release. Thus, this parameter has been used as an index of change in NO formation due to modulation of endothelium function. Increased mRNA expression of eNOS has been shown to stimulate release of NO; therefore reverse transcriptase polymerase chain reaction has been employed to assess the extent of

expression of eNOS. Atorvastatin in addition to its lipid lowering effect exerts pleiotropic effects on the vascular system. It has been reported to inhibit Rho-Kinase and activates eNOS to release nitric oxide that exerts a vasoprotective effect. Furthermore, PI3K $\gamma$  also stimulates Rac induced oxidative stress and endothelial dysfunction. Statins have been documented to exert the beneficial effect by attenuation of Rac mediated effects [20]. Thus it has been employed as a standard drug in the present study, further; it has been used as the standard agent in a number of studies to improve vascular endothelial dysfunction. PI3K was reported to play a role in angiotensin-II evoked smooth muscle contraction in two crucial, distinct signaling pathways. In response to angiotensin II, PI3K gamma is reported to be involved in the activation of Rac and the subsequent triggering of ROS production [21]. Furthermore, PI3K gamma is necessary to activate protein kinase B/Akt, which in turn, enhanced L-type Calcium channel mediated extracellular calcium entry. CAY 10505 is a potent inhibitor of PI3K, selectively inhibiting the Gamma ( $\gamma$ ) isoform ( $IC_{50}$ =30 nmol. L<sup>-1</sup>) more strongly than  $\alpha$ ,  $\beta$  and  $\delta$  isoforms ( $IC_{50}$ =0.94, 20, and 20 $\mu$ mol. L<sup>-1</sup>), respectively. The dose of CAY10505 (0.6 mg/kg, p.o) used was derived from the  $IC_{50}$ , and employed as a specific inhibitor of PI3K $\gamma$ . In the MCT and nicotine administered control groups increase in right ventricular hypertrophy was observed as compared to normal control group. However, CAY10505 and atorvastatin significantly attenuated the hypertrophy in MCT and nicotine administered rats. Treatment with CAY10505 and atorvastatin also decreases the oxidative stress assessed in terms of TBARS and superoxide anion generation in comparison to MCT and nicotine administered groups. Thus CAY10505 (PI3K gamma inhibitor) and atorvastatin treatment for 1 week after 3 weeks of administration of MCT and Nicotine significantly ameliorates pulmonary vascular endothelial dysfunction in rats assessed in terms of parameters employed. However there was no change in Sodium Nitro Prusside (SNP) induced endothelium independent vasorelaxation that shows effect is confined to endothelium. These data indicate that Cay 10505 (inhibitor of PI3K $\gamma$ ) ameliorates MCT and nicotine induced pulmonary vascular endothelial dysfunction. Thus, PI3K $\gamma$  may be a key transducer of the intracellular signals involved in pulmonary vascular endothelial dysfunction and pulmonary hypertension.

### Conclusion

Thus it may be concluded that CAY10505 a PI3K $\gamma$  inhibitor improves nicotine and monocrotaline-induced vascular endothelial dysfunction and up-regulates eNOS expression and nitric oxide production. Thus interventions designed for this pathway may provide therapeutics for pulmonary vascular complications.

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