

# The Possible Protective Effects of Zileuton against Pulmonary Fibrosis Induced by Amiodarone in Male Rats

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## Abstract:

### Introduction

Pulmonary fibrosis is characterized by cellular alteration of the alveolar region with lung inflammation. Leukotrienes play an important role in the progression of the disease. This study was designed to investigate the possible protective effect of zileuton against amiodarone-induced pulmonary fibrosis in male rats.

### Materials and methods

Pulmonary fibrosis was induced by administering amiodarone (100 mg/kg intraperitoneally i.p) and concomitantly treated with zileuton (30 mg/kg intraperitoneally i.p) for 15 days in wistar albino male rats. Biochemical parameters include TNF- $\alpha$ , IL-1 $\beta$ , 5-lipoxygenase; MDA and GSH in lung tissue were estimated, as well as lung histological studies.

### Results

In rats treated with amiodarone, levels of TNF- $\alpha$ , IL-1 $\beta$ , 5-lipoxygenase and MDA were significantly elevated ( $p < 0.05$ ) with reduction in GSH level when compared with those treated with saline only. Meanwhile; administration of zileuton in group 3 was concerned with significant ( $p < 0.05$ ) improvement in the biochemical parameters when compared with group 1 and 2. Furthermore, zileuton histologically reduced the number of inflammatory cells and ameliorate the destruction of lung architecture and pulmonary fibrosis induced by amiodarone.

### Conclusion

Zileuton ameliorate pulmonary fibrosis induced by amiodarone in male rats, by inhibiting 5-lipoxygenase activity and reducing leukotrienes synthesis.

**Keywords:** Zileuton, pulmonary fibrosis, amiodarone, rats.

## 1. Introduction

Pulmonary fibrosis is a disease marked by scarring in the lungs. Tissue deep in the lungs becomes thick, stiff and scarred, due to the accumulation of excess fibrous connective tissue that is called fibrosis. As the lung tissue becomes scarred and thickening of the walls, oxygen supply in blood reduced which interfere with a person's ability to breathe [1]. Amiodarone is an iodinated benzofuran derivative that is used to suppress ventricular and supraventricular tachyarrhythmias, but unfortunately pulmonary toxicity is among the most serious adverse effects of amiodarone. Several forms of pulmonary disease occur among patients treated with amiodarone, including interstitial pneumonitis, organizing pneumonia, acute respiratory distress syndrome (ARDS), diffuse alveolar hemorrhage (DAH), pulmonary nodules and solitary masses, and also (rarely) pleural effusion [2]. Several mechanisms have been reported by which amiodarone promotes pulmonary fibrosis [3]. Amiodarone impairs lipid metabolism, which increases cellular and phospho- lipid content, resulting in damage to the pulmonary endothelium. It can also produce toxic oxidants when exposed to high oxygen concentrations, further damaging cell membranes, which plays a part in the capillary leak syndrome related with adult respiratory distress syndrome [4]. Previous studies have been suggested that leukotrienes (LTs), which are arachidonic acid metabolites, are important regulators of pulmonary fibrosis [5, 6]. The synthesis of LTB<sub>4</sub> and cysteinyl LT (cysLT) are catalysed by 5- lipoxygenase. LTB<sub>4</sub> plays an important role in the host defense system against infection and invasion by foreign bodies [7]. LTB<sub>4</sub> is thought to be a cause of various inflammatory disorders, and it is produced by alveolar cells in patients with idiopathic pulmonary fibrosis (IPF) [8]. There are two LTB<sub>4</sub> receptor (BLTR) subtypes, BLT1 and BLT2; both subtypes are G protein-coupled receptors and are present on the cell surface [9]. BLT1 is primarily expressed in leukocytes whereas BLT2 is expressed more ubiquitously. Several studies have been proposed that proposed BLTR has a role in airway disease. It was reported that BLT1 mediates LTB<sub>4</sub>-induced T helper (Th) type 1 and Th2 cell chemotaxis and firm adhesion to endothelial cells exposed to flow, and mediates CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment into the airway in an asthma model [10]. These considerations represent a significant reason for the use of zileuton in the current study, however zileuton is a 5-lipoxygenase (5-LO) inhibitor; in which this enzyme is responsible for converting arachidonic acid into the leukotrienes LTB<sub>4</sub> and LTC<sub>4</sub>. These leukotrienes have inflammatory and fibrotic properties and are upregulated in the lungs of IPF patients [11]. Zileuton was approved for the treatment of asthma, and has also been shown to inhibit inflammation and fibrosis in the bleomycin model [12]. The current study was designed to determine the possible protective effect of zileuton against pulmonary fibrosis in male rats induced by amiodarone.

## 2. Materials and Methods

### Experimental protocol

The study included 36 wistar albino male rats weighing (150-200 gm); they were obtained from the animal house of the College of Pharmacy/Al-Mustansiriya University. The animals were maintained at controlled temperature ( $25\pm 2$  °C) with light schedule 12-12 hour's light/dark cycles, allowed free access to water, and fed standard rat chow *ad libitum*. Zileuton 600 mg tablets were provided by Abbott Laboratories pharmaceutical company, USA, while amiodarone vial (150 mg/ 3ml) was provided by APP pharmaceuticals, Germany. Animals were allocated randomly into 4 groups and treated as follows:

Group 1: includes 12 rats received saline i.p for 15 days and served as negative controls. Group 2: includes 12 rats received saline i.p for 5 days before and 10 days concomitant with 100 mg/kg amiodarone i.p, and served as positive control. Group 3: includes 12 rats received zileuton 30 mg/kg i.p for 5 days before and 10 days concomitant with 100 mg/kg amiodarone i.p.

One day later under light ether anesthesia, animals were sacrificed by cervical dislocation and lungs were obtained.

### Preparation of tissue homogenates

A 10% (w/v) lung tissue was prepared in phosphate buffer at 4°C, using metal head tissue homogenizer which adjusted at set 3 for one minute. The homogenate was then centrifuged at 10,000 g for 15 minutes and the supernatant formed was used for the biochemical estimations. All samples were kept frozen at (-18°C) unless analyzed immediately.

### Biochemical assays

#### Estimation of TNF- $\alpha$ and IL-1 $\beta$ levels in lung tissue

Sandwich enzyme immunoassay technique was performed for the quantification of TNF- $\alpha$  and IL-1 $\beta$  according to the instructions provided by Ray Biotech. [13].

#### Estimation of 5-lipoxygenase activity in lung tissue

The activity of 5-lipoxygenase enzyme was determined by homogenizing with lysis buffer to give 50% lung lysates. Dialysis buffer at pH 7.4 was Tris buffer. The activity of the enzyme was measured with an enzymatic colorimetric method using a diagnostic lipoxygenase inhibitor screening assay kit [14].

#### Lung tissue homogenate malondialdehyde (MDA) and glutathione (GSH) assay

The product of lipid peroxidation malondialdehyde (MDA) level was measured in tissue homogenates depending on the formation of pink chromophore because of the reaction between (MDA) and thiobarbituric acid (TBA), which can be measured spectrophotometrically according to the method of Buege and Aust [15]. Glutathione (GSH) levels were determined according to the method of Elman [16], in which 0.5 ml of 4% sulphosalicylic acid was added to the equal volume of tissue homogenate for precipitation of protein.

### Histological examination

After sacrificing the animals, lungs were excised from each animal immediately, placed in 10% formalin buffer solution for 24 hours at room temperature, blotted with filter paper and accurately weighed, followed by dehydration step, by immersing it in a gradually increasing concentration of alcohol. Lung tissues were kept in xylene for one hour under a temperature of 60°C, and then embedding it in paraffin wax. A rotary microtome was used to cut sections at 5  $\mu$ m in thickness, which sequentially mounted onto microscope slides, and stained with hematoxylin and eosin [17]. Slide sections were examined under a light microscope.

### Statistical analysis

The data were expressed as mean  $\pm$  standard deviation (SD) and statistically analyzed by using one way analysis of variance (ANOVA) followed by a post hoc test (LSD alpha) for multiple comparisons using SPSS (version 15) for data analysis. P-values less than 0.05 were considered significant for all data presented in the results.

## Results

### Effect of zileuton on TNF- $\alpha$ and IL-1 $\beta$ levels in lung tissue of rats treated by amiodarone

A significant ( $p < 0.05$ ) increase in TNF- $\alpha$  and IL-1 $\beta$  were recorded in rats received (100 mg/kg i.p) of amiodarone when compared with those rats received saline only (group 1), meanwhile those rats in group 3 which received zileuton (30 mg/kg i.p) 5 days before and 10 days concomitantly with amiodarone recorded significantly ( $p < 0.05$ ) reduced in TNF- $\alpha$  when compared with group 2 except IL-1 $\beta$  which recorded non significant different ( $p > 0.05$ ), however both TNF- $\alpha$  and IL-1 $\beta$  in group 3 recorded significantly higher ( $p < 0.05$ ) when compared with group 1 (table 1).

### Effects of zileuton on 5-lipoxygenase activity in lung tissue of rats treated by amiodarone

Rats in group 2 that subjected to amiodarone had higher significant 5-lipoxygenase activity ( $p < 0.05$ ) when compared with group 1 that treated by saline only. Administration of zileuton in group 2 was recorded a significant reduction ( $p < 0.05$ ) in 5-lipoxygenase activity when compared with amiodarone treated group only, meanwhile when compared with those animals in group 1 recorded a non significant different ( $p > 0.05$ ) (table 1).

### Effect of zileuton on MDA and GSH levels in lung tissue of rats treated by amiodarone

Administration of 100 mg/kg amiodarone in group 2 resulted in a significant elevation ( $p < 0.05$ ) of MDA level

and a significant ( $p < 0.05$ ) reduction of GSH level in lung tissue of rats in group 2 when compared with those rats treated with saline only (table 1). Meanwhile, administration of zileuton( 30mg/ kg) resulted in a significant( $p < 0.05$ ) reduction of MDA level and a non significant( $p > 0.05$ ) elevation of GSH level in brain tissue of rats in group 3 when compared with those animals in group 2, but still these values are significantly( $p < 0.05$ ) different when compared with values of group 1(table 1).

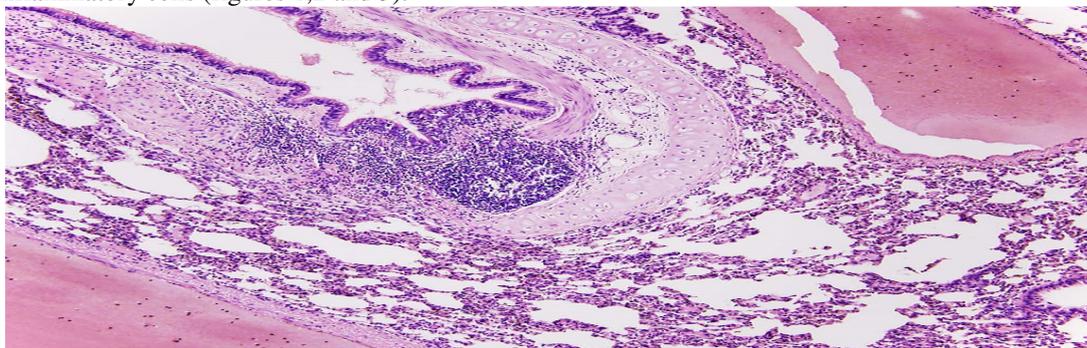
**Table 1:** The effect of 30 mg/kg zileuton on biochemical parameters in lung tissue homogenate of rats treated by 100 mg/kg amiodarone

Treatment groups	TNF- $\alpha$ pg/ gm tissue	IL-1 $\beta$ pg/ gm tissue	Lipoxygenase activity (nmol/min/g)	MDA nmol/gm tissue	GSH $\mu$ mol/gm tissue
Normal saline group n= 12	17.09 $\pm$ 1.87 <sup>a</sup>	10.59 $\pm$ 1.93 <sup>a</sup>	3.88 $\pm$ 0.78 <sup>a</sup>	43.5 $\pm$ 3.84 <sup>a</sup>	21.45 $\pm$ 2.18 <sup>a</sup>
Amiodarone (100 mg/ kg) n= 12	71.06 $\pm$ 4.5 <sup>b</sup>	46.32 $\pm$ 5.56 <sup>b</sup>	16.57 $\pm$ 1.23 <sup>c</sup>	72.87 $\pm$ 3.57 <sup>b</sup>	10.65 $\pm$ 5.27 <sup>b</sup>
Amiodarone (100 mg/ kg) + zileuton (30 mg/kg) n= 12	42.27 $\pm$ 1.04 <sup>c</sup>	40.33 $\pm$ 4.12 <sup>b</sup>	4.67 $\pm$ 0.53 <sup>a</sup>	59.62 $\pm$ 4.58 <sup>c</sup>	12.67 $\pm$ 2.33 <sup>b</sup>

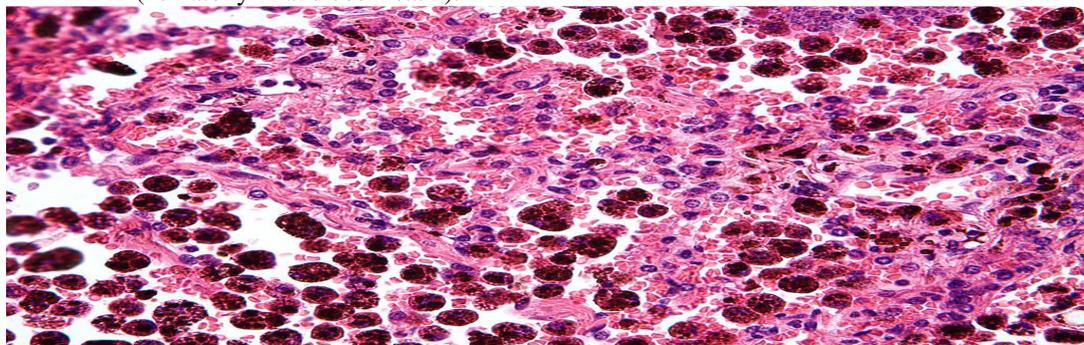
Data were expressed as mean  $\pm$  SD; n= number of animals, values with non identical superscripts (a,b,c) within the brain tissue were considered significantly different( $p < 0.05$ ).

**Histopathological study:**

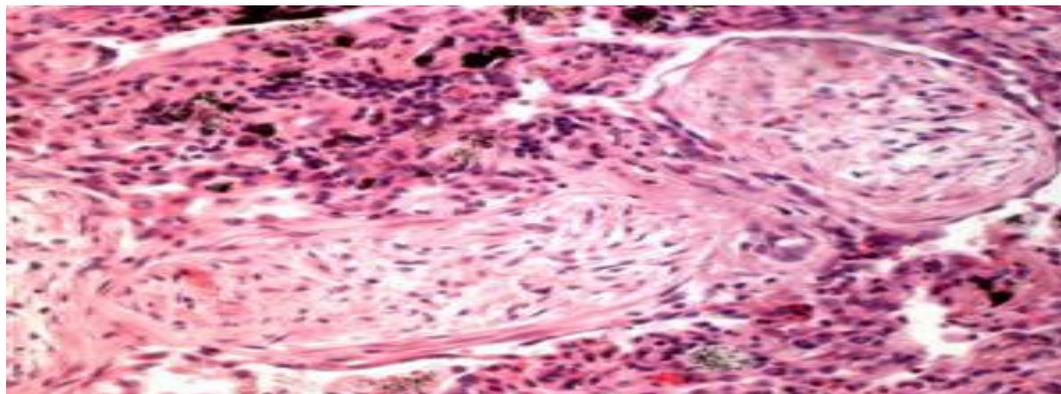
In the current study, the histopathological examination recorded normal architecture of lung tissue in those rats treated with saline only. However, administration of amiodarone in group 2 found patches of fibrosis, pulmonary hemorrhage with hemosiderin laden alveolar macrophages. Furthermore, treated rats by zileuton after exposure to amiodarone their lung tissues were showed a mild pulmonary fibrosis and lower level of infiltration with inflammatory cells (figures 1,2 and 3).



**Figure-1:** Lung section of rat in group 1 showing normal architecture with present of normal bronchiole in the centre of the image and the dark granular cells represent white blood cells. Magnification: 200X, (hematoxylin and eosin stain).



**Figure-2:** Section showing lung tissue of rat in group 2 treated with amiodarone 100 mg/kg with a marked pulmonary hemorrhage and hemosiderin laden alveolar macrophages. Magnification: 200X, (hematoxylin and eosin stain).



**Figure-3:** Lung tissue section of rat in group 3 treated with zileuton 30 mg/kg with a mild pulmonary fibrosis and lower level of infiltration with inflammatory cells. Magnification: 200X, (hematoxylin and eosin stain).

### Discussion

The results of the current study reported that zileuton attenuated pulmonary fibrosis induced by amiodarone, through reducing TNF- $\alpha$ , IL-1 $\beta$ , 5-lipoxygenase and MDA levels in lung tissue. Meanwhile, GSH level in lung tissue was significantly elevated. These findings are in line with previous studies [18, 19]. Pulmonary fibrosis is promoted by leukotrienes (LTs) [20, 21]. Histological sections of lungs with pulmonary fibrosis reported to be correlated with the extent of fibrosis [22, 23]. Inhibition of 5-lipoxygenase enzyme by zileuton was concerned with mediating inflammation and fibrosis which in line with previous study [24]. Furthermore, zileuton was reported as a tool for the evaluation of 5-lipoxygenase and leukotrienes activity *in vitro* and *in vivo* models of inflammation. Zileuton reported to show beneficial effects in attenuating a number of pathological conditions in animals, including inflammation of the upper airway such as chronic obstructive pulmonary disease [25]. Previous study also reported that using of 5-lipoxygenase inhibitor was concerned with reducing of TNF- $\alpha$  and interleukins which is in line with the current study [26]. Proinflammatory cytokines, tumor necrosis factor (TNF)- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) can additionally increase oxidative stress in humans [27], inducing production of reactive oxygen species (ROS), which have been suggested to act as second messengers [28]. Thus, used of zileuton in the current study which concerned with reducing the levels of tumor necrosis factor (TNF)- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) was reported to reduce oxidative stress. In conclusion, zileuton inhibited inflammation by the decrease in the inflammatory mediators including TNF- $\alpha$  and IL-1 $\beta$  in lung tissue. More importantly, the decrease in 5-lipoxygenase activity and MDA level with elevation in GSH content in lung tissue indicates mitigation in the development of pulmonary fibrosis induced by amiodarone in male rats.

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