Identification of Leads against Chronic Obstructive Pulmonary Disease from natural and synthetic sources

Thesis submitted by

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Dedicated to my Grandparents

Late Nishibhusan Sengupta

Late Priyabala Sengupta

Late Narayan Chaudhuri

Late Sushama Chaudhuri

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All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is 7%.

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DECLARATION

I hereby declare that my research work embodied in this PhD thesis entitled **"Identification of Leads against Chronic Obstructive Pulmonary Disease from natural and synthetic sources"** have been carried out by me in Cardiovascular Disease and Respiratory Disorders Laboratory, Division of Cell Biology and Physiology, CSIR-Indian Institute of Chemical Biology (Jadavpur and Saltlake TRUE Campus), Kolkata under the direct supervision of **Dr. Arun Bandyopadhyay**, Director, CSIR-Indian Institute of Chemical Biology, Kolkata-700032, India and **Prof. Pulok K. Mukherjee**, Director, Institute of Bioresources and Sustainable Development, Dept. of Biotechnology, Ministry of Science and Technology, Govt. of India, Takyelpat, Imphal-795001, Manipur , India & Professor (on lien), Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India. I also confirm that this work is original and has not been submitted partly or in full for any other degree or diploma to this or other University or Institute.

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CONTENTS

1

LIST OF ABBREVIATIONS

List of Abbreviations

AATD	deficiency of alpha-1 antitrypsin
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar Lavage
BPD	Bronchopulmonary dysplasia
COPD	Chronic Obstructive Pulmonary Disease
DALY	Disability-Adjusted Life Year
ETS	Environmental cigarette smoke
FEV1	Forced Expiratory Volume at 1 second
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HIV	Human Immunodeficiency Virus
HNE	Human Neutrophil Elastase
HLE	Human Leukocyte Elastase
LAMA	Long-acting muscarinic antagonists
LPS	Lipopolysaccharide
LVRS	Lung volume corrective surgery
MLI	Mean Linear Intercept
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
NE	Neutrophil Elastase
NIV	Noninvasive ventilation
NPPV	Noninvasive positive pressure ventilation
PPE	Porine Pancreatic Elastase
SAMA	Short-acting antimuscarinics

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ABSTRACT

Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are significant and growing global health issues. About 80–90% of all COPD cases worldwide are caused by smoking. Emphysema is predominantly brought on by neutrophil elastase in COPD, which results in the loss of lung tissue and the closure of tiny airways. When it comes to the advancement of the disease in COPD patients, neutrophil elastase has become a crucial target for therapeutic development. The Indian Sundarban regions are home to a variety of mangrove plants, including *Sonneratia apetala* Buch.-Ham. Fruit and leaf extracts have been demonstrated to alleviate the symptoms of cough and asthma, despite the fact that the plant's fruits also include antibacterial, antifungal, antioxidant, and astringent properties. This study aims to determine if fruit extracts of Sonneretia apetala inhibit neutrophil elastase and so stop the advancement of lung emphysema caused by neutrophil elastase. The IC_{50} of the hydroalcoholic extract of the fresh fruits of *Sonneratia apetala* Buch.-Ham. (SAM) was calculated using the neutrophil elastase enzyme kinetic test. The extract was standardised using gallic acid and ellagic acid as standards using the new HPLC technique. The extract underwent additional LC-MS2 profiling to determine the main phytochemicals and ten such compounds were identified. According to the HPLC calibration, *Sonneretia apetala* crude extract (SAM) contains 53 g/mg of gallic acid and 95 g/mg of ellagic acid. Human epithelial cells' in vitro morphological change caused by elastase was likewise reversed by SAM, and the vitality was determined by an MTT experiment which showed no toxicity of the herbal extracts. Furthermore, in the mice model, neutrophil elastase-induced alveolar collapse was lessened by 10mg/kg SAM. Thus, we discovered for the first time in this work that *Sonneretia apetala* fruit extract (SAM) has the ability to both *in vitro* and *in vivo* block human neutrophil elastase.

In a quest for novel synthetic neutrophil elastase inhibitors, we synthesised and tested seven brand-new benzoxazinone derivatives. By using an enzyme substrate kinetic assay and discovered that they were inhibitors of human neutrophil elastase. One of these substances, PD05, became the most effective inhibitor with a lower IC_{50} compared to the sivelestat control substance. ONO 6818 and AZD9668 are two well-known elastase inhibitors. However, PD05 demonstrated a high binding affinity for human neutrophil elastase (Kd=1.63nM) and a faster association and dissociation rate, and its interaction with human neutrophil elastase was totally reversible. In vitro preclinical pharmacokinetic investigations revealed a protein binding efficiency of 72%, a rapid recovery rate, an aqueous solubility of 194.7μM, a low permeability, and a favourable hERG. The chemical successfully inhibited elastase-induced rounding and retracted cell morphology and cell cytotoxicity, per the experiments with human lung epithelial cell lines. In a mouse model, neutrophil elastase-induced alveolar collapse can be decreased by PD05. In conclusion, we show that the newly synthesised benzoxazinone derivative PD05 has anti-elastase capability *in situ*, *in vitro*, and *in vivo*, making it a suitable option for additional research as a COPD treatment.

5

INTRODUCTION

INTRODUCTION

5.1 Chronic Obstructive Pulmonary Diseases (COPD)

5.1.1 Definition and Overview

5.1.1.1 Definition

Chronic obstructive pulmonary disease (COPD) is a widespread, preventable, and treatable illness that is characterised by recurrent respiratory symptoms and airflow restriction due to abnormalities in the airways and/or alveoli, which are typically brought on by prolonged exposure to toxic and harmful particles or gases and caused by environmental factors. The presence of serious comorbidities may affect morbidity and mortality. The chronic airflow restriction that is a hallmark of COPD is brought on by a combination of emphysema and small airways disease, the relative proportions of which differ from individual to individual. These changes don't usually happen at once; rather, they develop throughout time at various rates. Chronic inflammation alters the structure of the body, narrows the small airways, and destroys the lung parenchyma, which results in the loss of alveolar connections to the air passages and a reduction in the lung's elastic recoil. These modifications thus reduce the airways' capacity to stay open during expiration. Airflow restriction may also be caused by the loss of tiny airways, and mucociliary malfunction is a defining aspect of the illness. Spirometry is typically used to detect airflow restriction as it is most accessible and reliable lung function test. Emphysema is a clinical term that is frequently (but mistakenly) employed in clinical contexts and characterises only one of many anatomical abnormalities found in COPD patients. A useful term for clinical and epidemiological purposes is still "chronic

bronchitis," which is defined as having a persistent cough and sputum secretion for at least three months over two years.

5.1.1.2 Burden of COPD

The economic and social toll of COPD, a primary cause of morbidity and mortality worldwide, is significant and growing (Lozano et al.,2012;Vos et al.,2012). The prevalence, morbidity, and mortality of COPD vary between nations and among various populations within nations. In addition to genetics, airway hypersensitivity, and inadequate lung development throughout childhood, a number of host variables, including long-term prolonged exposure to toxic particles and gases, cause COPD (Lange et al., 2015; Stern et al., 2007; Tashkin et al., 1992).

5.1.1.2.1 Prevalence

Due to variations in survey techniques, diagnostic standards, and analytical procedures, the data on the prevalence of COPD in the literature varies greatly (Mathers et al., 2006) It's significant to note that all of the investigations used spirometry alone to determine COPD rather than a mix of symptoms and pulmonary function tests. The lowest prevalence estimates are those focused on self of a COPD or comparable ailment that has been diagnosed by a doctor. For instance, the majority of national data indicate that 6% of the elderly has received a diagnosis of COPD (Halbert et al., 2006). This is probably a result of the prevalent underdiagnosis and underrecognition of COPD (Quach et al., 2015).

5.1.1.2.2 Morbidity

Hospitalizations, Emergency visits, and doctor visits are all examples of traditional morbidity metrics. Although COPD database management systems for these outcome

characteristics are less accessible and typically less dependable than mortality data sets, studies on the data to date show that COPD-related morbidity increases with age (Halbert et al., 2006, Quach et al., 2015, Menezes et al., 2005), and in people with Copd, the progress of comorbid conditions may be seen at an early stage (Divo et al., 2018).

5.1.1.2.3 Mortality

The accuracy of mortality statistics is compromised by underdiagnosis and underrecognition of COPD (Walters et al.,2010; Walters et al., 2017), and COPD diagnosis codes documented in accessible medical databases may not be accurate. (Alfageme et al., 2006; Dransfield et al., 2012). The expanding smoking epidemic, decreased mortality from other major causes of death (such as ischemic heart disease and infectious diseases), ageing populations worldwide, particularly in high-income nations, and the lack of efficient disease-modifying treatments are the main causes of this rise in COPD-related mortality.

5.1.1.2.4 Economic Burden

Significant financial hardship is linked to COPD. COPD accounts for 56% (38.6 billion Euros) of the overall direct expenditures of respiratory disease in the European Union, which are projected to be around 6% of the total yearly healthcare budget (Thompson et al., 2021). Over the next 20 years, COPD-related costs in the United States are anticipated to rise, reaching $800.90billion, or $40 billion annually (Burge et al., 2000; Anthonisen et al., 1994). According to dynamic modelling, women will likewise be overrepresented, incur higher direct expenditures than men, and lose more years of quality-adjusted life (Anthonisen et al., 1994).

5.1.1.2.5 Social Burden

Finding additional, comparable, and quantitative metrics of disease burden within and between countries is desirable because mortality provides only a limited understanding of the human suffering of a disease. The Disability-Adjusted Life Year (DALY) is a composite measure of the burden of each health issue that was developed by the authors of the Global Burden of Disease (GBD) Study to be used in estimating the percentage of death and disability owing to major diseases and injuries (DALY) (Vestbo et al.,1999). In the year 2005, COPD was the eighth most common cause of DALYs lost worldwide, but by 2013, it has risen to the fifth most common cause (Fiore et al.,2009; Tashkin et al.,2008). After ischemic heart disease, COPD is the second most common cause of lower DALYs in the US (Celli et al., 2021).

5.1.1.3 Factors influencing Development and Progression of COPD

5.1.1.3.1 Genetic Factors

A severe hereditary deficiency of alpha-1 antitrypsin (AATD), a significant circulating inhibitor of serine proteases, is the genetic risk factor with the greatest documentation (O'Donnell et al., 2006). Although AATD deficiency only affects a small portion of the world's population, it demonstrates how genes and environmental exposures interact to increase a person's risk of COPD.

5.1.1.3.2 Age and Sex

A common risk factor of COPD is age. It is unknown whether normal ageing per se causes COPD or whether age simply represents the whole of one's lifetime exposures (O'Driscoll et al., 1992). Some of the anatomical alterations linked to COPD are mimicked by ageing of the bronchi and parenchyma. Sex-related changes in

immunological pathways and airway damage patterns may be observed and may have therapeutic significance. In this area, more work is required. Most studies in the past have shown that men are more likely than women to have COPD and die from it, but more recent statistics from developed nations have shown that this is no longer the case. This is likely due to changing tobacco smoking trends (Jenkins et al., 1987).

5.1.1.3.2 Lung growth and development

Lung development is influenced by events that take place during pregnancy, childbirth, and encounters during childhood and adolescence. Reduced maximal ascertained lung function (as determined by spirometry) may help identify people who are more likely to develop COPD (Regan et al., 2015; Stern et al., 2007). Any condition that has an impact on a person's ability to expand lungs throughout pregnancy and youth may raise their risk of getting COPD.

5.1.1.3.3 Socioeconomic status

Lower socioeconomic level is associated with a greater likelihood of developing COPD, and poverty is consistently linked to airflow obstruction (Townend et al.,2017). However, it is unclear if this trend reflects exposures to both indoor and outdoor air pollution, crowding, inadequate nutrition, illnesses, and other socioeconomically disadvantaged status-related issues.

5.1.1.3.4 Exposure to particles

The most prevalent risk factor for COPD found globally is cigarette smoking. Compared to non-smokers, smokers have a greater incidence of respiratory symptoms, anomalies in lung function, an increased annual rate of FEV1 decline, and a higher mortality risk from COPD (Kohansal et al., 2009). Marijuana (Tan et al. 2009) and other tobacco products,

such as pipes, cigars, and water pipes, are additional risk variables for COPD. By increasing the total load of inhaled particles and gases on the lungs, passive inhalation to cigarette smoke, commonly known as environmental cigarette smoke (ETS), may also lead to respiratory complaints and COPD (Yin et al.,2007). Smoking while pregnant may put the foetus at danger by influencing the lungs' development and growth in utero and perhaps the immune system's priming (Tager et al., 1995).

5.1.1.3.5 Asthma and hyper-reactivity

Chronic airflow restriction and COPD development could both be influenced by asthma. According to a data from long-term cohorts of the Tucson Epidemiological Study of Airway Obstructive Disease, after controlling for smoking, persons with asthma had a 12-fold increased risk of developing COPD over time compared to those without asthma (Silva et al., 2004). Another longitudinal research of asthmatics indicated that 20% of participants experienced lower *transfer coefficient and permanent* airflow limitation (Vonk et al., 2003).

5.1.1.3.6 Chronic bronchitis

Chronic bronchitis has additionally been linked to an elevated risk in the overall number as well as degree of exacerbations. Chronic bronchitis has also been linked to an increased risk in the whole number as well as frequency of exacerbations in younger individuals who smoke (Gauderman et al., 2004).

5.1.1.3.7 Infections

There is evidence that patients are at higher risk of COPD than HIV negative controls. A background of severe childhood lung infections has been linked to decreased lung function and exacerbated respiratory symptoms in adulthood (Bigna et al., 2018).

5.1.1.4 Pathophysiology, Pathology and Pathogenesis

Lung inflammation is brought either by smoking cigarettes or inhaling other harmful particles, such as biomass fuel smoke. Lung inflammation is a typical reaction that seems to be altered in people with COPD. Emphysema may develop from this persistent inflammatory response's damage of parenchymal tissue and interference with normal defence and healing processes. Gas entrapment and increasing airflow restriction are the results of these pathological alterations.

5.1.1.4.1 Pathophysiology

The pathophysiology in COPD and how it results in the recognisable physiological anomalies and symptoms are now well understood. For instance, diminished FEV1 is a result of peripheral airway inflammation and constriction (Hogg et al., 2004). Furthermore, emphysema-related parenchymal damage reduces gas transport and limits airflow. A growing body of research also points to the loss of tiny airways, which may result in the restriction of airflow in addition to airway constriction. (McDonough et al., 2011).

5.1.1.4.2 Pathology

The lungs' parenchyma, pulmonary vasculature, and the airways all exhibit pathological alterations that are typical of COPD (Hogg and Timens, 2009). Chronic inflammation, with elevated numbers of particular inflammatory cell types in various lung regions, and structural abnormalities brought on by repetitive injury and repair are two of the pathological changes seen in COPD. The severity of the condition tends to worsen the proinflammatory and structural alterations in the airways, which continue after smoking is stopped. The majority of pathology data originate from research on smokers, and when

other factors are at play, the same balance of airway and parenchymal illness cannot always be assumed. The numerous comorbid illnesses that are prevalent in COPD patients may be caused by systemic inflammation, which may be present and may have an impact (Barnes, 2016).

5.1.1.4.3 Pathogenesis

The inflammation in COPD patients' respiratory tracts appears to be a modified version of the respiratory tract's typical inflammatory response to long-term irritants like cigarette smoke. Although the exact causes of this increased inflammation are yet unknown, genetics may play a role in it at least in part. Despite the fact that certain patients can acquire COPD without smoking, it is still unclear what exactly causes the inflammatory response in these patients. Lung proteinases in excess and oxidative stress are likely to even further alter lung inflammation. These mechanisms working in concert may cause the pathological alterations that are typical of COPD. After quitting smoking, lung inflammation still exists for unknown reasons, though autoantigens and changes in the lung microbiome may also be involved (Sze et al.,2015). Similar mechanisms could be at play for chronic diseases.

5.1.2 Diagnosis and Initial Assessment

Any patient with dyspnea, a persistent cough or sputum production, a history of recurrent lower respiratory tract infections, and/or a history of exposure to risk factors for the illness should have their COPD evaluated. The objectives of a COPD evaluation are to identify the degree of airflow restriction, the effect of the illness on the patient's health condition, and the likelihood of future occurrences (such exacerbations, hospital admissions, or death), in order to direct therapy. Heart disease, muscle tissue dysfunction, metabolic disease, osteoporosis, depression, anxiousness, and lung cancer are common

concurrent chronic conditions in COPD patients. Active search and treatment should be made for these comorbidities.

5.1.2.1 Diagnosis

Any patient with dyspnea, a persistent cough or sputum production, as well as a history of exposure to risk factors for the disease, should be evaluated for COPD. Spirometry is necessary to establish the diagnosis in this clinical setting; in patients with the necessary symptoms and considerable exposure to noxious stimuli, the existence of a post-bronchodilator FEV1/FVC 0.70 establishes the presence of persistent airflow limitation and, consequently, of COPD. The WHO has established a minimal set of measures for the primary care diagnosis of COPD (Fletcher et al., 1977).

5.1.2.2 Symptoms

The most prominent sign of COPD is chronic and escalating breathlessness. In up to 30% of patients, a coughing fit results in sputum production. These symptoms may change from day to day (Kessler et al., 2011), and may appear many years before the onset of airflow limitation. People with these symptoms, especially those at risk for COPD, should be checked to rule out other underlying conditions (s). It is important to use these patient symptoms to inform the creation of effective therapies. Without chronic dyspnea, coughing, or sputum production, there may also be a significant airflow restriction, and vice versa (Montes de Oca et al., 2010).

5.1.2.3 Medical History

A new patient with known or suspected COPD should provide a thorough medical history that includes:

The degree to which the patient is exposed to risk factors, such as smoking and work-related or environmental hazards.

Previous medical conditions such as asthma, allergies, sinusitis, nasal polyps, childhood respiratory infections, HIV, TB, and other chronic respiratory and non-respiratory illnesses.

A history of COPD or another chronic respiratory illness in the family.

The majority of patients with COPD are aware of increased dyspnea, more frequent or protracted "winter colds," and some social constraint for a number of years before seeking medical attention. COPD normally develops in adulthood.

A history of respiratory illness hospitalizations or exacerbations is needed. Even if these periods have not been documented, patients may be aware of periodic exacerbation of their symptoms.

The existence of coexisting conditions, such as cancer, heart disease, osteoporosis, musculoskeletal diseases, anxiety, and depression, which may also limit activity.

The disease's effects on the patient's life, such as activity restrictions, lost wages and other financial consequences, effects on family routines, depressive or anxious moods, wellbeing, and sexual activity.

5.1.2.3.1 Physical Examination

A physical examination is rarely diagnostic for COPD while being an important component of patient management. Physical indicators of airflow limitation typically don't appear until severe lung function impairment has taken place (Holleman and Simel1995), and physical examination-based detection has a relatively low sensitivity and

specificity. There may be a variety of physical symptoms associated with COPD, but their absence does not rule out the diagnosis.

5.1.2.3.2 Spirometry

The most accurate and repeatable method of measuring airflow restriction is spirometry. It is a simple exam that is easily accessible. Peak expiratory flow measurement alone cannot be consistently utilised as the only diagnostic test while having a high sensitivity and a low specificity (Çolak et al., 2019).

5.1.2.4 Assessment

The objectives of a COPD assessment are to ascertain the degree of airflow restriction, its effect on the patient's health state, and the risk of future occurrences (such exacerbations, hospital admissions, or death), in order to ultimately guide medication.

5.1.2.4.1 Classification of severity of airflow limitation

Classification is done according to $FEV_1$and categorised into GOLD 1, GOLD 2, GOLD 3 and GOLD 4.

5.1.2.4.2 Assessment of symptoms

It is increasingly understood that COPD affects individuals in ways other than only dyspnea (Jones, 2001) Due to this, a thorough evaluation of symptoms rather than just a measurement of breathlessness is advised.

5.1.2.4.3 Choice of thresholds

The CATTM and CCQ give measurements of the symptomatic impact of COPD but do not classify patients into symptom severity categories for therapeutic purposes. The SGRQ is the most thoroughly studied comprehensive measure; scores below 25 are

exceedingly unusual in healthy people and are not prevalent in COPD patients who have been diagnosed (Agusti et al.,2010).

5.1.2.4.4 Assessment of exacerbation risk

Exacerbations of COPD are characterised by a sudden worsening of respiratory problems that necessitates extra treatment (Hurst and Wedzicha, 2007). These incidents are categorised as mild (treated only with short acting bronchodilators (SABDs), moderate (treated only with SABDs plus antibiotics and/or oral corticosteroids), or severe (treated alone with SABDs) (patient requires hospitalisation or visits the emergency room). Acute respiratory failure may also be linked to severe exacerbations.

5.1.2.4.5 Assessment of concomitant chronic diseases (comorbidities)

At the time of diagnosis, patients with COPD frequently also have significant coexisting chronic illnesses, and COPD is a significant contributor to the development of multimorbidity, especially in elderly patients in response to typical risk factors (e.g., aging, smoking, alcohol, diet and inactivity) (Soler-Cataluña et al., 2005).

5.1.2.4.6 Combined COPD assessment

Combining the patient's spirometric categorization and/or risk of exacerbations with their symptom assessment helps doctors determine how COPD will affect each particular patient. Because it incorporated patient-reported outcomes and emphasised the significance of exacerbation prevention in the management of COPD, the "ABCD" assessment tool of the 2011 GOLD update represented a significant improvement over the straightforward spirometric grading system of earlier versions of GOLD.

5.1.2.4.7 Alpha-1 antitrypsin deficiency (AATD)

screening for AATD (alpha-1 antitrypsin deficiency). All patients with a diagnosis of COPD should undergo a screening at least once, according to the World Health Organization, especially in regions with a high prevalence of AATD (Soler-Cataluña et al.,2005). Although the usual patient had panlobular basal emphysema and is under 45 years old, it is now known that some AATD patients were discovered when they were older and had a more typical arrangement of emphysema (centrilobular apical) (Parr et al., 2004).

5.1.2.4.8 Additional investigations.

Additional investigations include Imaging, Lung volumes, Diffusing capacity of the lungs for carbon monoxide (DLco), Oximetry and arterial blood gas measurement, Exercise testing and assessment of physical activity, Composite scores, Differential diagnoses and Biomarker assessment.

5.1.3 Prevention and Maintenance Therapy

5.1.3.1 Smoking Cessation

About 40% of COPD patients are current smokers, which has a detrimental effect on the disease's prognosis and progression. A large fraction of COPD patients continue to smoke even when they are aware they have the disease (Montes de Oca M, 2020). The best way to change how COPD develops naturally is to stop smoking. Long-term quit success rates of up to 25% are possible if effective time and resources are devoted to the effort (van Eerd et al., 2016). Legislative smoking restrictions are effective at boosting stop rates and lowering the risks associated with second-hand smoke exposure, in addition to personal approaches to quitting smoking (Frazer et al., 2016).

5.1.3.2 Vaccinations

5.1.3.2.1 Influenza vaccine

Infections of the lower respiratory tract that necessitate hospitalisation, such as influenza, can be prevented with vaccination in COPD patients (Poole).

5.1.3.2.2 Pneumococcal vaccine

For all individuals under the age of 65, PCV13 and PPSV23 pneumococcal immunizations are advised. Younger COPD patients with substantial concomitant illnesses, such as chronic heart or lung disease, are also advised to use the PPSV23 (Tomczyk et al., 2014).

5.1.3.2.3 Oral vaccines

The US Centers for Disease Control (CDC) advises adults with COPD who were not immunised during adolescence to get the Tdap vaccine (also known as dTaP/dTPa) to protect against whooping cough, tetanus, and diphtheria, and adults with COPD older than 50 should get the Zoster vaccine to prevent shingles.

In accordance with government recommendations, individuals with COPD should receive the COVID-19 immunisation (Thompson et al., 2021).

5.1.3.3 Pharmacological therapies for stable COPD

Pharmacological treatment for COPD is used to improve exercise tolerance and general health status, as well as to lessen symptoms and the frequency and severity of exacerbations. There is insufficient evidence from individual clinical studies to demonstrate that medication can slow the pace of FEV1 decline (Burge et al., 2000).

5.1.3.3.1Bronchodilators

Bronchodilators are drugs that alter other spirometric variables and/or raise FEV1. The gains in expiratory flow indicate wider airways rather than changes in lung elastic recoil because they work by changing the tone of the smooth muscles that line the airways. Exercise performance is generally enhanced by bronchodilators' tendency to lower dynamic hyperinflation both at rest and during exercise (O'Donnell et al., 2004). It is challenging to infer the magnitude of these changes from the improvement in FEV1 assessed at rest, particularly in people who have moderate and very severe COPD (Berger et al., 1988).

Beta2-agonists: By activating beta2-adrenergic receptors, which raises cyclic AMP and results in functional opposition to bronchoconstriction, beta2-agonists primarily relax airway smooth muscle. Beta2-agonists come in short-acting (SABA) and long-acting (LABA) varieties. SABAs often lose their effect after 4 to 6 hours (Higgins et al., 1991). SABAs enhance FEV1 and symptoms when used frequently and as needed. Levalbuterol does not appear to have any advantages over standard bronchodilators for single-dose, as-needed usage in COPD (Sestini et al.,2002) LABAs exhibit a duration of action of 12 hours or more and do not preclude further benefit from SABA therapy administered as needed (Cazzola et al., 2013).

The twice-daily LABAs formoterol and salmeterol significantly reduce FEV1 and lung volumes, dyspnea, health status, exacerbation rate, and hospitalizations (Kew et al., 2013), but had no impact on mortality or the rate of lung function decrease. A once-daily LABA called indacaterol helps with exacerbation rate, health status, and dyspnea (Han et al., 2013, Geake et al., 2015). After inhaling indacaterol, some individuals report

coughing (Geake et al., 2015). Additional once-daily LABAs that enhance lung capacity and complaints include vilanterol and oladaterol (Koch et al., 2014).

5.1.3.3.2 Antimuscarinic drugs

Antimuscarinic medications prevent acetylcholine's bronchoconstrictor effects on M3 cholinergic receptors expressed in smooth muscle of the airways. (Melani et al., 2015) Ipratropium and oxitropium, two short-acting antimuscarinics (SAMAs), also block the inhibitory neural receptor M2, which may lead to vagally induced bronchoconstriction. Long-acting muscarinic antagonists (LAMAs), including tiotropium, aclidinium, glycopyrronium bromide (also called glycopyrrolate), and umeclidinium, have prolonged binding to M3 muscarinic receptors and faster dissociation from M2 muscarinic receptors, extending the duration of bronchodilator effect (Melani et al., 2015).

5.1.3.3.3 Methylxanthines

Ongoing debate surrounds the precise effects of xanthine derivatives. Although they have been claimed to have a variety of non-bronchodilator activities, the importance of these actions is debatable. They may work as non-selective phosphodiesterase inhibitors. (Aubie 1988; McKay et al.,1993;Moxham, 1988) There is a dearth of information on the lengt

5.1.3.3.4 Combination bronchodilator therapy

In comparison to raising the dose of a single bronchodilator, combining bronchodilators with various mechanisms and durations of action may raise the extent of bronchodilation with a decreased chance of side effects (Cazzola and Molimard, 2010; Ray et al., 2019). Combos of SABAs with SAMAs are more efficient at increasing FEV1 and symptoms than either drug alone (Gross et al., 1998).

5.1.3.3.5 Inhaled corticosteroids

The low responsiveness of COPD-associated inflammation to corticosteroids is suggested by in vitro research. Additionally, some medications like beta2-agonists, theophylline, or macrolides may help COPD patients become more sensitive to corticosteroids (Barnes, 2013; Boardman et al., 2014). This effect's clinical utility has not yet been thoroughly proven.

5.1.3.3.6 Triple therapy (LABA/LAMA/ICS)

When contrasted to LAMA alone, LABA/LAMA, and LABA/ICS, it has been demonstrated that the step-up in inhalation treatment to LABA with LAMA plus ICS (triple therapy) improves lung capacity, patient reported outcomes, and reduces exacerbations. (Lipson et al.,2018; Papi et al.,2018; Vestbo et al.,2017; Welte et al.,2009; Singh et al.,2016)

5.1.3.3.7 Oral glucocorticoids

Steroid myopathy (Manson et al., 2009), a side effect of oral glucocorticoids, can cause muscle weakness, impaired functioning, and respiratory distress in people with very severe COPD. Systemic glucocorticoids have been demonstrated to enhance lung function and decrease shortness of breath when used to treat acute exacerbations in hospitalised patients or during visits to the emergency room (Walters et al., 2014).

5.1.3.3.8 Phosphodiesterase-4(PDE4) inhibitors

By preventing the breakdown of cytoplasmic cyclic AMP, PDE4 inhibitors work primarily to lessen inflammation (Rabe et al., 2011). The once daily oral drug roflumilast has no direct bronchodilator effects. Patients with chronic bronchitis, severe to very exacerbations of copd, and a history of exacerbations benefit from roflumilast's reduction

of serious and moderate exacerbations managed with systemic corticosteroids (Calverley et al., 2009).

5.1.3.3.9 Antibiotics

In earlier trials, prophylactic, ongoing antibiotic treatment had no effect on the incidence of COPD exacerbations (Francis et al., 1961), and a research that looked at the effectiveness of chemoprophylaxis throughout the winter over the course of five years found no benefit (Johnston et al., 1969). Recent research has indicated that using some antibiotics regularly may lower the exacerbation rate (Herath et al., 2013).

5.1.3.3.10 Mucolytic and Antioxidants

Regular use of mucolytics such carbocysteine and N-acetylcysteine (NAC) may lessen exacerbations in COPD patients who are not receiving ICS and marginally enhance health status (Cazzola et al., 2015). Erdosteine, however, may have a considerable impact on (mild) exacerbations regardless of whether ICS is being used concurrently. The currently available data do not allow one to pinpoint precisely the possible target group for antioxidant medicines in COPD due to the variability of investigated populations, treatment dose, and concurrent therapies (Poole et al., 2019).

5.1.3.3.11 Other drugs reducing exacerbations

Prior to 2005, two RCTs on the use of an immunoregulator in COPD patients examined both the severity and frequency of exacerbations (Collet et al., 1997). The long-term consequences of this medication in individuals receiving the currently advised COPD maintenance therapy need to be further investigated.

5.1.3.3.12 Other pharmacological treatments

Alpha-1-antitrypsin augmentation is the rational strategy to reduce the onset and progression of lung illness in AATD patients.

5.1.3.4 Rehabilitation, Education and Self-management

5.1.3.4.1 Pulmonary rehabilitation

Pulmonary rehabilitation should be viewed as a component of integrated patient management, and it frequently involves a variety of medical specialists to guarantee complete coverage of the many factors at play. Prior to enrolment, patients should undergo a thorough evaluation that identifies their goals, unique healthcare needs, smoking status, nutritional status, capacity for self-management, health literacy, psychological health status, social circumstances, comorbid conditions, as well as exercise capabilities and limitations (Vogiatzis et al.,2016).

5.1.3.4.2 Tele-rehabilitation

As a treatment for COPD, pulmonary rehabilitation (PR) can be administered as an in- or outpatient programme. (Lacasse et al., 2015) There is convincing evidence that self-management therapies and exercise training, along with disease-specific education, are essential PR components that can help practically all COPD patients (McCarthy et al., 2015).

5.1.3.4.3 Education, self management and integrative care

Patients are frequently "educated" by their healthcare practitioners by way of information and guidance, with the understanding that knowledge will result in behaviour modification. Although increasing patient knowledge is a crucial first step in changing behaviour, didactic group sessions fall short of effectively fostering self-management abilities.Iterative encounters between patients and healthcare providers who are skilled in

offering self-management treatments are necessary for the process to work. Utilizing behaviour modification strategies, patients' motivation, competence, and confidence are increased. Comprehensibility is improved through the employment of literacy-sensitive techniques (Effing et al., 2016). Given the complexity of COPD, numerous healthcare professionals must contribute and collaborate closely. Although there is conflicting evidence, it seems to reason that using a formal, organised programme to specify how each component is given should improve care's effectiveness and efficiency. An integrated care programme improved a lot of clinical outcomes, but not death, according to a meta-analysis of small trials (Kruis et al., 2013).

5.1.3.5 Supportive, End-of-life, Palliative and Hospital care

5.1.3.5.1 Symptom control and palliative care

Palliative care is a comprehensive phrase that includes methods for managing dying patients who are near to passing away as well as strategies for symptom control. Regardless of the disease stage or the need for further medicines, the aim of pain management is to prevent, relieve, and maintain the highest life quality for patients and their families. A lot of the symptoms associated with COPD, including fatigue, dyspnea, sadness, anxiety, and insomnia, call for symptom-based palliative therapy. Evidence suggests that individuals with COPD are much less likely than those with lung cancer to receive these services (Au et al., 2006; Levy et al., 2012).

5.1.3.5.2 Therapy relevant to all patients with COPD

Many COPD patients nonetheless endure severe dyspnea, reduced exercise capacity, exhaustion, panic, anxiety, and depression even while receiving the best medical care possible. Palliative therapies, which in the past have frequently only been used in instances of end-of-life care, can be used more widely to alleviate some of these symptoms (Han et al., 2016).

5.1.3.5.3 End of life and hospice care

In many individuals with COPD, the disease trajectory is characterised by a progressive deterioration in health status and an increase in symptoms, interspersed by acute episodes that are linked to an elevated risk of death (Murray et al., 2005). Although hospitalisation for a COPD acute exacerbation is associated with a decreasing death rate (Eriksen and Vestbo, 2010) reported rates still range from 23% (Groenewegen et al., 2003) to 80%. (Gudmundsson et al., 2012).

5.1.3.6 Other treatments

5.1.3.6.1 Oxygen therapy

It has been demonstrated that giving patients with chronic pulmonary failure oxygen for a prolonged period of time (more than 15 hours per day) increases their chance of surviving when they have severe resting hypoxemia (Cranston et al., 2005).

5.1.3.6.2 Ventilatory support

Noninvasive ventilation (NIV) in the form of noninvasive positive pressure ventilation (NPPV) is the standard of care for decreasing morbidity and mortality in patients hospitalized with an exacerbation of COPD and acute respiratory failure (Elliott and Nava, 2012).

5.1.3.7 Interventional therapy

5.1.3.7.1 Surgical interventions

Sections of the lungs are removed during a surgical operation called lung volume corrective surgery (LVRS), which increases the mechanical efficiency of the breathing muscles and lowers hyperinflation (Criner et al., 1998).

5.1.3.7.2 Bronchoscopic interventions

Less invasive bronchoscopic methods for lung reduction have been investigated because of the morbidity and mortality linked to LVRS (Criner et al, 2011). These methods include a variety of different bronchoscopic procedures (Criner et al., 2011). Although these methods differ greatly from one another, they all share the same goal of reducing thoracic volume in order to improve lung, chest wall, and respiratory muscle mechanics.

5.1.4 Management of Stable COPD

The assessment of symptoms and the potential for exacerbations in the future should serve as the foundation of the management approach for stable COPD.

5.1.4.1 Identification and reduction in exposure to risk factors

5.1.4.1.1 Tobacco smoke

A crucial strategy for all patients with COPD who still smoke is quitting. Healthcare professionals play a crucial role in communicating with patients about smoking cessation methods and messages, and they should constantly urge patients to stop smoking.

5.1.4.1.2 Indoor and outdoor air pollution

In order to reduce exposure to both indoor and outdoor air pollution, a mix of public policies, regional and federal funding, cultural shifts, and patient-specific safety measures are needed. To lower the incidence of COPD globally, it is essential to minimise exposure to biomass fuel smoke. The use of non-polluting cooking stoves, effective ventilation, and similar measures should be encouraged (Romieu et al., 2009; Liu et al., 2007).

5.1.4.1.3 Occupational exposures

No studies have been done to show that measures that lessen occupational exposures also lessen the burden of COPD, although it seems sense to advise patients to try to stay away from continual exposures to potential irritants.

5.1.4.2 Pharmacological Treatment of Stable COPD

Pharmacological treatments can enhance a patient's health condition and ability to tolerate exercise while reducing symptoms, exacerbation risk, and exacerbation intensity.

5.1.4.3 Non-pharmacological treatment of Stable COPD

The comprehensive care of COPD should include non-pharmacological treatment as a supplement to pharmaceutical treatment.

5.1.4.3.1 Education and self management

Self-management therapies are designed to encourage, involve, and guide patients in making positive changes to their health behaviour(s) and developing skills to more effectively manage their COPD on a daily basis (Effing et al., 2016). In order to assist patients acquire and practise sustainable self-management skills, doctors and other healthcare professionals must go beyond simple instruction and advice-giving (didactic) approaches.

5.1.4.3.2 Physical activity

A technique with definite benefits is pulmonary rehabilitation, including community- and home-based programmes. Promoting and maintaining physical exercise, though, is difficult. There is proof that people with COPD engage in less physical activity (Pitta et al., 2005).

5.1.4.3.3 Pulmonary rehabilitation programs

High symptom burden and exacerbation risk patients should be encouraged to participate in a formal rehabilitation programme that includes defining patient goals and is structuredly created and delivered, taking into account the individual's COPD characteristics and morbidities (Spruit et al., 2013, Vogiatzis et al., 2016, Garvey et al., 2016).

5.1.4.3.4 Exercise training

Exercise training alone or in combination with activity counselling significantly increased the amount of physical activity among COPD patients, according to a meta-analysis of RCTs. (Lahham et al., 2016) Strength training yields superior results when combined with either constant force or interval training (Ortega et al., 2002).

5.1.4.3.5 End-of-life and palliative care

Palliative care aims to reduce the pain of patients and families by thoroughly evaluating and treating the symptoms that patients experience physically, psychologically, and spiritually.

5.1.4.3.6 Nutritional support

As the severity of the disease worsens in COPD patients, losing weight and malnutrition set a terrible prognosis. In people with COPD, malnutrition is linked to lower quality of life, higher mortality, increased hospitalizations, decreased exercise tolerance, and compromised lung function (Collins et al., 2018; Schols et al., 2005).

5.1.4.3.7 Interventional bronchoscopy and surgery

A patient with emphysema may require surgical resection (lung volume reduction surgery, LVRS) or bronchoscopic lung reduction (endobronchial valve, coil implantation, or thermal ablation) to manage hyperinflation. These factors include the degree and type of emphysema detected on HRCT, the presence of interlobar collateral ventilation determined by fissure integrity on HRCT or physiological assessment (endoscopic balloon occlusion and flow assessment), the regional accessibility of the various therapies for clinical care, local proficiency in the execution of the procedures, and patient and provider preferences. The only lungs reduction therapy that has been documented to be carried out successfully at the segmental instead of lobar level is vapour ablation therapy (Herth et al., 2016).

5.1.5 COPD and Comorbidities

Comorbidities diseases that frequently accompany with COPD) may significantly affect prognosis. (Barnes et al., 2009; Campo et al., 2013; Miller et al., 2013) While some of these may develop independently of COPD, others may be causally connected, either due to common risk factors or because one disease raises the risk or exacerbates the intensity of the other. This mechanism shows a connection between COPD and a few of its complications because it's likely that characteristics of COPD are common with other diseases. The effects of COPD, such as decreased physical activity or continuing smoking, can raise the risk of comorbid illness.

5.1.5.1 Cardiovascular Disease (CVD)

Systolic or diastolic heart problems affects 20 to 70% of COPD patients, and its annual incidence is between 3-4%. A major and consistent predictor of mortality from all causes

is incident heart failure(Bhatt et al.,2013). Both COPD and cardiac arrhythmias are frequent conditions. The FEV1 is lowered and atrial fibrillation is common (Buch et al., 2003).

5.1.5.2 Lung Cancer

Epidemiological studies and observational cohorts studies have consistently supported the existence of a link between COPD and lung cancer (López-Encuentra et al., 2005; Mannino et al.,2003; de Torres et al.,2011).

5.1.5.3 Osteoporosis

A significant comorbidity that frequently goes undiagnosed (Madsen et al., 2010) and is linked to a poor health status and prognosis is osteoporosis.

5.1.5.4 Anxiety and depression

A poor prognosis (Ng et al., 2007; Eisner et al., 2010) younger age, female sex, smoking, poorer FEV1, cough, higher SGRQ score, and a background of cardiovascular disease are all related with anxiety and depression, which are significant comorbidities in COPD.

5.1.6 COVID-19 and COPD

The fear of contracting COVID-19 and the impact of the pandemic on social services and/or fundamental societal functions on COPD patients' health are added stressors to this disease. Due to decreased face-to-face consultations, challenges with spirometry, and restrictions on traditional rehabilitation and home healthcare programmes, the COVID-19 epidemic has made normal maintenance and identification of COPD more challenging. Medication shortages have also affected patients (Mahase 2020).

5.1.7 New Target identification, Drug discovery and current research

Inhaled long-acting bronchodilators are the cornerstone of the care of stable illness, but corticosteroids are most helpful for individuals who also have coexisting symptoms of asthma, such as eosinophilic inflammation and much more reversible airway blockage. Other than quitting smoking, no treatments slow the spread of the illness. To find new treatments that stop disease activity and development, as well as to truly understand disease mechanisms, further research is required.

New therapeutic targets have been discovered and treatments thereafter have been proposed. TLR blockers, inflammasome inhibitors, chemokine antagonists, kinase inhibitors, protease inhibitors and anti fibrotic agents are believed to change the course of the disease. Protease inhibitors include neutrophil elastase inhibitors and MMP9 inhibitors. In case of excessive inflammation alveolar macrophages get activated which in turn recruit neutrophil to the inflammatory sites. Those neutrophil release an enzyme, a serine protease which in excess degrades the lung cell wall causing emphysema and mucus overproduction. There are several reports that neutrophil elastase is a major factor in prognosis of COPD and thus inhibiting the enzyme can ultimately lead to decline in disease severity and help in improvement of patient's life.

5.2 Human Neutrophil Elastase

5.2.1 *Structure*

Human leukocyte elastase has been determined by x-ray crystallography to be a single-chain polypeptide of 218 amino acids containing four intramolecular disulfide linkages joining eight half-cystine residues in the following ways: Cys. 26 to 42, 122 to 179, 132 to 158, and 169 to 194. The protein is Nglycosylated at two locations, Asn-95 and Asn-

144, which were both independently verified by crystallographic evidence and inferred indirectly from the absence of an unidentified phenylthiohydantoin-conjugated amino acid at these locations during suitable peptide sequencing Since protein sequence analysis revealed no evidence of microheterogeneity, we conclude that the isoenzyme nature of neutrophil elastase preparations found by acid gel electrophoresis is caused by minor difference in carbohydrate (Sinha et al., 1986).

5.2.2 Mechanisms for pathogenesis of chronic lung disease

Elastase, cathepsin G, proteinase-3, cathepsins, and different metalloproteinases made by neutrophils and macrophages, as well as other proteinases, are key players in the pathophysiology of COPD. Alpha-1-antitrypsin, secretory leukocyte proteinase inhibitor, and tissue inhibitors of MMPs are the main anti-proteinases involved in the aetiology of COPD. When the delicate balance is upset by internal or external influences, the person is vulnerable to developing a chronic, lethal lung illness that will cause incapacity and eventually death (Demkow, U. & van Overveld, F. J. 2010).

5.2.3 Role in Airway Diseases

5.2.3.1 COPD

The activation of NET release into the airway environment by NE and MPO spreads the proteolytic and pro-inflammatory actions of NE and neutrophil granules (Genschmer et al., 2019). Increased exacerbations, reduced neutrophil phagocytosis, and lower lung function are all linked to NET predominance in the COPD airway (Dicker et al., 2017).

5.2.3.2 Asthma

Asthma and COPD deteriorate more quickly as a result of NE activity because the basement membrane thickens more.

5.2.3.3 Cystic Fibrosis

The primary cause of morbidity and mortality in CF, despite the fact that it affects multiple organ systems, is chronic lung illness brought on by infection and inflammation of the airways, which results in bronchiectasis and respiratory failure (Egan et al., 2020).

5.2.3.4 Bronchiectasis

A persistent and abnormal airway enlargement with mucus obstruction are characteristics of the condition known as bronchiectasis. Primary ciliary dyskinesia and primary immunodeficiencies are two examples of hereditary disorders that can result in bronchiectasis. However, mechanical airway obstruction, recurring insults including aspiration, secondary immunodeficiency, severe bacterial or viral pneumonia, and subsequent airway injury can all contribute to bronchiectasis (Boucher et al., 2020).

5.2.3.5 Bronchopulmonary Dysplasia

When a premature newborn needs supplementary oxygen at 36 weeks post-gestational age, the condition is known as bronchopulmonary dysplasia (BPD), a chronic lung illness. BPD is the outcome of a complex process where factors like gestational age, birth weight, ventilator support, and oxygen toxicity impair healthy lung development, which is ascribed to a stop in lung development (Voynow et al., 2017).

5.2.4 *Inhibitors*

The need for NE-targeted treatments is driven by mounting evidence that NE plays a significant role in the aetiology of chronic lung illnesses. Increased antiprotease activity is one approach that could directly address the protease-antiprotease imbalance present in these disorders. Based on evidence of NE inhibition, replacement medication has been approved for patients with 1-antitrypsin deficiency. Emphysema progression is halted by 1-antitrypsin infusion in three significant multicenter placebo-controlled, randomised, double-blind trials, as measured by CT scores during a 2-year time period.

Clinical trials have examined anti neutrophil elastase treatments. The only NE synthetic inhibitor with clinical approval is sivelestat, which is only available in Japan and Korea to treat acute lung damage and respiratory distress syndrome. The Data Safety Monitoring Board, which oversaw the study, however, forced an early end to studies testing Silvelestat as a therapy for acute respiratory distress syndrome in the US because to increased long-term mortality for Silvelestat subjects (Zeiher et al., 2004).

5.3 Herbal extracts and compounds targeting human neutrophil elastase

Polyphenolic substances like flavonoids are generated from plants. For the past 20 years, their antiprotease and anti-inflammatory activity has been researched. A number of flavonoid glucuronide compounds suppressed NE release from active neutrophils by 30–50% at 1 M and decreased ROS release by 50–70% at 10 M. With an IC_{50} in the micromolar range, several modified flavonoids also exhibit anti-elastase activity. These substances have not yet been studied in chronic lung illnesses that are characterised by inflammation that is predominately neutrophilic (Granica et al., 2013).

5.4 Synthetic molecules targeting human neutrophil elastase

Numerous novel mechanism-based inhibitors have recently been described as a result of ongoing attempts to find and improve them. These substances exhibit encouraging potency and selectivity profiles, but their intrinsic stability still places restrictions on their application. Two novel families of synthetic, nonelectrophilic, effective and selective human neutrophil elastase inhibitors with increased stability and general drug-like characteristics have recently been discovered. The most comprehensive drug in these classes, AZD9668, has been shown to have considerable effects on important biomarkers in populations of patients with cystic fibrosis and bronchiectasis.

6

REVIEW OF LITERATURE

Review of Literature

COPD

Chronic Obstructive Pulmonary Disease is increasing in an alarming rate all over the world. Every year, there are about three million deaths worldwide. (Mohsen. et al., 2015) The prevalence of COPD is predicted to increase over the next 40 years, and by 2060, there may be more than 5.4 million annual deaths from COPD and related illnesses due to rising smoking rates in emerging nations and ageing populations in high-income nations. (Lopez et al., 2006). Airflow issues were brought on by emphysematous lesions, which raised the resistance of the tiny airways and increased the compliance of the lung (Hogg et al., 2004). Dyspnoea and chronic sputum production are the main indicators and symptoms of COPD. A typical early sign of COPD is exertional dyspnea. Although a variety of reasons can cause dyspnoea, constriction of the airways is a significant contributing factor (O'Donnell et al., 1993).

There have been several COPD longitudinal studies have followed populations and groups for up to 20 years (Lange et al., 2005) to date no research findings have tracked the disease's progress throughout its entire course or taken into account the prenatal and perinatal years, which may be crucial in determining a person's future COPD risk. Therefore, there are still significant gaps in our understanding of the risk factors for COPD. Although smoking cigarettes is the major ecological risk factor for COPD, less than 50% of daily smokers ever acquire the disease (Rennard, S. I., & Vestbo, J., 2006).

The prevalence of AATD PiZZ genotypes in COPD patients ranged from 1 in 408 in Northern Europe to 1 in 1,274 in Eastern Europe, according to a systematic review of 20 studies in European populations (Blanco et al., 2020). Markers surrounding the alpha-

nicotinic acetylcholine receptor, hedgehog interacting protein (HHIP), and other genetic loci have been related with COPD (or FEV1 or FEV1/FVC as the symptom) in various genome-wide association studies. However, it is still unclear if these genes actually cause COPD or are only indicators for causal genes (Cho et al., 2010).

There are sex-based prevalence variations between WHO Global Burden of Disease sub-regions, according to a comprehensive study and meta-analysis of the worldwide incidence of COPD. In North America (8.07% vs 7.30%) and in metro regions (13.03% vs 8.34%), females had the highest frequency of COPD. According to the World Bank's income classifications, prevalence was highest for males in upper-middle income nations (9.00%) and for females in high-income countries. Obstructive sleep apnea and bronchiectasis are two comorbid diseases that might affect women with COPD (Montserrat-Capdevila, et al, 2021).

A significant portion of young people with COPD report having a family history of respiratory conditions and/or early life experiences (such as hospital admissions before the age of 5), which supports the idea that COPD may have early-life origins. (Cosío, et. al., 2020)

Sculptors, gardeners, and warehouse workers were among the occupations linked to a higher risk of COPD among never-smokers and non-asthmatics in a study of the demographically UK Biobank cohort (De Matteis et al., 2019) A cross-sectional cohort study showed that self-reported exposure to occupational dust and fumes is linked to greater emphysema and gas trapping in both men and women, as measured by computed tomography scans, in in addition to increased airflow limitation and breathing difficulties (Marchett et al., 2014). According to an examination of data from the National Health and Nutrition Examination Survey III, which included data on over 10,000 persons between

the ages of 30-75, the percentage of COPD that may be attributed to job exposures is 19.2% overall and 31.1% among non-smokers (Hnizdo et al., 2002).

The pathophysiology of chronic airflow restriction in non-smokers and smokers with asthma is noticeably different, indicating that the two disease categories may continue to be distinct even when they present with identical levels of decreased lung function (Silva,. et al., 2004).

The transcription factor Nrf2, which controls a number of antioxidant genes, may be less abundant in COPD patients, which could lead to a decrease in endogenous antioxidants. (Menezes et al., 2007).

Emphysema is thought to be characterised by the protease-mediated breakdown of elastin, a significant connective tissue component of the lung parenchyma; however, this characteristic may be more elusive in airway alterations (Stockley et al., 1999).

According to a study, local IgA deficiency is linked to airway remodelling, small airway inflammation, and bacterial translocation. (Massion et al., 2017)

In a prospective study of COPD patients followed for 10 years, it was discovered that increased telomere shortening, a sign of accelerated ageing, is associated with progressively deteriorating pulmonary gas exchange, lung hyperinflation, and extra pulmonary affection. Over this period of observation, telomere lengths that are consistently shorter increase the chance of death from all causes (Córdoba-Lanús et al., 2021).

Mucus hypersecretion is induced by a number of mediators and proteases, and many of them work by activating the epidermal growth factor receptor (EGFR) (Burgel, P. R., & Nadel, J. A. 2008). Patients with COPD frequently experience the release of

inflammatory mediators into the bloodstream, which can lead to or exacerbate diabetes, ischemic normocytic anaemia, heart disease, depression, osteoporosis, heart failure, and lung cancer (Barnes 2009). Reports suggest that adequate comorbidity management is essential for COPD patients. (Soriano et al., 2005)

Pharmaceutical and non-pharmacological treatments should aim to reduce symptoms, improve quality of life, increase exercise tolerance, stop the progression of the disease and accompanying conditions, and improve prognosis because COPD cases present with irreversible limitations in proper airflow in the lungs (Braido et al., 2015).

Current Treatments of COPD

In light of the improvement in dyspnea, a recent study recommends evaluating the effects of bronchodilators using changes in airway resistance in the resting tidal volume range rather than FEV1. (Santus et al., 2014)

Patients with mild intermittent symptoms of COPD may benefit from using short-acting 2 agonists and anticholinergics.(Ofir et al., 2008)

Tiotropium, glycopyrronium, aclidinium, and umeclidinium are examples of long-acting muscarinic drugs (LAMAs), also known as long-acting anticholinergics. These substances are more selective for M3 receptors than M2 receptors and disassociate from M3 receptors more slowly, which may result in longer-lasting bronchodilation. (Vogelmeier C & Banerji D., 2011, Gavalda et al., 2009 , Gross et al., 2004).

Indacaterol significantly increased the trough FEV1, according to randomised controlled trials. (Dahl R et al., 2011).

Theophylline plasma levels needed to be between 10 and 20 ng/ml for efficient bronchodilation. Theophylline levels must be closely monitored since they can be hazardous. Low-dose theophylline (approximately 5 g/ml at plasma level) has been shown to have anti-inflammatory effects in patients with COPD in recent years (Barnes PJ. 2006; Culpitt et al., 2002). This anti-inflammatory impact is probably brought on by rising HDAC2 expression and activity in COPD patients' alveolar macrophages (Ito et al., 2004).

Roflumilast significantly reduced the frequency of moderate to severe exacerbations and significantly increased the trough FEV1 in a 52-week randomised controlled trial involving 3091 COPD patients (Calverley et al., 2009).

Fluticasone-vilanterol modestly reduced the rates of moderate and severe exacerbation more than vilanterol alone, according to a multicenter, 24-week trial in which stable, moderate-to-severe COPD patients were enrolled and randomly assigned to receive fluticasone-vilanterol, fluticasone, vilanterol, or a placebo (Martinez et al., 2013).

Future Therapeutic Targets

The finding that a brief treatment of the antidepressant bupropion is an efficient adjuvant for smoking cessation in people with COPD represents a significant advancement (Buist et. al., 2001).

Microorganisms, uric acid, and extracellular ATP, acting through P2X purinergic receptor 7 (P2X7) and potassium efflux, may activate the NLRP3 inflammasome in COPD (Tashkin et al., 2010). Caspase 1 inhibitors (for which small-molecule inhibitors like VX 765 have been discovered) or P2X7 antagonists may target the inflammasome directly.

A decrease in HDAC2 activity and expression as a result of oxidative and nitrative stress may be the cause of corticosteroid resistance. The glucocorticoid receptor is more acetylated as a result of the decreased HDAC2 expression, which inhibits it from suppressing NF-B-driven inflammation. Reversing this corticosteroid resistance by raising the expression and activity of HDAC2 is a novel and promising treatment approach. (Ito et al., 2006).

At least 50% of COPD patients, especially those with severe disease, have bacterial colonisation of the lower airways, and these bacteria are the same ones linked to exacerbations. Although a notable advantage was seen in patients with purulent sputum, pulsed treatment with moxifloxacin for 5 days every 8 weeks failed to diminish exacerbations or lung function (Garcha, D. S. et al., 2012; Seemungal et al., 2008).

Hepatocyte growth factor (HGF; also referred to as scatter factor) is crucial for alveolar formation during the development of the lung and works by binding to the MET receptor. By stimulating stem cells, intranasal administration of HGF lessens elastase-induced emphysema in mice (Hegab et al., 2008).

In individuals with COPD, an inhaled EGFR inhibitor (BIBW 2948) failed to significantly lower gene expression or demonstrates any therapeutic effect (Woodrufft et al., 2010).

Fibroblast growth factors (FGF) 1 and 2 have been found in vascular and airway smooth muscle, with enhanced FGFR-1 in the intima of blood vessels. Vascular remodelling is recognised as an early characteristic of COPD (DaCosta et al., 2004).

The PDE4 inhibitor roflumilast, which is licenced for treating severe COPD exacerbations, improves lung function in patients with the disease, but this improvement

cannot be attributed to bronchodilation; rather, it is most likely the result of the medication's anti-inflammatory properties. In fact, neutrophils, T cells, and macrophages all express PDE4 as the main phosphodiesterase (Stevens, T. et al., 2011).

Neutrophil Elastase

Patients with various respiratory illnesses have remarkably high levels of neutrophil elastase in their BAL, sputum, and fluid (Oriano et. al., 2020; Kummarapurugu et. al., 2022; Margaroli et. al., 2022; Keir, H. R. et. al., 2022).

Collagen, elastin, laminin proteoglycans, and fibronectin are just a few examples of the structural proteins that can be broken down by HNE using its active serine to hydrolyze cleavable amide bonds (Pham 2008; Chua and Laurent, 2006). HNE is essential for chemotaxis and promotes neutrophil movement by dissolving adhesion molecules (Cepinskas et al., 1999; Hermant et al., 2003).

It is reported that human neutrophil elastase works by destroying pathogens to control inflammation and maintain tissue homeostasis (Pham C. T. 2006). Several reports also indicate that neutrophil elastase plays a pathophysiological role in airway hyperresponsiveness in addition to damaging tissue (Suzuki et. al., 1996).

HNE plays crucial roles in a wide range of other diseases, such as psoriasis and other skin illnesses (Meyer-Hoffert et al., 2004; Marto et al., 2018), rheumatoid arthritis (Cawston and Young, 2010), atherosclerosis (Henriksen and Sallenave, 2008), and different malignancies of the breast and lungs (Albrengues et al., 2006; Hunt et al., 2013).

Recent studies reveal the participation of key elements of neutrophil innate immunity as evaluated in COVID-19 patients (Guéant et al., 2021) and the potential contribution of neutrophil elastase to lung illnesses associated with COVID-19 (Mohamed et al., 2020).

Consequently, it is thought that human neutrophil elastase could be a useful therapeutic target (Henriksen et al., 2014).

Human neutrophil elastase (HNE) is a powerful stimulator of mucus release in the airways in addition to having elastolytic activity. In clinical trials involving COPD patients, a number of low-molecular-weight neutrophil elastase inhibitors did not produce positive results (Groutas et al., 2011). In animal models of neutrophil elastase-induced lung injury, inflammation brought on by cigarette smoke, and emphysema, the neutrophil elastase inhibitor AZ9668 has been successful (Stevens et al.,2011).

Elafin and secretory leukocyte protease inhibitor are two examples of NE inhibitors that are naturally produced at the sites of tissue damage or by the liver (in the case of a1-AT), and their production is enhanced in response to inflammatory stimuli. They are created by epithelial cells, macrophages, and neutrophils (Henriksenet al., 2014).

Natural Inhibitors of Human Neutrophil Elastase

A novel serine protease inhibitor was found by Luan *et al.* in the cDNA library of the Scolopendra Hainanum centipede's venom glands. This substance, known as ShSPI, has been discovered to be an atypical kazal-type protease inhibitor that may be turned into a brand-new medication for the treatment of diseases brought on by human elastase (Luan et al., 2019).

Trypsin, chymotrypsin, plasin, and human neutrophil elastase can all be inhibited by the spider peptide AvKTI (Araneus ventricosus). AvKTI exhibits a reduced neutrophil elastase inhibitory activity of roughly 44.4-fold, inhibiting neutrophil elastase with an IC50 value of 446.93 nM and plasmin with an IC50 value of 10.07 nM. AvKTI has a Ki

value of 4.89 nM against plasmin and 169.07 nM against neutrophil elastase. (Wan et al., 2013).

After the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) was added to the culture medium, the filamentous fungus Beauveria felina produced eight cyclodepsipeptides, three of which are brand-new substances, such as desmethylisaridin C2, desmethylisaridin E, and isaridin F (Chung et al., 2013).

AFUEI is an elastase inhibitor that Okumura *et. al.* isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis and Aspergillus fumigatus strain AFU-12 (Okumura et al., 2008).

Due to their secondary metabolites' typical restricted conformation, their associated metabolic stability, and their associated biological activities, cyanobacteria, which are ancient forms of life that are widely distributed on our planet, appear to be very interesting in order to promote pharmaceutical advancements (Mehner et al.,2008).

The Florida Atlantic coast plant Lyngbya confervoides produces lyngbyastatin 4, a depsipeptide with two unusual homotyrosine and Ahp residues that has the capacity to inhibit human neutrophil elastase with an IC_{50} value of 49 Nm (Salvador et al., 2013).

Taori *et al.* have identified the structure of Lyngbyastatin 7, a powerful chemical isolated from *Lyngbya spp.* It has been examined by Salvador *et al.* as an inhibitor against 68 proteases at a single concentration. It demonstrates particular activity against proteinase K, chymotrypsin, and serine protease elastase; its IC_{50} value for HNE is 23 nM (Salvador et al., 2013).

In two separate bacteria, tutuilamide A and tutuilamide B from the cyanobacterium *Schizothrix sp.* and tutuilamide C from *Coleofasciculus sp*., Keller et al. discovered three novel peptides with Ahp and Abu residues (Keller et al., 2020).

A convergent synthetic method comparable to the entire synthesis of lyngbyastatin 7 has been published for the total synthesis of tutuilamide A (Luo, D., 2006). Evaluation of its inhibitory efficacy against HNE revealed significant selectivity for this enzyme Compared to lyngbyastatin 7, tutuilamide A exhibits greater inhibitory action against PPE. Lyngbyastatin 7 appears to be equally effective as it in preventing HNE, if not more so Tutuilamide A and Lyngbyastatin 7 had respective IC50 values of 0.73 nM and 0.85 nM against HNE (Chen et al., 2020).

Loggerpeptins A–C and molassamide were discovered by Al-Awadhi *et al.* in the marine cyanobacterium DRTO–73 of Loggerhead Key, Florida. Previously obtained from the cyanobacterium Dichothrix utahensis, molassamide was studied by Gunasekera et al. for its structure and inhibitory efficacy against porcine pancreatic elastase, -chymotrypsin from the pancreas of cattle, and trypsin from the pancreas of pigs (Al-Awadhi et al., 2018).

Six cyclic depsipeptides called symplostatins 5–10 are produced from the red marine cyanobacterium Symploca sp. Their antiproteolytic efficacy against human neutrophil elastase was assessed by Salvador et al. Symplostatins 8–10 among them demonstrate a more potent and effective suppression of HNE (Salvador et al., 2013).

The strain *Nostoc insulare* was recognised by Mehner *et al.* as a significant source of HLE inhibitors. They discovered eight cyanopeptolins, known as insulapeptolides A to H, with IC_{50} values ranging from micro- to nanomolar values and the capacity to suppress HLE (Mehner et al., 2008).

The L-amino acid content of brunsvicamides A and B distinguishes them, whereas brunsvicamide C exhibits an N-methyl-NI-formylkynurenine produced from tryptophan in the D-configuration. With Ki values of 1.1 M, 0.70 M, and 1.6 M, respectively, evaluated using a competitive inhibition mechanism, and IC_{50} values of 3.12 M, 2.00 M, and 4.42 M, respectively, these compounds have selective activity against human leukocyte elastase (Sisay et al., 2009).

The fermented Gram-negative bacteria *Flexibacter* sp. strain Q17897 from a soil sample that Yasumuro *et al.* discovered to have cyclic depsipeptides which inhibit human leukocyte elastase, with respective IC_{50} values of 1.5 107 M and 3.0 107 M (Yasumuro et al., 1995).

Lee *et al*. extracted the derivatized peptides ixorapeptide I and II from the methanol extract of the aerial portion of Ixora coccinea. Only Ixorapeptide II, with an IC_{50} value of 0.27 g/mL, can prevent the release of elastase in an anti-inflammatory assay, suggesting potential anti-inflammatory effect on neutrophils (Lee et al., 2010).

Human neutrophil elastase can be inhibited by roseltide rT1, which was isolated from the medicinal plant Hibiscus sabdariffa, in a dose-dependent manner with an IC_{50} value of 0.47 M. The structure of roseltide rT1 contains disulfide bridges, which strengthen its resistance to the acid destruction facilitated by proteinases and human serum (Loo et al., 2016).

After being obtained from a natural source in a low yield, Cui *et. al.* recently outlined the chemical synthesis of cyclotheonellazole A to further explore its potential for use in pharmaceutical research as a lead molecule. Further testing revealed an IC_{50} value of 0.321 M for the synthesised Cyclotheonellazole A against human neutrophil elastase. As a result, they demonstrated that this substance is a more potent inhibitor of elastase than

sivelestat (IC_{50} = 0.704 M), which contradicts the findings of the earlier study by Issac et al. This is likely because the experimental conditions were different, such as the source and concentration of the enzyme, the substrate, and the incubation period (Cui 2022)

Seed extracts from W. filifera are effective in inhibiting the elastase enzymes implicated in skin aging (Era et al., 2021).

Locals regularly utilise fruits of *Sonneretia apetala* as medicine because of its pharmacological efficacy to treat conditions like asthma, cough, inflammation, hepatitis, diarrhoea, bruising, etc. Additionally, traditional healers in Bangladesh recommend *Sonneretia apetala* as an anti-inflammatory medication to lessen gastrointestinal issues like diarrhoea, dysentery, and cramps (Shefa et al., 2014; Bandaranayke 2002, Tea and Ravishankar, 2013).

Synthetic Inhibitors of Human Neutrophil Elastase

In a recent study, the synthesis of benzoxazinone analogues 3 was revealed. These compounds have a dual mechanism of action by preventing the production of superoxide anion and the release of HNE. It was discovered that chlorine's ring A substitution was crucial for HNE inhibition (Shreder et al.,2009).

It has been demonstrated that substituted azetidine-2, 4-diones 6 are extremely powerful and specific HNE inhibitors (Mulchande et al., 2010). A new class of HNE inhibitors, known as N-benzoylpyrazoles 7, work to block the enzyme by quickly acylating the serine in the active site (Schepetkin et al., 2007).

Sivelestat (Elaspol), marketed in Japan and Korea for the treatment of acute lung injury associated with systemic inflammatory response syndrome, continues to be the HNE acylating agent of choice (Hayakawa et al., 2010). It is also being investigated for a

variety of other indications; including sepsis associated with acute respiratory distress syndrome and disseminated intravascular coagulation.

In an observational study with 32 patients, sivelestat administration decreased neutrophil elastase activity and return to baseline after sivelestat cessation (Hashimoto, S., et al., 2008). In another single-center, single blind, placebo controlled trial Sivelestat decreased serum IL-6, IL-12, C-reactive protein (Akamoto et al., 2007).

During a randomized, double blind, placebo controlled, parallel group, phase IIa study including 56 patients with a clinical diagnosis of Cystic fibrosis treated with 27 AZD9668(neutrophil elastase inhibitor) and 29 placebos, there were significant reduction in inflammatory parameters (Elborn et al., 2012).

In a Randomized, double blind, placebo controlled, phase IIb, trial, AZD9668 caused no difference in lung function, symptoms, QoL, SGRQ score, or incidence of exacerbation(Kuna et al., 2012).

Another phase II clinical research using inhaled α1-AT in patients with cystic fibrosis had no impact on pulmonary function but did lower free NE activity, neutrophil count, PA bacterial load, IL-8 levels, and IgG levels (Griese et al., 2007).

Reports suggest that in animal model (mice), administration of Sivelestat after infection, cause a delayed mortality and there is a decrease in inflammatory cells in lungs (Yanagihara et al., 2007).

In animal model of PA lung infection EP-I-hNE4 caused no change in alveolar neutrophil recruitment (Honoré et al., 2004).

It has been reported that a new class of carbamylating compounds based on the cyclosulfamide scaffold that are selective for HNE include the R3NH(C=O) part of the inhibitor covalently linked to the active site serine (Yang 2008).

Drug discovery and design have made considerable use of mechanism-based inhibitors. An enzyme's catalytic system processes a mechanism-based inhibitor as a substrate, producing a reactive electrophilic species that, when further reacted with by a nucleophilic active site residue, causes the enzyme to become irreversibly inactive (Li et al., 2009).

In the patent literature, several new families of structurally different heterocyclic HNE inhibitors have been reported. When tested in a rat model of acute lung damage, 1, 4-Diarylpyrimidopyridazinyldiones and the structurally related 4-(4-cyano-2-thioaryl)-dihydropyrimidinones 12 both potently reduced HNE with IC_{50}s in the low nM or pM range, respectively (He et al., 2010).

In the patent literature, several new families of structurally different heterocyclic HNE inhibitors have been reported. However many fails in the clinical trials thus leaving practically no drugs to treat COPD.

7

AIMS & OBJECTIVES

*A*ims and Objectives

A. Identification of elastase inhibitor from natural sources

1. To identify potential herbal extract capable of inhibiting human neutrophil elastase.

2. To characterise herbal extract *in situ* and *in vitro* for its anti-elastase activity.

3. To investigate the anti-elastase activity of herbal extract *in vivo* mouse model of airway diseases.

B. Identification of elastase inhibitor from synthetic sources

1. To synthesise synthetic molecules targeted against human neutrophil elastase.

2. To screen molecules for their anti-elastase activity and lead identification.

3. To characterise the lead compound *in situ* and *in vitro* and perform pharmacokinetic and toxicity tests.

4. To investigate the anti-elastase activity of lead compound *in vivo* mouse model of airway diseases.

8

CHAPTER 1

8.1 Abstract

Chronic obstructive pulmonary disease (COPD) and asthma are significant and increasing global health issues. Tobacco use is responsible for approximately 80-90% of all COPD occurrences worldwide. COPD causes minor airway narrowing and lung tissue deterioration, resulting in emphysema, which is predominantly produced by neutrophil elastase. Neutrophil elastase is an important target for drug discovery because it plays an essential role in progression of the disease in COPD patients. *Sonneratia apetala* Buch.-Ham. is a mangrove plant native to India's Sundarbans. While the fruits of this species have antimicrobial, antifungal, antioxidant, and astringent properties, fruit and leaf extract have been demonstrated to alleviate asthma and cough symptoms. The goal of this study is to see if Sonneretia apetala fruit extracts block neutrophil elastase and hence halt the progression of neutrophil elastase-driven lung emphysema. The hydroalcoholic extract (Ethanol: water/90:10) of *Sonneratia apetala* Buch.-Ham. fresh fruits were utilised in the neutrophil elastase enzyme kinetic experiment, and the extract's IC50 was found. The extract was standardised using Gallic acid and Ellagic acid as standards, and the innovative HPLC method was established. The extract was then profiled using LC-MS2 to detect important phytochemicals. According to the HPLC calibration, the standardised *Sonneretia* apetala crude extract (SAM) includes 53g/mg of Gallic acid and 95g/mg of Ellagic acid. SAM also restored the elastase-induced structural change in human epithelial cells in vitro, and the vitality was determined using an MTT assay. Furthermore, 10mg/kg SAM decreased alveolar collapse mediated by neutrophil elastase in the mouse model. Thus, in this investigation, we reported for the first time that S. apetala fruit extract (SAM) has the capacity to inhibit human leukocyte elastase *in vitro* and *in vivo*.

8.2 Introduction

An important global health problem with rising morbidity and mortality rates is chronic obstructive pulmonary disease (COPD). An estimated 7% of people aged 30 and older are likely to have COPD. There may be up to 10% prevalence among individuals over the age of 65 (Fukuchi et al., 2004; Menezes et al., 2005; Halbert et al. 2006; Buist et al., 2007; Ford et al., 2013). The true incidence of COPD appears to be much higher than what has been recorded since it is underdiagnosed and underrecognized (Lopez et al., 2006) and as of today, it is the third-leading cause of death worldwide (Quaderi and Hurst, 2018). Through a prolonged inflammatory reaction in the bronchioles and lung parenchyma, chronic exposure to harmful substances or gases is connected to COPD. The majority of these cases of COPD are brought on by the damaging cigarette smoke particles that result in chronic, persistent inflammation. The airflow channel that makes up the pulmonary component is often progressive and not always very well reversible. Airflow issues were brought on by emphysematous lesions, which raised the resistance of the tiny airways and increased the compliance of the lung (Hogg et al., 2004). Dyspnoea and chronic sputum production are the main indicators and symptoms of COPD. A typical early sign of COPD is exertional dyspnoea. Although several reasons can cause dyspnoea, one important aspect that is connected to it is the narrowing of the airways (O'Donnell et al., 1993; O'Donnell et al., 2001). Fibrosis of the small airways cause thickening of the internal walls which results in narrowing of airways due to loss of elasticity and lung alveolar tissue rupture (Hogg et al., 1968; McDonough et.al., 2011). The increased generation of sputum is due to mucus gland hyperplasia and goblet cell proliferation (Lai and Rogers, 2010). Pharmaceutical and non-pharmacological treatments for COPD should aim to reduce symptoms, promote healthy lifestyles, increase exercise tolerance, stop the progression of the disease and concomitant conditions, and improve prognosis

because COPD cases present with unavoidable limitations in correct airflow in the lungs and their disease frequently progresses throughout life (Braido et al., 2015). To achieve these objectives, both pharmaceutical therapy and non-pharmacological measures should be used, such as stopping smoking, lowering other potential risks, vaccinations, oxygen therapy, and rehabilitation care. It is important to accurately diagnose the disease severity and to regularly check on patients to see if they are responding to treatment as intended. Patients with COPD frequently experience the release of inflammatory mediators into the bloodstream, which can lead to or exacerbate hyperglycaemia, ischemic normocytic anaemia, cardiovascular disease, anxiety, osteoporosis, heart failure, and lung cancer (Barnes 2009). Therefore, adequate comorbidity management is essential for COPD patients (Agusti et al., 2003; Soriano et al., 2005). It is vital to create effective therapeutic approaches because present medications do not alter the disease's growing burden (Barnes 2000). The development of small molecule inhibitors that specifically block new targets, the use of complete herbal extracts, or the isolation of novel substances from those extracts can all be helpful for treating COPD.

When the lungs respond to harmful particles and gases, it causes an abnormal elevation in the number of inflammatory cells in the airways like neutrophils, T lymphocytes, and macrophages. Directly recruited from the circulation, neutrophils release human neutrophil elastase to destroy the parenchymal layer. The development of COPD is significantly influenced by neutrophil elastase. Numerous clinical observations suggest that neutrophil elastase targeting may be advantageous in the prevention of inflammatory lung disease (Donnelly and Rogers, 2003). An imbalance in the protease-antiprotease ratio is brought on by a cytokine network that promotes neutrophil recruitment at the inflammatory site (Abboud and Vimalanathan, 2008). A member of the chymotrypsin superfamily, human neutrophil elastase (EC 3.4.21.37) is a serine protease that is mostly

retained inside the azurophilic granules of multinuclear neutrophils. During the inflammatory phases of COPD, neutrophils create excessive elastase, which is produced extracellularly. Human neutrophil elastase is composed of 218 amino acids and is held together by four disulfide bridges, weighing around 29 kDa (Sinha et al., 1987). Ser195, His57, and Asp102 residues make up its catalytic triad, which controls their activity. In the primary sequence, the trio is widely separated, but in the tertiary structure, they are brought together at the active site (Korkmaz et al., 2008; Bode et al., 1989). As a multifunctional enzyme, human neutrophil elastase fights infections to reduce inflammation and preserve tissue homeostasis (Pham 2006). HNE can degrade a number of structural proteins, such as collagen, elastin, laminin proteoglycans, and fibronectin, by hydrolyzing cleavable amide bonds using its active serine (Pham 2008; Chua and Laurent, 2006). HNE is essential for chemotaxis and promotes neutrophil movement by disintegrating adhesion molecules (Cepinskas et al., 1999; Hermant et al., 2003). Endogenous inhibitors, including 2-macroglobulin, 1-antitrypsin (1AT), secretory leukocyte protease inhibitors (SLPI), and elafin reduce tissue damage under physiological conditions that regulate inflammatory processes and minimise the harmful effects of extracellular HNE (Tremblay et al., 2003; Ohmoto et al., 2001; Heutinck et al., 2010). Extracellular HNE contributes to a number of diseases, including acute lung injury (ALI) (Kawabata et al., 2002), cystic fibrosis (Gifford and Chalmers, 2014), asthma (Guay et al., 2006), and acute respiratory distress syndrome (ARDS). HNE plays crucial roles in a

wide range of other diseases, such as psoriasis and other skin illnesses (Meyer-Hoffert et al., 2004; Marto et al., 2018), rheumatoid arthritis (Cawston and Young, 2010), atherosclerosis (Henriksen and Sallenave, 2008), and different malignancies of the breast and lungs (Albrengues et al., 2006; Hunt et al., 2013). Recent studies reveal the participation of key elements of neutrophil innate immunity as evaluated in COVID-19 patients (Guéant et al., 2021) and the potential contribution of neutrophil elastase to lung

illnesses associated with COVID-19 (Mohamed et al., 2020). Consequently, it is thought that human neutrophil elastase could be a useful therapeutic target (Henriksen 2014).

Neutrophil elastase inhibitors can be produced in the laboratory or derived naturally from herbal sources. Only Sivelestat (ONO-5046), a human neutrophil elastase antagonist used to treat patients with ARDS, is approved in Japan (Iwata et al., 2010). More recently, Sivelestat has also been proposed to cure acute lung injury in COVID patients despite the fact that many synthetic drugs are currently undergoing clinical trials (Sahebnasagh et al., 2020). The emergence of new neutrophil elastase blockers from natural origin, including plants, animals, fungi, bacteria, and sponges, could have a significant impact on the development of novel drugs to prevent and treat illnesses caused by the excessive elastase or the catastrophic breakdown of elastin (Marinaccio et al., 2022). A potent elastase inhibitor known as Cyclotheonellazole A (CTL-A) is a naturally occurring macrocyclic peptide (Cui et al., 2022). The toxic gland of the centipede *Scolopendra hananum* contains a unique elastase inhibitor called ShSPI (Luan et al., 2019). The 57 amino acid wide, cysteine-rich polypeptide guamerin, with a Ki value of 8.1M, can inhibit human neutrophil elastase (Jung et al., 1995). Compounds made from marine cyanobacteria also demonstrated potent anti-elastase action (Keller et al., 2020).

The use of plant-based natural products in the treatment of ailments has received more attention recently due to its accessibility and lack of adverse effects (Uchida et al., 2017; Lahouar et al., 2015). Sonneratia apetala (S. apetala), a member of the Lythraceae family and a mangrove plant, is found in coastal regions of India, Bangladesh, Malaysia, China, New Guinea, Myanmar, and other nations. It is also known locally as Keora (Patra et al., 2015; Hossain. et al., 2015). S. apetala is a rare mangrove species that was introduced to China from Sri Lanka and Bengal, India (Ren et. al., 2009). Due to its superior adaptability, rapid growth, improved crop setting rate, and other qualities, it is the

dominant species of mangrove in the southeast coastal regions of the country (Jia et al., 2014). The fruit of S. apetala is edible, and people who live in the coastal areas where it is found frequently prepare it for consumption. Additionally, it is manufactured and sold as fermented juice, soft drinks, and other consumables (Shefa et al., 2014; Mollik et al., 2010). Additionally, because of its medicinal significance, locals regularly utilise it as medicine to treat conditions like hepatitis, diarrhoea, bruising, etc. Additionally, traditional healers in Bangladesh recommend S. apetala as an anti-inflammatory medication to lessen gastrointestinal issues like diarrhoea, dysentery, and cramping (Shefa et al., 2014; Bandaranayke 2002, Tea and Ravishankar, 2013). S. apetala's fruit is also used as medicine in China to heal coughs and sprains in addition to the leaf and flower (Ji et al., 2005). The fruits and bark of S. apetala have been shown to be beneficial in treating sprains, bleeding, coughing up blood, swelling, and fever (Bandaranayake 1998). A broad range of biological activities, comprising antioxidant, anti-diabetic, anti-cancer, and antibacterial actions, have been linked to fruit extracts from S. apetala (Patra et al., 2015; Jaimini et al., 2011). Polyphenols, tannins, flavonoids, and carbohydrates make up the majority of S. apetala's bioactive components, which support the plant's antioxidant activity and make it a potential source of natural antioxidants (Hossain et al., 2015; Mun et al., 2010; Lin et al., 2009; Yi et al., 2017; Cao et al., 2015). In S. apetala, steroids, lactones, carboxylic acids, and triterpenes can also be detected (Bandaranayake 2002; Cao et. al., 2015).

Plant flavonoids have been shown to exhibit anti-elastase properties in the past (Jakimiuk et al., 2021). By directly activating the MUC5AC gene, neutrophil elastase also has a substantial role in mucus hyperproduction and mucin oversecretion in lung diseases (Voynow 1999). We anticipated that the fruits of Sonneretia apetala, which are high in flavonoids and historically used to treat wheeze, cough, and asthma, could be able to inhibit human neutrophil elastase. The impact of Sonneretia apetala fruit extract on

human neutrophil elastase as well as its implications in mice lung injury was first demonstrated in this work.

8.3 Materials and methods

8.3.1 Chemicals and equipment

Finar Ltd. in India provided the HPLC grade Methanol, and Fisher Chemicals provided the LC/MS grade Acetonitrile. Gallic acid, vanillic acid, syringic acid, and ascorbic acid Quercetin was provided by MP Biomedicals LLP, Germany, and ellagic acid by (TCI India) chemical Pvt. Ltd. Formic acid of ULC/MS grade was provided by Biosolve B.V. of the Netherlands. Merck supplied the ethanol. Shimadzu Prominence UFLC system (Shimadzu, Kyoto, Japan) with LC-20AD and LC-20AT prominence liquid chromatography pumps, DGU-20A3 prominence degasser, CBM-20A prominence communications bus module, and SPD-20A were used to perform the HPLC analysis. Significant UV/VIS detector. The LTQ XLTM Linear Ion Trap Mass Spectrometer was used to carry out the LC-MS/MS studies

8.3.2 Plant material

Dr. K. Karthigeyan of Botanical Gardens, Howrah has gathered and identified the plant. The plant was collected in Sundarbans, in the Hero - 1 Jharkhali area (GPS coordinates: 22 01. 448, 88 39. 88) of West Bengal, India. 65153 is the field specimen number.

8.3.3 Extraction

Fresh fruit weighing 500 grams was finely crushed and extracted for 24 hours using a 90:10 ratio of ethanol to water. The extract was then further collected, passed through Whitman filter paper, and vacuum-dried. A subsequent lyophilization of the extract

produced 18 g of brown amorphous powder. The sample that had been extracted was kept at -20°C.

8.3.4 HPLC analysis and method development for simultaneous detection of Gallic acid and Ellagic acid in S.apetala fruit extract

All seven standard compounds vis. (1) Ascorbic acid (AA), (2) Gallic acid (GA), (3) Vanillic Acid (VA), (4) Caffeic acid (CA), (5) Syringic acid (SA), (6) Ellagic acid (EA) and (7) Quercetin (QR), were accurately weighed and transferred to volumetric flasks and dissolved in HPLC grade Methanol to achieve.

1 mg/ml concentration. The overlap of HPLC chromatogram of standard compounds (1-7) and SAM (5mg/ml) showed that the standard compound (2) Gallic acid and (6) Ellagic acid was present in the extract(Hossain et al., 2016a), which was further used for the method development and standardization purpose (Figure 1). The extract (SAM) was dissolved in HPLC grade MeOH to achieve a 5 mg/ml concentration and filtered through a 0.22µM filter. This same stock concentration was used throughout the study. The standard (2) and (6) were then serially diluted to achieve the 6 concentrations vis 50 µg/ml, 100 µg/ml, 250 µg/ml, 500µg/ml, 750µg/ml, 1000µg/ml which were used for calibration and method development. Injection volume for standards and SAM was identical throughout the HPLC study i.e., 20µL. The aliquots of 20 µl were injected using SIL-20AC HT prominence autosampler. The separation was achieved on a Phenomenex reverse phase HPLC column (Luna® RP C_{18} column 4.6x260 mm), 5µ particle size, and elution was carried out using a mobile phase consisting of Methanol (A) and Water (0.1% Formic Acid) (B) using a gradient system using a flow rate of 0.8 ml/ min as described in (Figure 2) and (Table 1). The eluate was monitored at 260 nm. Data analysis was

performed in LC solution post-run analysis software (version 1.25) (Shimadzu, Kyoto, Japan).

8.3.5 Spectroscopy using LC-MS/MS

In order to create the SAM sample, 5 mg of extract was dissolved in LC-MS grade MeoH and put through a 0.22 M filter. The injection had a 5 l volume. Hypersil Gold C18 column (diameter: 100 2.1 mm, particle size: 1.9 M) was installed in the LC-MS. A gradient of solvents A (acetonitrile + 0.1% formic acid) and B (HPLC grade H2O + 0.1% formic acid) were utilised for the LC analysis. The capillary temperature was set to 320°C, the analyzer was in positive mode, the source voltage was 5.00 kV, the capillary voltage was 45.00 V, and the tube lens voltage (V) was 110.04. 32.00 min. MS Run Time. XcaliburTM Software from Thermo Scientific was used for data analysis.

8.3.6 Elastase activity and IC_{50} determination

To assess the effectiveness of suppressing human neutrophil elastase, 200 ng/mL of human neutrophil elastase (Calbiochem, USA) and 1.2 mM N-(OMe-succinyl)-Ala-Ala-Pro-Val-p-nitroanilide (Sigma-Aldrich) were utilised (pH 7.5). A 96-well microtiter plate was pre-incubated with SAM (1 l), 50 l of the previously produced elastase, and 24 L of Tris-NaCl for 15 minutes at room temperature (pH 7.5). At the conclusion of incubation, 25 L of substrate solutions were added to each well. The reaction was tracked by measuring the absorbance at 405 nm every 30 seconds for a total of two hours. After removing the blank from each, percentage inhibition was calculated using GraphPad Prism version 8.4 and compared to the norm (without the extract).

In order to determine the mechanism of inhibition, we incubated 4 g/ml HNE with three different concentrations of the substrate N-(OMe-succinyl)-Ala-Ala-Pro-Val-p-

nitroanilide (100 mM, 500 mM, and 1000 mM) as well as concentrations of 0 g/ml, 1 g/ml, 100 g/ml, and 1000 g/ml of the fruit The reaction was carried out with the Spectramax multimode plate reader following the same steps as previously mentioned. The reaction velocities were computed using GraphPad Prism version 8.4 after blank correction. Substrate titration plots and double reciprocal plots were made after that (Milicaj et al., 2022; Budnjo et al., 2014)

8.3.7 Cell culture procedures:

A549 cells were purchased from NCCS, Pune, and grown in F12K medium (Gibco) containing 10% foetal bovine serum (Life Technologies) at 37 °C in a humid environment.

8.3.8 Cell viability assays

Each well of a 96-well plate contained 5,000 A549 cells. The cells were treated to 100uM elastase or vehicle after being seeded for 24 hours. After that, these individuals received either DMSO, SAM, or sivelestat (Sigma-Aldrich). The MTT reagent was then added to each well at a concentration of 0.5 g/ml after another 12 hours of cell culture. Following the addition of DMSO as a solubilizing agent and 4 hours of MTT incubation, the absorbance at 550 nm was determined.

8.3.9 Cell detachment assay

Elastase, elastase+SAM, and elastase+Sivelestat were the treatments given to A549 cells after they were seeded in 6-cm cell culture dishes. After 12 hours, the medium was collected, and the segregated cells were pelleted using centrifugation. Attached cells were collected using trypsin (Thermo), and the cells were then pelleted. The cell pellet was redissolved in fresh medium, and trypan blue (0.04%) was then added. A

haemocytometer was used to count the cells in a 10 l aliquot of the material. The percent detachment was calculated using the number of detached cells and the overall number of cells present at the beginning of the experiment. The graphs were produced with Prism programme.

8.3.10 Cell Morphology and Cell Staining

On 6-cm dishes, A549 cells were planted. Vehicle, elastase, elastase+SAM, and elastase+Sivelestat were all used to treat the cells. After one, two, and three hours, cells were treated with two drops of DAPI (Thermo Fischer), and then rinsed with PBS after five minutes. Additionally, cells were stained with WGA (Thermo Fischer) in accordance with the instruction manual, and after each hour, they were checked for morphological changes using a fluorescence microscope (EVOS FL, Thermo Fischer).

8.3.11 Measurement of soluble ICAM-1 protein

Six-cm dishes were used to plate A549 cells. The cells were treated with vehicle, elastase, elastase+SAM, and elastase+Sivelestat in three separate experimental configurations. Media was gathered from the dishes after intervals of one hour, two hours, and three hours. To remove any particles and cells, the media was centrifuged. The sICAM ELISA Kit (Cat. No. EHICAM1, Invitrogen) was used to determine the concentration of soluble ICAM in cells from the supernatant in accordance with the manufacturer's instructions (Liu et al., 2018).

8.3.12 Mice grouping

The male C57/BL6 mice were acclimatised for one week at the IICB's animal house facility after being received from the institute's own breeding facility at CSIR-Indian Institute of Chemical Biology (IICB), Kolkata, when they were about 8–10 weeks old. All

animal studies were carried out in accordance with CPCSEA guidelines and were officially authorised by the Institutional Animal Ethics Committee at IICB (Reference Number IICB/AEC/Meeting/July/2021/6) (Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines). VEH/VEH (intratracheal vehicle, Tris buffer pH 7.4 administered and vehicle, diluted with saline, treated mice) and NE/VEH (intratracheal neutrophil elastase administered and vehicle-treated mice), as well as NE/Sonneretia apetala extracts at 1 mg/kg (c), 10 mg/kg/day (d), and 100 mg/kg/day (e).

8.3.13 Induction of emphysema in mice with human neutrophil elastase

In tris buffer pH 7.4, human neutrophil elastase (Cat. No. 324681, Calbiochem) was reconstituted. Mice were first momentarily sedated with isoflurane fumes before the elastase-induced emphysema experiment. Anesthetized mice were positioned on a piece of wood at a 45-degree angle, and the tongues were gently grabbed using blunt end forceps in an upward and leftward position. As shown in Figure 3, these mice were subsequently administered 10 ng of human neutrophil elastase (to the NE/VEH and NE/SAM groups) or a vehicle (to the VEH/VEH group) orally on day 1. Mice were held stationary for 15 seconds before being moved to a rehabilitation pad. From day 1 to day 6, SAM or vehicle was given twice daily. Mice were euthenized on day 6 and blood and lungs were collected..

H & E-stained lung slices from each animal were captured on camera using a Magnus MLX-i microscope with a 10x objective lens. At regular intervals, photographs were taken while wandering methodically across the surface of the lung parts. Utilizing the STEPanizer programme, a quadratic test system with 64 points, 8 horizontal and 8 vertical lines totaling m (d = m), was utilised to statistically analyse the images. (2011)

Tschanz et al. The mean linear intercept of the airspaces (Lm) of each lung slide was calculated using Pref (all points striking the parenchyma), Psep (points hitting the alveolar septa), and I (intersections of the test line system with the alveolar surface) [Salaets et al., 2020].

8.3.14 Statistical Evaluation

Statistics were carried out using the GraphPad Prism Software version 8.4. An unpaired, two-tailed Student's t-test was used to assess the statistically significant differences. Comparing more than two groups was done using one-way ANOVA and the Dunnett test. Statistical significance is defined as *P 0.05, **P 0.01 and ***P 0.001.

8.4 Results

8.4.1 LC-MS-MS Profile

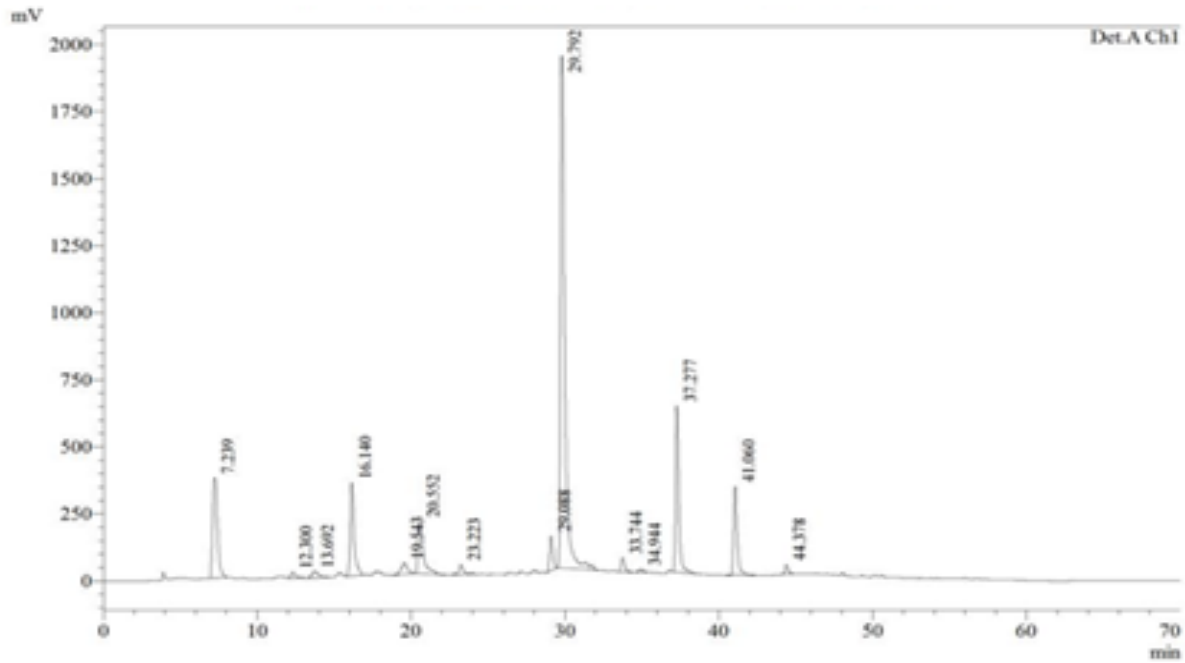


Figure 1: LC-MS/MS spectrum of *Sonneretia apetala* fruit extract

Table 1: Compounds identified from *S. apetala* fruit extract by LC-MS/MS spectroscopy

S.N.	*m/z* $(M+H)^+$	MS/MS fragments	Compound identified
1	295.23	263.16, 255.15, 236.16, 221.09	8,11-Octadecadienoic acid, methyl ester
2	455.22	478.30, 437.19, 427.08, 418.94, 395.25	Stigmasta-5,22-dien-3-ol, acetate
3	375.29	357.27, 343.18, 319.31, 251.17, 119.31	Sonneradon A
4	307.23	289.20, 275.13, 243.02, 151.11	Sonneradon C
5	277.13	259.13, 241.12, 221.07, 207.09, 175.02	Ranuncoside
6	171.03	143.01, 135.01, 125.85, 124.85, 113.84	Gallic acid
7	303.25	275.25, 257.06, 229.09, 215.08, 201.05	Ellagic acid
8	287.05	268.96, 244.99, 179.03	Luteoline
9	457.37	439.30, 411.24, 393.26, 275.16, 249.20, 217.16	Ursolic acid
10	413.38	395.35, 367.31, 297.20, 283.25, 255.16, 241.14, 201.17	Stigmasterol

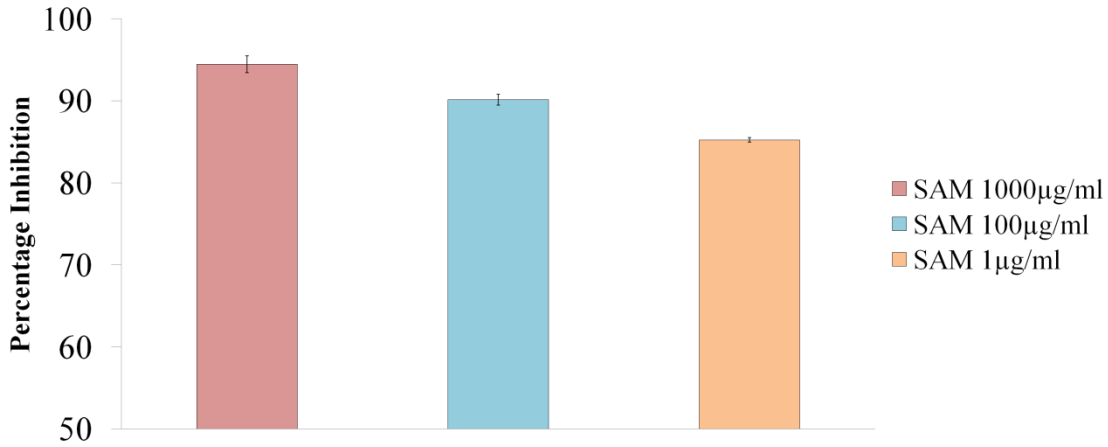
Using the prior literature and the SAM's LC-MS/MS spectrum (Figure 1), a total of 10 compounds were found to match the collected fragments. Gallic acid, ellagic acid, as well as other polyphenols, fatty esters, and terpenoids, were confirmed to be present by LC-MS/MS. The list of identified chemicals is (Table 1). By comparing the HPLC profiles of the SAM (5 mg/ml) sample with the calibration sample, it can be seen that gallic acid and ellagic acid are present at concentrations of 53 and 95 g/mg of extract, respectively.

8.4.2 In situ herbal extract characterisation

Table 2: IC_{50} values of *Sonneretia apetala* fruit extract (SAM) and Sivelestat

	IC_{50} (ng/ml)
SAM	371.8±3.73
Sivelestat	65.1±1.13

A



B

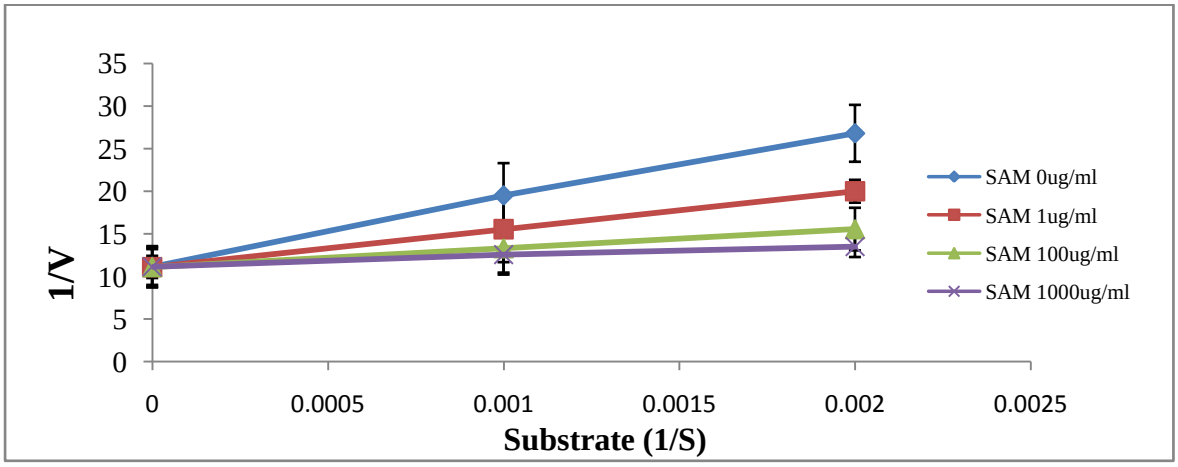
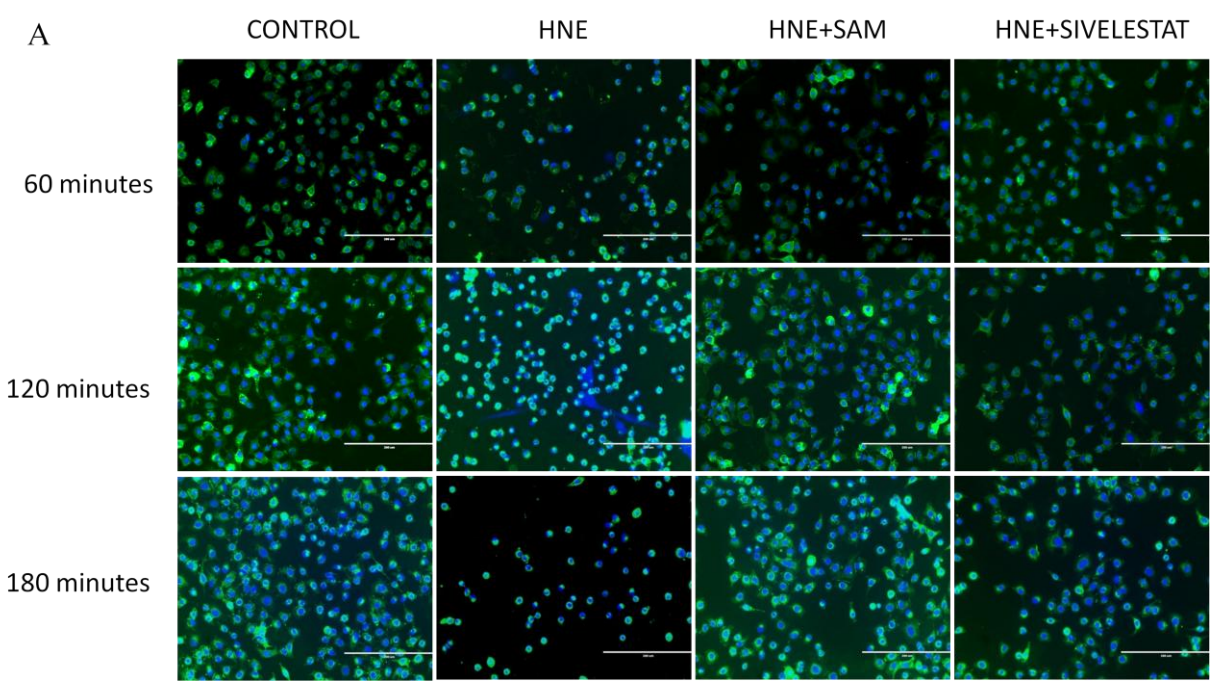


Figure 2: Dose dependant inhibition of Human neutrophil elastase by fruit extracts of *Sonneretia apetala*.(A). Mechanism of inhibition of fruit extracts on human neutrophil elastase (B)

When different doses of SAM were treated with human neutrophil elastase enzyme, dose-dependent inhibition was observed (Figure 2A). When comparing the inhibitory findings to the control medication, Sivelestat, the IC_{50} was computed and determined to be 371.8± 3.73 ng/ml (Table 2).

Kinetic tests were conducted to understand the manner of inhibition of synthetic medicines against elastase inhibition. The method of inhibition based on the IC_{50} was determined using SAM. The sequence of straight lines could be seen on the 1 / V Lineweaver Burk plot of the enzyme's kinetics for the substrate N-(O-Mesuccinyl) Ala-Ala-Pro-Val-p-nitroanilide 1 / (S) in the presence of various inhibitor dosages. SAM's Lineweaver Burk figure showed that Vmax remained constant while the gradient barely changed. Despite a little change in Vmax, Km remains constant as concentration (Figure 2B).

8.4.3 In vitro morphological study:



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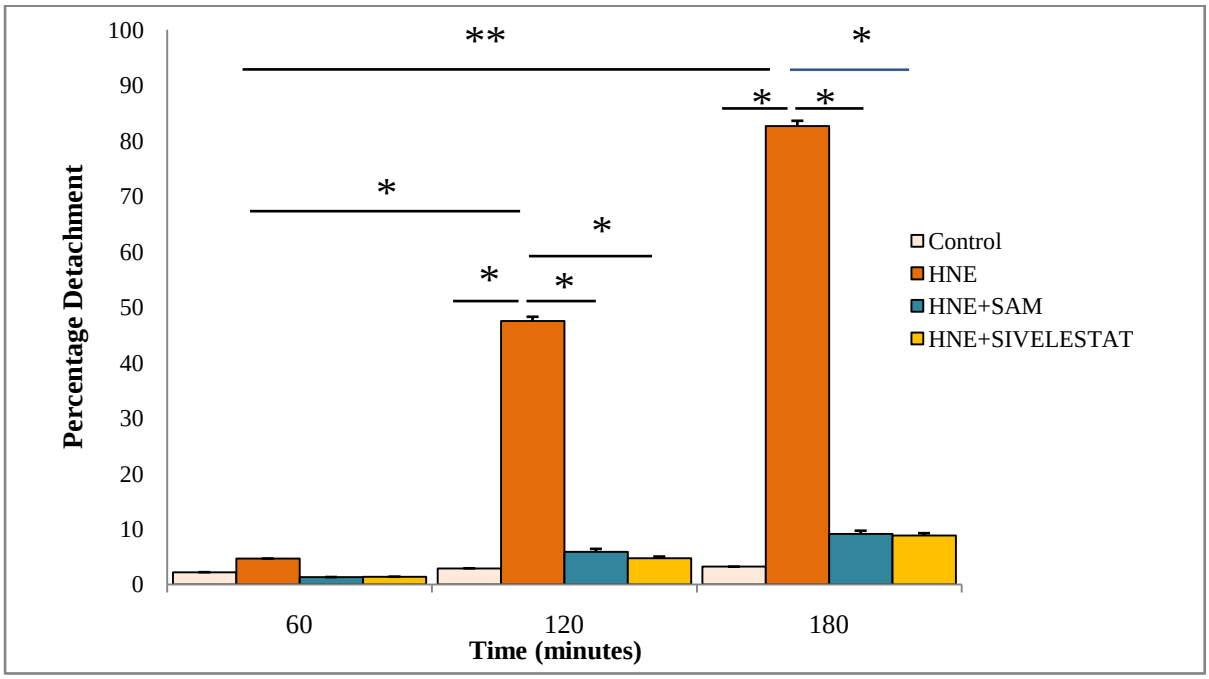
Percentage Detachment
100
90
80
70
60
50
40
30
20
10
0
60
120
180
Time (minutes)
Control
HNE
HNE+SAM
HNE+SIVELESTAT
**
*
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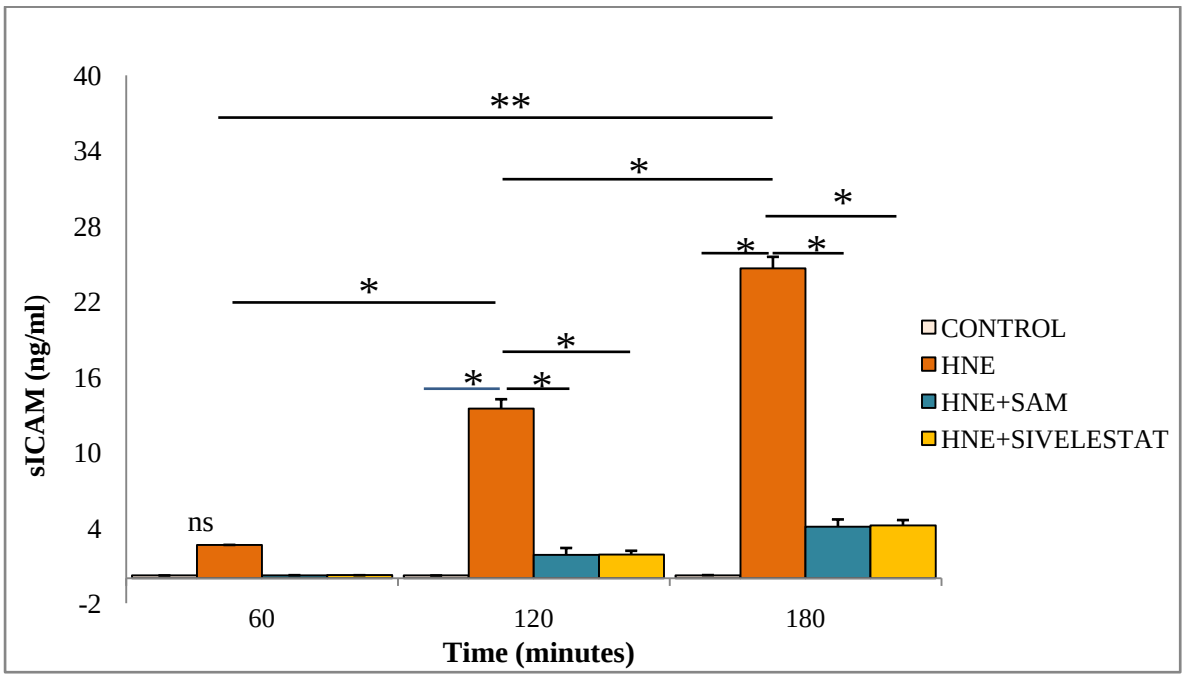
C


sICAM (ng/ml)
40
34
28
22
16
10
4
-2
ns
60
120
180
Time (minutes)
CONTROL
HNE
HNE+SAM
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D

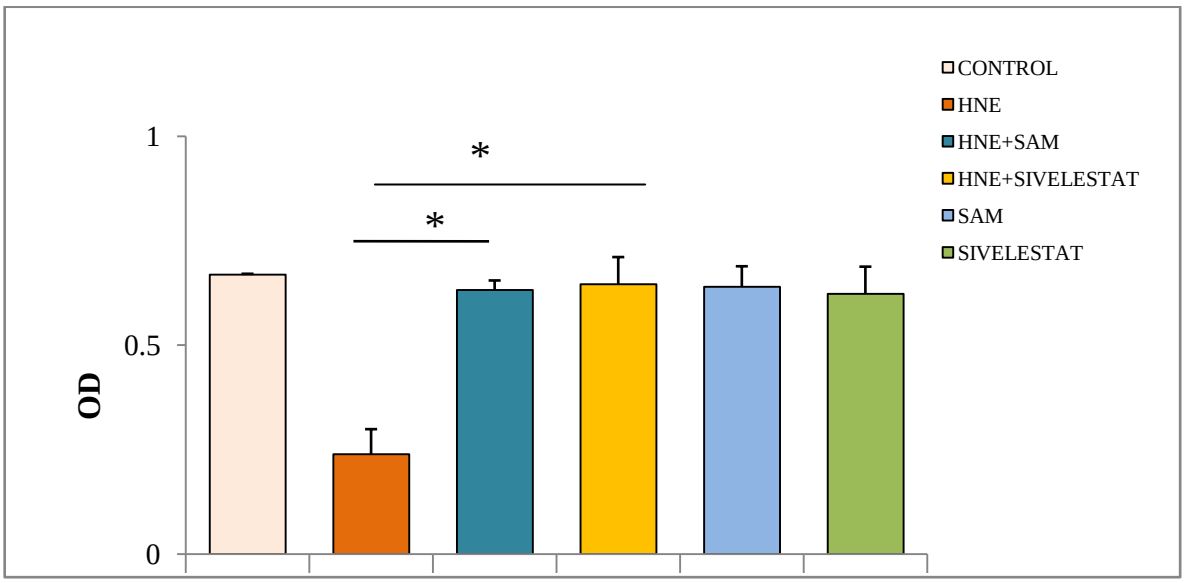


Figure 3 : *Sonneretia apetala* fruit extract exerts inhibitory effect on elastase-induced human lung epithelial cells (A) . Cell detachment assay representing the effect of Sonneretia apetala on lung epithelial cells (B). Sonnereta apetala's effect on sICAM levels in elastase induced lung epithelial cells. (C) Determination of cell viability by MTT assay (D),

In contrast to controls, after 1 hour of neutrophil elastase treatment, A549 cells started to gather, and after 2 hours, they started to detach from the cell culture dish. By combining the above incidence with SAM or sivelestat therapy, it was completely avoided. After 3 hours of treatment, however, all but a few of the cells had completely detached from the plate, suggesting that cell death or apoptosis may have occurred. Although there was a possibility of apoptosis in certain cases, treatment with SAM or sivelestat for two hours instead of three hours stopped the cells from desquamating, demonstrating that SAM is reversible and a free enzyme is the origin of the activity (Figure 3A).

The morphological transformation of A549 cells from the epithelium to a somewhat oval/rounded, constricted shape was the most fast and foretelling event that happened after elastase therapy (Figures 3A). Elastase begins to work within two to three hours,

indicating that cell death is not necessary for it to start working right away. The elastase inhibitor sivelestat served as a standard for comparisons.

Cell detachment caused by elastase and its inhibition by SAM and sivelestat were computed per hour. After 120 minutes, there had been a significant amount of cell detachment, with roughly 48% of the cells discovered floating in the medium. The detachment rose dramatically to 82% after three hours, but was successfully reversed by co-treating with fruit extract (Figure 3B). The variation in the percentage of dissociation at each hour is also significant.

As a result of the elastase treatment, which causes the cell to detach, sICAM is secreted in the cell culture media. As previously noted, by the end of the second hour, the levels of sICAM had dramatically increased to 13.49ng/ml. With elastase therapy, the levels increased to 24.64ng/ml by the experiment's conclusion and grew correspondingly throughout time(Figure 3C).

The viability of the cells was assessed using the MTT test, and after 12 hours of elastase treatment, co-treatment with SAM and sivelestat restored the viability of the cells. It's interesting to note that sivelestat, or SAM, had no noticeable effect on cells alone (Figure 3D).

8.4.5 In vivo study

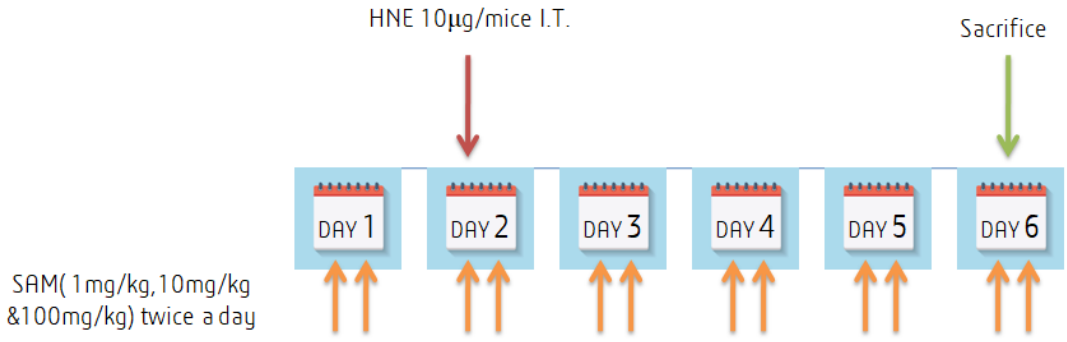


Figure 4: Mouse treatment protocol with Sonneretia apetala fruit extracts

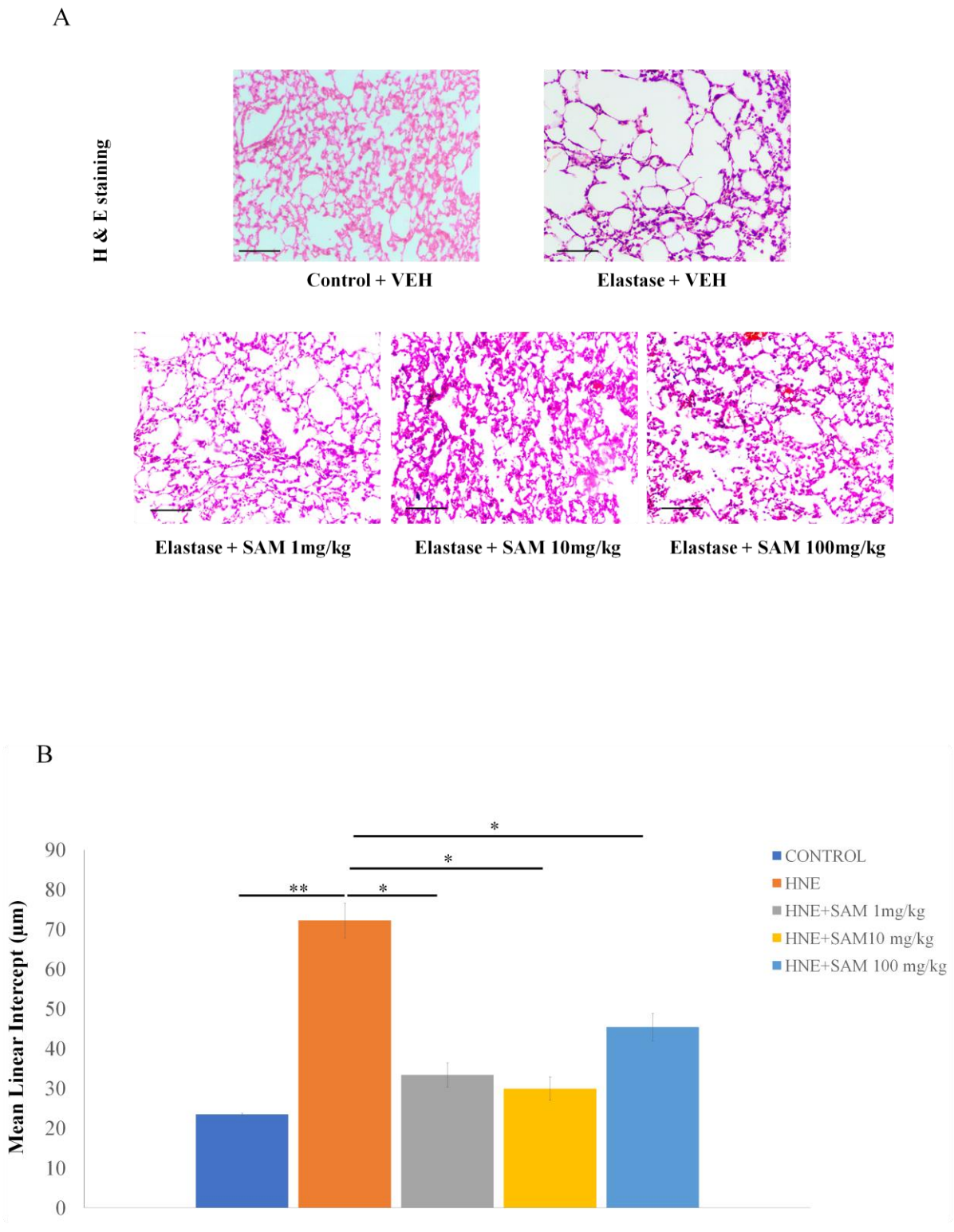


Figure 5: Mouse lung section stained with HNE (A) Mean linear intercept from corresponding lung tissue section (B)

Since we learned that SAM can potently inhibit neutrophil elastase and lessen elastase-induced cell detachment, we made the decision to investigate its effects in a mouse model of elastase-induced emphysema. To confirm this, we used the well-known elastase-induced emphysema model, shown in Figure 4 and previously discussed. Alveolar collapse occurred in the Elastase + VEH group compared to control mice, as seen in Figure 5A. But SAM treatment in various amounts reduced this collapse.

The test for the mean linear intercept served as evidence for this. Figure 5B shows that as compared to control mice and animals given elastase and SAM co-treatment, elastase + VEH mice displayed considerably larger Mean Linear Intercepts.

8.5 Discussion

The main causes of COPD—the third leading cause of death in the world today—include smoking, being exposed to air pollution, and burning biomass fuels. Neutrophil-mediated inflammation is a hallmark of COPD, much like it is in Cystic Fibrosis. Smoke from cigarettes and other irritants alters the protease-antiprotease ratio, which encourages neutrophil recruitment and starts an endless cycle of airway inflammation and alteration.(Barnes and Celli, 2009). The primary cause of illnesses and fatalities in COPD is acute bronchitis exacerbations caused on by bacterial or viral diseases, which are linked to higher NE levels (Beasley et al., 2012; Thulborn et al., 2019). According to research (Stănescu et al., 1996; O'Donnell et al., 2004), peripheral airway disruption and sputum neutrophil counts are connected to the deterioration of lung function. Neutrophil elastase levels in bronchioalveolar lavage, sputum, and fluid are noticeably elevated in patients with a variety of respiratory disorders (Oriano et al., 2020; Kummarapurugu et al., 2022; Margaroli et al., 2022; Keir et al., 2022); Due to its role in tissue degradation, which is primarily associated with respiratory diseases (Pham 2008; Chua and Laurent, 2006). The

pathogenic consequences of neutrophil elastase include mucus secretion, metaplasia involving secretary cells, and hyperresponsiveness in small airways (Suzuki et al., 1996). (Schuster et al., 1992). Among the signs include changes in interleukin-8 gene expression, modification of leukocyte adhesion, proliferating smooth muscle cells, and others (Cai and Wright 1996; Nakamura et al. 1992; Thompson and Rabinovitch 1996). Together, NE and other proteases play a significant role in controlling the protease-antiprotease activity in the COPD airway. For instance, there is compelling evidence that smoking causes the development of emphysema, which is dependent on MMP-12/macrophage elastase (Hautamaki et al., 1997). NE causes emphysema by activating cysteine cathepsin and MMPs, and it retains MMP activity by destroying TIMP-1, a key MMP inhibitor (Murphy 2011). Reactive oxygen radicals oxidise and inactivate -1-antitrypsin, which leads to unchecked NE activity (Taggart et al., 2000). The proteolytic and pro-inflammatory effects of NE and neutrophil granules are propagated by NE and MPO activating neutrophil extracellular trap (NET) releasing into the airway environment (Genschmer et al., 2019). Neutrophil elastase trap (NET) accumulation in the airway is associated with decreased neutrophil phagocytosis, increased exacerbations, and decreased lung function (Dicker et al., 2018). NE and MMP-12 (macrophage elastase) collaborate to increase the pathophysiology of the lungs associated with COPD brought on by tobacco smoke. The NE inhibitor -1 antitrypsin is destroyed by MMP-12, and the MMP-12 inhibitor TIMP1 is destroyed by NE (Jackson et al., 2010), resulting in uncontrolled protease activity (Hautamaki et al., 1997). These findings suggest that neutrophil elastase represents a potential therapeutic target for the management of respiratory disorders and that inhibition of elastase will eventually help to reduce pathological and functional problems.

Finding natural compounds that either directly block the enzyme or stop neutrophils from releasing it could be an attractive source for inhibitors. Plant extracts contain phenolic chemicals, flavonoids, and tannins that have strong inhibitory properties against human neutrophil elastase (Melzig et al., 2001). Lonicera japonica is a well-known botanical source of anti-inflammatory compounds (Lee et al., 1998). The entire plant, including the leaves and blooms, is extensively used in traditional medicine as an anti-inflammatory medication, notably for the treatment of conditions affecting the upper airways. L. japonica is a key ingredient in many complex lung inflammatory disease treatments found in ancient texts. Iridoids and flavonoids, the main components, have a potent anti-inflammatory action (Lee et al., 1995). Additionally, the alkaloid components of *Alstonia scholaris* and *Aconitum tanguticum* inhibited ALI in rats given low levels of LPS. Ginkgo biloba leaf extract, which has an anti-asthmatic effect, dramatically decreased lung inflammation in LPS-induced ALI when used at low doses (Babayigit et al., 2009). When used in the COPD model created using cigarette smoke, several plant extracts, including *Schisandra chinensis*, *Callicarpa japonica*, *Azadirachta indica*, *Euterpe oleracea*, *Juglans regia*, *Stemona tuberosa*, Galla chinensis, and *Cnidium monnieri*, were found to inhibit inflammatory changes in the lung (Zhong et al., 2015; Qamar and Sultana, 2011; Moura et al., 2012; Koul et al., 2012; Lee et al., 2014; Kwak and Lim, 2014). In Asia and Europe, bronchitis is commonly treated with *Pelargonium sidoides* (Matthys and Funk, 2008), *Hedera helix* (Guo et al., 2006), and *Echinacea purpurea* (Agbabiaka et al., 2008; Sharma et al., 2006).

Fast-growing evergreen *Sonneretia apetala* trees have trunks that are 20 to 30 cm in diameter and can grow as tall as 15 metres, with some specimens reaching 20 metres. Sonneretia apetala is known locally by the names Keora (Bengali), Chipi (Marathi), and Khirwa (Oriya), as well as MaramaMaram (Tamil), Kyalanki (Telugu), and others. Fruits

from this plant are used to make pickles and juices and are edible. Most of the Indian subcontinent, including Bangladesh, Sri Lanka, sections of Myanmar, and southern China, has coastal regions along the Bay of Bengal that are rich in tannins and polyphenols like flavonoids. Different pharmacological activity use different components of the plant. The plant's fruits have been used for a while to cure a variety of diseases. It's interesting to note that the fruits have historically been utilised as a folk medicine for symptomatic relief and to prevent coughing and wheezing caused by asthma and lung infections. There is no conclusive scientific research identifying the biomechanics and phytochemicals that are suppressing elastase. Using an *in situ*, *in vitro*, and *in vivo* elastase-induced lung injury model, we reported for the first time that fruit extracts may be a promising inhibitor of human neutrophil elastase.

We gathered fresh Sonneretia apetala fruits and used methanol to extract them. We examined the methanol extract's anti-elastase activity and discovered that it exhibits inhibitory effect against human neutrophil elastase at a range of doses (Figure 3A). We used a series of fruit extract dilutions to determine the extract's IC_{50} value, and we discovered that it is 0.3718 g/ml when compared to the control medication Sivelestat (Table 2), which is superior to the IC_{50} of certain popular herbal extracts with anti-elastase activities, such as Centenella asiatica (14.547 g/ml) (Nema et al., 2013), grape pomace extract polyphenols (14.7 g/ml) (Wittenauer et al., 2015), methanolic (ME) extracts from Washingtonia filifera (10.76 g/ml) (Era e (Maity et al., 2011).

The LC-MS/MS profiles confirm the content of gallic acid, ellagic acid, and other phenolic components in the extract and provide strong agreement with the HPLC spectrum. In light of the fact that S. apetala fruit extract lessened the harm caused by HNE in COPD, it is clear that polyphenols such gallic acid, elagic acid, luteoline, and ursolic acid as well as Sonneradon A and Sonneradon C, among others, may have some

bearing on the inhibition of HNE. Incidentally there are reports that ellagic acid, gallic acid and ursolic acid have potential anti-elastase activities (Singla, E.et al., 2020; Xing, X. Et al., 2016; Ying, Q. L. Et al., 1991).

In addition, recent data suggest that neutrophil elastase inhibitors may be used to treat patients with COVID-19 (Mohammed et al 2020). Hence this herbal extract can be used in herbal medicine clinical trial as a prophylactic treatment.

Inhibition is often characterised by data analysis techniques that linearize essentially nonlinear dynamics (either uncompetitive, noncompetitive or competitive). We used a substrate dilution test to determine the mechanism by which the fruit extract binds to the enzyme and discovered that it inhibits human elastase in a competitive manner (Figure.3B), as demonstrated in the Lineweaver-Burk plot. Ursolic, oleanolic, and 18-glycyrrhetinic acids are three plant triterpenes that exhibit competitive inhibition (Ros et al., 2000).

We also conducted a number of cell-based tests to better understand how elastase affects lung epithelial cell line. Fruit extract was discovered to be efficient against cell death and desquamation using fluorescence microscopy. Also noted was the inhibition of elastase-induced cell rounding by extracts and sivelestat treatment. (Figure.3A). To determine if fruit extracts could stop cells from shedding while neutrophil elastase was present, the cell detachment assay method was used (Figure 9B). These results were consistent with the previously described effects of Thai herbal extract on the treatment of lung cancer (Poofery et al., 2020).

There have been reports of the potential separation of lung cells because to decreased flexibility and adhesion molecules like ICAM. We were interested in determining how SAM affected the levels of sICAM after elastase treatment in cells because ICAM is

liberated as sICAM in the blood of COPD patients (Liu et al., 2018). We discovered that sICAM levels in cell culture media increased following elastase treatment, which suggests that cells that previously adhered via an adhesion molecule in cell membrane and detached after elastase therapy released ICAM as soluble ICAM into the media (Figure 3C). These outcomes are consistent with the cell detachment assay's observed results (Figure 3B). Although morphological changes have begun, the increase in sICAM levels after one hour as compared to control was non-significant, indicating that ICAM is not released into media as sICAM and the cells have not detached. Interestingly, SAM blocked the release of sICAM when combined with elastase, inhibiting cell separation like the control drug Sivelestat.

An MTT assay was used to gauge cell viability. Fruit extracts and the drug sivelestat both inhibited the viability of the cells caused by elastase alone. It is intriguing to note that neither the test nor the control inhibitor is hazardous to cells (Figure 3D). Fruit extracts of Sonneretia apetala are an excellent source of chemicals that can later be extracted and converted into semi-synthetic molecules because some earlier research have noted toxicity in herbal extracts (Nemati 2013).

Figures 5A and 5B from research using a mouse model of elastase-induced lung injury show a significant improvement in lung degradation. Here, we discovered that fruit extract of Sonneretia apetala at doses of 1 mg/kg/day, 10 mg/kg/day, and 100 mg/kg/day are protecting against the harm caused by elastase and can be compared to other studies on COPD where Paeonol, a vital component of Paeonia suffruticosa, at 10 mg/kg/day specifically reduced inflammation of the lungs in mice with a COPD model (Liu et al., 2014). When taken orally, extracts of Taraxacum officinale and Viola yedoensis' petroleum ether fraction demonstrated potent inhibitory activity against LPS-induced lung inflammation at modest doses of 3 mg/kg (Liu et al., 2010; Li et al., 2012). As a result,

like many other herbal preparations, Sonneretia apetala fruit extracts show a significant level of inhibitory activity against elastase-induced mice model of lung disorders.

8.6. Conclusions

Sonneretia apetala fruit extract has potential inhibitory effects against human neutrophil elastase and can act as an antagonist both *in vitro* (lung epithelial cell line) and *in vivo* (mouse emphysema model). The compounds identified from the extract having reported anti-elastase activities contribute to the inhibitory effects.

9

CHAPTER 2

9.1 Abstract

Neutrophil elastase, a powerful physiological defence tool, may serve as a therapeutic target for a variety of disorders, including chronic obstructive pulmonary disease (COPD) due to its bystander effect on host cells. Here, using an enzyme substrate kinetic experiment, we were able to identify seven new benzoxazinone derivatives as inhibitors of human neutrophil elastase. The most effective inhibitor of this class, PD05, showed a lower IC50 than the sivelestat control medication. Although the substrate dilution assay indicates that this inhibition is competitive, PD05 demonstrated higher binding affinity for human neutrophil elastase (Kd=1.63nM), faster association and dissociation rates, and fully reversible interaction with this enzyme when compared to well-known elastase inhibitors like ONO 6818 and AZD9668. In vitro preclinical pharmacokinetic investigations revealed a protein binding efficiency of 72%, a rapid recovery rate, an aqueous solubility of 194.7μM, a low permeability, and a favourable hERG having no cardiac toxicity. The chemical successfully inhibited elastase-induced rounded and retractable cell morphology and cell cytotoxicity, according to experiments with cell lines. In a mouse model, neutrophil elastase-induced alveolar collapse can be decreased by PD05. In conclusion, we show that the newly synthesised benzoxazinone derivative PD05 has anti-elastase capability in situ, in vitro, and in vivo, making it a suitable option for additional research as a COPD treatment.

9.2 Introduction

According to estimates, COPD, also known as chronic obstructive lung disease, affects around 7% of the world's population and has an exponentially rising incidence. Up to 10% of adults over the age of 65 have this condition. (Ford et al., 2013; Menezes et al., 2005; Herbert et al., 2006) The true prevalence of COPD appears to be higher than that indicated since it is both under-diagnosed and under-recognized. According to the WHO, COPD is the third leading cause of mortality and COPD-related fatalities are predicted to increase by more than 30% over the next ten years (Lucas et al., 2013). A persistent inflammatory responses in the pulmonary parenchyma and surrounding peripheral airways is connected to prolonged exposure to gases or noxious particles and results in COPD. Toxic substances in cigarette smoke are to blame for the majority of cases of COPD. A limitation of airflow that is typically progressive,, not entirely reversible, and distinguishes the pulmonary component. A restriction in airflow is caused by the small conducting airways' promotion of greater resistance and higher lung compliance (JC Hogg 2004). Dyspnea, which typically starts in the early stages of COPD, and continuous sputum production are the major features of the disease. Although there are several factors that contribute to the onset of COPD, airway constriction is the predominant one, mostly linked to dyspnea (Hogg et al., 2004; O'Donnell, D. E., and Webb, K. A. 1993; O'Donnell et. al., 2001).

As a result of the destruction of alveolar tissue and the subsequent loss of radial traction, airway narrowing is primarily brought on by thickening of the airway wall, lung fibrosis, and collapse of the airways during expiration (Hogg et al., 1968; McDonough et al., 2011). Increased goblet cells and mucous gland hyperplasia lead to an increase in sputum output (Lai, H. and Rogers, D. F. 2010). The airflow limits on COPD suitcases are irreversible, and the prognosis for their pathological diseases is typically gradual. In order

to achieve the objectives of avoiding COPD, pharmaceutical therapy as well as non-pharmacological measures, smoking cessation, the reduction of other variables, immunisation, and illnesses including adequate assessment of immunisation, oxygen therapy, and lung rehabilitation.

According to the most recent research, systemic inflammation caused by the "spill-over" of inflammatory cytokines from the lungs to the circulation in COPD patients leads to ischemic cardiovascular disease, heart failure, osteoporosis, and borderline anaemia. Systemic inflammation brought on by it may lead to lung cancer. Diabetes and depression worsen (Barnes, P. J. & B. R. Celli 2009). Therefore, it is crucial for COPD patients to control the relevant comorbidity. (August et al. 200 ; Soriano et al., 2005). It is essential to design an effective treatment plan because current therapies do not alter the disease's progressive nature [Barnes P. J. 2000]. Finding new targets and creating target-specific specific molecular inhibitors can be used to treat deadly diseases.

Neutrophil elastase targeting may be helpful in the prevention of inflammatory lung disease, according to numerous clinical observations. The COPD airway inflammation that results from the lungs' response to dangerous particles and gases is accompanied by an aberrant rise in inflammatory cells that line the airways, including neutrophils, T lymphocytes, and macrophages. By producing HNE, neutrophils are directly mobilised from the blood stream to cause damage to the parenchymal layer (Donnelly, L. E. and Rogers, D. F. 2003). Human Neutrophil Elastase (EC 3.4.21.37), a serine protease that is largely stored in the azurophilic granules of multinuclear neutrophils and is a member of the chymotrypsin superfamily. Neutrophils overproduce elastase during the inflammatory stage of COPD, which is released into the extracellular medium. Its catalytic triad, which consists of Ser195, His57, and Asp102 residues, controls their actions. The trio is widely separated in the primary sequence, but they are united at the active site in the tertiary structure (Korkmaz et al., 2008; Bode et al., 1989). Human Neutrophil Elastase is a

versatile enzyme that works against pathogens to control inflammation and maintain tissue homeostasis (Pham C. T. 2006). A variety of structural proteins, including collagen, elastin, laminin proteoglycans, and fibronectin, can be broken down by HNE using its active serine (Pham C. T. 2008; Chua, F., and Laurent, G. J. 2006). Through the breakdown of adhesion molecules, HNE plays a significant part in chemotaxis and aids in neutrophil migration (Cepinskas et al., 1999; Hermant et al., 2003). Through the breakdown of adhesion molecules, HNE plays a significant part in chemotaxis and aids in neutrophil migration (Cepinskas et al., 1999; Hermant et al., 2003). Under physiological circumstances that control inflammatory processes and prevent the negative consequences of extracellular HNE, endogenous inhibitors, such as 1-antitrypsin (1AT), 2-macroglobulin, elafin, and SLPI, lessen tissue damage. (Ohmoto et al., 2001; Heutinck et al., 2010; Tremblay et al., 2003). Extracellular HNE also plays a role in the development of lung diseases such as acute respiratory distress syndrome (ARDS) (Wang et. al. 2009), cystic fibrosis (Gifford, A. M., and Chalmers, J. D. 2014), acute lung injury (ALI) (Kawabata et. al., 2002) and asthma (Guay et. al., 2006). HNE plays vital roles in some other diseases like psoriasis and other skin diseases (Meyer-Hoffert et. al., 2004; Marto et. al., 2018), rheumatoid arthritis (Cawston T. E. and Young, D. A. 2010), atherosclerosis (Henriksen, P. A. and Sallenave, J. M. 2008) and various types of cancer including breast and lung cancer (Moroy et. al., 2012; Sato et. al., 2006). Neutrophil elastase inhibitors may be crucial in the management of COVID-19, according to recent research [Mohamed et al., 2020]. A wide range of functional problems and tissue damage are brought on by the broad specificity and proteolytic intensity of enzymes. So, according to P. A. Henriksen (2014), human neutrophil elastase is regarded as a prospective therapeutic target.

Arylating enzyme inhibitors (Lucas et al., 2013) and noncovalent inhibitors (Sjö, P. 2012), transition-state analogues (Edwards, P. D., and Bernstein, P. R. 1994), mechanism-

based inhibitors (Zhong, J., and Groutas, W. C. 2004), and noncovalent inhibitors are the four main inhibitor kinds. Recently, a number of thiazole-triazole acetamide hybrids with micromolar activity ranges were discovered as possible inhibitors of human neutrophil elastase (Butt et al., 2019). Additionally, deza analogue-containing inhibitors with pyrrolo-pyridine rings and indole derivatives were created (Crocetti et al., 2019; Crocetti et al., 2016).

Many pharmaceutical firms have worked hard to develop a powerful neutrophil elastase inhibitor. Sivelestat (ONO 5046), a medication used to treat acute lung injury that is typically accompanied by systemic inflammation, has only been approved for use in Japan (Iwata et al., 2010). Sivelestat has recently been suggested as a treatment for COVID patients with acute lung injury (Sahebnasagh et al., 2020).

For a very long time, it has been discovered that benzoxazinone compounds are highly effective against human neutrophil elastase (Krantz et al., 1990). The well-characterized substituted benzoxazin-4-ones that inhibit serine proteases work by interfering with an acyl enzyme intermediate (Hsieh et al., 2010). Using the structure-activity relationship, we define the newly synthesised benzoxazinone molecules in this work. The preclinical pharmacology of compound PD05 was described from research utilised for the development of the medication both *in vitro* and *in vivo*. Compound PD05 was discovered to be the most potent new selective NE inhibitor.

.9.3 Materials and Methods

9.3.1 General procedure for the synthesis of PD05

To a mixture of anthranilic acid (0.1 g, 0.54 mmol, 1 equiv) in pyridine (3 ml), chloroformate (0.116 ml, 2 equiv, 1.08 mmol,) was added and the resulting mixture was firmly stirred at 0° C for 4 hours in an atmosphere of nitrogen. Then 5ml of ice water was added to the mixture. The resultant mixture was then extracted with EtOAc (5 ml) and the

process was repeated twice. Organic layers, thus obtained, were then combined and dried over anhydrous sodium sulphate. It was then filtered followed by concentrating it under vacuum. The residue was washed with petroleum ether to give pure PD05.

9.3.2 Measurement of purity of Compounds by HPLC

The purity of PD05and its analogs as assessed by HPLC and the analysis was carried out in an HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-20AD and LC-20AT prominence liquid chromatography pump, DGU-20A3 prominence degasser, CBM-20A prominence communications bus module, SPD-20A, prominence UV/VIS detector and SPD-M20A PDA detector. Sample was dissolved in HPLC grade Methanol to achieve the concentration of 1mg/ml. An aliquot of 20 ml was injected using SIL-20AC HT prominence autosampler. The separation was achieved on a Phenomenex reverse phase HPLC column (Luna® RP C_{18} column 4.6x260 mm, 5µ particle size, column temperature; 25°C), and elution was carried out using mobile phase consisted of Water (0.1% Formic Acid) (A) and Methanol (B) and using a isocratic system, (0-30 min), B-80%, A- 20%. The elute was monitored at 254 nm and 380 nm. Data analysis was performed by LC solution version 1.25 (Shimadzu, Kyoto, Japan). Chromatogram and all other related data were presented in supplementary file as Figure S1-Figure S7.

9.3.3 Measurement of elastase activity and IC_{50} determination

The activity of inhibiting the human neutrophil elastase was measured using 200µg/mL human neutrophil elastase (Calbiochem,USA) and 1.2 mM *N*-(OMe-succinyl)-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma-Aldrich), both of which are prepared using 0.1 M Tris-NaCl buffer (pH 7.5). Test compounds (1 µL, DMSO), 50µL elastase (as prepared above) and 24µLTris-NaCl (pH 7.5) were pre-incubated in a 96-well microtiter plate at room temperature for 15 minutes. As incubation ended, 25 µL substrate solutions were added to each well. The reaction was observed by calculating the absorbance every 30 seconds for

a total of 2 hours, at 405 nm. Percentage inhibition was calculated after subtraction of blank from each curve as compared to standard (without drug) and IC_{50} was determined using GraphPad Prism v.8.4.

We incubated 4ug/ml HNE with three different concentrations (100µM,500µM and 1000µM) of the substrate *N*-(OMe-succinyl)-Ala-Ala-Pro-Val-*p*-nitroanilide and concentrations 0µM, 1µM, 10µM ,50µM and 100µM of the inhibitor PD05 (100µL total volume) in 96 well flat bottomed plates to determine the inhibition mechanism,. The reaction was carried out using the Spectramax multimode plate reader in the same process as described above. After blank correction the reaction velocities were determined and using GraphPad Prism v.8.4. The substrate titration plots and subsequent double reciprocal plots were then generated (Milicaj et. al., 2022; Budnjo et. al., 2014).

9.3.4 Binding Kinetics of PD05

To study the binding kinetics between PD05 and human neutrophil elastase assays were conducted using the BIAcore T100 instrument (GE Healthcare Biosciences AB). Human NE (100 µg/ml in 10 mM Tris-NaCl, pH 4.5) was pre-incubated with PD05 (1µ M) for 10 minutes to ensure that active site is available and was immobilized using a CM5 sensor chip surface (GE Healthcare Biosciences AB) by the method of amine coupling. Using ammonia coupling as a control surface the enzyme was immobilized on an activated and deactivated CM5 chip surface. Running buffer (0.1 M Tris-NaCl pH 7.5, with 1% dimethyl sulfoxide) was applied for attaining equilibrium and then PD05 was injected over the elastase enzyme at a flow rate of 50µL/min, and the reaction was observed and association rate was measured. Following the application of running buffer for 1 minute, the rate of dissociation was determined over 5 minutes. For PD05 (A) and immobilized NE (B), the complex (AB) formation is given by:

$d[AB]/dt=k_{on}[A][B]-k_{off}[AB]$

Using the T100 Evaluation software, we assessed the interaction data and determined the on (k_{on}) and off (k_{off}) rates, as well as KD values (k_{off}/k_{on}) based on the global fit (Stevens et. al., 2011).

9.3.5 ADME-Tox: Solution Properties

9.3.5.1Aqueous Solubility

All three aqueous solubility experiments in simulated gastric fluid, in PBS (pH 7.4) and in simulated intestinal fluid was carried out using shake flask technique with incubation of 24hours at room temperature and detected using HPLC-UV/VIS (Lipinski et. al., 2001).

9.3.5.2 Protein Binding

Equilibrium dialysis was used to measure protein binding with an incubation of 4 hours at 37°C using HPLC-MS/MS (Banker et. al., 2003).

9.3.6 ADME-Tox: In Vitro Absorption

Caco-2 cells were used to measure A-B permeability (pH 6.5/7.4) and B-A permeability (pH 6.5/7.4), incubation times of 0 and 60 minutes at 37°C, and detection with HPLC-MS/MS (Hidalgo et. al., 1989).

9.3.7 Cardiac Toxicity

Cardiac toxicity was assessed using an automated whole-cell patch clamp in hERG CHO-K1 cell line with an incubation of 5 minute at room temperature cumulatively (Mathes, C. 2006).

9.3.8 General Cell Culture Procedures

A549 cells were obtained from NCCS, Pune, and grown in F12K media (Gibco) containing 10% Fetal Bovine Serum (Life Technologies) at 37°C under a humidified environment.

9.3.9 Cell Viability Assay

5,000 cells/well of A549 were seeded in 96-well plate. The cells were treated with 100uM elastase or vehicle after 24 h of seeding. These were then co-treated with PD05 or sivelestat (Sigma-Aldrich) or DMSO. Prior to adding the MTT reagent at 0.5ug/ml concentration per well, the cells were incubated for further 12 hours. After 4 hours incubation with MTT, DMSO was added as solubilising agent and absorbance was taken at 550nm.

9.3.10 Cell Detachment and Morphology Change

In cell culture dishes (6-cm), A549 cells were seeded and were treated with vehicle+DMSO, elastase+DMSO and elastase+PD05 and elastase+Sivelestat. Using EVOS FL Microscope (10× magnification), brightfield images were taken at 3 hours, After 12 hours, the medium was collected and the separated cells were pelleted by means of centrifugation. Attached cells were collected by using trypsin (Thermo) and then pelleted. The cell pellet was resuspended in fresh medium and 0.04% trypan blue was added. Cells were counted using a haemocytometer using a 10 µl aliquot. The percent detachment was calculated based on the number of detached cells and the total number of cells present at the beginning of the experiment. Graphs were generated using prism software v. 8.4. We have used a varying micromolar concentrations of PD05 [Data not shown] to inhibit neutrophil elastase in cell line experiments. However at lower concentrations (2µM-5µM) PD05 couldn’t fully inhibit HNE (100nM) causing cell detachment but at 10µM we observed full inhibition of HNE which was in accordance with the already established and previously published methods (Salvador et. al., 2013; Misumi et. al., 2006).

9.3.11 Cell Staining

In 6-cm dishes, A549 cells were plated. Vehicle+DMSO, elastase+DMSO, elastase+PD05, and elastase+Sivelestat were used to treat the cells. After 3hours and 6 hours cell were treated with two drops of DAPI (Thermo Fischer) kept for 5 minutes and washed with PBS. Cells were observed under Fluorescence microscope as described above. Cells were also treated with WGA stain (Thermo Fischer) after 3 hours according to instruction manual and observed under microscope.

9.3.12 Mice grouping

The male C57/BL6 mice, which were about 8-10 weeks old, were obtained from in-house breeding facility at CSIR-Indian Institute of Chemical Biology (IICB), Kolkata and were acclimatized for one week at animal house facility, IICB. All the animal experiments were formally approved by Institutional animal ethics committee at IICB (Reference Number IICB/AEC/Meeting/July/2021/6). All the animal experiments were performed following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines).Mice were randomly divided into three groups: a) VEH/VEH (intratracheal vehicle, Tris buffer pH 7.4, administered and vehicle, 50% DMSO, diluted with saline, treated mice), b) NE/VEH (intratracheal neutrophil elastase administered and vehicle treated mice) and c) NE/PD-05 (intratracheal neutrophil elastase administered and PD-05 treated mice).

9.3.13 Induction of emphysema in mice with human neutrophil elastase

Human neutrophil elastase (Calbiochem, cat no. 324681) was reconstituted in tris buffer pH 7.4.For the induction of elastase induced emphysema, mice was first anesthetized by

brief isoflurane. Anesthetized mice were placed on a wood support at an angle of 45 degree and carefully grasped the tongue with an upward and leftward position using a blunt end forceps. These mice were then instilled with 10 µg human neutrophil elastase (to both NE/VEH and NE/PD-05 groups) or vehicle (VEH/VEH group) on day 1 through orotracheal route using a pipette as shown in Figure 1. Mice were maintained on the same position for 15 seconds and then placed on warm pad for recovery.PD-05 or vehicle was administered from day 1 to day 7, twice a day. We have used 1mg/kg dose based on our pilot experiments that were performed in LPS induced acute lung injury model where we have tested three different doses of 0.1 mg/kg, 1 mg/kg and 10 mg/kg (unpublished data). It also to be noted that LPS induced acute lung injury shares multiple features such as airway neutrophilia, alveolar injury etc. with human neutrophil elastase induced lung injury [Grommes, J., and Soehnlein, O. 2001; Zeiher et. al., 2004]. Therefore we had chosen 1mg/kg oral dose in mice study. Mice were euthanized on day 7 and blood and lungs were collected.

9.3.14 Lung histology

The lungs were collected and fixed with formalin and embedded in paraffin. 5µm sectionswere cut and Haematoxylin and Eosin (H & E) staining was performed.

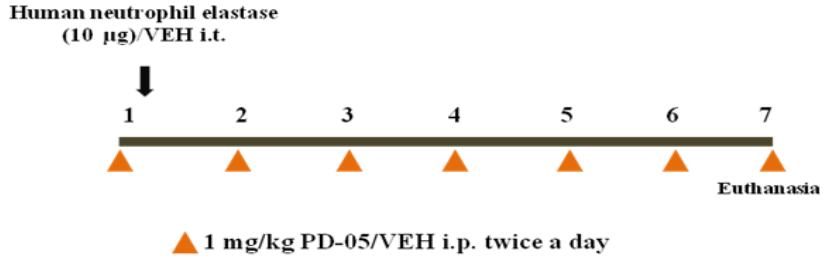


Figure 1: Schematic representation of mice experimental protocol

Images of H & E stained lung sections were captured for each animal using the Magnus MLX-i microscope at an objective lens magnification of 10x. The images were taken equally spaced and over the whole surface of the lung sections they were systematically placed meander-like. The images were quantitatively analyzed by a quadratic test system with 64 points and 8 horizontal lines and 8 vertical lines with a total length of µm (d = µm).via the STEPanizer software (Tschanz et. al., 2011). The mean linear intercept of the airspaces (Lm) of each lung slide was calculated based on P_{ref} (all points hitting the parenchyma), P_{sep} (points hitting the alveolar septa) and I (intersections of the test line system with alveolar surface) using the following formula: $Lm = 2*d* (\sum P_{ref} - \sum P_{sep} / \sum I)$ (Salaets et. al., 2020).

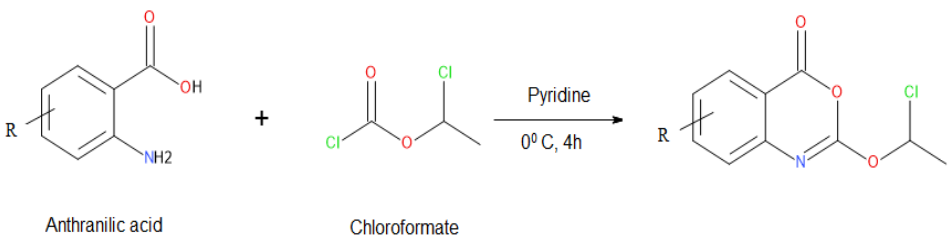
9.3.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism Software version 8.4. Unpaired, 2-tailed Student's t-test was used to calculate statistically significant differences. For comparison between more than two groups one-way ANOVA followed by Dunnett test was applied. *P < 0.05 was considered statistically significant, ** indicates P < 0.01, and *** indicates P< 0.001.

9.4 Results

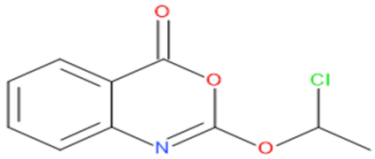
9.4.1 General Method of preparation of PD05 and its analogues

Scheme 1: General method of preparation of benzoxazinone derivatives



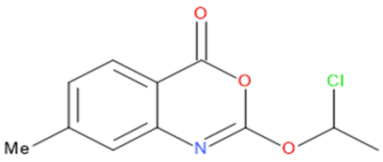
Anthranilic acid Chloroformate

List of benzoxazinone derivatives synthesised



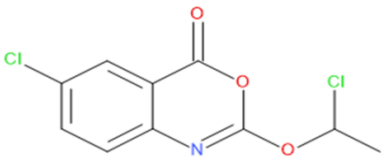
PD05

Chemical Formula: $C_{10}H_8ClNO_3$
Molecular Weight: 225.63
IUPAC Name: 2-(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 98.9% **Yield:** 97%



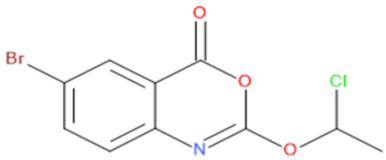
PD05a

Chemical Formula: $C_{11}H_{10}ClNO_4$
Molecular Weight: 255.65
IUPAC Name: 2-(1-chloroethoxy)-7-methyl-3H-benzoxazin-4-one
Purity: 97.19% **Yield:** 98%



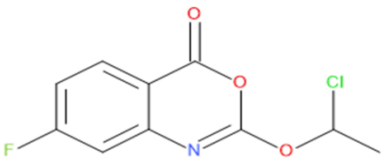
PD05b

Chemical Formula: $C_{10}H_7Cl_2NO_3$
Molecular Weight: 260.07
IUPAC Name: 6-chloro2--(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 100% **Yield:** 97%



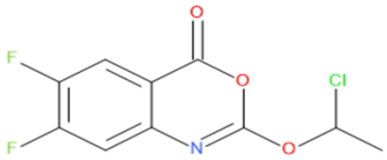
PD05c

Chemical Formula: $C_{10}H_7BrClNO_3$
Molecular Weight: 304.52
IUPAC Name: 6-bromo-2--(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 99.93% **Yield:** 97%



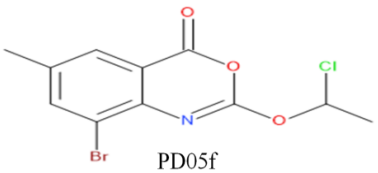
PD05d

Chemical Formula: $C_{10}H_7ClFNO_3$
Molecular Weight: 243.62
IUPAC Name: 2-(1-chloroethoxy)-7-fluoro-3H-benzoxazin-4-one
Purity: 96% **Yield:** 98%



PD05e

Chemical Formula: $C_{10}H_6ClF_2NO_3$
Molecular Weight: 261.61
IUPAC Name: 2-(1-chloroethoxy)-6,7-difluoro-3H-benzoxazin-4-one
Purity: 100% **Yield:** 99%



PD05f

Chemical Formula: $C_{11}H_9BrClNO_3$
Molecular Weight: 318.55
IUPAC Name: 8-bromo-2-(1-chloroethoxy)-6-methyl-3H-benzoxazin-4-one
Purity: 100% **Yield:** 98%

Figure 2A: Seven different benzoxazinone derivative as elastase inhibitors

NMR Data

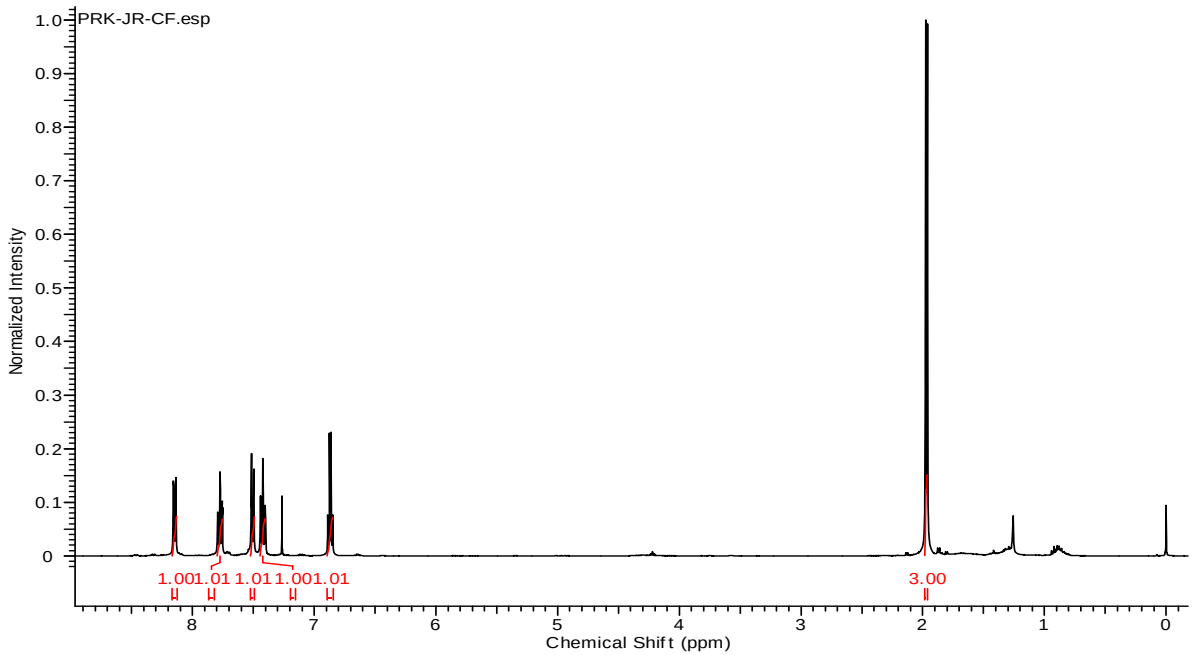


Figure 2B (I): NMR Profile of PD05

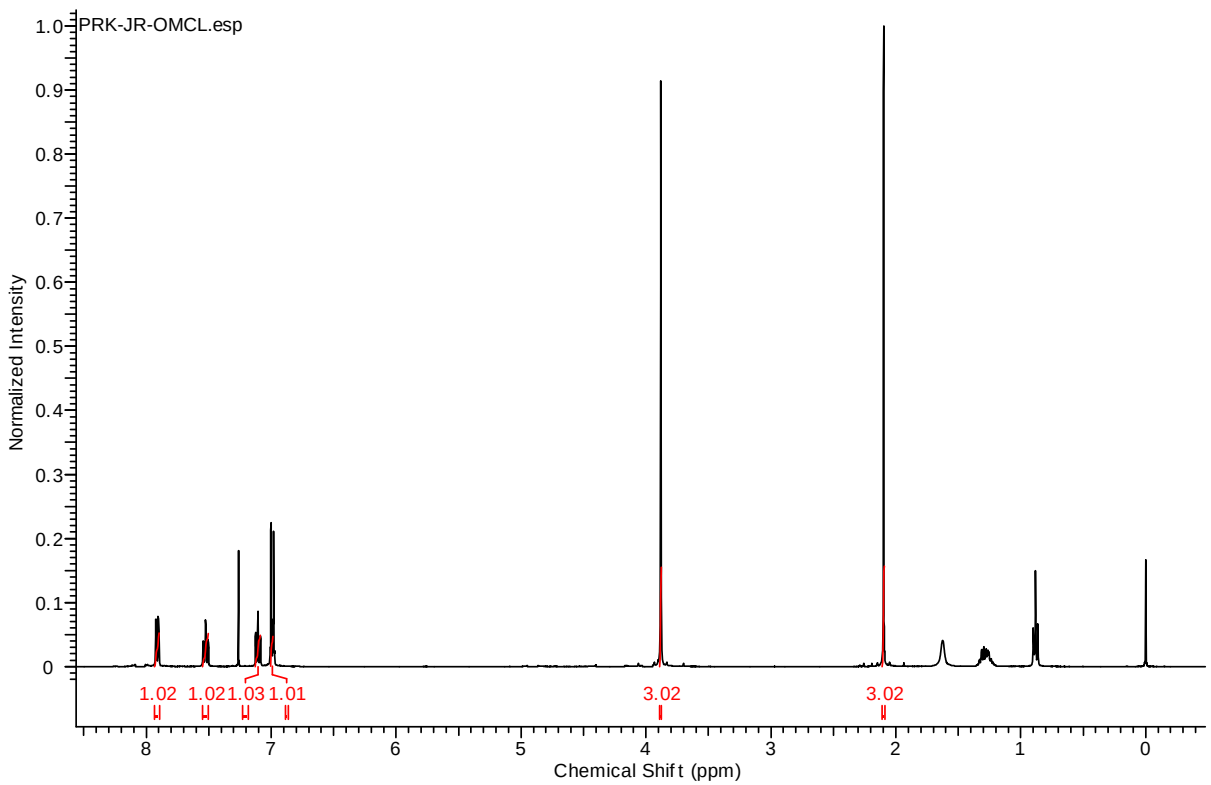


Figure 2B (II): NMR Profile of PD05a

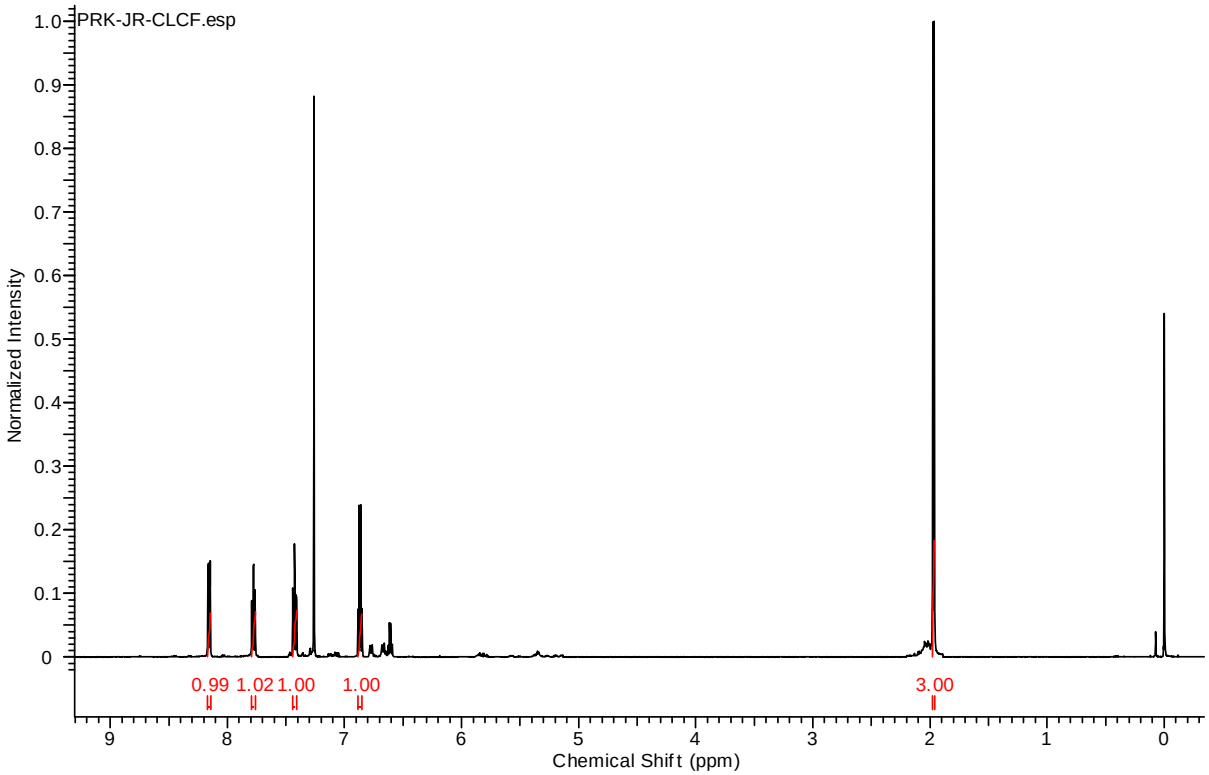


Figure 2B (III): NMR Profile of PD05b

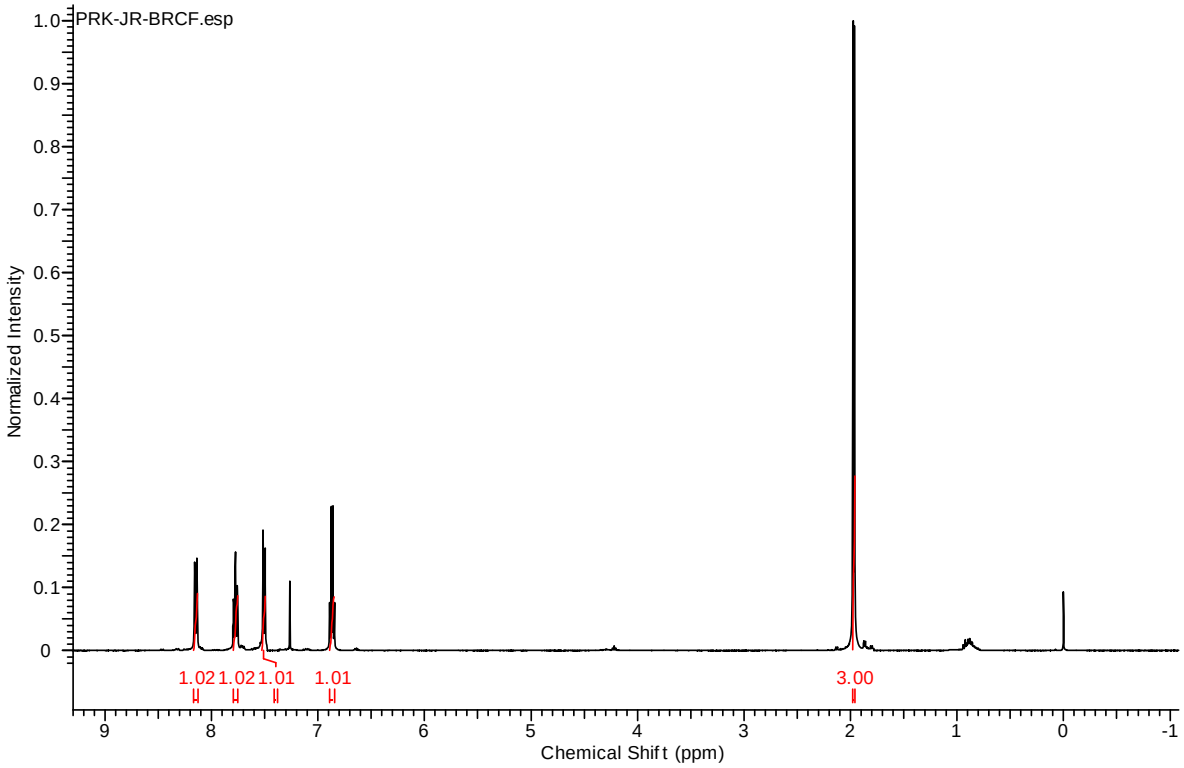


Figure 2B (IV): NMR Profile of PD05c

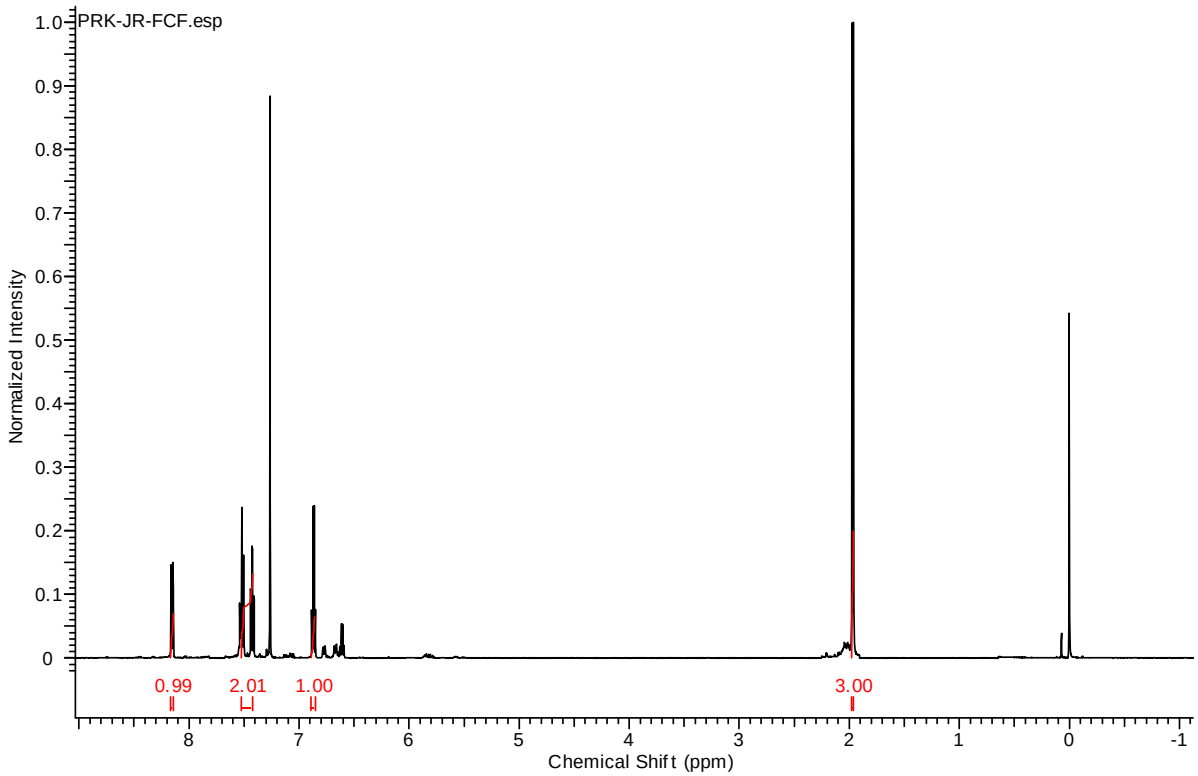


Figure 2B (V): NMR Profile of PD05d

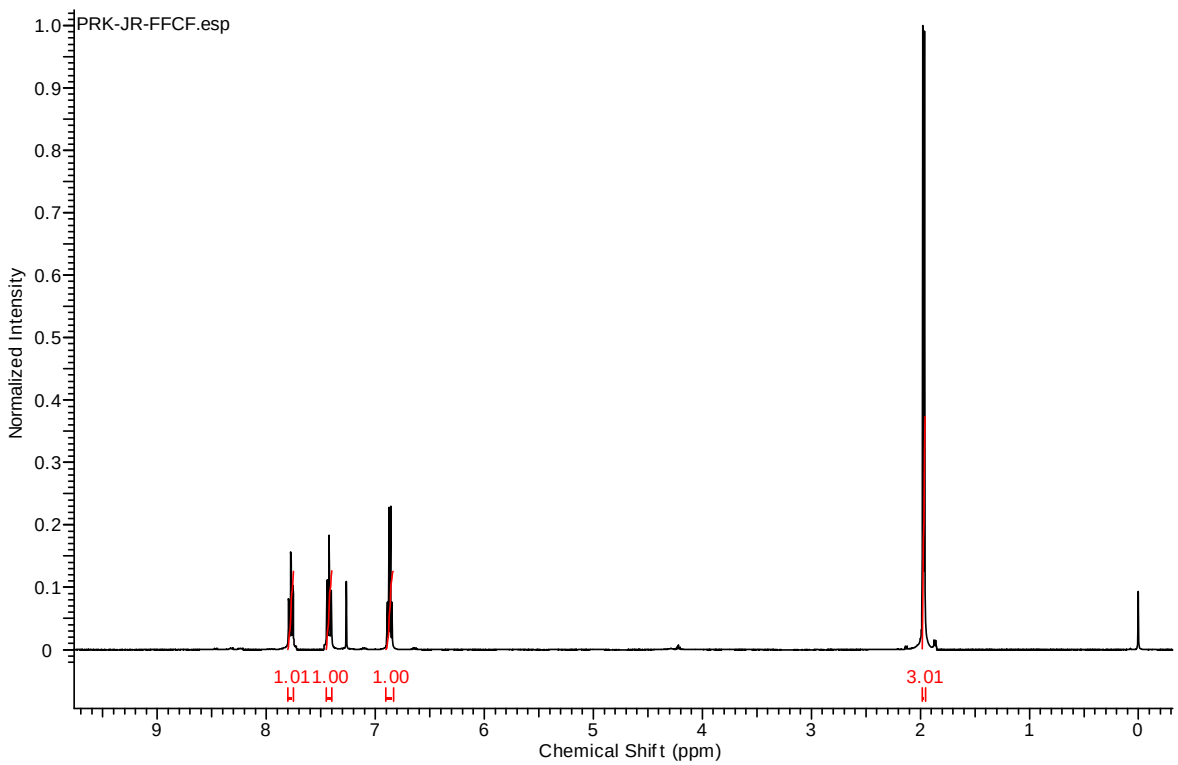


Figure 2B (VI): NMR Profile of PD05e

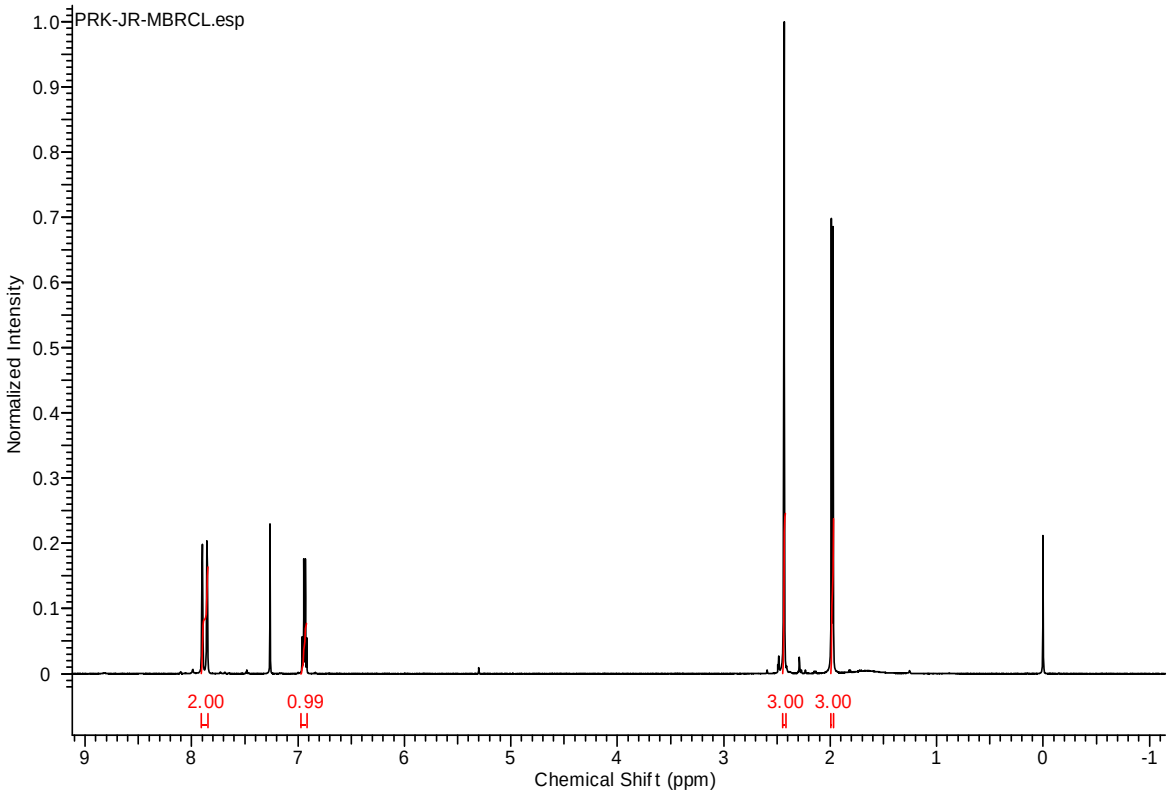
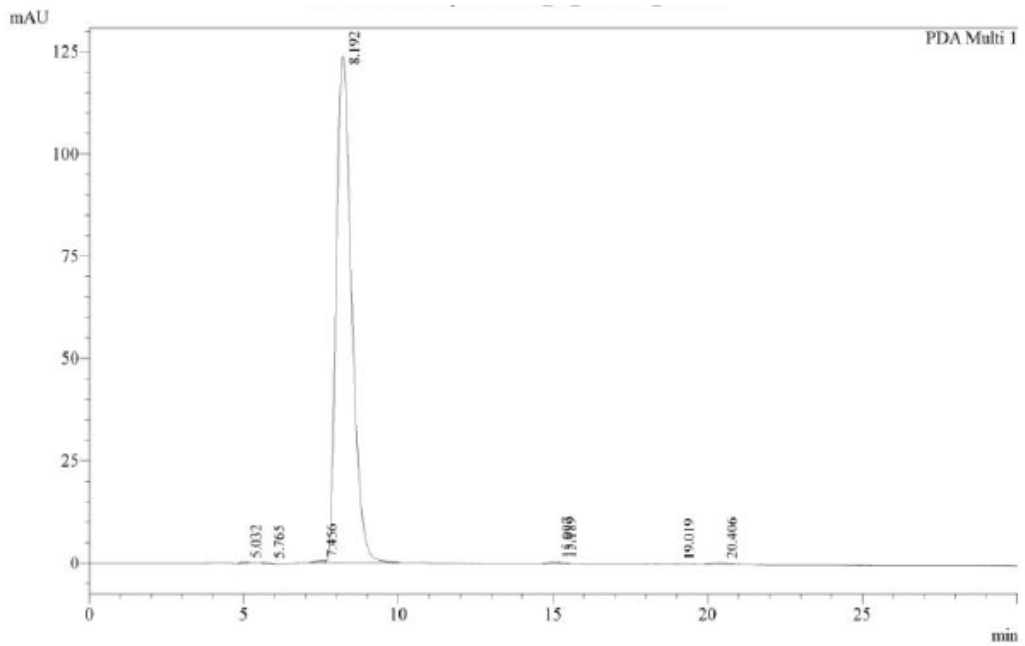


Figure 2B (VII): NMR Profile of PD05f



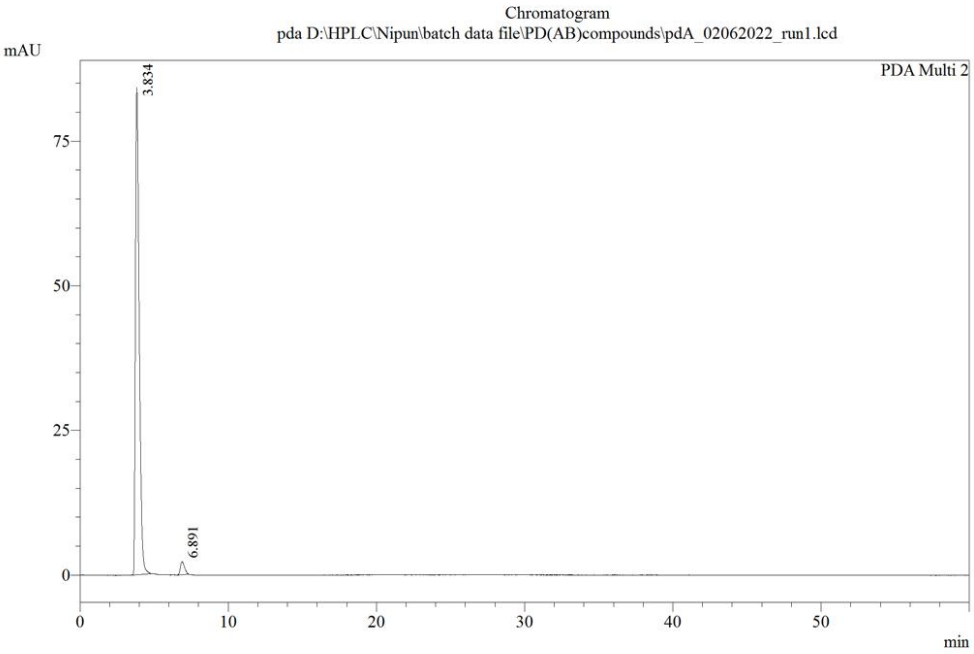
1 PDA Multi 1 / 380nm 4nm
2 PDA Multi 2 / 254nm 4nm

PeakTable D:\HPLC\Nipun\Data\PD_05_11062022_run2.lcd

PDA Ch1 380nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.032	5369	288	0.124	0.229
2	5.765	2880	176	0.067	0.140
3	7.456	8733	418	0.202	0.332
4	8.192	4278188	123685	98.904	98.282
5	15.097	8873	392	0.205	0.312
6	15.189	4893	355	0.113	0.282
7	19.019	1218	93	0.028	0.074
8	20.406	15448	441	0.357	0.350
Total		4325604	125847	100.000	100.000

Figure 2C (I): Chromatogram profile of PD05

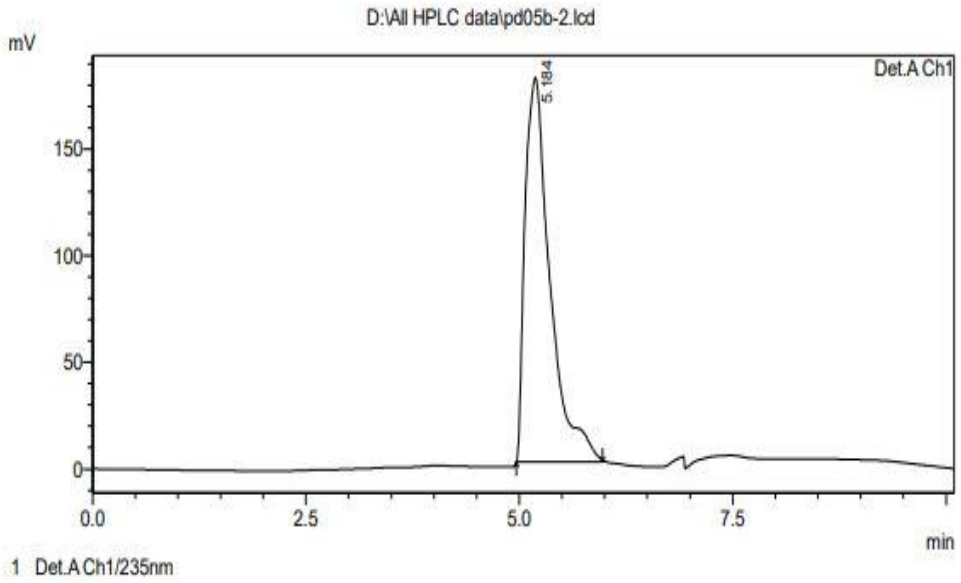


PeakTable D:\HPLC\Nipun\batch data file\PD(AB)compounds\pdA_02062022_run1.lcd

PDA Ch2 360nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.834	1471148	84170	97.190	97.445
2	6.891	42530	2207	2.810	2.555
Total		1513678	86377	100.000	100.000

Figure 2C (II): Chromatogram profile of PD05a

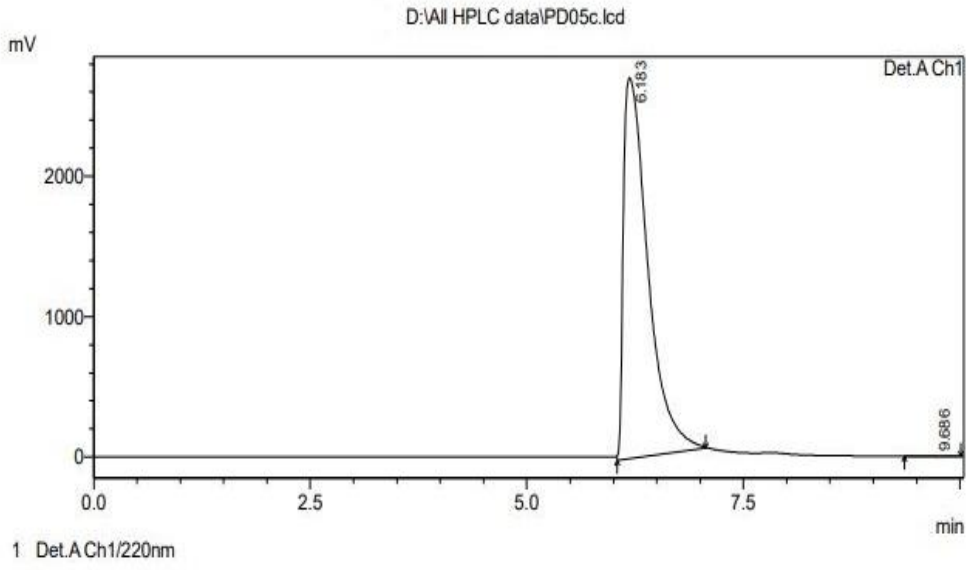


PeakTable

Detector A Ch1 235nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.184	3672626	180472	100.000	100.000
Total		3672626	180472	100.000	100.000

Figure 2C (III): Chromatogram profile of PD05b

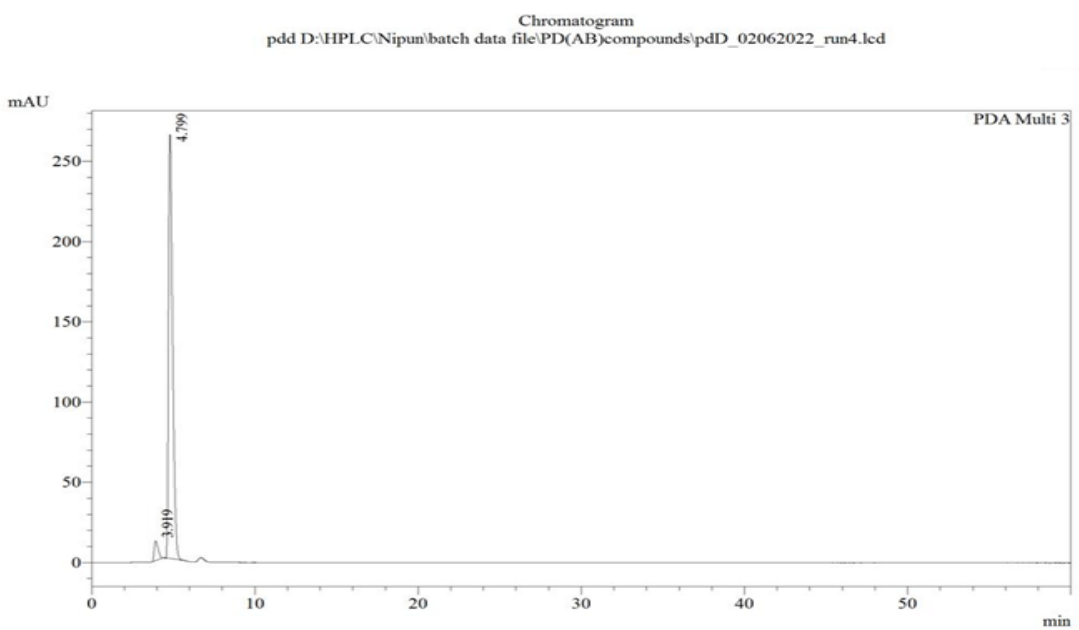


PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.183	53327446	2714046	99.931	99.937
2	9.686	36904	1698	0.069	0.063
Total		53364350	2715745	100.000	100.000

Figure 2C (IV): Chromatogram profile of PD05c

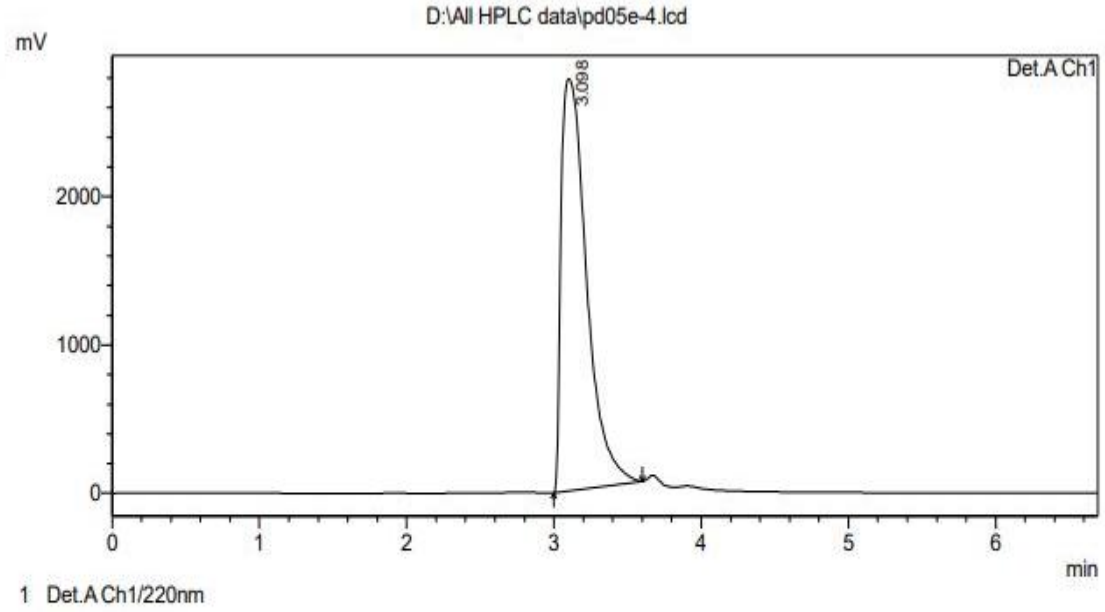


PeakTable

PDA Ch3 360nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.919	200966	12198	4.177	4.413
2	4.799	4610185	264188	95.823	95.587
Total		4811151	276386	100.000	100.000

Figure 2C (V): Chromatogram profile of PD05d

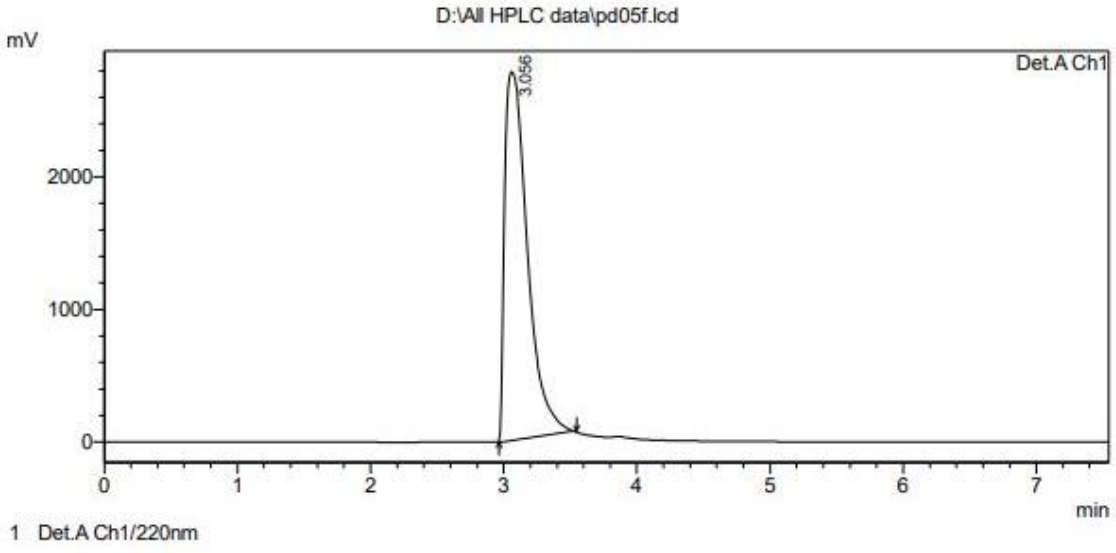


PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.098	33409027	2778689	100.000	100.000
Total		33409027	2778689	100.000	100.000

Figure 2C (VI): Chromatogram profile of PD05e



PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.056	32829258	2780760	100.000	100.000
Total		32829258	2780760	100.000	100.000

Figure 2C (VII): Chromatogram profile of PD05f

The general scheme for synthesis of benzoxazinone derivatives was given (Scheme 1) while the different structures of the compounds including nomenclature, molecular weight, Purity and yield of PD05 to PD05f were shown in Figure 2A. NMR data of all the seven molecules are shown in Figure 2B and purity data obtained from HPLC was shown in Figure 2C.

9.4.2 PD05 acts a neutrophil elastase inhibitor with a low IC_{50}

Table 1: Antiproteolytic Activity of Benzoxazinone derivatives

Compound	**PD05**	**PD05a**	**PD05b**	**PD05c**	**PD05d**	**PD05e**	**PD05f**	**Sivelestat**
IC_{50}(nM)	132.6 ± 3.21	186.4 ± 3.3	157.1 ± 3.06	166.6 ± 3.19	180.0 ± 3.15	173.8 ± 2.43	146.67 ± 3.48	138.76 ± 3.96

Table 1: Inhibitory concentrations (IC_{50}) of seven synthesised benzoxazinone derivatives tested against human neutrophil elastase. Data were expressed in nM as Mean±S.D.from three sets of experiments (n=3).

Enzyme Inhibition: We tested the anti proteolytic activity of seven benzoxazinone compounds against human neutrophil elastase and found that all compounds potently inhibited HNE with an IC_{50} value ranging from 132 nM to 180.03 nM. The most potent compound was found to be PD05 with an IC_{50} value of 132 nM as compared to only available known elastase inhibitor, sivelestat having IC_{50} value of 138.76 nM (Table 1).

Hence we selected PD05 which seemed to be a promising neutrophil elastase inhibitor and conducted all the further experiments with this molecule as a part of pre clinical drug discovery process.

9.4.3 PD05 inhibits neutrophil elastase in a competitive manner

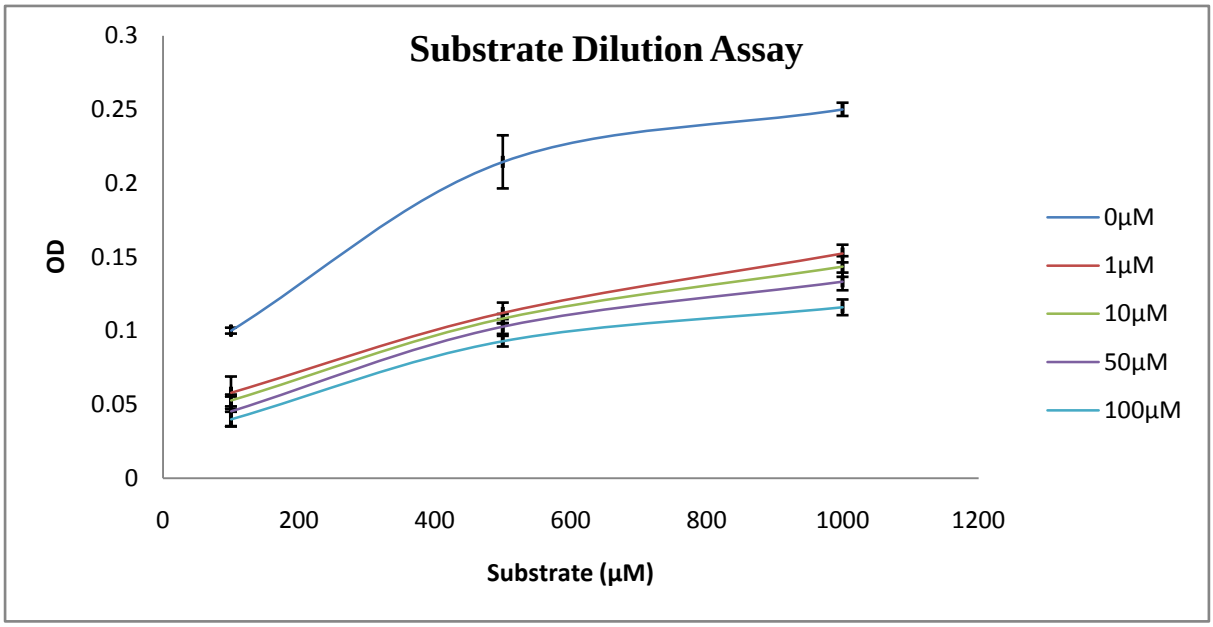


Figure 3A

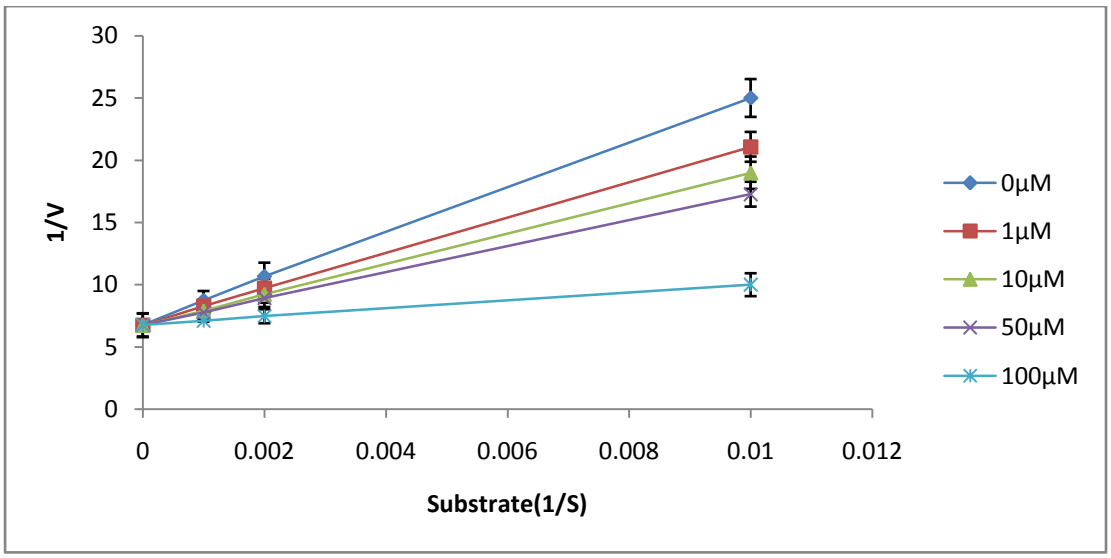


Figure 3B

Figure 3A: Substrate titration of steady-state rate of HNE in the presence of inhibitor PD05 (0-100 μM). Figure 3B: Double reciprocal format for Vmax determination. Data is represented from three independent experiments (n=3).

Kinetic studies were conducted to understand the style of inhibition of synthetic compounds against elastase inhibition. Based on the IC50, the most potent compound PD05 was selected to determine the mode of inhibition. Enzyme kinetic results from the 1 / V Lineweaver Burk plot to substrate N-(O-Mesuccinyl) Ala-Ala-Pro-Val-p-nitroanilide 1 / [S] in the presence of various concentrations of inhibitor revealed a series of straight lines. The Lineweaver Burk plot of compound PD05 showed that V_{max} remained the same without significantly affecting the gradient. K_m increases with increasing concentration, but V_{max} remains the same with slight differences. This behaviour indicates that the PD05 compound is a competitive inhibitor of the enzyme.

9.4.4 PD05 binds to neutrophil elastase with a low KD

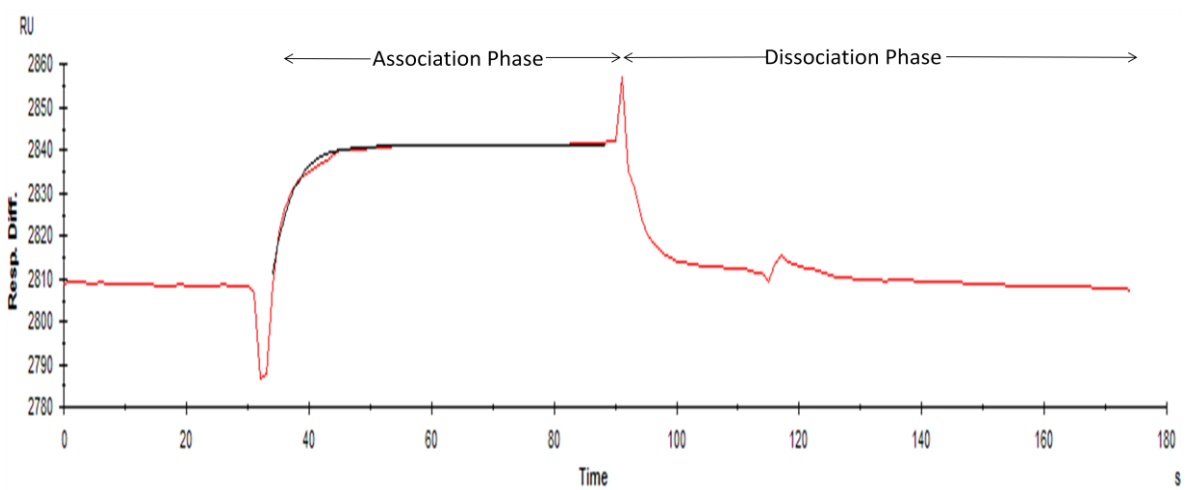


Figure 4

Figure 4: Biacore assay of PD05 binding to human NE. The y axis represents the amount of interaction and the x-axis is time in seconds. Data represent one of three different experiments (n=3). Kinetics data obtained from the graph are tabulated below (Table 2).

Table 2: PD05 binding kinetics and affinity for Human Neutrophil Elastase

Compound	PD05
ka(1/Ms)	3.06e4±0.02
kd (1/s)	5e-5±1.03
Rmax(RU)	30.3±2.9
RI(RU)	2.81e3±2.45
Concentration of Analyte (M)	1e-5
KA(1/M)	6.12e8±0.05
KD(M)	1.63e-9±1.9
Req (RU)	30.2±3.7
Kobs (1/s)	0.306±.04
Ki (pM)	4.4±0.9
Chi2	0.539±.002

Table 2: The kinetics data comprising of various parameters are reported as mean± S.D. obtained from three different experiments (n=3).

Binding kinetics: PD05 showed high binding affinity for HNE (KD = 1.63 nM) and strongly inhibited NE activity (Table2; Fig 4). K_{on} and K_{off} are 3.06e4 (1/Ms) and 5e-5(1/s) respectively. PD05 showed rapid rate of association and dissociation, and the interaction with NE was completely reversible.

9.4.5 Pharmacokinetic parameters of PD05

9.4.5.1 Protein binding of PD05

Using the peak area of the test compound in the buffer and test sample, the binding and recovery were calculated according to the following equation::

Protein binding (%) = $[(Area_p-Area_{b)}/Area_p] *100$

Recovery (%) = $[(Area_p+Area_b)/Area_c] *100$

Where,

$Area_p$ = Peak area of analyte in protein matrix

$Area_b$ = Peak area of analyte in buffer

$Area_c$ = Peak area of analyte in control sample

Table 3: Protein Binding (Plasma, human)

Compound	Test Concentration	% Protein Bound (Mean±S.D.)	%Recovery (Mean±S.D)
PD05	1.0E-05 M	72±3.27	97±1.21
Acetabutol	1.0E-05 M	3±0.07	99±0.07
Quinidine	1.0E-05 M	59±2.98	85±2.73
Warfarin	1.0E-05 M	98±1.23	86±4.39

Table 3: PD05 is compared with three different classes of drugs and Protein binding and recoveries have been given as percentage value (Mean±S.D.) from three different sets of experiments (n=3).

The protein binding percentage for PD05 was 68.5 and 74.6 resulting in a mean of 72. It was compared with Acetabutol, Quinidine and Warfarin whose results are 3, 59 and 98 respectively. The percentage recovery for PD05 was 97 which was similar to that of Acetabutol (99) and little higher than Quinidine (85) and Warfarin (86) (Table 3).

9.4.5.2 Aqueous solubility of PD05

The ratio of the peak area of the main peak of the calibration standard (200 µM) with the organic solvent (methanol / water, 60/40, v / v) to the peak area of the corresponding peak

of the buffer sample determines the water solubility (µM). The peak area of the main peak relative to the total integrated peak area of the calibration standard HPLC chromatogram defined the chromatographic purity (%).\

Table 4: Aqueous solubility

A

Compound	Test Concentration	Wavelength of Detection	Solubility(µM) (Mean±S.D.)	Chromatographic Purity %
Aqueous solubility (simulated intestinal fluid)				
PD05	2.0E-04 M	230	200±2.12	100
Aqueous solubility (PBS, pH 7.4)				
PD05	2.0E-04 M	230	194.7±1.38	100
Aqueous solubility (simulated gastric fluid)				
PD05	2.0E-04 M	230	200±1.73	100

B

Compound	Test Concentration	Wavelength of Detection	Solubility(µM) (Mean±S.D.)	Chromatographic Purity %
Aqueous solubility (PBS, pH 7.4)				
Haloperidol	2.0E-04 M	205	97.8±0.54	100
Ketoconazole	2.0E-04 M	205	125.5±2.82	100
Diethylstilbesterol	2.0E-04 M	230	6.5±0.03	100
Phenytoin	2.0E-04 M	230	89.7±3.2	100
Rifampicin	2.0E-04 M	230	192.3±2.49	100
Simvastatin	2.0E-04 M	230	21.5±1.1	100
Tamoxifen	2.0E-04 M	230	1.5±-.02	100

Table 4: Solubility Data expressed in µM (Mean±S.D.) of PD05 (Table 4A) has been tabulated along with seven other commonly used marketed drugs (Table 4B) obtained from three different sets of experiments (n=3).

The aqueous solubility in simulated gastric fluid and simulated intestinal fluid is 200µM while in PBS (pH 7.4) the solubility is 194.7µM for PD05. The solubility was compared with seven different drugs whose solubility varies from 6.5µM to 192.3µM (Table 4B).

9.4.6 In vitro absorption of PD05

Test compound's apparent permeability coefficient (Papp) was calculated as follows:

$$Papp\ (cm/s) = (V_R * C_{R,\ end})/\Delta t * [1/\ A*(C_{D,\ mid}-C_{R,\ mid})]$$

Where,

V_R means volume of the receiver chamber. $C_{R,end}$ represents concentration of the test compound in the receiver chamber at the end time point, Δt is the incubation time and A signifies the surface area of the cell monolayer. $C_{D,mid}$ is the calculated mid-point concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minute and the donor concentration at the end time point. $C_{R,\ mid}$ gives the mid-point concentration of the test compound in the receiver side, which is one half of the receiver concentration at the end time point. The test compound's concentration was expressed as the peak area of the test compound.

Recovery of the Test Compound from the Permeability Assay

The recovery of the test compound was measured as follows:

$$Recovery\ (\%) = [(V_D * C_{D,\ end} + V_R * C_{R,\ end})/\ V_D * C_{D0}] * 100$$

Where,

V_D and V_R represent the volumes of the donor and receiver chambers, respectively. $C_{D,\ end}$ is the concentration of the test compound in the donor sample at the end time point. $C_{R,\ end}$ is the concentration of the test compound in the receiver sample at the end time point. C_{D0} is the concentration of the test compound in the donor sample at time zero. The test compound's concentration was expressed as the peak area of the test compound.

Fluorescein assessment for Permeability Assays: As the cell monolayer integrity marker Fluorescein was used. Fluorescein permeability assessment (in the A-B direction at pH 7.4 on both sides) was carried out after the permeability assay for the test compound. The cell monolayer that had a fluorescein permeability of less than 1.5 x 10-6 cm/s for Caco-2 and MDR1-MDCKII cells and 2.5 x 10-6 cm/s for MDCKII cells was considered intact, and the permeability result of the test compound from intact cell monolayer is reported.

Table 5: Permeability

Compound	Test Concentration	Permeability (10^{-6}cm/sec) (Mean±S.D.)	%Recovery (Mean±S.D.)
A-B permeability (Caco-2, pH 6.5/7.4)			
PD05	1.0E-05 M	0.7±.002	78±2.98
Colchicine	1.0E-05 M	0.1±.0007	77±1.32
Labetalol	1.0E-05 M	8.8±0.9	74±3.09
Propranolol	1.0E-05 M	25±2.18	69±1.82
Ranitidine	1.0E-05 M	0.3±.0001	77±1.74
B-A permeability (Caco-2, pH 6.5/7.4)			
PD05	1.0E-05 M	2.5±0.07	79±2.1
Colchicine	1.0E-05 M	5.5±1.1	85±1.9
Labetalol	1.0E-05 M	35±2.47	84±2.9
Propranolol	1.0E-05 M	42.4±3.2	86±3.1
Ranitidine	1.0E-05 M	1.7±.006	97±4.87

Table 5 summarises the permeability values for PD05 as compared to 4 other known drugs like Colchinine, Labetalol, Propranolol and Ranitidine. Permeability and percentage recovery for both A-B and B-A are represented from three different sets of experiments (n=3), expressed as Mean±S.D.

The permeability values for PD05 were 0.65 and 0.73 with a mean value of 0.7 for A-B permeability. It was compared with Colchinine (0.1), Labetalol (8.8). Propranolol (25) and Ranitidine (0.3).The percentage recovery for PD05 was 78 which was comparable with Ranitidine of 77.

For B-A permeability the mean value for PD05 was 2.5 with a recovery rate of 79.

9.4.7 Cardiac toxicity profile of PD05

hERG (automated patch-clamp): By measuring the tail current amplitude, the degree of inhibition (%) was obtained, which is obtained by two second pulse to + 20 mV followed by a one second test pulse to - 40 mV, administered before and after drug incubation. The percent of inhibition was obtained by calculating the difference current normalized to control and then multiplied by 100. To generate estimates of the 50 % inhibitory concentration (IC50) concentration (log) response curves were generated and fitted to a logistic equation. Using the percentage reductions from current amplitude, the concentration response relationship for each of the compounds was constructed by sequential concentrations.

Table 6: Cardiac Toxicity

Compound	**Test concentration**	**% Inhibition of Tail current (Mean±S.D.)**
PD05	1.0E-07 M	1.8±0.076
PD05	1.0E-06 M	8.4±1.42
PD05	1.0E-05 M	15.8±1.02

Table 6 represents the % Inhibition of Tail current obtained from three different sets of experiments of three different concentrations (n=3 for each concentration).

PD05 displayed very low inhibition of 15.8, 8.4 and 1.8 at 100µM, 1mM and 10mM respectively (Table 6).

9.4.8 Cytoprotective Effects of PD05 against Elastase-Induced Cell Disjunction and Morphological Change

After3 hours of neutrophil elastase treatment A549 cells started to conglomerate and detach from the cell culture dish as compared to control.(Figure 5A). Co treatment with PD05 or sivelestat completely prevented the above event to happen. In contrast after 6 hours of treatment the cells are completely detached from the plate barring a few and suggesting a chance of cell death or apoptosis. As compared to three hours treatment with PD05 or sivelestat somewhat prevented the cells from desquamation but there is a hint of apoptosis suggesting that PD05 is reversible and free enzyme is responsible for the action.

Cytoprotective effect of PD05 against Neutrophil elastase induced cell desquamation

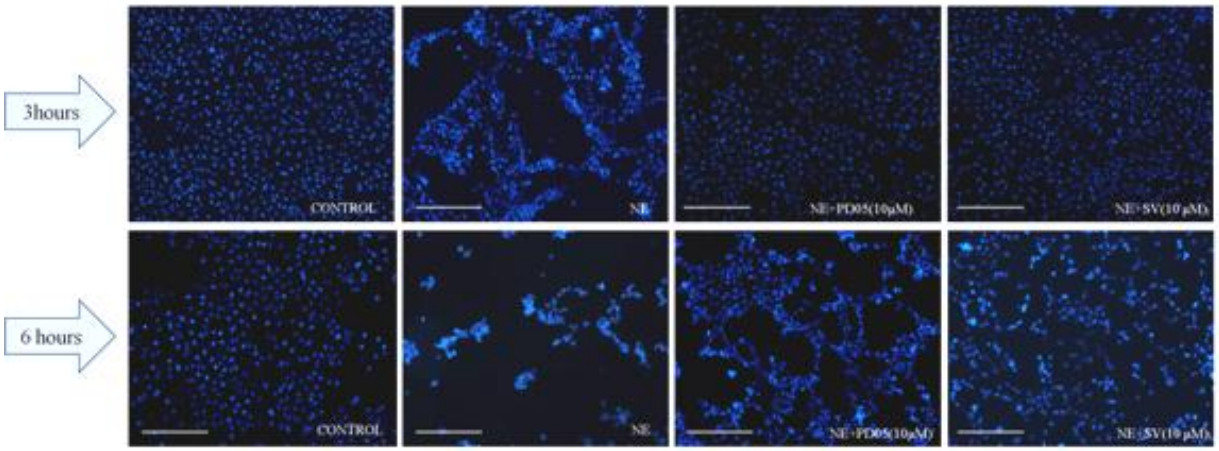


Figure 5A

PD05 inhibits rounding of epithelial cells under neutrophil elastase treatment (Brightfield Microscopy)

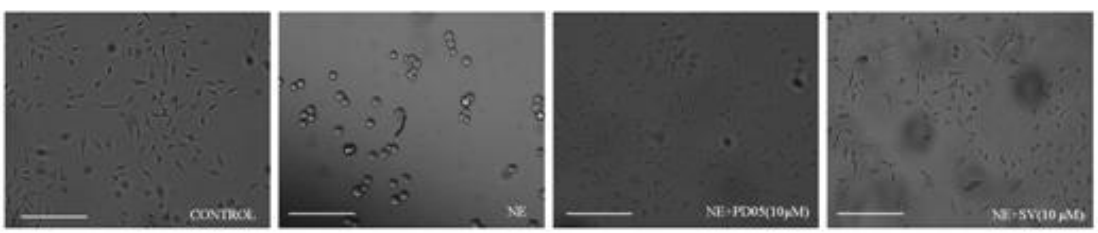


Figure 5B

PD05 inhibits rounding of epithelial cells under neutrophil elastase treatment (Fluorescence Microscopy)

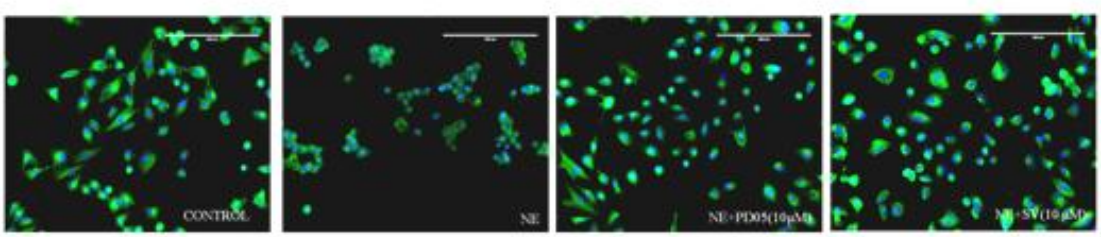


Figure 5C

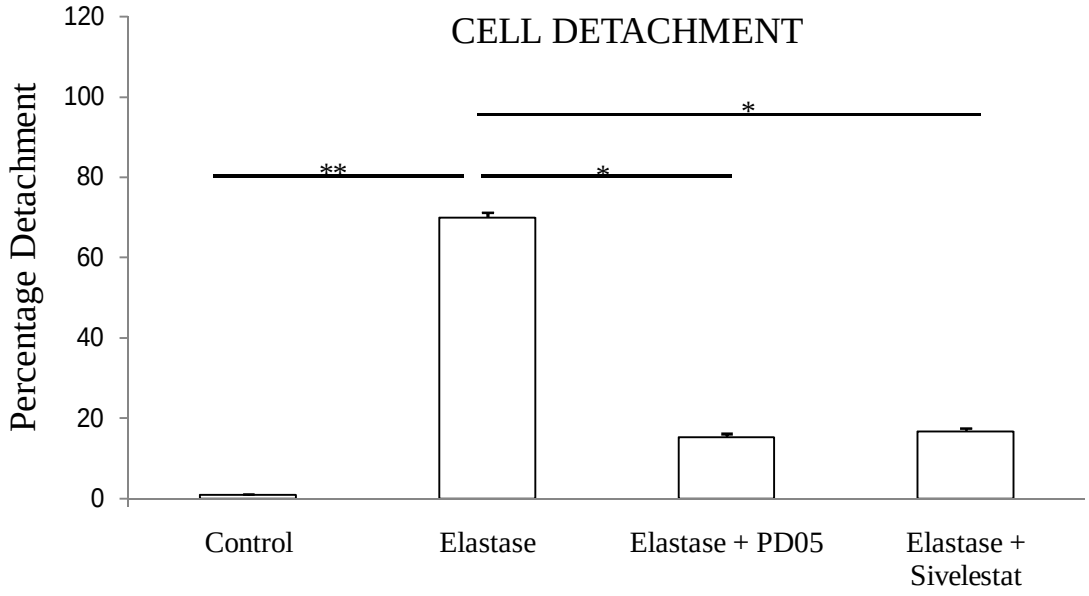


Figure 5D

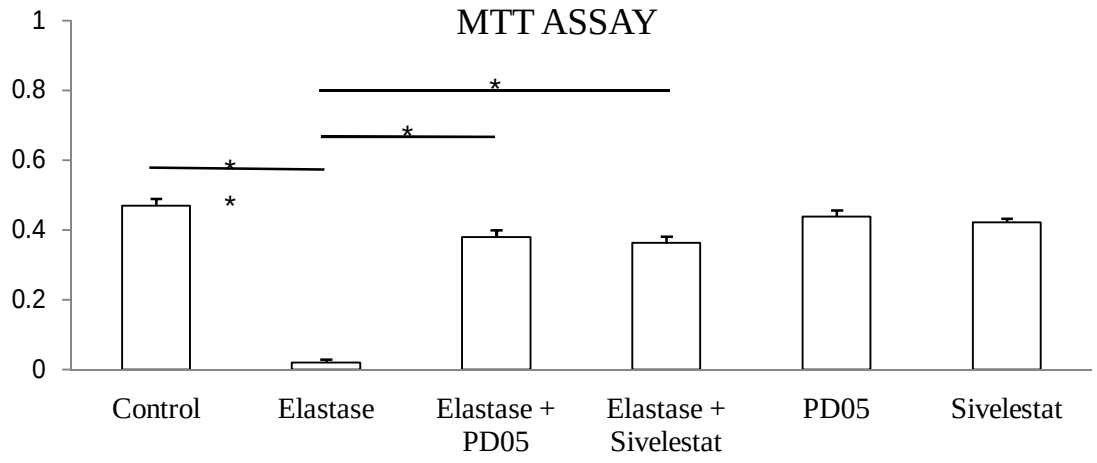


Figure 5E

Figure 5: Cytoprotective effect of PD05 and Sivelestat on elastase treated lung epithelial cells (A) PD05 protects against rounding of epithelial cells as seen under Brightfield microscope (B) PD05 protects against rounding of epithelial cells as seen under Fluorescence microscope (C) Cell detachment assay representing the effect of PD05 on lung epithelial cells (D) Determination of cell viability by MTT assay (E)

Elastase caused rounding of A549cells after 3 hours of incubation. Combination treatment with 10 μM PD05 or sivelestat prevented the effect of elastase as observed in the bright field under microscope (magnification 10x) (B) and with WGA(green) and DAPI (blue) stain (C) After 12 hours of incubation with elastase, a significant increase in cell exuviations was observed, which was

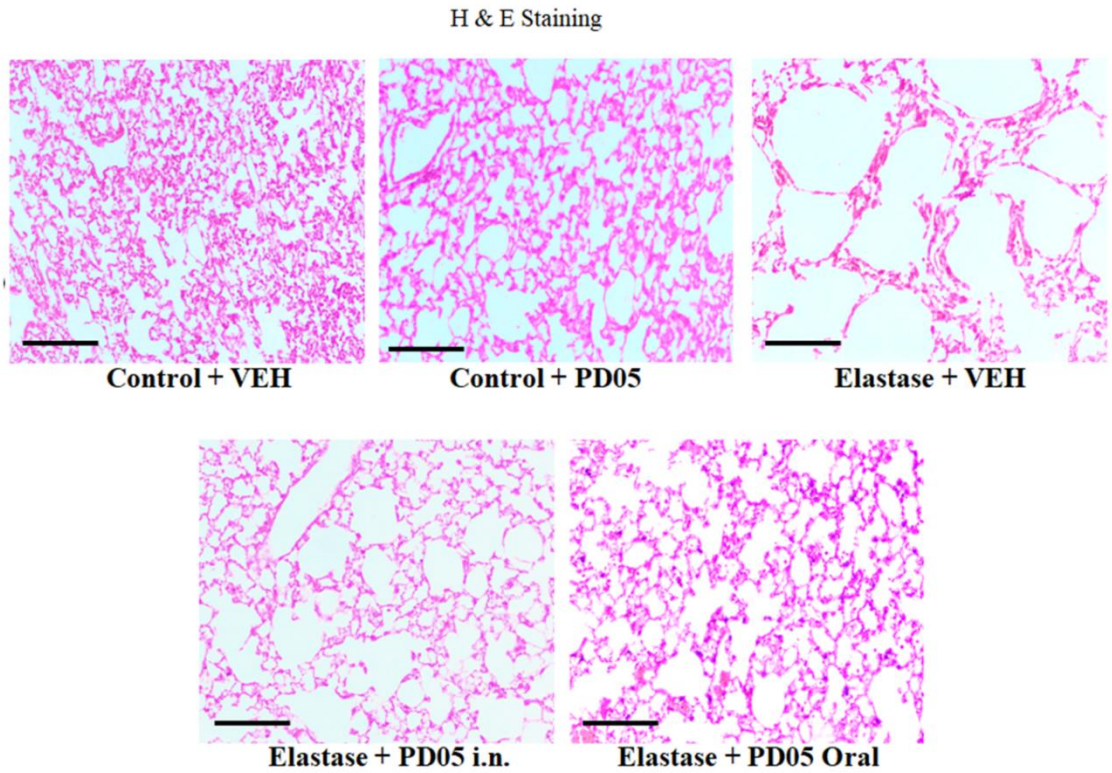
reversed by both PD05 and sivelestat..(D) Cell viability was decreased with elastase treatment which was reversed with cotreatment of PD05 and Sivelestat (E). All results are obtained from three different sets of experiments (n=3).[**P< 0.01and ***P< 0.05]

The morphological change of A549 cells from the epithelium to a somewhat oval/rounded, contracted appearance was the most predictive and expeditious event that took place after elastase treatment (Figures 5B and 5C). This effect of elastase is observed within 2-3 hours, suggesting that early onset is unrelated to cell death. Comparison was done with control elastase inhibitor sivelestat (SV).

A 7-fold increase in cell disjunction was caused by elastase from the cell culture dish matrix after 12 h, which was prevented by PD05 and sivelestat (Figure 5D).Cell viability was assessed by MTT assay and the viability after 12 hours of treatment made by elastase was reversed by co treatment of PD05 and sivelestat. Interestingly PD05 or sivelestat (SV) has no viable effect on cells alone (Fig 5E)

9.4.9 PD05 treatment reduced the emphysema like features in elastase model

A



B

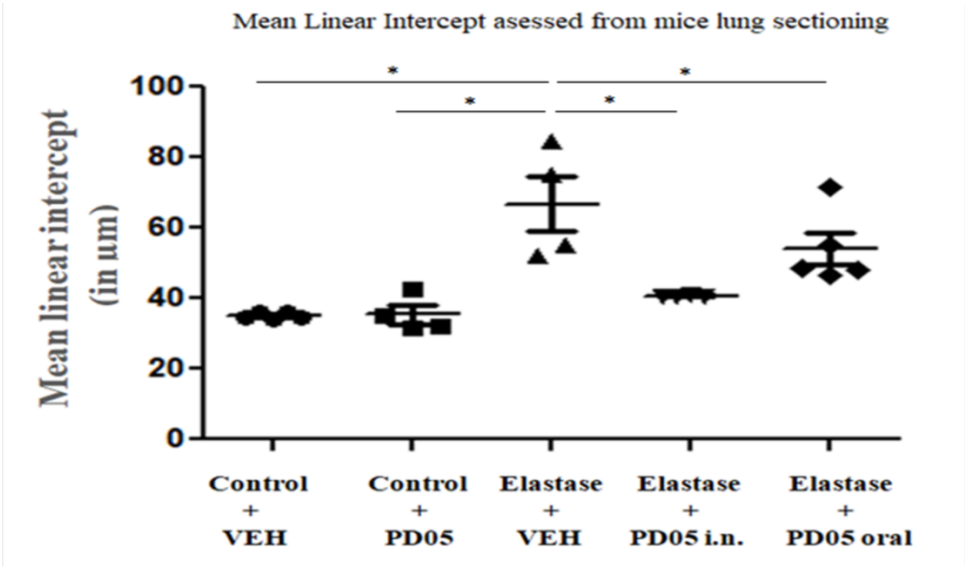


Figure 6: Mice lung section with H and E staining of different groups of mice (n=5 for each group) (6A) and mean linear intercept of mice lungs as assessed from sectioning (6B).

As we found that PD05 is able to inhibit neutrophil elastase in an effective manner and also reduce the elastase induced cell detachment, we wanted to determine its effects in elastase induced emphysema like model in mice. To determine the same, we have used well established elastase induced emphysema model as shown in Figure 1 as described earlier. As shown in Figure 6A, Elastase + VEH group had shown in alveolar collapse compared to the control mice. However, this collapse was reduced by treatment with either intranasal PD05 or oral PD05. This was confirmed by mean linear intercept assay. As shown in Figure 6B, Elastase + VEH mice had shown the significant increase of Mean Linear Intercept compared to control mice and also mice treated with only PD05. However, treatment with intranasal or oral PD05 reduced MLI in a significant manner ($P < 0.05$).

9.5 Discussion

Neutrophil elastase is remarkably elevated in BAL and sputum and fluid in patients with various respiratory disorders (Oriano et. al., 2020; Kummarapurugu et. al., 2022; Margaroli et. al., 2022; Keir, H. R. et. al., 2022) and therefore plays a role in tissue destruction which is primarily associated with various respiratory disorders (Pham C. T. 2008; Chua, F. and Laurent, G. J. 2006). The role of neutrophil elastase is not only tissue-destroying but also pathophysiological, causing airway hyperresponsiveness (Suzuki et. al., 1996), secretory cell metaplasia (Lucey et. al., 1990), mucus secretion (Schuster et. al., 1992) smooth muscle cell proliferation (Thompson, K.. and Rabinovitch, M. 1996), modulation of leukocyte adhesion (Cai, T. Q. and Wright, S. D. 1996), inducing interleukin-8 gene expression (Nakamura et. al., 1992) and impairment of host defences against bacteria (phagocytosis) (Tosi et. al., 1990).These findings suggest that neutrophil

elastase is a promising target for respiratory diseases and ameliorating elastase will eventually help in reducing pathological and functional disorders.

Recent publications on novel HNE inhibitors showed numerous sub groups, such as, b-lactams, benzoxazinone, succinimide-, azetidin-2-ones, electrophilic ketones saccharine and phthalimide-like compounds, coumarins as potential elastase inhibitors. Benzoxazinone play a crucial role in the inhibition of human neutrophil elastase (Hsieh et. al. 2010). More recent publications include a new series of stable sulphur fluoride exchange (SuFEx)able derivatives (Zheng et. al., 2019) and diazaborines were discovered as a new class of boron-based compounds for proteases inhibition [António et. al., 2018]. In this present study we reported the synthesis and characterization of seven novel benzoxazinone derivatives as neutrophil elastase inhibitor. The present design of the inhibitor and SAR development is inspired by previously described work on benzoxazinone analogues and also from the structure of Sivelestat. The structure of Sivelestat has two fragments, hippuric acid, and p-Hydroxybenzenesulfonamidopivalate ester moiety. We envisioned that the pivalate ester on p-hydroxybenzene, which is responsible for covalent binding with Ser195 on the receptor, can be replaced with an electrophilic chloroethoxy group as an isosteric replacement. The design of the inhibitors was also, facilitated by the ease of synthesis and derivatization. We also have ongoing plans to further modify the C2 and C6 positions.

Out of 7, PD05 was found to be the most effective and potent elastase inhibitor as compared to known elastase inhibitor, Sivelestat (Table 1). The results of the studies presented show that PD05 is a rapidly reversible, potent and specific inhibitor of human neutrophil elastase. Consistent with the role that elastase plays in COPD and other lung diseases; PD05 may suppress the reduction of inflammatory load and associated lung function parameters in a convenient and well-tolerated formulation. The potency of PD05

as an inhibitor of human neutrophil elastase activity *both in vitro and in vivo* is similar to or more potent than that of other synthesised synthetic inhibitors such as ICI-200880 (Williams et. al., 1991),ONO-5046 (Kawabata et. al., 1991), MDL101,146 (Durham et. al., 1994) and FK706 (Shinguh et. al.,1997).

Next we checked the way by which PD05 is binding to elastase by substrate dilution method (Figure 2A). Since the Vmax remaining the same for all the concentration used we can conclude that this is a competitive inhibition (Figure 2B).

The functional nature of binding interactions was investigated with the help of Surface Plasmon Resonance (SPR) which provides detailed kinetic information of small molecules. PD05 had a high binding affinity for human neutrophil elastase (Kd=1.63nM) and strongly inhibited NE activity. As compared to other notable elastase inhibitors like ONO 6818 and AZD9668, PD05 exhibited faster association and dissociation rate, and it has a fully reversible interaction with human neutrophil elastase. We have intentionally kept an electrophilic group at C2 position as envisioned that the designed compounds would act as covalent inhibitors. To understand the binding nature, we performed SPR study for Sivelestat and PD5. The SPR study showed, unlike Sivelestat, PD05 has a rapid rate of association and dissociation, and the interaction with NE was completely reversible. We feel that PD05 acts as a non-covalent mechanism based on the binding study.

A good pharmacokinetic profile makes a small molecule fit for a potential drug in future. ADME-Tox assay was performed at Cerep, France. Protein-binding affects drug activity in the following ways: either by changing the concentration of the drug at its main action site or by changing the elimination rate of the drug and therefore affecting the time duration which maintains effective concentrations. Plasma proteins having present in

higher concentrations have a control over the free drug concentration present in plasma and in compartments in equilibrium with plasma, resulting in the prevention of drug potency in vivo. PD05 has a high protein binding of 78% as compared to Acetabutol and Quinidine but lower than Warfarin. A high recovery rate of 98% indicates that PD05 will be excreted unaltered from the system smoothly.

For achieving required pharmacological response, solubility is primarily important variable to achieve desired concentration of drug to be available in systemic circulation .The aqueous solubility of PD05 in both simulated gastric and intestinal fluid was found to be 200. In PBS pH 7.4 the molecule was found to soluble at 194.7µM as compared to rifampicin of 192.3µM.As rifampicin is formulated in tablet, capsule and injection PD05 also has the potential to be developed into various pharmaceutical formulations.

To measure the rate of flux of a compound across polarised Caco-2 cell monolayers, permeability assay uses an established method from which in vivo absorption of drugs the can be predicted. In both A-B and B-permeability PD05 shows low permeability in comparison with ranitidine. In biopharmaceutical classification system PD05 will fall under class III with high solubility and low permeability like ranitidine.

We also performed some cell based assays to elucidate the effect of elastase in lung epithelial cell line. PD05 was found to be effective against cell apoptosis and desquamation (Figure 5A) under brightfield and fluorescence microscopy it was observed that elastase caused a rounding of the cell which was prevented with treatment of PD05 and sivelestat (Figure 5B and 5C). Cell detachment assay was performed to confirm that PD05 helps preventing the cell to detach as compared to sivelestat (Figure 5D).

MTT assay was performed and cell viability was checked. PD05 and sivelestat both stopped viability of the cells as caused by elastase alone. Interestingly PD05 itself has no toxic effects on cell (Figure 5E).

Studies conducted in elastase induced lung injury model in mice showed a significant improvement in lung degradation as observed in Figure 6A and 6B. PD05 also exerted similar effects via multiple route of administration and thus in future the compound can be formulated both as an oral medicine and as an inhalation agent.

9.6 Conclusion

PD05 is a benzoxazinone analogue synthesied in our laboratory which inhibits human neutrophil elastase in a competitive manner with a low IC_{50}. Morphological changes, in lung epithelial cells induced by elastase, were successfully altered with the administration of PD05. In a mouse model, the competitive and reversible inhibitor PD05 stops elastase-induced cellular morphological alterations and the development of emphysematous characteristics. This chemical has the potential to be further confirmed through clinical studies according to its positive pharmacokinetic characteristics.

11

SUMMARY

SUMMARY

Infections, cigarette use, second hand smoke, asbestos, radon, and other types of air pollution can all lead to respiratory illnesses. Chronic obstructive pulmonary disease (COPD), asthma, pneumonia pulmonary fibrosis, and lung cancer are examples of respiratory ailments, also known as pulmonary disease and lung disorder. A serious public health crisis is being created by the threat posed by chronic obstructive pulmonary disease (COPD). The worldwide population's ageing and the spectacular, if regrettable, success of the multinational tobacco companies in enforcing open global markets are the two main causes of this. The World Health Organization reports that COPD is currently the third most common cause of mortality in the world, but it is also an orphan illness that disproportionately affects the underprivileged and has gotten little attention from policymakers and academics. Despite the fact that cessation of smoking, pulmonary rehabilitation, and long-term oxygen therapy are all very cost-effective in the later stages of the disease, there are now just a few active treatments available.

Emphysema and chronic bronchitis are the two disorders that lead to COPD the most frequently. People with COPD may have these two conditions to varying degrees of severity, and they frequently coexist. Chronic bronchitis is characterised by inflammation of the bronchial airways' lining, which carry air into and out of the lungs' air sacs (alveoli). Its hallmark signs include frequent coughing and mucus (sputum) discharge. Emphysema is a condition that develops when the alveoli at the end of the bronchioles, the lungs' tiny air tubes, are destroyed as a result of exposure to hazardous cigarette smoke and other aggravating chemicals and particulate matter.

Common symptoms of COPD include increased dyspnea particularly during exercise or increased frequency at night, a chronic cough that produces phlegm , recurring chest

infections and continual wheezing. Although medication can help halt the progression, the symptoms typically worsen over time and make daily activities more challenging. Periods when patient's symptoms abruptly worsen are referred to as flare-ups or exacerbations. A few flare-ups per year are typical, especially in the winter. The majority of COPD sufferers do not exhibit any symptoms until they are in their late 40s or 50s. Weight loss, tiredness, swollen ankles from a build-up of fluid (oedema), chest pain and coughing up blood are less common symptoms.

Blood tests, chest x-rays, and spirometry are the main diagnostic tools for the illness. Other tests include EEG, blood oxygen measurement, ultrasound scan, ECG, CT scan, peak flow test and phlegm sample test. Although there is no known cure for chronic obstructive pulmonary disease (COPD), medication can help manage symptoms and limit the disease's progression. Current therapies include quitting smoking or supportive inhalers containing short acting bronchodilators (beta-2 agonist inhalers – such as salbutamol and terbutaline, antimuscarinic inhalers – such as ipratropium), long acting bronchodilators (beta-2 agonist inhalers – such as salmeterol, formoterol and indacaterol; antimuscarinic inhalers – such as tiotropium, glycopyronium and aclidinium). In the event that inhalalors do not produce any improvements, Theophylline, Mucolytic, antibiotics and steroid pills are utilised. Pulmonary rehabilitation including physical exercise, diet, councelling and improving core muscle strength are also useful. Roflumilast is sometimes used to treat flare-ups. Ventilation support, oxygen therapy, and in really serious situations, surgery, are all recommended.

New molecular targets and powerful inhibitors are urgently needed because existing medicines only offer transient comfort and cannot stop the spread of disease. The bronchial walls of the small airways exhibit the greatest degree of the chronic

inflammatory process in COPD, which involves both innate and adaptive immunity. There is noticeable variation in the inflammatory response in COPD. Acute exacerbations, which are frequently accompanied by increased inflammation, are a defining aspect of COPD. There are many different cell types that cause inflammation in COPD, but the macrophages, neutrophils, and lymphocytes are the most significant ones. The lung airways' acute exacerbations are when neutrophils are most noticeable since they are often more inflammatory than macrophages. The respiratory tract's neutrophil population and activation are both increased by smoking which produce proteases namely elastase and reactive oxygen species (ROS). According to recent findings, COPD patients' lungs have an influx of neutrophils that produce neutrophil elastase at the inflammatory site. Neutrophil elastase is a toxic and aggressive 29kDa protease that is mostly located in the azurophil granules of neutrophil granulocytes. Its action leads to the degradation of numerous extracellular matrix proteins, including fibronectin, laminin, proteoglycans, collagen, and elastin. Through its role in the inflammatory phase, mucus overproduction, and lung tissue degradation when the extracellular NE concentration exceeds the buffering capacity of endogenous inhibitors, it becomes engaged in the signs, symptoms, and prognosis of inflammatory lung diseases. The elevated elastase activity in COPD patients may promote the onset of emphysema by dissolving elastic fibres in the alveolar epithelium. It may also produce matrikines, which may help to reduce neutrophilic inflammation. This demonstrates that, especially in people with emphysema, it may be beneficial to either inhibit elastases or increase the activity of naturally occurring anti-elastases. The main endogenous elastase inhibitors are one-antitrypsin, secretory leukoprotease inhibitor, elafin, and tissue inhibitors of MMP (TIMPs). Gene therapy is unlikely to yield enough recombinant proteins because they need to be produced in enormous quantities. Recombinant proteins are therefore expected to be uneconomical.

Summary

The development of elastase inhibitors from medicinal herbs and the chemical synthesis of elastase antagonists are more feasible approaches that will be essential for managing the progression of COPD and other related respiratory conditions. The only neutrophil elastase inhibitor on the market is sivelestat. However, it is exclusively used in Japan and Korea to treat Acute Respiratory Distress Syndrome patients, and it is outlawed in America and Europe due to its high level of toxicity and lack of clinically significant results.

Flavonoids are naturally occurring elastase inhibitors. Fruits, barks, roots of plants rich in flavonoids are reported to inhibit elastase. Sonneretia apetala Buch-Ham is one such plant frequently rich in flavonoids and polyphenols, found in the Sunderban regions of India and Bangladesh, southern China, sections of Myanmar and Sri Lanka. The common name is Keora in Bengal. Fruits and barks of this plant have reportedly antioxidant, antibacterial, antifungal and astringent activity and used as tonic and to treat diarrhoea. Leaves of this plant can cure hepatitis, dysentery, sprains and bruises, open sores and eye problems. As there are claims that the fruits of this plant have been used historically to treat asthma and cough, we hypothesised that fruit extracts may be potential elastase inhibitor. For the hydroalcoholic extraction, fresh fruits were used, and HPLC was employed to characterise them. LC-MS-MS analysis was performed and ten compounds were identified namely 8,11-Octadecadienoic acid, methyl ester ; Stigmasta-5,22-dien-3-ol, acetate ; Sonneradon A ; Sonneradon C; Ranuncoside ; Gallic acid ; Ellagic acid ; Luteoline ; Ursolic acid and Stigmasterol. Among these identified compounds Gallic acid, Ellagic acid and ursolic acid are reported to be neutrophil elastase inhibitors and had been tested in various disease models including smoke induced lung emphysema model. According to *in situ* activity tests tests with this plant, the fruit extracts have significant anti-elastase properties as estimated from kinetics studies with an IC_{50} value of

371.8±3.73ng/ml and competitively block human neutrophil elastase. Human lung epithelial cells employed in in vitro investigations demonstrate that the extracts can affect the morphological alterations brought on by elastase treatment in cell culture and can greatly lower the percentage of cell detachment. Fruit extracts also have a negative impact on cell viability as measured by the MTT assay, although they have no harmful effects on a cell line. ICAM-1, VCAM-1, and other adhesion molecules are typically used to assist cells stick together. ICAM-1 is released as sICAM into the culture supernatant following elastase treatment. Fruit extracts from *Sonneretia apetala* successfully prevented the release of sICAM in elastase-treated cells, demonstrating that they directly bind to elastase to prevent its activity. Emphysematous characteristics, as determined by the mean linear intercept in mouse lung sections, were totally eliminated in the lungs of mice given elastase intratracheally, according to *in vivo* investigations with plant extracts. Extracts were orally administered at various dosage regimes of 1mg/kg, 10 mg/kg and 100mg/kg. At all doses extracts had similar effects as compared to control. Thus, for the first time, we reported how *Sonneretia apetala* Buch-Ham fruit extracts can be used to inhibit human neutrophil elastase.

Since benzoxazinone moieties have been shown to be inhibitors of human neutrophil elastase, we began developing them in our search for synthetic human neutrophil elastase. According to the structure-activity relationship, we created seven novel benzoxazinone derivatives, and their % yield was recorded. NMR was used to characterise all compounds, and HPLC was used to assess purity. Human neutrophil elastase was used for in situ screening with a two-hour kinetic test employing a recognised elastase substrate. PD05, the most potent inhibitor of the seven drugs, has an IC_{50} value of 132 nM when compared to the control medication sivelestat. Unlike other native enzymes like Porcine Pancreatic Elastase, Human Pancreatic Chymotrypsin and Bovine Pancreatic

Summary

Chymotrypsin PD05 was more specific towards neutrophil elastase. SPR data summarised the binding kinetics parameters of the newly synthesised compound with a KD of 1.63e-9±1.9M and Ki of 4.4±0.9 pM. Pharmacokinetic parameters were measured along with cardiac toxicity in a hERG setup. PD05 have 72% protein binding rate with a high recovery of 97%. The solubility is around 194µM with favourable permeability parameters. The compound PD05 showed no significant cardiac toxicity. Similar outcomes to those of plant extracts were seen in in vitro tests using a lung epithelial cell line. PD05 lessens cell rounding as observed under a fluorescence microscope stained with cell membrane stain WGA and reduces cell desquamation as seen with nuclei stained with DAPI. Additionally, PD05 assisted in lowering the proportion of elastase-induced detached cells, and the MTT experiment revealed no detectable cell damage. PD05 assisted in restoring lung function and morphology to those of control mice in *in vivo* model of elastase-induced lung emphysema model. Sivelestat served as the control in every trial. As a result, the novel benzoxazinone chemical PD05 was discovered as a potential inhibitor of human neutrophil elastase.

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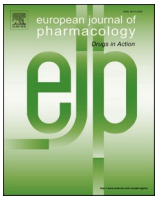

Novel benzoxazinone derivative as potent human neutrophil elastase inhibitor: Potential implications in lung injury

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ABSTRACT

Neutrophil elastase, a powerful physiological defence tool, may serve as drug target for diverse diseases due to its bystander effect on host cells like chronic obstructive pulmonary disease (COPD). Here, we synthesised seven novel benzoxazinone derivatives and identified that these synthetic compounds are human neutrophil elastase inhibitor that was demonstrated by enzyme substrate kinetic assay. One such compound, PD05, emerged as the most potent inhibitor with lower IC_{50} as compared to control drug sivelestat. While this inhibition is competitive based on substrate dilution assay, PD05 showed a high binding affinity for human neutrophil elastase (Kd = 1.63 nM) with faster association and dissociation rate compared to notable elastase inhibitors like ONO 6818 and AZD9668, and its interaction with human neutrophil elastase was fully reversible.Preclinical pharmacokinetic studies were performed *in vitro* where protein binding was found to be 72% with a high recovery rate, aqueous solubility of 194.7 μM, low permeability along with a favourable hERG. Experiments with cell line revealed that the molecule successfully prevented elastase induced rounding and retracted cell morphology and cell cytotoxicity. In mouse model PD05 is able to reduce the alveolar collapse induced by neutrophil elastase. In summary, we demonstrate the *in situ*, *in vitro* and *in vivo* anti-elastase potential of the newly synthesised benzoxazinone derivative PD05 and thus this could be promising candidate for further investigation as a drug for the treatment of COPD.

1. Introduction

COPD, or chronic obstructive pulmonary disease, causes a serious health concern globally with an exponential increase in incidence whose fatality rate affects roughly 7% of the population, according to estimates. Adults above the age of 65 years have a prevalence of up to 10%. [Halbert et al., 2006; Ford et al., 2013; Menezes et al., 2005]. As COPD is both under-diagnosed and under-recognized, the true prevalence appears to be higher than that reported. COPD-related deaths are expected to rise by more than 30% in the next ten years, making COPD the third biggest cause of death by the year 2030, according to the WHO [Lucas et al., 2013]. Prolonged exposure to gases or irritating particles is linked to a chronic inflammatory response in the lung parenchyma and surrounding peripheral airways and which leads to COPD. The majority of instances of COPD are caused by toxic chemicals found in cigarette smoke. The pulmonary component is marked by a lack of airflow which is not completely reversible and frequently progresses. The small conducting airways promote greater resistance and increased lung compliance consequently creates airflow limitation. [Hogg J. C. 2004]. COPD is mainly characterised by chronic sputum production and dyspnoea which is normally occurs at an early stage of COPD. Although numerous factors are involved in the development of COPD, airway narrowing is the leading factor, mainly associated with dyspnoea [Hogg et al., 2004; O'Donnell, D. E., and Webb, K. A. 1993; O'Donnell et al., 2001]. Airway narrowing is mainly caused by thickening of airway wall and fibrosis of lungs and collapse of the airways during exhalation as destruction of alveolar tissue results in the loss of radial traction [Hogg et al., 1968; McDonough et al., 2011]. Sputum production is increased due to increased goblet cells and mucous gland hyperplasia [Lai, H. and Rogers,

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Abbreviations

COPD	Chronic Obstructive Pulmonary Disease
HNE	Human Neutrophil Elastase
NE	Neutrophil Elastase
ARDS	Acute Respiratory Distress Syndrome
ALI	Acute Lung Injury
BAL	Bronchoalveolar Lavage
MLI	Mean Linear Intercept

D. F. 2010]. COPD suitcases have irreversible airflow limits, and their pathological diseases are usually gradually prognosis. To achieve the goals of preventing COPD, pharmacological therapy, as well as non-pharmacological interventions, heat reduction of smoking, reduction of other factors, vaccination, and diseases including appropriate evaluation of vaccination, oxygen therapy and lung rehabilitation. The latest observation in COPD patients, "Spill-over" of inflammatory mediators from the lungs to circulation causes systemic inflammation, resulting in ischemic heart disease, heart failure, osteoporosis, and normal anaemia. It may cause systemic inflammation that can cause lung cancer. It deteriorates diabetes and depression [Barnes, P. J. and Celli, B. R. 2009]. Therefore, control of the appropriate comorbidity is essential for COPD patients. [Agustí et al., 2003; Soriano et al., 2005]. Current treatments do not change the progressive load of disease, so it is necessary to develop an efficient treatment strategy [Barnes P. J. 2000]. Deadly diseases can be countered by finding new targets and developing target-specific small molecule inhibitors.

Many clinical observations have shown that neutrophil elastase targeting may be useful in the prevention of inflammatory lung disease. When the lungs react to harmful particles and gases, COPD airway inflammation occurs, resulting in an abnormal increase in airway inflammatory cells like neutrophils, T lymphocytes and macrophages. From blood stream neutrophils are mobilized directly causing destruction in parenchymal layer through the production of HNE [Donnelly, L. E. and Rogers, D. F. 2003]. Human Neutrophil Elastase (EC 3.4.21.37), belonging to the super family of chymotrypsin, is a serine protease is primarily stored in the azurophilic granules of multinuclear neutrophils. During the inflammatory phase of COPD, neutrophils release excess elastase into the extracellular medium. Human Neutrophil Elastase is 218 amino acids long, firmly held by four disulfide bridges, and has a molecular weight of 29–33 kDa [Sinha et al., 1987]. Its catalytic triad control their activities which consist of Ser195, His57, and Asp102 residues. In the primary sequence the triad is widely separated but in tertiary structure they are combined at the active site [Korkmaz et al., 2008; Bode et al., 1989]. Human Neutrophil Elastase is a multifunctional enzyme which helps in the regulation of inflammation and tissue homeostasis by attacking pathogens [Pham C. T. 2006]. By using its active serine HNE hydrolyze cleavable amide bonds and has the ability to degrade various structural proteins such as collagen, elastin, laminin proteoglycans and fibronectin [Pham C. T. 2008; Chua, F., and Laurent, G. J. 2006]. HNE plays a major role in the chemotaxis and helps in the migration of neutrophils through cleavage of adhesion molecules [Cepinskas et al., 1999; Hermant et al., 2003]. Endogenous inhibitors, including α1-antitrypsin (α1AT), α2-macroglobulin, elafin, and secretory leukocyte protease inhibitors (SLPI), reduce tissue damage under physiological conditions that regulate inflammatory processes and avoid harmful effects of extracellular HNE. [Tremblay et al., 2003; Ohmoto et al., 2001; Heutinck et al., 2010]. Extracellular HNE also plays a role in the development of lung diseases such as acute respiratory distress syndrome (ARDS) [Wang et al., 2009;], cystic fibrosis [Gifford, A. M., and Chalmers, J. D. 2014], acute lung injury (ALI) [Kawabata et al., 2002] and asthma [Guay et al., 2006]. HNE plays vital roles in some other diseases like psoriasis and other skin diseases [Meyer-Hoffert et al., 2004; Marto et al., 2018], rheumatoid arthritis [Cawston T. E. and Young, D. A. 2010], atherosclerosis [Henriksen, P. A. and Sallenave, J. M. 2008] and various types of cancer including breast and lung cancer [Moroy et al., 2012; Sato et al., 2006]. Recent reports have suggested that neutrophil elastase inhibitors can play a significant role in the treatment of COVID-19 [Mohamed et al., 2020]. Enzyme has a broad specificity and its proteolytic intensity causes tissue damage and other functional disorders. Hence human neutrophil elastase is considered as a promising therapeutic target [Henriksen, P. A. 2014].

The designing and synthesis of new HNE inhibitors is itself very challenging and mainly focussed to four inhibitor types: noncovalent inhibitors [Sjö, P. 2012], transition-state analogues [Edwards, P. D., and Bernstein, P. R. 1994], mechanism-based inhibitors [Zhong, J., and Groutas, W. C. 2004], and acylating-enzyme inhibitors [Lucas et al., 2013;]. Recently a number of thiazole-triazole acetamide hybrids were developed as potential human neutrophil elastase inhibitors with micromolar activity ranges [Butt et al., 2019]. Inhibitors containing pyrrolo-pyridine ring and indole derivatives as deza analogues was also developed [Crocetti et al., 2018; Crocetti et al., 2016].Numerous pharmaceutical companies have put their best efforts for a potent neutrophil elastase inhibitor. However, only Japan has approved Sivelestat (ONO 5046) for treating Acute Lung Injury which is generally associated with systemic inflammation [Iwata et al., 2010]. Sivelestat has been recently been proposed to treat Acute Lung Injury in COVID patients [Sahebnasagh et al., 2020]. A number of natural inhibitors have also been discovered showing inhibitory activities against neutrophil elastase [Marinaccio et al., 2022]. Cyclotheonellazole A (CTL-A), a naturally occurring macrocyclic peptide reported to be a potent elastase inhibitor [Cui et al., 2022]. ShSPI isolated from venomous gland of centipede *Scolopendra hananum* act as an atypical elastase inhibitor [Luan et al., 2019]. A 57 amino acid long cysteine rich polypeptide guamerin has the ability to inhibit human neutrophil elastase with a Ki value of 8.1×10^{-14} M [Jung et al., 1995]. Compounds isolated from marine cyanobacteria also showed potent anti-elastase activities [Keller et al., 2020].

Benzoxazinone derivatives have been found to be very effective against human neutrophil elastase from a very long time [Krantz et al., 1990]. Substituted benzoxazin-4-ones inhibit serine proteases and have been well characterized and act via mechanism involving an acyl enzyme intermediate [Hsieh et al., 2010]. In this present study we delineate the newly synthesised benzoxazinone compounds using structure activity relationship. Compound PD05 was found to be the most potent novel selective NE inhibitor and the preclinical pharmacology of PD05 was reported from studies used for the development of the drug both *in vitro* and *in vivo*.

2. Materials and methods

2.1. General procedure for the synthesis of PD05

To a mixture of anthranilic acid (0.1 g, 0.54 mmol, 1 equiv) in pyridine (3 ml), chloroformate (0.116 ml, 2 equiv, 1.08 mmol) was added and the resulting mixture was firmly stirred at 0 °C for 4 h in an atmosphere of nitrogen. Then 5 ml of ice water was added to the mixture. The resultant mixture was then extracted with EtOAc (5 ml) and the process was repeated twice. Organic layers, thus obtained, were then combined and dried over anhydrous sodium sulphate. It was then filtered followed by concentrating it under vacuum. The residue was washed with petroleum ether to give pure PD05.

2.2. Measurement of purity of compounds by HPLC

The purity of PD05and its analogues as assessed by HPLC and the analysis was carried out in an HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-20AD and LC-20AT prominence liquid chromatography pump, DGU-20A3 prominence degasser, CBM-20A prominence communications bus module, SPD-20A, prominence UV/VIS detector

and SPD-M20A PDA detector. Sample was dissolved in HPLC grade Methanol to achieve the concentration of 1 mg/ml. An aliquot of 20 ml was injected using SIL-20AC HT prominence autosampler. The separation was achieved on a Phenomenex reverse phase HPLC column (Luna® RP C_{18} column 4.6 × 260 mm, 5μ particle size, column temperature; 25 °C), and elution was carried out using mobile phase consisted of Water (0.1% Formic Acid) (A) and Methanol (B) and using an isocratic system, (0–30 min), B-80%, A- 20%. The elute was monitored at 254 nm and 380 nm. Data analysis was performed by LC solution version 1.25 (Shimadzu, Kyoto, Japan). Chromatogram and all other related data were presented in supplementary file as Fig. S1-Fig. S7.

2.3. Measurement of elastase activity and IC_{50} determination

The activity of inhibiting the human neutrophil elastase was measured using 200 μg/ml human neutrophil elastase (Calbiochem, USA) and 1.2 mM N-(OMe-succinyl)-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma-Aldrich), both of which are prepared using 0.1 M Tris-NaCl buffer (pH 7.5). Test compounds (1 μL, DMSO), 50 μL elastase (as prepared above) and 24μLTris-NaCl (pH 7.5) were pre-incubated in a 96-well microtiter plate at room temperature for 15 min. As incubation ended, 25 μL substrate solutions were added to each well. The reaction was observed by calculating the absorbance every 30 s for a total of 2 h, at 405 nm. Percentage inhibition was calculated after subtraction of blank from each curve as compared to standard (without drug) and IC_{50} was determined using GraphPad Prism v.8.4.

We incubated 4 μg/ml HNE with three different concentrations (100 μM, 500 μM and 1000μM) of the substrate *N*-(OMe-succinyl)-Ala-Ala-Pro-Val-*p*-nitroanilide and concentrations 0μM, 1μM, 10μM, 50μM and 100μM of the inhibitor PD05 (100 μL total volume) in 96 well flat bottomed plates to determine the inhibition mechanism,. The reaction was carried out using the Spectramax multimode plate reader in the same process as described above. After blank correction the reaction velocities were determined and using GraphPad Prism v.8.4. The substrate titration plots and subsequent double reciprocal plots were then generated [Milicaj et al., 2022; Budnjo et al., 2014].

2.4. Binding kinetics of PD05

To study the binding kinetics between PD05 and human neutrophil elastase assays were conducted using the BIAcore T100 instrument (GE Healthcare Biosciences AB). Human NE (100μg/ml in 10mM Tris-NaCl, pH 4.5) was pre-incubated with PD05 (1μM) for 10 min to ensure that active site is available and was immobilized using a CM5 sensor chip surface (GE Healthcare Biosciences AB) by the method of amine coupling. Using ammonia coupling as a control surface the enzyme was immobilized on an activated and deactivated CM5 chip surface. Running buffer (0.1M Tris-NaCl pH 7.5, with 1% dimethyl sulfoxide) was applied for attaining equilibrium and then PD05 was injected over the elastase enzyme at a flow rate of 50μL/min, and the reaction was observed and association rate was measured. Following the application of running buffer for 1 min, the rate of dissociation was determined over 5 min. For PD05 (A) and immobilized NE (B), the complex (AB) formation is given by:

$d[AB]/dt = k_{on}[A][B]\text{-}k_{off}[AB]$ Using the T100 Evaluation software, we assessed the interaction data and determined the on (k_{on}) and off (k_{off}) rates, as well as KD values (k_{off}/k_{on}) based on the global fit [Stevens et al., 2011].

2.5. ADME-Tox: solution properties

2.5.1. Aqueous solubility

All three aqueous solubility experiments in simulated gastric fluid, in PBS (pH 7.4) and in simulated intestinal fluid was carried out using shake flask technique with incubation of 24 h at room temperature and detected using HPLC-UV/VIS [Lipinski et al., 2001].

2.5.2. Protein binding

Equilibrium dialysis was used to measure protein binding with an incubation of 4 h at 37 °C using HPLC-MS/MS [Banker et al., 2003].

2.6. ADME-Tox: In vitro absorption

Caco-2 cells were used to measure A-B permeability (pH 6.5/7.4) and B-A permeability (pH 6.5/7.4), incubation times of 0 and 60 min at 37 °C, and detection with HPLC-MS/MS [Hidalgo et al., 1989].

2.7. Cardiac toxicity

Cardiac toxicity was assessed using an automated whole-cell patch clamp in hERG CHO–K1 cell line with an incubation of 5 min at room temperature cumulatively [Mathes, C. 2006].

2.8. General cell culture procedures

A549 cells were obtained from NCCS, Pune, and grown in F12K media (Gibco) containing 10% Fetal Bovine Serum (Life Technologies) at 37 °C under a humidified environment.

2.9. Cell viability assay

5000 cells/well of A549 were seeded in 96-well plate. The cells were treated with 100μM elastase or vehicle after 24 h of seeding. These were then co-treated with PD05 or sivelestat (Sigma-Aldrich) or DMSO. Prior to adding the MTT reagent at 0.5μg/ml concentration per well, the cells were incubated for further 12 h. After 4 h incubation with MTT, DMSO was added as solubilising agent and absorbance was taken at 550 nm.

2.10. Cell detachment and morphology change

In cell culture dishes (6-cm), A549 cells were seeded and were treated with vehicle + DMSO, elastase + DMSO and elastase + PD05 and elastase + Sivelestat. Using EVOS FL Microscope (10 × magnification), brightfield images were taken at 3 h, After 12 h, the medium was collected and the separated cells were pelleted by means of centrifugation. Attached cells were collected by using trypsin (Thermo) and then pelleted. The cell pellet was resuspended in fresh medium and 0.04% trypan blue was added. Cells were counted using a haemocytometer using a 10μl aliquot. The percent detachment was calculated based on the number of detached cells and the total number of cells present at the beginning of the experiment. Graphs were generated using prism software v. 8.4. We have used a varying micromolar concentrations of PD05 [Data not shown] to inhibit neutrophil elastase in cell line experiments. However at lower concentrations (2μM–5μM) PD05 couldn't fully inhibit HNE (100nM) causing cell detachment but at 10 μM we observed full inhibition of HNE which was in accordance with the already established and previously published methods [Salvador et al., 2013; Misumi et al., 2006].

2.11. Cell staining

In 6-cm dishes, A549 cells were plated. Vehicle + DMSO, elastase + DMSO, elastase + PD05, and elastase + Sivelestat were used to treat the cells. After 3 h and 6 h cell were treated with two drops of DAPI (Thermo Fischer) kept for 5 min and washed with PBS. Cells were observed under Fluorescence microscope as described above. Cells were also treated with WGA stain (Thermo Fischer) after 3 h according to instruction manual and observed under microscope.

2.12. Mice grouping

The male C57/BL6 mice, which were about 8–10 weeks old, were obtained from in-house breeding facility at CSIR-Indian Institute of

Chemical Biology (IICB), Kolkata and were acclimatized for one week at animal house facility, IICB. All the animal experiments were formally approved by Institutional animal ethics committee at IICB (Reference Number IICB/AEC/Meeting/July/2021/6). All the animal experiments were performed following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines).Mice were randomly divided into three groups: a) VEH/VEH (intratracheal vehicle, Tris buffer pH 7.4, administered and vehicle, 50% DMSO, diluted with saline, treated mice), b) NE/VEH (intratracheal neutrophil elastase administered and vehicle treated mice) and c) NE/PD-05 (intratracheal neutrophil elastase administered and PD-05 treated mice).

2.13. Induction of emphysema in mice with human neutrophil elastase

Human neutrophil elastase (Calbiochem, cat no. 324681) was reconstituted in tris buffer pH 7.4.For the induction of elastase induced emphysema, mice was first anesthetized by brief isoflurane. Anesthetized mice were placed on a wood support at an angle of 45° and carefully grasped the tongue with an upward and leftward position using a blunt end forceps. These mice were then instilled with 10 μg human neutrophil elastase (to both NE/VEH and NE/PD-05 groups) or vehicle (VEH/VEH group) on day 1 through orotracheal route using a pipette as shown in Fig. 1. Mice were maintained on the same position for 15 s and then placed on warm pad for recovery.PD-05 or vehicle was administered from day 1 to day 7, twice a day. We have used 1 mg/kg dose based on our pilot experiments that were performed in LPS induced acute lung injury model where we have tested three different doses of 0.1 mg/kg, 1 mg/kg and 10 mg/kg (unpublished data). It also to be noted that LPS induced acute lung injury shares multiple features such as airway neutrophilia, alveolar injury etc. with human neutrophil elastase induced lung injury [Grommes, J., and Soehnlein, O. 2001; Zeiher et al., 2004]. Therefore we had chosen 1 mg/kg oral dose in mice study. Mice were euthanized on day 7 and blood and lungs were collected.

2.14. Lung histology

The lungs were collected and fixed with formalin and embedded in paraffin. 5μm sections were cut and haematoxylin and Eosin (H & E) staining was performed.

Images of H & E stained lung sections were captured for each animal using the Magnus MLX-i microscope at an objective lens magnification of 10×. The images were taken equally spaced and over the whole surface of the lung sections they were systematically placed meander-like. The images were quantitatively analyzed by a quadratic test system with 64 points and 8 horizontal lines and 8 vertical lines with a total length of μm (d = μm).via the STEPanizer software [Tschanz et al., 2011]. The mean linear intercept of the airspaces (Lm) of each lung slide was calculated based on P_{ref} (all points hitting the parenchyma), P_{sep} (points hitting the alveolar septa) and I (intersections of the test line system with alveolar surface) using the following formula: $Lm = 2*d*(\sum P_{ref} - \sum P_{sep}/\sum I)$ [Salaets et al., 2020].

2.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software version 8.4. Unpaired, 2-tailed Student's t-test was used to calculate statistically significant differences. For comparison between more than two groups one-way ANOVA followed by Dunnett test was applied. *$P < 0.05$ was considered statistically significant, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

3. Results

3.1. General method of preparation of PD05 and its analogues

The general scheme for synthesis of benzoxazinone derivatives was given (Scheme 1) while the different structures of the compounds including nomenclature, molecular weight, Purity and yield of PD05 to PD05f were shown in Fig. 2. NMR data of all the seven molecules are added in Supplementary file from Figure S8 – Fig. S14.

3.2. PD05 acts a neutrophil elastase inhibitor with a low IC_{50}

Table 1: Inhibitory concentrations (IC_{50}) of seven synthesised benzoxazinone derivatives tested against human neutrophil elastase. Data were expressed in nM as Mean ± S.D.from three sets of experiments (n = 3).

Enzyme Inhibition: We tested the anti proteolytic activity of seven benzoxazinone compounds against human neutrophil elastase and found that all compounds potently inhibited HNE with an IC_{50} value ranging from 132 nM to 180.03 nM. The most potent compound was found to be PD05 with an IC_{50} value of 132 nM as compared to only available known elastase inhibitor, sivelestat having IC_{50} value of 138.76 nM (Table 1).

Hence we selected PD05 which seemed to be a promising neutrophil elastase inhibitor and conducted all the further experiments with this molecule as a part of pre clinical drug discovery process.

3.3. PD05 inhibits neutrophil elastase in a competitive manner

Kinetic studies were conducted to understand the style of inhibition of synthetic compounds against elastase inhibition. Based on the IC50, the most potent compound PD05 was selected to determine the mode of

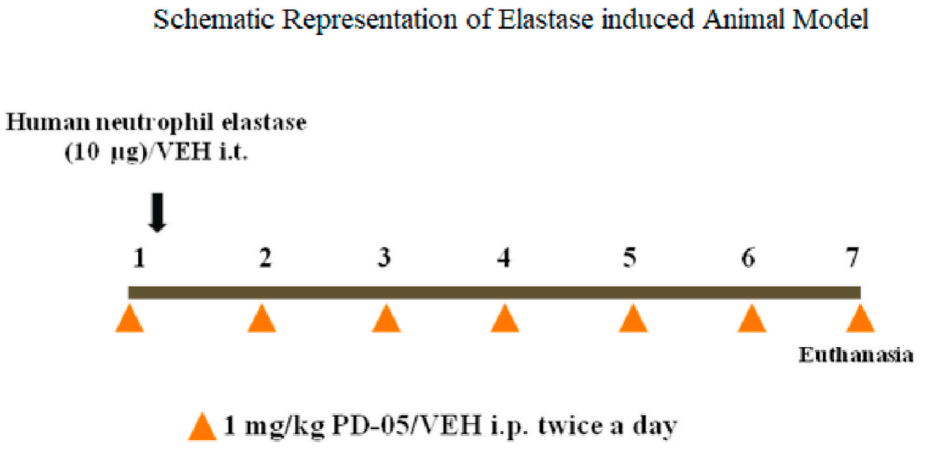
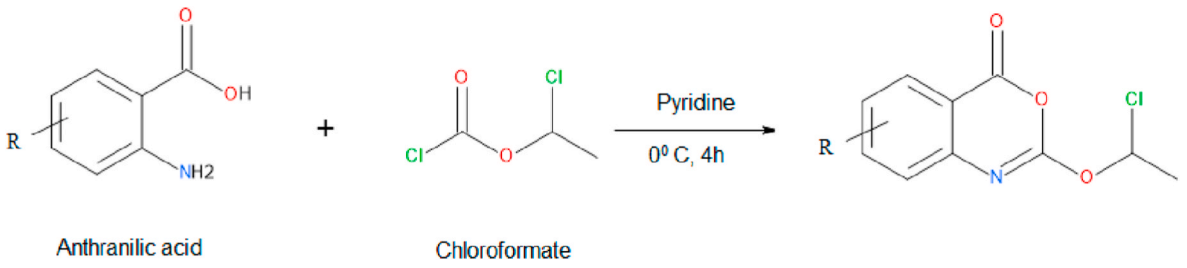
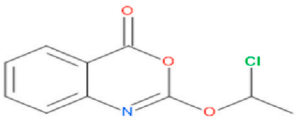


Fig. 1. Schematic representation of mice experimental protocol.



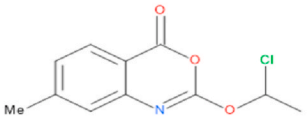
Scheme 1. General method of preparation of benzoxazinone derivatives.

List of benzoxazinone derivatives synthesised



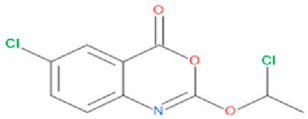
PD05

Chemical Formula: $C_{10}H_8ClNO_3$
Molecular Weight: 225.63
IUPAC Name: 2-(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 98.9% **Yield:** 97%



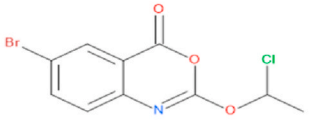
PD05a

Chemical Formula: $C_{11}H_{10}ClNO_4$
Molecular Weight: 255.65
IUPAC Name: 2-(1-chloroethoxy)-7-methyl-3H-benzoxazin-4-one
Purity: 97.19% **Yield:** 98%



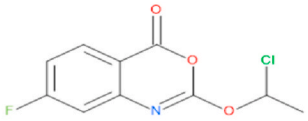
PD05b

Chemical Formula: $C_{10}H_7Cl_2NO_3$
Molecular Weight: 260.07
IUPAC Name: 6-chloro2--(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 100% **Yield:** 97%



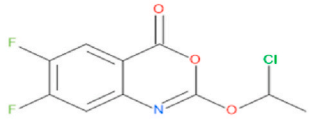
PD05c

Chemical Formula: $C_{10}H_7BrClNO_3$
Molecular Weight: 304.52
IUPAC Name: 6-bromo-2--(1-chloroethoxy)-3H-benzoxazin-4-one
Purity: 99.93% **Yield:** 97%



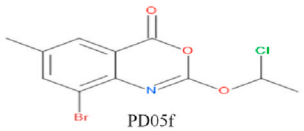
PD05d

Chemical Formula: $C_{10}H_7ClFNO_3$
Molecular Weight: 243.62
IUPAC Name: 2-(1-chloroethoxy)-7-fluoro-3H-benzoxazin-4-one
Purity: 96% **Yield:** 98%



PD05e

Chemical Formula: $C_{10}H_6ClF_2NO_3$
Molecular Weight: 261.61
IUPAC Name: 2-(1-chloroethoxy)-6,7-difluoro-3H-benzoxazin-4-one
Purity: 100% **Yield:** 99%



PD05f

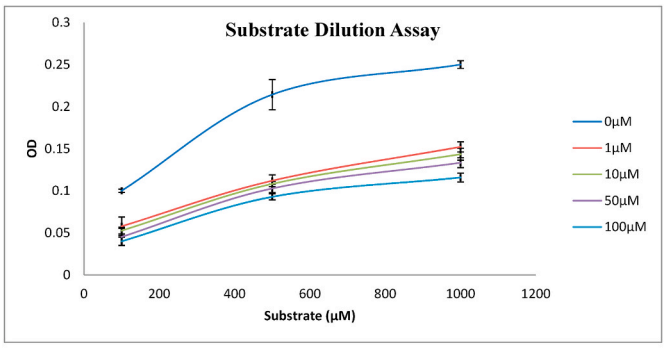
Chemical Formula: $C_{11}H_9BrClNO_3$
Molecular Weight: 318.55
IUPAC Name: 8-bromo-2-(1-chloroethoxy)-6-methyl-3H-benzoxazin-4-one
Purity: 100% **Yield:** 98%

Fig. 2. Seven different benzoxazinone derivative as elastase inhibitors.

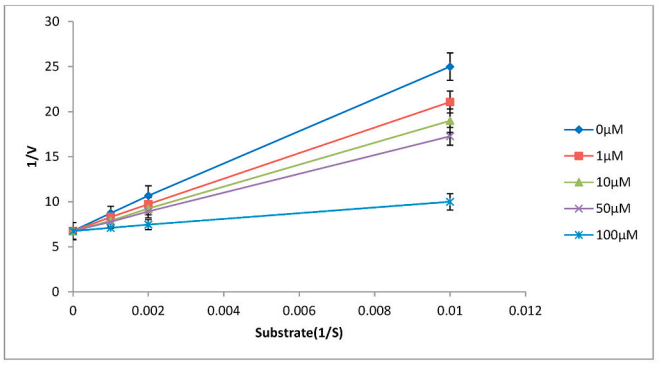
inhibition. Enzyme kinetic results from the 1/V Lineweaver Burk plot to substrate N-(O-Mesuccinyl) Ala-Ala-Pro-Val-p-nitroanilide 1/[S] in the presence of various concentrations of inhibitor revealed a series of straight lines. The Lineweaver Burk plot of compound PD05 showed that V_{max} remained the same without significantly affecting the gradient. K_m increases with increasing concentration, but V_{max} remains the same with slight differences. This behaviour indicates that the PD05 compound is a competitive inhibitor of the enzyme.

3.4. PD05 binds to neutrophil elastase with a low KD

Table 2: The kinetics data comprising of various parameters are reported as mean ± S.D. obtained from three different experiments (n = 3).



A



B

Fig. 3. A: Substrate titration of steady-state rate of HNE in the presence of inhibitor PD05 (0–100 μM). Fig. 3B: Data from panel A presented in double reciprocal format. Data is represented from three independent experiments (n = 3).

Binding kinetics: PD05 showed high binding affinity for HNE (KD = 1.63 nM) and strongly inhibited NE activity (Table 2; Fig. 4). K_{on} and K_{off} are 3.06e4 (1/Ms) and 5e-5(1/s) respectively. PD05 showed rapid rate of association and dissociation, and the interaction with NE was completely reversible.

3.5. Pharmacokinetic parameters of PD05

3.5.1. Protein binding of PD05

Using the peak area of the test compound in the buffer and test sample, the binding and recovery were calculated according to the following equation:

$$\text{Protein binding } (\%) = [(\text{Area}_p\text{-Area}_b)/\text{Area}_p] *100$$

$$\text{Recovery } (\%) = [(\text{Area}_p + \text{Area}_b)/\text{Area}_c] *100$$

Where.

Area_p = Peak area of analyte in protein matrix
Area_b = Peak area of analyte in buffer
Area_c = Peak area of analyte in control sample

Table 3: PD05 is compared with three different classes of drugs and Protein binding and recoveries have been given as percentage value (Mean ± S.D.) from three different sets of experiments (n = 3).

The protein binding percentage for PD05 was 68.5 and 74.6 resulting in a mean of 72. It was compared with Acetabutol, Quinidine and Warfarin whose results are 3, 59 and 98 respectively. The percentage recovery for PD05 was 97 which was similar to that of Acetabutol (99) and little higher than Quinidine (85) and Warfarin (86) (Table 3).

3.5.2. Aqueous solubility of PD05

The ratio of the peak area of the main peak of the calibration standard (200 μM) with the organic solvent (methanol/water, 60/40, v/v) to the peak area of the corresponding peak of the buffer sample determines the water solubility (μM). The peak area of the main peak relative to the total integrated peak area of the calibration standard HPLC

Table 2
PD05 binding kinetics and affinity for Human Neutrophil Elastase.

Compound	PD05
ka(1/Ms)	3.06e4±0.02
kd (1/s)	5e-5±1.03
Rmax(RU)	30.3 ± 2.9
RI(RU)	2.81e3±2.45
Concentration of Analyte (M)	1e-5
KA(1/M)	6.12e8±0.05
KD(M)	1.63e-9±1.9
Req (RU)	30.2 ± 3.7
Kobs (1/s)	0.306 ± .04
Ki (pM)	4.4 ± 0.9
Chi2	0.539 ± .002

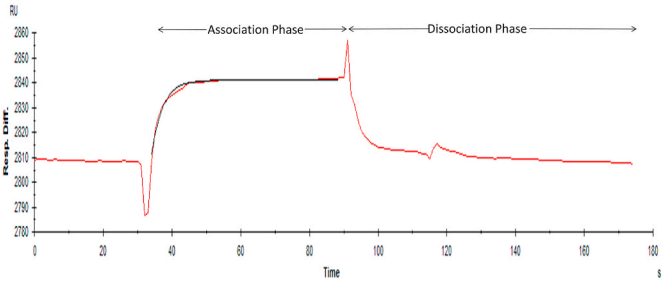


Fig. 4. Biacore assay of PD05 binding to human NE. The y axis represents the amount of interaction and the x-axis is time in seconds. Data represent one of three different experiments (n = 3). Kinetics data obtained from the graph are tabulated below (Table 2).

Table 3
Protein Binding (Plasma, human).

Compound	Test Concentration	% Protein Bound (Mean ± S.D.)	%Recovery (Mean ± S.D)
PD05	1.0E-05 M	72 ± 3.27	97 ± 1.21
Acetabutol	1.0E-05 M	3 ± 0.07	99 ± 0.07
Quinidine	1.0E-05 M	59 ± 2.98	85 ± 2.73
Warfarin	1.0E-05 M	98 ± 1.23	86 ± 4.39

Table 1
Antiproteolytic Activity of Benzoxazinone derivatives.

Compound	PD05	PD05a	PD05b	PD05c	PD05d	PD05e	PD05f	Sivelestat
IC_{50}(nM)	132.6 ± 3.21	186.4 ± 3.3	157.1 ± 3.06	166.6 ± 3.19	180.0 ± 3.15	173.8 ± 2.43	146.67 ± 3.48	138.76 ± 3.96

chromatogram defined the chromatographic purity (%).

Table 4: Solubility Data expressed in μM (Mean ± S.D.) of PD05 (Table 4A) has been tabulated along with seven other commonly used marketed drugs (Table 4B) obtained from three different sets of experiments (n = 3).

The aqueous solubility in simulated gastric fluid and simulated intestinal fluid is 200 μM while in PBS (pH 7.4) the solubility is 194.7 μM for PD05. The solubility was compared with seven different drugs whose solubility varies from 6.5 μM to 192.3 μM (Table 4B).

3.5.3. *In vitro absorption of PD05*

Test compound's apparent permeability coefficient (Papp) was calculated as follows:

$$Papp\ (cm/s) = (V_R{*}C_{R,\ end})/\Delta t\ *\ [1/\ A{*}(C_{D,\ mid}\text{-}C_{R,\ mid})]$$

Where.

V_R means volume of the receiver chamber. $C_{R,end}$ represents concentration of the test compound in the receiver chamber at the end time point, Δt is the incubation time and A signifies the surface area of the cell monolayer. $C_{D,mid}$ is the calculated mid-point concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 min and the donor concentration at the end time point. $C_{R,\ mid}$ gives the mid-point concentration of the test compound in the receiver side, which is one half of the receiver concentration at the end time point. The test compound's concentration was expressed as the peak area of the test compound.

Recovery of the Test Compound from the Permeability Assay.

The recovery of the test compound was measured as follows:

$$Recovery\ (\%) = [(V_D{*}C_{D,\ end} + V_R{*}C_{R,\ end})/\ V_D{*}C_{D0}]{*}100$$

Where.

V_D and V_R represent the volumes of the donor and receiver chambers, respectively. $C_{D,\ end}$ is the concentration of the test compound in the donor sample at the end time point. $C_{R,\ end}$ is the concentration of the test compound in the receiver sample at the end time point. C_{D0} is the concentration of the test compound in the donor sample at time zero. The test compound's concentration was expressed as the peak area of the test compound.

Fluorescein assessment for Permeability Assays: As the cell monolayer integrity marker Fluorescein was used. Fluorescein permeability assessment (in the A-B direction at pH 7.4 on both sides) was carried out after the permeability assay for the test compound. The cell monolayer that had a fluorescein permeability of less than 1.5 × 10-6 cm/s for Caco-2 and MDR1-MDCKII cells and 2.5 × 10-6 cm/s for MDCKII cells was considered intact, and the permeability result of the test compound from intact cell monolayer is reported.

Table 5 summarises the permeability values for PD05 as compared to 4 other known drugs like Colchinine, Labetalol, Propranolol and Ranitidine. Permeability and percentage recovery for both A-B and B-A are represented from three different sets of experiments (n = 3), expressed as Mean ± S.D.

The permeability values for PD05 were 0.65 and 0.73 with a mean value of 0.7 for A-B permeability. It was compared with Colchinine (0.1), Labetalol (8.8). Propranolol (25) and Ranitidine (0.3).The percentage recovery for PD05 was 78 which was comparable with Ranitidine of 77.

For B-A permeability the mean value for PD05 was 2.5 with a recovery rate of 79.

3.5.4. *Cardiac toxicity profile of PD05*

hERG (automated patch-clamp): By measuring the tail current amplitude, the degree of inhibition (%) was obtained, which is obtained by 2 s pulse to + 20 mV followed by a 1 s test pulse to - 40 mV, administered before and after drug incubation. The percent of inhibition was obtained by calculating the difference current normalized to control and then multiplied by 100. To generate estimates of the 50% inhibitory concentration (IC50) concentration (log) response curves were generated and fitted to a logistic equation. Using the percentage reductions from current amplitude, the concentration response relationship for

Table 5
Permeability.

Compound	Test Concentration	Permeability (10^{-6} cm/s) (Mean ± S.D.)	%Recovery (Mean ± S.D.)
A-B permeability (Caco-2, pH 6.5/7.4)			
PD05	1.0E-05 M	0.7 ± .002	78 ± 2.98
Colchicine	1.0E-05 M	0.1 ± .0007	77 ± 1.32
Labetalol	1.0E-05 M	8.8 ± 0.9	74 ± 3.09
Propranolol	1.0E-05 M	25 ± 2.18	69 ± 1.82
Ranitidine	1.0E-05 M	0.3 ± .0001	77 ± 1.74
B-A permeability (Caco-2, pH 6.5/7.4)			
PD05	1.0E-05 M	2.5 ± 0.07	79 ± 2.1
Colchicine	1.0E-05 M	5.5 ± 1.1	85 ± 1.9
Labetalol	1.0E-05 M	35 ± 2.47	84 ± 2.9
Propranolol	1.0E-05 M	42.4 ± 3.2	86 ± 3.1
Ranitidine	1.0E-05 M	1.7 ± .006	97 ± 4.87

Table 4
Aqueous solubility.

A

Compound	Test Concentration	Wavelength of Detection	Solubility(μM) (Mean ± S.D.)	Chromatographic Purity %
Aqueous solubility (simulated intestinal fluid)				
PD05	2.0E-04 M	230	200 ± 2.12	100
Aqueous solubility (PBS, pH 7.4)				
PD05	2.0E-04 M	230	194.7 ± 1.38	100
Aqueous solubility (simulated gastric fluid)				
PD05	2.0E-04 M	230	200 ± 1.73	100

B

Compound	Test Concentration	Wavelength of Detection	Solubility(μM) (Mean ± S.D.)	Chromatographic Purity %
Aqueous solubility (PBS, pH 7.4)				
Haloperidol	2.0E-04 M	205	97.8 ± 0.54	100
Ketoconazole	2.0E-04 M	205	125.5 ± 2.82	100
Diethylstilbesterol	2.0E-04 M	230	6.5 ± 0.03	100
Phenytoin	2.0E-04 M	230	89.7 ± 3.2	100
Rifampicin	2.0E-04 M	230	192.3 ± 2.49	100
Simvastatin	2.0E-04 M	230	21.5 ± 1.1	100
Tamoxifen	2.0E-04 M	230	1.5±-.02	100

each of the compounds was constructed by sequential concentrations.

Table 6 represents the % Inhibition of Tail current obtained from three different sets of experiments of three different concentrations (n = 3 for each concentration).

PD05 displayed very low inhibition of 15.8, 8.4 and 1.8 at 100 μM, 1 mM and 10 mM respectively (Table 6).

3.6. Cytoprotective effects of PD05 against elastase-induced cell disjunction and morphological change

After3 hours of neutrophil elastase treatment A549 cells started to conglomerate and detach from the cell culture dish as compared to control.(Fig. 5A). Co treatment with PD05 or sivelestat completely prevented the above event to happen. In contrast after 6 h of treatment the cells are completely detached from the plate barring a few and suggesting a chance of cell death or apoptosis. As compared to 3 h treatment with PD05 or sivelestat somewhat prevented the cells from desquamation but there is a hint of apoptosis suggesting that PD05 is reversible and free enzyme is responsible for the action.

The morphological change of A549 cells from the epithelium to a somewhat oval/rounded, contracted appearance was the most predictive and expeditious event that took place after elastase treatment (Fig. 5B and C). This effect of elastase is observed within 2–3 h, suggesting that early onset is unrelated to cell death. Comparison was done with control elastase inhibitor sivelestat (SV).

A 7-fold increase in cell disjunction was caused by elastase from the cell culture dish matrix after 12 h, which was prevented by PD05 and sivelestat (Fig. 5D).Cell viability was assessed by MTT assay and the viability after 12 h of treatment made by elastase was reversed by co treatment of PD05 and sivelestat. Interestingly PD05 or sivelestat (SV) has no viable effect on cells alone (Fig. 5E).

3.7. PD05 treatment reduced the emphysema like features in elastase model

As we found that PD05 is able to inhibit neutrophil elastase in an effective manner and also reduces the elastase induced cell detachment, we wanted to determine its effects in elastase induced emphysema like model in mice. To determine the same, we have used well established elastase induced emphysema model as shown in Fig. 1 as described earlier. As shown in Fig. 6A, Elastase + VEH group had shown alveolar collapse compared to the control mice. However, this collapse was reduced by treatment with either intranasal PD05 or oral PD05. This was confirmed by mean linear intercept assay. As shown in Fig. 6B, Elastase + VEH mice had shown the significant increase of Mean Linear Intercept compared to control mice and also mice treated with only PD05. However, treatment with intranasal or oral PD05 reduced MLI in a significant manner ($P < 0.05$).

4. Discussion

Neutrophil elastase is remarkably elevated in BAL and sputum and fluid in patients with various respiratory disorders [Oriano et al., 2020; Kummarapurugu et al., 2022; Margaroli et al., 2022;Keir, H. R. et al., 2022] and therefore plays a role in tissue destruction which is primarily associated with various respiratory disorders [Pham C. T. 2008; Chua, F. and Laurent, G. J. 2006]. The role of neutrophil elastase is not only tissue-destroying but also pathophysiological, causing airway hyperresponsiveness [Suzuki et al., 1996], secretory cell metaplasia [Lucey et al., 1990], mucus secretion[Schuster et al., 1992] smooth muscle cell proliferation[Thompson, K. and Rabinovitch, M. 1996], modulation of leukocyte adhesion [Cai, T. Q. and Wright, S. D. 1996], inducing interleukin-8 gene expression[Nakamura et al., 1992] and impairment of host defences against bacteria (phagocytosis) [Tosi et al., 1990].These findings suggest that neutrophil elastase is a promising target for respiratory diseases and ameliorating elastase will eventually help in reducing pathological and functional disorders.

Recent publications on novel HNE inhibitors showed numerous sub groups, such as, b-lactams, benzoxazinone, succinimide-, azetidin-2-ones, electrophilic ketones saccharine and phthalimide-like compounds, coumarins as potential elastase inhibitors. Benzoxazinone play a crucial role in the inhibition of human neutrophil elastase [Hsieh et al., 2010]. More recent publications include a new series of stable sulphur fluoride exchange (SuFEx)able derivatives [Zheng et al., 2019] and diazaborines were discovered as a new class of boron-based compounds for proteases inhibition [António et al., 2018]. In this present study we reported the synthesis and characterization of seven novel benzoxazinone derivatives as neutrophil elastase inhibitor. The present design of the inhibitor and SAR development is inspired by previously described work on benzoxazinone analogues and also from the structure of Sivelestat. The structure of Sivelestat has two fragments, hippuric acid, and p-Hydroxybenzenesulfonamidopivalate ester moiety. We envisioned that the pivalate ester on p-hydroxybenzene, which is responsible for covalent binding with Ser195 on the receptor, can be replaced with an electrophilic chloroethoxy group as an isosteric replacement. The design of the inhibitors was also, facilitated by the ease of synthesis and derivatization. We also have ongoing plans to further modify the C2 and C6 positions.

Out of 7 compounds, PD05 was found to be the most effective and potent elastase inhibitor as compared to known elastase inhibitor, Sivelestat (Table 1). The results of the studies presented show that PD05 is a rapidly reversible, potent and specific inhibitor of human neutrophil elastase. Consistent with the role that elastase plays in COPD and other lung diseases; PD05 may suppress the reduction of inflammatory load and associated lung function parameters in a convenient and well-tolerated formulation. The potency of PD05 as an inhibitor of human neutrophil elastase activity both *in vitro* and *in vivo* is similar to or more potent than that of other synthesised synthetic inhibitors such as ICI-200880 [Williams et al., 1991],ONO-5046 [Kawabata et al., 1991], MDL101,146 [Durham et al., 1994] and FK706 [Shinguh et al., 1997].

Next we checked the mechanism by which PD05 binds to elastase following substrate dilution method (Fig. 2A). Since the Vmax remaining the same for all the concentration used, it appears to be a competitive inhibition (Fig. 2B).

The functional nature of binding interactions was investigated with the help of Surface Plasmon Resonance (SPR) which provides detailed kinetic information of small molecules. PD05 had a high binding affinity for human neutrophil elastase (Kd = 1.63 nM) and strongly inhibited NE activity. As compared to other notable elastase inhibitors like ONO 6818 and AZD9668, PD05 exhibited faster association and dissociation rate, and it is a fully reversible interaction with human neutrophil elastase. We have intentionally kept an electrophilic group at C2 position as envisioned that the designed compounds would act as covalent inhibitors. To understand the binding nature, we performed SPR study for Sivelestat and PD5. The SPR study showed, unlike Sivelestat, PD05 a rapid rate of association and dissociation, and the interaction with NE was completely reversible. Thus, based on the binding study, it is likely that PD05 acts via a non-covalent mechanism for inhibiting neutrophil elastase.

A good pharmacokinetic profile makes a small molecule fit for a potential drug in future. ADME-Tox assay was performed at Cerep, France. Protein-binding affects drug activity in the following ways: either by changing the concentration of the drug at its main action site or by changing the elimination rate of the drug and therefore affecting the

Table 6
Cardiac toxicity.

Compound	Test concentration	% Inhibition of Tail current (Mean ± S.D.)
PD05	1.0E-07 M	1.8 ± 0.076
PD05	1.0E-06 M	8.4 ± 1.42
PD05	1.0E-05 M	15.8 ± 1.02

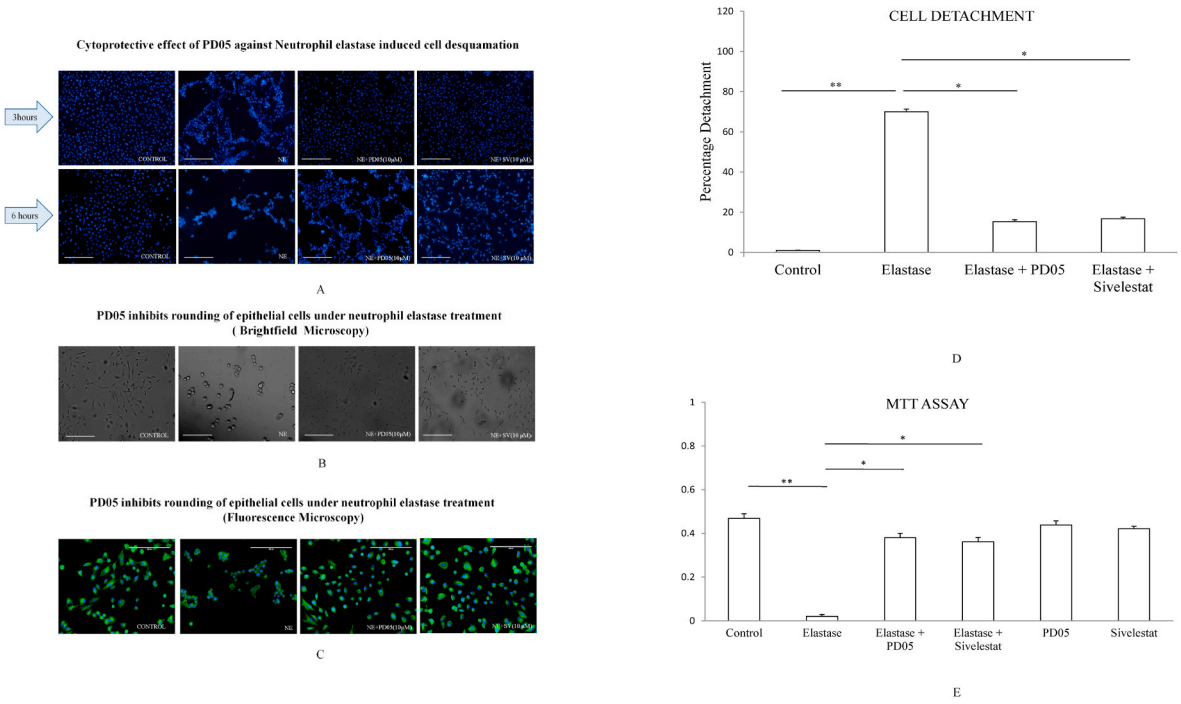


Fig. 5. Elastase behaves as sheddase promoting exuviations. (A) Elastase caused rounding of A549 cells after 3 h of incubation. Combination treatment with 10 μM PD05 or sivelestat prevented the effect of elastase as observed in the bright field under microscope (magnification 10×) (B) and with WGA(green) and DAPI (blue) stain (C) After 12 h of incubation with elastase, a significant increase in cell exuviations was observed, which was reversed by both PD05 and sivelestat.(D) Cell viability was decreased with elastase treatment which was reversed with cotreatment of PD05 and Sivelestat (E). All results are obtained from three different sets of experiments (n = 3).[**P < 0.01and ***P < 0.05].

time duration which maintains effective concentrations. Plasma proteins having present in higher concentrations have a control over the free drug concentration present in plasma and in compartments in equilibrium with plasma, resulting in the prevention of drug potency *in vivo*. PD05 has a high protein binding of 78% as compared to Acetabutol and Quinidine but lower than Warfarin. A high recovery rate of 98% indicates that PD05 will be excreted unaltered from the system smoothly.

For achieving required pharmacological response, solubility is primarily important variable to achieve desired concentration of drug to be available in systemic circulation.The aqueous solubility of PD05 in both simulated gastric and intestinal fluid was found to be 200. In PBS pH 7.4 the molecule was found to soluble at 194.7 μM as compared to rifampicin of 192.3 μM.As rifampicin is formulated in tablet, capsule and injection, PD05 also has the potential to be developed into various pharmaceutical formulations.

To measure the rate of flux of a compound across polarised Caco-2 cell monolayers, permeability assay uses an established method from which *in vivo* absorption of drugs the can be predicted. In both A-B and B-permeability PD05 shows low permeability in comparison with ranitidine. In biopharmaceutical classification system PD05 will fall under class III with high solubility and low permeability like ranitidine.

We also performed some cell based assays to elucidate the effect of elastase in lung epithelial cell line. PD05 was found to be effective against cell apoptosis and desquamation (Fig. 5A). Under brightfield and fluorescence microscopy it was observed that elastase caused a rounding of the cells which was prevented with treatment of PD05 and sivelestat (Fig. 5B and C). Cell detachment assay was performed to confirm that PD05 helps preventing the cell to detach as compared to sivelestat (Fig. 5D).

MTT assay was performed and cell viability was checked. PD05 and sivelestat both stopped viability of the cells as caused by elastase alone. Interestingly PD05 itself has no toxic effects on cell (Fig. 5E).

Studies conducted in elastase induced lung injury model in mice showed a significant improvement in lung degradation as observed in Fig. 6A and B. PD05 also exerted similar effects via multiple route of administration and thus in future the compound can be formulated both as an oral medicine and an inhalation agent.

5. Conclusions

Neutrophil elastase is a therapeutic target for the treatment of Chronic Obstructive Pulmonary Disease. In this study we have identified a novel benzoxazinone derivative, PD05, which was found to be a potential human neutrophil elastase inhibitor. PD05 is a competitive and reversible inhibitor which prevents elastase induced cellular morphological changes as well as emphysematous features in mice model. With promising pharmacokinetic features it can be concluded that this molecule has the potential to be further validated through clinical trials.

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Institutional review board statement

Animal experiments were carried out in accordance with the Institutional Ethics committee, Reference Number IICB/AEC/Meeting/July/2021/6. The animals were handled as per the guidelines of the animal ethics committee of CSIR-IICB and the Committee for the purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India.

CRediT authorship contribution statement

Sayantan Sengupta: Conceptualization, Methodology, Validation,

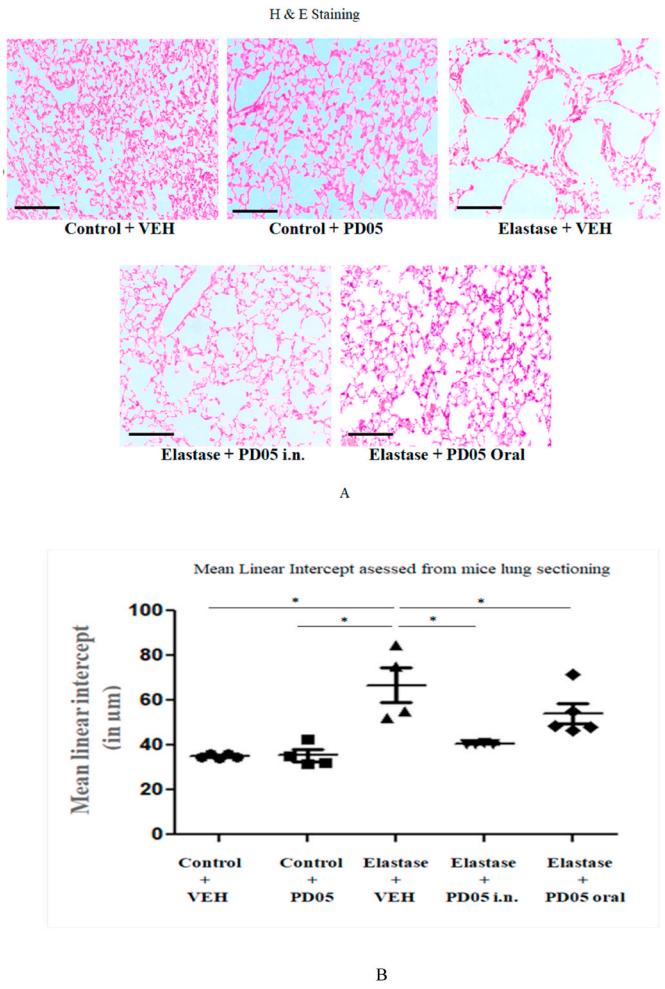

Fig. 6. Mice lung section with H and E staining of different groups of mice (n = 5 for each group) (6A) and mean linear intercept of mice lungs as assessed from sectioning (6B).

Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Jala Ranjith Reddy:** Methodology, Investigation, Formal analysis. **Nomula Rajesh:** Methodology, Investigation. **Ashish Jaiswal:** Methodology, Investigation. **Ulaganathan Mabalirajan:** Methodology, Writing – review & editing, Supervision. **Radha Krishna Palakodety:** Conceptualization, Methodology, Supervision. **Pulok Mukherjee:** Validation, Writing – review & editing, Visualization, Supervision. **Arun Bandyopadhyay:** Conceptualization, Validation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2022.175187.

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12.2

CERTIFICATES

Certificates of Oral / Poster Presentations

83rd Annual Meeting of Society of Biological Chemists (India)
CSIR-Central Food Technological Research Institute, Mysuru, INDIA
21st - 24th November 2016

POSTER PRESENTATION CERTIFICATE

This is to certify that Dr./Mr./Ms. Sayantan Sengupta, P Radha Krishna and Arun Bandyopadhyay

has participated and presented his/her research findings entitled "Novel neutrophil elastase inhibitor for management of chronic obstructive pulmonary disease"

poster presentation in the "83rd Annual Meeting of Society of Biological Chemists (India)" held during 21st to 24th November, 2016 at CSIR, Central Food Technological Research Institute, Mysuru, INDIA.

Dr. Alok K Srivastava
Chief Scientist, CSIR-CFTRI
Chairman, Poster Committee
SBCI Meet - 2016

Prof. Ram Rajasekharan
Director, CSIR-CFTRI
Chairman, Local Organizing Committee
SBCI Meet - 2016

5th International Conference on Molecular Signalling: Basics to Applications

Anna University KBC Research Centre, M.I.T Campus, Chromepet 600 044, Chennai

CERTIFICATE

This is to certify that ..... Sayantan Sengupta ..... was awarded the "Zydus Lipaglyn" second prize for oral presentation at the International Conference on Molecular Signalling: Basics to Applications held on 10th – 12th January 2017 organized by Anna University KBC Research Centre, Chennai, India.

Dr. P. Gautam
Director

Dr. Suvro Chatterjee
Convener

8th Convention
Society for Ethnopharmacology, India – 2021
23/3 Shaktigarh, Kolkata, India

National Seminar on
"Ethnopharmacology for wellness: Tradition to Translation"

Organized by
CSIR-Indian Institute of Chemical Biology
Kolkata, India

December 10, 2021

Certificate

This to certify that

Mr./Ms./Prof./Dr. Sayantan Sengupta participated & presented a paper in the Poster/ Oral session in the 8th Convention of SFE-India and National Seminar held at CSIR-IICB, Salt Lake Campus, Kolkata 700 091, India on December 10, 2021.

Mr. Birendra Kumar Sarkar
President
Society for Ethnopharmacology, India

Dr. Arun Bandyopadhyay
Chairman
8th Convention, SFE-India

Dr. Subhash C Mandal
Executive Secretary
Society for Ethnopharmacology, India

INDIA INTERNATIONAL SCIENCE FESTIVAL 2019

CERTIFICATE OF PARTICIPATION

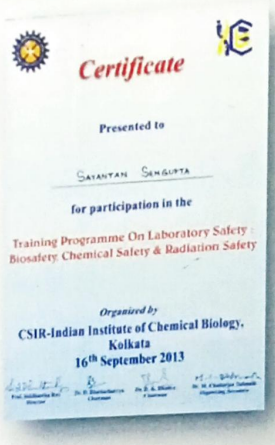
THIS IS TO CERTIFY THAT DR./PROF./MR./MRS./MS. SAYANTAN SENGUPTA

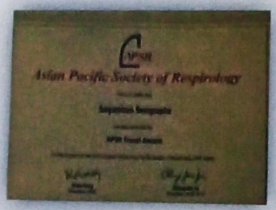
HAS ACTIVELY PARTICIPATED IN THE WELLNESS CONCLAVE AS PART OF 'INDIA INTERNATIONAL SCIENCE FESTIVAL - 2019' HELD AT BIDHA NAGAR CONVENTION CENTRE/SCIENCE CITY/BOSE INSTITUTE/CSIR-INDIAN INSTITUTE OF CHEMICAL BIOLOGY/SATYAJIT RAY FILM AND TELEVISION INSTITUTE, KOLKATA DURING NOVEMBER 5 TO 8, 2019. HE/SHE ALSO PRESENTED A PAPER ON THE THEME TITLED

PROF ASHUTOSH SHARMA
SECRETARY, DST

DR. VIJAY BHATKAR
PRESIDENT, VIGNANA BHARATI

Certificates of Participation in Conferences/Seminars/Workshops





Sayantan Sengupta

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Prof. Pulok K. Mukherjee

30/10/22