Asthma is an inflammatory disease of the airways that affects 5–10% of the general population. Epidemiological studies indicate that there is a global increase in the incidence, morbidity, and mortality caused by asthma despite an expanding repertoire of medications available for the treatment of this disease. The chronic inflammation is associated with airway obstruction and mucus production that leads to increased airway hyper-responsiveness resulting to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. Oxidative stress describes the damage that occurs when ROS overwhelm the antioxidant defences of the host. In asthma, this oxidative stress can have many detrimental effects on airway function, including airway smooth muscle contraction.

Natural products are diverse sources of important chemical constituents, most of them being heterocyclic. These molecules have diverse pharmacological actions. One of such heterocyclic compounds is vasicine. The alkaloid is derived from the highly reputed plant *Adhatoda vasica Nees*, family Acanthaceae, its leaves have been used in Indian system medicine for more than 2000 years. Vasicine was found to have bronchodilatory activity. It was reported to cause relaxation of tracheal muscle at low concentrations and contraction at high concentrations. It also exhibited protection against histamine induced bronchospasm.

![Figure: Structure of Vasicinone](image)

For the possibility that one of vasicine’s congeners could possess potent bronchodilator activity, various vasicine analogues were prepared and proved fruitful. Among the various congeners prepared, 7, 8, 9, 10-tetrahydroazepino [2, 1-b]-quinazoline-12 (6H)-one (TAZQ) showed potent bronchodilator activity as observed by a number of in vitro and in vivo experimental models viz. Relaxant effect of TAZQ on tracheal chain contracted by ACh or histamine or antigen and protection against bronchoconstriction induced by histamine aerosol and systemic anaphylaxis was studied and TAZQ was found to be potent as compared to theophylline. Thus the work was designed to study
the antiasthmatic potential of TAZQ in detail along with its anti-inflammatory and antioxidant potential in asthmatic subjects.

![Structure of TAZQ](image)

**Figure: Structure of TAZQ**

Literature survey reveals that the azepine ring with nitrogen at the bridgehead position is an important structural feature present in some of the recently explored alkaloids as antitussive studies *Stemona* alkaloids such as croomine, stemoninine, neotuberostemonine and tuberostemonine. In this context, 7, 8, 9, 10-tetrahydroazepino [2, 1-b]-quinazoline-12 (6H)-one was chemically modified to 2, 4-dibromo-7, 8, 9, 10-tetrahydroazepino [2, 1-b]-quinazoline-12 (6H)-one (DB-TAZQ) and both the compounds were tested for their antitussive activity.

**Combination drug therapy in asthma management**

For managing asthma attacks, symptomatic relief is foremost requirement. The two major conditions in asthma are bronchospasm that leads to breathlessness and inflammation in the lung tissues. Therefore the mainstream of antiasthmatic therapy aims to reduce bronchospasm and inflammation of the lung tissue. Many therapies have been studied and suggested including bronchodilators in combination with anti-inflammatory agents. Thus, bronchodilators and corticosteroids are used simultaneously for the effective treatment of acute and chronic asthma.

Basically, DB-TAZQ was synthesized with the idea of developing a moiety which could act as antitussive and antiasthmatic agent. Antitussive since it has nitrogen at bridgehead position with the quinazoline structure and antiasthmatic since the N-N-O triangle remains unaffected in the molecular model. Looking at the molecular model, the structure of DB-TAZQ resembles to the structure of ambroxol, which is an established mucolyte. Ambroxol was selected for the evaluation of its antiasthmatic study because of its structural resemblance to TAZQ, DB-TAZQ and vasicine. Vasicine and TAZQ are proved to possess bronchodilatory activity whereas no such study was
reported in the literature. TAZQ was again studied for antiasthmatic potential since no in-vivo data was reported compiling its anti-inflammatory potential in asthmatic animals.

DB-TAZQ was studied for its anti-inflammatory antiasthmatic activity but could not emerge as a potential agent since it could not produce in-vitro bronchodilation of the tracheal chain and poor anti-inflammatory action against inhaled antigen which was compared to the parent TAZQ moiety that showed great potential to possess both these properties. The combination of both again could not produce any synergism where as combination of TAZQ with AH displayed promising activity. With these results we tried to develop a formulation that could deliver TAZQ and AH to the lungs via inhalation. Numerous pre-formulation studies were performed to furnish the characteristics of the drugs and vigorous developmental experimentation was done to reach the optimum formulation that can be used to deliver the drugs into lungs through pulmonary administration.

METHODS

Animals

Male Hartley guinea pigs weighing 500 to 700 g and adult albino rats (Wistar origin) of either sex weighing 250 ± 20 g were purchased from IIIM, Jammu, India. The animals were maintained in ordinary animal cages in a constant 12-h light/dark cycle. Food and water were available ad libitum. All the experiments were approved by Institutional Animal Ethics Committee (IAEC).

SYNTHESIS OF TAZQ

TAZQ was synthesized by condensing Anthranilic acid and caprolactam in dry benzene, under anhydrous conditions. Further TAZQ was modified into DB-TAZQ. The synthesized compounds were characterized by melting point, IR and 1H-NMR spectrum.

EVALUATION OF ANTITUSSIVE ACTIVITY
All the compounds were evaluated for their antitussive effect using citric acid induced cough model in guinea pig. The animals were treated with the compounds on individual as well as combination basis in order to study the effect of various combinations.

**EVALUATION OF ANTIASTHMATIC ACTIVITY:**

Animals were actively sensitized by the intraperitoneal injection of ovalbumin Al(OH)$_3$ twice in a gap of seven days. These animals were randomly divided into further groups, (n=6). All the animals got daily treatment of drugs, dissolved in pyrogen free water, injected intraperitoneally for seven days. A group of unsensitized animals have also been included served as normal control.

**Evaluation of respiratory hyperreactivity**

The animals were challenged with ovalbumin aerosol (0.5% in normal saline) and airway abnormalities of the animals were visibly monitored. Evaluation of latency time (min) and the frequency of cough till 10 min after first cough stroke were measured.

**In-vivo antiasthmatic/ anti inflammatory activity**

Twenty four hours after final ovalbumin challenge, animals were sacrificed and the bronchoalveolar levag fluid was collected by a standardized technique. Also, the lung tissue was dissected out and homogenated in PBS. Homogenate and BAL samples were further used for biochemical estimations such as lipid peroxidation, Nitric oxide level by Griess reaction and determination of glutathione content.

**Assessment of airway inflammation:**

Total and differential leukocyte count in BAL fluid was performed manually with a hemocytometer.

**In-vitro bronchodilatory activity**

Spiral tracheal strips were obtained from sensitized guinea pigs (15 mm long and 3 mm wide) and concentration response curve of OVA ($10^{-9}$ to $10^{-4}$ M) was studied against various concentrations of drugs indivisually and in combination.

**Lung tissue histopathology:**
The lung tissue was fixed in 10% neutral buffered formalin. The lung tissue was embedded in paraffin, then cut into 4 mm thickness sections, stained with hematoxylin and eosin solution and observed under binocular microscope for histological changes.

**DEVELOPMENT AND CHARACTERIZATION OF TAZQ AND AMBROXOL LOADED LDPI SYSTEMS FOR PULMONARY DELIVERY**

**Preformulation studies:**

The synthesized compound was characterized by various sophisticated techniques such as IR and NMR. The melting point determination was carried out by DSC analysis. Various other parameters such as Partition-Coefficient and pKa was Determined.

**Analytical/ bioanalytical method development and validation**

A rapid, simple and sensitive HPLC method with UV detection was developed and validated for the determination of TAZQ and Ambroxol in plasma using Pantoprazole as an internal standard. Following protein precipitation with acetonitrile, the compounds were separated by the mobile phase Acetonitrile (ACN):Phosphate Buffer (PBS, pH 5.5) in the ratio of 40:60 (%v/v) at a flow rate of 0.6ml/min on a reverse-phase C-18 column (150mm×4.6mm i.d., 5μm particle size) and detection wavelength 267nm. The method was validated according to the ICH (Q-2) guidelines prescribed for analytical method validation.

**BCS Classification of the drug:**

Solubility studies:

Both the drugs were studied for their solubility behavior in aqueous and organic solvents. Ambroxol was procured as hydrochloride salt. The aqueous solubility at different pH was also studied and solubility class was determined according to USP guidelines.

Permeability studies:

Both the drugs were studied for permeability in order to study the extent of absorption property through biological membranes. Rat ilium was used to study the permeability behavior using Franz diffusion cell.
**Formulation development**

**Preparation of empty and drug loaded liposomal dispersion**

Empty and drug loaded liposomal dispersions were prepared by solvent stripping technique. Phosphatidyl choline, cholesterol and TAZQ film was prepared by evaporating chloroform. The thin film was then hydrated with PBS containing ambroxol HCl for 1 Hr. The molar ratios of phosphotidyl choline to cholesterol and drugs to lipid concentration was established in order to produce best fit liposomal structure desired for pulmonary administration. Various parameters like percent drug entrapment, drug loading capacity, particle size and polydispersity index were studied with liposomal formulations prepared using different ratios. Various process parameters like pH, volume of hydrating medium, time, temperature of hydration and instrumental parameters like RPM and vacuum were studied to reach the optimum formulation.

**Lyophilization of Liposomes**

Dry lipid film was hydrated using aqueous phase containing trehalose in mass ratio of lipid: sugar 1:12 for 1 Hr. The dispersions were frozen to −68°C and freeze drying was performed for 24 h. The lyophilized liposomal powder was mixed with lactose carrier in mass ratio of 1:5 and filled in capsules.

**Characterization of LDPI Formulation**

The formulated LDPI was studied for Angle of repose Moisture content, entrapment efficiency/Percentage drug encapsulation (PDE) before and after lyophilization Particle size, polydispersity index (PDI), zeta potential and Photomicrography. In vitro drug release from the liposomal dispersions before and after lyophilization was determined using dialysis bags 12 KD in type-II Tablet Dissolution Tester.

**In-vivo bioavailability studies:**

The developed lyophilized liposomal powders were studied for lung bioavailability and plasma pharmacokinetics.

Animals and Treatment:
Adult albino rats of either sex weighing 250 ± 20 g (n=18) were anaesthetized and the formulations were administered to the lungs with the help of cannula that was inserted up to the tracheal bifurcation.

Bio-availability studies:

The lungs of the animals were removed after 1, 2, 4, 8, 12 and 24Hrs after the drug administration. The blood samples (0.3-0.5mL) were collected from retro orbital plexus at in test tubes containing heparin at 1, 2, 4, 8, 12 and 24Hrs.

Plasma pharmacokinetic Analysis: The drugs plasma concentration after intra-tracheal administration was determined at different time points. The various pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-t} were determined.

RESULTS:

The synthesized compounds were characterised using sophisticated spectroscopic techniques like UV,IR,MASS and NMR and elucidated as shown in figure.

![Figure: Structures of TAZQ & DB-TAZQ](image)

EVALUATION OF ANTITUSSIVE ACTIVITY:

It has been demonstrated that the guinea pigs and humans respond to similar concentrations of citric acid and responses are well correlated with concentration-response relationship. Intraperitoneal Codeine as standard showed significant increase in cough latency and decrease in cough frequencies. TAZQ showed marked antitussive effect at 10, 20 mg/kg. DB-TAZQ 10mg/kg also showed marked antitussive effect in comparison to TAZQ 10mg/kg. The di-bromo substitution proved to be the most effective antitussive. AH failed to show any antitussive effect. The combination of TAZQ with DB-TAZQ failed to show any synergistic activity but the additive effect of the combination could be observed. The combination of AH with TAZQ did not
enhance any activity of TAZQ. Our study has thus given new dimensions to the molecules with azepinoquinazolone skeleton as antitussive agents.

EVALUATION OF ANTIASTHMATIC ACTIVITY:

Effect against antigen induced airway hyper responsiveness:

Animals treated with DB-TAZQ could resist the airway hyperreactivity with reduced cough frequency at 10 and 20 mg/kg doses respectively. TAZQ found to possess good antiasthmatic activity when compared to aminophylline at 50 mg/Kg dose. The combination treated animals (TAZQ+AH) showed fairly large increase in cough latency and half of the population didn’t even show any abnormalities with any signs of dyspnoea. On the other hand, combination with DB-TAZQ could not enhance the potency of TAZQ.

Assessment of airway inflammation

The present findings showed that regular administration of TAZQ-AH combination potently reduced eosinophillic accumulation into lungs and BAL fluid than the animals treated with both the drugs individually. TAZQ even at the dose of 10mg/kg significantly prevents eosinophillic infiltration proving to be potent anti-inflammatory agent. DB-TAZQ could not produce any reduction in eosinophillic infiltration, nor did its combination with TAZQ enhance the potency.

In present study we have found that, intraperitoneal AH protects lung lipids from oxidative stress. TAZQ at the dose of 20 mg/kg showed significant reduction in the concentration of TBARS in both BAL fluid and lung tissue homogenates whereas this property of TAZQ was not reported earlier. The low dose combination of TAZQ & AH showed marked synergistic reduction in TBARS level in sensitized guinea pigs which was not seen in TAZQ with DB-TAZQ combination. This effect may have contributed to less lung injury and better recovery in the combination treated group proving to be a relevant treatment for asthmatic patients which could be seen in lung tissue histopathology. Supporting the number of investigations, our data depicts that ambroxol at therapeutic concentration has beneficial therapeutic effects on concentration of GSH, a marker of oxidative stress in lung and BAL fluid. Ambroxol also reduces the release of reactive oxygen species by polymorphonuclear cells, indicating potential of free radicals
scavenging activity and prooxidative metabolism in inflammatory cells. On the other hand TAZQ is not reported to possess any free radical scavenging activity. The present study shows that TAZQ at the low dose combination with ambroxol synergistically improved the level of GSH in both BAL fluid and lung tissue homogenates. DB-TAZQ was studied for its anti-inflammatory antiasthmatic activity but could not emerge as a potential agent since it could not produce in-vitro bronchodilation of the tracheal chain and poor anti-inflammatory action against inhaled antigen which was compared to the parent TAZQ moiety that showed great potential to possess both these properties. The combination of both again could not produce any synergism where as combination of TAZQ with AH displayed promising activity.

The formulation development was a thorough investigation. Prepared liposomal formulations by using different PC to CHOL ratio were subjected to drug entrapment efficiency and particle size analysis. The best resulted liposomal dispersions were liophilised using different cryoprotectants to prepare the liposomal dry powder for inhalation that contains both TAZQ and AH. The optimised formulation variables can be seen in following table with which powder formulation desired for pulmonary administration was formulated.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>SPECIFICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC (mg)</td>
<td>52.5 (7M)</td>
</tr>
<tr>
<td>CHOLESTEROL (mg)</td>
<td>11. 4 (3M)</td>
</tr>
<tr>
<td>TAZQ (mg)</td>
<td>25 (0.12mM)</td>
</tr>
<tr>
<td>CHLOROFORM (ml)</td>
<td>10</td>
</tr>
<tr>
<td>AH (mg)</td>
<td>150 (0.36mM)</td>
</tr>
<tr>
<td>PHOSPHATE BUFFER pH 6.5 (mL)</td>
<td>10</td>
</tr>
<tr>
<td>HYDRATION TIME</td>
<td>1 Hr</td>
</tr>
<tr>
<td>TEMP °C</td>
<td>40</td>
</tr>
<tr>
<td>RPM (Film Preparation / Hydration)</td>
<td>100/25</td>
</tr>
<tr>
<td>VACCUME (in Hg)</td>
<td>40</td>
</tr>
<tr>
<td>CRYOPROTECTANT</td>
<td>Trehalose</td>
</tr>
<tr>
<td>LIPID TO CRYOPROTECTANT MASS RATIO</td>
<td>01:12</td>
</tr>
</tbody>
</table>

Table: Final formulation variables.

The lyophilized formulations were assayed using a validated HPLC method using Pantoprazole as an internal standard. Other than assay, various other parameters were studied like moisture content and angle of ripose. The lyophilized dry powder formulations prepared
using above variables were found to be best suited for pulmonary route, that was concluded with the results of Cascade impacter studies.

The bioavailability studies showed that liposomal formulations were able to retain the drug concentrations above therapeutic range in lung tissue even after 08 hrs, whereas minimum drug concentration was absorbed into the systemic circulation thereby minimizing the side effects of the drugs.