CHAPTER 5

DISCUSSION
Vasicine and vasicinone obtained from leaves of *Adhatoda vasica Nees* family Acanthaceae, have been reported for moderate degree bronchodilator activity. For the possibility that one of its congeners could possess potent bronchodilator activity, Zabeer et al 2006 took up a study 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.

Hermecz et al. [19] have reported the bronchodilator activity of bis- and tricyclic nitrogen bridgehead derivatives with a pyrimidine- 4(3H)-one ring. Moreover, 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 (Chung et al., 2003, Xu et al., 2006). 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.

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.
TAZQ was synthesized by condensing anthranilic acid with β-Caprolactam in dry benzene and the yield was found to be 51% as pale yellow crystals. TAZQ was further modified into DB-TAZQ and the yield was found to be 50.58%.

Antitussive potential of the synthesized compounds was evaluated in guinea pigs against citric acid induced cough (Laude et al., 1994). Both the compounds were found to possess antitussive activity. Decreased cough frequency along with increased cough latency time (Figure-19 & 20) was observed suggesting that the azepinoquinazolone skeleton represents an active moiety as far as antitussive activity is concerned, with the di-bromo derivative showing better activity than even codeine. Our study has thus given new dimensions to the molecules with azepinoquinazolone skeleton as antitussive agents.

Asthma is chronic lung disease typically associated with airway obstruction, chronic inflammation and mucus production which has long been considered an important cause of morbidity and mortality in asthma (Cluroe et al. 1989; Bhaskar et al 1988; Jeffery et al 1992). TAZQ is reported to inhibit antigen-induced mast cell degranulation and release of histamine from target tissue (Johri et al 2000) and was found to be a potent bronchodilator when studied in-vitro. Numerous studies have been carried out to study the effect of combination of bronchodilator with anti-inflammatory agent that reports the efficiency of the combinations. In this context present work was designed to study the effect of a combination of a bronchodilator with mucolytic agent. Ambroxol was selected to incorporate along with TAZQ because there are number of studies which demonstrated the anti-inflammatory, anti-oxidant effect of ambroxol. The protective effect of the drugs alone and in combination was studied on respiratory hyper reactivity, airway eosinophillic accumulation and bronchial inflammation induced by an exposure to OVA aerosol in asthmatic guinea pigs as it (Canning et al 2008) has been the most commonly used small animal species in preclinical studies related to asthma. The intraperitoneal injection of 20 µg OVA and 100 mg Al(OH)_3 twice in a gap of seven days (Andersson et al 1981) caused allergic reactions in male Hartley guinea pigs and the success rate of sensitization method was 90.66% (68 of 75 sensitized guinea pigs showed allergic reactions to inhaled antigen). Exposure to inhaled antigen developed pulmonary asthma-like response in antigen induced sensitized guinea pigs which is in connection with increased eosinophillic accumulation in the bronchial tissue and BAL fluid associated with chronic airway inflammation. TAZQ was evaluated and turned to be a
potent bronchodilator accompanied with notable antioxidant and anti-inflammatory activity at both 10 and 20 mg/kg doses. In present study we found that asthma-like response in antigen sensitized guinea pigs was inhibited synergistically by seven days treatment of TAZQ in combination with ambroxol (figure- 21 &22). The synergism may be because of TAZQ induced inhibition of release of histamine and other bronchoconstrictors from target tissue and enhanced smooth muscle relaxation due to bronchial airway clearance by ambroxol whereas there is no study that report ambroxol to produce bronchodilation or any effect on mast cell granulation and hence, the release of bronchoconstrictor and proinflammatory mediators by these cells. The airway hyperresponsiveness to the inhaled antigen was almost abolished with the pre-treatment of both the drugs in combination. Ambroxol on the other hand, at the dose of 50mg/kg, was not at all comparable with TAZQ when cough latency time and frequency of cough strokes were measured by behavioural symptoms. The combination of TAZQ with DB-TAZQ was also studied but there was no additive or synergistic effect found with this combination. DB-TAZQ alone found to possess effect on antigen induced hyper responsiveness but was not comparable to TAZQ on dose basis. DB-TAZQ may prevent hyper responsiveness due to its potent antitussive activity that reduced the cough frequency and improved the latency time.

Eosinophilic infiltration is known to be a hallmark of bronchial asthma and several studies had reported a significant eosinophilia in BAL fluid fluid of asthmatic patients (Bousquet et al 1990; Kirby et al., 1987; Lam et al 1985; Adelroth et al 1990; DeMonchy et al 1985; and; Foresi et al 1990 and Ellis et al 1908). The significant correlation between the concentration of major basic protein and the number of eosinophils in BAL fluid postulates the role of eosinophil-derived mediators in the development of bronchial hyperreactivity (Wardlaw et al 1988). As reported by Kirby et al 1987, our data depict that the number of eosinophils was markedly higher in Lung and BAL fluid of asthmatic animals than in healthy subjects. The present findings showed that regular administration of TAZQ-AH combination potently reduced eosinophilic accumulation into lungs and BAL fluid than the animals treated with both the drugs individually. Ambroxol at the dose of 100mg/Kg showed marked decrease in the eosinophilic infiltration into the BAL fluid that indicate its anti-inflammatory activity, whereas TAZQ even at the dose of 10mg/kg significantly prevents eosinophilic infiltration into the lung and BAL fluid proving to be potent anti-inflammatory agent.
Studies suggest that allergic subjects show elevations in Eosinophilic recruitment to lungs (Denburg et al. 2000, Wood et al. 1998) and produce NOx with the stimulation of interleukin (IL)-4 (Paoliello et al. 2005). So eosinophils may contribute to the production of NOx in alveoli during allergic inflammation. Therefore, the restoration of the oxidant/antioxidant imbalance and counteraction of nitrosative stress has been a desirable therapeutic option in various chronic inflammatory diseases (Beeh et al. 2008). Various tissues and cells like airway epithelium, vascular endothelium, neurons (Maarsingh et al. 2006), and immune cells (Ricciardolo et al. 2003) are known to produce and release NOx into the circulation, alveoli, and surrounding tissues during allergic asthma. In the present study concentration of NOx in BAL fluid and lung tissue homogenate of normal, sensitized and drug treated animal groups was measured. The levels of NOx in BAL fluid and in lung tissue homogenates were significantly elevated in OVA-sensitized and -challenged guinea pigs compared to normal control, whereas no increase of NOx was observed in BAL fluid and lung tissue of animals treated with TAZQ at both the doses and when combined with ambroxol at lower dose. Satisfactory correlation within elevated concentrations of NOx in BAL fluid and tissue with the levels of eosinophils in BAL fluid of OVA-sensitized and -challenged guinea pigs was found. DB-TAZQ was capable to enhance the Nox level showing its anti-oxidative potency at 10 and 20 mg/kg doses but not comparable to TAZQ.

Oxidative stress may play an important role in the pathophysiology of asthma and may be a final common pathway leading to tissue damage (Barnes 1990, Doelman 1990). Oxidative stress can have many detrimental effects on airway function, including airway smooth muscle contraction, induction of airway hyperresponsiveness (Rhoden et al. 1989, Katsumata 1990), mucus hypersecretion (Weiss 1986, Adler 1990), epithelial shedding (Phipps 1986) and vascular exudation (Doelman 1990, Del et al. 1981). TBARS, such as malondialdehyde, are end-products of cell membrane lipid peroxidation by reactive oxygen species and are considered reliable markers of oxidative tissue injury (Aruoma et al., 1989). In present study we have found that TBARS content in BAL fluid and lung tissue homogenates of the sensitized-challenged animals was significantly higher as compared to normal control. In support to the earlier report by Nowak et al. 1993, ambroxol at the intraperitoneal dose of 50 and 100 mg/kg once a day for 7 days 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
two drugs showed marked synergistic reduction in TBARS level in sensitized guinea pigs. 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. Glutathione, a sulfur-containing thiol effective in scavenging ROS, is the most abundant intracellular thiol-based antioxidant found to be distributed in the lung and BAL fluid of asthmatic subjects (Antonicelli et al 2002). Bronchoalveolar lavage fluid is reported to contain a 100-fold concentration of glutathione compared to blood (Van der Vliet et al 1999, Cantin et al 1987) and in high concentration in intracellular spaces, is indicative of oxidative stress in asthmatics (Halliwell et al 1999). GSH (Casoni et al 2003) have shown potential for beneficial effects on airway function both in humans and animals. Low GSH in the lung may amplify inflammation and hyper responsiveness (Rahman et al 2000). Supporting to the number of investigations, our data also shows 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 (Beeh et al 2008; Gillisen et al 1997). 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.

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.
The drug identification studies showed that AH supplied by Zoetic Pharmaceuticals resembles with the standards in various test including solubility, melting point, UV and IR.

Solubility of the drugs was determined in various solvents at room temperature. TAZQ was found to be freely soluble in CHCl$_3$, methanol, and acetonitrile whereas very slightly soluble in water (table-17). Being a hydrochloride salt, ambroxol showed maximum solubility in water. At pH 6.8 and 4.5 TAZQ showed slight solubility whereas AH was freely soluble. The solubility of AH decreased with the increase in pH in basic medium whereas TAZQ was found to be very slightly soluble at this condition. The partition coefficient of both the drugs showed their lipophilic nature (table-16).

A simple, rapid and stability indicating chromatographic method was developed using Waters 515 binary HPLC system equipped with 2998 PDA detector. The method was validated according to the ICH-Q1A (R2) guidelines and the conditions showed linear results for TAZQ at the concentration range of 0.1 to 10 µg/ml and 0.2 to 20 µg/ml for AH. Pantoprazole was employed as internal standard and showed proper separation under developed chromatographic conditions which was detected at UV 265nm. The chromatographic conditions showed good separation as shown in figure-35 and the method was employed for the simultaneous estimation of TAZQ and AH in the pre-formulation studies.

Based on the previous literature, the dose required to produce effect in humans was calculated from dose required in animals. The dosed quantity for humans was studied for solubility in different simulated body fluid in order to categorise the drug as per biopharmaceutical classification. The drug found to have very low solubility and high permeability of approximately 88% when studied using two different techniques. Based on the results, the drug was categorised as BCS class 2 since it has high permeability and low solubility.

The formulation development was a thorough investigation. Prepared liposomal formulations by using different PC to CHOL ratio were subjected to drug entrapment efficiency, particle size analysis. With the increase in PC: CHOL ratio, the size of liposomes was increased, however, by lowering the amount of cholesterol, PDI also decreased. As observed in F3, the liposomal dispersion prepared with the use of 07:03 ratio produced particle size of 2.18 µm that is optimum for pulmonary use. Cholesterol imparted fluidity to the vesicles. The different formulation depicting their percent (%) entrapment efficiency, particle size (µm), zeta potential (mv) and polydispersity index
are shown in table-35. Formulation prepared using different lipid ratios were subjected to entrapment efficiency and the ratio of 07:03 showed maximum drug entrapment with optimum particle size as shown in figure-39 and table-36. The dispersions prepared with incorporation of single drugs showed higher drug entrapment as compared to combination loaded liposomes. Thereby, the particle size was also increased in the combination loaded liposomal dispersions (table-35).

The loading efficiency of different lipid ratios was studied by incorporating different amount of drugs and the observations showed that the increase in TAZQ: Lipid mass ratio above 1:5 reduces the % entrapment while ratio to AH did not have any effect as it was found to increase with the increase in amount of AH into the hydration medium. Entrapment of drugs in the liposomes could be attributed to the lipophilic nature of the drug represented by lipid:aqueous phase ratio and acyl chain length of phospholipid. An increase in chain length of fatty acid and inclusion of cholesterol results in an increase in the encapsulation efficiency.

The effect of hydration medium volume on drug entrapment was studied and it was observed that the entrapment efficiency of the vesicles increases with the increase in hydration medium volume from 5 to 15 ml whereas it decreases with the use of 20 ml. The entrapment was quite consistent with the use of 10 and 15 ml hydration medium as shown in table-37.

The effect of vacuum on film formation was studied and it was inferred that 40 in of Hg was optimum since at low vacuum the film tends to retain the residual solvent and at high vacuum entrapment of air bubbles on lipid film surface were observed with the reduced drug entrapment.

The liposomal dispersions were purified using different purification methods and dialysis was found to be the most efficient technique as centrifugation caused the physical damage to the liposomal structure and purification through Sephadex G-50 suffered lack of precision.

The stability of liposomal dispersions was studied at different temperature conditions for 1 month. At all temperature conditions, the liposomal dispersions were found to be stable as drug content and particle size remained stable during the period.

During the freeze-drying process, liposomes constrict and get coated on the optimum surface of crystallized sugar. Hydration of polar head groups with the hydroxyl group of trehalose leads to stabilization of liposomes. If the sugar concentration is less than optimum, the crystallized sugar does not provide adequate surface for the adherence of
the constricted bilayer leading to drug leakage. Hence, the bulk concentration of sugar is required as cryoprotectant depends on the type of sugar selected and saturation of the polar head groups of the bilayer by drug or other formulation components.

The optimized liposomal dispersions were lyophilized using various cryoprotectant and optimised considering the percent drug retained (PDR) in lyophilized liposomal powder as described in table 39. Liposomes were best preserved in their structure with PDR using trehalose as cryoprotectant in mass ratio of lipid: trehalose at 1:12 with PDR of 82.09 ± 3.95 and 83.68 ± 4.23 for TAZQ and AH respectively. The PDR at both the mass ratios of 01:12 and 01:16 was observed with a very small difference, 01:12 lipid trehalose mass ratio was preferred in order to reduce the moisture content of lyophilized dry powder due to excess sugar content.

Lyophilized liposomes keeping the liposome: lactose weight ratio of 1:5 were evaluated, as high-energy adhesion sites (HA) of lactose may bind strongly to the carrier and low-energy adhesion sites (LA) may allow the formation of more reversible bonds with liposomal drug. This action results in efficient detachment of liposomal drug from the carrier as observed with plain DPI formulations. Liposomal drug powder adheres to carrier particles as seen in SEM photomicrographs of COMB LDPI formulations (figure 45). The studies of cascade impaction analysis revealed that LDPI had GSD 2.26 and MMAD 2.13 µm as shown in table 50, suggesting more effective liposomal drug deposition into lung.

In-vitro drug release from different formulations was monitored for 48 hrs and observed to be 59.076 ±1.33 and 64.02 ± 2.86 for TAZQ and AH respectively showing highest release that followed first order Higuchi kinetics.

Evaluation and control of flow and dispersion (de-aggregation) characteristics of the formulation are of critical importance in the development of DPI products. Inter-particle forces that influence flow and dispersion properties are particularly dominant in micronized or microcrystalline powders required for inhalation therapy (<5 µm). It has been demonstrated that powder adhesion, mediated in part by Van Der Waal forces, is directly related to particles <10 µm.

Moisture content determination is also important for drug stability upon storage and deaggregation upon inhalation. Incorporation of CHOL is known to cause strong reduction in the permeability of the liposome system and thus reduce leakage of drug from the liposomes. However, under the present anhydrous state of storage, the incorporation of CHOL reduces the permeability of the membrane, because in the
anhydrous state there is not any possibility of drug diffusion; therefore, drug retention cannot be increased by reducing the permeability alone. Flow and dispersion properties such as angle of repose and moisture content were characterized. The flowability and floodability expressed by angle of repose was 33.86 ± 0.38 (COMB LDPI), 35.98 ± 0.46 (TAZQ LDPI) and 37.19 ± 0.37 (AH LDPI) and moisture content 10.78 ± 2.22 % (COMB LDPI), 9.64 ± 2.1 % (TAZQ LDPI) and 12.09 ± 2.2 % (AH LDPI) respectively.

Following a single pulmonary administration of drug loaded liposomes to rats, the drug was detected in the plasma and lungs from 1 hr onwards up to 24 hrs. At each time point the drug concentrations were in detection limits.

Lung tissue distribution study of the developed lyophilized liposomal formulations was compared with the control (fine drug powder) table 51. All the developed liposomal formulations showed greater accumulation in the lungs when compared with the control. In the case of free drug inhalation, 74.02 ± 3.81 (%TAZQ) and 81.26 ± 4.18 (%AH) of the administered dose was found in the lungs at 1hr post-administration and was not detectable in the lungs after 08 hr. On the other hand 64.06 ± 3.04 % and 61.09 ± 4.32 % of TAZQ and AH were detected at first hour in liposomal dry powder which remained above therapeutic range even after 8 hours. This may be due to selective intervention and capture of negatively charged liposomes via scavenger receptors expressed on alveolar macrophages.

The observed values suggest that the DPI liposomes are not only effective in rapid attainment of high-drug concentrations in alveolar macrophages (lungs) but could also maintain the concentration over a prolonged period of time when compared against the free drug.

Pharmacokinetic studies were included to assess possible differences in the pulmonary absorption by evaluating area under the concentration time curves and $C_{\text{max}}$ estimates. This was feasible as area under the curve (AUC) estimates after pulmonary deposition reflect differences in the pulmonary drug availability (release from dosage form and absorption). Pharmacokinetic parameters obtained after non-compartmental analysis of the plasma concentration vs time data are listed in Table-51. The results further supported the effective delivery of drug from the liposomal systems.

In summary, TAZQ was found to be a potent bronchodilatory agent as reported along with significant anti-inflammatory activity that reduces eosinophilic infiltration into the lung tissue and BAL fluid of the sensitized guinea pigs. The antioxidant potential of
TAZQ was accessed by in-vitro measurement of TBARS, GSH and NOx content in lung tissue and BAL fluid of asthmatic guinea pigs and the results showed that it reduces the oxidative stress thereby prevents lung tissue damage. Incorporation of potent mucolytic, antioxidant and anti-inflammatory ambroxol with TAZQ at low dose have shown a substantial synergism in reducing asthma like reaction in OVA sensitized asthmatic guinea pigs. A notable synergistic effect on eosinophilic infiltration and antioxidant activity of TAZQ on combination with ambroxol was observed as both the drugs possess the activities. Mucolytics are known to be dispensed along with antibiotics in the treatment of many respiratory tract disorders for better potency. Synergistic activity of the combination may be because of potential mucolytic activity of ambroxol that causes enhanced bioavailability of the TAZQ into trachiobronchial tissue. For relief in asthma attacks i.e. reducing bronchial hyperreactivity and cough strokes, presently studied combination of bronchodilator TAZQ and mucolytic ambroxol may be an effective treatment of choice that will take care of bronchoconstriction, bronchial inflammation and restricted bronchial airflow due to mucus plugging into the bronchioles in acute and chronic asthma.

Lyophilization and DPI preparation is known to improve drug pharmacokinetics which is also supported by our findings. These values were significantly higher when compared to the administration of plain drug, which could be due to maintenance of concentration of drug within the therapeutic effective range for longer period of time from the encapsulated liposomal systems with the benefit of reduced blood concentration thereby reducing the toxicity of the drugs.