Chapter 4

In vivo evaluation of selected compounds for acute inflammatory conditions

4.1. Introduction

In vitro models employing cell lines can also give a clue about the rate or extent of drug absorption by specific cells. In vitro models can also give an idea about the possible effects of test compounds along with a probable mode of action. The effects of test compounds in cell line can be extrapolated to in vivo models, although conclusive results are possible only in animal experiments. Therefore, we decided to confirm the in vitro effects of the test compounds in cell lines, by employing suitable rodent models. In vivo
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models help in shortening the drug development period by economizing resources and giving a direction to clinical applications (Emami, 2006).

From the in vitro results, we could hypothesize the probable anti-inflammatory properties of our test compounds. Therefore, we tested them in preliminary rodent models. Among the several models used, chemically-induced acute paw inflammation method is one of the most common techniques used for screening of anti-inflammatory compounds. This method is a rapid and inexpensive in vivo model for evaluation of anti-inflammatory efficacy of small molecules (Vogel, 2002).

Carrageenan-induced acute inflammation includes extravasation of leukocytes from circulation to site of inflammation. Carrageenan injection into the air pouch activates infiltration and differentiation of cells connected to inflammatory responses and a parallel rise in exudate capacity. Development of an air pouch offers a cavity in which the inflammatory reaction may be examined. Progression of the air pouch leads to a pouch coating of granulation tissue, involving fibroblasts, macrophages and mast cells (Edwards et al., 1981). Carrageenan triggers the inflammatory cascade, which is accompanied by activation of mononuclear phagocytes that leads to release of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6 and IL-8 (Gambero et al., 2003, Dumas et al., 2012). Air-pouch is almost morphologically comparable to the synovium in joints (Romano et al., 1997). Therefore, this acute model could be an appropriate technique to study the effects of test compounds in the inflammation of joints. Arthritis is emerging as a major illness of modern life style.

Another easy and important method we followed to screen the selected test compound is an animal model that replicates human acute lung injury. Lung is constantly open to the outside environment and vulnerable to invasion by antigens. When antigens enters the pulmonary tract, cell migration and activation of the inflammatory cascade occurs. Lymphocytes on the surface of respiratory tract plays a protective role in immunity (Porter et al., 1999). Acute lung injury is dominated by neutrophils, whereas chronic infection is ruled by macrophages and lymphocytes. (Moldoveanu et al., 2009).

When bacteria overpower the local defenses, an acute infection develops in lungs. Due to insufficient innate defense, bacterial colonization and subsequent damage to pulmonary parenchyma, eventually result in chronic infection (Stockley, 1998). Bacteria dominates the host defense and releases ciliary toxins, pneumolysin and endotoxin, thereby disrupting mucociliary clearance (Wilson et al., 1987). When bacteria adhere to epithelium, dendritic cells, alveolar macrophages and epithelial cells get activated. The
pathogen markers are recognized through toll like receptors (TLRs) (Moldoveanu et al., 2009). LPS-induce acute lung-injury, developing a cellular response that can be detected in bronchoalveolar lavage (BAL). LPS-induced acute lung-injury in rodents shows almost the same pathological conditions that occur in organic-dust-stimulated lung infections in humans (Sandstrom et al., 1992a). Therefore, we evaluated the selected test compounds in these acute animal models.

4.2. Materials and Methods

4.2.1. Materials

0.22 mm syringe filter unit (Millipore, USA)
LPS - Lipopolysaccharide from Escherichia coli 0111:B4 (Cat # EL4391, Sigma).
NEDD- N-1-Naphthylethylene diamine dihydrochloride, LobaCheme.
O-dianisidine HCl, (Cat# D3252, Sigma)
O-Phosphoric acid (Cat#UN1805, Merck).
Sulphanilamide- (Cat#7995, Sulab reagents)
TNF-α ELISA kit (Cat# RTA00, Quantikine, R&D Systems),
λ-Carrageenan (Cat# 22049, Fluka)

Instruments:

Cooling micro-centrifuge (MIKRO 22R, Hettich, Germany)
Digital Plethysmometer (Ugo Basile, Italy)
Veterinary Blood Cell Counter (PCE-210VET, Erma Inc., Tokyo, Japan).

4.2.2. Methods

4.2.2.1. Animals

All the experiments were carried out on Sprague-Dawley rats of either sex weighing 180-230 g, obtained from Central Animal Research Facility, Manipal University. The rats were housed in plastic cages with 12 h light and dark cycle at a temperature and humidity of 26 ± 1 °C and 50 ± 5 % respectively. The standard food pellet and water ad libitum were provided to all the rats. The experiments were performed after approval of experimental protocol by the Institutional Animal Ethical Committee (No. IAEC/KMC/55/2013).

4.2.2.2 Acute toxicity test

The acute toxicity study was performed as per OECD 420 guidelines using female rats. The animals were administered a maximum dose of 2000 mg/kg orally and the animals
were observed individually for any symptom of toxicity every 30 min up to 4 h with care. Further, the animals were observed every 6-8 h for 14 days for further signs of toxicity.

4.2.2.3 Selection of optimum dose and route for test compounds in anti-inflammatory studies
We conducted a few preliminary studies in which acute doses of the most promising compounds did not show any significant reduction in paw edema. Since, many AMPK activators are acting in chronic dosing (Mathew and Unnikrishnan, 2015) we chose to employ a dosing schedule as described in the table below. The precise doses chosen for each test compound was based on results of acute toxicity and clues from in vitro models. We chose a prophylactic dosing schedule based on experience with similar AMPK activator identified and established for biological activity in our lab (Mathew et al., 2013, Kandadi et al., 2010).

Drug treatment regimen:

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Experiments</th>
<th>Days of Treatment</th>
<th>Dose of HMPH, HMBH and HPID</th>
<th>Dose of HPID-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Carrageenan-induced acute paw edema</td>
<td>5 days before carrageenan challenge.</td>
<td>25 and 50 mg/kg, i.p., o.d.</td>
<td>12.5 and 25 mg/kg, i.p., o.d.</td>
</tr>
<tr>
<td>2.</td>
<td>Carrageenan-induced acute air-pouch</td>
<td>-do-</td>
<td>12.5, 25 and 50 mg/kg, i.p., o.d.</td>
<td>6.25, 12.5 and 25 mg/kg, i.p., o.d.</td>
</tr>
<tr>
<td>3.</td>
<td>LPS-induced acute lung inflammation</td>
<td>-do-</td>
<td>25 and 50 mg/kg, i.p., o.d.</td>
<td>12.5 and 25 mg/kg, i.p., o.d.</td>
</tr>
</tbody>
</table>

Table 4.3.1. This table represents the treatment regimen. In all the studies, test compounds were administered for 5 days, once daily by intraperitoneal route. The reference standard (diclofenac, 10 mg/kg) was also administered for 5 days, once daily by oral route.

4.2.2.4. Carrageenan-induced paw-edema
HMPH, HMBH, HPID and HPID-1 was tested for acute paw inflammation as per the standard protocol (Mudgal et al., 2014). The test compounds were dosed (based on the results obtained in section 4.2.2.3) for five days. On the fifth day, 15 min after the last dose 0.1 ml of freshly prepared carrageenan in 1 % w/ v normal saline was injected to the sub-plantar region of the left hind paw of male SD rats to induce acute inflammation (naive control group was administered with normal saline in place of carrageenan).
The paw volume was measured (in milliliters) just before carrageenan injection, and successively at 1, 2, 3 and 5th hour after carrageenan challenge, using a digital plethysmometer. The difference in paw volumes were calculated by subtracting the basal paw volume from inflamed paw volume at each time point.

4.2.2.5. Carrageenan-induced air-pouch model in SD male rats
Air-pouch inflammation was developed as per established protocol (Talwar et al., 2011) with minor modifications. On day 1, the air-pouch was developed by subcutaneously injecting 20 ml of sterile air through 0.22 mm syringe filter unit (Millipore, USA) to the shaved dorsal part (below neck region) of male SD rats. Subsequently, on day 3 and 5, further 10 ml of air was injected into the same air-pouch. Rats were then administered with test compounds/standard at doses mentioned in table 4.3.1. 30 min after administering the test compounds/standard, 2 ml of 1 % w/v carrageenan in normal saline was injected to the air-pouch to trigger inflammation in all the groups except naive control group which received only normal saline in place of carrageenan.

Blood was collected from retro-orbital plexus of all the rats 6 h after carrageen challenge, following which the rats were sacrificed, air-pouch-lavage and spleen collected and tested for inflammatory markers. The protection by the test compounds and reference standard diclofenac were assessed based by comparing the level of inflammatory markers with the respective carrageenan control.

Estimation of inflammatory markers in plasma: After 6 h of carrageenan challenge, blood was withdrawn from retro-orbital plexus, collected in centrifuge tubes holding 10 % K2-EDTA. Plasma was separated by centrifugation (8000 RPM, 10 min at 4 °C) using cooling micro-centrifuge. Plasma was aliquoted and stored at -80 °C for future estimations.

Estimation of inflammatory markers in air pouch lavage: The rats were sacrificed after 6 h of carrageenan challenge and the air-pouch was washed three times with 5 ml of chilled sterile saline. The lavage was aspirated by syringe after a gentle massage. The volume of the lavage was measured and the total leucocyte count was quantified using Veterinary Blood Cell Counter. MPO, nitrite and TNF-α in air-pouch lavage were measured as per procedures described below. TNF-α was estimated by ELISA kit and followed manufacturer instructions.
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Estimation of MPO and nitrite in air pouch lavage and plasma: MPO activity in lavages and in plasma was determined by previously reported methods with minor amendments (Bradley et al., 1982). In brief, 125 µl of O-dianisidine HCl (ODA, 1.67 mg/10 ml in 50 mM phosphate buffer, pH 6) containing 0.5 % v/v hydrogen peroxide was added to 25 µl of lavage/plasma. After 30 min of incubation in dark at room temperature (RT), 25 µl of 4 M H₂SO₄ was added to stop the reaction and the absorbance was recorded (λ= 490 nm) using micro-plate reader. The percentage of MPO activity with respect to naive control was calculated.

Nitrite levels were estimated in air pouch lavage and in plasma by using Griess Reagent, adopting the established procedure (Mathew et al., 2013). In clear 96 well plate, 100 µl of Griess’ reagent was added to a 100 µl of supernatant medium, incubated at 37 °C for 20 min and absorbance was measured (λ=540 nm).

Preparation of Griess Reagent- 2.5 % phosphoric acid (stock 1), 1 % sulfanilamide in 2.5 % phosphoric acid (stock 2), 0.1 % NEDD in 2.5 % phosphoric acid (stock 3), Stock 2 and stock 3 were mixed in equal ratio, Griess reagent ready to use.

Splenomegaly: In rat air-pouch model, inhibition of splenomegaly was determined from the ratio of individual spleen weight to body weight. The average inhibition of splenomegaly of treatment groups were compared with carrageenan control group (Balague et al., 2012).

4.2.2.6. LPS-induced acute lung inflammation model

A standard procedure was followed with some modifications (Ulich et al., 1991). The test compounds and diclofenac were administered as per drug treatment regimen in table 4.3.1. On the 5th day of experiment, animals were treated with test compounds/standard. After 30 min of treatment, the rats were challenged with 50 µg/ml/kg of LPS in normal saline (by intra-tracheal route). After 4 h of LPS challenge, rats were anesthetized by ketamine + xylazine injection. The lungs were washed 3 times with chilled HBSS (4 ml each time) to obtain the brocho-alveolar lavage (BAL). The BAL was collected and the volume measured. BAL was centrifuged (4500 RPM for 10 min at 4 °C) and the pellets obtained were re-suspended in 2 ml of HBSS and the total leukocyte count was estimated by using Veterinary Blood Cell Counter. The aliquots of BAL were stored at -80 °C for further analysis. The prevention of the infiltration of inflammatory cells into the lungs, (in
percentage value) was compared with respective controls receiving test compound/standard.

4.2.2.7. Statistical Analysis
Statistical analysis was performed by using Prism 6.01 Software (trial version) (GraphPad Software Inc., La Jolla, USA). Data are represented as mean ± SEM and evaluated statistically using one-way ANOVA followed by Dunnett’s multiple comparisons test and two-way ANOVA followed by Dunnett’s multiple comparison test where p < 0.05 was considered statistically significant.
4.3. Figures and Tables
Effect of the test compounds on carrageenan-induced acute paw inflammation

Figure 4.3.2. HMPH, HMBH, HPID and HPID-1 were administered (see table 4.3.1 for doses and dosing schedule). On day 5, 15 min after administering test compound/standard, carrageenan (1 % w/v, 0.1 ml) was injected to the sub-plantar region of left hind paw of all rats except naïve control group, which received normal saline. Paw inflammation was measured at 0, 1, 2, 3 and 5th h after carrageenan injection. Graphs depict changes in paw volume (ml) at various time points. Data presented as mean ± SEM (n=6). # p<0.05 vs naïve control. ★ p<0.05 vs carrageenan control (two-way ANOVA followed by Dunnett’s multiple comparison test).
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Effect of the test compounds on air-pouch lavage cell count

Figure 4.3.3. Air-pouch was developed by subcutaneously injecting 20 ml of sterile air on day-1 and a further 10 ml on day 3 and 5. HMPH, HMBH, HPID and HPID-1 were dosed from day-1 to day-5 (see table 4.3.1). On day-5, after administering test compounds/standard, carrageenan (1 % w/v, 2 ml) was injected into the air pouch of all animals except naïve control group, which received normal saline. After 6 h of carrageenan challenge, rats were scarificed, air pouch lavage was aspirated and differential cell count was performed. % reduction in cell counts indicates anti-inflammatory efficacy. Data presented as mean ± SEM (n=5). ★ p < 0.05 vs carrageenan control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of the test compounds on MPO and nitrite in both lavage and plasma

Figure 4.3.4. Air-pouch was developed by subcutaneously injecting 20 ml of sterile air on day-1 and a further 10 ml on day 3 and 5. HMPH, HMBH, HPID and HPID-1 were dosed from day-1 to day-5 (see table 4.3.1). On day-5, after administering test compounds/standard, carrageenan (1 % w/v, 2 ml) was injected in the air pouch of all animals except naïve control group, which received normal saline. After 6 h of carrageenan challenge, blood was collected, plasma separated and then rats were sacrificed, air pouch lavage was aspirated. MPO and nitrite (data not shown) was estimated in plasma and air pouch lavage. % reduction in cell counts indicates anti-inflammatory efficacy. Data presented as mean ± SEM (n=5). * p<0.05 vs carrageenan control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of the test compounds on cell count in BAL and TNF-α in air-pouch lavage

Figure 4.3.5. On day-5, after 30 of test compound/standard treatment, all the rats were challenged with LPS (50 µg/ml/kg) except naïve control group, which received normal saline. After 4 h of LPS challenge lungs were washed with HBSS, BAL was collected, centrifuged (4500 rpm, 10 min at 4 °C), pellets re-suspended and differential cell count was performed. HMPH, HMBH, HPID and HPID-1 were dosed from day-1 to day-5 (see table 4.3.1). Panel a, b and c shows % reduction in cell counts indicates anti-inflammatory efficacy. Panel d represents the TNF-α in air pouch lavage (discussed in section 4.2.5). Data presented as mean ± SEM (n=5). ★ p<0.05 vs carrageenan control (one-way ANOVA followed by Dunnett’s multiple comparison test).
4.4. Results and Discussion

4.4.1. Safety profile in rats
HMPH, HMBH, HPID and HPID-1 did not produce any visible symptoms of toxicity in rats up to 72 h at 2000 mg/kg/oral and 400 mg/kg/intraperitoneal dose. These compounds did not cause any death in 30 days. These results propose a very safe window of doses.

4.4.2. Reduction of acute paw inflammation by test compounds
HMPH at 25 and 50 mg/kg (Fig. 4.3.2 a) and HPID-1 at 12.5 and 25 mg/kg (Fig. 4.3.2 d) decreased the inflammation significantly at 5th h. HMBH at 25 and 50 mg/kg (Fig. 4.3.2 b) reduced the edema at 3rd and 5th h whereas HPID at 25 and 50 mg/kg (Fig. 4.3.2 c) reduced at 2nd, 3rd and 5th h. The local inflammation was significantly reduced by diclofenac at 1st, 2nd, 3rd and 5th h. However, inhibition of inflammation began to decline from the 3rd h probably because of systemic clearance of diclofenac (diclofenac plasm $t_{max}$ varies from 1 to 4.5 h after single dose and half-life is 1.8 h) (Willis et al., 1979). HMPH, HMBH, HPID and HPID-1 produced significant reduction in inflammation after 3rd h which predict a possibly greater role against chronic inflammation by delayed onset and prolonged duration of action.

4.4.3. Attenuation of inflammatory markers in rat air-pouch model
Carrageenan-induced air-pouch model mimics the synovial cavity (a restricted environment) in which cell trafficking and inflammatory responses can be evaluated. Carrageenan triggers infiltration, differentiation of cells connected with the inflammatory reaction, rise in exudate volume, stimulation of the inflammatory cascade and concurrent rise of pro-inflammatory mediators (IL-1β, TNF-α, IL-6). A test compound which minimizes the specific inflammatory cell types can give a clue about the mechanism of action. Carrageenan-induced edema also comprises extravasation of polymorphonuclear leukocytes from circulation to the site of inflammation (Duarte et al., 2016). Therefore, leukocyte count at the site of inflammation is a reliable measure of inflammatory response.

The air-pouch model is self-possessed of a lining of cells that consists primarily of macrophages and fibroblasts which produces an inflammatory granulomatous reaction characterized by marked production of inflammatory mediators in the air-pouch lavage on induction of carrageenan (Masferrer et al., 1994). HMPH, HPID and HPID-1 have decreased significantly the total WBC count and lymphocytes in air-pouch lavage (Fig. 4.3.3). The absolute cell count in naïve-control and carrageenan-control was found to be
1.3 ± 0.24 and 27.3 ± 2.72 × 10^3/µl in total WBC, 0.93 ± 0.17 and 21 ± 1.68 × 10^3/µl in total lymphocytes, respectively. HMPH, HPID and HPID-1 plays a role in modulating the immune system by suppressing WBC and lymphocytes in air-pouch exudates. HMBH did not produce significant activity against increased cell counts.

4.4.3.1. Reversal of increased MPO and nitrite by test compounds in rat air-pouch model
An immunological reaction was observed with the elevated nitrite and MPO in air-pouch lavage and plasma. MPO is excessively expressed in neutrophils, granulocytes and produces HOCl (hypochlorous acid, cytotoxic) from hydrogen peroxide (H₂O₂) and chloride anion (Cl⁻) during neutrophil’s respiratory burst. MPO possesses antimicrobial activity by releasing it out into the extracellular space, from azurophilic granules of neutrophils. MPO helps neutralize invading pathogens. It is a marker of leukocyte infiltration to the site of inflammation. In circulating neutrophils, MPO is present in inactive form. During infection, neutrophils undergo activation. And some percent of MPO is lost during transit in blood. On reaching the site of inflammation, very low concentration of MPO from activated neutrophils remains available for maximum biological activity (Bradley et al., 1982).

HMPH dose-dependently decreased MPO in plasma (Fig. 4.3.4 b) and significantly in air-pouch lavage (Fig. 4.3.4 a) which was comparable to diclofenac. HPID significantly reduced MPO (dose dependent) in both lavage and plasma (Fig. 4.3.4 c and 4.3.4 d). HPID-1 (12.5 mg/kg) produced significant reduction in lavage and plasma MPO (Fig. 4.3.4 c and 4.3.4 d).

The rise in nitrite levels in the lavage mediated by iNOS, may also increase PGE₂, COX-1, COX-2 and 5-LOX in the infiltrating cells after carrageenan challenge (Turesin et al., 2003). Diclofenac, 10 mg/kg and HMPH (dose response) significantly reduced the elevated nitrite levels in the exudates (Fig. 4.3.4 e), demonstrating a probable mode of action by iNOS and PG pathway cascade (Fig. 4.3.3e). HMBH dose-dependently decreased nitrite in lavage (Fig. 4.3.4 f).

4.4.3.2. Effect on splenomegaly and TNF-α in air-pouch model
The test compounds at tested doses attenuated splenomegaly but was not statistically significant (data not shown). Splenomegaly observed in carrageenan-control group indicates the development of immunological response (Balague et al., 2012) which was annulled significantly by diclofenac.
TNF-α may be the primary cytokine which is synthesized by the stimulation of macrophages and neutrophils due to cell injury. As a result, TNF-α activates the release of other pro-inflammatory mediators which are also responsible for autoimmune disorders (Wu et al., 1999). HMPH (25 and 50 mg/kg) and HPID (25 and 50 mg/kg) significantly decreased TNF-α in a dose dependent manner in air-pouch lavage (Fig. 4.3.5 d), whereas diclofenac failed (Mudgal et al., 2014). HMBH (50 mg/kg) and HPID-1 (12.5 mg/kg) significantly reduced TNF-α in air-pouch lavage (Fig. 4.3.5 d), representing anti-inflammatory activity.

4.4.4. Effect on bronchoalveolar lavage in LPS-induced lung injury model
LPS causes cellular responses to develop symptoms of chronic lung injury in rats, which is almost similar to organic dust exposure in humans. Intra-tracheal LPS induction elevated the cell response, which was reflected proportionately in BAL (Sandstrom et al., 1992b). Some of the main pathological conditions of LPS-induced lung inflammation include an acute inflammatory reaction in alveoli and lung endothelium. Complications of these disorders may aggravate to pulmonary hypo/hypertension and myocardial depression. HMPH, HPID and HPID-1 demonstrated a dose dependent attenuation of lung injury by reducing total WBC, lymphocytes and granulocytes in BAL (Fig. 4.3.5 a, b and c). However, only the highest doses of HMPH, HPID, HPID-1 showed a statistically significant reduction in WBC, lymphocytes and granulocytes (Fig. 4.3.5 a, b and c). To sum up, test compounds demonstrated a protective role against the progression of chronic lung damage as illustrated by this model.

4.5. Conclusion
HMPH, HMBH, HPID and HPID-1 were safe up to a dose of 2 g/kg in rats. In acute local inflammation, HMPH and HPID-1 showed delayed onset of therapeutic action, whereas HMBH and HPID were fast acting. All four compounds decreased total and differential cell count in air-pouch lavage. Therefore, these compounds may possess immunomodulatory action. HMPH and HPID also decreased MPO in air-pouch lavage implying a role on immune cells. Also, HMPH, HPID and HPID-1 were more effective in reducing total and differential cell count in BAL. Immunomodulation by the test compounds were supported by significant attenuation of TNF-α in air-pouch lavage.
4.6. References


