Chapter 5

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Chapter – 5, *In-vivo* evaluation of best compounds for chronic immune-mediated inflammatory conditions

5.1. Introduction
Neutrophil (polymorphonuclear leukocytes, (PMN)) infiltration plays a central role in inflammation and is also a major cause of tissue damage (Bian et al., 2012). We observed a significant neutrophil infiltration in air-pouch lavage and BAL from our previous studies (see section 4.4.3. and 4.4.4 respectively in chapter-4). DSS-induced colitis (post-acute or chronic) model also revealed that enhancement of PMN infiltration and macrophage function are associated with markedly increased IL-17A in serum (Bian et al., 2012). With these evidences, we tested the activity of selected test compounds on colon TNF-α and IL-6 in DSS-induced IBD model.

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract, principally comprised of ulcerative colitis (UC) and Crohn's disease (CD), both involving a complex multifactorial etiology. Various experimental models of IBD have been developed, among which dextran sodium sulfate (DSS) model is widely used, simple and reproducible. DSS is a water soluble, negatively charged sulfated polysaccharide. DSS is administered orally with drinking water, which will damage epithelial lining of large intestines and increase pro-inflammatory signals. Both acute and chronic IBD models can be developed by varying the concentration of DSS and frequency of administration. Acute DSS model is useful in the study of innate immune system, contributing to the pathology associated with increased production of cytokines such as IL-6, IL-17 and TNF-α. On the other hand, the chronic DSS model is associated with adaptive immunity and affects T cells and macrophages (Randhawa et al., 2014, Chassaing et al., 2014, Perse and Cerar, 2012).
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Anti-TNF-α treatment is employed in both rheumatoid arthritis (RA) or Crohn’s disease, probably because these two pathologies share some common mechanisms. Imbalance in Th1/Th2 cytokines could be one of the shared aspects of such pathologies. (Boyer et al., 2001, Miossec and Berg, 1997).

Intestinal inflammation and arthritis, though affecting different parts of the body, could be linked because there is an association between enteric pathogens and both arthritis and IBD. Exposure of the lamina propria and systemic circulation to gut microflora and their metabolic products could result from proliferation of bacterial flora in the gut lumen and their enhanced invasive capability on account of increased mucosal permeability. Anaerobic bacteria and their byproducts present in the normal gut flora can induce chronic intestinal inflammation as well as arthritis. Despite such possibilities of pathological consequences, homeostatic mechanisms that are inherent to the gut can induce tolerance to pathogenic microflora and control inflammatory responses. The healthy host develops a tolerance to such bacteria and maintains homeostasis through a controlled inflammatory response and limited mucosal permeability (Sartor, 1997a).

Rheumatoid arthritis is a systemic inflammatory disorder (Bendele, 2001). RA involves a complex pathology driven by immune, genetic and environmental factors (Liu et al., 2009). The pathology of the RA is driven by inflammation, cartilage destruction and bone resorption. Different animal models are established for the pre-clinical evaluation of the drugs in RA. Rat adjuvant induced arthritis is one of the models and commonly used because it has a reliable onset and progression (Bendele, 2001). Complete Freund’s adjuvant (CFA) acts by prolonging lifetime of injected autoantigen, by stimulating its effective delivery to the immune system and by providing a complex set of signals to the innate compartment of the immune system, resulting in altered leukocyte proliferation and differentiation. CFA enhances phagocytosis, cytokines secretion and activates T cell proliferation production of prostaglandins, leukotrienes, adhesion molecules and matrix metalloproteinases (Billiau and Matthys, 2001).

Several cytokines also orchestrate the chronic inflammatory process, especially TNF and interleukins (Dinarello, 2000) TNF-α blockers are used to treat chronic inflammatory conditions, including rheumatoid arthritis, juvenile idiopathic arthritis, psoriasis and psoriatic arthritis, ankylosing spondylitis, and Crohn's disease (Bradley, 2008). Rheumatoid arthritis appears to be an autoimmune disease driven largely by activated T cells, giving rise to T cell–derived cytokines such as IL-1 and TNF-α. The naturally occurring human recombinant IL-1 receptor antagonist (IL-1ra; anakinra) inhibits the progression of
structural damage associated with active rheumatoid arthritis and other inflammatory conditions (Mertens and Singh, 2009). Similarly, TNF-α blockers (infliximab, etanercept, adalimumab, certolizumab, golimumab) are categorised under disease-modifying anti-rheumatic drugs (DMARDs) (Sarzi-Puttini et al., 2005).

NSAIDs are widely prescribed for RA and target prostaglandins synthesis by inhibiting cyclooxygenase (COX-1 and COX-2). COX-1 is constitutively present and important in production of prostaglandins with housekeeping functions. The COX-2 enzyme is inducible and has its role in inflammation. But the selective inhibition of COX-1/COX-2 results in gastrointestinal bleeding/thrombotic cardiovascular problems. Other biologicals such as infliximab (TNF-α monoclonal antibody) anti-CD20 therapy (rituximab) and interleukin-1-receptor antagonist are also used in RA.

AMPK activators like metformin and AICAR, are immune modulators with anti-inflammatory properties. Metformin modulates T17 and Treg cell activity and suppress collagen induced arthritis (Son et al., 2014). Metformin also suppressed the release of TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) by macrophages while enhancing the release of IL-10 and enhances autophagy in mice arthritis model (Yan et al., 2015).

Many of the natural products such as curcumin, resveratrol and quercetin are also effective in arthritis. Curcumin attenuates TNF-α, and IL-1β production and controls the expression of COX-2, MMPS and alters the transcriptional activity of NF-κB in arthritis model (Moon et al., 2010). In a pilot clinical study , a combination of curcumin and diclofenac showed better efficacy and safety than diclofenac alone in RA patients (Chandran and Goel, 2012).

Our test compounds demonstrated a significant activation of AMPK, characterized by AMPK-phosphorylation in skeletal muscle cell lines. Selected test compounds attenuated neutrophil infiltration in rat air-pouch and rat acute lung injury model. With these evidences from the above literatures, we evaluated selected test compounds in acute-immunological (DSS-induced IBD in mice) and chronic-immunological (CFA-induced arthritis in rat) models.

5.2. Materials and methods

5.2.1. Materials

- BCA - Bicinchoninic acid assay kit (Cat # 23227, ThermoFisher Scientific).
- Cell lytic-MT cell lysis reagent (Cat # C3228, Sigma).
- C-RP C-reactive protein (Cat# MBS268328, MyBioSource, USA).
- DSS- Dextran sodium sulfate (Cat# 160110, MP biomedicals).
Hemospot kit (a gift sample from Cat # HS 1050, Coral clinical systems, Tulip groups, Goa-403202).

IL-6 -Interleukin-6 (Cat # 583371, Cayman Chemicals).

Mycobacterium butyricum (Cat# 264010, Difco laboratories, Detroit, USA)

RF Rheumatoid factor ELISA kit (Cat# MBS260028, MyBioSource)

RIPA Buffer - Radioimmunoprecipitation Assay Buffer.

TNF-α Tumor necrosis factors- α ELISA kit (Cat# RTA00, Quantikine, R&D Systems)

TNF-α Tumor necrosis factors- α ELISA kit, (Cat #500850, Cayman Chemicals).

**Instruments:**

- Cooling Centrifuge (MIKRO 22R, Andreas Hettich GmbH & Co.KG, Tuttingen, Germany).
- Veterinary Blood Cell Counter (PCE-210VET, Erma Inc., Tokyo, Japan).
- X-ray (Model 2100, Kodak Co., Japan).

**Animals:** IBD and CFA-induced arthritis experiments were conducted in BALB/c mice (28-35 g) and Sprague-Dawley rats (180-230 g) of either sex respectively, obtained from Central Animal Research Facility, Manipal University. The mice/rats were housed in plastic cages with 12 h light/dark cycle at 26 ± 1 °C and 50 ± 5 % humidity and fed with standard food pellet and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee (Ref No. IAEC/KMC/55/2013).

**5.2.2. Evaluation of test compounds against DSS-induced IBD**

**5.2.2.1. Evaluation of the test compounds on Disease activity index in DSS-induced IBD**

**Standardization of the DSS-induced IBD model:** We followed the protocol (Chassaing et al., 2014) with minor modifications. Male BALB/c mice were randomized based on body weight and grouped (n=6). Dextran sodium sulfate (DSS) solution in drinking water at different concentrations, namely, 2.5 %, 4 % and 5 % were given to all the groups except naïve control group, which received only water for six days. This was carried out in order to determine the ideal strength of DSS solution for optimum induction of IBD. Body weight, haem-occult and stool consistency were recorded daily. On the sixth day, animals were sacrificed, colons were excised. Colon length and weight were recorded (Chassaing et al., 2014).
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Results of the standardization experiment yielded an optimum concentration of 4 % DSS for the induction of IBD. Therefore, 4 % of DSS was used. The severity of IBD was assessed by observing and recording decrease in body weight, looseness of stool consistency and increase in haem-occult score in stool and decreased colon length and weight. Of the above parameters, significant differences were obtained in stool consistency, increase in haem-occult score and decline in colon length and weight.

Treatment regimen for the test compounds: Based on body weight, mice were randomized and grouped (six mice per group, total 12 groups) into different treatment groups (Table 5.3.1). Naïve control group received animal drinking water. DSS control group received 4 % DSS dissolved in drinking water. Treatment groups received 4 % DSS along with test compounds (HMPH/HMBH/HPID/HPID-1) at doses mentioned in table 5.3.1. Suspension of test compounds and standard (dexamethasone) were prepared in 0.25 % methyl cellulose. Standard (diclofenac) drug solution was prepared in animal drinking water. All the groups were dosed with test compounds (intraperitoneal route) and standard drugs (oral route) form day-1 to day-5 (once daily, total 5 days). The details of dose are available in table 5.3.1.

Induction of DSS-induced colitis in mice: Colitis was induced by giving freshly prepared 4 % DSS in drinking water from day-0 to day-5 (total 6 days) continuously. Naïve control group received normal drinking water (without DSS) of same volume (Verma et al., 2014, Chassaing et al., 2014).
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Treatment regimen of the test compounds/standards in DSS-induced IBD.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatments</th>
<th>No. of Mice</th>
<th>Treatment day 0</th>
<th>Treatment day 1 to Day 5</th>
<th>Treatment day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naïve Control</td>
<td>Six</td>
<td>Normal drinking water</td>
<td>Normal drinking water</td>
<td>sacrifice</td>
</tr>
<tr>
<td>2</td>
<td>4% DSS Control</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + No treatment</td>
<td>sacrifice</td>
</tr>
<tr>
<td>3</td>
<td>Dexamethasone 0.1mg/Kg, PO, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + Dexamethasone</td>
<td>sacrifice</td>
</tr>
<tr>
<td>4</td>
<td>Diclofenac 10mg/Kg, PO, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + Diclofenac</td>
<td>sacrifice</td>
</tr>
<tr>
<td>5</td>
<td>HMPH 25mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HMPH, 25</td>
<td>sacrifice</td>
</tr>
<tr>
<td>6</td>
<td>HMPH 50mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HMPH, 50</td>
<td>sacrifice</td>
</tr>
<tr>
<td>7</td>
<td>HMBH 25mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HMBH, 25</td>
<td>sacrifice</td>
</tr>
<tr>
<td>8</td>
<td>HMBH 50mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HMBH, 50</td>
<td>sacrifice</td>
</tr>
<tr>
<td>9</td>
<td>HPID 25mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HPID, 25</td>
<td>sacrifice</td>
</tr>
<tr>
<td>10</td>
<td>HPID 50mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HPID, 50</td>
<td>sacrifice</td>
</tr>
<tr>
<td>11</td>
<td>HPID-1 12.5mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HPID-1, 25</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>12</td>
<td>HPID-1 25mg/Kg, IP, OD</td>
<td>Six</td>
<td>DSS</td>
<td>DSS + HPID-1, 50</td>
<td>Sacrifice</td>
</tr>
</tbody>
</table>

Table 5.3.1. This table represents the treatment regimen. Based on body weight, mice were randomized in to 12 different treatment groups. From day-0 to day-5, naïve control received normal drinking water, DSS control group received 4% DSS. All treatment groups received 4% DSS along with respective treatment from day-1 to day-5. From day-0 to day-5, body weight, stool consistency and haem-occult was recorded. On day-6, after 4-6 h of fasting, mice were bled, euthanized, colons were isolated, weighed, length measured and preserved to estimated immunological parameters.
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**Evaluation of general parameters of DSS-induced colitis in mice:** Evaluation of colitis was performed everyday (just before administration of test compounds/standards) by measuring changes in body weight, stool consistency and haem-occult (Chassaing et al., 2014, Verma et al., 2014). The feces were collected from individual mouse by placing them in an empty cage (without bedding materials) for 10-20 min. The softness of stool (stool consistency) was tested and haem-occult was examined using Hemospot kit (a gift sample from – Cat # HS 1050, Coral clinical systems, Tulip groups, Goa-403202).

Body weight was recorded (in grams) daily and % loss in body weight was calculated by the following formula.

\[
\text{\% decrease in body weight} = \frac{(\text{day “x” body weight} – \text{day 0 body weight})}{\text{day 0 body weight}} \times 100.
\]

% change in body weight, stool consistency and haem-occult were scored based on parameters in table 5.3.2 (blinded observation) (Verma et al., 2014). Disease activity index (DAI) was evaluated based on all these three parameters.

\[
\text{DAI} = \frac{(\% \text{ change in body weight} + \text{stool consistency} + \text{haem-occult})}{3}.
\]

**Scoring for % change in body weight, stool consistency and haem-occult in DSS-induced IBD (Verma et al., 2014).**

<table>
<thead>
<tr>
<th>% Loss in Body Weight</th>
<th>Stool Consistency</th>
<th>Haem-occult</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCORE</td>
<td>% LOSS</td>
<td>SCORE</td>
</tr>
<tr>
<td>0</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1 to 5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>6 to 10</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>11 to 15</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>16 to 20</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5.3.2. This table represents the scoring of body weight, stool consistency and haem-occult parameters. DAI was calculated from the scores in the 3 parameters above. DAI is an index of IBD severity.

**5.2.2.2. Molecular approach of the test compounds in DSS-induced IBD mice**

*Collection of blood and tissue samples:* On day-6, animals were fasted for 4-6 h and bled by retro-orbital plexus puncture. Blood was collected in two separate tubes for plasma separation (10 % K$_2$EDTA) and hematological evaluation. Plasma was separated by
centrifugation (10,000 RPM, 4 °C, for 8 min) and stored at -80 °C for future analysis. Hematological parameters were estimated by using veterinary blood cell counter. After blood collection, mice were euthanized and dissected, colon and spleen were isolated. With forceps, the colons were carefully pulled out up to cecum. The colons were cut at ileocecal junction (to separate it from small intestine) and at distal end of rectum. Then cecum was separated. Colon was flushed immediately with cold PBS using a 10-ml syringe to remove feces and blood. Colon length, weight, and spleen weight were recorded (Verma et al., 2014, Chassaing et al., 2014). Colon was cut into 3 pieces. The proximal and middle portion of the colon were frozen immediately for MPO/nitrite estimation and for estimation of IL-6 and TNF-α by ELISA respectively. The distal section of the colon was sent for histopathological analysis (stored in 10 % formalin buffer saline and submitted to histopathology lab). Remaining part was stored in PBS at -80 °C.

5.2.2.2.1. MPO and nitrite estimation in colon homogenate of DSS-induced IBD mice
For homogenization, the colon tissues were weighed and RIPA-buffer was added (five times of colon tissue weight) and homogenized using tissue homogenizer. The homogenate was centrifuged (5000 RPM, 10 min, 4 °C) and supernatant was collected. MPO (Bradley et al., 1982, Verma et al., 2014) and nitrite (Marcocci et al., 1994, Mathew et al., 2013) were estimated in the supernatant as previously described in the section 4.2.5 of chapter-4.

5.2.2.2.2. TNF-α and IL-6 estimation in colon homogenate of DSS-induced IBD mice
To measure the cytokine (TNF-α and IL-6) levels, colon homogenate was prepared separately (Verma et al., 2014). Colon tissues were weighed and homogenized using mortar pestle by adding liquid nitrogen. Cell lytic buffer (100 µl) was added and mixed thoroughly in the mortar. Homogenate was collected and centrifuged (5000 RPM, 10 min, 4 °C). Total protein estimation was carried out using BCA kit. IL-6 and TNF-α were estimated using ELISA kit method, by following manufacturer’s instruction.

5.2.2.3. Statistical analysis, in DSS-induced IBD mice. Statistical analysis was performed by using Prism 6.01 Software (trial version) (GraphPad Software Inc., La Jolla, CA, USA). Data are represented as mean ± SEM and evaluated statistically using one-way ANOVA and two-way ANOVA followed by Dunnett’s multiple comparison test where p<0.05 was considered statistically significant.
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5.2.3. Evaluation of test compounds against CFA-induced arthritis

5.2.3.1. Treatment regimen for the test compounds, in CFA-induced arthritis

Based on body weight, SD rats were randomized into different groups (six rats per group, total 15 groups) into different treatments (Table 4.3.3). AIA control group was administered with an intra-plantar injection of 0.1 ml of 5 mg/ml CFA, to the left hind paw and naive control group received 0.1 ml paraffin oil. Treatment groups received CFA along with test compounds (HMPH/HMBH/HPID/HPID-1). All the groups were dosed with test compounds and standard (diclofenac) from day-8 to day-21 (once daily, total 14 days). The details of doses administered is shown below in the table 5.3.3.

Test compounds/standard treatment regimen in CFA-induced arthritis

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatments</th>
<th>No. of Rats</th>
<th>Group #</th>
<th>Treatments</th>
<th>No. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naïve control</td>
<td>Six</td>
<td>10</td>
<td>HPID, 12.5 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>2</td>
<td>AIA control</td>
<td>Six</td>
<td>11</td>
<td>HPID, 25 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>3</td>
<td>Diclofenac, 10mg/kg, PO, OD</td>
<td>Six</td>
<td>12</td>
<td>HPID, 50 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>4</td>
<td>HMBH, 12.5 mg/kg, IP, OD</td>
<td>Six</td>
<td>13</td>
<td>HPID-1, 6.25 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>5</td>
<td>HMBH, 25 mg/kg, IP, OD</td>
<td>Six</td>
<td>14</td>
<td>HPID-1, 12.5 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>6</td>
<td>HMBH, 50 mg/kg, IP, OD</td>
<td>Six</td>
<td>15</td>
<td>HPID-1, 25 mg/kg, IP, OD</td>
<td>Six</td>
</tr>
<tr>
<td>7</td>
<td>HMPH, 12.5 mg/kg, IP, OD</td>
<td>Six</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>HMPH, 25 mg/kg, IP, OD</td>
<td>Six</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>HMPH, 50 mg/kg, IP, OD</td>
<td>Six</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3.3. This table represents the treatment regimen. On day-1, CFA (5 mg/ml, 0.1 ml) was injected to sub-plantar region of all rats except the naïve control group which received paraffin oil. Based on the ipsilateral paw volume, on day-7, rats were randomized into 15 treatment groups. Rats were dosed with respective treatments from day-8 to day-21.
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5.2.3.2. Induction of CFA-induced arthritis and evaluation of anti-arthritic effects of test compounds

The CFA was freshly prepared with Mycobacterium butyricum as suspension in paraffin oil. On day 1, SD male rats were administered with an intraplantar injection of 0.1 ml of 5 mg/ml CFA, to the left hind paw (ipsilateral paw) and naive control rats received 0.1 ml paraffin oil. Body weight, clinical scoring, ipsilateral and contralateral paw (non-induction paw) volume were measured on day 1, 7, 14 and 21 and difference in the paw volume was calculated by subtracting the basal paw volume on day 1 (before the CFA injection).

The severity of arthritis was evaluated by macroscopic inspection (such as clinical scoring), radiographic changes and hematological parameters (on day 21) using veterinary blood cell counter.

Rats were sacrificed on day 22 and the spleen, thymus, brain were isolated, blotted dry, weighed and tested for inflammatory markers (discussed in section 5.2.3.5). Serum MPO (performed as described in section 4.2.5 of chapter-4), serum C-RP, RF and TNF-α were estimated as per manufacturer instructions (Talwar et al., 2011, Balague et al., 2012, Page et al., 2010).

5.2.3.3. Clinical scoring and evaluation of arthritis development, in CFA-induced arthritis

From day 7 onwards, arthritis was evaluated by macroscopic parameters such as the ability to walk, redness and swelling of skin at ankle and wrist joints. The intensity of arthritis was scored by grading each paw from 0 to 4 based on erythema, swelling and deformity of joints (van Eden et al., 2001).

0 = No erythema or swelling
1 = Slight erythema or swelling of one of the toes or fingers
2 = Erythema and swelling of more than one toe or finger
3 = Erythema and swelling of the ankle or wrist
4 = Complete erythema and swelling of toes or fingers and ankle or wrist and inability to bend the ankle or wrist.

Example - All 4 legs are scored, so the highest possible arthritic index is 16.

5.2.3.4. Radiological and histopathological analysis, in CFA-induced arthritis

The radiological alterations were examined for severity of arthritis on day 21 at Oral Medicine and Radiology, Kasturba Medical College, Manipal University. The rats were anesthetized and radiographs of the ipsilateral and contralateral paws were taken using x-
ray. The machine was operated at 60 kV peak, 8 mA, exposure time was 0.08 sec and the focus distance was 40-50 inches) (Talwar et al., 2011).

Rats were sacrificed (day 22) by cervical dislocation. The amputated hind paw ankles were fixed in 10 % neutral-buffered formalin, decalcified in 10 % formic acid, dehydrated and then processed and embedded in paraffin. The 5 µ sections were stained with hematoxylin and eosin and evaluated in a blinded manner. Cellular infiltration, synovial hyperplasia, pannus formation, bone and cartilage erosion of the ankle joints were evaluated histopathologically (Balague et al., 2012).

5.2.3.5. Blood, spleen and thymus collection and processing, in CFA-induced arthritis
On day 21, blood was collected in centrifuge tubes with and without K2-EDTA. Plasma was separated by centrifugation (8000 RPM; 10 min, at 4 °C) using cooling micro-centrifuge and serum was separated through centrifugation (5000 RPM, 12 min). Whole blood was processed for hematological analysis before plasma/serum separation using the veterinary blood cell counter. Plasma/serum aliquots were stored in -80 °C before estimations.

On day 22, rats were sacrificed and the spleen, thymus, brain were isolated, blotted dry, weighed and tested for inflammatory markers (Balague et al., 2012).

Inhibition of splenomegaly was determined by the formula below:

\[ \text{Splenomegaly} = \frac{\text{spleen weight}}{\text{body weight}} \]

Inhibition of thymus atrophy was determined by the formula:

\[ \text{Thymus atrophy} = \frac{\text{thymus weight}}{\text{respective brain weight of the same rat}} \]

5.2.3.6. Statistical analysis, in CFA-induced arthritis. Statistical analysis was performed by using Prism 6.01 Software (trial version) (GraphPad Software Inc., La Jolla, CA, USA). Data are represented as mean ± SEM and evaluated statistically using one-way ANOVA and two-way ANOVA followed by Dunnett’s multiple comparison test where p<0.05 was considered statistically significant.
5.3. Tables and Figures

Effect of test compounds on body weight loss, in DSS-induced IBD mice

Fig. 5.3.4. IBD was induced in male BALB/c mice with 4% DSS in drinking water for 6 days. The above graphs present body weight changes (from day-1 to day-6) in different treatment groups. Data presented as mean ± SEM (n=6). p<0.05 is statistical significant (two-way ANOVA followed by Dunnett’s multiple comparison test).
Fig. 5.3.5. IBD was induced in male BALB/c mice with 4 % DSS in drinking water for 6 days. IBD was assessed by scoring the stool consistency, haem-occult and % decrease in body weight. Data presented as mean ± SEM (n=6). $ p<0.05$ vs naïve control. ★ $ p<0.05$ vs DSS control (two-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of test compounds on haem-occult, in DSS-induced IBD mice

Fig. 5.3.6. IBD was induced with 4 % DSS (day-0 to day-5) in all treatment groups except naïve control. IBD was assessed by scoring stool consistency, haem-occult and % decrease in body weight. Occult blood in stool was scored by using hemospot kit. Data presented as mean ± SEM (n=6). $ p<0.05$ vs naïve control. ★ $p<0.05$ vs DSS control (two-way ANOVA followed by Dunnett’s multiple comparison test).
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Effect of test compounds on DAI in DSS-induced IBD mice

Fig. 5.3.7. IBD was induced with 4% DSS (day-0 to day-5) in all treatment groups except naïve control. IBD was assessed by scoring stool consistency, haem-occult and % decrease in body weight. DAI was calculated from the average scores of 3 parameters namely stool consistency, haem-occult and % decrease in body weight. Data presented as mean ± SEM (n=6). $ p < 0.05$ vs naïve control. ★ $p<0.05$ vs DSS control (two-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of test compounds on colon length and weight in DSS-induced IBD mice

Table 5.3.8. IBD was induced with 4% DSS (day-0 to day-5) in all treatment groups except naïve control. Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Cecums were separated, immediately colons were flushed with chilled PBS. The colon weight and length were measured. Data presented as mean ± SEM (n=6). ★ p<0.05 vs naive control (one-way ANOVA followed by Dunnett’s multiple comparison test).
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Effect of test compounds on colon MPO in DSS-induced IBD mice

Fig. 5.3.9. IBD was induced with 4% DSS (day-0 to day-5) in all treatment groups except naïve control. Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Immediately colons were flushed with chilled PBS. Colon were cut into 3 pieces, proximal portion was frozen immediately for MPO estimation. Colon tissue weighed, homogenized with RIPA buffer, centrifuged (5000 rpm, 10 min, 4 °C) and MPO in the supernatant was estimated as described in section 4.2.5 of chapter-4. Data presented as mean ± SEM (n=6). ★ p<0.05 vs DSS control (one-way ANOVA followed by Dunnett’s multiple comparison test).
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**Effect of test compounds on colon nitrite in DSS-induced IBD mice**

Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Immediately colons were flushed with chilled PBS. Colon were cut into 3 pieces, proximal portion was frozen immediately for nitrite estimation. Colon tissue weighed, homogenized with RIPA buffer, centrifuged (5000 rpm, 10 min, 4 °C) and nitrite in the supernatant was estimated as described in section 4.2.5 of chapter-4. Data presented as mean ± SEM (n=6). ★ p<0.05 vs DSS control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Fig. 5.3.11. IBD was induced with 4% DSS (day-0 to day-5) in all treatment groups except naïve control. Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Immediately colons were flushed with chilled PBS. Colon were cut into 3 pieces, middle portion was frozen immediately for TNF-α estimation. Colons were weighed, homogenized with liquid nitrogen, mixed thoroughly with cell lytic buffer (100 µl), and centrifuged (5000 rpm, 10 minutes, 4 °C). Total protein was estimated and TNF-α was estimated by ELISA method. Data presented as mean ± SEM (n=6). $ p<0.05 vs naïve control. ★ p<0.05 vs DSS control (one-way ANOVA followed by Dunnett’s multiple comparison test).
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Effect of test compounds on colon IL-6 in DSS-induced IBD mice

Fig. 5.3.12. IBD was induced with 4 % DSS (day-0 to day-5) in all treatment groups except naïve control. Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Immediately colons were flushed with chilled PBS. Colon were cut into 3 pieces, middle portion was frozen immediately for IL-6 estimation. Colons were weighed, homogenized with liquid nitrogen, mixed thoroughly with cell lysate buffer (100 µl), and centrifuged (5000 rpm, 10 minutes, 4 °C). Total protein was estimated and IL-6 was estimated by ELISA method. Data presented as mean ± SEM (n=6). $ p <0.05$ vs naïve control. ★ $ p <0.05$ vs DSS control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of test compounds on colon-histopathological impression in DSS-induced IBD mice

Naïve control

DSS control

Dexamethasone (0.1 mg/kg)

Diclofenac (10 mg/kg)

HMPH (25 mg/kg)

HMPH (50 mg/kg)

Continue in next page.
Fig. 5.3.13. IBD was induced with 4% DSS (day-0 to day-5) in all treatment groups except naïve control. Test compounds were administered from day-1 to day-5. On day-6, after 4-6 h of fasting, mice were sacrificed, colons were isolated, cut from ileo-cecal junction to distal end of rectum. Immediately colons were flushed with chilled PBS, cut into 3 pieces and distal section stored in 10% formalin buffer saline and submitted for histopathological examination. Arrows (→) indicate epithelial erosion, asterisks (*) indicate immune cell infiltration and hash (#) indicates area of goblet cell depletion and distortion of crypt architecture (representative samples of each treatment group).
Effect of test compounds on body weight in CFA-induced arthritis

Fig. 5.3.14. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. Body weight, clinical score, ipsilateral and contralateral paw inflammation were measured on day 1, 7, 14 and 21. Data presented as mean ± SEM (n=6). $p<0.05$ vs naïve control ★$p<0.05$ vs AIA control (two-way ANOVA followed by Dunnett’s multiple comparison test).
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Effect of test compounds on clinical scoring in CFA-induced arthritis

Fig. 5.3.15. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. Body weight, clinical score, ipsilateral and contralateral paw inflammation were measured on day 1, 7, 14 and 21. In clinical scoring, the macroscopic parameters such as the ability to walk, redness and swelling of skin at ankle and wrist joints were scored (section 5.2.3.3). Data presented as mean ± SEM (n=6). $p<0.05$ vs naïve control $\$ p<0.05$ vs AIA control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of test compounds on ipsilateral paw inflammation in CFA-induced arthritis

Fig. 5.3.16. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. Ipsilateral paw inflammation were measured on day 1, 7, 14 and 21. Data presented as mean ± SEM (n=6). $p<0.05$ vs naïve control $\star p<0.05$ vs AIA control (two-way ANOVA followed by Dunnett’s multiple comparison test).
Fig. 5.3.17. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. Contralateral paw inflammation were measured on day 1, 7, 14 and 21. Data presented as mean ± SEM (n=6). $p<0.05$ vs naïve control ★p<0.05 vs AIA control (two-way ANOVA followed by Dunnett’s multiple comparison test).
Fig. 5.3.18. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. On day-21, blood was collected, (a) WBC, (b) lymphocytes, (c) monocytes and (d) granulocytes were estimated using Veterinary Blood Cell Counter. Data presented as mean ± SEM (n=6). *p<0.05 vs AIA control (one-way ANOVA followed by Dunnett’s multiple comparison test).
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**Effect of test compounds on C-RP, RF, TNF-α and thymus atrophy in CFA-induced arthritis**

![Graphs](image)

Fig. 5.3.19. Male SD rats, (randomized based on body weight) were divided into 15 groups. On day-1, freshly prepared CFA was administered to sub-plantar region of left hind paw (ipsilateral) of all rats except naïve control which received liquid paraffin oil. On day-7, rats were again randomized based on ipsilateral paw inflammation to 15 treatment groups (Table 5.3.3) and test compounds/standard administered from day-8 to day-21. On day-21, blood was collected, plasma/serum were separated and stored in -80 °C for future estimations. The above graphs represent: serum C-RP (panel-a), serum RF (panel-b), serum TNF-α (panel-c) and thymus atrophy (panel-d). Data presented as mean ± SEM (n=5). $p<0.05$ vs naïve control, ★$p<0.05$ vs AIA control (one-way ANOVA followed by Dunnett’s multiple comparison test).
Effect of selected test compounds on radiographic impression in CFA-induced arthritis

Fig. 5.3.20. Radiographic impressions of ipsilateral and contralateral paws of CFA-induced arthritic paws on day 21 (representative samples of each treatment group).
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Effect of selected test compounds on histopathological status in CFA-induced arthritis
Fig. 5.3.21. Black arrows indicate synovial joint surface with joint space and synovium. Blue arrow indicates the presence of dense inflammatory cells (lymphocytes and plasma cells). Red arrow depicts mild joint destruction (representative samples of each treatment group).
5.4. Results and discussion

5.4.1. Effect of selected test compounds in DSS-induced IBD mice

5.4.1.1. Effect of selected test compounds on DAI, colon weight and colon length of DSS-induced IBD mice

DSS treatment in mice generates symptoms that are similar to ulcerative colitis in humans. Symptoms such as weight loss, diarrhoea, bloody faeces, mucosal ulceration and shortening of colon are seen in humans suffering from ulcerative colitis. DSS carries a highly negative charge contributed by sulfate groups and is toxic to the colonic epithelia, and induce erosions and epithelial permeability. DSS is also an anticoagulant that aggravates intestinal bleeding (Lee and Lim, 2007, Verma et al., 2014). In our study, we experienced similar conditions in DSS control.

HMPH (25 mg/kg) improved the body weight (Fig. 5.3.4 b) from day-3 to day-6. However HMBH, HPID HPID-1, diclofenac and dexamethasone did not show any improvement in body weight (Fig. 5.3.4).

HMPH (25 mg/kg), HMBH (50 mg/kg), HPID (50 mg/kg) and HPID-1 (25 mg/kg) significantly diminished diarrheal symptoms from day-3 to day-6 (Fig. 5.3.5). However, the standards (diclofenac and dexamethasone) did not decrease the stool consistency score at all.

HMPH (25 mg/kg), HMBH (50 mg/kg), HPID (25 mg/kg) and HPID-1 (12.5 mg/kg) significantly decreased the occult blood in stool (Fig. 5.3.6).

HMPH (25 mg/kg), HMBH (50 mg/kg), HPID (25 mg/kg) and HPID-1 (12.5 mg/kg) decreased DAI which was significantly high in the control (Fig. 5.3.7).

To sum up, HMPH (25 mg/kg) consistently decrease the DAI, even more prominently than diclofenac and dexamethasone. On the other hand HMBH (50 mg/kg), HPID (25 mg/kg) and HPID-1 (12.5 mg/kg) decreased DAI, though not effectively as HMPH.

Colon length and weight (Fig. 5.3.8) was significantly decreased in DSS control. This could be due to acute inflammation in colon tissue (Chassaing et al., 2014). All the test compounds improved the colon length and weight but not significantly.

Enlargement of spleen correlates with intensity of inflammation and anemia (Chassaing et al., 2014). None of the test compounds, at the doses tested, decreased spleen weight significantly (data not shown).

5.4.1.2. Effect of selected test compounds on colon MPO and nitrite in DSS-induced IBD mice

Cytotoxicity of NO is implicated in the pathology of IBD, but evidence is conflicting on whether it is harmful or beneficial (Salas et al., 2002).
We found a significant increase in nitrite in colon homogenate of DSS control. HMPH (25 and 50 mg/kg), HMBH (25 and 50 mg/kg), HPID (25 and 50 mg/kg), HPID-1 (12.5 and 25 mg/kg) significantly attenuated the rise in nitrite in the colon homogenate (Fig. 5.3.10). Increased MPO in colon homogenate may be due to neutrophil infiltration into the colonic tissue (Chassaing et al., 2014). We observed a significant increase in MPO concentration in colon homogenate, which was significantly attenuated by HMPH (25 and 50 mg/kg), HMBH (25 and 50 mg/kg), HPID (25 and 50 mg/kg), HPID-1 (12.5 and 25 mg/kg) and diclofenac (Fig. 5.3.9). Test compounds were superior to dexamethasone, which did not significantly decrease MPO. Our test compounds, probably demonstrate an immunomodulatory action in DSS-colitis model.

5.4.1.3. Effect of selected test compounds on colon TNF-α and IL-6 in DSS-induced IBD mice
All these mediators actively contribute to the pathogenic cascade that initiates and perpetuates the inflammatory responses in the gut (Verma et al., 2014). IBD is accompanied by heightened synthesis and release of several pro-inflammatory mediators such as ROS, nitrogen metabolites, eicosanoids, platelet-activating factor and cytokines such as TNF-α, IL-6 and IL-1 (Sartor, 1997b, Podolsky, 2002, Kinoshita et al., 2006). HMPH (25 and 50 mg/kg), HMBH (50 mg/kg), HPID (25 and 50 mg/kg), HPID-1 (12.5 and 25 mg/kg) and diclofenac significantly attenuated the increased TNF-α in colon homogenate (Fig. 5.3.11). HMPH (25 and 50 mg/kg) and HPID (25 mg/kg), also significantly decreased the raised IL-6 in colon homogenate (Fig. 5.3.12).

5.4.1.4. Effect of test compounds on histopathological status, in DSS-induced IBD
There was a profound increase in histopathological scores in DSS control mice (Fig. 5.3.13). Epithelial erosion, neutrophils infiltration, goblet cell depletion and distortion of crypts are represented in figure 5.3.13. Among all the tested compounds, HMPH (25 and 50 mg/kg) and HPID (50 mg/kg) showed a significant improvement in histopathological features. Dexamethasone and diclofenac were not effective in improving histopathological scores. Diclofenac showed poor performance probably because COX-1/COX-2 inhibition interfered with homoeostasis. Dexamethasone, on the other hand, probably needed dose titration for better activity. Previous reports have shown that dexamethasone at both lower and higher doses can improve colitis symptoms (van Meeteren et al., 2000, Li et al., 2015). Better results with dexamethasone was perhaps possible if a wide range of doses were tested and the best dose was employed in our model.
5.4.2. Effect of selected test compound on CFA-induced arthritis

HMPH, HMBH, HPID and HPID-1 demonstrated anti-inflammatory activity in acute and sub-acute *in vivo* models of inflammation, by attenuating acute paw inflammation, leukocyte-infiltration (in air-pouch lavage and BAL), limiting TNF-α in air-pouch lavage and reducing TNF-α/IL-6 in IBD-colon homogenate. We thought it appropriate to consider an immune-mediated anti-inflammatory mechanism of our test compounds in chronic inflammation. Arthritis in rats was chosen because it has components of both local and systemic inflammation (Balague et al., 2012, Fonseca et al., 2009). CFA-induced arthritis pathology resembles human arthritis, involving cartilage destruction, bone resorption and bone formation, which are easily noticeable in our study (Bendele, 2001). Our test compounds attenuated acute and chronic inflammation and facilitated recovery as measured by decreasing paw inflammation, decreasing arthritic markers, increasing clinical scoring along with improving bone joint damage. The results are discussed below.

5.4.2.1. Effect of test compounds on body weight, clinical scoring and paw inflammation in CFA-induced arthritis

HMPH (at all doses), HPID (12.5 mg/kg, lowest dose) and HPID-1 (6.25 mg/kg, lowest dose) significantly improved the body weight from day-14 onwards. On day-21, HMPH (at all doses), HMBH (12.5 and 50 mg/kg), HPID (50 mg/kg) and HPID-1 (at all doses) significantly increased body weight towards naive control (Fig. 5.3.14). This demonstrates the improvement in the local paw inflammation by our test compounds, which is comparable to standard/diclofenac.

HMPH (all doses) and HMBH (25 and 50 mg/kg) showed a significant improvement in clinical scoring from day-17 onwards, which is comparable to standard/diclofenac. On day-21, we observed a significant progress in arthritic clinical scoring in HMPH, HMBH, HPID-1 (all the doses) and standard/diclofenac (Fig. 5.3.15). HPID showed the same effect at the lowest dose (12.5 mg/kg). (Fig. 5.3.15).

HMPH (at all doses) and HMBH (at all doses) significantly attenuated ipsilateral paw inflammation from day-14 onwards (just after 7-days of dosing) which was comparable to standard/diclofenac (Fig. 5.3.16). On day-14, HPID (12.5 and 50 mg/kg) and HPID-1 (6.25 and 25 mg/kg) significantly decreased ipsilateral paw inflammation. However, all the doses of HPID and HPID-1 significantly improved ipsilateral paw inflammation by the end of the study (day-21).
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HMPH, HMBH, HPID and HPID-1 (at all doses) significantly decreased contralateral paw inflammation on day-21 (Fig. 5.3.17) indicating that our test compounds are effective against both acute and chronic inflammation as well as immune-mediated inflammation. As in AIA model, development of paw-inflammation is biphasic, i.e., acute phase (day 1 to 8) and chronic phase (day 9 to 21). The contralateral paw inflammation develops during 2nd week after CFA injection (Hamada et al., 2000). The improvement in ipsilateral and contralateral paw inflammation may demonstrate the anti-arthritic and immunomodulating activity.

5.4.2.2. Effect of test compounds on neutrophil infiltration in CFA-induced arthritis
Total WBC count was restored towards normal levels only by HPID (50 mg/kg). However, lymphocyte count was improved by HMBH (12.5 mg/kg), HMPH (50 mg/kg), HPID (at all doses) and HPID-1 (at all doses) (Fig. 5.3.18). Granulocyte and monocyte counts were not significantly improved by any of the test compounds. However, Diclofenac reversed the total and differential cell count (Fig. 5.3.18). AIA is characterized by leukocytosis (lymphocytosis, monocytosis and granulocytosis), with extensive systemic neutrophilia (Balague et al., 2012). The corrections in hematological parameters demonstrate probable immuno-modulatory activity.

5.4.2.3. Effect of test compounds on serum C-RP, RF, TNF-α and thymus atrophy, in CFA-induced arthritis
C-RP is an acute-phase protein produced by hepatocytes that is elevated during tissue trauma, bacterial infections and inflammatory reactions in cartilage and bone. C-RP typically rises in conditions like rheumatoid arthritis (Talwar et al., 2011, Balague et al., 2012). There was nearly 4 fold increase in serum C-RP in AIA-control (Fig. 5.3.19 a), demonstrating the validity of the model which we employed. Interestingly, diclofenac, produced a 7 fold increase in C-RP (Salgia et al., 2015), which is even greater than the C-RP levels of AIA-control (Fig. 5.3.19 a).
We found that our test compounds, especially HPID (25 mg/kg) and HPID-1 (25 mg/kg) significantly reduced C-RP. Other test compounds did not affect C-RP levels. More surprisingly, HPID at higher doses (50 mg/kg) significantly increased C-RP (Fig. 5.3.19 a). We also found that lower doses of HPID (25 mg/kg), which did not raise the C-RP levels produced greater therapeutic effect in DSS-induced IBD. We also observed that diclofenac did not reverse the symptoms of DSS-induced IBD. The DAI of diclofenac treated animals was similar to that of DSS control. The poor response to diclofenac as against the significant
action by HPID and HPID-1 suggests that the mechanism of anti-inflammatory action of the two compounds differ substantially (McCarty, 2004). This difference could at least partly be attributed to the AMPK activating action of HPID and HPID-1, particularly at lower doses. The suppression of C-RP by HPID and HPID-1 is not surprising because previous reports have already suggested that AMPK activation brings down C-RP. There are also reports suggest that IL-6 plays a role in regulating C-RP (McCarty, 2004, van Erk et al., 2010). HPID (25 mg/kg) was also the most potent in reducing IL-6 among the test compounds in DSS-induced IBD.

In the present study there was more than 3 fold increase in RF in AIA-control (Fig. 5.3.19 b) implying a greater-probability of articular destruction with suspected autoimmune disease (Talwar et al., 2011). HMPH (50 mg/kg) and diclofenac significantly attenuated serum RF. HPID (25 mg/kg) and HPID-1 (at all doses) significantly decreased the serum RF (Fig. 5.3.19 b).

TNF-α levels being lower in serum than in inflammatory tissues, we were not surprised to find that TNF-α levels were below detectable limits in our experiment (Fig. 5.3.19 c) (Mudgal et al., 2014). We observed an increase in TNF-α in all the test compounds (at all doses) and diclofenac treated arthritic animals (Fig. 5.3.19 c). However, the TNF-α enhancing property of all test compounds and diclofenac suggests that both test compounds and diclofenac also share some mechanistic elements (Page et al., 2010). As NF-κB operates downstream AMPK activation, it is possible that both test compounds and diclofenac share some mechanistic aspects along downstream pathways in inflammation.

Previous reports have shown that elevation of TNF-α by NSAIDs is reversed either by addition of exogenous PGE2 or by a PGE2 EP2 receptor agonist. Therefore, inhibition of PGE2 by NSAIDs is probable reason for the excessive serum TNF-α levels in diclofenac treated rats. Our compounds have also increased TNF-α levels, suggesting a possible role mediated via PGE2/PGE2 EP2 receptor (Page et al., 2010). This is also a hint about NSAIDs and our compounds sharing some mechanistic elements in the suppression of inflammation, especially on downstream pathways of inflammation.

5.4.2.4. Effect of test compounds on radiographic and histopathological status, in CFA-induced arthritis
HMPH, HPID and diclofenac treated rats showed lower levels of soft tissue swelling, cystic enlargement of bone, extensive erosion and bone destruction as per the radiographic impression and histopathological study. The differences were substantial when compared to AIA-control. The efficacy of HMPH and HPID are comparable to diclofenac.
5.5. Conclusion
HMPH, HPID and HPID-1 (at lowest dose) and HMBH (at highest dose) decreased DAI. Among all test compounds, HMPH was consistently the most effective on DAI. All the test compounds increased colon length, weight and improved splenomegaly but not significantly. All the test compounds significantly attenuated nitrite and MPO in colon homogenate, demonstrating an immune-protective effect. Similarly, the test compounds were also effective in reducing colon-TNF-α levels. However, only HMPH and HPID were able to attenuate colon-IL-6 level and rectify epithelial damage of colon effectively. Body weight and clinical scoring were improved by our test compounds in CFA-induced arthritis. The ipsilateral and contralateral paw inflammations were attenuated by all the test compounds, implying significant anti-inflammatory action along with immunomodulatory property. Immunoprotective property was confirmed with the normalization of lymphocyte count. HPID and HPID-1 decreased serum C-RP in arthritis rats, highlighting their IL-6 reducing action, probably mediated by AMPK-activation and downstream NF-kB inhibition. Decrease in serum RF produced by HMPH, HPID and HPID-1 reflect in improved radiographical images. The rise in serum TNF-α in arthritic rats, induced by all the test compounds and diclofenac, demonstrate some probable mechanistic elements in common. Interestingly, the therapeutic effect of the some test compounds, especially HMPH and HPID were comparable to diclofenac in arthritic rats. All the test compounds presented its therapeutic value against DSS-induced IBD and CFA-induced arthritis. HMPH and HPID were the most effective.

5.6. References
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