Summary

The test compounds found effective in models of acute inflammation were evaluated in experimental models of chronic immune-mediated inflammatory conditions involving different T_H cells, such as colitis, arthritis and allergy. The efficacy of test compounds in TNBS-induced colitis indicates their therapeutic potential in T_H1 and T_H17-mediated conditions. In another model involving T_H2 and T_H17 cells viz. OVA-induced cutaneous anaphylaxis, DMFO significantly reduced dye extravasation, while, DHFO and DHPO reduced serum IgE levels significantly. In adjuvant-induced arthritis model, which stimulates T_H1 and T_H17 cell polarization, the test compounds showed variable action. In the contralateral paw, only DMFO significantly reduced paw oedema, while DHPO and DHFO increased pain threshold. On the other hand, in the ipsilateral paw, DMFO and DHFO improved pain threshold. The efficacy of test compounds in different models of chronic inflammation, involving different subsets of T_H cells, suggest that these compounds may be interfering with T_H cell polarization and is worth further evaluation.

4.1 Introduction

Chronic inflammation, which invokes the adaptive immune response, characterizes several diseases such as RA, CV diseases, cancer, pulmonary diseases, allergies etc (Weiss, 2008, Reuter et al., 2010). Immune-mediated chronic inflammation involves several important T_H cells, chiefly, T_H1, T_H2, T_H17, T_reg, etc (Yang and Gao, 2011), which reciprocally regulate one another (Kitching and Holdsworth, 2011).

IBD, chronic relapsing inflammatory conditions of the GIT, include two diseases viz. Ulcerative colitis (UC) and Crohn’s disease (CD). UC is a superficial continuous colonic mucosal inflammation, while CD is a transmural discontinuous GIT inflammation involving the entire intestinal wall. CD is T_H1-mediated, while UC is T_H2-mediated. T_H17 is also involved in the pathogenesis of IBD in general, as observed by T_H17-specific cytokines in the intestinal mucosa of both patients and animal models of IBD (Liu et al., 2009). The success of monoclonal antibodies against TNFα (infliximab) in IBD has resulted in a search for novel drugs especially those that target cytokines (Chaparro et al., 2012).
RA is a chronic relapsing and remitting inflammatory/autoimmune disorder (Sheibanie et al., 2007), involving the T helper subtypes $T_H1$ and $T_H17$ (Chen et al., 2010), resulting in a symmetric pattern of inflammation affecting multiple joints, coupled with systemic manifestations. There is pain, stiffness, swelling, warmth and redness and in severe cases, loss of joint shape and function. Literature reports that $T_H1$ and $T_H17$ are major players in RA (Schulze-Koops and Kalden, 2001). IL17 plays an important role in the development and progression of RA (Sheibanie et al., 2007). TGFβ and IL6 promote $T_H17$ differentiation, while IL23 is essential for maintaining the $T_H17$ phenotype. IL17 induces neutrophil proliferation and release of various chemokines and other inflammatory mediators from immune cells (Sheibanie et al., 2007). COX2 is also upregulated in the arthritic synovium (Sheibanie et al., 2007).

Allergy, an immediate (Type I) hypersensitivity reaction (Rang et al., 2003), has diverse manifestations. The incidence of allergic diseases is rising in developed countries (Arnold et al., 2011). $T_H$ cells, namely, $T_H2$ and $T_H17$ cells are involved in allergen-specific immune responses (Maggi, 2010, Arnold et al., 2011). Antigen-induced MC degranulation is a receptor-dependent process. Allergen cross-links IgE bound to FceR1 on MCs or basophils, activating several downstream protein kinases, leading to a signalling cascade that activates RAS-MAPK, PLCγ and PI3K pathways, finally resulting in calcium mobilization and degranulation (Siraganian, 2003). ROS also plays an active role in MC degranulation (Suzuki et al., 2005).

As the test compounds were found to be effective in acute models of inflammation without being ulcerogenic, we evaluated them in chronic immune-mediated inflammatory conditions such as colitis, arthritis and allergy, which involve differential polarization of $T_H$ cells. As $T_H$ cell subtypes ($T_H1$, $T_H2$, $T_H17$, $T_{reg}$, etc) are reciprocally cross-regulated between themselves,
the modulation of T<sub>H</sub> cell polarization could be a rational strategy for T<sub>H</sub> cell-dominated disorders (Bhattacharya et al., 2014, Bosnjak et al., 2011, Xiao et al., 2014, Chiang et al., 2007). Drugs targeting T cell subtypes are only in the early stages of development (Chen et al., 2010, Heijink and Van Oosterhout, 2005). However, there has not been much clinical success with this approach (Malmstrom et al., 2005), chiefly because monoclonal antibodies against T<sub>H</sub> cells in arthritis either failed (Tak et al., 1995) or significantly depleted T cells, resulting in severe adverse effects (Isaacs et al., 2001). T<sub>H</sub>1/T<sub>H</sub>2 balance has to be modulated with caution because T<sub>H</sub>1 is important in cell-mediated immunity and T<sub>H</sub>2 protects against larger parasites (Rockwell et al., 2012, Bhattacharya et al., 2014). Moreover, induction of T<sub>H</sub>2 predisposes to conditions such as atopy (Rockwell et al., 2012). Thus, there is a need for better agents that modulate T cell activation appropriately (Malmstrom et al., 2005).

4.1.1 Objectives:

The specific objectives of the present study were to evaluate the effect of test compounds in the following:

- TNBS-induced colitis (T<sub>H</sub>1 and T<sub>H</sub>17-involvement)
- Ovalbumin-induced active cutaneous anaphylaxis (T<sub>H</sub>2 and T<sub>H</sub>17-involvement)
- Freund’s complete adjuvant-induced arthritis (T<sub>H</sub>1 and T<sub>H</sub>17-involvement)

4.2 Materials and methods

4.2.1 Chemicals

2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), ovalbumin and aluminium hydroxide were from Sigma-Aldrich Co. LLC., USA, Difco™ Freund Adjuvant (5 mg/ml, Mycobacterium butyricum, catalog no. 264010) was from Difco Laboratories, Detroit, MI, USA). ELISA kits rat TNF-α (Invitrogen Corporation, Carlsbad, CA, USA), mouse IL-6 (Krishgen Biosystems, Mumbai, MH, India) and mouse IgE (GenWay Biotech, Inc, San
Diego, CA USA) kits were used. Ketotifen fumarate and diclofenac sodium were gift samples from Micro Labs Ltd, Bangalore, KA, India and Hetero Drugs Ltd., Hyderabad, AP, India, respectively. All other reagents and solvents used were of analytical grade.

4.2.2 Instruments

Automated intraoral X-ray processor (Medivance Instruments Limited, Harlesden, London), cooling microcentrifuge (MIKRO 22R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), Analgesy-Meter (Catalog No. 37215, UGO Basile, Srl, Comerio, VA, Italy)

4.2.3 Experimental Animals

Female BALB/c mice 6-8 week old (20-25 g) were used for TNBS-induced colitis and ovalbumin-induced cutaneous anaphylaxis models. Male Sprague-Dawley rats (180-220 g) were used for Freund’s adjuvant-induced arthritis studies. Experimental animals were maintained as in Section 3.2.2. The Institutional Animal Ethics Committee (IAEC), KMC, Manipal scrutinized and cleared (Ref No. IAEC/KMC/50/2011-2012) the experiment protocol. Dose selection was based on Section 3.2.5.

4.2.4 TNBS-induced colitis in mice

Mice were grouped and fasted overnight with water ad libitum (n=5) (Wirtz et al., 2007, Butzner et al., 1996, Billerey-Larmonier et al., 2008).

Group 1: 0.25% CMC (Saline Control)

Group 2: Sulfasalazine (100mg/kg p.o)

Group 3: DHPO (10mg/kg p.o)

Group 4: DHFO (200mg/kg p.o)

Group 5: DMFO (200mg/kg p.o)
Group 6: DMPI (200mg/kg p.o)

2% TNBS solution was prepared using 5% 2,4,6-TNBS (Sigma) and absolute alcohol as vehicle (Billerey-Larmonier et al., 2008). On day 1, 100 μL of 2%TNBS solution was administered intra-rectally under anesthesia using polyethylene cannula fitted to a 1mL syringe. The solution was administered slowly into the colon, 3 cm proximal to the anus, followed by a fixed volume of air (0.1mL) to ensure that the TNBS solution is completely removed from the cannula. The cannula was removed gently and the animal was kept in a head-down position for 1 minute, to ensure that TNBS solution remains in the colon. The normal control group received an equal volume of 0.9% saline. The body weight was recorded daily. On day 8, 100 μL of 2%TNBS solution was administered intrarectally. On days 8-11, animals were dosed once daily. On day 11, animals were weighed and sacrificed, colon was excised and the luminal contents were removed by flushing with sterile PBS at 37°C. Colon weight (mg) and length (cm) were determined and represented as mean of colon weight/length (mg/cm) ± SEM. The change in body weight during the study period is represented as mean decrease in body weight (day 0-11). Proximal colon was divided into two parts: one part was homogenized in sterile cold saline followed by centrifugation at 10000 rpm at 4°C for 10 minutes using cooling microcentrifuge and stored at -80 °C and used for estimating tissue levels of MPO, TBARS and IL6, the other part was used for colon damage assessment by histopathology.

The MPO levels in colonic tissue homogenate were determined as in Section 3.2.6. The result is expressed as mean % MPO ± SEM. Previously reported method was employed to determine the extent of lipid peroxidation as TBARS (Mihara and Uchiyama, 1978). Briefly, to 500μL of tissue homogenate, 500μL of TBA-TCA-HCl reagent (15% w/v TCA, 0.375%w/v TBA, 0.8% w/vBHT) was added and heated in a water bath at 80°C for 30min,
centrifuged at 10000 rpm for 10min. The absorbance of the supernatant was measured at 540nm. The result is expressed as mean % TBARS ± SEM. Commercially available ELISA kit for IL-6 was used according to manufacturers’ protocol to measure the levels in colon.

For histopathological assessment, the colon specimen was fixed in 10 % v/v neutral formalin solution and sections were prepared using conventional techniques (as in section 3.2.7) stained with eosin and hematoxylin, observed under microscope and scored (0-3) as reported in literature after slight modifications (Wirtz et al., 2007) as: 0 = no evidence of inflammation, 1 = low level of inflammation with scattered infiltrating mononuclear cells (1-2 foci), 2 = moderate level of inflammation with multiple foci, 3 = severe inflammation with transmural leucocyte infiltration.

4.2.5 Ovalbumin-induced active cutaneous anaphylaxis in mice

Active cutaneous anaphylaxis to ovalbumin was evaluated in mice (Teshima et al., 1998, Medeiros et al., 2008) by estimating Evan’s blue dye extravasation in the ear. Animals were grouped as follows (n=6):

Group 1: 0.25% CMC (Saline Control)
Group 2: Ketotifen fumarate (1mg/kg p.o)
Group 3: DHPO (10mg/kg p.o)
Group 4: DHFO (200mg/kg p.o)
Group 5: DMFO (200mg/kg p.o)
Group 6: DMPI (200mg/kg p.o)

On day 0, animals were sensitized to antigen by administering 100µL of 40µg ovalbumin in 1mg alum i.p. The normal control group received an equal volume of sterile saline. The
animals were dosed once daily orally. On day 14, the animals were anesthetized, blood withdrawn from the retro-orbital plexus, serum separated by centrifugation (6000 rpm at 25°C for 10 min) and stored at -80°C to measure IgE. The animals were then exposed to antigen by administering 20 µL of 10µg ovalbumin intradermally in ear followed by 0.3mL of 1% Evan’s blue saline solution i.v through the tail vein. After 30 min, animals were sacrificed, the ears were dissected out for measurement of extravasated dye (Yao et al., 2014).

Briefly, the ear was kept overnight in 1N KOH at 37°C. A 5:13 mixture of 0.6N phosphoric acid and acetone (5:13) was added and shaken vigorously. Following filtration, the absorbance was measured at 620nm. The amount of dye was quantified from Evan’s blue standard curve and expressed as Evan’s blue µM/mg of protein. Dye extravasation was expressed as mean ± S.E.M. The IgE in serum (ng/mL) was determined using commercially available mouse IgE ELISA kits according to manufacturer’s protocol. Serum IgE levels were expressed as mean serum IgE (ng/mL) ± S.E.M.

4.2.6 Freund’s complete Adjuvant-induced Arthritis in rats (Nagakura et al., 2003, Mudgal, 2013)

Rats were administered complete Freund’s adjuvant (100 mg of Mycobacterium butyricum triturated with 20 mL of light liquid paraffin & autoclaved for 20 min at 120°C) by intraplantar injection (0.1 mL/paw) into the right paw (ipsilateral paw) on day 0 after an initial measurement of paw volumes (ipsilateral and contralateral) using a digital plethysmometer. Normal control group received an equal volume of sterile saline. On day 14, the volumes of both ipsilateral and contralateral paws were measured. The paw oedema was calculated as change in paw volume (mL) (day14 –day0). The animals were then randomized into
treatment groups (n=6) such that the mean contralateral paw volume of each group was equal. After randomization, treatment was started once daily for 7 days from day 14 to day 21 (n=6).

Group 1: 0.25% CMC (Saline Control)
Group 2: Diclofenac Sodium (10mg/kg _p.o_)
Group 3: DHPO (10mg/kg _p.o_)
Group 4: DHFO (200mg/kg _p.o_)
Group 5: DMFO (200mg/kg _p.o_)
Group 6: DMPI (200mg/kg _p.o_)

Body weights were recorded daily and change in body weight is expressed as mean ± SEM on day 21. On day 21, the animals were anesthetized, blood withdrawn from retro-orbital plexus, and serum separated by centrifugation (6000 rpm at 25°C for 10 min) stored at -80°C. Corrected spleen weight (ratio of spleen weight to body weight) was calculated on day 21 and expressed as mean ± S.E.M. The paw oedema, is represented as mean increase in paw volume from day 0 to day 21 ± S.E.M.

Arthritis was evaluated based on (1) clinical score, (2) mobility score, and (3) joint stiffness. All scoring was performed by a blinded observer and final arthritis score was calculated as clinical score + joint stiffness – mobility score (Mudgal, 2013) and represented as mean ± S.E.M. (1) Clinical scoring for the severity of arthritis (0-4) was carried out as: 0 = no signs of arthritis, 1 = swelling and/or redness of the paw or 1 digit, 2 = 2 joints involved, 3 = >2 joints involved, 4 = severe arthritis of the entire paw and digits (Chen et al., 2010, Mudgal, 2013). (2) Mobility scoring was carried out as: 0 = no movement, 1 = crawls using fore paw,
2 = walks with protective behaviour towards both hind paws only partially using contralateral paw, 3 = walks with protective behaviour toward both hind paws, 4 = walks with protective behaviour towards ipsilateral paw only partially using the paw, 5 = walks with protective behaviour towards ipsilateral, 6 = normal walking (Nagakura et al., 2003). (3) Joint stiffness was scored as: 0 = freely moving joint, 1 = restriction of ankle movement in either bending or extension, 2 = restriction of ankle movement in both bending and extension (Nagakura et al., 2003).

Based on the method of Randall-Selitto, mechanical hyperalgesia (pain threshold) in both paws were measured using an analgesymeter. Pain threshold was assessed in terms of paw withdrawal response/attempts to increasing pressure (0-250g). The results are expressed as mean pain threshold (g) ± S.E.M. (n=6). The TNFα in serum (pg/mL) was determined using commercially available ELISA kits according to manufacturer’s protocol and results are expressed as mean ± S.E.M.

The soft tissue swelling and joint involvement in the animals were evaluated by radiography by an automated intraoral X-ray processor. Histopathological examination was carried out on the tibio-tarsal joints of both the hind paws which were dissected out, fixed in 10 % v/v neutral formalin solution, decalcified, embedded in wax and stained by H&E. The slides were observed under microscope and scored one point each for inflammatory cell infiltration (mild = 1, severe = 2), synovial hyperplasia, cartilage damage and bone resorption, pannus formation, joint space narrowing (mild = 1, severe = 2) (maximum score of 8) (Mudgal, 2013).
4.3 Results

4.3.1 TNBS-induced colitis in mice

![Graph showing change in body weight (Day 0-11) for different treatments.]

**Figure 4.1** Effect of test compounds on TNBS-induced colitis in mice

On day 1 and 8, 100 μL of an ethanolic solution of 2%TNBS was administered intra-rectally. On days 8-11, animals were dosed once daily. On day 11, animals were sacrificed and colon was excised and colon weight (mg) and length (cm) were determined. Colon segments were used for estimating tissue levels of MPO, TBARS, IL6 and histopathology.
Evaluation of test compounds in *in-vivo* models of chronic immune-mediated inflammation

Chapter 4

Figure 4.2 Histopathology of colon of TNBS-induced colitis in mice (low power)

Table 4.1 Effect of test compounds on TNBS-induced colitis in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation</th>
<th>Foci (Infiltrating mononuclear cells)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>No</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>TNBS Control</td>
<td>Moderate</td>
<td>Multiple</td>
<td>3</td>
</tr>
<tr>
<td>Sulfasalazine (100mg/kg)</td>
<td>Low</td>
<td>Scattered</td>
<td>1</td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>Low</td>
<td>Scattered</td>
<td>1</td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>Moderate</td>
<td>Scattered</td>
<td>2</td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>No</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>DMPI (200mg/kg)</td>
<td>Low</td>
<td>Scattered</td>
<td>1</td>
</tr>
</tbody>
</table>

On day 1 and 8, 100 μL of an ethanolic solution of 2%TNBS was administered intra-rectally. On days 8-11, animals were dosed once daily. On day 11, animals were sacrificed and colon was excised and colon weight (mg) and length (cm) were determined. Colon segments were used for estimating tissue levels of MPO, TBARS, IL6 and histopathology.
Evaluation of test compounds in *in-vivo* models of chronic immune-mediated inflammation

Figure 4.3 Effect of test compounds on TNBS-induced colitis in mice (contd)

On day 1 and 8, 100 μL of an ethanolic solution of 2%TNBS was administered intra-rectally. On days 8-11, animals were dosed once daily. On day 11, animals were sacrificed and colon was excised and colon weight (mg) and length (cm) were determined. Colon segments were used for estimating tissue levels of MPO, TBARS, IL6 and histopathology. Results are expressed as mean ± SEM (n=5) [*:  p < 0.05, compared to TNBS control; #: p < 0.05 compared to sulfasalazine].
Table 4.2 Effect of test compounds on TNBS-induced colitis in mice (contd)

<table>
<thead>
<tr>
<th>Group</th>
<th>Change in body weight (Day11-Day0) (g)</th>
<th>Colon weight/length (mg/cm)</th>
<th>%IL6</th>
<th>%MPO</th>
<th>%TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>2.25 ± 0.75</td>
<td>27.88 ± 1.07*</td>
<td>22.68 ± 7.37*</td>
<td>15.83 ± 9.33*</td>
<td>52.23 ± 5.17*</td>
</tr>
<tr>
<td>TNBS Control</td>
<td>0.14 ± 0.26</td>
<td>38.32 ± 3.06*</td>
<td>100 ± 0.00*</td>
<td>100 ± 14.77*</td>
<td>100 ± 6.43*</td>
</tr>
<tr>
<td>Sulfasalazine (100mg/kg)</td>
<td>0.17 ± 0.31</td>
<td>29.09 ± 0.88*</td>
<td>27.82 ± 6.62*</td>
<td>54.85 ± 3.01*</td>
<td>56.26 ± 3.68*</td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>-0.17 ± 0.79</td>
<td>33.01 ± 1.93</td>
<td>39.61 ± 9.73*</td>
<td>22.01 ± 6.06*</td>
<td>70.91 ± 10.44</td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>0.00 ± 0.32</td>
<td>32.17 ± 2.68</td>
<td>49.65 ± 15.74*</td>
<td>35.79 ± 17.29*</td>
<td>50.19 ± 11.61*</td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>0.40 ± 1.09</td>
<td>28.48 ± 1.95*</td>
<td>28.56 ± 0.16*</td>
<td>20.99 ± 6.68*</td>
<td>46.28 ± 3.40*</td>
</tr>
<tr>
<td>DMPI (200mg/kg)</td>
<td>0.67 ± 0.42</td>
<td>29.01 ± 1.91*</td>
<td>34.40 ± 0.04*</td>
<td>32.70 ± 6.17*</td>
<td>73.25 ± 11.80</td>
</tr>
</tbody>
</table>

On day 1 and 8, 100 μL of an ethanolic solution of 2%TNBS was administered intra-rectally. On days 8-11, animals were dosed once daily. On day 11, animals were sacrificed and colon was excised and colon weight (mg) and length (cm) were determined. Colon segments were used for estimating tissue levels of MPO, TBARS, IL6 and histopathology. Results are expressed as mean ± SEM (n=5) [*: p < 0.05, compared to TNBS control; #: p < 0.05 compared to sulfasalazine].
4.3.2 Ovalbumin-induced active cutaneous anaphylaxis in mice

Table 4.3 Effect of test compounds on ovalbumin-induced active cutaneous anaphylaxis in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Evan’s blue µM/mg of protein (n=6)</th>
<th>Serum IgE (ng/mL) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>3.35 ± 0.48*</td>
<td>438.08 ± 83.78*</td>
</tr>
<tr>
<td>Ovalbumin Control</td>
<td>8.99 ± 0.53*</td>
<td>1269.62 ± 186.46*</td>
</tr>
<tr>
<td>Ketotifen (1mg/kg)</td>
<td>3.79 ± 0.80*</td>
<td>583.06 ± 101.55*</td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>9.17 ± 0.80*</td>
<td>676.97 ± 142.25*</td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>7.87 ± 0.41*</td>
<td>745.13 ± 83.54*</td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>5.39 ± 0.50*</td>
<td>1003.47 ± 124.55</td>
</tr>
<tr>
<td>DMPI (200mg/kg)</td>
<td>7.97 ± 1.52*</td>
<td>785.46 ± 62.99</td>
</tr>
</tbody>
</table>

*: p < 0.05 compared to ovalbumin control; #: p < 0.05 compared to ketotifen.

Figure 4.4 Representative photograph of effect of test compounds on ovalbumin-induced active cutaneous anaphylaxis in mice

On day 0, animals were sensitized to antigen by administering 40µg of ovalbumin in 1mg of alum (100µL) i.p. Animals were dosed once daily. On day 14, serum was collected for IgE measurements and animals administered 20 µL of 10µg OVA intradermally in ear followed by 0.3mL of 1% Evan’s blue saline solution i.v. Thirty minutes later, animals were sacrificed, ears dissected out for measurement of extravasated dye. Results are expressed as mean ± SEM.
Figure 4.5 Effect of test compounds on ovalbumin-induced active cutaneous anaphylaxis in mice

On day 0, animals were sensitized to antigen by administering 40µg of ovalbumin in 1mg of alum (100µL) i.p. Animals were dosed once daily. On day 14, serum was collected for IgE measurements and animals administered 20 µL of 10ug OVA intradermally in ear followed by 0.3mL of 1% Evan’s blue saline solution an i.v. Thirty minutes later, animals were sacrificed, ears dissected out for measurement of extravasated dye. Results are expressed as mean ± SEM [*: p < 0.05 compared to ovalbumin control; #: p < 0.05 compared to ketotifen].
4.3.3 Freund’s complete Adjuvant-induced Arthritis in rats

**Figure 4.6** Effect of test compounds on (a) Ipsilateral and (b) Contralateral paw oedema, (c) Ipsilateral and (d) Contralateral paw pain threshold

**Figure 4.7** Effect of test compounds on adjuvant-induced arthritis in rats

Results are expressed as mean ± SEM (n=6) [*: p < 0.05 compared to adjuvant control; #: p < 0.05 compared to diclofenac]
Figure 4.8 Representative photograph of (a) Ipsilateral and (b) Contralateral paw of adjuvant-induced arthritic rats
Figure 4.9 Radiographic analysis of (a) Ipsilateral and (b) Contralateral paw of adjuvant-induced arthritic rats.
### Table 4.4 Effect of test compounds on adjuvant-induced arthritis in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Change in body weight (Day21-Day14) (g)</th>
<th>Spleen weight:Body weight (Day21)</th>
<th>Arthritis Scoring</th>
<th>Serum TNFα (pg/mL) (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>24.17 ± 9.74</td>
<td>0.255 ± 0.016*</td>
<td>-6 ± 0.00*</td>
<td>2.51 ± 2.51</td>
</tr>
<tr>
<td>Adjuvant Control</td>
<td>-9.17 ± 24.96</td>
<td>0.413 ± 0.048</td>
<td>5.17 ± 0.48*</td>
<td>18.8 ± 7.01</td>
</tr>
<tr>
<td>Diclofenac (10mg/kg)</td>
<td>-21.00 ± 7.98</td>
<td>0.403 ± 0.014</td>
<td>0.6 ± 0.87*</td>
<td>8.91 ± 8.43</td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>-12.00 ± 19.35</td>
<td>0.310 ± 0.041</td>
<td>1 ± 1.1*</td>
<td>10.79 ± 10.78</td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>-3.17 ± 16.90</td>
<td>0.355 ± 0.021</td>
<td>1.5 ± 0.99</td>
<td>16.95 ± 16.95</td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>-4.67 ± 16.57</td>
<td>0.374 ± 0.016</td>
<td>0.17 ± 1.25*</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DMPI (200mg/kg)</td>
<td>-5.20 ± 13.98</td>
<td>0.372 ± 0.03</td>
<td>1.4 ± 0.81</td>
<td>13.87 ± 5.39</td>
</tr>
</tbody>
</table>

On day 0, paw volumes were measured and 0.1 ml complete freund’s adjuvant was injected intraplantarly into the right paw (ipsilateral paw). On day 14, freund’s adjuvant-induced arthritic animals were randomized and treated once daily for 7 days from day 14 to day 21. On day 21, the animals were anesthetized, serum collected for TNFα estimation. Arthritis scoring was done based on clinical score, mobility score, joint stiffness. Change in body weight, paw oedema and corrected spleen weight over the treatment period were calculated. Mechanical hyperalgesia (pain threshold) in both paws were measured. Radiology and histopathology of the paws were performed. Results are expressed as mean ± SEM (n=6) [*: p < 0.05 compared to adjuvant control; #: p < 0.05 compared to diclofenac].
Table 4.5 Effect of test compounds on adjuvant-induced arthritis in rats (contd)

<table>
<thead>
<tr>
<th>Group</th>
<th>Ipsilateral paw oedema (mL)</th>
<th>Contralateral paw oedema (mL)</th>
<th>Ipsilateral pain threshold (g)</th>
<th>Contralateral pain threshold (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>0.17 ± 0.02*</td>
<td>0.14 ± 0.03*</td>
<td>113.33 ± 7.6*</td>
<td>108.33 ± 9.97*</td>
</tr>
<tr>
<td>Adjuvant Control</td>
<td>2.91 ± 0.30#</td>
<td>1.72 ± 0.32#</td>
<td>48.33 ± 4.01</td>
<td>48.33 ± 7.26#</td>
</tr>
<tr>
<td>Diclofenac (10mg/kg)</td>
<td>1.42 ± 0.14*</td>
<td>0.42 ± 0.08*</td>
<td>89 ± 12.08</td>
<td>118 ± 20.35*</td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>2.79 ± 0.41</td>
<td>0.89 ± 0.44</td>
<td>73.33 ± 7.92</td>
<td>106.67 ± 12.56*</td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>2.67 ± 0.34</td>
<td>0.71 ± 0.31</td>
<td>105.83 ± 12.87*</td>
<td>112.5 ± 13.4*</td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>2.33 ± 0.46</td>
<td>0.63 ± 0.23*</td>
<td>112.5 ± 8.92*</td>
<td>97.5 ± 12.89</td>
</tr>
<tr>
<td>DMOI (200mg/kg)</td>
<td>2.84 ± 0.40</td>
<td>0.74 ± 0.31</td>
<td>75 ± 11.40</td>
<td>93 ± 15.78</td>
</tr>
</tbody>
</table>

On day 0, paw volumes were measured and 0.1 ml complete freund’s adjuvant was injected intraplantarily into the right paw (ipsilateral paw). On day 14, freund’s adjuvant-induced arthritic animals were randomized and treated once daily for 7 days from day 14 to day 21. On day 21, the animals were anesthetized, serum collected for TNFa estimation. Arthritis scoring was done based on clinical score, mobility score, joint stiffness. Change in body weight, paw oedema and corrected spleen weight over the treatment period were calculated. Mechanical hyperalgesia (pain threshold) in both paws were measured. Radiology and histopathology of the paws were performed. Results are expressed as mean ± SEM (n=6) [\*: p < 0.05 compared to adjuvant control; \#: p < 0.05 compared to diclofenac].
Figure 4.10 Histopathological analysis of (a) Ipsilateral and (b) Contralateral paw of adjuvant-induced arthritic rats (low power)
### Table 4.6 Histopathological analysis of (a) Ipsilateral and (b) Contralateral paw of adjuvant-induced arthritic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ipsilateral paw</th>
<th>Score</th>
<th>Contralateral paw</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Control</td>
<td>Normal joint</td>
<td>0</td>
<td>Normal joint</td>
<td>0</td>
</tr>
<tr>
<td>Adjuvant Control</td>
<td>Severe inflammatory infiltrate</td>
<td>8</td>
<td>Severe inflammatory infiltrate</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Thick pannus protruding into joint space</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complete destruction of joint</td>
<td>7</td>
<td>Joint destruction</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Severe inflammatory infiltrate</td>
<td></td>
<td>Severe inflammatory infiltrate</td>
<td></td>
</tr>
<tr>
<td>Diclofenac (10mg/kg)</td>
<td>Severe inflammatory infiltrate</td>
<td>7</td>
<td>Severe inflammatory infiltrate</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Focal thickening of synovium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHPO (10mg/kg)</td>
<td>Mild narrowing of joint space</td>
<td>3</td>
<td>Normal joint</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHFO (200mg/kg)</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No inflammation</td>
<td></td>
<td>No inflammation</td>
<td></td>
</tr>
<tr>
<td>DMFO (200mg/kg)</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No inflammation</td>
<td></td>
<td>No inflammation</td>
<td></td>
</tr>
<tr>
<td>DMPI (200mg/kg)</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
<td>Mild narrowing of joint space</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No inflammation</td>
<td></td>
<td>No inflammation</td>
<td></td>
</tr>
</tbody>
</table>

On day 0, paw volumes were measured and 0.1 ml complete freund’s adjuvant was injected intrapantarily into the right paw (ipsilateral paw). On day 14, freund’s adjuvant-induced arthritic animals were randomized and treated once daily for 7 days from day 14 to day 21. On day 21, the animals were anesthetized, serum collected for TNFa estimation. Arthritis scoring was done based on clinical score, mobility score, joint stiffness. Change in body weight, paw oedema and corrected spleen weight over the treatment period were calculated. Mechanical hyperalgesia (pain threshold) in both paws were measured. Radiology and histopathology of the paws were performed.
4.4 Discussion

TNBS-induced colitis is a widely used and reproducible model of intestinal inflammation that mimics the pathological changes associated with IBD. TNBS haptenizes luminal proteins (Wirtz et al., 2007), while alcohol induces mucosal protein denaturation and dehydration, resulting in an inflammatory response, eventually damaging the mucosal barrier (Wang et al., 2010). DMFO and DMP1 significantly reduced colon weight/length ratio, indicating a reduction in the thickening/shortening of colon tissue, usually seen in colitis. DHFO and DMFO reduced TBARS, an indicator of lipid peroxidation, suggesting an inhibitory role on oxidative stress. All the test compounds effectively reduced the neutrophil activation marker (MPO), DMFO and DHPO being equally efficient. The test compounds significantly reduced IL6 levels in colonic tissue homogenate, DMFO being equally efficacious as sulfasalazine. The test compounds also reduced tissue damage in colon as observed histopathologically. As this model involves a $T_{H1}/T_{H17}$-mediated response (Daniel et al., 2008) resembling CD, characterized by a $T_{H1}$ immune response (Fichtner-Feigl et al., 2005, Heller et al., 2005), the test compounds may have a potential therapeutic benefit in CD.

The test compounds, being MC stabilizers (as reported in Chapter 2), we tested them for anti-allergic activity in ovalbumin-induced active cutaneous anaphylaxis in mice, involving $T_{H2}$ and $T_{H17}$ cells (Maggi, 2010, Arnold et al., 2011). Cutaneous anaphylaxis is a localized hypersensitivity reaction to an antigen injected into the skin. Anaphylactic reaction to ovalbumin is mediated by its binding to IgE antibody bound to MCs, resulting in MC degranulation and release of histamine, 5HT, etc (Singh et al., 1999). The released histamine increases vascular permeability at the
ovalbumin-sensitized cutaneous site and induces extravasation of Evan’s blue (Singh et al., 1999).

Antigen-induced MC degranulation is a receptor-dependent process involving cross-linking of allergen to IgE bound to FcεRI on MCs, leading to activation of signalling pathways that eventually culminate in degranulation (Siraganian, 2003). Among the test compounds, only DMFO, at 200mg/kg, significantly reduced Evan’s blue dye extravasation, with an efficacy comparable to ketotifen (1mg/kg) (Figure 4.4). DMFO and DMPI did not affect the IgE levels significantly, while ketotifen, DHFO and DHPO reduced IgE levels. This is in agreement with our previously published observations with reference to DHFO which was effective in sheep-serum-induced passive anaphylaxis in rats (Mathew et al., 2013). DMFO stabilized MCs in both antigen and non-antigen-induced models, suggesting a multimodal activity which was independent of IgE. Quite by contrast, DHFO and DHPO target IgE synthesis in addition to stabilizing MCs in-vitro, but not in-vivo. As ROS also plays a significant role in MC degranulation, whether antigen-mediated or otherwise (Suzuki et al., 2005, Nishikawa and Kitani, 2008), the antioxidant activity of test compounds could also partly explain the MC stabilizing activity.

Adjuvant-induced arthritis (a model of polyarthritis (Ryzewska, 1967) involving T_h cells – T_h1, T_h17 (Chen et al., 2010, Sheibanie et al., 2007)) result in inflammation of synovium, destruction of cartilage and bone, and narrowing of joint space. Mechanical allodynia and joint hyperalgesia, two important features of RA, develop as arthritis progresses (Nagakura et al., 2003). Only DMFO mildly reduced oedema in the ipsilateral paw, though not significantly. A similar observation was made in
contralateral paw where DMFO reduced oedema significantly. DHFO improved pain threshold in both paws, DMFO was effective in the ipsilateral paw and DHPO in the contralateral paw. The test compounds seemed to be reducing pain and disability score more effectively than reducing oedema. Interestingly, the oedema was not positively correlated to the pain threshold or arthritis score in all the treated animals, which is intriguing.

TNFα is a very important cytokine involved in acute and chronic inflammation (Popa et al., 2007), including RA progression (Brand et al., 2007). A very interesting observation was that DMFO reduced serum TNFα to undetectable levels. The success of monoclonal antibodies that neutralize TNFα in IBD suggests a significant therapeutic role for DMFO in RA (Chaparro et al., 2012).

Radiography confirmed soft tissue swelling in ipsilateral and contralateral paw in adjuvant-induced arthritic animals. Diclofenac, DHFO and DMFO reduced oedema in the ipsilateral paw, DMFO being most effective. This observation is mirrored in the photographs of paw and arthritis score. In the contralateral paw, diclofenac and all the test compounds reduced tissue swelling; with DMFO showing greatest efficacy. This observation is also in agreement with pain threshold, TNFα, arthritis score, etc.

Histopathological evaluation revealed that all compounds except DHPO effectively reduced synovial inflammation and joint space narrowing. Treatment with diclofenac/test compounds reduced inflammatory infiltration, joint space narrowing, bone destruction, synovial hyperplasia and pannus formation. DHFO, DMFO and
DMPI were more efficacious than diclofenac in the parameters assessed, with a more pronounced action on the contralateral paw.

The stomach was observed for ulcerogenicity following seven days chronic dosing. As in the acute model, DMPI showed a few minor lesions, while all the other test compounds were apparently safe. On the other hand, diclofenac produced severe ulcers in addition to one case of mortality.

Literature reports the upregulation of COX2 in the arthritic synovium (Sheibanie et al., 2007). Additionally, we have evidence that the test compounds may be interfering with the LPS-TLR4 signaling cascade in cell based assays. Therefore, COX2 expression was taken for further study.

The test compounds had divergent activity profiles in the different models of chronic inflammation as observed in Chapter 2 and 3. The pleiotropism arising from the structural identities/peculiarities of the test compounds may underlie the non-uniformity in biological activity.

Therapeutic dosing strategy (treatment after induction of inflammation) being more clinically relevant, was employed in all the chronic models, except cutaneous anaphylaxis (Bendele, 2001, McIntyre et al., 2003). On the contrary, we have found that prophylactic dosing is more popular in screening experiments (Broom et al., 2008, Pulichino et al., 2006). Considering the high efficacy and low toxicity of test compounds, a prophylactic study would be worthwhile. Overcoming toxicity being a greater challenge in the treatment of chronic conditions such as colitis and RA, non-
ulcerogenicity of test compounds is a particularly important advantage over prescribed drugs. Such compounds could also be used in combination with lower doses of existing drugs, thereby overcoming chronic toxicity and facilitating longer treatment duration.

4.5 Conclusions

- The test compounds significantly reduced MPO and IL6 in TNBS-induced colitis, thereby indicating their therapeutic potential in a Th1, Th17-mediated chronic inflammation. While only DMFO and DMPI reduced colon weight/length ratio, DHFO and DMFO reduced TBARS too.

- DMFO significantly reduced dye extravasation in ovalbumin-induced active cutaneous anaphylaxis, with an efficacy comparable to ketotifen, thereby indicating its therapeutic potential in a Th2, Th17-mediated allergen-induced immune response. DHFO and DHPO reduced serum IgE levels, whereas DMFO and DMPI did not affect serum IgE levels.

- The test compounds were efficacious in CFA-induced arthritis, thereby indicating their therapeutic potential in Th1, Th17-mediated chronic inflammation. The test compounds improved the arthritis score. In the ipsilateral paw, DMFO & DHFO improved pain threshold. In the contralateral paw, DHPO and DHFO increased pain threshold. DMFO was found to be the most potent as shown by reduced contralateral paw oedema, arthritis score, serum TNFα levels, and increased pain threshold. An interesting observation was that the test compounds were non-ulcerogenic even on chronic dosing.

- In all models tested, except cutaneous anaphylaxis, therapeutic dosing strategy was followed, making the compounds more therapeutically relevant.
4.6 References


Evaluation of test compounds in *in-vivo* models of chronic immune-mediated inflammation


