Chapter 5

Summary

&

Conclusion
Screening of plants for their anti-inflammatory properties and evaluation of natural compound for its anti-arthritic potency in CIA

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune inflammatory disorder characterized by chronic inflammation of the multiple synovial joints, inflammatory cell recruitment, and subsequent progressive destruction of articular tissue. Type II collagen-induced arthritis (CIA) is the most commonly used autoimmune animal model of RA. This model has been used in numerous studies to address questions of disease pathogenesis, to validate therapeutic targets and to evaluate potential therapeutic compounds (Cuzzocrea et al., 2000b). CIA shares clinical and pathological features, similar with those of human RA, including the involvement of inflammatory mediators in arthritic etiology. It is also known that when the body has any inflammation, usually level of C-reactive protein (CRP) in the blood increases. A part of this study was performed to examine whether level of CRP, a sensitive marker of disease activity in RA is associated with collagen induced arthritis in rats. The glycoproteomic analyses of this protein and other indicators of arthritis were carried out to establish co-relationship between disease severity and biochemical markers. Besides, activation of cell mediated immunity in CIA results in the secretion of Th1-related cytokines and free radicals that further contribute to articular degeneration. These cytokines induce and activate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), resulting in the production of PGE2 and nitric oxide (NO). Activation of macrophages also leads to the ingress of large numbers of polymorphonuclear cells (PMNs) into the synovial tissue and synovial fluid. Thus, it follows that suppression of these inflammatory mediators can be used to treat RA. The non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) have a track record in RA treatment but their long term usage is associated with several other potential side effects like gastrointestinal ulcer, renal morbidity, contracting infections etc., (Yeom et
This instigates the need for better and safer medicines. One solution to this problem is the use of herbal therapies, which have been considered safe and effective in alleviating chronic pain associated with arthritis. Therefore, this study is aimed to explore and evaluate the anti-inflammatory potential of extracts from some traditionally used medicinal herbs with unidentified anti-inflammatory activities and their mode of action. For the screening of such active components, pro-inflammatory TNF-α, IL-1β, IL-12 and enzyme-encoding genes, iNOS, and COX-2 are chosen as pro-inflammatory markers. The biological activities of the methanolic extracts against inflammation and their mode of action were examined in vitro using LPS-stimulated J774 murine macrophage cells as model. Previous work done in our lab has demonstrated the anti-inflammatory effects (through both in vitro and in vivo models of LPS-induced inflammation) of Rg1 or 3-(1′-1′-dimethyl-allyl)-6-hydroxyl-7-methoxy-coumarin isolated from the methanolic extract of R.graveolen’s plant. In this study, another potential anti-inflammatory coumarin Rg3 (8-methoxy-chromen-2-one) was isolated from the same plant and tested in cell culture for potential anti-inflammatory activity. Further, its therapeutic potency was evaluated in the collagen induced arthritic rat model for its use as a possible lead compound.

Following conclusions can be drawn from the present investigations.

- Methanolic extracts of four medicinal plants significantly reduced the protein levels of inducible NO synthase (iNOS) and the cyclooxygenase-2 (COX-2) as observed by Western blot analysis.
- Culture supernatants from cells treated with these extracts indicated 3–5-fold reduction of TNF-α.
- However, only G. robusta and Q. amara extracts significantly inhibited (by 50%) IL-1β and IL-12 secretions.
All these plant extracts were shown to prevent the LPS-mediated nuclear translocation of nuclear factor-κB.

Differential expression of protein spots was observed in CIA rat plasma. C-reactive protein and haptoglobin were found to be upregulated.

Monosaccharide pattern of these proteins as analyzed by HPAEC-PAD revealed that both the proteins were glycosylated along with fucosylation alteration in CIA rat plasma.

In both the proteins isolated from CIA rat plasma amount of fucose increased significantly (Table 3.1, Figure 3.11).

Rg3 was therapeutically administered to the CIA rats at two different dosages. Towards the end of the experiment, the CIA inhibitions were calculated to be 75.67% (2mg/kg) and 84.68% (20 mg/kg).

Rg3 treatment exerted a significant (p < 0.01) suppression of the arthritic score between days 27 and 36 post-immunization.

In the Rg3-treated groups, the degree of arthritis induced joint damage was significantly reduced. The synovial linings of the joints were smooth and normal, with slight indications of synovial hyperplasia or other characteristics of inflammation.

Rg3 controlled the synovial cell infiltration.

Radiographic analysis showed that Rg3 treatment markedly protected against bone resorption and soft tissue swelling.

Thus, Rg3 at both the doses has considerable protective effects on the cartilage and bone in the immunized rats.

The levels of pro-inflammatory cytokines (TNF-α, IL1β and IL6) were significantly (p<0.0001) suppressed in CIA rats treated with Rg3 at both the doses.

Rg3 showed no significant inhibition on the anti-CII antibody formation.
• Administration of Rg3 significantly (p<0.0001) reduced the nitrite concentration of plasma of the immunized rats. Treatment with indomethacin also yielded similar results (p<0.0001).

• Rg3-treated CIA rats showed significantly improved locomotor and exploratory behavior when compared with CIA (p<0.0001).

• All of these findings support the view that Rg3 attenuates the degree of arthritis and joint injury caused by CII in the rat.
**Suramin, a potent antioxidant and anti-inflammatory agent, ameliorates CIA, a model of RA**

Chemically, suramin is a hexasodium salt of 8,8’-(carbonyl-bis(imino-3,1-phenylene-carbonylimino(4-methyl-3,1-phenylene)carbonylimino))bis-1,3,5-naphthalene trisulfonic acid, is a polysulfonated naphthylurea, which is being used as an antiparasitic agent since 1920s. It has also been tested as a possible treatment for AIDS. Suramin also has demonstrated an ability to inhibit the activity of various growth factors *in vitro* and has therefore been used clinically to treat various cancers. The compound has also been noted to bind to a variety of cytoplasmic and intranuclear enzymes. Suramin is known to have anti-TNFα activity. TNF-α is a critical pleiotropic cytokine and its overproduction has been implicated in the pathogenesis of a number of inflammatory diseases including rheumatoid arthritis. Suramin specifically promotes dissociation of the biologically active trimeric form of TNF-α into inactive subunits, thus inhibiting the binding of TNF-α to its cellular receptors. It also blocks the activity of IL-6 as it interferes with the binding of IL-6 to its cell surface receptor. To the best of our knowledge, there was no report on the biochemical findings that support the amelioration of collagen-induced arthritis by suramin. The latter has also shown to suppress reactive oxygen and nitrogen species both *in vitro* and *in vivo*. So, the present study was carried out to gain mechanistic insights into the anti-oxidant as well as anti-rheumatic potential of suramin in established collagen-induced arthritis and the reduction in disease severity has also been evaluated.

The concluding points of the present study are:

- Suramin exhibited marked scavenging activity against free radicals generated in different cell free model systems.
Administration of suramin in CIA rats resulted in significant amelioration of inflammatory conditions and a gradual fall in clinical score up to 63% as compared to ‘CIA+vehicle’ group on day 42.

Anti-arthritic effect of suramin, measured in terms of CIA inhibition also demonstrated a significant inhibition (85%) of CIA at the end of the treatment.

Suramin treatment reduced bone erosion and maintained joint space in arthritic rats.

Besides, suramin treatment significantly reduced the arthritic severity accompanied by reduction in cartilage damage, and cell influx.

The high levels of pro-inflammatory cytokines were drastically reduced after suramin and indomethacin treatment.

Proteomic analysis of plasma proteins using both WGA bound and unbound plasma fractions showed the differential expression of many acute phase proteins like AGP, hemopexin, kininogen, clusterin in CIA rats. The level of kininogen was found to be highly increased (~6 fold) in CIA rats which was further validated using Western blotting.

The reversal of positive acute phase proteins after suramin treatment further substantiated its anti-inflammatory activity.

Increased oxidative stress observed in the arthritic animals was also found to be significantly restored in suramin-treated rats.
Evaluation of novel peptide inhibitor of human TNF-α using cell culture studies

To search for some small molecule peptide inhibitors against TNF-α, the phenomenon of TNF-α trimerization was targeted for designing of peptide inhibitors, as this is the most important step which renders the TNF-α active. The anti-TNF activity of four different peptides (peptide 1, 2, 3 and 4) was checked using in-vitro cell culture studies. Effect of peptide-2 was found to show higher and more significant anti-TNFα activity. TNF-α induced cell death of Wehi-164 cells was evaluated. The peptide-induced inhibition of TNF-α resulted in reduced cell death and increased viability of Wehi-164 cells, which was done through MTT assay. The TNF-α induced nuclear translocation of NF-κB was visualized using fluorescent microscopy technique for the immunocytochemical analysis of TNF-α stimulated A549 cells. The peptide-2 inhibited the TNF-induced activation, hence the translocation of NF-κB into the nuclei of A549 cells. The Western blotting of the nuclear lysate showed under expression of NF-κB in the TNF-stimulated cells with peptide inhibition as compared to the cells untreated with peptide-2. This result was fairly corroborated with the Electrophoretic Mobility Shift Assay (EMSA) test of the nuclear lysates of the experimental cells.

Peptide-2 showed a dose dependent inhibition on the TNF-α activity, with maximum effect at 200 μM of the peptide concentration (p < 0.05). No significant cytotoxicity was observed when stimulated with the peptides with the concentrations ranging between 50 and 400µM.

This anti-TNF peptide (peptide-2) has a potential of being a peptide drug, for which in-vivo studies and further trials are required.

The concluding points of the study are:

- Purified TNFα was active and leads to decrease in the viability of Wehi-164 cells.
• The decrease in viability due to TNFα was significantly inhibited by peptide 2 - PIYLGGVFAQ.

• Activation / nuclear translocation of NFκB due to TNFα stimulation was reduced with the peptide 2 in A549 cells.

• Peptide 2 showed higher anti-TNFα activity than other 3 peptides.