CHAPTER 1

INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis [RA] is an inflammatory disorder characterized by destruction of the synovium and progressive damage of the bones and cartilages. This leads to slow degradation of the joints thus causing chronic pain and inflammation around the joints throughout life [1, 2]. Figure 1.1 shows the distinct regions that are affected in RA when compared to normal joints. The condition is most likely triggered by a combination of factors including an abnormal immune response, genetic susceptibility, and some environmental or biologic trigger such as hormonal changes, viral infection or some toxic gas exposure [3].

Figure 1.1 An illustration of Normal versus RA joints
The typical disease is seen as a severe inflammation of the joints caused by immune cells which eventually leads to the destruction of cartilage, pannus formation and inflamed synovium membrane [4]. RA patients have difficulty in joint movements and this disease mostly affects women than men. It has been categorized as an autoimmune disorder and the disease can be managed for the side effects like pain, fever and loss of appetite but cannot be completely cured. It is a chronic disorder and most treatments help to manage the pain associated with the disease. Since the factors that lead to RA are ambiguous, the prevention of the disease is also not feasible [5].

1.1.1 STATISTICS

According to the Centre for Disease control and Prevention [CDC], 1% of the general population in the world suffers from RA. Forty one Americans out of every 100,000 Americans have RA (Figure 1.2). The data claims that the risk of Rheumatoid arthritis are higher in women than in men as well as the age of occurrence are much younger for women compared to men [6].

![Figure 1.2](image)

[Left] RA incidence in America compared to the world [right] Higher risk in women
RA has been on the rise in India with a 1993 statistic claiming incidence rate of 0.75 percent which is similar to other developing countries but higher than China [7].

### 1.1.2 Symptoms

RA always affects the joints. It makes them inflamed. The classic signs include [8]:

- Stiffness. The joint has clinical sign of stiffness with more pronounced signs lasting for longer time in the morning when compared to other form of arthritis.
- Swelling. Fluid enters the joint and makes it puffy and edematous.
- Pain. Inflammation inside a joint makes it sensitive and tender. Eventually, it causes damage and pain.
- Redness and warmth. The joints may be warmer and more pink or red than skin around it.

### 1.1.3 Histopathology

#### Synovium

The synovium, in normal joints, is a thin delicate lining that serves several important functions. The synovium serves as an important source of nutrients for cartilage since cartilage itself is avascular. In addition, synovial cells synthesize joint lubricants such as hyaluronic acid, as well as collagens and fibronectin that constitute the structural framework of the synovial interstitium.

1. Synovial lining or intimal layer: Normally, this layer is only 1-3 cells thick. In RA, this lining has been reported to be greatly hypertrophied (8-10 cells thick). Primary cell populations in this layer are fibroblasts and macrophages [9].
2. Subintimal area of synovium: This is where the synovial blood vessels are located; this area normally has very few cells. In RA, however, the subintimal area is heavily infiltrated with inflammatory cells, including T and B lymphocytes, macrophages, mast cells, and mononuclear cells that differentiate into multinucleated osteoclasts. The intense cellular infiltrate is also accompanied by new blood vessel growth (angiogenesis). In RA, the hypertrophied synovium called pannus invades and erodes contiguous cartilage and bone. As such, it can be thought of as a tumor-like tissue, although mitotic figures are rare and, of course, metastasis does not occur [9].

**Cartilage**

Composed primarily of type II collagen and proteoglycans, this is normally a very resilient tissue that absorbs considerable impact and stress. In RA, its integrity, resilience and water content gets impaired. This appears to be due to elaboration of proteolytic enzymes (collagenase, stromelysin) both by synovial lining cells and by chondrocytes themselves. Cytokines including IL1 and TNF drive the generation of reactive oxygen and nitrogen species and while increasing chondrocyte catabolic pathways and matrix destruction, also inhibit new cartilage formation. Polymorphonuclear leukocytes in the synovial fluid may also contribute to this degradative process [9].

**Bone**

Composed primarily of type I collagen, bony destruction is a characteristic of RA. This process is primarily driven by the activation of osteoclasts. Osteoclasts differentiate under the influence of cytokines especially the interaction of RANK with its ligand. The expression of RANK has been driven by cytokines including TNF and IL1, as well as other cytokines including IL-17 [9]. There may also be a contribution to bony destruction from mediators derived from activated synovial cells.
Synovial Cavity

The synovial cavity is normally only a “potential” space with 1-2ml of highly viscous (due to hyaluronic acid) fluid with few cells. In RA, large collections of fluid (“effusions”) occur which are, in effect, filtrates of plasma (and, therefore, exudative – i.e., high protein content). The synovial fluid is highly inflammatory. However, unlike the rheumatoid synovial tissue in which the infiltrating cells are lymphocytes and macrophages but not neutrophils, in synovial fluid the predominant cell is the neutrophils [9].

1.1.4 Propagation of Disease

T cell activation

Upon encounter with antigen in the context of MHC on an antigen presenting cell, a T lymphocyte is positioned for 3 possible fates: activation, anergy/tolerance, or apoptosis (death). T cell activation is only possible if the T cell receives a “second signal” through engaging additional cellular receptors. One of the most important of these second signals is delivered through the CD28 molecule on the surface of the T cell but many other second signals are also involved in this process of “costimulation”. Upon engagement of these receptors, a T cell usually becomes activated. Failure to engage the stimulatory receptors, or engagement of a down-regulator receptor will cause the cell to become tolerant to the antigen (eg. does not activate when exposed to the antigen) or to undergo programmed cell death through apoptosis. The process of T-cell costimulation is interrupted by abatacept, a biological therapy used to treat RA.

When T cells become activated, they will in turn proliferate and begin to secrete additional cytokines including IL-2 which furthers their proliferation, and depending on other exposures, cytokines such as IFN-γ, TNF, and IL-4. It is the effect of these T-cell derived cytokines that causes additional cells to get activated. T cells also directly interact through surface receptors with other cells to generate additional activation signals [10].
B Cell Activation and Autoantibodies

B cells become activated through interactions with T cells and through soluble cytokines that enhance their proliferation and differentiation. B cells express a number of receptors on their surfaces during their differentiation, including the molecule CD20, which is lost upon terminal differentiation to antibody-forming plasma cells. B cells and plasma cells can be found in rheumatoid synovium sometimes as lymphoid aggregates in the subsynovium. The effects of B cells extend beyond their roles in forming plasma cells including cytokine production, direct cellular interactions, and they themselves serve as antigen-presenting cells to T lymphocytes. The role of B cells in RA has been clearly demonstrated with the efficacy of rituximab which eliminates circulating B cells, though with limited impact on autoantibody formation.

One of the features of most autoimmune diseases is the presence of disease-specific autoantibodies that help to define disease phenotypes. Antibodies are made by plasma cells, which represent the terminal stage of differentiation for B lymphocytes. Rheumatoid arthritis is characterized by the presence of autoantibodies known as rheumatoid factors (RF) and anti-citrullinated peptide antibodies (ACPA, which includes the anti-cyclic citrullinated peptide antibody or anti-CCP). Rheumatoid factors have been long recognized as a feature of many patients with RA. These are autoantibodies in the classical sense; they are antibodies directed against native antibodies, most classically described as IgM antibodies that recognize the Fc portion of IgG molecules, but RF may also be of the IgG or IgA isotypes. Rheumatoid factors are not specific for the diagnosis of RA, but are seen in many other inflammatory and autoimmune conditions. These include Sjogren’s syndrome, chronic infections including tuberculosis and endocarditis, hepatitis C, chronic kidney or liver disease, lymphoproliferative diseases including myeloma, and other conditions. While the rheumatoid factor may be seen in other inflammatory conditions, ACPA are highly specific for rheumatoid arthritis and define a more aggressive disease phenotype [10].
Effector Cell Activation

While T cells and B cells represent the immunological aspects of RA, most of the damage from the disease is driven through effector cells and their products including cytokines and other mediators. The synovial lining in RA represents an expansion of fibroblast like cells and macrophages. It is the macrophage that has been seen as one of the master orchestrators of the effector damage in RA. Macrophages are rich sources and major producers of proinflammatory cytokines including TNF, IL-1, IL-6, IL-8, and GMCSF. These cytokines further stimulate the macrophage, as well as other cells in the microenvironment including fibroblasts and osteoclasts, and finally at distant sites in the body through cell surface receptors including the hepatocyte which is responsible for the generation of acute phase response proteins (such as C-reactive protein). Macrophages are also producers of prostaglandins and leukotrienes, nitric oxide, and other pro-inflammatory mediators with local and systemic effects. The synovial fibroblast also secretes cytokines including IL-6, IL-8 and GM-CSF, and other mediators including destructive proteases and collagenases [11].

Neutrophils are recruited in very large numbers to the rheumatoid cavity where they can be aspirated in the synovial fluid. The recruitment of neutrophils to the joint is likely driven by IL-8, leukotriene B4, and possibly localized complement activation through C5a. Neutrophils in the synovial fluid are in an activated state, releasing oxygen-derived free radicals that depolymerize hyaluronic acid and inactivate endogenous inhibitors of proteases, thus promoting damage to the joint.

Chondrocytes, like synovial fibroblasts, are activated by IL1 and TNF to secrete proteolytic enzymes. They may, therefore, contribute to the dissolution of their own cartilage matrix, thus explaining the progressive narrowing of joint spaces seen radiographically in this disease.
1.1.5 Inflammatory Mediators in RA

Cytokines

One of the most important group of mediators in RA are cytokines. The most prominent of these are TNF, IL-1, and IL-6. These cytokines, released in the synovial microenvironment have autocrine (activating the same cell), paracrine (activating nearby cells), and endocrine (acting at distant sites) effects and accounting for many systemic manifestations of disease. There are many shared functions of TNF, IL-1, and IL-6, and these cytokines in turn upregulate the expression of the others. Many other cytokines are increasingly described in RA. These include IL-8 which is involved in cellular recruitment, GM-CSF involved in macrophage development, IL-15 involved in T cell proliferation, IL-17 which has pleiotropic effects on multiple cell types including osteoblast expression of RANK leading to osteoclast activation, and IL-23 involved in increasing TH17 cell differentiation [11].

Soluble mediators of inflammation that may diffuse in from blood and/or be formed locally within the joint cavity include prostaglandins, leukotrienes, matrix metalloproteinases. Prostaglandins are involved in pain sensitization localized inflammation, and some effects on bone, and leukotrienes play roles in vascular permeability and chemotaxis. Matrix metalloproteinases (MMPs) are potent in their ability to enzymatically degrade the collagen matrix of cartilage. Kinins cause release of prostaglandins from synovial fibroblasts, and are also potent algesic (pain-producing) agents. Complement may be available for interaction with immune complexes to generate additional chemotactic stimuli. The neuropeptide substance P is a potent vasoactive, proinflammatory peptide that has also been implicated in RA [12].

1.1.6 Diagnosis

The 1987 American College of Rheumatology (ACR) classification criteria of morning stiffness, symmetrical arthritis, nodule, Rheumatoid factor and
X-ray changes have been normally used for diagnosis of Rheumatoid arthritis [13]. These include:

Criteria for diagnosis of active rheumatoid arthritis

1. Morning stiffness
2. Pain on movement or tenderness in a joint
3. Soft tissue swelling in a joint
4. Soft tissue swelling of another joint
5. Symmetrical soft tissue joint swelling simultaneously
6. Subcutaneous nodules
7. X-ray changes
8. Positive rheumatoid factor

3-4 criteria positive Probable rheumatoid arthritis
5-6 criteria positive definite rheumatoid arthritis
7-8 criteria positive Classical rheumatoid arthritis

1.1.7 Treatment of RA

Curing RA is still out of our reach, and the induction of immunologic tolerance will not be achieved until the driving autoantigen(s) in RA have been fully identified. This search for autoantigens is increasingly complicated because it has become clear that the development of RA is characterized by an accumulation of multiple autoantibody specificities (so-called ‘epitope spreading’), which differs in individual patients with RA. Currently the treatment of RA is by administering drugs for reducing inflammation, preventing damage to the bones and ligaments of the joint, preserving movement and by helping the patient to be free from side effects of these drugs for as long as possible [14]. The three types of treatments are Nonsteroidal Anti-Inflammatory Drugs [NSAIDS], Disease Modifying Anti Rheumatic Drugs [DMARDS] and Biologics.
**NSAIDS:** These drugs block prostaglandins, the substances that dilate blood vessels and cause inflammation and pain. There are dozens of NSAIDs:

- Over-the-counter NSAIDs include aspirin, ibuprofen (Motrin IB, Advil, Nuprin, Rufen), naproxen (Aleve), ketoprofen (Actron, Orudis KT).
- Prescription NSAIDs include naproxen (Naprosyn, Anaprox), flurbiprofen (Ansaid), diclofenac (Voltaren), tolmetin (Tolectin), ketoprofen (Orudis, Oruvail), dexibuprofen (Seractil). In 2004, a new NSAID, meloxicam (Mobic) was approved in the U.S. for the management and treatment of rheumatoid arthritis.

**DMARDS:** Widely used conventional DMARD drugs like Methotrexate and Leflunomide are usually given as a combination rather than singly to increase the effectiveness of the drugs [15].

**Biologics:** The other class of DMARDS is biologic response modifiers or “biologics.” They can target the parts of the immune system and the signals that lead to inflammation and joint and tissue damage [16].

Currently approved biologic response modifiers include:

- Etanercept (Enbrel). Etanercept is an anti-tumor necrosis factor (anti-TNF) drug. Approved in 1998, etanercept was the first biologic response modifier drug for treatment of rheumatoid arthritis. It is also approved for juvenile RA and psoriatic arthritis.
- Infliximab (Remicade). Approved in 1999, infliximab is also an anti-TNF drug. It is used in combination with methotrexate.
- Adalimumab (Humira). Adalimumab is another anti-TNF drug. First approved in 2002 as a second-line treatment for RA, adalimumab received additional approvals in 2005 as a first-line
treatment for RA and psoriatic arthritis. It is used alone or in combination with methotrexate or other DMARDs. It is also showing promising results in clinical trials for juvenile rheumatoid arthritis.

- **Anakinra (Kineret).** Approved in 2001, anakinra targets interleukin-1 (IL-1), another type of immune factor.

- **Abatacept (Orencia).** Approved in 2005 for adults with moderate-to-severe RA who have not responded to DMARD or anti-TNF drugs. Abatacept is known as a T cell co-stimulation modulator. It blocks T cell activation. It is used alone or in combination with other DMARDs aside from anti-TNF drugs.

- **Rituximab (Rituxan).** Approved in 2006, rituximab targets CD20-positive B cells and blocks their activation. It is used in combination with methotrexate for patients with moderate-to-severe RA who have not responded to anti-TNF therapies.[16]

The different treatment options for various modalities of RA. Previously, rheumatologists typically started treating patients with newly diagnosed RA with NSAIDs to reduce pain and joint swelling has been given in Figure1.3. Currently, such patients are treated more aggressively and joint damage is prevented with DMARDs, such as methotrexate (MTX), hydroxychloroquine, leflunomide, or sulfasalazine, and, depending on symptoms and stage of the disease, a combination of several DMARDs or a DMARD plus biological treatment has been demonstrated to be favorable for the disease outcome, resulting in 35% of patients with RA showing at least a 50% improvement in their American College of Rheumatology (ACR) criteria for treatment responses, and even 15 percent reaching the ACR70 response (indicating ≥70 percent improvement) [17].
1.1.8 Targets of DMARDs

Improved treatment outcome of RA is through better control of inflammation which is the key in managing RA. The mechanism of action of synthetic DMARDs is either through enzyme inhibition or by mediating a receptor—ligand pathway. The antimetabolite/cytotoxic drugs like MTX and azathioprine (AZT) were earlier thought to confer their therapeutic effects through cytolysis. Now, it has been found that these drugs act by inhibiting purine metabolism. In addition to the Pharmacokinetics of DMARDs, properties of ligand—enzyme interaction also influence the efficacy, dose and the dosing interval [18].

Most of the targets that are inhibited by DMARDs play a role in the cells as enzymes in metabolic pathways, signal transduction receptors, transcription control factors and lysosomes. The DMARDs thus present therapeutic benefits by interfering and blocking the metabolic, transcriptional and signal transducing pathways in the proliferating immune cells. All the targets of RA were evaluated by serendipity except DHODH which arose out of specifically designed protocols [18].

Dihydrofolate reductase and folate transporters play a role in purine metabolism, DHODH plays a role in pyrimidine synthesis, Lysosomes play a role in
antigen presenting cells and calcineurin in important for T-cell activation. Figure 1.4 gives the different conventional DMARDS and their mechanism of action. These targets are all related to roles in inflammation thus blockading of these targets results in lower inflammation.

<table>
<thead>
<tr>
<th>Enzyme inhibition</th>
<th>AZT: 6-thioguanine, a metabolite of AZT, has been demonstrated to interfere with CD28-dependent T-cell activation</th>
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<tbody>
<tr>
<td></td>
<td>MTX: Inhibits ACCAR and less likely DHFR</td>
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<tr>
<td></td>
<td>LEF: ATZ-1726 inhibits DHODH causing decrease in levels of rUMP and p53 activation</td>
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<tr>
<td></td>
<td>SSZ (MTX) folate transporter</td>
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<td>Cytotoxic actions</td>
<td>MTX and AZT</td>
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<tr>
<td>Inhibiting signal transduction</td>
<td>Cyclosporine: forms complex with cyclophilin (a cytoplasmic housekeeping protein), which subsequently binds with calcineurin (an intracellular phosphatase)</td>
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<td></td>
<td>D-pen: inhibits the DNA binding of the transcription factor AP-1, a dimer of the proto-oncogenes c-jun and c-fos, thereby inhibiting the cytokine production</td>
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<tr>
<td></td>
<td>Gold: action is similar to that of D-pen</td>
</tr>
<tr>
<td>Altering antigen presentation</td>
<td>Chloroquine and gold salts: interfere with lysosome function</td>
</tr>
<tr>
<td>Inhibiting nuclear transcription</td>
<td>Cyclosporine, LEF and corticosteroids: inhibit NF-kB transcriptional activity</td>
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</tbody>
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**Figure 1.4 Different modes of Intervention by DMARDS**

### 1.1.9 Dihydroorotate Dehydrogenase

Pyrimidine bases are essential for cellular metabolism and cell growth, and are considered as important precursors used in DNA (thymine and cytosine), RNA (uracil and cytosine), glycoproteins and phospholipids biosynthesis. The significance of DHODH in pyrimidines biosynthesis in DNA and RNA makes it an ideal target for pharmacological intervention.

The lymphocytes that cause inflammation, in rheumatoid arthritis utilize denovo pyrimidine synthesis pathway rather than the regular salvage pathway to obtain nucleotides for proliferation. The fourth step of this pathway is catalyzed by DiHydroOrotate Dehydrogenase enzyme [human DHODH] which converts dihydroorotate to orotate which is a precursor for Uridine-5’-monophosphate (Figure 1.5). This is a rate limiting enzyme of the pathway and thus used effectively for treatment of RA [19].
Human DHODH is a mitochondrial oxidoreductase enzyme in humans that has a two site ping pong mechanism to transfer electrons to ubiquinone for respiration cycle as well as convert dihydroorotate to orotate [20-22]. Leflunomide [active form-A771726] binds to the orotate site uncompetitively [23].
DHODHs, are divided into two large families, with the mammalian enzymes forming a tightly clustered group in family 2 which catalyze the only redox step in pyrimidine biosynthesis [25]. Oxidation of dihydroorotate (DHO) to orotate (ORO) and reduction of flavin mononucleotide (FMN) to dihydroflavin mononucleotide (FMNH2) comprise the two half reactions of the redox couple. For human DHODH, ubiquinone is the oxidant (Figure 1.6). In family 1 DHODHs, oxygen or some water-soluble molecules such as fumarate or NAD+ oxidize FMNH2 to regenerate FMN, and the enzymes are cytosolic. DHODH class 1 is seen in many microorganisms. DHODH is present in the inter membrane of the mitochondria surrounded by water. Full-length human DHODH has 396 residues and most constructs elucidated in the PDB database lacks the mitochondrial signal peptide and comprises Met30 in the C terminus (Figure 1.7).

**Figure 1.7 Cartoon Representation of Human DHODH**

DHODH is an alpha beta barrel shaped structure with two active sites one for FMN as a substrate for oxidative phosphorylation and another site where dihydroorotate forms orotate. Thus Human DHODH [human DHODH] follows the ping pong mechanism of catalysis [24].
Human DHODH protein has been annotated with alpha beta barrel architecture and TIM barrel topology [Q02127 in UNIPROTKB]. It performs oxidoreductase type of reactions on CH-CH type of donors. It’s a central enzyme with a large DHODH domain and binds to various substrates like FMN, Ubiquinone and dihydroorotate (Figure 1.8).
1.1.10 Inhibitor Binding Site of Human DHODH

The space between α1 and α2 forms a narrow tunnel in the hydrophobic patch, with the short α1–α2 loop at the narrow end. This slot forms the entrance to a tunnel that ends at the FMN cavity beneath the α1–α2 loop (Figure 1.9). This tunnel narrows as it goes into the proximal redox site, and several charged or polar side chains (Gln47, His56, Tyr356, Thr360 and Arg136) are located at the narrow end of the channel. Ubiquinone, which can readily diffuse in the mitochondrial inner membrane, undoubtedly uses this tunnel to approach FMNH2 for a redox reaction [25].

![Figure 1.9 Inhibitor Binding Site of human DHODH](image)

The inhibitor pocket consists of amino acids like Arg136, Ala55, Leu359, Ala59, Phe98, His56, Pro364, Met43, and Thr360. The ligand A771726 (active form of Leflunomide) forms a single hydrogen bond with Tyr356. Another potent inhibitor Brequinar, forms a hydrogen bond with Arg136 (Figure 1.10).
Figure 1.10 Residues present in the Inhibitor binding site of human DHODH

There are 35 different entries of human DHODH in PDB and resolutions varying from 1.23Å to 3.00Å. All the inhibitors listed in PDB for human DHODH bind to the same narrow channel. This narrow tunnel is missing in DHODH class1 structure and helps to understand the specificity of the inhibitors in different species of DHODH.

1.1.11 Conventional DMARDS

**Methotrexate.** Methotrexate (Rheumatrex, Trexall) acts as an anti-inflammatory drug and is now the most frequently used DMARD, particularly for severe disease. Methotrexate starts working within 3 - 6 weeks, but its full effect may not occur until after 12 weeks of treatment. This drug loses effectiveness, however, when used alone but may be more effective when used in combination with other DMARDs or other drugs. Methotrexate is often combined with hydroxychloroquine, sulfasalazine, or leflunomide. It may also be combined with various biological response modifier drugs, especially for treatment of patients with
early aggressive arthritis. The combination appears to work better than single drug therapy.

About 20 percent of patients withdraw from methotrexate because of its side effects. They include nausea and vomiting, rash, mild hair loss, headache, mouth sores, and muscle aches. Methotrexate reduces levels of folic acid (folate) in the body, which can lead to some of these side effects. Doctors may prescribe folic acid supplements to prevent side effects. However, some research suggests that folic acid may interfere with methotrexate’s effectiveness.

Methotrexate is usually given as pills. Patients who need higher doses can take it as an injection. Methotrexate has fewer serious toxic effects than many DMARDs. Although these severe reactions are rare, they may include:

- Kidney and liver damage. People at particular risk for liver damage from methotrexate include those with diabetes, obesity, and alcoholism. Patients should limit alcohol consumption to no more than 2 drinks per month while taking this drug.

- Increased risk for infections. Methotrexate should not be given to patients with active bacterial infections, active herpes-zoster viral infection, active or latent tuberculosis, or acute or chronic hepatitis B or C.

- Lung disease occurs in up to 5% of people. People who have poor lung function are most at risk.

- The drug increases the risk for birth defects and should not be taken by pregnant women. However, methotrexate will not harm a woman’s chance for future healthy pregnancy [26].

**Hydroxychloroquine.** Hydroxychloroquine (Plaquenil) was originally used for preventing malaria and is now also used for mild, slowly progressive arthritis. It starts to improve symptoms within 1 - 2 months, but may take up to 6 months to
achieve full benefit. It also does not appear to slow disease progression. Hydroxychloroquine usually causes less side effects than other DMARDs. The most common side effects are nausea and diarrhea, which typically improve over time or when the drug is taken with food. Less common side effects include skin rash or bleaching or thinning of hair. This drug used to be associated with eye and vision problems, but with current lower doses this side effect is rare. If vision problems occur, it is usually with people taking very high doses, those with kidney disease, or those over 60 years of age. Still, patients should have an eye exam (including retinal examination) within the first year of treatment. Patients with health risks (liver disease, retinal disease, over age 60) should have an annual eye exam. Patients should notify their doctors if they experience any sudden changes in vision [26].

**Sulfasalazine.** Sulfasalazine (Azulfidine) was developed in the 1930s for treating rheumatoid arthritis, but fell into disfavor when gold treatment emerged. It has regained popularity, however, and is now used for both adult and juvenile RA. It works best when the disease is confined to the joints. Symptom relief occurs within 1 - 3 months.

Side effects are common, particularly stomach and intestinal distress, which usually occur early in the course of treatment. (However, serious gastrointestinal side effects, such as stomach ulcers, occur less frequently with sulfasalazine than with NSAIDs.) A coated-tablet form may help reduce side effects. Other side effects include skin rash and headache. Sulfasalazine increases sensitivity to sunlight. Be sure to wear sunscreen (SPF 15 or higher) while taking this drug. People with intestinal or urinary obstructions or who have allergies to sulfa drugs or salicylates should not take sulfasalazine [26].

**Azathioprine.** Azathioprine (Imuran) suppresses immune system activity. It takes 6 - 8 weeks for early symptom improvement and up to 12 weeks for full benefit.
Azathioprine can cause serious problems with the gastrointestinal tract. About 10 - 15% of patients experience nausea and vomiting, often accompanied by stomach pain and diarrhea. (Taking the medication twice daily, instead of once daily, or taking it after eating may help avoid this problem.) Azathioprine can also cause problems with liver function and pancreas gland inflammation, and can reduce white blood cell count.

**Cyclosporine.** Like azathioprine, cyclosporine (Sandimmune, Neoral) is an immunosuppressant. It is used for people with RA who have not responded to other drugs. It can take a week before symptoms improve and up to 3 months for full benefit. The most serious and common side effects of cyclosporine are high blood pressure and kidney function problems. While kidney function usually improves once the drug is stopped, mild-to-moderate high blood pressure may continue. Cyclosporine can also cause gout or worsen gout in people who have this condition [26].

Other common side effects include headache, nausea, vomiting, stomach pain and upset, and swelling of hands and feet. About 10% of patients who take cyclosporine develop tremors, increased hair growth, muscle cramps, and numbing or tingling in hands and feet (neuropathy). Swelling of the gums is also common. Patients should practice good dental hygiene, including regular brushing and flossing.

**Leflunomide.** Leflunomide(Arava) is a FDA approved oral DMARD drug approved in 1998. The mechanism of action of its active metabolite, teriflunomide, is the inhibition of dihydroorotate dehydrogenase (DHODH), a mitochondrial flavin dependant enzyme that is central in the de novo synthesis of pyrimidines [27]. This pathway is used by highly dividing cells when the supply of nucleotides through the salvage pathway becomes limiting. Thus, teriflunomide acts as a general antiproliferative molecule and most specifically as an immunosuppressant as it inhibits proliferation of T- and B-activated lymphocytes. The efficacy of
leflunomide in RA is comparable with that of methotrexate [28]. Leflunomide has a long half life of several weeks in the body and about two weeks in the blood. This can be advantageous as missing a dose of drug is not going to affect the patient. On the other hand it can be disadvantageous as the patients suffering from side effects of Leflunomide are going to take longer time to recover [29]. Common side effects include hepatotoxicity, gastrointestinal upset, alopecia, and predisposition to infection [30]. Leflunomide is a known inhibitor of CYP2C9 which allows metabolic intermediates to persist and thus liver toxicity occurs. These symptoms are the major reason for the high rate of discontinuance of Leflunomide [31]. It also can lead to liver toxicity related diseases and at certain times cardiac related complications [32]. Various studies are available where they have taken analogues of Leflunomide [teriflunomide] and evaluated it as inhibitors of DHODH using different insilico workflow. Synthetic biaryl analogues of teriflunomide were developed using structure based methods and evaluated as potent in vitro and in vivo models [33]. Structure activity relationship based study of 4-Quinoline Carboxylic Acid Analogue of the inhibitor of brequinar was explored in another study [34]. Novel inhibitors of human DHODH were obtained from Molecular docking and QSAR methods. Other studies combined the two well known inhibitors of human DHODH like Leflunomide and Brequinar to obtain a common scaffold based on which new novel synthetic compounds were synthesized [35].

1.2 MEDICINAL PLANTS IN INDIA FOR RHEUMATOID ARTHRITIS

Plants have always been the principle tools of traditional medical systems. The WHO has estimated that majority of the world’s population depends on botanical medicines for basic healthcare needs [36]
Figure 1.11 Pie chart representing research on different human diseases in A: ayurveda and B: US pharmacopeia (1994)

This graph was represented in a study published in 2001 at the institute of economical botany, Newyork [36]. Figure 1.11 gives the various diseases for which ayurveda can be used as a cure. Compared to the DRUGS in the US pharmacopeia Ayurveda offers much higher benefits. Some of the common medicinal plants mentioned and used in ayurveda are

- *Commiphora wightii* (guggul) reducing obesity, as well as in the treatment of rheumatoid arthritis, osteoarthritis and sciatica.

- *Withania somnifera* (Indian ginseng) berries and leaves are applied externally to tumors, tubercular glands, carbuncles, and ulcers.
- *Ocimum tenuiflorum* (Holy Basil) It is an elixir for cough; the leaves when chewed after meals acts as a digestive, and when taken before and after cold water bath controls temperature in the stomach and prevents cold

- *Phyllanthus emblica* (Indian gooseberry) used for digestion, reducing fever, purifying blood, treat constipation, alleviate asthma, strengthen heart.

Among the many ayurveda plants used, *C. halicacabum Linn.* is an important medicinal twining herb distributed throughout India. Different parts of the plant are used in herbal medicine. [37] Roots and leaves are used to treat fever, arthritis and chronic rheumatism. Seeds are diaphoretic and used in tonics. The plant has sedative action on the central nervous system [38]. Direct antifilarial activity of the plant extract has also been reported [39]. This herb which is used to treat rheumatic arthritis from olden times is of great interest to the scientific community of south India as a number of publications related to its usefulness can be observed online.

### 1.2.1 *Cardiospermum halicacabum*

The biodiversity of India renders it with thousands of species that are known to have medicinal uses and different parts of several medicinal plants are used to cure specific ailments since ancient times [40]. Medicinal plants are valuable natural resources and regarded as potentially safe drugs and it is well known that most synthetic drugs have their origin from plant products [41]. Scientific interest in medicinal plants has flourished due to the increased efficiency of plant derived drugs and rising concern of synthetic drugs.

*Cardiospermum halicacabum* is a widely occurant shrub or climbers in most continents. The genus *Cardiospermum* L. of the Sapindaceae family consists of 17 species that are mostly occurring in the moist tropical and subtropical regions. *Cardiospermum* species have been extensively moved around the world for both
their medicinal [42,43] and ornamental [44] values. It is one among the "Ten Sacred Flowers of Kerala State in India, collectively known as Dasapushpam. *C. halicacabum* or Balloon vines is categorized as a weed by the FDA in America and other western countries like South Africa, and Australia. In India, *C. halicacabum* leaves are a commonly consumed leafy vegetable. Indian system of medicine namely ayurveda and siddha recommends *C. halicacabum* leaves for rheumatism, chronic bronchitis, stiffness of limbs, snakebite, earache and swellings. [46].

*Figure 1.12 (clockwise) Cardiospermum halicacabum* (balloon vine, Modukattan) plant, Fruit as an inflated balloon and the heart shaped indented seeds.

The plant is a perennial herbaceous shrub with a cosmopolitan distribution in India. The average length of *C. halicacabum* is 1–3 metres with white or yellow flowers.

*C. halicacabum* predominantly invades wood- and grasslands which highlight its threat to agricultural plantations [46]. The two ancient systems of medicine in India like the Ayurveda and the Siddha recommend the leaves of *C. halicacabum* for rheumatoid arthritis and nerve related disorders.

Either the tea brewed with these leaves or a vegetable made out of the leaves is believed to lessen the pain associated with the RA. Also a poultice made
from the leaves can lessen swelling as well as the juice can relieve earaches. In America, *C.halicacabum* is used as one of the ingredients in “Allergy Relief Liquid TM” and “Bioforce Pollinosan® Tabs” marketed by Bioforce USA as a natural relief for hay fever, allergies, sneezing, watery eyes, and allergic reactions. Another US based company, Boericke and Tafel produces “Florasone Cardiospermum Cream” for skin ailments such as swelling, scaling, blisters/vesicles, burning and pain. These products are supported by the various claims concerning the many medicinal properties of balloon vine. The Department of Ayurveda, Yoga, naturopathy, siddha and homeopathy [AYUSH], Govt. of India, categorizes *C. halicacabum* as one of 48 herbs that are economically beneficiary according to a report published in 2007[47].

### 1.3 INSILICO PHARMACOLOGY

It is a part of Rational drug Designing where the structure of the target protein is the prime factor that determines the course of action to be taken and suitable computational chemistry techniques to be chosen for decreasing the time taken for a hit to be converted to a drug. This Hit to Drug methodology using computers is *Insilico* Pharmacology.

![Figure 1.13 Insilico Pharmacology methods](image)
One of the new techniques that have been recently developed includes a structure based pharmacophore or an E-pharmacophore that abstracts the interactions of a known potent ligand of a receptor to specify steric and electronic features as a model to specify the activity of the ligand. This Hypothesis can then be used to screen potential novel chemical compounds to obtain a novel hits. This type of methodology was used in finding novel inhibitors for the NSP2 protein of chickungunya virus. The pharmacophore screening can be used for other drug designing aspects such as lead optimization, ADMET studies and chemogenomics [48].

Molecular docking comprises of following some scoring matrix to elucidate the optimal pose and conformation of a receptor and ligand in a binding site. Two aspects of docking are the pose generation and scoring evaluation which results in a list compound and its optimal poses that are near to the actual native structure. This molecular docking is used as a heuristic tool in virtual screening where a large numbers of small molecules from a database are screened and docked at the binding sit to obtain sizable hits which can be filtered and analyzed for optimal docking poses.

1.3.1 Computational Analysis of Phytochemicals for Treatment of Disease

Presently, there is a dramatic increase in interest in the field of natural product based drug research because of structural diversity, cost effectiveness, easy availability, better tolerability, lesser side effects, etc. It has been estimated that around 70% of new chemical entities launched into medical practice in the past 25 years were obtained directly or indirectly on the basis of natural products [49]. Initially in silico techniques were limited to synthetic molecules, but currently there is a lot of interest to use the same in medicinal plant based drug research. There are several databases available including, the Dictionary of Natural Products which provides structural information about bioactive metabolites that could be used in virtual screening to predict biological activity that could be confirmed through testing in an appropriate assay. Recent medicinal Natural products or phytocompounds are a gold mine of treatment for different disease. Among various types of Arthritis, Rheumatoid arthritis which is most prevalent, is a classic condition of the immune system being highly sensitized to self than non self and
leads to up regulation of proinflammatory compounds. There are many naturally occurring phytochemicals like flavanones, terpenes, quinines, catechins, anthocyanins, alkaloids and anthoxanthins are known antiinflammatories. Various in vitro and in vivo studies have been used in the past to characterize them as anti inflammatory in nature. However, nowadays various computational methods are used to shortlist a set of phytomolecules which are later proven using the conventional laboratory techniques.

1.4 AIM AND OBJECTIVES

To determine phytochemical inhibitors from Cardiospermum halicacabum for human dihydroorotate dehydrogenase – target for rheumatoid arthritis based on the already existent drug Leflunomide and evaluate whether the alternative phytomolecules would be efficient in activity with the natural variants of DHODH in population. In other words, new mechanism of action of C. halicacabum against Human DHODH is assessed using insilico methods.

OBJECTIVES

1. To screen known phytochemicals of Cardiospermum halicacabum against a E-pharmacophore built on existing drug Leflunomide (Active form-A771726)

2. Determining the behavior of active ingredients of Cardiospermum halicacabum against the receptor- DHODH through molecular docking and dynamics study and cross validate the docking results obtained.

3. The safety of the phytomolecules shortlisted is evaluated using a ligand based pharmacophore approach and drug likeness approach.

4. The activity of the phytomolecules is checked against other inflammatory targets of rheumatoid arthritis as well as compared to other phytocompounds used in RA

5. SNP Variants of DHODH – and its effect on inhibitory activity of lead like compounds.
1.5 OUTLINE OF THESIS

DHODH is a key Enzyme in the inflammatory mechanisms that’s helps in the proliferation of immune cells. Inhibition of DHODH is a key strategy in controlling RA. Traditionally used of *C.halicacabum* have good therapeutic potential in RA research.

Chapter 1 includes a brief introduction about RA, its symptoms, risk factors, histopathology, drugs used and targets of RA. The available inhibitors and their side effects are outlined. It also includes a short briefing on computer aided drug designing approaches.

Chapter 2 comprises of literature review related to Rheumatoid arthritis and different methodologies applied in Insilico Computer aided drug design.

Chapter 3 comprises of the materials and methods involved throughout the study. The workflow in HTVS, pharmacophore based screening and validation methods for the identification of novel inhibitors are presented in this chapter.

Chapter 4, To screen known phytochemicals of *Cardiospermum halicacabum* against a E-pharmacophore built on existing drug Leflunomide (Active form-A771726).Determining the behavior of active ingredients of *Cardiospermum halicacabum* against the receptor- DHODH through molecular docking and dynamics study and cross validate the docking results obtained.

Chapter 5, includes the results obtained from The safety of the phytomolecules shortlisted is evaluated using a ligand based pharmacophore approach and drug likeness approach.

Chapter 6 includes The activity of the phytomolecules is checked against other inflammatory targets of rheumatoid arthritis as well as compared to other phytocompounds used in RA

Chapter 7 includes effect of SNP on human DHODH on drug Binding

Chapter 8 concludes the findings from the thesis work.