In vitro screening of potential Carbonic Anhydrase-II inhibitors as novel anti-resorption therapeutic agents in Rheumatoid Arthritis

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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Dedication

I dedicate this humble

effort to my parents,

for their infinite love,

faith and support.

Acknowledgement

First and foremost, I would like to express my profound gratitude to Allah Almighty, the Omnipotent and the Omnipresent, for His countless blessings. This thesis marks the culmination of a very important phase of learning for me, and I would like to take this opportunity to acknowledge all those who have played a phenomenal role during this course of learning for me.

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Nida Ali Syed

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LIST OF ACRONYMS

Acronym	Abbreviation
1, 25$(OH)_2D_3$	1,25-dihydroxycholecalciferol/1,25-dihydroxyvitamin D3
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
Akt	Protein Kinase B
ATP	Adenosine Triphosphate
Bcl	B-cell lymphoma CLL
CA	Carbonic Anhydrases
Cad-11	Cadherin-11
CA-II	Carbonic Anhydrase-II
CAIs	Carbonic Anhydrase Inhibitors
CCR	Chemokine Receptor
CD	Cluster of Differentiation
cDNA	Complementary DNA
ClC	Chloride channel
CO_2	Carbon dioxide
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte Antigen 4
CXCR	C-X-C Chemokine receptor
DAS	Disease Activity Scoring
DEPC	Diethyl Pyrocarbonate
DMARD	Disease Modifying Anti-Rheumatic Drugs
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay

ESR	Erythrocyte Sedimentation Rate
EtBr	Ethidium Bromide
EULAR	European League Against Rheumatism
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FLS	Fibroblast like Synoviocytes
GM-CSF	Granulocyte-Macrophage Colony-Stimulating factor
H^+	Hydrogen ion
hCA-II	Human Carbonic Anhydrase-II
HCO_3^-	Hydrogen carbonate ion
HLA	Human Leukocyte Antigen
ICAM-I	Intercellular Adhesion Molecule-1
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-1RA	IL-1 Receptor Antagonist
JAK	Janus Kinase
LFA-I	Lymphocyte function associated antigen-1
LMA	Low Melting Agarose
MAPK	Mitogen Activated Protein Kinase
mL	Millilitre
MLS	Macrophage-like Synoviocytes
MMP	Matrix metalloproteinase
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Na^+	Sodium ion
NBC1	Sodium Bicarbonate Co-transporter 1
NF-kB	Nuclear Factor-Kappa B
NHE1	Sodium Hydrogen Antiporter 1
NMA	Normal Melting Agarose
NSAID	Non-steroidal Anti-inflammatory Drugs
PADI4	Peptidyl deiminase
PDGF	Platelet Derived Growth Factor
pH	Power of Hydrogen
PI3K	Phosphatidyl inositol-3 kinase
PTH	Parathyroid hormone
PTPN22	Protein tyrosine phosphatase non-receptor type-22
RA	Rheumatoid Arthritis
RA-FLS	Rheumatoid Arthritis-Fibroblast like Synoviocytes
RANK	Receptor Activator of Nuclear Factor Kappa-B
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RASF	Rheumatoid Arthritis-Synovial Fibroblasts
RF	Rheumatoid Factor
RNA	Ribose Nucleic Acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT- qPCR	Real Time- Quantitative Polymerase Chain Reaction
SAARD	Slowly Acting Anti-Rheumatic Drugs
SCID	Severe Combined Immunodeficiency
SDF-1	Stromal Cell Derived Factor-1
STAT	Signal transducer and activation of transcription
TGF- β	Transforming Growth Factor- β
Th	T-helper cell

TIMP	Tissue Inhibitors of Matrix Metalloproteinases
TLR	Toll like receptor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNF-α	Tumor Necrosis Factor-alpha
TRAF1	TNF receptor-associated factor 1
Ug	Microgram
uL	Microliter
uM	Micromolar
V-ATPase	Vacuolar Adenosine Triphosphatase
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLA4	Very Late Antigen 4
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XTT	(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

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ABSTRACT

Rheumatoid Arthritis is a systemic autoimmune disease characterized by chronic inflammation of joints, destruction of cartilage and autoantibody production. Oxidative stress has been recently implicated in the pathogenesis of Rheumatoid Arthritis and Carbonic Anhydrase II (CA-II) has been identified to be a major part of the nexus of pathophysiological components inducing reactive oxygen species, inflammation and autoimmunity which eventually leads to bone resorption and cartilage destruction. The aim of this project was to identify a novel role for Prazosin and Sulpiride as Carbonic Anhydrase-II inhibitors. This was done via *in vitro* testing of these inhibitors in MH-7A human arthritic cell line, followed by the evaluation of DNA damage induction and cytotoxicity via Comet Assay and MTT Assay respectively. Post-transcriptional expression levels of CA-II in conjunction with those of TNF-α and MMP-9 were determined via qRT-PCR. Prazosin and Sulpiride were both shown to have noncytotoxic and DNA protective effect on MH-7A cells. At the post-transcriptional level, only Sulpiride effectively downregulated the expression of CA-II. These findings will help potentiate their role as anti-resorptive therapeutic agents and open a new avenue of treatment options available to Rheumatoid Arthritis patients.

CHAPTER 1

INTRODUCTION

Rheumatoid Arthritis (RA) is the most common autoimmune disease marked by inflammation and joint destruction. Although it has an unknown etiology, an interaction between various environmental and genetics factors contributing towards the pathogenesis of Rheumatoid Arthritis has been reported. Primarily synovial joints are affected in RA, as one of the key initial events in its pathogenesis is chronic inflammation of synovium alongside destruction of articular cartilage and bone. Normal synovium is a one to three cell thick layer covering the edges of joints, which provides the essential nutrients and lubrication to cartilage. In RA, synovial hyperplasia occurs with an increased inflammation of the synovium, resulting in the formation of an invasive tissue 'pannus' which expands to adjacent cartilage and subchondral bone, resulting in bone and cartilage erosion (Xue *et al*., 2014).

Fibroblast-like synoviocytes form a major part of this inflamed synovium and resulting pannus tissue and are reported to play a role in both the initiation and progression of the disease, specifically in the destruction of articular structures. Rheumatoid Arthritis Fibroblast-like Synoviocytes (RA-FLS) have a tumor-like; transformed phenotype characterised by resistance to apoptosis, prolonged survival, increased proliferation rate and invasiveness. Resultantly, they directly invade cartilage and bone and destroy the cartilaginous matrix. Destruction of cartilage is an early manifestation of RA mediated by an increased activity of proteolytic enzymes such as matrix metalloproteinases. Therefore

inhibition of progressive joint damage mediated by RA-FLS is a key treatment goal in RA (Luczkiewicz, 2014).

1.1 Epidemiology of Rheumatoid Arthritis

Several prevalence and incidence studies for Rheumatoid Arthritis have been conducted so far that have highlighted a considerable variation in disease occurrence amongst different populations. Rheumatoid Arthritis is reported to affect 0.5-1.0% of the world's adult population. Incidence of RA is more in females than males, with the reported female to male ratio ranging from 2:1 to 3:1. Reproductive and hormonal factors are considered as a major influence on the occurrence of this disease, however due to conflicting experimental data, it's unclear as to how gender is a risk factor for RA. Specifically, estrogens have been shown to stimulate the immune system. It has a prevalence of 0.5-1.1% in Northern Europe and North America whereas 0.3-0.5% of the population in Southern Europe is reported to suffer from RA. Studies from developing countries have reported a relatively low prevalence of the disease, in the range of 0.1-0.5% and even lower for rural Africa. Time-bound prevalence studies have shown a general overall decline in the prevalence of RA globally. However this may be attributed to better access to healthcare facilities with the passage of time. Another important consideration in this regard is that the case identification and ascertainment criteria have changed over the time (Alamanos and Drosos, 2005).

1.2 Pathophysiology of Rheumatoid Arthritis

Rheumatoid Arthritis has been very aptly described as a syndrome since it harbours various disease subsets during the course of its pathogenesis, starting with inflammation

and culminating in persistent synovial inflammation and associated articular damage to cartilage and bone.

Inflammation is a direct consequence of overproduction of TNF-α and drives both synovial inflammation and joint destruction. TNF-α is overproduced due to an interaction between T and B lymphocytes, fibroblast-like synoviocytes and macrophages, which also leads to the production of various cytokines such as IL-6, a potent driver of persistent inflammation and joint destruction. Overproduction of another proinflammatory cytokine IL-1 is induced by a different process.

The most commonly affected cell populations in rheumatoid arthritis are synovial and cartilage cells. Synovial cells are of two main type; fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes (MLS). MLS are involved in overproduction of proinflammatory cytokines whereas FLS show abnormal behaviour in rheumatoid arthritis; invasion of cartilage and joint destruction. Osteoclast activation is a key event in the process of bone erosion, and inhibiting osteoclasts activation reduces joint destruction but does not alleviate inflammation. It is still not clear if arthritis starts within the bone and then spreads to the joints or it's the other way around. Altered behaviour of FLS in rheumatoid arthritis shows that they can spread between joints, suggesting how polyarthritis might actually develop. Extent of immune inflammation depends upon of the number and strength of various cell types in the joints (Figure 1.1).

Production of autoantibodies such as Rheumatoid Factor (RF) and Anti-citrullinated protein antibody (ACPA) is also a typical characteristic of RA. IgM and IgA rheumatoid factors are key pathogenic biomarkers against the Fc fragment of IgG. Usually, 50-80% of rheumatoid arthritis patients are RF positive as well as ACPA positive.

However, APCA is considered as a more specific and sensitive marker of disease diagnosis and prognosis such as progressive joint destruction. There is ongoing research for the identification of antibody specificities that correlate either to a specific disease stage or patient subset (Scott *et al.*, 2010).

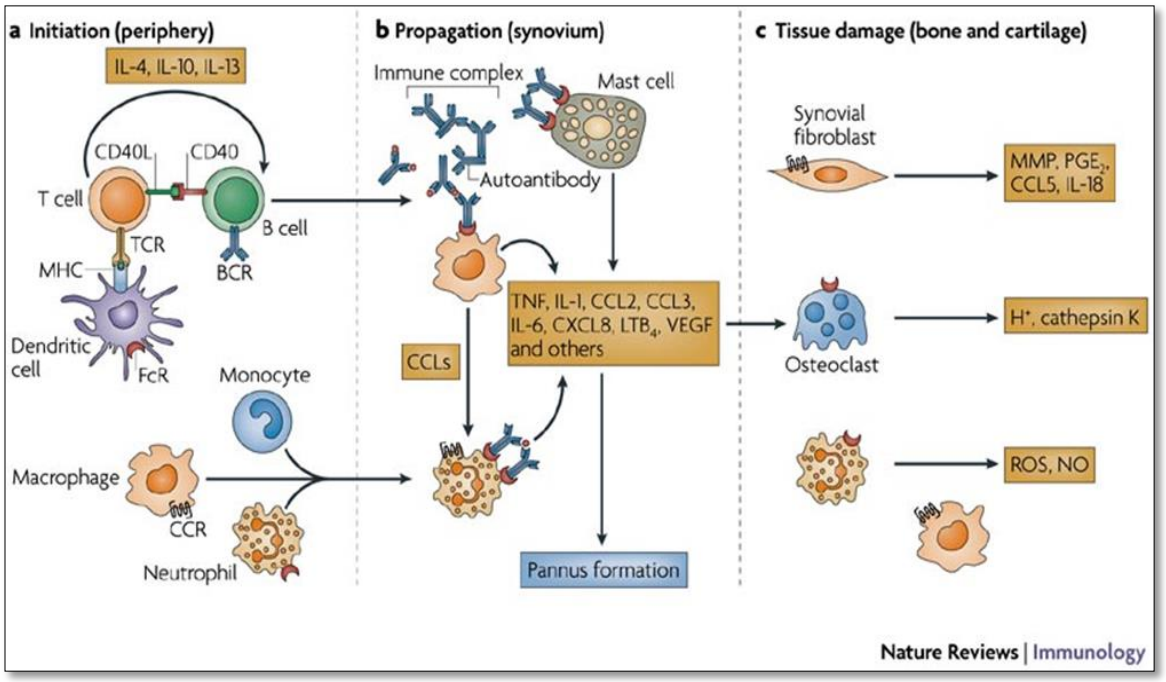


Figure 1.1. Stepwise progression of the development of Rheumatoid Arthritis. (A) Disease initiation takes place in peripheral lymphoid organs. (B) Disease propagation is mediated by immune complexes binding to Fc receptors (FcRs) on macrophages, neutrophils and mast cells. (C) High levels of cytokines and chemokines activate and recruit synovial fibroblasts, macrophages, osteoclasts and neutrophils, which release proteases, reactive oxygen species, nitric oxide and prostaglandin E2. Synoviocytes are also activated and recruited, and these cells invade cartilage. All steps collectively contribute to irreversible bone and cartilage destruction (Rommel *et al.*, 2007).

1.3 Diagnosis

Primary diagnosis of RA is made on the basis of clinical signs and patients symptoms. First ever criteria for the diagnosis of RA was established by the American Rheumatism Association in 1987 and underwent a modification in 2010 which is therefore referred to as the American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) criteria 2010 (Table 1.1). The ACR/EULAR 2010 criteria

allow physicians to make a more in-depth and early diagnosis based on the positive presence of RF, anti-CCP and any sign of early disease management and to administer effective therapy (Aletaha *et al*., 2010).

1.3.1 Disease Activity Scoring 28 system

The Disease Activity Scoring system, also referred to as DAS 28 is a system for evaluation of disease propensity in a patient. This evaluation is made on the basis of a scoring system based on 28 different joints in the body for evaluation of RA. The score is based on presence of inflammation in 28 joints which are most susceptible for RA, alongside serum levels of C-reactive protein (CRP) and Erythrocyte Sedimentation Rate (ESR) (Inoue, 2007).

Table 1.1: A comparison of the conventional and new classification criteria of rheumatoid arthritis (Scott *et al*., 2010).

ACR 1987 Criteria	Early Arthritis Prediction Criteria 2007	ACR/EULAR Criteria 2010
1. Morning stiffness (at least 1h)	1. Age (multiply by 0.02)	1. Joint involvement (0–5) • One medium-to-large joint (0) • Two to ten medium-to-large joints (1) • One to three small joints (large joints not counted) (2) • Four to ten small joints (large joints not counted) (3) • More than ten joints (at least one small joint) (5)
2. Arthritis of three or more joint areas	2. Sex (female 1)	2. Serology (0–3) • Negative RF *and* negative ACPA (0) • Low positive RF *or* low positive ACPA (2)

		• High positive RF *or* high positive ACPA (3)
3. Arthritis of hand joints (≥1 swollen joints)	3. Distribution of involved joints • Small joints hands and feet (0.5) • Symmetrical (0.5) • Upper limbs (1) or upper and lower limbs (1.5)	3. Acute-phase reactants (0–1) • Normal CRP *and* normal ESR (0) • Abnormal CRP *or* abnormal ESR (1)
4. Symmetrical arthritis	4. Morning stiffness (visual analogue scale) • 26-90mm (1) • >90mm (2)	4. Duration of symptoms (0–1) • Less than 6 weeks (0) • 6 weeks or more (1)
5. Rheumatoid nodules	5. Number of tender joints • Four to ten (0.5) • 11 or more	
6. Serum rheumatoid factor	6. Number of swollen joints • Four to ten (0.5) • 11 or more	
7. Radiographic changes (erosions)	7. C-reactive protein (mg/L) • 5-50 (0.5) • 51 or more (1.5)	
	8. RF positive (1)	
	9. ACPA positive (2)	
Four of these seven criteria must be present. Criteria 1–4 must have been present for at least 6 weeks.	Points are shown in parentheses. Cut-point for rheumatoid arthritis is 8 points or more.	Points are shown in parentheses. Cut-point for rheumatoid arthritis 6 points or more. Patients can also be classified as having rheumatoid arthritis if they have: (a) typical erosions; (b) long-standing disease previously satisfying the classification criteria.

ACR 1987 criteria were designed to classify established rheumatoid arthritis. 2010 ACR/EULAR criteria are intended to classify both early and established disease. Prediction models such as the van der Helm model were designed to identify patients with early undifferentiated arthritis who are most likely to subsequently meet criteria for rheumatoid arthritis.

1.4 Risk Factors in Rheumatoid Arthritis

Rheumatoid Arthritis is known to be a multifactorial disease since it includes an interplay between genetic and environmental factors which directly impact its prevalence. Several environmental factors have been studied with regards to their association with RA either as a risk factor for developing the disease or as a marker of worse prognosis but there is a lack of concrete data/findings in both of these aspects (Figure 1.2). On the other hand, sufficient epidemiological evidence stemming directly from research studies has accumulated in the recent years in support of various genetic factors being related directly to an increased risk of developing RA. Age of onset in RA has been frequently referred to as the fifth decade of life-span, and there are a few reports that suggest an even later age of onset. Furthermore, smoking is a recognized risk factor of disease severity and worse prognostic outcome of RA in a dose-dependent manner.

Socio-economic factors such as occupation, education, marital status and social group mainly influence the course and prognostic outcome of RA than the risk of developing the disease. Several reports have suggested an association of adverse socioeconomic status with worse disease prognostic outcome. Infectious agents such as Alphavirus, *Borrelia burgdorferi*, Epstein-Barr virus, *Escherichia coli*, Hepatitis B virus, human parvovirus B19, human retrovirus 5, *Mycobacterium tuberculosis*, Mycoplasma, *Proteus mirabilis*, *Porphyromonas gingivalis* and Rubella virus are amongst the pathogens reported to trigger rheumatoid arthritis (Tobón *et al.*, 2010). Some studies have suggested that infectious agents may trigger the disease in individuals that are already genetically susceptible to RA. In addition, increased titers of autoantibodies in RA patients have also been reported and attributed to the above mentioned infectious agents; however a distinct

causative role of infectious agents has not yet been identified. This is primarily due to absence of any relevant data, in terms of time or space cluster, regarding occurrence of infections or a specific infection in rheumatoid patients that would directly suggest an association between the two (Alamanos and Drosos, 2005).

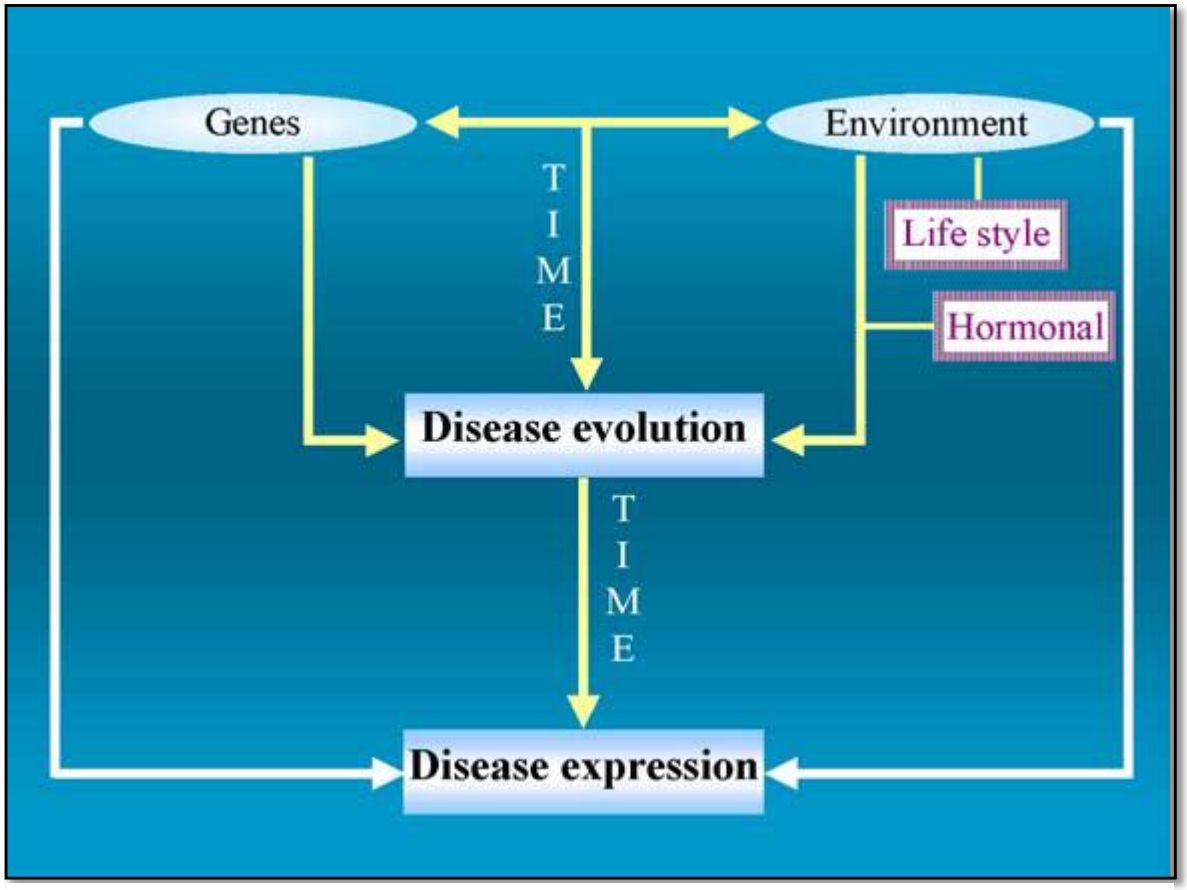


Figure 1.2. Hypothetical model of rheumatoid arthritis causation: interaction of genes with environmental factors, as well as with hormonal and lifestyle factors over time in an individual, may give rise to disease evolution and its expression (Alamanos and Drosos, 2005).

1.4.1 Genetic Risk Factors associated with Rheumatoid Arthritis

Genetic factors contribute to a 50% to 60% risk of developing Rheumatoid Arthritis. In this regard, the most strongly associated genetic factor with RA is the HLA-DRB1 gene in the major histocompatibility complex region, whose specific alleles in the DRB1*04 and

*01 clusters encode for the shared epitope sequences within the expressed DRB1 molecule (Gregersen *et al.*, 1987). One-third of the genetic susceptibility to RA has been attributed towards HLA-DRB1.

In addition to HLA-DRB1, another key susceptibility gene is the tyrosine-phosphatase gene PTPN22 located on chromosome 1. A missense C-to-T substitution at nucleotide position number 1856 results in the substitution of tryptophan (W) for arginine (R) at residue 620 of the final protein product. This leads to a gain of function in the form of an enhanced T-cell receptor signaling during thymic selection, which allows autoantigen-specific T cells to escape clonal deletion, therefore predisposing to autoimmunity (Begovich *et al.*, 2004). It is interesting to note that this polymorphism has not been reported in Asian population. The European population shows an exponential co-relation between smoking, shared epitope of HLA-DRB1 as well as PTPN22 polymorphism. It is pertinent to mention here that HLA-DRB1 shared epitope and PTPN22 represent the two key risk alleles that are only associated with the ACPA positive, RF positive or both ACPA and RF positive patients.

In addition to these, polymorphisms in TRAF1-C5 and TNFAIP3 have also been reported in RA. STAT4, IL-2/IL-21 and CTLA-4 have been identified as non-HLA risk factors in European population although a genome-wide association has not been shown (Plenge *et al.*, 2005; Remmers *et al.*, 2007).

In a comparison between populations, HLA-DRB1 is a common risk factor associated with RA in both Asian and European populations (Lee *et al.*, 2009). Polymorphisms in PTPN22 have been reported in European populations but are rare in Asian population. On the other hand, polymorphisms of PADI4, which encodes an enzyme

that converts the arginine residues of proteins into citrullines that are then recognized by ACPA in the sera of RA patients, are more common in Asian population than in European population (Lee *et al.*, 2009).

1.5 Role of Cytokines in Rheumatoid Arthritis

Cytokines act as chemical messengers between cells and several have been implicated in triggering or regulating the inflammatory response during the pathogenesis of Rheumatoid Arthritis. IL-1, IL-6, IL-8, IL-17, IL-21, tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are typically over-expressed in RA patients. These are involved in synovial membrane inflammation and osteocartilaginous resorption via osteoclastic mediators.

Autoreactive T-cells, such as Th1 and Th17 are the major mediator of autoimmunity in the pathogenesis of RA. A greater proportion of synovial fluid mononuclear cells expresses interferon gamma (IFNγ) whereas a lesser proportion of peripheral blood mononuclear cells expresses IFNγ. Levels of IL-12, a Th1 cytokine are elevated in both synovial fluid and serum and correlates with disease activity score. Levels of IL-17, a cytokine produced by Th17 cells are also elevated in both serum and synovial fluid of RA patients. Neutralisation of IL-17 has shown to reduce disease severity and progression in a collagen-induces arthritis mouse model for rheumatoid arthritis.

Elevated levels of IL-21 and IL-22 have also been observed in RA, both are produced by Th17 cells. IL-21 autoregulates its own synthesis in human CD4+ T-cells and also enhances the proliferation of Th17 cells. IL-23 has been shown to promote production of IL-17 in RA fibroblast-like synoviocytes, and in a loop, it is also upregulated by IL-17 via the PI3-kinase/Akt-, NF-kB-, and p38-MAPK-mediated pathways.IL-23 has been

demonstrated to induce osteoclast differentiation via upregulation of RANK and RANKL in FLS. RANK/RANKL signalling is required not only for the activation of osteoclast activation, differentiation and survival in normal bone remodelling as well as in pathological conditions. Expression of p19 subunit and RANKL have been positively co-related with in synovial tissue in RA.

A large number of innate immune cells produce IL-1, known to induce bone erosion and cartilage destruction. In conjunction with NF-kB, it regulates the production of proinflammatory cytokines in the inflamed synovium. NF-kB activation stimulated the production of MMPs in FLS, which degrade the collagen in cartilage and bone matrix, leading to joint deformity and pain in RA.

IL-6 is an important proinflammatory cytokine involved in the differentiation and activation of B and T cells, macrophages, osteoclasts, chondrocytes, endothelial cells and haematopoiesis in the bone marrow. Alongside TGF-β, it is also involve in induction of Th17 cells via STAT3.

TNFα is produced locally in the joints by synovial macrophages and the lymphocytes infiltrating the synovium. It is recognized as a key driver of pathogenic cytokine production that leads to tissue damage.

1.6 Bone Resorption in Rheumatoid Arthritis

Bone is a rigid tissue based component of the vertebrate endoskeleton that provides structure, support, protection and facilitates movement. It contains bone marrow from where most of the blood bells originate and also serve as a metabolic store for minerals, growth factors and fat. It is made up of two main types of cells: osteoblasts and osteoclasts.

Osteoblasts are mononuclear cells responsible for bone formation whereas osteoclasts are multinuclear cells responsible for bone resorption.

Normal bone homeostasis is achieved only when the activities of these two cells are well co-ordinated and a balance between bone formation and bone resorption is achieved. Disturbed bone homeostasis i.e. imbalance in the rates of bone formation and resorption is the singular underlying cause of bone loss in RA. Bone loss is an early manifestation of RA; marked with accelerated hand bone mineral density loss alongside progressive joint disease in the hands and feet in the first year of RA and its extent often co-relates with disease progression. On the cellular level, bone resorption depends on the ability of the osteoclasts to generate an acidic extracellular environment which is deemed necessary for the solubilisation of the alkaline salt and organic components of the bone matrix. RA presents as an ideal pathological model for gaining insight into the effect disturbed pH homeostasis on bone metabolism. Bone loss is a crucial target for developing effective therapeutic interventions which help alleviate early signs of RA; when the potential of reversibility is the also the greatest.

1.6.1 Role of Carbonic Anhydrase II in Bone Resorption

Carbonic Anhydrase (CA) is a family of enzymes having sixteen members or isoforms. Thirteen members are enzymatically active, recognised for catalyzing the reversible hydration of CO_2 and dehydration of carbonic acid, as represented by the equation below:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

These enzymes have been classified further into four subgroups on the basis of their cellular localisation: (1) cellular: CAI, II, III and IV (2) mitochondrial: CAV (3) membrane-bound: CAIV, IX, XII and XIV and (4) secreted: CAVI. Various studies conducted in the past decade have shown that these enzymes are involved in numerous physiological processes such as CO_2 transport, pH regulation, ion transport, bone resorption and calcification (Alver *et al*., 2011).

In the osteoclast cytoplasm, CAII primarily generates the H^+ ions which are then released into the extracellular space between the osteoclast and the bone surface via the H^+-ATPase pump and required levels of acidification for bone resorption and osteoclast activation are achieved. Acidification of the extracellular compartment is directly associated with increased osteoclast bone resorption activity (Heymann, 2002).

1.6.2 Carbonic Anhydrase Inhibitors

Carbonic Anhydrase inhibitors represent a class of pharmaceutical compounds that inhibit the ubiquitous family of enzymes-Carbonic Anhydrases. These comprise of a variety of chemical groups such as anions, imidazole, phenol, hydroxyurea, carboxylates, organic phosphates and phosphonates, and various sulfonamide derivatives such as sulfamates, hydroxysulfonamides and hydroxamates. Sulfonamides in particular and CAIs in general have found increased utility over the years as these have been extensively used as diuretics, anti-glaucoma, anti-convulsant, anti-obesity, anti-cancer, anti-pain, and anti-infective drugs apart from inhibiting Carbonic Anhydrases (Supuran, 2010).

1.7 Treatment of Rheumatoid Arthritis

Rheumatoid Arthritis therapy has undergone a dramatic change in the past twenty years. The earliest pharmacological interventions used for treatment was aspirin and other NSAIDs. This was replaced by the prolific Disease Modifying Anti-Rheumatic Drugs (DMARDs), which refer to a heterogeneous group of pharmacological agents that possess specific anti-rheumatic properties. These were earlier referred to as slowly acting anti-rheumatic drugs (SAARDs) since their therapeutic effect takes a long duration of administration to become apparent. Commonly used DMARDs include methotrexate, sulfasalazine, chloroquine and hydroxycholoroquine, leflunomide, injectible gold salts, auranofin, ciclosporin A and azathioprine. Most of these are administered in combination with methotrexate.

Prednisone belongs to the glucocorticoid class of drugs that have been used for RA therapy. The second class of therapeutic agents that have opened new horizons in RA treatment are collectively referred to as biologic agents. The term biologic agents or biologics refers to complex protein molecules created using molecular biology methods and produced in prokaryotic or eukaryotic cell cultures. These have predefined biological activities and are targeted towards a specific cytokine or cell surface molecule, hence it is possible to predict their activities both in terms of efficacy and toxicity. However, there are certain disadvantages associated with the use of biologics such as the associated use of regulators and quality control issues. Etanercept, infliximab, adalimumab, certolizumab pegol and golimumab are biologic agents that are being used as anti-TNF agents in RA therapy. When used in combination with methotrexate, almost complete inhibition of joint destruction has been observed.

IL-1RA (receptor antagonist) is the recombinant form of the naturally occurring IL-1 antagonist, commercially known as Anakinra. However, it is used for RA therapy less frequently due to its side-effects. Rituximab is a B-cell depleting agent commonly administered to patients who do not respond to anti-TNF agents. Abatacept is a recombinant dimerized form of cytotoxic T-lymphocyte antigen 4 on Ig frame, which is a natural inhibitor of T-cell activation and has shown superior clinical efficacy in patients that do not respond to anti-TNF agents. Tocilizumab is the most recent addition to the list of biologics; it is an anti-IL-6 receptor blocker and has shown clinical efficacy in patients who do not respond to DMARDs or anti-TNF agents or both.

1.8 Aims and Objectives

The aim of the current study is to explore a potential role of Carbonic Anhydrase inhibitors as novel anti-resorption therapeutic agents for Rheumatoid Arthritis. In order to achieve this aim, the following specific objectives were investigated:

1. *In vitro* screening of potential Carbonic Anhydrase inhibitors (CAIs) namely Prazosin and Sulpiride in MH-7A human arthritic cell line.
2. Assessment of DNA damage in MH-7A cells following treatment with CAIs.
3. Expression profiling at post-transcriptional level, of key markers of inflammation and resorption following treatment of MH-7A cells with CAIs.

1.9 Impact of Study

Prevalence of Rheumatoid Arthritis in Pakistan ranges from 0.5% to 0.75% and the female population is affected three-folds higher than the male population. The proposed project is expected to make a contribution to the current range of therapeutic drugs available for the treatment of Rheumatoid Arthritis, especially in the area of anti-resorptive therapy in order to ameliorate the current treatment options available to Rheumatoid Arthritis patients.

CHAPTER 2

REVIEW OF LITERATURE

Rheumatoid Arthritis (RA) is described as a chronic, systemic, autoimmune inflammatory disease that primarily affects synovial joints, bringing about articular destruction and functional disability. A typical presentation of Rheumatoid Arthritis is the symmetrical peripheral arthritis; affecting mainly the small joints of the hands and feet. RA develops directly as a result of the characteristic transformation of the tissue lining the edges of the joint namely the synovium. During pathogenesis, synovium is infiltrated by inflammatory cells; as a result of which the resident synoviocytes undergo profound changes that facilitate the inflammatory process and results in formation of a pathological invasive tissue that destroys articular cartilage and bone. Synovial fibroblasts have emerged as the key pro-inflammatory disease promoting cells in RA (Noss and Brenner, 2008).

2.1 Normal Synovium and its evolution during Rheumatoid Arthritis pathogenesis

Normal synovium is a one cell thick layer that covers the marginal surface of articular cartilage, attaches to the bone and the fibrous joint capsule. It synthesizes the synovial fluid that provides essential nutrients to cartilage chondrocytes and lubrication to joint. The synovium has two layers, the outer subintimal layer which is one to three cell thick and the inner intimal layer of connective tissue interspersed with blood and lymphatic vessels, fibroblasts, interstitial macrophages, mast cells and nerve endings (Edwards, 2000).

The synovium is structurally unique in the sense that it is neither made up of epithelial cells nor endothelial, and also lack a true basement membrane. The subintimal layer of the synovium is made up of type A synoviocytes, commonly referred to as macrophage like synoviocytes (MLS) whereas the intimal layer of type B synoviocytes, commonly referred to as fibroblast like synoviocytes (FLS) (Helder, 1973). MLS are phagocytic cells derived from myeloid lineage of bone marrow stem cells, characterized by presence of a large number of lysosomes (Henderson and Pettipher, 1985). Clearance of cellular debris from joint spaces and defense against invading microbes are functions typically associated with MLS. On the other hand, FLS are derived from mesenchymal precursor cells, characterized by the presence of an elaborate rough endoplasmic reticulum. FLS are mainly responsible for the synthesis of extracellular matrix components supporting the synovial lining such as hyaluronan and lubricin, these form the essential components of synovial fluid for lubrication of joints. The synovium itself is a one cell thick layer through which cellular connections between joint space and synovial sublining can communicate (Henderson and Pettipher, 1985).

During the pathogenesis of RA, the synovium is drastically modified due to the infiltration of inflammatory cells and synovial lining hyperplasia. Predominant immune system cells in such a scenario are neutrophils, followed by T-lymphocytes and macrophages and to a lesser extent B-cells, plasma cells, dendritic cells and mast cells that accumulate in the synovial sublining (Godfrey, 1984). Infiltration of synovium by these cells is facilitated by increased angiogenesis, extracellular matrix production and synovial fibroblast proliferation (Norton, 1965).

Synovial hyperplasia is marked by an increase in the number of both types of synoviocytes, alongside an increase in the size of the lining from one to three cells thick to up to fifteen cells thick (Mor *et al.*, 2005). As a result, the outer layer merges with the inner layer of cells, and this thickened synovium eventually forms an invasive tissue mass called 'pannus' that invades the subchondral bone and cartilage (Zvaifler *et al.*, 1994).

2.2 Immunobiology of Rheumatoid Arthritis

The innate immune system is involved in the pathogenesis of RA in multiple dimensions. It begins with the infiltration of the synovial lining by various cellular components of the innate immune system. This includes the recruitment and activation of neutrophils, macrophages and mast cells; along with the formation of immune complexes on the cartilage surface, complement activation and Toll like receptor (TLR) stimulation (Brentano *et al.*, 2005; Bromley and Woolley, 1984; Godfrey, 1984; Neumann *et al.*, 2002). These factors have been shown to drive the process of inflammation in RA in experiments based on genetic deletions and antibody blockades.

The activation of the innate immune system results in the over-production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Firestein, 2003; Miossec, 2004). A significant proportion of treatment options available for RA are biologic agents against these cytokines, with those targeting TNF-α and IL-6 proving to be particularly effective in amelioration of RA symptoms (Maini *et al.*, 2006; Maini *et al.*, 1998).

The adaptive immune system also plays a critical role in RA pathogenesis. In this regard, B-cells rose to prominence since they are responsible for the production of specific autoantibodies against Rheumatoid Factor (antibodies directed against the Fc protein of

IgG) and citrullinated proteins which are a marker of disease severity and progression in RA.

Autoantibodies have not been demonstrated to play a direct role in disease pathogenesis and their utility is limited to being the biological and prognostic markers of the disease. Development of biologic agents such as depleting monoclonal antibodies against B-cell molecules has led to a confirmation of the role played by B-cells in RA pathogenesis (Meyer *et al.*, 2003; Schellekens *et al.*, 2000; Zvaifler, 1973).

The understanding of the role of T-cells in the pathogenesis of RA is undergoing a dynamic evolution. This notion is supported by the efficacy of treatment options as abatacept [cytotoxic T-lymphocyte antigen immunoglobulin fusion protein (CTLA4Ig)], which specifically blocks T-cell co-stimulation (Genovese *et al.*, 2005).

Very few Th1 or Th2 cytokines have been found in the synovial fluid or tissues (Firestein and Zvaifler, 2002), and certain animal model based studies have shown that these exert disease ameliorating effects. However, recent characterization of Th17 cells subset has determined that the production of IL-17 is one possible way T-cells play a role in RA (Schmidt-Weber *et al.*, 2007). IL-17 is produced in abundance by RA synovium and acts as a potent stimulus. Studies conducted in disease models suggest that overexpression of IL-17 is associated with exacerbation of disease whereas its blockade ameliorates the disease (Miossec, 2007).

2.3 Cellular Signalling in Rheumatoid Arthritis

The resident joint cells also participate in the pathogenesis of RA alongside cells of the immune system. These include FLS, osteoclasts, osteoblasts and chondrocytes which, under normal conditions, function in bone homeostasis through maintenance of the joint

matrix. However, in RA, these are involved in joint destruction; with activated osteoclast carrying out bone resorption and FLS degrading the cartilage (Pap *et al.*, 2005; Sato K, 2006). These are two preliminary steps that mount up to local activation of the synovium; which is said to be the first crucial step in disease development. An example of this comes from the experimental development of a type-II collagen induced mouse model using type-II collagen and complete Freund's adjuvant in which synovial lining hyperplasia with fibrin deposition was observed well before leukocytes infiltrated the synovium and overt arthritis development took place. The resident cells, in particular the mesenchymal fibroblasts, have been shown to degrade cartilage and also maintain the immune response via secretion of various cytokines, chemokines and growth factors (Mor *et al.*, 2005; Ritchlin, 2000; Sweeney and Firestein, 2004).

2.4 Role of Fibroblast like synoviocytes in Rheumatoid Arthritis

2.4.1 Activation of Fibroblast like synoviocytes in Rheumatoid Arthritis

In the normal physiological conditions, Fibroblast like synoviocytes are the main synthetic cells that participate in tissue homeostasis through the production of extracellular matrix and synovial fluid components. However under pathological conditions such as RA, FLS are activated by a plethora of soluble factors and cell surface proteins, all of which participate in processes that clearly over-ride the normal homeostatic function of FLS. As a result, FLS synthesize such molecules that drive both inflammation and tissue destruction, as summarised in Table 2.1 (Lee, 2007).

Inflammatory mediators that stimulate FLS include soluble, membrane-associated, and extracellular matrix-associated cytokines, chemokines, growth factors, and bioactive

lipids synthesized by both infiltrating leukocytes and FLS. Key cytokines that act on FLS include TNF-α and IL-1β; produced by macrophages, IL-17 and IFN-γ produced by T-cells, and IL-6 and TGF-β produced by FLS themselves (Hirth, 2002; Hwang, 2004; Kehlen, 2003). FLS also express several chemokines receptors such as CCR2, CX3CR1, CXCR4 and CCR5 in addition to the chemokines secreted by various cells in the RA synovium (Koch, 2005). The interactions between these chemokines and their receptors increases fibroblast migration and cytokine stimulation *in vitro* (García-Vicuña *et al*., 2004).

In addition, various cell surface components' interactions with leukocytes have also been identified. Cell surface receptors for vascular cell adhesion molecule-1 (VCAM-1)/CD106 and intracellular adhesion molecule-1 (ICAM-1)/CD54 are highly expressed on activated FLS. These facilitate adhesion of inflammatory cells to FLS via their respective ligands such as very late antigen-4 (VLA-4)/ CD49dCD29 and lymphocyte function-associated antigen-1 (LFA-1)/ CD11aCD18 (Krzesicki *et al*., 1991); (Bombara *et al*., 1993). Cellular adhesion based interactions primarily upregulate the expression of inflammatory mediators and provide sustained contact for activation of other pathways (Miranda-Carús, 2004). Stimulation of FLS by cytokines such as IFN-γ has been shown to enhance the expression of both HLA-DR and the co-stimulatory molecule CD40; which in turn leads to the release of growth factors such as VEGF(vascular endothelial growth factor) and chemokines such as stromal cell derived factor-1 (SDF-1)/CXCL12 by FLS (Cho, 2007; Nanki *et al*., 2000).

Table 2.1. A summary of activators and products of Fibroblast like Synoviocytes implicated in synovial hyperplasia.

Fibroblast like Synoviocytes Activators	Fibroblast like Synoviocytes Products
Cytokines • TNF-α, IL-1β, IL-6, IL-17, IL-18, IFN-γ	**Cytokines** • IL-6, IL-15, IL-23, type I IFN's, IL-1β*, TNF-α*
Growth Factors • FGF, PDGF, TGF-β	**Growth Factors** • GM-CSF, VEGF, TGF-, PDGF, SCF
Chemokines • CCL-2, -5, -1, CXCL-2, CXC3CL I	**Chemokines** • CXCL-1, -5, -6, -8, -9, -10, -11,-12, -13; CCL-2, -3, -5; CXC3CL I
Bioactive Lipids • Prostaglandins and Leukotrienes	**Bioactive Lipids** • Prostaglandins
Cell Surface Ligand Interactions • CD40/CD40L, VLA-4/V-CAM-1, LFA-I/ICAM-I	**Cell Surface Receptor** • CD-40, VCAM-1, ICAM-1, TLRs, integrins, cytokine and chemokine receptors
Tissue Degradation Products • Endogenous TLR ligands, fibronectin and thrombin fragments, microparticles	**Degradative Enzymes** • MMPs, cathepsins
Hypoxia	
* Although IL-1β and TNF-α message and protein have been reported in some FLS cultures, FLS are not believed to be the major producers of these cytokines *in vivo*.	

2.4.2 Significance of Cadherin-11 in defining the role of Fibroblast like Synoviocytes in Rheumatoid Arthritis

Fibroblast like synoviocytes play an important role in organizing synovial lining structure and behavior, a feat that is contingent upon the action of cell-cell adhesion molecule Cadherin-11 (Cad-11). This enables them to play a unique role in both inflammation and joint destruction (Kiener, 2006); (Lee, 2007). Cadherins are a class of transmembrane glycoproteins that mediate homotypic cell to cell adhesion by binding of a cadherin on one cell to a cadherin of the same type on an adjacent cell; a quintessential example of juxtaposition dependant paracrine signalling (Nollet, 2000).

In the synovial lining, Cad-11 is only expressed in the FLS, whereby absence of Cad-11 results in a hypoplastic synovium, marked by reduced lining cell number and extracellular matrix (Lee, 2007). Transfection experiments of Cad-11 in a fibroblast cell line lacking endogenous expression of Cad-11 resulted in the formation of a lining of cells similar to the synovium in three dimensions in *in vitro* cell culture which points to the fact that FLS play a key role in maintaining synovium's architecture (Kiener, 2006). In addition, blockade of Cad-11 using monoclonal antibodies or a fusion protein construct not only prevented arthritis from developing but also proved to be a useful therapeutic for treating already developed arthritis. This is reminiscent of the fact that not only are FLS key effector cells in the pathogenesis of RA, having an influence on both inflammation and tissue destruction, they also present a target for developing a novel class of therapeutic agents for treating RA (Lee, 2007).

2.4.3 Consequences of Fibroblast like Synoviocytes' activation

There are multiple complementary pathways mediated by soluble pro-inflammatory molecules, cell-to-cell surface interactions, TLR agonists, and cellular debris of joint damage that result in FLS activation which, in turn, induces synthesis of pro-inflammatory molecules that act in an autocrine as well as paracrine fashion to further stimulate FLS and other cell types, promoting inflammation and tissue destruction (Abeles, 2006). Whether the stimulation of FLS takes place by cytokines, chemokines, costimulatory molecules, or TLR agonists, a pattern of activation is ignited that sustains synovial lining hyperplasia, surges synthesis of proinflammatory mediators, and endorses breakdown of the cartilage matrix.

2.4.4 Fibroblast like Synoviocytes hyperplasia

Hyperplasia of synovial layer is marked by an increase in the number of FLS, most likely a concomitant result of FLS sustained proliferation, reduced apoptosis, and recruitment from other fibroblast pools, all of which may be driven by FLS activation (Mor *et al.*, 2005; Zvaifler, 2006). FLS proliferation rates *in vitro* are augmented by various stimuli, including cytokines like TNF-α and IL-1β; chemokines like CCL5 and CXCL12 and growth factors such as TGF-β and FGF (García-Vicuña *et al.*, 2004). Fibroblast like synoviocytes have the capability to grow in anchorage-independent settings with loss of contact inhibition in the presence of serum or platelet derived growth factor (PDGF) (Lafyatis, 1989).

Numerous mechanisms appear to contribute to the FLS' resistance to apoptosis. This is exemplified by RA-FLS *in vivo*, that acquire loss of function mutations in the tumor

suppressor gene p53, potentially due to increased oxidative stress in the inflamed joint (Tak, 2000). As a result of loss of p53 function, cells harboring extensive DNA damage proliferate indefinitely instead of undergoing apoptosis. Moreover, TNF-α stimulation and nuclear factor-kB (NF-kB) activation both diminishes FLS apoptosis through mechanisms such as resistance to Fas ligand-induced signalling and up-regulation of anti-apoptotic molecules such as Bcl-1 (Baier, 2003).

2.4.5 Fibroblast like Synoviocytes production of pro-inflammatory mediators

Activation of FLS is followed by the synthesis of an expansive array of soluble and cell surface proinflammatory molecules with many assorted functions, as summarized in Table 2.1). Production of these molecules is achieved through the activation of manifold signal transduction pathways, including NF-kB, mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)/signal transducer and activation of transcription (STAT) factor families (Sweeney and Firestein, 2004). These pro-inflammatory molecules endorse various aspects of FLS such as further activation, recruitment, retention, and survival of different cell types in the synovium, including leukocytes, endothelial cells, and osteoclasts.

2.4.6 Fibroblast like Synoviocytes and Cartilage Destruction

Activation of Fibroblast like synoviocytes results in promotion of cartilage invasion and degradation (Firestein, 1996a). This is initiated by the transgression of FLS beyond the articular margin over to the cartilage and followed by digestion of the underlying matrix (Bromley and Woolley, 1984); (Pap *et al.*, 2005). RA-FLS undergo certain intrinsic changes in their behavior that enables them to erode the cartilage; a phenomenon that has

also been demonstrated by *ex vivo* models of study in which RA-FLS have been shown to perform bone erosion even in the absence of an intact immune system. As an example, histological evidence of FLS-mediated cartilage erosion was obtained in a model whereby patient derived RA-FLS co-implanted with human cartilage plugs under the renal capsule of severe combined immunodeficiency (SCID) mice. It was inhibited when matrix degrading enzymes such as MMP-1 or cathepsin L were also transfected into the site of implant (Rutkauskaite *et al.*, 2004); (Schedel *et al.*, 2004). In comparison, normal FLS implants showed no or minimal cartilage invasion in this assay (Geiler *et al.*, 1994).

Even though the specific mechanisms of FLS mediated bone erosion have not yet been fully elucidated, it can be stipulated on the basis of the above mentioned findings that changes in behaviour of FLS are caused by conditions such as chronic inflammation, increased attachment to cartilage, invasion through bone matrices, and synthesis of degradative enzymes (Meyer *et al.*, 2006) . β1-integrins are a major class of cell adhesion molecules central for cell attachment to extracellular matrix that are highly expressed on RA-FLS (Agarwal and Brenner, 2006; Wang *et al.*, 1997). In addition, RA-FLS have been shown to be inherently more invasive than normal FLS and numerous cytokines, chemokines, and growth factors have been found to both stimulate FLS migration and synthesis of matrix degrading enzymes such as matrix metalloproteinases and cathepsins that drive cartilage matrix breakdown and may also inhibit production of FLS collagen; eventually leading to a catabolic phenotype (Abeles, 2006; Firestein, 1996a; Rezzonico *et al.*, 1998; Rinaldi *et al.*, 2001).

2.5 Fibroblast like Synoviocytes' interaction with resident bone cells

Interaction of FLS with bone cells such as osteoclasts is critical for joint damage in RA. Bone resorption is primarily carried out by osteoclasts and experiments conducted in inflammatory arthritis models showed that inhibition of osteoclasts completely abrogates bone resorption as well (Kong *et al*., 1999; Pettit *et al*., 2001). RANKL (Receptor activator of NF-kB ligand) is the primary differentiation factor for osteoclastogenesis that originates from myeloid precursors. Its upregulated expression in RA synovium is mediated by activated T cells and FLS (Gravallese *et al*., 2000) . FLS have been found to produce RANKL locally in the invading pannus tissue as well; though its expression is restricted to sites of osteoclast-mediated degradation (Pettit *et al.*, 2006).

Osteoprotegerin, the natural inhibitor of RANKL, was least expressed in the sites of active pannus invasion, hence osteoclast activity is not stimulated in these locations. Apart from promotion of bone erosion via stimulation of osteoclasts, RA FLS also prevent bone repair by inhibition of bone forming cells; osteoblasts. Chondrocytes have not yet been established to interact with FLS however experimental evidence has pointed out that FLS-mediated cartilage degradation is enhanced in the presence of chondrocytes (Steenvoorden *et al*., 2007; Thomas *et al*., 2000).

2.6 Fibroblast like Synoviocytes as potential targets for treating Rheumatoid Arthritis

Rheumatoid Arthritis is a distinct autoimmune disease whose pathogenesis is a direct result of the synovial tissue's reaction to immune stimulation which mediates most of the chronic damage that limits the functionality of its patients. Fibroblast like Synoviocytes (FLS) provide the essential link between key components of the disease;

immune activation and tissue destruction. This distinctive function enables FLS to integrate multiple stimuli for the progression of disease pathogenesis, amplification of inflammation, and propagation of tissue damage through various interactions that sustain the recruitment, survival, and differentiation of various cell types found in the synovium (Fig. 2.1).

Presently approved treatments for RA primarily target components of the immune system, and efforts to combine newer, more potent biologic therapies to gain greater disease control have resulted in unacceptable increased infection risk by compromising the immunity (Weisman, 2002). Mesenchymal FLS are safer alternate targets for exerting a substantial impact on both inflammation and tissue destruction in the joint; the two major hallmarks of RA. In this regard, cadherin-11 that has been recently identified as a molecule over-expressed by FLS, pointing to a possibility of identifying similar compounds capable of altering fibroblast function in Rheumatoid Arthritis. This has been tested in mouse models of inflammatory arthritis whereby absence of cadherin-11 was associated with reduction in inflammation and protection against cartilage erosions (Lee, 2007). Furthermore to explore the potential of FLS as targets for treatment of established arthritis, administration of anti-cadherin-11 monoclonal antibodies following arthritis induction significantly lowered disease severity (Lee *et al.*, 2007), providing evidence that agents directed against mesenchymal cells in the joint may represent an important new class of therapeutic agents.

In order to extend development of effective, fibroblast-specific agents for the treatment of RA, a greater understanding of fibroblasts functioning in the synovium is required. Recent research findings have implicated FLS in RA pathogenesis primarily through the synthesis of chemokines, cytokines, and cell surface molecules that results in

leukocyte recruitment, retention, survival, and differentiation (Fig. 2.1). Furthermore, FLS have been shown to both promote angiogenesis to support synovial expansion and mediate tissue damage through direct erosion of cartilage and through interactions with chondrocytes and bone cells that promote cartilage and bone catabolism. However, current research findings appear to be disposed to treating all synovial fibroblasts as similar, in spite of a growing body of evidence pointing to existence of considerable fibroblast heterogeneity between tissues as well as within a given tissue (Fries *et al*., 1994; Smith, 2005).

Unlike skin or lung fibroblasts, cell adhesion molecule VCAM-1 is highly expressed in FLS which also synthesize proteoglycans such as hyaluronan and lubricin, which enable them to acquire a close interaction with MLS in the synovial lining and contribute to the lubricating ability of synovial fluid (Firestein, 1996b; Mor *et al*., 2005). In RA, lining FLS are in abundance in pannus tissue that directly invades cartilage and helps activate osteoclasts to mediate bone erosion. In contrast, sublining fibroblasts play a different role in RA pathogenesis. Lining FLS interact more closely with macrophages, osteoclasts, and chondrocytes whereas sublining FLS are in close proximity to B cells, T cells, and endothelial cells and may have an important stromal role in accumulation and activation of these cells. Therefore, understanding how location and microenvironment influences fibroblast behaviour in the synovium may be important for developing additional therapeutic agents in RA.

Fibroblasts are important in pathogenesis of several autoimmune diseases, not just RA, although their role in these diseases varies. In scleroderma and idiopathic pulmonary fibrosis, fibroblasts appear to drive tissue fibrosis in the skin and lungs, respectively

(Postlethwaite *et al.*, 2004). In contrast, in RA, although fibrosis occurs in end-stage disease, FLS mainly appear to contribute to ongoing disease by promoting inflammation and tissue destruction. These diverse fibroblast responses may represent dysregulation of what is a normal repair process. In wound repair models, fibroblasts are integral during the initial recruitment of inflammatory cells to the site of the wound in order to protect against infection. At later stages, fibroblasts downregulate the immune response to make way for laying down a new matrix that heals the tissue injury (Eming *et al.*, 2007). In case of RA, the pro-inflammatory response appears to dominate whereas other fibrotic diseases result from dysregulated tissue repair (Zvaifler, 2006);(Flavell *et al.*, 2008). A better understanding of fibroblasts function under both normal and pathological conditions and in the context of a particular disease may pave the way to exciting new treatments, not just for RA, but for many disabling diseases.

2.7 Osteoclast independent bone resorption by Fibroblast like synoviocytes

A growing body of evidence has implicated transformed appearing, activated fibroblast-like cells of mesenchymal origin in extracellular matrix degradation in various diseases. Bone resorption by hyperplastic fibrous synovial tissue is a typical and early characteristic of RA, and in this regard FLS have been critically implicated in bone and cartilage degradation (Pap *et al.*, 2000a). Being transformed with the SV-40 antigen, fibroblast like synoviocytes exhibit pro-tumorigenic phenotypes such as anchorage-independent proliferation (Lafyatis, 1989; Xue *et al.*, 1997), escape from contact inhibition (Grimbacher *et al.*, 1998), activation of proto-oncogenes (Xue *et al.*, 1997) and alterations in apoptosis (Franz *et al.*, 2000).

RA-FLS release matrix-degrading enzymes such as matrix metalloproteinases, membrane-type matrix metalloproteinases and cathepsins (Pap *et al.*, 2000b) which are potent mediators of bone resorption. These have also been shown to mediate the differentiation of macrophages into osteoclast-like cells (Pap *et al*., 2003). It has also been observed that the aggressive phenotype of FLS in RA is maintained even in the absence of inflammatory cytokines and this transformation is fairly permanent (Muller-Ladner, 1996).

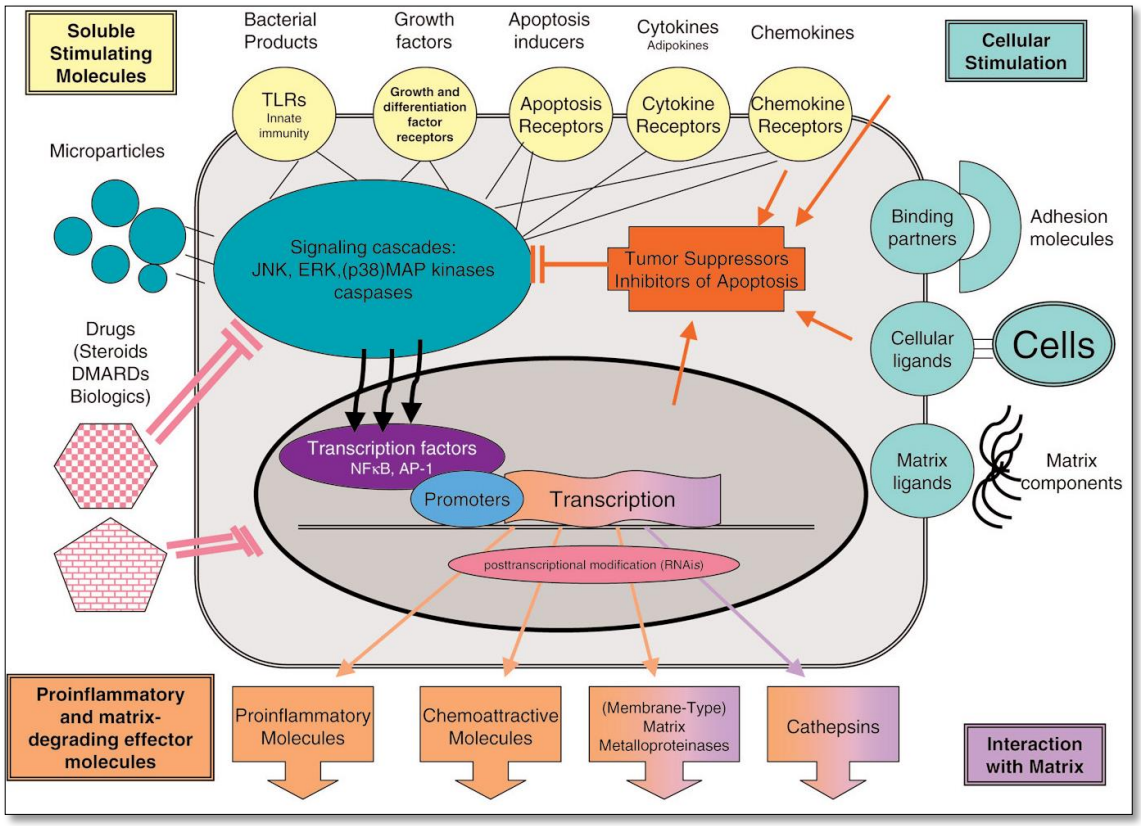


Figure 2.1. Representative image of various biological markers involved in a crossroad interaction in Rheumatoid Arthritis.

2.8 MH-7A Cell line

MH-7A is a fibroblast like synoviocytes cell line that has been derived from a primary synoviocytes culture immortalised with SV-40 large T antigen. The primary synoviocytes culture has been derived from human rheumatoid pannus of a 53 year old, female RA patient. In these cells, p42/p44 MAP kinase is constitutively expressed and is therefore associated with their transforming capability. These cells also express IL-1 type I receptor and have been shown to over-express p53. Resultantly, these cells can be stimulated with IL-1β, and induce the IL-1 signal transduction pathway which results in the expression of Stromelysin-1, NF-kB and ICAM-1 (Miyazawa *et al.*, 1998).

2.9 Potential role of Carbonic Anhydrase II in bone resorption

Carbonic Anhydrase (CA) is a family of enzymes having sixteen members or isoforms. Thirteen members are enzymatically active, recognised for catalyzing the reversible hydration of CO_2 and dehydration of carbonic acid, as represented by the equation below:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

These enzymes have been classified further into four subgroups on the basis of their cellular localisation: (1) cellular: CAI, II, III and IV (2) mitochondrial: CAV (3) membrane-bound: CAIV, IX, XII and XIV and (4) secreted: CAVI. Various studies conducted in the past decade have shown that CA bind with bicarbonate/anion transporters located in the cytoplasmic membrane and facilitate the process of ion exchange across the membrane. Such interactions involve the formation of a membrane-protein complex called a ‘transport metabolon’ which regulates bicarbonate metabolism and transport. Specifically

CAII has been shown to bind to and enhance the activity of Na^+/ H^+ antiporter NHE1. Such an interaction has also been reported between CAIV and NBC1; the Na^+/HCO_3^- co-transporter (Purkerson and Schwartz, 2005).

CAII facilitates the production of H^+ ions which brings about a drastic acidification of resorption lacuna. This is achieved by CAII working in conjunction with vacuolar H^+-ATPases (V-ATPases) which results in an active out flux of H^+ ions secreted by CAII into the resorption lacuna; which reduces its pH to 4.5-4.8.This protein complex is also referred to as a transport metabolon.Membrane-bound CAs such as CA IV, IX, XII and XIV are also counted as part of this metabolon since they act in synergy to CAII during acidification of resorption lacunae.

Loss of pH modulation systems directly disrupts the process of bone resorption and is a common feature of various pathological conditions such as osteopetrosis, which is also a clinical manifestation of a rare genetic condition marked by CAII deficiency and results in a decreased bone resorption.

CAII also interacts with bone resorption mediating hormones such as Parathyroid hormone (PTH) and 1, $25(OH)_2D_3$. Both of these hormones stimulate the expression of CAII, with 1, $25(OH)_2D_3$ having a direct interaction with a response element with CAII gene (Lehenkari *et al*., 1998). Acidification of the resorption lacuna is enhanced following treatment with parathyroid hormone and prostaglandin E2, both of which have also been shown to interact directly with CAII and promote its expression. Conversely, treatment with calcitonin inhibits acidification of the resorption lacuna, bringing about an increase in the pH in the range of 7.0-7.3.

Under normal conditions, production of alkaline products takes place after the acidification process and their removal to the extracellular environment usually occurs via bicarbonate/chloride exchangers (AE proteins). Extracellular pH levels also affect the activity of osteoclasts, as in vitro studies have revealed that osteoclasts are inactive at extracellular pH levels above 7.3 and exhibit optimum activity at pH 6.9. Bone resorption in itself is most sensitive to H^+ ion concentration changes at a pH of 7.

Apart from its role in bone resorption, CAII is crucial for maturation of osteoclasts and serves as a marker for early stage of osteoclasts differentiation essential for the formation of mature osteoclasts. It is mainly involved in regulation of intracellular pH of osteoclasts (Lehenkari *et al*., 1998).

2.9.1 Intracellular regulation of pH in Carbonic Anhydrase-II expressing osteoclasts

On the intracellular level, pH in osteoclasts is regulated by three key components namely V-ATPases, HCO_3^-/Cl^- transporters and Na^+/H^+ exchangers. V-ATPases are responsible for the acidification of the resorption lacuna as mentioned before whereas HCO_3^-/Cl^- transporters transfer excessive bicarbonate ions out of the cells and Na^+/H^+ exchangers facilitate recovery from acid loads. CAII has been reported to interact with V-ATPases and HCO_3^-/Cl^- transporters so both of these possess a consensus binding motif at their C-termini which allows them to directly bind to CAII. Inhibition of CAII *in vitro* resulted in a decreased bone resorption (Riihonen *et al*., 2007).

Maintenance of intracellular pH is crucial for both fusion and division of cells. CAII appears to play a key role here; however its dynamics differ in different cell types. Broadly put, in certain cell types its role is more generalised whereas in others its effects are specific

to the stage of cellular differentiation. Since both reaction products of CAII, HCO_3^- and H^+ ions have a neutralising effect on each other, and the reverse reaction of CO_2 hydration produces equally neutralising amounts of acid and base. Therefore it can be postulated that intracellular pH is more affected by the cell's activity rather than CAII i.e. if it has adapted itself as a bicarbonate/proton secretor, its ion exchange patterns and on its surrounding extracellular environment. However, expression of CAII enables cells to maintain a constant intracellular pH amidst variable extracellular pH conditions which is vital for their expression and survival. It has been reported that a decrease in extracellular pH induces an increase in the intracellular expression of CAII. And it has been demonstrated *in vitro* that a low extracellular pH is essential for optimal bone resorption and osteoclast formation.

Extracellular pH of osteoclasts is highly variable since the bone lining area where they are mostly present is rather poorly perfused. Furthermore, active osteoclasts maintain a low intracellular pH and Ca^{2+} influx which is facilitated by a low extracellular pH as prevalent in the bone lining. It is postulated that disturbance in the extracellular pH in resorption lacuna will cause drastic intracellular cytoplasmic alkalization of osteoclasts (Lehenkari *et al*., 1998).

Active bone resorption is subdivided into two main stages: acidification and proteolysis. Acidification of the resorption lacunae is mediated by the osteoclastic vacuolar adenosine triphosphatase (V-ATPase) via an out flux of H^+ ions, which is balanced by a transfer of Cl^- ions via either via HCO_3^-/Cl^- transporter or Cl^-/H^+ antiporter ClC-7, according to varied reports regarding the exact ion channel that is involved here. There are two key findings that support the ClC-7 channels here; first, they have been reported to be involved in lysosomal chloride transport and acidification in liver cells. Second, the loss of

ClC-7 has been associated with decreased bone resorption and osteopetrosis in both humans and mice.

Acidification of resorption lacuna serves to dissolve the inorganic bone matrix that is composed usually of alkaline salts of calcium and phosphorous. In the proteolytic phase, proteases such as cathepsin K, a cysteine protease, dissolve the organic bone matrix that is typically composed of type I collagen. CAII is reported to be highly expressed in osteoclasts and is a key element to the resorptive function of osteoclasts. Inhibition of CAII results in reduced resorption, mainly via a transient increase in cytosolic pH (Rousselle and Heymann, 2002); Henriksen *et al*., 2008).

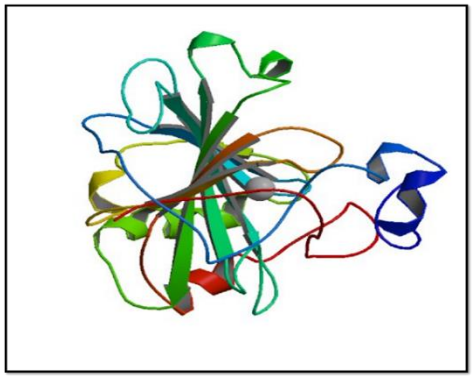


Fig 2.2 Biological assembly image of human Carbonic Anhydrase-II. Protein chains are coloured from the N-terminal to the C-terminal using a rainbow (spectral) colour gradient (Source: Protein Data Bank).

2.10 Carbonic Anhydrase Inhibitors- Current Knowledge

There are multiple factors that need to be taken into consideration for the development of CAI as therapeutic agents like the large number of their functional isoforms in humans, their rather diffused subcellular localisation and the lack of isozyme selectivity of the current available sulphonamide class of carbonic anhydrase inhibitors. Recent

studies have highlighted a potential of being anti-cancer, anti-infective and anti-obesity drugs for CAI's. (Scozzafava *et al*., 2006).

Classic CAIs belong to the sulphonamide/sulphamates class of drugs. Other classes of drugs that also act as carbonic anhydrase inhibitors include boronic acids, metal complexing anions and similar small molecules and phenols. Aliphatic, aromatic or heterocyclic sulphonamides have been used for therapeutically inhibiting certain α-carbonic anhydrases for a while now. These have found pharmacological utility as diuretics for the treatment of epilepsy, glaucoma, congestive heart failure, mountain sickness, gastric and duodenal ulcers, neuromuscular pathologies and various neurological disorders, hypokalaemia, tardive dyskinesia, genetic hemiplegic migraine and ataxia, essential tremor and Parkinson's disease and osteoporosis. Chemical compounds and drugs of this particular pharmacological class are under constant research and development as they have such broad therapeutic utility (Supuran, 2008).

Sulphonamides mainly inhibit CAII, CAIV and CAV. X-ray crystallographic studies have revealed that sulphonamides bind directly in a deprotonated (RSO_2NH^-) form to the Zn^{2+} ion of the enzyme which is also bound to three histidine residues of CA enzyme; His^{94},His^{96} and His^{119} giving it a tetrahedral conformation. Various interactions take place between the enzyme's active site and the bound inhibitor have been identified and are crucial for determining the affinity of this class's inhibitors towards different iso-enzymes. With exception of CAIII, all iso-enzymes belonging to the CA family; such as the cytosolic CAI, CAII and CAVII, the membrane bound forms CAIV, IX, XII and XIV, or the mitochondrial CAV all show high and similar binding affinities, ranging in micro and nanomoles, for sulphonamide inhibitors. This however is not completely desirable as

inhibition of CA in sites other than the target site may induce undesirable side effects. In spite of this, various derivatives of sulphonamides; such as acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide have been used successfully in clinical medicine for the past 45 years. Recently, two new derivatives namely dorzolamide and brinzolamide have gained significant therapeutic importance as ocular drugs in the treatment of glaucoma.

Recent studies have identified phenols as well as phenolic derivatives as potential CAIs. Phenolic compounds incorporate a hydroxyl group directly bonded to an aromatic hydrocarbon atom; the number and position of -OH groups on the aromatic ring varies in different compounds. Methoxyphenols have a low molecular weight, and are semi-volatile polar aromatic compounds. Guaiacol, also known as 2-methoxyphenol contains two oxygen containing functional groups: hydroxyl (-OH) and methoxy ($-OCH_3$). Four catalytically active isoforms of CA in humans; hCAI, hCAII, hCAIX and hCAXII have been shown to be inhibited by phenol and its derivatives derived from catechol and guaiacol (2-methoxyphenol) in micromolar range. All of these derivatives are unified by the presence of between two and four phenolic moieties. It is this phenolic moiety which enables these derivatives to bind to the Zn^{2+} ion in a manner diverse from the classical CAI's. These have been found to be effective against both cytosolic hCAI and hCAII and membrane-bound hCAIX and hCAXII iso-forms in concentrations ranging in micromoles (Scozzafava *et al.*, 2014).

Many studies have demonstrated for hCAII that its inhibition by any inhibitor depends upon its ability to mimic the intermediary tetrahedral state when binding to the catalytic Zn^{2+} ion present in the active site (Akbaba *et al.*, 2013).

2.11 Matrix Metalloproteinase-9

Matrix metalloproteinases is a family of zinc dependant endopeptidases that can degrade every component of the extracellular matrix and are the main proteases involved in destruction of articular cartilage. Most members of MMPs' mRNA has been shown to be expressed in the RA synovium including MMP-1, -2, -3, -9, -11, -13 and -15. The human MMP gene family has 24 known members which have been classified into four key subfamilies namely collagenases, gelatinases, stromelysins and the membrane-bound (Shingleton *et al.*, 1996). Collagenases include MMP-1, MMP-8 and MMP-13 which cleave triple helical collagen at specific sites and gelatinases include MMP-2 and MMP-9 which degrade denatured collagen. Stromelysins include MMP-3, MMP-7, MMP-10, and MMP-11 which cleave various molecules such as aggrecan, fibronectin, laminin and procollagens. Membrane bound MMPs include MMP-14, MMP-15, MMP-16, and MMP-17 which possess a transmembrane domain and a cytoplasmic tail at their C-terminal and are reputed for their expression in carcinomas (Moran *et al*., 2009).

Activation of MMPs' is controlled at various checkpoints; such as transcription of pro-MMPs, activation of pro-MMP enzymes, and inhibition of active MMPs. At the transcriptional level, MMP genes possess cis-regulatory elements corresponding to the activator protein-1 (AP-1) binding site, Ets binding site, Sp-1 binding site, and NF-kB binding site (Borden and Heller, 1997). It is interesting to note that these sites are shared regulatory sites between the natural inhibitors of active MMP's i.e. TIMPs (tissue inhibitors of matrix metalloproteinases). Activation of MMP's is achieved by proteolytic removal of MMP's propeptide which may even be performed by another MMP (Murphy *et al*., 1999).

MMP-2, also known as gelatinase A and MMP-9 known as gelatinase B are involved in digestion of denatured collagen (gelatin) generated by the collagenases. In addition to denatured collagen, these enzymes also degrade fibrillary collagen I and II and aggrecan which are predominantly found in the cartilage (Nagase *et al.*, 2006). Despite having similar substrate specificities; MMP-2 is constitutively expressed by most cell types; including synovial fibroblast and its expression is not induced by cytokines or growth factors. On the other hand, expression of MMP-9 has been detected exclusively in malignant and inflammatory cells such as RA synovial fibroblasts induced by inflammatory cytokines such as TNF-α (McQuibban *et al.*, 2000). Gelatinases also participate in the regulation of inflammation; based on their interactions with cytokines and chemokines. MMP-9 has stimulatory effects whereas MMP-2 has inhibitory effects on inflammation.

MMP-9 has been found to contribute to the prolonged survival and invasive and inflammatory properties of RA synovial fibroblasts where it works in conjunction with TNF-α, IL-6 and IL-8. It has a concurrent presence in the sites of inflammation (Le Goff *et al.*, 2010). MMP-9 contains the cis-regulatory element that is activated by NF-kB in its promoter region which further potentiates the role it plays in RA (Roman-Blas and Jimenez, 2008). The role of MMP-9 becomes obscure with progression of disease as the RASF's become more aggressive and invasive, reflective of a potential area that requires more research (Xue *et al.*, 2014).

2.12 Tumor Necrosis Factor-α

TNF-α is a proinflammatory cytokine that has been implicated in the differentiation and activation of osteoclasts for a long time. It is released mainly by activated macrophages and its functioning is mediated by TNF receptors. TNF-α has been shown to stimulate the

production of RANKL in activated T-cells which drives osteoclastogenesis and bone resorption. It also stimulates production of cytokines such as IL-6 and IL-1β which participates in an amplification loop and induces the production of RANKL and M-CSF, leading to increased osteoclastogenesis and bone resorption (Feldmann and Maini, 2003).

CHAPTER 3

METHODOLOGY

3.1 Revival of MH7A Cell Line

This project utilized MH7A, a human arthritic cell line as a research model. MH-7A, a human fibroblast-like synoviocytes cell line, was purchased from Riken Cell Bank, Japan. MH-7A cell line was cultured and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies) media supplemented by 10% heat inactivated Fetal Bovine Serum (FBS) (Biowest) , 100U/mL penicillin, 100µg/mL streptomycin and 250µg/mL Amphotericin B in a humidified incubator at 37℃, 95% air and 5% CO_2. T-25 and T-75 cell culture flasks were used for growing and maintaining the cells. The cells were passaged every three to four days. All the experiments were performed at passages 4 to 8.

The cryovial was taken out of the -140℃ freezer and immediately shifted to the -20℃ freezer where it underwent gradual thawing. It was wiped with 70% Ethanol and shifted to the laminar flow hood where its contents were transferred to a 15mL falcon tube containing pre-warmed RPMI 1640 media supplemented with 10% FBS. The cells were centrifuged for 5 minutes at 800rpm at 25℃. The supernatant was discarded and cells were re-suspended in FBS and centrifuged again at 800rpm for 5 mins at 25℃.The supernatant was discarded, the cells were resuspended in 10 ml of complete RPMI 1640 media supplemented with 10% FBS containing 1% Penicillin-Streptomycin and 2% Amphotericin B and transferred to a T-25 flask. These were placed in a humidified incubator at 37℃, 95% air and 5% CO_2.

3.2 Cell Counting

Cell counting is a vital pre-requisite step that needs to be performed before proceeding with any experiment. It is performed to determine the number of cells that are present in a culture flask. This number is then used to determine; via a series of mathematical calculations, the number of cells that are required to be plated for a given experiment or assay.

3.2.1 Manual cell counting using the glass haemocytometer

For performing manual cell counting using a glass haemocytometer, media was aspirated from cell culture flasks. Cells' monolayer was washed twice using ice-cold PBS. Cells were trypsinised using an appropriate volume of 1X Trypsin-EDTA solution (700µL for a T-25 flask) and incubating for 2-3 mins at 37°C. An appropriate volume of fresh complete media was added to the flask and it was ensured, by observing under the microscope, that the cells are in suspension. A volume of 10µl of the resulting cell suspension was transferred to a 1.5mL micro-centrifuge tube (Eppendorf®) and an equal volume of Trypan-blue dye was added to the tube. The cells were mixed with the dye by titurating the mixture. The haemocytometer was thoroughly cleaned with 70% ethanol and covered with a glass slide. 10µL volume of the cell suspension and dye mixture was loaded in both chambers of the haemocytometer using a micropipette; and cells were counted in the outer four and the central large squares in both the chambers. Trypan-blue stains dead cells as blue whereas the live cells appear as having blue peripheries and a bright shiny centre. Each reading was divided by five (number of large squares in which the cells were counted) and their average was taken.

The following formulae were used for further calculations:

$$\text{Average viable cell count/square} = \frac{\text{Total number of viable cells in 5 squares}}{5}$$

$$\text{Dilution Factor} = \frac{\text{Total Volume (Volume of sample + volume of diluting liquid)}}{\text{Volume of sample}}$$

Viable cells/mL=Average viable cell count per square X dilution factor X 10^4

Total viable cells/ sample = Viable cells/mL X original volume of media containing cell suspension from which the cell sample was removed.

Volume of media needed = (Number of cells needed/ total number of viable cells) X 1000

3.3 Selection of Carbonic Anhydrase-II inhibitors

The selection of Carbonic Anhydrase-II (CA-II) inhibitors was made after an *in silico* study carried out by Sidrah Anjum under the supervision of Dr Ishrat Jabeen. First the structure of the ligand binding pocket of CA-II was determined. On the basis of this binding pocket's structure and using MOE docking software; a pharmacophore model was generated. Using this pharmacophore model the DrugBank was screened for potential inhibitors in order to identify novel compounds that can be used to inhibit CA-II. As a result of this screening; 59 entries were initially selected which were further analysed by computing their chemical descriptor values and comparing these values with the chemical descriptor values of already known highly potent inhibitors of CA-II. The compounds having similar values as those of the putative inhibitors were selected as potential hits.

Three of these hits were selected as candidate compounds namely Alfuzosin, Prazosin and Sulpiride. Alfuzosin and Prazosin belong to the class of Quinazolinamines whereas Sulpiride is a Benzenesulphonamide. Out of these three candidate compounds, Prazosin and Sulpiride have been used in this study to assess their in vitro cytotoxic activity, ability to cause DNA damage and potential effect on the post-transcriptional levels of potent markers of bone resorption.

Prazosin hydrochloride and Sulpiride were purchased from Santa Cruz Biotechnologies. Stock solution of these drugs were prepared in the following manner:

1. Prazosin hydrochloride (Molecular weight: 419.87)
    a. For preparation of 1mM stock solution, 2.1mg of the drug were dissolved in 5 mL of DMSO.
    b. This stock was serially diluted, as shown in the table below to make further working concentrations of the drug.

Table 3.1. Preparation of working dilutions of Prazosin Hydrochloride.

Concentration to be prepared (µM)	**Volume to be prepared (mL)**	**Stock volume added (µL)**	**Dilution volume added (µL)**
10.0	1.0	10.0	990.0
20.0	1.0	20.0	980.0
40.0	1.0	40.0	960.0
60.0	1.0	60.0	940.0

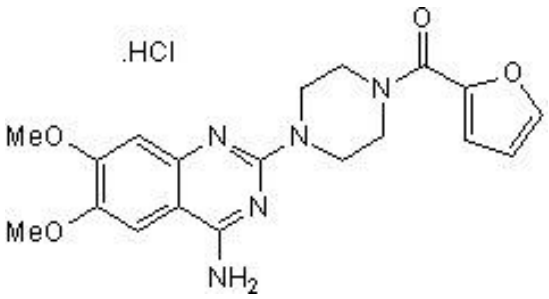


Figure 3.1 Prazosin hydrochloride

2. Sulpiride (Molecular weight: 341.42)
    a. For preparation of 1mM stock solution, 1.7mg of the drug were dissolved in 5 mL of DMSO.
    b. This stock was serially diluted, as shown in the table below to make further working concentrations of the drug.

Table 3.2. Preparation of working dilutions of Sulpiride.

Concentration to be prepared (µM)	**Volume to be prepared (mL)**	**Stock volume added (µL)**	**Dilution volume added (µL)**
10.0	1	10.0	990.0
20.0	1	20.0	980.0
40.0	1	40.0	960.0
60.0	1	60.0	940.0

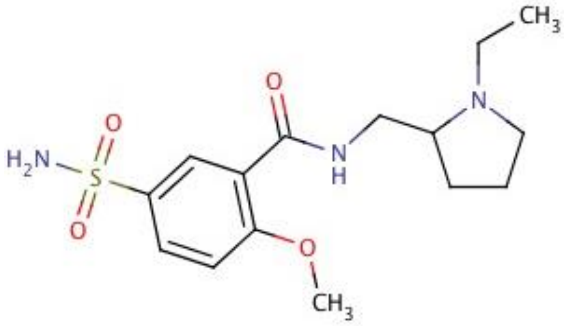


Figure 3.2 Sulpiride

3.4 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

It is a cell viability or *in vitro* toxicity assay used to determine the number of viable cells in the screening of compounds that are thought to have an effect on cell viability. However, it is not a reliable marker of cellular proliferation. Cell viability is mainly assessed by measuring some aspect of general metabolism or an enzymatic activity; this requires incubation of a reagent with a population of viable cells to convert a substrate to a coloured or fluorescent product that can be detected with an ELISA plate reader. Under standard conditions, this incubation will result in generation of a detectable signal that is measurable and proportional to the number of viable cells present. Since dead cells rapidly lose the metabolic/enzymatic ability to convert the substrate to product, this difference provides the basis for many commonly used cell viability assays.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay belongs to the class of Tetrazolium Reduction Assays which are based on the reduction of tetrazolium salts such as MTT, MTS, XTT and WST-1 into an insoluble dye called formazan (purple in colour). MTT is a positively charged compound which readily penetrates viable cells where it is reduced to formazan. MTT is a high throughput screening assay performed in a 96 well plate. The final concentration of MTT to be added to cells is 0.2-0.5 mg/mL, with an incubation of 1-4 hrs. The quantity of formazan (presumably proportional to the number of viable cells) is measured by recording absorbance at 490-570nm using a plate reader spectrophotometer since it has maximum absorbance near this wavelength. The formazan product accumulates as an insoluble precipitate inside the cells, near the cell surface and in the culture medium and must be solubilized before taking absorbance readings.

3.4.1 Protocol

1. Media was aspirated from cell culture flasks.
2. Cells' monolayer was washed twice using ice-cold PBS.
3. Cells were trypsinised using an appropriate volume of 1X Trypsin-EDTA solution (700µL for a T-25 flask) and incubated for 2-3 mins at 37°C.
4. An appropriate volume of fresh complete media was added to the flask and it was ensured, by observing under the microscope, that the cells are in suspension.
5. Cell count was performed and 1 x 10^6 cells in 100µL of media were seeded in each well of a 96 well plate and incubated overnight at 37°C. Only media was added to three wells to provide absorbance readings for blank.
6. MTT reagent was prepared by dissolving 5 mg of MTT powder in 1 mL of 1X PBS. It was then filtered using a 0.2micron filter. MTT is a light sensitive reagent; hence kept wrapped with Al foil and stored at -20°C for further use.
7. Following overnight incubation, 10µL volume of 10µM, 20µM, 40µM and 60µM concentrations of both inhibitors namely Prazosin and Sulpiride were added in triplicate wells and the cells were thus incubated for 24 hrs.
8. After 24 hrs, 10µL of MTT reagent was added to each well and the plate was incubated at 37°C for 3 hrs.
9. After 3 hrs, media was discarded from all the wells taking special care that the formazan crystals are not discarded in the process. 50µL of DMSO were added to each well to dissolve the formazan crystals and the plate was incubated at 37°C for 20-25 mins.
10. Absorbance readings were taken at 490nm using an ELISA plate reader.

Cell Viability was calculated using the following formula:

Cell Viability = $\frac{Absorbance\ of\ experimental\ well - Absorbance\ of blank}{Absorbance\ of\ Negative\ control - Absorbance\ of\ blank}$

Percentage Cell Viability = Cell Viability x 100

3.5 Comet Assay

Comet Assay is a microgel electrophoresis method first introduced by Ostling and Johanson in 1984 in order to measure the single stranded breaks of DNA. The alkaline version of the original protocol was published by Singh *et al* in 1988. The working principle behind Comet Assay combines the techniques of DNA electrophoresis and fluorescent microscopy.

3.5.1 Protocol

1. 1% Low Melting Agarose (LMA) was prepared by dissolving 0.1g LMA powder in 10ml of distilled water and dissipating it into 1ml aliquots.
2. Agarose precoated slides were prepared by dipping slides in 1% molten normal melting agarose and wiping one side of the slide clean. These were allowed to air-dry at room temperature.
3. Samples were prepared by taking pretreated cells in suspension in 1XPBS as it minimizes cell aggregation and inhibits DNA repair. Cells were centrifuged and supernatant was discarded. 0.4mL of cell suspension was mixed with 1.2mL of 1% LMA and loaded on to precoated glass slides in two parallel lines of 20µL droplets. The slides were covered with glass coverslips so as to ensure that the NMA and LMA merge and dry into a uniform layer.

4. Once dried, the coverslips were removed carefully so as not to remove the agarose and the slides were placed in the lysis buffer overnight (18-20 hrs) at 4ºC in the dark.
5. Following overnight incubation in the lysis buffer, slides were washed twice with distilled H_2O to remove any traces of salt and detergent.
6. The slides were subjected to electrophoresis in alkaline electrophoresis buffer at a voltage of 14V for 25 minutes.
7. Slides were then removed from the electrophoresis chamber and rinsed with distilled H_2O.
8. Slides were stained using a 10µg/mL EtBr solution by pipetting 100µL of the stain onto the slide and incubating it for 20 min. Excessive stain was removed by rinsing slides with dH_2O.
9. Slides were examined under a fluorescent microscope at a magnification of 10X and atleast 4 images were recorded from each slide and analysed for various parameters using the CaspLab® software.

3.6 Quantitative Real Time-PCR

3.6.1 RNA Extraction

1. Cells were seeded at a density of 3.56 x 10^6 cells in 500µL in a 24 well plate.
2. Equal volume of both inhibitors both inhibitors, Prazosin and Sulpiride i.e. 50µL was added in individual wells and cells were treated for 24 hours.
3. Cells were harvested in a 1.5mL microcentrifuge tube and pelleted out by centrifuging at 6000 rpm for 5 mins.

4. Supernatant containing media was discarded and cells' pellet was resuspended in 1 mL of ice-cold 1X PBS for PBS washing.
5. Cells were centrifuged at 6000 rpm for 5 mins.
6. Supernatant was discarded and 1 mL of TRIzol reagent was added to each 1.5 mL microcentrifuge tube. Then 20µL of 5N Glacial acetic acid was added in each tube. Lids were closed and tubes were manually shaken vigorously for complete dislodging and dissolution of cells' pellet. The tubes were incubated at room temperature for 5 mins.
7. A volume of 200µL of chilled Chloroform was added in each tube and tubes were manually shaken vigorously for 10-15 secs. The tubes were incubated at room temperature for 10 mins.
8. The tubes were centrifuged at 12000 rpm, 4º C for 15 mins. Three distinct phases were obtained.
9. A volume of 500µL of chilled Isopropanol was added to a new 1.5mL microcentrifuge tube.
10. The top-most clear layer was added to the Isopropanol containing tubes and the tubes were incubated without shaking or tituration at room temperature for 8-10 mins.
11. The tubes were centrifuged at 12000 rpm, 4º C for 15 mins to precipitate out the RNA.
12. Following centrifugation, a really tiny pellet appeared at the bottom of the tube. Isopropanol was carefully discarded and the pellets were resuspended in 75% Ethanol in DEPC treated water and stored at -80º C.

13. The RNA samples were quantified using a Nanodrop™ 2000 UV-Vis spectrophotometer.

3.6.2 cDNA synthesis

The following protocol is optimised to generate first-strand cDNA for use in two-step RT-PCR.

1. All components were mixed and briefly centrifuged after thawing and kept on ice at all times.
2. RNA template was added in a sterile PCR tube in the range of 0.1ng – 5µg; depending on the quantity of RNA.
3. 1µL (0.5µg; 100pmol) of oligo$(dT)_{18}$ primer were added in the tube.
4. Upto 12.5µL of DEPC treated H_2O was added in the tube; in order to make up the total volume.
5. The components were mixed gently and centrifuged briefly and incubated at 65ºC for 5 min in the thermocycler. After which it was again placed on ice and centrifuged briefly.
6. 4µL of 5X reaction buffer were added.
7. 0.5µL (20U) of RiboLock RNase inhibitor were added.
8. 2µL of dNTP mix, 10mM each were added to achieve a final concentration of 1mM.
9. Lastly, 1µL (200U) of Reverse Transcriptase were added.
10. All the components were mixed gently and centrifuged briefly.
11. The tubes were incubated at 42ºC for 60 min and for the termination of the reaction a final incubation of 10 min at 70ºC was given.
12. The cDNA product was stored at -20ºC until further use.

3.6.3 Quantitative Real Time-Polymerase Chain Reaction

For performing qRT-PCR, SYBR green master mix, primers, nuclease free H_2O and cDNA template were completely thawed on ice. All reagents were gently vortexed and briefly centrifuged. A cocktail of the following components was prepared in a 1.5mL microcentrifuge tube and equally distributed in real time PCR optical tubes so that each tube contained a final volume of each component as outlined below (Table 3.3) (for one sample):

Table 3.3. Description of reaction mixture prepared for one sample in qRT-PCR.

SYBR Green master mix	12.5 µL
Forward Primer	1.0 µL
Reverse Primer	1.0 µL
cDNA template	1.5 µL
Nuclease free water	9.0 µL
Total reaction volume	**25 µL**

The primer sequences were used in the expression profiling of their corresponding genes are given in Table 3.4. below:

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
CAII	ACTGGGGTTCACTTGATGGA	CTGCACAGCTTTCCCAAAT	119
TNFα	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC	217
MMP9	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA	232
β-actin	GGACTTCGAGCAAGAGATGG	TGTGTTGGCGTACAGGTCTTTG	229

3.7 Statistical Analysis

All the data obtained was compiled using Microsoft Excel and all the statistical analysis were performed using GraphPad Prism version 5.1. Students's t-test was used for the comparison of means between Prazosin treated, Sulpiride treated and Untreated groups and one way ANOVA was used for analysis of variance amongst Prazosin treated, Sulpiride treated and Untreated groups. Post hoc analysis was performed using Bonferroni's Multiple Comparison test. A 95% confidence interval was used throughout the statistical analysis and values having a p value of less than 0.05 ($p<0.05$) were considered to be statistically significant.

CHAPTER 4

RESULTS

4.1 Microphotography of MH7A cell line

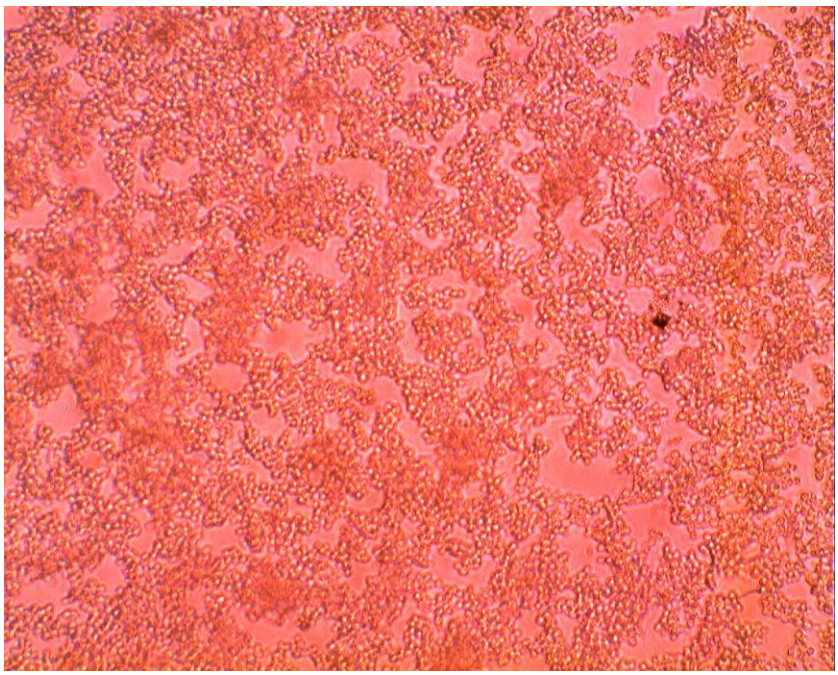


Figure 4.1. Microphotograph of MH7A showing a confluence of 90%. The image was taken at a magnification of 400X using a bright field microscope.

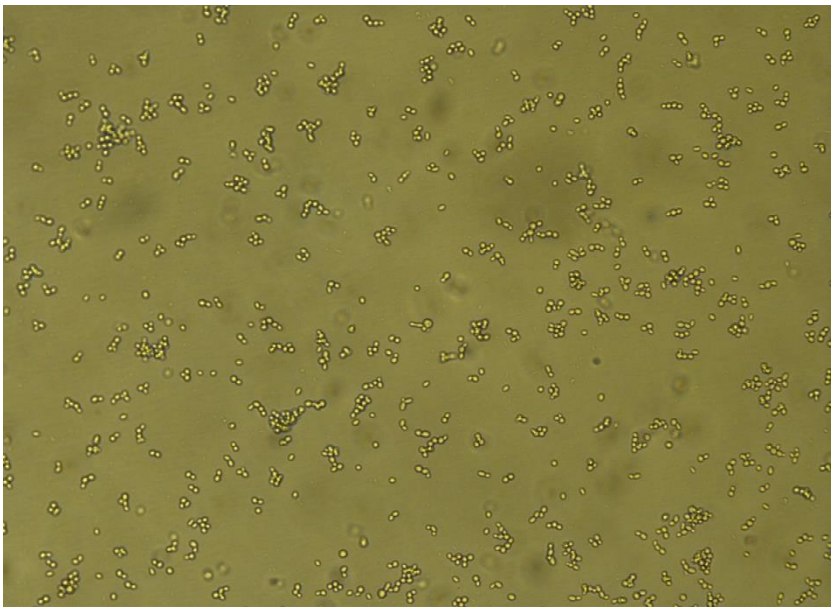


Figure 4.2. Microphotograph of MH7A cells showing a confluence of 25%, after passaging. The image was taken using a phase contrast microscope at a magnification of 200X.

4.2 Determination of Cytotoxicity of Prazosin through MTT Assay

The following graph represents the mean percentage cell viability values of various tested concentrations of Prazosin in comparison to DMSO treated and untreated samples (Figure 4.3):

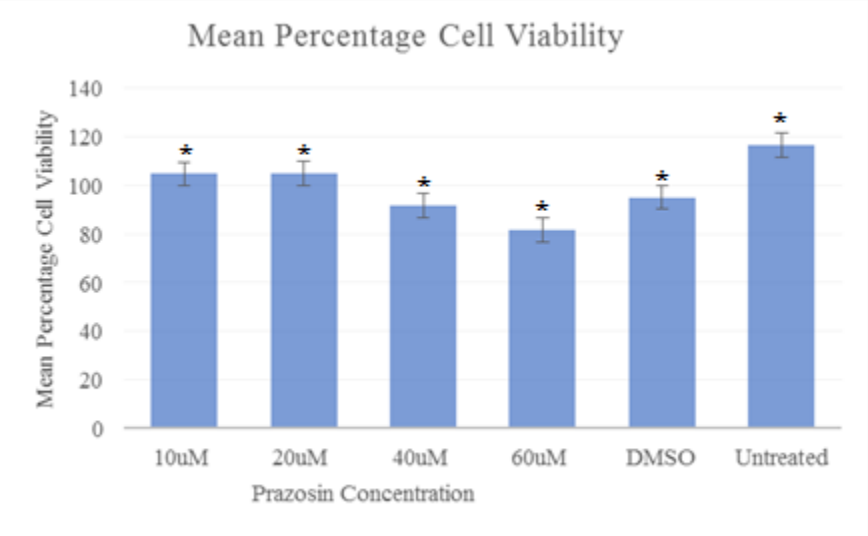


Figure 4.3. This graph represents the mean percentage cell viability values of various tested concentrations of Prazosin in comparison to DMSO treated and untreated samples. The error bars represent the percentage error in mean value.* represents $p < 0.05$ in Student's t-test.

According to the trend observed, cell viability decreases with an increase in the concentration of Prazosin. Furthermore, when the trendline is extrapolated; the minimum inhibitory concentration at which only 50% of the cells are viable is obtained to be ~140µM; as depicted in Figure 4.4.

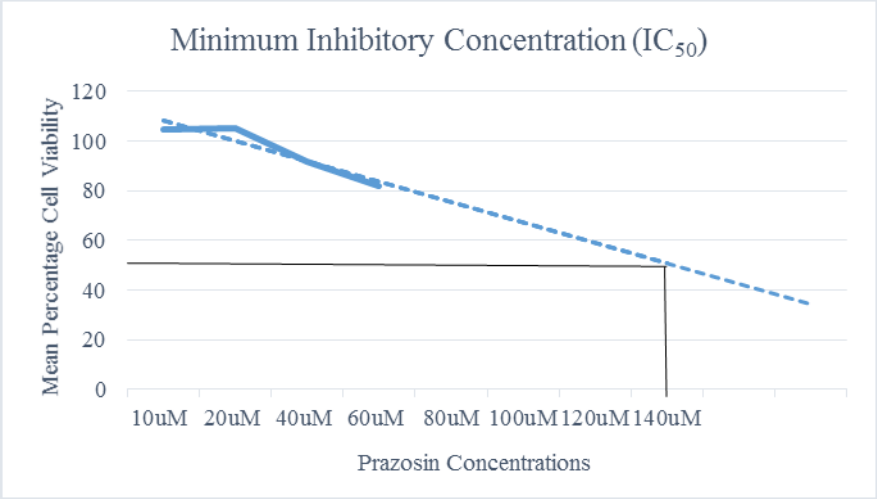


Figure 4.4. This graph represents the minimum inhibitory concentration of Prazosin at which the mean percentage cell viability is 50%.

The following tables show the results of statistical analysis performed on the mean percentage cell viability values obtained from Prazosin (Tables 4.1 and 4.2):

Table 4.1 Statistical analysis of mean percentage cell viability at various concentrations of Prazosin using Student's t-test.

t-Test: Paired Two Sample for Means		
	Mean Percentage Cell Viability	*Concentration*
Mean	99.15	3.5
Variance	149.894	3.5
Observations	6	6
Pearson Correlation	0.089064374	
Hypothesized Mean Difference	0	
Degree of freedom	5	
t Stat	19.17389455	
P(T<=t) one-tail	3.55752E-06	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	7.11505E-06	
t Critical two-tail	2.570581836	

Table 4.2. One way ANOVA results of Prazosin treated samples' cell viabilities vs. untreated sample.

One-way analysis of variance					
P value	0.4694				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	6				
F	1.046				
R squared	0.4658				
ANOVA Table	SS	df	MS		
Treatment (between columns)	0.1499	5	0.02998		
Residual (within columns)	0.1719	6	0.02866		
Total	0.3218	11			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Untreated vs 10uM Prazosin	0.1205	0.7118	No	ns	-0.4531 to 0.6941
Untreated vs 20uM Prazosin	0.1165	0.6882	No	ns	-0.4571 to 0.6901
Untreated vs 40uM Prazosin	0.25	1.477	No	ns	-0.3236 to 0.8236
Untreated vs 60uM Prazosin	0.35	2.068	No	ns	-0.2236 to 0.9236
Untreated vs DMSO	0.216	1.276	No	ns	-0.3576 to 0.7896

4.3 Determination of Cytotoxicity of Sulpiride through MTT Assay

The following graph represents the mean percentage cell viability values of various tested concentrations of Sulpiride in comparison to DMSO treated and untreated samples (Figure 4.5):

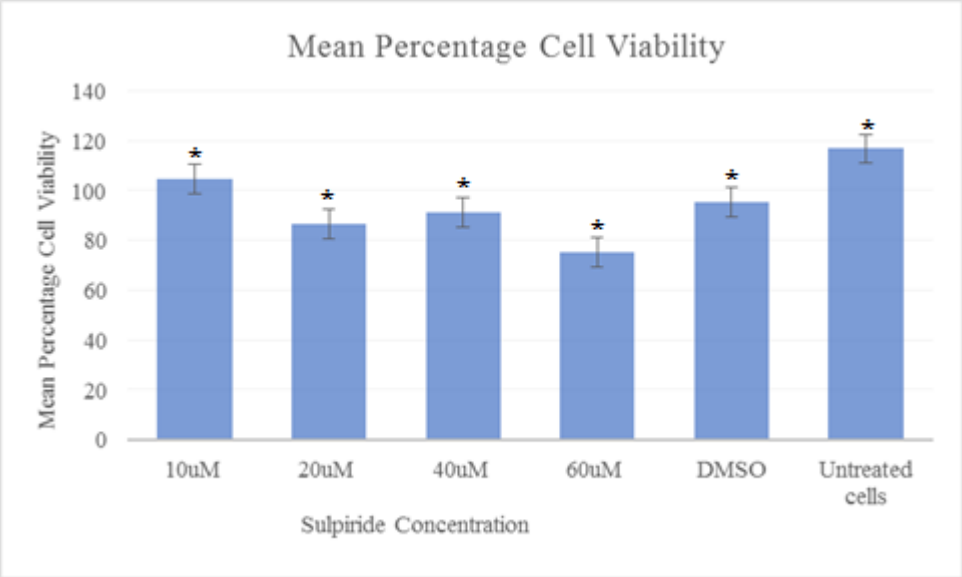


Figure 4.5. This graph shows the mean percentage cell viability values of various tested concentrations of Sulpiride in comparison to DMSO treated and untreated samples. The error bars represent the percentage mean error in the mean value. * represents $p < 0.05$ in Student's t-test.

According to the trend observed, cell viability decreases with an increase in the concentration of Sulpiride; with an exception at the concentration of 40µM. Furthermore, when the trendline is extrapolated; the minimum inhibitory concentration at which only 50% of the cells are viable is obtained to be ~120µM; as depicted in Figure 4.6.

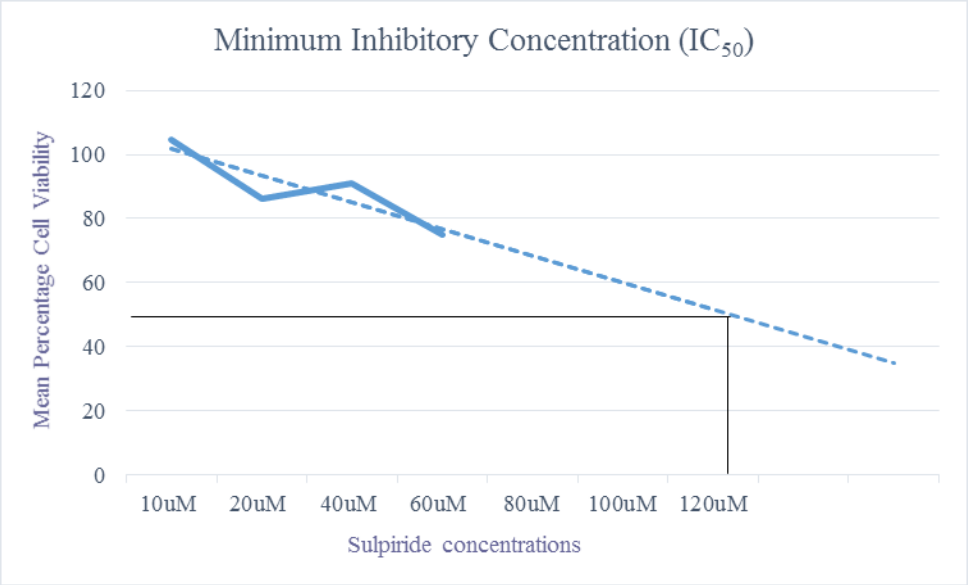


Figure 4.6. This graph represents the minimum inhibitory concentration of Sulpiride at which the mean percentage cell viability is 50%.

The following tables show the results of one tailed t-test and one way ANOVA performed on the mean percentage cell viability values obtained from Sulpiride (Tables 4.3 and 4.4):

Table 4.3. Statistical analysis of mean percentage cell viability at various concentrations of Sulpiride using Student's t-test.

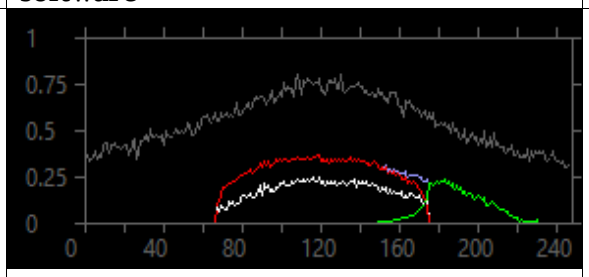
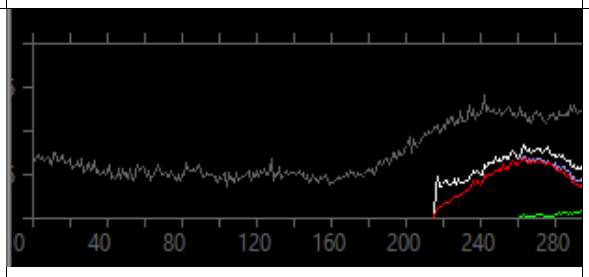
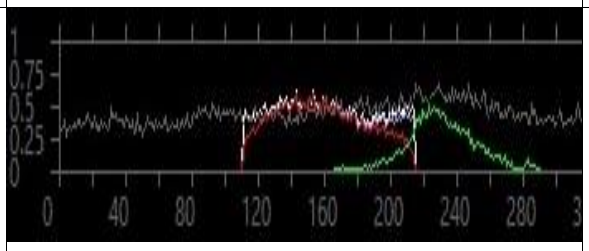
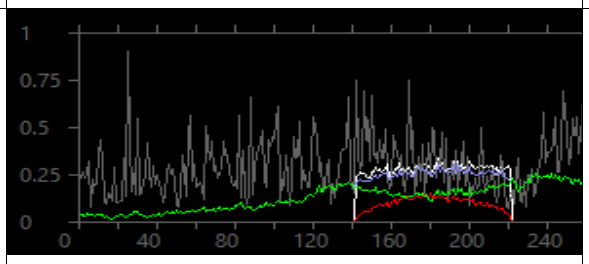
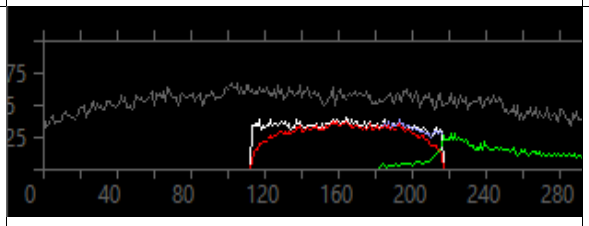
t-Test: Paired Two Sample for Means		
	Mean Percentage Cell Viability	*Concentration*
Mean	94.80833333	3.5
Variance	210.7684167	3.5
Observations	6	6
Pearson Correlation	0.260857343	
Hypothesized Mean Difference	0	
Degree of freedom	5	
t Stat	15.81117752	
P(T<=t) one-tail	9.20487 E-06	
t Critical one-tail	2.015048373	
P(T<=t) two-tail	1.84097 E-05	
t Critical two-tail	2.570581836	

Table 4.4. One way ANOVA results of Sulpiride treated samples' cell viabilities vs. untreated sample.

One way Analysis of Variance					
P value	0.4753				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	6				
F	1.032				
R squared	0.4624				
ANOVA Table	SS	df	MS		
Treatment (between columns)	0.2223	5	0.04446		
Residual (within columns)	0.2584	6	0.04307		
Total	0.4808	11			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Untreated vs 10uM Sulpiride	0.121	0.583	No	ns	-0.5822 to 0.8242
Untreated vs 20uM Sulpiride	0.3045	1.467	No	ns	-0.3987 to 1.008
Untreated vs 40uM Sulpiride	0.3055	1.472	No	ns	-0.3977 to 1.009
Untreated vs 60uM Sulpiride	0.417	2.009	No	ns	-0.2862 to 1.120
Untreated vs DMSO	0.216	1.041	No	ns	-0.4872 to 0.9192

4.4 Microphotographs of Comet Assay

Table 4.5 Representative images of Comet Assay.

	Graphical representation of DNA damage parameters using CaspLab software	Fluorescent representative microphotograph of a comet
Untreated control sample		
H_2O_2 treated sample		
Prazosin treated sample		
Sulpiride treated sample		
DMSO treated sample		

4.5 Determination of DNA damage effect of Prazosin through Comet Assay

The following graphs show the change in tail length (Figure 4.5), tail DNA (Figure 4.6) and tail moment (Figure 4.7) parameters of DNA damage at the tested concentrations of Prazosin. All three parameters are directly proportional to the extent of DNA damage in the cells and the observed trends in all the graphs here show that increasing concentrations of Prazosin have a DNA protective effect as tail length, tail DNA and tail moment all decrease with a concomitant decrease in Prazosin concentration.

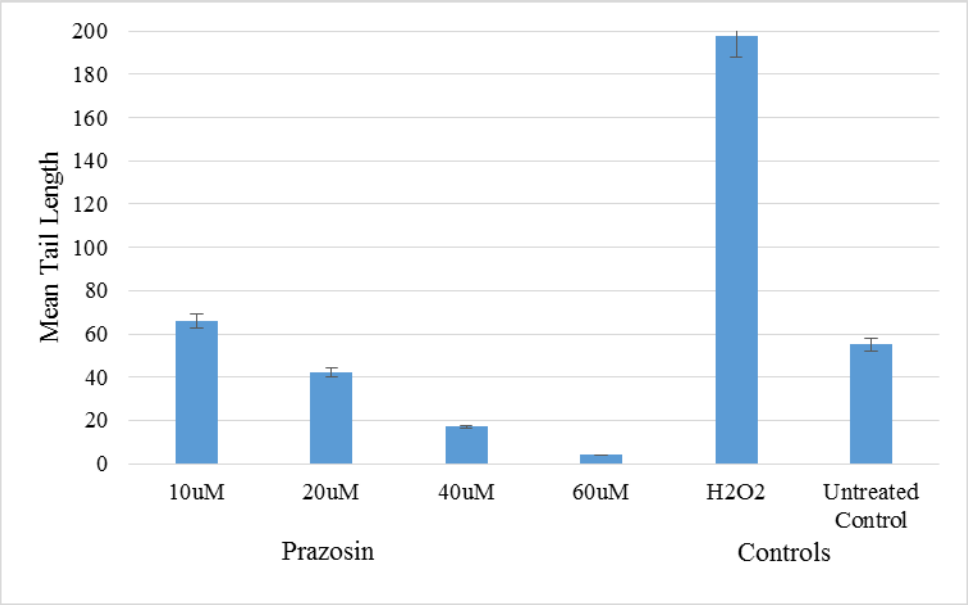


Figure 4.5. The graph shows the mean Tail Length for samples treated with various concentrations of Prazosin in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

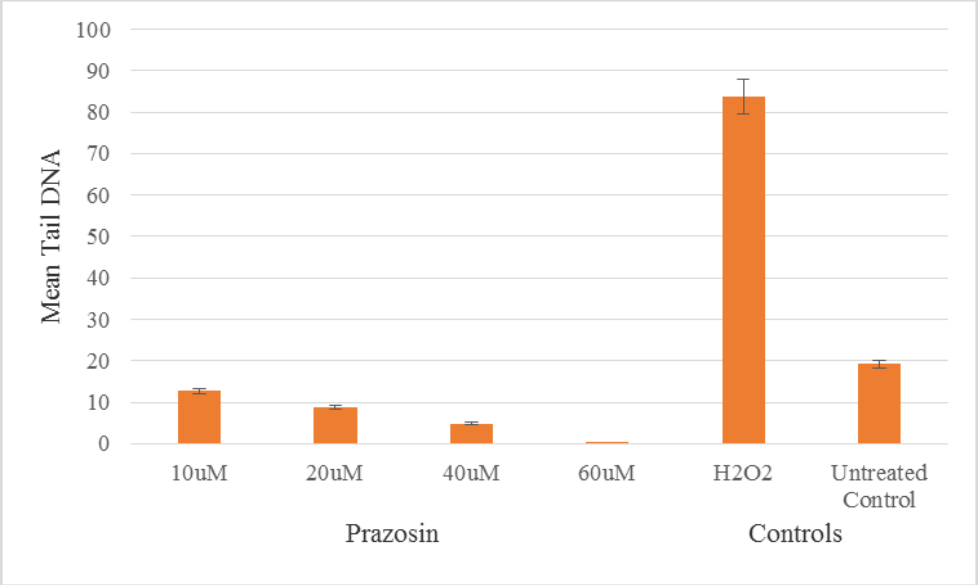


Figure 4.6. The graph shows the mean Tail DNA for samples treated with various concentrations of Prazosin in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

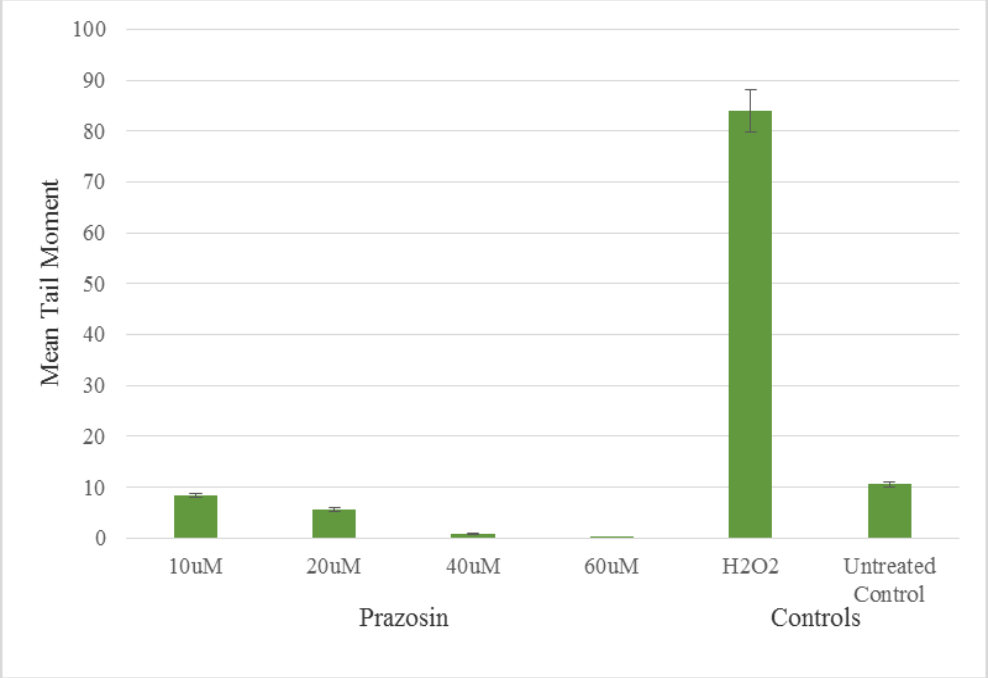


Figure 4.7. The graph shows the mean Tail moment for samples treated with various concentrations of Prazosin in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

The following table shows the statistical analyses of mean tail length, mean tail DNA and mean tail moment of Prazosin treated samples alongside H_2O_2 treated positive control and untreated negative control samples. The statistical test applied here is one way ANOVA followed by Bonferroni's Multiple Comparison test as the post-hoc test (Table 4.5).

Table 4.6. One way ANOVA results of Prazosin treated samples' mean tail length, mean tail DNA and mean tail moment vs. H_2O_2 treated positive control and untreated negative control sample.

One-way analysis of variance-Prazosin					
P value	0.2203				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	1.676				
R squared	0.1827				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	4.077				
P value	0.1302				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7705	2	3852		
Residual (within columns)	34470	15	2298		
Total	42180	17			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Mean Tail Length vs Mean Tail DNA	42.13	1.522	No	ns	-32.43 to 116.7
Mean Tail Length vs Mean Tail Moment	45.46	1.642	No	ns	-29.10 to 120.0
Mean Tail DNA vs Mean Tail Moment	3.327	0.1202	No	ns	-71.23 to 77.88

4.6 Determination of DNA damage effect of Sulpiride through Comet Assay

The following graphs show the change in tail length (Figure 4.8), tail DNA (Figure 4.9) and tail moment (Figure 4.10) parameters of DNA damage at the tested concentrations of Sulpiride. All three parameters are directly proportional to the extent of DNA damage in the cells and the observed trends in all the graphs here show that increasing concentrations of Sulpiride have a DNA protective effect as tail length, tail DNA and tail moment all decrease with a concomitant decrease in Sulpiride concentration.

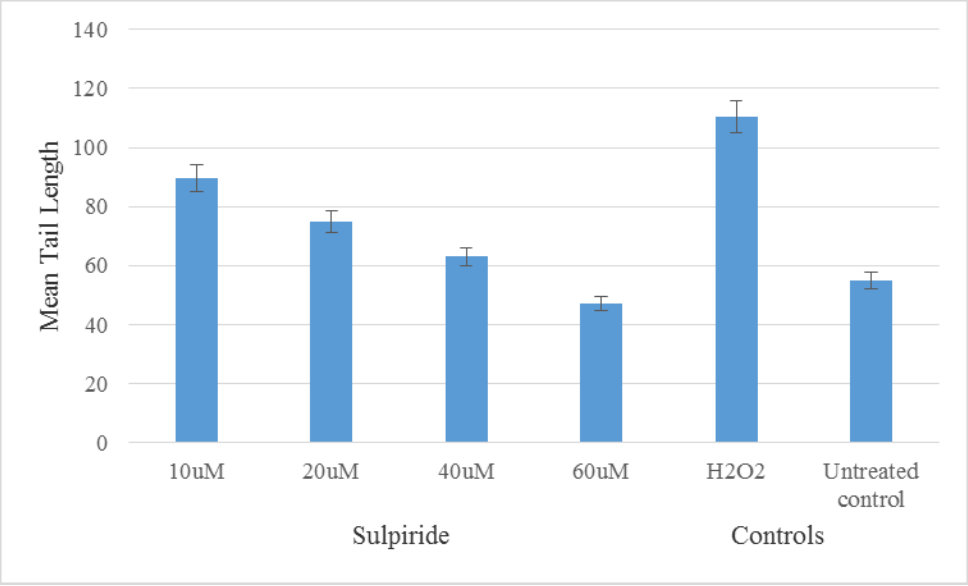


Figure 4.8. The graph shows the mean Tail Length for samples treated with various concentrations of Sulpiride in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

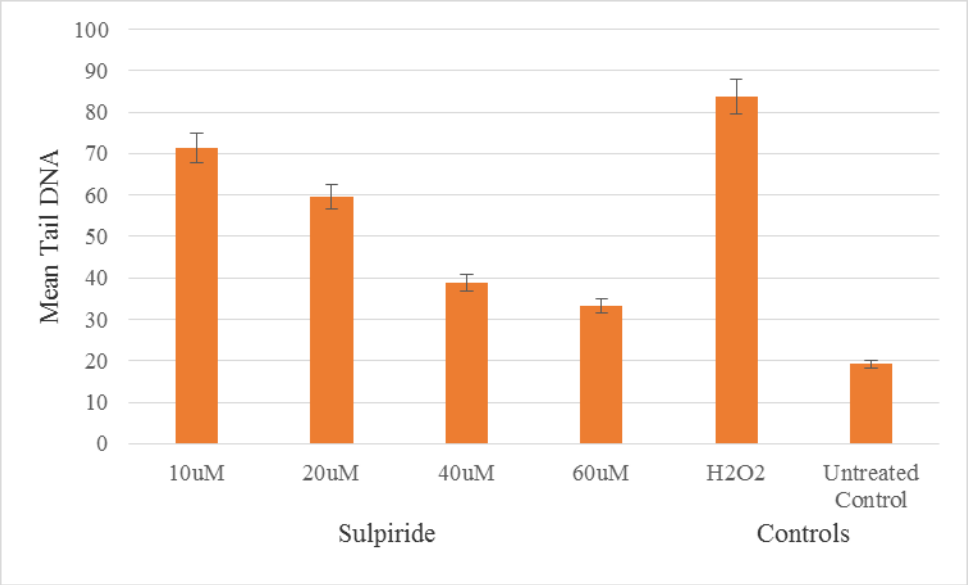


Figure 4.9. The graph shows the mean Tail DNA for samples treated with various concentrations of Sulpiride in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

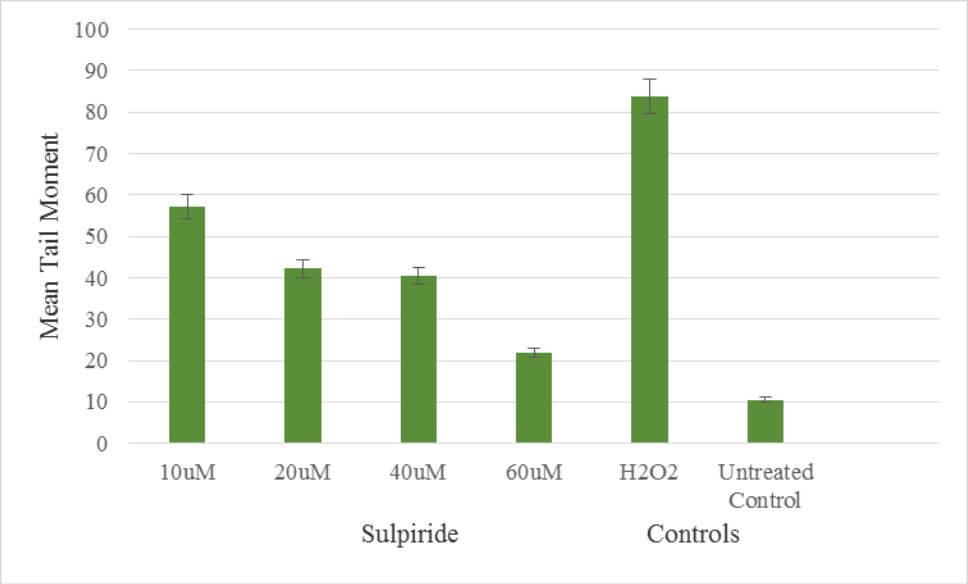


Figure 4.10. The graph shows the mean Tail moment for samples treated with various concentrations of Sulpiride in comparison to H_2O_2 and untreated sample. The error bars represent the percentage error in each mean value.

The following table shows the statistical analyses of mean tail length, mean tail DNA and mean tail moment of Sulpiride treated samples alongside H_2O_2 treated positive control and untreated negative control samples. The statistical test applied here is one way ANOVA followed by Bonferroni's Multiple Comparison test as the post-hoc test (Table 4.7).

Table 4.7. One way ANOVA results of Sulpiride treated samples' mean tail length, mean tail DNA and mean tail moment vs. H_2O_2 treated positive control and untreated negative control sample.

One-way analysis of variance-Sulpiride					
P value	0.1194				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	2.457				
R squared	0.2467				
Bartlett's test for equal variances					
Bartlett's statistic (corrected)	0.04367				
P value	0.9784				
P value summary	ns				
Do the variances differ signif. (P < 0.05)	No				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3010	2	1505		
Residual (within columns)	9190	15	612.6		
Total	12200	17			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Mean Tail Length vs Mean Tail DNA	22.34	1.563	No	ns	-16.15 to 60.83
Mean Tail Length vs Mean Tail Moment	30.62	2.143	No	ns	-7.876 to 69.11
Mean Tail DNA vs Mean Tail Moment	8.278	0.5793	No	ns	-30.22 to 46.77

4.7 Expression Profiling of *CA-II*

The following graph represents the mean fold change in the expression of *CA-II* in the Prazosin treated, Sulpiride treated and untreated groups of cells (Figure 4.11). In the Prazosin treated group; the expression of *CA-II* is only slightly reduced in comparison to the untreated group. On the other hand, the expression of *CA-II* is markedly reduced in the Sulpiride treated group in comparison to the Untreated group.

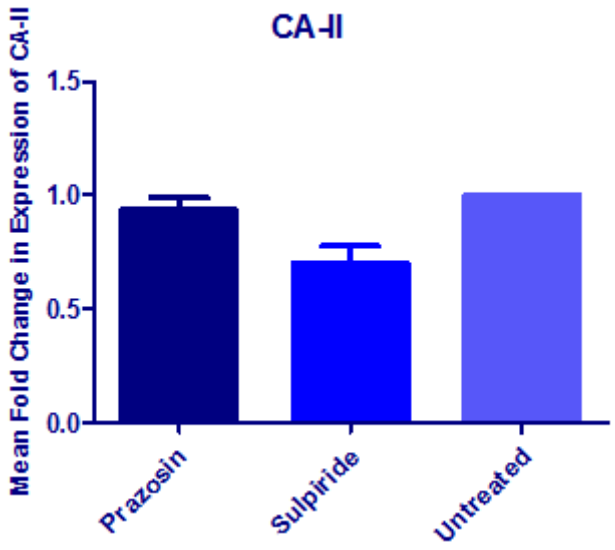


Figure 4.11. Relative expression of *CA-II* in Prazosin treated, Sulpiride treated and Untreated groups. Error bars represent the SEM (standard error of the mean).

The following table shows the results of One Way Analysis of Variance (ANOVA) statistical test which was performed to analyze the statistical significance of the mean fold change in the expression of *CA-II* across Prazosin treated, Sulpiride treated and untreated groups of cells. Bonferroni's Multiple Comparison test was used as the post-hoc test following ANOVA (Table 4.8).

Table 4.8. One way ANOVA results of the mean fold change in the expression of *CA-II* in Prazosin treated samples, Sulpiride treated samples and untreated samples.

One-way analysis of variance CA-II					
P value	0.3799				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	1.36				
R squared	0.4755				
ANOVA Table	SS	df	MS		
Treatment (between columns)	0.09921	2	0.0496		
Residual (within columns)	0.1094	3	0.03648		
Total	0.2086	5			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Prazosin vs Sulpiride	0.2353	1.232	No	ns	-0.6924 to 1.163
Prazosin vs Untreated	-0.06375	0.3338	No	ns	-0.9914 to 0.8639
Sulpiride vs Untreated	-0.299	1.565	No	ns	-1.227 to 0.6286

4.8 Expression Profiling of *MMP-9*

The following graph represents the mean fold change in the expression of *MMP-9* in the Prazosin treated, Sulpiride treated and untreated groups of cells (Figure 4.12). In the Prazosin treated group; the expression of *MMP-9* is reduced in comparison to the untreated group. On the other hand, the expression of *MMP-9* is markedly increased in the Sulpiride treated group in comparison to the untreated group.

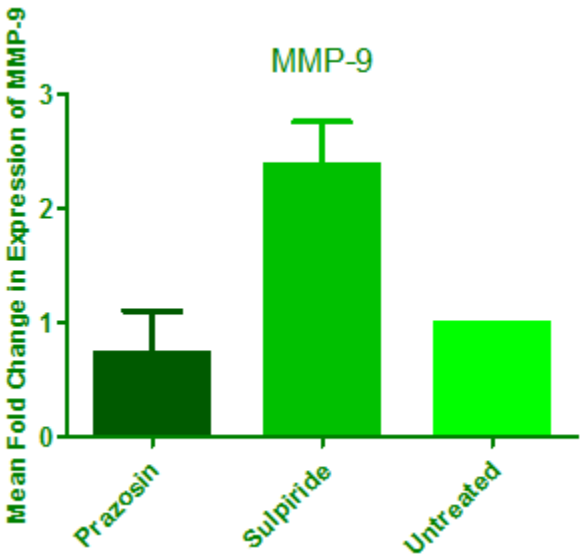


Figure 4.12. Relative expression of *MMP-9* in Prazosin treated, Sulpiride treated and Untreated groups. Error bars represent the SEM (standard error of the mean).

The following table shows the results of One Way Analysis of Variance (ANOVA) statistical test which was performed to analyze the statistical significance of the mean fold-change in the expression of *MMP-9* across Prazosin treated, Sulpiride treated and untreated groups of cells. Bonferroni's Multiple Comparison test was used as the post-hoc test following ANOVA (Table 4.9).

Table 4.9. One way ANOVA results of the mean fold change in the expression of *MMP-9* in Prazosin treated samples, Sulpiride treated samples and untreated samples.

One-way analysis of variance of MMP-9					
P value	0.0575				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	8.573				
R squared	0.8511				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3.165	2	1.583		
Residual (within columns)	0.5538	3	0.1846		
Total	3.719	5			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Prazosin vs Sulpiride	-1.656	3.854	No	ns	-3.743 to 0.4310
Prazosin vs Untreated	-0.2642	0.6149	No	ns	-2.351 to 1.823
Sulpiride vs Untreated	1.392	3.239	No	ns	-0.6952 to 3.478

4.9 Expression Profiling of *TNF-α*

The following graph represents the mean fold change in the expression of *TNF-α* in the Prazosin treated, Sulpiride treated and untreated groups of cells (Figure 4.13). In the Prazosin treated group; the expression of *TNF-α* is slightly increased in comparison to the untreated group. On the other hand, the expression of *TNF-α* is markedly increased in the Sulpiride treated group in comparison to the Untreated group.

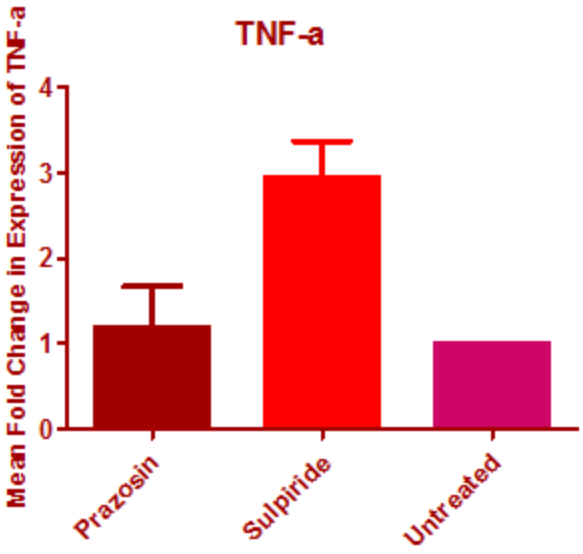


Figure 4.13. Relative expression of *TNF-α* in Prazosin treated, Sulpiride treated and Untreated groups. Error bars represent the SEM (standard error of the mean).

The following table shows the results of One Way Analysis of Variance (ANOVA) statistical test which was performed to analyze the statistical significance of the mean fold-change in the expression of *TNF-α* across Prazosin treated, Sulpiride treated and untreated groups of cells. Bonferroni's Multiple Comparison test was used as the post-hoc test following ANOVA (Table 4.10).

Table 4.10. One way ANOVA results of the mean fold change in the expression of *TNF-α* in Prazosin treated samples, Sulpiride treated samples and untreated samples.

One-way analysis of variance of TNF-α					
P value	0.061				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	3				
F	8.176				
R squared	0.845				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4.574	2	2.287		
Residual (within columns)	0.8392	3	0.2797		
Total	5.414	5			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Prazosin vs Sulpiride	-1.752	3.313	No	ns	-4.321 to 0.8167
Prazosin vs Untreated	0.1863	0.3521	No	ns	-2.383 to 2.755
Sulpiride vs Untreated	1.938	3.665	No	ns	-0.6304 to 4.507

CHAPTER 5

DISCUSSION

Carbonic Anhydrases (CA) represent a ubiquitous class of zinc containing metalloenzymes having sixteen mammalian isoforms. These participate in vital biological processes such as maintenance of pH homeostasis and catalysis of reversible hydration of carbon dioxide to yield bicarbonate and protons. Isoforms of CA enzymes have been found to partake in a plethora of key biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion. Many CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis (Maren, 1988; Supuran, 2008).

In view of this knowledge, the aim of the current study was to investigate the *in vitro* potential of candidate Carbonic Anhydrase-II (CA-II) inhibitors as novel anti-resorption therapeutic agents in Rheumatoid Arthritis. Two chemical inhibitors Prazosin and Sulpiride were selected upon the basis of an *in silico* study which screened the DrugBank database for chemical compounds that conformed to the structure of the binding pocket of CA-II. CA-II plays a key role in the initiation of the process of bone resorption as it performs the acidification of resorption lacunae in the bone via production of H^+ ions. This acidification in turn activates further processes down the line in a feed-forward loop that constitute important hallmarks during the pathogenesis of Rheumatoid Arthritis such as inflammation, bone and cartilage destruction and synovial hyperplasia (Arnett, 2007; Lehenkari *et al.*, 1998).

In the current study, results of MTT assay indicate that both inhibitors namely Prazosin and Sulpiride do not have a profound effect on the viability of cells as the range of mean percentage cell viability remained within the range of 70% to 110% for both inhibitors. Furthermore, the approximate values of IC_{50} were estimated to be 140uM for Prazosin and 120uM for Sulpiride respectively. Prazosin is known to act as a α_1-adrenergic receptor blocker belonging to the class of drugs known as quinazolinamines. It has been shown to induce apoptosis in prostate cancer cell lines at a concentration in the range of 10-100uM and the reported IC_{50} in this study is 10-30uM (Lin *et al.*, 2007). Sulpiride is a α_1-dopaminergic receptor blocker which has been reported to not induce apoptosis in normal pituitary gland lactotrophs and malignant lactotrophs in order to explore their potential as a therapeutic option for prolactinomas (Guo *et al.*, 1997; Radl *et al.*, 2008).

Rheumatoid Arthritis is frequently described as a disease of unknown etiology having strong underlying DNA damage connotations. This DNA damage is primarily due to an excessive generation of reactive oxygen species (ROS) by neutrophils and an impaired antioxidant activity during chronic inflammation during the course of pathogenesis in Rheumatoid Arthritis; also described as a state of oxidative stress (Altindag *et al.*, 2007). Oxidative damage to DNA has been associated with a number of pathologies including neoplastic, neurodegenerative, cardiovascular, and autoimmune diseases (Cooke *et al.*, 2006). Oxidative stress, which often arises as a result of an imbalance in the human oxidative/antioxidative status, has been implicated in aging and a number of diseases such as cancer, atherosclerosis, rheumatoid arthritis, osteoarthritis, fibromyalgia, and osteoporosis. Reactive oxygen species is produced at the site of synovitis by macrophage and polymorphonuclear cells or by mechanical reperfusion and may contribute to the

maintenance of inflammation through the activation of inflammatory molecules, leading to the destruction of articular cartilage in RA (Henrotin *et al.*, 2005). Another study has reported an association between disease activity and presence of oxidative stress in RA patients (Jikimoto *et al.*, 2002). It has been suggested that high level of DNA damage induced by oxidative stress was observed in human autoimmune diseases including RA (Bashir *et al.*, 1993). Pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, interleukin-6 and others have been shown to play pathologic roles in RA (Nishimoto, 2006).Hence amelioration of DNA damage represents a focal area of research in the treatment of Rheumatoid Arthritis.

In order to assess the DNA damaging/ protecting properties of CAIs Prazosin and Sulpiride, Comet Assay was performed. The results of Comet Assay indicate a protective effect towards DNA damage for both CAIs under investigation i.e. Prazosin and Sulpiride. This is evident from the fact that DNA damage decreased in proportion to an increase in the concentration of the inhibitor. DNA damage was measured by the parameters of mean tail length, mean tail DNA and mean tail moment; which are all directly proportional to DNA damage. The values of these parameters decreased with a concomitant increase in the concentration of the inhibitor under test.

CA-II, being the intended target of both inhibitors, showed a slightly reduced expression in Prazosin treated samples whereas a marked decrease in CA-II expression was observed in Sulpiride treated samples. This is corroborated by previous findings regarding the sulfonamide group of CAIs as Sulpiride itself belongs to the class of drugs known as benzenesulfonamides which have been reported to be effective inhibitors of Carbonic Anhydrases. Sulfonamides represent the third major class CAIs, the other two being

phenols and metal complexing anions (Innocenti *et al.*, 2008). Sulfonamides alongside its bioisosteres has been shown to selectively inhibit Carbonic Anhydrases by binding to the Zn^{2+} ions in their catalytic site. Other members of the sulfonamide class of CAIs include acetazolamide, methazolamide and ethoxzolamide which have long been used as diuretics, anti-glaucoma and anti-convulsant drugs and have recently been used to treat cancer and obesity as well (Supuran, 2008). Phenolic compounds and their derivatives have been recently investigated to determine their interaction with mammalian CA isoforms because of their already known antioxidant, ROS scavenging and anti-inflammatory properties (Innocenti *et al.*, 2008; Supuran and Scozzafava, 2007).

In addition to *CA-II*, expression profiling of *TNF-α* and *MMP-9* was also performed in Prazosin treated, Sulpiride treated and untreated samples. The expression of *MMP-9* was relatively reduced in Prazosin treated samples whereas in the case of Sulpiride treated samples, its expression was increased approximately two-fold in comparison to the untreated samples. The expression of *TNF-α* was slightly increased in Prazosin treated samples whereas in the case of Sulpiride treated samples a two-fold increase was observed in the expression of *TNF-α*. It can be postulated that in case of Sulpiride treated samples, a comparatively more effective inhibition of *CA-II* was obtained. Furthermore the concomitant increase in the expression of both *TNF-α* and *MMP-9* points in the direction of an underlying feed-forward loop which increases the expression of *TNF-α* and *MMP-9* in response to a reduction in *CA-II* expression. TNF-α has been reported to induce the expression of CA-II in human pancreatic adenocarcinoma cells in a time-dependent manner (Franz *et al.*, 1994). RANKL has been shown to induce the expression of both CA-II and MMP-9 in osteoclasts and the expression of CA-II is reported to induce the acidification

of the resorption lacunae which are then resorbed by collagenases such as Cathepsin K and MMP-9 (Fujisaki *et al.*, 2007; Teitelbaum, 2000). However, the direct mechanism of interaction of MMP-9 with CA-II is still unchartered as of now.

5.1 Conclusion

This study concludes that Prazosin and Sulpiride, two potential CA-II inhibitors are not cytotoxic in nature towards synovial fibroblasts nor do they cause DNA damage in these cells. They have demonstrated DNA protective effects on synovial fibroblasts as shown by the overall results of MTT and Comet Assays. Sulpiride is a relatively more potent inhibitor of CA-II in comparison to Prazosin, a change that is accompanied by a concomitant increase in the expression of TNF-α and MMP-9, markers of inflammation and cartilage destruction respectively. This is potentially due to the fact that the research model employed here is an arthritic cell line in which the disease onset has already reached its pinnacle. Prazosin and Sulpiride both represent promise as potential therapeutic agents for the treatment of Rheumatoid Arthritis due to their noncytotoxic and DNA protective properties.

5.2 Future Prospects

The expression profiling of additional markers of bone resorption like Cathepsin K, NFATc and Calpain-I will be pivotal towards unravelling the underlying pathways where CA-II interacts with various components of the bone resorption pathway. Furthermore, expression analysis at the post-translational level of these key proteins also needs to be performed in order to obtain conclusive results regarding the validity of Prazosin and Sulpiride as potential CA-II inhibitors as novel anti-resorption therapeutic agents in Rheumatoid Arthritis.

CHAPTER 6

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