Protein expression profiling of some osteoclastogenic markers in Rheumatoid arthritis: an in-vitro and in-silico study

BY

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Dedicated to my loving parents

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Acknowledgements

"In the name of Allah, the Entirely Merciful, the Especially Merciful."

"[All] praise is [due] to Allah, Lord of the worlds."

(The Holy Qur'an: *Surat Al-Fātiĥah*, Translation of *Ayaat*1-2)

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List of Abbreviations

A_acc	Hydrogen bond acceptors
a_don	Hydrogen bond donors
ACCP	Anti-cyclic Citrullinated Peptide
ACPA	Anti–citrullinated protein antibodies
ADMET	absorption, distribution, metabolism, and excretion - toxicity
aPKC	atypical protein kinase C
APS	Ammonium persulfate
b_rotN	Number of rotatable bonds
BM	Bone Marrow
CAII	Carbonic anhydrase II
CAIs	Carbonic anhydrase Inhibitors
CamKIV	Calcium/calmodulin-dependent protein kinase type IV
CAPN1	Calpain 1
CAPN2	Calpain 2
CAT	Catalase

List of Abbreviations

CCD	Charged coupled Device
CCL21	Chemokine (C-C motif) ligand 21
CCR6	Chemokine receptor 6
CD40	CD40 molecule, TNF receptor superfamily member 5
CEP	C-Terminally Encoded Peptide
CREB	cAMP response element-binding protein
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXCL12	C-X-C motif chemokine 12
DAP12	DNAX Activating Protein of 12KDa
DMARDs	Disease-modifying antirheumatic drugs
DMSO	Dimethyl sulfoxide
DW	Deep water
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FCGR3A	Gamma Fc region receptor III-A
FcRγ	Fc receptor Gamma
G-CSF	Granulocyte-colony stimulating factor
GH	Growth hormone

List of Abbreviations

GPx	Glutathione peroxidase
HAT	Histone acetyltransferase
hCAII	Human Carbonic anhydrase II
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon gamma
IKK	IκB kinase
IL	Interleukin
IRF5	Interferon regulatory factor
ITAMs	Immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N-terminal kinases
LIGHT	Tumor-necrosis factor (TNF) superfamily member (TNFSF14)
LMPA	Low-Melting-Point Alloy
MAPKs	Mitogen-activated protein kinase
MEKKs	Mitogen-activated protein kinase kinase kinase
MEKs	Mitogen-activated protein kinase kinase

List of Abbreviations

MMP	Matrix Metalloproteinase
NFATc3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kappa-B-inducing kinase
NMA	N-methyl-DL-aspartate
NOX4	NADPH oxidase 4
NSAIDs	Nonsteroidal anti-inflammatory drugs
OC	Osteoclasts
OD	Optical density
OSCAR	Osteoclast-associated immunoglobulin-like receptor
p38	P38 mitogen-activated protein kinases
p55	Palmitoylated membrane protein 1
p62	Nucleoporin p62
PAD14	peptidyl arginine deiminase, type IV
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
PLCγ2	Phospholipase Cγ2

List of Abbreviations

PTH	Parathyroid Hormone
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid Factor
RGD	Arginylglycylaspartic acid
ROS	Reactive oxygen species
SCGE	Single Cell Gel Electrophoresis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
TAB1	TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 1
TAB2	TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 2
TAK1	TGF-Beta-Activated Kinase 1
TEMED	Tetramethylethylenediamine
TGF-β	Tumor Growth Factor-Beta

List of Abbreviations

TNFSF14	Tumor necrosis factor ligand superfamily member 14
TNF-α	Tumor necrosis factor alpha
TPSA	Topological polar surface area:
TRAF1	TNF receptor-associated factor 1
TRAP	Tartrate-resistant acid phosphatase
TWEAK	TNF-Related Weak Inducer of Apoptosis
TWHF	Tripterygium wilfordii Hook. F

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Abstract

Rheumatoid Arthritis is a chronic polyarthritic autoimmune condition associated with joint erosion and synovial hyperplasia. Bone resorption in RA is a characteristic of disease pathogenesis. RANKL signaling pathway is the main pathway involved in bone resorption via osteoclastogenesis. Many factors activated downstream RANKL signaling pathway exhibit elevated expression levels in RA and have been targeted for therapeutic purposes. In this study, protein expression profiling of human carbonic anhydrase II, m-Calpain, NFATc3 and TNF-alpha have been elucidated by using the technique of western blotting. The results showeded their increased expression in the serum of RA patients. Furthermore, since these proteins are inflammatory markers and inflammation is tightly associated with oxidative stress, the DNA damage status due to this oxidative stress is assessed in this study using comet assay. The results showeded that lymphocytes from RA patients demonstrate detectable DNA damage. Moreover, in-silico virtual screening has been used to screen the DrugBank for the identification of new hit compounds exhibiting the potential to target human carbonic anhydrase II and inhibit its activity. For this, docking and pharmacophore modeling approaches were used. Three new hit compounds were elucidated. First one, Supiride, belongs to a class of Benzenesulphonamides which is the mostwell-studied class of compounds known till now. The other two compounds, alfuzosin and Prazosin are quinzolamines, which is a novel prediction since compounds of this class have never been reported as CAIs nor used for the treatment of RA.

Introduction

1.1. Rheumatoid Arthritis

Rheumatoid Arthritis (RA), affecting about 1% of the world population, is a chronic polyarthritic autoimmune condition characterized by colossal joint destruction as a consequence of synovial hyperplasia (McInnes *et al.,* 2011; Panati *et al.,*2012). In Fig 1.1 comparison between normal joints and those affected with Rheumatoid Arthritis is showedn. Although etiology of Rheumatoid Arthritis is not known per se, the "Bermuda triangle" of genetic, environmental factors and autoimmunity has been known to trigger the onset and perpetuation of synovitis underlying RA (Van der Woude *et al.,* 2010; Szodoray *et al.,* 2010; Klareskog *et al.,* 2007; Mahdi *et al.,* 2009). Research studies of rheumatoid synoviocytes conducted to gain insights into the pathogenesis of bone erosion have led to the unraveling of their altered phenotype in RA patients (Davis *et al.,*2003). Bone and articular cartilage are invaded by pannus which is a fibrovascular structure formed as a result of proliferation of synoviocytes. Radiographic exams reveal that the symptoms of joint erosion in RA arise in very early stages and exacerbate with the advancement of the disease causing acute structural and functional impairment associated with major odds of mortality and morbidity (Takeuchi *et al.,* 2013). The environmental causes include smoking, bronchial stress and infectious agents. Infiltration of synovial compartment by leukocytes, imbalanced levels of chemokines, synovial hyperplasia accompanied by hypoxic conditions and neoangiogenesis are all characteristics of synovitis underlying RA pathogenesis.

In addition, RA is also characterized by system complications like pulmonary, psychological and cardiovascular and brain-related problems (McInnes *et al.*, 2011).

1.2. Prevalence

Prevalence of RA is significantly diversified in both geographical and chronological context throughout the world. Around 0.5 to 1% of the world population is afflicted with the RA while 0.55 to 0.75% Pakistanis have been suffering from the disease (Silman and Pearson, 2002). People with ages between 30 and 50 are the most vulnerable ones for the onset of disease. Nevertheless, the disease symptoms can appear at any other age as well (Rindfleisch and Muller, 2005). Odds of RA incidence exhibit gender bias as the women are observed to be three times more exposed to the risk of suffering from the disease as compared to men. Furthermore, socioeconomic status also results in the fluctuations in RA prevalence rate. Results of epidemiological studies conducted in Serbia in 2013 showeded 0.35% RA prevalence which is close to that of France (0.31%) and lower than Lithuania (0.55%) and Pakistan (0.55%-0.75%) (Zlatković-Švenda *et al.*,2013). According to a recent study regarding the estimation of incidence rate of RA in Algeria showeded 0.15% prevalence. RA incidence rates are much lower in Sub-Saharan African countries (less than 0.05% to 0.9%) as compared to all other regions of the world (0.33% in Southern European countries, 0.50% in Northern European countries and 0.35% in developing countries) all having a female-to-male ratio around 3 (Slimani and Ladjouze-Rezig, 2014). Epidemiological study in Taiwan showeded the prevalence rate of 15.8 cases per 100,000 and a mortality rate of 14.7 deaths per 1000 person-years (Kuo *et al.*, 2013). Likewise, RA incidence lies in the same range (0.44%) as that of other European countries showeding a higher incidence rate in women as compared to men and older ages as compared to younger

people (Otsa *et al.*, 2013). Similarly, prevalence of RA in Sweden is found to be 41 per 100,000 with the rates spiking at the ages between 70 to 80 for both sexes (Eriksson *et al.*,2013).

1.3. Diagnosis

Following RA diagnostic criteria proposed by American College of Rheumatology (ACR) in 2010, needs to be satisfied for the definite RA diagnosis (Aletaha *et al.*, 2010):

- ✓ Presence of Rheumatoid Factor
- ✓ Presence of anti-CCP autoantibodies.
- ✓ Presence of synovitis in at least 1 joint.
- ✓ A total score of 6 or higher out of a total score of 10 from four separate domains which include:

1. Number and site of involved joints (score ranging from 0 to 5).
2. Serological aberrations (score ranging from 0 to 3).
3. Augmented acute phase reaction (score ranging from 0 to 1)
4. Duration of the disease (2 levels ranging from 0 to 1)

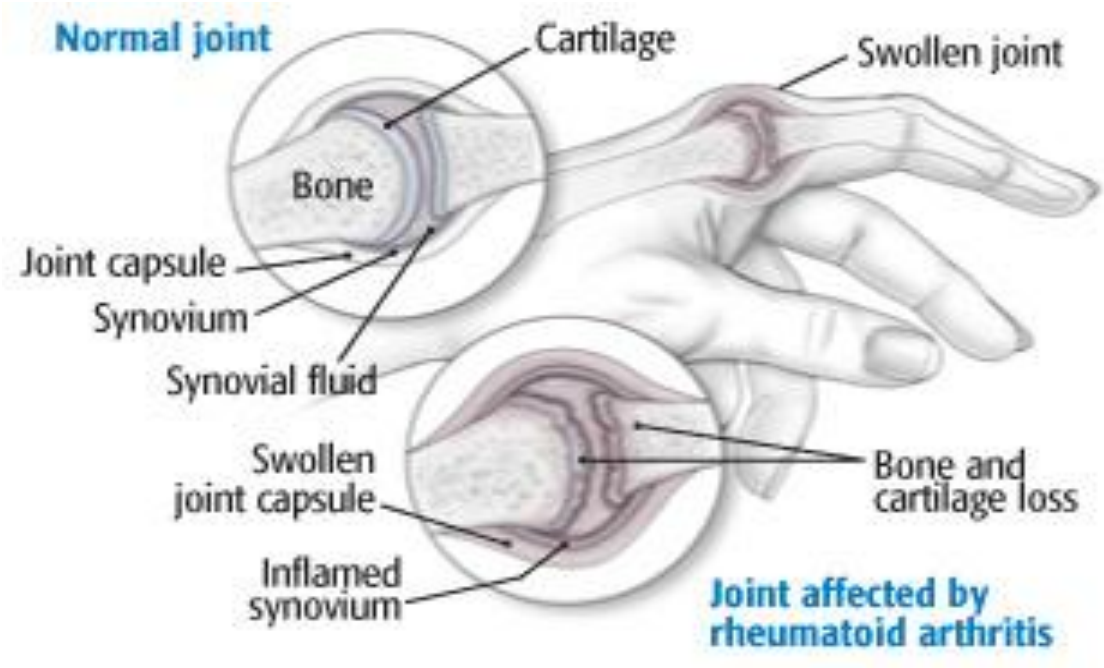

Figure 1.1. A comparison of a normal and RA affected joint (Van der Woude et al., 2010)

1.4. Risk factors

Although the exact etiology of Rheumatoid Arthritis still remains obscure, an intricate web of a multitude of factors have been reported to be involved in the pathogenesis of rheumatoid arthritis in a plethora of studies. These factors entail a broad horizon including the very basic one base pair mutations (SNPs) in various genes, gene-gene interactions, gene-environment interactions, autoimmunity and the most recently studied epigenetics.

1.4.1. Genetics

A large number of genetic factors have been associated with the pathogenesis of RA with 60% heritability (MacGregor *et al.,* 2000). Genetic factors involved in the disease production of RA can be broadly classified into HLA and non-HLA genes. HLA genes play prognostic and pathogenic roles in RA. In RA, HLA contributes about 30-50% to the disease development. An intricate hierarchy of risk factors is conferred by various HLA-DRB1 genotypes including HLA-DRB1*01, DRB1*04 and DRB1*10, DRB1*13 (HLA-DR13) and DRB*15 (HLA-DR15) (van der Helm-van Mil *et al.,* 2005; du Montcel *et al.,* 2005; van der Woude *et al.,* 2010). The prognostic role of HLA genes pertains to the presence of Citrulline in the antigen binding pockets of HLADRB1*1001 resulting in the T-cell mediated production of anti-CCP antibodies which are the main diagnostic marker of Rheumatoid arthritis (van der Helm-van Mil*et al.,* 2007). In addition to the HLA-DR, several non-HLA susceptible genes have also been reported. Among the 31 confirmed non-HLA loci involved in RA risk, probably the strongest associations have been found with PTPN22and PAD14 genes (Boissier *et al.,* 2012). Other significant, confirmed loci constitute TRAF1, CTLA4, IRF5, STAT4, FCGR3A, IL6ST, IL2RA, IL2RB, CCL21, CCR6, CD40 and others. In summary, more than 30 genes have been linked with the disease and these genetic factors comprise around 50 % of RA susceptibility linked genetic

variants (Bax *et al.,* 2011). However, because of the polygenic nature of the pathology, colossal genetic diversity and the intricacies associated with the interpretation of genome wide studies in RA, the exact genetic causes are being difficult to reach a concluding point. Furthermore, another finding that poses major doubts on genetics as the major causation of RA, is the low concordance rate in identical twins (only 15%) implying a prodigious role of other 'non-genetic' factors in the production of RA (Svendsen *et al.,* 2013).

1.4.2. Epigenetics

The alterations in gene activity/expression with no change in the primary sequence of DNA i.e. epigenetics has also been elucidated to play a role in the pathogenesis of rheumatoid arthritis. Various genomic regions in RA patients have been found to be differentially methylated. This includes the hypomethylation of the promoter region of *CXCL12* gene (Karouzakis *et al.,* 2011). Differential expression of methylated genes including HLA region, chromatin-modifying enzymes and increased HAT vs HDAC expression are all being observed in RA epigenetic expression profile leading to arthritic symptoms (Liu *et al.,* 2013; Glant *et al.,*2013).

1.4.3 Gene-environment interactions

A multitude of environmental factors have been found to impinge upon the genetic makeup thereby increasing the odds of RA occurrence. Correlation of oxidative stress with RA has been investigated in detail lately. Increased levels of reactive oxygen species including super oxides and hydrogen peroxides exhibit a momentous correlation with the disease perpetuation in RA by causing damage of articular damage associated with hyperplasia thereby implicating the potential of ROS as biomarkers for evaluation of disease extremity (Datta*et al.,* 2014). In

addition, as a result of inflammatory processes in RA, various pro-inflammatory cytokines also play their roles in oxidative damage. For instance, TGF-β, stimulates bone degradation in rheumatoid arthritis in a dual fashion (Yan*et al.,* 2001). Firstly, it instigates the NADPH oxidase 4 (NOX4) expression and secondly, it has an implicated role in the production of ROS like H_20_2 and hence the oxidative stress generation (Ismail*et al.,* 2009; Takac*et al.,* 2011). Other pro-inflammatory mediators secreted downstream NF-κB and IKKs entail pro-inflammatory enzymes responsible for synovial hyperplasia, generation of adhesion molecules, MMPs, leukotrienes and prostaglandins. Other comprise RNS, kinins, eicosanoids and pro-inflammatory cytokines like IL-1β, IL-12, TNF-α, IFN-γand IL-6 (Mirshafiey and Mohsenzadegan, 2008; Van Lint and Libert, 2007).

Smoking cigarettes, another recently well-established environmental factor responsible for RA, has been reported as a cause of citrullation and hence the anti-CCP antibodies (Pedersen*et al.,* 2006; Michou*et al.,* 2008). The risk is, however dependent only upon the HLA-DRB1*SEgenotype and is linked with the presence of anti-CEP-1 subtype of anti-CCP RA antibodies (van der Helm-van Mil*et al.,* 2007; Linn-Rasker*et al.,* 2006). Although the mechanistic aspects entailing how exactly the smoke leads to the production of anti-CEP-1 antibodies in RA remains obscure, the potential of smoke to trigger recruitment of immune cells, augmentation of CRP levels, fibrinogen, IL-6, IL-1α and TNF-α is well-documented (Costenbader and Karlson, 2006).

Likewise, infection is also an etiological environmental factor associated with RA. Infection with Porphyromonas (P.) gingivalis leads to the citrullination of the proteins thereby leading to the generation of anti-CCP antibodies (Mikuls,*et al.,* 2009). The mode of operation of the infectious agents in RA pathogenesis, however, remains obscure.

1.5. Pathogenesis of Rheumatoid arthritis

1.5.1. The immune system

Rheumatoid arthritis is characterized with the production of disease-specific autoantibodies against citrullinated proteins. These antibodies are themselves under the control of various gene-environment interactions. Mostly, the famous Rheumatoid factor, though not an immune auto-reactive specific to RA, has been used as part of the RA diagnostic criteria (Arnett *et al.,* 1988). Further studies have elucidated anti-keratin and anti-perinuclear factor exhibiting citrullin-specific reaction with mature filaggrin in Rheumatoid arthritis (Simon *et al.,* 1993; Schellekens *et al.,* 1998; Sebbag *et al.,* 1995). Anti-CCP antibodies tend to be recognized in the serum of patients even before the arthritic symptoms appear thus exhibiting tremendous prognostic potential (Wegner *et al.,* 2010).

Furthermore, pro-inflammatory cytokines like IL-1 and TNF-α have been observed to have elevated levels in RA synovium thereby indicating a significant correlation between immunity and synovitis. TNF-α, produced as a consequence of T-cells and stimulated macrophages, is majorly responsible for posing pro-inflammatory effects upon binding its receptor p55 (Sfikakis, 2010). IL-1, expressed at extremely elevated levels in RA, plays a significant role in inflammatory processes associated with RA (Bessis *et al.,* 2000). IL-21 is also reported to be involved in the promotion of osteoclastogenesis and a candidate for therapeutic intervention (Kwok *et al.,* 2012). Moreover, products of activated T-cells, like IL-17, TWEAK, IFN-gamma and G-CSF have been already been established as direct inducers of bone destruction via osteoclastogenesis (Kong *et al.,*1999; Takayanagi *et al.,* 2000; Udagawa, 2003; O'Gradaigh and Compston, 2004; Polek *et al.,* 2003). Another member of TNF super family, TNFSF14, which is a type II transmembrane protein, also known as LIGHT, has been showedn

to have elevated levels in RA RAW247.6 cells. LIGHT and TWEAK has been showedn to have induced osteoclastogenesis leading to systemic bone loss by RANKL-dependent pathway as well as by a mechanism independent of RANKL in RA (Edwards *et al.,* 2006; Park *et al.,* 2013). IL-17 is also involved in RANKL mRNA expression via osteoblast related synthesis of PGE2 which is dependent on COX2 pathway (Kotake *et al.,* 1999). IL-17 studies of bone articular and synovial explants from RA patients have showedn its implicated role in bone destruction process (Chabaud *et al.,* 2001). IL-23 induces the formation of osteoclasts from osteoclast precursor cells in an RANKL-independent process which is however dependent on IL-17, TNF-α and osteoprogerin (Yago *et al.,* 2007). IL-11 works for the sustenance of the osteoclastogenic process (Kawai *et al.,* 2006). Furthermore factors involved in osteoclastogenesis are up-regulated via the production of T-cells induced by IL-7 (Weitzmann *et al.,* 2000).

Another cytokine,TGF-β also promotes RANKL-induced osteoclastogenesis via augmented levels of alphaV integrin subunit, thereby playing a role in the cell-cell fusion of osteoclasts (Chin *et al.,* 2003). There is an accumulating evidence supporting the increased bone resorption as a result of increased ROS production and hence the impaired redox status (Garrett *et al.,* 1990; Hwangand Putney, 2011; Lee *et al.,* 2005; Sasaki *et al.,* 2009). Moreover, NOX4 deficit mice showed osteopetrotic symptoms accompanied by flawed activation of NFATc1 and c-Jun via RANKL induction, implicating its indispensable role in osteoclastogenesis (Goettsch *et al.,* 2013). An overview of factors involved in RA pathogenesis is showedn in Fig 1.2.

1.5.2 Molecular basis of osteoclastogenesis in RA: RANKL signaling pathway

Extensive research indicates that osteoclasts are the primary cells involved in the joint destruction in RA. Osteoclastogenesis takes place at a momentously accelerated rate in

Rheumatoid arthritis at a site at exterior to the marrow cavity, thereby leading to bone loss implicated in RA (Gravallese *et al.,* 2000). Consequently, abnormal bone resorption is the major pathology resulting in the functional impairment caused by rheumatoid arthritis (RA). It is supported by a multitude of research findings which suggest that synovial inflammatory tissue acts as a critical source of osteoclasts in RA by inducing localized osteoclastogenesis leading to loss in the bone mass (Fujikawa *et al.,* 1996; Takayanagi *et al.,* 1997; Gravallese *et al.,* 1998). The induction takes place as synovial tissue not only facilitates the population of osteoclast precursors, monocytes/macrophages, but also of the osteoclastogenic signal producing cells. Hence, overproduction of osteoclasts in RA suggests a significant connection between joint inflammation and structural damage. Hence, we would like to focus on the existing potential molecular agents involved in osteoclastogenesis which can be therapeutically targeted for the treatment and cure of RA.

Bone biology field has been greatly revolutionized since the unraveling of RANK-RANKL signaling pathway as the main pathway involved in osteoclastogenesis (Shigeyama *et al.,* 2000). Moreover, tremendous research in the area has provided insights into the molecular domains of osteoclast formation, differentiation and activation. This makes the expatiated comprehension of the processes of osteoclasts (OC) differentiation and activation inevitable. Fig 1.3 showeds the RANKL signaling pathway involved in bone resorption. Identifying the molecular potential therapeutic targets in the osteoclastogenesis will open new avenues into the treatment and diagnosis of the arthritic conditions.

Binding of RANKL to its receptor RANK leads to the activation of TRAF6 thereby inducing downstream classical and alternative NF-κB pathways. Classical pathway is induced via induction of p62 by aPKC. The alternative NF-κB pathway, is activated either via

phosphorylation of IKKβ by aPKC or by phosphorylation of IKK complex by NIK as a result of TRAF6/TAK1/TAB1/TAB2 complex formation. This also results in the activation of MAPKs like p38, ERK and JNK via MEKKs and MEKs which induce AP-1/c-fos. In addition to its activation by NF-kB, AP-1/c-fos is also activated by CREB after its phosphorylation by CamKIV. AP-1/c-fos induction leads to the activation of NFATc1 while SH3BP2 plays its role in its nuclear translocation. Activation of NFATc1 leads to the downstream expression of various osteoclast specific genes like Cathepsin K, carbonic anhydrase II, TRAP and β3 integrins. RANKL also induces downstream activation of src and OSCAR which lead to activation of Tec Kinases and ITAMs like DAP12 and FcRγ respectively, all essential for osteoclastogenesis. PLCγ2 forming complex with gab2 thereby phosphorylating it leads to the recruitment to RANK to its osteoclastogenic signaling complex. An overview of RANKL signaling pathway is showedn in Fig 1.3.

1.5.3 NF-κB pathway:

Rel or NF-κB, a transcription factor complex, plays significant role in numerous biological processes and hence implicated in a number of disorders including autoimmune disorders. It is activated by RANK and activates various downstream proteins involved in osteoclastogenesis. Activation of NF-kB is an immediate molecular event that takes place via recruitment of TRAF6 by RANK in osteoclastogenesis (Franzoso *et al.,* 1997). Among five members of the NF-kB family, p50 (NF-κB 1) and p52 (NF-κB2) together play a role in osteoclastogenesis. Phenotype of p50-/- or p52-/- mice showeds no bone related abnormally. However knockout mice for both of the genes showed osteopetrotic phenotype implicating the essential role of the two genes in osteoclastogenesis (Takayanagi *et al.,* 2002). NF-κB expression levels and its DNA binding activity have been demonstrated to be augmented in RA patients

(McGovern *et al.,* 2012). Studies showed that administration of an NF-κB inhibitor results in profound reduction in osteoclastogenesis during the initial stages of the process as compared to the later stages thereby explaining its implicated role in the activation of early-stage immediate genes downstream of RANKL (Ishida *et al.,* 2002). An inhibitor of NF-kB, has been showedn to ameliorate arthritic symptoms via down-regulation of the key regulator of osteoclastogenesis in mice with collagen induced arthritis without impinging on any upstream molecules like M-CSF and RANK. Similarly, a peptide, known as NBD, has been showedn to selectively inhibit NF-kB with significant reduction in osteoclast mediated-bone loss (Soysa *et al.,* 2009).

1.5.4 MAPK cascade

In addition to NF-kB/c-fos pathway, binding of RANK to its receptor RANK also leads to the subsequent activation of MAPK pathway including extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Li *et al.,* 2011). MAPK signaling in RA occurs via a three-level cascade of kinases called MAPKs. MAPKs or MAP kinases are the serine/threonine/tyrosine-specific protein kinases which belong to (CDK/MAPK/GSK3/CLK) group of kinases. Involvement of MAPK cascade has been reported in rheumatic conditions (Sweeney *et al.,* 2004; Schett *et al.,* 2000). MAPKKK or MEKKs are the most upstream kinases which work in connection with GTPases like Rho and Ras, the latter being associated with chronic synovial inflammation (Marinissen *et al.,* 2001). Nitrogen-containing biphosphate drugs inhibit Ras thereby showeding anti resorptive effects (Luckman *et al.,* 1998; Fisher *et al.,* 1999). MEKKs activate downstream MAPKKs which in turn, activate p38 and JNK pathways. MKK-7 specifically activates JNK pathway while MKK-4, in some cases also lead to phosphorylated activation of p38MAPK in addition to JNK (Yan *et al.,* 1994, Blank *et al.,* 1996, Gerwins *et al.,* 1997). MEKK-1 has been showedn to have elevated levels in RA patients as well as in cultured

synoviocytes, which also overly express MEKK-2 (Hammaker *et al.,* 2004). Other MAPKKKs like TAK1 and MTK specifically activate p38MPAK via induction of MKK-3 and MKK-6 (Takekawa *et al.,* 1997, Yamaguchi et al. 1995). TAK1 has been reported to have augmented levels in synovial tissues and synoviocytes in RA patients (Hammaker *et al.,* 2004). MKK-3 and MMK-6 have been reported to specifically induce p38 whereas MEK-1 and MEK-2 are basically responsible for the activation of ERKs (Boyle *et al.,* 2014; Fanger *et al.,* 1997). An inhibitor of MEK-1 has showedn to block differentiation of osteoclasts precursors thus implicating its role in osteoclastogenesis. Blocking of Mek ½ via a strong specific inhibitor ARRY-162 in AIA, which has entered phase-II clinical trials corroborates their therapeutic potential (Lindstrom *et al.,* 2010). Hence, pharmacological intervention which blocks their activity or JNK knockout transfection results in repression of RANKL-mediated osteoclast formation (Ikeda *et al.,* 2008).

MKKs, downstream to MAPKKKs, are responsible for phosphorylating p38MAPK. The most significant MKKs are MKK-3 and MKK-6 which is evident from the p38 lacking phenotype of the mice with double knockout mutation for both of these MKKs (Cong *et al.,* 1999). MKK-3 leads to the preferential induction of p38MAPK in synovial fibroblast as a result of their exposure to TNF and IL1 (the proinflammatory cytokines) (Inoue *et al.,* 2006, Moriguchi *et al.,* 1996). Overexpression of p38 also leads to the increased expression of MKK-3 and MKK-6 in the patients with RA (Chabaud-Riou *et al.,* 2004). As a result, p38 is strongly expressed in synovial membrane and in the osteoclasts at the site of synovial invasion (Hayer *et al.,* 2005). P38 and other MAPKs also control regulation of MMPs.

Hence increased MAPK levels implicate increased MMP and thus increased collagen destruction (Liacini *et al.,* 2003, Suzuki *et al.,* 2000). This is corroborated by alleviation of cartilage destructive symptoms via p38 blockade (Zwerina *et al.,* 2006) In addition; SCIO-469

another p38 inhibitor has showedn to block osteoclastogenic process by preventing formation of osteoclasts (Nguyen *et al.,* 2006). A JNK- specific inhibitor, SP600125, has also showedn to inhibit anti-apoptotic characteristics of osteoclasts (Ikeda *et al.,* 2008). Hence, the accumulating evidence suggests the therapeutic potential of MAPK cascade.

1.5.5 AP-1/c-fos

AP-1 is a transcription factor complex with c-Fos being an essential constituent of the AP-1 transcription factor complex activated via RANK induction (Wagner *et al.,* 2005, Johnson *et al.,* 1992, Teitelbaum, 2004). C-Fos belongs to the Fos family of transcription factors and is responsible for the induction of NFATc1, the master regulator of osteoclastogenesis. This makes c-Fos an indispensible factor for osteoclastogenesis. Complete blockage of osteoclastogenesis occurs as a result of c-Fos knockout mutations in mice thereby displaying severe osteopetrotic symptoms (Wang *et al.,* 1992, Kim *et al.,* 2013). Levels of AP-1 and c-fos have not only been reported to be higher in RA patients but the bone resorption process in RA is also implicated to be the ultimate result of c-fos overexpression (Han *et al.,* 1998). An inhibitor designed by pharmacophore modeling was showedn to be able to selectively inhibit AP-1/c-fos thereby resolving the arthritic condition (Harris *et al.,* 1990). This implies that function of these proteins is indispensible for osteoclastogenesis making them potential therapeutic targets (Takayanagi *et al.,* 2005, Asagiri *et al.,* 2005). An attempt to inhibit AP-1 expression at transcriptional level using a sequence specific AP-1 binding oligonucleotides in has already been showedn to be successful in alleviating bone erosion symtoms in a dose-dependent, sequence specific manner (Aliprantis *et al.,* 2008). A new drug, T-5224, has also been computationally designed to inhibit AP-1/c-fos suggesting their therapeutic potential (Miyazaki *et al.,* 2012).

1.5.6 Cartilage damage

The pathological alterations in RA entail destruction of the synovial membrane, formation of rheumatoid nodules accompanied by inflammation of the surrounding blood vessels (rheumatoid vasculitis). Normally, synovial fluid present in the synovial lining of joint capsule greases the joints. The synovial lining bears a width of 2 or 3 cells, which thickens in case of RA leading to inflammation and thus the irremediable impairment of the articular cartilage and joint capsule since a scar-tissue called "pannus" substitutes these structures. After the first prompting event, presumably exposure to an infection and an autoimmune reaction, macrophages and fibroblasts in the synovium commence uncontrolled proliferation accompanied by subsequent infiltration of perivascular areas by T-cells triggering the proliferation of endothelial cells. These endothelial cells have an implicated role in immune response. Moreover, angiogenesis takes place and small clots occlude the small blood vessels. With the passage of time, the eroded synovial tissue continues growing in an irregular pattern leading to the formation of pannus tissue which later protrudes over cartilage surface and hence the bone erosion (Goldring, 2000). This affects the adjacent muscles, skin and bones leading to their atrophy (Fig. 1.2).

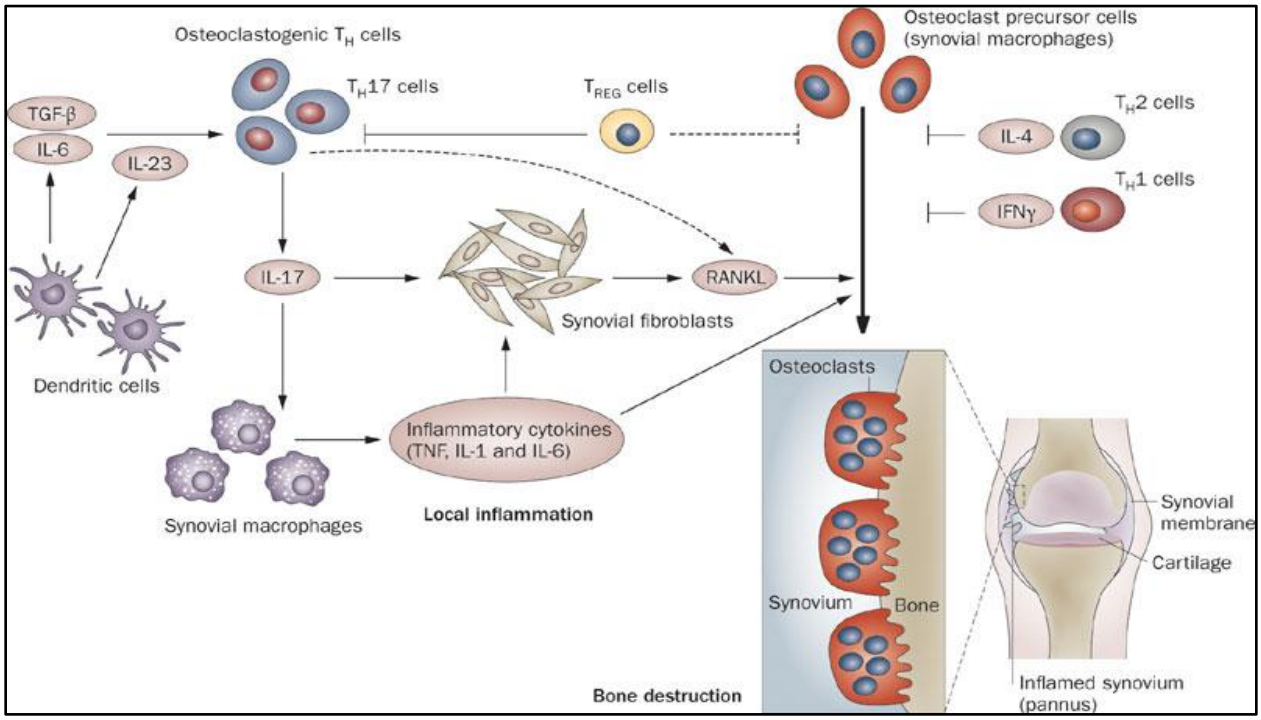

Figure 1.2. Pathogenesis of bone erosion, a characteristic of RA

The process involves the interplay between osteoclasts and immune cells at synovium. Various interleukins, pro-inflammatory cytokines, signaling molecules and transcription factors contribute to osteoclast-driven bone resorption in RA (Takayanagi, 2009).

1.6. RA and associated proteins

1.6.1 NFATc3

Nuclear factor of activated T-cells, cytoplasmic 3, belongs to NFAT family of transcription factors, which are involved in immune response. NFATc3 is the master regulator of the osteoclastogenesis owing to its indispensable role in the differentiation of osteoclasts (Takayanagi *et al.,* 2002, Li *et al.,* 2004, Kubota *et al.,* 2007). Activation of NFATc3 is mediated by calcineurin which is a specific calcium-dependent serine-threonine phosphatase which dephosphorylates NFATc3 leading to its translocation into the nucleus. Inhibition by calcineurin inhibitors like cyclosporine A and FK506 results in major blockage of osteoclastogenesis thereby relieving pain in Rheumatoid arthritis rat models (Takayanagi *et al.,* 2005, Magari *et al.,* 2003; de la Pompa *et al.,* 1998). Phenotype of mice deficient in NFATc3 exhibit acute osteopetrotic symptoms (Sitara and Aliprantis, 2010). In another study, in vitro experiments on NFATc3-/- demonstrate no differentiation of stem cells precursors into osteoclasts whereas in vivo analysis of NFATc3 knockout showed fatal results in the embryo implicating the crucial role of NFATc3 (Ranger *et al.,* 1998, Hou *et al.,* 2001). Phenotype of TRAF6 knockout mice exhibits impaired induction of NFATc1 implying that it is one of the major immediate targets of NF-kB during the initial stages of osteoclastogenesis (Li *et al.,* 2004). It is corroborated by the studies which showed that P50/p52-knockout mice showed no induction of NFATc3 (Yamasaki*et al.,* 2014). Expression profiling of RA patients showeds augmented levels of NFATc3 (Li *et al.,* 2012). Inhibiting effects of various compounds on NFATc3 activity have been studied including cinnamaldehyde, NGDA and obovatol (Kim *et al.,* 2013, Tsuji-Naito, 2008).

1.6.2 Carbonic anhydrase II

Carbonic anhydrase II belongs to carbonic anhydrase family of enzymes responsible for catalysis of carbon dioxide hydration in a reversible fashion. Bone resorption by osteoclasts requires a peculiar, poorly perfused, acidic, peripheral bone milieu which is achieved by the vacuolar proton pump activation (Vaananen *et al.*,1990). CAII facilitates the proton production from H_2O and CO_2 thereby enabling the radical acidification of the resorption space (AA and Al-Mashta, 1983). Disruption in CAII gene in vivo results in no bone resorption thereby implicating its critical role in optimal bone resorption process (Hall *et al.*, 1991). It has been showedn to be selectively activated downstream of RANKL (Takayanagi *et al.*, 2002). In mouse calvaria cultures, a specific inhibitor of CAII, acetazolamide, exhibits antiresorptive activity in a dose dependent, Ca2+ dependent pH-regulated manner (Kato *et al.,* 2013). Similar to acetazolamide, celecoxib and JTE-522 also inhibit osteoclast differentiation and activity which were tested in arthritic rat model for RA and showeded reduced bone resorption. Levels of CAII in Rheumatoid arthritis need to be checked since it has the potential to be therapeutically targeted.

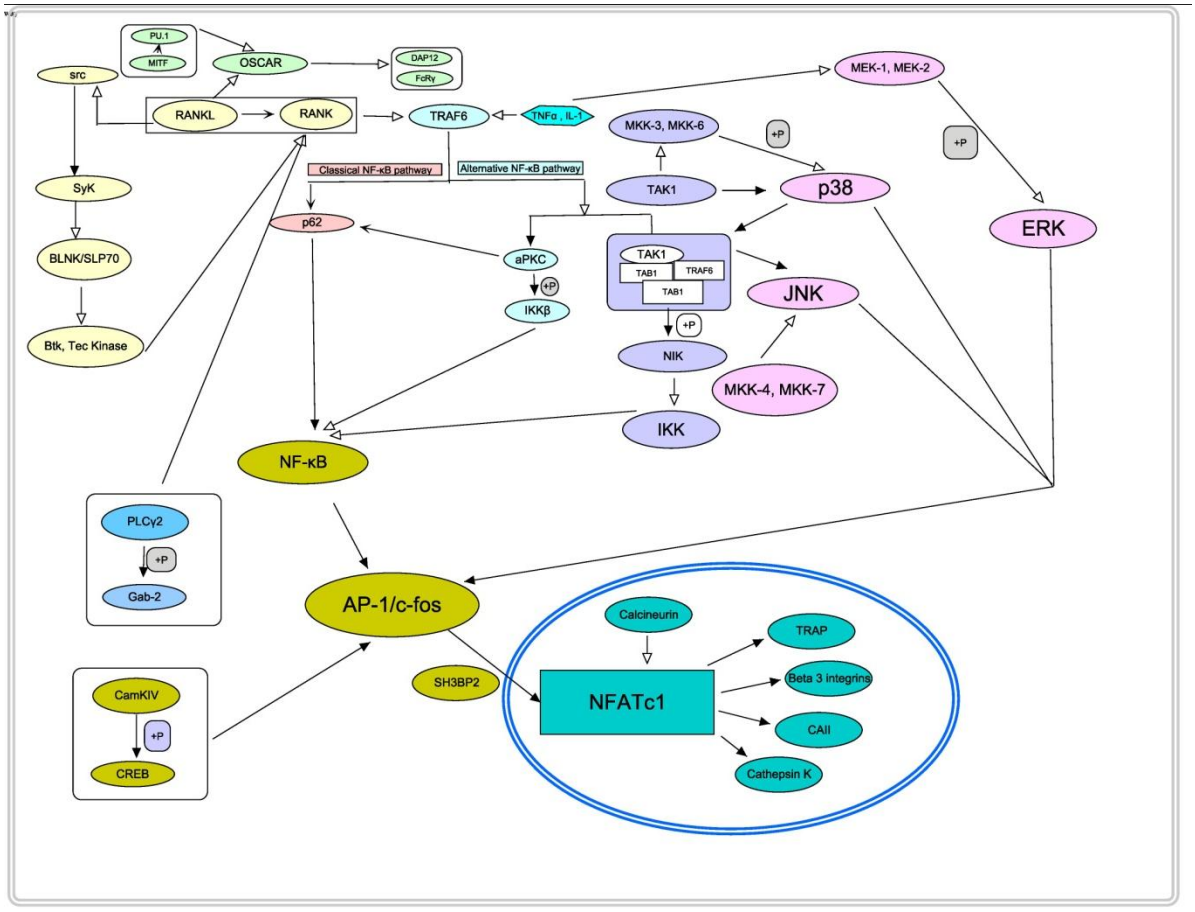

Figure 1.3 Osteoclastogenic factors in RANKL pathway

1.6.3 Calpain/calpastatin

Calpain, a calcium dependent cysteine protease, belongs to calpain superfamily, having two predominant isoforms; calpains 1 (μ-calpain) and 2 (m-calpain). Each of these calpains, differ on the basis of their sensitivity toCa^{2+} and comprise of two different polypeptide subunits. The larger subunit (80 kDa) is catalytically active, whereas the smaller, 30-kDa, subunit has a regulatory function. CAPN1 and CAPN2 are the genes which encode the large subunits of μ-calpain and m-calpain are encoded respectively**.** Calpain cleaves its specific substrates in-vitro including c-Fos, c-Jun, IkappaBa which are all reported to be key modulators in osteoclastogenesis and are activated downstream RANKL signaling pathway, the most important pathway involved in osteoclastogenesis (Xu *et al.,* 2011). There is cogent evidence for the fact that calpain is overexpressed in synovial fluid and membrane of RA patients thereby degrading articular cartilage. Calpastatin, natural inhibitor of calpain, on the other hand, is present as an autoantigen and has low levels compared to the enzyme and hence the perturbed enzyme/inhibitor ratio in RA patients (Ménard and El-Amine, 1996). Studies by Lee and fellows demonstrate that mu-calpain is activated by RANKL in RAW264.7 cells. Moreover, calpain inhibitors block the osteoclastogenesis in normal pre-osteoclasts. Furthermore, osteoclastogenic process was majorly augmented as a result of overexpression of mu-calpain in the cells thereby implying that calpain plays a crucial part in the process (Lee *et al.,* 2005).

1.7. Therapeutic approaches

A plethora of treatment options are available for RA to-date including drug therapy, physiotherapy, functional exercise and surgery. However, RA is a complex problem with various underlying causations. Physiotherapy and exercise work at the inceptive stages of the diseases, but more severe phases require drug therapy and possibly surgical therapy as well. Area of drug

therapy in RA has been undergoing expatiated research. NSAIDs are the first-line treatment option conventionally given to the patients at beginning stages (Scott *et al.,* 2007). DMARDs (Disease Modifying Anti-Rheumatic drugs) like methotrexate are also widely used owing to their role in alleviation of joint pain and inflammation and their effectiveness against progressive joint damage (Donahue *et al.,* 2008). Furthermore, biological agents have been elucidated for their therapeutic potential and have been targeted giving ameliorative outcomes in a great number of RA patients. For instance, inhibitors of TNF-α like Etanercept have been designed which have successfully blocked the target thereby preventing joint destruction (Mohler *et al.,* 1993). In addition to TNF-α, inhibitors of other cytokines like IL-1, IL-6 and RANKL have also been used for anti-arthritic therapy (Genovese *et al.,* 2008; Emery *et al.,* 2008). Similarly, B and T cells have also been targeted for destruction in order to control osteoclastogenesis and inflammation and hence the resultant bone destruction associated with RA (Dass *et al.,* 2006; Moreland *et al.,* 2002). Short-term Glucocorticoids have also been used for the temporary treatment of joint pain and swelling in RA. GCs however do exhibit serious side effects (Kirwan *et al.,* 2007). Chinese herbal medicine has long been used for the treatment of RA because of the anti-inflammatory, pain-alleviating properties. Tripterygium wilfordii Hook. F (TWHF), for example, occluded the expression of a large number of pro-inflammatory cytokines and induction of apoptotic effects in synovial fibroblasts. Other compounds sometimes used for the treatment of RA include antibiotics like tetracycline owing to their anti-inflammatory potential (Alarcón, 2000).

1.8. Hypothesis

Given the role of carbonic anhydrase II in osteoclast-driven bone resorption, it should have high protein expression profiles in patients of Rheumatoid arthritis. Furthermore, inhibiting CAII activity, should ameliorate arthritic symptoms.

1.9. Aims of study

The study is directed to following objectives:

1. To investigate protein expression profiles of carbonic anhydrase 2, calpain, TNF-alpha and NFATc3 in patients of Rheumatoid arthritis.
2. Development of a biomarker for early diagnosis of RA at molecular level. There is no prognostic or biomarker present specific for the detection of RA. The markers currently being used like RF are also present in other autoimmune disorders like SLE. Hence protein expression profiles of CAII in RA will provide as a potential prognostic marker for RA.
3. To elucidate a new class of compounds to inhibit CA2 for the treatment of RA using computational drug design approaches.

1.10 Impact of study

Protein expression levels of carbonic anhydrase II in Rheumatoid arthritis have never been elucidated as yet. Because of its momentous role in bone resorption, their high levels when proven, will provide as both a prognostic and diagnostic biomarker for the detection of Rheumatoid arthritis. Furthermore, inhibitors targeting CAII for anti-resorptive therapy in RA will be unraveled. Moreover, search of new class of compounds which can be used to selectively target CAII with better efficiency will be revealed.

Review of Literature

Rheumatoid arthritis (RA), affecting about 1% of the world population, is a chronic polyarthritic autoimmune condition characterized by colossal joint destruction as a consequence of synovial hyperplasia (McInnes *et al.,* 2011; Panati *et al.,* 2012). Although etiology of Rheumatoid arthritis is not known per se, the"Bermuda triangle" of genetic, environmental factors and autoimmunity has been known to trigger the onset and perpetuation of synovitis underlying RA (Van der Woude *et al.,* 2010; Szodoray *et al.,* 2010; Klareskog *et al.,* 2007; Mahdi *et al.,* 2009). Research studies of rheumatoid synoviocytes conducted to gain insights into the pathogenesis of bone erosion have led to the unraveling of their altered phenotype in RA patients (Davis *et al.,* 2003). Bone and articular cartilage are invaded by pannus which is a fibrovascular structure formed as a result of proliferation of synoviocytes. Radiographic exams reveal that the symptoms of joint erosion in RA arise in very early stages and exacerbate with the advancement of the disease causing acute structural and functional impairment associated with major odds of mortality and morbidity (Takeuchi *et al.,* 2013). On that account, obstructing the process of bone erosion in RA becomes the most challenging and indispensable target to achieve. Since RA is an autoimmune disease, it is clinically manifested by the presence of anti–citrullinated protein antibody (ACPA) and Rheumatic Factor (Aletaha *et al.,* 2010). Moreover, this autoimmunity is the result of underlying disturbed poise of the pro- and anti-inflammatory cytokines (McInnes *et al.,* 2007). In addition, epigenetic changes associated with the pathogenesis of RA have also been lately studied in detail (Pauley *et al.,* 2008). Synovium in RA patients is infiltrated by B cells, macrophages and $CD4^+$ cells in addtion to the increased

localized expression of a multitude of matrix degrading enzymes playing a role in articular cartilage damage (Firestein *et al.*, 2003). The environmental causes include smoking, bronchial stress and infectious agents. Infiltration of synovial compartment by leukocytes, imbalanced levels of chemokines, synovial hyperplasia accompanied by hypoxic conditions and neoangiogenesis are all characteristics of synovitis underlying RA pathogenesis. In addition, RA is also characterized by system complications like pulmonary, psychological and cardiovascular and brain-related problems (McInnes *et al.*, 2011). All these factors combined lead to the pathogenesis and perpetuation of the disease.

Bones are the rigid yet dynamic endoskeletal organs which undergo remodeling throughout one's life span without any alterations in the size or shape (Teitelbaum, 2000). Maintaining the normal mass in the adult skeleton requires précised poise between the bone formation and bone degradation by specific types of cells. Any change in the two processes leads to the alterations in bone mass leading to bone-related disorders (Soysa *et al.*, 2012) Fig. 2.1 showeds the stages of bone remodeling process.

Osteoclasts are the highly specialized, giant, unique bone resorbing polykaryons which originate from hematopoietic stem cells (Bar-Shavit, 2007). Myeloid progenitors undergo differentiation to form activated, bone resorbing osteoclasts via a process entailing the fusion of up to 20 mononuclear precursor cells, also known as syncytium (Vignery, 2005). Bone resorbing mechanism of osteoclasts is regulated by two fundamental instrumentaries (Teitelbaum, 2000). First, special enzymes responsible for the bone matrix degradation such as cathepsins and matrix metalloproteinases causing the cleavage of matrix molecules like collagen type-I and the consequent removal of the non-mineralized substances from the bone. Second, vacuolar ATPase, a proton/protein pump giving rise to acidic environs between part of the osteoclast plasma

membrane which demonstrates metabolic activity, the disheveled boundary and the bone surface. This acidification results in the solubilization of calcium from the bone matrix by the cells (Vanhille *et al.,* 1995; Li *et al.,* 1996) (Fig 2.2). The two aforementioned particularities allow osteoclasts to create a resorption pit by invading the bone. This resorption cavity can later be filled up by bone forming cells, osteoblasts. Osteoclasts are only found in the close proximity of bones because the mineralized tissue provides them pivotal signals for their differentiation (Schett, 2007). Osteoclastogenesis and bone remodeling , therefore, normally occur within the bone and bone marrow (BM), except in certain pathological bone diseases, such as rheumatoid arthritis.

Rheumatoid arthritis (RA), affecting about 1% of the world population, is a chronic polyarthritic autoimmune condition characterized by colossal joint destruction as a consequence of synovial hyperplasia (McInnes *et al.,* 2011). Research studies of rheumatoid synoviocytes conducted to gain insights into the pathogenesis of bone erosion have led to the unraveling of their altered phenotype in RA patients (Davis, 2003). Bone and articular cartilage are invaded by pannus which is a fibrovascular structure formed as a result of proliferation of synoviocytes. Radiographic exams reveal that the symptoms of joint erosion in RA arise in very early stages and exacerbate with the advancement of the disease causing acute structural and functional impairment (Fuchs *et al.,* 1989; Van Der Heijde *et al.,* 1992). On that account, obstructing the process of bone erosion in RA becomes the most challenging and indispensable target to achieve. Extensive research indicates that osteoclasts are the primary cells involved in the joint destruction in RA.

Osteoclastogenesis takes place at a momentously accelerated rate in Rheumatoid arthritis at a site exterior to the marrow cavity, thereby leading to bone loss implicated in RA.

Consequently, abnormal bone resorption is the major pathology resulting in the functional impairment caused by rheumatoid arthritis (RA). It is supported by a multitude of research findings which suggest that synovial inflammatory tissue acts as a critical source of osteoclasts in RA by inducing localized osteoclastogenesis leading to loss in the bone mass (Gravallese *et al.*, 1998). The induction takes place as synovial tissue not only facilitates the population of osteoclast precursors, monocytes/macrophages, but also of the osteoclastogenic signal producing cells. Hence, overproduction of osteoclasts in RA suggests a significant connection between joint inflammation and structural damage. Hence, we would like to focus on the existing potential molecular agents involved in osteoclastogenesis which can be therapeutically targeted for the treatment and cure of RA.

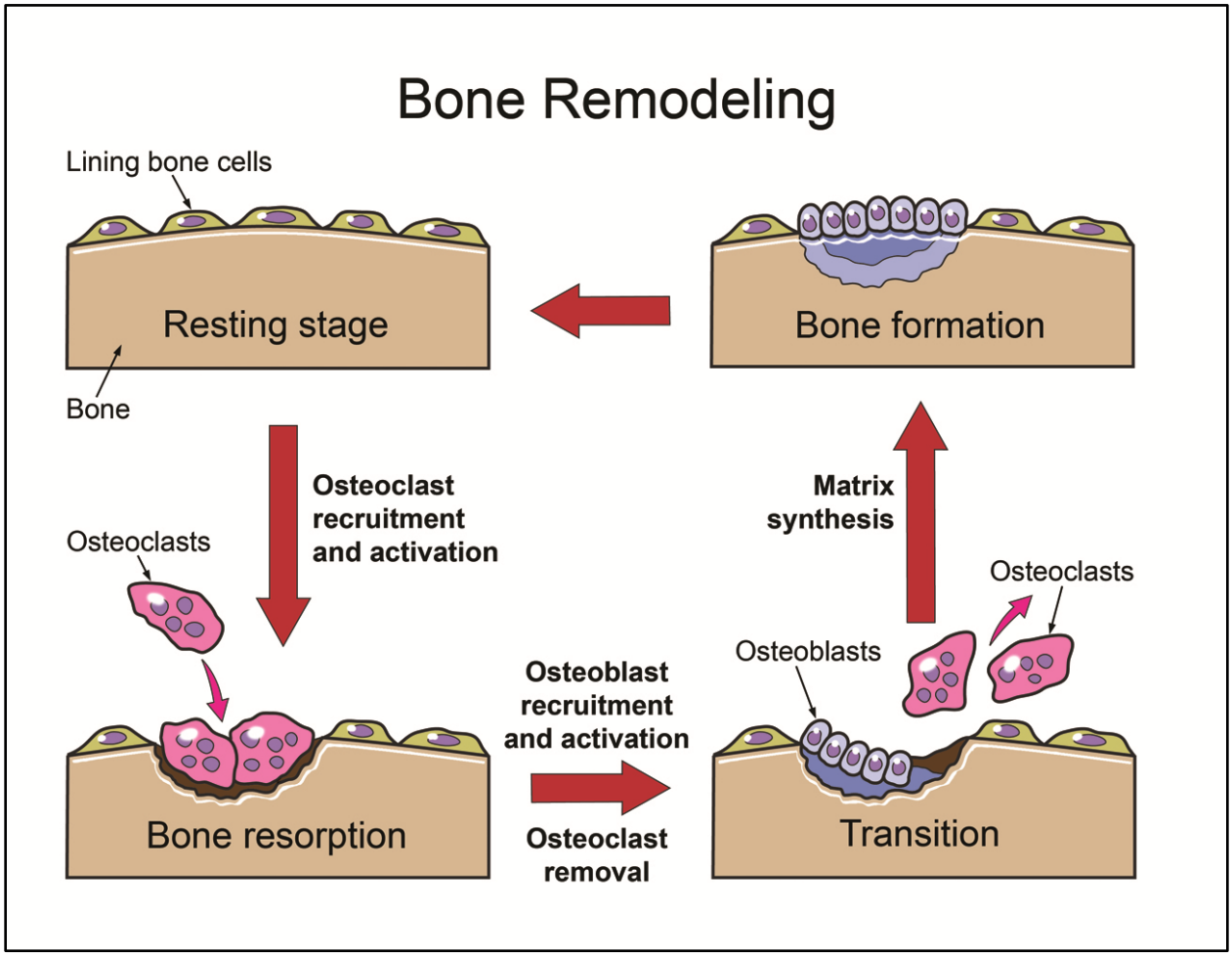

Figure 2.1. Normal bone remodeling mechanism

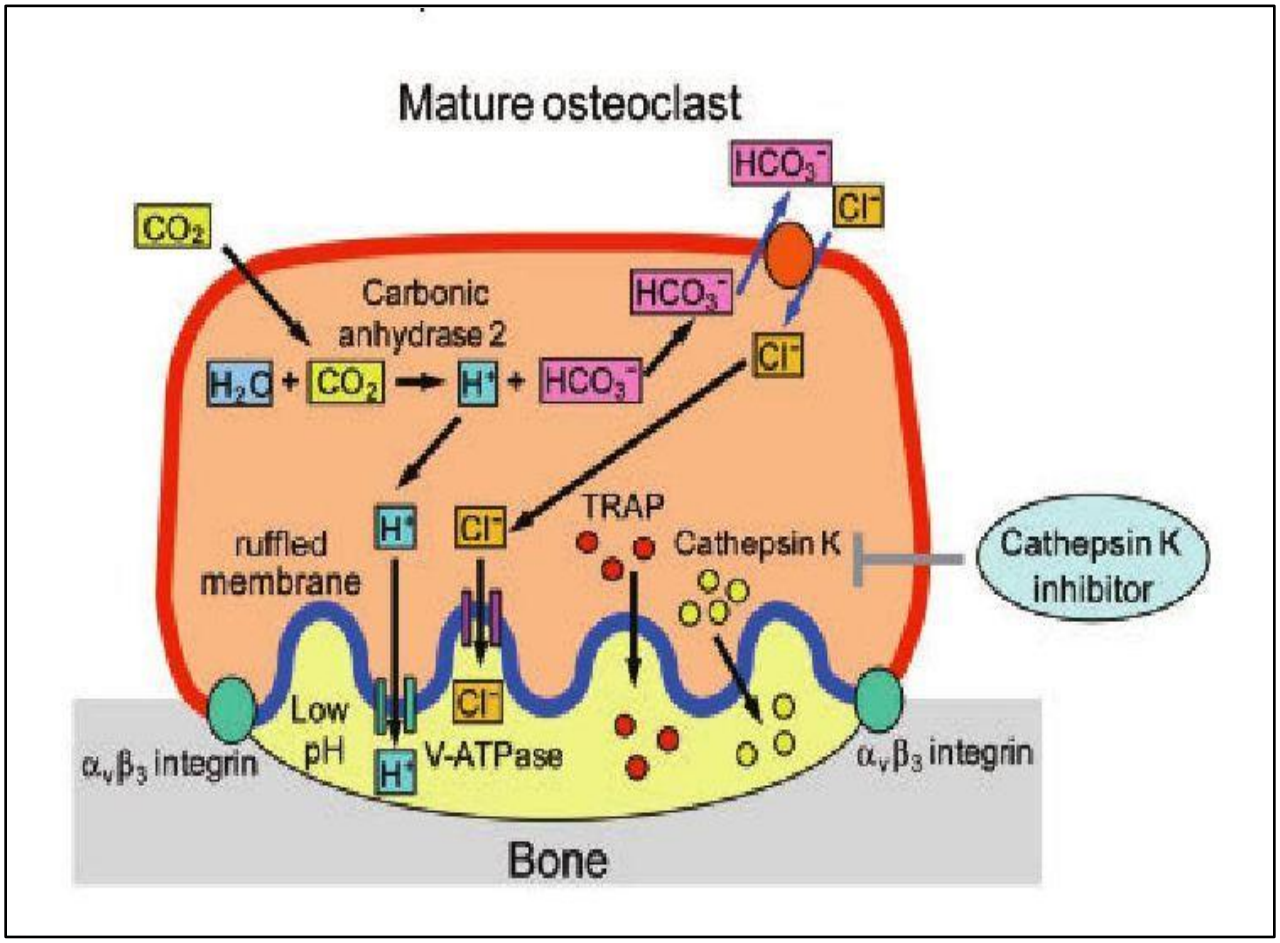

Figure 2.2. Mechanism of osteoclastic bone resorption.

The osteoclast adheres to bone via binding of RGD-containing proteins (green triangle) to the integrin $\alpha_v ß_3$, initiating signals that lead to insertion into the plasma membrane of lysosomal vesicles that contain cathepsin K (Ctsk). Consequently, the cells generate a ruffled border above the resorption lacuna, into which is secreted hydrochloric acid and acidic proteases such as cathepsin K. The acid is generated by the combined actions of a vacuolar H^+ ATPase (red arrow), its coupled Cl^- channel (pink box), and a basolateral chloride–bicarbonate exchanger. Carbonic anhydrase converts CO_2 and H_2O into H^+ and HCO_3^-. Solubilized mineral components are released when the cell migrates; organic degradation products are partially released similarly and partially transcytosed to the basolateral surface for release.

Bone biology field has been greatly revolutionized since the unraveling of RANK-RANKL signaling pathway as the main pathway involved in osteoclastogenesis (Kotake *et al.,* 1996). Moreover, tremendous research in the area has provided insights into the molecular domains of osteoclast formation, differentiation and activation. This makes the expatiated comprehension of the processes of osteoclasts (OC) differentiation and activation inevitable. Identifying the molecular potential therapeutic targets in the osteoclastogenesis will open new avenues into the treatment and diagnosis of the arthritic condition.

This research study points to two components the RANKL signaling pathway which has been showedn to play a role in osteoclastogenic bone resorption. First one is calpain, a calcium dependent cysteine protease which cleaves its specific substrates in-vitro including c-Fos, c-Jun, IkappaBa which are all reported to be key modulators in osteoclastogenesis (Johnson *et al.,* 1992; Kamolmatyakul *et al.,* 2004; Kawaji *et al.,* 1994). There are about 15 isoforms of calpains present in mammalian cells. Some of these isoforms happen to be ubiquitously present whereas others demonstrate tissue specific expression. Calpains1 and 2 are the most predominant of all

the isoforms. In order to carry out their proteolytic functions, they require micro and millimolar calcium ion concentrations. Increased calcium levels lead to increased localization of calpain from plasma to cytoplasm thereby activating it via interaction with phospholipids there (Kovács and Su, 2014). Given that calpain is dependent on calcium ion levels, any alterations in the environmental conditions like stress which lead to calcium ion influx, may lead to the unnecessary activation of calpain leading to its elevated levels (Padilla *et al.,* 2014).

In addition to the cleavage of a wide range of products, calpains are known to be involved a multitude of physiological processes including strokes, muscular dystrophies, cataract etc. Calpain also plays a role in increased osteoclast proliferation, survival, angiogenessis and migration which is a characteristic of Rheumatoid arthritis (Kovács and Su, 2014). There is an accumulating evidence for the fact that calpain is overexpressed in synovial fluid and membrane of RA patients thereby degrading articular cartilage.

Calpastatin, natural endogenous inhibitor of calpain specific for only 1 and 2 isoforms, on the other hand, is present as an autoantigen. It has binds to the targets with a greater affinity for the autoloyzed state of the calpain (Kovács and Su, 2014). Calpastatin exhibits lower levels as compared to the calpain and hence the perturbed enzyme/inhibitor ratio in RA patients. Studies by Lee and fellows demonstrate that mu-calpain is activated by RANKL in RAW264.7 cells. Calpain inhibitors block the osteoclastogenesis in normal pre-osteoclasts. Furthermore, osteoclastogenic process was majorly augmented as a result of overexpression of mu-calpain in the cells thereby implying that calpain plays a crucial part in the process. Furthermore, external intervention using laser in RA results in the decreased levels of calpain in the synovium (Barabás *et al.,* 2014).

A single calpastatin molecule comprises four inhibitory units each inhibiting one calpain molecule with different efficiency levels. Peptide sequences comprising only 20 amino acids have been derived from these inhibitory units are capable of inhibiting calpain, although at the low efficacy. Recently, the 3D structure of m-calpain (co-crystallized with one of the inhibitory units of calpastatin and Ca^{2+}) in part unraveled the clue; the structural flexibity of Calpastatin, which has been reported as "intrinsically unstructured", makes the tight binding to calpain possible, while, concurrently, allowing the adjacent various amino acids to loop out from the active site of calpain, thus keeping itself from proteolysis (Ono and Sorimachi, 2012).

Calpain is also a well-known inflammatory marker since a study demonstrated amelioration of acute inflammatory symptoms in rat models (Cuzzocrea *et al.,* 2000). This may help with the inflammation associated with RA if targeted for anti-inflammatory therapy.

Accumulating evidence supports the role of calpain in the re-arrangement of cytoskeleton via actin ring generation in the integrin-modulated pathway. As mentioned before, bone resorption by osteoclasts is associated with the contact between cell and the matrix thereby leading to the generation of sealing zone and the disheveled border specific to the osteoclasts. Inhibitors designed against calpain which are permeable to the cell membrane exhibit blockage of bone resorption by preventing the actin ring formation and hence the cytoskeletal reorganization (Hayashi *et al.,* 2005). Hence the role of calpain in bone resorption activity, its augmented levels in RA and targeting calpain for anti-resorptive therapy can opens new insights in the realm of therapeutics.

The evidence of calpain involvement in a multitude of human pathological phenomenon has expedited research in the realm of unraveling of calpain inhibitors as curative agents. Various calpain inhibitors have exhibited effectiveness in ameliorating disease symptoms.

However, most of the discovered compounds have calpain isoform selectivity issues. Calpain inhibitors are mainly categorized into allosteric, reversible and irreversible site directed inhibitors. However, only a few inhibitors exist due to lack of expatiated research on allosteric inhibitors. A mundane characteristic of active site targeting of calpain inhibitors is the electrophilic center present for the covalent interaction with the cysteine present in the catalytic site. A diverse set of calpain inhibitors are peptide based. These inhibitors are however vulnerable to degradation by extracellular and intracellular proteinases thereby compromising their in vivo usability. Several Calpastatin derivatives are also studied which showed greater cellular absorption. Substitution of residues at various positions of peptide inhibitors with non-peptides alternatives exhibit enhanced pharmacokinetic and physiochemical features. Implementation of the pro-drug idea to aldehyde inhibitors demonstrates better drug-like profiles, specificity and selectivity (Donkor, 2011).

Another evidence of calpain as a therapeutic target for inhibiting osteoclastogenesis is the study which illustrates the pain reducing potential of a calpain inhibitor in cancer-induced pain via osteoclastogenic blockage both in vitro and in vivo. It is majorly via inhibition of RANKL-induced osteoclastogenesis and maturity of bone resorbing function proved both in vitro and in vivo (Xu *et al.,* 2012). For the treatment of RA, it can be therefore considered. However, further research is needed to better elucidate the role of Calpain inhibitor in treating RA.

In addition to Calpastatin, calcitonin has also been reported to suppress osteoclastogenic bone resorption partly by down-regulating calpain activity. Calpain basically regulates the motility and bone resorption in osteoclasts, which upon inhibition leads to a greater number of osteoclasts due to increased cell survival (Marzia *et al.*, 2006). Hence calpains modulate osteoclastogenic activity in two nearly antagonistic fashions: firstly, by stimulating cytoskeletal

reorganizations and regulating cell adhesion thus inducing the spread of osteoclasts, their motility and bone degrading function and secondly, by putting a constraint on the life span of the cells. Both of these functions play their parts in the determination of pace of bone erosion and thus maintain the poise of skeletal homeostasis. Furthermore, it has been suggested that calcitonin poses its antiresorptive effects and causes osteoclast survival partly by down-regulating calpain activity, probably through PKC signaling pathway.

Signaling and transport of calcium ions is a crucial component of bone biology. Given that bones are the main repository of calcium and momentous modulatory organs for calcium homeostasis. Calcium-controlled signals sent via parathyroid hormones and metabolic products of vitamin D are the ones majorly responded by bone. However, even in the absence of PTH-mediation, bones have the ability to directly respond to extracellular calcium. Imbalance in bone mass has been showedn to be associated with defects in calcium transporters and other calcium-mediated phenomenon in various studies. Cells responsible for bone formation i.e. osteoblasts deposit calcium via strategies which entail transport of calcium transport accompanied with alkalinization thereby facilitating absorption of acid formed as a causatum of deposition of minerals. This mineralization results as a consequence of passive diffusion and generation of phosphate. Calcium transport by osteoclasts is controlled by secretion of acid as discussed earlier. Hence, both bone formation and resorption are under the control of calcium signals (Hwang and Putney, 2011). Since, calpain activity is dependent on calcium levels. Increased expression of calpain can be correlated to increased calcium levels in rheumatoid arthritis.

Calpains exhibit two peculiar features including their EF-hands resembling calmodulin and thiol protease activity. The mode of operation of calpains is quite intricate and the Ca^{2+}-dependent toggling between active and inactive states has remained obscure until the elucidation

of its 3D X-ray crystal structure. Unraveling of the crystal structure has led to new insights into the anomalous thiol protease fold and its relation with Ca^{2+}-binding domains through formation of heterodimers and a C2-like β-sandwich domain. The α-helical N-terminal anchor of the catalytic subunit does not cover the active site but blocks its assembly and controls the sensitivity to Ca2+- via association with the regulatory subunit. This Ca^{2+}-dependent activation mechanism is different from those of conventional proteases (Ono and Sorimachi, 2012).

Catalytic inactivity of calpain in the absence of calcium ions has been unraveled by crystallographic structural studies of m-calpain. This pertains to a significant distance between three amino acid residues which collectively make calpain catalytic triad, thereby preventing the assembly of a functionally active site. Due to the blockage of their active site by a propeptide, calpains are not considered as pro-enzymes. A multitude of animal models with dysfunctional small subunit of calpains lead to fatality whereas there are no significant effects of knocking mu-calpain down in terms of survival. Experimental attempts to unravel the concentration of Ca^{2+} atoms bound by the calpains, and which Ca^{2+} atoms are involved in inducing catalytic activity. It has been showedn that there are three sites residing on the calpain molecule where Calpastatin binds and binding to at least two of them is regulated by calcium signals. Calpain pathologies are mostly characterized by the absence of Ca^{2+} homeostasis by cells and inadequate or extraneous degradation of calpain substrates. It is broadly surmised that augmented levels of calcium ions results as the Ca^{2+} homeostasis is lost leading to the subsequent activation of Calpain (Goll *et al.*, 2003). Thus, overexpression of calpain in RA should be partly the result of elevated calcium concentrations.

Calpain is responsible for regulating RANKL signaling pathway since its blockage results in the subsequent blockage of RANKL signaling either by blocking the master regulator

of osteoclastogenesis i.e. NFATc1 or that of its downstream activated c-fos (Xu *et al.,* 2011). c-Jun protein, and AP1/PEA1 transcription factor component, is a typical short-lived protein, and like other short-lived proteins such as c-Fos, contains PEST regions. Calcium-dependent neutral protease (calpain), a candidate for the degradation of PEST-containing proteins, digests c-Jun and c-Fos efficiently in vitro (Hirai *et al.,* 1991).

Carbonic anhydrase II

The enzymes called carbonic anhydrases responsible for the catalysis of hydration of CO_2 are widely expressed throughout the body. These enzymes have atavistic roots since they exist as deepest of the evolutionary tree branches. Due to their pathological role in human diseases, the alpha class of carbonic anhydrases out of five various classes of carbonic anhydrases, has gained momentous attention. Physiological role of this class of carbonic anhydrases is to catalyze the reversible hydration of CO_2 thereby permitting the regulation of both intra- and extra-cellular CO_2, hydrogen and bicarbonate concentrations. Extensive research studies have confirmed the role of CAs in a wide spectrum of physiological phenomenon entailing gas exchange at the air water interface, transport of CO_2 and HCO_3 across membranes, biosynthetic reactions in metabolically active tissue, acid–base balance, secretion, calcification, signal transduction, oncogenesis, proliferation, among the many that have been reported (Chaput *et al.,* 2012; Brown *et al.,* 2012). These apparently diverse functions are controlled by specific isoforms in the'-CA family.

CA family comprises of sixteen isoforms discovered as yet which have their own idiosyncratic tissue-specific expression, kinetic features and inhibitor-specific sensitivity (Supuran, 2008). Out of these sixteen isoforms, eight reside in the cytosol and among them is the CAII isoform which if particularly of our interest because of its implicated role in RA and its

activation downstream of the RANKL signaling pathway. Furthermore, its role in the acidification and calcification can be correlated with the elevated levels of calcium and hence the calcium-dependent activation of calpain.

Bone resorption by osteoclasts requires a peculiar, poorly perfused, acidic, peripheral bone milieu which is achieved by the vacuolar proton pump activation (Vaananen *et al.*, 1990). CAII facilitates the proton production from H_2O and CO_2 thereby enabling the radical acidification of the resorption space (AA and Al-Mashta, 1983) (Fig. 2.2). Disruption in CAII gene in vivo results in no bone resorption thereby implicating its critical role in optimal bone resorption process (Hall *et al.*, 1991). It has been showedn to be selectively activated downstream of RANKL (Takayanagi *et al.*, 2002) (Fig. 2.2). In mouse calvaria cultures, a specific inhibitor of CAII, acetazolamide, exhibits antiresorptive activity in a dose dependent, Ca^{2+}-dependent pH-regulated manner (Kato *et al.*, 2013). Similar to acetazolamide, celecoxib and JTE-522 also inhibit osteoclast differentiation and activity which were tested in arthritic rat model for RA and showeded reduced bone resorption. Levels of CAII in Rheumatoid arthritis need to be checked since it has the potential to be therapeutically targeted.

It has been known that CA II is the most predominant an. active of all the isoforms because of its efficient kinetics. The protein 3D structures available for carbonic anhydrases elucidate that the active catalytic site of the enzyme is buried approximately 15˚ A deep inside the protein structure and is exposed to the solvent by an extensive open canonical cleft. There is a Zn^{+2} ion located at the base of the catalytic site which is tetrahedrally connected to three His residues and a solvent molecule (Fig. 3.1a, b). There is a division of residues in the catalytic site with one portion constituting hydrophobic and the other one comprising of hydrophilic faces. On spatial basis, hydrophobic site is further categorized into two types: The residues which line the

entrance of the active site and the others which make up the CO_2 binding site (Fig. 3.1b). The distal hydrophobic (residues 131, 135, 201, 202 and 204) with respect to the location of Zinc ion are known as the secondary hydrophobic binding sites whereas the proximal ones (residues 121, 143, 198, 207 and 209) are called primary hydrophobic binding sites (Krishnamurthy *et al.*, 2008; Lindskog and Silverman, 2000; Christianson and Fierke, 1996). The secondary hydrophilic binding site has its utmost significance in accommodating CO_2 inside the catalytic site and determining the binding efficacies of a multitude of CA inhibitors (Aggarwal *et al.*, 2013). HCAII has been extensively used for studying the mechanistic aspects of CA kinetics and catalysis. The reaction occurs in two sequential steps which are showedn in the following equation.

$$EZnOH^- + CO_2 \leftrightarrow EZn(OH^-)CO_2 \leftrightarrow EZnHCO_3^- \leftrightarrow EZnH_2O + HCO_3^-$$

$$EZnH_2O \leftrightarrow H^+EZnOH^- + B \leftrightarrow EZnOH^- + BH^+$$

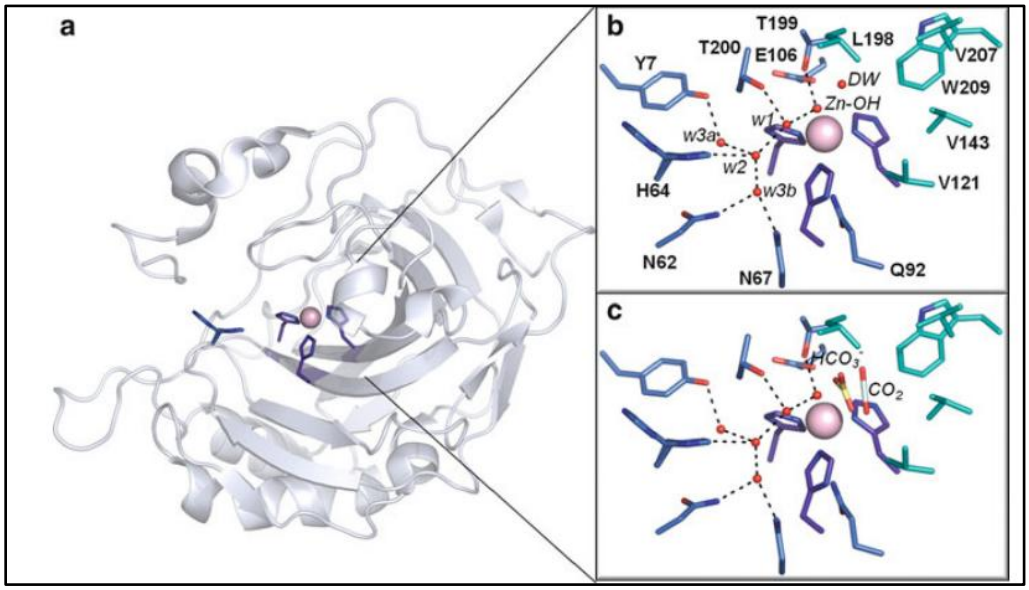

Figure 2.3 (a) The structure of HCA II

(PDB: 3KS3) showedn in cartoon representation with the C' backbone colored in silver, the Zn metal ion as a pink sphere, the coordinating histidine residues as purple sticksand the proton shuttle residue, His64, in blue**.(b)** Structure of the active site showeding the ordered water network (red spheres) hydrogen bond (dashed line) to their respective hydrophilic contacts (blue sticks). The hydrophobic residues important in coordinating CO_2in the active site are showedn as teal sticks. The C' backbone has been omitted for clarity**. (c)** Models of the bicarbonate (yellow) and CO2 (light blue) binding sites. Active site residues are colored and labeled as in (b).

Zn-OH launches a nucleophilic attack as a result of CO_2 binding in the hydrophobic pocket resulting in the subsequent generation of HCO_3. Later, this HCO_3 is dislocated by random water diffusion that occurs in the catalytic site. In the second phase, a proton is transferred from the water molecule bound to the zinc to an acceptor located in the bulk solvent. This is a requisite for the regeneration of hydroxide which is utilized later for subsequent catalytic reactions. This happens via His64, also referred to as proton shuttle reside (Fisher *et al.,* 2005; Silverman and Lindskog, 1988).

A multitude of inhibitors and structural analogs of HCA II have been studied. Crystallographic data reveals. Crystallographic studies elucidate that in the presence of these inhibitors specifically sulfonamides and their derivatives such as acetazolamide displace the Zn-OH molecule while maintaining the same tetrahedral link with the Zn. Furthermore, anionic inhibitors like cyanate exhibit the removal of deepwater (DW) molecule, which is coordinated by the amide nitrogen of Thr199, to create a new trigonal-bipyramidal coordination sphere of the Zn.

Sulfonamides are the most significant class of CAIs with various compounds such as acetazolamide, methazolamide, ethoxzolamide, sulthiame, dichlorophenamide, dorzolamide, brinzolamide, sulpiride and zonisamide in clinical use for many years, as diuretics, antiglaucoma agents, as well as antiepileptics (Alterio *et al.,* 2012; Supura, 2011; Supuran, 2010). Sulfamates such as topiramate, EMATE, and irosustat, although developed without considering their potential CA inhibitory properties, are also potent CAIs and are clinically used as antiepileptics/antiobesity agents or are in advanced clinical trials as dual, steroid sulfatase inhibitors/CAIs with anticancer effects (Woo *et al.,* 2011).

Most of the CA inhibitors exhibit side effects mainly because of the isoform specificity issues. These side effects entail bone loss, formation of kidney stones and metabolic acidosis. Infact, the inhibitors used as antiglaucoma agents target HCA 2, 4 and 12 (Alterio *et al.*, 2012). Here is when the scope of drug design comes into play. The major aim of state-of-the art drug design strategies is to develop isoform specific CAIs. This however is an arduous task since most of the active site architecture remains conserved in all the isoforms. All human CA2 isoforms have the three conserved His residues (His94, 96 and 119) as zinc ligands, two other conserved residues acting as "gate keepers", i.e., Thr199 (hydrogen bonded through its -OH group with the water molecule/hydroxide ion coordinated to the zinc) and Glu106 (hydrogen bonded to Thr199, as well as half the active site mainly lined with hydrophobic residues and the opposite one with hydrophilic residues) (Di Fiore *et al.,* 2009; Di Fiore *et al.,* 2010). However, there are also significant discrepancies in amino acid residues majorly at the center and at the entrance of towards binding pocket (Alterio *et al.,* 2012; Di Fiore *et al.,* 2009; Di Fiore *et al.,* 2010). Most of the aforementioned inhibitors, do not interact with them as more compact molecules tend to bind deep inside the catalytic site cavity which is rather similar in all mammalian isoforms (Alterio *et al.,* 2012; Di Fiore *et al.,* 2009; Di Fiore *et al.,* 2010). Furthermore, a diverse set of novel CAIs have been revealed from screening collections of natural products, most considerable being the coumarin and phenol containing NPs (Supuran, 2011). NPs constituting these fragments exhibit distinct profiles of CA inhibition when compared to classical inhibitors.

This study points at the in-silico design of new inhibitors which are specific to HCA 2 for the treatment of RA using various drug design strategies.

Materials and Methods

3.1 Sample collection

Fresh blood samples were collected from patients diagnosed with Rheumatoid arthritis and satisfying the American College of Rheumatology criteria 2011 for rheumatoid arthritis. In the present study, samples were collected from Rehmat Noor Clinic, Rawalpindi. Consent was taken from the patients. Patients history and clinical information including age, gender, duration of disease, erythrocyte sedimentation rate, disease activity score, growth hormone (GH), number of tender joints, number of swollen joints, family history , other diseases and anti-citrullinated protein antibodies (ACCP) were collected. About 3-5 cubic centimeter (CC) blood samples were collected in both 5 ml serum and EDTA vacutainer tubes (IMPROVACUTER®) and immediately used for serum separation.

3.2 Serum separation

- Serum was separated from fresh blood samples RA patients and controls by centrifugation at 4^{o}C for 10 minutes at 2500 rpm.
- Serum was isolated and stored at -80^{o}C until usage.

3.3 Protein quantification using Bradford's Assay

A standard curve with a standard known protein i.e. BSA was drawn. Later a reaction mixture of protein extract, Bradford reagent and water was made and its O.D was taken at 630

nm. The O.D of sample protein extract in the standard curve was plotted to get the µg/µl of protein in sample.

3.4 Western blotting

1. 20% SDS gel was used. 10% SDS gel preparation is showedn in Table 3.1.

2. 25 µg protein was loaded and added to an equal volume of 1x SDS gel-loading buffer. 1x SDS gel-loading buffer is showedn in Table 3.2. 2 x SDS gel-loading buffer lacking β-mercaptoethanol can be stored at room temperature. β- mercaptoethanol was then added, just before the buffer is used.

3. The sample was boiled (in loading buffer) at 100^{o}C for 3 – 5 minutes.

4. The samples were loaded for electrophoresis. For the first 10 minutes, 50 Volts were applied so that the samples settled equally and move through the stacking gel. After 10 minutes, the voltage was increased to 100 volts until the bromophenol blue reached the bottom of the resolving gel.

5. Gel was prepared for transfer in transfer buffer at 100 volts for 1.5 hours.

6. Western blotting transfer buffer (Table 3.3).

7. The membrane was blocked with blocking buffer overnight at 4^{o}C with gentle agitation on a shaker. Blocking solution is showedn in Table 3.4 and Phosphate-buffered saline (PBS) (pH 7.4, 1000 ml).

8. Blocking solution was discarded and PVDF membrane was immediately incubated with primary antibody.

9. 10 ml blocking solution was added. Blocking solution is showedn in Table 3.5.

10. Blocking solution was discarded and the membrane was washed 5 times (5 minutes each time) with 20 ml of PBST.

11. 1 ml of primary antibody (1:1000) was added in to blocking solution.

12. Incubation at $4^{o}C$ for 2 hours with gentle agitation on a shaker.

13. Primary antibody solution was discarded and washed with PBST for 5 times for 5 minutes each time

14. The membrane was washed with PBST wash buffer for 5 times for 5 minutes each time.

15. 1 ml of secondary antibody solution (1:2000) was added.

16. Incubation for 1 – 2 hours at room temperature with gentle agitation.

17. Secondary antibody solution was discarded and washed with PBST for 5 times for 5 minutes each time.

3.4.1 Autoradiography

The antibody labeled PVDF membrane was taken into a dark room, where a Bioluminol (Santa cruz) substrate (mix equal volumes of sol A with sol B) was added on top of the PVDF membrane and incubated without shaking for 10min. The PVDF membrane was then carefully lifted and dried by blotting on a tissue paper, after which it was placed in between two clean and clear plastic sheets and placed in an X-Ray cassette. Care was taken to ensure no bubbles are present between the plastic and PVDF membrane's surface, and the PVDF membrane should face up. All lights were closed and in total darkness a fresh X ray sheet (Koyoto films) was

placed ontop of the PVDF membrane in the cassette and the cassette closed. The cassette was left in darkness for 10seconds to 10minutes and then the film was carefully removed and immersed in developer solution (Agfa) for 10-20seconds. The film was removed and then washed in distilled water 3 times by immersion. The film was then fixed by immersing in a fixer solution (Agfa) for 1minute. The film was then washed in running water for 5minutes and then air dried. After air drying the films, pictures were taken of the bands using a Nikkon D5200 DSLR camera at an autofocus setting, distance from the film and the camera were kept constant by the help of a tripod. The pictures were then analyzed by the Studio Lite 4 software ver. 4.01. The band intensities were normalized using an Actin control and plotted on a bar graph.

Table 3.1. 20% SDS gel preparation reagent amount (µl)

	Separating gel (%10)	**Stacking gel (6%)**
Water	2250	1500
Tris-HCl	1575 (1.5 M, pH 8.8)	300 (1 M, pH 6.8)
SDS (20%)	40	**27**
Acr-Bis (30%)	2100	**450**
AP	25	**18**
TEMED	10	**5**
Sum	6000	**2300**

Table 3.2. 1x SDS gel-loading buffer for Western blotting

Water	3.55 ml
1 M Tris-HCl (pH 6.8)	1.25 ml
Glycerol	2.5 ml
20% SDS	2 ml
0.5% bromophenol blue	0.2 ml
Total volume	9.5 ml
Betamercaptoethanol (just before use)	0.5 ml

Table 3.3 Western blotting transfer buffer (1000 ml)

Tris	3.03g
Glycine	14.4g
Methanol	200 ml
SDS	2 ml

Table 3.4. Western blotting blocking buffer

Blocking solution	100 ml, in PBS
Nonfat dried milk	5%
Antifoam A	0.01%
Sodium azide	0.02%

Table 3.5. Blocking solution

Blocking solution	10 ml, in PBS (pH 7.4)
Nonfat dried milk	5%

3.5 Comet Assay

The single cell gel electrophoresis (SCGE)/ comet assay, developed by N.P. Singh, combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and crosslinking with the single cell approach typical of cytogenetic assays.

3.5.1 Preparation of Base Slides

Initially 1% (500 mg per 50ml PBS), 0.5% LMPA (250 mg per 50 ml PBS) and 1.0% NMA (500 mg per 50 ml in Milli Q water) were prepared. They were then heated until near boiling and the agarose dissolved. For LMPA, 5 mL samples were aliquot into scintillation vials (or other suitable containers) and refrigerated until needed. When needed, agarose was melted briefly in microwave. LMPA vial was placed in a 37ºC dry/water bath to cool and stabilize the temperature. The slides were dipped in methanol and they were burned over a blue flame to remove the machine oil and dust. While NMA agarose was hot, conventional slides were dipped up to one-third of the frosted area and gently removed. Underside of slide was wiped to remove agarose and the slide was laid in a tray on a flat surface to dry. The slides were air dried. The slides were stored at room temperature until needed; high humidity conditions were avoided. The slides were prepared the day before use.

3.5.2 Cell Isolation / Treatment

To the coated slide, 75 µL of LMPA (0.5%; 37ºC) mixed with ~10,000 lymphocytes were added in ~5-10 µL. Gently coverslip was slided off and a third agarose layer (80 µL LMPA) was added to the slide.Coverslip was replaced and returned to the slide tray until the agarose layer hardened (~5 to 10 minutes)

3.5.3 Viability Assay

In order to quantify cells 10µL of atleast 10^6 cells/ml were placed in a microcentrifuge tube, and 5µL of trypan blue dye was added. It was left to stand for at least two minutes and then placed on a slide and then coverslip was placed on top. Later 100 cells were scored and the number of viable cells (shiny) and dead cells (blue) were recorded.

3.5.4 Electrophoresis of Microgel Slides

The following procedure was performed for electrophoresis under pH>13 alkaline conditions. After at least 2hour at ~4ºC, the slides were gently removed from the Lysing Solution. The slides were placed side by side on the horizontal gel box near one end, sliding them as close together as possible. The buffer reservoirs were filled with freshly made pH>13 Electrophoresis Buffer until the liquid level completely covered the slides. The slides sat in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage. The Power supply was turned on to 24 volts (~0.74 V/cm) and the current was adjusted to 300 milliamperes by raising and lowering the buffer level. The slides were electrophoresed for 30 minutes. The power was turned off. Gently the slides were lifted from the buffer and placed on a drain tray. Drop wise the slides were coated with Neutralization Buffer and were let to sit for at least 5 minutes. The slides were drained and this step was repeated two more times. Slides were stained with 80µL 1X Ethidium Bromide, and left for 5 min and then dipped in chilled distilled water to remove excess stain. The coverslip were then placed over it and the slides were scored immediately and dried before staining. The slides were drained, then were kept for 20 min in cold 100% ethanol and alternatively in cold 100% methanol for dehydration. The slides were air dried and placed in oven at 500C for 30 min, then stored in a dry area.

When convenient, the slides were rehydrated with chilled distilled water for 30 min and stained with EtBr as in step 6 and covered with a fresh coverslip. Before viewing slides, excess liquid on the back and edges was blotted away. After scoring, coverslips were removed, rinsed in 100% alcohol to remove stain, dried, and stored for archival purposes if needed.

3.5.5 Evaluation of DNA Damage

For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on an Optika fluorescent microscope fitted with an excitation filter in the range of 460-550nm. Although any image analysis system may be suitable for the quantitation of SCGE data, a Cometscore image analysis software was used which was used to evaluate the quantitative and qualitative extent of DNA damage in the cells by measuring frequency of DNA damage (%age cells), Comet overall area (μm^2), comet length and height (µm), head DNA (%age), head diametric measurements (µm), comet tail DNA (%age), olive tail movement (OTM) and tail length (µm). These parameters were used to determine the quantification of DNA damage. Data was based upon 200 randomly selected cells per sample, taken in 50 different fields per sample.

3.6 Statistical Analysis

During the compilation of data Microsoft Office Excel Worksheet was used. For statistical evaluation the Statistical Package for Social Sciences IBM Ver. 18 Software was used. One way ANOVA was the statistical analysis of choice with Post-HOC analysis using Dunett's one sided and 2-sided tests where comparisons were required with a single control as well as Duncan's 2-sided test for comparisons amongst groups. Descriptive statistics using cross-tabulation were used to evaluate Data and T-tests were used for group-group significance and testing of hypotheses. A 95% Confidence interval was used throughout, and all values having a p value

showeding $p<0.05$ were taken to be statistically significant. Matlab was used for graphical interpretation and prediction of straight line graphs and equations.

3.7 *In-silico* virtual screening

3.7.1 Selection of target and ligands

The study was directed to unravel a new class of carbonic anhydrase 2 inhibitors and optimize the lead structures so that they are more selective, less toxic and more potent than the existing hCAII inhibitors. Most potent inhibitors existing yet belong to a class of sulfonamides. Biological activity data for hCAII inhibitors was downloaded from PDB. Six most active compounds having IC50 values less than 1.0 nM and which shared the same protocol for IC50 estimation (pH stat assay) were considered for further studies entailing pharmacophore modeling and docking. Target structure of human carbonic anhydrase (PDB ID: 4ITP) was downloaded from PDB for docking. This structure has a co-crystallized sulfonamide-based ligand and has already been used for docking purposes which makes it a reliable structure for use in our study. The structure was crystallized out using X-ray diffraction in 2013 covering 257 out of 260 residues with a resolution of 1.7 Angstroms.

3.7.2 Optimization of docking protocol

The protocol was optimized prior to docking of the actual ligands under study using the ligand IGD (2-phenyl-N-(4-sulfamoylbenzyl) acetamide) already co-crystallized with 4ITP. MOE software was used for this purpose. Firstly, water molecules were removed from the crystal structure of hCAII with the subsequent energy minimization of the ligand-bound structure using a force-field based algorithm called Amber99. 4ITP was subsequently protonated and the ligand was isolated. Docking simulations were then computed using all the combinations of the

placement methods and rescoring functions to find the optimal set of scoring functions to get most accurate docking results for sulfonamide binding into the active site of the enzyme. For this, surface area within 6 Angstroms near the ligand was isolated and used as a binding site for all subsequent docking simulations. With each docking simulation, 100 poses were generated and analyzed. The scoring function and placement method used were Proxy triangle and Alpha HB respectively.

3.7.3 Docking

Six compounds showeding high activity (IC50 value less than 1 nM) against CA2 were downloaded from CHEMBL database of compounds freely accessible at https://www.ebi.ac.uk/chembl/. Their 3D structures were then generated using MOE using their canonical *smiles* codes. These compounds along with their CHEMBL IDs, 3D structures and IC50 values are enlisted in table 3.1. All of these compounds were docked into the active site of CAII generating 100 different conformational poses for each compound. Subsequently, all the poses were analyzed and the best one was selected for further use in pharmacophore modeling.

3.7.4 Ligand-protein interactions

2D and 3D ligand-protein interactions for each of these highly active compounds were then computed in order to develop a generic pharmacophore model. At this step, protein residues interacting with the ligand and the type of interaction of various features in the ligand structure were analyzed.

3.7.5 Pharmacophore modeling

Based on the ligand protein interactions, a pharmacophore model was built using active analog approach thereby generating an overall model for all the compounds showeding significant activity against our target. Distances were computed between the pharmacophore features.

3.7.6 Pharmacophore model validation

The pharmacophore model generated was then validated using a test data set entailing 100 compounds out of which half were reported to be highly active whereas the rest of the compounds were reported to showed no inhibitory effect on carbonic anhydrase 2. The goal was to optimize the model so that it selects maximum number of actives and the least number of inactives.

3.7.7 Virtual screening

After the pharmacophore model was generated, it was used to screen all the drug compounds present in the DrugBank in order to elucidate novel hits which can be tested for their activity against cabonic anhydrase 2, efficiency, ADMET properties, toxicological profiles, and selectivity and off-target effects. The drug data was downloaded from DrugBank and all the structures were minimized to get lowest energy conformations. This data was then screened using the pharmacophore model generated in the previous step. Hit compounds were identified based on various descriptor values including LogP(o/w), number of rotatable bonds, number of H-bond acceptors, number of H-bond donors and molecular weight etc.

Results

In this investigation, physical parameters of a total of 30 RA patients in Pakistani population were collected in order to confirm the diagnosis. After that, protein expression levels of human carbonic anhydrase 2 in the serum of RA patients were analyzed and compared to those in 30 healthy controls.The resulting data was then statistically analyzed significant correction with RA. Furthermore, DNA damage was also analyzed from blood samples of the same RA patients and compared to those in controls with subsequent statistical analysis. In the end, computational drug designing approached were applied in order to unravel novel class of carbonic anhydrase 2 inhibitors as candidate drugs for isoform-selective targeting of CA2 for anti-resorptive therapy.

4.1. Physical parameters of patients

4.1.1. Age and gender

Age and gender tender to showed to play an important role in the RA pathogenesis. Generally, patients afflicted with RA have ages above 40. Among our samples, most of the patients had ages above 40, with females making a majority of the sample pool. All of the male patients were with ages above 45 (Fig. 4.1)

4.1.2. Concurrent ailments

Five of the patients among the 30 were also suffering from other ailments along with Rheumatoid arthritis Rest of the patients were not showeding any concurrent diseases. Out of

these five patients, 3 were suffering from hypertension whereas rest of the two had Diabetes Mellitus (Type 2) and severe depression respectively.

4.1.3 Family history

Some of the patients also had family history of Rheumatoid arthritis thus implying role of genetic factors in play. However, number of patients with family history was not very high (only 5/30) as compared to those with no family history (Fig 4.1).

4.1.4. Condition of joints

4.1.4.1 Tender joints

90% of the RA patients were showeding tender joints with at least one affected joint. However, number of tender joints varied from patient to patient and so did the joint morphology. Most of the patients had 1 to 14 tender joints with a very few having more than 14 tender joints.

4.1.4.2 Swollen joints

A large number of patients from our sample pool showeded swollen joints. The number varied from patient to patient. Some patients showeded 1 to 12 swollen joints while the rest had more than 12 swollen joints. All patients having swollen joints were also accompanied with joint tenderness.

4.2. Protein quantification by Bradford assay

Quantity of protein in each serum sample was determined using Bradford assay. Standard curve graph plotted for a representative gel using Microsoft Excel is showedn in Fig 4.1. Value

of R^2 came out to be 0.987 whereas the regression constant came out to be 0.015. Protein concentration in most of the samples ranges between 25 to 3025µg/25µl. Absorbance, protein concentration per microliter of sample and the amount of samples loaded for 25µg protein for each sample is showedn in Table 4.1.

4.3. Western Blotting

Use of western blotting technique elucidated the elevated levels of carbonic anhydrase II in case patients as compared to the healthy controls. A representative immune-blotted membrane for patients and controls is showedn in Fig 4.1(a) and (b).

4.4. Comet Assay

Results of comet assay indicate the DNA damage in cells from all of the RA patients as compared to no damage in case of healthy controls. Fig. 4.2 (a) and (b) represent the DNA damage status in patients and controls.

4.5. Selection of target and ligands

Crystal structure of our target CAII (PDB ID: 4ITP) with its co-crystallized ligand is showedn in Fig. 4.1. Six most active compounds having IC_{50} values less than 1.0 nM and which shared the same protocol for IC_{50} estimation (pH stat assay) were considered for further studies entailing pharmacophore modeling and docking are enlisted in Table 4.1.

4.6. Isolation of binding pocket

Using the co-crystallized ligand in the active site of the protein target, the surface of the binding site was computed using MOE which is showedn in Fig. 4.2.

4.7. Docking

All the compounds enlisted in Table 4.1 were docked into the active site of the CA2 structure. Best binding poses of each these compounds were identified and isolated for further use in pharmacophore modeling. The six docked structures are showedn in Fig. 4.3 to 4.8.

4.8. Estimation of ligand-protein interactions

Ligand-protein interactions between the target protein and all the aforementioned six compounds were computed using MOE. Glu106 and His119 were seen to showed metal/ion contact with the Zinc in case of all the active compounds. However, additional residues like Thr200, Thr199, His94 and Glu92 were also observed to interact in different cases. Fig. 4.9 (a) to 4.9 (f) and 4.10 (a) to 4.10 (f) are the diagrammatic representations of 2D and 3D ligand-protein interactions of all these compounds respectively.

In case of CHEMBL218490, in addition of involvement of Glu106 and His119 in metal/ion contact with zinc, His119 and His94 are also observed to showed side-chain donor interactions with the amino part in the sulfonamide of the ligand. His94 is also linked with sulphate linked to the aromatic ring in the ligand structure via side-chain donor interactions.

Sulphur attached to an aromatic ring in CHEMBL79939 and the sulphate group also showeds metal contacts with Zinc in addition to the residues His119 and Glu106. Thr200 showeds side-chain donor interactions with the oxygens in the sulphate linked to aromatic ring in the structure.

In CHEMBL69114, besides the metal/ion contacts of Glu106 and His119 with Zinc ion, amino group of the compound is also linked via its hydrogens to His119 and Glu106. Thr199 is also showedn to be involved in side-chain donor interactions by linking to oxygen in the sulphate in the sulfonamide part.

CHEMBL417975 interacts with the target by making side-chain donor interactions with Glu106 and His119 which in turn are also making metal contact with the zinc ion. In addition, amino group is also observed to be involved in metal ion contact with zinc ion with one of its hydrogens.

In case of CHEMBL543319, only Arg246 has been seen to interact with the ligand by linking to its sulphate via side-chain donor interaction.

Side-chain acceptor interactions are observed between His94 and the ligand CHEMBL553832. Moreover, oxygen of sulphate is also linked to Thr200 and Thr199 via backbone donor interactions. Like in case of all other compounds, Zinc is also attached to His119 and His94 via metal/ion contact. Amino group in the ligand is also linked to Glu106 via side-chain acceptor interactions.

4.9. Pharmacophore modeling and validation

By analyzing the overall interaction pattern of all these ligands with CAII, a generic pharmacophore model was built constituting three features. These features include H-bond acceptors, H-bond donors and an aromatic group. Fig. 4.11 represents the 3D pharmacophore model. Furthermore, distances were calculated between the pharmacophoric features which are represented in Fig. 4.12 and ae enlisted in Table 4.2.

In order to validate the pharmacophore model, a dataset of 100 compounds was used as a test test. This test set included 50 compounds known to be ‘active’ against CAII whereas rest of the 50 were known to be inactive. The database of these compounds was screened on the basis of the built pharmacophore model. The model selected all the actives as actives (no false negatives) and

only 5 out of 50 inactives as ‘actives’ (5 false positives) and was thus proved to be a fairly reliable model.

4.10. Virtual screening and hit identification

The pharmacophore model was used to screen all the existing drugs present in DrugBank. The model selected 59 entries in the DrugBank to be active against CAII. However, in order to identify the potential hits, all these 59 compounds were further analyzed by computing various chemical descriptors and comparing their values with the already known highly potent inhibitors of CAII. The list of shortlisted hits and their chemical descriptor values is enlisted in Table 4.3. The compounds showeding similar descriptor values as those of the known inhibitors were considered as potential hits. Three hits were selected as candidate compounds. These include Alfuzosin, Prazosin and Sulpiride. Sulpiride belongs to a class of benzenesulphonamides and Alfuzosin and Prazosin are quinazolinamines. Fig 4.14 showeds the chemical structures of the three hits.

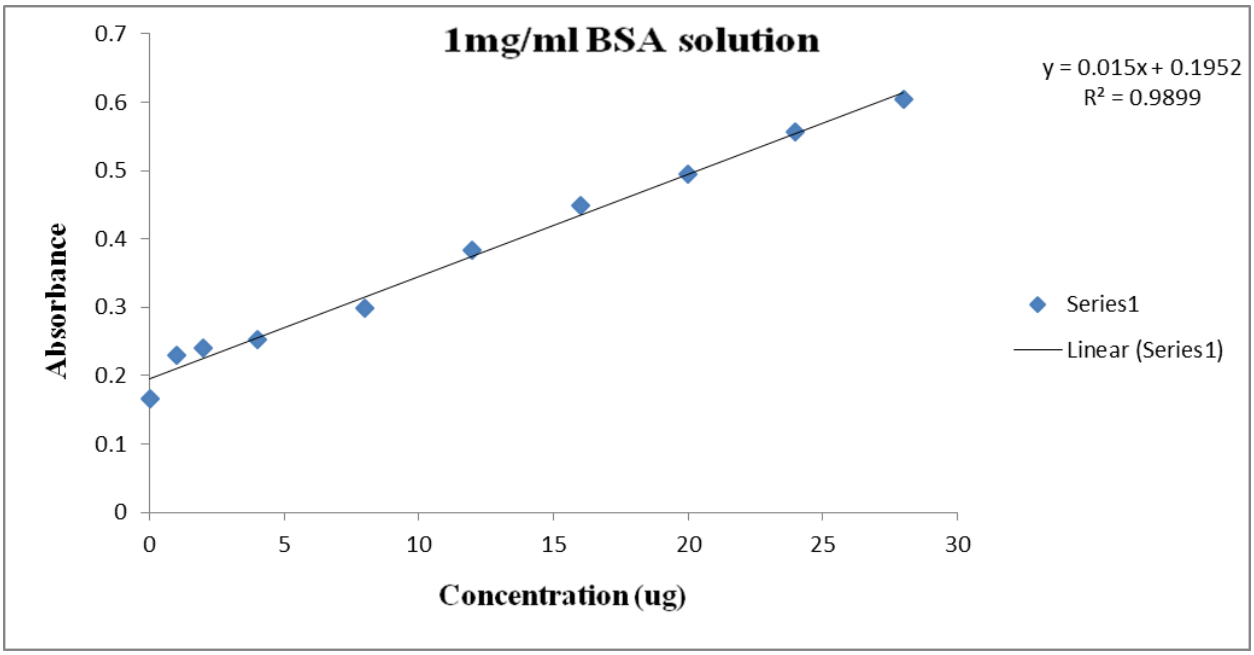

Figure 4.1: Standard Curve obtained using Bradford assay for protein quantification

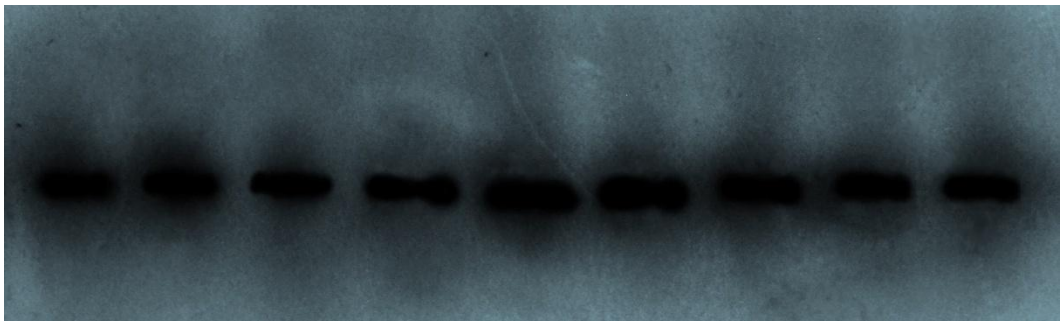

Figure 4.2. A representative immune-blotted membrane showeding the expression of actin in RA patients and controls.

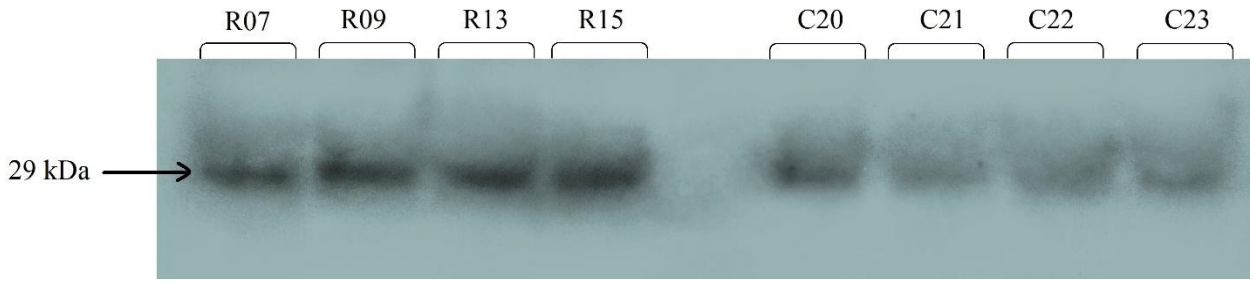

Figure 4.3. A representative immune-blotted membrane showeding the expression of CAII in RA patients and controls.

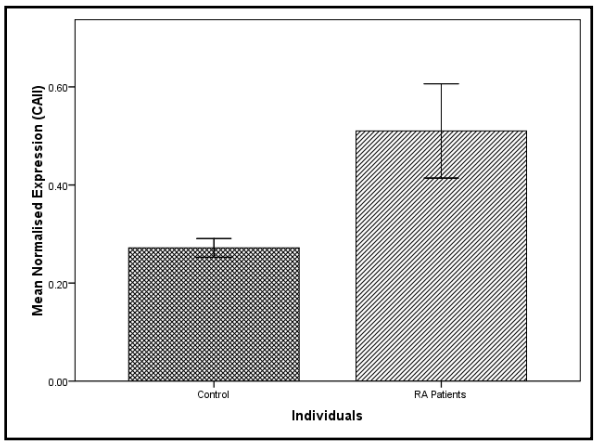

Figure 4.4. Graphic representation of mean normalized expression of CAII in RA patients and controls

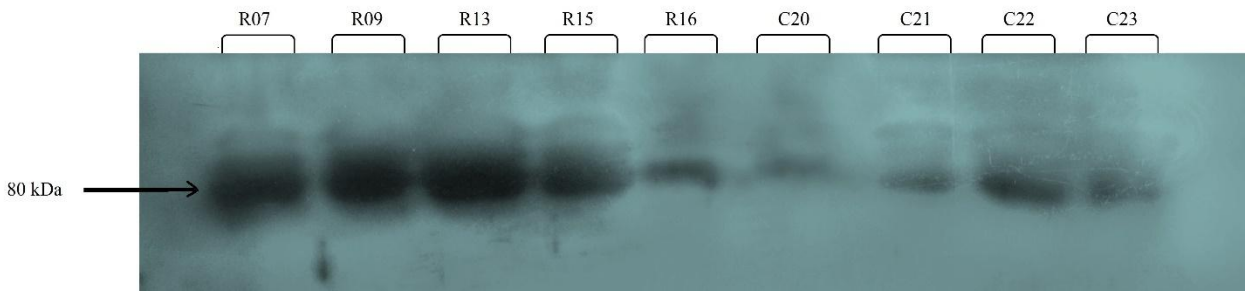

Figure 4.5. A representative immune-blotted membrane showeding the expression of Calpain in RA patients and controls.

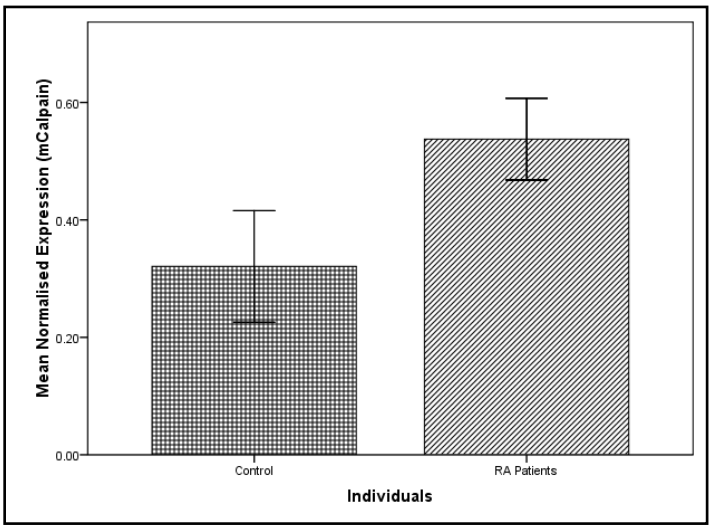

Figure 4.6. Graphic representation of mean normalized expression of Calpain in RA patients and controls

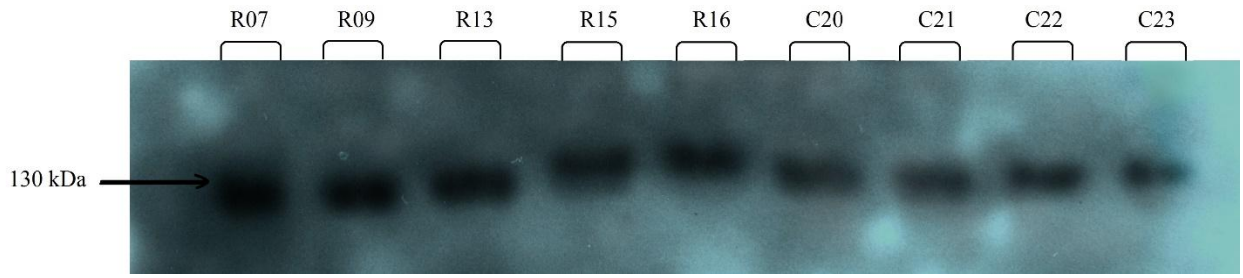

Figure 4.7. A representative immune-blotted membrane showeding the expression of NFATc3 in RA patients and controls.

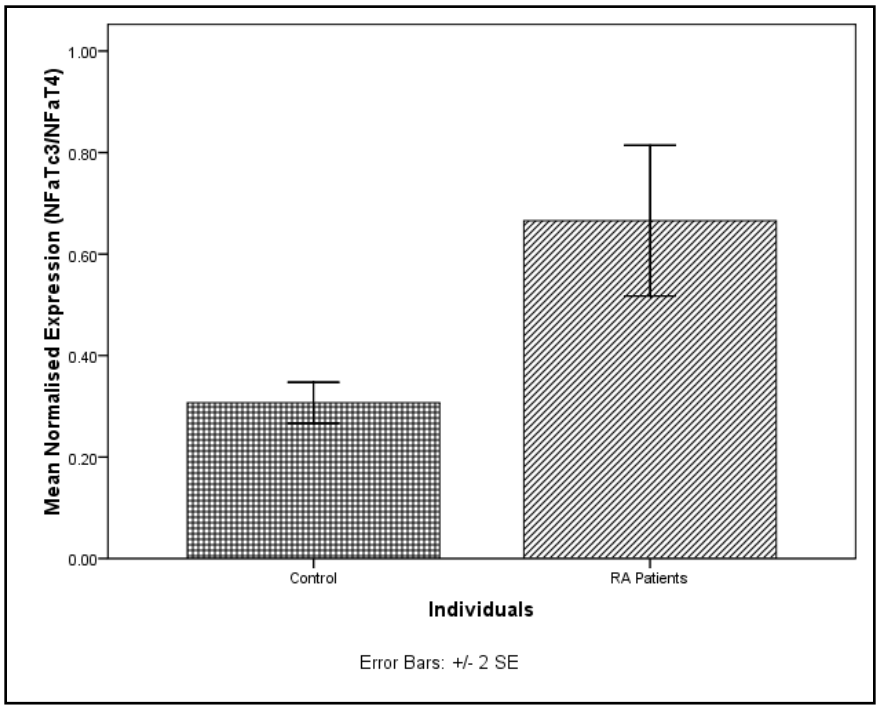

Fig 4.8. Graphic representation of mean normalized expression of NFATc3 in RA patients and controls

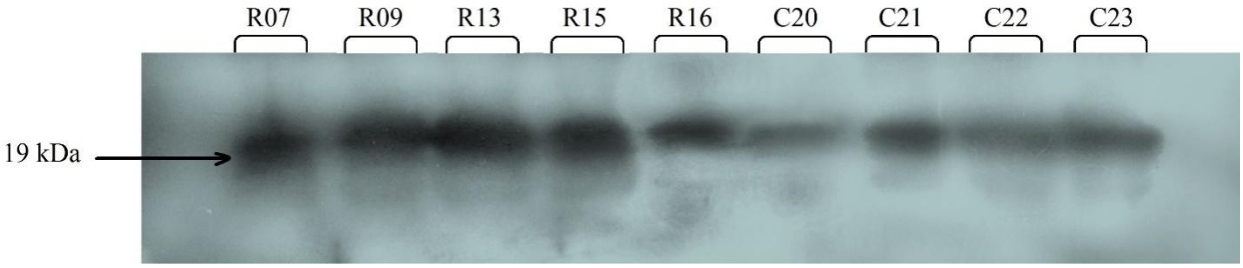

Figure 4.9. A representative immune-blotted membrane showeding the expression of TNF-α in RA patients and controls.

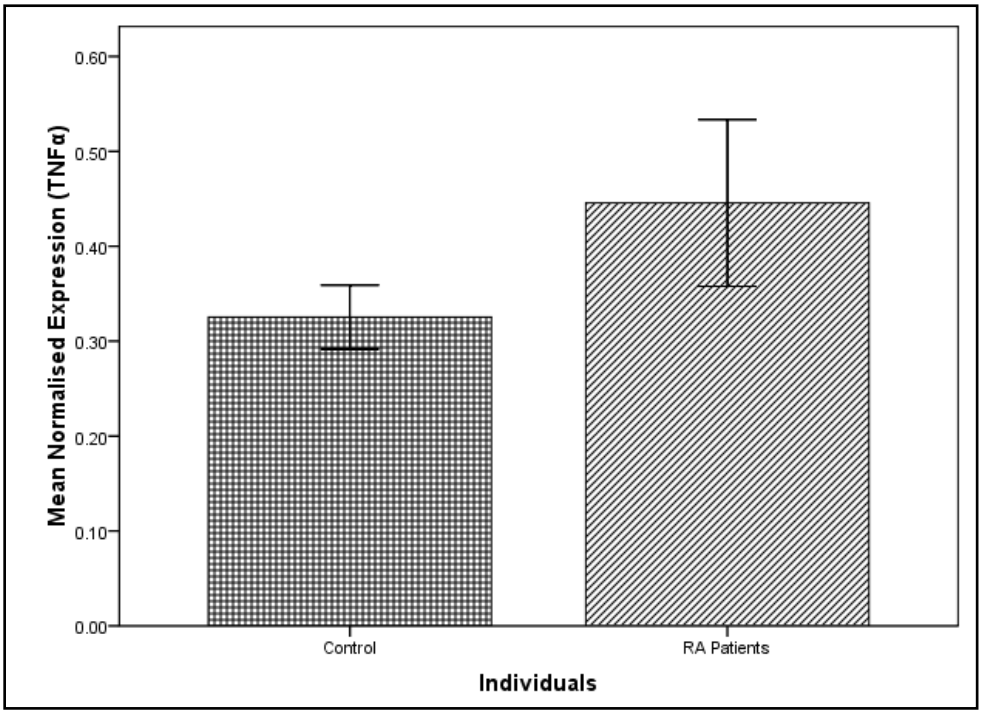

Figure 4.10. Graphic representation of mean normalized expression of TNF-α in RA patients and controls

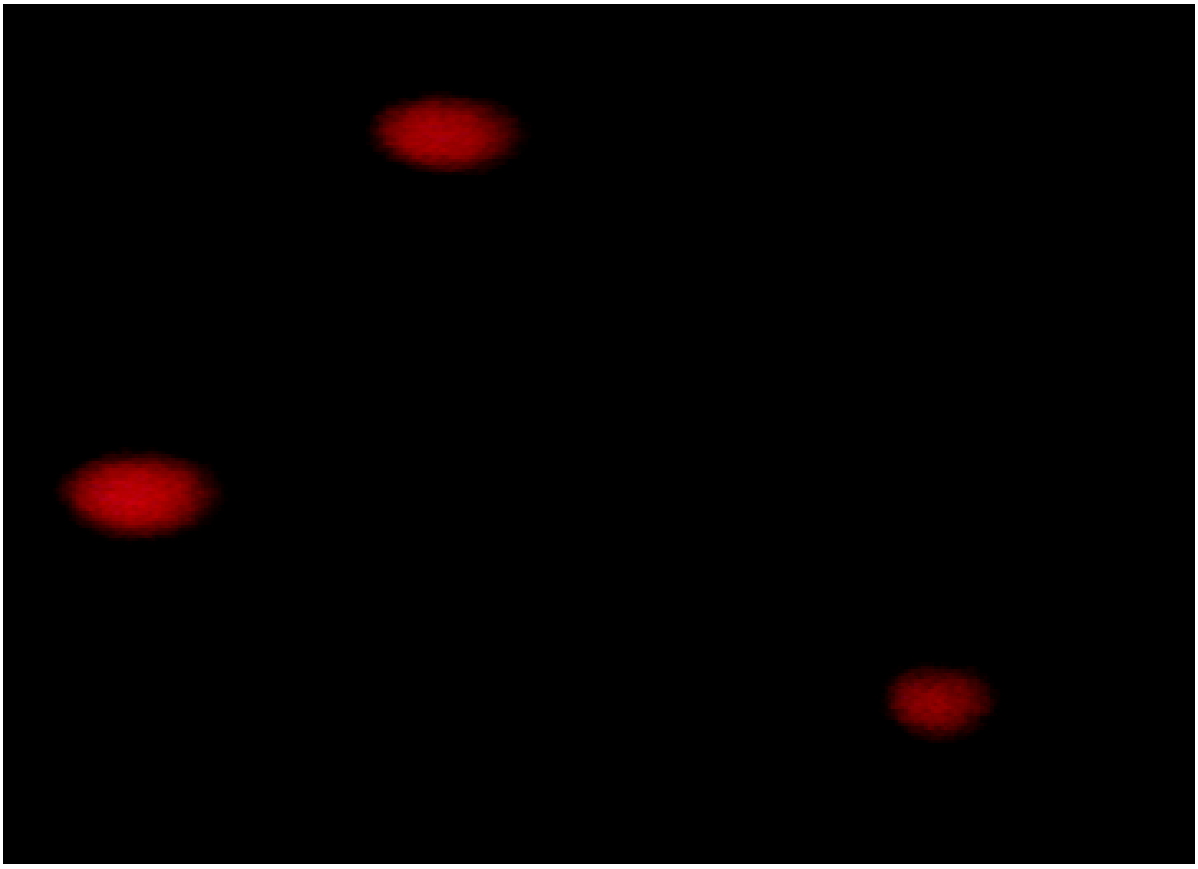

Figure 4.11. Comet Assay: A representative snapshot of a human lymphocyte from a RA patient showeding the DNA damage in the cells of the patient.

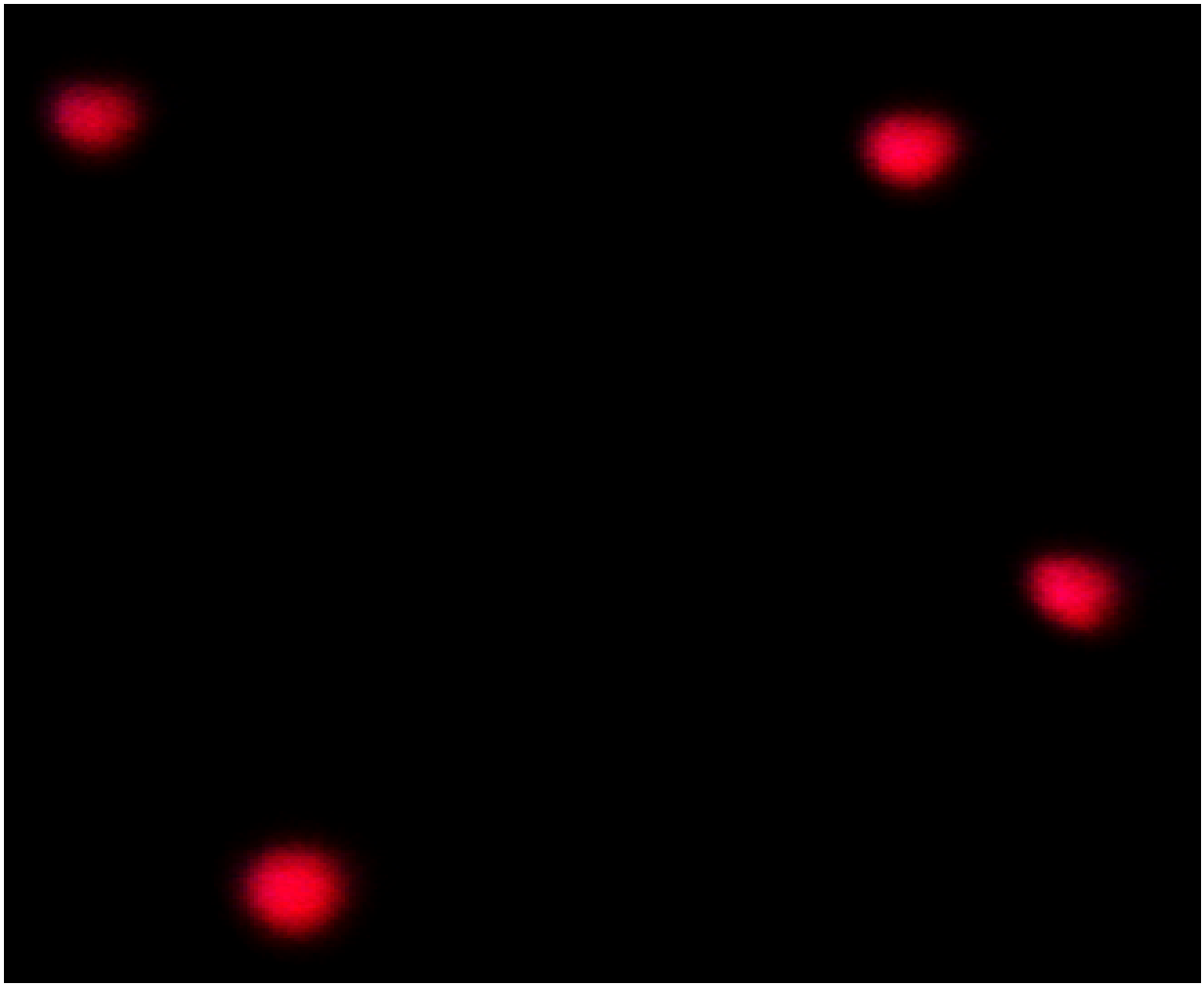

Figure 4.12 Comet Assay: A representative snapshot of a human lymphocyte from a healthy individual showeding no DNA damage in controls.

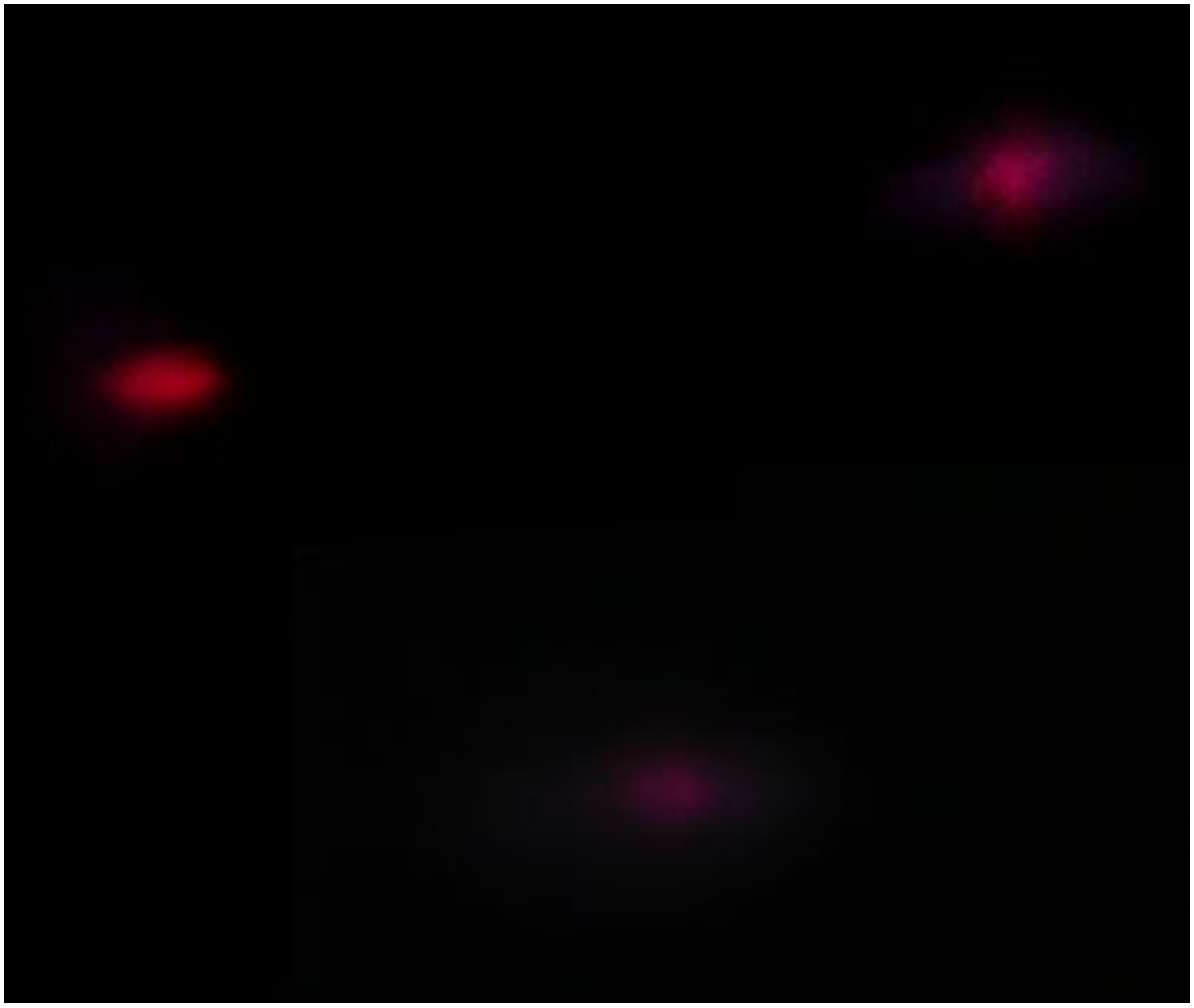

Figure 4.13 Comet Assay: A representative snapshot of a human lymphocyte from a healthy individual treated with H2O2 showeding DNA damage.

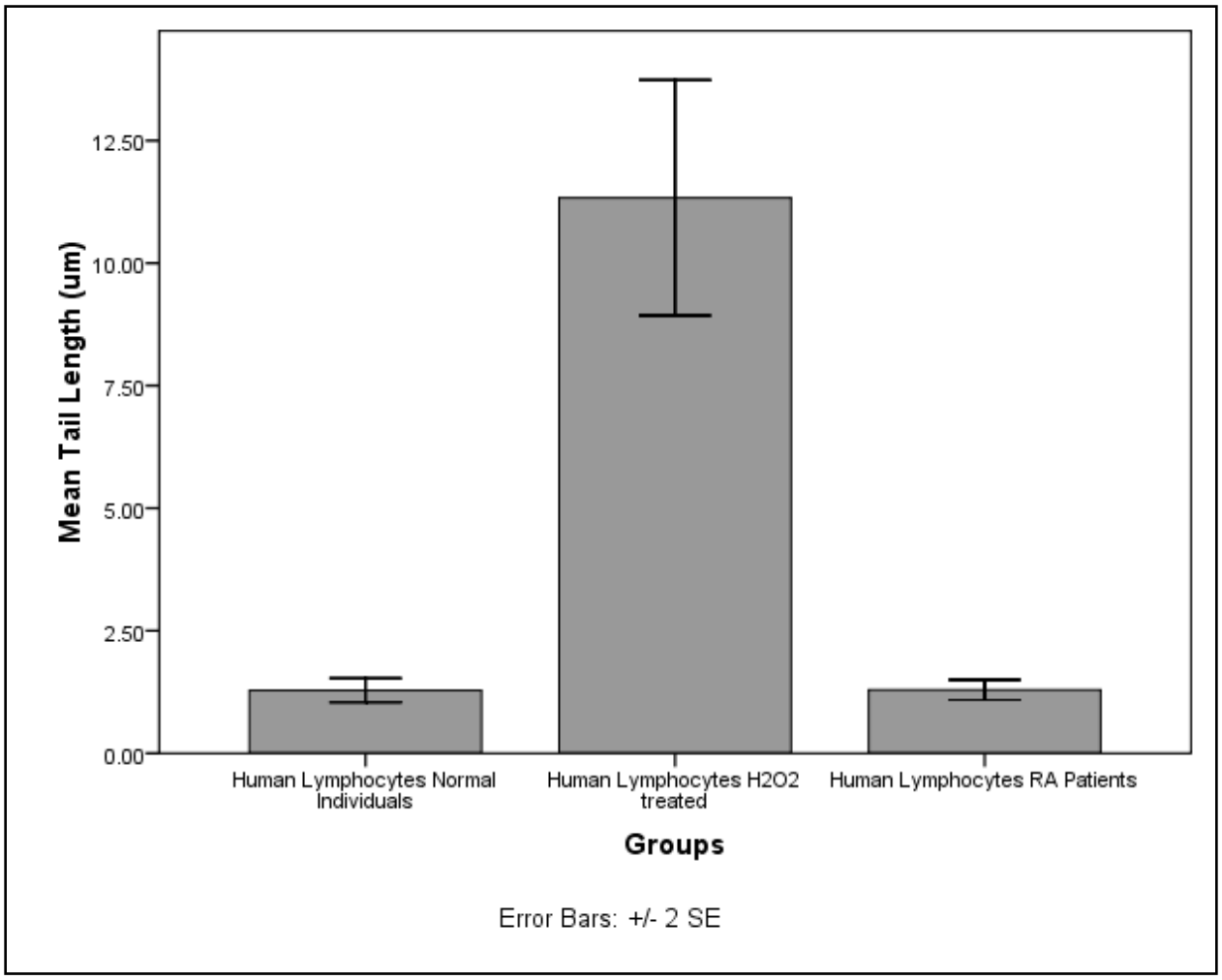

Figure 4.14 Graphic representation of mean tail length in micrometers obtained in comet assay for control, RA patients and H2O2 treated groups.

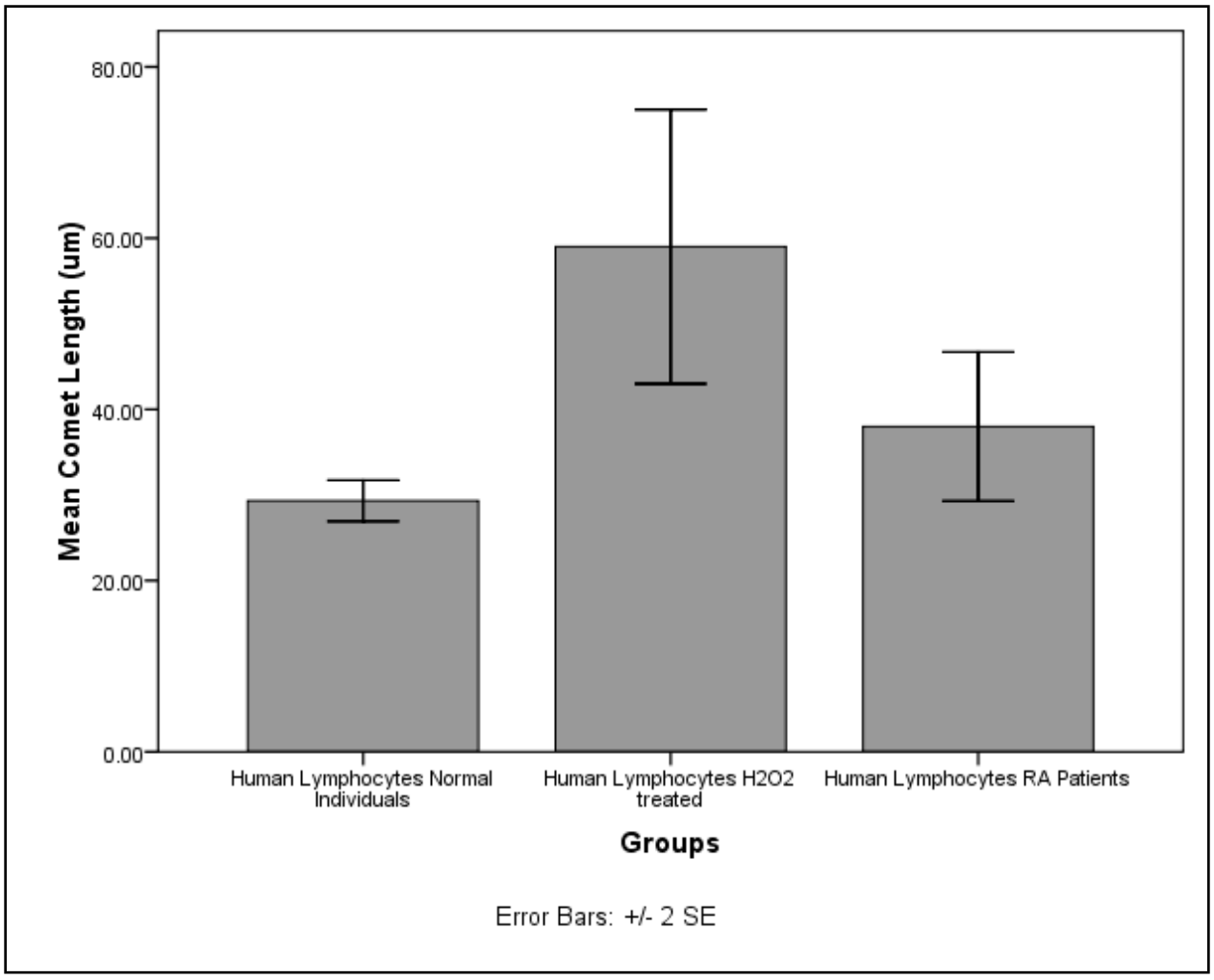

Figure 4.15 Graphic representation of mean comet length in micrometers obtained in comet assay for control, RA patients and H2O2 treated groups.

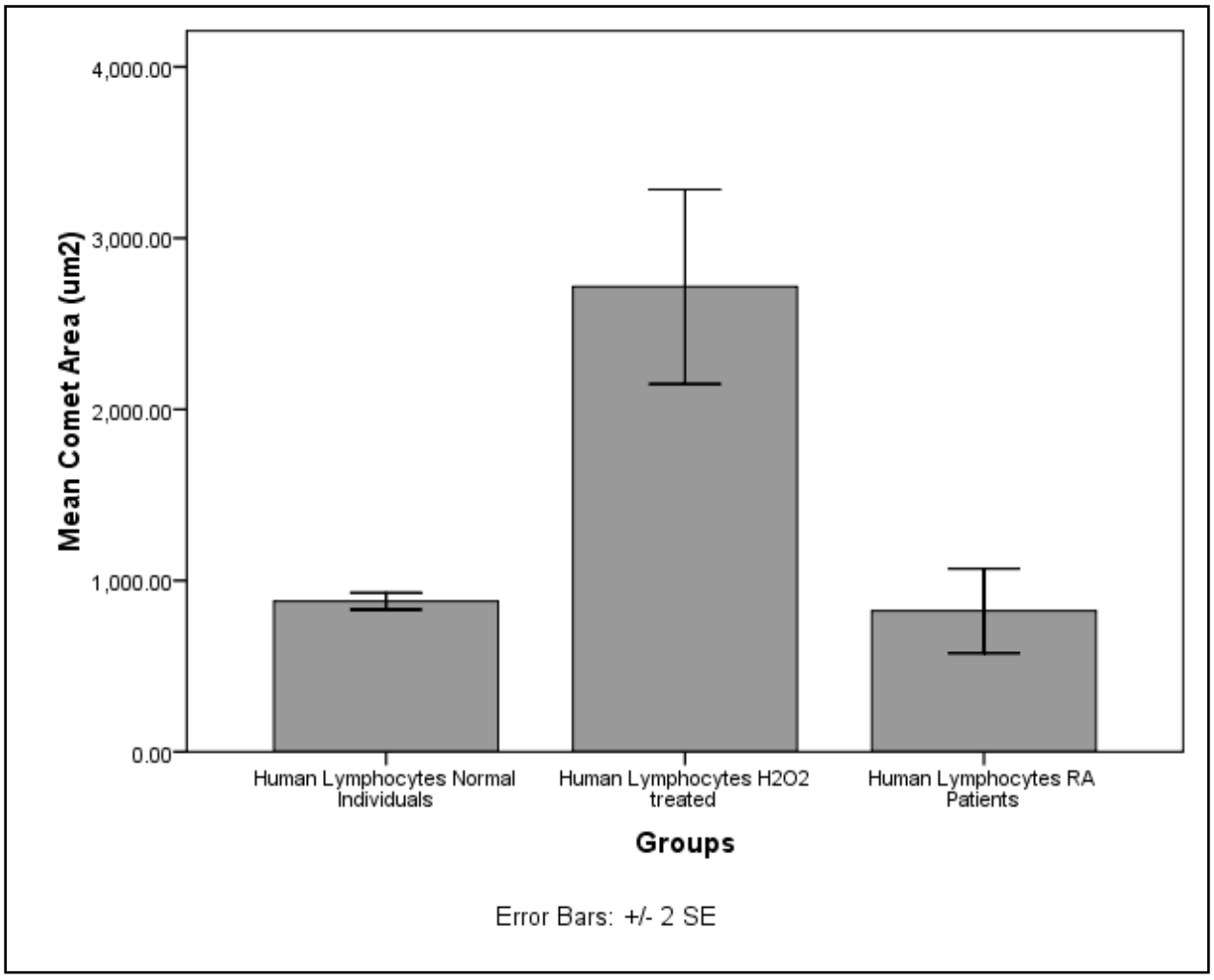

Figure 4.16 Graphic representation of mean comet area obtained in comet assay for control, RA patients and H_2O_2 treated groups.

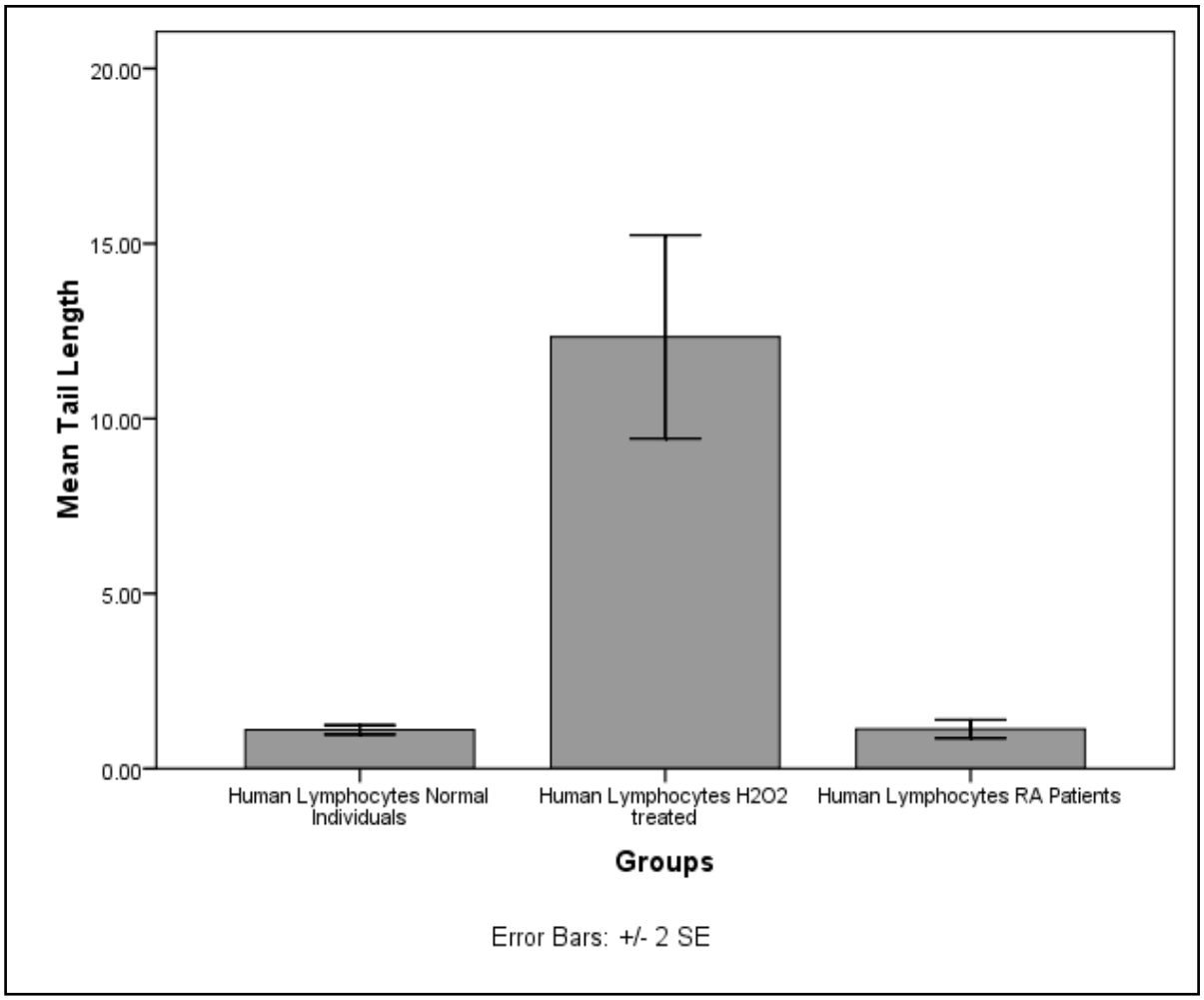

Figure 4.17 Graphic representation of mean percentage DNA in tail obtained in comet assay for control, RA patients and H2O2 treated groups.

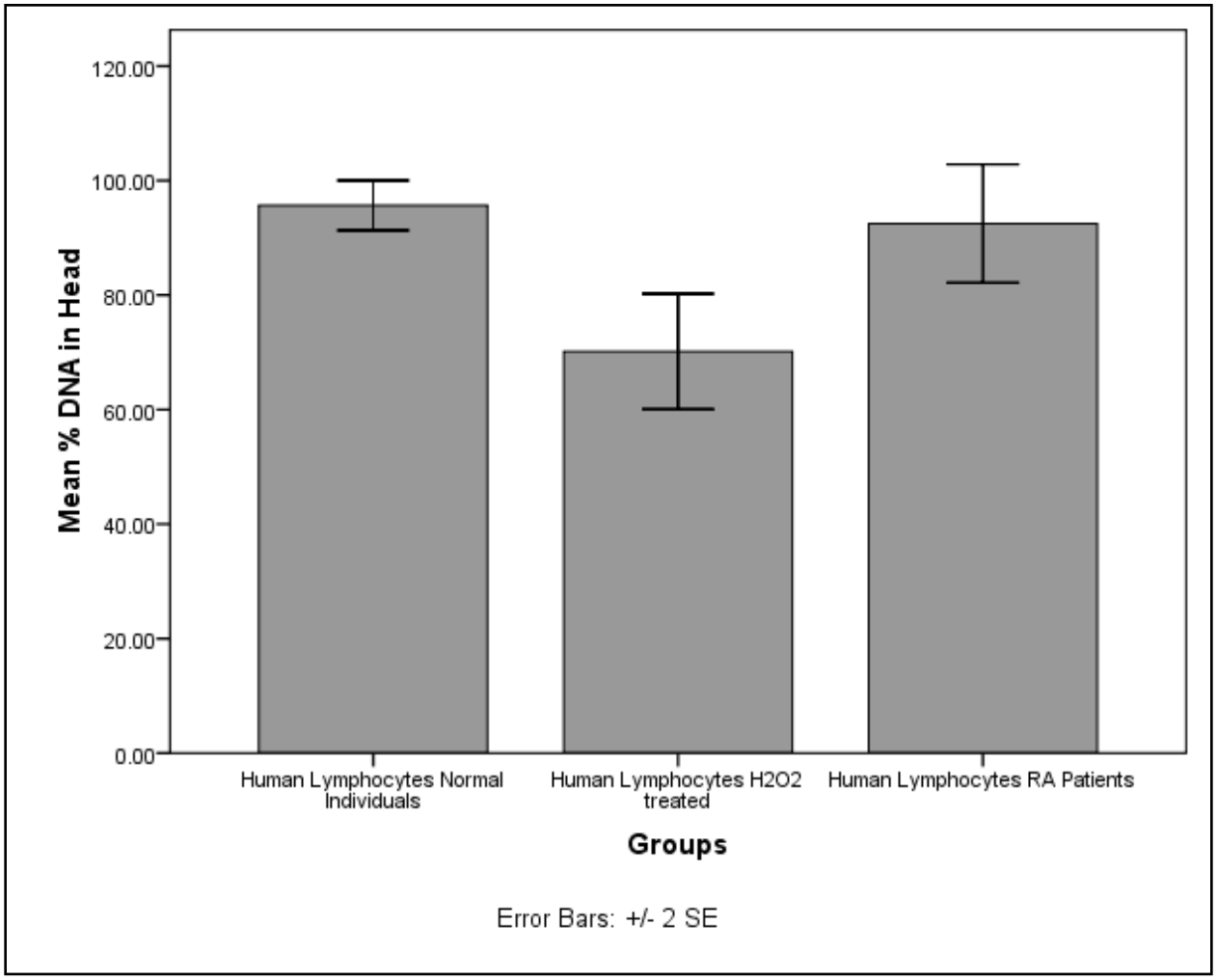

Figure 4.18. Graphic representation of mean percentage DNA in head obtained in comet assay for control, RA patients and H2O2 treated groups.

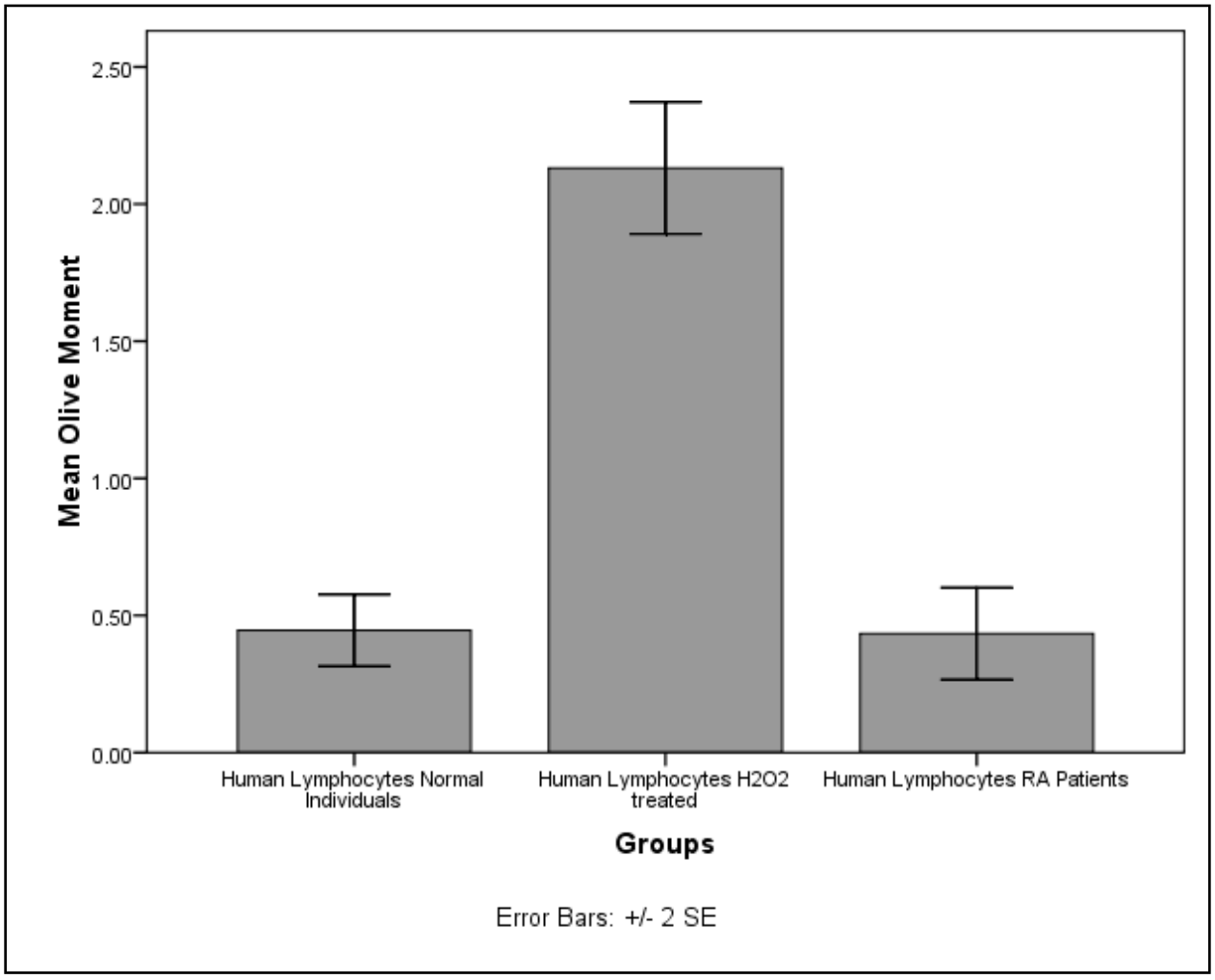

Figure 4.19. Graphic representation of mean olive moment obtained in comet assay for control, RA patients and H2O2 treated groups.

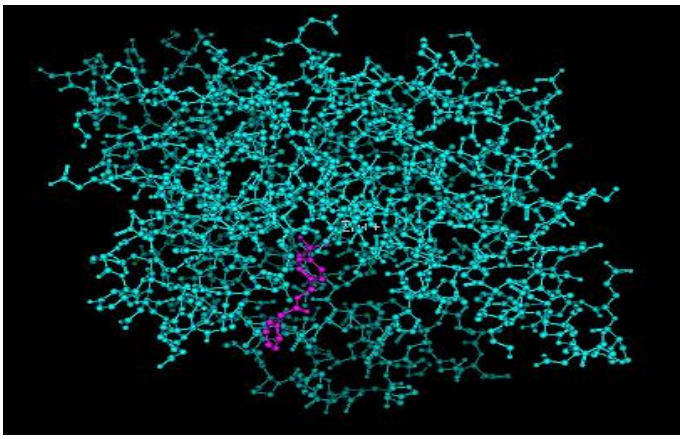

Figure 4.20. Carbonic anhydrase II with co-crystallized ligand (PDB ID: 4ITP).

Blue color indicates the structure of CA2 whereas the structures colored in pink showeds the bound ligand within the active site.

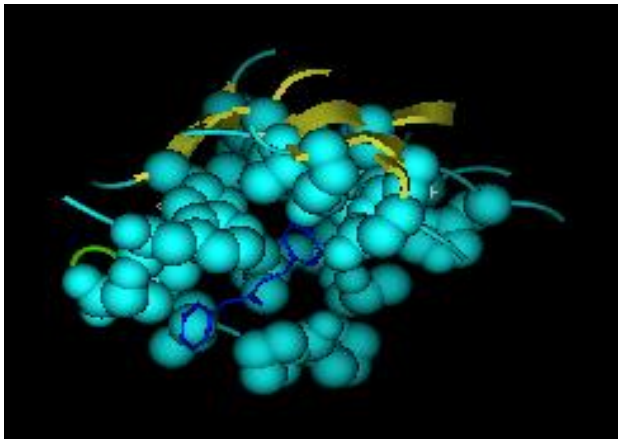

Figure 4.21. Ligand binding pocket of CAII isolated using MOE

Ligand binding pocket of CAII isolated using MOE covering an area of 6.0 Angstroms around the ligand. Dark blue structure represents the bound ligand whereas the light blue color showeds the active site of the target.

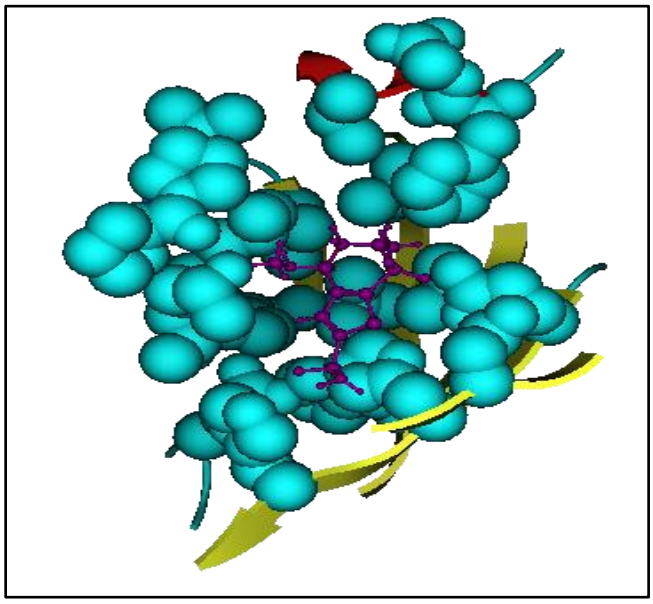

Figure 4.22. CHEMBL218490 docked in the active site of CA II

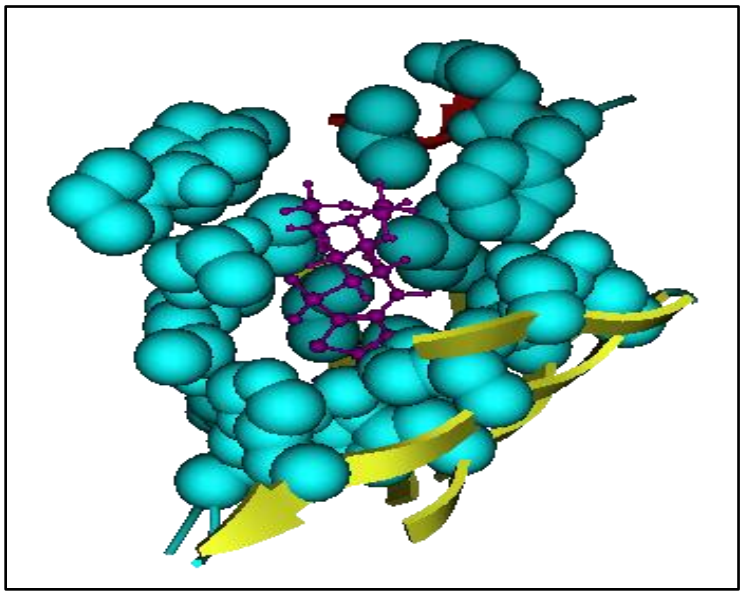

Figure 4.23. CHEMBL79939 docked in the active site of CA II

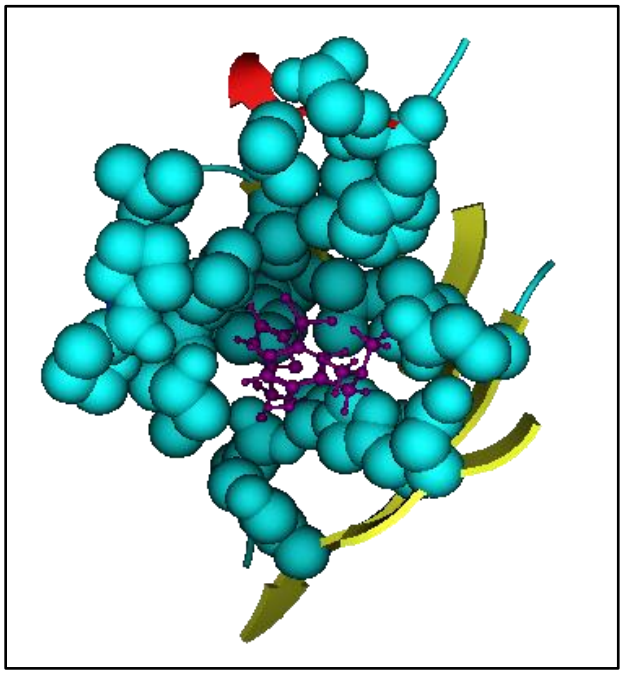

Figure 4.24. CHEMBL69114 docked into the active site of CA II

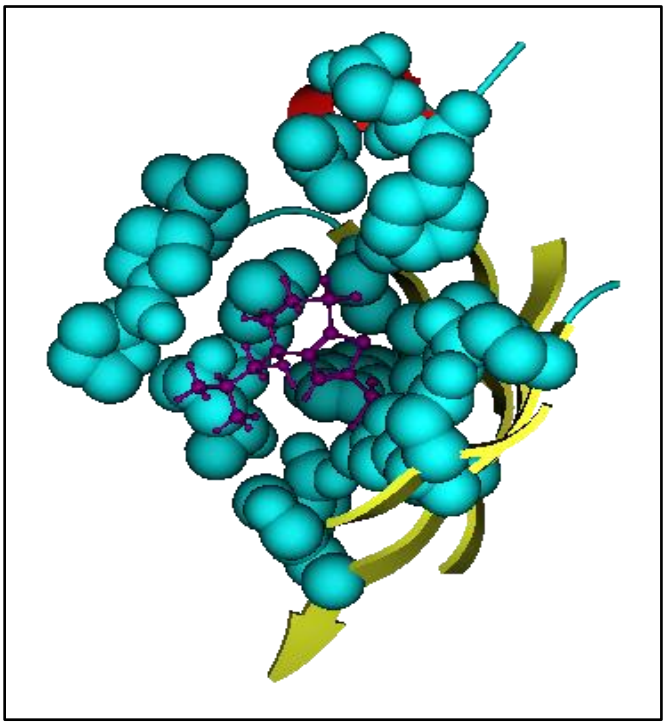

Figure 4.25. CHEMBL417975 docked into the active site of CA II

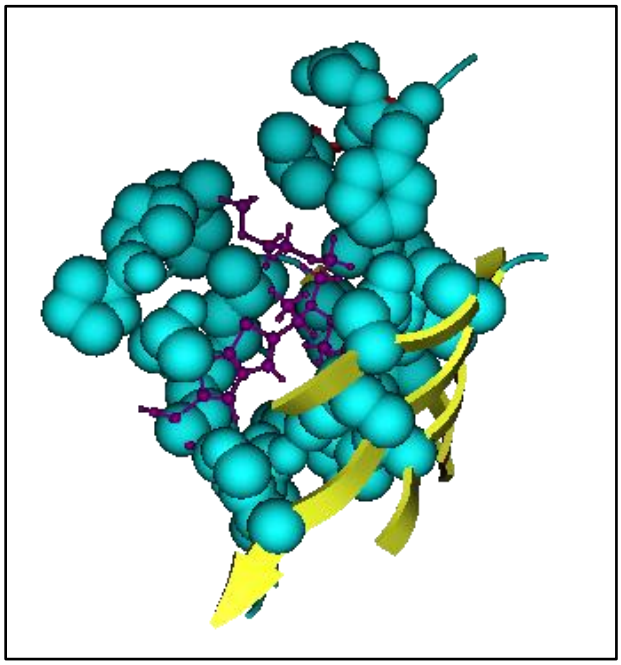

Figure 4.26. CHEMBL543319 docked into the active site of CA II

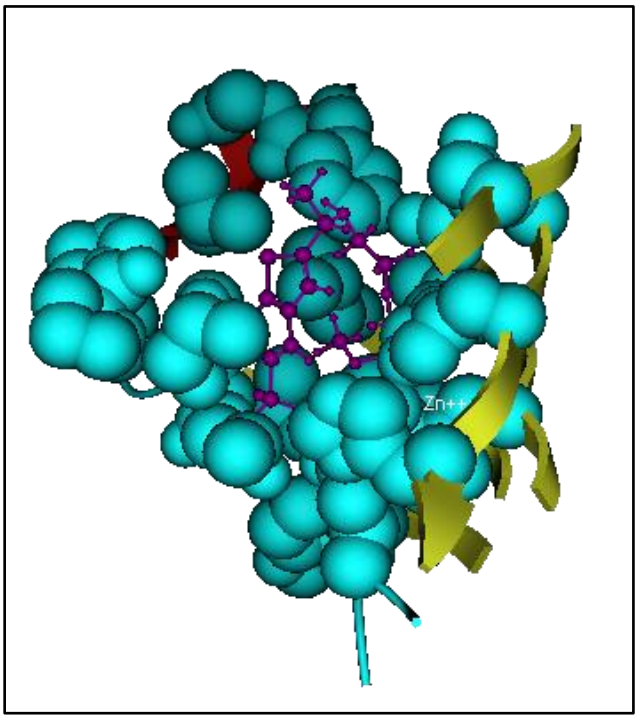

Figure 4.27. CHEMBL553832 docked into the active site of CA II

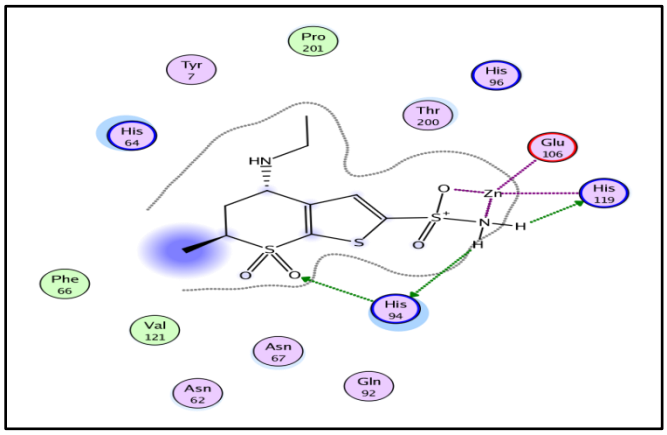

Figure 4.28. A 2D representation of ligand-protein interactions between CHEMBL218490 and CAII.

Green outgoing and incoming dashed lines represent side-chain acceptor and donor interactions respectively. Purple lines indicate polar interactions.

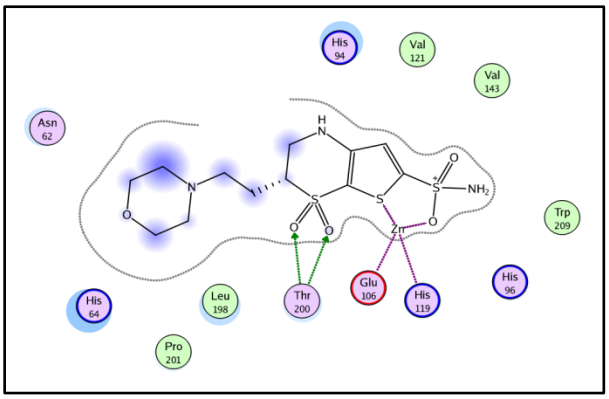

Figure 4.29. A 2D representation of ligand-protein interactions between CHEMBL79939 and CAII.

Green outgoing and incoming dashed lines represent H-bond acceptor and H-bond donor interactions respectively. Purple lines indicate polar interactions.

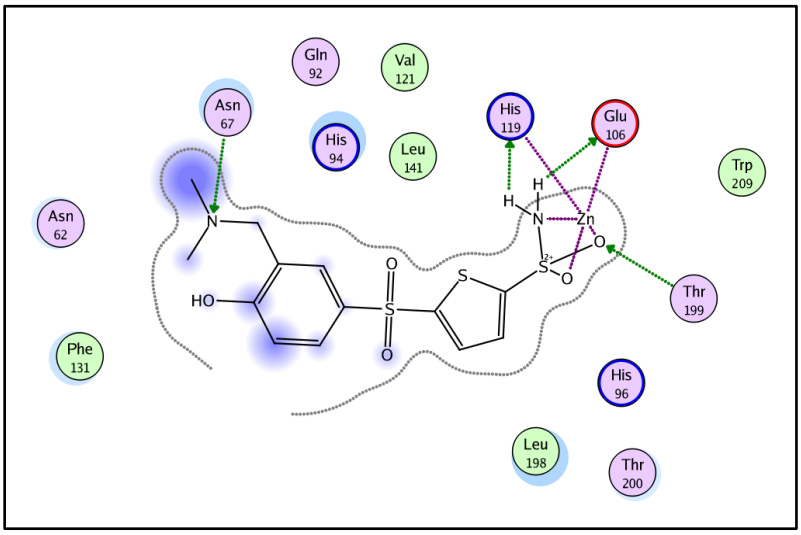

Figure 4.30. A 2D representation of ligand-protein interactions between CHEMBL69114 and CAII.

Green outgoing and incoming dashed lines represent H-bond acceptor and H-bond donor interactions respectively. Purple lines indicate polar interactions.

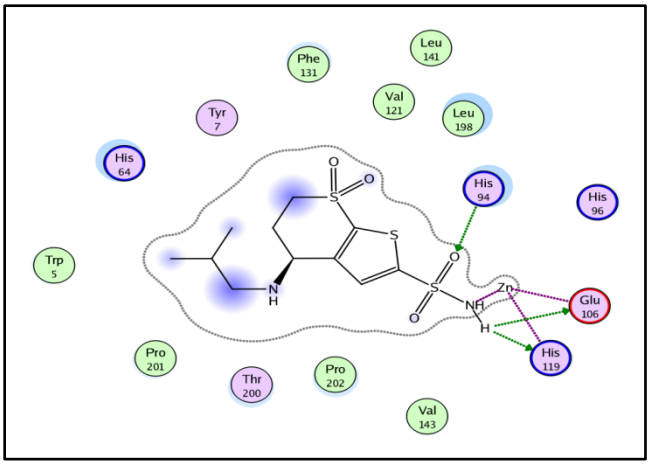

Figure 4.31. A 2D representation of ligand-protein interactions between CHEMBL417975 and CAII.

Green outgoing and incoming dashed lines represent H-bond acceptor and H-bond donor interactions respectively. Purple lines indicate polar interactions.

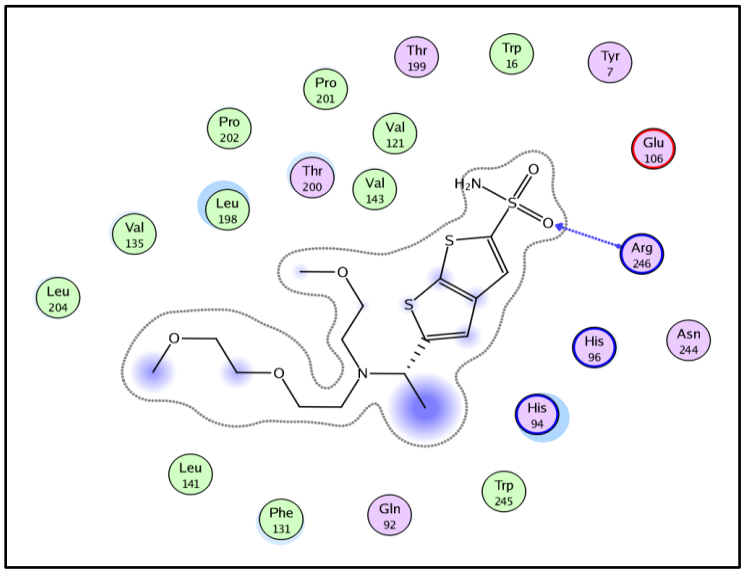

Figure 4.32. A 2D representation of ligand-protein interactions between CHEMBL543319 and CAII.

Green outgoing and incoming dashed lines represent H-bond acceptor and H-bond donor interactions respectively. Purple lines indicate polar interactions.

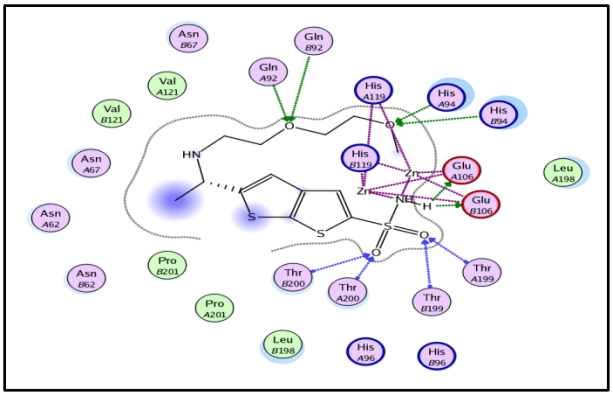

Figure 4.33. A 2D representation of ligand-protein interactions between CHEMBL553832 and CAII.

Green outgoing and incoming dashed lines represent H-bond acceptor and H-bond donor interactions respectively. Purple lines indicate polar interactions.

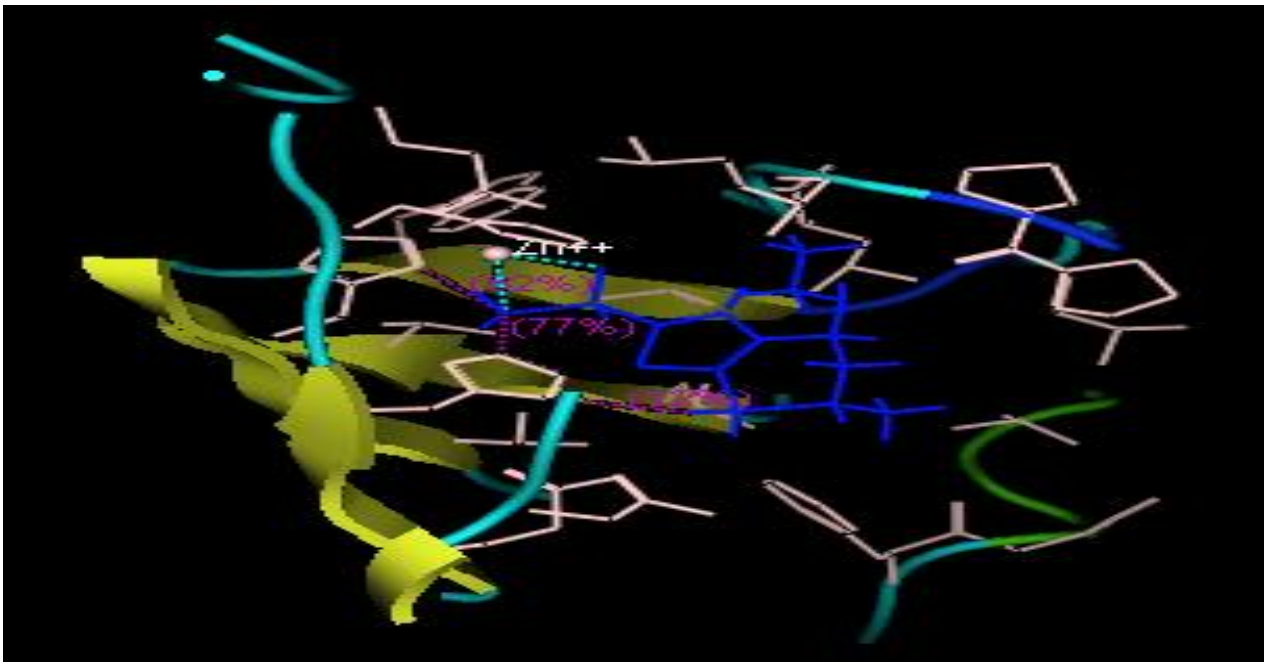

Figure 4.34. A 3D representation of ligand-protein interactions between CHEMBL218490 and CAII.

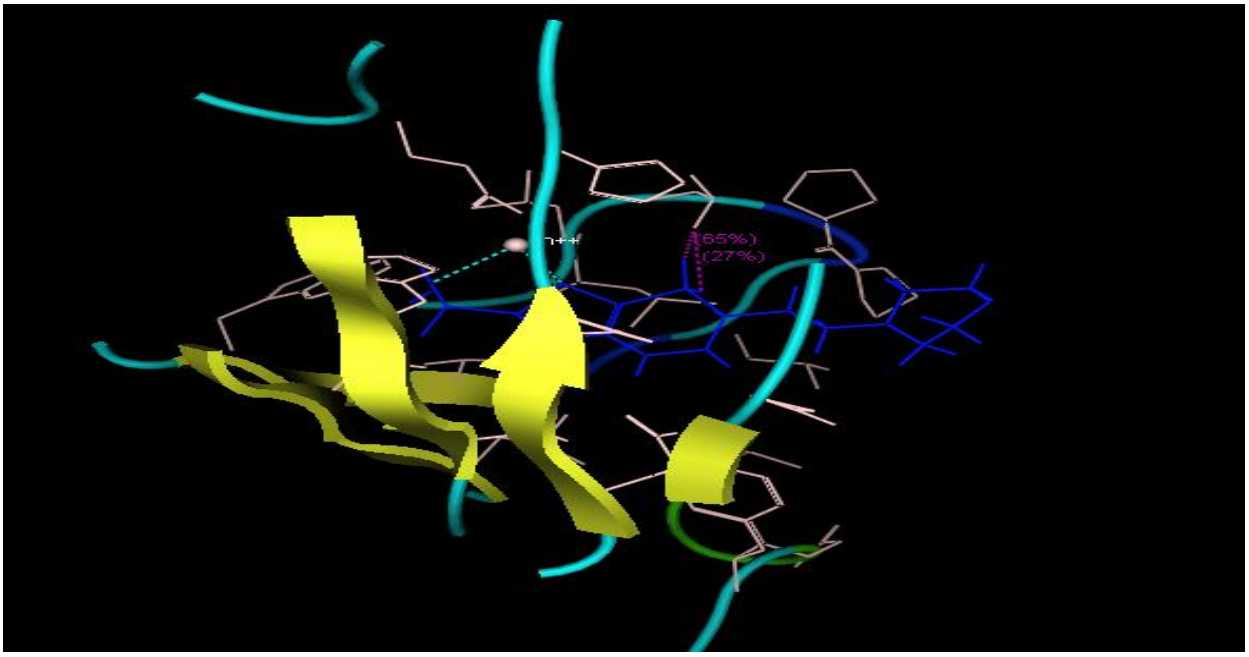

Figure 4.35. A 3D representation of ligand-protein interactions between CHEMBL79939 and CAII.

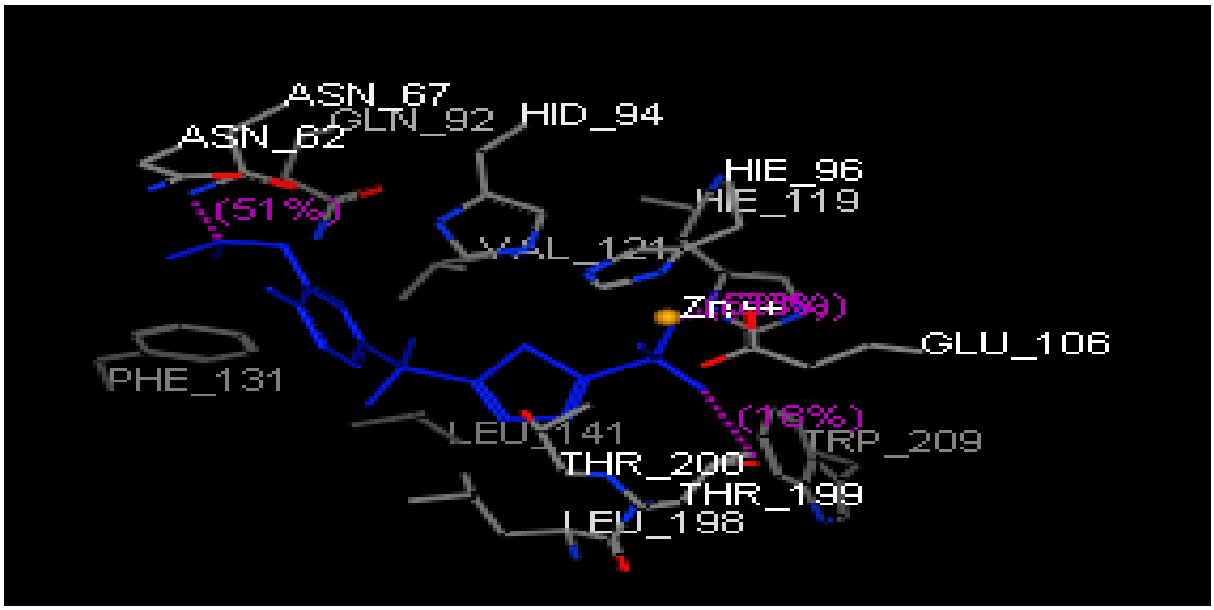

Figure 4.36. A 3D representation of ligand-protein interactions between CHEMBL69114 and CAII.

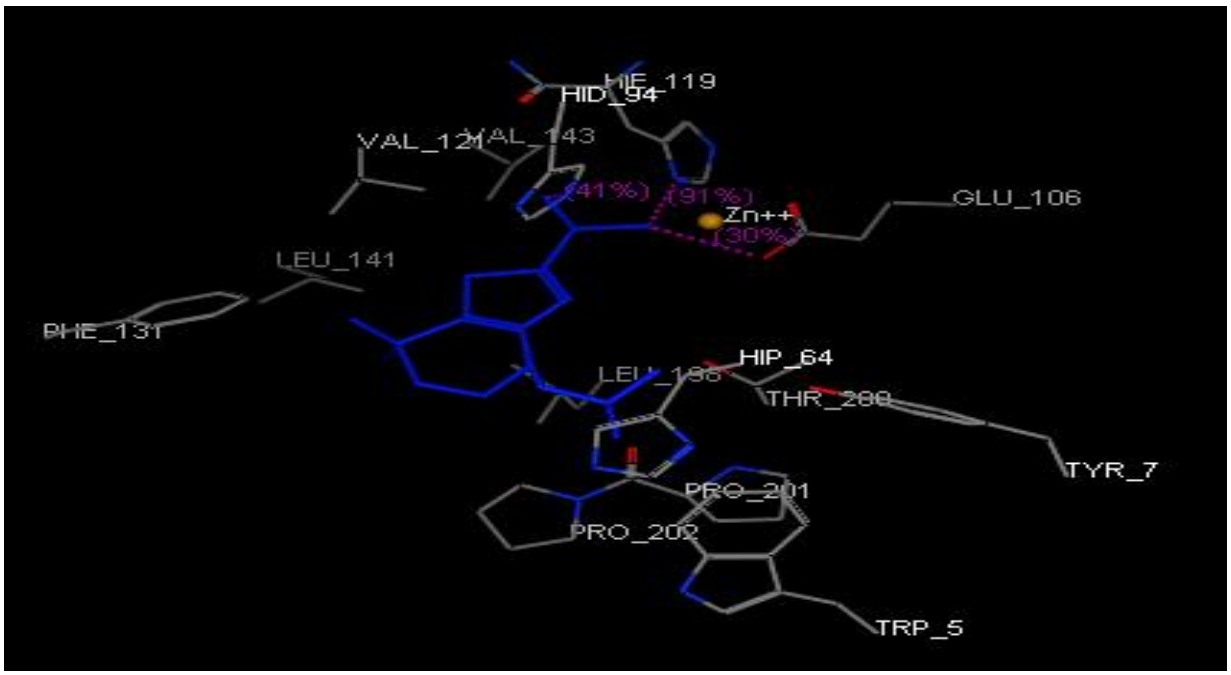

Figure 4.37. A 3D representation of ligand-protein interactions between CHEMBL417975 and CAII.

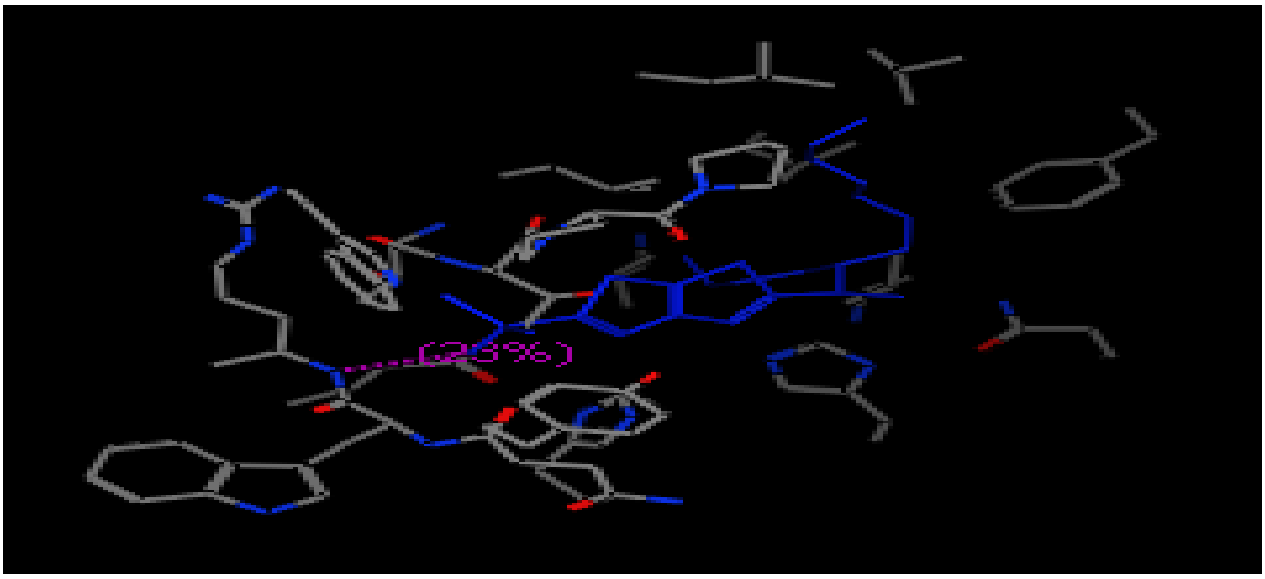

Figure 4.38. A 3D representation of ligand-protein interactions between CHEMBL543319 and CAII.

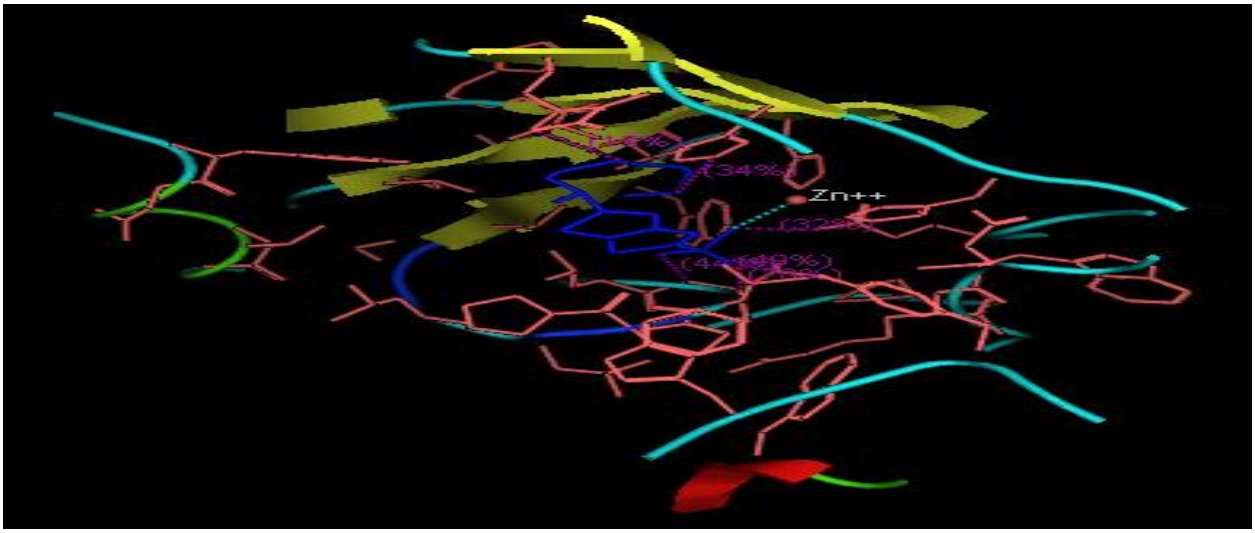

Figure 4.39. A 3D representation of ligand-protein interactions between CHEMBL CHEMBL553832 and CAII.

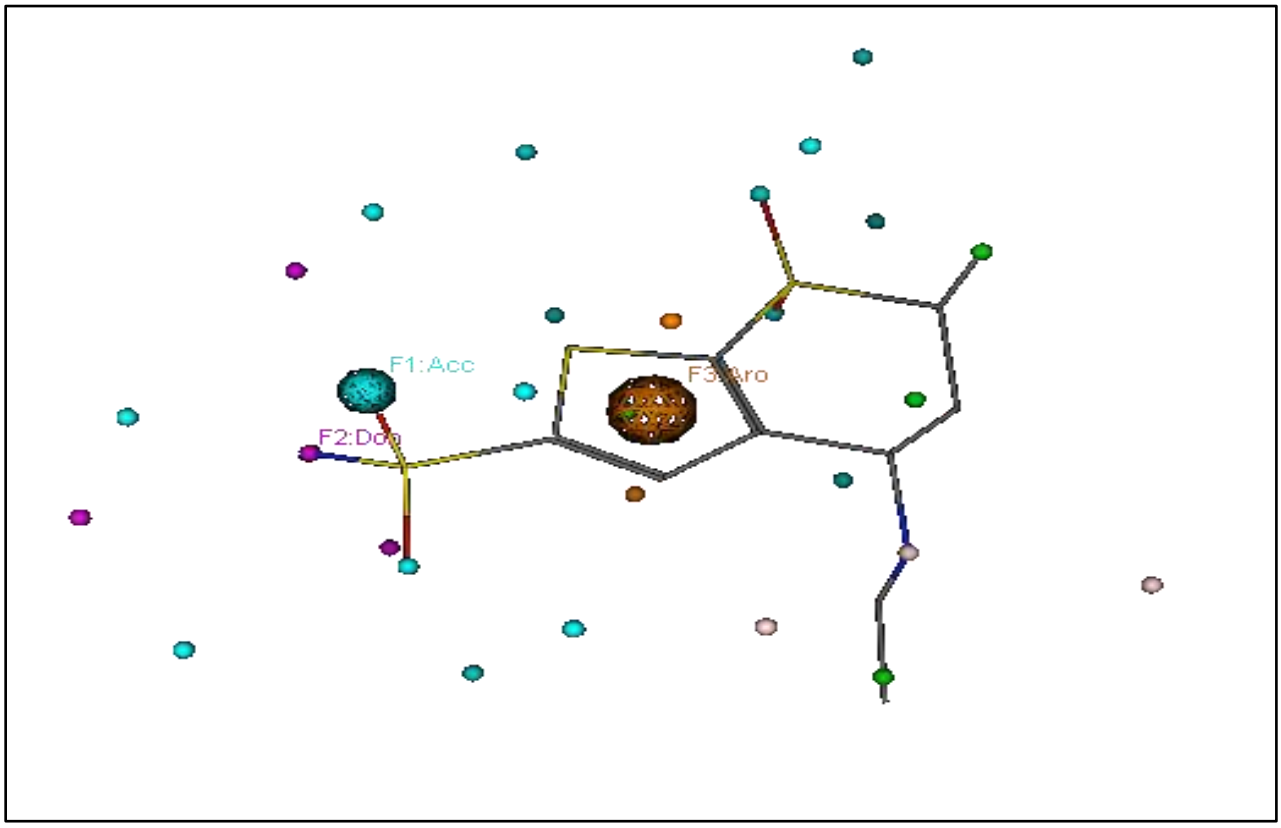

Figure 4.40. 3D pharmacophore model

F1 in blue represents the H-bond acceptor, F2 in pink represents H-bond donor and F3 in brown indicates the aromatic group.

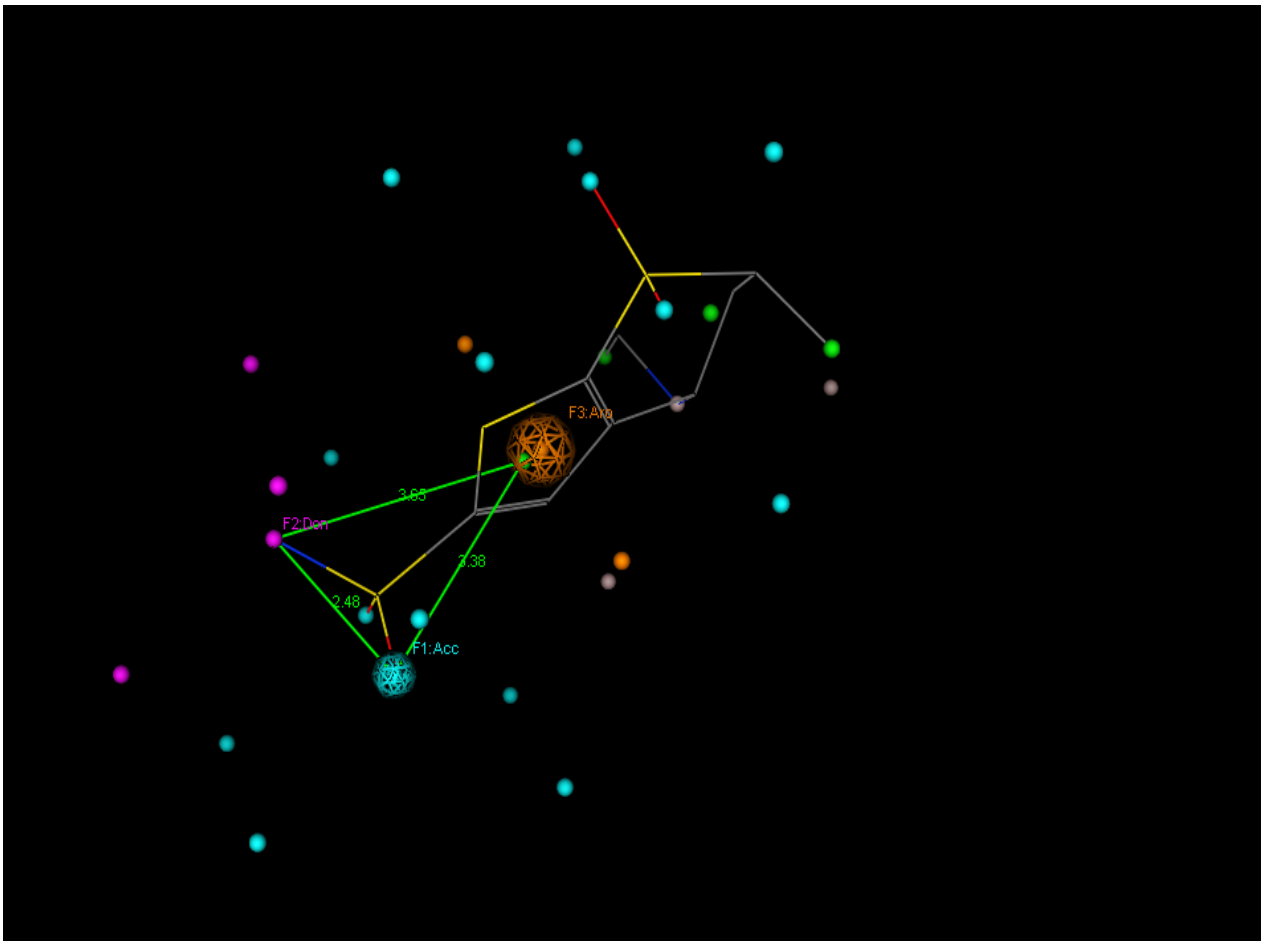

Figure 4.41. A visual representation of the distances computed between the pharmacophoric features of the pharmacophore model

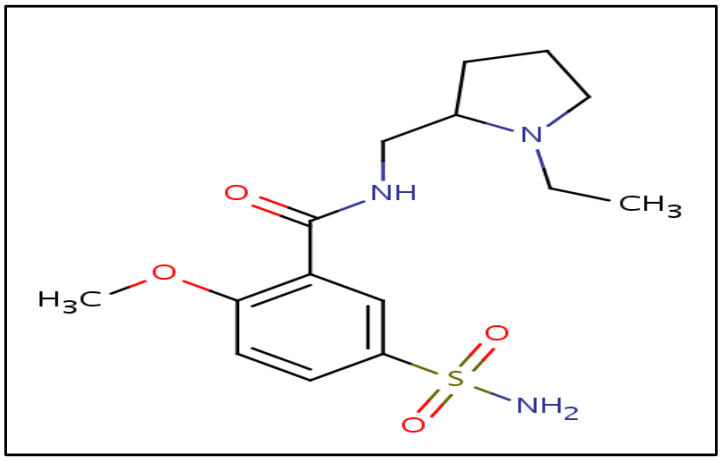

Figure 4.42 Chemical structure of Alfuzosin

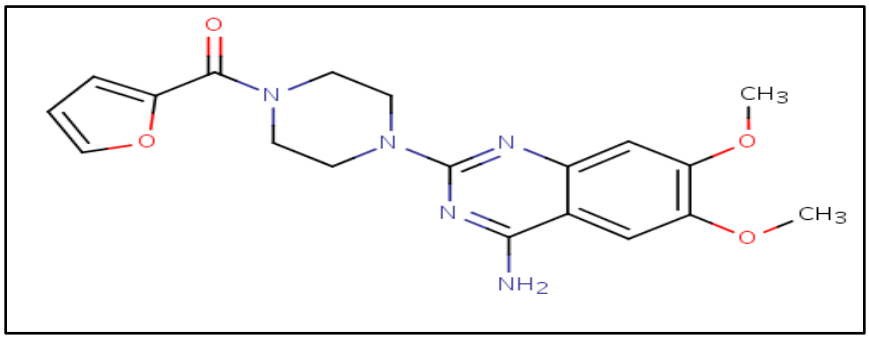

Figure 4.43 Chemical structure of Sulpiride

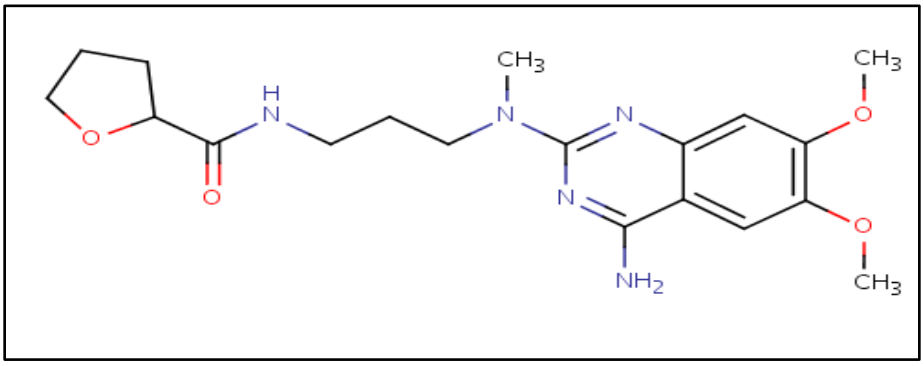

Figure 4.43 Chemical structure of Prazosin

Table 4.1 Protein concentrations in serum samples

Sample	Absorbance	Protein conc. ug/ul	Dilution factor	Actual conc.	Amount of sample loaded for 25 ug protein
R007	0.582	25.80	20.00	516.00	0.9689922
R09	0.629	28.93	20.00	578.67	0.8640553
R013	0.572	25.13	20.00	502.67	0.994695
R015	0.614	27.93	20.00	558.67	0.8949881
R016	0.510	21.00	20.00	420.00	1.1904762
C20	0.609	27.60	20.00	552.00	0.9057971
C21	0.556	24.07	20.00	481.33	1.0387812
C22	0.654	30.60	20.00	612.00	0.8169935
C23	0.604	27.27	20.00	545.33	0.9168704

Table 4.2 Paired Samples Statistics of CAII expression in RA patients and controls

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Individuals	1.50	24	.511	.104
	Normalised Expression	.3909	24	.16918	.03453

Table 4.3 Paired Samples Correlations of CAII expression in RA patients and controls

		N	Correlation	Sig.
Pair 1	Individuals & Normalised Expression	24	.720	.000

Table 4.4 Paired Samples Test for CAII expression in RA patients and controls

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Individuals - Normalised Expression	1.10909	.40626	.08293	.93754	1.28064	13.374	23	.000

Table 4.5 Paired Samples Statistics of Calpain expression in RA patients and controls

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Individuals	1.50	24	.511	.104
	Normalised Expression	.4292	24	.17928	.03660

Table 4.6 Paired Samples Correlations of Calpain expression in RA patients and controls

		N	Correlation	Sig.
Pair 1	Individuals & Normalised Expression	24	.617	.001

Table 4.7 Paired Samples Test for Calpain expression in RA patients and controls

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Individuals - Normalised Expression	1.07083	.42423	.08660	.89169	1.24997	12.366	23	.000

Table 4.8 Paired Samples Statistics of NFATc1 expression in RA patients and controls

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Individuals	1.50	24	.511	.104
	Normalised Expression	.4864	24	.26000	.05307

Table 4.9 Paired Samples Correlations of NFATc1 expression in RA patients and controls

		N	Correlation	Sig.
Pair 1	Individuals & Normalised Expression	24	.705	.000

Table 4.10 Paired Samples Test for NFATc1 expression in RA patients and controls

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Individuals - Normalised Expression	1.01360	.37590	.07673	.85487	1.17233	13.210	23	.000

Table 4.11 Paired Samples Statistics of TNF-α expression in RA patients and controls

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Individuals	1.50	24	.511	.104
	Normalised Expression	.3856	24	.12839	.02621

Table 4.12 Paired Samples Correlations of TNF-α expression in RA patients and controls

		N	Correlation	Sig.
Pair 1	Individuals & Normalised Expression	24	.478	.018

Table 4.13 Paired Samples Test for TNF-α expression in RA patients and controls

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Individuals - Normalised Expression	1.11442	.46325	.09456	.91881	1.31004	11.785	23	.000

Table 4.14 Descriptives for tail length obtained in comet assay for normal, RA and H2O2 treated cells.

Tail Length (um)								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Human Lymphocytes Normal Individuals	3	1.2833	.21385	.12347	.7521	1.8146	1.05	1.47
Human Lymphocytes H_2O_2 treated	3	11.3333	2.08167	1.20185	6.1622	16.5045	9.00	13.00
Human Lymphocytes RA Patients	3	1.2933	.17954	.10366	.8473	1.7393	1.09	1.43
Total	9	4.6367	5.13112	1.71037	.6925	8.5808	1.05	13.00

Table 4.15 ANOVA test for tail length obtained in comet assay for normal, RA and H2O2 treated cells.

Tail Length (um)					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	201.804	2	100.902	68.621	.000
Within Groups	8.823	6	1.470		
Total	210.627	8			

Table 4.16 Post Hoc Test: Multiple comparisons for tail length obtained in comet assay for normal, RA and H2O2

Tail Length (um)

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	10.05000*	.99010	.000	7.2156	12.8844
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	.01000	.99010	1.000	-2.8244	2.8444

*. The mean difference is significant at the 0.05 level.

Table 4.17 Descriptives for comet length obtained in comet assay for normal, RA and H2O2 treated cells.

Comet Length (um)								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Human Lymphocytes Normal Individuals	3	29.3333	2.08167	1.20185	24.1622	34.5045	27.00	31.00
Human Lymphocytes H2O2 treated	3	59.0000	13.85641	8.00000	24.5788	93.4212	43.00	67.00
Human Lymphocytes RA Patients	3	38.0000	7.54983	4.35890	19.2452	56.7548	30.00	45.00
Total	9	42.1111	15.42275	5.14092	30.2561	53.9661	27.00	67.00

Table 4.18. ANOVA test for comet length obtained in comet assay for normal, RA and H2O2 treated cells.

Comet Length (um)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1396.222	2	698.111	8.267	.019
Within Groups	506.667	6	84.444		
Total	1902.889	8			

Table 4.19 Post Hoc Test: Multiple comparisons for comet length obtained in comet assay for normal, RA and H2O2

Comet Length (um)

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	29.66667*	7.50309	.013	8.1872	51.1461
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	8.66667	7.50309	.453	-12.8128	30.1461

*. The mean difference is significant at the 0.05 level.

Table 4.20 Descriptives for comet area obtained in comet assay for normal, RA and H2O2 groups

Comet Area (um2)								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Human Lymphocytes Normal Individuals	3	8.7933E2	42.85246	24.74088	772.8819	985.7847	844.00	927.00
Human Lymphocytes H2O2 treated	3	2.5493E3	1253.71860	7.23835E2	-565.0763	5663.7430	1204.00	3685.00
Human Lymphocytes RA Patients	3	8.2300E2	213.56966	1.23305E2	292.4636	1353.5364	601.00	1027.00
Total	9	1.4172E3	1061.29741	3.53766E2	601.4368	2233.0076	601.00	3685.00

Table 4.21 ANOVA test for comet area obtained in comet assay for normal, RA and H2O2 treated cells.

Comet Area (um2)

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	5772300.222	2	2886150.111	5.347	.046
Within Groups	3238517.333	6	539752.889		
Total	9010817.556	8			

Table 4.22 Post Hoc Test: Multiple comparisons for comet area obtained in comet assay for normal, RA and H2O2

Comet Area (um2)

Dunnett t (2-sided)

		Mean			95% Confidence Interval	
(I) Groups	(J) Groups	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	1670.00000	5.99863E2	.055	-47.2572	3387.2572

Comet Area (um2)

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	1670.00000	5.99863E2	.055	-47.2572	3387.2572
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	-56.33333	5.99863E2	.994	-1773.5905	1660.9239

Table 4.23 Descriptives for percentage DNA in tail obtained in comet assay for normal, RA and H2O2 groups

%DNA in Tail

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Human Lymphocytes Normal Individuals	3	.0032	.00058	.00033	.0017	.0046	.00	.00
Human Lymphocytes H2O2 treated	3	11.1860	18.84308	10.87906	-35.6228	57.9948	.00	32.94
Human Lymphocytes RA Patients	3	.0099	.00857	.00495	-.0113	.0312	.00	.02
Total	9	3.7330	10.95492	3.65164	-4.6877	12.1537	.00	32.94

Table 4.24 ANOVA test for percentage DNA in tail obtained in comet assay for normal, RA and H2O2 treated cells.

%DNA in Tail					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	249.959	2	124.980	1.056	.405
Within Groups	710.124	6	118.354		
Total	960.083	8			

Table 4.25 Post Hoc Test: Multiple comparisons for percentage DNA in tail obtained in comet assay for normal, RA and H2O2

%DNA in Tail

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	11.18282	8.88271	.401	-14.2462	36.6118
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	.00678	8.88271	1.000	-25.4222	25.4358

Table 4.26 Descriptives for percentage DNA in head obtained in comet assay for normal, RA and H2O2 groups

% DNA in Head

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Human Lymphocytes Normal Individuals	3	95.6635	3.78619	2.18596	86.2581	105.0689	93.00	100.00
Human Lymphocytes H2O2 treated	3	70.1474	8.72639	5.03818	48.4698	91.8249	63.39	80.00
Human Lymphocytes RA Patients	3	92.4977	8.94620	5.16509	70.2741	114.7213	82.92	100.64
Total	9	86.1028	13.70069	4.56690	75.5716	96.6341	63.39	100.64

Table 4.27 ANOVA test for percentage DNA in head obtained in comet assay for normal, RA and H2O2 treated cells.

% DNA in Head					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1160.633	2	580.316	10.210	.012
Within Groups	341.039	6	56.840		
Total	1501.672	8			

Table 4.28 Post Hoc Test: Multiple comparisons for percentage DNA in head obtained in comet assay for normal, RA and H2O2

% DNA in Head

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	-25.51615*	6.15575	.011	-43.1385	-7.8938
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	-3.16585	6.15575	.832	-20.7882	14.4565

*. The mean difference is significant at the 0.05 level.

Table 4.29 Descriptives for olive moment obtained in comet assay for normal, RA and H2O2 groups

Olive Moment								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Human Lymphocytes Normal Individuals	3	.4459	.11339	.06546	.1642	.7275	.32	.54
Human Lymphocytes H2O2 treated	3	2.1314	.20853	.12040	1.6134	2.6494	1.95	2.36
Human Lymphocytes RA Patients	3	.4333	.14503	.08373	.0731	.7936	.29	.58
Total	9	1.0035	.85727	.28576	.3446	1.6625	.29	2.36

Table 4.30 ANOVA test for Olive moment obtained in comet assay for normal, RA and H2O2 treated cells.

Olive Moment					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	5.725	2	2.862	110.977	.000
Within Groups	.155	6	.026		
Total	5.879	8			

Table 4.31 Post Hoc Test: Multiple comparisons for percentage DNA in head obtained in comet assay for normal, RA and H2O2

Olive Moment

Dunnett t (2-sided)

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Human Lymphocytes H2O2 treated	Human Lymphocytes Normal Individuals	1.68552*	.13113	.000	1.3101	2.0609
Human Lymphocytes RA Patients	Human Lymphocytes Normal Individuals	-.01254	.13113	.993	-.3879	.3628

Table 4.32 List of six most active compounds against CAII (IC50 < 1 nM)

chEMBL ID	IC50 value	Canonical smiles
CHEMBL218490	0.18	s1c2S(=O)(=O)[C@H](C[C@@H](NCC)c2cc1S(=O)(=O)N)C
CHEMBL79939	0.58	s1c2S(=O)(=O)C(CNc2cc1S(=O)(=O)N)CCN1CCOCC1
CHEMBL69114	0.7	s1c(S(=O)(=O)c2cc(CN(C)C)c(O)cc2)ccc1S(=O)(=O)N
CHEMBL417975	0.54	s1c2S(=O)(=O)CC[C@@H](NCC(C)C)c2cc1S(=O)(=O)N
CHEMBL543319	0.5	s1c2sc(S(=O)(=O)N)cc2cc1C(N(CCOCCOC)CCOC)C
CHEMBL553832	0.7	s1c2sc(S(=O)(=O)N)cc2cc1C(NCCOCCOC)C

Table 4.33 Distances between pharmacophoric features

Features	H-Bond donor	H-bond acceptor	Aromatic
H-Bond donor	0		
H-bond acceptor	2.48	0	
Aromatic	3.65	3.38	0

Table 4.34 List of descriptors and their ranges observed in known 'actives'

Descriptors	Values
TPSA	90-118
log P(o/w)	1.05-1.27
Weight	324-422
a_acc	5-6
a_don	1-2
b_rotN	3-12

Table 4.35. List of identified hits along with their descriptor values

Compounds	DrugBank ID	log P(o/w)	Weight	a_acc	a_don	b_rotN	TPSA
Alfuzosin	DB00346	0.85	389	6	2	9	111
Sulpiride	DB00391	0.5	341	5	2	7	101
Prazosin	DB00457	0.18	383	5	1	5	106

Discussion

Rheumatoid arthritis affects around 1% of the entire adult population worldwide. Chronic inflammation of joints, erosion of articular cartilage and autoantibody as an autoimmune reaction are the key features associated with RA (McInnes and Schett, 2010). Furthermore,increased osteoclastogenesis is a major characteristic of RA. According to our investigation, the rate of occurrence of RA is much higher in females as compared to males. Out of a total of 30 RA samples collected, 25 were females whereas only five were male. Previous epidemiological studies in other populations of the world showeding a 2 to 4 times higher prevalence of RA in females also corroborate our findings. Likewise, the incidence of RA in females younger than 50 is 4 to 5 times higher as compared to males (Kvien *et al.*, 2006; Slimani and Ladjouze-Rezig, 2014). This gender bias in the prevelance of RA can be related to the sex hormones in males and females as these are implicated in the immune response. Estrogens are enhancers at least of the humoral immunity whereas androgens and progesterone are natural immune-suppressors. In male patients of rheumatoid arthritis (RA), androgen replacement ameliorates the disease and supports their involvement in the pathophysiology of the disease. The combination of androgens with cyclosporin A or methotrexate has been revealed to potentiate the apoptosis of monocytic inflammatory cells and reduce the cell growth at least in vitro. A significanteffort is devoted in the recent years to ensure whether the use of oral contraceptive pills (OCs) may have a protective effect on the risk of RA. Contradictory results arose from those controlled studies. Still nowadays no consensus has been reached regarding OCs administration and its relationship to

the prevention or development of RA. In addition, an association of estrogen receptor gene polymorphism with age at onset of RA was observed to explain inter-individual clinical and therapeutic-response variations more clearly. Local increased estrogen concentrations and decreased androgen levels wererevealed in RA synovial fluids and play a more vitalfunction in the immuneinflammatory local response (Cutolo *et al.,* 2002).

RANKL signaling pathway is the main pathway involved in bone biology. Most of the components of RANKL signaling pathway have been showedn to have elevated levels in RA patients. Carbonic anhyhyrase II responsible for the release of biocarbonate ions and the drastic acidification of bone environment leading to bone loss in RA is also activated downstream RANKL signaling pathway Takayanagi *et al.,* 2002). According to our investigation, levels of CAII have been demonstrated to be augmented in 27 out of 30 RA patients. Our results point to the significant role of CAII in RA. Previous studies to unravel the role of CAII in bone resorption also corroborate our findings .Disruption in CAII gene in vivo results in no bone resorption thereby implicating its critical role in optimal bone resorption process (Hall *et al.,* 1991). Likewise, studies related to the effect of a specific CAII inhibitor acetazolamide in mouse calvaria cultures demonstrate anti-resorptive activity in a dose dependent, Ca^{2+} dependent pH-regulated manner (Kato *et al.,* 2013). Similar to acetazolamide, celecoxib and JTE-522 also block osteoclast differentiation and activity which were tested in arthritic rat model for RA and showeded reduced bone resorption. Hence elevated levels of CAII in Rheumatoid arthritis can also be strongly related to the increased bone destruction and hence the disease progression.

It has been well-established that carbonic anhydrase leads to cerebral calcification. Furthermore, increased levels of CA2 also lead to renal acidosis. Thus from the results of our investigation, it is evident that CA2 leads to increased calcium levels and acidification in RA. Augmented

calcium levels can affect the body in a multitude of ways, one of them being changing the regulation of molecular components. Since, calpain, a cysteine protease, which is dependent on calcium levels for activation and is also activated downstream RANKL signaling pathway, has also showedn to have high levels in RA patients as compared to those in healthy controls. Our results point to the possibility that increased calcium levels as a result of increased CAII lead to increased activation of calpain.

Carbonic anhydrase 2 and calpain has been reported as an inflammatory marker and thus their elevated levels in RA point to increased inflammatory response in RA patients. There has been accumulating evidence suggesting that inflammatory response in cell leads to DNA damage in the cells. Our investigation also showeds that lymphocytes of all the RA patients exhibit detectable DNA damage. This is supported by the previous research that pro-inflammatory cytokines like TNF-α, IL-1, IL-6, and IFN-γ, which are also activated downstream RANKL signaling pathway have majorly elevated levels in RA (Sukkar and Rossi, 2004). In addition to the components of immune system, reactive oxygen and nitrogen species have also been observed in RA. Neutrophils, the most important cells present in the synovial fluid produce ROS, most specifically superoxide anion (Filippin*et al.*, 2008; Babior, 2000; Taysi*et al.*, 2002). Because of the increased oxidative stress, RA synovial fluid contains increased levels of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and contains low levels of iron, which is elevated in patients with RA. This may cause increased activity of Fenton and Haber-Weiss reactions, and oxidative damage (Johnson, 2001).Owing to their abnormally high concentrations, ROS damage lipids, proteins, membranes, and nucleic acids and also work to recruit nuclear factor kappa-B (NF-κB) which again is a momentous component of RANKL signaling pathway playing a role in

osteoclastogenesis. Oxidative stress produced within an inflammatory joint can stimulate the erosion of connective tissue in rheumatoid synovitis and other autoimmune systems (Kurien and Scofield, 2008). Hence, all these previous findings strengthen and justify the DNA damage observed in the current study.

Previous findings which corroborate the results of our current investigation implicate the potential of CAII as a target for anti-resorptive therapy in Rheumatoid arthritis. For this, various inhibitors for carbonic anhydrases already exist as approved drugs and some others being investigated in clinical trials. CAs have been targeted in glaucoma, epilepsy, obesity and various tumors. However, these drugs exhibit off-target effects and hence the side effects. So, it is inevitable to look for isoform-specific inhibitors and unravel new compounds which can be used for the treatment of RA by targeting CAII.

Consequently, the drug design of CA inhibitors (CAIs) is emerging as a very dynamic field.Sulfonamides and their isosteres (sulfamates/sulfamides) make up the main class of CAIs which showed binding to the Zinc metal ion in the CAII active site. Lately, dithiocarbamates exhibiting the same mechanistic approach, were categorized as a new CAI class. Other CAIs demonstrate a peculiar mechanism of action: some carboxylates, sulfocoumarins, phenols, polyamines and some carboxylates, bind to the zinc-bound water molecule. Coumarins and five/six-membered lactones are prodrug inhibitors, binding in hydrolyzed form at the entrance of the active site cleft. Novel drug design strategies have been showedn principally based on the tail approach for obtaining all these types of CAIs, which exploit more external binding regions within the enzyme active site (in addition to coordination to the metal ion), leading thus to isoform-selective compounds.

Both sulfonamides and sulfamates bind in deprotonated form, as anions, to the Zn(II) ion from the enzyme active site, which is in a tetrahedral geometry .This supports the results of our *in silico* prediction of ligand-protein interaction of six highly potent inhibitors of CAII. Furthermore, our results indicate the interaction of His119 with the Zinc metal ion in the active site cleft. This is corroborated by the findings which showed that His119 is one of the three conserved His residues ((His94, 96 and 119) which are considered as the Zinc ligands. Furthermore, Glu106 has also been found to showed interactions with the target. This is also supported by previous findings which suggest that Glu106 is one of the gate keepers (hydrogen bonded to Thr199, as well as half the active site mainly lined with hydrophobic residues and the opposite one with hydrophilic residues), the other being Thr199 (Supuran, 2012).

The three new compounds identified as potential hits include Alfuzosin, Prazosin and Sulpiride. Sulpiride belongs to a class of benzenesulphonamides and Alfuzosin and Prazosin are quinazolinamines.

Compounds belonging to the class of benzenesulphonamides (organic compounds containing a sulfonamide group that is S-linked to a benzene ring) have already been reported to have a potent inhibitory effect on the CAII (Di Fiore*et al.,* 2011). Sulpiride is known as a dopamine D2-receptor antagonist. It has been used therapeutically as an antidepressant, antipsychotic, and as a digestive aid (Cavallotti *et al.,* 2002). But its role has never been previously reported as a CA2 inhibitor or as an anti-resorptive therapy for the treatment of RA. From crystallographic aspects, benzenesulfonamide derivatives are the best studied class of CAIs. The comparative analysis of the hCA II—benzenesulfonamide inhibitor complexes, structurally characterized till now, suggest that the different tails can adopt various orientations within the binding pocket. Infact, some inhibitors bind with their tail within the hydrophobic

region of the CA II active site, some others bind with their tail in the hydrophilic half and some others yet in an intermediate position at the border between the hydrophobic and hydrophilic region. In general, the orientation adopted by the inhibitor tail depends on the nature of either the tail (hydrophobic/ hydrophilic/aromatic etc.) or the linker moiety. Therefore, the rationalization of the binding mode within the enzyme active site is difficult to be done except for some classes of compounds for which extensive structural data are available (De Simone *et al.*, 2013). Hence, in order to consider sulpiride as a new potential lead compound, its binding pattern by X-ray crystallography needs to be elucidated and further pharmacological research in the realm is required.

Alfuzosin and Prazosin belong to the class of quinazolinamines. These are heterocyclic aromatic compounds containing a quianazoline moiety substituted by one or more amine groups. Alfuzosin is a quinazoline-derivative alpha-adrenergic blocking agent used to treat hypertension and benign prostatic hyperplasia. Accordingly, Alfuzosin and Prazosin are a selective inhibitor of the alpha (1) subtype of alpha adrenergic receptors. In the human prostate, Alfuzosin antagonizes phenylephrine (alpha (1) agonist)-induced contractions, in vitro, and binds with high affinity to the alpha1a adrenoceptor, which is thought to be the predominant functional type in the prostate. The antihypertensive effects of Alfuzosin and Prazosin results from a decrease in systemic vascular resistance and the parent compound Alfuzosin is primarily responsible for the antihypertensive activity. Prazosin has also been used in conjunction with cardiac glycosides and diuretics in the management of severe congestive heart failure. It has also been used alone or in combination with β-blockers in the preoperative management of signs and symptoms of pheochromocytoma. Both of these drugs have not been previously reported as a carbonic anhydrase II inhibitors and as candidate leads for the treatment of RA.

Almost all reported carbonic anhydrase inhibitors comprise a zinc binding group in their structure of which the primary sulfonamide moiety (-SO2NH2) is the foremost example and to a lesser extent the primary sulfamate (-O-SO2NH2) and sulfamide (-NH-SO2NH2) groups. Current research findings have therefore elucidated a novel class of compounds which can be further studies in order to test various chemotypes in order to get CAII isoform selective inhibitors and can be tested for their efficacy in the treatment of Rheumatoid arthritis

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Conclusion

Rheumatoid arthritis (RA), affecting about 1% of the world population, is a chronic polyarthritic autoimmune condition characterized by colossal joint destruction as a consequence of synovial hyperplasia. Osteoclastogenesis is the major process associated with RA. In this study, protein expression profiles of carbonic anhydrase II, calpain, TNFα and NFATc1 were investigated in Rheumatoid arthritis. All these proteins, because of their role in bone resorption exhibited elevated levels in RA patients. Furthermore, since they are known as inflammatory markers and inflammation is tightly associated with DNA damage, RA samples were also investigated for any DNA damage. All the cells in RA patients showeded DNA damage. Hence, we conclude that all these proteins have significant roles in the pathogenesis of RA. Moreover, three new inhibitors were elucidated for targeting CAII. One, Sulpiride, belongs to a class of benzenesulfonamides. Compounds belonging to this class have been extensively studied against CAII but not with the aim to develop an anti-resorptive therapy for RA. Rest of the two newly identified drugs, Alfuzosin and Prazosin, belong to a class of quinazolinamines which has never been previously proposed to target CAII for any kind of treatment.

Future Perspectives

Protein expression profiles of all the aforementioned osteoclastogenic markers specifically calpain and carbonic anhydrase II can be used as a potential prognostic and diagnostic molecular biomarker for rheumatoid arthritis. Furthermore, the three drugs can be tested for their newly identified role in the inhibition of carbonic anhydrase II and their anti-resorptive effect can be studied. Not only these compounds can be improved by applying lead optimization strategies, but their derivatives can also be tested for better isoform-selectivity and ADMET properties.

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