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Anti-Rheumatic Activity of Celecoxib and Methotrexate in Collagen Induced Arthritis: A Proteomic Approach

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Abstract

Rheumatoid Arthritis (RA) is a chronic inflammatory disorder of the synovial membrane that results in the destruction of bone and cartilage in affected joints. In order to identify novel disease-related proteins and candidate biomarkers, we analyzed the changes in the serum proteome profiles of rats with RA that were treated with a COX-2 inhibitor, celecoxib and a DMARD, methotrexate. Serum samples were collected from the RA rats before and after the drug treatment. Following immunodepletion of major proteins, the proteins were digested. The proteins were identified using MALDI-TOF mass spectrometry. Several proteins are identified and the proteins that play a key role in RA are studied. These proteins are inhibited differentially by celecoxib and methotrexate. Although some of the proteins are known to be related to RA, several are currently unknown with respect to their relationship to RA and may be involved in the development of this disease. Our results may contribute to the identification of novel disease related proteins and enhance the understanding of the pathogenesis of RA.

Introduction

Rheumatoid Arthritis (RA) is an inflammatory condition with multi-organ involvement. It is a relatively common condition and about 0.5% to 1% of the population is effected [1]. Evidence suggests that it becomes more prevalent correlating with increasing latitude and when it is compared between rural to urban populations [2]. RA is destructive and it affects the small joints predominantly. Increased prevalence of atherosclerotic disease and reduced participation in society are observed in RA patients [3]. Reduced work capacity as a result of RA puts an indirect cost to society and has been estimated to be as high as € 41.6 billion in the United States and € 45.3 billion in Europe [4].

It was initially thought that RA was infection driven that led to the use of gold and sulpha drugs with limited success. As research progressed in the treatment of RA, use of steroids as therapeutic agents gained popularity among physicians [5]. As our understanding of the disease has improved, we have moved on to more targeted therapies. The onset of RA is thought to be dependent on both environmental and genetic factors. Of these, the major modifiable environmental risk factor identified is that of smoking [6]. Therapies currently employed to combat RA include traditional treatments such as glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatic drugs (DMARDs), classified broadly as synthetic (encompassing the traditional DMARDs and newer therapies such as JAK-2 inhibitors) and biological drugs.

Marked symptomatic relief was seen with the introduction of NSAIDs. Though not a DMARD, its beneficial effects have been long documented with Willow and poplar bark, the substrates for salicylic acid, commonly used in the treatment of inflammatory arthopathies [7]. Aspirin was in use by the early twentieth century in the treatment of RA, making it the oldest of the medications currently in use [8]. We now have a vast selection of NSAIDs available, all working to inhibit the cyclo-oxygenase pathway. Prostaglandins are made by two different enzymes, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The prostaglandins made by the two different enzymes have slightly different effects on the body. COX-2 inhibitors are NSAIDs that selectively block the COX-2 enzyme and not the COX-1 enzyme. Blocking this enzyme impedes the production of prostaglandins by the COX-2 which causes the pain, swelling and other painful conditions. Because they selectively block the COX-2 enzyme and not the COX-1 enzyme, these drugs are uniquely different from traditional NSAIDs which usually block both COX-1 and COX-2 enzymes. Although NSAIDs are effective analgesics, they do not have any demonstrable effect on disease course and further they have side effects that include gastric irritation and nephrotoxicity [9].

The term DMARD (Disease modifying anti-rheumatic drug) is applied to medications which can alter the course of disease and thus prevent joint erosion [10]. The mechanisms through which DMARDs act are varied but a collective outcome is to help stem the destructive process of intertwined inflammatory cascades resulting in the degradation of soft tissue, cartilage and bone.

The advent of proteomics technologies has enabled large-scale analysis of proteins to identify biomarkers that delineate disease subtypes of rheumatoid arthritis, and to gain insights into the mechanisms underlying these subtypes. The aim of this study is to identify proteins that are differentially expressed between control and arthritis sera and to further look at the protein profiles of the COX-2 inhibitor, celecoxib vs DMARD, methotrexate, and treated sera profiles by proteomics approach. The aim of this study is to figure out differences at the protein level for the above two drugs since celecoxib is known for its anti-inflammatory properties in arthritis and methotrexate is known to target the cytokines.

Materials and Methods

Unless otherwise noted, all chemicals were purchased from Sigma (Sigma Chemicals, St. Louis, MO) and were of analytical grade. Trypsin is purchased from Promega (sequencing grade modified trypsin, Catalog No. V5111, Mannheim, Germany).

Animals

Male Lewis rats (150-180 gm) were obtained from Harlan Sprague Dawley (Indianapolis, IN), housed in micro isolator cages (Lab Products, Maywood, NJ), and supplied with sterilized standard rodent chow diet and acidified water (pH 2.8-3.0). The animals were handled under clean conditions and were acclimated to the new environment for a week before use.

Preparation of type II collagen

Native type II collagen was extracted from bovine articular cartilage, after digestion with papain. The extraction and purification procedures for collagen have been described previously [11]. The purified preparation was stored in O.05M acetic acid at -20°C.

Induction of arthritis

The mean body weight and mean hind paw volume of rats in each group were very similar at the start of every experiment. Type II collagen arthritis was induced by an intradermal injection, at the base of the tail, of an emulsion of 100 pg bovine type II collagen in 0.05M acetic acid with an equivalent volume of incomplete Freund's adjuvant (ICFA).

Intravenous treatment with the antigens

Type II collagen (stock solution in O.05M acetic acid) was adjusted to pH 7, diluted with phosphate buffered saline (PBS), and stirred vigorously at 0°C to avoid any possible clumping. The collagen solution (0.5 mg) was injected intravenously into the tail vein of each rat, without the use of any anesthetic and with precautions to minimize trauma. The denatured type II collagen solution was prepared by heating native type II collagen at 60°C for 30 minutes. Some animals received M tuberculosis (2 mg in PBS) intravenously in the same fashion. These treatments were given to rats either 3 days prior to or 7 or 10 days after the immunization for induction of arthritis.

Evaluation of inflammatory response

The development and severity of arthritis were mainly assessed by the quantitation of hind paw swelling of the rats. Both hind paws of each rat were dipped in a mercury bath and the volume of displacement of mercury was measured twice a week [12,13]. Along with the swelling of hind paws, visual disfigurement, restricted movement, and radiologic evaluation of the joints were taken into consideration to determine the degree of arthritis.

Administration of celecoxib and methotrexate to arthritic rats

Seven rats with collagen induced arthritis were given 10 mg/kg per day of celecoxib for two weeks from the onset of arthritis. Seven rats with collagen induced arthritis were given 10 mg/kg per day of methotrexate for two weeks from the onset of arthritis. Seven rats with collagen induced arthritis served as normal controls and are not given either of these drugs.

Serum protein solubilization and sample application

Serum collected from normal rats, arthritic rats, and arthritic rats treated with celecoxib and arthritic rats treated with methotrexate, were processed as follows. The serum sample is passed through albumin/globulin removal columns (commercially supplied by Amersham). The eluate from the column is collected and the protein amount is determined. The protein is suitably solubilized in the 2-d gel buffers. To each 4-7 IPG strip, 300 µg protein is added and the strips are allowed to rehydrate overnight along with the sample.

2-d Gel electrophoresis

The IPG strips are rehydrated with the sample overnight and electrophoresis is carried out in the first dimension the following day. The strips are then placed on a regular SDS-PAGE gel and the electrophoresis is carried out in the second dimension. The gels are then silver stained.

2-d Gel analysis

After 2-D gels are stained, the protein patterns are digitized and analyzed across multiple gels (Control rat sera, arthritis rat sera, celecoxib treated rat sera and methotrexate treated rat sera) by Computer-assisted image analysis which is an indispensable tool for the evaluation of complex 2-D gels. PDQuest™ 2-D analysis software from Biorad is used to pick the spots and compare the spots of interest across the gels.

Spot picking, protein digestion and MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) analysis

Using the Ettan spot handling workstation (Amersham Biosciences) selected spots were automatically cut from the gels, destained and enzymatically digested with trypsin. The tryptic peptides were then spotted onto a MALDI target plate. The MALDI target plates were loaded in a Micromass M@ldi MALDI-TOF mass spectrometer (Waters, Milford, USA) for analysis of the peptide masses.

Database search

Peptide masses retrieved from MALDI-TOF analysis spectra were submitted to a database (Mascot) for protein identification.

Results and Discussion

The rats receiving an intradermal injection of native type II collagen began to exhibit swelling of the hind paws in approximately 15-17 days, with maximum swelling occurring at days 21-25. Thereafter, the inflammation subsided slightly and remained constant until day 30. Then the hind paws were measured. Each treatment reduced inflammation, but both celecoxib and methotrexate prevented bone loss adjacent to inflamed joints and significantly decreased bone resorption. In contrast, no treatment affected bone formation parameters.

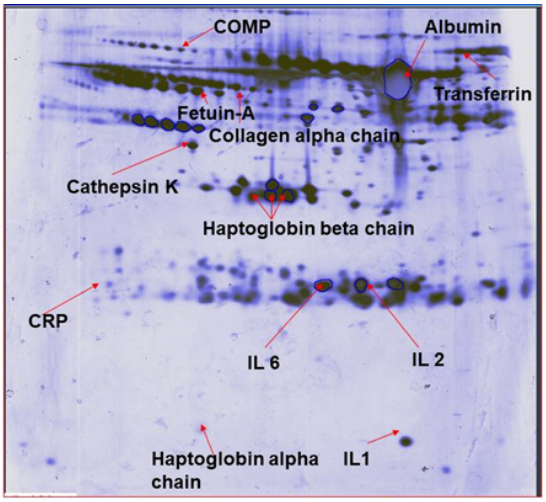
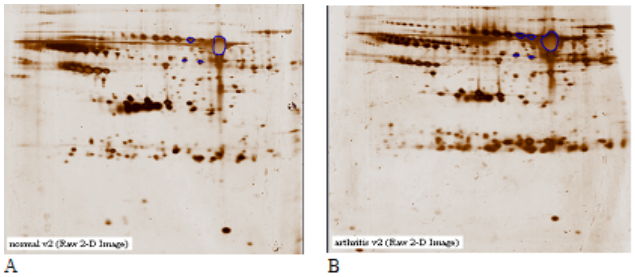


Figure 1: Proteins from rat serum that are of significance in arthritic condition are labeled on the 2-d gel.

Some important proteins of interest in arthritis are labelled on the 2-d gel picture (Figure 1). The analysis of serum protein profiles from 2-d gels of normal and arthritic rats clearly shows that several proteins are up-regulated and several proteins are down regulated in arthritic animals compared to normal animals (Figure 2, Tables 1 and 2). A pie chart (Figure 3) shows the protein pattern observed in this study and their distribution. We can see that some proteins are differentially expressed between control and arthritis and some proteins are common between these conditions. Both Celecoxib and Methotrexate are found to be effective in inhibiting some of the proteins that are responsible for arthritis and are over expressed in arthritic condition. The 2-d gel serum protein patterns of celecoxib treated and methotrexate treated rats are shown along with normal and arthritis serum profiles (Figure 4).



A B

Figure 2: 2-d gel electrophoresis protein profiles between A) Normal and B) Arthritis rat sera. Proteins separated on 4-7 IPG strips.

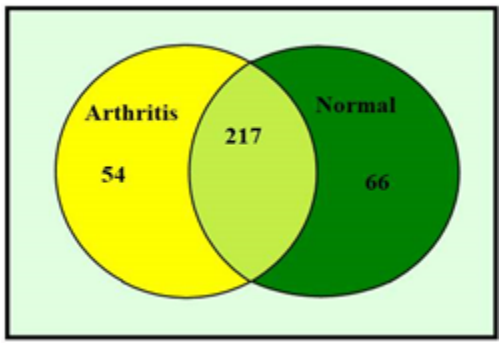


Figure 3: Image Analysis of some proteins analyzed after 2-d gel electrophoresis. Image shows proteins that are differentially expressed between normal and arthritic conditions and proteins that are common in normal and arthritic sera.

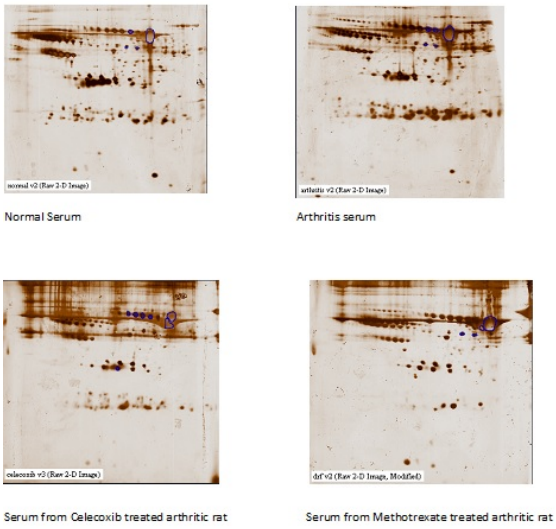


Figure 4. 2-d gel showing the effects of Celecoxib and Methotrexate on arthritic protein profiles.

SPOT NO	SWISS PROT ID	PROTEIN IDENTIFIED	MW
2304	P97776	A disintegrin and metalloproteinase domain 18	48199
7901	Q91ZY8	SNAP-25-interacting RING finger protein	79206

7405	Q9Z1A5	Amyloid protein-binding protein 1	60382
7610	P55063	Heat shock protein 1A	70549
5904	P41738	Aryl hydrocarbon receptor [Precursor]	96226
5201	P36506	MAP kinase kinase 2	44281
6911	Q63788	Phosphatidylinositol 3-kinase regulatory beta subunit	85000
7704	P28573	Sodium-dependent proline transporter	71090
4307	Q63639	Retinaldehyde-specific dehydrogenase type 2	54739
6909	P55063	Heat shock 70 kDa protein 3	70549
7613	P05178	Cytochrome P450 2C6	56002
7502	Q02955	Interleukin-1 receptor, type I [Precursor]	66758
4903	P13596	Neural cell adhesion molecule 1	94658
8102	P15473	Insulin-like growth factor binding protein 3	31680
6209	P19945	60S acidic ribosomal protein	34215
5512	P18588	Interferon-induced GTP-binding protein Mx1	74469
3615	Q63616	Vacuolar protein sorting 33B	70693
6412	P33274	Cytochrome P450 4F1	59868
7606	Q6UE39	Polypeptide N-acetylgalactosaminyltransferase	63948
7512	P80204	TGF-beta receptor type I	55999
7408	P12939	Cytochrome P450	57076
4202	P02571	Gamma-actin	41793
4110	P63086	Mitogen-activated protein kinase 1	41275
9102	Q63199	Tumor necrosis factor receptor superfamily member 6 (Precursor)	36835
3108	Q9R1T3	Cathepsin Z	34194
7107	P46418	Glutathione S-transferase Yc-2	25216
7508	P49088	Asparagine synthetase	64000
3603	Q02955	Interleukin-1 receptor, type I	66758
2108	P06300	Proenkephalin B	28078
7407	Q02955	Interleukin-1 receptor, type I	66758
4108	Q02346	Myoblast determination protein 1	34359
4509	O88599	Myosin-binding protein H	52656
1910	P24054	SPARC-like protein 1 [Precursor]	70633
5302	Q99PW5	Sialidase 3	46980
5409	O08628	Procollagen C-proteinase enhancer protein	50185
4206	Q63199	Tumor necrosis factor receptor superfamily member 6	36835

Table 1: Protein up-regulated more than 2-fold in Arthritis condition.

SPOT NO.	SWISSPROT ID	PROTEIN IDENTIFIED	MW
5307	P17246	Transforming growth factor beta 1	44329
5301	Q8K5E0	Lysophosphatidic acid receptor	40286
5402	P49001	Bone morphogenetic protein 2 precursor	60000
1903	O70531	Sulfate transporter	82027
510	P50430	Protein-arginine deiminase type I	53320
5308	P50430	Arylsulfatase B [Fragment]	47002
5205	O70210	Cartilage leucine-rich protein	40403
509	Q07116	Sulfite oxidase	54354
1102	P37996	ADP-ribosylation factor-like protein 3	20456
9208	P08033	Gap junction beta-1 protein	32003
507	P50137	Transketolase	67643
603	P20781	Glycine receptor beta chain	55927
504	Q6P6V1	Polypeptide N-acetylgalactosaminyltransferase 11	69039
2303	P54313	G protein beta 2 subunit	37331
9207	O55145	Fractalkine Precursor	41976
6208	P53610	Geranylgeranyl transferase type I beta subunit	42414
7001	P07490	Progonadoliberin I [Precursor	10500
4407	Q9JKY3	Zinc finger protein 238	58310
9204	Q9R1A7	Orphan nuclear receptor PXR	49660
6307	O88599	Myosin-binding protein H	52656
3209	Q63495	Advanced glycosylation end product-specific receptor	42663
6204	Q9QX79	Fetuin-B [Precursor]	41532
3103	P13444	Methionine adenosyltransferase	43698
1104	P35738	2-oxoisovalerate dehydrogenase beta subunit, mitochondrial [Precursor]	40561
503	P00185	Cytochrome P450 1A1	59393
2003	P06911	Epididymal secretory protein I	20670
604	O55096	Dipeptidyl-peptidase III	83038
7302	P97711	G protein coupled receptor kinase 6	65962
7302	P06762	Heme oxygenase 1	33005
1105	P07154	Cathepsin L	37660
3310	O34598	Guanine deaminase	51016

Table 2: Proteins down-regulated more than 2-fold in Arthritis condition.

Cathepsin K is a cysteine protease that plays an essential role in osteoclast function and in the degradation of protein components of the bone matrix by cleaving proteins such as collagen type I, collagen type II and osteonectin. Cathepsin K therefore plays a role in bone remodelling and resorption in diseases such as osteoporosis, osteolytic bone metastasis and rheumatoid arthritis. We found increased levels of cathepsin K compared with a healthy control group and found a significant correlation with radiological destruction. Inhibition of different proteins responsible for arthritic condition by celecoxib and Methotrexate Figures 5 (A-F).

A valuable approach to monitor arthritis would be by measuring biological markers of cartilage degradation and repair to reflect variations in joint remodeling. One such potential biological marker of arthritis is cartilage oligomeric matrix protein (COMP). In various studies, COMP has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment. We found increased levels of COMP in arthritic sera.

C-reactive protein (CRP) is one of the biomarkers for the diagnosis and assessment of disease activity in rheumatoid arthritis (RA). CRP is not only the by-product of inflammatory response, but also plays proinflammatory and prothrombotic roles.

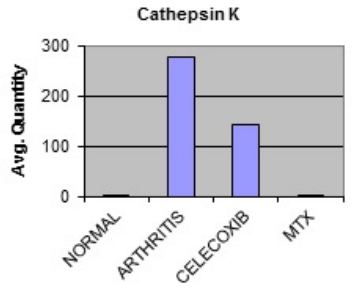


Figure 5A: Methotrexate was found to be a better inhibitor of cathepsin compared to celecoxib.

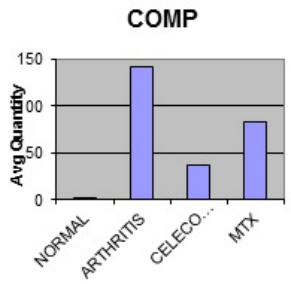


Figure 5B: This protein is potentially inhibited upon celecoxib and methotrexate treatments, where celecoxib was found to be more effective compared to methotrexate.

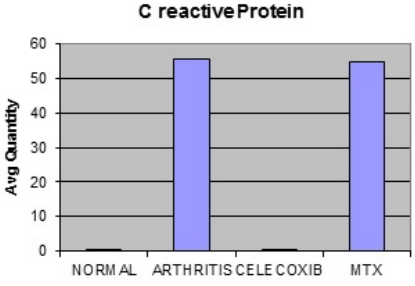


Figure 5C: In our study CRP is significantly up-regulated in arthritic condition and it is better inhibited by celecoxib when compared to methotrexate.

Interleukin 6 (IL-6) is a pleiotropic cytokine with a pivotal role in the pathophysiology of rheumatoid arthritis (RA). It is found in abundance in the synovial fluid and serum of patients with RA and the level correlates with the disease activity and joint destruction. IL-6 can promote synovitis and joint destruction by stimulating neutrophil migration, osteoclast maturation and vascular endothelial growth factor (VEGF)-stimulated pannus proliferation. IL-6 may also be mediating many of the systematic manifestations of RA including inducing the acute-phase reaction [including C-reactive protein (CRP)], anemia through hecipidin production, fatigue via the hypothalamic-pituitary-adrenal (HPA) axis) and osteoporosis from its effect on osteoclasts. In addition, IL-6 may contribute to the induction and maintenance of the autoimmune process through B-cell maturation and TH-17 differentiation. All of the above makes IL-6 blockade a desirable therapeutic option in the treatment of RA.

IL-2 is generally considered a pro-inflammatory cytokine that exacerbates Th1-mediated disease states, such as autoimmune arthritis.The collagenases, MMP-1 and MMP-13, have predominant roles in RA and because they are rate limiting in the process of collagen degradation. MMP-1 is produced primarily by the synovial cells that line the joints, and MMP-13 is a product of the chondrocytes that reside in the cartilage. In addition to collagen, MMP-13 also degrades the proteoglycan molecule, aggrecan, giving it a dual role in matrix destruction. Expression of other MMPs such as MMP-2, MMP-3 and MMP-9, is also elevated in arthritis and these enzymes degrade non-collagen matrix components of the joints. Significant effort has been expended in attempts to design effective inhibitors of MMP activity and/or synthesis with the goal of curbing connective tissues destruction within the joints. To date, however, no effective clinical inhibitors exist.

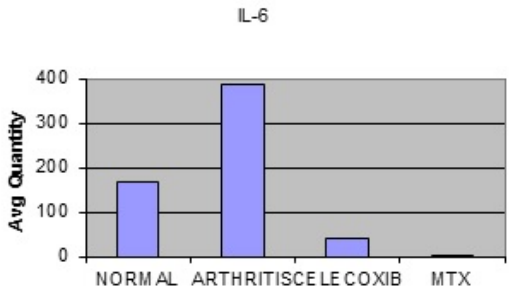


Figure 5D: The increased levels of IL-6 are effectively inhibited by D both celecoxib and methotrexate treatments, with methotrexate showing more inhibitory effect compared to celecoxib.

Increasing our knowledge of the crystal structures of these enzymes and of the signal transduction pathways and molecular mechanisms that control MMP gene expression may provide new opportunities for the development of therapeutics to prevent the joint destruction seen in arthritis. In our study we studied inhibition of total MMPs by celecoxib and methotrexate treatments.

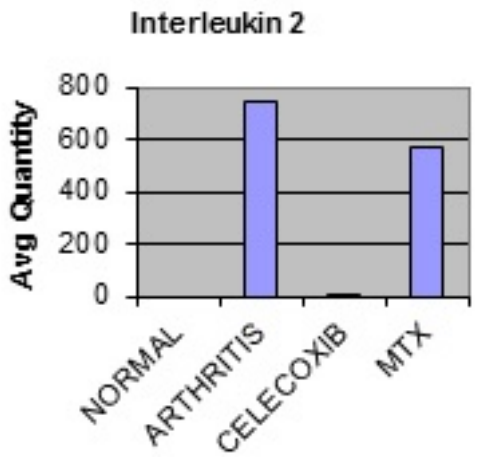


Figure 5E: Increased levels of IL-2 are found in arthritic sera and it is inhibited to a great extent by celecoxib and to a moderate extent by methotrexate.

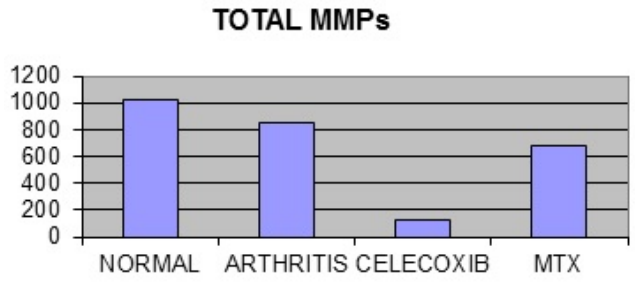


Figure 5F: Celecoxib to be a better inhibitor of MMPs compared to methotrexate.

Conclusion

In conclusion, this study provides a comparative profile of the effects of therapeutic doses of celecoxib and methotrexate on arthritic rats. The results indicate that the two antiarthritics have varying degrees of side effects on bone metabolism, and these findings may help physicians figure out whether appropriate measure will be needed to better prevent the occurrence of osteopenia in RA treatment.

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