Ph.D. THESIS

PHARMACOLOGICAL RESEARCHES AND VIRTUAL PREDICTIVE MODELS ON THE IMPLICATION OF SOME DERIVATIVES FROM OXICAM CLASS ON OXIDATIVE STRESS IN PATIENTS WITH OSTEOARTHRITIS

ABSTRACT

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Key words: piroxicam, tenoxicam, meloxicam, osteoarthritis, oxidative stress, malondialdehyde, nitric oxide, superoxide dismutase, glutathione peroxidase, myeloperoxidase, cyclooxygenase, molecular docking.

1. Introduction
Osteoarthritis, a major public health problem that ranks second among chronic conditions after the cardiovascular affections, is a heterogeneous group of diseases causing joint manifestations associated with alterations of cartilage integrity, accompanied by changes in subchondral bone and periarticular structures. Reactive oxygen and nitrogen species are playing significant roles in the patogenesis of osteoarthritis.

2. Osteoarthritis
Osteoarthritis (OA) is defined as a heterogeneous group of diseases that cause joint manifestations associated with alterations in the integrity of cartilage, subchondral bone and periarticular structures (capsule, ligaments, tendons, muscles). OA incidence is increasing mainly due to higher life expectancy and has significant consequences in society. Changes in the articular cartilage affected by OA come from the imbalance between anabolic and catabolic processes influenced by biomechanical forces and defective autocrine, paracrine and endocrine regulation at cellular level, resulting in an imbalance in the normal turnover of articular tissue.

3. Implications of oxidative stress in osteoarthritis
Formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been suggested to play significant roles in various diseases, including OA. There are many studies that have focused on the destructive effects of oxidative and nitrosative agents in the pathogenesis of OA. Oxidative stress is involved in cartilage destruction in OA and also has indirect action by activating colagenases and up-
regulating genes encoding enzymes involved in matrix degradation and cytokines production.

Lipid peroxidation is initiated by many reactive species among which superoxide and hydroxyl radical. Lipid peroxides act as damaging substances by altering cellular, lysosomal and mitochondrial membrane properties leading to alterations of osmotic, electric and chemical gradients. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and a marker of ROS mediated damage and oxidative stress. MDA can react with DNA bases resulting adducts which affect DNA structure and functions. Increased oxidative stress accelerates chondrocyte senescence and initiates apoptosis, reducing chondrocytes’ ability to maintain and repair cartilage.

Due to an increased oxidative stress, results a higher possibility of interaction between nitric oxide (NO) and ROS, leading to RNS responsible for indirect effects, such us nitrosylation, nitration or oxidation of biomolecules.

Inflammation and mechanical stress are associated with up-regulation of NO, which may have adverse effects on chondrocytes, such as collagen and proteoglycans synthesis inhibition, modulation of cytokines expression, matrix metalloproteinase (MMP) activation, suppression of chondrocyte proliferation and apoptosis.

NO is an important promoter of chondrocytes catabolic activity, inhibiting cartilage matrix synthesis, accelerating chondrocyte-mediated matrix degradation, promoting chondrocyte apoptosis and inflammatory responses, leading to loss of cartilage matrix.

Thus, in pathological circumstances, NO and ROS contribute to cartilage destruction through direct degradation of matrix components, increasing catabolic cytokines activity and reducing cartilage repair ability.

4. Use of oxicams in osteoarthritis

According with OARSI (Osteoarthritis Research Society International) recommendations, cyclooxygenase (COX) non-selective and selective oral NSAIDs are included among pharmacological treatment modalities for OA. NSAIDs influence prostaglandin (PG) synthesis through inhibition of the COX enzyme. There are two COX isoforms that have been well-recognized: COX-1 and COX-2. COX-1 is a constitutively expressed isoform and tends to have a homeostatic function, whereas COX-2 is inducible during inflammation and facilitate the inflammatory response. Non-selective COX inhibitors may determine side effects, while selective COX-2 inhibitors reduce inflammation with fewer side effects. NSAIDs affect ROS formation, some attenuate whereas others enhance ROS generation.

Oxicams are a NSAIDs class of compounds comprising enolic acids structure. These substances can act as nonselective COX inhibitors (piroxicam, tenoxicam, lornoxicam) and as COX-2 selective inhibitors (meloxicam).

5. Molecular modeling studies. Virtual predictive models

QSAR field can be synthetically defined as: "Starting from a given set of molecules, L_i, i = 1, 2, ..., N, that is known for a certain type of biological activity, A_i, a relationship between this activity and some structural properties of the molecules considered will be found". Basically, it seeks an equation (preferably linear) between the
intensity of biological activity, \( A_i \), of each molecule and quantitative measures of structural properties and \( q_{ij}, j = 1, 2, \ldots, M \), outlined for the entire set of molecules.

"Molecular Docking" is a method that can be used to predict favorable orientations of a molecule (ligand) that binds to a second molecule (receptor) to form a stable complex.

6. Motivation of the study and current working hypotheses

The effects of NSAIDs are well studied in OA patients, especially in terms of action by COX inhibition. OA is a complex disease with many other elements involved in its pathogenesis, including oxidative stress, thus study other possible mechanisms of action of NSAIDs is a topic of interest. Research on the involvement of reactive oxygen and nitrogen species in different conditions such as arthritis, atherosclerosis, diabetes, etc. is accelerating rapidly and understanding of their potential utility is increasing. NSAIDs being studied today as free radicals scavengers and for slowing the progression of Alzheimer's disease or cancer. In recent years a growing number of studies have evaluated the role of oxidative stress in OA, synovitis being considered a secondary phenomenon in cartilage destruction, so that the efficacy of NSAIDs may not be due only to inhibition of COX and PG synthesis, but of more complex interference of these drugs with other processes involved in the pathogenesis of osteoarthritis, including oxidative stress. Although it was found that some NSAIDs were interfering with oxidative stress, the mechanisms involved have not been exactly established. These facts open ways to create new NSAIDs, which in addition to anti-inflammatory effect, demonstrate antioxidant activity. All these considerations support the opportunity to conduct such a study.

7. Study objectives

Evaluation of oxidative stress markers: malondialdehyde as an index of lipid peroxidation, nitric oxide, the antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in patients with OA of the knee.

Functional evaluation using patients’ self reported measures: WOMAC score (Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index LK3.1), Oswestry questionnaire, Health Assessment Questionnaire – Disability Index (HAQ-DI), pain using visual analogue scale, Lequesne's functional index for osteoarthritis.

The levels of these parameters were assessed at baseline and after 20 days of treatment with piroxicam (20 mg po daily), tenoxicam (20 mg po daily) and meloxicam (15 mg po daily).

Assessing and comparing the influence of studied oxicams on these parameters before and after 20 days of treatment.

Modeling oxicams’ chemical structure and docking them in the active site of myeloperoxidase and cyclooxygenase.

Comparison of oxicams binding interactions in the active site of MPO and COX.

The aim of this research is to evaluate the antioxidant capacity of piroxicam, tenoxicam, and meloxicam and to elucidate a possible mechanism of action for these effects.
8. Pharmacological research of some derivatives from oxicam class on the involvement of oxidative stress in patients with osteoarthritis

Patients with OA of the knee enrolled in this study were recruited from the Medical Clinic No. 1, Emergency County Hospital, Craiova. Distribution of patients in groups was made according to a study protocol approved by the Ethics Committee of University of Medicine and Pharmacy of Craiova. 30 patients with OA of the knee were selected to assess blood MDA, NO, superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels at baseline and after 20 days of treatment with piroxicam, tenoxicam or meloxicam; 10 patients were treated with piroxicam 20 mg po daily, 10 patients with tenoxicam 20 mg po daily and the rest with meloxicam 15 mg po daily.

All patients completed the study.

For the statistical analysis was used Statistics Package for Social Sciences (SPSS) and results were expressed as mean ± SD (standard deviation). Differences between the three groups at baseline were assessed by independent sample t test. Changes observed before and after treatment were assessed by the paired sample t test. A p value of <0.05 was considered statistically significant.

The baseline MDA levels in tenoxicam and meloxicam groups did not have significant difference when compared with each other. In the piroxicam group, MDA baseline levels were slightly higher then in the other two groups. Results showed that MDA levels were slightly decreased in all three groups of patients, but none of the three NSAIDs belonging to the oxicam group had a statistically significant influence on MDA.

The baseline NO levels in piroxicam and tenoxicam groups did not have significant difference when compared with each other. In the tenoxicam group NO baseline levels were slightly higher then in the other two groups. Piroxicam-treated patients had a significant decrease in NO levels (p=0.006), tenoxicam-treated patients had no significant change in NO levels (p=0.213), while meloxicam-treated patients had an unchanged NO levels (p=0.915).

Results showed that SOD activity increased significantly in piroxicam treated group (p = 0.04) and had no significant variations in tenoxicam and meloxicam treated groups (p = 0.85, p = 0.11 respectively).

In this study it was found that in all three groups GPx values slightly increased, but the change was not statistically significant (p>0.05).

Western Ontario and McMaster Universities (WOMAC) LK3.1 Osteoarthritis Index was performed at baseline and at the end of treatment. The WOMAC Osteoarthritis Index LK3.1 is a validated multidimensional questionnaire and consist in 24 questions (5 on pain, 2 on stiffness, 17 on physical function) each scored on a 5-point Likert scale (0 to 4, 0 representing none, 4 representing extreme). Significant improvement was observed in the WOMAC pain, stiffness and physical function scores in all treated groups (p<0.01).

A slight, but not significant difference in the baseline of HAQ-DI scores was observed in all three groups. The reported values were between 1 and 2 which means a moderate to severe disability. After treatment in all three groups was found a statistically significant improvement (p<0.01) and the values were between 0 and 1, which means mild to moderate difficulty.
Pain intensity was assessed using a visual analog scale (VAS, 0-100 mm, 0 representing no pain, 100 representing worst pain imaginable). Significant improvement was observed in the pain-VAS scores in all treated groups (p<0.01).

In the evaluation using Lequesne score after the treatment it can be observed a significant decrease (p <0.01) in all three groups.

According to the Oswestry questionnaire there is a significant improvement (p <0.01) of life quality in all three groups after the treatment.

9. Molecular modeling studies. Virtual predictive models

The confidence degree of the observed and predicted values can lead to the observation that the type Hansch equation established in this study can be used to predict biological activity of new derivatives in the series.

Piroxicam, tenoxicam, and meloxicam were tested as potential inhibitors of myeloperoxidase and it was observed that the molecular complex, Meloxicam-MPO had the lowest energy value, which makes meloxicam a potential inhibitory agent of myeloperoxidase.

The calculated data showed that tenoxicam is the best inhibitor of COX-1 and meloxicam inhibits more COX-2 isoform, with binding energy values of -41.41, and -36.14 respectively.

10. Final conclusions

1. The studied oxicams show antioxidant effects in varying degrees in osteoarthritis, affecting oxidative stress in the following order: piroxicam > meloxicam > tenoxicam.

2. After 20 days treatment with therapeutic doses of oxicams, plasma levels of malondialdehyde, a marker of oxidative stress, decreased, but the differences were not statistically significant. The order of decreasing this biochemical parameter has been: piroxicam > meloxicam > tenoxicam.

3. The study shows that these NSAIDs did not significantly affect lipid peroxidation in patients with osteoarthritis.

4. Nitric oxide, an important marker in osteoarthritis, was significantly decreased by piroxicam, indicating interferences in nitric oxide pathways, while tenoxicam and meloxicam had no significant effect on NO. The order of NO decreasing potentials is: piroxicam >>> meloxicam > tenoxicam.

5. By influencing the levels of NO, which is involved in cartilage catabolism and reduce anabolic processes in the OA joint, oxicams seems to have an additional mechanism of action - reducing oxidative stress.

6. Increased SOD activity was statistically significant in piroxicam treated group. Tenoxicam and meloxicam did not alter significantly the activity of superoxide dismutase. Order on the superoxide dismutase activity was: piroxicam >>> meloxicam > tenoxicam.

7. The activity of glutathione peroxidase, another member of the antioxidant systems, has increased after 20 days of treatment with oxicams, but not significantly. Order on the glutathione peroxidase activity was: meloxicam > piroxicam > tenoxicam.
8. Comparing the effects of these three oxicams on oxidative stress markers in OA patients, it can be concluded that piroxicam has the best antioxidant properties and tenoxicam the lowest.

9. Although during prostaglandin synthesis from arachidonic acid under the influence of COX are generated free radicals, COX inhibition alone does not seem to fully explain the antioxidant effects demonstrated by oxicams in this study, supporting the hypothesis of an additional mechanism on oxidative stress, independent of COX inhibition.

10. Analyzing the therapeutic effects of piroxicam, tenoxicam, and meloxicam reported by the patients was evident that meloxicam has the best effect on WOMAC pain and stiffness scores, and meloxicam the lowest.

11. Assessment of pain using visual analog scale confirmed meloxicam better action obtained in WOMAC pain score. At the end of the treatment, piroxicam and tenoxicam reduced pain almost identical, while meloxicam had a more pronounced analgesic action.

12. Using Lequesne score it can be observed a significant decrease in all three groups, with better scores for piroxicam.

13. The values obtained using HAQ-DI, are initially placed between 1 and 2, which means a moderate to severe disability. After the treatment, in all three groups was noted a statistically significant improvement in HAQ-DI score, with values between 0 and 1, signifying mild to moderate difficulty.

14. According to the Oswestry questionnaire there is a significant improvement in life quality in all three groups, the most significant improvement occurring in the meloxicam treated group, piroxicam and tenoxicam-treated groups showing approximately the same initial and final scores.

15. "Molecular docking" technique is an effective tool in selecting potential therapeutic agents, assessing how the suitable ligand molecule is binding in the active site of the biological receptor and calculating the minimum binding energy. That helps researchers to find the best potential therapeutic agents for synthesis and reduce financial costs.

16. Results showed that the complex Meloxicam-MPO has the lowest energy value, thus meloxicam is the best inhibitor of myeloperoxidase when compared with piroxicam and meloxicam. According to the calculated energy values tenoxicam is the best inhibitor of the cyclooxygenase-1 and meloxicam inhibits more cyclooxygenase 2 isoform

17. Net loads on the atoms of the studied molecules, arising from the formation of chemical bonds, describe the nature and reactivity of atoms in molecules and indicate atoms that contribute to the biological response in the class of studied substances. Thus, at the inhibitory activity shown by compounds of the oxicam class, oxygen (sp²) and sulfur (sp³) atoms contributes the most.

18. Contrary to the docking technique, QSAR / QSPR technique does not require information about biological receptor, but allows the correlation of biological activity shown by compounds from oxicam class with various structural parameters (desciptors). Because does not require any alignment of the ligand or suitable interaction conformation, this technique is fast and can be used as virtual screening for large molecular databases.
19. Chemical structure - property correlation for this series of substances showed the potential role of some chemical structure, i.e. the involvement of these descriptors to aqueous/lipid phase partition (biological membrane) characterized by partition coefficients log P.

20. Hansch equation established in this study allows the calculation of partition coefficient of a new derivative before it is tested and its biological activity is determined experimentally by virtual chemical modulation. Thereby, inhibition of MPO and COX activity characteristic of some new compounds could be predicted by the Hansch equation established in this study.

23. Finally, by clinical trials and docking and QSAR/QSPR techniques, the study argues the reduction of oxidative stress as an additional mechanism of oxicams action in osteoarthritis.

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