The Effects of a 5-Chromene-yl-thiazolidin-2,4-dione Derivative in Alleviating Oxidative Stress in Adjuvant-Induced Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that reduces life quality and requires long-life therapy. Quercetin (Que) is a natural flavonoid with antioxidant and anti-inflammatory effects. (3-(2-(4-Chlorophenyl)-2-oxoethyl)-5-((6-methyl-4-oxo-4H-chromen-3-yl)methylene) thiazolidine-2,4-dione (TZD) is a thiazolidinedione derivative synthesized in our laboratory. This study was designed to investigate the antioxidant effects of the Que and TZD derivative administration in adjuvant-induced arthritic (AIA) rats. AIA was induced in Wistar rats by the intraplantar injection of Freund's complete adjuvant (FCA), unilaterally in the right hind paw. The control non-arthritic rats and the arthritic rats were treated with Que (30 mg/kg/day) or TZD derivative (12 mg/kg/day) for 21 days. The antioxidant effects of 5-chromen-yl- thiazolidinedione were compared to Que. The serum levels of malondialdehyde (MDA) and protein carbonyl (PC) groups, the superoxide dismutase (SOD) and catalase (CAT) activity were assessed. AIA rats showed significatly increased oxidative stress parameter levels in the blood. The results indicated that the TZD derivative decreased the blood oxidative stress parameters in the treated arthritic rats, compared to Que. The antioxidant effects of 5-chromen-yl-thiazolidinedione in AIA suggest its therapeutic properties for the clinical treatment of RA.

Keywords: oxidative stress; adjuvant-induced arthritis; quercetin, 5-chromenyl-thiazolidinedione.

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease, of known ethiology and autoimmune pathogeny, characterized by an inflammatory arthropaty with a chronic, progressive, deforming, distructive evolution and with multiple systemic manifestations [1,2].

RA ethiology is still uncertain, but an important role is played by the oxidative stress, which is responsible for the occurrence of proliferative and destructive synovitis. The increased level of the reactive oxygen species (ROS) in the synovial cavity increases the inflammation and the joint damage [3,4]. m The stimulation of the inflammatory cells is done to discharge ROS and the proinflammatory cytokines responsible for the progression of RA. The excessive production of ROS dirupts the redox balance and amplifies the inflammatory response via the nuclear factor-κB (NF-κB). This is the central regulator of the cellular inflammatory response, which controls multiple genes involved in inflammation, inducing the genic transcription of some pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin-(IL)-1 and IL-6 [3].

Freund's complete adjuvant (FCA) is used for the induction of the adjuvant-induced arthritis (AIA), a widely used rodent model in the studies addressing RA, as a typical inflammatory arthritic pain and joint disease model, owing the similarity of its pathological characteristics to human RA [1,4, 5].

Quercetin is the major dietary flavonol present in apples, onion, citrics, forrest fruits, red grapes, broccoli, root bark, flowers and tea, manifesting *in vitro* and *in vivo* antioxidant properties [6,7]. Recently, *in vitro* and *in vivo* studies demonstrated its effects in reducing the inflammation and the oxidative stress in RA. It prevents or reduces inflammatory reactions by adjusting the NF-κB activity, inhibits the transcription of joint synovial inflammatory factors and affects the generation of inflammatory factors. On the other hand, quercetin plays an important role in relieving RA inflammation and preventing pannus formation [8].

Thiazolidinediones are a class of compounds known for their beneficial role in diabetes. These substances proved, during the time, good anti-inflammatory [9] and antioxidant effects [10].

The research nowadays is focused on discovering new pharmacological and non-pharmacological resources with antioxidant effects, in order to ameliorate the symptomatology and prevent the complications in RA. One of the main research directions is the testing of some natural or synthetic products with anti-inflammatory and antioxidant potential, which can be very useful in the treatment of RA. Based on our previous experience in the synthesis [11,12] and the investigation of the biological potential of new thiazolidinedione derivatives [13], we present here a study which aims to see whether (3-(2-(4-chlorophenyl)-2- oxoethyl)-5-((6-methyl-4-oxo-4H-chromen-3-yl)methylene)thiazolidine-2,4-dione and quercetin, respectively, decrease the oxidative stress in arthritic rats.

Experimental part

Materials and methods

Drugs and Chemicals

Freund's complete adjuvant (FCA), quercetin (3,3',4',5,7-pentahydroxyflavone dihydrate, >98% purity powder), indomethacin, thiazolidinedione, 2-bromo-1-(4-chlorophenyl)ethanone, 6-methyl-4-oxo-4*H*-chromene-3-carbaldehyde, were purchased from Sigma-Aldrich Chemical Company Inc., (Gillingham, Dorset, UK). The oral treatment with quercetin (Que), indomethacin (IND)

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and thiazolidinedione derivative (TZD) were performed by gavages with 0.5% suspensions of the tested compounds, prepared in carboxymethylcellulose (CMC) as vehicle.

*Synthesis of 3-(2-(4-chlorophenyl)-2-oxoethyl)-5-((6-methyl-4-oxo-4H-chromen-3-yl) methylene)thiazolidine-2,4–dione **2** (TZD)*

1 mmol of 5-chromenyl-2,4-thiazolidinedione **1** was stirred for 30 min, at room temperature, with 1.1 mmol (0.062 g) of anhydrous potassium hydroxide, in 6 mL of dimethylformamide (DMF). After the potassium salt was formed, 1.1 mmol of 2-bromo-1-(4-chlorophenyl)ethanone were added. The crude product was filtered under reduced pressure, washed with water and purified by recrystallization from ethanol.

Induction of rat adjuvant-arthritic model

Male Wistar rats (250±20 g) were purchased from the Experimental Animal House of the Faculty of Medicine within Iuliu Hatieganu University of Medicine and Pharmacy of Cluj-Napoca, Romania. The animals were given standard rat pellets diet and water *ad libitum*. All the experiments were performed according to the approved animal protocols of the Ethical Committee on Animal Welfare of Iuliu Hatieganu University (No. 43/13.03.2017), in accordance with the Romanian Ministry of Health and complying with the Guidelines in the Use of Animals in Toxicology. The animals were randomly divided into eight experimental groups (n=10). Adjuvant-induced arthritis (AIA) was induced in rats as a typical inflammatory arthritic pain model [14]. The forty Wistar rats used in the present investigations underwent chronic, unilateral inflammation. The remaining forty Wistar rats served as untreated (non-inflammated) control animals. Arthritis was induced by the single intra-dermal injection of 0.1 mL FCA into a metatarsal footpad of the right hind paw. Seven days later, AIA was confirmed by clinical evaluation.

The experiment included eight experimental groups: the first group (control+CMC; CO+CMC)-healthy, non-arthritic control rats treated with CMC; the second group (control+quercetin, CO+Que)-healthy, non-arthritic control rats treated with quercetin; the third group (control+indomethacin, CO+IND)-healthy, non-arthritic control rats treated with indomethacin; the fourth group (control+5-chromen-yl-thiazolidinedione, CO+TZD)-healthy, non-arthritic control rats treated with 5-chromen-yl-thiazolidinedione; the fifth group (arthritic+CMC, AR+CMC)-arthritic control rats treated with CMC; the sixth group (arthritic+Que, AR+Que)-arthritic control rats treated with quercetin; the seventh group (arthritic+IND, AR+IND)-arthritic control rats treated with indomethacin; the eighth group (arthritic+TZD, AR+TZD)-arthritic rats treated with TZD derivative.

The rats from the groups CO+CMC and AR+CMC were treated with CMC (0.6 mL/rat) administered orally, once a day, for 21 consecutive days. The quercetin (30 mg/kg body weight), indomethacin (2 mg/kg body weight) and TZD derivative (12 mg/kg body weight) were orally administered by gavages, once a day, for 21 consecutive days, the administration started seven days later after the FCA administration.

Measurement of the Biochemical Parameters of Oxidative Stress

At the end of the experiment, all animals were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/rat) and sacrificed by cervical decapitation. The venous blood samples were collected from the rats' retro-orbital sinuses. Immediately after sampling, the blood was centrifuged to separate the serum, which was frozen with liquid nitrogen and stored in a -80 °C refrigerator, until biochemical assays. By measuring the free radical production, the levels of the oxidative stress in serum were indirectly assessed.

Estimation of the lipid peroxidation

The lipids are one of the primary targets of ROS. The peroxidation of lipids produces highly reactive aldehydes, including malondialdehyde (MDA), a primary biomarker of the free radical mediated lipid damage and oxidative stress. The MDA levels were measured in serum, using the fluorimetric method with 2-thiobarbituric acid, described by the Conti method [15] The MDA was spectrofluorimetrically determined in the organic phase, using a synchronous technique with excitation at 534 nm and emission at 548 nm. The MDA levels were expressed as nanomole per milliliter (nmol MDA/mL).

Estimation of the protein carbonylation

The protein carbonylation under ROS action was estimated from serum, by measuring the protein carbonyl (PC) group levels. The protein carbonyl derivatives that are produced through the protein oxidative damage were determined using the fluorimetric method with 2,4-dinitrophenyl-hydrazine (DNPH) [16]. The readings were performed using a spectrophotometer at 355-390 nm and in order to calculate the remaining carbonyl fragments, the molar extinction coefficient with a value of 22,000/M/cm, was used. The levels of the carbonyl-derivative groups were expressed as nanomole per milligram of protein (nmol/mg protein).

The activities of some antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) were determined in the animal blood.

Estimation of the superoxide dismutase activity

The SOD activity was determined by the enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA). The SOD activity was expressed as units per gram of protein (U/g protein).

Estimation of the catalase activity

The CAT activity was assayed by the method proposed by Pippenger et al. [17]. This consists in following the change in absorbance of a solution of H2O2 10 mM in potassium phosphate buffer 0.05 M, *p*H = 7.4, at 240 nm. One unit of CAT is defined as the amount of enzyme which induces reduction in the absorbance of 0.43, at 25 °C, for 3 min. The CAT activity was expressed as units per gram of protein (U/g protein).

Statistical Analysis

The statistical analysis was performed using the SPSS software package (version 17.0, SPSS Inc., Chicago, IL 60606-6412, USA). The data were reported as mean ± SD. One-way analysis of variance (ANOVA) was used to compare differences between groups, and two-way ANOVA for repeated measurements, followed by Tukey's multiple posttest comparisons, to compare the responses to quercetin and TZD derivative in AIA rats. Differences were considered significant if $p<0.05$.

Results nand discussions

Chemistry

5-(Chromene-3-yl)methylene-2,4-thiazolidinedione **1** was obtained by the condensation of 6-methyl-4-oxo-4*H*-

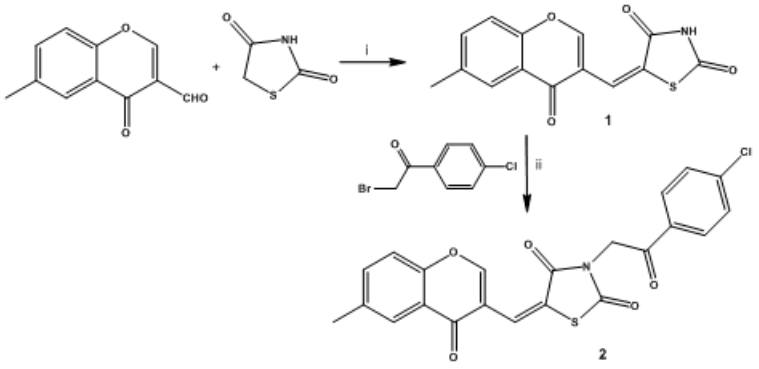

Fig. 1. Synthesis of new *N*-substituted 5-(chromene-3-yl)methylene-2,4-thiazolidinedione i: anhydrous sodium acetate/acetic acid, 3h reflux; ii: anhydrous potassium hydroxide, DMF, 30 minutes, stirring, room temperature

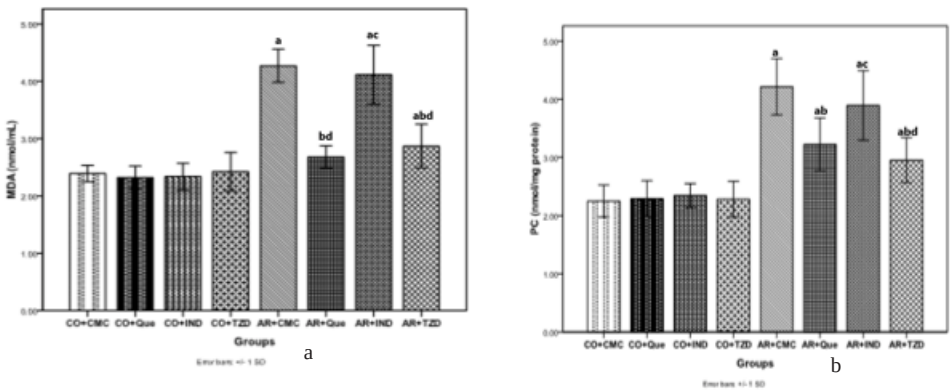

Fig. 2. The effects of TZD on (a) lipid peroxidation (MDA) (nmol/mL) and (b) protein carbonyl groups (PC) (nmol/mg protein) levels in the serum of control and arthritic rats. Results are the means ± SD for ten animals in each group in the CO+CMC (healthy, non-arthritic control rats treated with CMC), CO+Que (healthy, non-arthritic control rats treated with quercetin), CO+IND (healthy, non-arthritic control rats treated with indomethacin), CO+TZD (healthy, non-arthritic control rats treated with 5-chromen-yl-thiazolidinedione), AR+CMC (arthritic control rats treated with CMC), AR+Que (arthritic control rats treated with quercetin), AR+IND (arthritic control rats treated with indomethacin), AR+TZD (arthritic rats treated with 5-chromen-yl-thiazolidinedione) groups. Results are mean ± SD of 10 rats per each group. Statistically significant differences are indicated by the symbols: [a] $p<0.05$ vs. CO+CMC, [b] $p<0.05$ vs. AR+CMC group; [c] $p<0.05$ vs. AR+Que, [d] $p<0.05$ vs. AR+IND group.

chromene-3-carbaldehyde with 2,4-thiazolidinedione, according to the literature data (fig. 1) [20]. Then, it reacted with 2-bromo-1-(4-chlorophenyl)ethanone, in order to obtain the new *N*-substituted derivative 2.

3-(2-(4-chlorophenyl)-2-oxoethyl)-5-((6-methyl-4-oxo-4H-chromen-3-yl)methylene)thiazolidine-2,4–dione ***(2)*** Yield 70%. Pale yellow powder, mp: 224 °C. ^{1}H NMR (DMSO-*d*6, 500 MHz, ppm): δ 2.45 (s, 2H, -CH3-); 4.21 (s, 2H, -CH2-); 7.10 (d, 2H, phenyl); 7.65 (d, 1H, C8-chromone-H); 7.70 (dd, 1H, C7-chromone-H); 7.72 (s, 1H, C=CH); 7.94 (s, 1H, C5-chromone-H); 8.06 (d, 2H, phenyl); 8.93 (s, 1H, C2-chromone-H). ^{13}C NMR (DMSO-*d*6, 500 MHz, ppm): δ 20.9 (CH3); 40.5 (CH2); 118.1 (chromone-C8); 118.9 (chromone-C4a); 120.1-130.2 (6C, phenyl); 123.1 (CH); 123.2 (chromone-C5); 125.3 (chromone-C3); 126.8 (chromone-C6); 136.7 (thiazolidinedione-C5); 140.7 (chromone-C7); 154.1 (chromone-C8a); 162.3 (chromone-C2); 166.2 (thiazolidinedione-C4); 167.4 (thiazolidinedione-C2); 169.1 (-C=O); 175.3 (chromone-C4). Anal. Calcd. (%) for C22H14ClNO5S (439.87): C, 60.07; H, 3.21; N, 3.18; S, 7.29. Found: C, 60.10; H, 3.21; N, 3.17; S, 7.28. MS (EI, 70 eV): m/z 440 [M+].

Oxidative Stress in blood of the Rats of All Experimental Groups

As a measurement of the oxidative stress, we determined the malondialdehyde (MDA) and protein carbonyl groups (PC) levels in the serum of non-arthritic control rats and arthritic rats, measured on day 28 of AIA (fig. 2 a and b). The data in figures 2a and 2b demonstrate that the serum levels of MDA and PC increased significantly ($p<0.05$) after the induction of AIA in the arthritic control rats treated with CMC (AR+CMC group) compared to the non-arthritic control rats (CO+CMC, CO+Que and CO+TZD groups). The AIA rats treated with quercetin (AR+Que group), for 21 days, exhibited a significant decrease ($p<0.05$) of the levels of MDA and PC in their serum, when compared to the arthritic control rats treated with CMC (AR+CMC group). The administration of indomethacin (AR+IND group) in AIA rats for 21 days non-significantly reduced ($p>0.05$) the serum levels of MDA and PC, compared to the arthritic control rats treated with CMC (AR+CMC group). The arthritic rats treated with TZD for 21 days (AR+TZD group) showed significantly reduced MDA and PC levels ($p<0.05$) in the serum, as compared with the arthritic control rats treated with CMC (AR+CMC group). The results showed that TZD decreased the MDA and PC levels in the serum in a manner similar to Que.

Figure 3 show the effects of quercetin, indomethacin and 5-chromen-yl-thiazolidinedione on the activities of superoxiddismutase (SOD) and catalase (CAT) in the blood of the non-arthritic control rats and arthritic rats, measured on day 28 of AIA. The activities of SOD (fig. 3a) and CAT (fig. 3b) were significantly lowered ($p<0.05$) 28 days after the FCA administration (AR+CMC group). Quercetin treatment for 21 days significantly increased the SOD and CAT activities in the blood of the arthritic rats (AR+Que group) when compared to the arthritic control rats treated with CMC (AR+CMC group). The AIA rats treated with indomethacin (AR+IND group) for 21 days, exhibited a non-significant increase ($p>0.05$) of the SOD and CAT

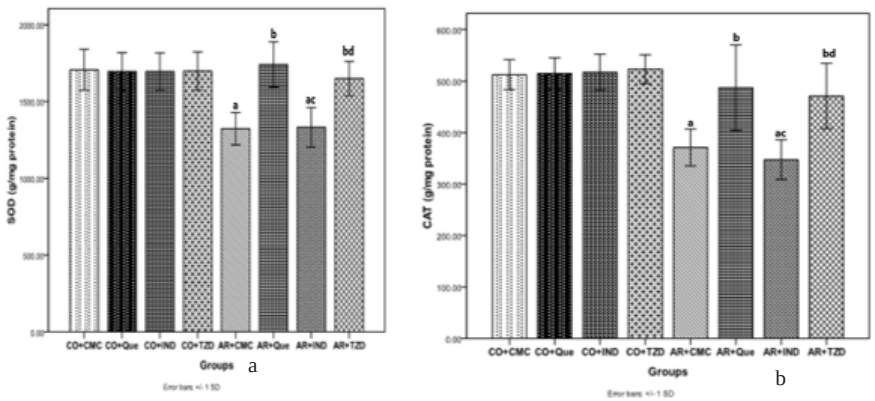

Fig. 3. The effects of TZD on the on the activities of (a) superoxide dismutase (SOD) (g/mg protein) and (b) catalase (CAT) (g/mg protein) in the blood of control and arthritic rats. Results are the means ± SD for ten animals in each group in the CO+CMC (healthy, non-arthritic control rats treated with CMC), CO+Que (healthy, non-arthritic control rats treated with quercetin), CO+IND (healthy, non-arthritic control rats treated with indomethacin), CO+TZD (healthy, non-arthritic control rats treated with 5-chromen-yl-thiazolidinedione), AR+CMC (arthritic control rats treated with CMC), AR+Que (arthritic control rats treated with quercetin), AR+IND (arthritic control rats treated with indomethacin), AR+TZD (arthritic rats treated with 5-chromen-yl-thiazolidinedione) groups. Results are mean ± SD of 10 rats per each group. Statistically significant differences are indicated by the symbols: [a] $p<0.05$ vs. CO+CMC, [b] $p<0.05$ vs. AR+CMC group; [c] $p<0.05$ vs. AR+Que, [d] $p<0.05$ vs. AR+IND group.

activities in the blood, compared with the arthritic control rats treated with CMC (AR+CMC group). The SOD and CAT activities in the blood significantly increased ($p<0.05$) in the arthritic rats treated with TZD (AR+TZD group), when compared to the arthritic control rats treated with CMC (AR+CMC group).

Rheumatoid arthritis (RA) is characterized as an autoimmune chronic systemic inflammatory disease characterized by inflammation of peripheral joints and damage of the articular and periarticular structure [12]. For the *in vivo* animal models, Freund's complete adjuvant (FCA) is used, to induce the adjuvant-induced arthritis (AIA). This *in vivo* animal model has been used for the evaluation of the anti-inflammatory, antinociceptive and antioxidant effects of natural and synthetic compounds on chronic arthritis [1,2]. AIA involves immune responses mediated by pro-inflammatory mediators and proteolytic enzymes such as interleukin (IL)-1β, IL-6, IL-8, IL-10, cyclo-oxygenase (COX)-2, collagenase (MMP-1), gelatinase A (MMP-2), etc., and it plays a crucial role in inflammation and joint damage of RA [1, 2,17,18]. The earliest symptoms in RA are pain and inflammation, followed by various degrees of joint destruction. Inflammation is initiated and propagated by the production of cytokines, chemokines and cell adhesion molecules [19]. This research was performed to determine the potential of a TZD derivative in treating AIA, comparing with quercetin and indomethacin. The results regarding the therapeutic roles against the blood oxidative status are promising.

In the etiopathogenesis of RA, the primary and dominant processes are the immunological mechanisms, while the oxidative stress is involved in this chronic autoimmune disease [8]. The present research has examinated the positive effects that quercetin and TZD have on the oxidative stress in the serum of AIA rats. This study found that the oxidative stress was higher in the serum of AIA rats, evidenced by increased MDA and PC levels and decreased antioxidant activities of SOD and CAT in the blood. The present study highlights the capacity of quercetin to improve the antioxidant defenses in AIA rats. The results suggest that quercetin, owing to its effects of decreasing the MDA and PC levels in serum, as markers of lipid peroxidation and protein oxidation, increased the antioxidant enzymes activities in the blood and therefore, alleviated the oxidative stress in the blood. Recent studies proved that quercetin reduced the inflammation and decreased the oxidative stress in AIA [5,8].

In our study, after 21 days of TZD administration, MDA and PC levels in serum significantly decreased and the antioxidant activities significantly increased in the blood of AIA rats. These results demonstrate the beneficial antioxidant effects of TZD to FCA-induced oxidative stress.

Conclusions

In the present study, we concluded that TZD administration into FCA-induced arthritis protected rats against the oxidative stress, by ameliorating FCA-dysregulated oxidative related enzymes. These results suggested the antioxidant effects of TZD derivative against RA.

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