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Original research article

# Myelosuppressive and hepatotoxic potential of leflunomide and methotrexate combination in a rat model of rheumatoid arthritis

Shymaa E. Bilasy [a], Soha S. Essawy [b], Mohamed F. Mandour [c], Eman A.I. Ali [d], Sawsan A. Zaitone [e,*]

[a] Department of Biochemistry, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt
[b] Department of Pharmacology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
[c] Department of Clinical Pathology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
[d] Department of Histology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
[e] Department of Pharmacology & Toxicology, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt

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ABSTRACT

*Background:* Safety of the combination of leflunomide and methotrexate was examined in several studies with inconclusive results. The present study was designed to compare the efficacy and safety of the combination of leflunomide and methotrexate in adjuvant-induced arthritis (AIA) in rats focusing on immunosuppressive and hepatotoxic effects.
*Methods:* Eighty four rats were divided into seven groups. Group 1: Sham control, group 2: the vehicle control, group 3: methotrexate group, group 4–5: leflunomide (5 and 10 mg/kg/day) groups, group 6–7: combination 1 and 2 [methotrexate + leflunomide (5 and 10 mg/kg/day)] groups, respectively.
*Results:* The current results indicated that combination therapies improved the ankle circumference and clinical scores compared to monotherapies; histopathological examination confirmed these findings. The myelosuppressive effect of leflunomide (10 mg/kg/day) was comparable to that produced by methotrexate as indicated by the complete blood count and bone marrow cellularity; however their combination resulted in greater toxicity. Furthermore, methotrexate greatly affected the splenic histopathology compared to leflunomide and the combination therapy produced a greater effect compared to leflunomide not methotrexate. Differently, assessment of the hepatotoxic potential of the two drugs highlighted that leflunomide induced a dose-dependent increase in the fibrosis score which was higher in their magnitude than that induced by methotrexate. Leflunomide (10 mg/kg/day) and combination 2 groups showed the greatest degree of liver fibrosis.
*Conclusions:* In rats with AIA, current drug combinations provided higher therapeutic benefit compared to monotherapies, however, greater toxicities were observed. Therefore, continuous monitoring of hematologic parameters and liver function will be recommended in clinical settings.


## Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic arthritis leading to progressive joint erosions with subsequent damage and destruction [1]. Several studies revealed the significant role for the inflammatory cytokines such as tumor-necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 [2] as well as angiogenesis [3] in the pathogenesis of RA.

Diseases modifying anti-rheumatic drugs (DMARDs) are the cornerstone of treatment as they suppress underlying immune-mediated inflammation and joint damage and prevent long-term morbidity and mortality. Initial DMARD therapy is typically methotrexate monotherapy or a combination of other DMARD agents [4]. Low-dose methotrexate therapy slows the rate of joint destruction and improves the patient's quality of life; therefore, it is considered to be the gold standard for treatment of RA.

*Abbreviations:* AIA, adjuvant-induced arthritis; CBC, complete blood count; CFA, complete Freund's adjuvant; CMC, carboxymethyl cellulose; COX 2, cyclooxygenase 2; DMARDs, disease modifying anti-rheumatic drugs; IL-1β, interlukin-1β; RA, rheumatoid arthritis; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

* Corresponding author.
*E-mail addresses:* Sawsan_zaytoon@pharm.suez.edu.eg, sawsan_zaytoon@yahoo.com (S.A. Zaitone).

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Methotrexate competitively inhibits the dihydrofolate reductase enzyme that leads to the inhibition of enzymes involved in the metabolism of purine [5]. Additionally, methotrexate is suggested to promote the apoptosis of activated T cells, an action that would be complementary to the effect of leflunomide to limit T cell proliferation [6]. However, the use of methotrexate has been limited by some of its toxic manifestations. Methotrexate therapy is sometimes accompanied with side effects including gastrointestinal toxicity, myelosuppression and liver function abnormalities [7].

Leflunomide is an orally effective DMARD that was introduced for use principally as a single agent in RA. Leflunomide inhibits the mitochondrial enzyme dihydroorotate dehydrogenase thus inhibiting the de novo synthesis of pyrimidine synthesis and limits proliferation of activated T lymphocytes [8]. Leflunomide's active metabolite, A77 1726, interferes with T-cell production of inflammatory cytokines by preventing activation and gene expression of nuclear factor-κB required for expression of inflammatory cytokines. Leflunomide has been successfully used for the treatment of RA and has been shown to improve the physical status in RA patients. The adverse effects of leflunomide include gastrointestinal symptoms, decreased leukocyte count and elevation of alanine aminotransferase. The incidence of adverse effects and the rate of withdrawal due to adverse effects are lower for leflunomide than for methotrexate [9]. Currently, leflunomide is often combined with other DMARDs after failure of the initial monotherapy. Leflunomide is usually used in lower doses as an adjunct to methotrexate or other DMARDs [10].

Data examining the benefit of combining leflunomide with methotrexate are limited. The safety profile of these drugs, however, has been re-examined in several studies, with non-conclusive results [11–14]. Clinical studies have the disadvantage of lacking histopathological data and the inclusion of untreated controls, at the same time, little is known from experimental studies. Therefore, the present animal study was designed to determine the efficacy of the combination of both drugs through clinical, macroscopic and histopathological examination. In the current study, the immunosuppressive and hepatotoxic potential of this combination was determined in rats with AIA to assess its safety.

## Materials and methods

### *Animals*

The experiment was carried out using healthy male Wistar rats, weighing 250–300 g (8–10 weeks) supplied by the Modern Veterinary Office for Laboratory Animals (Cairo, Egypt). Rats were housed in groups of four in polypropylene cages with food and tap provided water ad libitum. Rats were maintained at $22 \pm 3$ °C under normal light and dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University (Ismailia, Egypt).

### *Drugs and chemical*

Complete Freund's adjuvant (CFA) was purchased from Sigma–Aldrich (MO, USA). Methotrexate powder was provided by T3A Pharmaceutical Company (Cairo, Egypt) and dissolved in phosphate buffered saline (pH = 7.4). Leflunomide powder was provided by Sigma Pharmaceutical Co. (Quesna, Egypt) and suspended in 0.5% carboxymethyl cellulose (CMC) solution. All the used solvents were of analytical grades and were supplied by Al-Nasr Company for Chemical Industries (Cairo, Egypt).

### *Experimental design*

Eighty four rats were divided into seven groups, twelve animals each. Group 1 served as sham control and received an injection of 0.25 ml of paraffin oil into the left hind paw. All the other rat groups received a single subcutaneous injection of 0.25 ml of CFA in the palmar surface of the left hind paw to produce AIA [15]. Four days after induction of arthritis, rats started various drug regimens that continued up to 4 weeks. Group 2: rats received 0.5% CMC (2 ml/kg, *po*) as the vehicle control group. Group 3: rats received methotrexate (0.05 mg/kg/day, *ip*). Groups 4 and 5: rats received leflunomide (5 or 10 mg/kg/day, *po*), respectively. Group 6 and 7: rats received a combination of methotrexate (0.05 mg/kg/day; *ip*) and leflunomide (5 or 10 mg/kg/day, *po*) [combination 1 group and combination 2 group, respectively]. The selected doses of leflunomide were greater than those clinically used in treating patients with RA as reported in the literature [1,11,16] and the preliminary study conducted in our laboratory confirmed that those doses were effective against experimentally induced arthritis. In general, leflunomide was administered orally in a total volume of 2 ml/kg. Regarding methotrexate, the daily schedule was chosen rather than the weekly schedules to minimize the mortality among rats and to increase the chance for survival; this allowed studying the adverse effects of the drug combinations in the surviving animals.

### *Clinical assessment of AIA*

Starting from day 4, the clinical signs of arthritis were assessed, by a single observer blind to the treatment group. Rats were re-examined every 4 days for two clinical parameters: ankle circumference and articular score measurements of the injected paw. For ankle circumference determination, two perpendicular diameters of the joint were measured every other day with a caliper (Lange Caliper, Cambridge Scientific Industries, Cambridge, MA, UK). Ankle circumference was determined using the geometric formula:

$$\text{Circumference} = 2\pi\sqrt{\frac{a^2 + b^2}{2}}$$

where $\pi$ (Pi) = 3.14, $a$ is the medio-lateral diameter, and $b$ is the antero-posterior diameter, as described previously [17].

Joint swelling was assessed with a semi-quantitative clinical score (articular index) from 0 to 4 where (0 = no swelling, 1 = weak swelling and/or erythema, 2 = mild swelling, 3 = moderate swelling, 4 = severe swelling of the toes and ankle) [18].

### *Blood collection and serum separation*

At the end of the experiment (day 32), rats were anesthetized with thiopental sodium (50 mg/kg) [19] and killed by decapitation. A midline incision was made and blood samples were withdrawn from the heart by cardiac puncture. One blood sample (1 ml) was collected in a tube containing ethylenediaminetetraacetic acid solution (29 μg/ml blood) for complete blood count (CBC) and analyzed within 2 h in an automated cell counter (Cell-DYN 1700, Model: CD-1700, ABOTT Diagnostics, USA). Another blood sample was collected in a dry centrifuge tube and allowed to stand for 30 min before centrifugation at $2000 \times g$ for 15 min. Sera were separated and stored at −80 °C until use.

### *Determination of serum level of TNF-α, IL-6, COX 2, and VEGF*

Enzyme Linked Immunosorbent Assay (ELISA) kits for TNF-α (Biosource International Inc., Camarillo, CA, USA), IL-6 (Glory Science Co., Ltd, Del Rio, TX, USA), cyclooxygenase 2 (COX 2; Glory Science Co., Ltd, Del Rio, TX, USA) and vascular endothelial growth