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Research paper

Methotrexate prodrugs sensitive to reactive oxygen species for the improved treatment of rheumatoid arthritis

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ABSTRACT

Methotrexate (MTX) is the standard of care in the treatment of rheumatoid arthritis (RA), a common autoimmune disease that is characterized by chronic inflammation in the synovial membrane of joints. Unfortunately, MTX suffers from high discontinuation rates due to a large variability in efficacy and, in particular, adverse effects. As inflammation is associated with elevated levels of reactive oxygen species (ROS) like H_2O_2, we propose to improve treatment through site-selective delivery of MTX to inflammatory tissue by use of a H_2O_2 sensitive MTX prodrug. To establish proof proof-of-concept, two novel H_2O_2 sensitive, thiazolidinone-based MTX prodrugs were synthesized and evaluated for this purpose. MTX-γ-thiazolidinone (MTX-γ-TZ) exhibited the most promising properties – good to high chemical and metabolic stability, excellent aqueous solubility, while being activated when subjected to patho-physiological concentrations of H_2O_2. *In vivo*, MTX-γ-TZ exhibited comparable efficacy to MTX in a murine collagen type II-induced arthritis (CIA) model while treated mice showed indications of reduced toxicity as their body weight decreased less towards the end of the study, compared to the MTX-treated group.

1. Introduction

1.1. Background

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic synovial inflammation and is associated with progressive disability, systemic complications, early death, and socioeconomic costs [1]. The pathogenesis of RA is still incompletely understood but involves a complex interplay of genetic and environmental factors. It is estimated that 0.5–1% of the adult populations in developed countries suffer from RA, with a prevalence three times greater for women than men [2].

Currently, no cure for RA exists and treatment strategies consist of life-long palliative care, primarily using disease-modifying antirheumatic drugs (DMARDs) to relieve inflammatory symptoms and retard joint destruction. Since the 1980s, methotrexate (MTX, **1**) has been the standard of care in the treatment of RA (Fig. 1). MTX was originally developed as a folic acid antagonist for high-dose cancer therapy (HD-MTX: 1000–5000 mg/week). However, low-dose treatment (LD-MTX: 5–25 mg/week), administered either orally, subcutaneously, or intravenously, has shown potent

Abbreviations: ADMET, absorption, distribution, metabolism, excretion, toxicity; CFA, Complete Freund's Adjuvant; CIA, collagen-induced arthritis; CL'_{int}, apparent intrinsic clearance; DBA, dilute brown non-agouti; dec., decompose; DMA, dimethylacetamide; DMARD, disease-modifying antirheumatic drug; HD, high-dose; i.p., intraperitoneal; LD, low-dose; MTX, methotrexate; PBS, phosphate buffered saline; RA, rheumatoid arthritis; ROS, reactive oxygen species; SEM, standard error of the mean; TNF, tumor necrosis factor; TZ, 1,3-thiazolidin-2-one.

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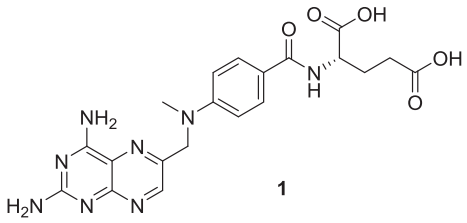


Fig. 1. Methotrexate (**1**) is the standard of care for treatment of RA.

anti-inflammatory effects in patients suffering from RA [3–5].

Unfortunately, in spite of LD-MTX being the standard of care in managing RA, treatment is still unsatisfactory for many patients. Low-dose treatment is associated with several prominent adverse effects, in particular gastrointestinal toxicities but also hepatotoxicity, lethargy, fatigue, nodulosis, hepatic and pulmonary fibrosis, renal insufficiency, anemia, and neutropenia [4]. Furthermore, LD-MTX suffers from high interindividual variability, leading to unpredictable treatment outcomes and, in many cases, poor patient response or lack of efficacy [4,6]. Consequently, nearly half of patients discontinue treatment within 3 years of therapy [7]. Currently, the best alternative is combinational therapy using MTX with newer biological therapeutics such as the anti-TNF-α agents etanercept or infliximab. However, such treatments are extremely costly and are associated with additional side effects [3,4]. Thus, there is still an urgent need for improving the efficacy and safety of RA therapies.

Prodrugs are inactive forms of pharmaceuticals that undergo chemical or enzymatic conversion *in vivo* to release the active agent [8–10]. Prodrug design is typically implemented to improve undesirable absorption, distribution, metabolism, excretion, toxicity (ADMET) properties, typically poor solubility or absorption, but it may also be exploited to increase tissue selectivity. While several prodrugs, drug conjugates, and drug delivery systems for MTX have been reported in the literature over the years, so far none have been approved for clinical use [11].

Reactive oxygen species (ROS), e.g. H_2O_2, $O_2^{\cdot-}$, HO$^{\cdot}$, and HOCl, serve important physiological roles that include signaling functions, host defense, and oxidative biosynthesis [12,13]. However, elevated levels of ROS can lead to harmful oxidative stress, which is a central component of the pathogenesis of chronic inflammation [14–17], including autoimmune disease such as RA [18,19]. H_2O_2 is the most stable of the ROS and under pathological conditions, extracellular concentrations up to 1 mM of H_2O_2 have been measured [12,20–23]. This increased concentration of H_2O_2 can potentially serve as a stimulus for site-selective drug delivery of ROS sensitive prodrugs. Development of such prodrugs has emerged as a novel approach to potentially increase target selectivity of drugs. Examples of H_2O_2 sensitive promoieties include phenylboronic acids and esters [24–29], phenylsulfonate esters [30,31], *N*-(2,5-dihydroxyphenyl)acetamides [32], α-boryl ethers, carbonates, and acetals [33], and 1,3-thiazolidin-2-one has also been proposed as a promising ROS labile promoiety [34].

Recently, our group published promising preliminary results for the treatment of RA using a boronic acid based MTX prodrug *in vivo* [35]. Based on these results and the promising ROS sensitive properties of the 1,3-thiazolidin-2-one moiety (henceforward simply thiazolidinone or TZ), we here propose a new strategy for the treatment of RA using a thiazolidinone-based MTX prodrug for site-selective delivery of MTX. Our aim is to localize and accumulate MTX in inflammatory tissue in order to improve the safety profile and potentially the efficacy of the drug. We herein report the synthesis, *in vitro* pharmacokinetic and physiochemical studies, and *in vivo* evaluation in a murine collagen-induced arthritis (CIA) model.

1.2. Strategy

The thiazolidinone group can be introduced through coupling to a carboxylic acid. As MTX contains two carboxylic acids (α and γ), we planned at first, to synthesize three MTX prodrugs: MTX-α-TZ (**2**), MTX-γ-TZ (**3**), and MTX-TZ_2 (**4**) to explore the potentially different behavior of the compounds (Fig. 2).

2. Results and discussion

2.1. MTX-TZ_2 (**4**)

2.1.1. Synthesis

As a preliminary test of the hypothesis, MTX-TZ_2 (**4**) was initially synthesized to investigate its properties for use as a prodrug. Starting from commercially available MTX, MTX-TZ_2 was obtained in one step using DCC and DMAP for the coupling (Scheme 1).

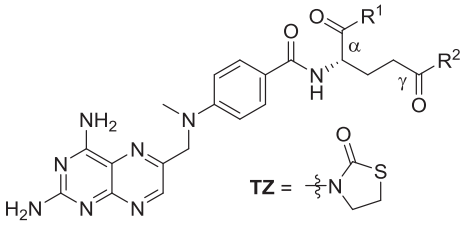
2.1.2. Preliminary evaluation of prodrug stability

As a first measure of prodrug potential, the aqueous stability of the synthesized prodrug candidate was examined in PBS (pH 7.4) at 20 °C using RP-UPLC-MS analysis. Interestingly, the α-thiazolidinone moiety of MTX-TZ_2 (**4**) was highly labile and rapidly hydrolyzed to release MTX-γ-TZ (**3**) within 30 min (Figure S1, SI). On the other hand and to our delight, MTX-γ-TZ (**3**) was significantly more stable and no hydrolysis of this compound was observed even after 24 h. Based on the short half-life of the α-thiazolidinone moiety, MTX-α-TZ (**2**) was deemed to likely be a poor prodrug candidate and we decided to pursue MTX-γ-TZ as the most promising of the three thiazolidinone-based MTX prodrugs shown in Fig. 2.

2.2. MTX-γ-TZ (**3**)

2.2.1. Synthesis

MTX-γ-TZ (**3**) was synthesized from the commercially available pteridine alcohol **5** (Scheme 2). The commercially available hydrochloride salt of **5** was first neutralized with aqueous NaOH and then converted to the corresponding α-bromo species *in situ* using PPh_3Br_2 followed by nucleophilic substitution with 4-(methylamino)benzoic acid to form **6**. Coupling of H-Glu (OMe)-O*t*Bu with **6** using PyBOP and Et_3N with subsequent hydrolysis of the γ-methyl ester using $Ba(OH)_2$, afforded MTX-α-O*t*Bu (**7**). The thiazolidinone moiety was then introduced to the γ position through a DCC/DMAP-mediated coupling with the unprotected γ-carboxylic



MTX-α-TZ (**2**): R^1 = TZ, R^2 = OH
MTX-γ-TZ (**3**): R^1 = OH, R^2 = TZ
MTX-TZ_2 (**4**): R^1 = TZ, R^2 = TZ

Fig. 2. The three targeted thiazolidinone (TZ) based MTX prodrug candidates: Methotrexate-α-thiazolidinone (MTX-α-TZ, **2**), methotrexate-γ-thiazolidinone (MTX-γ-TZ, **3**), and methotrexate-α,γ-dithiazolidinone (MTX-TZ_2, **4**).