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Auxiliary *in vitro* and *in vivo* biological evaluation of hydrogen peroxide sensitive prodrugs of methotrexate and aminopterin for the treatment of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes severe joints damage and other extra-articular alterations. Despite the efficacy of low-dose methotrexate (LD-**MTX**) in RA treatment, adverse effects are the predominant reasons for discontinuation of therapy. As a therapeutic targeting strategy, the presence of increased concentrations of reactive oxygen species (ROS) in the inflammatory environment can serve as the stimulus for prodrug activation in site-selective drug delivery systems. Our group has previously reported novel ROS sensitive prodrugs (**1**–**3**) of **MTX** and aminopterin (**AMT**) for site-selective delivery to inflammatory tissue associated with RA, with the aim of reducing side effects in RA therapy. Herein, we investigate the effect and toxicity of the same prodrugs in a rat CIA (collagen-induced arthritis) model of RA. We find that prodrug **1**, an arylboronic acid ROS-sensitive **MTX**-prodrug, displays similar *in vivo* efficacy as **MTX** at an equimolar dose, while avoiding adverse effects known to restrict **MTX** treatment. To further characterize prodrug **1** and its ROS mediated activation, we synthesized compound **4**, a negative control lacking the boronic acid moiety. We then investigated the effect of molecules on cell proliferation and cytotoxicity in the presence of the ROS scavenger pyruvate, as well as their stability in buffer and cell media, demonstrating a direct correlation between ROS concentration and the prodrug activity. Moreover, the *in vitro* ADME properties were investigated, including permeability, rat plasma and microsomal stability.

Keywords

Rheumatoid arthritis; Inflammation; Methotrexate; Aminopterin; Prodrug; Boronic Acids; Reactive oxygen species

Introduction

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory joint disease and is associated with joints damage and progressive disability.[1] RA is considered a burden for both the individual and society and the factors causing disease remain unknown.[2] Advances in understanding the pathogenesis of the disease have brought new treatment strategies, but several unmet needs persist.[3] Aminopterin (**AMT**) was initially tested for RA treatment[4] but its toxicity and low therapeutic index led to the development of methotrexate (**MTX**) as an alternative antifolate for cancer therapy, which later showed antiarthritic properties as a disease modifying antirheumatic drug (DMARD).[5] Despite the advent of effective biologic DMARDs, **MTX** remains the anchor drug for the treatment of

RA.[6] Folate antagonism is an important mechanism of action for **MTX**, but other anti-inflammatory activities contribute to the complex pharmacology that makes low-dose methotrexate efficacious in the treatment of inflammatory disorders.[7,8] One of the drawback of these complex and multifaceted mechanisms of action is the variety of side effects that are often associated with **MTX** therapy and responsible for the discontinuation of treatment in many cases (e.g. gastrointestinal side effects, hepatotoxicity, nephrotoxicity, pulmonary and hematologic toxicity).[9]

Reactive oxygen species (ROS, *e.g.* H_2O_2), have important physiological roles, and oxidative stress has also been described to be an important factor in the pathogenesis of inflammation in RA.[10] In order to counter balance this oxidative dysfunction, large amounts of antioxidants like glutathione (GSH) are recruited in the inflammatory region, contributing to a change in the local microenvironment that has been suggested as a means to control release of therapeutic agents in RA treatment.[11,12]

Prodrugs are chemically modified molecules that possess little or no pharmacological activity and that are converted to the active parent drug *in vivo* by enzymatic or chemical reactions.[13] The presence of increased concentrations of ROS (among which H_2O_2 is the most stable species[14]) in the inflammatory environment found in cancer and inflammatory diseases has been extensively exploited recently as stimulus for prodrug activation in site-selective drug delivery systems.[15]

In this regard, our group has previously reported promising preliminary results for the treatment of RA using boronic acid and thiazolidinone based **MTX** or **AMT** ROS-sensitive prodrugs in a collagen-induced arthritis (CIA) mouse model of RA.[16,17] Among the set of compounds previously synthesized and investigated (Figure 1), prodrugs **1**, **2**, and **3** showed good physicochemical and pharmacokinetic properties *in vitro*, as well as equivalent efficacy and lower toxicity in comparison to parental drugs *in vivo*.

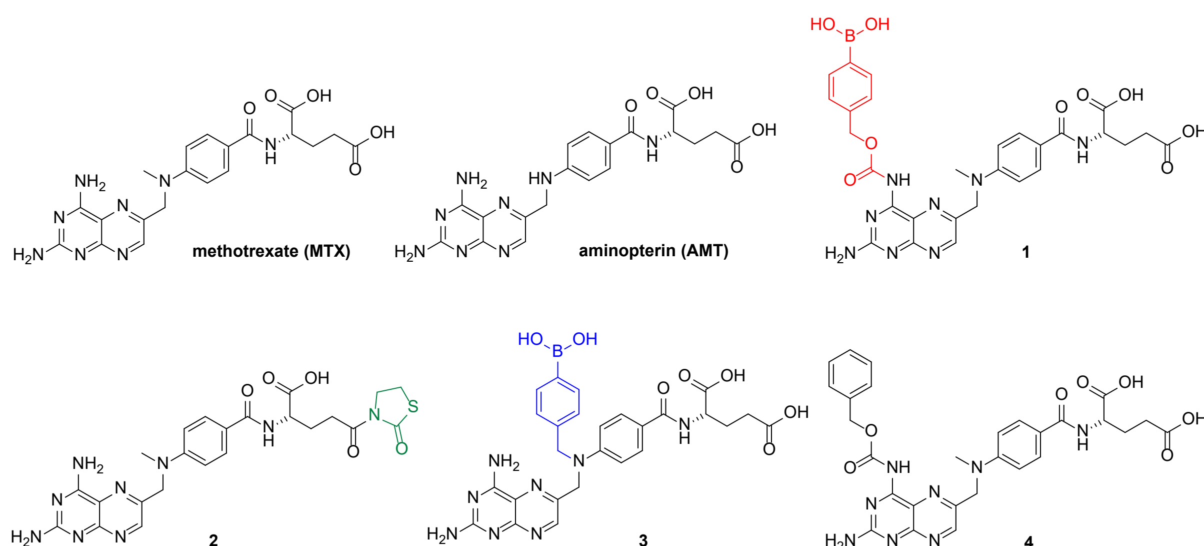

Figure 1: Structures of compounds investigated in the current study.[16,17]

Following these results, we herein present additional *in vivo* studies and further *in vitro* biological evaluation to assess the prodrugs activation by ROS and their biological profile compared to the parent drugs. In this regard, we also present the synthesis and biological evaluation of the negative control of prodrug **1**, compound **4**, lacking the ROS sensitive boronic acid moiety.

Results and discussion

In vivo efficacy validation in rat CIA model of RA and preliminary toxicity

The aim of this study was to investigate the efficacy of the prodrugs **1**, **2**, **3**, and their parent drugs **MTX** and **AMT** in a rat CIA animal model. This model was chosen because it mimics, to a large extent, the criteria used for diagnosis of rheumatoid arthritis in humans.[18]

A dosing regimen equimolar to 0.3 mg/kg **MTX** with daily intraperitoneal (*i.p.*) injections was chosen and to minimize the anti-inflammatory effects of DMSO,[19] its concentration was kept to 2% (v/v). Therapeutic intervention was initiated at the onset of the disease (when a mean arthritis severity score > 2 was reached, on day 17) and terminated at day 31 (Supporting Information). Disease progression was followed macroscopically throughout the experiment and the results reported in Figure 2 (A, B). As evident from the figure, animals treated with prodrug **1** showed significantly ameliorating effects on disease until termination of the treatment, similarly to **MTX** treatment. Comparatively, animals treated with prodrug **2** had significantly higher arthritis severity.

As a first estimate of drug tolerability, the body weight of the rats was evaluated as an indicator of general health (Figure 2, B). Animals treated with prodrugs **1**, **2**, and **3** had a significantly higher weight compared with **MTX** treated animals. Animals treated with prodrug **3** had significantly higher arthritis severity compared with the **AMT** group, but also a higher average body weight. No adverse effects from treatments were observed in animals treated with the prodrugs **1**, **2**, and **3**. **AMT**-administered animals displayed a severe decline in general health and were removed from the study pre-termination on day 20–21. Animals treated with **MTX** also lost weight from day 23, declined in general health and were removed pre-termination from days 24–29.

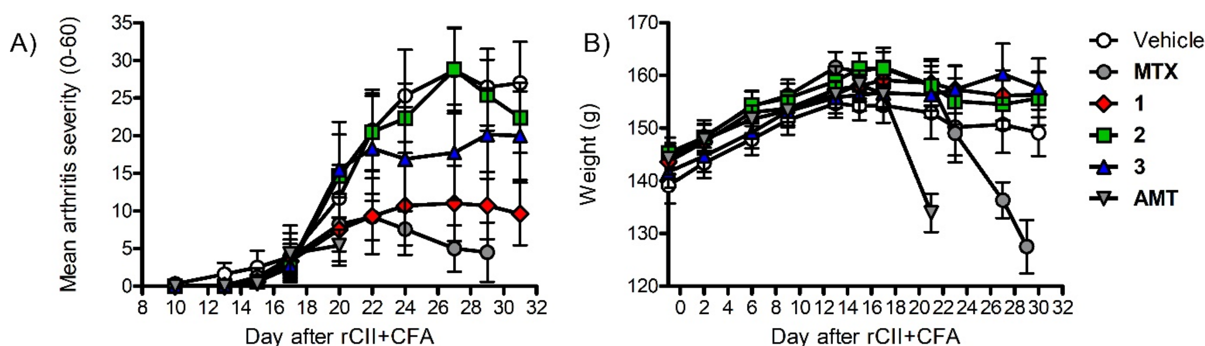

Figure 2: A) suppression of CIA development in rats after treatment with **MTX** (0.30 mg/kg, *i.p.*), prodrug **1** (0.42 mg/kg, *i.p.*), prodrug **2** (0.36 mg/kg, *i.p.*), prodrug **3** (0.38 mg/kg, *i.p.*), **AMT** (0.29 mg/kg, *i.p.*), and vehicle (n = 8 per group, n = 10 for vehicle). Dark agouti (DA) rats were given the indicated amounts of compound daily, starting at onset of disease (day 17), and disease progression was evaluated three times per week. A macroscopic scoring system of the four limbs ranging from 0 to 15 (1 point per swollen toe, 1 point per swollen foot knuckle, and 5

points for swollen ankle) was used for a maximal score of 60 per rat. Data represents mean values of arthritic score ± SEM. B) the general health of mice was evaluated as the average body weight during collagen-induced arthritis as an indication of drug tolerability. Measurements were performed three times per week. Five animals, one in the vehicle group, two in prodrug **2** group and two in prodrug **3** group were sacrificed pre-termination, due to a high score. Animals in the **MTX** group were removed on days 24, 27–29 and the animals in the **AMT** group were removed on day 22 due to a decline in general health. Data represents mean values of body weight ± SEM. Please note that statistical analysis and comparison between compounds of interest and parental drug can be found in Supporting Information (Figure S1 and Figure S2).

Micronuclei are extra-nuclear bodies that contain chromosomes fragments and/or whole chromosomes that come from DNA breakage or disruption of the mitotic apparatus and their presence is a good indication of genotoxicity of the compounds under study.[20] A micronucleus assay was performed according to the protocol reported in the Supporting information. The experiment demonstrates a significantly lower micronuclei count in animals treated with prodrug **1** in comparison to all other treatments including **MTX** (Figure 3).

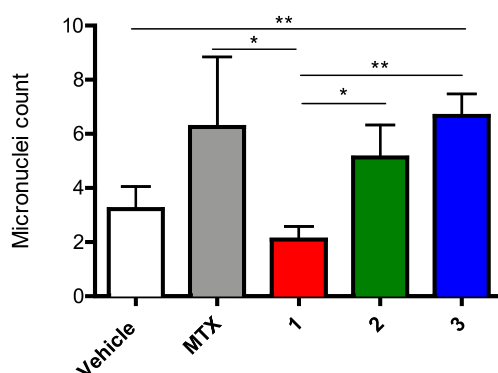

Figure 3: Evaluation of genotoxicity by bone marrow cell extraction and staining was performed according to the procedure reported in the Supporting Information. Observations were made with a Leica microscope (Epi-Fluorescence- DMRE) with a 100x oil immersion objective and micronuclei were manually counted from five images from a central area of the smear acquired from each animal using LARS.4.8 software. Graph represents the mean of total micronuclei count per animal for each treatment group. * represents a p–value < 0.05, ** represents a p–value < 0.01. Micronuclei example pictures can be found in Supporting Information (Figure S3).

Cytotoxicity – cell viability and induction of apoptosis

In order to better understand the results observed for the *in vivo* study and the data previously reported,[16,17] we further investigated the biological profile and *in vitro* activity of prodrugs **1**, **2**, and **3**. The negative control of prodrug **1**, compound **4** (Figure 1) lacking the boronic acid moiety, was synthesized (synthesis and characterization in the Supporting Information, Figure S4) to demonstrate the role of the boronic acid moiety in ROS activation and release of **MTX**.[21,22]

It is important to show that the prodrugs are not cytotoxic in cells when not activated by ROS. To assess this issue, the cytotoxicity of the compounds of interest at different ROS concentrations was assessed. HL-60 (human leukemia cancer cell line), HEK293 (human embryonic kidney cell line), and

HeLa (cervical cancer cell line) were chosen for their different capacity of ROS production and scavenging.[23–25] Moreover, pyruvate was used as an active ROS scavenger[26,27] and ROS levels were investigated by using fluorescence from the ROS probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Supporting Information). As supported by Figure S5, HEK293 cell line has a similar ROS production regardless of whether pyruvate is added to the media.[24] The scavenging capacity of pyruvate is much more evident in the HL-60 cell line, where an 8.7-fold decrease in the ROS level was observed after 48 hours in the presence of pyruvate. Cell viability after 48 h of cells treated with prodrug **1**, **2**, **3**, their parental drugs **MTX**/**AMT**, and compound **4**, was evaluated for HL-60, HEK293 and HeLa cell lines, also using pyruvate as ROS scavenger (Table 1).

Table 1: IC_{50} (mean of at least three replicates ± SEM, nM) values of compounds **1**–**4**, **MTX**, and **AMT** in HL-60, HEK293, and HeLa.

Compd.	IC_{50} (mean ± SEM, nM)					
	HL-60		HEK293		HeLa	
	(+) pyr	(-) pyr	(+) pyr	(-) pyr	(+) pyr	(-) pyr
1	203.2 ± 38.8	40.3 ± 5.6	191.8 ± 9.5	62.9 ± 7.6	193.5 ± 17.3	156.0 ± 25.0
2	36.0 ± 3.9	40.0 ± 7.0	nd	nd	207.2 ± 127.6	163.7 ± 15.1
3	38.3 ± 6.9	7.5 ± 0.8	nd	nd	42.2 ± 5.0	19.2 ± 3.3
4	nd	> 1000	nd	> 1000	nd	nd
MTX	9.8 ± 1.3	8.9 ± 1.5	35.0 ± 0.1	28.6 ± 4.3	59.3 ± 0.1	21.0 ± 0.1
AMT	3.0 ± 0.4	2.5 ± 0.2	nd	nd	3.6 ± 0.9	8.3 ± 0.8

nd = not determined

From the results reported in Table 1, there is a clear difference in cytotoxicity between prodrugs **1**–**4** and their parent drugs **MTX** and **AMT**. Interestingly, cytotoxicity of the prodrugs toward all the cell lines was significantly lower with added pyruvate, presumably because the ROS scavenger inhibited the release of the parent drug. For prodrugs **2** and **3**, the IC_{50} fold difference to **MTX** and **AMT** respectively is not large, but for prodrug **1**, the difference to **MTX** is up to 20 fold in HL-60 (+) pyruvate (Figure 4). As expected, pyruvate does not seem to play any role in the mechanism of toxicity for **MTX** and **AMT**, while, especially for prodrug **1**, the difference between (+) and (-) pyruvate is 5 fold in HL-60 and HEK293 cell lines (Figure 4). To have a more detailed and realistic comparison, compound **4** was tested in HL-60 and HEK293 cell lines (-) pyruvate. It afforded a much higher IC_{50} value (> 1 μM) compared to **MTX** and **AMT**, confirming the importance of the boronic acid moiety and its self-immolative, oxidative deboronation as the mechanism of prodrug activation.

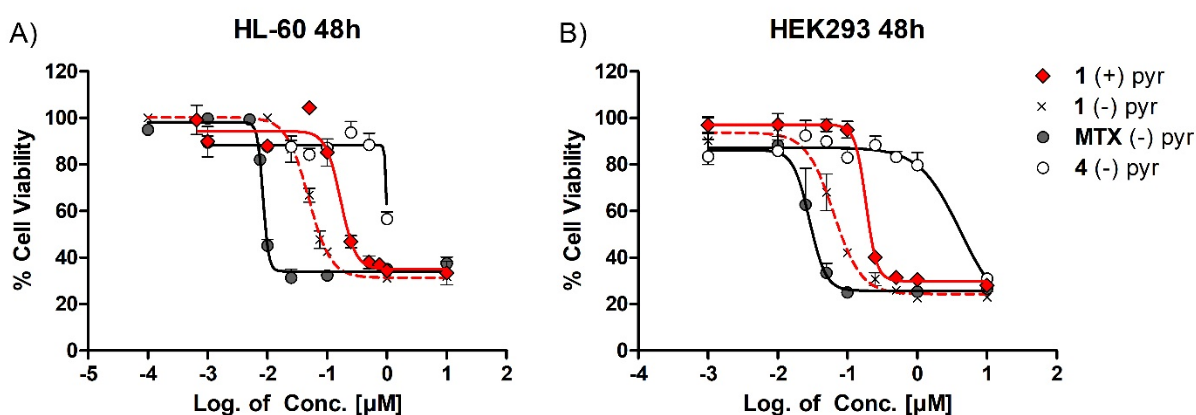

Figure 4: Cell viability of prodrug **1** (± pyruvate), **MTX** (- pyruvate), and negative control **4** (- pyruvate) in HL-60 (A) and HEK293 (B) cell lines. Results were calculated as mean of at least three replicates (mean ± SEM, n = 3).

The mechanism by which **MTX** exhibits its anti-inflammatory effect in RA have been connected to the induction of apoptosis.[28] The induction of apoptosis by **MTX** and prodrug **1** were investigated by flow cytometry in HL-60 cells (-) pyruvate treated with 10 μM, 1 μM, and 0.1 μM of compound, similar to previous experiments conducted for MTX.[29] As shown in Figure 5, the percentage of apoptotic cells gradually increased from around 3% of the vehicle (DMSO 0.1%) to around 17% as the HL-60 cells were treated with increasing concentration of prodrug **1** and **MTX**. The difference in apoptotic cells percentage between cells treated with prodrug **1** and **MTX** was highest at 0.1 μM, where **MTX** resulted in 14% apoptotic cells and treatment with **1** afforded only 7%. The flow cytometry results confirm the viability measurements reported in Table 1 obtained using the Alamar blue assay.

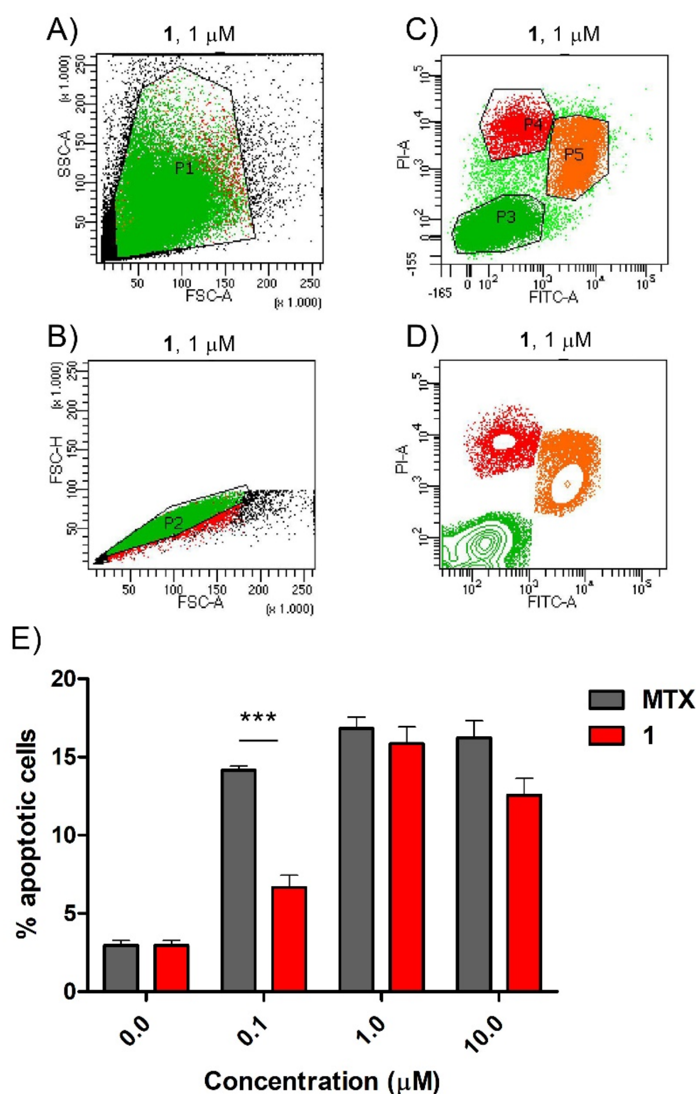

Figure 5: Flow cytometric analysis of AnV and PI stained HL-60 cells in RPMI media, treated with prodrug **1** (1 μM) for 48 hours (for **MTX** treated cells and other concentrations, see Supporting Information Figure S6): (a) shows all the data obtained when samples were analyzed at 100 000 events per second; (b) P2 indicates cell singlets; (c) indicates different cell populations: P3 is viable cells, while P4 and P5 are dead cells; (d) contour plot

of the cell populations; (e) percentage of apoptotic cells (P4 + P5) as a function of the concentration of **1** and **MTX**. Results were calculated as mean of at least triplicates (mean ± SD, n ≥ 3). Statistics were calculated using a two-tailed unpaired t-test, where $p < 0.05$ was considered significant. *** represent a p-value < 0.001.

To account for the relatively high activity of prodrugs **1**–**3** (IC_{50} values of 7–200 nM) as well as the ability of **1** to induce apoptosis, the presence of residual ROS in the media and the chemical stability of the molecules during the assay conditions were investigated.

In vitro evaluation

Among all the different prodrugs synthesized, **1** was considered the most promising both in terms of efficacy in the rat CIA model, but also when comparing the relative toxicity of the prodrug and its parent drug. In order to further explain the *in vivo* and cellular results, we investigated the stability and ADME profile of **1** compared to **MTX** and the control compound **4** (Table 2). The experiments were designed to investigate the ROS activation of **1** and to understand if the activity stems from unspecific hydrolysis or can be attributed to selective activation by ROS.

Table 2: Pharmacokinetic and physiochemical properties of **1**, **4**, and **MTX**. Values are an average of at least three measurements ± standard deviation (SD).

	H_2O_2 activation (1/0.25 mM) $t_{1/2}$ (h)	PBS stab. $t_{1/2}$ (h)	Culture media stab.[a] $t_{1/2}$ (h)	Culture media stab. in the presence of HEK293[b] $t_{1/2}$ (h)	Culture media stab. after pre-incubation with HEK293[c]	Culture media stab. in the presence of HEK293 and PQ (10 mM)[b] $t_{1/2}$ (h)	Rat Plasma stab. $t_{1/2}$ (h)	Rat microsomes stab., CL'_{int} (mL/min/kg)	PAMPA (10^{-6} cm/s)	clogP[d]
1	0.29/2.34[16]	>24[16]	3.4 ± 0.6	107.2 ± 41.3	16.1 ± 8.9	63.2 ± 11.6	16.4±4.1	1.8±0.8	< 1.50	-0.59
4	> 24	nd	>24	nd	nd	nd	nd	nd	< 1.50	1.10
MTX	nd	>24	>24	>24	nd	nd	nd	1.5±0.7	< 1.50	-0.42

[a] media used is without pyruvate and the measurements are in the absence of cells
[b] media used is without pyruvate and the media is sampled and analyzed at different time points
[c] media used is without pyruvate and pre-incubated with HEK293 cells for 24 h before removal of cells and addition of compound
[d] consensus log $P_{o/w}$ calculated with SwissADME[30]

Our group has previously reported the *in vitro* activation profile of prodrug **1** with H_2O_2.[16] Compound **4** was subjected to similar conditions (Table 2, Figure S7), demonstrating that compound **4** was not converted to **MTX** over 24 h. This experiment supports the specific activation of prodrug **1** by ROS *in vitro* and its stability to unspecific background hydrolysis.

MTX was stable for 24 h in PBS and culture media both in the presence and absence of HEK293 cells (Table 2, Figure S8). Similarly, prodrug **1** was stable in PBS, but its half-life in culture media was reduced to 3.4 h, with only 1% of **1** remaining after 24 h (Table 2, Figure S9). In contrast, **4** was stable in culture media for 24 h. A likely explanation for this observation is the presence of ROS in the media.[31,32]

Interestingly, **1** displayed better chemical stability in culture media after pre-incubation with cells and in media where HEK293 cells are present - up to 80% of the prodrug remained in the media after 24 h (Table 2, Figure S9). As previously reported,[33] this can likely be explained by antioxidants released by the cells that help maintaining ROS homeostasis, resulting in reduced activation of prodrug **1** to **MTX**.[34]

To further link activation of **1** with ROS, media stability during HEK293 cell growth with added paraquat (PQ) was tested (Table 2, Figure S9). PQ is a bipyridyl quaternary ammonium salt known to induce cellular ROS production,[35] which was confirmed using the DCF assay, in which the relative ROS levels in HEK293 cells with and without 10 mM PQ were measured (Table 2, Figure S10). The stability of prodrug **1** in culture media in the presence of HEK293 with PQ (10 mM) in the first 24 h was similar to the one of **1** without PQ and a small difference could be observed only after 48 h (Figure S9), in accordance with previous reports of PQ induction of ROS levels for prochelators.[36]

Plasma stability was examined in reconstituted freeze dried rat plasma diluted 1:1 with PBS to ensure a pH of 7.4.[37] At 37 °C, the half-life of **1** was determined as 16 ± 4 h indicating good plasma stability (Table 2, Figure S11).

Metabolic stability was evaluated by determining the apparent intrinsic clearances (CL'_{int}) in pooled rat liver microsomes. The apparent intrinsic clearance was calculated to be 1.8 ± 0.8 mL/min/kg for prodrug **1** and 1.5 ± 0.7 mL/min/kg for **MTX** indicating low intrinsic clearance and a profile similar to **MTX**[38] (Table 2, Figure S12).

The parallel artificial membrane permeability assay (PAMPA) assay gives a quick, simple determination of passive transport through biological membranes.[39] A new artificial membrane that consists of a lipid/oil/lipid tri-layer in the porous filter was used, as it is known to give good predictability and agreement with data obtained for absorption in the Caco-2 cell line.[40] The results of the permeability are reported as a permeability coefficient (Pe, for high permeability, Pe > 1.50×10^{-6} cm/s) and compared to caffeine, known as a well-permeable molecule.[40,41] As shown in Table 2, **MTX**, prodrug **1**, and negative control compound **4** displayed no permeability.[42] The hydrophilicity of prodrug **1** is similar to **MTX** (clogP, Table 2) explaining the low permeability. **MTX** is actively transported across membranes by different transporters[7,43] and, having ruled out passive diffusion, prodrug **1** is likely activated to **MTX** by extracellular ROS and then internalized in cells as **MTX**.

Conclusions

In summary, we have described further *in vivo* and *in vitro* studies to support the use of different prodrugs, and in particular **1**, as a ROS sensitive strategy for targeted **MTX** therapy in RA.

These findings confirmed that **1** is activated by ROS and is efficacious in a rat CIA model of arthritis, exhibiting lower toxicity than **MTX**. Although the activity of prodrug **1** in our *in vivo* model resembled **MTX**, it did not surpass it, and further studies will be needed to distinguish between a targeted therapy and a systemic slow-release of **MTX**. In this regard, ROS sensitive functionalities, as the boronic acid, are labile functionalities, sensitive to ROS but in some extent also to background hydrolysis. Our cellular studies demonstrate that the activity of prodrug **1** is clearly attenuated compared to the parent drug while the *in vivo* efficacy is maintained with significantly reduced toxicity. Nonetheless, it is virtually impossible to avoid some non-specific activation *in vivo* and it must be expected that the

prodrug can be activated in tissues other than synovium, also by mechanisms that are independent of ROS levels. The consequence of this is that the biodistribuition of the targeted therapy can be unpredictable, also because boronic acids can react with endogenous nucleophiles, leading to unfavorable pharmacokinetics.[44–47]

For these reasons, evidence for accumulation, activation and retention of prodrugs at the site of inflammation is needed to support the theory of targeted therapy. The synovial fluid concentrations of a small molecule or a delivery system is determined by synovial blood flow, plasma concentration, microvascular permeability, and lymphatic drainage. Unfortunately, the primary obstacle preventing use of rodent synovial fluid for accumulation studies is the lack of reliable methods for its harvesting.[48,49] Some methods have been developed recently,[48,49] but have been mainly used to quantify inflammatory mediators and cells.[50] Moreover, quantification of **MTX**, other DMARDs and derivatives has mostly been done in human synovial fluid[51] or in larger model animals such as rabbits.[52,53]

Further investigations that address the points discussed above are important for the successful advancement of prodrug strategies relying on ROS activation and should be taken into account as early as possible in discovery.

Acknowledgments

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