

SHORT REPORT

In vitro evaluation of disease-modifying antirheumatic drugs against rheumatoid arthritis associated pathogens of the oral microflora

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ABSTRACT

Objectives In the past, the human microbiome has consistently been associated with rheumatoid arthritis (RA) and disease activity. Here, we investigate the antimicrobial activity of disease-modifying antirheumatic drugs (DMARDs) against typical representatives of the oral microflora that have been associated with RA.

Methods DMARDs were screened for antimicrobial activity against bacteria that are associated with the pathogenesis of the disease and/or frequently isolated from the oral microflora of patients with RA. Screening was done by an agar diffusion assay and minimum inhibitory concentrations (MICs) of antimicrobial active substances were then determined by broth dilution.

Results Aurothiomalate and sulfasalazine demonstrated broad-spectrum antimicrobial activity, but with MICs ranging from 18 to >280 µg/mL and 150 to >600 µg/mL, respectively, only at supratherapeutic concentrations. Methotrexate showed antimicrobial activity only against *Fusobacterium nucleatum* and *Viridans streptococci*. The corresponding MICs were 3.75 to >30 µg/mL and 0.5–15 µg/mL, respectively, thus at least for streptococci, within the therapeutically achievable range. No other DMARD tested showed antimicrobial activity in the agar diffusion screening assay.

Conclusion Methotrexate, sulfasalazine and aurothiomalate showed antimicrobial activity against a broad spectrum of RA associated pathogens of the oral microflora. While methotrexate showed relevant antimicrobial activity, and to a more limited extent aurothiomalate, sulfasalazine was active only at far supratherapeutic systemic concentrations. Nevertheless, given the highly species-dependent antimicrobial activity and the multiple ways it can affect the human microbiome, our results suggest a link between antimicrobially active antirheumatic drugs and their potential effect in the treatment of RA.

INTRODUCTION

In recent years, there has been an increasing body of literature demonstrating a link between distinct microbial infections, periodontal disease (PD) and the initiation and

Key messages

What is already known about this subject?

- A growing body of literature indicates a microbial origin for the pathogenesis of rheumatoid arthritis.

What does this study add?

- Disease-modifying antirheumatic drugs (DMARDs) possess a strongly species-dependent antimicrobial activity against organisms of the oral microflora.
- While methotrexate and, to a lesser extent, aurothiomalate showed relevant antimicrobial activity, all other DMARDs tested were active only at far supratherapeutic concentrations.

How might this impact on clinical practice or further developments?

- Future studies linking target site pharmacokinetics, mucosal immune response and oral microflora alterations are needed.

perpetuation of rheumatoid arthritis (RA).^{1–3} Some of the hypothesised pathogenic mechanisms are the generation of neoautoantigens by citrullination of proteins through bacterial enzymes, as well as triggering autoantibody production by molecular mimicry and the expression of bacterial superantigens.^{2 3} It has been shown that *Porphyromonas gingivalis*, one of the most common causative pathogens of PD, is the only prokaryotic organism with the ability to generate citrullinated autoantigens using its unique peptidylarginine deiminase. This was supported by an experimental arthritis model in mice, which showed that *P. gingivalis* increased levels of citrullinated proteins and autoantibodies, resulting in accelerated progression and aggravated disease severity.⁴ Furthermore, in patients with RA, the levels of anticyclic citrullinated peptide antibodies and rheumatoid factor were shown to correlate with *P.*

gingivalis antibodies and even direct DNA detection of periodontitis pathogens from serum and synovial fluid was achieved.^{2,5} In this context, the oral microflora and especially common periodontal pathogens have been extensively studied. In addition, more common infectious agents such as *Proteus mirabilis*, a frequently isolated urinary tract pathogen, or *Staphylococcus aureus*, have also been described in the context of RA.^{2,3}

First-line treatment of RA consists of conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) such as methotrexate (MTX) or sulfasalazine (SSZ), both of which have been shown to have antimicrobial activity against various prokaryotes.³ In addition, several studies demonstrated the utility of tetracycline antibiotics for treatment of RA, supporting a potential mechanism of action based on antimicrobial activity against disease-initiating and/or disease-exacerbating pathogens.³ Furthermore, although different in aetiology, the ability of bacterial infections to trigger aberrant immune responses is clearly evident also from diseases such as reactive arthritis.

Despite this evidence and the abundance of studies examining the oral and/or gut microbiome and subsequent changes in composition caused by specific antirheumatic drugs, there are few data on the direct antimicrobial activity of DMARDs, particularly against periodontal pathogens.^{6–14} One study conducted a high-throughput drug screening of 835 non-antibiotic agents to evaluate their potential antimicrobial activity against representative bacterial species of the human gut microbiome, but only two DMARDs, namely auranofin and MTX, were included.¹⁴ Overall, this in vitro study and previous microbiome studies demonstrated highly genus-dependent and/or species-dependent antimicrobial activity. Therefore, the potential antimicrobial activity of DMARDs against periodontal pathogens and commensals of the oral microflora cannot be extrapolated from the existing literature.

METHODS

Bacterial strains

We selected candidate organisms associated with the pathogenesis of RA or enriched in the oral microbiome of patients with active RA.^{2,3,6,9,15} In addition, *S. aureus*, *P. mirabilis* and *Escherichia coli*, which have also been described as potential disease-initiating and/or disease-exacerbating pathogens, were investigated. All bacteria used in the present study were clinical isolates of the oral microbiome, kindly provided by the Department of Clinical Microbiology of the Medical University of Vienna. In addition following standardised strains from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) were used: *Actinomyces naeslundii* DSMZ-43013, *Capnocytophaga sputigena* DSMZ-7273, *Eikenella corrodens* DSMZ-8340, *E. coli* ATCC-25922, *Fusobacterium nucleatum* DSMZ-15643, *Parvimonas micra* DSMZ-20468, *P. gingivalis*

DSMZ-20709, *Prevotella intermedia* DSMZ-20706, *P. mirabilis* ATCC 14273, *S. aureus* ATCC-29213 and *Streptococcus salivarius* ATCC-7073.

DMARDs and controls

For this study, csDMARDs were selected for which antimicrobial activity was considered likely, based either on the existing literature or their structure: azathioprine, D-penicillamine, sodium-aurothiomalate-hydrate (ATM), SSZ (SSZ; all from Sigma-Aldrich, Austria) and chloroquine-diphosphate, hydroxychloroquine-sulfate, leflunomide, MTX, tofacitinib (all from Selleckchem, Germany). Sodium-penicillin-G (Sigma-Aldrich, Vienna, Austria) and sodium-cefuroxime (Fresenius-Kabi, Austria) served as positive controls for the agar diffusion assay.

In vitro susceptibility testing

Agar diffusion screening assay

Bacterial inocula were prepared by culturing anaerobic bacteria (*Actinomyces* spp., *Capnocytophaga* spp., *E. corrodens*, *F. nucleatum*, *P. micra*, *P. gingivalis* and *P. intermedia*) on prereduced Brucella agar plates with 5% horse blood for 72 hours at 36°C–37°C in an anaerobic chamber. Streptococci were cultured on Mueller-Hinton fastidious agar plates supplemented with 5% horse blood for 24 hours at 36°C–37°C in a CO₂ enriched environment while aerobic bacteria (*E. coli*, *P. mirabilis* and *S. aureus*) were cultured on Mueller-Hinton agar plates for 24 hours at 36°C–37°C. Colonies were suspended in prereduced brucella broth to a concentration equivalent to a McFarland 1.0 for anaerobic bacteria while aerobic bacteria and streptococci were suspended in sterile saline to a concentration equivalent to a McFarland 0.5. After inoculation of the respective agar plates with the bacterial inocula, 6 mm filter disks, impregnated with 20 µL of the tested drugs at concentrations of ×10 and ×100 Cmax for DMARDs and ×1 and ×10 Cmax for antibiotic controls were placed on the surface (online supplemental table S1). Inhibitory zones were measured after an anaerobic incubation of 48–72 hour, CO₂ enriched incubation of 24–48 hours and aerobic incubation of 24 hours at 36°C–37°C, depending on the respective organism. Anaerobic and CO₂-enriched environmental conditions were generated using AnaeroGen or CO₂Gen sachets in combination with an anaerobic jar (all Thermo Fisher Scientific/Oxoid, Austria).

Determination of minimum inhibitory concentrations

Bacterial inocula were prepared as described for the agar diffusion assay, except of the bacterial concentrations of anaerobic bacteria which were prepared at a McFarland turbidity of 0.5 for these experiments. As previously described, a serial tube dilution assay was used for minimum inhibitory concentrations (MIC) determination of anaerobic bacteria.¹⁶ For testing of aerobic bacteria and streptococci a microdilution assay in 96-well u-bottom microtiterplates with a total volume of 200 µL cation-adjusted Mueller-Hinton broth

Table 1 Antimicrobial activity of aurothiomalate, methotrexate and sulfasalazine against RA associated pathogens

	ATM	MTX	SSZ
Cmax	1.4–4 µg/mL ^{17 23}	0.2–0.6 µg/mL ²⁴	6.0 µg/mL(*)
<i>Anaerobic bacteria</i>			
<i>Actinomyces</i> spp (n=6)	>280 µg/mL (70; >280)	n.a.	>600 µg/mL (300; >600)
<i>Capnocytophaga</i> spp (n=6)	35 µg/mL (18; >280)	n.a.	>600 µg/mL (600; >600)
<i>Eikenella</i> spp (n=6)	>280 µg/mL (18; >280)	n.a.	>600 µg/mL (300; >600)
<i>Fusobacterium</i> spp (n=6)	53 µg/mL (18; 280)	30 µg/mL (3.75; >30)	300 µg/mL (150; 300)
<i>Parvimonas</i> spp (n=6)	210 µg/mL (70; >280)	n.a.	n.a.
<i>Prevotella</i> spp (n=6)	>280 µg/mL (>280; >280)	n.a.	n.a.
<i>Porphyromonas</i> spp (n=6)	53 µg/mL (17.5; 140)	n.a.	450 µg/mL (150; >600)
<i>Viridans Streptococci</i>			
<i>Streptococcus</i> spp (n=8)	>280 µg/mL (>280; >280)	2.3 µg/mL (0.5; 15)	n.a.
<i>Aerobic bacteria</i>			
<i>Escherichia coli</i> (n=6)	>280 µg/mL (140; >280)	n.a.	n.a.
<i>Proteus mirabilis</i> (n=6)	>280 µg/mL (>280; >280)	n.a.	n.a.
<i>Staphylococcus aureus</i> (n=6)	>280 µg/mL (>280; >280)	n.a.	n.a.

Minimum inhibitory concentrations, presented as median (min; max) µg/mL for disease-modifying anti rheumatic drugs which demonstrated antimicrobial activity in the diffusion disk screening assay.

Cmax, maximum serum concentrations from diverse pharmacokinetic studies, as shown in online supplemental table 1; n.a., not applicable because no antimicrobial activity was observed in the agar diffusion screening assay; For the calculation of median minimum inhibitory concentrations, the highest concentrations tested were used if no inhibition was observed.

*Pharmacokinetic data were taken from the summary of product characteristics (Sulfasalazin-Heyl, Germany).

ATM, Aurothiomalate; MTX, Methotrexate; RA, rheumatoid arthritis; SSZ, Sulfasalazine.

(CA-MHB) or CA-MHB supplemented with 5% lysed horse blood and 20 mg/L β-nicotinamide adenine dinucleotide were used, respectively. MICs were read after an incubation period of 18–24 hours for aerobic bacteria and streptococci as well as after 48–72 hours for anaerobic bacteria.

RESULTS

The agar diffusion screening assay revealed an antimicrobial activity of ATM against at least one isolate of all bacterial species tested. MTX demonstrated activity against *F. nucleatum* and *Viridans streptococci* whereas SSZ was active against *Actinomyces* spp, *Capnocytophaga* spp, *E. corrodens*, *F. nucleatum* and *P. gingivalis*. No other DMARD demonstrated antibacterial activity, whereas the antimicrobial controls penicillin-G and cefuroxime revealed high activity against all respective bacterial isolates tested.

MICs of ATM, MTX and SSZ are presented in table 1, online supplemental figure S1 and online supplemental table S2. Highest activity was observed for MTX against certain species of *V. streptococci* which were inhibited at concentrations ≥0.5 µg/mL. ATM showed highest activity against *Capnocytophaga* spp, *E. corrodens*, *F. nucleatum* and *P. gingivalis*, with MICs of >18 mg/L. All other substances revealed antimicrobial concentrations far above their maximum serum concentrations (data not shown).

DISCUSSION

In this study, ATM, MTX and SSZ demonstrated antimicrobial activity against a wide range of bacterial species associated with the pathogenesis of RA or frequently found in the oral microbiome of patients with the disease. MTX demonstrated relevant activity against *V. streptococci*, considering doses up to 20–30 mg/week. ATM also showed potentially relevant activity against some isolates of *Capnocytophaga* spp, *E. corrodens*, *F. nucleatum* and *P. gingivalis* but to a lesser extent than MTX against streptococci and only when considering concentrations achieved after parenteral administration of high doses in the range of 50 mg.¹⁷ In contrast to that, antimicrobially active concentrations of SSZ and the other DMARDs tested, are not reached systemically in sera of patients with RA receiving conventional doses (online supplemental table S1).

As recently shown by Nayak *et al* MTX, like its antimicrobial counterpart trimethoprim, acts by inhibiting the bacterial dihydrofolate reductase (DHFR), resulting in reduced purine, pyrimidine and amino acid biosynthesis and thus inhibition of replication.¹⁸ Considering possible resistance mechanisms to trimethoprim, conclusions can probably be drawn about the cause of the strongly species-dependent antimicrobial activity of MTX due to the similar mechanism of action. These comprise impaired permeability, intrinsic or acquired insensitive DHFR, compensatory increased production of target enzymes or the expression of efflux pumps.^{18 19}

It must be emphasised that some of the drugs used in the present study have multiple metabolites that may even possess increased antimicrobial activity.¹² Therefore, their potential influence on the microbiome and thus their potential role in the treatment of RA might be underestimated given the limited antimicrobial activity observed in the present study. Sulfapyridine has a close relationship with sulfonamide antibiotics and thus an expectation of one of the highest antimicrobial activity among all DMARDs. Nevertheless, some studies have shown only low activity against group-A streptococci and pneumococci.^{7 11} In addition, a study examining the antimicrobial activity of gold compounds against *Lactobacillus* spp and *Neisseria gonorrhoeae* and a study investigating the activity of sulfapyridine against *Mycobacterium avium* subspecies showed a highly variable antimicrobial activity.^{10 13}

Given the highly species-dependent antimicrobial activity observed in the present study, DMARDs might indirectly influence RA-initiating and/or RA-exacerbating pathogens through changes in the community composition of the human microflora. In addition to changes in the microbiome due to the direct antimicrobial activity of DMARDs, anti-inflammatory activity could also contribute via a variety of changes in the gut bacterial nutritional milieu and the resulting competition between beneficial and harmful pathogens.²⁰ As demonstrated by using a gnotobiotic mouse model, MTX-induced shifts in the gut microbiome, which primarily included decreased abundances of Bacteroidetes members, led to decreased activation of the host immune system and thus reduced inflammation.¹⁸ This is consistent with previous data in patients with active RA showing enrichment of proinflammatory pathogens such as *Prevotella copri* and reduced abundances of bacteria such as *Bacteroides* spp, *Blautia* spp and *Lachnospiraceae* clades possessing anti-inflammatory properties including the induction of regulatory T cells.²¹ This has also been supported by several studies among patients with RA showing an association between treatment efficacy of DMARDs and changes in the composition or partial restoration of the gut microbiome.^{6 8 15} Interestingly, a recent study found evidence that, particularly in patients with RA, the vast majority of oral microbes are transmissible to and subsequently colonise the colon.²² This suggests even further reaching implications of changes in the composition of the oral microflora induced by antimicrobially active DMARDs. Although the aforementioned mechanisms could contribute to the effect of DMARDs in the treatment of RA, no corresponding conclusions can be drawn based on the in vitro design of the present study.

In summary, ATM, MTX and SSZ demonstrated antimicrobial activity against a broad spectrum of RA-associated bacterial species but mostly at supratherapeutic systemic concentrations, although a potential biologically relevant effect through target site/organ enrichment must be considered. In this context, our results encourage future investigation of target site pharmacokinetics as

well as circumstantial mechanisms such as interactions of DMARDs with the host mucosal immune system and the specific influences of DMARDs on the human microflora over time in form of longitudinal case-control studies.

Contributors MK, SW and DA conceived this study and participated in its design and coordination. MK, MO and KS designed the various experiments including agar diffusion screening assay, tube dilution and broth microdilution. MK drafted the manuscript and SW and DA participated in the data analysis and revision of the manuscript. All authors read and approved the final manuscript.

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Patient consent for publication Not applicable.

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