

# Therapeutic Inhibition of Murine Collagen-Induced Arthritis by Non-Antibacterial Derivatives of Minocycline

P. J. Higgins, J. Zhang-Hoover \*, M. Mammolenti, R. Chang, T. Bowser, A. Verma, P. Abato, A. Kasper, K. Larson, S. Kumar and S. K. Tanaka  
Paratek Pharmaceuticals, Inc., 75 Kneeland Street, Boston, MA 02111



## Abstract

Clinical studies have demonstrated that minocycline can improve disease symptoms in rheumatoid arthritis (RA) patients. We generated non-antibacterial analogues of minocycline and tested them in the murine model of the disease, collagen-induced arthritis (CIA). Male DBA/1 mice were immunized intradermally with 200 µg of bovine type II collagen and boosted with collagen three weeks later. Minocycline and four non-antibacterial minocycline derivatives were administered i.p. beginning after disease onset. Paw thickness was measured and animals were scored daily. Treatment of CIA with dexamethasone and methotrexate inhibited paw inflammation by 82% and 45% at doses of 4 mg/kg and 12 mg/kg, respectively. Minocycline inhibited the disease by 22% at 25 mg/kg/day and 45% at 50 mg/kg/day. The minocycline derivatives each inhibited CIA more potently than minocycline, ranging from 60 to 81% inhibition of paw swelling at 25 mg/kg/day. The EC<sub>50</sub>s for CIA inhibition for minocycline derivatives were lower than those of minocycline and methotrexate. Footpad tissue levels of cytokines (IL-1, IL-6, RANKL and MCP-1) and a matrix metalloproteinase (MMP-9) were decreased after therapeutic treatment of mice with dexamethasone and methotrexate, but not with minocycline. Two minocycline derivatives, however, inhibited the level of these biomarkers in the footpad tissue. These compounds may be effective for the oral treatment of RA as alternatives to commonly-used cytotoxic drugs, without the adverse effects associated with chronic administration of antibacterial drugs.

## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune condition that is characterized by synovial infiltration of activated inflammatory cells, synovial membrane hyperplasia, neoangiogenesis, and progressive destruction of cartilage and bone. Conventional first line therapy for RA includes nonsteroidal anti-inflammatory drugs (NSAIDs) followed by disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate and hydroxychloroquine. Minocycline, a member of tetracycline family of antibiotics, has shown some beneficial effects in treating RA. A number of double-blind, placebo-controlled trials have concluded that early seropositive (<1 year of disease) RA patients respond positively to a 3-6 month minocycline treatment after 6 month, 1 year and 4 year follow-ups. The efficacy of minocycline in RA is postulated to be linked to its immunomodulatory characteristics via inhibition of metalloproteinases and suppression of macrophage and T cell activation. However, long term use of minocycline would have undesirable consequences (e.g. gastrointestinal upset) due to its anti-bacterial activity.

From a number of minocycline derivatives synthesized by Paratek, we tested four with no antibacterial activity for their therapeutic effect in a murine collagen-induced arthritis CIA model which shares several clinical, histological, and immunological features with RA.

## Methods

### Murine collagen-induced arthritis model and compound dosing protocol

- Male DBA/1 mice were immunized i.d. with an emulsion of 200 µg bovine type II collagen in Complete Freund's Adjuvant.
- On day 21, mice received i.d. boost of 100 µg collagen in Incomplete Freund's Adjuvant.
- Compounds were administered i.p. daily for 7 days starting from disease onset (day 3-4 after boost).
- Foot swelling was measured by an engineering micrometer and disease severity was scored accordingly (1, erythema and mild swelling confined to the tarsal or ankle joint; 2, erythema and mild swelling extending from the ankle to the mid-foot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot, and digits).
- Change (Δ) of paw thickness = sum of paw thickness from 4 paws of a mouse (experimental) – sum of baseline paw thickness from 4 paws of the same mouse.
- % inhibition = cumulative Δ paw thickness (disease group – compound-treated group) / cumulative Δ paw thickness (disease group)

### Paw extract preparation and biomarkers ELISA assay

- Paws were collected from mice after 5-7 days of dosing and dissected free of skin.
- The paws were then homogenized in ice-cold PBS (2 ml/4 paws/mouse) containing 1x protease inhibitor using a Polytron homogenizer.
- Debris and particles were removed from the homogenized samples by centrifugation.
- Liquid layers were collected for MMP-9, IL-1, IL-6, RANKL, MCP-1, and TNFα analysis using ELISA kits from R & D System.

## Results

### 1. Minocycline derivatives (PTK-RA-1, PTK-RA-2, PTK-RA-3, and PTK-RA-4) have no anti-bacterial activity compared to minocycline, and are bio-available after oral dosing.

Compound	Antibacterial Activity		PK Parameters (Rat)	
	<i>E. coli</i> -MIC <sup>a</sup> (µg/ml)	Protein Synthesis Inhibition <sup>b</sup> IC <sub>50</sub> (µM)	T <sub>1/2</sub> (hr)	% F <sup>c</sup>
Minocycline	1	1.9	3.6	31
PTK-RA-1	>64	>100	3.0	52
PTK-RA-2	>64	>100	2.5	65
PTK-RA-3	>64	>100	3.1	77
PTK-RA-4	>64	>100	6.4	42

a. *E. coli*-MIC (minimal inhibitory concentration) was determined by broth micro-dilution method performed according to CLSI guidelines. *E. coli* ATCC25922 (tetracycline sensitive) was grown in cation-adjusted Mueller Hinton broth to a 0.5 McFarland standard. Turbidity was measured using a Microscan turbidity Meter.

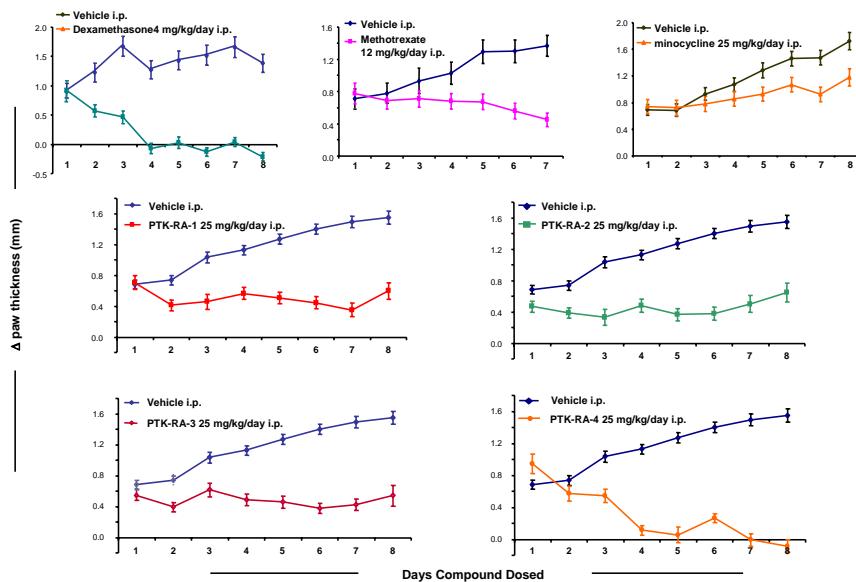
b. Protein Synthesis Inhibition was measured using an *in vitro* transcription/translation assay system, *E. coli* S30 Extract System for Circular DNA from Promega, that detects microbial ribosome activity.

c. PK, Pharmacokinetics; All the samples were analyzed on LC-MS/MS and parameters were calculated using WinNonLin program.

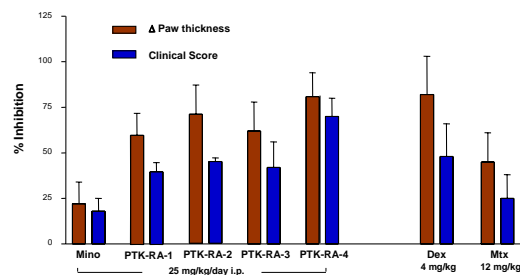
d. % F, fraction of absorption after oral dosing of 5 mg/kg of compound

### 2. Minocycline-derivatives (PTK-RA-1, PTK-RA-2, PTK-RA-3, and PTK-RA-4) inhibit joint inflammation when administered after disease onset in a murine collagen induced arthritis (CIA) model. Effects on reduction of paw swelling and clinical score are greater when compared to either minocycline or methotrexate.

#### 2a. *In vivo* efficacy of methotrexate (Mtx), minocycline (Mino), and minocycline derivatives (PTK-RA-1, PTK-RA-2, PTK-RA-3, and PTK-RA-4) in reducing disease severity in the CIA model.



#### 2b. Comparison of disease severity (paw swelling and clinical score) in CIA mice that were treated with minocycline vs. minocycline derivatives.

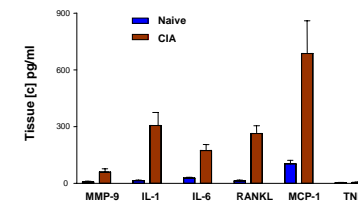


#### 2c. Comparison of EC<sub>50</sub>s of minocycline vs. minocycline derivatives in inflammation suppression in CIA mice.

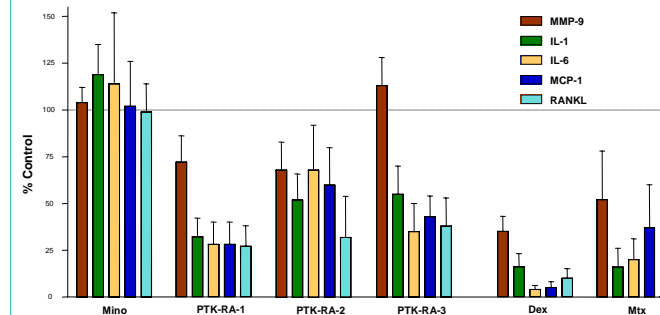
Compounds	EC <sub>50</sub> (mg/kg/day i.p.)
Minocycline	>50
PTK-RA-1	20
PTK-RA-2	14
PTK-RA-3	12
PTK-RA-4	12

### 3. Minocycline derivatives (PTK-RA-1, PTK-RA-2, and PTK-RA-3) inhibit inflammatory/osteoclastic cytokines better than minocycline.

#### 3a. ELISA analysis of inflammatory biomarkers using paw extracts from CIA mice.



#### 3b. Comparison of inflammatory biomarker expression in paws of CIA mice treated with experimental compounds.



## Summary

- Minocycline derivatives (PTK-RA-1, PTK-RA-2, PTK-RA-3, and PTK-RA-4) had no antibacterial activity in an *in vitro* microbial protein synthesis system and the MIC assay.
- These four minocycline derivatives were bio-available when they were dosed orally in rat.
- These non-antibiotic minocycline derivatives were successful in reducing joint inflammation when administered after disease onset in a collagen induced arthritis model.
- The minocycline derivatives were more efficacious compared to both minocycline and methotrexate in the collagen induced arthritis model based on their cumulative inhibitions and EC<sub>50</sub>s.
- The inhibition of arthritis development by these minocycline derivatives was correlated with their ability to reduce local tissue levels of inflammatory/osteoclastic cytokines (MMP-9, IL-1, IL-6, MCP-1, RANKL) *in vivo*.

## Conclusion

Non-antibacterial minocycline derivatives, PTK-RA-1, PTK-RA-2, PTK-RA-3, and PTK-RA-4, may be considered as oral drug candidates for the treatment of RA, without the adverse effects associated with chronic administration of antibacterial drugs.