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Translational Biomarkers and Ex Vivo Models of Joint Tissues as a Tool for Drug Development in Rheumatoid Arthritis

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Objective. **Rheumatoid arthritis (RA) is a chronic and degenerative autoimmune joint disease that leads to disability, reduced quality of life, and increased mortality. Although several synthetic and biologic disease-modifying antirheumatic drugs are available, there is still a medical need for novel drugs that control disease progression. As only 10% of experimental drug candidates for treatment of RA that enter phase I trials are eventually registered by the Food and Drug Administration, there is an immediate need for translational tools to facilitate early decision-making in drug development. In this study, we aimed to determine if the inability of fostamatinib (a small molecule inhibitor of Syk) to demonstrate sufficient efficacy in phase III of a previous clinical study could have been predicted earlier in the development process.**

Methods. **Biomarkers of bone, cartilage, and interstitial matrix turnover (C-telopeptide of type I collagen [CTX-I], matrix metalloproteinase–derived types I, II, and III collagen neoepitopes [C1M, C2M, and C3M]) were measured in 450 serum samples from the Oral Syk Inhibition in Rheumatoid Arthritis 1 study (OSKIRA-1, a phase III clinical study of the efficacy of fostamatinib in RA) at baseline and follow-up. Additionally, the same biomarkers were subsequently measured in conditioned media from osteoclast, cartilage, and synovial membrane cultured with the active metabolite of fostamatinib, R406, to assess the level of suppression induced by the drug.**

Results. **In OSKIRA-1 serum samples and osteoclast and cartilage cultures, fostamatinib suppressed the levels of CTX-I and C2M. In OSKIRA-1 serum samples and synovial membrane cultures, fostamatinib did not mediate any clinical or preclinical effect on either C1M or C3M, which have previously been associated with disease response and efficacy.**

Conclusion. **These data demonstrate that translational biomarkers are a potential tool for early assessment and decision-making in drug development for RA treatment.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease estimated to affect 1.3 million adults in the US (1). The first-line disease-modifying antirheumatic drug (DMARD) for RA treatment is usually methotrexate (MTX), which is often combined with conventional or biologic DMARDs as second-line therapy (2,3). However, as some patients do not respond sufficiently to either therapy, there is a need for drugs with alternative mechanisms of action. Despite significant efforts, only 10% of RA drugs that enter phase I clinical trials are likely to be approved by the Food and Drug Administration (4,5).

Syk is a nonreceptor tyrosine kinase and is considered a possible target for RA treatment due to its diverse biologic roles within inflammatory signaling, cellular adhesion, pathogen recognition, bone metabolism, tissue damage, and vascular effects (6,7). It is expressed by hematopoietic cells including T and B

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lymphocytes, neutrophils, macrophages, and mast cells, where it is involved with intracellular signaling related to classic immunoreceptors (8). In addition, Syk is expressed by cells found in joint tissues (bone, cartilage, and synovium), where it is involved in downstream tumor necrosis factor α (TNF) receptor signaling (9–11). Fostamatinib is a small-molecule inhibitor of Syk, and has been tested as a potential novel DMARD for RA therapy.

In the Oral Syk Inhibition in Rheumatoid Arthritis 1 study (OSKIRA-1), both patient groups treated with fostamatinib exhibited statistically significant responses, meeting the American College of Rheumatology criteria for 20% improvement (ACR20) (12) at week 24 in comparison to placebo; however, no effect on the progression of structural damage relative to placebo was observed. The totality of the OSKIRA phase III program indicated that the profile of this molecule was insufficient to warrant further development for RA (13,14). As phase III clinical programs require considerable investment from patients, physicians, and sponsors, our goal in the present investigation was to determine whether the lack of structural efficacy in the fostamatinib OSKIRA-1 study could have been predicted earlier in the clinical development process. If translational preclinical models are able to reveal clinically relevant biomarker effects, an early "go-or-no-go" decision would contribute to improved success rates in the approval of novel drugs.

Biomarkers are increasingly being used to inform clinicians regarding drug development decisions and understanding of modes of action (15,16). Serologic biomarkers can be used to help elucidate the effects of a drug through preclinical models, as well as in clinical studies (17). This presents an opportunity to identify translational biomarkers in preclinical models of disease-relevant target tissues or cell types, and translate the effect into clinical studies and guide decision-making during the drug development process.

Biomarkers of joint tissue turnover have been used successfully to support and improve the understanding of clinical modes of action in RA (18). They are considered to include 3 main classes: bone, cartilage, and synovial inflammation. Bone resorption can be assessed with C-telopeptide of type I collagen (CTX-I), a type I collagen fragment generated by the main osteoclast protease cathepsin K (19,20). Bone formation can be assessed with osteocalcin and N-terminal type I procollagen propeptide (PINP). Osteocalcin is produced by mature bone-forming osteoblasts, and PINP is released upon incorporation of type I procollagen into mature collagen (21,22). Matrix metalloproteinase (MMP) degradation of type II collagen can be assessed with the MMP-derived type II collagen neoepitope C2M (a measure of cartilage degradation), as type II collagen is predominantly found in cartilage (23,24). Finally, synovitis can be assessed with total MMP-3 and acMMP-3, a biomarker for active MMP-3 (25,26).

The connective tissue of the joint contains mainly types I and III collagen (27,28). During joint inflammation, MMPs are increased, leading to increased degradation of extracellular matrix (ECM) components (29). C1M and C3M are neoepitopes of MMP-2 and MMP-9–degraded types I and III collagen, respectively. They serve as biomarkers of interstitial membrane inflammation (30,31). We have previously shown that C1M and C3M are up-regulated in RA, osteoarthritis (OA), spondyloarthritis, and ex vivo in synovial membrane explants in response to TNF (24,32–36). Importantly for the context of this study, C1M has been shown to be prognostic for disease progression in RA (33) and C1M and C3M levels are correlated with disease activity (37,38), emphasizing the significance of interstitial tissue inflammation for progression of disease. Acute systemic inflammation is estimated using levels of C-reactive protein (CRP) and of interleukin-6 (IL-6), a proinflammatory cytokine that correlates with clinical disease activity in RA; chronic tissue inflammation can be assessed with the neoepitope of MMP-degraded CRP, CRPM (37,39,40).

Efficacious therapies such as tocilizumab (TCZ), etanercept, MTX, adalimumab, and tofacitinib positively modulate biomarkers of joint tissue turnover in RA and ankylosing spondylitis (18,24,37,38,41). Therefore, we tested whether the lack of efficacy of fostamatinib on structural end points in the OSKIRA-1 phase III study could have been predicted by the translation of biomarkers of joint tissue turnover, measured in preclinical models.

PATIENTS AND METHODS

Study design. This study was undertaken to investigate whether a translational model using serum-based biomarkers in ex vivo and in vitro cultures could have predicted the insufficient clinical effect of fostamatinib on joint structure. In order to investigate this, the clinical data from the OSKIRA-1 study of fostamatinib in combination with MTX (13), a phase III, multicenter, randomized, double-blind, placebo-controlled, parallel-group study of RA patients whose illness did not adequately respond to MTX therapy, were used. In vitro human osteoclasts and ex vivo cultures of bovine cartilage and human synovium were used to test the effect of the active metabolite of fostamatinib, R406, on 4 biomarkers of ECM degradation that were also measured in OSKIRA-1.

OSKIRA-1 was carried out in full accordance with the principles of the Declaration of Helsinki and with the laws and regulations of the countries in which the research was conducted (which include Argentina, Australia, Belgium, Brazil, Chile, Estonia, France, Hungary, India, Mexico, Peru, Poland, Slovakia, Ukraine, the UK, and the US). All patients

provided written informed consent, and the trials were approved by all relevant institutional ethics committees or review bodies and were conducted in accordance with the principles of Good Clinical Practice and the AstraZeneca Policy on Bioethics (https://www.astrazeneca.com/content/dam/az/PDF/Bioethics_policy.pdf). Briefly, the OSKIRA-1 study compared 2 patient cohorts (groups A and B) who were receiving different dosing regimens of fostamatinib with a placebo group. The study was placebo-controlled for the initial 24-week period, and all study patients continued to receive MTX therapy. For the 24-week period, group A received 100 mg fostamatinib twice daily in combination with MTX and group B received 100 mg fostamatinib twice daily in combination with MTX for the first 4 weeks, followed by 150 mg fostamatinib once-daily maintenance in combination with MTX; the placebo group received MTX only. Placebo-treated patients who did not achieve a satisfactory response (improvement in disease activity according to the ACR20) by week 12 were able to transfer to active treatment. Optional consent for serum collection at baseline and week 24 was obtained from a subset of patients and used for biomarker analysis. None of the placebo-treated patients who were included in the biomarker analysis had transferred to active treatment.

The statistical analysis plan was defined prior to database lock and included an analysis of 9 exploratory biomarkers, including assessment of bone balance (CTX-I:osteocalcin ratio). Changes in biomarker levels and bone balance from baseline to week 24 were assessed in each treatment group, and changes in the active treatment groups were compared with those in the placebo group. The biomarker analyses were based on those patients in the full analysis set who consented to optional biomarker sampling and had evaluable data available at baseline and at week 24.

Human osteoclast cultures. Human osteoclasts were generated from CD14+ monocytes isolated from peripheral blood, as described previously (42). Briefly, CD14+ cells were isolated from human super buffy coats received from the local hospital, using CD14-coated Dynabeads M-450 (catalog no. 111.49D; Invitrogen). The CD14+ cells were then cultured in α-minimum essential medium (α-MEM) (catalog no. 041-94723M; Gibco) with 1% penicillin/streptomycin (catalog no. P4333; Sigma-Aldrich), thymidine (catalog no. A2265; AppliChem), 10% fetal calf serum (FCS), and 25 ng/ml of macrophage colony-stimulating factor (M-CSF) (catalog no. 216-MC; R&D Systems) for the first 3–4 days. Thereafter, the media were modified to α-MEM containing penicillin/streptomycin, thymidine, 10% FCS, 25 ng/ml M-CSF, and 25 ng/ml RANKL (catalog no. 390-TN; R&D Systems) and were incubated for 10–12 days for osteoclastogenesis, changing every 2–3 days.

Resorption was assessed by seeding 25,000 mature osteoclasts per bovine bone slice 2 hours prior to the start of the experiment. The mature osteoclasts were treated with DMSO as a negative control, 100 n*M* diphyllin as a positive control inhibitor, and R406 (catalog no. HY-12067; MedChem) at 9 μ*M*, down to 0.3 μ*M* in a 3-fold dilution. Three wells containing bone slices without cells were included as a background control. The medium was changed on day 3, and resorption stopped on day 6. The conditioned media from day 6 were stored at –20°C until measurements of Ca^{2+} and CTX-I were obtained. Metabolic activity was assessed with alamarBlue (catalog no. DAL1100; Invitrogen) on day 6. Briefly, the osteoclasts were incubated with 10% alamarBlue in α-MEM containing penicillin/streptomycin, thymidine, 10% FCS, 25 ng/ml M-CSF, and 25 ng/ml RANKL for 3 hours. Fluorescence was then measured at 540 nm excitation and 590 nm emission on a SpectraMax (Molecular Devices).

Explant cultures. Intact knees from cows ages 1–2 years were used to make full-depth cartilage explants. The cow knees were retrieved from the local butcher (Harald Hansens) at a maximum of 24 hours postmortem. The full-depth cartilage explants were punched from the lateral and medial femoral condyle with a biopsy puncher of 3 mm (catalog no. MTP-33-32; Scandidact) and released from the bone with a scalpel. They were placed in 96-well plates with Dulbecco's modified Eagle's medium (DMEM)/F-12 (catalog no. 31331-093; Invitrogen) with 1% penicillin/streptomycin, 24 hours prior to treatment. The full-depth cartilage explants were cultured for 3 weeks with DMEM/F-12, 1% penicillin/streptomycin, and DMSO, with 10 ng/ml oncostatin M (OSM), 2 ng/ml TNF, and DMSO, or with OSM and TNF together with 2.5 μ*M*, 0.625 μ*M*, or 0.156 μ*M* R406.

Synovial membrane explants were made using synovial membrane biopsy specimens obtained from OA patients undergoing total knee replacement at Gentofte University Hospital. All patients provided written informed consent, and the Danish scientific ethical commission approved the trials (H-D-2007-0084). Fat was removed from the synovial membrane specimens, which were cut into explants of 30 mg (±5 mg). The synovial membrane explants were incubated overnight in DMEM/F-12 and 1% penicillin/streptomycin prior to treatment start. They were cultured for 14 days with DMEM/F-12, 1% penicillin/streptomycin, and DMSO, with 10 ng/ml TNF and DMSO, or with 10 ng/ml TNF and 5 μ*M*, 0.5 μ*M,* or 0.05 μ*M* R406 (6 patients) or 10 ng/ml TNF and 5 μ*M*, 2.5 μ*M*, 1.25 μ*M*, or 0.63 μ*M* R406 (5 patients). Between 1 and 4 technical replicates were included per patient and a total of 11 patients were used to test R406. The conditioned media were changed 3 times per week with the addition of fresh treatment for all explant cultures and were stored at –20°C until measurement of C2M, C1M, C3M, and acMMP-3.

Biochemical biomarker assays and calcium measurements. Assays for osteocalcin, PINP, and CTX-I were conducted on serum acquired from patients at baseline and week 24. CTX-I, PINP, and osteocalcin in serum were measured individually with an Elecsys 2010 analyzer (Roche Diagnostics) using CrossLaps, total PINP, and N-terminal midfragment, respectively. Conditioned media CTX-I was measured with CrossLaps for Culture CTX-I (catalog no. AC07F1; IDS) according to the instructions of the manufacturer.

Quantitative competitive enzyme-linked immunosorbent assays were used to measure the biomarkers C1M, C2M, and C3M in the OSKIRA-1 study at baseline and week 24 and in conditioned media from cartilage or synovium ex vivo cultures as described previously (23,30,31,39). Briefly, streptavidin-coated 96-well microtiter plates were coated with a biotinylated peptide specific for each biomarker. After washing to eliminate unbound biotinylated peptide, standards, controls, samples, and horseradish peroxidase (HRP)–conjugated monoclonal antibodies specific for each biomarker were added to the assay plates. Following a wash to remove unbound HRP-conjugated antibody, the substrate solution 3,3′,5,5′-tetramethylbenzidine was added. Color development was terminated by adding sulfuric acid, and the intensity of the color was measured with SpectraMax (Molecular Devices). The concentration of total calcium was measured in culture supernatants after resorption using a colorimetric assay and a Hitachi 912 Automatic Analyzer (Roche Diagnostics).

Statistical analysis. In the OSKIRA-1 study, the analysis methods were determined a priori and were recorded in an exploratory analysis plan. As the biomarker data were not normally distributed, they were log transformed prior to analysis. The mean change was derived on the original scale. The log-transformed data were analyzed using an analysis of covariance model on the change from baseline, including terms for baseline as a continuous covariate and treatment and country as factors. The results were displayed as back-transformed ratios to baseline and ratios between treatments. Due to an insufficient amount of material for biomarker measurements, CTX-I was measured in 447 patients, osteocalcin in 448 patients, PINP in 447 patients, C1M in 448 patients, IL-6 in 445 patients, and MMP-3 in 448 patients. The missing values were not imputed in the statistical analysis.

For the preclinical study, the biomarker levels from the ex vivo data were plotted as a function of time of culture, and the total release of biomarkers was quantified by calculating the area under the curve (AUC) using GraphPad Prism version 6.07. CTX-I, Ca^{2+}, and metabolic activity in the osteoclast culture were measured on day 6. The AUC values, CTX-I, Ca^{2+}, and metabolic activity were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test, performed with GraphPad Prism version 6.07. For all biomarker measurements, any values below the lower limit of the measurement range were imputed as the lower limit of the measurement range.

RESULTS

Patient population. In the OSKIRA-1 study, 923 patients were randomized to receive fostamatinib (group A, n = 311; group B, n = 306) or placebo (n = 306), all of whom had also received MTX (13). Of these patients, 450 had provided consent for serum collection and had evaluable samples at baseline and at week 24 (group A, n = 164; group B, n = 153; placebo, n = 133), representing 43–53% of patients from each treatment arm. This subset of patients was used for biomarker analysis, although 5 of the 450 samples did not contain sufficient material to allow measurement of all biomarkers. Baseline clinical and demographic data for this subset of patients for biomarker analysis suggest that these patients were a reasonable representation of the overall intent-to-treat population (13). Demographic characteristics of the patients in the OSKIRA-1 study are summarized in Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40527/abstract).

Decrease in serum levels of bone biomarkers after fostamatinib treatment. In the OSKIRA-1 study, there was a 30% decrease in CTX-I from baseline to week 24 in group A and a 26% decrease in group B, as well as a small decrease in the placebo group (3%) (Figure 1A). Compared with patients who had received placebo, group A showed a 28% decrease ($P < 0.001$) and group B showed a 23% decrease ($P < 0.001$). Similarly, osteocalcin levels from baseline to week 24 decreased by 20% in group A, by 16% in group B, and by 2% in the placebo group (Figure 1B). Compared with the placebo group, osteocalcin levels were decreased by 18% in group A ($P < 0.001$) and by 14% in group B ($P < 0.001$). The CTX-I:osteocalcin ratio is considered to be the bone balance, which can be used to assess the net gain or loss of bone (43). Despite an overall suppression of bone turnover, the bone balance favored net bone formation (osteocalcin) over bone resorption (CTX-I) in groups A and B (Figure 1C). As an alternate marker of bone formation and consistent with the reduction in osteocalcin levels, the geometric mean levels of PINP decreased by 24% and 18% from baseline to week 24 in groups A and B, respectively (Figure 1D). Compared with geometric mean levels of PINP in the placebo group (1%), levels in groups A and B were significantly decreased (by 24% and 19%, respectively; $P < 0.001$ for both).

Changes in joint tissue degradation biomarker levels following fostamatinib treatment. Changes in the levels of joint tissue degradation biomarkers from baseline to week 24 are summarized in Figure 2. There were no statistically significant differences in C1M or C3M in the fostamatinib treatment groups versus the placebo group (Figures 2A and C). C2M levels decreased from baseline by

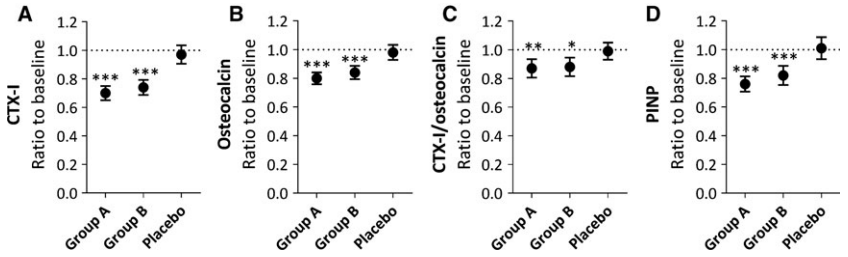

Figure 1. Reduction of bone biomarkers and bone balance by fostamatinib. The ratios of the levels of C-telopeptide of type I collagen (CTX-I) (**A**), osteocalcin (**B**), and N-terminal type I procollagen propeptide (PINP) (**D**) and the CTX-I:osteocalcin ratio (**C**) at 24 weeks compared to baseline are shown. Patients in group A received 100 mg fostamatinib twice daily in combination with methotrexate (MTX), group B began with 100 mg fostamatinib twice daily in combination with MTX for the first 4 weeks, followed by 150 mg fostamatinib once daily maintenance in combination with MTX, and the placebo-treated patients received MTX only. Values are the mean ± SEM. * = $P = < 0.05$; ** = $P = < 0.01$; *** = $P = < 0.001$, versus baseline.

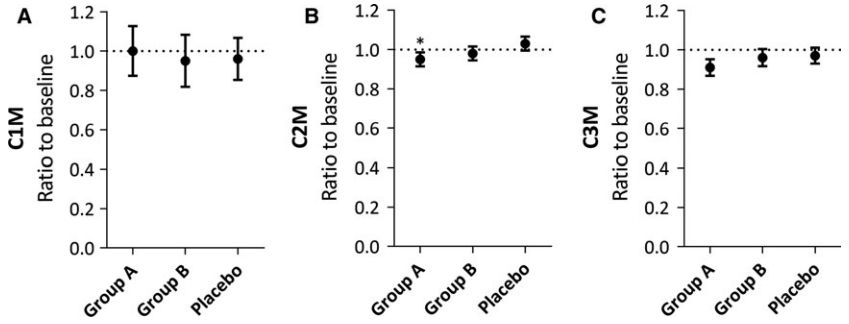

Figure 2. Joint tissue degradation. The ratios of the levels of matrix metalloproteinase–derived type I collagen neoepitope (C1M) (**A**), C2M (**B**), and C3M (**C**) at 24 weeks compared to baseline are shown. Patients in group A received 100 mg fostamatinib twice daily in combination with methotrexate (MTX), group B began with 100 mg fostamatinib twice daily in combination with MTX for the first 4 weeks, followed by 150 mg fostamatinib once daily maintenance in combination with MTX, and the placebo-treated patients received MTX only. Values are the mean ± SEM. * = $P = < 0.05$.

5% in group A and 2% in group B, while a small increase was observed in the placebo group (3%) (Figure 2B). The difference from placebo was statistically significant only in group A (8%; $P = 0.027$). Three additional biomarkers—CRPM, IL-6, and total MMP-3—were also measured. Fostamatinib had no effect on CRPM; however, lower levels of IL-6 were demonstrated in group A, and levels of total MMP-3 decreased in both groups A and B. (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40527/abstract).

Back-translation of the effect of fostamatinib on biomarker release ex vivo. Fostamatinib is an oral prodrug, which is rapidly converted to the active metabolite, R406. The effect of R406 on joint tissue turnover was retrospectively investigated in in vitro and ex vivo models of bone, cartilage, and synovial tissue after the OSKIRA-1 study was conducted.

Diphyllin, a control for inhibition of bone resorption, significantly reduced resorption by mature human osteoclasts on bovine bone slices, as compared with DMSO. This was measured with Ca^{2+} and CTX-I release ($P = 0.002$) (Figures 3A and B). Similarly, R406 decreased the release of Ca^{2+} and CTX-I into the conditioned media in a dose-dependent manner, with significance at 1 μM ($P = 0.037$ and $P = 0.026$ for Ca^{2+} and CTX-I, respectively) (Figures 3A and B). The decrease of Ca^{2+} and CTX-I in response to R406 was accompanied by a dose-dependent decrease in metabolic activity. At 3 μM, R406 significantly decreased metabolic activity ($P = 0.009$). Diphyllin did not affect osteoclast metabolic activity (Figure 3C).

Bovine cartilage explants treated with OSM and TNF released C2M in the late stage of the culture period (days 14–21) (Figure 4A). The total release of C2M was significantly higher in bovine cartilage explants treated with OSM and TNF compared to that observed without the treatment ($P = 0.008$). R406 inhibited total C2M release in a dose-dependent manner, with significance at 1.25 μM ($P < 0.001$) (Figure 4B).

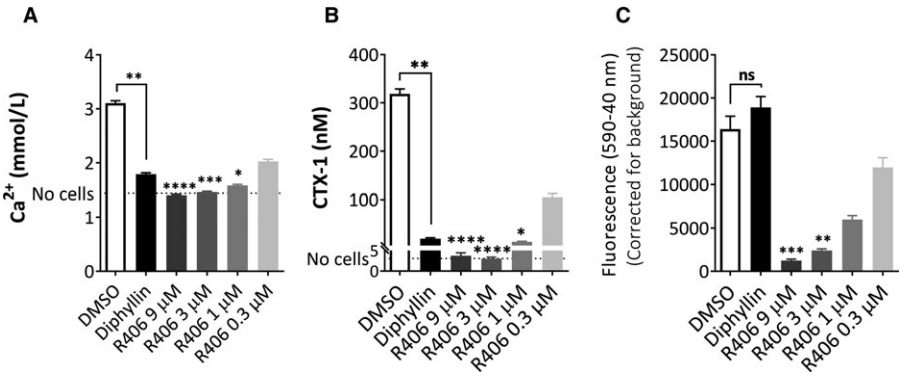

Figure 3. Effect of R406 on bone resorption and osteoclast metabolic activity. Bone resorption was measured in conditioned media from mature human osteoclasts cultured in bovine bone slices for 6 days, and levels of Ca^{2+} (**A**) and C-telopeptide of type I collagen (CTX-I) (**B**) were measured. Dotted lines represent the value of conditioned media from wells with bone slices but no osteoclasts. Metabolic activity of the human osteoclasts on the last day of culture was measured with alamarBlue (**C**). Bars show the mean ± SEM from 6 replicates. * = $P = < 0.05$; ** = $P = < 0.01$; *** = $P = < 0.001$; **** = $P = < 0.0001$, versus DMSO treatment. NS = not significant.

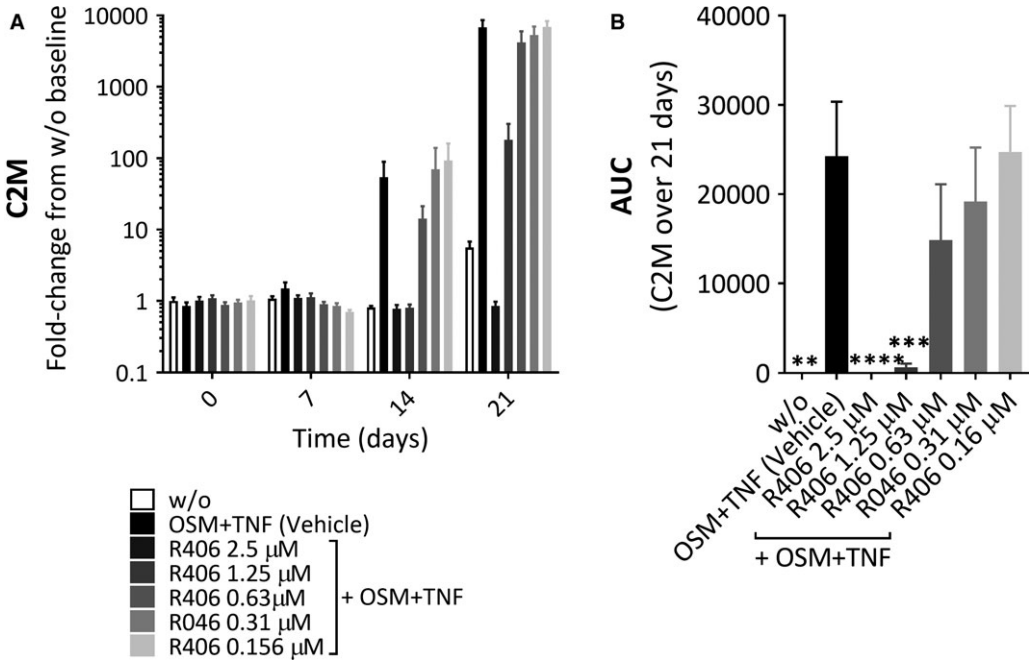

Figure 4. R406 inhibits the release of matrix metalloproteinase (MMP)–derived type II collagen neoepitope (C2M) ex vivo. Cartilage MMP degradation was quantified by measurement of C2M levels in conditioned media from bovine cartilage explants (**A**). C2M is plotted as the fold change versus without treatment (w/o) (baseline) over time after treatment start. Area under the curve (AUC) for C2M release over 21 days was determined (**B**). Bars show the mean ± SEM from 12 replicates in 2 experiments. ** = P = < 0.01; *** = P = < 0.001; **** = P = < 0.0001, versus baseline. OSM = oncostatin M; TNF = tumor necrosis factor.

TNF increased the release of C1M and C3M from human synovial membrane explants, with a peak at approximately days 5–10 of the culture period (Figures 5A and B), and the total release of C1M and C3M was significantly increased by TNF compared to that observed without TNF treatment (P = 0.001 and P < 0.001, respectively) (Figures 5C and D). R406 inhibited the release of C1M and C3M in a dose-dependent manner, but the degree of inhibition was significant only at 5 μM (P = 0.030 and P = 0.046 for C1M and C3M, respectively) (Figure 5C). Levels of acMMP-3 in the synovial membrane explants increased from day 5 in response to TNF, resulting in a significant total release of acMMP-3 (P = 0.005) compared to that observed without TNF treatment acMMP-3 (see Supplementary Figures 2A and B, available at http://onlinelibrary.wiley.com/doi/10.1002/art.40527/abstract). R406 tended to decrease acMMP-3 release at 5 μM (P = 0.051).

DISCUSSION

It was recently found that fostamatinib, a small molecule inhibitor of Syk, did not meet the coprimary structural end point of the OSKIRA-1 phase III study (13), and further development for its usage for treatment of RA was consequently discontinued. This was despite the fact that fostamatinib had shown a promising effect on structure in preclinical trials using animal models (44). Fostamatinib thereby joined the large number of potential RA therapeutic drugs that enter clinical development but are never approved for treatment of RA (4,5). There is a need for novel translational tools to identify an RA treatment that can provide patients with sufficient joint protection before they are exposed to a drug in a phase III study. In the present study, we investigated findings from the OSKIRA-1 study. Utilizing translational biomarkers of joint tissue turnover, we found inhibition of bone resorption, minimal inhibition of cartilage degradation, and no effect on inflammatory interstitial matrix degradation in clinical and preclinical models of the 3 main joint tissues.

The role of Syk in cartilage turnover is not well described. Syk is involved in stimulation of OA chondrocytes by basic calcium phosphate crystals (10), indicating that chondrocytes do express functional Syk. C2M was used to quantify cartilage degradation, as serum C2M has been shown to predict early response in patients receiving treatment with TCZ and has recently been shown to predict development of erosive disease in patients with early RA (18,45,46). Ex vivo, R406 inhibited TNF- and OSM-stimulated C2M release.

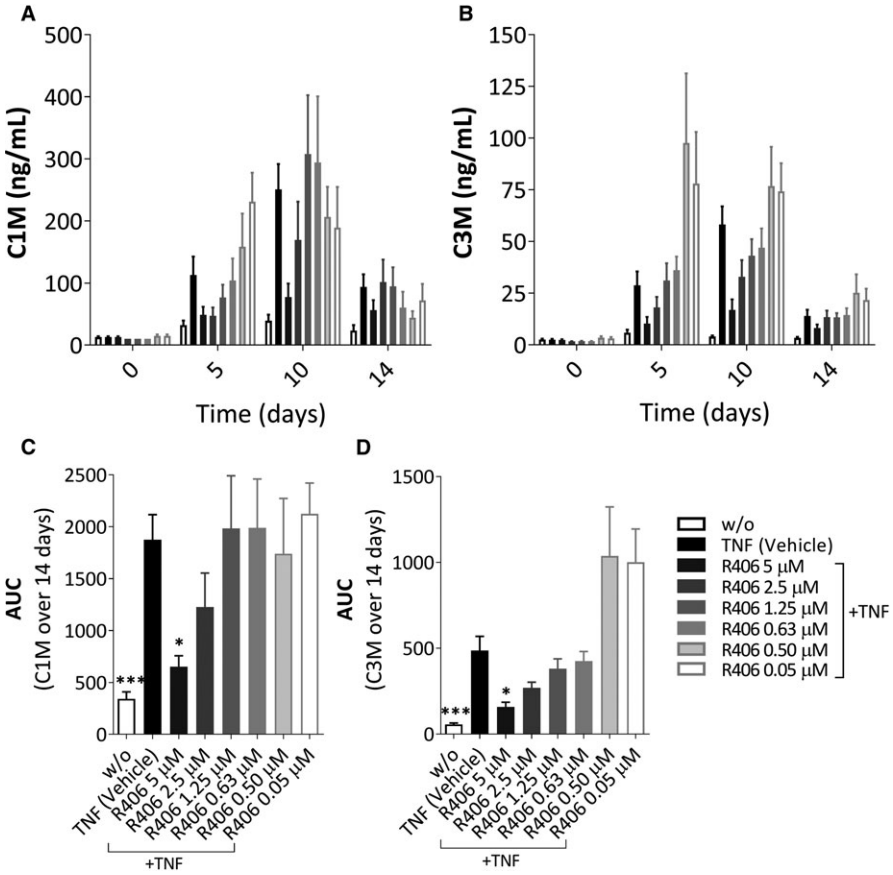

Figure 5. R406 inhibits ex vivo release of joint tissue biomarkers only at high concentrations. MMP degradation of type I collagen (**A**) and type III collagen (**B**) in conditioned media from human synovial explants was quantified with MMP-derived C1M and C3M, respectively, and plotted as ng/ml released over time. AUC for biomarker release of C1M (**C**) and C3M (**D**) from human synovial explants over 14 days was determined. Bars show the mean ± SEM from 6 patients. * = $P = < 0.05$; *** = $P = \leq 0.001$, versus treatment with TNF only. See Figure 4 for definitions.

This inhibition translated to the OSKIRA-1 study, in which serum C2M levels were significantly decreased in patient group A compared to the placebo group. However, minimal effect on clinical cartilage erosion was observed in the OSKIRA-1 study (13). This might be due to the concentration of R406 in the cartilage of the patients. Aqueous fostamatinib (160 mg, twice daily) reaches a steady state at ~1 μ*M* R406 in plasma, and a single dose of 75 mg fostamatinib as a tablet reaches a maximum concentration of 0.8–1.3 μ*M* (47). Group A patients in the OSKIRA-1 study would have had ~1 μ*M* R406 in circulation. However, as cartilage is an avascular tissue, the concentration of R406 in cartilage is likely to be <1 μ*M*, and possibly in the range where R406 has limited inhibitory effect on cartilage degradation. This correlates with the finding that group A had 5% lower C2M levels compared to the placebo group, indicating some inhibition of cartilage degradation (although too little to have a clinical effect on cartilage erosion). We have previously shown that a 12% decrease in C2M levels corresponds with efficacious clinical results of TCZ treatment (24).

Inflammatory interstitial tissue degradation can be monitored by C1M and C3M levels (both biomarkers that are up-regulated in RA) and correlated with inflammatory status in OA patients (32,34,37,38,48). C1M and C3M were included in the prospective analysis plan for OSKIRA-1, since C1M has been shown to be prognostic of RA progression, and both C1M and C3M have been shown to be associated with efficacy of treatment (18,33,38,46). In the present study, we did not see any effect of fostamatinib on C1M or C3M concentrations in any of the treatment groups. Ex vivo, we found that R406 at 5 μ*M* decreased C1M and C3M levels. However, at a clinically relevant concentration (~1 μ*M*), it had no effect on TNF-stimulated C1M or C3M release. In the Tocilizumab Safety and the Prevention of Structural Joint Damage study, TCZ decreased C1M levels by 20–40% compared to placebo at the 2 doses tested, and C3M

levels by ~25% at the highest dose compared to placebo (24,33). Additionally, in a study of a Japanese population, we showed that MTX and TCZ reduced C1M concentrations, and MTX, TCZ, adalimumab, and tofacitinib reduced C3M concentrations, to levels comparable to those in healthy subjects (38). C1M and C3M can be considered to be effective putative biomarkers of joint structure protection, and therefore we would expect an efficacious treatment to decrease both. However, as no effect of fostamatinib on either C1M or C3M was observed in OSKIRA-1 serum samples or ex vivo at relevant concentrations, fostamatinib would not be expected to have an efficacious protective effect on joint structure.

It is well-documented that in bone, Syk plays an important role in osteoclast function through osteoclastogenesis and organization of the cytoskeleton (11,49,50). Additionally, there are data indicating that Syk is involved in osteoblast differentiation (51). Consistent with these findings, our study demonstrates that the inhibition of Syk by fostamatinib significantly decreased the release of CTX-I both in vitro and clinically. In vitro, bone resorption (measured with both CTX-I and Ca^{2+}) by mature osteoclasts was significantly reduced in a dose-dependent manner by the active metabolite of fostamatinib, R406. Additionally, R406 decreased the metabolic activity of the osteoclasts. This observation has not been previously reported, and might explain the in vitro reduced bone resorption observed in the present study. Consistent with human osteoclast data, CTX-I was decreased by 23–31% in both treatment groups compared to the placebo group in the OSKIRA-1 study. This effect is comparable to both the effect of TCZ (decreasing CTX-I to ~25%) and the range of CTX-I reduction previously demonstrated to improve bone quality in phase III clinical studies of osteoporosis (43,52,53).

Treatment with fostamatinib also reduced bone formation as measured with osteocalcin and PINP. This is most likely due to the strong coupling between bone resorption and bone formation, in which a decrease in bone formation is observed secondarily to a decrease in bone resorption (54). However, the resultant bone balance (CTX-I:osteocalcin ratio) decreased significantly in the fostamatinib treatment groups (by 10–12% compared to the placebo group) and favored a net gain in bone.

We have demonstrated back-translation of the effect of fostamatinib on joint tissue remodeling, including decreased levels of biomarkers of bone resorption and, in part, cartilage degradation, and reproduced the lack of effect on biomarkers of inflammatory interstitial matrix degradation. Use of similar models as decision tools in early drug development would enable earlier go/no-go decision points in RA drug development.

Our study had some limitations. Biomarker analyses were derived from patients with both baseline and week 24 biomarker data available, rather than from the complete set of patients who had consented to provide samples for biomarker analysis. This analysis set therefore comprised patients with a generally more favorable outcome of their randomized treatment. In the OSKIRA-1 study, patients could transfer to a long-term extension study to receive fostamatinib 100 mg twice daily if they did not achieve a satisfactory response by week 12. Potential bias may therefore have been introduced into the biomarker analyses, given that a higher proportion of patients who were treated with fostamatinib completed 24 weeks than those treated with placebo. Additionally, since the assessment of biomarkers in the OSKIRA-1 study was an exploratory end point, the study was not prospectively sized for the analyses of biomarker data and anticipated treatment effects, and might therefore have been overpowered. Hence, caution should be applied when interpreting statistical significance of these biomarker analyses, as clinical significance is not necessarily implied.

The tissue culture, species, and disease-dependent differences between models and clinical measurements should also be taken into account when interpreting the data. Generally, the ex vivo models focus on single tissues. Thus, they do not take into account delivery of compounds. Our in vitro and ex vivo models were conducted after OSKIRA-1 was completed, and therefore we do not know how our data might have influenced the clinical study design.

In conclusion, it is recommended for future development of drugs for the treatment of RA, the value of exploratory biomarker analyses should be considered as part of phase II clinical studies. In addition, the potential impact of these findings should be fully considered in decision-making for later-stage clinical development.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Ms. Kjelgaard-Petersen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data. Kjelgaard-Petersen, Platt, Braddock, Jenkins, Graham, Slynn, Weinblatt, Karsdal, Thudium, Bay-Jensen.

ROLE OF THE STUDY SPONSOR

AstraZeneca facilitated the clinical study design and clinical data analysis and reviewed and approved the manuscript prior to submission. The authors independently collected the data, interpreted the results, and had the final decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by AstraZeneca.

ADDITIONAL DISCLOSURES

Authors Kjelgaard-Peterson, Musa, Karsdal, Thudium, and Bay-Jensen are employees of Nordic Bioscience.

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