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# Serum level of soluble triggering receptor expressed on myeloid cells-1 as a biomarker of disease activity in relapsing polychondritis

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**Abstract**
*Objectives* We aimed to identify a serum biomarker for evaluating the disease activity of relapsing polychondritis (RP).
*Methods* We measured and compared serum levels of 28 biomarkers potentially associated with this disease, including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), high-sensitivity C-reactive protein (hs-CRP), and cartilage oligomeric matrix protein (COMP), in 15 RP patients and 16 healthy donors (HDs). We divided the 15 RP patients into active RP ($n = 8$) and inactive RP ($n = 7$) groups, depending on the extent of the disease, and compared candidate markers between groups. The localization of membrane-bound TREM-1 in the affected tissue was examined by immunohistochemistry.
*Results* Serum levels of sTREM-1, interferon-γ, chemokine (C–C motif) ligand 4, vascular endothelial growth factor, and matrix metalloproteinases-3 were significantly higher in RP patients than HDs. Among these markers, sTREM-1 had the highest sensitivity and specificity (86.7 and 86.7 %, respectively). Furthermore, the serum level of sTREM-1 was significantly higher in active RP patients than inactive RP patients ($p = 0.0403$), but this was not true for hs-CRP or COMP. TREM-1 was expressed on endothelial cells in RP lesions.
*Conclusions* The serum level of sTREM-1 may be a useful marker of disease activity in RP.

**Keywords** Relapsing polychondritis · Serum marker · Soluble triggering receptor expressed on myeloid cells-1

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## Introduction

Relapsing polychondritis (RP) is a rare inflammatory disorder of unknown etiology; it is characterized by recurrent, widespread chondritis of systemic cartilages, specifically those in the ear, eye, nose, large airways, and joints [1–3]. RP is occasionally life-threatening, as its progression leads to fatal dyspnea due to cartilage destruction in large airways. To detect such disease progression, the accurate assessment of disease activity is important. Today, this assessment is performed by analyzing a combination of clinical manifestations, laboratory findings, and imaging results.

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However, it is still difficult to conduct proper evaluations. This is partly because there are no established biomarkers for evaluating the disease activity of RP, although several potential biomarkers—such as CRP, antibody to type II collagen, and cartilage oligomeric matrix protein (COMP)—have been reported previously [3–7]. For example, CRP is the most commonly used marker of inflammation, and its serum level is frequently used to assess RP disease activity [3, 4]. However, RP patients with normal CRP levels are often observed to experience advanced fibrosis of the airways, suggesting insidious chronic inflammation in those tissues, which is difficult to detect by CRP [8]. It has also been reported that antibodies to type II collagen reflect RP disease activity [6]. However, these antibodies were only detected in 30–50 % of RP patients [6, 9]. Furthermore, it has been reported that this measure lacks sensitivity and specificity [10]. Therefore, in the current study, we aimed to identify more sensitive biomarkers that would be able to detect those small differences that cannot be detected by antibodies to type II collagen or CRP.

To do so, this study excluded highly active RP patients. We measured 28 candidate markers that had been previously shown to be involved in RP, inflammation, or cartilage destruction. The levels of these markers were compared not only between RP patients and healthy donors (HDs) but also between active RP and inactive RP patients. Our results showed that the serum level of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is most suitable as a disease-activity marker in RP.

TREM-1 is a type I transmembrane receptor of the immunoglobulin superfamily. The soluble form of TREM-1 (sTREM-1) is thought to be released from TREM-1-expressing cells by proteolytic cleavage of membrane-bound TREM-1 [11]. The serum level of sTREM-1 has been found to be elevated in patients with sepsis and has therefore been considered as a marker of microbial infection [12].

## Materials and methods

### Patients and samples

Fifteen patients (8 women and 7 men) diagnosed with RP according to Damiani's criteria [13, 14] and 16 healthy donors (HD) serving as age-matched and sex-matched controls (Table 1) were recruited from St. Marianna University Hospital, Kanagawa, Japan. They were enrolled between November and December 2009. In this study, we used the patient information (disease condition, disease duration, medication, etc.) obtained at the time of enrollment (Table 1). None of the patients had any other inflammatory disorders, such as overt infections or collagen diseases. To detect small differences that cannot be detected by CRP, this study enrolled RP patients in the chronic phase—not the acute phase—and further excluded patients who had highly active RP, such as those with acute respiratory failure. From among them, we divided the 15 RP patients into two groups (active RP and inactive RP) according to the definition by Lekpa et al. [7]. Briefly,

**Table 1** Demographics, clinical characteristics, and medication of subjects

| | HD | RP | | |
|---|---|---|---|---|
| | ($n$ = 16) | Total ($n$ = 15) | Active ($n$ = 8) | Inactive ($n$ = 7) |
| Demographics | | | | |
| Age (years)[a] | 40.5 [27–67] | 47 [10–81] | 50.5 [10–74] | 44 [27–81] |
| Female sex | 50.0 % | 53.3 % | 50.5 % | 57.1 % |
| Clinical characteristics | | | | |
| Disease duration (years)[a] | | 5 [1–19] | 12 [4–19] | 4 [1–8] |
| Auricular chondritis | | 46.7 % | 62.5 % | 28.6 % |
| Nasal chondritis | | 40.0 % | 62.5 % | 14.3 % |
| Laryngotracheal chondritis | | 66.7 % | 87.5 % | 42.9 % |
| Ear symptoms | | 53.3 % | 87.5 % | 14.3 % |
| Arthritis | | 46.7 % | 75.0 % | 14.3 % |
| Ocular inflammation | | 33.3 % | 50.0 % | 14.3 % |
| Medication | | | | |
| Prednisolone | | 86.7 % | 87.5 % | 85.7 % |
| Methotrexate | | 33.3 % | 50.0 % | 28.6 % |
| Azathioprine | | 20.0 % | 25.0 % | 14.3 % |

*HD* healthy donor, *RP* relapsing polychondritis

[a] Data are expressed as median [range]

patients were defined as having active RP if they were affected with chondritis involving at least two of three sites (auricular, nasal, or laryngotracheal cartilage) at the time of blood collection or if they were affected in one of these sites and also had two other manifestations, which could include ocular inflammation, audiovestibular symptoms, or seronegative inflammatory arthritis. Fourteen patients with HTLV-1-associated myelopathy (HAM), 10 with progressive systemic sclerosis (PSS), 19 with systemic lupus erythematosus (SLE), and 20 with rheumatoid arthritis (RA) also participated in this study.

All blood and cartilage samples were obtained with written informed consent and full ethical approval. The study protocol was approved by the Ethics Committee of St. Marianna University School of Medicine.

Measurement of serum levels of marker candidates

High-sensitivity CRP (hs-CRP) was determined by nephelometry using N-latex CRP II (Siemens Healthcare Diagnostics, Tokyo, Japan). Serum concentrations of sTREM-1; matrix metalloproteinases (MMP)-1, MMP-2, MMP-3, MMP-13; cartilage oligomeric matrix protein (COMP); interleukin (IL)-17A; and anti-type II collagen antibody (α-COLII Ab) were measured using commercially available ELISA kits (sTREM-1, MMP-1, and MMP-2: R&D Systems, Minneapolis, MN, USA; MMP-3: Daiichi Fine Chemical, Toyama, Japan: MMP-13: GE Healthcare, Chalfont St Giles, UK; COMP: Abnova, Taipei, Taiwan; IL-17A: Gen-Probe, San Diego, CA, USA; α-COLII Ab: Chondrex, Redmond, WA, USA). Serum concentrations of

**Table 2** Serum concentrations of biomarker candidates in healthy donors and patients with RP

| Biomarker candidates[a] | Units | Methods of measurement | HD ($n$ = 16) Mean ± SD | RP ($n$ = 15) Mean ± SD | $p$* |
|---|---|---|---|---|---|
| sTREM-1 | pg/ml | ELISA | 92.48 ± 56.45 | 281.87 ± 150.42 | **0.0002** |
| IFN-γ | pg/ml | CBA | N.D.[c] | 5.65 ± 6.25 | **0.0035** |
| CCL4 | pg/ml | CBA | 64.38 ± 66.03 | 133.76 ± 68.13 | **0.0075** |
| VEGF | pg/ml | CBA | 131.03 ± 104.66 | 267.46 ± 187.03 | **0.0212** |
| MMP-3 | ng/ml | ELISA | 35.96 ± 29.23 | 243.12 ± 313.50 | **0.0229** |
| CXCL10 | pg/ml | CBA | 154.72 ± 91.72 | 229.50 ± 114.03 | 0.0552 |
| CCL5 | ng/ml | CBA | 2.70 ± 1.43 | 37.66 ± 15.66 | 0.0582 |
| hs-CRP | ng/ml | Nephelometry | 0.04 ± 0.05 | 0.30 ± 0.50 | 0.0643 |
| IL-17A | pg/ml | ELISA | 1.17±1.52 | 0.33 ± 0.79 | 0.0673 |
| TNF | pg/ml | CBA | N.D.[c] | 0.76 ± 2.01 | 0.1646 |
| IL-4 | pg/ml | CBA | N.D.[c] | 0.80 ± 2.13 | 0.1671 |
| IL-6 | pg/ml | CBA | N.D.[c] | 1.27 ± 3.38 | 0.1686 |
| COMP | ng/ml | ELISA | 14.38 ± 4.28 | 24.33 ± 26.72 | 0.1750 |
| MMP-13 | ng/ml | ELISA | 0.31 ± 0.04 | 0.28 ± 0.09 | 0.2367 |
| MMP-2 | ng/ml | ELISA | 125.01 ± 10.45 | 133.01 ± 28.45 | 0.3191 |
| IL-1α | pg/ml | CBA | N.D.[c] | 0.54 ± 2.09 | 0.3343 |
| IL-1β | pg/ml | CBA | N.D.[c] | 0.58 ± 2.24 | 0.3343 |
| IL-10 | pg/ml | CBA | N.D.[c] | 0.69 ± 2.69 | 0.3343 |
| IL-12p70 | pg/ml | CBA | N.D.[c] | 0.35 ± 1.36 | 0.3343 |
| CX3CL1 | pg/ml | CBA | N.D.[c] | 6.55 ± 25.38 | 0.3343 |
| CXCL8 | pg/ml | CBA | 12.93 ± 11.52 | 16.24 ± 7.05 | 0.3413 |
| MMP-1 | ng/ml | ELISA | 5.19 ± 3.15 | 4.30 ± 3.67 | 0.5129 |
| CCL2 | pg/ml | CBA | 67.08 ± 43.78 | 72.29 ± 59.36 | 0.7842 |
| αCOLII Ab[b] | U/ml | ELISA | 51.75 ± 37.95 | 263.93 ± 577.87 | 0.2109 |

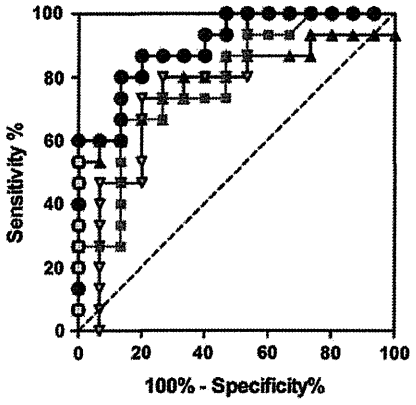
*HD* healthy donor, *RP* relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *CBA* cytometric bead array, *ND* not detected, *CCL* chemokine (C–C motif) ligand, *VEGF* vascular endothelial growth factor *MMP* matrix metalloproteinase, *CXCL* chemokine (C–X–C motif) ligand, *hs-CRP* high-sensitivity C-reactive protein, *IL* interleukin, *TNF* tumor necrosis factor, *COMP* cartilage oligomeric matrix protein, *CX3CL* chemokine (C–X3–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's $t$ test. $p$ values of less than 0.05 are indicated in boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (HD: $n$ = 13, RP: $n$ = 13)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries




| symbol | candidate markers | AUC | 95%CI |
|---|---|---|---|
| ●— | sTREM-1 | 0.90 | 0.80 to 1.01 |
| □— | IFN-γ | 0.77 | 0.59 to 0.94 |
| ▽— | CCL4 | 0.79 | 0.62 to 0.96 |
| ■— | VEGF | 0.78 | 0.62 to 0.95 |
| ▲— | MMP-3 | 0.80 | 0.63 to 0.97 |

---- reference line

**Fig. 1** Receiver operating characteristic (ROC) analysis of marker candidates of relapsing polychondritis (RP). We compared the sensitivity and specificity of soluble triggering receptors expressed on myeloid cells-1 (sTREM-1), interferon (IFN)-γ, chemokine (C–C motif) ligand 4 (CCL4), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-3 (MMP-3) for discriminating RP patients from healthy donors (HDs) using ROC analysis. Closer proximity of the ROC curve to the upper left corner indicates higher sensitivity and specificity of the marker

IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70; interferon (IFN)-γ; tumor necrosis factor (TNF); chemokine (C–C motif) ligand (CCL) 2, CCL3, CCL4, CCL5; chemokine (C–X–C motif) ligand 8 (CXCL8), CXCL10; chemokine (C–X3–C motif) ligand 1 (CX3CL1); granulocyte–macrophage colony-stimulating factor (GM-CSF); and vascular endothelial growth factor (VEGF) were measured using a cytometric bead array (CBA; BD Biosciences, San Jose, CA, USA). All assays were conducted according to the respective manufacturers' instructions.

### Immunohistochemistry

Biopsy specimens from three patients with RP chondritis were subjected to immunohistochemical analysis. Formalin-fixed tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were processed for antigen retrieval by a standard microwave-heating technique and incubated with anti-TREM-1 antibody (Sigma), followed by detection with streptavidin–biotin-horseradish peroxidase (Dako Cytomation Japan, Tokyo, Japan). All sections were visualized using 3,3′-diaminobenzidine (DAB).

### Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to plot graphs and perform statistical analyses. Mean serum concentrations of biomarker candidates were compared between RP patients and HDs using Welch's *t* test (Table 2). Receiver operating characteristic (ROC) analysis was used to examine the sensitivity and specificity of the selected markers (Fig. 1). Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP were analyzed by Welch's *t* test (Table 3). To compare serum sTREM-1 levels between healthy donors and patients with some inflammatory diseases (Fig. 3), we employed the Kruskal–Wallis test followed by Dunn's post hoc test. In all analyses, statistical significance was set at $p < 0.05$.

## Results

### Serum biomarker candidates in RP patients

First, we measured the serum levels of 12 cytokines, 7 chemokines, 4 MMPs, VEGF, hs-CRP, sTREM-1, COMP, and anti-type II collagen antibody in RP patients and age- and sex-matched HDs (Table 1), and compared the results from these two groups (Table 2). Serum samples from RP patients showed significantly higher concentrations of five molecules (sTREM-1, IFN-γ, CCL4, VEGF, and MMP-3) than the samples from HDs (Table 2). The serum levels of several other molecules (including hs-CRP, COMP, and anti-type II collagen antibody) tended to be higher in RP patients than in HDs, though the differences were not statistically significant.

Then, using ROC analysis, we compared the performances of the above five molecules in distinguishing RP patients from HDs. As shown in Fig. 1, the ROC analysis demonstrated that sTREM-1 had the highest sensitivity and specificity of the five molecules (area under the ROC curve [AUC] = 0.90; 95 % confidence interval [CI] 0.80–1.01; $p = 0.0002$). A sTREM-1 cut-off value of 158 pg/ml had a sensitivity of 86.7 % with a specificity of 86.7 %.

**Table 3** Serum concentrations of biomarker candidates in patients with active RP and patients with inactive RP

| Biomarker candidates[a] | Units | Active RP ($n = 8$) Mean ± SD | Inactive RP ($n = 7$) Mean ± SD | $p$* |
|---|---|---|---|---|
| sTREM-1 | pg/ml | 353.39 ± 158.03 | 200.14 ± 95.11 | **0.0403** |
| VEGF | pg/ml | 339.19 ± 218.10 | 185.48 ± 106.88 | 0.1066 |
| hs-CRP | ng/ml | 0.48 ± 0.64 | 0.10 ± 0.08 | 0.1342 |
| TNF | pg/ml | 1.43 ± 2.65 | N.D.[c] | 0.1708 |
| IL-6 | pg/ml | 2.38 ± 4.45 | N.D.[c] | 0.1752 |
| IL-17A | pg/ml | 0.05 ± 0.14 | 0.71 ± 1.14 | 0.2129 |
| MMP-3 | ng/ml | 334.71 ± 400.33 | 138.44 ± 135.59 | 0.2254 |
| MMP-1 | ng/ml | 5.35 ± 4.35 | 3.07 ± 2.51 | 0.2658 |
| MMP-13 | ng/ml | 0.30 ± 0.11 | 0.26 ± 0.05 | 0.3469 |
| IL-1α | pg/ml | 1.01 ± 2.86 | N.D.[c] | 0.3506 |
| IL-1β | pg/ml | 1.09 ± 3.07 | N.D.[c] | 0.3506 |
| IL-10 | pg/ml | 1.30 ± 3.68 | N.D.[c] | 0.3506 |
| IL-12p70 | pg/ml | 0.66 ± 1.87 | N.D.[c] | 0.3506 |
| CX3CL1 | pg/ml | 12.29 ± 34.75 | N.D.[c] | 0.3506 |
| MMP-2 | ng/ml | 139.68 ± 25.79 | 125.38 ± 31.39 | 0.3589 |
| COMP | ng/ml | 30.26 ± 35.31 | 17.56 ± 10.53 | 0.3598 |
| CXCL10 | pg/ml | 251.14 ± 110.78 | 204.78 ± 121.20 | 0.4563 |
| IFN-γ | pg/ml | 4.54 ± 7.29 | 6.93 ± 5.06 | 0.4703 |
| CXCL8 | pg/ml | 17.31 ± 6.34 | 15.01 ± 8.11 | 0.5571 |
| CCL2 | pg/ml | 80.59 ± 78.04 | 62.80 ± 30.33 | 0.5660 |
| CCL4 | pg/ml | 141.68 ± 90.46 | 124.71 ± 33.26 | 0.6332 |
| IL-4 | pg/ml | 0.83 ± 2.36 | 0.76 ± 2.02 | 0.9509 |
| CCL5 | ng/ml | 37.87 ± 17.21 | 37.42 ± 15.05 | 0.9585 |
| αCOLII Ab[b] | U/ml | 382.34 ± 808.48 | 162.44 ± 311.65 | 0.5525 |

*RP* relapsing polychondritis, *sTREM-1* soluble triggering receptor expressed on myeloid cells-1, *VEGF* vascular endothelial growth factor *hs-CRP* high-sensitivity C-reactive protein, *TNF* tumor necrosis factor, *N.D.* not detected, *IL* interleukin, *MMP* matrix metalloproteinase, *CX3CL* chemokine (C–X3–C motif) ligand, *COMP* cartilage oligomeric matrix protein, *CXCL* chemokine (C–X–C motif) ligand, *IFN* interferon, *CCL* chemokine (C–C motif) ligand, *αCOLII Ab* anti-type II collagen antibody

* By Welch's $t$ test. $p$ values of less than 0.05 are indicated by boldface

[a] The serum levels of IL-2, IL-5, GM-CSF, and CCL3 were below the detection limits in all cases

[b] The sample size of this item is different from that of the others due to the lack of some serum samples (active RP: $n = 6$, inactive RP: $n = 7$)

[c] For the statistical analyses, values of zero were substituted for the "N.D. (not detected)" entries

## Identification of serum markers of disease activity in RP

Next, to identify a serum marker that correlates with RP disease activity, we divided the 15 RP patients into two groups based on the extent of inflammation (see "Methods" for details) (Table 1): active RP ($n = 8$) and inactive RP ($n = 7$). We then compared serum levels of all tested molecules in the two RP groups. The results showed that only serum sTREM-1 level was significantly higher in active RP patients than in the inactive RP patients ($p = 0.0403$) (Table 3). Moreover, to investigate the association of serum sTREM-1 level with disease activity in RP, we examined the clinical course of one patient with active RP. As shown in Fig. 2, treatment with methotrexate (MTX) provided symptomatic improvement in this case; simultaneously, the patient's abnormally high sTREM-1 level was reduced to almost the same level as healthy donor (720.5 pg/ml in Nov 2009 → 106.6 pg/ml in June 2011). Importantly, before the MTX treatment, the patient's CRP level was almost normal, even when the sTREM-1 level was abnormally high (CRP 0.41 mg/dl, sTREM-1 720.5 pg/ml).

## Serum levels of sTREM-1 in patients with other immunological disorders

To investigate the disease specificity of sTREM-1, we measured the serum levels of this molecule in patients with other immunological disorders, including HTLV-1-associated

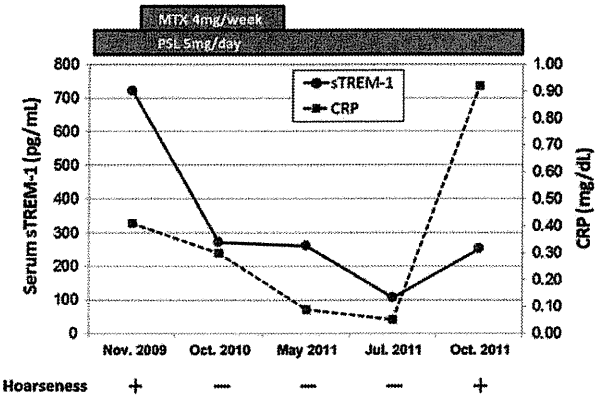



**Fig. 2** Clinical course of a patient who was classified as having active RP at the time of enrollment, in 2009. The *line chart* shows the time courses of the serum sTREM-1 level (*closed circles, solid line*) and the CRP level (*closed squares, dashed line*) in an RP patient treated with prednisolone (PSL) and methotrexate (MTX). A plus sign (+) indicates the presence of hoarseness as a respiratory tract symptom, while a minus sign (−) indicates the absence of that symptom

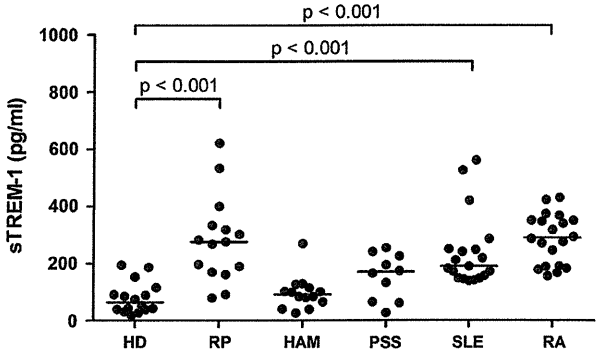



**Fig. 3** Comparison of serum sTREM-1 levels between HDs and patients with other immunological disorders, including RP. Individual values are plotted, and the *bars* represent medians of the values. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post hoc tests. *HAM* HTLV-1-associated myelopathy, *PSS* progressive systemic sclerosis, *SLE* systemic lupus erythematosus, and *RA* rheumatoid arthritis

myelopathy (HAM), progressive systemic sclerosis (PSS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). Serum sTREM-1 levels were higher by a statistically significant amount in patients with RP and in patients with SLE or RA when compared to the levels in HDs (Fig. 3). This result indicates that elevation of the serum sTREM-1 level is not specific to RP.

TREM-1 expression in chondritis-affected areas of RP patients

Finally, we examined the expression of membrane-bound TREM-1 in chondritis-affected areas of RP patients. Immunohistochemistry demonstrated that TREM-1 was expressed on vascular endothelial cells in perichondral granulation foci but not on chondrocytes (Fig. 4). No positive cells were observed in a control sample (nonspecific inflammatory granulation tissue derived from a ruptured epidermal cyst) (Fig. 4).

## Discussion

In this study, we identified serum sTREM-1 level as a novel biomarker for RP. We produced several results indicating the strength of this candidate marker: first, our results indicated that serum sTREM-1 level could discriminate RP patients from HDs more successfully than could other candidate biomarkers (Table 2; Fig. 1). Second, serum sTREM-1 level gave better discrimination between active RP patients and inactive RP patients than 27 other tested molecules, including hs-CRP, COMP, and anti-type II collagen antibody (Table 3). Third, the time course of serum sTREM-1 level was associated with the clinical course in an RP patient who was treated with prednisolone and MTX (Fig. 2). However, sTREM-1 showed some limitations in disease specificity, as its serum level was also elevated in patients with SLE or RA (Fig. 3). These results suggest that serum sTREM-1 level is suitable for use as a disease-activity marker for RP, but not as a diagnostic marker for the disease.

TREM-1, as the name suggests, has been shown to express on myeloid cells such as neutrophils and monocytes/macrophages [15]. Recently, it has been reported that TREM-1 is also expressed on endothelial cells (a type of non-myeloid cell) in liver tissue from lipopolysaccharide-treated mice [16]. In this study, our immunohistochemical analyses demonstrated that TREM-1 is expressed on human endothelial cells in chondritis-affected areas of RP patients (Fig. 4). The increase in sTREM-1 in the blood of RP patients might be due to its presence on the surfaces of endothelial cells in those inflammatory lesion sites. This hypothesis is supported by the finding that there was no difference in the expression level of TREM-1 on peripheral blood mononuclear cells between healthy donors and RP patients (data not shown). However, further investigations are needed to clarify the source of the increased sTREM-1.

It was previously reported that the expression of TREM-1 is induced by bacterial infection and that levels of circulating sTREM-1 are important as a diagnostic and prognostic marker of sepsis [17–19]. More recently, however, it has been reported that the serum sTREM-1 level is elevated in non-infectious chronic inflammatory diseases such as RA and inflammatory bowel diseases [20, 21]. Therefore, our finding that serum samples from patients with chronic inflammatory diseases (including RP, RA, and

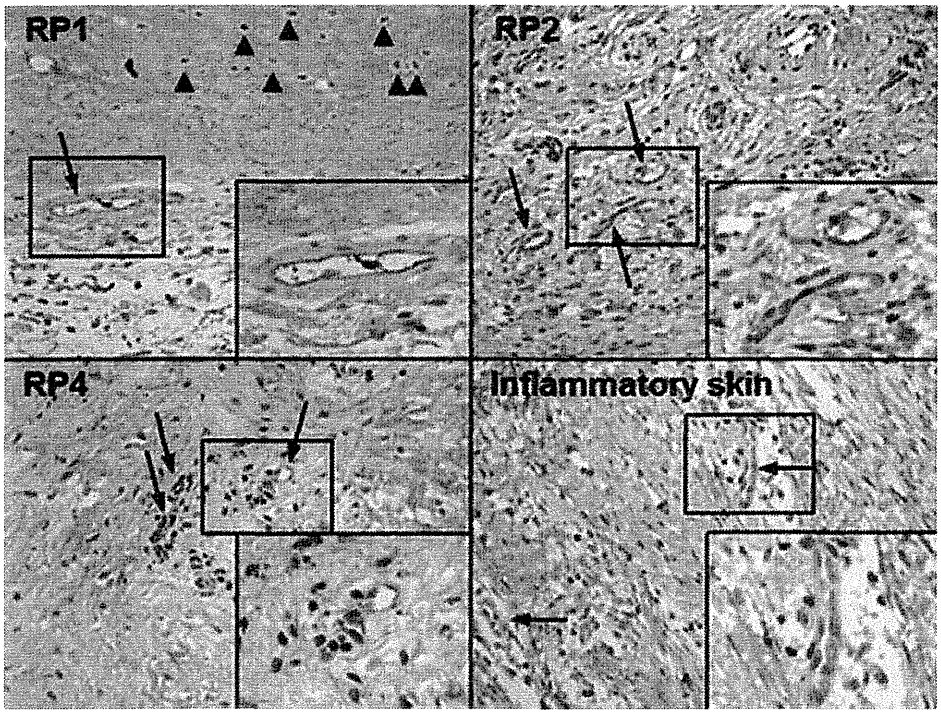



**Fig. 4** Immunohistological staining showing the expression of TREM-1 in chondritis-affected areas. Inflammatory granulation tissue from a patient with a ruptured epidermal cyst was used as a negative control (*lower right panel*: inflammatory skin). TREM-1-positive cells were stained brown using 3,3′-diaminobenzidine (DAB) and are displayed at a higher magnification in the *lower right inset*. *Arrows* and *arrowheads* indicate vascular endothelial cells and chondrocytes, respectively

SLE) had significantly higher concentrations of sTREM-1 is consistent with previous reports. On the other hand, serum level of sTREM-1 in patients with HAM—a chronic inflammatory neurologic disease caused by human T cell leukemia virus-1—was not significantly higher than the level in HDs. This indicates that the serum level of sTREM-1 differs among patients with different chronic inflammatory diseases. Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a chronic inflammatory disease. Patients with AAV show elevated levels of serum sTREM-1 [22]. Intriguingly, as in RP, sTREM-1 levels in active AAV have been shown to be significantly higher than those for inactive AAV [22]. Thus, elevated levels of serum sTREM-1 have been observed in several chronic inflammatory diseases.

Such disorders with elevated sTREM-1 levels often overlap in the same patient. For example, 14 % of patients with RP have clinically evident vasculitis [23] and 35.5 % of patients have other collagen diseases, such as RA or SLE [24]. These examples imply the existence of common mechanisms in the pathogenesis of these disorders. In this regard, because TREM-1 works as an amplifier of inflammatory responses through the production of multiple pro-inflammatory cytokines and chemokines, TREM-1 may play an important role in the common pathomechanisms of these disorders [15, 21, 25, 26]. A previous study provided in vivo evidence that the blockade of TREM-1 can ameliorate collagen-induced arthritis in mice [27].

One of the molecules that has been reported as a disease-activity marker for RP is COMP [7]. This is a non-collagenous protein found in the matrix of cartilage. Lekpa et al. reported that serum COMP levels during the active phase were significantly higher than those seen during the inactive phase in the same patients. However, our results showed no significant differences in the serum levels of this molecule in active RP patients compared to inactive RP patients (Table 3). This discrepancy could be attributed to the different study designs employed, including differing disease conditions of the RP patients, sample sizes, and measurement methods.

To further characterize this molecule, we checked for correlations between serum levels of COMP and the other tested molecules. Interestingly, serum COMP levels in RP patients had a strong positive correlation only with serum MMP-3 levels ($rs = 0.7357$, $p = 0.0018$, by Spearman rank correlation test, data not shown). This suggests that serum levels of MMP-3 and COMP might reflect the degree of cartilage destruction in RP patients, since serum

MMP-3 level is considered a predictor of the degree of cartilage destruction in patients with early RA [28].

In conclusion, this study suggests that serum sTREM-1 level can serve as a more sensitive marker for disease activity in RP patients than other candidate molecules, such as CRP, COMP, and anti-type II collagen antibody.

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**Conflict of interest** None.

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ORIGINAL ARTICLE

# The Japanese version of the modified ACR Preliminary Diagnostic Criteria for Fibromyalgia and the Fibromyalgia Symptom Scale: reliability and validity

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**Abstract**
*Purpose* The aim of this study is to investigate the reliability and validity of the Japanese version of the modified American College of Rheumatology (ACR) Preliminary Diagnostic Criteria for Fibromyalgia (mACR 2010-J) and the Fibromyalgia Symptom Scale (mFS-J).
*Methods* According to the ACR 1990 classification criteria, patients with chronic pain were divided into the fibromyalgia group and nonfibromyalgia group (rheumatoid arthritis and osteoarthritis). Patients in both groups were assessed using mACR 2010-J and mFS-J.
*Results* 294 of 462 (64 %) patients in the fibromyalgia group met mACR 2010-J, whereas 4 % (9/231) of the nonfibromyalgia group did, with sensitivity of 64 %, specificity of 96 %, positive predictive value of 97 %, negative predictive value of 56 %, and positive likelihood ratio of 16.3. Mean total scores on mFS-J significantly differentiated the fibromyalgia from the nonfibromyalgia group. According to the value of the Youden index, the best cutoff score for the mFS-J was 9/10.
*Conclusion* Our findings indicate that mACR 2010-J as a positive test and mFS-J as a quantification scale might be suitable for assessing fibromyalgia among Japanese chronic pain populations.

**Keywords** Diagnostic criteria · Fibromyalgia · Symptom scale · Modified ACR Preliminary Diagnostic Criteria for Fibromyalgia

## Introduction

Fibromyalgia (FM) is characterized by widespread musculoskeletal chronic pain, fatigue, poor sleep, frequent psychological difficulties, and multiple tender points on physical examination [1, 2]. In 1990, the American College

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of Rheumatology (ACR) presented FM criteria (ACR 1990) that required tenderness on pressure (tender points) in at least 11 of 18 specified sites and the presence of widespread pain for diagnosis [1]. Widespread pain was defined as axial pain, both left- and right-sided and with upper and lower segment pain. However, ACR 1990 had the serious problem of little variation in symptoms. To improve this shortcoming, new clinical criteria, which integrate variations in symptoms with severity scale (2010 ACR Preliminary Diagnostic Criteria for FM, ACR 2010) [3], have been presented. The diagnostic criteria for FM are satisfied if the following three conditions are met: (1) Widespread Pain Index (WPI) $\geq$7 and Symptom Severity Score (SS) $\geq$5, or WPI of 3–6 and SS $\geq$9; (2) symptoms have been present at a similar level for at least 3 months; and (3) the patient does not have a disorder that would otherwise explain the pain. The publication of ACR 2010 eliminated the tender point examination, thus making it possible to study FM in survey and clinical research.

Accordingly, we have validated the Japanese version of ACR 2010 [4]. In addition, we have originally validated the Japanese version of the Fibromyalgia Symptom Scale with the sum of WPI and the original SS, i.e., fatigue, waking unrefreshed, cognitive symptoms, and somatic symptoms in general consisting of 41 symptoms of the FS-J [4]. Both ACR 2010-J and FS-J have high reliability and validity, and are useful for assessing fibromyalgia among Japanese chronic pain populations.

Recently, Wolfe et al. [5] proposed a modification of the ACR 2010 (mACR 2010), deleting 38 out of 41 somatic symptoms in general from the original SS. Consequently, complete self-administration has become possible. Furthermore, they created the Fibromyalgia Symptom Scale with the sum of WPI and the new SS (FS). They reported that the criteria properly identified diagnostic groups, and that FS score $\geq$13 best separated criteria+ and criteria− patients.

The aim of this study is to investigate the reliability and validity of the Japanese version of the mACR 2010 (mACR 2010-J) and the Japanese version of the FS (mFS-J). Furthermore, our questions are whether mACR 2010-J would be more useful than ACR 2010-J for assessing fibromyalgia among Japanese chronic pain populations, and whether mFS-J is more suitable than FS-J as a positive test.

## Subjects and methods

An experienced rheumatologist and an experienced psychiatrist had translated the mACR 2010 into Japanese with the author's permission and produced forward- and back-translations to create the mACR 2010-J.

We recruited FM patients who met the previous criteria of the ACR 1990 and were without psychiatric disorders according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [6] in a clinic specialized for FM, the Kasumigaseki Urban Clinic, in Tokyo, Japan, between August 1, 2010 and July 31, 2011. During the study period, other patients with diseases associated with chronic pain such as rheumatoid arthritis (RA) and osteoarthritis (OA) who had not been diagnosed previously with FM were recruited as control patients. To adjust the imbalance of number of patients, control patients were additionally recruited from May 30 to July 2, 2012. The diagnoses of RA and OA were made according to the 2010 rheumatoid arthritis classification criteria [7] and the American College of Rheumatology criteria for classification and reporting of osteoarthritis of the hand, hip, and knee [8–10]. The experienced rheumatologist and the experienced psychiatrist familiar with FM assessed these patients. This study was approved by the Institutional Review Board of Kasumigaseki Urban Clinic.

After obtaining informed consent from study participants, the rheumatologist rated patients with the mACR 2010-J. In order to assess interrater reliability, another rater independently rated a subset of the same subjects ($N = 19$) while blind to the diagnoses and scores of the other rater. The raters in this study were already fully trained in use of the scale and quite experienced in use of it. We therefore decided that only a small subsample was needed to reevaluate consistency across raters.

### Statistics

Data were analyzed using SPSS 17.0-J software. Differences among groups in demographic and clinical characteristics were calculated with the unpaired $t$ test. If data were not sampled from Gaussian distributions, a nonparametric test (Mann–Whitney $U$ test) was used. To compare categorical data, we used Fisher's exact test.

In the present study, the control group was not healthy volunteer but consisted of chronic pain patients with RA and OA. It has been reported that the age-specific incidence of RA peaked in the 60–64 and 70–74 year age groups for females and males, respectively, in Taiwan [11]. Similarly, it has been reported that the peak prevalence of knee OA in women and men was $\geq$80 years in Japan [12]. In contrast, we have reported that the frequent age of onset of FM in women was 35–55 years based on our FM database including 3,500 Japanese patients with FM [13]. Among Asians, thus, patients with FM are much younger than those with RA and OA. Therefore, matching age of control patients with age of FM patients seems to be rather arbitrary. Accordingly, to control for the effect of age on the rate of patients meeting the mACR 2010-J, patients were divided into three age categories, i.e., 20–39, 40–59, and $\geq$60 years.

There were only eight FM patients and one non-FM patients less than 20 years of age, and there were only two FM patients and three non-FM patients 80 years or older. Then, the Mantel–Haenszel method was used to test the difference in the percentage of patients meeting the mACR 2010-J between the two groups. Also, to control for the effect of age on the score on the mFM-J, one-way analysis of covariance was used. The internal consistency for the mFM-J was calculated with Cronbach's $\alpha$. Interrater reliability was measured with the intraclass correlation coefficient (ICC) for pairs of independent raters. Cutoff scores for the mFS-J were determined using receiver-operator characteristic (ROC) analyses to determine the Youden index when comparing the FM group with all non-FM subjects. Positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio (sensitivity/1 − specificity) were also calculated. All statistical tests were two-tailed. Statistical significance was set at $p < 0.05$.

## Results

A total of 462 patients meeting the ACR 1990 (the FM group) and a total of 231 non-FM patients (RA patients, 196; OA patients, 35; the non-FM group) were enrolled. Demographic and clinical characteristics of the groups are presented in Table 1, showing that 294 of 462 (64 %) patients in the FM group met the mACR 2010-J, whereas 4 % (9/231) of the non-FM group did, including 4 % (8/196) of RA patients and 3 % (1/35) of OA patients. The percentage of patients meeting the mACR 2010-J criteria in the FM group was significantly higher than that of the non-FM group after adjusting for age (estimated odds ratio, 35.7, $p < 0.0001$; Table 1). The sensitivity, specificity, PPV, NPV, and positive likelihood ratio for comparison of the FM group with all non-FM subjects were 64, 96, 97, 56, and 16.3 %, respectively. The ICC between the two independent raters was very high for the mACR 2010-J, at 0.877.

The mean score (standard deviation, SD) of mFS-J in the FM group was 16.7 (6.5), while that in the non-FM group was 3.7 (4.1). The mean score of mFS-J in the FM group was significantly higher than that of the non-FM group after adjusting for age ($F = 605.1$, $p < 0.0001$; Table 1). Internal consistency was not high, with a Cronbach's $\alpha$ coefficient for the mFS-J (WPI + the modified SS) of 0.603. ROC analyses were performed for the mFS-J, comparing the FM group with the non-FM group. Table 2

**Table 1** Demographic and clinical characteristics of the fibromyalgia group and nonfibromyalgia group

| Group | Fibromyalgia ($N = 462$) | Nonfibromyalgia ($N = 231$) | | $p$ |
|---|---|---|---|---|
| | | RA ($N = 196$) | OA ($N = 35$) | |
| Mean age (SD), years | 50.6 (14.8) | 61.3 (13.9) | | <0.0001 |
| | | 60.6 (14.4) | 65.5 (10.2) | |
| Sex (female), $N$ (%) | 389 (84) | 188 (81) | | 0.39 |
| | | 160 (82) | 28 (80) | |
| Patients meeting the mACR 2010-J,[a] $N$ (%) | 294 (64) | 9 (4) | | <0.0001[c] |
| | | 8 (4) | 1 (3) | |
| Mean score (SD) of mFS-J[b] | 16.7 (6.5) | 3.7 (4.1) | | <0.0001[d] |
| | | 3.7 (4.2) | 3.9 (3.3) | |

[a] The Japanese version of the modified 2010 ACR Preliminary Diagnostic Criteria for Fibromyalgia

[b] The Japanese version of the Fibromyalgia Symptom Scale (WPI + modified SS)

[c] To control for the effect of age on the rate of patients meeting the mACR 2010-J, patients were divided into three age categories. Then, the Mantel–Haenszel method was used to test the difference in the percentage of patients meeting the mACR 2010-J between the two groups

[d] To control for the effect of age on the score of the mFM-J, one-way analysis of covariance was used

*SD* standard deviation

**Table 2** Sensitivity and specificity of the Japanese version of the Fibromyalgia Symptom Scale (mFS-J), based on receiver-operating characteristics (ROC) analysis: fibromyalgia group versus nonfibromyalgia (RA and OA) group

| Cutoff score | Sensitivity (%) | Specificity (%) | Positive likelihood ratio | Youden index |
|---|---|---|---|---|
| 8.5 | 87.7 | 89.2 | 8.1 | 0.769 |
| 9.5 | 84.8 | 92.2 | 10.9 | 0.770 |
| 10.5 | 82.0 | 92.2 | 10.5 | 0.742 |

shows the sensitivity, specificity, positive likelihood ratio, and Youden index for ROC analysis at various cutoff scores for the mFS-J. According to the value of the Youden index, the best cutoff score for the mFS-J was 9/10.

## Discussion

This is the first study to validate the mACR 2010-J and mFS-J, which is the quantification scale of the mACR 2010-J. The positive likelihood ratio of 16.3 for the mACR 2010-J is sufficiently high as a positive test. We cannot directly compare the likelihood ratios for the mACR 2010-J and the ACR 2010-J, as the present study group is quite different from that used in the study of the ACR 2010-J [4]. However, the value of mACR 2010-J is sufficiently high compared with that of ACR 2010-J, for which the positive likelihood ratio was 8.8. Therefore, the modification of ACR 2010-J may be superior to the original ACR 2010-J as a positive test.

The best cutoff score for the mFS-J was 10, which is just the same as the FS-J [4]. Furthermore, the positive likelihood ratio for the mFS-J (Table 2) is as high as that for the FS-J at the cutoff score [4]. As the mFS-J is simpler than the FS-J, mFS-J may be superior to FS-J based on the original ACR 2010-J as a quantification scale. Meanwhile, the best cutoff score of 10 in the present study is smaller than that of the original study on the FS performed in the USA (cutoff score 13) [5]. One explanation for this difference is in patient characteristics. In the present study, comorbid psychiatric disorders were excluded, while in the previous study they were not. Patients with major depressive disorder, panic disorder, or anxiety disorder usually have somatic symptoms similar to those of ACR 2010, and comorbidity of major depressive disorder, panic disorder, or anxiety disorder is not rare [1]. Therefore, the population in the previous study may have been modified by comorbid psychiatric disorders. Thus, the cutoff score of 10 in the present study might reflect fibromyalgia itself more than that of 13 in the previous study. Another possible explanation is cross-cultural differences in expression or rating of symptoms.

The internal consistency with a Cronbach's $\alpha$ coefficient for the mFS-J (WPI + the modified SS) of 0.603 is lower than that for the FS-J (WPI + the original SS) of 0.747 [4]. The modified SS consists of fatigue, waking unrefreshed, cognitive symptoms, plus having pain/cramps in the abdomen, depression, and headache, resulting from 38 somatic symptoms in general having been deleted from the original SS. Therefore, the modified SS values neuropsychiatric symptoms more than the original SS. In contrast, WPI is the number of pain areas, which is simply somatic. Accordingly, the internal consistency for the mFS-J (WPI + the modified SS) might have been lower than that for the FS-J (WPI + the original SS). As FM shows a variety of symptoms and is suspected of involving not only musculoskeletal but also central nervous system [14], the not so high value of internal consistency for the mFS-J may not necessarily be a shortcoming.

Thus, mACR 2010-J as a positive test and mFS-J as a quantification scale might be suitable for assessing fibromyalgia among Japanese chronic pain populations. A strength of the present study is that the findings represent real clinical practice in Japan, since the study was performed in a clinic specialized in FM which is visited by the largest number of FM patients in Japan. A limitation of this study is that the findings may not be applicable to all patients, since FM patients with other musculoskeletal diseases, such as spondylitis, were not included in it. Further studies with patients of other countries or ethnicities will be needed to determine cross-cultural or ethnic differences in expression or rating of symptoms.

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**Conflict of interest** None.

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# RING-finger type E3 ubiquitin ligase inhibitors as novel candidates for the treatment of rheumatoid arthritis

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**Abstract.** Rheumatoid arthritis (RA) significantly affects quality of life. We recently cloned synoviolin, a RING-type E3 ubiquitin ligase implicated in the endoplasmic reticulum-associated degradation (ERAD) pathway. Synoviolin is highly expressed in rheumatoid synovial cells and may be involved in the pathogenesis of RA. Inhibition of synoviolin activity is a potentially useful therapeutic approach for the treatment of RA. We conducted a high-throughput screen of small molecules to find inhibitors of synoviolin autoubiquitination activity. We identified two classes of small molecules, named LS-101 and LS-102, which inhibited synoviolin activity. LS-102 selectively inhibited synoviolin enzymatic activity, while LS-101 inhibited a broad array of RING-type E3 ligases. Moreover, these inhibitors suppressed the proliferation of rheumatoid synovial cells, and significantly reduced the severity of disease in a mouse model of RA. Our results suggest that inhibition of synoviolin is a potentially useful approach in the treatment of RA.

## Introduction

Rheumatoid arthritis (RA) is the most common chronic inflammatory joint disease, affecting ~0.5-1% of people in the industrialized world (1). Clinically, the disorder is characterized by joint pain, stiffness, and swelling due to synovial inflammation and effusion. The clinical features of RA are based on several pathological processes including chronic inflammation, overgrowth of synovial cells, bone and joint destruction, and fibrosis. Currently, the goal of RA treatment is the control of underlying inflammatory process to prevent joint damage using non-steroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARD). The most widely used small molecule DMARD is methotrexate, which shows the highest retention rate compared with other agents (2). In recent years, biological agents such as inhibitors of tumor necrosis factor (TNF) signaling have become available for clinical use; however, this therapy is prohibitively expensive, and although TNF inhibitors are clinically as effective as methotrexate, the frequency and extent of response are more restricted. In fact, many patients can lose the clinical response to TNF inhibition, highlighting the need for other treatment modalities to further improve the outcome of RA (3,4).

To address this need, we have been investigating the mechanism of outgrowth in rheumatoid synovial cells (RSCs). First, we demonstrated the crucial role of Fas antigen-induced apoptosis in synovial cell hyperplasia (5). Then, while studying cellular functions of RSCs, we cloned synoviolin from these cells (6). Synoviolin, a mammalian homolog of Hrd1p/Der3p (7-9), is an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase with a RING motif that is involved in ER-associated degradation (ERAD) pathway. Synoviolin is also highly expressed in synoviocytes of patients with RA (6,10-12). Overexpression of synoviolin in transgenic mice leads to advanced arthropathy caused by reduced apoptosis of synoviocytes (6). We postulated that hyperactivation of the ERAD pathway by overexpression of synoviolin prevents ER-stress-induced apoptosis, leading to synovial hyperplasia (13). Synoviolin$^{+/-}$ knockout mice showed resistance to the development of collagen-induced arthritis (CIA) due to enhanced apoptosis of synovial cells (6). Consistent with our hypothesis, cells from these mice show impaired ERAD due to the lack of synoviolin. In addition, synoviolin ubiquitinates and sequesters the tumor suppressor p53 in the cytoplasm, thereby negatively regulating its biological functions in transcription, cell cycle regulation, and apoptosis by targeting it instead for proteasomal degradation (14). Therefore, synoviolin regulates apoptosis in response to ER stress (through ERAD) as well as p53-dependent apoptosis.

Together, these studies implicated synoviolin as a candidate pathogenic factor in arthropathy, and suggested that the gene dosage of this protein correlates with the onset of arthropathy. Furthermore, elevated synoviolin levels were identified in

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circulating monocytes in association with resistance to treatment with infliximab (a monoclonal antibody against TNF) (10). Therefore, blocking the function of synoviolin could be clinically beneficial in RA patients. This study attempted to identify an inhibitor of synoviolin that acts by blocking its enzymatic activity.

## Materials and methods

*Screening of synoviolin inhibitor.* Purified glutathione S-transferase (GST)-synoviolin Δ transmembrane domain (TM) was mixed with glutathione-SPA beads (Amersham Pharmacia Biotech) in buffer (50 mM Tris-HCl, pH 7.4, Protease inhibitor cocktail, 14 mM β-mercaptoethanol, 0.5 μl cell lysate/well, 0.2 mg SPA bead/well) and incubated for 30 min at room temperature. Glutathione-SPA beads were washed twice, and then mixed with the candidate synoviolin inhibitor compounds in buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM NaF, and 10 nM okadaic acid) in the presence of ATP (2 mM), $^{33}$P-labeled ubiquitin (0.38 μg/well), E1 (25 ng/well) (Affiniti Research), and E2 (0.3 μg/well) (UbcH5c). After incubation for 90 min at room temperature, buffer comprising 0.2 M boric acid, pH 8.5, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2% Triton-X100 was added to stop the reaction. The beads were allowed to settle and the amount of $^{33}$P-ubiquitin incorporated into the GST-synoviolin beads was determined using a Microbeta Scintillation counter.

The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Compound mixtures showing potential activity in the primary screen were then rescreened at one compound per well to determine the active compound within the mixture. Three equivalents of a single compound per well follow-up screening were evaluated. Reconfirmed active compounds were resynthesized and tested in a dose-response experiment to determine potency.

*In vitro ubiquitination assay.* The *in vitro* ubiquitination assay used in this study was described previously (15). Briefly, 40 ng of E1 (Affiniti Research), 0.3 μg of E2 (UbcH5c), 0.75 μg of $^{32}$P-labeled ubiquitin (a gift from T. Ohta), and 1 μg of recombinant E3 ubiquitin ligases were incubated for 30 min at 37°C. Samples were analyzed as described above.

*Cells.* HeLa cells were obtained from ATCC. Synovial cells were isolated from synovial tissue obtained patients with rheumatoid arthritis (RA) who met the American College of Rheumatology criteria for RA at the time of orthopedic surgery. These cells were cultured in Dulbecco's modified Eagle's medium (Sigma).

*Proliferation assay.* The proliferation of rheumatoid synovial cells (RSCs) was evaluated using Alamar blue (BioSource International) according to the manufacturer's instructions.

*Induction of CIA.* CIA was induced as described previously (6). Briefly, bovine type II collagen (Collagen Research Center) was dissolved overnight in 0.05 M acetic acid at 4°C, and then emulsified in complete Freund's adjuvant (Difco) to a final concentration 1 mg/ml. DBA/1 male mice (7-week-old) were immunized by subcutaneous injections containing 100 μg of collagen emulsion. After 3 weeks, mice were boosted with 200 μg collagen emulsion in Freund's complete adjuvant. Then, the mice were treated daily for 4 weeks with the inhibitor compounds at 1.3, 4.0, and 12.0 mg/kg/day in olive oil, vehicle control intraperitoneally, or oral administration of 0.25 mg/kg/day dexamethasone in methylcellulose as a positive control.

The mice were monitored daily for signs of arthritis using an established scoring system (16): 0, no swelling or redness; 1, swelling, redness of paw or 1 joint; 2, two joints involved; 3, more than two joints involved; 4, severe arthritis of entire paws and joints. All paws were evaluated in each animal and the maximum score per animal was 16.

*Histological studies.* The knee and elbow joints were fixed in 4% paraformaldehyde. After decalcification with EDTA, the joints were embedded in paraffin, and 4-μm sections were prepared for staining with hematoxylin and eosin. The extent of arthritis in the joints was assessed according to the method reported by Tomita *et al* (17): 0, normal synovium; 1, synovial membrane hypertrophy and cell infiltration; 2, pannus and cartilage erosion; 3, major erosion of cartilage and subchondral bone; 4, loss of joint integrity and ankylosis.

*Statistical analysis.* All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student's t-test. A P-value $<0.05$ denoted the presence of a statistically significant difference.

*Ethical considerations.* The ethics committee for Animal Experiments of St. Marianna University School of Medicine approved the mice experiments described in this study. Furthermore, all the experimental protocols described in this study were approved by the Ethics Review Committee of St. Marianna University School of Medicine (Approval number 01008), and the written informed consent was obtained from all patients.

## Results

*High-throughput compound screening for inhibitors of synoviolin.* To identify small molecule inhibitors of synoviolin autoubiquitination, we screened the Lead Discovery Service program of Pharmacopeia, which includes more than four million compounds from Pharmacopeia's Compound Collection (18). Herein we monitored $^{33}$P-autoubiquitinated synoviolin in cell lysates containing GST-synoviolinΔTM in the presence of ATP, E1, E2, and $^{33}$P-labeled ubiquitin (Fig. 1A). The primary screen was conducted with multiple compounds per well (10-20 compounds per well) at an estimated screening concentration of 2-10 μM. Mixtures of compounds showing potential activity in the primary screen were then rescreened individually. Compounds demonstrating activity in this reconfirmation assay were resynthesized and retested. Two unique compounds, termed LS-101 and LS-102, inhibited the autoubiquitination of synoviolin with a 50% inhibitory concentration value ($IC_{50}$) of ~15 μM (Fig. 1B) and 20 μM (Fig. 1C), respectively.

*LS-101 and LS-102 inhibit the autoubiquitination of synoviolin.* Further evaluation of LS-101 and LS-102 in an *in vitro* ubiqui-

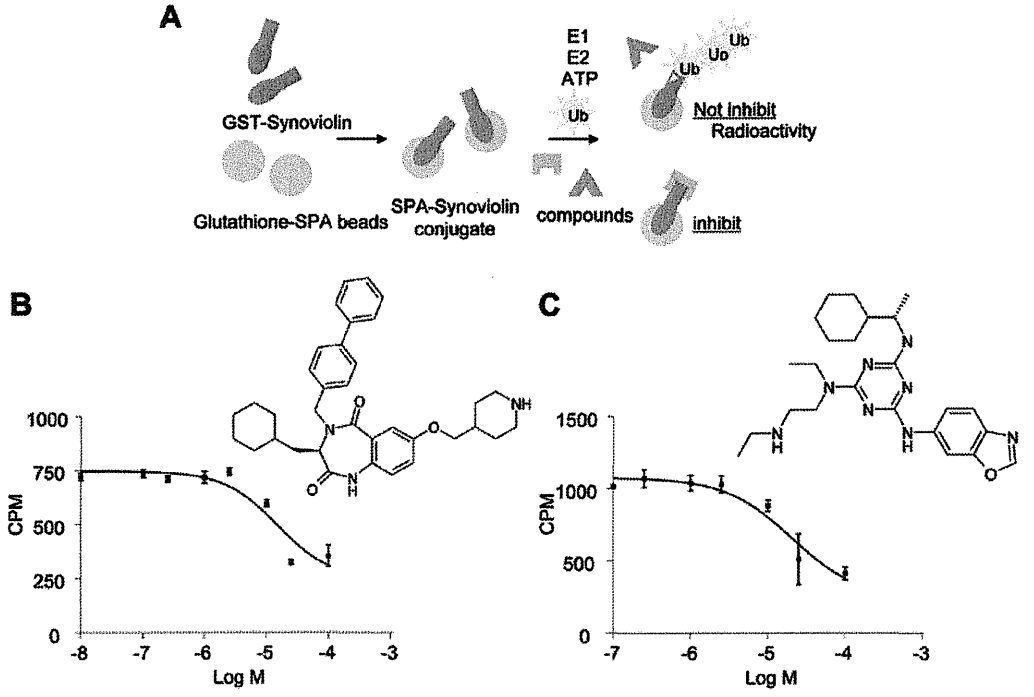


Figure 1. Screening for synoviolin inhibitors. (A) Scheme of high-throughput screening of synoviolin-induced ubiquitination assay. (B) Inhibition of synoviolin $^{33}$P-polyubiquitination by LS-101 and chemical structure of LS-101. (C) Inhibition of synoviolin $^{33}$P-polyubiquitination by LS-102 and chemical structure of LS-102.

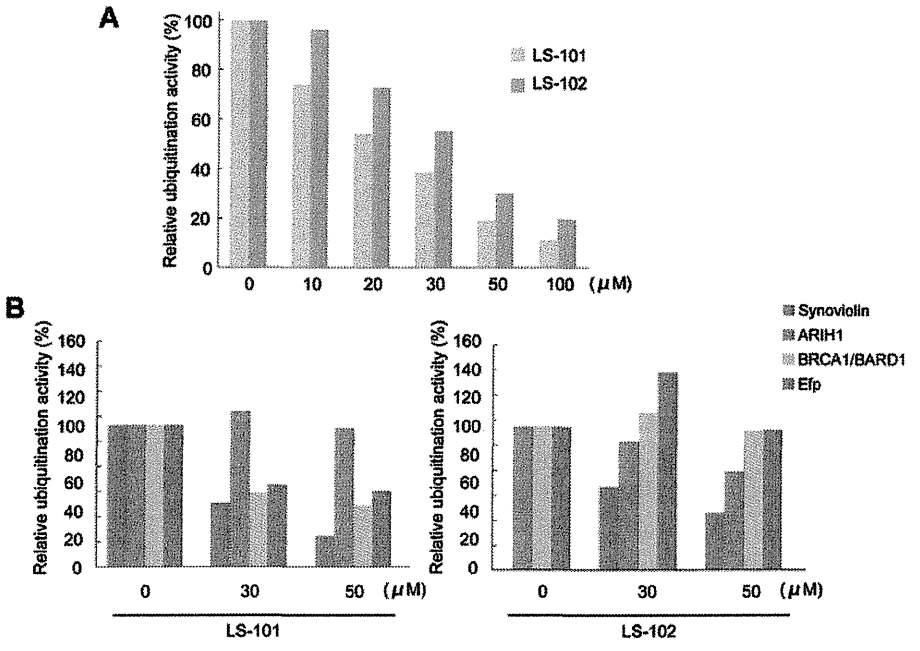


Figure 2. Effects of LS-101 and LS-102 on *in vitro* ubiquitination. (A) Both LS-101 and LS-102 inhibited the autoubiquitination of synoviolin in a dose-dependent manner. The $IC_{50}$ of LS-101 was 20 $\mu$M and that of LS-102 was 35 $\mu$M. (B) Selectivity of LS-101 (left) and LS-102 (right) against other E3 ubiquitin ligases. LS-102 inhibited synoviolin selectively compared with LS-101. Data are mean ± SEM of 3 experiments.

tination assay showed that the inhibition of synoviolin activity by both LS-101 and LS-102 was dose-dependent (LS-101; $IC_{50}$=20 $\mu$M, LS-102; $IC_{50}$=35 $\mu$M) (Fig. 2A). To assess the selectivity of the compounds for other E3 ubiquitin ligases, we determined the effects of LS-101 and LS-102 on the enzymatic activity of the following RING-finger type E3 ubiquitin ligases: ariadne, *Drosophila*, homolog of, 1 (ARIH1) (19), breast cancer 1 gene (BRCA1)/BRCA1-associated RING domain 1 (BARD1) (20), and estrogen-responsive RING-finger protein (Efp) (21). LS-101 inhibited the activity of BRCA1/BARD1 and Efp

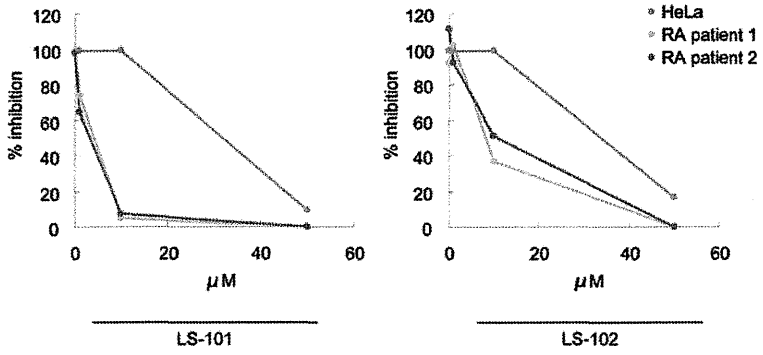


Figure 3. Effects of LS-101 and LS-102 on cell growth of RSCs. HeLa cells and RSCs derived from two RA patients were treated with synoviolin inhibitors for 12 h at the indicated concentrations. LS-101 and LS-102 repressed the proliferation of each RSC population tested. Data are expressed as the mean percentage of inhibition of the vehicle-treated control group ± SEM; (n=3).

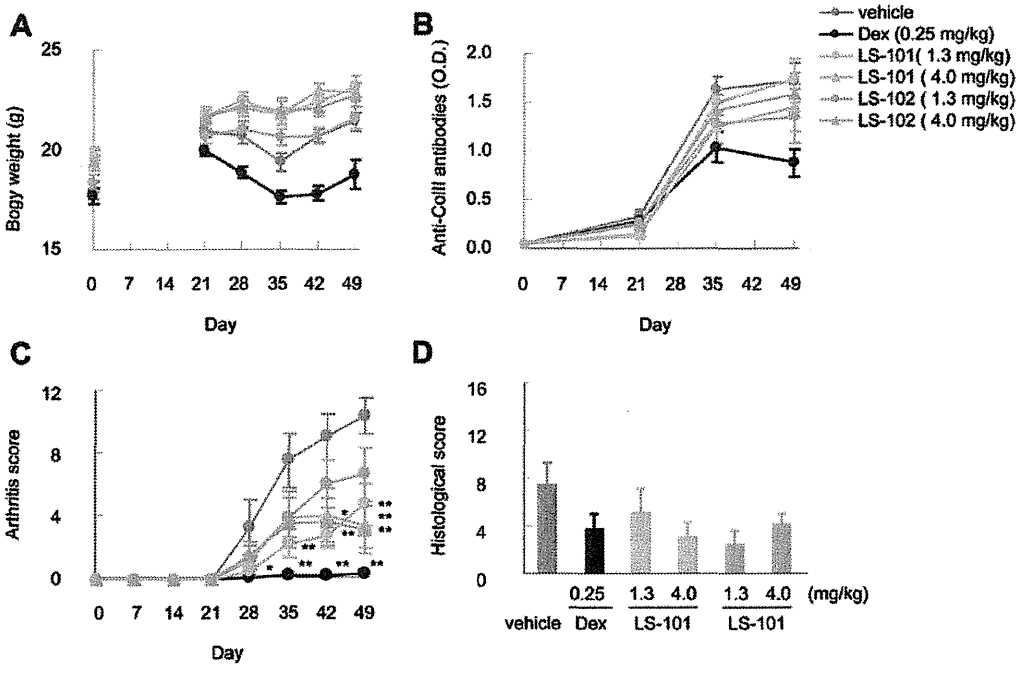


Figure 4. Effects of LS-101 and LS-102 in mouse CIA. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were treated with the vehicle alone, 0.25 mg/kg dexamethasone (Dex), or with 1.3, 4.0 mg/kg LS-101 or LS-102 from day 21 to 49. (A) Change in body weight. (B) The level of anti-type II collagen antibodies. (C) Total arthritis score. (D) Histological arthritis score. Data are mean ± SEM (initial n=12; final n=7). $^{*}P<0.05$, $^{**}P<0.01$.

(Fig. 2B), although this effect was weaker than that observed with synoviolin (Fig. 2B). Moreover, LS-101 had no effect against the enzymatic activity of ARIH1 (Fig. 2B). On the other hand, LS-102 did not inhibit the activity of other E3 ubiquitin ligases, only affecting synoviolin (Fig. 2B). These results suggested that LS-102 is a more selective synoviolin inhibitor than LS-101.

*LS-101 and LS-102 inhibit proliferation of RSCs.* We next tested LS-101 and LS-102 for their effects on the proliferation of RSCs, using HeLa cells as a control. LS-101 and LS-102 inhibited HeLa cell growth only at very high concentrations (LS-101; $IC_{50}$=31.3 μM, LS-102; $IC_{50}$=32.7 μM). However, treatment of RSCs with these compounds suppressed synovial cell growth dose-dependently and with much greater potency than that observed in HeLa cells (Fig. 3). A similar effect was also observed in another line of RSCs (Fig. 3). In addition, LS-101 inhibited synovial cell proliferation more potently than LS-102 (LS-101; $IC_{50}$=4.2 μM, LS-102; $IC_{50}$=5.4 μM). These results demonstrated that blockade of synoviolin function reduced the proliferation of RSCs, and that RSCs are more susceptible to this effect than HeLa cells. Consistent with these findings, higher expression levels of synoviolin were observed in RSCs than in HeLa cells (6).

*LS-101 and LS-102 reduce clinical severity scores in a CIA model.* To evaluate the *in vivo* efficacy of synoviolin inhibitors, we tested LS-101 and LS-102 in a mouse model of arthritis over a period of 28 days. No reduction of body weight was observed during the administration of these compounds (Fig. 4A). Moreover, the production of anti-type II collagen antibodies resulting from type II collagen immunization in both the LS-101 and LS-102 group was comparable to that

observed in the vehicle control group (Fig. 4B). Intraperitoneal treatment with LS-101 or LS-102 starting on day 21 reduced the clinical severity scores compared to vehicle controls (Fig. 4C). The efficacy was observed at both 1.3 mg/kg and 4.0 mg/kg doses in this experiment, although the protective effect of LS-101 at 1.3 mg/kg against CIA was stronger than the same dose of LS-102. At 4.0 mg/kg, there was no difference in the effects between LS-101 and LS-102. Finally, histological analysis showed lower histological arthritis scores in mice treated with the synoviolin inhibitors compared with wild-type mice (Fig. 4D).

## Discussion

The selective degradation of proteins in eukaryotic cells is carried out by the ubiquitin proteasome system (UPS), whereby proteins are targeted for degradation by covalent ligation to small polypeptide ubiquitin (22,23). This reaction requires the sequential actions of three enzymes: E1, E2, and E3 ligases (22,23). E3 ligases are responsible for conferring selectivity to ubiquitination by recognizing specific substrates. Bioinformatic analysis has identified over 600 E3 ligases, with RING-type E3 ligases constituting the largest subfamily within this group (24). Accordingly, RING E3 ligases have been linked to the control of multiple cellular processes and to many human diseases such as diabetes mellitus, polyglutamine disease, and Parkinson's diseases (24-26). In the UPS, the proteasome inhibitory agent bortezomib (Velcade) was recently approved for the treatment of multiple myeloma and mantle cell lymphoma (27). Bortezomib induces apoptosis of a wide variety of cancer cells, and is the first proteasome inhibitor to gain FDA approval (28-30). However, widespread clinical use of bortezomib continues to be hampered by the appearance of dose-limiting toxicities, drug-resistance, and interference by some natural compounds (31). Thus, despite the efficacy of bortezomib for treating lethal diseases such as cancer, the associated toxicities prevent its use for the treatment of chronic diseases such as RA. Thus, it is important to develop inhibitors of the ubiquitin-proteasome enzymatic cascade upstream from the proteasome to impact fewer cell processes and reduce toxicity. E3 ligases are attractive such targets given their large number and substrate specificity. We recently cloned the E3 ubiquitin ligase synoviolin, which localizes to the ER lumen and has enzymatic activity. We have also demonstrated that this protein plays crucial roles in the pathological processes of RA (6), and could therefore be a candidate novel therapeutic target of RA (32).

In this study, we identified two potent small compounds as inhibitors of synoviolin enzymatic activity using high-throughput screening (Fig. 1). Moreover, *in vivo* studies showed no serious toxicity associated with these compounds in terms of survival and weight loss during treatment (Fig. 4A). Biochemical characterization of the two compounds, LS-101 and LS-102, demonstrated that they both inhibit the autoubiquitination activity of synoviolin *in vitro* (Fig. 2), with LS-101 showing stronger efficacy ($IC_{50}$=20 $\mu$M) than LS-102 ($IC_{50}$=35 $\mu$M), but less selectivity (Fig. 2). It was unclear from this study why LS-101 showed a weak inhibitory effect on BRCA1/BARD1 and Efp activity, and further study is needed to understand the molecular basis for this observation. LS-101 and LS102 inhibited the proliferation of RSCs and to a much lesser extent, HeLa cells (Fig. 3). The difference in cell sensitivities to these compounds could be, at least in part, due to the expression level of synoviolin, namely, high levels of synoviolin in RSCs would contribute to the cell overgrowth and therefore, inhibition of synoviolin in these cells would in turn suppress proliferation. These cells may also have different requirements for synoviolin, such that repressing synoviolin activity in RSCs would lead to growth suppression. Prophylactic administration of either LS-101 or LS-102 also significantly reduced the severity of murine CIA (Fig. 4C). Since LS-101, a nonselective inhibitor, reduced clinical severity scores in CIA similarly to LS-102, blocking synoviolin enzymatic activity seems crucial in the pathological process of CIA. These findings suggest that the suppression level of synovial cell growth and incidence of arthritis reflect the efficacy of these compounds rather than their selectivity, and that in RA, synoviolin might have an indispensable role among E3 ligases.

RA comprises multiple processes such as chronic inflammation, overgrowth of synovial cells, joint destruction, and fibrosis. During the course of inflammation, synovial cells, macrophages, T cells, and B cells all contribute to the production of cytokines such as interleukin (IL)-1, IL-6, IL-10, TNF, and transforming growth factor β (TGF-β) (33,34). These cytokines, in turn, stimulate the overgrowth of synovial cells to form a mass of synovial tissue, called pannus, which invades and destroys the bone and cartilage through osteoclast activation and protease production (33-37). This chronic inflammation state ultimately leads to fibrosis. Our study proved that synoviolin is, at least in part, involved in the overgrowth of synovial cells (6) and fibrosis (38) among these processes. The IL-17 induction of synoviolin may also contribute to RA chronicity (39), and synoviolin has been shown to target misfolded MHC class I heavy chains (40). In this study, antibody titers were elevated in synoviolin inhibitor-treated mice to levels comparable to those in vehicle controls (Fig. 4B). Thus, as with the study of synoviolin$^{+/-}$ knockout mice in CIA, it is difficult to clarify the function of synoviolin with respect to the chronicity of inflammation, because suppressing synoviolin blocks synovial cell outgrowth directly due to sequential events following immunization of type II collagen (6). Our results confirm that further studies of the association between chronic inflammation and synoviolin are clearly warranted.

Eight biological agents are currently approved for clinical use in treatment of RA, and these drugs have dramatically changed the outcome of RA during the past decade (3,4). However, some patients still fail to respond to the biological treatment or develop adverse effects such as an increased risk of infection. Moreover, these agents are associated with high costs and discomfort arising from the subcutaneous or intravenous administration. Thus, there is a clear need for the development of cheaper, orally administered therapies with fewer side effects. In this regard, spleen tyrosine kinase (Syk) inhibitor, an orally administered drug, has been developed for the treatment of RA (41,42). Dual blockade of TNF and IL-17 was also reported recently as a strategy for halting RA disease from progression to the extent seen when only one cytokine is blocked (43). The involvement of synoviolin in both the TNF and IL-17 pathways further implicates inhibitors of this enzyme as potential candidate drugs for treatment of RA.

In conclusion, we identified two strong synoviolin inhibitors, and confirmed that synoviolin is an ideal molecular target for RA for disease modification and treatment. We are now proceeding with the optimization of LS-101 and LS-102, and hope our research will lead to the development of a new therapy for RA.

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