The Stat3 inhibitor F0648-0027 is a potential therapeutic against rheumatoid arthritis

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Abstract

Background

Rheumatoid arthritis (RA) is a disease characterized by chronic joint inflammation, pain and joint destruction, leading to alteration in activities of daily living, yet pathological mechanisms underlying the condition are not fully clarified. To date, various therapeutic agents have been developed as RA therapy including DMARDs and/or biological agents that target inflammatory cytokines or inhibit JAK. Here we asked whether inhibiting signal transducer and activator of transcription 3 (Stat3) activity would antagonize RA.

Methods

Stat3 forms dimers when activated and undergoes nuclear translocalization; thus we screened approximately 4.9 million small compounds as potential blockers of protein-protein interactions required for Stat3 dimerization using in silico screening. We then tested candidate Stat3-inhibiting activity in vitro by analyzing expression of IL-6, a Stat3 target, in compound-treated fibroblasts. We also analyzed expression of RANKL, a cytokine essential for osteoclastogenesis, in vitro. We then evaluated anti-arthritis effects of candidate compounds in vivo in collagen-induced arthritis model mice. Effects of the candidate compounds on inhibiting Stat3 phosphorylation and nuclear localization following IL-6 stimulation of fibroblasts were analyzed by an immune histocochemical analysis as well as western blotting.

Results

We identified 15 as strong candidates as potential blockers of protein-protein interactions required for Stat3 dimerization using in silico screening from approximately 4.9 million small compounds. Four of the 15 significantly inhibited IL-6 as well as RANKL expression induced by IL-6. One compound, F0648-0027, significantly inhibited arthritis development without apparent adverse effects. F0648-0027 also significantly blocked Stat3 phosphorylation and nuclear localization following IL-6 stimulation of fibroblasts.

Conclusions

These data suggest that Stat3 is a target for RA, and that F0648-0027 could serve as a therapeutic reagent against the condition.

Introduction

Chronic joint inflammation and destruction are characteristic features of rheumatoid arthritis (RA) that limit patients’ activities of daily living (ADL) due to severe joint pain and deformity[1, 2]. Both genetic
factors and infectious agents have been proposed to underlie RA development[3, 4], but molecular mechanisms remain unknown. In particular, mechanisms allowing sustained joint inflammation and destruction are unidentified.

We previously used mouse arthritis models to demonstrate that signal transducer and activator of transcription 3 (Stat3) is required for chronic joint inflammation and destruction[5, 6]. Stat3 is activated by either IL-6, TNFα or IL-1, all of which are major inflammatory cytokines in RA patients, and activated Stat3 stimulates IL-6 expression in a positive feedback manner[7]. Activated Stat3 further promotes expression of receptor activator of nuclear factor kappa B ligand (RANKL), which is required for osteoclastogenesis[5], and osteoclasts are required for joint destruction in RA[8]. Thus, Stat3 is a target for both inflammation and joint destruction. Currently, patients with RA are treated with disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate (MTX), and/or with biological reagents such as neutralizing antibodies against inflammatory cytokines or JAK inhibitors; however, currently no Stat3 inhibitors are available for use as RA treatment.

The Stat family includes six proteins (Stat1-6) that translocate into the nucleus and serve as transcription factors to promote target gene expression, a process requiring Stat homo- or hetero dimerization[9]. Stat protein-protein interaction occurs at particular sites identified by structural analysis, and those sites could be targeted to block activation and nuclear translocation[10, 11]. Most Stats are activated and undergo dimerization by cytokine stimulation[12]. Thus, as RA treatments, the most accessible targets have been considered to be soluble cytokines or cell surface molecules, or JAK kinases, which phosphorylate Stat proteins. Stat proteins themselves have been considered less targetable since they are transcription factors, although strategies have been devised to successfully target other transcription factors like hypoxia inducible factor 1 alpha[13].

*In silico screening* is currently a powerful tool used to develop pharmaceutical therapies[14, 15]. *In silico* computational analysis or artificial intelligence approaches now enable us to screen very large numbers of compounds in a short period[16, 17].

Here, we identify and characterize potential Stat3 inhibitors based on an *in silico* screen of 4.9 million compounds. Among candidates identified, we found that treatment of collagen-induced arthritis (CIA) model mice with one, F0648-0027, significantly inhibited arthritis development. We also show that *in vitro* F0648-0027 inhibits Stat3 phosphorylation and nuclear translocation. Taken together, our data suggest a novel approach in treatment of RA.

**Materials And Methods**

**Mice.** All 5-week DBA/1 J mice were purchased from Sankyo Labo Service (Tokyo, Japan) and placed under specific pathogen-free conditions in animal facilities certified by the Keio University Institutional Animal Care and Use Committee. Animal protocols were approved by that committee. Animal experiments were carried out in accordance with the Guidelines of the Keio University Institutional Animal
Care and Use Committee and Institutional Guidelines on Animal Experimentation at Keio University. This study is reported in accordance with ARRIVE guidelines.

**CIA model.** Experimental CIA models were generated in 5-week-old male mice by injecting 200 µl of emulsion containing 100 µg of type II collagen (CII) (Collagen Research Center, Tokyo, Japan) intradermally at the proximal end of the tail. To create CIA models, all mice received a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection as anesthesia, and euthanasia of CIA models was performed unconsciously. Emulsion composition was 1mg/ml bovine CII dissolved in PBS plus an equal volume of complete Freund’s adjuvant (CFA, Difco, Detroit, MI, USA). Following the first injection, a second immunization of CII/CFA was administered. Clinical symptoms of arthritis were evaluated visually in each limb and graded on a scale of 0–4: 0, no erythema or swelling; 0.5, swelling of one or more digits; 1, erythema and mild swelling of the ankle joint; 2, mild erythema and swelling involving the entire paw; 3, erythema and moderate swelling involving the entire paw and 4, erythema and severe swelling involving the entire paw. We defined the clinical score for each mouse as an overall “arthritis score” calculated as the sum of scores for all four limbs (maximum = 16).

**Chemicals, drugs, reagents.**

Drugs selected by *in silico* screening were purchased from following companies.

UZI/2602234 was purchased from Zelensky, Russia; P2000N-15185 and P2000N-29959 from Pharmeks, Russia; Z1082961568 from Enamine, Kyiv, Ukraine, AP-113/15379001 from SPECS, Germany; P2001S-254605, STK576506, STK587698, and STK591642 from VITAS-M LABORATORY, Russia; Z24581406, Z90677708 from Enamine, Kyiv, Ukraine; and F0648-0027, F0648-0476, F0648-0479 and F0648-0589 from Life Chemicals Inc, Canada.

*In silico* screening

We retrieved a crystal structure of Stat3 (PDB ID: 3CWG)[18] from the Protein Data Bank ([www.rcsb.org][19]). The protein structure was prepared for *in silico* analysis using Protein Preparation Wizard in Maestro of Schrödinger Suite 2015-2 (Schrödinger, LLC, New York, USA). *In silico* fragment mapping was then performed according to the previous studies[20, 21]. Briefly, residues in the dimerization interface of Stat3 were defined as the mapping surface. The surface was then searched for similar topographies to the protein substructures (subsites) stored in our *in house* fragment-subsite structure database[20]. When a local topography similar to the subsite was found on the target protein, the corresponding fragment was mapped on its matching subsite. The 3D pharmacophore model was then constructed according to the physicochemical properties of the mapped fragments as well as characteristics of fragment-protein interactions, using the UNITY program of SYBYL-X 1.3 (Certara, Princeton, NJ, USA). This model was used to screen approximately 4.9 million compounds from a commercially available compound database (Namiki201404HTS; constructed in April 2014 by Namiki Shoji Co. Ltd., Tokyo, Japan) using the UNITY Flex Search program of SYBYL-X 1.3. For ligand docking, a receptor grid was generated using the grid generation feature of Glide (Schrödinger Suite 2015-2). Multiple conformations of compounds were then
generated using LigPrep (Schrödinger Suite 2015-2), followed by ConfGen (Schrödinger Suite 2015-2). All docking calculations were performed with Glide and run in standard precision (SP) mode. The ligand van der Waals radius for non-polar atoms was scaled by a factor of 0.8. The conformers generated above were docked into the receptor grid, and the top pose for each compound, determined by the Glide docking score (cutoff: -5.0), was collected.

Cell culture and in vitro screening of drugs by a Stat3 inhibition assay.

The NIH3T3 cell line was maintained in DMEM (Sigma–Aldrich Co.) containing 10% fetal bovine serum (FBS) with penicillin G and streptomycin. Adherent cells were collected and cultured in 24-well plates (5 × 10⁴ cells per well) and subjected to serum starvation in the presence or absence of IL-6 (100 ng/ml, R & D Systems) and sIL-6R (100 ng/ml, R & D Systems). Eight hours later, mRNA was collected to analyze IL-6 and RANKL expression, as described below in realtime PCR analysis.

RNA isolation and real-time PCR.

Total RNAs were isolated from cultured NIH3T3 cells using TRizol reagent (Invitrogen Corp) using general procedures. cDNA synthesis was then performed with oligo(dT) primers and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix ExTaq® reagent and a DICE Thermal cycler (Takara Bio Inc.). Stored data was analyzed using a calibrator. β-actin (Actb) expression served as internal control. Primer sequences for realtime PCR were as follows.

β-actin-forward: 5′-TCCTCCTGGAGAAGAGCTATG-3′

β-actin-reverse: 5′-TGCCACAGGATTCCATACCCAG-3′

IL6-forward: 5′-GTCCCTAGCCACTCCTTCTG-3′

IL-6-reverse: 5′-CAAAGCCAGAGTCCTTCAGAG-3′

RANKL-forward: 5′-GCATCGCTCTGTTCCTGTACTTT-3′

RANKL-reverse: 5′-CGTTTTCATGGAGTCTCAGGATT-3′

Histopathology analysis in vivo.

Ankle tissues from CIA mice were fixed in 10% neutral-buffered formalin and embedded in paraffin, and tissue blocks were cut into 4-µm sections. For other analyses, lower ankles were decalcified in 10% EDTA, pH 7.4, before embedding. Safranin-O staining was performed using standard procedures, and then the Mankin score, a histopathological arthritis score, was evaluated using a BioRevo microscope and corresponding software (Keyence, Tokyo, Japan).

Fluorescent immunohistochemistry analysis in vitro.
NIH3T3 cells were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) and then incubated with PBS buffer containing 0.5% Triton. Cells were blocked using 5% BSA for 20 minutes at room temperature and then stained with rabbit anti-Stat3 (1:100 dilution; #12640, Cell signaling Technology, Danvers, MA, USA) or mouse anti-pSTAT3 (1:100 dilution; #4113, Cell signaling Technology, Danvers, MA, USA), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:100 dilution; Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 488 goat anti-mouse IgG (1:100 dilution; Invitrogen, Carlsbad, CA, USA), respectively. After staining in secondary antibodies for 1 h at room temperature, DAPI (1:1000; Wako Pure Chemicals Industries, Osaka, Japan) was used as a nuclear stain.

Immunoblotting analysis.

Whole-cell lysates were prepared from cell cultures using RIPA buffer (1% Tween 20, 0.1% SDS, 150 mM NaCl, 10mM Tris-HCl (pH 7.4), 0.25 mM phenylmethylsulfonylfluoride, 10µg/mL aprotinin, 10µg/mL leupeptin, 1 mM Na3VO4, 5 mM NaF (Sigma-Aldrich Co.)). Equivalent amounts of proteins were separated by SDS–PAGE and transferred to a PVDF membrane (EMD Millipore Corp, Burlington, MA, USA.). Proteins were detected by using anti-pSTAT3 (#9134), anti-STAT3 (#4904) (Cell Signaling Technology, Danvers, MA, USA) or anti-actin antibody (Sigma-Aldrich Co., St Louis, MO, USA).

Statistical analysis.

Results are expressed as means ± s.d. Statistical significance of differences between compared groups was evaluated using Student's t-test (*p < 0.05; **p < 0.01; ***p < 0.001; NS, not significant, throughout the paper).

Results

In silico screen for Stat3 inhibitors

Stat3 is known to homo-dimerize or hetero-dimerize with other Stat proteins, an interaction required for activation and nuclear translocation[22]. Thus, we undertook in silico screen for inhibitors that might block Stat3 protein-protein interaction (Fig. 1) and identified 4,032 candidates by a 3D pharmacophore model-based 1st screen of approximately 4.9 million compounds (Fig. 1). Of those, 39 were identified by the docking-based 2nd screen. Finally, 15 of those were selected for analysis based on their availability from vendors (Fig. 1 and Table 1).

In vitro screen for Stat3 inhibitors

We then tested the 14 candidate Stat3 inhibitors identified above in vitro (Fig. 2). Stat3 is activated by IL-6/soluble IL-6 receptor (sIL-6R) stimulation and promotes IL-6 expression in a positive feedback manner[7, 23]. Thus, we tested Stat3 inhibiting activity of the 14 candidates by evaluating whether the presence of the inhibitor blocked upregulation IL-6 mRNA transcript levels in response to IL-6/sIL-6R treatment (Fig. 2). To do so, we cultured NIH3T3 cells fibroblasts in the presence or absence of IL-6/sIL-
6R with or without each candidate Stat3 inhibitor in vitro (Fig. 2) and then 24 hours later analyzed IL-6 expression by realtime PCR (Fig. 2). Of the 14 candidates, 4 (STK591642, F0648-0027, F0648-0476 and F0648-0589) blocked IL-6 upregulation stimulated by IL-6/sIL-6R in NIH3T3 cells (Fig. 2). Moreover, among those 4, F0648-0027, F0648-0476 and F0648-0589 blocked IL-6 upregulation dose-dependently (Fig. 3). We also observed that expression of RANKL, a Stat3 target[24, 25] required for osteoclastogenesis[26] and also induced by IL-6/sIL-6R, was also significantly inhibited when cells were treated with either F0648-0027, F0648-0476 or F0648-0589 (Fig. 4).

**In vivo screen for Stat3 inhibitors**

We then tested the 3 remaining candidate Stat3 inhibitors (F0648-0027, F0648-0476 or F0648-0589) for potential therapeutic activities against RA in vivo using a CIA mouse model, which is a well-established RA model (Fig. 5). To do so, we established CIA models by injecting a collagen cocktail into five-week-old wild-type DBA/1 mice followed by injection of the candidate compound subcutaneously every day starting three weeks after the initial collagen injection (when mice were eight weeks old) for two more weeks (Fig. 5A). We then calculated an arthritis score (see Materials and Methods) in each mouse every other day, starting two weeks after mice first received candidate inhibitor (Fig. 5B). Arthritis development, based on analysis of arthritis scores, was effectively blocked following treatment with each candidate Stat3 inhibitor, with F0648-0027 showing the most potent effect (Fig. 5B).

Thus, histological analysis was performed in mice administered F0648-0027 (Fig. 6). Arthritis development determined by Mankin score was also effectively blocked by treatment with F0648-0027 (Fig. 6A and B)

**F0648-0027 blocks Stat3 phosphorylation and nuclear translocation**

Finally, we asked whether F0648-0027 inhibited phosphorylation and/or nuclear translocation of Stat3 induced by IL-6/sIL-6R. To do so, we treated NIH3T3 cells with or without IL-6/sIL-6R in the presence or absence of the concentrations of F0648-0027 indicated in Fig. 7 and then analyzed Stat3 phosphorylation and nuclear translocation based on western blotting and immune-histochemistry. Stat3 nuclear translocation and phosphorylation in response to IL-6/sIL-6R treatment were both significantly blocked by F0648-0027 dose-dependently (Fig. 7A, B and C). Stat3 phosphorylation following IL-6/sIL-6R stimulation was significantly inhibited by F0648-0027 treatment dose-dependently based on western blotting analysis (Fig. 7C, D and E).

**Discussion**

RA causes joint pain and deformities and limits patients’ ADL. Thus better control of this condition is critical. The cause of RA has not been identified, although genetic factors or infectious conditions may be associated with disease development[27, 28]. RA patients are now treated symptomatically, but development of molecularly-targeted biological reagents against RA, many targeting the inflammatory cytokine TNFα, has allowed a greater number of patients to reach disease remission status[29, 30].
Nonetheless some RA patients or those experiencing RA recurrence may not respond well to biological reagents. Other treatments have been developed to target cell surface IL-6R and CD80/86[31], while others focus on intracellular Jak1, 2 and 3 kinases[32, 33]. Here we propose a novel approach to target and inhibit their downstream effector Stat3, a transcription factor, as RA therapy. Transcription factors have been considered difficult to target by drugs, but several such as hypoxia inducible factor 1 alpha (HIF1α) have been successfully targeted[6, 13, 34, 35]. Here we identified a Stat3-inhibiting compound that targets Stat3 dimerization using an in silico screen. The compound identified here could serve as the basis for a potential therapeutic reagent for RA.

Our previous studies reported that Mx1 Cre/Stat3\textsuperscript{floxflo}x mice were significantly resistant to arthritis in CIA models[6], suggesting that inhibiting Stat3 could antagonize joint inflammation and destruction by inhibiting IL-6 and RANKL expression in adult mice. Stat3 is expressed ubiquitously during embryogenesis and development, and global Stat3-deficient mice exhibited embryonic lethality, indicating that Stat3 is required for embryogenesis[36, 37]. In contrast, global Stat3 knockout in adult mice using Stat3-flox and Mx1 Cre (Mx1 Cre/Stat3\textsuperscript{floxflo}) did not promote severe adverse effects such as lethality and body weight loss[6]. Hyperactivation of macrophages in response to LPS were demonstrated in Mx1 Cre/Stat3\textsuperscript{floxflo}x as well as LysM Cre/Stat3\textsuperscript{floxflo}x mice[38].

**Conclusion**

Thus, we concluded that targeting Stat3 would be an effective therapy against RA.

**Abbreviations**

RA  
Rheumatoid arthritis  
Stat3  
Signal transducer and activator of transcription 3  
ADL  
Activities of daily living  
RANKL  
Receptor activator of nuclear factor kappa B ligand  
DMARDs  
Disease-modifying anti-rheumatic drug  
CIA  
collagen-induced arthritis  
sIL-6R  
soluble IL-6 receptor

**Declarations**
Ethical Approval and Consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of supporting data: The supporting data of the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Conceptualization: SO, KM, SK, KH, SH, MM, MN, YN, and TM (Miyamoto). Acquisition of the data: YK, SO, YS, and TK. Analysis of the data: YK, TM (Matsumoto), and TM (Miyamoto). Interpretation of the data: YK, TM (Matsumoto), KM, and TM (Miyamoto). Original draft preparation: YK, SO, and TM (Miyamoto). Writing—reviewing and editing: SO, KM, SK, KH, MM, MN, YN, and TM (Miyamoto). Supervision: SO, SK, KH, MM, SH, MN, YN, and TM (Miyamoto). All authors approved the final version of the manuscript.

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**Tables**

Table1. List of potential Stat3 inhibitors selected through *in silico* screening

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**Figures**
Identification of Stat3 inhibitors through \textit{in silico} screening.

We conducted \textit{in silico} fragment mapping applying 3CWG. From among 4,900,000 candidates, we identified 4,032 compounds from the database through construction of a 3D pharmacophore model. Through docking-based 2ndary screening, candidates were then limited to 39. Based on commercial availability, 15 compounds were tested in STAT3 inhibitor assays in the last step of the procedure.
**Figure 2**

**In vitro analysis of 15 potential Stat3 inhibitors.**

We then performed an *in vitro* Stat3 inhibition assay in NIH3T3 cells using the 15 remaining inhibitor candidates shown at the bottom of Figure 1. To do so, semi-confluent cells were cultured with serum free media and stimulated with or without IL-6/sIL6-R (10 ng/ml each) in the presence or absence of 10μM of each candidate drug. After 8 hours, mRNAs were collected, and expression of *IL-6*, a Stat3 target gene, was analyzed by realtime PCR. Data represent mean *IL-6* levels ± SD in each sample relative to control (IL-6/sIL6-R (-), drug (-)).

Dotted line indicates the threshold used to select candidate Stat3 inhibitors for the next round of analysis, selected drugs are indicated by red arrows. Compound names are shown below the bar chart.
Figure 3

Inhibition of *IL-6* expression by four Stat3 inhibitor candidates.

Compounds STK591642, F0648-0027, F0648-0476 and F0648-0589 were assessed in a Stat3 inhibition assay. Semi-confluent serum-starved NIH3T3 cells were treated with or without IL-6/sIL6-R (10 ng/ml each) in the presence or absence of indicated concentrations (10μM, 30μM, or 50μM) of candidate
compounds. After 8 hours of treatment, mRNAs were collected for analysis of IL-6 expression. Data represent mean IL-6 transcript levels ± SD in each sample relative to control (IL-6/sIL6-R (-), drug (-)/vehicle).

Figure 4

Selected Stat3 inhibitors block RANKL upregulation in response to IL-6/sIL6-R treatment.
Compounds tested in Figure 3 (STK591642, F0648-0027, F0648-0476 and F0648-0589) were tested for ability to block IL-6/sIL6-R-stimulated upregulation of RANKL expression. Semi-confluent serum-starved NIH3T3 cells were stimulated with or without IL-6/sIL6-R (10 ng/ml each) in the presence or absence of indicated concentrations (10μM, 30μM, or 50μM) of candidate compounds. After 8 hours of treatment, mRNAs were collected and analyzed for RANKL expression. Data represent mean RANKL transcript levels ± SD in each sample relative to control (IL-6/sIL6-R (-), drug (-)/vehicle).

Figure 5
Figure 5

Potential Stat3 inhibitors block RA progression of a CIA model in vivo.

(A) Schematic showing protocol used analyze effects of potential Stat3 inhibitors F0648-0027, F0648-0476, F0648-0589 or vehicle in CIA model mice. The first injection of CII emulsion was performed in 5-week-old male mice to initiate the model and then three weeks later mice received a second CII injection, at which time subcutaneous injection of candidate Stat3 inhibitors was begun for 14 consecutive days. (B) An arthritis score was calculated every other day, starting when mice were 8 weeks old, for a period of 2 more weeks.

Figure 6

Safranin O staining of ankle joint of vehicle- or F0648-0027-treated CIA mice.

(A) Safranin O staining of ankle joints of CIA mice treated two weeks with vehicle or F0648-0027 was performed to detect articular cartilage when mice were 10-week old. Bar, 100μm. (B) A Mankin score was
evaluated in each group treated for the same period with vehicle or F0648-0027.

Figure 7

**Treatment of fibroblasts with F0648-0027 blocks Stat3 activation by IL-6/sIL-6R *in vitro.*
(A-C) Semi-confluent serum-starved NIH3T3 cells were stimulated with or without IL-6/sIL6-R (10 ng/ml each) in the presence or absence of indicated concentration of F0648-0027. (A) Five minutes later, cells were fixed and stained with rabbit anti-mouse Stat3 or rabbit anti-mouse phosphorylated Stat3 (pStat3) followed by Alexa488 conjugated goat anti-rabbit antibody. Nuclei were stained with DAPI, and cells were observed under a fluorescence microscope. (B) Cells showing nuclear or cytoplasmic Stat3 were counted and a ratio of nuclear/cytoplasmically stained cells was calculated as an indicator of Stat3 nuclear translocation. Data represents mean nuclear/cytoplasmic Stat3 ± SD (*p < 0.05). (C) Data shows mean fluorescent intensity of pStat3 ± SD (ns, not significant, *p < 0.05). (D) Cells were treated as above and after 5 minutes, cell lysates were prepared for western blotting to detect Stat3 and pStat3. Actin served as an internal control. (E) Band intensities levels were quantified using image J and shown as mean band intensity ± SD (*p < 0.05).