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Successful Treatment of Animal Models of Rheumatoid Arthritis with Small-Molecule Cyclin-Dependent Kinase Inhibitors

Chiyoko Sekine,* Takahiko Sugihara,*† Sachiko Miyake,‡ Hiroshi Hirai,§ Mitsuaki Yoshida,§ Nobuyuki Miyasaka,† and Hitoshi Kohsaka2*†

Intraarticular gene transfer of cyclin-dependent kinase (CDK) inhibitors to suppress synovial cell cycling has shown efficacy in treating animal models of rheumatoid arthritis. Endogenous CDK inhibitors also modulate immune function via a CDK-independent pathway. Accordingly, systemic administration of small molecules that inhibit CDK may or may not ameliorate arthritis. To address this issue, alvocidib (flavopiridol), known to be tolerated clinically for treating cancers, and a newly synthesized CDK4/6-selective inhibitor were tested for antiarthritic effects. In vitro, they inhibited proliferation of human and mouse synovial fibroblasts without inducing apoptosis. In vivo, treatment of collagen-induced arthritis mice with alvocidib suppressed synovial hyperplasia and joint destruction, whereas serum concentrations of anti-collagen type II (CII) Abs and proliferative responses to CII were maintained. Treatment was effective even when therapeutically administered. Treated mice developed arthritis after termination of treatment. Thus, immune responses to CII were unimpaired. The same treatment ameliorated arthritis induced by K/BxN serum transfer to lymphocyte-deficient mice. Similarly, the CDK4/6-selective inhibitor suppressed collagen-induced arthritis. Both small-molecule CDK inhibitors were effective in treating animal models of rheumatoid arthritis not by suppressing lymphocyte function. Thus, the two small-molecule CDK inhibitors ameliorated arthritis models in a distinct way, compared with other immunosuppressive drugs.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia with massive infiltration of inflammatory cells in the affected joints. This hyperplasia leads to degeneration of cartilage, erosion of bone, and ultimately functional loss of joints. Although the etiology of RA remains elusive, T cells recognizing unknown autoantigens have been proposed to initiate inflammation in synovial tissues. This mechanism is followed by local recruitment of leukocytes, which are further activated in the inflamed sites (1). Immune complexes, complement activation, and activation of lymphocytes, macrophages, neutrophils, mast cells, and synovial fibroblasts are part of the aberrant humoral and cellular network (2).

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2 Address correspondence and reprint requests to Dr. Hitoshi Kohsaka, Department of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, and 21st Century Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo, Japan; Departments of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; and *Banyu Tsukuba Research Institute, Banyu Pharmaceutical, Tsukuba, Japan

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and tissue-degrading enzymes involved in rheumatoid pathology. Intra-articular gene transfer of p16\textsuperscript{INK4a} or p21\textsuperscript{Cip1} ameliorated disease in animal models of RA, including collagen-induced arthritis (CIA) in mice and adjuvant arthritis in rats (7–9). These studies have indicated that induction of p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1} expression in synovial tissue should hold promise as a new therapeutic strategy for RA treatment. Notably, because cell cycle regulation therapy suppresses a phase of rheumatoid pathology distinct from that suppressed by anti-inflammatory drugs, combination with another anti-inflammatory drug might act synergistically.

To apply gene transfer to treatment of patients with RA, vectors and protocols need to be optimized to avoid unexpected tragic complications (10). An alternative to gene therapy as a way to inhibit CDK activity is the use of small-molecule (sm) compounds that inhibit CDK. Because uncontrolled CDK activity drives neoplastic cell cycling in some tumor cells, CDK activity represents an attractive target in development of anti-cancer therapeutics (11). In response to these smCDK inhibitors, some tumor cells stop proliferation and then also undergo apoptotic cell death (12–14). Thus, many smCDK inhibitors have been developed as candidate oncostatic drugs with a prototype being alvocidib (formerly called flavopiridol) (11). This synthetic flavone, the first of multiple CDKs such as CDK1, 2, 4, 6, and 7 (15). Structure–activity studies have shown that alvocidib interferes with binding of ATP to the adenine-binding pocket of CDK2 (16). However, although alvocidib generally proved safe in clinical trials, its efficacy was limited except for a subtype of malignant cells (11, 17). Other smCDK inhibitors, such as staurosporine, R-roscovitine, and BMS-387032, have also been studied in clinical trials, showing good tolerability (11).

The primary known role for CDKI is suppression of CDKs required for cell cycle progression. However, our group as well as other investigators have shown that p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1} also have immunomodulatory effects (7, 18, 19). Notably, overexpression of p21\textsuperscript{Cip1} in RA FLs reduced production of inflammatory cytokines and tissue-degrading enzymes involved in rheumatoid pathology. Thus, our department of CDK1 to undergo clinical trials (11), inhibits the kinase activity of multiple CDKs such as CDK1, 2, 4, 6, and 7 (15). Structure–activity studies have shown that alvocidib interferes with binding of ATP to the adenine-binding pocket of CDK2 (16). However, although alvocidib generally proved safe in clinical trials, its efficacy was limited except for a subtype of malignant cells (11, 17). Other smCDK inhibitors, such as staurosporine, R-roscovitine, and BMS-387032, have also been studied in clinical trials, showing good tolerability (11).

Materials and Methods

Reagents

Alvocidib was provided by Aventis Pharmaceuticals. A stock solution of alvocidib prepared in DMSO was diluted to working concentrations before each experiment. In addition, a CDK4/6-selective inhibitor compound A (N-[5-{2-[(cyclohexyloxy)-6-methylpyrimidin-4-yl]-1,3-thiazol-2-yl}-5-{(4-methylpirazin-1-yl)methyl]pyrazin-2-amine}, which was characterized as compound 4 in the previous report (20), was synthesized at Merck-Banyu. It was dissolved in DMSO for in vitro analyses and in 5% glucose containing 10 mM citrate buffer (pH 4) for treatment of mice. Human and mouse IL-1\textbeta and TNF-\alpha were purchased from WAKO.

Cells

FLSs were prepared from synovial tissues of patients with RA who underwent total joint replacement surgery or synovectomy at Tokyo Medical and Dental University Hospital (Tokyo, Japan) or National Shimoshizu Hospital (Chiba, Japan). RA was diagnosed according to the criteria of the American College of Rheumatology (21). Written consent forms concerning experimental use of resected tissues were completed by patients before surgery. All procedures in the present studies were approved by the ethics committees of RIKEN and Tokyo Medical and Dental University. Mouse fibroblast-like synoviocytes (MFLSs) were prepared from synovial tissues from knee joints of CIA mice as previously described (22). Although FLSs were used from 7 to 11 passages, MFLSs were used from 6 to 11 passages. Both were cultured in DMEM (Sigma-Aldrich) supplemented with 1-glutamine, penicillin, streptomycin, and 10% FBS (Sigma-Aldrich). Purity of the fibroblasts was assessed with flow cytometry (23). CD14 or HLA class II were not expressed by FLSs, suggesting that macrophages and dendritic cells were not present in FLSs.

Proliferation assays

From 2000 to 5000 cells/well were grown overnight in 96-well plates and stimulated with 10 ng/ml IL-1\textbeta and 10 ng/ml TNF-\alpha. These cytokine concentrations were determined to be optimal in preliminary experiments. Alvocidib (1–300 \textmuM), compound A (0.01–10 \textmuM), or DMSO alone was placed in wells in the presence of 10% FBS. After 24–48 h, BrdU was added and culture was continued for 20 h. Then incorporation of BrdU was quantified by ELISA with a BrdU cell proliferation ELISA kit (Exalpha Biologicals).

Cell cycle analysis

Cells were stimulated for 24 h in the presence or absence of smCDK inhibitors, washed with PBS, and resuspended in 0.15% Triton X-100/PBS before staining with propidium iodide (50 \textmug/ml) and examination for DNA content with a FACSCalibur flow cytometer (BD Biosciences).

Animal models of arthritis

CIA was induced in 7–8-week-old male DBA/1 mice purchased from Japan Charles River Breeding Laboratories. Each mouse was immunized with 200 \mug of bovine type II collagen (CII; Collagen Research Center) emulsified with CFA (Difco) by injection at the tail base. Immunization was repeated 21 days after primary immunization.

Serum-transfer arthritis was induced by transfer of serum from arthritic K/BxN mice, which spontaneously develop arthritis resembling RA. These mice were provided by Drs. C. Benoist and D. Mathis (Joslin Diabetes Center, Boston, MA) (24). Preliminary titration experiments showed that injection of 300 \muM of serum into the peritoneal cavity transferred arthritis consistently in RAG2-null mutant (RAG2\textsuperscript{-/-}) and C57BL/6 wild-type mice. All procedures met institutional regulations for animal experiments.

Assessment of arthritis

Arthritis in each limb of arthritic mice was assessed clinically by visual scoring from 0 to 4: 0, no swelling; 1, detectable swelling in one joint; 2, non-severe swelling in two or more joints; 3, severe swelling in two or more joints; and 4, severe swelling in two or more joints including digital swelling. Maximal score for an individual animal was 16. Joint swelling was quantified by measuring hindpaw thickness and the ankle width within a micrometer (Ozaki Manufacturing). Arthritis scores were analyzed statistically with unpaired Student’s t test.

In histological examination, hindpaws were obtained and fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections (4 \muM) were stained with H&E for histologic examination. Infiltration of inflammatory cells, transformation of synovial lining, cartilage destruction, and pannus formation were independently scored in a blind manner from 0 to 3 as previously described (25). Maximum histological score was 12. Histological scores were analyzed statistically with unpaired Student’s t test. Radiographs of formalin-fixed hindpaws were obtained with a cabinet soft x-ray apparatus (CMB-2; Softex).

Measurement of serum levels of anti-CII Abs

Serum samples were collected at indicated time points and examined for anti-CII Ab concentrations by ELISA. Briefly, wells of 96-well plates were
coated overnight at 4°C with bovine CII in PBS, washed with PBS-0.05% Tween 20, and blocked with 2% BSA in PBS. Samples were incubated for 2 h at room temperature. After washing, HRP-conjugated rabbit anti-mouse IgG1, IgG2a, or IgG2b Ab (Zymed Laboratories) was added. Reaction product formed with 3,3′,5,5′-tetramethylbenzidine was measured as OD at 450 nm. A standard mixture serum from arthritic mice was placed on each plate in serial dilution. Arbitrary units for anti-CII IgG1, IgG2a, and IgG2b Abs were determined with this standard curve.

**Western blot analysis**

Whole-cell lysates (15 μg of protein) were separated by SDS-PAGE on 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Anti-phospho RB (Ser807/ Ser811) Ab (catalog no. 9308; Cell Signaling Technology) at 1/1000 dilution and anti-RB mAbl (clone G3-245; BD Pharmingen) at 1 μg/ml were used as primary Abs. Bound Abs were visualized with peroxidase-conjugated secondary Ab (Amer sham Biosciences) at 1/5000 dilution and ECL system (ECL; Amer sham Biosciences).

**Immunohistochemistry**

Paraffin-fixed tissue sections were deparaffinized and incubated with anti-phospho RB (Ser807/ Ser811) Ab. They were then incubated with biotinylated anti-rabbit IgG Ab and with ABC reagent (Vectorstain Elite ABC kit; Vector Laboratories). Color was developed with diaminobenzidine (Kirkegaard & Perry Laboratories), whereas the sections were counterstained with hematoxylin. Diaminobenzidine-positive cells and stained nuclei were counted by observers in a blind manner at three independent sites per slide to calculate percentage of nuclei with phosphorylated RB gene product. The data were analyzed statistically with unpaired Student’s t test.

**Results**

**Alvocidib suppresses proliferation of synovial fibroblasts**

FLSs from the inflamed joints of rheumatoid patients were isolated and cultured in the presence of alvocidib, a pan-CDKI. MFLSs from arthritic joints of CIA mice were isolated and treated in the same way. When alvocidib was present in culture medium, growth of FLS and MFLS was inhibited in a concentration-dependent manner (Fig. 1A). No cell death was visually apparent at concentrations below 300 nM in FLSs and 150 nM in MFLSs. We next analyzed the cell cycle in these fibroblasts after treatment with alvocidib. In either human or mouse cells, alvocidib increased cell populations at the G0/G1 phase of cell cycle without increasing the DNA content and percentage of population at each phase of the cell cycle are presented. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested and one line was tested twice in two independent experiments. The growth inhibitory effect as alvocidib concentrations increase is shown as declining BrdU incorporation relative to incorporation into cells incubated without alvocidib. DMSO, which was used to solubilize alvocidib, had no effects on incorporation at concentrations used in these experiments. Mean percentage is derived from three wells with error bars representing SD. Data are representative of three independent experiments. B, Cellular DNA content was assessed cytometrically as a means of cell cycle analysis. FLSs and MFLSs pre-stimulated as in A were cultured for 18 h with either 100 nM alvocidib or DMSO as a control before flow cytometry. Histograms of DNA content and percentage of population at each phase of the cell cycle are presented. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for A and B. One line was tested twice in two independent experiments. The two lines responded quite similarly; calculated IC50 of alvocidib were 130 and 90 nM in one FLS sample and 75 nM in the other sample. Cells in the G0/G1 phase of cell cycle increased to 81 and 80% in one FLS sample and 75 nM in the other sample.
These results agreed well with specimen radiographic findings in the feet. DMSO-treated control mice displayed severe bone destruction, most characteristically as cystic changes and ankylosis of the articular bones. These changes were inhibited dose-dependently by alvocidib (Fig. 2, H–J).

**Immune responses to CII are not impaired by alvocidib treatment**

CIA is triggered by host immune responses to CII. Ab response is of central importance because B cell-deficient mice do not develop the disease, whereas transfer of mAbs against CII can induce full-blown arthritis (26). Immune responses require activation and proliferation of lymphocytes. Although peripheral lymphocyte counts were not affected by the dose of alvocidib used in the present study (data not shown), lymphocyte activation might be blocked by alvocidib. To assess immune suppression by alvocidib treatment, we determined serum concentrations of anti-CII Abs in the treated mice. When anti-CII-specific Abs of IgG1, IgG2a, and IgG2b subclasses were quantified separately with a specific ELISA, alvocidib-treated mice had serum IgG concentrations comparable to
those in control mice (Fig. 3). No difference was seen in relation to dose or time during the experiment. We then studied effect of terminating alvocidib treatment, following the clinical disease. In a group of CIA mice given 2.5 mg/kg of alvocidib twice weekly, treatment was terminated after 10 days. Arthritis became evident ~8 days later, and then progressed rapidly until the disease score approached that in untreated mice (Fig. 2C). The results showed that the underlying immune reaction against CII was not impaired during alvocidib treatment.

**Alvocidib shows efficacy in treating a lymphocyte-independent arthritis**

To substantiate that alvocidib can inhibit arthritis without suppressing lymphocyte function, we investigated its effect on a K/BxN serum-induced arthritis model induced in lymphocyte-deficient RAG2−/− mice. K/BxN mice spontaneously develop arthritis similar to RA in several aspects (24). Transfer of serum from these arthritic mice induces arthritis depending upon the genetic background of recipient mice (27). Pathology in the joints is characterized by acute edema in synovial tissues followed by proliferative and erosive arthritis that can be induced in lymphocyte-deficient mice (28). Making use of this model, we induced arthritis in RAG2−/− mice and began i.p. administration of 2.5 mg/kg of alvocidib on the day after serum transfer, continuing for 10 days. Treatment reduced edematous swelling of the joints in the early phase, and then abrogated proliferative arthritis (Fig. 4). Thus, suppression of immune responses is not required for the antiarthritic effect of alvocidib.

**A newly synthesized CDK4/6-selective inhibitor suppresses synovial fibroblasts growth and CIA**

Alvocidib was tolerated well in clinical trials except for a few occurrences of diarrhea when administered as a prolonged infusion; its toxicity profile was dose- and schedule-dependent (11). This agent has broad activity, inhibiting all CDKs and some other kinases (29). To study further whether cell cycle inhibition by CDK4/6 inhibition had an important role in ameliorating arthritis, a selective inhibitor, compound A, was synthesized (20). It is an aminothiazole CDKI that is structurally unrelated to alvocidib (Fig. 5A). It selectively inhibited CDK4 and CDK6, both of which have been shown to have indistinguishable activities in vitro (20). The IC50 for CDK4 and CDK6 was 9.2 and 7.8 nM, respectively, which were at least 100-fold less than concentrations for other CDKs.

As alvocidib did, compound A inhibited growth of FLSs and MFLSs in a concentration-dependent manner (Fig. 5B) and induced cell cycle arrest at the G1 phase (Fig. 5C). It suppressed RB expression of immune responses is not required for the antiarthritic effect of alvocidib.

![FIGURE 5. Compound A suppresses proliferation of synovial fibroblasts. A. Chemical structure of compound A and alvocidib. B. Proliferation was assessed as in Fig. 1. FLSs or MFLSs were stimulated with TNF-α and IL-1β for 24 h, and then cultured for 24 h with indicated concentrations of compound A. C. RNA content of FLSs and MFLSs were analyzed as in Fig. 1. Instead of alvocidib, compound A (2.5 μM) was used. Histograms of DNA content and the percentage of population at each phase of the cell cycle are indicated. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for B and C as was in Fig. 1. Again, they responded similarly; calculated IC50 of compound A were 1.0 and 0.6 μM in one FLSs sample and 1.8 μM in the other sample. Cells in the G0/G1 phase increased to 88 and 87% in one FLSs sample and 85% in the other sample. D. RB gene product phosphorylation of FLSs and MFLSs treated as in C were analyzed by Western blot using specific Ab to phospho-RB (pRB) or RB. The results are representatives of two samples.](http://www.jimmunol.org/content/187/4/1958/F5)
phosphorylation at Ser\(^{807}/811\), which are among residues specifically targeted by CDK4/6 in FLS and MFLS (Fig. 5D). Titration experiments showed that 0.25 \(\mu\)M compound A, which was below IC\(_{50}\) for other CDKs, started inhibiting the RB phosphorylation and also increasing cells at the G\(_0/G_1\) phase; 83.0% in FLS and 72.0% in MFLS.

Compound A (200 mg/kg) was given orally twice daily for 7 days, beginning 24 days after the initial CII immunization. This treatment reduced arthritis score, ankle, and paw swelling of the CIA mice (Fig. 6, A–C). The dose could be reduced to 30 mg/kg once daily i.p. injection without loss of therapeutic effects (Fig. 6D). This treatment was started after the onset of clinical arthritis. Five-day treatments repeated after a day hiatus suppressed ongoing arthritis. Histological analysis of the synovial tissues revealed that CDK1/2/5 inhibitor, R-roscovitine, suppressed characteristic features of pathology in the arthritis (Fig. 6, E–G). When nuclei with phosphorylated RB gene product in the synovial tissues were stained, their number was decreased in the compound A-treated mice (Fig. 6, H and I). Their ratio in the total nuclei was lower in the treated mice (Fig. 6J), showing that CDK4/6 was inhibited in vivo.

We found no abnormality by physical or behavioral observation in association with administration of compound A. At completion of the therapeutic experiment, inguinal LNs cells were isolated and stimulated with CII. No difference in proliferative response was observed between the compound A-treated and the nontreated mice (Fig. 6G). The preservation of T cell response was also observed in the alvocidib-treated mice (data not shown). Thus, inhibition of the lymphocytes did not play an important role in the therapeutic effects of the smCDK inhibitors.

Discussion
We have demonstrated that systemic administration of the two CDKIs exerted an antiarthritic effect without critical impairment of lymphocyte responses. Such an inhibitor proved effective in treating a lymphocyte-independent arthritis. Although alvocidib is known as a pan-CDKI, CDK4/6-selective inhibitor showed a comparable effect. This finding suggested that inhibition of CDK4/6 played an important role in the antiarthritic activity. It was reported that a CDK1/2/5 inhibitor, R-roscovitine, suppressed a passively induced arthritis model by promoting apoptosis of inflammatory cells (30). Alvocidib induces apoptotic cell death in various tumor cells (13, 14, 31, 32), likely by inhibition of other kinases than CDK (29). In this regards, TUNEL of the joint tissues treated with the two inhibitors did not show increase of apoptotic synovial cells (data not shown). This suggested that local induction of apoptosis in the joints was not responsible for the effect. Although we could not formally exclude effects of these agents on lymphocytes, the results argue that effects on nonlymphoid cells including synovial...
fibroblasts and innate immune cells should account for the therapeutic activity.

A reported immunomodulatory action of endogenous CDKI does not depend upon CDK inhibition. This immune modulation could be at least partly responsible for therapeutic effects seen in CDKI gene therapy (18, 19). However, the present study demonstrated that inhibition of CDK activity alone is sufficient to ameliorate arthritis.

A major concern with systemic delivery of smCDK inhibitors is possible inhibition of normal cell turnover, which is essential for maintenance of organ homeostasis. Reassuringly, alvocidib, the prototype CDKI, was tolerated relatively well in clinical trials involving cancer (33). Diarrhea, the most frequent side effect in these trials, was not observed in any mice during our experiments. We suspect that proliferating cells involved in the arthritis were more sensitive than tumor cells because 2.5 mg/kg or less of alvocidib sufficed to treat arthritis, whereas at least 5 mg/kg was required to treat tumors (34, 35). Although alvocidib was injected i.v. for tumor treatment, oral administration of compound A was effective in treating arthritis. Although toxic effects might not be tolerated by patients without malignancies, route and dosage could be reconsidered in RA patients being treated in clinical trials. RA might respond to much lower doses than do tumors as is true in treatment with methotrexate.

Compound A is a more selective inhibitor acting primarily upon CDK4 and CDK6. Like alvocidib, it induced cell cycle arrest of synovial fibroblasts at the G1 phase. The cell cycle arrest was accompanied by inhibition of RB phosphorylation. In selectivity testing of compound A against other serine/threonine or tyrosine kinases beyond those of the CDK family, only 6 of 45 kinases were inhibited >75% by compound A at 1 μM. No kinase among these six showed a IC50 comparable to those of CDK4 and CDK6. Partly because of structural irrelevance, alvocidib and compound A do not share the same kinases as off-target kinases. Accordingly, inhibition of cell cycling by CDK4/6 inhibition seemed important in suppressing arthritis. It has been proposed that MAPKs are potential therapeutic targets in RA (36). When their inhibitors showed efficacy, they suppressed Ab responses (37, 38). This was in contrast to the treatment with the CDKIs.

We assume that CDKIs might represent a new class of anti-rheumatic drugs. Based on the results of clinical trials of alvocidib as well as those of the present experiments, patients with RA easily should tolerate an antiarthritic dose of alvocidib because alvocidib seems to exert cytotoxic adverse effects primarily via inhibition of other kinases at antineoplastic doses (29). Alvocidib exhibited higher protein binding in human serum than in FBS, which led to development of an effective administration schedule (17). The optimal dose should be carefully considered in RA patients being treated in clinical trials. RA might respond to much lower doses than do tumors as is true in treatment with methotrexate.

The results of the present studies should encourage further development, clinical testing, and use of the smCDK inhibitors to treat human RA.

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Disclosures
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