Immunopharmacology and clinical safety and efficacy of iguratimod, a novel anti-rheumatic drug

Jiang Gao¹*, Lin-Yu Mei¹, Xiao-Min Li²

¹ Research Centre of New Drug Evaluation, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China
² Institute of Pharmaceutical Research, Sincere Pharmaceuticals Group, Nanjing, 210042, China

Abstract
Iguratimod (T-614) has been shown to have an anti-inflammatory effect and to improve abnormal immunological findings in rheumatoid arthritis. Pharmacological studies of T-614 have been shown to display a steroid-like improvement in several autoimmune animal models, such as collagen-induced arthritis. Therefore, it has attracted attention as a disease-modifying anti-rheumatic drug (DMARD) under development. In this review, in order to understand the mechanism of action and clinical safety and effect of iguratimod, we introduced the recent information on immunopharmacology, clinical safety and efficacy of iguratimod.

Key words: Anti-rheumatic drug; Immunopharmacology; clinical safety; efficacy; iguratimod

Introduction
Early in 1992, Tanaka et al’s study has been showed that the antiinflammatory, analgesic and antipyretic activities of T-614 (iguratimod, (3-formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one)), a novel antiinflammatory drug with different pharmacological properties from that of the reference drugs, were investigated in various animal models and compared with those of nimesulide, indomethacin and ibuprofen. The antiinflammatory potency of T-614 on carrageenin-induced paw edema, paper disk granuloma and established adjuvant arthritis was greater than that of ibuprofen, but slightly lower than those of nimesulide and indomethacin. Although the analgesic activity of T-614 was hardly demonstrated in writhing tests in mice, its potency against the inflammatory pain such as Randall-Selitto test, adjuvant-induced hyperalgesia and antigen-induced arthritic pain in rats was superior to that of ibuprofen. Moreover, it had a potent analgesic effect on urate-induced synovitis in dogs.¹ Pharmacological studies of T-614 have been shown to display a steroid-like improvement in several
autoimmune animal models, such as collagen-induced arthritis, MRL-lpr/lpr mice\(^2^3\) and experimental autoimmune encephalomyelitis in rats.\(^4^5\) Therefore, it has attracted attention as a disease-modifying anti-rheumatic drug (DMARD) under development.\(^6^7\)

In this review, in order to understand the mechanism of action and clinical safety and effect of iguratimod, a new anti-rheumatic drug, we introduced the recent information on immunopharmacology, clinical safety and efficacy of iguratimod.

**Effects of T-614 on the production of IL-1 and/or IL-6**

Tanaka et al examined the effect of T-614 and other anti-rheumatic drugs on a mouse model of adenocarcinoma-induced cachexia. Cachexia was induced in BALB/c mice by s.c. inoculation of colon 26/clone 20 cells. The drugs were administered p.o. daily from day 0 for 15 days for prophylactic experiments and from day 7 for 8 days for therapeutic experiments. Serum biochemical parameters and wasting of adipose tissue and muscle were evaluated as the nutritional condition in tumor-bearing mice at day 14. IL-6 levels in serum and tumor tissue of those mice were also quantified. Administration of T-614 did not inhibit the tumor growth, but it resulted in attenuation of cachexia symptoms, such as the reduction in epididymal fat and gastrocnemius muscle, and the decrease of serum albumin. Furthermore, T-614 decreased the serum levels of IL-6, and reduced its gene expression in the tumor tissues. Exogenously administered IL-6 nullified the suppressive effect of T-614. Prednisolone prevented the weight loss and the wasting without inhibiting tumor growth. Methotrexate and indomethacin did not exert any preferable effects in a therapeutic dosing schedule. The results demonstrated that T-614 exerts an anticachectic effect in tumor-bearing mice through the inhibition of IL-6 gene expression.\(^8\)

*In vitro* effects of T-614 on the production of IL-1 and/or IL-6 by human monocytes and the THP-1 cells of a human monocytic cell line, were examined. T-614 inhibited the release of immunoreactive IL-1 beta from these cells stimulated with lipopolysaccharides (LPS) in a dose-dependent manner (0.3-30 μg·mL\(^{-1}\)). The release of IL-6 from THP-1 cells, as determined by the assays for its hepatocyte-stimulating activities and immunoreactivities, was inhibited by T-614 with the IC50 values of 2.0 and 6.6 μg·mL\(^{-1}\), respectively. Northern blotting analysis using LPS-stimulated THP-1 cells indicated that the inhibitory effect of T-614 on IL-1 beta production is caused by the suppression of IL-1 beta mRNA expression. The inhibition of cytokine production by T-614 may provide an important insight into the additional mechanisms contributing to its antiinflammatory activities.\(^9\) T-614 shows potent anti-arthritic activity in animal models of rheumatoid arthritis. The aim of the present investigation was to characterize the anti-arthritic activity of T-614 in terms of regulation of the nuclear transcription factor NF-kB, which is associated with expression of many immune and inflammatory genes. THP-1 cells (human monocyctic leukemia cell line) were used throughout this in vitro study, and lipopolysaccharide (LPS) and TNF-α were employed for activation of the cells. The mRNA levels were determined by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Assessment of the NF-kB DNA binding activity was performed by an electrophoretic mobility shift assay (EMSA) using a digoxigenin (DIG)-labeled double-stranded oligonucleotide containing κB-binding site. Degradation kinetics of the cytosolic NF-kB inhibitor α (IκBα) were studied by Western blot analysis. T-614 inhibited LPS-stimulated production of TNF-α, IL-6, and IL-8 in a concentration-dependent manner with decreasing mRNA levels (IL-6 and IL-8). EMSA study showed that T-614 prevented TNF-α as well as LPS-stimulated activation of NF-kB, and Western blot analysis proved that T-614 did not affect degradation of IκBα protein. These results suggest that the inhibitory effect of T-614 on the production of TNF-α, IL-6 and IL-8 in LPS-stimulated THP-1 cells may involve transcriptional regulation through suppression of NF-kB activation without interfering with IκBα degradation.\(^10\) T-614 has been determined for the treatment of patients with rheumatoid arthritis (RA). RA synovial fibroblast-like cells were cultured with tumor necrosis factor-α (TNF-α, 10 ng·mL\(^{-1}\)) in the presence or absence of T-614. After incubation, cytokine production was measured by ELISA. Expression of
IL-6 and IL-8 mRNA was examined by real-time quantitative reverse transcriptase-polymerase chain reaction analysis and TNF-α induced (NF-kB activation was observed using immunostaining with an antibody against NF-kB p65). T-614 suppressed TNF-α induced production of IL-6, IL-8, and monocyte chemoattractant protein 1, and also reduced the accumulation of IL-6 and IL-8 mRNA in a concentration dependent manner. T-614 interfered with the TNF-α induced translocation of NF-kB to the nucleus from the cytoplasm. Inhibition of NF-kB activation and transcription of proinflammatory cytokines by T-614 contributes to its clinical antirheumatic effect. To investigate the effect of T-614 on TNF-α mRNA expression and TNF-α production, and on the activity of NF-kB in the rat alveolar macrophage cell line activated by LPS. NR8383 cells were pretreated with T-614 (13.4, 26.7, 53.4 μmol·L⁻¹), then were stimulated with LPS. T-614 inhibited LPS-stimulated mRNA expression and production of TNF-α in a concentration-dependent manner, as well as the activity of NF-kB. The IC₅₀ value of effect of T-614 on TNF-α level was 26.2μmol·L⁻¹. The inhibitory effect of T-614 on the production of TNF-α in LPS-stimulated NR8383 cells may be mediated by suppression of NF-kB activity.

To clarify the pharmacological action of an anti-rheumatic agent T-614, researchers investigated its effects on immunoglobulin (Ig) production by cultured B cells and Ig secretion from synovial tissues of patients with rheumatoid arthritis (RA) using SCID mice engrafted with human RA tissue (SCID-HuRAg). Murine B cells were prepared from mouse spleen by a T-cell depletion method. The cells were cultured with lipopolysaccharide (LPS) and/or IL-4 in the absence or presence of T-614. Human B cells were isolated from peripheral blood of healthy donors and the Ig production was induced by co-culture with autologous T cells and anti-CD3 antibody. SCID-HuRAg was prepared according to our previous method. T-614 was orally administered to the mice once daily for 4 weeks starting on the fourth week after the implantation. Then, peripheral blood was obtained and the implanted tissues were removed. In murine B-cell cultures, T-614 significantly decreased not only the IgM production stimulated with LPS but IgG1 production induced by LPS and IL-4. Regarding human B cells stimulated with T cells, it also inhibited IgM and IgG production. In SCID-HuRAg mice, high concentrations of polyclonal human IgG were detectable in the sera of all mice. A significant decrease in the IgG level was observed in the T-614-treated group compared with the control group. The study showed that T-614 inhibited Ig production by the cultured B cells and also decreased the high level of human IgG observed in SCID-HuRAg mice. These results may support its effect on plasma Ig in RA patients and provide insights into the mechanisms of its anti-rheumatic effect.

Researchers found that T-614 stimulated osteoblastic differentiation of stromal cell line (ST2) and preosteoblastic cell line (MC3T3-E1) in the presence or absence of recombinant human bone morphogenetic protein-2 (rhBMP-2). Calcium content of mineralized nodules was 14-fold elevated by the addition of T-614 in the presence of rhBMP-2 in ST2 but not MC3T3-E1. Oral administration of T-614 to mice also promoted rhBMP-2 induced bone formation in vivo. Northern blot analysis showed that transcriptional level of ostex, an essential transcription factor for osteoblastic differentiation, was 3-fold increased by T-614 with rhBMP-2 in ST2. Taken together, these results suggested that T-614 possessed anabolic effects on bone metabolism, besides suppressor of bone resorption, by increased expression of ostex.

Sawada et al examined the action of T614 on Leu-OME mediated killing of THP-1, a human monocytic cell line. We revealed that T614 and phenylmethyl sulfonyl fluoride (PMSF), a serine protease inhibitor, inhibited Leu-OME mediated killing of THP-1 cells. All the other mNSAIDs, including nimesulide, flusulide, FK3311 and NS398, also rescued THP-1 from Leu-OME mediated killing, although to a lesser degree. Of the classical NSAIDs tested, a protective effect was observed with diclofenac at high concentration, but not with naproxen or indomethacin. Unlike conventional lysosomal inhibitors, such as chloroquine and ammonium chloride, T614 and PMSF did not raise lysosomal pH, as measured by flow cytometry using fluorescein isothiocyanate dextran (FITC-dextran). Therefore, the mechanism whereby T614 and PMSF inhibit Leu-OME killing is distinct from that of
chloroquine or NH₄Cl. Based on the similarity of T614 and PMSF, we suggest that, besides their roles as COX-2 inhibitors, T614 and other mNSAIDs may act as lysosomal protease inhibitors.[15]

The present study was undertaken to investigate the immunoregulatory effects of T-614 on synovial cells in vitro. Synovial cells were cultured with T-614 in the presence or absence of various cytokines. After incubation, the costimulatory molecule expression on synovial cells and cytokine production in culture supernatants were analyzed by an indirect immunofluorescence method and enzyme-linked immunosorbent assay, respectively. Kawakami et al also examined the effect of T-614 on the function of synovial cells as antigen-presenting cells (APCs). The costimulatory molecules including CD54, CD58, and CD106 were constitutionally expressed on the surface of synovial cells. However, neither CD80 nor CD86 nor CD102 was found on the surface, and these costimulatory molecules could not be induced by any cytokines. T-614 itself did not affect the costimulatory molecule expression and cytokine production of unstimulated synovial cells. The stimulation of synovial cells with interferon-gamma (IFN-γ), interleukin-1beta, or 12-O-tetradecanoyl phorbol 13-acetate enhanced the expression of costimulatory molecules and the proinflammatory cytokine production of these cells. Both the up-regulated expression of these costimulatory molecules and the enhanced production of proinflammatory cytokines were significantly inhibited by T-614. Autologous T cell proliferation in response to purified protein derivative by IFN-gamma-treated synovial cells was significantly suppressed by T-614. T-614 has considerable immunosuppressive effects on synovial cells by inhibiting the costimulatory molecule expression and cytokine production of these cells and the antigen-specific T cell proliferation mediated by the synovial cells. These results suggest that T-614 plays an important immunoregulatory role in rheumatoid synovial tissues.[16]

Aikawa et al examined the therapeutic effects of T-614 on a T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE). T-614 dose-dependently suppressed the development of active EAE induced in Lewis rats by immunization with myelin basic protein (MBP) when administered for 2 weeks starting on the day of immunization (day 0 to 14). Amelioration of clinical signs was also obtained by the treatment at the effector phase (day 7 to 14) of the disease. Furthermore, T-614 treatment of recipient rats that had received MBP-sensitized lymphoid cells resulted in suppression of the clinical severity of EAE. Immunohistological examination revealed that the number of TCR alpha beta-expressing T cells and the extent of MHC class II expression in the spinal cord of rats treated with T-614 was markedly reduced. In vitro study using MBP-specific T cells showed that the addition of T-614 inhibited the proliferative responses of T cells and the production of pro-inflammatory cytokines such as IFN-γ, IL-6 and TNF produced by T and accessory cells. These findings imply that T-614 suppresses the development of EAE by inhibiting the proliferation of autoreactive T cells and pro-inflammatory cytokine production not only by T cells but also by macrophages/microglia. This may be attributable to the result that T-614 is more effective at the effector phase rather than the induction phase. Thus, this drug has a potential value for the treatment of various T cell-mediated autoimmune diseases including multiple sclerosis as well as rheumatoid arthritis.[17]

Selective COX-2 inhibitory action of T-614

Two forms of COX activity are involved in the synthesis of prostaglandins, prostacyclins, and thromboxanes in mammalian cells. There is now convincing evidence, obtained with a number of structurally distinct inhibitors, that selective COX-2 inhibitors possess anti-inflammatory effects with an improved gastrointestinal tolerability compared with conventional nonsteroidal anti-inflammatory drugs (NSAIDs) affecting both COX-1 and COX-2. As more selective COX-2 inhibitors are being developed, assays with a high degree of sensitivity to inhibition are needed to compare the relative effects of compounds on COX-1 activity. In Riendeau et al presented report, they described a sensitive assay for the inhibition of human COX-1 based on the production of prostaglandin E₂ by microsomes from U937 cells incubated with a subsaturating concentration of arachidonic acid.[18] More than 45 NSAIDs and selective COX-2 inhibitors were tested.
in this assay. IC$_{50}$ values ranged from 1 nM for flunixin and flurbiprofen to about 200-500 μM for salicylate and acetaminophen. Potent and nonselective NSAIDs such as sulindac sulfide, diclofenac, and indomethacin showed IC$_{50}$ values of < 20 nM. Among the compounds that have been reported to show selectivity for COX-2, the rank order of potency against COX-1 was DuP 697 > SC-58451 > celecoxib > nimesulide-meloxicam-piroxicam-NS-398-RS-57067 > SC-57666 > SC-58125 > flosulide > etodolac > L-745,337 > DFU-T-614, with IC$_{50}$ values ranging from 7 nM to 17 μM. A good correlation was obtained between the IC$_{50}$ values for the inhibition of microsomal COX-1 and both the inhibition of TXB2 production by Ca$^{++}$ ionophore challenged platelets and the inhibition of prostaglandin E2 production by CHO cells stably expressing human COX-1. However, the microsomal assay was more sensitive to inhibition than cell-based assays and allowed the detection of inhibitory effects on COX-1 for all NSAIDs and selective COX-2 inhibitors examined with discrimination of their potency under conditions of limited availability of arachidonic acid.\[18\]

Tanaka et al elucidated the mechanism for the selective inhibition of prostaglandin E$_2$ (PGE$_2$) production in inflammatory tissue by T-614 and its effects on both the activity and the induction of COX-2 were investigated in vitro. T-614 inhibited the activity of purified COX-2 enzyme (IC50: 7.7 μg·mL$^{-1}$), but was inactive against both COX-1 activities of microsomal and purified enzymes (IC$_{50}$> 300μg·mL$^{-1}$). On the other hand, when the inhibition of PGE$_2$ production by T-614 was examined in the cultured fibroblasts stimulated with bradykinin, T-614 at 1μg·mL$^{-1}$ or less inhibited PGE$_2$ release more effectively than that in the above cell-free system. Therefore, we examined which of the COX enzymes was expressed in bradykinin-stimulated fibroblasts by using both the reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analyses. As a result, COX-1 mRNA was constitutively expressed in the cells, whereas COX-2 mRNA was not detected without stimulation with bradykinin, but was expressed within 30 min when stimulated. Furthermore, it was found that the addition of T-614 reduced the COX-2 mRNA levels in 30 min after stimulation. These studies suggest that at least some of inhibitory effects of T-614 on prostanoids production are mediated by the synergy of the inhibition of COX-2 activity and the inhibition of induction, and such an action of T-614 may explain the pharmacological properties of this drug.\[19\]

In order Tanaka et al also elucidated the analgesic mechanism of T-614, its effects on the kinin-forming system were examined both in vivo and in vitro. T-614 at doses more than 10 mg·kg$^{-1}$ p.o., exhibited a significant inhibitory effect on the increased levels of bradykinin released into the pouch fluid of kaolin-induced inflammation in rats. In the kaolin-induced writhing response in mice, which is shown to be mainly dependent on the action of bradykinin, T-614 reduced not only the writhing frequency but also the peritoneal levels of bradykinin in a dose-dependent manner. Whereas, in the zymosan-induced writhing response in which prostaglandin I$_2$ (PGI$_2$) is shown to be an important mediator, it did not exert an obvious inhibition on either writhing responses or peritoneal PGI$_2$ levels at a highest dose of 100 mg·kg$^{-1}$. T-614 did not inhibit the activities of serine proteases, such as trypsin, thrombin, kallikrein and plasmin. Furthermore, it did not affect the kinin-forming enzymes of rat plasma in vitro. The above results suggest that the analgesic effects of T-614 may be partly mediated by the inhibition of bradykinin release in the local inflamed tissue.\[20\]

Inhibitory effect of T-614 on the production of TNF-α, IL-6 and IL-8, T-614 shows potent anti-arthritic activity in animal models of rheumatoid arthritis. The aim of the present investigation was to characterize the anti-arthritic activity of T-614 in terms of regulation of the nuclear transcription factor NF-κB, which is associated with expression of many immune and inflammatory genes. THP-1 cells were used throughout this study in vitro, and lipopolysaccharide (LPS) and tumor necrosis factor (TNF-α) were employed for activation of the cells. The mRNA levels were determined by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Assessment of the NF-κB DNA binding activity was performed by an electrophoretic mobility shift assay (EMSA) using a digoxigenin (DIG)-labeled double-stranded oligonucleotide containing κB-binding site. Degradation
kinetics of the cytosolic NF-κB inhibitor α (IκBα) was studied by Western blot analysis. Their study showed that T-614 inhibited LPS-stimulated production of TNF-α, IL-6, and IL-8 in a concentration-dependent manner with decreasing mRNA levels (IL-6 and IL-8). EMSA study showed that T-614 prevented TNF-α as well as LPS-stimulated activation of NF-κB, and Western blot analysis proved that T-614 did not affect degradation of IκBα protein. These results suggest that the inhibitory effect of T-614 on the production of TNF-α, IL-6 and IL-8 in LPS-stimulated THP-1 cells may involve transcriptional regulation through suppression of NF-κB activation without interfering with IκBα degradation.[21]

Tanaka et al.’s study was to examine the effect of iguratimod and other anti-rheumatic drugs on a mouse model of adrenocarcinoma-induced cachexia. Cachexia was induced in BALB/c mice by s.c. inoculation of colon 26/clone 20 cells. The drugs were administered p.o. daily from day 0 for 15 days for prophylactic experiments and from day 7 for 8 days for therapeutic experiments. Serum biochemical parameters and wasting of adipose tissue and muscle were evaluated as the nutritional condition in tumor-bearing mice at day 14. IL-6 levels in serum and tumor tissue of those mice were also quantified. Administration of T-614 did not inhibit the tumor growth, but it resulted in attenuation of cachexia symptoms, such as the reduction in epididymal fat and gastrocnemius muscle, and the decrease of serum albumin. Furthermore, T-614 decreased the serum levels of IL-6, and reduced its gene expression in the tumor tissues. Exogenously administered IL-6 nullified the suppressive effect of T-614. Prednisolone prevented the weight loss and the wasting without inhibiting the tumor growth. Methotrexate and indomethacin did not exert any preferable effects in a therapeutic dosing schedule. The results demonstrate that T-614 exerts an anticachectic effect in tumor-bearing mice through the inhibition of IL-6 gene expression.[22]

Jiang et al. studied the effect of iguratimod on TNFα, mRNA expression and TNF-α production, and on the activity of nuclear factor-κB (NF-κB) in the rat alveolar macrophage cell line activated by LPS. In this study, NR8383 cells were pretreated with T-614 (13.4, 26.7, 53.4 μmol L⁻¹), then were stimulated with LPS. The production of TNF-α in the supernatant of NR8383 was assayed by enzyme-linked immunosorbent assay. The TNF-α mRNA level was determined by a semi-quantitative PCR assay. Assessment of the NF-κB DNA binding activity was performed by an ELISA kit. T-614 inhibited LPS-stimulated mRNA expression and production of TNF-α in a concentration-dependent manner, as well as the activity of NF-κB. The IC₅₀ value of the effect of T-614 on TNF-α level was 26.2 μmol·L⁻¹. Their results showed that the inhibitory effect of T-614 on the production of TNF-α in LPS-stimulated NR8383 cells may be mediated by suppression of NF-κB activity.[12]

In vitro effects of T-614 on the production of IL-1 and/or IL-6 by human monocytes and the THP-1 cells of a human monocytic cell line, were examined. T-614 inhibited the release of immunoreactive IL-1β from these cells stimulated with LPS in a dose-dependent manner (0.3-30 μg·mL⁻¹). The release of IL-6 from THP-1 cells, as determined by the assays for its hepatocyte-stimulating activities and immunoreactivities, was inhibited by T-614 with the IC₅₀ values of 2.0 and 6.6 μg·mL⁻¹, respectively. Northern blotting analysis using LPS-stimulated THP-1 cells indicated that the inhibitory effect of T-614 on IL-1β production is caused by the suppression of IL-1β mRNA expression. The inhibition of cytokine production by T-614 may provide an important insight into the additional mechanisms contributing to its antiinflammatory activities.[9]

Inhibition of Ig production and the isotype switch in murine B cells

In order to examine the effect of T-614 on maturation of B cells to plasma cells, murine B cells were incubated with LPS and/or IL-4, and the resultant levels of IgM and IgG1 in culture supernatants were measured. In non-stimulated and IL-4-stimulated conditions, T-614 did not affect, or only marginally affected, IgM production. However, at 3 and 30 μg·mL⁻¹, T-614 significantly inhibited the elevation induced by the addition of LPS of 4 μg·mL⁻¹. The IgM secretion induced by the simultaneous addition of LPS and IL-4 was also decreased to a non-stimulated level by T-614.
addition, T-614 inhibited IgG1 production stimulated with the simultaneous addition of LPS and IL-4. The inhibition rates at 3 and 30 µg•mL⁻¹ of T-614 were 73 and >90%, respectively.⁹ T-614, at concentrations of 3 µg•mL⁻¹ or more, inhibited both IgM and IgG1 production by the B cells.

The proliferation assay by XTT revealed that both LPS and IL-4 showed an increasing effect on the number of cells, and in conditions of the simultaneous addition of LPS and IL-4, there was a 28-fold increase compared with non-stimulated cultures. T-614 at 30 µg•mL⁻¹ inhibited the LPS/IL-4-induced proliferation by about 50%; however, at 3 µg•mL⁻¹, which showed a significant inhibition of Ig production, it did not affect the cell growth induced by LPS, IL-4 or both.²² It is of note that the inhibition of IgG1 production by T-614 was more effective than that of IgM, indicating that T-614 may affect the process of class switch induced by IL-4. Since T-614 at 3 µg•mL⁻¹ had no effect on B-cell proliferation, it is unlikely that its cellular toxicity or the suppression of proliferation was related to the effect on Ig production. Thus, for the inhibition of Ig production by T-614, a different mechanism from the suppression of proliferation might be involved. Although the mechanism of T-614 for the effect on B-cell function has not been clarified, this study suggests that T-614 may have an inhibitory effect on the signal transduction of IL-4. Further studies will be required to investigate the effect of T-614 on cellular signal transduction in the IL-4-induced class switch in B cells.

Matsumoto et al have reported a mouse model for RA in which the pathology is driven almost entirely by the Ig recognizing a ubiquitously expressed protein, glucose-6-phosphate isomerase.²⁴ They have proposed that some forms of RA may be developed by a mechanism fundamentally different from the currently approved paradigm of joint-specific T-cell response, i.e. a revival of the B-cell paradigm for RA pathogenesis.²⁴ Recently, it has also been found that 64% of humans with RA had increased concentrations of anti-GPI IgG in their sera. Moreover, Edwards and Cambridge have shown a clinical efficacy of B-lymphocyte depletion by a monoclonal anti-CD20 antibody in RA patients and it has been suggested that RA is critically dependent on B lymphocytes. Thus, the inhibition of Ig production by T-614 may be directly involved in its clinical efficacy in RA patients, although this is only a speculation.²⁵ In conclusion, T-614 inhibited IgM production and the isotype switch to IgG1 induced by IL-4 via a direct effect on B cells. Furthermore, the IgM and IgG production by human B cells was also inhibited in a dose-dependent manner. In SCID-HuRAg mice, human IgG was detected in the serum at a high concentration and T-614 treatment resulted in a significant suppression of the IgG accumulation in the mouse serum. These results may support its improvement effect on plasma Igs in clinical trials on RA patients and provide insights into the mechanism for its anti-rheumatic effect.

In 2003, Tanaka et al investigated the effects on immunoglobulin (Ig) production by cultured B cells and Ig secretion from synovial tissues of patients with rheumatoid arthritis using SCID mice engrafted with human RA tissues (SCID-HuRAg).¹³ Murine B cells were prepared from mouse spleen using a T-cell depletion method. The cells were cultured with LPS and/or IL-4 in the absence or presence of T-614. Human B cells were isolated from the peripheral blood of healthy donors and the Ig production was induced by the co-culture with autologous T cells and anti-CD3 antibody. SCID-HuRAg was prepared according to their previous method. T-614 was orally administered to the mice once daily for 4 weeks starting on the fourth week after the implantation. Then, the peripheral blood was obtained and the implanted tissues were removed. Igs in the culture media or the sera were determined by enzyme-linked immunosorbant assay (ELISA). In murine B-cell cultures, T-614 significantly decreased not only the IgM production stimulated with LPS but IgG1
production induced by LPS and IL-4. Regarding human B cells stimulated with T cells, it also inhibited IgM and IgG production. In SCID-HuRAG mice, high concentrations of polyclonal human IgG were detectable in the sera of all mice. A significant decrease in the IgG level was observed in the T-614-treated group compared with the control group. The results showed that T-614 inhibited Ig production by the cultured B cells and also decreased the high level of human IgG observed in SCID-HuRAG mice. These results may support its effect on plasma Igs in RA patients and provide insights into the mechanism of its anti-rheumatic effect.[13]

In order to examine the effect of various concentrations of T-614 on Ig production by human B cells, peripheral B cells were stimulated by a 6-day co-culture with autologous T cells and anti-CD3 antibody, and Ig levels were measured, though IgG could not be assessed separately by specific isotypes. T-614 suppressed the production of IgM and IgG in a dose-dependent manner with the IC50 values of 0.58 ± 0.26 and 0.55 ± 0.12 µg•mL\(^{-1}\), respectively.[15] Since such an effect was observed in the T-cell-depleted culture, it suggests that the inhibition of Ig production by T-614 is due to its direct effect on B cells. Furthermore, in human B cells stimulated with anti-CD3-activated T cells, T-614 also suppressed IgM and IgG production at a lower concentration. Using a SCID-HuRAG mouse model, the researchers examined whether B cells existing in synovial tissues from RA patients persistently produced Igs and whether T-614 inhibited Ig production in this model. In the present study, a high level of human polyclonal IgG was detected in sera of all the mice after the implantation. Human IgM was also found, but the level was much lower. The high level of human IgG in mouse serum must derive from CD20-positive B cells and histologically discriminated plasma cells in the implanted tissues.

Aikawa et al examined the therapeutic effects of T-614 on a T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE)[21]. T-614 dose-dependently suppressed the development of active EAE induced in Lewis rats by immunization with myelin basic protein (MBP) when administered for 2 weeks starting on the day of immunization (day 0 to 14). Amelioration of clinical signs was also obtained by the treatment at the effector phase (day 7 to 14) of the disease. Furthermore, T-614 treatment of recipient rats that had received MBP-sensitized lymphoid cells resulted in suppression of the clinical severity of EAE.

The immunohistological examination revealed that the number of TCR \(\alpha \beta\)-expressing T cells and the extent of MHC class II expression in the spinal cord of rats treated with T-614 were markedly reduced. The study in vitro using MBP-specific T cells showed that the addition of T-614 inhibited the proliferative responses of T cells and the production of pro-inflammatory cytokines such as IFN-\(\gamma\), IL-6 and TNF produced by T and accessory cells. Taken together, these findings imply that T-614 suppresses the development of EAE by inhibiting the proliferation of autoreactive T cells and the pro-inflammatory cytokine production not only by T cells but also by macrophages/microglia. This may be attributable to the result that T-614 is more effective at the effector phase than the induction phase. Thus, this drug has a potential value for the treatment of various T cell-mediated autoimmune diseases including multiple sclerosis (MS) as well as rheumatoid arthritis. In order to elucidate the analgesic mechanism of T-614, its effects on the kinin-forming system were examined both in vivo and in vitro. T-614, at doses more than 10 mg•kg\(^{-1}\) p.o., exhibited a significant inhibitory effect on the increased levels of bradykinin released into the pouch fluid of kaolin-induced inflammation in rats. In the kaolin-induced writhing response in mice, which is shown to be mainly dependent on the action of bradykinin, T-614 reduced not only the writhing frequency but also the peritoneal levels of bradykinin in a dose-dependent manner. Whereas, in the zymosan-induced writhing response in which PGI\(_2\) is shown to be an important mediator, it did not exert an obvious inhibition on either writhing responses or peritoneal PGI\(_2\) levels at a highest dose of 100 mg•kg\(^{-1}\). T-614 did not inhibit the activities of serine proteases, such as trypsin, thrombin, kallikrein and plasmin. Furthermore, it did not affect the kinin-forming enzymes of rat plasma in vitro. The above results suggest that the analgesic effects of T-614 may be partly mediated by the inhibition of bradykinin release in the local inflamed tissue.[22]

T-614 at a daily dose of 100 mg•kg\(^{-1}\) showed a significant inhibition of human IgG produced by the
implanted tissues. This is the first experimental evidence that supports the clinical improvement effect of DMARDs on plasma Ig levels of RA patients. The dose of T-614 inhibiting the IgG production exceeded the dose (50 or 75 mg·d⁻¹) yielding clinical efficacy. However, the pharmacokinetics of T-614 was found to have species differences and the data revealed that the area under the curve (AUC) in mice was about 20-fold lower than that in humans when given an identical dose. In fact, it was reported that its effective doses in mouse arthritis models, such as collagen-induced arthritis and MRL/lpr mice, were 10 mg·kg⁻¹ or more.⁶⁰ Therefore, the dose used in this study is considered to be reasonable, and the sufficient efficacy of T-614 on Ig production by synovial tissues may be expected in clinical studies. T-614 did not induce apparent histological changes in RA synovial tissues in this model; however, it has already been reported that among well-recognized DMARDs including salazosulphapyridine, only methotrexate inhibits synovitis by inducing apoptosis.⁶¹

**Immunoregulatory mechanism and role**

Whether it has immunomodulatory or disease-modifying properties and its mechanism of action are largely undetermined. Rats with collagen-induced arthritis (CIA) were treated with T-614 (5 and 20 mg·kg⁻¹) daily. Animals receiving methotrexate (1 mg·kg⁻¹ every 3 days) and the nonsteroidal anti-inflammatory agent nimesulide (10 mg·kg⁻¹ per day) were used as controls. A combination therapy group was treated with both T-614 (10 mg·kg⁻¹ per day) and methotrexate (1 mg·kg⁻¹ every 3 days). Hind paw swelling was evaluated and radiographic scores calculated. Serum cytokine levels were assessed by Bio-plex analysis. Quantitative PCR was used to evaluate expression of mRNA for interferon-gamma, IL-4 and IL-17. Serum IL-17 and anti-type II collagen antibodies (total IgG, IgG1, IgG2a, IgG2b and IgM) were measured using ELISA. Oral T-614 inhibited paw swelling and offered significant protection against arthritis-induced cartilage and bone erosion, comparable to the effects of methotrexate. CIA rats treated with T-614 exhibited decreases in both mRNA expression of IL-17 in peripheral blood mononuclear cells and lymph node cells, and circulating IL-17 in a dose-dependent manner. T-614 also reduced serum levels of tumor necrosis factor-alpha, IL-1β and IL-6. A synergistic effect was observed for the combination of methotrexate and T-614. In addition, T-614 (20 mg·kg⁻¹ per day) depressed production of anti-type II collagen antibodies and differentially affected levels of IgG2a subclasses in vivo, whereas IgM level was decreased without any change in the IgG1 level. Together, the findings presented here indicate that the novel agent T-614 has disease-modifying effects against experimental arthritis, as opposed to nimesulide. The study data suggested that T-614 is an effective disease-modifying agent that can prevent bone/cartilage destruction and inflammation in in CIA rats. Combination with methotrexate markedly enhances the therapeutic effect of T-614.⁶²

Kawakami *et al.* presented a study to investigate the immunoregulatory effects of T-614 on synovial cells in vitro. Synovial cells were cultured with T-614 in the presence or absence of various cytokines. After incubation, the costimulatory molecule expression on synovial cells and the cytokine production in culture supernatants were analyzed by an indirect immunofluorescence method and enzyme-linked immunosorbent assay, respectively. They also examined the effect of T-614 on the function of synovial cells as antigen-presenting cells (APCs). The costimulatory molecules including CD54, CD58 and CD106 were constitutionally expressed on the surface of synovial cells. However, neither CD80 nor CD86 nor CD102 was found on the surface, and these costimulatory molecules could not be induced by any cytokine. T-614 itself did not affect the costimulatory molecule expression and cytokine production of unstimulated synovial cells. The stimulation of synovial cells with interferon-gamma (IFN-γ), interleukin-1β or 12-O-tetradecanoyl phorbol 13-acetate enhanced the expression of these costimulatory molecules and the enhanced production of proinflammatory cytokines. Both the up-regulated expression of these costimulatory molecules and the enhanced production of proinflammatory cytokines were significantly inhibited by T-614. The autologous T cell proliferation in response to purified protein derivatives by IFN-γ-treated synovial cells was significantly suppressed by T-614. T-614 has
considerable immunosuppressive effects on synovial cells by inhibiting the costimulatory molecule expression and cytokine production of these cells and the antigen-specific T cell proliferation mediated by the synovial cells. These results suggest that T-614 plays an important immunoregulatory role in rheumatoid synovial tissues.\[32-34\]

In order to investigate the mechanism of the immunosuppressive effect of T-614 on clinical efficacy that has been determined for the treatment of patients with rheumatoid arthritis (RA), RA synovial fibroblast-like cells were cultured with tumor necrosis factor-alpha (TNF-\(\alpha\), 10 ng·mL\(^{-1}\)) in the presence or absence of T-614. The expression of interleukin 6 (IL-6) and IL-8 mRNA was examined by the real-time quantitative reverse transcriptase-polymerase chain reaction analysis and the activation of inducing the nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) by TNF-\(\alpha\) was observed using immunostaining with an antibody against NF-\(\kappa\)B p65. T-614 suppressed the production of IL-6, IL-8 and monocyte chemoattractant protein 1 induced by TNF-\(\alpha\), and also reduced the accumulation of IL-6 and IL-8 mRNA in a concentration-dependent manner. T-614 interfered with TNF-\(\alpha\) induced the translocation of NF-\(\kappa\)B to the nucleus from the cytoplasm. The inhibition of NF-\(\kappa\)B activation and transcription of proinflammatory cytokines by T-614 contributes to its clinical antirheumatic effect.\[39\] In clinical trials on Japanese patients with rheumatoid arthritis (RA), T-614 has been found to yield a good therapeutic effect on the clinical symptoms and laboratory indices, such as erythrocyte sedimentation rate (ESR), plasma C-reactive protein (CRP), levels of plasma IgG and IgM.\[8\] The efficacy and tolerability of T-614 in RA patients are being compared with those of salazosulphapyridine, an established DMARD with a similar pharmacological profile, in a double-blind randomized clinical study. However, the mechanism for anti-rheumatic actions of T-614 and its immunopharmacological effect on B lymphocytes are not well understood. This is the first experimental evidence that supports the clinical improvement effect of DMARDs on plasma Ig levels of RA patients. The dose of T-614 inhibiting the IgG production exceeded the dose (50 or 75mg·d\(^{-1}\)) yielding clinical efficacy.\[2\]

The molecular mechanisms by which T-614 alters an ongoing immune response in vivo are not yet clear. Rheumatoid arthritis is a complicated and treatment refractory autoimmune disease that is characterized by a chronic inflammatory infiltrate of immune cells, in particular T cells, which represent approximately 40% of the synovial cellular infiltration and participate in a number of inflammatory and destructive events, such as synovial hyperplasia, pannus formation, cartilage and bone erosion, and joint malformation.\[35-38\]

IL-17-producing T cells, and not IFN-\(\gamma\) CD4\(^{+}\) effector T cells, are pathogenic in collagen-induced arthritis (CIA)\[39,40\]. Ligation of the IL-17 receptor, which is expressed on several cell types (including epithelial cells, endothelial cells, and fibroblasts), induces the secretion of IL-6, IL-8, granulocyte colony-stimulating factor, monocyte chemotactic protein-1, prostaglandin E2, TNF-\(\alpha\) and IL-1\(\beta\), as well as neutrophil chemotaxis and granulopoiesis\[41-44\]. IL-17 also induces the expression of matrix metalloproteinase-1 and -13 in RA synovial cells and osteoblasts\[45,46\], and induces the expression of RANKL (receptor activator of nuclear factor-\(\kappa\)B ligand), which contributes to bone resorption.\[47\]

Relative to other experimental arthritis models, CIA has been demonstrated to resemble human RA more closely in terms of clinical, histological and immunological features, as well as genetic linkage.\[47,48\] Oral T-614 inhibited rat paw swelling and offered significant protection against arthritis-induced cartilage and bone erosion, comparable to the effects of methotrexate. CIA rats treated with T-614 exhibited decreases in both mRNA expression of IL-17 in peripheral blood mononuclear cells and lymph node cells, and circulating IL-17 in a dose-dependent manner. T-614 also reduced serum levels of tumor necrosis factor-\(\alpha\), IL-1\(\beta\) and IL-6. A synergistic effect was observed for the combination of methotrexate and T-614. In addition, T-614 (20 mg·kg\(^{-1}\) per day) depressed production of anti-type II collagen antibodies and differentially affected levels of IgG2\(\alpha\) subclasses in vivo, whereas IgM level was decreased without any change in the IgG1 level. Together, the findings presented here indicate that the novel agent T-614 has disease-modifying effects against experimental arthritis, as opposed to nimesulide. The study data suggested that T-614 is an effective disease-modifying agent that can prevent
bone/cartilage destruction and inflammation in in CIA rats. Combination with methotrexate markedly enhances the therapeutic effect of T-614.[69]

Clinical safety and efficacy of T-614

Rheumatoid arthritis (RA) is an autoimmune disease characterized by severe inflammation of the joints, resulting in destruction of cartilage, bone and tendon. Current treatments for RA emphasize the early use of disease-modifying anti-rheumatic drugs (DMARDs) to minimize or prevent joint damage. Unfortunately, DMARDs can rarely be given for long periods in RA owing to lack of sustained efficacy or to toxicity. Reflecting laboratory findings, they observed significant improvements in RA in clinical trials.[7] T-614 has been shown to have an anti-inflammatory effect and to improve abnormal immunological findings in RA. Lü et al assessed the safety and efficacy of T-614 versus placebo in patients with active RA they conducted a 24-week clinical study in 280 Chinese patients. In a multicenter, randomized, double blind, placebo controlled study, 280 patients were randomly assigned to receive placebo (n = 95) or T-614 at 50 mg (n = 93) or 25 mg (n = 92) daily. Active disease was defined by 4 of the following 5 criteria: > or = 5 tender joints, > or = 3 swollen joints, morning stiffness lasting for > or = 60 minutes, and Westergren erythrocyte sedimentation rate (ESR) > or = 28 mm·h⁻¹, the assessment of pain at the rest by patient as moderate or severe. Clinical and laboratory parameters were analyzed at baseline, 2, 4, 6, 12, 18 and 24 weeks. The primary efficacy variable at week 24 was the American College of Rheumatology (ACR) response rate using the intent-to-treat population. The ACR response rate was significantly higher in the T-614 treatment group compared with the placebo group within 8 weeks after the initiation of treatment. After 24 weeks, the 25 mg·d⁻¹ and 50 mg·d⁻¹ dosage groups and the placebo group showed 39.13%, 61.29% and 24.21% in ACR20 and 23.91%, 39.13%, 61.29% and 24.21% in ACR50, respectively. A time-response in ACR response was observed, with clear superiority for the 25 mg·d⁻¹ and 50 mg·d⁻¹ dosage groups compared to placebo (P < 0.0001), and the 50 mg·d⁻¹ dose compared to the 25 mg·d⁻¹ dose (P < 0.05) when using the ACR response analyses after 24 weeks. ESR and c-reactive protein (CRP) were significantly different in the treatment groups after 24 weeks. The incidence of adverse events (AEs) was not significantly higher with T-614 than with placebo, but upper abdominal discomfort, leucopenia, elevated serum alanine aminotransferase (sALT), skin rash and/or pruritus were more common in the 50 mg and 25 mg dosage groups. T-614, a new slow-acting drug, is effective in treatment of rheumatoid arthritis and is well tolerated.[7]

References


