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Title: COMPOSITIONS AND METHODS FOR TREATING INFLAMMATORY ARTHRITIS

Abstract: Methods for treating or preventing one or more symptoms of rheumatoid arthritis or other types of inflammatory arthritis involves administering a composition containing an effective amount of arsenic trioxide to an affected patient. The composition comprising arsenic trioxide can be administered orally, for example, as a solution, suspension, syrup, emulsion, tablet, or capsule.
COMPOSITIONS AND METHODS FOR TREATING
INFLAMMATORY ARTHRITIS

FIELD OF THE INVENTION

The present disclosure generally relates to the field of methods for treating rheumatoid arthritis and other inflammatory arthritis using arsenic trioxide.

BACKGROUND OF THE INVENTION

Rheumatoid arthritis (RA) is an autoimmune arthropathy associated with systemic inflammatory manifestations. In RA, the synovium, the inner lining of synovial joints, is abnormal. There is infiltration of acute inflammatory cells in the synovium, which undergoes hypertrophy, resulting in effusion of the joint. Hyperplastic synovium may also lead to erosion of the adjacent cartilage and bone, causing joint destruction. The end result is joint inflammation, effusion, joint and adjacent ligament destruction, joint deformity and, finally, loss of function. RA is the most common inflammatory joint disease, affecting virtually all populations in the world. It is a serious worldwide health problem.

Traditional treatment of RA centers on the use of anti-inflammatory drugs to suppress joint inflammation and production of cytokines. Immunomodulatory drugs may also be effective, including immunosuppressive drugs such as corticosteroids, methotrexate and cyclosporine (Turner, et al., Nat. Clin. Pract. Rheumatol., 3:336-45 (2007)). Other disease modifying medications including penicillamine and gold may also be used.

The pathogenesis of RA is complex and has not been fully defined. However, a number of cytokines, including interleukin (IL)-6, IL-15, IL-17, and tumor necrosis factor, have been implicated in causing joint and systemic inflammation (Turner, et al., Nat. Clin. Pract. Rheumatol., 3:336-45 (2007)). The cytokine signaling pathways incriminated in RA pathogenesis include the tumor necrosis factor alpha (TNF-α) pathway, the mitogen activated protein kinase (MAPK) pathway, the Janus kinases (JAK) pathway, and the signal transducer and activator of transcription (STAT) pathways.
An important molecule downstream of TNF-α pathway is the transcription factor nuclear factor kappa B (NFκB). These pathways play important parts in the synovial proliferation, and the consequent joint damage characteristic of RA (Tarner, et al., Nat. Clin. Pract. Rheumatol., 3:336-45 (2007)). Recently, it has also been shown that the synovium plays an active role in joint inflammation. Fibroblast-like synoviocytes are the most important cellular component of the synovium. Synoviocytes are necessary for the initiation and propagation of the inflammation observed in RA, mediating both cartilage damage and acute and chronic inflammation (Lipsky, N. Engl. J. Med., 356:2419-20 (2007)). The importance of the synoviocytes is further shown by the finding that mouse models deficient in a functional synovium are resistant to experimental arthritis (Amano, et al., Genes Dev., 17:2436-49 (2003); Lee, et al., Science, 315:1006-10 (2007)).

Targeting of two of the key pathways involved putatively in RA pathogenesis has shown that this may be a potential therapeutic strategy (Choy, et al., N. Engl. J. Med., 344:907-16 (2001)). Treatment with infliximab, a chimeric monoclonal antibody against TNF-α, results in significant improvement in joint inflammation and halts the progression of joint damage (Lisky, et al., N. Engl. J. Med., 343:1594-602 (2000)). Treatment with tocilizumab, an antibody against the IL-6 receptor (IL-6R), leads to significant control of symptoms in RA patients (Smolen, et al., Lancet, 371:987-97 (2008)).

The use of infliximab, however, has been associated with tuberculosis (Keane, et al., N. Engl. J. Med., 345:1098-104 (2001)), and other opportunistic infections including pneumocystis pneumonia (Harigai, et al., N. Engl. J. Med., 357:1874-6 (2007))) and cerebral toxoplasmosis (Young, et al., N. Engl. J. Med., 353:1530-1 (2005)). Serious infections have also happened to patients on tocilizumab treatment (Nishimoto, et al., Ann. Rheum Dis., (2008)). These medications are also expensive. Moreover, because they are given intravenously, they are expensive and cumbersome for the patient.
Therefore, it is an object of the invention to provide compositions and methods for suppressing the growth and activity of synoviocytes in rheumatoid arthritis. Because synoviocytes may also play important pathogenetic roles in other inflammatory arthritis, it is another object of the invention to provide compositions and methods for suppressing the growth and activity of synoviocytes in other inflammatory arthritis.

It is yet another object of the invention to provide compositions and methods for treating inflammatory arthritis.

**SUMMARY OF THE INVENTION**

It has been discovered that As$_2$O$_3$ reduces the growth of synoviocytes associated with rheumatoid arthritis. As$_2$O$_3$ leads to a decrease in levels of gp130, a component of the IL-6 receptor complex, by targeting gp130 to destruction in the lysosome, thereby disrupting an autocrine IL-6 loop in synovial cells.

Methods for inhibiting or reducing the growth of synoviocytes using As$_2$O$_3$ are provided. Also provided are methods for treating or preventing one or more symptoms of rheumatoid arthritis and other inflammatory arthritis using compositions containing As$_2$O$_3$.

Specifically, the present application provides the following embodiment:

**Embodiment 1.** A method for treating or preventing one or more symptoms of rheumatoid arthritis and other inflammatory arthritis comprising administering to a subject in need thereof a composition comprising arsenic trioxide in an effective amount to reduce or treat one or more symptoms of rheumatoid arthritis.

**Embodiment 2.** A method for reducing or inhibiting the proliferation of synoviocytes or of promoting the apoptosis of synoviocytes comprising contacting the synoviocytes with an amount of arsenic trioxide effective to reduce or inhibit their proliferation or promote their apoptosis.

**Embodiment 3.** A method for reducing cell surface expression of the IL-6 receptor in a cell comprising contacting the cell with an amount of arsenic trioxide effective to promote the degradation of the IL-6 receptor by targeting it to the lysosome.
Embodiment 4. The method of embodiment 3, wherein the IL-6 receptor is expressed in a synoviocyte.

Embodiment 5. The method of embodiment 2 or 4, wherein the synoviocytes are in a patient with rheumatoid arthritis.

Embodiment 6. The method of embodiment 2 or 4, wherein the synoviocytes are in a patient with inflammatory arthritis.

Embodiment 7. The method of embodiment 5, wherein the arsenic trioxide is administered to the patient orally.

Embodiment 8. The method of embodiment 5, wherein the arsenic trioxide is administered locally to a synovial joint.

Embodiment 9. The method of embodiment 1, further comprising administering a steroidal or non-steroidal anti-inflammatory agent.

Embodiment 10. A pharmaceutical composition for treating one or more symptoms of rheumatoid arthritis or other inflammatory arthritis in a subject comprising an effective amount of arsenic trioxide to inhibit the proliferation or promote the apoptosis of synoviocytes, and, optionally, a pharmaceutically acceptable excipient.

Embodiment 11. The pharmaceutical composition of embodiment 10, wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for oral administration.

Embodiment 12. The pharmaceutical composition of embodiment 10, wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for local administration to a synovial joint.

Embodiment 13. The pharmaceutical composition of any of embodiments 10-12, wherein the arsenic trioxide is present in an amount from 1 to 10 mg.

Embodiment 14. The pharmaceutical composition of embodiment 13, wherein the arsenic trioxide is present in an amount from 5 to 10 mg.

Embodiment 15. The pharmaceutical composition of embodiment 10 in a unit dosage form for oral administration, wherein the unit dosage form is selected from the group consisting of solutions, suspensions, emulsions, syrups, tablets and capsules.
Embodiment 16. Use of arsenic trioxide in preparation of a medicament for treating or preventing one or more symptoms of rheumatoid arthritis and other inflammatory arthritis.

Embodiment 17. Use of arsenic trioxide in preparation of a medicament for reducing or inhibiting the proliferation of synoviocytes or of promoting the apoptosis of synoviocytes.

Embodiment 18. Use of arsenic trioxide in preparation of a medicament for reducing cell surface expression of the IL-6 receptor in a cell.

Embodiment 19. The use of embodiment 18, wherein the IL-6 receptor is expressed in a synoviocyte.

Embodiment 20. The use of embodiment 17 or 19, wherein the synoviocytes are in a patient with rheumatoid arthritis.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1A** is a line graph showing the effects of increasing concentrations of arsenic trioxide (As₂O₃) on growth of MH7A cells when treated for 24 (-●-), 48 (-▲-) or 72 (-■-) hours, as assessed by MTT assays. Data are expressed as cell viability (%) as a function of As₂O₃ concentration (µM). Error bars represent the standard error of the mean (SEM) (n = 3) (# = P<0.001)

**Figure 1B** is a bar graph showing the percent of apoptosis in the absence or presence of 5 µM As₂O₃, as determined by FACS analysis of MH7A cells following propidium iodide (PI) and annexin-V staining.

**Figure 1C** is a line graph showing viability of MH7A cells in the presence (-●-) or absence (-■-) of the pan-caspase inhibitor Z-VAD-FMK (25 µM) and in the presence of increasing concentrations of As₂O₃ over 72 hours, as assessed by MTT assays. Data are expressed as cell viability (%) as a function of As₂O₃ concentration (µM). Error bars represent the standard error of the mean (SEM) (n = 3) (** = P<0.01).

**Figure 2A** is a bar graph showing viability of MH7A cells in the presence of increasing concentrations of IL-6 for 72 hours, as assessed by MTT assays. Data are expressed as cell viability (%) as a function of IL-6
concentration (ng/ml). Error bars represent the standard error of the mean (SEM) (n = 3).

**Figure 2B** is a bar graph showing viability of MH7A cells in the presence of increasing concentrations of anti-IL-6 Ab for 6 days, as assessed by MTT assays. Data are expressed as cell viability (%) as a function of anti-IL-6 Ab concentration (µg/ml). Error bars represent the standard error of the mean (SEM) (n = 3) (** = P<0.001).

**Figure 2C** is a line graph showing the effects of increasing concentrations of As₂O₃ on expression of gp130 when treated for 24 hours, as assessed by Western blot. Data are expressed as gp130 expression (%) as a function of As₂O₃ concentration (µM). Error bars represent the standard error of the mean (SEM) (n = 3) (# = P<0.001; ** = P<0.01).

**Figure 2D** is a line graph showing the effects of 5 µM As₂O₃ on expression of gp130 when treated for 0, 3, 6, 12 or 24 hours, as assessed by Western blot. Data are expressed as gp130 expression (%) as a function of time (hours). Error bars represent the standard error of the mean (SEM) (n = 3) (** = P<0.01).

**Figure 2E** is a bar graph showing the effects of 5 µM As₂O₃ on expression of gp130 when treated for 0, 3, 6, 12 or 24 hours, as assessed by quantitative-polymerase chain reaction (Q-PCR). Error bars represent the standard error of the mean (SEM) (n = 3).

**Figure 3A** is a bar graph showing the effect of the lysosome inhibitor NH₄Cl (2.5 mM) on suppression of gp130 expression by 5 µM As₂O₃. Error bars represent the standard error of the mean (SEM) (n = 3) (* = P<0.05).

**Figure 3B** is a line graph showing levels of gp130 immunoprecipitated (IP) from MH7A cells following treatment with 100 µM As₂O₃ for 0, 4 or 6 hours. Antibody to gp130 was used for immunoprecipitation (IP) of total cell lysates, followed by Western blot analysis with anti-ubiquitin antibodies FK1 and FK2. IP with non-immune rabbit serum (NIS) served as control. As₂O₃-induced gp130 ubiquitination was shown by FK2 that recognized both mono- and poly-ubiquitinated proteins, but not FK1 that recognized poly-ubiquitinated proteins. Error bars
represent the standard error of the mean (SEM) (n = 3) (* = P<0.05; # = P<0.001).

**Figure 4A** is a line graph showing phosphorylation of JNK following treatment of MH7A cells with 5 \( \mu M \) As\(_2\)O\(_3\) for 0, 3, 6, 12 or 24 hours as determined by Western blot. Values represent mean signal density ± SEM (n = 3) (* = P<0.05; ** = P<0.01).

**Figure 4B** is a bar graph showing viability of MH7A cells in the presence or absence of the JNK inhibitor SP600125 (30 \( \mu M \)) and in the presence of 0, 30 or 100 \( \mu M \) As\(_2\)O\(_3\) over 24 hours, as assessed by MTT assays. Data are expressed as cell viability (%). Error bars represent the standard error of the mean (SEM) (n = 3) (** = P<0.01).

**Figure 5A** is a bar graph showing the effect of the lysosome inhibitor NH\(_4\)Cl (2.5 mM) on activation of JNK phosphorylation by 5 \( \mu M \) As\(_2\)O\(_3\). Error bars represent the standard error of the mean (SEM) (n = 3) (\( * = P<0.05 \)).

**Figure 5B** is a line graph showing the effect of treatment of MH7A cells with 0.5 \( \mu g/ml \) anti-IL-6 Ab for 24 hours on phosphorylation of JNK as determined by Western blotting. Error bars represent one-way ANOVA with Dunnett’s post-tests (n = 3) (* = P<0.05).

**Figure 5C** is a bar graph showing the effect of the JNK inhibitor SP600125 (30 \( \mu M \)) on phosphorylation of p53 on serine-46 by 5 \( \mu M \) As\(_2\)O\(_3\), as determined by Western blotting. Error bars represent one-way ANOVA with Dunnett’s post-tests (n = 3) (* = P<0.05).

**Figure 6A** is a line graph showing the effects of increasing concentrations of As\(_2\)O\(_3\) on expression of NF\(_{\kappa}\)B when treated for 24 hours, as assessed by Western blot. Data are expressed as NF\(_{\kappa}\)B expression (%) as a function of As\(_2\)O\(_3\) concentration (\( \mu M \)). Error bars represent the standard error of the mean (SEM) (n = 3) (# = P<0.001; ** = P<0.01).

**Figure 6B** is a line graph showing the effects of 5 \( \mu M \) As\(_2\)O\(_3\) on expression of NF\(_{\kappa}\)B when treated for 0, 6, 12 or 24 hours, as assessed by Western blot. Data are expressed as NF\(_{\kappa}\)B expression (%) as a function of
time (hours). Error bars represent the standard error of the mean (SEM) (n = 3) (# = P<0.001; ** = P<0.01).

DETAILED DESCRIPTION OF THE INVENTION

I. Compositions Comprising Arsenic Trioxide

A. Arsenic Trioxide

Arsenic trioxide is very useful in the treatment of refractory promyelocytic (M3) subtype of acute myeloid leukemia. An oral arsenic trioxide (As₂O₃) is highly efficacious for relapsed acute promyelocytic leukemia. (Au, et al., Blood, 112:3587-90 (2008)) Oral As₂O₃ causes a smaller prolongation of QT intervals, and therefore is a much safer drug for treating leukemia. Recently, it was also demonstrated that oral As₂O₃ produces minimal QT prolongation in the heart, meaning that it is safe for prolonged use (Siu, et al., Blood, 108:103-6 (2006)).

B. Compositions

The following delivery systems are representative of compositions for administering the As₂O₃.

1. Parenteral compositions

Injectable drug delivery systems include pharmaceutically acceptable solutions, suspensions, gels, microspheres and implants. Typically these will be in the form of distilled water, phosphate buffered saline, or other vehicle for injection intravenously or subcutaneously.

2. Enteral compositions

Oral delivery systems include solutions, suspensions, and solid dosage forms such as tablets (e.g., compressed tablets, sugar-coated tablets, film-coated tablets, and enteric coated tablets), capsules (e.g., hard or soft gelatin or non-gelatin capsules), blisters, and cachets. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc). The solid dosage forms can be coated using coatings and techniques well known in the art.
Oral liquid dosage forms include solutions, syrups, suspensions, emulsions, elixirs (e.g., hydroalcoholic solutions), and powders for reconstitutable delivery systems. The compositions can contain one or more carriers or excipients, such as suspending agents (e.g., gums, zanthans, cellulosics and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG, glycerin, and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, TWEENs, and cetyl pyridine), emulsifiers, preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anticaking agents, coating agents, chelating agents (e.g., EDTA), flavorants, colorants, and combinations thereof.

3. **Topical compositions**

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Dermal delivery systems include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer.
II. Methods of treatment
   A. Treatment of rheumatoid arthritis

Compositions comprising $\text{As}_2\text{O}_3$ are administered to an individual having RA in an effective amount to inhibit or reduce the proliferation of synoviocytes, to inhibit or reduce cell surface expression of the IL-6 receptor in synovial cells, or in an effective amount to treat or reduce the risk of developing one or more symptoms of rheumatoid arthritis. The disclosed compositions comprising $\text{As}_2\text{O}_3$ can be administered therapeutically or prophylactically.

Therapeutically effective amounts of the disclosed compositions comprising $\text{As}_2\text{O}_3$ refers to amounts effective to delay progression, expedite remission, induce remission, augment remission, speed recovery, increase efficacy of or decrease resistance to alternative therapeutics, or a combination thereof. Therapeutically effective amounts can be effective in reducing the severity of symptoms, reducing the severity of an acute episode, reducing the number of symptoms, reducing the incidence of disease-related symptoms, reducing the latency of symptoms, ameliorating symptoms, reducing secondary symptoms, reducing secondary infections, prolonging patient survival, or a combination thereof.

Prophylactically effective amounts of the compositions comprising $\text{As}_2\text{O}_3$ refers to amounts effective to delay the onset of symptoms, prevent relapse to a disease, decrease the number or frequency of relapse episodes, increasing latency between symptomatic episodes, or a combination thereof.

The examples below use MH7A as a model of RA synovium, since MH7A retains most of the biological properties of fibroblast-like synoviocytes, including surface immunophenotype and response to cytokines (Miyazawa, et al., *J. Biochem.*, 124:1153-62 (1998)). Cellular signaling and gene expression patterns of MH7A cells are also similar to primary RA synoviocytes (Kitano, et al., *Arthritis Rheum.*, 54:742-53 (2006)). Using this model, it was discovered that $\text{As}_2\text{O}_3$ is effective to inhibit the proliferation of fibroblast synoviocytes implicated in the development of rheumatoid arthritis.
The examples below also demonstrate that an autocrine IL-6 loop functions in MH7A cells, as shown by expression of both IL-6 and IL-6 receptor, and that IL-6 neutralization significantly inhibits MH7A cellular proliferation. IL-6 signals by binding to the IL-6 receptor complex, which comprises an 80-kDa IL-6 binding protein (IL-6 receptor α) and a 130 kDa signal transducer gp130 (Naka and Kishimoto, *Arthritis Res.*, 4:154-6 (2002)).

The examples also demonstrate that an autocrine IL-6 loop functions in MH7A cells, as shown by expression of both IL-6 and IL-6 receptor, and that IL-6 neutralization significantly inhibits MH7A cellular proliferation. IL-6 signals by binding to the IL-6 receptor complex, which comprises an 80-kDa IL-6 binding protein (IL-6 receptor α) and a 130 kDa signal transducer gp130 (Naka and Kishimoto, *Arthritis Res.*, 4:154-6 (2002)). The examples demonstrate that As₂O₃ treatment leads to a time and dose dependent decrease in gp130 cell surface expression post-transcriptionally. Through the use of specific inhibitors, the examples show that As₂O₃-mediated decreases of gp130 are regulated by the lysosome and not the proteasome or caspase systems. The examples also show that gp130 degradation is initiated by mono-ubiquitination, a process that provides a biologic signal leading to sorting of the receptors for degradation in the lysosomes (Marmor and Yarden, *Oncogene*, 23:2057-70 (2004)).

**B. Methods of administration**

The compositions comprising As₂O₃ can be administered before, during or after the onset of symptoms associated with rheumatoid arthritis. Any acceptable method known to one of ordinary skill in the art can be used to administer the disclosed compositions comprising As₂O₃ to a subject.

The administration can be localized (i.e., to a particular region, physiological system, tissue, organ, or cell type) or systemic. The compositions comprising As₂O₃ can be administered by different routes, such as oral, parenteral and topical. The compositions comprising As₂O₃ can also be administered directly to a joint, especially a synovial joint. The particular route of administration selected will depend upon factors such as
the particular composition, the severity of the state of the subject being
treated, and the dosage required to induce an effective immune response.

In a preferred embodiment, the compositions comprising As₂O₃ are
administered orally. Effective oral dosages of As₂O₃ range from about 0.5
mg to about 1 to 10 mg, typically about 5 to 10 mg depending on the age if
the subject and their kidney function. As₂O₃ is excreted via the kidneys, and
therefore As₂O₃ dosage has to be adjusted according to the renal function,
lowering the dosage where renal function of the patient is impaired.

An effective level of the composition comprising As₂O₃ can be
ideally obtained after one single administration. In certain circumstances, it
can be beneficial to administer two or more doses of compositions
comprising As₂O₃.

C. Combination therapy

The compositions comprising As₂O₃ can be administered alone or in
combination with one or more additional therapeutic or prophylactic agents,
or can be coupled with surgical, radiologic, or other approaches in order to
affect treatment. For example, the compositions comprising As₂O₃ can be
administered in combination with one or more anti-inflammatory agents.

Anti-inflammatory agents can be non-steroidal, steroidal, or a
combination thereof. Representative examples of non-steroidal anti-
inflammatory agents include, without limitation, oxicams, such as piroxicam,
isoxicam, tenoxicam, sularoxicam; salicylates, such as aspirin, disalcid,
benorylate, trilisate, safapryn, solprin, diflunisal, and fendosal; acetic acid
derivatives, such as diclofenac, fenclofenac, indomethacin, sulindac, tolmelin,
isoxepac, furofenac, tiopinac, zidometacin, acematacin, fentiazac, zomepirac,
clindanac, oxeponic, felbinac, and ketorolac; fenamates, such as mefenamic,
meclomenamic, flufenamic, niflumic, and tolfenamic acids; propionic acid
derivatives, such as ibuprofen, naproxen, benoxaprofen, flurbiprofen,
ketoprofen, fenoprofen, fenbufen, indopropfen, piprofen, carprofen,
oxaprozin, pranoprofen, miroprofen, tioxaprofen, suprofen, alminoprofen,
and tiaprofenic; pyrazoles, such as phenylbutazone, oxyphenbutazone,
feprazone, azapropazone, and trimethazone. Mixtures of these non-steroidal anti-inflammatory agents can also be employed.

Representative examples of steroidal anti-inflammatory drugs include, without limitation, corticosteroids such as hydrocortisone, hydroxy-triamcinolone, alpha-methyl dexamethasone, dexamethasone-phosphate, beclomethasone dipropionates, clobetasol valerate, desonide, desoxymethasone, desoxycorticosterone acetate, dexamethasone, dichlorisone, diflorasone diacetate, diflucortolone valerate, fluadrenolone, flucorolone acetonide, fludrocortisone, flumethasone pivalate, flusinolone acetonide, fluocinonide, flucortine butylesters, flucortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone, fludrocortisone, difluorocone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, chlorprednisone acetate, cloprednol, clescinolone, dichlorisone, diflurprednate, flucoronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone valerate, hydrocortisone cyclopentylpropionate, hydrocortamate, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate, triamcinolone, and mixtures thereof.

Examples

Example 1. Inhibition of MH7A cell proliferation by As₂O₃.

Materials and Methods:

Fibroblast-like synoviocyte MH7A cell line

MH7A was purchased from the Riken BioResource Center (Tsukuba, Japan). Cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen) in 5% CO₂ at 37°C.

Reagents and antibodies

Reagents included As₂O₃ and dimethylsulphoxide (DMSO) (Sigma, St. Louis, MO, USA), ammonium chloride (NH₄Cl) (Amresco, Solon, OH,
USA), the proteasome inhibitor MG115, pan-caspase inhibitor Z-VAD-FMK, JNK inhibitor SP600125 and its negative control (Merck, Darmstadt, Germany), and recombinant human IL-6 (Peprotech, Rocky Hill, NJ, USA). Primary antibodies included rabbit anti-IL-6 receptor α (IL-6Rα) and gp130 (C-20) antibodies (Santa-Cruz Biotechnology, Santa Cruz, CA, USA), rabbit-IL-6 antibody (Merck), rabbit anti-phosphorylated JNK (Thr\textsuperscript{183}/Tyr\textsuperscript{185}), anti-caspase 3, anti-β-actin and anti-NFκB antibodies (Cell Signaling Technology, Beverly, MA, USA), and mouse anti-ubiquitin antibodies FK1 and FK2 (Biomol, Plymouth Meeting, PA, USA). Secondary antibodies included horseradish peroxide conjugated goat anti-rabbit IgG and rabbit anti-mouse IgG (Invitrogen).

**MTT assay**

Cellular proliferation was assessed by an MTT assay (GE Healthcare, Piscataway, NJ, USA) (Cheung et al., *Cancer Lett.*, 246:122-8 (2007)). Cells (2 × 10\textsuperscript{4} cells/well in 96-well plates) were incubated overnight before treatment with controls or reagents for 2–6 days. The treated cells were then incubated with the MTT labeling solution (10 μL/well). After 4 hours of incubation, cells were lysed, and formazan crystals solubilized overnight at 37\textdegree C. Formazan signal was detected at 570 nm (μ-quant\textsuperscript{TM} microplate spectrometer, Bio-Tek Instruments Inc., VT, USA). Data obtained were analyzed by the KC junior software (BLD Science, Garner, NC, USA).

**Western blot analysis**

Western blot analysis was performed (Pang et al., *Gastroenterology*, 132:1088-103 (2007)). Cell lysis and protein collection was conducted according to standard protocols. Protein samples (typically 30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% resolving gel and electro-transferred to nitrocellulose membranes (400 mA for 2 hours). After blocking with tris-buffered saline-TWEEN (TBS-T) containing 5% non-fat milk at room temperature for 30 minutes, membranes were incubated with the primary antibody and TBS-T with 5% bovine serum albumin at 4\textdegree C overnight. The membranes were then washed thrice with TBS-T, and incubated for 1 hour at room
temperature with 1:2000 horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia Biotechnology, Piscataway, NJ, USA). Immunoreactive bands were detected with chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL, USA), and visualized on X-ray films. Densitometric quantification of band signals was performed using ImageJ 1.36b software (National Institutes of Health, USA). All experiments were performed in triplicates.

**Annexin V apoptosis assay**

For apoptosis assay, 1 x 10^6 cells were incubated in 10-cm plates overnight, before treatment with controls or reagents for 24 hours. Cells were then trypsinized, washed twice with ice-cold phosphate buffered saline (PBS), re-suspended in 500 μL of binding buffer, and incubated on ice for 10 minutes with FITC-conjugated annexin-V and propidium iodide (PI) (Immunotech; Fullerton, CA, USA). Apoptotic cells (annexin-V-positive, PI-negative) were enumerated by flow cytometry in triplicates (Epics, Beckman Coulter, Fullerton, CA, USA) after appropriate color compensation. Data analysis was performed by the WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA, USA).

**Results:**

As₂O₃ induced a dose and time dependent inhibition of growth of MH7A cells (Figure 1A), with a 50% inhibitory concentration at about 5 μM. Flow cytometric analysis confirmed that inhibition of cellular growth was mediated by induction of apoptosis. MH7A cells were incubated with As₂O₃ (5 μM) for 0 – 24 hours, and stained with propidium iodide (PI) and annexin-V to distinguish between apoptotic cells and dead cells. In a representative assays, 13% of control cells were annexin-V positive, propidium iodide negative (apoptotic) and 2.36% were annexin-V positive, propidium iodide positive (dead), while 38.2% of treated cells were annexin-V positive, propidium iodide negative (apoptotic) and 5.76% were annexin-V positive, propidium iodide positive (dead). Results of the FACS analysis shows an increase in apoptotic cells after As₂O₃ treatment.
Western blot analysis showed that As$_2$O$_3$ induced a dose and time dependent activation of caspase 3. Inhibition of caspase 3 by the caspase inhibitor Z-VAD-FMK (25 μM) significantly reduced but did not totally ameliorate the cytotoxic effect of As$_2$O$_3$ (Figure 1B). The results implied that both caspase-dependent and caspase-independent pathways were involved in As$_2$O$_3$-induced cytotoxicity in MH7A.

**Example 2. Inhibition of MH7A cell proliferation by an IL-6 neutralizing antibody.**

**Materials and Methods:**

Reverse transcription polymerase chain reaction (RT-PCR) Total RNA was prepared using TRIzol (Invitrogen). Reverse transcription PCR (RT-PCR) was performed (Cheung, et al., *Cancer Lett.*, 246:122-8 (2007)). RNA was reverse transcribed with the Superscript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. Reaction mixes contained 1.2 μL of cDNA, 18 μL of Platinum® PCR Supermix (Invitrogen) and 200 nM of each pair of primers. Annealing temperatures and number of amplification cycles performed for each set of primers were listed in the following table.
### Table 1. Primers and conditions for RT-PCR

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**Immunoblot Analysis**

Immunoblot analysis was performed with gp130 ubiquitylation.

MH7A cells are incubated with 100 μM As₂O₃ for 0, 4 or 6 hours. Antibody to gp130 is used for immunoprecipitation (IP) of total cell lysates, followed by Western blot analysis with anti-ubiquitin antibodies FK1 and FK2. IP with non-immune rabbit serum (NIS) serves as control.
Quantitative RT-PCR

Quantitative-RT-PCR (Q-PCR) for GP130 was performed with the Assays-on-Demand™ Gene Expression System (AssayID: Hs00174360_m1, PE Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The reaction mix contained 2 μL of cDNA, 10 μL of TaqMan® Universal PCR Master Mix, 1 μL of Assay-on-demand™ Gene Expression Assay Mix, and RNase-free water to a volume of 20 μL. Q-PCR was performed with the ABI Prism 7700 Sequence Detector (PE Biosystems). Thermal cycling was initiated with an initial setup for 50°C for 2 minutes, followed by a first denaturation step at 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). GAPDH was used as an internal control for cDNA input. For GAPDH, amplification was performed with an initiation step of 2 minutes at 50°C, a first denaturation step of heating at 95°C for 10 minutes, and then 40 cycles of 95°C for 20 seconds (denaturation) and 62°C for 1 minute (annealing and extension). Real-time PCR amplification data were collected continuously and analyzed with the ABI Prism 7700 Sequence Detector. Relative gene expression to the control calibrator, and normalized to the internal control, was calculated by the ΔΔC_T method (ABI user bulletin number 2, PE Biosystem) (Cheung, et al., Cancer Lett., 246:122-8 (2007)). All experiments were performed in triplicates.

Results:

To investigate if MH7A proliferation might be IL-6 dependent, the effects of IL-6 on cellular proliferation were investigated. Exogenous IL-6 did not increase the proliferation of MH7A cells (Figure 2A). The effect of an IL-6 neutralizing antibody on cellular proliferation was then investigated. Interestingly, the results indicated that IL-6 neutralization led to significant inhibition of MH7A proliferation (Figure 2B). As2O3-induced gp130 ubiquitination is shown by FK2 that recognizes both mono- and poly-
ubiquitinated proteins, but not FK1 that recognizes poly-ubiquitinated proteins. These results demonstrate that gp130 is mono-ubiquitinated.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed that the IL-6 gene was actively transcribed in MH7A cells. These results suggest that an autocrine IL-6 loop might be involved in the proliferation of MH7A.

Example 3. \( \text{As}_2\text{O}_3 \) post-transcriptionally down-regulates gp130 of the IL-6 receptor complex.

Materials and Methods:

Materials and methods were as described in Examples 1-3, above.

Results:

To investigate if the inhibitory actions of \( \text{As}_2\text{O}_3 \) might be mediated via targeting of the IL-6 autocrine pathway, the effect of \( \text{As}_2\text{O}_3 \) on IL-6 transcription was first investigated. The results showed that \( \text{As}_2\text{O}_3 \) did not affect IL-6 gene transcription. The effects of \( \text{As}_2\text{O}_3 \) on the IL-6 receptor, comprising the two subunits IL-6 receptor \( \alpha \) and gp130, were then examined. Western blot analysis showed that both subunits were expressed. Treatment with \( \text{As}_2\text{O}_3 \) resulted in a dose and time dependent decrease of gp130, with IL-6 receptor \( \alpha \) remaining unchanged. Semi-quantitative RT-PCR showed that IL-6 receptor \( \alpha \) and gp130 were actively transcribed, and that treatment with \( \text{As}_2\text{O}_3 \) had no effect on the transcription of both genes. To further verify these results, real-time quantitative polymerase chain reaction for the \( GP130 \) gene was performed. The results confirmed that \( \text{As}_2\text{O}_3 \) treatment had no effect on \( GP130 \) gene transcription (Figure 2E). These findings implied that \( \text{As}_2\text{O}_3 \) targeted the IL-6 signaling pathway in MH7A, by down-regulation of the gp130 component of the IL-6 receptor complex at the post-transcriptional level.
Example 4. As$_2$O$_3$ induces degradation of gp130 through the lysosomal pathway.

Materials and Methods:
Materials and methods were as described in Examples 1-3, above.

Results:
The As$_2$O$_3$-mediated post-transcriptional down-regulation of gp130 suggested that degradation of gp130 might be enhanced. To address this issue, three pathways of protein degradation, lysosomal, proteasomal, and caspase-dependent proteolysis, were examined. Pre-incubation of MH7A cells with 2.5 mM NH$_4$Cl, a lysosome inhibitor, significantly prevented As$_2$O$_3$-induced decrease in gp130 (Figure 3A). On the other hand, pre-incubation with the proteasome inhibitor MG115 (10 μM) and the caspase inhibitor Z-VAD-FMK (25 μM) had no effect on As$_2$O$_3$-induced decrease of gp130. These findings suggested lysosomal degradation to be involved in As$_2$O$_3$-induced decrease of gp130.

Example 5. As$_2$O$_3$ induces ubiquitination of gp130.

Materials and Methods:

Immunoprecipitation

Immunoprecipitation was performed by standard procedures (Pang et al., Gastroenterology, 132:1088-103 (2007)). Cells were washed with ice-cold PBS supplemented with 1 mM sodium orthovanadate and Complete protease inhibitor cocktail (Complete; Roche Molecular Biochemicals), and lysed with buffer (50 mM Tris-HCl, pH: 7.5, 100 mM NaCl, 1% Triton X-100, 4 μg/mL aprotinin, 100 μM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 2 μg/mL leupeptin, 1 mM dithiothreitol, and 1X Complete) at 4°C for 15 minutes. Lysates were collected and centrifuged. Proteins were assayed (Bio-Rad Protein Assay Kit, Philadelphia, PA, USA), and adjusted to 1 μg/μL (typically 800 to 1000 μg). Immunoprecipitation was preformed by incubating protein samples with the appropriate antibodies (typically 4 μg) or control non-immune sera at 4°C overnight with gentle shaking. The antibody-protein complex was precipitated by incubation with 30 μL of rec-Protein G SEPHAROSE beads® (Invitrogen) at 4°C for 2
hours with gentle shaking. Protein G beads were then washed 3 times with 500 μL of ice-cold lysis buffer. The supernatant was aspirated, and 50 μL of 2X Laemmli buffer added. The antibody-protein complex was released from the beads by heating at 95°C for 10 minutes. The immunoprecipitates were then analyzed by Western blotting in triplicates.

Results:

The addition of mono-ubiquitin moieties to a protein enables it to be sorted to lysosomes where it is finally degraded. To investigate if As₂O₃ increased gp130 ubiquitination, cell lysates from MH7A cells before and after As₂O₃ treatment were immunoprecipitated with an anti-gp 130 antibody, followed by Western blot analysis with the antibodies FK1 that recognized poly-ubiquitinated proteins, and FK2 that recognized both mono-ubiquitinilated and poly-ubiquitinated proteins. The results showed that ubiquitinated gp130 was only detected (as a high molecular weight smear) in immunoblots with FK2, and not with FK1. Therefore, As₂O₃ induced mono-ubiquitination of gp130. Moreover, an increased amount of ubiquitinated gp130 was obtained with longer As₂O₃ treatment, suggesting a time-dependent mono-ubiquitination (Figure 3B). These findings further confirmed that As₂O₃ induced lysosomal degradation of gp130.

Example 6. As₂O₃ activation of the c-Jun-terminal-N kinase (JNK) is involved in suppression of IL-6 signaling.

Materials and Methods:

Materials and methods were as described in Examples 1-5, above.

Results:

One of the important IL-6 signaling cascades is the MAPK pathway, including activation of JNK (Heinrich, et al., Biochem. J., 374:1-20 (2003)). Interestingly, in other cellular systems, arsenic has also been shown to activate JNK (Davison, et al., Blood, 103:3496-502 (2004)). The possible involvement of JNK activation in As₂O₃-induced suppression of IL-6 signaling in MH7A cells was therefore investigated. As₂O₃ treatment resulted in significant increases in JNK phosphorylation and hence its activation (Figure 4A). Pre-treatment with the JNK inhibitor SP600125 (30
μM) prevented As$_2$O$_3$-induced increase in JNK phosphorylation. JNK activation was biologically relevant, as inhibition of As$_2$O$_3$-induced JNK activation significantly prevented As$_2$O$_3$-mediated suppression of MH7A cellular proliferation (Figure 4B).

To examine if JNK activation was related to suppression of IL-6 signaling, MH7A cells were treated with the lysosomal inhibitor NH$_4$Cl (2.5 mM), which had previously been shown to rescue gp130 from As$_2$O$_3$-induced suppression. In the presence of NH$_4$Cl, As$_2$O$_3$-induced JNK activation was almost totally abrogated (Figure 5A), suggesting that suppression of the IL-6 signaling pathway via degradation of gp130 was necessary for JNK activation. This point was directly demonstrated by treatment of MH7A cells with an IL-6 neutralizing antibody (0.5 μg/mL). As shown in Figure 5B, IL-6 neutralization induced JNK activation. These results indicated that disruption of IL-6 signaling was needed for JNK activation in MH7A cells.

**Example 7. As$_2$O$_3$-induced JNK activation might induce growth arrest via the p53 pathway.**

**Materials and Methods:**

**Cell cycle analysis**

Cell cycle analysis was performed as described (Pang et al., *J. Pathol.* 210:19-25, (2006)). Cells were trypsinized, washed twice with ice-cold PBS, re-suspended in 500 μL PBS, and stained with PI for 10 minutes on ice (DNA Prep™, Beckman Coulter). Cell cycle was determined by flow cytometry, and data analyzed by the WinMDI 2.8 software.
Results:
To investigate if the growth inhibitory effect of As$_2$O$_3$-induced JNK activation might be mediated through caspase-3, MH7A cells were treated with the JNK inhibitor SP600125 (30 µM) before As$_2$O$_3$ treatment. JNK inhibition did not affect As$_2$O$_3$-induced caspase-3 activation, suggesting that a caspase-independent pathway leading to growth inhibition might be involved in As$_2$O$_3$-induced JNK activation. This observation was consistent with the results shown in Figure 1. As JNK is known to phosphorylate p53, and phosphorylation of p53 is a critical step in inducing growth arrest (Wu, Cancer Biol. Ther., 3:156-61 (2004)), the effects of As$_2$O$_3$ on p53 phosphorylation were examined. As shown in Figure 5C, As$_2$O$_3$ treatment significantly increased p53 phosphorylation at serine 46, an important site for transactivation of pro-apoptotic genes (Wu, Cancer Biol. Ther., 3:156-61 (2004)). Inhibition of JNK activation with SP600125 significantly suppressed As$_2$O$_3$-mediated p53 phosphorylation, confirming p53 phosphorylation to be a down-stream effector of As$_2$O$_3$-induced JNK activation. Analysis of cell cycle showed that As$_2$O$_3$ treatment led to a significant G2M arrest (Figure 5D). Histograms were used to show the effect of treatment of MH7A cells with 5 µM As$_2$O$_3$ over 24 hours, as determined by cell labeling with PI and flow cytometry.

Example 8. As$_2$O$_3$ suppresses the JNK cross-talk partner nuclear factor-kappaB (NFkB).

Materials and Methods:
Materials and methods were as described in Examples 1-7, above.

Results:
Another important cross-talk partner of JNK is NFkB (Liu and Lin, Oncogene, 26:3267-78 (2007)). NFkB is also an important pro-inflammatory factor in arthritis (Simmonds, et al., Rheumatology, 47:584-90 (2008)). As shown in Figure 6, NFkB was constitutively expressed in MH7A. As$_2$O$_3$ treatment significantly decreased the level of NFkB in a dose (Figure 6A) and time (Figure 6B) dependent manner.
 Modifications and variations will be apparent to those skilled in the art and are intended to come within the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A method for treating or preventing one or more symptoms of rheumatoid arthritis and other inflammatory arthritis comprising administering to a subject in need thereof a composition comprising arsenic trioxide in an effective amount to reduce or treat one or more symptoms of rheumatoid arthritis.

2. A method for reducing or inhibiting the proliferation of synoviocytes or of promoting the apoptosis of synoviocytes comprising contacting the synoviocytes with an amount of arsenic trioxide effective to reduce or inhibit their proliferation or promote their apoptosis.

3. A method for reducing cell surface expression of the IL-6 receptor in a cell comprising contacting the cell with an amount of arsenic trioxide effective to promote the degradation of the IL-6 receptor by targeting it to the lysosome.

4. The method of claim 3, wherein the IL-6 receptor is expressed in a synoviocyte.

5. The method of claim 2 or 4, wherein the synoviocytes are in a patient with rheumatoid arthritis.

6. The method of claim 2 or 4, wherein the synoviocytes are in a patient with inflammatory arthritis.

7. The method of claim 5, wherein the arsenic trioxide is administered to the patient orally.

8. The method of claim 5, wherein the arsenic trioxide is administered locally to a synovial joint.

9. The method of claim 1, further comprising administering a steroidal or non-steroidal anti-inflammatory agent.

10. A pharmaceutical composition for treating one or more symptoms of rheumatoid arthritis or other inflammatory arthritis in a subject comprising an effective amount of arsenic trioxide to inhibit the proliferation or promote the apoptosis of synoviocytes, and, optionally, a pharmaceutically acceptable excipient.
11. The pharmaceutical composition of claim 10, wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for oral administration.

12. The pharmaceutical composition of claim 10, wherein the arsenic trioxide is in a pharmaceutically acceptable carrier for local administration to a synovial joint.

13. The pharmaceutical composition of any of claims 10-12, wherein the arsenic trioxide is present in an amount from 1 to 10 mg.

14. The pharmaceutical composition of claim 13, wherein the arsenic trioxide is present in an amount from 5 to 10 mg.

15. The pharmaceutical composition of claim 10 in a unit dosage form for oral administration, wherein the unit dosage form is selected from the group consisting of solutions, suspensions, emulsions, syrups, tablets and capsules.

16. Use of arsenic trioxide in preparation of a medicament for treating or preventing one or more symptoms of rheumatoid arthritis and other inflammatory arthritis.

17. Use of arsenic trioxide in preparation of a medicament for reducing or inhibiting the proliferation of synoviocytes or of promoting the apoptosis of synoviocytes.

18. Use of arsenic trioxide in preparation of a medicament for reducing cell surface expression of the IL-6 receptor in a cell.

19. The use of claim 18, wherein the IL-6 receptor is expressed in a synoviocyte.

20. The use of claim 17 or 19, wherein the synoviocytes are in a patient with rheumatoid arthritis.

21. The use of claim 17 or 19, wherein the synoviocytes are in a patient with inflammatory arthritis.

22. The use of claim 20, wherein the arsenic trioxide is administered to the patient orally.

23. The use of claim 20, wherein the arsenic trioxide is administered locally to a synovial joint.
24. The use of claim 16, further comprising administering a steroidal or non-steroidal anti-inflammatory agent.
Figure 1A

Figure 1B
Figure 1C

Figure 2A
Figure 2D

Figure 2E
Figure 3A

Figure 3B
Figure 5C

Figure 6A
Figure 6B
## INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC: A61K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI; EPODOC; CNPAT; CNKI; BIOSIS; MEDLINE and keywords: rheumatoid arthritis, inflammatory arthritis, arsenic trioxide, synoviocyte?, IL-6, interleukin-6, receptor?, synovium, gp130 etc.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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* Further documents are listed in the continuation of Box C.  
* See patent family annex.

**Date of the actual completion of the international search**
20 Jul. 2010 (20.07.2010)

**Date of mailing of the international search report**
26 Aug. 2010 (26.08.2010)

Name and mailing address of the ISA/CN  
The State Intellectual Property Office, the P.R. China  
6 Xitucheng Rd., Jiemian Bridge, Haidian District, Beijing, China 100088  
Facsimile No. 86-10-62019451

Form PCT/ISA/210 (second sheet) (July 2009)

Authorized officer  
WANG Boli

Telephone No. (86-10) 82245036
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<td>CHEUNG, W.C. et al., Arsenic trioxide targets the interleukin-6 cascade in multiple myeloma by promoting lysosomal degradation of the interleukin-6 receptor complex, Blood, 2005, vol. 106, No.11, pages 373B-373B</td>
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<td>HAN, F. et al., The effects of As₂O₃ on the apoptosis of synoviocytes of rheumatoid arthritis—a report of 18 cases, New Medicine, February 2009, vol. 40, No.2, pages 80-82</td>
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### INTERNATIONAL SEARCH REPORT

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **☒** Claims Nos.: 1-9 because they relate to subject matter not required to be searched by this Authority, namely: Although claims 1-9 involve methods for treatment of diseases of the human or animal body (see Article 17(2) (a) (i) and Rules 39.1(iv)), the search has been carried out and based on the alleged effects of the compounds/compositions.

2. **☐** Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **☐** Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. **☐** As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. **☐** As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of additional fee.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on protest**

**☐** The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

**☐** The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

**☐** No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)
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Continuation of: CLASSIFICATION OF SUBJECT MATTER

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A61P 19/02 (2006.01) i
A61P 29/00 (2006.01) i