DISEASE MODIFYING ANTI-ARTHRITIC ACTIVITY OF 2-METHOXYESTRADIOL
KRANKHEITSMODIFIZIERENDE ANTIARTHRITISCHE WIRKUNG VON 2-METHOXYESTRADIOL
2-MÉTHOXYESTRADIOL PRÉSENTANT UNE ACTIVITÉ ANTI-ARTHRITIQUE POUVANT MODIFIER L’ÉVOLUTION DE LA MALADIE

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References cited:

Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

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The present invention relates to compositions comprising 2-methoxyestradiol, in combination with methotrexate. More particularly, the present invention relates to the use of the combination for treating rheumatoid arthritis and related rheumatic diseases.

BACKGROUND OF THE INVENTION

Rheumatoid arthritis (RA) is a rheumatic disease characterized by persistent synovial tissue inflammation. In time, this persistent inflammation can lead to bone erosion, destruction of cartilage, and complete loss of joint integrity. Eventually, multiple organs may be affected (Rindfleish et al. American Family Physician (2005), 72(6):103746). Joint damage is initiated by proliferation of synovial macrophages and fibroblasts after a triggering incident, possibly autoimmune or infectious. This is followed by infiltration of the perivascular regions by lymphocytes and endothelial cell proliferation. Over time, inflamed synovial tissue begins to grow irregularly, forming invasive pannus tissue. The pannus invades and destroys cartilage and bone. Multiple cytokines, interleukins, proteinases, and growth factors are released causing further joint destruction and the development of systemic conditions (Ruddy et al. eds. Kelly’s Textbook of Rheumatology. 7th ed. Philadelphia: W.B. Saunders, 2005:996-1042). Destruction of joints can begin within a few weeks of symptom onset. Early diagnosis is imperative as early treatment is effective in slowing disease progression. However, there are currently no diagnostic tests that can conclusively confirm rheumatoid arthritis.

The management of rheumatoid arthritis typically consists of medication and non-medication based treatments. Treatments aimed at reversing the course of the disease have so far been largely unsuccessful. Instead, therapeutic goals typically focus on preservation of function and quality of life, minimization of pain and inflammation, joint protection, and control of systemic complications (Harris, (2005) and American College of Rheumatology Subcommittee on Rheumatoid Arthritis Guidelines, Arthritis Rheum (2002), 46:328-46). A typical treatment regimen includes administration of nonsteroidal anti-inflammatory drugs (NSAIDs) for control of pain, with selective use of oral and intra-articular glucocorticosteroids, and initiation of one or more disease-modifying anti-rheumatic drugs (DMARDs). DMARDs commonly used include methotrexate, hydroxychloroquine, sulfasalazine, and leflunomide. In a recent reversal of therapeutic paradigms, early and aggressive treatment with one or more DMARDs is now favored. While this more intensive regime has shown promise when treated early, only a fraction of patients achieve the ideal goal of halted progression and/or elimination of clinical activity (Machold et al., Arthritis Research & Therapy (2006), 8:1-6). A number of new biologics are also available for treating rheumatoid arthritis including infliximab (Remicade®), a chimeric tumor necrosis factor alpha (TNF-α) specific antibody; etanercept (Enbrel®), a soluble dimerized human p72 receptor/Fc fusion protein that competitively binds TNF-α; and anakinra, an interleukin-1 receptor blocker. While this new class of anti-rheumatic drugs has shown promise as a substitute or complementary form of treatment, infectious complications have been observed following treatment (Imaizumi et al., Intern. Med. (2006), 48(10):685-88).

There is an increasing appreciation for the role that angiogenesis plays in RA initiation and progression (Koch, Ann Rheum Dis (2000), 59(Suppl. I):i65-i71 and Veale et al., Best Practice & Research Clinical Rheumatology (2006), 20(5):941-47). Chronic inflammation and angiogenesis are codependent, with the proliferation, migration and recruitment of tissue and inflammatory cells capable, through direct and indirect means, of stimulating angiogenesis. Likewise, angiogenesis contributes to inflammatory pathology through the creation of new blood vessels that sustain the chronic inflammatory state by transporting inflammatory cells and supplying nutrients and oxygen to the inflamed tissue (Jackson et al., FASEB Journal, (1997), 11:457-65). Several angiogenic inducers have been identified as having a role in RA,

\[ \text{[0005]} \quad \]
including FGF2; VEGF; TGFβ; TNFα; chemokines, such as IL8, IL18, and IL1; soluble adhesion molecules, such as E-selectin and soluble VCAM-1; glycoconjugates, such as the soluble 4A11 antigen, soluble CD 146 and the angioipoetine-Tie system (Koch, Ann. Rheum. Dis. (2003), 62(Suppl. II):i60-i67).

[0007] Joints affected by RA have been shown to be hypoxic. Contributing factors to hypoxia in RA joints include the high metabolic demand of inflamed synovial tissue and the rapid rate of synovial proliferation which quickly outgrows the supporting vasculature (Taylor et al., Current Opinion in Rheumatology (2005), 17:293-98). Tissue hypoxia in a rheumatoid joint results in increased VEGF mRNA stability and enhanced VEGF gene expression through the binding of hypoxia inducible factor-1 (HIF-1) (Richard et al., Biochem Biophys Res Commun. (1999), 266:718-22). HIF-1, which is made up of HIF-1α and hydroxycarbon nuclear transclocator (ARNT), controls many transcription responses to hypoxia by binding the hypoxia response elements in target genes like the VEGF gene (Jones et al., Cancer J Sci Am. (1998), 4:209-17). HIF-1 is overexpressed in the synovial lining and stromal cells of RA patients relative to synovial tissues from individuals without RA (Hollander et al., Arthritis Rheum. (2001), 44:1540-44 and Giatromanolaki et al., Arthritis Res Ther. (2003), 5:R193-R201). VEGF is also intimately linked with the processes of immune regulation as a number of cytokines and growth factors regulate its expression in different cell types including interleukin 1β, TGFβ, FGF-2, and TNFα. Studies have shown a synergistic interaction between growth factors and hypoxia in VEGF induction (Brenchley, Ann Rheu Dis (2001), 60:iii71-iii74).

[0008] Several compounds have been used to inhibit angiogenesis. One such compound is 2-methoxyestradiol (2ME2). 2ME2 is a naturally occurring derivative of estradiol and has been shown to be an orally active, well-tolerated, small molecule that possess anti-proliferative and anti-angiogenic activity (Pribluda et al., Cancer Metastasis Rev. (2000), 19(1-2):173-9). 2ME2 has low affinity for estrogen receptors, α and β, and its anti-proliferative activity is independent of the interaction with those receptors (LaVallee et al. Cancer Research (2002), 62(13):3691-7). Several mechanisms have been proposed for 2ME2 activity, including those mediated by its ability to bind to the colchicines binding site of tubulin (Cushman et al., 1995; D’Amato et al., 1994), destabilization of microtubules and inhibition of HIF-1α nuclear accumulation (Mabjeesh et al., Cancer Cell, (2003) 3:363-75), induction of the extrinsic apoptotic pathway through upregulation of Death Receptor 5 (LaVallee et a1., Cancer Research (2003),63(2): 469-75) and induction of the intrinsic apoptotic pathway, potentially through the inhibition of superoxide dismutase enzymatic activity (Huang et al., Trends Cell Biology (2001), 11 (8):343-8).

[0009] US2005/0148496 describes that the symptoms of an inflammatory disease, such as rheumatoid arthritis, may be alleviated by administering a compound that inhibits the activity of H1F-1α.

[0010] What is needed are methods and compositions capable of stopping progression and/or reversing the progression of both early and late stage rheumatic diseases without unwanted or undesirable complications or side effects.

SUMMARY OF THE INVENTION

[0011] The present invention provides compositions for treating rheumatic diseases. The compositions comprises 2-methoxyestradiol, in combination with methotrexate. 2-Methoxyestradiol is a powerful antiangiogenic agent and has the ability to enhance the effects of other anti-rheumatic agents through its own anti-angiogenic and anti-proliferative capabilities.

[0012] Another disclosed embodiment comprises the combination of a compound having the formula

\[
\text{R} = -\text{OCH}_3
\]

and methotrexate for use in treating rheumatic diseases.

[0013] Another embodiment comprises a compound for use in treating rheumatic diseases. The composition comprise a compound having the formula
wherein \( R_a \) is selected from \(-\text{OCH}_3\) and methotrexate

Accordingly, it is an object of the present invention to provide an improved combination and composition for use in treating rheumatic diseases.

Another object of the present invention is to provide an improved combination and composition for use in treating rheumatoid arthritis.

A further object of the present invention is to provide a combination and composition for use in treating rheumatic diseases which has few or no unwanted or undesirable side effects.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of disclosed embodiments and the appended drawing and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph which shows that administration of 2ME2 decreases the arthritic score in the Athrogen-CIA model of RA.

Fig. 2 is two photographs of a control and 100 mg/kg treatment which show administration of 2ME2 inhibits synovial inflammation in the Athrogen-CIA model of RA.

Fig. 3 is two photographs of a control and 100 mg/kg treatment which show the inhibition of periarticular inflammatory infiltrate and fibrosis by 2ME2 in the Athrogen-CIA model of RA.

Fig. 4 is two photographs of a control and 100 mg/kg treatment which show that 2ME2 prevents loss of proteoglycan in articular cartilage and induction of osteoclast activity.

Fig. 5 is a series of graphs which show that administration of 2ME2 can inhibit the severity of RA progression in the Athrogen-CIA model of RA.

Fig. 6 is a table which show the assessment of histomorphometric alterations following 2ME2 treatment in the Athrogen-CIA model of RA.

Fig. 7 is a graph and a table which show that administration of 2ME2 in combination with methotrexate results in an unexpected increase in percent inhibition of arthritic score of Athrogen-CIA model of RA.

Fig. 8 is two photographs which show radiographic evidence of 2ME2’s anti-rheumatic effect in Arthrogen-CIA model of RA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises a combination and compositions for use in treating rheumatic diseases. In a disclosed embodiment of the present invention, the composition comprises an anti-angiogenic and anti-proliferative agent in combination with methotrexate. The anti-angiogenic and anti-proliferative agent is 2ME2, and derivatives thereof, as shown in Formula I below.

2-Methoxyestradiol

The process or processes by which 2ME2 exhibits its anti-proliferative and anti-angiogenic activities remains unclear, however, a number of studies have implicated various mechanisms of action and cellular targets. 2ME2 induced changes in the levels and activities of various proteins involved in the progression of the cell cycle. These include cofactors of DNA replication and repair, e.g., proliferating cell nuclear antigen (PCNA) (Klauber, N., Parangi, S., Flynn, E., Hamel, E. and D’Amato, R.J. "Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and Taxol," Cancer Research, (1997) 57:81-86; Lottering, M-L., de Kock, M., Viljoen, T.C., Grobler, C.J.S. and Seegers, J.C. "17β-Estradiol metabolites affect some regulators of the MCF-7 cell cycle," Cancer Letters,)

[0021] The anti-angiogenic and anti-proliferative portion of the composition according to the disclosed embodiment of the present invention comprises a compound of Formula I:

\[
\begin{align*}
\text{wherein } R_a \text{ is selected from } -\text{OCH}_3. \text{ In cases where stereoisomers are possible, both } R \text{ and } S \text{ stereoisomers are envisioned, as well as any mixture of stereoisomers.}
\end{align*}
\]

Anti-Rheumatic Agents

[0022] The anti-rheumatic agent used in the present invention is methotrexate.
In accordance with the present invention, the compounds of Formula I may be mixed with methotrexate into a single formulation. The compounds of Formula I and methotrexate may also be formulated and delivered separately.

The compositions described herein can be provided as physiologically acceptable formulations using known techniques, and the formulations can be administered by standard routes. In general, compounds of Formula I and methotrexate can be administered by topical, oral, rectal or parenteral (e.g., intravenous, subcutaneous or intramuscular) route. In addition, the compositions can be incorporated into polymers allowing for sustained release, the polymers being implanted in the vicinity of where delivery is desired, for example, at the site of inflammation or within or near an affected joint, or the polymers can be implanted, for example, subcutaneously or intramuscularly or delivered intravenously or intraperitoneally to result in systemic delivery of compounds of Formula I and/or methotrexate. Other formulations for controlled, prolonged release of therapeutic agents useful in the present invention are disclosed in U.S. Patent No. 6,706,289.

The formulations in accordance with the present invention can be administered in the form of a tablet, a capsule, a lozenge, a cachet, a solution, a suspension, an emulsion, a powder, an aerosol, a suppository, a spray, a pastille, an ointment, a cream, a paste, a foam, a gel, a tampon, a pessary, a granule, a bolus, a mouthwash, or a transdermal patch.

The formulations include those suitable for oral, rectal, nasal, inhalation, topical (including dermal, transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intraocular, intratracheal, and epidural) or inhalation administration. The formulations can conveniently be presented in unit dosage form and can be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and a pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil emulsion, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding, in a suitable machine, a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide a slow or controlled release of the active ingredient therein.

Formulations suitable for topical administration in the mouth include lozenges comprising the ingredients in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Formulations suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising the ingredient to be administered in a pharmaceutical acceptable carrier. In one embodiment the topical delivery system is a transdermal patch containing the ingredient to be administered.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of 20 to 500 microns which is administered in the manner in which snuff is taken; i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations, wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing, in addition to the active ingredient, ingredients such as carriers as are known in the art to be appropriate.

Formulation suitable for inhalation may be presented as mists, dusts, powders or spray formulations containing, in addition to the active ingredient, ingredients such as carriers as are known in the art to be appropriate.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Formulations suitable for parenteral administration also include, but are not limited to, nanoparticle formulations made by numerous methods as disclosed in U.S. Patent Application No. 10/392,403 (Publication No. US 2004/0033267), U.S. Patent Application No. 10/412,669 (Publication No. US 2003/0219490), U.S. Patent No. 5,494,693, 2004/0033267, U.S. Patent Application No. 10/412,669 (Publication No. US 2003/0219490), U.S. Patent No. 5,494,693,
By forming 2-methoxyestradiol nanoparticles, the compositions disclosed herein are shown to have increased bioavailability. Preferably, the particles are comprised of the compounds of Formula I and/or methotrexate alone or in combination, with accessory ingredients or in a polymer for sustained release. The particles of the compounds of the present invention have an effective average particle size of less than about 2 microns, less than about 1900 nm, less than about 1800 nm, less than about 1700 nm, less than about 1600 nm, less than about 1500 nm, less than about 1400 nm, less than about 1300 nm, less than about 1200 nm, less than about 1100 nm, less than about 1000 nm, less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 250 nm, less than about 200 nm, less than about 150 nm, less than about 100 nm, less than about 75 nm, or less than about 50 nm, as measured by light-scattering methods, microscopy, or other appropriate methods well known to those of ordinary skill in the art. It is understood that the particle sizes are average particle sizes and the actual particle sizes will vary in any particular formulation. Often, surface stabilizers are used to form stable nanoparticles; however, this method of forming nanoparticles is only one of many different methods of forming effective nanoparticle compositions. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in freeze-dried (lyophilized) conditions requiring only the addition of a sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kinds previously described.

In one embodiment, the compounds of Formula I and methotrexate can be administered simultaneously. In another embodiment, they can be administered separately (i.e.; compounds of Formula I dosage in the morning, methotrexate dosage in the evening).

If the 2-methoxyestradiol formulation and the methotrexate are to be administered sequentially, the amount of time between administration of the 2-methoxyestradiol formulation and the methotrexate will depend upon factors such as the amount of time it take the 2-methoxyestradiol formulation to be fully incorporated into the circulatory system of the host and the retention time of the 2-methoxyestradiol formulation in the host's body. In one embodiment, dosage formulations for 2-methoxyestradiol are disclosed in U.S. Patent Application Serial No. 11/288,989, filed November 29, 2005. The methotrexate is administered in a therapeutically effective amount. This amount will be determined on an individual basis and will be based, at least in part, on consideration of the host's size, the specific disease to be treated, the severity of the symptoms to be treated, the results sought, and other such considerations. An effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

It should be understood that, in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents, and nanoparticle formulations (e.g.; less than 2000 nanometers, preferably less than 1000 nanometers, most preferably less than 500 nanometers in average cross section) may include one or more than one excipient chosen to prevent particle agglomeration.

Pharmaceutical Preparations

Also contemplated by the present invention are implants or other devices comprised of the formulation in accordance with the disclosed embodiments, or prodrugs thereof, or other compounds included by reference where the drug or prodrug is formulated in a biodegradable or non-biodegradable polymer for sustained release. Non-biodegradable polymers release the drug in a controlled fashion through physical or mechanical processes without the polymer itself being degraded. Biodegradable polymers are designed to gradually be hydrolyzed or solubilized by natural processes in the body, allowing gradual release of the admixed drug or prodrug. The drug or prodrug can be chemically linked to the polymer or can be incorporated into the polymer by admixture. Both biodegradable and non-biodegradable polymers and the process by which drugs are incorporated into the polymers for controlled release are well known to those skilled in the art. Examples of such polymers can be found in many references, such as Brem et al., J. Neurosurg 74: pp. 441-446 (1991). These implants or devices can be implanted in the vicinity where delivery is desired, for example, at the site of a tumor or a stenosis, or can be introduced so as to result in systemic delivery of the agent.

Because anything not formed in the body as a natural component may elicit extreme and unexpected responses, such as blood vessel closure due to thrombus formation or spasm, and because damage to blood vessels by the act of insertion of a vascular stent may be extreme and unduly injurious to the blood vessel surface, it is prudent to protect against such events. Restenosis is a re-narrowing or blockage of an artery at the same site where treatment, such as
an angioplasty or stent procedure, has already taken place. If restenosis occurs within a stent that has been placed in
an artery, it is technically called "in-stent restenosis," the end result being a narrowing in the artery caused by a build-
up of substances that may eventually block the flow of blood. The compounds that are part of the present invention are
especially useful to coat vascular stents to prevent restenosis. The coating should preferably be a biodegradable or non-
biodegradable polymer that allows for a slow release of a compound of the present invention thereby preventing the
restenosis event.

[0044] The invention also relates to compositions including the prodrugs of the present invention.
[0045] In one aspect, the present invention provides a conjugated prodrug of an estradiol compound, preferably
compounds of Formula I, conjugated to a biological activity modifying agent.
[0046] Alternatively, the conjugated prodrug according to the present invention includes the compounds of Formula
I, conjugated to a peptide moiety.
[0047] The incorporation of an estradiol compound, such as the compounds of Formula I, into a disease-dependently
activated pro-drug enables significant improvement of potency and selectivity of this anti-angiogenic agent.
[0048] A person skilled in the art will be able by reference to standard texts, such as Remington’s Pharmaceutical
Sciences 17th edition, to determine how the formulations are to be made and how these may be administered.
[0049] In a further aspect of the present invention there is provided use of compounds of Formula I in combination
with an anti-rheumatic agent according to the present invention for the preparation of a medicament for the prophylaxis
or treatment of rheumatic diseases.
[0050] Pharmaceutically acceptable salts of the compounds of the Formula I, can be prepared in any conventional
manner, for example from the free base and acid. In vivo hydrolysable esters, amides and carbamates and other
acceptable prodrugs of Formula I can be prepared in any conventional manner.
[0051] 100% pure isomers are contemplated by this invention; however a stereochemical isomer (labeled as α or β,
or as R or S) may be a mixture of both in any ratio, where it is chemically possible by one skilled in the art. Also
contemplated by this invention are both classical and non-classical bioisosteric atom and substituent replacements,
such as are described by Patani and Lavoie ("Bio-isosterism: a rational approach in drug design" Chem. Rev. (1996) p.
3147-3176) and are well known to one skilled in the art. Such bioisosteric replacements include, for example, but are
not limited to, substitution of =S or =NH for =O.
[0052] A particularly useful formulation in the present invention is a nanoparticulate liquid suspension of 2-methox-
yestradiol disclosed in U.S. Patent Application Serial No. 10/392,403, filed March 20, 2003. This formulation is available
from EntreMed, Inc., Rockville, Maryland, under the designation Panzem® NCD.
[0053] Known compounds that are used in accordance with the invention and precursors to novel compounds according
to the invention can be purchased, e.g., from Sigma Chemical Co., Steraloids or Research Plus. Other compounds
according to the invention can be synthesized according to known methods from publicly available precursors.

EXAMPLES

[0054] The data present in the Examples and the following table indicates that 2ME2 can be used in combination with
a wide range of anti-rheumatic agents. The characteristics of 2ME2 and compounds of Formula I are such that they can
be combined with anti-rheumatic agents at the maximally tolerated or maximally effective dose and schedule of the anti-
rheumatic agent. In some embodiments, combination with 2ME2 can be used to maintain the effectiveness while reducing
the dose of the anti-rheumatic agent. Such reduction in dose can result in reduction of toxicity or reduction in any
unacceptable effect or side-effect of the anti-rheumatic agent.
<table>
<thead>
<tr>
<th>Study Number</th>
<th>Dose, Schedule, Route</th>
<th>Mode of Intervention</th>
<th>Endpoint(s) Measured</th>
<th>Main Findings</th>
</tr>
</thead>
</table>
| EntreMed SMP04-040 Mouse (Balb/C), Female, n=10/group Arthrogen Collagen MAB-Induced Arthritis | 1, 10, 100 mg/kg/d; Vehicle control PO | Prevention | • Footpad swelling  
• Clinical arthritic score  
• Histopathology & histomorphometric analysis of joint  
• Immunohistochemistry | • Dose-dependent inhibition of clinical arthritic score, inflammation, cartilage degradation, bone resorption, and pannus formation  
• Inhibition of angiogenesis |
| EntreMed SMP05-010 Mouse (Balb/C), Female n=10/group Arthrogen Collagen MAB-Induced Arthritis | 10, 25, 50, 75, 100 mg/kg/d; Vehicle control PO | Prevention | • Clinical arthritic score  
• Histopathology & histomorphometric analysis of joint  
• PK | • Dose-dependent inhibition of clinical arthritic score, inflammation, cartilage degradation, bone resorption, and pannus formation  
• Steady-state AUC$_{0.24}$ for 25, 50, and 75mg/kg were 66, 117, and 301 ng•hr/mL, respectively |
| EntreMed SMP05-009 Mouse (Balb/C), Female, n=10/group Arthrogen Collagen MAB-induced Arthritis | 100 mg/kg/d, 100 mg/kg/week, 100 mg/kg twice a week; Vehicle control PO | Prevention | • Clinical arthritic score  
• Histopathology & histomorphometric analysis of joint | • Regimen-dependent inhibition of clinical arthritic score, inflammation, cartilage degradation, bone resorption, and pannus formation |
| EntreMed SMP05-046 Mouse (Balb/C), Female, n=10/group Arthrogen Collagen MAB-Induced Arthritis | 2ME2 (10 or 100 mg/kg/d) PO MTX (0.1 or 1 mg/kg/d) IP; Vehicle control | Prevention | • Clinical arthritic score  
• Liver histopathology | • Additive inhibition of clinical arthritic score upon combination of the two drugs  
• No pathology in the liver with either drug alone or in combination |
<table>
<thead>
<tr>
<th>Study Number</th>
<th>Dose, Schedule, Route</th>
<th>Mode of Intervention</th>
<th>Endpoint(s) Measured</th>
<th>Main Findings</th>
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<tr>
<td>Rat (Louvain) Female, n=12/group Collagen-Induced Arthritis</td>
<td>Prevention: 30, 100 mg/kg/d; Vehicle control PO Treatment: 10, 30, 100 mg/kg/d; Vehicle control PO</td>
<td>Prevention&lt;sup&gt;a&lt;/sup&gt;&amp; Treatment&lt;sup&gt;b&lt;/sup&gt;</td>
<td>• Clinical arthritis score • Radiographic score</td>
<td>• Dose dependent inhibition of clinical arthritis score and bone erosion • Delays onset of disease</td>
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<td>UCLA Ernie Brahn Study #2</td>
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<tr>
<td>Rat (Louvain) Female, n=12/group Collagen-Induced Arthritis</td>
<td>50 mg/kg BID, 100 or 300 mg/kg/d; Vehicle control PO OR 60 mg/kg/d SC osmotic pumps; Vehicle control SC osmotic pumps</td>
<td>Treatment&lt;sup&gt;b&lt;/sup&gt;</td>
<td>• Clinical arthritis score • Radiographic score • Histopathology &amp; histomorphometric analysis of joint • Immunohistochemistry</td>
<td>• Dose dependent inhibition of clinical arthritis score, inflammation, and bone erosion, cartilage degradation and pannus formation. • Inhibition of angiogenesis</td>
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<tr>
<td>Rat (Lewis) Male, n=5-9 Adjuvant-Induced Arthritis</td>
<td>30, 100 mg/kg/d; Vehicle control (1.2% HPC, 0.06% DOSS in sterile water) PO</td>
<td>Prevention&lt;sup&gt;c&lt;/sup&gt; &amp; Treatment&lt;sup&gt;b&lt;/sup&gt;</td>
<td>• Clinical arthritis score • Histopathology of joint • Histological evaluation of spleen, liver, lung, lymph nodes • Immune cell migration</td>
<td>• Inhibition of clinical arthritis score and cartilage degradation at both doses and regimens • Decreased splenic abscess formation, giant cells, and lymphoid hyperplasia • Blocked immune cell migration to the joint</td>
</tr>
<tr>
<td>Dalhousie University Andrew Issekutz Study #2</td>
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<tr>
<td>Rat (Lewis) Male, n=5 Adjuvant-Induced Arthritis (Adoptive Transfer Model)</td>
<td>3, 30 mg/kg/d; Vehicle control (1.2% HPC, 0.06% DOSS in sterile water) SC</td>
<td>Prevention&lt;sup&gt;c&lt;/sup&gt; &amp; Treatment&lt;sup&gt;b&lt;/sup&gt;</td>
<td>• Clinical arthritis score • Histopathology of joint • Immune cell migration • Lymphocyte proliferation</td>
<td>• Dose dependent inhibition of clinical arthritis score, inflammation, and bone destruction • Inhibition of cellular infiltration to the joints • Inhibition of migration of PMNs and splenocytes to dermal sites only in response to TNFα and PPD</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates treatment started 1 day following the induction of arthritis; clinical signs of arthritis are not evident
<sup>b</sup> Indicates treatment started 10 days following the induction of arthritis, clinical signs of arthritis are present
<sup>c</sup> Indicates treatment started 6 days following the induction of arthritis; clinical signs of arthritis are not evident
Effect of 2ME2 Treatment of Clinical Arthritis Severity

Example 1

Effect of 2ME2 Treatment of Clinical Arthritis Severity

Example 2

Effect of 2ME2 treatment on neutrophil migration to arthritic joints and dermal inflammatory reactions

Example 2

Effect of 2ME2 Treatment of Clinical Arthritis Severity

Example 1

Effect of 2ME2 Treatment of Clinical Arthritis Severity

Example 2

Effect of 2ME2 treatment on neutrophil migration to arthritic joints and dermal inflammatory reactions

Example 2

Effect of 2ME2 Treatment of Clinical Arthritis Severity
mulated isotope in the tissue was expressed as cpm/10^6 cpm injected i.v.

[0062] We have previously shown that radiolabeled blood PMNL migration to the joints of rats with AA develops rapidly during the second week post-immunization and peaks by day 14-15, this being most intense in the hind limb joints, i.e., the talar and metatarsal joints, with lesser accumulation in the forelimb carpal and metacarpal joints (Issekutz et al. (1991) 2ME2 treatment initiated during the preclinical phase at day 6, at either 100 or 30 mg/kg/d, significantly decreased the accumulation of ^51^Cr-labeled PMNL in the most intensely involved joints of the hind limbs, and to a lesser degree and somewhat more variably in the forelimb joints. However, although there was a tendency for a decrease in the group receiving delayed 2ME2 treatment starting on day 10, when arthritis had clinically developed, this did not reach significance.

[0063] In the same rats, the migration of PMNL to dermal inflammatory reactions was measured, these reactions being initiated at the time of radiolabeled PMNL injection. Thus the migration quantitated the acute reaction during the first two hours of the lesions. 2ME2 treatment did not affect PMNL migration to the complement chemotactic factor C5a_des-arg or to E. coli LPS. In contrast, the PMNL migration to sites injected with rat TNF-α was inhibited by approximately 50% in rats that were treated with 2ME2 at either dose tested, starting at day 6 post-immunization. There was a tendency for inhibition by 2ME2 in the group in which treatment was delayed to day 10, but this did not reach significance. It should be noted that rats with AA develop a leukocytosis with marked neutrophilia (Taurog (1988) and Issekutz (1991) This response was not affected by 2ME2 treatment initiated either on day 6 or day 10 at either dose tested. Thus the observed effects could not be attributed to differences in blood PMNL counts. Furthermore, there was no difference between any of the groups in the level of radiolabeled ^51^Cr PMNL in the circulation during the 2-h migration period.

Example 3

Effect of 2ME2 treatment on joint histology and cartilage damage

[0064] Tissue samples, including joints, were fixed in 10% phosphate-buffered formalin or AFA fixative (75% ethyl alcohol, 2% formalin, 5% glacial acetic acid, 20% water), the latter for immunostaining. Samples were then decalcified in formic or acetic acid and paraffin embedded. Sections (5 μm) were stained with hematoxylin-eosin (H&E) using routine techniques. To stain cartilage proteoglycan, separate sections were stained with safranin O (Difco, Detroit, MI) for 1 min. as described previously (Issekutz, (2001). To assess microvessel density in the synovium, rabbit anti-mouse laminin polyclonal IgG antibody (Cedarlane Laboratories Limited, Hornby, ON) was used to outline vessels, with detection using biotinylated goat anti-rabbit IgG antibody and avidin-biotin complexed with HRPO according to manufacturer’s recommendations ( Vectastain Elite ABC kit; Vector Laboratories Canada Inc., Burlington, ON).

[0065] Joints from a vehicle-treated rat showed that the synovium is markedly thickened and intensely infiltrated by leukocytes with marked synovial expansion. Even by the H&E staining the cartilage damage is visible on the articular surfaces and margins. This is more profoundly illustrated by the safranin O staining with marked focal proteoglycan loss and fragmentation. 2ME2-treated rats that received 100 mg/kg/d initiated at day 6 post-immunization, showed less intense leukocyte infiltration of the synovium when compared to vehicle treated, although clearly synovial infiltration and expansion have occurred. However, most notable is the preservation of articular cartilage surfaces and proteoglycan staining. Similar changes were observed in rats treated with 30 mg/kg/d from day 6, but in rats in which 2ME2 treatment was delayed to day 10, the leukocyte cellularity in the synovium was less noticeably affected. Nevertheless, cartilage preservation was still observed.

Example 4

Effect of 2ME2 treatment on synovial vascularity

[0066] To assess synovial vascularity and angiogenesis, sections were immunostained for laminin to reliably delineate microvessels as well as medium-sized vessels. This technique was found to be more reliable than staining for von Willebrand factor (factor VIII related antigen), since it was observed that staining for von Willebrand factor was diminished or absent in areas where there was intense leukocyte infiltration, even though microvessels were obviously present. The other commonly used endothelial cell marker, CD31, was also evaluated on the synovial tissue. Although a monoclonal antibody to rat CD31 clearly stained vascular endothelium, CD31 was also present on infiltrating leukocytes, which complicated the interpretation. The antibody to mouse laminin used here specifically stained vessels of capillary size and larger, presumably reacting with the basement membrane laminin. The immunohistochemistry of synovium from a vehicle-treated rat, revealing the intense vascularity of the leukocyte-infiltrated synovium. The vascularity in the synovium of a rat treated with 100 mg/kg/d of 2ME2 starting on day 6 post-immunization showed less intense leukocyte infiltrate in the synovium, but synovial vascularity is still prominent. This vascularity was quantitated in synovia from rats treated with the various protocols and compared to vehicle-treated animals. The synovia were examined for small (1-2 RBC
Example 5

Effect of 2ME2 treatment on endothelial cell proliferation under minimal and optimal growth conditions

[0067] Because of the lack of a clear effect of 2ME2 therapy on synovial angiogenesis, the effect of 2ME2 on endothelial cell proliferation in vitro under optimal endothelial cell growth stimulatory conditions, likely to be present in vivo in the synovium during arthritis, was examined. The endothelial inhibitory effect of 2ME2 has generally been evaluated under conditions of restricted serum and growth factor as a means of optimally observing the effect of 2ME2 on endothelial growth. The inhibition of endothelial cell proliferation by 2ME2 is dose-dependent under conditions of restricted serum and growth factor with significant inhibition at 0.25 μg/mL of 2ME2. Under conditions of optimal serum and endothelial cell growth supplements, 2ME2 was still capable of inhibiting endothelial cell proliferation in a dose-dependent fashion, although this required approximately a two-fold higher concentration of 2ME2. Under these optimal growth conditions, the IC50 was approximately 0.5 μg/mL (approximately 1.5 x 10^-6M). These findings demonstrate that 2ME2 inhibited endothelial cell proliferation even under optimal growth conditions, despite the fact that it seemed to have little observed effect on angiogenesis in the arthritic synovium during in vivo treatment.

Example 6

Effect of 2ME2 treatment on splenomegaly

[0068] During the course of AA in the rat, splenomegaly is a prominent feature due to splenitis, lymphoid hyperplasia and even abscess formation. Therefore the splenic enlargement in these animals was monitored for any effect of 2ME2 on this parameter. In rats that were treated with vehicle, spleen weights increased dramatically, increasing nearly three-fold. This was completely inhibited by 2ME2 treatment started on day 6 post-immunization by the high dose, but also by the more moderate dose of 30 mg/kg/d. Furthermore, even when 2ME2 treatment was initiated during the late stage of AA development, i.e., at day 10, the splenic enlargement was still markedly inhibited.

[0069] A histological examination of the spleens was undertaken in the different groups. The appearance of a normal rat spleen (A) and vehicle-treated spleen (B). The latter shows the severe splenitis involving the red pulp and even the capsule. Furthermore, the spleen demonstrated extensive PMNL and mononuclear cell accumulation with abscess formation and some cellular necrosis. There were also increased numbers of multinucleated giant cells and lymphoid hyperplasia in the white pulp. Only mild splenitis was visible in a rat treated with 2ME2 (30mg/kg/d) from day 6-14 post-immunization, with no increase in giant cells or abscess formation, and there was no lymphoid hyperplasia observed.

Example 7

[0070] Effect of 2ME2 treatment on lymphocyte response to mitogens and antigen. Because of the effect of 2ME2 treatment on arthritis severity and the response in the spleen during AA, the T lymphocyte reactivity to mitogens the Mycobacterial purified protein derivative (PPD) antigen was evaluated. Lymph node cells showed a proliferative response to Con A, PHA and PPD from rats with full-blown AA harvested at day 14 post-immunization. Lymphocytes from naive, non-immunized rats had no significant proliferative response to PPD above medium control. However, lymphocytes from rats with AA had a strong proliferative response to mitogens at 3 days and also to PPD at 6 days, measured by 3H-thymidine incorporation. The effect of various concentrations of 2ME2 in vitro on this proliferative response was evaluated. Addition of 2ME2 to the culture on the day of initiation dose-dependently inhibited lymphocyte response to PPD, with an IC50 of about 0.3 μg/mL. The 2ME2 also decreased the spontaneous proliferation in unstimulated cultures. A comparable inhibitory effect of 2ME2 in vitro on the proliferation induced by the mitogens was observed. There was no effect on viability since viability (≥85% live cells) in all cultures was comparable. To further assess the effect of 2ME2 on lymphocyte responsiveness, lymph node cells harvested from rats treated with 2ME2 (30 mg/kg/d) from day 6-14 post-immunization were tested for proliferative response to PPD and the mitogens, Con A and PHA and compared to lymphocytes from vehicle-treated animals. DNA incorporation was increased by nearly five-fold in vehicle-treated rats. This response was significantly reduced with lymphocytes from rats that received 2ME2 treatment. In contrast, no difference in the proliferation induced by Con A or PHA was observed when the rats were treated with 2ME2 and cultures did not contain any added 2ME2. This suggests that 2ME2 had an immunomodulatory effect in vivo on antigen responsiveness.
Claims

1. A therapeutic agent having the structure

\[
\begin{align*}
\text{OH} \\
\text{R}_a \\
\text{HO}
\end{align*}
\]

wherein R\text{a} is -OCH\text{3}, for use in administration with methotrexate for the treatment of rheumatic diseases.

2. The therapeutic agent of Claim 1, wherein the therapeutic agent and methotrexate are for administration in a single formulation.

3. The therapeutic agent of Claim 1, wherein the therapeutic agent and methotrexate are for administration in two or more separate formulations.

4. A composition for use in treating rheumatic diseases comprising:
   a) a compound having the formula

\[
\begin{align*}
\text{OH} \\
\text{R}_a \\
\text{HO}
\end{align*}
\]

wherein R\text{a} is -OCH\text{3}; and
b) methotrexate.

5. The therapeutic agent or composition of any one of the preceding claims, wherein the rheumatic disease is osteoarthritis, fibromyalgia, spondyloarthropathies, gout, polymyositis, bursitis, tendonitis, rheumatoid arthritis, systemic lupus erythematosus, polymyalgia rheumatica, scleroderma, or psoriatic arthritis.

6. The therapeutic agent or composition of any one of the preceding claims, wherein said therapeutic agent and methotrexate are for administration together.

7. The therapeutic agent or composition of any one of the preceding claims, wherein said therapeutic agent and methotrexate are for administration separately.

8. Use of a therapeutic agent having the structure
wherein Ra is -OCH₃ and methotrexate or the composition of any one of the preceding claims in the manufacture of a medicament for treating rheumatic diseases.

9. The composition of claim 4 or the use of claim 8 wherein the therapeutic agent having the structure

wherein Ra is -OCH₃ is administered by oral delivery in an amount of 100mg/Kg of body weight per day and methotrexate is administered by intraperitoneal injection in an amount of 1mg/Kg of body weight per day.

Patentansprüche

1. Therapeutikum mit der folgenden Struktur

wobei Ra -OCH₃ ist, zur Verwendung beim Verabreichen mit Methotrexat zum Behandeln von rheumatischen Erkrankungen.

2. Therapeutikum nach Anspruch 1, wobei das Therapeutikum und Methotrexat zum Verabreichen in einer einzigen Formulierung vorgesehen sind.

3. Therapeutikum nach Anspruch 1, wobei das Therapeutikum und Methotrexat zum Verabreichen in zwei oder mehreren separaten Formulierungen vorgesehen sind.

4. Zusammensetzung zur Verwendung beim Behandeln von rheumatischen Erkrankungen, Folgendes umfassend:
a) eine Verbindung mit der folgenden Formel

\[
\begin{align*}
\text{R}_a \cdot \text{OCH}_3 & \quad \text{wobei } \text{R}_a \cdot \text{OCH}_3 \text{ ist; und} \\
b) \text{Methotrexat.}
\end{align*}
\]


6. Therapeutikum oder Zusammensetzung nach einem der vorhergehenden Ansprüche, wobei das Therapeutikum und Methotrexat zum gemeinsamen Verabreichen vorgesehen sind.

7. Therapeutikum oder Zusammensetzung nach einem der vorhergehenden Ansprüche, wobei das Therapeutikum und Methotrexat zum separaten Verabreichen vorgesehen sind.

8. Verwendung eines Therapeutikums mit der Struktur

\[
\begin{align*}
\text{R}_a \cdot \text{OCH}_3 & \quad \text{wobei } \text{R}_a \cdot \text{OCH}_3 \text{ ist, und Methotrexats oder der Zusammensetzung nach einem der vorhergehenden Ansprüche beim Herstellen eines Medikaments zum Behandeln von rheumatischen Erkrankungen.}
\end{align*}
\]

9. Zusammensetzung nach Anspruch 4 oder Verwendung nach Anspruch 8, wobei das Therapeutikum mit der Struktur
Revendications

1. Agent thérapeutique de structure

![Image of molecule](image)

où $R_a$ est -OCH$_3$, pour une utilisation lors de l'administration avec du méthotrexate pour le traitement de maladies rhumatismales.

2. Agent thérapeutique selon la revendication 1, où l'agent thérapeutique et le méthotrexate sont destinés à une administration dans une seule formulation.

3. Agent thérapeutique selon la revendication 1, où l'agent thérapeutique et le méthotrexate sont destinés à une administration dans deux formulations séparées ou plus.

4. Composition pour une utilisation dans le traitement de maladies rhumatismales comprenant :

   a) un composé de formule

   ![Image of molecule](image)

   où $R_a$ est -OCH$_3$ ; et

   b) du méthotrexate.

5. Agent thérapeutique ou composition selon l'une quelconque des revendications précédentes, où la maladie rhumatismale est l'ostéo-arthrite, la fibromyalgie, les spondylarthropathies, la goutte, la polymyosite, la bursite, la tendinite, la polyarthrite rhumatoïde, le lupus érythémateux systémique, la polymyalgie rhumatismale, la sclérodermie ou le rhumatisme psoriasique.

6. Agent thérapeutique ou composition selon l'une quelconque des revendications précédentes, où ledit agent thérapeutique et le méthotrexate sont destinés à une administration conjointe.

7. Agent thérapeutique ou composition selon l'une quelconque des revendications précédentes, où ledit agent thérapeutique et le méthotrexate sont destinés à une administration séparée.

8. Utilisation d’un agent thérapeutique de structure
où $R_a$ est $-\text{OCH}_3$, et du méthotrexate ou de la composition selon l'une quelconque des revendications précédentes dans la fabrication d'un médicament pour le traitement de maladies rhumatismales.

9. Composition selon la revendication 4 ou utilisation selon la revendication 8, où l'agent thérapeutique de structure

où $R_a$ est $-\text{OCH}_3$, est administré par voie orale dans une quantité de 100 mg/kg de poids corporel par jour et le méthotrexate est administré par injection intrapéritonéale dans une quantité de 1 mg/kg de poids corporel par jour.
FIG. 1

- Vehicle Control
- 2ME2 1mg/kg
- 2ME2 10mg/kg
- 2ME2 100mg/kg

Arthritic Source
0 = no signs
1 = redness/red spots on paw
2 = partial swelling, difficulties with stretching paw
3 = swelling, no loading on paw
4 = maximally swollen paw, no loading

* p < 0.05
FIG. 5
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total Osteoclast Numbers</th>
<th>Articular cartilage area without proteoglycan staining (%)</th>
<th>Damaged articular cartilage surface (%)</th>
<th>Thickness of total articular cartilage area (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>33.9</td>
<td>17.23</td>
<td>47.35</td>
<td>26.06</td>
</tr>
<tr>
<td>2ME2 1mg/ kg</td>
<td>21.9</td>
<td>11.21</td>
<td>37.07</td>
<td>27.55</td>
</tr>
<tr>
<td>2ME2 10 mg/ kg</td>
<td>5.7</td>
<td>6.71</td>
<td>27.91</td>
<td>27.34</td>
</tr>
<tr>
<td>2ME2 100 mg/kg</td>
<td>0</td>
<td>0.18</td>
<td>0</td>
<td>32.64</td>
</tr>
</tbody>
</table>

p < 0.05

**FIG. 6**
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>% Inhibition of arthritic score (day 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>2ME2</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>MTX + 2ME2</td>
<td></td>
<td>98</td>
</tr>
</tbody>
</table>

Expected inhibition in arthritic score = 85%
2ME2 100mg/kg
(treatment arm)

Vehicle Control
REFERENCES CITED IN THE DESCRIPTION

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