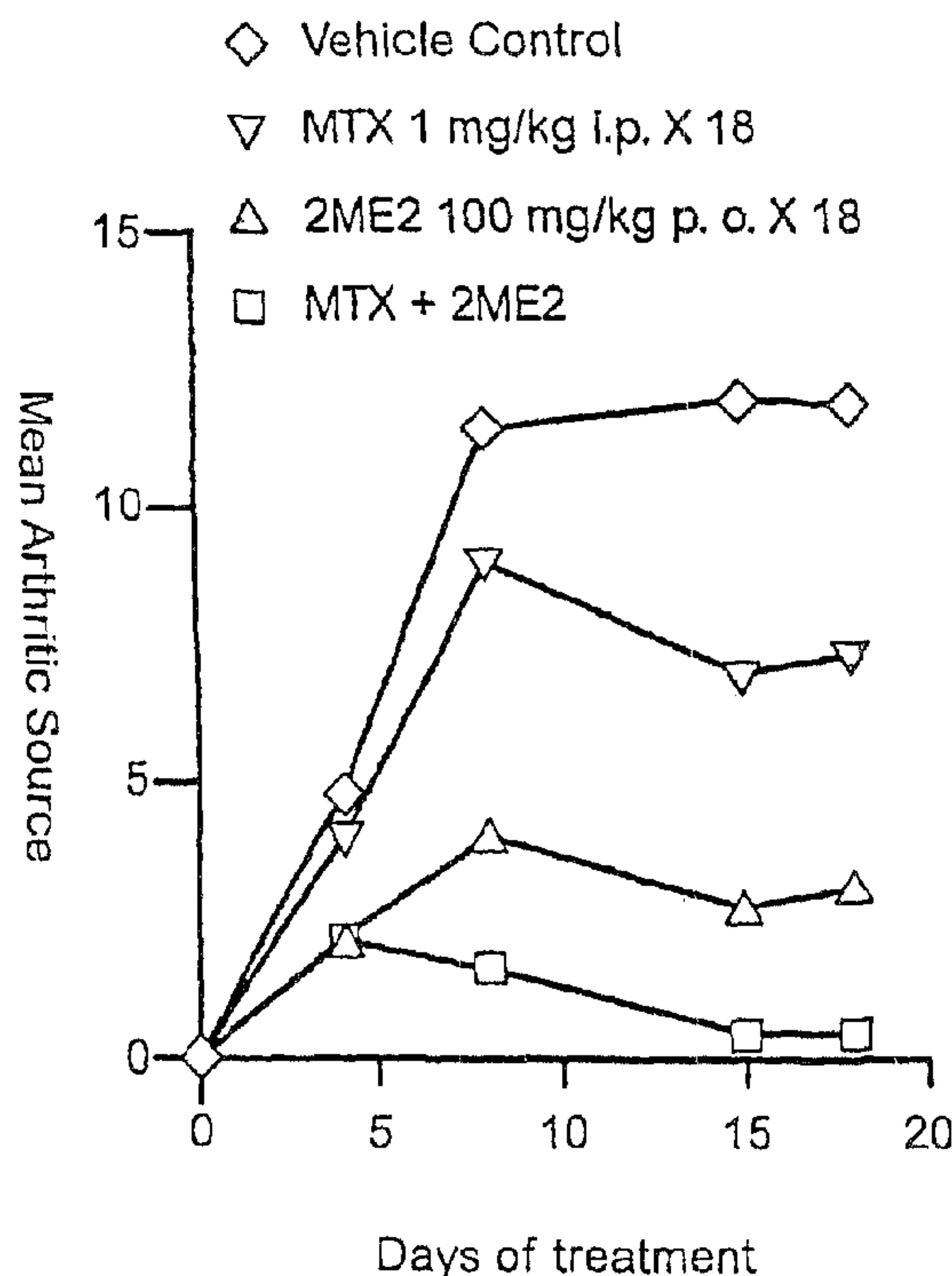




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(54) Titre : 2-METHOXYESTRADIOL PRESENTANT UNE ACTIVITE ANTI-ARTHRITIQUE POUVANT MODIFIER
L'EVOLUTION DE LA MALADIE
(54) Title: DISEASE MODIFYING ANTI-ARTHRITIC ACTIVITY OF 2-METHOXYESTRADIOL



Treatment	Dose (mg/kg)	% Inhibition of arthritic score (day 18)
MTX	1	39
2ME2	100	75
MTX + 2ME2		98

Expected inhibition in arthritic score
85%

(57) **Abrégé/Abstract:**

The present invention relates to a composition for treating rheumatic diseases. The composition comprises 2-methoxyestradiol, or its derivatives, and one or more anti-rheumatic agents. A method of using the composition for treating rheumatic diseases in humans and animals is also disclosed.



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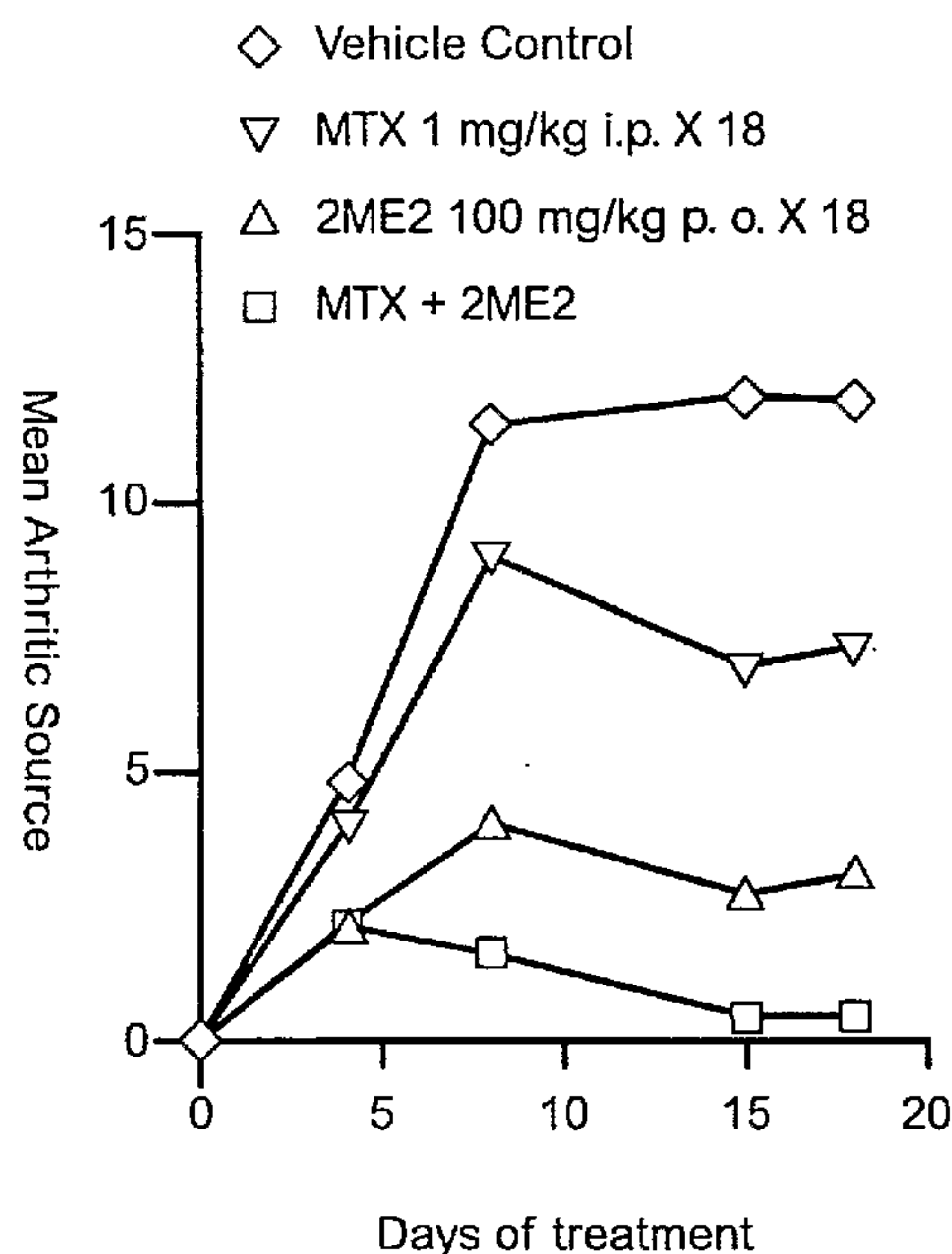
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(54) Title: DISEASE MODIFYING ANTI-ARTHRITIC ACTIVITY OF 2-METHOXYESTRADIOL



Treatment	Dose (mg/kg)	% Inhibition of arthritic score (day 18)
MTX	1	61
2ME2	100	75
MTX + 2ME2		98

Expected inhibition in arthritic score
85%

(57) Abstract: The present invention relates to a composition for treating rheumatic diseases. The composition comprises 2-methoxyestradiol, or its derivatives, and one or more anti-rheumatic agents. A method of using the composition for treating rheumatic diseases in humans and animals is also disclosed.

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DISEASE MODIFYING ANTI-ARTHRITIC ACTIVITY OF 2-METHOXYESTRADIOL

5

FIELD OF THE INVENTION

The present invention relates generally to compositions comprising anti-angiogenic agents in combination with anti-rheumatic agents and to methods of
10 using the same. More specifically, the present invention relates to compositions comprising 2-methoxyestradiol, and derivatives thereof, in combination with anti-rheumatic agents. More particularly, the present invention relates to methods of treating rheumatoid arthritis and related rheumatic diseases by administering 2-
15 methoxyestradiol, and derivatives thereof, in combination with one or more anti-rheumatic agents.

BACKGROUND OF THE INVENTION

Rheumatic diseases are characterized by inflammation and loss of function
20 in one or more connecting or supporting structures of the body. Those structures especially affected are joints, tendons, ligaments, bones and muscles and in some cases internal organs. Some rheumatic diseases are classified as connective tissue disorders and include osteoarthritis, fibromyalgia, spondyloarthropathies, gout, polymyositis, bursitis and tendonitis. Other rheumatic diseases are classified as
25 automimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, polymyalgia rheumatica, scleroderma, and psoriatic arthritis. An estimated 45 million people in the United States have arthritis or other rheumatic conditions and rheumatic diseases are the leading cause of disability among adults age 65 and older. While the pathogenesis of the diseases may vary, their
30 characteristic inflammatory symptoms often share common inflammatory mediators.

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*
5 *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
10 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).

The symptoms of rheumatoid arthritis present as pain and stiffness in
15 multiple joints. Symptoms can emerge over weeks and are often accompanied by anorexia, weakness, or fatigue. Joints most commonly affected are those with the highest ratio of synovium to articular cartilage, including the wrists and the proximal interphalangeal and metacarpophalangeal joints (Ruddy *et al. eds. Kelly's Textbook of Rheumatology* 7th ed. Philadelphia: W.B. Saunders, 2005:996-1042).
20 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
25 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
30 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
5 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
10 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,
15 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 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
25 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, including FGF2; VEGF; TGF β ; TNF α ; chemokines, such as IL8, IL18, and IL1; soluble adhesion molecules, such as E-selectin and soluble VCAM-1; glycoconjugates, such as the
30 soluble 4A11 antigen, soluble CD 146 and the angiopoietin-Tie system (Koch, *Ann. Rheum. Dis.* (2003), 62(Suppl. II):ii60-ii67).

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-1 α and hydroxycarbon nuclear translocator (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 1 β , TGF β , FGF-2, and TNF α . Studies have shown a synergistic interaction between growth factors and hypoxia in VEGF induction (Brenchley, *Ann Rheu Dis* (2001), 60:iii71-iii74).

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-1 α nuclear accumulation (Mabjeesh *et al.*, *Cancer Cell*, (2003) 3:363-75), induction of the extrinsic apoptotic pathway

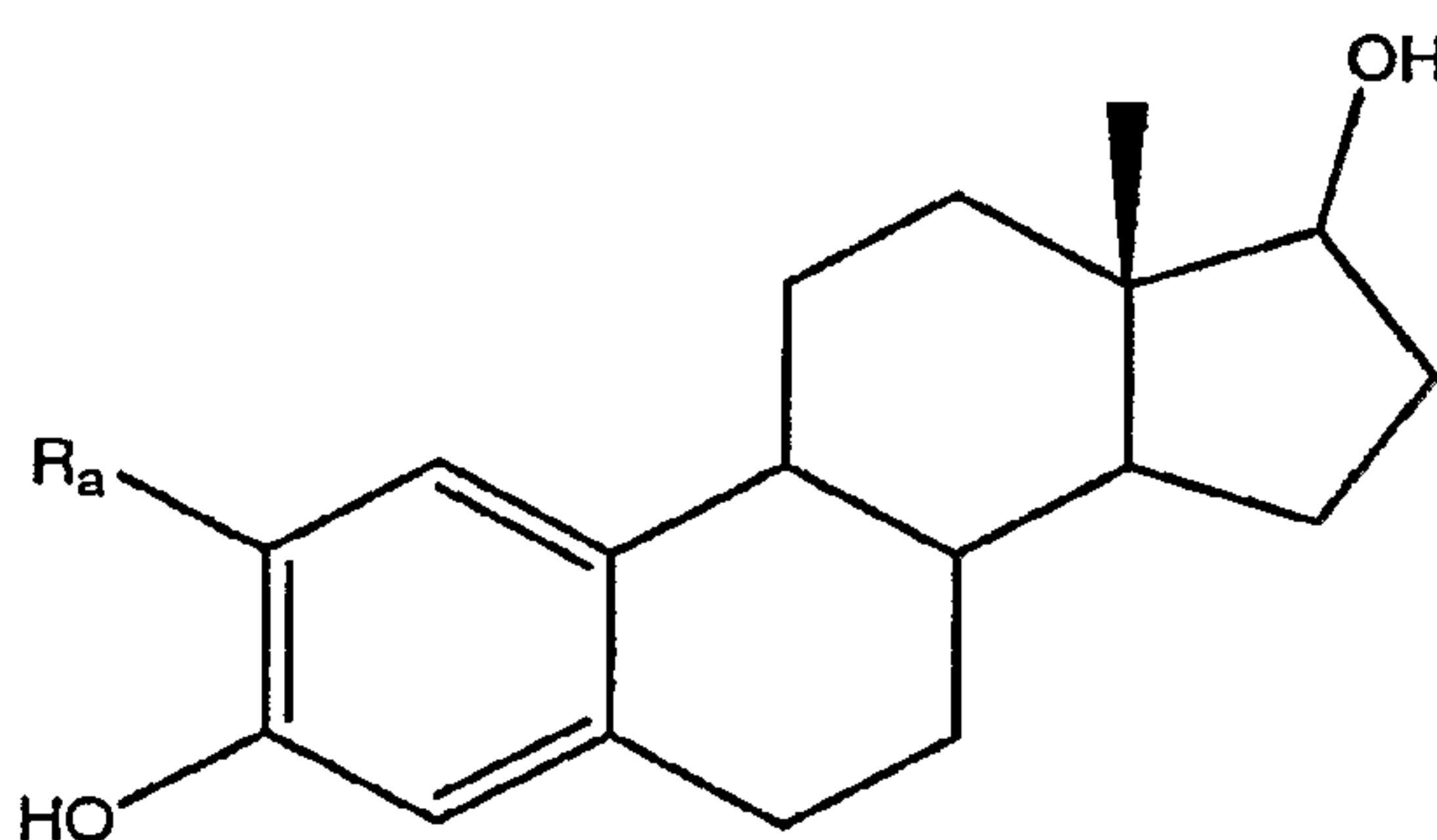
through upregulation of Death Receptor 5 (LaVallee et al., *Cancer Research* (2003), 63(2): 469-75) and induction of the intrinsic apoptotic pathway, potentially through the inhibition of superoxide dismutase enzymatic activity (Huang et al., *Trends Cell Biology* (2001), 11(8):343-8).

5 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

10 The present invention comprises methods and compositions for treating rheumatic diseases. In a disclosed embodiment, the composition comprises 2-methoxyestradiol, and derivatives thereof, in combination with anti-rheumatic agents. 2-Methoxyestradiol is a powerful antiangiogenic agent and has the ability to enhance the effects of other anti-rheumatic agents through its own anti-
15 angiogenic and anti-proliferative capabilities.

Another disclosed embodiment comprises a method of treating rheumatic diseases comprising administering to a human or an animal an amount of a compound having the formula

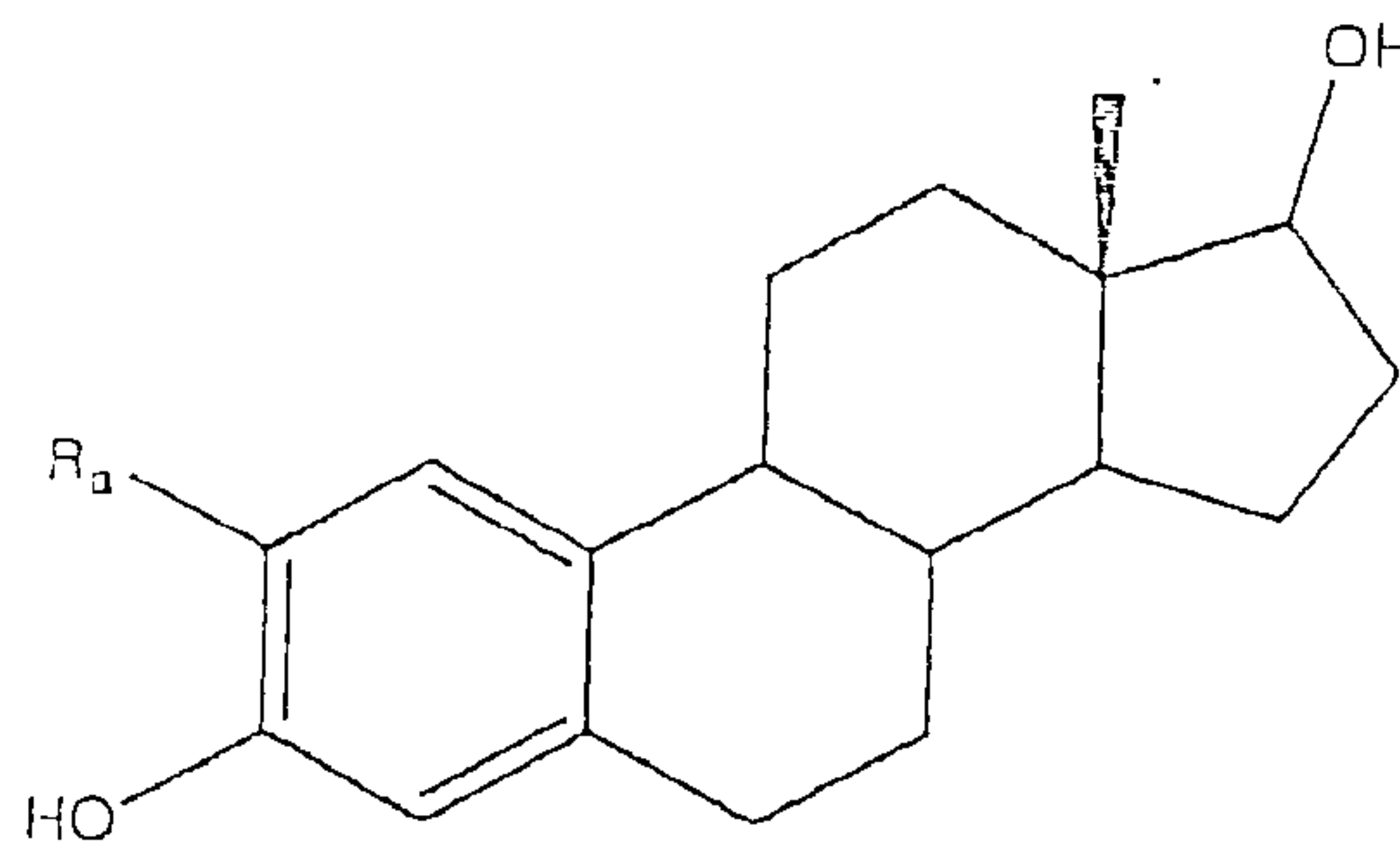


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wherein R_a is selected from $-\text{OCH}_3$, $-\text{OCH}_2\text{CH}_3$, $-\text{CH}_3$, $-\text{CH}_2\text{CH}_3$, $-\text{CCCH}_3$, $-\text{CHCH}-\text{CH}_3$, or $\text{CH}_2-\text{CHCH}_2$; and one or more anti-rheumatic agents.

Another embodiment comprises a composition for treating rheumatic diseases. The composition comprise a compound having the formula

6



wherein R_a is selected from $-OCH_3$, $-OCH_2CH_3$, $-CH_3$, $-CH_2CH_3$, $-CCCH_3$, $-CHCH-CH_3$, or CH_2-CHCH_2 ; and one or more anti-rheumatic agents.

5 Accordingly, the present invention seek to provide an improved method and composition for treating rheumatic diseases in humans and animals.

Another aspect of the present invention seeks to provide an improved method and composition for treating rheumatoid arthritis in humans and animals.

10 A further aspect of the present invention seeks to provide a method and composition for treating rheumatic diseases in humans or animals which has few or no unwanted or undesirable side effects.

A further aspect of the present invention seeks to provide the use of the therapeutic agent and the second compound for treating rheumatic diseases. The therapeutic agent having the afore described structure and in particular R_a is $-OCH_3$; and
 15 the second compound is methotrexate. In an embodiment of the present invention the first therapeutic agent and the second compound are in administrable formulation and in synergistic amounts for treating rheumatoid arthritis; the synergistic amounts result in an inhibition of arthritic score that is greater than an expected inhibition of arthritic score.

20 Another aspect of the present invention provides for a composition for use in treating rheumatic diseases in including the afore mentioned formula in that R_a is $-OCH_3$ and a second compound being methotrexate. The first and second compounds are provided in synergistic amounts effective for treating rheumatoid arthritis. The synergistic amounts result in an inhibition of arthritic score that is greater than an expected inhibition of arthritic score.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of
5 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 Arthrogen-CIA[®] model of RA

10 Fig. 2 is two photographs of a control and 100 mg/kg treatment which show administration of 2ME2 inhibits synovial inflammation in the Arthrogen-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 Arthrogen-
15 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
5 inhibit the severity of RA progression in the Arthrogen-CIA® model of RA.

Fig. 6 is a table which show the assessment of histomorphometric alterations following 2ME2 treatment in the Arthrogen-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
10 inhibition of arthritic score of Arthrogen-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

15 The present invention may be understood more readily by reference to the following detailed description of specific embodiments included herein. Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention. The entire text of the
20 references mentioned herein may be referenced for further details.

The present invention comprises methods and compositions for treating rheumatic diseases in humans and animals. In a disclosed embodiment of the present invention, the composition comprises an anti-angiogenic and anti-proliferative agent in combination with one or more anti-rheumatic agents. The
25 anti-angiogenic and anti-proliferative agent is 2ME2, and derivatives thereof, as shown in Formula I below.

2-Methoxyestradiol and Derivatives Thereof

The process or processes by which 2ME2 exhibits its anti-proliferative and
30 anti-angiogenic activities remains unclear, however, a number of studies have implicated various mechanisms of action and cellular targets. 2ME2 induced

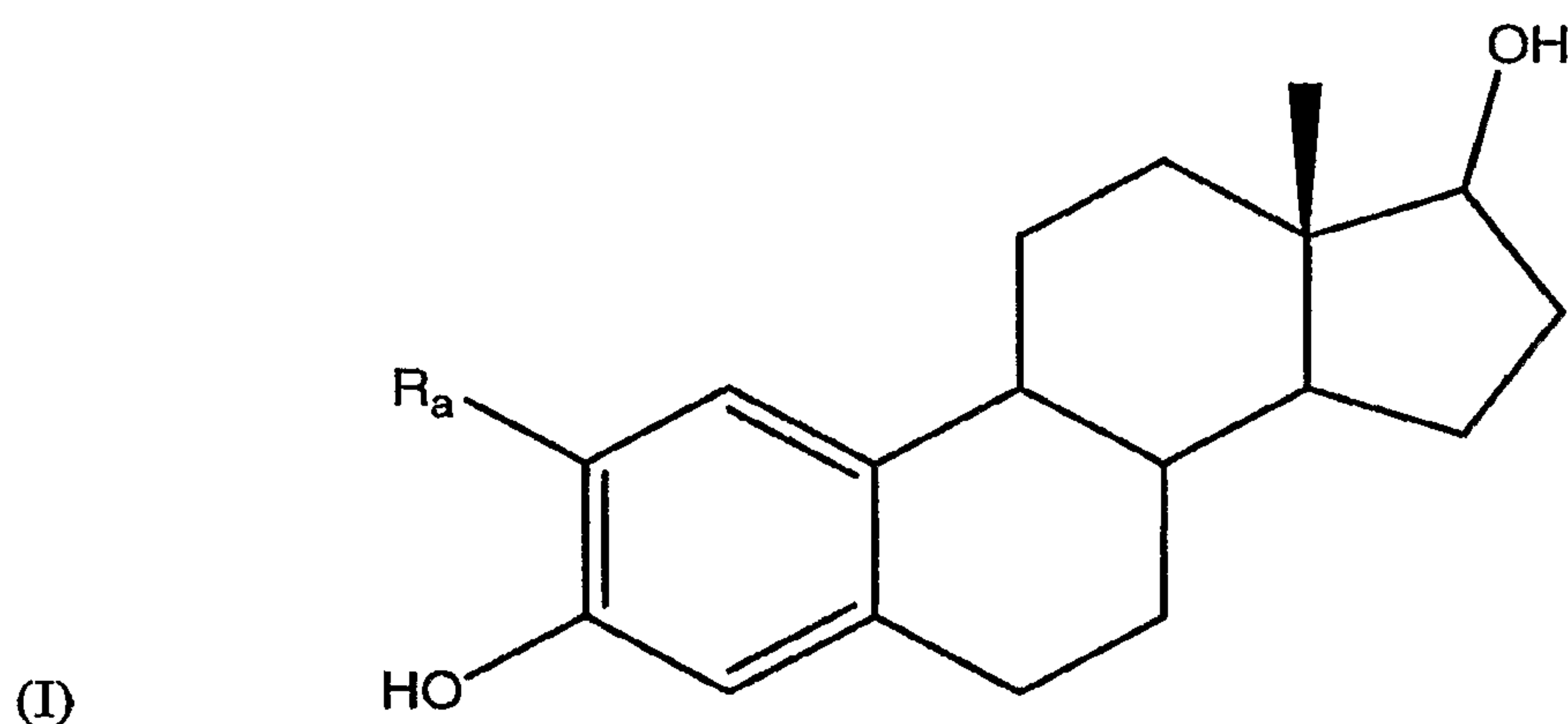
changes in the levels and activities of various proteins involved in the progression of the cell cycle. These include cofactors of DNA replication and repair, *e.g.*, proliferating cell nuclear antigen (PCNA) (Klauber, N., Parangi, S., Flynn, E., Hamel, E. and D'Amato, R.J. "Inhibition of angiogenesis 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. "17 β -Estradiol metabolites affect some regulators of the MCF-7 cell cycle," *Cancer Letters*, (1996) 110:181-186); cell division cycle kinases and regulators, *e.g.*, p34^{cdc2} and cyclin B (Attalla, H., Mäkelä, T.P., Adlercreutz, H. and Andersson, L.C. "2-Methoxyestradiol arrests cells in mitosis without depolymerizing tubulin," *Biochemical and Biophysical Research Communications*, (1996) 228:467-473; Zoubine, M.N., Weston, A.P., Johnson, D.C., Campbell, D.R. and Banerjee, S.K. "2-Methoxyestradiol-induced growth suppression and lethality in estrogen- responsive MCF-7 cells may be mediated by down regulation of p34cdc2 and cyclin B1 expression," *Int J Oncol.*, (1999) 15:639-646); transcription factor modulators, *e.g.*, SAPK/JNK (Yue, T-L., Wang, X., Loudon, C.S., Gupta, L.S., Pillarisetti, K., Gu, J-L., Hart, T.K., Lysko, P.G. and Feuerstein, G.Z. "2-Methoxyestradiol, an endogenous estrogen metabolite induces apoptosis in endothelial cells and inhibits angiogenesis: Possible role for stress-activated protein kinase signaling pathway and fas expression," *Molecular Pharmacology*, (1997) 51:951-962; Attalla, H., Westberg, J.A., Andersson, L.C., Aldercreutz, H. and Makela, T.P. "2-Methoxyestradiol-induced phosphorylation of bcl-2: uncoupling from JNK/SAPK activation," *Biochem and Biophys Res Commun.*, (1998) 247:616-619); and regulators of cell arrest and apoptosis, *e.g.*, tubulin (D'Amato, R.J., Lin, C.M., Flynn, E., Folkman, J. and Hamel, E. "2-Methoxyestradiol, and endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site," *Proc. Natl. Acad. Sci. USA*, (1994) 91:3964-3968; Hamel, E., Lin, C.M., Flynn, E. and D'Amato, R.J. "Interactions of 2-methoxyestradiol, and endogenous mammalian metabolite, with unpolymerized tubulin and with tubulin polymers," *Biochemistry*, (1996) 35:1304-1310), p21^{WAF1/CIP1} (Mukhopadhyay, T. and Roth, J.A. "Induction of apoptosis in human lung cancer

cells after wild-type p53 activation by methoxyestradiol,” *Oncogene*, (1997) 14:379-384), bcl-2 and FAS (Yue *et al.* (1997); Attalla *et al.* (1998)), and p53 (Kataoka, M., Schumacher, G., Cristiano, R.J., Atkinson, E.N., Roth, J.A. and Mukhopadhyay, T. “An agent that increases tumor suppressor transgene product coupled with systemic transgene delivery inhibits growth of metastatic lung cancer *in vivo*,” *Cancer Res.*, (1998) 58:4761-4765; Mukhopadhyay *et al.* (1997); Seegers, J.C., Lottering, M-L., Grobler C.J.S., van Papendorp, D.H., Habbersett, R.C., Shou, Y. and Lehnert B.E. “The mammalian metabolite, 2-methoxyestradiol, affects p53 levels and apoptosis induction in transformed cells but not in normal cells,” *J. Steroid Biochem. Molec.Biol.*, (1997) 62:253-267). The effects on the level of cAMP, calmodulin activity and protein phosphorylation may also be related to each other. More recently, 2ME2 was shown to upregulate Death Receptor 5 and caspase 8 in human endothelial and tumor cell lines (LaVallee TM, Zhan XH, Johnson MS, Herbstritt CJ, Swartz G, Williams MS, Hembrough WA, Green SJ, Pribluda V.S. “2-Methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway,” *Cancer Res.* (2003) 63(2):468-75), destabilize microtubules and inhibit HIF-1 α nuclear accumulation (Mabjeesh *et al.*, *Cancer Cell*, (2003) 3:363-75), and interact with superoxide dismutase (SOD) 1 and SOD 2 and to inhibit their enzymatic activities (Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. “Superoxide dismutase as a target for the selective killing of cancer cells,” *Nature*, (2000) 407:390-5). All cellular targets described above are not necessarily mutually exclusive to the inhibitory effects of 2ME2 in actively dividing cells.

The more relevant mechanisms described above have been extensively discussed in Victor S. Pribluda, Theresa M. LaVallee and Shawn J. Green, *2-Methoxyestradiol: A novel endogenous chemotherapeutic and antiangiogenic* in The New Angiotherapy, Tai-Ping Fan and Robert Auerbach eds., Human Press Publisher.

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:

10



wherein R_a is selected from $-OCH_3$, $-OCH_2CH_3$, $-CH_3$, $-CH_2CH_3$, $-CCCH_3$, $-CHCH-CH_3$, or CH_2-CHCH_2 . In cases where stereoisomers are possible, both R and S stereoisomers are envisioned, as well as any mixture of stereoisomers.

Those skilled in the art will appreciate that the invention extends to other 2-methoxyestradiol analogs within the definitions given and in the claims below, having the described characteristics. These characteristics can be determined for each test compound using assays known in the art.

Anti-Rheumatic Agents

Anti-rheumatic agents that may be used with the disclosed embodiment of the present invention include, but are not limited to, disease-modifying anti-rheumatic drugs, non-steroid anti-inflammatory drugs, corticosteroids, tumor necrosis factor inhibitors, selective B-cell inhibitors, interleukin-1 inhibitors and mixtures or combinations thereof. Each of these anti-rheumatic agents is discussed further below.

Disease-Modifying Anti-Rheumatic Drugs

Disease-modifying anti-rheumatic drugs that may be used with the disclosed embodiment of the present invention include, but are not limited to, allopurinol, amitriptyline hydrochloride, auranofin (oral gold), azathiopine, chlorambucil, colchicine, cyclobenzaprine cyclophosphamide, cyclosporine, duloxetine, fluoxetine, gold sodium thiomalate (injectable gold),

hydroxychloroquine sulfate, leflunomide, methotrexate, minocycline, mycophenolate mofetil, probenecid, sulfasalazine, tamadol and mixtures or combinations thereof.

5 Non-Steroid Anti-Inflammatory Drugs

Non-steroid anti-inflammatory drugs that may be used with the disclosed embodiment of the present invention include, but are not limited to, traditional NSAIDS, such as diclofenac potassium, diclofenac sodium, diclofenac sodium with misoprostol, diflunisal, etodolac, fenoprofen calcium, flurbiprofen, ibuprofen, 10 indomethacin, ketoprofen, meclfenamate sodium, mefenamic acid, meloxicam, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, and tolmetin sodium; COX-2 inhibitors, such as celecoxib, rofecoxib, and valdecoxib; salicylates, such as aspirin, choline salicylate, salsalate, and sodium salicylate; bisphosphonates, such as alendronate, ibandronate, and risedronate.

15

Corticosteroids

Corticosteroids that may be used with the disclosed embodiment of the present invention include, but are not limited to, betamethasone, cortisone acetate, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone and 20 mixtures or combinations thereof.

Tumor Necrosis Factor Inhibitors

Tumor necrosis factor inhibitors that may be used with the disclosed embodiment of the present invention include, but are not limited to, adalimumab, 25 etanercept, infliximab, abatacept and mixtures or combinations thereof.

Selective B-cell Inhibitors

Selective B-cell inhibitors that may be used with the disclosed embodiment of the present invention include, but are not limited to, rituximab.

30

Interleukin-1 Inhibitors

Interleukin-1 inhibitors that may be used with the disclosed embodiment of the present invention include, but are not limited to, anakinra.

5 Administration

In accordance with the present invention, the compounds of Formula I may be mixed with one or more anti-rheumatic agents into a single formulation. The compounds of Formula I and the anti-rheumatic agent may also be formulated and delivered separately.

10 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 the anti-rheumatic agent can be administered by topical, oral, rectal or parenteral (e.g., intravenous, subcutaneous or intramuscular) route. In addition, the compositions
15 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
20 Formula I and/or anti-rheumatic agent. 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 disclosure of which may be referred to for further details.

The formulations in accordance with the present invention can be
25 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,
30 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
5 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
10 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,
15 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
20 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
25 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
30 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
5 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.

10 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,
15 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
20 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.
25 10/412,669 (Publication No. US 2003/0219490), U.S. Patent No. 5,494,683, U.S. Patent Application No. 10/878,623 (Publication No. US 2005/0008707), U.S. Patent No. 5,510,118, U.S. Patent No. 5,524,270, U.S. Patent No. 5,145,684, U.S. Patent No. 5,399,363, U.S. Patent No. 5,518,187, U.S. Patent No. 5,862,999, U.S. Patent No. 5,718,388, and U.S. Patent No. 6,267,989, all of which may be referred
30 to for further details. A review of drug formulation technology is

provided in "Water Insoluble Drug Formulation" by Rong Liu, editor, pp. 1-633, (2000) CRC Press LLC, which may be referred to for further details.

By forming 2-methoxyestradiol nanoparticles, the compositions disclosed herein are shown to have increased bioavailability. Preferably, the particles are
5 comprised of the compounds of Formula I and/or anti-rheumatic agents 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
10 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
15 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
20 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.
25 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 the anti-rheumatic agent can be administered simultaneously. In another embodiment, they can be administered separately (*i.e.*; compounds of Formula I dosage in the morning, anti-
30 rheumatic agent dosage in the evening). Mixtures of more than one anti-rheumatic agent can, of course, be administered. Indeed, it is often desirable to use mixtures

or sequential administrations of different anti-rheumatic agents to treat rheumatic disease, especially anti-rheumatic agents from the different classes.

5 If the 2-methoxyestradiol formulation and the anti-rheumatic agent are to be administered sequentially, the amount of time between administration of the 2-methoxyestradiol formulation and the anti-rheumatic agent 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.

10 The anti-rheumatic agent 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.

15 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
20 section) may include one or more than one excipient chosen to prevent particle agglomeration.

Pharmaceutical Preparations

25 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
5 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,
10 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
15 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
20 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
25 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.

The present invention also relates to conjugated prodrugs and uses thereof. More particularly, the invention relates to conjugates of steroid compounds, such as
30 compounds of Formula I, and the use of such conjugates in the prophylaxis or treatment of conditions associated with rheumatic or related inflammatory diseases.

The invention also relates to compositions including the prodrugs of the present invention.

In one aspect, the present invention provides a conjugated prodrug of an estradiol compound, preferably compounds of Formula I, conjugated to a
5 biological activity modifying agent.

Alternatively, the conjugated prodrug according to the present invention includes the compounds of Formula I, conjugated to a peptide moiety.

The incorporation of an estradiol compound, such as the compounds of Formula I, into a disease-dependently activated pro-drug enables significant
10 improvement of potency and selectivity of this anti-angiogenic agent.

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.

In a further aspect of the present invention there is provided use of
15 compounds of Formula I, or prodrugs thereof, 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.

In a still further aspect of the present invention there is provided a method of prophylaxis or treatment of a rheumatic disease, said method including
20 administering to a patient in need of such prophylaxis or treatment an effective amount of compounds of Formula I, or prodrugs thereof, in combination with an anti-rheumatic agent according to the disclosed embodiment, as described herein. It should be understood that prophylaxis or treatment of said condition includes amelioration of said condition.

25 Pharmaceutically acceptable salts of the compounds of the Formula I, or the prodrugs thereof, 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.

30 100% 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-
5 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.

A particularly useful formulation in the present invention is a nanoparticulate liquid suspension of 2-methoxyestradiol disclosed in U.S. Patent Application Serial No. 10/392,403, filed March 20, 2003 (the disclosure of which
10 may be referenced for further details). This formulation is available from EntreMed, Inc., Rockville, Maryland, under the designation Panzem® NCD.

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
15 according to the invention can be synthesized according to known methods from publicly available precursors.

The compositions and methods are further illustrated by the following non-limiting examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood
20 that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the scope of the present invention.

EXAMPLES

25 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,
30 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 1.
Summary of In Vivo Anti-Arthritic Activity of 2ME2 (Panzem[®] NCD) Formulation

5

Study Number	Dose, Schedule, Route	Mode of Intervention	Endpoint(s) Measured	Main Findings
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 ^a	• 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 ^a	• 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 AUC₀₋₂₄ for 25, 50, and 75 mg/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 ^a	• 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 ^a	• 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
UCLA Ernie Brahn Study #1 Rat (Louvain) Female, n=12/group Collagen- Induced Arthritis	Prevention: 30, 100 mg/kg/d; Vehicle control PO Treatment: 10, 30, 100 mg/kg/d; Vehicle control PO	Prevention ^a & Treatment ^b	• Clinical arthritic score • Radiographic score	• Dose dependent inhibition of clinical arthritic score and bone erosion • Delays onset of disease
UCLA Ernie Brahn Study #2 Rat (Louvain) Female, n=12/group Collagen- Induced Arthritis	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	Treatment ^b	• Clinical arthritic score • Radiographic score • Histopathology & histomorphometric analysis of joint • Immunohistochemistry	• Dose dependent inhibition of clinical arthritic score, inflammation, and bone erosion, cartilage degradation and pannus formation. • Inhibition of angiogenesis

Dalhousie University Andrew Issekutz Study #1 Rat (Lewis) Male, n=5-9 Adjuvant-Induced Arthritis	30, 100 mg/kg/d; Vehicle control (1.2% HPC, 0.06% DOSS in sterile water) PO	Prevention ^c & Treatment ^b	• Clinical arthritis score • Histopathology of joint • Histological evaluation of spleen, liver, lung, lymph nodes • Immune cell migration	• Inhibition of clinical arthritic 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
Dalhousie University Andrew Issekutz Study #2 Rat (Lewis) Male, n=5 Adjuvant-Induced Arthritis (Adoptive Transfer Model)	3, 30 mg/kg/d; Vehicle control (1.2% HPC, 0.06% DOSS in sterile water) SC	Prevention ^c & Treatment ^b	• Clinical arthritic score • Histopathology of joint • Immune cell migration • Lymphocyte proliferation	• Dose dependent inhibition of clinical arthritic 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

a. Indicates treatment started 1 day following the induction of arthritis; clinical signs of arthritis are not evident

b. Indicates treatment started 10 days following the induction of arthritis, clinical signs of arthritis are present

5 c. Indicates treatment started 6 days following the induction of arthritis; clinical signs of arthritis are not evident

The following examples provide a detailed description of the protocols used in the Dalhousie University Studies 1 and 2, in Table 1 above and are
 10 representative of the general protocols employed in assessing the disease

modifying characteristics of 2ME2 alone and in combination with other anti-rheumatic agents.

Example 1

5 Effect of 2ME2 Treatment of Clinical Arthritis Severity

Adjuvant arthritis was induced in 6-8 week old inbred male Lewis rats (175-200g; Charles River Canada, St-Constant, QC) by s.c. immunization with 0.5 mg of *Mycobacterium butyricum* (Gibco, Detroit, MI) in 0.05 mL mineral oil at two sites on each side of the base of the tail. Arthritis was scored clinically from 0-
10 4 points per limb and tail based on severity of swelling, erythema and limitation of movement as described by Taurog *et al.* (Methods Enzymol. (1988), 162:339-55).

Rats were treated with 2ME2 at two stages in the development of AA. 2ME2 was provided by EntreMed, Inc. (Rockville, MD). Treatment was initiated either 6 days (d) following immunization, with doses of 30 mg/kg/d or 100
15 mg/kg/d or vehicle given i.p. in a volume of 0.5 ml, for a total of 8 d, i.e., to 14 d post-immunization. This protocol was used to start 2ME2 therapy following the initiation of the immune response but prior to the development of clinical arthritis, which normally develops by day 9 or 10. Additional groups of animals were treated with 2ME2 (100 mg/kg/d i.p.) starting on day 10 post-immunization,
20 corresponding to the first signs of clinical arthritis (redness, usually starting in the hind paws, and limping). Treatment was continued to day 14 and all measurements were concluded by day 15 post-immunization, which was the time the animals were sacrificed.

In Lewis rats, polyarticular arthritis, typically most severe in the hind limbs, develops 9-10 days following immunization with killed *M. butyricum* (or *M. tuberculosis*) in mineral oil. The arthritis is rapidly progressive, reaching a peak clinical arthritis severity score by day 14-15 post-immunization. Rats treated with vehicle developed arthritis reaching a clinical score of 11. The vehicle-treated group developed arthritis at the same rate and maximum severity as untreated rats.
30 Rats treated with 2ME2 at 30 mg/kg/d or 100 mg/kg/d, initiated on day 6 post-immunization, had significantly less arthritis severity by day 14 (mean = 7.4 and

6.5, respectively) than the vehicle-treated group. Furthermore, rats treated with the higher dose of 2ME2 (100 mg/kg/d) initiated at the onset of clinical arthritis on day 10 post-immunization also had an attenuation of arthritis severity which was comparable to the groups in which treatment was initiated during the preclinical phase (day 6). However, the modification of arthritis severity in a group receiving the lower dose of 2ME2 (30 mg/kg/d) initiated on day 10 post-immunization did not reach statistical significance, suggesting that the higher dose was required once clinical arthritis had developed.

10

Example 2

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

Rat blood PMNL were isolated from a donor arthritic rat by hydroxyethyl starch (Hespan; Dupont Merck, Wilmington, DE) exchange transfusion performed through a needle inserted in a femoral vein. The exchanged blood was collected into heparin and acid-citrate-dextrose (ACD, formula A; Fenwal-Travenol, Malton, ON) and the leukocyte-rich plasma was harvested during 1 g sedimentation of red blood cells. The PMNL were then isolated by centrifugation on discontinuous Percoll density gradients of 63% Percoll above 74% Percoll with 10% autologous plasma and calcium and magnesium-free Tyrode's solution (TyS⁻) for the balance of the suspending medium. After centrifugation (350 × g, 30 min at 22°C) the purified PMNL (>95%) were harvested from the layer above the 74% plasma-Percoll cushion and after washing with TyS⁻-10% plasma, the cells were radiolabeled with 75 µCi/5 × 10⁷ cells of Na₂⁵¹CrO₄ (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC). After the cells were washed, they were injected i.v. into rats at a dose of 10⁷ ⁵¹Cr-labeled PMNL per animal bearing approximately 1-2 × 10⁵ cpm. These cells were allowed to circulate and migrate for 2 h prior to sacrifice of the rats.

PMNL accumulation in skin sites in the arthritic rats was measured by inducing local inflammation by intradermal (i.d.) injection (0.05 ml) of rat TNF-α (Peprotech, Rocky Hill, NJ), *E. coli* endotoxin (LPS; List Biologicals, Campbell,

CA) or zymosan-activated serum (rat) as a source of C5a_{desArg}.²² 2 h before sacrifice, administered in the doses indicated at the same time as the i.v. injection of radiolabeled PMNL. The accumulation of PMNL in these sites was quantified by biopsy of the injection sites using a 12-mm punch biopsy taken following
5 sacrifice of the animals.

At the time the rats were killed, cardiac blood was obtained to measure the blood leukocyte count and the leukocyte and plasma associated radioactivity. Joints were dissected and segments of limbs sectioned to include carpal, metacarpal, talar and metatarsal joints for analysis as previously reported (Issekutz
10 *et al.* Lab Invest. (1991), 64:656-63 and Issekutz *et al.* Arthritis Rheum. (2001), 44:1428-37). The content of ⁵¹Cr in the joints, as well as in dermal skin sites and internal organs, was determined with a Wallac LKB 1280 gamma counter (Fisher Scientific, Nepean, ON). Accumulated isotope in the tissue was expressed as cpm/10⁶ cpm injected i.v.

15 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
20 preclinical phase at day 6, at either 100 or 30 mg/kg/d, significantly decreased the accumulation of ⁵¹Cr-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
25 developed, this did not reach significance.

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
30 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
5 (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.

10

Example 3

Effect of 2ME2 treatment on joint histology and cartilage damage

Tissue samples, including joints, were fixed in 10% phosphate-buffered formalin or AFA fixative (75% ethyl alcohol, 2% formalin, 5% glacial acetic acid,
15 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
20 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).

25 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
30 100 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

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 diameters), medium (3-5 RBC diameters) and large (diameter greater than 5 RBC diameters or $>30\text{ }\mu\text{m}$) vessels. Only vessels that were in cross-section were scored. There was no significant difference in the vessel density per 0.8 mm^2 between the various treatment groups at any of the vessel sizes. This confirmed the impression

from the immunohistochemical staining that, although leukocyte infiltration was attenuated by 2ME2 treatment, neovascularization was not appreciably modified.

Example 5

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

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 15 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 1.5×10^{-6} M). These 20 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

25 Effect of 2ME2 treatment on splenomegaly

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 30 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.

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

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 naïve, 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

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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.

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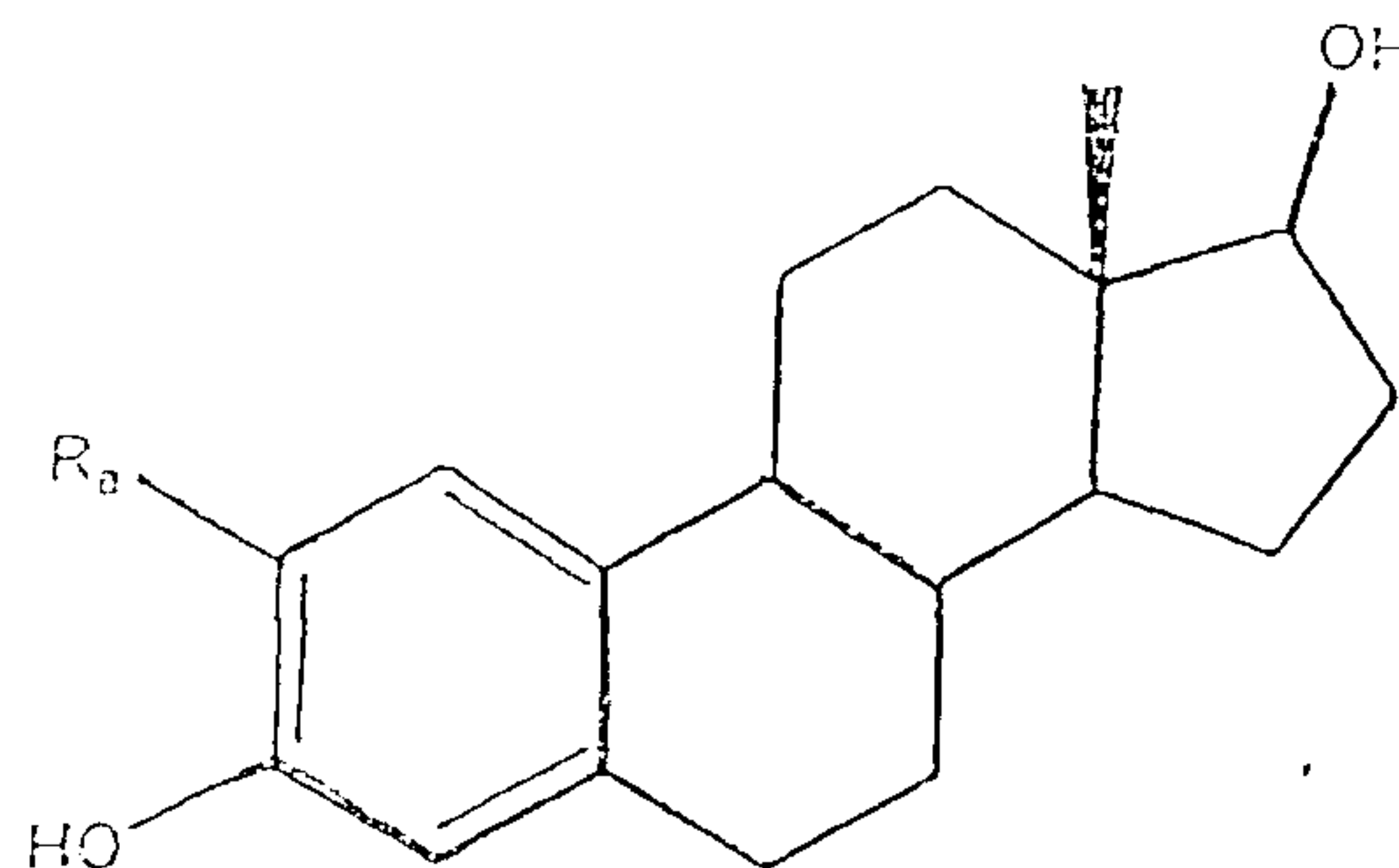
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WHAT WE CLAIM IS:

1. Use of a therapeutic agent and a second compound for treating rheumatic diseases comprising:

a) the therapeutic agent having the structure



wherein R_a is -OCH₃; and,

b) the second compound being methotrexate;

wherein the first therapeutic agent and the second compound are in administrable formulation and in synergistic amounts for treating rheumatoid arthritis; and, wherein the synergistic amounts result in an inhibition of arthritic score that is greater than an expected inhibition of arthritic score.

2. The use of claim 1, wherein the therapeutic agent and the anti-rheumatic agent are in a single administrable formulation.

3. The use of claim 1, wherein the therapeutic agent and the anti-rheumatic in two or more administrable separate formulations.

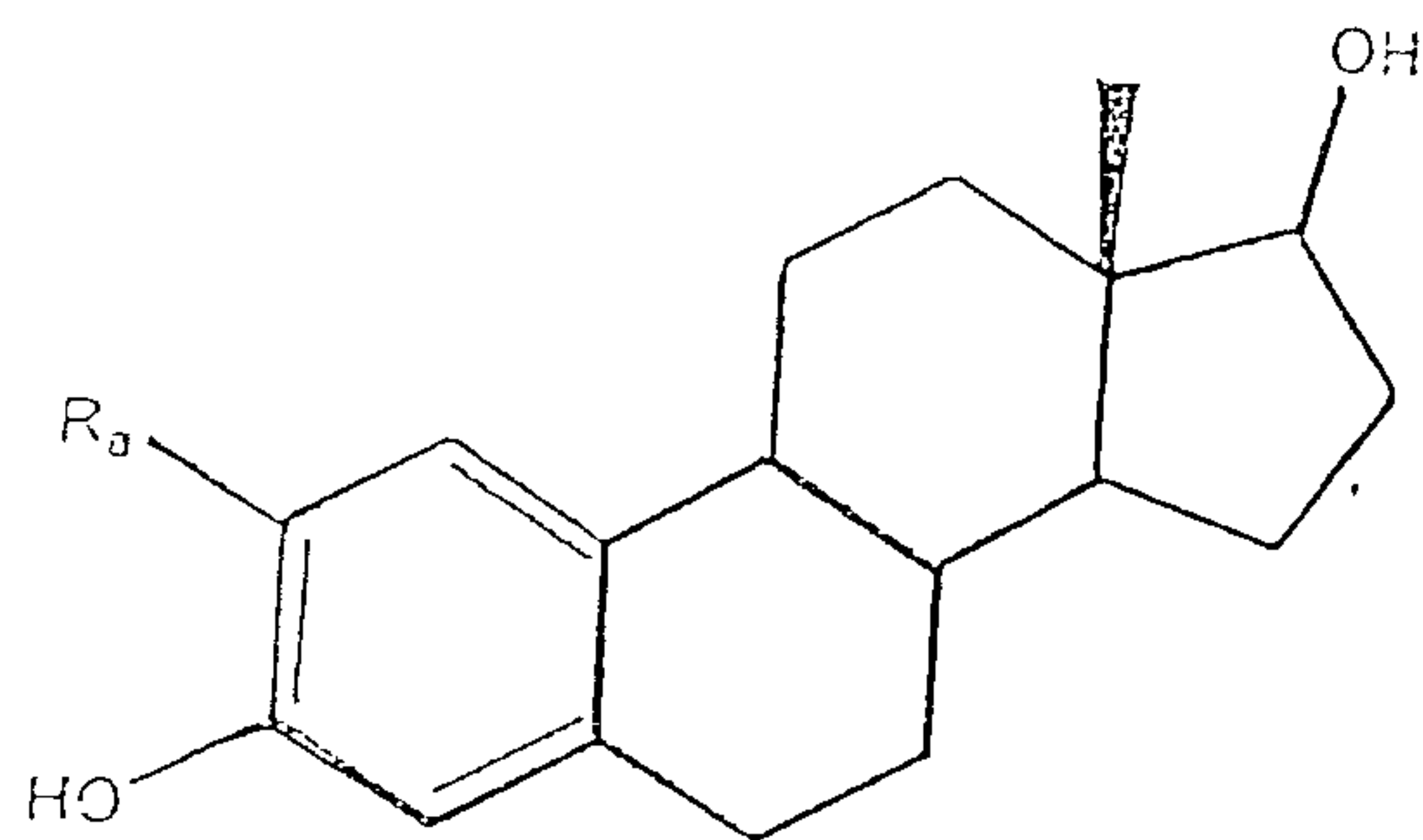
4. The use of claim 1, wherein the first therapeutic agent and the methotrexate are applied together for administration.

5. The use of claim 1, wherein the first therapeutic agent and the methotrexate are applied separately for administration.

6. The use as defined in claim 1, wherein the therapeutic agent is in a formulation of 100 mg/kg of body weight and the second compound in a formulation of 1 mg/kg of body weight.

7. A composition for use in treating rheumatic diseases comprising;

a) a compound having the formula



wherein R_a is $-\text{OCH}_3$; and

b) a second compound being methotrexate,

wherein the first and second compounds are provided in synergistic amounts effective for treating rheumatoid arthritis; and, wherein the synergistic amounts result in an inhibition of arthritic score that is greater than an expected inhibition of arthritic score.

8. The composition of claim 7, wherein the composition comprises:

approximately 100 mg/kg of body weight of the first compound; and

approximately 1 mg/kg of body weight of the second compound.

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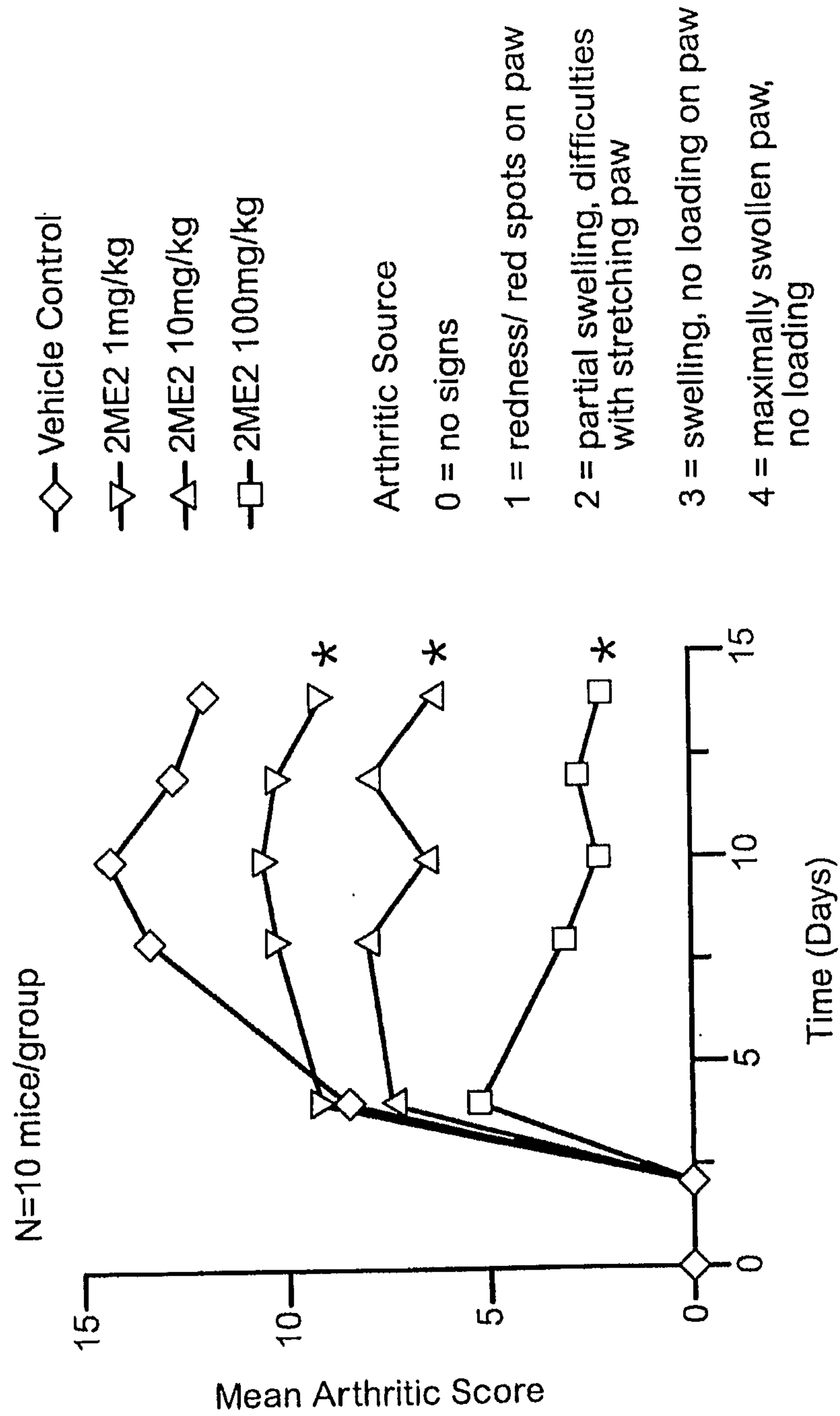
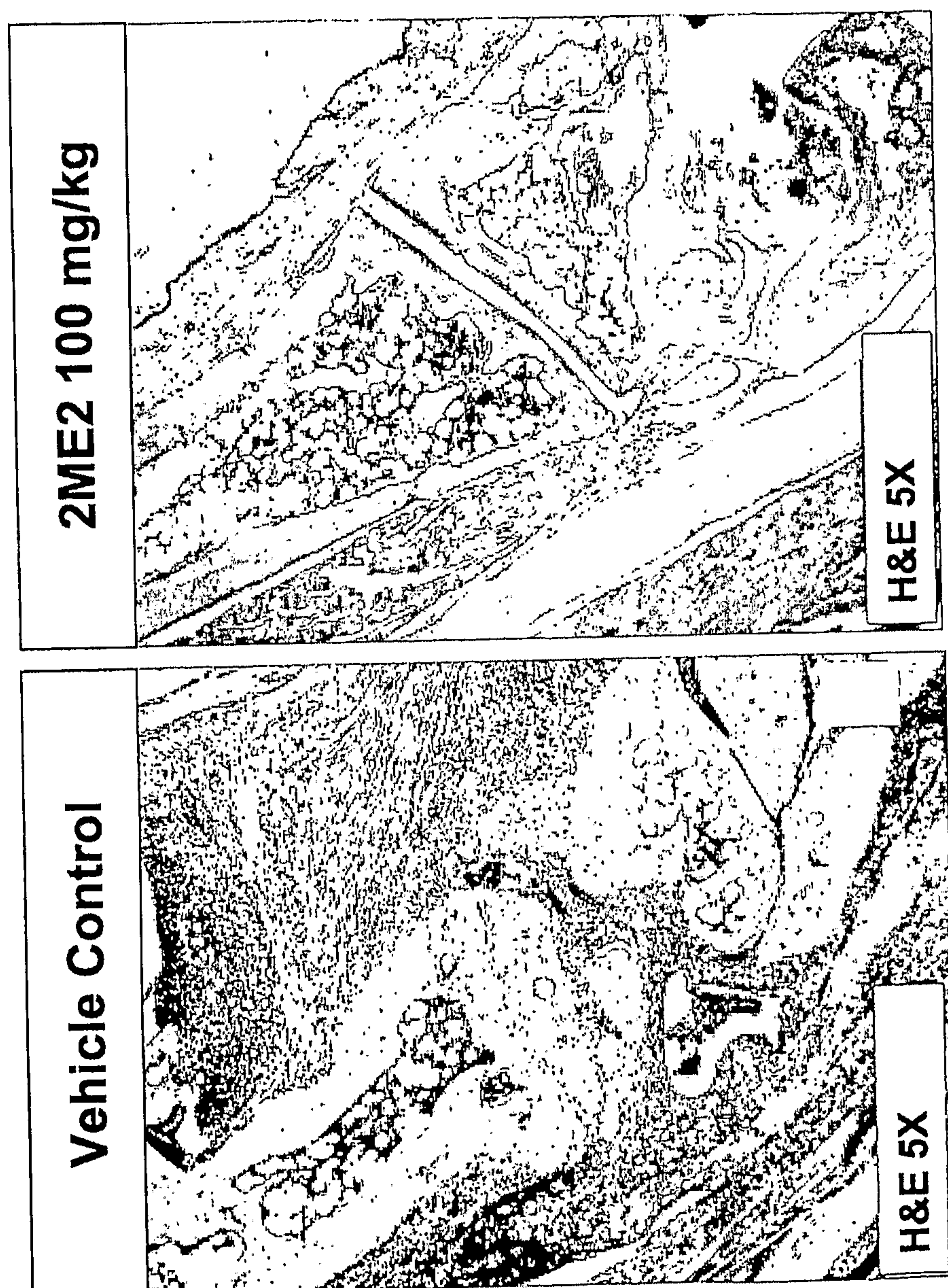
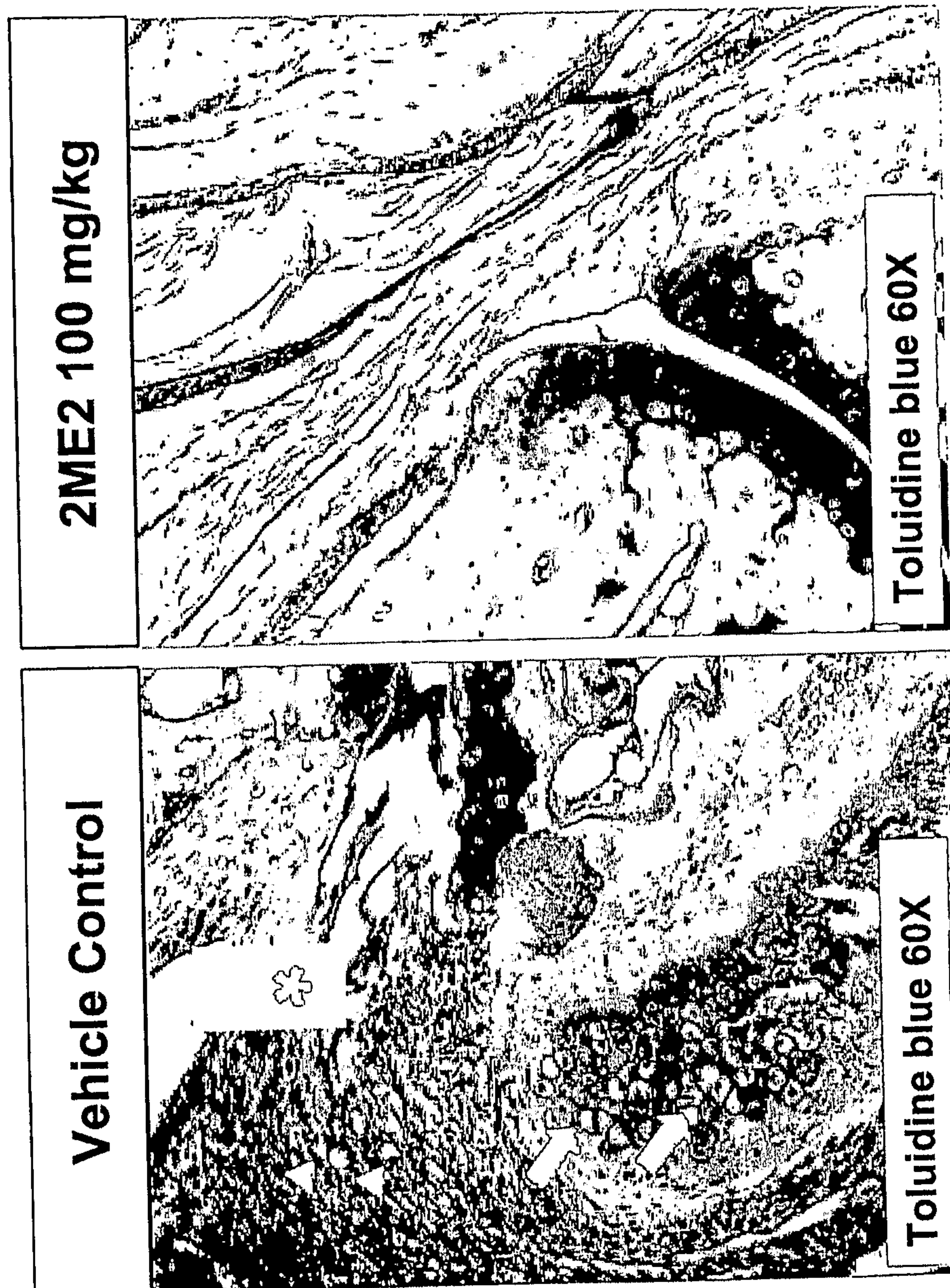


FIG. 1

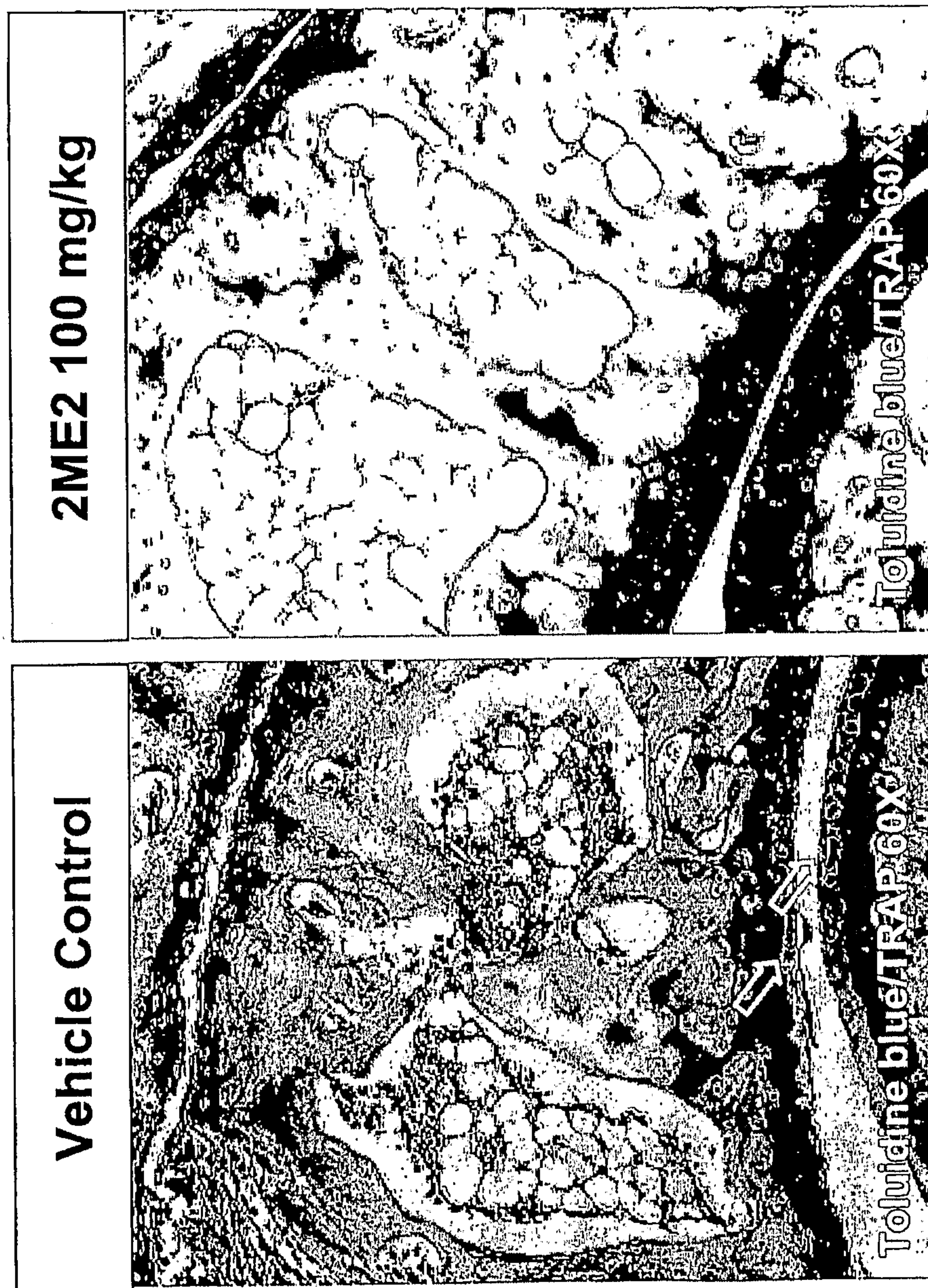
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**FIG. 2**

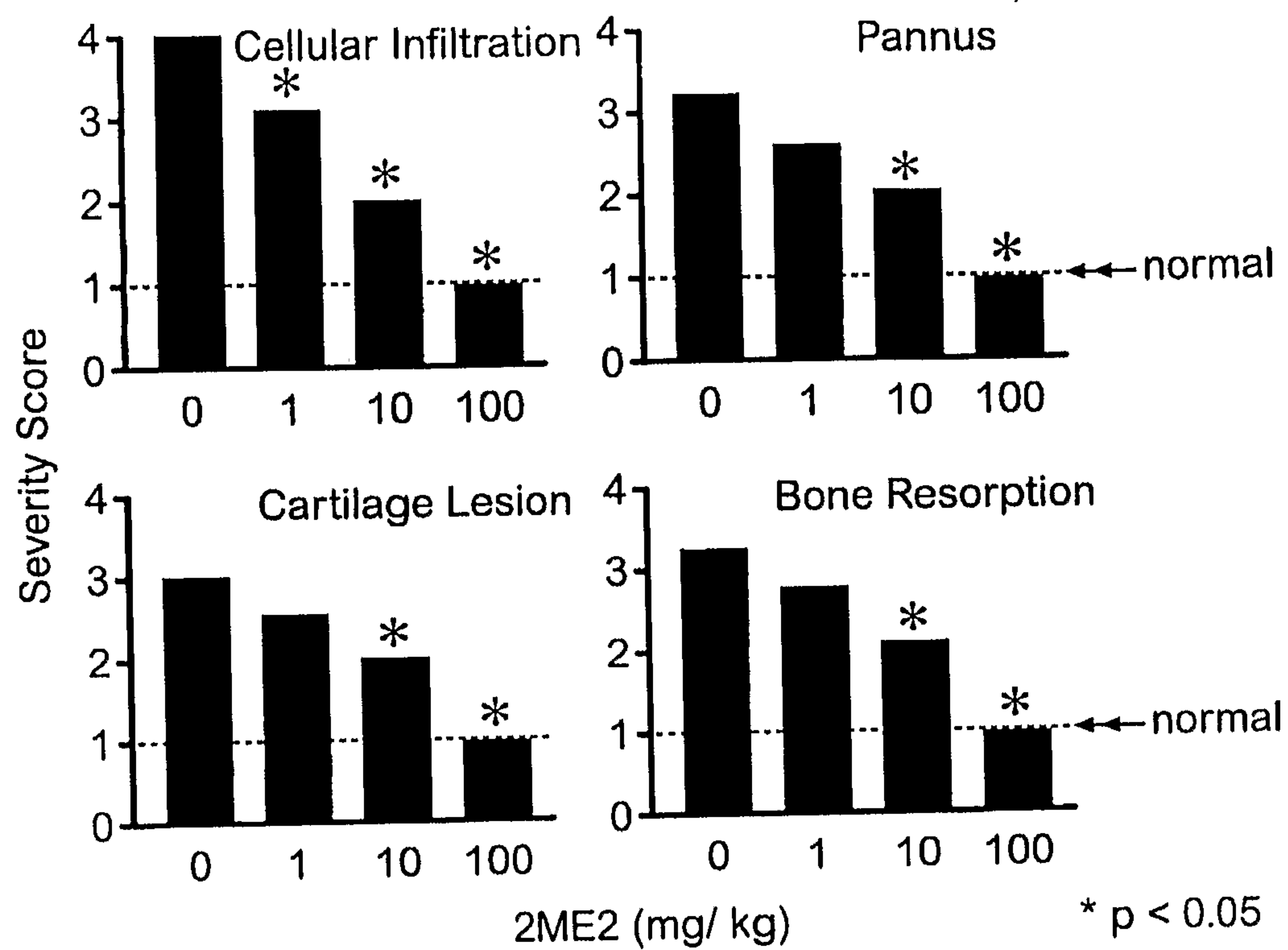
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**FIG. 3**

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**FIG. 4**

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**FIG. 5**

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Treatment Group	Total Osteoclast Numbers	Articular cartilage area without <i>proteoglycan</i> staining (%)	Damaged articular cartilage surface (%)	Thickness of total articular cartilage area (μM)
Vehicle Control	33.9	17.23	47.35	26.06
2ME2 1mg/ kg	21.9	11.21	37.07	27.55
2ME2 10 mg/ kg	5.7	6.71	27.91	27.34
2ME2 100 mg/kg	0	0.18	0	32.64

 $p < 0.05$ **FIG. 6**

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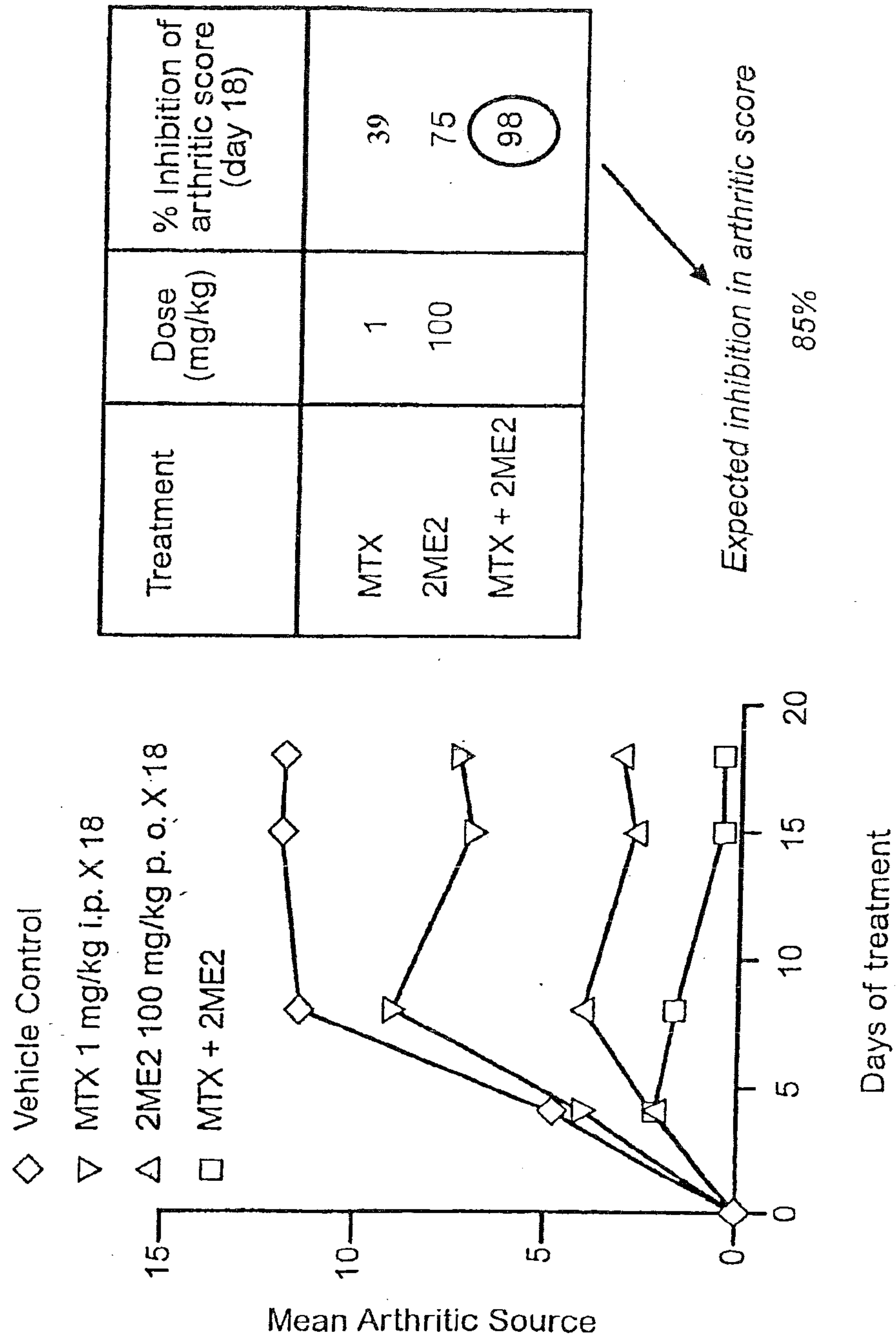
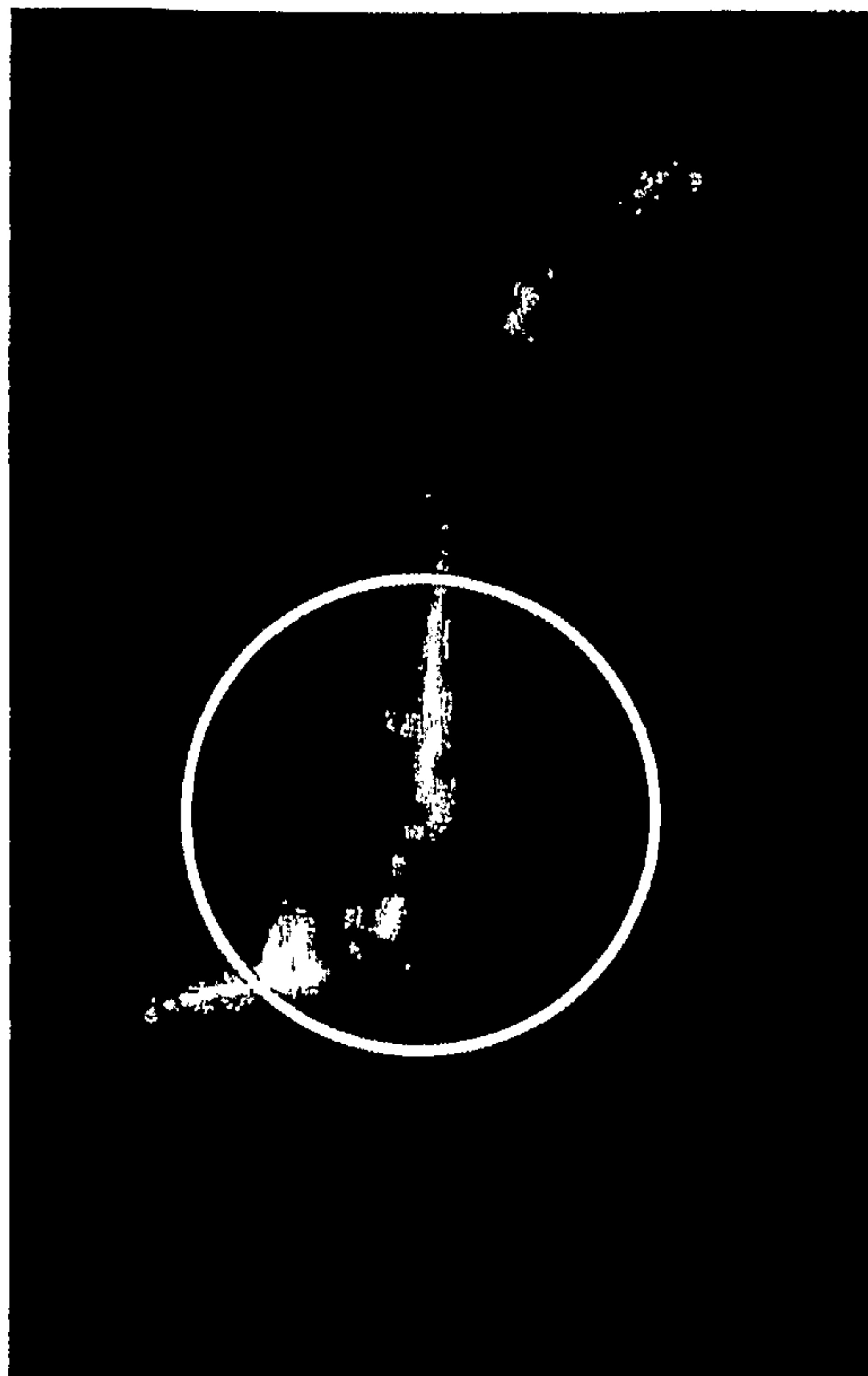


FIG. 7

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2ME2 100mg/kg
(treatment arm)



Vehicle Control

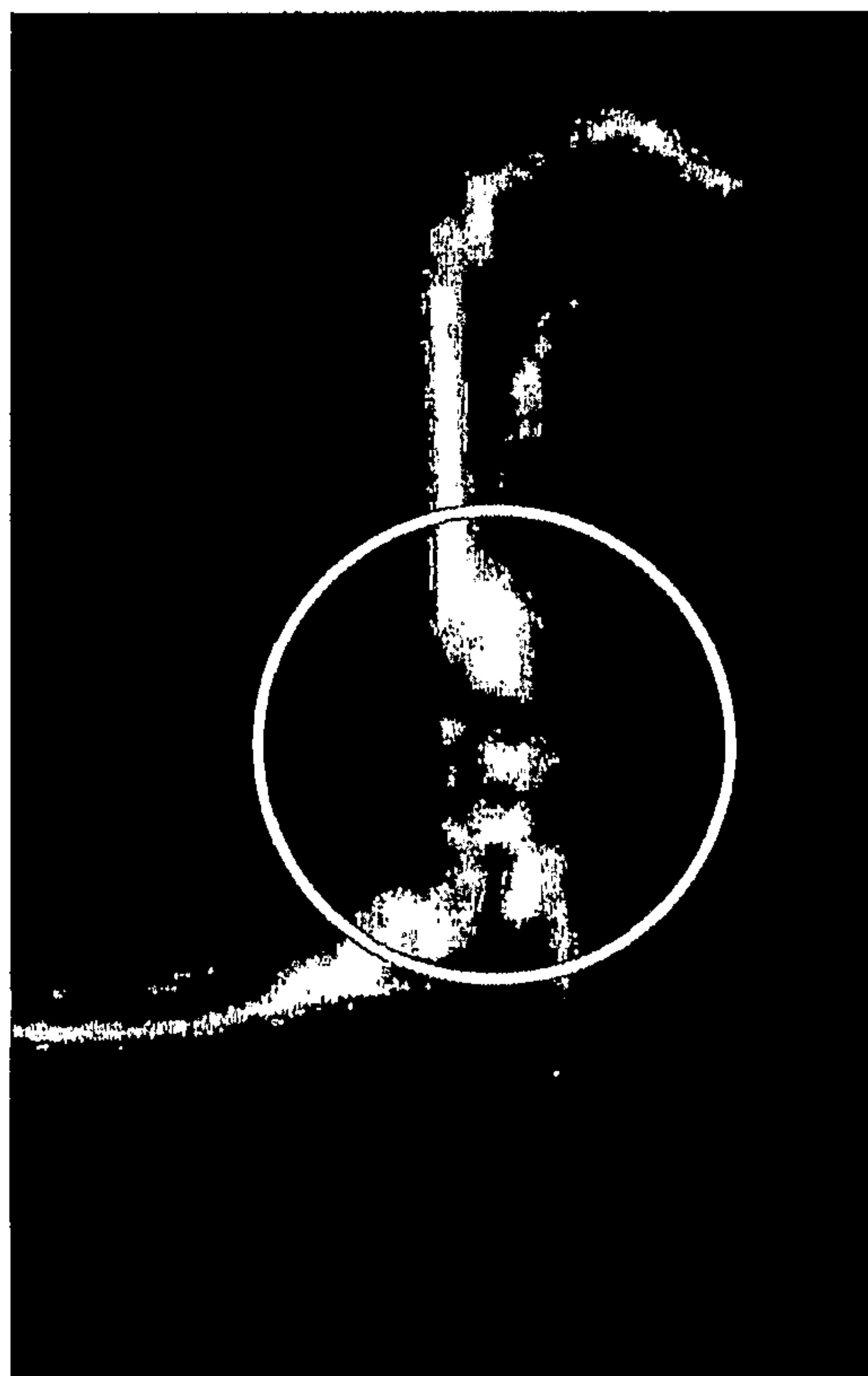
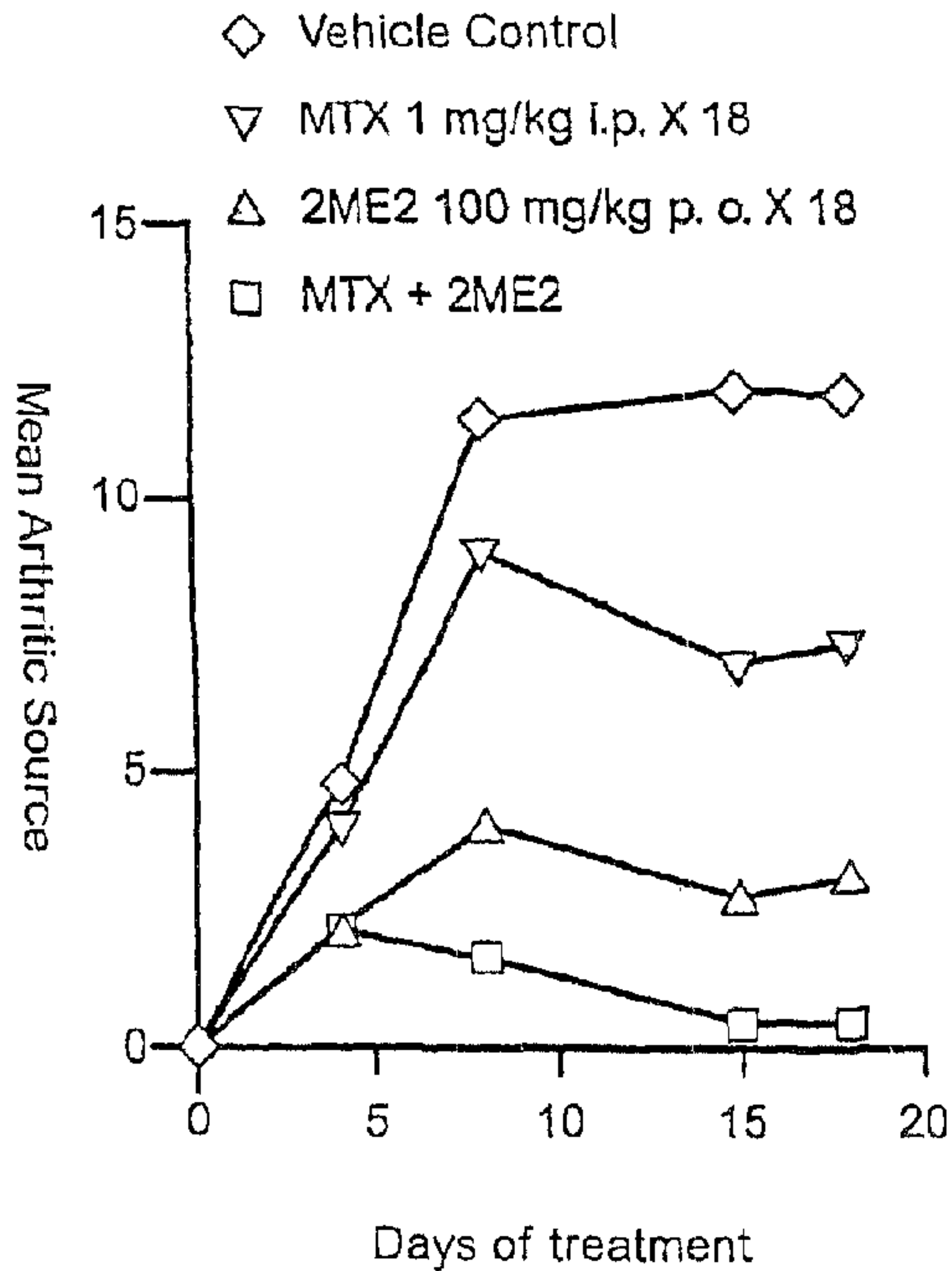


FIG. 8



Treatment	Dose (mg/kg)	% Inhibition of arthritic score (day 18)
MTX	1	39
2ME2	100	75
MTX + 2ME2		98

Expected inhibition in arthritic score

85%