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(54) **Title:** METHOD OF USING PYRUVATE AND/OR ITS DERIVATIVES FOR THE TREATMENT OF CYTOKINE-MEDIATED INFLAMMATORY CONDITIONS

(57) **Abstract:** This invention is directed to a method of using a therapeutic composition comprising a compound of an alpha-ketoalkanoic acid (pyruvate) and/or its derivatives for the treatment of cytokine-mediated inflammatory conditions. The compound is an alpha-ketoalkanoic acid, a physiologically acceptable salt of an alpha-ketoalkanoic acid, an ester of an alpha-ketoalkanoic acid, or an amide of an alpha-ketoalkanoic acid. A component for inducing and stabilizing the enol resonance form of the ester at physiological pH values is also disclosed. The cytokine-mediated inflammatory conditions are mediated by, for example, an "early" (Tumor Necrosis Factor (TNF), interleukin-1β) or "late" (high mobility group B-1 (HMGB-1)) mediator of inflammation. Exemplary cytokine-mediated inflammatory conditions include, but are not limited to, local and systemic inflammation, inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis, asthma (including status asthmaticus), sepsis or septic shock, also including inflammatory skin conditions, for example, psoriasis and eczema.

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TITLE OF THE INVENTION
METHOD OF USING PYRUVATE AND/OR ITS DERIVATIVES FOR THE TREATMENT OF CYTOKINE-MEDIATED INFLAMMATORY CONDITIONS

BACKGROUND OF THE INVENTION
During inflammation, cytokines are secreted by a myriad of cell types in response to endotoxin or other pro-inflammatory stimuli. Exemplary cytokines include tumor necrosis factor (TNF), interferon-gamma (IFN-γ), interleukin-1β (IL-1β) and high mobility group B-1 (HMGB-1). TNF functions as a cell-associated or secreted protein that interacts with a receptor of the TNF receptor family, which in turn communicates with the interior of the cell, with components of TNF receptor associated factors.

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The cytokines are important and critical inflammatory mediators of a wide variety of disease states and conditions. For example, TNF-mediated inflammation results in tissue injury, causing a variety of cellular damage, by generating procoagulant activity on endothelial cells (Pober et al., *J. Immunol.* (1986) 136(5):1680-7), increasing the adherence of neutrophils and lymphocytes (Pober et al., *J. Immunol.* (1987) 138(10):3319-24), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi et al., *J. Exp. Med.* (1987) 166(5):1390-404).

Excessive or unregulated TNF production or activity has been implicated in mediating or exacerbating rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis, gouty arthritis and other arthritic conditions, sepsis, septic shock, endotoxic shock, gram negative sepsis, toxic shock syndrome, adult respiratory distress syndrome, cerebral malaria, chronic pulmonary inflammatory disease, silicosis, pulmonary sarcoidosis, bone resorption diseases, reperfusion injury, graft v. host rejection, allograft rejections, fever and myalgia due to infection, cachexia secondary to infection or malignancy, cachexia secondary to acquired immune deficiency syndrome (AIDS), AIDS related complex (ARC), keloid formation, scar tissue formation, Crohn's disease, ulcerative colitis and psoriasis.

For example, to expand on a specific cytokine-mediated condition, disseminated bacterial infection causes sepsis, a catastrophic clinical syndrome that develops in 750,000 people, and is associated with mortality in about 30% of those affected. (Angus et al., *Crit. Care Med.,* (2001) 29(7):1303-10.) Antibiotics can eradicate infection, but

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mediators released systemically by the innate immune system
mediate the characteristic signs of sepsis, including
microvascular hyperpermeability, coagulopathy, organ
failure, tissue injury and lethal· shock. (Marshall et al.,
5   *Crit. Care Med.* (2001) 29(7 Supp):S99-106.) Significant
advances have been made in understanding the activities and
mechanisms of specific immune-derived mediators, and the
role of timing in the resultant cytokine cascade.
Nonetheless, the development of effective clinical
10 therapeutics for sepsis has been hampered by observations
that previously described agents must be given prior to the
development of sepsis or very soon after onset of the
syndrome in order to improve survival, a scenario that is
obviously difficult to apply to typical clinical situations.
15   The systemic inflammatory ·mediators of lethal sepsis
have "early" and "late" components. TNF is the predominate
early mediator of lethal shock and tissue injury; it is
released within two hours after the onset of acute
infection, and it activates the release of other
20 proinflammatory mediators in a "cytokine cascade." High
mobility group B-1 (HMGB-1), a cytosolic and nuclear protein
first identified as a non-chromosomal DNA-binding protein,
was recently implicated as a "late" mediator of sepsis-
related lethality. HMGB-1 is released by endotoxin-
25 stimulated macrophages, but only after a delay of 12-18
hours; a similar delay in HMGB-1 appearance is observed in
the serum of mice during endotoxemia, an experimental model
that induces a sepsis-like systemic cytokine response. (Wang
et al., *Science* (1999) 285:248-251.) Anti-HMGB-1 antibodies
30 confer significant protection against delayed endotoxin
lethality, even when antibody dosing is initiated at a time

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after the early cytokine responses have peaked and resolved. (Wang et al., *Science* (1999) 285:248-251.) Other cytokine activities of HMGB-1 include stimulation of macrophages to release TNF, IL-1β, and other inflammatory products,

5   inducing chemotaxis of smooth muscle cells, and mediating acute lung injury and lethality. (Abraham et al., *J. Exp. Med.* (2000) 165:2950-2954.) Most drug targets available today are so-called "alarm phase" cytokines, such as TNF and IL-1β, that are released early in the inflammatory response.

10 In the clinical environment, it typically takes many hours to recognize sepsis and institute specific treatment; accordingly, it is not surprising that agents directed against these early proinflammatory cytokines have proved to be ineffective when tested in large clinical trials.

15 (Abraham et al., *Lancet* (1998) 351(9107):929-33; Fisher et al., *JAMA* (1994) 271(23):1836-43.)

It would be useful to specifically ameliorate cytokine-mediated inflammatory conditions with a composition that inhibits, suppresses or antagonizes the production or

20 activity of cytokines, both early and late phase mediators.

SUMMARY OF THE INVENTION

This invention is directed to a method of ameliorating deleterious cytokine effects during

25 inflammation and other cytokine-mediated conditions. In one aspect, the invention prevents endotoxin-induced lethality by attenuating the release of "early" (tumor necrosis factor (TNF) and interleukin-1β (IL-1β)) and "late" (high mobility group B-1 (HMGB-1)) phase mediators

30 of pathogenic systemic injuries. The method of the

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invention also provides for a wider treatment window than previously described.

The present invention features administering to a patient a composition comprising alpha-ketoalkanoic acid and/or one of its derivatives, which specifically includes a physiologically-acceptable salt of alpha-ketoalkanoic acid, an ester of alpha-ketoalkanoic acid, or an amide of alpha-ketoalkanoic acid for the treatment of a cytokine-mediated inflammatory condition. Suitable alpha-ketoalkanoic acids include C_3-C_8 straight chained or branched alpha-ketoalkanoic acids, for example, pyruvic acid. Physiologically acceptable salts, of α-ketoalkanoic acids include Na^+, K^+, Ca^{++}, Mg^+, NH_4^+ and the like.

Preferably, the therapeutic agent used in the method disclosed herein is an effective amount of an ester of an alpha-ketoalkanoic acid, for example, a C_3-C_8 straight-chained or branched alpha-ketoalkanoic acid ester. Examples include alpha-keto-butyrate, alpha-ketopentanoate, alpha-keto-3-methyl-butyrate, alpha-keto-4-methyl-pentanoate or alpha-keto-hexanoate. Pyruvate is preferred. A variety of groups are suitable for the ester position of the molecule, e.g., alkyl, aralkyl, alkoxyl, carboxyalkyl, glyceryl or dihydroxy acetone. Specific examples include ethyl, propyl, butyl, carboxymethyl, acetoxymethyl, carbethoxymethyl and ethoxymethyl. Ethyl esters are preferred. Thiolesters (e.g., wherein the thiol portion is cysteine or homocysteine) are also included.

In a preferred embodiment, the pharmaceutical composition used in the disclosed method comprises ethyl pyruvate, propyl pyruvate, carboxymethyl pyruvate, acetoxymethyl pyruvate, carbethoxymethymethyl pyruvate,

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ethoxymethyl pyruvate, ethyl alpha-keto-butyrate, ethyl
alpha-keto-pentanoate, ethyl alpha-keto-3-methyl-butyrate,
ethyl alpha-keto-4-methyl-pentanoate, or ethyl alpha-keto-
hexanoate. Ethyl pyruvate is more preferred.

5   In yet another aspect, the therapeutic agent used in
the method disclosed herein is an effective amount of an
amide of an alpha-ketoalkanoic acid. Suitable amides of
alpha-ketoalkanoic acids for use in the method of the
present inventions include compounds having the following
10   structural formula: $RCOCONR^1R^2$. R is an alkyl group; R^1 and
R^2 are independently -H, alkyl, aralkyl, alkoxyalkyl,
carboxyalkyl or $-CHR^3COOH$; and R^3 is the side chain of a
naturally occurring amino acid. Preferably, the amide of an
alpha-ketoalkanoic acid is a pyruvamide.

15   In another aspect, when the ester derivative is used, a
component for inducing and stabilizing the enol resonance
form of the ester at physiological pH values is included in
the composition. The stabilizing component is a cationic
material, preferably a divalent cation, and most preferably
20   calcium or magnesium. This composition further comprises a
pharmceutically acceptable inert carrier substance in which
the enol resonance form of the ester is stabilized at
physiological pH values.

           The pharmaceutically acceptable inert carrier in the
25   composition of the invention can be any carrier substance
generally recognized as safe for administering a therapeutic
agent to a mammal, e.g., a buffer solution for infusion, a
tablet for oral administration or in gel, micelle or
liposome form for on-site delivery. Examples of delivery
30   solutions   include   isotonic   or   hypertonic   saline;

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bicarbonate, phosphate or citrate buffer, plasma extender, or a microcolloid or microcrystalline solution.

The conditions treatable by the method of the invention include, but are not limited to, local and
5 systemic inflammation, inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis, asthma (including status asthmaticus), sepsis and septic shock. The method of the invention also includes the treatment of inflammatory skin conditions, e.g., psoriasis
10 and eczema.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred
15 embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Figs. 1A-B show the structures of the preferred alpha-ketoalkanoic acid esters and amides in the composition of the invention;

20 Fig. 2 depicts a bar graph showing the effect of ethyl pyruvate on the permeability of Caco-2 monolayers incubated for 48 hours with a cocktail of cytokines ("cytomix") ("Cyto") containing IFN-γ (1000 U/ml), IL-1β (1 nM) and TNF-α (10 ng/ml). REPS blocks cytokine-induced intestinal
25 epithelial hyperpermeability;

Figs. 3A-C depict the effect of ethyl pyruvate pretreatment in preventing endotoxin lethality by attenuating TNF and HMGB-1 release *in vivo*. (a) Kaplan-Meier plot mice exposed to lethal doses of endotoxin (5 mg LPS/kg,
30 i.p. at time = 0 hr) after pretreatment with EP (single EP dose of 40 mg/kg; i.p. at t=-30 minutes). EP conferred

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significant protection against lethality (P<0.05). (b) Ethyl pyruvate pretreatment significantly attenuates the release of serum TNF at 2 hours after LPS. (c) Ethyl pyruvate significantly inhibits serum HMGB-1 levels at 20 hr after

5   LPS. P<0.05 for TNF and HMGB-1 of EP as compared to vehicle treated endotoxemic animals; n=5 for an experiment that was repeated twice;

Figs. 4A-C depict the effects of delayed administration of ethyl pyruvate in attenuating the lethality of

10 endotoxemia and sepsis HMGB-1. (a) Kaplan-Meier plot of mice subjected to a lethal dose of LPS (3.75 mg LPS/kg; i.p.) and delayed administration of ethyl pyruvate (40 mg/kg, i.p.), starting 4 hours after LPS injection and repeatedly dosed at 8, 12, 24 and 30 h. (b) HMGB-1 release was analyzed by

15 western blot of serum collected at 20 hrs. LPS induces the release of HMGB-1 in vivo (lanes 4,5,6), and delayed treatment with ethyl pyruvate (lanes 7,8,9) significantly prevents the release of serum HMGB-1. (c) Kaplan-Meier plot of septic mice with cecal perforation treated with ethyl

20 pyruvate (40 mg/kg, i.p.) beginning 24 hours after the onset of peritonitis induced by cecal perforation at time = 0 hr. Note that in both endotoxemia and CLP models, delayed administration of ethyl pyruvate significantly increased survival;

25   Figs. 5A-B show that ethyl pyruvate inhibits TNF transcription and synthesis. (a) The influence of ethyl pyruvate on TNF release by LPS-stimulated macrophage cultures was measured by ELISA on macrophage conditioned media stimulated with 50 or 100 ng/ml of LPS. Note that the

30 concentration of ethyl pyruvate that inhibited 50% of the TNF response (ethyl pyruvate EC_{50}) from macrophage cultures

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was 3.4 mM, a clinically achievable concentration. (b) Ethyl
pyruvate prevents the transcription of the TNF gene. The
effect of ethyl pyruvate on TNF transcription was analyzed
by Ribonuclease protection assay (RPA III™, Ambion, Austin,
5   TX). Note that ethyl pyruvate prevents LPS-induced
transcription of TNF in an ethyl pyruvate concentration
dependent fashion;

Figs. 6A-D show that ethyl pyruvate prevents LPS-
induced activation of NF-κB and p38MAPK pathways in
10   macrophage cultures. (a) RAW264.7 cells were stimulated with
LPS in the presence of the indicated concentrations of ethyl
pyruvate. The activation of NF-kB was analyzed by EMSA at 15
min and 4 hours using a ^{32}P-labeled NF-κB probe. Ethyl
pyruvate suppressed activation of NF-κB in an ethyl pyruvate
15   concentration dependent fashion. The experiment was
repeated 3 times. (b) Supershift and competition studies.
Nuclear extract of macrophages stimulated with LPS and
incubated with antibody against p65-Rel to induce specific
supershift of NF-κB complex. Nuclear extracts were incubated
20   with 100-fold molar excess of unlabeled (cold) NF-κB or HIF-
1 probe for competition analysis. (c) RAW264.7 cells were
stimulated with LPS in the presence of the indicated
concentration of ethyl pyruvate. The phosphorylation of
p38MAPK was analyzed by western blot using antibodies
25   against phosphorylated (thr180/tyr182) p38MAPK (in
accordance with the manufacturer (New England Biolabs, Inc
Cat. # 9210, Beverly, MA). (d) The total amount of p38 MAPK
is visualized by western blot. Ethyl pyruvate prevents the
phosphorylation of p38MAPK in macrophage cultures without
30   affecting the intracellular level of total p38MAPK; and

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Figs. 7A-B show that ethyl pyruvate inhibits HMGB-1 release. (a) Ethyl pyruvate inhibits release of HMGB-1 from macrophage cultures as evidenced by western blot of conditioned culture media 24 hrs after addition of LPS. (b)

5   Analysis of the effect of ethyl pyruvate on total cell associated levels of HMGB-1. Note that ethyl pyruvate did not affect the intracellular stability and levels of HMGB-1 either at 10 or 20 h after treatment, suggesting that ethyl pyruvate specifically prevents the release of HMGB-1.

10

DETAILED DESCRIPTION OF THE INVENTION

Unexpectedly, it has been discovered that a composition comprising alpha-ketoalkanoic acid and/or one of its derivatives can ameliorate a cytokine-mediated inflammatory

15   condition. The method of the invention has been tested, e.g., in a standard mouse model of murine sepsis, in which it was shown that ethyl pyruvate rescues mammals from lethal sepsis caused by peritonitis, even when the first doses of a derivative of pyruvate were administered 24 hours after

20   sepsis was established. The precise mechanism(s) responsible for the protective actions of pyruvate and/or its derivatives remain to be elucidated. Nevertheless, an exemplary derivative, ethyl pyruvate, is shown herein to inhibit the release of both TNF and HMGB-1 from LPS-

25   stimulated macrophages and to down-regulate activation of the pro-inflammatory p38 MAP kinase and NF-κB signal transduction pathways. These data suggest that treatment using pyruvate and/or its derivatives in patients with established sepsis can be deferred even after the onset of

30   clinical signs of inflammation.

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In an effort to obtain some clues regarding the basis for the therapeutic benefit afforded by ethyl pyruvate in murine models of acute endotoxemia and lethal polymicrobial bacterial peritonitis, the effect of the compound on

5   activation of two important pro-inflammatory signaling cascades was assessed. These studies revealed that the incubation of LPS-stimulated macrophages with ethyl pyruvate inhibits signal transduction through two essential pathways, p38 MAP kinase and NF-κB. Macrophage activation by

10   endotoxin, cytokines, and products of cell injury lead to the nuclear translocation of NF-κB, a transcription factor that enhances the transcription of TNF and other products of the activated macrophage (Senftleben et al., *Crit. Care Med.* (2002) 30(1 Suppl):S18-26). Macrophage activation also leads

15   to phosphorylation of p38 MAP kinase, a pathway that has been implicated in stabilizing TNF mRNA, and increasing TNF translation efficiency (Adams et al., *Prog. Med. Chem.* (2001) 38:1-60).

The late stage mediator of delayed endotoxin lethality,

20   HMGB-1, is a cytosolic and nuclear protein known previously only as a transcription factor that stabilizes nucleosome structure. HMGB-1 is released by activated macrophages, but only after a significant lag of 12-18 hours; a similar kinetic course is observed during lethal endotoxemia (Wang

25   et al., *Science* (1999) 285:248-251). This delayed kinetic of HMGB-1 release enables the application of anti-HMGB-1 antibodies downstream of the early TNF response, suggesting a therapeutic approach that can target a late mediator of lethality. The present study found that ethyl pyruvate

30   significantly inhibited HMGB-1 release from macrophages, and decreased serum HMGB-1 levels in septic mice. Macrophages

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contain large quantities of HMGB-1 in the cytosol that provide a pre-formed pool of HMGB-1 that can be released during activation. The mechanism(s) that regulate HMGB-1 release from macrophages is enigmatic, but the present data indicate that ethyl pyruvate inhibits the release of the cytosolic protein from activated macrophages. Inhibition of HMGB-1 release by ethyl pyruvate was specific, because ethyl pyruvate did not affect intracellular HMGB-1 protein expression or stability. The present studies show that ethyl pyruvate inhibition of signaling through NF-κB and p38 MAPK pathway may underlie the ability of ethyl pyruvate to suppress HMGB-1 release from LPA-stimulated cells. Additionally, inhibition of TNF in the local macrophage milieu contributes to inhibition of HMGB-1 release, because TNF is a potent inducer of HMGB-1 release.

Together, these results reveal that pyruvate and/or its derivatives have the therapeutic potential for disease mediated by an excess of TNF-α and HMGB-1. Preferably, ethyl pyruvate is a relatively non-toxic food additive, and the observed effects occur in therapeutically achievable and safe levels. Ethyl pyruvate can be administered in Ringer's-type crystalloid fluid, a calcium- and potassium-containing balanced salt solution that is widely used as resuscitating agent for the treatment of shock.

Accordingly, it is a primary object of this invention to provide new method of treating inflammatory conditions, particularly those that are mediated by cytokines, using an improved composition containing alpha-ketoalkanoic acid and/or its derivatives.

To achieve the foregoing object and in accordance with the purpose of the invention, as embodied and broadly

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described herein, one composition of this invention comprises a alpha-ketoalkanoic acid ester, in accordance with the molecular structures shown in Fig. 1, admixed with a sufficient concentration of biologically safe organic or inorganic cations to induce enolization of the alpha-keto functionality of the ester at physiological pH values. In a preferred embodiment, the composition comprises an alkyl ester of alpha-ketopropionic acid (pyruvic acid), the ester is the ethyl analog and the cation is a divalent cation, particularly either calcium or magnesium. In a particularly preferred formulation of the composition of the invention, the ester compound is ethyl pyruvate admixed with calcium ion in a Ringer's solution at a pH of about 7-8.

The therapeutic compositions of the invention may be administered orally, topically (e.g., ointment, gel or cream), or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, intraluminally, intra-arterially, intravaginally, transurethrally or rectally) by routine methods in pharmaceutically acceptable inert carrier substances. For example, the therapeutic compositions of the invention may be administered in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels, liposomes, or a buffer solution.

Preferably, the pharmaceutical composition is administered as an infusate at a concentration of, e.g., 10 mM to 200 mM, preferably 20 mM to 90 mM of alpha-ketoalkanoic acid, at a rate of 1 mg/kg body weight/day to 200 mg/kg body weight/day, in a buffer solution as described herein. More preferably, the pharmaceutical composition is

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administered as an infusate at a concentration of about 28 mM of alpha-ketoalkanoic acid, at a dose of 100 mg/kg body weight/day to 150 mg/kg body weight/day of alpha-ketoalkanoic acid, in a buffer solution. In bolus form, the

5   active agent can be administered at a similar dosage, e.g., 1 mg/kg body weight/day to 200 mg/kg body weight/day of alpha-ketoalkanoic acid, where the dosage is divided into aliquots and delivered 1 to 4 times daily (for a total dosage of 1 mg/kg body weight/day to 200 mg/kg body

10 weight/day), with the concentration of alpha-ketoalkanoic acid adjusted accordingly. The enolization agent in the composition of the invention is at an appropriate concentration to induce enolization of the alpha-keto functionality of the amount of active ester agent in the

15 administered composition. Optimal dosage and modes of administration can readily be determined by conventional protocols.

Exemplary cytokine-mediated inflammatory conditions include, but are not limited to, local and systemic

20 inflammation, inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis, asthma (including status asthmaticus), endotoxemia, sepsis and septic shock, also including inflammatory skin conditions, for example, psoriasis and eczema.

25

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit

30 the scope of the disclosure.

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Exemplary Materials and Methods

Animal Experiments. Male 6-8 week old Balb/C mice (20-25g) were purchased from Harlen Sprague-Dawley (Indianapolis, IN) and allowed to acclimate for 7 days housed at 25 ^{0}C on

5   a 12 h light/dark cycle. Animals were randomly grouped (10 mice per group) and assigned to a specific experiment. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee

10   of North Shore University Hospital and New York University Medical School.

Endotoxin shock. Mice were injected with endotoxin (*Escherichia coli* lipopolysaccharide, LPS 0111:B4; Sigma

15   Chemical Co, St. Louis, MO) that was dissolved in sterile pyrogen-free saline at stock concentrations of 10 mg/ml. LPS solutions were sonicated for 20 min immediately prior to use for each experiment. Mice received an LD$_{75}$ dose of LPS (5 mg/kg, i.p.). Blood was collected at different times after

20   LPS administration, allowed to clot for 2 h at room temperature, then centrifuged for 20 min at 1,500 g. Serum samples were stored at 20 ^{0}C prior to analysis. Mortality was recorded for up to 3 weeks after injection to ensure that no additional late deaths occurred.

25

Cecal ligation and puncture (CLP). Cecal ligation and puncture (CLP) was performed as described by (Wichterman et al., *J. Surg. Res.* (1980) 29(2):189-201). Briefly, mice were anesthetized with 20 mg/ml xylazine intramuscularly at a

30   dose of 1.0 g/kg, a midline incision was performed, and the cecum was isolated. A 6-0 prolene suture ligature was

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placed at a level 5.0 mm from the cecal tip away from the ileocecal valve. The ligated cecal stump was then punctured once with a 22-gauge needle, and stool extruded (1 mm) to ascertain patency of the puncture site. The cecum was then

5   placed back into its normal intra-abdominal position, and the abdomen was closed with a running suture of 6-0 prolene in two layers, peritoneum and fascia separately, to prevent leakage of fluid. All animals received an antibiotic (primexin 0.5 mg/kg s/c) 12 hr after surgery as a single

10   dose. All animals received resuscitation with normal saline 24 hr after surgery as a single injectate (20 ml/kg of body weight). Mortality was recorded for up to 1 week after the procedure; survivors were followed for 3 weeks to ensure no late mortalities had occurred.

15

Ethyl Pyruvate Solution: Ethyl pyruvate was prepared in solution with sodium (130 mM), potassium (4 mM), calcium (2.7 mM), chloride (130 mM), and ethyl pyruvate (28 mM); pH 7.0). For injections in mice solutions were diluted so that

20   each injection volume was 0.4 ml per dose.

Cell Culture. BALB/c murine macrophage-like RAW 264.7 cells obtained from the American Type Culture Collection (ATCC Number TIB-71, ATCC, Rockville, MA) (61), were cultured in

25   RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Gemini, Calabasas, CA), 2 mM glutamine (Cat. # 25030-149, Gibco BRL, Rockville, MA) and antibiotic-antimycotic mix (Cat. # 15240-062, Gibco BRL, Rockville, MA) in a humidified incubator

30   with 5% CO_2. Cells were mechanically removed and resuspended

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in serum-free Opti-MEM I medium (Life Technology, Grand Island, NY) to perform experiments at 75% confluence.

Cytokine measurements. TNF concentration in mouse serum and
5   in conditioned media from RAW 264.7 cell cultures was measured by ELISA (minimum detectable concentration = 10 pg/ml). Recombinant mouse TNF standards were obtained from R&D Systems (Inc., Minneapolis, MN) and dissolved in 0.1% bovine serum albumin solution (BSA, low endotoxin grade from
10   Sigma Chemical Co., St. Louis, MO). Monoclonal antibody to mouse TNF was purchased from Biosource International, Inc., Camarillo, CA. Human TNF monoclonal antibody, human TNF antiserum and mouse TNF antiserum were prepared and contributed by Dr. Christine Metz. Mouse serum IL-6 and IL-
15   1β levels were measured using ELISA kits (R&D Systems, Inc., Minneapolis, MN). HMGB-1 was analyzed by western blot as previously described by Wang et al, 1999. Briefly, serum or cell culture conditioned media was first filtrated through centricon YM-100 (Millipore Corp., Bedford, MA) to clear the
20   samples from cell debris and macromolecular complex formed during clotting. Then, samples were concentrated 15 folds by using centricon YM-30 and subjected to 12% SDS-polyacrylamide gels (SDS-PAGE). Protein was electroblotting to Immun-blot PVDF membrane (Bio-rad, Hercules CA) and HMGB-
25   1 was analyzed using polyclonal 241 antibody and secondary anti-rabbit horseradish peroxidase (Amersham, Pitscataway, NJ). Standard curves were constructed using r-HMGB-1, and the intensity of the 30kD band analyzed by densitometry.

30   *Nuclear extract preparation.* The day before use, cells were plated at a density of $1x10^6$ per well in 6 well tissue

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culture plates. After stimulation at indicated times, cells were removed from the incubator and place on ice immediately. Cells were washed once with 2 ml phosphate buffered saline (PBS) then harvested in 1 ml of PBS

5   containing 2 % FBS using rubber scrapper. The cells were transferred to a 1.5 ml tube and centrifuged at 14,000 g for 10s; the pellet was resuspended in 600 µL of buffer I (10mM pH=7.8 Tris.HCL, 10 mM KCL, 1.5 mM $MgCl_2$, 0.3M sucrose, 500 µM phenylmethylsulfonyl fluoride, 1.0 mM sodium

10 orthovanadate, 1 mM dithiothreitol, and protease inhibitor cocktail (Sigma-Aldrich Catalog# p-8340) and incubated for 15 min. 38.3 µl 10% NP40 was added and the tube was vortexed at full speed for 10 s. Nuclei were isolated by centrifugation at 300g for 3 min. The supernatant was

15 aspirated, and the nuclear pellet was gently resuspended in 80 µL of buffer II (10mM pH=7.8 Tris. HCL, 420 mM KCL, 1.5 mM $MgCL_2$, 20% glycerol). Following a 15 min incubation, nuclear extracts were cleared by centrifugation at 14,000g for 10 min. The supernatant was transferred to a new tube,

20 protein concentration was determined using a commercially available Bradford assay (Bio-Rad Protein Assay, Hercules, CA). Nuclear extracts were frozen at -80°C.

Electrophoretic Mobility Shift Assays (EMSA). The sequence

25 of the double-stranded NF-κB oligonucleotide was as follows: Sense: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', antisense: 3'-TCA ACT CCC CTG AAA GGG TCC G-5' (Promega, Catalog # E3291). The oligonucleotides were end-labeled with $^{\gamma-32}P$ adenosine triphosphate (New England Nuclear, Boston, MA) using T4

30 polynucleotide kinase (Promega, Madison, Wis). 3 µg of nuclear protein/reaction was incubated with $^{\gamma-32}P$-labeled NF-

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κB probe in bandshift buffer (13 mM pH=8.0 HEPES, 65 mM NaCl, 1mM DTT, 0.14 mM EDTA, 8% glycerol) in the presence of 1 ng of calf thymus DNA for 20 min at room temperature. For competition reactions 100 fold molar excess of cold
5   oligonucleotide was added simultaneously with labeled probe. Supershift assays were performed by incubating nuclear extracts with 2 μL of anti-p65 and anti-p50 (Santa Cruz Biotechnology) for 1 h prior to the addition of radiolabeled probe. The binding reaction mixture was electrophoresed on
10 4% nondenaturing polyacrylamide electrophoresis (PAGE) gels. After PAGE, the gels were dried and exposed to XAR-5 film (Kodak, Rochester, NY) at -80°C overnight using an intensifying screen.

15 *Total RNA extraction and RNase protection assay.* Total RNA was extracted from cultured cells by using RNAzol B in accordance with the manufacturer's instructions (Tel-Test "B" Inc, Friendswood, TX). The integrity of the RNA was verified by electrophoresis on 1.2% agarose/17%
20 formaldehyde gels. The levels of TNF and cyclophilin mRNA in RAW cells were measured using an RNase protection assay kit from PharMingen in accordance with the manufacturer's instructions (San Diego, CA). The antisense RNA probe was labeled with α-^{32}P-UTP (800 Ci/mmol, Amersham, IL) using T7
25 RNA polymerase. The protected transcript of TNF is 287 bp, and the control transcript (cyclophilin) is 105 bp. Molecular weight markers were prepared using pBR-322 plasmid DNA digested with MSP I (New England Bio Labs, Beverly, MA) and Klenow end-labeled (Strategene, La Jolla,
30 CA) with α-^{32}P-dCTP (800 Ci/mmol, Amersham); mRNA levels were measured with an InstantImager (Packard Instrument

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Co., Meriden, CT).

Statistical analysis. All data in the figures and text are expressed as mean ± SEM for at least 20 animals per
5 condition. Significance of Differences between groups were determined by two-tailed Student's t-test, one way ANOVA followed by the least significant difference test or Fisher's LSD Exact Test or the Chi-square test, as appropriate. FD4 clearance data were logarithmically
10 transformed prior to performing statistical analyses. Differences were considered significant for $p<0.05$.

EXAMPLE I
Stability of pyruvate esters in solution

15   Ethyl pyruvate affords a more stable aqueous solution than sodium pyruvate in the presence of calcium salts, and this observation can be extended to the study of other pyruvate ester analogs, as shown in Fig. 1A, by dissolving them in Ringer's solution containing at least 0.2
20 equivalents of calcium per molar equivalent of pyruvate analog titrated with sodium hydroxide, or other suitable inorganic alkali, to physiological pH values. A specific "pyruvated" Ringer's solution for use in NMR, stability, and subsequent biological studies is shown in Table 1. Another
25 exemplary buffer solution is given in Table 2.

It is to be understood that the pyruvate analog in the instant example may be substituted with any of the analogs shown in Fig. 1 at any concentration sufficient to afford a homogenous solution or substituted by control substances for
30 comparative purposes, such as pyruvic acid, lactic acid (as would be the case in "lactated" Ringer's solution and other

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reference or inactive ketoacid analogs. The calcium cation may also be substituted, e.g., with magnesium or any other biologically safe cation capable of substituting for calcium and stabilizing the formation of transient coordination complexes with pyruvate ester enolates in aqueous solution.

TABLE 1. Constituents of a Ringer's Ethyl Pyruvate Solution (REPS)

Component	Composition	Range
Isotonic saline	75cc	-- (fixed)
KCl	11.25	-- (fixed)
$CaCl_2$	7.5 mg	5-20 mg
Ethyl pyruvate	0.781 ml	0.5-1.5 ml
NaOH	To pH 7.5	7.35-7.55 (pH)

TABLE 2. Constituents of a Citrate Ethyl Pyruvate Solution

Component	Composition
Isotonic saline (NaCl)	102 mM
KCl	4 mM
$CaCl_2$	2.7 mM
Ethyl pyruvate	10 mg/ml
Sodium citrate	200 mM

(Adjusted to pH 4-5.)

EXAMPLE II

Ethyl pyruvate effect against inflammation in an *in vitro* model of intestinal epithelial injury

To test for the effect of ethyl pyruvate on a stabilizing calcium salt solution (REPS) in a cytokine-induced intestinal epithelial hyperpermeability condition, an *in vitro* model of intestinal epithelial injury due to inflammation, such as might occur in cases of inflammatory bowel disease (i.e., Crohn's disease or ulcerative colitis) was used.

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Exemplary materials and methods include the following, while minor modifications can be made: Caco-2 human enterocytes (10^5 cells/well) were plated on permeable filters in 12-well Transwell bicameral chambers (COSTAR,

5   Corning, NY) and fed biweekly. Permeability studies were carried out using confluent monolayers between 21-28 d after seeding. The permeability probe was FITC-labeled dextran (4400 Da; FD4). A sterile stock solution of FD4 (25 mg/ml) was prepared by dissolving the compound in HEPES-buffered

10   DMEM complete medium (pH 6.8) and passing it through a filter (0.45 μm pore size). For permeability studies, the medium was aspirated from the apical and basolateral sides of the Transwell chambers. FD4 solution (200 μl) was added to the apical compartments. The medium on the basolateral

15   side of the Transwell chambers was replaced with 500 μl of control medium or medium containing cytomix (1 ng/ml IL-1β + 10 ng/ml TNF-α + 1000 U/ml IFN-γ), or medium with cytomix and graded concentrations of ethyl pyruvate. After 24 and 48 h of incubation, 30 μl of medium was aspirated from the

20   basolateral   compartments   for   spectrofluorometric determination of FD4 concentration as previously described. (Menconi et al., *Am. J. Physiol.* (1997) 272:G1007-G1021.) The permeability of monolayers was expressed as a clearance (C), which was calculated as previously described. (Menconi

25   et al., *Am. J. Physiol.* (1997) 272:G1007-G1021.) Results at 48 h are depicted in the figure (* indicates P<0.01 versus cytomix alone).

As shown in Fig. 2, both of the highest concentrations of ethyl pyruvate significantly decreased cytomix-induced

30   hyperpermeability (P<0.001). Ethyl pyruvate prevents the

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increase in permeability, which is observed when human Caco-
2 enterocytic monolayers are incubated with a cocktail of
pro-inflammatory cytokines.

5   **EXAMPLE III**

**Ethyl pyruvate pretreatment prevents endotoxin lethality by
attenuating TNF and HMGB1 release *in vivo***

        The initial evaluation of ethyl pyruvate (EP) was
10   conducted as a therapeutic solution in lethal systemic
     inflammation in a standard model of murine endotoxemia.
     Balb/c mice received a single dose of ethyl pyruvate (40 mg
     ethyl pyruvate /kg mouse) 30 minutes before the onset of
     lethal endotoxemia (*E. coli* lipopolysaccharide, LPS, 5 mg
15   LPS/kg, i.p.). Ethyl pyruvate pretreatment protected BALB/c
     mice from lethal endotoxemia (ethyl pyruvate survival =
     20/20 versus vehicle control survival = 3/20; P<0.005) (Fig.
     3A). Ethyl pyruvate pretreatment significantly prevented the
     clinical manifestations of endotoxin morbidity, including
20   lethargy, diarrhea, piloerection, huddling, and depressed
     spontaneous mobility. Late deaths in ethyl pyruvate-treated
     animals did not occur during a subsequent 3 week period of
     observation, indicating that ethyl pyruvate confers a
     complete and lasting protection against lethal endotoxemia,
25   and does not merely delay the onset of lethal pathology.
     Pre-treatment of endotoxemic mice with ethyl pyruvate
     significantly attenuated the serum levels of both TNF
     (vehicle treated = 17 +/- 4 ng TNF/ml versus ethyl pyruvate
     treated = 39 +/- 6 ng TNF/ml; P<0.05 (Fig. 3B) and serum
30   HMGB1 measured 20 hours after the onset of endotoxemia (Fig.
     3C). Ethyl pyruvate also attenuated the peak serum levels of
     IL-1α and IL6 (data not shown), indicating that ethyl

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pyruvate prevents endotoxin lethality by attenuating the release of early (TNF) and late (HMGB-1) mediators of lethality.

5   **EXAMPLE IV**

Delayed administration of ethyl pyruvate prevents the lethality of endotoxemia by inhibiting HMGB1 release

The therapeutic efficacy of the delayed administration
10   of ethyl pyruvate after the onset of endotoxemia was assessed. Ethyl pyruvate dosing in endotoxemic mice was initiated 4 hours after the onset of endotoxemia, when clinical signs were already evident, including diarrhea, piloerection, and depressed spontaneous activity (Fig. 4A).
15   Notably, this first dose was administered after the early peak in serum TNF, which occurs within the first 1-2 hours after the onset of endotoxemia. In this delayed treatment protocol (40 mg ethyl pyruvate/kg, i.p., at 4, 8, 12, 24 and 30 hours after LPS), ethyl pyruvate significantly
20   increased survival as compared to treatment with vehicle (vehicle survival = 5/20; ethyl pyruvate survival = 17/20; P<0.005). In agreement with the role of HMGB-1 as a mediator of delayed endotoxin lethality, delayed treatment of endotoxemic mice with ethyl pyruvate beginning 4 hours post-
25   LPS injection significantly attenuated the systemic release of HMGB-1 measured at 20 hours after the onset of endotoxemia (Fig. 4B). Ethyl pyruvate treatment reversed clinical signs of morbidity, and no late deaths occurred during the 3 week period of observation, indicating that
30   delayed treatment with ethyl pyruvate confers significant protection against lethal endotoxemia.

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EXAMPLE V

Delayed administration of ethyl pyruvate prevents the lethality of sepsis

5   The efficacy of ethyl pyruvate as treatment for lethal sepsis in a standardized model of peritonitis caused by surgical perforation of the cecum was tested. Mice subjected to peritonitis received ethyl pyruvate (40 mg ethyl pyruvate/kg, i.p.) beginning 24 hours *after* the onset of
10   peritonitis, a time at which 10% of the mice in each group had already died. Survival in vehicle-treated controls during the ensuing three weeks of monitoring was 5/17; in contrast, ethyl pyruvate treatment significantly improved survival of septic mice, resulting in survival of 14/17;
15    $P<0.005$. The protection against lethal sepsis was ethyl pyruvate dose-dependent, because lower ethyl pyruvate doses failed to confer significant protection against death (Fig 4C). To our knowledge there have not been any other experimental therapeutic agents reported to rescue septic
20   mice when applied after 24 hours of established sepsis.

EXAMPLE VI

Ethyl pyruvate inhibits the synthesis of TNF in macrophage cultures

25
        To determine the effect of ethyl pyruvate on TNF synthesis by macrophages, murine macrophage-like RAW 264.7 cells were stimulated with endotoxin, and TNF measured in the conditioned media. Ethyl pyruvate significantly
30   inhibited TNF release in macrophage cultures (Fig 5A). The concentration of ethyl pyruvate that suppressed 50% of the TNF response was 3.4 mM, a clinically achievable concentration. At the highest doses of ethyl pyruvate

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tested, the synthesis of TNF was not completely inhibited, indicating that even in the presence of saturating amounts of this agent, macrophages can still produce some level of TNF. Ethyl pyruvate inhibition of TNF in macrophages also
5   significantly reduced the amount of TNF mRNA (Figure 5B), indicating that ethyl pyruvate acts to suppress the steady-state levels of the TNF gene transcript in activated macrophages.

10   **EXAMPLE VII**

        **Ethyl pyruvate inhibits signal transduction via NF-κB and
                            p38 MAP kinase**

        Signal transduction through NF-κB and p38 MAPK pathways
15   is a critical step in macrophage activation that leads to increased expression of TNF and other proinflammatory cytokine genes. The suppression of TNF mRNA suggested the possibility that ethyl pyruvate might inhibit the nuclear translocation of NF-κB, because this transcription factor is
20   a predominate regulator of TNF gene transcription. In agreement with previous studies, LPS induced a significant activation of NF-κB as showed by EMSA at 15 minutes and at 4 hours after LPS exposure (Fig. 6A). Ethyl pyruvate significantly prevented LPS-induced activation of NF-κB
25   signaling in a concentration dependent fashion (Fig 6A). Ethyl pyruvate inhibition of NF-κB activation persisted for at least 4 hours after LPS exposure, indicating that inhibition is complete, and does merely represent a delay of pathway activation. The specificity of the ethyl pyruvate
30   effect on NF-κB was established by additional supershift and competition assays (Fig. 6B), in which anti-p65 antibodies

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induced a supershift in the electrophoretic mobility of the complex consistent with the presence of Rel p65 in the complex. Pretreatment of nuclear extracts with a 100-fold molar excess of unlabeled (cold) NF-κB, but not unlabeled

5   HIF1, prevented the electrophoretic shift of the radioactive probe. Ethyl pyruvate also prevented the LPS-induced phosphorylation and subsequent activation of p38 MAPK in an ethyl pyruvate concentration dependent fashion (Fig. 6C). To determine specificity of inhibiting the activation of p38

10   MAPK by ethyl pyruvate, the intracellular stability and concentration of total p38MAPK was assessed, and observed that ethyl pyruvate did not significantly influence the total levels of p38 MAPK (Fig. 6D). Together, these results indicate that ethyl pyruvate interferes with the activation

15   of macrophages by suppressing signal transduction through these critical pathways.

EXAMPLE VIII
Ethyl pyruvate attenuates the release of HMGB-1

20   To determine whether ethyl pyruvate directly inhibits HMGB-1 release from macrophages, HMGB-1 release was measured in media conditioned by overnight endotoxin-exposed macrophage cultures. Ethyl pyruvate significantly prevented

25   LPS-induced HMGB-1 release in a concentration dependent manner (Figure 7A). Most of the HMGB-1 released by activated macrophages exists as a preformed cell associated protein, so to distinguish between the possibilities that ethyl pyruvate either inhibited the release of HMGB1 or else

30   suppressed the total cellular levels of HMGB-1, total cell associated HMGB1-levels in macrophages was measured.

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RAW264.7 cells were treated with 1, 5 or 10 mM of ethyl
pyruvate, and total cell associated HMGB-1 protein levels
analyzed (Fig. 7B). Ethyl pyruvate did not significantly
influence total intracellular HMGB1 protein concentration
5   indicating that ethyl pyruvate specifically prevented HMGB1
release without affecting protein synthesis or stability.

      While the invention has been described in conjunction
with specific embodiments thereof, it is evident that many
10   alternatives, modifications, and variations will be apparent
to those skilled in the art in light of the foregoing
description. Accordingly, it is intended that the invention
shall be directed to all such alternatives, modifications
and variations as fall within the spirit and broad scope of
15   the appended claims.

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CLAIMS

What is claimed is:

5   1.   A method of treating a patient having a cytokine-
mediated inflammatory condition, said method comprising
the steps of:
       (a) providing said patient having an inflammatory
condition mediated by a cytokine; and
10   (b) administering to said patient an effective
amount of a composition comprising an alpha-ketoalkanoic
acid compound, wherein said compound is an alpha-
ketoalkanoic acid, a physiologically acceptable salt of an
alpha-ketoalkanoic acid, an ester of an alpha-ketoalkanoic
15   acid, or an amide of an alpha-ketoalkanoic acid in a
pharmaceutically acceptable inert carrier substance.

       2.   The method of claim 1, wherein said cytokine is an
early mediator in said inflammatory condition.
20
       3.   The method of claim 1, wherein said cytokine is a
late mediator in said inflammatory condition.

       4.   The method of claim 1, wherein said composition is
25   administered 24 hours after onset of said inflammatory
condition.

       5.   The method of claim 1, wherein said cytokine-mediated
inflammatory condition is mediated by tumor necrosis
30   factor.

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6.   The method of claim 1, wherein said cytokine-mediated inflammatory condition is mediated by interleukin-1β.

7.   The method of claim 1, wherein said cytokine-mediated inflammatory condition is mediated by high mobility group B-1.

8.   The method of claim 1, wherein said alpha-ketoalkanoic acid compound is C3 to C8, straight chained or branched.

9.   The method of claim 8, wherein said alpha-ketoalkanoic acid compound may be selected from the group consisting of alpha-keto-butyrate, alpha-ketopentanoate, alpha-keto-3-methyl-butyrate, alpha-keto-4-methyl-pentanoate or alpha-keto-hexanoate.

10.   The method of claim 1, wherein said alpha-ketoalkanoic acid compound is pyruvic acid.

11.   The method of claim 1, wherein said physiologically-acceptable salt may be selected from the group consisting of Na^+, K^+, Ca^{++}, Mg^+, and NH_4^+.

12.   The method of claim 1, wherein said alpha-ketoalkanoic acid compound may be selected from the group consisting of alkyl, aralkyl, alkoxyl, carboxyalkyl, glyceryl or dihydroxy acetone.

13.   The method of claim 1, wherein said ester of alpha-ketoalkanoic acid compound may be selected from the group

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consisting of ethyl, propyl, butyl, carboxymethyl, acetoxymethyl, carbethoxymethyl and ethoxymethyl.

14.   The method of claim 1, wherein said ester of alpha-
5   ketoalkanoic acid compound is an ethyl pyruvate.

15.   The method of claim 1, wherein said amide of alpha-ketoalkanoic acid compound has the structural formula, wherein said formula comprises $RCOCONR^1R^2$,

10   wherein:

          R is an alkyl group;

          R^1 and R^2 are independently -H, alkyl, aralkyl, alkoxyalkyl, carboxyalkyl or $-CHR^3COOH$; and

          R^3 is the side chain of a naturally occurring
15   amino acid.

16.   The method of claim 1, wherein said amide of an alpha-ketoalkanoic acid compound is a pyruvamide.

20   17.   The method of claim 1, wherein said carrier substance further comprises a biologically safe component for inducing and stabilizing enolization of the alpha-keto functionality of said acid at physiological pH values.

25   18.   The method of claim 17, wherein said component for inducing and stabilizing enolization of the alpha-keto functionality of said ester is an inorganic, divalent cation.

30   19.   The method of claim 18, wherein said divalent cation is calcium or magnesium.

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20. The method of claim 1, wherein said alpha-ketoalkanoic acid portion of said ester is alpha-ketopropionic acid.

5

21. The method of claim 1, wherein said alpha-ketoalkanoic acid ester compound is ethyl pyruvate, said divalent cation is calcium and said inert carrier substance is Ringer's solution in a pH range of 7-8.

10

22. The method of claim 1, wherein said inert carrier substance is a Ringer's solution of isotonic saline supplemented with potassium ion.

15 23. The method of claim 1, wherein said alpha-ketoalkanoic acid ester compound is selected from the group consisting of ethyl 2-keto-butyrate, ethyl 2-ketopentanoate, ethyl 2-keto-3-methyl-butyrate, ethyl 2-keto-4-methyl-pentanoate and ethyl 2-keto-hexanoate.

20

24. The method of claim 1, wherein said alpha-ketoalkanoic acid ester compound is admixed in a saline solution, said solution containing a cation selected from the group consisting of calcium and magnesium.

25

25. The method of claim 1, wherein said inflammatory condition comprises local and systemic inflammation, inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis, asthma (including status

30 asthmaticus), sepsis or septic shock.

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26. The method of claim 1, wherein said inflammatory condition is a skin condition.

27. The method of claim 26, wherein said skin condition
5 is psoriasis or eczema.

1 / 7
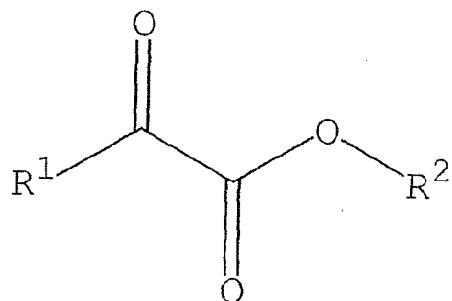
R^1 = methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, terbutyl, pentyl, 4-methylpentyl, 3-methylpentyl, hexyl, heptyl, octyl, 1-phenylmethyl, 2-phenyl-ethyl

R^2 = ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, terbutyl, pentyl, 4-methylpentyl, ethoxymethyl, 2-ethoxyethyl, carboxymethyl, ethoxycarbonylmethyl

FIG. 1A
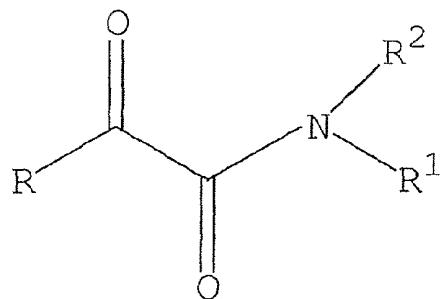
R is alkyl group

R^1 and R^2 are independently H, alkyl, aralkyl, alkoxyalkyl, carboxyalkyl, -CHR3COOH

R^3 is the side chain of a naturally occurring amino acid

FIG. 1B
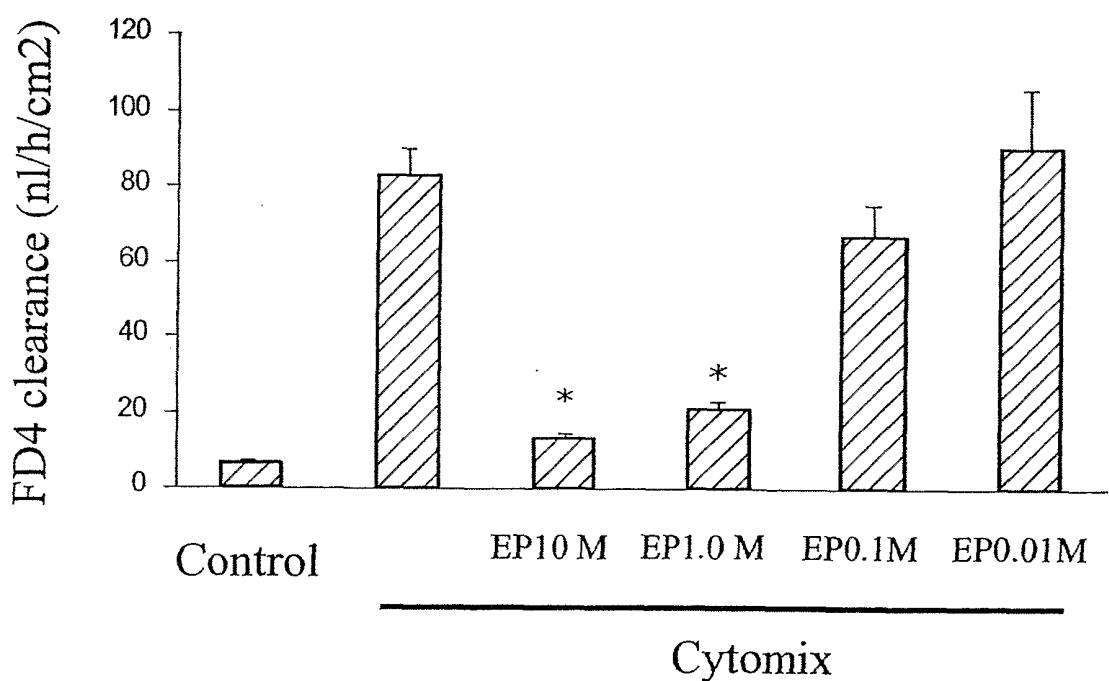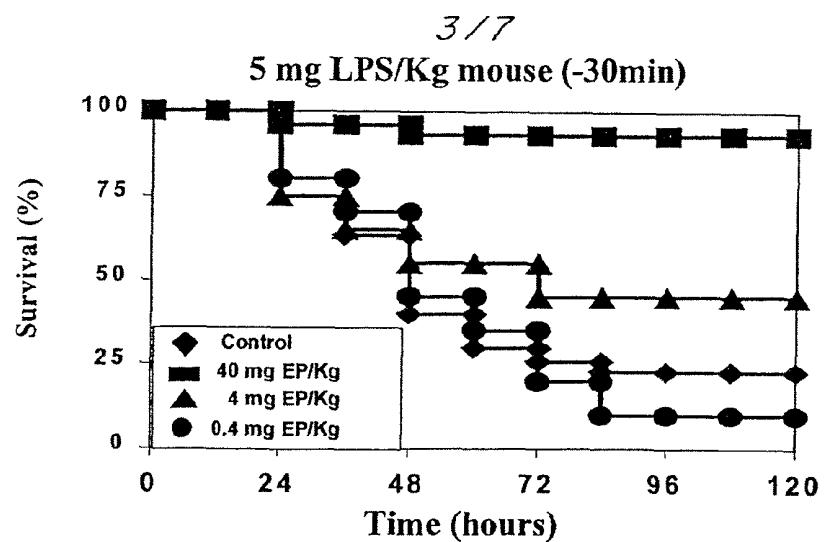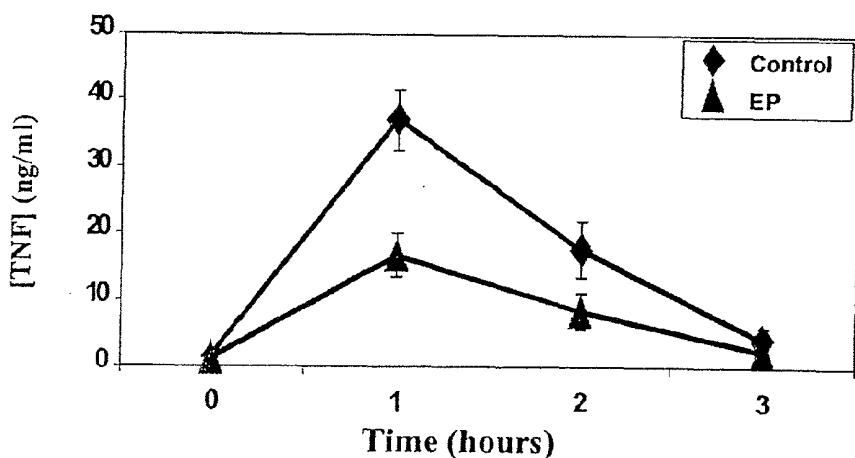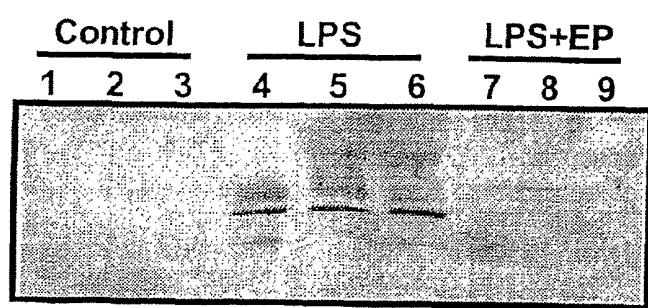
FIG. 2

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5 mg LPS/Kg mouse (-30min)

FIG. 3A

FIG. 3B

HMGB1

FIG. 3C

4/7

FIG. 4A

HMGB1

FIG. 4B

FIG. 4C

[ethyl pyruvate] (mM)

FIG. 5A

TNF mRNA

FIG. 5B

LPS Time   -   [15 min]   -   [4 hours]

[EP] (mM)   _   -   1   5   -   -   1   5

FIG. 6A

Control   Anti-p65   "cold" NF-kB   "cold" HIF-1

FIG. 6B

[LPS] (ng/ml) -   [50]   [100]

[EP] (mM)   -   -   1   5   10   -   1   5   10
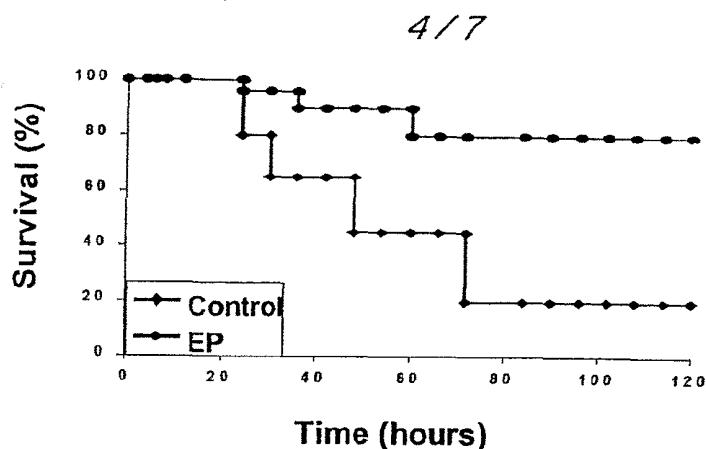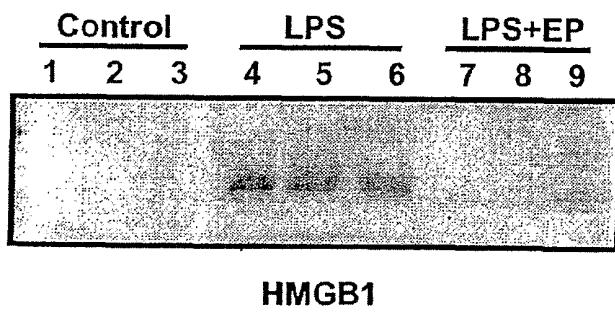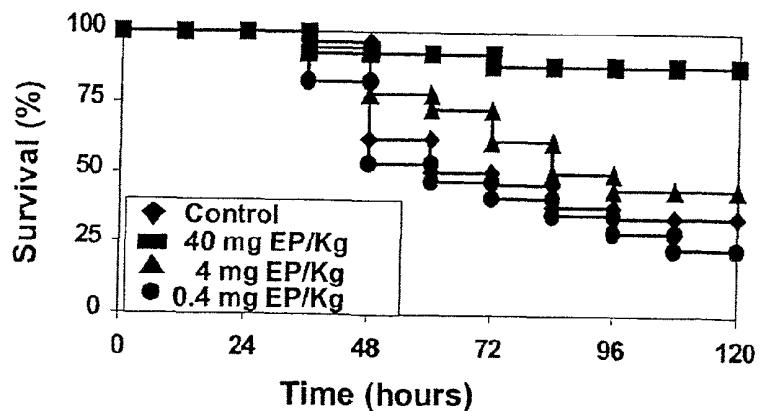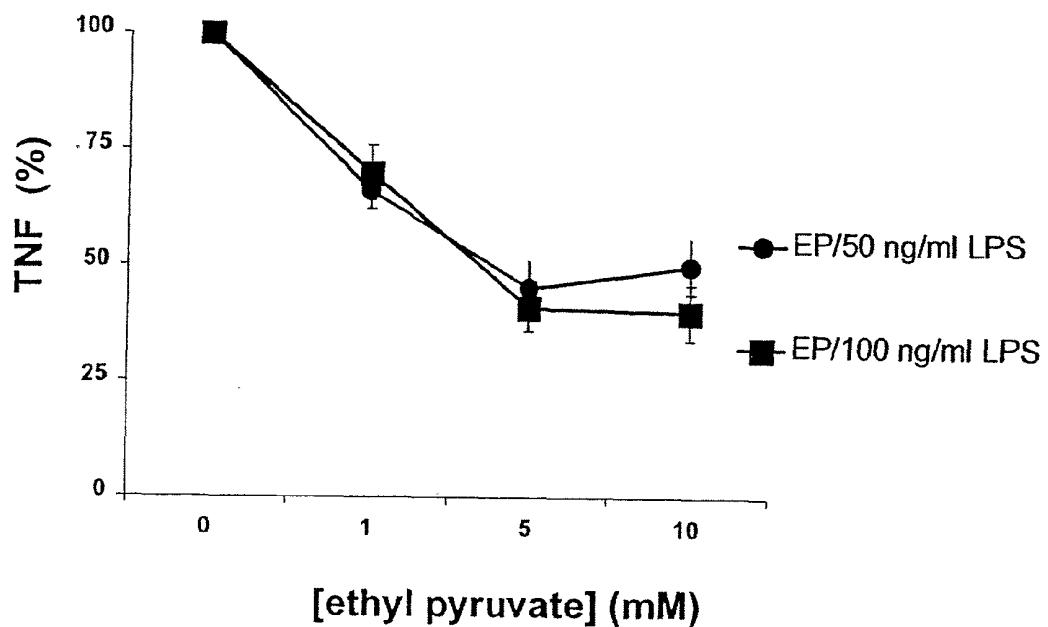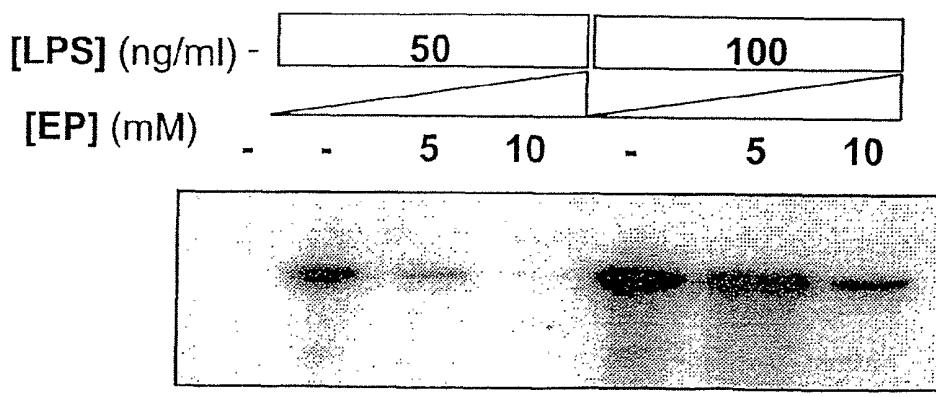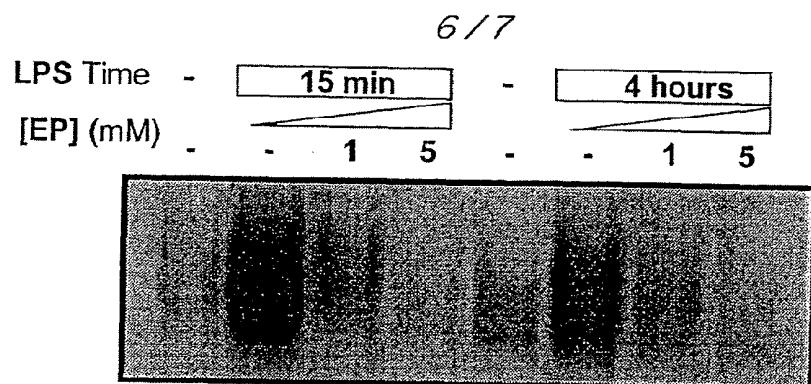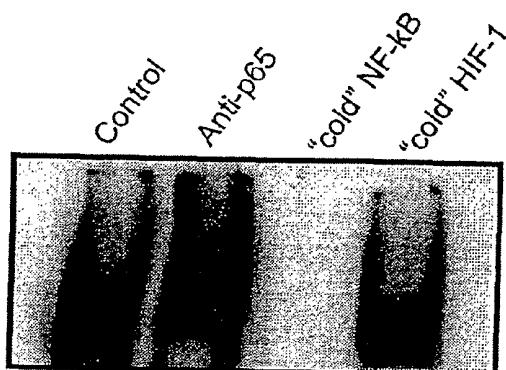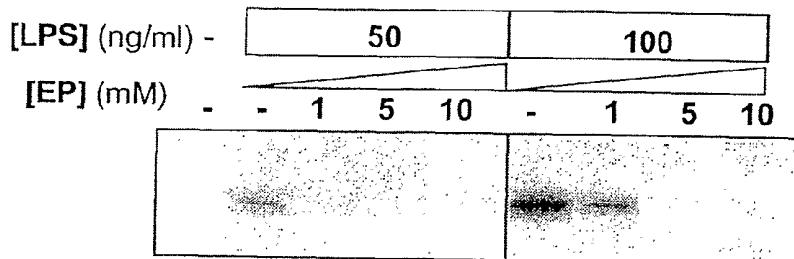
Phospho - p38 MAPK

FIG. 6C
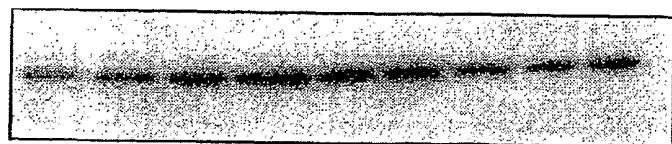
Total p38 MAPK

FIG. 6D
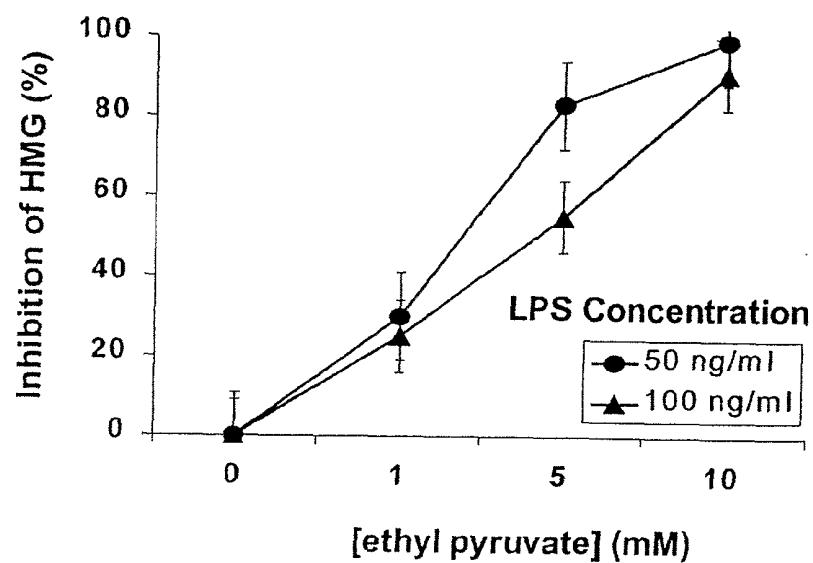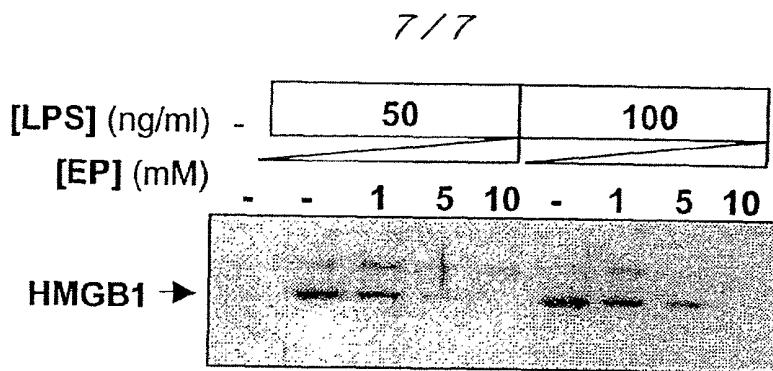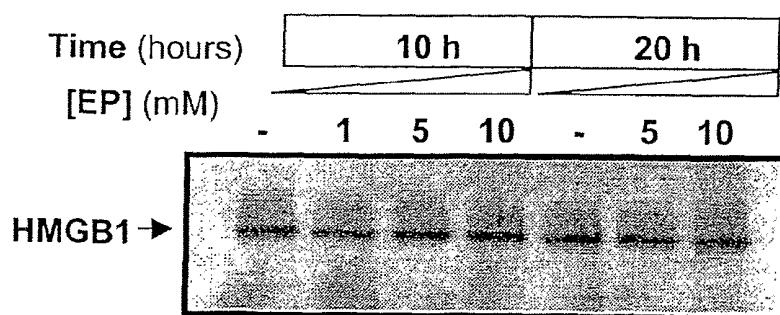
7/7

[LPS] (ng/ml) -   50   100

[EP] (mM)   -   -   1   5   10   -   1   5   10

HMGB1 →

FIG. 7A

Time (hours)   10 h   20 h

[EP] (mM)   -   1   5   10   -   5   10

HMGB1 →

FIG. 7B

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :A61K 31/19, 31/195
US CL   :514/557, 561

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   514/557, 561

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,105,782 A (YU et al.) 08 August 1978 (08.08.78), see the entire document.	1-27
A	US 4,234,599 A (VAN SCOTT et al.) 18 November 1980 (18.11.80), see the entire document.	1-27
A	US 5,714,515 A (BUNGER) 03 February 1998 (03.02.98), see the entire document.	1-27

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 MAY 2002	2 8 JUN 2002

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