METHODS AND PHARMACEUTICAL COMPOSITIONS FOR STIMULATING THE IMMUNE SYSTEM AND/OR TREATING CANCER

A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method comprises administering to a subject in need thereof at least one agent in an amount so as to induce H2O2 generation at a concentration effective in inducing immune stimulation and or providing cancer therapy.
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STIMULATING THE IMMUNE SYSTEM AND/OR TREATING
CANCER

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a methods and pharmaceutical compositions useful in inducing immune stimulation and/or treating cancer. More particularly, the present invention relates to methods and pharmaceutical compositions for inducing immune stimulation and/or for treating cancer, which comprise H₂O₂ and/or an H₂O₂ generating agent, such as metalloccenes (e.g., ferrocene) and their derivatives.

Compounds that stimulate the immune system in a non specific manner, have potential for treating tumors that are resistant to conventional therapy. Biological response modifiers such as IL-2, IFN-α IFN-γ and TNF-α, are all possible candidates. Clinical use of some of these compounds resulted in limited response and has been associated with marked toxicity (1-5).

Agonistic immune stimulatory antibodies, such as anti-CD3, were also evaluated for their anti-tumor activity in humans, with limited success (6,7).

Bacillus Calmette-Guerin (BCG) preparations known to activate the immune system are effective upon administration intravesically against urinary bladder transitional cell carcinoma (TCC) (8).

The immune-stimulatory properties of agents that generate free radicals, including iron-containing compounds such as hemin were previously described (9-13). Hemin, by itself, does not exhibit anti-tumor activity but shows this effect in combination with IL-2 (14).

The protooncogene p21^{ras} has been identified as a key molecular switch involved in regulating T cell activation triggered by different mitogens (15-19). A breakthrough in understanding the stimulatory activity mechanism of reactive free radicals occurred when it was discovered that p21^{ras} is the primary target of these agents (including iron-containing compounds) and cellular redox stress.

These studies of p21^{ras} have identified a single cysteine residue, cys118, which is modified by free radicals. This modification triggers the activation of
p21\textsuperscript{ras} and leads to downstream signaling events such as the activation of mitogen-activated protein kinase, activation of phosphatidylinositol 3'-kinase (PI-3K) and transcription factor activation (20-24).

Metalloccenes, such as ferrocene are stable iron-containing compounds which generate free radicals. Metalloccenes were so far never suggested as immune stimulants and/or an anti-cancer agents.

While conceiving the present invention, it was hypothesized that metalloccenes, such as, for example, ferrocene, could stimulate the immune system and/or provide for anti-cancer therapy. While reducing the present invention to practice this hypothesis was tested in various systems.

**SUMMARY OF THE INVENTION**

According to one aspect of the present invention there is provided a method of determining whether a metalloccene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining an immune stimulation/cancer therapy dose-response curve for the metalloccene derivative and determining whether the dose-response curve has an optimum with respect to immune stimulation/cancer therapy within a predetermined range of concentrations.

According to further features in preferred embodiments of the invention described below, the immune stimulation is determined in vivo.

According to still further features in the described preferred embodiments, immune stimulation is determined in vitro.

According to still further features in the described preferred embodiments, the immune stimulation is determined with respect to macrophages.

According to still further features in the described preferred embodiments, the immune stimulation is determined with respect to lymphocytes.
According to still further features in the described preferred embodiments, the immune stimulation is determined by a cell proliferation assay.

According to still further features in the described preferred embodiments, the cell proliferation assay includes a thymidine incorporation assay.

According to still further features in the described preferred embodiments, the immune stimulation is determined by a cellular response assay.

According to still further features in the described preferred embodiments, the cell response assay is selected from the group consisting of an NO production assay, a TNF-α production assay and an oxygen burst assay.

According to another aspect of the present invention there is provided a method of determining whether a metalloocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining a p21<sup>ras</sup> signal transduction pathway component activity dose-response curve for the metalloocene derivative and determining whether the dose-response curve has an optimum with respect to p21<sup>ras</sup> signal transduction pathway component activity within a predetermined range of concentrations.

Preferably, the p21<sup>ras</sup> signal transduction pathway component is selected from the group consisting of p21<sup>ras</sup> and NF-κB.

Hence, according to yet additional aspects of the present invention there is provided (i) method of determining whether a metalloocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining p21<sup>ras</sup> oxidation dose-response curve for the metalloocene derivative and determining whether the dose-response curve has an optimum with respect to p21<sup>ras</sup> oxidation within a predetermined range of concentrations; (ii) A method of determining whether a metalloocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining a GTPase activity dose-response curve for the
metallocene derivative and determining whether the dose-response curve has an optimum with respect to the GTPase activity within a predetermined range of concentrations; and (iii) A method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining an NF-κB activity dose-response curve for the metallocene derivative and determining whether the dose-response curve has an optimum with respect to the NF-κB activity within a predetermined range of concentrations.

According to still another aspect of the present invention there is provided a method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining H₂O₂ generation catalyzed by the metallocene derivative, and determining whether the rate is within a predetermined range.

According to an additional aspect of the present invention there is provided a method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising monitoring H₂O₂ generation catalyzed by the metallocene derivative, and determining whether the generation is above a predetermined value.

According to yet an additional aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, the method comprising administering to a subject in need thereof at least one agent in an amount so as to induce H₂O₂ generation at a concentration effective in inducing immune stimulation and or providing cancer therapy. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount so as to induce H₂O₂ generation at a
concentration effective in inducing immune stimulation following administration thereof.

According to a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight.

According to yet a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in activating a $p21^{ras}$ signal transduction pathway component. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in activating a $p21^{ras}$ signal transduction pathway component following administration thereof.

According to still a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing $p21^{ras}$ oxidation. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit
of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing p21ras oxidation following administration thereof.

According to still a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing GTPase activity. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing GTPase activity following administration thereof.

According to another aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing NF-κB activity. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing NF-κB activity following administration thereof.

According to further features in preferred embodiments of the invention described below, the at least one agent comprises at least one ferrocene and/or metalloocene derivative.

According to still further features in the described preferred embodiments the amount is between 0.025 - 10 mg/kg body weight.

According to still further features in the described preferred embodiments the amount is between 0.1 - 5 mg/kg body weight.
According to still further features in the described preferred embodiments the amount is between 0.2 - 3 mg/kg body weight.

According to still further features in the described preferred embodiments the at least one agent is administered orally and hence the pharmaceutical composition is formulated for oral administration.

According to still further features in the described preferred embodiments the at least one agent is administered by injection and hence pharmaceutical composition is formulated for administration by injection.

According to still further features in the described preferred embodiments the cancer is selected from the group consisting of all malignant tumors, including, but not limited to, carcinoma, sarcoma, lymphoma or hematological malignancies, hence, the pharmaceutical composition is packaged and identified for the treatment of all malignant tumors, including, carcinomas, sarcomas, lymphomas or hematological malignancies. According to still further features in the described preferred embodiments the disease, syndrome or condition is selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency, hence, the pharmaceutical composition is packaged and identified for the treatment of a disease, syndrome or condition selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

According to still further features in the described preferred embodiments the metallocene or metallocene derivative has a general formula:
wherein, M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

According to still further features in the described preferred embodiments, the method further comprising co-administering at least one additional agent having immune stimulation activity. Accordingly, the pharmaceutical composition, further comprises at least one additional agent having immune stimulation activity, such as, but not limited to, a cytokine (e.g., IL-2, TNF, interferon), a chemokine, an immune-stimulatory antibody (e.g., anti-CD3, anti-CTLA-4) or an anti-tumor vaccine.

According to still further features in the described preferred embodiments, the method further comprising co-administering at least one additional agent having anti-cancer activity. Accordingly, the pharmaceutical composition, further comprises at least one additional agent having anti-cancer activity, such as, but not limited to, chemotherapeutic agents, anti-metabolites, signal transduction inhibitors (e.g., protein tyrosine kinase inhibitors), anti-angiogenesis compounds, antibodies (e.g., anti-receptors, anti-tumor determinants), hormones and hormone antagonists, retinoids, steroids, pro-apoptotic agents, differentiating agents or radiation.
According to another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer in a body cavity, the method comprising administering into the body cavity H$_2$O$_2$ in an amount effective in immune stimulation. Preferably, the amount is of 10-2000 ml of 50-2500 μM H$_2$O$_2$ administered in a single or multiple administrations. The body cavity can be, for example, the peritoneal cavity or the pleural cavity.

According to still another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer, the method comprising exposing immune cells ex vivo to at least one ferrocene and/or metalloocene derivative, and administering the cells to a subject in need thereof.

According to yet another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer, the method comprising exposing immune cells ex vivo to H$_2$O$_2$ in a concentration effective in inducing immune stimulation and administering the cells to a subject in need of thereof.

The present invention successfully addresses the shortcomings of the presently known configurations by providing new methodologies with which to induce immune stimulation and/or treat cancer.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the
description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-B demonstrate stimulation by ferrocene and IL-2 of ³H-thymidine incorporation in mouse splenocytes, in vitro. 1A - Mouse splenocytes were incubated in the present of ferrocene, at different concentrations, for 3 days. ³H-Thymidine incorporation during the last 20 hours of incubation was determined. Results are expressed as means CPM±SD of triplicate cultures. 1B - Same as 1A, but the cells were cultured in the presence of IL-2 (50 U/ml).

FIG. 2 demonstrates stimulation by ferrocene of ³H-thymidine incorporation in mouse splenocytes, in vivo. Mice were injected intraperitoneally with ferrocene at different doses. Three days later, splenocytes were isolated and incorporation of ³H-thymidine during 30 hours, was determined. Two mice were included in each group. Results are expressed as means CPM ±SD of triplicate cultures from each mouse.

FIG. 3 demonstrates activation by ferrocene of mouse peritoneal macrophages, in vitro. Peritoneal macrophage monolayers were prepared (as described in the Examples section that follows) and co-incubated with splenic lymphocytes in the presence of ferrocene at different concentrations. The ratio of lymphocytes to macrophages was 10:1. After incubation for 3 days, the non-adherent lymphocytes were removed by washing with PBS. Production of H₂O₂ as a measurement of oxygen burst was determined in the presence of phorbol myristate acetate (PMA) (100 ng/ml), using the aminotriazole-catalase inhibition test (see Examples section that follows). Results are expressed as means ± SD of triplicate cultures.

FIG. 4 demonstrates activation by ferrocene of mouse peritoneal macrophages, in vivo. Mice were injected intraperitoneally with ferrocene at different doses (per mouse). After 3 days, peritoneal macrophages were obtained and oxygen burst was determined in the presence and absence of PMA
(100 ng/ml). As a positive control, mice were injected with 1 ml of NaIO₄ (5 mM). NaIO₄ was previously shown to activate peritoneal macrophages, in vivo (39). Four mice were included in each experimental group and the results are expressed as means ±SD.

FIGS. 5A-C demonstrate activation by ferrocene of mouse peritoneal macrophages in vivo: TNF-α production (5A), oxygen burst (5B), nitric oxide (5C) production. Mice were injected intraperitoneally with ferrocene (1 mg/mouse) or with NaIO₄ (0.15 M, 1 ml), as a positive control. Three days later the peritoneal cavity was washed with PBS and macrophage monolayers (10⁶ cells/6 mm plate) were obtained as described in the Examples section that follows. LPS (10 µg/ml) was added, and after 6 hours, supernatants were collected and TNF-α (5A) and nitric oxide (5C) levels were determined as described in the Examples section that follows. Oxygen burst (H₂O₂ production, 5B) was determined in the absence and presence of PMA.

FIG. 6 demonstrates anti-tumor effect of ferrocene administered intraperitoneally by a single injection (T₁) at different doses in B-16 melanoma bearing mice. Ferrocene was injected intraperitoneally one day post B-16 melanoma inoculation (T₁) via the tail vein. Mice were sacrificed 25 days post tumor inoculation and the pair of lungs from each mouse was weighed. 4-6 mice were included in each experimental group. Results are expressed as the means of the weights of the pairs of lungs of each mouse ±SD.

FIG. 7 demonstrates anti-tumor effect of ferrocene at different doses, administered intraperitoneally by multiple injections. Mice were inoculated with B-16 melanoma (T₀). Ferrocene was administered intraperitoneally 3 times (T₁, T₈ and T₁₅) each, at a dose specified in the Figure. Mice were sacrificed 25 days post tumor inoculation and the pair of lungs from each mouse was weighed. 4-6 mice were included in each group. Results are expressed as the means of the weights of the pair of lungs of each mouse ±SD.

FIG. 8 demonstrates the anti-tumor effect of ferrocene. Photographs were taken of pairs of lungs from the experiments depicted in Figures 6 and 7.
FIGs. 9A-B demonstrate the effect of ferrocene, administered in the drinking water (1 μg/ml) on established lung metastases in B-16 melanoma bearing mice. Mice, inoculated with B-16 melanoma were supplied with drinking water (ad libidum) containing 1 μg/ml ferrocene. A mouse drinks approximately 5 ml water/day, so the overall dose of ferrocene was approximately 0.25 mg/kg body weight/day. One group of mice was supplied with ferrocene in the drinking water for 1 week followed by replacing it with plain water, the second and third groups were supplied with ferrocene in the drinking water for 2 and 3 weeks, respectively. Mice were sacrificed 25 days post tumor inoculation and tumor load was assessed by weighing the lungs. 4-6 mice were included in each group. Results are expressed as the means of the weight of the pair of lungs of each mouse ±SD (9A). Photographs of lungs of the mice that were sacrificed 25 days post tumor inoculation (9B).

FIGs. 10A-B demonstrate anti-tumor effect of ferrocene administered in drinking water at different concentrations. For experimental details see Table 1 below. Photographs of lungs taken 25 days post tumor inoculation. Mice were supplied with drinking water containing ferrocene for 1 week (10A) or 2 weeks (10B).

FIG. 11 demonstrates the effect of ferrocene administered in drinking water (1 μg/ml) for one week on survival of B-16 melanoma bearing mice.

FIG. 12 demonstrates anti-tumor effect of ferrocene in mice bearing Lewis lung carcinoma. Ferrocene was administered intraperitoneally 3 times (T₁, T₈ and T₁₅), each at a dose of 0.025, 0.05 and 0.2 mg/kg body weight. Mice were sacrificed 28 days post tumor inoculation and tumor load was assessed by weighing the lungs. Four mice were included in each group and the results are expressed as the means of lungs' weight ±SD.

FIG. 13 demonstrates anti-tumor effect of combined treatment with ferrocene and IL-2 in B16 melanoma bearing mice. Ferrocene was administered intraperitoneally at a single dose on day T₁. IL-2 was administered intraperitoneally 3 times on days T₁, T₂ and T₂ each, at a dose of
25 x 10^4 U/kg body weight). 5-6 mice were included in each group. Mice were sacrificed 25 days post tumor inoculation and tumor load was assessed by weighing the lungs. Results are expressed as the means of lungs' weight ±SD.

FIGs. 14A-B demonstrate an adoptive transfer experiments: Anti-tumor effect of immunocytes from ferrocene-treated mice. 14A - Donor cells were obtained from B-16 melanoma bearing mice which were treated on day T0 with a single injection of ferrocene (0.2 mg/kg body weight). Cells were collected from donor mice 25 days post tumor inoculation. Splenocytes (10^8 cells/mouse) and peritoneal mononuclear cells ("macrophages") (5 x 10^6 cells/mouse) were administered intravenously to B16 melanoma inoculated recipient mice on day T4. Recipient mice were sacrificed 25 day post tumor inoculation and tumor load was assessed by weighing the lungs. 2-4 recipient mice were included in each experiment and results are expressed as means ±SD. 14B - Photographs of lungs from recipient mice treated with donor ("macrophages") peritoneal mononuclear cells.

FIG. 15 demonstrates an adoptive transfer experiments: Anti-tumor effect of immunocytes from ferrocene-treated mice. Donor cells were obtained from B-16 melanoma bearing mice which were treated on day T0 with a single injection of ferrocene (0.2 mg/kg body weight). Cells were collected from donor mice 25 days post tumor inoculation. Splenocytes (10^8 cells/mouse) and peritoneal mononuclear cells ("macrophages") (5 x 10^6 cells/mouse) were administered intravenously to B16 melanoma inoculated recipient mice on day T4. Recipient mice were sacrificed 25 day post tumor inoculation and tumor load was assessed by weighing the lungs. 2-4 recipient mice were included in each experiment. Photographs of lungs from recipient mice treated with donor ("macrophages") peritoneal mononuclear cells.

FIG. 16 demonstrate production of H2O2 in ferrocene solution. H2O2 production was measured by the aminotriazole-catalase inhibition test (see Examples section that follows).
FIG. 17 demonstrates the effect of N-acetyl cysteine (NAC) on the anti-tumor effect of ferrocene in B-16 melanoma bearing mice. Ferrocene was injected intraperitoneally at a single dose of 0.25 mg/kg body weight (optimal) or 2.5 mg/kg body weight (supraoptimal), on day T1. N-acetylcysteine was injected at a dose of 500 mg/kg body weight, twice a day, for 3 days (T1, T2, T3). Mice were sacrificed 25 days post tumor inoculation and the tumor load was assessed by weighing the lungs. Five mice were included in each group and the results are expressed as means of lungs' weight ± SD.

FIG. 18 demonstrates that ferrocene induces phosphorylation of ERK1/2 in wild type Jurkat cells and not in C118S Jurkat cells. Jurkat cells (wild type and C118S) were incubated (2 x 10⁶ cells/ml) for 24 hours in RPMI 1640 containing 0.5 % FCS. Ferrocene was added at a final concentration of 0.05, 0.2, 1 and 5 μM. PHA was added at a final concentration of 20 μg/ml. After incubation for 5 minutes at 37 °C, cells were centrifuged and suspended in lysis buffer. Western blot analysis for the detection of activated (phosphorylated) ERK1/2 was done as described in the Examples section that follows.

FIG. 19 demonstrates the effects of ferrocene on NF-κB activity. Cells were treated with the indicated concentrations of ferrocene for 4 hours prior to isolation of nuclei and assay for NF-κB binding activity as described in the Examples section that follows. Arrows indicate the protein/DNA complex.

FIG. 20 demonstrates the anti-tumor effect of H₂O₂ administered intraperitoneally on established lung metastases in B-16 melanoma-bearing mice. H₂O₂ was administered intraperitoneally, twice a day, each, at a dose of 5 μmoles/kg body weight, on day T1, T2, and T3 (1 cycle), or on days T1-T3, T₈-T₁₀ and T₁₅-T₁₇ (3 cycles). Mice were sacrificed 25 days post tumor inoculation and tumor load was assessed by lungs' weight. Four mice were included in each group. Results are expressed as means of lungs' weight ± SD.

FIGs. 21A-B demonstrate the anti-tumor effect of H₂O₂ intraperitoneally. The experimental design was similar to that depicted in Figure 20, except that 6
mice were included in each group. Scoring of tumor load was done by counting
and measuring the size of lungs’ metastases as follows:

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Size</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conglomerate</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>3-5 mm</td>
<td>3</td>
</tr>
<tr>
<td>Medium</td>
<td>2-3 mm</td>
<td>2</td>
</tr>
<tr>
<td>Small</td>
<td>&lt;2 mm</td>
<td>1</td>
</tr>
</tbody>
</table>

FIG. 22 shows photographs of lungs from mice inoculated with B-16
melanoma. Experimental data is outlined in Figures 21A-B.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and pharmaceutical compositions
which can be used for inducing immune stimulation and/or treating cancer.
More specifically, the present invention is of methods and pharmaceutical
compositions for inducing immune stimulation and/or for treating cancer, which
comprise H₂O₂ and/or an H₂O₂ generating agent, such as metallocenes (e.g.,
ferrocene) and their derivatives.

The principles and operation of the present invention may be better
understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is
to be understood that the invention is not limited in its application to the details
set forth in the following description or exemplified by the Examples. The
invention is capable of other embodiments or of being practiced or carried out
in various ways. Also, it is to be understood that the phraseology and
terminology employed herein is for the purpose of description and should not be
regarded as limiting.

Oxidizing agents have been shown to regulate protein function by
modification of cysteine residues. Their protein targets include Ras, calcium
dependent potassium channels, N-methyl-D-aspartate and caspas (28). Ras is
a key element of various signaling pathways and is implicated in the regulation
of proliferation and differentiation by tyrosine kinase and G protein-coupled receptors (29). It was previously shown that p21\textsuperscript{ras} is a common signaling target of reactive free radicals and cellular redox stress (21) and the immune-stimulatory properties of agents that generate free radicals, including iron-containing compounds such as hemin was described (9-13). Hemin, by itself, does not exhibit anti-tumor activity but shows this effect in combination with IL-2 (14).

While conceiving the present invention, it was hypothesized that metallocenes, stable metal-containing compounds, and metallocene derivatives could stimulate the immune system and/or provide for anti-cancer therapy. While reducing the present invention to practice this hypothesis was tested in various systems. Hence, it is demonstrated herein that ferrocene, induces \textit{in vitro} and \textit{in vivo} activation of mouse splenocytes and peritoneal macrophages. Ferrocene also has a marked anti-tumor effect in mice bearing B-16 melanoma and Lewis lung carcinoma.

The immune stimulatory and anti-tumor effects of ferrocene follow a bell shaped curve, namely ferrocene at high, supraoptimal concentrations, is far less effective. The inhibiting effects of ferrocene at high concentrations may be due to oxidation of thiol moieties and other susceptible molecules that are not relevant to the stimulatory process. Reactive oxygen species have been implicated in damaging biomolecular components including lipids, proteins and DNA.

Previous reports indicated that ferrocene at doses of 20-500 mg/kg body weight did not inhibit Ehrlich ascites tumor in mice (30), whereas other metallocene derivatives at high doses (180-300 mg/kg body weight) exhibited anti-tumor activity. The LD\textsubscript{50} dose of these compounds ranged between 240-400 mg/kg body weight. It should be noted that, as reported herein, the effective dose of ferrocene that elicit an anti-tumor effect is approximately 2000 fold less than that reported for the anti-tumor effect of metallocene derivatives. The LD\textsubscript{50} of ferrocene is 440 mg/kg body weight (30).
The inhibitory effects of ferrocene derivatives, at high doses, on solid tumors in mice and two cell lines (31, 32) were demonstrated under conditions exhibiting immunotoxic activities (33).

It is plausible that the anti-tumor activity of metalloocene derivatives, at high dose, resulted from a direct effect on the tumor itself.

It is postulated that the anti-tumor activity of ferrocene at low doses, 0.05-0.2 mg/kg bodyweight (administered intraperitoneally) and 0.5-2 µg/ml in drinking water, is mediated by immune-stimulation. This is based on the findings that ferrocene, at low doses, activates p21\textsuperscript{ras} and ERK1/2, stimulates lymphocyte proliferation and activates peritoneal macrophages. Most importantly, adoptive transfer experiment demonstrated that mouse peritoneal monocellular cells from ferrocene-treated mice have an anti-tumor effect in mice that were otherwise not treated with this compound.

Anti-tumor effects of agents known to stimulate the immune-system have been previously reported (1-5). They include IL-2, cytokines and agonistic antibodies such as anti CD3 (6,7) and anti BAT monoclonal antibody (34). Of interest are the findings described herein that H\textsubscript{2}O\textsubscript{2} administered intraperitoneally, at a low dose, has an anti-tumor effect. H\textsubscript{2}O\textsubscript{2}-induced activation of the MAPK and NF\textkappaB pathways has been previously reported (35-37). Hydrogen peroxide was also reported to activate T lymphocytes (38).

In several independent assays treatment with ferrocene showed a bell-shaped (optimum) response curve. Hence, methods of determining whether a metalloocene derivative is an effective immunostimulant and/or effective in cancer therapy constitute an embodiment of the present invention.

Hence, according to one aspect of the present invention there is provided a method of determining whether a metalloocene derivative is an effective immunostimulant and/or effective in cancer therapy. The method according to this aspect of the present invention comprises determining an immune stimulation/cancer therapy dose-response curve for the metalloocene derivative and determining whether the dose-response curve has an optimum with respect
to immune stimulation/cancer therapy within a predetermined range of concentrations. As is further exemplified in the Examples section, immune stimulation can be determined \textit{in vivo} or \textit{in vitro} (e.g., \textit{ex vivo}). As is still further exemplified in the Examples section the immune stimulation can be determined with respect to macrophages and/or lymphocytes, by a cell proliferation assay, such as a thymidine incorporation assay or by a cellular response assay, such as an NO production assay, a TNF-\(\alpha\) production assay and/or an oxygen burst assay.

According to another aspect of the present invention there is provided a method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy. The method according to this aspect of the invention comprises determining a p21\(^{\text{ras}}\) signal transduction pathway component activity dose-response curve for the metallocene derivative and determining whether the dose-response curve has an optimum with respect to p21\(^{\text{ras}}\) signal transduction pathway component activity within a predetermined range of concentrations. Preferably, the p21\(^{\text{ras}}\) signal transduction pathway component is p21\(^{\text{ras}}\) and/or NF-\(\kappa\)B.

Hence, according to yet additional aspects of the present invention there is provided (i) method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining p21\(^{\text{ras}}\) oxidation dose-response curve for the metallocene derivative and determining whether the dose-response curve has an optimum with respect to p21\(^{\text{ras}}\) oxidation within a predetermined range of concentrations; (ii) a method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy, the method comprising determining a GTPase activity dose-response curve for the metallocene derivative and determining whether the dose-response curve has an optimum with respect to the GTPase activity within a predetermined range of concentrations; and (iii) a method of determining whether a metallocene derivative is an effective immunostimulant and/or effective in cancer therapy,
the method comprising determining an NF-κB activity dose-response curve for
the metalloccene derivative and determining whether the dose-response curve
has an optimum with respect to the NF-κB activity within a predetermined
range of concentrations.

Without being bound by a theory, it is postulated that ferrocene and
metalloccene derivatives are effective immunostimulant as they catalyze the
generation of H₂O₂ which reacts with susceptible molecules, or react directly
with thiol moieties such as C118 in p21ras.

Ferrocene at 5-500 nM under the experimental system described in the
Examples section that follows, generated 9-12 micromolar H₂O₂. It is hence
assumed that metalloccene derivatives with a similar capabilities of H₂O₂
generation are effective immunostimulants and/or effective in cancer therapy.

Thus, according to still another aspect of the present invention there is
provided a method of determining whether a metalloccene derivative is an
effective immunostimulant and/or effective in cancer therapy. The method
according to this aspect of the present invention comprises monitoring the rate
of H₂O₂ generation catalyzed by the metalloccene derivative, and determining
whether the rate is within a predetermined range.

Kinetic parameters determination assay is a multi data points assay and
hence less amenable for highthroughput screening. However, based on the
preferred range of parameters a single data point assay (a highthroughput assay)
for H₂O₂ generation catalyzed by metalloccene derivatives can be developed by
those of skills in the art so as to be used in determining whether a metalloccene
derivative is an effective immunostimulant and/or effective in cancer therapy.
Hence, according to an additional aspect of the present invention there is
provided a method of determining whether a metalloccene derivative is an
effective immunostimulant and/or effective in cancer therapy. The method
according to this aspect of the invention comprises monitoring H₂O₂ generation
catalyzed by the metalloccene derivative, and determining whether the
generation is above a predetermined value.
According to yet an additional aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one agent in an amount so as to induce H$_2$O$_2$ generation at a concentration effective in inducing immune stimulation and or providing cancer therapy. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprises, as an active ingredient, at least one agent in an amount so as to induce H$_2$O$_2$ generation at a concentration effective in inducing immune stimulation following administration thereof.

According to a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprises, as an active ingredient, at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight.

According to yet a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one agent in an amount effective in activating a p21$^{ras}$ signal transduction pathway component. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit
of the pharmaceutical composition comprises, as an active ingredient, at least one agent in an amount effective in activating a p21ras signal transduction pathway component following administration thereof.

According to still a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one agent in an amount effective in inducing p21ras oxidation. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprises, as an active ingredient, at least one agent in an amount effective in inducing p21ras oxidation following administration thereof.

According to still a further aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one agent in an amount effective in inducing GTPase activity. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer, each dose-unit of the pharmaceutical composition comprises, as an active ingredient, at least one agent in an amount effective in inducing GTPase activity following administration thereof.

According to another aspect of the present invention there is provided a method of treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or treating cancer. The method according to this aspect of the present invention comprises administering to a subject in need thereof at least one agent in an amount effective in inducing NF-κB activity. Accordingly, there is also provided a pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial
and/or treating cancer, each dose-unit of the pharmaceutical composition comprises, as an active ingredient, at least one agent in an amount effective in inducing NF-κB activity following administration thereof.

According to a presently preferred embodiment of the present invention the agent(s) used in the methods and compositions described above comprises at least one ferrocene and/or metalloocene derivative and the amount thereof in a dose-unit is 0.025 - 10 mg/kg body weight, which translates to 0.25 - 1000 mg per dose-unit for individuals weighting 10-100 kg. Preferably, the amount of the ferrocene and/or metalloocene derivative in a dose-unit is 0.1 - 5 mg/kg body weight, which translates to 1 - 500 mg per dose-unit for individuals weighting 10-100 kg. Still preferably, the amount of the ferrocene and/or metalloocene derivative in a dose-unit is 0.2 - 3 mg/kg body weight, which translates to 2 - 300 mg per dose-unit for individuals weighting 10-100 kg.

Preferably the agent(s) used according to the present invention for treating a disease, syndrome or condition, in which immune stimulation is beneficial and/or for treating cancer are administered orally or by injection. Hence, the pharmaceutical compositions described herein are preferably formulated for oral administration or administration by injection. Other modes of application are further described herein.

The present invention offers treatment for any and all cancers. These include, for example, carcinomas (e.g., colon, lung, breast, prostate, ovary), sarcomas (e.g., soft tissue, bone), lymphomas (e.g., Hodgkin lymphoma, non-Hodgkin lymphoma), hematological malignancies (e.g., myelogenous leukemia, lymphocytic leukemia). Hence, the pharmaceutical composition is preferably packaged and identified for the treatment of carcinomas (e.g., colon, lung, breast, prostate, ovary), sarcomas (e.g., soft tissue, bone), lymphomas (e.g., Hodgkin, non-Hodgkin), hematological malignancies (e.g., myelogenous leukemia, lymphocytic leukemia).

The present invention further offers treatment for acquired immune deficiency, such as AIDS, radiotherapy induced immune deficiency,
chemotherapy induced immune deficiency and congenital immune deficiency (e.g., SKID). Hence, the pharmaceutical composition can be packaged and identified for the treatment of a disease, syndrome or condition, such as an acquired immune deficiency, a radiotherapy induced immune deficiency, a chemotherapy induced immune deficiency and a congenital immune deficiency.

The metalloocene or metalloocene derivatives used in the methods and pharmaceutical compositions of the present invention has a a general formula:

![Chemical structure](image)

wherein, M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and R_1-R_8 are each independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryl and arloxy.

As used herein, the term "alkyl" refers to a saturated aliphatic hydrocarbon including straight chain and branched chain groups. Preferably, the alkyl group has 1 to 10 carbon atoms. Whenever a numerical range; e.g., "1-10", is stated herein, it means that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 10 carbon atoms. More preferably, it is a medium size alkyl having 1 to 6 carbon atoms. Most preferably, it is a lower alkyl having 1 to 4 carbon atoms. The alkyl group may be substituted or unsubstituted. When substituted,
the substituent group can be, for example, cycloalkyl, aryl, hydroxy, alkoxy, aryloxy, cyano, halo, carbonyl, nitro, silyl, or amino.

A "cycloalkyl" group refers to an all-carbon monocyclic or fused ring (i.e., rings which share an adjacent pair of carbon atoms) group wherein one of more of the rings does not have a completely conjugated pi-electron system. Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, cycloheptane, cycloheptatriene, and adamantane. A cycloalkyl group may be substituted or unsubstituted. When substituted, the substituent group can be, for example, cycloalkyl, aryl, hydroxy, alkoxy, aryloxy, cyano, halo, carbonyl, nitro, silyl, or amino.

An "alkenyl" group refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon double bond.

An "alkynyl" group refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon triple bond.

An "alkoxy" group refers to both an -O-alkyl and an -O-cycloalkyl group, as defined herein.

An "aryl" group refers to an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups having a completely conjugated pi-electron system. Examples, without limitation, of aryl groups are phenyl, naphthalenyl and anthracenyl. The aryl group may be substituted or unsubstituted. When substituted, the substituent group can be, for example, halo, trihalomethyl, cycloalkyl, aryl, hydroxy, alkoxy, aryloxy, cyano, carbonyl, nitro, silyl, or amino.

An "aryloxy" group refers to an -O-aryl as defined herein.

A "halo" group refers to fluorine, chlorine, bromine or iodine.

A "trihalomethyl" group refers to a -CX₃ group wherein X is a halo group as defined herein.

A hydroxy group refers to a -OH group.

An "Amino" group refers to an -NH₂ group.
A "nitro" group refers to an -NO\textsubscript{2} group.

A "cyano" group refers to a -C≡N group.

A "carbonyl" group refers to a -C(=O)-R' group, where R' is hydrogen, alkyl, cycloalkyl or aryl, as defined herein.

A "silyl" group refers to a -Si(R')\textsubscript{3}, where R' is hydrogen, alkyl, cycloalkyl or aryl, as defined herein.

Depending on the disease, syndrome or condition to be treated, according to a preferred embodiment of the present invention, the method further comprises co-administering at least one additional agent having immune stimulation and/or anti-cancer activity. Accordingly, the pharmaceutical composition, further comprises at least one additional agent having immune stimulation and/or anti-cancer activity. Agents having immune stimulation activity include, for example, cytokines (e.g., IL-2, TNF, interferon), chemokines, immune-stimulatory antibodies (e.g., anti-CD3, anti-CTLA-4) or an anti-tumor vaccine. Agents having anti-cancer activity include, for example, chemotherapeutic agents, anti-metabolites, signal transduction inhibitors (e.g., protein tyrosine kinase inhibitors), anti-angiogenesis compounds, antibodies (e.g., anti-receptors, anti-tumor determinants), hormones and hormone antagonists, retinoids, steroids, pro-apoptotic agents, differentiating agents or radiation.

It is shown herein that ferrocene activity with respect to immune stimulation and/or cancer therapy is mediated, at least in part, by H\textsubscript{2}O\textsubscript{2} generation. Hence, according to another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer in a body cavity. The method according to this aspect of the present invention comprises administering into the body cavity of a subject in need thereof H\textsubscript{2}O\textsubscript{2} in an amount effective in immune stimulation. Preferably, the amount is of 10-2000 ml of 50-2500 μM H\textsubscript{2}O\textsubscript{2} in a single or multiple administrations. The body cavity can be, for example, the peritoneal cavity or the pleural cavity.
It is further shown herein that cells derived from mice treated with ferrocene are effective in inducing immune stimulation and/or treating cancer in adoptive transfer experiments. Hence, according to still another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer. The method according to this aspect of the present invention comprises exposing immune cells ex vivo to at least one ferrocene and/or metalloocene derivative, and administering the cells to a subject in need thereof. According to yet another aspect of the present invention there is provided a method of inducing immune stimulation and/or treating cancer. The method according to this aspect of the present invention comprises exposing immune cells ex vivo to $\text{H}_2\text{O}_2$ in a concentration effective in inducing immune stimulation and administering the cells to a subject in need of thereof.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of metalloocene or metalloocene derivatives (including salts and acids thereof) described herein, with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" includes compounds directly accountable for a biological effect.

Hereinafter, the terms "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.
Techniques for formulation and administration of drugs may be found in "Remington’s Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intrapleural, intranasal, or intraocular injections.

Alternately, one may administer a preparation in a local rather than systemic manner, for example, via injection of the preparation directly into a solid tumor often in a depot or slow release formulation, such as described below.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a tumor specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank’s solution, Ringer’s solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be
permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets; pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or
suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuos infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may
also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, a preparation of the present invention may also be formulated for local administration, such as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the preparation may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives such as sparingly soluble salts. Formulations for topical administration may include, but are not limited to, lotions, suspensions, ointments gels, creams, drops, liquids, sprays emulsions and powders.

According to a preferred embodiment of the present invention, the pharmaceutical composition is designed for a slow release of the active ingredients. The composition includes particles including a slow release carrier (typically, a polymeric carrier), such as, for example, polylactic acid, and the active ingredients. Slow release biodegradable carriers are well known in the art. These are materials that may form particles that may capture therein an active compound(s) and slowly degrade/dissolve under a suitable environment (e.g., aqueous, acidic, basic, etc.) and thereby degrade/dissolve in body fluids and release the active compound(s) therein. The particles are preferably nanoparticles (i.e., in the nanometer range, e.g., in the range of about 1 to about 500 nm in diameter, preferably about 50-200 nm in diameter, most preferably about 100 nm in diameter).
The pharmaceutical compositions herein described may also comprise suitable solid of gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Many of the compounds in the claimed preparations of the present invention may be provided as physiologically acceptable salts wherein the compound may form the negatively or the positively charged species.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of preparation effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC$_{50}$ as determined in cell culture (i.e., the concentration of the test compound, which achieves a half-maximal activity). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the IC$_{50}$ and the LD$_{50}$ (lethal dose causing death in 50 % of the tested animals) for a subject compound. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary
depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the therapeutic effects, termed the minimal effective concentration (MEC). The MEC will vary for each preparation, but can be estimated from in vitro data; e.g., the concentration necessary to achieve 50-90 % activity may be ascertained using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using the MEC value. Preparations should be administered using a regimen, which maintains plasma levels above the MEC for 10-90 % of the time, preferable between 30-90 % and most preferably 50-90 %.

It is noted that, in the case of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration. In such cases, other procedures known in the art can be employed to determine the effective local concentration.

Depending on the severity and responsiveness of the condition to be treated, dosing can also be a single administration of a slow release composition described herein above, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one
or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions are listed hereinabove.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

MATERIALS AND EXPERIMENTAL METHODS

Ferrocene:

Ferrocene (98 % pure) was obtained from Sigma, Israel (Cat. No. F40-8). Stock solutions (5 mg/ml) were made in absolute ethanol and dilutions were made in NaCl. 0.9 % Hydrogen peroxide (H₂O₂), ACS reagent, 30 % in
water was obtained from FLUKA, Israel (Cat. No. 95300). Recombinant human IL-2 (2 x 10^7 IU/mg in NaCl 0.9 %) was obtained from ReproTech, Inc., N.J., USA.

Mice:

C57BL female mice, 6-8 weeks of age, were obtained from the Animal Breeding Center of the Tel Aviv University.

Tumor Models:

Two mouse tumors grown in C57BL mice were used: B-16 melanoma and 3LL (Lewis lung carcinoma). B-16 and 3LL were obtained from large stocks of frozen cells and were grown in cultures. Culture medium included RPMI 1640, supplemented with 5 % FCS. The B-16 and 3LL cell stocks were prepared from in vivo subcutaneous growth in C57BL mice. To generate the stocks, B-16 cells were injected intravenously at 5 x 10^4 cells/mouse, whereas 3LL cells were injected intravenously at 2 x 10^5 cells/mouse.

Isolation of cells:

Mouse splenocytes: Spleens were removed aseptically and crushed with the blunt end of a syringe in PBS. The cells were then centrifuged, washed twice with PBS and resuspended in RPMI 1640, supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5 x 10^5 2-mercaptoethanol, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.03 % fresh glutamine, and 5 % heat-inactivated fetal calf serum. Cells (2 x 10^6/ml) were distributed (0.2 ml aliquots) in flat bottomed microwells and incubated at 37 °C, 5 % CO_2 for 68 hours. ^3H-Thymidine incorporation (2 μCi/well) into DNA during the final 20 hours of incubation was determined. The means ±SD of CPM of triplicate cultures were determined.

Mouse peritoneal macrophages: C57BL female mice were anesthetized by ether and their peritoneal cavity washed with 3-5 ml of Ca^{++} and Mg^{++} free PBS. The washing fluid was centrifuged and the cells in the pellet were suspended in RPMI 1640 containing 20 % FCS and distributed into microwells.
After incubation for 2 hours at 37 °C, the non-adherent cells were removed by washing (3 times) with Ca\(^{++}\), Mg\(^{++}\) free PBS.

**Assessment of oxygen burst in macrophages by determination of H\(_2\)O\(_2\) produced, using a sensitive assay:**

The assay was done essentially as described by Scanonne et al., (25) and is based on the findings that H\(_2\)O\(_2\) irreversibly inactivates catalase in the presence of 3-amine-1,2,4-triazole (26). Sodium perborate served as a substrate for catalase and its amount was determined by titration with KMnO\(_4\).

**TNF-α determination:**

TNF-α was determined using a bioassay, as previously described (27). A9 cells (a TNF-α sensitive cell line) were incubated for 24 hours in serum free DMEM medium containing cycloheximide (50 μg/ml) in the presence of experimental aliquots. Recombinant TNF-α was used to generate a standard curve. Cytotoxicity was assessed using the neutral red vital staining assay.

**Nitric oxide assay:**

Nitric oxide levels in experimental aliquots was determined colorimetrically using a Griess reagent, essentially as described (27).

**Western blot analysis:**

Cells were solubilized with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 % TRITON X-100, 3 mM EGTA, 12 mM β-glycerophosphate, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium vanadate, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin and 0.1 % 2-mercaptoethanol. The cell lysates were then applied to a 10 % polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking with 5 % (w/v) of non fat dry milk, the membranes were incubated with a mouse monoclonal anti-MAP kinase, activated (Diphosphorylated ERK1 and ERK2), clone MAPK-YT (Sigma M-8159). The membrane was then incubated with HRP-conjugated goat anti-mouse antibody (Jackson) and developed with the enhanced chemiluminescence mixture.
**GTPase assay:**

The GTPase assay was carried out as follows: Pure recombinant P21ras (1 μM) was added to assay tubes on ice containing 50 μl of NaCl, 200 mM; Tris, 25 mM; MgCl₂, 10 mM; and EGTA; 0.5 mM, pH 7.4. To this was added 10 μl of each of DTT, 20 mM; ATP, 10 mM; adenosine 5'-(β, γ-imido) triphosphate, 5 mM; creatine phosphate, 100 mM; and creatine kinase, 500 U/ml. The final reaction volume was 100 μl and therefore the final concentration of components was 10-fold less than that added. Then, 100,000 dpm of GTP-γ²³P (5000 Ci/mmole) were added as well as any test reagent, and tubes transferred to a shaking 37 °C water bath for 10 minutes. At the end of the assay period, 0.8 ml of an acid charcoal solution was added (1 N HCl containing 10 % (v/v) Norit A) and samples were spun for 3 minutes at 14,000 x g. The supernatants (450 μl) were counted in a liquid scintillation counter. Data are expressed as fmol PO₄⁻ released per minute per mg.

**Electromobility shift assay:**

PBMC cells (5 x 10⁶/ml) in 1 ml were treated for 4 hours at 37 °C in RPMI 1640 containing 5 % heat inactivated FCS and the indicated treatments. Cells were pelleted, washed once with cold PBS and resuspended in 250 μl of 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 10 % glycerol. Cells were left on ice for 10 minutes and Nonidet P-40 was added to a final concentration of 0.04 %. After 5 minutes on ice, nuclear extracts were pelleted at 10,000 x g for 5 minutes. Supernatants were removed and 100 μl of 20 mM HEPES, pH 7.9, 200 mM KCl, 20 % glycerol, 0.5 mM PMSF, and 1 mM EDTA was added to the pellets. After another hour on ice, pellets were spun at 100,000 x g for 20 minutes. Supernatants were stored at -70 °C until use. Protein concentrations were determined by the method of Lowry (Gaunce,AP and D'Iorio,Λ., Microdetermination of protein by a automated Lowry method. Anal Biochem,37:204-207,1970).
The NF-κB DNA probe used contained the 206 to -195 region of the IL-2 promoter (5'-CCAAGAGGGATTTCACTAAATCC-3', SEQ ID NO:1).

Approximately 10⁴ cpm (0.2 ng) of 5'-end labeled DNA was added to 5 μg nuclear protein and 2 μg Poly (dI-dC) in 20 μl of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5 % glycerol at room temperature for 20 minutes. Protein-DNA complexes were resolved on 4 % polyacrylamide gels in 45 mM tris, 45 mM borate, and 1 mM EDTA pH 8.3 at 150 V for 1.5 hours at room temperature. Gels were dried and exposed to x-ray film. The nonspecific probe used contained the -93 to -69 region of the IL-2 promoter (5'-TTACAAAATGTATAATGTGTTAAA-3', SEQ ID NO:2).

**Jurkat T-cells stably expressing p21ras C118S (1-189):**

Jurkat T-cells stably expressing p21ras C118S (1-189), in which cysteine at position 118 was replaced by serine, were obtained from Dr. Harry M. Lander, Department of Biochemistry, Cornell University College of Medicine, New York. These cells did not respond to stimulation by nitric oxide (NO) as assessed by activation of ERK1/2 but did so in response to PHA. These transfected cells expressed 7-10-fold more p21ras than the wild-type cells as determined by Western blotting with the anti-p21ras antibody, Y13-259. This antibody cannot distinguish between wild-type and p21ras C118S, suggesting that although endogenous p21ras was not specifically inhibited, ectopic expression of high levels of mutant p21ras apparently prevented its signaling. This dominant negative activity of p21rasC118S toward NO action may be due to its high level of expression (22).

**EXPERIMENTAL RESULTS**

**Immune-stimulation properties of ferrocene:**

Ferrocene stimulated ³H-thymidine incorporation in mouse splenocytes upon incubation for 3 days, in vitro (Figure 1A). Maximal stimulation was obtained by ferrocene at a dose of 0.05-0.2 μM. Ferrocene further enhanced stimulation of splenocytes that had been treated with interleukin-2 (Figure 1B).
Ferrocene also stimulates $^{3}$H-thymidine incorporation upon administration in vivo. Splenocytes that were isolated from mice that had been injected 3 days earlier with ferrocene (0.05-1 mg/kg body weight) showed increased $^{3}$H-thymidine incorporation above controls (Figure 2). Purified peritoneal macrophages were not stimulated by ferrocene in vitro. Ferrocene activated peritoneal macrophages in vitro upon their incubation in the presence of mouse lymphocytes (Figure 3). Macrophage activation was assessed by oxygen burst in the presence of phorbol myristate acetate (PMA). Ferrocene also stimulated mouse peritoneal macrophages upon administration in vivo. Peritoneal macrophages were isolated 3 days following a single intraperitoneal ferrocene injection and their activation was assessed by measuring oxygen burst in the presence of PMA (Figure 4).

Activation of mouse peritoneal macrophages in vivo was also assessed by production of TNF-α, NO and eliciting an oxygen burst (H$_2$O$_2$) (Figures 5A-C). Mice were injected intraperitoneally with ferrocene (50 μg/kg body weight). Three days later, macrophages were isolated and TNF-α and NO levels were determined in the supernatants after incubation in vitro for 6 hours in the presence of LPS (10 μg/ml). Oxygen burst was assessed by determination of H$_2$O$_2$ production in the presence of PMA.

**Anti-tumor properties of ferrocene:**

The anti-tumor effect of a single intraperitoneal administration of ferrocene, at different doses, in B-16 melanoma bearing mice was investigated (5 mice were included in each group). Mice were sacrificed 25 days post tumor inoculation and the lungs were weighed. Weight of lungs in B16 inoculated mice above controls represents tumor load.

Results indicated that ferrocene at a single dose of 20 μg/kg body weight was most effective in eliciting an anti-tumor effect. Ferrocene at higher doses (1 and 5 mg/kg body weight) was significantly less effective (Figure 6). A similar pattern of results was obtained upon multiple administrations of ferrocene at different doses, each at T$_1$, T$_8$ and T$_{15}$ (T$_0$, the day of tumor
inoculation) (Figure 7). However, ferrocene at a dose of 50 μg/kg body weight, was more effective upon administration at $T_1$, $T_8$, and $T_{15}$ compared to administration at $T_1$, alone (Figure 8).

Ferrocene also has an anti-tumor effect upon administration in drinking water. The effect of ferrocene in drinking water on established lung metastases in B-16 melanoma bearing mice is depicted in Table 1, Figure 9A and Figure 9B. Maximal anti-tumor effect was attained upon administration of ferrocene in drinking water (1 μg/ml) for 1 week. Administration for 2 weeks was less effective and for 3 weeks had no anti-tumor effect.

### Table 1

**Anti-tumor effect of ferrocene in drinking water on established lung metastases of B-16 melanoma**

<table>
<thead>
<tr>
<th>Ferrocene in drinking water (μg/ml)</th>
<th>One week</th>
<th>Two Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lungs' weight mg ±SD</td>
<td>Scoring Index* ±SD</td>
</tr>
<tr>
<td>0</td>
<td>459±49</td>
<td>75±23</td>
</tr>
<tr>
<td>0.5</td>
<td>255±47</td>
<td>26±16</td>
</tr>
<tr>
<td>1</td>
<td>272±19</td>
<td>20±16</td>
</tr>
<tr>
<td>2</td>
<td>239±27</td>
<td>8±5</td>
</tr>
</tbody>
</table>

*Scoring index was determined by assessment of tumor load by counting and measuring lungs' metastases as follows: conglomerate - 20; large (> 5 mm) - 3; medium (2-5 mm) - 2; small (< 2 mm) - 1. Mean ±SD of lungs' weight (mg) of normal mice (n=6) was 203 ±7.*

The anti-tumor effect on B-16 melanoma of ferrocene in drinking water (at different concentrations) administered for one and two weeks was investigated. The most effective anti-tumor protocols were the administration of ferrocene in drinking water for 2 weeks at a concentration of 0.5 μg/ml and
administration in drinking water for 1 week at a concentration of 2 μg/ml. Other protocols were also effective to different degrees (Table 1 and Figures 10A and 10B).

Ferrocene administered in drinking water (1 μg/ml) for one week prolonged the survival of B-16 melanoma bearing mice (Figure 11). Ferrocene also has an anti-tumor effect in Lewis lung carcinoma bearing mice.

Intraperitoneal administration of ferrocene at a dose of 50 μg/kg body weight, 3 times each, on days T1, T8 and T15 markedly reduced established lung metastases of Lewis lung carcinoma (Figure 12). Ferrocene at a higher dose (200 μg/kg body weight) was less effective.

The anti-tumor effect of combined treatment with ferrocene at a low dose (50 μg/kg body weight at T1) along with IL-2 (250 x 10^3 U/kg body weight on days T1, T2 and T3) against B-16 melanoma was more pronounced than by treatment with each of the agents alone (Figure 13).

Adoptive transfer experiments

Based on the assumption that ferrocene mediates its effect by immune stimulation, whether immunocytes from ferrocene treated mice could elicit an anti-tumor effect in otherwise untreated tumor-inoculated mice was investigated.

Donor mice were inoculated with B-16 melanoma and treated at day T2 with ferrocene (200 μg/kg body weight). Mice were sacrificed at T25 splenocytes and peritoneal cells (containing 50 % lymphocytes and 50 % macrophages) were isolated and injected intravenously into B-16 inoculated mice (T1). As depicted in Figures 14A and 14B, the peritoneal mononuclear cells from ferrocene-treated mice were most effective in eliciting an anti-tumor effect. Figure 15 represents an additional experiment illustrating the anti-tumor effect of peritoneal mononuclear cells from ferrocene-treated mice.

Mechanisms of action of ferrocene:

It is postulated that the primary molecular target of ferrocene is p21ras which has been identified as a key molecule in T lymphocyte activation
(15-19). p21<sup>ras</sup> has previously been shown to be activated by cellular redox stress (21, 22).

The following experiments support this postulation.

1. Auto-oxidation of ferrocene in PBS generates H<sub>2</sub>O<sub>2</sub> (Figure 16). H<sub>2</sub>O<sub>2</sub> was measured using the aminotriazole-catalase inhibition assay (see Materials and Experimental Methods section above). The effect of N-acetylcysteine (NAC), a free radical scavenger, on ferrocene-induced anti-tumor effect (B-16 melanoma) was investigated.

Ferrocene at an optimal dose of 0.25 mg/kg (250 μg/kg) body weight or at a supraoptimal dose of 2.5 mg/kg body weight) was injected intraperitoneally to C57BL mice which had been inoculated with B16-melanoma on day T<sub>1</sub>. NAC was injected intraperitoneally at a dose of 500 mg/kg body weight twice a day for 3 consecutive days (T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>)

The anti-tumor effect of ferrocene at an optimal dose of 250 μg/kg body weight was markedly inhibited by N-acetylcysteine. In contrast, NAC enhanced the anti-tumor effect in mice that were treated with ferrocene, at a single-supraoptimal dose of 2.5 mg/kg body weight which, by itself, had a very little anti-tumor effect (Figure 17). These results are in accordance with the findings presented herein that the anti-tumor effect of ferrocene follows a "bell shape" curve, namely it is most effective at an optimal dose and is less active at higher doses.

3. To establish that p21<sup>ras</sup> cys118 is the primary target site for oxidation by ferrocene, the stimulatory effect of ferrocene on ERK1/2 (which is mediated by ras) was investigated in wild type Jurkat cells and in Jurkat T cells in which p21<sup>ras</sup> cys118 was replaced by a serine residue (referred to as p21<sup>ras</sup> C118S, see Materials and Experimental Methods), which is not susceptible to redox-stress modification.

As depicted in Figure 18, ferrocene activated ERK1/2 in wild type Jurkat cells but failed to do so in Jurkat C118S. On the other hand, PHA which does not activate p21<sup>ras</sup> via redox stress, phosphorylated ERK1/2 in both cells.
Levels of activated ERK1/2 in the cell lysates were determined by Western blot analysis using anti-phosphorylated ERK1/2.

4. Ferrocene stimulates GTPase activity catalyzed by pure recombinant p21ras in a bell-shaped pattern (Table 2). Ferrocene, at a high concentration (50 nM) was inhibitory.

<table>
<thead>
<tr>
<th>Ferrocene (nM)</th>
<th>ras GTPase* ( % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100±2</td>
</tr>
<tr>
<td>5</td>
<td>121±2</td>
</tr>
<tr>
<td>10</td>
<td>145±6</td>
</tr>
<tr>
<td>50</td>
<td>12±3</td>
</tr>
</tbody>
</table>

* The effect of ferrocene on GTPase activity of p21ras was determined as outlined in Materials and Experimental Methods.

5. Ferrocene also activates and translocates NFκB in human PBM cells (Figure 19), a pathway that is mediated by ras. Binding of NFκB to a DNA fragment was demonstrated in an electromobility shift assay as described in Materials and Experimental Methods.

**Anti-tumor effect of H₂O₂, administered intraperitoneally**

Ferrocene generates hydrogen peroxide (H₂O₂) upon auto-oxidation (see Figure 16). Following the observation that ferrocene elicits an anti-tumor effect, whether H₂O₂ itself will share this property was investigated. H₂O₂ was injected intraperitoneally, twice a day at T1, T2 and T3 (1 cycle) or at day T₁-T₃, T₅-T₁₀ and T₁₅-T₁₇ (3 cycles), each time at a dose of 5 μM/kg body weight. Mice were sacrificed 25 days post tumor inoculation and tumor load was assessed.

Four mice were included in each experiment and the results are expressed as means of lungs' weight ±SD (Figure 20). An additional
experiment depicted in Figures 21A, 21B and 22 also demonstrated the anti-tumor effect of \( \text{H}_2\text{O}_2 \). The experimental protocol in this experiment was similar to that outlined in Figure 20. Six mice were included in each group and the results are expressed as the means of lungs' weight ±SD (Figures 21A-B). Photographs of the lungs are shown in Figure 22.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.
REFERENCES CITED
(Additional references are cited in the text)


WHAT IS CLAIMED IS:

1. A method of determining whether a metallocene derivative is an effective immunostimulant, the method comprising determining an immune stimulation dose-response curve for the metallocene derivative and determining whether said dose-response curve has an optimum with respect to immune stimulation within a predetermined range of concentrations.

2. The method of claim 1, wherein said immune stimulation is determined in vivo.

3. The method of claim 1, wherein said immune stimulation is determined in vitro.

4. The method of claim 1, wherein said immune stimulation is determined with respect to macrophages.

5. The method of claim 1, wherein said immune stimulation is determined with respect to lymphocytes.

6. The method of claim 1, wherein said immune stimulation is determined by a cell proliferation assay.

7. The method of claim 6, wherein said cell proliferation assay includes a thymidine incorporation assay.

8. The method of claim 1, wherein said immune stimulation is determined by a cellular response assay.

9. The method of claim 8, wherein said cell response assay is selected from the group consisting of an NO production assay, a TNF-α production assay and an oxygen burst assay.
10. A method of determining whether a metalallocene derivative is an effective immunostimulant, the method comprising determining a p21\(^{ras}\) signal transduction pathway component activity dose-response curve for the metalallocene derivative and determining whether said dose-response curve has an optimum with respect to p21\(^{ras}\) signal transduction pathway component activity within a predetermined range of concentrations.

11. The method of claim 10, wherein said p21\(^{ras}\) signal transduction pathway component is selected from the group consisting of p21\(^{ras}\) and NF-κB.

12. A method of determining whether a metalallocene derivative is an effective immunostimulant, the method comprising determining p21\(^{ras}\) oxidation dose-response curve for the metalallocene derivative and determining whether said dose-response curve has an optimum with respect to p21\(^{ras}\) oxidation within a predetermined range of concentrations.

13. A method of determining whether a metalallocene derivative is an effective immunostimulant, the method comprising determining a GTPase activity dose-response curve for the metalallocene derivative and determining whether said dose-response curve has an optimum with respect to said GTPase activity within a predetermined range of concentrations.

14. A method of determining whether a metalallocene derivative is an effective immunostimulant, the method comprising determining an NF-κB activity dose-response curve for the metalallocene derivative and determining whether said dose-response curve has an optimum with respect to said NF-κB activity within a predetermined range of concentrations.

15. A method of determining whether a metalallocene derivative is an effective immunostimulant, the method comprising determining \(\text{H}_2\text{O}_2\)
50
generation catalyzed by the metallocene derivative, and determining whether
the rate is within a predetermined range.

16. A method of determining whether a metallocene derivative is an
effective immunostimulant, the method comprising monitoring \( \text{H}_2\text{O}_2 \) generation
catalyzed by the metallocene derivative, and determining whether said
generation is above a predetermined value.

17. A method of determining whether a metallocene derivative is a
candidate for cancer therapy, the method comprising determining a cancer
therapy dose-response curve for the metallocene derivative and determining
whether said dose-response curve has an optimum with respect to said cancer
therapy within a predetermined range of concentrations.

18. The method of claim 17, wherein said cancer therapy is
determined in vivo.

19. The method of claim 17, wherein said cancer therapy is
determined in vitro.

20. The method of claim 17, wherein said cancer therapy is
determined in a test animal inoculated with cancer cells.

21. A method of determining whether a metallocene derivative is a
candidate for cancer therapy, the method comprising determining a \( p21^{ras} \) signal
transduction pathway component activity dose-response curve for the
metallocene derivative and determining whether said dose-response curve has
an optimum with respect to \( p21^{ras} \) signal transduction pathway component
activity within a predetermined range of concentrations.
22. The method of claim 21, wherein said $p21^{ras}$ signal transduction pathway component is selected from the group consisting of $p21^{ras}$ and NF-κB.

23. A method of determining whether a metalloocene derivative is a candidate for cancer therapy, the method comprising determining $p21^{ras}$ oxidation dose-response curve for the metalloocene derivative and determining whether said dose-response curve has an optimum with respect to $p21^{ras}$ oxidation within a predetermined range of concentrations.

24. A method of determining whether a metalloocene derivative is a candidate for cancer therapy, the method comprising determining a GTPase activity dose-response curve for the metalloocene derivative and determining whether said dose-response curve has an optimum with respect to said GTPase activity within a predetermined range of concentrations.

25. A method of determining whether a metalloocene derivative is a candidate for cancer therapy, the method comprising determining an NF-κB activity dose-response curve for the metalloocene derivative and determining whether said dose-response curve has an optimum with respect to said NF-κB activity within a predetermined range of concentrations.

26. A method of determining whether a metalloocene derivative is a candidate for cancer therapy, the method comprising determining determining $H_2O_2$ generation catalyzed by the metalloocene derivative, and determining whether the rate is within a predetermined range.

27. A method of determining whether a metalloocene derivative is a candidate for cancer therapy, the method comprising monitoring $H_2O_2$ generation catalyzed by the metalloocene derivative, and determining whether
said generation is above a predetermined value under predetermined experimental conditions.

28. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one agent in an amount so as to induce \( \text{H}_2\text{O}_2 \) generation at a concentration effective in inducing immune stimulation.

29. The method of claim 28, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

30. The method of claim 29, wherein said amount is between 0.025 - 10 mg/kg body weight.

31. The method of claim 29, wherein said amount is between 0.1 - 5 mg/kg body weight.

32. The method of claim 29, wherein said amount is between 0.2 - 3 mg/kg body weight.

33. The method of claim 29, wherein administering said at least one ferrocene and/or metalloocene derivative is by oral administration.

34. The method of claim 29, wherein administering said at least one ferrocene and/or metalloocene derivative is by injection.

35. The method of claim 29, wherein said disease, syndrome or condition is selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.
36. The method of claim 29, wherein said metalloocene or metalloocene derivative has a general formula:

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and arylxoy.

37. The method of claim 29, further comprising co-administering at least one additional agent having immune stimulation activity.

38. The method of claim 37, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-timor vaccine.

39. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one ferrocene and/or metalloocene derivative, in an amount of 0.025 - 10 mg/kg body weight.
40. The method of claim 39, wherein said amount is between 0.1 - 5 mg/kg body weight.

41. The method of claim 39, wherein said amount is between 0.2 - 3 mg/kg body weight.

42. The method of claim 39, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

43. The method of claim 39, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

44. The method of claim 39, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

45. The method of claim 39, wherein said metallocene or metallocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and arylxy.

46. The method of claim 39, further comprising co-administering at least one additional agent having immune stimulation activity.

47. The method of claim 46, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

48. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one agent in an amount effective in activating a p²¹ras signal transduction pathway component.

49. The method of claim 48, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

50. The method of claim 49, wherein said p²¹ras signal transduction pathway component is selected from the group consisting of p²¹ras and NF-κB.

51. The method of claim 49, wherein said amount is between 0.1 - 5 mg/kg body weight.

52. The method of claim 49, wherein said amount is between 0.2 - 3 mg/kg body weight.
53. The method of claim 49, wherein administering said at least one ferrocene and/or metalloocene derivative is by oral administration.

54. The method of claim 49, wherein administering said at least one ferrocene and/or metalloocene derivative is by injection.

55. The method of claim 49, wherein said disease, syndrome or condition is selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

56. The method of claim 49, wherein said metalloocene or metalloocene derivative has a general formula:

\[
\begin{array}{c}
\text{R}_1 - \text{R}_4 - \text{M} - \text{R}_5 - \text{R}_6 \\
\text{R}_7 - \text{R}_8
\end{array}
\]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.
57. The method of claim 49, further comprising co-administering at least one additional agent having immune stimulation activity.

58. The method of claim 57, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

59. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing p21ras oxidation.

60. The method of claim 59, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

61. The method of claim 60, wherein said amount is between 0.1 - 5 mg/kg body weight.

62. The method of claim 60, wherein said amount is between 0.2 - 3 mg/kg body weight.

63. The method of claim 60, wherein administering said at least one ferrocene and/or metalloocene derivative is by oral administration.

64. The method of claim 60, wherein administering said at least one ferrocene and/or metalloocene derivative is by injection.

65. The method of claim 60, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.
66. The method of claim 60, wherein said metalloocene or metallocone derivative has a general formula:

\[
\begin{array}{c}
\text{M} \\
R_1 - R_8
\end{array}
\]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and R_1-R_8 are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and arylxoy.

67. The method of claim 60, further comprising co-administering at least one additional agent having immune stimulation activity.

68. The method of claim 67, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

69. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing GTPase activity.
70. The method of claim 69, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

71. The method of claim 70, wherein said amount is between 0.1 - 5 mg/kg body weight.

72. The method of claim 70, wherein said amount is between 0.2 - 3 mg/kg body weight.

73. The method of claim 70, wherein administering said at least one ferrocene and/or metalloocene derivative is by oral administration.

74. The method of claim 70, wherein administering said at least one ferrocene and/or metalloocene derivative is by injection.

75. The method of claim 70, wherein said disease, syndrome or condition is selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

76. The method of claim 70, wherein said metalloocene or metalloocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

77. The method of claim 70, further comprising co-administering at least one additional agent having immune stimulation activity.

78. The method of claim 77, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-timor vaccine.

79. A method of treating a disease, syndrome or condition, in which immune stimulation is beneficial, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing NF-κB activity.

80. The method of claim 79, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.
81. The method of claim 80, wherein said amount is between 0.1 - 5 mg/kg body weight.

82. The method of claim 80, wherein said amount is between 0.2 - 3 mg/kg body weight.

83. The method of claim 80, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

84. The method of claim 80, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

85. The method of claim 80, wherein said disease, syndrome or condition is selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

86. The method of claim 80, wherein said metallocene or metallocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

87. The method of claim 80, further comprising co-administering at least one additional agent having immune stimulation activity.

88. The method of claim 87, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

89. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one agent in an amount so as to induce H₂O₂ generation at a concentration effective in inducing immune stimulation.

90. The method of claim 89, wherein said at least one agent comprises at least one ferrocene and/or metallocene derivative.

91. The method of claim 90, wherein said amount is between 0.025 - 10 mg/kg body weight.

92. The method of claim 90, wherein said amount is between 0.1 - 5 mg/kg body weight.

93. The method of claim 90, wherein said amount is between 0.2 - 3 mg/kg body weight.
94. The method of claim 90, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

95. The method of claim 90, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

96. The method of claim 90, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

97. The method of claim 90, wherein said metallocene or metallocene derivative has a general formula:

```
     R2
    /   \\  \\
   /     \\ M
  R1    R4
     \\   \\
    R3  R5
       / \\
      /   \\
     R6  R7
         / \\
        /   \\
       /     \\
      R8   R9
```

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and wherein R1-R8 are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.
98. The method of claim 90, further comprising co-administering at least one additional agent having anti-cancer activity.

99. The method of claim 98, wherein said at least one additional agent is an immunostimulant.

100. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight.

101. The method of claim 100, wherein said amount is between 0.1 - 5 mg/kg body weight.

102. The method of claim 100, wherein said amount is between 0.2 - 3 mg/kg body weight.

103. The method of claim 100, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

104. The method of claim 100, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

105. The method of claim 100, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

106. The method of claim 100, wherein said metallocene or metallocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

107. The method of claim 100, further comprising co-administering at least one additional agent having anti-cancer activity.

108. The method of claim 107, wherein said at least one additional agent is an immunostimulant.

109. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in activating a p21\textsuperscript{ras} signal transduction pathway component.

110. The method of claim 109, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

111. The method of claim 110, wherein said p21\textsuperscript{ras} signal transduction pathway component is selected from the group consisting of p21\textsuperscript{ras} and NF-κB.
112. The method of claim 110, wherein said amount is between 0.1 - 5 mg/kg body weight.

113. The method of claim 110, wherein said amount is between 0.2 - 3 mg/kg body weight.

114. The method of claim 110, wherein administering said at least one ferrocene and/or metalloocene derivative is by oral administration.

115. The method of claim 110, wherein administering said at least one ferrocene and/or metalloocene derivative is by injection.

116. The method of claim 110, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

117. The method of claim 110, wherein said metalloocene or metalloocene derivative has a general formula:

```
R1
R2
R3
R4

M
```

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and
R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

118. The method of claim 110, further comprising co-administering at least one additional agent having anti-cancer activity.

119. The method of claim 118, wherein said at least one additional agent is an immunostimulant.

120. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing p21\textsuperscript{ras} oxidation.

121. The method of claim 120, wherein said at least one agent comprises at least one ferrocene and/or metallocene derivative.

122. The method of claim 121, wherein said amount is between 0.1 - 5 mg/kg body weight.

123. The method of claim 121, wherein said amount is between 0.2 - 3 mg/kg body weight.

124. The method of claim 121, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

125. The method of claim 121, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

126. The method of claim 121, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.
127. The method of claim 121, wherein said metalloocene or metalloocene derivative has a general formula:

![Chemical Structure](image)

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

128. The method of claim 121, further comprising co-administering at least one additional agent having anti-cancer activity.

129. The method of claim 128, wherein said at least one additional agent is an immunostimulant.

130. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing GTPase activity.

131. The method of claim 130, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.
132. The method of claim 131, wherein said amount is between 0.1 - 5 mg/kg body weight.

133. The method of claim 131, wherein said amount is between 0.2 - 3 mg/kg body weight.

134. The method of claim 131, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

135. The method of claim 131, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

136. The method of claim 131, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

137. The method of claim 131, wherein said metallocene or metallocene derivative has a general formula:

\[
\begin{array}{c}
\text{R}_2 \quad \text{R}_3 \\
\text{R}_1 \quad \text{R}_4 \\
\text{M} \\
\text{R}_5 \quad \text{R}_6 \\
\text{R}_7 \quad \text{R}_8
\end{array}
\]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.
138. The method of claim 131, further comprising co-administering at least one additional agent having anti-cancer activity.

139. The method of claim 138, wherein said at least one additional agent is an immunostimulant.

140. A method of treating a cancer, the method comprising administering to a subject in need thereof at least one agent in an amount effective in inducing NF-κB activity.

141. The method of claim 140, wherein said at least one agent comprises at least one ferrocene and/or metallocene derivative.

142. The method of claim 141, wherein said amount is between 0.1 - 5 mg/kg body weight.

143. The method of claim 141, wherein said amount is between 0.2 - 3 mg/kg body weight.

144. The method of claim 141, wherein administering said at least one ferrocene and/or metallocene derivative is by oral administration.

145. The method of claim 141, wherein administering said at least one ferrocene and/or metallocene derivative is by injection.

146. The method of claim 141, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

147. The method of claim 141, wherein said metallocene or metallocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

148. The method of claim 141, further comprising co-administering at least one additional agent having anti-cancer activity.

149. The method of claim 148, wherein said at least one additional agent is an immunostimulant.

150. A method of inducing immune stimulation in a body cavity, the method comprising administering into the body cavity H₂O₂ in an amount effective in immune stimulation.

151. The method of claim 150, wherein said amount is of 10-2000 ml of 50-2500 µM H₂O₂.
152. The method of claim 150, wherein said body cavity is selected from the group consisting of peritoneal and pleural cavities.

153. A method of treating cancer in a body cavity, the method comprising administering into the body cavity $\text{H}_2\text{O}_2$ in an amount effective in immune stimulation.

154. The method of claim 153, wherein said amount is of 10-2000 ml of 50-2500 $\mu$M $\text{H}_2\text{O}_2$.

155. The method of claim 153, wherein said body cavity is selected from the group consisting of peritoneal and pleural cavities.

156. A method of inducing immune stimulation, the method comprising exposing immune cells ex vivo to at least one ferrocene and/or metallocene derivative, and administering said cells to a subject in need of immune stimulation.

157. A method of treating cancer, the method comprising exposing immune cells ex vivo to at least one ferrocene and/or metallocene derivative, and administering said cells to a subject in need of such treatment.

158. A method of inducing immune stimulation, the method comprising exposing immune cells ex vivo to $\text{H}_2\text{O}_2$ in a concentration effective in inducing immune stimulation and administering said cells to a subject in need of immune stimulation.

159. A method of treating cancer, the method comprising exposing immune cells ex vivo to $\text{H}_2\text{O}_2$ in a concentration effective in inducing immune stimulation and administering said cells to a subject in need of such treatment.
160. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount so as to induce $\text{H}_2\text{O}_2$ generation at a concentration effective in inducing immune stimulation following administration thereof.

161. The pharmaceutical composition of claim 160, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

162. The pharmaceutical composition of claim 161, wherein said amount is between 0.025 - 10 mg/kg body weight.

163. The pharmaceutical composition of claim 161, wherein said amount is between 0.1 - 5 mg/kg body weight.

164. The pharmaceutical composition of claim 161, wherein said amount is between 0.2 - 3 mg/kg body weight.

165. The pharmaceutical composition of claim 161, wherein said pharmaceutical composition is formulated for oral administration.

166. The pharmaceutical composition of claim 161, wherein said pharmaceutical composition is formulated for administration by injection.

167. The pharmaceutical composition of claim 161, packaged and identified for treatment of a disease, syndrome or condition selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.
168. The pharmaceutical composition of claim 161, wherein said metallocene or metallocene derivative has a general formula:

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

169. The pharmaceutical composition of claim 161, further comprising at least one additional agent having immune stimulation activity.

170. The pharmaceutical composition of claim 169, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-timor vaccine.

171. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one
ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight.

172. The pharmaceutical composition of claim 171, wherein said amount is between 0.1 - 5 mg/kg body weight.

173. The pharmaceutical composition of claim 171, wherein said amount is between 0.2 - 3 mg/kg body weight.

174. The pharmaceutical composition of claim 171, wherein said pharmaceutical composition is formulated for oral administration.

175. The pharmaceutical composition of claim 171, wherein said pharmaceutical composition is formulated for administration by injection.

176. The pharmaceutical composition of claim 171, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

177. The pharmaceutical composition of claim 171, wherein said metallocene or metallocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

178. The pharmaceutical composition of claim 171, further comprising at least one additional agent having immune stimulation activity.

179. The pharmaceutical composition of claim 178, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-timor vaccine.

180. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in activating a p21ras signal transduction pathway component following administration thereof.

181. The pharmaceutical composition of claim 180, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

182. The pharmaceutical composition of claim 181, wherein said p21^{ras} signal transduction pathway component is selected from the group consisting of p21^{ras} and NF-κB:

183. The pharmaceutical composition of claim 181, wherein said amount is between 0.1 - 5 mg/kg body weight.
184. The pharmaceutical composition of claim 181, wherein said amount is between 0.2 - 3 mg/kg body weight.

185. The pharmaceutical composition of claim 181, wherein said pharmaceutical composition is formulated for oral administration.

186. The pharmaceutical composition of claim 181, wherein said pharmaceutical composition is formulated for administration by injection.

187. The pharmaceutical composition of claim 181, packaged and identified for treatment of a disease, syndrome or condition selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

188. The pharmaceutical composition of claim 181, wherein said metallocene or metallocene derivative has a general formula:

![Diagram]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and
R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

189. The pharmaceutical composition of claim 181, further comprising at least one additional agent having immune stimulation activity.

190. The pharmaceutical composition of claim 189, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

191. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing p21ras oxidation following administration thereof.

192. The pharmaceutical composition of claim 191, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

193. The pharmaceutical composition of claim 192, wherein said amount is between 0.1 - 5 mg/kg body weight.

194. The pharmaceutical composition of claim 192, wherein said amount is between 0.2 - 3 mg/kg body weight.

195. The pharmaceutical composition of claim 192, wherein said pharmaceutical composition is formulated for oral administration.

196. The pharmaceutical composition of claim 192, wherein said pharmaceutical composition is formulated for administration by injection.
197. The pharmaceutical composition of claim 192, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

198. The pharmaceutical composition of claim 192, wherein said metalloocene or metalloocene derivative has a general formula:

```
R_2
 R_1
  R_4
   M
 R_3
   R_5
    R_6

R_7
```

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R_1-R_8 are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

199. The pharmaceutical composition of claim 192, further comprising at least one additional agent having immune stimulation activity.

200. The pharmaceutical composition of claim 199, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-timor vaccine.
201. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing GTPase activity following administration thereof.

202. The pharmaceutical composition of claim 201, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

203. The pharmaceutical composition of claim 202, wherein said amount is between 0.1 - 5 mg/kg body weight.

204. The pharmaceutical composition of claim 202, wherein said amount is between 0.2 - 3 mg/kg body weight.

205. The pharmaceutical composition of claim 202, wherein said pharmaceutical composition is formulated for oral administration.

206. The pharmaceutical composition of claim 202, wherein said pharmaceutical composition is formulated for administration by injection.

207. The pharmaceutical composition of claim 202, packaged and identified for treatment of a disease, syndrome or condition selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

208. The pharmaceutical composition of claim 202, wherein said metalloocene or metalloocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

209. The pharmaceutical composition of claim 202, further comprising at least one additional agent having immune stimulation activity.

210. The pharmaceutical composition of claim 209, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

211. A pharmaceutical composition for treating a disease, syndrome or condition, in which immune stimulation is beneficial, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing NF-κB activity following administration thereof.

212. The pharmaceutical composition of claim 211, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.
213. The pharmaceutical composition of claim 212, wherein said amount is between 0.1 - 5 mg/kg body weight.

214. The pharmaceutical composition of claim 212, wherein said amount is between 0.2 - 3 mg/kg body weight.

215. The pharmaceutical composition of claim 212, wherein said pharmaceutical composition is formulated for oral administration.

216. The pharmaceutical composition of claim 212, wherein said pharmaceutical composition is formulated for administration by injection.

217. The pharmaceutical composition of claim 212, packaged and identified for treatment of a disease, syndrome or condition selected from the group consisting of cancer, acquired immune deficiency, radiotherapy induced immune deficiency, chemotherapy induced immune deficiency and congenital immune deficiency.

218. The pharmaceutical composition of claim 212, wherein said metalloocene or metalloocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

219. The pharmaceutical composition of claim 212, further comprising at least one additional agent having immune stimulation activity.

220. The pharmaceutical composition of claim 219, wherein said at least one additional agent is selected from the group consisting of a cytokine, a chemokine, an immune-stimulatory antibody and an anti-tumor vaccine.

221. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount so as to induce H₂O₂ generation at a concentration effective in inducing immune stimulation following administration thereof.

222. The pharmaceutical composition of claim 221, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

223. The pharmaceutical composition of claim 222, wherein said amount is between 0.025 - 10 mg/kg body weight.

224. The pharmaceutical composition of claim 222, wherein said amount is between 0.1 - 5 mg/kg body weight.
225. The pharmaceutical composition of claim 222, wherein said amount is between 0.2 - 3 mg/kg body weight.

226. The pharmaceutical composition of claim 222, wherein said pharmaceutical composition is formulated for oral administration.

227. The pharmaceutical composition of claim 222, wherein said pharmaceutical composition is formulated for administration by injection.

228. The pharmaceutical composition of claim 222, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

229. The pharmaceutical composition of claim 222, wherein said metallocene or metallocene derivative has a general formula:

![Diagram of metallocene derivative]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.
230. The pharmaceutical composition of claim 222, further comprising co-administering at least one additional agent having anti-cancer activity.

231. The pharmaceutical composition of claim 230, wherein said at least one additional agent is an immunostimulant.

232. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one ferrocene and/or metallocene derivative, in an amount of 0.025 - 10 mg/kg body weight.

233. The pharmaceutical composition of claim 232, wherein said amount is between 0.1 - 5 mg/kg body weight.

234. The pharmaceutical composition of claim 232, wherein said amount is between 0.2 - 3 mg/kg body weight.

235. The pharmaceutical composition of claim 232, wherein said pharmaceutical composition is formulated for oral administration.

236. The pharmaceutical composition of claim 232, wherein said pharmaceutical composition is formulated for administration by injection.

237. The pharmaceutical composition of claim 232, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.
238. The pharmaceutical composition of claim 232, wherein said metalloccene or metalloccene derivative has a general formula:

\[
\begin{array}{c}
\text{R}_2 \\
\text{R}_1 \\
\text{M} \\
\text{R}_4 \\
\text{R}_3 \\
\end{array}
\]

\[
\begin{array}{c}
\text{R}_8 \\
\text{R}_7 \\
\text{R}_6 \\
\text{R}_5 \\
\end{array}
\]

wherein,

\(M\) is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

\(R_1-R_8\) are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

239. The pharmaceutical composition of claim 232, further comprising co-administering at least one additional agent having anti-cancer activity.

240. The pharmaceutical composition of claim 239, wherein said at least one additional agent is an immunostimulant.

241. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in activating a p21ras signal transduction pathway component following administration thereof.
242. The pharmaceutical composition of claim 241, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

243. The pharmaceutical composition of claim 242, wherein said p21\textsuperscript{ras} signal transduction pathway component is selected from the group consisting of p21\textsuperscript{ras} and NF-\kappaB.

244. The pharmaceutical composition of claim 242, wherein said amount is between 0.1 - 5 mg/kg body weight.

245. The pharmaceutical composition of claim 242, wherein said amount is between 0.2 - 3 mg/kg body weight.

246. The pharmaceutical composition of claim 242, wherein said pharmaceutical composition is formulated for oral administration.

247. The pharmaceutical composition of claim 242, wherein said pharmaceutical composition is formulated for administration by injection

248. The pharmaceutical composition of claim 242, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

249. The pharmaceutical composition of claim 242, wherein said metalloocene or metalloocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

250. The pharmaceutical composition of claim 242, further comprising co-administering at least one additional agent having anti-cancer activity.

251. The pharmaceutical composition of claim 250, wherein said at least one additional agent is an immunostimulant.

252. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing p21ras oxidation following administration thereof.

253. The pharmaceutical composition of claim 252, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

254. The pharmaceutical composition of claim 253, wherein said amount is between 0.1 - 5 mg/kg body weight.
255. The pharmaceutical composition of claim 253, wherein said amount is between 0.2 - 3 mg/kg body weight.

256. The pharmaceutical composition of claim 253, wherein said pharmaceutical composition is formulated for oral administration.

257. The pharmaceutical composition of claim 253, wherein said pharmaceutical composition is formulated for administration by injection.

258. The pharmaceutical composition of claim 253, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

259. The pharmaceutical composition of claim 253, wherein said metalloocene or metalloocene derivative has a general formula:

\[
\begin{array}{c}
R_1 \quad R_2 \\
\text{M} \\
R_3 \quad R_4 \\
R_5 \quad R_6 \\
R_7 \quad R_8
\end{array}
\]

wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R_1-R_8 are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.
260. The pharmaceutical composition of claim 253, further comprising co-administering at least one additional agent having anti-cancer activity.

261. The pharmaceutical composition of claim 260, wherein said at least one additional agent is an immunostimulant.

262. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing GTPase activity following administration thereof.

263. The pharmaceutical composition of claim 262, wherein said at least one agent comprises at least one ferrocene and/or metallocene derivative.

264. The pharmaceutical composition of claim 263, wherein said amount is between 0.1 - 5 mg/kg body weight.

265. The pharmaceutical composition of claim 263, wherein said amount is between 0.2 - 3 mg/kg body weight.

266. The pharmaceutical composition of claim 263, wherein said pharmaceutical composition is formulated for oral administration.

267. The pharmaceutical composition of claim 263, wherein said pharmaceutical composition is formulated for administration by injection.

268. The pharmaceutical composition of claim 263, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.
269. The pharmaceutical composition of claim 263, wherein said metallocene or metallocene derivative has a general formula:

\[
\begin{array}{c}
\text{R}_2 \\
\text{R}_3 \\
\text{R}_1 \\
\text{R}_4 \\
\text{M} \\
\text{R}_5 \\
\text{R}_6 \\
\text{R}_7 \\
\text{R}_8
\end{array}
\]

wherein,

- \( M \) is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and
- \( \text{R}_1 \text{-R}_8 \) are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

270. The pharmaceutical composition of claim 263, further comprising co-administering at least one additional agent having anti-cancer activity.

271. The pharmaceutical composition of claim 270, wherein said at least one additional agent is an immunostimulant.

272. A pharmaceutical composition for treating a cancer, each dose-unit of the pharmaceutical composition comprising, as an active ingredient, at least one agent in an amount effective in inducing NF-κB activity following administration thereof.
273. The pharmaceutical composition of claim 272, wherein said at least one agent comprises at least one ferrocene and/or metalloocene derivative.

274. The pharmaceutical composition of claim 273, wherein said amount is between 0.1 - 5 mg/kg body weight.

275. The pharmaceutical composition of claim 273, wherein said amount is between 0.2 - 3 mg/kg body weight.

276. The pharmaceutical composition of claim 273, wherein said pharmaceutical composition is formulated for oral administration.

277. The pharmaceutical composition of claim 273, wherein said pharmaceutical composition is formulated for administration by injection.

278. The pharmaceutical composition of claim 273, wherein said cancer is selected from the group consisting of a carcinoma, a sarcoma, a lymphoma and a leukemia.

279. The pharmaceutical composition of claim 273, wherein said metalloocene or metalloocene derivative has a general formula:
wherein,

M is a metal selected from the group consisting of Fe (iron), Cr (chromium), V (vanadium), Mn (manganese), Co (cobalt), Zn (zinc), Ni (nickel), Ti (titanium), Zr (zirconium), Hf (hafnium) and Nb (niobium); and

R₁-R₈ are each independently selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, alkoxy, aryl and aryloxy.

280. The pharmaceutical composition of claim 273, further comprising co-administering at least one additional agent having anti-cancer activity.

281. The pharmaceutical composition of claim 280, wherein said at least one additional agent is an immunostimulant.
Fig. 1b
Fig. 2

Fig. 3
Fig. 4
**Fig. 5a**

**Fig. 5b**
Nitric Oxide

Fig. 5c

Fig. 6
Fig. 7

Weight of lungs (g)

Fig. 8

Ferrocene 50 µg/kg
3 cycles

Ferrocene 50 µg/kg
1 cycle

PBS
**Fig. 9a**

Ferrocene (1 μg/ml) in drinking water

**Fig. 9b**

Comparison of lung weight over time:
- **PBS**
- 1 week
- 2 weeks
- 3 weeks
- Control (no tumor)
Fig. 10a

Fig. 10b
Fig. 11

Fig. 12
Fig. 13

Fig. 14a
Donor

Macrophages (ferrocene treated mice)

Macrophages (ferrocene untreated mice)

Control

Fig. 14b

Donor

Macrophages (ferrocene treated mice)

Control

Fig. 15
Fig. 18
Fig. 19
Fig. 20
Fig. 21a

Weight of lungs (g)

PBS  |  H2O2  |  Control (3 cycles) (no tumor)

Fig. 21b

Scoring of metastases

PBS  |  H2O2 (3 cycles)
Fig. 22

H₂O₂ - 5 umoles/kg
3 cycles

PBS
SEQUENCE LISTING

<110> Novogrodsky, Abraham

<120> METHODS AND PHARMACEUTICAL COMPOSITIONS FOR STIMULATING THE IMMUNE SYSTEM AND/OR TREATING CANCER

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