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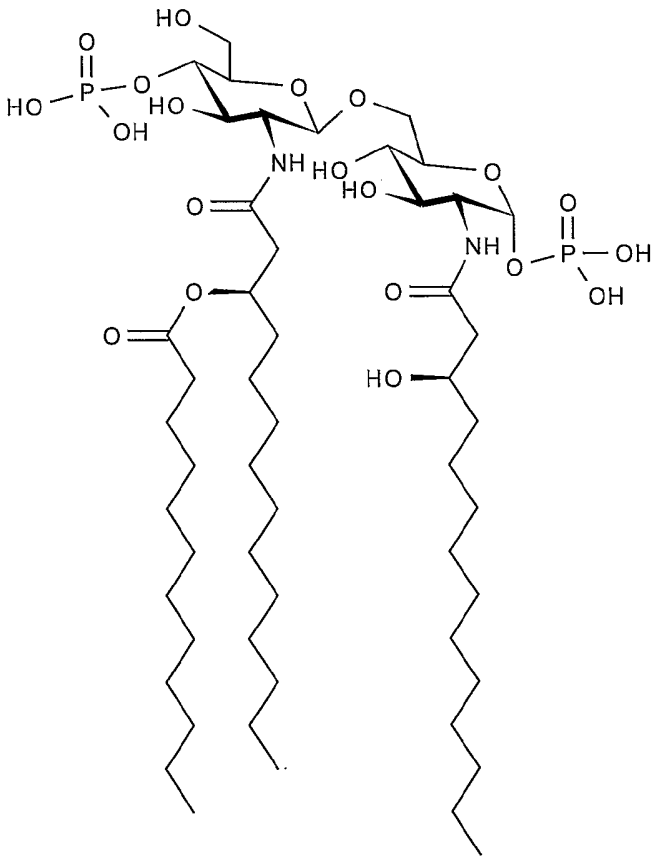

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(57) Abstract: This invention relates to anticancer therapy and more precisely to the immunological control of cancer. Specifically this invention relates to pharmaceutical compositions incorporating as the active ingredient a glucosamine disaccharide OM- 174 of formula(I) together with known antineoplastic agents, selected principally from the group consisting of alkylating agents and antimetabolic agents, in conjunction or admixture with an inert non toxic pharmaceutically acceptable diluent or carrier. This invention also relates to ionic derivatives of OM- 174.

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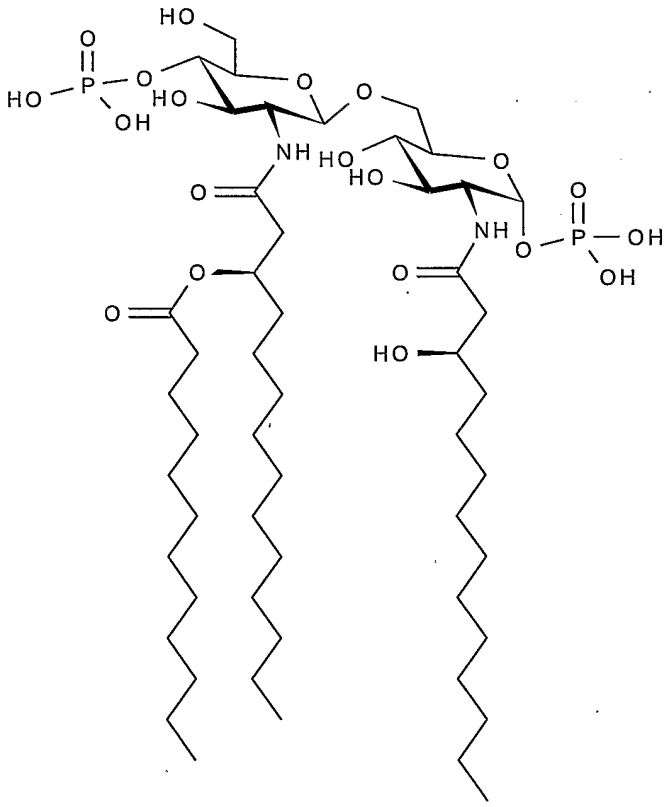
COMBINATION ANTICANCER THERAPY OR OM-174 AND PHARMACEUTICAL COMPOSITIONS THEREFOR

This invention relates to the immunological control of cancer in humans or other warm-blooded animals.

This invention relates to pharmaceutical compositions increasing or improving the efficacy of known antineoplastic agents by stimulating the cancer patients immune system.

More precisely this invention relates to pharmaceutical compositions incorporating as the active ingredients a combination of an immunomodulating agent and a known or experimental antineoplastic agent in admixture or combination with a diluent, an excipient, a carrier or a vehicle intended for oral or injectable way.

More specifically, the present invention has, as a subject matter, pharmaceutical compositions combining as the active ingredient an immunostimulating agent :a triacylated diphosphorylated lipid A derivative (OM-174) of formula (I)



(I)

together with a known antineoplastic chemotherapeutic agent selected from the group consisting of alkylating agents and anti-metabolite agents, in conjugation or admixture with an inert non-toxic pharmaceutically acceptable diluent or carrier.

The invention also relates to the salts of OM-174 with a mineral or organic base and namely a pharmaceutically acceptable base such as the sodium, potassium, calcium, magnesium, triethylamine or triethanolamine salt.

This invention also relates to a pharmaceutical composition wherein the immunologically-active compound is OM-174 in combination with standard or experimental chemotherapies in admixture or combination with one or more non-toxic, inert, pharmaceutically-acceptable diluent(s) or carrier(s).

This invention also relates to methods for treating cancer in warm blooded animals comprising humans suffering from cancer, which consists in administering to them a combination of a therapeutically effective amount of a mixture of OM-174 of formula (I) and a known antineoplastic agent selected from the group consisting of an alkylating agent, and an antimetabolite agent, in a pharmaceutically-acceptable carrier excipient or vehicle suitable for the oral, parenteral, such as intravenous, intratumoral, subcutaneous and rectal, topical or sub-mucosal ways of administration.

The active ingredients may be given either simultaneously mainly in a single unit dosage, or separately or sequentially in separate unit dosages, mainly as a kit containing in separate containers the active ingredients.

These pharmaceutical compositions and the method using the same are based on well established antineoplastic agents as well as newly developed methods for treating neoplastic diseases.

PRIOR ART

Control of cancer by the immune system

Healthy cells normally divide, grow, and finally die when necessary in a patterned and well controlled manner. Often during a life-time, it may happen incidentally that an individual cell starts to divide without control. Since nature is well prepared, the generated uncontrolled cells concomitantly generally express on their surface modified antigens (tumor associated antigens) which normally are not present on non-tumor cells, allowing thus in the vast majority of the cases, the immune system to prevent the occurrence of many cancers.

Cancer cells may escape immune recognition

However, some cancer antigens are tissue-specific molecules shared by cancer cells and healthy cells. Thus, these weak antigens do not typically elicit immunity. In addition, tumors have several features that make their recognition and destruction by the immune system, difficult. Indeed cancer cells are known to release immunosuppressive substances (such as e.g. the cytokine TGF-beta or the prostaglandin PGE_2) to escape immune recognition.

If the immune system, for any reason, fails to recognize the risk and to destroy the proliferating cells, cancer and metastases appear.

Combining immunotherapy with standard chemotherapeutic drugs

When cancer is established, it is unfortunately often incompletely treated by rather aggressive chemotherapeutic drugs or radiotherapeutic methods which may further damage the already weakened human immune system.

The general practice today is to use immunostimulation (e.g by filgrastim or NEUPOGEN®, a medication that stimulates blood cells proliferation to fight the potential complications of neutropenia), principally to restore the immune system often severely damaged by the chemotherapeutic agent used, or after radiotherapy. The common standard rational is to use immunostimulating agents in order to restore "normal" blood cellular

formulas to avoid as much as possible opportunistic infections in cancer patients undergoing an anticancer therapy.

In contrast thereof, it is proposed in this application that a clinical treatment with OM-174, a triacylated diglucosamine diphosphate (WO 95/14026; PCT/EP94/03852), takes place as an immunostimulating agent before, concomitantly, or after the use of well-established standard or experimental anticancer cytotoxic drugs in order to improve the efficacy of the anticancer treatment as shown in the experimental part.

The burden of cancer

Cancer presently refers to a family of related proliferative diseases, which kill millions of persons each year. Despite recent progresses such as the use of Gleevec®, effective therapeutic agents to fight cancer, continue to be lacking, and cancer rates could further increase by 50% to 15 million new cases in the year 2020, (World Cancer Report, 2003) if no counteraction is attempted.

In the year 2000, malignant tumors were responsible for 12 per cent of the nearly 56 million deaths worldwide from all causes. In 2000, 5.3 million men and 4.7 million women developed a malignant tumor and altogether 6.2 million died from the disease. Cancer remains the third lethal cause, after infectious and parasitic diseases on one part and coronary and heart diseases on the other part.

Lung cancer is the most common cancer worldwide, accounting for 1.2 million new cases per year ; followed by cancer of the breast, just over 1 million cases; colorectal, 940,000; stomach, 870,000; liver, 560,000; cervical, 470,000; esophageal, 410,000; head and neck, 390,000; bladder, 330,000; malignant non-Hodgkin lymphomas, 290,000; leukemia, 250,000; prostate and testicular, 250,000; pancreatic, 216,000; ovarian, 190,000; kidney, 190,000; endometrial, 188,000; nervous system, 175,000; melanoma, 133,000; thyroid, 123,000; pharynx, 65,000; and Hodgkin disease, 62,000 cases.

The three leading causes of cancer are different than the three most common forms, lung cancer being responsible for 17.8 per cent of all cancer deaths, stomach, 10.4 per cent and liver, 8.8 per cent.

Main Treatments to combat cancer

Most cancers are classically treated with :

- surgery,
- radiation therapy,
- chemotherapy,
- and/or biological therapy.

Surgery :

During this procedure, solid tumoral masses are removed from the body. However if metastases have already spread out, this treatment procedure becomes usually useless.

Radiation therapy

This method, also called radiotherapy, refers to the use of high-energy radiation from X-rays, gamma rays, neutrons, and other sources to kill cancer cells and shrink tumors.

It may be given before surgery (neoadjuvant therapy) to shrink a tumor so that it is easier to remove. In other cases, radiation therapy is given after surgery (adjuvant therapy) to destroy any cancer cells that may remain in the area.

Chemotherapy :

Chemotherapy is usually given in cycles: a treatment period, one or more days, followed by a recovery period, several days or weeks, then another treatment period, and so on. Here, it is suggested that in between or concomitantly to these chemotherapeutic cycles (designed to shrink the tumor and reveal tumor antigens), stimulation of the immune system by OM-174 could be performed.

The rational behind chemotherapy :

Any efficient and safe chemotherapy drug should kill the cancer cells and not harm the adjacent healthy cells. This can in theory be achieved by

characterizing properties specific to cancer cells which are not found on normal tissues.

The strategy behind the clinical use of chemotherapeutic drugs, is based on the simple factual observation that most cancer cells grow faster than normal cells. Therefore targeting specifically some enzymes or cellular elements involved in the cell growth cycle, seems reasonable. This cytotoxic strategy implies that fast growing cells would be most affected, and slow growing cells would be less disturbed. This rational was indeed applied for the development of many chemotherapeutics currently used clinically.

Chemotherapeutic agents are mainly active during the S and M phases of the cell cycle.

The limits of chemotherapy:

Chemotherapy has still largely insufficient clinical efficacy. This strategy has its own toxicological limitations, because some normal cells (such as e.g. proliferating T and B cells) need also to divide when necessary. Indeed, when a patient suffers from kidney or liver damage and can therefore not eliminate normally a chemotherapeutic agent, administering the recommended amount of drug may prove to be too toxic in a patient unable to metabolize and/or excrete it. Therefore dose adjustments are absolutely necessary to avoid non-acceptable toxicities or sub-therapeutic dosing.

The pharmacokinetics for cancer patients are often very complex, and sometime limits the patient's chemotherapy options.

How to enhance the efficacy of chemotherapy and reduce side-effects:

It is contemplated here that an adequate and timely controlled clinical combined therapy with well-recognized or experimental chemotherapeutic drugs, used first to shrink and kill some cancer cells (and thus potentially reveal tumor-associated antigens), followed by an unspecific immunostimulation with OM-174 enhances the efficacy of oncostatic drugs, and allows the acquisition of an immunological (specific) memory to

get rid of cells bearing the tumor associated antigen, and also to limit the level of the side effects observed, by allowing e.g. to reduce the number of administrations and/or the doses of the chemotherapeutic drug.

Moreover the immunotherapeutic agent OM-174 increases the production of cytokines in human beings (see figure 1 for an example with TNF-α). TNF-α is known to affect tumor vessel destruction and improves vascular permeability to drugs such as Melphalan, as shown in clinical trials (Lejeune et al., 1988, Lejeune 2002).

It will therefore be postulated that the increase in tumor vessels permeability would facilitate the accessibility of chemotherapeutic agents such as Melphalan in cases of transit limb melanoma metastases or soft-tissue sarcomas (Lejeune et al. 1998, 2002).

Major chemotherapeutic drugs:

The Information below has been adapted from the work published by Julia Draznin Maltzman, 2003.

Chemotherapeutic agents can be divided into the following classes :

- *Alkylating agents :*

  For example, Alretamine, BCNU, Busulfan, Carmustin, CCNU, Chlorambucil, Chlormethin, Carboplatin, Cisplatin, Cyclophosphamide, Dacarbazine, Estramustin, Fotemustin, Ifosphamide, Lomustin, Maphosphamide, Melphalan, Mitomycin, Nimustin, Oxaliplatin, Procarbazine, Streptozocin, Thiotepa, Lobaplatin, Miboplatin, and so on.

- *Intercalants/topoisomerase II inhibitors and topoisomerase I inhibitors*

  Asacrin, Dactinomycin, Daunorubicin, Doxorubicin, Elliptinium Acetate, Epirubicin, Idarubicin, Irinothecan, Mitoxanthrone, Pirarubicin, Plicamycine, Topothecan, Vabrubicine, Zorubicine.

  Despite their well established anticancer activities, they are generally known for possessing a high and irreversible cardio-toxicity.

- *Antimetabolites*

  They are classified into antifolic agents, purine analogs, pyrimidine analogs

Examples thereof are Capecitabine, Cladribine, Cytarabine, Fludarabine, Fluorouracil (5-FU), Gemcitabine, Mercaptopurine, Methotrexate, Thioguanin and the like.

- *Agents acting on tubules: (e.g alcaloids and toxoids)*

Paclitaxel, Docetaxel, Taxol, Vinblastine, Vincristine, Vindesine, Vinorelbine and the like.

- *Tyrosine kinase inhibitors:*

Protein kinase inhibitors are used as anticancer therapeutic agents and biological tools in cell signaling. Two representative members of this family of compounds are Imatinib Mesylate (Gleevec®) and Gefitinib (Iressa®).

- *Other chemotherapeutic agents:*

They are numerous enzymes or antibiotics which have been tested as anticancer agents.

Alkylating agents :

Alkylating agents share a common mechanism of action to the poisonous nitrogen mustards compounds originally developed for military use. It is therefore not surprising that in addition such agents display a full array of adverse events.

They act on the negatively charged sites on DNA. By linking to DNA, replication and transcription are altered, cellular activity is stopped, and cells start to die. This class of anticancer drugs is very powerful and is used in many types of cancer (both solid tumors and leukemia). Unfortunately, the noted side effects are considerable (mainly decreased sperm production, cessation of menstruation, and possibly cause permanent infertility). Alkylating agents can cause secondary cancers. The most common secondary cancer is a leukemia (Acute Myeloid Leukemia) that may occur years after the end of the therapy.

Metal derivatives such as the platinum derivatives and salts, for example cisplatin, have demonstrated some activity against cancer, mainly against lung and testicular cancer. The most significant toxicity of cisplatin is kidney damage. Second-generation platinum derivatives, called carboplatin, have fewer kidney side effects, and may be an appropriate substitute for regimens containing cisplatin. Oxaliplatin is a third-

generation platinum that is active in colon cancer and has no renal toxicity. However, its major side effects are neuropathies.

Examples in different models are provided, in which the use of OM-174 after treatment with alkylating agents such as cyclophosphamide, cisplatin, and oxaliplatin display a very good synergistic antitumoral activity. In the "in vivo" examples provided, using specific conditions, each agent when taken individually does not give satisfactory anticancer results, and quite unexpectedly, a non specific boost of the immune system by OM-174 after a first non specific chemotherapeutic treatment provides encouraging anticancer results worth to be tested in clinical anticancer trials.

Intercalants/topoisomerase II inhibitors and topoisomerase I inhibitors:

Intercalants/topoisomerase II inhibitors form a complex with the enzyme and the DNA, and therefore inhibit DNA re-ligation. They are used to treat mainly malignant hemopathies, breast cancer, digestive tract cancers, genital cancers, bronchial, or conjunctive sarcomas. Their main adverse events are myelo-suppression, vomiting, cardiotoxicity, and alopecia.

Topoisomerase I inhibitors inhibit specifically topoisomerase-I, and thus transcription and replication during the S-phase of the cell-cycle. They are mainly used to fight colorectal cancers. Their main adverse events are myelo-suppression, neutropenia, vomiting, alopecia, and cholinergic syndromes.

Antimetabolites:

They are used mainly against trophoblastic carcinomas, breast cancer, ovarian cancer, acute leukemia, osteosarcomas, lymphomas...

Their main adverse events concern mainly myelosuppression, mucites, cutaneous toxicity, diarrhea, vomiting...

In 1948 Farber demonstrated that a folic acid analog could induce remission in childhood leukemia . Then other analogs inhibiting key enzymatic reactions were synthetized. Antimetabolites interfere with normal metabolic pathways, including those necessary for making new DNA (phase S of the cell cycle). The most widely used antifolate in cancer

therapy with activity against leukemia, lymphoma, breast cancer, head and neck cancer, sarcomas, colon cancer, bladder cancer and choriocarcinomas is Methotrexate which inhibits a crucial enzyme (dihydrofolate reductase) required for DNA synthesis.

Another widely used antimetabolite that disturbs DNA synthesis is the pyrimidin analogue 5-Fluorouracil, which is transformed in fluorodeoxiuridin monophosphate (5-FdUMP) which blocks the enzyme thymidilate synthase, needed for the endogenous synthesis of pyrimidin bases (C and T). As an example, a combination of OM-174 with 5-Fluorouracil to treat colon cancer will be provided below. The compound has a wide range of activities including colon cancer, breast cancer, head and neck cancer, pancreatic cancer, gastric cancer, anal cancer, esophageal cancer and hepatomas. However, 5-Fluorouracil is metabolized by the enzyme dihydropyrimidine dehydrogenase (DPD), which is not expressed by a small population of patients. When these patients are challenged with this chemotherapeutic drug, they get acute and severe toxicity (bone marrow suppression, severe GI toxicities, and neurotoxicities which may include seizures and even coma). Capecitabine is a derivative of 5-fluorouracil i.e. an oral pro-5-Fluorouracil compound that has similar side-effect potentials. Premetrexed is an antifolate antineoplastic agent impeding cell replication intended for injection (Alimta®), produced by Eli Lilly and Company.

Other antimetabolites that inhibit DNA synthesis and DNA repair include: Cytarabine, Gemcitabine (Gemzar®), 6-mercaptopurine, 6-thioguanine, Fludarabine, and Cladribine.

Agents acting on tubules: (e.g alcaloids and toxoids)

Alcaloids such as Vinblastine , Vincristine, Vindesine, or Vinorelbine bind to tubulin, a cytoplasmic protein and therefore impede the formation of the mitotic spindle and block mitosis in the metaphase.

Vincristine, vinblastine, and vinorelbine were extracted from the leaves of a periwinkle plant, Vinca rosea. They are mainly used to treat malignant hemopathies (including Hodgkin), aero-digestive cancers, nephroblastomas, breast cancers...

Their main adverse effects are myelosuppression, nausea, vomiting, alopecia, causticity, neuropathy and neurotoxicity.

Taxanes, first isolated from the bark of the Pacific yew tree Taxus brevifolia in 1963, are specific for the M phase of the cell cycle. The familly includes paclitaxel and docetaxel. Taxanes bind with high affinity to the microtubules and inhibit their normal function. They are efficient against breast cancer, lung cancer, head and neck cancer, ovarian cancer, bladder cancer, esophageal cancer, gastric cancer and prostate cancer. These drugs however decrease the number of blood cells.

Their main adverse effects are mainly myelosuppression (neutropenia), and lymphoedema

Tyrosine kinase inhibitors

The Tyrosine kinase inhibitor Gefitinib (Iressa®, AstraZeneca) is used for the treatment of advanced non-small cell lung cancer (NSCLC), the most common form of lung cancer in the United States.

Gefitinib blocks the action of the EGF receptors on the cells of certain lung cancers and has shown some effects against these cancers.

Some most common side effects with Iressar® include among others: diarrhea, rash, acne, dry skin, nausea, vomiting, itching, loss of appetite, weakness, and weight loss.

The tyrosine kinase inhibitor Imatinib Mesylate (Gleevec®, Novartis) has been approved for the treatment of patients with positive inoperable and/or metastatic malignant gastrointestinal stromal tumors (GISTs) and for the treatment of chronic myeloid leukemia (CML).

Imatinib Mesylate is a signal transduction inhibitor that acts by targeting the activity of tyrosine kinases. The activity of one of these tyrosine kinases, known as c-kit, is thought to drive the growth and division of most GISTs. Imatinib is an inhibitor of the receptor tyrosine kinases for platelet-derived growth factor (PDGF) and stem cell factor (SCF), c-kit, and inhibits PDGF- and SCF-mediated cellular events. In vitro, imatinib inhibits proliferation and induces apoptosis in GIST cells, which express an activating c-kit mutation.

The majority of patients who received Gleevec® in clinical studies did experience side effects, such as nausea, fluid retention (swelling around the eyes, of the legs, etc.), muscle cramps, diarrhea, vomiting,

hemorrhage, muscle and bone pain, skin rash, headache, fatigue, joint pain, indigestion, and shortness of breath.

Other chemotherapeutic agents :

Bleomycin is a small peptide isolated form the fungus Streptomyces verticillus. Its mechanism of action is similar to that of anthracyclines. Free oxygen radicals are formed that result in DNA breaks leading to cancer cell death. This drug is rarely used by itself, rather in conjunction to other chemotherapies. Bleomycin is an active agent in the regimen for testicular cancer as well as Hodgkin's lymphoma. The most frequent side effect of this drug is lung toxicities due to oxygen free radical formation.

Asparaginase catalyses the hydrolysis of asparagin into aspartic acid and ammonia, and therefore can kill cancer cells sentitive to a lack of asparagine-synthetase (lymphocytes and cells of lymphoid origin). It is used to treat hemopathies (acute leukemias, non Hodgkin lymphomas..). Its main adverse events are hepatic toxicity, nausea, and some anaphylactic shocks.

Biological Therapy :

This section has been divided in 3 parts: Monoclonal antibodies, cytokines, and immunostimulation by bacterial agents. The compounds of this invention belong to this latter class of agents.

Monoclonal antibodies :

Mouse, chimeric, humanized and human monoclonal antibodies (huMoAb) are used for treatment of human cancer [Untch M et al, 2003).

About 20 antibodies will be presumably in clinical use by the year 2010.

The use of monoclonal antibodies involves the development of specific antibodies directed against antigens located on the surface of tumor cells. Samples of the patient's tumor cells are taken and processed to produce specific antibodies to the tumor-associated antigens. In order for this approach to operate, a sufficient amount of antigens specific to the tumor cells must be present. In addition, the tumor antigens must be sufficiently different from the antigens elaborated by normal cells to provoke an antibody response.

These antibodies (recognizing cancer cells) can be used either alone to kill cancer cells or as carriers for other substances used for either therapeutic

or diagnostic purposes. For example, chemotherapeutic agents can be bound to monoclonal antibodies to deliver high concentrations of these toxic substances directly to the tumor cells. In theory, this approach is less toxic and more effective than conventional chemotherapy because it reduces the delivery of harmful agents to normal tissues.

Erbitux (cetuximab) is a monoclonal antibody that targets epidermal growth factor receptor (EGFR), and thus regulates cell growth. Erbitux is believed to interfere with the growth of cancer cells by binding to EGFR so that endogeneous epidermal growth factors cannot bind and stimulate the cells to grow. Erbitux is used to treat metastatic colon or rectum cancers. The infusion of Erbitux can cause serious side-effects, which may include difficulty in breathing and low blood pressure, which are usually detected during the first treatment. Infrequent interstitial lung disease (ILD) has also been reported. Other more common side effects of Erbitux treatment are:, rash (acne, rash, dry skin), tiredness/weakness, fever, constipation, and abdominal pain.

Rituximab (anti-CD20) was the first registered MAB for the therapy of follicular lymphoma. Impressive results have been seen in combination with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone) in follicular and high-grade lymphomas.

Other marketed monoclonal antibodies are: Alemtuzamab (Campath®, targets CDw52 expressed on lymphoid tumors); Gemtuzumab-ozogamicin (Mylotarg® targets CD33 expressed on myeloid leukemia blasts), and Tositumab (Bexxar®).

Cytokines :

Examples of cytokines tested for the treatment of cancer are Tumor Necrosis Factor-α, Interleukin-2 and Interferons.

Tumor necrosis alpha:

Between 1985 and 1988, recombinant TNF-α (rTNF-α) was made available to medical oncology (for review see ten Hagen et al. 2001; Lejeune 2002; Leist and Jaattela 2002). Unfortunately, systemic application in advanced cancer patients showed a very low maximal tolerated dose and tumor response was seen rarely, also with unacceptable side effects such as hypotension and organ failure (Lejeune et al. 1998).

In 1998, Lejeune et al. had the idea of infusing high-dose TNF-α in patients with locally advanced melanomas and sarcomas of the limbs. Angiographic and histologic studies revealed that its effect was due to selective destruction of the tumor associated vessels and that vessels in normal tissues were spared.

Heterogeneous tumor perfusion, vascular permeability, and increased interstitial pressure restricted the penetration of chemotherapeutic agents from the circulation into the tumor tissue (Jain 1994, 1999). Lejeune and others have shown that TNF exerts two distinct effects that are selective for angiogenic vessels, namely an early increase of tumor vessels permeability, which can be induced with a low dose of TNF, and a later increase in vascular apoptosis, which appears to require high doses (Lejeune et al. 1998, 2002).

Figure 1 shows that even a single i.v. dose of OM-174 increases the plasmatic levels of TNF-α in human beings. This induced and controlled production of TNF-α strongly suggests that OM-174 would allow a better bio-availability, of chemotherapeutic drugs such as Melphalan, towards the target cancer cells.

Interleukin-2 :

Interleukin-2 (IL-2) is a substance produced by lymphocytes. In addition to being an essential growth factor for T cells, IL-2 increases various NK and T-cell functions. IL-2 also activates lymphokine-activated killer (LAK). LAK cells destroy tumor cells and improve the recovery of immune function in certain immunodeficiency states. Patients with renal cell cancer, melanoma, and non-Hodgkin's lymphoma have demonstrated some responses to IL-2 therapy.

The most severe toxicities result from IL-2's ability to increase capillary permeability. This may cause hypotension, ascites, generalized body edema, and pulmonary edema. Chills and fever also frequently occur within a few hours after IL-2 administration. Headache, malaise, and other flu-like symptoms are also common. Gastrointestinal effects include nausea, vomiting, loss of appetite, diarrhea, and mucositis.

Interferons :

Interferons (IFNs) are small proteins that inhibit viral replication and promote the cellular (T-cell) immune response. There are currently three major types of IFNs: alpha, beta, and gamma. Each type has similar but distinctive abilities for altering biological responses. Alpha-IFN main indication is for use in treatment of hepatitis C, but it is currently also prescribed for use in the treatment of hairy cell leukemia and AIDS-associated Kaposi's sarcoma. It also displays some therapeutic effectiveness against hematologic diseases such as low-grade Hodgkin's lymphoma, cutaneous T-cell lymphoma, chronic myelogenous leukemia, and multiple myeloma. It is also somewhat effective on some solid tumors, such as renal cell cancer.

Beta-interferon is currently in use for treatment of multiple sclerosis.

One of the most common side effects of IFN therapy is a flu-like syndrome. Symptoms include fever, chills, tachycardia, muscle aches, malaise, fatigue, and headaches.

Other common side effects to IFN include a decrease of the white blood cell count, anemia (with prolonged therapy), and decreased platelets. Gastrointestinal symptoms such as a loss of appetite, nausea, vomiting, and diarrhea may also be present. Central nervous system toxicities range from mild confusion and sleepiness to seizures. Acute kidney failure is rare, but can occur. Loss of hair may also be a problem.

Immunostimulation by bacterial agents :

After promising results in animal studies, searchers initiated large-scale clinical trials to stimulate cancer patients' immune systems using bacterial agents such as Corynebacterium parvum (C. parvum) and Bacillus Calmette-Guerin (BCG). Unfortunately, the results of these early immunotherapy trials were discouraging, and cancer treatment using immunostimulating drugs per se lost momentum.

The toxicity of extrinsic immuno-stimulants strongly limited their use in cancer patients. In 1976, Morales et al introduced intravesical Bacillus Calmette-Guérin (BCG) to treat superficial bladder cancer (Morales et al. 1976 and 2002). BCG, a non-specific immunotherapeutic agent for superficial bladder cancer may be regarded as the most successful of all immunotherapies in man (for recent review see Boyd, 2003).

The antitumor effect of lipopolysaccharides (LPS) has been well established. In the 19th century Coley developed a cancer therapy based on bacterial toxins (see Coley WB, 1909). In the 1940's it was shown that bacterial lipopolysaccharide (LPS) was at least partially responsible for the observed anti-tumor activity in Coley's toxins. More recent publications have shown anti-tumoral effects of LPS in animal models and a very limited number of studies have been carried out in man. Because LPS is very toxic and can lead to endotoxic shock, the therapeutic window appears to be very small, and patients can only be treated using very small amounts of LPS that are often too low to produce the desired beneficial effects.

The biological and toxic activities of LPS are associated with its lipid moiety, called lipid A. Different bacterial species synthesize different lipid A structures and these have varying degrees of toxicity. This fact suggests that by modifying the structure of the native bacterial lipid A, it would be possible to prepare derivatives that have attenuated toxicity but retain this beneficial biological activity. A number of different lipid A derivatives have been tested in animal models of cancer with some success.

Presently it is evidenced that immunostimulation with OM-174 would help the body's immune system to achieve a coordinated combination of nonspecific and specific responses to tumor associated antigen if these are revealed first or concomitantly by a classical chemotherapeutic agent as those described above.

Once the first chemotherapeutic treatment has been performed, it would be necessary to initiate an inflammatory response to boost first the nonspecific host defense. Then, specific immune responses would be elicited by the presence of the revealed tumor associated antigen. These specific memory responses are generally divided into humoral (immunity conferred by the antibodies produced by B-lymphocytes) and cell-mediated immunity (immunity conferred by T-lymphocytes). Other important cells are antigen presenting cells (APC) such as macrophages and natural killer (NK) cells. Macrophages bind to an antigen and "present" the antigen to naive T-cells. These, in turn, become activated and produce mature lymphocytes. NK cells are cytotoxic to tumor cells and virus-infected cells.

Contemplated combined treatments with OM-174 :

The goal of the present therapeutic strategy to fight cancer is to first attack cancer cells with standard or experimental chemotherapeutic drugs, and thus reveal "in situ" cancer antigens, and to subsequently boost the immune system to prepare an appropriate immunological response. Alternatively, chemotherapeutic drugs and OM-174 could be administered concomitantly, because it is postulated that OM-174, by increasing plasmatic TNF-α levels, would allow a better distribution of the anticancer drug towards the tumoral site(s).

The invention resides in the fact that, when the nonspecific drug OM-174 (activating innate immunity) is combined with the nonspecific standard antineoplastic drugs claimed in this application the resulting combination leads to an increased (synergistic) efficiency of the combined anticancer treatment .

The present invention resides in the fact that OM-174 could be used therapeutically to treat many forms of cancer in combination with the compounds and drugs listed below.

OM-174 in oncology trials

The product was well tolerated in cancer patients. Doses higher than 1 mg OM-174/m^2 by i.v. infusion were reached without unacceptable toxicity according to non-haematological grade III and haematological grade IV NCI Common Toxicity Criteria.

The analyzed cytokines (TNF-α, IL-1b, IL-1 ra, IL-6, IL-8, sTNF-RI, sTNF-RII, IL-10, IL-2, IL-2sRa, IFN-γ) showed a secretion profile consistent with that of immunostimulating agents. Secretion occurred in all steps, and appeared more “patient”- than “dose”-dependent. (see figure I for the TNF-α profile induced by OM-174).

The results of this single i.v. injection study led to the selection of three doses (0.6, 0.8, and 1.0 mg OM-174/m^2) for repeated i.v. injections (5 to 15 injections, two injections per week), used in a phase Ib study, in which 6 cytokines are monitored. The results obtained in the patients included in this ongoing Phase Ib study show that even after 15 injections the plasmatic levels of TNF-α, IL-6, IL-8, and IL-10 are transiently increased by OM-174.

Pharmacokinetic data in man (clearance, volume of distribution, and half-live) are summarized in the Table 1 for OM-174.

Table 1: Summary of pharmacokinetics data of OM-174 in man

	Healthy volunteers median and (range)		Cancer patients median and (range)	
CL (ml/hr)	169	(116 – 202)	102	(55 – 173)
V_{ss} (l)	5.1	(4.3 – 6.8)	2.9	(1.9 – 4.8)
$T_{1/2}$ (hr)	23	(18 – 32)	20	(12 – 28)

List of Drugs likely to be combined with the compounds of the invention:

Alemtuzamab; Alretamine; Asacrin; Asparaginase (Elspar®); Anastrozole (Arimidex®), Bevacizumab (Avastin®); Bicalutamide (Casodex®); Bleomycin (Blenoxane®); Bortezomib (Velcade®); Busulfan (Myleran); Capecitabine (Xeloda®); Carboplatin (Paraplatin); Carmustine (BCNU, BiCNU); Cetuximab (Erbitux®); Chlorambucil (Leukeran); Chlormethin; Cisplatin (Platinol®); Cladribin; Cyclophosphamide (Cytoxan®, Neosar®); Cytarabine (Cytosar-U®, Ara-C); Dacarbazine (DTIC-Dome); Dactinomycin (Cosmegen®); Daunorubicin (Cerubidine®); Dexrazoxane (Zinecard®); Docetaxel (Taxotere®); Doxorubicin (Adriamycin, Rubex); Erbitux (cetuximab), Elliptinium acetate; Epirubicin; Estramustin; Etoposide (VePesid®, VP-16®); Fentanyl Citrate (Actiq); Floxuridine (FUDR®, Fluorodeoxyuridine); Fotemustin; Fludarabine (Fludara®); Fluorouracil (Adrucil, 5-FU); Flutamide (Eulexin®); Fulvestrant (Faslodex®); Gefitinib (Iressa®) Gemcitabine (Gemzar®); Gemtuzumab; Goserelin acetate implant (Zoladex®); Hydroxyurea (Hydrea®); Idarubicin (Hydrea®); Ifosfamide (IFEX®); Imatinib Mesylate (Gleevec , STI-571); Irinotecan (Camptosar®, CPT-11); Leucovorin; Leuprolide acetate for depot suspension (Lupron®); Lomustine (CCNU, CeeNU®); Maphosphamide; Mechlorethamine (Mustargen®, Nitrogen Mustard); Melphalan (Alkeran®, L-PAM); Mercaptopurine (Purinethol® , 6-MP); Methotrexate (MTX); Mitomycin (Mitomycin C, Mutamycin); Mitotane (Sodren); Mitoxantrone (Novantrone); Nilutamide (Nilandron®); Oxaliplatin (Eloxin®); Paclitaxel (Taxol); Pamidronate (Aredia); Pentostatin (Nipent); Pirarubicin; Plicamycin

(Mithracin, Mithramycin); Premexetred (Alimta®); Procarbazine (Mutalane); PROCRIT (Epoetin alfa); Polifeprosan 20 with carmustine implant (GLIADEL®); Rituximab (Rituxan®); Streptozocin (Zanosar); Tamoxifen (Nolvadex®); Teniposide (Vumon); Tepotecan; Thioguanine (6-TG, Thioguanine Tabloid®) Thiotepa (Thioplex); Tositumomab (Bexxar®); Toxaliplatin (Elotaxin®); Vinblastine (Velban); Vincristine (Oncovin); Vindesine; Vinorelbine (Navelbine)

DESCRIPTION OF THE INVENTION

The compounds of the invention are obtained according to the process described in WO 95/14026.

The compounds of the invention can be in the form either of the acid form or of any acceptable salt suitable for injection in warm blooded animals and human beings. Compounds will be administered parenterally (i.v. preferentially) after (or concomitantly in any suitable formulation) a preliminary therapy involving standard classical or experimental chemotherapeutic drugs.

In humans first, tumors would be treated conventionally with well defined or experimental chemotherapeutic agents to reveal the patients tumor antigens. Then (or concomitantly to allow a TNF-α-mediated increase in vascular permeability) immunostimulation with the compounds of the invention (preferentially 1 to 7 injections/per week and at least 5 parenteral injections) will be performed. Cycle of conventional therapies could then optionally be performed with decreased doses.

It has been known from previous work as disclosed in WO 95/14026 that when tested *per se* as an immunotherapeutic agent, OM-174 displays a strong therapeutic activity even when treatment, in the BDIX/ProB colon model of cancer, is started up to 14 days after tumor induction. Such a treatment leads either to cure or to cause a strong inhibition of tumor development. In the case of complete remission, animals are immunized specifically against the tumor, and re-implantation leads to rejection. Treatment consisted of repeated injections of OM-174, the schedule of administration being more critical than the dose for the therapeutic effect of the drug.

It is shown below that there is potentially a major advantage in combining the effects of immunotherapy (induced by OM-174) with those of chemotherapy. Thus, an initial treatment for cancer by -for example- chemotherapy (alkylating agents such as cisplatin analogues or cyclophosphamide, or antimetabolite agents such as 5-FU), will reduce the tumor mass and viability, and by damaging the tumor cells, may also make them more immunogenic. This initial non specific treatment could be followed by non-specific immunotherapy using the compounds of the invention, which would be more effective as a result of the initial chemotherapy. Immunotherapy will lead to the specific rejection of remaining tumor cells by the immune system and to the prevention of any tumor regrowth and metastatic growth.

This combination of treatments potentially offers a very powerful method to fight cancer as described in the examples below.

The impact of such an invention is extended, when the number of anticancer agents and cancer types is considered.

Advantages and improvement due to the specified therapy will more clearly appear from the examples attached herewith and the appended claims.

EXAMPLES

Example 1 : Enhancement of the curative effect of cyclophosphamide by OM-174 in the melanoma B16 model.

Introduction

To present knowledge, no experimental studies have been disclosed on the effects of combining OM-174 with standard chemotherapeutic drugs as those claimed in this document.

In this example, it is shown that OM-174 *per se* partially inhibits tumor progression (Figure 2) and slightly extends the survival time of mice in the B16 melanoma experimental model (Figure 3). In the conditions used in the study, OM-174 antitumor activity is comparable to that of cyclophosphamide (CY), a reference cytostatic drug.

Interestingly, and this is a part of the invention , more striking effects are achieved by means of the combination of the two agents in a protocol consisting of a single administration of cyclophosphamide (200 mg/Kg,

i.p.) followed by five injections of OM-174 (1 mg/Kg, i.p.). See Figures 2 and 3.

Immunological studies of treated and control mice revealed that the antitumor activity of OM-174, alone or in combination with cyclophosphamide, is mediated by the stimulation of natural killer (NK) and cytotoxic T lymphocyte (CTL) responses as well as by a significant increase in the absolute number of NK1.1, CD4 and CD8 positive cells. OM-174 therefore increases the anticancer effect of the well-known chemotherapeutic drug cyclophosphamide and is therefore a candidate for association with chemotherapy in the treatment of human cancers.

Animals and tumor cells

Four to six weeks-old male C57BL/6 mice have been purchased from Charles River (Calco, Corno, Italy). B16 melanoma tumor cells, were serially passaged subcutaneously (s.c.) in syngenic mice. On day 0, mice were injected s.c. in the right flank with 2 x 10^5 B16 melanoma cells. Tumor growth was measured daily in each mouse, using calipers, and mean tumor diameter per day was calculated. At day 7 after tumor injection, all mice with s.c. tumors of about 2-3 mm diameter were divided into different experimental groups, i.e. phosphate buffered saline (PBS)-injected control 3, CY, OM-174 or CY with OM-174.

Drugs and treatments

Cyclophosphamide (Sigma, St. Louis, MO) was dissolved at 20 mg/ml in PBS immediately before use, and 0.2 ml per mouse were injected intraperitoneally . Each treated animal received a single dose of 200 mg/Kg CY on day 7. This dose was chosen on the basis of previous experiments as the most active one, that did not lead to observable toxicity in this strain of mice.

For the study of tumor growth and survival, each mouse (20/group) received OM-174 i.p. (1 mg/kg) on days 8, 13, 18, 23 and 28 after tumor inoculation. The analysis of the spleen cell cytotoxic activities and lymphocyte subsets of different experimental groups (5 animals/group) was performed on day 14 after tumor injection, i.e. after two treatments with OM-174 (on days 8 and 13).

Spleen cell preparation :

Mice were sacrificed by cervical dislocation on day 14 after tumor inoculation. Spleen cells were obtained by gently teasing the individual spleens in RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, U.K.). Cells were filtered through a 10 μm Nytex mesh, then washed twice and resuspended in Complete Medium (CM): RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 200 mM L-glutamine, 25 mM HEPES, penicillin 50 U/ml and streptomycin 50 μ ml (all from Flow Laboratories).

Cytotoxicity assays :

In vitro-passaged YAC-1 cells (a Moloney-virus induced mouse T cells lymphoma of A/SN origin), and in vivo-passaged B16 melanoma cells, were used as target cells in a chromium-release assay. B16 melanoma cells were obtained from tumor-bearing mice, seeded in cell-culture flasks (Falcon, Becton Dickinson and Co., Plymouth, England) and used within the first week of culture in CM. B16 and YAC-1 cell lines were obtained from the laboratory collection, and were originally obtained from the American Tissue Culture Collection (ATCC).

The cytotoxic activity of the effector cells collected from individual mice was measured by a standard 4-hour ^{51}Cr-release assay. Briefly, target cells were harvested from the cultures, washed twice, resuspended at 5 x 10^6 cells in 0.9 ml of CM and labeled with 100 μ Ci (^{51}Cr) sodium chromate (New England Nuclear, Boston, MA) for 1 hour at 37°C in a 5% CO_2 incubator. After labeling, the cells were washed three times in RPMI 1640 and seeded in U-shaped 96-well microtiter plates (Flow Laboratories) at 1 x 10^4 cells/well. The effector cells suspension was added to quadruplicate wells to give three E/T ratios (i.e. 100:1, 50:1, 25:1) in a final volume of 200 μl per well. The plates were then incubated for 4 hours at 37°C in a 5% CO_2 incubator, 100 μl of supernatants were collected from each well, and the radioactivity was measured using a gamma counter. Total mean cytotoxicity ± S.E.M. were calculated from quadruplicate cpm values from individual spleens.

Immunofluorescence staining and flow cytometric analysis of spleen cell subsets

Spleenocytes from individual mice were analyzed by flow cytometry. The following monoclonal antibodies were used for double fluorescence analysis of spleen cell subsets: fluorescein (FITC)-conjugated anti-mouse NK1.1 PE (PharMingen, San Diego, CA), PE-conjugated anti-mouse CD4

(PharMingen), FITC-conjugated anti-mouse CD8 (PharMingen). Approximately 1 x 10^6 spleen cells were resuspended in 50 ml of CM and staining was performed at 4°C for 30 minutes. Cells were then washed twice in PBS containing 0.02% sodium azide and flow cytometric analysis was performed using a FACscan flow cytometer (Becton Dickinson).

Fluorescence data were collected using a 488 nm excitation wavelength from a 15 mW air-cooled argon-ion laser. Emission was collected through a 585/420 nm band pass filter. A minimum of 5,000 events were collected on each sample and acquired in list mode by a Hewlett Packard 9000 computer. To exclude dead cells, debris, non lymphoid cells, and cell aggregates, data collection was gated on live spleen lymphocytes by forward and side angle scatter. Data are represented as the percentage of positive cells over the total number of cells counted.

Statistical analysis

Kaplan-Meier method was used to estimate the survivor functions and Log-rank test was performed for testing the homogeneity of survival functions across the four groups (control, CY, OM-174, CY + OM-174).

Tumor growth was analyzed by T-test for unpaired data.

Student's T-test was employed to analyze mean control values in the other experiments. Values of less than 0.05 were considered significant.

Results

Tumor growth

As shown in Figure 2, both cyclophosphamide (CY) and OM-174, when used individually, inhibited slightly but significantly B16 tumor growth as compared to the untreated controls. Importantly, the combination OM-174 and CY leads to a better inhibition of tumor growth rate, which was significantly better than that obtained by means of the single treatments.

Survival time

Both CY and OM-174, when used alone, increased slightly but significantly the mean survival time (MST) of mice with respect to the untreated controls. The combined treatment with CY and OM-174, induced better results in terms of survival of mice, which was significantly higher than that of control mice but also of mice receiving CY or OM-174

alone. Figure 3 shows the percentage of animals surviving in each treatment group during the whole period of observation.

NK activity

Tumor cell elimination is known to be mediated in part by the cytotoxic activity of NK cells. It has been therefore measured the cytotoxic activity of spleenocytes against NK-sensitive (YAC-l) tumor cells. Spleen cells were obtained from normal mice or from tumor-bearing mice that had been treated with PBS, CY, OM-174, or CY in combination with OM-174. Results are represented graphically in Table 2.

Table 2: **Effect of treatment on NK and CTL activities**

Treatment group	NK % cytotoxicity (E/T ratio 25:1)	CTL % cytotoxicity (E/T ratio 25:1)
Normal (N)	3.73 ± 0.4	<1
N + OM-174	10.39 ± 1.0#	<1
Tumor (T)	2.69 ± 0.3	2.71 ± 0.3
T + OM-174	6.3 ± 1.7	5.6 ± 0.9
T+CY	2.3 ± 0.4	2.63 ± 0.2
T+CY+OM-174	12.4 ± 2.0*	9.95 ± 1.6*

# p<0.001 vs. normal control mice.
*p<001 vs. all the other groups of mice injected with B16 melanoma tumor.

On day 14 post-tumor injection, five mice per group were killed and cytotoxic NK and CTL activities were measured as described in Materials and Methods. Results are expressed as mean percentage cytotoxicity ± S.E., derived from five individually tested mice per group.

In normal mice the treatment with OM-174 induced a dramatic increase of NK cell activity with respect to the untreated controls. The same dramatic increase of NK activity was observed also in B16 melanoma-injected mice. On day 14 both control and CY-treated tumor-bearing mice showed a decreased NK activity when compared to the untreated normal controls. OM-174 was always able to fully restore the NK activity over the levels

observed in untreated normal controls. $p<0.001$ for T+CY+OM-174 vs. all other groups.

Cytotoxic activity against autologous tumor cells

Cytotoxic T lymphocytes (CTLs) also play an important role in the elimination of tumor cells. It has been tested from spleen cells from normal and tumor-bearing mice for specific cytotoxic activity against autologous tumor cells using in-vivo B16 melanoma cells as target. The results of these experiments are shown in Table 2 above. As expected, it has been found that spleen cells from normal mice showed no detectable cytotoxic activity against B16 cells. On the contrary, spleenocytes from tumor-bearing mice showed an appreciable cytotoxic activity against autologous tumor cells, which appeared not to be increased by CY treatment. The administration of OM-174 was capable of inducing a marked stimulation of CTL activity in tumor-bearing mice (two-fold increase). Interestingly, in mice treated with the combination of OM-174 and CY, the highest levels of cytotoxic activity against autologous tumor cells has been shown to be increased 4-fold with respect to those of tumor controls and 2-fold with respect to those of tumor mice treated with OM-174 alone.

Analysis of spleen cell subset

To assess the impact of the different treatments on lymphocyte subsets of the experimental mice and their correlation with the results obtained on tumor growth, survival time, and cytotoxic activities, the percentages of spleen cells expressing CD4, CD8, and NK1.1. have been measured.

As shown in Table 3, tumor-bearing mice showed a significant reduction in all the spleen cell subsets tested compared to normal controls. The treatment with OM-174 increased the percentages of $CD4^+$, $CD8^+$ and NK 1.1 positive cells both in normal and in tumor-bearing mice. As already mentioned for the other parameters analyzed, the highest percentages of $CD4^+$, $CD8^+$ and NK1.1 positive cells were found in mice treated with CY + OM-174, which were over the values found in normal mice.

Table 3: Effect of treatment on spleen lymphocyte subsets (%).

Treatment group	$CD4^+$(%)	$CD8^+$ (%)	NK (%)
Normal (N)	28.5 ± 3.1	10.2 ± 1.6	9.2 ± 1.7
N + OM-174	34.0 ± 2.5	12.6 ± 1.5	11.6 ± 2.1
Tumor (T)	18.9 ± 1.4	6.3 ± 0.9	5.4 ± 0.7
T + OM-174	27.0 ± 2.0	9.3 ± 1.8	7.5 ± 0.5
T+CY	21.7 ± 1.8	6.4 ± 1.4	4 ± 0.5
T+CY+OM-174	32.7 ± 2.2*	15.8 ± 1.9*	10.9 ± 1.1*

*p<005 vs. all the other groups of mice injected with B16 melanoma tumor.

On day 14 post-tumor injection mice were killed and cells obtained from individually processed spleens were stained with monoclonal antibodies for FACS analysis. Results are expressed as mean percentages of positive cells vs total spleen cells ± S.E.M derived from five individually tested mice.

Conclusion

The present combined treatment is highly effective in the model of B-16 melanoma, ascertaining the efficacy of immunochemotherapeutic protocols for the lipid-A derivative OM-174. Indeed, the results obtained on the stimulation of cytotoxic activities (non specific NK and cancer specific CTL) of spleen cells and on the increase of NK, $CD4^+$ and $CD8^+$ phenotypes following treatment with OM-174, alone or in combination with CY, correlate with the delay in tumor growth and with the prolonged survival time .

Based on these results, OM-174 may thus be considered as a candidate for association with chemotherapeutic regimens in the treatment of cancer at clinical level.

Example 2: Antitumor Activity of Intratumoral OM-174 Combined with Intraperitoneal Cyclophosphamide on Advanced PROb Subcutaneous Colon Tumors in BDIX Rats.

In this experiment, it was studied in a colorectal model of cancer cells the effect of a combined sequential therapy using first the well-recognized chemotherapeutic drug cyclophosphamide, to reduce the tumor-induced immunosuppression, followed by unspecific intratumoral immunostimulation with OM-174. In contrast to the results obtained with other immunostimulating drugs such as CpG or BCG, it is thus demonstrated here that the antitumoral activity of cyclophosphamide (CY) was highly increased when this standard treatment was followed by intratumoral injections of OM-174.

MATERIAL, METHODS AND STATISTICS

Animals

Female inbred BDIX-strain rats 4 to 6 months old, weighing 200-250 g, were bred in constant conditions of temperature, hygrometry and exposure to artificial light.

Chemical and drugs

OM-174, was from OM PHARMA, cyclophosphamide (CY) from Sigma-Aldrich (L'Isle d' Abeau, France), intradermic BCG (BCG Vaccine) from Pasteur Vaccins (Lyon, France). CpG (synthetic polynucleotides) was synthesized internally in the laboratory of Prof Chauffert (Dijon, France).

Cancer cells and tumor model

The DHD/K12 cells originated from a dimethylhydrazine-induced colon tumor in BD IX rats. The PROb clone was chosen for its regular tumorigenicity when injected into syngeneic rats. PROb cells were maintained in culture in Ham's F10 medium supplemented with 10% fetal bovine serum. Cells were detached with trypsin and EDTA and centrifuged in the presence of complete culture medium with fetal bovine serum to inhibit trypsin. Cells (2×10^6/rat) were suspended in 0.1 ml of serum-free Ham's F10 medium then s.c. inoculated in the anterior thoracic area of anesthetized rats.

Treatments of animals

Female BDIX rats treatment started at day 36 after the s.c. inoculation of PROb cancer cells, when the tumor volume was about 1 cm^3. Experiments consisted of 8 groups of rats (6 animals in each group). Control group received no treatment. The other groups received either an unique injection of CY by the i.p. route (25 mg/kg in 5 ml of a sterile NaCl

solution), or immunostimulants by the intratumoral (i.t.) route starting at day 43, or i.p. CY at day 36 combined with i.t. immunostimulant starting at day 43. i.t. Injections were done at day 43 and 50 for BCG (100 µl of the reconstituted solution + 100 µl NaCl for every intratumoral injection). CpG (100 µg/injection in 200 µl NaCl) and OM-174 (200 µg/injection in 200 µl NaCl), were i.t. injected three times a week for 4 weeks (12 injections). Tumor diameter was measured once a week with a calliper.

RESULTS AND DISCUSSION

Intratumoral immunostimulants alone (OM-174, BCG, CpG) have no antitumoral effect comparatively to untreated animals on these large, established PROb tumors (figure 4). In contrast, i.p. cyclophosphamide caused a transient regression of the subcutaneous tumors, followed by a growth resumption in all animals. This was in accordance with the known chemosensitivity of the PROb cells to alkylating agents (Chauffert et al, 1992). However, CY alone was unable to cure animals. BCG had a deleterious effect, since its association to CY was less active than CY alone. CpG did not modify the CY activity. In contrast to the other immunostimulants, OM-174 strongly enhanced the antitumor affect of CY. All tumors regressed at a greater extent than in animals treated with CY alone and a complete and lasting tumor regression was obtained in 4/6 animals in this group (see Table 4).

Table 4: Number of cured animals after various treatments

Treatment	Number of cured animals/total number of animals
Control	0/6
BCG	0/5
CpG	0/6
OM-174	1/6
CY	0/6
CY + BCG	1/6
CY + CpG	0/6
CY + OM-174	4/6

In conclusion, these results demonstrate that OM-174 enhanced the antitumor effect of cyclophosphamide on advanced subcutaneous tumors in rats. In the present experiment, two other immunostimulants, BCG and CpG, worsened or did not improve at all the effect of cyclophosphamide alone, respectively.

Example 3: Enhancement of the anticancer effect of the chemotherapeutic agent cisplatin in combination with OM-174

Introduction

The antitumoral effect of the immunostimulating agent OM-174 has been demonstrated many times in the past in the BDIX/ProB model of peritoneal carcinomatoses in the rat (e.g. Onier et al., 1999). It has been shown that the beneficial effect is maximal (90% of complete remissions) when the treatment starts 14 days (D14) after the injection of the cancer cells (syngenic Prob cells). In contrast, the efficacy of the product is diminished when the treatment starts on D21, or D28, and completely disappears when treatment starts on D35 (when the tumor nodules are larger than 1 cm in diameter).

In order to find a therapy which could be adapted to humans, a combination of OM-174 with the platin oncostatic alkylating agent cisplatin was tested, by selecting experimental conditions in which OM-174 *per se* is marginally active. As it will be presented below, the

results suggest that the combination cisplatin/OM-174 may have a therapeutic effect in humans, since when cisplatin (3 mg/kg, i.v.) is provided on D21, OM-174 is still highly effective , even when injected for the first time on D28, or D35.

The following procedure was followed:

Cancer cells

Colon cancer PROb cells were originally obtained from a tumor of a BDIX rat induced by 1,2-diméthylhydrazine.

The BDIX strain of rats was established in 1937 by H. Druckrey. Nowadays these rats come from Iffa-Credo (L'Asbresle, France).

BDIX rats, 4 months ± 1 month at the beginning of the experiment, 7 animals /group, received i.p. cultured syngenic PROb cells (i.p) on day 0. Cisplatin (3 mg/kg) was injected i.v. on day 21, and OM-174 treatment (1 mg/kg, 3 injections i.v. per week in the penile vein) started either on days 28 or 35. Survival was followed until day 72 in the example presented here.

Results :

OM-174 *per se* is fully able to display anticancer effects when treatment (1 mg/kg, up to 15 injections i.v. every 2nd day) starts until 2 weeks after tumor inoculation. However the anticancer effect is lost when treatment is started later (day 28 or day 35 as shown in figure 5). This less favorable condition is certainly closer to the real clinical situation encountered in many cancerous patients.

In this example, cisplatin (3 mg/kg i.v) is given on day 21. A further immunostimulating treatment with OM-174 is started only on day 28 or 35 (1 mg/kg, 5 injections i.v. every 5th day). The survival curves are shown in figure 4.

Conclusion

The combination of OM-174 treatment with cisplatin, in this very unfavorable environment, gave a much stronger antitumor activity than either treatment alone.

Cisplatin treatment, as shown here, displays only partial efficacy, but when boosted by OM-174 immunostimulation, it reveals a strong antitumor effect.

Example 4: Enhancement of the anticancer effect of the chemotherapeutic agent oxaliplatin in combination with OM-174

Introduction

Oxaliplatin is a clinically successful member of a recent generation of anticancer chemotherapeutic agents of the platinum complex series.

Cisplatin and carboplatin, were until recently the only platinum compounds used clinically against solid tumors, such as testicular, ovarian, bladder and lung carcinomas. Unfortunately their use was limited by severe toxicity including nephrotoxicity, severe gastrointestinal intolerance (with nausea and vomiting), ototoxicity, and myelosuppression.

Really, it appears that the benefit/risk ratio of oxaliplatin is higher that those of the previously described platinium salts.

The purpose of this study was therefore to test the efficacy of various doses of OM-174 (0.1; 0.3; and 1 mg/kg) in association or not with oxaliplatin (3 mg/kg) in rats with colon cancer metastasis.

The following procedure was followed:

BDIX rats, 4 months ± 1 month at the beginning of the experiment, 10 animals /group, received i.p. cultured syngenic PROb cells (i.p) on day 0. Oxaliplatin (3 mg/kg) was injected i.v. on day 21, and OM-174 treatment (0.1; 0.3; or 1 mg/kg, from day 28 to 60, 3 injections per week for a total of 15 i.v. injections). Control injections were performed with physiological serum. Survival was followed for 112 days in the example presented below.

Results :

As previously shown in example 3 (figure 5), whatever the dose used, the anticancer effect of OM-174 *per se* is lost or marginal when treatment is started on day 28 (see the triangles in figure 6). The efficacy of oxaliplatin

alone was also marginal at the dose of 3 mg/kg (only a slight delay in mortality is initially observed, see the black squares in Figure 6).

In striking contrast (figure 7), the combination of 3 mg/kg of oxaliplatin + OM-174 (0.1, 0.3, or 1 mg/kg) appears to be a powerful anticancer therapy, especially when the doses of 0.3 or 1 mg/kg of OM-174 are used.

Conclusion

The combination of OM-174 (even at the lowest dose tested) treatment with oxaliplatin (3 mg/kg), in this very advanced stage of cancer, gave a much stronger antitumor activity than either treatments alone, as revealed by the striking survival of the majority of the animals cured by the oxaliplatin/OM-174 combination.

Example 5: Enhancement of the anticancer effect of the chemotherapeutic agent 5-Fluouracil (5-FU) in combination with OM-174.

Introduction

Antimetabolites interfere with normal metabolic pathways, including those necessary for making new DNA (phase S of the cell cycle). This class of molecules is often used to treat cancer.

A clinically efficient antimetabolite drug that disturbs DNA synthesis is 5-FU, used since at least four decades (see e.g Rich et al., 2004). It has a wide range of activity including colon cancer, breast cancer, head and neck cancer, pancreatic cancer, gastric cancer, anal cancer, oesophageal cancer and hepatomas.

An adequate and timely controlled clinical combined therapy with a well-recognized chemotherapeutic drug such as 5-FU, used first to shrink and kill some cancer cells (and thus potentially reveal tumor-associated antigens), followed by an unspecific immunostimulation with OM-174 will probably enhance the efficacy of the oncostatic drug, and permits the acquisition of an immunological (specific) memory to get rid of cells bearing the tumor associated antigen, and also to limit the level of the side effects observed, by allowing e.g. to reduce the number of administrations and/or the doses of the chemotherapeutic drug.

This experiment was aimed to check the efficacy of the combination of 5-FU with OM-174 in a rat model of colon cancer.

Material and Methods

The following procedure was followed:

The products: OM-174-DP was tested in association or not with 5-FU as decribed below:

On day 0 (D0), 10^6 PROb cells were injected i.p. to each rat. 5-FU was administered i.p. at the dose of 30 mg/kg on days 7 and 14. OM-174 was injected at the dose of 1 mg/kg i.v. from day 21 three times a week for a total of 10 injections.

Readouts

All rats (controls and treated) were sacrificed by CO_2 on day 61. The efficacy of the treatment was determined by read-outs such as survival (Figure 5) and measure of the classes of cancer given depending on the number and the size of the nodules, and also by ascites measurements.

Carcinomatoses were evaluated blindly. As it is impossible to measure the volume of a carcinomatosis, they were classified according to the number and diameter of the nodules:

- Class 0: no visible nodule
- Class 1: some countable nodules with a diameter from 0.1 to 0.3 cm
- Class 2: many uncountable nodules with a diameter from 0.1 to 0.3 cm
- Class 3: some nodules with a diameter of 1 cm invade the peritoneal cavity
- Class 4: the cavity is completely invaded by tumor masses of several cm.

The ascite volume was measured by double weights of the rats.

Results :

see the Table 5 and Figure 7:

Table 5: carciomatosis classes and ascites volumes after treatments

Groups/read-outs	**Nomber of rats in each carcinomatosis classes (0, 1, 2, 3, and 4)**					**Ascites (ml)**
	0	**1**	**2**	**3**	**4**	
Control	0	0	1	0	8	57
5-FU	2	0	0	0	8	44
OM-174	2	3	2	2	1	1
OM-174 + 5-FU	8	0	0	0	1	0

Concerning the classes:

The Mann-Whitney test shows a significant difference between Control and OM-174 groups as well as between Control and 5-FU + OM-174 groups. No significant difference has been shown for 5-FU versus Control groups. There is a significant difference in the median scores between the Control group and both the OM-174-DP and the 5-FU + OM-174-DP groups (DP means diphosphorylated derivative).

The corresponding survival curve is shown on Figure 8.

Conclusion

The combination OM-174 + 5-FU is better in term of carcinomatosis classes and survival time than both agents taken individually in this model of cancer.

General conclusion

In summary these results appear promising and suggest that non-specific immuno-stimulation by the well tolerated compound OM-174 have strong potential to improve the anticancer effects obtained by well-established or experimental chemotherapeutic anticancer therapies.

Immunotherapy with OM-174 in any appropriate formulation, dose, frequency of administration will be applied in humans repeatedly parenterally, preferentially by the intravenous or intratumoral routes. The prefered chemtherapeutic treatment will be applied each time according to standard practice (formulation, dose, frequency and route), either before, concomitantly, or after immunotherapy.

The needed dosages of OM-174 range from 0.05 to 100 mg/m^2 in humans and preferably from 0.1 to 20 mg/m^2.

The needed dosages of the antineoplastic agent very broadly vary depending on the nature of the tumor, the delay in tumor growth and the mode of action of the antineoplastic agent. These dosages may extend from 0.1 to 200mg/kg.

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World Cancer Report, 2003, World Health Organization

http://www.who.int/mediacentre/news/releases/2003/pr27/en/

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Figure legends

Figure 1

Kinetics of human plasma TNF-α in cancer patients treated i.v. with a single injection of OM-174 (1000 μg/m^2). The analysis of TNF-α was performed by ELISA.

Figure 2

Growth curve of B16 melanoma tumors in control and treated C57BL/6 mice (20/group).
On day 0, C57BL/6 mice were injected s.c. in the right flank with 2 x 10^5 B16 melanoma cells. Tumor growth was measured daily in each mouse, using calipers, and mean tumor diameter was calculated. At day 7 after tumor injection, all mice with s.c. tumors of about 2-3 mm diameter were divided into different experimental groups, i.e. phosphate buffered saline (PBS)-injected control, cyclophosphamide (CY), OM-174 or CY with OM-174. Each treated animal received a single dose of CY, i.p. (200 mg/Kg) on day 7, or/and OM-174, i.p. (1 mg/kg) on days 8, 13, 18, 23 and 28 after tumor inoculation. Data are from one out of three separate experiments, which gave similar results.

Figure 3.

Effect of treatment on survival in mice (20 animals/group) injected with B16 melanoma cells, and treated as described for Figure 2. Data are from one out of three separate experiments, which gave similar results.

Figure 4

Effect of cyclophosphamide (CY) combined either with OM-174, or Bacillus Calmette-Guerin (BCG), or unmethylated cytidine-guanosine dinucleotides (CpG) in rat tumor growth.
Female BDIX rats treatment started at day 36 after the s.c. inoculation of PROb cancer cells, when the tumor volume was about 1 cm^3. Experiments consisted of 8 groups of rats (6 animals in each group). Control group received no treatment. The other groups received either an unique injection of CY by the i.p. route (25 mg/kg), or immunostimulants by the intratumoral (i.t.) route starting at day 43, or i.p. CY at day 36 combined with i.t. immunostimulant starting at day 43. Injections were done at day 43 and 50 for BCG (100 μl of the reconstituted solution + 100 μl saline for every intratumoral injection). CpG (100 μg/injection in 200 μl saline) and OM-174 (200 μg/injection in 200 μl saline), were i.t. injected three times a week for 4 weeks (12 injections). Tumor diameter was measured once a week with a calliper.

Figure 5

7 BDIX rats/group received cultured syngenic PROb cells (i.p) on day 0. Cisplatin (cis, 3 mg/kg) was injected i.v. once on day 21, and the animals were treated or not i.v. with OM-174 (1 mg/kg, 3 injections per week) either from day 28 or day 35. Survival was followed until day 72.

Figure 6

10 BDIX rats/group received cultured syngenic PROb cells (i.p) on day 0. Oxaliplatin (3 mg/kg) was injected i.v. on day 21, and OM-174 treatment (0.1; 0.3; or 1 mg/kg, from day 28 to 60, 3 injections per week for a total of 15 i.v. injections). Control injections were performed with saline. Survival was followed for 112 days in the example presented.

Figure 7

10 BDIX rats/group received cultured syngenic PROb cells (i.p) on day 0. Oxaliplatin (3 mg/kg) was injected i.v. on day 21, with or without OM-174 (0.1; 0.3; or 1 mg/kg) which was given i.v., from day 28 to 60, 3 injections per week for a total of 15 i.v. injections. Control injections were performed with physiological serum. Survival was followed for 112 days in the example presented.

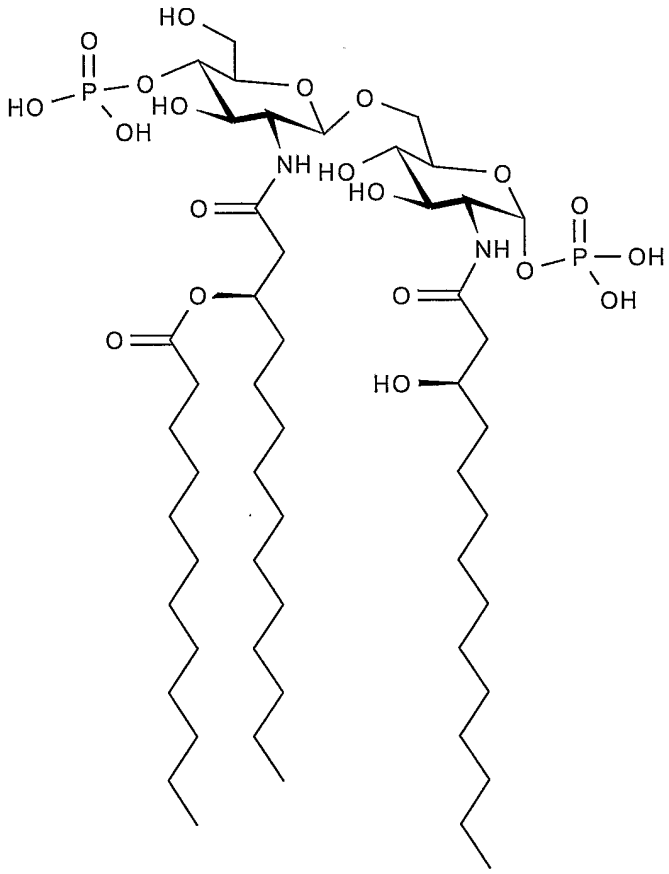
Figure 8

10 BDIX rats/group received cultured syngenic PROb cells (i.p) on day 0. They were treated i.p. on days 7 and 14 with 5-fluouracil (5-FU, 30 mg/kg), and/or from day 21 with OM-174 i.v. (1 mg/kg) 3 injections per week, for a total of 10 injections. Survival was followed.

The examples presented in the figures show that OM-174 is an immunomodulating agent in humans and that when combined with alkylating agents or antimetabolite agents, the resulting anti-cancer activities of the combinations is surprisingly higher then the activities observed when the compounds are given alone.

WHAT IS CLAIMED IS

1°) A pharmaceutical composition intended for treating warm-blooded animals including humans, suffering from a proliferative disease, comprising a non specific immuno modulating agent coded as OM-174, of general formula I



(I)

together with one or more known antineoplastic agents selected from the groups consisting of alkylating or antimetabolite agents, in conjugation or admixture with an inert non-toxic pharmaceutically acceptable diluent or carrier.

2°) A pharmaceutical composition according to claim 1,

wherein OM-174 is in the protonated or unprotonated form.

3°) A pharmaceutical composition according to claim 1 or claim 2 wherein the compound OM 174 is in the form of a salt with a mineral or organic base.,

4°) A pharmaceutical composition according to claim 1 to 3,

wherein the compound OM 174 is in the form of a sodium, potassium, calcium, triethylamine or triethanolamine salt.

5°) A pharmaceutical composition according to claim 1 to 3,

wherein the alkylating agent is selected from the group consisting of cyclophosphamide and its derivatives.

6°) A pharmaceutical composition according to claim 1 to 3, wherein the antimetabolites are selected from the group consisting of 5-fluorouracyle (5-FU) and its derivatives.

7°) A process for treating cancer in warm-blooded animals including humans, which consists in administering a combination of a therapeutically effective amount of a mixture of compounds according to claim 1 in a pharmaceutically-acceptable carrier, excipient or formulation.

8°) The method of claim 7,

wherein the active ingredient OM-174 and the well-established or experimental chemotherapeutic anticancer treatment,

are simultaneously administered.

9°) The method of claim 7,

wherein the pharmaceutical compound OM-174 and the well-established or experimental chemotherapeutic anticancer treatment ,

are sequentially administered.

10°) The method of claim 7,

wherein the pharmaceutical compound OM-174 and the well-established or experimental anticancer treatment ,

are applied in situ or into the tumors.

11°) The method of claim 7,

wherein the a pharmaceutical compound OM-174 and the well-established or experimental anticancer treatment,

are administered with a carrier insuring a controlled or sustained delivery.

12°) A pharmaceutical composition according to claim 1 and claim 2,

wherein OM-174 and the antineoplastic agent are present within a single container.

13°) A pharmaceutical composition according to claim 1 and claim 2, wherein the OM-174 and the antineoplastic agent are disposed within distinct containers.

14°) A pharmaceutical composition according to claim 1 and claim 2 wherein the needed dosages of OM 174 range from 0.05 to 100mg/m^2 in humans.

15°) A pharmaceutical composition according to claim 14 wherein the needed dosages of OM 174 range from 0.1 to 20 mg/m^2.

16°) A pharmaceutical composition according to claim 1 or claim 2 wherein the antineoplastic agent is used at doses very broadly ranging from 0.1 to 200mg/kg.

FIGURE 1/8

Figure 1

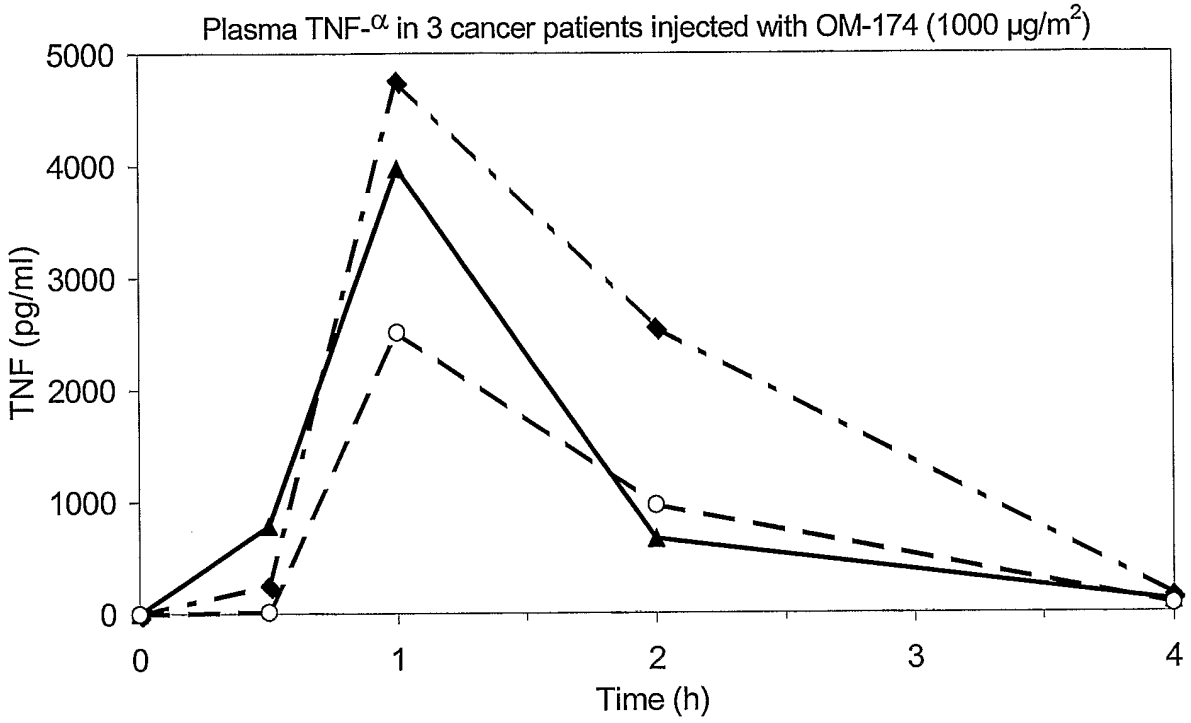


FIGURE 2/8

Figure 2

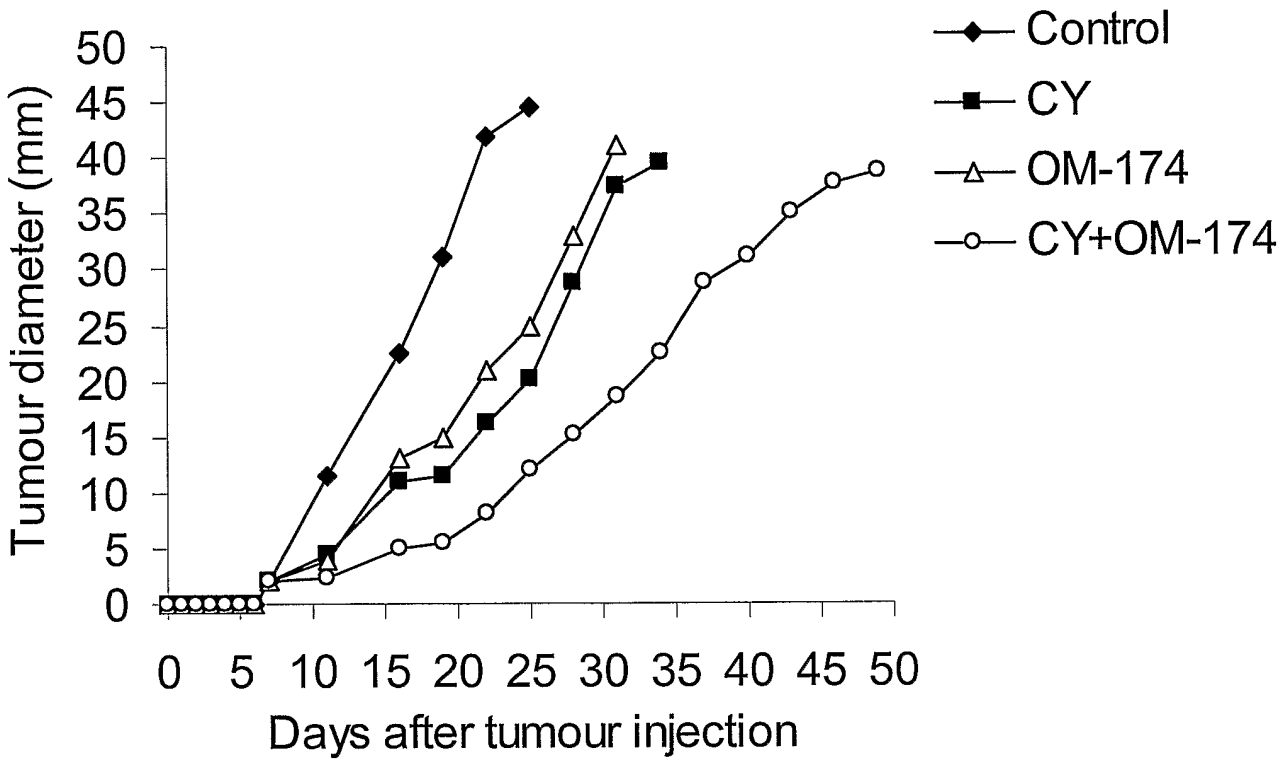


FIGURE 3/8

Figure 3

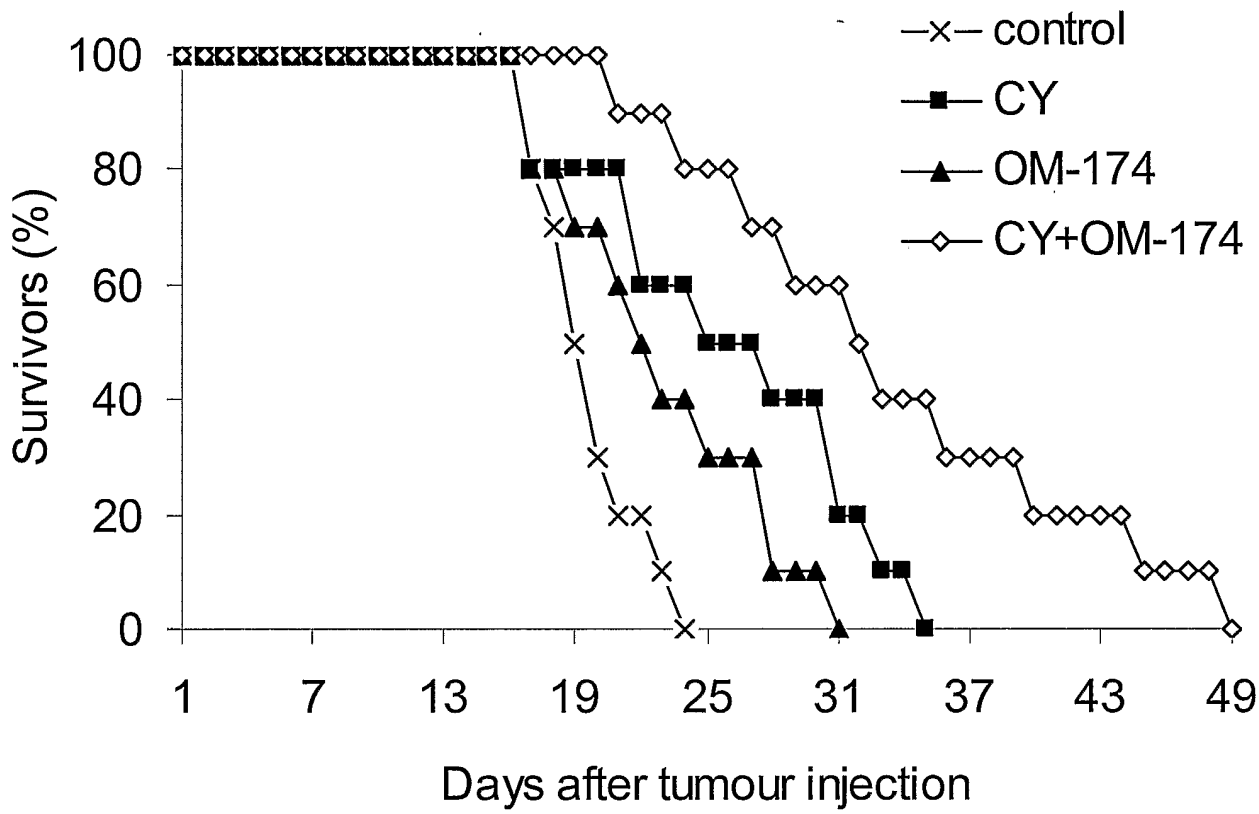


FIGURE 4/8

Figure 4

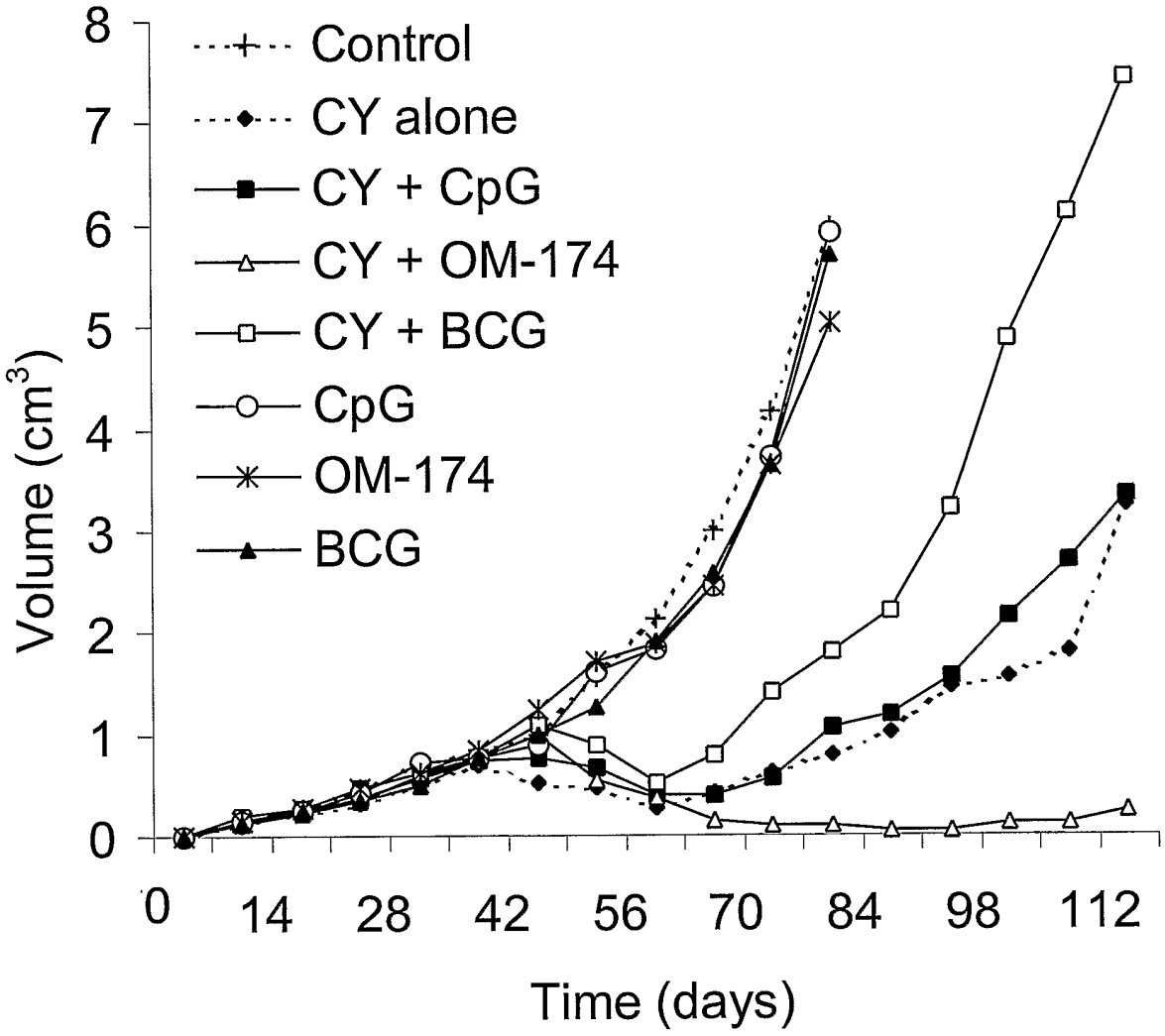


FIGURE 5/8

Figure 5

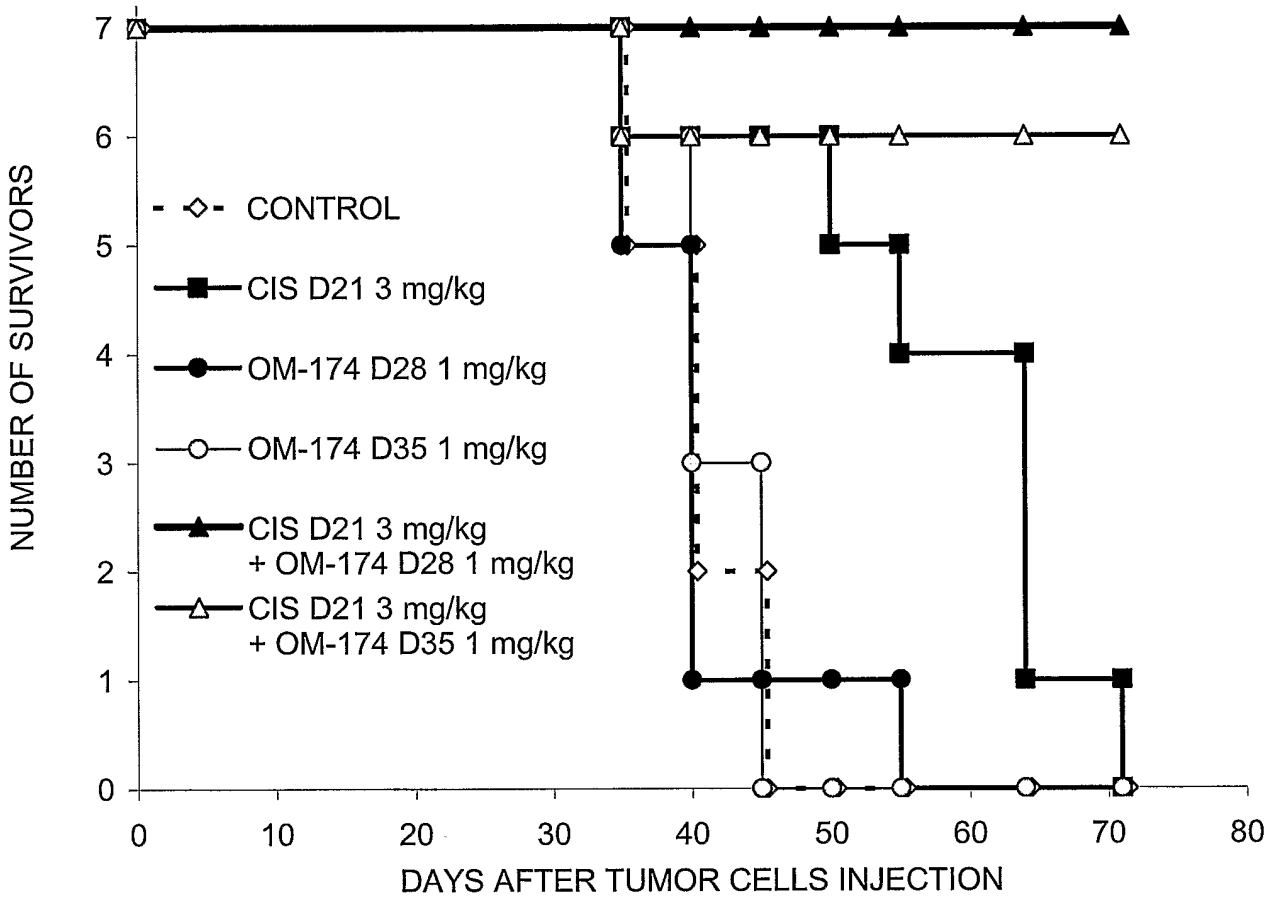


FIGURE 6/8

Figure 6

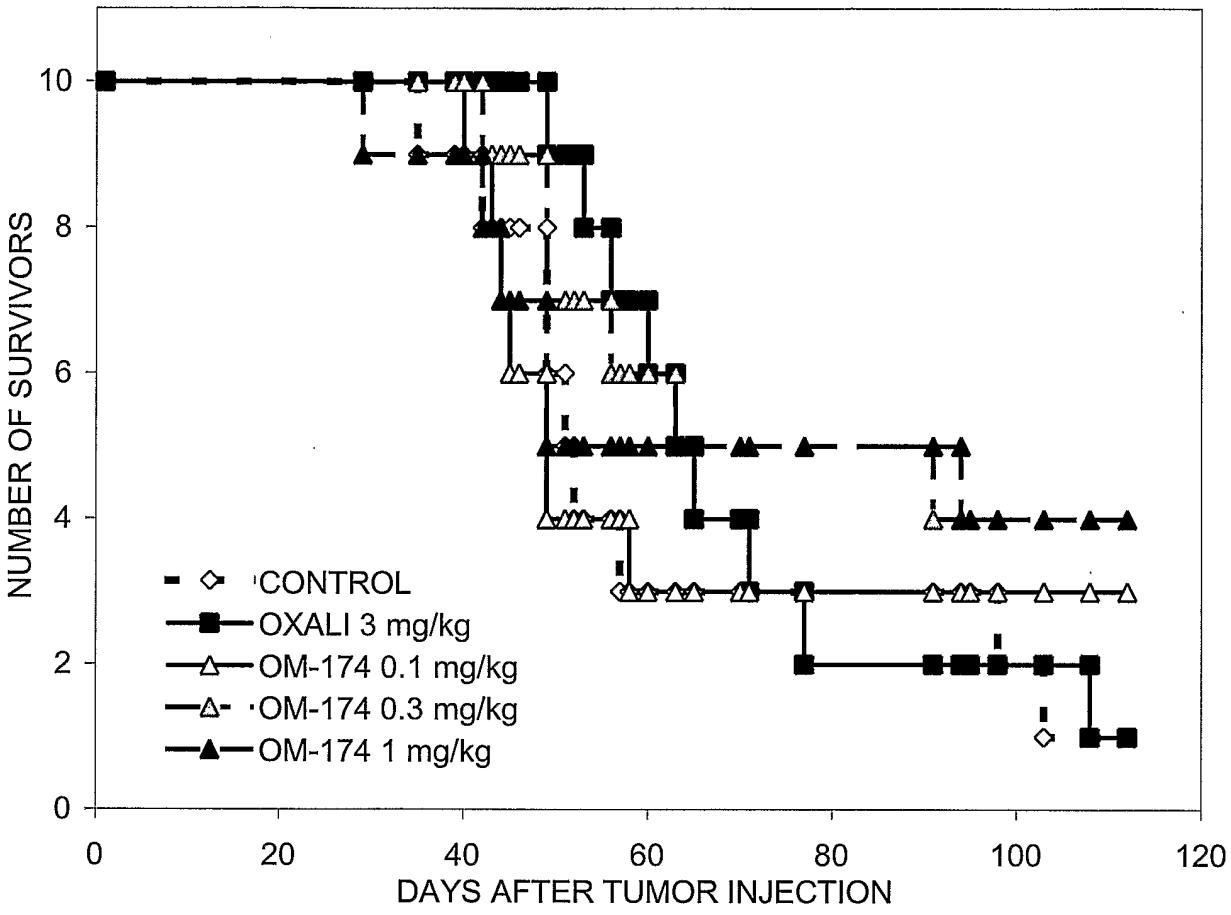


FIGURE 7/8

Figure 7

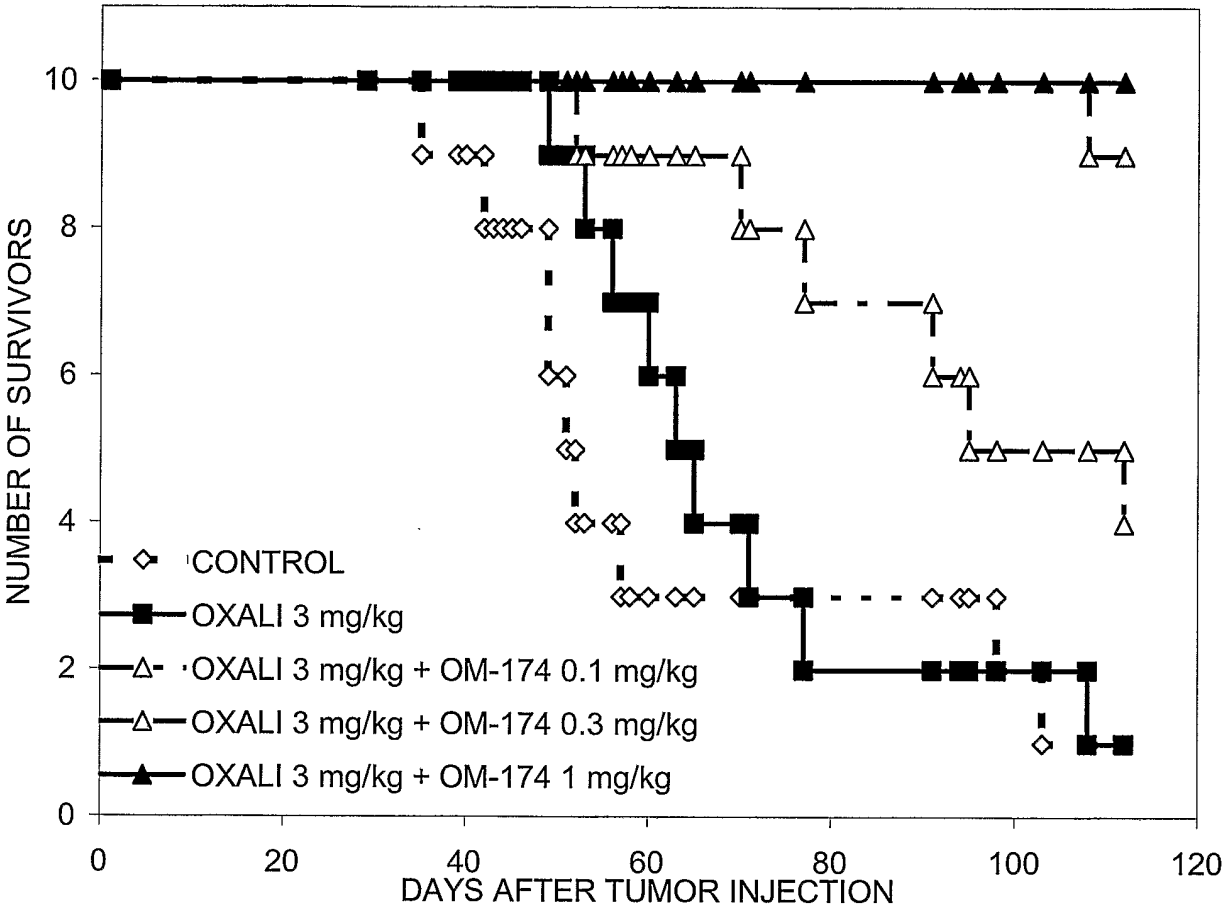
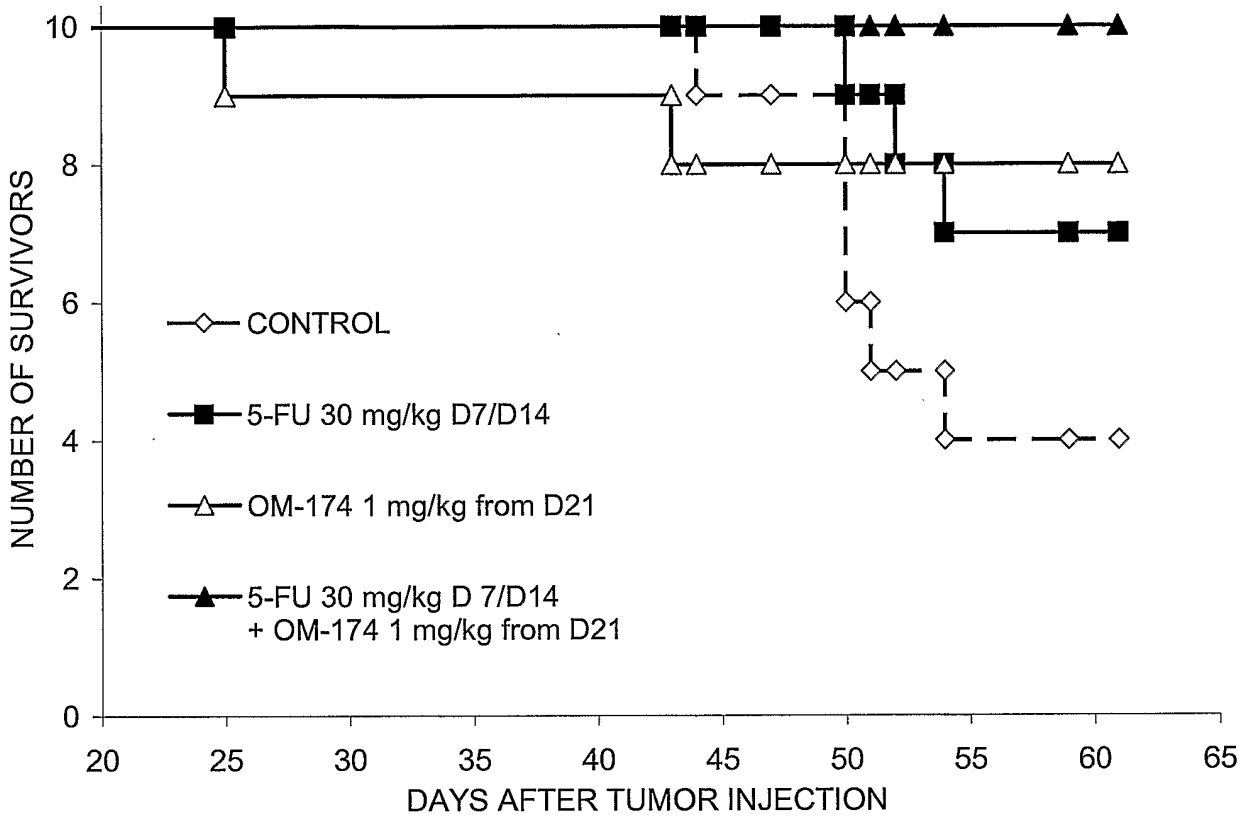


FIGURE 8/8

Figure 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/001180

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/282 A61K31/513 A61K31/675 A61K31/7028 A61K33/24
A61K45/06 A61P35/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	D'AGOSTINI C ET AL: "Antitumour effect of OM-174 and Cyclophosphamide on murine B16 melanoma in different experimental conditions" INTERNATIONAL IMMUNOPHARMACOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 5, no. 7-8, July 2005 (2005-07), pages 1205-1212, XP004898776 ISSN: 1567-5769 the whole document	16
A	US 2002/156033 A1 (BRATZLER ROBERT L ET AL) 24 October 2002 (2002-10-24) the whole document, in particular paragraphs [12], [0159] and [0162]	1-16

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2006	03/08/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Albrecht, S

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2006/001180

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ONIER N ET AL: "Expression of inducible nitric oxide synthase in tumors in relation with their regression induced by lipid A in rats" INTERNATIONAL JOURNAL OF CANCER, vol. 81, no. 5, 31 May 1999 (1999-05-31), pages 755-760, XP002316983 ISSN: 0020-7136 page 757, column 1, paragraph 2 page 759, column 1, paragraph 2 - column 2, paragraph 1 page 760, column 1, paragraph 2 - column 2, paragraph 1	1-16
A	US 6 005 099 A (DAVIES ET AL) 21 December 1999 (1999-12-21) the whole document	1-16
A	& WO 01/46126 A (OM PHARMA; BAUER, JACQUES; MARTIN, OLIVIER, RICHARD; RODRIGUEZ, SYLVAI) 28 June 2001 (2001-06-28) cited in the application	1-16
A	EP 0 668 289 A (SUNTORY KABUSHIKI KAISHA) 23 August 1995 (1995-08-23) the whole document	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2006/001180

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7-11
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 7-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

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

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2006/001180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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