

USE OF ANGELOYL-SUBSTITUTED INGENANES IN COMBINATION WITH OTHER AGENTS TO TREAT CANCER

BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates generally to therapeutic protocols for the treatment of cancer in subjects including humans. More particularly, the present invention provides a method for treating cancer comprising the administration of an angeloyl-substituted ingenane or a
10 pharmaceutically acceptable salt, derivative, homolog or analog thereof and at least one other agent. Preferably, the latter agent is an anti-cancer agent or has or induces an anti-cancer effect.

DESCRIPTION OF THE PRIOR ART

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Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an
20 acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Natural product screening is a term applied to the screening of natural environments for bioactive molecules. Particularly sought after bioactive molecules are those having
25 potential as useful therapeutic agents. Natural environments include plants, microorganisms, soil, coral and marine animals. The search for potential therapeutic agents for the treatment of cancer remains an important focus.

The Euphorbiaceae family of plants covers a wide variety of plants including weeds of
30 *Euphorbia* species. One intensively studied species of this group is *Euphorbia pilulifera* L

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(synonyms *E. hirta* L., *E. capitata* Lam.), whose common names include pill-bearing spurge, snakeweed, cat's hair, Queensland asthma weed and flowery-headed spurge. The plant is widely distributed in tropical countries, including India, and in Northern Australia, including Queensland. *Euphorbia peplus* is another species from which ingenol angelates with anti-cancer properties have been isolated (See US Patent Nos. 6,432,452, 6,787,161 and 6,844,013). PEP005 is an ingenol angelate extracted and purified from *E. peplus*, and is useful, *inter alia* in the treatment of actinic keratoses and non-melanoma skin cancer (NMSC) by short term topical administration. The cytotoxicity of PEP005 has been shown for many cell lines *in vitro* and its efficacy *in vivo* has been clinically established. The chemical name of PEP005 is ingenol-3-angelate.

Cancer is a cellular disease which occurs when a cell population hyper-proliferates. In the US alone, 2,604,650 people died from cancer between 1990-1994, with more men (53%) than women (47%) affected. The most numerous cancer deaths were the result of cancer of the lung (~30%), colon and rectum (~11%), breast (~8%), and prostate (~6.5%). Among women, the most commonly occurring cancers are breast (~31%), lung (~12%), colon and rectum (~12%), uterus (~6%) and ovary (~4%). It is estimated that 570,280 people will die from some form of cancer in 2005-2006.

Cancer treatment generally requires a therapeutic protocol comprising one or more of surgery, radiation and/or chemotherapy. Chemotherapy is a particularly common and well-established treatment for cancer. However, despite the fact that anti-cancer regimes provide significant benefit to patients, their use can be restricted because of problems with toxicity and adverse reactions. In fact, patients who are suffering from late-stage disease sometimes choose not to undergo active treatment because of the severe impact on quality of life.

When treating malignant disease, low molecular weight (LMW) anti-cancer drugs demonstrate deficiencies in therapeutic efficacy, highlighting a growing need for a multi-disciplinary approach to cancer therapy (Jain, *J Natl Cancer Inst* 81:570, 1989; Jain, *Science* 271:1079, 1996). New ideas on controlling the pharmacokinetics,

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- pharmacodynamics, non-specific toxicity, immunogenicity, biorecognition, and efficacy of drugs have been generated and have resulted in the development of new drug delivery systems based on inter-disciplinary approaches of polymer science, pharmaceuticals, bioconjugate chemistry and molecular biology. The combination of drugs with
- 5 macromolecules has created a new class of therapeutics which are drug delivery systems based on water-soluble polymeric carriers, micelle-forming block copolymers, nanospheres, microspheres, liposomes, dendrimers and hydrogels (Kim and Lim, *Arch Pharm Res* 25:229, 2002). However, unlike their LMW counterparts, macromolecular drugs often encounter significant permeability barriers that can limit achievement of the
- 10 above desirable properties. Thus, while tumor selectivity is often heightened due to a tumor's poorly formed vasculature and the consequent passive accumulation of macromolecules within the malignant mass, a phenomenon known as enhanced permeability and retention (EPR) effect (Maeda *Adv Enzyme Regul* 41:189, 2001), the tumor cell membrane can still constitute a formidable barrier for those macromolecules
- 15 that must first enter their target cells in order to cause cell death. Increased intra-tumoral pressure can also compromise delivery of macromolecular drugs to sites deep within a malignant mass if the tumor's lymphatic drainage is poorly developed (Swartz *et al*, *J Biomech* 32:1297, 1999).
- 20 Another important drug delivery system involves combination therapy where multiple agents are administered to treat different symptoms or to enhance the activity of one or multiple agents. The ability to reduce the toxicity of anti-cancer agents by co-administering with other compounds has been a long term goal in cancer therapy.
- 25 There is a need, therefore, to develop formulations and protocols for the treatment of cancer with enhanced efficacy.

SUMMARY OF THE INVENTION

The present invention provides a therapeutic protocol for the treatment of cancer. The protocol comprises the administration of an angeloyl-substituted ingenane (i.e. an ingenol angelate) or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and at least one or more other agents. The latter agents may have direct anti-cancer properties or may induce or facilitate the reduction in size, growth and/or spread (e.g. metastasis) of cancer cells or tissue. The present invention contemplates, therefore, combination therapy for the treatment of cancer comprising the administration of an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof together with, or associated with, an agent which exhibits one or more properties selected from: having anti-cancer activity; stimulates neutrophils or other immune cells or immune effector molecules (e.g. antibodies) in an immune response against cancer cells; has the ability to mediate antibody-dependent cellular cytotoxicity (ADCC) towards cancer cells; stimulates production of antibodies capable of binding to cancer cells; and/or has cytotoxic T-cell activity against cancer cells.

The present invention provides a combined chemotherapeutic protocol or a combined chemotherapeutic and immunotherapeutic effect (i.e. a chemoimmunotherapeutic effect) to induce anti-cancer activity to treat subjects with cancer. It is proposed that the combined therapeutic protocol enhances the anti-cancer activity or efficacy of the protocol compared to the use of either an ingenol angelate or other agent alone.

Reference to "anti-cancer activity" includes the apoptosis, necrosis, lysis, death, senescence and/or cell cycle arrest of cancer cells. It also includes inhibition of metastasis of a cancer and the induction of immunological memory against cancer cells. "Anti-cancer efficacy" includes anti-cancer activity as well as other properties such as *inter alia* rate, timing and effectiveness of inducing cancer cell cytotoxicity, apoptosis, necrosis, senescence, and/or cell cycle arrest; an ability to treat or inhibit metastasis; inducing or stimulating an immunological response against cancer cells; inducing immunological memory against recurrence of cancer cells; reducing the individual amounts of one or other

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of the angeloyl-substituted ingenane or agent required; reducing the toxic side-effects of the anti-cancer therapy to a subject; and reducing cancer growth following a period of remission.

- 5 Reference to a "cancer" includes solid and blood borne cancers such as tumors, leukemias, sarcomas or carcinomas. The combination of ingenol angelate and other agents may be given in a single formulation or in separate formulations admixed together prior to administration or may be administered simultaneously or sequentially from separate formulations. The terms "cancer" and "tumor" may be used interchangeably in the subject
10 specification. A "neoplastic" disorder is also a form of cancer or tumor.

Accordingly, one aspect of the present invention contemplates a therapeutic protocol for treating a subject with cancer or suspected of having cancer, said protocol comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically
15 acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

- (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;
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 - (ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;
 - (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating
25 such antibodies;
 - (iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;
- 30 wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

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An example of T-cell mediated inhibition or death of cancer cells is cytotoxic T-cell-mediated inhibition or cell death.

5 The present invention further provides a method of treating a subject with cancer or suspected of having cancer, said method comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

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(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

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(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

20

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

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Hence, the total amount of both agents when administered together may be less than when administered separately or may be additive but nevertheless more therapeutically efficacious in the sense of having improved or enhanced anti-cancer activity or efficacy.

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The present invention further provides a therapeutic protocol for treating cancer in a subject, said protocol comprising administering an ingenol angelate or a pharmaceutically

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acceptable salt, derivative, homolog or analog thereof and at least one other agent which exhibits one or more of the following properties:

(i). induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest
5 of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

10 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;
15 wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone;

wherein the combination of ingenol angelate and agent results in a combination index (CI)
20 of less than 1 where the CI is determined using the equation:

$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2]$$

wherein $(C_x)_1$ is the concentration of one of ingenol angelate or agent to produce x percent
25 effect of that drug alone, $(C)_1$ is the concentration of one of ingenol angelate or agent to produce the same x percent effect in combination with $(C)_2$ which is the other of the ingenol angelate or agent and α is a constant whereby if the mode of anti-cancer activity of ingenol angelate and agent is mutually exclusive or non-exclusive, α is 0 and 1, respectively.

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The present invention also provides a therapeutic protocol which involves the

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administration of an ingenol angelate and one or more of: (1) a second anti-cancer agent; (2) anti-cancer T-cells such as anti-cancer cytotoxic T-cells; (3) a neutrophil-stimulating agent; (4) an ADCC-facilitating agent (including neutrophils); (5) anti-cancer cell antibodies; and/or (6) an agent which induces, stimulates or causes to generate one or more
5 of (1) to (5).

Another aspect of the present invention provides a multi-part pharmaceutical formulation having a first part comprising an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and having a second part comprising an agent or a
10 pharmaceutically acceptable salt thereof, said parts further optionally comprising one or more pharmaceutically acceptable carriers, diluents and/or excipients wherein said agent exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest
15 of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;
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(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;
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wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

The present invention further provides a formulation comprising an ingenol angelate or a
30 pharmaceutically acceptable salt, derivative, homolog or analog thereof and an agent or a pharmaceutically acceptable salt thereof and one or more pharmaceutically acceptable

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carriers, diluents and/or excipients wherein said agent exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest
5 of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

10 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;
15 wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

Still another aspect of the present invention is directed to the use of an ingenol angelate or
20 a pharmaceutically acceptable salt, derivative, homolog or analog thereof and an agent or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of cancer wherein said agent exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest
25 of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

30 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

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(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

5 wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

In relation to formulations and uses, in a preferred embodiment, the CI of the combination of ingenol angelate and agent is less than 1. Reference to a CI value of less than 1 includes
10 values of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98 and 0.99 as well as values in between.

In a preferred embodiment, the ingenol angelate is ingenol-3-angelate which is referred to herein as "PEP005". Reference to "ingenol angelate", "ingenol-3-angelate", "PEP005" or
15 anti-cancer agent includes naturally occurring isolated forms, chemically modified forms, chemically synthesized forms as well as pharmaceutically acceptable salts, derivatives, homologs or analogs thereof.

Anti-cancer agents contemplated by the present invention include cytotoxic, necrotic,
20 apoptotic, cell cycle arresting and static agents useful in the treatment of neoplastic disorders such as solid and blood borne cancers. Examples of particular anti-cancer agents are listed in Table 1. The term "anti-cancer agent" also includes cytotoxic T-cells and other T-cell types and other immune cells as well as antibodies and neutrophil-stimulating agents and ADCC facilitating agents and agents which induce any one or more of the
25 above.

The subject may be any animal, including a mammal such as a human.

The present invention further contemplates delivery systems for the subject formulations.
30 In one embodiment, the ingenol angelate and agent are maintained separately and are admixed together prior to administration. In this embodiment, the formulation may be

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administered through a single body tissue-invasive inlet device or through multiple body tissue-invasive inlet devices such as but not limited to syringes and needles. Consequently, simultaneous or sequential administration of the ingenol angelate and an agent, in either order, is part of the present invention.

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TABLE 1

Examples of agents for use as a co-medicaments with an angeloyl-substituted ingenane for the treatment of cancer

Alkylating Agents:
Alkeran – Melphalan Alkeran Injection -Melphalan BiCNU- Carmustine CeeNU – Lomustine Cycloblastin – Cyclophosphamide Endoxan (containing sodium chloride) - Cyclophosphamide Endoxan (new formulation without sodium chloride) – Cyclophosphamide liadel Implant - Carmustine Holoxan - Ifosfamide Leukeran - Chlorambucil Muphoran - Fotemustine Myleran - Busulfan Temodal - Temozolomide Thiotepa – Thiotepa
Antibiotic cytotoxics:
Adriamycin Solution - Doxorubicin hydrochloride Blenamax - Bleomycin sulfate Blenoxane - Bleomycin sulfate Bleomycin Sulfate for Injection (DBL) - Bleomycin sulfate Caelyx - Doxorubicin hydrochloride Cosmegen - Dactinomycin Daunorubicin Injection - Daunorubicin hydrochloride DaunoXome - Daunorubicin hydrochloride Docetaxel Doxorubicin Hydrochloride Injection (DBL) - Doxorubicin hydrochloride Doxorubicin Hydrochloride Injection USP - Doxorubicin hydrochloride Epirubicin Hydrochloride Injection (DBL)- Epirubicin hydrochloride Fludara- Fludarabine phosphate Mitomycin C Kyowa- Mitomycin Mitozantrone Injection - Mitozantrone hydrochloride Novantrone- Mitozantrone hydrochloride

Onkotrone- Mitozantrone hydrochloride Pharmorubicin- Epirubicin hydrochloride Zavedos- Idarubicin hydrochloride
Antimetabolites:
Cytarabine (DBL) – Cytarabine Cytarabine Injection - Cytarabine Efudix – Fluorouracil (5 Fluorouracil) Fluorouracil Injection BP – Fluorouracil Fluorouracil Injection BP – Fluorouracil Gemzar - Gemcitabine hydrochloride Hycamtin - Topotecan hydrochloride Hydrea - Hydroxyurea Lanvis - Thioguanine Ledertrexate - Methotrexate Leunase - Colaspase Leustatin - Cladribine Methoblastin - Methotrexat Methotrexate Injection and Tablets (DBL) - Methotrexate Methotrexate Injection BP - Methotrexate Puri-Nethol - Mercaptopurine Tomudex - Raltitrexed Xeloda – Capecitabine
Hormonal antineoplastic agents:
Anandron – Nilutamide Androcur-100 - Cyproterone acetate Arimidex - Anastrozole Aromasin - Exemestane Chem mart Tamoxifen - Tamoxifen citrate Cosudex - Bicalutamide Cyprone - Cyproterone acetate Cyprostat-100 - Cyproterone acetate Cytadren - Aminoglutethimide Depo-Provera - Medroxyprogesterone acetate Depo-Ralovera - Medroxyprogesterone acetate Eulexin - Flutamide Fareston - Toremifene citrate Femara - Letrozole Flutamin - Flutamide Fugerel - Flutamide Genox - Tamoxifen citrate GenRx Tamoxifen - Tamoxifen citrate healthsense Tamoxifen - Tamoxifen citrate Lucrin - Leuporelin acetate Lucrin Depot - Leuporelin acetate Medroxyhexal - Medroxyprogesterone acetate

Megace - Megestrol acetate
 Nolvadex, Nolvadex-D - Tamoxifen citrate
 Procur - Cyproterone acetate
 Provera - Medroxyprogesterone acetate
 Ralovera - Medroxyprogesterone acetate
 Sandostatin - Octreotide
 Tamosin - Tamoxifen citrate
 Tamoxen - Tamoxifen citrate
 Tamoxifen Hexal - Tamoxifen citrate
 Tamoxifen-BC - Tamoxifen citrate
 Terry White Chemists Tamoxifen - Tamoxifen citrate
 Zoladex 10.8 mg Implant - Goserelin acetate
 Zoladex 3.6 mg Implant - Goserelin acetate

Other antineoplastic agents:

Anzatax Injection - Paclitaxel
 Agrylin - Anagrelide hydrochloride
 Amsidyl - Amsacrine
 Camptosar - Irinotecan hydrochloride
 Carboplatin Injection - Carboplatin
 Carboplatin Injection (DBL) - Carboplatin
 Cisplatin Injection - Cisplatin
 Cisplatin Injection (DBL) - Cisplatin
 D.T.I.C. - Dacarbazine
 Dacarbazine for Injection (DBL) - Dacarbazine
 Eloxatin - Oxaliplatin
 Etopophos - Etoposide phosphate
 Etoposide Injection - Etoposide
 Etoposide Injection (DBL) - Etoposide
 Glivec - Imatinib mesylate
 Herceptin - Trastuzumab
 Hexalen - Altretamine
 Mabthera- Rituximab
 Natulan- Procarbazine hydrochloride
 Proleukin- Aldesleukin (rbe)
 Sodium Iodide (131I) Capsules (Therapy)- Sodium iodide (131I)
 Taxol – Paclitaxel
 Taxotere - Docetaxel
 Vepesid- Etoposide
 Vesanoid- Tretinoin
 Vumon- Teniposide

Vinca alkaloids:

Eldisine - Vindesine sulfate
 Navelbine - Vinorelbine tartrate
 Oncovin - Vincristine sulfate
 Velbe - 21

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Vinblastine Sulfate Injection (DBL) - Vinblastine sulfate Vincristine Sulfate Injection - Vincristine sulfate Vincristine Sulfate Injection (DBL) - Vincristine sulfate Vinorelbine
Other agents
Antibodies T-cells Cytotoxic T-cells Cancer antigen (vaccine) Neutrophils Agents which stimulate, activate or cause to generate anti-cancer antibodies, cytotoxic T-cells or neutrophils which facilitate or mediate ADCC

Hence, the agents listed in Table 1 include chemotherapeutic compounds and molecules in addition to antibodies to cancer-specific antigens or to combinations of antigens specific for cancer cells as well as a range of other biologically- or chemically-synthesized

5 cytotoxic, apoptotic, necrotic, static and cell cycle arresting agents.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the

10 exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is

15 provided in Table 2. A sequence listing is provided after the claims.

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TABLE 2
Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Primer for IL-1 β -5'
2	Primer for IL-1 β -3'
3	Primer for TNF α -5'
4	Primer for TNF α -3'
5	Primer for glyceraldehyde-3-phosphate dehydrogenase-5'
6	Primer for glyceraldehyde-3-phosphate dehydrogenase-3'
7	Primer for macrophage inflammatory protein 2-5'
8	Primer for macrophage inflammatory protein 2-3'

BRIEF DESCRIPTION OF THE FIGURES

Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

Figure 1 is a graphical representation showing effects of the course of experiments with PEP005 given for 1 hour, 24 hours and 48 hours in a panel of cell lines.

Figure 2 is a graphical representation showing PEP005 cytotoxicity in two leukemic cell lines, K562 and CCRF-CEM after administration for 24, 48 and 72 hours.

Figure 3 represents photographs and graphs of cell cycle changes over 0, 1, 5, 12, 24 and 48 hours of exposure to PEP005.

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Figures 4A and B are a representation of apoptosis induction (A) sub-G1 DNA content, PI staining and (B) PI-AnnexinV staining.

Figure 5 is a graphical representation showing comparative analysis of PEP005 cytotoxicity with other anti-cancer drugs in a panel of cell lines. Data presented as (IC₅₀-IC₅₀ average) for HT29 (1), HCT116 (2), HCC2998 (3), COLO205 (4), MCF7 (5), MDA435 (6), HOP62 (7), HOP92 (8), IGROV1 (9) and OVCAR3 (10) cell lines.

Figure 6 is a graphical representation showing interaction of PEP005 and 5FU in the human COLO205 cancer cell line. PEP-5FU; PEP005 followed by 5FU; 5FU-PEP: 5FU followed by PEP005; PEP+5FU: simultaneous administration of PEP005 and 5FU. Calculation of a combination index below 0.8 indicates synergy, above 1.2 antagonism while a combination index between 0.8 and 1.2 corresponds to an additive effect.

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Figure 7 is a graphical representation showing the interaction of PEP005 and Doxorubicin in the human COLO205 cancer cell line. PEP-Doxorubicin: PEP005 following by Doxorubicin; Doxorubicin-PEP: Doxorubicin followed by PEP005; PEP+ Doxorubicin: simultaneous administration of PEP005 and Doxorubicin.

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Figures 8A and B are a graphical representation showing (A) the interaction of PEP005 and Oxaliplatin in the human COLO205 cancer cell line. PEP-24h-oxa: PEP005 24 hours followed by post-incubation in R10 medium for 24 hours followed by 24 hours of Oxaliplatin; PEP-72h-oxa; PEP005 1 hour, 5 hour, 24 hours followed by post-incubation in R10 medium for 72 hours followed by 24 hours of Oxaliplatin. (B) PEP-Oxaliplatin combination without interval. Isoblograms showing the interaction of PEP005 and Oxaliplatin in the human COLO205 cancer cell line.

Figure 9 is a graphical representation showing the interaction of PEP005 and Gemcitabine in the human COLO205 cancer cell line. PEP-Gemcitabine: PEP005 followed by Gemcitabine; Gemcitabine-PEP: Gemcitabine followed by PEP005; PEP+Gemcitabine: simultaneous administration of PEP005 and Gemcitabine.

Figure 10 is a graphical representation showing the interaction of PEP005 and Vinorelbine in the human COLO205 cancer cell line. PEP-Vinorelbine: PEP005 followed by Vinorelbine; Vinorelbine-PEP: Vinorelbine followed by PEP006; PEP+Vinorelbine: simultaneous administration of PEP005 and Vinorelbine.

Figure 11 is a graphical representation showing the interaction of PEP005 and Docetaxel in the human COLO205 cancer cell line. PEP-Docetaxel: PEP005 followed by Docetaxel; Docetaxel-PEP: Docetaxel followed by PEP005; PEP+Docetaxel: simultaneous administration of PEP005 and Docetaxel.

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Figure 12 is a graphical representation showing the interaction of PEP005 and Cisplatin in the human COLO205 cancer cell line. PEP-Cisplatin: PEP005 followed by Cisplatin; Cisplatin-PEP: Cisplatin followed by PEP005; PEP+ Cisplatin: simultaneous administration of PEP005 and Cisplatin.

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Figures 13A to D are graphical representations of effects of PEP005 on endothelial cells and neutrophils superoxide generation and antibody production. (A) Activation of human endothelium by PEP005. Endothelial cells were treated for 4 hours with PEP005 prior to addition of neutrophils and adhesion to the endothelial cell layer was assessed. 100 U/ml TNF α was used as a positive control. Data are mean $\pm$ SE of five separate experiments. ANOVA analysis demonstrated a significant effect of the dose of PEP005 on the levels of neutrophil adhesion ($p = 0.003$). (B) Killing of melanoma cells by PEP005-activated human neutrophils *in vitro*. Human MM96L melanoma cells were co-cultured with neutrophils and treated with PEP005 for 24 hours. The neutrophils and PEP005 were removed and melanoma cell survival was determined after 6 days. Killing is expressed as a percentage of the survival of tumor cells in the absence of PEP005 and neutrophils, which is shown as 100% ($\square$) at a neutrophil : MM96L ratio of 0:1. (C) Superoxide generation by PEP005-treated neutrophils. Human neutrophils were cultured with PEP005 for 2 hours and generation of superoxide was determined using a lucigenin based assay. (D) Antibody responses following PEP005 treatment of B16 tumors. B16 tumors growing on C57BL/6 mice were cured with PEP005 treatment after they had reached 30-60 mm³, (one tumor per mouse) and B16-specific IgG1 and IgG2a antibody titres were measured by standard ELISA using blood taken day 11 post treatment initiation ($n=6$) ($\blacksquare$) or day 135 post treatment initiation ($n=5$) ($\blacktriangle$). Antibody titres for placebo-treated animals whose tumors had reached $121.5 \pm \text{SD } 20.1$ ($n=4$) by day 11 are also shown ($\circ$). Naïve mice ($n=5$) received no tumors ($\square$).

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Figures 14A and B are graphical representations of PEP005 rescued IL-2-deprived activated CD8⁺ T-cells from apoptosis. (A) Forward/side scatter plots of untreated cells and cells cultured with IL-2 (25 U/ml). Cells gated in R1 were plotted in lower panels for their active caspase-3 expression represented by FITC fluorescence. Bold black line indicates irrelevant control. Proportion of cells undergoing apoptosis was calculated as the percentage of events under marker 1 (M1) out of total gated cells, from which the same proportion under M1 in the irrelevant control was subtracted. (B) Percentage of apoptosis in cells given IL-2 (closed bars) and deprived of IL-2 (open bars) was plotted against increasing concentrations of PEP005. Data was obtained from three individual experiments. Values are plotted as Mean $\pm$ SD. Statistical analysis was performed using unpaired two-tailed Student *t*-test with *p* value indicated, CI = 95% was taken and * denotes level of significance.

Figures 15A to D are graphical representations of PEP005 induced proliferation and rescue of apoptosis at 0.01 μ g/ml (20nM). Cells receiving different treatments were pulsed with BrdU and stained for BrdU and caspase as described in the methods section. Percentage of proliferating cells at 0, 24 and 48 hours of the experiment (A) Percentage of total apoptotic cells analyzed on day 2 (B) Percentage of apoptotic cells at day 2 among those proliferating at the stated time points (C) Percentage of apoptotic cells among those which were non-proliferating at the stated time-points was analyzed on day 2 (**D**).

DETAILED DESCRIPTION OF THE INVENTION

In describing and claiming the present invention, the following terminology is used in accordance with the definitions set forth below.

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It is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of components, manufacturing methods, dosage regimes, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

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The terms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a cancer" includes a single cancer or two or more cancers; reference to "an angeloyl-substituted ingenane" or "an ingenol angelate" includes a single compound, as well as two or more compounds; reference to "the
15 formulation" includes a single formulation or two or more formulations and so forth.

The terms "compound", "agent", "active agent", "chemical agent", "pharmacologically active agent", "medicament", "active" and "drug" are used interchangeably herein to refer to a chemical compound, protein (e.g. antibody) or cells which induces a desired
20 pharmacological and/or physiological effect. The terms also encompass pharmaceutically acceptable and pharmacologically active ingredients of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms "compound", "agent", "active agent", "chemical agent" "pharmacologically active agent", "medicament", "active" and "drug" are
25 used, then it is to be understood that this includes the active agent *per se* as well as pharmaceutically acceptable and pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs and the like. The desired pharmacological or physiological effect is anti-cancer activity or efficacy which includes cytotoxicity, apoptosis, necrosis, senescence and/or cell cycle arrest of cancer cells or cells which have a propensity to become
30 cancerous. The present invention is the combination of at least two agents which leads to greater anti-cancer efficacy compared to the use of either agent alone. Such greater anti-

cancer efficacy includes *inter alia* reduced toxicity of the anti-cancer agents, greater efficacy in inducing cancer cell death or growth arrest, reduced side effects to a subject, reduced metastasis or treatment of same, and/or an immunological response or immunological memory against cancer cells.

5

The above terms (i.e. "compound", "agent", "active agent", "chemical agent", "pharmacologically active agent", "medicament", "active" and "drug") are collectively referred to as "agents" which include anti-cancer chemotherapeutic agents, antibodies and T-cells (such as cytotoxic T-cells) and other immune cells as well as neutrophil-stimulating agents and ADCC-facilitating agents as well as agents which induce any of the latter effects. Hence, the agent used in combination with the angeloyl-substituted ingenane may be a chemotherapeutic agent which induces one or more of cytotoxicity, apoptosis, necrosis, senescence or cell cycle arrest of cancer cells; T-cells (e.g. cytotoxic T-cells) capable of inducing T-cell-mediated inhibition or death of cancer cells or an agent capable of inducing same; an antibody specific for an antigen on a cancer cell or a combination of antigens predominately or exclusively on cancer cells; or neutrophils or an agent which induces neutrophil-facilitated antibody-dependent cellular cytotoxicity (ADCC) of cancer cells.

Reference to a "compound", "agent", "active agent", "chemical agent" "pharmacologically active agent", "medicament", "active" and "drug" includes combinations of two or more actives such as two or more ingenol angelate or two or more agents. A "combination" also includes multi-part compositions such as a two-part composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation. For example, a multi-part pharmaceutical pack may have two or more active agents separately maintained. This also applies to the term "anti-cancer agent" and "chemotherapeutic agent" and other agents such as cells (e.g. cytotoxic T-cells or neutrophils) or antibodies.

The terms "effective amount" and "therapeutically effective amount" of an agent as used herein mean a sufficient amount of the agent (e.g. an ingenol angelate or agent) to provide

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the desired therapeutic or physiological effect or outcome which is anti-cancer activity or efficacy and in particular death or ablation or apoptosis or senescence or stasis or cell cycle arrest or necrosis of cancer cells reduced recurrence of cancer growth following remission, reduced or treated metastasis, immune-based anti-cancer activity immunological memory
5 against cancer cells, and/or reduced toxicity to a subject. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of
10 administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation.

Hence, as used herein, an "effective amount" refers to an amount of active agent that
15 provides the desired effect of reducing cancer cell growth when administered according to a suitable dosing regime. Preferably, the combined amount of active agent administered is an amount that provides the desired cytotoxicity, senescence, cell cycle arrest, necrosis and/or and ablation of cancer cells or other indicators of anti-cancer efficacy. Dosing may occur at intervals of several minutes, hours, days, weeks or months. Suitable dosage
20 amounts and regimes can be determined by the attending physician or veterinarian.

In one embodiment, the effective amount of a combination of ingenol angelate and agent is proposed to be less than when either is used in the absence of the other. In another embodiment, the combined amounts are additive even if the therapy is more efficacious.
25 Hence, the combination of angeloyl-substituted ingenane is proposed to provide greater anti-cancer activity or efficacy compared to the activity or efficacy of either agent alone. The anti-cancer activity or efficacy includes rate, timing and effectiveness of inducing cancer cell cytotoxicity, apoptosis, necrosis, senescence, and/or cell cycle arrest; an ability to treat or inhibit metastasis; inducing or stimulating an immunological response against
30 cancer cells; inducing immunological memory against recurrence of cancer cells; reducing the amounts of one or other of the angeloyl-substituted ingenane or agent required;

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reducing the toxic side-effects of the anti-cancer therapy to a subject; and reduced cancer growth following a period of remission.

In one embodiment, the effective amount may be calculated to give a combination index (CI) of less than 1 wherein the CI is determined using the equation:

$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2]$$

wherein $(C_x)_1$ is the concentration of one of ingenol angelate or agent to produce x percent effect of that drug alone, $(C)_1$ is the concentration of one of ingenol angelate or agent to produce the same x percent effect in combination with $(C)_2$ which is the other of the ingenol angelate or agent and α is a constant whereby if the mode of anti-cancer activity of ingenol angelate and anti-cancer agent is mutually exclusive or non-exclusive, α is 0 or 1, respectively.

15

Reference to a CI value of less than 1 includes values of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98 and 0.99 as well as values in between.

20 By "pharmaceutically acceptable" carrier, excipient or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH
25 buffering agents, preservatives, and the like.

Similarly, a "pharmacologically acceptable" salt, ester, amide, prodrug or derivative of a compound as provided herein is a salt, ester, amide, prodrug or derivative that this not biologically or otherwise undesirable.

30

The terms "treating" and "treatment" as used herein refer to reduction in severity and/or

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frequency of symptoms of the condition being treated, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms of the condition and/or their underlying cause and improvement or remediation or amelioration of damage following a condition.

5

"Treating" a subject may involve prevention of a condition or other adverse physiological event in a susceptible individual as well as treatment of a clinically symptomatic individual by ameliorating the symptoms of the condition. The term "treating" also applies to secondary or metastatic cancers, other than the primary cancer, which is the cancer which
10 may originally have been treated. It also covers the induction of immunological memory against cancer cells. The treatment may be referred to as a therapeutic protocol to induce improved or enhanced anti-cancer activity or efficacy as hereinbefore defined.

A "subject" as used herein refers to an animal, preferably a mammal and more preferably
15 human who can benefit from the pharmaceutical formulations and methods of the present invention. There is no limitation on the type of animal that could benefit from the presently described pharmaceutical formulations and methods. A subject regardless of whether a human or non-human animal may be referred to as an individual, patient, animal, host or recipient. The compounds and methods of the present invention have
20 applications in human medicine, veterinary medicine as well as in general, domestic or wild animal husbandry.

As indicated above, the preferred animals are humans or other primates such as orangutangs, gorillas, marmosets, livestock animals, laboratory test animals, companion
25 animals or captive wild animals, as well as avian species.

Examples of laboratory test animals include mice, rats, rabbits, simian animals, guinea pigs and hamsters. Rabbits, rodent and simian animals provide a convenient test system or animal model. Livestock animals include sheep, cows, pigs, goats, horses and donkeys.

30

The present invention is directed in part to a therapeutic protocol comprising the

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administration to a subject with cancer or suspected of having cancer at least two agents wherein at least one agent is an angeloyl-substituted ingenane (i.e. an ingenol angelate) or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and at least one other agent exhibits one or more of the following properties:

5

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

10 (ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

15 (iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

20

The therapeutic protocol includes, therefore, the administration to a subject of an ingenol angelate and one or more of (1) another anti-cancer agent; (2) cancer-specific T-cells (such as cytotoxic T-cells); (3) a neutrophil-stimulating agent; (4) an ADCC-facilitating agent (including neutrophils); (5) anti-cancer cell antibodies; and/or (6) an agent which induces, stimulates or causes to generate one or more of (1) to (5).

25

The present invention extends to an agent having only one property of (i) through (iv) or two or more or three or more or all four properties.

30 The therapeutic protocol of the present invention further extends to the use of the ingenol angelate to lower the threshold of neutrophils prior to triggering ADCC of cancer cells.

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An example of a neutrophil-stimulating agent includes an ingenol angelate (e.g. PEP005) or an interleukin (e.g. IL-6).

- 5 An example of a T-cell in part (ii) is a cytotoxic T-cell.

An example of an ADCC-facilitating agent includes an antibody. Agents which modulate complement-mediated cell death are also contemplated by the present invention.

- 10 Reference to "an ingenol angelate" includes compounds isolated from plants such as from a species of the family Euphorbiaceae as well as derivatives, homologs, analogs or functional equivalents thereof as well as chemically synthesized forms thereof as well as pharmaceutically acceptable salts thereof. This also applies to PEP005.
- 15 Reference herein to a member of the Euphorbiaceae family includes reference to species from the genera *Acalypha*, *Acidoton*, *Actinostemon*, *Adelia*, *Adenocline*, *Adenocrepis*, *Adenophaedra*, *Adisca*, *Agrostistachys*, *Alchornea*, *Alchorneopsis*, *Alcinaeanthus*, *Alcoceria*, *Aleurites*, *Amanoa*, *Andrachne*, *Angostyles*, *Anisophyllum*, *Antidesma*, *Aphora*, *Aporosa*, *Aporosella*, *Argythamnia*, *Astrococcus*, *Astrogyne*, *Baccanrea*, *Baliospermum*,
20 *Bernardia*, *Beyeriopsis*, *Bischofia*, *Blachia*, *Blumeodondron*, *Bonania*, *Bradleia*, *Breynia*, *Breyniopsis*, *Briedelia*, *Buraveia*, *Caperonia*, *Caryodendron*, *Celianella*, *Cephalocroton*, *Chaenotheca*, *Chaetocarpus*, *Chamaesyce*, *Cheilosa*, *Chiropetalum*, *Choriophyllum*, *Cicca*, *Chaoxylon*, *Cleidon*, *Cleistanthus*, *Cluytia*, *Cnesmone*, *Cnidoscolus*, *Coccoceras*, *Codiaeum*, *Coelodiscus*, *Conami*, *Conceveiba*, *Conceveibastrum*, *Conceveibum*, *Corythea*,
25 *Croizatia*, *Croton*, *Crotonopsis*, *Crozophora*, *Cubanthus*, *Cunuria*, *Dactylostemon*, *Dalechampia*, *Dendrocousinsia*, *Diaspersus*, *Didymocistus*, *Dimorphocalyx*, *Discocarpus*, *Ditaxis*, *Dodecastingma*, *Drypetes*, *Dysopsis*, *Elateriospermum*, *Endadenium*, *Endospermum*, *Erismanthus*, *Erythrocarpus*, *Erythrochilus*, *Eumecanthus*, *Euphorbia*, *Euphorbiodendron*, *Excoecaria*, *Flueggea*, *Calearia*, *Garcia*, *Gavarretia*, *Gelonium*,
30 *Giara*, *Givotia*, *Glochidion*, *Clochidionopsis*, *Glycydendron*, *Gymnanthes*, *Gymnosparia*, *Haematospermum*, *Hendecandra*, *Hevea*, *Hieronima*, *Hieronyma*, *Hippocrepantra*,

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- Homalanthus, Hymenocardia, Janipha, Jatropha, Julocroton, Lasiocroton, Leiocarpus, Leonardia, Lepidanthus, Leucocroton, Mabea, Macaranga, Mallotus, Manihot, Mappa, Maprounea, Melanthesa, Mercurialis, Mettenia, Micrandra, Microdesmis, Microelus, Microstachy, Maocroton, Monadenium, Mozinna, Neoscortechinia, Omalanthus,*
- 5 *Omphalea, Ophellantha, Orbicularia, Ostodes, Oxydectes, Palenga, Pantadenia, Paradrypeptes, Pausandra, Pedilanthus, Pera, Peridium, Petalostigma, Phyllanthus, Picrodendro, Pierardia, Pilinophytum, Pimeleodendron, Piranhea, Platygyne, Plukenetia, Podocalyx, Poinsettia, Poraresia, Prosartema, Pseudanthus, Pycnocomia, Quadrasia, Reverchonia, Richeria, Richeriella, Ricinella, Ricinocarpus, Rottlera, Sagotia, Sanwithia,*
- 10 *Sapium, Savia, Sclerocroton, Sebastiana, Securinega, Senefeldera, Senefilderopsis, Serophyton, Siphonia, Spathiostemon, Spixia, Stillingia, Strophoblachia, Synadenium, Tetracoccus, Tetraplandra, Tetrorchidium, Thyrsanthera, Tithymalus, Trageia, Trewia, Trigonostemon, Tyria and Xylophylla.*
- 15 A preferred genus and particularly suitable for the practice of the present invention is the genus *Euphorbia*. Particularly useful species of this genus include *Euphorbia aaron-rossii, Euphorbia abbreviata, Euphorbia acuta, Euphorbia alatocaulis, Euphorbia albicaulis, Euphorbia algomarginata, Euphorbia aliciae, Euphorbia alta, Euphorbia anacampseros, Euphorbia andromedae, Euphorbia angusta, Euphorbia anthonyi, Euphorbia antiguensis,*
- 20 *Euphorbia apocynifolia, Euphorbia arabica, Euphorbia ariensis, Euphorbia arizonica, Euphorbia arkansana, Euphorbia arteagae, Euphorbia arundelana, Euphorbia astroites, Euphorbia atrococca, Euphorbia baselicis, Euphorbia batabanensis, Euphorbia bergeri, Euphorbia bermudiana, Euphorbia bicolor, Euphorbia biformis, Euphorbia bifurcata, Euphorbia bilobata, Euphorbia biramensis, Euphorbia biuncialis, Euphorbia*
- 25 *blepharostipula, Euphorbia blodgetti, Euphorbia boerhaavioides, Euphorbia boliviana, Euphorbia bracei, Euphorbia brachiata, Euphorbia brachycera, Euphorbia brandegeei, Euphorbia brittonii, Euphorbia caesia, Euphorbia calcicola, Euphorbia campestris, Euphorbia candelabrum, Euphorbia capitellata, Euphorbia carmenensis, Euphorbia carunculata, Euphorbia cayensis, Euphorbia celastroides, Euphorbia chalicophila,*
- 30 *Euphorbia chamaerhodos, Euphorbia chamaesula, Euphorbia chiapensis, Euphorbia chiogenoides, Euphorbia cinerascens, Euphorbia clarionensis, Euphorbia colimae,*

- Euphorbia colorata*, *Euphorbia commutata*, *Euphorbia consoquitlae*, *Euphorbia convolvuloides*, *Euphorbia corallifera*, *Euphorbia creberrima*, *Euphorbia crenulata*, *Euphorbia cubensis*, *Euphorbia cuspidata*, *Euphorbia cymbiformis*, *Euphorbia darlingtonii*, *Euphorbia defoliata*, *Euphorbia degeneri*, *Euphorbia deltoidea*, *Euphorbia*
5 *dentata*, *Euphorbia depressa* *Euphorbia dictyosperma*, *Euphorbia dictyosperma*,
Euphorbia dioeca, *Euphorbia discoidalis*, *Euphorbia dorsiventralis*, *Euphorbia drumondii*,
Euphorbia duclouxii, *Euphorbia dussii*, *Euphorbia eanophylla*, *Euphorbia eggersii*,
Euphorbia eglandulosa, *Euphorbia elata*, *Euphorbia enalla*, *Euphorbia eriogonoides*,
Euphorbia eriophylla, *Euphorbia esculaeformis*, *Euphorbia espirituensis*, *Euphorbia*
10 *esula*, *Euphorbia excisa*, *Euphorbia exclusa*, *Euphorbia exstipitata*, *Euphorbia exstipulata*,
Euphorbia fendleri, *Euphorbia filicaulis*, *Euphorbia filiformis*, *Euphorbia florida*,
Euphorbia fruticulosa, *Euphorbia garber*, *Euphorbia gaumerii*, *Euphorbia gerardiana*,
Euphorbia geyeri, *Euphorbia glyptosperma*, *Euphorbia gorgonis*, *Euphorbia gracilior*,
Euphorbia gracillima, *Euphorbia gradyi*, *Euphorbia graminea*, *Euphorbia graminea*
15 *Euphorbia grisea*, *Euphorbia guadalajarana*, *Euphorbia guanarensis*, *Euphorbia*
gymnadenia, *Euphorbia haematantha*, *Euphorbia hedyotoides*, *Euphorbia heldrichii*,
Euphorbia helenae, *Euphorbia helleri*, *Euphorbia helwigii*, *Euphorbia henricksonii*,
Euphorbia heterophylla, *Euphorbia hexagona*, *Euphorbia hexagonoides*, *Euphorbia*
hinkleyorum, *Euphorbia hintonii*, *Euphorbia hirtula*, *Euphorbia hirta*, *Euphorbia hooveri*,
20 *Euphorbia humistrata*, *Euphorbia hypericifolia*, *Euphorbia inundata*, *Euphorbia involuta*,
Euphorbia jaliscensis, *Euphorbia jejuna*, *Euphorbia johnston*, *Euphorbia juttiae*,
Euphorbia knuthii, *Euphorbia lasiocarpa*, *Euphorbia lata*, *Euphorbia latazi*, *Euphorbia*
latericolor, *Euphorbia laxiflora* *Euphorbia lecheoides*, *Euphorbia ledienii*, *Euphorbia*
leucophylla, *Euphorbia lineata*, *Euphorbia linguiformis*, *Euphorbia longecornuta*,
25 *Euphorbia longepetiolata*, *Euphorbia longeramosa*, *Euphorbia longinsulicola*, *Euphorbia*
longipila, *Euphorbia lupulina*, *Euphorbia lurida*, *Euphorbia lycioides*, *Euphorbia*
macropodoides, *macvaughiana*, *Euphorbia manca*, *Euphorbia mandoniana*, *Euphorbia*
mangleti, *Euphorbia mango*, *Euphorbia marylandica*, *Euphorbia mayana*, *Euphorbia*
melanadenia, *Euphorbia melanocarpa*, *Euphorbia meridensis*, *Euphorbia mertonii*,
30 *Euphorbia mexiae*, *Euphorbia microcephala*, *Euphorbia microclada*, *Euphorbia*
micromera, *Euphorbia misella*, *Euphorbia missurica*, *Euphorbia montana*, *Euphorbia*

- montereyana*, *Euphorbia multicaulis*, *Euphorbia multiformis*, *Euphorbia multinodis*,
Euphorbia multiseta, *Euphorbia muscicola*, *Euphorbia neomexicana*, *Euphorbia*
nephradenia, *Euphorbia niqueroana*, *Euphorbia oaxacana*, *Euphorbia occidentalis*,
Euphorbia odontodenia, *Euphorbia olivacea*, *Euphorbia olowaluana*, *Euphorbia*
5 *ophthalmica*, *Euphorbia ovata*, *Euphorbia pachypoda*, *Euphorbia pachyrhiza*, *Euphorbia*
padifolia, *Euphorbia palmeri*, *Euphorbia paludicola*, *Euphorbia parciflora*, *Euphorbia*
parishii, *Euphorbia parryi*, *Euphorbia paxiana*, *Euphorbia pediculifera*, *Euphorbia*
peplidion, *Euphorbia peploides*, *Euphorbia peplus*, *Euphorbia pergamena*, *Euphorbia*
perlignea, *Euphorbia petaloidea*, *Euphorbia petaloidea*, *Euphorbia petrina*, *Euphorbia*
10 *picachensis*, *Euphorbia pilosula*, *Euphorbia pilulifera*, *Euphorbia pinariona*, *Euphorbia*
pinetorum, *Euphorbia pionosperma*, *Euphorbia platysperma*, *Euphorbia plicata*,
Euphorbia poeppigii, *Euphorbia poliosperma*, *Euphorbia polycarpa*, *Euphorbia*
polycnemoides, *Euphorbia polyphylla*, *Euphorbia portoricensis*, *Euphorbia portulacoides*
Euphorbia portulana, *Euphorbia preslii*, *Euphorbia prostrata*, *Euphorbia pteroneura*,
15 *Euphorbia pycnanthema*, *Euphorbia ramosa*, *Euphorbia rapulum*, *Euphorbia remyi*,
Euphorbia retroscabra, *Euphorbia revoluta*, *Euphorbia rivularis*, *Euphorbia robusta*,
Euphorbia romosa, *Euphorbia rubida*, *Euphorbia rubrosperma*, *Euphorbia rupicola*,
Euphorbia sanmartensis, *Euphorbia saxatilis* M. Bieb, *Euphorbia schizoloba*, *Euphorbia*
sclerocyathium, *Euphorbia scopulorum*, *Euphorbia senilis*, *Euphorbia serpyllifolia*,
20 *Euphorbia serrula*, *Euphorbia setiloba* Engelm, *Euphorbia sonora*, *Euphorbia soobyi*,
Euphorbia sparsiflora, *Euphorbia sphaerosperma*, *Euphorbia syphilitica*, *Euphorbia*
spruceana, *Euphorbia subcoerulea*, *Euphorbia stellata*, *Euphorbia submammilaris*,
Euphorbia subpeltata, *Euphorbia subpubens*, *Euphorbia subreniforme*, *Euphorbia*
subtrifoliata, *Euphorbia succedanea*, *Euphorbia tamaulipasana*, *Euphorbia telephioides*,
25 *Euphorbia tenuissima*, *Euphorbia tetrapora*, *Euphorbia tirucalli*, *Euphorbia tomentella*,
Euphorbia tomentosa, *Euphorbia torralbasii*, *Euphorbia tovariensis*, *Euphorbia*
trachysperma, *Euphorbia tricolor*, *Euphorbia troyana*, *Euphorbia tuerckheimii*, *Euphorbia*
turczaninowii, *Euphorbia umbellulata*, *Euphorbia undulata*, *Euphorbia vermiformis*,
Euphorbia versicolor, *Euphorbia villifera*, *Euphorbia violacea*, *Euphorbia whitei*,
30 *Euphorbia xanti* Engelm, *Euphorbia xylopoda* Greenm., *Euphorbia yayalesia* Urb.,
Euphorbia yungasensis, *Euphorbia zeravschanica* and *Euphorbia zinniiflora*.

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Particularly preferred species of the genus *Synadenium* include *Synadenium grantii* and *Synadenium compactum*.

- 5 Particularly preferred species of the genus *Monadenium* include *Monadenium lugardae* and *Monadenium guentheri*.

A preferred species of the genus *Endadenium* is *Endadenium gossweileni*.

- 10 *Euphorbia peplus* is particularly useful in the practice of the present invention in terms of providing a source of ingenol angelates. Reference herein to "*Euphorbia peplus*" or its abbreviation "*E. peplus*" includes various varieties, strains, lines, hybrids or derivatives of this plant as well as its botanical or horticultural relatives. Furthermore, the present invention may be practiced using a whole Euphorbiaceae plant or parts thereof including
15 sap or seeds or other reproductive material may be used. Generally, for seeds or reproductive material to be used, a plant or plantlet is first required to be propagated.

- Reference herein to a Euphorbiaceae plant, a *Euphorbia* species or *E. peplus* further encompasses genetically modified plants. Genetically modified plants include transgenic
20 plants or plants in which a trait has been removed or where an endogenous gene sequence has been down-regulated, mutated or otherwise altered including the alteration or introduction of genetic material which exhibits a regulatory effect on a particular gene. Consequently, a plant which exhibits a character not naturally present in a Euphorbiaceae plant or a species of *Euphorbia* or in *E. peplus* is nevertheless encompassed by the present
25 invention and is included within the scope of the above-mentioned terms.

- The ingenol angelates are generally found in extracts of the Euphorbiaceae plants. An extract may comprise, therefore, sap or liquid or semi-liquid material exuded from, or present in, leaves, stem, flowers, seeds, bark or between the bark and the stem. Most
30 preferably, the extract is from sap. Furthermore, the extract may comprise liquid or semi-liquid material located in fractions extracted from sap, leaves, stems, flowers, bark or other

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plant material of the Euphorbiaceae plant. For example, plant material may be subject to physical manipulation to disrupt plant fibres and extracellular matrix material and inter- and intra-tissue extracted into a solvent including an aqueous environment. All such sources of ingenol angelates are encompassed by the present invention including
5 compounds obtained by chemically synthetic routes.

The most preferred compound of the present invention is referred to chemically as ingenol-3-angelate and is also referred to herein as "PEP005". Reference herein to "ingenol-3-angelate" or "PEP005" includes naturally occurring as well as chemically synthetic forms
10 and pharmaceutically acceptable salts, derivatives, homologs and analogs thereof.

One example of an agent used in combination with angeloyl-substituted ingenane is a chemotherapeutic agent such as anti-metabolites, anti-tumor antibiotics, mitotic inhibitors, steroids, sex hormones, alkylating agents, nitrogen mustards, nitrosources, hormone
15 agonists, and microtubule inhibitors.

Anti-metabolites interfere with the body's chemical processes, such as protein or DNA synthesis required for cell growth and reproduction. Anti-metabolite drugs can prevent cell division which is a requirement in cancer treatment. Examples include Azaserine, D-
20 Cycloserine, Mycophenolic acid, Trimethoprim, 5-fluorouracil, capecitabine, methotrexate, gemcitabine, cytarabine (ara-C) and fludarabine.

Anti-tumor antibiotics interfere with DNA by stopping enzymes and mitosis or altering the membranes that surround cells. These agents work in all phases of the cell cycle. Thus,
25 they are widely used for a variety of cancers. Examples of anti-tumor antibiotics include dactinomycin, daunorubicin, doxorubicin (Adriamycin), idarubicin, and mitoxantrone.

Mitotic inhibitors are plant alkaloids and other compounds derived from natural products. They can inhibit, or stop, mitosis or inhibit enzymes for making proteins needed for
30 reproduction of the cell. These work during the M phase of the cell cycle. Examples of mitotic inhibitors include paclitaxel, docetaxel, etoposide (VP-16), vinblastine, vincristine,

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and vinorelbine.

Steroids are natural hormones and hormone-like drugs that are useful in treating some types of cancer (such as but not limited to lymphoma, leukemias and multiple myeloma) as well as other illnesses. When these drugs are used to kill cancer cells or slow their growth, they are considered anti-cancer drugs. They may also be combined with other types of chemotherapy drugs to increase their effectiveness. Examples include prednisone and dexamethasone.

Sex hormones, or hormone-like drugs, alter the action or production of female or male hormones. They are used to slow the growth of breast, prostate, and endometrial (lining of the uterus) cancers, which normally grow in response to hormone levels in the body. Examples include anti-estrogens (tamoxifen, fulvestrant), aromatase inhibitors (anastrozole, letrozole), progestins (megestrol acetate), anti-androgens (bicalutamide, flutamide), and LHRH agonists (leuprolide, goserelin).

Alkylating agents work directly on DNA to prevent the cancer cell from reproducing. As a class of drugs, these agents are not phase-specific (in other words, they work in all phases of the cell cycle). These drugs are active against chronic leukemias, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and certain cancers of the lung, breast, and ovary. Examples of alkylating agents include busulfan, cisplatin, carboplatin, chlorambucil, cyclophosphamide, ifosfamide, dacarbazine (DTIC), mechlorethamine (nitrogen mustard), and melphalan.

Nitrogen mustard in the form of its crystalline hydrochloride it is used as a drug in the treatment of Hodgkin's disease, non-Hodgkin's lymphomas and brain tumors. Nitrogen mustards cause mutations in the genetic material of cells, thereby disrupting mitosis, or cell division. Cells vary in their susceptibility to nitrogen mustards, with rapidly proliferating tumor and cancer cells most sensitive; bone marrow, which produces red blood cells, is also sensitive, and depression of red blood cell production is a frequent side effect of nitrogen mustard therapy. The nitrogen mustards also suppress the immune response.

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Other types include the aromatic mustards melphalan and chlorambucil.

Nitrosoureas act in a similar way to alkylating agents. They interfere with enzymes that help repair DNA. These agents are able to travel to the brain so they are used to treat brain
5 tumors as well as non-Hodgkin's lymphomas, multiple myeloma, and malignant melanoma. Examples of nitrosoureas include carmustine (BCNU) and lomustine (CCNU).

Examples of hormone agonists include Leuprolide (Lupron, Viadur, Eligard) such as for prostate cancer, Goserelin (Zoladex) for breast and prostate cancers and Triptorelin
10 (Trelstar) for ovarian and prostate cancers and nafarelin acetate (Synarel).

Microtubule inhibitors include "Vinca" alkaloids, taxoids and benzimidazoles. Examples of such anti-cancer agents include but are not limited to anti-cancer drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin,
15 bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-
20 mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES).

25

The agents of the present invention also include antibodies or other immunointeractive molecules. For example, antibodies to particular cancer-specific antigens may be administered or an antigen is provided to induce antibody formulation directed to a cancer-specific antigen. The antibodies represent an example of an agent given with an angeloyl-
30 substituted ingenane in an anti-cancer protocol. Furthermore, a subject's T-cells or other immune cells may be isolated, expanded in culture and then returned to the subject. With

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respect to the latter, the present invention extends to a protocol involving selecting a cancer patient, isolating T-cells or a sample comprising T-cells, expanding the T-cells in *in vitro* culture, optionally stimulating the cells with an interleukin (e.g. IL-6) or other cytokine or an ingenol angelate (e.g. PEP005) and returning the expanded T-cell population to the
5 subject. The subject would also be subject to ablation of the cancer with an angeloyl-substituted ingenane (e.g. PEP005). In another embodiment, T-cells are raised by vaccination or other immune potentiating agents or are adoptively transferred to a patient. PEP005 or other ingenol angelate is then used to improve T-cell activity.

10 With respect to the former, the antibodies may be selected to enhance ADCC of cancer cells or to facilitate a cytotoxic T-cell response or other response from immune cells.

With respect to antibodies, polyclonal antibodies may conveniently be used, however, the use of monoclonal antibodies is particularly preferred because of the ability to produce
15 them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production is derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation (i.e. comprising a cancer antigen).

20 The present invention further provides the application of biochemical techniques to render an antibody derived from one animal or avian creature substantially non-immunogenic in another animal or avian creature of the same or different species. The biochemical process is referred to herein as "deimmunization". Reference herein to "deimmunization" includes processes such as complementary determinant region (CDR) grafting, "reshaping" with
25 respect to a framework region of an immunointeractive molecule and variable (v) region mutation, all aimed at reducing the immunogenicity of an immunointeractive molecule (e.g. antibody) in a particular host (e.g. a human subject). In the present case, the preferred immunointeractive molecule is an antibody such as a polyclonal or monoclonal antibody specific for a cancer cell. In a most preferred embodiment, the immunointeractive
30 molecule is a monoclonal antibody, derived from one animal or avian creature and which exhibits reduced immunogenicity in another animal or avian creature from the same or

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different species such as but not limited to humans.

Reference to "substantially non-immunogenic" includes reduced immunogenicity compared to a parent antibody, i.e. an antibody before exposure to deimmunization processes. The term "immunogenicity" includes an ability to provoke, induce or otherwise facilitate a humoral and/or T-cell mediated response in a host animal. Particularly convenient immunogenic criteria include the ability for amino acid sequences derived from a variable (v) region of an antibody to interact with MHC class II molecules thereby stimulating or facilitating a T-cell mediating response including a T-cell-assisted humoral response.

By "antibody" is meant a protein of the immunoglobulin family that is capable of combining, interacting or otherwise associating with an antigen. An antibody is, therefore, an antigen-binding molecule. An "antibody" is an example of an immunointeractive molecule and includes a polyclonal or monoclonal antibody. The preferred immunointeractive molecules of the present invention are monoclonal antibodies. The term "antibody" also includes engineered antibodies such as bi-specific antibodies to two different antigens on cancer cells.

The term "antigen" is used herein in its broadest sense to refer to a substance that is capable of reacting in and/or inducing an immune response. Reference to an "antigen" includes an antigenic determinant or epitope or a cancer cell.

By "antigen-binding molecule" is meant any molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins (e.g. polyclonal or monoclonal antibodies), immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity. The terms "antibody" and "antigen-binding molecules" include deimmunized forms of these molecules.

By "antigenic determinant" or "epitope" is meant that part of an antigenic molecule against which a particular immune response is directed and includes a hapten. Typically, in an

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animal, antigens present several or even many antigenic determinants simultaneously. A "hapten" is a substance that can combine specificity with an antibody but cannot or only poorly induces an immune response unless bound to a carrier. A hapten typically comprises a single antigenic determinant or epitope.

5

Although the preferred antibodies of the present invention are deimmunized forms of murine monoclonal antibodies for use in humans, the subject invention extends to antibodies from any source and deimmunized for use in any host. Examples of animal and avian sources and hosts include humans, primates, livestock animals (e.g. sheep, cows, horses, pigs, donkeys), laboratory test animals (e.g. mice, rabbits, guinea pigs, hamsters), 10 companion animals (e.g. dogs, cats), poultry bird (e.g. chickens, ducks, geese, turkeys) and game birds (e.g. pheasants).

Immunization and subsequent production of monoclonal antibodies can be carried out using standard protocols as for example described by Köhler and Milstein (Kohler *et al*, 15 *Nature* 256:495-499, 1975 and Kohler *et al*, *Eur. J. Immunol.* 6(7):511-519, 1976, Coligan *et al*, *Current Protocols in Immunology*, 1991-1997 or Toyama *et al*, *Monoclonal Antibody, Experiment Manual*, published by Kodansha Scientific, 1987). Essentially, an animal is immunized with an antigen-containing (e.g. cancer antigen) or fraction thereof by 20 standard methods to produce antibody-producing cells, particularly antibody-producing somatic cells (e.g. B lymphocytes). These cells can then be removed from the immunized animal for immortalization. The antigen may need to first be associated with a carrier.

By "carrier" is meant any substance of typically high molecular weight to which a non- or 25 poorly immunogenic substance (e.g. a hapten) is naturally or artificially linked to enhance its immunogenicity.

Immortalization of antibody-producing cells may be carried out using methods, which are well-known in the art. For example, the immortalization may be achieved by the 30 transformation method using Epstein-Barr virus (EBV) [Kozbor *et al*, *Methods in Enzymology* 121:140, 1986]. In a preferred embodiment, antibody-producing cells are

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- immortalized using the cell fusion method (described in Coligan *et al, supra*, 1991-1997), which is widely employed for the production of monoclonal antibodies. In this method, somatic antibody-producing cells with the potential to produce antibodies, particularly B cells, are fused with a myeloma cell line. These somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals, preferably rodent animals such as mice and rats. In the exemplary embodiment of this invention mice, spleen cells are used. It would be possible, however, to use rat, rabbit, sheep or goat cells, or cells from other animal species instead.
- Specialized myeloma cell lines have been developed from lymphocytic tumors for use in hybridoma-producing fusion procedures (Kohler *et al, supra*, 1976; Kozbor *et al, supra*, 1986; and Volk *et al, J. Virol.* 42(1):220-227, 1982). These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas from unfused and similarly indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumor cells to produce their own antibodies. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing endogenous light or heavy immunoglobulin chains are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g. P3X63-Ag8, P3X63-AG8.653, P3/NS1-Ag4-1 (NS-1), Sp2/0-Ag14 and S194/5.XXO.Bu.1. The P3X63-Ag8 and NS-1 cell lines have been described by Köhler and Milstein (Kohler *et al, supra*, 1976). Shulman *et al, Nature* 276:269-270, 1978, developed the Sp2/0-Ag14 myeloma line. The S194/5.XXO.Bu.1 line was reported by Trowbridge, *J. Exp. Med.* 148(1):220-227, 1982.

- Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1

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proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promotes the fusion of cell membranes. Fusion methods have been described (Kohler *et al, supra*, 1975; Kohler *et al, supra*, 1976; Gefter *et al, Somatic Cell Genet.* 3:231-236, 1977; and Volk *et al, supra*, 1982). The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG).

Because fusion procedures produce viable hybrids at very low frequency (e.g. when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every 1×10^5 spleen cells), it is preferable to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary. Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the unfused myeloma cells, which normally would go on dividing indefinitely. The-somatic cells used in the fusion do not maintain long-term viability in *in vitro* culture and hence do not pose a problem. In the example of the present invention, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) were used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

Several weeks are required to selectively culture the fused cell hybrids. Early in this time period, it is necessary to identify those hybrids which produce the desired antibody, so that they may subsequently be cloned and propagated. Generally, around 10% of the hybrids obtained produce the desired antibody, although a range of from about 1 to about 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques as, for example, described in US Patent No. 6,056,957.

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Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal.

- 5 The injected animal will then develop tumors that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated *in vitro* in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody
10 can be harvested by decantation, filtration or centrifugation, and subsequently purified.

- The cell lines are tested for their specificity to detect the antigen of interest by any suitable immunodetection means. For example, cell lines can be aliquoted into a number of wells and incubated and the supernatant from each well is analyzed by enzyme-linked
15 immunosorbent assay (ELISA), indirect fluorescent antibody technique, or the like. The cell line(s) producing a monoclonal antibody capable of recognizing the target antigen but which does not recognize non-target epitopes are identified and then directly cultured *in vitro* or injected into a histocompatible animal to form tumors and to produce, collect and purify the required antibodies.

20

Thus, the present invention provides polyclonal and monoclonal antibodies which specifically interact with cancer cells and induce *inter alia* ADCC of the cancer cells or otherwise facilitate cell death or stasis.

- 25 The monoclonal antibody is then generally subjected to deimmunization means. Such a process may take any of a number of forms including the preparation of chimeric antibodies which have the same or similar specificity as the monoclonal antibodies prepared according to the present invention. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from
30 immunoglobulin variable and constant region genes belonging to different species. Thus, in accordance with the present invention, once a hybridoma producing the desired

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monoclonal antibody is obtained, techniques are used to produce interspecific monoclonal antibodies wherein the binding region of one species is combined with a non-binding region of the antibody of another species (Liu *et al*, *Proc. Natl. Acad. Sci. USA* 84:3439-3443, 1987). For example, the CDRs from a non-human (e.g. murine) monoclonal antibody can be grafted onto a human antibody, thereby "humanizing" the murine antibody (European Patent Publication No. 0 239 400, Jones *et al*, *Nature* 321:522-525, 1986; Verhoeyen *et al*, *Science* 239:1534-1536; 1988 and Richmann *et al*, *Nature* 332:323-327, 1988). In this case, the deimmunizing process is specific for humans. More particularly, the CDRs can be grafted onto a human antibody variable region with or without human constant regions. The non-human antibody providing the CDRs is typically referred to as the "donor" and the human antibody providing the framework is typically referred to as the "acceptor". Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e. at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized antibody, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. Thus, a "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A donor antibody is said to be "humanized", by the process of "humanization", because the resultant humanized antibody is expected to bind to the same antigen as the donor antibody that provides the CDRs. Reference herein to "humanized" includes reference to an antibody deimmunized to a particular host, in this case, a human host.

It will be understood that the deimmunized antibodies may have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions.

Exemplary methods which may be employed to produce deimmunized antibodies according to the present invention are described, for example, in Chou *et al*, *supra*, 1988; US Patent No. 6,056,957; Queen *et al* (US Patent No. 6,180,377); Morgan *et al* (US Patent No. 6,180,377), and Chothia *et al*, *J. Mol. Biol.* 196:901, 1987.

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Accordingly, particular agents contemplated for use with an ingenol angelate include those listed in Table 1. However, the present invention extends to any agent which:

5 (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

10 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils.
15

It is proposed that the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

20 Hence, the present invention contemplates a protocol of treating a subject with cancer or suspected of having cancer, said protocol comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

25

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of
30 cancer cells or is capable of stimulating or activating such T-cells;

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(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of
5 cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

10 The present invention further provides a method of treating a subject with cancer said method comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

15 (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

20

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of
25 cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

30 The amounts provided may be less than the individual amounts added together or may be additive. In either event, the therapeutic outcome is proposed to be more efficacious. This is referred to as its "anti-cancer efficacy" which includes one or more of cytotoxicity,

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apoptosis, necrosis, senescence or cell cycle arrest of cancer cells; cytotoxic T-cells capable of inducing T-cell-mediated inhibition or death of cancer cells or an agent capable of inducing same; an antibody specific for an antigen on a cancer cell or a combination of antigens predominately or exclusively on cancer cells; or neutrophils or an agent which
 5 induces neutrophil-facilitated antibody-dependent cellular cytotoxicity (ADCC) of cancer cells.

The "anti-cancer effect" includes cytotoxicity, necrosis, stasis, senescence, cell cycle arrest and ablation of cancer cells. This term also extends to antibodies, neutrophils and
 10 cytotoxic T-cells facilitating anti-cancer activity or agents which induce same.

The present invention also provides a therapeutic protocol for treating cancer in a subject, said protocol comprising administering an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and at least one other agent or its
 15 pharmaceutically acceptable salt wherein the combination of ingenol angelate and agent results in a combination index (CI) of less than 1 wherein the CI is determined using the equation:

$$CI = [(C)/(C_x)]_1 + [(C)/(C_x)]_2 + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2]$$

20

wherein $(C_x)_1$ is the concentration of one of ingenol angelate or agent to produce x percent effect of that drug alone, $(C)_1$ is the concentration of one of ingenol angelate or agent to produce the same x percent effect in combination with $(C)_2$ which is the other of the ingenol angelate or agent and α is a constant whereby if the mode of anti-cancer activity or
 25 efficacy of ingenol angelate and agent is mutually exclusive or non-exclusive, α is 0 or 1, respectively.

The ingenol angelate and agent may be administered separately either sequentially or simultaneously or formulated into a single composition which is administered to the
 30 subject. The ingenol-substituted ingenane and agent may have the same or different mode of administration such as both may be provided *via*, for example, intravenous, sub-

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cutaneous, topical, inter- or intra-lesional, intraperitoneal or intra-nasal administration or each may be provided *via* different routes of administration.

In a particularly preferred embodiment, the present invention provides a protocol of
5 treating a subject with cancer or suspected of having cancer, said protocol comprising administering to said subject an ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

10 (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

15

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of
20 cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the ingenol-3-angelate and agent is greater than either the ingenol-3-angelate or agent alone.

25 Another aspect of the present invention provides a method of treating a subject with cancer said method comprising administering to said subject an ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

30 (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

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(ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

5 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

10 wherein the anti-cancer activity or efficacy of the combination of the ingenol-3-angelate and agent is greater than either the ingenol-3-angelate or agent alone.

The present invention provides a therapeutic protocol for treating cancer in a subject, said
15 protocol comprising administering an ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and at least one other agent selected from the list in Table 1 wherein the combination of ingenol-3-angelate and agent selected results in a combination index (CI) of less than 1 wherein the CI is determined using the equation:

20
$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2]$$

wherein $(C_x)_1$ is the concentration of one of ingenol-3-angelate or agent to produce x percent effect of that drug alone, $(C)_1$ is the concentration of one of ingenol-3-angelate or agent to produce the same x percent effect in combination with $(C)_2$ which is the other of
25 the ingenol-3-angelate or agent and α is a constant whereby if the mode of anti-cancer activity of ingenol-3-angelate and agent is mutually exclusive or non-exclusive, α is 0 or 1, respectively.

In another embodiment, the present invention is directed to a formulation comprising an
30 ingenol angelate and an agent which has an anti-cancer effect, said formulation further comprising one or more pharmaceutically acceptable carriers, diluents and/or excipients.

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In particular, the present invention is directed to a formulation comprising an ingenol angelate and an agent which has one or more properties selected from:

- 5 (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;
- (ii) mediates T-cell (e.g. cytotoxic T-cell) mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;
- 10 (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;
- (iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;
- 15

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the ingenol angelate or agent alone,

- 20 said formulation further comprising one or more pharmaceutically acceptable carriers, diluents and/or excipients.

Still in another embodiment, the present invention provides a multi-part formulation, said formulation comprising at least one part comprising an ingenol angelate and at least one
25 other part comprising an agent. These parts or additional parts may further comprise at least one pharmaceutically acceptable carrier, diluent and/or excipient.

Reference to an anti-cancer effect or an anti-cancer treatment is not to be construed that the agent (or combination of angeloyl-substituted ingenol and agent) is selectively cytotoxic to
30 cancer cells alone although such selectivity is within the scope of the present invention.

The agent may be a chemically synthesized or isolated molecule or it may be a biological molecule such as an antibody or a cell such as a cytotoxic T-cell or other immune cell or it may be a vaccine to induce antibodies to cancer cells or it may be a molecule which induces any of the desired properties.

5

The phrase "pharmaceutically acceptable salt, derivative, homologs or analogs" is intended to convey any pharmaceutically acceptable tautomer, salt, pro-drug, hydrate, solvate, metabolite or other compound which, upon administration to the subject, is capable of providing (directly or indirectly) the compound concerned or a physiologically (e.g. analgesically) active compound, metabolite or residue thereof. An example of a suitable derivative is an ester formed from reaction of an OH or SH group with a suitable carboxylic acid, for example $C_{1-3}\text{alkyl-CO}_2\text{H}$, and $\text{HO}_2\text{C-(CH}_2)_n\text{-CO}_2\text{H}$ (where n is 1-10 such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, but preferably 1-4), and $\text{CO}_2\text{H-CH}_2\text{phenyl}$.

15 Thus, the active compounds may be in crystalline form, either as free compounds or as solvates (e.g. hydrates). Methods of solvation are generally known within the art.

The salts of the active compounds of the invention are preferably pharmaceutically acceptable, but it will be appreciated that non-pharmaceutically acceptable salts also fall within the scope of the present invention, since these are useful as intermediates in the preparation of pharmaceutically acceptable salts. Examples of pharmaceutically acceptable salts include salts of pharmaceutically acceptable cations such as sodium, potassium, lithium, calcium, magnesium, ammonium and alkylammonium; acid addition salts of pharmaceutically acceptable inorganic acids such as hydrochloric, orthophosphoric, sulfuric, phosphoric, nitric, carbonic, boric, sulfamic and hydrobromic acids; or salts of pharmaceutically acceptable organic acids such as acetic, propionic, butyric, tartaric, maleic, hydroxymaleic, fumaric, citric, lactic, mucic, gluconic, benzoic, succinic, oxalic, phenylacetic, methanesulphonic, trihalomethanesulphonic, toluenesulphonic, benzenesulphonic, salicylic, sulphanilic, aspartic, glutamic, edetic, stearic, palmitic, oleic, lauric, pantothenic, tannic, ascorbic and valeric acids.

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The term "pro-drug" is used herein in its broadest sense to include those compounds which can be converted *in vivo* to the compound of interest (e.g. by enzymatic or hydrolytic cleavage). Examples thereof include esters, such as acetates of hydroxy or thio groups, as well as phosphates and sulphonates. Processes for acylating hydroxy or thio groups are known in the art, e.g. by reacting an alcohol (hydroxy group), or thio group, with a carboxylic acid. Other examples of suitable pro-drugs are described in *Design of Prodrugs*, H. Bundgaard, Elsevier, 1985, the disclosure of which is included herein in its entirety by way of reference.

10 The term "metabolite" includes any compound into which the active agents can be converted *in vivo* once administered to the subject. Examples of such metabolites are glucuronides, sulphates and hydroxylates.

It will be understood that active agents as described herein may exist in tautomeric forms.

15 The term "tautomer" is used herein in its broadest sense to include compounds capable of existing in a state of equilibrium between two isomeric forms. Such compounds may differ in the bond connecting two atoms or groups and the position of these atoms or groups in the compound. A specific example is keto-enol tautomerism.

20 The compounds of the present invention may be electrically neutral or may take the form of polycations, having associated anions for electrical neutrality. Suitable associated anions include sulfate, tartrate, citrate, chloride, nitrate, nitrite, phosphate, perchlorate, halosulfonate or trihalomethylsulfonate.

25 The active agents may be administered for therapy by any suitable route. Suitable routes of administration may include oral, rectal, nasal, inhalation of aerosols or particulates, topical (including buccal and sublingual), transdermal, vaginal, intravesical, intralesional and parenteral (including subcutaneous, intramuscular, intravenous, intrasternal, intrathecal, epidural and intradermal).

Reference herein to "cancer" includes solid and blood borne cancers, leukemias, sarcomas and carcinomas. Examples of cancers which may be treated using the protocol of the present invention include but are not limited to ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute lymphocytic leukemia, acute myeloid leukemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancers, cutaneous T-cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynecological cancers, hematological malignancies, hairy cell leukemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, li-fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer (NMSC), non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile

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cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, rothmund-thomson syndrome, salivary gland cancer, sarcoma, schwannoma, sezary syndrome, skin cancer, small cell lung cancer (SCLC), small intestine
5 cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis/-ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia or Wilms' tumor. The terms
10 "cancer" and "tumor" may be used interchangeably throughout the subject specification. A "neoplastic disorder" is also a form of cancer or tumor.

The present invention also relates to compositions comprising an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof, and an agent
15 together with one or more pharmaceutically acceptable additives and optionally other medicaments. The pharmaceutically acceptable additives may be in the form of carriers, diluents, adjuvants and/or excipients and may include all conventional solvents, dispersion agents, fillers, solid carriers, coating agents, antifungal or antibacterial agents, dermal penetration agents, surfactants, isotonic and absorption agents and slow or controlled
20 release matrices. The active agents may be presented in the form of a kit of components adapted for allowing concurrent, separate or sequential administration of the active agents. Each carrier, diluent, adjuvant and/or excipient must be "pharmaceutically acceptable" in the sense of being compatible with the other ingredients of the composition and physiologically tolerated by the subject. The compositions may conveniently be presented
25 in unit dosage form and may be prepared by methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier, which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers, diluents, adjuvants and/or excipients or finely divided solid carriers or
30 both, and then if necessary shaping the product.

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Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous phase or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. inert diluent, preservative disintegrant, sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Compositions suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the composition isotonic with the blood of the intended subject; and aqueous and non-aqueous sterile suspensions which may include suspended agents and thickening agents. The compositions may be presented in a unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

30

Compositions suitable for topical administration to the skin, i.e. transdermal

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administration, may comprise the active agents dissolved or suspended in any suitable carrier or base and may be in the form of lotions, gels, creams, pastes, ointments and the like. Suitable carriers may include mineral oil, propylene glycol, waxes, polyoxyethylene and long chain alcohols. Transdermal devices, such as patches may also be used and may
5 comprise a microporous membrane made from suitable material such as cellulose nitrate/acetate, propylene and polycarbonates. The patches may also contain suitable skin adhesive and backing materials.

The active compounds of the present invention may also be presented as implants, which
10 may comprise a drug bearing polymeric device wherein the polymer is biocompatible and non-toxic. Suitable polymers may include hydrogels, silicones, polyethylenes and biodegradable polymers.

The compounds of the subject invention may be administered in a sustained (i.e.
15 controlled) or slow release form. A sustained release preparation is one in which the active ingredient is slowly released within the body of the subject once administered and maintains the desired drug concentration over a minimum period of time. The preparation of sustained release formulations is well understood by persons skilled in the art. Dosage forms may include oral forms, implants and transdermal forms. For slow release
20 administration, the active ingredients may be suspended as slow release particles or within liposomes, for example.

The pharmaceutical compositions of the present invention may be packaged for sale with other active agents or alternatively, other active agents may be formulated with the ingenol
25 angelate and agent or their pharmaceutical salts, derivatives, homologs or analogs thereof.

Thus, a further particular aspect of the present invention provides a system for the controlled release of both active agents or at least one of the active agents or a pharmaceutically acceptable salt, derivative, homolog or analog thereof wherein the
30 system comprises:

- (a) a deposit-core comprising an effective amount of the active substance and

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having defined geometric form, and

(b) a support-platform applied to the deposit-core, wherein the deposit-core contains at least the active substance, and at least one member selected from the group consisting of:

- 5 (i) a polymeric material which swells on contact with water or aqueous liquids and a gellable polymeric material wherein the ratio of the swellable polymeric material to the gellable polymeric material is in the range 1:9 to 9:1, and
- (ii) a single polymeric material having both swelling and gelling properties, and wherein the support-platform is an elastic support, applied to the
- 10 deposit-core so that it partially covers the surface of the deposit-core and follows changes due to hydration of the deposit-core and is slowly soluble and/or slowly gellable in aqueous fluids.

The support-platform may comprise polymers such as hydroxypropylmethylcellulose, plasticizers such as a glyceride, binders such as polyvinylpyrrolidone, hydrophilic agents

15 such as lactose and silica, and/or hydrophobic agents such as magnesium stearate and glycerides. The polymer(s) typically make up 30 to 90% by weight of the support-platform, for example about 35 to 40%. Plasticizer may make up at least 2% by weight of the support platform, for example about 15 to 20%. Binder(s), hydrophilic agent(s) and

20 hydrophobic agent(s) typically total up to about 50% by weight of the support platform, for example about 40 to 50%.

The tablet coating may contain one or more water insoluble or poorly soluble hydrophobic excipients. Such excipients may be selected from any of the known hydrophobic cellulosic

25 derivatives and polymers including alkylcellulose, e.g. ethylcellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, carboxymethyl cellulose, and derivatives thereof; polymethacrylic polymers, polyvinyl acetate and cellulose acetate polymers; fatty acids or their esters or salts; long chain fatty alcohols; polyoxyethylene alkyl ethers; polyoxyethylene stearates; sugar esters; lauroyl macrogol-32 glyceryl, stearyl macrogol-

30 32 glyceryl, and the like. Hydroxypropylmethyl cellulose materials are preferably selected from those low Mw and low viscosity materials such as E-Type methocel, and 29-10 types

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as defined in the USP.

Other agents or excipients that provide hydrophobic quality to coatings may be selected from any waxy substance known for use as tablet excipients. Preferably they have a HLB
5 value of less than 5, and more preferably about 2. Suitable hydrophobic agents include waxy substances such as carnauba wax, paraffin, microcrystalline wax, beeswax, cetyl ester wax and the like; or non-fatty hydrophobic substances such as calcium phosphate salts, e.g. dibasic calcium phosphate.

10 Preferably the coating contains a calcium phosphate salt, glyceryl behenate, and polyvinyl pyrrolidone, or mixtures thereof, and one or more adjuvants, diluents, lubricants or fillers.

Preferred components in the coating are as follows, with generally suitable percentage amounts expressed as percentage weight of the coating.

15

Polyvinyl pyrrolidone (Povidone) is preferably present in amounts of about 1 to 25% by weight or the coating, more particularly 4 to 12%, e.g. 6 to 8%.

Glyceryl behenate is an ester of glycerol and behenic acid (a C22 fatty acid). Glyceryl
20 behenate may be present as its mono-, di-, or tri-ester form, or a mixture thereof. Preferably it has an HLB value of less than 5, more preferably approximately 2. It may be present in amounts of about 5 to 85% by weight of the coating, more particularly from 10 to 70% by weight, and in certain preferred embodiments from 30 to 50%.

25 Calcium phosphate salt may be the dibasic calcium phosphate dihydrate and may be present in an amount of about 10 to 90% by weight of the coating, preferably 20 to 80%, e.g. 40 to 75%.

The coating may contain other common tablet excipients such as lubricants, colorants,
30 binders, diluents, glidants and taste-masking agents or flavorants.

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Examples of excipients include colorants such a ferric oxide, e.g. yellow ferric oxide; lubricants such as magnesium stearate; and glidants such as silicon dioxide, e.g. colloidal silicon dioxide. Yellow ferric oxide may be used in amounts of about 0.01 to 0.5% by weight based on the coating; magnesium stearate may be present in amounts of 1 to 20%
5 by weight of the coating, more preferably 2 to 10%, e.g. 0.5 to 1.0% ; and colloidal silica may be used in amounts of 0.1 to 20% by weight of the coating, preferably 1 to 10%, more preferably 0.25 to 1.0%.

The core comprises in addition to a drug substance, a disintegrating agent or mixtures of
10 disintegrating agents used in immediate release formulations and well know to persons skilled in the art. The disintegrating agents useful in the exercise of the present invention may be materials that effervesce and or swell in the presence of aqueous media thereby to provide a force necessary to mechanically disrupt the coating material.

15 Preferably a core contains, in addition to the drug substance, cross-linked polyvinyl pyrrolidone and croscarmellose sodium.

The following is a list of preferred core materials. The amounts are expressed in terms of percentage by weight based on the weight of the core.

20

Cross-linked polyvinyl pyrrolidone is described above and is useful as a disintegrating agent, and may be employed in the core in the amounts disclosed in relation to the core.

Croscarmellose sodium is an internally cross-linked sodium carboxymethyl cellulose (also
25 known as Ac-Di-Sol) useful as a disintegrating agent.

Disintegrating agents may be used in amounts of 5 to 30% by weight based on the core. However, higher amounts of certain disintegrants can swell to form matrices that may modulate the release of the drug substance. Accordingly, particularly when rapid release is
30 required after the lag time it is preferred that the disintegrants is employed in amounts of up to 10% by weight, e.g. about 5 to 10% by weight.

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The core may additionally comprise common tablet excipients such as those described above in relation to the coating material. Suitable excipients include lubricants, diluents and fillers, including but not limited to lactose (for example, the mono- hydrate), ferric
5 oxide, magnesium stearates and colloidal silica.

Lactose monohydrate is a disaccharide consisting of one glucose and one galactose moiety. It may act as a filler or diluent in the tablets of the present invention. It may be present in a range of about 10 to 90%, preferably from 20 to 80%, and in certain preferred
10 embodiments from 65 to 70%.

As stated above, it is an important aspect of the present invention that core is correctly located within the coating to ensure that a tablet has the appropriate coating thickness.

15 In this way, lag times will be reliable and reproducible, and intra-subject and inter- subject variance in bioavailability can be avoided.

In another embodiment, a multi-particulate release ingenol angelate/agent composition for oral administration is provided. The formulation is made by complexing the active agents
20 or a pharmaceutically acceptable salt, derivative, homolog or analog thereof optionally with an ion-exchange resin in the form of small particles, typically less than 150 microns. To prepare a multi-particulate formulation, one or more of the following types of particles are formulated into a final dosage form: (a) immediate release particles, prepared by coating drug-containing particles with a polymer that is insoluble in the neutral medium of
25 saliva, but dissolves in the acid environment of the stomach; (b) enteric coated particles, prepared by coating drug-containing particles with a polymer that is insoluble in the acidic environment of the stomach but dissolves in the neutral environment of the small intestines; (c) extended release particles, prepared by coating drug-containing particles with a polymer that forms water insoluble but water permeable membrane; (d) enteric
30 coated-extended release particles, prepared by coating extended release drug particles with an enteric coating; (e) delayed release particles, prepared by coating drug-containing

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particles with a polymer that is insoluble in the acidic environment of the stomach and the environment of the upper small intestines, but dissolves in the lower small intestines or upper large intestines.

5 For the treatment of cancer, the formulations may also include compounds which assist in reducing resistance to the therapeutic agent. Examples of such compounds are those which inhibit P-glycoprotein or other cell mechanisms which are involved in excluding intracellular accumulation of drugs.

10 The formulation may also contain carriers, diluents and excipients. Details of pharmaceutically acceptable carriers, diluents and excipients and methods of preparing pharmaceutical compositions and formulations are provided in Remington's *Pharmaceutical Sciences* 18th Edition, 1990, Mack Publishing Co., Easton, Pennsylvania, USA.

15

Hence, the present invention contemplates the use of an angeloyl-substituted ingenane such as PEP005 and an agent such as an agent listed in Table 1 which includes antibodies, cancer antigens and T-cells (e.g. cytotoxic T-cells) or other immune cells to treat a subject with cancer. The "treatment" may result in cancer cell death or it may arrest cancer cell growth or help prevent metastasis and/or induce immunological memory against cancer. The treatment may, therefore, result in cancer cell death, necrosis, cytotoxicity, senescence, cell cycle arrest or stasis.

20

The present invention will now be further described with reference to the following examples, which are intended for the purpose of illustration only and are not intended to limit the generality of the subject invention as hereinbefore described.

25

In the Examples, the following materials and methods are employed:

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Cell Lines

- All cell lines were obtained from the ATCC (Rockville, MD, USA). Cells were grown as monolayers in RPMI, DMEM or McCoy's medium supplemented with 10% v/v fetal calf serum (Invitrogen, Cergy-Pontoise, France), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All cells were split twice a week using trypsin/EDTA (0.25% w/v and 0.02% w/v, respectively Invitrogen, Cergy-Pontoise, France) and seeded at a concentration of 2.5×10^4 cells/ml. All cell lines were tested regularly for *Mycoplasma* contamination by PCR using a Stratagene kit (La Jolla, CA).
- Twelve cell lines were analyzed and their characteristics are summarized in Table 3 (data from NCI cell line screen).

TABLE 3

Cell line	PEP005	p53	MDR	MMR	HER2/neu	EGFR
Colon						
HT29	R	mut	Low	High	Low	High
HCT116	R	mut	Low	Low	Low	High
COLO205	S	mut	Low	High	High	High
HCC2998	S	mut	Low	Low	High	High
Breast						
MCF7	R	wt	Low	Low	Low	Low
MDA-MB-435	S	mut	Low	Low	High	Low
Ovarian						
OVCAR3	R	mut	Low	High		High
IGROV1	R	wt	Low	Low	High	High
Lung						
Hop62	S	mut	High	High	Low	Low
Hop92	R	mut	Low	Low	High	High
Leukemia						
K562	R	mut	Low	High	Low	Low
CCRF-CEM	S	mut	High	Low	Low	Low

wt: wild-type; mut: mutation

Drug

Purified PEP005 (ingenol-3-angelate) was supplied by Peplin Limited (Brisbane, Australia). A 60 mM stock solution was prepared in DMSO and stored in the dark at 20°C.

***In vitro* growth inhibition assay (MTT assay)**

The MTT assay was carried out as previously described (Hansen *et al*, *J Immunol Methods* 119(2):203-210, 1989). In brief, cells were seeded in 96-well tissue culture plates at a density of 2×10^3 cells/well. Cell viability was determined after 120 hours incubation by the colorimetric conversion of yellow, water-soluble tetrazolium MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, Saint-Quentin Fallavier, France), into purple, water-insoluble formazan. This reaction is catalyzed by mitochondrial dehydrogenases and is used to estimate the relative number of viable cells (Mosmann, *J. Immunol. Methods* 65(1-2):55-63, 1983). Cells were incubated with 0.4 mg/ml MTT for 4 hours at 37°C. After incubation, the supernatant was discarded, the cell pellet was resuspended in 0.1 ml of DMSO and the absorbance was measured at 560 nm by use of a microplate reader (Dynatech, Michigan). Wells with untreated cells or with drug-containing medium without cells were used as positive and negative controls, respectively. Growth inhibition curves were plotted as a percentage of untreated control cells.

15

Single agent study

The cells were seeded at 2×10^3 cells/well in 96-well plates and treated 24 hours later with increasing concentrations of PEP005. After 1 hour, 24 hours or 48 hours incubation the cells were washed and post-incubated in drug-free medium for 72 hours. Growth inhibition were then determined by the MTT assay.

20

Simultaneous exposure of PEP005 with other drugs

For simultaneous drug exposure, cells were seeded at 2×10^3 cells/well in 96-well plates and treated 24 hours later with increasing concentrations of PEP005 alone or with another drug in various concentrations corresponding to the IC_{20} , IC_{40} or IC_{60} values. After approximately four doubling times (120 hours), the growth inhibitory effect were measured by the MTT assay.

25

Sequential exposure to PEP005 and other drugs

Cells were seeded at 2×10^3 cells/well in 96-well plates and allowed to grow for 24 hours. Cells were then exposed to various concentrations of the first drug for 1 hour/24 hours/48

30

- 60 -

hours, the drug was removed, the cells were washed and the second drug was added. After an additional drug exposure, the second drug was removed, the cells were washed and post-incubated in drug-free medium for 72 hours. Growth inhibition was then determined by the MTT assay.

5

Statistical analysis and determination of synergistic activity

Effects of drug combinations were evaluated using the Chou and Talalay method which is based on the median-effect principle (Chou and Talalay, *Adv Enzyme Regul* 22:27-55, 1984). This involves plotting dose-effect curves for each drug and for multiple diluted, fixed-ratio combinations, using the equation: $f_a/f_u = (C/C_m)^m$, where C is the drug concentration, IC_{50} the concentration required for a half-maximal effect (i.e. 50% inhibition of cell growth), f_a the cell fraction affected by the drug concentration C (e.g. 0.9 if cell growth is inhibited by 90%), f_u the unaffected fraction, and m the sigmodicity coefficient of the concentration-effect curve. On the basis of the slope of the curve for each drug in a combination, it can be determined whether the drugs have mutually non-exclusive effects (e.g. independent or interactive modes of action). The combination index (CI) is then determined by the equation:

20

$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2],$$

25

30

where $(C_x)_1$ is the concentration of drug 1 required to produce an x percent effect of that drug alone, and $(C)_1$, the concentration of drug 1 required to produce the same x percent effect in combination with $(C)_2$. If the mode of action of the drugs is mutually exclusive or non-exclusive, then α is 0 or 1, respectively. CI values are calculated by solving the equation for different values of f_a (i.e. for different degrees of cell growth inhibition). CI values of <1 indicate synergy, the value of 1 indicates additive effects, and values >1 indicate antagonism. Data were analyzed on an IBM-PC computer using concentration-effect analysis for microcomputer software (Biosoft, Cambridge, UK). For statistical analysis and graphs Instat and Prism software (GraphPad, San Diego, USA) were used. The dose-effect relationships for the drugs tested, alone or in paired combinations, were subjected to median-effect plot analysis to determine their relative potency (IC_{50}), shape

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(m), and conformity (r) in each selected cell line. As stated above, the IC_{50} and m values were respectively used to calculate synergism and antagonism on the basis of the CI equation. Results were expressed as the mean $\pm$ standard deviation of at least three experiments performed in duplicate. In each experiment, cells were exposed to the paired combinations for 38 hours as described above. Means and standard deviations were compared using Student *t*-test (two-sided *p* value).

Cell cycle analysis and apoptosis

Cell cycle analysis and the measurement of the percentage of apoptotic cells were assessed by flow cytometry. In brief, cells were seeded onto 25 cm³ flasks and not treated or treated with various concentrations of PEP005. At various time-points adherent and non-adherent cells were recovered, washed with PBS, fixed in 70% v/v ethanol and stored at +4°C until use. Cells were dehydrated in PBS, incubated for 20 minutes at room temperature with 250 µg/ml RNase A with Triton X-100 and 20 minutes at +4°C with 50 µg/ml propidium iodide in the dark. The cell cycle distribution and percentage of apoptotic cells were determined with FACScan flow cytometer.

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EXAMPLE 1***Single agent study***

Figure 1 shows the effects of time course experiments with PEP005 given for 1 hour, 24
 5 hours and 48 hours in a panel of cell lines. Based on data, 48 hours exposure was selected
 for further evaluations of PEP005 in our panel of cancer cell lines. This duration of
 exposure was shown to be optimal to observe the antiproliferative effects of PEP005 in the
 most sensitive human cancer cell lines. Each point is the average of at least three
 individual experiments each done in duplicate. PEP005 displayed cytotoxic effects against
 10 human colon COLO205 and HCC2998 cells, breast cancer cell line MDA-MB-435 and
 COLO205 being the most sensitive.

IC₅₀s for 48 hours exposure are shown in Table 4.

15

TABLE 4

Cell Line	IC₅₀ (μM)
COLO205	0.01 ± 0.002
MDA435	2.6 ± 0.52
HCC2998	30 ± 6
HOP92	85 ± 17
HOP62	110 ± 22
HCT116	120 ± 24
HT29	140 ± 28
MCF7	180 ± 36
OVCAR3	200 ± 40
IGROV1	200 ± 42

As a control, leukemia cell lines were used that were previously shown to be sensitive to
 PEP005.

20

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PEP005 has been shown to inhibit K562 and CCRF-CEM leukemia cell lines survival (Figure 2) with IC_{50} s $0,006 \pm 0,001 \mu M$ and $60 \pm 14 \mu M$, respectively.

Two other schedules of PEP005 administration were tested: 3 hours for three days and 3 hours for five days. The results of these experiments have shown an increase of the sensitivity of COLO205 and MDA under some conditions. IC_{50} for 3 hours x three days were 0.1 (COLO205) and 1 μM (MDA). For 3 hours x five days they were 0.002 and 0.0015 μM for COLO205 and MDA respectively. These two administration modalities do not change the sensitivity profile to PEP005 of other cell lines.

10

EXAMPLE 2

Changes of cell cycle and apoptosis induction of PEP005 in COLO205 cells

The effect of PEP005 on cell cycle progression was studied by flow cytometry which allowed the assessment of fragmented DNA which elutes from apoptotic cells when permeabilized with PI. In a time course experiment, COLO205 cells were incubated with 0.03 μM , 0.3 μM and 1 μM PEP005 for 1 hour, 5 hours, 12 hours, 24 hours and 48 hours. Constant PEP005 exposure led to a dose dependent accumulation of cells in G0-G1 phase with an almost complete block of the phase S (Figure 3).

20

PEP005 effects also resulted in a progressive generation of particles corresponding to hypoploid DNA content (sub-G1 fraction) that was considered characteristic of apoptosis induction. Quantification of the apoptotic fraction is plotted as percentage of total cell number (Figure 4). These results were confirmed by AnnexinV-staining. After 24 hours of PEP005 treatment no more apoptosis but necrosis was shown.

25

EXAMPLE 3***Comparative study of PEP005 cytotoxicity with other anti-cancer drugs***

Figure 5 shows the comparative analysis of PEP005 cytotoxicity with oxaliplatin, cisplatin,
5 doxorubicin, gemcitabine, ara-C and 5FU in a panel of cell lines. Results are presented as
(IC₅₀- IC₅₀ average).

EXAMPLE 4***Combination agent studies***

10

The effect of sequential and simultaneous exposure to PEP005 with oxaliplatin, cisplatin,
doxorubicin, docetaxel, gemcitabine, vinorelbine and 5FU was determined using
combination indexes that represent an affected fraction for the concentration of drugs
corresponding to IC₅₀. Calculation of a combination index below 0.8 indicates synergy,
15 above 1.2 antagonism while a combination index between 0.8 and 1.2 corresponds to an
additive effect.

EXAMPLE 5***5FU-PEP005 combinations***

20

In COLO205, the combination of PEP005 and 5FU leads to various degrees of synergistic
activity (Figure 6) when 5FU administered before or after PEP005. Each spot represents
one experiment performed in triplicate. 5FU given either prior or after PEP005 led to
combination index values <1 demonstrating synergistic effects between those two
25 compounds.

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EXAMPLE 6***Docetaxel-PEP005 combinations***

As shown in Figure 11, additive and synergistic effects were observed when docetaxel was
5 given before or after PEP005. Antagonistic effects were observed when docetaxel was
given concomitant with PEP005.

EXAMPLE 7***Doxorubicin-PEP005 combinations***

10

Synergy is obtained in immediate sequential exposition. The other schedules are mostly
antagonistic (Figure 7).

EXAMPLE 8

15

Oxaliplatin-PEP005 combinations

PEP005 combination with oxaliplatin (concomitant or immediately sequential exposition)
is antagonistic (Figure 8A). This antagonism between oxaliplatin and PEP005 is reversed
if COLO205 is exposed to oxaliplatin 24 to 72 hours after PEP005, in which case the
20 sequence is highly synergistic (Figure 8B).

EXAMPLE 9***Gemcitabine-PEP005 combinations***

25 The sequence gemcitabine followed by PEP005 is synergistic contrasting with an
antagonism when the drugs are administered in a concomitant setting or PEP005 before
gemcitabine. This antagonism is reversed if gemcitabine is administered 24 hours after the
end of PEP005 exposition, in this case the combination is synergistic with a $CI < 1$ for
most concentrations (Figure 9).

30

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EXAMPLE 10

Combinations Vinorelbine-PEP005

This combination reproduced the same results observed when PEP005 is combined with
5 docetaxel. Synergistic activity is obtained with the sequence vinorelbine followed by
PEP005. All the other schedules are antagonistic (Figure 10)

EXAMPLE 11

Cisplatin-PEP005 combinations

10

Contrasting with oxaliplatin; the antagonism observed in concomitant or immediately
sequential expositions is maintained with a 24 hours washout between the two drugs. The
results are shown in Figure 12.

15

EXAMPLE 12

Anti-cancer effects of PEP005 in combination with other agents

The above Examples showed that PEP005 displayed an anti-proliferative effect in human
colon COLO205 and breast MDA435 cancer cells. In human sensitive cancer cells, the
20 anti-proliferative effects of PEP005 was associated with cell cycle changes (diminution of
cells in S phase), apoptosis associated with caspase 3 activation and cellular necrosis.
These effects seem to be optimal between 24 and 48 hours of exposure to the drug.

PEP005 displays a unique cytotoxic profile. Combination with other anti-cancer agents
25 showed additive and/or synergistic effects with several classical cytotoxic drugs such as
5FU, doxorubicin, oxaliplatin, gemcitabine, vinorelbine, docetaxel, and cisplatin. Some
evidence of schedule dependency were observed. This suggested that one drug might
somehow protect from cytotoxic effects of another. Interestingly, considering cell cycle
blockage induced by PEP005, consideration was also given in the experiment to introduce
30 a lag time of 24 hours between the administrations of PEP005. It was observed that in
most circumstances, schedules in which PEP005 was separated by 24 hours from the

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administration of the second drug appear more efficient to induce cytotoxic effects. Although preliminary, time/schedule dependency for PEP005-combinations needs clearly to be explored.

5

EXAMPLE 13***Anti-cancer effects of PEP005 in combination with various chemotherapeutic agents***

In this Example, the combined application (given simultaneously) of PEP005 with three (on cell line U937) or four (on cell line K562) chemotherapeutic agents was investigated.
10 Chemotherapeutic agents were applied at 10 different concentrations in half-log increments and PEP005 was added simultaneously at two fixed concentrations, respectively.

The study design is shown in Table 5.

15

TABLE 5***Study design***

Cell Line	PEP005[ng/ml]	Standard agents (10 conc. Half-log increments)
U937 (lymphoma)	3, 10	Daunocrubicin, Cytarabine, Etoposide
K562 (CML)	0.3, 1, 3	Adriamycin, activated Cyclophosphosphamide, Vincristine, Etoposide

20 The objective of this Example was to investigate the *in vitro* anti-cancer activity of PEP005 combined with various chemotherapeutic agents in the lymphoma cell line U937 and the chronic myeloid leukemia (CML) cell line K562.

The two cell lines were grown at 37°C in a humidified atomosphere (95% v/v air, 5% v/v
25 CO₂) in RPMI 1640 medium (PAA, Germany) supplemented with 10% v/v fetal calf serum (PAA, Germany) and 0.1 mg/ml gentamicin (Invitrogen, Karlsruhe, Germany). Cells were routinely passaged once weekly. They were maintained no longer than 20

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passages in culture.

Both cell lines were purchased from ATCC (Rockville, USA). U937 was established from a patient with histiocytic lymphoma (Sundstrom and Nilsson, *Int. J. Cancer* 17:565-577, 1976). The continuous cell line K562 was established by Lozzio and Lozzio from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises (Lozzio and Lozzio, *Blood* 45:321-334, 1975).

A modified propidium iodide (PI) assay (Dengler *et al*, *Anticancer Drugs* 6:522-532, 1995) was used to assess the effects of PEP005 on the chemotherapeutic agents' activity in the two test cell lines. Briefly, cells were harvested from exponential phase cultures by centrifugation, counted and plated in 96 well flat-bottomed microtiter plates at a cell density of 20,000 cells/well. After a 24 hour recovery period to allow the cells to resume exponential growth, 10 μ l of culture medium (six control wells/plate) and culture medium containing the chemotherapeutic agents (six wells/concentration) were added to the cells. Immediate thereafter, 10 μ l of culture medium containing PEP005 at one fix concentration was added. On each plate PEP005 alone (at the fix concentration) is given in six wells. After that cells were incubated continuously over a period of four days.

Thereafter, 50 μ l of an aqueous PI solution was added to the wells (final PI concentration of 7 μ g/ml). After a 30 minute incubation period, fluorescence (FU1) was measured using the Cytofluor 4000 microplate reader (excitation 530 nm, emission 620 nm) giving a direct measurement of dead cells. Microplates were then kept at -20°C for 24 hours resulting in a total cell kill. After thawing of the plates and a second fluorescence measurement (FU2), the amount of viable cells was calculated by subtraction of FU1 from FU2.

In each experiment, treated groups (PEP005 alone as well as the combinations) and untreated control cells were determined in sextuplicate. For calculations the mean of sextuplicate data was used. Growth inhibition was expressed as $\text{Test/Control} \times 100$ (%T/C). Based on the T/C values IC_{50} and IC_{70} values, being the drug concentrations necessary to inhibit cell growth by 50% (T/C=50%) and 70% (T/C=30%), respectively,

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were determined by plotting compound concentration *versus* cell viability (T/C), and IC-values were calculated based on the principle of point-to-point curve fit. To illustrate effects on dose-response relation, concentration-response curves of the chemotherapeutic agent alone and the combined application with the two fixed PEP005 concentrations were
5 fitted together in one graph.

PEP005 was provided by Peplin Limited, Brisbane, Australia, as a dry powder. A stock solution of 10 mg/ml was prepared in DMSO and aliquots were stored at -20°C. An aliquot of the stock solution was thawed on the day of use and stored at room temperature
10 prior to and during dosing. Intermediate dilution steps were carried out using RPMI 1640 cell culture medium and resulted in solutions of 15-fold the final concentration. For the final dilution step (1:15), 10 µl of these solutions were directly added in wells to the 140 µl medium on top of the cultured test cells.

15 Daunorubicin (#D8809), Cytarabine (#C1768), Etoposide (E1383) and Vincristine (V8879) were supplied by Sigma (Taufkirchen, Germany). 4-Hydroperoxy-cyclophosphamid (activated cyclophosphamide) was supplied by Asta Medica (Frankfurt, Germany) and Adriamycin was used as the clinical preparation from the pharmacy (Medac, Hamburg, Germany).

20

The results of the combination studies are summarized in Table 6 (cell line U937) and Table 7 (cell line K562).

The cell line U937 was treated with PEP005 in combination with the Topoisomerase II
25 inhibitors Daunorubicin and Etoposide (VP16), as well as the DNA damaging agent Cytarabine. Table 6 shows the results of the three independent experiments. PEP005 was applied at a fix concentration of 3 and 10 ng/ml, respectively. The effect of PEP005 alone was in the range of T/C= 62 % to T/C = 89%. Positive interactions of PEP005 were detected with Cytarabine and VP16. PEP005 at a concentration of 10 ng/ml effected a
30 reduction of the IC₇₀ value from 0.74 µg/ml (Cytarabine alone) down to 0.037 µg/ml (reduction to 5% compared to Cytarabine alone) in experiment GF578, from 0.24 µg/ml

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- down to 0.074 $\mu\text{g/ml}$ (reduction to 31%) in the second experiment (GF624), and from 0.3 $\mu\text{g/ml}$ down to 0.173 $\mu\text{g/ml}$ (58%) in the third experiment (GF769). PEP005 effected a shift of the concentration-response curve to the left, resulting in decrease of IC_{50} and IC_{70} values. The combination of PEP005 at a concentration of 10 ng/ml with VP16 resulted in
- 5 a reduction of IC_{70} value from 1.67 $\mu\text{g/ml}$ (VP16 alone) down to 0.24 $\mu\text{g/ml}$ (reduction of IC_{70} value down to 14%) in experiment GF578, from 1.15 $\mu\text{g/ml}$ to 0.19 $\mu\text{g/ml}$ (reduction to 17%) in the second experiment (GF624), and from 2.22 $\mu\text{g/ml}$ to 1.32 $\mu\text{g/ml}$ (reduction to 59%) in the third experiment (GF769).
- 10 No significant positive interaction was found in the CML cell line K562 (Table 7). K562 cells were treated with PEP005 in combination with the chemotherapeutic agents Adriamycin (Topoisomerase II inhibitor), activated Cyclophosphamide (alkylating agent), Vincristine (tubulin binder) and VP16 (Topoisomerase II inhibitor). Three independent experiments were performed and PEP005 alone effected Test/Control values between 79%
- 15 and 108%. Significant reduction of IC_{70} values (to less than 50% of IC_{70} value of the chemotherapeutic agent alone) by PEP005 were not detected in this cell line.

This Example demonstrated that PEP005 caused a marked increase of anti-tumor activity *in vitro* in combination with Cytarabine and VP16 in the lymphoma cell line U937.

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TABLE 6
In vitro anti-cancer activity of PEP005 in combination with 3 chemotherapeutic agents in the lymphoma cell line U937

Cell line	exp. no.	PEP005 [ng/ml]	IC70 [µg/ml] of st. agent alone or of PEP005 in comb. with							
			PEP ¹⁾	+ Dauno		PEP ¹⁾	+ Cytara		PEP ¹⁾	+ VP16
U937	GF578	-		0,024			0,740			1,67
(lymphoma)	GF578	3	74	0,043	-	60	0,125	++	76	4,93 -
	GF578	10	67	0,026	-	62	0,037	+++	70	0,24 ++
U937	GF624	-		0,027			0,241			1,15
(lymphoma)	GF624	3	89	0,040	-	81	0,084	+	89	0,30 ++
	GF624	10	62	0,009	+	66	0,074	+	70	0,19 ++
U937	GF769			0,028			0,300			2,22
(lymphoma)	GF769	3	81	0,030	-	85	1,316	-	87	2,28 -
	GF769	10	73	0,033	-	74	0,173	-	79	1,32 -
Total positive interaction				1/3		2/3		2/3		

1) T/C[%] of PEP005 alone

5

Evaluation positive interaction between PEP005 and chemotherapeutic agents:

-, IC₇₀ (combination) >50% of IC₇₀ chemotherapeutic agent alone

+, IC₇₀ (combination) ≤50% of chemotherapeutic agent alone

++, IC₇₀ (combination) ≤30% of chemotherapeutic agent alone

10 +++ , IC₇₀ (combination) ≤10% of chemotherapeutic agent alone

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TABLE 7

In vitro anti-cancer activity of PEP005 in combination with 4 chemotherapeutic agents in the cell line K562 (chronic myeloid leukaemia ,CML)

Cell line	exp.no.	PEP005 [ng/ml]	IC70 [μ g/ml] of st. agent alone or of PEP005 in comb. with							
			PEP ¹⁾	+ADR	PEP ¹⁾	+CYACT	PEP ¹⁾	+VCR	PEP ¹⁾	+VP16
K562 (CML)	GF751			0,222		21,6		0,045		n.e.
	GF751	0,3	96	0,219 -	86	13,6 -	98	0,132 -		n.e.
	GF751	1	95	0,448 -	86	15,1 -	87	0,047 -		n.e.
K562 (CML)	GF770			0,100		8,5		0,030		4,05
	GF770	1	96	0,132 -	99	12,7 -	102	0,030 -	98	3,00 -
	GF770	3	83	0,084 -	84	7,7 -	82	0,050 -	89	2,28 -
K562 (CML)	GF791			0,208		21,0		0,055		7,09
	GF791	1	105	0,256 -	108	15,9 -	104	0,520 -	91	8,42 -
	GF791	3	92	0,388 -	91	22,5 -	89	0,089 -	79	3,82 -
Total positive interaction				0/3		0/3		0/3		0/2

1) T/C[%] of PEP005 alone

n.e.: not evaluable

5

Evaluation positiveinteraction between PEP005 and chemotherapeutic agents:

-, IC₇₀ (combination) >50% of IC₇₀ chemotherapeutic agent alone

+, IC₇₀ (combination) <=50% of chemotherapeutic agent alone

10 ++, IC₇₀ (combination) <=30% of chemotherapeutic agent alone

+++, IC₇₀ (combination) <=10% of chemotherapeutic agent alone

EXAMPLE 14***Neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC)***

This Example provides evidence that neutrophils are required to prevent relapse of skin tumors following topical treatment with PEP005. Topical PEP005 treatment induces primary necrosis of tumor cells, potently activates protein kinase C, and was associated with an acute T-cell-independent inflammatory response characterized by a pronounced neutrophil infiltrate. In *Foxn1^{nu}* mice depleted of neutrophils and in CD18 deficient mice (in which neutrophil extravasation is severely impaired) PEP005 treatment was associated with a >70% increase in tumor relapse rates. NK cell or monocyte/macrophage deficiency had no effect on relapse rates. Both *in vitro* and in mice PEP005 induced MIP-2/IL-8, TNF α and IL-1 β , all mediators of neutrophil recruitment and activation. *In vitro* PEP005 activated human endothelial cells resulting in neutrophil adhesion, and also induced human neutrophils to generate tumoricidal reactive oxygen intermediates. Treatment of tumors with PEP005 significantly elevated the level of anti-cancer antibodies, which were able to promote neutrophil mediated antibody dependent cellular cytotoxicity (ADCC) *in vitro*. PEP005 treatment of tumors grown in SCID mice was also associated with >70% increase in tumor relapse rates. Taken together these data suggest a central role for neutrophil-mediated ADCC in preventing relapse. PEP005-mediated cure of tumors, therefore, appears to involve initial chemoablation followed by a neutrophil-dependent ADCC-mediated eradication of residual disease, illustrating that neutrophils can be induced to mediate important anti-cancer activity with specific chemotherapeutic agents.

Materials and Methods***Cells and cell culture***

The B16 mouse melanoma line (ATCC CRL-6322), the LK2 UV-induced mouse squamous cell carcinoma (SCC) line (Cavanagh and Halliday, *Cancer Res* 56:2607-2615, 1996), and the human melanoma lines MM96L (Parsons and Hayward, *Photochem Photobiol* 42:287-293, 1985) and Me10538 (Larizza *et al*, *Clin Exp Metastasis* 7:633-644, 1989) were cultured at 37°C and 5% CO₂ in RPMI 1640 medium (Life Technologies, Inc.,

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Rockville, MD) supplemented with 10% v/v fetal calf serum (FCS) (CSL Biosciences, Parkville, Australia), 100 µg/ml streptomycin and 100 IU/ml penicillin (Life Technologies, Inc., Rockville, MD) (complete medium). Human epidermal keratinocytes were isolated from newborn foreskin and cultured in the presence of a mitomycin C-treated 3T3 feeder layer as described previously (Hotchin and Watt, *J Biol Chem* 267:14852-14858, 1992). Human skin fibroblasts were isolated from tissue digests taken at the time of joint replacement surgery and were grown out over several weeks in defined medium as described previously (Parsonage *et al*, *Thromb Haemost* 90:688-697, 2003). Human neutrophils were isolated from peripheral blood by percoll density centrifugation as described previously (de Boer and Roos, *J. Immunol* 136:3447-3454, 1986) and purity was assessed by Giemsa staining and was routinely >95%.

PEP005 treatment and histology

LK2 cells (10^6) were injected s.c. (two tumor sites/mouse and two mice per group) into the flanks of 6-10 week-old *Foxn1^{nu}* (BALB/c nu⁻/nu⁻) mice (Animal Resource Centre, Perth, Australia). The tumors and two patches of normal skin on the opposite flanks were treated once topically with PEP005 or placebo (isopropanol-based gel). PEP005 was dissolved in 100% v/v acetone and diluted in an isopropanol-based gel composed of 25% v/v isopropyl alcohol and 25% w/w propyl alcohol in water (pH 4-6), and 10 µg of PEP005 was applied in 10 µl of gel. PEP005 powder was obtained from Peplin Limited (Brisbane, Australia) at greater than 98.5% purity. Mice were euthanized at 1, 2, 6, 24 and 48 hours post-PEP005 treatment and treated sites were excised, formalin fixed, paraffin wax embedded and processed for histology using standard H & E staining. Slides were examined using the ScanScopeT2 slide scanner (APerio Technologies).

25

PEP005 treatment in neutrophil depleted mice

LK2 cells (10^6) were injected s.c. (four tumor sites/mouse) into the flanks of 6-10 week-old *Foxn1^{nu}* mice (n=3 mice and n=12 tumors per group). After two weeks (when tumors had reached ≈ 14 mm³), six mice were given i.p. injections of rat anti-Ly-6G anti-granulocyte antibody (100 µg in PBS) (RB6-8C5; BD Biosciences, San Diego, CA). Another six mice were injected i.p. with an isotype matched control antibody (100 µg of

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rat IgG2b in PBS) (A95-1; BD Biosciences). Antibody was injected on days -2, 0 and 2 relative to initiation of PEP005 or placebo treatment on day 0. A further six control mice received no antibody. The tumors on a total of nine mice (n=3 no antibody, n=3 A95-1 treated, and n=3 RB6-8C5 treated) were topically treated daily for three days with PEP005 as above. Another nine mice, grouped as above, were treated with the same volume of placebo (isopropyl-based gel). Tumor and erythema size were measured with calipers. Blood was taken from tail tips twice weekly, smeared and air dried on glass slides before being stained with Quick Dip (Fronine Laboratory Supplies, Melbourne, Australia). In all tumor experiments mice were euthanized when the cumulative tumor burden per mouse exceeded 1000 mm³.

PEP005 treatment in CD18 deficient mice

B6.129S7-*Itgb2*^{tm1Bay/J} mice (The Jackson Laboratory, Bar Harbor, ME) are CD18 hypomorphic mice on the B6 background and have 2-16% of normal CD18 expression on granulocytes (Wilson *et al*, *J Immunol* 151:1571-1578, 1993). B16 cells (5x10⁵) were injected s.c. (one tumor site/mouse) into the flanks of 6-10 week-old mice (n=9 mice per group) and after six days (when tumors had reached ≈14 mm³) mice were treated with PEP005 or placebo. Tumor and erythema size were measured as above.

PEP005 treatment in NK depleted mice

LK2 cells (10⁶) were injected s.c. (four tumor sites/mouse) into the flanks of 6-10 week-old *Foxn1*^{nu} mice (n=3 mice and n=12 tumors per group). After two weeks when the tumors had reached ≈14 mm³, mice were treated with PEP005 or placebo (on days 0, 1 and 2) and tumor volumes monitored as above. The mice received on days -2 and 2 (relative to initiation of PEP005 treatment) anti-asialo GM1 polyclonal anti-serum (50 µl i.p.) (Cedarlane, Ontario, Canada). NK depletion (>90%) was confirmed two days after antibody administration using splenocytes from a parallel group of animals and FACs analysis (using the pan NK antibody DX5, BD Biosciences Pharmingen).

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PEP005 treatment in op/op mice

B16 cells (5×10^5) were injected s.c. (four tumor sites) into the flanks of 6-10 week-old op/op hypomorphic mice (Herston Medical Research Centre, Brisbane, Australia) (n=3 and n=12 tumors per group). Tumors were treated with PEP005 or placebo and monitored as
5 above.

PEP005 treatment in SCID mice

LK2 cells (10^6) were injected s.c. (four tumor sites/mouse) into the flanks of 6-10 week-old SCID mice (Animal Resource Centre) (n=3 mice and n=12 tumors per group). Tumors
10 were treated with PEP005 or placebo and monitored as above.

Statistical analysis

SPSS 13.0 for Windows (release 13.0) was used for statistical analysis. Cox regression analysis was used to compare relapse rates between different groups and was adjusted for
15 the mouse effect when four tumors per mouse were used. The analysis showed that the influence of mouse on time to relapse was not significant (p=0.88 and p=0.315), illustrating that each tumor essentially behaves independently.

In vivo cytokine responses

20 LK2 cells were injected, as above, into the flanks of 6-10 week-old *Foxn1^{mu}* mice (two tumor sites/mouse). After two weeks (when the tumors had reached $\approx 14 \text{ mm}^3$) the tumors and two skin sites on the opposite flanks of the animals were treated once with PEP005 as above. Parallel mice (n=2 per time point) were euthanized 0, 1, 2 and 6 hours after treatment, and the tumor and skin sites excised. Total RNA was extracted and purified
25 from skin and tumor tissue as per the manufacturer's instructions (RNeasy Protect-midi kit; Qiagen, Clifton Hill, Australia). Total RNA (4 ug) was reverse transcribed using (Oligo d(T₁₅)) and Superscript III (Life Technologies). PCR product intensity after gel electrophoresis and staining was determined to be linear for the number of cycles used, thus the analysis was deemed to be semi-quantitative. The PCR reactions were conducted
30 using the GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT), DyNAZyme II DNA polymerase (Finnzymes, Espoo, Finland) and Hot Star Taq DNA polymerase (Qiagen) as

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described previously (Mateo *et al*, *Intervirology* 43:55-60, 2000). The primers were IL-1 β , 5' CAG GAT GAG GAC ATG AGC ACC [SEQ ID NO:1], 3' CTC TGC AGA CTC AAA CTC CAC [SEQ ID NO:2]; TNF α 5' CCA GAC CCT CAC ACT CAG AT [SEQ ID NO:3], 3' GGT AGA GAA TGG ATG AAC AC [SEQ ID NO:4]; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5' TGA AGG TCG GTG TGA ACG GAT TTG GC [SEQ ID NO:5], 3' CAT GTA GGC CAT GAG GTC CAC CAC [SEQ ID NO:6]; Macrophage inflammatory protein 2 (MIP-2) 5' TCC AGA CTC CAG CCA CAC TTC AGC [SEQ ID NO:7], 3' TCT CAG ACA GCG AGG CAC ATC AGG [SEQ ID NO:8].

10 *Cytokine production by PEP005-treated human cells*

Cultures of Me10538 cells, keratinocytes, fibroblasts (75% confluent at $\approx 5 \times 10^5$ cells per well of a 24 well plate) and neutrophils (10^4 cells per well of a 96 well plate) were incubated for 6 hours in the absence or presence of PEP005 (1-100 ng/ml). The supernatants were harvested and analyzed for the presence of the following cytokines; 15 TNF α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and GM-CSF using a multiplex detection kit (Biosource International, Nivelles, Belgium).

PEP005-induced activation of vascular endothelium

Adherence of neutrophils to vascular endothelium was measured using a standard static 20 adhesion assay (Butler *et al*, *Exp Cell Res* 310:22-32, 2005). First passage human umbilical vein endothelial cells (HUVEC) were grown to confluence on glass cover slips in the wells of 24 well plates using Medium 199 (Life Technologies, Edinburgh, UK) supplemented with 20% v/v fetal bovine serum (FBS) (Sigma, St Louis, MO), 1 ng/ml epidermal growth factor (Sigma, St Louis, MO), 1 μ g/ml hydrocortisone (Sigma, St Louis, 25 MO), 28 μ g/ml gentamycin (David Bull Laboratories, Warwick, UK) and 2.5 μ g/ml amphotericin B (Sigma, St Louis, MO). PEP005 was added to culture wells at 1 - 100 ng/ml and TNF α was used as a positive control at 100 units/ml. Endothelial cells were exposed to TNF α or PEP005 for 4 hours prior to thorough washing to remove PEP005 or TNF α . Human neutrophils (7.5×10^5 /well) were then added to the endothelial cells and 30 allowed to adhere for 5 minutes at 37°C. Cells were then washed, fixed in 2% w/v

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glutaraldehyde and examined by phase contrast microscopy to determine the number of neutrophils adhering to the endothelial cell monolayer.

MM96L cell killing by neutrophils in vitro

- 5 Human neutrophils were added to MM96L human melanoma cells (5000 cells per 96 U-well plate in triplicate), with and without 10 ng/ml of PEP005. After 24 hours the cultures were washed with PBS to remove neutrophils and PEP005, and were then maintained for a further six days in complete medium. The cells were washed with phosphate buffered saline (PBS), fixed in methanol and the total protein of the adherent MM96L cells
10 determined using sulfo-rhodamine B as described previously (Skehan *et al*, *J Natl Cancer Inst* 82:1107-1112, 1990). Cell survival was expressed as a percentage of total protein measured from wells containing only melanoma cells.

Lytic mediator release by neutrophils in vitro

- 15 Neutrophils (10^7) were treated with 10 ng/ml PEP005 for 2 hours and assessed for generation of (i) superoxides measured using a lucigenin based assay (Gyllenhammar, *J Immunol Methods* 97:209-213, 1987), (ii) release of soluble TRAIL measured by ELISA using a commercial kit (Biosource International) and (iii) release of defensins 1-3, measured by ELISA using a commercial kit (Cell Sciences Inc, Canton, MA).

20

Anti-cancer antibody measurements

- B16 cells (10^6) were injected s.c. (one tumor/mouse) into the flanks of 6-10 week-old C57BL/6 mice (Animal Resource Centre) and when tumors had reached ≈ 30 -60 mm³ they were cured with PEP005 as above. On day 11 and day 135 sera was taken and analyzed by
25 ELISA for antibodies specific for B16. A group of B16 bearing animals, which were not treated with PEP005 and a naïve group were included. B16 cells were sonicated in carbonate buffer (pH=9) and absorbed onto Immuno Maxisorp 96 well plates (Nunc) overnight and dried. The plates were blocked with 5% v/v FBS, 0.01% v/v Tween in PBS for one hour at 37°C. Test sera was serially diluted in duplicate and probed with rat anti-
30 mouse biotinylated primary antibody (BD Biosciences Pharmingen) and HRP-labeled

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streptavidin (Biosource International, Camarillo, CA) followed by ABTS substrate (Sigma) and measurement of OD at 405 nm.

In vitro neutrophil ADCC assay

5 LK2 tumors ($\approx 14 \text{ mm}^3$) were established in *Foxn1^{nu}* mice (four tumor sites/mouse) and were cured with PEP005 as described above. On day 11 after treatment initiation antisera was collected by heart puncture and pooled. A parallel group of mice were not treated and sera was also collected on day 11, by which time the tumors had grown to $\approx 50 \text{ mm}^3$. Sera from naïve animals was taken at the same time (n=3 per group). LK2 cells were seeded in
10 triplicate (4×10^3 /well) in 96 round bottom wells and were incubated for 2 hours at 4°C with a 1 in 3 dilution of the antisera (total volume 13 μl). Guinea Pig complement (Gibco) (final dilution 1 in 10), PEP005 (final concentration 10/ng/ml) or medium (Control) was then added with murine neutrophils at an effector:target ratio of 100:1 (final total volume 200 μl). The plates were kept at 4°C and spun at 50 g for 5 minutes. The plates were then
15 incubated at 37°C for four days with two medium changes, and total cellular protein of the adherent LK2 cells was then measured using crystal violet staining as previously described (Antalis *et al*, *J. Exp. Med.* 187:1799-1811, 1998). Murine neutrophils were prepared as described previously (Bergman *et al*, *Cancer Immunol. Immunother.* 49:259-266, 2000) except that mice received two intraperitoneal injections of casein separated by 18 hours
20 prior to peritoneal lavage, and red blood cells were not lysed.

Results

Neutrophil recruitment and tissue morphology after PEP005 treatment

The UV-induced murine squamous cell carcinoma line, LK2 was grown as s.c. tumors in
25 *Foxn1^{nu}* mice. Histological examination of tumor sites and normal skin treated topically with placebo (isopropanol gel) using H&E staining revealed normal morphology with small numbers of leukocytes present. However, skin obtained 6 hours after topical application of PEP005 showed decreased integrity of hair follicles and sebaceous glands, as well as increased dilation of local blood vessels. A modest increase in neutrophil
30 numbers was observed in the treated area. Similar results were also seen in the PEP005-treated tumor sites at this time. Twenty four hours after treatment with PEP005 a large

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number of neutrophils and some macrophages were found in treated skin and in and around the treated tumor site, with neutrophils abundant at the periphery of the tumor. Some hemorrhage of local blood vessels was also apparent at this time. Higher magnification images of areas surrounding the tumor mass clearly show numerous cells with multi-lobed nuclei and red staining cytoplasm, which are characteristic of neutrophils. A similar pattern of neutrophil infiltration was also observed when this experiment was repeated using C57BL/6 mice and B16 melanomas (data not shown). The acute inflammatory response that follows PEP005 treatment was, therefore, characterized by a pronounced neutrophil infiltrate.

As reported previously the cosmetic effect after PEP005 treatment in was very favorable. Approximately three weeks after PEP005 treatment the skin at the treatment site was similar to untreated skin and had normal elasticity, and by 2-3 months few if any signs of scarring or erythema were apparent (Ogbourne *et al*, *Cancer Res* 64:2833-2839, 2004).

Tumor relapse in neutrophil-depleted mice after topical chemotherapy with PEP005

It has been shown that PEP005 was able to cure, without significant relapse, a number of different tumor types grown in *Foxn1^{nu}* mice, illustrating that T-cells are not required for effective treatment (Ogbourne *et al*, *supra* 2004). Neutrophils are fully active in *Foxn1^{nu}* mice and their importance for the anti-cancer activity of PEP005 was investigated by using the anti-Ly-6G antibody (RB6-8C5) to deplete these cells in mice bearing LK2 tumors. The tumors grew only marginally slower in control animals (that had received no antibody) and animals receiving control antibody (A95-1), compared with animals given the anti-Ly-6G (RB6-8C5) antibody.

In a separate experiment, LK2 tumors were established in control animals, animals receiving control antibody and animals receiving anti-Ly-6G antibody. When the tumors had reached $\approx 14 \text{ mm}^3$ they were treated topically with PEP005 daily for three days. Initial chemoablation of the tumors was apparent in all groups; however, after day 25 in the anti-Ly-6G antibody treated group tumors began to re-emerge. Data from this experiment are also presented as a percentage of tumors relapsing in each group over time. In control

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animals only 1/12 (8.3%) tumors relapsed, and none of the tumors relapsed in animals that had received the control antibody. However, in animals whose neutrophils had been depleted with anti-Ly-6G antibody (see below), a significant increase in the relapse rate to 83% was observed (Cox regression analysis $p=0.005$, Wald statistic = 7.92, for anti-Ly-6G antibody verses control antibody). These data suggest that neutrophils are required to prevent relapse following PEP005 treatment.

Together with the PEP005-treated animals a parallel group of animals was established that were treated with placebo (instead of PEP005). Acute erythema was absent in all placebo-treated animals, but apparent in all PEP005-treated tumor site. However, the peak erythema on day 2 was approximately 50% lower in the mice that received the anti-Ly-6G antibody compared to control antibody-treated animals ($p<0.001$, unpaired Student *t*-test). The neutrophil infiltrate thus appeared to contribute significantly to the PEP005-induced inflammatory response.

15

The neutrophil counts in blood were determined for each of the animal groups. The white cell counts in placebo-treated mice, mice treated with PEP005 and mice treated with the control antibody, were essentially identical. In contrast, the percentage of neutrophils in the blood fell dramatically from $\approx 80\%$ of total leukocytes to $\approx 3\%$ following anti-Ly-6G antibody treatment in both placebo treated and PEP005 treated animals. Neutrophil counts were restored to normal levels approximately 10 to 12 days after the last antibody injection.

20

Rapid relapse after PEP005 treatment of B16 tumors in CD18 deficient mice.

Neutrophil extravasation into inflamed sites is severely impaired in CD18 deficient mice (Schruefer *et al*, *J Vasc Res* 43:1-11, 2005; Wilson *et al*, *supra* 1993; Arnaout, *Immunol Rev* 114:145-180, 1990), whereas antibody and some T-cell responses remain intact (Lee *et al*, *Nat Med* 9:1281-1286, 2003). B16 tumors grew faster in CD18 deficient mice compared to C57BL/6 mice, so PEP005 treatment was initiated earlier post tumor cell implantation in the former so that tumor sizes were comparable in CD18 deficient and C57BL/6 mice. Although 100% of B16 tumors with an average volume of $\approx 14 \text{ mm}^3$ were

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cured with three topical applications of PEP005 in C57BL/6 mice, all tumors of a similar size treated in CD18 deficient mice rapidly relapsed after the same PEP005 treatment. An acute erythema was apparent after PEP005 treatment, and the area was similar in CD18 deficient and wild type mice, although the intensity was considerably less in the former
5 animals. The erythema in CD18 deficient mice was mainly a discoloration with slight reddening seen on some mice, whereas wild type mice showed pronounced reddening in all treated sites.

Thus in a second neutrophil defective model, PEP005 treated tumors relapsed, and the
10 intensity of the PEP005-induced inflammation was reduced. Although multiple leukocyte interactions can be affected by CD18 deficiency, these data support the view that an acute inflammatory response with a dominant neutrophil infiltrate is required for the anti-tumor efficacy of PEP005.

15 *The role of NK cells and macrophages*

NK cells and macrophages are present in *Foxn1^{mu}* mice and these cell types can be activated by PKC activating agents (Borrego *et al*, *Immunology* 97:159-165, 1999; Martin and Edwards, *J Immunol* 150:3478-3486, 1993). To determine their potential contribution to the anti-tumor effects of PEP005, the experiment was repeated using a polyclonal anti-
20 asialo GM1 polyclonal antibody to deplete NK cells. (Tumors grew between 1.5 and 2 times faster in NK-depleted animals compared to controls). The erythema and relapse rates were not significantly different between these groups (p=0.47). Thus NK cells did not appear to have a significant role in preventing relapse after PEP005 treatment.

25 The experiment using B16 tumors was repeated in *Csfm*(op)/*Csfm*(op) (op/op) mice, which lack functional macrophage-colony stimulating factor, and are, therefore, severely monocytopenic (Schonlau *et al*, *J Leukoc Biol* 73:564-573, 2003). PEP005 treatment was able to cure, without relapse, 100% of tumors, indicating that macrophages do not have an important role in preventing relapse following PEP005 treatment.

Relapse of PEP005 treated tumors in SCID mice

To determine whether successful relapse free PEP005 treatment requires B-cells and antibody production, LK2 tumors were grown in SCID mice and were treated with PEP005 and monitored for relapse. The relapse rate of PEP005-treated LK2 tumors grown in SCID mice was very similar to that seen for PEP005-treated LK2 tumors grown in granulocyte depleted *Foxn1^{mu}* mice, with a >80% of tumors relapsing by day 40. In contrast, nearly all the tumors were cured when similar sized LK2 tumors grown in *Foxn1^{mu}* mice were treated with PEP005 (Cox regression analysis $p=0.006$, Wald statistic = 7.68, for relapse rates in SCID compared to *Foxn1^{mu}* mice. LK2 tumors grew at similar rates in SCID and *Foxn1^{mu}* mice and the PEP005-induced erythema was similar in the two mouse strains. The high relapse rates in SCID mice indicates that anti-cancer antibody production following PEP005 treatment is also required to prevent relapse.

Induction of MIP-2, TNF α and IL-1 β after PEP005 treatment in vivo.

To determine whether the rapid inflammation induced by PEP005 was associated with production of pro-inflammatory mediators, RT-PCR was used to assess IL-6, KC/Gro α , MIP-2, TNF α and IL-1 β mRNA expression in PEP005-treated tumor sites and PEP005-treated normal skin. mRNA isolated from full thickness mouse skin that was treated with PEP005 showed a ≈ 250 fold increase in MIP-2 mRNA, a ≈ 8 fold increase in TNF α and a ≈ 2 fold increase in IL-1 β mRNA within 6 hours of treatment. Tumor tissue was separated from surrounding skin and dermal tissue and showed a ≈ 17 fold increase in MIP-2 mRNA, a ≈ 2 fold increase in TNF α and a ≈ 1.5 fold increase in IL-1 β mRNA within six hours of treatment. No changes in IL-6 and KC/Gro α transcripts were detected. Thus in both skin and tumor tissue *in vivo* PEP005 treatment induced MIP-2, TNF α and to a lesser extent IL-1 β ; all mediators involved in neutrophil migration and activation (Ferrante, *Immunol Ser* 57:417-436, 1992; Cataisson *et al*, *J Immunol* 174:1686-1692, 2005).

Pro-inflammatory cytokine induction in human keratinocytes, fibroblasts, neutrophils and melanoma cells after PEP005 treatment in vitro.

To determine whether the inflammatory mediator profile induced in mice following PEP005 treatment also applies to human cells, the induction of cytokines and chemokines

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following PEP005 treatment of human keratinocytes, neutrophils and a human melanoma cell line (Me10538) was assessed *in vitro*. Culture medium was analyzed for cytokines and chemokines 6 hours after treatment with PEP005. IL-8, the human counterpart of MIP-2, was induced in all cells tested, with keratinocytes and neutrophils producing maximal levels at 5 ng/ml PEP005 (Table 8). The reduced response of neutrophils at 10 ng/ml of PEP005 (compared to 5 ng/ml) was probably due to apoptosis, as PEP005 was a potent inducer of neutrophil activation (see below), which leads to activation-induced cell death. TNF α levels were induced $\approx$ 60 fold in keratinocytes in the presence of 1 ng/ml PEP005, and IL-6 was marginally induced in fibroblasts after treatment with >10 ng/ml PEP005 (Table 8). No IL-2, IL-4, IL-10, IL-12, or GM-CSF was detected in any cell type tested after PEP005 treatment. Thus PEP005 induced production of the neutrophil chemoattractant IL-8 in keratinocytes, fibroblasts, neutrophils and tumor cells, and potently induced TNF α production in keratinocytes.

15 *Activation of human endothelial cells by PEP005.*

Recruitment of neutrophils to the site of inflammation requires activation of the vascular endothelium to promote neutrophil binding, which is a pre-requisite for extravasation and tissue infiltration (Juttila *et al*, *Transplantation* 48:727-731, 1989). PEP005 was able to activate vascular endothelial cells in a dose dependent manner, with significant neutrophil binding occurring at 10 ng/ml PEP005. TNF α is known to activate vascular endothelium, and neutrophil binding induced by TNF α shown as a positive control (Figure 13A).

Direct anti-tumor activity of PEP005-activated neutrophils

To determine whether the neutrophils recruited to the PEP005 treated tumor site might have direct anti-tumor activity, human neutrophils were co-cultured with MM96L human melanoma cells in the presence and absence of PEP005 (10 ng/ml). Neutrophils and drug were removed after 24 hours and the cells cultured for a further six days. In the absence of PEP005, the neutrophils had no effect on the viability of MM96L cells. However, in the presence of PEP005 the neutrophils were able to reduce the viability of MM96L cells by $\approx$ 50% at a neutrophil to target ratio of 3:1, and >90% at a neutrophil to target ratio of 100:1 (Figure 13B).

The production of potential anti-tumor agents by PEP005-stimulated human neutrophils was investigated. PEP005 concentrations >10 ng/ml were able to induce marked superoxide production by human neutrophils (Figure 13C). At 100 ng/ml (but not 10
5 ng/ml) PEP005 induced a modest release of defensins, which are neutrophil granule proteins that have been reported to have some anti-cancer activity (Lichtenstein *et al*, *Cell Immunol* 114:104-116, 1988). Soluble TNF-related apoptosis-inducing ligand (sTRAIL/Apo-2 ligand) (Tecchio *et al*, *Blood* 103:3837-3844, 2004) production was not induced at PEP005 concentrations of 1-100 ng/ml. Thus PEP005 by itself did not
10 efficiently induce neutrophil degranulation, whereas PEP005 was able to induce generation of reactive oxygen species.

MM96L human melanoma cells are known to be sensitive to reactive oxygen species (Parsons *et al*, *Cancer Res.* 42:3783-3788, 1982) and these mediators when secreted by
15 neutrophils can be cytotoxic for tumor cells (Lichtenstein, *Blood* 67:657-665, 1986; Di Carlo *et al*, *Chem Immunol Allergy* 83:182-203, 2003). To determine whether PEP005-induced reactive oxygen species could mediate anti-tumor activity, human neutrophils were co-cultured with MM96L cells in the presence and absence of PEP005 (10 ng/ml). Neutrophils and drug were removed after 24 hours and the cells cultured for a further six
20 days. In the absence of PEP005 the neutrophils had no effect on the viability of MM96L cells. However, in the presence of PEP005 the neutrophils were able to reduce the viability of MM96L cells by $\approx 50\%$ at a neutrophil to target ratio of 3:1, and >90% at a neutrophil to target ratio of 100:1.

25 *Increased anti-cancer antibody levels after PEP005 treatment of tumors*

Adoptively transferred anti-tumor antibodies are known to promote neutrophil-mediated ADCC of tumor cells (Hernandez-Ilizaliturri *et al*, *Clin Cancer Res* 9:5866-5873, 2003; Niitsu *et al*, *Clin Cancer Res* 11:697-702, 2005; De Carlo *et al*, *supra* 2003). To determine whether PEP005 treatment leads to increased anti-tumor antibody levels, B16 tumors
30 growing in C57BL/6 mice were cured with PEP005. As early as day 11 post-PEP005 treatment of 30-60 mm³ tumors, anti-cancer antibody levels were elevated, with responses

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significantly exceeding those seen in untreated animals that had large tumor burdens at this time point (Figure 13D) [$p=0.045$ at 1/100 dilution, unpaired Student *t*-test]. Thus PEP005-mediated cure of cancer appears to increase the levels of anti-cancer antibodies.

5 *Murine neutrophil-mediated ADCC of LK2 cells in vitro*

The data suggest that tumor relapse following PEP005 treatment of LK2 tumors on *Foxn1^{mu}* mice is prevented by neutrophil-mediated ADCC. *Foxn1^{mu}* mice cannot generate significant IgG responses, but they do generate IgM responses that can fix complement and thereby trigger neutrophil degranulation *via* complement receptors (Nielsen *et al*, *Eur. J. Immunol.* 27:2914-2919, 1997). It was thus sought to determine whether sera from *Foxn1^{mu}* mice, whose LK2 tumors had been cured with PEP005, would be able to mediate ADCC *in vitro*. Anti-serum from such mice was able to reduce significantly ($p=0.006$, unpaired Student *t*-test) the viability of LK2 cell in the presence of murine neutrophils and added complement. Neither anti-serum from *Foxn1^{mu}* mice, whose LK2 tumors had not been treated, nor anti-serum from naïve *Foxn1^{mu}* mice, was able to reduce significantly the viability of LK2 cell under the same conditions. The lower levels of complement present in the mouse serum (which was not heat inactivated) was insufficient under these assay conditions to activate the anti-cancer activity of the neutrophils. However, when 10 ng/ml PEP005 was added, anti-cancer activity was again seen for the PEP005 LK2 anti-serum, but not the LK2 or naïve serum. In the absence of PEP005 or added complement, none of the antisera were able to reduce significantly the viability of LK2 cells. Thus anti-serum from *Foxn1^{mu}* mice, whose LK2 tumors had been cured by PEP005 treatment, was able to induce neutrophil-mediated ADCC of LK2 cells *in vitro* in the presence of added complement or low levels of PEP005.

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TABLE 8
Induction of pro-inflammatory cytokines in human cells in vitro. Cells were incubated with the indicated concentration of PEP005 for 6 hours and the supernatants analyzed for the indicated cytokines. (ND – not detectable. nt – not tested).

PEP005 ng/ml	Keratinocytes			Fibroblasts			Melanoma			Neutrophils		
	IL-8	TNF α	IL-6	IL-8	TNF α	IL-6	IL-8	TNF α	IL-6	IL-8	TNF α	IL-6
0	995 $\pm$ 48	8 $\pm$ 1	ND	20 $\pm$ 1	ND	76 $\pm$ 6	4 $\pm$ 0.3	ND	ND	644 $\pm$ 271	ND	ND
1	3910 $\pm$ 148	510 $\pm$ 26	ND	79 $\pm$ 3	ND	81 $\pm$ 4	<2	ND	ND	7089 $\pm$ 1293	ND	ND
5	4775 $\pm$ 178	847 $\pm$ 37	ND	160 $\pm$ 14	ND	85 $\pm$ 4	210 $\pm$ 6	ND	ND	nt	ND	ND
10	3895 $\pm$ 198	498 $\pm$ 29	ND	215 $\pm$ 12	ND	141 $\pm$ 6	737 $\pm$ 26	ND	ND	2241 $\pm$ 684	ND	ND
100	2950 $\pm$ 108	335 $\pm$ 21	ND	239 $\pm$ 9	ND	205 $\pm$ 5	390 $\pm$ 18	ND	ND	617 $\pm$ 52	ND	ND

EXAMPLE 15***Potentiating immune response with PEP005***

The anti-apoptotic effect of PEP005 on effector T-cells indicated a potential utility for use
5 of PEP005 for potentiating T-cell responses in vaccine responses or immunotherapy
against tumors. The aim of this example was to further develop an understanding of these
observations and determine the potential utility of systemic PEP005 as a T-cell
immunopotentiating agent.

10 Experimental plan:

- Determine the longevity of the effect of PEP005 treatment on CD8 T-cell survival
in vitro.
- Investigate which T-cell subset is primarily affected by PEP005 (i.e. is PEP005
active during the expansion phase or effector phase?).
- 15 - Test the effect of PEP on killing of target cells by CTL.

The objectives of this example were four-fold:

- 1) To test if PEP005 can inhibit apoptosis of CD8⁺ activated T-cells;
- 20 2) To investigate the efficacy and duration of the anti-apoptotic effect of PEP005;
- 3) To test if there is a difference in the effect of PEP005 on subsets of CD8⁺ T-cells; and
- 4) To test how PEP005 affects cytotoxicity of CD8⁺ T-cells.

In vitro assays are used to test the effect of PEP005 on the cytokine withdrawal-induced
25 apoptosis of CD8⁺ T-cells. Subsequently a time course of the effects of PEP005 on T-cell
apoptosis is studied as well as a comparison of the effects on T-cells derived from isolated
naïve and memory CD8⁺ T-cells. For this purpose a cytotoxicity assay using T-cell clones
and antigen loaded target cells is used.

30 PEP005 stored dry at -70°C weighed into 1-2 mg aliquots, reconstituted in DMSO was
used at a strength of 0.0001-10 µg/ml.

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A single dose to *in vitro* system was employed.

CD8⁺ T-cell lines were generated by isolation of CD8⁺ T-cells from peripheral blood of healthy human donors. These cells are dependent on the presence of anti-apoptotic
5 cytokines such as IL2. If deprived of these, they rapidly enter apoptosis. In the assay system used cells were cultured in the absence and presence of IL-2 with varying concentrations of PEP005 to test the effects on apoptosis.

CD8⁺ T-cells were isolated by negative selection using a MACS-based system. Purified
10 cells were stimulated with PHA at 10 µg/ml and irradiated EBV transformed B-cells were used to supply costimulatory signals. Cells maintained in the presence of 25 U/ml IL2 were restimulated at seven day intervals and frozen down at passage 3 in aliquots. These were brought up from liquid nitrogen for each experiment, restimulated and used in survival assays on day 7 to maintain a reproducible cell source. For the survival assays
15 cells were cultured with or without IL2 (25 U/well) at a density of 1x10⁶/ml and treated with 0.01 to 1 µg/ml PEP005 for 48 hours. The percentage of apoptotic cells was measured by flow-cytometric detection of activated caspase-3 as a marker for apoptosis. Experiments were performed in triplicate.

20 Detection of cell proliferation by analysis of bromodeoxyuridine (BrdU) incorporation was coupled to a survival assay. BrdU is an analog of thymidine and can be incorporated into DNA when cells are in the S (synthesis) phase of the cell cycle. Using fluorochrome-conjugated antibodies recognizing BrdU-incorporated DNA epitopes, flow cytometry can then be used to monitor the proportion of cycling cells at different time-points during a
25 survival assay which usually take two days.

Passage 5 (day 7 post-stimulation) CD8⁺ T-cells isolated by Ficoll-Paque were washed twice in RPMI 1640 and cultured in a 96-well plate at 2x10⁶/ml, in the same way as the survival assay set-up. Cells were either given PEP005 at 0.01 µg/ml (20 nM) or medium.
30 At 0, 24 hour and 48 hours from the start of each experiment, each well of cells was pulsed with 10 µM BrdU for 2 hours at 37°C. Cells were then washed twice and resuspended in

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medium in the presence/absence of IL2 and PEP005 (hereafter called supplementary medium) corresponding to their original culture condition. Supplementary medium was made at the start of each experiment and incubated for the same time as the cell culture. After the BrdU-pulse at 48 hour, cells were resuspended and washed once in PBS and
5 stained for BrdU and active caspase-3 using the BrdU flow kit (BD Biosciences, UK) as per manufacturer's instructions. FACS data were analyzed using CellQuest Pro (BD Biosciences, UK).

At the start of the CTL assay, effector CD8⁺ T-cells were resuspended at 1×10^6 cells/ml in
10 medium (RPMI 1640 + 10% heat inactivated (HI) fetal calf serum (HIFCS)) containing different concentrations of PEP005 diluted in RPMI 1640 with 10% HIFCS for 30 minutes at 37°C, 5% CO₂ in 15 ml tubes. During the PEP005-treatment of effector cells, lymphoblastoid cell line (LCL) target cells were labeled with 5 μ Ci Na⁵¹CrO₄ and sensitized with latent or lytic EBV antigenic peptides dissolved in DMSO and diluted in
15 RPMI 1640 for 1.5 hours at 37°C, 5% CO₂; dilutions of DMSO solvent were used as controls. PEP005-treated effector cells were washed once in RPMI 1640, resuspended in RPMI + 10% HIFCS, diluted at different effector/target ratios and transferred to 96 V-well assay plates. ⁵¹Cr-labeled, peptide sensitized target cells were washed twice with RPMI
20 V-well assay plates at 2500 cells/well. Target cells were also incubated with medium alone or 1% w/v Sodium Dodecyl Sulfate (SDS) for spontaneous lysis and maximum lysis controls. Plates were then incubated at 37°C, 5% CO₂ for 4 hours. After the 4 hour incubation, plates were centrifuged at 1300 rpm for 5 minutes to pellet the cells. 100 μ l supernatant of each well was transferred to Fluorescence Activated Cell Sorting (FACS)
25 tubes and radioactivity measured. Percentage of specific lysis of samples using a specific target was calculated as:

$$(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{spontaneous lysis}}) / (\text{CPM}_{\text{maximum lysis}} - \text{CPM}_{\text{spontaneous lysis}}) \times 100$$

30 Control and test triplicate counts were meaned and compared using Student *t*-test.

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One of the aims of this study was to test if apoptosis of activated CD8⁺ T-cells was inhibited. Activated CD8⁺ T-cells normally undergo apoptosis in the absence of survival
5 inducing cytokines. This is shown here by flow cytometric analysis of caspase-3 staining of cells in the survival assays. The effect of PEP005 on CD8⁺ T-cell survival was titrated using different concentrations of PEP005 on passage 5, day 7 post-stimulation CD8⁺ T-cells. Increasing concentrations of PEP005 significantly reduced the percentage of apoptosis of IL-2-deprived CD8⁺ T-cells (Figure 14B, open bars). Percentage of apoptosis
10 of IL2-deprived CD8⁺ T-cells was decreased to a minimum of 19% when PEP005 at 0.01 µg/ml was used, compared to 42.47% in untreated cells.

From the results of the survival assays, it was observed that PEP005 induced a small degree of apoptosis in CD8⁺ T-cells treated with IL-2 (Figures 14A and B). Since IL-2 has
15 a direct effect on stimulating proliferation, whether proliferating cells induced by IL-2 are also induced to undergo apoptosis by PEP005 was investigated. The co-staining technique of BrdU and active caspase-3 allows us to trace whether apoptotic cells found on day 2 were previously proliferating at any given time in the course of the survival assay. The proportion of apoptotic cells that proliferated at different time-points of the total
20 proliferating population is shown in Figure 15 for different treatments; the non-proliferating counterparts is shown in Figure 15D.

Levels of apoptosis of both proliferating and non-proliferating cells not treated with IL-2 remained high, around 75%. Among the cells that were treated with PEP005, the
25 proportion of apoptotic cells which were not proliferating (Figure 15D) and the cells which were proliferating underwent (Figure 15C) apoptosis at similar percentages. These data suggested that the high apoptosis in cells treated with both IL-2 and PEP005 seen in the previous survival assays were not due to the proliferation caused by IL2 in this system itself.

30

In most experiments, percentage of apoptosis in survival assays was analyzed on day 2.

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However, to assess the duration of the anti-apoptotic effect, it was necessary to investigate how long the survival enhancement effected by PEP005 can last in the survival assays. In order to study this aim, the survival assay was extended to day 7 and percentage of apoptosis was measured daily using caspase-3 staining. Apoptosis of cells cultured with
5 IL-2, PEP005 or a combination of these was significantly delayed by 2-3 days. This observation is similar to those made with other survival enhancing cytokines in the group.

Previous publications suggested that the PKC activator PMA can increase CD8⁺ T-cell cytotoxicity under suboptimal conditions which include cytotoxicity towards target cells
10 with weak or no antigen-specificity (Russell, *J. Immunol.* 136(1):23-7, 1986). It was tested if PEP005 shared this effect with PMA. For this purpose, cytotoxicity assays were used with antigen-specific CD8⁺ T-cell clones (SY30C23, IM106 and SY3/2) and peptide pulsed LCL cell lines. Decreasing concentrations of HLA-restricted EBV antigenic peptides loaded onto target cells were titrated and then tested the effect of varying
15 concentrations of PEP005 on the cytotoxicity of CD8⁺ T-cells.

Peptide loading concentration at 5 µg/ml allowed untreated effector cells to give optimal specific lysis of target cells. When 1 µg/ml peptide was used for target cell sensitization, PMA-treated CD8⁺ T-cell clones exhibited higher cytotoxicity than untreated cells, this
20 observation confirms the previously published observation that PMA can increase cytotoxicity only under sub-optimal conditions. At 0.1 µg/ml peptide there was only a low level of cytotoxicity in all conditions. Concentrations of PEP005 ranging between 2 nM and 2 µM did not affect cytotoxicity of CD8⁺ clones.

25 The data indicate that PEP005 extends the lifespan of both CD4⁺ and CD8⁺ activated T-cells. The anti-apoptotic effect works on proliferating and non-proliferating cells to an equal extent. Neither an increase nor a decrease of cytotoxic activity was detected. Therefore, the conclusion from the *in vitro* work is that increased *in vivo* anti-tumor activity is due to the anti-apoptotic effect of PEP005 rather than an increase in cytotoxicity.

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Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in
5 this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A therapeutic protocol for treating a subject with cancer or suspected of having cancer, said protocol comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

2. The therapeutic protocol of Claim 1 wherein the combination of ingenol angelate or pharmaceutically acceptable salt, derivative, homolog or analog thereof and agent results in a combination index (CI) of less than 1 when the CI is determined using the equation:

$$CI = [(C)_1/(C_x)_1] + [(C)_2/(C_x)_2] + [\alpha (C)_1(C)_2/(C_x)_1(C_x)_2]$$

wherein $(C_x)_1$ is the concentration of one of ingenol angelate or agent to produce x percent effect of that drug alone, $(C)_1$ is the concentration of one of ingenol angelate or agent to

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produce the same x percent effect in combination with $(C)_2$ which is the other of the ingenol angelate or agent and α is a constant whereby if the mode of ingenol angelate and agent anti-cancer activity is mutually exclusive or non-exclusive, α is 0 or 1, respectively.

3. The therapeutic protocol of Claim 1 or 2 wherein the ingenol angelate is ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof.
4. The therapeutic protocol of Claim 3 wherein the ingenol angelate is ingenol-3-angelate.
5. The therapeutic protocol of Claim 4 wherein the ingenol-3-angelate is obtainable from *Euphorbia peplus*.
6. The therapeutic protocol of Claim 4 wherein the ingenol-3-angelate is generated by chemical synthetic means.
7. The therapeutic protocol of Claim 1 wherein the agent is selected from the list given in Table 1.
8. The therapeutic protocol of Claim 1 wherein the agent is an anti-cancer chemotherapeutic agent.
9. The therapeutic protocol of Claim 7 wherein the agent is a cytotoxic T-cell or is capable of stimulating or activating cytotoxic T-cells against cancer cells.
10. The therapeutic protocol of Claim 7 wherein the agent is an anti-cancer cell antibody or is capable of stimulating the generation of anti-cancer cell antibodies.
11. The therapeutic protocol of Claim 10 wherein the agent is an anti-cancer cell antigen vaccine.

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12. The therapeutic protocol of Claim 10 wherein the agent mediates antibody-dependent cellular cytotoxicity of cancer cells.
13. The therapeutic protocol of Claim 12 wherein the agent comprises neutrophils or is capable of stimulating or activating neutrophils.
14. The therapeutic protocol of Claim 1 wherein the subject is a human.
15. The therapeutic protocol of Claim 1 or 14 wherein the cancer is selected from the list consisting of ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute lymphocytic leukemia, acute myeloid leukemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancers, cutaneous T-cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynecological cancers, hematological malignancies, hairy cell leukemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, li-fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell

cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, nephroblastoma, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer (NMSC), non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, rothmund-thomson syndrome, salivary gland cancer, sarcoma, schwannoma, sezary syndrome, skin cancer, small cell lung cancer (SCLC), small intestine cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis-/ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia and Wilms' tumor.

16. A therapeutic protocol for treating a subject with cancer or suspected of having cancer, said protocol comprising administering to said subject an ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

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(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the ingenol-3-angelate and agent is greater than either the ingenol-3-angelate or agent alone.

17. The therapeutic protocol of Claim 16 wherein the agent is selected from the list given in Table 1.

18. The therapeutic protocol of Claim 17 wherein the agent is an anti-cancer chemotherapeutic agent.

19. The therapeutic protocol of Claim 17 wherein agent is a cytotoxic T-cell or is capable of stimulating or activating cytotoxic T-cells against cancer cells.

20. The therapeutic protocol of Claim 17 wherein the agent is an anti-cancer cell antibody or is capable of stimulating the generation of anti-cancer cell antibodies.

21. The therapeutic protocol of Claim 20 wherein the agent is an anti-cancer cell antigen vaccine.

22. The therapeutic protocol of Claim 20 wherein the agent mediates antibody-dependent cellular cytotoxicity of cancer cells.

23. The therapeutic protocol of Claim 22 wherein the agent comprises neutrophils or is capable of stimulating or activating neutrophils.

24. The therapeutic protocol of Claim 16 wherein the subject is a human.

25. The therapeutic protocol of Claim 16 or 24 wherein the cancer is selected from the list consisting of ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute

lymphocytic leukemia, acute myeloid leukemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancers, cutaneous T-cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynecological cancers, hematological malignancies, hairy cell leukemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, li-fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, nephroblastoma, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer (NMSC), non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, rothmund-thomson syndrome, salivary gland cancer, sarcoma, schwannoma, sezary syndrome, skin cancer,

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small cell lung cancer (SCLC), small intestine cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis/-ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia and Wilms' tumor.

26. A method for treating a subject with cancer or suspected of having cancer, said method comprising administering to said subject an angeloyl-substituted ingenane or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and administering to said subject an agent which exhibits one or more of the following properties:

- (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;
- (ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;
- (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;
- (iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

27. The method of Claim 26 wherein the agent is selected from the list given in Table 1.

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28. The method of Claim 27 wherein the agent is an anti-cancer chemotherapeutic agent.
29. The method of Claim 27 wherein agent is a cytotoxic T-cell or is capable of stimulating or activating cytotoxic T-cell against cancer cells.
30. The method of Claim 27 wherein the agent is an anti-cancer cell antibody or is capable of stimulating the generation of anti-cancer cell antibodies.
31. The method of Claim 30 wherein the agent is an anti-cancer cell antigen vaccine.
32. The method of Claim 30 wherein the agent mediates antibody-dependent cellular cytotoxicity of cancer cells.
33. The method of Claim 32 wherein the agent comprises neutrophils or is capable of stimulating or activating neutrophils.
34. The method of Claim 26 wherein the subject is a human.
35. The method of Claim 26 or 34 wherein the cancer is selected from the list consisting of ABL1 protooncogene, AIDS related cancers, acoustic neuroma, acute lymphocytic leukemia, acute myeloid leukemia, adenocystic carcinoma, adrenocortical cancer, agnogenic myeloid metaplasia, alopecia, alveolar soft-part sarcoma, anal cancer, angiosarcoma, aplastic anemia, astrocytoma, ataxia-telangiectasia, basal cell carcinoma (skin), bladder cancer, bone cancers, bowel cancer, brain stem glioma, brain and CNS tumors, breast cancer, CNS tumors, carcinoid tumors, cervical cancer, childhood brain tumors, childhood cancer, childhood leukemia, childhood soft tissue sarcoma, chondrosarcoma, choriocarcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancers, cutaneous T-cell lymphoma, dermatofibrosarcoma-protuberans, desmoplastic-small-round-cell-tumor, ductal carcinoma, endocrine cancers, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extra-hepatic bile

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duct cancer, eye cancer, eye: melanoma, retinoblastoma, fallopian tube cancer, fanconi anemia, fibrosarcoma, gall bladder cancer, gastric cancer, gastrointestinal cancers, gastrointestinal-carcinoid-tumor, genitourinary cancers, germ cell tumors, gestational-trophoblastic-disease, glioma, gynecological cancers, hematological malignancies, hairy cell leukemia, head and neck cancer, hepatocellular cancer, hereditary breast cancer, histiocytosis, Hodgkin's disease, human papillomavirus, hydatidiform mole, hypercalcemia, hypopharynx cancer, intraocular melanoma, islet cell cancer, Kaposi's sarcoma, kidney cancer, Langerhan's-cell-histiocytosis, laryngeal cancer, leiomyosarcoma, leukemia, li-fraumeni syndrome, lip cancer, liposarcoma, liver cancer, lung cancer, lymphedema, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, male breast cancer, malignant-rhabdoid-tumor-of-kidney, medulloblastoma, melanoma, merkel cell cancer, mesothelioma, metastatic cancer, mouth cancer, multiple endocrine neoplasia, mycosis fungoides, myelodysplastic syndromes, myeloma, myeloproliferative disorders, nasal cancer, nasopharyngeal cancer, nephroblastoma, neuroblastoma, neurofibromatosis, nijmegen breakage syndrome, non-melanoma skin cancer (NMSC), non-small-cell-lung-cancer-(NSCLC), ocular cancers, oesophageal cancer, oral cavity cancer, oropharynx cancer, osteosarcoma, ostomy ovarian cancer, pancreas cancer, paranasal cancer, parathyroid cancer, parotid gland cancer, penile cancer, peripheral-neuroectodermal-tumors, pituitary cancer, polycythemia vera, prostate cancer, rare-cancers-and-associated-disorders, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, rothmund-thomson syndrome, salivary gland cancer, sarcoma, schwannoma, sezary syndrome, skin cancer, small cell lung cancer (SCLC), small intestine cancer, soft tissue sarcoma, spinal cord tumors, squamous-cell-carcinoma-(skin), stomach cancer, synovial sarcoma, testicular cancer, thymus cancer, thyroid cancer, transitional-cell-cancer-(bladder), transitional-cell-cancer-(renal-pelvis-/ureter), trophoblastic cancer, urethral cancer, urinary system cancer, uroplakins, uterine sarcoma, uterus cancer, vaginal cancer, vulva cancer, Waldenstrom's-macroglobulinemia and Wilms' tumor.

36. A multi-part pharmaceutical formulation having a first part comprising an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and having a second part comprising an agent or a pharmaceutically acceptable salt thereof,

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said parts further optionally comprising one or more pharmaceutically acceptable carriers, diluents and/or excipients wherein said agent exhibits one or more of the following properties:

- (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;
- (ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;
- (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;
- (iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

37. A formulation comprising an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and an agent or a pharmaceutically acceptable salt thereof and one or more pharmaceutically acceptable carriers, diluents and/or excipients wherein said agent exhibits one or more of the following properties:

- (i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;
- (ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;
- (iii) exhibits properties of anti-cancer cell antibodies or is capable of generating

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such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

38. The formulation of Claim 36 or 37 wherein the ingenol angelate is ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof.

39. The formulation of Claim 38 wherein the ingenol angelate is ingenol-3-angelate.

40. The formulation of Claim 38 or 39 wherein the ingenol-3-angelate is obtainable from *Euphorbia peplus*.

41. The formulation of Claim 38 or 39 wherein the ingenol-3-angelate is generated by chemical synthetic means.

42. The formulation of Claim 36 or 37 wherein the agent is selected from Table 1.

43. The formulation of Claim 42 wherein the agent is an anti-cancer chemotherapeutic agent.

44. The formulation of Claim 42 wherein the agent is a cytotoxic T-cell or is capable of stimulating or activating cytotoxic T-cells against cancer cells.

45. The formulation of Claim 42 wherein the agent is an anti-cancer cell antibody or is capable of stimulating the generation of anti-cancer cell antibodies.

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46. The formulation of Claim 45 wherein the agent is an anti-cancer cell antigen vaccine.

47. The formulation of Claim 45 wherein the agent mediates antibody-dependent cellular cytotoxicity of cancer cells.

48. The formulation of Claim 47 wherein the agent comprises neutrophils or is capable of stimulating or activating neutrophils.

49. Use of an ingenol angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof and an agent or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of cancer wherein said agent exhibits one or more of the following properties:

(i) induces apoptosis, necrosis, senescence, cytotoxicity and/or cell cycle arrest of cancer cells;

(ii) mediates T-cell mediated inhibition or death of cancer cells or is capable of stimulating or activating such T-cells;

(iii) exhibits properties of anti-cancer cell antibodies or is capable of generating such antibodies;

(iv) mediates neutrophil-facilitated antibody-dependent cellular cytotoxicity of cancer cells or is capable of generating such neutrophils;

wherein the anti-cancer activity or efficacy of the combination of the angeloyl-substituted ingenane and agent is greater than either the angeloyl-substituted ingenane or agent alone.

50. Use of Claim 49 wherein the ingenol angelate is ingenol-3-angelate or a pharmaceutically acceptable salt, derivative, homolog or analog thereof.

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51. Use of Claim 50 wherein the ingenol angelate is ingenol-3-angelate.
52. Use of Claim 50 or 51 wherein the ingenol-3-angelate is obtainable from *Euphorbia peplus*.
53. Use of Claim 50 or 51 wherein the ingenol-3-angelate is generated by chemical synthetic means.
54. Use of Claim 49 wherein the agent is selected from Table 1.
55. Use of Claim 54 wherein the agent is an anti-cancer chemotherapeutic agent.
56. Use of Claim 54 wherein the agent is a cytotoxic T-cell or is capable of stimulating or activating cytotoxic T-cells against cancer cells.
57. Use of Claim 54 wherein the agent is an anti-cancer cell antibody or is capable of stimulating the generation of anti-cancer cell antibodies.
58. Use of Claim 57 wherein the agent is an anti-cancer cell antigen vaccine.
59. The formulation of Claim 57 wherein the agent mediates antibody-dependent cellular cytotoxicity of cancer cells.
60. The formulation of Claim 59 wherein the agent comprises neutrophils or is capable of stimulating or activating neutrophils.