Title: THIAZOLONE COMPOUNDS FOR TREATMENT OF CANCER

The present invention provides concerns a thiazoline compound 5-[(4 methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof for the treatment of cancers. This compound induces apoptosis in cancer cells without killing of normal healthy cells. In addition, 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof is able to overcome multidrug resistance in cancer cells. Thus, the present invention provides a thiazoline compound 5-[(4-methylphenyl)methylene]-2-(phenylamino) 4(5H)-thiazolone or an analog thereof as a therapeutic agent for treating cancers.
DESCRIPTION

THIAZOLONE COMPOUNDS FOR TREATMENT OF CANCER

BACKGROUND OF THE INVENTION

This application claims the benefit of priority to U.S. Application Serial No. 60/546,479 filed February 20, 2004 and U.S. Application Serial No. 60/549,723 filed March 3, 2004, which all have the same title and inventors as the present application. Each of these applications is hereby incorporated by reference in their entirety.

The United States Government owns rights in the present invention pursuant to grant numbers RO1 CA98582, RO1 CA92487 and CA16672 from The National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the fields of cancer biology and cancer therapeutics. More particularly, it concerns the use of a thiazolone compound 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, also referred to herein as MMPT, and analogs thereof, in the treatment of cancer.

2. Description of Related Art

Microtubules play critical roles in intracellular transport, morphogenesis, and cell division. Consequently, the major protein component of microtubules, tubulin, has been the subject of intensive molecular anticancer research (Checchi et al., 2003; Jordan et al., 1998). Various agents targeting microtubules have been reported to cause cell cycle arrest at mitosis and induce apoptosis in cancer cells. The mechanism by which these drugs disrupt microtubule dynamics has led to the discovery and development of potent agents for the treatment of cancer. Microtubule interacting agents such as vinca alkaloids, colchicine, nocodazole, and taxanes are well known in the art. Despite their different effects on microtubule polymerization activity, these agents all cause cell-cycle arrest at the same phase, namely, the metaphase-to-anaphase transition, and lead to induction of apoptosis.

Although antimitotic drugs such as the taxanes and vinblastine have been used clinically to inhibit the progression of some cancers, acquired resistance to these drugs usually arises from multiple cycles of treatment and abolishes the drugs' efficacy (Drukman and Kavallaris, 2002). One of the mechanisms by which neoplastic cells acquire resistance is overexpression of efflux pumps, such as the protein regulating multidrug resistance, p-glycoprotein (P-gp). A
transmembrane pump, P-gp binds to many antitumor drugs and prevents their intracellular accumulation by increasing their efflux out of cells. Many microtubule-interacting drugs such as the taxanes and vinblastine are well-known substrates of P-gp (Bosch and Croop, 1996; Smyth et al., 1998; Goldman, 2003). Therefore, the discovery of new compounds that are effective against drug-resistant cells that overexpress P-gp is an important step for cancer therapy.

Another major drawback of taxanes and vinca alkaloids in clinical application is the development of neurotoxicity. These drugs interfere with the function of microtubule axons, which mediate the neuronal vesicle transport. New synthetic small molecules that bind to tubulin, but are neither a substrate of transmembrane pumps nor interfere with the function of axonal microtubules, would provide potential therapeutics in treating cancer. Furthermore, such candidate therapeutic agents need to exhibit minimal toxicity to normal human cells. Thus, there remains a need for effective therapeutic agents in the art.

**SUMMARY OF THE INVENTION**

The present invention is directed to a cancer therapeutic that overcomes the deficiencies in the art of current therapies in combating cancer. The present invention addresses the need for more desirable therapeutic agents to overcome toxicity, side effects or resistance offered by current chemotherapeutic agents. Thus, the present invention provides small, synthetic, heterocyclic chemotherapeutic agents, 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or analogs thereof, for the treatment of cancer with minimal toxicity, side effects or resistance. Also provided are methods of inhibiting cell growth, and inducing apoptosis in the cell.

The cell as contemplated in the present invention may be a cancer cell such as, but not limited to, a breast cancer cell, lung cancer cell, head and neck cancer cell, bladder cancer cell, bone cancer cell, bone marrow cancer cell, brain cancer cell, colon cancer cell, esophageal cancer cell, gastrointestinal cancer cell, gum cancer cell, kidney cancer cell, liver cancer cell, nasopharynx cancer cell, ovarian cancer cell, prostate cancer cell, skin cancer cell, stomach cancer cell, testis cancer cell, tongue cancer cell, or uterine cancer cell. In some embodiments, the cancer cell may be a lung cancer cell such as a non-small cell lung cancer cell. In some embodiments, the invention contemplates any hyperproliferative cell. Such cells may also undergo microtubule polymerization activity and may include autoimmune cells or psoriasis cell, or cardiovascular cell, but is not limited to such. In particular embodiments of the present invention, the cancer cell may be a multidrug-resistant cancer cell such as, but not limited to, a
paclitaxel-resistant cancer cell. The cell may be located in cell culture or tissue culture. In a further embodiment of the invention, the cell may be located in a mammal such as a human.

Any analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is contemplated for inclusion in the present invention. The definition of “analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone” is defined below in the specification. Exemplary analogs of MMPT include 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone may be 5-[(4-methylphenyl)methylene-2-(methylthio)-4-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5([(3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-methoxy-4-(2-propenylxyloxy)phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 3-[(4-oxo-2-(phenylamino)-5(4H)-thiazyloylidene) methyl]-benzoic acid, 5-[(4-hydroxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone, N-[4-[(4-oxo-2-(phenylamino)-5(4H)-thiazyloylidene) methyl]phenyl]-acetamide, 5-[(2-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 2-(phenylamino)-5-[(2-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 5-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2-(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 2-[(4-methylphenyl)amino]-5-(phenylmethylene)-4(5H)-thiazolone, 5-benzylidene-2-(3,4-dichlorophenyl)iminio)-4-thiazolidinone, 2-[(4-hydroxy phenyl) amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(4-chlorophenyl)methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, 5-[(2,3-dimethoxyphenyl)methylene]-2-[(4-methylphenyl)amino]-4(5H)-thiazolone, or 5-[(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin, or an analog of any of these agents. In certain particular embodiments, the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin or an analog of 5-[(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

In another particular embodiment of the invention, there is provided a method of inhibiting microtubule polymerization activity in a cell comprising providing to the cell 5-[(4-
methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof. Inhibiting microtubule polymerization activity in a cell further comprises inducing cell cycle arrest in the cell. The analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone may, for example, be any of those analogs set forth above.

In yet another particular embodiment, the present invention provides a method of treating a cancer in a subject comprising administering to the subject a therapeutically effective amount of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof to inhibit cell growth. The method of treating cancer in a subject further comprises inhibiting microtubule polymerization activity and inducing cell cycle arrest.

Any analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is contemplated for inclusion in the methods of the present invention. Exemplary analogs of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone are set forth above. In certain particular embodiments, the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin or an analog of 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

In some embodiments of the invention, the cancer to be treated may be a breast cancer, lung cancer, head and neck cancer, bladder cancer, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, or uterine cancer.

In a particular embodiment of the invention, the cancer to be treated may be a lung cancer such as, but not limited to, a non-small cell lung cancer. In yet another embodiment of the invention, the cancer to be treated may be a multidrug-resistant cancer such as a paclitaxel-resistant cancer, but is not limited to such.

It is contemplated in the present invention that the therapeutic 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof may be administered to a subject by any method known to those of ordinary skill in the art. For example, therapeutic agent may be administered intravenously, intradermally, intramuscularly, intraarterially, intralesionally, percutaneously, subcutaneously, or by aerosol. It is further contemplated in the present invention, that an additional therapeutic agent may be administered to the subject. The additional therapeutic agent may be, for example, an additional therapeutic 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof. The additional therapeutic agent may also be a chemotherapeutic agent that is not a thiazolone, a
radiotherapeutic agent, or a microtubule-interacting agent. Microtubule-interacting agents may be vinca alkaloids or taxanes but are not limited to such.

In some embodiments, the subject may be a mammal such as a human.

The 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or analog thereof of the present invention may be administered more than once, or before the additional therapeutic agent, or after the additional therapeutic agent, or at the same time as the additional therapeutic agent.

In yet another embodiment of present invention, the additional therapeutic agent may be administered intravenously, intradermally, intramuscularly, intraarterially, intraleasonally, percutaneously, subcutaneously, or by an aerosol. In a further embodiment, the additional therapeutic agent may be administered more than once.

In yet another particular embodiment, the present invention provides a method of assaying for cancer cell sensitivity comprising (a) providing a cancer cell; (b) contacting the cancer cell with 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof; (c) analyzing the cancer cell for inhibition of growth; and (d) comparing the inhibition of growth in the cancer cell from step (c) with the inhibition of growth in the cancer cell in the absence of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof, wherein growth inhibition by 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof indicates that the cancer cell is susceptible to the candidate substance.

Any analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is contemplated for inclusion in the methods of the present invention. Exemplary analogs of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone are set forth above. In certain particular embodiments, the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin or an analog of 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

In another particular embodiment, the present invention provides a method of identifying a candidate 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone substance having anticancer activity comprising (a) providing a cancer cell; (b) contacting the cancer cell with the candidate substance; (c) analyzing the cancer cell for inhibition of growth; and (d) comparing the inhibition of growth in the cancer cell from step (c) with the inhibition of growth in the cancer cell in the absence of the candidate substance, wherein growth inhibition by the candidate 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone substance indicates that the cancer
cell is susceptible to the candidate 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone substance.

In further embodiments of the invention it is contemplated that the candidate substance identified may be an organic molecule, an inorganic molecule, or an organopharmaceutical, but is not limited to such.

The present invention also provides a method of analyzing for growth inhibition by XTT assay. In other embodiments, the present invention further comprises a method of assaying for microtubule polymerization activity in a cancer cell comprising analyzing for α-tubulin expression. The assaying methods of the present invention may further comprise analyzing for induction of apoptosis by FACS.

In some embodiments of the present invention, assaying may further comprise determining the phosphorylation status of cell cycle molecules selected from the group consisting of MPM-2, cdc25C, and histone H3.

In yet another particular embodiment, the present invention provides a pharmaceutical composition comprising 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof in a pharmaceutically acceptable excipient.

Any analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is contemplated for inclusion in the pharmaceutical compositions of the present invention. Exemplary analogs of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone are set forth above. In certain particular embodiments, the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin or an analog of 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

The pharmaceutical composition may further comprise an additional therapeutic agent such as an organopharmaceutical, a chemotherapeutic agent, or specifically a microtubule-interacting agent. Microtubule-interacting agents may be a vinca alkaloid or a taxane, but is not limited to such.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention,
are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1.** Chemical structure of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone (MMPT; also known as 10086-7B).

**FIGS. 2A-2B.** Effects of MMPT and paclitaxel on proliferation of non-small cell lung cancer (NSCLC) cell lines and fibroblast cells (NHFB). **FIG. 2A** — Cells were treated with various concentrations of MMPT and paclitaxel for 72 hr and then their viability was determined by XTT assay. Cells treated with DMSO were used as a control, with viability set at 100%. Each data point represents the mean ± SD of three independent studies. **FIG. 2B** — Cells treated in a dose-dependent manner with MMPT.

**FIGS. 3A-3C.** MMPT induced G2-M phase cell-cycle arrest in NSCLC cells. **FIG. 3A** - Flow cytometric analysis of asynchronous H1299 and H460 cells treated with 15-μM MMPT for the indicated times. **FIG. 3B** - Flow cytometric analysis using various concentration of MMPT for 12 hr. After treatment, the cells were harvested and fixed with ice-cold 70% ethanol. Cells were then resuspended in PBS containing 10 μg/ml of PI and 10 μg/ml of RNase A at 37°C for 30 min. **FIG. 3C** - Percentages of cells in the G1, S, and G2-M phase.

**FIG. 4.** Induction of M-phase cell-cycle arrest. Flow cytometric analysis of H1299 and H460 cells treated with 10 μM MMPT for the indicated times. The cells were harvested, fixed, and stained with the anti-mitotic MPM-2 antibody and PI. **Top panel** - histograms represent DNA contents. **Bottom panel** - dot plots show that the proportions of mitotic and 4N cells increased after MMPT treatment. The cells staining positively for MPM-2 reveal increased fluorescence (inset rectangles). The numbers represent the percentages of MPM-2-positive cells.

**FIG. 5.** Cell cycle analysis of NSCLC cells after MMPT treatment. H1299 and H460 cells were exposed to 15 μM MMPT for 3, 6, 9, 12 and 24 hr. 0 = control.

**FIGS. 6A-6D.** Effect of various antimitotic agents on α-tubulin in cancer cells. Control (FIG. 6A), 50 nM nocodazole (FIG. 6B), 15 μM MMPT (FIG. 6C), or 50 nM taxol (FIG. 6D).
FIG. 7A-7B. Induction of apoptosis in NSCLC cells and in vivo antitumor activity.

FIG. 7A - The percentage of cells in sub-G0 phase after treatment with 15 μM MMPT for the indicated times in H1299 and H460 cells. Values are means ± SD of three separate studies.

FIG. 7B – H460 lung cancer cells were inoculated subcutaneously on day 1. On days 11, 14, and 17, when tumors grew to 3-5 mm in diameter, mice were treated with 40 mg/kg of MMPT (closed triangles; n = 6 mice), solvent (closed circles; n = 5 mice), or 4 mg/kg paclitaxel (open circles; n = 5 mice) by i.p. injection. Tumor volume was monitored over time. The value represents mean ± SE. The difference in tumor growth was significant between MMPT treated and solvent treated groups (p ≤ 0.05).

FIGS. 8A-8D. Effect of various antimitotic agents on mitotic spindles in H460 cells. Mitotic spindles were visualized by indirect immunofluorescence after 5 hr of treatment with DMSO control (FIG. 8A), 50 nM nocodazole (FIG. 8B), 15 μM MMPT (FIG. 8C), or 50 nM paclitaxel (FIG. 8D).

FIGS. 9A-9B. Inhibition of tubulin polymerization. FIG. 9A - immunoblots for α-tubulin and β-actin in soluble (S) and particulated (P) fractions of H460 cells treated with DMSO, nocodazole, MMPT, and paclitaxel, at the concentrations indicated for 5 hr. FIG. 9B - the intensity of each band of the immunoblot was determined by the NIH Image program, and the ratio of polymerized to depolymerized tubulin and actin was calculated for each treatment. Values are the mean ± SD ratios from three independent studies.

FIGS. 10A-10C. The effect of MMPT on tumor cell lines. FIG. 10A – Dose response curves of various cancer cell lines. FIG. 10B – Means graph of the cancer cell lines in FIG 10A. FIG. 10C – Dose response curve of all cancer cell lines.

FIG. 11A-11B. Chemical structure of 5-(2,4-dihydroxybenzylidene)-2-(phenylmino)-1,3-thiazolidin (DBPT) (FIG. 11A) and its analog 2-[(4-methylphenyl)amino]-5-(phenylimethylene)-4(5H)-Thiazolone (MAPT ) (FIG. 11B).

FIG. 12A-12C DBPT induced apoptosis in colon cancer cells by activating caspases. FIG. 12A - DLD-1 and wild-type HCT116 cells were treated with 3 and 5 μM DBPT, respectively, for the indicated times and stained with fluorescein isothiocyanate-labeled annexin V and PI. Stained cells were analyzed by flow cytometry to determine the apoptotic ratio. Data represent mean ± SD of three independent experiments. FIG. 12B - DLD-1 cells were pretreated with 10, 50, or 100 μM z-VAD-fmk for 30 min and treated with DBPT for 24 or 48 h. Cell growth inhibition was determined by XTT assay. Data represent mean ± SD of three independent experiments. *, P< 0.01 compared with DBPT treatment alone. FIG. 12C - DLD-1 cells were
pretreated with 50 or 100 μM z-VAD-fmk for 30 min and treated with DBPT for 24 or 48 h. The percentage of cells in sub-G1 phase was determined by flow cytometry.

**FIG. 13A-13B.** Effect of MAPK inhibitors on DBPT-induced apoptosis. **FIG. 13A** - DLD-1 cells were treated with 3 μM DBPT in the presence or absence of 50 μM PD98059 (ERK inhibitor) or 40 μM SB202190 (p38 inhibitor). The apoptotic ratio of stained cells with PI was determined by flow cytometry. Data represent mean ± SD from three independent experiments performed in triplicate. **FIG. 13B** - DLD-1 cells were treated with 3 μM DBPT in the presence or absence of 50 μM SP600125 (JNK inhibitor) for 24 h. The apoptotic ratio of stained cells with PI was determined by flow cytometry. Data represent mean ± SD from three independent experiments performed in triplicate. *, P< 0.01 compared with DBPT treatment alone.

**FIG. 14A-14B.** JNK1 activation has a crucial role in DBPT-induced apoptosis. DLD-1 cells stably transfected with dnJNK1 or dnJNK2 were treated with 3 μM DBPT for 24 or 48 h. Cell viability was determined by XTT assay (**FIG. 14A**), and apoptotic ratio was determined by flow cytometry (**FIG. 14B**). Data represent mean ± SD of three independent experiments.

**FIG. 15.** JNK activation alone has no effect on cell proliferation. DLD-1 cells were pretreated with AdGFP or AdMKK7DN at the indicated MOI for 24 or 48 h. The effect of JNK activation on cell growth inhibition was determined by XTT assay. Data represent mean ± SD of three independent experiments.

**FIG. 16.** Core chemical structure of MMPT.

**FIG. 17A-17C.** To test whether analogues that selectively killed tumor cells were effective for these P-glycoprotein over-expressing cells, the cell killing effect of MMPT and three tumor selective analogues A, G and R were compared in parental H460 cells (**FIG. 17A**), H460/TaxR cells (**FIG. 17B**) and H460/VinR cells (**FIG. 17C**). Dose response in the three cancer cell lines are shown in Fig.17A-17C.

**FIG. 18.** Synthetic route of MMPT.

**FIG. 19.** Synthetic route of analog G.

**DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

I. The Present Invention

Microtubules are important targets of cancer chemotherapy. Various microtubule-interacting agents have been evaluated for their anticancer functions and some have been used
clinically as anticancer drugs, including paclitaxel and the vinca alkaloids vincristine or vinorelbine. However, in order to overcome the deficiencies in the art in treating such diseases as cancer, new therapeutic agents are sought which are more effective, less toxic, and not affected by the p-glycoprotein status of a cell. Thus, the present invention provides a synthetic microtubule-interacting agent for the treatment of cancer.

By screening a chemical library from Chembridge, Inc. (San Diego, CA), a synthetic microtubule-interacting agent, 5-[(4-methylphenyl)methylene]-2-(phenylamino-4(SH)-thiazolone (MMPT; 10086-7B) was identified as having the ability to kill cancer cells more effectively than it kills normal human fibroblasts. Subsequently, the antitumor properties and mechanisms of action of MMPT was investigated in a number of human cancer cell lines. For example, MMPT inhibited the growth of human non small-cell lung cancer (NSCLC) cell lines, with 50% inhibitory concentrations ranging from 4.9 to 6.7 μM. This inhibitory effect was found to be independent of p53 status. Although p53 plays an important role in cell cycle regulation and induction of apoptosis, some reports have shown that paclitaxel induced apoptosis is independent of p53 status (Blagosklonny et al., 1997; Halder et al., 1997). The results of the cytotoxicity assay demonstrated that MMPT was effective in H1299 cells, which are p53 null, in H460 cells, which are p53 wild-type, and in H322 cells, which are p53 mutant type. These results suggest that MMPT-induced cell death of cancer cells is not correlated with p53 status. Nevertheless, some recent studies have reported that functional p53 might interfere with the formation of hyperploid cells after cell cycle arrest at mitosis caused by nocodazole treatment (Verdoordt et al., 1999; Tsuiki et al., 2001). It was found in the present invention that induction of hyperploidy by MMPT is more effective in H1299 cells than in H460 cells (data not shown), although both cell lines were susceptible to MMPT.

Moreover, MMPT had almost no toxic effects on normal human cells at the 50% inhibitory concentration for NSCLC cells. Furthermore, MMPT showed similar antiproliferative effects against both parental H460 and paclitaxel-resistant H460 cells, which have a high level of P-glycoprotein expression, suggesting that MMPT is not affected by P-glycoprotein status. Treatment of NSCLC cells with MMPT led to a time- and dose-dependent accumulation of cell cycle arrest at mitosis, modulated levels of G2-M-phase checkpoint proteins such as cyclin B1, cdc25C, Bcl-2, and histone H3, and induced apoptotic cell death with caspase activation. Additionally, MMPT induced abnormal spindle formation and prevented tubulin polymerization in mitotic cancer cells.

Thus, the present invention demonstrates that MMPT has a potent antitumor effect on multidrug resistant cancer cells that overexpress P-glycoprotein. Moreover, MMPT inhibits
microtubule polymerization in cancer cells, induces cell-cycle arrest at the M-phase of the cell cycle followed by the induction of apoptosis. These results indicate that MMPT, a small heterocyclic molecule, has microtubule depolymerization activity and is a potent, safe anticancer agent.

II. Thiazolone and Analogs Thereof as Therapeutic Agents

The present invention relates to a heterocyclic compound 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone (MMPT) or an analog thereof that shows unexpected, potent anti-cancer activity in vitro. This compound inhibits cell growth and is therefore useful in the treatment of diseases of uncontrolled proliferation, such as cancer. Thus, the present invention provides MMPT or an analog thereof as a therapeutic agent for treating a cancer in a subject.

Diseases contemplated for treatment with the thiazolone MMPT or an analog thereof include, but are not limited to, cancer, autoimmune disease, psoriasis or any hyperproliferative disease. Examples of cancers contemplated for treatment with the thiazolone compound MMPT or analog thereof include breast cancer, lung cancer, head and neck cancer, bladder cancer, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, or uterine cancer. In some instances the cancer to be treated using MMPT or an analog thereof may be a multidrug-resistant cancer such as, but not limited to, a paclitaxel-resistant cancer.

To kill cells, inhibit microtubule polymerization activity, induce cell cycle arrest, inhibit cell growth, inhibit metastasis, inhibit angiogenesis or otherwise reverse or reduce the malignant phenotype of cancer cells, using the methods and compositions of the present invention, one would generally contact a cell with the thiazolone MMPT or analogs thereof. The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic agent is delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, the therapeutic agent is delivered to a cell in an amount effective to inhibit microtubule polymerization activity, induce cell cycle arrest, inhibit cell growth and induce apoptosis in the cell.

MMPT or an analog thereof as a therapeutic agent may be administered to a subject more that once and at intervals ranging from minutes to weeks. In instances where multiple delivery of MMPT or an analog thereof is needed, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent would still be able to exert an advantageous effect on the cell. In some situations, it may be desirable to extend the
time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several
weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Administration of MMPT or an analog thereof to a subject may be by any method known
in the art for delivery of a therapeutic agent to a subject. For example, such methods may
include, but are not limited to, oral, nasal, intramuscular, or intraperitoneal administration.
Methods of administration are disclosed in detail elsewhere in this application.

A. Chemical Definitions

Following long-standing patent law convention, the words “a” and “an”, when used in the
specification including the claims, denotes one or more.

An “alkyl” group refers to a saturated aliphatic hydrocarbon, including straight-chain,
branched chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More
preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl
group may be substituted or unsubstituted. When substituted, the substituted group(s) is
preferably hydroxyl, cyano, alkoxy, =O, =S, NO₂, N(CH₃)₂, amino, or SH.

An “alkenyl” group refers to an unsaturated hydrocarbon group containing at least one
carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups.
Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from
1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or
unsubstituted. When substituted, the substituted group(s) is preferably hydroxyl, cyano, alkoxy,
=O, =S, NO₂, N(CH₃)₂, halogen, amino, or SH.

An “alkynyl” group refers to an unsaturated hydrocarbon group containing at least one
carbon-carbon triple bond, including straight-chain, branched chain, and cyclic groups.
Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from
1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or
unsubstituted. When substituted, the substituted group(s) is preferably hydroxyl, cyano, alkoxy,
=O, =S, NO₂, N(CH₃)₂, amino, or SH.

An “alkoxy” group refers to an “—O-alkyl” group, where “alkyl” is defined above.

An “aryl” group refers to an aromatic group which has at least one ring having a
conjugated pi electron system, and includes carbocyclic aryl, heterocyclic aryl, and biaryl
groups, all of which may be optionally substituted. Preferably, the aryl is a substituted or
unsubstituted phenyl or pyridyl. Preferred aryl substituent(s) are halogen, trihalomethyl,
hydroxyl, SH, OH, NO₂, amine, an ester (e.g., COOH), thioether, cyano, alkoxy, alkyl, and
amino groups.
An “alkylaryl” group refers to an alkyl (as described above), covalently joined to an aryl group (as described above). Preferably, the alkyl is a lower alkyl.

“Carbocyclic aryl” groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted with preferred groups as described for aryl groups above.

“Heterocyclic aryl” groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thiienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazoyl, and the like, all optionally substituted.

An “amide” refers to a —C(O)—NH—R, where R is either alkyl, aryl, alkylaryl, or hydrogen.

A “thioamide” refers to a —C(S)—NH—R, where R is either alkyl, aryl, alkylaryl, or hydrogen.

An “ester” refers to a —C(O)—OR’, where R’ is either alkyl, aryl, alkylaryl, or hydrogen.

An “amine” refers to a —N(R’’)R’’, where R’’ and R’’’ is each independently either hydrogen, alkyl, aryl, or alkylaryl, provided that R’’ and R’’’ are not both hydrogen.

A “thioether” refers to —S—R, where R is either alkyl, aryl, or alkylaryl.

A “sulfonyl” refers to —S(O)2—R, where R is aryl, C(CN)=C—aryl, CH2—CN, alkylaryl, NH—alkyl, NH—alkylaryl, or NH—aryl.

B. MMPT Analogs

An aspect of the present invention relates to a compound having the structure:

```
O
\|--N--R1
|      |
R2----R3
```

wherein R1 is chosen from the group consisting of

```
\|--N--R4
|      |
S----R5
```

and

```
\|--O--R4
|      |
S----R5
```

wherein R2 is alkyl; and

wherein R3 is aryl; and
wherein $R_5$ is selected from the group consisting of alkyl and hydrogen.

"MMTP analogs" is defined herein as including compound described by the above structure (excluding MMTP) as well as any compound that is involved in the synthesis of MMTP but which is not described by the above structure (e.g., certain compounds in Table 9) and any compound described herein which is indicated to be a MMTP analog but which is not described by the above structure (e.g., certain compounds in Table 1). All compounds described by the above structure (excluding MMTP) are examples of "MMTP analogs".

In certain embodiments, $R_1$ is

In certain embodiments, $R_5$ is hydrogen.

In certain embodiments, $R_2$ is lower alkyl (e.g., CH).

$R_4$ may be heterocyclic aryl or carbocyclic aryl. In certain embodiments, $R_4$ is

wherein $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are each independently selected from the group consisting of hydrogen, halogen, alkyl, alkoxy, OH, trihalomethyl, NO$_2$, ester, amide, and amine. In certain embodiments, $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are each independently selected from the group consisting of hydrogen, alkoxy, and OH.

$R_3$ may be heterocyclic aryl or carbocyclic aryl. In certain embodiments, $R_3$ is

wherein $X_6$, $X_7$, $X_8$, $X_9$, and $X_{10}$ are each independently selected from the group consisting of hydrogen, halogen, alkyl, alkoxy, OH, trihalomethyl, NO$_2$, ester, amide, and amine.
In certain embodiments, \(X_6, X_7, X_8, X_9,\) and \(X_{10}\) are each independently selected from the group consisting of hydrogen, alkoxy and OH. In certain embodiments, \(X_8\) is \(\text{CH}_3\). In certain embodiments, \(X_3\) is OH.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is hydrogen, \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is OH, \(X_7\) is hydrogen, \(X_8\) is OH, \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is hydrogen, \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is \(\text{-O-CH}_2\text{-CH}_3\), \(X_7\) is hydrogen, \(X_8\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is hydrogen, \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is hydrogen, \(X_7\) is \(-\text{O-CH}_3\), \(X_8\) is \(\text{OH}\), \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is hydrogen, \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is \(-\text{O-CH}_3\), \(X_7\) is hydrogen, \(X_8\) is \(-\text{O-CH}_3\), \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is OH, \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is hydrogen, \(X_7\) is \(-\text{O-CH}_3\), \(X_8\) is \(\text{OH}\), \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is \(-\text{O-CH}_3\), \(X_8\) is \(\text{OH}\), \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In a certain embodiment, \(X_1\) is hydrogen, \(X_2\) is hydrogen, \(X_3\) is \(-\text{O-CH}_3\), \(X_4\) is hydrogen, \(X_5\) is hydrogen, \(X_6\) is hydrogen, \(X_7\) is \(-\text{O-CH}_3\), \(X_8\) is hydrogen, \(X_9\) is hydrogen, and \(X_{10}\) is hydrogen.

In certain embodiments, \(R_3\) and \(R_4\) are each independently selected from the group consisting of

\[
\begin{align*}
\text{Y}_1 & \quad \text{Y}_2 \\
\text{Y}_3 & \quad \text{Y}_2
\end{align*}
\]

wherein \(Y_1, Y_2,\) and \(Y_3\) are each independently selected from the group consisting of halogen, alkyl, alkoxy, OH, trihalomethyl, \(\text{NO}_2\), ester, amide, and amine. In certain embodiments, \(Y_1, Y_2,\) and \(Y_3\) are each independently selected from the group consisting of
alkoxy and OH. In certain embodiments, Y₁, Y₂, and Y₃ are each independently selected from the group consisting of -O-CH₃ and OH.

C. Examples of MMPT Analogs

In certain embodiments of the present invention, one may select analogs or derivatives of MMPT having the structural attributes based on a pharmacophore of the MMPT molecule including the general formula of either of the thiazolone structures below (which are tautomers):

\[
\text{NH} \quad \text{N} \quad \text{N} \quad \text{O} \\
\text{S} \quad \text{S}
\]

It is thus recognized that structures of compounds of the present invention may be in either tautomeric form.

The MMPT pharmacophore can be used to rationally choose or design analogs or derivatives with the same attributes as the lead compound that may be more effective as therapeutic agents. One would choose or design compounds having a chemical modification of the NH group (or N) at position 2 of the pharmacophore and/or at the carbon at position 5 of the pharmacophore. Such modifications as contemplated in the present invention may include aromatic compounds such as a benzene or benzene derivative, or a heterocyclic or heterocyclic aromatic compound such as a pyridine, thiophene, pyrrole, or furan. In some embodiments of the invention, the aromatic or heterocyclic compound may further include an alkane, alkene or alkyne compound. In a further embodiment, the aromatic or heterocyclic compound may include an alcohol, ester, ether, amine, aldehyde, ketone, carboxylic acid, amide or haloalkane compound. In some embodiments, the modification may include an alkylamine, aromatic amine, or heterocyclic amine compound; an acyl compound such as a carboxylic acid derivative, ester, or amide; a nitrile, aldehyde or ketone compound; an alcohol or ether compound; an organic halide such as a benzyl halide or alkyl halide compound. However, it is noted that the above examples are not meant to be limiting.

Examples of preferred modification to the NH group (or N group) at position 2 of the pharmacophore and/or at the carbon at position 5, to produce analogs or derivatives of MMPT, are provided in Table 1. Other examples of MMPT analogs are set forth in the Example section below. However, these examples are not meant to be limiting.
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The term "drug" is intended to refer to a chemical entity, whether in the solid, liquid, or gaseous phase which is capable of providing a desired therapeutic effect when administered to a subject. The term "drug" should be read to include synthetic compounds, natural products and macromolecular entities such as polypeptides, polynucleotides, or lipids and also small entities such as ligands, hormones or elemental compounds. The term "drug" is meant to refer to that compound whether it is in a crude mixture or purified and isolated.

### III. Screening to Identify Analogs of the Thiazolone Compound MMPT

In a particular embodiment, the present invention provides methods for identifying analogs of the thiazolone compound MMPT. MMPT may be used as a target in screening for similar compounds that inhibit microtubule polymerization activity, induce cell cycle arrest, inhibit cell growth or induce apoptosis in cells such as cancer cells. Assays may focus on particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to mimic the effect of MMPT. In some instances, libraries may be randomly screened for candidate substances. By effect, it is meant that one may assay for inhibition of microtubule polymerization activity, cell cycle arrest, growth inhibition, or induction of apoptosis in a hyperproliferative cell such as a cancer cell.
To identify a MMPT analog, one generally will determine the anticancer activity in the presence and absence of the candidate substance, wherein an analog is identified by its ability to inhibit microtubule polymerization activity, induce cell cycle arrest, inhibit cell growth or induce apoptosis in cells such as cancer cells. For example, a method may generally comprise:

a) providing a cell;
b) contacting the cell with a candidate substance;
c) analyzing the cell for inhibition of growth; and
d) comparing the inhibition of growth in the cell from step (c) with the inhibition of growth in the cell in the absence of the candidate substance, wherein growth inhibition in the presence of the candidate substance indicates that said cell is susceptible to the candidate substance.

Assays may be conducted in isolated cells, or in organisms including transgenic animals. It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. These assays may be performed at a lab bench by a human operator, via mechanized high through-put screening, or any other manner known in the art. The candidate substance(s) tested may be an individual candidate or one or more of a library of candidates and may be obtained from any source and in any manner known to those of skill in the art.

As used herein the term "candidate substance" or "candidate compound" refers to any MMPT compound that may potentially inhibit microtubule polymerization activity, induce cell cycle arrest, inhibit cell growth or induce apoptosis. Any compound or molecule described in the methods and compositions herein (e.g., MMPT or an MMPT analog) may be a candidate substance or compound.

The MMPT compound of the present invention may be used in rational drug design to produce structural analogs of biologically active compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for the MMPT compound of the invention or a portion thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches. An alternative approach, involves the random replacement of functional groups throughout the MMPT compound, and the resulting affect on the function of the analog or derivative determined.

Thus, one may design drugs which have improved biological activity, such as for example, inhibition of microtubule polymerization activity, cell cycle arrest, growth inhibition,
or induction of apoptosis, relative to starting MMPT compound. By virtue of the chemical isolation procedures and descriptions herein, sufficient amounts of the MMPT compounds of the invention can be produced to perform crystallographic studies. In addition, knowledge of the chemical characteristics of these compounds permits computer employed predictions of structure-function relationships. Computer models of various chemical structures are also available in the literature or computer databases. Such databases may be used by one of ordinary skill in the art to identify MMPT analogs.

The term "drug" is intended to refer to a chemical entity, whether in the solid, liquid, or gaseous phase which is capable of providing a desired therapeutic effect when administered to a subject. The term "drug" should be read to include synthetic compounds, natural products and macromolecular entities such as polypeptides, polynucleotides, or lipids and also small entities such as ligands, hormones or elemental compounds. The term "drug" is meant to refer to that compound whether it is in a crude mixture or purified and isolated.

IV. Combined Cancer Therapy with Microtubule-Interacting Agents and/or Other Anticancer Agents

In the context of the present invention, it is contemplated that the thiazolone, MMPT or analogs thereof may be used in combination with an additional therapeutic agent to more effectively treat a cancer. Cancers contemplated by the present invention include, but are not limited to, breast cancer, lung cancer, head and neck cancer, bladder cancer, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, or uterine cancer.

Additional therapeutic agents contemplated for use in combination with the thiazolone compound MMPT or analogs thereof include but are not limited to microtubule-interacting agents and anticancer agents. Anticancer agents may include but are not limited to, radiotherapy, chemotherapy, gene therapy, hormonal therapy or immunotherapy that targets cancer/tumor cells.

To kill cells, inhibit microtubule polymerization activity, induce cell-cycle arrest, inhibit cell growth, inhibit metastasis, inhibit angiogenesis or otherwise reverse or reduce the malignant phenotype of cancer cells, using the methods and compositions of the present invention, one would generally contact a cell with MMPT or analogs thereof in combination with an additional therapeutic agent such as a microtubule-interacting agent. These compositions would be provided in a combined amount effective to inhibit cell growth and/or induce apoptosis in the
cell. This process may involve contacting the cells with MMPT or analogs thereof in combination with an additional therapeutic agent or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the MMPT or derivatives thereof and the other includes the additional agent.

Alternatively, treatment with MMPT or analogs thereof may precede or follow the additional agent treatment by intervals ranging from minutes to weeks. In embodiments where the additional agent is applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hr of each other and, more preferably, within about 6-12 hr of each other, with a delay time of only about 12 hr being most preferred. In some situations, it may be desirable to extend the treatment period significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either MMPT or analogs thereof in combination with an additional therapeutic agent such as a microtubule-interacting agent or anticancer agent will be desired. Various combinations may be employed, where MMPT or analogs thereof is "A" and the additional therapeutic agent is "B", as exemplified below:

A/B/A  B/A/B  B/B/A  A/A/B  B/A/A  A/B/B  B/B/A  B/B/A/B  A/A/B/B  A/B/B/A  B/B/A/A  B/B/A/B  B/B/A/B  B/B/A/A  A/B/A/B  A/B/B/B  B/A/B/B  B/A/B/A  A/B/A/B

Other combinations are contemplated. Again, to achieve cell killing by the induction of apoptosis, both agents may be delivered to a cell in a combined amount effective to kill the cell.

A. Microtubule-interacting Agents

Tubulin-containing structures are important for diverse cellular functions, including chromosome segregation during cell division, intracellular transport, development and maintenance of cell shape, cell motility, and possibly distribution of molecules on cell membranes. The agents that interact with tubulin are heterogenous in chemical structure (Angerer, 2000).
However, a common characteristic of these agents is the while binding to tubulin, they cause its precipitation and sequestration to interrupt many important biologic functions that depend on the microtubular class of subcellular organelles. Most microtubule-interacting agents that interfere with normal spindle formation by inducing microtubule polymerization or depolymerization, can cause cell-cycle arrest at the metaphase to anaphase transition (Sorger et al., 1997).

Although most microtubule-interacting agents induce apoptosis in cancer cells, the mechanisms by they do so remain uncharacterized. It is speculated that different mechanisms may be involved in the induction of apoptosis by MMPT. First, MMPT may induce apoptosis directly after mitotic arrest. For example, endonucleolytic cleavage of DNA is observed only in the cells at G2-M-phase arrest after epothilone and paclitaxel treatment (Wang et al., 2000). Moreover, it has been shown that paclitaxel-induced cell death in cancer cells might derive from apoptosis directly after mitotic arrest (Wood et al., 1995; Basu et al., 1998). Several investigators have also reported that Bcl-2 phosphorylation may be involved in one of the multiple pathways of antimitotic drug-induced apoptosis (Blagosklonny et al., 1997; Wang et al., 1999). For example, in the present invention phosphorylated Bcl-2 was increased in H460 cells after treatment with MMPT. The time course of regulation of the phosphorylation status of Bcl-2 in H460 cells appeared to be correlated with that of MPM-2, cdc25C, and histone H3.

Taxanes such as taxol and taxotere and vinca alkaloids such as vincristine and vinblastine are considered antimicrotubule agents that interfere with cell division by disrupting the normal functionality of the cellular microtubules. Thus, microtubule interacting agents are classified into two major groups: the first, which includes agent such as vinca alkaloids, colchicine and nocodazole, interferes with mitosis and changes the balance of the tubulin in a microtubule to a depolymerized state; the second group includes agents such as the taxanes, which change the balance of the tubulin to a polymerized state. Vinca alkaloids, such as vinorelbine and vincristine have been widely used as antitumor drugs for the treatment of hematopoietic neoplasia and some solid tumors (Jordan et al., 1998; Correia and Lobert, 2001; Ngan et al., 2000; Masters et al., 1998). The taxanes, including paclitaxel and docetaxel are newer cytotoxic antitumor agents that are used clinically for the treatment of several solid tumors of the head and neck, breast, lung, ovary, and bladder (Rowinsky, 1997; Sandercock et al., 1998; Fossella et al., 1994), taxanes prevent the disassembly of microtubules.

1. Vinca alkaloids

The microtubule interacting agents, vinca alkaloids are a type of plant alkaloid identified to have pharmaceutical activity. Vinca alkaloids include, but are not limited to, vinblastine,
vincristine, vindoline, vinodesine, vinleurosine, vinrosidine, vinorelbine, or derivatives thereof (see, e.g., the Merck Index, 11th Edition (1989) entries 9887, 9891, and 9893, for vinblastine, vincristine, and vindoline). See also U.S. Patents 5,620,985 and 6,127,377. These agents are known in the art to disrupt mitosis and change the balance of the tubulin in a microtubule to a depolymerized state.

a. Vinblastine

Vinblastine is an example of a vinca alkaloid that is known in the art for the treatment of cancer and precancer. This agent is known to cause dissolution of the microtubules in cells. Unpredictable absorption has been reported after oral administration of vinblastine. At the usual clinical doses the peak concentration of this agent in plasma is approximately 0.4 mM. Vinblastine is known to bind to plasma proteins and is extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes.

After intravenous injection, vinblastine has a multiphasic pattern of clearance from the plasma; after distribution, it disappears from the plasma with half-lives of approximately 1 and 20 hr. Vinblastine is metabolized in the liver to a biologically active derivative, desacetylvinblastine. Approximately 15% of an administered dose is detected intact in the urine, and about 10% is recovered in the feces after biliary excretion. In treating patients with hepatic dysfunction, the dosage is reduced. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Vinblastine sulfate is available in preparations for injection. When the drug is given intravenously; special precautions must be taken against subcutaneous extravasation, since this may cause painful irritation and ulceration. The drug should not be injected into an extremity with impaired circulation. After a single dose of 0.3 mg/kg of body weight, myelosuppression reaches its maximum in about 7 days to about 10 days. If a moderate level of leukopenia (approximately 3000 cells/mm$^3$) is not attained, the weekly dose may be increased gradually by increments of about 0.05 mg/kg of body weight. In regimens designed to cure testicular cancer, vinblastine is used in doses of about 0.3 mg/kg about every 3 weeks irrespective of blood cell counts or toxicity.

An important clinical use of vinblastine is with bleomycin and cisplatin in the curative therapy of metastatic testicular tumors. Beneficial responses have been reported in various lymphomas, particularly Hodgkin's disease, where significant improvement may be noted in 50 to 90% of cases. The effectiveness of vinblastine in a high proportion of lymphomas is not diminished when the disease is refractory to alkylating agents. It is also active in Kaposi's
sarcoma, testis cancer, neuroblastoma, and Letterer-Siwe disease (histiocytosis X), as well as in carcinoma of the breast and choriocarcinoma in women.

Doses of about 0.1 mg/kg to about 0.3 mg/kg can be administered or about 1.5 mg/m² to about 2 mg/m² can also be administered. Alternatively, about 0.1 mg/m², about 0.12 mg/m², about 0.14 mg/m², about 0.15 mg/m², about 0.2 mg/m², about 0.25 mg/m², about 0.5 mg/m², about 1.0 mg/m², about 1.2 mg/m², about 1.4 mg/m², about 1.5 mg/m², about 2.0 mg/m², about 2.5 mg/m², about 5.0 mg/m², about 6 mg/m², about 8 mg/m², about 9 mg/m², about 10 mg/m², to about 20 mg/m², can be given.

b. Vincristine

Another well known mitorubule-interacting agent belonging to the family of vinca alkaloids is vincristine, also known as leurocristine sulfate, 22-oxovincaleukoblastine, Kyocristine, vincosid, vincrrex, oncovicin, Vincasar PFS™, or VCR, is commercially available from any of a number of sources (e.g., Pharmacia & Upjohn, Eli Lilly, IGT, etc.). Vincristine is often supplied as vincristine sulfate (e.g., 1 mg/ml) for intravenous injection.

This agent is known to blocks mitosis and produces metaphase arrest. Most of the biological activities of this drug is explained by its ability to bind specifically to tubulin and to block the ability of protein to polymerize into microtubules. Through disruption of the microtubules of the mitotic apparatus, cell division is arrested in metaphase. The inability to segregate chromosomes correctly during mitosis presumably leads to cell death.

The relatively low toxicity of vincristine for normal marrow cells and epithelial cells make this agent unusual among anti-neoplastic drugs, and it is often included in combination with other myelosuppressive agents.

Similar to that of vinblastine, unpredictable absorption of vincristine has been reported after oral administration. At the usual clinical doses the peak concentration of this agent in plasma is about 0.4 mM. Vincristine is also known to bind to plasma proteins and is extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes. Vincristine has a multiphasic pattern of clearance from the plasma; the terminal half-life is about 24 hr. The drug is metabolized in the liver, but no biologically active derivatives have been identified. In treating patients with hepatic dysfunction, the dosage is reduced. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than about 3 mg/dl (about 50 mM).

Vincristine used together with corticosteroids is presently the treatment of choice to induce remissions in childhood leukemia; the optimal dosages for these drugs appear to be vincristine, intravenously, about 2 mg/m² of body-surface area, weekly; and prednisone, orally,
about 40 mg/m², daily. Adult patients with Hodgkin's disease or non-Hodgkin's lymphomas usually receive vincristine as a part of a complex protocol. When used in the MOPP (mechloretamine, prednisone, and procarbazine) regimen, the recommended dose of vincristine is about 1.4 mg/m². High doses of vincristine seem to be tolerated better by children with leukemia than by adults, who may experience severe neurological toxicity. Administration of the drug more frequently than every 7 days or at higher doses seems to increase the toxic manifestations without proportional improvement in the response rate. Precautions should also be used to avoid extravasation during intravenous administration of vincristine. Vincristine (and vinblastine) can be infused into the arterial blood supply of tumors in doses several times larger than those that can be administered intravenously with comparable toxicity.

Vincristine has been effective in Hodgkin's disease and other lymphomas. Although it appears to be somewhat less beneficial than vinblastine when used alone in Hodgkin's disease, when used with mechloretamine, prednisone, and procarbazine (the so-called MOPP regimen), it is the preferred treatment for the advanced stages (III and IV) of this disease. In non-Hodgkin's lymphomas, vincristine is an important agent, particularly when used with cyclophosphamide, bleomycin, doxorubicin, and prednisone. Vincristine is more useful than vinblastine in lymphocytic leukemia. Beneficial response have been reported in patients with a variety of other neoplasms, particularly Wilms' tumor, neuroblastoma, brain tumors, rhabdomyosarcoma, small cell lung, and carcinomas of the breast, bladder, and the male and female reproductive systems.

Doses of vincristine include about 0.01 mg/kg to about 0.03 mg/kg or about 0.4 mg/m² to about 1.4 mg/m² can be administered or about 1.5 mg/m² to about 2 mg/m² can also be administered. Alternatively, in certain embodiments, about 0.02 mg/m², about 0.05 mg/m², about 0.06 mg/m², about 0.07 mg/m², about 0.08 mg/m², about 0.1 mg/m², about 0.12 mg/m², about 0.14 mg/m², about 0.15 mg/m², about 0.2 mg/m², about 0.25 mg/m² can be given as a constant intravenous infusion.

2. Taxanes

Another class of microtubule-interacting agents contemplated for use in the present invention are the taxanes. These agents play a role in the polymerization state of tubulin. Taxanes and related active ingredients are produced by plants of the Taxus species and are constituents of different parts of such plants. Taxanes, such as taxol (paclitaxel), are cyclotoxic diterpenes obtained from the yew tree. Taxanes are known in the art to inhibit cell replication on
a molecular basis, in that they inhibit growing cells in the G2/M phase of the cell cycle. Thus, taxanes have an anti-tumor effect and are used increasingly for the treatment of a series of carcinomas (ovarian, breast, bronchial and lung carcinomas).

a. Paclitaxel/Taxol

Paclitaxel, also known as taxol is a diterpene alkaloid thus it possesses a taxane skeleton in its structure. Paclitaxel is extracted from the bark of the Pacific yew (Taxus brevifolia) as a natural compound having anti-cancer activity (Fuchs and Johnson, 1978). Paclitaxel works against cancer by interfering with mitosis. Paclitaxel is a taxoid drug, widely used as an effective treatment of primary and metastatic cancers.

Paclitaxel (Taxol) is widely used in the treatment of breast, ovarian, and other solid tumors. Randomized clinical trials have shown a survival advantage among patients with primary breast cancer who received paclitaxel in addition to anthracycline-containing adjuvant chemotherapy (Eifel et al., 2001). Furthermore, paclitaxel is effective for both metastatic breast cancer (Holmes et al., 1991; Nabholtz et al., 1996; Bishop et al., 1999) and advanced ovarian cancer (McGuire et al., 1996; Piccart et al., 2000). Due to the antimitotic activity of paclitaxel it is a useful cytotoxic agent in treating several classic refractory tumors. It may also be used in treating head and neck cancer, Kaposi’s sarcoma and lung cancer, small cell and non-small cell lung cancer. It may also slow the course of melanoma. Response rates to taxol treatment varies among cancers. Advanced drug refractory ovarian cancer responds at a 19-36% rate, previously treated metastatic breast cancer at 27-62%, and various lung cancers at 21-37%. Taxol has also been shown to produce complete tumor remission in some cases (Guchelaar et al., 1994).

The antitumor activity of paclitaxel is unique because it promotes microtubule assembly and stabilizes the microtubules, thus preventing mitosis (Huizing et al., 1995). Paclitaxel does this by reversibly and specifically binding to the beta subunit of tubulin, forming microtubule polymers thereby stabilizing them against depolymerization and thus leading to growth arrest in the G2/M phase of the cell cycle (Gotaskie and Andreassi, 1994). This makes taxol unique in comparison to vincristine and vinblastine which cause microtubule disassembly (Gatzemeier et al., 1995). Additionally, recent evidence indicates that the microtubule system is essential to the release of various cytokines and modulation of cytokine release may play a major role in the drug's antitumor activity (Smith et al., 1995).

However, some patients are resistant to paclitaxel therapy. Previous reports have demonstrated that paclitaxel resistance is due to a variety of mechanisms such as up-regulation of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-XL (Tang et al., 1994); up-regulation of membrane transporters (e.g., mdr-1), resulting in an increased drug efflux (Huang
et al., 1997); mutations in beta-tubulin resulting in abolishment of paclitaxel binding (Giannakakou et al., 1997); and up-regulation of ErbB2 (HER2) through inhibition of cyclin-dependent kinase-1 (Cdk1), resulting in delayed mitosis (Yu et al., 1998).

Paclitaxel is given intravenously since it irritates skin and mucous membranes on contact. It is typically administered intravenously by a 3 to 24 hour infusion three times per week (Guchelaar et al., 1994).

Paclitaxel (which may include formulations, prodrugs, analogues and derivatives such as, for example, TAXOL™, TAXOTEREM™, docetaxel, 10-desacetyl analogues of paclitaxel and 3‘N-desbenzoyl-3‘N-t-butoxy carbonyl analogues of paclitaxel) are known to those skilled in the art (see, e.g., Schiff et al., 1979; Long and Fairchild, 1994; Ringel and Horwitz, 1991; Pazdur et al., 1993; PCT Applications. WO 94/07882; WO 94/07881; WO 94/07880; WO 94/07876; WO 93/23555; WO 93/10076; WO94/00156; WO 93/24476; EP 590267; WO 94/20089; U.S. Pat. Nos. 5,294,637; 5,283,253; 5,279,949; 5,274,137; 5,202,448; 5,200,534; 5,229,529; 5,254,580; 5,412,092; 5,395,850; 5,380,751; 5,350,866; 4,857,653; 5,272,171; 5,411,984; 5,248,796; 5,422,364; 5,300,638; 5,362,831; 5,440,056; 4,814,470; 5,278,324; 5,352,805; 5,059,699; 4,942,184; or obtained from a variety of commercial sources, including for example, Sigma, St. Louis, MO (T7402—from Taxus brevifolia).

b. Docetaxel/Taxotere

Docetaxel is another microtubule-interacting, antineoplastic agent belonging to the taxoid family. Docetaxel has primarily been used to treat breast cancer, lung cancer, non-small cell lung cancer. In addition, it may be used to treat head and neck cancer, small cell lung cancer, mesothelioma, ovarian cancer, prostate cancer, and urothelial transitional cell cancer. Docetaxel interferes with the growth of cancer cells, which are eventually destroyed. However, as discussed above in regards to paclitaxel therapy, some patients are resistant to docetaxel therapy.

Docetaxel is a semi-synthetic drug derived from precursor extracted from the needles of the European yew tree, Taxus baccata. The chemical name for docetaxel is (2R,3S)-N-carboxy-3-phenylisoserine, N-tert-butyl ester, 13-ester with 5b-20-epoxy-12a,4,7b,10b,13a-hexahydroxymethoxytetra-11-en-9-one 4-acetate 2-benzoate, trihydrate. It acts by disrupting the microtubular network that is essential for mitotic and interphase cellular functions. It promotes the assembly of tubulin into stable microtubules and inhibits their disassembly, causing inhibition of cell division and eventual cell death. Both docetaxel and paclitaxel bind to the same microtubule site, although the affinity of docetaxel is 1.9-fold higher. Cross-resistance between docetaxel and paclitaxel does not occur consistently. Docetaxel is a radiation-sensitizing agent. It is cell cycle phase-specific (G2/M phase).
c. Other Taxanes

The present invention also contemplates any microtubule interacting agents belonging to the taxane family of compounds for use in combination with the thialozone, MMPT or analogs thereof. Such compounds may have a taxane skeleton as is known to one of ordinary skill in the art, and may include taxane analogues or derivatives having anticancer activity. The taxane compounds contemplated in the present invention are those compounds that are useful for inhibiting microtubule polymerization activity and inhibiting tumor growth in subjects (patients) having cancer. The term ‘taxane compound’ also is intended to include pharmaceutically acceptable salts of the compounds. Taxane compounds have previously been described in U.S. Patent Nos. 5,641,803, 5,665,671, 5,380,751, 5,728,687, 5,415,869, 5,407,683, 5,399,363, 5,424,073, 5,157,049, 5,773,464, 5,821,263, 5,840,929, 4,814,470, 5,438,072, 5,403,858, 4,960,790, 5,433,364, 4,942,184, 5,362,831, 5,705,503, 5,278,324, 5,840,929, 5,773,464, 5,248,796, 5,821,263, 4,814,470, 5,438,072, 4,960,790, 4,942,184, 5,433,364, 5,278,324, 6,362,217, 6,017,935, 5,977,376, 5,912,264, 5,773,464, 5,739,539, 5,698,712, 5,250,722, 6,284,746; U.S. Patent Appln. Nos. 20030144344, 20030130341, 20030134793, 20030130170, 20030130178, 20030124055, and 20020016356; and PCT Applns. WO 95/33740, 96/03394, 95/33736, 93/02067, 94/15929 and 94/15599; all of which are incorporated herein by reference.

Other taxanes may include water soluble compositions of paclitaxel and docetaxel formed by conjugating the paclitaxel or docetaxel to a water soluble polymer such as polyglutamic acid, poly-aspartic acid or poly-lysine (U.S. Patent Application 20030147807). Derivatives of paclitaxel possess varying degrees of pharmacological activity. Since the discovery of paclitaxel, over one hundred compounds having a related structure have been isolated from various species of Taxus and/or made synthetically.

One exemplary paclitaxel derivative having desirable antitumor properties, is the compound, 7-O-methylthiomethyl paclitaxel (herein referred to as "$7-O$-MTM paclitaxel") which differs structurally from paclitaxel at the C-7 position on the taxane ring. 7-O-MTM paclitaxel is a known antitumor agent currently under study in clinical trials. Studies involving 7-O-MTM paclitaxel have shown promising results in the treatment of gastrointestinal and colorectal cancers where paclitaxel has been found to be less effective. It is known that 7-O-MTM paclitaxel may be produced by synthetic processes (U.S. Patent 5,646,176, and WO 96/00724; content of which are each incorporated herein by reference).

Representative examples of paclitaxel derivatives or analogues contemplated in the present invention may include, but are not limited to, 7-deoxy-docetaxol, 7,8-cyclopropataxanes, N-substituted 2-azetidones, 6,7-epoxy paclitaxels, 6,7-modified paclitaxels, 10-desacetoxytaxol,
10-deacetyltaxol (from 10-deacetylbaccatin III), phosphonoxy and carbonate derivatives of taxol, taxol 2',7-di(sodium 1,2-benzenedicarboxylate, 10-desacetoxy- 11,12-dihydotaxol- 10,12(18)-diene derivatives, 10-desacetoxytaxol, Protaxol (2'-and/or 7-O-ester derivatives), (2'- and/or 7-O-carbonate derivatives), asymmetric synthesis of taxol side chain, fluoro taxols, 9- deoxotaxane, (13-acetyl-9-deoxobaccatine III, 9-deoxotaxol, 7-deoxy-9-deoxotaxol, 10- desacetoxy-7-deoxy-9-deoxotaxol; derivatives containing hydrogen or acetyl group and a hydroxy and tert-butoxycarbonylamino; sulfonated 2'-acryloyltaxol and sulfonated 2'-O-acetyl acid taxol derivatives, succinyltaxol, 2'-γ-aminobutyryltaxol formate, 2' -acetyl taxol, 7-acetyl taxol, 7-glycine carbamate taxol, 2'-OH-7-PEG(5000) carbamate taxol, 2'-benzoyl and 2',7- dibenzoyl taxol derivatives; other prodrugs (2'-acetyltaxol; 2',7-diacetyltaxol; 2'succinyltaxol; 2'-(beta-alanyl)-taxol); 2'gamma-aminobutyryltaxol formate; ethylene glycol derivatives of 2'- succinyltaxol; 2' -glutaryltaxol; 2'-(N,N-dimethylglycyl) taxol; 2'-(2-(N,N-dimethylamino)propionyl)taxol; 2'orthocarboxybenzoyl taxol; 2'aliphatic carboxylic acid derivatives of taxol; prodrugs {2'(N,N-diethylaminopropionyl)taxol, 2'(N,N-dimethylglycyl)taxol, 7(N,N-dimethylglycyl)taxol, 2',7-di-(N,N-di methylglycyl)taxol, 7(N,N-diethylaminopropionyl)taxol, 2',7-di(N,N-diethylaminopropionyl)taxol, 2'-(L-glucyl)taxol, 7-(L-glycyl)taxol, 2',7-di-(L-glycyl)taxol, 2'-(L-alanyl)taxol, 7-(L-alanyl)taxol, 2',7-di(L-alanyl)taxol, 2'-(L-leucyl)taxol, 7-(L-leucyl)taxol, 2',7-di(L-leucyl)taxol, 2'-(L-isoleucyl)taxol, 7-(L-isoleucyl)taxol, 2',7-di(L-isoleucyl)taxol, 2'-(L-valyl)taxol, 7-(L-valyl)taxol, 2',7-di(L-valyl)taxol, 2'-(L-phenylalanyl)taxol, 7-(L-phenylalanyl)taxol, 2',7-di(L-phenylalanyl)taxol, 2'-(L-prolyl)taxol, 7-(L-prolyl)taxol, 2',7-di(L-prolyl)taxol, 2'-(L-lysyl)taxol, 7-(L-lysyl)taxol, 2',7-di(L-lysyl)taxol, 2'-(L-glutamyl)taxol, 7-(L-glutamyl)taxol, 2',7-di(L-glutamyl)taxol, 2'-(L-arginyltaxol, 7-(L-arginyltaxol, 2',7-di(L-arginyltaxol}; taxol analogs with modified phenylisoserine side chains, taxotere, (N-debenzoyl-N-tert-(butoxycaronyl)-10- deacetyltaxol, and taxanes (e.g., baccatin III, cephalomannine, 10-deacetylbaccatin III, brevifoliol, yunantaxusin and taxusin); and other taxane analogues and derivatives, including 14-beta hydroxy-10 deacetylbaccatin III, debenzoyl-2-acyl paclitaxel derivatives, benzoate paclitaxel derivatives, phosphonoxy and carbonate paclitaxel derivatives, sulfonated 2'-acryloyltaxol; sulfonated 2'-O-acetyl acid paclitaxel derivatives, 18-site- substituted paclitaxel derivatives, chlorinated paclitaxel analogues, C4 methoxy ether paclitaxel derivatives, sulfinamide taxane derivatives, brominated paclitaxel analogues, Girard taxane derivatives, nitrophenyl paclitaxel, 10-deacetylated substituted paclitaxel derivatives, 14- beta-hydroxy-10 deacetylbaccatin III taxane derivatives, C7 taxane derivatives, C10 taxane derivatives, 2-debenzoyl-2-acyl taxane

3. Other microtubule interacting agents

A wide variety of anti-microtubule agents can be utilized within the context of the present invention. Representative examples of such anti-microtubule agents includes colchicine, LY290181, glycine ethyl ester, aluminum fluoride, and CI 980 (Allen et al., 1991; Ding et al., 1990; Gonzalez et al., 1991; Stargell et al., 1992; Garcia et al., 1995), discodermolide (ter Haar et al., 1996), D-24851 and D-64131 (Bacher et al., 2001), podophyllotoxins as well as analogues and derivatives of any of these (see also PCT/CA97/00910 (WO 98/24427), incorporated by reference, for a list of additional anti-microtubule agents). Such compounds can act by either depolymerizing microtubules or by stabilizing microtubule formation and thus may be used in combination with the thialozone, MMPT or analogs thereof in treating cancer.

B. Other Anticancer Agents

1. Chemotherapeutic Agents

The present invention also contemplates the use of chemotherapeutic agents in combination with MMPT or an analog thereof in the treatment of cancer. Examples of such chemotherapeutic agents may include, but are not limited to, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosourea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, gemcitabine, navelbine, farnesyl-protein transference inhibitors, cisplatinum, 5-fluorouracil and methotrexate, or any analog or derivative variant of the foregoing.

2. Radiotherapeutic Agents

Radiotherapeutic agents may also be used in combination with the compounds of the present invention in treating a cancer. Such factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the
assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

3. **Immunotherapeutic Agents**

Immunotherapeutics may also be employed in the present invention in combination with MMPT or analogs thereof in treating cancer. Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

Generally, the tumor cell must bear some marker that is amenable to targeting, i.e., is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155.

4. **Inhibitors of Cellular Proliferation**

The tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16 and C-CAM are described below.

High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein et al., 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue.
Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G1. The activity of this enzyme may be to phosphorylate Rb at late G1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16\textsuperscript{INK4} has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano et al., 1993; Serrano et al., 1995). Since the p16\textsuperscript{INK4} protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

p16\textsuperscript{INK4} belongs to a newly described class of CDK-inhibitory proteins that also includes p16\textsuperscript{B}, p19, p21\textsuperscript{WAF1}, and p27\textsuperscript{KIP1}. The p16\textsuperscript{INK4} gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16\textsuperscript{INK4} gene are frequent in human tumor cell lines. This evidence suggests that the p16\textsuperscript{INK4} gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the p16\textsuperscript{INK4} gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas et al., 1994; Cheng et al., 1994; Hussussian et al., 1994; Kamb et al., 1994; Kamb et al., 1994; Mori et al., 1994; Okamoto et al., 1994; Nobori et al., 1995; Orlow et al., 1994; Arap et al., 1995). Restoration of wild-type p16\textsuperscript{INK4} function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

Other genes that may be employed according to the present invention include Rb, mda-7, APC, DCC, NF-1, NF-2, WT-1, MEN-1, MEN-II, zac1, p73, VHL, MMAC1/PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (e.g., COX-1, TFPI), PGS, Dp, E2F, ras, myc, neu, raf, erb, fps, trk, ret, gsp, hst, abl, E1A, p300, genes involved in angiogenesis (e.g., VEGF, FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.
5. Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential process in cancer therapy (Kerr et al., 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi et al., 1985; Cleary and Sklar, 1985; Cleary et al., 1986; Tsujimoto et al., 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Members of the Bcl-2 that function to promote cell death such as, Bax, Bak, Bik, Bim, Bid, Bad and Harakiri, are contemplated for use in combination with MMPT or an analog thereof in treating cancer.

6. Surgery

It is further contemplated that a surgical procedure may be employed in the present invention. Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and micropically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

7. Hormonal Therapy

Hormonal therapy may also be used in conjunction with the MMPT or analog thereof as in the present invention, or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones.
such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

8. **Other agents**

It is contemplated that other agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, or agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas / Fas ligand, DR4 or DR5 / TRAIL would potentiate the apoptotic inducing abilities of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increased intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

V. **Formulations and Routes for Administration of MMPT or Analogs Thereof**

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions of MMPT or analogs thereof, or any additional therapeutic agent disclosed herein in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention in an effective amount may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase
"pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. In certain embodiments, pharmaceutical preparations are formulated for administration to a subject. The subject may be a mammal, such as a human. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The composition(s) of the present invention may be delivered orally, nasally, intramuscularly, intraperitoneally, or intratumorally. In some embodiments, local or regional delivery of MMPT or analogs thereof, alone or in combination with an additional therapeutic agent, to a patient with cancer or pre-cancer conditions will be a very efficient method of delivery to counteract the clinical disease. Similarly, chemo- or radiotherapy may be directed to a particular, affected region of the subject’s body. Regional chemotherapy typically involves targeting anticancer agents to the region of the body where the cancer cells or tumor are located. Other examples of delivery of the compounds of the present invention that may be employed include intra-arterial, intracavity, intravesical, intrathecal, intrapleural, and intraperitoneal routes.

Intra-arterial administration is achieved using a catheter that is inserted into an artery to an organ or to an extremity. Typically, a pump is attached to the catheter. Intracavity administration describes when chemotherapeutic drugs are introduced directly into a body cavity such as intravesical (into the bladder), peritoneal (abdominal) cavity, or pleural (chest) cavity. Agents can be given directly via catheter. Intravesical chemotherapy involves a urinary catheter to provide drugs to the bladder, and is thus useful for the treatment of bladder cancer. Intrapleural administration is accomplished using large and small chest catheters, while a Tenkhoff catheter (a catheter specially designed for removing or adding large amounts of fluid from or into the peritoneum) or a catheter with an implanted port is used for intraperitoneal chemotherapy. Abdomen cancer may be treated this way. Because most drugs do not penetrate the blood/brain barrier, intrathecal chemotherapy is used to reach cancer cells in the central nervous system. To do this, drugs are administered directly into the cerebrospinal fluid. This method is useful to treat leukemia or cancers that have spread to the spinal cord or brain.

Alternatively, systemic delivery of the chemotherapeutic drugs may be appropriate in certain circumstances, for example, where extensive metastasis has occurred. Intravenous
therapy can be implemented in a number of ways, such as by peripheral access or through a vascular access device (VAD). A VAD is a device that includes a catheter, which is placed into a large vein in the arm, chest, or neck. It can be used to administer several drugs simultaneously, for long-term treatment, for continuous infusion, and for drugs that are vesicants, which may produce serious injury to skin or muscle. Various types of vascular access devices are available.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes but is not limited to, oral, nasal, or buccal routes. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described supra. The drugs and agents also may be administered parenterally or intraperitoneally. The term "parenteral" is generally used to refer to drugs given intravenously, intramuscularly, or subcutaneously.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention may be administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH, exact concentration of the various components, and the pharmaceutical composition are adjusted according to well known parameters. Suitable excipients for formulation with MMPT or analogs thereof include croscarmellose sodium, hydroxypropyl
methylcellulose, iron oxides synthetic), magnesium stearate, microcrystalline cellulose, polyethylene glycol 400, polysorbate 80, povidone, silicon dioxide, titanium dioxide, and water (purified).

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent(s) of the present invention is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation or (ii) elimination of tumor cells. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

VI. Therapeutically Effective Amounts of MMPT and Analogs Thereof

A therapeutically effective amount of MMPT or analogs thereof, alone or in combination with an additional therapeutic agent, such as a microtubule-interacting agent or an anticancer agent, as a treatment varies depending upon the host treated and the particular mode of administration. In one embodiment of the invention the dose range of the MMPT or analogs thereof, alone or in combination with an additional agent used, will be about 0.5mg/kg body weight to about 500mg/kg body weight. The term "body weight" is applicable when an animal is being treated. When isolated cells are being treated, "body weight" as used herein should read to mean "total cell weight". The term "total weight may be used to apply to both isolated cell and animal treatment. All concentrations and treatment levels are expressed as "body weight" or simply "kg" in this application are also considered to cover the analogous "total cell weight" and "total weight" concentrations. However, those of skill will recognize the utility of a variety of dosage range, for example, 1mg/kg body weight to 450mg/kg body weight, 2mg/kg body weight to 400mg/kg body weight, 3mg/kg body weight to 350mg/kg body weight, 4mg/kg body weight to 300mg/kg body weight, 5mg/kg body weight to 250mg/kg body weight, 6mg/kg body weight to 200mg/kg body weight, 7mg/kg body weight to 150mg/kg body weight, 8mg/kg
body weight to 100mg/kg body weight, or 9mg/kg body weight to 50mg/kg body weight. Further, those of skill will recognize that a variety of different dosage levels will be of use, for example, 1mg/kg, 2mg/kg, 3mg/kg, 4mg/kg, 5mg/kg, 7.5mg/kg, 10 mg/kg, 12.5mg/kg, 15mg/kg, 17.5mg/kg, 20mg/kg, 25mg/kg, 30mg/kg, 35mg/kg, 40mg/kg, 45 mg/kg, 50mg/kg, 60mg/kg, 70mg/kg, 80mg/kg, 90mg/kg, 100mg/kg, 120mg/kg, 140mg/kg, 150mg/kg, 160mg/kg, 180mg/kg, 200mg/kg, 225 mg/kg, 250mg/kg, 275mg/kg, 300mg/kg, 325mg/kg, 350mg/kg, 375mg/kg, 400mg/kg, 450mg/kg, 500mg/kg, 550mg/kg, 600mg/kg, 700mg/kg, 750mg/kg, 800mg/kg, 900mg/kg, 1000mg/kg, 1250mg/kg, 1500mg/kg, 1750mg/kg, 2000mg/kg, 2500mg/kg, and/or 3000mg/kg. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention. Any of the above dosage ranges or dosage levels may be employed for MMPT or analogs thereof in combination with an additional therapeutic agent.

"Therapeutically effective amounts" are those amounts effective to produce beneficial results, particularly with respect to cancer treatment, in the recipient animal or patient. Such amounts may be initially determined by reviewing the published literature, by conducting in vitro tests or by conducting metabolic studies in healthy experimental animals. Before use in a clinical setting, it may be beneficial to conduct confirmatory studies in an animal model, preferably a widely accepted animal model of the particular disease to be treated. Preferred animal models for use in certain embodiments are rodent models, which are preferred because they are economical to use and, particularly, because the results gained are widely accepted as predictive of clinical value.

As is well known in the art, a specific dose level of active compounds such as MMPT or analogs thereof, alone or in combination with an additional therapeutic agent, for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy. The person responsible for administration will determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

In some embodiments, MMPT or analogs thereof alone, or in combination with an additional therapeutic agent will be administered. When an additional therapeutic agent is administered, as long as the dose of the additional therapeutic agent does not exceed previously quoted toxicity levels, the effective amounts of the additional therapeutic agent may simply be
defined as that amount effective to inhibit microtubule polymerization activity and/or reduce the cancer growth when administered to an animal in combination with the MMPT or analogs thereof. This may be easily determined by monitoring the animal or patient and measuring those physical and biochemical parameters of health and disease that are indicative of the success of a given treatment. Such methods are routine in animal testing and clinical practice.

In some embodiments of the present invention chemotherapy may be administered, as is typical, in regular cycles. A cycle may involve one dose, after which several days or weeks without treatment ensues for normal tissues to recover from the drug's side effects. Doses may be given several days in a row, or every other day for several days, followed by a period of rest. If more than one drug is used, the treatment plan will specify how often and exactly when each drug should be given. The number of cycles a person receives may be determined before treatment starts (based on the type and stage of cancer) or may be flexible, in order to take into account how quickly the tumor is shrinking. Certain serious side effects may also require doctors to adjust chemotherapy plans to allow the patient time to recover.

VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

MATERIALS AND METHODS

Cells and Culture Conditions

The human NSCLC H1299, H322, H460 cell lines, which have an internal homozygous deletion of the p53 gene, a mutated p53 gene, or the wild-type p53 gene (Nishizaki et al., 2001), respectively, were routinely propagated in monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml of penicillin, and 100 mg/ml streptomycin. Normal human fibroblasts (NHFB) were maintained in Dulbecco's modified Eagle medium (DMEM) with the same supplements. Normal human mesenchymal stem cells (MSC) were grown in Alpha-MEM with 20% heat-inactivated fetal calf serum and the same supplements. All cells were maintained in the presence of 5% CO₂ at 37°C.
Chemicals

A chemical library with 10,000 compounds, including MMPT (FIG. 1), was obtained from Chembridge Corporation (San Diego, CA). The chemicals in the library are provided as 5 mg/ml in DMSO. MMPT has a molecular weight of 295.42 Da, and was dissolved in DMSO to a stock concentration of 10 mM and stored at 4°C. Paclitaxel was purchased from Bristol-Myers Squibb Company (Princeton, NJ). Vinorelbine was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in DMSO to a concentration of 10 mM and stored at -20°C, and protected from light. An equal volume of DMSO as of the drugs was used as a control.

Cell Proliferation Assay

The inhibitory effects of MMPT and paclitaxel on cell growth were determined by the XTT assay. Cells (2-8 × 10^3 cells in 100 μl of culture medium/well) were seeded in 96-well flat-bottomed plates and treated the next day with the drugs at the indicated concentrations. After 72 hr, cells were washed once with PBS, and cell viability was determined by colorimetric assay with the tetrazolium dye XTT using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. The studies were performed at least three times for each cell line. Cell viability was calculated using the following formula: cell viability = 100 × A_{treatment}/A_{control} (%). The IC₅₀ value was the concentration of MMPT that cause a 50% inhibition in absorbance according to the Curve Fit 1.3 program.

The cytotoxicity profile of MMPT was also evaluated at the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD) against 43 cell lines in the NCI drugs screening panel. Cell growth inhibitio (GI₅₀) of compounds was determined by sulforhodamine B (SRB) assay as described previously (Monks et al., 1991).

Flow Cytometry Analysis

To analyze the intracellular DNA content, cells treated with various concentrations of MMPT were harvested in 0.125% trypsin and washed twice in PBS, and fixed in 70% methanol at -20°C for several hours. The cells were then resuspended in PBS containing 10 μg/ml propidium iodide (PI) (Roche Diagnostics) and 10 μg/ml RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell-cycle analysis was performed using an EPICS Profile II flow cytometer (Coulter...
Corp., Hialeah, FL) with the Multicycle Phoenix Flow Systems program (Phoenix Flow Systems, San Diego, CA). All studies were repeated three times.

For detection of cells at mitosis, fixed cells were resuspended in PBS containing 5 μg/ml mouse monoclonal anti-MPM-2 antibody (Upstate Biotechnology, Lake Placid, NY). The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (BD Biosciences Pharmingen, San Diego, CA), 10 μg/ml of PI, and 10 μg/ml of RNase A at 37°C for 1 hour in the dark. Stained cells were analyzed with the EPICS Profile II flow cytometer using the Multicycle Phoenix Flow Systems program. All studies were repeated at least twice.

Western Blot Analysis

The following antibodies were used in Western blot analysis: anti-caspase-3 and P-glycoprotein (Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase-8 (MBL International, Woburn, MA), anti-caspase-9 (Cell Signaling, Beverly, MA), and anti-COX-4, cytochrome c, and poly (ADP ribose) polymerase (PARP) (Pharmingen), and anti-β-actin (Sigma).

For preparation of whole-cell extracts, cells were washed twice in cold PBS, collected, and then lysed in lysis buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, and 10% glycerol) containing 1 x proteinase-inhibitor cocktail (Roche). The lysates were spun at 14,000 x g in a microcentrifuge at 4°C for 10 minutes. The supernatants were used as whole cell extracts. For preparation of cytosolic and mitochondrial extracts, cells were washed twice in cold PBS, collected, and then lysed in lysis buffer contained in the ApoAlert Cell Fraction Kit (Clontech, Palo Alto, CA) and cytosolic and mitochondrial extracts were isolated according to the manufacturer’s protocol. Protein concentration was determined using the BCA-protein determination method (Pierce, Rockford, IL). Equal amounts (50-100 μg) of proteins were used for immunoblotting as described previously (Teraishi et al., 2003). Proteins were subjected to electrophoresis under reducing conditions on 8-12.5% (w/v) polyacrylamide gels and then electrophoretically transferred to Nitrocellulose transfer membranes (Amersham). The membranes were incubated with primary antibody followed by peroxidase-linked secondary antibody. An electrochemiluminescence Western blotting system (Amersham) was used to detect secondary probes.

Immunological Assays

H460 cells were grown on a glass chamber slide (Becton Dickinson and Company, Franklin Lakes, NJ) and treated with 0.1% DMSO, 15-μM MMPT, 100-nM nocodazole, or 100-
nM paclitaxel for 14 hr. Cells were then washed with PBS and permeabilized with microtubule-stabilizing buffer [80 mM piperazine-N,N'-bis (2-ethanesulfonic acid)-KOH (pH 6.8), 5-mM egtazicacid, and 1-mM MgCl$_2$ containing 0.5% Triton X-100] for 5 min at room temperature before being fixed with chilled absolute methanol for 10 min at -20°C as described previously (Sasaki et al., 2002). After being washed, cells were incubated with monoclonal mouse anti-α-tubulin antibody (Sigma) for 1 hour at ambient temperature followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (BD Biosciences Pharmingen). After extensive washing, cells were rinsed once in water mounted with Vectashield (Vector Laboratories, Burlingame, CA), and observed with a Nikon BX61 microscope (Nikon, Inc., Melville, NY).

**Measurement of Soluble and Insoluble Tubulin in vivo**

Soluble (depolymerized) tubulin and insoluble (polymerized) tubulin were measured as described previously with some modifications (Sasaki et al., 2002). Briefly, cells were treated with DMSO, MMPT, nocodazole, or paclitaxel for 5 hr. The cells were then washed with PBS, lysed with 100 µl of lysis buffer (20 mM Tris-HCl [pH 6.8], 0.5% NP40, 1-mM MgCl$_2$, 2-mM egtazicacid, and 4.0 µg/ml paclitaxel), mixed with a vortex mixer, and spun a centrifuge at 12,000 × g for 10 min at 4°C. Supernatants containing depolymerized tubulin were separated from pellets containing polymerized tubulin and placed in separate tubes. The pellets were resuspended in 100 µl of water. The cytosolic and cytoskeletal fractions were dissolved in lysis buffer and separated by SDS-PAGE. Immunoblots were probed with both monoclonal mouse anti-α-tubulin and anti-β-actin antibody (Sigma). The band of each sample was quantified by using the NIH Image program (Bethesda, MD), and the ratios of depolymerized to polymerized tubulin were calculated for each treatment condition. The ratios of depolymerized to polymerized actin were also determined as an internal control.

**Animal Experiments**

Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (NIH publication number 85-23) and the institutional guidelines of M. D. Anderson Cancer Center. Subcutaneous tumors were established in 4- to 6-week-old female nude mice (Charles River Laboratories Inc., Wilmington, MD) by inoculation of 1.5 × 10$^6$ H460 cells into the dorsal flank of each mouse. After the tumors grew to 3-5 mm in diameter, the mice were treated with intraperitoneal administration of MMPT 40 mg/kg/injection (dissolved in 0.5 ml solvent containing 5.7% DMSO, 9.6% Cremophore EL, and 9.6% ethanol), solvent alone or paclitaxel (4mg/kg/injection) (Lin et al., 2003). Tumor volumes were calculated by using the
formula a x b^2 x 0.5, where a and b represented the larger and smaller diameters, respectively (Lin et al., 2002). Mice were killed when the tumors grew to 15 mm in diameter. To evaluate the toxicity of treatment, blood samples were collected from the tail vein before MMPT treatment and on day 21, 4 days after the last treatment, and serum alanine transaminase, aspartate transaminase, alkaline phosphatase, blood urea nitrogen, and creatinine levels were determined as described elsewhere (Gu et al., 2000; Gu et al., 2002). Hematopoietic toxicity was monitored by counting red blood cells, white blood cells, and platelets.

Statistical Analysis

Differences among the treatment groups were assessed by analysis of variance (ANOVA) using statistical software (StatSoft, Tulsa, OK, USA). Differences among the results for the experiment of tumor growth in vivo were assessed by ANOVA with a repeated measurement module. P values < 0.05 were regarded as significant.

EXAMPLE 2

The initial screening of the chemical library from Chembridge, led to the identification of MMPT as an agent potentially more effective in killing lung cancer cell lines than NHB

Library Screening for Cytotoxic Compounds Effective for P-Glycoprotein-Overexpressing Cells. In order to identify agents that are effective for chemoresistant cancer cells, paclitaxel- and vinorelbine-resistant lung cancer cells H460/TaxR and H460/VinR were established by repeated treatments of H460 cells with paclitaxel or vinorelbine. The cells were initially treated with 5 nM paclitaxel or vinorelbine. Surviving cells were then treated with increased doses of paclitaxel or vinorelbine, up to 100 nM. H460/TaxR10 cells had tolerance to 10 nM paclitaxel. H460 parental cells had little expression of P-glycoprotein, however, H460/TaxR10, H460/TaxR, and H460/VinR cells expressed high levels of P-glycoprotein. The expression of P-glycoprotein in H460/TaxR or H460/VinR cells was at least 10-fold greater than H460 parental cells.

Parental H460, H460/TaxR cells and normal human fibroblasts were then used to screen a chemical library from Chembridge Corporation for compounds that can kill both H460 and H460/TaxR cells but not fibroblasts. For this purpose, 5 x 10^3 and 1 x 10^4 cells were seeded into each well of a 96-well plate and then treated with each compound at a final concentration of about 5 µg/ml. Cells treated with solvent (DMSO) were used as controls. Changes in cell morphology were then observed under microscope and cell viability was determined by XTT assay 2-4 days after the treatment. The compounds that were initially observed to kill both H460 and H460/TaxR cells but not fibroblasts were undergone two additional screenings to confirm
the observation. After three round of screening, 5-([(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone (MMPT) (FIG. 1) was found to kill both H460 and H460/TaxR cells but not fibroblasts at the concentration of about 5 μg/ml.

**Effect of MMPT on Cell Proliferation**

The effect of MMPT on cell proliferation in NSCLC cell lines was then determined using an XTT assay. H1299, H460, and H322 cell lines and NHFB were treated with MMPT at concentrations ranging from 0.03 to 68 μM and cell viability was determined 72 hr after the treatment (FIGS. 2A-2B). MMPT effectively inhibited growth of H1299, H460, and H322 cells, with IC$_{50}$ values ranging from 4.9 to 8.0 μM (Table 2). Because p53 is the wild-type in H460 cells but mutant or homologously deleted in H322M (Table 2) and H1299 cells, this result suggested that the action of MMPT is p53 independent. Moreover, MMPT effectively inhibited growth of H460 cells that overexpressed P-gp and were resistant to paclitaxel (Table 2). The IC$_{50}$ values for parental H460 and paclitaxel-resistant H460/TaxR cells were comparable, suggesting that the anticancer activity of MMPT is not affected by P-gp status.

Thus, the results showed that paclitaxel-resistant H460/TaxR cells, which have high P-gp expression, were 80 times as resistant to paclitaxel as parental H460 cells. However, the effect of MMPT was not influenced by the P-gp status in H460/TaxR cells. These findings indicated that MMPT is a potential substrate of P-gp, that may be used for the treatment of paclitaxel resistance in tumor cells in patients.

The dose effect of cell killing by MMPT in NHFB and human bone marrow mesenchymal stem cells (MSC) was then determined. The IC$_{50}$ values in these two cell types were approximately 6 times higher than those observed in cancer cells. For comparison, IC$_{50}$ values for paclitaxel in NHFB and MSC were 10.6 and 3.9 nM, respectively, similar to those observed in H1299, H460, and H322 cells (ranged from 2.9 to 6.3 nM). This result suggests that MMPT may have a larger therapeutic window than that of paclitaxel.
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>p53 status</th>
<th>MMPT (µM)</th>
<th>Paclitaxel (nM)</th>
<th>Vinorelbine (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1299</td>
<td>null</td>
<td>6.7 ± 2.9</td>
<td>6.3 ± 0.5</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>H322</td>
<td>mutant</td>
<td>4.9 ± 0.9</td>
<td>2.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>H460</td>
<td>wild</td>
<td>5.6 ± 1.0</td>
<td>3.3 ± 0.2</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>H460/TaxR</td>
<td></td>
<td>5.7 ± 1.3</td>
<td>260.6 ± 11.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.0)(^a)</td>
<td>(80.0)(^a)</td>
<td></td>
</tr>
<tr>
<td>H460/VinR</td>
<td></td>
<td>8.0 ± 0.8</td>
<td>ND</td>
<td>513.3 ± 39.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.4)(^a)</td>
<td></td>
<td>(213.9)(^a)</td>
</tr>
<tr>
<td>NHFB</td>
<td></td>
<td>&gt; 50</td>
<td>10.6 ± 4.8</td>
<td>5.9 ± 2.2</td>
</tr>
<tr>
<td>MSC</td>
<td></td>
<td>39.2 ± 4.3</td>
<td>3.9 ± 0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) The relative resistant factors were calculated as follows; IC\(_{50}\) in resistant cells/ IC\(_{50}\) in parental cells

The cell killing effect of MMPT in NHFB and human bone marrow mesenchymal stem cells was also evaluated. The IC\(_{50}\) values in these two cell types were approximately 6 times higher than those observed in cancer cells. For comparison, the growth-inhibitory effect that the existing anticancer agent paclitaxel elicited in H1299, H460, and H322 cells, IC\(_{50}\) values ranged from 2.9 to 6.3 nM. Nevertheless, the IC\(_{50}\) values for paclitaxel in NHFB and mesenchymal stem cells were 10.6 and 3.9 nM, respectively. This result suggested that MMPT may have a larger therapeutic window than paclitaxel.

To further evaluate antitumor activity of the MMPT, we send MMPT to the Developmental Therapeutics Program of the National Cancer Institute for testing its effect on a panel of cancer cells. A test on 42 cancer cell lines derived from leukemia, NSCLC, colon
cancer, central nerve system (CNS) cancer, melanoma, ovarian cancer, renal cancer, prostate
cancer, and breast cancer by NCI’s Drug Discovery and Development Program showed that
average concentration required to suppress 50% growth ($GI_{50}$) after a 48 treatment is 2.51 $\mu$M.
12 of the 42 cell lines had $GI_{50}$ values lower than the average (Table 3), suggesting that MMPT
may be effective for various other cancer cells.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Tumor Types</th>
<th>$GI_{50}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI H322M</td>
<td>NSCLC</td>
<td>0.35</td>
</tr>
<tr>
<td>NCI H460</td>
<td>NSCLC</td>
<td>1.62</td>
</tr>
<tr>
<td>NCI H522</td>
<td>NSCLC</td>
<td>1.95</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colon Cancer</td>
<td>0.41</td>
</tr>
<tr>
<td>KM12</td>
<td>Colon Cancer</td>
<td>0.13</td>
</tr>
<tr>
<td>SF-295</td>
<td>CNS Cancer</td>
<td>0.04</td>
</tr>
<tr>
<td>SF-268</td>
<td>CNS Cancer</td>
<td>2.29</td>
</tr>
<tr>
<td>M14</td>
<td>Melanoma</td>
<td>1.20</td>
</tr>
<tr>
<td>IGROV1</td>
<td>Ovarian Cancer</td>
<td>0.44</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>Ovarian Cancer</td>
<td>0.07</td>
</tr>
<tr>
<td>OVCAR-5</td>
<td>Ovarian Cancer</td>
<td>1.95</td>
</tr>
<tr>
<td>NCI/ADR-RES</td>
<td>Breast Cancer</td>
<td>0.41</td>
</tr>
</tbody>
</table>

**Table 3. 50% Growth Inhibition ($GI_{50}$) on various cancer cells**

**EXAMPLE 3**

**Induction of M-Phase Arrest by MMPT**

To elucidate the mechanisms of MMPT induced growth inhibition in cancer cells, the
effects of MMPT on cell-cycle progression were tested. H1299 and H460 cells were treated with
MMPT or DMSO and harvested at various time points. Cells were then analyzed for cell-cycle
distribution by flow cytometric analysis. As shown in FIG. 3A, treatment with MMPT resulted
in an accumulation of cells at the G$_2$-M phase in a time-dependent manner. G$_2$-M-phase arrest
occurred as early as 6 hr after treatment and increased over time. Results of dose-effect analysis
of cycle changes 12 hr after treatment with MMPT (0.3 $\mu$M-10 $\mu$M) showed that effective arrest
during the G$_2$-M phase can be elicited with only a 3 $\mu$M concentration of MMPT (FIGS. 3B-3C).
The accumulation of cells at G2-M phase was accompanied by synchronous decreases of cells in the G1 and S phases.

To determine whether cell-cycle arrest occurred at the G2 or M phase, a flow cytometric analysis of MMPT-treated H1299 and H460 cells using PI and an anti-MPM-2 antibody, which is specific for the phosphoproteins that appear at the M phase, was performed (Vandere and Borisy, 1989). Anti-mouse IgG was used as a control antibody, and H460 cells treated with 50-µM paclitaxel were used as a positive control for MPM-2. The results showed that MMPT induced the accumulation of MPM-2 positive cells. The peak values of mitotic arrest for H1299 and H460 cells were 42.7% and 50.5%, respectively, when tested 12 hr after treatment with 10-µM MMPT (FIG. 4).

**EXAMPLE 4**

**Induction of G2-M Phase Arrest in NSCLC Cells**

To further elucidate the mechanisms of MMPT induced growth inhibition in cancer cells, the effects of MMPT on cell-cycle progression were tested. H1299 and H460 cells were treated with 15 µM MMPT for 3, 6, 9, 12, and 24 hours and harvested at various time points. DMSO was used as a control. Cells were then analyzed for cell-cycle distribution by flow cytometric analysis. As shown in FIG. 5, treatment with MMPT resulted in an accumulation of cells at the G2-M phase in a time-dependent manner. G2-M-phase arrest occurred as early as 6 hr after treatment and increased over time. The accumulation of cells at G2-M phase was accompanied by synchronous decreases of cells in the G1 and S phases.

To determine whether cell-cycle arrest occurred at the G2 or M phase, a flow cytometric analysis of MMPT-treated H1299 and H460 cells using DAPI and an α-tubulin antibody, or a combination of both agents (Merge) was performed. H460 cells were treated with 50 nM nocodazole (FIG. 6D), 15 µM 10086-7B (FIG. 6C), or 50 nM taxol (FIG. 6D). DMSO was used as a control (FIG. 6A). The results showed that MMPT induced cycle arrest in non small cell lung cancer cells (FIG. 6C).

**EXAMPLE 5**

**MMPT-Induced Expression and Phosphorylation Status of Regulatory Proteins**

To evaluate molecular changes in cells treated with MMPT, the effect of MMPT on changes in the expression of proteins related to the G2-M phase in H460 cells was examined. Western blot analysis showed that the level of cyclin A was noticeably reduced 24 hr after
treatment with 10 μM MMPT. In addition, a slower migrating form of the cdc25C phosphatase was detected in cells after 6-24 hr of treatment. Phosphorylated histone H3, a mitotic marker, was also dramatically induced 6-24 hr after treatment with MMPT. The level of cdc2 remained unchanged, whereas an obvious increase in the level of phospho-form Bcl-2 was observed after treatment with MMPT. These results further indicated that MMPT induced M-phase arrest and possibly apoptosis. The changes of the phosphorylated proteins are consistent with mitotic arrest (Vandre et al., 1989; Morris et al., 2000; Giet et al., 2001).

**EXAMPLE 6**

**Induction of Apoptosis in Cancer Cells and Antitumor Effect In Vivo**

**Induction of Apoptosis in Cancer Cells.** Results of the cell cycle analysis shown in FIGS. 3A-3C demonstrated that a substantial proportion of H1299 and H460 cells treated with MMPT underwent apoptosis.

To assess the ability of MMPT to induce apoptosis, the caspase activation and PARP cleavage in H460 cells treated with MMPT was next examined. Cells were treated with various concentration of MMPT for 48 hours and then cell lysates were subjected to 8-12.5% SDS-PAGE, followed by Western blotting with caspase and PARP antibodies. Cleavages of caspase-3, caspase-8, caspase-9, and PARP were easily detectable in H460 cells treated with 15 μM MMPT. Next, we determined the apoptotic cells by using FACS analysis. H1299 and H460 cells were treated with 15 μM MMPT for 12, 24, and 48 hours. Cells were then harvested for quantification of apoptotic subdiploid cells by flow cytometry (FIG. 7). At 48 hours, 38% of H460 cells were in sub-G1 phase. The portion of sub-G1 cells in H460 cells was gradually increased in time-dependent manner, however, it reached a peak at 24 hours in H1299 cells. In contrast, only background levels (less than 5%) of sub-G1 cells were seen in H1299 and H460 cells treated with solvent (data not shown).

Apoptosis-induction by MMPT in P-glycoprotein overexpressing H460/TaxR and H460/VinR cells was also tested. For this purpose, H460, H460/TaxR and H460/VinR cells were treated with 15 μM MMPT for indicated time periods and then activation of caspase-3, caspase-8, and caspase-9 was analyzed by Western blotting. Treatment with MMPT caused apparent cleavages of caspase-3, caspase-8, and caspase-9 in all three cell lines. Moreover, Western blotting for cytochrome c showed that treatment with MMPT resulted in release of cytochrome c to the cytosol in H460 and H460/TaxR cells after 24 hours. These results clearly showed that MMPT could induce apoptosis in chemosensitive and chemoresistant cancer cells via caspase activation.
Antitumor Effect in Vivo. To test in vivo effect of MMPT, subcutaneous H460 tumors were established in nude mice. When tumors reached to 3-5 mm in diameter, mice were treated with three sequential intraperitoneal injection of MMPT (40 mg/kg/injection), solvent, or paclitaxel (4 mg/kg/injection). Tumor volume was then monitored over time. In comparison with animals treated with solvent, tumor growth was significantly inhibited in mice treated with MMPT (p≤0.05) (Fig. 4). One week after the last treatment, the tumor volume in animals treated with solvent was 1435 ± 406 mm³ (n = 5), while the mean tumor volumes in animals treated with MMPT or paclitaxel were 755 ± 229 mm³ (n = 6) and 913 ± 424 mm³ (n = 5), respectively. The inventors also monitored the changes of mouse body weight, red blood cells, white blood cells, platelets, serum alanine transaminase, aspartate transaminase, alkaline phosphatase, blood urea nitrogen, and creatinine levels before and after the treatment. No obvious change in body weight was found in all the animals among the groups. Blood cell counts and all the serum tests showed that the values were in normal ranges in all the animals tested. Together, these results indicated that MMPT effectively inhibits the growth of H460 human lung carcinoma models in vivo without noticeable acute toxicity.

EXAMPLE 7
Effect of MMPT on Mitotic Spindles in Human Cancer Cell Lines

The ability of MMPT to induce M phase arrest led to the investigation of its effect on mitotic spindles because most microtubule-binding drugs have the ability to inhibit mitosis by disrupting the organization of mitotic spindles. Therefore, the status of mitotic spindles in H460 cells was examined 14 hr after treatment with nocodazole, MMPT, or paclitaxel. A normal spindle formation in transition from metaphase to anaphase was identified in untreated H460 cells (FIG. 8A). In nocodazole treated cells, many mitotic cells had depolymerization of the mitotic spindles or monster spindles (FIG. 8B). These abnormal spindle formations were also identified in cells treated with MMPT; however, some of the later cells had two- or three-spindle polar organization (FIG. 8C). In contrast, paclitaxel treatment caused the destruction of the mitotic spindles and aggregated spindle formation as a result of increased microtubule polymerization (FIG. 8D). Similar results were observed in NSCLC cells treated with MMPT, nocodazole or taxol (FIGS 6A-6D). These results suggest that MMPT functions as a microtubule depolymerization agent.
EXAMPLE 8

Inhibition Microtubule Polymerization In Vivo

To further evaluate the effect of MMPT on microtubule polymerization, the effect of MMPT on tubulin polymerization was tested using an in vivo tubulin polymerization assay. Tubulin in soluble and insoluble fractions from H460 cells treated with DMSO, nocodazole, MMPT, and paclitaxel for 5 hr, was prepared and visualized using immunoblotting for α-tubulin and β-actin (FIG. 9A). At a 50-μM concentration, MMPT induced tubulin depolymerization in a manner very similar to that of nocodazole. The ratios of polymerized to depolymerized tubulin were 0.26 ± 0.01 for MMPT-treated cells, 0.01 ± 0.01 for nocodazole-treated cells, 3.87 ± 0.67 for paclitaxel-treated cells, and 0.83 ± 0.03 for DMSO-treated control cells (FIG. 9B). Thus, MMPT dramatically depolymerized tubulin 5 hr after treatment. This result suggests that MMPT functions by directly inhibiting tubulin polymerization.

EXAMPLE 9

The Effect of MMPT on Cell Proliferation in Cancer Cell Lines

Sixty human cancer cell lines were treated as described above, in Example 1, with five concentrations at 10-fold dilutions of MMPT. The cells were harvested after 48 hr and a sulforhodamine B (SRB) protein assay was conducted to estimate cell viability or growth. FIG. 10A shows the dose-dependent curves of various leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer cell lines. FIG. 10B shows the mean graph of each cell line. This allowed visual scanning of the data for potential patterns of selectivity for particular cell lines. FIG. 10C shows the combined dose-dependent curves of all cancers tested. These results demonstrated that sensitivity to MMPT occurs in a variety of cancer cells lines and causes inhibition of cell growth and induction of apoptosis. Further, these results indicate that MMPT is a candidate compound for treating cancer in a subject.

EXAMPLE 10

Identification of Novel Synthetic Compound Capable of Inducing c-Jun N-Terminal Kinase-Dependent Apoptosis in Human Colon Cancer Cells

Materials and Methods

Cells and Culture Conditions. The human colon cancer cell lines DLD-1 and LoVo and the non-small-cell lung cancer cell lines H1299 and H460 were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin,
and 100 mg/ml streptomycin. HCT116 colon carcinoma cells (p53 wild-type and p53<sup>−/−</sup>;
generously provided by Dr. Bert Vogelstein, The Johns Hopkins University, Baltimore, MD)
(Bunz et al., 1998) were cultured in RPMI 1640 medium with 10% fetal calf serum. Normal
human fibroblasts (NHFBs) were maintained in Dulbecco’s modified Eagle medium with the
same supplements. All cells were maintained in the presence of 5% CO<sub>2</sub> at 37°C.

**Chemicals and Antibodies.** A chemical library with 10,000 compounds, DBPT (5-(2,4-
dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin), and DBPT analog MAPT (2-[(4-
methylphenyl)amino]-5-(phenylmethylene)-4(5H)-thiazolone) were obtained from ChemBridge
(San Diego, CA). The chemical structures of DBPT and MAPT are shown in FIG. 11A and FIG.
11B. The compounds were dissolved in dimethyl sulfoxide to a concentration of 10 mM and
stored at 4°C as a master stock solution. The JNK-specific inhibitor SP600125, the ERK
inhibitor PD98059, and the p38 inhibitor SB202190 were purchased from Calbiochem (La Jolla,
CA), dissolved in dimethyl sulfoxide, stored at −20°C, and protected from light. The general
caspase inhibitor z-VAD-fmk was obtained from R&D Systems (Minneapolis, MN). Antibodies
to the following proteins were used for western blot analysis: caspase-3 and P-glycoprotein (P-
gp) (Santa Cruz Biotechnology, Santa Cruz, CA); caspase-8 (MBL International, Woburn, MA);
poly(ADP-ribose) polymerase (PARP) and cytochrome c (BD PharMingen, San Diego, CA);
JNK, phosphorylated JNK (p-JNK), ERK, phosphorylated ERK (p-ERK), p38, phosphorylated
p38 (p-p38), phosphorylated c-Jun (p-c-Jun), and caspase-9 (Cell Signaling, Beverly, MA); and
hemagglutinin (HA) and β-actin (Sigma, St. Louis, MO).

**Cell Proliferation Assay.** The antiproliferative effects of DBPT on various cancer cell
lines and NHFBs were examined using cell proliferation assays. Cells were seeded in 96-well
flat-bottomed plates (3-5 × 10<sup>3</sup> in 100 μl of culture medium per well) and treated the next day
with the indicated concentrations of compounds. An equal volume of dimethyl sulfoxide was
used as a control. After 72 h, cells were washed once with phosphate-buffered saline, and cell
viability was determined by XTT assay using a Cell Proliferation Kit II (Roche Molecular
Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. The experiments were
performed at least three times for each cell line. Cell viability was calculated as 100 × A<sub>treatment</sub>/
A<sub>control</sub>, where A is the absorbance measured by using a microplate reader (Model MRX;
Dynatech Laboratories, Chantilly, VA) at 450 nm with a reference wavelength at 650 nm. The
concentrations of DBPT that inhibited absorbance by 50% and 80% (IC<sub>50</sub> and IC<sub>80</sub>, respectively)
were calculated by using the CurveExpert Version 1.3 program.
**Apoptosis Assay.** For detection of apoptosis, fixed cells were suspended in phosphate-buffered saline containing 10 μg/ml propidium iodide (PI) (Roche Diagnostics, Indianapolis, IN) and 10 μg/ml RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell-cycle analysis was performed using an epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA) with MultiCycle software (Phoenix Flow Systems, San Diego, CA). Accumulation of sub-G1 cells, a known indicator of DNA fragmentation and apoptosis, was used to quantify apoptosis. The percentage of cells undergoing apoptosis was also determined by annexin V staining. Briefly, cells were collected by using 0.125% trypsin and washed in RPMI 1640 medium with 10% fetal calf serum. After centrifugation, 1 × 10^5 cells were resuspended in reaction buffer and stained with fluorescein isothiocyanate-labeled annexin V and PI using the Annexin V-FITC Apoptosis Detection Kit (PharMingen). Stained cells were analyzed using the flow cytometer and software described above. All experiments were repeated at least three times.

**Plasmid Transfection and Adenovirus Vector Transduction.** The plasmids pLNCX-3X HA-p46JNK1alpha (dnJNK1; encodes an HA-tagged, dominant-negative JNK1 mutant) and pLNCX-3X HA-p54JNK2alpha (dnJNK2; encodes an HA-tagged, dominant-negative JNK2 mutant) (Wojtaszek et al., 1998) were provided by Dr. L. E. Heasley (University of Colorado Health Sciences Center, Denver, CO). Plasmid transfection was performed using FuGENE6 reagent (Roche Diagnostics), and cells were selected for growth in the presence of 500 μg/ml G418. The adenoviral vector AdMKK7DN, which encodes a dominant-negative, constitutively active MKK7 mutant, was provided by Dr. Yibin Wang (University of California, Los Angeles, CA) (Wang et al., 1998). The vector encoding green fluorescence protein (AdGFP) has been reported previously (Gu et al., 2000).

**Western Blot Analysis.** For preparation of whole-cell extracts, cells were washed twice in cold phosphate-buffered saline, collected, and lysed in lysis buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, and 10% glycerol) containing 1× proteinase-inhibitor cocktail (Roche Diagnostics). The lysates were spun at 14,000 × g in a microcentrifuge at 4°C for 10 min., and the resulting supernatants were used as whole-cell extracts. For preparation of cytosolic and mitochondrial extracts, cells were washed twice in cold phosphate-buffered saline, collected, and lysed in lysis buffer from the ApoAlert Cell Fractionation Kit (Clontech, Palo Alto, CA). Cytosolic and mitochondrial extracts were isolated according to the manufacturer’s protocol. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts (30-50 μg) of proteins were used for immunoblotting as described previously (Teraishi et al., 2003; specifically incorporated herein in its entirety). Briefly,
proteins were subjected to electrophoresis under reducing conditions on 8-12.5% (w/v) polyacrylamide gels and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were incubated with primary antibody followed by peroxidase-linked secondary antibody, which was detected using the ECL Western Blotting System (Amersham).

**Statistical Analysis.** Differences among the treatment groups were assessed by analysis of variance using StatSoft statistical software (Tulsa, OK). \( P < 0.05 \) was considered significant.

**Results**

**DBPT Inhibited Proliferation of Human Colon Cancer Cells.** In a primary screen of 10,000 compounds, the cytotoxic effects of these compounds was tested on human colon cancer cell lines DLD1 and LoVo, human lung cancer cell lines H1299 and H460 and normal human fibroblasts. Cells seeded in 96 well plates were treated with each compound at a final concentration of about 5 \( \mu \text{g/ml} \). Cells treated with solvent (DMSO) were used as controls. Cytotoxic effects were then determined by observation under microscope and by cell viability assay. This screening led us to identify DBPT as an agent that had cytotoxic effects in the four cancer cell lines tested and but not in NHFBs. Human colon cancer cells were then treated with DBPT at various concentrations for 72 h and its effect on cell viability was examined using XTT assays. DBPT effectively inhibited the growth of DLD-1 and HCT116 cells with IC\(_{50}\) values ranging from 1.6 to 5.9 \( \mu \text{M} \) (Table 4). This cytotoxic effect is p53-independent because DBPT inhibited the growth of both wild-type and p53-deficient HCT116 cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>P-gp Status</th>
<th>IC(_{50}) (( \mu \text{M} ))</th>
<th>IC(_{80}) (( \mu \text{M} ))</th>
</tr>
</thead>
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<tr>
<td>DLD-1</td>
<td>+++</td>
<td>1.6 ( \pm ) 0.3</td>
<td>2.7 ( \pm ) 0.3</td>
</tr>
<tr>
<td>HCT116 (p53(^+))</td>
<td>( \pm )</td>
<td>4.9 ( \pm ) 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>HCT116 (p53(^-))</td>
<td>( \pm )</td>
<td>5.9 ( \pm ) 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>NHFB</td>
<td>ND</td>
<td>22.9 ( \pm ) 7.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 4. IC\(_{50}\) and IC\(_{80}\) for DBPT in Human Colorectal Cancer Cell Lines and NHFBs
"p53\textsuperscript{+}, wild type; p53\textsuperscript{-}, p53 deficient; ND, not determined. Each value in the DBPT columns represents the mean \pm SD of three independent experiments.

In addition, the expression level of P-gp, a product of the human multidrug resistance MDR1 gene (Ambudkar et al., 1999), was measured in three colon cancer cell lines (Table 3). P-gp levels were evaluated by western blot analysis. The amount of P-gp in DLD-1 cells was markedly higher than in HCT116 cells, but the sensitivity of DLD-1 cells to DBPT was also much higher than that of HCT116 cells, indicating that the activity of DBPT in colon cancer cells was not affected by P-gp status.

The dose-response effect of DBPT in NHFB cells was also evaluated (Table 4). The IC\textsubscript{50} in NHFB cells was 4 to 14 times higher than in cancer cells, indicating that DBPT may selectively induce cytotoxic effects in cancer cells. Interestingly, MAPT, a DBPT analog with similar chemical structure (FIG. 11), a compound with did not induce any cytotoxic effects in any of the cells tested, even at 31 \textmu M, the highest concentration tested (data not shown).

**DBPT Induced Apoptosis and Caspase Activation in Colon Cancer Cells.** To elucidate the mechanisms by which DBPT induces cytotoxicity, flow cytometry was used to analyze apoptosis in DLD-1 and HCT116 cells after DBPT treatment (FIG. 12A). DBPT induced a marked, time-dependent increase in the percentage of annexin V-positive DLD-1 and HCT116 cells, with peak values of 65.2\% and 44.5\% after 48 h of treatment with 3 \textmu M and 5 \textmu M DBPT, respectively. To further examine the ability of DBPT to induce apoptosis, DLD-1 cells were treated with 3 \textmu M DBPT for 6, 12, 24, or 48 h and evaluated caspase activation by western blot analysis. Activation of caspases and PARP were detected 24 and 48 h after DBPT treatment. Cleavage of caspase-3, caspase-8, caspase-9, and PARP was easily detectable after 48 h.

Studies were then conducted to investigate whether inhibition of caspase activation affects DBPT-induced apoptosis. DLD-1 cells were pretreated with the general caspase inhibitor z-VAD-fmk at 10, 50, or 100 \textmu M for 30 min and then treated with DBPT for another 24 to 48 h. The cells were analyzed for viability by XTT assay and for apoptosis by flow cytometry (FIG. 12B and FIG. 12C). Pretreatment with z-VAD-fmk blocked DBPT-mediated cell growth inhibition in a dose-dependent manner. The most significant difference in cell viability was observed between cells treated with DBPT alone (42.6\%) and those pretreated with 100 \textmu M z-VAD-fmk and then treated with DBPT for 48 h (81.3\%) (P < 0.01, FIG. 12B). Consistently, pretreatment with z-VAD-fmk markedly diminished the DBPT mediated apoptotic (Sub-G1) cells (FIG. 12C). These results indicate that DBPT-induced apoptosis in DLD-1 cells was related to caspase activation, although z-VAD-fmk did not completely block apoptosis.
DBPT Activated MAPK Signaling. Many cellular stresses and stimuli induce apoptosis and modulate MAPK signaling pathways (Shtil et al. 1999; Stone et al., 2000). However, the role of these pathways in cell death is not completely understood. The activation of the MAPKs JNK, ERK, and p38 were examined after DBPT treatment. Lysates of DBPT-treated cells were analyzed by western blotting for MAPK phosphorylation, a hallmark of MAPK activation. Control cells were treated with MAPT. Treatment of DLD-1 cells with DBPT increased the amounts of phosphorylated JNK and p38, which were easily detectable at 24 and 48 h after the treatment. In contrast, treatment with MAPT did not result in detectable activation of JNK and p38. No significant change in p-ERK was found in cells treated with either compound.

JNK Inhibitor SP600125 Blocked DBPT-induced Apoptosis. To further investigate the role of JNK and p38 activation in DBPT-induced apoptosis, DLD-1 cells were treated with DBPT in the presence or absence of the ERK-specific inhibitor PD98059, p38 inhibitor SB202190, or JNK inhibitor SP600125 (Bennett et al., 2001) and analyzed the sub-G1 population by flow cytometry. Treatment with DBPT alone resulted in an approximately 38% increase in sub-G1-phase cells. The ERK and p38 inhibitors had no notable effect on DBPT-induced apoptosis (FIG. 13A). In contrast, SP600125 significantly (P< 0.01) blocked DBPT-induced apoptosis (FIG. 13B). Whereas the percentage of apoptotic cells was 7.2% with 50 µM SP600125 alone and 37.3% with 3 µM DBPT alone, only about 4% of cells were apoptotic with the combination of SP600125 and DBPT. In addition, DLD-1 cells treated with DBPT alone showed the typical morphological changes of cytopathies, and became rounded and detached from the plate; these changes were abrogated by the presence of SP600125.

Western blot analysis showed that pretreatment with SP600125 diminished both DBPT-induced JNK activation and the phosphorylation of c-Jun, a substrate of JNK. SP600125 pretreatment also reduced DBPT-mediated cleavage of caspase-8, caspase-9, and caspase-3. Additionally, DBPT-mediated release of cytochrome c from mitochondria was substantially attenuated in the presence of SP600125. These results indicate that DBPT-mediated caspase activation and cytochrome c release were JNK dependent, which in turn suggests that JNK activation had a key role in DBPT-mediated apoptosis.

To determine whether DBPT-induced JNK activation was associated with p53 status, wild-type and p53-deficient HCT116 cells were treated with DBPT at two different concentrations, and the expression of p-JNK and p-c-Jun was monitored. DBPT activated JNK and c-Jun after 24 h in both cell lines, suggesting that DBPT-mediated JNK activation was independent of p53. Interestingly, treatment with DBPT also led to the accumulation of p53 in
HCT116 (p53 +/-) cells. Whether is accumulation of p53 is also related to JNK activation is not clear.

**JNK1 Activity Was Required for DBPT-Induced Apoptosis.** To further investigate the role of JNK activation in DBPT-induced apoptosis, DLD-1 cells were stably transfected with plasmid expressing either of the dominant-negative mutant dnJNK1 or dnJNK2, treated the cells with 3 μM DBPT, and analyzed cell viability and apoptosis. The expression of dnJNK1 in DLD-1 cells markedly blocked DBPT-induced growth inhibition after 24 and 48 h, whereas dnJNK2 had no effect as compared with the same treatment but with an empty control vector (FIG. 14A). Flow cytometric analysis of cells treated with 3 μM DBPT for 24 h showed that DBPT-mediated apoptosis was markedly reduced in two dnJNK1 transfectants but not in any dnJNK2 transfectants as compared with an empty control vector (FIG. 14B). These results indicate that JNK1 activation is crucial for DBPT-mediated apoptosis and that different JNK isoforms (or their respective genes) have different roles in DBPT-induced apoptosis.

**Constitutive JNK Activation Alone Did Not Replicate the Effects of DBPT.** To determine whether ectopic JNK activation can itself affect cell proliferation, DLD-1 cells were treated with an expression vector encoding MKK7, a kinase upstream of JNK. Treatment with the AdMKK7DN vector activated JNK and c-Jun, but not ERK and p38, after 24 and 48 h. An XTT assay revealed that AdMKK7DN did not inhibit the growth of DLD-1 cells after 24 and 48 h compared with the control vector AdGFP (FIG. 15). These findings indicate that although JNK activation is required for DBPT-induced apoptosis, activation of JNK alone is not sufficient to induce apoptosis. Additional apoptotic signal transduction might be required for DBPT-mediated apoptosis.

**EXAMPLE 11**

**Cytotoxic Effect of MMPT Analogues**

1. **Chemical structure of MMPT analogues**

To test whether MMPT analogues can also induce tumor-selective cytotoxic effects, the chemical database was searched and several compounds were identified that have similar chemical structures as MMPT. Twenty of the analogues were obtained from ChemBridge Corp. The core structure of MMPT is shown in FIG. 16. For convenience, the two aromatic cyclic-ring structures, where modification occurs, were designated as ring A and ring B. The modifications at different positions of these two rings are summarized in Table 5. They are divided into two groups according to the position of substitutive groups in ring A and ring B. The first group

71
contains 13 analogues that substitutive groups in the ring A. The analogues that have substitutive
groups position in the ring B or both A and B are grouped to Group II. Analogue A in Table 5 is
DBPT (see Example 10).

### Table 5. MMPT and Analogues from ChemBridge

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Name</th>
<th>CAS# Formula</th>
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<th>Substitutive Group</th>
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<td>4-CH3</td>
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<td>A</td>
<td>356800-74-3 C16 H12 N2 O3 S 2.974</td>
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<td>2-OH 4-OH</td>
</tr>
<tr>
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<td>B</td>
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<td>34798-17-9 C17 H14 N2 O5 S 4.511</td>
<td>2-[[4-methylphenylamino]-5-(phenylmethylene)-4(5H)-thiazolone</td>
<td>4'-CH3</td>
</tr>
</tbody>
</table>

72
2. Cytotoxic effect of MMPT analogues

The cytotoxic effects of MMPT analogues were evaluated by cell viability assays. For this purpose, human lung cancer cell line H460 and H1299, human colon cancer cell line Lovo and DLD-1, human breast cancer cell line MDA-MB-231, and normal human fibroblasts were seeded in 96 well plate and treated the MMPT and its analogues as listed in Table 4 at concentrations ranging from 0.1 μM ~31μM. Cell viability was then determined by a quadruplet XTT assay at 4 days after treatment. The experiment was repeated at least three times. Cells treated with DMSO were used as controls. Dose-responses and IC₅₀ values of MMPT analogues in these cell lines were then determined by CurveExpert software. The IC₅₀ of these compounds obtained by XTT assay are summarized in Table 6.

<table>
<thead>
<tr>
<th>Name</th>
<th>NHFB</th>
<th>DLD1</th>
<th>MDA-MB-231</th>
<th>H460</th>
<th>H1299</th>
<th>LOVO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPT</td>
<td>11.5±2.12</td>
<td>1.9±0.07</td>
<td>2.4±0.70</td>
<td>2.8±0.09</td>
<td>2.0±0.14</td>
<td>10.5±0.70</td>
</tr>
</tbody>
</table>

**Table 6. IC₅₀ of MMPT and Its Analogues**

**GROUP A**

<table>
<thead>
<tr>
<th>Name</th>
<th>NHFB</th>
<th>DLD1</th>
<th>MDA-MB-231</th>
<th>H460</th>
<th>H1299</th>
<th>LOVO</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>H</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>J</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>K</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>L</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>N</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
<tr>
<td>O</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
</tr>
</tbody>
</table>

**GROUP B**

<table>
<thead>
<tr>
<th>Name</th>
<th>NHFB</th>
<th>DLD1</th>
<th>MDA-MB-231</th>
<th>H460</th>
<th>H1299</th>
<th>LOVO</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>13.4±3.46</td>
<td>6.2±0.14</td>
<td>&gt;31</td>
<td>9.6±1.56</td>
<td>5.2±1.56</td>
<td>2.4±0.21</td>
</tr>
<tr>
<td>P</td>
<td>22.2±3.35</td>
<td>7.8±0.21</td>
<td>13.4±1.77</td>
<td>&gt;31</td>
<td>13.4±1.48</td>
<td>1.75±0.21</td>
</tr>
<tr>
<td>S</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>20.5±13.4</td>
<td>2.6±0.42</td>
</tr>
<tr>
<td>Name</td>
<td>NHFB</td>
<td>DLD1</td>
<td>MDAMB-231</td>
<td>IC\textsubscript{50} (\textmu M) ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>------</td>
<td>-----------</td>
<td>---------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMPT</td>
<td>11.5±2.12</td>
<td>1.9±0.07</td>
<td>2.4±0.70</td>
<td>2.8±0.09, 2.0±0.14, 10.5±0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>&gt;31</td>
<td>5.2±0.07</td>
<td>&gt;31</td>
<td>26.3±1.69, 1.6±0.42, 1.9±0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>&gt;31</td>
<td>&gt;31</td>
<td>8.5±0.92</td>
<td>25.7, &gt;31, 16.7±4.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>16.5±5.51</td>
<td>&gt;31</td>
<td>7.6±0.28</td>
<td>21.4±2.12, &gt;31, 6.5±0.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**GROUPPC**

| A | 17.5±4.94 | 0.51±0.35 | 4.4±0.07 | 3.4±0.07, 1.6±2.10, 3.9±3.96 |
| B | 5.3±0.42  | >31   | 4.1±0.78 | 2.6±1.14, 3.3±0.71, 1.3±0.21 |
| C | 3.4±0.42  | 1.9±1.2 | 3.3±0.42 | 5.9±0.49, 2.3±0.28, 2.9±0.35 |
| D | 15.8±1.06 | 7.8±0.78 | 5.6±0.78 | 4.1±1.27, 4.8±0.42, 5.4±1.20 |
| G | >31   | 0.8±0.08 | 12.3±14.3| 2.5±0.42, 0.48±0.21, 0.73±0.09 |
| Q | 6.9±2.89 | 3.5±0.21 | 0.73±0.15 | 3.4±0.28, 3.5±0.21, 1.8±0.07 |
| R | 18.7±1.77 | 1.1±0.07 | 0.83±0.67 | 2.6±0.35, 15.9±3.32, 0.4±0.07 |

The result showed that 7 analogues in Group A did not kill any of the 6 cell lines, either cancer cells or normal cells, at the concentration ranging 0.1 \textmu M to 31 \textmu M. Six analogues in Group B killed some of cancer cell lines or normal cells, but their IC\textsubscript{50} values were higher than that of MMPT. Seven analogues in Group C were more potent than MMPT in inducing cytotoxic effects, or have lower IC\textsubscript{50} values. Among them, four (A, D, G and R) were less toxic to normal human fibroblast than MMPT.

The analogues J and K differ from MMPT in the substitutive groups in position 4 of ring A. Interestingly, substitute 4-CH\textsubscript{3} with 4-OH or 4-NHAc abolished the cytotoxic effects of MMPT. Moreover, changing the methyl group of position 4 in ring A to position 4 in ring B (analogue O) also abrogates the cytotoxic effect of the compound. Analogues A, B and R that have different substitutive groups in position 2 and 4 of the ring A, all of the three analogues induce cytotoxicity in various cells. However, analogues A and R have tumor selectivity. Analogues G and D have the same substitutive groups at the positions 3 and 4 in ring A and both induced tumor selective cytotoxicity. However, G contains an additional -OH group in position 4 of ring B, and is more potent in inducing cell killing and more tumor specific. Thus, size and polarity or H-bond potency of substitutive groups may play key role to the cell-killing activity and cell-type specificity.

3. **Cell-killing activity in chemoresistant cancer cells**

These results indicate that MMPT can effectively kill paclitaxel- or vinorelbine-resistant human lung cancer cells, H460/TaxR and H460/VinR, that overexpress P-glycoprotein, a transmembrane ATP-dependent drug efflux pump that is expressed in a wide variety of tumors and is responsive for multi-drug resistance. To test whether analogues that selectively killed tumor cells were also effective for these P-glycoprotein over-expressing cells, the cell killing effect of MMPT and three tumor selective analogues A, G and R were compared in parental
H460, H460/TaxR and H460/VinR cells. Dose response in the three cancer cell lines are shown in Fig.17A-17C. Their IC$_{50}$ and IC$_{90}$ were listed in Table 7.

**Table 7. IC$_{50}$ and IC$_{90}$ of MMPT Analog to Cancer Cell Line H460 Resistance**

<table>
<thead>
<tr>
<th>MMPT and Analog</th>
<th>H460/TaxR</th>
<th>H460/VinR (µM)</th>
<th>H460</th>
<th>NIHFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPT</td>
<td>2.0/&gt;31</td>
<td>1.1/&gt;31</td>
<td>2.8/&gt;31</td>
<td>12.1/&gt;31</td>
</tr>
<tr>
<td>A</td>
<td>1.1/17</td>
<td>2.5/16</td>
<td>3.4/20</td>
<td>16/&gt;31</td>
</tr>
<tr>
<td>G</td>
<td>1.9/14</td>
<td>2.2/6.8</td>
<td>2.5/18</td>
<td>&gt;31</td>
</tr>
<tr>
<td>R</td>
<td>0.36/5.9</td>
<td>0.15/0.79</td>
<td>2.6/25</td>
<td>19.9/&gt;31</td>
</tr>
</tbody>
</table>

Similar to MMPT, the analogues A and G were effective for all the three cell lines, suggesting that their cytotoxic effects were not affected by P-glycoprotein status. Interestingly, the analog R is more effective in killing the chemo-resistant cells when compare with parental cells. The data listed in Table 7 provide evidence that MMPT and its 3 analogues are effective to kill both chemosensitive and chemoresistant H460 cancer cells.

4. **Uptake of the compound by cancer cells.**

The uptake of analogues G and R by H460 and P-glycoprotein overexpressing H460/TaxR and H460/VinR cells was also tested. For this purpose, cells were tested with 10µg/ml concentrations of analogues G or R. Five or seventeen hours later, cells were harvested, lysed, disrupted compound-protein interaction/binding by diluted acid solution, and cell proteins removed by acid precipitate method. Analogues G and R were extracted by solid phase extraction methods. The extractive was then subjected to HPLC and the amount of the compounds within $10^7$ cells was determined. The results are summarized in Table 8.

**Table 8. Permeability of Analogues G and R in H460 cells**

<table>
<thead>
<tr>
<th>Cancer Cell Line</th>
<th>Treatment Duration (hr)</th>
<th>Concentration in Cell (n mole/10$^7$ cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>H460/TaxR</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>13.7</td>
</tr>
<tr>
<td>H460/VinR</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>36.5</td>
</tr>
<tr>
<td>Cancer Cell Line</td>
<td>Treatment Duration (hr)</td>
<td>Concentration in Cell (nmole/10^7 cell)</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>H460</td>
<td>5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>20.6</td>
</tr>
</tbody>
</table>

The results showed that uptake of analogues G and R in parental H460, H460/TaxR and H460/VinR cells were increased over time from 5 hr and 17 hr. Overall, the uptake of the analogue R in these three cancer cell lines is much better than analogue G, although water-solubility of R3G (logP=2.536) is higher than R3R (logP=3.882). The results indicate that accumulation these compounds in the cancer cells were not affected by the levels of drug efflux pump P-glycoprotein.

**EXAMPLE 12**

**Synthesis and Biological Activity of MMPT and its Analogues**

Sufficient amounts of these compounds are required in order to further characterize the antitumor activity and potential toxicity of MMPT and its analogues. However, commercial supply of these compounds is quite limited. Therefore, studies were undertaken to synthesize some of these compounds. As a first step for chemical synthesis, a small-scale synthesis of MMPT and analog G was performed. The synthetic route of MMPT is depicted in FIG. 18, and the synthetic route of analog G is depicted in FIG. 19.

Compounds 2, 4, and 5 (see FIG. 18 and FIG. 19) and MMPT have been separated and purified by silica gel column chromatography based on polarity of the compounds. The compounds were then analyzed by high performance liquid chromatography-mass spectrography (LC-MS). The cytotoxic effects of these compounds was then evaluated in human colon cancer cell lines DLD1 and Lovo. For this purpose, DLD1 and Lovo cells were treated with 10 µg/ml of the compounds listed in Table 9. Cytopathological changes, such as cell death, change in morphology, and detachment from culture plates were examined 4 days after the treatment. The preliminary results showed that compounds 2 and 5 caused obvious cell death in both cells, whereas compound 4 caused growth inhibition or partial cell death in both cells. In contrast, Compound 1 did not cause any change in cell viability and morphology when compared with control cells treated with DMSO. Compound 3 caused mild growth inhibition in Lovo cells only. These results suggested that phenylamino group (PhNH-) in MMPT and phenolamino group (PhOH-NH) in analog G can be substituted by methylthio(CH₃S-) group without dramatic impact on their cytotoxic effects. However, antitumor activity of these intermediates need to be
further evaluated in order to compare their antitumor activity and toxic effect with that of MMPT and its analogues.

Table 9. Cytocixic Effect of Starting Compounds, Intermediates and Resulting Products in Chemical Synthesis

<table>
<thead>
<tr>
<th>No.</th>
<th>ACS# Formula MW</th>
<th>NAME</th>
<th>Bio-activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DLD-1</td>
</tr>
<tr>
<td>Compound 1</td>
<td>33992-80-2 C11 H9 N O S2 MW=235</td>
<td>5-[(4-methylphenyl)methylene]-2-thioxo-4-thiazolidinone</td>
<td>no</td>
</tr>
<tr>
<td>Compound 2</td>
<td>32723-65-2 C12 H11 N O S2 MW=249</td>
<td>5-[(4-methylphenyl)methylene]-2-(methylthio)-4-Thiazolone</td>
<td>Cell death</td>
</tr>
<tr>
<td>MMPT</td>
<td>101444-37-5 C17 H14 N O S MW=294</td>
<td>5-[(4-methylphenyl)methylene]-2-(phenylamino)-4-thiazolone</td>
<td>Cell death</td>
</tr>
<tr>
<td>Compound 3</td>
<td>5447-37-0 C11 H9 N O S2 MW=267</td>
<td>5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone</td>
<td>no</td>
</tr>
<tr>
<td>Compound 4</td>
<td></td>
<td>5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone</td>
<td>Growth inhibition</td>
</tr>
<tr>
<td>Compound 5</td>
<td>464221-74-7 C13 H13 N O S2 MW=295</td>
<td>5-[(3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone</td>
<td>Cell death</td>
</tr>
</tbody>
</table>

The effects of an analog of compound 1 on certain cardiovascular parameters has been reported in Tawab et al., 1959. The effect of compound 3 as an antiinflammatory and antiallergy agent has been reported (Eur. Patent Appln. 343643).

***************

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may
be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.
REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent 3,988,315
U.S. Patent 4,039,736
U.S. Patent 4,127,714
U.S. Patent 4,144,329
U.S. Patent 4,169,142
U.S. Patent 4,316,011
U.S. Patent 4,737,583
U.S. Patent 4,814,470
U.S. Patent 4,814,470
U.S. Patent 4,814,470
U.S. Patent 4,857,653
U.S. Patent 4,942,184
U.S. Patent 4,942,184
U.S. Patent 4,942,184
U.S. Patent 4,960,790
U.S. Patent 4,960,790
U.S. Patent 5,059,699
U.S. Patent 5,157,049
U.S. Patent 5,200,534
U.S. Patent 5,202,448
U.S. Patent 5,229,529
U.S. Patent 5,248,796
U.S. Patent 5,248,796
U.S. Patent 5,250,722
U.S. Patent 5,254,580
U.S. Patent 5,272,171
U.S. Patent 5,274,137
U.S. Patent 5,278,324
U.S. Patent 5,278,324
European Appln. 590267
Haar et al., Biochemistry, 35:243-250, 1996.
PCT Appln. CA 97/00910
PCT Appln. WO 93/02067
PCT Appln. WO 93/10076
PCT Appln. WO 93/23555


CLAIMS

1. A method of inhibiting growth of a cell comprising providing to the cell 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof.

2. The method of claim 1, wherein the analog is a compound having the structure:

   
   
   
   wherein \( R_1 \) is chosen from the group consisting of

   
   
   
   wherein \( R_2 \) is alkyl; and
   wherein \( R_3 \) is aryl; and
   wherein \( R_5 \) is selected from the group consisting of alkyl and hydrogen.

3. The method of claim 2, wherein \( R_1 \) is

   
   
   

4. The method of claim 3, wherein \( R_5 \) is hydrogen.

5. The method of claim 4, wherein \( R_2 \) is lower alkyl

6. The method of claim 5, wherein \( R_2 \) is CH.

7. The method of claim 2, wherein \( R_4 \) is heterocyclic aryl

8. The method of claim 6, wherein \( R_4 \) is carbocyclic aryl.

9. The method of claim 2, wherein \( R_4 \) is
wherein $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are each independently selected from the group consisting of hydrogen, halogen, alkyl, alkoxy, OH, trihalomethyl, NO$_2$, ester, amide, and amine.

10. The method of claim 9, wherein $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ are each independently selected from the group consisting of hydrogen, alkoxy and OH.

11. The method of claim 2, wherein $R_3$ is heterocyclic aryl.

12. The method of claim 8, wherein $R_3$ is carbocyclic aryl.

13. The method of claim 9, wherein $R_3$ is

wherein $X_6$, $X_7$, $X_8$, $X_9$, and $X_{10}$ are each independently selected from the group consisting of hydrogen, halogen, alkyl, alkoxy, OH, trihalomethyl, NO$_2$, ester, amide, and amine.

14. The method of claim 13, wherein $X_6$, $X_7$, $X_8$, $X_9$, and $X_{10}$ are each independently selected from the group consisting of hydrogen, alkoxy and OH.

15. The method of claim 14, wherein $X_5$ is CH$_3$.

16. The method of claim 14, wherein $X_3$ is OH.
17. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is hydrogen, X₄ is hydrogen, X₅ is hydrogen, X₆ is OH, X₇ is hydrogen, X₈ is OH, X₉ is hydrogen, and X₁₀ is hydrogen.

18. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is hydrogen, X₄ is hydrogen, X₅ is hydrogen, X₆ is OH, X₇ is hydrogen, X₈ is -O-CH₂-CH₃, X₉ is hydrogen, and X₁₀ is hydrogen.

19. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is hydrogen, X₄ is hydrogen, X₅ is hydrogen, X₆ is hydrogen, X₇ is OH, X₈ is hydrogen, X₉ is hydrogen, and X₁₀ is hydrogen.

20. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is hydrogen, X₄ is hydrogen, X₅ is hydrogen, X₆ is hydrogen, X₇ is -O-CH₃, X₈ is OH, X₉ is hydrogen, and X₁₀ is hydrogen.

21. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is hydrogen, X₄ is hydrogen, X₅ is hydrogen, X₆ is hydrogen, X₇ is -O-CH₃, X₈ is hydrogen, X₉ is -O-CH₃, and X₁₀ is hydrogen.

22. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is OH, X₄ is hydrogen, X₅ is hydrogen, X₆ is hydrogen, X₇ is -O-CH₃, X₈ is OH, X₉ is hydrogen, and X₁₀ is hydrogen.

23. The method of claim 14, wherein X₁ is hydrogen, X₂ is hydrogen, X₃ is -O-CH₃, X₄ is hydrogen, X₅ is hydrogen, X₆ is hydrogen, X₇ is -O-CH₃, X₈ is hydrogen, X₉ is hydrogen, and X₁₀ is hydrogen.

24. The method of claim 5, wherein R₃ and R₄ are each independently selected from the group consisting of

```
\begin{tikzpicture}
  \node[circle, draw] (A) at (0,0) {Y₁};
  \node[circle, draw] (B) at (1,0) {Y₂};
  \node[circle, draw] (C) at (2,0) {Y₃};

  \draw (A) -- (B);
  \draw (B) -- (C);
\end{tikzpicture}
```

and

```
\begin{tikzpicture}
  \node[circle, draw] (A) at (0,0) {Y₁};
  \node[circle, draw] (B) at (1,0) {Y₂};
  \node[circle, draw] (C) at (2,0) {Y₃};

  \draw (A) -- (B);
  \draw (B) -- (C);
\end{tikzpicture}
```

wherein Y₁, Y₂, and Y₃ are each independently selected from the group consisting of halogen, alkyl, alkoxy, OH, trihalomethyl, NO₂, ester, amide, and amine.
25. The method of claim 24, wherein \( Y_1, Y_2, \) and \( Y_3 \) are each independently selected from the group consisting of alkoxy and OH.

26. The method of claim 25, wherein \( Y_1, Y_2, \) and \( Y_3 \) are each independently selected from the group consisting of -O-CH\(_3\) and OH.

27. The method of claim 1, wherein inhibiting comprises inducing apoptosis in the cell.

28. The method of claim 1, wherein the cell is a cancer cell.


30. The method of claim 28, wherein the cancer cell is a lung cancer cell.

31. The method of claim 30, wherein the lung cancer cell is a non-small cell lung cancer cell.

32. The method of claim 28, wherein the cancer cell is a multidrug-resistant cancer cell.

33. The method of claim 32, wherein the multidrug-resistant cancer cell is a paclitaxel-resistant cancer cell.

34. The method of claim 1, wherein the cell is located in a cell culture.

35. The method of claim 1, wherein the cell is located in a tissue culture.

36. The method of claim 1, wherein the cell is located in a mammal.

37. The method of claim 36, wherein the mammal is a human.

38. The method of claim 1, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4([5H]-thiazolone is 5-[(4-methylphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5[(3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)- 4([5H]-thiazolone, 5-[(4-ethoxy-2-
hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(3-methoxy-4-(2-propenyl)oxy)phenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 3-[(4-oxo-2-(phenylamino)-5(4H)-thiazolylidene) methyl]-benzoic acid, 5-[(4-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, N-[4-[[4-oxo-2-(phenylamino)-5(4H)-thiazolylidene]methyl]phenyl]-acetamide, 5-[(2-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 2-(phenylamino)-5-[(2-hydroxyphenyl)methylene]-4(5H)-thiazalone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(3,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazalone, 5-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazalone, 2-[(4-methylphenyl)amino]-5-(phenylmethylene)-4(5H)-thiazalone, 5-benzylidene-2-(3,4-dichlorophenyl)methylene)-4-thiazolidinone, 2-[(4-hydroxy phenyl)amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazalone, 5-[(4-chlorophenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazalone, 5-[(2,3-dimethoxyphenyl) methylene]-2-[(4-methylphenyl)amino]-4(5H)-thiazalone, or 5-(2,4-dihydroxybenzylidene)-2-(phenylamino)-1,3-thiazolidin.

39. The method of claim 1, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone, or 2-[(4-hydroxyphenyl)amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazalone.

40. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazalone.
41. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(3-ethoxy-2-hydroxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

42. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(3-hydroxy-phenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

43. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

44. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dimethoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

45. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone.

46. The method of claim 39, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 2-[(4-hydroxyphenyl) amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone.

47. The method of claim 38, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

48. A method of inhibiting microtubule polymerization activity in a cell comprising providing to the cell 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone.

49. The method of claim 48, wherein inhibiting comprises inducing cell cycle arrest in the cell.

50. A method of treating a cancer in a subject comprising administering to the subject a therapeutically 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof to inhibit cell growth.
51. The method of claim 50, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazole is 5-[(4-methylphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-methoxy-4-(2-propenylxyloxy)phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 3-[(4-oxo-2-(phenylamino)-5(4H)-thiazolylidene) methyl]-benzoic acid, 5-[(4-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, N-[(4-oxo-2-(phenylamino)-5(4H)-thiazolylidene)methyl][phenyl]-acetamide, 5-[(2-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 2-(phenylamino)-5-[(2-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, 5-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, 2-[(4-methylphenyl)amino]-5-(phenylmethylene)-4(5H)-thiazolone, 5-benzylidene-2-(3,4-dichlorophenylamino)-4-thiazolidinone, 2-[(4-hydroxy phenyl) amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(4-chlorophenyl)methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, 5-[(2,3-dimethoxyphenyl) methylene]-2-[(4-methylphenyl)amino]-4(5H)-thiazolone, or 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

52. The method of claim 50, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxy-phenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(2,4-dimethoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, or 2-[(4-hydroxyphenyl) amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone.
53. The method of claim 51, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin or an analog thereof.

54. The method of claim 50, further comprises inhibiting microtubule polymerization activity.

55. The method of claim 54, comprising inducing cell cycle arrest.

56. The method of claim 50, wherein the cancer is a breast cancer, lung cancer, head and neck cancer, bladder cancer, bone cancer, bone marrow cancer, brain cancer, colon cancer, esophageal cancer, gastrointestinal cancer, gum cancer, kidney cancer, liver cancer, nasopharynx cancer, ovarian cancer, prostate cancer, skin cancer, stomach cancer, testis cancer, tongue cancer, or uterine cancer.

57. The method of claim 50, wherein the cancer is a lung cancer.

58. The method of claim 57, wherein the lung cancer is a non-small cell lung cancer.

59. The method of claim 50, wherein the cancer is a multidrug-resistant cancer.

60. The method of claim 59, wherein the multidrug-resistant cancer is a paclitaxel-resistant cancer.

61. The method of claim 50, wherein administering is intravenous, intradermal, intramuscular, intraarterial, intralesional, percutaneous, subcutaneous, or by aerosol.

62. The method of claim 50, wherein the subject is a mammal.

63. The method of claim 62, wherein the mammal is a human.

64. The method of claim 50, further comprising administering to the subject an additional therapeutic agent.

65. The method of claim 64, wherein the additional therapeutic agent is a chemotherapeutic agent.

66. The method of claim 64, wherein the additional therapeutic agent is a radiotherapeutic agent.
67. The method of claim 64, wherein the additional therapeutic agent is a microtubule-interacting agent.

68. The method of claim 67, wherein the microtubule-interacting agent is a vinca alkaloid.

69. The method of claim 67, wherein the microtubule-interacting agent is a taxane.

70. The method of claim 50, wherein the thiazolone or analog thereof is administered more than once.

71. The method of claim 50, wherein the thiazolone or analog thereof is administered before the additional therapeutic agent.

72. The method of claim 50, wherein the thiazolone or analog thereof is administered after the additional therapeutic agent.

73. The method of claim 50, wherein the thiazolone or analog thereof is administered at the same time as the additional therapeutic agent.

74. The method of claim 64, wherein the additional therapeutic agent is administered intravenously, intradermally, intramuscularly, intraarterially, intralesionally, percutaneously, subcutaneously, or by an aerosol.

75. The method of claim 64, wherein the additional therapeutic agent is administered more than once.

76. A method of assaying for cancer cell sensitivity to a 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof comprising:

   a) providing a cancer cell;
   b) contacting the cancer cell with 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof;
   c) analyzing the cancer cell for inhibition of growth; and
   d) comparing the inhibition of growth in the cancer cell from step (c) with the inhibition of growth in the cancer cell in the absence of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof,
wherein growth inhibition by 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof indicates that said cancer cell is susceptible to 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof.

77. The method of claim 76, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-methylphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-methoxy-4-(2-propenyl)oxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 3-[[4-oxo-2-(phenylamino)-5(4H)-thiazolylidene]methyl]-benzoic acid, 5-[(4-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, N-[[4-[[4-oxo-2-(phenylamino)-5(4H)-thiazolylidene]methyl]phenyl]-acetamide, 5-[(2-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 2-(phenylamino)-5-[(2-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 5-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 2-[(4-methylphenyl)amino]-5(phenylmethylene)-4(5H)-thiazolone, 5-benzylidene-2(3,4-dichlorophenylaminoo)-4-thiazolidinone, 2-[(4-hydroxy phenyl)amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(4-chlorophenyl)methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone, 5-[(2,3-dimethoxyphenyl) methylene]-2-[(4-methylphenyl)amino]-4(5H)-thiazolone, or 5-[(2,4-dihydroxybenzylidene)-2-(phenylamino)-1,3-thiazolidin.

78. The method of claim 76, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxy-phenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone.
thiazolone, 5-[(2,4-dimethoxyphenyl) methylene]-2-(phenylamino)- 4(5H)-thiazolone, 5-
[(4-hydroxy-3-methoxyphenyl) methylene]-2-[(4-hydroxyphenyl) amino]- 4(5H)-
thiazolone, or 2-[(4-hydroxyphenyl) amino]-5-[(3-hydroxyphenyl)methylene]- 4(5H)-
thiazolone.

79. The method of claim 77, wherein the analog of 5-[(4-methylphenyl)methylene]-2-
(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-
thiazolidin.

80. The method of claim 76, wherein analyzing for growth inhibition is by XTT assay.

81. The method of claim 76, further comprising assaying for microtubule polymerization
activity in a cancer cell.

82. The method of claim 81, comprising analyzing for α-tubulin expression.

83. The method of claim 81, comprising analyzing for β-tubulin expression

84. The method of claim 76, wherein assaying further comprises analyzing for induction of
apoptosis.

85. The method of claim 84, wherein induction of apoptosis is analyzed by FACS.

86. The method of claim 76, wherein assaying further comprises determining the
phosphorylation status of cell cycle molecules selected from the group consisting of
MPM-2, cdc25C, and histone H3.

87. A method of identifying a candidate 5-[(4-methylphenyl)methylene]-2-(phenylamino)-
4(5H)-thiazolone substance having anticancer activity comprising:
a) providing a cancer cell;
 b) contacting the cancer cell with the candidate substance;
c) analyzing the cancer cell for growth inhibition; and
d) comparing the inhibition of growth in the cancer cell from step (c) with the
inhibition of growth in the cancer cell in the absence of the candidate substance,
wherein growth inhibition by the candidate 5-[(4-methylphenyl)methylene]-2-
(phenylamino)-4(5H)-thiazolone substance indicates that said cancer cell is susceptible to
the candidate 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone
substance.
88. The method of claim 87, wherein the candidate substance is an organic molecule.

89. The method of claim 87, wherein the candidate substance is an inorganic molecule.

90. The method of claim 87, wherein the candidate substance is an organopharmaceutical.

91. The method of claim 87, wherein analyzing for growth inhibition is by XTT assay.

92. The method of claim 87, further comprising assaying for microtubule polymerization activity in a cancer cell.

93. The method of claim 92, comprising analyzing for α-tubulin expression.

94. The method of claim 92, comprising analyzing for β-tubulin expression.

95. The method of claim 87, wherein assaying further comprises analyzing for induction of apoptosis.

96. The method of claim 95, wherein induction of apoptosis is analyzed by FACS.

97. The method of claim 87 wherein assaying further comprises determining the phosphorylation status of cell cycle molecules selected from the group consisting of MPM-2, cdc25C, and histone H3.

98. A pharmaceutical composition comprising 5-{[4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone or an analog thereof in a pharmaceutically acceptable excipient.

99. The pharmaceutical composition of claim 98, wherein the analog of 5-{[4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-{[4-methylphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-{[4-hydroxy-3-methoxyphenyl)methylene]-2-thioxo-4-thiazolidinone, 5-{[4-hydroxy-3-methoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5{[(3,4-dimethoxyphenyl)methylene]-2-(methylthio)-4-thiazolone, 5-{[2,4-dihydroxyphenyl)methylene]-2-(phenylamino)- 4(5H)-thiazolone, 5-{[4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)- 4(5H)-thiazolone, 5-{[3-hydroxy- phenyl)methylene]-2-(phenylamino)- 4(5H)-thiazolone, 5-{[4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)- 4(5H)-thiazolone, 5-{[3-methoxy-4-(2-propenylxy)phenyl)methylene]-2-(phenylamino)- 4(5H)-thiazolone, 3-{[4-oxo-2-
(phenylamino)-5(4H)-thiazolylidene]methyl]-benzoic acid, 5-[(4-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, N-[4-[[4-oxo-2-(phenylamino)-5(4H)-thiazolylidene]methyl]phenylacetamide, 5-[[2-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 2-(phenylamino)-5-[(2-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[[2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[[3,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[[4-hydroxy-3-methoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 5-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 2-[(4-methylphenyl)amino]-5-(phenylmethylene)-4(5H)-thiazolone, 5-benzylidene-2-(3,4-dichlorophenylamino)-4-thiazolidinone, 2-[(4-hydroxyphenyl)amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone, 5-[(4-chlorophenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, 5-[(2,3-dimethoxyphenyl)methylene]-2-[(4-methylphenyl)amino]-4(5H)-thiazolone, or 5-(2,4-dihydroxybenzyldiene)-2-(phenylamino)-1,3-thiazolidin.

100. The method of claim 98, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(3-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(2,4-dimethoxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone, 5-[(4-hydroxy-3-methoxyphenyl)methylene]-2-[(4-hydroxyphenyl)amino]-4(5H)-thiazolone, or 2-[(4-hydroxyphenyl)amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone.

101. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dihydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone.

102. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-ethoxy-2-hydroxyphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone.
103. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(3-hydroxy-phenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

104. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

105. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(2,4-dimethoxyphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone.

106. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-[(4-hydroxy-3-methoxyphenyl) methylene]-2-[(4-hydroxyphenyl) amino]-4(5H)-thiazolone.

107. The method of claim 100, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 2-[(4-hydroxyphenyl) amino]-5-[(3-hydroxyphenyl)methylene]-4(5H)-thiazolone.

108. The pharmaceutical composition of claim 99, wherein the analog of 5-[(4-methylphenyl)methylene]-2-(phenylamino)-4(5H)-thiazolone is 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin.

109. The pharmaceutical composition of claim 98, further comprising an additional therapeutic agent.

110. The pharmaceutical composition of claim 109, wherein the additional therapeutic agent is an organopharmaceutical.

111. The pharmaceutical composition of claim 109, wherein the additional therapeutic agent is a chemotherapeutic agent.

112. The pharmaceutical composition of claim 109, wherein the additional therapeutic agent is a microtubule-interacting agent.

113. The pharmaceutical composition of claim 112, wherein the microtubule-interacting agent is a vinca alkaloid.
114. The pharmaceutical composition of claim 112, wherein the microtubule-interacting agent is a taxane.
5-((4-methylphenyl)methylene)-2-(phenylamino)-4(5H)-thiazolone (MMPT)
**H1299**

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**H460**

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concentration (μM)

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**FIG. 10B**
MMPT Analogues (Summary)

Fig. 1 MMPT Analog Structure

![MMPT Analog Structure Diagram]

FIG. 16
Synthetic route of MMPT:

**Compound 1** → **Compound 2** → **MMPT**

FIG. 18
Synthetic route of analogue G:

Compound 3 → Compound 4 → Analog G

Compound 5

FIG. 19
# INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/US05/05496

## A. CLASSIFICATION OF SUBJECT MATTER

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.: 514/372, 439; 436/64

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

none

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

Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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### Further documents are listed in the continuation of Box C.

- **Special categories of cited documents:**
  - “A” document defining the general state of the art which is not considered to be of particular relevance
  - “E” earlier application or patent published on or after the international filing date
  - “L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - “O” document referring to an oral disclosure, use, exhibition or other means
  - “P” document published prior to the international filing date but later than the priority date claimed
  - “T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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  - “Z” document member of the same patent family

### Date of the actual completion of the international search

23 July 2005 (23.07.2005)

### Date of mailing of the international search report

4 AUG 2005

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Alexandria, Virginia 22313-1450

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**Authority**

Cybille Delbecq-Muirheid

**Telephone No.** 571-272-0572

Form PCT/ISA/210 (second sheet) (January 2004)
Continuation of B. FIELDS SEARCHED Item 3:
STN: registry, licplus, uspatfull
structure search; cancer, tumor, carcinoma, microtubule polymerization, melanoma, adenocarcinoma, lymphoma, leukemia