Abstract: The present invention is directed methods for the treatment of cancer and inhibition of cancer cell proliferation through the use of polymethoxyflavones. The polymethoxyflavones inhibit cell proliferation without resulting in cell death.
The Use of Flavonoids for the Inhibition of Cellular Growth

[0001] This application claims priority to U.S. Provisional Application No. 60/801,682, filed May 19, 2006, the disclosure of which is hereby incorporated by reference in its entirety.

Background of the Invention

[0002] Cancer is a disease of inappropriate tissue accumulation. Traditional chemotherapeutic agents share one characteristic: they are usually more effective in killing or damaging malignant cells than normal cells. However, the fact that they do harm normal cells indicates their potential for toxicity. Animal tumor investigations and human clinical trials have shown that drug combinations produce higher rates of objective response and longer survival than single agents. Combination drug therapy is, therefore, the basis for most chemotherapy employed at present (De Vita, V. T. et al., 1995, Cancer 35:98).

[0003] Cancer treatment requires inhibitions of a variety of factors including tumor cell proliferation, metastatic dissemination of cancer cells to other parts of the body, invasion, tumor-induced neovascularization, and enhancement of host immunological responses and cytotoxicity. Conventional cancer chemotherapeutic agents have often been selected on the basis of their cytotoxicity to tumor cells. However, some anticancer agents have adverse effects on the patient's immune system.

[0004] Cytotoxic anticancer drug efficacy is contingent upon differences in drug sensitivity between normal and tumor tissue (i.e. therapeutic index). The therapeutic index of these drugs is often small, with little difference between the dose required for optimal antitumor activity (effective dose) and that which produces unacceptable toxicities in normal tissues (toxic dose). The ideal anticancer agent would exert minimal adverse effects on normal tissues with maximal capacity to kill tumor cells and/or inhibit tumor growth (i.e. with large therapeutic indices). Targeting tumor-associated molecules is one way to enhance therapeutic index. However, a molecularly targeted agent has maximal antitumor effect and minimal host toxicity only when its target is the single cause of disease or is only found in tumor tissues. Another approach is to target a physiological process essential to tumor growth such as cellular proliferation, but not processes involved
in survival. With this approach, tumor cell proliferation can be reduced or halted while cell viability is relatively unimpaired (cytostasis). Most normal tissues would be less functionally impeded by cytostatic agents, thus increasing the gap between effective and toxic doses. Even if affected, cell viability and the capacity to quickly return to normal function would remain more intact in non-tumor tissues. With less dependence on biochemical differences between tumor and normal cells, cytostatic agents potentially have an important role to play in anticancer chemotherapy.

[0005] Because they are inherently low in toxicity, naturally occurring constituents of the human diet such as flavonoids (low molecular weight polyphenols ubiquitously found in plants) are of particular interest as potential sources of compounds with cytostatic activity. There is a correlation between diets high in fruits and vegetables and decreased risk of cancer. High consumption of fruits and vegetables is significantly protective against risk of lung, colon, ovarian, esophageal, breast, cervix, bladder, pancreas, oral cavity and stomach cancer. Flavonoids have demonstrated good potential as anticancer agents by their antiproliferative activity against human tumor cell lines both in vitro and as xenografts in immunocompromised mice.

[0006] The term "flavonoid" includes, but is not limited to, polymethoxylated flavonoids and refers to any member of the group of aromatic, oxygen-containing, heterocyclic pigments found in the derivatives of the invention and includes for example members of the chemical subgroups 1) catechols, 2) leucoanthocyanidins and flavanones, 3) flavanins, flavones, and anthocyanins, and 4) flavonols.

[0007] Some flavonoids are present in citrus juices. For example, orange juice contains hesperetin, grapefruit juice includes naringenin and tangerine juice includes tangeretin and nobiletin.

[0008] Two polymethoxylated flavones (PMFs), 4',5,6,7,8-pentamethoxyflavone (tangeretin) and 3',4',5,6,7,8-hexamethoxyflavone (nobiletin), are among the most effective citrus flavonoids at inhibition of human cancer cell proliferation. Tangeretin and nobiletin have been found to inhibit proliferation of human cancer cell lines derived from squamous cell carcinoma, gliosarcoma, leukemia, melanoma, colorectal cancer, gastric carcinoma, and lung carcinoma, addition, they have been reported to decrease the incidence of
chemically induced tumors in mice and rats. Although the chemopreventive and antiproliferative mechanisms of action may differ, the in vivo tumor preventive activity of tangeretin and nobiletin demonstrate the potential for their safe pharmacological use.

[0009] At the cellular and molecular level, flavonoids have a broad range of effects and interactions that contribute to their chemopreventive, anticarcinogenic and antiproliferative activities. The modulation of cellular processes such as cell cycle and apoptosis contributes to their antiproliferative effects. At the molecular level, flavonoids interact with receptors, enzymes, and kinases. These cellular and molecular effects also mediate the antiproliferative effect of tangeretin and nobiletin. Both of these flavonoids have been shown to induce cell cycle arrest and apoptosis in tumor cell lines. Even though flavonoids are considered non-toxic constituents of the diet, their antiproliferative action reportedly relies on induction of cell death.

[0010] Inhibition of proliferation of human cancers without inducing cell death (or minimizing cell death) may be advantageous in treating human tumors in their natural environment, as it would restrict proliferation in a manner less likely to cause cytotoxicity and induce death in non-tumor tissues. There is little evidence to date that tangeretin and nobiletin can significantly inhibit tumor cell proliferation in a purely cytostatic, non-cytotoxic manner. This mode of action could potentially avoid complications of toxicity to normal tissues. Thus it would be greatly advantageous if a cancer therapy or treatment could be developed that would afford non-cytotoxic protection against factors that might lead to progression of tumors.

[0011] The inventors of the present invention have addressed this issue, by developing a method in which tangeretin and nobiletin inhibit proliferation of cancer cells in the absence of, or with minimal cytotoxicity.

**Summary of the Invention**

[0012] The present invention is directed to a method for the treatment of cancer by administration of a polymethoxyflavone composition to a patient in need thereof. The polymethoxyflavone composition is administered in an effective amount to inhibit cell proliferation without causing apoptosis or without significantly causing apoptosis.
[0013] The present invention is also directed to a method for inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective concentration of polymethoxyflavone to inhibit proliferation of the cancer cells without causing apoptosis to the cancer cells or without significantly causing apoptosis to the cancer cells.

[0014] In one embodiment of the present invention, the polymethoxyflavone composition comprises tangeretin, nobiletin, genistein, quercitin, silibinin, apigenin, hesperetin, naringenin or combinations thereof.

[0015] In another embodiment of the present invention, the polymethoxyflavone composition comprises tangeretin, nobiletin or combinations thereof.

[0016] In another embodiment of the present invention, the polymethoxyflavone is tangeretin. In a preferred embodiment, the effective concentration of tangeretin to inhibit cell proliferation without causing apoptosis when contacted with a cancer cell is from about 5 mcg/ml to about 60 mcg/ml or from about 15 mcg/ml to about 30 mcg/ml. In more preferred embodiments, the concentration of tangeretin is about 20 mcg/ml.

[0017] In another embodiment of the present invention, the polymethoxyflavone is nobiletin. In a preferred embodiment, the effective concentration of nobiletin to inhibit cell proliferation without causing apoptosis when contacted with a cancer cell is from about 5 mcg/ml to about 100 mcg/ml; from about 20 mcg/ml to about 90 mcg/ml; or from about 24 mcg/ml to about 80 mcg/ml. In more preferred embodiments, the concentration of nobiletin is about 40 mcg/ml.

[0018] In yet another embodiment of the present invention, the proliferation of cancer cells is inhibited by about 40% to about 100%; about 60% to about 95%; or about 50% to about 70% at about 12 hours after contact with the cancer cells.

[0019] In another embodiment of the present invention, the cancer cells being treated are breast cancer cells, colon cancer cells, nasopharyngeal cancer cells, gastric cancer cells, hepatocellular cancer cells, colorectal cancer cells, or any other types of cancer cells recognized in humans.
When used herein, "PMF" is an abbreviation for polymethoxyflavones. "PI" is an abbreviation for propium iodide. "Tan" is an abbreviation for tangeretin. "Nob" is an abbreviation for nobiletin.

Apoptosis (cell suicide) and cell death are used interchangeably herein.

**Brief Description of the Drawings**

Fig. 1 depicts the structure of tangeretin and nobiletin.

Fig. 2 depicts the dose-dependent inhibition of proliferation by tangeretin (A) and nobiletin (B) in MDA-MB-435 (●), MCF-7 (T), and HT-29 (■). Cells were exposed to a range of concentrations of tangeretin and nobiletin as shown and cell numbers were determined after four days. Mean cell numbers from triplicate measurements are expressed as a % of DMSO control ± SEM, and are representative of three separate experiments.

Fig. 3 depicts the inhibition of proliferation by tangeretin and nobiletin in MDA-MB-435 (A,D), MCF-7 (B,E), and HT-29 (C,F) over time. Cells were exposed to tangeretin [Panels A-C: DMSO (●) and tangeretin (●) 20 µg/ml], and nobiletin [Panels D-F: DMSO (■) and nobiletin (○) 24µg/ml for MCF-7 and HT-29, 40µg/ml for MDA-MB-435] for four days. Cell numbers were determined at 6, 12, 24, 48, 72 and 96 hours. Mean cell numbers ± SEM are from quadruplicate measurements, and are representative of three to four separate experiments.

Fig. 4 depicts cell cycle distribution in flavonoid-treated MDA-MB-435 (A,D), MCF-7 (B,E) and HT-29 (C,F). Cells were exposed to tangeretin 20 µg/ml (panels A-C, white bars), nobiletin 40 and 80 µg/ml (panel D, white and grey bars, respectively), nobiletin 24 and 80 µg/ml (panels E-F, white and grey bars, respectively) and DMSO control (all panels, black bars) for 24, 48 and 72 hours. Cell nuclei were stained with propidium iodide, and DNA content analyzed by flow cytometry. Percent of total cell population values were obtained in triplicate for each time point, and are expressed as...
means ± SEM. Graphs are representative of three separate experiments. *, significantly different from corresponding DMSO controls (P < 0.05).

Fig. 5 depicts cell death in flavonoid-treated MDA-MB-435 (A,D), MCF-7 (B,E) and HT-29 (C,F). Cells were exposed to tangeretin 20 µg/ml (Tan)(panels A-C), nobiletin 24 µg/ml (Nob)(panels E-F), nobiletin 40 µg/ml (Nob)(panel D), DMSO control (VC) and positive control (PC) (cycloheximide 100 µg/ml for MDA-MB-435 and MCF-7, or cisplatin 20 µM for HT-29) for 48 and 72 hours. Cells were labeled with annexin V-FITC and propidium iodide and analyzed by flow cytometry. Graphs are representative of two separate experiments. *, significantly different annexin V staining (black bars), and **, significantly different annexin V plus PI staining (grey bars) compared to controls (P < 0.05).

Fig. 6 depicts proliferation of MDA-MB-435 (A), MCF-7 (B) and HT-29 (C) for four days following removal of tangeretin and nobiletin. Cells were exposed to tangeretin 20 µg/ml (A), nobiletin 40 µg/ml (†), media (‡) or DMSO (§) controls for four days. Treatment media was removed and replaced with flavonoid- and DMSO-free growth medium and grown for an additional four days. Cell numbers were determined each day following media renewal. Day four cell numbers are considered baseline and cell numbers from subsequent days are expressed as fold increases of this number.

Fig. 7 depicts cell cycle distribution of MDA-MB-435 (A), MCF-7 (B) and HT-29 (C) 24 and 96 hours following removal of tangeretin or nobiletin. Cells were exposed to tangeretin 20 µg/ml (panels A-C, white bars) and nobiletin 40 µg/ml (panel A, grey bars) or 24 µg/ml (panels B-C, grey bars), or DMSO control (all panels, black bars) for four days. Treatment media was removed and replaced with flavonoid- and DMSO-free growth medium and cells were grown for an additional four days. 24 and 96 hours following media change, cell nuclei were prepared and stained with propidium iodide, and DNA content analyzed by flow cytometry. Graphs are representative of 3 separate experiments.*, significantly different from DMSO control (P < 0.05).
Detailed Description of the Preferred Embodiments

[0026] The present invention is directed to compositions and methods for the treatment of cancer and inhibition of cancer cell proliferation without causing apoptosis with the use of polymethoxyflavones.

[0027] Cancers that can be prevented and/or treated by the methods of the present invention include, but are not limited to, human sarcomas and carcinomas, e.g. carcinomas, e.g., colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chondroma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wiim's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendrogloma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease.

[0028] The present invention involves a variety of polymethoxyflavones, including but not limited to tangeretin, nobiletin, genistein, quercitin, silibinin, apigenin, hesperetin, naringenin or combinations thereof. In preferred embodiments, the polymethoxyflavones are tangeretin, nobiletin or combinations thereof.

[0022] The polymethoxyflavones utilized in the present invention may be contained in an oral dosage form together with a pharmaceutically acceptable ingredient.
Pharmaceutically acceptable ingredients may include, but are not limited to acidifying agents, antimicrobial agents, alkalinizing agents, antioxidants, antiseptic agents, bacteriostatic agents, binders, buffering agents, coating agents, desiccants, diluents, dispersing agents, emollients, emulsifying agents, fillers, film-formers, flavoring agents, gelling agents, granulating agents, lubricants, plasticizers, preservatives, solubilizing agents, stiffening agents, suspending agents, sweetening agents, viscosity increasing agents, wetting agents, and the like.

In certain preferred embodiments, the polymethoxyflavones may be contained in an immediate release oral dosage form. In other preferred embodiments, the polymethoxyflavones may be contained in a controlled-release dosage form. The dosage forms of the present invention may include, but are not limited to tablets and soft or hard gelatin capsules.

In certain embodiments, polymethoxyflavones may be contained within multiparticulates that can be compressed into tablets or filled into a soft or hard gelatin capsules. The multiparticulates may be spheroids, beads, pellets, rods, microparticles, e.g., microspheres, and the like.

In certain embodiments, the oral dosage form may be a controlled-release dosage form, wherein the polymethoxyflavones are contained in a controlled-release matrix. In other embodiments, the polymethoxyflavones may be contained within controlled-release multiparticulates. In yet another embodiment, the polymethoxyflavones may be contained in an immediate release dosage form that has a controlled-release coating.

The multiparticulates of the present invention may be compressed into tablets or filled into soft or hard gelatin capsules to provide for an oral solid dosage form. In certain embodiments, the tablets and capsules of the present invention may be coated with an immediate release coating, a controlled-release coating or an enteric coating.

In another embodiment, the polymethoxyflavones may be coated onto beads to provide immediate release beads. In certain embodiments, the beads may be coated with an immediate release coating. In other embodiments, the beads may be coated with a controlled-release coating.
In certain other embodiments, the polymethoxyflavones may be contained in an oral solution, emulsion, suspension, and the like.

While oral dosage forms are preferred, it is contemplated that the polymethoxyflavones may be administered parenterally as an injection or nasogastrically as a solution, suspension, an emulsion and the like.

When combination therapy is contemplated, the agents may be administered in the same or different dosage forms, and by the same or by different routes of administration.

When treating or preventing these cancers, the polymethoxyflavones of the invention can either be used individually or in combination.

In certain embodiments of the present invention, the polymethoxyflavones used to inhibit proliferation of tumor cells without inducing cell death are combined with other chemotherapeutic agents. These agents may include, e.g., alkylating agents-that work directly on the cancer cell’s DNA to prevent it from replicating, such as busulfan, cyclophosphamide and melphalan; Nitrosureas-that inhibit a cancer cell’s enzymes needed for DNA repair, such as carmustine and lomustine; antimetabolites-that interfere with both a cancer cell’s DNA and RNA, such as 5-Fluorouracil, methotrexate and fludarabine; antitumor antibiotics-that also interfere with a cancer cell’s DNA in addition to changing its cellular membrane-the outside layer of protective coating, such as bleomycin, doxorubicin and darubicin; and mitotic inhibitors-which are plant alkaloids that inhibit enzymes needed for protein synthesis in the cancer cell, such as docetaxel, etoposide and vinorelbine. This list is not exhaustive and is meant only as an exemplary list of possible chemotherapeutic agents which may be used in conjunction with the present invention.

Example

Materials and Methods

Cell culture

MDA-MB-435, MCF-7 (human ductal breast carcinoma and adenocarcinoma, respectively) and HT-29 (human colorectal adenocarcinoma) cell lines (American Type Culture Collection, Manassas, VA) were grown in mc-MEM plus nucleosides
supplemented with 10% fetal bovine serum (growth medium) and maintained at 37°C, in a humidified atmosphere of 5% CO₂ in air.

**Proliferation assay**

[0031] Cells were plated in 25-cm² tissue culture flasks at 1x10⁵ cells/flask and allowed to attach overnight. Cells were treated with a range of concentrations of tangeretin and nobiletin, or DMSO controls, for 4 days. Final DMSO concentrations did not exceed 0.27% (v/v). After 4 days, cells were trypsinized from replicate flasks and were counted using a Beckman Coulter Z1 particle counter (Miami, FL). Cell numbers were expressed as a percent of vehicle control.

**Recovery of proliferation assay following removal of PMFs**

[0032] Cells were plated in 6-well tissue culture plates at 1.25x10⁴ cells/well and allowed to attach overnight. Tangeretin (20 µg/ml), nobiletin (40 µg/ml for MDA-MB-435, and 24 µg/ml for MCF-7 and HT-29), and DMSO controls were added to cells and incubated for 4 days. On day 4, treatment and control media were replaced with fresh growth medium. Cells were incubated and replicate wells were counted each day for an additional 4 days.

**Flow cytometry for cell cycle distribution**

[0033] Cells were plated in 75-cm² tissue culture flasks at 0.6-1x10⁶ cells per flask and incubated overnight to allow attachment. Cells were treated with tangeretin (20 mcg/ml), nobiletin (40 and 80 mcg/ml for MDA-MB-435, and 24 and 80 mcg/ml for MCF-7 and HT-29), and DMSO control for 24, 48, and 72 hours. Bare nuclei from cells were prepared for flow cytometric analysis of DNA content using a modification of a previous method (Vindelov, L.L., et al. Cytometry, (1983) 3, 323-7). Cells were trypsinized to detach and washed 3x in a solution containing 250 mM sucrose, 5% v/v DMSO, 40 mM trisodium citrate, pH 7.6. After each wash, cells were centrifuged 5 minutes, 180 x g at room temperature to pellet cells. Cytoplasmic proteins were stripped by resuspending cells in 250 mc ltR trypsin in a solution containing 1.5 mM spermine, 0.1% v/v NP40, 0.5 mM Tris, 3.4 mM citrate, pH 7.6 (citrate buffer) and incubating for 10 minutes at room temperature. Trypsin was inactivated by adding 200 mc 1trypsin inhibitor (408 Units/ml) in citrate buffer containing RNase A and incubating for 10 minutes at room temperature. Bare,
unfixed nuclei were stabilized and stained by adding 200 μl of a solution containing propidium iodide (PI) (0.42 mg/ml) plus spermine (4.8mM) in citrate buffer and incubating for 10 minutes at room temperature. Cellular debris was removed using 30-mc m cell strainer cap tubes (VWR, Mississauga, ON). Samples were kept on ice in the dark and analyzed on a Beckman Coulter Epics XL-MCL Flow Cytometer (Miami, FL) within 2-3 hours of preparation. A minimum of 25,000 events were collected for each sample.

Resumptions of cell cycling following treatment with PMFs

[0034] Cells were plated in 75-cm² tissue culture flasks at 1 x 10^4 cells per flask and allowed to attach overnight. Tangeretin (20 mcg/ml), nobiletin (40 μg/ml for MDA-MB-435, and 24 μg/ml for MCF-7 and HT-29), and DMSO controls were added to cells and incubated for 4 days. On day 4, treatment and control media were replaced with fresh growth medium, and cells were grown for an additional 4 days. Cell cycle distribution was determined on days 5 and 8 (1 day and 4 days post flavonoid removal) using the method outlined above.

Annexin V/PI staining for apoptosis

[0035] Cells were plated in 25-cm² tissue culture flasks at 1x10^5 cells per flask, incubated overnight, and treated with tangeretin (20 mcg/ml), nobiletin (40 μg/ml for MDA-MB-435, and 24 μg/ml for MCF-7 and HT-29) and DMSO control for 48 and 72 hours. At the end of incubation, treatment and control media was collected since it could contain dead or dying cells that had detached from the flasks. Adherent cells were collected by trypsinization and added to the non-adherent population. Cells were washed 2x with ice cold phosphate buffered saline (136 mM NaCl, 2.8 mM KCl, 10 mM Na_2HPO_4, 1.6 mM KH_2PO_4, pH 7.4) and resuspended in binding buffer (140 mM NaCl, 10 mM HEPES, 2.5 mM CaCl, pH 7.4) at a concentration of 1x10^5 cells per 100 mc1. Cells were stained by adding annexin V-FITC and PI and incubating for 15 minutes in the dark at room temperature. FITC and PI fluorescence were measured by flow cytometry on a Beckman Coulter Epics XL-MCL Flow Cytometer within one hour of annexin V/PI staining. A minimum of 10,000 events were collected for each sample.

Statistical analysis
All results are presented as mean ± SEM. Significant differences between means of three or more data sets were determined by one-way analysis of variance (One-way ANOVA) with post hoc Tukey test. Differences between two data sets were determined by student's t-test. Significance levels were set a priori at P < 0.05.

Results

Inhibition of proliferation

Tangeretin and nobiletin (Figure 1) inhibited proliferation of MDA-MB-435, MCF-7 and HT-29 cells in a dose-dependent manner, with 60-95% inhibition in treated cells compared to control cells (Figure 2). Single concentrations of tangeretin and nobiletin that inhibited proliferation 50-70% were chosen for all subsequent experiments: for tangeretin, 20 µg/ml in all three cell lines; for nobiletin, 24 µg/ml in MCF-7 and HT-29 and 40 µg/ml in MDA-MB-435. These concentrations significantly inhibited proliferation of all cell lines beginning at 12 h and continuing to 4 days (Figure 3).

Effects on cell cycle and apoptosis

The inhibition of proliferation observed for tangeretin and nobiletin could be the result of cell cycle effects or induction of apoptosis or a combination of the two. Treatment of all three cell lines with tangeretin and nobiletin resulted in significant accumulation of cells in the G1 phase compared to vehicle controls (Table 1). This arrest was significant for tangeretin- and nobiletin-treated MDA-MB-435 and MCF-7 cells by 24 h, continuing to 72 h (Figure 4, panels A-B, D-E). In HT-29, for tangeretin and the lower concentration of nobiletin (24 or 40 mcg/ml depending on the cell line), G1 arrest was seen at 48 and 72 h, following a transient accumulation of cells in S phase at 24 h (Figure 4, panels C, F). This transient S phase accumulation was not observed at the higher nobiletin concentration (80 mcg/ml) where cells were arrested in G1 by 24 h, with the arrest being maintained up to 72 h (Figure 4, panel F). Both PMFs maintained cell cycle arrest up to 96 h in all three cell lines (data not shown). With nobiletin, the percentage of cells arrested was directly proportional to concentration (Figure 4, panels D-F) however, there was no apparent relationship with time as the percentage of cells arrested in G1/S remained constant over time.
To determine whether apoptosis was involved in the inhibition of proliferation by tangeretin and nobiletin, apoptotic cells were quantified using flow cytometry. There was no significant difference between annexin V-positive (apoptotic) or annexin V + PI-positive (dead or dying) populations of control and tangeretin- or nobiletin-treated cells (Figure 5). Apoptosis or cell death did not occur at either time-point following treatment of MDA-MB-435, MCF-7 and HT-29 cells with tangeretin and nobiletin at concentrations that were shown to inhibit proliferation over 50%. The absence of apoptosis or cell death
by other means was not due to an inability of these cell lines to undergo induced cell death as appreciable apoptosis or cell death was achieved using other drugs (Figure 5). These data suggest that, at these concentrations, tangeretin and nobiletin inhibit proliferation in a purely cytostatic manner, without killing tumor cells.

Resumption of proliferation and cell cycling

[0061] If these flavonoids were exerting a cytostatic effect, instead of a cytotoxic effect, then cells should regain their capacity to proliferate and cycle following removal of the flavonoids. To demonstrate this, treatment medium was replaced with flavonoid-free growth medium, proliferation was assessed daily for four days following media renewal, and cell cycle distribution was assessed one and four days after media renewal. By the end of four days, proliferation of tangeretin- and nobiletin-exposed cells was similar to that of control cells in all three cell lines (Figure 6). Tangeretin- and nobiletin-exposed MDA-MB-435 and MCF-7 cells returned to normal cycling within a day of flavonoid removal and this was maintained up to four days (Figure 7, panels A-B). Tangeretin-exposed HT-29 cells resumed cell cycling comparable to untreated controls within a day of flavonoid removal as well however, nobiletin-treated HT-29 cells did not resume normal cycling until four days after media renewal (Figure 7, panel C). These data indicate that these cancer cells lines can recover and resume normal proliferation and cycling when no longer exposed to tangeretin and nobiletin.

Conclusion

[0062] Both tangeretin and nobiletin inhibited proliferation in a dose- and time-dependent manner, and blocked cell cycle progression at G1 in all three cell lines. At concentrations that resulted in significant inhibition of proliferation and cell cycle arrest, neither flavonoid induced apoptosis or necrosis in any of the tumor cell lines. The data above indicates that, in these cell lines at concentrations that inhibit proliferation up to 80% over four days, tangeretin and nobiletin are purely cytostatic and significantly suppress proliferation by cell cycle arrest without apoptosis. Such an agent could be expected to spare normal tissues from toxic side effects.
[0063] Both tangeretin and nobiletin exerted their effects quickly (earlier than 12 h after administration of compounds) (Figure 3). Tangeretin (20 mcg/ml) induced accumulation of cells in G1 or S phase of the cell cycle in all three cell lines within 24 h, indicating a pronounced cytostatic effect (Figure 4, panels A-C). Certain differences between cell lines were apparent. For example, HT-29 cells accumulated in S rather than G1 after 24 h (Figure 4, panel C), whereas both breast tumor cell lines accumulated in G1 rather than S (Figure 4, panels A,B). Nevertheless, accumulation at the G1/S boundary was common among all cell lines, and G1 accumulation (and S depletion) was apparent at 48 and 72 h of tangeretin exposure in all 3 lines. Nobiletin had the same effect, at concentrations ranging from 24 to 80 mcg/ml (Figure 4, panels D-F), with the same HT-29-specific S-phase accumulation and G1 depletion at 24 h, at least at the lowest concentration of nobiletin (Figure 4, panel F). This is consistent with previous reports of G1 arrest induced by tangeretin in COLO 205 human colorectal tumor cells and nobiletin in TMK-I gastric tumor cells in vitro. Tangeretin and nobiletin have also been reported to induce arrest in G2/M in HL-60 promyelocyte leukemia and HepG2 hepatocellular human tumor cell lines, respectively. This effect was not evident in the breast and colon tumor cell lines assessed here. AU concentrations of tangeretin or nobiletin either had no consistent effect on the percentage of cells in G2/M, or resulted in decreased G2/M accumulation.

[0064] Other flavonoids, such as quercetin and silibinin have also been reported to induce both G1 and G2/M arrest. In these studies the cell cycle phase in which cells accumulated depended on the concentration of the flavonoid. For example, treatment of nasopharyngeal cancer cells with quercetin induced G2/M arrest at a lower concentration (14.8 mcg/ml) and G1 arrest at a higher concentration (52.1 mcg/ml). Silibinin on the other hand, induced G1 arrest at lower concentrations (50 and 75 mcg/ml) and both G1 and G2/M arrest at a higher concentration (100 mcg/ml). Examples of flavonoids that arrest in G2/M are genistein and apigenin. G2/M arrest is typically associated with DNA damage and affords the cell time to repair before proceeding with mitosis, thereby preventing the persistence of genomic mutations. Therefore, the absence of G2/M accumulation reported in the present study suggests that damage events induced by tangeretin or nobiletin are minimal or absent, at least in some human tumor cell lines, and that G1/S but not G2/M accumulation (consistent with a cytostatic effect), appears to be the consequence of treatment of human tumor cell lines of divergent origin with both flavonoids.
[0065] In addition to cell cycle arrest, nobiletin has been reported to induce apoptosis in
hitman TMK-1 gastric, HepG2 hepatocellular and Colo 320 colorectal cancer cell lines. Tangeretin has been reported to induce apoptosis in HL-60 leukemia cells which have been suggested to be particularly susceptible to damage and death. Further reports of tangeretin effects on apoptotic events are sparse, likely due to its hydrophobic nature which limits its solubility in water to concentrations of 50-60 μM or less; a feature shared, to a lesser degree, by nobiletin. Interestingly, at concentrations that substantially reduced proliferation, neither flavonoid induced cell death in any tested human tumor cell line in the present study (Figure 5). The concentrations of nobiletin reported to induce apoptosis in the cited studies were over twice the amount used in the present invention (0.2-1 mM), with the exception of one qualitative report of apoptosis at 60 μM nobiletin, the lowest concentration used in the present study. Although flavonoids may induce apoptosis at high concentrations, the lack of any significant cell death at concentrations that have profound inhibitory effects on proliferation and induce clear cytostatic effects (G1/S accumulation) suggests that cytostasis and not cytotoxicity is the most relevant biological effect of these compounds.

[0066] When flavonoid-treated, growth-suppressed cells were re-cultured in flavonoid-free medium, those cells quickly recovered their ability to proliferate (Figure 6) and regained cell cycle distribution comparable to controls (Figure 7). Neither tangeretin nor nobiletin inflicted damaging effects sufficient to result in reduced capacity to survive and proliferate. The observation that cells recovered normal proliferation and cell cycling within a day of flavonoid removal supports the hypothesis that both flavonoids exert a purely cytostatic effect without inducing damage likely to delay or suppress growth and/or survival.

[0039] The present invention demonstrates that the flavonoids tangeretin and nobiletin reduced proliferation of human tumor cell lines of disparate origin, that the reduction in proliferation involved cell cycle effects that resulted in accumulation of cells in the G1/S cell cycle compartment (most consistent with a cytostatic rather than a cytotoxic mode of action), and did not involve induction of cell death/apoptosis. The recovery and growth of cells after exposure to flavonoids was indistinguishable from the growth of control cells.
further supporting the contention that tangeretin and nobiletin, at concentrations that substantially reduce human tumor cell proliferation, are purely cytostatic and non-toxic. The ability of these compounds to selectively inhibit growth in the absence of toxicity suggests that they interact selectively with mediators of cell cycle events, and that interactions promoting toxicity are excluded. Tangeretin and nobiletin may induce selective changes in the expression of genes mediating cell cycle events, particularly in advance of accumulation of cells in G1/S. This is currently under investigation. These and other compounds that inhibit proliferation of human cancers without inducing cell death may be advantageous in treating human tumors by restricting proliferation in a manner unlikely to induce cytotoxicity and death in normal, non-tumor host cells.
What is claimed is:

1. A method for inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective concentration of polymethoxyflavone to inhibit proliferation of the cancer cells without causing apoptosis to the cancer cells.

2. The method of claim 1, wherein said polymethoxyflavone is selected from the group consisting of tangeretin, nobiletin and combinations thereof.

3. The method of claim 2, wherein said polymethoxyflavone is tangeretin.

4. The method of claim 3, wherein said cancer cells are contacted with tangeretin in a concentration of from about 5 mcg/ml to about 60 mcg/ml.

5. The method of claim 3, wherein said cancer cells are contacted with tangeretin in a concentration of from about 15 mcg/ml to about 30 mcg/ml.

6. The method of claim 3, wherein said cancer cells are contacted with tangeretin in a concentration of about 20 mcg/ml.

7. The method of claim 2, wherein said polymethoxyflavone is nobiletin.

8. The method of claim 7, wherein said cancer cells are contacted with nobiletin in a concentration of from about 5 mcg/ml to about 100 mcg/ml.

9. The method of claim 7, wherein said cancer cells are contacted with nobiletin in a concentration of from about 20 mcg/ml to about 90 mcg/ml.

10. The method of claim 7, wherein said cancer cells are contacted with nobiletin in a concentration of from about 24 mcg/ml to about 80 mcg/ml.

11. The method of claim 7, wherein said cancer cells are contacted with nobiletin in a concentration of about 40 mcg/ml.
12. The method of claim 1, wherein said proliferation of cancer cells is inhibited by about 40% to about 100% at about 12 hours after contact.

13. The method of claim 1, wherein said proliferation of cancer cells is inhibited by about 60% to about 95% at about 12 hours after contact.

14. The method of claim 1, wherein said proliferation of cancer cells is inhibited by about 50% to about 70% at about 12 hours after contact.

15. The method of claim 1, wherein said cancer cells are breast cancer cells.

16. The method of claim 1, wherein said cancer cells are colon cancer cells.

17. A method for the treatment of cancer comprising administration of a composition comprising a polymethoxyflavone in an effective amount to inhibit proliferation of cancer cells without causing apoptosis to a patient in need thereof.

18. The method of claim 17, wherein said polymethoxyflavone is selected from the group consisting of tangeretin, nobiletin and combinations thereof.

19. The method of claim 18, wherein said polymethoxyflavone is tangeretin.

20. The method of claim 18, wherein said polymethoxyflavone is nobiletin.

21. A method for inhibiting the proliferation of cancer cells without causing apoptosis to the cells comprising contacting cancer cells with an effective concentration of polymethoxyflavone to inhibit proliferation of the cancer cells without causing apoptosis to the cancer cells.

22. A method for the treatment of cancer without causing apoptosis comprising administration of a composition comprising a polymethoxyflavone in an effective amount to inhibit proliferation of cancer cells without causing apoptosis to a patient in need thereof.
Fig. 1.

Tangeretin

Nobiletin
Fig. 2.
Fig. 4.

Tangeretin

24h  48h  72h

A

B

C

Nobiletin

24h  48h  72h

D

E

F

Cell cycle phase

Percent of cells

G1  S  G2M  G1  S  G2M  G1  S  G2M  G1  S  G2M  G1  S  G2M

*
Fig. 5.

![Graph showing the effects of Tangeretin and Nobiletin on cell percentages at 48h and 72h for different cell lines.]

- **A** shows cell percentages for Tangeretin at 48h and 72h for a control (PC), vitamin C (VC), and Tangeretin (Tan) with a marked decrease at 72h.
- **B** shows cell percentages for Tangeretin at 48h and 72h for MDA-MB-435, with a marked decrease at 72h.
- **C** shows cell percentages for Tangeretin at 48h and 72h for HT-29, with a marked decrease at 72h.
- **D** shows cell percentages for Nobiletin at 48h and 72h for MDA-MB-435, with a marked decrease at 72h.
- **E** shows cell percentages for Nobiletin at 48h and 72h for MCF-7, with a marked decrease at 72h.
- **F** shows cell percentages for Nobiletin at 48h and 72h for HT-29, with a marked decrease at 72h.