

Original Article

Catalytic Therapy of Cancer with Ascorbate and Extracts of Medicinal Herbs

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Catalytic therapy (CT) is a cancer treatment modality based on the generation of reactive oxygen species (ROS) using a combination of substrate molecules and a catalyst. The most frequently used substrate/catalyst pair is ascorbate/Co phthalocyanine (PcCo). In the present work, herb extracts containing pigments have been studied as a catalyst in place of PcCo. Extracts from herbs are expected to have efficiency comparable with that of phthalocyanines but as natural products, to exhibit fewer side effects. The present studies demonstrate that a combined use of ascorbate and herbal extracts results in ROS production and a significant decrease in the number of cancer cells after a single *in vitro* treatment. Treatment with ascorbate in conjunction with extracts prepared from several medicinal herbs stimulated apoptosis and disrupted the cell cycle. The number of cells accumulating in the sub-G₀/G₁ stage of the cell cycle was increased 2- to 7-fold, and cells in G₂/M increased 1.5- to 20-fold, indicating that the treatment protocol was highly effective in suppressing DNA synthesis and potentially reflecting DNA damage in the tumor cells. In addition, 20–40% of the cells underwent apoptosis within 24 h of completing treatment. Our results suggest that herbal extracts can function as CT catalysts in the treatment of cancer.

Introduction

Although significant progress has been made over the last several decades in cancer diagnosis, prevention and treatment, the development of effective treatment regimens remains one of the greatest challenges in medical technology. There is presently a number of anticancer treatment techniques used in medical practice including chemotherapy, catalytic therapy (CT), radiotherapy and photodynamic therapy (PDT). Each of the techniques has both beneficial and detrimental effects. Recently, CT, an anticancer therapy with activity similar to that of many chemotherapeutic agents, has emerged as an efficacious cancer treatment approach. Similar to photodynamic and radiotherapies, a significant mechanism underlying the

antitumor activity of this approach is the production of reactive oxygen species (ROS). Potentially, ROS generated in response to these treatments interact with critical cellular moieties resulting in the oxidative degradation of molecules and organelles in cancer cells.

The anticancer effects of CT are elicited through harnessing the energy from an exogenous source. Analogous to the use of light in the case of PDT, donation of energy derived from chemically reactive agents is used to activate 'sensitizing drugs', which enables the generation of ROS by these intermediate molecules. In CT, a transition–metal complex acts as the sensitizing drug or catalyst and a second molecule as the reactant or substrate (1–6). The resultant radical attack on critical cellular molecules ultimately leads to tumor growth suppression, apoptosis and necrosis. The benefits of treatment with CT are becoming increasingly well recognized. In fact, in many instances, CT offers distinct advantages when compared with PDT for anticancer therapy. For example, PDT requires illumination with

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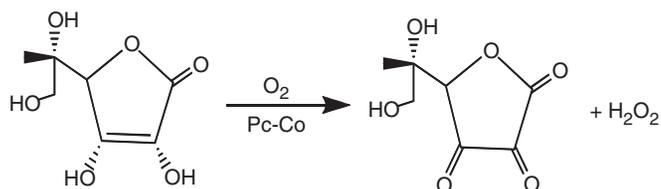
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light sources capable of emitting specific wavelengths of light, a process that limits the use of PDT for treatment of many inaccessible tumors. Also, poorly localized neoplasms and tumors often cannot be effectively treated with PDT. In contrast, the effectiveness of CT for a diverse array of tumor types has been demonstrated *in vitro* in experiments conducted in Japan, the USA and Russia. In these studies, the nutrients ascorbate and vitamin B12 were used. Ascorbate in this approach acts to chemically activate B12 and its derivatives, or Co phthalocyanine (PcCo) to become free radical generators (1–12). Furthermore, *in vivo* studies using the same CT system has been successfully conducted in Russia and the USA (9–21) and clinical trials have been started in Russia (22).

First attempts using CT for anticancer application were reported in 1983 by Kimoto *et al.* (23). In recent years, several promising new CT systems have been developed. In these studies, it was found that a combination cobalt or iron phthalocyanine and sodium ascorbate is highly active in inducing cytostasis and tumor cell deaths. These CT systems have been patented as agents for binary CT of malignant tumors (1–2,24–28). In our previous research (29), we have demonstrated that porphyrins are promising CT agents as well. Currently, ascorbate is considered the most suitable substrate for CT; it is widely accepted because it is a dietary component and many human tumors accumulate ascorbate to a greater extent than do normal tissues (30,31). Ascorbate is easily oxidized by oxygen in the presence of transition metal ions and produces ROS such as H_2O_2 , O_2^- and OH^- . Moreover, some products of ascorbate oxidation have significant antitumor effects themselves.

In addition, in previous studies, we have shown that ascorbate dramatically increases the efficiency of PDT. Apparently, the efficient generation of oxygen radicals by ascorbate intensifies the antitumor effect. These findings led to the hypothesis that ascorbate along with photosensitizers may act by direct tumor cell damage resulting in apoptosis (32,33). Recently, it has been demonstrated that CT can achieve efficiency comparable with that of PDT (1). Similarities observed in the responses elicited by these treatments support the thesis that common processes, potentially mediated by ROS, may underlie both treatment regimens. It is our hypothesis that CT, like PDT, is effective in cancer treatment through eliciting a sequela of effects including direct cell killing, ROS generation, vascular damage, inducing inflammatory responses and the stimulation of nonspecific or specific immune effector cells (34–47). The process of catalytic oxygenation of ascorbate by cobalt (II) octa-4,5-carboxyphthalocyanine (PcCo) is shown in Scheme 1 (48).

At the current time, ~36–40% of cancer patients in USA use complementary alternative medicine (CAM) approaches, including therapy with herbs; unfortunately the scientific research in this area is far from



Scheme 1. The process of catalytic oxygenation of ascorbate by cobalt (II) octa-4,5-carboxyphthalocyanine (Pc Co).

comprehensive. Undeniably, there is a pressing need to use objective procedures to test the effectiveness of putative remedies and help distinguish therapeutically induced changes in underlying pathologic conditions from the subjective relief that might follow any intervention (49–52). Hypericin from St. John's Wort is one herbal remedy commonly used in CAM. Hypericin has an array of demonstrated and claimed physiological effects; however, its activity as a photosensitizer that facilitates ROS generation in response to light is well documented. Interestingly, inclusion of this herb in a CT binary system excited the chemiluminescent oxidation of luciferin by the luciferase (53). In addition, it has been shown that treatment with Hypericin (HY) and Hyperforin (HP) (abundant components of St John's Wort) inhibits the growth of K562 and U937 leukemia cells, and the brain glioblastoma cell line LN229. In these studies, treatment of tumor cells with HP resulted in a dose-dependent generation of apoptotic oligonucleosomes, DNA-laddering and apoptosis-specific morphological changes (54,55). Also, recently there has been increasing interest in potential pharmaceutical activities of anthocyanins. These pigments have been implicated in cancer chemoprevention, acting through several mechanisms, including modulation of ROS levels, an effect that is important for the prevention of carcinogenesis, as well as by inducing apoptosis in tumor cells. In addition, in current studies, the bioavailability and structure–activity relationship of anthocyanins have been reviewed (56–58).

In the present studies, total herbal extracts were examined. The use of total extracts rather than isolated pigment fractions was based on findings demonstrating that purified Hypericin have only weak inhibitory effects on cell growth and no apparent role in apoptotic cell death, whereas the *Hypericum* flower extract elicited a significant concentration-dependent and long-lasting inhibition of cell growth and induced apoptosis. This work unveiled a potential role of *Hypericum perforatum L.* in cancer therapy and strongly supports the hypothesis that agents, other than Hypericin, present in the total extract may contribute to the antitumor activity.

These findings prompted us to examine the activity of extracts containing Hypericin and anthocyanin pigments including preparations derived from the herbs St John's Wort (*Hypericum perforatum*), Bilberry (*Vaccinium myrtillus*) and Blue Malva (High mallow) for use in

CT. In the present study, we tested the hypotheses that extracts from whole herbs contained Hypericin and anthocyanin pigments could replace the cobalt (II) octa-4,5-carboxyphthalocyanine in CT, and that herbs containing these pigments would be effective in activating catalytic systems.

Materials and Methods

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma (St Louis, MO, USA).

Cell Cultures

MCF-7 human breast cancer cells, PAM 212 murine keratinocytes, and B-16 mouse melanoma cells were purchased from ATCC (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal serum (FCS) (HyClone, Logan, UT, USA), 2 mM l-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and maintained at 37°C in an atmosphere containing 5% CO₂. Cells were seeded on 6-well plates (10 000 cells/well) 24 h before treatment for cell growth assays. In some experiments, CT was initiated a day after cell seeding at concentrations indicated in the text. Cell proliferation was evaluated at various intervals 1–6 days after treatment. Duplicate plates were used as controls in all experiments. For studies focusing on apoptotic cell death and the effects of treatments on cell cycle distribution, cells were plated in 6-well plates (10 000 cells/well) and treatment with CT was initiated 4 days after cell seeding (before cells achieved full confluence); drugs were used at concentrations indicated in the text. Experiments evaluating apoptosis and cell cycle status were conducted 2–24 h after CT treatment.

Herb Extracts Preparation and Treatment

We recognize that extracts from whole herbs contain numerous moieties in addition to pigments and we cannot exclude the possibility that other components of the herbal extracts may influence our results. In fact in many instances, it has been demonstrated that pure extracts from plants often exhibit diminished efficiency when compared with whole extracts. Therefore, in the present studies we have examined whole herbal extracts.

St John's Wort extracts were prepared from fresh wild flowers (obtained at Sandy Hook, NJ, USA), the identity of the herb was positively established according to the Herb Book by John Lust (59), extracts were prepared using pure St John's Wort flower; Bilberry extracts were

prepared using pulverized, dried whole bilberry berries or flowers (Alvita, American Fork, UT, USA); Blue Malva extracts were derived from Blue Malva Tea containing only tea flowers (Kroeger Herb Products Co. Inc., Boulder, CO, USA). The extracts contained pure Blue Malva or St John's Wort flowers, and hydrated in 5 ml distilled water or in 70% ethanol/0.08 g dry flowers. Twenty milliliters of water extract and/or 5 µl of ethanol extract were added to each well of a 6-well plate containing MCF-7 -Human breast adenocarcinoma cell line, PAM 212- murine keratinocytes SJW- St. John's Wort or B-16 cells in 0.5 ml of phenol red-free DMEM. Extracts from Bilberry flowers were resuspended in water (0.08 g dry on 5 ml) and 20 µl was added to each well of a 6-well plate containing MCF-7, PAM or B-16 cells in 0.5 ml of clear DMEM. Cells were incubated with the herbal preparations for 1 h then the medium was exchanged for fresh DMEM (without herbal extracts) and the catalytic mixture (ascorbic acid, 0.07 mM and CuSO₄, 0.07 mM) was added to the wells. Cells were then incubated with the catalytic mixture for an additional hour and the medium then exchanged to growth DMEM. For growth experiments, the cells were allowed to proliferate for up to 1 week; in some experiments cells were analyzed at various intervals 30 min–24 h for evidence of apoptosis and alterations in the cell cycle.

For these experiments optimized conditions were employed; optimal concentrations of the extracts, and treatment durations were determined using various concentrations of the extracts and components of activation mixture (data not shown). The time points used for the analysis (1 week for cell growth and 24 h for cell cycle and apoptosis) have been chosen according the common practice for these procedures.

Statistical Analysis

All experiments were repeated at least three times in the course of this study. Three controls groups have been used: (i) untreated cells, (ii) treated by activation mixture, (iii) treated by herb extracts without activation mixture. Statistical variation was determined using the Student's *t*-test calculation. In some instances, representative results are presented. Plus or minus standard deviation (\pm SD) was calculated where appropriate.

Ground State Absorption Spectra

Ground state absorption spectra were recorded on an HP-8452A UV-visible spectrophotometer with 2 nm resolution. Spectra of extract from Blue Malva were determined using 0.08 g of dry flowers in 5 ml phosphate buffered saline (PBS), 0.01 mM ascorbate and 0.02 mM CuCl₂.

Cell Growth Suppression

Cells were seeded in 6-well plates 24 h prior to CT treatment. CT treatment was carried out as described earlier in this article. Sample size was 1 sample/well with 3–6 parallel measurements. Cells proliferation was determined 6 days after treatment using a Coulter Counter model Z B1, Coulter Electronic Inc., Hialiah, FL, USA. Baseline variability was not significant in any of the experiments.

Cell Cycle Analysis

Cells were seeded in 6-well plates 4 days prior to CT treatment. CT treatment was carried out as described earlier in this article. Control and treated cells were harvested within the next 24 h, washed twice in PBS, fixed with 70% ethanol for 30 min at room temperature, washed three times with PBS, resuspended in the staining buffer (10 mM PIPES, pH 6.8, 0.1 M NaCl, 0.1% Triton X-100, 2 mM MgCl₂), treated with DNase-free RNase I (100 µg/ml) for 30 min at room temperature, and stained with propidium iodide (PI, Molecular Probes, Eugene, OR, USA) at a concentration of 10 µg/ml. Stained cells were analyzed by flow cytometry using a Beckman Coulter flow cytometer.

Annexin V and PI Staining

Cells were seeded in 6-well plates 4 days prior CT treatment. CT treatment was carried out as described earlier in this article. Both adherent and detached cells from untreated and CT exposed samples were collected, washed twice in PBS and incubated with the fluorescent indicator dyes Annexin V and PI according to the manufacturer's protocol (Sigma, St Louis, MO, USA). Control populations consisted of unstained cells and cells individually stained with only Annexin V or PI. Cells were analyzed by flow cytometry using a Beckman Coulter flow cytometer. The fluorescence emission of 10 000 cells/treatment was collected in each channel.

ROS Measurement

The generation processes of ROS can be monitored using the luminescence analysis or also fluorescence methods. The intracellular ROS generation of cells can be investigated using 2',7'-dichlorfluorescein-diacetate (DCFH-DA) as a well-established compound to detect and quantify intracellular produced H₂O₂. The conversion of the non-fluorescent DCFH-DA to the highly fluorescent compound 2',7'-dichlorfluorescein (DCF) happens in several steps. First, DCFH-DA is transported across the cell membrane and deacetylated by esterases to form the non-fluorescent 2',7'-dichlorfluorescein (DCFH). This compound is trapped inside of the cells.

Next, DCFH is converted to DCF through the action of peroxidase rated by the presence of peroxidase.

Immediately after CT treatment, cells were incubated for 30 min with 5 µM DCFH-DA in phenol and serum-free medium. Cells were analyzed on Coulter Profile flow cytometer.

Results

Ground State Absorption Spectra

Full-width half-maximum (FWHM) of the 620 nm band in the UV/VIS spectra of Blue Malva under CT has been changed from 170 to 100 nm (Table 1). The ground state absorption which has been recorded on an HP-8452A UV-visible spectrophotometer with 2 nm resolution has been changed from 0.42 to 0.11 units. It indicated the bleaching of pigment under CT influence.

Cell Growth Suppression

Data were calculated using the formula 1: X (data in Table 2) = $[(O \times 100\%)/C]$. Here, O is the number of cells following treatment by CT; C is the number of cells treated by activation system alone (control cells) and X is the difference between the cells treated by CT in comparison with cells treated by the activation system only (%). St John's Wort alone caused little decreasing in

Table 1. Ground state absorption spectra of Blue Malva

| Mixtures | FWHM (nm), λ 620 nm | Absorbance, λ 620 nm |
|---|------------------------|----------------------|
| Blue Malva in PBS | 170 | 0.42 |
| Blue Malva in PBS + activation mixture | 100 | 0.11 |

Table 2. CT with St John's Wort inhibits cell proliferation (data has been calculated by formula 1)

| Parameters | St John's Wort alone | St John's Wort with activation mixture |
|---|-------------------------|---|
| PAM | | |
| Cell count (mean,%) | 94.1 ± 3.1 | 68.9 ± 7.6 |
| <i>P</i> | | < 0.006 |
| Repetition of experiment | | |
| Cell count (mean,%) | 93.9 ± 2.7 | 71.1 ± 6.1 |
| <i>P</i> | | < 0.004 |
| <i>P</i> (between 1st and 2nd experiments) | | < 0.64 |
| Human keratinocytes | | |
| Cell count (mean,%) | 96.7 ± 3.2 | 94.7 ± 4.1 |
| <i>P</i> | | < 0.54 |

cell counting 94.1 ± 3.1 . But St John's Wort with activation mixture significantly decreased the number of cells more than 30%. There were no difference between this experiment and its repetition ($P < 0.64$). The CT with Bilberry also demonstrated the ability of CT to decrease the number of cancer cells (Fig.1).

However, the number of normal cells (human keratinocytes) has not been affected by CT (Table 1).

Cellular DNA and Early Stages of Apoptosis Analysis

The formula 2: X (data in Table 3) = $O - A$ was used in our calculations; in this formula O is the experimental data (CT with herbs), and A is the control data (CT with activation mixture alone). The number of cells accumulating in the sub-G0/G1 stage of the cell cycle was increased 2- to 7-fold for St John's Wort, and cells in G₂/M increased 1.5- to 20-fold for Bilberry and Blue Malva (Table 3). As demonstrated in Table 4, treatment by CT using herbs significantly increased the number of dead (PI staining cells); 2-folds for St John's Wort and 12-fold for Bilberry. Also, the number of cells undergoing

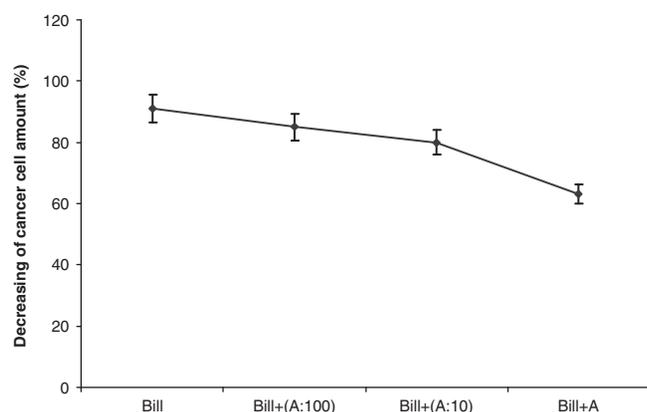


Figure 1. Decreases in the numbers of B-16 cells after CT with Bilberry (Bill, data have been calculated according to formula 1) with various concentrations of activation mixture A and A dissolved in 10 or 100 times [Bill alone, Bill+ (A:100), Bill + (A:10), Bill + A]. Cells were seeded in 6-well plates 24 h prior to CT treatment. CT treatment was carried out as described above. Sample size was 1 sample/well with 3–6 parallel measurements. Cells proliferation was determined 6 days after treatment using a Coulter Counter model Z B1, Coulter Electronic Inc., Hialiah, FL, USA. Baseline variability was not significant in any of the experiments.

early stage of apoptosis (Annexin staining cells): 6.3-fold for St John's Wort; 4.2-fold for Bilberry and 3.4 for Blue Malva. We could see on the Fig. 3A that cells had been damaged by CT with St John's Wort plus activation mixture. All cells on the picture show some signs of apoptosis (some apoptotic cells are pointed by arrows a1; a2; a3; a4; a5) which includes: cell shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton; the cytoplasm appears dense, and the organelles appear tightly packed; chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis; the nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA; the cell membrane shows irregular buds known as blebs; the cell breaks apart into several vesicles called apoptotic bodies. Figure 3B shows that activation mixture does not damage the cells and Fig. 3C shows that St. John's Wort alone is damaging only for few cells (one apoptotic cell is pointed by arrow c1).

The measurements of ROS by DCFH method have been shown the increase in ROS generation during CT with St. John's Wort and Blue Malva up to 14- and 8-fold, respectively.

Discussion

In the process of CT with phthalocyanines, active agents, known to kill cancer cells, are produced including the ROS OH^\bullet (hydroxyl radical) and H_2O_2 . We hypothesized that reaction of herbal pigments with CT would also result in the generation of ROS (Scheme 1); therefore, cellular ROS generation in response to herbal extracts and CT was examined. In previous studies, different experimental methods have been used to evaluate ROS generation in CT. An approach of particular interest because it also yields information concerning the photochemistry of these compounds is the examination of the spectral properties of various types of pigments with and without CT. In this regard, it is well known that active oxygen species are involved in the photo-bleaching process. When degraded by the attack of the self-generated active oxygen species during PDT, the photosensitizer forms photoproducts, many of which can be identified by dramatic decreases in the visible spectral

Table 3. CT influence on the cell cycle parameters

| Parameters | St John's Wort | St John's Wort + AM | Bill | Bill + AM | Malva | Malva + AM |
|---------------|----------------|---------------------|---------------|----------------|----------------|---------------|
| Sub G0/G1 | | | | | | |
| Mean \pm SD | 1.2 ± 0.3 | 15.7 ± 2.5 | 3.3 ± 0.6 | 5.1 ± 1.9 | 1.00 ± 0.3 | 1.3 ± 0.5 |
| <i>P</i> | | <0.001 | | <0.2 | | <0.4 |
| G2/M | | | | | | |
| Mean \pm SD | 4.0 ± 2.6 | 5.4 ± 1.3 | 1.0 ± 0.5 | 20.1 ± 6.8 | 1.0 ± 0.6 | 3.9 ± 0.9 |
| <i>P</i> | | <0.5 | | < 0.008 | | <0.01 |

Table 4. CT with herbs induces unscheduled cell death and apoptosis in MCF-7 breast cancer cells: flow cytometric evaluation of PI and Annexin V fluorescence

| Parameters | St John's Wort | St John's Wort + AM | Bill | Bill+AM | Malva | Malva + AM |
|---|----------------|---------------------|---------------|----------------|----------------|----------------|
| Dead (PI staining) cells, (%) | | | | | | |
| Mean \pm SD | 18.5 \pm 4.5 | 39.5 \pm 4.6 | 0.8 \pm 0.1 | 9.8 \pm 2.2 | 15.1 \pm 4.4 | 23.2 \pm 5.4 |
| <i>P</i> | <0.005 | | <0.002 | | <0.11 | |
| Apoptotic (Annexin staining cells), (%) | | | | | | |
| Mean \pm SD | 2.9 \pm 0.6 | 18.3 \pm 3.9 | 6.3 \pm 2.1 | 13.3 \pm 3.1 | 5.6 \pm 1.5 | 19.2 \pm 2.9 |
| <i>P</i> | <0.002 | | <0.03 | | <0.002 | |

bands (so-called self-sensitized photo-oxidation). Similarly, bleaching of pigments in the process of CT is also caused by self-generated ROS. Therefore, we reasoned that a loss of absorption of the model mixtures during CT is likely to represent pigment bleaching. This has been termed self-sensitized catalytic oxidation. In our first studies, self-sensitized bleaching of Blue Malva in CT was examined (Table 1). Treatment with ascorbate along with Cu^{2+} ions resulted in changes in FWHM of the 620 nm band in the UV/VIS spectra indicating that CT treatment induced bleaching (Table 1). This self-catalytic bleaching indicates that ROS produced during the treatment resulted in pigment bleaching, demonstrating that CT with Blue Malva is mediated by ROS generation. The direct measurement of ROS in cells treated with CT will be done in the next research. Also, antioxidants will be tested to prove the ROS mechanism.

As demonstrated in Table 2, our preparations of St John's Wort and Bilberry alone had little effect on cell viability. In contrast, when activated by CT we observed a dramatic decrease in tumor cell numbers. In a manner similar to the activation of cobalt (II) octa-4, 5-carboxyphthalocyanine (PcCo) by ascorbate, in these experiments responses to CT were mediated by the catalytic oxygenation of ascorbate by the herbal pigments (Scheme 1). No significant differences were observed between the numbers of cells receiving no treatment and of cells treated with the activation mixture alone (data not shown). Proliferation of cells treated with activation mixture alone was determined using the calculation according to formula 1. CT treatment with activated St. John's Wort resulted in decreases of approximately 30% in the number of PAM 212 squamous carcinoma cells; similar results were observed in experiments analyzing the effects of CT with St John's Wort on B-16 melanoma cells. Repetition of the experiments with St John's Wort clearly demonstrated significant reproducibility ($P < 0.64$). In additional studies the effects of Bilberry in CT were also examined. As exhibited in Fig. 1, CT treatments using Bilberry resulted in dose-dependent decreases in tumor cell numbers. Similar to our studies using St. John's Wort, in these experiments the herb extract without the CT activation mixture had minimal effects on tumor cell proliferation. In these

experiments, activated Bilberry extracts dramatically inhibited cell proliferation. Tumor cell numbers were depleted up to 40% when examined 6 days following treatment, an effect that was dependent on the concentration of the herbal extracts (100 \times dilution of the herbal mixture to undiluted extract). Taken together, these data demonstrated that both St John's Wort and Bilberry are effective in CT and that inhibition of proliferation in response to the herbal CT mixtures is dependent on the concentration of the herbal extracts.

Although more than 50 years have elapsed since progression through the cell cycle was first described for mouse sertoli cells, analysis of the diverse paths of tumor cells traversing the cycle still proves an effective mechanism for obtaining information about the progression of neoplastic diseases (39). In fact, alterations to the rate at which cellular DNA levels multiply from a singlet complement (G1) to fully duplicated, ready to divide G2M levels, is perhaps one of the most efficient measures of the efficacy of anticancer drugs. Therefore, we next examined the effects of CT treatment with herbs on the cell cycle progression of cells from two tumor cell lines, PAM 212 squamous cell carcinoma and B16 melanoma cells. In these experiments the influence of the herbal extracts from St John's Wort, Bilberry and Blue Malva in combination with activation mixture on the cell cycle were examined using PI in conjunction with flow cytometry. Cell DNA content was analyzed and levels reflecting the G0/G1, S, G2/M stages of the cell cycle as well as and sub-G0/G1 cells (apoptotic cells) were defined.

As demonstrated in Table 3 and Fig. 2, treatment with herb extracts, and with the extracts in combination with activation mixtures, increased the percentage of cells with less than a singlet component of DNA (the sub-G0/G1 fraction), a fraction presumed to contain a significant proportion of apoptotic cells, and the percentage of cells in the G2/M fraction, a process indicative of cell cycle arrest. Potentially, damage to DNA and other critical cellular molecules produced by CT treatment mediated these effects. In this regard, it is well recognized that the cellular responses to the injury and stress induced by treatment with anticancer agents are key determinants of drug activity. Checkpoint proteins that control the normal passage of cells through the cell cycle play a

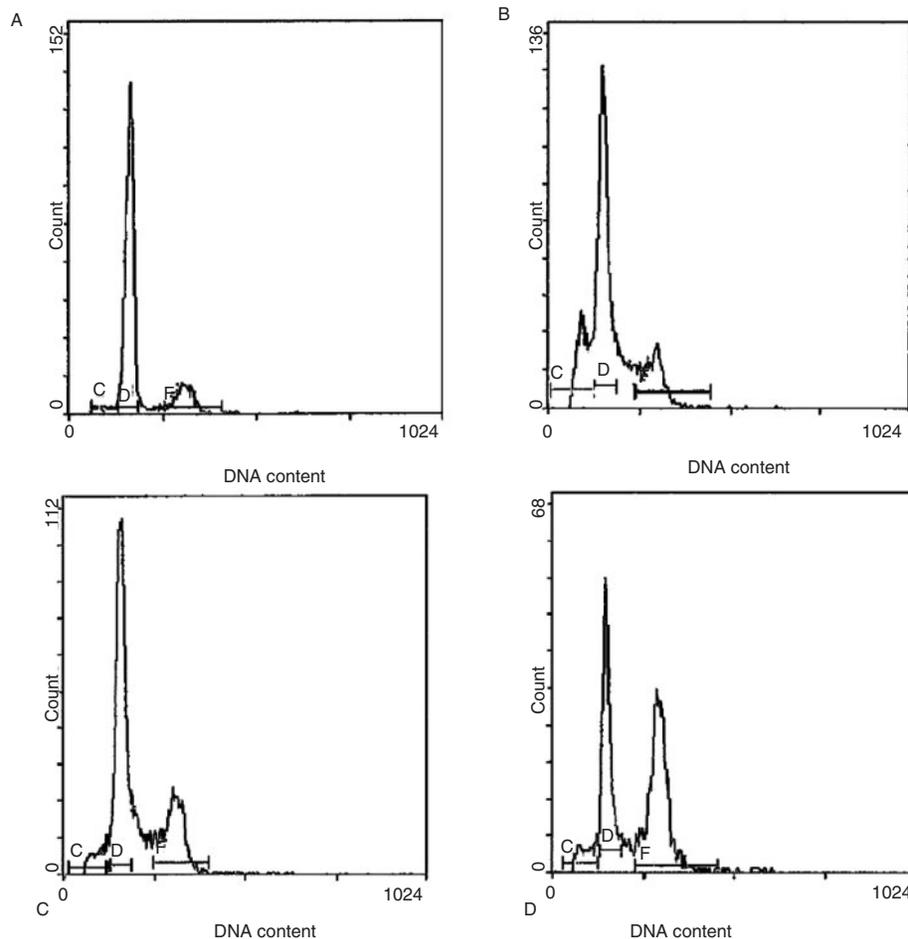


Figure 2. Cell cycle of herbs extracts. (A) Control (activation mixture). (B) St John's Wort with activation mixture. (C) Malva with activation mixture. (D) Bilberry with activation mixture. Cells were seeded in 6-well plates 4 days prior CT treatment. CT treatment was carried out as described above. Control and treated cells were harvested within the next 24h. Stained cells were analyzed by flow cytometry using a Beckman Coulter flow cytometer.

pivotal role in the responses to injury. In fact, increasing evidence indicates that defects in one or more cell cycle checkpoints often act to enable transformed cells to proliferate. However, these same defects may also render some cancer cells more vulnerable to inhibition of a second checkpoint, thereby enhancing the overall sensitivity to some anticancer treatments. (58). Our findings indicate that these processes may underlie the responses to St. John's Wort. Treatment with this herb alone increased the percentage of cells unable to traverse the G2/M point, however, CT with the St. John's Wort extract not only enhanced this effect, this treatment also dramatically increased the number of sub-G0/G1 (apoptotic) cells (Fig. 3). Interestingly, Hostanska (54) and Schempp (55) previously reported that St. John's Wort alone exhibits low levels of inhibition to cancer cell proliferation, a finding that may be reflected in our findings of increases in G2/M cells following treatment with St. John's Wort without activation mixture. Furthermore, our findings that CT with St. John's Wort treatment significantly increased the number of

apoptotic cells (sub-G0/G1) cells, and that CT with Bilberry and Blue Malva also caused G2/M checkpoint arrest, clearly indicate that when used in CT these herbal extracts are potent modulators of the cell cycle. Potentially, the unique pigment molecules in each herbal extract may mediate distinct responses. These findings prompted us to further evaluate the apoptotic effects of CT treatment. Changes in the plasma membrane of cell surface are among the earliest identifiable features of cells undergoing apoptosis, and the exclusion of PI is a sensitive indicator of cell viability. In cells undergoing apoptosis the membrane phospholipid phosphatidylserine (PS) are translocated from the inner to the outer leaflet of the plasma membrane thereby exposing PS. In these studies, the early stages of apoptosis of cancer cells after CT treatment were evaluated by flow cytometry using Annexin V, a fluorescent indicator die which binds to phosphatidyl serine in the cell membrane, in conjunction with the viable die PI (Table 4).

As demonstrated in Table 4, treatment by CT using herbs significantly increased the number of dead

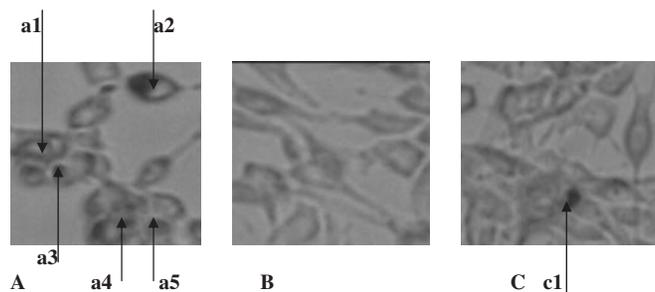


Figure 3. B-16 mouse melanoma cells with CT with St John's Wort. (A) St John's Wort+ activation mixture. (B) Activation mixture. (C) St John's Wort. For these experiments optimized conditions were employed; optimal concentrations of the extracts, and treatment durations were determined using various concentrations of the extracts and components of activation mixture (data not shown). The time points used for the analysis (1 week for cell growth and 24 h for cell cycle and apoptosis) have been chosen according to the common practice for these procedures. We could see on the (A) that cells had been damaged by CT with St John's Wort plus activation mixture. All cells on the picture show some signs of apoptosis (some apoptotic cells are pointed by arrows a1; a2; a3; a4; a5), which includes: cell shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton; the cytoplasm appears dense and the organelles appear tightly packed; chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis; the nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA; the cell membrane shows irregular buds known as blebs; the cell breaks apart into several vesicles called apoptotic bodies. (B) shows that activation mixture does not damage the cells and (C) shows that St John's Wort alone is damaging only for few cells (one apoptotic cell is pointed by arrow c1).

(PI staining cells); in addition, these treatments also resulted in significant increases in the number of cells undergoing apoptosis (Annexin staining cells). Of the herbal extracts examined, St John's Wort extracts had the greatest effects on the G1 point of cell cycle whereas Blue Malva and Bilberry were more active in regulating accumulation in G2/M (Tables 3 and 4 and Fig. 2). However, CT using each of three herbal extracts led to significant increases in the numbers of apoptotic cells. Unique proteins regulate the G1 and G2 checkpoints; our findings that distinct herbal extracts differentially alter passage through these checkpoints may reflect distinct effects elicited by different herbal extracts on these proteins. Similar to hypericin, pigments from St John's Wort are large planar molecules with little structural flexibility, whereas the blue anthocyanin pigments found in Bilberry and Blue Malva are smaller and flexible. These characteristics may be important for specific molecular interactions with different cell cycle regulators and underlie the differences in the activity of the pigment sub-classes. In addition, the gross structural dichotomies may also render some pigments more susceptible to destruction by ROS generated during CT. Our findings indicate that CT with herbal extracts generates ROS, increases the percentage of apoptotic cells and decreases the proliferation of cancer cells. Cell damage and

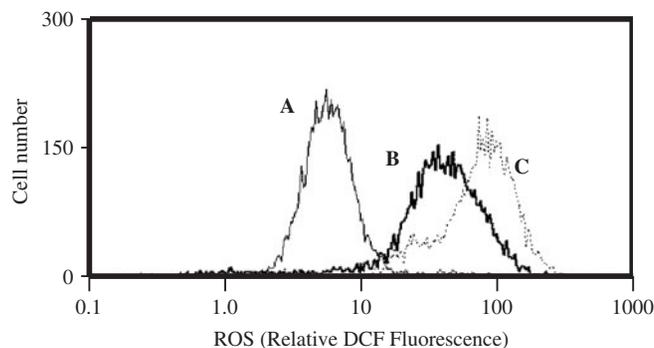


Figure 4. ROS levels in cancer cells after CT: A—control, B—CT with Blue Malva, C—CT with St John's Wort. The intracellular ROS generation of cells has been investigated using 2',7'-dichlorofluoresceindiacetate (DCFH-DA) as a well-established compound to detect and quantify intracellular produced H_2O_2 . Immediately after CT treatment cells were incubated for 30 min with 5 μ M DCFH-DA in phenol and serum-free medium. Cells were analyzed on Coulter Profile flow cytometer.

inhibition of tumor cell proliferation were found to be dependent on the presence of the catalytic mixture. Taken together, the results of these studies indicate that CT treatment with pigmented herbal extracts results in extensive death in tumor cells and that all of the extracts used were effective in activating apoptosis.

It has been previously demonstrated that CT leads to ROS generation in cancer cells (29). As indicated in the flow cytogram exhibited in Fig. 4, the DCFH peak position indicates that enhanced levels of ROS are found in PAM 212 cells after CT with SJW (from 7.2 to 101 fluorescence units) and after CT with Malva (from 7 to 58.7 channel units) (Fig. 4). In these studies, the data are presented on a four-decade log scale and indicate that ROS levels have been increased 14- and 8-folds, respectively (Table 5).

We have employed rigorous and scientifically valid approaches for evaluating the effects of CT with herbal extracts on tumor cells. In the present studies, our preliminary results suggested that whole extract pigments are efficacious as anticancer agents. The effects of purified pigments will be examined in future research. Also, we did not analyze direct ROS production by all of the preparations examined in live cells. However, indirect evidence has been obtained from spectral bleaching studies. In future studies, we plan to examine the effects of individual extract pigments on cellular ROS production.

In future studies, we will examine the effects of purified individual pigments as well as those of herbal extracts on intracellular oxidant levels. We plan to measure the ROS production in the cells and sample mixtures by flow cytometry using dichlorofluorescein diacetate (DCFH-DA).

Another important issue with CT as well as with all other drug therapies is the potential for discrepancies

Table 5. ROS production by CT

| DCFH staining cells, fold from control | | |
|--|-----------------|-----------------|
| | SJW | Blue Malva |
| Mean \pm SD | 14.08 \pm 0.8 | 8.18 \pm 1.25 |
| P with control | < 0.001 | < 0.001 |

between the results of *in vitro* and *in vivo* studies. Drug efficacy can be decreased *in vivo* due to metabolism of the drug or interactions with other biological structures, or due to small accumulation of components in tumor cells. Thus, it is necessary to transport drugs (e.g. catalysts and the activation mixture) to tumors safely without changing, destroying, or decreasing the concentration, as well as to achieve a high concentration of components in tumor cells. We have solved this problem in one of our previous studies using magnetically guided liposomes loaded with photosensitizer in *in vivo* PDT experiments (60). It has been shown that this guided transport dramatically increased the concentration of photosensitizer at the tumor site and PDT efficiency. Most recently this method has been undertaken by researchers at University at Buffalo where they demonstrated an approach for drug delivery in which an applied magnetic field directs the accumulation in tumor cells. Adaptation of this technique to CT may lead to treatments that exploit the advantages of PDT and yet have the potential to reduce drug accumulation in normal tissues (61). Increased effectiveness of CT may result from the use of the same guided delivery system for distinct treatment components. Moreover, new products such as polymeric and ceramic-based nanoparticles, combined with specific antibodies may be tested as well. This complete system of guided transport (magnetically guided, antibody combined drug-loaded nanoparticles) may act to facilitate delivery of the components of CT to tumors with little change or loss. Using these techniques to improve the pharmacokinetic profiles of CT components is likely to facilitate *in vivo* CT studies, and we will investigate these approaches in our future research.

Conclusions

Our findings clearly indicate that CT treatments with herb extracts is effective in suppressing tumor cell proliferation and inducing both unscheduled and apoptotic cell death in cancer cells, effects potentially due to damage to critical cellular molecules. However, many of the molecular mechanisms underlying these responses remain poorly defined. It is our contention that the present results demonstrate the efficacy of St John's Wort, Blue Malva and Bilberry extracts as potential anticancer agents, and that these studies also suggests the need for further investigations into the anticancer activities of these herbs.

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