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The flavonoid quercetin transiently inhibits the activity of taxol and nocodazole through interference with the cell cycle

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Abstract

Quercetin is a flavonoid with anticancer properties. In this study, we examined the effects of quercetin on cell cycle, viability and proliferation of cancer cells, either singly or in combination with the microtubule-targeting drugs taxol and nocodazole. Although quercetin induced cell death in a dose dependent manner, 12.5-50 μ M quercetin inhibited the activity of both taxol and nocodazole to induce G2/M arrest in various cell lines. Quercetin also partially restored drug-induced loss in viability of treated cells for up to 72 hours. This antagonism of microtubule-targeting drugs was accompanied by a delay in cell cycle progression and inhibition of the buildup of cyclin-B1 at the microtubule organizing center of treated cells. However, quercetin did not inhibit the microtubule targeting of taxol or nocodazole. Despite the short-term protection of cells by quercetin, colony formation and clonogenicity of HCT116 cells were still suppressed by quercetin or quercetin-taxol combination. The status of cell adherence to growth matrix was critical in determining the sensitivity of HCT116 cells to quercetin. We conclude that while long-term exposure of cancer cells to quercetin may prevent cell proliferation and survival, the interference of quercetin with cell cycle progression diminishes the efficacy of microtubule-targeting drugs to arrest cells at G2/M.

Keywords

quercetin; cell cycle; taxol; nocodazole; drug-diet interaction; flavonoid

Introduction

Consumption of foods of plant origin, especially fruits, vegetables and whole grains is associated with a reduced risk of different types of cancer, including those of the lung, oral cavity, esophagus, stomach, prostate and colon (Gonzalez, Pera et al. 2006; Kirsh, Peters et al. 2007; Lunet, Valbuena et al. 2007; Millen, Subar et al. 2007). Dietary compounds are being intensively studied for their chemopreventive, chemotherapeutic, or adjuvant potential in cancer management. Dietary polyphenolic compounds, in particular, have attracted much attention because of their abundance and due to well documented bioactivity that includes their antioxidant effects.

Quercetin is one of the most abundant dietary flavonoids. Quercetin and its derivatives constitute about 99% of the flavonoids in apple peel (He and Liu 2008), and it is also one of

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the major constituents in foods consumed in the United States (Harnly, Doherty et al. 2006; Huang, Wang et al. 2007). Numerous in vitro and animal model studies using quercetin alone or quercetin in combination with other bioactive compounds have shown the anti-cancer activities of the compound (Gee, Hara et al. 2002; Huynh, Nguyen et al. 2003; Nguyen, Tran et al. 2004; Ong, Tran et al. 2004; Zhang, Huang et al. 2004; Kim, Bang et al. 2005; Mertens-Talcott and Percival 2005; Granado-Serrano, Angeles Martin et al. 2008).

While much is known about the bioactivities and the major signaling pathways modulated by quercetin (see ref in (Ramos 2008)), less is known about the potentials of the compound as a complementary supplement once the cancer has established itself and therapy has been implemented to treat the cancer. The benefits and dangers of the concomitant use of antioxidants and chemotherapeutic agents has been controversial (Keith I. Block 2008). This has especially been true for therapeutic agents that induce oxidative stress as the mechanism of action, as antioxidants may also reduce the side effects of chemotherapeutic agents. A definitive recommendation is still lacking as to whether or when antioxidants should at all be used in the course of chemo- or radiation-therapy (Bairati, Meyer et al. 2005; Block, Koch et al. 2007; Keith I. Block 2008; Lawenda, Kelly et al. 2008). Drug-diet interactions among antioxidants and classes of drugs that act through non-oxidative mechanisms is not well known.

We examined the effect of the co-treatment of cancer cells with the flavonoid quercetin and two anti-microtubule drugs, namely taxol and nocodazole. We analyzed cells treated with single agents or a drug-flavonoid combination. We hypothesized an additive or a synergistic effect with this drug-flavonoid combination, but unexpectedly, quercetin protected cells from the activity of these anti-microtubule drugs, and sustained the viability of the cells. However, prolonged exposure of the cells to the highest protective dose of quercetin was still able to prevent cell proliferation. Thus, we identify bimodal activity of the flavonoid quercetin, a short term activity which is cytoprotective against chemotherapeutic drugs, and a long term activity which is inhibitory to cancer cell growth.

Materials and Methods

Cell culture and treatments

The human colorectal cancer HCT116 cell lines (wild type and p53 null) were generous gifts from Dr Bert Vogelstein (Johns Hopkins). The cells were maintained in McCoy's medium (Lonza, Walkersville, MD) supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin/Streptomycin. Prostate cancer PPC1 cells (gift from Dr John C. Reed, Burnham Institute) were grown in RPMI medium (Invitrogen, Carlsbad, CA), supplemented with 10% FBS and Penicillin/Streptomycin. RKO colorectal cancer cells (ATCC, Manassas, VA) were maintained in similarly supplemented DMEM. MCF7 cells were kindly provided by Dr Leslie Wilson (UC Santa Barbara) and were maintained in DMEM. For most experimental treatments, cells were seeded in 96-well, 6-well or 6-cm dishes, at approximate densities of 10^3 , 10^4 , or 10^5 cells per well, respectively. For experiments requiring longer than 48 hours, the cell numbers for the entire experimental set up were reduced by half. All cell cultures were incubated at 37°C and 5% CO₂ in a humidified incubator. Cells were synchronized by the double thymidine block method. Exponentially growing cells were treated overnight with 2mM thymidine in growth medium. The next morning, culture medium was removed and the monolayer was washed 3 times with plain growth medium to remove thymidine. The cells were allowed to grow in complete medium for 8 hours and were treated again overnight with 2mM thymidine. The synchronized monolayer cells were washed again and released into complete growth medium. Cell cycle was analyzed at different time points after the release.

Reagents

Quercetin (Q0125), nocodazole, and taxol were purchased from Sigma (St. Luis, MO). A stock solution of 50mM quercetin was prepared in DMSO, aliquoted in single use portions, and stored at -20°C . Unused portions of any thawed aliquots were discarded. Nocodazole and taxol were also dissolved in DMSO as 5mM and 10mM stock solutions. Working dilutions of the stock were prepared in culture medium. Polyclonal antibodies to cyclin-B1 (AHF0062) and CDK1 (AHZ0112) were purchased from Invitrogen. Monoclonal antibody to α -tubulin (clone DM1A) was purchased from Sigma (St Louis, MO).

Colony formation and clonogenic assays

Colony formation assay was performed by seeding approximately 500 cells per well of a 12-well dish. Depending on the cell types or experimental designs, cells were allowed to adhere for up to 18 hours and then treated with quercetin, or they were directly seeded in culture medium containing quercetin. Culture medium was changed every 48 hours until discreet colonies were visible to the naked eye, after which they were stained with 10% crystal violet in methanol, washed and air-dried. Clonogenic assay was performed as described (Franken, Rodermond et al. 2006). The number of cells in colonies was counted microscopically (200X magnification), whereas the number of established clonal colonies was counted using a stereo microscope. Due to their small sizes, HCT116 cells were allowed to grow up to about 130 cells per colony before staining with crystal violet.

Flow cytometry

Cells were harvested and prepared for flow cytometry as described, with some modifications (Samuel, Okada et al. 2005). Cells were harvested by trypsinization using 0.25% trypsin EDTA. Prior to trypsinization, floating or loose cells were harvested by gentle manual rocking of the culture dishes and transferring the culture medium containing the cells into centrifuge tubes. Trypsinized and loose cells were then combined and centrifuged. Pellets were resuspended in 300 μl phosphate buffered saline, fixed by the addition of 700 μl 100% ethanol while vortexing, and stored at -20°C for at least overnight. Fixed cells were centrifuged, and stained in FACS staining solution (320 mg/ml RNase A, 0.4 mg/ml propidium iodide) in PBS without calcium and magnesium for 30 minutes at 37°C . Stained cells were filtered through a 70 microns pore sized filter and analyzed by flow cytometry (FACScalibur® Beckton Dickinson and C6 Accuri® flow cytometers). Data was analyzed and histograms were prepared using CellQuest and CFlow software.

MTT/MTS and BrdU incorporation assays

MTT reagent was obtained from American Type Culture Collection (ATCC), whereas the MTS assay was performed using CellTiter 96® AQueous One Solution cell proliferation assay kit from Promega® (Madison, WI, USA). The assays were performed on cells seeded in triplicates in 96-well plates, according to the manufacturer's instructions. Absorbance was recorded at 570nm (MTT) or 490nm (MTS) using Synergy HT multimode plate reader or PowerWave XS2 (BioTek®, Winooski, VT). To account for absorbance of quercetin at 490nm, during each MTT or MTS experiment, separate wells were set where quercetin was diluted in culture medium without cells. The average A490 readings from wells containing quercetin in culture medium were subtracted from the readings of treated cells. To calculate MTT viability index, absorbance readings from DMSO treated control wells were set at 100% and the relative A490 was calculated as a percentage of the control.

BrdU incorporation ELISA

BrdU incorporation was analyzed by Cell Proliferation BioTrak ELISA (GE Lifesciences) according to the manufacturer's instructions. For this assay, about 5×10^3 cells were seeded

per well of 24-well plates. After allowed to adhere for about 12 hours, cells were serum deprived for about 24 hours by culturing them in serum-free medium. Cells were then released into serum containing culture medium, and after 3 hours treated with quercetin, taxol, or quercetin and taxol. Five hours after the treatment, BrdU labeling reagent was added to the culture medium to label those cells synthesizing DNA. Cells were labeled overnight, fixed the next morning, and processed for BrdU ELISA as recommended. Absorbance readings were taken at 405nm using PowerWave XS2 plate reader (BioTek®).

Cell monolayer immunocytochemistry

HCT116 cells were seeded in 4-well chamber slides and allowed to adhere for about 16 hours. Then, cells in each well were treated with control (DMSO), single agents (quercetin or taxol or nocodazole), or a combination of quercetin and taxol or quercetin and nocodazole. About six hours after treatment, the culture medium was removed and the cells were fixed in 4% formaldehyde for 15 minutes at room temperature. The fixed cells were washed with PBS and processed for immunocytochemical staining at the immunohistochemistry lab of the College of Veterinary Medicine, Nursing and Allied Health (CVMNAH), Tuskegee University. Cyclin-B1 primary antibody (Invitrogen AHF0062) was used at 15mg/ml concentration. Peroxidase conjugated secondary antibody (Envision+ Dual Link System, Dako®, Carpinteria, CA) and DAB+ Chromogen (Dako®) were used for the detection. Mayer's Hematoxylin (Lillie's modification, Dako®) was used as counter stain. Slides were mounted using Micromount mounting medium (Surgipath® Richmond, IL) and cover slips.

Immunofluorescent staining and Microscopy

Images of unstained live cells and immunocytochemically stained cells were taken at 20X and 40X magnification objectives using Leica or Olympus microscopes fitted with digital image capture cameras (Digital Microscopy Lab, CVMNAH). Photographs saved in TIFF format were directly imported to Microsoft PowerPoint and cropped or adjusted for brightness, contrast, or grayscale conversion. MCF7 cells for immunofluorescent staining were grown in 4-well chamber slides. Staining was performed as described (Samuel, Okada et al. 2005). Confocal images were taken at the Carver Research Center at Minority Institutions (RCMI, Tuskegee University) core-facility using Olympus DSU spinning disk confocal microscope using 40X dry objective. Images were captured using Metamorph Premium® software and further processed in Adobe Photoshop®.

Immunoblotting

Cell lysates were prepared in NP-40 lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% Glycerol and 0.2% NP-40 plus protease inhibitor cocktail) and protein concentrations were determined using NanoVue spectrophotometer (GE Healthcare Life Sciences, Piscataway, NJ). Samples containing equivalent protein concentrations were mixed with Laemmli buffer, and boiled for 5 minutes. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes (GE Healthcare Life Sciences) and blocked in 5% non-fat dry milk. Primary antibodies used were rabbit anti cyclin-B1 (Invitrogen) at 1:200, rabbit anti-CDK1 (Invitrogen) at 1:500, β -actin (Cell Signaling) at 1:1000. Peroxidase conjugated anti-rabbit and anti-mouse IgG secondary antibodies were purchased from GE Healthcare Life Sciences and used at 1:5000 dilution. Chemiluminescent detections were done using LumiSensor™ Chemiluminescent HRP Substrate (Genescript, Piscataway, NJ).

Results

We examined the bioactivity of quercetin singly and in combination with two chemotherapeutic drugs known to act via disruption of the microtubule dynamics, namely taxol and nocodazole. Both drugs induce G2/M arrest as phenotype.

Dose dependent induction of apoptosis by quercetin

To examine the apoptosis-inducing activity of quercetin, we exposed human colorectal tumor HCT116 cells to increasing doses of quercetin, and analyzed the cell cycle profile of the cells at 24 and 48 hours post treatment. As shown in Fig. 1 A, quercetin induced apoptosis, which was evident as increased sub-G1(s-G1) population, most significantly by 48 hours of exposure, accompanied by reduction in the G2/M population. We also examined the proapoptotic effect of quercetin on an adherent PPC1 prostate cancer cell line. PPC1 cells were treated with 0 to 100 μ M quercetin in growth medium. As shown in Fig. 1B, by 48 hours of exposure to 25 μ M and 50 μ M quercetin, the sub-G1 population of PPC1 cells began to increase. The increase in apoptosis was concurrent with the reduction in the proportion of cells at G1 as well as G2/M phases of the cell cycle. At the dose level of 100 μ M, over 40% of the cells were in sub-G1 state (apoptotic), indicating that higher doses of quercetin are cytotoxic. Similar results on the cell death inducing potential of quercetin have previously been reported (Murtaza, Marra et al. 2006). From these data, the bioactivity of quercetin appears to be similar in both colorectal and prostate cancer cells, though the latter seemed to be more sensitive to the flavonoid.

Inhibition of microtubule-acting drugs by quercetin

Bioactive compounds with antioxidant properties have been suggested to antagonize the activity of chemotherapeutic agents that induce oxidative stress (Lawenda, Kelly et al. 2008). However, it is not well known if flavonoids may enhance or inhibit the activities of other classes of anti-cancer drugs. We investigated the bioactivity of quercetin in the presence of microtubule-targeting chemotherapeutic drugs. Since quercetin alone induced apoptosis in colon and prostate cancer cells, we hypothesized the cell cycle inhibitory activity of the anti-microtubule drugs nocodazole and taxol would be enhanced by co-treatment with quercetin. To test this, we first examined the effect of combination treatment of nocodazole, a microtubule-destabilizing agent, and quercetin on HCT116 colon cancer cells. Adherent wild type and p53-null HCT116 cells were treated with the carrier alone (DMSO), with single agents (nocodazole 10 μ M, or quercetin 50 μ M), or with a combination of both agents. Cell morphology was examined by microscopy, and cell cycle profile was analyzed by flow cytometry. While HCT116 cells treated with nocodazole alone were completely rounded as expected, surprisingly, cells treated with a combination of quercetin and nocodazole were morphologically indistinguishable from the control cells (Fig. 2A-D). HCT116 cells treated with quercetin alone did not show any major morphological alteration within 24 hours. Additionally, flow cytometric analysis showed that while 10 μ M nocodazole induced 70-90% G2/M accumulation of cells, co-treatment with 50 μ M quercetin completely abolished the G2/M arrest induced by nocodazole in both wild type and p53-null cells (Fig. 2 G-I). Quercetin at 25 μ M dose showed moderate inhibition of nocodazole activity in wild type cells within 24 hours. Within this time frame, lower doses of quercetin had neither inhibitory nor enhancing effects on nocodazole activity (G2/M arrest). Additionally, to assess the inhibitory effect of quercetin on another microtubule-targeting drug, we tested the combination of taxol and quercetin on colon cancer cells. Unlike nocodazole, taxol prevents cell cycle progression by stabilizing the microtubules. We performed similar single (quercetin or taxol) and combination (quercetin and taxol) treatments of HCT116 cells with the two agents. As with nocodazole, the cells treated with the combination of quercetin and taxol were morphologically indistinguishable from control DMSO treated cells (Fig. 2E, F)

and displayed cell cycle profile similar to the control cells (not shown). This suggested that quercetin-treated cells may not have responded to the cell cycle effects of the microtubule targeting drugs.

To further test that quercetin protected cells from taxol activity, we treated PPC1 prostate cancer cells with taxol or with taxol and quercetin, and examined the cells by flow cytometry. To this end, we treated the cells overnight with increasing doses of taxol (0-400nM) with or without co-treatment with 50 μ M quercetin. The cell cycle profiles of treated and untreated cells were analyzed by flow cytometry. As with HCT116 cells, co-treatment of PPC1 prostate cancer cells with quercetin completely abolished the prominent G2/M arrest induced by the drug taxol (Fig. 3A, B).

Since we found that quercetin blocked the cell cycle arrest induced by nocodazole and taxol, we became interested in examining if the viability of cells treated with the microtubule acting drugs would be restored by quercetin. To assess this, we performed MTT assay on singly (quercetin or nocodazole) or doubly (quercetin and nocodazole) treated cells at 24, 48, and 72 hours after the treatments. The MTT viability index showed that quercetin alone in doses above 50 μ M reduced the viability of both wild type and p53-null HCT116 cells (Fig. 4A, B). However, doses of quercetin as low as 3.13 μ M attenuated the activity of nocodazole, while nocodazole (10 μ M) alone reduced the viability of the treated cells (Fig. 4C, D). At 72 hours after treatment, the viability index of nocodazole treated HCT116 cells was about 65%, whereas the viability index of cells treated with nocodazole plus 50 μ M quercetin was comparable to that of the carrier treated control cells. Quercetin at 100 μ M dose was less protective than 50 μ M, suggesting the cytotoxicity of quercetin at higher doses.

To assess if the viability of cells treated with quercetin and taxol was accompanied with cell cycle progression, we performed BrdU incorporation assay as an indicator of cellular DNA synthesis, and analyzed BrdU incorporation in singly or combination treated cells. As shown in Fig. 4E, cells treated with the combination of taxol and quercetin incorporated BrdU to a degree comparable to singly treated cells. Therefore, it appears that the sustained viability of quercetin-taxol combination treated cells may not necessarily be accompanied by DNA replication, but by steady state maintenance of viability.

To further test the effect of quercetin on cell cycle progression, we synchronized HCT116 cells at G1-S boundary by the double thymidine block method and released them into culture medium containing 50 μ M quercetin. Progression of the released cells through the cell cycle was assessed by flow cytometry of cells harvested at different time points after the release. We found that cells released into quercetin medium showed marked delay in cell cycle. By 9 hours after release, most cells in the control medium were in G1 phase of the next cell cycle, whereas the majority of the cells in quercetin medium were still in S-G2 phase of the first cell cycle after the release (Fig. 4F).

Quercetin does not interfere with microtubule targeting of taxol and nocodazole

The inhibition of the activity of taxol and nocodazole by quercetin led us to speculate that quercetin might interfere with the uptake, intracellular distribution, or microtubule targeting of the two drugs. To rule out this possibility, we examined the α -tubulin architecture in MCF7 cells treated with taxol or nocodazole in the presence or absence of quercetin. Similar to HCT116 and PPC1 cells, treatment of MCF7 cells with taxol and nocodazole in the presence of quercetin also resulted in absence of G2/M arrest of the cells. However, unlike HCT116 and PPC1 cells, 50 μ M and 25 μ M quercetin were cytotoxic to MCF7 cells, whereas 12.5 μ M was protective against the G2/M arrest of cells (not shown). Confocal images of cells immunostained for α -tubulin showed that in the presence of quercetin nocodazole and taxol were still able to destabilize or stabilize microtubules, respectively (FIG. 5). Since the

drugs target microtubule dynamics in the presence of quercetin, we conclude that the absence of G2/M arrest of combination-treated cells is not due to lack of uptake or increased efflux of the anti-microtubule drugs.

Taxol/nocodazole and quercetin combination treatment prevents accumulation of cyclin-B1 at the microtubule organizing center (MTOC)

As shown above, cells treated with quercetin and taxol or quercetin and nocodazole did not accumulate at the G2/M phase of the cell cycle. Since mitotic entry is regulated mainly by the cell cycle dependent subcellular dynamics and stability of cyclin-B1 and its partner CDK1 through the MTOC (Jackman, Lindon et al. 2003), we examined the localization of these proteins in HCT116 cells treated singly with quercetin or taxol or nocodazole or by a combination of quercetin and taxol or quercetin and nocodazole for 8 hours. Monolayers of HCT116 cells grown in chamber slides were immunohistochemically stained using an antibody against cyclin-B1. Interestingly, combination-treated cells showed weak to no detectable accumulation of cyclin-B1 at the MTOC in contrast to those cells treated with either the drugs or quercetin alone (Fig. 6A). This indicates that the lack of cell cycle arrest by taxol and nocodazole in the presence of quercetin is accompanied by the absence of proper mobilization of cyclin-B1-CDK complex to the MTOC to initiate mitosis. However, since the cells did not accumulate in S-phase, combination treated cells could also be blocked at other phases of the cell cycle. Indeed, as shown above (Fig. 4E), cells treated with quercetin alone or quercetin-taxol combination did not incorporate BrdU more than taxol treated cells, suggesting quercetin treatment may have stalled the progression of the cell cycle also before the S-phase. The decrease in the levels of cyclin-B1 in combination-treated cells was also confirmed by immunoblotting. While taxol-treated cells accumulated cyclin-B1 as expected, taxol-quercetin treated cells had markedly low levels of cyclin-B1 (Figs. 6B)

Quercetin inhibits colony formation of both wild type and p53-null colorectal tumor cells

It is estimated that more than 50% of human cancers carry p53 protein mutations, almost all of which have been cataloged (Magali Olivier 2002; Christophe Bérout 2003). As p53 is also a key protein regulating the apoptotic and cell cycle signaling, we became interested to examine if the anti-proliferative activity of quercetin would be dependent on the p53 status of colon cancer cells.

To address this, we exposed wild type and the isogenic p53-null human colorectal tumor HCT116 cells to varying concentrations of quercetin, and examined growth of the cells by colony formation assay. Both wild type and p53-null cells were seeded in the presence of 0 – 100µM concentrations of quercetin under two different conditions. In one instance, the cells were allowed to adhere for overnight before adding quercetin, and under the second instance, the dissociated cells were seeded in the presence of quercetin. Growth medium was replaced at 72 hours intervals with a fresh supplementation of quercetin at the same concentration as the initial dose.

As shown in Fig. 7A-B, long term exposure to quercetin (50µM or more) inhibited colony formation in both p53 positive and negative cells at a comparable dose, which suggests that the long term cell proliferation inhibitory effect of quercetin probably does not require cellular p53. Moreover, the same dose of quercetin (50µM) that abrogated the G2/M arrest by taxol and nocodazole also inhibited colony formation by HCT116 cells. Additionally, we observed that both wild type and p53-null cells were more sensitive to the activity of quercetin when the cells were seeded in the presence of the flavonoid. While 50µM quercetin was needed to inhibit colony formation of adherent HCT116 cells, 12.5µM

quercetin was sufficient to achieve an even stronger inhibition of colony formation of both wild type and mutant cells when they were treated before they adhered to the culture dishes.

To examine if quercetin provided long-term survival advantage to cancer cells exposed to anti-microtubule drugs, we performed clonogenicity assays on wild type HCT116 cells treated with only quercetin or a combination of quercetin and taxol. The numbers of clonal colonies formed and the number of cells per colony were compared. As shown in Fig. 7C and D, quercetin doses (25 μ M and 50 μ M) that interfered with taxol and nocodazole still inhibited the clonogenicity of HCT116 cells. Moreover, the number of cells per colony was lower in cells treated with 12.5 μ M or higher quercetin, compared to control cells, suggesting that quercetin may have interfered with cell cycle progression and therefore limited the rate of cell proliferation or survival. When we tested the clonogenicity of HCT116 cells treated with 25 μ M quercetin and taxol (0.6nM to 5nM) combinations, we observed that quercetin provided no clonogenicity advantage to cells. On the contrary, the combination of quercetin with taxol consistently suppressed the clonogenic survival of treated cells, and sensitized the cells to lower doses of taxol which did not inhibit clonogenic survival. Cells treated with 1.25nM and 0.6nM taxol retained clonogenicity, while combination of 25 μ M quercetin with the same doses of taxol markedly inhibited clonogenic survival of the cells (Fig. 7E).

Discussion

We have found that quercetin, a ubiquitous flavonoid abundantly available in green vegetables and fruits, has pleiotropic effects on cancer cell survival as a single agent and when combined with conventional chemotherapeutic drugs that target the microtubules. While we initially predicted that quercetin would enhance the activity of taxol or nocodazole, we unexpectedly found that quercetin antagonized the G2/M arrest induced by both drugs. We also found that even in the presence of quercetin the uptake of nocodazole or taxol was not inhibited, as shown by the distinctive effects of the drugs on the microtubules. The antagonistic activity of quercetin on taxol and nocodazole was accompanied by the absence of recruitment of cyclin-B1 to the MTOC in combination-treated cells. Cyclin-B1 and CDK1 are partner proteins crucial for mitotic entry (Jackman, Lindon et al. 2003). At the end of the S phase, cyclin-B1 protein level is elevated, cyclin-B1 – CDK complexes are formed, and the CDK component is activated. Activated cyclin B1-CDK complex phosphorylates substrate proteins, including those at the MTOC, to drive cells into mitosis. We propose that quercetin's interference with the cell cycle progression inhibits the activity of the two microtubule-acting drugs to arrest cells at G2/M.

Although we found that quercetin interfered with the mitotic arrest induced by microtubule-targeting drugs, we did not find evidence to suggest that the cells continue to synthesize DNA and proliferate when combination-treated. Indeed, quercetin by itself inhibited the long-term growth and survival of cells at the same concentrations that interfered with anti-microtubule drugs. Though our in vitro observations are limited, our data suggest that the continued presence of quercetin in the cellular environment may attenuate the activity of microtubule acting agents in the short run. Since the viability of cells in the presence of microtubule disrupting drugs was maintained even by low concentration of quercetin (3.13 μ M or higher in our study), the co-administration of quercetin during treatment with anti-microtubule agents such as paclitaxel may diminish drug activity. In vivo studies need to be performed to elucidate the relevance of this interference. However, our clonogenic assays suggest that long term administration of high doses of quercetin alone or even low doses of quercetin in combination with taxol may not promote the clonogenic survival of colorectal cancer cells.

The current thought on the bioactivity of quercetin and other flavonoids is that these compounds act by scavenging free radicals induced by endogenous and exogenous pro-oxidants (Valko, Rhodes et al. 2006). These pro-oxidant agents include DNA damaging chemotherapeutic drugs and irradiation. However, recent studies suggest that polyphenolic compounds and antioxidants may antagonize diverse groups of chemotherapeutic drugs. Liu et al. (Liu, Agrawal et al. 2008) showed that dietary flavonoids, especially quercetin, inhibit bortezomib-induced apoptosis in malignant B-cell lines and primary chronic lymphocytic leukemia (CLL) cells, by direct association with bortezomib. The authors also found that the inhibitory effect of quercetin was abolished by boric acid, thereby restoring the apoptotic effect of bortezomib on CLL cells. Similarly, Golden et al. (Golden, Lam et al. 2009) found that green tea polyphenols blocked the activities of bortezomib and other boronic acid-based proteasome inhibitors through direct interference. Our data adds taxol and nocodazole to the list of drugs potentially antagonized by quercetin.

It is not clear, however, if the antioxidant properties of flavonoids explain all of such anti-drug bioactivity. For example, a recent study on vitamin C -another antioxidant dietary compound - showed that vitamin C significantly attenuated the activity of diverse classes of chemotherapeutic compounds such as doxorubicin, cisplatin, vincristine, methotrexate, and imatinib, independent of its anti-oxidant potential (Heaney, Gardner et al. 2008). The chemotherapeutic compounds used in the study and found to be inhibited by vitamin C are known to target cellular DNA, the cytoskeleton, or diverse cell signaling mechanisms.

These results and our data suggest that compounds such as quercetin, other polyphenols, and vitamin C may have hitherto unknown bioactivities that may be independent of their antioxidant properties. Competitive interference of polyphenols with bortezomib for proteasome inhibition has been documented (Liu, Agrawal et al. 2008; Golden, Lam et al. 2009), but mechanisms of antagonism of polyphenols against other drugs remain unknown. In the cases of taxol and nocodazole, the effects of quercetin do not appear to stem from the inhibition of uptake of the drugs. Also, unlike bortezomib, the two drugs are not known to directly target the proteasome, excluding the possibility of competitive proteasomal inhibition. Therefore, it is possible that the cell cycle inhibitory effects of quercetin and the resulting lack of cycling cells may explain the antagonistic effect of quercetin on taxol and nocodazole.

We also observed that the bioactivity of quercetin varies with the adherence status of the treated cells. In colony formation assay, non-adherent colon carcinoma cells were inhibited by a dose of quercetin fourfold less than that required for the adherent cells. This observation, together with lack of a major difference between p53 wild type and p53-null HCT116 cells suggests that the adherence status rather than the p53 status renders tumor cells more sensitive to the bioactivity of quercetin. Moreover, the observation that adherent cell lines are also more sensitive to quercetin before they attach to surfaces suggests that the mechanisms and pathways that support cell attachment may confer a degree of resistance to the growth inhibitory effects of quercetin. This in turn may imply that cells may be more sensitive to the actions of the flavonoid quercetin if they are detached from their anchor, as it may occur during metastasis. However, this possible mechanism of action can't explain the cancer preventive activities of flavonoids such as quercetin because metastatic events occur during later stages of oncogenesis. The chemopreventive mechanisms of dietary levels of quercetin and other flavonoids remain to be elucidated.

In conclusion, quercetin appears to have a bimodal bioactivity where it may provide a short-term transient survival benefit to cells exposed to taxol and nocodazole, but has a long-term anti-cell proliferative effect. The anti-proliferative effects appear to be strong especially when the cells have lost their attachment to the growth matrix. Although quercetin

attenuated the cell cycle effects of taxol and nocodazole in the short term, we observed diminished survival and clonogenicity of cancer cells exposed to combinations of quercetin and taxol, which suggests no long-lasting antagonistic effects. Further studies are needed to examine the in vivo effects co-administration of quercetin or other flavonoids with microtubule-acting drugs.

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Abbreviations

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
BrdU	bromodeoxyuridine
DAB	diaminobenzidine
PVDF	polyvinylidene fluoride
CDK1	cyclin dependent kinase 1
MTOC	microtubule organizing center
DAPI	4',6-diamidino-2-phenylindole

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A

	s-G1		G1		S		G2/M	
	24h	48h	24h	48h	24h	48h	24h	48h
<i>DMSO</i>	0.7	1.3	35.4	37.8	31	29.6	32.9	27.2
<i>NOC 10μM</i>	10.4	30.4	6.0	12.1	16.3	8.4	67.3	47.3
<i>TAX 100nM</i>	17.5	27.4	6.5	12.5	10.1	14.2	65.9	38.8
<i>Qctn 6.25nM</i>	0.6	13.2	33.4	50.1	33.0	25.2	31.4	8.5
<i>Qctn 12.5nM</i>	0.9	9.6	40.6	46.7	29.3	28	27.7	13.2
<i>Qctn 25nM</i>	2.9	16.4	48.2	39.8	25.9	25.9	21.0	13.3
<i>Qctn 50nM</i>	1.7	16.5	35.1	39.7	20.4	26.2	38.8	14.4
<i>Qctn 100nM</i>	3.6	26.9	24.6	16.4	38.9	25.3	29.9	26.5

B

Figure 1. The effect of quercetin on the cell cycle profile of HCT116 colorectal and PPC1 prostate cancer cells

A, HCT116 cells were treated with 10μM nocodazole, 100nM taxol, or with the indicated concentrations of quercetin for 24 hours or 48 hours. Cells were harvested and analyzed by flow cytometry. The proportions of cells in each phase of the cell cycle (sub-G1, G1, S, G2/M) for each treatment are indicated in the table. B, PPC1 cells are treated with 0 to 100μM quercetin (as shown) for 24 hours. Cells were harvested and analyzed by flow cytometry. Histograms of the cell cycle profiles of the cells are shown on the upper panel. The lower panel shows the proportion of cells in phases of the cell cycle (sub-G1, G1, S, G2/M) for each dose of quercetin. Representative data from two independent experiments are shown.

Figure 2. Quercetin blocks the activity of nocodazole and taxol
A-F, HCT116 cells were treated with carrier DMSO (A), 50μM quercetin alone (B), 10μM nocodazole (C), 10μM nocodazole plus 50μM quercetin (D), 100nM taxol (E), or 100nM taxol plus 50μM quercetin (F). Cells remained under treatment for 24 hours (A-D), or 16 hours (E, F), and phase contrast images were taken at 200X magnification. G-I, Quercetin inhibits G2/M arrest in HCT116 cells. Wild type (G) and p53-null (H) HCT116 cells were treated with DMSO, 50μM quercetin, 10μM nocodazole, or the indicated decreasing concentrations of quercetin in the presence of 10μM nocodazole as shown. 50μM quercetin effectively blocked the cell cycle effect of nocodazole on both cell types, while lower concentration showed weaker or no inhibition. I. Tabular presentation of the data in G and H.

Figure 3. Quercetin inhibits the activity of taxol on PPC1 prostate cancer cells
PPC1 cells were treated with 0 - 400nM taxol as shown (A) or a combination of 0 – 400nM taxol and 50µM quercetin (B), and incubated for 12 hours. Cells were harvested and analyzed by flow cytometry. The histograms in upper panels show the cell cycle profiles of the cells, and the lower panels (tables) show the numerical proportion of cells in each phase of the cell cycle for each treatment in A and B. One of three independent experiments is shown.

Figure 4. Quercetin maintains the viability of colorectal cancer cells treated with nocodazole but delays cell cycle progression

A-D, effect of quercetin or quercetin-nocodazole combination on the viability of HCT116 cells. Wild type or p53-null HCT116 cells were treated with quercetin alone (A, B) or with combinations of 10 μ M nocodazole and increasing doses of quercetin (C, D) as shown. Cell viability was measured after 24, 48, and 72 hours by MTT assay. Cell viability is plotted as MTT index, relative to that of the control DMSO treated cells. E. BrdU uptake in wild type HCT116 cells treated with DMSO, 100nM taxol, 50 μ M quercetin, or a combination of taxol and quercetin was measured by BrdU incorporation ELISA. Relative BrdU uptake is shown as a percentage of uptake by the control cells. The difference in BrdU incorporation between taxol, quercetin, and combination treated cells was not significant. F. RKO colorectal cancer cells were synchronized by double thymidine (2mM) block, and released into growth medium containing DMSO (Contr.) or quercetin (Qctn). Aliquots of cells growing asynchronously or at different time points (at release (t0), 2 hours, 4 hours or 9 hours) after release from the block were analyzed by flow cytometry. Cell cycle profiles are shown as histograms in the top panels, and the proportion of cells in G1, S, or G2 at the time points are shown in the lower panels (tables). Cells exposed to quercetin medium showed considerable delay (underlined values) in cell cycle progression compared to control cells.

Figure 5. Quercetin does not interfere with microtubule targeting of taxol and nocodazole
MCF7 cells were treated for 16 hours (overnight) with carrier (DMSO), quercetin (QCTN, 10 μ M), taxol (TAX, 50nM), nocodazole (NOC, 10 μ M) or combinations of taxol and quercetin (TAX + QCTN) or nocodazole and quercetin (NOC + QCTN) as shown. Cells were then fixed and immunofluorescently stained for tubulin (upper row). DAPI was used as a counterstain for nuclei (middle row). Merged images (tubulin and DAPI) are shown in the bottom row. Confocal images were taken using a 40X dry objective.

Figure 6. Treatment of HCT116 cells with a combination of quercetin and taxol disrupts the localization of cyclin-B1 at the MTOC

A, HCT116 wild type cells grown in chamber slides were exposed to DMSO, 50µM quercetin (Qctn), 100nM taxol (TAX), or 50µM quercetin and 100nM taxol combination (TAX+Qctn). After 8 hours of treatment, cell monolayers were stained with anti cyclin-B1 antibody by immunocytochemistry. Arrows indicate the localization of cyclin-B1 at the MTOC. B, HCT116 cells grown in 6 cm diameter dishes were treated with DMSO, 50µM quercetin, 100nM taxol or a combination of 50µM quercetin and 100nM taxol for 8 hours. Cell lysates were prepared as described in the Materials and Methods section. Cyclin-B1, CDK1, and β-actin proteins were detected by immunoblotting. * Indicates a non specific band.

Figure 7. Continued exposure of HCT116 cells to quercetin inhibits colony formation

A. Wild type and p53-null HCT116 cells were seeded in 12-well cell culture dishes and allowed to adhere to the plate for about 16 hours. Adherent cells were treated with the indicated concentrations of quercetin and colony formation was examined over 8 days as described under materials and methods. B. Wild type and p53-null HCT116 cells were seeded in 12-well cell culture dishes in the presence of the indicated concentrations of quercetin in culture medium. Colony formation was examined as described. C-E, Quercetin does not provide lasting clonogenicity and survival advantage to HCT116 cells. Clonogenicity of HCT116 cells exposed to 6.25 μ M -100 μ M quercetin was examined by clonogenicity assay (Franken, Rodermond et al. 2006). The colonies that formed after the treatments, and the number of cells per colony for each treatment are shown in C and D, respectively, relative to the numbers from control cells. Doses of quercetin that antagonized taxol or nocodazole still inhibited clonogenic survival of the cells. E. Clonogenic survival of HCT116 cells treated with quercetin (25 μ M) or quercetin in combination with taxol (0.6nM – 5nM). Clonogenicity of the cells is shown as the number of colonies that formed relative to the control (DMSO) treatment. Quercetin in combination with taxol provided no clonogenic advantage; on the contrary, combination treated cells had the poorest clonogenic survival.