

The Flavonoid Quercetin Protects MDCK Cells against Toxicity of CPT by Induction of Autophagy

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Abstract

In chemotherapy, two or more pharmaceuticals targeting different metabolic pathways are often combined in the expectation that they will synergize or function additively against the tumor. Flavonoids, typically polycyclic plant pigments, have many beneficial effects in humans, including being putatively anticancer, inducing apoptosis by undefined means. Camptothecin (CPT), a powerful chemotherapeutic natural product used in Chinese traditional medicine is a topoisomerase inhibitor. In this study, we investigated whether two recommended flavonoids from plants, baicalein and quercetin, affected the capability of CPT to kill cells. We exposed MDCK (Madin Darby Canine Kidneys) cells to each flavonoid in the presence or absence of CPT; the flavonoids themselves were very modestly toxic at 50-100 µg/ml. However, quercetin but not baicalein reduces cell death induced by CPT. The flavonoids induced autophagy, which can protect cells against CPT and other stressors and is likely to be a major factor protecting cells from toxins like CPT. This potential effect indicates that not all putatively pro-apoptotic agents will synergize with others, and they may even restrain the oncolytic toxicity of other chemotherapeutic drugs. Presumptively beneficial natural products used in folk medicine may even antagonize the oncolytic effect of the chemotherapy.

Keywords: Flavonoids; Quercetin; Baicalein; Autophagy; Apoptosis; Cancer

Introduction

Flavonoids

Natural products and chemicals deriving from folk medicine are frequently taken as supplements to maintain health or to augment the potential of formal medicine to treat disease. Among these products, flavonoids are considered to be beneficial against cancer. Flavonoids, a group of more than 4,000 related polyphenolic compounds from plants that are commonly found in human diet [1] are antioxidants, antibacterial, anti-inflammatory, anti-cancer, and anti-viral compounds [2,3] and are often considered to be potential anti-neoplastic compounds, to be combined with more conventional chemotherapeutic agents. Unfortunately, as we demonstrate here, they potentially can antagonize chemotherapeutic agents, protecting cells against the toxicity of the agents. Flavonoids can act as anti-viral agents, inhibiting, for instance, Dengue NS2B-NS3 protease [4-6]. They also affect generation of inflammatory signals can cause cell cycle arrest, leading to their employment as antitumor agents. Quercetin and Baicalein are considered to have substantial medicinal value [7-9]. Baicalein (5, 6, 7-trihydroxyflavone), a flavone extracted from the root of the skullcap mint *Scutellaria baicalensis*, likewise has anti-inflammatory, antioxidant and anti-cancer properties [10-12]. Quercetin (3,3',4',5,7-pentahydroxyflavone), found in many fruits and some vegetables, is a major flavonol in human diet [3]. As an oncolytic agent, it prevents repair of radiation-induced double strand DNA breaks in models of human ovarian cancer by inducing p53-mediated endoplasmic reticulum stress, thus increasing the radiosensitivity of

these tumors [10]. It is also an antioxidant, scavenging free radicals and blocking tumor formation [11]. However, as we report below, quercetin can protect cells against camptothecin.

Camptothecin, an oncolytic compound

20 (S)-Camptothecin (CPT), a pentacyclic alkaloid extracted from stem wood of a Chinese plant, is a topoisomerase inhibitor [13], used against gastric carcinoma, hepatoma and leukemia [14-17]. It may also activate reactive oxygen species (ROS) and ATM-Chk2-CDC25C.

Autophagy mitigates the toxicity of camptothecin

We have previously demonstrated that autophagy, stimulated by some viruses, rapamycin or other agents can protect cells against camptothecin [18,19]. As described below, quercetin induces autophagy and protects MDCK cells from the CPT.

The biology of autophagy

Autophagy is a highly conserved homeostatic recycling mechanism that is believed to have preceded apoptosis in evolution [20]. It is defined by the uptake of endogenous or exogenous particles or proteins by cytosolic vesicles followed by degradation in the lysosomal compartment [21]. The most widely studied form of autophagy, macroautophagy, is a surveillance process where macromolecules (proteins, lipids) and organelles are packaged in double-membrane vesicles (autophagosomes) and transported to lysosomes where they undergo acidic degradation [22,23]. In a normal or healthy cell, mammalian target of rapamycin complex 1 (mTORC1) limits autophagy to a basal level by keeping Unc-51-like kinase (ULK1/Ser 757) and ATG13 deactivated through inhibitory phosphorylation

[24-34]. Under extreme conditions as starvation, infection or other forms of stress, regulatory molecules such as PTEN, AMPK, and TSC2 remove this block [35-37]. Deregulation of autophagy is likely to be important in several pathophysiological conditions. However, autophagy is a defense mechanism for stressed cells, permitting them to survive otherwise lethal challenges. We suggest here that this defense mechanism can countermand the toxicity of other oncology.

Materials and Methods

Cell culture and treatment

MDCK (a gift of Dr. Anastasia Gregoriades, Queens College, Flushing, NY, USA) and MDCK-LC3-GFP (MDCK cells with GFP tagged LC3 gene, provided by Guido Kroemer, Institute Gustave-Roussy, Villejuif, France) cells were grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (penicillin/streptomycin #P4333, Sigma Aldrich) at 37°C under a 5% CO₂ atmosphere. To block autophagy we added class I/III PI3K inhibitor Wortmannin (wort) (#681675, Calbiochem), which was applied at 1 µM. To stimulate autophagy, we added rapamycin (#R0395, Sigma) at 50 nM. In these cases, cells were incubated with inhibitors dissolved in the maintenance media. Camptothecin (CPT, 5 µM final concentration) (C9911, Sigma) was used as an inducer of apoptosis. These were all applied in fresh media, and the cells were incubated for 24 h or otherwise as indicated. The two flavonoids were baicalein (#465119, Sigma-Aldrich) and quercetin (#Q4951, Sigma-Aldrich). To prepare the baicalein and quercetin stock solutions, 20 mg of baicalein or quercetin were dissolved in 1 mL dimethyl sulfoxide and the solution filtered. The stock solutions were added to the cell maintenance medium as indicated in each experiment.

Assessment of cell viability

Cells were treated with flavonoid or toxin (such as CPT) and incubated for an additional 12-24 h as indicated at 37°C, 5% CO₂. At the end of treatment, the medium with floating cells was collected first, and the attached cells were collected by trypsin digestion. The collected samples were pelleted and stained with 0.4% trypan blue (T8154, Sigma-Aldrich) and incubated at room temperature for 2-5 min in 1 × PBS. We have shown previously a direct correlation between trypan blue exclusion and other viability assays. On a hemocytometer 100 to 300 live (white) and dead (blue) cells were counted from each sample for each of three separate experiments. Cell viability is expressed as percent dead cells. Statistical significance of the results was calculated by Student's t-test; values of P and t<0.05 were considered significant.

Cytochemical analysis

For cytochemical analysis, MDCK LC3 GFP cells were seeded onto flame-sterilized glass coverslips, allowed to attach overnight and infected and treated as above. After 24 h of incubation, the cells were washed with PBS, fixed using paraformaldehyde (04042, Fisher, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min, rinsed once with 1 × PBS, and then embedded in Fluoromount (F4680, Sigma). The cells were observed by fluorescence microscopy (Leitz, subsidiary of Leitz, Buffalo Grove, IL, USA). Generation of a punctate pattern of green fluorescent protein (GFP) is indicative of LC3 translocation and autophagosome formation. Mock-infected cells were also analyzed to ensure that LC3-GFP expression alone did not cause autophagy.

Results

Flavonoids selectively protect cells from different cell-lethal toxins

We investigated how flavonoids affected the toxicity of camptothecin (CPT). We first established an EC₅₀ for flavonoids on cells. MDCK cells were exposed for 24 h to 20, 50 and 100 µg/mL of either baicalein or quercetin. Cell viability was assessed by trypan blue exclusion. At concentrations of 50 and 100 µg/mL quercetin is moderately toxic, but little cell death is found with treatment with baicalein (Figure 1A).

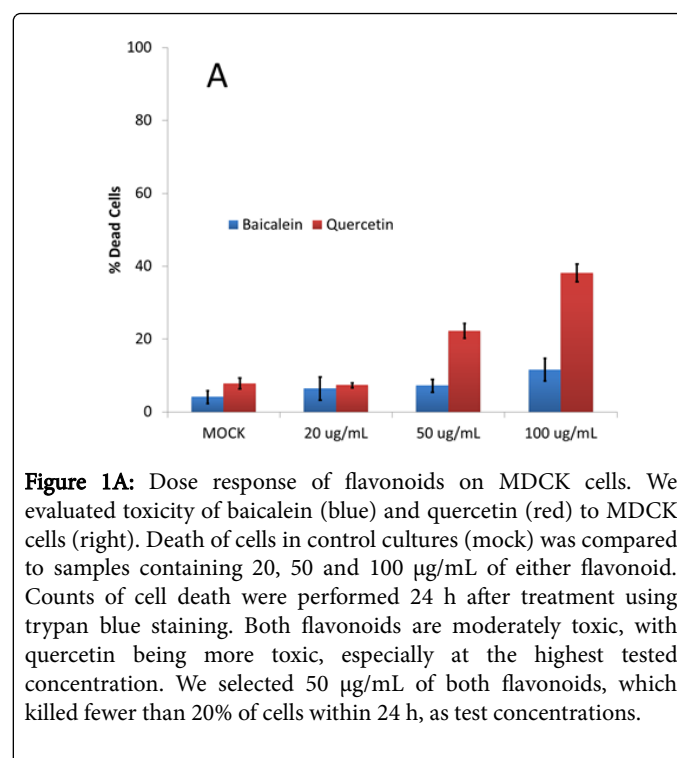


Figure 1A: Dose response of flavonoids on MDCK cells. We evaluated toxicity of baicalein (blue) and quercetin (red) to MDCK cells (right). Death of cells in control cultures (mock) was compared to samples containing 20, 50 and 100 µg/mL of either flavonoid. Counts of cell death were performed 24 h after treatment using trypan blue staining. Both flavonoids are moderately toxic, with quercetin being more toxic, especially at the highest tested concentration. We selected 50 µg/mL of both flavonoids, which killed fewer than 20% of cells within 24 h, as test concentrations.

Cell morphology also did not change significantly with the concentrations 20-50 µg/ml, while cells with 100 µg/ml of either flavonoid looked rounded and stressed. We therefore used the lower concentration of 50 µg/ml for our experiments.

To evaluate the toxicity of CPT in the presence of flavonoids we incubated the cells with and without baicalein or quercetin prior to and during incubation with CPT. 5 µM CPT kills 65% of the cells within 24 h (Figure 1B).

Baicalein does not affect this toxicity, but quercetin significantly reduces killing, reducing the number of dead cells by almost 40% (Figure 1B).

Thus we see no evidence that the flavonoids synergize with CPT. In fact, if anything quercetin appears to counteract the lethality of CPT and therefore may diminish the effect of chemotherapy.

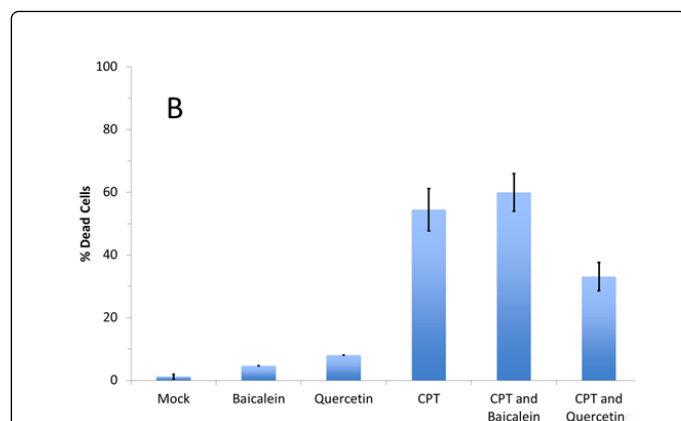


Figure 1B: Effect of flavonoids on survival of CPT-treated cells. MDCK cells were exposed to either baicalein or quercetin (50 µg/ml) for 24 h, followed by treatment with 5 µM CPT for another 24 h in the continued presence of the flavonoid. Although quercetin is itself moderately toxic, it suppresses cell death almost 40% compared to CPT alone. Baicalein had no effect on the survival of CPT-treated cells. Error bars represent the SEMs for three separate experiments.

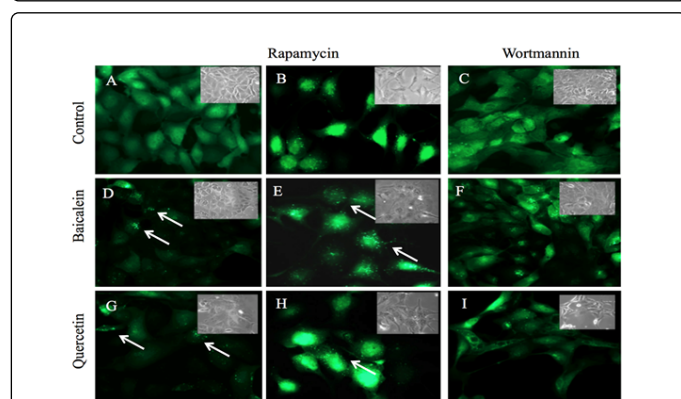


Figure 2: Cytochemical analysis of MDCK-LC3-GFP cells: Quercetin and baicalein induce autophagy in MDCK cells. MDCK-LC3-GFP cells were exposed to quercetin or baicalein (50 µg/mL) in the presence of 100 nM rapamycin (autophagy inducer) or 1 µM wortmannin (autophagy inhibitor). Controls include cells with (A) no treatment; (B) cells treated with 100 nM rapamycin alone; or (C) 1 µM wortmannin alone; (D-F) Cells exposed to 50 µg/ml baicalein as well as the inducer or inhibitor; (G-I) Cells exposed to quercetin as well as the inducer or inhibitor. Both baicalein and quercetin induced autophagy as indicated by puncta (arrows). The autophagy was enhanced by rapamycin and suppressed by wortmannin.

Autophagy is likely involved in protection from cell death

We and others have argued that viral infections protect cells by inducing autophagy [19,38]. Here we asked if autophagy could explain the protection by flavonoids. We first asked if flavonoids induced autophagy. We assessed induction of autophagy by sequestration of LC3II (light chain 3 variety 2, 16 kD), which can be identified by localization (puncta) within cells. MDCK LC3 GFP cells were exposed

baicalein or quercetin at 50 µg/mL by themselves; in combination with an inducer of autophagy, 50 nM rapamycin; or in combination with an inhibitor of autophagy, 1 µM wortmannin. Very few puncta were produced by either flavonoid, a slight increase over that of untreated cells (Figure 2).

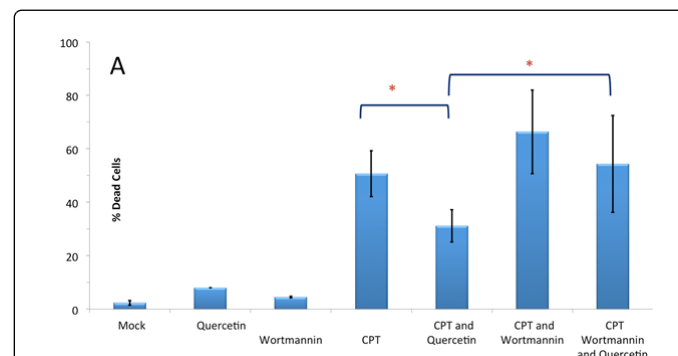


Figure 3A: Quercetin protects MDCK cells from CPT in an autophagy-dependent manner. MDCK cells were exposed to 5 µM CPT, 50 µg/ml quercetin, and 1 µM wortmannin, and incubated for 24 h. Controls of both experiments include mock samples without any treatments, samples treated with baicalein or quercetin, samples treated with CPT only, and a sample with wortmannin alone. Cell death counts were conducted using trypan blue staining and a hemocytometer. The addition of wortmannin, which blocks autophagy, blocks the protection afforded by quercetin.

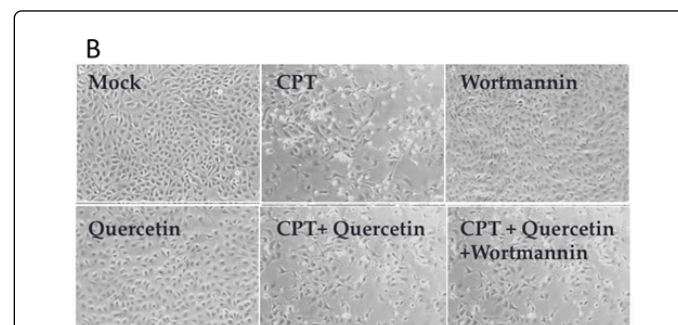


Figure 3B: Morphology of MDCK cells treated as follows: control (no treatment), 5 µM CPT, and 1 µM wortmannin, 50 µg/ml of baicalein or quercetin and combinations of those as indicated. By both morphology and counts of dead cells, wortmannin suppresses the protection against CPT afforded by quercetin.

Addition of rapamycin (middle column) substantially increased the number of puncta, and in all cases wortmannin inhibited the formation of puncta (right column). To compare the protection of cells by quercetin to the induction of autophagy we induced cell death in MDCK cells using 50 µM CPT, and exposed the cells to 50 g/ml quercetin, in the presence of 1 µM wortmannin. Control samples included no additions; quercetin alone; CPT alone; and wortmannin alone. After 24 h there was no significant cell death more than that seen in untreated samples when cells were exposed to either wortmannin or quercetin alone (Figure 3A).

CPT-induced cell death is reduced when quercetin is present. The toxicity of CPT is not altered by the addition of wortmannin, but this

inhibitor of autophagy abolishes the protection from cell death induced by quercetin (Figure 3A). The morphological analyses confirm this evaluation as well (Figure 3B).

These results suggest that, as is the case with viral infections, induction of autophagy by quercetin protects cells from CPT.

Discussion

Neither baicalein nor quercetin is toxic to MDCK cell at 20-50 µg/ml. However, quercetin can effectively block the toxicity of CPT. While baicalein and quercetin both induce autophagy, quercetin diminished CPT-induced cell death but baicalein did not, suggesting that the flavonoids influence different pathways or vary in specificity of action. Wortmannin negates the protective effect of quercetin, indicating that the protection operates through the increase in autophagy, as we know that autophagy induced by several means including by rapamycin can protect cells against CPT [19]. Quercetin induces protective autophagy in gastric cancer cells and HeLa cells, while disruption of autophagy by genetic knockout or blockage of autophagy by 3-methyladenine or chloroquine induced apoptosis in these cells [39,40]. Quercetin also induces autophagy through ER stress in ovarian cancer cells [41]. In these cases, quercetin induced apoptosis

especially if autophagy was blocked. Although autophagy was once considered to be a primary mechanism of cell death, more recent research emphasizes its protective effect [19,22,23,38]. To briefly summarize this growing literature, autophagy is activated in cells stressed by any of several challenges. The autophagy consumes mitochondria and damaged organelles, thus reducing energy demand, removing potentially toxic metabolites, and generating a bit more energy for the cell. Cells that can activate autophagy during a transient stress survive longer, thus potentially outlasting the stress. If the stress persists or is more severe, the cell can consume too much of itself, leading to death by apoptosis or other means [19,22,23,38]. We speculate that quercetin can protect cells by inducing autophagy, and it therefore may counteract conventional chemotherapeutic agents, as described in Table 1 and Appendix 1. Because natural products frequently vary in composition and patients are often reluctant to discuss accurately their use of these products, controlled and validated experiments are difficult to locate. Nevertheless, a Canadian group has assembled a list of interactions reported as anecdote, case study, or theoretical possibility of the interactions of natural products with chemotherapeutic and other pharmacological treatments. The list was presented as a lecture that is available on the Internet as a PDF of a PowerPoint presentation and is summarized in Table 1.

| Natural product | Interacts with (agent) | Mechanism |
|-------------------------------|------------------------------------|--|
| St. John's Wort | Imotecan (chemotherapy) | Inhibits Cytochrome P450 (CYP3A4) |
| St. John's Wort | Docetaxel, Imatinib (chemotherapy) | Blocks CYP3A4 induction |
| Grapefruit | Docetaxel (Chemotherapy) | Inhibits CYP3A4 |
| Grapefruit | Etoposide (Chemotherapy) | Blocks P-glycoprotein |
| Probiotics | Docetaxel (chemotherapy) | Increase risk of infection |
| Green Tea | Bortezomib (proteasome inhibitor) | Antagonizes proteo. inh. (PI) |
| Ascorbic acid | Bortezomib (proteasome inhibitor) | Complexes with PIs |
| Melatonin | Radiation | Antioxidant |
| Vitamin E | Radiation | Antioxidant |
| Flaxseed | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Black Cohosh | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Dong Quai | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Evening Primrose | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Soy | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Red Clover | Tamoxifen (anti-estrogen) | Phytoestrogen |
| Dihydroepiandrosterone (DHEA) | Anti-androgens (prostate cancer) | An androgen |
| Ginkgo | Dalteparin (anticoagulant) | Interferes with decrease of platelet aggregation |
| Garlic | Dalteparin (anticoagulant) | Interferes with decrease of platelet aggregation |
| Ginseng | Dalteparin (anticoagulant) | Interferes with decrease of platelet aggregation |

Table 1: Interactions of natural products with medicinal pharmacologic agents

This presentation listed a series of references consisting of reviews, case studies, and theoretical possibilities of interaction, which is copied

into Appendix 1. Undoubtedly, other studies exist in the literature from Asia, not currently known in Europe or the Americas. Readers familiar

with such studies are encouraged to make them known to Western scientists and physicians. While much remains to be examined regarding dosages, kinetics, and mechanisms for the roles of flavonoids in inducing autophagy, apoptosis, and the resistance of cells to the toxicity of oncolytics, we present this preliminary report as a warning that the use of natural or folk products in the hope that they will synergize with conventional chemotherapeutic drugs may be a false hope and in fact may diminish the effectiveness of the conventional drug.

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