Protection of human colon epithelial cells against deoxycholate by rottlerin

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Abstract:

The bile salt, deoxycholate (DOC), can harm cells and cause disease. Hence, there is interest in identifying compounds capable of protecting cells against DOC. In HCT-116 colon epithelial cells, DOC increased generation of reactive oxygen species and caused DNA damage and apoptosis. These effects of DOC were inhibited by rottlerin, which is a phenolic compound of plant origin. In elucidating its mechansim, rottlerin prevented the release of cytochrome c from mitochondria into cytosol, and also prevented the cleavage of caspase-3. Yet, rottlerin by itself markedly decreased mitochondrial membrane potential and increased mitochondrial superoxide production, but this did not result in cytochrome c release or in caspase-3 cleavage. At a higher test concentration, two other phenolic phytochemicals, namely, quercetin and resveratrol, were each able to largely prevent the occurrence of apoptosis in cells exposed to DOC. In contrast, epigallocatechin gallate, curcumin, and genistein were ineffective.

Keywords:

Apoptosis - Deoxycholate - Quercetin - Resveratrol - Rottlerin

Article:

INTRODUCTION

Bile acids are both useful and harmful. An important physiological role of bile acids is to facilitate the digestion and subsequent absorption of dietary fats. On the other hand, bile acids are potentially cytotoxic. For example, when not processed normally by the hepatobiliary system, bile acids cause the development of cholestatic liver disease [1]. Furthermore, when present at undesirable concentrations, bile acids may become problematic in the intestinal tract. Using fecal water for analysis, total bile acid concentrations as high as 1 mM have been found, and deoxycholate (DOC) approached concentrations as high as 0.8 mM in human subjects given additional fat in the diet [2]. There is support for the notion that high concentrations of bile acids in the large intestine may promote colonic abnormalities associated with inflammation. In particular, secondary bile acids such as DOC are pro-inflammatory, as demonstrated in a recent study [3], where supplementing the diet of mice with DOC resulted in colitis. It is thought [4] that DOC is a carcinogen, based on the association of high concentrations of bile acids with several gastrointestinal forms of cancer. Therefore, it would seem desirable to discover protective agents against DOC.

At the cellular level, exposing cultured cells to bile acids produces genotoxic and cytotoxic effects. Bile acids induce the generation of reactive oxygen species (ROS) in rat hepatocytes [5], although the mechanism is unclear. Previously, we reported that DOC induced DNA damage and apoptosis in HCT-116 human colon epithelial cells [6]. Hence, ROS may have been involved in causing the effects of DOC, leading one to suspect that antioxidants could protect the cells against DOC. Previously, however, we found that neither N-acetylcysteine nor α-tocopherol could counteract DOC, at least in regards to DOC's ability to upregulate the GADD153 gene that is often associated with oxidative stress, DNA damage, and apoptosis [7]. In the present

study focused on several phenolic phytochemicals, we have discovered that rottlerin, which is a pigmented plant product isolated from Mallotus philippinensis and originally reported to be a protein kinase inhibitor [8], most effectively protects HCT-116 cells against DOC.

MATERIALS AND METHODS

Materials

HCT-116 human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). DOC, diphenylpicrylhydrazyl (DPPH), rottlerin and the other phenolic phytochemicals, as well as all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell culture and treatment

HCT-116 cells were propagated in McCoy's 5A medium that was supplemented with 10% fetal bovine serum, 0.2% amphotericin B (250 µg/ml stock), and 1% penicillin/streptomycin mixture (10,000 units penicillin & 10 mg streptomycin/ml stock). Upon reaching 70–80% confluency, the cells were exposed to 300 µM or 600 µM DOC for 1–24 h, depending on the experiment. These relatively high concentrations of DOC, which is within the broad range found in human fecal water samples [2], were selected to ensure that the HCT-116 cells would be maximally challenged by DOC so that the phenolic phytochemicals could be severely tested. Thus, in other experiments, cells were pre-treated with rottlerin, quercetin, resveratrol, curcumin, genistein, or epigallocatechin gallate (EGCG) for 0.5 h, before exposing them to DOC.

Comet assay

Cellular DNA damage was assessed by performing alkaline gel electrophoresis with visualization by fluorescence microscopy (comet assay), as described previously [6]. For each sample, each of 50 randomly selected nucleoids was scored on a numerical scale of 0–4 to arrive at the total comet score.

Assessment of overall ROS production, mitochondrial superoxide anion generation, and mitochondrial membrane potential (MMP) status

Overall cellular ROS production was assessed with an Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Inc., Eugene, OR). Mitochondrial superoxide anion generation and MMP status were assessed with the MitoSOX Red reagent (Molecular Probes, Inc., Eugene, OR) and DePsipher reagent (Trevigen, Inc., Gaithersburg, MD), respectively. All three fluorescence microscopic procedures were performed according to the supplied instructions from the vendors.

Staining of mitochondria and immunocytofluorescence detection of cytochrome c

Cells were grown and treated in Lab-Tek chamber slides. Mitochondria in cells were stained with MitoTracker Red CMXRos dye according to the protocol provided by the vendor (Molecular Probes, Inc., Eugene, OR). After fixation with 3.7% formaldehyde in complete media, the fixed cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) and then blocked with 3% bovine serum albumin in PBS containing 0.5% Tween 20. The cells were then incubated overnight with a 1:400 dilution of a mouse antibody against cytochrome c (BD Pharmingen, Inc., San Diego, CA), followed eventually by a 1:400 dilution of a rabbit anti-mouse secondary antibody conjugated to AlexaFluor 488 (Molecular Probes, Inc., Eugene, OR) before viewing by fluorescence microscopy.

Western blotting analysis of cytochrome c and caspase-3

A procedure based on Dounce homogenization of cultured cells [9] was followed for isolation of cytosol to detect the presence of cytochrome c in this cell fraction. Aliquots of cytosol (30 µg protein) was subjected to electrophesis (Novex 4–12% NuPage gels) and then blotted to nitrocellulose membrane. After blocking with 5% skim milk, the membrane was probed with 1:5,000 dilution of mouse anti-cytochrome c (BD Pharmingen, Inc., San Diego, CA) and 1:100,000 dilution of goat anti-mouse/HRP secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Also, whole cell extracts were prepared for similar processing to detect

the presence of cleaved or activated caspase-3. This was accomplished with a rabbit polyclonal antibody (#9661 from Cell Signaling Technology, Danvers, MA), which is said to recognize the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to aspartic acid residue 175. Target protein detection was with SuperSignal West Femto chemiluminescent reagent system (Pierce Biotechnology, Rockford, IL). Processed membranes were stripped and reprobed for β-actin as a loading control.

Annexin V-Alexa Fluor 488 binding assay

HCT-116 cells that had been grown and treated in Lab-Tek chamber slides were washed twice with cold phosphate-buffered saline. The washed cells were equilibrated for 10 min in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). After equilibration, the binding buffer was removed from the chamber slide. Then, fresh binding buffer but containing annexin V-Alexa Fluor 488 conjugate (Molecular Probes, Inc., Eugene, OR) was pipetted into the chamber slide for 15 min of incubation in the dark (CO2 incubator). After washing with binding buffer, cells on the slides were viewed by fluorescence microscopy.

Determination of chromatin condensation/fragmentation

After washing and fixation of the cells, they were stained with 4′,6-diamidine-2-phenylindole (DAPI) and then examined by fluorescence microscopy, as detailed previously [6]. The percentage of cells having chromatin condensation/fragmentation was calculated after examining 100 randomly selected cells over the entire surface of the slide.

Determination of free radical-scavenging capability of the test compounds

The ability of rottlerin and the five phenolic phytochemicals to scavenge the stable free radical, DPPH, was evaluated spectrophotometrically [10] with slight modification. The appropriate concentrations of both DPPH and test compounds were empirically established to permit comparison of the relative capabilities of the test compounds to scavenge DPPH. To semi-micro cuvets was added 1.0 ml of a 0.1 mM solution of DPPH (dissolved in absolute ethanol), and then, the absorbance at 516 nm was recorded (time point zero). Next, to the cuvets was added 0.004 ml of a 1.25 mM solution of rottlerin, quercetin, resveratrol, curcumin, epigallocatechin gallate (EGCG), or genistein (each dissolved in absolute ethanol). Immediately after quick mixing, the absorbance of the samples in the cuvets was recorded at 2-min intervals thereafter for 12 min.

RESULTS

Effects of DOC and rottlerin on cellular ROS production and DNA structural integrity

To determine if DOC induces oxidative stress in HCT-116 cells, we first performed an assay that detects the oxidation by ROS of the reduced form of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (non-fluorescent) to a product having green fluorescence (Fig. 1a). It is apparent from the images that DOC markedly increased overall production of ROS in the cells, but this effect of DOC was prevented by pre-treatment of the cells with rottlerin. Next, the comet assay was performed to see if DOC causes DNA damage, and more importantly, if rottlerin can stop DOC from causing any such DNA damage. As can be seen by the set of images (Fig. 1b) and scoring results (Fig. 1c), untreated control cells had nucleoids that were spherical, reflecting no DNA damage and producing a comet score of 0 ± 0. In contrast, DOC-treated cells had nucleoids with the typical comet appearance, reflecting DNA single-strand breaks and producing a comet score of 72 ± 30. Most notable, however, pre-treatment of the cells with rottlerin reduced the extent of the DNA damage caused by DOC, as reflected by a markedly lower comet score of 6 ± 7.

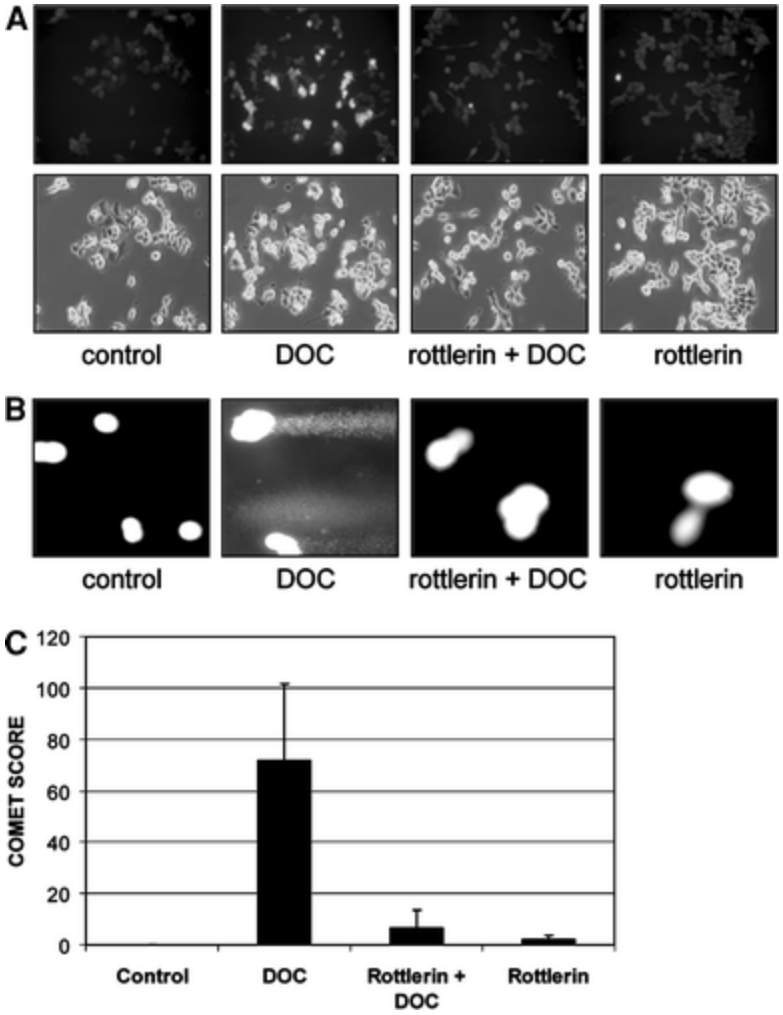

Fig. 1 Attenuating effect of rottlerin on DOC-induced ROS production and DNA damage. HCT-116 cells were pre-treated with 20 μM rottlerin for 0.5 h and then exposed to 600 μM DOC for 1 h. Cells were stained to detect the presence of ROS by fluorescence microscopy (a, upper images), with matching images of the stained cells obtained in the phase-contrast microscopic mode (a, lower images). Also, the comet assay was performed with capture of the fluorescence images (b) and scoring for comets (c). All images are representative of at least three different experiments, and the bar graph values are the average ± SEM for three different experiments

Effects of DOC and rottlerin on mitochondrial superoxide anion production, functioning of mitochondria, cytochrome c release, and caspase-3 cleavage

To determine if mitochondria of HCT-116 cells are adversely affected by DOC and/or rottlerin, assays were done to look for evidence of mitochondrial dysfunction. Based on a more specific ROS detection assay using MitoSOX Red reagent, it would appear that DOC increased mitochondrial superoxide anion generation in the cells, but rottlerin had a greater effect in this regard (Fig. 2a). Moreover, pre-treatment of the cells with rottlerin did not attenuate the induction of mitochondrial superoxide anion generation caused by DOC, as might be expected in view of the inducing effect of rottlerin per se on mitochondrial superoxide anion generation.

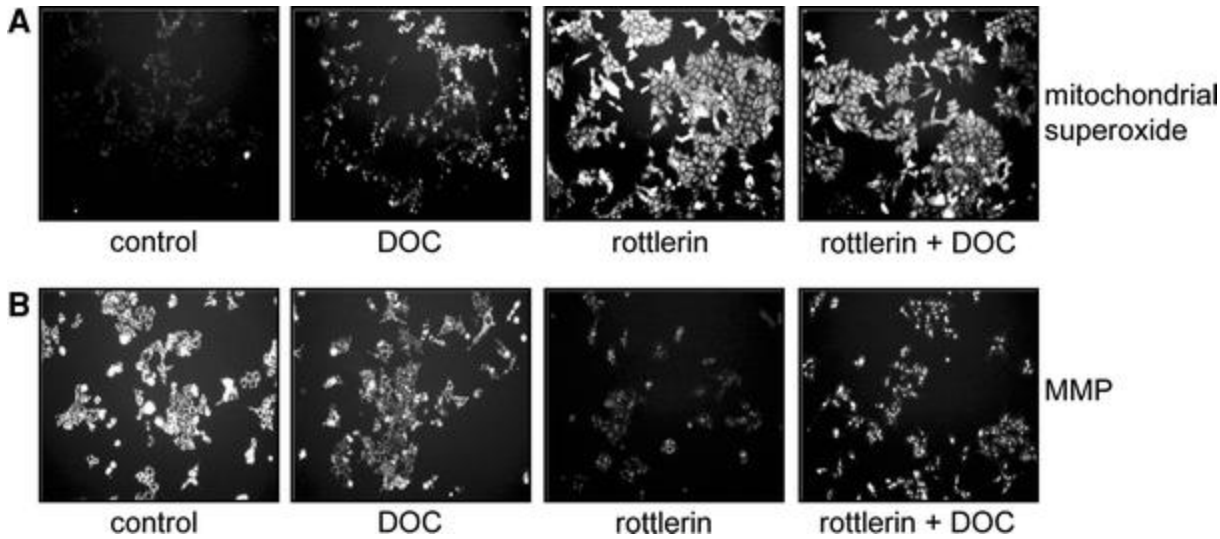

Fig. 2 Increase of mitochondrial superoxide anion generation and loss of mitochondrial membrane potential in cells exposed to rottlerin and/or DOC. HCT-116 cells were pre-treated with 20 μM rottlerin for 0.5 h and then exposed to 600 μM DOC for 1 h. Cells were stained to detect the presence of mitochondrial superoxide anion (a) and status of the mitochondrial membrane potential (b). All images are representative of at least three different experiments

In assessing MMP status with the DePsipher reagent (Fig. 2b), DOC caused a loss of MMP as indicated by the reduction in fluorescence intensities of the DOC-treated cells in comparison to control cells. But, rottlerin produced an even greater loss of MMP. Thus, as might be anticipated, pre-treatment of the cells with rottlerin did not inhibit DOC from decreasing MMP.

To determine if the above events caused by DOC and rottlerin are associated with release of a specific mitochondrial membrane protein known to be involved in initiating apoptosis, the localization of cytochrome c was initially visualized along with mitochondria by immunocytofluorescence microscopy (Fig. 3a). As shown in the first row of images, the mitochondria become visible only if they are functioning normally that permits the MitoTracker Red CMXRos reagent to fluorescently stain the active mitochondria, which visually seem to surround the nuclei of cells as evident in the control sample only. In DOC-treated cells, the mitochondria appear to be abnormally distributed and condensed. It is apparent that rottlerin-treated cells, as well as DOC-treated cells pre-treated with rottlerin, stained poorly, which would indicate that the mitochondria of these cells were functionally impaired or inactive. The second row of images shows the antibody-based detection of cytochrome c. When the first and second rows of images are merged, it becomes apparent that cytochrome c is localized in the mitochondria of control cells. However, in DOC-treated cells, a noticeable amount of cytochrome c seems to be present outside mitochondria, that is, in the cytoplasm. As further shown, cytochrome c rather unexpectedly remained in the mitochondria of rottlerin-treated cells, and most interesting, also in DOC-treated cells that had been pre-treated with rottlerin.

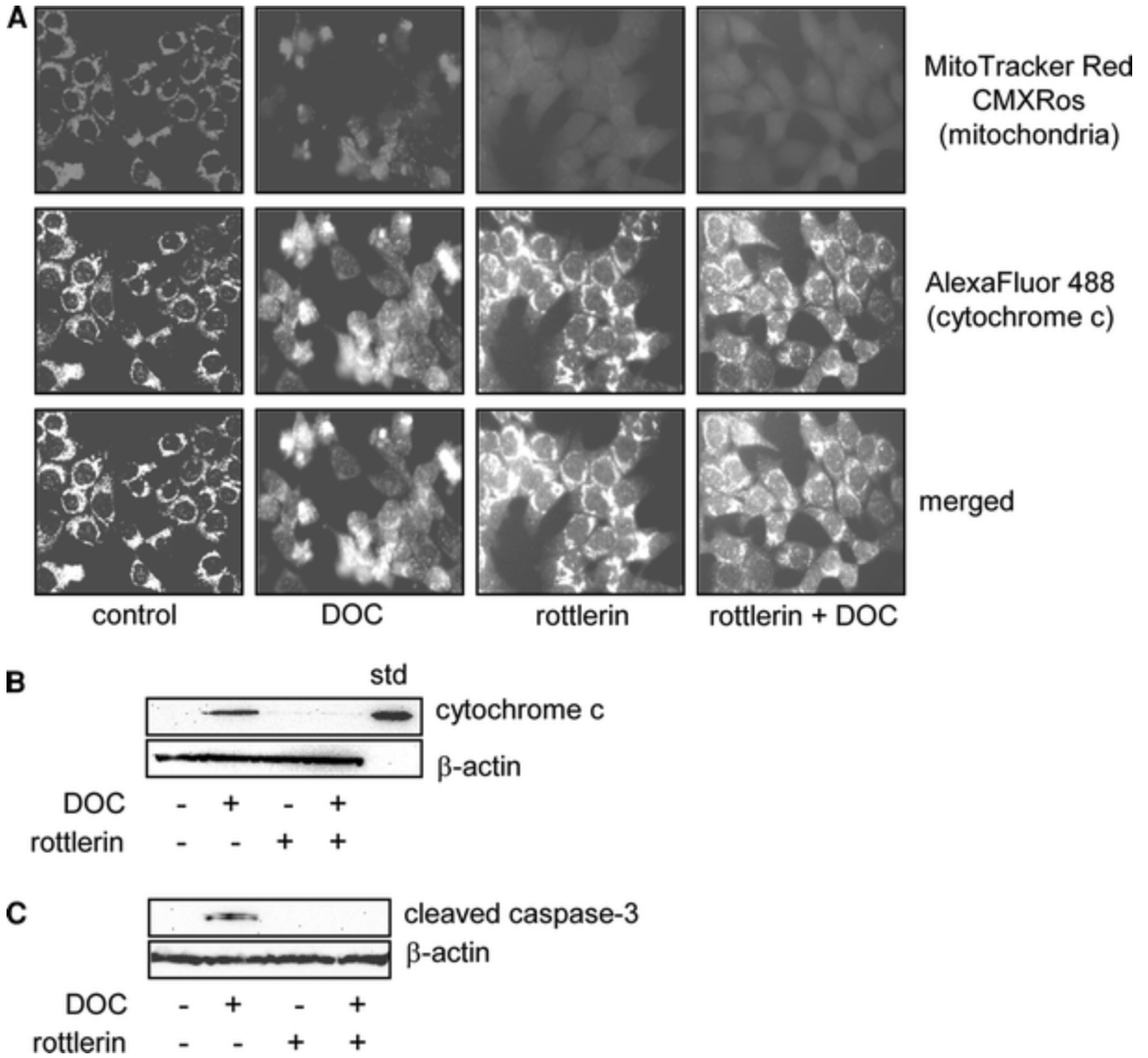

Fig. 3 Prevention by rottlerin of DOC-induced cytochrome c release and caspase-3 cleavage. HCT-116 cells were pre-treated with 20 μM rottlerin for 0.5 h and then exposed to 600 μM DOC for 1 h. Cells were stained with MitoTracker Red CMXRos to visualize active mitochondria, followed by immunocytofluorescence staining of cytochrome c (a). Additionally, cytosolic fractions and whole cell lysates were separately prepared from harvested cells for Western-blotting analysis of cytochrome c (b) and cleaved fragment of caspase-3 (c), respectively, with β-actin as a loading control. All images are representative of at least three different experiments

To complement the above data, Western-blotting analysis of the cytosolic fraction prepared from harvested cells was performed (Fig. 3b). The results revealed that cytochrome c was not present in the cytosol of control cells. In contrast, a band coinciding with purified cytochrome c was clearly detected in the cytosolic fraction from DOC-treated cells, but not rottlerin-treated cells or DOC-treated cells that had been pre-treated with rottlerin.

In determining if the above cytochrome c release is accompanied by caspase activation as assessed by Western-blotting analysis of whole cell extracts (Fig. 3c), a primary antibody that recognizes the 17/19 kDa proteolytic fragment of cleaved or activated caspase-3 was used. A band corresponding to the appropriate size was detected for DOC-treated cells, but not rottlerin-treated cells and also DOC-treated cells that had been pre-treated with rottlerin.

Examination of cells exposed to DOC and/or rottlerin for later features characteristic of apoptosis

When used at 600 μM, DOC caused morphological changes to HCT-116 cells that were consistent with programmed cell death. Membrane blebbing and formation of apoptotic bodies were prominent (Fig. 4a). Of much greater importance as can further be seen, pre-treatment of the cells with rottlerin prevented DOC from

causing these changes in morphology. Another feature supporting the actual occurrence of apoptosis in DOC-treated HCT-116 cells under our experimental conditions is the translocation of phosphatidylserine from the inner to outer leaflet of the plasma membrane (Fig. 4b). That is, more of the DOC-treated cells than the untreated control cells bound the annexin V-Alexa Fluor 488 fluorescence probe at the cell surface. Most importantly, however, cells pre-treated with 20 μM rottlerin and then treated with 600 μM DOC showed annexin V-Alexa Fluor 488 binding similar to the control cells. Moreover, as shown by other fluorescence images (Fig. 4c), the nuclei of a significant number of cells exposed to DOC contained condensed and/or fragmented chromatin, but not when DOC-treated cells were first pre-treated with rottlerin. More specifically (Fig. 4d), about 30% of DOC-treated cells had chromatin condensation/fragmentation, whereas the value dropped to less than 1% as a result of the rottlerin pre-treatment.

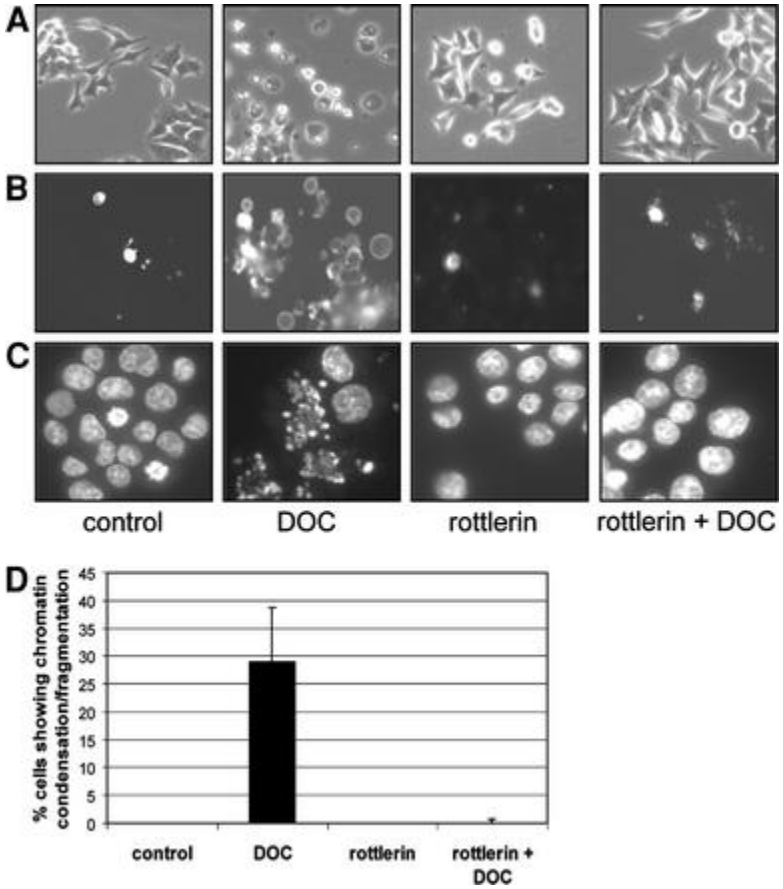

Fig. 4 Cytoprotective effect of rottlerin against DOC. HCT-116 cells were pre-treated with 20 μM rottlerin for 0.5 h and then exposed to 600 μM DOC for 3 h. The cells were examined by phase-contrast microscopy (a), assayed for annexin V-Alexa Fluor 488 binding (b), and stained with DAPI (c) that enabled counting of the cells having abnormal chromatin (d). The images are representative of at least three different experiments, and the bar graph values are the average ± SEM for three different experiments

To further assess the effect of rottlerin on HCT-116 cells exposed to DOC, similar experiments as above were performed but pre-treating the cells with a much lower concentration of rottlerin (0.25 μM) and then exposing them to only 300 μM DOC for either 3 h (Fig. 5a, b) or 24 h (Fig. 5c, d). Under these different experimental conditions, rottlerin was still able to noticeably reduce the occurrence of membrane blebbing and formation of apoptotic bodies (Fig. 5a, c), and also chromatin condensation/fragmentation (Fig. 5b, d), as caused by DOC.

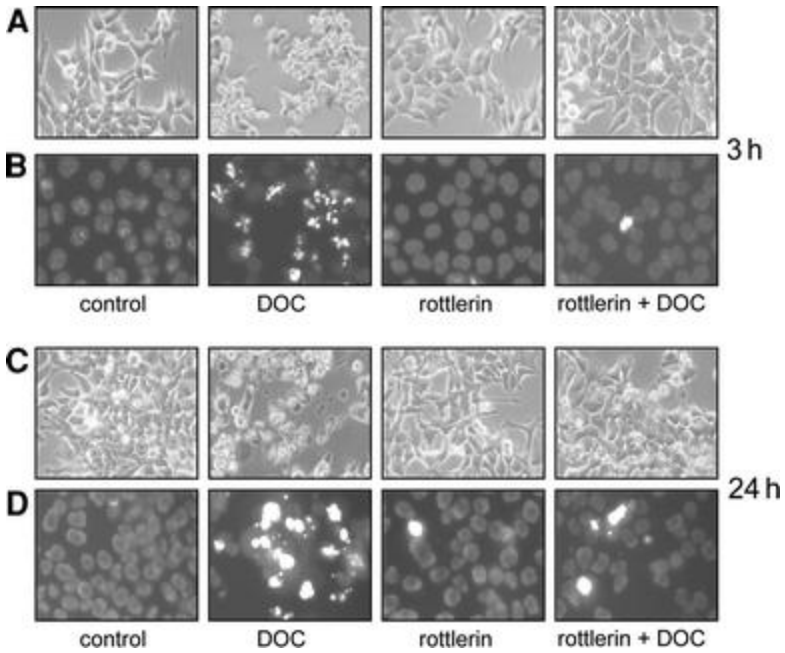

Fig. 5 Effects of lower concentrations of rottlerin and DOC on HCT-116 cells. The cells were pre-treated with 0.25 μM rottlerin for 0.5 h and then exposed to 300 μM DOC for either 3 h (a, b) or 24 h (c, d). The cells were examined by phase-contrast microscopy (a, c) and also stained with DAPI (b, d) to visualize the chromatin in nuclei. The images are representative of at least three different experiments

Evaluation of some other plant phenolic compounds for protective effects against DOC

The early findings (Fig. 1) that rottlerin can inhibit DOC-induced DNA damage, along with the observation that DOC increased ROS production, suggested that rottlerin has antioxidant activity. Because rottlerin's molecular structure, particularly its two phenolic ring systems, somewhat resembled that of some more recognizable and frequently studied phenolic phytochemicals (Fig. 6), a simple assay was performed to see if rottlerin in comparison to the five other depicted phenolic phytochemicals could scavenge the stable free radical, DPPH (Fig. 6). In principle, the spectrophotometric absorbance of the DPPH solution decreases if the test substance is able to scavenge DPPH. Thus, in this particular test for relative antioxidant activity, it turned out that EGCG had the greatest potency in scavenging DPPH. Quercetin was the next potent, followed by rottlerin and curcumin that were about equal. Resveratrol had little scavenging ability, whereas genistein had no activity under the experimental conditions.

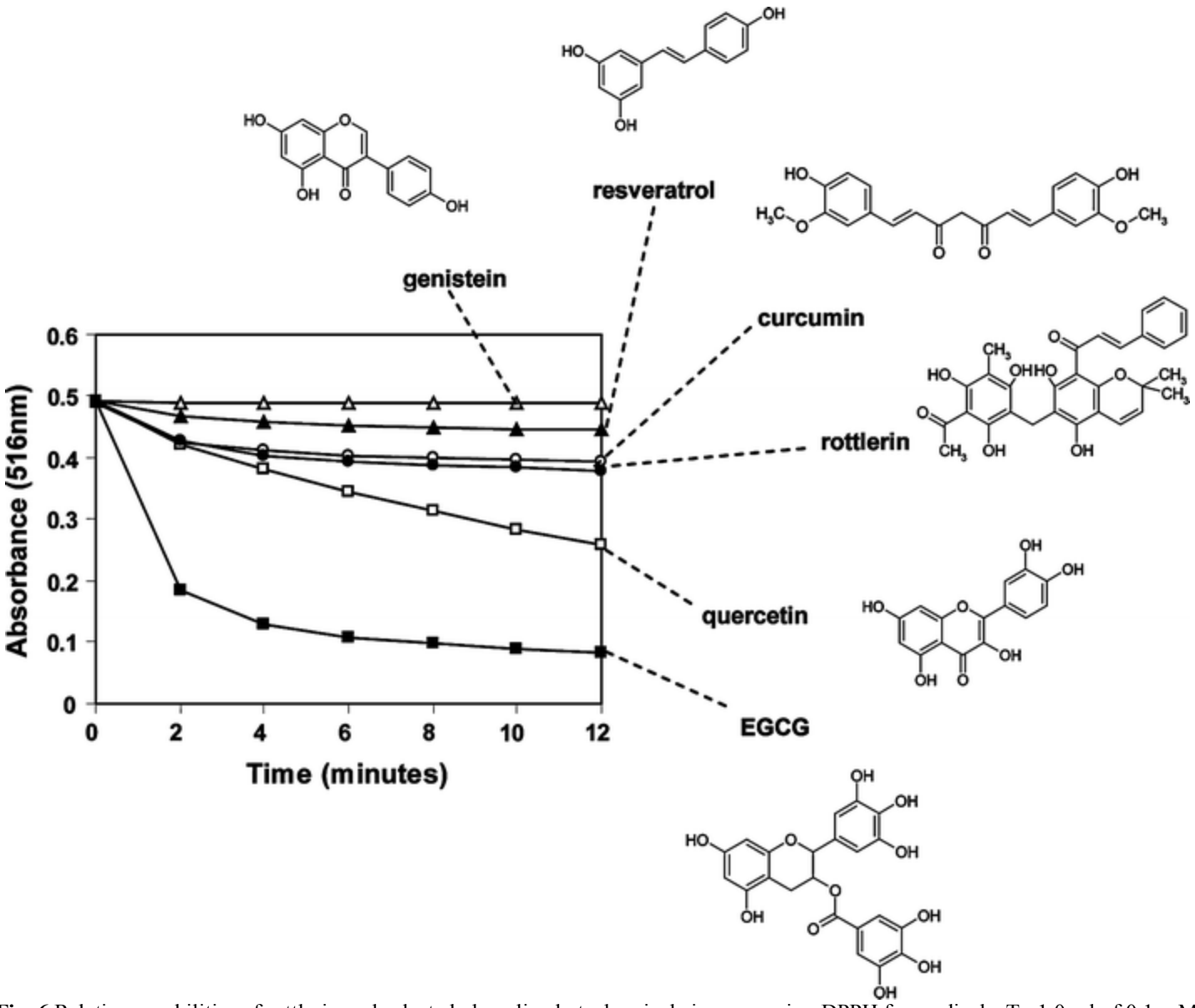

Fig. 6 Relative capabilities of rottlerin and selected phenolic phytochemicals in scavenging DPPH free radicals. To 1.0 ml of 0.1 mM DPPH was added 0.004 ml of 1.25 mM of rottlerin, quercetin, curcumin, epigallocatechin gallate (EGCG), reseveratrol, or genistein. Spectrophotometric analysis was then performed on the samples. Each of the 7 points making up each of the six distinct plots represents the average value for 4 different determinations. Two additional experiments with triplicate determinations but conducted for up to 30 min yielded very similar results in terms of relative DPPH scavenging capabilities of the test compounds

The results so far imply that rottlerin might protect HCT-116 cells against DOC by acting as an antioxidant. Therefore, because of the antioxidant activity found for most of the phenolic phytochemicals examined, we next evaluated them for protective effects against DOC in HCT-116 cells. However, at the same concentration (20 μM) that was initially used for testing of rottlerin, the five other phenolic phytochemicals were essentially ineffective in protecting the cells against DOC. Thus, the test concentration was increased to enable further evaluation of the phenolic phytochemicals, excluding rottlerin of course. A 5-fold higher concentration (100 μM) of two of them (quercetin and resveratrol) did produce some notable effects. As shown by the images in Fig. 7a, quercetin and resveratrol by themselves slightly changed the morphological appearance of HCT-116 cells. Nevertheless, quercetin prevented, and resveratrol attenuated, the incidences of membrane blebbing and formation of apoptotic bodies caused by DOC. Furthermore, as seen in Fig. 7b and c, quercetin prevented, and resveratrol attenuated, the incidences of chromatin condensation/fragmentation caused by DOC. On the other hand, 100 μM EGCG, genistein, and curcumin were each unable to protect HCT-116 cells against DOC-induced cytotoxicity (data not shown).

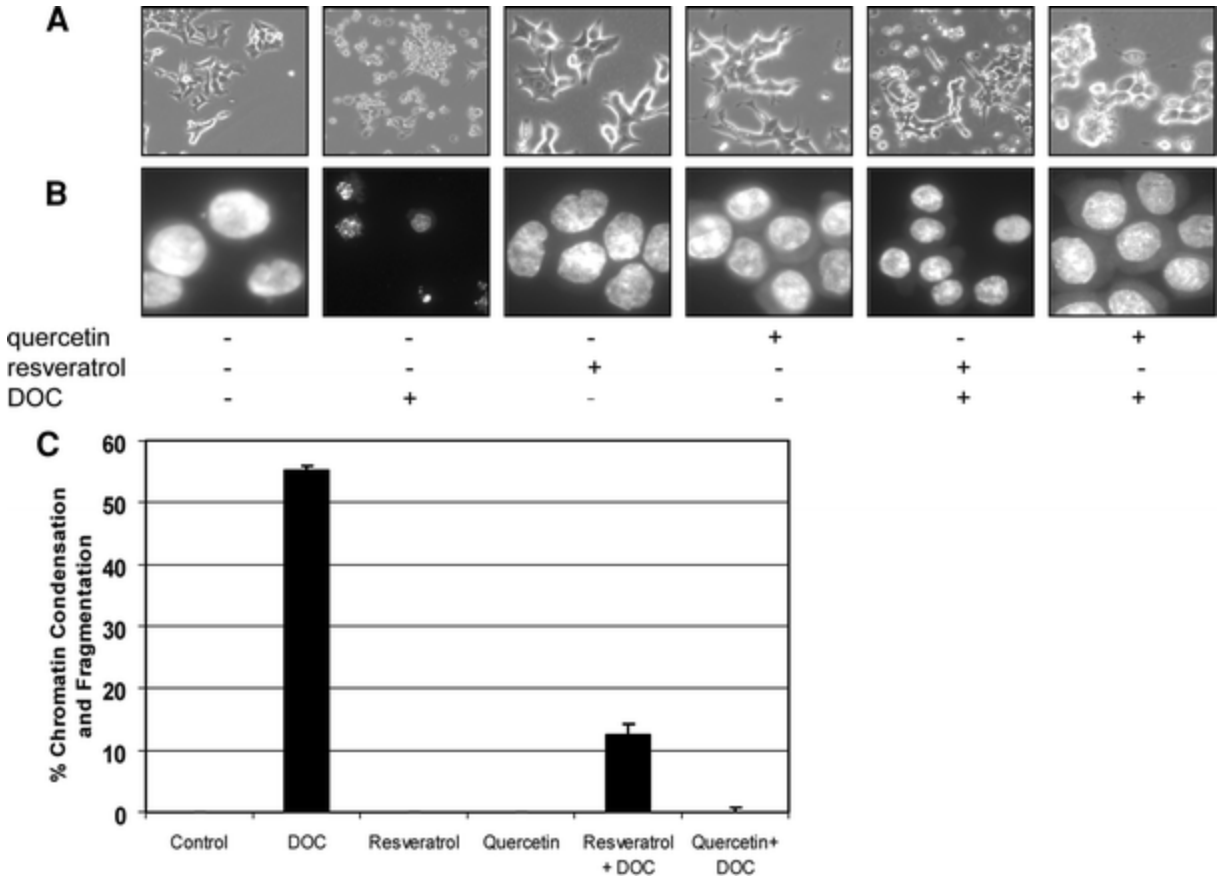

Fig. 7 Effect of quercetin and resveratrol on DOC-induced cytotoxicity. HCT-116 cells were pre-treated with 100 μM quercetin or resveratrol for 0.5 h and then exposed to 600 μM DOC for 3 h. The cells were examined by phase-contrast microscopy (a), and then, they were processed for nuclear staining with DAPI (b) that enabled counting of the cells having abnormal chromatin (c). The images are representative of at least three different experiments, and the bar graph values are the average ± SEM for three different experiments

When 100 μM quercetin and resveratrol were evaluated regarding their capabilities to protect against DOC-induced DNA damage, disparate results were obtained (Fig. 8a and b). Whereas resveratrol attenuated DOC-induced DNA damage, quercetin did not produce such an effect but by itself actually caused DNA damage. Interestingly, the most potent phenolic phytochemical tested in terms of DPPH-scavenging capacity, namely, EGCG, was unable to protect HCT-116 cells against the genotoxic effects of DOC, as was also found to be the case with curcumin and genistein (data not shown). The lack of a protective effect by EGCG, curcumin, and genistein against DOC-induced damage is consistent with their inabilities to stop DOC from killing the cells under the existing experimental conditions.

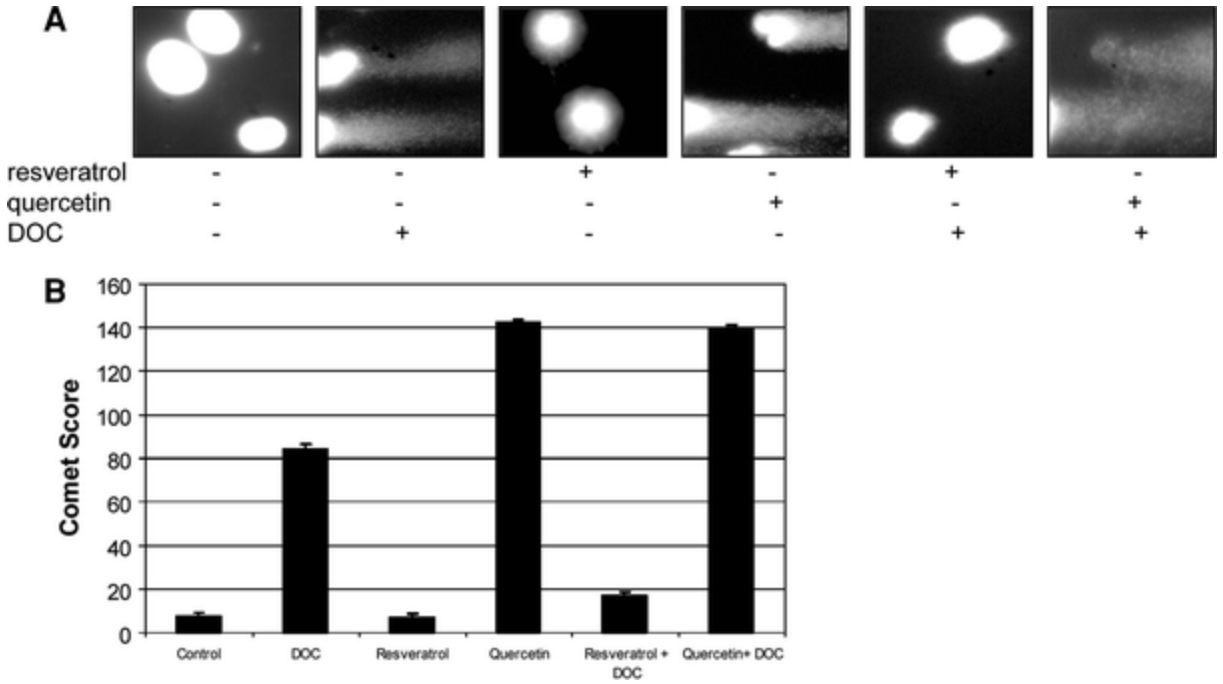

Fig. 8 Effect of quercetin and resveratrol on DOC-induced DNA damage. HCT-116 cells were pre-treated with 100 μM quercetin or resveratrol for 0.5 h and then exposed to 600 μM DOC for 1 h. The comet assay was performed with capturing of the fluorescence images (a) and scoring for comets (b). The images are representative of at least three different experiments, and the bar graph values are the average ± SEM for three different experiments

DISCUSSION

Certain bile acids are often studied to better understand the capability of some of them to adversely affect cells, so that perhaps one can identify ways to nullify the undesirable cellular effects of such bile acids. In particular, there is general interest in the toxic effects of DOC on colon epithelial cells. Consistent with earlier studies [6, 11, 12], DOC appeared to initiate programmed cell death in HCT-116 cells under our experimental conditions. The DOC-treated HCT-116 cells displayed several recognizable features characteristic of apoptosis, namely, surface membrane blebbing, breakup of the cells into small apoptotic bodies, surface membrane phospholipid translocation, and chromatin condensation/fragmentation. But, dramatically, rottlerin prevented these apoptotic features from appearing in DOC-treated HCT-116 cells.

In explaining the ability of rottlerin to stop the process of apoptosis caused by DOC, cytochrome c seems most important. Release of cytochrome c from mitochondria into cytosol is known to be a pivotal step for initiating caspase-mediated apoptosis [13]. The released cytochrome c enables assembly of the apoptosome, and consequently, caspase activation. In a recent study [14], exposing HCT-116 cells to DOC initially caused cytochrome c release and caspase-9 activation, which was followed by activation of caspase-3 and then caspase-8. In the present study, DOC caused release of cytochrome c from mitochondria into the cytosol, and also caused caspase-3 cleavage that is indicative of caspase-3 activation. But, as a main novel finding, rottlerin evidently prevented these two critical events from occurring. Interestingly, rottlerin alone caused a rather dramatic loss of MMP, which is consistent with several other studies [15–17], but no cytochrome c release into cytosol was detected within the timeframe of our experiments. Recently, it was reported that in HeLa cells rottlerin down-regulated protein expression of caspase-2, but not caspase-3, caspase-7, caspase-8, or caspase-9 [18]. However, it required at least 10 h of incubation for rottlerin to noticeably exert its effect on caspase-2.

Although rottlerin to a large extent clearly prevented the occurrence of apoptosis in HCT-116 cells exposed to DOC under the present experimental conditions, it should be noted that rottlerin by itself at micromolar concentrations can cause extensive apoptosis upon prolonged incubation with HCT-116 cells (data not shown). The apoptotic effect of rottlerin is also known from previous studies, but the requirement for protein kinase C (PKC) and caspases for cell death has been inconsistent. In one study with HL60 cells, Jurkat cells, and RAW

264.7 cells [16], rottlerin induced multiple features characteristic of apoptosis, including MMP depolarization and activation of caspase-3 and caspase-9. Plasmid-mediated overexpression of PKC-delta attenuated MMP depolarization and cell death in rottlerin-treated cells. However, in a study with HT1080 fibrosarcoma cells [19], rottlerin induced apoptosis and also autophagy. There was MMP depolarization, but caspases 3 and 9 were unaffected. Moreover, PKC-delta overexpression did not prevent cell death.

Exactly how rottlerin prevents DOC from initiating apoptosis in HCT-116 cells is not clear. But, at first glance, there is one explanation that appears to be reasonable involving PKC. Rottlerin was originally reported [8] to be a protein kinase inhibitor with some specificity for PKC but especially the isoform, PKC-delta. As a result, rottlerin has been widely used over the years with the historical assumption that this compound is a selective inhibitor of PKC-delta. In light of this information, it is also noteworthy that activation of PKC has been reported in DOC-treated HCT-116 cells [20] and DOC-treated human gastric epithelial cells undergoing apoptosis [21]. Moreover, DOC promotes nuclear translocation of PKC-delta [22], and PKC-delta is involved in apoptosis as has been reviewed [23]. Interestingly, rottlerin reduced the apoptotic effect of etoposide, which was shown to work through PKC-delta, in C6 glioma cells [24]. Hence, collectively, these above studies would conjure up the thought that the protective effect of rottlerin against DOC might be attributed primarily to rottlerin inhibiting PKC, or more specifically PKC-delta, thereby impeding a signaling pathway leading to apoptosis. However, there is a major problem with this line of thinking. It has been reported [25] that rottlerin does not inhibit PKC-delta activity but does inhibit the activity of several other protein kinases outside of the PKC family. Because of this and other unexpected effects, such as causing uncoupling of mitochondrial respiration [26], rottlerin is currently considered neither an appropriate nor effective PKC-delta inhibitor [27].

Bile acids create a state of oxidative and nitrosative stress, resulting in lipid peroxidation [5] and protein nitrosylation [28] due to the action of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Thus, it is probable that the DNA damage detected in DOC-treated HCT-116 cells can be attributed mainly to ROS and/or RNS. Antioxidants scavenge ROS and RNS, thereby helping to preserve genomic and cellular integrity. A recent study has reported that the antioxidant, ascorbate, can decrease the genotoxicity of DOC in oesophageal cells [29]. Other studies have reported that other antioxidants, namely, α-tocopherol, ebselen, and idebenone [30], as well as beta-carotene [31], decreased the number of rat hepatocytes undergoing apoptosis upon exposure to another bile acid known as glycochenodeoxycholic acid.

In the present study, rottlerin was shown to have antioxidant activity, more or less than a few other phenolic phytochemicals tested. Rottlerin was also able to largely protect HCT-116 cells against the genotoxic effects of DOC, markedly lessening the DNA damage caused by DOC. Thus, in regards to a possible mechanism of rottlerin in providing cytoprotection against DOC, it would seem logical to conclude that rottlerin may have acted to some extent as a direct antioxidant.

On the other hand, other findings suggest that rottlerin may have protected HCT-116 cells independent of an antioxidant effect. In support of this notion, EGCG and quercetin were shown to be more potent than rottlerin as a free radical scavenger, but were either ineffective or not as potent as rottlerin in protecting against DOC. Moreover, quercetin had weaker cytoprotective capability but better DPPH-scavenging capability than rottlerin. However, in complete contrast to rottlerin, quercetin alone caused substantial cellular DNA damage although this event did not result immediately in apoptosis under our experimental conditions. EGCG, which was the most potent phenolic phytochemical tested in terms of scavenging DPPH, was unable to protect HCT-116 cells against the genotoxic and cytotoxic effects of DOC. Compared to rottlerin, the disparate effects of quercetin and EGCG may be attributed to their abilities to react unexpectedly in the presence of cell culture media to generate ROS, namely hydrogen peroxide [32], which is to say that they can act as prooxidants rather than antioxidants depending on the experimental conditions. It was previously reported that relatively high concentrations of quercetin and EGCG can cause DNA damage in Jurkat T-lymphocytes [33]. In the case of resveratrol, it had some capacity to not only prevent DOC from damaging the DNA in HCT-116 cells but also from killing them. Yet, resveratrol was a relatively weak DPPH scavenger. Therefore, taken together, these data indicate that the effects of rottlerin, quercetin, and resveratrol against DOC cannot be explained by their antioxidant activities

alone. Besides, in light of the experiments where sub-micromolar rottlerin inhibited DOC-induced apoptosis, it would be hard to conclude that the effect of rottlerin could be attributed primarily to its moderate antioxidant activity.

In summary, rottlerin, quercetin and resveratrol have been identified as natural substances capable of decreasing to varying degrees the cytotoxic and/or genotoxic effects of DOC on colon epithelial cells. This knowledge might be useful in devising strategies to reduce the potentially harmful effects of high concentrations of bile acids present in certain abnormal conditions.

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