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MODULATION OF ADRIAMYCIN® ACCUMULATION AND EFFLUX BY FLAVONOIDS IN HCT-15 COLON CELLS

ACTIVATION OF P-GLYCOPROTEIN AS A PUTATIVE MECHANISM

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Abstract—Since P-glycoprotein (P-gp) in normal tissues may serve as a cellular defense mechanism against naturally occurring xenobiotics, we considered whether physiologically active components of commonly ingested plant foods could influence P-gp function. To examine this possibility, a series of flavonoids commonly found in plant foods was tested for their ability to modulate [^{14}C]Adriamycin® ([^{14}C]ADR) accumulation and efflux in P-gp-expressing HCT-15 colon cells. Many flavonoids, in the micromolar range, inhibited the accumulation of [^{14}C]ADR. Detailed experiments utilizing flavonoids with the greatest activity in reducing [^{14}C]ADR accumulation, i.e. galangin, kaempferol, and quercetin, revealed that the efflux of [^{14}C]ADR is increased markedly in the presence of these compounds. Flavonoid-induced stimulation of efflux was rapid and was blocked by the multidrug-resistant (MDR) reversal agents verapamil, vinblastine, and quinidine. The magnitude of flavonoid-stimulated efflux in sodium butyrate-treated cells with a 4-fold induction of P-gp protein was similar to that in uninduced cells. [^{3}H]Azidopine photoaffinity labeling of P-gp in crude membrane preparations revealed mild to no competition for binding by flavonoids possessing either activity or inactivity in reducing ADR accumulation. Although flavonoid hydrophobicity was found to be unrelated to flavonoid activity in altering [^{14}C]ADR accumulation, certain structural features were associated with enhancement or diminution of activity. Finally, the significance of flavonoid-related reduction of [^{14}C]ADR accumulation was underscored in cell growth studies, showing partial protection by quercetin against ADR-induced growth inhibition. It is concluded that certain naturally occurring plant flavonoids may acutely up-regulate the apparent activity of P-gp.

Key words: P-glycoprotein; flavonoids; quercetin; Adriamycin; HCT-15 cells

The transmembrane protein P-gp† is well-known for its involvement in the ATP-dependent efflux of a broad range of cytotoxic drugs from MDR tumor cells [1]. In addition to its prominent role in the MDR phenotype, P-gp is also expressed in many normal human tissues, including the brush border of the small and large bowels, adrenal cortex, kidney proximal convoluted tubules, epithelial cells of the pancreatic ducts, and brain and testicular endothelium [2]. Accordingly, one of the postulated physiological functions of P-gp is the clearance of naturally occurring cytotoxic compounds from epithelial cells [3, 4]. Indeed, P-gp induction has been shown to accompany carcinogen exposure in preneoplastic liver nodules in rodents [5], and recent evidence directly implicates P-gp in mediating the efflux of benzo[*a*]pyrene and dimethylbenz[*a*]-anthracene from ADR-resistant human breast MCF-7 cells [6, 7]. Hence, in certain tissues P-gp may constitute an important component of cellular defenses against naturally occurring carcinogens.

Due to the potential importance of P-gp in cellular defense against environmental carcinogens [6, 7], more information is needed regarding the regulation of P-gp expression and activity. To date, research on P-gp has emphasized the quest for agents that reverse the MDR phenotype, because these agents may be useful for cancer chemotherapy. An array of compounds that interfere with P-gp activity has been discovered, thereby increasing the accumulation of chemotherapy drugs and increasing cytotoxicity [8–11]. In contrast, stimulation of P-gp function has received little attention. Although P-gp expression can be increased by differentiating agents [12, 13], heat shock [14, 15] and chemical insults [16], the increased expression does not necessarily translate into function as indicated by increased drug efflux or decreased drug accumulation and cytotoxicity [12, 17]. Clearly, the identification of naturally occurring effectors of P-gp is relevant to the understanding of the role of P-gp in normal cell physiology.

One important source of potential P-gp effectors is the dietary milieu to which P-gp-expressing intestinal epithelial cells are directly exposed. Human and animal diets that include significant amounts of plant foods contain not only essential nutrients, but also a complex array of non-nutrient plant natural products, indigestible materials, and potentially

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† Abbreviations: P-gp, P-glycoprotein; ADR, Adriamycin®; MDR, multidrug-resistant; and DMEM, Dulbecco's modified Eagle's medium.

harmful compounds. The flavonoids, a group of over 4000 polyphenolic, widely substituted polycyclic compounds that occur to an extent of approximately 1 g per day in western diets, are prominent constituents of this milieu [18]. Due to their ubiquitous distribution in human diets, their wide range of physiological effects [19], their low toxicity, their often-hydrophobic nature, and their chemopreventative activity [20], flavonoids were examined in the present study to determine possible interactions with P-gp functional activity. These studies showed that the flavonoids quercetin, kaempferol, and galangin are potent stimulators of ADR efflux in HCT-15 colon cells, apparently via a P-gp-mediated mechanism.

MATERIALS AND METHODS

Reagents and materials. The following compounds were purchased from the Sigma Chemical Co. (St. Louis, MO): vinblastine, quinidine, (±)-verapamil, flavone, quercetin, naringin, naringenin, rutin, morin, chrysin, (+)-catechin, (−)-epicatechin, phlorizdin, phloretin, biochanin A, hesperidin, hesperetin, rhoifolin, myricetin, diosmin, taxifolin, kaempferol, apigenin, genistein, ADR and sulforhodamine B. Chemicals obtained from the Aldrich Chemical Co. (Milwaukee, WI) included galangin, chalcone, and fisetin. Amersham (Arlington Heights, IL) was the supplier of [^{14}C]ADR HCl (sp. act. 50–60 mCi/mmol) and [^{3}H]azidopine (sp. act. 48 Ci/mmol). HCT-15 colon carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD).

Accumulation and efflux of [^{14}C]*ADR.* HCT-15 cells, passages 5–20 in our hands, were grown at 37° in a humidified 5% CO_2 atmosphere in DMEM supplemented with 15% fetal bovine serum. For [^{14}C]ADR accumulation and efflux experiments, cells were seeded in 6-well plates at a density of approximately 10^6 cells per well, which resulted in confluency after 3 days. Experiments were performed on confluent cultures 3 or 4 days following plating. For accumulation studies, cultures were exposed to [^{14}C]ADR (final concentration 0.9 μM, at 0.05 μCi/mL of medium) in fresh medium for up to 2 hr. Flavonoids (dissolved in DMSO) were added to cultures at the same time as, or 30 min prior to, [^{14}C]ADR. The final concentration of flavonoids was 100 μM for most experiments, and the final concentration of DMSO 0.5% (v:v) for all experiments and controls. Following accumulation of [^{14}C]ADR cells were washed twice in cold PBS (pH 7.4) without calcium and magnesium, and then harvested in 1.0% SDS (w:v) for liquid scintillation counting. For efflux experiments, cells were loaded for 60 min with [^{14}C]ADR in the absence of flavonoids, and then the medium was replaced with medium containing flavonoids, MDR reversal agents, combinations of the two, or the vehicle (DMSO). Following efflux intervals of up to 30 min, the medium was removed and the cells were washed twice with cold PBS and prepared for liquid scintillation counting (as above).

Cell growth and ADR cytotoxicity. HCT-15 cells were plated at a density of 5000 cells per well in 96-well plates, using DMEM with 15% fetal bovine serum. After 24 hr, additional medium was added which provided ADR at final concentrations up to 3.0 μM, with or without flavonoids at 50 μM. At 24-hr intervals, plates were collected and cell density was measured using a sulforhodamine B staining procedure [21]. Briefly, the cells were fixed with cold 10% trichloroacetic acid, rinsed five times with water, and allowed to air dry. Staining was with 0.4% (w:v) sulforhodamine B in 1.0% acetic acid, followed by four washes with 1.0% acetic acid and air drying. The bound stain was then solubilized with 10 mM unbuffered Tris base and quantitated by reading the absorbance at 570 nm.

Sodium butyrate exposure and western blotting. HCT-15 cells were plated and then grown to confluency in 6-well plates in the presence or absence of 2 mM sodium butyrate. In the case of sodium butyrate, cells were seeded at twice the cell number so that cell density after 3 days would be comparable to that of the control. After 3 days of growth, cells were exposed to [^{14}C]ADR and assessed for accumulation and efflux activity (as described above) under control conditions and in the presence of flavonoids or MDR reversal agents. Corrections for actual cell density after 3 days of growth were made using the staining procedure referred to above [21]. Cells in other wells were harvested and analyzed via western blotting for their content of P-gp. First, crude membranes were electrophoresed (SDS–PAGE, 7.5%) and electroblotted onto PVDF membrane (Immobilon, Millipore, Bedford, MA). The membrane was blocked with nonfat dry milk, exposed to an anti P-gp antibody (C219, Centocor Diagnostics, Malvern, PA), treated with goat anti-mouse conjugated to horseradish peroxidase, and then developed using enhance chemiluminescence (ECL, Amersham). Quantitation of the film image was achieved by scanning densitometry.

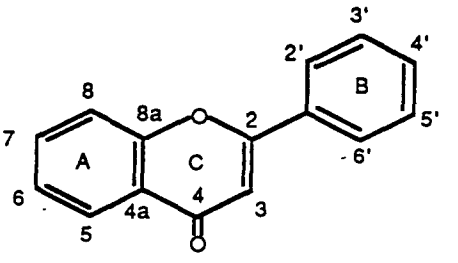
Photoaffinity labeling of P-gp. Crude membranes from HCT-15 cells were isolated and photoaffinity labeled with [^{3}H]azidopine essentially as described by Yang *et al.* [22]. Reaction mixtures were 50 μL, and contained 100 μg protein. Flavonoids were added prior to [^{3}H]azidopine addition, using a 200-fold molar excess relative to total azidopine (100 and 0.5 μM, respectively). Following a 1 hr incubation at 23° in the dark, the samples were irradiated at 254 nm (Spectronics Corp, Westbury, NY, model ENF-280C) on ice for 15 min. The labeled preparations were subjected to SDS–PAGE (4–20%), and the gels were prepared for fluorography with preflashed Kodak X-omat AR film.

Thin-layer chromatography. For the estimation of flavonoid hydrophobicity, compounds were resolved on silica gel plates (Analtech, silica gel G) with an ascending solvent system consisting of chloroform : ethyl acetate : acetic acid (16 : 8 : 1). Plates were pre-run with solvent and allowed to dry prior to sample application. Resolved flavonoids were visualized using $CuSO_4$/phosphoric acid and then heat.

RESULTS

Flavonoid modulation of [^{14}C]*ADR accumulation,*

Table 1. Flavonoid structures and flavonoid modulation of [^{14}C]ADR accumulation over a 2 hr period



Flavone

Class/name	2,3 bond	Substitutions relative to flavone*	Accumulation of [^{14}C]ADR† (% of DMSO control)
Flavones	Unsat		
Flavone		None	99.3 ± 2.1
Chrysin		5-OH, 7-OH	98.6 ± 1.6
Apigenin		5-OH, 7-OH, 4'-OH	54.0 ± 1.6
Rhoifolin		5-OH, 7-OHes, 4'-OH	67.9 ± 1.0
Diosmin		5-OH, 7-ORut, 3'-OH, 4'-OCH_3	157.4 ± 2.5
Flavonols	Unsat		
Galangin		3-OH, 5-OH, 7-OH	40.1 ± 1.7
Fisetin		3-OH, 7-OH, 3'-OH, 4'-OH	49.2 ± 2.5
Morin		3-OH, 5-OH, 7-OH, 2'-OH, 4'-OH	70.9 ± 1.7
Quercetin		3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH	42.8 ± 2.1
Rutin		3-ORut, 5-OH, 7-OH, 3'-OH, 4'-OH	64.5 ± 1.3
Kaempferol		3-OH, 5-OH, 7-OH, 4'-OH	41.6 ± 1.5
Myricetin		3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH, 5'-OH	75.6 ± 2.6
Flavanones	Sat		
Taxifolin		3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH	92.2 ± 2.1
Naringenin		5-OH, 7-OH, 4'-OH	76.9 ± 1.4
Naringin		5-OH, 7-ORha, 4'-OH	88.2 ± 4.0
Hesperetin		5-OH, 7-OH, 3'-OH, 4'-OCH_3	73.5 ± 2.1
Hesperidin		5-OH, 7-ORha, 3'-OH, 4'-OCH_3	87.9 ± 2.3
Chalcones	Unsat		
Chalcone		None	109.0 ± 2.3
Phloretin		5-OH, 7-OH, 8a-OH, 4'-OH	76.5 ± 2.0
Phlorizdin		5-OGlu, 7-OH, 8a-OH, 4'-OH	95.0 ± 1.0
Isoflavones	Unsat		
Genistein		5-OH, 7-OH, 4'-OH	67.6 ± 2.2
Biochanin A		5-OH, 7-OH, 4'-OCH_3	88.5 ± 1.4
Flavanols	Sat		
Catechin		3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH	95.5 ± 1.7
Epicatechin		3-OH, 5-OH, 7-OH, 3'-OH, 4'-OH	98.1 ± 1.9
Verapamil			179.1 ± 3.2
Vinblastine			163.4 ± 6.1

* Hes, hesperodiside; Rut, rutinoside; Rha, rhamnoglucoside; and Glu, glucose.

† HCT-15 cells were treated with 100 μM flavonoid or verapamil or vinblastine (DMSO as control) for 30 min, and then exposed to [^{14}C]ADR for 2 hr during continued exposure to the test compounds. Accumulation of [^{14}C]ADR in the presence of these compounds is expressed as a percentage of the control value. Values are the means ± SEM from four separate experiments.

and flavonoid structures. Chemical structures of the flavonoids and their activity in modulating [^{14}C]-ADR accumulation are shown in Table 1. Structures are based on the unsubstituted parent compound flavone, and are representative of six classes of flavonoids: flavones, flavonols, isoflavones, flavanones, chalcones, and flavanols. Each flavonoid (100 μM) was present 30 min prior to, and during the 2 hr of exposure to [^{14}C]ADR. Many flavonoids were found to inhibit the net accumulation of [^{14}C]-ADR, with the most active compounds being members of the flavonol class: galangin, kaempferol, and quercetin. Each of these flavonoids inhibited [^{14}C]ADR accumulation to a level of approximately 40% of the control. Structural properties enhancing this activity included hydroxylation at the 3 position and desaturation of the 2,3 bond. In contrast, hydroxylation at the 2' or 5' position, or the addition of a carbohydrate to the 3-hydroxyl, was associated with a marked reduction in apparent activity.

Kinetics and concentration dependence of flavonoid-induced reduction of ADR accumulation. The

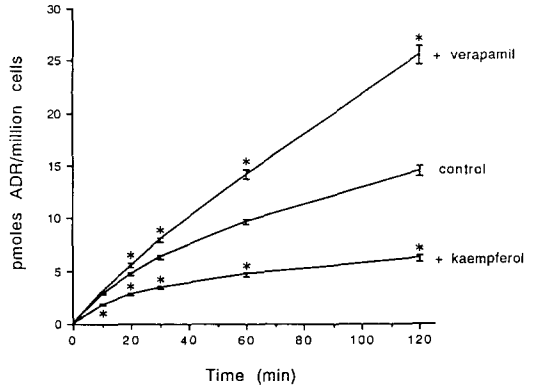


Fig. 1. Effect of kaempferol and verapamil on accumulation of [^{14}C]ADR. Cells were exposed for up to 2 hr to [^{14}C]-ADR plus either kaempferol (100 μM), verapamil (100 μM) or DMSO (0.5%) as control, and then were assessed for the accumulation of cellular radioactivity. Radioactivity was converted to actual picomoles ADR, and expressed per million cells. Data represent the means ± SEM of four experiments. Asterisks denote a difference from the control value at $P < 0.05$, using a two-tailed t-test.

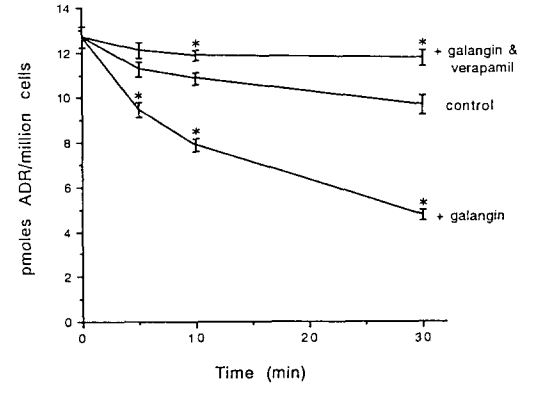


Fig. 2. Efflux of [^{14}C]ADR from HCT-15 cells as modified by galangin. Cells were loaded with [^{14}C]ADR for 1 hr, and then treated with fresh medium containing galangin (100 μM), galangin plus verapamil (100 μM each), or control (0.5% DMSO). Cellular radioactivity was determined at the indicated time points, and expressed as picomoles ADR per million cells. Values are means ± SEM of three experiments. Asterisks denote a difference from the control value at $P < 0.05$, using a two-tailed t-test.

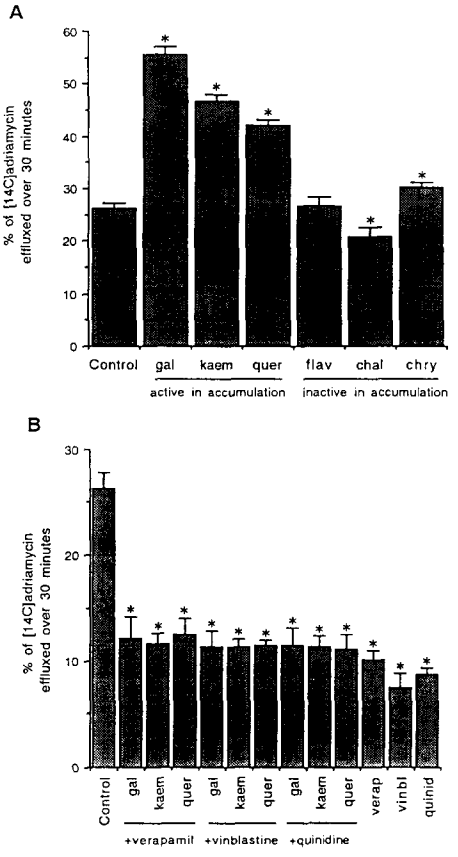


Fig. 3. (A) Effect of accumulation-active versus accumulation-inactive flavonoids (Table 1) on the efflux of [^{14}C]-ADR. HCT-15 cells were loaded with [^{14}C]ADR for 1 hr, and then allowed to efflux for 30 min in the presence of flavonoids (100 μM) or DMSO (0.5%) as control. Values (means ± SEM from four experiments) are expressed as a percentage of accumulated cellular radioactivity effluxed over the 30-minute period. Asterisks denote a difference from the control value at $P < 0.05$, using a two-tailed t-test. Abbreviations: gal, galangin; kaem, kaempferol; quer, quercetin; flav, flavone; chal, chalcone; and chry, chrysin. (B) Effect of combining MDR reversal agents with flavonoids that stimulated [^{14}C]ADR efflux as shown in panel A. After the cells were loaded with [^{14}C]ADR for 1 hr, they were allowed to efflux into medium containing combinations of individual flavonoids (100 μM) and individual reversal agents (verapamil, vinblastine, and quinidine, all at 100 μM). Some cells received the reversal agents only (100 μM). As in panel A, values (means ± SEM from four experiments) are expressed as a percentage of accumulated cellular radioactivity effluxed over the 30-min period. Asterisks denote a difference from the control value at $P < 0.05$, using a two-tailed t-test. Abbreviations: gal, galangin; kaem, kaempferol; quer, quercetin; verap, verapamil; vinbl, vinblastine; and quinid, quinidine.

kinetics of [^{14}C]ADR accumulation in the presence or absence of active flavonoids were determined, with the effect of kaempferol shown in Fig. 1. In this case, kaempferol was used without a 30-min preincubation period since the effect was found to take place nearly immediately. Thus, kaempferol attenuated [^{14}C]ADR accumulation early in the course of the 2-hr period, with a measurable difference from the control at 10 min post-exposure to a combination of [^{14}C]ADR and 100 μM kaempferol. As a positive control, the MDR reversal agent verapamil (100 μM) was tested and found to effectively increase the time-dependent accumulation of [^{14}C]ADR relative to the control (Fig. 1).

Flavonoid- and MDR reversal agent-modulation of [^{14}C]ADR efflux. Experiments measuring efflux of [^{14}C]ADR from HCT-15 cells revealed acceleration of efflux by the same flavonoids that were active in

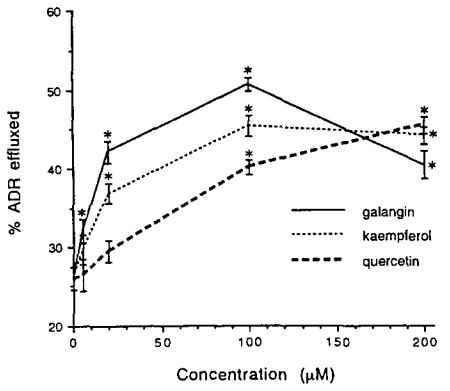


Fig. 4. Concentration dependence of flavonoid-stimulated [^{14}C]ADR efflux. Following a 1 hr accumulation of [^{14}C]-ADR, cells were exposed to medium containing 0–200 μM galangin, kaempferol, or quercetin. Data are expressed as percent [^{14}C]ADR effluxed during a 30-min period. Values are means ± SEM of four experiments. Asterisks denote a difference from the control level of efflux, that is, the level of efflux with no flavonoid addition (0 μM flavonoid). The difference reflects a P value of <0.05, using a two-tailed *t*-test.

attenuating accumulation. For example, Fig. 2 shows that [^{14}C]ADR efflux was accelerated in the presence of galangin-containing medium in comparison to control medium. Also shown in Fig. 2 is the ability of verapamil to block completely galangin-related stimulation of [^{14}C]ADR efflux. To get a broader view of flavonoid stimulation of [^{14}C]ADR efflux, additional efflux experiments were conducted using a 30-min fixed time interval for efflux. It was found that the accumulation-active flavonoids galangin, kaempferol, and quercetin each stimulated [^{14}C]-ADR efflux relative to the control (Fig. 3A). In contrast, the accumulation-moderate to inactive flavonoids flavone, chalcone, and chrysin did not alter [^{14}C]ADR efflux.

However, a direct demonstration that flavonoid stimulation of ADR efflux through a P-gp-mediated mechanism is essential. We further examined the P-gp-mediated efflux of ADR by flavonoids in MDR human breast cancer MCF-7 cells derived against ADR with high P-gp-expressing and the drug-sensitive, none P-gp-expressing MCF-7 wild-type (WT) cells [6, 7]. In MCF-7 WT cells, flavonoids had no effect on [^{14}C]ADR efflux. However, in MDR cells (R65, 65-fold resistant to ADR), a marked effect was seen with flavonoids. The ratio of ADR efflux in R65/WT was increased from 4.7 to 7.6 with galangin at a 100 μM concentration. We also found that kaempferol stimulated the efflux of ADR in MDR cells but not in WT. At a 50 μM concentration of kaempferol, a 2-fold increase in ADR (5 μM) efflux in MDR but no effect in WT cells was observed by Adherent Cell Analysis and Sorting measurement [23]. Thus, in an established MDR cell line, the flavonoid effect on ADR efflux was dependent on the expression of P-gp.

To determine the relevance of this stimulation to P-gp, we examined the effects of combining each of three flavonoids with each of three reversal agents (Fig. 3B). The stimulation of [^{14}C]ADR efflux provided by galangin, kaempferol, or quercetin was eliminated completely by the concurrent addition of either verapamil, vinblastine, or quinidine. Moreover, these combinations resulted in a level of efflux that was nearly as low as that seen in the presence of the MDR reversal agents alone. The stimulation of [^{14}C]ADR efflux by these flavonoids

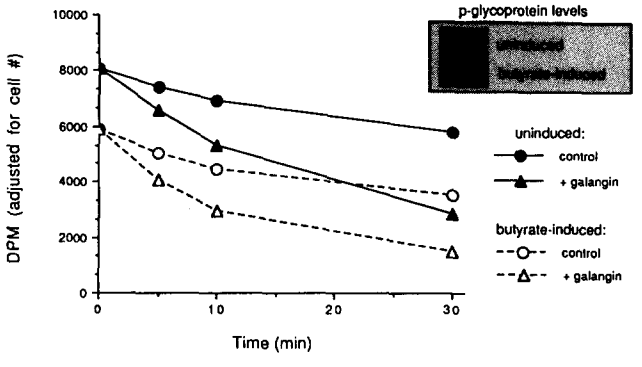


Fig. 5. Efflux of [^{14}C]ADR from HCT-15 cells induced with sodium butyrate, compared with that from uninduced cells. Cells were exposed to 2 mM sodium butyrate for 3 days, beginning from the time of plating. Relative to control cells, butyrate-treated cells were plated at twice the density so that the final cell density after 3 days would be similar. After 3 days of growth, all cells were exposed to [^{14}C]ADR for 1 hr, followed by efflux of [^{14}C]ADR for up to 30 min (see Materials and Methods). The values for the induced cells were normalized to correspond to the protein content of uninduced cells, as measured by sulforhodamine B staining (see Materials and Methods). Inset of P-glycoprotein levels shows immunoblots of membrane preparations from butyrate-induced and uninduced cells (10 μg protein each). These results represent a typical result from one of two experiments done in duplicate (both experiments demonstrated virtually identical results).

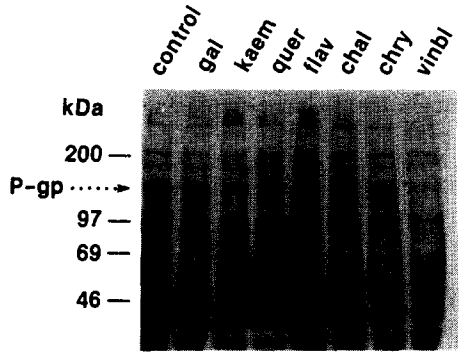


Fig. 6. Effect of flavonoids on [^{3}H]azidopine labeling of crude membranes. Photoaffinity labeling of P-glycoprotein was carried out in the presence of a 200-fold molar excess of flavonoids relative to [^{3}H]azidopine, using DMSO and vinblastine (200-fold) as controls. Abbreviations: gal, galangin; kaem, kaempferol; quer, quercetin; flav, flavone; chal, chalcone; chry, chrysin; and vinbl, vinblastine.

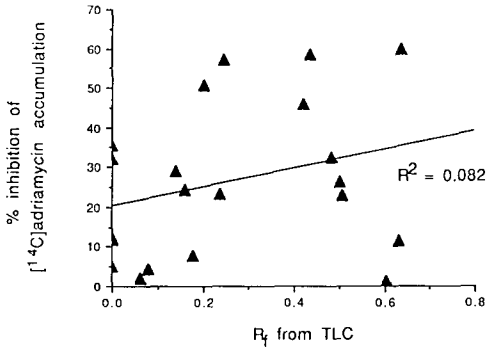


Fig. 7. Relationship of flavonoid hydrophobicity to activity in inhibiting [^{14}C]ADR accumulation. Flavonoids were resolved using thin-layer chromatography, and their R_f values were taken as an estimate of hydrophobicity. R_f values are plotted against inhibitory activity derived from Table 1.

was also found to be concentration dependent, with a measurable response at 20 μM (Fig. 4). The maximal response for kaempferol and galangin was at approximately 100 μM, while the quercetin-induced response continued upward beyond 100 μM.

[^{14}C]*ADR accumulation and efflux in cells with butyrate-induced P-gp augmentation.* As determined by western blotting, P-gp levels in HCT-15 cells were elevated approximately 4-fold in response to a 3-day exposure to 2 mM sodium butyrate (Fig. 5, inset). Concomitant to this induction was 25% less accumulation of [^{14}C]ADR, but virtually the same efflux pattern for [^{14}C]ADR was observed (Fig. 5). Cells induced to overexpress P-gp showed a similar magnitude of [^{14}C]ADR efflux compared with the uninduced cells, and efflux was stimulated to approximately the same extent by galangin. Verapamil also displayed similar efflux-blocking activity in butyrate-induced and uninduced cells (data not shown).

Photoaffinity labeling of P-gp, and the relationship of flavonoid hydrophobicity to activity. Active and moderate to inactive flavonoids were tested for their ability to compete with or enhance [^{3}H]azidopine photoaffinity labeling of P-gp in crude membrane preparations (Fig. 6). Both groups of flavonoids displayed mild to no competition with [^{3}H]azidopine binding. Hence, the interaction of flavonoids with P-gp photoaffinity labeling appeared to be unrelated to their activity in stimulating ADR efflux or reducing its accumulation. We further examined whether activity in modulating [^{14}C]ADR accumulation is a function of flavonoid hydrophobicity by resolving flavonoids using thin-layer chromatography and taking their R_f values as an index of hydrophobicity. A plot of the R_f values against activity in lessening [^{14}C]ADR accumulation showed a lack of relationship between flavonoid hydrophobicity and activity (Fig. 7).

Effect of flavonoids on ADR cytotoxicity. The growth inhibition of HCT-15 cells was determined

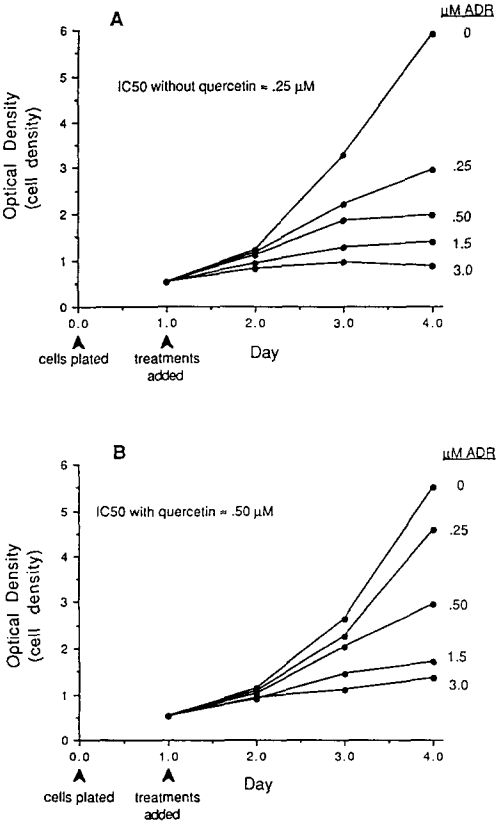


Fig. 8. Partial protection against ADR toxicity afforded by quercetin. HCT-15 cells were plated in 96-well plates and, after 24 hr were treated with 0–3 μM ADR without (A) or with (B) 50 μM quercetin. Using sulforhodamine B staining, cell density was determined at 24 hr intervals for 3 days. The IC_{50} was estimated from cell density values at day 4 of growth.

in response to increasing concentrations of ADR with or without added quercetin, galangin, or kaempferol (50 μM). Figure 8 shows growth resulting from ADR exposure alone (panel A) or ADR plus 50 μM quercetin (panel B). The addition of quercetin was associated with elevated cell number (decreased toxicity) over the range of concentrations of ADR tested (0.25 to 3.0 μM), with an approximate doubling of the IC_{50} when quercetin was present ($\approx$0.50 μM vs $\approx$0.25 μM). The IC_{50} was estimated from values obtained at day 4 of growth. Analogous results were obtained with galangin and kaempferol, where protection against ADR was particularly evident at the high concentration of ADR (data not shown). However, it should be noted that the flavonoids alone, at 50 μM, caused measurable growth inhibition. Comparing the two 0 μM ADR curves in Fig. 8, it can be seen that this effect of quercetin was small but noticeable. The growth inhibition caused by 50 μM kaempferol or galangin was greater than that seen for quercetin (data not shown). In spite of measurable growth inhibition by all three of these flavonoids, they still conferred a decreased cytotoxicity of ADR.

DISCUSSION

Although P-gp-mediated efflux of drugs is well accepted, the functional role of P-gp in normal tissues has not been resolved. It has been suggested that P-gp may function as an efflux mechanism for protection against environmental carcinogens; we recently provided direct experimental support of this concept [6, 7], finding that MDR human breast cancer MCF-7 cells efflux benzo[*a*]pyrene and dimethylbenz[*a*]anthracene via a P-gp-dependent mechanism. In the present study, we sought to determine whether P-gp activity in HCT-15 colon cells could be modulated by naturally occurring plant-derived dietary factors such as flavonoids. HCT-15 colon cells were chosen because they express measurable levels of P-gp [12, 24] and have a high level of intrinsic drug resistance that is blocked by low concentrations of the MDR reversal agent verapamil [24].

These experiments revealed that the flavonoids quercetin, kaempferol, and galangin potently reduce the accumulation, and stimulate the efflux, of ADR in HCT-15 human colon cells. The inhibition of accumulation was rapid, i.e. no preincubation period was necessary, and there was a concentration dependence showing activity in the low micromolar range (Fig. 2). It appears that the reduced ADR accumulation in response to flavonoids is due to acceleration of ADR efflux. As in the case of ADR accumulation, the stimulation of ADR efflux by flavonoids was virtually immediate. The involvement of P-gp in this stimulation of efflux is supported not only by the presence of P-gp in these cells [12], but more compellingly by the finding that flavonoid-stimulated efflux is blocked completely by any of three MDR reversal agents that were tested: verapamil, vinblastine, and quinidine (Fig. 3B). That is, in an experiment using three flavonoids and three reversal agents, the stimulation of efflux by each of the flavonoids was blocked by each of the reversal agents.

The potential functional importance of this newly identified flavonoid–ADR relationship was illustrated by the fact that quercetin partially protected cells against ADR cytotoxicity. Presumably, this protective effect is afforded by the reduction in cellular ADR accompanying flavonoid exposure. In contrast to the protection against ADR afforded by quercetin reported here, several other studies have reported enhancement of chemotherapeutic drug effectiveness in the presence of quercetin *in vitro*, but the mechanism remains unclear. Thus, enhancement by quercetin was observed for busulphan [25], *cis*-diammine-dichloroplatinum(II) [26], and cytosine arabinoside [27], drugs that are not generally considered to be substrates of P-gp. In contrast, the protection against ADR by flavonoids described here may be specifically due to the efflux of ADR by P-gp, and the apparent stimulation of P-gp activity by flavonoids. These observations on relationships between quercetin and different pleiotropic drugs for cancer chemotherapy may be relevant to the clinical management of multidrug resistance. For example, the withholding of dietary flavonoids in cases where the chemotherapeutic drug of choice is a substrate of P-gp could potentially enhance the efficacy of the drug.

Butyrate induction of P-gp expression was chosen as an additional tool to study the linkage between P-gp and flavonoid-stimulation of ADR efflux since butyrate treatment of HCT-15 cells has been shown to result in production of functional P-gp [12]. Functionality was inferred from decreased [^{3}H]-vinblastine accumulation, which was reversible with verapamil, rather than increased efflux [12]. We reasoned that the presence of increased levels of functional P-gp would result in greater baseline efflux of ADR and greater stimulation by flavonoids. In fact, baseline efflux and galangin-stimulated efflux appeared to be unchanged relative to the uninduced state (Fig. 5). This finding is in the face of 4-fold more P-gp and 25% less ADR accumulation in butyrate-treated cells compared with uninduced cells (Fig. 5). These observations raise the possibility that butyrate treatment resulted not only in enhanced P-gp expression, but also in membrane changes that retarded the uptake of ADR. Hence, these data suggest that the P-gp produced in response to butyrate may not be functional in terms of actual efflux, and that this model of P-gp induction will not provide clear information concerning the role of P-gp in flavonoid-stimulated efflux of ADR. The use of other P-gp-overexpressing models, such as MDR or transfected cell lines, should provide further information regarding the relationship between P-gp and flavonoid-stimulation of ADR efflux. For example, experiments using MCF-7 wild type and MCF-7 cells resistant to ADR support a linkage between P-gp expression and the ability of flavonoids to stimulate ADR efflux.

With regard to flavonoid structure–activity relationships, we identified structural features associated with positive or negative effects on the ability of flavonoids to reduce cellular accumulation

of ADR, and also determined that overall hydrophobicity is not a key to activity. The most prominent structural features favoring activity appear to be hydroxylation at the 3 position and desaturation of the 2,3 bond, both of which are characteristics of flavonols as a group. It therefore seems reasonable to predict that other flavonols not tested in this experiment will also possess appreciable activity. Speculation over the functional significance of structural specifics may be premature insofar as we have not identified a definitive molecular target mediating this newly described activity. However, it is interesting to note that desaturation of the 2,3 bond has the steric effect of rendering the molecule planar, and that the 3-hydroxyl in proximity to the 4 keto group has been reported to promote radical scavenging activity [28].

The molecular mechanism(s) underlying flavonoid stimulation of P-gp remains to be determined. While it is conceivable that flavonoids could bind directly to an allosteric site on P-gp, perhaps more likely is that they act indirectly via other factors such as protein kinases or unidentified helper proteins. For example, evidence exists for the inhibition by certain flavonoids of growth factor-associated protein tyrosine kinases [29], protein kinase C [30], and phosphatidylinositol-3-kinase [31]. Considering reports that P-gp has numerous phosphorylation sites, and that the phosphorylation state of P-gp affects efflux activity [17], it is certainly feasible that flavonoids work via altering the phosphorylation state of P-gp. Also, it should be emphasized that in the present study we addressed the short-term effect of flavonoids; future research may reveal long-term (hours to days) effects as well.

In closing, it should be noted that flavonoids are found in abundance in diets rich in fruits, vegetables, and plant-derived beverages such as tea. Even the typical western diet, which falls short of recommendations to consume five servings of fruits plus vegetables per day [32], has been estimated to contain approximately 1 g of flavonoids per day [18]. While it is difficult, if not impossible, to ascribe the cancer-protective effects of diets rich in fruits and vegetables to any single dietary component, the present findings are suggestive of a role for certain flavonoids in facilitating the removal of xenobiotics from colon cells. The extent to which the efflux of other xenobiotics (e.g. benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene) is affected by flavonoids [7] and the role of P-gp in this system are currently under investigation in our laboratory.

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