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Effect of Quercetin on Hoechst 33342 Transport by Purified and Reconstituted P-Glycoprotein

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ABSTRACT. Multidrug resistance due to P-glycoprotein is a serious impediment to successful chemotherapy of cancer. Numerous compounds are known that inhibit the drug-exporting function of P-glycoprotein. Understanding the mechanisms of action of these chemosensitizers is made difficult by the complexity of the *in vivo* cell systems usually employed. To examine the direct effects of chemosensitizers, we have developed a system in which purified and reconstituted P-glycoprotein transports Hoechst 33342 from the lipid membrane to the aqueous interior of proteoliposomes, requiring ATP hydrolysis (Shapiro AB and Ling V, *J Biol Chem* **270:** 16167–16175, 1995). Here, we use this system to understand the effect on P-glycoprotein of quercetin, one of three flavonoids that have been reported to have the unique property of stimulating drug transport by P-glycoprotein *in vivo* (Phang *et al.*, *Cancer Res* **53:** 5977–5981, 1993). Since flavonoids are abundant in food, it is important to understand their effects on the function of P-glycoprotein because of the implications for cancer chemotherapy. In our hands, quercetin inhibited P-glycoprotein-mediated Hoechst 33342 efflux and enhanced accumulation, as measured by flow cytometry, by multidrug-resistant CH^RC5 cells. In the purified system, quercetin strongly inhibited Hoechst 33342 transport by P-glycoprotein, at least in part by inhibiting the ATPase activity of P-glycoprotein required for transport. We conclude that the previously reported stimulatory effect of quercetin on drug efflux from multidrug-resistant cells is not a direct effect on P-glycoprotein. The ATPase domain of P-glycoprotein may be an attractive target for new chemosensitizing agents. BIOCHEM PHARMACOL 53;4:587–596, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; P-glycoprotein; quercetin; Hoechst 33342; 7,12-dimethylbenz[*a*] anthracene; Adriamycin

Multidrug resistance is a formidable problem for cancer chemotherapy. Multidrug resistance often results from expression of P-glycoprotein in the plasma membranes of cells, due either to selection for overexpressing cells during chemotherapy or to normal high levels in certain tissues [1–3]. P-Glycoprotein is a 170-kDa integral membrane protein belonging to the large ABC† superfamily of proteins, also known as traffic ATPases [4, 5]. It is believed to function as an ATP hydrolysis-dependent transporter of broad specificity, recognizing a wide variety of structurally diverse lipophilic compounds and pumping them out of cells [3]. By reducing the intracellular concentrations of cytotoxic compounds, P-glycoprotein prevents them from killing the cells. Our measurements of the transport activity of purified and reconstituted P-glycoprotein support the hypothesis that P-glycoprotein functions as a drug pump [6]. The substrates of P-glycoprotein include many compounds used in cancer chemotherapy, such as *Vinca* alkaloids, anthracyclines, taxol, and epipodophyllotoxins [3].

Because of the deleterious effect of P-glycoprotein on chemotherapeutic efficacy, compounds that modify its function are of potential clinical value. Numerous modulators, or chemosensitizers, are known that inhibit the ability of P-glycoprotein to maintain subtoxic intracellular drug concentrations [3]. Examples include calcium channel blockers like verapamil and trifluoperazine, detergents like Triton X-100, and immunosuppressants like cyclosporin A.

In contrast to chemosensitizers, three flavonoids, kaempferol, galangin, and quercetin, have been found to *stimulate* efflux of two P-glycoprotein substrates, 7,12-dimethylbenz[*a*]anthracene and Adriamycin, from multidrug-resistant cells [7, 8] and to increase Adriamycin resistance [8]. This remarkable observation could be of importance for chemotherapy because flavonoids are very common constituents of food, and substances that increase the activity of P-glycoprotein could potentially reduce the efficacy of chemotherapy. On the other hand, Scambia *et al.* [9] reported that quercetin inhibits Adriamycin resistance of multidrug-resistant cells and reduces Rhodamine

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† *Abbreviations:* ABC, ATP Binding Cassette; and Tricine, *N*-tris(hydroxymethyl)methylglycine.

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123 efflux. This discrepancy is, as yet, unexplained. Whether quercetin stimulates or inhibits drug efflux, it is not known whether or not these flavonoids exert their effect directly on P-glycoprotein, since they have pleiotropic effects on cells. Documented effects of quercetin, for example, include inhibition of protein tyrosine kinases and serine/threonine kinases [10, 11], 5′-nucleotidase [10], phospholipase A_2 [12], and glutathione-S-transferase [13]; antioxidant effects [14]; regulation of heat shock protein synthesis [15]; and inhibition of nitric oxide synthase [16].

We have developed a system to measure ATP hydrolysis-dependent drug transport by purified P-glycoprotein reconstituted into artificial lipid bilayer vesicles, using Hoechst 33342 as a substrate [6]. Hoechst 33342, a topoisomerase I inhibitor with potential use in chemotherapy [17], has been shown to be a substrate of P-glycoprotein *in vivo* [17, 18]. Preparatory to using our system to look for direct effects of the flavonoid quercetin on P-glycoprotein, we tested the effect of quercetin on Hoechst 33342 accumulation and efflux by Chinese hamster ovary multidrug-resistant cells. Quercetin appeared to inhibit Hoechst 33342 efflux from multidrug-resistant CH^RC5 cells. The transport system with purified P-glycoprotein, by eliminating all other cellular constituents and metabolic pathways, enabled us to study the direct effects of quercetin on Hoechst 33342 transport by P-glycoprotein.

MATERIALS AND METHODS

Flow Cytometry

Aux B1 and CH^RC5 cells were grown in monolayer culture in plastic flasks in α-Minimum Essential Medium supplemented with 10% fetal bovine serum at 37° and 5% CO_2. Cells were harvested by trypsinization. Serum was removed by twice pelleting the cells by mild centrifugation and resuspending in medium without serum. Hoechst 33342 (Molecular Probes, Eugene, OR, U.S.A.) was added to 10 μM to cells at a density of 2×10^6 cells/mL. The cells were incubated on a roller wheel at 37° for 30 min. For uptake experiments, 100 μM quercetin was included with a portion of the cells during incubation with Hoechst 33342. For efflux experiments, after incubation with Hoechst 33342 the cells were pelleted and resuspended at the same density in serum-free medium containing 0 or 100 μM quercetin, and then were incubated for various lengths of time at 37°. For both experiments, the cells were finally pelleted and resuspended in ice-cold, serum-free medium without phenol red and stored on ice. Cells were kept in an atmosphere of 5% CO_2 in air throughout the experiment.

Hoechst 33342 fluorescence measurements of individual cells were performed with a Becton–Dickinson FACStar Plus fluorescence-activated cell sorter (Mississauga, Ontario, Canada) equipped with an ultraviolet laser. Analysis was gated to include only live, single cells and was based on acquisition of data from 10,000 such cells.

Hoechst 33342 Transport Assay with Purified, Reconstituted P-Glycoprotein

P-Glycoprotein was purified from highly drug-resistant Chinese hamster ovary CH^RB30 cells as described in Shapiro and Ling [19] and reconstituted as in Shapiro and Ling [6]. The assay for the transport of Hoechst 33342 was carried out as described previously [6]. Briefly, liposomes were prepared from crude soybean L-α-phosphatidylcholine with or without P-glycoprotein incorporated into the membrane with the cytoplasmic domains external. The buffer both inside and outside the liposomes consisted of 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, and 1 mM EDTA. Liposome suspensions were placed in a cuvette in an Amin-co–Bowman Series 2 Luminescence Spectrometer (Spectronic Instruments, Rochester, NY, U.S.A) and held at 37°. Hoechst 33342 was added to 25 μM. When quercetin was used, it was added at the same time as Hoechst 33342 to 25 μM from a 300-fold concentrated stock solution in ethanol. The final concentration of ethanol, 0.3%, had no effect on the transport assay. After 15 min, MgATP was added to final $MgCl_2$ and Na_4ATP concentrations of 3 and 1.5 mM, respectively. Dithiothreitol was present at 2 mM. Fluorescence was monitored continuously for 40 min. The excitation and emission wavelengths were 355 and 457 nm, respectively, with 0.5 and 16 nm bandwidths, respectively. Fluorescence traces were normalized to a value of 1 arbitrary unit at the time just prior to addition of MgATP. Traces from control liposomes prepared without P-glycoprotein were subtracted from traces for P-glycoprotein-containing liposomes. The subtracted traces were then subjected to a 15-sec moving window averaging procedure to reduce the signal noise.

Displacement of Hoechst 33342 from Lipid by Quercetin

Samples of 1 mL were prepared consisting of 0.55 mg/mL of sonicated, crude soybean L-α-phosphatidylcholine in 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, and 1 mM EDTA. Hoechst 33342 and/or quercetin were added to 25 and 80 μM, respectively. The liposome suspensions were centrifuged at 100,000 *g* for 45 min at 22° to pellet the liposomes. Absorbance spectra of the supernatants were taken between 250 and 550 nm. The supernatant absorbance spectrum of the Hoechst 33342-treated liposomes showed that 10% of the Hoechst 33342 remained in the supernatant. The amount of Hoechst 33342 in the supernatant of liposomes treated with both Hoechst 33342 and quercetin was measured by subtracting the absorbance due to quercetin. To do this, the absorbance spectrum of 80 μM quercetin in buffer was used to determine the extinction coefficient at each wavelength. Hoechst 33342 has no significant absorbance at 420 nm, so the 420 nm absorbance of the supernatant from liposomes treated with Hoechst 33342 and quercetin was due to quercetin alone. The 420 nm quercetin absorbance in the supernatant spectrum was

used to generate the full quercetin absorbance spectrum in the supernatant, as follows. The quercetin absorbance at each wavelength was equal to the quercetin absorbance at 420 nm in the supernatant sample multiplied by the ratio of the quercetin extinction coefficient at each wavelength to the quercetin extinction coefficient at 420 nm. The quercetin spectrum was subtracted from the Hoechst 33342 + quercetin spectrum, yielding the spectrum of Hoechst 33342 in the supernatant.

Quenching of Fluorescence by Quercetin

Hoechst 33342, Adriamycin, or 7,12-dimethylbenz[*a*]anthracene was added to 25 μM to 0.5 mg/mL of sonicated, crude soybean L-α-phosphatidylcholine in a buffer consisting of 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, and 1 mM EDTA. Quercetin was added to various concentrations from a 200- or 300-fold concentrated stock solution in ethanol. Hoechst 33342 fluorescence was measured at ambient temperature with a Perkin–Elmer LS3 Luminescence Spectrometer (Norwalk, CT, U.S.A.). Adriamycin and 7,12-dimethylbenz[*a*]anthracene fluorescence was measured at ambient temperature with a Perkin–Elmer LS50B Luminescence Spectrometer. The excitation and emission wavelengths were 355 and 457 nm, respectively, for Hoechst 33342; 305 nm (2.5 nm bandwidth) and 480 nm (2.5 nm bandwidth), respectively, for 7,12-dimethylbenz[*a*]anthracene; and 475 nm (5 nm bandwidth) and 560 nm (8 nm bandwidth), respectively, for Adriamycin. The excitation and emission path lengths in the cuvette were 2 and 10 mm, respectively. Quercetin was not fluorescent at the wavelengths used.

For measurements of 7,12-dimethylbenz[*a*]anthracene fluorescence quenching by quercetin, the absorbance of excitation light by quercetin necessitated correction for the inner filter effect. This was done by measuring the reduction in the fluorescence of 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (Molecular Probes) covalently linked to dithiothreitol (DTT-IAEDANS), a non-lipophilic compound, by each quercetin concentration under the same conditions used for the 7,12-dimethylbenz[*a*]anthracene measurements. The 7,12-dimethylbenz[*a*]anthracene measurements were multiplied by correction factors consisting of the ratio of the DTT-IAEDANS fluorescence without quercetin to the fluorescence at each quercetin concentration.

Fluorescence Lifetime Measurements

Fluorescence lifetimes were measured with an SLM 48000 DSCF Lifetime Spectrofluorometer (Spectronic Instruments) with a 500 W Hg/Xe lamp. The excitation bandpasses were 32 and 1 nm. Emission was filtered by a KV370 filter. Data were acquired at each frequency using 5 sample/reference pairs at 100 averages each. No polarizers were used. The reference sample was glycogen in water. The excitation wavelengths were 290 nm for Hoechst 33342 and 300 nm for Adriamycin and 7,12-dimethylbenz[*a*]anthracene.

ATPase Assays

Measurements of ATP hydrolysis by purified, reconstituted P-glycoprotein were made according to Shapiro and Ling [19] except that P-glycoprotein was reconstituted into liposomes consisting of crude soybean L-α-phosphatidylcholine. One hundred microliters of liposomes was mixed with 50 μL of 100 mM Tricine-NaOH (pH 7.4), 168 mM NaCl, 40 mM $MgCl_2$, 20 mM dithiothreitol, 2% ethanol, and four times the desired final concentration of quercetin, or no quercetin. The quercetin remained soluble under these conditions. The ethanol was present because the quercetin was added from a stock solution in ethanol. ATPase reactions were initiated by the addition of 50 μL of Na_4ATP at four times the desired final concentration and placement of the samples at 37°. Incubation was for 1 hr. The final concentration of ethanol, 0.5%, had no effect on the ATPase activity of P-glycoprotein. Liposomes prepared without P-glycoprotein were used as blanks at each ATP concentration.

[^{3}H]Azidopine Photolabeling of P-Glycoprotein

Sucrose gradient-purified plasma membranes from CH^RB30 cells were photolabeled with [^{3}H]azidopine as described in Georges *et al.* [20]. One microliter of quercetin dissolved in ethanol at fifty times the desired final concentration was added to a total volume of 50 μL of membranes containing 0.2 μM [^{3}H]azidopine (Amersham, Oakville, Ontario, Canada) with a specific activity of 52 Ci/mmol. The final ethanol concentration was 3% because the azidopine was also dissolved in ethanol.

RESULTS

Since our transport assay with purified and reconstituted P-glycoprotein from Chinese hamster ovary cells uses Hoechst 33342 as a substrate, we studied the effect of quercetin on Hoechst 33342 accumulation and efflux from Chinese hamster ovary cells. Multidrug-resistant CH^RC5 cells were derived by stepwise selection with colchicine from drug-sensitive Aux B1 cells [21, 22]. CH^RC5 cells exhibit a high level of multidrug resistance relative to Aux B1 cells due to reduced intracellular drug accumulation [21], amplification of the *pgp1* gene [23], and overexpression of P-glycoprotein in the plasma membrane [24]. As expected, the fluorescence of Hoechst 33342 in CH^RC5 cells following a 30-min incubation with 10 μM Hoechst 33342 was much lower than the fluorescence of identically treated Aux B1 cells (Fig. 1), consistent with P-glycoprotein-mediated efflux of Hoechst 33342 from CH^RC5 cells.

Treatment of the cells with 100 μM quercetin during Hoechst 33342 uptake resulted in a reduction of Hoechst 33342 fluorescence in Aux B1 cells, but an increased fluo-

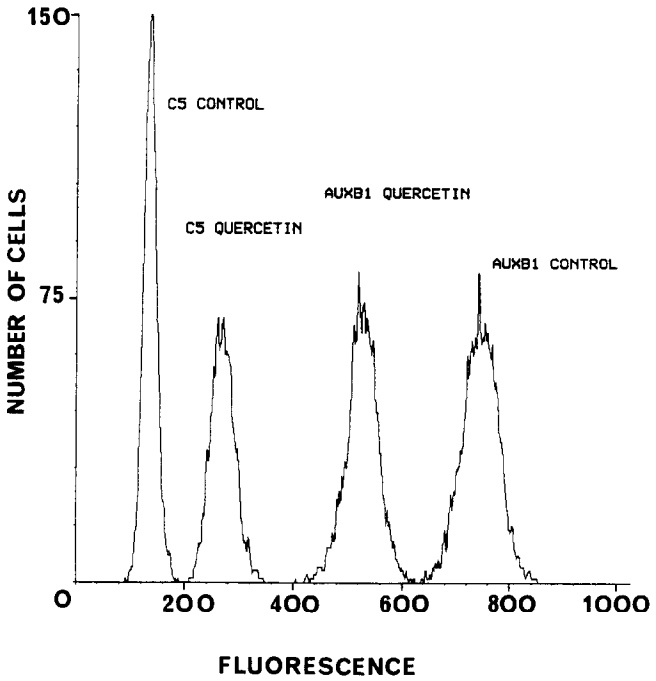

FIG. 1. Effect of quercetin on Hoechst 33342 fluorescence accumulation of Aux B1 and CH^{R}C5 cells. The experiment was performed as described in Materials and Methods. Control: cells incubated with Hoechst 33342 alone. Quercetin: cells treated with Hoechst 33342 and quercetin.

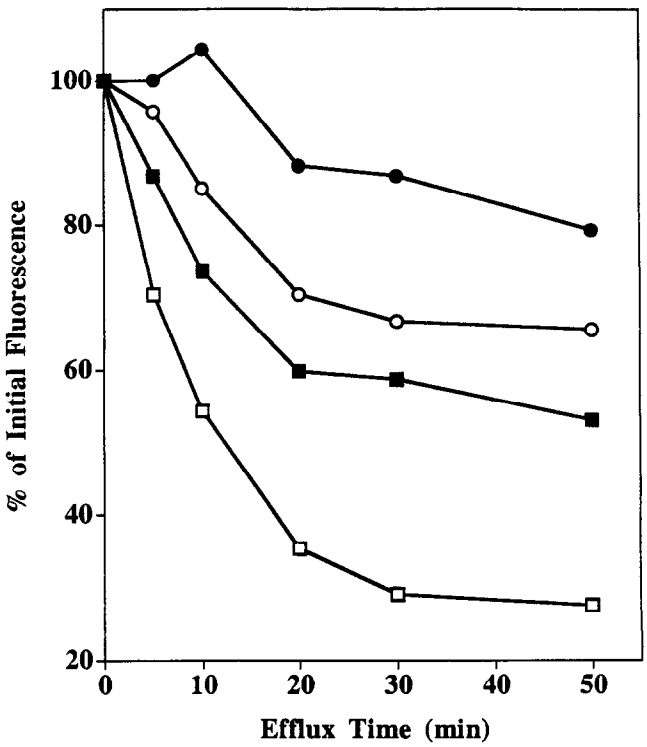

FIG. 2. Effect of quercetin on Hoechst 33342 fluorescence efflux from Aux B1 and CH^{R}C5 cells. The experiment was performed as described in Materials and Methods. Key: (■,□) no quercetin; (●,○) 100 μM quercetin; (○,□) CH^{R}C5 cells; and (●,■) Aux B1 cells. Initial mean fluorescence levels in arbitrary units, were 337 and 470 for AuxB1 cells with and without quercetin, respectively, and 45.3 for C5 cells both with and without quercetin.

rescence in CH^{R}C5 cells (Fig. 1). Increased Hoechst 33342 uptake by CH^{R}C5 cells suggests a possible inhibition of P-glycoprotein by quercetin. On the other hand, reduced Hoechst 33342 accumulation by Aux B1 cells suggests a separate effect of quercetin. Since Aux B1 cells express P-glycoprotein only at a very low level, it is unlikely that the quercetin effect on Hoechst 33342 transport is mediated by P-glycoprotein in these cells.

The effect of quercetin on the rate of Hoechst 33342 efflux from the cells was also measured. The cells were allowed to accumulate Hoechst 33342 for 30 min, after which external Hoechst 33342 was removed. Efflux then occurred in the presence or absence of quercetin. Figure 2 shows that Hoechst 33342 fluorescence of CH^{R}C5 cells decreased faster and to a greater extent than that of Aux B1 cells, as expected if efflux is due to P-glycoprotein. Quercetin reduced the rate and extent of the decrease of Hoechst 33342 fluorescence of both cell lines, however. The reduced rate of Hoechst 33342 efflux from Aux B1 cells in the presence of quercetin is surprising considering that quercetin also appeared to reduce the accumulation of Hoechst 33342 in Aux B1 cells.

Clearly, quercetin did not have the stimulatory effect on Hoechst 33342 efflux from CH^{R}C5 cells that it had on Adriamycin and 7,12-dimethylbenz[*a*]anthracene efflux from MCF-7 and HCT-15 cells, respectively [7, 8]. To the contrary, quercetin appears to have had an inhibitory effect, as seen by Scambia *et al.* [9]. The effect of quercetin may depend on features of both the substrate of transport and the cells in which transport occurs, as well as the level of P-glycoprotein expression. A complication for the interpretation of the effects of quercetin on drug transport by P-glycoprotein, however, is the odd effect of quercetin on Aux B1 cells.

Aqueous Hoechst 33342 is virtually nonfluorescent, but its fluorescence is enhanced enormously upon binding to DNA. Fluorescence microscopy of Hoechst 33342-treated cells shows that a significant level of fluorescence is found only in the nucleus (not shown). Differences in Hoechst 33342 fluorescence between different cell lines or cells that have or have not been treated with quercetin could be due to differences in the total intracellular amount of Hoechst 33342 or to redistribution of Hoechst 33342 between the nucleus, where it fluoresces, and the cytoplasm or other organelles, where it does not fluoresce. Fluorescence changes of intracellular Adriamycin (doxorubicin) in multidrug-resistant cells due to redistribution between the nucleus and the cytoplasm have been described [25]. Furthermore, quercetin could directly alter the fluorescence of Hoechst 33342 (see below). Thus, interpretation of the effects of quercetin on Hoechst 33342 fluorescence of live cells is complex.

Our drug transport assay using purified P-glycoprotein reconstituted into artificial lipid vesicles (liposomes) and

Hoechst 33342 as a substrate [6] provides a simplified model system for examining the direct effect of quercetin on Hoechst 33342 transport by P-glycoprotein. In our system, the P-glycoprotein is present in the lipid membranes exclusively in an inside-out orientation, i.e. with cytoplasmic domains facing the external aqueous medium. The cytoplasmic domains contain the ATP binding sites. P-Glycoprotein is dependent upon ATP hydrolysis for drug transport, so the transport phase of the assay can be initiated by the addition of MgATP to a suspension of liposomes preincubated with Hoechst 33342. The Hoechst 33342 becomes almost completely bound to the lipid membranes, where it is fluorescent. Upon addition of MgATP to the Hoechst 33342-containing liposomes, the fluorescence signal decreases as P-glycoprotein transports the Hoechst 33342 to the interior aqueous compartment of the liposomes, where it is not fluorescent (Fig. 3, lower trace). Radioactive P-glycoprotein substrates are unsuitable for use with this system because the high lipid concentration results in a very high background level of binding to the membrane. Unlike radioactive substrates, Hoechst 33342 can be used to monitor the change from lipid to aqueous environments upon transport, obviating the background problem. In addition, an entire time course of transport is obtained with a single sample using continuous fluorescence monitoring. Radioactive substrate-based assays, in contrast, yield only a single data point per specimen.

In the presence of 25 μM quercetin, added at the same time as the Hoechst 33342, the fluorescence decrease in the transport assay after ATP addition was largely abrogated (Fig. 3, upper trace), indicating that Hoechst 33342 transport out of the lipid membranes by P-glycoprotein was strongly inhibited. This experiment demonstrates that quercetin directly inhibits Hoechst 33342 transport by P-glycoprotein.

During the transport experiments shown in Fig. 3, the Hoechst 33342 fluorescence level prior to the addition of MgATP was about 50% lower in the presence of 25 μM quercetin than in its absence (data not shown). The effect was the same for both P-glycoprotein-containing liposomes and control liposomes without P-glycoprotein. This observation suggested two possibilities: either quercetin competed with Hoechst 33342 for binding to the lipid membrane or quercetin quenched Hoechst 33342 fluorescence. Either effect could contribute to inhibition of Hoechst 33342 transport by P-glycoprotein. Since our data [6] support the hypothesis that P-glycoprotein recognizes its substrates within the lipid membrane [3], quercetin could prevent Hoechst 33342 transport by excluding Hoechst 33342 from the membrane, thus lowering the substrate concentration. On the other hand, formation of a complex between quercetin and Hoechst 33342 would quench Hoechst 33342 fluorescence and probably place the Hoechst 33342 in a non-transportable form.

To determine whether quercetin competes with Hoechst 33342 for binding to lipid, we followed the procedure described in Materials and Methods to determine the concentration of Hoechst 33342 in the aqueous phase in the presence and absence of 80 μM quercetin. At 80 μM, quercetin increased the amount of unbound Hoechst 33342 from about 10% to about 20% of total Hoechst 33342 (data not shown). Since only 25 μM quercetin was used in the transport assay, displacement of Hoechst 33342 from the lipid by quercetin cannot have contributed significantly to the 50% reduction of the initial Hoechst 33342 fluorescence by quercetin prior to MgATP addition.

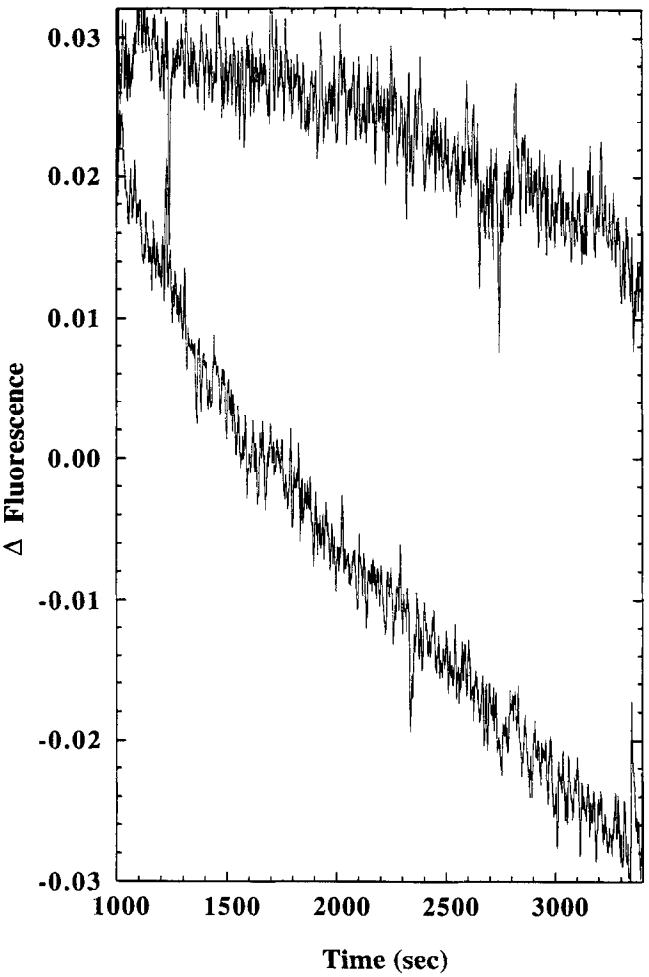

FIG. 3. Effect of quercetin on Hoechst 33342 transport by purified, reconstituted P-glycoprotein. The experiment was performed as described in Materials and Methods. Subtracted traces for P-glycoprotein-containing liposomes minus control liposomes without P-glycoprotein are shown from the point at which MgATP was added to initiate transport. A decrease in fluorescence intensity with time signifies Hoechst 33342 transport from the lipid bilayer to the internal aqueous space. Lower trace: no quercetin; upper trace: 25 μM quercetin.

Next we measured quenching of Hoechst 33342 fluorescence by quercetin. Hoechst 33342 at 25 μM was mixed with 0.55 mg/mL liposomes and 0–100 μM quercetin. The reduction of Hoechst 33342 fluorescence as a function of quercetin concentration followed a linear Stern–Volmer quenching relationship (Fig. 4), indicating the formation of

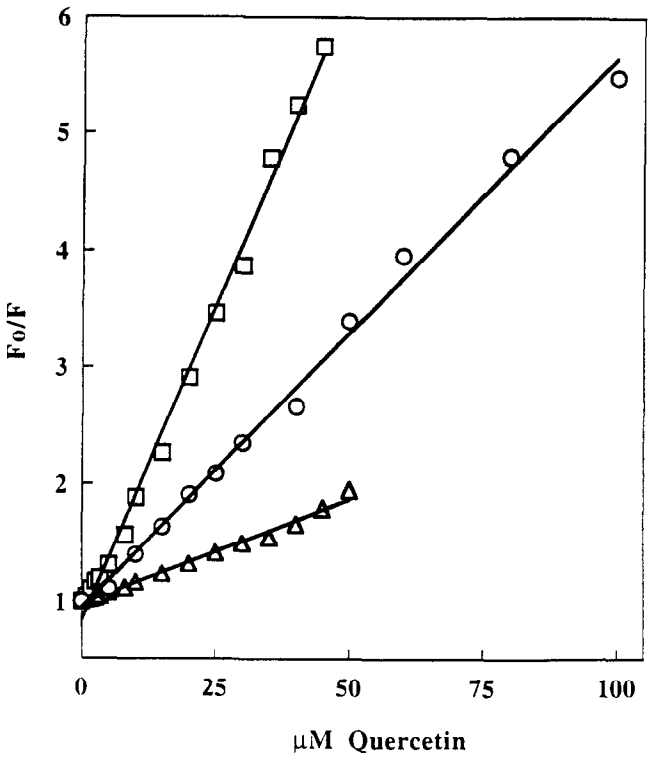

FIG. 4. Stern–Volmer plot of Hoechst 33342 (○), 7,12-dimethylbenz[a]anthracene (□), and Adriamycin (△) fluorescence quenching by quercetin. The experiment was performed as described in Materials and Methods. The ratio F_o/F is the fluorescence in the absence of quercetin divided by the fluorescence in the presence of quercetin.

a complex within the lipid membrane between Hoechst 33342 and quercetin. Figure 4 shows that 25 μM quercetin caused about 50% quenching ($F_o/F = 2$) of Hoechst 33342 fluorescence, which matches the level seen in the transport experiment. The extent of quenching was not affected by the optical path length and was not a linear function of quercetin concentration (data not shown), showing that the quenching was not a trivial effect of absorption of the excitation light or Hoechst 33342 fluorescence by quercetin. Dithiothreitol at 2 mM, as present in the Hoechst 33342 transport measurements with purified and reconstituted P-glycoprotein, had no effect on quenching of Hoechst 33342 fluorescence by quercetin.

We also measured the quenching of fluorescence of 7,12-dimethylbenz[*a*]anthracene and Adriamycin by quercetin. In both cases, linear Stern–Volmer plots were obtained, although for 7,12-dimethylbenz[*a*]anthracene it was necessary to correct for the inner filter effect. Quercetin produced 50% quenching of 7,12-dimethylbenz[*a*]anthracene and Adriamycin fluorescence at about 10 and 60 μM, respectively (Fig. 4).

There are two types of fluorescence quenching, static and dynamic [26]. Static quenching arises from formation of a quenching complex between fluorophore and quencher. Dynamic quenching arises from collision between fluorophore and quencher, without complex formation. Dynamic quenching by quercetin would not be expected to cause inhibition of Hoechst 33342 transport by P-glycoprotein. The two types of quenching can be distinguished by comparison of absorbance spectra and fluorescence lifetimes of the fluorophore in the presence and absence of the quencher. Static quenching usually results in a change in the absorbance spectrum of the fluorophore but no change in the fluorescence lifetime, whereas dynamic quenching does not change the absorbance spectrum of the fluorophore but reduces its fluorescence lifetime.

The absorbance spectrum of 10 μM Hoechst 33342 bound to 0.5 mg/mL liposomes in 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, and 1 mM EDTA was measured in the presence and absence of 20 μM quercetin. The absorbance spectrum of quercetin alone was subtracted from the absorbance spectrum of quercetin + Hoechst 33342 to give the spectrum of Hoechst 33342 in the presence of quercetin. The absorbance spectra of Hoechst 33342 was unchanged by the presence of quercetin (data not shown).

The fluorescence lifetimes of Hoechst 33342, 7,12-dimethylbenz[*a*]anthracene, and Adriamycin were measured by the phase modulation method in the presence and absence of quercetin (Table 1). Each fluorophore revealed two or three lifetime components, and the lifetime of each component was reduced by quercetin, indicative of dynamic quenching. The existence of two lifetimes of the fluorophores results from their presence in two different environments, aqueous and lipid. The third lifetime for 7,12-dimethylbenz[*a*]anthracene is probably due to a contaminant.

The absorbance spectra and lifetime measurements demonstrated conclusively that fluorescence quenching by quercetin is dynamic. Inhibition of P-glycoprotein-mediated Hoechst 33342 transport by quercetin, therefore, is not due to formation of a non-substrate complex between quercetin and Hoechst 33342.

Quercetin is known to inhibit enzymatic reactions involving ATP hydrolysis, such as phosphorylation [10, 11]. Since drug transport by P-glycoprotein is dependent upon ATP hydrolysis [3, 6, 27, 28], we checked for inhibition of the ATPase activity of P-glycoprotein by quercetin. Figure 5 shows that, indeed, quercetin inhibits the ATPase activity of P-glycoprotein. The extent of inhibition at a given concentration of quercetin depends upon the ATP concentration in a complex manner. At 1.5 mM ATP, the concentration used in the transport assay, the ATPase activity of P-glycoprotein was inhibited 33% by 25 μM quercetin. Since ATP hydrolysis is required for Hoechst 33342 transport by purified P-glycoprotein [6], this amount of ATPase inhibition should result in decreased Hoechst 33342 transport. It is possible that small decreases in the rate of ATP hydrolysis translate into large effects on the rate of Hoechst 33342 transport in this assay. Hoechst 33342 itself had a slightly stimulatory effect on ATP hydrolysis by P-glycoprotein (not shown).

TABLE 1. Fluorescence lifetime measurements of Adriamycin, 7,12-dimethylbenz[a]anthracene, and Hoechst 33342 in the presence and absence of quercetin

Fluorophore	Lifetimes (nsec)		
Hoechst 33342	3.4 (67%)	1.0 (33%)	
Hoechst 33342 + quercetin	2.9 (75%)	0.4 (25%)	
Adriamycin	4.3 (9%)	1.3 (91%)	
Adriamycin + quercetin	2.3 (39%)	0.9 (61%)	
7,12-Dimethylbenz[*a*]anthracene	19.5 (91%)	4.8 (5%)	0.9 (3%)
7,12-Dimethylbenz[*a*]anthracene + quercetin	12.2 (77%)	3.4 (17%)	0.4 (6%)

One micromolar 7,12-dimethylbenz[*a*]anthracene or 25 μM Adriamycin or Hoechst 33342 was added to 0.5 mg/mL sonicated, crude soybean L-α-phosphatidylcholine liposomes in 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, and 1 mM EDTA. Quercetin was added to 20 μM with 7,12-dimethylbenz[*a*]anthracene and Hoechst 33342 and to 50 μM with Adriamycin. The quercetin concentrations were chosen to give about 50% fluorescence quenching. Values in parentheses are the percent of fluorophore with the given lifetime. Each lifetime component of each fluorophore was reduced by quercetin.

When the P-glycoprotein was reconstituted into crude sheep brain phosphatidylethanolamine instead of crude soybean phosphatidylcholine, 25 μM quercetin caused about 90% inhibition of the ATPase activity (data not shown). Since quercetin is lipophilic, the lipid composition and concentration will influence the partitioning of quercetin into the lipid phase, and hence the effective concentration. The somewhat greater lipid concentration in the Hoechst 33342 transport assay than in the ATPase assay may have resulted in a greater effective quercetin concentration in the transport assay than in the ATPase assay, hence greater inhibition.

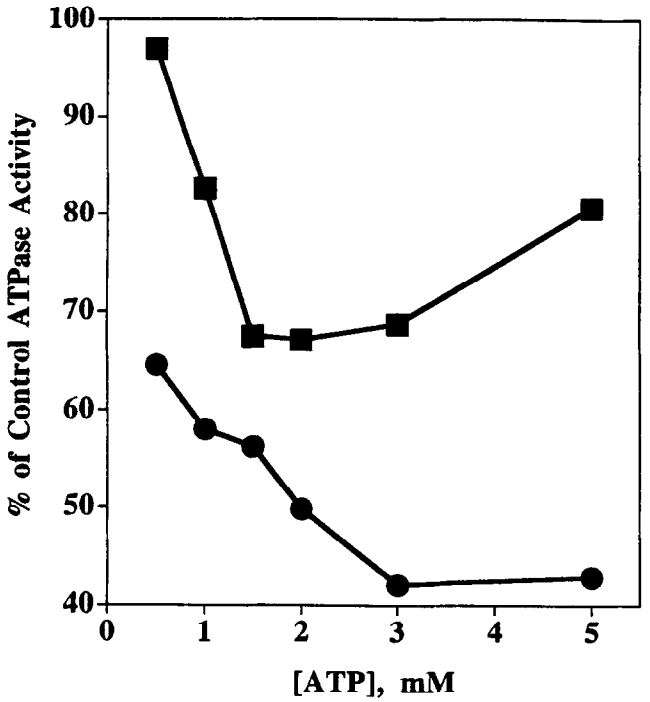

FIG. 5. ATP dependence of quercetin inhibition of the ATPase activity of purified, reconstituted P-glycoprotein. The experiment was performed as described in Materials and Methods. Key: (■) 25 μM quercetin; (●) 50 μM quercetin. The control ATPase activities in the absence of quercetin were 3.3, 5.4, 8.0, 10.8, 13.0, and 14.4 nmol/hr/100 μL of proteoliposomes for the 25 μM quercetin experiment and 7.0, 10.2, 12.1, 13.2, 14.6, and 13.4 nmol/hr/100 μL of proteoliposomes for the 50 μM quercetin experiment for 0.5, 1, 1.5, 2, 3, and 5 mM ATP, respectively.

Another way quercetin could inhibit Hoechst 33342 transport is by competing with Hoechst 33342 for binding to P-glycoprotein. Hoechst 33342 competes with azidopine for photolabeling of P-glycoprotein [6], suggesting that Hoechst 33342 shares a drug binding site with azidopine, a P-glycoprotein substrate and chemosensitizer [29]. Quercetin at concentrations up to at least 100 μM had no effect on azidopine photolabeling of P-glycoprotein, however (data not shown; [8]). This result suggests that quercetin does not compete with Hoechst 33342 for binding to P-glycoprotein at the azidopine binding site, but it is possible that competition occurs at a site other than the azidopine binding site. We currently have no direct way to measure binding of Hoechst 33342 or quercetin to P-glycoprotein.

We have shown that quercetin directly inhibits Hoechst 33342 transport by P-glycoprotein by using a simplified system consisting of purified P-glycoprotein reconstituted into artificial lipid membrane bilayer vesicles. With this system, we were able to determine that quercetin inhibits Hoechst 33342 transport, at least in part by inhibiting the ATPase activity of P-glycoprotein which is essential for drug transport.

DISCUSSION

Because of the complexity of living cells, the effects of added chemical compounds on specific cellular components can be difficult to discern. The compound may affect sev-

eral components, or its effect on a single component may produce several additional cellular effects that confuse the interpretation. This situation applies to the effect of quercetin on P-glycoprotein-mediated transport of Hoechst 33342.

Quercetin has effects on numerous cellular processes, as listed above. Figures 1 and 2 showed that quercetin affected Hoechst 33342 fluorescence accumulation and efflux in Chinese hamster ovary cells in both P-glycoprotein-dependent and -independent manners. By studying the effects of quercetin on P-glycoprotein-mediated Hoechst 33342 transport by purified, reconstituted P-glycoprotein, we were able to discern the nature of these effects.

First, quercetin quenched Hoechst 33342 fluorescence. Fluorescence quenching will affect the flow cytometry experiments in Figs. 1 and 2, reducing the Hoechst 33342 fluorescence but not necessarily the amount of intracellular Hoechst 33342. This may account for the lower fluorescence of Aux B1 cells, which express an insignificant amount of P-glycoprotein, following 30 min of Hoechst 33342 accumulation in the presence of quercetin (Fig. 1). In addition, this same effect would apply to the Hoechst 33342 fluorescence accumulation of CH^RC5 cells, so that the enhanced accumulation of Hoechst 33342 caused by quercetin would be underestimated.

During the Hoechst 33342 efflux phase of the experiment (Fig. 2), quercetin accumulation during Hoechst 33342 efflux may have caused an apparent enhancement of the rate of fluorescence decrease in both Aux B1 and CH^RC5 cells due to increased quenching with time of the fluorescence of the remaining intracellular Hoechst 33342. Inhibitory effects of quercetin on Hoechst 33342 efflux would thus have been underestimated.

Quenching of Hoechst 33342 fluorescence by quercetin can thus be seen to severely complicate interpretation of the flow cytometry data. In the Hoechst 33342 transport assay with purified, reconstituted P-glycoprotein, however, quenching of Hoechst 33342 fluorescence did not affect the results because the fluorescence was normalized to a value of one arbitrary unit for each assay just prior to the addition of MgATP, after a prolonged incubation in the presence of quercetin, such that the fluorescence decrease after MgATP addition was measured as a percentage change from the initial fluorescence. The only result of the quenching was to increase slightly the noise in the trace relative to the quercetin-free trace (Fig. 3), because the initial fluorescence level was reduced in the presence of quercetin.

The second effect of quercetin on P-glycoprotein-mediated transport of Hoechst 33342 was more direct. Quercetin inhibited ATP hydrolysis by P-glycoprotein, which is required for Hoechst 33342 transport [6]. In the Hoechst 33342 accumulation experiment (Fig. 1), quercetin-treated CH^RC5 cells had higher Hoechst 33342 fluorescence, at least in part because quercetin inhibited the ATPase activity of P-glycoprotein, impairing its transport function. In the efflux experiment (Fig. 2), inhibition of the ATPase activity of P-glycoprotein probably contributed to the reduced rate of loss of Hoechst 33342 fluorescence by CH^RC5 cells treated with quercetin.

Quercetin stimulated the efflux rate of Adriamycin and 7,12-dimethylbenz[*a*]anthracene from multidrug-resistant HCT-15 and MCF-7 cells [7, 8], but quercetin was also found to inhibit Rhodamine 123 efflux from MCF-7 cells and render them more Adriamycin sensitive [9]. It was noteworthy, therefore, that quercetin inhibited P-glycoprotein-mediated transport of Hoechst 33342. There are several possible explanations for this discrepancy. The aforementioned pleiotropic effects of quercetin, which may differ depending on cell type and species (MCF-7 and HCT-15 are human cell lines, whereas Aux B1 and CH^RC5 are hamster cell lines), could account for some of the difference. Another potentially important factor could be differences between the cell lines in cytoplasmic ATP concentrations, which may also be affected by quercetin. As Fig. 5 shows, the degree to which quercetin inhibits P-glycoprotein ATPase activity depends upon the ATP concentration. Furthermore, since the K_m for ATP of P-glycoprotein, about 1 mM, is similar to the cytoplasmic ATP concentration, the drug transport activity of P-glycoprotein could be increased or decreased by raising or lowering the cytoplasmic ATP concentration. Moreover, effects of flavonoids on redox modification of 7,12-dimethylbenz[*a*]anthracene and Adriamycin by cytochrome P450 may contribute to flavonoid-mediated stimulation of efflux of these compounds. No such modification may be involved in Hoechst 33342 or Rhodamine 123 transport. Evaluation of the effects of compounds on P-glycoprotein function *in vivo* must take into account the specific cell type and P-glycoprotein substrate involved.

Fluorescence quenching by quercetin could potentially confound results of fluorescence-based drug transport measurements. The study by Critchfield *et al.* [8] of Adriamycin transport by HCT-15 cells used radiolabeled Adriamycin, so it would not have been affected. The 7,12-dimethylbenz[*a*]anthracene transport experiments [7], however, may have suffered from fluorescence quenching by quercetin. We do not know whether any of the other flavonoids used in that paper are also quenchers. In efflux experiments, if quercetin accumulation occurs during drug efflux, the rates of efflux could be overestimated and any inhibitory effects of quercetin underestimated. In the paper by Scambia *et al.* [9], Rhodamine 123 was used as the probe of drug transport activity. We do not know whether Rhodamine 123 fluorescence is affected by quercetin. Cell proliferation assays to measure drug resistance, which showed decreased resistance to Adriamycin by MCF-7 cells [9] in one case and increased resistance to Adriamycin by HCT-15 cells [8] in another, are unaffected by fluorescence quenching.

An interesting question is how quercetin inhibits the ATPase activity of P-glycoprotein. Since quercetin inhibits many ATP-dependent enzymes, it might be expected to act as a competitive inhibitor, binding to the catalytic ATP

binding site or sites. Michaelis–Menten kinetic analysis of the ATPase inhibition data for 50 μM quercetin in Fig. 5, however, demonstrates that inhibition was neither purely competitive nor purely noncompetitive (not shown). This may reflect the fact that quercetin partitions into the lipid membrane where it may also interact with drug binding sites of P-glycoprotein or alter the interaction of P-glycoprotein with the surrounding lipids, producing noncompetitive inhibition of the ATPase activity. Verapamil, a lipophilic chemosensitizer that stimulates the ATPase activity of P-glycoprotein, also affected both the V_{max} and K_m for ATP hydrolysis by P-glycoprotein [19]. Effects of various P-glycoprotein-directed compounds that alter its ATPase activity can be complex, and are likely to be a fruitful area of future research with purified, reconstituted P-glycoprotein.

The quercetin result demonstrates the potential therapeutic value of inhibiting the ATPase activity of P-glycoprotein, as opposed to competing for substrate binding and transport, as is the case with most chemosensitizers. In the Hoechst 33342 transport assay with purified, reconstituted P-glycoprotein, Hoechst 33342 transport was inhibited completely by agents that inhibit ATP hydrolysis, such as *N*-ethylmaleimide and vanadate [6] or quercetin. In contrast, chemosensitizers, such as verapamil and amiodarone, were unable to reduce transport of Hoechst 33342 added to 25 μM. Inhibitory effects of chemosensitizers became apparent only when the Hoechst 33342 concentration was reduced to 2 μM [6] or less. Compounds that inhibit the ATPase activity of P-glycoprotein may be of use, therefore, against P-glycoprotein-mediated multidrug resistance.

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