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Inhibition of Drug Transport by Genistein in Multidrug-Resistant Cells Expressing P-Glycoprotein

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ABSTRACT. It has been claimed that the flavonoid genistein could be used to distinguish multidrug-resistant tumors expressing the multidrug resistance-associated protein (MRP) from those expressing P-glycoprotein (Pgp). Genistein would block drug transport by MRP without affecting Pgp-mediated drug transport. However, we found that exposure to 200 μM genistein elicited an elevation in intracellular accumulation of rhodamine 123 (R123) and daunorubicin (DNR) in Pgp-expressing cell lines. Genistein inhibited R123 efflux in a rapidly reversible manner (*ca.* 2 min). The flavonoid also decreased photoaffinity labeling of Pgp by [^{3}H]azidopine, a Pgp substrate. The present results show that genistein interacts with Pgp and inhibits Pgp-mediated drug transport. Hence, genistein cannot be used in simple assays to distinguish MRP- and Pgp-expressing cells. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:89–93, 1997.

KEY WORDS. multidrug resistance; drug transport; photoaffinity labeling; MRP; tyrosine kinase; phosphorylation

Overexpression of Pgp† in the plasma membrane causes resistance against a variety of chemically unrelated drugs [1]. The available evidence strongly suggests that Pgp is a pump that catalyzes the efflux of drugs from the cells, reducing drug accumulation and hence the access of cytotoxic drugs to their targets [1]. Recently, MRP, another membrane protein that causes multidrug resistance, has been identified. Both Pgp and MRP belong to the ATP-binding-cassette superfamily of membrane proteins [1, 2]. Multidrug resistance produced by overexpression of MRP is phenotypically similar, but not identical, to that caused by overexpression of Pgp [2–4].

It has been reported that the tyrosine kinase inhibitor genistein, as well as other isoflavonoids, selectively reverses MRP-associated multidrug resistance and drug accumulation, without effects on Pgp-expressing cells [5]. Based on these results, it was suggested that elevation in drug accumulation by genistein denotes non-Pgp-mediated multidrug resistance [5, 6]. The aim of the present experiments was to determine whether or not genistein lacks effects on Pgp-mediated drug transport, and hence the validity of its use as a specific probe for MRP-associated drug resistance.

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† *Abbreviations:* Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; PSS, physiologic salt solution; R123, rhodamine 123; DNR, daunorubicin; F_{R123}, intracellular R123 fluorescence; RT-PCR, reverse transcription-polymerase chain reaction.

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MATERIALS AND METHODS

Cell Lines

The human breast cancer cell lines MCF-7 (drug-sensitive [7], BC19/3 (MCF-7 cells transfected with human MDR1, cDNA) [7], and MCF-7/VP (MCF-7 cells selected with etoposide, which overexpress MRP) [8], were grown in improved Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. Doxorubicin, 0.01 μM, was added to the culture medium of BC19/3 cells. The mouse fibroblast cell lines BALB/c-3T3 (drug sensitive) and BALB/c-3T3-1000 (transfected with human *MDR1* cDNA) were grown in Dulbecco's Modified Eagle's Medium with 10% FBS, 1% streptomycin/penicillin, and 1.1 μM vinblastine. Cells were plated on coverslips 1 day prior to the experiments, in the absence of chemotherapeutic agents.

Steady-State R123 and DNR Levels

Accumulation of R123 and DNR were measured essentially as described [9]. Briefly, cells plated on rectangular coverslips were exposed to either 10 μM R123 or 1 or 10 μM DNR for 1 hr, at 37°, in the presence or absence of 200 μM genistein. The control incubation solution, PSS, contained (in mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.5 sodium phosphate, and 8 glucose, was equilibrated with 95% O_2/5% CO_2, and had a pH of 7.42 to 7.43. After washing for 30 sec with an ice-cold probe-free solution containing 100 μM verapamil (to block R123 and DNR efflux during washing) [9], the coverslip-attached cells loaded with R123 or DNR were placed in a cuvette with distilled

water or 1-butanol, respectively. In the experiments using R123 as a probe, emitted light was measured at 535 nm and excitation was at 495 nm. In the experiments using DNR, excitation and emission were 480 and 590 nm, respectively. Measurements were carried out on a CM1T1 spectrofluorometer (Spex Industries, Edison, NJ).

Unidirectional Efflux of R123

Efflux of R123 was performed as previously described [9]. Monolayers, *ca.* 80% confluent, on round coverslips No. 1, were loaded with R123 in the presence or absence of genistein. The concentration of R123 during loading was 2 μM (MCF-7 and BALB/c-3T3) or 10 μM (BC19/3, BALB/c-3T3-1000). Once loading was completed (1 hr in most experiments), the coverslips were mounted in a chamber that was placed on the stage of an inverted microscope (Nikon Diaphot, Nikon, Tokyo) coupled to a spectrofluorometer (CM1T1, Spex Industries). Excitation was at 495 nm, and fluorescence was measured at 535 nm. The decay in F_{R123} from 100 to 150 cells was determined during superfusion with R123-free PSS (≈20 mL/min).

The rate constants (k) for the decay in F_{R123} were calculated from fits of Equation (1) to the data:

$$F_{R123} = F_0 + F_{R123}(0)e^{-kt} \quad (1)$$

where F_0 is the background fluorescence, and $F_{R123}(0)$ is F_{R123} at $t = 0$.

At the fluorescence levels of the present experiments, we have shown that the decrease in F_{R123} follows a single-exponential decay as a function of time, that the rate constants are independent of R123 loading, and that R123 fluorescence is not quenched by dye accumulation in mitochondria [9]. Under the conditions of the present experiments (low fluorescence levels and rapid superfusion to remove extracellular R123), k represents the rate constant for unidirectional efflux of R123 [9].

Preparation of Membranes

Membranes were prepared from cells grown in tissue culture flasks. All steps were performed at 4°. Cells were scraped from the flasks, collected by centrifugation at 1000 *g* for 10 min, and resuspended in homogenizing medium containing 20 mM Tris-HCl, pH 7.2, 250 mM sucrose, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, 10 μM leupeptin, and 0.1 μM pepstatin A. Homogenization was carried out in a Potter-Elvehjem homogenizer with a Teflon pestle (20 strokes at 2500 rpm). The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant was saved. The pellet was resuspended in homogenizing solution, and the centrifugation was repeated. The supernatants were mixed and centrifuged at 10,000 *g* for 20 min to separate mitochondria. The resulting supernatant was centrifuged at 270,000 *g* for 30 min, and the pellet, containing the cell membranes, was stored at −80° in 100 mM mannitol and 25 mM Tris-HCl, pH 7.2.

Western Blot Analysis and Photoaffinity Labeling of Pgp

Immunoblots were performed as described [10] on membranes subjected to 7% SDS-PAGE, and using the anti-Pgp monoclonal antibody C219 (Signet, Dedham, MA). For photoaffinity labeling of Pgp, 50 μg of membrane proteins was incubated with 100 μM vinblastine, 200 μM genistein, or solvent alone (DMSO, 0.1%, v/v). After 30 min, [^{3}H]azidopine (55 Ci/mmol, Amersham, Arlington Heights, IL) was added to a final concentration of 0.4 μM, and the incubation proceeded for 60 min in the dark. Then, the samples were irradiated for 2 min (total energy = 2.4 mJ) at 254 nm (Stratalinker, Strategene, La Jolla, CA). After irradiation, the samples were centrifuged at 270,000 *g* for 30 min, and the pellet was resuspended in SDS buffer and electrophoresed (7 or 10% SDS-PAGE). Gels were fixed in 7% acetic acid/25% methanol, incubated for 30 min with AMPLIFY® (Amersham), and dried. Autoradiographies were performed at −70° for 1–3 days.

Expression of mRNA by RT-PCR

Poly(A)$^+$ mRNA was isolated with the Oligotex Direct mRNA Kit (Qiagen, Chatsworth, CA) and 200 ng poly(A)$^+$ mRNA was reverse transcribed with the RT-PCR Kit (Stratagene), according to the manufacturers' instructions. Target sequences of MRP, MDR1 and β-actin were amplified by PCR using the following primers: 5′-GATGGACAGGATATTAGGACC-3′ (sense MDR1), 5′-ATGGCACAAAATACACCAACA-3′ (antisense MDR1), 5′-GTCACGTGGAATACCAGCAAC-3′ (sense MRP, primer set 1) and 5′-CTCATTCAGCTCGTCTTGTCC-3′ (sense MRP, primer set 2), and 5′-GTCCACAGACATGAGGTTGAC-3′ (antisense MRP, primer sets 1 and 2). PCR was performed with 20% of the reverse transcription reaction, using *Taq* polymerase (Promega, Madison, WI), 30 cycles of 1 min at 94°, 1 min at 45°, and 2 min at 72°. One-tenth of each PCR reaction was electrophoresed on a 1% agarose gel, and stained with ethidium bromide.

Statistics

Data are presented as means ± SEM. Statistical comparisons were done by Student's *t*-tests for paired or unpaired data as appropriate. Differences were considered significant when $P < 0.05$.

RESULTS

The immunoblots shown in Fig. 1A confirmed the expression of Pgp in the multidrug-resistant cell lines BC19/3 and BALB/c-3T3-1000, and the absence of detectable expression in the parental cell lines MCF-7 and BALB/c-3T3 [7,

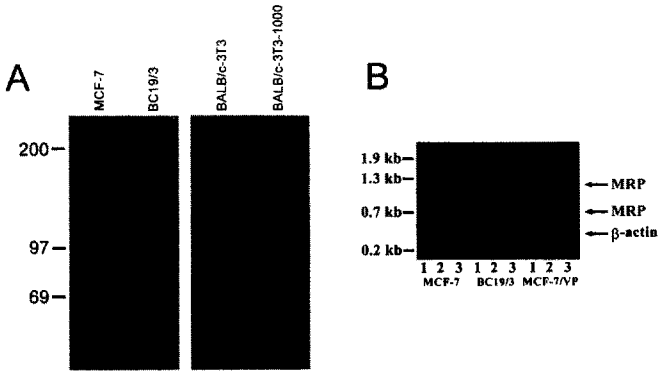

FIG. 1. (A) Western blot analysis of membrane proteins with the anti-Pgp antibody C219. Molecular weights (kDa) are indicated on the left. BC19/3: 25 μg; MCF-7: 200 μg; BALB/c-3T3: 200 μg; BALB/c-3T3-1000: 12.5 μg. (B) Expression of MRP mRNA in drug-sensitive and multidrug-resistant human breast cancer cells. cDNAs were obtained from 200 ng of poly(A)$^+$ mRNA, and specific target sequences were amplified by PCR. One-tenth of each PCR reaction was electrophoresed in a 1% agarose gel and stained with ethidium bromide. For all cell lines, lanes 1 and 2: PCR with MRP primers, sets 1 and 2, respectively (see Materials and Methods). Lane 3: PCR with β-actin primers. The two bands labeled MRP have approximately 1.2 and 0.7 kb, the expected size for DNAs amplified using specific MRP primer sets 1 and 2, respectively (see Materials and Methods). A fragment from β-actin cDNA was amplified to test for mRNA integrity and provide with an approximate reference point to estimate mRNA MRP presence.

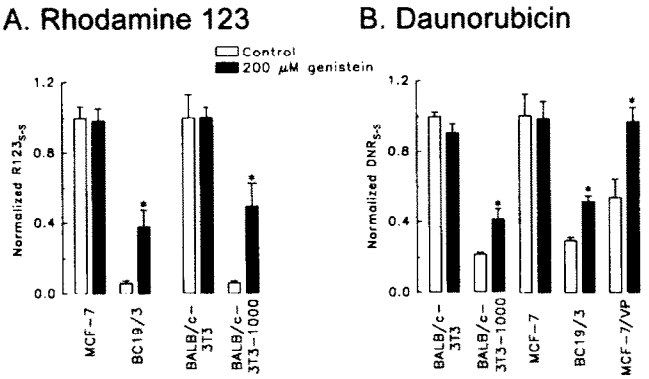

FIG. 2. Steady-state intracellular levels of R123 (A) and DNR (B). Experiments were performed in the absence (DMSO, 0.1%, v/v) or presence of 200 μM genistein. The concentrations of R123 and DNR in the loading solutions were 10 and 1 μM, respectively. Results with 10 μM DNR were similar (data not shown). Drug accumulation was normalized to the average uptake in the corresponding parental cell line. (*) $P < 0.05$ compared with control. Data are means ± SEM of 5–19 experiments.

10]. The absence of MRP mRNA detection in BC19/3 cells is illustrated in Fig. 1B. Under the conditions of the experiments, MRP mRNA was detected in multidrug-resistant MCF-7-derived cells that overexpress MRP (MCF-7/VP [8]), but not in MCF-7 and BC19/3 cells. Using the same methodology, MDR1 mRNA was readily detected in BC19/3 and BALB/c-3T3-1000 cells, but not in MCF-7, MCF-7/VP, or BALB/c-3T3 cells (data not shown). The data in Fig. 1B indicate that the multidrug resistance phenotype in BC19/3 cells is not associated with overexpression of MRP, as expected for a cell line derived from MCF-7 cells and transfected with *MDR1* cDNA. Figure 2 illustrates the effects of genistein on drug accumulation. Genistein elevated R123 accumulation only in the multidrug-resistant cell lines. The increases in R123 accumulation in BC19/3 and BALB/c-3T3-1000 were *ca.* 7- and 10-fold, respectively. It has been claimed that genistein at a concentration of 200 μM has no effect on DNR accumulation in cells expressing Pgp [5, 6]. To determine whether or not the effect of genistein was specific for R123 accumulation, we measured the effects of genistein on steady-state levels of DNR in mouse and human breast cancer cell lines. Figure 2 shows that genistein elevated DNR accumulation in the multidrug-resistant Pgp-expressing cells (BC19/3 and BALB/c-3T3-1000), as well as in the MRP-expressing MCF-7/VP cells, without effects in drug-sensitive cells (MCF-7, and BALB/c-3T3).

Because Pgp expression results in elevation of R123 efflux without effect on unidirectional influx [9], we determined whether the elevation in steady-state R123 levels by genistein could be ascribed to inhibition of R123 efflux. Panels A and B of Fig. 3 show that genistein inhibited R123 efflux in BALB/c-3T3-1000 cells. Figure 4A illustrates the rapid reversibility of the inhibition of R123 efflux by genistein. In the experiment shown, the cells were loaded with R123 in the presence of genistein, and the decrease in F_{R123} (a function of the efflux rate) was initially measured in the

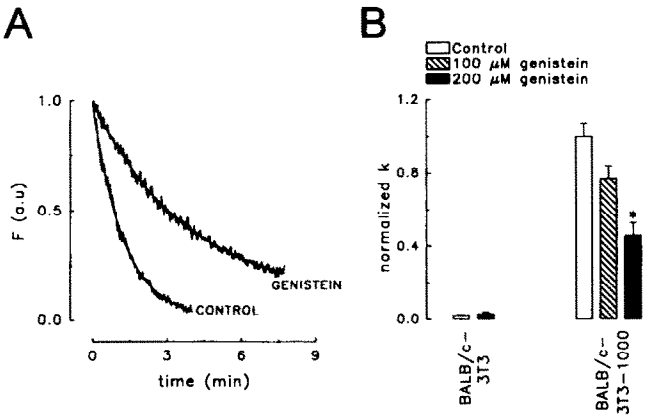

FIG. 3. Effects of genistein on R123 efflux. (A) Time courses of decay of F_{R123} in BALB/c-3T3-1000 in the absence or presence of 200 μM genistein. Cells were loaded for 1 hr in PSS with 10 μM R123, with or without genistein, and washed with PSS with or without genistein, without R123. Single-exponential fits are superimposed on the data. (B) Rate constants (k) for R123 efflux (see Materials and Methods). Experiments were performed in the absence (DMSO) or presence of genistein. Rate constants were normalized to the average value in BALB/c-3T3-1000 in the absence of inhibitors (0.76 ± 0.08 min^{-1}, N = 12). Key: (*) $P < 0.05$ compared with control. Data are means ± SEM of 4–16 experiments.

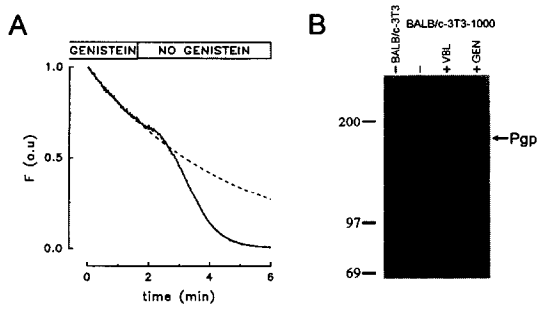

FIG. 4. Reversibility of the effect of genistein on R123 efflux and effect of genistein on photoaffinity labeling of Pgp by [^{3}H]azidopine. (A) BALB/c-3T3-1000 cells were loaded with R123 in the presence of genistein, as in Fig. 2A, and initially washed with a solution containing genistein. At 1.5 min, genistein was removed. The dashed line is the best fit of Equation 1 to the data in the presence of genistein. The trace is representative of 8 experiments. (B) Photoaffinity labeling of membranes (50 μg) with [^{3}H]azidopine, in the absence (solvent alone, DMSO, -) and presence of 100 μM vinblastine (+VBL) or 200 μM genistein (+GEN). Molecular weights (kDa) are indicated at the side of the blots.

presence of genistein. The tyrosine kinase inhibitor was then removed, which resulted in a rapid increase (<2 min) in the rate of decrease of F_{R123}.

Because of the hydrophobic and aromatic nature of genistein, it is possible that it could interact directly with Pgp [11, 12]. Hence, we determined the effect of genistein on photoaffinity labeling of Pgp by the substrate [^{3}H]azidopine. The results of these experiments, shown in Fig. 4B, indicated that genistein produced an inhibition of the labeling of Pgp by [^{3}H]azidopine. Densitometric analysis showed 75 ± 4 and 42 ± 15% inhibition of [^{3}H]azidopine labeling of Pgp by 100 μM vinblastine and 200 μM genistein, respectively (N = 3; both changes were significant, $P < 0.05$).

DISCUSSION

Versantvoort *et al.* [5, 6] have reported an increase in DNR accumulation and changes in intracellular drug distribution by the isoflavonoid genistein in MRP-expressing cells [5, 6]. No effects of genistein were observed in Pgp-expressing cells, and it was therefore suggested that genistein could be used to distinguish MRP- from Pgp-expressing tumors [5, 6]. Our observations differ from those of Versantvoort *et al.*, since we observed an increase in steady-state R123 and DNR accumulation as well as a corresponding inhibition of Pgp-mediated R123 efflux in human breast cancer cells and mouse fibroblasts. It could be argued that a complete reversal of the decrease in DNR accumulation of MRP-expressing cells, as opposed to a partial effect on Pgp-expressing cells, could be of value in distinguishing different types of multidrug resistance (see Fig. 2). However, incomplete reversal of the decrease in DNR accumulation in MRP-expressing cells has been described previously [5]. Therefore, genistein cannot be used reliably to distinguish Pgp- and MRP-expressing multidrug-resistant cells.

In MRP-expressing cells, block of drug transport by genistein via inhibition of tyrosine kinases is unlikely, because structurally related compounds that produce poor inhibition of tyrosine kinases also increase DNR accumulation [5]. Recent experiments also showed that phosphoserine, but not threonine and tyrosine are phosphorylated in MRP *in vivo* [14]. The inhibition of tyrosine kinase activity by genistein is also unlikely to play a role in the block of Pgp-mediated drug transport in our experiments. This conclusion is based on the lack of phosphorylation of tyrosine residues of Pgp *in vivo* [15], and on the rapid reversal of the block of Pgp-mediated R123 efflux by genistein (Fig. 4A). The block of R123 efflux was fully reversed in *ca.* 2 min, which seems too fast for a phosphorylation-mediated effect [15]. In this context, the potent tyrosine kinase inhibitor lavendustin A (10 μM [16]) had no effect on R123 accumulation in BALB/c-3T3-1000 cells (data not shown), further supporting a mechanism of Pgp inhibition independent of inhibition of tyrosine kinases.

Since increased drug accumulation by genistein has been documented [5, 6] (see also Fig. 2), a possible interpretation of the present results would be that part of the multidrug-resistance phenotype of BC19/3 and BALBc-3T3-1000 is due to MRP overexpression. In this case, the elevation in drug accumulation by genistein would be the result of MRP inhibition. Several pieces of information argue against this hypothesis. First, BC19/3 and BALBc-3T3-1000 cells were obtained by transfection of *MDR1* cDNA with subsequent drug selection, and not by drug selection alone. Second, BC19/3 and BALBc-3T3-1000 cells extrude R123 very efficiently, and R123 is known to be a poor MRP substrate [17] (Schneider E, Altenberg GA and Cowan K, unpublished data). Third, verapamil is an efficient modulator of drug resistance in BC19/3 cells. MRP-associated multidrug resistance is not reversed efficiently by classic multidrug-resistance modulators such as verapamil [4]. Fourth, overexpression of MRP mRNA could not be detected by RT-PCR in BC19/3 cells. The available evidence strongly suggest that MRP plays no role in the multidrug-resistance phenotype of BC19/3 and BALBc/3T3-1000 cells.

In MRP-expressing cells, genistein acts as a competitive inhibitor of DNR efflux, and also reduces intracellular ATP levels. The latter effect did not occur at concentrations lower than 400 μM [6]. This ATP depletion by genistein was observed in only one of the two Pgp-expressing cells studied (KB8-5, but not SW-1573/2R160) [6]. One possible explanation for the observed inhibition of Pgp-mediated R123 efflux in our studies could be that the cell lines that we employed are more sensitive to the ATP-depleting effect of genistein that those used in previous studies [6]. However, the demonstration that genistein blocked Pgp-photoaffinity labeling by [^{3}H]azidopine, measured *in vitro* in the absence of ATP, suggests that genistein could block Pgp-mediated drug efflux by direct interaction with Pgp.

The lower efficiency of genistein, as compared with vinblastine, in blocking [^{3}H]azidopine labeling of Pgp, correlates well with the smaller inhibition of R123 efflux by genistein (*ca.* 50–60% at 200 μM), compared with vinblastine (95–100% at 100 μM, see Fig. 3 and Ref. 9).

A decrease in drug accumulation by some flavonoids has been observed previously in HCT-15 cells, which express Pgp [18], and has been ascribed to activation of Pgp by hydroperoxides [19]. On the other hand, quercetin and other flavonoids potentiated the cytotoxicity of doxorubicin and reduced R123 efflux in Pgp-expressing human breast cancer cells [20]. The results of the present work, together with previous observations by others [5, 6, 18–20] indicate that flavonoids have multiple effects, including ATP depletion and elevation of hydroperoxides, and in addition they possibly bind to Pgp. These effects can result in variable responses to flavonoids in different cell types.

In summary, the present results show that genistein can inhibit Pgp-mediated drug transport, and therefore it cannot be used to distinguish MRP- from Pgp-expressing tumors in simple drug accumulation assays. A possible mechanism for the inhibition of R123 efflux by genistein is direct interaction with Pgp, as suggested by the inhibition of photoaffinity labeling of Pgp by [^{3}H]azidopine, but other mechanisms cannot be ruled out.

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