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Quercetin potentiates the effect of Adriamycin in a multidrug-resistant MCF-7 human breast-cancer cell line: P-glycoprotein as a possible target

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Abstract. This study demonstrates that the flavonoid quercetin (Q), a plant-derived compound with low toxicity in vivo, greatly potentiates the growth-inhibitory activity of Adriamycin (ADR) on MCF-7 ADR-resistant human breast cancer cells. The effect of Q was dose-dependent at concentrations ranging between 1 and 10 μ*M*. Since ADR resistance in these cells is associated with the expression of high levels of P-glycoprotein (Pgp), we evaluated the effect of Q and related flavonoids of Pgp activity in cytofluorographic efflux experiments with the fluorescent dye rhodamine 123 (Rh 123). Our results indicate that Q and 3-OMe Q (3′,4′,7-trimethoxyquercetin) but not the 3-rhamnosylglucoside of Q (rutin) inhibit the Pgp pump-efflux activity in a dose-related manner. Moreover, 10 μ*M* Q reduces the expression of the immunoreactive Pgp in MCF-7 ADR-resistant cells as evaluated by cytofluorimetric assay. In conclusion, these findings provide a further biological basis for the potential therapeutic application of Q as an anticancer drug either alone or in combination with ADR in multidrug-resistant breast tumor cells.

Key words: Quercetin – Adriamycin – Multidrug resistance – Human breast cancer cells

Introduction

Recently we demonstrated that the flavonoid quercetin (Q), a plant-derived compound [13] with low toxicity in vivo [15], has a powerful antiproliferative activity on fresh human leukemic cells [20] and on several human cancer cell lines in vitro [30, 33, 34, 37], including a multidrug-resistant human breast-cancer cell line [36]. Moreover, Q synergizes with some chemotherapeutic agents such as cisplatin [16, 35, 37] and cytosine arabinoside (ara-C) [39]. Among the various anticancer agents available, anthracyclines have a place of primary importance, but their clinical utility is often limited by the development of drug resistance, which represents a major reason for treatment failures in a variety of cancers [14]. One type of drug resistance – multidrug resistance (MDR) – is associated with overexpression of a membrane protein termed P-glycoprotein (Pgp), which leads to the efflux of drugs from the cells [1, 17].

The aim of the present study was to verify whether Q could synergize the inhibitory action of Adriamycin (ADR) by reversing ADR resistance in vitro. We used the human breast-cancer cell line MCF-7, which does not express Pgp, and its ADR-resistant variant MCF-7 ADRr, which expresses the MDR phenotype.

Materials and methods

Cell culture. The breast-cancer cell line MCF-7 was originally obtained from the Michigan Cancer Foundation (Detroit, Mich.); the variant MCF-7 ADRr, selected as previously described [41], was kindly provided by Dr. Kenneth H. Cowan (National Cancer Institute, NIH, Bethesda, Md.). Both cell lines were grown in monolayer culture in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 200 units of penicillin/ml. Cells were trypsinized weekly and plated at a density of 8×10^4 cells/ml. They were incubated at 37° C in an atmosphere of 5% CO_2 95% air and high relative humidity.

Growth experiments. Cells were plated in six-well flat-bottom plates (Falcon 3046; Becton Dickinson, Lincoln Park, N. J.) at a concentration of 10^5 cells/ml in MEM supplemented as described above. After 24 h, the medium was replaced with fresh medium containing the compounds to be tested. Q (3,3′,4′,5,7-pentahydroxyflavone) and rutin (the 3-rhamnosylglucoside of Q) were purchased from Aldrich (Steinhein, Germany); 3-OMe Q (3′,4′,7-trimethoxyquercetin) was kindly provided by Bergamon (Ariccia, Rome). The compounds were

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added from an absolute ethanol (Q) or dimethylsulfoxide (DMSO; rutin, 3-OMe Q) stock solution and the control cells were treated with the same amount of vehicle alone. The final ethanol and DMSO concentrations never exceeded 1% and 0.5% (v/v), respectively. Stock solutions of ADR were made in distilled water and frozen at –20° C until their use. Quadruplicate hemocytometer counts of triplicate culture dishes were performed at various times.

Staining with rhodamine 123. Cells were labeled by incubation with Rhodamine 123 (Rh 123; Sigma, St. Louis, Mo.) at 0.5 μg/ml for 30 min at 37° C [22]. After incubation, cells were washed twice with ice-cold complete medium and used for efflux-pump studies. Efflux-kinetics studies were performed by incubating cells (10^6 cells/ml) in Rh 123-free complete medium for 3 h at 4° and 37° C. Efflux-inhibition studies were performed by incubating cells (10^6 cells/ml) in Rh 123-free complete medium for 3 h at 37° C in the presence of Q, 3-OMe Q, rutin, or vehicle alone.

Flow-cytometric analysis of Rh 123-labeled cells. MCF-7 ADRr cells were analyzed by a Facscan flow cytometer (Becton Dickinson) operating at 488 nm; a minimum of 10,000 events were acquired in the list mode. The analysis was performed according to the method reported by Coon et al. [6]. Briefly, the marker was placed immediately to the left of the green fluorescence peak of cells stained with Rh 123 at time zero (cells washed with Rh 123-free ice-cold medium). The marker was kept at the same location and cells that had shifted to the left of the marker after 3 h of incubation were considered to have effluxed Rh 123.

Immunocytofluorimetric assay. MCF-7 ADRr cells were plated at a concentration of 6×10^4 cells/ml in MEM supplemented as described above. At 24 h after plating, the medium was replaced with fresh medium containing 10 μ*M* Q or vehicle alone. After 24 h, cells were detached with a cell scraper, adjusted to a concentration of 3×10^5 cells/ml, and centrifuged at 1,500 *g* for 5 min at 20° C. The cell pellet was resuspended in 20 μl anti-p170 antibody (MM 4.17) [2] as a supranatant diluted 1:10 in phosphate-buffered saline (PBS) and then incubated at 4° C for 30 min under protection from light. The cells were next washed in ice-cold PBS containing 10 m*M* ethylenediaminetetraacetic acid (EDTA) by centrifugation at 1,500 *g* for 7 min. After the washing step, the cell pellet was incubated under protection from light at 4° C for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG diluted 1:10 in PBS. At the end of the incubation period, the cells were resuspended in 500 μl PBS and then centrifuged and 10 μg propidium iodide/ml (Sigma) was added to test cell viability. Only the cells excluding the dye were analyzed by the Facscan flow cytometer (Becton Dickinson).

Statistical analysis. Student's *t*-test for unpaired samples was performed to evaluate the significance of the shift for the change in intracellular Rh 123 levels after treatment with different flavonoids. The Kolmogorov-Smirnov statistics test [42] was used to evaluate the difference between the cumulative frequencies of Pgp fluorescence intensities from control and Q-treated samples.

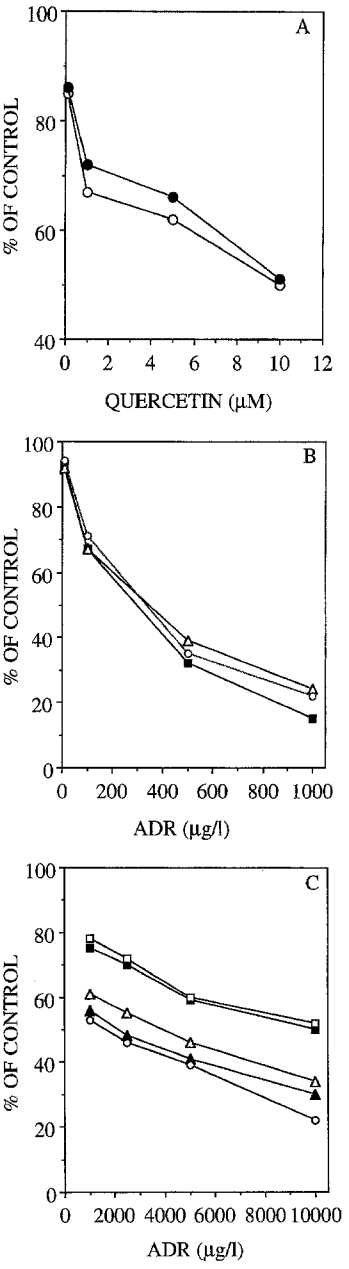

Fig. 1A. Effect of various concentrations of Q on MCF-7 (–●–) and MCF-7 ADRr (–○–) cell proliferation. Effect of various concentrations of ADR alone (■) or in combination with 1 (△), 5 (▲), and 10 μ*M* Q (○) and 10 μ*M* rutin (□) on MCF-7 (**B**) and MCF-7 ADRr (**C**) cell proliferation. Cell counts were performed after 3 days of exposure to the compounds as described in Materials and methods. Each point represents the mean value for three different experiments performed in triplicate; the SD of the means were less than 10% and were omitted

Results

Q produced a dose-dependent growth inhibition of MCF-7 and MCF-7 ADRr cells (Fig. 1A). In MCF-7 cells, Q at fixed concentrations (1 and 10 μ*M*) did not synergize the growth-inhibitory action of ADR in the concentration range between 10 and 1000 μg/l (Fig. 1B). In MCF-7 ADRr cells, Q at fixed concentrations (1, 5, and 10 μ*M*) potentiated the growth-inhibitory action of ADR (Fig. 1C). Rutin, which was ineffective in inhibiting MCF-7 ADRr cell growth (data not shown), did not potentiate the growth-inhibitory action of ADR (Fig. 1C).

Figure 2 shows that Q produced a dose-dependent decrease in the ADR IC_{50} (the ADR concentration capable of inhibiting 50% of cell growth) in MCF-7 ADRr cells. The same figure shows the reversal of ADR resistance by Q, expressed as the ratio of the IC_{50} for ADR alone to the IC_{50} for ADR in the presence of Q (MDR ratio). Q enhanced the MDR ratio in a dose-dependent manner (from 1 to 10 μ*M*). In contrast, Q at both 1 and 10 μ*M* did not modify either the IC_{50} for ADR or the MDR ratio in MCF-7 cells (data not shown).

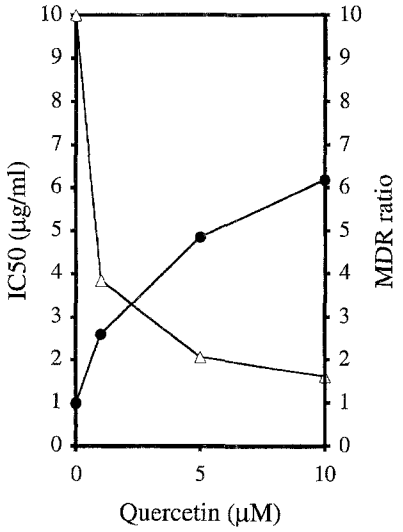

Fig. 2. Potency of the ADR-sensitizing activity of Q in MCF-7 ADRr cells. On the *left y-axis* is shown the decrease in IC_{50} (△, the ADR concentration capable of inhibiting 50% of cell growth) as a function of Q concentration; on the *right y-axis* is shown the MDR ratio (●, the IC_{50} for ADR alone divided by the IC_{50} for ADR in the presence of Q) as a function of Q concentration. Each point represents the mean value for triplicate experiments performed in duplicate; the SD of the means were less than 10% and were omitted

Since ADR resistance has been reported to be associated with increased production of Pgp [38], we studied the effect of Q on Pgp activity by flow cytometry using the fluorescent dye Rh 123. The cytofluorimetric pattern of an efflux experiment on MCF-7 ADRr cells loaded with Rh 123 is shown in Fig. 3. The cells were incubated at 4° and 37° C, and the cells that shifted to the left of the cursor (set at 4° C) were considered to have effluxed Rh 123. In a dose-related manner, Q reduced the number of cells that shifted to the left of the cursor at 37° C. We also tested 3-OMe Q and rutin in similar efflux experiments. Figure 4 shows that at 37° C, 3-OMe Q as well as Q blocked the exclusion of Rh 123 in MCF7-ADRr cells in a dose-related manner (1–10 μ*M*), maintaining the intracellular levels of Rh 123 at a value similar to that observed at 4° C. In contrast, rutin was completely ineffective.

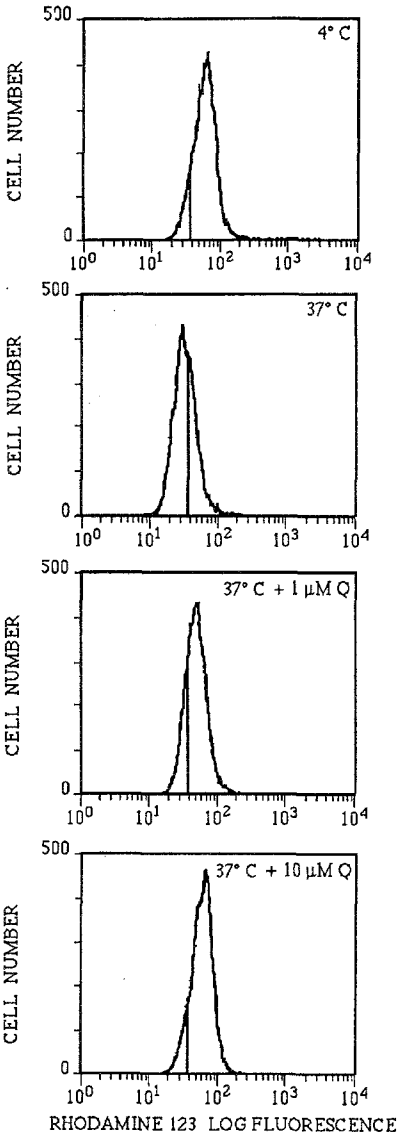

Fig. 3. Fluorescence histograms showing the Rh 123 efflux from MCF-7 ADRr cells either untreated or treated with 1 or 10 μ*M* Q for 3 h. *Cells to the left of the cursor* (set at 4° C) were considered to have effluxed Rh 123. All histograms represent the results obtained in one of three similar experiments performed

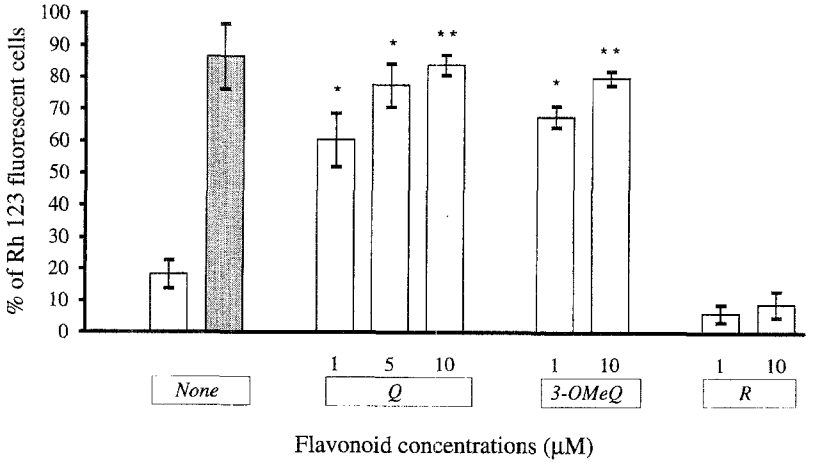

Fig. 4. Effect of various concentrations of Q, 3-OMe Q, and rutin (*R*) on Rh 123 efflux from MCF 7 ADRr cells at 37° C. The results are expressed as the percentage of Rh 123-fluorescent cells relative to the total number of cells counted. Control columns (vehicle alone, *None*) indicate the percentage of Rh 123-fluorescent cells observed at 37° C (*light column*) and 4° C (*dark column*). The results are the mean values ± SD for 3 separate experiments. * $P < 0.01$, ** $P < 0.001$ (Student's one-sided *t*-test)

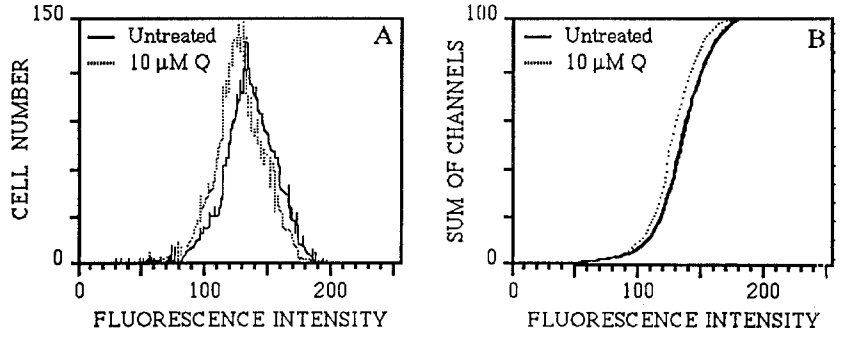

Fig. 5. **A** Fluorescence histograms of MCF-7 ADRr cells either untreated or treated with 10 μ*M* Q for 24 h and then reacted with anti-Pgp monoclonal antibody MM4.17. **B** The curves of the cumulative frequencies of Pgp fluorescence intensities for control and Q-treated cells were calculated from the data shown in **A**

For evaluation of Pgp expression, MCF7-ADRr cells were analyzed immunocytofluorimetrically with an anti-Pgp monoclonal antibody (MM 4.17). This antibody is highly suitable for flow-cytometric analysis, as it is directed against an external epitope of Pgp and can therefore be applied to unfixed cells in suspension [2]. As shown in Fig. 5 A, the fluorescence histogram of Q-treated cells was shifted to the left as compared with that of untreated cells. In a comparison of the curves of the cumulative frequencies (Kolmogorov-Smirnov test, $P < 0.05$) of fluorescence intensities from the control and Q-treated cells (Fig. 5 B), it appeared that 10 μ*M* lowered the amount of immunoreactive Pgp.

Discussion

Our results show for the first time that Q synergizes the inhibitory activity of ADR on the growth of MCF-7 ADRr human breast cancer cells. In these cells, Q used at concentrations ranging from 1 to 10 μ*M* displays a dose-response relationship for reversing ADR resistance. In contrast, Q does not potentiate the growth-inhibitory activity of ADR in the parental cell line, which does not express Pgp. These findings suggest a role of Pgp in determining the capacity of Q to synergize the growth-inhibitory effect of ADR.

Pgp does have an important role in the expression of the ADR-resistant phenotype [38], and MCF-7 ADRr cells express high levels of Pgp [11]. Thus, we evaluated the effect of Q on Pgp activity in efflux experiments with Rh 123. The fluorescent dye Rh 123 has been found to be transported by Pgp [10, 27] and is now recognized as a useful tool for studying Pgp efflux activity by flow cytometry. This technique, unlike other biochemical methods, provides information concerning the activity of Pgp.

Although the mechanism by which Q reverses ADR resistance in vitro remains to be fully clarified, our results indicate that this flavonoid can act by modulating the activity of Pgp in MCF-7 ADRr cells. Our cytofluorimetric results showed that Q and 3-OMe Q reduced the efflux of Rh 123 from MCF-7 ADRr cells in a dose-dependent manner. Furthermore, rutin, which was ineffective in synergizing the inhibitory activity of ADR on cell growth, did not prevent Rh 123 efflux.

Treatment strategies to overcome drug resistance have included various chemosensitizers [3, 9, 12, 21, 25, 28, 31]. Several in vitro studies have suggested that the mechanism of reversing MDR with these agents include competition with cytotoxic drug-binding sites on Pgp [31]; modulation of other intracellular targets such as topoisomerase [8], DNA polymerase [24], or enzymes necessary to the synthesis of glutathione (GSH) [7, 19, 32]; and alterations of cell-membrane lipid integrity [29].

At present, our results do not explain the mechanism by which Q modulates Pgp activity and reverses MDR. A direct interaction between Q and Pgp may be possible. Alternatively, the capacity of flavonoids to block Pgp activity may be explained, as in the case of other MDR modulators such as antiestrogens [4, 5], by an interaction with the membrane phospholipids altering the lipid packing density and, thereby, the diffusion rate of certain drugs. In this respect, the reported stabilizing effect of Q on cell membranes [23] is noteworthy.

Recently it was demonstrated in human HepG2 cells that Q at 100 μ*M* suppressed the transcription activation of the *mdr1* gene after exposure to arsenite [18]. Accordingly, our preliminary immunocytofluorimetry studies indicate that Q reduces the amount of Pgp. Thus, it is possible that besides reducing Pgp efflux activity, this flavonoid can modulate Pgp expression. It should be noted that these cells contain type II estrogen-binding sites, which bind Q [30, 33, 34], and it therefore cannot be excluded that these receptor sites may mediate flavonoid activity on Pgp expression.

Recent clinical interest has largely focused on the reversal of MDR by drugs that interact with the MDR transport system [25, 28, 31]. Although a variety of agents reverse MDR in vitro, virtually all suffer from a common problem: the concentrations of unbound drug necessary to reverse MDR in vitro cannot be maintained or achieved in humans without exceeding the maximum tolerated dose of the sensitizing agent. Verapamil, one of the calcium-channel blockers most thoroughly investigated as a modulator of MDR, has been used to assist doxorubicin treatment [26, 31]; nevertheless, its cardiotoxicity has prevented the use of adequate doses [40].

The identification of new, less toxic chemosensitizers should open new perspectives in cancer treatment. Interestingly, a plasma concentration of 12 μ*M* Q, which is similar to that effective in vitro in modulating Pgp activity, was obtained following the i.v. injection of 100 mg Q without producing any apparent side effect [15].

In conclusion, our data provide a further biological basis for the potential therapeutic application of Q and related flavonoids in cancer therapy, either alone or in combination with other conventional cytotoxic drugs.

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