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Quercetin inhibits the growth of a multidrug-resistant estrogen-receptor-negative MCF-7 human breast-cancer cell line expressing type II estrogen-binding sites

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Summary. It has been demonstrated that the flavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone; Q) inhibits the growth of several cancer cell lines. There is evidence suggesting that the antiproliferative activity of this substance is mediated by the so-called type II estrogen-binding site (type II EBS). We looked for the presence of type II EBS and the effect of Q on the proliferation of an Adriamycin-resistant estrogen-receptor-negative human breast-cancer cell line (MCF-7 ADRr). By whole-cell assay using estradiol labelled with 6,7-tritium ([^{3}H]-E2) as a tracer, we demonstrated that MCF-7 ADRr cells contain type II EBSs. Competition analysis revealed that diethylstilbestrol (DES) and Q competed with similar potency for [^{3}H]-Es binding to type II EBSs. The antiestrogen tamoxifen (TAM) competed for type II EBSs, albeit to a lesser extent than either DES or Q. Growth experiments demonstrated that Q and DES exerted a dose-dependent inhibition of cell proliferation in the range of concentrations between 10 nM and 10 μM, whereas TAM was less effective. Q could also inhibit colony formation in a clonogenic assay. Our results indicate that multidrug-resistant estrogen-receptor-negative MCF-7 cells express type II EBSs and are sensitive to the inhibitory effect of Q. This substance could be the parent compound of a novel class of anticancer agents.

Introduction

Flavonoids are a large class of natural substances with a wide variety of biological actions [8]. In particular, the flavonoid quercetin (Q) exerts powerful growth-inhibitory activity on human leukemic cells [18] and on several human cancer cell lines [21, 29, 30]. Therefore, it has been suggested that this substance might be the parent compound of a novel class of anticancer agents. This is further supported by the following observations: (1) a dietary supplement of Q inhibits the development of 7-12-dimethylbenzanthracene- and *N*-nitrosomethylurea-induced rat mammary cancer [34], suggesting that Q could also be active in vivo; moreover, Q and certain related flavonoids may be inhibitors of experimental skin carcinogenesis [4]; and (2) Q exhibits a synergistic antiproliferative effect with cisplatin (C-DDP) and busulphan both in vitro [13, 14, 31] and in vivo [15].

It has been reported that Q binds to the so-called type II estrogen-binding sites (type II EBSs) originally described by Clark et al. [5] in the rat uterus. Although these sites display the same steroid and tissue specificity shown by the "true" estrogen receptor (ER) they are distinct from the latter. They are reported to occur at higher concentrations than ERs, although they exhibit a lower apparent affinity dissociation constant (K_d, 10–20 nM) for E2 than do ERs (K_d, 0.2–1 nM). Moreover, their presence has been described in many primary human tumors [2, 18, 20, 26, 29].

Although the mechanism underlying the antineoplastic activity of Q remains to be fully clarified, there is evidence suggesting that the action of this substance is mediated by its interaction with type II EBSs. This possibility is supported by the demonstration that in rat uterus, type II EBSs are occupied in vivo by a flavonoid-like ligand with growth-inhibitory activity [19]. Furthermore, it has recently been shown that in the human breast-cancer cell line MCF-7, flavonoids bind to type II EBSs and inhibit cell growth by mimicking the endogenous ligand [22].

To gain more information on the therapeutic potential of Q, we used a variant of MCF-7, which is Adriamycin-resistant and expresses the phenotype of multidrug resistance (MCF-7 ADRr) [6]. Moreover, this cell line is associated with a loss of the ER, resulting in resistance to hormonal agents and the expression of hormone-independent tumor formation [35]. In the present study we demonstrated that MCF-7 ADRr cells express type II EBSs and that their growth is inhibited by Q.

Materials and methods

Cell culture. The breast-cancer cell line MCF-7 ADRr, selected as previously described [35], was kindly provided by Dr. K. H. Cowan

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(National Cancer Institute, NIH, Bethesda, Md.). Cells were grown in monolayer culture in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 200 IU penicillin/ml. Cells were trypsinized weekly and plated at a density of 8 × 10 E4 cells/ml; they were then incubated at 37 °C in an atmosphere containing 5% CO_2 and 95% air under high humidity.

Growth experiments. Cells were plated in 6-well flat-bottom plates (Falcon 3046; Becton Dickinson, Lincoln Park, N. J.) at a concentration of 1 × 10 E5 cells/ml in MEM supplemented as described above. After 24 h, the medium was replaced with fresh medium containing the compounds to be tested. Diethylstilbestrol (DES) was obtained from Sigma (Deisenhofen, FRG), tamoxifen (TAM) was a gift from ICI-Italia (Milano, Italy) and Q (3,3′,4′,5,7-pentahydroxy-flavone) was purchased from Aldrich (Steinhein, FRG). The compounds were added from an absolute ethanol stock solution and the control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceeded 1% (v/v) in either control or treated samples. Quadruplicate hemocytometer counts of triplicate culture dishes were performed at the times indicated in the figures.

Culture assay for colony-forming cells. In all, 1 ml under-layer constituting 0.5% agar in Iscove modified Dulbecco's medium (IMDM) containing 10% FCS, 200 IU penicillin/ml and the compounds to be tested were plated in 35-mm plastic petri dishes. Before plating, MCF-7 ADRr were passed through 25-gauge needles to yield a single-cell suspension and then suspended in 0.3% agar in Iscove medium supplemented as above and containing the drugs to be tested at the indicated concentrations. Each culture received 2 × 10 E3 cells/ml. Cultures were incubated at 37° C in a humidified atmosphere containing 7.5% CO_2 and were examined with an inverted-phase microscope. At the 2nd and 6th day of culture, the medium with 20% FCS and drugs was added to the double layer of agar. The colonies (aggregations of ⩾30 cells) appeared at 14–20 days after plating.

Type II EBS analysis in MCF-7 ADRr cells. Type II EBSs were measured by a whole-cell assay as previously described [27], with slight modifications. MCF-7 ADRr cells were plated into Multiwell TM (Falcon 3047) at a concentration of 5 × 10 E4 cells/ml in MEM supplemented as described above. After 24 h, the cells were incubated at 4° C for 2.5 h in fresh medium without serum that contained increasing concentrations (4–50 nM) of [^{3}H]-E2 (40 Ci/mmol; Amersham, UK) either alone or in the presence of a 100-fold molar excess of DES. At the end of the incubation period, cells were rapidly washed twice with ice-cold MEM and then incubated in 1 M NaOH for 30 min at 50° C. Radioactivity was measured by a liquid scintillation spectrometer (LS-7000; Beckman, Palo Alto, Calif.).

Specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence of DES (non-specific binding). Non-specific binding was linear and accounted for about 40% of total binding at saturating steroid concentrations (i.e. 35–40 nM). Results were expressed as the number of binding sites per cell. The conversion of radioactivity to the number of sites per cell was accomplished by determining the amount of [^{3}H]-E2 bound in aliquots derived from a known number of cells and then applying Avogadro's number.

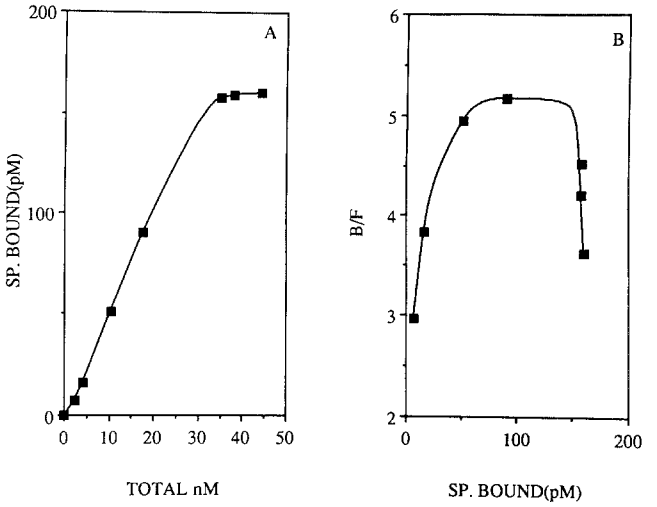

Fig. 1. A Specific binding of [^{3}H]-E2 as a function of tracer concentration in MCF-7 ADRr cells. Specifically bound (*SP. BOUND*) [^{3}H]-E2 was measured as described in Materials and methods. **B** Scatchard analysis of data from Fig. 1 A

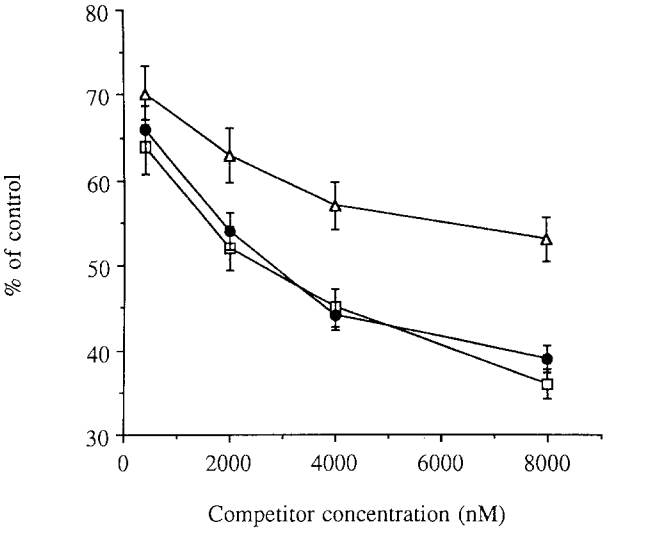

Fig. 2. Competition of DES (□), Q (●) and TAM (△) for type II EBS in MCF-7 ADRr cells. Cells were incubated at 4° C for 2.5 h with [^{3}H]-E2 (40 nM) plus or minus the indicated concentrations of competitors. Results are expressed as the percentage of [^{3}H]-E2 bound in the absence (100%) or presence of competitors. Each value represents the mean ± SD of triplicate experiments performed in duplicate

Results

Figure 1 shows a representative example of the saturation curve and Scatchard analysis of [^{3}H]-E2 binding to MCF-7 ADRr cells. The [^{3}]-E2 saturation analysis carried out by a whole-cell assay at 4° C for 2.5 h resulted in a sigmoid curve, with saturation occuring between 35 and 45 nM [^{3}H]-E2 (Fig. 1 A). As predicted from the biphasic nature of the saturation curve, Scatchard analysis resulted in a concave plot (Fig. 1 B). Since an accurate estimate of both the K_d value and the number of EBSs cannot be made from a curvilinear Scatchard plot, these parameters were obtained from the saturation curve [5, 27]. For the experiment shown in Fig. 1, the number of type II EBSs calculated from the saturation curve at maximal binding was about 640,000 sites/cell. The K_d value determined from the [^{3}H]-E2 concentration required for half-saturation was about 17 nM. In four similar experiments, the number of sites/cell and the K_d values were 520,000 ± 130,000 and 15 ± 3 nM, respectively.

Table 1. Steroid specificity of type II EBSs in MCF-7 ADRr cells

Competing steroids[a]	% Binding
None	100
17-β-Estradiol	39± 4
Diethylstilbestrol	33± 7
Progesterone	102± 4
5-a-Dihydrotestosterone	99± 8
Androstenedione	96±11
Dexamethasone	91± 8

Values represent the mean ± SD of three different experiments performed in triplicate

[a] All competing steroids were used at a 100-fold molar excess with respect to [^{3}H]-E2 (40 nM)

Table 2. Effect of Q on the clonogenic efficiency of MCF-7 ADRr cells

Treatment	Number of colonies	CE[a]	% of control
None (1% ethanol)	56	2.8	100
0.1 μM Q	46	2.3	82
1 μM Q	35	1.7	62.5
10 μM Q	26	1.3	47

CE, Clonogenic efficiency

[a] CE = (number of colonies/2,000 plated cells) × 100. Values indicate the results of 1/4 similar experiments

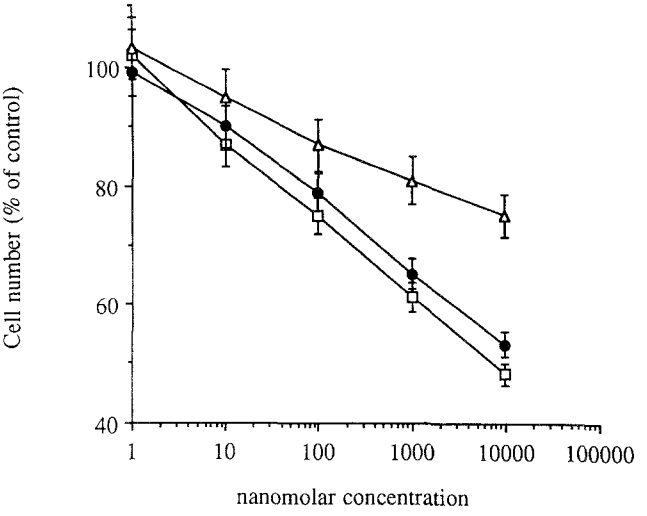

Fig. 3. Effect of various concentrations of DES (□), Q (●) and TAM (△) on MCF-7 ADRr cell proliferation. Cell counts were performed after 3 days of exposure to compounds. Each value represents the mean ± SD of three different experiments performed in triplicate

Among the steroids tested, only estrogenic compounds competed for [^{3}H]-E2 binding to type II EBSs in MCF-7 ADRr cells (Table 1). Furthermore, data shown in Fig. 2 demonstrate that Q could compete with [^{3}H]-E2 for type II EBSs, showing a potency similar to that of DES. TAM also competed with [^{3}H]-E2 for type II EBSs, albeit to a lesser extent than either DES or Q (Fig. 2).

Since MCF-7 ADRr cells contain type II EBSs, it seemed conceivable that they would be sensitive to Q. Indeed, Q produced a dose-dependent growth inhibition in these cells (Fig. 3). DES and TAM also inhibited the proliferation of MCF-7 ADRr cells (Fig. 3), and the magnitude of their growth-inhibitory effect corresponded to their capacity to bind type II EBSs. The inhibitory effect of DES, TAM and Q was not attributable to a cell-killing action of these substances. In fact, the cell viability after 3 days' culture did not vary between control and treated cells, being greater than 85%. Furthermore, the inhibitory effect appeared to be reversible since after the removal of DES, TAM and Q from the culture medium, the treated cells regrew like untreated cells (data not shown).

For further characterization of the antiproliferative properties of Q, the ability of this substance to inhibit colony formation was tested by a clonogenic assay. As shown in Table 2, Q inhibited colony formation in a dose-related manner.

Discussion

The development of drug resistance is one of the major obstacles to effective cancer chemotherapy. Two pharmacological approaches are being followed in efforts to overcome this problem. One involves the use of agents that enhance the chemosensitivity of known antineoplastic agents [9]. The other approach involves the screening of novel compounds against resistant cell lines.

Our results show that MCF-7 ADRr cells express type II EBSs and are sensitive to the antiproliferative effect of Q. Q was also active in a clonogenic assay, suggesting that this compound can inhibit the proliferation of the tumor stem cells. Therefore, multidrug-resistant human breast-cancer cells do not develop cross-resistance to the antiproliferative effect of Q. This is a further demonstration of the possible therapeutic potential of this substance.

Although the mechanism underlying the antiproliferative activity of Q remains to be clarified, our data suggest that as in other human cancer cell lines [30], this flavonoid may regulate cell growth through a binding interaction with type II EBSs. Although bioflavonoids affect a variety of enzymes [1, 10, 11, 16, 17, 24, 25, 32], the concentrations eliciting these effects lie in the range of 50–100 μM. Conversely, Q both interacts with type II EBSs and becomes effective as a cell-growth inhibitor at concentrations as low as 0.01 μM.

Observations that type II EBSs are expressed in several human primary tumors [2, 18, 20, 26, 29] suggest that Q can also be active in vivo. It is also noteworthy that a plasma concentration of 12 μM Q, which is similar to that effective in vitro for the inhibition of cancer-cell growth, was obtained following an intravenous injection of 100 mg without producing any apparent side effect [12].

Interestingly, TAM interacts with type II EBSs and its binding affinity correlates well with its growth-inhibitory effect on MCF-7 ADRr cells. A previous study [35] reported that MCF-7 ADRr cells were resistant to the inhibitory activity of TAM; however TAM was applied in those experiments at 0.1 μM, a concentration that was also minimally active under the experimental conditions used in the present study.

Our results further confirm that TAM may exert a growth-inhibitory effect that is not mediated by the ER

[23, 28, 33, 36]. Moreover, the clinical response to TAM obtained in the same ER-negative breast cancers is well recognized [7]. It is possible that this ER-independent antiproliferative activity of TAM could at least in part be ascribed to a binding interaction with type II EBSs. Similarly, our data might also provide an explanation for the clinical response previously observed following the administration of estrogen to breast cancer patients [3].

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