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QUERCETIN DOWN-REGULATES SIGNAL TRANSDUCTION IN HUMAN BREAST CARCINOMA CELLS

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SUMMARY: Signal transduction activity was markedly elevated in cancer cells as shown by the increased activity of enzymes utilizing 1-phosphatidylinositol, PI (PI 4-kinase and PI-4-phosphate 5-kinase) for the production of the second messenger inositol 1,4,5-trisphosphate, IP_3, in rat hepatomas (Cancer Res. 54: 2611; 5574, 1994) and in human ovarian and breast carcinoma cells (Life Sci. 55:1487, 1994). Quercetin, a flavonoid, in human breast carcinoma MDA-MB-435 cells produced growth inhibition (IC_{50} = 55 μM) and cytotoxicity (LC_{50} = 26 μM). Quercetin inhibited PI kinase activity in extracts of breast carcinoma cells (IC_{50} = 6 μM) and in cultured cells (IC_{50} = 10 μM) with a minor inhibition of PIP kinase activity. IP_3 concentration decreased in parallel with PI kinase activity. In time sequence studies quercetin in breast carcinoma cells brought down PI kinase and IP_3 concentration in 60 min to 5 and 6%, respectively; PIP kinase activity was at 63% of controls. The results demonstrate for the first time in proliferating human breast carcinoma cells a reduction by quercetin of the increased capacity for signal transduction, thus providing a novel and sensitive target in cancer cells.

From 1990 to 2000 more than 1.5 million women in the United States will be newly diagnosed with invasive breast carcinoma and about 30% will eventually die from the disease (1). About 1 in every 8 women will develop carcinoma of the breast in her lifetime. Breast cancer patients with clinically evident distant metastases have no cure available. Therefore,

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid; IC_{50}, concentration of drug that inhibits 50% of growth of the cells; IP_3, inositol 1,4,5-trisphosphate; LC_{50}, concentration of drug that kills 50% of cells; MEM, minimum essential medium; PI, 1-phosphatidylinositol; PI kinase, 1-phosphatidylinositol 4-kinase (EC 2.7.1.67); PIP kinase, 1-phosphatidylinositol-4-phosphate 5-kinase (EC 2.7.1.68).

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there is a pressing need for developing new targets and drugs for an improved treatment approach.

Recently it was discovered that the signal transduction capacity was markedly elevated in rat hepatomas, human ovarian epithelial carcinomas and human breast carcinoma cells in comparison with their respective normal tissues of origin (2-4). The increased capacity for signal transduction was shown by the markedly increased activities of the enzymes which utilize PI through the action of the first committed enzyme (PI kinase) and the second enzyme (PIP kinase) leading to the production of the second messenger, IP_3. The steady-state specific activities of PI kinase and PIP kinase increased 7.3- and 2.3-fold respectively in human breast carcinoma MDA-MB-435 cells grown in nude mice as solid tumors as compared to activities in normal human parenchymal breast HMEC cells in log phase. When these breast carcinoma cells were grown in culture in the log phase, PI and PIP kinase activities were 95.8- and 15.5-fold higher than those in normal human breast cells (4). The increased enzymic capacities for signal transduction should confer selective advantages for the breast carcinoma cells; therefore, PI and PIP kinase activities should be sensitive targets for chemotherapy (2-5).

In this paper we provide evidence that quercetin, 3, 3', 4', 5, 7-pentahydroxyflavone (Fig. 1), is antiproliferative and cytotoxic in human breast carcinoma MDA-MB-435 cells. The mechanism of action is due to, in part at least, a dose- and time-dependent reduction in PI kinase activity and IP_3 concentration. Since quercetin is cytotoxic to human breast carcinoma cells, it might be of interest for the clinical treatment of disseminated breast carcinoma.

MATERIALS AND METHODS

Drugs and chemicals. Quercetin and all chemicals of the purest grade available were purchased from Sigma Chemical Co., St. Louis, MO. Hyperfilm MP and [γ^{32}P] ATP (3000 Ci/mmol) were from Amersham Corp., Arlington Heights, IL. Quercetin was solubilized in 95% ethanol fresh for each experiment.

Human breast carcinoma. MDA-MB-435 human breast carcinoma cells were the kind gift of Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX). The cells were implanted in female athymic nude mice and grown as solid tumors. Cells were also cultured in MEM medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals Inc., Norcross, GA), penicillin (100 U/ml) and streptomycin (100

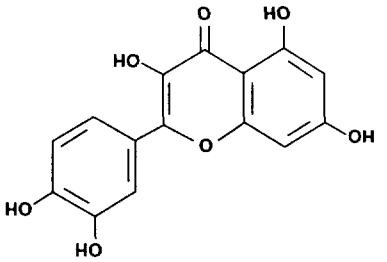

Figure 1. Structure of quercetin.

μg/ml). Cells were cultivated as monolayers in 5% CO_2: 95% humidified air at 37°C. For subculture, cells were dispersed with 0.25% trypsin containing 1 mM EDTA at 37°C for 5 min, centrifuged and suspended in fresh medium. The cells were seeded at a density of 1 x 10^5 cells/ml in 75 cm^2 culture flasks, harvested and centrifuged for 5 min at 1000 rpm. Pellets were suspended in a homogenizing buffer for assaying PI and PIP kinase activities. In some experiments MDA-MB-435 breast carcinoma cells in log phase were incubated for 60 min in presence of various concentrations of quercetin.

Preparation of particulate fraction, protein, enzyme and IP_3 assays. Cell suspensions were homogenized for 30 sec at 600 rpm using a motor driven Teflon pestle in 0.25 M sucrose, 50 mM TRIS-HCl (pH 7.4), 1 mM EGTA, and 10 mM dithiothreitol containing 10 mM benzamide, 0.5 mM phenylmethylsulphonyl fluoride, 1 μl/ml leupeptin, and 10 μg/ml aprotinin. The homogenates were centrifuged at 1,200 rpm for 10 min at 4°C. The supernate was decanted and centrifuged at 45,000 rpm for 30 min. The resulting pellet (particulate fraction) was suspended in 25 mM ß-glycerophosphate buffer containing 1 mM EGTA and protease inhibitors in the same concentration as in the homogenization buffer. PI kinase and PIP kinase activities were assayed in the particulate fraction as described (2,3). Optimum enzyme kinetics were achieved with activities proportionate with enzyme amount added and time elapsed. Since enzyme activities were measured under 0 order conditions the elevated enzymic activities should reflect increased enzyme amounts in the cancer cells.

For IP_3 assay MDA-MB-435 carcinoma cells were extracted immediately with ice cold 10% perchloric acid. After centrifugation at 10,000 x g for 10 min at 4°C, the clear supernatant was neutralized with 10 N KOH. The precipitate of potassium perchlorate was removed by centrifugation at 4°C and the supernatant fluid was used for the IP_3 assay (3).

Protein concentration was measured by a routine method using crystalline bovine serum albumin as standard (6).

Expression and evaluation of results. Enzymic activities were calculated in nmol product formed per h per mg protein (specific activity); IP_3 concentration and the kinase activities in MDA-MB-435 carcinoma cells were also expressed in pmol/cell x 10^{-6}. Results are presented as means ± SE of 3 or more independent experiments, each performed in duplicate unless otherwise stated. Data are also expressed in percentages of controls. Results were statistically evaluated by the *t* test for small samples. Differences between means yielding a probability of < 5% were considered as significant.

RESULTS AND DISCUSSION

Growth inhibition and cytotoxicity of quercetin in MDA-MB-435 human breast carcinoma cells. In breast carcinoma cells, the growth inhibition assay for quercetin yielded IC_{50} = 55 μM. Whereas 10 or 20 μM quercetin produced little effect, 50, 75 and 100 μM progressively inhibited cell proliferation; quercetin stopped cell growth at 150 μM (Fig. 2). In the clonogenic assay quercetin yielded LC_{50} = 26 μM.

In vitro inhibition by quercetin of PI kinase in breast carcinoma cell extracts. When various concentrations of quercetin were added to the assay mixture and preincubated for 15 min at 20°C, PI kinase activity in the particulate extracts was inhibited yielding IC_{50} = 6 μM. At 100 μM, quercetin inhibited enzyme activity by 75-80% of controls (Fig. 3). In contrast, PIP kinase activity was not affected. The in vitro inhibition of PI kinase by this flavonoid is in agreement with our data in human ovarian carcinoma OVCAR-5 cells (not shown) and with the report of Nishioka et al. on A431 cell membrane extracts (7).

Dose-response effect of quercetin on PI and PIP kinase activities and IP_3 concentration in proliferating breast carcinoma cells. When breast carcinoma cells in log phase were

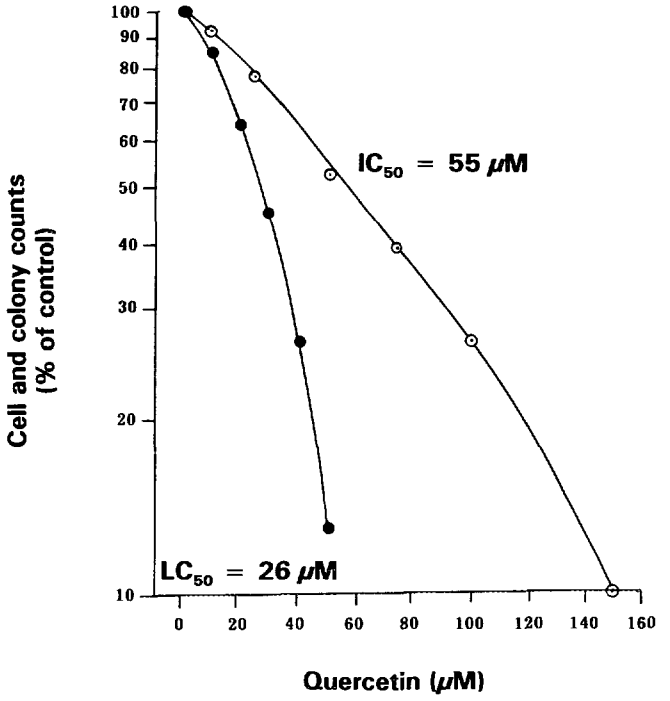

Figure 2. Antiproliferative and cytotoxic action of quercetin in MDA-MB-435 human breast carcinoma cells. IC_{50} is the drug concentration that inhibits 50% growth of cells; LC_{50} is the drug concentration that kills 50% of cells.

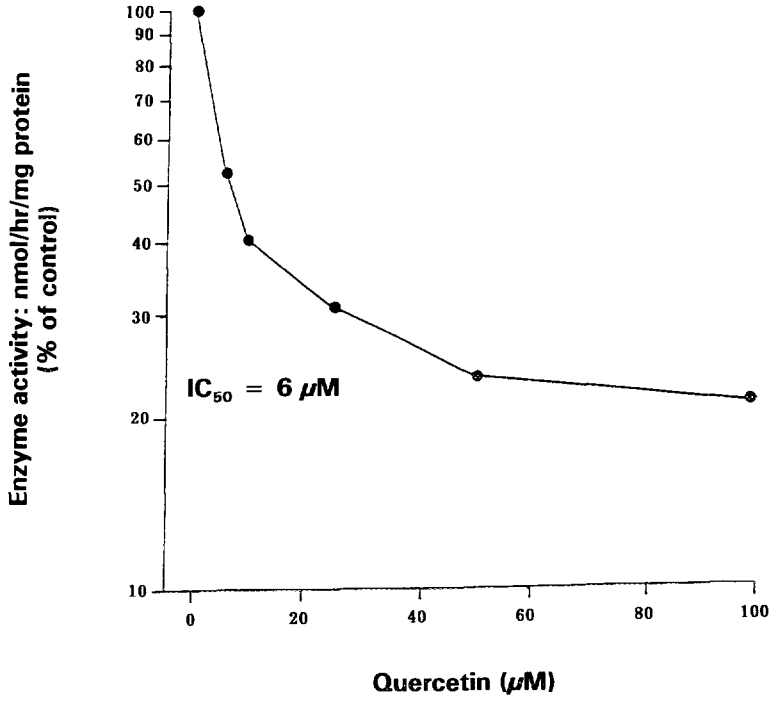

Figure 3. Various amounts of quercetin were added to the assay mixture and preincubated at 20°C for 15 min before [^{32}P]ATP and Mg^{2+} were added to start the reaction. Values are means of two experiments in duplicate.

incubated for 60 min in presence of 10, 50 or 100 μM quercetin, PI kinase activity declined to 53, 49 and 20% of controls, respectively (Table 1). Since at 10 and 50 μM quercetin the IP_3 concentration dropped to 58 and 31%, respectively, but PIP kinase activity did not change significantly, the IP_3 content appears to be linked with the reduction in PI kinase activity. The activity of PIP kinase was less sensitive to quercetin as it decreased significantly only with the highest drug concentration used (100 μM).

Time-sequence action of quercetin in proliferating breast carcinoma cells on PI and PIP kinase activities and IP_3 concentration. Table 2 shows that when quercetin (100 μM) was added to breast carcinoma cells in log phase, at 60 min PI kinase activity and IP_3 concentration fell to 5 and 6%, respectively. PIP kinase activity decreased to 63% of controls. Thus, the data demonstrate that the quercetin-produced growth inhibition of breast carcinoma cells was accompanied primarily by a marked decline in PI kinase activity and IP_3 levels.

Quercetin action on PI and PIP kinase activities and IP_3 concentration in human breast carcinoma cells. The increased activities of PI and PIP kinases and the high IP_3 concentration revealed an elevated signal transduction capacity in breast carcinoma cells (4). Because of a report of Umezawa and associates showing that quercetin in vitro inhibited PI kinase activity in extracts of A431 cells (7) we explored the impact of quercetin on cytotoxicity and signal transduction in breast carcinoma cells. Quercetin, an active compound against human large cell carcinoma of the lung (8), human ovarian carcinoma cells (14) and rat hepatoma cells (Y.A. Yeh and G. Weber, to be published), has low toxicity in rat (8, 9) and in human (10). In current clinical Phase 1 trials quercetin had low toxicity in 35 patients treated for a variety of tumors refractory to conventional treatment (15). Quercetin has anticarcinogenic and

Table 1
Dose-response effect of quercetin on PI and PIP kinase activities and IP_3 concentration in breast carcinoma cells

Quercetin μM	PIP kinase	PI kinase	IP_3
	pmol/h/cell x 10^{-6}		pmol/cell x 10^{-6}
Control	149.0 ± 9.0 (100)	9.6 ± 0.8 (100)	56.8 ± 3.9 (100)
10	78.9 ± 5.9 (53)*	8.3 ± 0.3 (86)	33.6 ± 3.1 (58)*
50	72.3 ± 4.5 (49)*	8.4 ± 0.4 (88)	20.3 ± 2.4 (31)*
100	29.1 ± 3.2 (20)*	6.3 ± 0.2 (66)*	5.2 ± 1.1 (9)*

Means ± SE of 3 separate determinations are given. Incubation time was 60 min.
*Statistically significant difference from controls ($p = < 0.05$).

Table 2
Time sequence of quercetin action on PI and PIP kinase activities and IP_3 concentration in breast carcinoma cells

Time (min)	PI kinase	PIP kinase	IP_3
	pmol/h/cell x 10^{-6}		pmol/cell x 10^{-6}
Control	120.0 ± 9.8 (100)	7.1 ± 1.1 (100)	55.3 ± 2.3 (100)
30	14.0 ± 2.8 (8)*	5.0 ± 0.03 (70)	8.0 ± 1.2 (14)*
60	5.6 ± 0.2 (5)*	4.5 ± 0.04 (63)*	3.3 ± 0.4 (6)*

Each value is the mean ± S.E. of 3 or more separate determinations. Exponentially growing cells were seeded at a density of 1 x 10^5 cells/ml and incubated at 37°C in 5% CO_2: 95% humidified air. Twenty-four h later, quercetin (100 μM) was added and incubated for 30 and 60 min. Cells were then harvested for determining enzyme activities and IP_3 levels.

*Significantly different from controls ($p < 0.05$).

antineoplastic properties (11-13); it also enhanced the antiproliferative action of platinum and nitrogen mustard (8). Several mechanisms of action have been proposed which include decreased incorporation of thymidine in DNA and arrest of cancer cells in G_1-phase (13). However, there has been little attempt to compare the antiproliferative, cytotoxic and biochemical action of quercetin in the same cell type. In carrying out such an investigation we are reporting that the mechanism of quercetin action may be based, in part at least, on its reduction of PI kinase activity and IP_3 concentration. Our studies showed selective inhibition of PI kinase with minor effect on PIP kinase activity. The dose- and time-dependent reductions of PI kinase activity and IP_3 concentrations were linked in proliferating breast carcinoma cells treated with quercetin. These observations, which also apply to the action of quercetin in human ovarian carcinoma OVCAR-5 cells (14), emphasize the role of PI kinase which is at the fountainhead of PI utilization as the first committed enzyme in the pathway of IP_3 production.

Novel aspects of this work include the following: a. Quercetin was shown to be anti-proliferative and cytotoxic in human breast carcinoma cells. b. Quercetin inhibited PI kinase activity in vitro in extracts and also when incubated with breast carcinoma cells. c. In a dose-response and time-dependent fashion in breast carcinoma cells, quercetin rapidly and markedly reduced PI kinase activity and IP_3 concentration with only a minor effect on PIP kinase. d. Since quercetin proved to be antiproliferative and cytotoxic in breast carcinoma cells and because progress has been made in understanding the mechanism of action of this drug, quercetin might be considered in the treatment of relapsed, inoperable breast carcinoma

cases. PI kinase activity and IP_3 concentration provide new targets in breast chemotherapy and these parameters may also be used as monitors of the responsiveness to quercetin of these cancer cells in clinical treatment.

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