



MOLECULAR MECHANISMS IN THE ANTIPROLIFERATIVE ACTION OF QUERCETIN

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Summary

A single treatment with quercetin (5.5 μ M), a plant flavonoid, activated both apoptosis and differentiation programs in K562 human leukemia cells. K562 cells expressed commitment to apoptosis after 1 h exposure, however, at least 12 h of drug exposure was needed to induce differentiation. Early (1 h) down-regulation of the *c-myc* and *Ki-ras* oncogenes and rapid reduction of inositol-1,4,5-trisphosphate (IP₃) concentration (IC₅₀ = 9 μ M, 1 h incubation) are part of the antiproliferative action of quercetin and appear to relate to induction of differentiation and/or apoptotic program of K562 leukemia cells treated with quercetin.

Key Words: quercetin, K562 leukemia cells, apoptosis, cell differentiation, *c-myc*, *Ki-ras*, IP₃

Quercetin (3,3',4',5,7-pentahydroxyflavone) (Fig. 1), a plant flavonoid, is widely distributed in the plant kingdom and occurs naturally in a wide range of fruits and vegetables (1). Quercetin inhibits the growth of malignant cells, arresting them in the late G₁ phase of the cell cycle (2). It blocks signal transduction pathways by inhibiting protein tyrosine kinase, PI- and PIP-kinases (1-phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase) resulting in a reduction of IP₃ concentration which should decrease the release of Ca²⁺ from intracellular sources (3). Quercetin is well known to inhibit various tyrosine protein kinases and serine/threonine protein kinases (4, 5). However, the exact mechanisms responsible for the antitumor effect of quercetin are not yet completely understood.

The purpose of this investigation was to gain a deeper insight into the molecular mechanisms of the antiproliferative action of quercetin. Here we report that *c-myc* and *Ki-ras* oncogenes, which are overexpressed in the K562 cells, are down-regulated after quercetin treatment, concurrently the drug induced inhibition of the phosphatidylinositol cascade. To further evaluate the mechanisms of drug action we also extended our observations to induction of apoptosis and cell differentiation.

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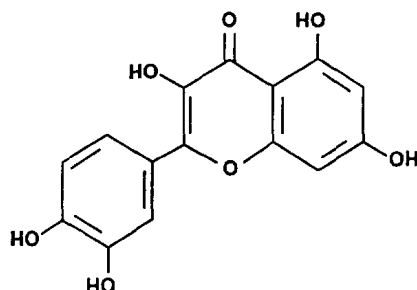


Fig. 1
Structure of Quercetin.

Materials and Methods

Chemicals. Quercetin and all other reagents of purest grade available were purchased from Sigma (St. Louis, MO). Quercetin was solubilized in 95 % ethanol for a 10 mM stock solution. Drug was added to the cultures in small volumes (50 μ l) to dilute ethanol. The same volume of ethanol was added to the solvent-only control group. Culture media were from GIBCO (Grand Island, NY).

Cell line, tissue culture and growth inhibition assay. K562 human myeloid leukemia cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% bovine calf serum and 2 mM glutamine. The clonogenic agar assay for K562 cells was carried out as described (6, 14). Briefly, cells were treated with various concentrations of quercetin for 1 and 12 h. At the end of the incubation period cells were rinsed with PBS, then 1500 cells/ml of the RPMI-1640 medium were plated in a plastic 35 mm Petri dish. The medium was supplemented with 20 % fetal calf serum, penicillin (100 U/ml), streptomycin (50 μ g/ml) and 0.33 % agar. Fourteen days later the colonies were scored under an inverted microscope. Survival of treated cells was calculated as percentage of colonies formed by untreated cells. No difference was seen in survival for the control groups with or without ethanol. For measurement of cytotoxicity, 5×10^4 log phase cells/ml were seeded in culture flasks. Cell viability was monitored daily by the trypan-blue dye exclusion test. The number of treated cells was plotted as percentage of that of the control cells.

Morphological signs and assessment of apoptosis. Cells at 5×10^4 starting density were treated with quercetin for 15 min, 30 min, 1, 3, 6, 12, 18 and 24 hr. At the end of each incubation period cells were rinsed with PBS, then cytocentrifuged onto microscopic slides, and stained with hematoxylin-eosin. One-thousand cells per sample were analyzed under light microscope for appearance of morphological signs (12) of apoptosis. Internucleosomal DNA fragmentation was determined as described (6). Briefly, cells were washed, collected, counted and lysed, DNA was extracted and precipitated with standard methods. DNAs were run on 1.6% agarose gel (10 μ g/lane). In parallel experiments quantitative analysis of internucleosomal DNA fragmentation was carried out as described (7). Briefly, the fragmented DNA in the cell lysate was separated from intact chromatin by centrifugation (20,000 \times g for 30 min at 4 $^{\circ}$ C). DNA contents of the supernatant fluid and the pellet were determined spectrophotometrically.

Cellular differentiation. The capacity of K562 leukemia cells to undergo erythroid differentiation was measured by counting the percentage of benzidine positive cells. For each sample 1,000 cells were examined for benzidine staining by microscopic analysis.

IP₃ assay. K562 cells were extracted with ice cold 10% perchloric acid. After centrifugation at 10,000 g for 10 min at 4 °C, the clear supernatant was neutralized with 10 N KOH. The potassium perchlorate precipitate was removed by centrifugation at 4 °C and the supernatant fluid was used for IP₃ radioassay (Amersham TRK-1000) (8).

Northern blot analysis. Total cellular RNA was electrophoresed in formaldehyde gels, and blots containing 15 µg RNA per lane were prepared on Hybond-N⁺ membrane (Amersham). Membranes were hybridized with ³²P-labeled genomic probes for *Ki-ras* and *c-myc*, then reprobod for glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the latter one was used as a control for lane loading. Quantitative densitometry of X-ray films was performed using a Molecular Dynamics laser densitometer.

Results

Cytotoxic and antiproliferative action of quercetin. Lethal effects of the drug were measured by cytotoxicity assay. Short-term treatment of quercetin was effective in K562 leukemia cells. When quercetin was washed out from the cultures after 1 h treatment, the lethal effect of the drug was still increased with the time elapsed between treatment and counting (Fig 2a). Growth inhibition of quercetin was measured by clonogenic assay. After 1 and 12 h treatment of cells with various concentrations of quercetin, the cells were incubated for 14 days to have colonies developed. Survival curves are shown in Fig 2b. The IC₅₀ values in the clonogenic assay for 1 and 12 h treatments were 13.5 µM and 3.4 µM, respectively.

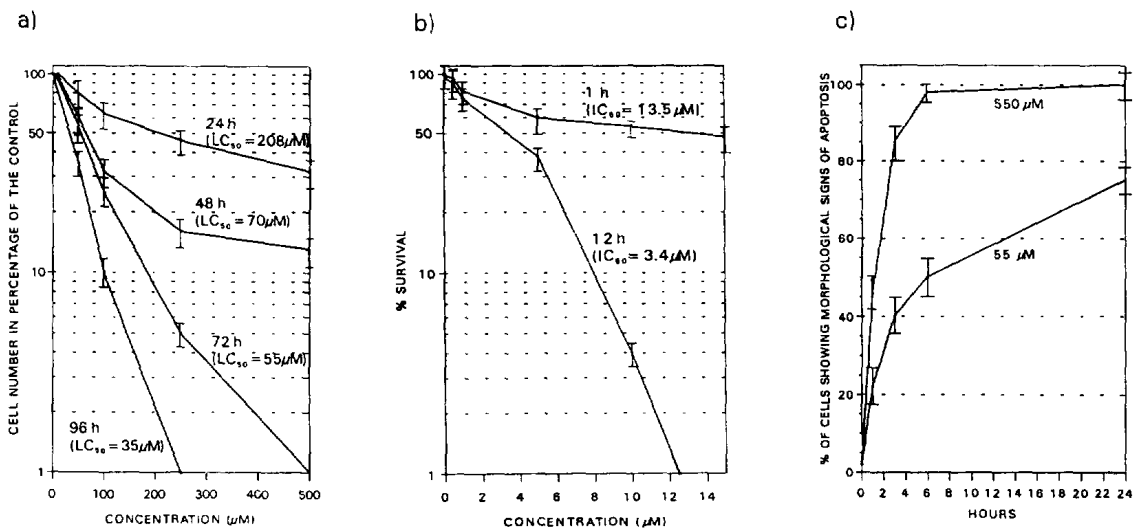


Fig. 2

- a) Cytotoxicity of quercetin. K562 cells were treated with quercetin for 1 h, then drug was withdrawn and cells were counted at 24, 48, 72 and 96 h.
- b) Growth inhibition of quercetin measured by clonogenic assay. Cells were treated with the drug for 1 and 12 h, colonies were counted after 14 days incubation in drug-free medium. Means of 3 or more assays are given.
- c.) Frequency of cells showing morphological signs of apoptosis. Means of 3 or more assays are given.

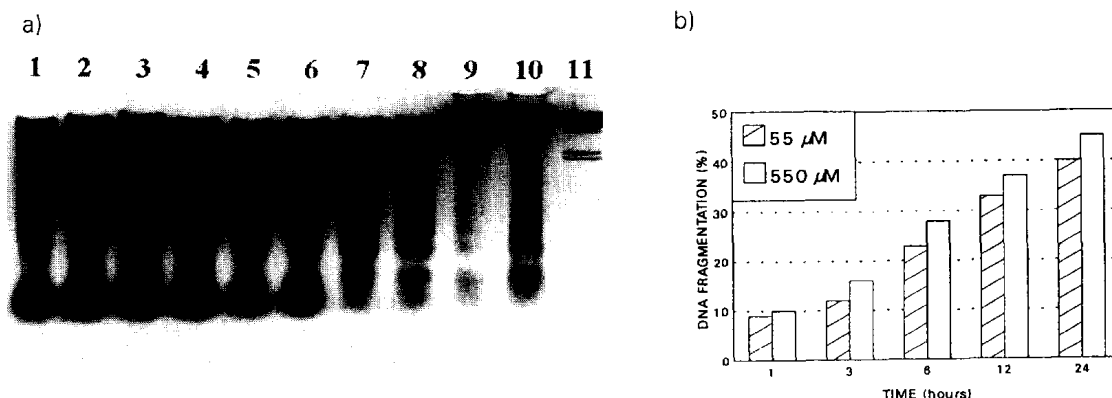


Fig. 3

a) Internucleosomal DNA fragmentation caused by quercetin in K562 leukemia cells. Cells were exposed to 55 μM quercetin for 15 min to 24 h. Lane 1: control cells; Lanes 2 - 10: 15 min, 30 min, 45 min, 1, 3, 6, 12, 18, 24 h; Lane 11: marker DNA (Boehringer).

b) Percentage of DNA fragmentation caused by quercetin in K562 human leukemia cells. Striped bars represent 55 μM , white bars 550 μM treatments. Means of 3 experiments are given. S.E. was less than $\pm 10\%$.

TABLE I
Induction of Erythroid Differentiation in K562 Human Leukemia Cells by Quercetin

Drug	Drug concentration μM	Benzidine positive cells %	-Fold increase
None, control	-	6 \pm 0.2	1.0
Quercetin	0.55	9 \pm 0.4	1.5
	5.50	12 \pm 0.3	2.0
Hemin	30.00	38 \pm 1.9	6.3

Means \pm S.E. of triplicate experiments. Cells were seeded at 5×10^4 cells/ml in RPMI-1640 medium containing 10% bovine calf serum in presence of drugs. The percentage of benzidine positive cells was determined at 120 h of treatment.

Induction of apoptosis. Quercetin treatment induced cellular changes associated with programmed cell death. The proportion of cells with these features was dose- and time-dependent (Fig 2c). Quercetin exposure (55 μM and 550 μM) resulted in a characteristic ladder of DNA fragments of approximately 180 base-pair multiples after 1 h or longer treatments (Fig. 3a). This reveals that cells were undergoing programmed cell death. Quantitative analysis of internucleosomal DNA fragmentation indicated that higher concentrations (550 μM) and prolonged exposure (up to 24 h) resulted in 45% of DNA fragmentation (Fig. 3b). No DNA ladder formation was observed after treatment with 5.5 μM of quercetin (not shown).

Induction of erythroid differentiation. The ability of quercetin to induce erythroid differentiation was evaluated. K562 cells were incubated with drug for 120 h, this exposure time with 55 μM quercetin concentration proved to be toxic for K562 cells, therefore 10-fold and 100-fold lower concentrations (5.5 μM and 0.55 μM) were used for induction of differentiation. Quercetin proved to be a moderately effective inducer of erythroid differentiation as compared to hemin, a known inducer of differentiation of these cells which was included for comparison. (Table I).

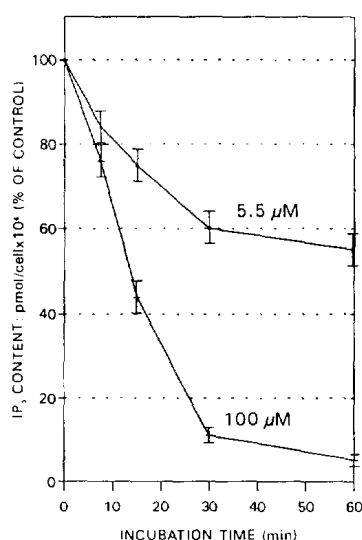


Fig. 4

Effect of quercetin on IP₃ content of K562 cells. Doses of quercetin were 5.5 μM and 100 μM. Exponentially growing cells were seeded at a density of 1 × 10⁵ cells/ml and incubated at 37 °C. Twenty-four h later quercetin was added and the cells were incubated for 7.5, 15, 30 and 60 min. Cells were then harvested for determination of IP₃ levels. Each value is the mean ± SE of three separate determinations.

TABLE II
Effect of Quercetin Concentration on IP₃ Content of K562 Cells

Quercetin (μM)	IP ₃ (pmol/cell × 10 ⁵)	% of control
0	51.5 ± 3.5	100
5	36.6 ± 2.6	71*
10	23.0 ± 2.1	45*
50	10.7 ± 1.6	21*
100	2.6 ± 0.5	5*

Incubation time: 60 min. Mean ± S.E. are of 3 separate determinations.

*Significantly different from untreated control (p < 0.05)

Down-regulation of IP₃ concentrations in K562 cells. Quercetin was incubated with cells in concentrations of 5.5 and 100 μM for 7.5, 15, 30 and 60 min. The reduction in IP₃ concentration was rapid (t_{1/2} = 14 min, 100 μM) and dose-dependent, reaching a nadir of 5% of control at 100 μM concentration by 60 min (Fig. 4 and Table II). The IP₃ level did not change during the next 6 h (data not shown).

Down-regulation of oncogene expression. Exposure of K562 cells to 55 μM of quercetin resulted in early (1 h) down-regulation of both *c-myc* and *Ki-ras* oncogenes (Fig. 5). Densitometric analysis of the blots showed that by 6 h of treatment there was an 85 % maximal decrease of *c-myc* RNA level. After 12 h of treatment *c-myc* expression started to increase and by 24 h it almost reached the initial (control) level. *Ki-ras* RNA expression showed only 33 % decrease and returned to the initial level sooner (by 12 h) (Fig. 5). Treatment with 5.5 μM quercetin resulted in down-regulation of *c-myc* oncogene by 64% at 6 h (data not shown).

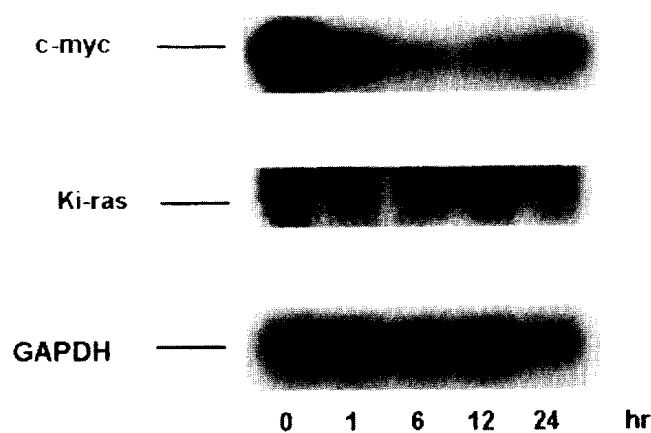


Fig. 5

Down-regulation of *c-myc* (upper panel) and *Ki-ras* (middle panel) oncogenes in K562 human leukemia cells treated with quercetin (55 μ M). Northern analysis was done as described in Materials and Methods. As a control the filter was probed for the consecutively expressed GAPDH mRNA.

Discussion

In the present study we observed induction of both apoptosis and differentiation in K562 human leukemia cells as a result of quercetin treatment. Apoptosis is an important mode of death for cells treated with certain chemotherapeutic drugs (9). Our studies indicate that quercetin has a major impact on induction of apoptosis in K562 leukemia cells. DNA ladder formation (internucleosomal DNA fragmentation) characteristic of apoptosis appeared after short (1 h) drug exposure but at concentrations of 55 μ M and above. On the basis of our results it appears that a complex sequence of molecular events prepares cells for programmed cell death and/or differentiation induced by quercetin. This starts with metabolic changes (decrease in IP₃ concentration), and with down-regulation of certain "proliferation-linked" oncogenes (*c-myc*, *Ki-ras*). The products of these genes are also required for induction of apoptosis (10, 11). Decreased expression of HSP70 gene after quercetin treatment was previously reported in various cells undergoing apoptosis (13). This indicates that different molecular mechanisms may operate in the induction of apoptosis by quercetin.

As we have shown recently, quercetin inhibits elevated PI and PIP kinase activities in human ovarian carcinomas resulting in a reduction of IP₃ concentration (8). We reported previously that *Ki-ras* and *c-myc* oncogenes, which are overexpressed in the K562 cells, were down-regulated in the early precommitted period of differentiating K562 cells treated with tiazofurin (14). In the present studies we detected an early (1 h) marked down-regulation of *c-myc* oncogene parallel with the decrease of IP₃ concentration. A trend similar to that observed for *c-myc* transcripts, although to a lesser extent, was seen in *Ki-ras* mRNA expression. Unlike *c-myc* and *Ki-ras* transcripts that returned to the initial level after treatment, no such recovery of IP₃ concentration was observed. Our results suggest a link of inhibition of phosphatidylinositol cascade with the irreversible induction of apoptosis.

It has been shown that *c-myc* overexpression inhibits differentiation of various cell types and when differentiation occurs it results in a transient *c-myc* down-regulation (15, 16). This decrease may reflect an early response of this gene to cessation of cell proliferation. Alternatively, down-regulation of *c-myc* may be required for induction of the early differentiation program, which in part might be shared with the initiation of the apoptotic program.

Novel aspects of this study include the following. 1. This is the first report on the impact of quercetin to induce both apoptosis and differentiation in the same cells. 2. Low concentrations (0.55 μM and 5.5 μM) of the drug induced erythroid differentiation after at least 12 h treatment. 3. High concentrations (55 μM and above) of quercetin but short (1 h) exposures were needed to induce DNA ladder formation. 4. The irreversible decrease of IP_3 concentrations paralleled the induction of apoptosis. 5. Transient down-regulation of *c-myc* and *Ki-ras* oncogenes might be linked to both apoptosis and cellular differentiation induced by quercetin.

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