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In vitro evaluation of newly developed chalcone analogues in human cancer cells

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Abstract *Purpose*: Among flavonoids, chalcones have been identified as interesting compounds having chemopreventive and antitumor properties. We studied a panel of newly developed chalcone analogues (**S1**–**S10**) using MDA-MB 231 and MCF-7 ADRr breast cancer cells and the T-leukemic Jurkat cell line. Quercetin was used as the reference compound. *Methods*: Antiproliferative activity was evaluated by cell counts performed after 72 h of exposure to the drugs. DNA analysis and redox activity were evaluated using flow cytometry. Apoptosis was assessed by morphological analysis, using YOYO-1 as DNA dye; p-glycoprotein function was ascertained by quantitating the efflux of rhodamine 123. *Results*: All cells were sensitive to chalcone analogues yielding IC_{50} in micromolar concentrations with the following order regardless of the multidrug resistance (MDR) status: **S1** > **S2** > quercetin. **S1** and **S2**, the most active compounds, were selected to evaluate their effect on the cell cycle, apoptosis, redox activity, and modulation of the p-glycoprotein function. No significant perturbation in cell cycle was seen with concentration up to 1 μM after 24 h. After 72 h a slight increase in G_2/M block and DNA fragmentation occurred at 10 μM. Morphological analysis of apoptosis showed that chalcone analogues induced apoptosis to a higher extent than quercetin. Redox analysis demonstrated that all substances were able to increase intracellular thiol levels, which returned to baseline value after 24 h for all drugs except quercetin. Production of reactive oxygen species was essentially unaffected by all compounds. Finally, in MDR-positive MCF-7 ADRr cells chalcone analogues were unable to modulate p-glycoprotein function while quercetin was able to. *Conclusions*: Newly developed **S1** and **S2** chalcones have a different but higher antitumor activity than quercetin and could be considered as potential new anticancer drugs.

Key words Chalcones · Quercetin · MDR Cancer cell lines

Abbreviations *CMFDA* Chloro-methyl-fluorescein diacetate · *DCHFDA* Dichloro-dihydro-fluorescein diacetate · *MDR* Multidrug resistance · *P-gp* P-Glycoprotein · *Rh123* Rhodamine 123 · *ROS* Reactive oxygen species

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Introduction

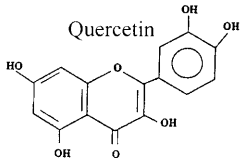
Over the past 10 years research for new drugs to be used in oncology has refocused on natural products. This rediscovery of natural products has yielded promising compounds such as taxanes and camptothecins. Moreover, recent reports have indicated the possible use of natural products as a source of potential chemopreventive agents [4, 15, 20].

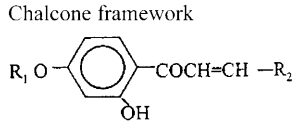
In particular, interest has intensified in the class of flavonoids – compounds present in normal human diet and in many folk medicines still in use. Chalcones, considered as the precursor of flavonoids and isoflavonoids, are abundant in edible plants. Chemically they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. The vast majority of naturally

occurring chalcones are polyhydroxylated [3]. Chalcones have multiple biological actions which include anti-inflammatory [13], analgesic and antipyretic properties, antimutagenic effect [26], cytotoxic, and tumor-reducing and antioxidant activity [11, 19].

Of particular interest, the effectiveness of chalcones against cancer has been investigated. Several compounds have been shown to have antimitotic activity against tumor cells in vitro [6, 7]. Chalcones inhibit the proliferation of both established and primary ovarian cancer cells [5]. An anti-invasive activity in vitro has been demonstrated, and this has frequently been found among chalcones having a prenyl group [18]. The chalcone butein exerts a potent cytotoxic effect on human colon cancer cells [30]. In vivo chalcones have been demonstrated effective as antitumor agents in skin carcinogenesis [21, 29] and chemopreventive agents in several experimental models [15, 20, 27].

To our knowledge, there are no reports investigating the antitumor activity of chalcones on multidrug resistant (MDR) cells or their potential to modulate P-glycoprotein (P-gp) protein activity. Thus the identification of new chalcone analogues will be important in the continued development of this class of agents as antitumor drugs. Here we studied a panel of newly developed chalcones derived by the framework of isocordoin, a naturally occurring chalcone first isolated from *Lonchocarpus* spp. (Leguminosae) and previously characterized in our laboratory for its antitumor activity [5]. Semisynthesis was performed by adding electrons donor/acceptor side chains.





Compound	R_1	R_2
S1	3-methyl-2-buten-1-yl	pyrid-4-yl
S2	3-methyl-2-buten-1-yl	pyrid-3-yl
S3	3-methyl-2-buten-1-yl	p-acetamidophenyl
S4	2-methylpropen-3-yl	p-dimethylaminophenyl
S5	2-methyl-2-buten-3-yl	indol-3-yl
S6	2-methylpropen-3-yl	indol-3-yl
S7	propyn-3-yl	indol-3-yl
S8	2-methylpropen-3-yl	pyrid-3-yl
S9	propyn-3-yl	m-methoxyphenyl
S10	propyn-3-yl	pyrid-3-yl

Fig. 1 General structures of quercetin and chalcone analogs

Materials and methods

Chemicals

A series of ten new chalcone analogues were provided by Dr. Bombardelli (Indena, Milan, Italy). The chemical structures of these compound are shown in Fig. 1. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) was obtained from Sigma (St. Louis, Mo., USA). All compounds were dissolved in dimethylsulfoxide (DMSO). The control cells were treated with the same amount of vehicle alone. The final DMSO concentration never exceeded 0.1% (v/v), in either control or treated samples. Previous experiments have shown that DMSO at this concentration does not modify the cellular activities that we were analyzing.

Cell culture

Two breast cancer cell lines (MDA-MB 231 and MCF-7 ADRr) and one human leukemic cell line (Jurkat) were used to assess the growth inhibitory activity of these chalcone analogues. MDA-MB 231 cells were grown in minimum essential medium; the MDR line MCF-7 ADRr cells, selected to Adriamycin (doxorubicin) resistance were grown in RPMI 1640. supplemented with 10% fetal calf serum (FCS) and 200 U/ml penicillin. The MDR phenotype was maintained by culturing cells in the presence of 10 μM doxorubicin as previously described [24]; doxorubicin was removed at least 15 days prior to perform experiments. Cells propagated as monolayer culture in 75-cm^2 tissue-culture flasks, were trypsinized weekly and plated at a density of 8×10^4 cells/ml. Jurkat cells were grown in RPMI 1640 supplemented with 10% FCS and 200 U/ml penicillin. Cells were seeded at $2–3 \times 10^5$ cells/ml and split at a ratio of 1:3 every day. All cells were incubated at 37 °C under 5% CO_2 95% air in a high humidity atmosphere. The MCF-7 ADRr cell line exhibited the classical MDR phenotype with overexpression of mdr1 mRNA and increased P-gp expression.

Growth experiments

MDA-MB 231 and MCF-7 ADRr cells were plated in six-well flat-bottom plates (Falcon 3046, Becton Dickinson, Lincoln Park, N.J., USA) at a concentration of 8×10^4 cells/ml in the specific medium as described above. After 24 h the medium was replaced with fresh medium containing the compounds to be tested at various concentrations (range 1 *nM*–10 μM). Jurkat cells were plated in six-well flat-bottom plates at a concentration of 1×10^5 cells/ml in the specific medium supplemented as above either with or without the compounds to be tested. Quadruplicate hemocytometer counts of triplicate culture dishes were performed after 3 days of exposure to the drugs. Results are expressed as the concentration of test compound able to induce the half-maximal growth inhibition (IC_{50}). The IC_{50} values were calculated by fitting the concentration-effect curve data obtained in the three independent experiments with the sigmoid-Emax model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect [17].

Cell viability

To assess cell viability an ethidium bromide (EtBr) exclusion test was performed. Cells were washed once and then incubated for 15 min at room temperature with 4 μg/ml EtBr (Molecular Probes, Eugene, Ore., USA), a specific DNA dye. In addition, such analysis allows the identification of apoptotic cells in the earliest stages of the process [8]. The percentage of viable cells was calculated for the untreated control and for each culture condition in triplicate samples. Results are expressed as a percentage of the untreated control.

Reactive oxygen species production and intracellular thiol level

To investigate the redox state in the cells, 5-chloro-methyl-fluorescein diacetate (CMFDA, Molecular Probes) and 2,7-dichlorodihydro-fluorescein diacetate (DCHFDA, Molecular Probes) were used according to the flow cytometric technique as previously described [9].

Cell cycle analysis

Cells were plated at a concentration of 10^5 cells/ml in their specific medium. After 24 h the medium was replaced with fresh medium containing compounds to be tested or vehicle alone. After 24 h of treatment cells were harvested and nuclei isolated and stained using a solution containing 0.1% Na^+ citrate (w/v), 0.1% NP40 (v/v), 4 mM EDTA, and 50 μg/ml propidium iodide as DNA dye [8]. Incubation of the cells with staining solution lasted a minimum of 12 h at 4 °C. Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 20,000 nuclei with an Epics-XL flow cytometer (Coulter Immunology, Miami, Fla., USA). DNA fluorescence was collected in linear mode and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using a Multicycle software package (Phoenix, San Diego, Calif., USA).

Functional cytometric analysis of P-gp

Rhodamine 123 (Rh123) fluorescent probe (Molecular Probes) served to measure the functionality of the P-gp efflux pump according to the protocol of the National Cancer Institute Drug Screen [14] with minor modifications. Briefly, MCF-7 ADRr cells were loaded at 37 °C with 0.5 μg/ml of the dye in PBS supplemented by 0.2% of BSA. After 15 min cells were transferred onto ice and washed twice to remove free Rh123 from the medium. After washing, 10 μM of the potential P-gp inhibitors was added, and cells were kept at 37 °C for 30–120 min. The positive control was represented by quercetin, a well known inhibitor of the P-gp function. An aliquot of cells was maintained on ice to prevent dye efflux (control at 4 °C), and the maximal efflux was established by adding the vehicle DMSO 0.1% and allowing the efflux at 37 °C. Flow cytometric acquisition was performed by acquiring a minimum of 20,000 cells with an Epics-XL flow cytometer with standard collection filters and electronics. Mean channel of the Rh123 fluorescence was calculated for each condition and time point. The ratio of mean channel between control at 37 °C and control at 4 °C was considered the control dye efflux. Similarly, the mean channel of Rh123 fluorescence of treated cells was divided over the control at 4 °C. This ratio was divided over the control dye efflux to establish the potency of P-gp inhibition. Results from two independent experiments have been averaged.

Morphological analysis of apoptosis

Morphological features of apoptosis were assessed by scoring control and flavonoids treated cultures (concentration range 1–10 μM) seeded in six-well flat bottom plates using an inverted Diavert fluorescence microscope (Leica, Wetzlar, Germany). Cultures treated as previously described [8] were stained with the green DNA dye YOYO-1 (Molecular Probes). Image analysis was performed using the IAS2000 system (Delta Sistemi, Rome, Italy).

Statistical analysis

In some experiments statistical analysis used one-way analysis of variance. Post-hoc analysis of interaction effects used Tukey's "honestly significantly different" test. The Statistica 6.0 software package (Statsoft, Tulsa, Okla., USA) was used for all analyses.

Results

Growth inhibition effect

Human breast cancer cell lines were sensitive to the antiproliferative effect of the new chalcone analogues in micromolar concentrations. After calculating the IC_{50} following 72 h of continuous drug exposure for all cell lines **S1** was the most active agent ($IC_{50} = 1.2$ μM and 2.2 μM for MDA-MB231 and MCF-7 ADRr, respectively), followed in decreasing order by **S2** ($IC_{50} =$ 1.9 μM and 2.8 μM for MDA-MB231 and MCF-7 ADRr, respectively; see Table 1).

On the basis of these data we selected the two most active chalcone analogues (**S1** and **S2**) to study their effect on the proliferation of Jurkat cells, an additional P-gp negative model. Quercetin was used as the reference compound. The magnitude of the antiproliferative effect after 72 h of continuous treatment follows the order: **S1** > **S2** > quercetin ($IC_{50} = 2.2$, 2.7, and 5.6 μM, respectively), similar to that obtained with the breast cancer cell lines.

Although no definitive structure-activity relationship was determined, we were able to draw the following conclusion for the structural changes which might influence antiproliferative activity: (a) the presence in R_1 of the 3-methyl-2-buten-1-yl leads to a higher antitumor activity than other shorter side chains: replacing this group with either a propyn-3-yl or a 2-methyl-propen-3-yl caused a slight decrease in activity; (b) the replacement of the pyridyl group in R_2 decreases cytotoxicity in both breast cancer cell lines.

DNA analysis

We performed DNA analysis to establish whether the growth inhibition effect causes a specific block in the cell cycle. On the basis of the data obtained on cell growth inhibition we selected the most potent chalcone analogues to study their effect on the cell cycle in Jurkat cells.

Table 1 Tumor cell growth inhibition by chalcone analogues

Compound	IC_{50} (μM)	
	MDA-MB231	MCF-7 ADRr
Quercetin	3.4	9
S1	1.2	2.2
S2	1.9	2.8
S3	> 10	7
S4	> 10	9.4
S5	> 10	> 10
S6	> 10	> 10
S7	> 10	> 10
S8	5.4	6.0
S9	5.0	8.9
S10	3.7	3.7

After 24 h of treatment with various concentrations (range 0.1–10 μM) of the compounds there was no significant perturbation in the cell cycle distribution up to a dose of 1 μM. Interestingly, DNA analysis revealed that an increase in G_2/M block and DNA fragmentation, suggestive of apoptosis, occurred at the highest concentration of 10 μM (Table 2). In cells treated by both **S1** and **S2** this block was associated with a parallel increase in S phase. The order in the potency of inducing block and DNA fragmentation followed this order: **S2** > **S1** > quercetin. Similar results were obtained with MCF-7 ADRr cells (data not shown).

Assessment of reactive oxygen species production and intracellular thiol level

As chalcones have previously been shown to be antioxidant agents [1], we decided to investigate their ability to modulate the intracellular balance between reactive oxygen species (ROS) production and intracellular thiols. Flow cytometry and fluorescent probes for ROS (DCHFDA) and thiols (CMFDA) were used. The intracellular balance was compared to that produced by quercetin, previously reported as an antioxidant agent [16]. As shown in Fig. 2, after just 1 h of culture in the presence of **S1**, **S2**, and quercetin (range 0.1–10 μM) Jurkat cells showed a significant dose-dependent increase in intracellular thiol levels, in the following order of magnitude: quercetin > **S1** > **S2**. After 4 h this increase consistently subsided. After 24 h only quercetin was still able to significantly increase thiol levels (Fig. 2B). Analysis of ROS production showed these compounds had no affect until 24 h (Fig. 2A), where only a slight, nonsignificant increase in ROS production was detected only for the highest dose of **S1** and **S2** (10 μM). This increase was likely due to the massive increase in dead cells, as depicted in Fig. 3.

Table 2 Cell cycle modification induced by chalcone analogues in Jurkat cells after 24 h of treatment. DNA fragmentation was calculated as the percentage of events falling in the sub-G_{0-1} region and served as an indirect measurement of apoptosis. These events are excluded from cell cycle analysis

Compound	G_1	S	G_2	Percentage DNA fragmentation
DMSO 0.1%	79.6	17.0	3.4	8.5
S1 (0.1 μM)	81.2	15.2	3.6	8.9
S1 (1 μM)	83.8	13.0	3.2	9.9
S1 (10 μM)	65.7	27.1	7.2	26.6
S2 (0.1 μM)	81.1	16.2	2.7	9.2
S2 (1 μM)	83.7	12.7	3.6	10.5
S2 (10 μM)	67.9	24.4	7.7	34.1
Quercetin (0.1 μM)	83.2	12.9	3.9	8.4
Quercetin (1 μM)	81.5	15.3	3.2	8.5
Quercetin (10 μM)	77.3	15.9	6.8	14.0

Cell viability and evaluation of apoptosis

Cell viability was assessed by the EtBr exclusion test after 1, 4, and 24 h of continuous exposure to drugs. After 1 and 4 h no significant changes were evident, revealing that even at the highest concentrations these compounds were unable to induce a sudden necrotic cell death. After 24 h a significant decrease in viable cells was evident (Fig. 3), particularly at the highest doses of **S1**, **S2**, and quercetin (in this order). The occurrence of cell death and DNA fragmentation observed only at the highest compound concentrations led us to hypothesize that apoptosis is occurring in this cellular system. To test this hypothesis morphological analysis in Jurkat cell line was performed to identify nuclei with typical apoptotic features. Several signs of chromatin alterations were found in cells treated with chalcone analogues and quercetin. An increased chromatin granularity along the nuclear membrane was the prevalent morphological pattern found in quercetin-treated cells. In this condition only few figures of advanced apoptosis were visible. On the other hand, chromatin condensation in one of the cellular poles was the most common pattern in cells treated with **S1** and **S2**. In addition, particularly in samples treated with **S1**, classical apoptotic nuclei with fragmented chromatin were observable (Fig. 4). Taking all these data together, we concluded that these chalcone analogues were able to induce apoptosis in this model.

Rhodamine 123 efflux

Since is frequently associated with the MDR phenotype, we studied the effect of quercetin and the most active chalcones on P-gp using the efflux activity of the fluorescent dye Rh123 and flow cytometry. We used as a model MCF-7 ADRr cells which express high levels of P-gp. Results are reported in Fig. 5. As previously reported [24], quercetin (10 μM) induced MCF-7 ADRr cells to retain over time a consistent amount of Rh123, while **S1** and **S2** were unable significantly to modulate P-gp efflux activity.

Discussion

Among the number of substances identified from plants, flavonoids represent one of the most important and interesting class of biologically active compounds. The common synthone of the flavonoid family is that of chalcones, which in the cyclized form generates flavanones, flavones, isoflavones, and flavonols such as quercetin.

The present study was undertaken to assess and compare the biological activity of several synthetic chalcone analogues, using different established human cancer cell lines. Quercetin was used as the reference compound due to its potential as a new antitumor agent [2, 28]. Quercetin exerts a wide variety of biological

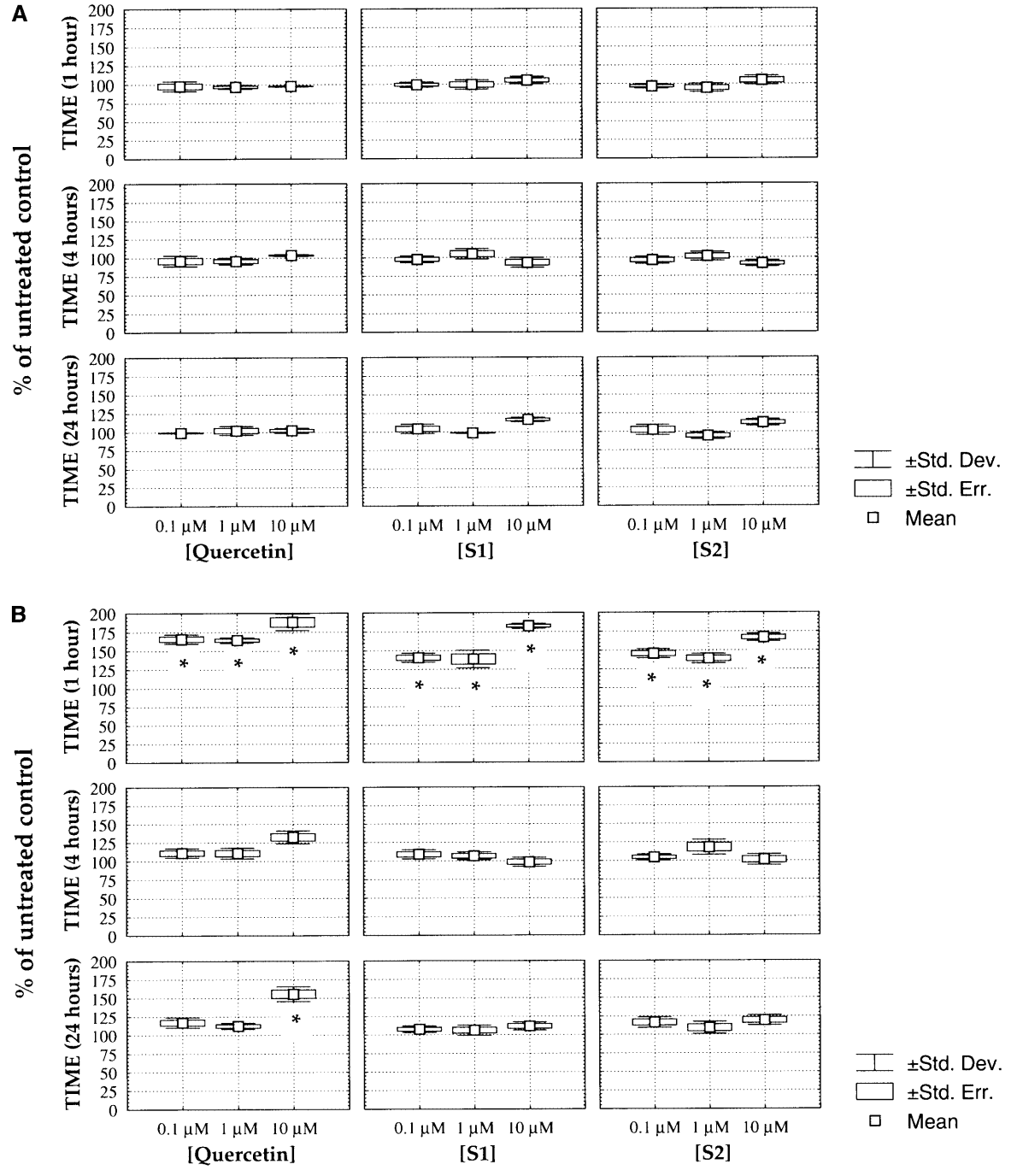


Fig. 2 Box-whisker plots showing ROS production (**A**) and intracellular thiol level (**B**) in Jurkat cells after 1, 4, and 24 h of continuous exposure to **S1**, **S2**, and quercetin. Mean channel of DCHFDA and CMFDA fluorescence for ROS and thiol level, respectively, was calculated for each culture in triplicate samples. Data are expressed as the percentages of the untreated control. *Bars and boxes* Standard deviation and error standard of triplicates, respectively. $*P < 0.05$

actions, such as an antiproliferative effect [22, 23, 24, 31], inhibition of protein kinase C [25], and induction of apoptosis [28]. Furthermore, quercetin may act synergistically with common chemotherapeutic agents both in vitro and in vivo [12, 23] and is also able to act as chemopreventive agent in a rat model [15]. A phase I clinical trial has been concluded demonstrating that quercetin is well tolerated, and that some clinical response is obtained in a restricted number of patients [10].

In this work we have shown that the three cancer cell lines tested are sensitive to the antiproliferative effect of some chalcone analogues in a range of micromolar concentrations. Interestingly, we have identified new compounds (**S1** and **S2**) more active than quercetin in terms of growth inhibition of both MDR-positive and MDR-negative cancer cell lines. Remarkably, the

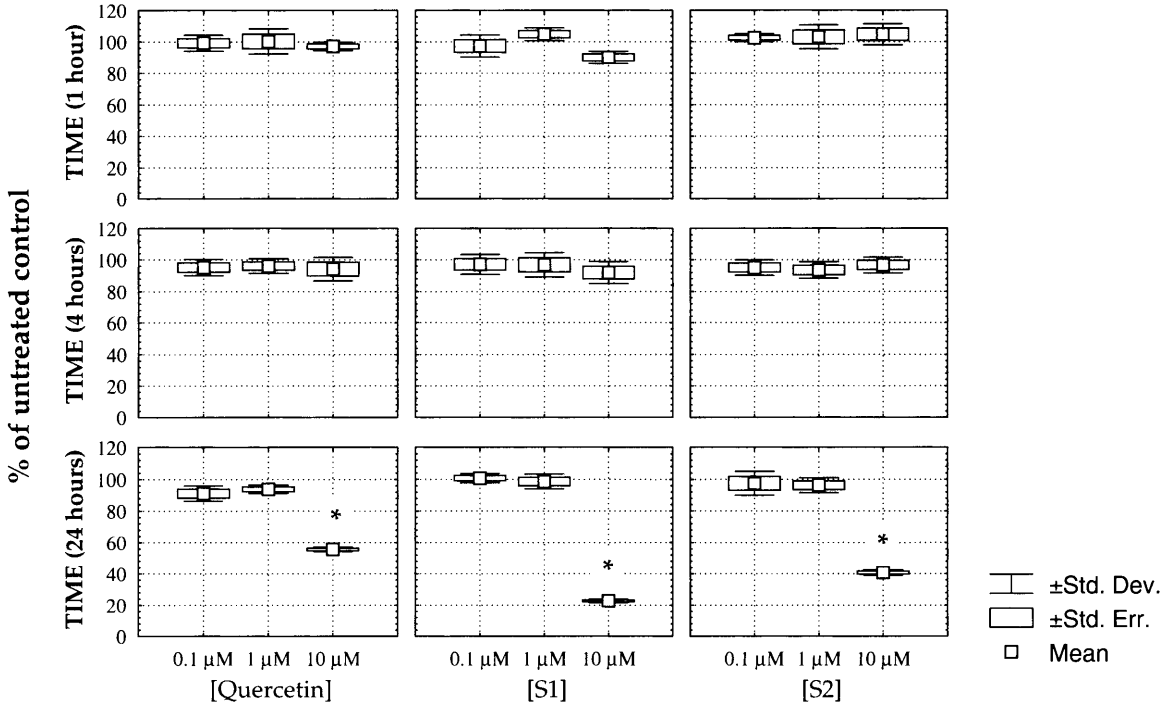


Fig. 3 Box-whisker plots showing cell viability in Jurkat cells after 1, 4, and 24 h of continuous exposure to **S1**, **S2**, and quercetin. The percentage of viable cells has been calculated using the EtBr exclusion test in triplicate samples. Data are expressed as the percentages of untreated control. *Bars and boxes* Standard deviation and error standard of triplicates, respectively. $*P < 0.05$

difference in the activity of **S1** on multidrug resistant MCF7-ADRr cells, as compared to MDA-MB 231 and Jurkat cells was very limited, indicating that the MDR phenotype would not play a substantial role in resistance to these drugs.

From a comparison of our results with values reported in the literature, it is interesting that IC_{50} values of **S1** and **S2** show a growth inhibition effect in the concentration range (1–2 µM) of the most active chalcone analogues previously tested [6, 30].

The mechanism of the antitumor activity of chalcones remains to be fully clarified. To gain further insight into

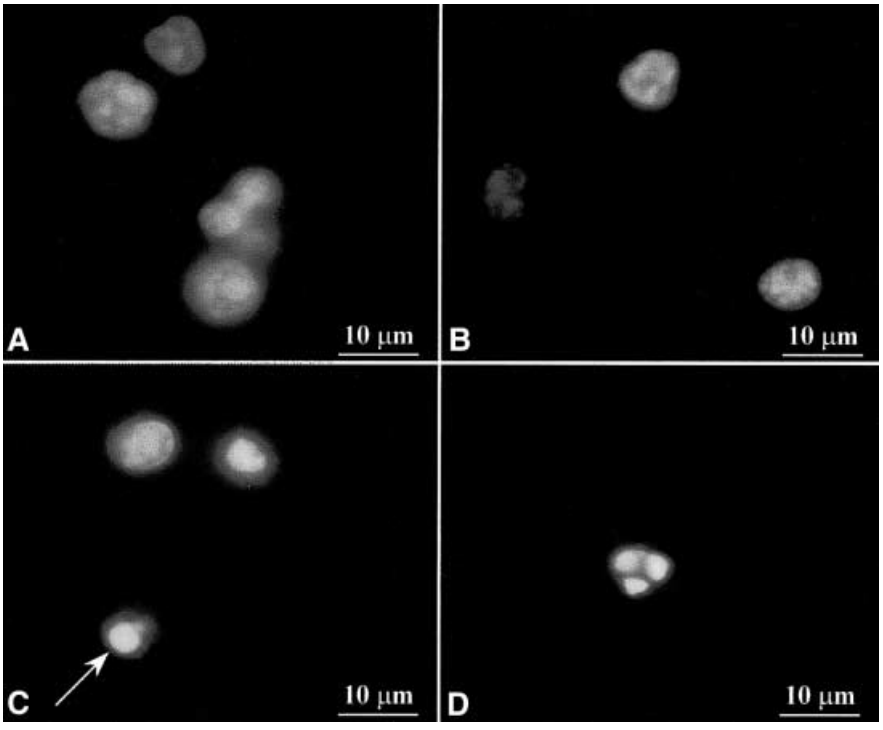


Fig. 4A–D Morphological analysis of apoptosis (cell magnification, ×1250). **A** Control (DMSO 0.1%) cells. **B** Cells with chromatin particularly along the nuclear envelope (quercetin 10 µM). **C** Condensation and marginalization of chromatin (*arrow*; **S1** 10 µM). **D** Late apoptosis with complete chromatin fragmentation (**S2** 10 µM)

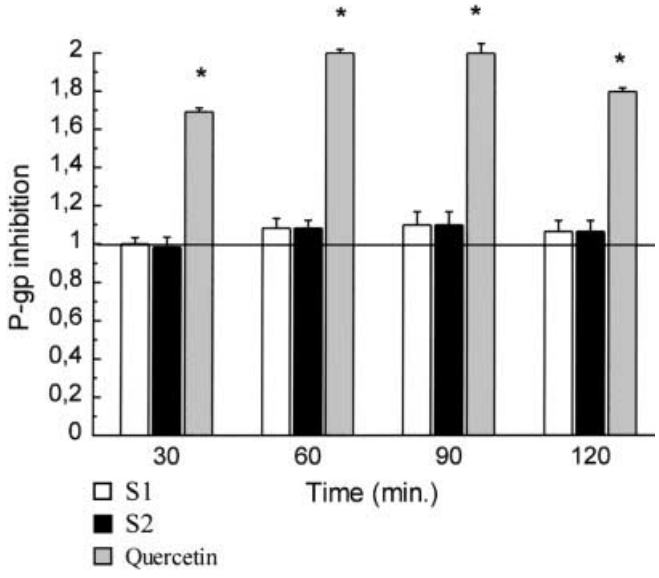


Fig. 5 Bar chart of P-gp inhibition of the panel of flavonoids. MCF-7 ADRr cells were loaded with Rh123 (0.5 μg/ml). After 15 min cells were transferred onto ice and washed twice in cold PBS/BSA 0.2%. Onto ice was added 10 μM of all the tested drugs or the vehicle, DMSO 0.1%. Cells were then kept at 37 °C to allow the maximal dye efflux. An aliquot of control cells was maintained on ice to have the maximal dye uptake. At each time point (from 30–120 min), Rh123 fluorescence was assessed by flow cytometry. The ratio between control at 37 °C and control at 4 °C served to calculate the control dye efflux. The same procedure was employed for each drug. This ratio was divided by the amount of the control dye efflux to establish the P-gp inhibition. A value higher than 1 means that drug acts as P-gp inhibitor. Results obtained from the mean of two independent experiments are shown. *Bars* Standard deviations. $*P < 0.05$ vs. control (*continuous line*)

this aspect we selected the most active compounds, **S1** and **S2**, to evaluate their effect on the cell cycle, apoptosis, redox activity, and modulation of the P-gp function, always using quercetin as a reference compound. The EtBr exclusion test after 24 h of exposure to the drugs indicated an increased amount of dead cells at the highest doses (10 μM) of **S1**, **S2**, and quercetin (in this order). The occurrence of this massive cell death led us to hypothesize the presence of apoptosis in this system. This view was supported by cell cycle analysis of the **S1** and **S2** analogues showing that there was a G_2/M cell cycle arrest concomitant with the increase in DNA fragmentation at the same doses at which a massive rate of dead cells was found. To verify the presence of apoptosis morphological analysis was performed, and this revealed that apoptosis occurs in this system. Again, in this model, **S1** and **S2** were able to induce apoptosis to a greater extent than quercetin. Considering together the IC_{50} values and the assessment of apoptosis, we concluded that these newly developed compounds have an antiproliferative activity greater than that of quercetin.

Although these newly developed compounds possess a partial similarity to the structure of quercetin, several lines of evidence suggest that there are qualitative differences in the activity and antitumor performance of the chalcone analogues as compared with quercetin: (a) Chalcone analogues did not modulate P-gp function in our experimental model whereas quercetin did. (b) ROS production was essentially unaffected by these compounds, and intracellular thiol levels were increased in a short period of time by all compounds. However, this level returned to baseline value after 24 h of culture in the presence of the newly developed chalcones, whereas it was still increasing in quercetin-treated cells, confirming previous observations of quercetin as a potent antioxidant [16]. (c) The morphological pattern of apoptosis induced by chalcones differs from that of quercetin. The hallmark of quercetin toxicity is a large and sparse chromatin distress, whereas with the newly developed analogues apoptosis reaches the later phases of the process with the consequent appearance of nuclei characterized by extremely condensed and fragmented chromatin.

These differences are important, and they may account for a diverse use of these newly developed chalcone analogues with respect to quercetin.

Moreover, an interesting property of this class of chalcones is their reported selective toxicity toward neoplastic rather than normal tissues [6, 18], thereby suggesting that in vivo high doses of these drugs should be administered without severe general side effects. Further studies are now in progress in our laboratory to identify molecular pathways, such as ras-farnesylation, possibly involved in the antitumor activity of the compounds.

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