



## Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells

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### Abstract

Effects of three flavonoids, quercetin (QU), galangin (GA), and chrysin (ChR) on cisplatin (cis-Pt)-induced apoptosis of human promyelocytic leukemia HL-60 cells and murine leukemia L1210 cells were investigated. The quantitative analysis of apoptotic DNA fragmentation was used to show that preincubation of cells with flavonoids can influence cis-Pt-induced apoptosis in different way. ChR had no effect, QU enhanced, and GA reduced apoptotic DNA fragmentation. It is also shown that combined treatment with QU and cis-Pt showed synergistic effect, however, GA combined with cis-Pt exhibited antagonism on cytotoxicity in L1210 murine leukemia cells. We assume that tested flavonoids affect the important biological activities connected with cancer chemotherapy and chemoprevention as they differently modulated the sensitivity of cells to cis-Pt treatment. QU is presented as pro-apoptotic agent and GA as agent with anti-apoptotic potential. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Quercetin; Galangin; Chrysin; Cisplatin; Apoptosis; HL-60; L1210

### 1. Introduction

Flavonoids are polyphenolic compounds that occur naturally in foods of plant origin. They have a variety of biological activities, such as anti-allergic, anti-inflammatory, anti-oxidative, free radical scavenging, and anti-mutagenic activities [1]. Many studies have demonstrated that flavonoids are also potent inhibitors of key enzymes taking part in signal transduction. They inhibit several kinases such as PKC, tyrosine kinases, or lipid kinases [2], affect various metabolic pathways such as activation of glycolytic enzymes or protein synthesis [3], promote cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> or G<sub>2</sub>/M phase, depending on their structure and on the cellular model [4–6], interact with estrogen type II binding sites to regulate mammary cell growth [7], and induce apoptosis in different cell lines [8,9]. Some flavonoids are more selective towards cancer cells and may have the

potential for reducing side-effects compared with other drugs [10].

Because of extensive intake of flavonoids by humans, it was essential to analyze their potential effect on chemotherapy treatment. Previous reports showed that some flavonoids could potentiate anti-proliferative effects of some chemotherapeutics [11,12], however, there was no evidence that they also intervene with chemotherapy-induced apoptosis. As the biological activities of chemicals are dependent on the individual structure, we have investigated effects of QU, GA and ChR, three structurally related flavonoids (Fig. 1) on cell viability and cisplatin (cis-Pt)-induced apoptosis of human promyelocytic HL-60 and murine leukemia L1210 cell lines. The aim of the present work was to examine the eligibility of presented agents in cancer chemotherapy or prevention.

### 2. Materials and methods

#### 2.1. Drugs

Platidium (cis-Pt) was obtained from Lachema (Brno, Czech Republic) as a solution for injection. Flavonoids (QU, GA, and ChR) were purchased from Sigma (St. Louis,

*Abbreviations:* HL-60, human promyelocytic leukemia cell line; L1210, murine leukemia cell line; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; QU, quercetin; GA, galangin; ChR, chrysin; PKC, protein kinase C; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; PARP, poly(ADP-ribose) polymerase

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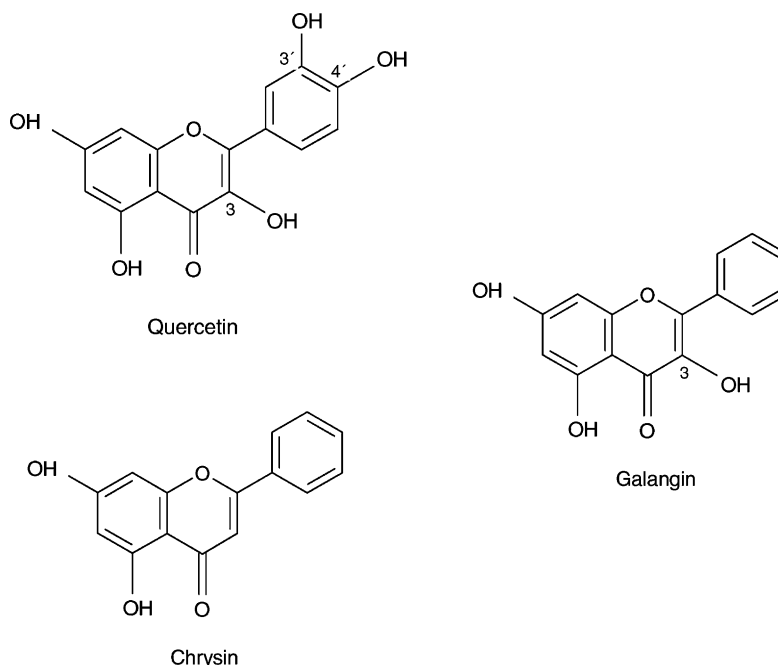


Fig. 1. Chemical structure of the flavonoids, QU, GA and ChR.

MO, USA) and dissolved in dimethyl sulphoxide (DMSO, Sigma). The stock solutions of flavonoids (0.1 M) were stored at  $-20^{\circ}\text{C}$ . The final concentration of DMSO in the medium was  $<0.02\%$  and did not affect cell growth [13].

## 2.2. Cell culture

Human promyelocytic leukemia HL-60 cells were kindly provided by Dr. P. Ujházy (Roswell Park Cancer Institute, Buffalo, USA) and murine leukemia cell line L1210 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY, USA), 100 U/ml Penicillin G, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mM L-glutamine (Sebac, Germany) in an atmosphere of 5%  $\text{CO}_2$  in humidified air at  $37^{\circ}\text{C}$ . In all experiments, exponentially growing cells were used.

## 2.3. Cell viability assay

Cell viability was assayed in triplicate using a MTT chemosensitivity assay [14]. Briefly, exponentially growing cells ( $1.5 \times 10^5$  cells/ml) were incubated in 96-well microtiter plates with medium containing various concentrations of drugs and their combinations in the final volume of 200  $\mu\text{l}$ . After 24 h drug incubation at  $37^{\circ}\text{C}$ , 50  $\mu\text{l}$  of MTT (1 mg/ml, Sigma) was added to each well followed by 4 h incubation at  $37^{\circ}\text{C}$ . Cells were centrifuged, the supernatant was discarded and the pellet was dissolved in DMSO (150  $\mu\text{l}$ ).  $\text{OD}_{540\text{nm}}$  was determined by Microplate

Autoreader (Labsystem Multiscan Multisoft, Finland) as cited [15]. Simultaneously, number of surviving cells was counted by Trypan blue exclusive method.

## 2.4. Analysis on drug combination

The MTT data were analyzed using Calcsyn program to determine the  $\text{IC}_{50}$  of each drug alone. The combination index (CI)-isobologram by Chou and Talalay [16] was used to analyze the drug combination. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate).  $\text{CI} > 1$ ,  $\text{CI} = 1$ , and  $\text{CI} < 1$  indicate antagonism, additive effect, or synergism, respectively.

## 2.5. Induction of apoptosis

Exponentially growing cells were harvested by centrifugation and resuspended in fresh medium to achieve a culture density of  $4 \times 10^5$  cells/ml. The cells were induced to apoptosis by 250  $\mu\text{M}$  cis-Pt for 4 h (HL-60 cells) and 1–4  $\mu\text{M}$  cis-Pt for 22 h (L1210 cells). When flavonoids were tested, they were added 40 min before cis-Pt addition to achieve a sufficient effect.

## 2.6. Electrophoretic analysis of DNA fragmentation

The untreated cells (control) and drug-treated cells ( $1 \times 10^6$ ) were harvested, washed in phosphate-buffered saline

(PBS) and then lysed in 100  $\mu$ l of solution (10 mmol/l TRIS, 10 mmol/l EDTA, 0.5% Triton X-100) supplemented with proteinase K (1 mg/ml, Serva, Germany). Samples were then incubated at 37 °C for 1 h and heated at 70 °C for 10 min. Following lysis, RNA-ase (200  $\mu$ g/ml, Serva) was added and followed by repeated incubation at 37 °C for 1 h. The samples were electrophoresed at 40 V for 3 h in 2% (w/v) agarose gels (Sigma) complemented with ethidium bromide (1  $\mu$ g/ml, Sigma) [17]. Separated DNA fragments (DNA ladders) were visualised using UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA). The size of DNA fragments was determined by comparing to the DNA molecular weight markers, Superladders-Mid2 200 bp ladder (Advanced Biotechnologies, UK).

### 2.7. Quantification of DNA fragmentation

The extent of DNA fragmentation of cellular DNA of treated cells was determined by the method of Rauko et al. [18]. Briefly, equal amounts of DNA samples (from  $1 \times 10^6$  cells) were electrophoresed and visualized as described above. Photographs of gels were made using digital camera Olympus CAMEDIA C-1400 L (Japan) and elaborated by the software Olympus C-W95. Determination of a relative DNA intensity at the area of DNA ladders (width of area  $>200$  to  $<1200$  bp) was performed using the software UTH-SCSA Image Tool for Windows (Version 1.28). The values of DNA ladder intensities are presented as the percentage of DNA ladder intensity of cis-Pt treated cells.

## 3. Results

### 3.1. Effects of flavonoids on cisplatin-induced apoptosis of HL-60 cells

Effects of three structurally related flavonoids (QU, GA, and ChR) on cis-Pt-induced apoptosis were examined by incubating HL-60 cells with 250  $\mu$ M cis-Pt for 4 h after 40 min preincubation with different concentrations of flavonoids (2.5, 10, 20  $\mu$ M). We monitored the intensity of apoptotic DNA fragmentation by agarose gel electrophoresis. No effect on apoptotic DNA fragmentation was observed in cells treated with combination of ChR and cis-Pt. Both QU and GA affected cis-Pt-induced apoptosis of HL-60 cells, however by opposite modes (Fig. 2a). Effects of increasing concentrations of flavonoids tested on cis-Pt-induced apoptotic DNA fragmentation are shown in Fig. 2b. Combined treatment with 20  $\mu$ M QU and cis-Pt increased apoptotic DNA fragmentation (148.20% of cis-Pt treated cells). Treatment with cis-Pt and 20  $\mu$ M GA decreased the intensity of DNA ladders (46.18% of cis-Pt treated cells). The stimulation/reduction of apoptotic DNA fragmentation was concentration-dependent. Flavonoids themselves did not induce apoptotic DNA fragmentation after treatment of HL-60 cells for 4 h (data not shown).

### 3.2. The effect of time-different treatment with flavonoids on cisplatin-induced apoptosis of HL-60 cells

In studies on the time dependence of treatment with flavonoids (QU and GA), we found that the time of flavonoid addition (20  $\mu$ M) is important for maximal enhancement (QU) or reduction (GA) of cis-Pt-induced apoptotic DNA fragmentation.

The significant effects were achieved when flavonoids were added 30 min before (QU) or simultaneously (GA) with cis-Pt. After flavonoid pretreatment periods of 40 min and longer, no further enhancement or reduction of cis-Pt-induced apoptotic DNA fragmentation occurred. Simultaneous treatment (QU) or post-treatment (QU and GA) with flavonoids had little or no effect on cis-Pt-induced apoptosis. Results are shown in Fig. 2c.

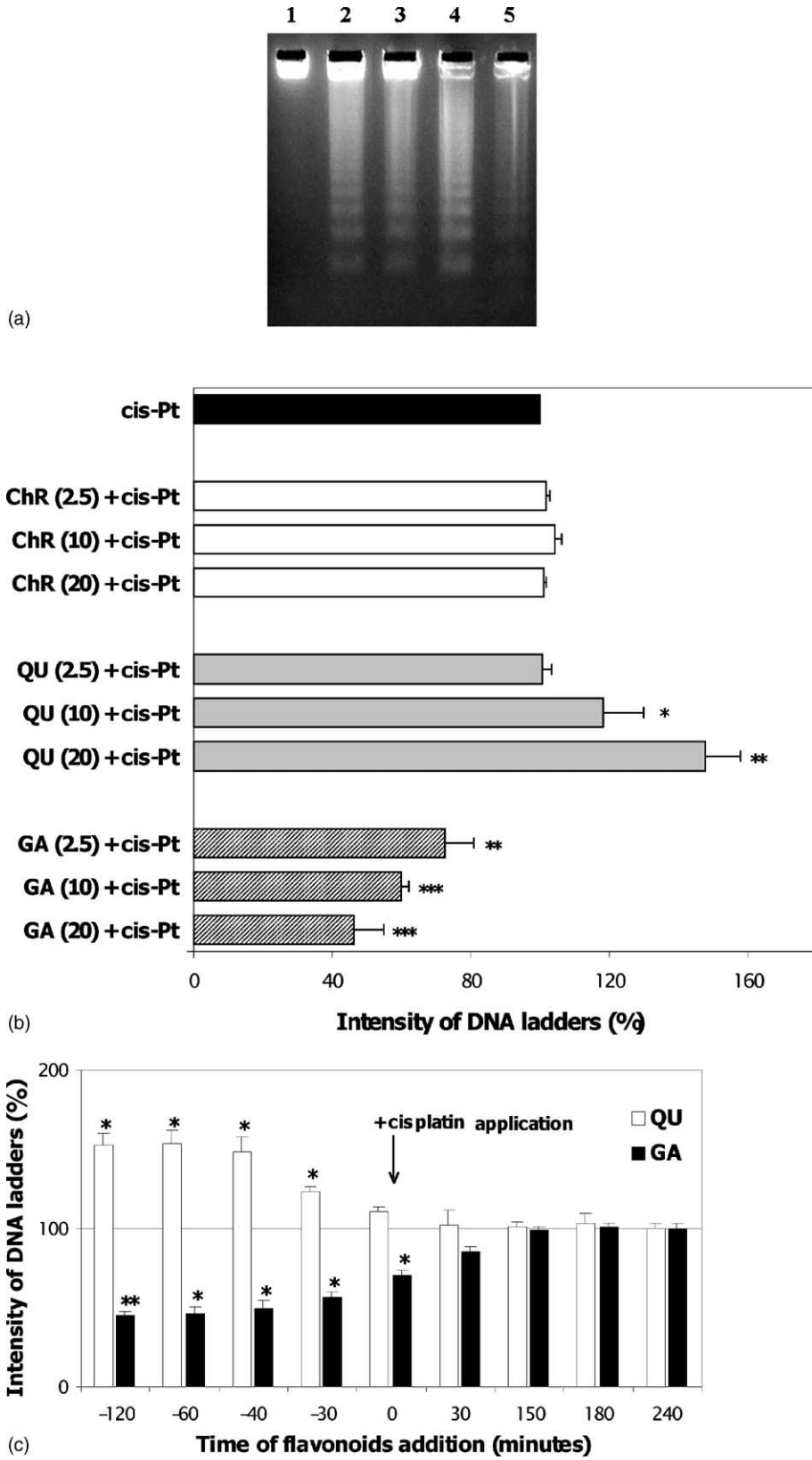
### 3.3. The effect of QU on cisplatin-induced apoptosis of L1210 cells

To prove the ability of QU to potentiate cis-Pt-induced apoptosis, L1210 cells were treated for 22 h with cis-Pt (1–4  $\mu$ M) alone or combined with various doses of QU (5–10  $\mu$ M). As indicated in Fig. 3a and b, QU and cis-Pt induced dose-dependent apoptosis of L1210 cells. Intensity of apoptotic DNA fragmentation of cells treated with drug combinations was significantly increased in comparison to that obtained in single drug treated cells. These results proved synergistic effect of QU and cis-Pt on apoptosis of L1210 cells, corresponding to results obtained with HL-60 cells (see Fig. 2b).

### 3.4. Opposite effects of QU and GA combined with cisplatin on cytotoxicity in L1210 cells

To determine the correlation between cytotoxicity and cis-Pt-induced apoptosis modulated by flavonoids, MTT chemosensitivity test and Trypan blue assay were used. Drug combination studies by MTT test demonstrated that QU combined with cis-Pt synergistically enhanced cell death (CI  $< 1$ ), however, GA combined with cis-Pt exhibited antagonism as far as cell death was concerned (CI  $> 1$ , Table 1).

Dose-dependence study of combined application of QU and cis-Pt by MTT test did not reveal any enhancement of synergism when higher concentrations of QU were used. On the contrast, Trypan blue assay proved concentration-dependent enhancement of synergism. CI values obtained by Trypan blue assay and MTT test after the combined treatment with QU and cis-Pt are presented in Table 2. These results correlate with significantly increased apoptotic DNA fragmentation in L1210 cells (see Fig. 3a and b). Therefore, Trypan blue assay is more eligible than MTT test for examination of combined treatment with flavonoids and cis-Pt, because of possible intervention of flavonoids in reduction of MTT.



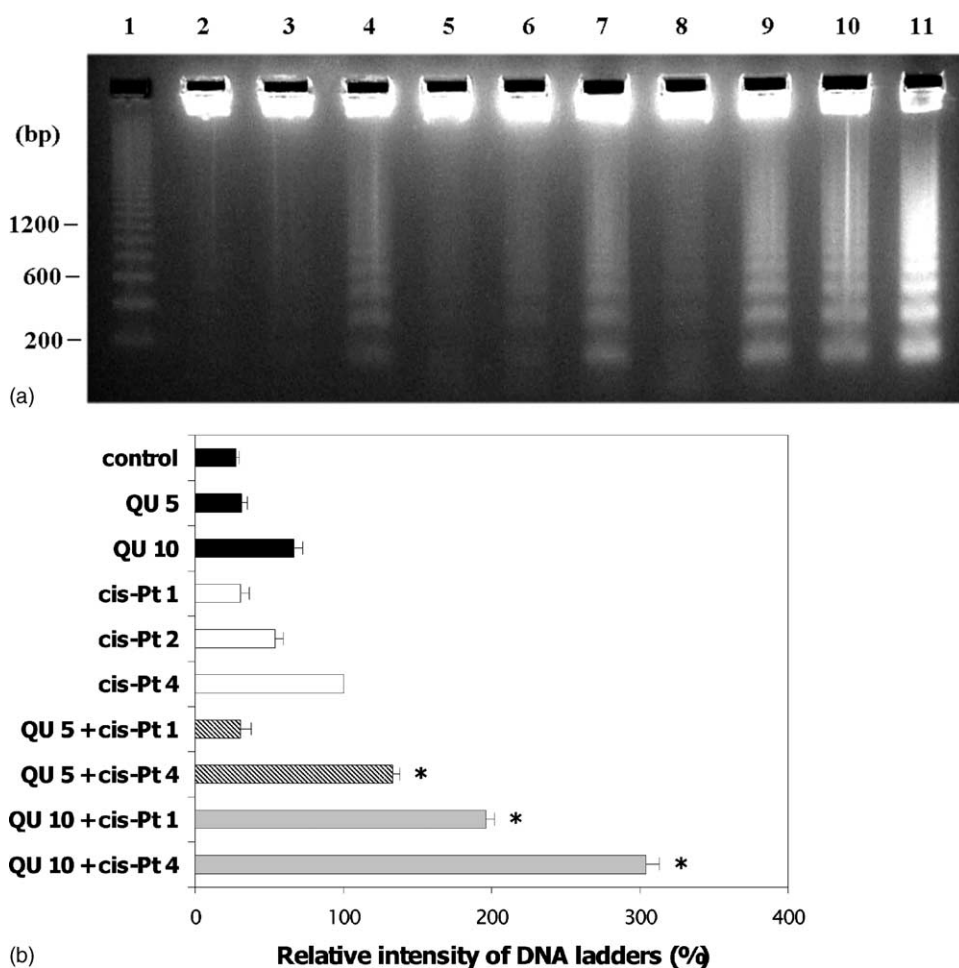


Fig. 3. (a) Apoptotic DNA fragmentation of L1210 leukemia cells after 22 h application of cis-Pt or QU alone or in combination. Lane 1, size marker DNA (super ladder-Mid2); lane 2, untreated cells; lanes 3 and 4, cells treated 22 h with 5 and 10  $\mu\text{M}$  QU; lanes 5–7, cells treated 22 h with 1, 2 and 4  $\mu\text{M}$  cis-Pt; lanes 8 and 9, cells 40 min preincubated with 5  $\mu\text{M}$  QU and then coincubated 22 h with 1 and 4  $\mu\text{M}$  cis-Pt; lanes 10 and 11, cells 40 min preincubated with 10  $\mu\text{M}$  QU and then coincubated 22 h with 1 and 4  $\mu\text{M}$  cis-Pt, respectively. (b) Densitometric evaluation of DNA ladder content obtained after treating of L1210 cells with QU and cis-Pt (cis-Pt) alone and in combination. The cells were preincubated with 5 and 10  $\mu\text{M}$  QU for 40 min and then treated with 1, 2 and 4  $\mu\text{M}$  cis-Pt for 22 h (see Fig. 3a). Apoptotic DNA fragmentation was monitored by agarose gel electrophoresis and intensity of DNA ladders was quantificated as described in Section 2. The values of DNA intensities are presented as the percentage of DNA ladder intensity of cis-Pt (4  $\mu\text{M}$ ) treated cells. The columns represent mean  $\pm$  S.D. from three independent experiments. Note: \* $P < 0.05$  as compared with single drug treated cells.

Fig. 2. (a) Detection of apoptotic DNA fragmentation in treated HL-60 leukemia cells by agarose gel electrophoresis. Cells were exposed to 250  $\mu\text{M}$  cis-Pt for 4 h after 40 min preincubation with or without 20  $\mu\text{M}$  flavonoids (ChR, QU, GA). Lane 1, negative control (non-treated cells); lane 2, cells treated with cis-Pt without flavonoid; lane 3, cells preincubated with ChR and then treated with cis-Pt; lane 4, cells preincubated with QU and then treated with cis-Pt; lane 5, cells preincubated with GA and then treated with cis-Pt. (b) The different effect of flavonoids on cis-Pt-induced apoptosis of HL-60 cells. The cells were preincubated with 2.5, 10 and 20  $\mu\text{M}$  flavonoids (ChR, QU, GA) for 40 min and then treated with 250  $\mu\text{M}$  cis-Pt for 4 h. Apoptotic DNA fragmentation was monitored by agarose gel electrophoresis and intensity of DNA ladders was quantificated as described in Section 2. The columns represent mean  $\pm$  S.D. from three independent experiments. Note: \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  as compared with cis-Pt treated cells. (c) The effect of pre- or coincubation of two flavonoids (QU and GA) on intensity of apoptotic DNA fragmentation in cis-Pt treated HL-60 cells. Cells exposed to 250  $\mu\text{M}$  cisplatin (0 min) were either preincubated (30, 40, 60 and 120 min) or coincubated (0, 30, 150, 180 and 240 min) with 20  $\mu\text{M}$  flavonoids. Then the cells were harvested and intensity of DNA ladders was determined as described in Section 2. The values of DNA ladder intensities are presented as the percentage of DNA ladder intensity of cis-Pt treated cells without flavonoid application. Data represent mean  $\pm$  S.D. from three separate experiments. Note: \* $P < 0.05$  and \*\* $P < 0.01$  as compared with cis-Pt treated cells.

Table 1  
Combined effects of cis-Pt and QU or GA on viability of L1210 cells monitored by MTT test

Single drug treatment		Drug combination		CI <sup>a</sup>
Agent (μM)	Viability (% of control)	Agent (μM)	Viability (% of control)	
Cis-Pt 1	99.9 ± 4.2	Cis-Pt 1 + QU 5	80.2 ± 5.6	0.537
Cis-Pt 2	99.9 ± 3.5	Cis-Pt 1 + QU	1054.6 ± 2.4	0.702
Cis-Pt 4	92.3 ± 2.9	Cis-Pt 1 + QU 20	46.7 ± 3.7	1.212
Cis-Pt 6	81.6 ± 5.7	Cis-Pt 1 + GA 5	99.9 ± 4.2	1.649
QU 5	99.9 ± 2.8	Cis-Pt 1 + GA 10	99.8 ± 4.2	1.983
QU 10	71.9 ± 4.6	Cis-Pt 1 + GA 20	95.7 ± 6.0	1.126
QU 20	55.6 ± 5.3	Cis-Pt 2 + QU 5	77.6 ± 2.8	0.665
GA 5	99.9 ± 0.6	Cis-Pt 2 + QU 10	54.6 ± 4.9	0.810
GA 10	99.8 ± 3.6	Cis-Pt 2 + QU 20	48.9 ± 7.3	1.336
GA 20	95.6 ± 10.7	Cis-Pt 2 + GA 5	99.9 ± 2.4	2.435
		Cis-Pt 2 + GA 10	99.7 ± 3.9	2.294
		Cis-Pt 2 + GA 20	95.3 ± 6.3	1.339
		Cis-Pt 4 + QU 5	81.3 ± 4.5	1.019
		Cis-Pt 4 + QU 10	48.7 ± 2.1	0.968
		Cis-Pt 4 + QU 20	39.6 ± 5.3	1.409
		Cis-Pt 4 + GA 5	93.1 ± 4.2	1.063
		Cis-Pt 4 + GA 10	89.9 ± 4.2	1.092
		Cis-Pt 4 + GA 20	81.5 ± 6.0	1.117
		Cis-Pt 6 + QU 5	69.4 ± 1.9	1.119
		Cis-Pt 6 + QU 10	38.3 ± 3.5	1.051
		Cis-Pt 6 + QU 20	32.2 ± 2.3	1.472
		Cis-Pt 6 + GA 5	82.9 ± 4.2	1.102
		Cis-Pt 6 + GA 10	72.1 ± 4.2	1.011
		Cis-Pt 6 + GA 20	67.9 ± 6.0	1.136

Data are presented as mean ± S.D. of triplicate determinations.

<sup>a</sup> CI was calculated as described in Section 2. Antagonism (CI > 1); synergism (CI < 1).

Table 2  
Combined effect of QU and cis-Pt on cytotoxicity after their application on L1210 cells measured by two methods (MTT test and Trypan blue assay)

Agents (μM)	CI <sup>a</sup> value (MTT test)	CI <sup>a</sup> value (Trypan blue assay)
QU 5.0 + cis-Pt 1.0	0.537	0.569
QU 10.0 + cis-Pt 1.0	0.702	0.327
QU 5.0 + cis-Pt 2.0	0.665	0.269
QU 10.0 + cis-Pt 2.0	0.810	0.176
QU 5.0 + cis-Pt 4.0	1.019	–
QU 10.0 + cis-Pt 4.0	0.968	–

<sup>a</sup> CI was calculated as described in Section 2. Antagonism (CI > 1); synergism (CI < 1).

#### 4. Discussion

The anti-neoplastic effects of some agents can influence cells by many different mechanisms, either by preventing initiation and promotion of cancerogenesis or by elimination of abnormal cells by apoptosis. In the process of chemotherapy some agents cooperate with other anti-neoplastic drugs to induce apoptosis and thus may increase/decrease drug efficacy [19,20]. Therefore, the identification of inhibitors or activators of apoptosis may help in providing more effective strategies for therapeutic intervention.

In our laboratory, agents that either prevent induction of damages to DNA by radical scavenging and metal chelating [20,21] or eliminate the abnormal cells by apoptosis are studied [18,22]. In the present work, we studied effects of some flavonoids on cis-Pt-induced apoptosis and cytotoxicity in HL-60 and L1210 leukemia cells.

GA was presented as the agent with anti-oxidative, free radical scavenging, anti-mutagenic and enzyme modulating activities [19]. Results from in vitro and in vivo studies indicate that GA is capable of suppressing the mutagenicity and clastogenicity of *N*-methyl-*N*-nitrosourea [23]. Therefore, it may be useful as a cancer chemopreventive agent against potential long-term health effects from genotoxic environmental compounds [19,23]. Up to now, only a few studies have been conducted on apoptosis for GA. The ability of GA to block apoptosis was observed with polycyclic aromatic hydrocarbon-induced pre-B-cell apoptosis. This inhibition of apoptosis is specific and was not determined if C<sub>2</sub>-ceramide and H<sub>2</sub>O<sub>2</sub> were used as inducers of apoptosis [24]. The failure of GA to block H<sub>2</sub>O<sub>2</sub> and C<sub>2</sub>-ceramide-induced pre-B cell apoptosis indicates that radical scavenging within pre-B cells by GA does not contribute to apoptosis inhibition in this system and signals distal to ceramide generation, such as caspase 8 and caspase 3 activation [25], are not likely to be targeted by this flavonoid. Our results showed that GA acts also as an inhibitor of cis-Pt-induced apoptosis. At present, the reason

why the pretreatment of cells with GA results in inhibition of cis-Pt-induced apoptosis is unknown. We assume, this is due to the modulation of enzyme activities involved in chemoprevention.

Another anti-neoplastic effect of flavonoids is associated with the elimination of drug-treated cells by apoptosis [8,9]. As it was presented earlier, QU sensitizes cells to the cytotoxic potential of cis-Pt [26,27], synergistically enhances the anti-proliferative activity of cis-Pt in vitro by inhibition of PKC [28], increases the anti-tumourous activity of cis-Pt in vivo [29], and are able to induce apoptosis by activating the pre-existing apoptosis machinery, such as induction of caspase-3 activity and degradation of PARP [9]. Therefore, the combination of QU and cis-Pt (also other cytostatic drugs) might be of therapeutic benefit. In our experiments, QU is presented as agent with increased effect on cis-Pt-induced apoptosis and cytotoxicity. Data on agarose gel electrophoresis clearly showed that pretreatment with QU enhanced cis-Pt-induced apoptotic DNA fragmentation in HL-60 and L1210 leukemia cells. Although the exact mechanism of QU intervention to cis-Pt-induced therapy is unclear, we assume that this process related to the application of QU in pretreatment is associated with the regulation of apoptosis.

In conclusion, we presented GA as anti-apoptotic agent that had a negative effect on cis-Pt-induced apoptosis. However, QU is presented as pro-apoptotic agent with the perspective potential for cis-Pt-induced apoptosis. Despite of structural similarity of tested flavonoids, we found their modulating effects on cis-Pt efficacy to be contrary. Elucidation of the molecular mechanisms by which flavonoids modulate cis-Pt-induced apoptosis is the topic to be addressed. Further investigations of new flavonoids as agents with cancer-preventive or therapy-increasing effects are required as well.

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