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Effects of Selected Flavonoids and Carotenoids on Drug Accumulation and Apoptosis Induction in Multidrug-resistant Colon Cancer Cells Expressing MDR1/LRP

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Abstract. *The effects of various flavonoids and carotenoids on Rhodamine 123 accumulation in multidrug-resistant Colo 320 human colon cancer cells expressing MDR1/LRP were studied. The Colo 205 cell line was used as a drug-sensitive control. Rotenon, Catechin, Neohesperidin, Naringin, Robinin, Phloridzin, Robinetin, Dihydrobinetin, Dihydrofisetin, Kampferol, Dihidroquercetin, Sakuranin and Sakuranetin were tested on Colo 320 cells: only Rotenon was found to be effective as regards multidrug resistance (MDR) reversal, while a majority of the flavonoids, such as Catechin, Neohesperidin, Naringin, Robinin, Phloridzin, Dihydrobinetin and Sakuranetin, had only marginal effects on Rhodamine 123 accumulation. The tested carotenoids (β-Cryptoxanthin, Luteoxanthin, Anteroxanthin, Violeoxanthin, Apple peel fetoxanthin, Lutein, Violaxanthin and Neoxanthin) were able to increase Rhodamine 123 accumulation in Colo 320 cells. Verapamil was applied as a resistance-modifying positive control. The levels of apoptosis induction in drug-resistant and -sensitive cell lines were also compared. The results indicated that the tested flavonoids were weak apoptosis inducers on MDR and parent cells, without significant differences. A majority of the carotenoids induced only early apoptosis, but apoptosis and cell death were not induced in MDR colon cancer cells.*

Cross-resistance between different cytostatic agents, which are structurally and functionally unrelated, is a common phenomenon, called multidrug resistance (MDR). There are several ways that cancer cells develop resistance against anticancer drugs (1-3). One of them is the function of efflux pumps, whereby cells pump out drugs from the cytoplasm (4-6). Early attempts to characterize such efflux pumps were reviewed by Gottesman and Pastan (7). The best-characterized mechanism of MDR involves P-glycoprotein (P-gp), which belongs to the ATP-binding cassette (ABC) family of transporter molecules (8). Numerous compounds reverse MDR in experimental systems (9). This has led to the theory of the concomitant administration of chemotherapy and a MDR modulator to reverse clinical drug resistance. Phenothiazines are among the compounds known to modify MDR mediated by P-gp in various kinds of cancer cell lines (10,11). The interactions of MDR modulators such as phenothiazines and isoflavones with phospholipid liposomes and multilamellar lipid structures have also been studied (12). The MDR modulators act mainly as "competitive" or "non-competitive" inhibitors, resulting in an increased concentration of cytotoxic drugs in tumor cells. Modulators such as Verapamil and cyclosporin A serve as substrates for P-gp, supporting the hypothesis that they behave as competitive ligands (13,14). Recently, it was found that a large number of newly synthesized and plant-derived compounds increase the intracellular drug accumulation in MDR cancer cells *in vitro* (15). It is known that flavonoid - and carotenoid -rich foods can be associated with a reduced risk of the development of chronic diseases such as cancer (16). In addition, some of these compounds have been shown to undergo high-affinity binding to MDR P-gp (17,18).

Various mechanisms of action have been identified in the reversal of MDR and apoptosis induction (19,20). We decided to investigate the MDR-reversal effects and apoptosis induction of two large groups of plant-derived compounds, flavonoids and carotenoids, on MDR1/LRP-expressing colon cancer cells.

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Materials and Methods

Chemicals. The carotenoids β-Cryptoxanthin, Luteoxanthin, Anteroxanthin, Violeoxanthin, Apple peel fetoxanthin, Lutein, Violaxanthin and Neoxanthin were isolated by Péter Molnar, while the flavonoids Rotenon, Catechin, Neohesperidin, Naringin, Chrysin, Robinin, Phloretin, Phloridzin, Robinetin, Dihydrobinetin, Kampferol, Dihydrofisetin, Dihydroquercetin, Sakuranin and Sakuranetin were isolated and identified earlier by Sandor Antus. 12H-Benzo[α]phenothiazine (M627) was synthesized by Motohashi *et al.* (21). The stock solutions were prepared in dimethylsulfoxide (DMSO). Verapamil was from EGIS (Hungarian Pharmaceutical Company, Budapest, Hungary), Rhodamine 123 from Sigma (St. Louis, MO, USA) and Annexin-V (human, recombinant) fluorescein isothiocyanate (FITC) from Alexis Biochemicals (ALEXIS DEUTSCHLAND Gmbh, Grünberg, Germany). The human colon adenocarcinoma cell lines, ATCC-CCL-220.1 (Colo 320) and CCL-222(Colo 205), were purchased from LGC Promochem, Teddington, England.

Cell cultures. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cell lines were incubated in a humified atmosphere (5% CO_2, 95% air) at 37˚C. The semi-adherent human colon cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C.

Assay for reversal of MDR in tumor cells. The cells were adjusted to a density of $2x10^6$/mL, resuspended in serum-free RPMI 1640 medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added to the samples at various concentrations in different volumes (2.0-20 μL) of the 1.0 mg/mL stock solutions and the samples were then incubated for 10 min at room temperature. Ten μL (5.2 μM final concentration) Rhodamine 123 indicator was added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for the analysis. The fluorescence of the cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the Rhodamine 123 exclusion experiments (22). The percentage of the control mean fluorescence intensity (fluorescence activity ratio, FAR) was calculated *via* the following equation on the basis of the measured fluorescence values:

$$FAR = \frac{\text{MDR-treated /MDR control}}{\text{parental-treated / parental control}}$$

The shift of the maximum fluorescence peak was measured and compared with the fluorescence maxima of the non-treated cells. When the compounds were tested at various increasing concentrations, the mean fluorescence of the cell population was continuously increased and, in addition, the shift of the maximum fluorescence peak was also increased.

Assay of drug activity on the induction of apoptosis. The cells were adjusted to a density of $2x10^5$/mL and were distributed in 1.0 mL aliquots into microcentrifuge tubes. The apoptosis inducer 12H-Benzo[α]phenothiazine was added to the samples as a positive control at a final concentration of 50 μg/mL. In the case of control cultures, 10 μL DMSO was added. The cells were incubated at 37˚C for 45 min. The samples were then centrifuged and washed with PBS, and the cells were resuspended in 1 mL culture medium. The drugs used for treatment were added to the samples at a final concentration of 10 μg/mL. After incubation for 24 h at 37˚C, the cells were transferred from a 24-well plate into Eppendorf centrifuge tubes, centrifuged and resuspended in 1.0 mL binding buffer. The samples were mixed and centrifuged and supernatant was removed from each tube. Annexin-V-FITC (3 μL/mL samples) was added to the 200 mL samples remaining in the tubes. Controls without Annexin-V were also prepared. The samples and controls were incubated at room temperature for 30 min in the dark. Before the measurement of fluorescence activity, 10 μL propidium iodide (from a 20 μg/mL stock solution) was added to the samples and the apoptosis of the cells was then investigated. In some cases we had no complete apoptosis, however, positivity with only Annexin-V was found, but not with double staining with propidium iodide and Annexin-V simultaneously. This first membrane alteration due to phosphatidylserin translocation was termed as early apoptosis.

Table I. *The effects of various flavonoids on the drug accumulation (Rhodamine 123) in MDR1/LRP-expressing human colon cancer cells (Colo 320).*

Compounds	Final concentration μg/mL	Fluorescence activity ratio	Peak channel
Verapamil	10	21.62	598
DMSO	-	1.09	18
Rotenon	4	13.58	881
	40	40.04	1433
Catechin	4	1.48	41
	40	1.57	59
Neohesperidin	4	1.60	35
	40	1.55	50
Naringin	4	1.91	44
	40	1.18	16
Robinin	4	1.43	36
	40	1.18	18
Phloridzin	4	1.26	24
	40	2.81	27
Robinetin	4	1.10	26
	40	0.83	15
Dihydrobinetin	4	2.01	52
	40	1.48	40
Dihydrofisetin	4	1.32	57
	40	1.21	21
Kampferol	4	0.46	9
	40	0.81	14
Dihydroquercetin	4	0.69	16
	40	0.91	15
Sakuranin	4	0.59	12
	40	0.52	11
Sakuranetin	4	0.60	11
	40	5.2	27

Table II. *The effects of various carotenoids on the drug accumulation (Rhodamine 123) in MDR1/LRP-expressing human colon cancer cells (Colo 320).*

Compounds	Final concentration. μg/mL	FL-1	Fluorescence activity ratio	Peak channel
Verapamil	10	383.31	14.36	319
DMSO	-	37.72	1.41	35
β-Cryptoxanthin	4	39.33	1.47	30
	40	117.95	4.41	66
Luteoxanthin	4	111.45	4.17	79
	40	167.89	6.29	181
Anteroxanthin	4	143.39	5.37	85
	40	352.50	13.20	307
Violeoxanthin	4	359.17	13.45	296
	40	437.58	16.39	385
Apple peel fetoxanthin	4	248.41	9.30	235
	40	498.86	18.69	414
Lutein	4	216.36	8.10	214
	40	418.79	15.69	403
Violaxanthin	4	72.40	2.71	42
	40	379.00	14.20	336
Neoxanthin	4	24.93	0.93	11
	40	84.84	3.17	69

Table III. *The effects of various flavonoids on apoptosis in MDR1, and LRP-expressing human colon cancer cells (Colo 320).*

Samples	Conc. μg/mL	Apoptosis and cell death: Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Annexin-V		4.7	0.09	0.00
12H-Benzo[α]phenothiazine	50	10.07	24.68	0.33
Rotenon	10	11.83	4.82	1.80
Catechin	10	6.65	6.22	1.37
Neohesperidin	10	11.27	5.21	2.16
Naringin	10	6.91	3.21	0.99
Chrysin	10	11.62	4.55	1.41
Robinin	10	8.82	5.64	1.37
Phloretin	10	12.89	6.04	1.17
Phloridzin	10	9.59	6.71	4.03
Robinetin	10	6.99	4.25	1.21
Dihydrobinetin	10	7.97	4.40	3.24
Kampferol	10	10.82	7.03	1.95
Dihydrofisetin	10	10.52	6.99	0.98
Dihydroquercetin	10	8.16	6.20	1.36
Sakuranin	10	9.60	4.83	0.77
Sakuranetin	10	0.71	1.41	5.29

Results

The effects of various flavonoids on the reversal of the MDR of Colo 320 cells were studied. Verapamil was used as a control MDR modifier in these experiments. Among the tested flavonoids, Rotenon was found to be the most effective resistance modulator. Catechin, Neohesperidin, Naringin, Robinin, Phloridzin, Dihydrobinetin and Sakuranetin had only marginal effects, while Robinetin and Dihydrofisetin were ineffective. Kampferol, Dihydroquercetin and Sakuranin were not able to increase the Rhodamine 123 accumulation. The peak channel of Rotenon was shifted from 881 to 1433, that of Catechin from 41 to 59, that of Neohesperidin from 35 to 50 and that of Sakuranetin from 11 to 27, while the peak channel of Verapamil was found at 598. The results indicate that the increase in drug accumulation is dose-dependent and the change in the curve or the maximum fluorescence represents the fluorescence of a great majority of the treated cell population (Table I).

Another group of plant-derived hydrophobic compounds, the carotenoids, were also tested on the reversal of MDR on Colo 320 cells. All of the tested carotenoids increased the Rhodamine 123 accumulation of MDR colon cancer cells by inhibition of the efflux pump. The concentration-dependent effect is shown in Table II. It should be noted that the cell size and the intracellular structures of the carotenoid-treated cells were not modified during the short-term experiments. In all cases, the shift of the maximum fluorescence was increased to various extents by increasing carotenoid concentrations. In the control experiments, the cells were treated with carotenoids and the fluorescence was measured without Rhodamine 123 administration to check the autofluorescence of the treated cells. In this experiment, none of the carotenoids induced fluorescence in the cells (data not shown).

The most effective carotenoids in elevating the Rhodamine 123 accumulation were Anteroxanthin, Violeoxanthin, Apple peel fetoxanthin, Lutein and Violaxanthin. Moderate increases in drug accumulation were found in the presence of Luteoxanthin and β-Cryptoxanthin. Neoxanthin was shown to be a less effective carotenoid on the drug accumulation of Colo 320 cells.

The effects of the flavonoids tested above were also studied in Colo 320 MDR and Colo 205-sensitive cells. In these experiments, moderate early apoptosis characteristics were induced by Rotenon, Neohesperidin, Chrysin, Phloretin, Kampferol, Dihydrofisetin and 12H-Benzo[α]phenothiazine as control. It is interesting that these early apoptosis characteristics were not followed by apoptosis, and none of the compounds induced noteworthy cell death in the MDR Colo 320 cells, except Phloridzin, Sakuranetin and Dihydrobinetin (Table III).

Table IV. *The effects of various carotenoids on apoptosis in MDR1/LRP-expressing human colon cancer cells (Colo 320).*

Samples	Conc. μg/mL	Apoptosis and cell death: Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Annexin-V		4.8	0.15	0.00
12H-Benzo[α]phenothiazine	50	37.08	27.40	1.98
β-Cryptoxanthin	10	4.59	6.71	5.90
Luteoxanthin	10	3.95	4.09	3.58
Anteroxanthin	10	5.39	5.36	2.12
Violeoxanthin	10	6.91	4.88	1.94
Apple peel fetoxanthin	10	8.32	4.80	1.23
Lutein	10	7.09	4.78	2.01
Violaxanthin	10	7.35	4.15	3.53
Neoxanthin	10	6.23	5.23	3.38

Table V. *The effects of various flavonoids on apoptosis in human colon cancer cells (Colo 205).*

Samples	Conc. μg/mL	Apoptosis and cell death: Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Annexin-V		9.9	2.52	0.62
12H-Benzo[α]phenothiazine	50	15.70	80.94	0.42
Rotenon	10	34.24	3.60	1.84
Catechin	10	25.45	6.91	3.35
Neohesperidin	10	11.73	4.09	12.56
Naringin	10	20.08	3.33	2.44
Chrysin	10	13.75	3.52	1.51
Robinin	10	13.63	3.88	2.65
Phloretin	10	31.24	5.37	2.09
Phloridzin	10	22.70	3.70	2.15
Robinetin	10	12.99	4.73	1.95
Dihydrobinetin	10	27.59	3.82	2.50
Kampferol	10	29.59	4.46	2.20
Dihydrofisetin	10	15.34	4.45	5.05
Dihydroquercetin	10	9.92	4.51	6.07
Sakuranin	10	9.42	3.72	5.51
Sakuranetin	10	18.60	4.42	2.95

The effects of some selected carotenoids were also tested on the apoptosis of Colo 320 MDR1/LRP cells. A weak early apoptosis was observed on Colo 320 MDR1/LRP cells at 10 μg/mL Violeoxanthin, Apple peel fetoxanthin, Lutein, Violaxanthin and Neoxanthin, while in the 12H-Benzo[α]phenothiazine-treated sample marked apoptosis and early apoptosis were induced. The early apoptosis was less frequent than after treatment with the flavonoids. The results may point to a particular membrane effect of carotenoids. The carotenoids and flavonoids were not able to induce appreciable cell death at the concentrations applied (Table IV).

For comparison, the effects of the various flavonoids were studied on drug-sensitive Colo 205 cells. A marked early apoptosis was observed in all of the treated cells. However, real apoptosis induction was found only in the 12H-Benzo[α]phenothiazine-treated cells. In the carotenoid-treated cells, the frequency of apoptosis varied between 3 and 6 per cent. It must be noted, that Neohesperidin-induced cell death was more than 10 per cent (Table V). The apoptosis induction of the carotenoids was also checked on drug-sensitive colon cancer cells. The spontaneous early apoptosis was hardly modified in the presence of the different carotenoids, except for Lutein and 12H-Benzo[α]phenothiazine. The apoptosis-inducing effect generally varied between 3 and 7 per cent, whereas 12H-Benzo[α]phenothiazine led to over 70 per cent apoptosis. The cell death of the carotenoid- treated cells was found to be between 4 and 6 per cent, except for Luteoxanthin (Table VI).

In conclusion, we may summarize the most important results found on the reversal of MDR in colon cancer cells. A majority of the tested flavonoids had no effect, while a large number of the carotenoids displayed marked drug accumulation in the MDR colon cancer cells. None of the flavonoids or carotenoids were able to induce significant apoptosis and cell death in the colon cancer cells. In the drug-sensitive colon cancer cells, the early apoptosis was higher than in the MDR cells, but still not significant.

Discussion

After successful surgery and/or radiotherapy, the success of chemotherapy depends ultimately on the rate and extent of drug-resistant cancer cell populations, which either develop with time or by the selection of a few MDR cells from the original, heterologous tumor. Prevention, circumvention or reversal of the MDR phenomenon remains an elusive goal of research (23). The flavonoids constitute an interesting group of polyphenolic compounds with wide distribution in fruits and vegetables. Different biological activities have been described *e.g.* antiproliferative and anticarcinogenic (24).

Various compounds have been investigated in different laboratories for the reversal of MDR, including synthetic and naturally occurring, plant-derived compounds (25, 26). Among the plant-derived compounds, the flavonoids and carotenoids play a prominent role in cancer prevention (27), but a reversal of MDR has been described only by using retinoids (28). *In vivo* research has demonstrated that quercetin can increase the antitumor activity of cisplatin and busulfan, and it was proposed for use in conjunction with doxorubicin and etoposide (29). The use of carotenoids, and particularly β-carotene, in cancer treatment has been

Table VI. *The effects of various carotenoids on apoptosis in human colon cancer cells (Colo 205).*

Samples	Conc. μg/mL	Apoptosis and cell death: Early apoptosis (%)	Apoptosis (%)	Cell death (%)
Annexin-V		4.0	0.95	0.50
12H-Benzo[α]phenothiazine	50	25.91	72.78	0.32
β-Cryptoxanthin	10	11.06	3.58	5.20
Luteoxanthin	10	14.35	5.44	9.83
Anteroxanthin	10	8.18	7.92	4.40
Violeoxanthin	10	14.47	5.52	5.11
Apple peel fetoxanthin	10	11.11	3.88	5.76
luthein	10	18.76	5.49	5.81
Violaxanthin	10	12.11	4.31	6.83
Neoxanthin	10	9.66	3.33	6.26

reviewed (30) and general immunostimulant effects have been found for β-carotene (31).

Since carotenoids and flavonoids have been tested for antiproliferative effects in various tumor cell lines, a systematic study was initiated in our laboratory in the direction of the reversal of MDR and apoptosis induction in colon cancer cells. In our study, the flavonoid Rotenon was found to be the most effective resistance modulator. Catechin, Neohesperidin, Naringin, Robinin, Phloridzin, Dihydrobinetin and Sakuranetin had marginal effects, while Robinetin, Dihydrofisetin, Kampferol, Dihydroquercetin and Sakuranin were ineffective. Our results indicate that the MDR reversal effect needs a particular chemical structure, which exists in Rotenon, but is missing from all the other tested flavonoids.

A majority of the tested carotenoids were able to increase the Rhodamine 123 accumulation in Colo 320 MDR cancer cells by inhibition of the efflux pump. In this group of compounds, we were not able to make a clear distinction on the basis of the structure-activity relationships. Some carotenoids and flavonoids have been reported to influence programmed cell death *in vitro* (32, 33). A majority of the flavonoids and carotenoids had little effect on the apoptosis of the MDR colon cancer cells, but large numbers of compounds from the two main groups induced early apoptosis in the cell cultures. Since the early apoptosis events were not followed by apoptosis, we presume that the Annexin-V-positivity of flavonoid-or carotenoid-treated cells is a consequence of the structural alteration in the cell membrane, which results in the translocation of phosphatidylserine molecules from the inside to the outer surface of the membrane. Despite the relative ineffectiveness of the compounds with regards to apoptosis induction, it was not surprising that the drug-sensitive colon cancer cells were more sensitive to apoptosis induction than their MDR1/LRP-expressing counterparts.

A comparison of the MDR-reversal and apoptosis-inducing activities of the tested carotenoids reveals no correlation between the two biological effects of the carotenoids. The results concerning the reversal of MDR by the various compounds can be exploited. With an increased number of effective resistance modifiers, there is a rational perspective to improve the effectiveness of traditional chemotherapy. On the basis of current diagnostic procedures to determine the MDR phenotype of tumor cells, this is a realistic goal in clinical practice. In the histological routine, the chemosensitivity / chemoresistance of tumor cell samples is determined. In MDR tumors, the clinician may decide on the type of chemotherapy by considering the possible beneficial effects of new types of combined chemotherapy.

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