in vivo *19*: 367-374 (2005)

In Vitro Search for Synergy Between Flavonoids and Epirubicin on Multidrug-resistant Cancer Cells

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Abstract. *The drug accumulation of a human multidrug resistance 1 (mdr1) gene-transfected mouse lymphoma cell line and a multidrug resistance protein (MRP)-expressing human breast cancer cell line MDA-MB-231 was compared in the presence of sixteen flavonoids and five isoflavonoids. The expression of the 170-kDa P-glycoprotein (P-gp) (MDR1) and 190-kDa multidrug resistance protein (MRP) in both cell lines was confirmed by immunocytochemistry. The rhodamine 123 accumulation of the P-glycoprotein (P-gp)-expressing cells increased up to 46.4, while 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) accumulation of the MRP-expressing cells increased up to 1.6, in fluorescence activity ratio (FAR). Major P-gp-mediated efflux pump modifiers are formononetin, amorphigenin, rotenone and chrysin, while MRP-mediated efflux pump modifiers are formononetin, afrormosin, robinin, kaempferol and epigallocatechin. In antiproliferative assay, afrormosin, amorphigenin, chrysin and rotenone exhibited the strongest antiproliferative effects in L5178 (max. ID_{50}: 19.70) and MDA-MB-231 cell lines (max. ID_{50}: 55.47). In a checkerboard microplate method in vitro, furthermore, the most effective multidrug resistance (MDR) resistance modifiers, amorphigenin, formononetin, rotenone and chrysin, were assayed for their antiproliferative effects in combination with epirubicin. Rotenone and afrormosin showed additive effects. Chrysin and amorphigenin on the mouse lymphoma cell line and formononetin on the MDA-MB-231 cell line synergistically enhanced the effect of epirubicin.*

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Key Words: Multidrug resistance, P-glycoprotein, flavonoids, isoflavonoids.

Multidrug resistance (MDR) is one of the main causes of failure in the chemotherapeutic treatment of malignant tumors. Several mechanisms of MDR have been identified (1). Resistance to numerous anticancer agents is associated with expression of the 170-kDa P-glycoprotein (P-gp) (MDR1) and/or the 190-kDa multidrug resistance protein (MRP). Both belong to the ATP-binding cassette (ABC) superfamily of membrane transporters (2). P-gp-negative MDR cells frequently express lung resistance protein (LRP), related to the major vault protein and believed to be involved in nucleo-cytoplasmic transport (3). Breast cancer resistance protein (BCRP) belongs also to the ABC transporter family of efflux proteins (4). Expression of BCRP is induced by various chemotherapeutics, such as doxorubicin or mitoxanthrone, resulting in a drug resistance profile different from that of other membrane transporters (5).

There is sufficient evidence that diets rich in vegetables and fruits have cancer-preventive effects (6). Plant-derived foods are generally good sources of important nutrients including carotenoids, vitamin C, folate, minerals, fiber and many less well-characterized bioactive compounds (phytochemicals). Flavonoids, the most common group of plant phenolics, are mainly found in citrus fruits, apple skin, celery, onions, tea, berries, soy and paprika (7). Some epidemiological studies have suggested that people regularly drinking several cups of tea a day have a lower risk of breast cancer. Tea polyphenols are known to inhibit the large multi-catalytic proteases and metalloproteinases, which are involved in tumor survival and the formation of metastases (8). Polyphenols, furthermore, inhibit the activities of tumor-associated protein kinases and some cancer-related proteins that regulate DNA replication and tissue transformation (9). Additionally, certain studies have indicated that one group of flavonoids, the isoflavones, inhibits the MRP-mediated efflux in human erythrocytes (10).

In this study, MRP and MDR efflux pumps were compared in MDA-MB-231 (HTB-26) human breast cancer cells and *mdr1* gene-transfected mouse lymphoma cells treated with various flavonoids and isoflavonoids. Furthermore,

antiproliferative effects and drug synergism with epirubicin were examined. We report on the modulation of drug accumulation, antiproliferative activities and drug synergism in tumor cells by flavonoids and isoflavonoids.

Materials and Methods

Tested compounds. Rotenone, catechin, neohesperidin, naringin, chrysin, robinin, floretin, floridzin, robinetin, dihydrorobinetin, kaempferol, dihydrofisetin, dihydroquercetin, sakuranin and sakuratenin were provided by Sandor Antus, Department of Organic Chemistry, University of Debrecen, Hungary. Formononetin, amorphigenin, afrormosin, 6a,12a-dehydroamorphigenin and (+)-12-hydroxyamorphigenin were provided by Judit Hohmann, Department of Pharmacognosy, Faculty of Pharmacy, Albert Szent-Györgyi Medical Center, University of Szeged, Hungary. Epigallocatechin was purchased from Sigma (St Louis, MO, USA). All of the tested compounds were dissolved in dimethylsulfoxide (SERVA, Feinbiochemica, Heidelberg, Germany).

Cell cultures. The MDA-MB-231 (ATCC: HTB-26) breast cancer cells were grown in Leibovitz's L-15 medium with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics. The L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described (11). MDR1-expressing cell lines were selected by culturing the infected cells with 60ng/mL colchicine to maintain the expression of the MDR phenotype. L5178 (parent) mouse T-cell lymphoma cells and the human *mdr1*-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. Both cell lines were cultured at 37˚C. The mouse lymphoma cell line was maintained in a 5% CO_2 atmosphere; the MDA-MB-231 cell line does not require CO_2.

Immunocytochemistry. The cells were harvested and resuspended in serum-free medium; the density of the cell suspension was $5x10^5$/mL. One hundred mL was cytocentrifuged for 5 min at 1000 rpm. The samples were fixed in 4˚C acetone for 10 min and washed in TBS buffer (pH 7.6) for 5 min. Endogenous peroxidases were quenched in 0.3% H_2O_2 for 15 min. The samples were incubated with primary antibodies (MRP: MRPm6, monoclonal (ICN) and P-gp: NCL-PGLYm in suitable dilutions (1:5, 1:10, 1:20) for 1 h at room temperature. The samples were washed in TBS buffer three times for 5 min and were incubated with the secondary antibody (DAKO EnVision™ System, CA, USA) for 30 min. The samples were washed in TBS three times for 5 min. Diaminobenzidine (DAKO) was used as the chromogen. The sections were counterstained with hematoxylin and mounted.

Reversal of MRP drug resistance in the MDA-MB-231 (HTB-26) cell line. The cells were distributed onto a 6-well plate, each well containing $2.5x10^5$ cells in 5 mL of culture medium. These cells were grown for 72 h for the experiment. The culture medium was then changed to 1 mL of serum-free medium per well and the tested compounds were added in various amounts (4-40 μL) from a 1.0 mg/mL stock solution. The cells were incubated for 10 min at room temperature. Next, 10 μL of 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) (Sigma) was added to the samples to give a final concentration of 2.6 μM. The cells were incubated for a further 20 min at 37˚C, washed in phosphate-bufferd saline (PBS) and detached with trypsin-versen solution [0.25%(w/v) trypsin – 0.03%(w/v) EDTA] to harvest the cells from the 6-well plate. The cells from each well were transferred to Eppendorf centrifuge tubes and washed once in culture medium and PBS. The samples were resuspended in 0.5 mL of PBS for measurement. The fluorescence of the cell population was analyzed by flow cytometry using a Beckton Dickinson FACScan instrument. Indomethacin (Sigma) at a final concentration of 20 μM was used as a positive control in the experiments. The fluorescence activity was determined as the ratio of the fluorescence activities of treated and untreated samples.

Assay for reversal of MDR in mouse lymphoma cells. The L5178 MDR and L5178Y parent cell lines were grown in McCoy's 5A medium containing 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of $2x10^6$/mL, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volumes (2.0-20.0 μL) of the 1.0-10.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μM final concentration) of the indicator rhodamine 123 (Sigma) 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 PBS for analysis. The fluorescence of the cell population was measured with a Beckton Dickinson FACScan flow cytometer. Verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary) was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. An activity ratio R was calculated by the following equation (12), on the basis of the measured fluorescence values:

$$R = \frac{MDRtreated/MDRcontrol}{parental\ treated/parental\ control}$$

Assay for antiproliferative effect. The effects of increasing concentrations of the drugs alone and their combinations with resistance modifiers on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μL. Then, $1x10^4$ cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37˚C for 72 h; at the end of the incubation period, 20 μL of MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37˚C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) (Sigma) solution (10%) was measured into each well and the plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \frac{ODsample/ODmediumcontrol}{ODcellcontrol/ODmediumcontrol} \times 100$$

A previously described (13), the checkerboard microplate method was applied to study the interactions between multidrug resistance modifiers and epirubicin on the cancer cells.

The effects of the anticancer drug epirubicin and the multidrug resistance modifiers in combination were studied on various cancer cell lines. The dilutions of epirubicin (A) were made in a horizontal direction, and the dilutions of multidrug resistance modifiers (B) vertically in the microtiter plate in a 100 µL volume. The cell suspension in the tissue culture medium was distributed into each well in 100 µL containing $5x10^4$ cells. The plates were incubated for 48h at 37˚C in a CO_2 incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a micro ELISA reader. Drug interactions were evaluated according to the following system:

$FIC_A = ID_{50A \text{ in combination}} / ID_{50A \text{ alone}}$
$FIC_B = ID_{50B \text{ in combination}} / ID_{50B \text{ alone}}$
ID = inhibitory dose
FIC = fractional inhibitory concentration
$FIX = FIC_A + FIC_B$

FIX: 0.51<1	Additive effect
FIX: < 0.5	Synergism
FIX: 1 < 2	Indifferent effect
FIX: > 2	Antagonism

FIX=fractional inhibitory index

Results

The drug accumulation of the human *mdr1* gene-transfected mouse lymphoma and breast cancer cell line carrying MRP were compared in the presence of naturally-occurring plant-derived flavonoids and isoflavonoids.

The presence of MRP on MDA-MB 231 was detected by immunocytochemistry with MRPm6, monoclonal antibody (Figure 1). Antibody MRPm6 reacts with an internal epitope of MRP. It does not cross react with the human MDR1 and MDR3 gene product. A high level of P-gp expression was also detected on the membrane of L5178Y human *mdr1* transfected mouse lymphoma cells by NCL-PGLYm mouse monoclonal antibody that reacts with the human P-glycoprotein G-terminal cytoplasmic domain (Figure 2).

Of the isoflavonoids, amorphigenin and formononetin proved to have the strongest MDR-reversal effects. Afrormosin, (+)-12a-hydroxyamorphigenin and 6a,12a-dehydroamorphigenin were moderately effective on the human *mdr1* gene-transfected mouse lymphoma cells (Table I).

When the same compounds were tested on MRP, the carboxyfluorescein accumulation of the MDA-MB-231 breast cancer cells was measured. BCECF-AM, a fluorescent dye used as an intracellular pH indicator, is an especially good MRP substrate (14). The MRP expression and its function were assessed by flow cytometry (15), although the sensitivity of this method was lower than the rhodamine 123 exclusion test in the mouse lymphoma cells. Formononetin increased the drug accumulation in both cell lines as compared with the control. Afrormosin was only moderately effective on the MRP reversal of the tumor cells. 6a,12a-Dehydroamorphigenin and (+)-12a-hydroxyamorphigenin had only marginal effects as compared with indomethacin-treated MRP cells (Table II).

When the flavonoids were tested on the drug accumulation of the breast cancer cells, three groups of compounds could be distinguished. In the first group, chrysin, robinin, kaempferol, dihydroquercetin and epigallocatechin were as effective as the indomethacin control, elevating the BCECF-AM accumulation by 20-60%. In the second group, neohesperidin, naringin, floretin, floridzin, robinetin, dihydrorobinetin and dihydrofisetin were practically ineffective. In the third group, rotenone, catechin and sakuranin reduced the drug accumulation in the cells (Table III).

When the same flavonoids were tested on the mouse lymphoma cells, rotenone, chrysin, floretin and epigallocatechin displayed a dose-dependent increase in the fluorescence activity ratio (FAR). These compounds caused a much higher drug accumulation than that of the verapamil control. Catechin, neohesperidin, naringin, robinin and sakuratin had moderate effects. Dihydroquercetin, dihydrofisetin, dihydrorobinetin and floridzin reduced the rhodamine 123 accumulation in the cells. Kaempferol and robinetin had no effect on the rhodamine 123 accumulation of the human *mdr1* gene-transfected mouse lymphoma cell line (Table IV).

As Table V shows, low concentrations of the isoflavonoids amorphigenin and rotenone inhibited the cell proliferation or were most toxic on both cell lines. The breast cancer cells were apparently more sensitive than the MDR lymphoma cells to the selected flavonoids.

In further experiments, the enhanced antiproliferative activity of combinations of the tested compounds with epirubicin was examined. Of the resistance modifiers (formononetin, amorphigenin, rotenone, chrysin and epigallocatechin), chrysin and amorphigenin were able to enhance the antiproliferative activity of epirubicin on mouse lymphoma cells. Although rotenone significantly increased the rhodamine 123 drug accumulation, it had only a marginal additive antiproliferative effect in combination with epirubicin. On the MDA-MB-231 breast cancer cell line, formononetin and kaempferol exhibited synergism. Some compounds, *e.g.* afrormosin and robinin, also had weak effects in combination with epirubicin. Interestingly, they were ineffective on the human *mdr1* transfected mouse lymphoma cell line. Amorphigenin treatment likewise resulted in different effects on the two cell lines (Table VI).

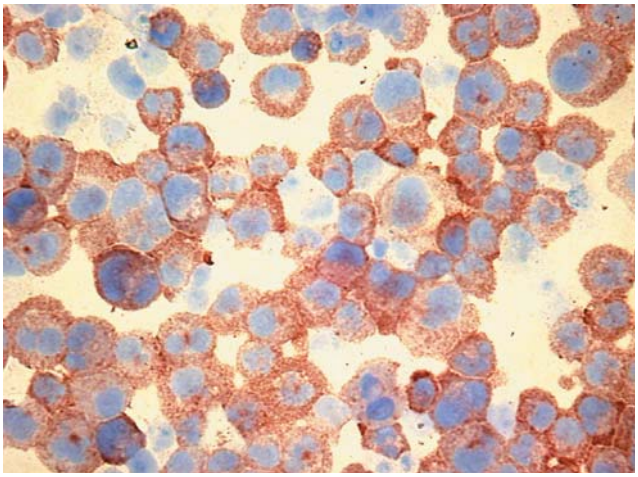

Figure 1. *MRP staining of MDA-MB-231 breast cancer cell line.*

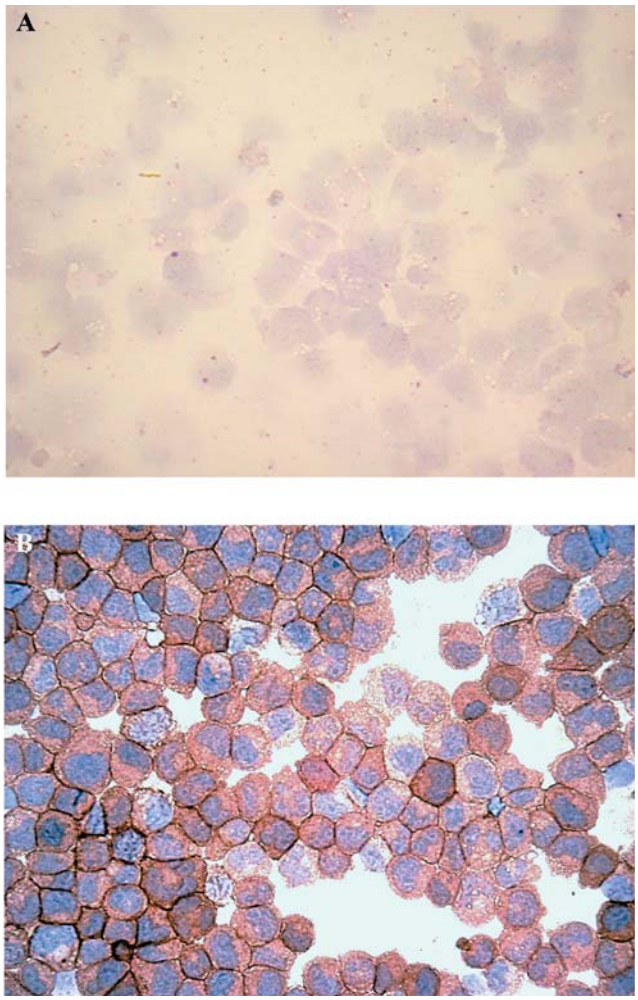

Figure 2. *MDR1 staining of parental L5178Y mouse lymphoma cells (A) and its human mdr1 transfected subline (B)*

Table I. *Effects of isoflavonoids on rhodamine 123 accumulation of human mdr1 gene-transfected mouse lymphoma cell line.*

Compounds	μg/mL	Fluorescence activity ratio
Verapamil	5	17.1
Formononetin	4	0.8
	40	18.3
Amorphigenin	4	12.4
	40	46.4
Afrormosin	4	0.5
	40	3.1
6a,12a-Dehydroamorphigenin	4	1.1
	40	3.0
(+)-12-Hydroxyamorphigenin	4	0.4
	40	2.8
DMSO control		0.9

Table II. *Effects of isoflavonoids on BCECF-AM accumulation of MDA-MB-231 (HTB-26) cell line.*

Compounds	μg/mL	Fluorescence activity ratio
Indomethacin	10	2.4
Formononetin	4	1.0
	40	1.5
Amorphigenin	4	1.0
	40	1.0
Afrormosin	4	1.3
	40	1.4
6a,12a-Dehydroamorphigenin	4	1.0
	40	1.2
(+)-12-Hydroxyamorphigenin	4	1.1
	40	1.1
DMSO control		1.0

Table III. *Effects of flavonoids on BCECF-AM accumulation of MDA-MB-231 (HTB-26) cell line.*

Compounds	μg/mL	Fluorescence activity ratio
Indomethacin	10	1.3
Rotenone	4	0.3
	40	0.3
Catechin	4	0.6
	40	0.6
Neohesperidin	4	0.8
	40	0.9
Naringin	4	1.0
	40	0.8
Chrysin	4	1.0
	40	1.2
Robinin	4	1.4
	40	1.6
Floretin	4	0.7
	40	1.0
Floridzin	4	0.8
	40	0.9
Robinetin	4	0.8
	40	0.9
Dihydrorobinetin	4	0.8
	40	1.1
Kaempferol	4	0.9
	40	1.3
Dihydrofisetin	4	1.0
	40	1.0
Dihydroquercetin	4	0.9
	40	1.3
Sakuranin	4	0.8
	40	0.7
Sakuranetin	4	0.8
	40	0.8
Epigallocatechin	4	1.1
	40	1.3
DMSO control		0.9

Table IV. *Effect of flavonoids on rhodamine 123 accumulation of human mdr1 gene-transfected mouse lymphoma cell line.*

Compounds	μg/mL	Fluorescence activity ratio
Verapamil	5	8.9
Rotenone	4	27.7
	40	28.6
Catechin	4	2.7
	40	2.9
Neohesperidin	4	2.3
	40	2.8
Naringin	4	2.6
	40	2.3
Chrysin	4	3.7
	40	14.6
Robinin	4	2.0
	40	1.5
Floretin	4	0.8
	40	4.9
Floridzin	4	0.7
	40	0.6
Robinetin	4	0.7
	40	0.7
Dihydrorobinetin	4	0.8
	40	0.7
Kaempferol	4	0.8
	40	0.8
Dihydrofisetin	4	0.7
	40	0.5
Dihydroquercetin	4	0.7
	40	0.6
Sakuranin	4	0.7
	40	0.8
Sakuranetin	4	0.8
	40	2.4
Epigallocatechin	4	0.6
	40	36.1
DMSO control		0.8

Table V. *Antiproliferative effects of selected flavonoids and isoflavonoids on tumor cells.*

Compounds	MDR cell line ID_{50} (μg/mL)	MDA-MB-231 ID_{50} (μg/mL)
Epigallocatechin	1.8	2.6
Rotenone	0.01	0.0005
Kaempferol	55.47	20.59
Robinin	38.51	18.6
Dihydroquercetin	59.32	10.19
Chrysin	15.51	5.61
Formononetin	19.7	17.54
Amorphigenin	1.04	0.01
Afrormosin	48.78	20.5
6a,12a,-Dehydroamorphigenin	9.8	13.51
12a-Hydroxyamorphigenin	2.77	14.04
DMSO	12.5	32.76

Table VI. *Antiproliferative effects of selected flavonoids in combination with epirubicin on tumor cells.*

Cell line	Samples	ID_{50} (μg/mL)	FIX	Interaction
L5718	Chrysin	15.51		
	Epirubicin control	1.4		
	Chrysin + Epi*	0.606	0.471	synergism
	Rotenone	0.01		
	Epirubicin control	1.4		
	Rotenone + Epi	0.006	0.604	additive effect
	Amorphigenin	1.04		
	Epirubicin control	3.46		
	Amorphigenin + Epi	0.18	0.120	synergism
	Formononetin	19.7		
	Epirubicin control	3.46		
	Formononetin + Epi	3.64	1.230	no interaction
MDA-MB-231	Amorphigenin	0.01		
	Epirubicin control	0.11		
	Amorphigenin + Epi	0.01	1.090	no interaction
	Formononetin	17.54		
	Epirubicin control	0.11		
	Formononetin + Epi	0.02	0.181	synergism
	Afrormosin	20.5		
	Epirubicin control	0.11		
	Afrormozin + Epi	0.06	0.548	additive effect
	Robinin	18.6		
	Epirubicin control	0.04		
	Robinin + Epi	0.02	0.501	additive effect
	Epigallocatechin	26.01		
	Epirubicin control	0.05		
	Epigallo. + Epi	0.05	1.001	no interaction
	Kaempferol	55.47		
	Epirubicin control	0.05		
	Kaempferol + Epi	0.02	0.440	synergism

Epi* = Epirubicin

Discussion

A number of plant extracts and chemically characterized plant compounds have previously been tested for MDR reversal in our laboratory (16, 17). In the present study, we examined the effects of flavonoids and isoflavonoids on the P-gp- and MRP-mediated MDR mechanisms in mouse lymphoma and human breast cancer cell lines. Their interactions with the drug transporters P-gp and MRP, which are frequently expressed in human tumors, may be of relevance to their use as anticancer agents, alone or in combination with cytotoxic agents (18, 19). Differences in the activities of the P-gp and the MRP-mediated efflux pump were found in the presence of flavonoids and isoflavonoids. A short-term intracellular drug accumulation and a long-term viability assay were used to gain insight into various aspects of the effects of flavonoid compounds on tumor cells.

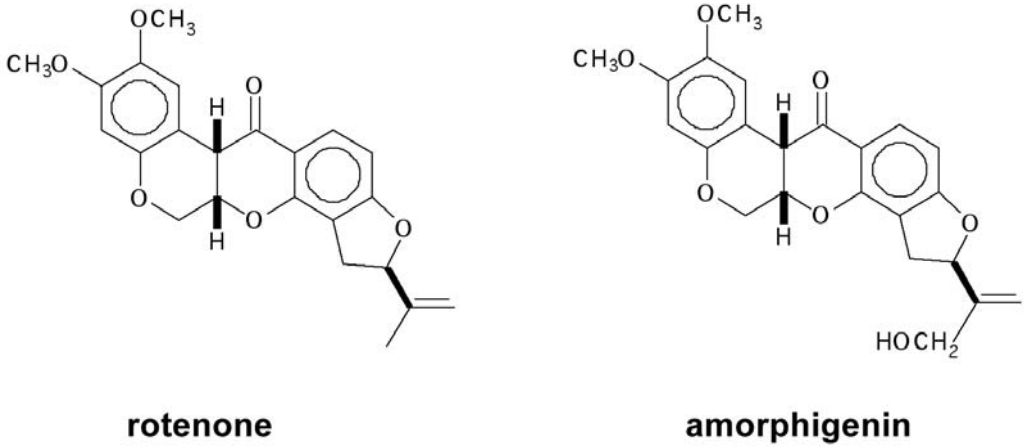

Figure 3. *Chemical structures of rotenoid derivatives, rotenone and amorphigenin.*

In the drug accumulation studies, the P-gp inhibition was more significant than the MRP inhibition. Furthermore, some compounds in combination with epirubicin were more effective than expected on the MRP-expressing human breast cancer cell line. Thus, the MTT assay allows an assessment of other cellular effects of the tested compounds, or these differences can also be explained by structural and functional mechanisms which might not be related to P-gp and MRP.

One of the differences between the two efflux systems is that P-gp has been shown to bind directly the transported drug to which P-gp confers resistance, whereas MRP pumps out some compounds through a co-transport mechanism with reduced glutathione (10, 20, 21). Other studies have demonstrated that some dietary flavonoids may modulate organic anion and GSH transport, ATPase and drug resistance (21). The soybean isoflavone genistein was found to act as an inhibitor of drug accumulation in MRP-overexpressing cells (19, 22), and also to interact with P-gp and inhibit the P-gp-mediated drug transport (23). However, contradictory results have been reported concerning the MDR-modulating activity of the flavonoid polyphenols kaempferol and quercetin, which stimulated the P-gp-mediated efflux of doxorubicin on the adriamycin-resistant subline of the MCF-7 breast cancer cell line (24).

In our experiments, kaempferol was ineffective in P-gp inhibition; however, chrysin had a significant inhibitory effect. Rotenoid derivatives, amorphigenin and rotenone, had MDR modulating activity, with a structural difference of hydroxyl(-OH) group at the position of 29th carbon (Figure 3). The concentrations of the test compounds had great effects on the outcome of the experiments. A low concentration of quercetin, for example, activated the activity of P-gp, whereas a high concentration inhibited that of P-gp. A similar biphasic effect has been reported for kaempferol (25).

It is concluded that the sensitivities of the MDR and MRP-mediated drug resistance mechanisms to the inhibitory flavonoids and isoflavonoids differ, based on the chemical structures, in good agreement with other studies (18, 23).

Acknowledgements

This study was supported by the Szeged Foundation of Cancer Research (Szegedi Rakkutatatasért Alapitvany) and the COST B16 Action of the EU Commission, and the Hungarian National Scientific Research Fund (OTKA T034250).

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Received November 1, 2004
Accepted January 7, 2004