Reversal of Multidrug Resistance by Natural Substances from Plants

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Abstract: The multidrug resistance (MDR) proteins that belong to the ATP-binding cassette superfamily such as P-glycoprotein (P-gp) and MRP1, are present in a majority of human tumors and constitute an important cause of therapeutic failure. Selective inhibitors of the MDR-efflux proteins may improve the effectiveness of cancer chemotherapy. Their mechanism of action was believed to be a competition between resistance modifiers and drugs for the same binding site of P-gp. In our previous work we studied modulation of MDR in cancer cells expressing P-gp or MRP1 by selected carotenoids, flavonoids and extracts from medically important Chinese plants. Capsanthin and capsorubin, carotenoids isolated from paprika, were identified as potent P-gp inhibitors, while lycopene, lutein, antheraxanthin and violaxanthin induced moderate effects. Among flavonoids, effective modulators were rotenone, chrysin, phloretin and sakuranetin. Some chloroform extracts of Chinese herbs were also found to inhibit MDR efflux pumps. The effects of the modulators on P-gp activity were studied by measuring rhodamine 123 uptake in several cancer cells such as the human *MDR1* gene-transfected mouse lymphoma cells (L1210) and human breast cancer cells MDA-MB-231 expressing the MRP1 pump (HTB26). Additionally, the ability to alter biophysical properties of lipid bilayers by selected carotenoids was studied by differential scanning calorimetry. The antiproliferative effects as well as the MDR reversal activity of the studied compounds, applied in combination with anticancer drugs, were also discussed.

Keywords: Carotenoids, Flavonoids, Human breast- and colon cancer, Mouse lymphoma cells transfected with human *MDR1* gene, Multidrug resistance (MDR), Resistance modifiers, Synergistic interactions, Traditional Chinese medicinal plants.

INTRODUCTION

The discovery of chemotherapeutic agents from plant extracts led to the identification of new chemotherapeutic agents or to the isolation of compounds that enhance the anti-tumour activity of chemotherapeutic drugs by inhibiting multidrug resistance (MDR) of cancer cells. Extracts from paprika fruits contain many compounds such as carotenoids, flavonoids, isoflavones and xanthenes, active against ABC transporters. The inhibition of ABC transporters, responsible for MDR of cancer cells, is an important effect of the plant-isolated compounds because efflux inhibitors applied in combination with anticancer drugs may reverse the resistance of various types of tumor cells [1].

MDR cancer cells are resistant to many anticancer drugs with different chemical structures and mechanisms of action. In cancer patients who do not respond to chemotherapy, MDR is usually mediated by the over-expression of various ATP-dependent efflux pumps displaying a broad substrate specificity, such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) [2]. Expression of ABC transmembrane pumps leads to lowering of the intracellular concentration of drugs, enabling tumor cells to survive despite chemotherapeutic treatment. MDR inhibitors may block or slow down the pumps therefore the chemotherapeutic drugs could remain longer inside the cells. Many other molecular mechanisms of drug resistance also exist, for example the increased repair of chemotherapy-induced DNA damage [3].

Considering the growing need to identify MDR revertant agents with high potency and low toxicity, we studied the ability of natural products to counter drug resistance. In our systematic studies, extracts of green, yellow, red and black paprika fruits, one of the most popular vegetables, were studied and the most effective inhibitors of ABC transporters were subsequently identified.

Six different fractions extracted from green paprika with hexane, acetone and methanol were identified as containing MDR inhibitors [4]. A similar extraction procedure of red paprika fruits yielded altogether twelve fractions that exerted resistance reversal effect [5]. In case of black paprika, thirteen effective fractions were found [6]. Based on these studies, we supposed that some kinds of pigments present in green [4], black [6,7] and red paprika [5,8] could be responsible for the MDR reversal effects.

The inhibition of MDR transporters by modulators can be exerted in three different ways: *i*) by competitive binding at the same site as the substrates (competitive inhibition); *ii*) by

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allosteric modification of the active conformation of P-gp through a site far from the substrate binding site (non-competitive inhibition), *iii*) by modulation of the membrane lipid bilayer in which P-gp is embedded. Resistance modifiers may also bind to the extra-membrane domains of P-gp: *iv*) cytoplasmic, ATP-binding sites are sensitive to certain flavonoids, and *v*) the polylactosamine moiety, located on the first loop of extra-cellular domain may bind tomato lectin [9].

However, a therapeutic effect of carotenoids was only observed in the precancerous state and in promyelocytic leukemia [10]. The reduced risk of developing ageing-related diseases was ascribed to a beneficial effect of carotenoids, such as β-carotene [11]. Indeed, the higher intake of carotenoid-containing food is associated with reduced risk of developing several chronic diseases, such as coronary heart disease, cancer and cataract [12]. In animal experiments, it was shown that a carrot-rich diet can reduce tumor incidence in rats and mice treated with carcinogenic chemicals [13]. Both β- and α-carotene showed significant antitumor activity in the two stage carcinogenesis model [14].

Recently, carotene-containing paprika extracts were fractionated by Motohashi and coworkers. Some of the fractions could apparently reverse MDR of tumor cells [4]. Considering previous results and the contradictory findings obtained with saffron compounds on resistant cancer cells [1], it seemed promising to study the MDR reversal effects of selected carotenoids isolated from paprika and other vegetables.

CAROTENOIDS

Literature data suggest the potential importance of carotenoids as resistance modifiers in cancer chemotherapy [1]. Previously, our group isolated and identified several carotenoids [1,4,15]. Taking into account the blocking effect of hexane and acetone fractions of paprika extracts on the ABC transporters, the effects of carotenoids on the drug accumulation in cancer cells were worth studying [1].

A study on rhodamine 123 uptake in *MDR1* gene-transfected mouse lymphoma (L1210) cells allowed us to classify the studied carotenoids into three different groups based on their MDR reversal activity: inactive, moderately active, or very active (Table **1**). The most effective carotenoids were capsanthin, capsorubin and lycophyll. Violaxanthin, lycopene, lutein, zeaxanthin, antheraxanthin, α- and β-cryptoxanthines were moderately active, while α- and β-carotene had no effect. Thus, effective P-gp modulators were identified among carotenoids isolated from different kinds of paprika. The differential sensitivity of cancer cells to the various carotenoids may be explained by the differences in their diffusion properties or distribution in membrane.

Rhodamine 123 accumulation in the presence of carotenoids was also studied in human breast cancer HTB26 cells that do not contain P-gp but only MRP1 [1]. The rhodamine 123 accumulation in these cells was not modified after carotenoid treatment. The results indicated that some carotenoids, that exerted MDR reversing effect in P-gp-expressing mouse lymphoma cells, were not able to modify the drug accumulation in human breast cancer cells (data not shown). Interestingly, MDR reversing effects of carotenoids, tested in two different cell lines, were apparently related to some properties of their chemical structures.

The majority of carotenoids hydroxylated at the right cyclohexene ring (β- and ε-end group) revealed moderate MDR reversing effect. Antheraxanthin, lutein and zeaxanthin are some examples (Fig. (**1**)).

The hydroxylation at the right cyclopentane ring (κ-end group), in addition to the hydroxylation at the left ring (β-end group in capsanthin; κ-end group in capsorubin) could be responsible for the very high resistance reversal effect of capsanthin and capsorubin (Fig. (**2**)).

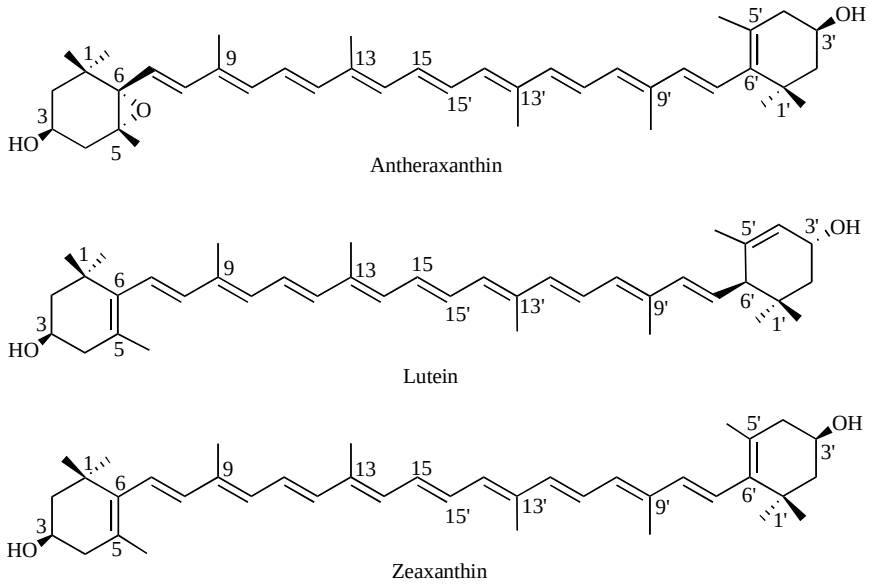
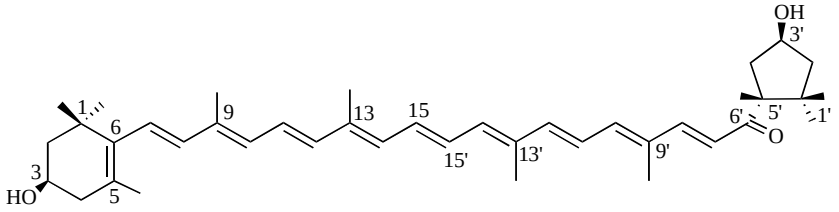
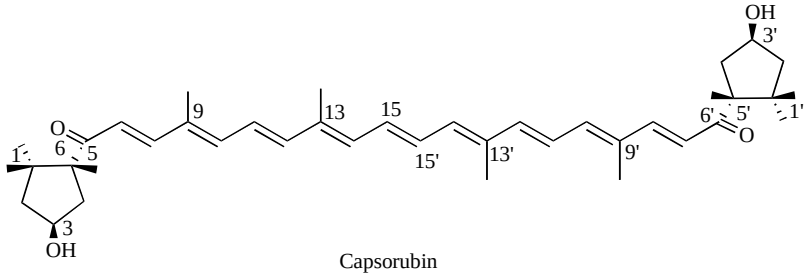


Fig. (1). Chemical structures of carotenoids which are "moderately active" MDR reversal agents.



Capsanthin



Capsorubin

Fig. (2). Chemical structures of carotenoids which are "very active" MDR reversal agents.

Table 1. MDR Reversal Effects of Carotenoids in Human *MDR1* Gene Transfected Mouse Lymphoma Cells (L1210)

Compound	Conc [μg/mL]	FAR[a]	References
Verapamil[b]	5	5.73	[1]
		inactive	
β-Carotene	20	0.56	[1]
α-Carotene	20	0.81	[1]
	moderately active		
Lycopene	20	9.70	[1]
β-Cryptoxanthin	20	9.65	[1]
α-Cryptoxanthin	20	4.42	[1]
Zeaxanthin	20	9.67	[1]
Lutein	20	10.06	[1]
Antheraxanthin	20	10.17	[1]
Violaxanthin	20	5.97	[1]
	very active		
Capsanthin	20	27.59	[1]
Capsorubin	20	24.46	[1]
Lycophyll	20	25.2	[1]

[a] FAR (Fluorescence Activity Ratio): mean fluorescence ratio for treated/untreated samples. [b] Reference compound.

Further studies on rare types of carotenoids revealed other effective MDR modulators [15]. Mutatochrome and diepoxy-β-carotene were moderately active on MDR reversal activity in the L5178 cell line (Table **2**). Flavoxanthin plus chrysanthemaxanthin, (5*R*,8*R*)-capsochrome, (5*R*,8*S*)-capsochrome, (5*S*,8*S*)-capsochrome, (5*S*,8*R*)-capsochrome, monoepoxy-β-carotene, (8'*R*)-luteoxanthin, (13*Z*)-lutein+(13'*Z*)-lutein, (9*Z*)-zeaxanthin, and (9*Z*)-violaxanthin, displaying extremely high fluorescence activity ratio, showed high P-gp inhibition activity. 15,15'-Dehydro-diepoxy-β-carotene and mono-epoxy-α-carotene were ineffective.

A much lower inhibitory effect of the same carotenoids was found in MCF7/MDR1 cells [15]. All compounds were able to moderately increase rhodamine 123 accumulation, with exception of 15,15'-dehydro-diepoxy-β-carotene that was inactive (Table **2**). The different sensitivities of L5178

Table 2. MDR Reversal Effects of Rare Types of Carotenoids in Human *MDR1* Gene Transfected Mouse Lymphoma Cells (L5178) and Human Breast Cancer Doxorubicin resistant Cells (MCF7/KCR) Expressing *MDR1* Gene.

Compound		FAR[a]		FAR[a]	References
	Conc [μg/mL]	L5178/MDR1 cell line	Conc [μg/mL]	MCF7/MDR1 cell line	
Verapamil[b]	10	15.70	5	11.9	[15]
	inactive				
15,15`-Dehydro-diepoxy-β-carotene	40	0.58	40	0.62	[15]
Monoepoxy-α-carotene	40	0.56	40	-	[15]
	moderately active				
Diepoxy-β-carotene	40	5.72	40	2.08	[15]
Mutatochrome	40	7.47	40	-	[15]
	very active		*moderately active*		
Flavoxanthin plus Chrysanthemaxanthin	40	30.27	40	-	[15]
(5*R*,8*R*)-Capsochrome	40	37.92	40	1.95	[15]
(5*R*,8*S*)-Capsochrome	40	56.81	40	1.67	[15]
(5*S*,8*S*)-Capsochrome	40	41.52	40	1.99	[15]
(5*S*,8*R*)-Capsochrome	40	53.54	40	1.38	[15]
Monoepoxy-β-carotene	40	37.59	40	2.37	[15]
(8'*R*)-Luteoxanthin	40	61.81	40	1.9	[15]
(13*Z*)+(13'*Z*)-Lutein	40	31.42	40	2.0	[15]
(9*Z*)-Zeaxanthin	40	20.54	40	-	[15]
(9*Z*)-Violaxanthin	40	57.74	40	2.2	[15]

[a] FAR (Fluorescence Activity Ratio): mean fluorescence ratio for treated/untreated samples. [b] Reference compound.

and MCF7/MDR1 cells to carotenoids suggested that P-gp was likely differentially expressed in these cell lines, in spite of the fact that the drug accumulation in the presence of verapamil was similar in both cell lines. We also supposed that the cellular membrane properties differed in the two cell lines explaining their different sensitivity to hydrophobic carotenoids.

In fact, carotenoids are extremely hydrophobic, and sensitive to oxidation or photooxidation, so that their investigation is laborous and time-consuming [16-18]. Therefore, complexes of carotenoids with cyclomalto-oligosaccharides as water soluble micelles have been recommended as a convenient and inexpensive solubilizing technique [18,19].

Since there are many studies suggesting that interactions of MDR modulators with the lipid bilayer of biological membranes might play an important role in MDR reversal [20], the ability of selected carotenoids to alter biophysical properties of membranes was investigated by differential scanning calorimetry (DSC) on the dimyristoylphosphatidylcholine (DMPC) model (Table **3**). DSC is a precise method to study the influence of chemical compounds on thermotropic properties of phospholipid bilayers.

All the studied compounds abolished the pretransition parameters of DMPC and reduced the temperature of main phospholipid phase transition when present in the model membrane at a drug:lipid ratio 0.04. The melting temperature reduction ranged from 0.5 °C for capsorubin and β-carotene to 1.5 °C for the most active (9*Z*)-violaxanthin. Additionally, a carotenoid-induced broadening of calorimetric peaks was observed, being the smallest for β-carotene and capsorubin and the biggest for (9*Z*)-violaxanthin and antheraxanthin. Such an effect indicated a reduction of transition cooperativity. Some compounds also diminished the transition enthalpy. The most pronounced effect was observed for (9*Z*)-violaxanthin and capsanthin, a smaller effect for antheraxanthin and violaxanthin, while capsorubin and β-carotene did not affect this parameter (Table **3**).

All studied carotenoids were observed to reduce the transition temperature and to decrease transition cooperativity, while their influence on transition enthalpy was weaker. The obtained results are in accordance with previously published data for other carotenoids [21]. The changes of biophysical properties of model membranes exerted by carotenoids were, according to the empirical classification introduced by Jain and Wu [22], typical for modifiers that interacted mainly

Table 3. Influence of Selected Carotenoids on Transition Parameters of Dimyristoylphosphatidylcholine (DMPC). Drug:Lipid Molar Ratio was 0.04. Mean ± S.D. of Eight Experiments is Shown

Compound	Transition Temperature [°C]	Transition Enthalpy [kJ/mol]	Peak Half-Height Width ($T_{1/2}$) [°C]
DMPC	23.10 ± 0.09	26.0	0.44 ± 0.02
DMPC +			
Capsanthin	21.85 ± 0.09	13.59 ± 2.38	0.82 ± 0.05
Capsorubin	22.48 ± 0.09	23.52 ± 0.75	0.74 ± 0.07
Antheraxanthin	21.79 ± 0.07	16.26 ± 1.61	1.38 ± 0.11
Violaxanthin	21.88 ± 0.04	16.44 ± 1.85	0.91 ± 0.05
(9Z)-Violaxanthin	21.62 ± 0.16	13.21 ± 1.19	1.10 ± 0.17
β-Carotene	22.49 ± 0.03	25.03 ± 2.19	0.67 ± 0.04

with the hydrophobic/hydrophilic interface of the membrane and that penetrated only partially the hydrocarbon region. The following modes for carotenoid-membrane interactions were postulated: *i*) carotenoid molecule could span lipid bi-layer with polar groups located at two opposite sides of the molecule anchored in two opposite polar zones of the bi-layer; *ii*) carotenoid molecule (especially *cis*-isomer) could adopt a tilted conformation and their two polar groups would interact with polar region of the same leaflet of a membrane [21].

The moderately effective MDR modulators (9*Z*)-violaxanthin and antheraxanthin were the most active compounds in changing main DMPC phase transition parameters. Among the least membrane-perturbing compounds, β-carotene and capsorubin were identified (Table **3**). β-Carotene was also found to be non-active in P-gp modulation; in contrast capsorubin, was identified among the most active MDR modulators. The above findings suggested that the interaction of carotenoids with P-gp, was not influenced by the binding of these compounds with lipid membrane.

Additionally, the effect of combined treatment (selected carotenoids and doxorubicin) on resistant cancer cells was tested in order to check whether their MDR would be reduced [15]. The results were encouraging as concomitant use of the anticancer drug and carotenoids resulted in synergism of the antiproliferative action in both human *MDR1* gene-transfected mouse lymphoma L5178 and MCF7/MDR1 human breast cancer cell lines.

Multiple Pharmacological Targets of Carotenoids

The wide variety of carotenoids can influence multiple targets. Antiproliferative effects of lycopene, a carotenoid from tomato, on various prostate cancer cells have been studied and potent antitumor properties were demonstrated [18]. Lycopene was then studied *in vivo*, in healthy subjects and in patients with cervical, breast, ovarian and colorectal cancer. The results indicated that high serum concentrations of carotenoid were associated with a decreased risk of recurrence [23]. The growth inhibition of MCF7 (mammary cancer) cells was accompanied by a slowing down of cell cycle progression through the G1-S phases *via* reduction of cycline-D1 [19]. It was found that lycopene inhibited the growth of MCF7 cells and endometrial cancer by interfering with insulin-like growth factor (IGF) signal transduction, due to a decrease in IGF-induced tyrosine phosphorylation of the insulin receptor [24,25]. These growth factors act on the oncogenes [26], affect the activity of apoptosis pathways [27] and protect against membrane damage. In addition to the discussed mechanism of actions, carotenoids exert immunomodulatory effects through complex mechanisms, such as immune-stimulation, which may involve antioxidant and singlet oxygene quenching capacities of the carotenoids having nine or more conjugated double bonds [28].

The dietary absorption and bioavailability of lutein and zeaxanthin were studied and epimeric isomers were found in the human serum [29]. It was suggested that a high intake of carotenoid-containing fruits and vegetables was associated with a reduced risk of lung cancer. Studies showed that α-carotene rather than β-carotene might be responsible for this effect [30]. It was shown that numerous carotenoids exert an inhibitory action on free radical generation in leukocytes [31]. Some of the carotenoids, e.g. capsanthin, isolated from the fruits of red paprika *Capsicum annum L*. remarkably inhibited early tumour antigen expression of Epstein-Barr virus (EBV) after Tissue Polypeptide Antigen (TPA) induction [32]. This antitumour effect of paprika carotenoids was observed both *in vitro* and *in vivo*. Data demonstrated that the increased consumption of carotenoids reduces the risk of cancers especially in the respiratory and digestive tracts [33]. A carotenoid-rich diet can suppress prostate cancer development by inducing apoptotic cell death, a process in which the proteins Bcl-2 and Bax, members of the Bcl-2 family of apoptosis-associated proteins are involved [34]. Carotenoids are also capable of reducing cell cycle progression from G1 to S phase induced by growth factors [35].

FLAVONOIDS

Flavonoids have been studied as MDR reverting agents in several tumor cell lines overexpressing MDR proteins.

The accumulation of rhodamine 123 in mouse lymphoma cells transfected with the human *MDR1* gene and in human breast cancer cells expressing MRP1 (HTB26) was com-

pared in the presence and absence of naturally occurring plant-derived isoflavonoids (Fig. (**3**)), taking into account the results of our former studies [36]. Amorphigenin and formononetin proved to be the most effective MDR reversal agents in transfected mouse lymphoma cell line. Afrormosin, (+)-12a-hydroxyamorphigenin and 6a,12a-dehydroamorphigenin were moderately effective in P-gp expressing mouse lymphoma cells (Table **4**).

The same flavonoids were tested in HTB26 cells by measuring the fluorescent accumulation of 2',7'-bis'(carboxyethyl)-5-(6')-carboxyfluorescein (BCECF), a specific MRP1 substrate. MRP1 expression and transport activity were analysed by flow cytometry [15], although the sensitivity of this method is lower than rhodamine 123 exclusion test. All tested compounds moderately increased BCECF accumulation in HTB26 cells (Table **4**).

The ability of another set of flavonoids to inhibit MDR-related efflux pumps has also been tested [36] in different cell lines. This set included dihydroflavonols (dihydrofisetin, dihydrorobinetin, dihydroquercetin and sakuranin), flavonols (kaempferol, robinetin and robinin), flavanons (naringin, neohesperidin and sakuranetin), dihydrochalcones (phloretin and phloridzin), one flavon (chrysin), one rotenoid (rotenone) and flavan-3-ols (catechin, epigallocatechin) (Fig. (**4**)).

In P-gp expressing mouse lymphoma cells, rotenone, chrysin, and epigallocatechin increased rhodamine 123 accumulation and displayed a dose-dependent increase in the fluorescence activity ratio (Table **5**). These flavonoids were better enhancers of fluorescent probe accumulation than the reference compound verapamil. Catechin, neohesperidin, naringin, robinin, phloretin and sakuranetin displayed moderate effects, while dihydroquercetin, dihydrofisetin, dihydrorobinetin, phloridzin, kaempferol, sakuranin, robinetin and dihydrorobinetin had no effect.

The effects of flavonoids on the MRP1 pump were tested by measuring BCECF accumulation in HTB26 cells [36]. Chrysin, robinin, kaempferol, dihydroquercetin, dihydrorobinetin, dihydrofisetin and epigallocatechin displayed the same effect as indomethacin, viz. increasing BCECF accumulation. On the other hand, neohesperidin, naringin, phloretin, phloridzin, robinetin, dihydrofisetin, rotenone, catechin, sakuranin and sakuranetin showed a poor activity.

The effect of different flavonoids on rhodamine 123 accumulation in P-gp-expressing human colon cancer cells (Colo320) was also studied [36]. Rotenone was the most effective inhibitor of the P-gp efflux pump. Sakuranetin, dihydrorobinetin, phloridzin, naringin, neohesperidin, catechin, robinin, dihydrophysetin and epigallocatechin had only moderate effects, while robinetin, kaempferol, dihydroquercetin and sakuranin displayed a poor effect (Table **5**).

Finally, an enhanced antiproliferative activity of flavonoids in combination with epirubicin was observed in both P-gp and MRP1 expressing cells [36]. When such a combinatory treatment was applied to drug-resistant mouse lymphoma cells and antiproliferative effects were measured, a synergistic effect was observed in the case of chrysin and amorphigenin and an additive one in the case of rotenone. In HTB26 cells, formononetin and kaempferol displayed synergistic antiproliferative effects when they were combined with epirubicin, while afrormosin and robinin affected proliferation in an additive way [36].

The drug accumulation studies have proven that P-gp inhibition by flavonoids is higher than MRP1 inhibition. However, in combination with epirubicin some compounds were more effective than expected in the MRP1-expressing human breast cancer cell line. P-gp and MRP1 may not be equally sensitive to inhibition by flavonoids and the antiproliferative effects of simultaneously applied anticancer drugs could be different in cell lines expressing different efflux pumps. One of the differences between the two efflux systems is that P-gp binds the unmodified transported drug, whereas MRP1 predominantly pumps out drugs conjugated

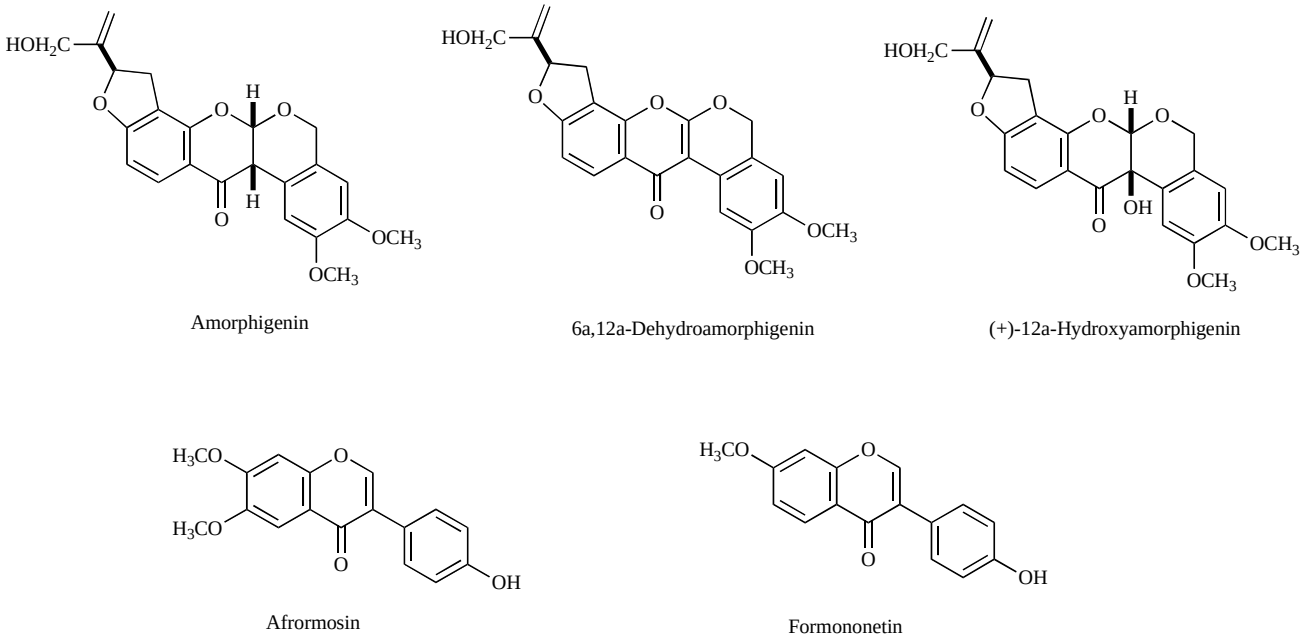
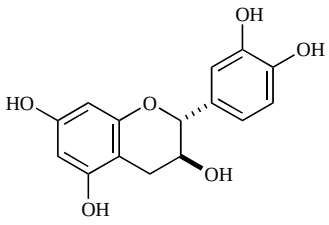


Fig. (3). Isoflavonoids which are MDR reverting agents.

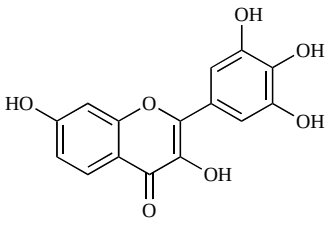
Table 4. MDR Reversal Effects of Isoflavonoids in Human *MDR1* Gene Transfected Mouse Lymphoma Cells and HTB26/MRP1 Cells

Compound	Conc [μg/mL]	FAR[a]		References
		Rhodamine 123 accumulation	*BCECF accumulation*	
		Mouse lynphoma/ MDR1 cells	**HTB26/MRP1 cells**	
Verapamil[b]	5	17.1		[36]
Indomethacin[c]	10		2.4	[36]
Formononetin	40	18.3	1.5	[36]
Amorphigenin	40	46.4	1.0	[36]
Afrormosin	40	3.1	1.4	[36]
6a,12a-Dehydroamorphigenin	40	3.0	1.2	[36]
(+)-12a-Hydroxyamorphigenin	40	2.8	1.1	[36]

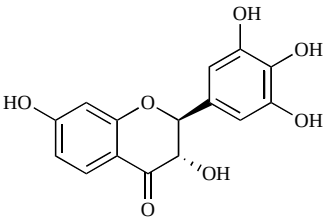
[a] FAR (Fluorescence Activity Ratio): mean fluorescence ratio for treated/untreated samples. [b] Reference compound of mouse lymphoma/MDR1 cells. . [c] Reference compound of HTB26/MRP1 cells.



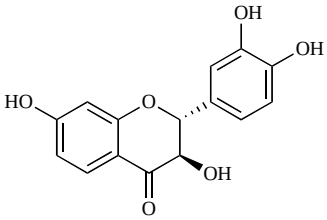
Catechin



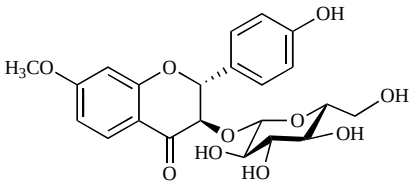
Robinetin



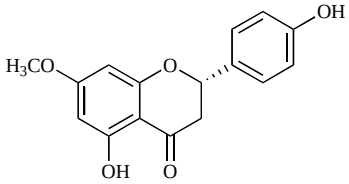
Dihydrorobinetin



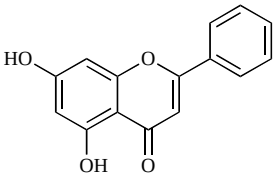
Dihydrofisetin



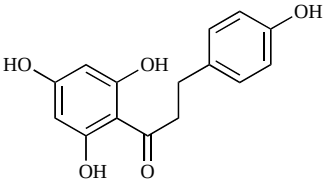
Sakuranin



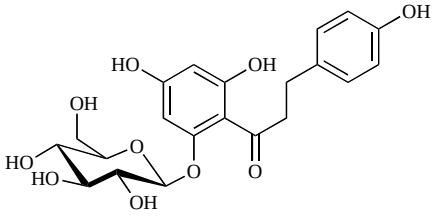
Sakuranetin



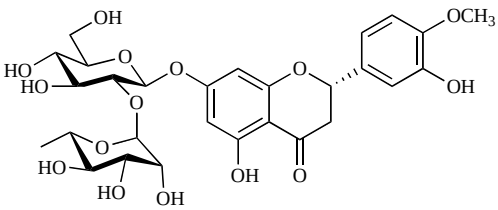
Chrysin



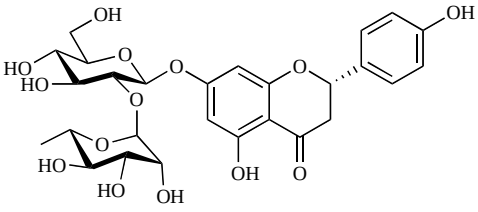
Phloretin



Phloridzin



Neohesperidin



Naringin

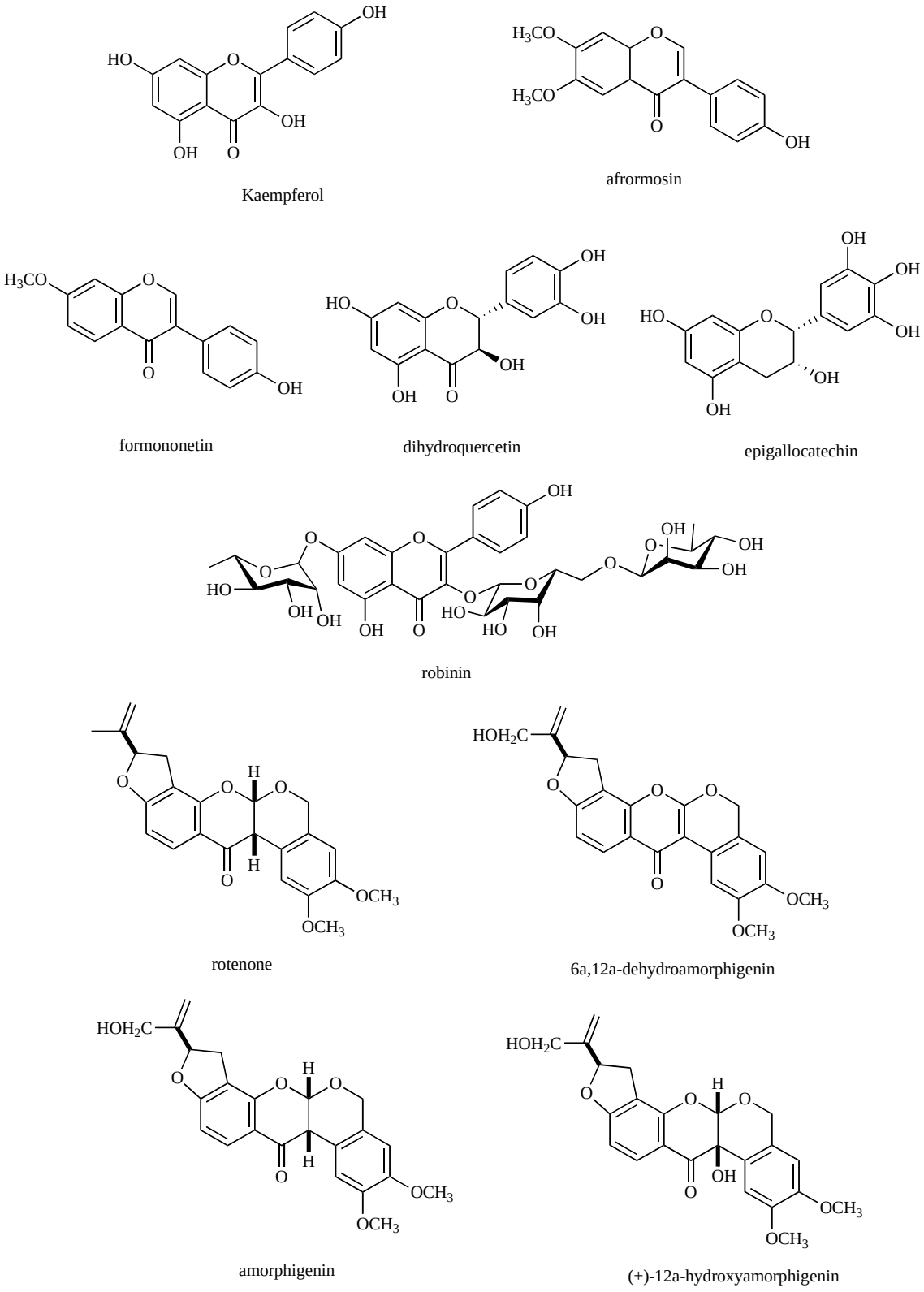


Fig. (4). Some flavonoid subclasses which are MDR reverting agents.

with glutathione (GSH). Its substrates are often anionic compounds [37]. Other studies have demonstrated that certain dietary flavonoids may modulate MRP1-mediated organic anion and GSH transport, its ATPase activity and drug resistance [38]. Genistein was found to be an inhibitor of drug accumulation in MRP1-overexpressing cells [39], and also to decrease the transport activity of P-gp [40]. However, contradictory results have been reported for the MDR-

Table 5. MDR Reversal Effect of Flavonoids in Human *MDR1* Gene Transfected Mouse Lymphoma cells, Colo320/MDR1 Cells and HTB26/MRP1 Cells

Compound	Conc [μg/mL]	FAR[a]			References
		Rhodamine 123 accumulation		*BCECF accumulation*	
		Mouse lymphoma/MDR1 cells	COLO320/MDR1 cells	HTB26/MRP1 cells	
Verapamil[b]	5	8.9	21.6		[36]
Indomethacin[c]	10			1.3	[36]
Rotenone	40	28.6	40.04	0.3	[36]
Catechin	40	2.9	1.57	0.6	[36]
Neohesperidin	40	2.8	1.55	0.9	[36]
Naringin	40	2.3	1.18	0.8	[36]
Chrysin	40	14.6		1.2	[36]
Robinin	40	1.5	1.18	1.6	[36]
Phloretin	40	4.9		1.0	[36]
Phloridzin	40	0.6	2.81	0.9	[36]
Robinetin	40	0.7	0.83	0.9	[36]
Dihydrorobinetin	40	0.7	1.48	1.1	[36]
Kaempferol	40	0.8	0.81	1.3	[36]
Dihydrofisetin	40	0.5	1.21	1.0	[36]
Dihydroquercetin	40	0.6	0.91	1.3	[36]
Sakuranin	40	0.8	0.52	0.7	[36]
Sakuranetin	40	2.4	5.2	0.8	[36]
Epigallocatechin	40	36.1	3.80	1.3	[36]

[a] FAR (Fluorescence Activity Ratio): mean fluorescence ratio for treated/untreated samples. [b] Reference compound of mouse lymphoma/MDR1 cells. . [c] Reference compound of HTB26/MRP1 cells.

modulating activity of the flavonoids kaempferol and quercetin, which were previously found to stimulate P-gp-mediated efflux of doxorubicin in a resistant colon cancer cell line [41]. In our experiments, kaempferol was ineffective whereas chrysin showed a significant inhibitory effect [36]. Rotenoid derivatives, amorphigenin and rotenone, had significant MDR-modulating activity. The concentrations of the test compounds were crucial. For example, at low concentrations quercetin activated the activity of P-gp whereas at high concentrations it inhibited this pump [36]. A similar biphasic effect has been observed for kaempferol. The effects of various flavonoids on drug accumulation were also studied in resistant human colon cancer cells and their drug sensitive counterparts [36]. In these cell lines, rotenone was an effective inhibitor of drug efflux whereas phloridzin, catechin, neohesperidin, naringin, and robinin influenced rhodamine 123 accumulation slightly.

The main conclusion drawn from our studies was that flavonoids and isoflavonoids differentially affected P-gp- and MRP1-mediated drug resistance and that the chemical structure of modulators was of great importance. This was in agreement with other studies. P-gp was inhibited by flavonoids such as formononetin, amorphigenin, rotenone and chrysin [40,41]. MRP1-mediated carboxyfluorescein efflux was reduced by afrormosin, formononetin, amorphigenin, rotenone, chrysin, robinin, kaempferol and epigallocatechin [38,39]. The effect was probably an inhibition of ATPase activity [38,42]. In addition, some flavonols stimulated the efflux of carcinogens in P-gp-expressing breast cancer cells [43].

By comparison of the fluorescence activity ratios and chemical structures of the studied compounds, a certain correlation between MDR modulating activity and physicochemical parameters could be found. The majority of carotenoids may have a special binding site on P-gp, in contrast flavonoids are likely to posses multiple targets, whereas only the ATP binding site contributes to P-gp inhibition.

EXTRACTS FROM CHINESE MEDICINAL HERBS

Traditional Chinese Medicine (TCM) is a complete medical system that has been used to diagnose, treat, and prevent illnesses for more than 2,000 years. The principal Fu Zhen herbs *(astragalus, ligustrum, ginseng, codonopsis,*

atractylodes, and ganoderma) are believed to strengthen the body's nonspecific immunity and to increase the functions of T-cells. Herbal antitoxin therapies, also regularly used, contain many herbs that have been found to inhibit tumor growth by a variety of mechanisms. *Kelp* and *pokeroot (Phytolacca americana)* are among the herbs used to treat tumors in Chinese herbal therapy. The most highly praised blood tonic in the East, *Tang kuei (Angelica sinensis)*, has been used clinically in China to treat cancer of the esophagus and liver with good results. There are plenty examples of the effects of the recommended plant extracts [44]. In recent years there has been a trend to integrate TCM treatment with standard Western medicine in an attempt to further optimize treatment outcome, minimize the side effects of surgery, radiation and chemotherapy, increase immune function and improve survival [45].

Certain compounds extracted from Chinese herbal medicines have been reported to be capable of reversing MDR in tumor cells [46]. We have studied the anti-MDR properties of Chinese herbal extracts obtained from twelve selected medicinal plants, using different solvents such as methanol, chloroform, ethyl acetate and water [47]. The following representative plant species were selected on the basis of their possible antitumor and imunomodulation activities as described in TCM: *Astragalus membranaceus* var. *mongholicus*, *Glehnia littoralis*, *Asparagus cochinchinensis*, *Ligustrum lucidum*, *Aconitum carmichaeli*, *Astragalus membranaceus, Selaginella doederleinii*, *Atractylodes macrocephala*, *Poria cocos*, *Asarum heteropoides* var. *mandshuricum*, *Paris polyphylla* var. *chinensis*.

When the four types of extracts (methanol, chloroform, ethyl-acetate and water) were studied in resistant mouse lymphoma cells in a search for putative MDR modulators, chloroform extracts demonstrated the highest P-gp-inhibitory activity [47]. The most effective resistance modifiers were found in chloroform extracts from *Ligustrum lucidum*, *Selaginella doederleinii*, *Atractylodes macrocephala*, and *Asarum heteropoides* var. *mandshuricum* (Table **6**). Some extracts exhibited very high ID_{50} values (> 100 μg/mL) suggesting that they could contain compounds that were substrates of P-gp. The aqueous extracts were practically ineffective (data not shown).

The ability of Chinese plant extracts to reverse multidrug resistance in P-gp expressing mouse lymphoma cells in combination with doxorubicin has been also studied [47]. *Asarum heteropoides* var. *mandshuricum* revealed high anti-MDR activity not only when applied alone. When it was combined with doxorubicin, a synergistic effect was observed. The other MDR-reversal extract (from *Selaginella doederleinii*) applied together with the anticancer drug displayed only an additive effect [47]. Previous studies have shown that polysaccharides from *Astragalus membranaceus* are capable of inhibiting tumor development, decreasing the toxic effect of chemotherapy, enhancing the immune function of the organism and improving the quality of life in patients with malignant tumors [48]. Moreover, it has been reported that injection of *Astragalus membranaceus* extract can enhance the antitumor metastatic action of dentritic cells, and can effectively promote the immune response of the tumor-bearing host; it therefore exerts an inhibitory effect on cancer metastasis *in vivo* [49]. *Atractyloides macrocephala* has long been used to induce cancer remission [50].

The human cytomegalovirus (CMV) preferentially infects tumor tissues and the accumulated CMV immediate

Table 6. MDR Reversal Effect of Methanol, Chloroform and Ethyl-Acetate Extracts of CHINESE Plants (20 μg/mL of Each Extract was Used) in *MDR1* Gene-Transfected Mouse Lymphoma Cells

Chinese plants	FAR[a]			Reference
	Extracts			
	Methanol	Chloroform	Ethyl-acetate	
root of *Astragalus membranaceus* var. *mongholicus*	1.48	2.24	1.26	[47]
root of *Glehnia littoralis*	1.03	1.27	1.18	[47]
tuber of *Asparagus cochinchinensis*	1.09	1.78	0.94	[47]
fruit of *Ligustrum lucidum*	1.85	3.54	1.07	[47]
tuber of *Aconitum carmichaeli*	1.01	2.19	10.35	[47]
Massa medicata fermentata *Astragalus membranaceus*	1.01	1.57	0.94	[47]
herb of *Selaginella doederleinii*	2.17	16.69	5.10	[47]
rhizome of *Atractylodes macrocephala*	1.66	3.93	3.54	[47]
sclerotium of *Poria cocos*	1.69	1.51	0.95	[47]
entire plant of *Asarum heteropoides* var. *mandshuricum*	1.12	27.15	1.28	[47]
root of *Paris polyphylla* var. *chinensis*	1.31	1.51	1.59	[47]
root of *Astragalus membranaceus*	1.19	1.82	1.13	[47]

early antigen (IE) may lead to tumor promotion and progression [51,52]. The development of strategies to inhibit the expression and/or function of human IE antigen is an important goal to prevent and treat certain forms of cancer associated with human CMV. Human CMV was used in a modified *in vitro* model [53,54] for the screening of selected Chinese plant extracts for antitumor-promoting activity [47]. The concentrations of test extracts greatly influenced the results of the experiments. A low extract concentration, for example, increased the IE antigen expression of CMV, whereas a high concentration decreased it. Ukiya *et al.* [55] demonstrated that certain lanostane-type triterpenoids from *Poria cocos* may inhibit tumor promotion and progression, and markedly suppress the Epstein-Barr virus (EBV) early antigen activation. This finding is in contrast with our results [47], where chloroform extracts of *P. cocos* did not modify the IE antigen expression of CMV in human lung cancer (A459) cells.

The integration of Western and oriental medicinal knowledge will lead to optimization of the therapeutic uses of Chinese medicinal plants in the foreseeable future. These plants contain many compounds responsible for various biological effects, and it is therefore important to know what types of compounds exert anticancer or antitumor-promoting activity.

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