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Reversal of Multidrug Resistance of Cancer Cells *In Vitro*: Modification of Drug Resistance by Selected Carotenoids

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Abstract. *The development of multidrug resistance (MDR) causes difficulties in the chemotherapy of of human cancer. Investigation of the possibility of reversal of MDR has been greatly aided by the use of cell lines with aquired resitance to anticancer agents in vitro or transfected with the mdr1 gene.The aim of this study was to examine new perspectives of chemotherapy focused on natural, carotenoid compounds, in connection with the modification of MDR. The function of the MDR protein was examined via the R123 drug accumulation of both cell lines in the presence of carotenoids. The fluorescence of the cell population was measured by flow cytometry. The most effective resistance modifiers Monoepoxy-b-carotene, (5S, 8S)-capsochrome, (8'S) Luteoxanthin, (9Z)-Violaxanthin, (9Z)-Zeaxanthin, (13Z)-Zeaxanthin were assayed for their antiproliferative effects in combination with the anti-cancer drug epirubicin. (13Z)-Zeaxanthin was able to enhance the antiproliferative effect on human mdr1 gene transfected mouse lymphoma and anthracycline resistant human breast cancer cell line MCF7. (8'S)-luteoxanthin, (5S, 8S)-capsochrome and (9Z)-zeaxanthin treatment revealed synergism with epirubicin on resistant mouse lymphoma. The enhanced antiproliferative activity of epirubicin combinated with (9Z)-Violaxanthin was more significant on MCF7 cells resistant to anthracycline.*

Many potent antitumor drugs loose their effectiveness as a consequence of the development of multidrug resistance (MDR) in tumor cells.

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Membrane-bound ABC transporters including the P-glycoprotein (P-gp)have been found to be responsible for the MDR phenotype of cancer cells. Several antitumor compounds isolated from plant extracts or synthesized (1-3) have shown activity towards the modification of the action of the P-glycoprotein (P-gp) and membrane proteins responsible for drug efflux and MDR(3).

Carotenoids, which are widely distributed plant pigments and well-known antioxidants, belong to the terpenoids. The most potent antioxidant carotenoid lycopene inhibits the proliferation of several types of cancer cells. Lycopene inhibits cell growth by interfering with growth factor receptor signalling and cell cycle progression, specifically in prostate and breast cancer, without evidence of toxic effects or apoptosis on the cells (4, 5).

The MDR reversal effects of carotenoids on were previously studied (2). Capsanthin and capsorubin enhanced the rhodamine 123 accumulation 30-fold relative to non-treated lymphoma cells. Lycopene, lutein, antheraxanthin and violaxanthin had moderate effects, while alfa- and beta-carotene had no effect on the reversal of MDR in the tumor cells. Apoptosis was induced in human MDR1 transfected mouse lymphoma cells and human breast cancer MDA-MB-231 (HTB-26) cell lines in the presence of lycopene, zeaxanthin and capsanthin. The data suggest the potential importance of carotenoids as possible resistance modifiers in cancer chemotherapy. The (*E*/*Z*)-isomerism of carbon-carbon double bonds is an interesting feature of stereochemistry of carotenoids. The polyene system is extremely susceptible to oxidative degradation and to geometrical isomerization brought about by light, heat or acids. The chemical and physical properties of carotenoids may be modified by interactions with other molecules (*e.g.* proteins, lipids). These interactions may be critical to the actions of the carotenoid molecule in membranes (6).

This study will focus on an investigation of the MDR reversal effects of several stereosomeric forms of previously

studied carotenoids (Figure 1) on mouse lymphoma cells transfected by human *mdr1* gene in model experiments and on a anthracycline-resistant subline of the human breast cancer cell line MCF7.

The classification of studied carotenoids was listed as follows mutatochrome, aurochrome, flavoxanthin, chrisanthemaxanthin, capsochrome-epimers (4 carotenoids) are carotenoid-5,8-epoxides (furanoid-oxides). Mutatochrom is an 5,8-monoepoxy-carotenoid with unsymmetrical structure. Aurochrome is a 5,8,5',8'-diepoxy-carotenoid with symmetrical structure. Flavoxanthin and Chrysanthemaxanthin are the two epimers of the 3,3'-dihydroxy- 5,8-moepoxy-alpha-carotene (beta,epsilon-carotene). They are unsymmetrical carotenoid-5,8-epoxides.Capsochrome-epimers are the natural (5R,8R and 5R8S)- and the semisynthetic (5S,8S and 5S,8R) epimers of the 3,3'-dihydroxy-5,8-epoxy-beta-kappa-carotene with unsymmetrical structure. Monoepoxy-alpha- and monoepoxy-beta-carotene are carotenoid-5-6-monoepoxides with unsymmetrical structure. Diepoxy-beta-carotene is an 5,6,5',6'-diepoxy-carotenoid with symmetrical structure. 15,15'-Dehydrodiepoxy-beta-carotene is the central acetylenic derivative of diepoxy-beta-carotene and this carotenoid has symmetrical structure. Luteochrome and epimers of luteoxanthin are 5,6-and 5,8-carotenoid-epoxides with unsymmetrical structures. Luteoxanthin-epimers are the corresponding 3,3'-dihydroxy-derivatives of luteochrome. The last five carotenoids which can be seen in Figure 1 are (Z)-carotenoids (cis-carotenoids). The polyene chain of these carotenoids contains a cis-double bond.

Materials and Methods

Chemicals. Mutatochrome, aurochrome, flavoxanthin, chrysanthemaxanthin, monoepoxy-β-carotene, diepoxy-β-carotene, 15,15'-dehydrodiepoxy-β-carotene, monoepoxy-α-carotene, (5*R*,8*R*)-capsochrome, (5*R*,8*S*)-capsochrome, (5*S*,8*S*)-capsochrome, (5*S*,8*R*)-capsochrome, luteochrome, (8'*R*)-luteoxanthin, (8'*S*)-luteoxanthin, (13*Z*)-lutein (13'*Z*)-lutein, (13*Z*)-zeaxanthin, (9*Z*)-zeaxanthin (9*Z*)-violaxanthin and (9'*Z*)-neoxanthin (Figure 1) were obtained from our collection. The samples were dissolved in dimethylsulfoxide (DMSO) (SERVA, Feinbiochemica, Heidelberg, Germany).

Cell culture. The L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described (7). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicines. L5178 (parent) mouse T-cell lymphoma cells and its human mdr1 gene transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum L-glutamine and antibiotics. The anthracycline-resistant subline of human breast cancer MCF7 cells selected to doxorubicine and its parental cell line were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and antibiotics. In the case of the drug-resistant cell line, 1 μM doxorubicin was added to the medium to maintain the P-gp expression; this cell line was a kind gift from Dr. Zoltan Kiss (CanCure, Michigan, USA). The adherent human cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min. The cell lines were incubated in a humified atmosphere (5% CO_2, 95% air) at 37˚C.

Reversal of MDR of tumor cells. The cells were adjusted to a density of 2 x 10^6/mL, resuspended in serum-free medium and distributed in 0.5-mL aliquots into Eppendorf centrifuge tubes. Various volumes of the 1.0 mg/mL stock solutions of the tested compounds were then added, and the cells were incubated for 10 min at room temperature. Ten μL (5.2 μM final concentration) rhodamine 123 indicator was then added, and the cells were incubated for an additional 20 min at 37˚C, washed twice, and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured by flow cytometry, using a Beckton-Dickinson FACScan instrument. Verapamil was used as a positive control. The activity ratio was calculated from the measured fluorescence values by using the following formula (2, 3):

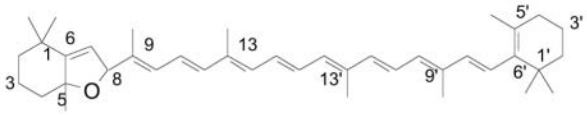
$$R = \frac{MDRtreated \,/\, MDRcontrol}{parental\ treated \,/\, parental\ control}$$

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 100 μL. Then, 1x10^4 cells in 50 μL 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 mL of MTT (thiazolyl blue tetrazolium bromide, Sigma, St Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37˚C for 4 h, 100 mL of sodium dodecylsulfate (SDS) (Sigma, St Louis, MO, USA) solution (10%) was addad 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:

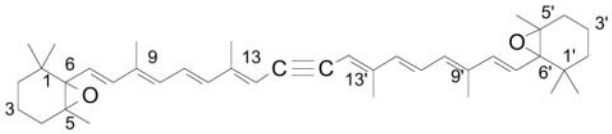
$$100 - \left[\frac{ODsample - ODmediun\ control}{ODcell\ control - ODmediun\ control} \right] \text{x } 100$$

A previously described (3) checkerboard microplate method was applied to study the interactions between MDR modifiers and the anthracycline derivatives, epirubicin or doxorubicin, on the cancer cells.

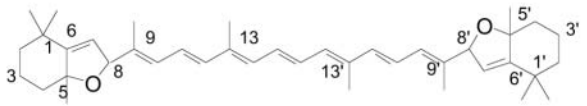
The effects of the anticancer drug and the MDR modifiers in combination were studied on various cancer cell lines. The dilutions of the anticancer drug (A) were made in a horizontal direction, and the dilutions of the MDR modifiers (B) vertically in the microtiter plate in a 150-μL volume. Each well was seeded 500 mL medium containing 1x10^4 cells. The plates were incubated in a humified CO_2 incubator for 72 h at 37˚C. 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:



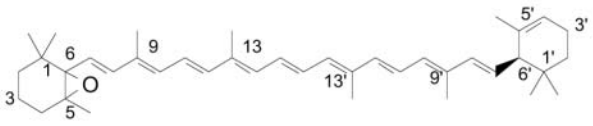
Mutatochrome ;



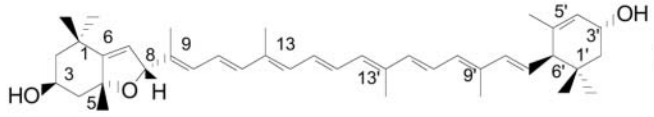
15,15'-Dehydro-diepoxy-β-carotene ;



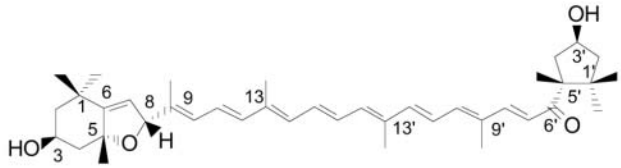
Aurochrome;



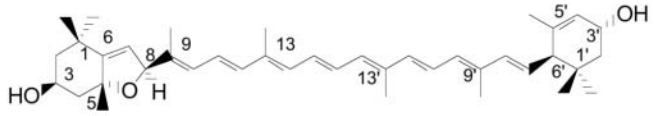
Monoepoxy-α-carotene;



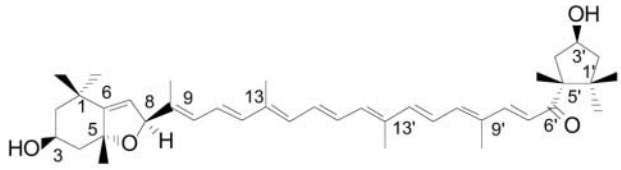
Flavoxanthin;



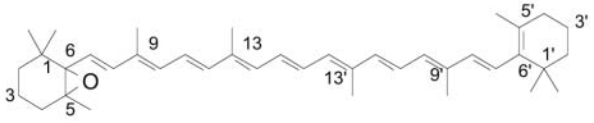
(5*R*,8*R*)-Capsochrome;



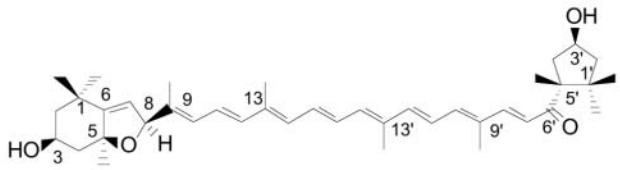
Chrysanthemaxanthin ;



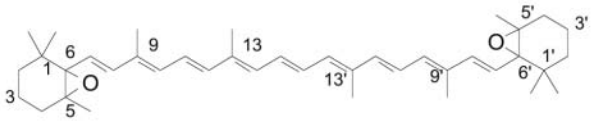
(5*R*,8*S*)-Capsochrome



Monoepoxy-β -carotene;



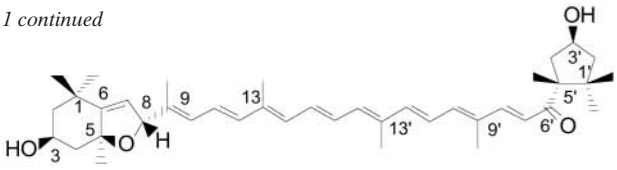
(5*S*,8*S*)-Capsochrome ;



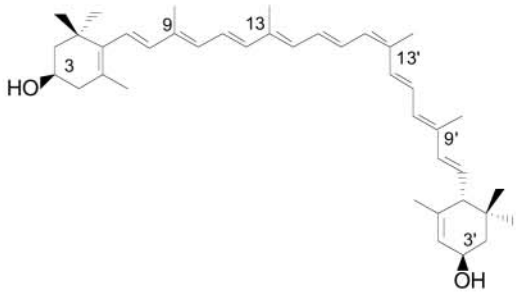
Diepoxy-β-carotene ;

Figure 1. *Chemical structure of carotenoids tested for reversal of MDR on human mdr1 gene transfected mouse lymphoma and anthracycline resistant human breast caqncer cells MCF7.*

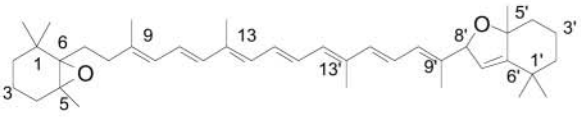
Figure 1 continued



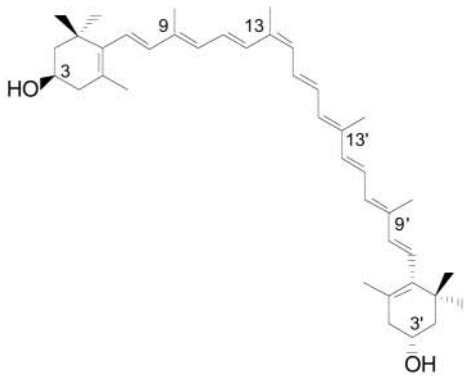
(5*S*,8*R*)-Capsochrome;



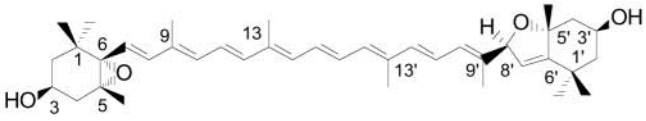
(13'*Z*)-Lutein



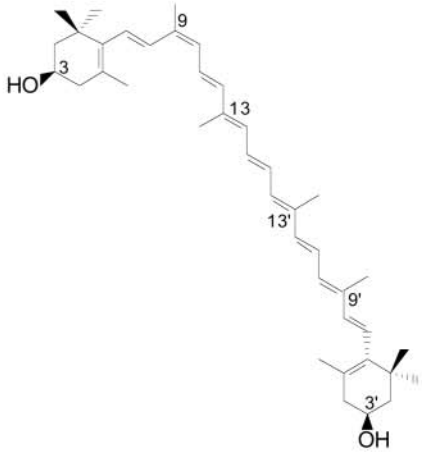
Luteochrome ;



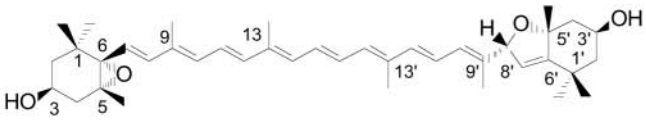
(13*Z*)-Zeaxanthin



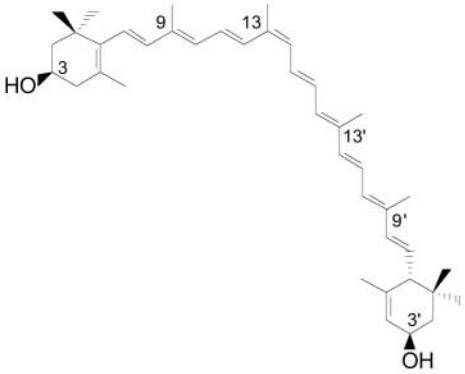
(8*R*)-Luteoxanthin ;



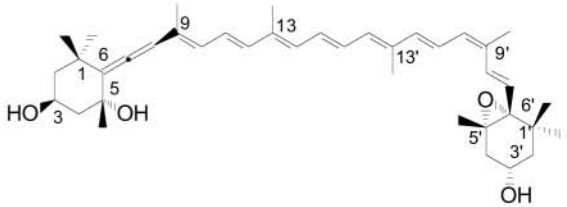
(9*Z*)-Zeaxanthin



(8*S*)-Luteoxanthin;



(13*Z*)-Lutein



(9'*Z*)-Neoxanthin

$FIC_A = ID_{50A \text{ in combination}} / ID_{50A \text{ alone}}$
$FIC_B = ID_{50B \text{ in combination}} / ID_{50B \text{ alone}}$

where 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
and FIX = fractional inhibitory index.

Immunocytochemistry. The cells were harvested and resuspended in serum-free medium at a cell density of $5x10^5$/mL. One hundred mL were cytocentrifuged for 5 min at 1000 rpm. The samples were fixed in 4°C acetone for 10 min and then 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 antibody P-gp: NCL-PGLYm in suitable dilutions (1:5, 1:10 and 1:20) for 1 h at room temperature then washed in TBS buffer three times for 5 min, and were incubated with the secondary antibody (DAKO EnVisionTM System, CA, USA) for 30 min. The samples were again washed in TBS three times for 5 min. Diaminobenzidine (DAKO, CA, USA) was used as the chromogen. Sections were counterstained with hematoxylin and mounted.

Results

The extent of drug accumulation in the human *mdr1* gene-transfected mouse lymphoma cell and the breast cancer cell line expressing MDR1 was tested in the presence or absence of the carotenoids.

The presence of P-gp was detected on the membrane surface of the drug-resistant subline of MCF7 by immunocytochemistry with the NCL-PGLYm mouse monoclonal antibody that reacts with the human P-gp G-terminal cytoplasmic domain. A high level of P-gp expression was also detected on the membrane of the L5178Y human *mdr1*-transfected mouse lymphoma cells.

Some carotenoids *e.g.* mutatochrome, aurochrome and diepoxy-β-carotene were moderately active in the P-gp inhibition (Table I). Treatment of the cancer cells with flavoxanthin + chrysanthemaxanthin, (5*R*,8*R*)-capsochrome, (5*R*,8*S*)-capsochrome, (5*S*,8*S*)- capsochrome, or (5*S*,8*R*)-capsochrome resulted in an extremely high fluorescence activity ratio. 15,15-Dehydrodiepoxy-β-carotene and monoepoxy-α-carotene in the same concentrations were ineffective. Luteochrome was hardly effective as a reversing agent, but the structurally related compounds (8'*R*)- and (8'*S*)-luteoxanthin, (13*Z*)-zeaxanthin, (13*Z*)-lutein, (9*Z*)-zeaxanthin, (9*Z*)-violaxanthin and (9'*Z*)-neoxanthin exhibited a high fluorescence activity ratio on mouse lymphoma cells (Table I).

A lower inhibition of the MDR efflux pumps was found for the drug-resistant MCF7 human breast cancer cell line than for the human *mdr1* gene transfected mouse lymphoma cells in the presence of the same carotenoids. As shown in Table II, the rhodamine accumulation was enhanced only moderately, from a fluorescence activity ratio of 1.1 to 2.2, which means that the rhodamine uptake was enhanced from 10 to 120 per cent in the human breast cancer cells. Some compounds were inactive, *e.g.* (9'Z)-neoxanthin, monoepoxy-α-carotene and 15,15'-dehydrodiepoxy-β-carotene (Table II).

Comparison of the sensitivities of the two cell lines demonstrated that the same human *mdr1*-gene-encoded P-gp efflux pump had different sensitivities to carotenoids, despite the fact that the drug accumulation in the verapamil control was similar for both cell lines. It may be suggested that the P-gp target in the cell membrane has different sensitivities to the extremely hydrophobic carotenoids in various cell lines.

In further experiments the checkerboard microplate method was applied to examine the effects of carotenoids in combination with anthracycline derivatives (Table III). Verapamil was also used as a functionally positive control in this experiment. The treatment resulted in a synergistic effect between verapamil and doxorubicin for cell lines with MDR resistance. Similarly (13*Z*)-zeaxanthin was able to enhance the antiproliferative effect of epirubicin synergistically on both cell lines. (8'S)-luteoxanthin, (5*S*, 8*S*)-capsochrome and (9*Z*)-zeaxanthin treatment revealed synergism with epirubicin on resistant mouse lymphoma. The enhanced antiproliferative activity of epirubicin combined with (9*Z*)-Violaxanthin was more significant on MCF7 cell line with anthracycline resistance.

(5*S*,8*S*)-Capsochrome exhibited an antagonistic effect on the drug-resistant breast cancer cell line. Monoepoxy-β-carotene increased the rhodamine 123 accumulation in both cell lines, but remained almost ineffective in combination treatments (Table III). Despite the relatively low degree of inhibition on the P-gp efflux pump of the drug-resistant subline of MCF7 human breast cancer cells, the results were encouraging; the combinations of epirubicin with some carotenoids resulted in synergism in the antiproliferative action of epirubicin on the two cell lines. Maintance of the carotenoids in solution at –20°C for a few months, resulted in a decrease MDR reversing activity, probably due to oxidation.

Discussion

Although verapamil one of the first P-gp inhibitors initially exhibited a promising *in vitro* activity, its maximum tolerated dose is too low for the effective inhibition of P-gp (8, 9). Recently, an increasing number of studies have focused on the possibility of P-gp modulation, with new more effective and less toxic inhibitors (10).

Among natural compounds, various fractions of paprika extracts have been show to reverse the MDR of cancer cells (11). The effectiveness of the hexane and acetone fractions

Table I. *The effects of carotenoids in reversing multidrug resistance of mdr1 gene transfected mouse lymphoma cells.*

Samples	Conc. (μg/ml)	Fluorescence activity ratio
Verapamil	10	15.70
Mutatochrome	4	2.02
	40	7.47
Aurochrome	4	0.94
	40	3.34
Flavoxanthin + chrysanthemaxanthin	4	4.12
	40	30.27
Diepoxy-β-carotene	4	1.44
	40	5.72
15,15'-Dehydrodiepoxy-β-carotene	4	0.75
	40	0.58
Monoepoxy-α-carotene	4	0.72
	40	0.56
Monoepoxy-β-carotene	4	9.54
	40	37.59
(5*R*,8*S*)-Capsochrome	4	11.03
	40	37.92
(5*R*,8*R*)-Capsochrome	4	14.04
	40	56.81
(5*S*,8*S*)-Capsochrome	4	40.13
	40	41.52
(5*S*,8*R*)-Capsochrome	4	23.25
	40	53.54
Luteochrome	4	1.54
	40	2.83
(8'*R*)-Luteoxanthin	4	56.90
	40	61.95
(8'*S*)-Luteoxanthin	4	35.94
	40	63.57
(13*Z*)+(13'*Z*)-Lutein	4	11.64
	40	31.42
(13*Z*)-Zeaxanthin	4	3.58
	40	35.24
(9*Z*)-Zeaxanthin	4	4.86
	40	20.54
(9*Z*)-Violaxanthin	4	34.01
	40	57.74
(9'*Z*)-Neoxanthin	4	51.21
	40	59.55
DMSO control		0.74

*We can observed that the carotenoids containing –OH substituents in the position C-3 and C-3' are effective; and carotenoids without –OH substituents in positions C-3 and C-3' are ineffective or they show very small effect. In addition the epoxy substitution has sme role in the MDR reversal as the results show in Table I and Table II

Table II. *The effects of carotenoids in reversing multidrug resistance on doxorubicin-resistant MCF7 human breast cancer cell line*

Samples	Conc. (μg/ml)	Fluorescence activity ratio[a]
Verapamil	5	11.9
Mutatochrome	4	1.1
	40	1.9
Aurochrome	4	0.9
	40	0.8
Flavoxanthin + chrysanthemaxanthin	4	1.0
	40	1.1
Diepoxy-β-carotene	4	1.07
	40	2.08
15,15'-dehydro-diepoxy- β-carotene	4	0.89
	40	0.62
Monoepoxy-α-carotene	4	0.94
	40	0.69
Monoepoxy-β-carotene	4	1.17
	40	2.37
(5*S*,8*R*) Capsochrome	4	1.27
	40	1.38
(5*S*,8*S*)-Capsochrome	4	1.77
	40	1.99
(5*R*,8*R*)-Capsochrome	4	1.34
	40	1.67
(5*R*,8*S*)-Capsochrome	4	1.43
	40	1.95
Luteochrome	4	2.1
	40	1.3
(8'*R*)-Luteoxanthin	4	1.2
	40	1.9
(8'*S*)-Luteoxanthin	4	1.1
	40	2.1
(13*Z*)+(13'*Z*)-Lutein	4	1.2
	40	2.0
(13*Z*)-Zeaxanthin	4	1.1
	40	2.0
(9*Z*)-Zeaxanthin	4	1.4
	40	2.2
(9*Z*)-Violaxanthin	4	1.7
	40	2.2
(9'*Z*)-Neoxanthin	4	0.89
	40	0.69
DMSO control		0.6

[a]Fluorescence activity ratio: mean fluorescence ratio for treated/ untreated samples

of paprika on the ABC transporter, suggested that it would be worthwhile to study the effects of various well-defined carotenoids on the drug accumulation in tumor cells.

The MDR-reversing effects of the carotenoids tested in the present experiments exhibited a structure-activity relatiopnship. Interestingly, neoxanthin was ineffective in our previous study (2), whereas (9'*Z*)-neoxanthin was able to increase the drug accumulation (59.55) on an *mdr1*- transfected mouse lymphoma cell line, and similar differences were found for violaxanthin and, (9*Z*)-violaxanthin and zeaxanthin, (9*Z*)-zeaxanthin. It is possible that the configuration of (9*Z*) compounds has a

Table III. *Interaction between epirubicin and some carotenoid resistance modifiers.*

Samples	L5178 mouse lymphoma MDR1 resistant cell line		Drug-resistant subline of MCF7 breast cancer cells	
	FIX	Interaction	FIX	Interaction
Verapamil as positive control	0.162	synergism[a]	0.231	synergism[a]
Monoepoxy-β-carotene	0.678	additive	1.512	indifferent
(5*S*,8*S*)-Capsochrome	0.467	synergism	2.855	antagonism
(8'*S*)-Luteoxanthin	0.448	synergism	0.803	additive
(9*Z*)-Violaxanthin	0.568	additive	0.279	synergism
(9*Z*)-Zeaxanthin	0.239	synergism	0.976	additive
(13*Z*)-Zeaxanthin	0.494	synergism	0.426	synergism

[a]In the case of the verapamil control doxorubicin was used as the cytotoxic drug instead of epirubicine

stronger binding capacity toward the P-gp than the linear (all-E) forms of neoxanthin, violaxanthin and zeaxanthin. A majority of the carotenoids that have a 3-hydroxy group on the right six-membered ring (a 3-hydroxy-b-end group) exert a moderate MDR reversal effect. Additionally, the presence of the 3-hydroxy- and 3'-hydroxy-κ-end group is possibly responsible for the high resistance reversal effect in the human *mdr1* gene-transfected mouse lymphoma cells.

Recent studies suggest that the membrane structure may play a critical role in the MDR activities, the resistance modulator – lipid interaction may be an important factor in the mechanism of drug resistance reversal (12-15). The carotenoids seem to be selectively absorbed by the membranes, depending on the carotenoid structural features (size, shape and polarity), and on the membrane characteristics (16).These properties determine the incorporation yield and the abilities of the carotenoids to fit into the membrane bilayer (17, 18). It has also been suggested that there are differences between the membrane insertion of polar (*e.g.* lutein or zeaxanthin) and nonpolar (*e.g.* β-carotene) carotenoids, polar carotenoids generally being incorporated better than β-carotene (18). This phenomenon could explain the higher activity of polar carotenoids in MDR reversal. Moreover, polar carotenoids are preferentially located in the region of unsaturated lipids of the membrane (19), and it may be presumed that there are differences in the ratio of saturated and unsaturated fatty acids in different cell lines, which may be responsible for the variation in the MDR-modulating effect of carotenoids on the mouse lymphoma and resistant MCF7 cell lines in the present experiments.

Several studies have been performed on the relationship between differences in membrane properties and MDR (13, 20, 21). These data emphasized the importance of the increased membrane fluidity and rarely rigidity (13) in the inhibition of P-gp. It is suggested that the carotenoids partition into the hydrophobic core of the membrane and cause a decrease in lipid fluidity (22). The reduced membrane fluidity may sterically hinder diffusion of drugs and decrease the kinetics of drug excretion (15).

In conclusion, several of the tested carotenoids inhibited the P-gp activity and enhanced the effects of the anthracycline derivatives on both cell lines. On the basis of these results the use of the most effective carotenoids may be considered for *in vivo* experiments.

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