Putative Supramolecular Complexes Formed by Carotenoids and Xanthophylls with Ascorbic Acid to Reverse Multidrug Resistance in Cancer Cells

JÓZSEF MOLNÁR[1], JULIANNA SERLY[1], ROZÁLIA PUSZTAI[1], IRÉN VINCZE[2], PÉTER MOLNÁR[3], GYÖRGYI HORVÁTH[3], JÓZSEF DELI[4], TAKASHI MAOKA[5], ATTILA ZALATNAI[6], HARUKUNI TOKUDA[7] and HOYOKU NISHINO[8]

[1]*Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary;*
[2]*Department of Organic Chemistry, University of Szeged, Szeged, Hungary;*
[3]*Department of Pharmacognosy, University of Pécs, Pécs, Hungary;*
[4]*Deparment of Biochemistry and Medical Chemistry, University of Pécs, Pécs, Hungary;*
[5]*Research Institute for Production Development, Food Function and Chemistry, Kyoto, Japan;*
[6]*First Department of Pathology and of Experimental Cancer Research, Semmelweis University, Budapest, Hungary;*
[7]*Department of Complementary and Alternative Medicine, Clinical R&D, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan;*
[8]*Ritsumeikan Global Innovation Research Organization, Ritsumeikan University, Kusatu, Japan*

Abstract. *Background: The molecular basis of interaction of selected carotenoids and xanthophylls with ascorbic acid on cancer cells was studied to determine their anticancer effects. Materials and Methods: Drug accumulation was measured in a human ABCB1 gene-transfected mouse lymphoma cell line and in a human lung cancer cell line by flow cytometry; furthermore, their anticancer effects were determined in mice in vivo. Results: Several carotenoids inhibited the multidrug resistance of cancer cells. Ascorbic acid improved the effect of certain xanthophylls, but the effect of capsanthin was not modified. Capsanthin had weak (12%) but capsorubin (85%) had a remarkable antiproliferative effect on A549 lung cancer cells. Capsorubin reduced immediate-early tumor antigen expression, while capsanthin was not effective. Capsorubin accumulates selectively in the nuclei of cancer cells. Conclusion: The Authors suggest a special complex formation between membrane-bound capsorubin and ascorbic acid, which can be exploited in experimental chemotherapy.*

Correspondence to: Professor József Molnár, MD, Ph.D., Department of Medical Microbiology and Immunobiology, University of Szeged, Dóm tér 10., Szeged 6720, Hungary. Tel: +36 62545114, Fax: +36 62545113, e-mail: molnarj@comser.szote.u-szeged.hu

Key Words: MDR reversal, cancer cells, capsorubin, capsanthin, electronic charge transfer complex, vitamin A, vitamin C.

Numerous vegetables contain carotenoids and vitamins that are essential components to preserve health by reducing aging damage (1). Therefore, the interactions between antioxidant effects of nutrient carotenoids and ascorbic acid were of interest for investigation in an *in vitro* study.

Carotenoids are fat-soluble antioxidants which can be electron donors in the excited state, and can be divided into two main groups: carotenes and xanthophylls. Carotenes contain hydrocarbon groups on both ends, xanthophylls are oxygenated carotenoids.

Ascorbic acid is a water-soluble electron donor antioxidant, and was first isolated from paprika (*Capsicum annuum*, Solanaceae) by Ilona Banga and Albert Szent-Györgyi, a Nobel laureate scientist from the University of Szeged, Hungary in the early 1930s (2). According to our previously published results, a large dose of ascorbic acid has a beneficial effect on 5-fluorouracil (5FU) chemotherapy because it enhances the apoptosis of cells both *in vitro* and *in vivo* (3). There was some other encouraging but not overwhelming support for ascorbic acid as a protector against aging of normal cells and as a contributing factor to cancer therapy (4).

This study attempted to find evidence for interaction between ascorbic acid and carotenoids for the drug accumulation of cells expressing ATP-binding cassette member B1 (ABCB1) transporter protein, explaining it with supramolecular complex formation. Selective membrane permeability can be induced by carrier molecules and membrane channels transporting charged and neutral molecules. Transmembrane channels allow the passage of molecules by flow or site-to-site hopping. Electron channels as transmembrane 'wires' work as mobile electron

carriers, coupling chemical potentials during signal transduction. The coupled transfer of charges at the molecular level opens perspectives for the functional features of supramolecular chemistry *via* molecular recognition between complementary binding compounds. The conformational differences in membrane alignment of antioxidants such as ascorbic acid and carotenoids may help to explain their biological activity. The precise transmembrane alignments in the lipid bilayer of cells exposed to xanthophylls has demonstrated efficacy in the case of water-dispersible capsanthin both *in vitro* and *in vivo* (5).

The reddish-colored astaxanthin is known as an antioxidant that has single and double electron oxidant action and is a chain-breaking scavenger of free radicals itself (4). In addition, the transmembrane alignment of carotenoids likely provides proximity to cofactors such as ascorbic acid, which serves as a sink for accepting free radical cations by effectively recharging the electron transfer capacity of carotenoids by improving the exhausted acceptor (or rather the electronic conductive) property of carotenoids (4). The transmembrane orientation of polar carotenoids is known to facilitate electron shuttling and physicochemical interaction of antioxidant compounds with cell membranes responsible for several biological actions (6). The transmembrane alignments provide exposure of the polar hydrophilic ends of the long carotenoid molecule to the internal cytoplasm and to the aqueous environment external to the cell. The same situation exists in mitochondrial matrix and intermembrane space of mitochondria, facilitating electron transfer *via* the conjugated double bonds of the polyene chain of carotenoids.

Based on the measurements in chemical reaction between carotenoids and ascorbic acid, evidence was found for the formation of charge transfer complexes (7). Nonpolar carotenoids, such as lycopene and β-carotene, disorder the membrane bilayer and stimulate lipid peroxidation in the membrane. On the contrary, polar carotenoids (xanthophylls), such as astaxanthin and capsanthin, preserve the membrane structure, show antioxidant activity and can indirectly modify the functionally active conformation of ABCB1 by specific binding to the ABCB1–lipid bilayer complex, hence, the antioxidant activity of carotenoids was suggested to be dependent on their distinct membrane–lipid interactions.

In the membrane of multidrug-resistant (MDR) cancer cells, electrical activation of lipid bilayer–ABCB1 complex can be important to produce effective pumping of the anticancer drugs (8). This effect depends on the orderly coordinated spatial transfer of electric current from the cell interior of the cell surface to the extracellular region by a functionally active ABCB1 conformation. To obtain evidence for electron paramagnetic resonance, electronuclear double resonance spectroscopy and molecular orbital calculations were applied to determine the formation of carotenoid radical cations and dications and their electron transfer properties under different conditions (9-11).

The action of carotenoid isomers on cancer can be realized in several ways *e.g.* direct antitumor effect, apoptosis induction and chemoprevention *via* inhibition of Epstein-Barr virus, adenovirus or cytomegalovirus induced early tumor antigen expression in immortalized cells; this mechanism is called an antitumor-promoting effect (12).

Carotenoids can serve as membrane stabilizers due to their charged character (12). Carotenoids inhibit MDR of cancer cells and induce apoptosis (13). The antitumor-promoting effect was measured by inhibition of the Epstein-Barr virus (12) and cytomegalovirus by the inhibition of early tumor antigen expression induced by phorbol esters *in vitro* (11). Capsanthin and its esters exhibited MDR reversal effect in cancer cells and anti-promotion effect *in vitro* and *in vivo* (12, 14). Capsanthin and some structurally related xanthophylls had antitumor promoting activity, as was shown by inhibition of Epstein-Barr virus immediate-early antigen activation *in vitro* (12).

Cancer promotion was inhibited by other plant carotenoids such as crocin in two-stage carcinogenesis mouse skin assays by using anthracene as inducer and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as promoting agent (15). As explanation of the mentioned effect, the singlet oxygen quenching activity of carotenoid was shown and there was also inhibition of free radical-induced lipid peroxidation, and anticancer activities (12, 16, 17).

It was shown that carotenoids altered the electron density associated with the membrane hydrocarbon core over a broad area from the centre of the membrane (16). Carotenoids with all-*trans* (all-*E*) polyene chain make the membrane more rigid by increasing the crystalline state of the membrane compartment, while carotenoids with *cis* (Z) polyene chain disorganize the lipid bilayer and increase the liquid state of membrane (16, 18, 19). The structure–activity relationships were assessed by flow cytometry and paramagnetic carotenoid radical cations and diamagnetic radical dications, purported to be related with differences in electron transfer, during isomerization (9). Based on the former studies, the modification of MDR of cancer cells was studied in the presence of carotenoids.

In the present study, we describe the MDR-reversing effect of some selected xanthophylls and the interaction between carotenoids and ascorbic acid on MDR mediator ABCB1 in human *ABCB1* gene-transfected mouse lymphoma (MDR) cells.

In addition, the antiproliferative effect of various carotenoids and accumulation of capsorubin in the nuclei of human lung cancer cells (A549) were studied. The chemopreventive effect of capsorubin was shown by the reduction of early tumor antigen-expressing cells. Putative explanation of the theoretical approach and the biological importance of the interaction between some carotenoids and ascorbic acid are presented.

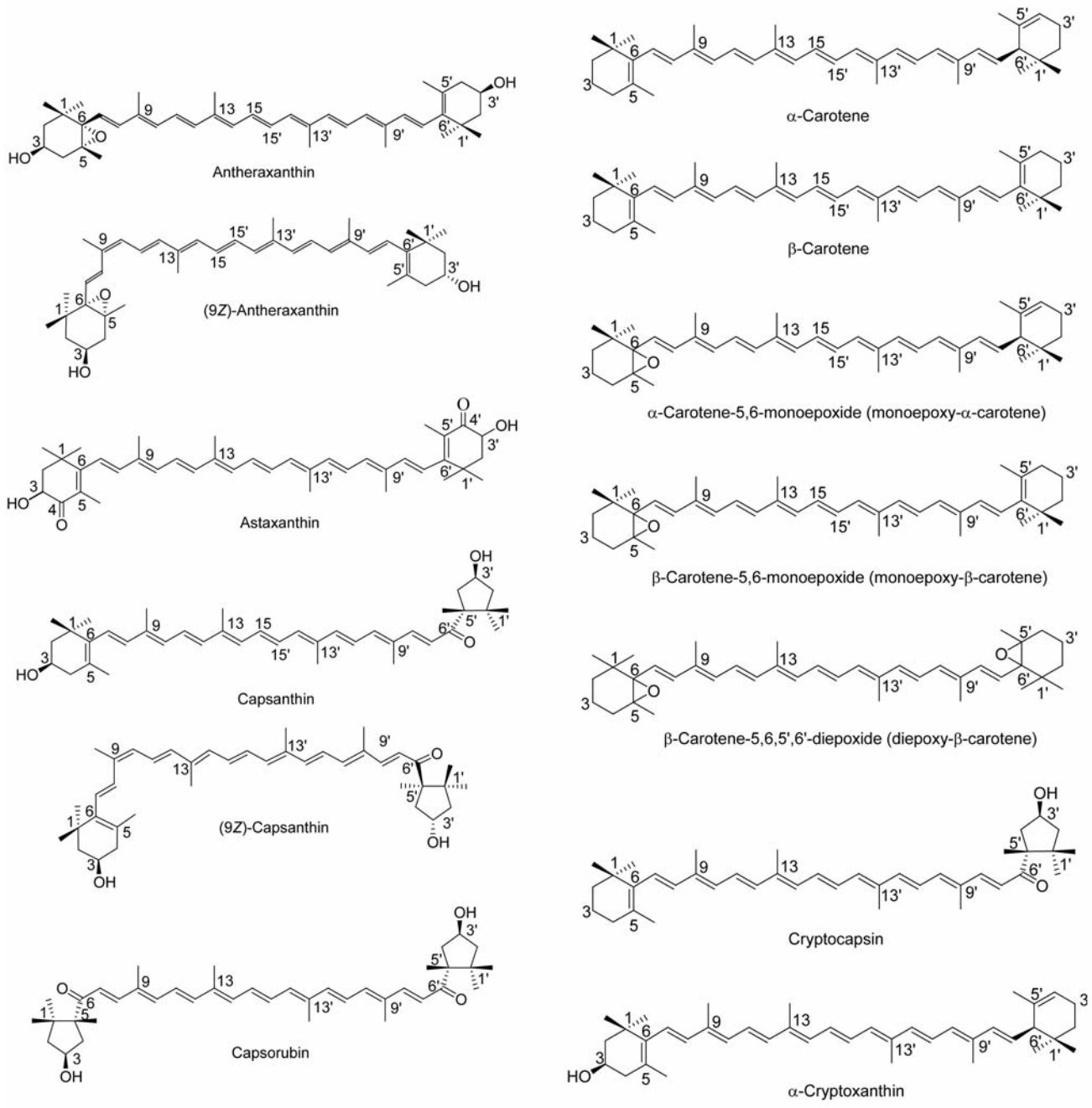

Figure 1. *Continued*

Materials and Methods

Chemicals. Carotenoids of plant origin were isolated in highly pure (>95%) crystalline state in our laboratory. Antheraxanthin (*Lilium candidum*, *Viola tricolor*); (9Z)-antheraxanthin (*Lilium candidum*); astaxanthin (Fluka AG., Buchs SG, Switzerland); capsanthin (*Capsicum annuum*); (9Z)-capsanthin (prepared from capsanthin by iodine-catalyzed photoisomerization) (20, 21); capsorubin (*Capsicum annuum*); α-carotene (*Daucus carota*); β-carotene (*Daucus carota*); α-carotene-5,6-monoepoxide (monoepoxy-α-carotene; prepared semisynthetically from α-carotene); β-carotene-5,6-monoepoxide (monoepoxy-β-carotene; prepared semisynthetically from β-carotene); β-carotene-5,6,5',6'-diepoxide (diepoxy-β-carotene; prepared semisynthetically from β-carotene); cryptocapsin (*Capsicum annuum*); α-cryptoxanthin (*Capsicum annuum*); β-cryptoxanthin (*Citrus aurantium*); 15,15'-dehydro-β-carotene-5,6,5',6'-diepoxide (synthetic; F. Hofmann-La-Roche Ltd., Basel, Switzerland); lutein (Tagetes erecta); lutein-5,6-epoxide (*Helianthus*

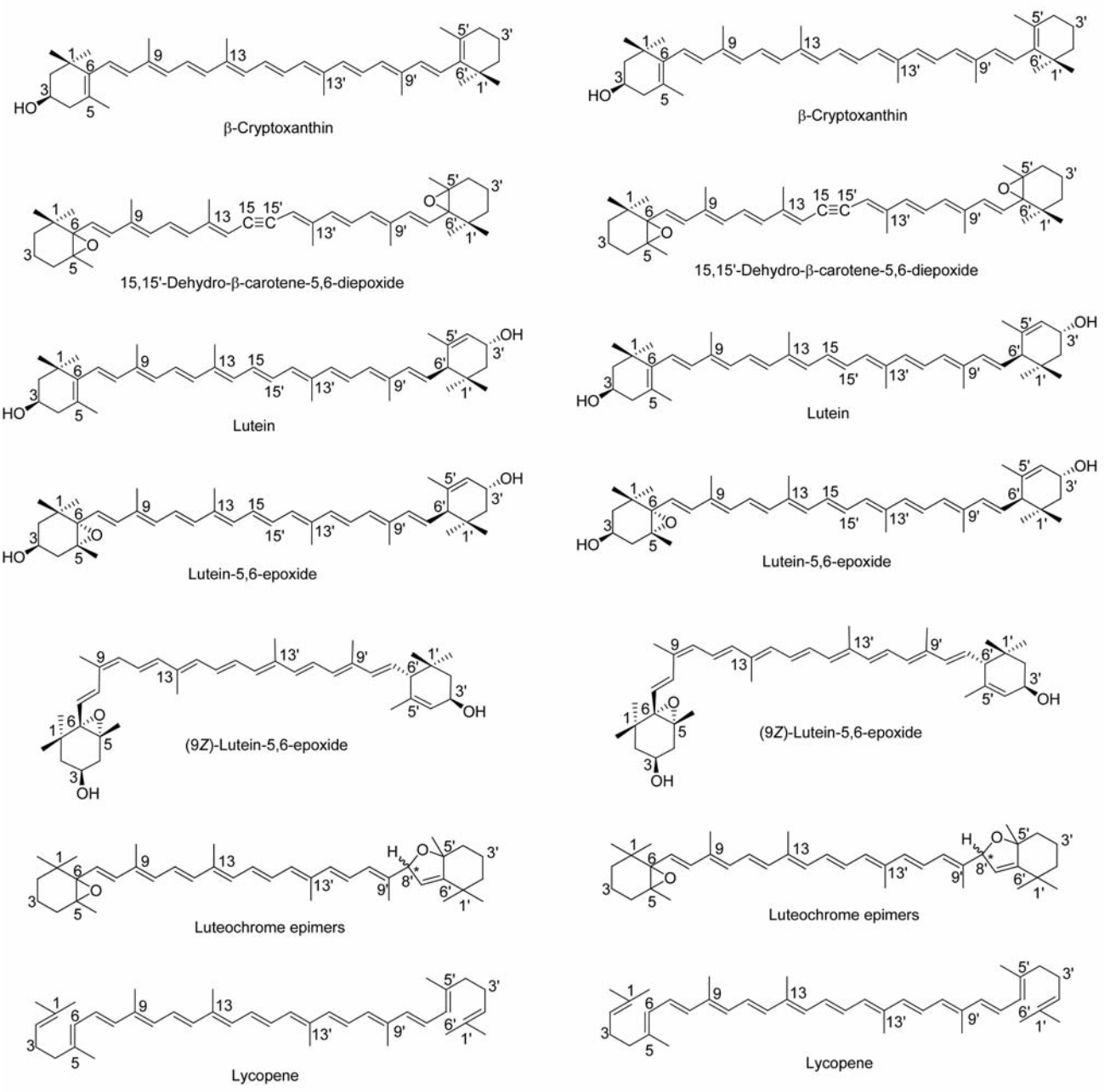

Figure 1. *Continued*

annuus); (9*Z*)-lutein-5,6-epoxide (*Helianthus annuus*); luteochrome (semisynthetic; prepared from β-carotene-5,6,5',6'-diepoxide); lycopene (*Lycopersicon esculentum*); mutatochrome (semisynthetic; prepared from β-carotene-5,6-monoepoxide); violaxanthin (*Viola tricolor* and *Citrus aurantium*); (9*Z*)-violaxanthin (*Viola tricolor* and *Citrus aurantium*); (9*Z*)-violaxanthin-diacetate (prepared from (9*Z*)-violaxanthin by acetylation); vitamin A (retinol; synthetic; Fluka AG); vitamin A-aldehyde (retinal; synthetic; Fluka AG); zeaxanthin (*Lycium halimifolium*); (9*Z*)-zeaxanthin (prepared from zeaxanthin by iodine-catalyzed photoisomerization) (5). The structures of the carotenoids examined in this study are shown in Figure 1.

Cell lines. Parental mouse T-cell lymphoma cells (ECACC cat. no. 87111908; U.S. FDA, Silver Spring, MD, USA) were transfected with pHa MDR11/A retrovirus, as described previously (22, 23). The ABCB1-expressing cell line L5178Y was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. The parental mouse T-cell lymphoma cell line (PAR) and the human *ABCB1* gene-transfected subline (MDR) were cultured at 37˚C in McCoy's 5A medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (H1270; Sigma-Aldrich Co.), 200 mM L-glutamine (Invitrogen Corp., Carlsbad, CA, USA) and penicillin-

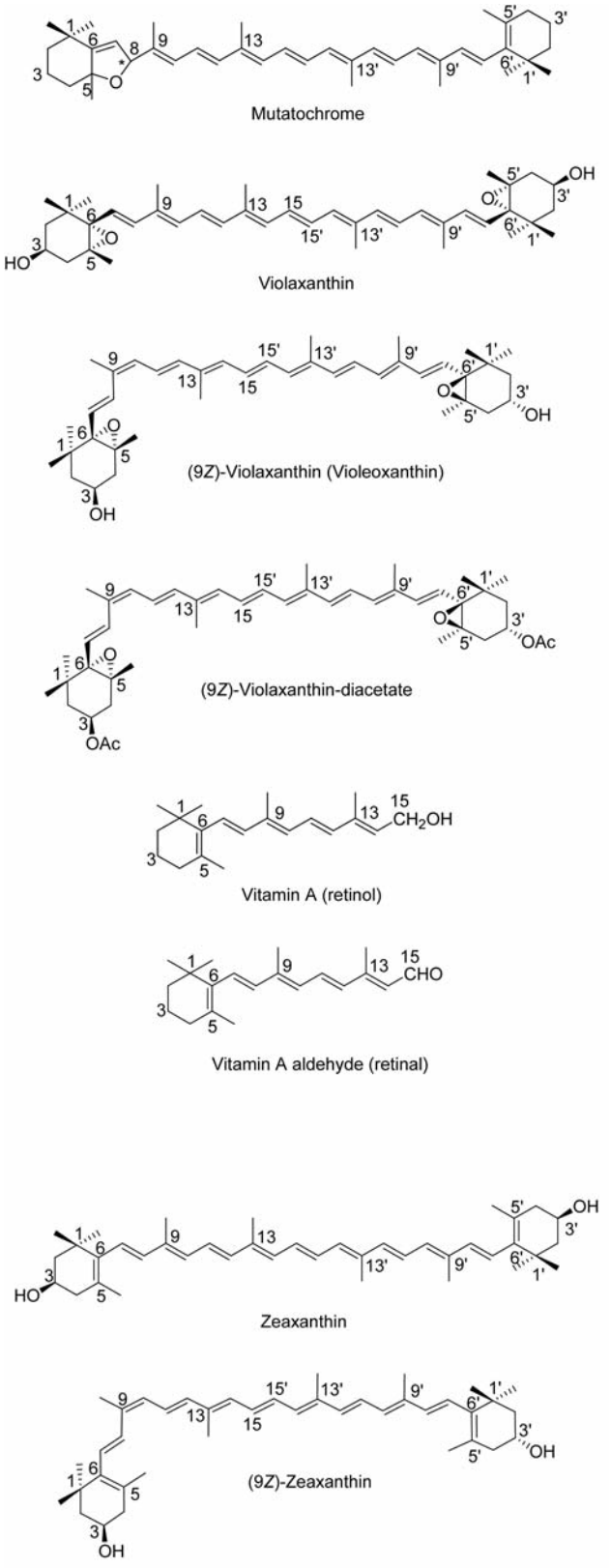

Figure 1. *Chemical structures of the investigated carotenoids.*

streptomycin mixture (Sigma-Aldrich Co.) in 100 U/l and 100 mg/l concentration, and the media for culturing the *ABCB1* gene-transfected cell line was complemented with colchicine (60 ng/ml; Sigma-Aldrich Co.). The mouse lymphoma cell lines were maintained in a 5% CO_2 atmosphere at 37˚C.

The A549 human alveolar adenocarcinoma cell line (ECACC cat no. 86012804) was obtained from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan) (24, 25). It was maintained in Eagle's minimal essential medium (EMEM) (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO, Invitrogen Corp.), 200 mM L-glutamine and penicillin-streptomycin mixture in 100 U/l and 100 mg/l concentration, maintained at 37˚C in 5% CO_2.

Assay for reversal of MDR in mouse lymphoma cells. The cells were adjusted to a density of 2×10^6 cells/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 different final concentrations (4.0 and 40 μg/ml), and the samples were incubated for 10 min at room temperature. Indicator rhodamine 123 (R123) (Sigma-Aldrich Co.) was added to each sample to a final concentration of 10 μg/ml 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 analysis. The fluorescence of the cell population was measured with a FACS Star Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil (EGIS Pharmaceuticals PLC, Budapest, Hungary) was used as a positive control in the R123 exclusion experiments at final concentration of 10 μg/ml (26). The percentage mean fluorescence intensity was calculated for the treated MDR/PAR cell lines as compared with the untreated cells. Fluorescent activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{MDR\,treated \,/\, MDR\,control}{Parental\,treated \,/\, Parental\,control}$$

The results presented are obtained from a representative flow cytometric experiment in which 1×10^4 individual cells of the population were evaluated for the amount of R123 retained are first presented by the FACS Star Plus flow cytometer as histograms and the data were converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total and in the gated populations (27).

Growth inhibition of human lung cancer cells (A549) by carotenoids and xanthophylls. After 72 h pre-incubation, the medium was removed and the cells were cultured in EMEM supplemented with 10% FCS. The carotenoids were dissolved in dimethylsulfoxide (DMSO), and then were added to the cultured medium in 10, 20 and 40 μM final concentration. Viable cells were assessed with trypan blue dye after incubation of 24 to 72 h. Viability was expressed as a percentage of the viable cell number of control cultures.

Quantitative analysis of capsorubin and capsanthin in the nucleus of A549 cells. A549 cells were incubated in 35 μM capsorubin or capsanthin for 24 h at 37˚C in 5 % CO_2. The nucleus of A549 cells was fractionated by treatment with *ProteoExtract*® Subcellular Proteome Extraction Kit (cat. no. 539790; Merck KGaA, Darmstadt, Germany). The nucleus was extracted with acetone. After

evaporation of the solvent, the residual was dissolved in 200 μl of acetone and filtrated through a 0.45 μm polytetrafluoroethylene membrane filter, then 20 μl solution was subjected to high-performance liquid chromatography (HPLC) on Wakosil 5C18 (Wako Pure Chemical Industries Ltd., Osaka, Japan) (ODS) column (250 mm × 4.6 mm *i.d.*) with a mobile phase of $CHCl_3$:CH_3CN (1:9), detected at 470 nm. Capsorubin and capsanthin were quantified relative to calibration with a standard sample.

Assay for human cytomegalovirus (CMV) immediate-early (IE) gene expression by immunofluorescence. The immunofluorescence assay is described elsewhere (14). Cells were grown for 24 h on glass coverslips in 24-well plates initially containing 2×105 cells/well, then cells on the coverslips were infected with Towne strain of HCMV at a multiplicity of infection of 2.4. The infected A549 cells were centrifuged for 60 min at 1200 rpm at room temperature, and then incubated for 1 h at 37˚C. The cells were washed three times with serum-free EMEM medium, then were cultured in medium (EMEM with 1% FCS and antibiotics) and the appropriate concentration of a compound or DMSO. After 48 h incubation, the cells were washed twice with cold PBS and fixed with a mixture of cold acetone:ethanol (1:1) for 20 min at –20˚C. The fixed cells were stored at –20˚C until immunofluorescence assays were performed.

HCMV IE antigen was detected in the nuclei of infected cells by immunostaining using monoclonal antibody (MAB810; Chemicon International Inc., Temecula, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (F0257; Sigma-Aldrich Co.). The number of IE antigen-positive cells was counted in 30 microscopic fields, containing 400 cells of each sample. The frequency of IE antigen-expressing cells in the treated cultures is shown as a percentage that of the control.

In vivo experiments. These experiments with mice were performed in compliance with the appropriate laws and institutional guidelines approved by the Budapest and Pest County Agricultural Office (permission number: 22.1/1159/3/2010). Ten artificially immune suppressed CBA/J male mice (4 weeks old, b.w. 20 g ±1.6 g) bearing subcutaneously growing human pancreatic cancer xenografts (PZX-40/46) were treated with 2.5 mg/kg b.w. capsanthin for 1 week. The compound was dissolved in DMSO, and each animal was given 0.1 ml of solution subcutaneously every day. Control animals were given DMSO only. At the end of the experiments the animals have been sacrificed by neck dislocation and the freshly removed tumors were fixed in 4% neutral buffered formalin.

Immunohistochemistry. Paraplast-embedded tissues from human pancreatic cancer xenograft (PZX-40/46) sections were subjected to immunostaining. Sections were cut at 4 μm, deparaffinized, rehydrated and then rinsed in tap water for 5 minutes. Antigen retrieval was completed in 10 mM citrate buffer at pH 6.0 by microwaving the slides. Antigen retrieval was completed in 10 mM citrate buffer at pH 6.0 by microwaving the slides. Endogeneous peroxidase activity was quenched by incubation in methanol containing 0.3% hydrogen peroxide at room temperature for 30 minutes. Slides were then washed in PBS for 5 minutes and blocked with normal horse serum for 20 minutes. To localize the ABCB1 slides were incubated with specific mouse monoclonal primary antibody (clone: C494; LABVISION, Fremont, CA, USA) diluted in PBS for 45 minutes at room temperature. Thereafter, tissue sections were washed with PBS for 5 minutes and incubated with an anti-mouse peroxidase-labeled rabbit IgG reagent ImmPRESS (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 minutes. After washing in PBS, each section was subjected to diaminobenzidine (DAB)-chromogen/substrate reagent and counterstained with hematoxylin. Adrenal cortex was used as positive control.

Results

The effect of carotenoids on the reversal of MDR in mouse lymphoma. In our study, a specific interaction was found between some (9*Z*)-carotenoids [(9*Z*)-zeaxanthin, (9*Z*)-violaxanthin, (9*Z*)-violaxanthin-diacetate, (9*Z*)-capsanthin, (9*Z*)-lutein-5,6-epoxide, (9*Z*)-antheraxanthin], capsanthin, capsorubin and ascorbic acid as measured by the rhodamine 123 accumulation of MDR cancer cells (Tables I and II). Capsanthin and lycopene were exceptions, since their effect was not modified by ascorbic acid in the MDR reversal experiment.

The interactions between the two selected xanthophylls, capsorubin and capsanthin, and ascorbic acid were studied. Ascorbic acid significantly enhanced the effect of capsorubin in a dose-dependent manner, but the effect of capsanthin and retinal compounds was not modified. Based on this difference, a charge transfer and/or a supramolecular complex formation is suggested. The possible explanation of our results based on the supramolecular complex formation is that vitamin A induces isomerization, and the most effective resistance modifiers were retinol and vitamin A aldehyde. The potency of isomerization was evaluated by comparing the MDR-reversal effect of some modeled carotens and retinol derivatives *in vitro* (Table II).

Growth inhibition of A549 cells by carotenoids in vitro. Capsorubin and capsorubin diester remarkably inhibited the growth of human lung cancer cells (A549). On the other hand, capsanthin, capsorubin diester, lycopene, and β-carotene did not inhibit the growth of A549 cells. As the results show in Figure 2, the growth-inhibitory effects of carotenoids at a concentration of 20 μM on A549 cells were different. Furthermore, capsorubin inhibited the growth of A549 cells in a dose-dependent manner (Figure 3).

CMV IE gene expression changes following treatment with carotenoids. Evidence was found for the particular binding differences of capsanthin and capsorubin to cancer cells when the chemopreventive effects of the two compounds were studied. Capsorubin reduced the IE tumor antigen expression in the CMV-infected fibroblast cells, but capsanthin enhanced the expression of IE antigens in the transfected cells.

The antipromotory effects of xanthophyll capsorubin and α-carotene were shown, however, in the preliminary experiments, capsanthin had the opposite effect (Figure 4). The binding of capsorubin and capsanthin to biological membranes and nucleic acids might be basically different in the A549 cell line.

Table I. *Effects of carotenoids and ascorbic acid on the inhibition of ABCB1 membrane transporter measured by R123 fluorescence intensity (FL-1) and fluorescence maxima (peak channel) in PAR/MDR tumor cells.*

Sample	Final cc (μM)	FAR	Peak channel
PAR cells, control	-	-	1144
MDR cells, control	-	-	12
Verapamil	22	8.43	62
(9Z)-Zeaxanthin	70	40.72	626
(9Z)-Zeaxanthin +	70 + 113.6	41.35	582
vitamin C	70 + 568	67.83	1144
(9Z)-Violaxanthin	66	66.84	947
(9Z)-Violaxanthin +	66 + 113.6	59.52	827
vitamin C	66 + 568	61.75	991
(9Z)-Violaxanthin-diacetate	62.4	55.32	813
(9Z)-Violaxanthin-diacetate +	62.4 + 113.6	53.67	798
vitamin C	62.4 + 568	61.49	1074
(9Z)-Capsanthin	68.4	57.97	850
(9Z)-Capsanthin +	68.4 + 113.6	59.15	798
vitamin C	68.4 + 568	55.58	889
(9Z)-Lutein-5,6-epoxide	68.4	52.76	784
(9Z)-Lutein-5,6-epoxide +	68.4 + 113.6	62.66	938
vitamin C	68.4 + 568	60.54	930
(9Z)-Anti-antheraxanthin	68.4	46.87	704
(9Z)-Anti-antheraxanthin	68.4 + 113.6	49.81	743
+ vitamin C 5 mg/ml	68.4 + 568	53.21	820

cc, Concentration. FAR, Fluorescence activity ratio (see equation in the Materials and Methods). The results are from one representative flow cytometric experiment in which 1×10^4 individual cells were investigated. Carotenoids were solved in DMSO; DMSO was used as a negative control (data not shown); vitamin C was dissolved in distilled water. Treated PAR cells did not show significant difference in FL-1 values (data not shown).

Table II. *Effects of capsanthin/capsorubin and ascorbic acid on the inhibition of ABCB1 membrane transporter measured by R123 fluorescence intensity (FL-1) and fluorescence maxima (peak channel) in PAR/MDR tumor cells.*

Sample	Final cc (μM)	FAR	Peak channel
PAR cells, control	-	-	842
MDR cells, control	-	-	12
Verapamil	22	5.15	42
Capsanthin	400	40.76	749
Capsanthin +	400 + 284	38.67	704
vitamin C	400 + 568	32.49	643
	400 + 1136	32.34	523
Capsorubin	400	29.77	491
Capsorubin +	400 + 284	34.33	582
vitamin C	400 + 568	40.57	777
	400 + 1136	28.44	491
Vitamin C	284	0.86	13
	568	0.61	9
	1136	0.96	12
β-Citraurin	22.7	1.26	11
Vitamin A (retinol)	27.9	33.7	392
Vitamin A aldehyde (retinal)	28.1	15.4	224
Vitamin A acetate	24.4	17.1	186

cc, Concentration. FAR, Fluorescence activity ratio (see equation in the Materials and Methods). The results are from one representative flow cytometric experiment in which 1×10^4 individual cells were investigated. Carotenoids were solved in DMSO; DMSO was used as a negative control (data not shown); vitamin C was dissolved in distilled water. Treated PAR cells did not show significant difference in FL-1 values (data not shown).

Quantitative analysis of capsorubin and capsanthin in the nucleus of A549 cells. It was observed that the nuclei of A549 cells were stained red. HPLC analysis revealed that capsorubin was detected in the nucleus of A549 cell at a concentration of 0.18 μmol/μg protein after 24 h treatment, and 0.21 μmol/μg protein after 48 h treatment. These results showed that capsorubin was taken into the nucleus of A549 in a time-dependent manner. Capsanthin was also taken into the nucleus but showed less affinity than capsorubin (0.05 μmol/μg protein of capsanthin was detected in the nucleus after 48 h treatment).

In vivo effects of capsanthin and capsorubin. The effect of the treatment had to be terminated after the first week because of the severe side-effects of capsanthin. Within two days, cutaneous necrosis developed near the site of injections, and on the following days, multiple, irregular, sometimes confluent skin ulcerations appeared on the back, far from the injection sites. Histologically, there was no sign of thrombus formation in the small vessels. During treatment, the tumors grew continuously, and histologically, retained their original, well-differentiated ductal adenocarcinoma morphology. In the tumorous tissue, 70-75% of tumor cells expressed ABCB1, and this feature proved to be constant during single treatments.

Discussion

Interaction between ascorbic acid and carotenoids. Ascorbic acid is a five-membered lactone, containing an ene-diol system, its acidic hydroxyl groups are easily activated into free radicals, being an antioxidant in a water-soluble system it acts as electron donor and this antioxidant effect was demonstrated by Albert Szent-Györgyi in 1937 (28, 29). Most of the carotenoids are highly lipophilic and water insoluble. But xanthophylls have a hydrophilic oxygen function, which is suitable for hydration, and have oral bioavailability by improved solubility.

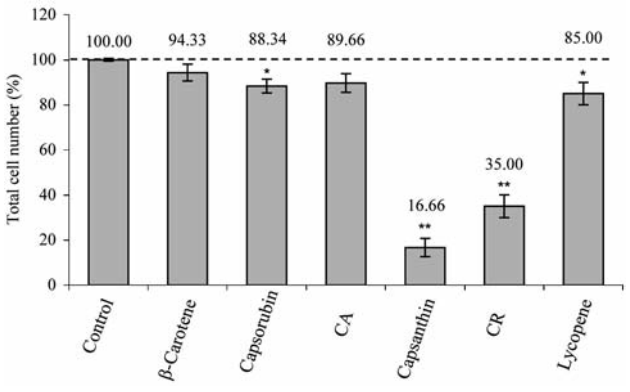

Figure 2. *Growth inhibitory effect of carotenoids on A549 cells. Compounds were applied in 20 μM final concentrations. CA: Capsanthin diester; CR: capsorubin diester. The proportion of total cells in the treated culture is presented as a percentage that of the control (broken line). Significance: *p<0.05, **p<0.01 vs. Control.*

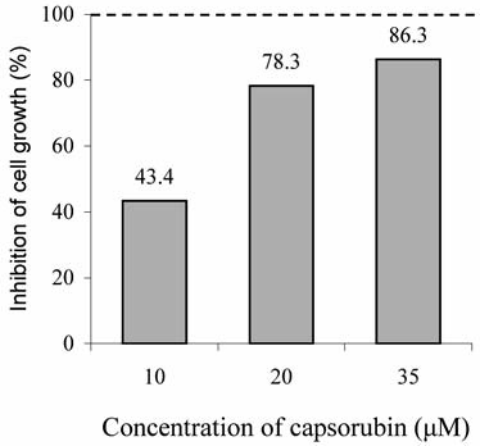

Figure 3. *Growth inhibition of A549 cells by capsorubin. Initial seeding density was 2×10^5 cell/25 cm^2 flask. The treatment with capsorubin was started 24 h after the preparation of initial seeding, and lasted for three days.*

Anti-MDR activity. Anticancer drugs and MDR modifiers can bind to specific targets in several ways. The first is the binding to ABCB1 by targeting the channel and binding to the tyrosine residue, such as occurs with verapamil; the second is to bind ATP-binding sites, as isoflavones do; the third is the destabilization of physiological active conformation through the first glycosylated transmembrane loop (30). There is another opportunity to change the active conformation by changes in the membrane structures with carotenoids chemically, by increasing the transient state of the membrane. The electronic structure of membranes, polarization, depolarization and conductance may also be modified.

The membrane potential depends on transport proteins in which ion channels and ion pumps are involved, the ions can passively diffuse without expenditure of energy. Some ion pumps are electrogenic, such as the Na^+/K^+-ATPase, they produce charge imbalance across the cell membrane. This pump uses energy to function resulting in pK equilibrium potentials of –80 mV (inside negative). The equilibrium potential of Na^+ related to Cl^- is 61 mV. The resting potential is not an equilibrium potential, but relies on constant expenditure of energy. The resting potential of ionic species has the greatest conductance across the membrane. These interventions lead to a field where thermodynamics and electrobiology meet with cancer resistance of mutatochrome, aurochrome, diepoxy-β-carotene, monoepoxy-α-carotene and luteochrome. The inhibition of free radical-induced lipid peroxidation and anticancer activities of capsorubin and structurally related carotenoids have been shown (12, 31).

The electron migration mediated by carotenoid in the presence of ascorbic acid depends on the differences of activation energy values of carotenoids and ascorbic acid in the cascade of the electron flow. Only the xanthophylls show

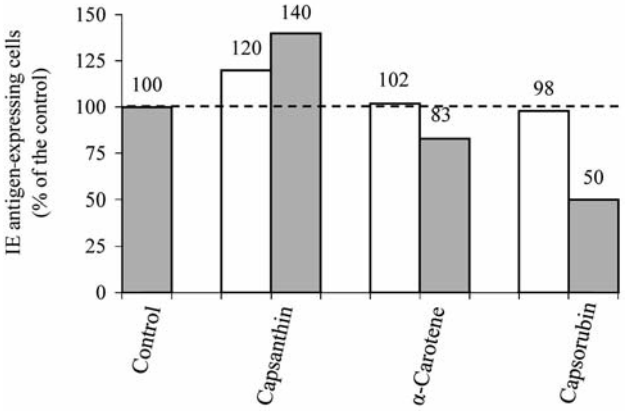

Figure 4. *The effect of selected carotenoids on CMV IE antigen expression in A549 cells. All of the compounds were tested at two final concentrations: at 1 μg/ml (white column) and 10 μg/ml (grey column). The IE antigen expression was detected by immunofluorescence using MAB810 monoclonal antibody. The proportion of IE antigen-expressing cells in the treated culture is presented as a percentage that of the control (broken line).*

synergism with ascorbic acid, which has two polar ends, one of which contains a five-membered ring with a 1,4-hydroxyl-ketone, and the other an epoxide group. Enolization of the ketone group is hindered in the direction of the ring by the neighbouring quaternery carbon atom; consequently, its radical activation is easier than ionic activation. Atomic activation can be initiated by the presence of the ascorbic acid in the case of the capsorubin–ascorbic acid interaction.

It was shown that carotenoids altered the electron density associated with membrane hydrocarbon core over a broad area from the center of the membrane that can be considered

as a supramolecular complex (32, 33). Carotenoids with (all-*E*) polyene chain make the membrane more rigid by increasing the crystalline state of the membrane compartment, while *cis* (Z)-isomers of carotenoids disorganize the lipid bilayer and increase the liquid state (flexibility) of the membrane (16, 18). Carotenoids also affect several transport processes in cell membranes (34, 35) and a large number of carotenoids bind to biological membranes similarly. As an example of general interaction between cellular membranes and carotenoids, the all-*trans* (all-*E*)-isomers enhance the crystalline phase, while *cis* (*Z*)-isomers are more flexible and can increase the liquid phase of the lipid bilayer (16, 18, 19).

The structure–activity relationships were assessed by flow cytometry and paramagnetic carotenoid radical cations and diamagnetic radical dications, and were found to be related to differences in electron transfer during isomerization (9-11, 17-19, 20, 35). Theoretical explanation of the biological importance of these plant-derived compounds was studied by flow cytometry with rhodamine 123 accumulation in MDR cancer cells.

Many herbal extracts from Chinese traditional medicine have antiproliferative effects on various tumor cells *in vitro* and some of them showed chemopreventive effects in human CMV-infected human lung cancer cells *in vitro* (36, 37).

Electronic conductance. The electronic conductance in biological systems and macromolecules is known as possible signal transmitter submolecular level in case of DNA and protein conductance. Szent-Györgyi suggested in 1941 that electronic delocalization in proteins may be responsible for their energy and charge transport properties (28). His idea at that time revolutionized science and gave birth to quantum biology. Indeed, the interactions between biopolymers strongly depend on the charge transfer between electron acceptors and donors, and the resulting charge transfer among the biopolymers themselves, as was stated by Szent-Györgyi (28, 29). Ladik described that the electron acceptors transform proteins from insulators to conductors. Based on this, the problem of interactions between charged polymers with partially filled bands was re-evaluated (38).

After examining all the calculated interactions between DNA and protein chains, one can look into the changes caused in the interactions by the binding of chemical carcinogens to DNA. Ladik proposed that if the carcinogen can cause a charge transfer reaction, this would change the position of the Fermi level of the macromolecular chains, and in this way, the interaction energy between them would change as well due to supramolecular interaction (39).

Thus, knowing the electron–photon interaction terms, one can calculate binding energy using time-dependent perturbation theory and an appropriate solution of the Boltzmann transport equation for different transport properties of electrons and holes in different biopolymers, as DNA and proteins (38). Ladik supposed that if one could find the basis of the quantum-mechanical investigations of how various carcinogens bound to DNA, it would enable the deblocking of previously masked genetic information regarding cancer (39, 40).

Gutmann and co-workers established models for charge transfer and supramolecular complexes in biological systems (32). DNA itself can conduct charges both *in vitro* and *in vivo*, the DNA duplex soaks up charge and transports it over long distances. The easiest target for oxidative damage is guanine, since one of its electrons is more weakly bound than any of those in the other three bases. The loss of electron from guanine results in an oxidized base that can easily react with water leading to formation of 8-oxo derivative that incorrectly pairs with adenine, not only with cytosine, where a cytosine should be, resulting in a mutation and leading to cancer and other diseases. If DNA were a conductor, then the oxidative damage might skip along the strands; the loss of an electron creates a vacancy or a 'hole' that migrates through a conductor as if it was a tangible object from some distance from the initial oxidative attack. Charge transfer may have another function; not only traveling electron holes exist, but free electrons do the same, which is important in DNA repair. According to Szent-Györgyi, if a charge transfer reaction is a member of a chain reaction, then an electron may be transferred by a third molecule to the donor filling its hole (41). The practical importance of this process is the effective application of antioxidants (32), which can be more easily activated to free radicals than other compounds in the membrane, having a lower redox potential. For example, quercetin process 300 mV, and ascorbic acid process 282 mV standard redoxpotential in the membrane. These values are lower than that of arachidonic acid or other unsaturated fatty acids. Accordingly, this means that charge transfer plays a vital role in DNA repair in living cells also (42).

Based upon numerous experiments, retinoids have a great importance in cancer research and perspectives in oncology regarding various steps of tumor development (36). As general effects of retinoids on cell physiology, the inhibition of cell proliferation, angiogenesis, induction of differentiation and suppression of oncogene expression were shown. These effects are mediated by interactions of retinoids with cellular membranes and with nuclear receptors (43).

When the chemical structures and diverse biological effects were compared, we suppose the explanation of the findings may be that both bipolar ends of capsorubin may form bidentate H-bridges or other supramolecular attachments with the amide bond of a protein molecule, which can serve as a carrier supramolecular complex, transporting the carotene molecule into the cell. Apparently, there are very specific bindings of specific carotenoids similarly to (11*Z*)-isomer of vitamin A aldehyde [(11*Z*)-retinal] and unique physiological functions in which supramolecular complexes of carotenoids have a key role in color vision (32). The contribution to the

color vision and other cell functions depends on retinoid binding and carotenoid isomerization (44).

The action of carotenoid isomers on cancer cells can be realized on several ways *e.g.* direct antitumor effect, apoptosis induction and chemoprevention *via* inhibition of virus-(Epstein-Barr virus, adenovirus or cytomegalovirus) induced IE tumor antigen expression in immortalized cells, called antipromotion effect (12).

Carotenoids such as caroviologens can be incorporated into phospholipid vesicles, by electron transfer occurring between an external reducing phase and an internal oxidizing phase of the zwitterions. It may be that carotenoids produce functional molecular 'wires' that affect electron conduction in supramolecular-scale systems (42).

It is known that retinal-carotenoids are activated by light and bind after *trans-cis (E/Z)*-isomerization *via* converting proton absorption by the chromophore into protein conformational changes. Retinal molecule covalently linked to a membrane-embedded apoprotein as photosensory receptor is known in visual systems. When a polypeptide folds into seven transmembrane helices and its binding alters the conformation of the protein, it activates signal transduction and facilitates photoaffinity of membrane-bound receptors, possibly resulting in formation of a supramolecular complex (32, 44, 45). It is supposed that the binding of capsorubin is similar to a membrane compartment that facilitates the particular signal for beneficial effect of capsorubin.

The explanation of the observed biological effects can be that carotenoids can integrate into cell membrane as lipophilic compounds do into the lipid bilayer. The intercalated carotenoids can be oxidized easily during membrane binding. If ascorbic acid donates one electron towards the oxidized carotenoid, the recharged capsorubin can function as a specific structure, which may account for electron flow and distort the functionally active conformation of the ABCB1 in the cell membrane resulting in reduced drug efflux.

The MDR reversal activity of carotenoids is a more general biological effect than the found particular interaction of some carotenoids and ascorbic acid, but there are differences between the various carotenoids in this particular effect. The membrane affinity of capsorubin is stronger than the other carotenoids as it was shown by selective accumulation in the nuclei. The strong membrane binding can be stabilized by H-bridge or electron transfer complex formation in the presence of ascorbic acid. The electrochemical interaction can result a strong inhibition. The changes in the activity of ABCB1 transporter protein can be related to the modified electric potential in the functionally active confirmation.

Interaction between carotenoids and ascorbic acid investigated in this study refers to complexity of the possibilities and may be promising for *in vivo* examinations in the future.

Acknowledgements

We thank to Dr. Fumio Enjo for his contribution to the research and Mrs. Anikó Vigyikánné Váradi for her technical assistance. This work was supported by the European Union and the European Regional Fund (TÁMOP-4.2.1/B-09/1/KONV-2010-0005), the Hungarian Scientific Research Fund (OTKA K 76176, OTKA K 83898), the Research Fund of the University of Pécs (PTE-ÁOK-KA) (Hungary), proBRAIN (Ministry of Agriculture, Forestry and Fisheries of Japan) and the Foundation for Cancer Research Szeged (Hungary).

References

1 Cutler RG: Antioxidants and aging. Am J Clin Nutr *53*: 373S-379S, 1991.

2 Banga I and Szent-Györgyi A: The large scale preparation of ascorbic acid from Hungarian pepper (*Capsicum annuum*). Biochem J *28*: 1625-1628, 1934.

3 Nagy B, Mucsi I, Molnár J, Varga A and Thurzó L: Chemosensitizing effect of vitamin C in combination with 5-fluorouracil *in vitro*. In Vivo *17*: 289-229, 2003.

4 Pashkov FJ, Wattumall DG and Campbel CL: Astaxanthin: a novel potential treatment for oxidative stress and inflammations in cardiovascular disease. Am J Cardiol *101*: 58D-68D, 2008.

5 Britton G, Liaaen-Jensen S and Pfandr H (eds.): Carotenoids Vol. 1A: Isolation and Analysis. Birkhäuser Verlag: Basel-Boston-Berlin, 1995.

6 Di Mascio P, Devasagayam TP, Kaiser S and Sies H: Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. Biochem Soc Trans *18*: 1054-1056, 1998.

7 Deli J and Molnár P: Paprika carotenoids: analysis, isolation, structure, elucidation. Curr Org Chem *6*: 1197-1219, 2002.

8 Molnár J, Gyémánt N, Tanaka M, Hohmann J, Bergmann-Leitner E, Molnár P, Deli J, Didiziapetris R and Ferreira MJU: Inhibition of multidrug resistance of cancer cells by natural diterpenes and carotenoids. Curr Pharm Des *12*: 287-311, 2006.

9 Focsan AL, Bowmann MK, Konovalova TA, Molnár P, Deli J, Dixon DA and Kispert LD: Pulsed EPR and DFT characterization of radicals produced by photooxidation of zeaxanthin and violaxanthin on silica-alumina. J Phys Chem B *112*: 1806-1819, 2008.

10 Lawrence J, Focsan AL, Konovalova T, Molnár P, Deli J, Bowman MK and Kispert LD: Pulsed ENDOR studies of carotenoid oxidation in Cu(II)-substituted MCM-41 molecular sieves. J Phys Chem B *112*: 5449-5457, 2008.

11 Focsan AL, Molnár P, Deli J and Kispert LD: The structure and properties of 9'-*cis* neoxanthin carotenoid radicals by EPR measurements and DFT calculations: present in LHC II? J Phys Chem B *113*: 6087-6096, 2009.

12 Maoka T, Mochida K, Kozuka M, Ito Y, Fujiwara Y, Hashimoto K, Enjo F, Ogata M, Nobukuni Y, Tokuda H and Nishino H: Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L. Cancer Lett *172*: 103-109, 2001.

13 Molnár J, Gyémánt N, Mucsi I, Molnár A, Szabó M, Körtvélyesi T, Varga A, Molnár P and Tóth G: Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. In Vivo *18*: 237-244, 2004.

14 Pusztai R, Ferreira MJU, Duarte N, Engi H and Molnár J: Macrocyclic lathyrane diterpenes as antitumor promoters. Anticancer Res *27*: 201-206, 2007.

15 Konoshima T, Takasaki M, Tokuda H, Morimoto S, Tanaka H, Kawata E, Xuan LJ, Saito H, Sugiura M, Molnár J and Shoyama Y: Crocin and crocetin derivatives inhibit skin tumor promotion in mice. Phytother Res *12*: 400-404, 1998.
16 McNulty H, Jacob RF and Mason RP: Biologic activity of carotenoids related to distinct membrane physicochemical interactions. Am J Cardiol *101*: 20-33, 2008.
17 Kispert LD, Konovalova T and Gao Y: Carotenoid radical cations and dications: EPR, optical, and electrochemical studies. Arch Biochem Biophys *430*: 49-60, 2004
18 Forster C and Pfander H: Carotenoid-phospholipid monolayers at the air/water interface. Proceedings of the Eleventh International Symposium on Carotenoids, August 18-23 1996, Leiden, the Netherlands. Abstract page 25, poster presentation.
19 Molnár P, Matus Z, Szabolcs J and Körtvélyesi T: Kinetic studies on the thermal (*Z*/*E*)-isomerisation of C40-carotenoids. J Chem Res (S) *4*: 120-121, 1997
20 Molnár P and Szabolcs J: (*Z*/*E*)-photoisomerization of C_{40}-carotenoids by iodine. J Chem Soc Perkin Trans *2*: 261-266, 1993.
21 Molnár P, Kawase M and Motohashi N: Isolation, crystallization and handling of carotenoids and the (*E*/*Z*)-isomerization of carotenoids. *In*: Functional Polyphenols and Carotenoids with Antioxidative Action. Motohashi N. (ed.). RSFLASH: Kerala, India, pp. 111-131, 2006.
22 Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC: A retrovirus carrying an *MDR1* cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Natl Acad Sci USA *85*: 4486-4490, 1988.
23 Choi K, Frommel TO, Stern RK, Perez CF, Kriegler M, Tsuruo T and Roninson IB: Multidrug resistance after retroviral transfer of the human *MDR1* gene correlates with P-glycoprotein density in the plasma membrane and is not affected by cytotoxic selection. Proc Natl Acad Sci USA *88*: 7386-7390, 1991.
24 Lieber M, Smith B, Szakal A, Nelson-Rees W and Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer *17*: 62-70, 1976.
25 Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H and Parks WP: *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst *51*: 1417-1423, 1973.
26 Candussio L, Decorti G, Crivellato E, Granzotto M, Rosati A, Giraldi T and Bartoli F: Toxicologic and pharmacokinetic study of low doses of verapamil combined with doxorubicin. Life Sci *71*: 3109-3119, 2002.
27 Kars MD, Iseri OD, Gündüz U, Ural AU, Arpaci F and Molnár J: Development of rational *in vitro* models for drug resistance in breast cancer and modulation of MDR by selected compounds. Anticancer Res *26*: 4559-4568, 2006.
28 Szent-Györgyi A: Towards a new biochemistry? Science *27*: 609-611, 1941.
29 Szent-Györgyi A: Electron biology and its relation to cancer. Life Sci *15*: 865-875, 1974.
30 Molnár J, Kars MD, Gündüz U, Engi H, Schumacher U, Van Damme EJ, Pneumans WJ, Makovitzky J, Gyémánt N and Molnár P: Interaction of tomato lectin with ABC transporter in cancer cells: glycosylation confers functional conformation of P-gp. Acta Histochem *111*: 329-333, 2009.
31 Maoka T, Goto Y, Isobe KK, Fujiwara Y, Hashimoto K, Mochida K: Antioxidative activity of capsorubin and related compounds from paprika (*Capsicum annuum*). J Oleo Sci *50*: 663-665, 2001.
32 Gutmann F, Keyzer H, Johnson C and Molnár J: Chapter 8: Charge transfer complexes of lipids, lipid systems and vitamins. *In*: Charge Transfer Complexes in Biological Systems. Marcel Dekker, Inc: New York, USA, pp. 319-382, 1997.
33 Lehn JM: Transport processes and carrier design. *In*: Supramolecular Chemistry: Concepts and Perspectives. Anton U. (ed.). Wiley-VCH: Weinheim, Germany *1*: 69-80, 1995.
34 Chaturvedi VK and Kurup CK: Effect of lutein on the transport of Ca across phospholipid bilayer and mitochondrial membrane. Biochem Int *12*: 373-377, 1986.
35 Kupisz K, Sujak A, Patyra M, Trebacz K and Gruszecki WI: Can membrane-bound carotenoid pigment zeaxanthin carry out a transmembrane proton transfer? Biochim Biophys Acta *1778*: 2334-2340, 2008.
36 Pusztai R, Motohashi N, Párkányi C, Aaron JJ, Rao BK and Molnár J: Relationhip between tumor (T) antigen expression and substituent effects on benzo[a]phenothiazines. Anticancer Res *16*: 2961-2964, 1996.
37 Engi H, Hohmann J, Gang G, Pusztai R, Rédei D, Kovács O, Schelz Zs and Molnár J: Chemoprevention and inhibition of P-glycoprotein in cancer cells by Chinese medicinal herbs. Phytother Res *22*: 1671-1676, 2008.
38 Ladik J: Energy-band structure and charge transfer in biopolymers. Int J Quant Chem *10*: 237-246, 1976.
39 Ladik J, Suhai S and Seel M: Electronic structure of biopolymers and possible mechanisms of chemical carcinogenesis. Int J Quant Chem *5*: 35-49, 1978.
40 Ladik J: Toward the electronic structure of real DNA. Int J Quant Chem *2*: 33-143, 1975.
41 Szent-Györgyi A: Charge transfer and electronic mobility. Proc Natl Acad Sci USA *58*: 2012-2014, 1967.
42 Lawton G: Live Wire. New Scientist *177*: 38-39, 2003.
43 Bollag W: Retinoids in oncology: experimental and clinical aspects. Pure Appl Chem *66*: 995-1002, 1994.
44 Yan B, Nakanishi K and Spudich JL: Mechanism of activation of sensory rhodopsin I: evidence for a steric trigger. Proc Natl Acad Sci USA *88*: 9412-9416, 1991.
45 Li H, Liu Y, Fang K and Nakanishi K: A simple photoaffinity labeling protocol. Chem Commun pp. 365-366, 1999.

Received November 8, 2011
Revised December 14, 2011
Accepted December 16, 2011