Multidrug resistance reversal effect of organosilicon, terpenoid and flavonoid derivatives on various tumor cell lines *in vitro*

Ph.D. Thesis

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JOURNAL ARTICLES RELATED TO THE THESIS

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II. Valente C, Ferreira MJU, Abreu PM, Gyémánt N, Ugocsai K, Hohmann J, Molnár J: Pubescenes, new jatrophane diterpenes with multidrug resistance reversing activity of mouse lymphoma cells from *Euphorbia pubescens. Planta Med* 70: 81-84, 2004. IF: 1.639

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IV. Molnár J, Gyémánt N, Mucsi I, Molnár AM, Szabó M, Körtvélyesi T, Varga A, Molnár P, Tóth G: Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. *In Vivo* 18: 237-244, 2004. IF: 0.811

V. Motohashi N, Wakabayashi H, Kurihara T, Fukushima H, Yamada T, Kawase M, Sohara Y, Tani S, Shirataki Y, Sakagami H, Satoh K, Nakashima H, Molnar A, Spengler G, Gyemant N, Ugocsai K, Molnar J: Biological activity of barbados cherry (acerola fruits, fruit of Malpighia emarginata DC) extracts and fractions. *Phytother Res* 18:212-23, 2004. IF: 0.975

VI. Molnar J, Mucsi I, Nacsa J, Hever A, Gyemant N, Ugocsai K, Hegyes P, Kiessig S, Gaal D, Lage H, Varga A.: New silicon compounds as resistance modifiers against multidrug resistant cancer cells. *Anticancer Res* 24:865-71, 2004. IF: 1.395

VII. Molnar P, Kawase M, Satoh K, Sohara Y, Tanaka T, Tani S, Sakagami H, Nakashima H, Motohashi N, Gyémánt N, Molnár J.:Biological activity of carotenoids in red paprika, *Valencia orange* and *Golden delicious* apple. *Phytother Res* 19,(accepted for publication), 2005. IF: 0.975

VIII. Ferreira MJ, Gyemant N, Madureira AM, Tanaka M, Koós K, Didziapetris R, Molnar J: The effects of jatrophane derivatives on the reversion of MDR1 and MRP-mediated multidrug resistance in the MDA-MB-231 (HTB-26) cell line. *Anticancer Res.* 25, (accepted for publication) 2005. IF: 1.395

JOURNAL ARTICLES NOT RELATED TO THE THESIS

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II. Gyemant N, Molnar A, Spengler G, Mandi Y, Szabo M, Molnar J: Bacterial models for tumor development. Mini-review. *Acta Microbiol Immunol Hung*.51:321-332, 2004.

III. Richter M, Gyemant N, Molnar J, Hilgeroth A: Comparative effects on intestinal absorption in situ by P-glycoprotein-modifying HIV protease inhibitors. *Pharm Res* 21: 1862-1866, 2004.

ABSTRACTS ON HUNGARIAN AND INTERNATIONAL CONGRESS

Molnár J, Molnár A, Gyémánt N, Molnár P, Tóth G: Modulation of multidrug resistance on cancer cells selected carotenoids

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Molnár J, Gyémánt N, Tanaka M, Mucsi I, Ugocsai K, Hohmann J, Ferreira MJU, Molnár P and Didiziapetris R: Reversal of multidrug resistance: modification of rhodamine accumulation by selected flavonoids and carotenoids and related compounds

Ferreira MJU, Gyémánt N, Madureira AM, and Molnár J: The effects of jatrophane derivatives on the reversion of multidrug resistance on the HTB-26 cell line.

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In vitro MDR reversal effects of plant derived compounds

Molnar J, Gyémánt N, Tanaka M, Scheltz Z, Hohmann J, Umbelino Ferreira MJ, Molnár P and Didziapetris R:Reversal of resistance of Cancer cells by inhibition of efflux pumps and QSAR studies

Ferreira MJU, Duarte N, Gyémánt N and Molnar J:In vitro evaluation of pentacyclic triterpenes as potencial modulators of multidrug resistance on mouse lymphoma cells

Molnar J, Gyémánt N, Spengler G, Tanaka M, Schelz Zs, Ugocsai K, Ferreira MJU, Molnár P and Didziapetris R: Inhibition of resistance of cancer cells by plant derived compounds

LIST OF ABBREVIATIONS

DME: Drug metabolizing enzymes

ABC: ATP-binding cassette

ATP: Adenozine-triphosphate

MDR: Multidrug resitance

P-gp: P-glycoprotein (MDR1)

MRP: Multidrug resistance related protein

BCRP: Breast cancer resistant protein

LRP: Lung resistant protein

GSH: Glutathione

GST: Glutathione-*S*-transferase

MGMT: O^6-methylguanine DNA methyltransferase

TM: Transmembrane domain

NBD: Nucleotide-binding domain

FAR: Fluorescence Activity Ratio

TPSA: Total polar surface area

LogP: Octanol/water partition coefficient

SAR: Structure activity relationship

BCECF-AM: 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester

MTT: Thiazolyl Blue Tetrazolium Bromide

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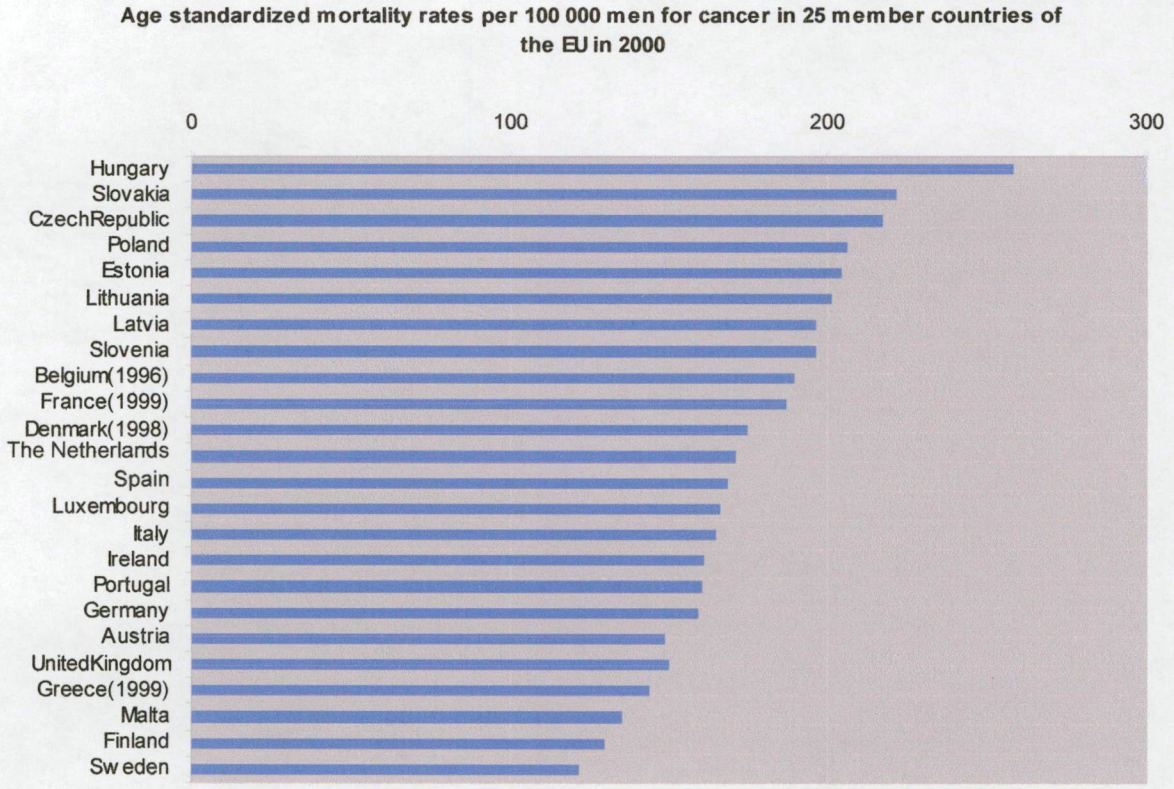
1. INTRODUCTION

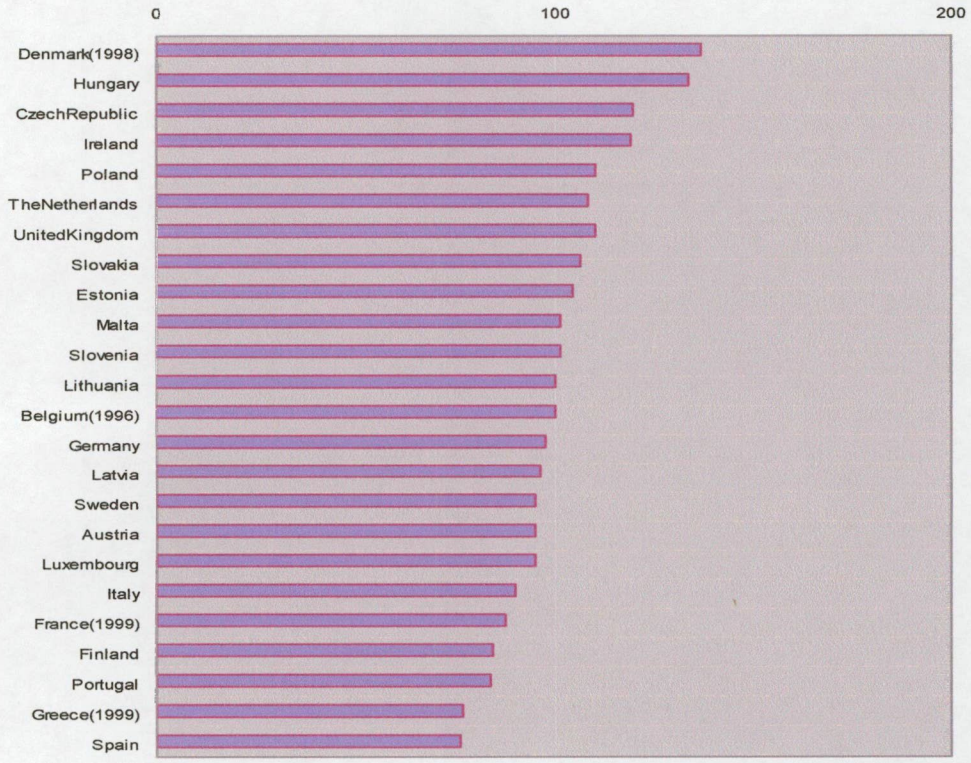
1.1. Cancer mortality in Europe and Hungary

In European countries, the second leading cause of death after cardiovascular diseases is cancer. Around in three Europeans will develop cancer during his or her lifetime. Cancer can occur at any age, but the risk of developing cancer increases with age (1). Hungary has currently the highest cancer mortality rates for all cancer sites combined among men. It has especially unfavorable rates for cancer of the lung and the intestines, as well as for leukaemias.



Among women Hungary has the second highest mortality for all cancers together (2). In Hungary mortality rates for 100 000 women are the highest for mouth and pharynx, larynx and intestine. Breast cancer was 22.60 per 100 000 women in 2000 (2). Breast cancer had a tendency to decline moderately over the last decade; however it has remained the most frequent cause of death among cancers (2).

Age standardized mortality rates per 100 000 women for cancer in 25 member countries of the EU in 2000



The inclusion of data from accession countries has led to some increases in average cancer mortality and to some (quantitatively) less favorable trends, because the rates in Central and Eastern European countries that entered the EU in 2004 were higher than in the existing EU member countries (3,4) . Furthermore, the difference in cancer mortality rates across various EU countries has become substantial, calling for urgent action toward cancer control in most of the former accession countries from Central and Eastern Europe. These actions include control of both tobacco and alcohol consumption; wider availability of favorable components of diet, such as vegetables, fiber, and fruit; and wider and more uniform adoption of earlier diagnosis and treatment procedures (5,6).

Considering the currently available cancer treatment antineoplastic drugs are usually effective for the treatment of various tumors, they may prove to be relatively ineffective in the treatment of some primary and recurrent neoplasias. The identification of factors that might effectively predict the response of patients to treatment is a constant challenge in oncology.

1.2. Drug resistance mechanisms

Overcoming of drug resistance is very complex due to host toxicity, and **tumor characteristics** including kinetic resistance, heterogeneity of cell subpopulations, hypoxia, mutation and gene amplification. **Kinetic resistance** is defined as dependence of kinetics of cell population and cell cycle

state of tumor cells *e.g.* tumor is in a slow growth phase or plateau phase with small growth fraction, timing of drug administration, when tumor starts (7).

Biochemical resistance is when tumor inactivates or extrudes drugs by different mechanisms. **Drug-metabolizing enzymes (DME)** play a key role in the activation and deactivation of drugs, including a number of cytotoxics, and can therefore influence the susceptibility of **organs and tissues** to their therapeutic and toxic effects (8-10).

Displaying both genetic polymorphism and inducibility in some cases DME are subjected to significant inter- and intraindividual variability with several genes, including those of the cytochrome P450 (11), glutathione-*S*-transferase (GST) (12, 13), uridine diphospho-glucuronosyltransferase (14), superfamilies, thiopurine methyltransferase and dihydropyrimidine dehydrogenase (11). An understanding of the differential expression and activity of DME within tumoral and nontumoral tissues may provide opportunities for modulation and hence improved therapeutic outcome.

Cell resistance to drugs is a determinant of the response to chemotherapy and radiotherapy and its detection has clinical relevance (15). Treatment failure can be explained partly by pharmacokinetic mechanisms that reduce the time length or exposure to drug of tumor cell, also known as **pharmacokinetic resitance** (16). Pharmacological resistance is based on poor adsorption, increased excretion, catabolism and drug interaction.

1.3. Classification of intracellular MDR mechanisms

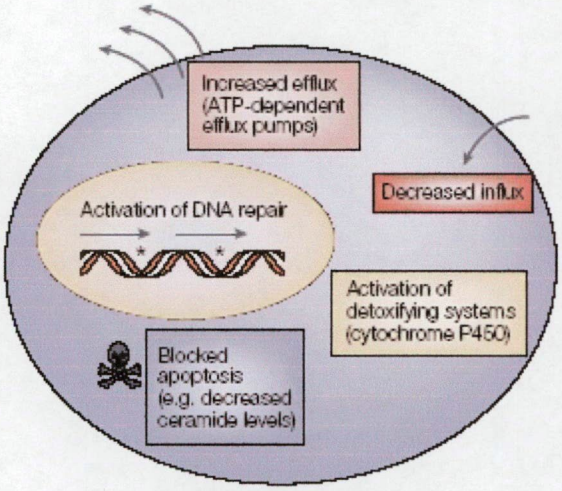


Fig. 1 (ref: 17)

1. **Alteration in drug target:** This mechanism of MDR is best characterized by alteration in the expression and function of DNA topoisomerase (18, 19). Topo II family members are targets for several classes of chemotherapeutic drugs, including anthracyclines (20).

2. **Increasing in DNA repair mechanisms**: The DNA repair enzyme MGMT (O^6-methylguanine DNA methyltransferase) catalyzes the removal of methyl adducts of the O^6 of guanine resulting from treatment with nitrosoureas (21). Levels of MGMT enzymatic activity have been shown to correlate with sensitivity to nitrosourea mediated cell death (22), suggesting that MGMT should be an important effector of drug resistance.

3. **Defective apoptotic pathways**: These pathways may occur as a result of malignant transformation; in cancers with mutant or non-functional p53 (23). The Bcl-2 family of proteins has been demonstrated to play a major role in the regulation of programmed cell death (24) and has recently been shown to correlate with MDR (25, 26, 27).

4. **Reduced intracellular accumulation** has historically been associated with overexpression of the ABC (ATP-binding cassette) transporter, P-gp/MDR-1 (28) and related drug transporters, including MRP protein 1 (29) and breast cancer resistance protein (30). This family of proteins is characterized by reduced drug accumulation at the target involving the transport of natural product agents, like vincristine, doxorubicin and Taxol. (31). Additionally, the major vault protein, lung resistance protein, has also been associated with drug resistance via a redistribution of doxorubicin from the nucleus to cytoplasm without overall changes in total cellular drug accumulation (32).

1.4. ABC transporters

Over 200 proteins involved in the transport of substrates across biological membranes are members of the ABC (ATP-binding cassette) superfamily of proteins, also known as the traffic ATPases (33, 34).

A typical ABC transporter protein (Fig. 2) consists of four units, two membrane-bound domains (TM) with six transmembrane segments at each side and two nucleotid-binding domains (NBD), which bind and hydrolyze ATP.

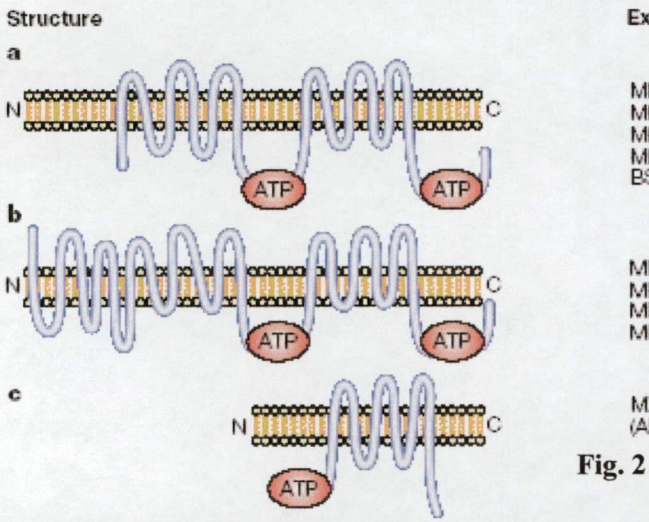


Fig. 2 (ref: 17)

Two sequence motifs located 100–200 amino acids apart in each NBD, designated Walker A and Walker B, are conserved among all ABC transporter superfamily members, as well as numerous other ATP-binding proteins (35). Furthermore it is unique to ABC proteins, that a third, highly conserved amino acid sequence (ALSGGQ) located between the Walker A and B motifs, having been referred to as the ABC signature motif (or C motif). The precise function of this sequence has not yet been determined although it has been directly implicated in the recognition, binding, and hydrolysis of ATP (34).

The most common member of ABC transporter is the 170 kDa **multidrug resitance protein 1 (MDR1) also called P-glycoprotein (P-gp)** (encoded by *ABCB1*) (36). The isolation of a second distantly related ABC protein, the 190-kDa **multidrug resistance related protein 1 (MRP1)** (encoded by ABCC1) facilitated the discovery of eight more genes within the same ABC subfamily, at least six of which are potentially involved in mediating drug resistance MRP2 (encoded by ABCC2), MRP3 (encoded by ABCC3), MRP4 (encoded by ABCC4), MRP5 (encoded by ABCC5), MRP6 (ABCC6) and MRP7 (encoded by ABCC10) (29, 37, 39, 40). Two additional members, MRP8 (ABCC11) and MRP9 (ABCC12) have been reported recently (41, 42).

The third drug transporter, also distantly related to P-glycoprotein and the MRPs, is the **breast cancer resistance protein (BCRP)** (encoded by ABCG2) that was originally isolated from a multidrug-resistant breast cancer cell line co-selected in doxorubicin and verapamil (43, 44).

Although the **lung resistance protein (LRP)** is not an ABC transporter, it is frequently included in discussions of drug resistance, as it is expressed at high levels in drug-resistant cell lines and in some tumor (32,45). LRP is a major vault protein found in the cytoplasm and on the nuclear membrane. Vaults are large ribonucleoprotein particles that present in all eukaryotic cells. They might also confer drug resistance by redistributing drugs away from intracellular targets.

In addition to their role in drug resistance, there is substantial evidence that these efflux pumps have overlapping functions in tissue defense. Collectively, these proteins are capable of transporting a vast and chemically diverse array of toxicants including bulky lipophilic cationic, anionic, and neutrally charged drugs and toxins. Conjugated organic anions that encompass dietary and environmental carcinogens, pesticides, metals, metalloids, and lipid peroxidation products are also transported this way. P-glycoprotein, MRP1, MRP2, and BCRP/ABCG2 are expressed in tissues important for absorption (*e.g.*, lung and gut) metabolism and elimination (liver and kidney) (34). In addition, these transporters have an important role in maintaining the barrier function of sanctuary site tissues (*e.g.*, blood–brain barrier, blood–cerebral spinal fluid barrier, blood–testis barrier and the maternal–fetal barrier or placenta). Thus, these ABC transporters are increasingly recognized for their ability to modulate the absorption, distribution, metabolism, excretion, and toxicity of xenobiotics (34).

The **clinical relevance** of these proteins in multidrug resistance has not yet established except for expression of MRP1 and P-glycoprotein which has been reported in a variety of hematological and solid tumors, suggesting a significant role for these transport proteins in clinical drug resistance (46). Studies show that P-gp expression is widespread in clinical cancer. In some tumor types the expression at low levels can be demonstrated to increase by following the development of resistance. In several studies P-gp expression is showed to confer a poor prognosis at the time of diagnosis. Nevertheless the progress of reversal agents towards definitive clinical studies has been enhanced by the development of surrogate assay which confirm that P-gp antagonism can occur in patients (47-50).

It has been found that MRP messenger RNA (mRNA) is expressed in most cell and tumor types (51). Levels of expression in tumors are frequently low. In non-small-cell lung cancer, high levels of MRP protein expression are observed in approximately one-third of cases, and in lung cancer cell lines, expression of MRP appears to correlate with resistance to MRP substrates including doxorubicin, vincristine and VP-16 (52-54). In primary breast cancer, significant MRP protein expression has been reported in 25–30% of samples by immunohistochemistry, and in a series of 259 patients, appeared to confer an increased risk of treatment failure (55-57).However the role of MRP in clinical drug resistance remains poorly defined. The question in a clinical setting requires identification of an MRP-specific inhibitor. Nevertheless, redundancy with the other MRPs may not allow direct determination of this question.

1.5. Substrate specifity of MDR and MRP transporters

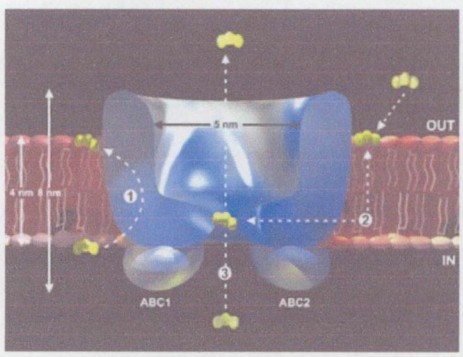
Hundreds of compounds have been identified as ''substrates'' for the P-gp, usually by indirect means. MDR spectrum compounds include a large number of anticancer drugs (anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxanes), as well as other cytotoxic agents, linear and cyclic peptides, steroids, fluorescent dyes, and the γ-emitting radiopharmaceutical 99mTc-SESTAMIBI (48, 49).

A ''typical'' compound in the **MDR substrate spectrum** (Table 1.) is large (M*r* > 400), hydrophobic, amphipathic, with a planar ring system, and often carries a positive charge at physiological pH (33, 51). However, not all putative P-gp substrates fall into this category; many are uncharged at physiological pH (*e.g.*, colchicine), and several uncharged cyclic and linear hydrophobic peptides and ionophores have recently been described as P-gp substrate (58-62).

Table 1. P-glycoprotein substrates Ref.:(63)

Anthracyclines	**Vinca alkaloids**	**Epipodophyllotoxins**
Daunorubicin	Vinblastine	VP-16 (Etoposide)
Doxorubicin	Vincristine	VM-26 (Teniposide)
Epirubicin	Vinorelbine	
	Vindesine	**Tubulin polymerizing drugs**
Anthracenes		Colchicine
Bisantrene	**Camptothecin derivates**	Paclitaxel
Mitoxantrone	CPT-11	Docetaxel
Fluorophores	**HIV-1 protease inhibitors**	**Chromopeptide antibiotic**
Calcein-AM	Ritonavir	Actinomycin D
Fluo-3 AM	Saquinavir	
Fura-2 AM	Indinavir	
Hoechst 33342/33258		
Rhodamine 123		

There are evidences that the transporter interacts directly with non-polar substrates within the membrane environment. Drugs with a high degree of polarity or high charge may gain access to the P-gp from the aqueous phase. It is possible to act as a **drug-flippase,** moving drugs from the inner to the outer leaflet of the bilayer (33, 64). This transporter could be very sensitive for changing of membrane structure, membrane fluidity. Alterations in membrane fluidity were able to reverse MDR and several chemosensitisers were observed to alter membrane, even if they are not substrate for MDR, such as Tween 80 (65).



Unlike P-gp that mostly targets and transport hydrophobic drugs, **MRP proteins can transport** hydrophilic molecules even organic anions. They also transport neutral drugs conjugated with

glutathione (GSH) glucuronid, or sulfate (66) and anticancer agents, which are not metabolized into glutathione conjugates by co-transport with free (GSH) (Fig.3)(67, 68).

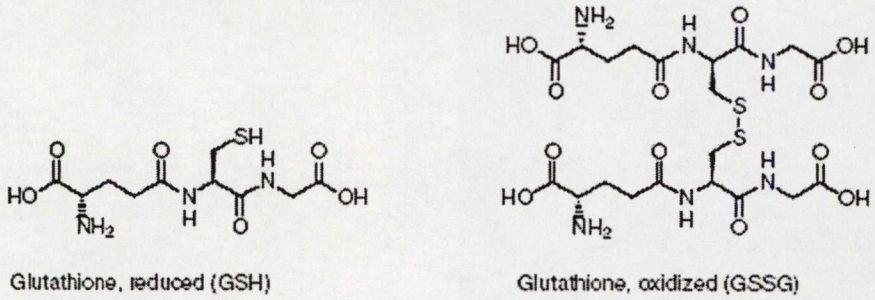


Fig.3.: Structures of reduced (GSH) and oxidized (GSSG) glutathione

GSH conjugation can occur either spontaneously or through catalysis by a glutathione S-transferase, and may contribute to the MDR phenotype. Indeed a significant increase has been demonstrated in glutathione concentration and in glutathione transferase activity of adriamycin-resistant cells (69, 70). However, it is important that the mechanism of GSH-conjugates formation and transport is far from being fully understood.

It is well accepted that substrates for MRP include doxorubicin, vincristine and etoposide (VP-16). MRP overexpression has emerged in cell lines exposed to these compounds, and developing non-P-gp-mediated drug resistance. However, mice in which MRP has been genetically deleted display only an increased sensitivity to etoposide (71). This finding is perhaps easily explained by the overlap in substrate specificity between MRP and P-gp (Fig 4) and VP-16 being a better substrate for MRP than for P-gp.

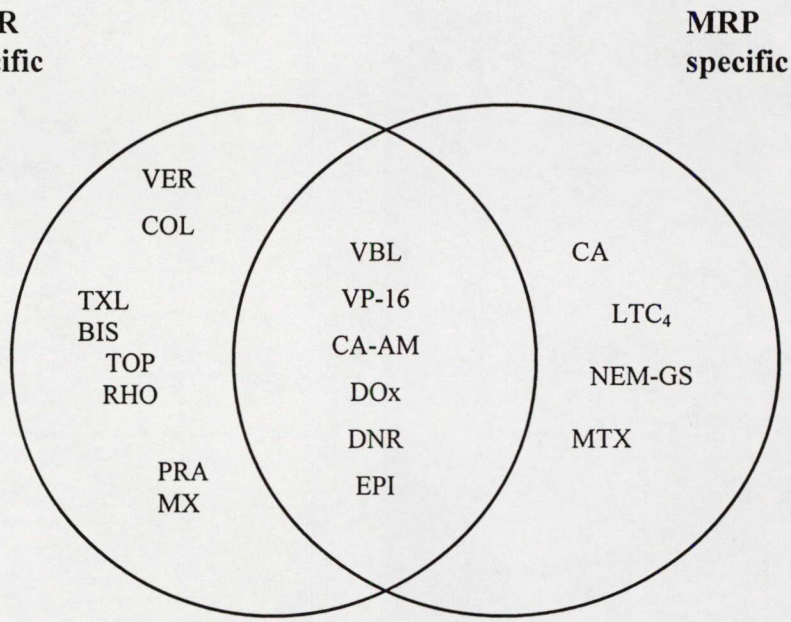
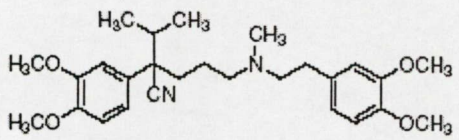


Fig 4. Venn diagram is showing the substrate overlaps between MDR and MRP. Abbreviations: BIS, bisantrene; CA, calcein;CA-AM, calcein-AM ester; COL, colhcicine; DNR, daunorubicin; DOX, doxorubicin; EPI, epirubicin; LTC4, leukotriene C4; MTX, methotrexate; MX,

mitoxantrone; NEMGS,*N*-ethyl maleimide glutathione; PRA, prazosin; RHO, rhodamine 123; TXL, taxol; TOP, topotecan; VBL, vinblastine; VER, verapamil; VP-16, etoposide. (Ref:63)

1.6. MDR modulators

The **first generation** of P-gp modulators, including verapamil, cyclosporin (cyclosporin A), tamoxifen, and several calmodulin antagonists were identified in the 1980s (72,73).



Verapamil

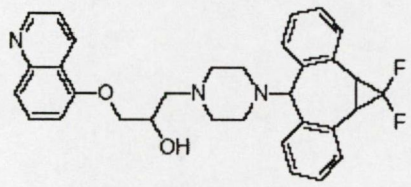
They were classified as substrates for P-gp and thus worked by competing with the cytotoxic compounds for efflux by the P-gp pump; therefore, high serum concentrations of the chemosensitizers were necessary to produce adequate intracellular concentrations of the cytotoxic drug (37). In vivo use of high doses from them results in unacceptable toxicity. Moreover unpredictable pharmacokinetic interaction was detected in the presence of chemoterapy agents (72) *e.g.* increased myelosupression, hyperbilirubinemia and altered disposition of the cytototoxins were observed using cyclosporine A therapy to modulate P-gp mediated multidrug resistance (74).

The **second generation P-gp** modulators include dexverapamil, dexniguldipine, valspodar (PSC 833), and biricodar (VX-710). These agents are more potent than their predecessors and also less toxic (73). Second-generation P-gp inhibitors have a better pharmacologic profile than the first generation compounds, but they also retain some characteristics that limit their clinical usefulness. These compounds significantly inhibit the metabolism and excretion of cytotoxic agents, thus leading to unacceptable toxicity that has required dose reductions in clinical trials (72). In fact, many of the cytotoxic agents that are substrates for P-gp are also substrates for the cytochrome P450 isoenzyme 3A4. The competition between cytotoxic agents and these P-gp modulators for cytochrome P450 3A4 activity has resulted in unpredictable pharmacokinetic interactions (75). Many of the early-generation P-gp modulators inhibited several other ABC transporters as well as the P-gp transporter. For example, valspodar and biricodar are not specific solely to P-gp; both of these agents affect MRP1 (76, 77). It is possible that this inhibition of non-target transporters may lead to greater adverse effects of anticancer drugs, including neutropenia and other myelotoxic effects.

The stereoselectivity of enantiomers of methotrimeprazine, clopenthixol and butaclamol was tested previously by our group. We hypothesize that the CNS inactive member of stereoisomer pairs can be used as a resistance modifier without any risk in patients suffering from drug resistant cancer (78). Moreover the substrate sensitivity of P-gp depends on its location and surroundings. For example, the tomato lectin sensitivity of P-gp was different in human mdr1 gene transfected mouse lymphoma and human brain capillary endothelial cells (79).

The **third-generation** modulators that specifically and potentially inhibit P-gp function have been developed by using structure-activity relationships (SAR) and combinatorial chemistry to overcome the limitations of the second generation P-gp modulators (73). They have high specifity for P-gp and

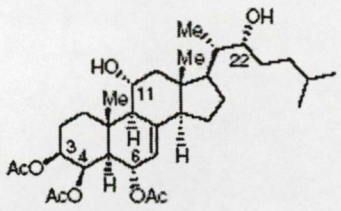
not for other ABC transporters. This phenomenon minimizes the possibility of the blockade of more than one pump might result in altered bioavailability or excretion of the chemotherapy agents (72).



LY-335979

The third generation P-gp inhibitors currently in clinical development include the anthranilamide derivative tariquidar (XR9576),(80), the cyclopropyl-dibenzosuberane zosuquidar (LY335979), (81, 82) laniquidar (R101933),(83) and the substituted diarylimidazole ONT- 093 (84).

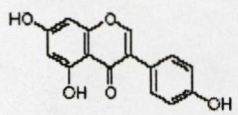
1.7. MRP modulators



agosterol A

Agosterol A is a natural polyhydroxylated sterol which was isolated in 1998 from a marine sponge and for which an MRP1-mediated MDR reversing activity was reported (85). The total synthesis of agosterol A as well as of several analogs has been described, and few of them were found to be powerful modulators (86, 87).

Flavonoids have been largely explored as modulators of P-gp-mediated MDR (88). In 1993, Versantvoort et al.(89, 90) have reported that genistein (4 0,5,7-trihydroxyisoflavone) can inhibit the efflux of daunorubicin in MRP1-overexpressing small cells from human lung cancer. In the last few years, a growing number of reports have been published concerning to the use of dietary flavonoids as MRP1 inhibitors.



Genistein

The **nonsteroidal estrogen receptor mixed agonist/antagonist**, raloxifene, which acts as a nonsteroidal estrogen receptor agonist/antagonist, was used by Eli Lilly as a pharmacophore model to conceive selective modulators of MRP1-mediated MDR. This approach led to the discovery of a series of raloxifene analogs exhibiting both in vitro and in vivo activities (91, 92).

In an independent and unrelated study, Benyahia et al. reported the ability of the antiinflammatory drug, **indomethacin** to chemosensitize MRP1-overexpressing cells to vincristine and etoposide (93). However, micromolar concentration range (5–50 μM) was required to achieve effective results.

Compounds with dual MRP1/P-gp inhibitor property were also reported. In 2002, Degenhardt and Eickhofft have reported the use of **N,N-disubstituted piperazines** for inhibition of MDR. These compounds inhibit both P-gp and MRP1 as shown by accumulation of fluorescent substrates and sensitization assays (92, 94). Wang et al. reported the synthesis and SAR studies of **quinazolinones** as dual inhibitors of P-gp and MRP1. By using quinazolinone 1 as the core structure, SAR was examined at the N-3 and C-2 positions. Drug accumulation and potentiation assays are revealed potent dual inhibitory activities against both P-gp and MRP1 (95).

Despite the fact that large numbers of chemicals were synthesized and evaluated, there is no resistance modifier to-date in the medical practice. Edible plants, vegetables and well defined compounds are therefore considered to play a role in improvement the efficacy of treatment of cancer due to the modification of sensitivity of cancer cells to chemotherapeutics and chemoprevention (96). Natural compounds of edible plants and vegetables are expected to be incomparably less toxic on normal cells, tissues of the host than synthetic compounds. Consequently, jatrophane diterpenes carotenoids and flavonoids are promising to study their MDR reversing effect on various tumor cell lines with multidrug resitance.

2. AIMS OF THE STUDY

The aim of our study was to examine new perspectives of chemotherapy in connection with modification of multidrug resistance of cancer. Our purpose was to find a less toxic multidrug resistant reversal agent than verapamil among synthethic organosilicons and natural plant derivative carotenoids, flavonoids, tri- and diterpenoid compounds.

Before starting the multidrug resistance studies the presence of various efflux pumps were studied by using monoclonal antibodies in immuncytochemistry on following cell lines: mouse lymphoma cells transfected by human *mdr1* gene as a model cell line for MDR1 reversal studies obtained from FDA; drugresistant, doxorubicine selected subline of breast cancer cell line MCF7 (KCR); MDA-MB-231 (ATCC:HTB-26) breast cancer cells with MRP resistance and human laryngeal carcinoma Hep2 cell.

In the next steps:

- The antiproliferative effect of above mentioned compounds were studied by MTT test.
- Modification of intracellular drug accumulation was evaluated by flowcytometry
- Interactions of resistance modifiers and anticancer drug modeling combination therapy was analysed in checkerboard experiments.
- Correlation between reversal of drug resistance and chemichal structure were studied by computer program of Pharma Algorythm.

Main goals of the study in details:

1. Examined cell lines were characterized to multidrug resistant properties by immuncytochemistry
2. Antiproliferative effects of potential resistant modifiers were studied
3. MDR1 reversal effects of organosilicon, terpenoid (jatrophane diterpens and carotenoids) and flavonoid compounds was systematically studied by human mdr1 gene transfected mouse lymphoma cell line
4. Reversal of MDR1 was also studied on drug resistant human breast cancer cell line KCR by carotenoid compounds
5. Reversal of MRP was investigated on human breast cancer cell line MDA-MB-231 (HTB-26) by terpenoid (jatrophane diterpens and carotenoids) and flavonoid compounds.
6. Reversal of multidrug resistance by organosilicon compounds on human larynx cancer Hep2C cell line
7. Combinations of possible resistance modifiers and anticancer drugs in vitro were examined by checkerboard microplate method.
8. Comparison of the structure – activity relationship by physico-chemical parameters of carotenoids was analyzed by Computer model ADME/Boxes (computer program Adsorption, Distribution, Metabolism, Excretion).
9. Apoptosis induction as additional contributing factor of induced cancer cell death.

3. MATERIALS AND METHODS

3.1. Chemicals

Synthetic compounds Compounds alis-409 (1,3-dimethyl-1,3-p-fluorophenyl-1,3(3-morfolinopropyl)-1,3 disiloxan dihydrochlorid) and alis-421 (1,3-dimethyl-1,3-(4-fluorophenyl)-1,3-[3(4-buthyl)-(1piperazinyl)-propyl]-1,3-disiloxan-tetrahydrochlorid). The compounds were originally synthesized by Hegyes *et. al.* (101). The silicion substituted alis compounds were dissolved in dimethylsulfoxide (DMSO) by preparing stock solutions at a concentration 1.0 mg/ml.

Natural compounds:

Ten macrocyclic diterpenes of the jatrophane type and rearranged polycyclic derivatives were involved in the study as follows:

(1) pubescene A (3β,9α,15β-triacetoxy-7β-benzoyloxy-14-oxojatropha-5*E*,12*E*-diene); **(2)** pubescene B (3β,9α,15β-triacetoxy-7β-butanoyloxy-14-oxojatropha-5*E*,12*E*-diene); **(3)** pubescene C (3β,9α-diacetoxy-7β-benzoyloxy-15β-hydroxy-14-oxojatropha-5*E*,12*E*-diene); **(4)** pubescene D (3β,9α-diacetoxy-7β-benzoyloxy-15β-hydroxy-14-oxo-2βH-jatropha-5*E*,12*E*-diene); **(5)** 1b,5a,14a,17a-tetraacetoxy-3b-benzoyloxy-15b-hydroxy-9-oxo-paraliane; **(6)** portlandicine (2a,5a,14a,17a-tetraacetoxy-3b-benzoyloxy-15b-hydroxy-9-oxo-paraliane; **(7)** segetalol-1,5,14-triacetate-3-benzoate; **(8)** euphopubescenol [5α,8α,15β-triacetoxy-3α-benzoyloxy-4α-hydroxy-9,14-dioxo-13βH-jatropha-6(17),11*E*-diene]; **(9)** euphopubescene (3β,7β,8β,9α,14α,15β-hexaacetoxy-2βH-jatropha-5*E*,11*E*-diene); **(10)** pubescenol [5α, 8β,15β-triacetoxy-3β-benzoyloxy- 4α-hydroxy-9,14-dioxo-13βH-jatropha-6(17),11*E*-diene].

Compounds **1-4** and **8-10** were isolated from the methanol extract of *Euphorbia pubescens.* Compounds **7** and **8** and compound **9** were isolated from the acetone extracts of *Euphorbia segetalis* L. and *Euphorbia portlandica* respectively. Their structures were characterized by high-field NMR spectroscopic methods (IR, ^{1}H-NMR, ^{13}C-NMR and MS) including 2D NMR techniques (COSY, HMQC, HMBC and NOESY). The purity of the compounds was more than 95 % by HPLC analysis. All compounds were dissolved in DMSO.

Carotenoids: Lycopene, capsanthin, capsorubin, antheraxanthin, violaxanthin, α-carotene, β-carotene, α-cryptoxanthin, β-cryptoxanthin, zeaxanthin, lutein, neoxanthin, violeoxanthin, apple peel phytoxanthins and lycophill were isolated and identified earlier by our group (97-100), and were taken from our store. In case of the newly isolated and identified carotenoids mutatochrome, aurochrome, flavoxantin, chrisanthemaxanthin, monoepoxy-β-carotene, diepoxy-β-carotene, 15,15`-dehydro-diepoxy-β-carotene, monoepoxy-α-carotene, capsochrome "upper" epimer, from natural (anti)-capsanthin-5,6-epoxide, capsochrome "lower" epimer, from natural (anti)-capsanthin-5,6-epoxide, capsochrome "upper" epimer, from semisynthetic (syn) capsanthin-5,6-epoxid, capsochrome "lower" epimer from semisynthetic (syn) capsanthin-5,6-epoxid, lutechrome, (8'R)-luteoxanthin, (8'S)-luteoxanthin, (13Z)-lutein (13'Z)-lutein, (13Z)-zeaxanthin, (9Z)-violaxanthin and (9'Z)-neoxanthin. Stock solutions were prepared in DMSO.

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

3.2. Cell cultures

3.2.1. The L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described (102). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. L5178 (parent) mouse T-cell lymphoma cells and the human MDR1-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum L-glutamine and antibiotics.

3.2.2. The drug-resistant subline of breast cancer MCF7 cells and their parental cell line were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics. In the case of the drug-resistant cell line, 1 μM doxorubicin was added to the medium so as to maintain the P-gp expression. This cell line was a kind present from Dr. Zoltan Kiss (CanCure, Michigan).

3.2.3. The breast cancer MDA-MB-231 (ATCC: HTB-26) cells were grown in Leibovitz's L-15 medium with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics. The adherent human cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min.

3.2.4. The Hep2 (ATCC: CCL-23) cell line was originally thought to be derived from an epidermoid carcinoma of larynx, but based on isoenzyme analysis, HeLa cell contamination was found. Hep2 cells were desribed as radio- and chemoresistant cell line by Nagy *et al* (103).These cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics.

The cell lines were incubated in a humified atmosphere (5% CO_2, 95% air) at 37 °C. The MDA-MB-231 cell line does not require CO_2.

3.3. Immunocytochemistry

Cells were harvested and resuspended in serum-free media; the density of the cell suspension was 5×10^5/ml. One hundred μl was cytocentrifugated for 5 minutes on 1000 rpm. The samples were fixed in 4°C acetone for 10 minutes and were washed in TBS buffer (pH 7, 6) for 5 minutes. Endogenous peroxidases were quenched in 0.3% H_2O_2 for 15 minutes. The samples were incubated with primary antibodies (MRP: MRPm6, monoclonal (ICN), P-gp: NCL-PGLYm, monoclonal (Novocastra) in suitable dilutions (1:5, 1:10, 1:20) for one hour at room temperature.

The samples were washed in TBS buffer for 3×5 minutes and were incubated with the secondary antibody (DAKO EnVision™ System; CA, USA) for 30 minutes. The samples were washed in TBS for 3×5 minutes. Diaminobenzidin (DAKO, CA, USA) was used as the chromogen. Sections were counterstained with haematoxylin and mounted.

3.4. Assay for antiproliferative and cytotoxic effect

The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μL medium. Then, 1×10^4 cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h. (In case of cytotoxicity test the cell number were 3×10^4 and the incubatuion time was 24 h.) At the end of the incubation period, 20 μL of MTT (thiazolyl blue, 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 μL of Sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO, USA) solution (10%) was measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100-\left[\frac{OD\,sample-OD\,medium\,control}{OD\,cell\,control-OD\,medium\,control}\right]\times100$$

3.5. Assay for reversal of MDR1 in mouse lymphoma cells

The L5178 MDR and L5178Y parent cell lines were grown in McCoy's 5A medium containing 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of 2×10^6/mL, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volumes (2.0-20.0 μL) of the 1.0-10.0 mg/mL stock solutions and the samples were incubated for 10 min. at room temperature. Next, 10 μL (5.2 μM final concentration) of the indicator rhodamine 123 (Sigma, St Louis, MO, USA) was added to the samples and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Beckton Dickinson FACScan flow cytometer. Verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary) was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. An activity ratio R was calculated *via* the following equation (102, 104) on the basis of the measured fluorescence values:

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

3.6. Assay for reversal of MDR1 drug-resistant subline of breast cancer MCF7 cells

See at assay for reversal of MDR in mouse lymphoma cells.

3.7. Reversal of MRP in the MDA-MB-231 (HTB-26) cell line

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

3.8. Checkerboard microplate method as a model for combination therapy

It was applied to study the effects of drug interactions between resistance modifiers and cytotoxic compound on cancer cells.

The effects of the anticancer drug epirubicin and the resistance modifiers in combination were studied on various cancer cell lines. The dilutions of epirubicin (A) were made in a horizontal direction, meanwhile the dilutions of resistance modifiers (B) vertically in the microtiter plate in 100μL volume. The cell suspension in the tissue culture medium was distributed into each well in 100 μL containing 5×10^4 cells. The plates were incubated for 72 h at 37 °C in a CO_2 incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a Dynatech MRX vertical beam ELISA reader. Drug interactions were evaluated according to the following system:

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

ID=inhibitory dose
FIC=fractional inhibitory concentration
FIX=fractional inhibitory index

$FIX = FIC_A + FIC_B$

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

3.9. Assay of induction of apoptosis

The cells were adjusted to a density of $2x10^5$/mL and were distributed in 1.0 mL aliquots into microcentrifuge tubes. The apoptosis inducer 12H-Benzo[a]phenothiazine was added to the samples as a positive control at a final concentration of 50 μg/mL. In the case of control cultures, 10 μL DMSO was added. The cells were incubated at 37 °C for 45 min. The samples were than centrifuged and washed with PBS, and the cells were resuspended in 1 mL culture medium. The drugs used for treatment were added to the samples at a final concentration of 10 μg/mL. After incubation for 24 h at 37 °C, the cells were transferred from a 24-well plate into Eppendorf centrifuge tubes, centrifuged and resuspended in 1.0 mL binding buffer. The samples were mixed and centrifuged and supernatant was removed from each tube. Annexin-V-FITC (3 μL/mL samples) was added to the 200 μL samples remaining in the tubes. Controls without Annexin-V were also prepared. The samples and controls were incubated at room temperature for 30 min in the dark. Before the measurement of fluorescence activity, 10 μL propidium iodide (from a 20 μg/mL stock solution) was added to the samples and the apoptosis of the cells was then investigated. In some cases we had no complete apoptosis, however, the positivity only with Annexin V was found, but neither with double staining nor with propidium iodide. This first membrane alteration due to phosphatidylserin translocation was called as early apoptosis.

3.10. The prediction of P-glycoprotein substrate specificity for clinical studies

For tested compounds values of Hydrogen donors, Hydrogen acceptors, Abraham's alpha, Abraham's beta, Total Polar Surface Area (TPSA), logP and McGowan volume were calculated by ADME/Boxes (computer program) available from Pharma Algorithms Inc., 2004 Canada; www-ap-algorithms.com (105). Fisher's Exact Test and linear regression were used for analizing the correlations between FAR values, physico-chemical parameters of compounds and inhibitor probability.

4. RESULTS

4.1 Immuncytochemistry of cell lines

The presence of MRP on MDA-MB 231 was detected by immunocytochemistry with MRPm6, monoclonal antibody (Fig 5). Antibody MRPm6 reacts with an internal epitope of MRP. It does not cross react with the human MDR1 and MDR3 gene product. High level of P-gp expression was also detected on the membrane of L5178Y human *mdr1* transfected mouse lymphoma cells by NCL-PGLYm mouse monoclonal antibody which reacts with the human P-glycoprotein G-terminal cytoplasmic domain (Fig 6). The presence of P-gp was detected on the membrane surface of the drug-resistant subline of MCF7 by immunocytochemistry with NCL-PGLYm mouse monoclonal antibody which usually reacts with the human P-gp G-terminal cytoplasmic domain (Fig 7). High expression of both MDR1 and MRP proteins were detected on cell surface of human larynx carcinoma Hep2C cell line by the above mentioned antibodies (Fig 8).

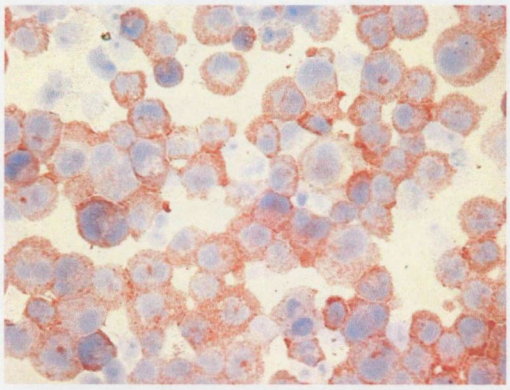


Figure 5. MRP staining of MDA-MB-231 breast cancer cell line

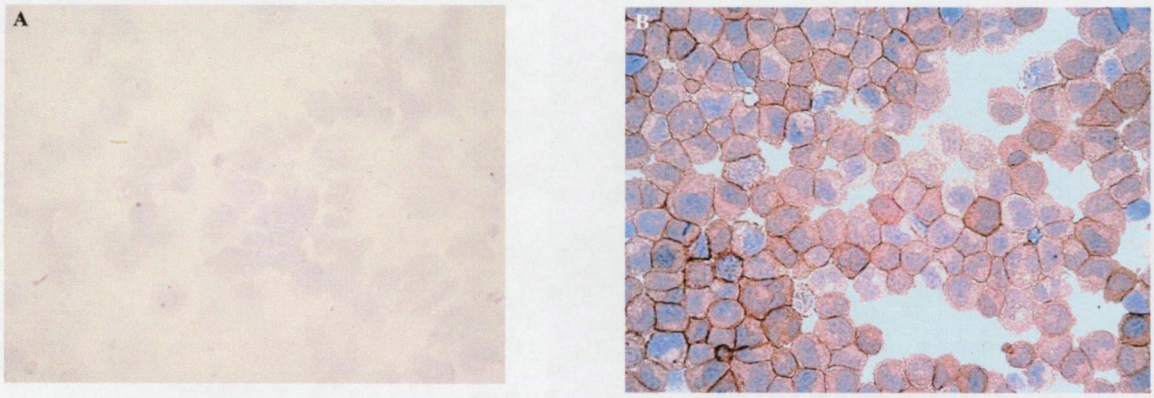


Figure 6. MDR1 staining of parental L5178Y mouse lymphoma cells (A) and its human *mdr1* transfected subline (B)

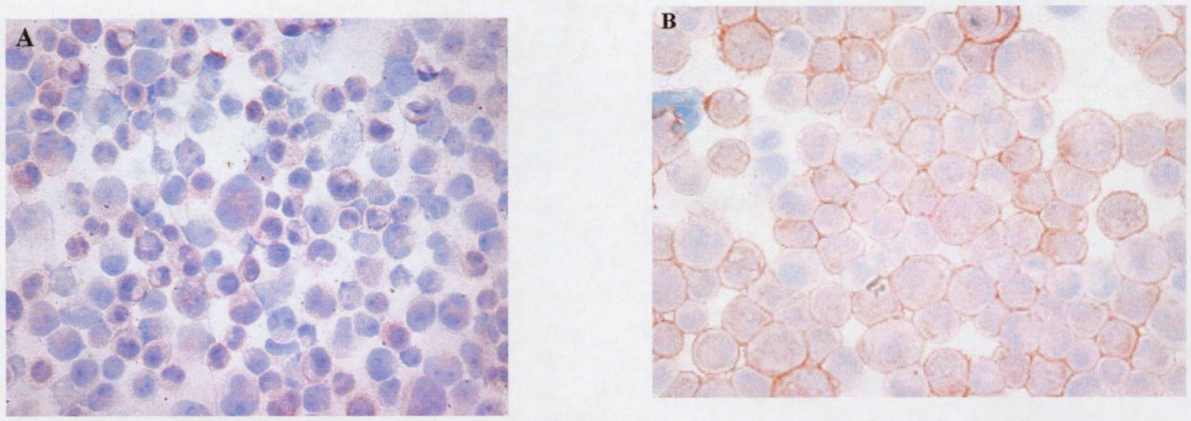


Figure 7. MDR staining of human breast cancer MCF7 (A) and its doxorubicine selected, drugresistant (B) subline

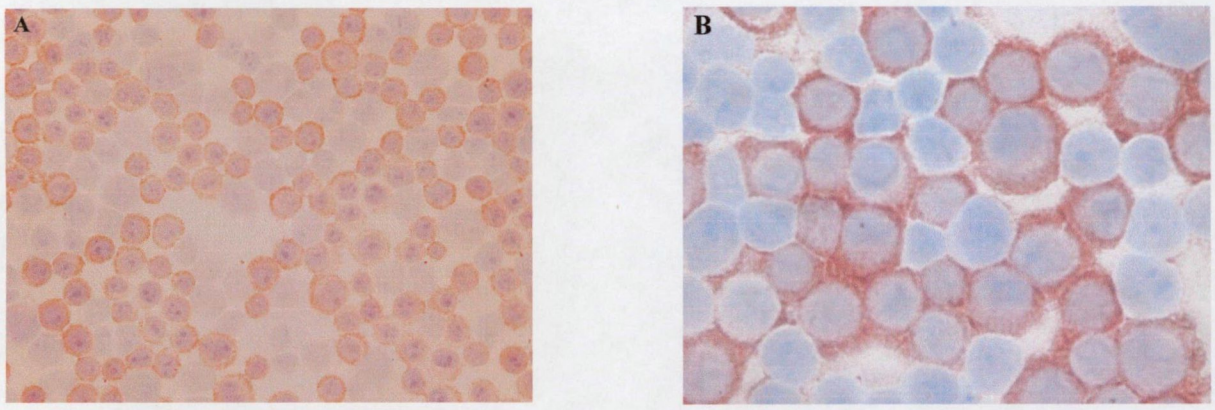


Figure 8. MDR1 (A) and MRP (B) staining of Hep2C cell line

4.2 Antiproliferative and cytotoxic effect of potential resistant modifiers

As discovery of less toxic MDR modifiers suggests, it is important to study the antiproliferative and toxic property of various compounds before a time-consuming experiment such as *in vitro* combination study. The long time antiproliferative and/or short time cytotoxic effect were studied in each examined compounds by MTT test.

As shown in table 2 antiproliferative effects may vary on different cell lines. Because of the limited length of the thesis and considering the large number of studied compounds only some representative chemicals are demonstrated in the table below.

Some organosilicon compounds are well known cytostatic drugs (106,107,108). Organosilicon compounds also have similar, but not so significant antiproliferative effect. The mouse lymphoma seems (120) to be more sensitive for these compounds than two human cell lines (Table 2).

In further experiments the effect of natural compounds on cell proliferation was determined.

Several studies indicated that diterpene and triterpene derivatives have apoptosis inducing activity (116-119).

Table 2. Antiproliferative effect of organosilicon compounds on MDA-MB-231 breast cancer cell and human larynx cancer cell line Hep2C

	MDA-MB-231 ID_{50} µg/ml	Hep2C ID_{50} µg/ml
Alis-409	8	10
Alis-421	10	5

Considering this property the cytotoxic effect was examined on human *mdr1* gene transfected mouse lymphoma and human breast cancer cell line by macrocyclic jatrophane diterpene polyesters, pubescences A-D and rearranged jatrophane diterpenes isolated from *Euphorbia* species (Table 3). ID_{50} values of most of diterpene derivatives are very similar or higher than DMSO control, thus these compounds have no significant cytotoxic effect on the above mentioned cell line. In some case on mouse lymphoma pubescene B and C, compound 7 and pubescenol had some toxic effect.

Table 3. Cytotoxic effect of some selected jatrophane diterpens on MDA-MB-231 breast cancer and mouse T lymphoma cell line

Samples	MDA-MB-231 ID_{50}(µg/ml)	L5178 ID_{50} values (µg/ml)
Pubescene A (1)	37.59	19.11
Pubescene B(2)	64.65	24.01
Pubescene C(3)	55.45	10.01
Pubescene D(4)	44.17	13.97
Compound (5)	44.66	18.12
Portlandicine (6)	45.76	22.37
Compound 7	39.89	14.78
Euphopubescenol (8)	94.7	28.69
Euphopubescene (9)	47.21	32.98
Pubescenol (10)	39.48	16.29
DMSO	33.73	19.35

Cancer chemopreventive effect of carotenoids was reported by several studies (109-111) (9Z)zeaxanthin and (5S, 8S) capsocrome have considerable antiproliferative effect on drugresistant tumor cell line *in vitro*, as it was also observed in Alis compounds mouse lymphoma cells except 13(Z) zeaxanthin. However, its difference in the antiproliferative action on the two cell line is negligible (Table 4).

Cancer protective effect of polyphenolic flavonoid derivatives is also common by their apoptosis inductive and growth arrest effect on cancer cell (112, 113). The most effective cell proliferation inhibitor was the mitochondrial electron transport inhibitor, rotenone (114) which could be responsible for caspase dependent and independent cell death (115). This may be the explanation for its very high antiproliferation and cytotoxic activity on both cell lines. The effect of epigallocatechin and afrormosin are also very significant (Table 5).

As the tables show the antiproliferative effect and cytotoxic effect varie. Generally the human *mdr1* gene transfected mouse lymphoma is more sensitive than human cancer cell lines. This complex effect

on cell proliferation and viability may depend on the chemical structure of each derivatives and sensitivity of cell lines.

Table 4. Antiproliferative effect of some selected carotenoids on mouse T lymphoma cell line and drugresistant subline of MCF7 (KCR).

Samples	L5178 ID_{50} values (μg/ml)	KCR ID_{50} values (μg/ml)
Monoepoxy-β-carotene	8.54	12.6
(5*S*,8*S*)-Capsochrome	5.50	5.50
8'SLuteoxanthin	8.71	16.69
9(Z)Violaxanthin	12.03	18.17
9(Z)Zeaxanthin	1.90	9.27
13(Z)Zeaxanthin	7.27	6.27

Table 5. Antiproliferative effects of selected flavonoids and isoflavonoids on tumor cells

Compounds	L5178 MDR ID_{50} (μg/mL)	MDA-MB-231 ID_{50} (μg/mL)
Epigallocatechin	1.80	2.60
Rotenone	0.01	0.0005
Kaempferol	55.47	20.59
Robinin	38.51	18.60
Dihydroquercetin	59.32	10.19
Chrysin	15.51	5.61
Formononetin	19.70	17.54
Amorphigenin	1.04	0.01
Afrormosin	48.78	20.50
6a,12a,-Dehydroamorphigenin	9.80	13.51
12a-Hydroxyamorphigenin	2.77	14.04
DMSO	12.5	32.76

4.3 Reversal of MDR1 on mouse lymphoma

4.3.1 Reversal of multidrug resistance by jatrophane diterpenes

The macrocyclic jatrophane diterpene polyesters, Pubescene A-D **1-4** and compounds **8-10** were isolated from the whole dried plant of *Euphorbia pubescens*. The rearranged jatrophane-type diterpenes **5-7** were isolated from *Euphorbia portlandica* and *Euphorbia segetalis*. The dose dependent effect of compounds was studied in a μM concentration rang for easier comparison of dose dependent biological effect. The jatrophane diterpenes were examined for MDR–reversing activity on L5178 mouse lymphoma cells and the results are displayed in table 6. Pubescences A-D were shown to enhance drug retention in the cells by inhibiting the efflux-pump activity mediated by P-glycoprotein. The results showed concentration dependence for all the compounds. Pubescene A and D exhibit the highest effect in reversing activity ratios (Table 6) and mainfold activity when compared to that of the positive control **verapamil** (FAR=21.28 in 20μM concentration).

Table 6. Fluorescence Activity Ratios in presence of jatrophane diterpens (8.16.32.64 μM) on MDR in mouse lymphoma cell line

Name	FAR values at various concentration				
	8μM	16 μM	32μM	64μM	80μM
Pubescene A (1)	86.98	79.78	126.76	88.16	110.02
Pubescene B(2)	4.06	19.76	32.12	60.22	60.85
Pubescene C(3)	20.01	16.51	24.01	63.04	95.06
Pubescene D(4)	24.41	32.93	111.01	105.78	101.96
Compound (5)	2.73	3.21	15.89	32.67	69.69
Portlandicine (6)	1.31	2.12	2.35	2.57	23.25
Compound 7	3.65	15.59	27.26	52.92	74.96

4.3.2 Reversal of multidrug resistance by carotenoids on mouse lymphoma

In these experiments the aim was to find less toxic compounds with well-defined MDR reversal effect. The drug uptakes by human MDR-1 gene-transfected mouse lymphoma cells were examined in the presence of selected carotenoids (Table 7).

Table 7. Fluorescence Activity Ratios in presence of carotenoids (2 and 20μg/ml) on human MDR-1-transfected mouse lymphoma cells

Samples	FAR values at various concentration		
	2 μg/ml	20 μg/ml	5 μg/ml
Verapamil			5.73
Lycopene	1.10	9.70	
β-Carotene	0.66	0.56	
α-Carotene	0.76	0.81	
β-Cryptoxanthin	4.41	9.65	
α-Cryptoxanthin	0.93	4.42	
Zeaxanthin	5.21	9.67	
Lutein	8.61	10.06	
Antheraxanthin	11.13	10.17	
Violaxanthin	0.90	5.97	
Capsanthin	30.98	27.59	
Capsorubin	33.78	24.46	
Lycophyll	1.40	25.20	
DMSO control		1.10	

Carotenoids were classified into three different groups according to their MDR reversal activity: inactive, moderately active or very active. As shown in table 7, alpha- and beta-carotene exhibited no effect. The most effective compounds were capsanthin, lycophyll and capsorubin. Moderately effective carotenoids were violaxanthin, lycopene, lutein, zeaxanthin and antheraxanthin. Those results suggest that the MDR-reversing effect depended on the chemical structure.

Besides mouse lymphoma cells the rhodamine accumulation was also studied in human breast cancer MDA-MB-231/MRP cells in the presence of carotenoids. The rhodamine accumulation was not

modified in these cells after carotenoid treatment however these cell line containing P-gp which was detected by immunocytochemistry. But these proteins remained ineffective *in vitro* because the accumulation of R123 defaulted even if in case of verapamil (97).

The majority of carotenoids having hydroxylation on the right six membered ring exert a moderate effect on the MDR reversal. We found very high resistance reversal effect on the human MDR1 gene transfected mouse lymphoma cells in case of capsanthin which has hydroxylation on the left ring and in case of capsorubin which has a hydroxylation on the right five membered ring (see chemical structures).

According to these results we tried to focus on the effect of isomeric configuration of carotenoids on the effect of MDR modification. Some carotenoids were moderately active in drug accumulation *e.g.* mutatochrome, aurochrome and diepoxy-β-carotene (Table 8).

Table 8. Fluorescence Activity Ratios in presence of different isomers of carotenoids (4 and 40 µg/ml) on human MDR-1 gene-transfected mouse lymphoma cells

Samples	FAR values at various concentration	
	4µg/mL	40 µg/mL
Mutatochrome	2.02	7.47
Aurochrome	0.94	3.34
Flavoxanthin	4.12	30.27
Chrisanthemaxanthin	4.12	30.27
ß-caroten-monoepoxid	9.54	37.59
ß-caroten-diepoxid	1.44	5.72
15,15`-dehydro-diepoxy- ß-carotene	0.75	0.58
monoepoxy-a-carotene	0.72	0.56
(5R,8S)-Capsochrome	11.03	37.92
(5R,8R)-Capsochrome	14.04	56.81
(5S,8S)-Capsochrome	40.13	41.52
(5S,8R)-Capsochrome	23.25	53.54
Luteochrome	1.52	2.83
(8'R)-luteoxanthin	56.9	61.95
(8'S)-luteoxanthin	35.94	63.57
(13Z)-lutein	11.64	31.42
(13'Z)-lutein	11.64	31.42
(13Z)-zeaxanthin	3.58	35.24
(9Z)-zeaxanthin	4.86	20.54
(9Z)-violaxanthin	34.01	57.74
(9'Z)-neoxanthin	51.21	59.55

Treatment of the cancer cells with flavoxanthin, + chrysanthemaxanthin mixture, (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. (8'*R*)- and (8'*S*)-luteoxanthin, (13*Z*)-lutein, (9*Z*)-violaxanthin and (9'*Z*)-neoxanthin exhibited a high fluorescence activity ration on mouse

lymphoma cells. However structurally related luteochrome was hardly effective as a reversing agent. (Table 8).

4.3.3 Reversal of multidrug resistance by flavonoids

When flavonoids were tested on the mouse lymphoma cells, rotenone, chrysin, floretin and epigallocatechin displayed a dose-dependent increase in the fluorescence activity ratio (FAR). These compounds caused a much higher drug accumulation than that of the verapamil control. Catechin, neohesperidin, naringin, robinin and sakuratin had moderate effects. Dihydroquercetin, dihydrofisetin,

Table 9. Fluorescence Activity Ratios in presence of flavonoids (4 and 40 µg/ml) on human MDR-1-transfected mouse lymphoma cells

Samples	FAR values at various concentration	
	4µg/mL	40 µg/mL
Rotenone	**27.7**	**28.6**
Catechin	2.7	2.9
Neohesperidin	2.3	2.8
Naringin	2.6	2.3
Chrysin	3.7	14.6
Robinin	2.0	1.5
Floretin	0.8	4.9
Floridzin	0.7	0.6
Robinetin	0.7	0.7
Dihydrorobinetin	0.8	0.7
Kaempferol	0.8	0.8
Dihydrofisetin	0.7	0.5
Dihydroquercetin	0.7	0.6
Sakuranin	0.7	0.8
Sakuranetin	0.8	2.4
Epigallocatechin	0.6	36.1
DMSO control		0.8

dihydrorobinetin and floridzin reduced rhodamine 123 accumulation in the cells. Kaempferol and robinetin had no effect on rhodamine 123 accumulation of the human *mdr1* gene transfected mouse lymphoma cell line (Table 9).

4.3.4 Reversal of multidrug resistance by isoflavonoids

Of the isoflavonoids, amorphigenin and formononetin proved to have the strongest MDR-reversal effects. Afrormosin, (+)-12a-hydroxyamorphigenin and 6a,12a-dehydroamorphigenin were moderately effective on human *mdr1* gene-transfected mouse lymphoma cells (Table 10).

Table 10. Fluorescence Activity Ratios in presence of isoflavonoids (4 and 40 µg/ml) on human MDR-1-transfected mouse lymphoma cells

Samples	FAR values at various concentration	
	4µg/mL	40 µg/mL
Formononetin	0.8	18.3
Amorphigenin	12.4	46.4
Afrormosin	0.5	3.1
6a,12a-Dehydroamorphigenin	1.1	3
(+)-12-Hydroxyamorphigenin	0.4	2.8
DMSO control		0.9

4.4 Reversal of MDR1 on doxorubicine selected drugresistant subline of MCF7

4.4.1. Reversal of multidrug resistance by carotenoids on doxorubicine selected drugresistant subline of MCF7 (KCR).

Previously the resistance was tested on this cell line in comparison with its parental MCF7 cells. ID_{50} value in presence of doxorubicin on KCR was 50 fold higher than in parental MCF7.

Table 11. Fluorescence Activity Ratios in presence of different isomers of carotenoids (4 and 40 µg/ml) on human drugresistant breast cancer cells (KCR).

Samples	FAR values at various concentration		
	4µg/mL	40 µg/mL	5µg/mL
Verapamil			11.90
Mutatochrome	1.10	1.90	
Aurochrome	0.90	0.80	
Flavoxanthin	1.00	1.10	
Chrisanthemaxanthin	1.00	1.10	
Diepoxy-β-carotene	1.07	2.08	
15.15`-dehydro-diepoxy- β-carotene	0.89	0.62	
Monoepoxy-α-carotene	0.94	0.69	
Monoepoxy-β-carotene	1.17	2.37	
(5S.8R) Capsochrome	1.27	1.38	
(5S.8S)-Capsochrome	1.77	1.99	
(5R.8R)-Capsochrome	1.34	1.67	
(5R.8S)-Capsochrome	1.43	1.95	
Lutechrome	2.10	1.30	
(8'R)-luteoxanthin	1.20	1.90	
(8'S)-luteoxanthin	1.10	2.10	
(13Z)-lutein	1.20	2.00	
(13'Z)-lutein	1.20	2.00	
(13Z)-zeaxanthin	1.10	2.00	
(9Z)-zeaxanthin	1.40	2.20	
(9Z)-violaxanthin	1.70	2.20	
(9'Z)-neoxanthin	0.89	0.69	

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

When the carotenoid isomers were tested on KCR cell line, much less inhibition of MDR efflux pumps was found than for that in human *mdr*1 gene transfected mouse lymphoma cells in the presence of the carotenoids. As revealed by Table 11 the rhodamine accumulation was enhanced only moderately, from a fluorescence activity ratio of 1.1 to 2.2. This means that the rhodamine uptake was enhanced from 10 to 120 per cent in human breast cancer cells. Additionally, some compounds were inactive, *e.g.* (9'*Z*)-neoxanthin, monoepoxy-α-carotene and 15,15'-dehydrodiepoxy-β-carotene (Table 11).

The comparison of the sensitivities of the mouse lymphoma and breast cancer cell lines demonstrated that the same human *mdr*1 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 must be presumed that the P-gp target in the cell membrane in which the p-glycoprotein is embedded, has different sensitivities to the extremely hydrophobic carotenoids in various cell lines.

4.5 Reversal of MRP on breast cancer cell line MDA-MB-231 (HTB-26)

4.5.1 Reversal of multidrug resistance by jatrophane diterpenes on MDA-MB-231 cell line

Ten macrocyclic diterpenes of the jatrophane type and rearranged polycyclic derivatives of which majority are able to strongly enhance the rhodamine 123 accumulation of MDR cells were examined for their effects on the reversion of MDR on breast cancer MDA MB 231 (HTB-26) cell line by the flow cytometry.

Table 12. Fluorescence Activity Ratios in presence of jatrophane diterpenes (5 and 20 µM) on human breast cancer cells MDA-MB-231with MRP resistance

Name	Fluorescence Activity Ratio values at various concentration	
	5 µM	20 µM
Pubescene A (1)	0.80	11.50
Pubescene B(2)	0.80	0.70
Pubescene C(3)	0.90	0.80
Pubescene D(3)	0.90	3.90
Compound (5)	0.90	1.80
Portlandicine (6)	1.04	1.50
Compound 7	0.80	1.20
Euphopubescenol (8)	0.80	12.70
Euphopubescene (9)	1.25	4.40
Pubescenol (10)	0.90	5.80
DMSO		0.75

The HTB26 cells simultaneously express MDR1 and MRP. R123 accumulation was very low in case of 1 and 4, it was only 20%. However the presence of MDR1 was detected by specific monoclonal antibody but it seemed that this protein remained ineffective, because neither verapamil nor pubescences could enhance the fluorescence activity ratio (data not shown).

The same compounds were tested on MRP, the carboxyfluorescein (BCECF-AM) accumulation of the MDA-MB-231 breast cancer cells was measured (Table 12). Indomethacin, a known MRP1 inhibitor was used as a reference compound.

BCECF-AM, a fluorescent dye was used as an intracellular pH indicator is an especially good MRP substrate (121). The MRP expression and its function were assessed by the flow cytometry (122), although the sensitivity of this method was lower than rhodamine 123 exclusion test in the mouse lymphoma cells. This means that the values of fluorescence activity ratio are not so high. Increased fluorescence activity was shown by some compounds such as **1. 8. 9** (Table 12). Indometacin was also used as a positive control in the MRP accumulation test by Bobrowska-Hägerstrand M *et al* (123).

When the carotenoid compounds were tested on MRP, the carboxyfluorescein accumulation of the MDA-MB-231 breast cancer cells was measured. Several compounds like mutatochorme, 15-15'dehidroβcarotene, monoepoxy-α-carotene, (8'S)-luteoxantin and (13Z)-lutein (13'Z)-lutein caused higher accumulation than positive control indometacin (Table 13).

Table 13. Fluorescence Activity Ratios in presence of different isomers of carotenoids (4 and 40 μg/ml) on human breast cancer cells MDA-MB-231 with MRP resistance.

Samples	FAR values at various concentration		
	4μg/mL	40 μg/mL	5μg/mL
Indometacin			**1.4**
Mutatochrome	4.0	2.1	
Aurochrome	1.0	1.2	
Flavoxanthin	1.0	1.3	
Chrisanthemaxanthin	1.0	1.3	
Diepoxy-β-carotene	0.9	1.0	
15.15`-dehydro-diepoxy- β-carotene	1.5	1.7	
Monoepoxy-α-carotene	1.1	2.0	
Monoepoxy-β-carotene	0.9	1.0	
(5R,8S)-Capsochrome	0.8	1.3	
(5R,8R)-Capsochrome	0.9	1.2	
(5S,8S)-Capsochrome	0.9	1.0	
(5S,8R)-Capsochrome	0.7	0.9	
Lutechrome	1.0	1.4	
(8'R)-luteoxanthin	1.2	1.2	
(8'S)-luteoxanthin	1.4	1.5	
(13Z)-lutein	1.3	1.6	
(13'Z)-lutein	1.3	1.6	
(13Z)-zeaxanthin	1.1	1.3	
(9Z)-zeaxanthin	1.0	1.2	
(9Z)-violaxanthin	1.1	1.3	
(9'Z)-neoxanthin	1.3	1.3	
DMSO control			**1.0**

4.5.2 Reversal of multidrug resistance by flavonoids on MDA-MB-231 cell line

When the flavonoids were tested on the drug accumulation of the breast cancer cells three groups of compounds could be distinguished. He members of the first group: chrysin, robinin, kaempferol, dihydroquercetin and epigallocatechin were as effective as the indometacin control, by elevating the BCECF-AM accumulation 20-60%. The second group, neohesperidin, naringin, floretin, floridzin, robinetin, dihydrorobinetin and dihydrofisetin were practically ineffective. Meanwhile the members of the third group: rotenone, catechin and sakuranin reduced the drug accumulation in the cells (Table 14).

Table 14. Fluorescence Activity Ratios in presence of flavonoids (4 and 40 µg/ml) on human breast cancer cell line with MRP resistance

Samples	Fluorescence Activity Ratio values at various concentration		
	4µg/mL	40 µg/mL	10 µg/mL
Indomethacin			**1.3**
Rotenone	0.3	0.3	
Catechin	0.6	0.6	
Neohesperidin	0.8	0.9	
Naringin	1.0	0.8	
Chrysin	1.0	1.2	
Robinin	1.4	1.6	
Floretin	0.7	1.0	
Floridzin	0.8	0.9	
Robinetin	0.8	0.9	
Dihydrorobinetin	0.8	1.1	
Kaempferol	0.9	1.3	
Dihydrofisetin	1.0	1.0	
Dihydroquercetin	0.9	1.3	
Sakuranin	0.8	0.7	
Sakuranetin	0.8	0.8	
Epigallocatechin	1.1	1.3	
DMSO control			**0.9**

4.5.3 Reversal of multidrug resistance by isoflavonoids on MDA-MB-231 cell line

When the isoflavonoids were tested on MRP the carboxyfluorescein accumulation of the MDA-MB-231 breast cancer cells was measured. Formononetin increased the drug accumulation in both cell lines as compared with the control. Afrormosin was only moderately effective on the MRP reversal of the tumor cells. 6a12a-Dehydroamorphigenin and (+)-12a-hydroxyamorphigenin had only marginal effects as compared with indomethacin-treated MRP cells (Table 15).

Table 15. Fluorescence Activity Ratios in presence of isoflavonoids (4 and 40 µg/ml) on human breast cancer cells MDA-MB-231with MRP resistance

Samples	Fluorescence Activity Ratio at various concentration		
	4µg/mL	40 µg/mL	10 µg/mL
Indomethacin			2.4
Formononetin	1.0	1.5	
Amorphigenin	1.0	1.0	
Afrormosin	1.3	1.4	
6a,12a-Dehydroamorphigenin	1.0	1.2	
(+)-12-Hydroxyamorphigenin	1.1	1.1	
DMSO control			1

4.6 Reversal of multidrug resistance by organosilicon compounds on human larynx cancer Hep2C cell line

The drug accumulation of Hep2C cells was studied in the presence of different concentration of Alis 409 in MDR and MRP specific systems. The rhodamine 123 accumulation was investigated by using verapamil as positive control (Table 16). MRP activity was investigated by the carboxyfluorescein accumulation by using indomethacin as positive control (Table 17). Apparently the rhodamine and carboxyfluorescein accumulation was increased in the presence of low concentration of Alis 409 remarkably while the rhodamine accumulation was increased moderately in the presence of 0.4 µg/ml Alis 409. In case of Alis 421 the lower concentrations have higher activity, probably due to some toxical effects at higher concentration.

Table 16. Fluorescence Activity Ratios in presence of alis-compounds (0.4-40µg/ml) on human larynx carcinoma Hep2C cell line by R123 accumulation

Samples	Fluorescence Activity Ratio values at various concentration				
	0.4µg/ml	2µg/ml	4µg/ml	10µg/ml	40µg/ml
Verapamil		1.21		1.03	
Alis 409	1.36		1.02		0.56
Alis 421			0.90		0.40

Table 17. Fluorescence Activity Ratios in presence of alis-compounds (0.4-40µg/ml) on human larynx carcinoma Hep2C cell line by BCECF-AM accumulation

Samples	Fluorescence Activity Ratio values at various concentration					
	0.4 µg/ml	0.5 µg/ml	4 µg/ml	5 µg/ml	10 µg/ml	40 µg/ml
Indometacin		1.42		1.07	1.90	
Alis 409	1.58		1.20			0.88
Alis 421			1.00			0.80

4.7 In vitro combinations (checkerboard method)

4.7.1 In vitro effects of organosilicon compounds in combination with cytotoxic drugs

Based on the synergism having been found between epirubicin and the mdr-reversing compounds alis-409 and alis-421 *in vitro* on human *mdr1* gene transfected mouse lymphoma cell (120), the nature of the interactions between epirubicin and the resistance modifiers was also studied on MDA-MB-231 (HTB-26) breast cancer cell line. No significant interaction was found between the Alis compounds and epirubicin in these non-MDR-mediated human tumor cells. The two new organosilicon compounds exerted strong inhibitory effects on the efflux pump activity of the MDR (mdr-1) tumor cells.

However, in the short term accumulation assays the Alis compounds seemed to be ineffective. Meanwhile in the longer combination both of them could enhanced the effect of epirubicin on human larynx cancer Hep2C cell line (Table 18).

Table 18. In vitro effects of Alis compounds in combination with epirubicin on various tumor cell lines

	HTB-26/MRP		Hep2C	
Samples	**FIX**	**Interaction**	**FIX**	**Interaction**
Alis 409	1.27	no interaction	0.40	synergism
Alis 421	1.50	no interaction	0.48	synergism

4.7.2 In vitro effects of jatrophane diterpenes in combination with doxorubicin

The Pubescene A showed the highest activity against efflux pump MRP; however, it had only moderate effect in longer *in vitro* combination assay on MDA-MB-231 cell line. Both of the Pubescences enhanced the effect of epirubicin on human *mdr1* gene transfected mouse lymphoma cell line (Table 19).

Table 19. In vitro effects of jatrophane diterpenes in combination with doxorubicin on human mdr1 gene transfected mouse lymphoma and MDA-MB-231 human breast cancer cell lines

	MDA-MB-231		MDR	
Samples	**FIX**	**Interaction**	**FIX**	**Interaction**
Pubescene A (1)	0.543	Additive	0.387	Synergism
Pubescene D(3)	0.305	Synergism	0.212	Synergism

4.7.3 In vitro effects of carotenoids in combination with cytotoxic drugs

In further experiments the checkerboard microplate method was applied to examine the effects of carotenoids in combination with anthracycline derivative cytotoxic drugs (Table 20). Verapamil and indometacin (MDA-MB-231 cell line) were also used as functionally positive controls in these experiments. The treatment resulted in a synergistic effect between verapamil and doxorubicin for both cell lines. Of the possible resistance modifiers (13*Z*)-zeaxanthin was able to enhance the antiproliferative effect of epirubicin synergistically (8'*S*)-Luteoxanthin, (9*Z*)-violaxanthin and (9*Z*)-

zeaxanthin treatment revealed synergism in at least one of the cell lines. Monoepoxy-β-carotene increased the rhodamine 123 accumulation in both cell lines, but remained almost ineffective in combination treatment (Table 20).

Table 20. Interaction between epirubicin and some carotenoid resistance modifiers on human mdr1 gene transfected mouse lymphoma and drugresistant human MCF7 (KCR) cell line

Samples	L5178 MDR1		KCR	
	FIX	Interaction	FIX	Interaction
Verapamil as positive control	0.162	synergism*	0.231	synergism*
Monoepoxy-β-carotene	0.678	additive	1.512	indifferent
(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

*In the case of verapamil control doxorubicin was used as cytotoxic drug instead of epirubicin

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 are encouraging; the combinations of epirubicin and some carotenoids resulted in synergism in the antiproliferative action of epirubicin on the two cell lines. Carotenoids mutatochrome, 15-15'dehidroβcarotene, monoepoxy-α-carotene and (8'S) Luteoxanthin which had higher activity of MRP inhibition were only moderately effective *in vitro* combination on MDA-MB-231 cell line (Table 21).

Table 21. Interaction between epirubicin and some carotenoid resistance modifiers on MDA-MB-231 breast cancer cell line

Samples	MDA-MB-231	
	FIX	Interaction
Indometacin as positive control	0.210	synergism
mutatochrome	0.746	additive
15-15'dehidroβcarotene	0.910	additive
monoepoxy-α-carotene	0.663	additive
(8'S) Luteoxanthin	1.286	indifferent

4.7.4 In vitro effects of mdr modifier flavonoids and isoflavonoids in combination with epirubicin

The enhanced antiproliferative activity of combinations of the tested compounds with epirubicin was examined. Of the resistance modifiers (formononetin, amorphigenin, rotenone, chrysin and epigallocatechin) chrysin and amorphigenin were able to enhance the antiproliferative activity of epirubicin on mouse lymphoma cells. Although rotenone significantly increased the rhodamine 123 drug accumulation it had only a marginal additive antiproliferative effect in combination with epirubicin. On the MDA-MB-231 breast cancer cell line, formononetin and kaempferol exhibited

synergism. Some compounds, e.g. afrormosin and robinin also had weak effects in combination with epirubicin. Interestingly, they were ineffective on the human *mdr1* transfected mouse lymphoma cell line. Amorphigenin treatment likewise resulted in different effects on the two cell lines (Table 22).

Table 22. In vitro effects of mdr modifier flavonoids and isoflavonoids in combination with epirubicin

Cell lines	Samples	FIX	Interaction
L5718	Chrysin	0.471	synergism
	Rotenone	0.604	additive effect
	Amorphigenin	0.120	synergism
	Formononetin	1.230	no interaction
MDA-MB-231	Amorphigenin	1.090	no interaction
	Formononetin	0.181	synergism
	Afrormosin	0.548	additive effect
	Robinin	0.501	additive effect
	Epigallocatechin	1.001	no interaction
	Kaempferol	0.440	synergism

4.8 Comparison of the structure – activity relationship by physico-chemical parameters of carotenoids was analyzed by Computer model ADME/Boxes (computer program Adsorption, Distribution, Metabolism, Excretion)

Prediction of *P-glycoprotein substrate specificity* ($S_{P\text{-}GP}$) or P-glycoprotein inhibitor properties can be a very important part of pharmaceutical drug design (105, 124). This task is difficult to achieve due to several factors that have raised many contradictory opinions:

(i) the disparity between the $S_{P\text{-}GP}$ values obtained in different assays,

(ii) the confusion between P-gp substrates and inhibitors,

(iii) the confusion between lipophilicity and amphiphilicity of P-gp substrates,

(iv) and the dilemma of describing class-specific relationships when P-gp has no binding sites of high ligand specificity.

ADME computer model system is competent in giving the probability of P-gp inhibitor properties of examined compounds by determination physico-chemical parameters of the tested compounds. The following factors were examined in case of carotenoid derivatives:

- Molar weight (MW) or volume
- H+ acceptor and donor properties
- Abraham's a: H-bond acidity (H-donating)
- Abraham's b: H-bond basicity (H-accepting)
- TPSA: Total polar surface area
- LogP: octanol/water partition coefficient

Based on the fluorescence activity ratio values and the chemical structures of the studied compounds some correlation was found between the resistance modifier action and physicochemical parameters of the tested compounds (Fig 9). Non-inhibitors (0) are in lower FAR range, meanwhile , in case of inhibitors, where the value of prediction is 1, the FAR values are higher.

Correlaion was studied by Fisher exact test (Table 23) and the odds for higher FAR value with inhibitor property (which was given by computer model system) was 7.815 fold higher than the odds lower FAR value with inhibition property.

Figure 9. Correlation of FAR and structure activity relationship (SAR) by computer analysis

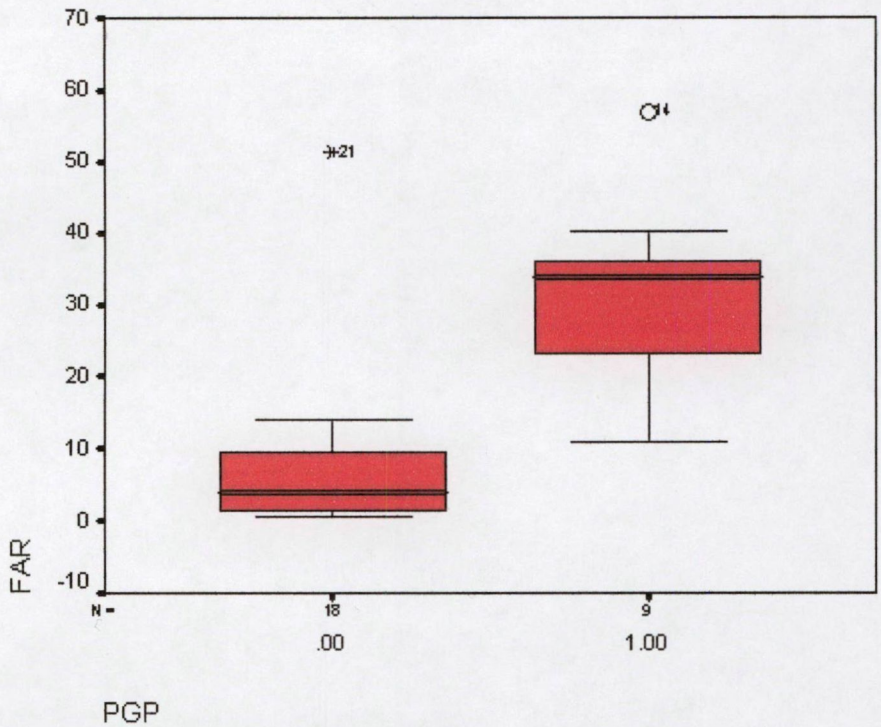


Table 23. Crosstable for Fisher's Exact Test

	Non inhibitor (0)	*Inhibitor (1)*	*Total number*
Small FAR value 0-11	16	2	18
Higher FAR value 11-...	2	7	8
Total number	18	9	

In further analysis we decided which physico-chemical property is the most significant in describing of MDR inhibitor capacity of tested carotenoids. The parameters were adjusted by multiple linear regression system. Our finding that TPSA is one of the most important factors in describing the capacity of MDR inhibitor however, TPSA in itself, is not enough, because the relevancy was R^2=0,63 (Fig 11) when we compared the regression to log values of FAR.

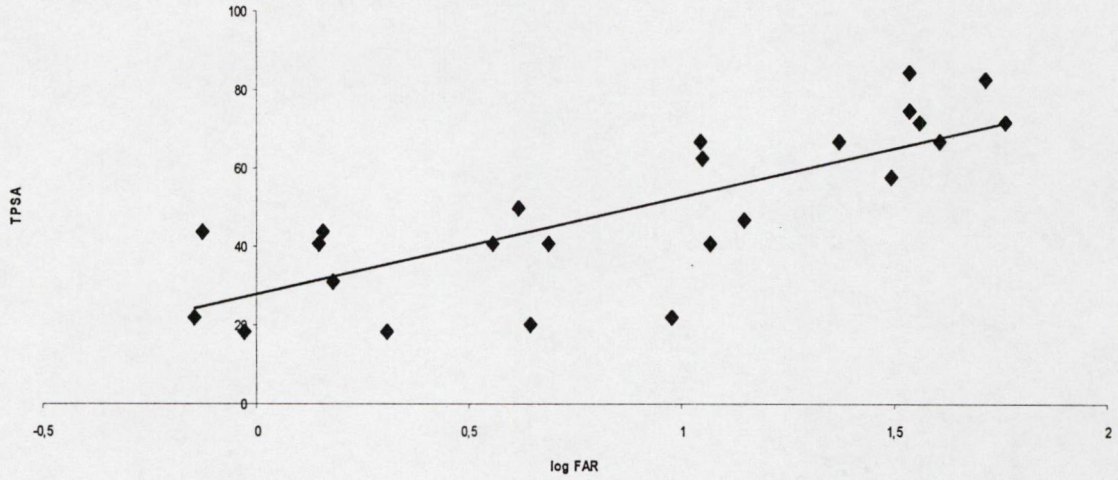


Figure 10. Linear regression between log FAR and TPSA values

4.9 Apoptosis induction by some selected carotenoid derivatives

The "early" apoptosis-inducing effects of carotenoids were high in the presence of lycopene, zeaxanthin and capsorubin, while lutein and capsanthin had moderate activities. Alpha- and beta-cryptoxanthin, lutein, antheraxanthin and violaxanthin did not modify the total apoptosis, since the frequency was the same as in the control untreated cells. None of the tested carotenoids induced noteworthy cell death in the cultures of MDR mouse lymphoma cells (97).

When apoptosis and cell death were investigated in the human breast cancer cells, the highest rate of "early" apoptosis induction was observed in the presence of lycopene and antheraxanthin. The total apoptosis was highest in the presence of lycopene, zeaxanthin and capsanthin, comparable to that for the positive control, 12H-benzo(*a*)phenothiazine (M627). In these cells, death rate increased in the samples treated with lycopene, violaxanthin and capsanthin (Table 24).

Table 24. Apoptosis induction by carotenoids on MDA-MB-231 (HTB26) human breast cancer cells.

Samples	Conc. (μg/mL)	Early apoptosis (%)	Total apoptosis (%)	Cell death
Cell control Double staining		9.07	15.64	4.79
Lycopene	2.00	12.70	27.90	13.24
β-Cryptoxanthin	2.00	5.90	11.50	1.06
α-Cryptoxanthin	2.00	10.5	8.00	2.01
Zeaxanthin	2.00	9.10	35.30	6.18
Lutein	2.00	10.60	15.00	3.88
Antheraxanthin	2.00	17.30	10.10	1.31
Violaxanthin	2.00	7.70	22.70	10.10
Capsanthin	2.00	7.10	40.30	8.02
Capsorubin	2.00	8.20	17.30	7.04
M-627	50.00	1.40	97.00	1.20

5. DISCUSSION

The clinical efficacy of anticancer therapy is severely limited by the inability to predict the outcomes for the patient accurately in terms of both tumor response and toxicity. Despite the advances in molecular medicine resulting in discovery of new drugs with special cellular targets, there are still major issues in drug resistance and individual patient variability. Resistance to chemotherapy in cancer cells is primarily mediated by overexpression of P-glycoprotein. Meanwhile there are cases related to another type of plasma membrane ABC transporters, which extrudes drugs at the expense of ATP hydrolysis.

Since P-glycoprotein is involved in the acquired MDR of tumor cells, the selective killing of cancer cells and the expression of P-gp can be improved by the simultaneous administration of anticancer drugs and resistance modifiers in combination chemotherapy. This can be achived by increasing the concentrations of cytostatic in cancer cells. Theoretically cancer or organ-specific efflux pump inhibitors can reduce the general toxic effect of cytostatic drugs by the induced selective accumulation of cytostatic in the cancer cells even at reduced doses.

In general P-gp can be inhibited on direct and indirect ways: directly a) blocking drug binding site either competitively, non-competitively or allosterically (125), b) interfering ATP hydrolysis (126) and indirectly altering integrity of cell membrane lipids (127).

Before starting the multidrug resistance studies, the presence of various efflux pumps by using monoclonal antibodies in immunocytochemistry were analyzed. The presence of P-gp was detected all of the examinated cell lines except for parental mouse T lymphoma. However the MDR1 on MDA-MB-231 (HTB-26) is seemed to be extinct in test experiments, having been proved by the observation after the treatment that rhodamine accumulation was not modified in these cells by possible resistance modifiers which were effective on mouse lymphoma cell line *e.g.* carotenoids. It is possible that the MDR protein is defective in this cell line because of the defaulted accumulation of R123 even in case of verapamil. Nevertheless other studies did not report the over expression of P-gp in MDA-MB-231 cell line (128).

Four groups of compounds were studied

- synthetic **organosilicon;**
- natural terpenoid derivatives such as
    - **jatrophane-type diterpenes** from Euphorbia species and
    - **carotenoids** from edible plants;
- polyphenolic **flavonoid derivatives** common in plants and some of them are well-known components of green tee.

Some **organosilicon compounds** are well-known cytostatic drugs: 2.6-cis-diphenylcyclotetrasiloxane (Cisobitan) was found to be partially effective in the *in vivo* treatment of patients with prostate

carcinoma (106-108). Cisobitan, with the generic name quarosilan, is an organosilicon drug with high estrogenic activity. Other synthesized fluoro-organo-silicon complexes displayed cytotoxic effects in human ovarian carcinoma cell cultures *in vitro* and one compound prolonged the life of mice with MX-11 tumor (129).

A similar silicon compound, as 2-piperidoethyl-phenyldimethylsilane, exhibited an inhibitory effect on Ehrlich sarcoma-180 and Lewis lung carcinoma in mice and rats. This latter compound was also able to increase the delayed type cellular hypersensitivity (130). The high toxicity and side effects of organosilicon compounds (131) limited their application in the therapy of cancer. Organosilicon compounds were isolated from the mycobacterium *Sorangium cellulosum* and their antitumor effect was shown (132).The possible application of organosilicon compounds is a new approach for the reversal of MDR. 1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis(3-morpholinopropyl)-disiloxane-dihydrochloride (ALIS 409) and 1,3-dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-[1(4-butylpiperazinyl}]propyl)disiloxane-tetrahydrochlorid (ALIS 421) display marked effectivity for the reversal of the MDR of mdr-1 cells (120). The toxicity of these compounds was similar on each cell line, however the mouse lymphoma seems to be more sensitive for them. Both P-gp and MRP inhibitory effects were very low on human cancer cell lines in the short term drug accumulation assay however significant synergism was observed *in vitro* combination of Alis compounds and epirubicin at least on human larynx carcinoma Hep2C cell line.

A number of plant extracts and chemically characterized plant compounds have been previously tested for MDR reversal in our laboratory (133, 134). It seems to be controversial to use poisoning Euphorbia species derivatives **pubescences** for this purpose. Various Euphorbia species were used in traditional medicine (135,136), however these plants contain phorbol esters and tumor-promoting diterpene derivatives, bound to the C1 domain in Protein kinase C, as they do diacyl-glycerols and activate the enzyme in a phospholipid-dependent manner (137). The examined members of diterpene group were different from phorbol esters, as they were newly synthesized jatrophane-type diterpenes. New ingredients of Euphorbia pubescens showed antitumor activity on cancerous cell lines (138). In our experiment only pubescene B and C, compound 7 and pubescenol had some toxic effect.

Several derivatives of polyfunctional diterpenes isolated from Euphorbiaceae have been considered to be potent inhibitors of multidrug resistance (139). The high anti-MDR potency of jatrophanes, pubescene A and D were also very effective in *in vitro* combination with epirubicin. This indicates that this type of diterpenes is considered to be a new chemotype of P-gp inhibitors, regarded as promising lead compounds for drug development programs. Moreover, Pubescene D enhanced the antiproliferative activity of doxorubicin on MDA-MB-231 MRP by expressing cell line and having significant affinity to both MRP and MDR1 molecules for inhibition.

Recently, G. Corea's group also reported jatrophane diterpenes from Mediterranean *Euphorbia* species as a powerful MDR-modifier (140-143). The biological activities of the diterpenes were

assayed through their ability to inhibit P-gp-mediated daunomycin efflux of cancer cells, related to cyclosporine A. In these experiments three structurally homogeneous series of jatrophanes were obtained and evaluated for their structure-activity relationships.

Comparing certain pairs of the isolated compounds having been evidenced the importance of different parts of the molecule, such as substitutions on ring A and at C-5, C-8, C-9, C-14 and C-15. Although the results of this study are useful for identifying efficacious sets of compounds, they do not allow us to draw conclusions about the ideal structure of a jatrophane with the best P-gp inhibitory activity. Therefore, 3D QSAR studies of *Euphorbia* diterpenes about their made of action, a better understanding is needed to gain. It is well known that conformational flexibility of medium-sized rings is strongly influenced by the steric interactions of the substituents. Double bonds, pseudo-rotation of the ester groups and transannular interactions of the substituents have significant effects on the stereostructure of the molecules. In conclusion, jatrophanes and modified jatrophanes have most probably a common pharmacophore, which is dramatically affected by changes of the oxygenation pattern.

Carotenoids It is known that retinoids, a class of compounds structurally related to vitamin A, have a role in cancer chemoprevention (144). Retinoic acid has multiple effects; for example, it is able to induce differentiation of cells by increasing the percentage of G1 phase cells and to reduce the percentage of cells in the S phase (145). Some carotenoids exhibit, depending on their chemical structures, chemopreventive, anti-carcinogenic, resistance-modifying and apoptosis-inducing effect (146). In addition to the general chemopreventive and anti-tumor effects, retinol is known to have effects on our visions.

Epidemiological studies have indicated that a high consumption of vegetables and fruits rich in flavonoids and carotenoids reduces the risk of various cancers. The common fruits and vegetables contain approximately 40-50 carotenoids and many of them can be selected as chemopreventive agents. Apart from β- and α-carotene, the rarer types of carotenoids from red paprika, *e.g.* prenigroxanthin (147), cycloviolaxanthin (148) and many others (149), have been chemically characterized, but their effects on cancer cells still have not been studied in detail.

In an investigation of the anti-proliferative effect of carotenoids, lycopene was found to be the most effective on various prostate cancer cells. The growth inhibition was accompanied by a slowing-down of the cell cycle progression through phases G1-S in mammary cancer MCF7 *via* the reduction of cycline D1. It was found that lycopene inhibits cell growth by interfering with growth factor signal transduction IGF (Insulin-like Growth Factor) in breast cancer, MCF7 and endometrial cancer due to a decrease in the IGF-induced tyrosine phosphorylation of the insulin receptor substrate. It is assumed that the anti-carcinogenic activity of some natural carotenoids, such as β-cryptoxanthin, is related to the stimulation of the expression of anti-oncogenes (150). A retinoic acid derivative was found to exert a high anti-proliferative action with similar inhibitory potency in drug-resistant and parental cell lines

of colon and mammary cancer (151).

Among natural compounds, various fractions of paprika extracts reverse the MDR of cancer cells (133). The effectiveness of the hexane and acetone fractions 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 having been tested in the present experiments exhibited a structure-activity relatiopnship. Interestingly, neoxanthin was ineffective 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 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-β-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 *mdr*1 gene-transfected mouse lymphoma cells.

Recent studies suggest that the membrane structure may play a critical role in MDR activities; the resistance modulator – lipid interaction may be an important factor in the mechanism of drug resistance reversal (152,153,33,154). 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 (155).These properties determine the incorporation yield and the abilities of the carotenoids to fit into the membrane bilayer (156,157). 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 are generally incorporated better than β-carotene (157). This phenomenon can 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 (158) and it may be presumed that there are differences in the ratio of saturated and unsaturated fatty acids in different cell lines. This may be responsible for the variation in the MDR-modulating effect of carotenoids on the mouse lymphoma and resistant MCF7 cell lines in our experiments.

Several studies have been performed on the relationship between differences in membrane properties and MDR (153,159,160). These data emphasized the importance of the increased membrane fluidity and rare rigidity (153) in the inhibition of P-gp. It suggested the carotenoids partition into the hydrophobic core of the membrane and it caused a decrease in lipid fluidity (161). The reduced membrane fluidity may sterically hinder diffusion of drugs and decrease the kinetics of drug excretion (154).

While (Z) stereoisomeric form of carotenoids, (13*Z*)-zeaxanthin, (13*Z*)-lutein, (9*Z*)-zeaxanthin and (9*Z*)-violaxanthin were more effective on P-gp inhibition, they were ineffective on MRP inhibition which can also been explained by functional and structural differencies of two efflux pump.

Carotenoids mutatochrome, 15-15'dehidroβcarotene, monoepoxy-α-carotene and (8'S) luteoxanthin, which had higher activity of MRP inhibiton were only moderately effective *in vitro* epirubicin combination on MDA-MB-231 cell line.

P-gp transports the most of the natural compounds (such as peptides and alkaloids) and drugs of many therapeutic areas (37, 162-165). Several authors (166, 167) stressed the importance of predicting P-gp substrate specificity (SP-GP) in the frameworks of "property-based design" and "pharmaceutical profiling" in global drug discovery. This task is difficult to be achieved due to the large uncertainty of experimental data that leads to the contradictory results of SAR analysis when different data sets are used.

The large uncertainty of experimental data can be associated with the fact that P-gp has different specificities towards the substrates and inhibitors. Many clinically relevant P-gp substrates are poor inhibitors, and many inhibitors are poor substrates (124). On the other hand, compounds that inhibit P-gp at higher concentrations may become substrates at lower concentrations (168, 169). This leads to the highly contradictory situation when the same compound may be qualified as a substrate, non-substrate, or inhibitor, depending on its concentration in a given assay. It is believed that substrate and inhibitory properties depend on multiple factors, including logP, MW or surface area (SA), aromaticity, amphiphilicity, proton basicity, and H-bond accepting (105). Prediction of *P-glycoprotein substrate specificity* ($S_{P\text{-}GP}$) or P-glycoprotein inhibitor properties can be a very important part of pharmaceutical drug design (33, 124, 105). This task is difficult to be achieved due to the several factors that raise many contradictory opinions:

(v) the disparity between the $S_{P\text{-}GP}$ values obtained in different assays,

(vi) the confusion between P-gp substrates and inhibitors,

(vii) the confusion between lipophilicity and amphiphilicity of P-gp substrates,

(viii) and the dilemma of describing class-specific relationships when P-gp has no binding sites of high ligand specificity.

The computer analisys of probability of carotenoids P-gp inhibition properties is based on ADME (Adsorption, Distribution, Metabolism, Excretion) computer modell system. We found quite good correlation between the range of FAR and SAR data.

TPSA total polar surface area seems to be the most relevant factor among physico-chemical properties of carotenoids which is derived from polar fragments with nitrogen and oxygen moreover "slightly polar" fragments containing phosphorus and sulphur (105). Thus the polarity has a very important role in P-gp inhibitory activity of carotenoids, however it is not enough for prediction in itself.

Many classes of antitumor drugs, including naturally occurring and pharmaceutical compounds, induce apoptosis in cancer cells. Apoptosis is typically induced by the activation of membrane receptors, cell cycle arrest, p53 activation by DNA damaging agents and mitochondria pore transition permeability (PMT) (170). Caspases and Bcl-2 famiy of proteins also have determinant role in the regulation of progress of cell death (170, 171).

The apoptotic process is characterized by particular morphological and ultrastructural features, which can be evidenced by several assays, including terminal deoxynucleotidyltransferase nick-end labeling (TUNEL), FITC-conjugated annexin V method, acridin orange assay and DNA fragmentation.

There is a growing amount of literature on the role of β-carotene and other carotenoids in human chronic diseases, including cancer. Numerous *in vitro* and *in vivo* studies reported that carotenoids are able to inhibit the growth of cancer cells (172). It could be an interesting question if the carotenoids also promote apoptotic signals beside their MDR reversal effect. According to several *in vitro* and *in vivo* studies tumor cells are much more sensitive to the pro-apoptotic effects of the carotenoids than normal cells, which, in contrast, are often protected from apoptosis by these molecules (173-176). Moreover our results are good agreement with other observation of Palozza *et al*, that pro-apoptotic effect of β-carotene are deeply influenced by the cell type probably because of the fact that cells differ in their ability to incorporate the carotenoid. In our study the MDA- MB-231 human breast cancer cell line was less sensitive than human *mdr1* gene transfected mouse lymphoma and the group of the most effective pro-apoptotic carotenoids was also different on two cell lines. However, the levels of total apoptosis were much lower in every case than positive control M-627. While, in case of lutein and lycopene higher level of "early" apoptosis was observed and it was hardly ever followed by apoptosis event on both cell lines. Similar results were observed in case of flavonoids on colon cancer cells (177). It was also presumed that the annexin V positivity of flavonoid or carotenoid-treated cells could be a consequence of the structural alteration in cell membrane, which results in the translocation of phosphatidylserine molecules from the inside to the outer surface of the membrane (177).

The effects of **flavonoids** and isoflavonoids were also examined on the P-gp- and MRP- mediated MDR mechanisms in mouse lymphoma and human breast cancer cell lines. Differences in the activities of the P-gp and the MRP-mediated efflux pump were found in the presence of flavonoids and isoflavonoids.

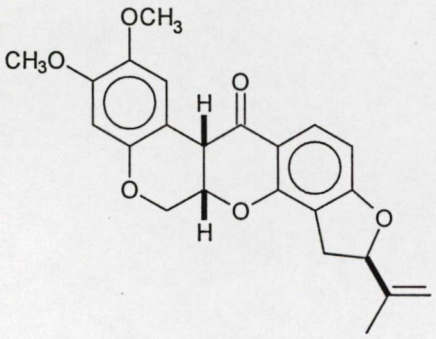
A short-term intracellular drug accumulation and a long-term viability assay were used to gain insight into various aspects of the effects of flavonoid compounds on tumor cells.

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

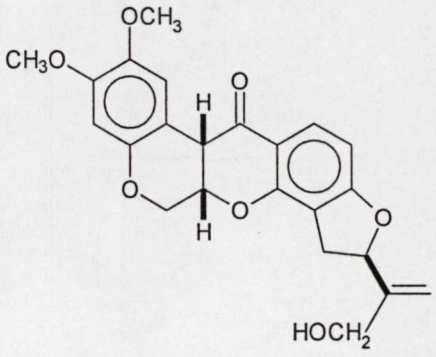
One of the differences between the two efflux systems is that P-gp has been shown to bind directly the transported drug to which P-gp confers resistance, whereas MRP pumps out some compounds through a co-transport mechanism with reduced glutathione (33, 123, 178). Other studies have demonstrated that some dietary flavonoids may modulate organic anion and GSH transport, ATPase and drug resistance (178). The soybean isoflavone genistein was found to act as an inhibitor of drug

accumulation in MRP-overexpressing cells (89, 123, 152), and also to interact with P-gp and to inhibit the P-gp-mediated drug transport (179).

Contradictory results have been reported regarding the MDR-modulating activity of the flavonoid polyphenols. Kaempferol and quercetin stimulated the P-gp-mediated efflux of doxorubicin on the adriamycin-resistant subline of the MCF-7 breast cancer cell line (180). In our experiments, kaempferol was ineffective in P-gp inhibition; however, chrysin had a significant inhibitory effect. Rotenoid derivatives, amorphigenin and rotenone, had MDR modulating activity, with a structural difference of hydroxyl(-OH) group at the position of 29th carbon. The most effective cell proliferation inhibitor was the mitochondrial electron transport inhibitor rotenone (114) which also could be responsible for caspase dependent and independent cell death (115). This may be the explanation for its very high antiproliferation and cytotoxic activity on human mdr1 gene transfected mouse lymphoma and human breast cancer cell line MDA-MB-231. This structural difference moderated the toxicity of amorphigenin compared to rotenone, although it remained significant.



rotenone



amorphigenin

The concentrations of the test compounds had great effects on the outcome of the experiments. A low concentration of quercetin, for example, activated the activity of P-gp, whereas a high concentration inhibited that of P-gp. A similar biphasic effect has been reported for kaempferol (169).

6. NEW STATEMENTS

1. Detecting P-gp molecule by mABs is not always equivalent with increased efflux activity, P-gp resitant phenotype of cancer cells. In our experiments immunocytochemy signed high presence of P-gp on MDA-MB-231; however, *in vitro* experiments these protein remained non-functional (or with very low activity) in comparison to transfected cell line or other cells with acquired resistance.
2. Beside the human *mdr1* gene transfected mouse lymphoma the **organosilicon** compounds seem to be effective on human Hep2 cell line with chemo and radioresistance. *In vivo* investigations are in progress with organosilicon compounds on Hep2C transplanted SCID mice.
3. There is not enough data to draw a conclusion on the ideal structure of **jatrophane diterpenes** with the best P-gp inhibitory activity. However, the high anti-MDR potency of jatrophanes pubescen A and D which was also very high in combination with epirubicine *in vitro*, moreover pubescen D also had shown significant MRP inhibition. The results indicated that this type of diterpenes are considered as a new chemotype of MDR1 and MRP1 inhibitors, which can be regarded as promising lead compounds for drug development programs.
4. It seems that the effect of **carotenoids** on MDR reversal and apoptotic effect of tumor cells depends on the chemical structure, polarity as well as membrane incorporation ability of carotenoid derivatives.
5. The importance of computer analysis of structure-activity relationship (SAR) should be emphasized in connection with MDR reversal. This method is used in predicting P-gp substrate specificity in "pharmaceutical profiling" of drug design. Further P-gp reversal experiment also is beneficial to use ADME computer program to predict the P-gp inhibitory probability to save money and time.
6. While effective carotenoids interact indirect with P-gp through membrane – lipid and protein interactions, flavonoids (especially rotenone and amorphigenin) rather affect directly the ATP binding domain of protein.
7. Membrane mediated mechanisms of MDR reversal through a putative modulation of membrane fluidity are likely to play a key role in reversal of resistance by carotenoids. The membrane mediated conformational changes of P-gp can be blocked by interaction of some carotenes changing the membrane fluidity.

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