Perspectives in cancer chemotherapy, *in vitro* and *in vivo* experiments

Ph.D. Thesis

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2007

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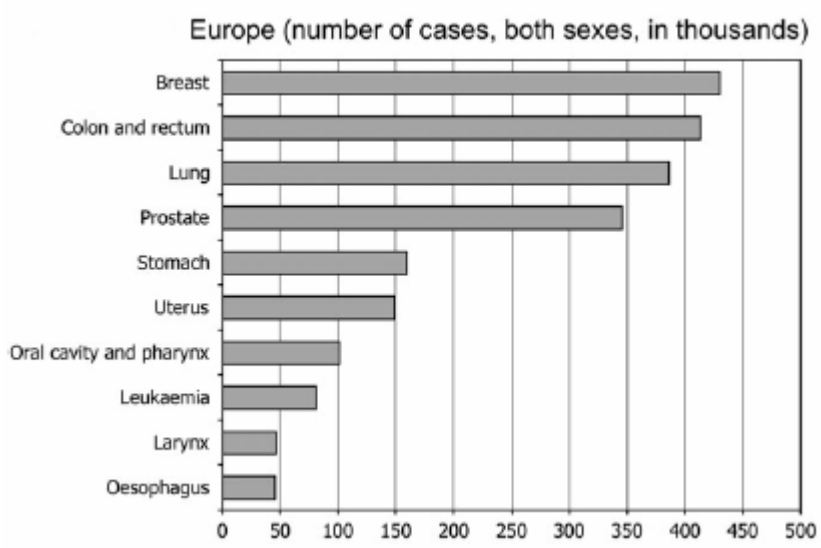
1. INTRODUCTION

1.1 Cancer incidence and mortality in Europe and Hungary

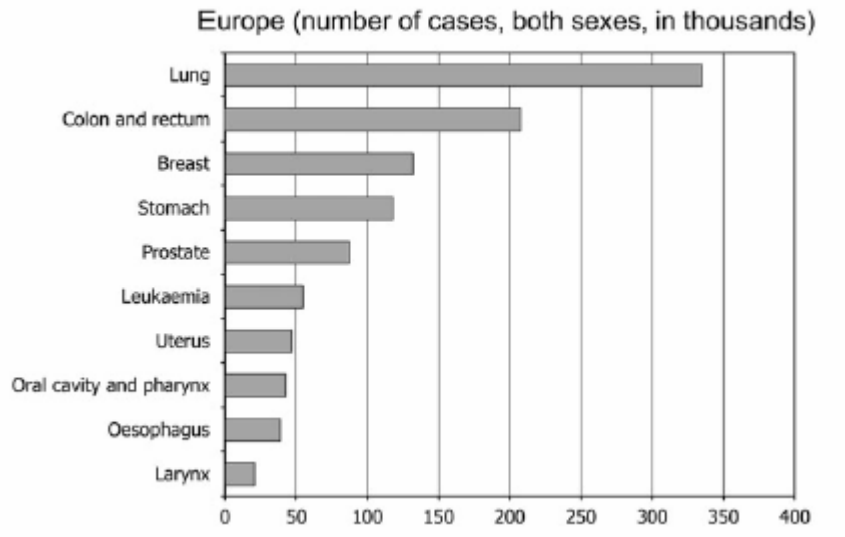
Cancer is a major public health problem worldwide. Currently, after cardiovascular diseases, cancer is the second leading cause of death in European countries (1).

Figure 1. Estimated incidence of cancer in Europe, 2006 (Ref.: 2)



In 2006 in Europe, there were an estimated more than 3 million incidence cases of cancer diagnosed (2). Breast cancer has now become the most common form of cancer diagnosed in Europe, both sexes combined (Figure 1).

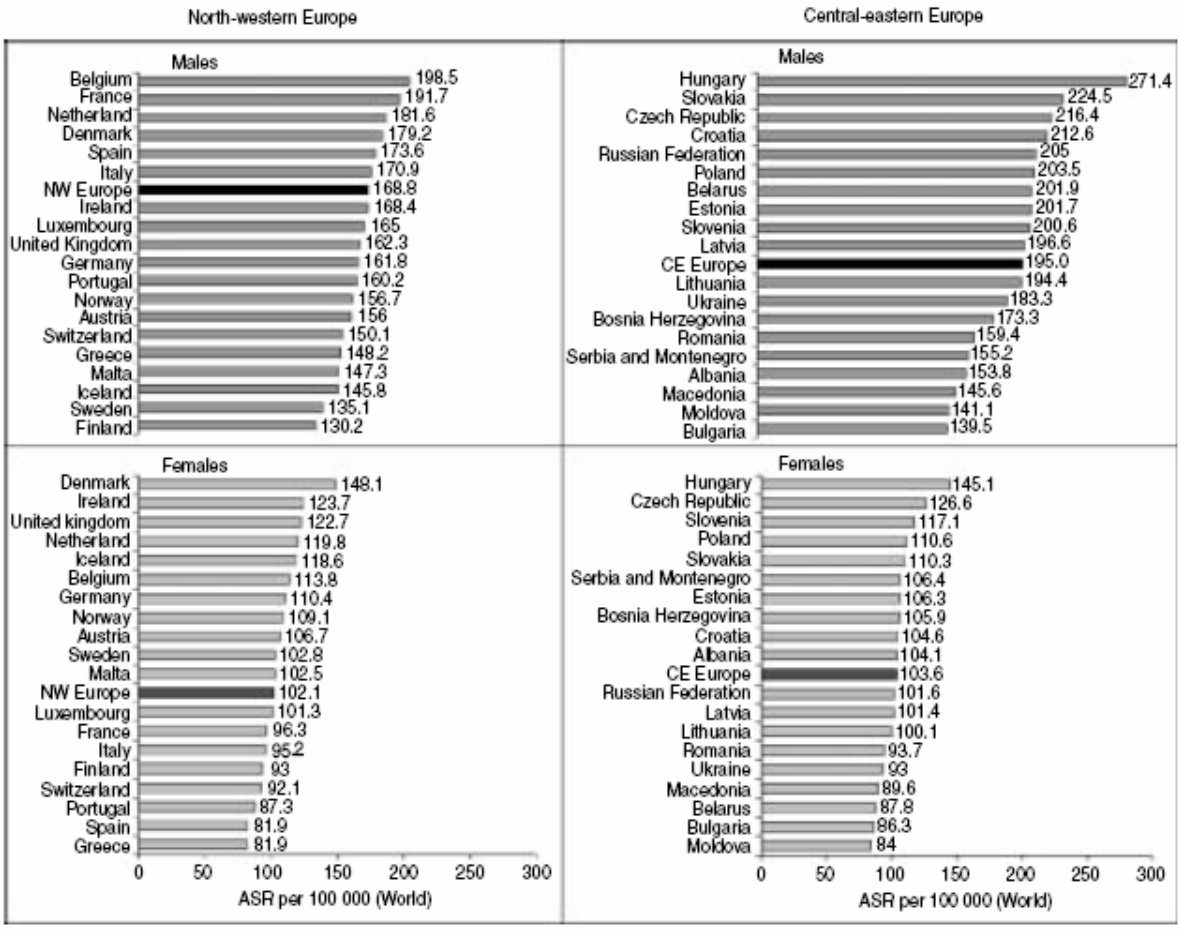
Figure 2. Estimated mortality from cancer in Europe, 2006 (Ref.: 2)



It is not only a problem for the elderly, but is also an important cause of premature mortality in the productive adult years.

Overall, 1.7 million cancer death are estimated in Europe in 2006. The most common cause of cancer death is lung cancer (Figure 2). Colorectal cancer was the second major cause of cancer death, followed by breast cancer, which now ranked higher than stomach cancer (2). Hungary has currently the highest cancer mortality rates for all cancer sites combined among men (Figure 3). Compared to Hungary, the situation in the Czech Republic is better and only Denmark produce worse data than Hungary (3).

Figure 3. Estimated age-standardized mortality rates by area and country in Europe, 2002 (Ref.: 3)



In Hungary (Table 1), the cancer mortality list is topped by lung and colorectal cancer among men and by breast and colorectal cancer among women (4).

40 % of cancer can be prevented by a healthy diet, physical activity and not using tobacco. Tobacco use is the single most important risk factor for cancer and causes a large variety of cancer types such as lung, larynx, oesophagus, stomach, bladder, oral cavity and others (5). Environmental tobacco smoke (passive smoking) causes lung cancer. Although there are still

some open questions, there is sufficient evidence that dietary factors also play an important role in causing cancer. This applies to obesity as a compound risk factor per se as well as to the composition of the diet such as lack of fruit and vegetables and high salt intake. Lack of physical activity has a distinct role as risk factor for cancer. There is solid evidence about alcohol causing several cancer types such as oesophagus, pharynx, larynx, liver, breast, and other cancer types (6).

Table 1. Newly reported cancer cases in Hungary according to sexes in 2001 (based on the National Cancer Registry)

	Men			*Women*	
	Cancer number	29 982		Cancer number	28 790
	Patient number	25 660		Patient number	25 476
1	Lung	6 099	1	Breast	5 610
2	Colorectal	3 981	2	Colorectal	3 619
3	*Skin	3 072	3	*Skin	3 307
4	Oral cavity	2 369	4	Lung	2 728
5	Prostatic	2 304	5	Lymphatic and haematopoetic system	1 598
6	Urinary bladder	1 436	6	Cervical	1 132
7	Lymphatic and haematopoetic system	1 436	7	Uterine	1 119
8	Stomach	1 248	8	Ovarian	1 027
9	Laryngeal	1 043	9	Stomach	927
10	Kidney	886	10	Pancreas	703
			11	Melanoma	701

*without malignant melanoma

One-fifth of cancers worldwide are due to chronic infections, mainly from Hepatitis B viruses HBV (7) (causing liver), human papilloma viruses HPV (8) (causing cervix), Helicobacter pylori (9) (causing stomach) and human immunodeficiency virus HIV (10) (Kaposi sarcoma and lymphomas).

1.2 Strategies for cancer treatment

Cancer is a class of diseases characterized by an imbalance in the mechanisms of cellular proliferation (growth) and apoptosis (programmed cell death). When left untreated, this imbalance results in the growth of cancerous malignancies, including solid tumours and blood-borne disease, among others, and the resulting death of the host organism. Once cancer is detected, it is removed, if possible (in the case of accessible solid tumours), and treatment is initiated. Surgery, radiotherapy, immunotherapy, hormonal therapy and chemotherapy are common treatment methods (11). Approximately 40 % of cancer patients can be treated surgically, and the great majority of patients need chemotherapy. A great number of anticancer agents are under clinical investigation at this moment. Some of them belong to

classic groups of chemotherapy (Table 2), but others are the first of new families of drugs (12).

Table 2. Useful chemotherapeutic agents (Ref. : 13)

Alkylating agents	**Antimetabolites**	**Natural products**
Nitrogen mustard (Chlorambucil, Cyclophosphamide)	Folic acid analogue (Methotrexate)	Mitotic inhibitor (Vinblastine, Vincristine)
Nitrosourea (Carmustine, Lomustine)	Pyrimidine analogue (Fluorouracil)	Microtubule polymer stabilizer
Metal salt (Cisplatin, Carboplatin)	Purin analogue (Fludarabine)	*Podophyllum* derivative (Etoposide)
Hormone / hormone antagonists (Tamoxifen, Prednisone, Flutamide,Leuprolide)	**Miscellaneous agents** (Hydroxyurea, Procarbazine, Mitotane)	Antibiotic (Daunorubicin, Doxorubicin, Epirubicin)

Some anticancer drugs are **cell cycle phase-specific** (13) *e.g.* Antimetabolites (Cytarabine, Fluorouracil, Methotrexate, Thioguanine, Fludarabine); Miscellaneous (Hydroxyurea); Natural products (Etoposide, Paclitaxel, Vinblastine, Vincristine); other are **cell cycle-specific** *e.g.* Antibiotics (Daunorubicin, Doxorubicin); **or cell cycle-non-specific agents** *e.g.* Alkylating agents (Nitrogen mustard, Nitrosourea).

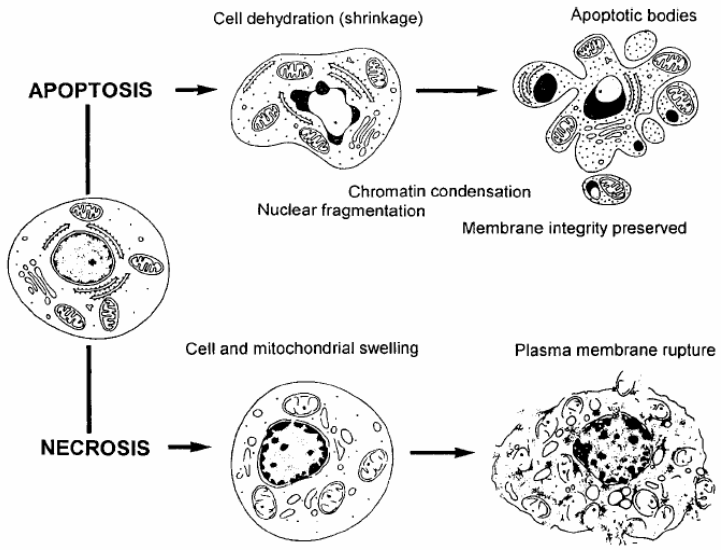
Although a number of anticancer drugs have been commercialized, the need for more effective ones continues to exist, because the most common tumours are resistance to available drugs. In fact, multidrug resistance (MDR) remains a significant problem for the effectiveness of cancer treatment (see Section 1.3).

An alternative mechanism for cancer therapy is the selective **induction of apoptosis** by various compounds (14). Several studies have shown that most chemotherapeutic agents exert their anticancer activity by inducing programmed cell death, which is an essential physiological process required to eliminate abnormal cells (15-17). Compounds, which regulate and overcome the apoptosis deficiency of cancer cells, are of great therapeutic importance, and the development of apoptosis-modulating agents has become an important approach for the discovery of new antitumour drugs.

Induction of apoptosis in tumour cells can occur by at least three different pathway involving one extrinsic (cell surface receptors), and two intrinsic pathways (the mitochondria and the endoplasmic reticulum) which, may or may not be initiated separately or concurrently (18, 19). Chemotherapeutic drugs (20), cellular conditions (serum starvation, radiation), the presence of mutated p53 can trigger the apoptotic response via one of the three pathways (21).

The apoptosis defines a genetically encoded cell death program, which is morphologically, biochemically and molecularly distinct from necrosis (Figure 4).

Figure 4: Morphological and biochemical changes during apoptosis and necrosis (Ref.: 22)



While apoptosis is characterized by an active participation (23) of the affected cell in its own demise (*e.g.* activation of endonucleases, which cleave DNA at the internucleosomal section), necrosis is a passive, catabolic and degenerative process, which always triggers an inflammatory reaction in the tissue.

1.3 Cellular drug resistance mechanisms

The ability of cancer cells to become cross-resistance to structurally and functionally unrelated anticancer drugs is known as multidrug resistance (MDR). MDR is a major cause for the failure of chemotherapy in the treatment of cancer patients (24). It has been recognized that MDR is multifactorial and factors that affect response to anticancer chemotherapy include cell kinetic (25), pharmacokinetic (26) and cellular drug resistance mechanisms (Table 3).

Table 3. (Ref.: 24)

Mechanisms of drug resistance	
Mechanism	**Individual process**
Cell kinetic resistance	Tumor growth
Pharmacokinetic resistance	Poor absorption
	Excessive metabolism
	Poor penetration to certain sites
	Blood supply of the tumor
	Drug diffusion
Cellular drug resistance	Increased drug efflux
	Decreased drug uptake
	Sequestration of drugs
	Alterations in drug targets
	Activation of detoxifying systems
	Increased repair of drug-induced DNA damage
	Blocked apoptosis
	Disruption in signaling pathways
	Alterations of factors involved in cell cycle regulation

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Cellular mechanisms are categorized in terms of alterations in the biochemistry of malignant cells (27). Such mechanisms can be further classified into two major categories: non-classical MDR phenotypes and transport-based classical MDR phenotypes.

I. *Non-classical MDR phenotypes*

The term non-classical MDR is used to describe non-transport based mechanisms that affect multiple drug classes. This type of resistance can be caused by altered activity of specific enzyme systems (such as glutathione S-transferease (GST) and topoisomerases (Topo I and II)), which can decreased the cytotoxic activity of drugs in a manner independent of intracellular drug concentrations, which remain unaltered. In addition, changes in the apoptotic pathways can also reduce chemosensitivity since most anticancer drugs are exert their cytotoxic effects via apoptotic processes (27).

1. **Drug resistance mediated by detoxification mechanism of the drug in the cells**

The cellular GST system is a critical component of detoxification of cytostatics in the cells. Glutathione (GSH), a non-protein thiol, can interact via its thiol with the reactive site of a drug, resulting in conjugation of the drug with GSH (28). The conjugate is less active and more water-soluble, and it is excluded from the cell with the participation of transporter proteins. Activation of these enzymes can cause cellular drug resistance. Increased levels of GSH were found in several resistance cell lines (29, 30).

2. **Drug resistance mediated by alterations of drug targets or by enhancement of target repair**

Topoisomerases are intrinsically involved in the process of DNA replication (31). Consequently, these enzymes constitute therapeutic targets in rapidly dividing tumour cells for anticancer drugs (32). Cells become resistant to topo II inhibitors such as anthracyclines either to the" under-expression" of Topo II (33) or topo II gene mutations (34).

Enhanced DNA repair is probably implicated in drug resistance to the drugs interacting with DNA, for example, to nitrosomethylurea or platinum derivatives. Resistance to BCNU (carmustine) is correlated with the expression of chloro-O-6-alkylguanine-DNA-alkyl-transferase. Changes in the quantities of proteins recognizing or repairing DNA injury were found in cultured cells with altered sensitivity to platinum complexes (35).

3. **Drug resistance mediated by defective apoptotic pathways**

Anticancer drugs typically induced apoptosis. Resistance may develop with loss of genes required for cell death as p53 anti-oncogene (36) or overexpression of genes (bcl-2) that block cell death (37).

II. *Transport-based classical MDR phenotypes*

Reduced intracellular accumulation has historically been associated with overexpression of the ATP-binding cassette (ABC) transporters (38). These ABC transporters serve to pump

anticancer drugs out of the cells, resulting in lack of intracellular levels of drug necessary for effective therapy (39).

1.4 ABC transporters

The ABC transporter superfamily is among the largest and most broadly expressed protein superfamilies known. In humans, 48 ABC genes that are organized into seven subfamilies (A-G) have been described, several of which are involved in well-defined genetic disorders (40).

ABC transport proteins that have been implicated in multidrug resistance include the 170 kDa **multidrug resistance protein 1** (**MDR1)** also called **P-glycoprotein (P-gp)** (encoded by ABCB1), the 190 kDa **multidrug resistance related protein 1 (MRP1)** (encoded by ABCC1). Other MRP family members that have the potential to confer multidrug resistance are MRP2 (encoded by ABCC2), MRP3 (encoded by ABCC3), MRP4 (encoded by ABCC4), MRP5 (encoded by ABCC5), MRP6 (ABCC6) and MRP7 (encoded by ABCC10) (28, 41-42). Two additional members, MRP8 (ABCC11) and MRP9 (ABCC12) have been reported recently (43, 44).

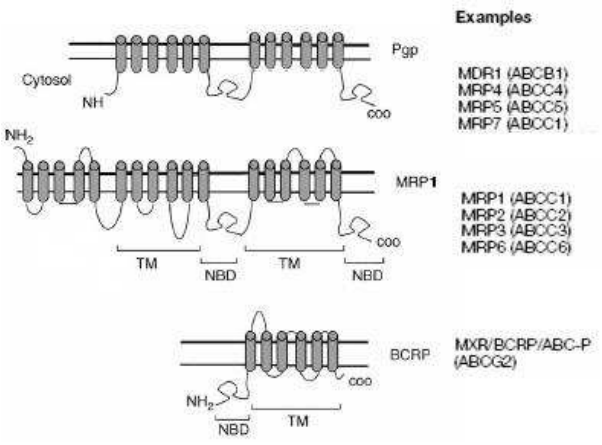
Studies in human cancer cell lines selected for mitoxanthrone resistance resulted in the characterization of another member of the G subfamily of ABC transporters. The transporter called as **breast cancer resistance protein (BCRP)** or mitoxanthrone resistance gene (MXR) (encoded by ABCG2) (45, 46). The **lung resistance protein (LRP),** also known as the major vault protein, is not an ABC transporter but it is frequently expressed at high levels in drug-resistant cell lines and tumour samples (47). Vaults are ribonucleoprotein particles that are present in the cytoplasm of most eukaryotic cells and might confer drug resistance by transporting drugs away from their intracellular targets and/or by the sequestration of drugs (48).

The role of P-gp in MDR is best characterized. P-gp occurrence in clinical tumours has been shown to occur both during diagnosis as well as during relapse. For example, P-gp was detected at the time of diagnosis in leukaemias (49); lymphomas (50); adult and childhood sarcomas (51), and neuroblastomas (52). P-gp detection also appears to correlate well with poor response to chemotherapy (53).

Although these transporters have been identified as drug-resistance proteins, they are all expressed in normal tissues important for absorption (*e.g.*, lung and gut) metabolism and elimination (liver and kidney) (39). In addition, they 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).

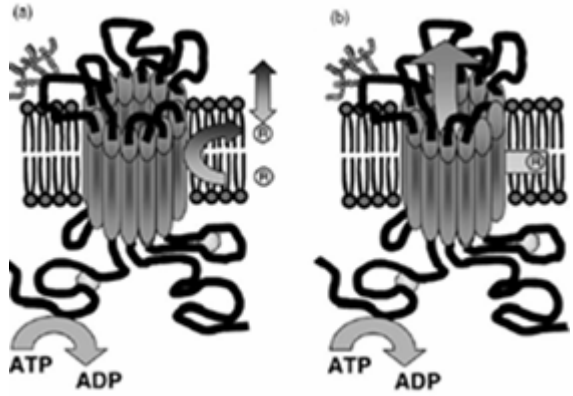
The structures of the ABC transporters. The functional unit of typical ABC transporters consists of two **nucleotide-binding domains (NBD),** which bind and hydrolyze ATP and two **transmembrane domains (TM),** each with **six transmembrane segments** (Figure 5). The NBD harbours several conserved sequence motifs (55). From NH_2 to COOH terminal, these are **Walker A** (P-loop), the family-specific **ABC signature (LSGGQ) motif** (also called C-loop), the **Walker B motif**, and a conserved His (His-switch). The lysine residue in the Walker A motif is involved in the binding of the ß-phosphate of ATP while the aspartic acid residue in the Walker B motif interacts with Mg^{2+} (56). The ABC signature motif is diagnostic for the family as it is present only in ABC proteins, and its facilitates the formation of the nucleotide sandwich dimmer (57-59). Finally, the Ser residue in each of the ABC signature sequence motifs appears necessary for the interaction between the Walker A and ABC signature sequences and potentially interacts with the γ-phosphate of ATP (57).

Figure 5. (Ref.: 54)



Mechanisms of drug efflux. The mechanism by which P-gp and other ABC transporters couple the hydrolysis of ATP to movement of drug across the plasma membrane is not well-defined (60). Several models have been proposed to conceptualise P-gp pump function (61, 62), the most widely accepted being ”flippase” (a) and ”hydrophobic vacuum cleaner” (b) models, (Figure 6). In ”lipase” model, substrates are flipped from the inner leaflet of the lipid belayed, to either the outer leaflet of the plasma membrane where they diffuse into the extracellular region (63). A”hydrophobic vacuum cleaner” extracting hydrophobic compounds embedded in inner leaflet of the plasma membrane and pumping them directly to the external aqueous medium (64). Experimentally it is difficult to distinguish between the two models (65, 66). Irrespective of the models proposed, efflux occurs as a consequence of ATP binding or hydrolysis and involves four distinct phases: (i) loading with drug and ATP, (ii) inducing of conformational

Figure 6. (ref: 50)



changes and altered affinity of the binding sites (drug release), (iii) ATP hydrolysis/release and (iv) a return to its starting conformation (60).

1.5 Transported substrates of ABC transporters

Based on a great deal of clinical and experimental work, it has been established that ABC transporters recognize a very large range of drug substrates, include a large number of anticancer drugs (anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxanes). Although recognized substrates are mostly hydrophobic compounds, MDR pumps are also capable to extrude a variety of amphipathic anions and cations.

P-gp (MDR1) preferentially extrudes large (M*r* > 400) hydrophobic, amphipathic molecules with a planar ring system, and often carries a positive charge at physiological pH (67), 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 (67) (Table 4).

Table 4. P-gp substrates included in the multidrug resistance spectrum (Ref.: 67)

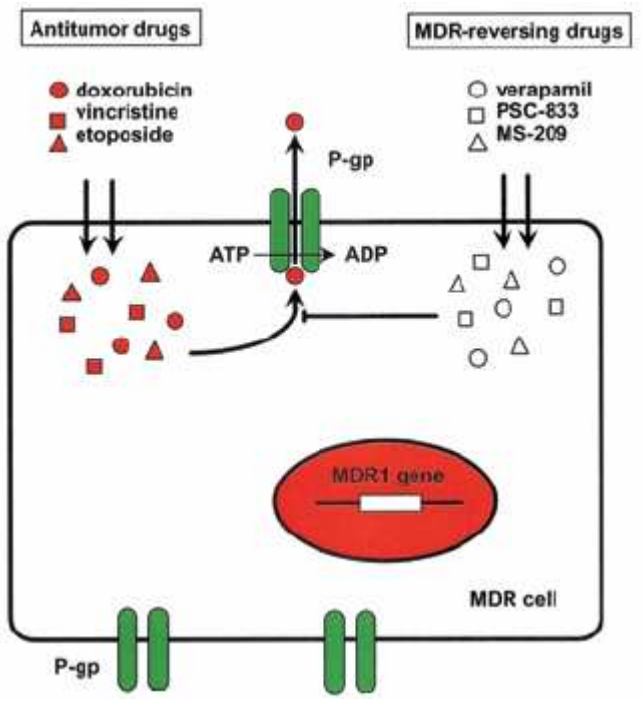
Anthracyclines	Cytotoxic agents	Steroids
Doxorubicin	Colchicine	Aldosterone
Daunorubicin	Emetine	Dexamethasone
	Actinomycin D	
Vinca alkaloids	Puromycin	Miscellaneous
Vinblastine	Mitoxantrone	Rhodamine 123
Vincristine		Hoechst 33342
	Linear and cyclic peptides	Triton X-100
Epipodophyllotoxins	NAc-Leu-Leu-norLeu-al	Prenyl-Cys methyl esters
Etoposide	NAc-Leu-Leu-Met-al	Calcein acetoxymethylester
Teniposide	Leupeptin	^{99m}Tc-SESTAMIBI
	Pepstatin A	
Taxanes	Gramicidin D	
Paclitaxel	Nonactin	
Docetaxel	Yeast *a*-factor	

MRP1 and BCRP can transport both hydrophobic drugs and large anion compounds, e.g. drug conjugates (glutathione or glucoronide conjugates) (68, 69). This ”promiscuous” character, and the additional overlapping substrates recognition by the three major classes of MDR ABC transporters, provide an amazing network of drug resistance capacity in cancer cells.

1.6 Reversal of MDR

Numerous compounds have been shown to inhibit the drug efflux function of P-gp (70, 71) and therefore, reverse cellular resistance (Figure 7). Also in our laboratory, a variety of studies have been previously performed to find MDR modulators, which, in combination of anticancer drugs, increased the anticancer effect (72-75). The first attempt was undertaken by Zamora *et al.,* and much of them simple framework is still utilized in drug development (77). Fifteen years after this investigation Wang *et al.* was described a new set of criteria for P-gp substrates (78). The criteria included (i) lipid solubility with a log P<2.92, (ii) a molecule size of approximately 18 atom length, (iii) nucleophilicity and (iv) at least 1 tertiary basic amine. Unfortunately, the pharmacophoric profile for P-gp inhibitors was not sufficiently stringent to facilitate drug development.

Figure 7. (Ref.:76)



Many of **the first generation chemosensitizers** (e.g. verapamil (79), cyclosporine A (80), tamoxifen (81), and several calmodulin antagonists (82)) were themselves substrates for P-gp and competed with the cytotoxic drugs for efflux by the P-gp pump. Therefore, high serum concentrations of these agents were needed to produce sufficient intracellular concentration. At these elevated doses, compounds exhibited severe and sometimes life-threatening toxicities (83). These dose-limiting toxic effects have precluded their application as effective MDR modulators for *in vivo* applications, particularly in the clinical practice.

The second generation modulators include either stereoisomers of their first generation racemic counterparts, such as dexverapamil (R-enantiomer of verapamil), dexniguldipine (R-enantiomer of niguldipine), or structural analogs of first generation agents (73, 84-85), such as valspodar (PSC-833) or biricodar (VX-710), quinoline derivative (MS-209). All of these compounds exhibited decreased inherent toxicity while retaining MDR reversal efficacy compared to their parent compounds (86, 87), but they also retain some characteristics that limit their clinical usefulness. Many of the second generation MDR chemosensitizers are also

substrates for cytochrom P450 3A4 and metabolized by this enzyme. The competition between cytotoxic agents and these P-gp modulators for cytochrome P450 3A4 activity has resulted in unpredictable pharmacokinetic interactions (88). Many of these MDR modulators inhibited several other ABC transporters. Inhibition of non-target transporters may enhance adverse effects of anticancer drugs (89). Side effects, due to modulation of MDR protein in normal tissues, especially blood-brain barrier should be monitored carefully to avoid neurology responses (90, 91).

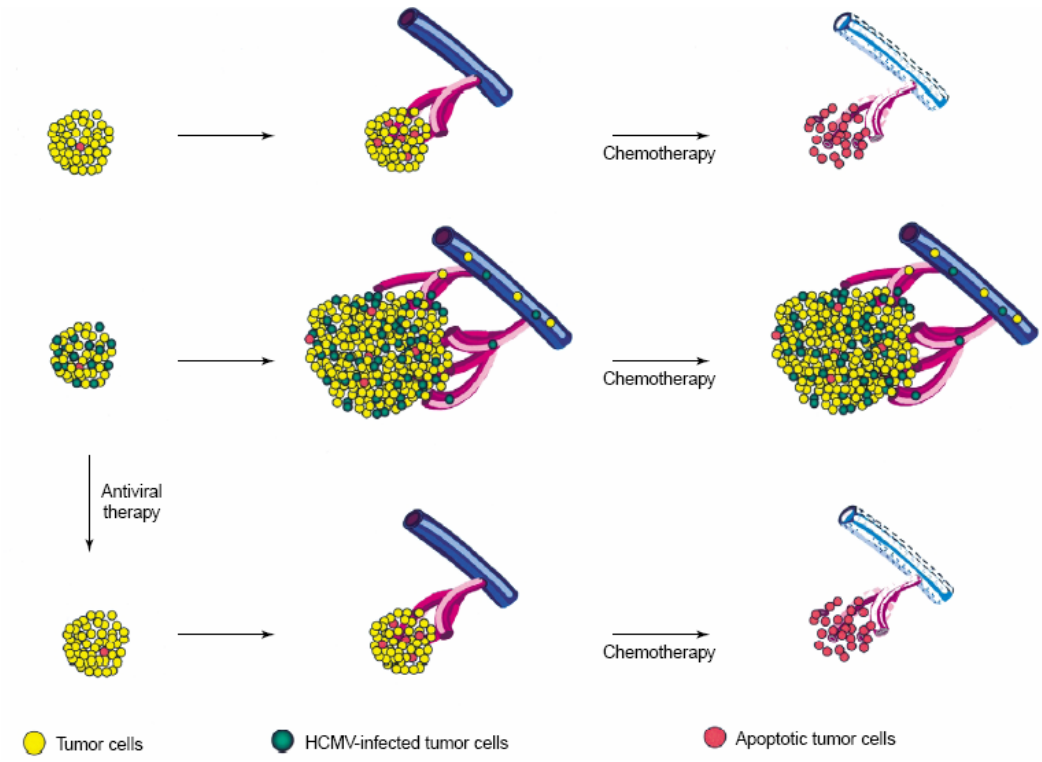
Third generation molecules have been developed to overcome the limitations of the second generation MDR modulators. They are not metabolized by cytochrome P450 3A4 and they do not alter the plasma pharmacokinetics of anticancer drugs. Third generation agents specifically and potently inhibit P-gp and do not inhibit other ABC transporters (92). These agents exhibit effective reversing at concentrations in the nanomolar range (20-100 nM), thus requiring low doses to achieve effective MDR reversal *in vivo*. Examples include specific P-gp blockers such as anthranilamide derivative tariquidar (XR9576) (93), laniquidar (R101933) (94) and the substituted diarylimidazole OC144-093 (95), the cyclopropyl-dibenzosuberane zosuquidar (LY335979) (96). Clinical trials with these new third generation agents are ongoing with the aim for a longer survival in cancer patients. This effort continues, but none of them has found a general clinical use so far (92).

1.7 Cancer chemoprevention; the oncomodulatory effect of human cytomegalovirus

Human cytomegalovirus (CMV) is a ß-herpes virus that persistently infects 50 % to 90 % of the adult population. Studies indicated the CMV infection might be associated with the pathogenesis of several human malignancies (97-99). A high frequency of CMV genome and antigens in tumour samples of patients with different malignancies such as Epstein–Barr virus (EBV) negative Hodgkin's disease, colon cancer, and malignant neuroblastoma, is well documented (98-99).

CMV infection can modulate multiple cellular regulatory and signalling pathways in a manner similar to that of oncoproteins of small DNA tumour viruses such as human papilloma virus or adenoviruses (100). However, in contrast to these DNA tumour viruses, CMV infections fail to transform susceptible normal human cells. CMV infection modulates properties of tumour cells such as growth, apoptosis, production of angiogenic factors, cell invasion and immunogenic properties (101). Inhibition of virus production through treatment of CMV-infected tumour cells with the antiviral agent, ganciclovir, restored the sensitivity to chemotherapy, lowered Bcl-2 expression, and facilitated sensitivity of apoptosis (Figure 8).

Figure 8. Tumour progression induced by CMV infections (Ref.: 102)



The oncomodulatory effects are mediated mainly by the activity of CMV regulatory proteins and rely on the persistence of viral infection in malignant cells. The sequential expression of the CMV genome has been divided into three phases: immediate early (IE), early (E) and late (L) antigen based on the appearance of the respective mRNA or protein (100). Both latent infection and reactivation are determined by the activity of IE gene products. IE gene products accumulate in infected cells causing disturbance of host cell functions.

The development of strategies to inhibit human CMV IE antigen expression and/or function is an important goal to prevent and treat certain forms of cancers associated with human CMV.

2. AIMS OF THE STUDY

In the past few years, different studies have been performed with the aim of developing effective chemosensitizers to overcome the multidrug resistance of human cancer cells. In spite of the great number of MDR modulators known, no effective inhibitor without side effects is still available for clinical practice.

Our purpose was to find MDR reversal agents less toxic than verapamil among various synthetic compounds: cinnamylidene ketones; 1,4-dihydropyridines; phenothiazines; HSP90 inhibitor peptides; betti base derivative of tylosin and some naturally occurring plant derived jatrophane and lathyrane-type diterpenes.

The effect of selected MDR reversal compounds were studied in combination with anticancer chemotherapeutics both *in vitro* and *in vivo*. Apoptosis induction and inhibition of tumour promotion were studied as well.

Main goals of the study in details:

I. Antiproliferative and cytotoxic effect of tested compounds

Before starting, the multidrug resistance studies the antiproliferative and cytotoxic properties of the compounds were determined and compared on various cell lines

II. Reversal of MDR1 by flow-cytometric assay

1. Reversal of multidrug resistance on human *mdr1* gene-transfected mouse lymphoma cell line (L 5178 Y) by various compounds (cinnamylidene ketones, 1,4-diphenyl-1,4-dihydropyridines, phenothiazines, HSP90 inhibitor peptide derivatives and Betti base of tylosin derivative)
2. MDR1 reversal effect of cinnamylidene ketones, 1,4-diphenyl-1,4-dihydropyridines, phenothiazines and certain *Euphorbia* diterpenes on human colon cancer cells (COLO 320)

III. Interaction of resistance modifiers with anticancer drugs

1. Antiproliferative effect of the combination of some representative resistance modifiers and some group representative anticancer drugs (doxorubicin, epirubicin, paclitaxel, vincristine) were examined by checkerboard microplate method *in vitro*
2. To investigate the *in vivo* P-glycoprotein inhibition of the most effective MDR-reversal Betti base of Tylosin derivative, N-tylosil aminonaphtol (TBN), DBA/2 mice were used bearing syngenic resistance T-cell lymphoma L 5178 Y. The doxorubicin

accumulation in tumour in the presence or absence of modulator was compared with *in vitro* results

IV. Apoptosis induction of some resistance modifiers

1. As an alternative means of cytotoxic effects, programmed cell death induction was studied of the most effective resistance modifiers: two cinnamylidene derivatives, two dihydropyridines and one *Euphorbia* jatrophane diterpene by flow cytometry using human *mdr1* gene-transfected mouse lymphoma cells
2. Analysis of apoptotic events by staining with ethidium bromide and acridine orange, on human cervical adenocarcinoma cell line (HeLa) was also examined in the presence of one representative jatrophane diterpene

V. Anti-promotion effect of plant derivatives

To search for antitumour promoters from plants sources, the effect of some macrocyclic lathyrane-type diterpenoids, were studied on the expression of human cytomegalovirus immediate-early (IE) antigen in human lung cancer cells (A549)

3. MATERIALS AND METHODS

Chemicals

Synthetic compounds

- **Cinnamylidene derivatives** (See Appendix 1.)
- **1,4-Diphenyl-1,4- dihydropyridine derivatives** (See Appendix 2.)
- **Phenothiazine derivatives:** Perphenazine (Bracco-Italy), promethazine hydrochloride (EGYT-Hungary), oxomemazine (Rhone-Poulence-France), methotrimeprazine maleate (EGYTE-Hungary), trifluoropromazine hydrochloride (Squibb-England), trimeprazine (Rhone-Poulence-France), prochlorperazine dimaleate (Farmitalia) (See Appendix 3.)
- **Heat Shock Protein 90 (HSP90) inhibitor peptide derivatives**: *D*-Trp-Phe-*D*-Trp-Leu-AMB (**1**), *p*-HOPA-*D*-Trp-Phe-*D*-Trp-Leu-ψ(CH_2NH)-Leu-NH_2 (**2**), *D*-Trp-Phe-*D*-Trp-OH (**3**), Suc-*D*-Trp-Phe-*D*-Trp-Leu-AMB (**4**), *D*-Tyr-Phe-*D*-Trp-Leu-AMB (**5**), *D*-Arg-*D*-Trp-Phe-*D*-Trp-Leu-Leu-NH_2 (**6**), Leu-ψ (CH_2NH)-Leu-NH_2x2HCl (**7**), Phe-Trp-Phe-Trp-Leu-Leu-NH_2 (**8**), Tyr-Trp-Phe-Trp-Leu-Leu-NH_2 (**9**) and Tyr-*D*-Trp-Phe-*D*-Trp-Leu-Leu-NH_2 (**10**)
- Betti-base derivative of tylosin (**TBN**) (103) (See Appendix 4.)

Natural compounds

- ***Euphorbiaceae* diterpenes**: The compounds were isolated from the lipophilic phase of methanol extracts of *Euphorbia esula* (compounds **1**, **2** and **3**), *E. peplus (*compounds **4** and **5**), *E. villosa* (compound **6**) and *E. serrulata* (compound **7**, **8** and **9**) (See Appendix 5.)
- **Macrocyclic lathyrane-type diterpenes**: Compounds were isolated from the methanol extract of *Euphorbia lagascae* (See Appendix 6.)

Other chemicals were used in *in vitro* assays:
Verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary); phosphate-buffered saline and dimethyl sulfoxide (PBS and DMSO; Sigma-Aldrich Ltd., Budapest, Hungary); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, sodium dodecyl sulphate and Rhodamin 123 (MTT, SDS and R123; Sigma, St Louis, MO, USA); Doxorubicin hydrochloride (Wako Pure Chem, Ind, Osaka, Japan); epirubicin hydrochloride (Farmitalia Carlo Erba, Milano, Italy);
Annexin-V-FITC (Annexin-V; human recombinant-FITC; Alexis Biochemical, Grünberg, Germany); propidium iodide and ethidium bromide/acridine orange (PI and EB/AO; Sigma-Aldrich Ltd., Budapest, Hungary);
Monoclonal antibody (MAB810; Chemicon International Inc., Temecula, CA, USA); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich Ltd., Budapest, Hungary);

The following compounds were used in *in vivo* assays:
Natrium-pentobarbital (Nembutal, Cave-Sante Animale, Brussels); Doxorubicin HCl and daunorubicin HCl were purchased as a powder for injection from Teva Pharma (Wilrijk, Belgium) and Aventis Pharma (Brussels, Belgium); water was purified by the Milli-Q

system (Millipore, Milford, USA) and acidified water was prepared by the addition of perchloric acid Sigma –Aldrich (Steinheim, Germany);

3.2 Cell cultures

3.2.1 The L 5178 Y mouse T-cell lymphoma cells (obtained from Prof. Gottesmann, NCI and FDA, USA) were transfected with pHa MDR1/A retrovirus, as previously described (106). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. The parent L5178 Y (PAR) mouse T-cell lymphoma cells and the transfected subline (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum (HS; Gibco, Auckland, New Zealand) L-glutamine and antibiotics.

3.2.2 The human colon cancer cells (COLO 320) were cultured in RPMI 1640 medium (Gibco BRL, Gland Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosci, Lenexa, KS, USA), 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes.

3.2.3 The human cervical adenocarcinoma cells (HeLa) were cultivated in Eagle's MEM (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids and an antibiotic-antimycotic mixture.

3.2.4 The human lung alveolar epithelial cells (A549) were cultivated in Eagle's MEM supplemented with 10% fetal calf serum (FCS) and for immunofluorescence studies cells were grown on glass coverslips in 24 well plates containing $2x10^5$ cells/ well.

3.2.5 Normal human lung fibroblast (MRC 5) were cultivated in Eagle's MEM supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10% FBS supplemented with antibiotics

The cell lines were incubated in a humified atmosphere (5% CO_2, 95% air) at 37 °C. The semiadherent human cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37 °C.

3.3 Animals

DBA/2 inbred mice (female, 5-7 weeks old) were obtained from Charles River Laboratory (France). After an experiment, the animals were killed by cervical dislocation. All aspects of the animal experiment and husbandry were carried out in compliance with national and European regulations and were approved by the Animal Care and Use Committee of K. U. Leuven.

3.4 Virus

The stock of human CMV laboratory-adapted strain "Towne" was propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics. The infectivity titer was determined by plaque assay with the inoculation of confluent MRC 5 in 24 well plates.

3.5 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 as described in Publication I and V.

(In case of cytotoxicity test, the cell number was higher (3×10^4/well) and the incubation time was only 48 h.) 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 (Labsystems, Helsinki, Finland). Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \left[\frac{OD\,sample - OD\,medium\ control}{OD\,cell\ control - OD\,medium\,control} \right] \times 100 \qquad \text{(Equation 1)}$$

3.6 Flow-cytometric assay for R123 accumulation in tumour cells

Reversal of MDR was examined by using a standard functional assay with R123. The fluorescence of the cell population was measured by flow cytometry using a Beckton Dickinson FACScan instrument (cell sorter, Oxford, U.K.) as described previously (Publication I, II and Ref.:74).

The fluorescence of activity ratio (FAR) was calculated (107), based on the measured fluorescence intensities (FL1):

$$FAR = \frac{FL1\,treated\ cells}{FL1\,untreated\ cells} \qquad \text{(Equation 2)}$$

The results are one representative experiment of flow cytometry in which 10 000 individual cells were investigated, because experiments cannot be repeated exactly.

3.7 Checkerboard microplate methods 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 and the resistance modifiers in combination were studied on various cancer cell lines (see Publication I, IV, VI and Ref.: 75). The cell growth rate was determined after MTT staining and the intensity of the blue colour was measured on a micro ELISA reader. Drug interactions were evaluated according to the following expressions:

$FIC_A = ID_{50A\ in\ combination} / ID_{50A\ alone}$ (Equation 3)

$FIC_B = ID_{50B\ in\ combination} / ID_{50B\ alone}$ (Equation 4)

where ID is the inhibitory dose, and FIC is the fractional inhibitory concentration.

The fractional inhibitory index, $FIX = FIC_A + FIC_B$, demonstrates the effect of the combination of the anticancer drug and the tested compound. It is accepted that, if the value of FIX is 0.51-1.00, it is an additive effect; if FIX is <0.50, it is a synergistic one; a FIX value in the interval 1.00-2.00 is considered an indifferent effect, while a value >2.00 indicates an antagonistic effect (108).

3.8 Cellular accumulation and cytotoxicity of doxorubicin *in vitro*

Parental or transfected L5178 Y cells were plated using 24 well plates ($4x10^6$ cell/1.5ml per well) in serum-free medium. TBN (10μM) was added (or not) 30 min before exposing the cells to 40 μM doxorubicin at 37 °C for 1 h. The cell suspensions were centrifuged on 4500 rpm for 5 min and washed twice with ice-cold PBS. After resuspending the cells in water, doxorubicin was extracted (using 60% ethanol +0.3 M HCl) and quantified by high performance liquid chromatography (HPLC). For the toxicity assay, the cell suspensions were also centrifuged on 4500 rpm for 5 min and washed twice in serum-free medium. The cells were cultured for 48 h at 37 °C using 96 well plates (10^5 cell/0.15ml per well) in serum

supplemented medium. Cell proliferation was evaluated by the above mentioned MTT test. Statistical analysis was performed by using an ANOVA model.

3.9 *In vivo* studies

3.9.1 *In vivo doxorubicin accumulation*

DBA/2 mice were anesthetized and injected subcutaneously with 4 x 10^6 cells of the parental or transfected L 5178 Y cells. Tumours were allowed to grow to ca 0.5 cm diameter, after which the animals were treated once with various regimens of TBN (10 and 50 mg/kg) or vehicle, and doxorubicin (10 mg/kg) or vehicle, administered either i.p. or i.v. via a lateral tail vein. Tumours were excised 24 h after i.p. or i.v. administration and stored at -20°C until extraction and HPLC analysis.

3.9.2 Sample extraction and doxorubicin quantification

The amount of doxorubicin in frozen tumour quantified as described by Asperen *et al.* (109). Samples were thawed and thoroughly homogenized. Dilutions of the homogenate were prepared in water (100 mg/mL), and 200 µL aliquots were diluted with an equal volume of 6% (w/v) borate buffer (pH 9.5) and 50 µL internal standard (daunorubicin) working solution. After vortexing, the analytes were extracted with 1 mL of a chloroform/propanol mixture (4:1, v/v) by mixing for 5 min, followed by centrifugation for 10 min at 4°C (6000 rpm). The aqueous layer and the pellet were removed by suction, and the isolated organic layer evaporated using a Speed-Vacuum system (Reciprotor, Denmark) at 43°C. The residue was reconstituted in 75 µL of acetonitrile-tetrahydrofuran (40:1, v/v), vortexed for 20 s and then sonicated for 5 min. After adding 125 µL acidified water (pH 2.05), a 20 µL aliquot was analyzed by LC. The LC system consisted of a Hitachi Elite LaChrom L-2130 solvent delivery module and a Hitachi Elite LaChrom L-2480 fluorescence detector (Hitachi High-Technologies Corporation Tokyo, Japan). The LiChroCART 250-4 analytical column packed with 5µm Purospher STAR material (Merck, Darmstadt, Germany) was protected by a LiChrospher guard column (4x4mm) (Merck, Darmstadt, Germany). The mobile phase was composed of acidified water (pH 2.05)/acetonitrile/THF (64:35:1, v/v/v) and was degassed by ultrasonication. A flow rate of 0.8 ml/min was used. The column eluent was monitored fluorimetrically at 460 nm (ex) and 550 nm (em).

Statistical analysis. Statistical analysis of the data was performed with Graphpad Prism 2.01 (Graph Pad Software, San Diego, CA, USA).

3.10 Apoptosis assays

3.10.1 Flow-cytometric assay for 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 12*H*-benzo[α]phenothiazine (M627) was added to the samples as a positive control at a final concentration of 5 or 25 µg/mL. The M627 was synthesized by Motohashi *et al.* (110). In the cases of control cultures, 10 µL DMSO was added. The compounds used for treatment was added to the samples at a final concentration of 2 or 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 washed with PBS, and resuspended in 195 µL binding buffer. The samples were mixed, centrifuged and supernatant was removed from each tube. 5 µL Annexin V was added to the tubes. Controls without Annexin V were

also prepared. The samples and controls were incubated at room temperature for 10 min in the dark, then centrifuged, washed with PBS, and resuspended in 190 µL binding buffer. Before the measurement of fluorescence activity, 10 µL of 20 µg/mL PI was added to the samples and the apoptosis of the cells was investigated. The fluorescence activity (FL-1 and FL-2) of the cells was measured and analysed on a Beckton Dickinson FACScan instrument. In each analysis, 10 000 events were recorded, and the percentages of the cells in the different states were calculated by using winMDI2.8 (111).

3.10.2 Ethidium bromide and acridine orange (EB/AO) staining for apoptosis

Staining with EB/AO was carried out in a 96-well plate format after 24 h of treatment in order to visualize the basic morphological events (112). Plates were centrifuged at 1000 rpm for 5 min, and 8 µL of staining solution (0.1 mg/mL for both AO and EB in PBS) was added to each well. After 10 min, the cells were washed with PBS, and the cells were viewed with a Nikon Eclipse inverted microscope at 200x magnification with a 500/20 nm excitation filter, a cut-on 515 nm LP dichromatic mirror, and a 520 nm LP barrier filter (Chroma Technology, Rockingham, VT, USA). Pictures were taken with a Nikon Coopix 4500 digital camera (Nikon, Tokyo, Japan).

3.11 Anti-promotion experiments

One-day-old A549 cultures on the coverslips were infected with the "Towne" strain of CMV as described previously (Publication V). Human CMV IE1-72 antigen was detected in the nuclei of infected cells by immunostaining using monoclonal antibody (MAB810) and FITC-conjugated rabbit anti-mouse IgG. The number of IE antigen-positive cells was counted in 30 microscopic fields containing 400 cells for each sample. The frequency of IE antigen-expressing cells in the treated cultures is shown as a percentage of the control.

4. RESULTS

4.1 Antiproliferative and cytotoxic effect of tested compounds

In the first stage of study, the antiproliferative and toxic property of various compounds were determined of possible MDR modifiers. The inhibitory dose fifty (ID_{50}) values were studied in each examined compounds by MTT test.

Considering the large number of studied compounds, only some representative chemicals are shown in the table below.

As shown in Table 1, there are some interesting compounds, *e.g.:* cinnamylidene **1**, **2**, **4,** and **10,** which had higher ID_{50} values for the MDR cells than for the PAR cell line. It is possible that these compounds are substrates of the P-gp-mediated efflux pump. COLO 320 has a moderate sensitivity for the antiproliferative effects of the cinnamylidene ketones.

Table 1. Comparison of the antiproliferative effects of cinnamylidene ketones on PAR, MDR and COLO 320 cell lines

Compounds	ID_{50} values (µg/mL) ± S.E.M*		
	L 5178 Y MDR	L 5178 Y PAR	COLO 320
1	98.99 ± 0.89	60.00 ± 0.99	10.83 ± 0.39
2	43.75 ± 0.65	41.04 ± 0.44	6.00 ± 0.11
3	3.31 ± 0.12	11.76 ± 0.12	0.75 ± 0.02
4	49.00 ± 0.55	25.89 ± 0.88	16.80 ± 0.19
5	9.97 ± 0.05	20.18 ± 0.15	5.05 ± 0.78
6	2.31 ± 0.03	65.34 ± 0.34	8.72 ± 0.67
7	15.21 ± 0.19	89.35 ± 0.98	7.05 ± 0.03
8	37.26 ± 0.39	72.87 ± 0.23	8.58 ± 0.93
9	9.91 ± 0.34	22.23 ± 0.03	6.36 ± 0.77
10	51.76 ± 0.99	46.87 ± 0.56	8.94 ± 0.56
11	3.99 ± 0.78	67.44 ± 0.42	4.58 ± 0.88
12	12.68 ± 0.11	33.31 ± 0.30	9.36 ± 0.93
13	52.35 ± 0.02	62.53 ± 0.74	13.22 ± 0.16
14	4.88 ± 0.44	13.51 ± 0.12	0.68 ± 0.09
15	15.17 ± 0.22	65.00 ± 0.94	3.11 ± 0.07
16	17.34 ± 0.98	70.00 ± 0.22	17.02 ± 0.85
DMSO	23.50 ± 0.50	52.50 ± 0.11	11.34 ± 0.46

*All values are expressed as mean ± S.E.M (standard errors of mean) from parallel experiments (n=2-4)

The cytotoxicity of dihydropyridines was also examined by using seven human cell lines (Publication II). The results confirmed the tumour specificity of some compounds. The dihydropyridine derivatives were more cytotoxic against four oral tumour cell lines as

compared with normal cells. However, the tumour cell lines display considerable variation in sensitivity. On the other hand, the normal cells were all comparable in sensitivity.

Plants of the genus *Euphorbia* are known to produce a large variety of diterpenoids; some of them display tumour-promoting activity, while others exhibit cytostatic activity (113). In order to prepare the long-term combination experiments, the antiproliferative effect of several new jatrophane diterpene polyesters were tested on COLO 320 cell line. The similar effect on human *mdr1* gene-transfected mouse lymphoma cell line was earlier studied (114, 115). The applied chemotherapeutic, epirubicin reduced the proliferation of COLO 320 cell line (ID_{50}=0.10±0.03); a majority of the compounds exhibited a moderate antiproliferative effect. ID_{50} values of most of diterpene derivatives are similar (between 5 and 17).

The cytotoxicity of macrocyclic lathyrane diterpenes on the human lung cancer (A549) and on the normal human lung fibroblast (MRC 5) was measured (Table 2). In order to assess the preferential toxicity for malignant cells, tumour specificity (TS) was calculated for each compounds. The TS values indicated that **Latilagalascene C** and **Jolkinol B** had the most noteworthy tumour-specific cytotoxic action. The cancer cells were apparently more sensitive than the MRC5 cell line to the selected diterpenes.

Table 2. Cytotoxic effects of lathyrane-type diterpenes on A549 lung cancer cell line and on MRC 5 normal lung fibroblasts

Samples	A549 ID_{50} (μg/mL)	MRC 5 ID_{50} (μg/mL)	TS*
Latilagascence A (1)	33.45	49.72	1.48
Latilagascence B (2)	43.33	66.40	1.53
Latilagascence C (3)	43.26	>150	> 3.46
Latilagascence D (4)	150.39	106.87	0.71
Latilagascence E (5)	51.74	112.87	2.18
Jolkinol B (6)	52.02	>200	> 3.88

*TS= ID_{50} (normal) / ID_{50} (tumour)

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

4.2 Reversal of MDR1 by flow-cytometric assay

The standard assay to evaluate the potency of various compounds as MDR modulators is based on the increased accumulation of Rhodamin 123 in MDR and sensitive cells.

4.2.1 Reversal of multidrug resistance on human *mdr1* gene-transfected mouse lymphoma cell line (L 5178 Y)

4. 2. 1. 1 Cinnamylidene ketones

Some of the cinnamylidene derivatives were able to enhance the drug accumulation of MDR cells markedly: **1, 2**, **4, 7, 8, 10** and **11** (Table 3). The compounds displayed dose-dependent inhibition of the MDR P-gp. At the same time, certain cinnamylidene derivatives (**5**, **6**, **9, 13** and **16)** were hardly effective in inhibiting the naturally occurring MDR of the mouse lymphoma cells. The FAR value of reference compound, verapamil was 4.235 in 10 µg/mL concentration.

Table 3. The effects of cinnamylidene derivatives on R123 accumulation on L 5178 Y cell line

Samples	FAR values at various concentration	
	4 µg/mL	40 µg/mL
1	1.60	18.94
2	1.32	23.61
3	2.66	14.60
4	4.19	32.73
5	1.90	2.97
6	1.13	2.05
7	3.09	26.17
8	8.32	61.30
9	1.16	1.60
10	3.28	27.34
11	10.53	19.86
12	6.52	12.34
13	1.09	2.33
14	1.82	7.73
15	8.50	13.51
16	2.09	2.18

4. 2. 1. 2 Dihydropyridine derivatives

The effects of the forty-one substituted 1,4-diphenyl-1,4-dihydropyridine derivatives on MDR reversal were investigated by treating the mouse lymphoma L 5178 Y cells transfected with the human *mdr1* gene. Certain dihydropyridines at the higher concentration of 40 µg/mL used were found to be toxic: the cell size and the intracellular structures of the cells were changed during the short-term experiments (Publication II). Then non-toxic concentration (0.4 µg/mL) was applied for experiments on R123 accumulation. At some representative samples, the results are presented in Table 4. Among them, **DL8-10**, **DL12**, **DL13** and **DL15** were found to be the most effective MDR modulator.

Table 4. The effects of 1,4-diphenyl-1,4-dihydropyridine derivatives on R123 accumulation on L 5178 Y cell line			
Samples	**FAR values at various concentration**		
	0.4 µg/mL	**4 µg/mL**	**40 µg/mL**
DL1	9.53	24.50	12.09
DL2	11.01	47.34	33.88
DL3	20.80	49.81	30.27
DL4	16.43	45.51	28.70
DL5	5.33	20.22	10.31
DL8	10.00	115.67	178.03
DL9	*	54.49	104.58
DL10	*	4.99	139.61
DL12	*	7.50	77.03
DL13	*	12.64	91.07
DL15	5.69	144.00	158.00
DP70	5.09	40.00	38.89
DP80	16.93	41.62	40.91
DP82	8.03	52.08	19.73
DP89	1.07	50.14	8.14

The FAR value of reference compound, verapamil was 12.77 in 10 µg/mL concentration.
*the MDR reversal effect of these compounds tested only in 4 and 40 µg/mL concentrations

4. 2. 1. 3 Phenothiazines

Seven phenothiazine derivatives were tested on MDR mouse lymphoma cells (Table 5).

Table 5. The effects of phenothiazine derivatives on R123 accumulation on L 5178 Y cell line		
Samples	**FAR values at various concentration**	
	4 µg/mL	**40 µg/mL**
Oxomemazine	1.14	4.58
Perphenazine	10.66	1.14*
Prometazine hydrochloride	1.23	4.03*
Methotripromazine maleate	2.18	5.03*
Prochlorperazine dimaleate	11.74	1.24*
Trifluoropromazine hydrochloride	4.11	1.59*
Trimeprazine	2.04	1.20*

Numbers with the superscript * indicate cytotoxic effect induced by the particular phenothiazine derivative

At concentration of 4 µg/mL all phenothiazines used were moderately active, however only two of them (**perphenazine** and **prochlorperazine dimaleate**) were more powerful MDR inhibitors than verapamil (FAR= 8.34 at 10 µg/mL concentration). Apparently, at four concentrations used (0.5, 1, 2 and 4 µg/mL) **perphenazine** and **prochlorperazine dimaleate** affect MDR in mouse lymphoma cells (Table 6).

Table 6. The effects of perphenazine and prochlorperazine dimaleate on R123 accumulation on L 5178 Y cell line					
Samples	**FAR values at various concentration**				
	0.5 µg/mL	**1 µg/mL**	**2 µg/mL**	**4 µg/mL**	**10 µg/mL**
Verapamil					12.30
Perphenazine	1.73	5.52	9.00	12.60	
Prochlorperazine dimaleate	1.44	7.28	7.97	9.45	

Moreover, the perphenazine stimulating effect on Rhodamin 123 accumulation was stronger than that of Verapamil.

4. 2. 1. 4 HSP 90 Inhibitor Peptide derivatives

When peptide derivatives were tested on the mouse lymphoma cells, peptide **1** and **4** displayed a dose-dependent increase in the FAR value (Table 7). The majority of antiproliferative peptide practically ineffective in MDR reversal.

Table 7. The effects of HSP90 inhibitor peptide derivatives on R123 accumulation on L 5178 Y cell line		
Samples	**FAR values at various concentration***	
	8 µg/mL	**40 µg/mL**
Peptide **1**	12.15	13.00
Peptide **2**	1.18	1.31
Peptide **3**	1.10	0.92
Peptide **4**	1.22	10.47
Peptide **5**	1.07	1.03
Peptide **6**	0.97	0.83
Peptide **7**	0.89	0.98
Peptide **8**	1.01	1.05
Peptide **9**	1.07	1.41
Peptide **10**	0.81	0.84

* Verapamil (FAR=7.85 in 10 µg/mL concentration)

Compound **5** proved ineffective in inhibiting the ABC transporters, but was selected as a negative control in the flow-cytometric experiments because of its somewhat similar chemical structure to that of peptide derivative **4.** Apparently, derivatives having D-Trp or D-Tyr residue in the N-terminal position of the peptide were the effective compounds that mean some sequence specificity of peptide-P-gp interaction.

4. 2. 1. 5 Betti base of Tylosin

TBN and its two building blocks, i.e. Betti-base and tylosin, were examined in a R123 accumulation assay for their P-gp inhibitory effect, using human *mdr1* gene transfected L 5178 Y mouse lymphoma cells. The results are shown in Table 8. **TBN** resulted near 100 fold higher R123 accumulation in mouse lymphoma cells than its two building blocks still at low concentration, while its precursors shown only limited effect. In these experiments, no sign of toxicity or cell damage were observed.

Table 8. The effects of TBN and its two precursor moiety on R123 accumulation on L 5178 Y cell line

Samples	FAR values at various concentration*	
	4 µg/mL	40 µg/mL
Betti base	10	8
Tylosin	1	5
TBN	105	94

*FAR value of Verapamil was 10 in 10 µg/mL concentration

4.2.2 MDR1 reversal effect on human colon cancer cells (COLO 320)

4. 2. 2. 1 Cinnamylidene ketones

When the cinnamylidene ketones were tested for the reversal of MDR in the human colon cancer cells, three groups could be distinguished, as may be see in Table 9.

Table 9. The effects of cinnamylidene derivatives on R123 accumulation on COLO 320 cell line

Samples	FAR values at various concentration*	
	4 µg/mL	40 µg/mL
1	0.75	8.08
2	0.80	3.16
3	1.95	13.20
4	3.36	13.06
5	0.44	0.51
6	0.66	1.38
7	1.65	20.03
8	4.27	16.05
9	0.73	0.74
10	2.19	15.08
11	1.56	12.42
12	3.24	3.89
13	0.86	1.35
14	1.89	18.37
15	2.27	6.79
16	1.58	2.07

*FAR value of Verapamil was 4.245 in 10 µg/mL concentration

The first group contains the most effective compounds, **1, 3, 4, 7, 8, 10, 11** and **14**. The compounds in the second group, **5, 6, 9** and **12,** were practically ineffective. In the third group, moderate increases in drug accumulation were found in the presence of compounds **2, 13, 15** and **16.**

4. 2. 2. 2 Dihydropyridine derivatives

The dihydropyridines displayed lower FAR values in human colon cancer cells than in human *mdr1* gene-transfected mouse lymphoma cell line, with a few exceptions (such as **DL12**, and **13).** Some representative samples revealed by Table 10 (Publication II). At higher concentration of the compounds (40 μg/mL), most cells died due to the toxicity of the chemicals. It was shown that most of the derivatives were able to increase the R123 accumulation without toxic effects.

Table 10. The effects of 1,4-diphenyl-1,4-dihydropyridine derivatives on R123 accumulation on COLO 320 cell line

Samples	FAR values at various concentration		
	0.4 μg/mL	4 μg/mL	40 μg/mL
DL1	4.26	12.52	18.32
DL2	6.33	21.89	26.08
DL3	4.13	27.79	24.51
DL4	6.69	23.34	28.36
DL5	2.57	14.38	17.81
DL8	6.59	60.27	76.13
DL9	*	40.63	69.04
DL10	*	4.69	140.12
DL12	*	3.34	109.35
DL13	*	18.18	144.09
DL15	2.5	44.45	64.82
DP70	3.56	33.26	40.33
DP80	2.55	36.22	27.80
DP82	2.14	31.04	36.73
DP89	10.73	36.38	29.37

The FAR value of reference compound, Verapamil was 14.69 in 10 μg/mL concentration.

*the MDR reversal effect of these compounds tested only in 4 and 40 μg/mL concentration

4. 2. 2. 3 Phenothiazines

Some phenothiazines (**perphenazine**, **prochlorperazine dimaleate** and **trifluoropromazine hydrochloride**) were found to be toxic at the applied higher concentration in COLO 320 cell line, the extent that it was not able to examine the cells under the test conditions (Table 11). If

the drug concentration was reduced obviously, it behaves as an effective MDR inhibitor and did not cause cell death (Table 12).

Table 11. The effects of phenothiazine derivatives on R123 accumulation on COLO 320 cell line

Samples	FAR values at various concentration**	
	4 µg/mL	40 µg/mL
Oxomemazine	1.74	3.04
Perphenazine	2.77	2.17*
Prometazine hydrochloride	1.58	2.41
Methotripromazine maleate	2.29	2.10
Prochlorperazine dimaleate	3.45	2.12*
Trifluoropromazine	2.56	0.83*
Trimeprazine	1.91	2.09

Numbers with the superscript * indicate cytotoxic effect induced by the particular phenothiazine derivative

** verapamil (FAR= 4.52)

On the other hand, in COLO 320 cell line the observed effect of phenothiazines did not differ from that of verapamil positive control, while both phenothiazines had stimulated Rhodamin 123 efflux in cytotoxic concentrations from these cells due to their membrane desintegration.

Table 12. The effects of perphenazine and prochlorperazine dimaleate on R123 accumulation on COLO 320 cell line

Samples	FAR values at various concentration				
	0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL	10 µg/mL
Verapamil					6.89
Perphenazine	1.02	3.21	4.48	4.50	
Prochlorperazine dimaleate	0.99	3.04	3.38	4.47	

4. 2. 2. 4 *Euphorbia* diterpenes

Our research group previously reported that certain *Euphorbia* diterpenes may reverse MDR by inhibiting P-gp in human *mdr1* gene-transfected mouse lymphoma cells (116). Their MDR reversal effects on human colon cancer cell line were also studied (Publication VI).

Compounds **6**, **7**, **8** and **9** were found to be very strong inhibitors (FAR = 1.49-2.20 at 4 µg/mL). For compound **7**, the effect was almost the same at the two concentrations, meaning that both of the applied concentrations were close to the saturation zone. Compounds **1-5** were moderately effective in modulating the P-gp on the resistant human colon cell line.

4.3 Interaction of resistance modifier with anticancer drugs

4.3.1 *In vitro* effects of cinnamylidene derivatives in combination with doxorubicin

As a result of the MDR-reversing experiments, some effective resistance modifier cinnamylidene ketones were selected for combined chemotherapy in the checkerboard microplate method. The results can be seen in Tables 13.

Table 13. Interaction between resistance modifier cinnamylidene ketones with doxorubicin on human *mdr1* gene-transfected mouse lymphoma (L 5178 Y) and human colon cancer (COLO 320) cell line

Samples	L 5178 Y		COLO 320	
	FIX ± S. E.M*	Interaction	FIX ± S. E.M*	Interaction
1	0.83 ± 0.16	additive	0.56 ± 0.09	additive
2	0.88 ± 0.12	additive	0.99 ± 0.02	additive
3	1.26 ± 0.17	indifferent	1.18 ± 0.14	indifferent
4	0.36 ± 0.04	synergism	0.41 ± 0.02	synergism
7	1.20 ± 0.18	indifferent	1.03 ± 0.03	indifferent
8	0.41 ± 0.05	synergism	0.36 ± 0.05	synergism
10	1.18 ± 0.14	indifferent	0.74 ± 0.19	additive
11	1.04 ± 0.03	indifferent	1.14 ± 0.13	indifferent
14	1.11 ± 0.03	indifferent	1.16 ± 0.05	indifferent

*FIX-fractional inhibitory index; S.E.M were derived at least three FIX values

Synergy was found between doxorubicin and compounds **4** and **8** on L 5178 Y cells. It was interesting that the FIX values of the cinnamylidene-cycloalkanones **(1** and **2)** revealed additive effects, while compounds **3, 7, 10, 11** and **14** in combination with doxorubicin were indifferent.

When the same experiment was performed on the COLO 320 cells, only compounds **4** and **8** were able to enhance the antiproliferative activity of doxorubicin, compounds **1, 2** and **10** were shown only marginally additive.

4.3.2 Interaction between dihydropyridine derivatives in combination with doxorubicin

The nature of the interactions between doxorubicin and the resistance modifier dihydropyridines also studied on L 5178 Y and COLO 320 cell line. All the selected compounds were able to enhance antiproliferative activity of doxorubicin (Table 14).

Synergy was found between doxorubicin and **DL4** on *mdr1* gene-transfected mouse lymphoma cell line, but the other combinations resulting in additive effect on both cells.

Table 14. Interaction between resistance modifier dihydropyridines with doxorubicin on human *mdr1* gene-transfected mouse lymphoma (L 5178 Y) and human colon cancer (COLO 320) cell line				
Samples	**L 5178 Y**		**COLO 320**	
	FIX ± S. E.M	**Interaction**	**FIX ± S. E.M**	**Interaction**
DL4	0.47 ± 0.05	synergism	0.58 ± 0.07	additive
DL12	0.55 ± 0.04	additive	0.71 ± 0.07	additive
DL13	0.51 ± 0.05	additive	0.56 ± 0.05	additive
DP89	0.55 ± 0.06	additive	0.59 ± 0.05	additive

4.3.3 Antiproliferative effects of MDR modifier HSP90 inhibitor peptide derivatives in combination with epirubicin *in vitro*

Three of the MDR inhibitor peptide derivatives and the anticancer chemotherapeutic epirubicin were studied on multidrug resistant cancer cells in checkerboard assays (Table 15).

Table 15. Interaction between epirubicin and some peptide resistance modifiers		
Samples	**FIX ± S. E.M**	**Interaction**
Peptide 1	0.37 ± 0.12	synergism
Peptide 4	0.63 ± 0.11	additive
Peptide 5	0.52 ± 0.07	additive

These MDR inhibitors increased the antiproliferative effect of epirubicin on human MDR1 gene-transfected mouse lymphoma cells resulting from synergism for compound **1** and additive antiproliferative effects for compounds **4** and **5**. The effect depends on chemical structures.

4.3.4 Effects of TBN with doxorubicin, paclitaxel and vincristine

P-gp inhibitory effect of Betti base derivative of tylosin was tested in the presence of representative anticancer agents, such as doxorubicin, paclitaxel and vincristine, on human *mdr1* gene-transfected mouse lymphoma cells (Table 16).

Table 16. *In vitro* effects of TBN in combination with doxorubicin, paclitaxel and vincristine on human *mdr1* gene transfected mouse lymphoma cells		
Anticancer agents	**FIX ± S. E.M**	**Interaction**
Doxorubicin	0.16 ± 0.05	synergism
Paclitaxel	0.61 ± 0.11	additive
Vincristine	0.35 ± 0.14	synergism

Strong synergistic effect were observed between TBN and doxorubicin, however the interaction with paclitaxel was additive.

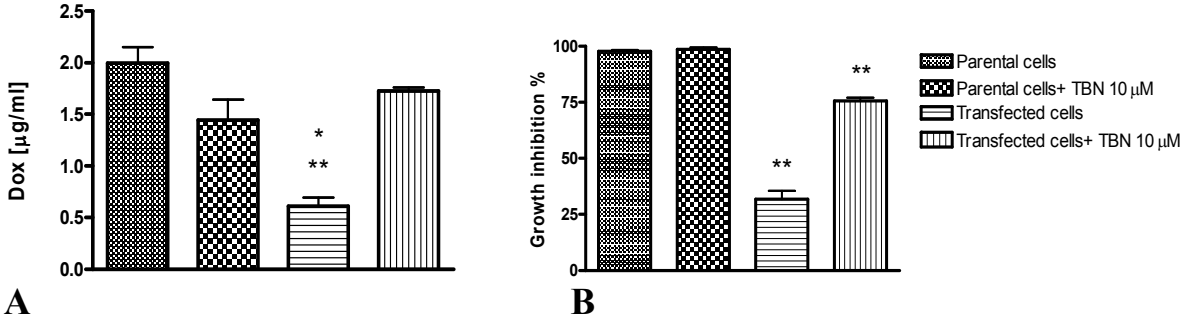
4.3.5 Interaction between *Euphorbia* diterpenes with epirubicin on COLO 320 cells

The enhanced antiproliferative activity of combinations of the diterpene compounds with epirubicin was examined. Synergistic interaction was found between epirubicin and compound **8** (Publication VI). Although compound **9** significantly increased the Rhodamin 123 drug accumulation, did not enhance the antiproliferative effect of the anticancer drug, epirubicin. Diterpenes **3**, **6** and **7** had only marginal additive effects.

4.4 Cellular accumulation and cytotoxicity of doxorubicin in mouse lymphoma cell line *in vitro*

Tumour cells with multidrug resistance are able to retain less anticancer drug than their sensitive counterparts. To test whether increased cytotoxicity correlated with enhanced intracellular concentrations of doxorubicin, we exposed the parental and transfected tumour cells to 40 μM doxorubicin for 1 h in the presence or absence of **TBN** (10 μM).

Figure 1. Doxorubicin accumulation and *in vitro* toxicity after 1h doxorubicin exposition on L 5178 Y non-resistant and resistant mouse lymphoma cell lines



Significant differences are indicated by * $p < 0.01$ and ** $p < 0.001$. Means of "resistant" was found significantly different from non-resistant and treated resistant group respectively in both cases

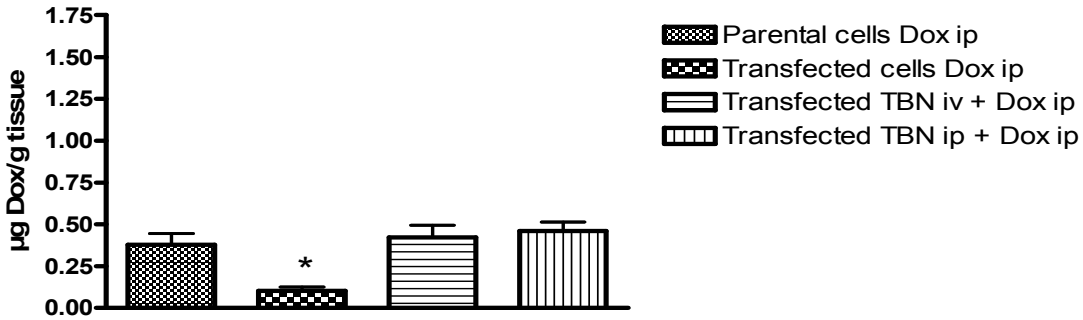
More than double difference was found between doxorubicin accumulation of the resistant and non-resistant cells. As it was expected **TBN** treatment was able to restore the sensitivity of resistant cell line to doxorubicin (Figure 1A), whereas it had no significant effect on the accumulation of non-resistant ones.

For the toxicity (Figure 1B) test, the cell density was measured by MTT assay. We found that in this short time experiment (the exposition time was only 1h) the correlation is linear between the accumulation and toxicity *in vitro*.

4.5 *In vivo* doxorubicin accumulation study in mouse MDR cells

When doxorubicin was injected intraperitoneal (i.p.), low levels of doxorubicin were detected in the tumour (Figure 2).

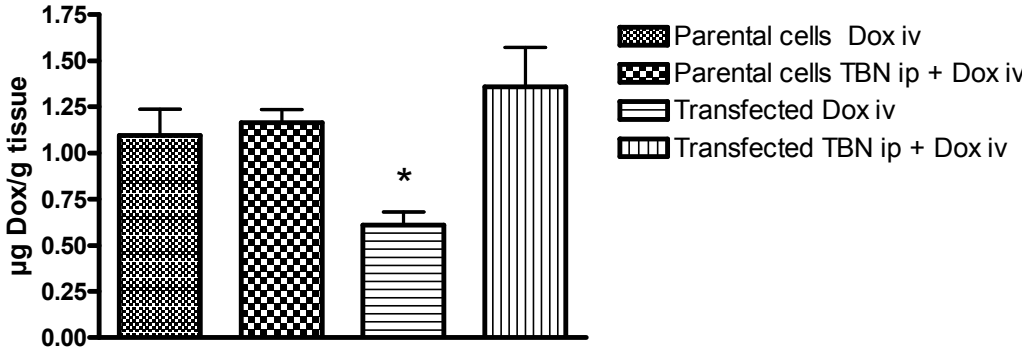
Figure 2. Accumulation of doxorubicin in tumour samples after i.p. administration of doxorubicin



Bars present means±SE from seven experiments. Significant difference (*P < 0.05) was found in doxorubicin accumulation of resistant tumour with non-resistant counterpart and both treated samples respectively

TBN was able to enhance the drug accumulation when it was administered intravenously (i.v.) 1 h or i.p. 3 h before doxorubicin. However, when **TBN** and doxorubicin were simultaneously injected, no increase of the doxorubicin could be observed (data not shown). After administering doxorubicin i.v., the recovered amount of drug was much higher as compared to the results obtained after i.p. injections (Figure 3).

Figure 3. Accumulation of doxorubicin in tumour samples after i.v. administration of doxorubicin



Bars present means±SE from seven experiments. Level of significance was evaluated by ANOVA analysis (* means P < 0.05).

Furthermore, also in these conditions i.p. administered TBN was able to restore the doxorubicin accumulation in MDR1-expressing tumours to the level of that in tumours consisting of non-resistant cells.

4.6 Apoptosis induction of some resistance modifiers

An evaluation of the capacity of the most effective resistance modifiers, as apoptosis inducer, was demonstrated by Annexin-V in flow cytometric analysis and by staining with ethidium bromide and acridine orange, using human *mdr1* gene-transfected mouse lymphoma cells and a human cervix adenocarcinoma cell line (HeLa).

4.6.1 Apoptosis induction with FITC-labelled Annexin V and Propidium iodide

Based on the Rhodamin 123 accumulation and drug interaction experiments, the most effective MDR modifiers cinnamylidene ketones, dihydropyridine derivatives and one *Euphorbia* diterpene were evaluated as concern apoptosis induction on L 5178 Y cell line (Table 17). The death rate in the presence of the compounds is extremely low. Also low level at early apoptosis was observed in all of the treated cells.

Table 17. Apoptosis induction by the most effective resistance modifier on L 5178 Y cells

Samples	Conc. (µg/mL)	Early apoptosis (%)	Total apoptosis (%)	Cell death (%)
Cell control without	-	1.14	1.22	0.06
Cell control + Annexin-V[b]	-	8.05	11.43	1.97
Cell control +PI[c]	-	0.31	1.18	2.91
Cell control double	-	4.83	6.93	2.98
12H-benzo(α) phenothiazine	5	77.67	99.74	0.04
	25	26.25	99.55	0.03
Diterpene 8	2	4.04	5.80	3.36
	10	3.02	6.14	3.02
Cinnamylidene 4	2	3.33	5.50	3.01
	10	2.89	3.91	1.93
Cinnamylidene 8	2	4.69	6.30	1.79
	10	5.37	7.16	2.48
Dihydropyridine 12	2	5.56	7.89	3.05
	10	2.94	4.57	1.68
Dihydropyridine 13	2	4.71	7.03	2.64
	10	6.15	12.07	2.37

[a]Annexin-V - negative/propidium iodide-negative samples = intact viable cells (98-100%)
[b]Annexin-V - positive/propidium iodide-negative = apoptotic cells
[c]Annexin-V - negative/propidium iodide-positive = necrotic cells
[d]Annexin-V - positive/propidium iodide-positive = apoptotic/necrotic cells
The results are one representative experiment of flow cytometry in which 10 000 individual cells were investigated

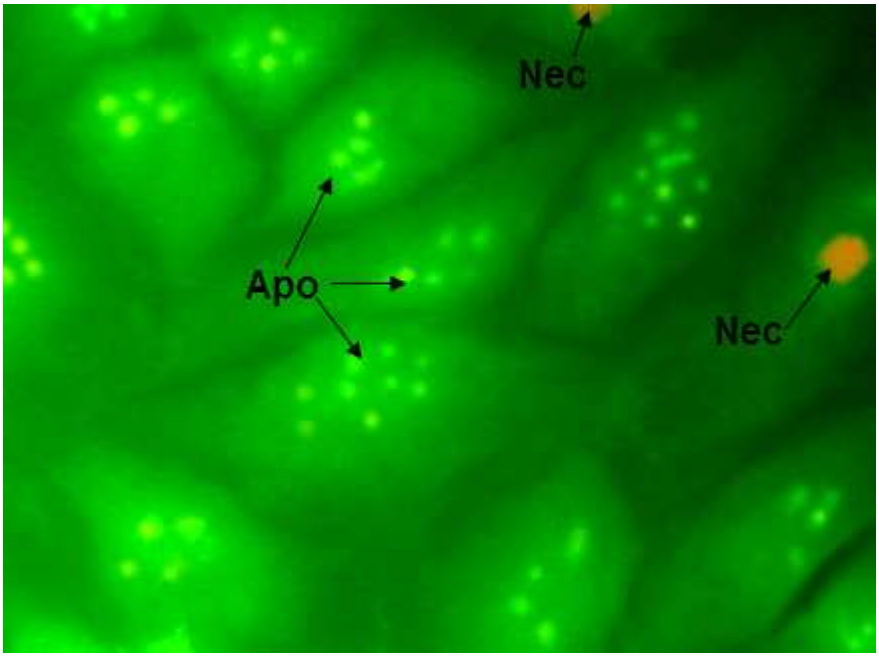
The apoptosis inducing effect generally varied between 3 and 13 percent, whereas 12H-benzo(α)-phenothiazine led to almost 100 percent apoptosis.

According to this data, all the tested compounds can be considered as moderate apoptosis inducer, except **dihydropyridine 13**, which showed an increased apoptosis effect at higher concentration.

4.6.2 The identification of apoptotic and necrotic cells by staining with ethidium bromide and acridine orange (EB/AO)

The EB/AO staining of the HeLa cells (monolayer) allowed the identification of live, apoptotic and necrotic cells in situ, without trypsin treatment. AO permeates all cells and makes the nuclei appear green. EB is taken up by cells only when the cytoplasmic membrane integrity has been lost; it stains the nucleus red. Even at a lower concentration of compounds (2 μg/mL), treatment of HeLa cells with possible MDR modifiers led to the typical morphological features of apoptosis, including increased cell membrane permeability, cellular shrinkage and granulation in the nucleus. Figure 4. shows the fluorescent microscopic pictures of one representative samples, diterpene **8**-treated HeLa cells after a 24 h incubation.

Figure 4. EB/AO staining of diterpene 8-treated HeLa cells



"Apo" indicates cellular shrinkage and nuclear granulation characteristic of apoptosis; "Nec" indicates necrosis evidence by fluorescence of ethidium bromide

4.7 Anti-promotion effect of plant derivatives

As a model for prevention of cancer progression by CMV infection, the antitumour-promotion effects of some lathyrane-type diterpenoids were studied *in vitro*.

To develop an objective method for studying antipromotion effect, the compounds had to be applied in non-toxic doses. Based on the ID_{50} values, then ID_1 and ID_{10} inhibitory doses were tested for modification of CMV IE antigen expression in the different lathyrane-treated lung cancer cells (A549) cells.

The results are summarized in Table 18. Latilagascenes B-C (**2**-**3**), E (**5**) and jolkinol B (**6**) showed inhibitory activity in a dose dependent manner against IE antigen expression of CMV. Latilagascene A (**1**) in dose ID_1 was more effective than ID_{10} in inhibition of production of IE antigen. It is assumed that both doses might be in the dose saturation region concerning the biological effect. This explanation is supported by the lowest ID_{50} value of the latilagascene A (**1**). Latilagascene D (**4**) was inactive due to the steric properties.

The inhibition of IE antigen expression of CMV in the presence of non-toxic doses of lathyranes was evaluated as antipromoting effect that can reflect the chemopreventive activity of a compound.

Table 18. The effects of lathyrane diterpenes 1-6 on human cytomegalovirus IE gene expression

Compounds	ID[a]	IE antigen expressing cells (% of control)
Latilagascene A (1)	1	61
	10	79
Latilagascene B (2)	1	91
	10	80
Latilagascene C (3)	1	74
	10	63
Latilagascene D (4)	1	95
	10	95
Latilagascene E (5)	1	74
	10	51
Jolkinol B (6)	1	69
	10	54

[a]ID: Inhibition dose for A549 cells

5. DISCUSSION

The development of pharmacological agents able to counteract the mechanisms of multidrug resistance in oncology has remained a major goal for the past ten years. Several mechanisms are thought to be involved in drug resistance, including those associated with drug transport, detoxification and apoptosis.

The first part of this thesis was focused on the inhibition of MDR through inhibition of the MDR P-glycoprotein in various cell lines. Six groups of compound were involved in the P-gp efflux pump inhibitory studies: cinnamylidene ketones; 1,4-dihydropyridines derivatives; phenothiazines; HSP90 inhibitor peptide derivatives; Tylosin Betti base derivative and *Euphorbia* diterpenes.

The cytotoxic effects of some **cinnamylidene ketones** and their derivatives against P388 lymphocytic leukaemia and murine L1210 lymphoid leukaemia were described by Dimmock *et al.* (117, 118). 2-(2-methoxycinnamylidene)indan-1-one (**4**), 2-cinnamylidene-3,4-dihydro-2H-naphthalen-1-one (**7**), 2-(2-methoxycinnamylidene)-3,4-dihydro-2H-naphthalen-1-one (**8**), 6-cinnamylidene-6,7,8,9-tetrahydrobenzocyclohepten-5-one (**10**) and 6-(2-methoxycinnamylidene)-6,7,8,9-tetrahydrocyclohepten-5-one (**11**) display marked effectivity for the reversal of the MDR on human *mdr1* gene-transfected mouse lymphoma cells and human colon cancer cell line (COLO 320). In the case of the COLO 320 cells, which express the *mdr1* gene, the degree of Rhodamin 123 accumulation was lower than on the artificially constructed extremely sensitive mouse lymphoma cells, in which P-gp is overexpressed (106). This means that the effectivity of the reversal compounds also depends on the "type" of cancer cells, which express more or less vulnerable P-gp.

Two of the tested compounds, namely, 2-(2-methoxycinnamylidene) indan-1-one (**4**) and 2-(2-methoxycinnamylidene)-3,4-dihydro-2H-naphthalen-1-one (**8**) were able to enhance the antiproliferative activity of doxorubicin in a synergistic way. Similar studies illustrated that combined application of above mentioned two compounds with paclitaxel, docetaxel or vincristine exerted significant antiproliferative effects on the resistance sublines of MCF7 (119).

1,4-Dihydropyridines are well known as Ca^{2+} channel blockers and as drugs for the treatment of cardiovascular diseases, including hypertension (120). The 1,4-dihydropyridine

heterocyclic ring is a common feature of various bioactive compounds, such as vasodilator, bronchodilator, antitubercular agents (121, 122), and more recently MDR modulators and antitumour agents (123-125).

The MDR-reversing effects of the dihydropyridines having been tested in MDR reversal experiments. Most of the 1,4-dihydropyridines considered as potent inhibitors of multidrug resistance. The high anti-MDR potency of selected dihydropyridines (**DL4**, **DL12**, **DL13** and **DP89**) were also very effective in combination with doxorubicin. Some dihidropyridines at the higher concentrations used were found to be toxic, but at low doses most of the derivatives were able to increase the R123 accumulation without toxic effects.

Phenothiazines, apart from their wide range of biological activity (126) have been described as effective MDR modifiers. During the last few years, many new phenothiazine derivatives have been synthesized and their activity has been extensively studied (127-129).

The tested phenothiazines were moderately active resistance modulators, especially in the low concentration. The higher concentration, appeared cytotoxic, however, this effect was more profoundly accentuated in MDR1-expressing mouse T-cell lymphoma cell line than in COLO 320 cell line. The toxic effect and MDR reversal of phenothiazine derivatives on tumour cells has been described previously by Wesołowska *et al.* (130), who reported that the phenothiazine maleates were more toxic than other derivatives tested. These observations are in agreement with our results, where **methotripromazine maleate** and **prochlorperazine dimaleate** caused decreased cell survival of the two cell lines studied. According to the observations of Flores *et al.* (131) phenothiazine hydrochlorides are also characterized by high cytotoxicity. Similarly, in our experiment, **trifluoropromazine hydrochloride** was toxic. The above-mentioned authors claim that the phenothiazines, exemplified by phenothiazine hydrochlorides, are able to modify cell membrane properties by disorganization of functionally active conformation of P-gp and the phospholipids; translocation of antitumour drug complexes through the membrane. On the other hand, the same ability could also benefit in modulation of MDR. Actually, if the drug concentration were reduced, obviously it would behave as an effective MDR inhibitor and did not cause cell death. According to the Flores *et al.* (131), the MDR reversal in L5178 Y and COLO 320 cell lines by phenothiazines might be mediated by two alternative mechanisms: by changing the conformation of lipids in the membranes, and, when combined with other drugs, by acting as carriers of insoluble antitumour agents to target proteins of cell interior.

Heat shock protein 90 (Hsp90) inhibitor peptides arrests the growth of cancer cells (132). Their ability to reverse the MDR of tumour cells was also investigated, in order to obtain additional evidence concerning the relationship between chemical structure and biological activity. A structure-activity relationship was observed in the MDR reversal effect since two of the three strongly hydrophobic compounds (**1** and **4**) were the most potent. The correlation between the inhibition of the ABC transporter and the chemical properties of the active compounds is possibly similar to that for a certain group of carotenoids in which the total polar surface areas of the hydrophobic compounds exhibited some correlation with the MDR-reversal effects (133). *D*-Trp-Phe-*D*-Trp-Leu-AMB (**1**), which has a relatively high apolar character, and its succinylated derivative, Suc-*D*-Trp-Phe-*D*-Trp-Leu-AMB (**4**) which is slightly more water soluble demonstrated the highest fluorescence activity ratios. Interestingly, replacing the *N*-terminal *D*-Trp of **1** by *D*-Tyr to obtain *D*-Tyr-Phe-*D*-Trp-Leu-AMB (**5**) caused a dramatic change in the fluorescence activity ratio, indicating that the indole side-chain at this position is indispensable for the biological effect, and for the reversal of MDR of cancer cells. The low activity compounds revealed that introduction of a free carboxamide or carboxyl group at the *C*-terminus (peptide derivatives **2**, **3** **6**, **7**, **8**, **9** and **10**) or introduction of a reduced peptide bond in the chain (peptide derivatives **2** and **7**) were not functionally important structural factors. It should be noted that the activity of the peptide derivatives in reversing the MDR did not depend upon the length of the peptide chain: rather, it depended on the stacking ability of tryptophan, and the tetrapeptide structure proved best from this respect. Orosz *et al.* reported that the 10 tested peptide derivatives exerted noteworthy antiproliferative effects on various human small-cell-lung cancer, colorectal and mammary adenocarcinoma cells. It was also found in the *in vivo* model experiments that growth inhibitors inhibited tumour growth in nude mice bearing the xenografts without general toxicity (134, 135).
These MDR inhibitors increased the antiproliferative effect of epirubicin on human *mdr1* gene-transfected mouse lymphoma cells resulting from synergism for compound **1** and additive antiproliferative effects for compounds **4** and **5**.

In order to get better insight into structure activity relationship *in vitro* P-gp inhibitory effects of **N-Tylosil-1-α-amino-(3-bromophenyl)-methyl-2-naphtanol (TBN)** and its precursor moieties (tylosin and Betti base) were also studied. Since macrolide antibiotic erythromycin was reported as a possible P-gp inhibitor (136) it was supposed that tylosin part of **TBN** has a crucial role in P-gp modulator activity. Interestingly, only slight effect of tylosin was

observed in inhibition of drug efflux and *in vitro* interaction studies compared to **TBN**. Differences were very appreciable in their effect on R123 efflux studies between precursors and **TBN**. **TBN** treatment was resulted significantly higher activity in P-gp inhibition than its two-precursor moiety. This finding also suggests that the molar weight and lipophilicity of compound has a crucial role in P-gp substrate specifity and/or inhibition as it was previously reported by Didziapetris *et al.* (137). Further experiments were focused on the effect of **TBN**. Significant synergism was observed *in vitro* combination of **TBN** and doxorubicin and/or vincristine on mouse lymphoma cell line.

Before *in vivo* studies, we attempted to determine the relationship between doxorubicin accumulation and toxicity. Linear correlation was found between the toxicity and increased accumulation of doxorubicin however the cells were exposed to higher concentration of doxorubicin in short time incubation. Smaller differences were achieved in doxorubicin accumulation between resistant and non-resistant or **TBN** treated and none treated cells if it is compared to the results of R123 accumulation study (data not shown). Since the lipid peroxidation activity of anthracyclines is appreciable under defined condition (138) in this experiment the results of doxorubicin accumulation and toxicity might be influenced by membrane damage of the cells. An important site of cytotoxic action of doxorubicin is the nucleus, where doxorubicin intercalated into DNA, forming DNA adducts and inhibition topoisomerase II (139).

The *in vitro* activity of **TBN** was confirmed in further *in vivo* efficacy studies. The combination of doxorubicin with TBN increased the doxorubicin concentration in the tumours compared with doxorubicin alone using DBA/2 mice bearing syngeneic L 5178 Y tumours. In case of applied regimens of **TBN** on resistant tumours, the doxorubicin level was similar as in non-resistant tumours due to the inhibited efflux of MDR cells. Independently from doxorubicin administration (i.v. or i.p.), **TBN** treatment was able to restore the doxorubicin sensitivity of resistant tumours. The efficacious dose of **TBN** seemed relatively low and co-administration with doxorubicin is well tolerated. Our study suggests that **TBN** is a very promising P-glycoprotein inhibitor with low toxicity and relatively high efficacy.

***Euphorbia* species** have been used in traditional medicine in many countries to treat cancer and warts (140). Besides the presence of highly skin-irritant compounds (phorboids) (141, 142), *Euphorbia* species are of further considerable interest owing to a large diversity of

structurally unique and non-irritant jatrophane and lathyrane diterpenoid constituents, which have been considered to be potent modulators of MDR (143, 144). Our research group previously reported that extracts containing macrocyclic diterpenes may reverse MDR by inhibiting P-gp in human *mdr1* gene-transfected mouse lymphoma cells (114-116).

Three of the tested diterpene derivatives (**6**, **8** and **9**) displayed a significant concentration-dependent effect in inhibiting the efflux pump activity on the COLO 320 cell line. The most effective synergistic effect was found between compound **8** and epirubicin. The strong activity of this derivative can be explained by its high lipophilicity, but other parameters, such as the presence of functional groups, may also be involved in the synergistic effect and in the interaction with P-gp. The numbers of double bonds, pseudorotation of the ester groups and transannular interactions of the substituents have significant effects on the stereostructure and the activity of the molecules. It is known that the conformational flexibility of the twelve-membered ring of the jatrophanes is strongly influenced by the steric interactions of the substituents. On comparison of the efficacies of compounds **7** and **8**, it can be presumed that the presence of a hydroxy group instead of peracylation is favourable as concerns the antiproliferative activity in combination with epirubicin.

Although compound **9** significantly increased the R123 accumulation in COLO 320 cells, in combination with epirubicin it had no antiproliferative effect on the tested cell line. The differences between the dose-dependent antiproliferative and the MDR-reversal effects of studied derivatives can be consequences of the various experimental procedures, including the different incubation times, and possible metabolisms of compounds.

Many classes of antitumour drugs, including naturally occurring and pharmaceutical compounds, induce **apoptosis** in cancer cell (15, 16). Apoptosis is typically induced by the activation of membrane receptors, cell cycle arrest, p53 activation by DNA damaging agents and mitochondria pore transition permeability (11). The apoptotic process is characterized by particular morphological and ultrastructural features, which can be evidenced by several assays, including FITC-conjugated annexin V method or acridine orange assay (111).

In our study the tested compounds (cinnamylidene **4** and **8**, dihydropyridine **12** and *Euphorbia* diterpene **8**) were able to induce moderate apoptosis in human *mdr1* gene-transfected mouse lymphoma cell line. The most promising apoptosis inducer was the dihydropyridine substituted compound **13**, however, the levels of total apoptosis were much lower in every case than positive control 12*H*-benzo[α]phenothiazine (M-627). The annexin V positivity of treated-cells by various compounds 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 (111). The apoptosis inducing effect of MDR reversal compounds can have special importance in experimental chemotherapy.

Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves initiation, usually from DNA damaging agents, promotion, during which cell proliferation is increased, and progression, involving additional genetic alterations (145). **Cancer chemoprevention** is defined as the use of natural, synthetic, or biological agents to prevent, suppress, or reverse either the initiation phase of carcinogenesis or the carcinogenic progression (146) and promotion.

The mechanism of action of chemopreventive compounds acting as antitumour-promoters is not exactly known. However, a significant number of them have been considered to inhibit the tumour promotion stage by interacting with the protein kinase C which plays a crucial role for the regulation of cell growth (147).

In the present work, the human CMV was used in a modified *in vitro* model for characterizing lathyrane compounds with antipromotion effect on human lung cancer cells (A549). The ability of human CMV to preferentially infect tumour tissues suggests a unique character of mutual interaction between the mechanisms of tumour cells and human CMV (101). IE gene products of the virus accumulate in the infected cells causing disturbances of host cell functions. The oncomodulatory effects of human CMV infection may lead to a shift to more malignant phenotype of tumour cells contributing to tumour progression (148).

The structures of the six studied compounds differ in the substitution pattern of ring A having compound **5** also a different substitution at C-20 where the methyl group is oxidized, having been replaced by a $-CH_2OH$**.** This structural feature appears to be important justifying the activity difference between latilagascene D (**4**) and latilagascene E (**5**) which showed the highest activity as anti-promoters. The comparison of the activity of compounds **4** and **1**, whose structures differ only at the ester group at C-16, suggests that the presence of the benzoyl moiety in the former has a negative action in the inhibitory effect.

This study revealed that certain macrocyclic lathyrane-type diterpenoids may be promising lead compounds for natural product-based drug development. Similar results were found an inhibition of adenovirus and EBV early antigen expression both *in vitro* (149) and *in vivo* (150) by inhibition of skin tumour promotion in mice.

6. NEW STATEMENTS

I. Based on the antiproliferative or cytotoxic effect differences were found between sensitive and MDR tumour cells.

II. During my PhD study, several groups of compounds were identified as MDR modulators (cinnamylidene ketones; 1,4-dihydropyridines; phenothiazines; HSP90 inhibitor peptides; Betti base derivative of tylosin and some naturally occurring plant derived jatrophane and lathyrane-type diterpenes).

A majority of the tested **cinnamylidene derivatives** were able to increase the R123 accumulation in human *mdr1* gene-transfected mouse lymphoma cell line (L 5178 Y) and also in human colon cancer cells (COLO 320). The MDR-reversing effect of tested of cinnamylidenes were markedly lower in COLO 320 cells as compared with L 5178 Y cell line. The reason for this phenomenon could be the fact that P-glycoprotein expression in COLO 320 cell line is lower than in L 5178 Y cells.

Numerous tested **1,4-diphenyl-1,4-dihydropyridine derivatives** reversed the MDR of tumour cells more effectively than the reference compound, verapamil.

Two of the **phenothiazine derivatives** namely **perphenazine** and **prochlorperazine dimaleate** proved to be effective inhibitors of Rhodamin 123 efflux. Other tested phenothiazine derivatives also modulated intracellular drug accumulation in resistant cell lines.

Among the synthetic **HSP90 inhibitor peptide derivatives** two of the strongly hydrophobic compounds (compound **1** and **4**) were the most potent in MDR reversal.

TBN (*N*-tylosil-1-α-amino-(3-bromophenyl)-methyl-2-naphthol), and not the individual Betti-base and tylosin moieties, is able to increase the cellular uptake of the fluorescent P-gp substrate Rhodamin 123 in human *mdr1* gene transfected mouse T-cell lymphoma drug resistant cell line L 5178 Y, and fully reversed the cellular resistance against doxorubicin.

It seems that the effect of ***Euphorbia*** **diterpenes** on MDR reversal effect of tumour cells depends on the chemical structure. The presence of hydroxy group instead of peracylation is favourable on COLO 320 cells.

III. Generality, the newly identified MDR modifiers were able to enhance the antiproliferative activity of selected anticancer drugs (e.g. doxorubicin, paclitaxel and vincristine) in a synergistic or additive way *in vitro* on MDR cells.

The *in vivo* results shown, that co-administration of **TBN** with doxorubicin significantly increased the antitumour activity of doxorubicin in DBA/2 mice.

IV. As an alternative way of antitumour effect, apoptosis inductions of resistance modifiers were studied.

The substituted **dihydropyridine 13** was the most promising apoptosis inducer on L 5178 Y cells during the 1 hour incubation.

Typical apoptotic markers were observed after a 24 h treatment with the tested jatrophane derivative **8** by staining with ethidium bromide and acridine orange on HeLa cells.

V. As a model for prevention of cancer progression by CMV infection, the antitumour-promotion effects of some resistance modifiers were studied. All the six **macrocyclic lathyrane-type diterpenoids**, could reduced the promotion *in vitro*, except latilagascene D, decreased IE-antigen expression of CMV to prevent progression of tumour malignancy.

7. ÖSSZEFOGLALÓ

A daganatos megbetegedések elleni küzdelem hatásosságában a multidrog rezisztencia (MDR) kérdés megoldása fontos szerepet játszik. Növekvő igény mutatkozik olyan új vegyületek kifejlesztésére, melyek képesek gátolni a különböző rezisztencia mechanizmusokat.

Célul tűztük ki a szintetikus (cinnamilidén ketonok, 1,4-dihidropiridin és fenotiazin származékok, HSP90 gátló peptidek és Tilozin betti bázisú származék), valamint természetes növényi alkotókból (*Euphorbia* diterpének) kivont származékok MDR-efflux pumpa gátlás vizsgálatát különböző sejtvonalakon, remélve, hogy kevésbé toxikus, de annál hatásosabb rezisztenciamódosító vegyületeket találunk. A különböző MDR módosító vegyületeket *in vitro* és *in vivo* kísérletekben kombináltuk rákellenes szerekkel.

A **cinnamilidén ketonok** közül a, 2-(2-metoxicinnamilidén) indán-1-on (**4**), 2-cinnamilidén-3,4-dihidro-2H-naftalén-1-on (**7**), 2-(2-metoxicinnamilidén)-3,4-dihidro-2H-naftalén-1-on) (**8**), 6-cinnamilidén-6,7,8,9-tetrahidrobenzocikloheptén-5-on (**10**) és 6-(2-methoxicinnamilidén)-6,7,8,9-tetrahidrocikloheptén-5-on (**11**), jelentős MDR visszafordító hatást fejtett ki a humán *mdr1* gén-transzfektált egérlymphoma (L 5178 Y), valamint humán vastagbélrák sejteken (COLO 320). Közülük, kettő (**4 és 8-as** jelölésű vegyület) szinergizálta a doxorubicin sejtszaporodásgátló hatását.

Az **1,4-dihidropiridinek** többsége hatásos MDR revertáló vegyületnek bizonyult. Néhányuk magasabb koncentrációban toxikus hatást mutatott, azonban alacsonyabb dózisban legtöbbjük képes volt a Rhodamin 123 sejtekben való felhalmozódás fokozására, sejtkárosodás nélkül. Kiválasztott dihidropiridin származékok **(DL4, DL12, DL13 és DP89)** szintén hatásosak voltak doxorubicinnel kombinálva.

Fenotiazinok közül a **perfenazin** és **proklorperazin dimaelat** jelentős Rhodamin 123 efflux gátlást mutatott L 5178 Y és COLO 320 sejtvonalakon. A többi fenotiazin származék mérsékelt hatású volt, magasabb koncentrációban toxicitásuk is megjelent.

Hősokk fehérje 90 (Hsp90) gátló peptidek közül a *D*-Trp-Phe-*D*-Trp-Leu-AMB (**1**), mely viszonylag jelentős apoláris tulajdonsággal rendelkezik, és szuccinilált származéka, Suc-*D*-Trp-Phe-*D*-Trp-Leu-AMB (**4**), mely vízoldékonyabb, a legmagasabb fluoreszcencia értékeket mutatták kísérleteinkben.

A vizsgált **diterpén származékok** közül három (**6**, **8** és **9**), szignifikáns koncentrációfüggő efflux pumpa gátló hatást váltott ki COLO 320 sejtvonalon. A legjelentősebb szinergizálló hatást epirubicin és *Euphorbia* diterpén **8** együttes alkalmazásánál tapasztaltuk. Annak

ellenére, hogy a **9**-es vegyület vastagbélrák sejtekben megnövelte az R123 felhalmozódását, az epirubicinnel való kombináció alkalmával nem mutatott kölcsönhatást a rákellenes szerrel, a vizsgált sejtvonalon. A dózis-függő antiproliferatív és MDR gátló kísérletekben tapasztalt különbségekért, a különböző kísérleti módszerek, beleértve az inkubációs idők hosszát és a vegyületek lehetséges metabolitikus változását, tehetők felelősé.

Kísérleteinkben modellként használt egérlymphoma sejtvonal, melyben fokozott MDR1 expresszió figyelhető meg, érzékenyebbnek bizonyult a vizsgált vegyületekre, mint a humán vastagbélrák sejtvonal, ugyanis már a legkisebb pumpa gátló hatás is érzékelhető benne.

TBN (*N*-tilozil-1-α-amino-(3-bromofenil)-metil-2-naftol) kezelés jelentős P-gp gátló hatást mutatott L 5178 Y sejteken és fokozta a doxorubicin illetve vincristin sejtszaporodásgátló hatását *in vitro* kísérleteinkben. Megnövelte rezisztens sejtek érzékenységét doxorubicinnel szemben, de nem idézett elő szignifikáns változást a nem-rezisztens sejteknél. Egyenes arányosságot tapasztaltunk a doxorubicin felhalmozódás és toxicitás között. Doxorubicinnel való együttes alkalmazásánál, megnövelte annak felhalmozódását L 5178 Y tumorsejtvonalat hordozó DBA/2 egerekben, függetlenül a doxorubicin adagolás módjától (i.v. vagy i.p.).

Megvizsgáltuk egyes vegyületeink apoptózis indukáló hatását is. Kísérletünkben a cinnamilidén **4** és **8**, dihidropiridin **12** illetve *Euphorbia* diterpén **8** mérsékelt apoptotikus hatást fejtett ki humán *mdr1* génnel-transzfektált egérlymphoma sejtvonalon. A leghatásosabb apoptózis indukáló a dihidropiridin **13** volt, annak ellenére, hogy az általa kiváltott teljes apoptózis mértéke alacsonyabb volt, mint a pozitív kontrol 12*H*-benzo α]fenotiazinak (M-627).

Számos esetben leírták, hogy a humán cytomegalovírus (CMV) képes tumorszövetek fertőzésére, a vírus korai (IE) antigénjei felszaporodnak a tumorsejtekben és a daganat további malignizációját segítik. CMV korai antigén expresszió gátlása arra utalhat, hogy az antipromóciós hatás érvényesült. *In vitro* modell kísérletünkben, humán tüdőrák sejtvonalon, a vizsgált makrociklikus latrán típusú diterpén vegyületek többsége képes volt a CMV korai antigén kifejeződés gátlására.

8. ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor **Professor József Molnár** for his continuous support and encouragement throughout my PhD studies. I also express many thanks to his kind help, skilful guidance and inspiration.

I greatly acknowledge to **Professor Yvette Mándi** for providing working facilities at the department of Medical Microbiology and Immunobiology.

I owe **Professor Rozália Pusztai** my thanks for her help and constructive comment.

I am grateful to **Dr. Imre Ocsovszki** for performing the flow-cytometric measurements.

I would like to show gratitude to my colleague, **Dr. Nóra Gyémánt** for her scientific help.

I thank **Anikó Váradi Vigyikán** for her excellent technical assistance in the laboratory work.

I wish to thank all **my co-workers, colleagues and staff members** at the Department of Microbiology and Immunobiology, University of Szeged, are gratefully thanked for creating a supportive and pleasant working environment.

Finally, I heartfully thank **my parents**, **my love** and **my friends** for their support, love and patience through all these years.

This thesis was supported financially by the **Foundation for Cancer Research of Szeged** (Szegedi Rákkutatásért Alapítvány) and **Flemish-Hungarian Intergovernmental Cooperation Program** for 2005-2006 (B-31/04).

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