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

Ph.D. Thesis

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2007

1. INTRODUCTION

Cancer is a major public health problem worldwide. Currently, after cardiovascular diseases, cancer is the second leading cause of death in European countries.
Approximately 40 % of cancer patients can be treated surgically, and the great majority of patients need chemotherapy. 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.

1.1 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). It has been recognized that MDR is multifactorial and factors that affect response to anticancer chemotherapy include cell kinetic, pharmacokinetic and cellular drug resistance mechanisms. Cellular mechanisms are categorized in terms of alterations in the biochemistry of malignant cells:

I. *Non-classical MDR phenotypes*
- Drug resistance mediated by detoxification mechanism of the drug in the cells
- Alterations of drug targets or enhancement of target repair
- Defective apoptotic pathways

II. *Transport-based classical MDR phenotypes*
- Overexpression of ATP-binding cassette (ABC) transporters

1.2 ABC transporters

The ABC transporter superfamily is among the largest and most broadly expressed protein superfamilies known. 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)**; the 190 kDa **multidrug resistance protein 1 (MRP1)**; the **breast cancer resistance protein (BCRP)** and the **lung resistance protein (LRP),** also known as the major vault protein. LRP is not an ABC transporter but it is frequently expressed at high levels in drug-resistant cell lines and tumour samples. These ABC transporters serve to pump anticancer drugs out of the cells, resulting in lack of intracellular levels of drug necessary for effective therapy.
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). In addition, they have an important role in maintaining the barrier function of sanctuary site tissues (*e.g.* blood–brain barrier).

1.3 Substrates and modulators of ABC transporters

ABC transporters recognize a very large range of drug substrates; include a large number of anticancer drugs (anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxanes). Recognized substrates are mostly hydrophobic compounds, but MDR pumps are also capable to extrude a variety of amphipathic anions and cations.

Many of **the first generation chemosensitizers** (*e.g.* verapamil, cyclosporine A) 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.
The second generation modulators (*e.g.* dexverapamil, dexniguldipine, valspodar, biricodar) exhibited decreased inherent toxicity while retaining MDR reversal efficacy, but they also retain some characteristics that limit their clinical usefulness. Many of these

MDR modulators are also substrates for cytochrom P450 3A4 and metabolized by this enzyme or inhibited several other ABC transporters.
Third generation agents specifically and potently inhibit P-gp and do not inhibit other ABC transporters. Clinical trials with these new third generation agents (*e.g.* tariquidar, laniquidar zosuquidar) 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.

1.4 The oncomodulatory effect of human cytomegalovirus

Studies indicated that, the human cytomegalovirus (CMV) infection might be associated with the pathogenesis of several human malignancies. CMV infection modulates properties of tumour cells such as growth, apoptosis, production of angiogenic factors, cell invasion and immunogenic properties. CMV infections fail to transform susceptible normal human cells.
The oncomodulatory effects are mediated mainly by the activity of CMV regulatory proteins. Immediate early (IE) antigen expression is based on the appearance of the respective mRNA or protein and rely on the persistence of viral infection in malignant cells. 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 can be an important goal to prevent and treat certain forms of cancers associated with human CMV.

2. AIMS OF THE STUDY

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 and some naturally-occurring plant derived compounds.
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 derivative of Tylosin)
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 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 citotoxic effects, programmed cell death induction was studied of the most effective resistance modifiers 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

3. MATERIALS AND METHODS

3.1 Chemicals

Synthetic compounds

Sixteen **cinnamylidene derivatives** (**1-16**); forty-one substituted **1,4-diphenyl-1,4-dihydropyridines** (**DL1-20 and DP70-90**); seven **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); ten **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**); *N*-tylosil-1-α-amino-(3-bromophenyl)-methyl-2-naphthol (**TBN**);

Natural compounds

Nine ***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**)**;**
Six **macrocyclic lathyrane-type diterpenes**: Compounds were isolated from the methanol extract of *Euphorbia lagascae* (latilagascene A (**1**), latilagascene B (**2**), latilagascene C (**3**), latilagascene D (**4**), latilagascene E (**5**), jolkinol B (**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 (transfected with pHa MDR1/A retrovirus)
3.2.2 The human colon cancer cells (**COLO 320**)
3.2.3 The human cervical adenocarcinoma cells (**HeLa**)
3.2.4 The human lung alveolar epithelial cells (**A549**)
3.2.5 Normal human lung fibroblast (**MRC 5**)

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). 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. The compounds were diluted from high to low concentrations horizontally in the plates. Cells were seeded into each well (1×10^4 cell/well)) with the exception of the medium control wells. The plates were incubated at 37 °C for 72 h, (in case of cytotoxicity test the cell number were 3×10^4 /well and the incubation time was 48 h) and 15 μL of MTT solution (from a 5 mg/mL stock) was then added to each well. After incubation at 37 °C for 4 h, 100 μL of SDS solution (10%) was measured into each well. The plates were further incubated at 37 °C overnight to allow the dissolution of formazan crystals produced by the mitochondrial enzymes of living cells. The extend of inhibition of cell proliferation was determined by measuring the optical density (OD) of the chromogenic products 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, in which OD cell control means the untreated cells:

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

3.6 Flow-cytometric assay for R123 accumulation in tumour cells

The cells were adjusted to a density of 2×10^6/mL, resuspended in serum-free medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volumes, from the 1.0-10.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μM final concentration) of the indicator R 123 was added to the samples and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Beckton Dickinson FACScan instrument (cell sorter, Oxford, U.K.).Verapamil was used as a positive control.

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

$$FAR = \frac{FL1\,treated\,cells}{FL1\,untreated\,cells}$$

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 dilutions of anticancer drug were made in a horizontally and the dilutions of resistance modifiers vertically in the microtiter plate in 100 μL volume. The cell suspension in the tissue culture medium was distributed into each well in 50 μL containing 1×10^4 cells. The plates were incubated for 72 h at 37 °C in a CO_2 incubator. The cell growth rate was determined after MTT staining and the intensity of the blue colour was measured on a micro ELISA reader. Drug interactions were evaluated according to the following expressions:

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

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

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.

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 4×10^6 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. 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.

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. 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 and 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 next investigated. The fluorescence activity (FL-1 and FL-2) of the cells was measured and analysed on a Beckton Dickinson FACScan instrument.

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. 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 evaluated with a Nikon Eclipse inverted microscope. 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 “Towne” strain of CMV at a multiplicity of infection of 2.4. The infected A549 cultures were centrifuged for 60 min at 1200 rpm in a Heraeus, Megafuge 1.0 at room temperature and then incubated for 1h at 37 ºC. The unabsorbed virus was removed and the cells were washed three times with

serum-free medium. After washing, the cells were overlayed with medium containing the appropriate concentration of a compound or DMSO. After 48 hours incubation, the cells were washed twice with cold PBS and fixed with cold acetone: ethanol for 20 min at –20 ºC. The fixed cells were stored at –20 ºC until immunofluorescence assays were performed.
Human CMV IE1-72 antigen was detected in the nuclei of infected cells by MAB810 and fluorescein isothiocyanate (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 AND DISCUSSION

4.1 Antiproliferative and cytotoxic effect of tested compounds

In the first stage of study the antiproliferative and cytotoxic property of various compounds were determined of possible MDR modifiers. The inhibitory dose fifty (ID_{50}) are essential data to design combination experiments with several anticancer drugs. The 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 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-diphenyl-1,4-dihydropyridine derivatives; phenothiazines; HSP90 inhibitor peptide derivatives; Betti base derivative of Tylosin and *Euphorbia* diterpenes.
Among the tested **cinnamylidene ketones,** 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. 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.

Most of the **1,4-diphenyl-1,4-dihydropyridine derivatives** considered as potent inhibitors of multidrug resistance. The 1,4-dihydropyridine heterocyclic rings is a common feature of various bioactive compounds, such as vasodilator, bronchodilator, antitubercular agents and more recently MDR modulators and antitumour agents. 1,4-Dihydropyridines are well known as Ca^{2+} channel blockers and as drugs for the treatment of cardiovascular diseases, including hypertension. 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.

Phenothiazines, apart from their wide range of biological activity have been described as effective MDR modifiers.
The tested phenothiazines were moderately active resistance modulators, especially in the low concentration. Two of the phenothiazine derivatives namely **perphenazine** and

prochlorperazine dimaleate proved to be effective inhibitors of Rhodamin efflux. 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. **Methotripromazine maleate** and **prochlorperazine dimaleate** caused decreased cell survival of the two cell lines studied. Similarly, **trifluoropromazine hydrochloride** was toxic. It is reported, that phenothiazine hydrochlorides are able to modify cell membrane properties by disorganization of the phospholipids and 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 was reduced, obviously it would behave as an effective MDR inhibitor and did not cause cell death.

Heat shock protein 90 (Hsp90) inhibitor peptides arrests the growth of cancer cells. *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 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. In the *in vivo* model experiments, it is reported that these peptides inhibited tumour growth in nude mice bearing the xenografts.

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. **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.

Besides the presence of highly skin-irritant compounds, ***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.
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.

4.3 Interaction of resistance modifier with anticancer drugs

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.

The high anti-MDR potency of selected dihydropyridines (**DL4**, **DL12**, **DL13** and **DP89**) were also very effective in combination with doxorubicin.

Significant synergism was observed *in vitro* combination of **TBN** and doxorubicin and/or vincristine on mouse lymphoma cell line.

The most effective synergistic effect was found between **Euphorbia diterpene 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.
On comparison of the efficacies of compounds **7** and **8**, it can be presumed that the presence of a hydroxy group 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.

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

In the absence of **TBN**, More than double difference was found between doxorubicin accumulation of the resistant and non-resistant cells. **TBN** treatment was able to restore the sensitivity of resistant cell line to doxorubicin, whereas it had no significant effect on the accumulation of non-resistant ones. Linear correlation was found between the toxicity and accumulation of doxorubicin however the cells were exposed to higher concentration of doxorubicin in short time incubation.

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

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. Independently from doxorubicin administration (i.v. or i.p.), **TBN** treatment was able to restore the doxorubicin sensitivity of resistant tumours.

4.6 Apoptosis induction of some resistance modifiers

Many classes of antitumour drugs, including naturally occurring and pharmaceutical compounds, induce apoptosis in cancer cell.
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.

The ethidium bromide and acridine orange (EB/AO) staining of the HeLa cells (monolayer) allowed the identification of live, apoptotic and necrotic cells, *in situ*. Even at a lower concentration of **diterpene 8** (2 µg/mL), treatment of HeLa cells with possible MDR modifiers led to the typical morphological features of apoptosis.

4.7 Anti-promotion effect of plant derivatives

The human CMV was used in a modified *in vitro* model for characterizing lathyrane compounds with antipromotion effect on human lung cancer cells (A549). All the compounds, except **latilagascene D**, decreased IE antigen expression of CMV.
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.

5. NEW STATEMENTS

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

II. During my Ph.D study several groups of compounds were identified as MDR modulators.

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 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, 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 induction of resistance modifiers was studied.

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. ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor **Professor József Molnár** for his continuous support and encouragement throughout my Ph.D 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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