INHIBITION OF DRUG RESISTANCE IN TWO CANCER CELL LINES (MDR AND A2780CIS) *IN VITRO*; AND THE ROLE OF SELECTED SINGLE NUCLEOTIDE POLYMORPHISMS IN CANCER

Ph.D. Thesis

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Szeged

2012

INTRODUCTION

Death from cancer represents around one eighth of all deaths in the world. In the past 30 years, the yearly detected new cases of cancer patients have been doubled. In 2008 approximately 12.7 million new patients were diagnosed with cancer, and an estimated 7.6 million people died of cancer in the world. In the WHO European Region 3.4 million people were diagnosed with and 1.8 million people died of cancer. This represents more than 23% of the people who died from cancer all over the world. However, the population of this region represents less than 15% of the world's population. The higher ratio is the consequence of the aging population with a bulge-number of middle age and decreasing number of births in the younger age categories.

In Hungary, the number of cancer patients has been dramatically increased in the 20th century due to the aging and civilization harms, but it has been more or less stabilized in the last 20 years at around 70.000. At present, there are about 300.000 cancer patients and 32.460 people died of malignant neoplastic diseases in Hungary in 2010. Cancer is the second most frequent cause of death following cardiovascular diseases with 47.743 deaths in 2010 in Hungary according to the KSH (Hungarian Central Statistical Office) data.

Due to the aging of the population predicted for the next decades, cancer incidence will dramatically raise. Therefore, scientific discovery into new approaches to cancer treatment and prediction is even more urgent.

A complete understanding of the mechanisms of the cancer development is very unlikely to come about in the foreseeable future making impossible reliance on a single approach to prevent cancer and deaths from the disease. Pharmacological research is of paramount importance from basic research to clinical phase studies. The development of new anticancer drugs can not only improve the efficacy of treatment, but it can reduce the side effects with the understanding of the mechanism of action. In cancer research, genetic aspects have become even more important for revealing the molecular basis of the disease that can have a predictive value in cancer development and can contribute to forecast the efficacy of treatment.

Cancer cell resistance to drugs is of paramount importance when evaluating response to chemotherapy; therefore, its relevance is unambiguous in clinical practice. Treatment failure can be due to pharmacokinetic mechanisms, also called pharmacokinetic resistance; the consequence is decreased drug exposure of cancer cells. **Multidrug resistance (MDR)** is the main reason for the reduced effect of chemotherapy in many cancer types. Experimentally, tumor cells *in vitro* exposed to one cytotoxic agent develop cross-resistance to a range of structurally and functionally unrelated compounds. A number of different mechanisms can mediate the development of drug or multidrug resistance in cancer. Here we focused our research on **membrane transporters** and **single nucleotide polymorphisms (SNPs)**.

In **ovarian cancer**, up to two-thirds of tumor specimens have been found to overexpress **ABCB1** (ATP-binding transporter B1) membrane transporter and this overexpression has been shown to correlate with poor overall survival. However, in case of the A2780cis cisplatin-resistant ovarian cancer cell line, ABCB1 overexpression does not seem to be important in cisplatin resistance. There are other gene families that are supposed to be responsible for cisplatin resistance: **copper transporters and P-type transporters.** Their members are localized in the plasma membrane such as CTR1 (copper transporter 1), or localized in the intracellular membranes such as CTR2 (copper transporter 2) or ATP7A and ATP7B (P-type copper transporters α and β).

ABC transporters couple the hydrolysis of ATP to move drugs and xenobiotics unidirectional out of the cells, thereby promoting drug resistance. These transporters are membrane proteins consisting of both transmembrane domains and distinctive nucleotide binding domains. The nucleotide binding domains generate energy from ATP hydrolysis to actively transport a variety of compounds across the plasma membrane. Nowadays, twenty ABC transporters have been associated with multidrug resistance. In our investigation we focused on a human ABCB1-transfected mouse lymphoma cell line. Normally, **ABCB1** is expressed in the apical membrane of many secretory cell types, such as kidney, liver, intestine, adrenal gland, and the blood-brain barrier where the normal function involves the excretion of drugs and

their metabolites. The expression of ABCB1 in this cell line is constitutively high enough to be a good model cell line to examine the ABCB1-linked drug resistance. Due to the nature of ABCB1 as an efflux pump for cell protection against a variety of substances, its substrates vary greatly in size, structure and function, ranging from small molecules, such as organic cations, carbohydrates, amino acids, and some antibiotics to macromolecules, such as polysaccharides and proteins.

Current evidence suggests that one component of cisplatin uptake is mediated by the CTR1 and CTR2. CTR1 has been described as the major copper influx transporter that controls the uptake of platinum-containing drugs, such as cisplatin, carboplatin and oxaliplatin *via* coupled-transport. According to recent studies, it has been shown that mammalian cells express a second structurally related transporter, CTR2, whose function is less well-defined. CTR2 is predominantly localized in endosomes and lysosomes in mammalian cells; however, when expressed at the cell surface, CTR2 is capable of mediating copper uptake as well. A number of studies have demonstrated that besides CTRs, ATP7A and B modulate the export of cisplatin. These Cu-ATPases represent a large superfamily of P-type membrane transporters which use the energy of ATP hydrolysis to transport copper and cisplatin across cell membranes.

Several compounds have been found as promising **MDR modifiers** *e.g.* flavonoids and phenothiazines such as chlorpromazines, stilbenes, di- and triterpenes and carotenoids at the Department of Medical Microbiology and Immunobiology in the last 30 years. Furthermore, specifically modified compounds such as **metal coordination complexes** of tricyclic compounds have also been investigated, since it has been assumed that metals induce some changes in binding features to ABCB1. At present, a number of metal-based compounds, such as ruthenium(II) organometallic arene complexes or tetrahedral Au(I) phosphine complexes, are known to have promising antiproliferative effects in a wide range of tumors.

Therefore, we tried to **combine the advantages of platinum- and copper-based coordination complexes and resistance modifier steroid compounds** in a wish to overcome drug resistance in a cisplatin-resistant human ovarian cancer cell line (A2780cis) directly through copper transporters and in a human ABCB1 gene-expressing mouse T-lymphoma cell line (MDR). Other **compounds of synthetic** (hydantoin derivatives) **and natural origin** (steroidal alkaloids, saponins and phenolic components) were also **investigated to reverse drug resistance**.

Cancer is a consequence of genetic changes in certain tumor suppressor genes and proto-oncogenes. Resistance can also be caused by SNPs of membrane transporters which can be hereditary in many cancer types, such as breast and ovarian cancer. SNPs can also alter the ability of a protein to bind its substrates or inhibitors and change the subcellular localization of proteins. These small genetic changes may be responsible for medicinal drug deposition and disease susceptibility, which have already been verified in certain diseases, such as age-related cortical cataract, diabetes mellitus and cancer. Therefore, another objective of our research was to **define the relationship between certain SNPs and cancer** complementing our knowledge about sequence – protein function relationships of the examined genes and encoded proteins.

AIMS

The main aim of our recent study was to evaluate the effects of synthetic and natural compounds on drug resistance in a hormone-independent cisplatin-resistant human ovarian cancer cell line (A2780cis) and in a human *ABCB1* gene-transfected doxorubicin-resistant mouse T-lymphoma cell line (MDR). Furthermore, distributions of certain single nucleotide polymorphisms in cancer-related genes were also investigated.

The goals of the study are as follows:

1. **Characterization of the examined cell lines (A2780/A2780cis and PAR/MDR) by immunocytochemistry** by using specific monoclonal antibodies to determine the expression of certain proteins, such as ABCB1, MRP1, estrogen and progesterone receptors.
2. **Determination of the antiproliferative effects** of compounds (steroids and platinum/copper complexes, hydantoin derivatives, steroidal alkaloids of *Veratrum lobelianum*, *V. nigrum* and *Peganum nigelastrum,* saponins and phenolic components of *Tribulus terrestris* and *Smilax excelsa*) in A2780cis and/or MDR cells.
3. **Evaluation of the MDR reversal effects** of compounds (steroids and platinum/copper complexes, hydantoin derivatives, steroidal alkaloids of *Veratrum lobelianum*, *V. nigrum* and *Pergamum nigelastrum,* saponins and phenolic components of *Tribulus terrestris* and *Smilax excelsa*) on A2780cis and/or MDR cell lines.
4. **Combination of selected resistance modifiers and some anticancer agents doxorubicin and cisplatin to determine their mode of interaction** by checkerboard microplate method in A2780cis and/or MDR cells.
5. **Apoptosis induction in A2780cis and MDR cells** by selected substituted steroids, and their platinum- and copper-complexes.
6. **Evaluation of allele distributions of single nucleotide polymorphisms of cancer-related genes** by PCR-RFLP.

MATERIALS AND METHODS

Cell cultures

PAR/MDR. Parental (PAR) mouse T-cell lymphoma cells (ECACC cat. no. 87111908) were transfected with pHa ABCB1/A retrovirus. The human ABCB1-expressing cell line L5178Y was selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. The PAR and the human *ABCB1* gene-transfected subline (MDR) were cultured at 37°C in McCoy's 5A media (Sigma-Aldrich) supplemented with 10% heat-inactivated horse serum (H1270, Sigma-Aldrich), L-glutamine 200 mM (Invitrogen) and penicillin-streptomycin mixture (Sigma-Aldrich) in 100U/l and 100 mg/l concentration, respectively. The media for culturing the MDR cell line was complemented with colchicine (60 ng/ml, Sigma-Aldrich). These mouse lymphoma cell lines were maintained in a 5% CO_2 incubator at 37°C.

A2780/A2780cis. A2780cis cell line (ECACC cat. no. 93112517) was developed by chronic exposure of the parental cisplatin-sensitive A2780 human ovarian cancer cell line to increasing concentrations of cisplatin. These cell lines were cultured in RPMI 1640 media (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated fetal bovine serum (10106-169, GIBCO, Invitrogen), L-glutamine (200mM) and penicillin-streptomycin mixture in 100U/l and 100 mg/l concentration, respectively. In order to retain resistance of A2780cis cells, cisplatin (Teva Pharma) was added to the media every 2-3 passages at 1 μM final concentration. The ovarian cancer cell lines were maintained in a 5% CO_2 incubator at 37°C.

Compounds

Semisubstituted steroids and their derivatives in complex with platinum and copper salt (I). We examined thirty-five modified steroid (1-35) derivatives provided by Prof. Irén Vincze (Department of Organic Chemistry, University of Szeged) and Dr. Csaba Somlai (Department of Medical Chemistry, University of Szeged). Then, we evaluated three selected compounds (4, 5, 25) and their copper (4C, 5C, 25C) and platinum coordination complexes (4P, 5P, 25P), these compounds were provided by Dr. Imre Labádi (Department of Inorganic and Analytical Chemistry, University of Szeged).

Hydantoin derivatives (II). We investigated thirty hydantoin derivatives (SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, P10, P11, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2, GL-1, GL-7, GL-14, GL-16, GL-18) which were provided by Dr. Jadwiga Handzlik and Prof. Katarzyna Kieć-Kononowich (Department of Technology and Biotechnology of Drugs, Jagiellonian University, Cracow, Poland).

Steroidal alkaloids of Veratrum and Peganum species (III, IV). We examined nine alkaloids (A1-9): neogermitrine (A1), verabenzoamine (A2), veratroilzigadenine (A3), 15-O-(2-methylbutyroyl)germine (A4), veralosinine (A5), veranigrine (A6), deoxypeganine (A7), harmine (A8) and peganine (A9) isolated prevoiusly from the roots and rhizomes of three Mongolian species (*Veratrum lobelianum, V. nigrum, Melanthiaceae; Peganum nigelastrum, Nitrariaceae*) by Veselin Christov *et al.* and Antoaneta Ivanova *et al.* (Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria).

Saponins and phenolic components of Tribulus terrestris and Smilax excelsa (V). We evaluated six pure steroidal saponins (S-4, S8, S9), a mixture of three saponin isomers (S5-7), and two phenolic components (S10, S11) isolated from the saponin fractions of *Tribulus terrestris (Zygophyllaceae)* and *Smilax excelsa (Smilacaceae)* by Antoaneta Ivanova *et al.* (Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria).

Methods

Immunocytochemistry. The immunocytochemical investigations were carried out at the National Institute of Oncology (Budapest, Hungary). Cancer cells were cultured and resuspended in serum-free media to a density of 1×10^6 cells/ml. 100 µl cell suspension was centrifuged at 1.000 rpm for 5 min by a Shandon Cytospin 3 centrifuge (Thermo Fisher Scientific), then Giemsa-stain was applied for one of each sample. The slides were fixed in acetone at -20°C for 10 min and were washed for 5 min in TBS. The investigations were performed in a wet chamber.

The following monoclonal antibodies (MABs) were applied to detect the cell membrane proteins: NCL-JSB1 (for ABCB1; Novocastra™, Leica Biosystems), NCL-PGLYm (for ABCB1; Novocastra™), NCL-MRP1 (for MRP1; Novocastra™), NCL PGR 312 (for progesterone receptor; Novocastra™), SP1 RM 9101-S (for estrogen receptor; Neomarkers, Thermo Fisher Scientific). MCF7 breast cancer cell line was applied as a control. The smears were washed with PBS. Endogenous peroxidases were quenched in 0.03% H_2O_2 + NaN_3 for 10 min and washed with PBS. The samples were incubated for 1 h at room temperature with primary MABs, 100 µl of the MABs (anti-ABCB1, anti-MRP1 10x; anti-estrogen 200x; anti-progesterone 180x diluted in PBS) and the positive control cytokeratinase (25x diluted in PBS) were added. Then, DAKO Cytomation EnVision+ System-HRP (AEC) kit (DAKO North America) was applied according to manufacturer's instructions to detect the red end-product of the cleavage by horseradish peroxidase under microscope. Finally, haematoxylin-eosin staining was carried out and cover slips placed over the slides containing the samples (wet mounts) with Dako Cytomation Faramount Adequous Mounting. Then, photographed with a Nikon Microphot - SA + UFX-DX instrument (Nikon) to yield 100x, 200x, 400x magnifications.

Assay for antiproliferative effect. The effects of increasing concentrations of the drugs on cell growth were examined in 96-well flat-bottomed microtitre plates. The stock compounds were dissolved in DMSO to yield a starting concentration of 50 µg/ml in a final volume of 150 µl. Two-fold dilutions with 2% DMSO and 50 µg/ml compounds were made; the final concentration of DMSO was 0.0156% and the final concentration of the compounds were 1.5625 µg/ml. DMSO was used as a control. A total of 6×10^3 cells in 50 µl of media were then added to each well, with the exception of the media control wells. The culture plates were further incubated at 37°C for 72 h, at the end of which 15 µl of MTT solution (3-(4,5-dimethyiltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) thiazolyl blue solved in PBS to a final concentration of 5 mg/ml) were added to each well. After further incubation at 37°C for 4 h, 100 µl of sodium dodecyl sulphate (SDS) solution (10% + 1 N HCl) were added to each well and the plates were further incubated overnight in a 5% CO_2 incubator at 37°C. Cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Fisher Scientific). Inhibition of cell growth was determined as a percentage according to the formula:

$$100-\left[\frac{OD\,treated\,cells-OD\,medium\,control}{OD\,cell\,control-OD\,mediumcontrol}\times 100\right]$$

Assay for reversal of MDR in tumor cells by flow cytometry. The cells were adjusted to a density of 2×10^6 cells/ml, resuspended in serum-free media and distributed in 0.5 ml aliquots (1×10^6 cells) into 1.5 ml Eppendorf centrifuge tubes. The cells were incubated in the presence of compounds for 10 min at room temperature. Then, indicator ABCB1-substrate rhodamine 123 (R123) (Sigma-Aldrich) was added to each sample at a final concentration of 10 μg/ml and the cells were incubated for a further 20 min at 37°C in water bath, washed twice and resuspended in 0.5 ml PBS for analysis. The fluorescence of the cell population was measured in FL1 at 540 nm (ref. 630 nm) wavelength with a FACS Star Plus flow cytometer (Beckton, Dickinson and Company). Verapamil hydrochloride (EGIS) was used as a positive control at a final concentration of 10 μg/ml.

The results presented are obtained from a representative flow cytometric experiment in which 1×10^4 individual cells of the population were evaluated for the amount of the retained R123. The percentage mean fluorescence intensity was calculated for the PAR/MDR and A2780/A2780cis cell lines, treated cells were compared to the untreated ones. Fluorescent activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

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

Assay for combination of chemotherapy. A checkerboard microplate method was applied to study the effects of interactions between the resistance modifiers and the anticancer agent doxorubicin (Ebewe) on MDR cells or cisplatin (EGIS) on A2780cis cells as an *in vitro* model of combination chemotherapy. The combinations were carried out in a final volume of 200 μl. Doxorubicin or cisplatin (A) was measured into the 96-well microtitre plate in a horizontal direction in two-step dilutions, the highest final concentrations were 8 μg/ml or 20 μg/ml, respectively. The resistance modifiers (B) were diluted vertically in two-step dilutions. The cell suspension in tissue culture media (supplemented McCoy'5A without colchicine or RPMI 1640) was distributed into each well in 50 μl containing 1×10^4 MDR cells or 2×10^4 A2780cis cells. The plates were incubated in a CO_2 incubator at 37°C for 48 h. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured with a Multiscan EX ELISA reader. Drug interactions were evaluated according to the following system:

$$FIC_A = IC_{50A\ combination} / IC_{50A\ alone}$$
$$FIC_B = IC_{50B\ combination} / IC_{50B\ alone}$$

where IC = inhibitory concentration and FIC = fractional inhibitory concentration. FIX = FIC_A+FIC_B, where FIX = fractional inhibitory index. FIX < 0.5 indicates synergism, 0.5 < FIX < 4 indicates no interaction (addition), and FIX > 4 indicates antagonism between the anticancer drug and the potential MDR modifier.

Assay for apoptosis induction. Apoptosis induction in the presence of various compounds on MDR and A2780cis cells was determined. The MDR cells were adjusted to a density of 1×10^6 cells/ml and were distributed in 0.5 ml aliquots into microcentrifuge tubes. Then, drugs were added to the samples to determine their effect in apoptosis induction. 12H-benzo(*a*)phenothiazine (M627) was used as a positive control of apoptosis at a final concentration of 50 μg/ml. The MDR cells were incubated at 37°C for 45 min in water bath, then washed twice with PBS and resuspended in 0.5 ml McCoy's 5A media. The 24h incubation was carried out in a 5% CO_2 incubator at 37°C.

The suspension of A2780cis ovarian cancer adherent cells were diluted to 1.5×10^6 cells/ml and distributed into 24-well culture plates for 4 h in 750 μl aliquots to let the cells attach. Then, the compounds were added, M627 was used as a control for apoptosis at a final concentration of 50 μg/ml, and paraformaldehide was used as a control for necrosis. Apoptosis induction on the A2780cis cells was conducted over a period of up to 3 h in the presence and absence of the compounds; the samples were

then washed with PBS and resuspended in RPMI media. The 24 h incubation was carried out in a 5% CO_2 incubator at 37°C.

MDR cells were transferred into 1.5 ml Eppendorf-tubes. The apoptosis assay was carried out according to the Rapid Annexin V Binding Protocol of Annexin V-FITC Apoptosis Detection Kit (Calbiochem, Merck). Briefly, after transferring the MDR cells into Eppendorf-tubes, 10 µl of Media Binding Buffer was added to each sample, then 1.2 µl Annexin V (AV)-FITC (fluorescein isothiocyanate) was measured to the samples. In case of A2780cis cells, 15 µl of Media Binding Buffer and 1.2 µl AV-FTIC was added into the wells of the 24-well plate. The samples (both MDR and A7820cis) were incubated at room temperature for 15 min in the dark. Then, after washing the cells in PBS, the samples were centrifuged at 2000 g for 3 min at room temperature, and the media was removed. The cells were resuspended in 0.5 ml ice-cold 1x Binding Buffer, then, stored on ice. 10 µl propidium iodide (PI) was added to the samples at a final concentration of 1.5 µg/ml, and the samples were analyzed immediately by measuring the fluorescence activity. AV was measured in FL1 at 540 nm, and PI was measured in FL3 at 680 nm wavelength. 2×10^4 cells of the gated population were measured in each sample by a FACS Star Plus flow cytometer (Beckton, Dickinson and Company).

DNA extraction from human blood for PCR. Ethical Permission was approved by the Human Investigation Review Board at the Albert Szent-Györgyi Clinical Centre. All patients gave their inform consents to the examination. In our experiments most of the cancer patients (69 of 88) selected for SNP analysis had been treated by chemotherapy previously, the therapies were carried out according to the variable protocols of certain cancer types. The patients were divided into two groups: gynecological cancer group (ovarian and cervical cancer, n=31), and a mixed cancer group (n=57). A group of healthy volunteers (n=99) was applied as control.

Venous blood was taken into 3 ml tubes anticoagulated with EDTA at the Regional Blood Transfusion Centre (University of Szeged). The DNA extraction was carried out by a modified salting out protocol. Briefly, 400 µl blood was resuspended in a 1.5 ml Eppendorf centrifugation tube with 600 µl distilled water and 250 µl of nuclei lysis buffer (50 µM Tris-HCl pH 7.5, 22.7% Triton X-100, 20 µM $MgCl_2$ and 140 µM saccharose). After washed twice with 600 µl distilled water (10.000 rpm, 3 min), the cell lysates were digested overnight at 37°C with 40 µl of 10% SDS, 80 µl 5x proteinase-K buffer (0.3 M NaCl and 0.1 M EDTA pH 8.0) and 20 µl of a protease K (20 mg/ml, Fermentas) solution. After digestion, 150 µl of 5M Na-acetate was added to each tube and shaken vigorously for 15 sec, and centrifuged at 13.000 rpm for 3 min. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 1.5 ml Eppendorf centrifugation tube. 600 µl of pre-cooled isopropanol (-20°C) was added to each tube and the tubes were inverted several times until the DNA precipitation. After centrifugation at 13.000 g for 3 min, the supernatant was discarded and 800 µl of pre-cooled ethanol (-20°C, 70%) was added to the pellet. After the final centrifugation step at 13.000 g for 3 min, the supernatant was discarded again, and the DNA was dried at room temperature. For the PCR experiments, DNA was dissolved in 25 µl PCR-clean DNAse and RNAse free distilled-water (Fermentas). The concentration of the DNA was measured by NanoDrop™ 1000 (Thermo Fisher Scientific).

Evaluation of SNPs by PCR-RFLP. These experiments were carried out at the Institute of Integrative and Comparative Biology (University of Leeds, United Kingdom). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) was applied to identify *ABCB1* (rs2032582, rs1045642), *P53* (rs1042522), *BCL2* (rs2279115) and *BAX* (rs4645878) SNPs. Each PCR was performed in a 20 µl reaction mixture containing 10-100 ng of genomic DNA templates, 0.5 pM of each primer (Sigma-Aldrich), 2 mM of each deoxynucleoside triphosphate (Promega), 10x PCR buffer, 2.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase (BIOTAQ™ Red DNA Polymerase, Bioline). The PCR profile consisted of an initial denaturation step of 96°C for 5 min, 35 cycles of 96°C for 45 s, 56°C for 40 s and 72°C for 30 s and a final extension step of 72°C for 10 min in a 2720 Thermal Cycler (Applied Biosystems).

ABCB1, *BAX*, *BCL2* and *P53* polymorphisms were examined by the digestion of 5 µl of PCR product by 2 U of restriction enzyme (New England Biolabs) in a final volume of 10 µl at 37°C overnight (BanI, DpnII, MspI, BccI) or at 60°C for 4 h (BstuI), then evaluated by gel electrophoresis on 2% agarose

gel (Sigma-Aldrich). The sequences of the primers and fragment length of selected SNPs are presented in Table I.

Table I. Cancer-related single nucleotide polymorphisms selected for SNP studies examined by PCR-RFLP.

Gene	SNP	primer	sequence	RE	size (bp)
ABCB1	rs2032582	MDR-9 (f)	5`-TgCAggCTATAggTTCCAgg-3`	BanI	198, 26; 224
		MDR-10 (r)	5`-TTTAgTTTgACTCACCTTCCCg-3`		
	rs1045642	MDR-11 (f)	5`-TgTTTTCAgCTgCTTGATgg-3`	DpnII	158, 39; 197
		MDR-12 (r)	5`-AAggCATgTATgTTggCCTC-3`		
BAX	rs4645878	BAX1 (f)	5`-CATTAgAgCTgCgATTggACCg-3`	MspI	20, 89; 109
		BAX2 (r)	5`-gCTCCCTCgggAggTTTggT-3`		
BCL2	rs2279115	BCL1 (f)	5`-CTgCCTTCATTTATCCAgCA-3`	BccI	189, 111; 300
		BCL2 (r)	5`-ggCggCAgATgAATTACAA-3`		
P53	rs1042522	P53A (f)	5`-ATCTACAgTCCCCCTTgCCg-3`	BstUI	169, 127; 296
		P53B (r)	5`-gCAACTgACCgTgCAAgTCA-3`		

Rs: reference sequence number. (f) and (r) represents forward and reverse primers. RE: restriction endonuclease. Bp: base pair. Product size after digestion: wild type alleles digested, variant alleles undigested in italics underlined. Bases: A – adenine, C – cytosine, g – guanine, T – thymine.

Statistical analysis. Statistical analyses for comparison of genotype frequencies between groups was performed by using the χ^2 (chi-square) test, and Fisher's exact test when the values in any of the cells of a contingency table was below 10 with only one degree of freedom. The relationship between genotypes and cancer is presented as the odds ratio (OR), with a 95% confidence interval (CI). Genotype distributions and deviation from Hardy-Weinberg equilibrium (HWE) were tested separately for both cases and controls with SPSS (SPSS for Windows 16.0) and HWE calculator. All statistical tests were two-sided, a *P* value of 0.05 was considered significant. Power and sample size (PS) was determined by the PS program version 3.0.

RESULTS AND DISCUSSION

Immunocytochemistry

High level of ABCB1 expression was detected in the membrane of MDR cells; whereas in the A2780cis cells and in the control cells ABCB1 expression could not be shown. Specific staining of the cytoplasm was detected when using MRP1 specific MAB on both MDR and A2780cis cell lines. Estrogen and progesterone hormone receptors were undetectable in the examined cell lines; however, in the case of control breast cancer MCF7 cell line they were detectable.

In our experiments it was revealed that this A2780cis cell line does not overexpress either of the two hormone receptors of estrogen and progesterone. Hence, we assume that the effects of our extensively examined steroid-type compounds can be basically different from that of hormone-dependent tumors. The mechanism of cisplatin resistance in the A2780cis cells is still not fully determined, therefore, further investigation is required. The resistance is supposedly due to a multifactorial mechanism including altered transporter expression patterns, mismatch repair and cell-cycle modification.

Effect of selected compounds on cancer cells

Various compounds have been investigated for their ability to reverse drug and multidrug resistance, including synthetic and natural plant-derived compounds. In our experiments substituted steroids (I) and their platinum and copper complexes, hydantoin derivatives (II), alkaloids of *Veratrum* and *Peganum* species (III, IV), and saponins and phenolic components of *Tribulus terrestris* and *Smilax excelsa* (V) were evaluated.

Semisubstituted steroids and selected platinum and copper coordination complexes (I). A number of steroid-type molecules have been described previously to prevent drug and multidrug resistance in cancer. Furthermore, specifically modified compounds have also been investigated, such as metal coordination complexes of tricyclic compounds, since it has been assumed that metals induce some changes in binding features to ABCB1. A number of studies have demonstrated that copper transporters CTR1 and CTR2,

and the ATPase copper efflux transporter α and β (ATP7AB) are responsible for transport of cisplatin. Therefore, we tried to combine the advantages of platinum and copper-based coordination complexes and resistance modifier steroid-based compounds in a wish to overcome drug resistance.

In our study, thirty-five estrone- and androstane-type compounds (1-35) and copper and platinum complexes of selected compounds (4, 5, 25) were examined in a cisplatin-resistant human ovarian cancer cell line (A2780cis) and in a human ABCB1-expressing mouse lymphoma cell line (MDR). Eighteen compounds were effective as proliferation inhibitors with IC_{50} under 50 μg/ml on A2780cis cell line, and fourteen were found to inhibit cell proliferation on MDR cell line. Only two compounds (9 and 34) were active on both cell lines.

Then, the compounds were examined as potent resistance inhibitors. Seven of the substituted steroids were found to be effective in inhibition of resistance on both cell lines. On the basis of the activity of the steroid samples, certain tendencies seem to be present. Both androstane derivatives and estrone ethers can be found among the active and inactive groups of compounds, consequently, the activity does not depend on the structure of the skeleton. Thus, it is rather the nature and arrangement of the functional groups that determine the activity of the compound. Compounds containing a primary amino function with O-acetyl or N-acetyl groups were cytotoxic for both cell types. Very polar compounds containing two or more hydroxyl groups or secondary amino groups were ineffective in our examinations. But most of the samples, where these groups are arranged on the D-ring of the skeleton and the O or N atom of the functional group was at 1,3-position to D-ring were found to be active in both cell types. This structural arrangement may serve as a binding moiety on the polar protein-glycan surface of the cells and can result in an effect independent of the structure of the A ring. The distance between X and Y (300-400 pm) may allow a supramolecular connection on the peptide chain of the cell surface by polar-polar interaction (Figure 1). Our *in vitro* experimental data indicate that some of the steroid-type synthetic molecules can be regarded as promising structures for rational drug design as resistance inhibitors with a novel mechanism of action.

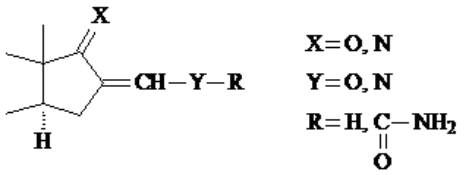

Figure 1. Structure of the most active five semisubstituted steroid compounds examined in the MDR reversal assay on both A2780cis and MDR cell lines. Their common feature is the O or N atom in 1,3-position to D-ring. The distance between X and Y (300-400 pm) may allow a supramolecular connection on the peptide chain of the cell surface by polar-polar interaction.

Then, we evaluated the interaction between anticancer agents and steroid-derivatives. On A2780cis cell line, we did not find synergistic interaction between cisplatin and any of the steroid-type compounds. On MDR cell line, one compound (11) showed synergism with doxorubicin (FIX = 0.45) and another compound (25) was found to be weakly synergistic (FIX = 0.47).

After the evaluation of these thirty-five steroids in both cell types, three compounds (4, 5, 25) were selected to determine their effect in platinum and copper complexes. Copper-steroid complexes seem to be better inhibitors of cell proliferation than platinum-steroid complexes on both cell lines according to the IC_{50} values. In A2780cis cells, none of the complexes were found to be better resistance inhibitors than pure steroids at a final concentration of 40 μg/ml. In MDR cells, both platinum and copper complexes were effective resistance inhibitors at 40 μg/ml concentration. In these experiments, all compounds were evaluated at ten-fold lower concentrations (4 μg/ml) as well, and the FAR values were found to be about four-fold lower. This means that these compounds act in a concentration-dependent manner, and at the higher concentration the membrane-embedded ABCB1 transporters might be oversaturated.

In the apoptosis induction experiments none of the tested compounds induced apoptosis in A2780cis human ovarian cancer cells or in MDR mouse lymphoma cells. However, a non-significant increase of the percentage of apoptotic population was observed when evaluated in A2780cis cells, but the higher dose of compounds 25 and 25C only resulted in an increase of non-apoptotic dead cell population. Most likely these selected compounds at higher concentrations directly alter membrane structure rather than modify apoptotic pathways, thus, inducing necrosis and avoiding complete apoptosis in these two examined cell lines.

Hydantoin derivatives (II). Various aspects of the biochemical and pharmacological properties of hydantoin derivatives have been studied such as fungicidal, herbicidal and anti-inflammatory properties. Previously, the cytotoxic activity of spirohydantoin derivatives has been tested on ovarian and breast cancer cell lines. Furthermore, it has been shown that two spirohydantoin derivatives have inhibited cell growth and induced apoptosis in leukemia and colon cancer cells. Recent studies have shown that some diversely substituted diazaspirohydantoins has antiproliferative effect against MCF-7 breast carcinoma, HepG-2 hepatocellular carcinoma, HeLa cervix carcinoma and HT-29 colon carcinoma cell lines. The result of a recent study provided evidence to support the concept that introducing accessory metal centers is a viable strategy for augmentation of the cytotoxic activity of platinum drugs. Further data about the *in vitro* cytotoxicity of new platinum(II) complexes with hydantoin ligands have shown that 5-methyl-5-phenylhydantoin, as well as the previously synthesized spirohydantoin compounds cyclopentane-spirohydantoin and cyclohexane-spirohydantoin, exert concentration-dependent cytotoxic activity on a panel of tumor cell lines, although less pronounced than that of the reference drug cisplatin.

In our experiments, thirty hydantoin derivatives were evaluated. First, the IC_{50} values were determined; they varied widely between 3 and 450 μg/ml. Non-toxic compounds with IC_{50} values under 100 μg/ml were selected out of the 30 hydantoin derivatives for the MDR reversal experiments. Compound BS-1 was the most potent inhibitor of resistance (FAR = 77.68), this compound was active with FAR value of 46.71 at very low 0.4 μg/ml concentration. From the most effective compounds with high FAR values, nine compounds (AD-26, AD-29, RW-13, KF-2, BS-1, MN-3, RW-15b, JH-63 and SZ-7) were chosen to determine the interaction with doxorubicin, out of these, most of the compounds except SZ-7 showed synergistic interaction on MDR cell line.

All of the most active nine compounds contained aromatic substituents as well as some tertiary amine fragments. Furthermore, based on literature data, new platinum(II)-hydantoin complexes would be interesting to examine as possible anticancer agents.

Steroidal alkaloids of Veratrum and Peganum species (III, IV). Alkaloids have extensive pharmacological actions including anticancer, anti-Alzheimer's disease, anti-diabetes, antimicrobial and anti-inflammatory activities. Vinca alkaloids are most famous for being anticancer agents, since they are well-known substrates of the ABCB1 transporter. Furthermore, it has been demonstrated that the cytotoxic effects of vinca alkaloids as well as those of podophyllotoxin against certain human tumor cells have been antagonized by cisplatin. Therefore, in our examinations, newly extracted alkaloids were considered to be promising as possible ABCB1-inhibitors.

The present study was undertaken to access the inhibition of MDR of cancer cells by alkaloids (A1-9) isolated from *Veratrum lobelianum*, *V. nigrum* and *Peganum nigelastrum*. Plants from genus *Veratrum* and genus *Peganum* were traditionally used as medicinal substances in Mongolia for a long time. The main chemical ingredients in *V. lobelianum*, *V. nigrum* and *P. nigelastrum* species are steroidal and indole-type alkaloids.

In this experiment, some steroidal alkaloids moderately inhibited the cell proliferation of MDR cells. The IC_{50} values were found to be between 20.76 and 26.07 μg/ml for compounds A1-3, A5 and A6. The MDR reversal effects of the alkaloids were examined on MDR cells and the most active compounds were verabenzoamine (A2), veralosinine (A5) and veranigrine (A6) and with FAR values of 87.00, 93.26 and 88.81 at final cc. of 40 μg/ml, respectively.

The mechanism by which these steroidal alkaloids inhibit ABCB1 activity is currently unknown. However, structurally, these steroidal alkaloids are small lipophilic molecules with planar polycyclic skeleton and tertiary nitrogen atom and are likely to be able to enter cells by passive diffusion. Although, it is difficult to find structural features that are common to a large number of chemosensitisers, it has been suggested that resistance modifier agents are hydrophobic, contain two or more planar aromatic rings and tertiary nitrogen. The structure of steroidal alkaloids fits this profile.

Saponins and phenolic components of Tribulus terrestris and Smilax excelsa (V). Saponins are generally found in the roots, flowers and seeds of plants and have long been used against various diseases to enhance natural resistance. In a recent study it has been found that tubeimoside I, a triterpenoid saponin extracted from *Bolbostemma paniculatum (Cucurbitaceae)*, sensitizes to cisplatin in cisplatin-resistant

A2780cis human ovarian cancer cells. Hence, we evaluated eleven saponins and structurally related compounds (S1-S11) isolated from *T. terrestris* and *S. excelsa* in order to find new resistance modifiers.

All of the examined compounds were found to be moderate proliferation and MDR inhibitors on MDR cell line. Furthermore, in the combination experiment with doxorubicin it was found that one of these eleven saponins, methyloprototribestin (S2) can act in a synergistic manner.

When evaluating the structure-activity relationship, it was found that the activity of these saponins appears to correlate the position of sulphur-containing substituents; this may refer to the role of charge transfer complex formation while binding of these compounds to ABCB1. Since it has already been demonstrated that saponins can sensitize A2780cis cancer cell to cisplatin, these new compounds isolated from *T. terrestris* and *S. excelsa* would be promising candidates as adjuvants in cisplatin-resistant cancer therapy.

SNP analysis to determine their role in cancer

Complex SNP studies may be a way of personal risk assessment and could serve as a possible treatment predictive marker for chemotherapy in several cancer types. Certain hereditary SNPs in cancer patients are assumed to increase the development of cancer, for instance, by inducing conformational changes in the encoded protein. Therefore, we evaluated some SNPs in cancer-related genes to determine cancer risk.

In our experiments, most of the cancer patients (69 of 88) selected for SNP analysis had been treated with chemotherapy, the therapies were carried out according to the variable protocols of certain cancer types. The patients were divided into two groups: gynecological cancer group (ovarian and cervical cancer, n = 31), and a mixed cancer group (n = 57), and a group of healthy volunteers (n = 99) was applied as control group.

The correlation of anticancer therapies and the success of treatment are not discussed in this thesis because the sample size is not big enough to draw the inference in statistical analysis. Since, we focused on hereditary SNPs that have been already included in the genome of healthy volunteers and patients before cancer developed, genomic DNA was extracted from blood, but not from the cancer tissue itself. No significant differences in *ABCB1* and *BCL2* genotype frequencies were found between cancer patients and the control group. However, it has already been verified previously that these examined SNPs are in correlation with ABCB1 levels and uptake of its substrates and disease outcome. Significant differences in *BAX* genotype frequencies with allele G ($P < 0.001$, OR = 3.561), or positive trend in *P53* genotype frequencies with allele C ($P = 0.05917$, OR =1 .622) were found between the mixed cancer group and the control group.

The data obtained in our study suggest that the allelic variants of *BAX* may be predictive marker of cancer. However, in this preliminary study only a limited number of samples were included. Further investigation is required to determine the correlation between *ABCB1* allelic variants and their expression levels in chemotherapy-resistant cancer.

NEW RESULTS RELATED TO THE THESIS

1. Immunocytochemistry

We demonstrated that the cisplatin-resistant A2780cis cell line does not express any of the ABCB1 and MRP1 transporters, and any of the hormone receptors of estrogen and progesterone.

2. Effects of compounds in A2780cis and MDR cancer cell lines

2.1. Antiproliferative effect

Several compounds were found to be good proliferation inhibitors in A2780cis and MDR cell lines. Copper-steroid coordination complexes seem to be better inhibitors of cells proliferation than platinum-steroid coordination complexes in both A2780cis and MDR cell lines.

Numerous compounds were found to significantly inhibit cell proliferation of MDR cells including semisubstituted steroids, hydantoin derivatives, steroidal alkaloids and saponins and phenolic components.

2.2 Reversal of drug resistance

Several substituted steroids were effective in the MDR reversal experiments in A780cis and MDR cell lines. Some steroids (2, 5, 6, 12, 22, 25, 27) were found to be effective proliferation inhibitors in both A2780cis and MDR cells. When comparing structure, it seems that the activity does not depend on the structure of the skeleton, but it is rather the nature and arrangement of the functional groups that determine the activity.

Numerous hydantoin derivatives and natural compounds (steroidal alkaloids and phenolic components, and saponins) reversed resistance of MDR mouse lymphoma cells.

2.3 Interaction of selected compounds and anticancer agents

None of the complexes were found to be in synergistic interaction with the anticancer agent cisplatin in A2780cis cells. In MDR cells, one the steroid-type molecules with androst-5-ene structure (11) was found to have a synergistic effect in combination with doxorubicin; compound 25 showed some synergism as well.

Eight hydantoin derivatives (AD-26, AD-29, RW-13, KF-2, BS-1, MN-3, RW-15b, JH-63) containing aromatic substituents and some tertiary amine fragments showed synergistic interaction with doxorubicin in MDR cells.

The most active alkaloids in the combination experiments in MDR cells were veralosinine (A5) and veranigrine (A6). Both are hydrophobic compounds containing two or more planar aromatic rings and a tertiary nitrogen.

Methylprototribestin (S2) was found to be the only saponin that had some synergistic interaction with doxorubicin in MDR cells.

2.4 Apoptosis induction

None of the steroid-type platinum and copper complexes was able to induce apoptosis either in A2780cis or in MDR cells.

3. SNP analysis to determine their role in cancer

The SNP nr. rs2279115 in *BAX* gene may be predictive of cancer development, allele G might increase the probability of developing cancer. In case of the other four SNPs of *ABCB1*, *BCL2* and *P53* genes, no significant differences were found between cancer patient and healthy volunteer groups. In case of *ABCB1*, strong linkage disequilibrium was measured between its two SNPs nr. rs2032582 and rs1045642.

ACKNOWLEDGEMENT

I wish to thank my supervisor, **Professor József Molnár** for the opportunity to work in his group at the Department of Medical Microbiology and Immunobiology. Furthermore, I wish to thank for his valuable comments and inspiration. The supportive work environment created by him considerably contributed to the success of my work.

I would like to express my greatest gratitude to **Professor Yvette Mándi** for the possibility to work in her Department of Medical Microbiology and Immunobiology.

I would like to acknowledge to **Professor Irén Vincze**, to **Dr. Csaba Somlai** and to **Dr. Imre Labádi** for their contribution to the steroid work.

I would like to thank to **Professor Leonard Amaral** and **Professor Maria José Umbelino Ferreira** from the University of Lisbon, and to **Professor Neven Zarkovic** from the University of Zagreb the opportunity to work in their laboratories as a guest early-stage researcher.

Here, I would like to acknowledge to **Dr.Veronika Szekeres** (Regional Transfusion Centre of the Hungarian National Blood Transfusion Service, University of Szeged), **Dr. Katalin Tápai** (Regional Transfusion Centre of the Hungarian National Blood Transfusion Service, University of Szeged), **Dr. Beatrix Nagy** (Keszthely City Hospital), **Dr. Éva Szilágyi** (Division A, Department of Oncotherapy, University of Szeged) and **Dr. László Hodoniczki** (Division B, Department of Oncotherapy, University of Szeged) the DNA samples for the SNP examinations.

I am really grateful to **Dr. Rupert Quinnell**, my English co-supervisor from the University of Leeds for the opportunity to work in his lab at the Institute of Integrative and Comparative Biology within the confines of a short-term Marie Curie scholarship.

I would like to thank **Dr. Imre Ocsovszki** for the flow cytometric experiments and to **Dr. Krisztina Boda** for her help in the statistical calculation.

I wish to thank to **Dr. Ilona Mucsi** for the kind talks, for which I am really grateful as well to my colleagues, namely to **Dr. Gabriella Spengler**, **Dr. Ana Martins, Dr. Cátia Ramalhete, Mariana Reis** and **Zoltán Gábor Varga**, who gave me not only their wholehearted support in work but they are friends of mine, too. I really thank to **Anikó Vigyikánné Váradi** and **Mariann Ábrahám** for their technical help and for the deep talks during coffee breaks. Here, I wish to thank to my **colleagues** and **staff members** at the Department of Medical Microbiology and Immunobiology for creating a supportive and pleasant environment.

Finally, I would like to thank to all my **friends** and **family** for their selfless help and support during my whole life.

FINANCIAL SUPPORT

This thesis was supported financially by the following organizations and grants: TÁMOP 4.2.1/B-09/1/KONV-2010-0005 (European Union, European Social Fund, University of Szeged); National Innovation Office grants, contrac nr. OMFB-1004/2007 and OMFB-00389/2008; Department of Medical Microbiology and Immunobiology (University of Szeged); Foundation for Cancer Research Szeged; National Institute of Oncology; Marie Curie fellowship of the European Union.

PUBLICATIONS

1. Publications related to the thesis

I. **Serly, J.**; Vincze, I.; Somlai, Cs.; Hodoniczki, L.; Molnár, J. Synthesis and comparison of the antitumor activities of steroids on ABCB1-transfected mouse lymphoma and human ovary carcinoma. *Lett. Drug Des. Dis.*, 2011, *8*: 138-147. IF: 0.668

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ΣIF = 6.112

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ΣIF = 15.269

Cumulative impact factor: 21.381