

**Modulation of efflux pumps in tumour cells as a possible
way of reversal multidrug resistance**

Ph.D. Thesis

Zoltán Baráth M.D.

Department of Medical Microbiology and Immunobiology

*Faculty of Medicine
University of Szeged*

Szeged

2008

1. INTRODUCTION

1.1. Epidemiological factors of cancer mortality

The mortality of cancer has increased dramatically during the last decades. The estimated number of new cases each year is expected to rise from 10 million in 2000 to 15 million by 2020. Some 60% of all these new cases will occur in the less developed parts of the world.

Cancer mortality rate in Hungary is the highest in Europe and an analysis of the past 40 years has revealed a worsening trend. Apart from the high fat consumption, the insufficient intake of vegetables, fruits and smoking could be identified as major, convincing risk factors. This background plays a role in the development of mouth and pharynx, esophagus, lung, stomach, colon and rectum cancers. Cancer causes every fourth death in Hungary. In spite of the recent improvements, the premature death rate among under 65-year old Hungarians is the highest in the WHO European Region, for both males and females. The 45–59-year old Hungarian population has the highest mortality in the WHO European Region, stagnating for males and increasing for females. Hungarians have the highest death rates in the WHO European Region for lip, colorectal, laryngeal, tracheal, bronchial and lung cancers. Pancreatic and breast cancer mortality are the second and fourth highest in the region, respectively. For several cancers, the Hungarian rates are either stagnating (skin, liver and oesophageal) or increasing (colorectal, lip and pancreatic).

1.2. Oral cancers

Oral cancer holds the eighth position in the cancer incidence worldwide, but there is epidemiologic variation in various geographic regions: it is the third most common malignancy in south-central Asia.

It can be observed that the new cases of malignant neoplasms of lip, oral cavity and pharynx are constantly increasing in Csongrád county due to social and environmental factors. In Hungary the changes are not so expressed as we experienced in case of the data of Csongrád county.

1.3. Resistance mechanisms of cancer cells

Drug resistance was first documented experimentally in mouse leukemic cells that acquired resistance to 4-amino-N¹⁰-methyl-pteroylglutamic acid in a laboratory model in 1950. In 1973, *Dano* discovered active outward transport of daunomycin by drug-resistant cells that were cross-resistant to other chemotherapeutic agents, such as vinca alkaloids and other anthracyclines. Moreover, when tumour resistance developed against a single particular chemotherapeutic agent, in many cases the resulting phenotype shows a wide range or multidrug resistance pattern. The term multidrug resistance (MDR) was defined as „cellular resistance to anticancer agents due to a decreased concentration of active drug at the target sites that is caused by increased metabolism or altered transport or routing of the active drug species”.

Anticancer drugs may act at different levels: cancer cells, endothelium, extracellular matrix, the immune system or host cells. The tumour cell can be targeted at the DNA, RNA or protein level. Most classical chemotherapeutic agents interact with tumour DNA, whereas monoclonal antibodies and small molecules are directed against proteins. The endothelium and extracellular matrix may be affected also by specific antibodies and small molecules. Classically, anticancer drugs were grouped as chemotherapy, hormonal therapy and immunotherapy. Chemotherapy included a number of families defined by both their chemical

structure and mechanism of action: alkylating agents, antibiotics, antimetabolites, topoisomerase I and II inhibitors, mitosis inhibitors, platinum compounds and others.

1.4. The main mechanisms of drug resistance

- *Decrease of drug accumulation*
- *Drug resistance mediated by detoxification of the drug in the cell*
- *Alterations of drug targets or by enhancement of target repair*
- *Alterations of genes controlling apoptosis*

1.5. Transporter proteins as one of the most important resistance mechanisms

The principal mechanism of multidrug resistance is the active transport of drugs out of the cells. The transporter proteins show high specificity for their substrates, however, they have broad specificity for a wide range of chemically unrelated molecules. Transporters can be divided into different protein superfamilies based on their amino acid sequence, structure and evolutionary origin.

Structures have been obtained for multidrug transporters from five distinct transporter superfamilies:

1. **ABC family** (ATP-binding cassette transporters)
2. **RND family** (resistance-nodulation-division superfamily)
3. **MFS family** (major facilitator superfamily)
4. **SMR family** (small multidrug resistance superfamily)
5. **MATE family** (multidrug and toxic compound extrusion superfamily)

1.6. The ATP-binding cassette (ABC) transporter superfamily: structure, function, distribution in normal tissues and in tumour cells

ABC proteins have been identified in each genome sequenced, and they typically form large families with 30–100 members in various organisms. ABC proteins are named after a conserved, specific ABC domain, which can bind and hydrolyze ATP. The ABC unit (also called nucleotide binding domain or NBD) harbours several conserved sequence motifs. From NH₂ to COOH terminal, these are the Walker A (P-loop), a glycine-rich sequence; a conserved glutamine (Q-loop), the family-specific ABC-signature (LSGGQ) motif (also called the C-loop), the Walker B motif, and a conserved His (His-switch). The ABC-signature motif is diagnostic for the family as it is present only in ABC proteins, while Walker A and B motifs are found in many other ATP-utilizing proteins.

In humans, the three major types of multidrug resistance (MDR) proteins include members of the **ABCB** (**ABCB1/MDR1/P-glycoprotein**), the **ABCC** (**ABCC1/MRP1, ABCC2/MRP2**, probably also **ABCC3–6**, and **ABCC10–11**), and the **ABCG** (**ABCG2/MXR/BCRP**) subfamily. On the basis of a great deal of clinical and experimental work, it has been established that these pumps recognize a very wide range of drug substrates. ABCB1 preferentially extrudes large hydrophobic molecules, while ABCC1 and ABCG2 can transport both hydrophobic drugs and large anionic compounds, e.g., drug conjugates.

1.7. Inhibition of P-glycoprotein

Inhibiting P-gp and other ABC transporters has been extensively studied for more than two decades. **The first generation of MDR modulators** include many agents with different structure, such as calcium channel blockers, calmodulin antagonists, steroidal agents, protein

kinase C inhibitors, immunosuppressive drugs, antibiotics, antimalarials, psychotropic phenothiazines and indole alkaloids, detergents and surfactants. First-generation MDR drugs had other pharmacological activities and were not specifically developed for inhibiting MDR. Their affinity was low for ABC transporters and necessitated the use of high doses, resulting in high toxicity and side effects.

Second-generation chemosensitizers were designed to reduce the side effects of the first generation drugs. They have better pharmacological profile, however, they still retain some characteristics that limit their clinical usefulness. Many of them are substrates both for ABC transporters and for P450 isoenzyme 3A4. The competition between anticancer agents and MDR modulators for cytochrome P450 3A4 activity may result in unpredictable pharmacokinetic interactions. Another aspect is the well defined physiologic roles of ABC transporters in normal tissues (e.g. central nervous system, testes, placenta), and the inhibition of these transporters could reduce the ability of normal cells and tissues to protect themselves from cytotoxic agents. **Third-generation** molecules have been developed to overcome the limitation 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. These agents inhibit specifically and potently the P-glycoprotein and do not inhibit other ABC transporters. A non-immunosuppressive cyclosporin D derivative (**PSC-833**; **Valspodar**, Novartis AG) was the first of these drugs to be studied, but unfortunately due to pharmacokinetic interactions the development of PSC-833 was discontinued. **Biricodar (VX710)**, from the third-generation drugs, has been shown to reverse MDR *in vitro* and *in vivo* by acting on both Pgp and MRP1. **Laniquidar (R101933**; NCI/EORTC Inc.) and the substituted diarylimidazole **ONT-093** (Ontogen Inc.) have a high potency and specificity for the P-gp transporter despite having diverse chemical structures and origins. R101933 and ONT-093 were shown to inhibit P-gp pump with no effect on the pharmacokinetics of docetaxel and paclitaxel. **LY335979** is an extremely potent P-gp, and not MRP1 or MRP2, modulator and has a significantly lower affinity for CYP3A than for P-gp.

1.8. The background of the present study

Professor Joseph Molnár's research group has been working for many decades on the field of reversal of multidrug resistance in bacteria and cancer cells. Bacteria and cancer cells develop resistance to more than one agent as a consequence of being exposed to ineffective levels of the agent for a prolonged period of time. The resistance of these cells is mediated by overexpressed efflux pumps that have the ability to extrude a large variety of unrelated chemicals. The experimental work focused on multidrug resistance of tumour cells is based on development of new resistance modifiers of synthetic or natural origin.

Various phenothiazines, benzo[a]phenothiazines and their derivatives had been tested in regard to different aspects. 12H-benzo[alpha]phenothiazine [M627] induced apoptosis in mouse lymphoma cells, in the parent cell line and in the P-glycoprotein overexpressing subline as well. The *MDR1* gene expression of the mouse lymphoma cells (which were transfected with the human *MDR1* gene) could be reduced by phenothiazines such as promethazine and trifluoperazine. 4-phenyl-3, 5-diacetyl-1, 4-dihydropyridines substituted at the phenyl ring were synthesized and compared for their cytotoxic activity and multidrug resistance (MDR)-reversing activity in *in vitro* assay systems. Among them, compound [G7] showed the highest cytotoxic activity against human promyelocytic leukemia HL-60 and human squamous cell carcinoma HSC-2 cells.

Furthermore, natural compounds such as tomato lectin, feijoa peel extracts, kiwifruit peel extracts, *Anastasia green* sweet pepper, morphine alkaloids, *Allium victorialis* L. extracts and natural compounds from *Euphorbia* species received special consideration.

The main goal of Professor Molnár's research activity is to select resistance modifiers using *in vitro* and *ex vivo* systems and to test the most potent compounds in *in vivo* experiments founding the base for further clinical applications.

2. AIMS OF THE STUDY

Multidrug resistance (MDR) of cancer cells can be the result of different mechanisms. One of the most important among them concerns altered membrane transport in tumour cells, often referred to as classic MDR. This mechanism is related to the overexpression of a variety of proteins. The main aim of this study was to look for new effective modulators of MDR1 efflux pumps. The development of pharmacological agents that reverse multidrug resistance is a very promising way to overcome the difficulties in chemotherapy.

The effects of MDR modulators were demonstrated at three levels:

- The antiproliferative affect of the compounds were studied on different cancer and normal cells including the oral cells by MTT test
- The modification of intracellular drug accumulation was evaluated by flow cytometry using rhodamine 123 accumulation assay
- The interaction of resistance modifiers and anticancer drugs modelling combined chemotherapy was analysed by checkerboard microplate method

The main goals of the study in details:

1. Effect of 3-formylchromones (FC) on proliferation of tumour cells and reversal of multidrug resistance

1.1. Antiproliferative effect of 3-formylchromones on human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

1.2. Effect of 3-formylchromones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 by flow cytometry using rhodamine 123 accumulation assay.

1.3. Combination of 3-formylchromones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells *in vitro*.

2. Effect of conjugated arylidene ketones on proliferation of tumour cells and reversal of multidrug resistance

2.1 Antiproliferative effect of conjugated arylidene ketones on human oral cancer and human normal oral cells, human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

2.2. Effect of conjugated arylidene ketones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 by flow cytometry using rhodamine 123 accumulation assay.

2.3. Combination of conjugated arylidene ketones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells *in vitro*.

3. Effect of α,β -unsaturated cyclic ketones on proliferation of tumour cells and reversal of multidrug resistance

3.1. Antiproliferative effect of α,β -unsaturated cyclic ketones on human oral cancer and human normal oral cells, human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

3.2. Effect of α,β -unsaturated cyclic ketones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells.

3.3. Combination of α,β -unsaturated cyclic ketones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 *in vitro*.

3. MATERIALS AND METHODS

Cell cultures:

Human oral cancer cells (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG) and **human normal oral cells** (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. Human promyelocytic leukaemia **HL-60** cells were cultured in RPMI1640 + 10% FBS. Normal cells were prepared from the periodontal tissues, according to the guideline of Meikai University Ethic Committee after obtaining the informed consent from the patients.

L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described. *Mdr-1*-expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178 (parent) mouse T-cell lymphoma cells and the human *mdr1*-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. Both cell lines were cultured at 37°C. The mouse lymphoma cell line was maintained in a 5% CO₂ atmosphere.

The human colon cancer cells (COLO320) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cell lines were incubated in a humidified atmosphere (5% CO₂, 95% air) at 37 °C. The semiadherent human colon cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37 °C.

Medium: McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics; **RPMI 1640** medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. **DMEM** medium supplemented with 10% heat- inactivated FBS.

Compounds: Rhodamine 123 (Sigma, St Louis, MO, USA); verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO, USA); SDS (sodium dodecylsulfate; Sigma, St Louis, MO, USA); and doxorubicin (Ebewe, Austria). **3-Formylchromones** (synthesized by Prof. **Professor Noboru Motohashi**, Department of Medicinal Chemistry, Meiji Pharmaceutical University, Tokyo, Japan), **conjugated arylidene ketones** (synthesized by **Professor Jonathan Dimmock** (College of Pharmacy and Nutrition, University of Saskatchewan, Canada), **α,β -unsaturated cyclic ketones** (synthesized by **Professor Jonathan Dimmock** (College of Pharmacy and Nutrition, University of Saskatchewan, Canada)

Assay for reversal of MDR in tumour cells: The cells were adjusted to a density of 2×10^6 /ml, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-ml aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in

different volumes (2.0-20.0 μ l) of the 1.0-10.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature. Next, 10 μ l (5.2 μ M final concentration) of the indicator rhodamine 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 flow cytometer. Verapamil was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. An activity ratio R was calculated via the following formula, on the basis of the measured fluorescence values:

$$R = \frac{MDR_{treated} / MDR_{control}}{parental_{treated} / parental_{control}}$$

Assay for antiproliferative effect: The effects of increasing concentrations of the drugs alone and their combinations with resistance modifiers on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μ L. Then, 1×10^4 cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h; at the end of the incubation period, 20 μ L of MTT (thiazolyl blue, Sigma, St Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μ L of Sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO, USA) solution (10%) was measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \left[\frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}} \right] \times 100$$

A checkerboard microplate method was applied to study the effects of drug interactions between resistance modifiers and cytotoxic compound on cancer cells. The effects of the anticancer drug doxorubicin and the resistance modifiers were studied in combination on various cancer cell lines. The dilutions of doxorubicin (A) were made in a horizontal direction, and the dilutions of resistance modifiers (B) vertically in the microtiter plate in 100 μ L volume. The cell suspension in the tissue culture medium was distributed into each well in 100 μ L containing 5×10^4 cells. The plates were incubated for 72 h at 37 °C in a CO₂ incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a micro ELISA reader. Drug interactions were evaluated according to the following system:

ID=inhibitory dose

FIC=fractional inhibitory concentration

FIX=fractional inhibitory index

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

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

$FIX = FIC_A + FIC_B$

$FIX = 0.51 - 1$ Additive effect

$FIX < 0.5$ Synergism

$1 < FIX < 2$ Indifferent effect

$FIX > 2$ Antagonism

4. RESULTS

1. EFFECT OF 3-FORMYLCHROMONES (FC) ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

1.1. Antiproliferative effect of 3-formylchromones on human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

When the antiproliferative effect of 3-formylchromones was measured on MDR mouse lymphoma cells, three different groups could be distinguished on the basis of the ID₅₀ values. The ID₅₀ value shows the 50% inhibition of cell proliferation. In the *first* group, FC 3, with an ID₅₀=1.9 µg/mL, was the most active. The compounds in the *second* group, FC 2, 4, 6, 7, 8, 9, 10 and 11, were moderately active with ID₅₀ values between 4.27-7.13. Those in the *third* group, FC 5 and FC 12-16 had very high ID₅₀ values in the range from 21.42-79.14 µg/mL.

The effects of the tested compounds on the proliferation of human colon cancer cells were also investigated. The compounds FC 2, 3 and FC 5-10 exhibited a moderate antiproliferative effect, as measured on Colo 320 cells (ID₅₀=2.02-8.70 µg/mL).

1.2. Effect of 3-formylchromones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 by flow cytometry using rhodamine 123 accumulation assay.

A further investigation on the activity of the MDR efflux pump of mouse lymphoma cells transfected with the human *MDR1* gene was also conducted. The effects of the compounds on the drug accumulation of the MDR cancer cells in non-toxic concentrations were studied using the rhodamine accumulation test. The most effective compounds proved to be FC 1, 3, 4, 6, 7, 8, 10 and 11.

The effects of these compounds on the activity of the MDR efflux pump of human Colo 320 colon cancer cells were investigated. The most effective compounds were found to be FC 1, 6, 7, 8, 9, 10 and 11.

1.3. Combination of 3-formylchromones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells *in vitro*.

From the most effective compounds, 6,8-dibromo-3-formylchromone was chosen to determine its interaction with the anticancer drug doxorubicin. 6,8-dibromo-3-formylchromone was combined with doxorubicin on *MDR1*-gene transfected mouse lymphoma cells. 6,8-dibromo-3-formylchromone acted synergistically with doxorubicin on mouse lymphoma cells in checkerboard assay.

2. EFFECT OF CONJUGATED ARYLIDENE KETONES ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

2.1. Antiproliferative effect of conjugated arylidene ketones on human oral cancer and human normal oral cells, human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

Thus 1a was considered a lead molecule with demonstrated cytotoxicity without short-term marked toxicity. The main goal of *Dimmock's* research group was to prepare a small group of prototypic molecules related to 1a with a view to determining whether selective toxicity to malignant cells and reversal of multidrug resistance (MDR) would be demonstrated.

All of the compounds in series **1–4** were evaluated against four neoplastic cell lines, namely squamous cell carcinoma **HSC-2**, **HSC-3**, submandibular gland carcinoma **HSG** and human promyelocytic leukaemia **HL-60** cells as well as the nonmalignant gingival fibroblast cells (**HGF**), pulp cells (**HPC**) and periodontal ligament fibroblast cells (**HPLF**). The average CC_{50} (CC_{50} : concentration of the compound to kill 50% of the cells) of the lead compound **1a** to HSC-2, HSC-3, HSG and HL-60 cells was 1.75 μ M or 85% of the average potency of doxorubicin towards these four cell lines.

2.2. Effect of conjugated arylidene ketones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 by flow cytometry using rhodamine 123 accumulation assay.

The data revealed that **1a–c,f**, **2a,b** displayed a remarkable inhibition of the MDR of human MDR1 gene-transmitted mouse lymphoma cells. These cells overexpress the P-gp 170 protein responsible for drug efflux. The same compounds were also the most effective on the elevated drug accumulation of human colon cancer Colo320 cells but in each case the FAR values were lower than for the murine lymphoma cells.

2.3. *In vitro* combination of conjugated arylidene ketones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells

From the most effective compounds, **2b** was chosen to determine its interaction with the anticancer drug doxorubicin. **2b** was combined with doxorubicin on *MDR1*-gene transfected mouse lymphoma cells. **2b** exerted indifferent effect (**FIX: 1.22**) with doxorubicin on mouse lymphoma cells in combination with doxorubicin.

3. EFFECT OF α,β -UNSATURATED CYCLIC KETONES ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

3.1. Antiproliferative effect of α,β -unsaturated cyclic ketones on human oral cancer and human normal oral cells, human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320.

Of the 10 compounds studied, **1**, **2** and **8** showed potent antiproliferative effect on mouse lymphoma cells. Compound **6**, **7**, and **9** were moderately effective in the antiproliferative assay on mouse lymphoma cells.

The proliferation of human colon cancer cells could be inhibited efficiently by compound **1**, **7** and **8**.

Based on our results, compound **1** seemed to be the most promising compound, because it was the most effective compound against four neoplastic cell lines such as HSC-2, HSC-3, HSG and HL-60 (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma and human promyelocytic leukaemia, respectively). However, it has no effect against nonmalignant normal oral cells HGF, HPC and HPLF cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast cells, respectively).

Compound **1**, **6** and **8** exerted the most effective antiproliferative effect on human MDR1 gene transfected mouse lymphoma cells and human colon cancer cells as well.

3.2. Effect of α,β -unsaturated cyclic ketones on modulation of human MDR1-gene encoded P-glycoprotein in human MDR1-gene transfected mouse lymphoma cells.

Compound **1**, **6**, **9** and **10** could reverse dose dependently the P-gp coupled multidrug resistance in mouse lymphoma cells, furthermore compound **8** has the same effect on both concentrations applied (4 and 40 $\mu\text{g/mL}$).

3.3. Combination of α,β -unsaturated cyclic ketones and doxorubicin using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells and MDR1-expressing human colon cancer cell line Colo 320 *in vitro*.

From the most effective compounds, two compounds were chosen to determine their interaction with the anticancer drug doxorubicin. Compound **2,7-Bis-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone** and **2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone** were combined with doxorubicin on *MDR1*-gene transfected mouse lymphoma cells and human colon cancer cell line Colo320. Compound **2,7-Bis-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone** and **2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone** exerted additive effect with doxorubicin on mouse lymphoma cells, and surprisingly compound **2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone** interacted synergistically with doxorubicin on human colon cancer cells.

5. DISCUSSION

1. EFFECT OF 3-FORMYLCHROMONES (FC) ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

The chromone moiety forms an important component of pharmacophores of a number of biologically active molecules of synthetic as well as natural origin, and many of them have useful medicinal applications. Chromone and coumarin derivatives are of great interest because of their antimicrobial, antitumour and antiviral activity. Phosphorohydrazone derivatives of coumarin and chromone demonstrated antitumour activity against leukemia L1210. Among chromone derivatives, cytotoxic activity against leukemia P388 has been recognized in phosphorohydrazinecarbonylic derivative.

In our studies 3-formylchromone derivatives were investigated. It is interesting to note that the substituents had great influence on the antiproliferative effect. The result of the antiproliferative assay revealed the most effective compounds against the mouse lymphoma cells as **FC 3, 8, 9** and **11**, and against human colon cancer cells as **FC 2, 5, 6, 7, 8, 9** and **10**. There is apparently a strong structure-activity relationship between the MDR reversal activity and the chemical structures of the compounds studied.

A few derivatives were able to reverse the MDR as tested the rhodamine 123 accumulation assay. The most effective compounds against mouse lymphoma cells were **FC 1, 3, 4, 6, 7, 8, 10** and **11**, while the compounds found to be very effective against the Colo 320 cancer cells were **FC 1, 4, 6, 7, 8, 9, 10** and **11**.

We found synergism between formylchromone derivative **6,8-dibromo-3-formylchromone** and doxorubicin on mouse lymphoma cell line transfected with human *MDR1* gene, and this result could be applied as a basis for further *in vivo* experiments in mice to improve the success of the combined chemotherapy.

A special ground state dipole moment is probably important for the biological effect of the above compounds on P-glycoprotein. Additionally, the TPSA (total polar surface area) of the highly lipophilic compounds must be taken into consideration.

2. EFFECT OF CONJUGATED ARYLIDENE KETONES ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

The average potencies of the other compounds prepared in our study were less than **1a** revealing that 3,4,5-trimethoxy substitution in aryl ring B was optimal (**1a** > **1b–f**), the presence of ring C increases potency (**1a** > **2a**), and a six-membered alicyclic ring was preferred to a five-membered one (**1a** > **3a**). In order to obtain an answer to the first query as to whether preferential toxicity for malignant cells was displayed, a selectivity index (SI) value was calculated for each of the compounds in series **1–4**.

A review of the SI data revealed the following structure–activity relationships (SAR). First, marked selective potencies for malignant cells were noted in series **1** and **2**, but not in **3** and **4**, indicating the importance of a six-membered rather than a five-membered alicyclic ring. Second, in general, the presence of ring C in series **1** enhanced selectivity since the SI figures of **1a,b,d–f** were greater than **2a,b,d–f**, respectively. Third, **1a** and **2a** possessed the highest SI figures in series **1** and **2**, respectively, indicating that the 3,4,5-trimethoxy substitution pattern was optimal.

1a–c,f, **2a,b** displayed a remarkable inhibition of the MDR of human MDR1 gene-transmitted mouse lymphoma cells. These cells overexpress the P-gp 170 protein responsible for drug efflux. The same compounds were also the most effective on the elevated drug accumulation of human colon cancer Colo320 cells, but in each case the FAR values were lower than for the murine lymphoma cells. In general, the SAR of the compounds inhibiting MDR were the same as the SI values generated, namely the presence of ring C was beneficial (**1a–f** > **2a–f**), a six-membered alicyclic ring was preferable to the 2,5-disubstituted cyclopentanones (**2a,d** > **4a,b**) and in series **1** and **2**, maximum inhibition of MDR was found in **1a** and **2a**, having the 3,4,5-trimethoxy groups in ring B.

We have to emphasize that this study of several series of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has led to novel, potent cytotoxins, some of which demonstrated remarkable selective toxicity to malignant cells and the ability to reverse MDR. The reasons for these important findings may have included the fact that the theory of sequential cytotoxicity was verified and/or preferential reduction of the nitro group took place in those tumours in which greater hypoxia exists.

3. EFFECT OF α,β -UNSATURATED CYCLIC KETONES ON PROLIFERATION OF TUMOUR CELLS AND REVERSAL OF MULTIDRUG RESISTANCE

The 4-nitrophenylmethylene group is not essential for inducing MDR-reversing properties. Thus, replacement of this functionality in **1** by a 3,4,5-trimethoxyphenylmethylene group led to **6** having a similar MDR reversing potency. In fact, on occasions the 4-nitrophenylmethylene substituent exerted a dystherapeutic effect, for example, excision of this group from the inactive compounds **3** and **4** led to **9** and **10**, respectively, which possess MDR reversing properties.

Secondly, an attempt was made to find reasons for the differences in MDR-reversing properties between **1** (which is an active compound) and **3** and **4** (which are devoid of this property).

Thirdly, both compounds containing a sole methoxy group in aryl ring A viz. **2** and **5** were devoid of MDR-reversing properties.

Fourthly, all three bis compounds **6–8** reverse MDR. In particular, the remarkable potency of **8** establishes it as a very useful lead compound which is likely due, at least in part, to the favorable spatial arrangement of the 3,4,5-trimethoxyphenylmethylene groups.

Fifthly, removal of one of the 3,4,5-trimethoxyphenylmethylene groups of **7** yielding **9** led to an increase in potency, suggesting that only one of these structural moieties may be required to confer MDR reversing properties in these compounds.

Finally, in considering the possible structural features which may influence the magnitude of MDR reversal in mouse L-5178 cells, two other physicochemical parameters were considered.

Compounds **6–10** are novel MDR-reversing agents which have been developed from the lead compound **1**. In particular, the remarkable potency of **8** was discovered in this study. Future work will involve placing one or more 3-(3,4,5-trimethoxyphenyl)-2-propenoyl groups onto a variety of acyclic, alicyclic, aryl, and heteroaryl scaffolds in an attempt to find correlations between the topography of these molecules and potencies in this assay. Most of the compounds possess noteworthy cytotoxic potencies and display greater lethality to some neoplasms than normal cells. Activation of different caspases which induced apoptosis was shown in representative molecules to be at least one way whereby cytotoxicity was mediated.

We found synergism between compound **8** and doxorubicin on Colo 320 human colon cancer cell line, and this result might be a promising way for combined chemotherapy of colon cancer patients.

6. NEW STATEMENTS OF THE THESIS

The main goal of this study was to look for new effective modulators of MDR1 efflux pumps. The development of pharmacological agents that reverse multidrug resistance could improve the efficiency of tumour chemotherapy. In our studies three groups of compounds were tested such as 3-formylchromones, conjugated arylidene ketones and α,β -unsaturated cyclic ketones on various tumour cell lines. We found that the above mentioned compounds could be valuable resistance modifiers applied in cancer chemotherapy.

1. 3-Formylchromones

Chromone and coumarin derivatives are of great interest because of their biological activity. The pharmacological activities of many chromone derivatives such as anti-inflammatory, antiviral and anti-neoplastic activities have been extensively investigated.

1.1. The results obtained in our study demonstrate that 3-formylchromones can modify the multidrug resistance of various cancer cells. Concerning the antiproliferative effect **3-formyl-6-isopropylchromone** was the most active compound. The compounds **FC 2, 3** and **FC 5,6,7,8,9,10** exhibited a moderate antiproliferative effect on Colo 320 cells.

1.2. A further investigation on the activity of the MDR efflux pump of mouse lymphoma cells transfected with the human *MDR1* gene showed that some of the 3-formylchromones are modifiers of P-glycoprotein: the most effective compounds proved to be **FC 1, 3, 4, 6, 7, 8, 10** and **11**. When the structure-activity relationship was analysed, the most effective compounds were those substituted at position 6 of the aromatic ring. A CH_3 or NO_2 group reduced the biological activity. A substituent Cl at position 6 and a CH_3 at position 7 resulted in toxic compounds. A special ground state dipole moment is probably important for the biological effect of the above compounds on P-glycoprotein. Additionally, the TPSA (total polar surface area) of the highly lipophilic compounds must be taken into consideration.

We can assume that **3-formyl-6-isopropylchromone** is the most important compound, because it modifies the multidrug resistance in mouse lymphoma cells and in human Colo 320 colon cancer cells as well. **3-formyl-6-isopropylchromone** may represent a lead compound for the design of novel, safe and potent MDR chemosensitizers.

1.3. Synergism was found between formylchromone derivative **6,8-dibromo-3-formylchromone** and doxorubicin on mouse lymphoma cell line transfected with human *MDR1* gene. This information could lead to further *in vivo* experiments to investigate the combined effect of fromylchromone derivatives with different anticancer drugs.

2. Conjugated arylidene ketones

2.1. The cytotoxic effect of the conjugated arylidene ketones was studied and compared on normal oral and oral cancer cell lines. Among the arylidene ketones compounds **1a, 1b, 1e, 2a, 2c** and **2g** exhibited the highest cytotoxic activity against the human oral cancer cells (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG) and HL-60 cells. These four human tumour cell lines showed similar sensitivities to these compounds. On the other hand, three normal cell lines such as gingival fibroblast (HGF), pulp cell (HPC) and periodontal ligament fibroblast (HPLF) cells were resistant to these derivatives.

The normal cells were relatively resistant to the derivatives **1a, 1b, 1d, 1e, 2a, 2d** and **2f**. The preferential toxicity for malignant cells, the so called selective toxicity was calculated for each compounds. The SI values indicated that **1a, 1b, 1e, 2a, 2c** and **2g** had the highest tumour specific cytotoxic activities among the substituted arylidene ketones.

2.2. **1a–c,f** and **2a,b** displayed a remarkable inhibition of the MDR of human *MDR1* gene-transmitted mouse lymphoma cells. These cells overexpress the P-gp 170 protein responsible for drug efflux. The same compounds were also the most effective on the elevated drug accumulation of human colon cancer Colo 320 cells, but in each case the FAR values were lower than the ones for the murine lymphoma cells.

2.3. Marked selective potencies for malignant cells were noted in series **1** and **2**, but not **3** and **4**, indicating the importance of a six-membered rather than a five-membered alicyclic ring. The presence of ring C in series **1** enhanced selectivity, and we proved that the 3,4,5-trimethoxy substitution pattern was optimal for selectivity.

We have to emphasize that this study of several series of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has led to novel, potent cytotoxins, some of which demonstrated remarkable selective toxicity to malignant cells and the ability to reverse MDR.

3. α,β -unsaturated cyclic ketones

Based on our results, compound **1** seemed to be the most promising compound, because it was the most effective compound against four neoplastic cell lines such as HSC-2, HSC-3, HSG and HL-60 (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma and human promyelocytic leukaemia, respectively). However, it has no effect against nonmalignant normal oral cells HGF, HPC and HPLF cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast cells, respectively).

3.1. α,β -unsaturated cyclic ketone **1, 6** and **8** exerted the most effective antiproliferative effect on human *MDR1* gene transfected mouse lymphoma cells and human colon cancer cells as well.

3.2. α,β -unsaturated cyclic ketone **1, 6, 9** and **10** could reverse dose dependently the P-gp coupled multidrug resistance in mouse lymphoma cells.

3.3. α,β -unsaturated cyclic ketone **7** and **8** exerted additive effect with doxorubicin on mouse lymphoma cells. We found synergism between compound **8** and doxorubicin on Colo 320 human colon cancer cell line and this result might be a promising way for combined chemotherapy of colon cancer patients.

Most of the compounds possess noteworthy cytotoxic potencies and display greater lethality to some neoplasms than normal cells and α,β -unsaturated cyclic ketones can be recommended for animal experiments as good candidates for MDR-reversal agents.

7. SUMMARY

The number of new cases of cancer has increased dramatically during the last decades. Cancer mortality rate in Hungary is the highest in Europe, and an analysis of the past 40 years has revealed a worsening trend.

Cancer cells develop resistance to more than one agent as a consequence of being exposed to ineffective levels of the agent for a prolonged period of time. When tumour resistance developed against a single particular chemotherapeutic agent, in many cases the resulting phenotype shows a wide range or multidrug resistance pattern. The **main mechanisms of drug resistance** of tumour cells are the following: decrease of drug accumulation by efflux pumps, drug resistance mediated by detoxification of the drug in the cell, alterations of drug targets or by enhancement of target repair, alterations of genes controlling apoptosis.

In our studies we investigated the principal mechanism of multidrug resistance which is the active transport of drugs out of the cells. In tumour cell lines, multidrug resistance is often associated with an ATP-dependent decrease in cellular drug accumulation which was originally attributed to the overexpression of a single protein, the 170-kDa **ABC drug transporter P-glycoprotein** encoded by human MDR1 gene. MDR is associated with a reduced intracellular drug accumulation and an increased cellular drug efflux.

The main aim of this study was to look for new effective modulators of MDR1 efflux pumps, which can increase the intracellular drug accumulation by modifying efflux pumps. We found that some of the **3-formylchromones** are modifiers of P-glycoprotein, and **3-formyl-6-isopropylchromone** is the most important compound, because it modifies the multidrug resistance in mouse lymphoma cells and in human Colo 320 colon cancer cells as well. Formylchromone derivative **6,8-dibromo-3-formylchromone** showed synergistic effect with doxorubicin on human MDR1 gene transfected mouse lymphoma cells. **Conjugated arylidene ketones** displayed a remarkable inhibition of the MDR of human MDR1 gene-transmitted mouse lymphoma cells. Based on our results obtained in studies with arylidene ketones, a new group of compounds has been developed called **α,β -unsaturated cyclic ketones**. Some α,β -unsaturated cyclic ketones exerted effective antiproliferative effect on human MDR1 gene transfected mouse lymphoma cells and human colon cancer cells as well, and they could reverse dose dependently the P-gp coupled multidrug resistance in mouse lymphoma cells. We found synergism between **compound 2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone** and doxorubicin on Colo 320 human colon cancer cell line.

We conclude that 3-formylchromones, conjugated arylidene ketones and α,β -unsaturated cyclic ketones can be recommended as good candidates for anticancer drug development.

8. ACKNOWLEDGEMENTS

First of all I would like to express my deep and sincere gratitude to my supervisor, **Professor József Molnár**. His wide knowledge, understanding, encouraging and personal guidance served as excellent basis for the preparation of the present Ph.D. thesis.

I am deeply grateful to **Professor Katalin Nagy, Dr. Márta Radnai, Professor András Fazekas** (Faculty of Dentistry), who supported me in my research work.

I would like to express my sincere gratitude to **Professor Yvette Mándi**, chair of the Department of Medical Microbiology and Immunobiology, for providing possibility to work at the department.

I would like to give my special thanks to **Helga Engi, Anikó Váradi Mrs. Vigyikán, Zsuzsanna Schelz** and **Gabriella Spengler** from the Department of Medical Microbiology and Immunobiology. I want to thank them for all their help, support and interest shown.

I would like to thank **Dr. Imre Ocsovszki** for performing the flow-cytometric measurements.

I thank **Professor Jonathan Dimmock** (College of Pharmacy and Nutrition, University of Saskatchewan, Canada) and **Professor Noboru Motohashi** (Department of Medicinal Chemistry, Meiji Pharmaceutical University, Tokyo, Japan) for their help, support and constructive comments

I feel a deep sense of gratitude for my family, my wife, my parents and my sister for always being ready to help.

I would like to express thankfulness to all those who made it possible for me to complete this thesis.

9. PUBLICATIONS RELATED TO THE THESIS

I Dimmock JR, Das U, Gul HI, Kawase M, Sakagami H, Barath Z, Ocsovsky I, Molnar J: 3-Arylidene-1-(4-nitrophenylmethylene)-3,4-dihydro-1H-naphthalen-2-ones and related compounds displaying selective toxicity and reversal of multidrug resistance in neoplastic cells.

Bioorg Med Chem Lett. 15(6):1633-1636, 2005.

IF: 2,478

II Barath Z, Radics R, Spengler G, Ocsovszki I, Kawase M, Motohashi N, Shirataki Y, Shah A, Molnar J: Multidrug resistance reversal by 3-formylchromones in human colon cancer and human *mdr1* gene-transfected mouse lymphoma cells. In Vivo, 20(5):645-649, 2006.

IF: 1,273

III Das U, Kawase M, Sakagami H, Ideo A, Shimada J, Molnar J, Barath Z, Bata Z, Dimmock JR: 3-(3,4,5-Trimethoxyphenyl)-1-oxo-2-propene: a novel pharmacophore displaying potent multidrug resistance reversal and selective cytotoxicity. Bioorg Med Chem.15(10):3373-3380, 2007.

IF: 2,624

Baráth Z., Radics R., Ocsovszky I., Kawase M., Motohashi N., Das U., Inci Gul H., Dimmock JR., Molnar J.: Inhibition of Multidrug Resistance in Mouse Lymphoma and Human Colon Cancer Cell Lines by Formyl Chromone and Alpha-Beta-Unsaturated Cyclic Ketones. pp. 325. (poster)

16th International Congress on Anti-Cancer Treatment

Paris, France, February 1-4 2005