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

Ph.D. Thesis

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PUBLICATIONS RELATED TO THE THESIS

1. 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

2. 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

3. 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

Poster

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
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ABBREVIATIONS

ABC	ATP-binding cassette
BCRP	breast cancer resistance protein
CMOAT	canalicular multi-organic anion transporter
FAR	fluorescence activity ratio
FC	formyl-chromones
FIC	fractional inhibitory concentration
FIX	fractional inhibitory index
FRET	Fluorescence Resonance Energy Transfer
GSH	glutathione
GST	glutathione-S-transferase
HGF	human gingival fibroblast
HIV	human immunodeficiency virus
HL-60	human promyelocytic leukaemia
HPC	human pulp cell
HPLF	human periodontal ligament fibroblast
HSC	human squamous cell carcinoma
HSG	human submandibular gland carcinoma
ID	inhibitory dose
LRP	lung resistance protein
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFS	major facilitator superfamily
MRP	MDR related protein
NBD	nucleotide binding domain
MSD	membrane spanning domain
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide
RND	resistance-nodulation-division
SDR	standardized death rate
P-gp	P-glycoprotein
SAR	structure–activity relationship
SCC	squamous cell carcinoma
SDS	sodium dodecylsulfate
SMR	small multidrug resistance
TM	transmembrane
TMD	transmembrane domain
TPSA	total polar surface area
TSG	tumour suppressor gene
WHO	World Health Organisation

INTRODUCTION

1. Epidemiological factors of cancer mortality

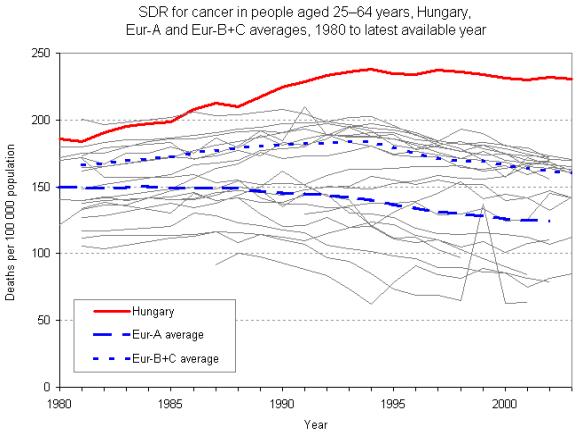
The mortality of cancer has increased dramatically during the last decades. The estimated number of new cases each year is expected to rise from 10 million in 2000 to 15 million by 2020. Some 60% of all these new cases will occur in the less developed parts of the world. Concerning the biological characteristics of cancer cells, we have to emphasize the proliferation of abnormal cells and failure of programmed cell death (apoptosis) which promote cancer progression (1).

Cancer occurs because of mutations in the genes responsible for cell multiplication and DNA repair. The activation of oncogenes and inactivation of tumour suppressor genes (TSGs) have been implicated in the development of many human and animal malignancies. Changes in certain specific genes have been shown to be of potential value for diagnosis and prognosis, as well as treatment, of some cancers. Aberrant oncogene expression is the driving force behind the development of all cancers. Importantly, if oncogene-induced DNA damage is a hallmark of premalignant lesions, then tumor progression in the presence of a potent oncogene may require the inactivation of DNA damage response pathways (2).

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 the 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 (3,4). 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 (**Figure 1**)(5). Hungarians have the highest death rates in the WHO European Region for lip, colorectal, laryngeal, tracheal, bronchial and lung cancers (**Figure 2, 3**)(4,5,6). 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). The death rates from breast, prostate, bladder, lymphoid and haematopoietic cancers are also

above the Eur-A and Eur-B+C averages, but the mortality rates have declined since the mid-to-late 1990s (**Figure 1**)(4).

Figure 1. Standardized death rate in people in Hungary aged 25-64



www.euro.who.int/eprise/main/WHO/Progs/CHHHUN/mortality/20050524_18

Figure 2. Standardized death rate for larynx, bronchus and lung cancer in males in Hungary aged 25-64

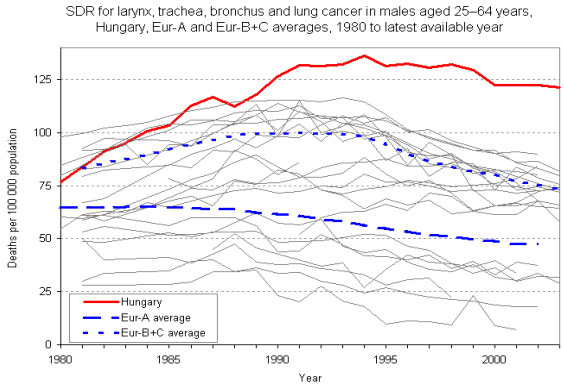


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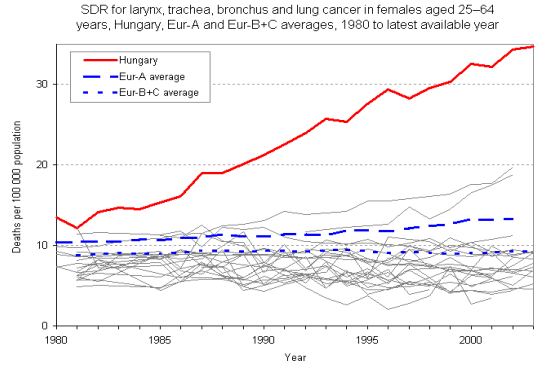
Figure 3. Standardized death rate for larynx, bronchus and lung cancer in females in Hungary aged 25-64



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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 (7).

It can be observed that the new cases of malignant neoplasms of lip, oral cavity and pharynx are constanly 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 (**Figure 4**) (8).

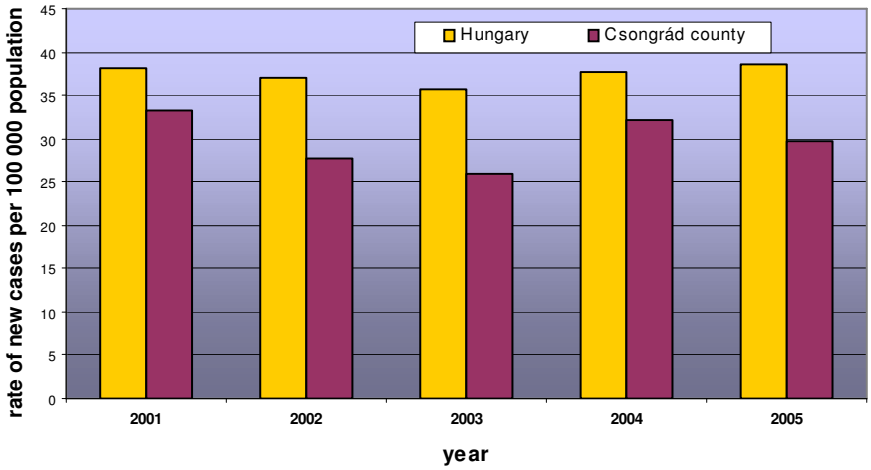


Figure 4. New cases of malignant neoplasms of lip, oral cavity and pharynx per 100 000 population 2001-2005: male and female

In some western European countries, such as Belgium, Denmark, Greece, Portugal and Scotland, there has been an upward trend in the incidence of oral squamous cell carcinomas. Increasing mortality rates have been detected for at least 2 decades in Eastern Europe (5). Apparently, there is no prognostic difference between males and females (9, 10) and most authors report higher mortality in smokers and alcohol drinkers (11).

Squamous cell carcinomas (SCC) encompass at least 90% of all oral malignancies (12). According to the reports, the correlation of prognosis with age seems controversial. Some authors found no relationship between them (13), whereas others show worse prognosis in elder patients (11). Survival rates are lower in patients with concomitant oral SCC and

disorders such as congestive heart failure, arrhythmias, peripheral vascular disease, pulmonary and renal diseases, and other cancers; either treated and untreated (14).

The cancer cells have altered cellular physiology and several genetic aberration have been identified in oral SCC, most frequently in chromosomes 3, 9, 11, 13 and 17. The inactivation of tumour suppressor genes such as *p16* and *p53*, the overexpression of oncogenes, and the alteration of genes involved in the metabolism of carcinogens or DNA repair seem to play a role in the carcinogenesis of oral squamous cell carcinomas (15). Moreover, most oral carcinomas are telomerase-activity positive (16).

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 (17). 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 (18). 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 (19). 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”(20).

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 (21). 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 (**Table 1**).

Table 1. Classification of anticancer drugs (22,23)

Families of anticancer drugs	Examples of drugs	Some mechanisms of action	Examples of drug targets
Chemotherapeutic agents against tumours with low therapeutic index (cytotoxic)	1.1methotrexate, fluorouracil, cytosar, 5-azacytosine, 6-mercaptopurine, gemcitabine	1.1 Inhibition of enzymes participating in DNA and RNA synthesis	1.1 Dihydrofolate reductase, thymidylate synthetase, etc.
	1.2 nitrozureas, temozolomid, dacarbazin	1.2 Binding with DNA, breaks and inappropriate links between DNA strands	
	1.3 irinotecan, etoposide	1.3 Topoisomerase inhibition	1.3 Topoisomerase II and I
	1.4 vinca alkaloids, taxans	1.4 depolimerization of microtubules, damage to mitotic spindle	1.4 Cytoplasmic microtubules, mitotic spindle
2. Chemotherapeutic agents against tumours with high therapic index (cytostatic)	2.1 gefitinib, imatinib, flavopiridol	2.1 Modifiers of intermedier mechanism	
	2.2 geldanamycin	2.2 Inhibitors of proteosoma and HSP	
	2.3 Isotretionin	2.3 Modifiers of chromatin operation	
	2.4 Asparaginase	2.4 Inhibitors of protein synthesis	
	2.5 Celecoxib	2.5 Affect according to other mechanism	
3. Hormone derivatives	3.1 flutamid 3.2 tamoxifem, toremifen 3.3 exemestan, letrozol 3.4 goserelin, buserelin 3.5 octreocid	3.1 Anti-androgen 3.2 Anti- oestrogen 3.3 Aromatas inhibitor 3.4 LH-RH compounds 3.5 Somatostatin derivatives	
4. Citokins	Interferons, interleukins		
5. Therapeutics inhibiting neovascularisation	Endostatin, angiostatin, bevacizumab		
6. Antimetastatic medicines	Bisphosphonat, marimastat		
7. Others	Chemoprotective modulators, vaccines		

The mulidrug resistance can be divided into two main groups such as **1) intrinsic resistance** and **2) acquired resistance:**

1) Intrinsic MDR: MDR is not necessarily the result of only of the treatment of the cells by a drug moreover it is connected with the type of the cell differentiation or the localization of the cells in an organism. The phenomenon called **intrinsic MDR** describes cells which are

drug resistant before the drug treatment. For example brain tumours are resistant to chemotherapy due to the blood-brain barrier. Intrinsic MDR may be connected with a genetical change that initiated the tumour.

2) Acquired drug resistance can be caused by chemotherapy. Rare genetic variants of drug resistant cells can occur in a tumour cell population under the influence of cytostatic drugs, and these cells can multiply if they have selective advantage (22,24).

The main mechanisms of drug resistance of tumour cells are the following (**Table 2**):

1. Decrease of drug accumulation

The ABC transporter family reduces drug accumulation of the target molecules. Many studies have reported that the multidrug resistance gene 1 (MDR1) encoding human P-gp and the MRP gene may play an important role in the multidrug resistance of breast cancer (25,26,27,28).

2. Drug resistance mediated by detoxification of the drug in the cell

Glutathione transferases are often up-regulated in tumours and have been suggested to play an important role in multiple drug resistance in cancer chemotherapy (29,30,31).

3. Alterations of drug targets or by enhancement of target repair

Topoisomerase II has been identified as the primary cellular target for a number of antitumour drugs currently being used in cancer chemotherapy. Topoisomerase I has also been found to be the target of camptothecin, an antitumour drug currently under development. Topoisomerases normally solve the topological problems of DNA, which are generated during replication, transcription and recombination, by breaking and rejoining the DNA strands. Alterations of the topoisomerase enzymes have been described as associated with the development of drug resistance to topoisomerase inhibitors. The best known alterations are reduced gene expression and mutations in the genes (32,33,34).

4. Alterations of genes controlling apoptosis

Programmed cell death, particularly adhesion-dependent regulation of cell survival and apoptosis, is recognized as one of the main homeostatic mechanisms designed to control cell positioning, eliminate misplaced cells and block metastatic dissemination. Highly metastatic cancer cells exhibit a higher resistance to the programmed cell death compared to their poorly metastatic counterparts (35). Although studies on Bcl-2 and p53 established the importance of apoptosis in carcinogenesis, it is now clear that mutations in many cancer-related genes can disrupt apoptosis. For example, the Fas/CD95 receptor normally controls cell numbers in the

immune system by eliminating cells through apoptosis, and disruption of this pathway can lead to lymphoproliferative disorders and even cancers (36,37).

Table 2. Main mechanisms of drug resistance of tumour cells (taken from Stavrovskaya AA: Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc). 65(1):95-106, 2000)

No.	Steps in the cytostatic action of drugs	Alterations resulting in drug resistance	Mechanisms of drug resistance (examples)
1.	drug uptake by the cell ↓	decrease of drug accumulation by the cell	activation of transporter proteins (P-glycoprotein, MRP, etc.)
2.	activation or preservation of activity of a drug in the cell ↓	detoxification of the drug or inability of drug-activating systems	activation of the enzymes of the glutathione system, sequestration of the drug in intracellular vesicles
3.	Damage to drug target ↓	alteration of drug target, increased reapir of the damaged target	mutations of the genes coding for topoisomerases, enhancement of DNA repair
4.	arrest of cell cycle and/or death of the cell ↯	abrogation of apoptosis or cell cycle arrest: alteration of the genes controlling apoptosis	mutations of p53 gene, activation of BCL-2 gene

4. 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 (24).

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

1. **the ABC family** (ATP-binding cassette: Sav1866 from *Staphylococcus aureus* and mammalian P-glycoprotein). ATP-binding cassette (ABC) transporters are present in all cells of all organisms and use the energy of ATP binding/hydrolysis to transport substrates across cell membranes (38,39). P-glycoprotein was the first multidrug transporter for which structural data were obtained (40,41).

2. **the RND family** (resistance-nodulation-division: AcrB form *E.coli*): RND proteins are found in both prokaryotic and eukaryotic cells and have diverse substrate specificities and physiological roles. They are secondary transporters, in contrast to the ABC-transporters, energized by the proton movement down the transmembrane electrochemical gradient (42,43,44).

3. **the MFS family** (major facilitator superfamily: EmrD from *E. coli*)

As secondary transporters they are energized by the electrochemical proton gradient. Only in 2003 were the first X-ray structures for MFS transporters determined, for LacY (45) and GlpT (46) from *E. coli*. Fortunately, Emrd from *E. coli* extrudes a range of cytotoxic molecules from the cell and is homologous to two other MFS multidrug transporters: LmrP from *Lactococcus lactis* and MdfA from *E. coli* (47).

4. **the SMR family** (small multidrug resistance: EmrE from *E.coli*)

SMR proteins are a relatively small family of transporters, restricted to prokaryotic cells. They are also the smallest multidrug transporters, with only four transmembrane α-helices (50,51,52).

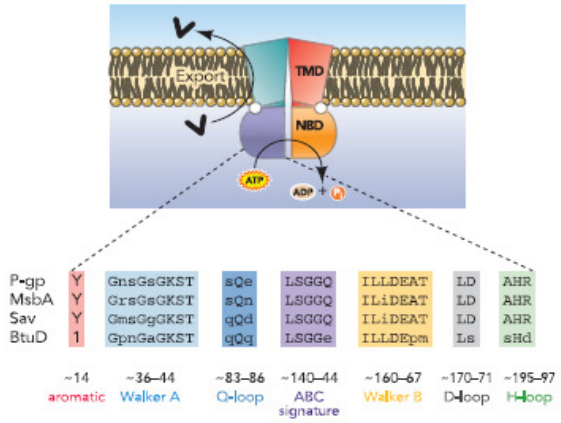
5. **the MATE family** (multidrug and toxic compound extrusion)

Multidrug and toxic compound extrusion (MATE) proteins, are widely distributed in all kingdoms of living organisms. The bacterial MATE-type transporters that have been characterized, function as exporters of cationic drugs, plant MATE-type transporters are involved in the detoxification of secondary metabolites and mammalian4 MATE-type transporters are responsible for the final step in the excretion of metabolic waste and xenobiotic organic cations in the kidney and liver (48,49).

5. 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 (53), which can bind and hydrolyze ATP. The ABC unit (also called nucleotide binding domain or NBD) harbours several conserved sequence motifs. From NH_2 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 (**Figure 5**)(54, 55, 56,56).

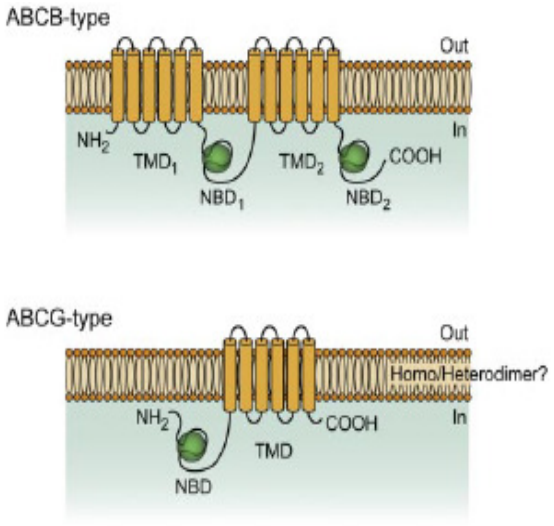
Figure 5. A minimal ABC transporter has four domains
(Figure taken from Linton KJ: Structure and function of ABC transporters. Physiology 22:122-130, 2007.)



Two transmembrane domains (TMDs) bind ligand, and the transport is driven by ATP binding and hydrolysis by the two nucleotide binding domains (NBDs). The TMDs from different subfamilies of ABC transporters are not necessarily homologous. The NBDs are homologous throughout the family. Each NBD has seven highly conserved, but not invariant, motifs.

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 (**Figure 6).** 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 (54).

Figure 6. Membrane topology models of the ABCB1 (MDR1/P-gp) and ABCG2 (MXR/BCRP) proteins
(Figure taken from Sarkadi B, Homolya L, Szakács G, Váradi A: Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol. Rev. 86: 1179-1236, 2006.)

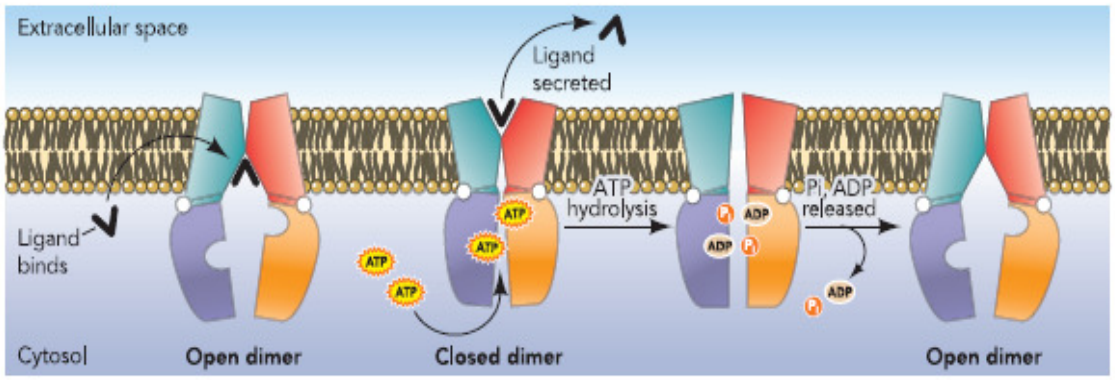


1. ABCB1 (P-GLYCOPROTEIN, MDR1): THE CLASSICAL HUMAN MDR-ABC TRANSPORTER

Structurally, the human 170 kDa P-gp consists of 1280 amino acids residues forming two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). The ABC-ATPase (NBD) domain has two short peptide motifs, a glycine-rich Walker-A and a hydrophobic Walker B motif. A third consensus sequence is named ABC signature (**Figure 5**) (24,56).

The membrane topology of MDR1/Pgp has been elucidated by epitope insertion experiments (58,59), fully supporting the original topology model of six TM helices in both TMDs of the protein (60). The linker region connecting the two halves of the protein plays a critical role in ensuring proper interaction of two subunits. ATP binding and cleavage occur at the ABC units, and the close interaction of two ABC units results in the formation of a fully competent ATP-hydrolytic site (61). MDR1/Pgp (as all ABC transporters) differs from P-type ATPases in that it does not show a high-affinity ATP binding and does not utilize a covalently phosphorylated protein intermediate. Theoretically, the ATPase cycle can be described as containing the following basic steps: ATP binding, cleavage of the terminal phosphate bond, and release of the catalytic products (P_i and ADP) (**Figure 7**)(62).

Figure 7. A simple ATP-switch mechanism powers ABC transporters
(Figure taken from Linton KJ: Structure and function of ABC transporters. Physiology 22:122-130, 2007)



MDR1/Pgp confers resistance to a vast array of clinically and toxicologically relevant compounds, including anticancer drugs, human immunodeficiency virus (HIV)-protease inhibitors, antibiotics, antidepressants, antiepileptics, and analgesics (64, 65). Studies using fluorescent substrates and FRET have confirmed that the drug binding sites are located within

the membrane plane. There are strong indications that this type of recognition makes the MDR1 protein a highly effective multidrug resistance pump, preventing the cellular entry of toxic compounds (63). It was demonstrated that verapamil (66), trifluoperazine (67) and quinidine (68) could act as chemosensitizers, inhibiting MDR conferred by P-gp.

Table 3. Selected substrates of MDR1/P-gp (64, 65)

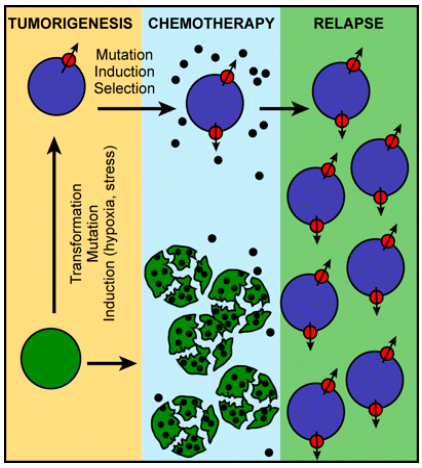
Anticancer drugs	
Vinca alkaloids (vincristine, vinblastine)	Anthracyclines (doxorubicin, daunorubicin, epirubicin)
Epipodophyllotoxins (Etoposide, Teniposide)	Paclitaxel (taxol)
Actinomycin D	Topotecan
Mithramycin	Mitomycin C
Other cytotoxic agents	
Colchicine	Emetine
Ethidium bromide	Puromycin
Cyclic and linear peptides	
Gramicidin D	Valinomycin
N-Acetyl-leucyl-leucyl-norleucine	Yeast a-factor pheromone
HIV protease inhibitors	
Ritonavir	Indinavir
Saquinavir	
Other compounds	
Hoechst 33342	Rhodamine 123
Calcein-acetoxymethyl ester	

Table 4. Drugs which act as P-glycoprotein antagonists (64)

Calcium channel blockers	
Verapamil	Diltiazem
Calmodulin antagonists	
Trifluoperazine	Chlorpromazine
Antiarrhytmics	
Quinidine	Amiodarone
Propafenone	
Antihypertensives	
Reserpine	Propranolol
Hormones	
Progesterone	Tamoxifen
Hydrophobic peptides	
Cyclosporin A	SDZ PSC 833
Valinomycin	Gramicidin D
Antihistaminic drugs	
Promethazine	Terfenadine
Antimalarial drugs	
Mefloquine	Quinine
Other drugs	
Amitriptyline (antidepressant)	Dipyridamole (anticoagulant)
Fucidin (antibiotic)	Staurosporin (protein kinase C antagonist)

Tumour cells arise by a complex mutation and induction pathway. Cells that do not express multidrug transporters are sensitive to chemotherapy and are eliminated. MDR is associated with a reduced intracellular drug accumulation and an increased cellular drug efflux. Resistance to multiple anticancer agents is a major impediment to the successful treatment of many forms of malignant disease. 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 *ABCB1* gene) (64). In the course of chemotherapy, further mutations and selection may greatly increase the expression of multidrug transporters, which protect the tumour cells against chemotherapy (**Figure 8**) (54).

Figure 8. Evolution of MDR1/Pgp positive cancer
(Figure from Sarkadi B, Homolya L, Szakács G, Váradi A: Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol. Rev. 86: 1179-1236, 2006.)



MDR1/Pgp shows high expression in the apical (luminal) membranes of epithelial cells lining the lower gastrointestinal tract, in the apical surface of proximal tubule cells of the kidney, in the canalicular membranes of hepatocytes, and in the capillary endothelial cells in the brain and testes. P-glycoprotein is expressed in many cell types in the brain including choroid plexus, astrocytes, microglia, and capillary endothelium. Lower levels are expressed in the placenta, the adrenal cortex, and CD34+ hematopoetic stem cells (70,71,73,74,75).

The most important physiological effect associated with P-glycoprotein expression in the blood-brain barrier appears to be in the lumenal plasma membrane of the capillary endothelium, where the protein prevents the passage of drugs and toxins across the capillary membrane into the brain (69,70).

2. ABCC (ABCC1/MRP1, ABCC2/MRP2) EFFLUX PUMPS

In 1992, *Susan Cole* and *Roger Deeley* observed amplification and increased expression of a novel gene, the **MRP1 (ABCC1) (MDR related protein)** gene in a non-P-gp MDR cell line, namely a small-cell lung carcinoma cell line (76,77). Overexpression of MRPs, other ABC proteins, causing MDR in mammalian cells has been also observed in several other non-P-gp MDR cell lines.

The detoxification of xeno- and endobiotic electrophiles, including genotoxic carcinogens and mutagens, frequently involves their conjugation with glutathione (GSH). These conjugation reactions, catalyzed by glutathione *S*-transferases (GSTs), have evolved to remove the amphiphilic conjugates from the cell by ATP-dependent efflux transporters that include several members of the MRP family of membrane-associated transport proteins (72). MRP1 has three membrane spanning domains, two nucleotide binding domains and extracellular N-terminal. Both the structure and drug resistance spectra of MRP1 and P-gp are similar except taxanes which are poor substrates for MRP1. The isolation of a second distantly related ABC protein, the 190-kDa multidrug resistance protein 1 (MRP1) (encoded by *ABCC1*) facilitated the discovery of eight more genes within the same ABC subfamily of which at least four: MRP2 (encoded by *ABCC2*), MRP3 (encoded by *ABCC3*), MRP4 (encoded by *ABCC4*), and MRP5 (encoded by *ABCC5*), are potentially involved in mediating drug resistance (73).

The second member of the multidrug resistance protein (MRP) (ABCC) family is called the canalicular multi-organic anion transporter **(CMOAT, MRP2)** which is involved in bilirubin glucuronide transport and confers resistance to MRP1 substrates and cisplatin. **MRP3** is expressed in liver and involved in the efflux of organic anions from the liver into the blood in case of biliary obstruction. **MRP4** and **MRP5** transport nucleosides and confer resistance to antiretroviral nucleoside analogs. **MRP6** is a lipophilic anion pump with a wide spectrum of drug resistance. Among the members of MRP family, only MRP1 has been widely accepted to cause clinical drug resistance (78). In glioma cell lines, MDR1 P-glycoprotein and ABCC1 were shown to confer resistance to various anticancer drugs (79,80,80)

3. THE ABCG2 (MXR/BCRP) PROTEIN

ABCG2, a 655-amino acid glycoprotein, was cloned independently from two drug-selected cell lines and a human cDNA library and was given three different names. ABCG2

cloned from a heavily drug-selected breast cancer cell line was named breast cancer resistance protein (**BCRP**) (82). ABCG2 is physiologically expressed in a variety of tissues, most abundantly in the liver and intestinal epithelia, the placenta, the blood-brain barrier and various stem cells. ABCG2 is a plasma membrane glycoprotein, in polarized cell types localizing to the apical regions (83,83).

ABCG2 is a drug transporter with a wide substrate specificity, that includes large molecules, both positively and negatively charged, with amphiphilic character. The reported cytotoxic drugs extruded by the wild-type human ABCG2 are mitoxantrone, topotecan, the active metabolite (SN-38) of irinotecan, camptothecin, flavopiridol, and methotrexate. The transported substrates of this protein also include sulfated hormone metabolites, antibiotics such as nitrofurantoin, antihelmintic benzimidazoles, various flavonoids, the food carcinogen PhIP, the chlorophyll metabolite pheophorbide a, fluorescent dyes such as Hoechst 33342 and BODIPY-prazosin, or the H2 receptor antagonist cimetidine (54,65, 85,86,87,88,89).

Altough the **lung resistance protein (LRP)** is not an ABC transporter, it is expressed at high levels in drug-resistant cell lines and in some tumour (90). LRP is a major vault protein found in the cytoplasm and on the nuclear membrane. Vaults are barrel-shaped cytoplasmic ribonucleoprotein particles composed of multiple copies of three proteins. The mammalian vault complex is made up of major vault protein (MVP or LRP), vault poly ADP-ribose polymerase (VPARP), and telomerase-associated protein 1 (TEP-1) that are associated with small 88- to 141-bp fragments of untranslated RNA (91,92,93,94).

To summarize the most important multidrug transporters involved in anticancer drug resistance, the basic differences and similarities are the following (54,65):

- MRP1 and MRP2 belong to the same ABC subfamily and share 49% amino acid identity with each other. In contrast, P-glycoprotein and BCRP/ABCG2 belong to separate subfamilies of the ABC superfamily and share very limited amino acid identity (<20%) with each other and with MRP1 or MRP2.
- P-glycoprotein has a four-domain structure, as is typical of most eukaryotic ABC transporters, in contrast, BCRP/ABCG2 is an atypical so-called “half-transporter”, consisting of a single hydrophobic membrane spanning domain predicted to contain 6 TM helices. MRP1 and MRP2 are also structurally atypical in that they are composed of five domains with an extra NH_2-proximal membrane spanning domain (MSD) which has five TM segments and an extracytosolic NH_2-terminus.

- Despite these differences in domain organization, there is considerable overlap in the spectrum of drugs to which P-glycoprotein, BCRP/ABCG2, and MRP1 (and MRP2) confer resistance. Thus, P-glycoprotein confers high levels of resistance to bulky amphipathic natural product type drugs such as paclitaxel, *Vinca* alkaloids, anthracyclines, camptothecins, and epipodophyllotoxins. The spectra of drugs transported by MRP1 and MRP2 are similar to each other and to P-glycoprotein.
- The mechanisms by which MRP1/2 and P-glycoprotein transport many unconjugated xenobiotics and thereby decrease cellular drug accumulation are very different. P-glycoprotein has been shown to be a *primary active transporter* of drugs to which it confers resistance. In contrast, while also ATP-dependent transporters, MRP1 and MRP2 efflux xenobiotics such as vincristine and daunorubicin through a *co-transport* mechanism with reduced glutathione (GSH).

Inhibiting P-gp and other ABC transporters has been extensively studied for more than two decades (95, 96). **The first generation of MDR modulators** include many agents with different structure, such as calcium channel blockers (97), calmodulin antagonists (98), steroidal agents (99), protein kinase C inhibitors (100), immunosuppresive drugs (101), antibiotics (102), antimalarials (103), psychotropic phenothiazines and indole alkaloids (104,105), detergents and surfactants (106). 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 (78).

Second-generation chemosensitizers were designed to reduce the side effects of the first generation drugs. They have better pharmacological profile, however still they 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. An other 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 (69,78).

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 (95). The anthranilamide derivative **tariquidar (XR9576)** is a potent, specific, non-competitive inhibitor of P-glycoprotein. Tariquidar inhibits the ATPase activity of P-glycoprotein, suggesting that the modulating effect is derived from the inhibition of substrate binding, inhibition of ATP hydrolysis or both. In clinical trials, tariquidar is tolerable and does not have significant pharmacokinetic interaction with chemotherapy (107). **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 (108). **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 (109,110). **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 (111).

6. 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 (112,113,114). 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. The correlation between the carcinogenic activity of benz[c]acridines and the electron density has been investigated (115). The 6-methyl-5-oxo-5H-benzo[a]phenothiazine enhanced the expression of viral oncogene product (tumor antigen) in the adenovirus infected cells (116). 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. (117). The alkylene-urea substituted phenothiazines affected

the growth and inhibited the growth rate of AIDS-related lymphoma cells (118). 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 (119). 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 (120).

Furthermore, natural compounds such as tomato lectin (121), feijoa peel extracts (122), kiwifruit peel extracts (123,124), *Anastasia green* sweet pepper (125), morphine alkaloids (126), *Allium victorialis L.* extracts (127) and natural compounds from *Euphorbia* species (128) 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.

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 some 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*.

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 (129, 130). *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% CO_2 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 humified atmosphere (5% CO_2, 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-dimethythiazol-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 μl) of the 1.0-10.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature. Next, 10 μl (5.2 μM final concentration) of the indicator rhodamine 123 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 (131), 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 μL. 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 μL of MTT (thiazolyl blue, Sigma, St Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of Sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO, USA) solution (10%) was measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

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

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 100µL volume. The cell suspension in the tissue culture medium was distributed into each well in 100 µL containing 5×10^4 cells. The plates were incubated for 72 h at 37 °C in a CO_2 incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a 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

RESULTS

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

Chromone and its derivatives are widely distributed in plant life, mostly as pigments in plant leaves and flowers. They have considerable interest in terms of their biological potential as drugs. The pharmacological activities of many chromone derivatives such as anti-inflammatory, antiviral and anti-neoplastic activities have been extensively investigated (132).

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 ID_{50} values. The ID_{50} value shows the 50% inhibition of cell proliferation. In the ***first*** group, **FC 3,** with an ID_{50}=1.9μg/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 ID_{50} values between 4.27- 7.13. Those in the ***third*** group, **FC 5** and **FC 12-16** had very high ID_{50} values in the range from 21.42-79.14 μg/mL (**Table 5**).

Table5. Antiproliferative effect of 3-formylchromones (**1-16**) on mouse lymphoma cells transfected with human MDR1 gene.

Compounds	ID_{50}
FC1	**5.76**
FC2	**4.27**
FC3	**1.9**
FC4	**5.04**
FC5	**36.76**
FC6	**7.1**
FC7	**7.13**
FC8	**4.84**
FC9	**4.95**
FC10	**6.4**
FC11	**5.97**
FC12	**45.84**
FC13	**41.87**
FC14	**40.75**
FC15	**79.14**
FC16	**21.42**

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 (ID_{50}=2.02-8.70 μg/mL). The compounds **FC 1,4,11** and **FC 13-16** were less effective, with ID_{50} values in the range 10.53-84.61 μg/mL The majority of the compounds were moderately effective in the antiproliferative effect measured on Colo 320 cells: **FC 5-10, FC 2,3**.

The following compounds were less effective: **FC 1, 4, 11, 13, 14, 15, 16** having the ID_{50} value between 10-84.61 μg/ml (**Table 6**).

Table6. Antiproliferative effects of 3-formylchromones (**1-16**) on Colo 320 colon cancer cells line

Compounds	**ID_{50}**
FC1	10.53
FC2	3.83
FC3	8.70
FC4	10.61
FC5	4.32
FC6	4.43
FC7	2.07
FC8	2.68
FC9	3.31
FC10	2.02
FC11	18.95
FC12	84.61
FC13	19.43
FC14	15.88
FC15	26.37
FC16	30.15

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 assays.

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 (**Table 7**). The most effective compounds proved to be **FC 1,3,4,6,7,8,10** and **11**, while compounds **FC 2** and **FC 5** were hardly effective, but were toxic at 40 μg/mL. Compounds **FC 12-16** were ineffective in the mouse lymphoma cells transfected with the human *MDR1* gene.

Table 7. Effect of 3-formylchromones (**1-16**) on the activity of MDR efflux pump of mouse lymphoma cells transfected with human *MDR1* gene.

Compounds	*concentration μg/mL*	*Fluorescence activity ratio (FAR)*
FC1	**4**	2.05
	40	23.25
FC2	**4**	1.25
	40	4.21
FC3	**4**	11.76
	40	TOXIC
FC4	**4**	1.36
	40	23.89
FC5	**4**	1.11
	40	2.72
FC6	**4**	1.67
	40	19.59
FC7	**4**	6.25
	40	26.44
FC8	**4**	6.65
	40	48.35

FC9	**4**	7.69
	40	TOXIC
FC10	**4**	1.37
	40	15.90
FC11	**4**	1.37
	40	27.38
FC12	**4**	0.79
	40	0.95
FC13	**4**	0.66
	40	1.01
FC14	**4**	0.64
	40	1.07
FC15	**4**	0.78
	40	0.73
FC16	**4**	0.63
	40	1.05

When the structure-activity relationship was analyzed, 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 **(Annex 1).**

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**, while compounds **FC 2** and **FC 4** were moderately effective. Compounds **FC 3, 12, 13, 14** and **16** were ineffective (**Table 8**). In case of colon cancer cells, the effects of these compounds depended mainly on the electron-withdrawing substituent at position 6. Compounds **FC 2** and **4** were exceptions (**Table 8**). A bulky substituent at position 3 resulted in ineffective compounds, *e.g.* **FC 12-16**.

Table8. Effect of 3-formylchromones (**FC1 - FC16**) on rhodamine accumulation of Colo 320 colon cancer cells.

Compounds	*concentration µg/mL*	*Fluorescence activity ratio (FAR)*
FC1	**4**	2.92
	40	3.89
FC2	**4**	2.72
	40	2.02
FC3	**4**	1.52
	40	0.78
FC4	**4**	1.08
	40	2.13
FC5	**4**	0.98
	40	1.79
FC6	**4**	2.41
	40	3.64
FC7	**4**	4.69
	40	4.53
FC8	**4**	4.83
	40	5.41
FC9	**4**	4.56
	40	2.70
FC10	**4**	3.13
	40	3.29
FC11	**4**	2.01
	40	4.31
FC12	**4**	0.74
	40	0.69
FC13	**4**	0.69
	40	0.51
FC14	**4**	0.78
	40	0.39
FC15	**4**	0.84
	40	1.11
FC16	**4**	1.13
	40	1.36

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. The interaction is shown in **Table 9**. **6,8-dibromo-3-formylchromone** acted synergistically with doxorubicin on mouse lymphoma cells in checkerboard assay.

Table9. Interaction between **6, 8-dibromo-3-formylchromone** and doxorubicin on *MDR1*-gene transfected mouse lymphoma cell line

Compound	FIX values	Interaction
FC11	**0.054**	**Synergism**

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

Conjugated arylidene ketones react preferentially with thiols rather than hydroxy and amino groups (133). Thus these enones may exert their bioactivities without interactions with the amino and hydroxy portions of nucleic acids thereby avoiding the genotoxic problems of a number of anticancer drugs (134). In addition, the theory of sequential cytotoxicity states that successive chemical attacks by candidate antineoplastic agents may be more harmful to malignant cells rather than the corresponding normal tissues (135,136). On the basis of this hypothesis, a number of 1,3-diarylidene-2-tetralones were prepared by *Prof. Dimmock* and his group which had different electron densities on the olefinic carbon atoms which may permit sequential thiol addition by cellular constituents (137). In general, these compounds were more than twice as cytotoxic as the corresponding 1-arylidene-2-tetralones suggesting that the theory of sequential cytotoxicity was worthy of further consideration. In particular, the aryl substituents of **1a** were chosen for the following reasons. Under the hypoxic conditions of certain tumours, the nitro group could be reduced to one or more toxic metabolites this process would occur more readily in such tumours rather than normal cells. Second, the 3,4,5-trimethoxyphenyl group is an integral part of a number of anticancer agents such as podophyllotoxin (138). These considerations were validated when **1a** displayed excellent cytotoxicity when evaluated against 56 human tumour cell lines having an average IC_{50} value

of 0.59 μM. Furthermore, mice tolerated doses up to and including 300 mg/kg of this compound (137).

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. If such properties were noted, an indication of those structural features which contributed to these favourable bioactivities would be sought.

The design of analogues of **1a** was based on the following reasoning. First, in order to ensure a differential between the fractional positive charges on C^A and C^B, the 4-nitrophenylmethylene group was retained while, in general, electron-releasing substituents were placed in ring B. Second, further molecular modifications of series **1** were undertaken in order to glean some idea of the contributions to bioactivity of ring C as well as the size of the alicyclic ring; such considerations led to the decision to prepare series **2–4 (Annex 2)**.

All of the compounds in series **1–4** were evaluated against four neoplastic cell lines, namely HSC-2, HSC-3, HSG and HL-60 cells as well as the nonmalignant HGF, HPC and HPLF cells.The potency of the lead compound **1a** towards four cancer cell lines is revealed in **Table 10**. The average CC_{50} (CC_{50}: concentration of the compound to kill 50% of the cells) figure of **1a** to HSC-2, HSC-3, HSG and HL-60 cells was 1.75 μM or 85% of the average potency of doxorubicin towards these four cell lines.

Table 10. The cytotoxicities of substituted arylidene ketones on normal oral cells and oral cancer cell lines

Compound	Aryl substituents[a]				CC_{50}[b] (μM)							
	R^1	R^2	R^3	Δσ[c]	HSC-2[e]	HSC-3[e]	HSG[e]	HL-60[e]	HGF[e]	HPC[e]	HPLF[e]	SI[d]
1a	OCH_3	OCH_3	OCH_3	0.81	0.55	2.9	3.0	0.55	>400	>400	>400	>229
1b	OCH_3	OCH_3		0.93	0.92	4.2	4.4	0.56	>400	>400	>400	>159
1c	OCH_3	OH		1.03	1.45	3.8	5.8	0.95	33	83	21	15
1d		OCH_3		1.05	11	32	47	4.4	>400	>400	>400	>17
1e	OCH_2O			1.10	2.6	7.6	5.0	2.2	>400	>400	>400	>92
1f		OH		1.15	5.2	14	11	2.5	85	113	75	12
2a	OCH_3	OCH_3	OCH_3	0.81	0.76	4.7	8.3	1.1	336	347	378	95
2b	OCH_3	OCH_3		0.93	2.3	4.4	13	4.3	53	82	95	13
2c	OCH_3	OH		1.03	2.8	5.2	11	3.4	165	>400	171	>44
2d		OCH_3		1.05	>400	>400	>400	>400	>400	>400	>400	1.0
2e	OCH_2O			1.10	308	189	>400	>400	>400	391	330	1.2
2f		OH		1.15	8.1	13	>400	4.4	>400	>400	>400	3.8
2g		Cl		0.55	1.5	4.6	15	4.1	191	345	332	46
3a	OCH_3	OCH_3	OCH_3	0.81	221	>400	>400	318	89	153	>400	0.6
4a	OCH_3	OCH_3	OCH_3	0.81	>400	>400	>400	>400	>400	>400	>400	1.0
4b		OCH_3		1.05	63	>400	>400	>400	>400	>400	>400	1.3
Doxorubicin	—	—	—	—	0.68	2.9	1.9	0.47	233	414	>400	>235

[a] For clarity, hydrogen atoms are omitted.
[b] Concentrations of compound to kill 50% of the cells.
[c] Δσ refers to the differences in the σ values between the substituents in rings A and B in series **1**–**4**. The sigma values used in these calculations were taken from Ref. (139).
[d] The letters SI indicate the selectivity index which was calculated as follows:
$SI = [CC_{50}(HGF)+CC_{50}(HPC)+CC_{50}(HPLF)]/[CC_{50}(HSC\text{-}2)+CC_{50}(HSC\text{-}3)+CC_{50}(HSG)+CC_{50}\ (HL\text{-}60] \times 4/3$.

[e]Human tumour 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. Near confluent cells were incubated for 24 h with or without various concentrations of each compound and the relative viable cell number, that is, the absorbance of 540 mm of the MTT-stained cell lysate, was determined by the MTT method. The viable cell number of HL-60 cells was obtained by trypan blue exclusion. The CC_{50} values were determined from the dose–response curves.

The normal cells were relatively resistant to the derivatives **1a, 1b, 1d, 1e, 2a, 2d** and **2f**. The preferential toxicity for malignant cells, the selective toxicity was calculated for each compound. The SI values indicated that **1a, 1b, 1e, 2a, 2c** and **2g** had the highest tumour specific cytotoxic activities among the substituted arylidene ketones (**Table 10**). In order to address the second question as to the efficacy or otherwise of these novel cytotoxins to MDR, murine lymphoma L-5178 cells transfected with the human MDR1 gene as well as human

colon cancer Colo320 neoplasms were treated with two concentrations of the compounds in series **1**, **2** and **4**.The accumulation of rhodamine 123 was measured in the treated and untreated cells. The data are summarized in **Table 11**. In those cases where the fluorescence activity ratio (FAR) values are greater than 1, reversal of MDR has taken place. MDR is due, inter alia, to an increase in the efflux of a compound from the cell. Consequently a reversal of MDR means that the exodus of rhodamine 123 from the cells will be either the same or lower than in the parental cells. The data in **Table 11** reveal 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.

Table11. Effect of conjugated arylidene ketones **1a**–**f**, **2a**–**g** and **4a**, **b** on multidrug resistance in murine L-5178 cells and human colon cancer Colo 320 neoplasms

Compound	**Fluorescence activity ratios (FAR) at different concentrations**[a]			
	L-5178		**Colo320**	
	Concentration			
	4 µg/mL	**40 µg/mL**	**4 µg/mL**	**40 µg/mL**
1a	134	120	10.9	13.0
1b	44.1	55.9	7.19	11.4
1c	51.8	123	3.38	6.20
1d	4.36	7.68	2.22	4.30
1e	3.27	3.87	1.95	2.27
1f	135	39.3	1.99	11.7
2a	15.6	75.1	7.43	4.38
2b	20.4	47.3	6.12	4.62
2c	1.50	0.83	1.98	1.13
2d	0.76	0.82	0.50	0.58
2e	0.91	1.23	0.67	0.59
2f	0.94	0.97	1.15	0.60
2g	1.16	0.94	0.60	0.53
4a	3.06	6.62	1.96	1.28
4b	0.59	0.69	0.63	0.37

[a] The fluorescence activity ratio (FAR) values are the ratios of the fluorescence intensities of the treated cells/untreated cells. The FAR value of a reference compound verapamil was 38.2 and 8.05 using L-5178 and Colo320 cells, respectively, when a concentration of 10 µg/mL of verapamil was used (limitations of solubility precluded a higher concentration of verapamil being utilized).

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

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

A preliminary paper from Professor *Dimmock's* laboratory disclosed that 6-(4-nitrophenylmethylene)-2-(3,4,5-trimethoxyphenylmethylene)cyclohexanone **1** reversed MDR in murine L-5178 cells and demonstrated greater toxicity toward various neoplasms than non-malignant cells (1). In this series of compounds, the 3,4,5-trimethoxyphenyl group was optimal, for example, under the test conditions utilized, the 4-methoxy analog **2** was bereft of MDR-reversing and cytotoxic properties (1). A subsequent study confirmed the good potency of **1** (but not **2**) towards additional neoplastic cell lines (140) **(Annex 3)**.

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.

Sakagami, Ideo and *Shimada* evaluated the compounds with preferential cytotoxicity for malignant cells used HSC-2, HSC-3, and HSC-4 squamous cell carcinomas and HL-60 promyelocytic leukemia cells as well as the following non-malignant cells such as HGF gingival fibroblasts, HPLF periodontal ligament fibroblasts, and HPC pulp cells. The results for these assays are portrayed in **Table 12**.

Table 12. The cytotoxicities of α,β-unsaturated cyclic ketones (**1**–**10**)substituted on normal oral cells and oral cancer cell lines

Compound	**CC_{50}[a] (μM)**								
	HSC-2	**HSC-3**	**HSC-4**	**HL-60**	**Ave[b]**	**HGF**	**HPLF**	**HPC**	**SI[c]**
1[d]	0.76	4.7	1.5	1.1	< 2.0	336	378	347	>176
2[d]	>400	>400	>400	>400	>400	>400	>400	>400	1.0
3	116	279	100	266	190	256	370	198	1.4
4	<3.1	5.7	3.8	6.4	<4.8	16	106	94	>15
5	6.3	16	12	25	15	81	193	141	9.3
6	34	62	36	46	45	335	388	285	7.5
7	113	132	67	46	90	229	250	276	2.8
8	19	89	58	21	47	240	281	247	5.5
9	214	200	269	31	179	262	291	277	1.6
10	55	72	41	47	54	159	263	188	3.8
Melphalan[e]	35	115	81	6	59	>200	>200	>200	>3.4

[a] The CC_{50} figure is the concentration of compound required to kill 50% of the cells and is the average of two independent determinations.
[b] Average CC_{50} value of the compound toward HSC-2, HSC-3, HSC-4, and HL-60 neoplastic cells.
[c] The letters SI refer to the selectivity index. [d] With the exception of the figure of HSC-4 cells, the data were shown in the section **„Conjugated arylidene ketones"**
[e] Solubility problems of melphalan in dimethylsulfoxide precluded the use of concentrations above 200 μM.

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**, 5, **6** and **8** exerted the most effective antiproliferative effect on human MDR1 gene transfected mouse lymphoma cells and human colon cancer cells as well.

Of the 10 compounds studied, **1, 2** and **8** showed potent antiproliferative effect on mouse lymphoma cells. Compound **6**,**7**, and **9** were moderate effective in the antiproliferative assay on mouse lymphoma cells (**Table 13**).

Table 13. Antiproliferative effect of α, β-unsaturated cyclic ketones (**1-10**) on mouse lymphoma cells transfected with human *MDR1* gene

Compounds	ID_{50}
1	**1.41**
2	**1.21**
3	**11,85**
4	**14.57**
5	**13.67**
6	**8.31**
7	**7.53**
8	**2.65**
9	**7.24**
10	**10.71**

The proliferation of human colon cancer cells could be inhibited efficiently by compound **1**, **7** and **8.** Compound **2, 6** and **9** exerted moderate antiproliferative effect (**Table 13**).

Table14. Antiproliferative effects of α,β-unsaturated cyclic ketones (**1-10**) on Colo 320 colon cancer cells line

Compounds	ID_{50}
1	**0.18**
2	**11.96**
3	**43,85**
4	**48.56**
5	**37.55**
6	**8.31**
7	**2.35**
8	**3.15**
9	**11.19**
10	**43.95**

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.

The ability of these compounds to reverse MDR was assessed using murine lymphoma L-5178 cells transfected with the human MDR1 gene and the data are presented in **Table 15**. 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 μg/mL).

Table15. Effect of α,β-unsaturated cyclic ketones on the activity of MDR efflux pump of mouse lymphoma cells transfected with human *MDR1* gene

Compounds	**Fluorescence activity ratio FAR**	
	Concentration	
	4 μg/mL	**40 μg/mL**
1	15.6	75.1
2	0.76	0.82
3	1.10	1.36
4	0.82	1.89
5	1.38	1.34
6	13.3	69.3
7	4.30	13.8
8	96.3	107
9	1.15	39.0
10	1.37	48.3

3.3. Combination of α, β-unsaturated cyclic ketones and doxorubicine using checkerboard microplate method on human MDR1-gene transfected mouse lymphoma cells and MDR1/LRP-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 **7** and **8** were combined with doxorubicin on *MDR1*-gene transfected mouse lymphoma cells and human colon cancer cell line Colo320. The interactions are shown in **Table 16 and 17**. Compound **7** and **8** exerted additive effect with doxorubicin on mouse lymphoma cells, and surprisingly compound **8** interacted synergistically with doxorubicin on human colon cancer cells.

Table 16. Interaction between α, β-unsaturated cyclic ketones **7 and 8** with doxorubicin on *MDR1*-gene transfected mouse lymphoma cell line

Compound	FIX values	Interaction
7	**0.52**	**Additive**
8	**0.81**	**Additive**

Table 17. Interaction between α, β-unsaturated cyclic ketones **7 and 8** with doxorubicin on Colo 320 human colon cancer cell line

Compound	FIX values	Interaction
7	**1.41**	**Indifferent**
8	**0.35**	**Synergism**

DISCUSSION

Over the past 20 years two general approaches have been adopted, relatively unsuccessfully, to overcome drug resistance in the clinic. First, there have been considerable efforts to modify drugs and antibiotics chemically so that they are no longer substrates for multidrug transporters. The second approach to overcome multidrug resistance -the development of specific inhibitors of transporters- has also proved unsatisfactory. There is frequently more than one multidrug transporter that can confer resistance to a specific drug, thus more than one inhibitor may be required. Another difficulty could be the side effects caused by inhibiting P-glycoprotein's normal physiological function in healthy tissues (24,78). Pharmacological investigations progressed to rational design using the 1st generation compounds as a template structure. Inherent toxicity of the drugs was reduced; however, pharmacokinetic interactions with the anticancer drugs were unsustainable. Generation of the most recent of inhibitors employed combinatorial chemistry to produce a handful of potent and selective P-gp inhibitors. Some of these drugs have progressed to clinical trials with poor results or in some cases, undisclosed progress (141).

Many compounds have been obtained through extensive **screening** of compounds of natural origin and drug collections or by molecular modification of known MDR modulators. Selection of the compounds to screen was usually based on the few general features that were

considered important for MDR antagonism: high lipophilicity, presence of one protonable nitrogen atom and of two or more aromatic rings (142). This situation may change now, as some features of the recognition site of ABC transporters are emerging. Possibly, this will allow a more rational design of inhibitors even if they will maintain a quite heterogeneous structure (143).

The modulators of ABC transporters can be divided into three groups based on former results of Professor Joseph Molnár's group: 1. natural plant extracts, 2. well defined natural compounds, 3. synthetic compounds.

1. Natural plant extracts: For instance fractions of **barbados cherry** (acerola fruit, *Malpighia emarginata*) were investigated in our former studies and the hexane extracts H3 and HE3 showed higher cytotoxic activity against tumour cells such as human oral squamous cell carcinoma (HSC-2) and human submandibular gland carcinoma (HSG), when compared with that against normal cells such as human periodontal ligament fibroblasts (HPLF) and human gingival fibroblast (HGF). The hexane extracts H3 and HE3, which displayed high tumour specific cytotoxicity also showed higher multidrug resistance reversal activity than verapamil as positive control (144).

2. Natural compounds: **Carotenoid** fractions were extracted from **red paprika**, ***Valencia Orange*** peel and the peel of ***Golden Delicious*** apple. The hypophasic carotenoids PM3 (orange) and PM4 (apple) showed slightly higher cytotoxic activity against three human tumour cell lines (human oral squamous cell carcinoma HSC-2, HSC-3; human submandibular gland carcinoma HSG; human promyelocytic leukemic HL-60 cells) than against three normal human oral cells (human gingival fibroblast HGF; human pulp cell HPC; human periodontal ligament fibroblast HPLF). The same carotenoids PM3 and PM4 displayed higher MDR-reversing activity than verapamil (145).

Jatrophane-type diterpenes isolated from ***Euphorbia portlandica*** and ***Euphorbia segetalis*** revealed to be active in MDR reversal assay. The difference of activity observed for the compounds may be related with the substitution pattern of ring A that may induce small conformational changes in the molecule (146). Some diterpenes based on the jatrophane skeleton were able to strongly enhance the rhodamine accumulation of human MDR1 gene transfected mouse lymphoma cells. In HTB-26 human breast cancer cells, which simultaneously express MDR1 and MRP proteins, the jatrophane derivatives enhanced the accumulation of the MRP-specific indicator carboxyfluorescein (147).

3. ***Synthetic compounds:*** The inhibition of the activities of MDR1 and MRP proteins was investigated via administration of two new organosilicon compounds, **ALIS-406** and **ALIS-421**. The rhodamine uptake was increased in human MDR1 gene transfected mouse lymphoma cells and in Colo320/MDR1-LRP cells. The MRP-mediated carboxyfluorescein accumulation in HTB-26/MRP human breast cancer cells was not modified by these organosilyl derivatives (151). 3,5-Dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (**DP7**) exhibited MDR reversing activity and this compound may represent a lead compound for the development of potent dihydropyridine MDR chemosensitizers devoid vascular effects (152).

To summarize our former studies, the inhibition of the ABC transporters of tumour cells is an opportunity to overcome multidrug resistance. The treatment of MDR cancer cells can be improved by the administration of traditional chemotherapeutics in combination with resistance modifiers. Large numbers of natural plant compounds (145,146,148,149,150,146, 153,154,155) and synthetic molecules have been shown to block MDR pump efflux activity. The synthetic molecules include phenothiazines, aza-oxafluorenes, acridine, coumarin and naphthyridine derivatives (151,156,157,158,159).

1. 3-FORMYLCHROMONE DERIVATIVES

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 (160). Chromone and coumarin derivatives are of great interest because of their antimicrobial, antitumour and antiviral activity (160,161,162). Phosphorohydrazone derivatives of coumarin and chromone demonstrated antitumour activity against leukemia L1210 (161). Among chromone derivatives, cytotoxic activity against leukemia P388 has been recognized in phosphorohydrazinecarbonylic derivative (163).

In our studies 3-formylchromone derivatives were investigated. Regarding the antiproliferative effect of the 3-formylchromone (**FC**) derivatives, the relevant ID_{50} values are presented in **Table 5** and **Table 6**. It is interesting to note that the substituents had a 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** (**Table 5**) and against human colon cancer cells as **FC 2, 5, 6, 7, 8, 9** and **10** (**Table 6**). 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, 11** (**Table 7**), 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** (**Table 8**).

We found synergism between formylchromone derivative **FC11** 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 (**Table 9**).

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. CONJUGATED ARYLIDENE KETONES

Dimmock and his research group prepared a number of arylidene derivatives of alicyclic ketones and some corresponding oximes, oxime esters, and related compounds to be studied as candidate cytotoxic agents. All of the compounds were evaluated against murine L1210 lymphoid leukemia cells. In general, cytotoxicity was greatest with the α, β-unsaturated ketones and diminished with the oximes, and the oxime esters had little or no activity in this screen. When the same compounds were examined in both the *in vitro* L1210 and P388 leukemia screens, in the majority of cases the L1210 cells were more sensitive to these derivatives. The cytotoxicity screening and stability studies of representative compounds suggested that the ketones, oximes, and oxime esters were stable under the conditions of bioevaluation. X-ray crystallography of four representative compounds revealed structural features associated with cytotoxicity which may be considered in the design of future candidate cytotoxins (164).

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**. These figures are presented in **Table 10** which reveal that within series **1** and **2**, many of the compounds displayed excellent selectivity. In particular, **1a** rivals doxorubicin in this regard. 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.

The data in **Table 11** reveal 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. 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. α,β-UNSATURATED CYCLIC KETONES

The evaluation of two concentrations of the enones **1**–**10** as MDR reversing agents is summarized in **Table 15**. In this assay, rhodamine 123 is employed and its concentration in treated and untreated cells is compared from which data, fluorescence activity ratio (FAR) values are computed. Compounds with a FAR value of 1 or more indicate that reversal of MDR has taken place. MDR modulators likely bind in the transmembrane domains of P-gp thereby inducing a conformational change in Pgp which inhibits the action of ABC transporters (153).

Firstly, 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). **Figure 9** reveals the differences in the relative locations of not only the aryl rings A and B but the olefinic carbon atoms as well, suggesting that the topography of these molecules impacts considerably on the magnitude of the biological responses.

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.

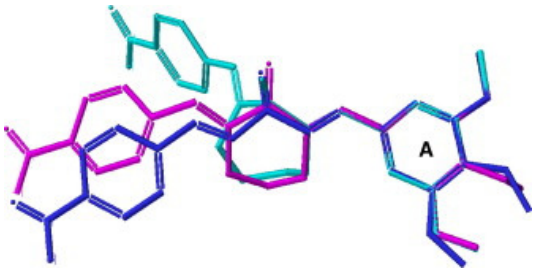


Figure 9. The α,β-unsaturated cyclic ketones: overlap of ring A in **1** (pink), **3** (green), and **4** (blue). Hydrogen atoms were removed for clarity.

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.

Nóra Gyémánt and her coworkers reported synergism between flavonoids and epirubicin on multidrug resistant cancer cells. Chrysin and amorphigenin on the mouse lymphoma cell line and formononetin on the MDA-MB-231 (HTB-26) cell line enhanced the effect of epirubicin synergistically (148). However, contradictory results have been reported concerning the MDR-modulating activity of the flavonoid polyphenols kaempferol and quercetin, which stimulated the P-gp mediated efflux of doxorubicin on the adriamycin-resistant subline of the MCF-7 breast cancer cell line (165).

The overall survival of colon cancer has improved only marginally in recent decades despite advances in surgery and early detection. Potentially curative resection at disease presentation can be performed only in 70–80% of the patients, and overall survival at 5 years is less than 60%. Treatment for advanced colorectal cancer has nevertheless made progress in the last few years. Systemic chemotherapy doubles the survival of these patients compared to untreated controls. For nearly four decades, fluorouracil (5FU) has been the mainstay of treatment. New compounds active against colorectal cancer are now available (166,167,168). 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.

To summarize the novel approaches of multidrug resistance reversal, one group of studies consists of trials designed to inhibit MDR mechanisms in novel ways, the other group focuses on trials to circumvent MDR mechanisms (78).

There are several studies to inhibit mechanisms involved in regulation of MDR transporters. MDR protein gene expression in tumour cells is induced upon treatment with cytotoxic drugs, whereas this gene expression is inhibited by several pharmacological inhibitors that affect the signaling pathways. The above mentioned new resistance modifiers could give a new perspective for the chemotherapy. However, in spite of advances in cancer chemotherapy and developing a few good candidates to modulate MDR, we are still too far to conclude that these agents could be applied clinically. Hopefully, the development of new drugs will continue, and the challenge to improve the effectiveness of cancer chemotherapy will be still the aim of many new *in vitro* and *in vivo* studies in the future.

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 formylchromone 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. The most effective compound in selective toxicity was 1a.

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 **6-(4-nitrophenylmethylene)-2-(3,4,5-trimethoxyphenylmethylene)cyclohexanone** 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 **2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone** 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.

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. The **1a** compound was 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 for the murine lymphoma cells and it demonstrats remarkable selective toxicity to malignant cells and the ability to reverse MDR. The **2b compound** exerted indifferent effect (**FIX: 1.22**) with doxorubicin on mouse lymphoma cells in combination with doxorubicin.

Based on our results obtained in studies with arylidene ketones, a new group of compounds has been developed called **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). 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 **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.

ÖSSZEFOGLALÓ

Az utóbbi évtizedekben a rákos megbetegedések száma drámaian megnőtt. Az európai helyzetet tekintve Magyarországon a legmagasabb a rák mortalitása, és az utóbbi negyven év egyre romló tendenciát mutat. Ha a rákos sejteket hosszú ideig nem a hatásos koncentrációjú rákellenes szerrel kezelik, a sejtek nemcsak az adott szerre, hanem más kemoterápiás szerre is rezisztenssé válhatnak. Így ha egy adott kemoterápiás szerre kifejlődött a rezisztencia, ez sok esetben több kemoterápiás szerre rezisztens fenotípust eredményez.

A **tumorsejtek drogrezisztenciájának** fő mechanizmusai a következők: csökkent drogfelhalmozódás az efflux pumpák következtében, detoxifikációhoz kapcsolt rezisztencia, a gyógyszer célmolekulájának vagy a javítómechanizmusoknak a megváltozása, az apoptózist befolyásoló gének megváltozása. Vizsgálataink során a multidrog rezisztencia legfőbb mechanizmusát, az efflux pumpák működését tanulmányoztuk. Ebben az esetben az adott gyógyszert aktív transzporttal távolítja el a rákos sejt. Tumorsejt vonalakban a multidrog rezisztencia ATP-függő, csökkent celluláris drogakkumulációhoz kapcsolt, amelyet eredetileg egyetlen fehérje, a 170-kDa nagyságú **ABC transzporter, a P-glikoprotein** overexpressziójának tulajdonítottak, melyet az emberi MDR1 gén kódol.

A dolgozat fő célja az MDR1 efflux pumpák új, hatékony módosítóinak vizsgálata, amelyek megnövelik a sejten belüli gyógyszerfelhalmozódást az efflux pumpák módosítása révén. Azt találtuk, hogy néhány **formil-chromon** származék módosítja a P-glikoprotein működését. Ezek közül a **3-formyl-6-isopropylchromone** származék a leghatékonyabb, mert módosítja a humán MDR1 génnel transzfektált egér limfóma sejtek és az emberi Colo 320 vastagbél karcinóma sejtek multidrog rezisztenciáját is.

A **6,8-dibromo-3-formylchromone** származék szinergizmust mutatott doxorubicinnel humán MDR1 génnel transzfektált egér limfóma sejteken.

A másik vizsgált vegyületcsoport, a **konjugált arilidén ketonok** jelentősen gátolták az emberi MDR1 génnel transzfektált egér limfóma sejtek P-glikoproteinhez kapcsolt efflux mechanizmusát. **Conjugated arylidene ketones** displayed a remarkable inhibition of the MDR of human MDR1 gene-transmitted mouse lymphoma cells. The **1a** compound was 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 for the murine lymphoma cells and it demonstrats remarkable selective toxicity to malignant cells and the ability to reverse MDR. The **2b compound** exerted indifferent effect (**FIX: 1.22**) with doxorubicin on mouse lymphoma cells in combination with doxorubicin.

Az arilidén ketonokkal kapcsolatos kísérleti eredmények alapján új vegyületcsoport került kifejlesztésre, az **α,β-telítetlen ciklikus ketonok** csoportja. Az eredmények alapján az 1 származék tűnt a legígéretesebbnek, mert a négy szájüregi daganatos sejtvonallal szemben a leghatásosabbnak bizonyult, míg a normál szájüregi sejteketre nem fejtett ki hatást.

Több α,β-telítetlen ciklikus keton hatékony antiproliferatív tulajdonsággal rendelkezett humán MDR1 génnel transzfektált egér limfóma sejteken és az emberi Colo 320 vastagbél karcinóma sejteken, valamint koncentrációfüggést mutatva gátolták az egér limfóma sejtek P-glikoproteinhez kapcsolt rezisztenciáját. Azt találtuk, hogy a **2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone származék** szinergizmust mutatott doxorubicinnel emberi Colo 320 sejteken.

Kijelenthetjük, hogy a 3-formil-chromonok, a konjugált arilidén ketonok és az α,β-telítetlen ciklikus ketonok új lehetőséget teremthetnek rákellenes gyógyszerek kifejlesztésére**.**

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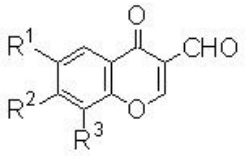
ANNEX

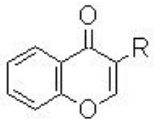
CHEMICAL STRUCTURES OF COMPOUNDS STUDIED

1. 3-FORMYLCHROMONE DERIVATIVES



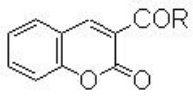
1-11

Compd	R^1	R^2	R^3	Calcd log P
1	H	H	H	0.35
2	CH_3	H	H	0.86
3	i-Pr	H	H	1.76
4	CH_3O	H	H	0.56
5	NO_2	H	H	-
6	F	H	H	0.60
7	Cl	H	H	1.17
8	Br	H	H	1.32
9	Cl	CH_3	H	1.67
10	Cl	H	Cl	1.91
11	Br	H	Br	2.21



12, 13

Compd	R	Calcd log P
12	CN	0.45
13	H	1.01



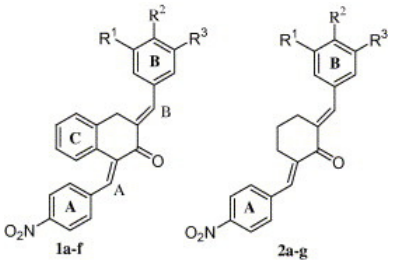
14-16

Compd	R	Calcd log P
14	CH_3	1.15
15	OH	1.54
16	OC_2H_5	1.91

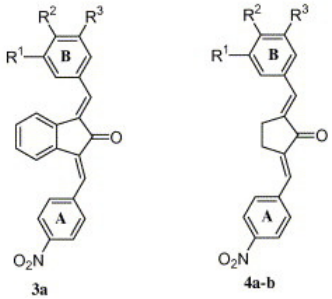
Figure. 1. Chemical structures and calculated log P values of chromone (**1**-**13**) and coumarin (**14**-**16**) derivatives.

3-formylchromone (**FC1**)(MW=174), 3-formyl-6-methylchromone (**FC2**)(MW=188), 3-formyl-6-isopropylchromone (**FC3**)(MW=216), 3-formyl-6-nitrochromone (**FC5**)(MW=219), 6-chloro-3-formylchromone (**FC7**)(MW=208.5), 6-bromo-3-formylchromone **(FC8**)(MW=253), 6-chloro-3-formyl-7-methylchromone (**FC9**)(MW=222.5), 6,8-dichloro-3-formylchromone (**FC10**)(MW=243), 6,8-dibromo-3-formylchromone (**FC11**)(MW=332), 3-cyanochromone (**FC12**)(MW=171), chromone (**FC13**)(MW=146), 3-acetylcoumarin (**FC14**)(MW=188), coumarin-3-carboxylic acid (**FC15**)(MW=190) and ethyl coumarin-3-carboxylate (**FC16**)(MW=218) (Aldrich Chemical Co. Inc. Milwaukee, USA),
6-Methoxy-(**FC4**)(MW=204) and 6-Fluoro-3-formylchromone (**FC6**)(MW=192) were synthesized as described in the literature (169)

2. CONJUGATED ARYLIDENE KETONES



1a-f 2a-g



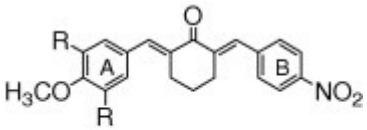
3a 4a-b

Compound	Aryl substituents[a]			
	R^1	R^2	R^3	$\Delta\sigma$[b]
1a	OCH_3	OCH_3	OCH_3	0.81
1b	OCH_3	OCH_3		0.93
1c	OCH_3	OH		1.03
1d		OCH_3		1.05
1e	OCH_2O			1.10
1f		OH		1.15
2a	OCH_3	OCH_3	OCH_3	0.81
2b	OCH_3	OCH_3		0.93
2c	OCH_3	OH		1.03
2d		OCH_3		1.05
2e	OCH_2O			1.10
2f		OH		1.15
2g		Cl		0.55
3a	OCH_3	OCH_3	OCH_3	0.81
4a	OCH_3	OCH_3	OCH_3	0.81
4b		OCH_3		1.05

The aryl substituents of the compounds in series **1**–**4**

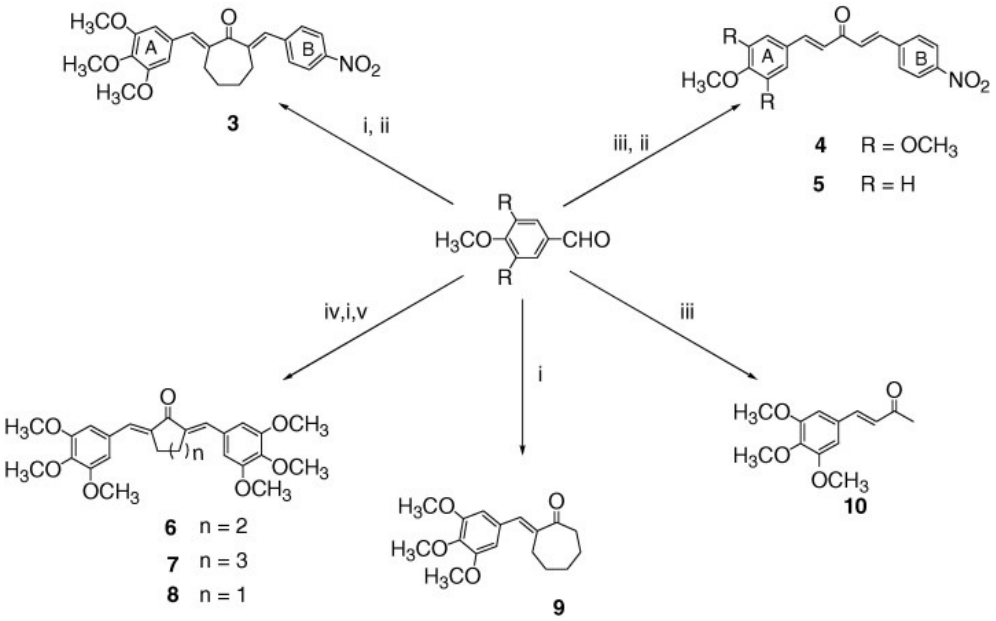
[a] For clarity, hydrogen atoms are omitted.
[b] $\Delta\sigma$ refers to the differences in the σ values between the substituents in rings A and B in series **1**–**4**. The sigma values used in these calculations were taken from (139).

3. α,β-UNSATURATED CYCLIC KETONES



1 R = OCH_3
2 R = H

6-(4-nitrophenylmethylene)-2-(3,4,5-trimethoxyphenylmethylene)cyclohexanone **1**
2-(4-Methoxyphenylmethylene)-6-(4-nitrophenylmethylene)-cyclohexanone **2**



The synthetic chemical scheme used in preparing **3–10**. The reagents used were as follows, i, cycloheptanone/NaOH; ii, 4-nitrobenzaldehyde/NaOH; iii, acetone/NaOH; iv, cyclohexanone/HCl; v, cyclopentanone/HCl.

2-(4-Nitrophenylmethylene)-7-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone (**3**)
1-(4-Nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-1,4-pentadien-3-one (**4**)
1-(4-Methoxyphenyl)-5-(4-nitrophenyl)-1,4-pentadien-3-one (**5**)
2,6-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclohexanone (**6**)
2,7-Bis-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone (**7**)
2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone (**8**)
2-(3,4,5-Trimethoxyphenylmethylene)-cycloheptanone (**9**)
4-(3,4,5-Trimethoxyphenyl)-3-buten-2-one (**10**)

PUBLICATIONS