in vivo *20*: 645-650 (2006)

Multidrug Resistance Reversal by 3-Formylchromones in Human Colon Cancer and Human *mdr1* Gene-transfected Mouse Lymphoma Cells

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Abstract. *Several new 3-formylchromone derivatives proved to be modifiers of multidrug resistance in mouse lymphoma cells and in human Colo320 colon cancer cells. There is apparently a structure-activity relationship between the antiproliferative multidrug resistance-reversing effect and the chemical structure of the 3-formylchromones. The total polar surface areas and the ground state dipole moments of the molecules are presumed to play a key role in the multidrug resistance-reversing effect. The log P values can provide an adequate explanation for the selective cytotoxicity against cancer cells.*

Broad-spectrum resistance to chemotherapeutic agents in human cancer has been termed multidrug resistance (MDR). The resistance of tumor cells to multiple chemotherapeutic drugs is a barrier to the treatment of human cancer. The main cause of this resistance is mediated by the overexpression of a 170 kDa integral plasma membrane protein, known as P-glycoprotein (Pgp). It has been suggested that Pgp operates as a drug efflux pump for numerous structurally unrelated natural or synthetic chemotherapeutic drugs (1).

Pgp can be found in normal cells, where it is responsible for maintaining physiological barriers. In normal tissues, the distribution of Pgp is restricted to various organs, such as the blood brain-barrier, adrenal cortex, the bile canaliculus, the small intestine, the colon and the proximal renal tubules. It functions as an efflux detoxification pump that extrudes hormones and xenobiotics (2). However, there are some differences between the sensitivities of the normal efflux pump to resistance modifiers, such as phenothiazines and lectins in human brain capillary endothelial and MDR cancer cells (3).

The physiological role of Pgp is known to operate through lipid flippase activity (4, 5). Inhibition of the activity of this efflux mechanism results in the reversal of MDR. The question arises as to how MDR cells become hypersensitized to chemotherapeutics delivered together with MDR modifiers. There is a possibility that the internalization of lipophilic carotenes reduces the biological function of the MDR and sensitive cells (6). A second possibility is that the cytoplasmic vesicles of MDR cells are more easily permeabilized by resistance modifiers than vesicles in sensitive cells in which doxorubicin, or other anticancer drugs accumulate (24).

MDR can be reversed by co-treatment of resistant cells with nontoxic concentrations of hydrophobic or hydrophilic drugs known to be chemosensitizers with certain values of total polar surface area (TPSA) (16, 24). As chemosensitizers are positively charged hydrophobic compounds, which affect biological membranes, MDR chemosensitizers induce substantial changes in the cell membrane, such as in the critical micelle concentrations of detergents. Due to this fact, the modulators not only reverse MDR in Pgp-overexpressing cells, but potentiate the cytotoxicity of anticancer drugs in wild-type cells that are devoid of Pgp. On the basis of our previous studies, a number of cyclic ketones and structurally related chromones with different electron densities were prepared (7), which permit charge transfer or micellar-type interactions with cellular proteins, including Pgp.

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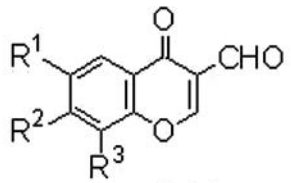
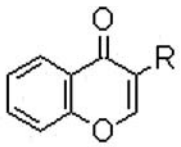
Key Words: Formylchromones, multidrug resistance-reversing effect.



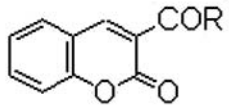
1-11

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.*

In the present paper, the selective toxicity of some 3-formylchromones and related compounds on MDR human colon cancer and mouse lymphoma cells transfected with the human MDR1 gene, based on antiproliferative and MDR-reversal effects, is reported.

Materials and Methods

Tested compounds and chemicals (Figure 1). 3-Formylchromone (**1**) (MW=174), 3-formyl-6-methylchromone (**2**) (MW=188), 3-formyl-6-isopropylchromone (**3**) (MW=216), 3-formyl-6-nitrochromone (**5**) (MW=219), 6-chloro-3-formylchromone (**7**) (MW=208.5), 6-bromo-3-formylchromone (**8**) (MW=253), 6-chloro-3-formyl-7-methylchromone (**9**) (MW=222.5), 6,8-dichloro-3-formylchromone (**10**) (MW=243), 6,8-dibromo-3-formylchromone (**11**) (MW=332), 3-cyanochromone (**12**) (MW=171), chromone (**13**) (MW=146), 3-acetylcoumarin (**14**) (MW=188), coumarin-3-carboxylic acid (**15**) (MW=190) and ethyl coumarin-3-carboxylate (**16**) (MW=218) were obtained from Aldrich Chemical Co. Inc. Milwaukee, USA.

6-Methoxy-3-formylchromone (**4**) (MW=204) and 6-fluoro-3-formylchromone (**6**) (MW=192) were synthetized as described in the literature (10).

The following compounds were purchased from the corresponding suppliers: rhodamine 123 (Sigma, St Louis, MO, USA); verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary); MTT (thiazolyl blue; Sigma); SDS (sodium dodecylsulfate; Sigma); and doxorubicin (Ebewe, Austria).

Cell cultures

a) L5178 mouse T-cell lymphoma cells. The cells were transfected with pHa MDR1/A retrovirus, as previously described (8). The *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.

b) Human colon cancer cells (COLO320). The cells 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 semi-adherent human colon cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C.

Assay for reversal of MDR in tumor cells. The cells were adjusted to a density of $2x10^6$/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 were added to the samples and the cells were incubated for an additional 20 min at 37˚C, were washed twice and were resuspended

Table I. *Antiproliferative effects of tested compounds on mouse lymphoma cells transfected with the human mdr1 gene.*

Compounds	ID_{50}
1	5.76
2	4.27
3	1.9
4	5.04
5	36.76
6	7.1
7	7.13
8	4.84
9	4.95
10	6.4
11	5.97
12	45.84
13	41.87
14	40.75
15	79.14
16	21.42

Table II. *Antiproliferative effects of tested compounds on the Colo 320 colon cancer cell line.*

Compounds	ID_{50}
1	10.53
2	3.83
3	8.70
4	10.61
5	4.32
6	4.43
7	2.07
8	2.68
9	3.31
10	2.02
11	18.95
12	84.61
13	19.43
14	15.88
15	26.37
16	30.15

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 (FAR) was calculated *via* the following formula (9), on the basis of the measured fluorescence values:

$$FAR = \frac{MDR_{treated}/MDR_{control}}{parental\ treated/parental\ control}$$

Determination of log P values. The log P values were calculated by CLOGP (Pomona College Medicinal Chemistry Project, Claremont, CA, USA).

Results

When the antiproliferative effects of the tested compounds were measured on MDR mouse lymphoma cells, three different groups could be distinguished on the basis of the ID_{50} values. In the first group, **3,** with an ID_{50}=1.9 μg/ ml, was the most active. The compounds in the second group, **1, 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, **5** and **12-16**, had very high ID_{50} values in the range from 21.42-79.14 μg/ml (Table I).

The effects of the tested compounds on the proliferation of human colon cancer cells were also investigated. The compounds **2**, **3** and **5-10** exhibited a moderate antiproliferative effect, as measured on Colo 320 cells (ID_{50}=2.02-8.70 μg/ml). The compounds **1, 4, 11** and **13-16** were less effective, with ID_{50} values in the range 10.53-84.61 μg/ml (Table II).

A further investigation of 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 III). The most effective compounds proved to be **1, 3, 4, 6, 7, 8, 10** and **11**, while compounds **2** and **5** were hardly effective. Compounds **3** and **9** at low concentration were effective, but were toxic at 40 μg/ml. Compounds **12-16** were ineffective in the mouse lymphoma cells transfected with the human *mdr1* gene.

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 (Figure 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 **1, 6, 7, 8, 9, 10** and **11**, while compounds **2** and **4** were moderately effective. Compounds **3, 12, 13, 14, 15, 16** were ineffective (Table IV). In the case of colon cancer cells, the effects of these compounds depended mainly on the electron-withdrawing substituent at position 6. Compounds **2** and **4** were exceptions (Table IV). A bulky substituent at position 3 resulted in ineffective compounds, *e.g.* **12-16**.

Discussion

The cell efflux pump, involving the ABC transporters, can be regarded as an enzyme with extremely wide substrate specificity. Inhibition of the ABC transporters of tumor cells

Table III. *Effects of tested compounds on the activity of the MDR efflux pump of mouse lymphoma cells transfected with the human mdr1 gene.*

Compounds	μg/ml	Fluorescence activity ratio (FAR)
1	4	2.05
	40	23.25
2	4	1.25
	40	4.21
3	4	11.76
	40	toxic
4	4	1.36
	40	23.89
5	4	1.11
	40	2.72
6	4	1.67
	40	19.59
7	4	6.25
	40	26.44
8	4	6.65
	40	48.35
9	4	7.69
	40	toxic
10	4	1.37
	40	15.90
11	4	1.37
	40	27.38
12	4	0.79
	40	0.95
13	4	0.66
	40	1.01
14	4	0.64
	40	1.07
15	4	0.78
	40	0.73
16	4	0.63
	40	1.05

Table IV. *Effects of the tested compounds on rhodamine accumulation in Colo 320 colon cancer cells.*

Compounds	μg/ml	Fluorescence activity ratio (FAR)
1	4	2.92
	40	3.89
2	4	2.72
	40	2.02
3	4	1.52
	40	0.78
4	4	1.08
	40	2.13
5	4	0.98
	40	1.79
6	4	2.41
	40	3.64
7	4	4.69
	40	4.53
8	4	4.83
	40	5.41
9	4	4.56
	40	2.70
10	4	3.13
	40	3.29
11	4	2.01
	40	4.31
12	4	0.74
	40	0.69
13	4	0.69
	40	0.51
14	4	0.78
	40	0.39
15	4	0.84
	40	1.11
16	4	1.13
	40	1.36

is a promising opportunity to overcome MDR. 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 (11-18) and synthetic molecules have been shown to block the MDR pump efflux activity (19-23). The synthetic molecules include phenothiazines, aza-oxafluorene, acridine, coumarin and naphthyridine derivatives.

The effect of various arylidine derivatives were recently studied on the proliferation and drug accumulation of cancer cells, normal gingival fibroblast and other cells. Some of these compounds displayed noteworthy inhibition on normal and malignant cells isolated from human oral tissues and MDR mouse lymphoma cells *in vitro* (7).

The normal gingival fibroblastic cell line, however, was not affected (7). Regarding the antiproliferative effects of the 3-formylchromone derivatives, the relevant ID_{50} values are presented in Tables I and II. It is interesting to note that the substituents had a great influence on the antiproliferative effect. The results of the antiproliferative assay revealed the most effective compounds against the mouse lymphoma cells as **3, 8, 9** and **11** (Table I) and against human colon cancer cells as **2, 5, 6, 7, 8, 9** and **10** (Table II). 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 with the rhodamine 123 accumulation assay. The most effective compounds against mouse lymphoma cells were **1, 3, 4, 6, 7, 8, 10, 11** (Table III), while those compounds found to be very effective against the Colo320 cancer cells were **1, 4, 6, 7, 8, 9, 10** and **11** (Table IV).

A special ground state dipole moment is probably important for the biological effect of the above compounds on Pgp. Additionally, the TPSA of the highly lipophilic compounds must be taken into consideration. The present *in vitro* results provide a basis for future *in vivo* experiments.

Acknowledgements

This study was supported by the Szeged Cancer Research Foundation (Szegedi Rákkutatásért Alapítvány), the COST B16 action of the European Commission and a Japanese source. The authors are also grateful for the skillful technical assistance with the flowcytometry of Mrs. A. Váradi.

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Received March 29, 2006
Accepted July 7, 2006