in vivo *20*: 119-124 (2006)

Cinnamylidene Ketones as Potential Modulators of Multidrug Resistance in Mouse Lymphoma and Human Colon Cancer Cell Lines

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Abstract. *The resistance to chemotherapy of cancer cells is mediated by the overexpression of P-glycoprotein, as an ATP-dependent membrane efflux pump. Two families of compounds have been screened, the cinnamylidenecycloalkanones and cinnamylidenebenzocycloalkanones, as promising multidrug resistance (MDR) reversal agents on mouse lymphoma and human colon cancer (COLO320) cell lines. The antiproliferative effects of the cinnamylidene derivatives were tested with the MTT method. The MDR effect on drug accumulation was tested by flow cytometry. Combinations of resistance modifiers and cytostatics were tested on the two cell lines to obtain evidence for additive or synergistic interactions. Verapamil was applied as a resistance-modifying positive control. The best effects in the reversal of MDR in both cell lines were exhibited by the methoxy derivatives 2-(2-methoxycinnamylidene)indan-1-one, 2-(2-methoxycinnamylidene)-3,4-dihydro-2H-naphthalen-1-one, 6-(2-methoxycinnamylidene)-6,7,8,9-tetrahydrocyclohepten-5-one), 2-cinnamylidene-3,4-dihydro-2H-naphthalen-1-one and 6-cinnamylidene-6,7,8,9-tetrahydrobenzocyclohepten-5-one. 2-(2-methoxycinnamylidene) indan-1-one and 2-(2-methoxy-cinnamylidene)-3,4-dihydro-2H-naphthalen-1-one were able to enhance the antiproliferative activity of doxorubicin in a synergistic way.*

Hopes for curing most cancers lie in systematic treatments such as chemotherapy and immunotherapy (1). Approximately 40% of cancer patients can be treated surgically, and the great majority of patients need chemotherapy. However, the effectiveness of chemotherapy is limited by the emergence of multidrug resistance (MDR) (2). Resistance to anticancer drugs is often mediated by the overexpression of a membrane pump called P-glycoprotein (P-gp), while MDR is encoded by a gene, *mdr1* (3).

P-gp was first reported by Juliano and Ling in 1976 (4). It functions as an ATP-dependent membrane efflux pump that reduces the intracellular drug concentration to below cytotoxic levels (5). P-gp is also present in various normal cells (*e.g.* the capillary endothelium in brain, colon, kidney and liver cells) and is associated with physiological functions. In these tissues, P-gp is responsible for the transport of toxic compounds into the cerebrospinal fluid, bile, urine, *etc*. (2).

The 170 kDa P-gp is a member of a superfamily of ABC transporters that share a high degree of homology with a large number of both prokaryotic and eukaryotic membrane transport proteins. Other ABC transport proteins that have been implicated in MDR include the MRP-associated proteins (MRP1–5), the lung resistance protein (LRP) and the breast cancer resistance protein (BCRP). Tumours may co-express several of these transport proteins (6, 7).

A variety of studies have been performed to find MDR modulators which, in combination with anticancer drugs, increase the anticancer effect (8, 9). The proposed mechanism of action of the chemosensitizers is *via* binding to P-gp, by antagonizing the binding of the anticancer drugs or by inhibiting their efflux from the cells (10, 11).

Potent P-gp inhibitors are being investigated in clinical trials, including calcium channel blockers such as verapamil and immunosuppressants such as cyclosporin A (12). However, clinical application has not been attained to date

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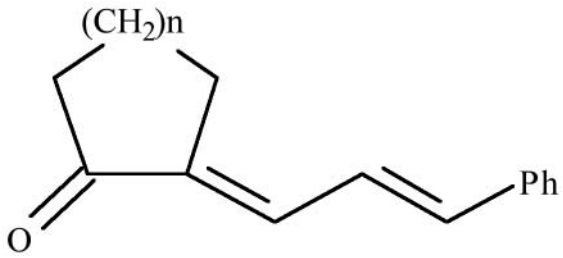
Key Words: Multidrug resistance, MDR reversal, efflux pump, P-glycoprotein, cinnamylidene ketones.

because of the toxicity, undesirable side-effects and/or low efficacy of the resistance modifiers in the host organism (13).

To improve the prospects of treatment, we decided to investigate the MDR reversal of the newly-synthesized cinnamylidene ketones, which are similar to carotenoids. The cytotoxic effects of some cinnamylidene ketones and their derivatives against P388 lymphocytic leukaemia and murine L1210 lymphoid leukaemia were described by Dimmock *et al.* (14, 15). The aim of this study was to examine the potential of the cinnamylidenecycloalkanones (**1,2**) and cinnamylidenebenzocycloalkanones (**3-16**) as MDR-reversing agents, and to compare the drug accumulation of a human *mdr1* gene-transfected mouse lymphoma cell line and that of a human colon cancer (COLO320) cell line *in vitro* in the presence of cinnamylidene ketones.



Compound	n
1	1
2	2

Figure 1. *Chemical structures of cinnamylidenecycloalkanones.*

Materials and Methods

Tested compounds and chemicals. The cinnamylidenecycloalkanones and cinnamylidenebenzocycloalkanones tested were prepared according to literature methods (16): **1**: 2-cinnamylidenecyclopentanone; **2**: 2-cinnamylidenecyclohexanone; **3**: 2-cinnamylideneindan-1-one; **4**: 2-(2-methoxycinnamylidene)indan-1-one; **5**: 2-(2-nitrocinnamylidene)indan-1-one; **6**: 2-(β-methylcinnamylidene)indan-1-one; **7**: 2-cinnamylidene-3,4-dihydro-2H-naphthalen-1-one; **8**: 2-(2-methoxycinnamylidene)-3,4-dihydro-2H-naphthalen-1-one; **9**: 2-(2-nitrocinnamylidene)-3,4-dihydro-2H-naphthalen-1-one; **10**: 6-cinnamylidene-6,7,8,9-tetrahydrobenzocyclohepten-5-one; **11**: 6-(2-methoxycinnamylidene)-6,7,8,9-tetrahydrocyclohepten-5-one; **12**: 6-(2-nitrocinnamylidene)-6,7,8,9-tetrahydrobenzocyclohepten-5-one; **13**: 3-cinnamylidene-chromanone; **14**: 3-cinnamylidene-1-thiochromanone; **15**: 3-cinnamylideneflavanone; and **16**: 3-cinnamylidene-1-thioflavanone (Figures 1-3). For administration, they were dissolved in DMSO (dimethyl sulphoxide). Other chemicals used were: 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. L5178 mouse T-cell lymphoma cells were transfected with pHa MDR1/A retrovirus, as previously described (17). *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.

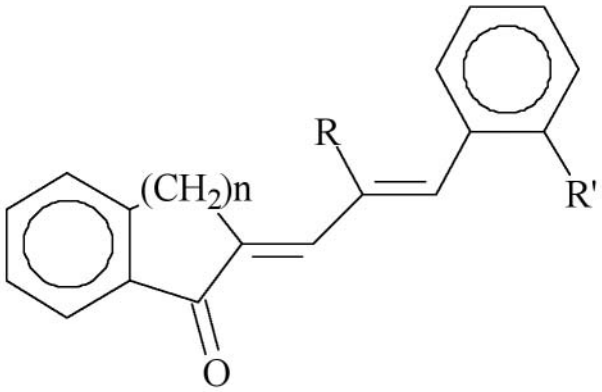
The human colon cancer cells (COLO320) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cell lines were incubated in a humidified atmosphere (5% CO_2, 95% air) at 37˚C. The semi-adherent human colon cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C.

Assay for reversal of MDR in tumour 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 test 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 mM 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 phosphate-buffered saline (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 equation (18), 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 microtitre plates. The compounds were diluted in a volume of 50 μL. Then, $1x10^4$ 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 mL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37˚C for 4 h, 100 μL of SDS solution (10%) was measured into each well and the plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

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



Comp.	n	R	R'
3	1	H	H
4	1	H	OMe
5	1	H	NO_2
6	1	Me	H
7	2	H	H
8	2	H	OMe
9	2	H	NO_2
10	3	H	H
11	3	H	OMe
12	3	H	NO_2

Figure 2. *Chemical structures of cinnamylidenebenzocycloalkanones.*

Checkerboard microplate method. The microplate method was applied to study the effects of drug interactions between resistance modifiers and doxorubicin on cancer cells.

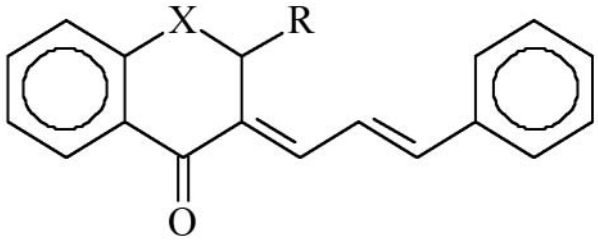
The effects of the anticancer drug doxorubicin and the resistance modifiers in combination were studied on various cancer cell lines. The dilutions of doxorubicin (A) were made in a horizontal direction, and the dilutions of resistance modifiers (B) vertically in the microtitre plate, in a volume of 100 μL. The cell suspension in the tissue culture medium was distributed into each well in 100 mL containing $5x10^4$ cells. The plates were incubated for 48 h at 37˚C in a CO_2-incubator. The cell growth rate was determined after MTT staining and the intensity of the blue colour was measured on a micro ELISA reader. Drug interactions were evaluated according to the following system:

$FICA = ID_{50A \text{ in combination}} / ID_{50A \text{ alone}}$
$FICB = ID_{50B \text{ in combination}} / ID_{50B \text{ alone}}$

ID = inhibitory dose
FIC = fractional inhibitory concentration:

	$FIX = FIC_A + FIC_B$
FIX = 0.51-1	additive effect
FIX < 0.5	synergism
1 < FIX < 2	indifferent effect
FIX > 2	antagonism

FIX = fractional inhibitory index



Comp.	X	R
13	O	H
14	S	H
15	O	Ph
16	S	Ph

Figure 3. *Chemical structures of cinnamylidenebenzocycloalkanones.*

Table I. *Comparison of the antiproliferative effects of cinnamylidene ketones on PAR/ MDR and COLO320 cell lines.*

Samples	MDR ID_{50} (nmol/mL)	PAR ID_{50} (nmol/mL)	COLO320 ID_{50} (nmol/mL)
1	499	303	54.6
2	206	193	28.3
3	13.4	47.8	3.05
4	177	93.7	60.8
5	34.2	69.3	17.3
6	8.88	251	33.5
7	58.4	343	27.1
8	128	251	29.6
9	32.5	72.8	20.8
10	188	171	32.6
11	13.1	222	15.1
12	39.7	104	29.3
13	200	238	50.4
14	17.5	48.5	2.44
15	44.8	192	9.19
16	48.9	198	48.0
DMSO	299	672	145

Results

The antiproliferative effects of cinnamylidene derivatives. The antiproliferative effects of cinnamylidene derivatives **1-16** were first tested and compared on doxorubicin-sensitive mouse lymphoma cells (PAR), the multidrug-resistant subline (MDR) and the human colon cancer cell line (COLO320) (Table I).

It was found that the 16 tested compounds exerted antiproliferative effects on the 3 cell lines. The effect

Table II. *Reversal of multidrug resistance in mouse lymphoma cells by cinnamylidene ketones.*

Samples	nmol/mL	Fluorescence activity ratio
PAR	-	139.98
PAR	-	131.74
MDR	-	1.105
MDR mean	-	0.995
Verapamil	11.0	4.235
1	20.2	1.60
	202	18.94
2	18.8	1.32
	188	23.61
3	16.2	2.66
	162	14.60
4	14.5	4.19
	145	32.73
5	13.7	1.90
	137	2.97
6	15.4	1.13
	154	2.05
7	15.4	3.09
	154	26.17
8	13.8	8.32
	138	61.30
9	13.1	1.16
	131	1.60
10	14.6	3.28
	146	27.34
11	13.1	10.53
	131	19.86
12	12.5	6.52
	125	12.34
13	15.3	1.09
	153	2.33
14	14.4	1.82
	144	7.73
15	11.8	8.50
	118	13.51
16	11.3	2.09
	113	2.18
DMSO control	256	0.95
MDR	-	0.885

Table III. *Reversal of multidrug resistance in human colon cancer cells by cinnamylidene ketones.*

Samples	nmol/mL	Fluorescence activity ratio
COLO320 + verapamil + R123	11.0	4.245
1	20.2	0.75
	202	8.08
2	18.8	0.80
	188	3.16
3	16.2	1.95
	162	13.20
4	14.5	3.36
	145	13.06
5	13.7	0.44
	137	0.51
6	15.4	0.66
	154	1.38
7	15.4	1.65
	154	20.03
8	13.8	4.27
	138	16.05
9	13.1	0.73
	131	0.74
10	14.6	2.19
	146	15.08
11	13.1	1.56
	131	12.42
12	12.5	3.24
	125	3.89
13	15.3	0.86
	153	1.35
14	14.4	1.89
	144	18.37
15	11.8	2.27
	118	6.79
16	11.3	1.58
	113	2.07
DMSO control	256	0.84

depended on the chemical structure. There are some interesting compounds, *e.g.*: **1, 2, 4** and **10**, which had higher ID_{50} values for the MDR cells than for the PAR cell line. Apparently, these compounds were less effective on the MDR cells than on the PAR cell line. In some other cases, the compounds had much higher ID_{50} values on the PAR mouse lymphoma cells than on the MDR cells, *e.g.*: **3, 5, 6, 7, 8, 9, 11, 12, 14, 15** and **16** (Table I). It is interesting that a majority of the compounds had nearly the same ID_{50} values on the COLO320 cells, with a few exceptions, such as **3** and **14**. In the case of the COLO320 cell line, compounds **3** and **14** were most effective. The studies indicated that COLO320 has a moderate sensitivity to the antiproliferative effects of the cinnamylidene ketones.

Reversal of MDR in tumour cells. On the basis of the antiproliferative activity results, the drug accumulation of the mouse lymphoma and human colon cancer cells were systematically investigated by flow cytometry. Verapamil was used as a positive control in the experiments.

Some of the compounds were able to enhance the drug accumulation of MDR cells markedly, *e.g.*: **1, 2, 4, 7, 8, 10** and **11** (Table II). The compounds displayed dose-dependent inhibition of the MDR P-gp. At the same time, certain cinnamylidene derivatives (**5, 6, 9, 13** and **16**) were hardly effective in inhibiting the naturally occurring MDR

Table IV. *Interaction between resistance modifier cinnamylidene ketones with doxorubicin on a mouse lymphoma cell line.*

Samples	FIX values	Comments
1	0.83	Additive
2	0.88	Additive
3	1.26	Indifference
4	0.36	Synergism
7	1.20	Indifference
8	0.41	Synergism
10	1.18	Indifference
11	1.04	Indifference
14	1.11	Indifference

FIX, fractional inhibitory index.

Table V. *Interaction between resistance modifier cinnamylidene ketones with doxorubicin on a human colon cancer cell line.*

Samples	FIX values	Comments
1	0.56	Additive
2	0.99	Additive
3	1.18	Indifference
4	0.41	Synergism
7	1.03	Indifference
8	0.36	Synergism
10	0.74	Additive
11	1.14	Indifference
14	1.16	Indifference

FIX, fractional inhibitory index.

of the mouse lymphoma cells. Compounds **3, 12, 14** and **15** were only moderately effective on the MDR cells.

When the cinnamylidene ketones were tested for the reversal of MDR in the human colon cancer cells, three groups could be distinguished, as may be seen in Table III. The first group contained the most effective compounds, **1, 3, 4, 7, 8, 10, 11** and **14**. The compounds in the second group, **5, 6, 9** and **12**, were practically ineffective. In the third group, moderate increases in drug accumulation were found in the presence of compounds **2, 13, 15** and **16**.

Combined chemotherapy in checkerboard microplate method. As a result of the MDR-reversing experiments, some very effective resistance modifier cinnamylidene ketones were selected for combined chemotherapy in the checkerboard microplate method. The results can be seen in Tables IV and V.

In these experiments, various concentrations of the most effective MDR-reversing cinnamylidene ketones (**1, 2, 3, 4, 7, 8, 10, 11** and **14**) and doxorubicin were tested in combination. In the mouse lymphoma MDR cells, strong synergy was found between doxorubicin and compounds **4** and **8**. It was interesting that the FIX values of the cinnamylidenecycloalkanones **1** and **2** revealed additive effects, which were not shown for compounds **3, 7, 10, 11** and **14** in combination with doxorubicin (Table IV).

When the same experiment was performed with COLO320 cells, only compounds **4** and **8** were able to enhance the antiproliferative activity of doxorubicin, compounds **1, 2** and **10** were only marginally additive, while compounds **3, 7, 11** and **14** did not show any additive effect in combination with the anticancer drug (Table V).

Discussion

This work has demonstrated that some cinnamylidene compounds have MDR reversal effects. The transported substrates of P-gp 170 form a wide group, ranging from a large polypeptide (HlyB) to a simple ion ("cystic fibrosis transmembrane conductance regulator", CFTR), and include sugars (MalEFGK and AraFGH), amino acids (HisJMPQ and GlnHPQ) and cytotoxic drugs. As shown above, the main cause for the inefficiency of many modulators is their behaviour as "pseudo-substrates" (19). A large number of chemically unrelated compounds, ranging from calcium channel blockers (20) to various naturally occurring compounds in plants (21), are able to block the MDR efflux pump.

The indanone derivative (**3**) and the thiochromanone derivative (**14**) exhibited almost the lowest ID_{50} values for the three cell lines in antiproliferative experiments. Several compounds had rather low ID_{50} values against two different cell lines. As regards the activity against the COLO320 cell line, this was not influenced greatly by the structures of the compounds examined, but the tendency was the same for all three cell lines. Some ketones (**1, 2, 4** and **10**) have higher ID_{50} values for the MDR cells than the mouse lymphoma cell line. It is possible that these compounds are substrates of the P-gp-mediated efflux pump. Cinnamylidene ketones, which have much higher ID_{50} values on the PAR cell line than on the MDR cells, are especially promising for the reversal of MDR, because they cannot be substrates for the ABC transporters, based on the ID_{50} differences between the two cell lines.

The degree of drug accumulation was lower for the COLO320 cell line than the mouse lymphoma cells. The MDR reversal experiments indicated that **4** (2-(2-methoxycinnamylidene)indan-1-one), **7** (2-cinnamylidene-3,4-dihydro-2H-naphthalen-1-one), **8** (2-(2-methoxycinnamylidene)-3,4-dihydro-2H-naphthalen-1-one), **10** (6-cinnamylidene-6,7,8,9-tetrahydrobenzocyclohepten-5-one) and **11** (6-(2-methoxycinnamylidene)-6,7,8,9-tetrahydrocyclohepten-5-one) increased the drug accumulation, whereas compounds **5, 6, 9** and **13** were less effective in both cell lines as compared with the selected verapamil control, which had no structural relationship to the tested cinnamylidene

ketones. Although **7** (2-cinnamylidene-3,4-dihydro-2H-naphthalen-1-one) and **11** (6-(2-methoxycinnamylidene)-6,7,8,9-tetrahydrocyclohepten-5-one) significantly increased the rhodamine 123 drug accumulation, in combination with doxorubicin they had no antiproliferative effect on the two tested cell lines. The differences between the dose-dependent antiproliferative and the MDR reversal effects of the cinnamylidene derivatives may be consequences of the various experimental procedures, including the different incubation times.

The results of these checkerboard experiments lead us to suppose that the reversal of MDR depends on the chemical structure of the resistance modifiers. However, a detailed analysis of the structure-activity relationship will be the subject of further studies. Our preliminary experiments suggest that the methoxy substitution is important in the most effective compounds (R' = OMe), as was shown for compounds **4, 8** and **11**. It may be noted that the molecular masses are quite close to each other.

In the case of the human colon cancer cells, which express the *mdr1* gene, the compounds were less effective than on the artificially constructed, extremely sensitive human *mdr1* gene-transfected mouse lymphoma cells, in which P-gp is overexpressed. This means that the effectiveness of the reversal compounds also depends on the "type" of cancer cells, which express more or less vulnerable P-gp. P-gp overexpression to a lowered intracellular drug accumulation is due to an increased efflux of anticancer drugs. Inhibition of the efflux mechanism results in an elevated anticancer drug concentration in the cells.

Acknowledgements

This study was supported by the Foundation of Cancer Research of Szeged (Szegedi Rákkutatásért Alapítvány) and by the Hungarian National Research Foundation (Grand No. OTKA T-049468), for which our gratitude is expressed. The technical assistance of Mrs. A. Váradi and Mrs. M. Nagy was highly appreciated.

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Received May 13, 2005
Accepted September 16, 2005