Antitumour Properties of Acridone Alkaloids on a Murine Lymphoma Cell Line

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Abstract. *The aim of the present study was to investigate the anticancer properties of a set of furanoacridone alkaloids, arborinine and evoxanthine, including the inhibitory effect of P-glycoprotein (Pgp) and the apoptosis-inducing capacity. The tested alkaloids were evaluated for multidrug resistance (MDR)-reversing activity on human Pgp-transfected L5178 mouse lymphoma cells, using the rhodamine-123 (Rh-123) assay. The antiproliferative effects of natural compounds and their interactions with doxorubicin were determined in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. Apoptosis-inducing activity was additionally measured by means of dual annexin V and propidium iodide staining. RT-PCR was used to test the expression of Pgp mRNA after acridone treatment. All of the acridones investigated increased the accumulation of Rh-123. Gravacridonetriol and gravacridonediol monomethyl ether increased the antiproliferative effect of doxorubicin on resistant L5178 cells. Treatment with these agents resulted in a decrease in Pgp mRNA levels. Naturally occurring acridone alkaloids exhibit a beneficial combination of anticancer effects and, accordingly, the acridone skeleton can be considered useful in the design of novel antiproliferative agents.*

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Multidrug resistance (MDR), a consequence of overexpression of ATP-binding cassette (ABC) transporters, is one of the greatest current challenges in chemotherapy (1). P-glycoprotein (Pgp, also known as ABCB1) is a member of the large ABC superfamily called traffic ATPases (2). The human MDR gene family is known to include two members, *MDR1* and *MDR2*. Pgp is coded by the *MDR1* gene and expressed as a single chain containing two homologous portions of equal length, each containing six transmembrane domains and two ATP-binding regions (3). ATP binding and hydrolysis appear to be essential for the correct functioning of Pgp, including drug transport (4).

The physiological role of Pgp and related pumps is the extrusion of xenobiotics, protecting tissues with specialized excretory, secretory and barrier functions against toxic insults (5). The intracellular levels of many chemotherapeutic drugs are decreased by the activity of Pgp or related pumps which are up-regulated in drug-resistant cancer cells. Accordingly, the development of agents which inhibit the Pgp-mediated efflux of drugs, and hence reverse MDR, has been intensively pursued.

The extensive search for chemosensitizers has resulted in a variety of agents that inhibit the physiological function of Pgp. The first-generation modulators (such as verapamil, cyclosporine A, reserpine, *etc.*) were not specifically developed for the inhibition of MDR, they were discovered by chance (6). The unacceptable toxicity characteristic of these agents (*i.e.* the primary pharmacological effect) was eliminated by the introduction of the second-generation agents, produced *via* chemical modification of the earlier set of pharmacons. These modulators displayed better tolerability, but they also participated in unpredictable

pharmacokinetic interactions that disturbed the anticancer therapy. The third-generation compounds (tariquidar, zosuquidar, laniquidar, *etc*.) that were subsequently designed lacked other pharmacological effects and conferred greater selectivity and specificity for Pgp. Several compounds from this group are currently undergoing clinical trials (7, 8).

Verapamil was one of the first in-depth investigated Pgp inhibitors found to be effective in clinical trials, though the promising early results were not reinforced later (9). Many agents exhibit a capacity to inhibit Pgp *in vitro*, whereas the clinical results are contradictory or even disappointing (10). This failure can be explained by either dose-limiting toxicity or by a disturbing pharmacokinetic interaction with the basic anticancer agent. Non-toxic and non-interacting Pgp inhibitors are therefore needed; the ideal candidate should not exert any other marked pharmacological effect, and should not affect the activity of the physiologically expressed transporters.

Recent findings have indicated that resistance to chemotherapy might correlate not only with the MDR mechanisms, but also with inactivation of the apoptosis machinery (11). Apoptosis is an essential function responsible for the elimination of harmful or unneeded cells, and for the maintenance of tissue homeostasis in all multicellular organisms (12). Since apoptotic self-demolition involves the protection of surviving cells from the liberation of inflammatory mediators, apoptosis induction is a key feature of most anticancer drugs now being developed (13). Biochemical features are the internucleosomal cleavage of DNA, the proteolytic cleavage of a number of intracellular substrates and phosphatidylserine (PS) externalization (14, 15). In the early stage of apoptosis, plasma membrane alterations occur at the cell surface and PS is translocated from the inner side of the plasma membrane to the outer layer as a consequence of the inhibition of aminophospholipid translocase. PS in the outer surface has been identified as a trigger stimulating the phagocytosis of apoptotic cells by macrophages, thereby preventing secondary necrosis and inflammation of the surrounding tissue (16).

Plants serve as an extensive source of potentially clinically usable natural anticancer drugs and indicate lead structures for the development of semisynthetic agents (17). Acridone alkaloids are found exclusively in plants belonging in the Rutaceae family and can be characterized by their pharmacological effects against human pathogens, including herpes virus, Leishmania and Plasmodium species (18, 19). As concerns the anticancer effects of these alkaloids, the tricyclic glyfoline, one of the most widely investigated acridones, has been reported to induce apoptosis and arrest the cell cycle in the G_2/M phase (20). Besides the natural and synthetic tricyclic acridones, compounds containing an additional heterocycle comprise a special group, one member of which, the pyranoacridone-type acronycine, has been evaluated in clinical trials; a structure–activity relationship has been described following the synthesis and testing of many analogues (21, 22).

A number of naturally occurring and also synthetic acridones were tested earlier in Pgp-mediated drug accumulation assays, with encouraging results (23-25). Tricyclic ring-containing compounds such as acridine, acridine orange and quinacrine potentiate drug-induced cytotoxicity, emphasizing the importance of the aromatic moiety of the acridine structure relative to the side groups (3). Abundant information is available on the occurrence and activity of arborinine and evoxanthine (26, 27), but furan ring-containing acridones are uncommon, appearing among the alkaloids of *R. graveolens* L. and *Thamnosma rhodesica* Bak. f. (28). Natural furanoacridones have been poorly investigated in spite of the promising data. Gravacridonediol and rhodesiacridone have been reported to exhibit antileishmanial effects, while a group of furanoacridones from *R. graveolens* inhibit the proliferation of human adherent cancer cell lines (28, 29).

In our ongoing search for natural antitumour agents, acridones have proven to exert activity against adherent human cancer cell lines (29). In the present study, human *MDR1* gene-transfected mouse lymphoma cells were treated with a set of furanoacridones isolated from *R. graveolens* and rhodamine-123 (Rh-123) accumulation was assessed by flow cytometry. Furthermore, the antiproliferative effects and drug interactions with doxorubicin were examined by the checkerboard method. The effects of the most active acridones on the expression of *MDR1* at the mRNA level were also tested by RT-PCR. The apoptosis-inducing activities of these compounds were additionally assayed by means of dual annexin V and propidium iodide staining, followed by flow cytometry.

Materials and Methods

Chemicals. The tested acridone alkaloids (arborinine (**1**), evoxanthine (**2**), isogravacridone chlorine (**3**), rutacridone (**4**), gravacridonediol (**5**), gravacridonetriol (**6**) and gravacridonediol monomethyl ether (**7**), Figure 1) were isolated from the roots and aerial parts of *Ruta graveolens*, as described earlier (30, 31). A 10 mM stock solution of the acridones was prepared in dimethyl sulfoxide (DMSO). Substances were purchased, if otherwise not specified, from Sigma-Aldrich, Budapest, Hungary.

Cell lines and culture conditions. The MDR mouse lymphoma cell line L5178 MDR transfected with pHa MDR1/A retroviral vector (32) was maintained in the presence of 60 ng/ml colchicine. The parent (PAR) L5178 mouse T-cell lymphoma cells and the human *MDR1* gene-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, 1% L-glutamine and antibiotic-antimycotic. Cell lines were obtained from Professor M.M. Gottesman, National Cancer Institute, Bethesda, MD, USA. Both types of cells were cultured at 37˚C in a humidified CO_2 incubator.

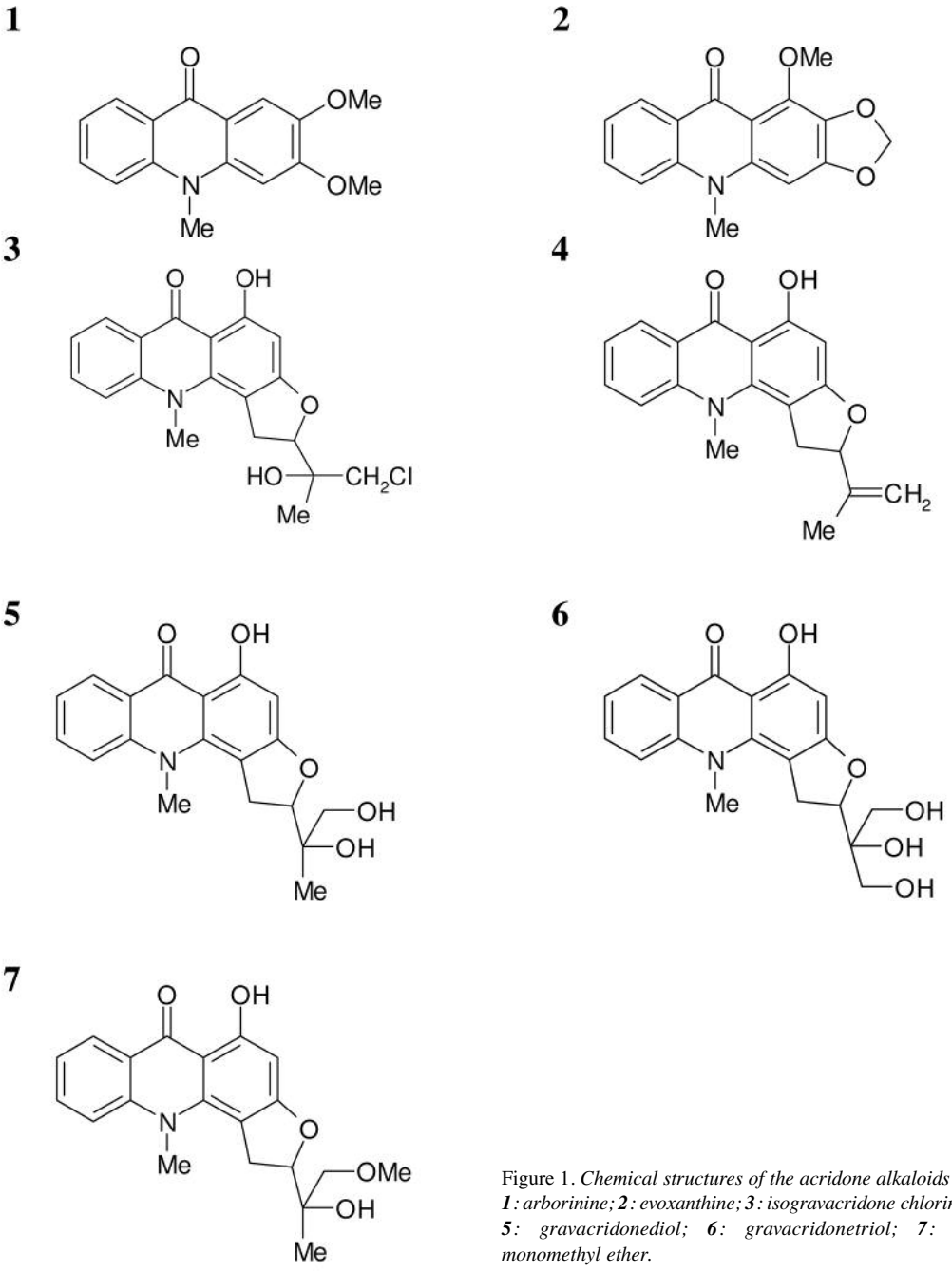

Figure 1. *Chemical structures of the acridone alkaloids investigated here.* ***1****: arborinine;* ***2****: evoxanthine;* ***3****: isogravacridone chlorine;* ***4****: rutacridone;* ***5****: gravacridonediol;* ***6****: gravacridonetriol;* ***7****: gravacridonediol monomethyl ether.*

Rhodamine-123 accumulation assay. Rh-123 is a substrate for Pgp and is widely used as an indicator in tests of the activities of Pgp. A total of 10^6/ml L5178/MDR cells were treated with 40 or 400 μM acridone and incubated for 10 min at room temperature. Ten 10 μl of 1 mg/ml Rh-123 was added to the cells and the mixture was incubated for 20 min at 37˚C. The cell pellet was washed twice with phosphate-buffered saline (PBS) and analysed with a FACStar (Becton-Dickinson, Mountain View, CA, USA). Verapamil was used as positive control in a final concentration of 40.6 μM. The percentage mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio (FAR) was calculated on the basis of the measured fluorescence values: $FAR = MDR_{treated}/MDR_{control}$.

Antiproliferative assay. The cytostatic effects of the acridones were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) assay after incubation for 72 h (33). The cells were seeded into 96-well plates at a density of 10^4/well and exposed to one of the acridones for 72 h. The precipitated formazan crystals were solubilized in 10% sodium dodecylsulfate

Table I. *Effects of acridones on the Rh-123 accumulation assay in L5178 MDR mouse lymphoma cells. Each datum is the mean±SEM of the results of three experiments.*

Compound	μM	FAR	Compound	μM	FAR
1	40	1.72±0.16	**5**	40	105.10±16.34
	400	28.02±4.08		400	130.30±20.79
2	40	1.76±0.47	**6**	40	16.11±7.80
	400	20.17±4.80		400	20.84±8.18
3	40	18.69±1.53	**7**	40	2.54±0.12
	400	20.89±3.34		400	43.77±9.71
4	40	17.93±2.77	Verapamil	40.6	2.18±0.40
	400	10.96±2.21	DMSO	4%	0.94±0.07

and the absorbance was read at 545 nm. Sigmoidal dose–response curves were fitted to the measured points by GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA), and the IC_{50} values were calculated. Doxorubicin was used as positive control.

Analysis of drug combinations. The checkerboard microplate method was applied to characterize the effect of combinations of these acridone alkaloids and the cytotoxic compound doxorubicin on the L5178 MDR cancer cell line (34). A series of 2-fold dilutions of the acridones were tested in combination with 2-fold dilutions of doxorubicin. The dilutions of doxorubicin (A) were made in the horizontal direction, and the dilutions of the resistance modifiers (B) vertically in the microtitre plate in a volume of 100 μl. After incubation for 3 days, the MTT assay was applied, and the drug interactions were evaluated *via* the following relationship:

$FICA=IC_{50A \text{ in combination}} / IC_{50A \text{ alone}}$
$FICB=IC_{50B \text{ in combination}} / IC_{50B \text{ alone}}$
$FIX=FIC_A + FIC_B$

FIX<0.5	synergism
0.51<FIX<1	additive effect
1<FIX <2	indifferent effect
FIX>2	antagonism

where FIC and FIX are the fractional inhibitory concentration and the fractional inhibitory index, respectively.

Apoptosis assay. Apoptosis was assessed by staining with the PS-binding annexin V and the nucleic acid-binding propidium iodide (35). Annexin V detects PS translocation on the cell surface, a hallmark of early apoptosis, while late apoptosis and necrosis reveal additional positive nuclear staining with propidium iodide. In brief, the 24-h-treated L5178 MDR cells were washed with PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC was added to the cells for 10 min in the dark at room temperature. After washing, propidium iodide (20 μg/ml) was added and the cells were analysed on the FACStar. 12H-benzo[α]phenothiazine (M627) was used as positive control (36).

RT-PCR study. The effects of the tested compounds on the mRNA expression pattern of *MDR1* were determined by the RT-PCR technique. After the experimental treatment (48 h), 10^6 cells were treated with denaturing solution (37). Following precipitation with isopropanol, the RNA was washed with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 μl DNase- and RNase-free distilled water and the RNA concentrations were determined spectrophotometrically. A sample of 0.5 μg RNA was denatured at 70˚C for 5 min in a reaction mixture containing 20 μM oligo(dT), 20 U RNase inhibitor, 200 μM dNTP in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$ in a final reaction volume of 20 μl. After the mixture had been cooled to 4˚C, 20 U moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco, Paisley, UK) and RNase H Minus (Promega, Southampton, UK) were added, and the mixture was incubated at 37˚C for 60 min. The PCR was carried out with 5 μl cDNA, 25 μl ReadyMix REDTaq PCR reaction mix, 2 μl sense and antisense primer and 16 μl DNase- and RNase-free distilled water with a PCR Sprint thermal cycler (Hybaid, Middlesex, UK). Rat glyceraldehyde-3-phosphate dehydrogenase primers were used as internal control in all the samples (38). The products were separated on 2% agarose gels, stained with ethidium bromide and photographed. The sequences of the oligonucleotide primers for *MDR1* were the same as reported previously (39).

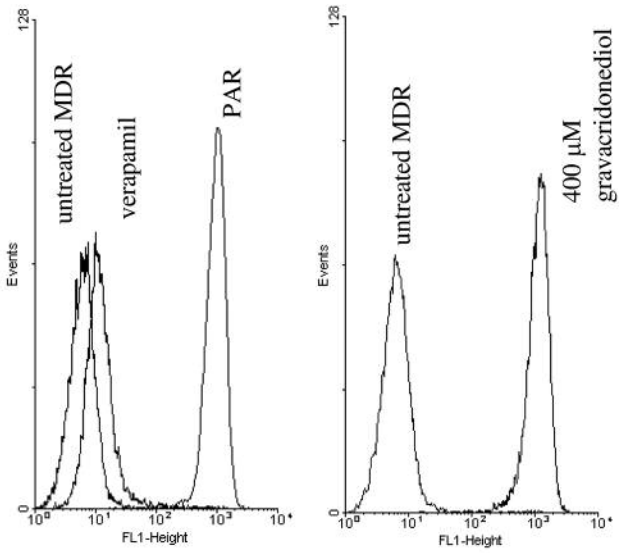

Figure 2. *Effect of gravacridonediol (**5**) on intracellular Rh-123 accumulation in L5178 MDR cells. Cells were treated with **5** for 30 min, and assayed for Rh-123 accumulation. Left panel: A representative histogram of non-treated parent (PAR) and MDR cells and 40.6 μM verapamil-treated L5178 MDR cells. Right panel: The effects of 400 μM **5** on the intracellular Rh-123 accumulation.*

Results

Effects on the intracellular accumulation of Rh-123. The results of the Rh-123 accumulation assay are presented in Table I. Compounds **3**, **4**, **5** and **6** at 40 μM inhibited the pump function of Pgp more efficiently than the positive control verapamil (40.6 μM). Compounds **1** and **2** at 40 μM did not exert any effect on the drug accumulation. At 400 μM,

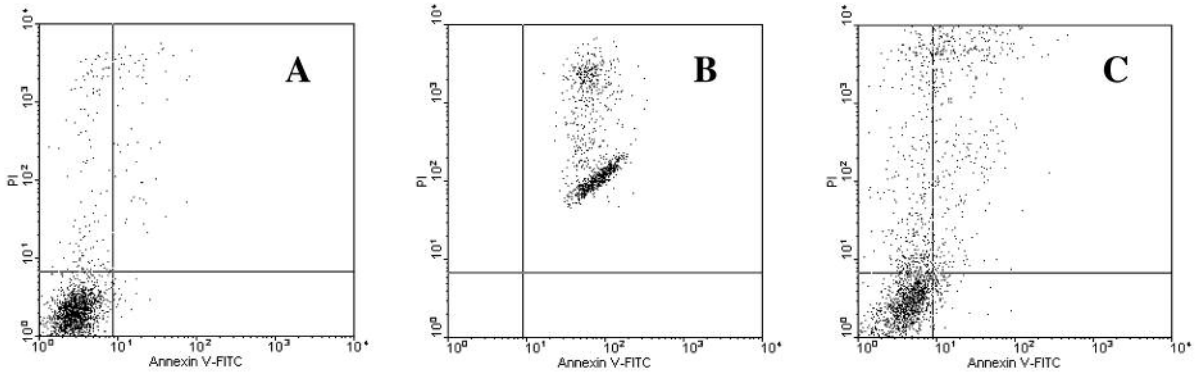

Figure 3. *Flow cytometric plots of annexin V and propidium iodide (PI) staining of L5178 MDR cells, untreated (panel A), treated for 24 h with M627 (panel B) and with 60 μM gravacridonediol monomethyl ether (**7**) (panel C).*

all of the tested acridones caused a substantial increase in the FAR. From among these compounds, **5** proved to have the most efficient MDR-reversal effect, resulting in a more than 100-fold increase in Rh-123 accumulation, even at the lower concentration (Figure 2).

Cytostatic effects and combination studies of the acridones. The antiproliferative effects of the tested acridones are summarized in Table II. The sequence of antiproliferative potency was **3>4>2≥5>7>6≥1**; compound **3**, with the lowest IC_{50}, was comparable in effect with doxorubicin (IC_{50}=1.097 μM).

In the combination studies, the capacity to enhance the antiproliferative effect of doxorubicin was examined with the checkerboard method. Compounds **6** and **7** substantially enhanced the antiproliferative activity of doxorubicin on the L5178 MDR cell line; their interactions proved to be synergistic. Although **5** was the most effective agent in the MDR reversal test, the combination surprisingly resulted in only an additive antiproliferative effect. Additive interactions were also observed for the tricyclic acridones **1** and **2**. Compounds **3** and **4** exerted antagonism with doxorubicin, reducing its cytostatic effect (Table II).

Analysis of apoptosis. Representative flow cytometric results are presented in Figure 3. Acridone-treated L5178 MDR cells were stained with annexin V (early apoptosis) and propidium iodide (late apoptosis and necrosis) and measured flow cytometrically (Table III). A substantial increase in the late apoptotic L5178 MDR cell population (annexin V+, propidium iodide +) was found after 24-h treatment with the acridones.

Effects of acridones on MDR1 expression. In view of the results of the combination experiments gravacridonetriol and gravacridonediol monomethyl ether were selected in order to test their effects on Pgp expression during a longer exposure. Both alkaloids at 15 μM significantly reduced the level of Pgp mRNA over 48 h incubation (Figure 4).

Table II. *Calculated IC_{50} values and the results for the doxorubicin combinations.*

Compound	IC_{50}	FIX	Interaction
1	69.57	0.85	Addition
2	33.22	0.71	Addition
3	0.062	37.29	Antagonism
4	16.02	2.19	Antagonism
5	33.97	0.76	Addition
6	67.21	0.41	Synergism
7	43.65	0.03	Synergism

Table III. *Effects of acridones on apoptosis induction in human MDR1 gene-transfected mouse lymphoma cells. Cells were incubated for 24 h with 60 μM acridones and measured for apoptosis by flow cytometry. Early apoptosis (lower right, LR), late apoptosis (upper right, UR) and necrosis (upper left, UL) were distinguished. M627 was used as positive control.*

Compound	Concentration	LR	UR	UL
Control		3.11±2.45	2.94±1.34	4.42±1.19
M627	50 μg/ml	1.62±0.60	99.11±0.38	0.23±0.16
1	60 μM	1.24±0.67	15.21±3.99	8.94±2.63
2	60 μM	1.45±0.31	19.84±5.85	8.91±1.89
3	60 μM	1.93±0.29	12.33±1.96	3.85±1.72
4	60 μM	2.87±0.07	15.08±3.99	4.53±0.34
5	60 μM	2.02±0.50	5.94±0.52	2.92±0.27
6	60 μM	2.42±1.90	14.11±2.97	4.49±0.52
7	60 μM	1.52±0.08	26.97±2.38	9.07±2.43
DMSO	1%	2.51±1.78	2.41±1.05	3.46±0.53

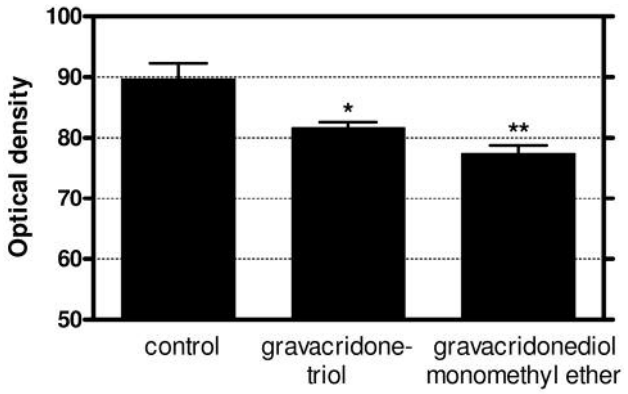

Figure 4. *RT-PCR assay for MDR1 in L5178 MDR cells. Gravacridonetriol (**6**) and gravacridonediol monomethyl ether (**7**) were used at 15 μM for 48 h. * and **indicate differences at the levels p<0.05 and p<0.01 when compared with the control value, respectively.*

Discussion

One of the investigated compounds, isogravacridone chlorine, displayed outstanding cytostatic activity, while others exhibited moderate effects (IC_{50}: 16.02-69.57 μM) and all of the tested compounds increased the late apoptotic cell population. However, no obvious relationship was found between the calculated IC_{50} values and the apoptosis-inducing activity. The sequence of antiproliferative potency was **3>4>2>5>7>6>1**. Comparisons of the results for the furanocridones (**3-7**) with previous results on the HeLa, MCF7 and A431 cell lines revealed that **6** was always less active than the other furanocridones, while **3** and **4** exhibited the leading IC_{50} values. Independently of the direct antiproliferative effect, the acridone skeleton has been reported to be a structure of value for the design of inhibitors of ABC transporters conferring MDR (40, 41).

All of the tested compounds enhanced the intracellular Rh-123 accumulation. The most effective compound was **5**, which at the tested concentration resulted in a similar extent of drug accumulation as for the drug-sensitive PAR cell line.

In the combination study, some compounds were more or less effective than expected from the accumulation assay. This method is frequently used to detect interactions between antimicrobial agents, but can be applied in cytotoxic studies as well (42). The combination of doxorubicin and compound **5** resulted only in an additive effect, whereas **6** and **7** displayed synergistic effects with doxorubicin. Since the duration of exposure in the Rh-123 accumulation test was short (30 min), it is unlikely that acridones act by down-regulating *MDR1* transcription, therefore reducing the amount of cellular Pgp. Accordingly, the effects of the two furanoacridones that gave the most promising results in the combination assay on the expression of Pgp at the mRNA level after a longer (2-day) incubation were examined.

A decrease in the expression of the efflux pump responsible for resistance is presumed to be more advantageous than direct inhibition of the pump. ET-743 (ecteinascidin-743), a tetrahydroisoquinoline alkaloid isolated from a marine tunicate, exhibited a unique spectrum of anticancer actions, including a direct cytostatic effect, even against resistant carcinomas, and also inhibition of the chemotherapy-induced expression of Pgp (43). Two of the tested foranoacridones, gravacridonediol and gravacridonediol monomethyl ether, reduced the expression of Pgp at mRNA level which is reported here for the first time.

Taken together, these data indicate that the acridones increase intracellular drug levels by modulating the Pgp activity, and the most effective furanoacridones are able to decrease the expression of Pgp at the mRNA level. These *in vitro* experimental data indicate that the acridone skeleton, especially when it contains an additional heterocycle, can be regarded as a promising starting structure for the design of Pgp inhibitors with a novel mechanism of action.

Acknowledgements

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