Pharmaceutical Research, Vol. 26, No. 1, January 2009 (© 2008)
DOI: 10.1007/s11095-008-9736-7

Research Paper

Impact of Novel MDR Modulators on Human Cancer Cells: Reversal Activities and Induction Studies

Claudius Coburger,[1] Hermann Lage,[2] Josef Molnár,[3] and Andreas Hilgeroth[1,4]

Received August 13, 2008; accepted September 22, 2008; published online October 30, 2008

Purpose. Novel multidrug resistance (mdr) modulators have been proved as inhibitors of P-glycoprotein (P-gp). We first investigated the *in vitro* effects of selected compounds in human cancer cells on multidrug resistance reversal effects compared to drug standards and on P-gp induction to characterize the potential of the compounds as clinical candidates.
Methods. The uptake of daunorubicin into a parental cancer cell line and P-gp expressing subcell line in presence of the modulators was characterized by flow cytometry. Induction of P-gp was investigated in P-gp expressing and non-expressing cancer cell lines on the RNA level by real-time quantitative polymerase chain reaction (RTQ-PCR) and protein quantification. Results were additionally confirmed by northern blot techniques and functionality assays in selected cell lines.
Results. The novel modulators showed activities as mdr reversers in a P-gp specific human cancer cell model with mainly increased uptake rates of daunorubicin into the drug-resistant cell line. H17 proved to be more active than cyclosporine A as a known strong mdr modulator. The induction studies revealed practically no induction potential of the compounds in usual short-time drug application regimes in all cell lines. Furthermore, the novel modulators did not increase the efflux of a P-gp model substrate in the functionality model assay. This confirmed the results of non-P-gp induction which was observed on both the RNA and the protein levels.
Conclusions. The novel mdr modulators proved as perspective candidates for further clinical studies because they turned out to be highly active in human cancer cell models. Furthermore, they showed no potential to induce the transmembrane efflux pump P-gp. This is a significant advantage compared to modulators which failed in clinical trials because of induction-effects that increase cellular resistances and, moreover, side effects in normal cells.

KEY WORDS: flow cytometry; induction studies; mdr modulators; P-glycoprotein; reversal effects.

INTRODUCTION

The occurrence of multidrug resistance (mdr) is one great problem in cancer treatment of today (1,2). Drug-sensitive cancer cells develop such a resistance under short-time therapies with various anticancer drugs (3,4). Not only established cytostatics like anthracycline derivatives, topoisomerase inhibitors or mitomycin C turned out to be non-effective after some time (3,4), but also novel cytostatics like imatinib as a tyrosin receptor kinase inhibitor, gemtuzumab oxagamicin as monoclonal antibody or bortezumib as proteasome inhibitor show the resistance phenomenon under therapeutical applications (5–7).

Transmembrane efflux pumps like P-glycoprotein (P-gp) or the multidrug-resistance associated proteins (MRPs) have been identified as main causative agents of the clinically significant mdr problem (1,3,8–10). These efflux pumps transport drugs out of the cells so that insufficient therapeutic drug levels result (8,11,12).

The most critical problem is the broad substrate diversity of these efflux pumps. So in the case of P-gp almost all mdr substrates are substrates of this efflux pump (13). The MRPs are known to additionally transport organic anions like glucuronides and glutathione-conjugates out of the cells (14). The reason for the observed substrate diversity lies in the existence of various substrate binding sites and the different affinities of the substrates to these binding sites (13,15). Presently, almost four of these binding sites are known in the case of P-gp (13).

From all the numerous efforts to overcome mdr like a transcriptional control of P-gp expression the most promising access has been the development of mdr modulators. The mdr modulators are able to increase the intracellular drug-levels in co-application with mdr substrates by the effect of efflux pump inhibition (16,17). Early mdr modulators which

[1] Institute of Pharmacy, Martin-Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Straße 4, 06120 Halle, Germany.
[2] Institute of Pathology, University Hospital Charité, Charitéplatz 1, 10117 Berlin, Germany.
[3] Department of Medical Microbiology, University of Szeged, Dom tér 10, 6720 Szeged, Hungary.
[4] To whom correspondence should be addressed. (e-mail: andreas.hilgeroth@pharmazie.uni-halle.de)

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have been discovered by drug-screening efforts showed other pharmacological activities beside P-gp inhibition (1). Thus, they were strongly dose-limited in therapy with resulting toxic effects (1). Following compounds were developed as derivatives of the early generations but partly showed reduced activities as modulators (17).

We developed novel cage dimeric 1,4-dihydropyridines as highly effective P-glycoprotein inhibitors. Early toxicological *in vitro* studies promised no additional side effects under application concentrations (18). Two of these candidates were first evaluated in human cancer cell lines by investigating their potential to overcome mdr. Moreover, detailed induction studies are reported which investigate the potential to induce P-gp under short-time therapy in comparison to recent studies which will be referred to with respect to clinical applications.

MATERIALS AND METHODS

Chemicals

Daunorubicin was purchased from Farmitalia Carlo Erba (Freiburg, Germany), verapamil as Isoptin® injection solution from Abbott GmbH & Co. KG (Wiesbaden, Germany), cyclosporine A and other chemicals for cell culture from Sigma–Aldrich (Steinheim, Germany). Primers for RTQ-PCR were obtained from MWG Biotech AG (Ebersberg, Germany). The synthesis of the cage dimeric 1,4-dihydropyridines has been described (19).

Cell Culture

The human gastric carcinoma cell line EPG85–257P and its daunorubicin-resistant subline EPG85–257RDB were cultivated in Leibovitz L15 medium (Bio Whittaker, Verviers, Belgium) supplemented by 10% FCS (Biochrom AG, Berlin, Germany), 1 mM Ultraglutamine (Bio Whittaker, Verviers, Belgium), 1.1 g/l $NaHCO_3$, 1% minimal essential vitamins, 0.225 g/l glucose, 80 IE/l insulin (Insuman® Rapid, Hoechst Marion Roussell, München, Germany), 5000 KIE Trasylol® (Bayer AG, Leverkusen, Germany), 2.5 mg/ml transferrin and 6.25 mg/l fetuin in a humidified atmosphere of 5% CO_2 at 37°C (20,21). The daunorubicin-resistant cell line has been derived by induction under exposure to increasing concentrations of daunorubicin (21). To ensure the MDR phenotype, cell culture medium for the P-gp-expressing line EPG85–257RDB was supplemented with daunorubicin (2.5 μg/ml). The human colon carcinoma cell line COLO-320 was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM Na-pyruvate and 100 mM Hepes as described (22). The mouse T lymphoma cell line L5178Y and the L5178Y *mdr* subline were grown in McCoys 5A medium with 10% heat-inactivated horse serum albumin, 2 mM glutamine and antibiotics (23).

MDR Reversal

The cultured gastric carcinoma cells were adjusted to a concentration of 1.2×10^5 cells per ml and seeded out in six-well plates to a concentration of 2.4×10^5 cells per well. After 24 h the medium was removed and fresh medium containing several concentrations of the test compounds was added and cells were incubated for 15 min at room temperature. Then 100 μl of daunorubicin (5 μM final concentration) were added to the samples and incubated for further 60 min at 37°C. After removing the medium cells were washed twice with phosphate-buffered saline (PBS), trypsinized and transferred to 15 ml tubes. After centrifugation (5 min, 1000 rpm, 5°C) cells were washed again, resuspended in 0.5 ml FACS-PBS and stored on ice. The fluorescence of the cell population (1×10^4 cells) was measured with a Becton Dickinson flow cytometer. The cultured mouse T lymphoma cells were treated under same conditions. Rhodamine 123 was used instead of daunorubicin with a final concentration of 5.2 μM.

RTQ PCR Studies

Gastric carcinoma cells and COLO-320 cells were grown to a density of 80% and seeded out in six-well plates to a concentration of 2.4×10^5 cells per well. After 24 h the medium was removed and fresh medium containing the compounds H17 (10 μM), JW41 (10 μM) and DMSO (0.5%), respectively, was added and cells were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37°C.

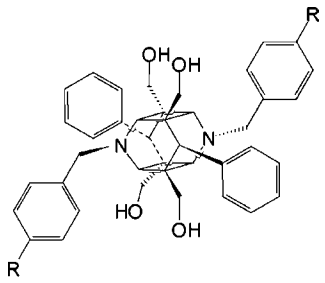
To analyze quantitative mRNA expression, real time quantitative RT-PCR was carried out using 2 μg of total RNA (see Northern Blot Analysis). The SuperScript Kit (Invitrogen GmbH, Carlsbad, USA) was used to synthesize first-strand cDNA. For further analysis the samples were diluted 1:10 and measured by a LightCycler instrument and SYBR-green fluorescent dye (Light Cycler-FastStart DNA Master SYBR-Green I Kit, Roche Diagnostics, Mannheim, Germany). Primers used for amplification were MDR1-fwd (5′-CAG CTA TTC GAA GAG TGG GC-3′) and MDR1-rev (5′-CCT GAC TCA CCA CAC CAA TG-3′). Furthermore primers yielding an amplification product specific for a housekeeping gene that encodes aldolase were used named Aldolase-fwd (5′-ATC GTG GCT GCA CAT GAG TC-3′) and Aldolase-rev (5′-GCC CTT GTC TAC CTT GAT GC-3′) and acted as a control. First step of polymerase chain reaction was an enzyme activation at 95°C for 10 min, followed by 45 cycles of amplification shown below. Specificity of amplification products was confirmed by melting curve analyses. Each sample was measured at least for three times.

Amplification steps for real-time quantitative RT-PCR

Temperature	Time	Step
95°C	15 s	Denaturation of DNA
58°C (56°C)	5 s	Primer hybridization P-gp (aldolase)
72°C	15 s	Elongation
84°C	5 s	Fluorescence measurement

Western Blot Analysis

For investigation in changes in protein expression, cells were pretreated as described (see "RTQ PCR Studies"). After removing the medium, cells were washed twice and cell proteins were detached by using triton x-100. Cell suspension was transferred to Eppendorf tubes and proteins were denatured for 10 min at 95°C. After centrifugation the



H17 R = H
JW41 R = OCH_3

Fig. 1. Structures of investigated cage dimeric 1,4-dihydropyridines H17 and JW41.

supernatant was stored at −80°C. To determine protein concentration samples were measured in a spectrophotometer at 630 nm after treatment with amido black solution. 20 µg of the extracted membrane proteins were separated on 4% stacking and 6% resolving SDS-PAGE gels and transferred to a 0.2 µm cellulose nitrate membrane. The membrane was blocked in 5% skim milk in TBS overnight. For detection of human P-gp the blocking buffer was removed and the membrane was incubated for 2 h with mouse monoclonal antibody C219 (Alexis Biochemicals, San Diego, USA) which was diluted 1:100 in 1% skim milk. Afterwards the membrane was treated with peroxidase-conjugated anti-mouse IgG (Perbio Science, Bonn, Germany) diluted 1:10,000 for visualization of the protein–antibody complexes by chemoluminescence (ECL-Kit, Amersham Pharmacia Biotech, Freiburg, Germany). As control for equivalent protein loading, the membrane was also incubated with a mouse monoclonal antibody directed against the actin protein (Chemicon, Hofheim, Germany) diluted 1:50,000.

Northern Blot Analysis

From pretreated COLO-320 cells (see "RTQ PCR Studies") total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). To determine the RNA concentration the samples were measured in a spectrophotometer at 260 nm. 10 µg RNA was separated on 1% agarose-formaldehyde gels and transferred onto Hybond-N^+ membranes (Amersham Pharmacia Biotech, Freiburg, Germany). By using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech, Freiburg, Germany) a MDR1 cDNA fragment was randomly labelled with [^{32}P] dCTP. The blots were hybridized with 25 ng of the labelled MDR1 cDNA fragment in ExpressHyb™ hybridization solution (Clontech, Heidelberg, Germany) for 60 min at 58° C. Afterwards the membranes were washed several times and the exposure followed with a BioMax MR film (Kodak, Stuttgart, Germany).

Functionality Assay for Induction Studies

COLO-320 cells were adjusted to a density of 1×10^5 cells per ml and seeded out in six-well plates to a concentration of 2×10^5 cells per well. After 24 h the medium was removed and fresh medium containing several concentrations of the cage dimers H17 and JW41 was added. After an incubation time of 72 h the medium was discarded, cells were trypsinized, resuspended in fresh medium and each sample was divided into two Eppendorf centrifuge tubes. Half of the samples were preincubated with a high concentration of verapamil (100 µM) for 10 min at 37°C to prove the functionality of P-gp as control. Next, 10 µl of the P-gp substrate rhodamine 123 (5.2 µM final concentration) was added to all samples and the cells were incubated for further 20 min at 37°C, washed twice with PBS and resuspended in 0.5 ml PBS for analysis. The fluorescence of the cell population (1×10^4 cells) was measured with a Becton Dickinson flow cytometer. Results of flow cytometry were calculated as *R* values that indicate the ratios of the fluorescence intensities of the treated to untreated cells following described protocols (24,25).

Data Analysis

From all single values the arithmetical mean and the standard error of the mean (SEM) have been calculated. Studies for quantitative real time PCR and mdr reversal were performed in triplicates (n=3). Statistical significance of correlation was determined using one-way ANOVA (Origin 6.1) with $p<0.05$. Results of western blot and northern blot analyses were additional quantified by densitometric analysis using ImageJ 1.4 (http://rsb.info.nih/ij). Values were calculated as the ratio of actin (aldolase) control to P-gp and are displayed in relation to untreated control which is set up to a value of one.

Table I. P-gp Inhibiting Properties of Compounds H17 and JW41 Evaluated in the Model Cell Line (Mouse T Lymphoma) and the MDR Reversal of Daunorubicin after Treatment with H17 and JW41 in Human Cancer Cells (Gastric Carcinoma) Determined as *R* Values by Flow Cytometry

	R values mouse T lymphoma cells		*R* values gastric carcinoma cells
Compound	1 µM	10 µM	10 µM
H17	4.10±0.3[a]	14.64±3.10[a,b]	2.68±0.13[a]
JW41	3.50±0.6[a]	8.82±1.20[a,b]	1.47±0.01[a]
Verapamil	2.10±0.1[a]	2.85±0.78	2.19±0.23[a]
Cyclosporine A	n.d.[c]	n.d.[c]	2.56±0.74

Each value indicates the mean±SEM of n=3 as number of individual measurements
[a] $p<0.05$, significant difference
[b] Saturation
[c] *n.d.* not determined

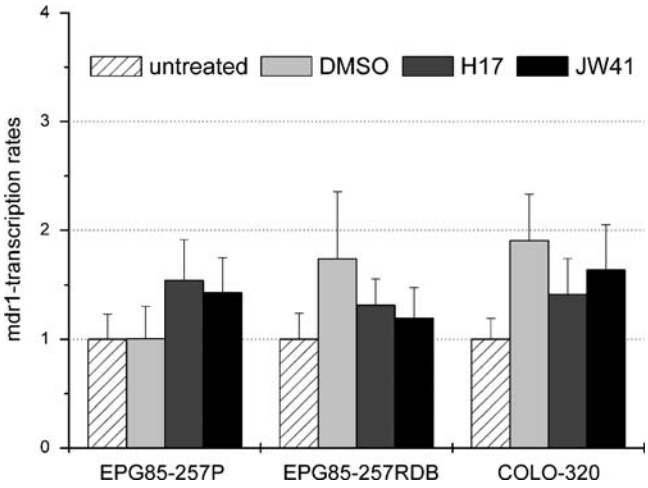


Fig. 2. Alterations of MDR1-mRNA expression using RTQ PCR after treatment with compounds H17, JW41 and DMSO 0.5% (control), respectively, for 72 h. Samples are in relation to the untreated control cell line with a transcription rate set up to a value of one. The data are expressed as mean±SEM of $n=3$. $*p<0.05$, significant difference.

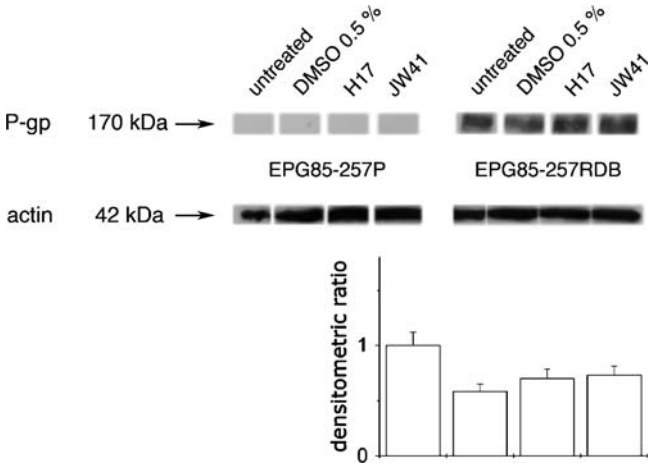


Fig. 4. Western blot analyses of the gastric carcinoma cell line (EPG85–257P) before induction (*line 1*), with 0.5% DMSO (*lane 2*), after incubation with compounds H17 (*lane 3*) and JW41 (*lane 4*), respectively, after induction with daunorubicin (*line 5*) and after treatment of the induced cell line with 0.5% DMSO (*line 6*), incubation with compound H17 (*lane 7*) and with JW41 (*lane 8*), respectively. The densitometric analysis of the blots is shown in the lower part. The data are expressed as mean±SEM of $n=3$. $*p<0.05$, significant difference.

RESULTS AND DISUSSION

Mdr Reversal Studies

Recent studies of first cage dimeric 1,4-dihydropyridines as mdr modulators have been restricted to non-human cell lines (23). We evaluated the potential to inhibit P-gp in a mouse T lymphoma cell line by comparison of the activities in the mdr1 resistant subline and the parental cell line. Although the expressed P-gp was encoded by the human mdr1 gene which was transferred to the parental cell line by gene transfection the effect of the cage dimers as mdr modulators had to be investigated in human cell lines to estimate their potential for further clinical applications. Furthermore, the effect was investigated with a clinically relevant cytostatic agent instead of the P-gp substrate rhodamine 123 which was used as model substrate in the mouse T lymphoma model cell line. In the study we used a human gastric carcinoma cell line EPG85–257P and the P-gp expressing subline EPG85–257RDB. This mdr1 resistant cell line exclusively expresses P-gp and, moreover, the expression resulted from induction of P-gp under short-time treatment with daunorubicin.

We selected two cage dimers H17 and JW41 (Fig. 1) which differ just in one aromatic substitution to find relations of these structural features to the observed activities. Both compounds showed strong P-gp inhibiting activities in the mouse T lymphoma cell lines determined as fluorescence

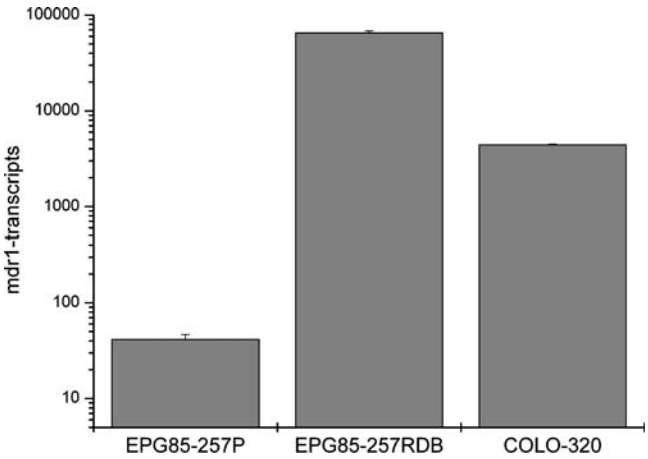


Fig. 3. Mdr1-mRNA expression of the untreated gastric cancer cell line EPG85–257P (*left*), after treatment with P-gp inducer daunorubicin (*middle*) and the P-gp expressing colon carcinoma 320 cell line (*right*). Displayed are absolute values given from RTQ-PCR. The data are expressed as mean±SEM of $n=3$.

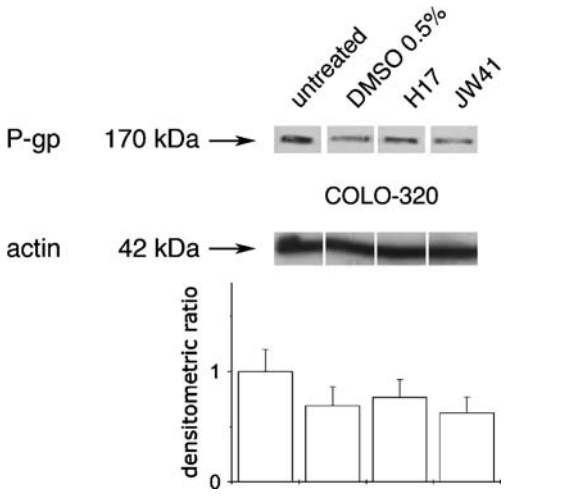


Fig. 5. Western blot analyses of the colon carcinoma cell line (*COLO-320*) before induction (*line 1*), with 0.5% DMSO (*lane 2*) and after incubation with compounds H17 (*lane 3*) and JW41 (*lane 4*), respectively. The densitometric analysis of the blots is shown in the lower part. The data are expressed as mean±SEM of $n=3$. $*p<0.05$, significant difference.

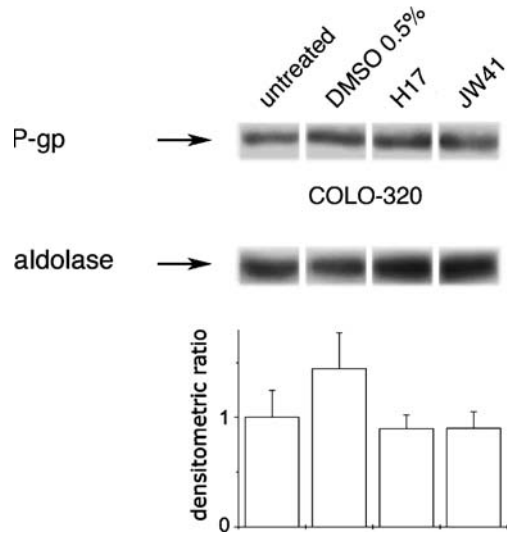


Fig. 6. Northern blot analyses of the colon carcinoma cell line (*COLO-320*) before induction (*line 1*), with 0.5% DMSO (*lane 2*) and after incubation with compounds H17 (*lane 3*) and JW41 (*lane 4*), respectively. The densitometric analysis of the blots is shown in the lower part. The data are expressed as mean±SEM of $n=3$. *$p<0.05$, significant difference.

activity ratios (*R* values, Table I). At the lowest inhibitor concentrations of 1 μM both compounds H17 and JW41 showed better activities than the verapamil standard. The activities of both compounds were much stronger than those of verapamil at the higher concentrations of 10 μM with a saturation of the P-gp inhibition for each H17 and JW41. JW41 showed about half of the activity than H17 at this saturation concentration. Verapamil did not even reach the inhibition potential of the cage dimers at a concentration of 30 μM with a determined *R* value of 7.3±0.3.

The mdr reversal effects in the human cancer cell lines which result from specific P-gp inhibition have been determined with daunorubicin in competition studies with each of our two cage dimers compared to known inhibitors. The daunorubicin uptake directly reflects the mdr reversal effect as increased cellular daunorubicin fluorescence. At the lowest concentrations of 1 μM all compounds were not active in comparative studies with daunorubicin (*R* values<1.0). At the highest inhibitor concentrations H17 led to an almost threefold increased cellular uptake of daunorubicin (Table I). JW41 again showed half of the activity than H17. H17 was more effective than the verapamil standard. Cyclosporine A which is known to be a better P-gp inhibitor than verapamil (17) proved to be more active than verapamil, but the best activity of H17 was not reached by cyclosporine A. So it can be concluded that the P-gp inhibiting effect of our cage dimers in the mouse T lymphoma model cell line was confirmed in the human gastric cancer cell lines leading to strong mdr reversing effects which were proved by mainly increased cellular daunorubicin uptake.

Induction Studies

The major problem of effectively fighting mdr in clinical studies is the induction of resistance under short-time therapies with either cytostatic agents or mdr modulators (16,23,26,27). So the application of even originally effective modulators failed due to the induced over-expression of functional resistance-causing transmembrane efflux pumps. In *in vitro* studies short-time exposures to mdr modulators of 8 up to 48 h led to increased mRNA- and following protein levels. An extended efflux of transmembrane substrates like cytostatics or mdr modulators was observed and caused the inactivation of the mdr reversers (16,24,26,27).

First, we investigated the induction effects of both compounds H17 and JW41 in our cancer cell lines on the RNA level using the RTQ PCR method. We started with the non P-gp expressing gastric carcinoma cell line EPG85–257P. The determined transcription-rates of the mdr1 gene were related for each compound to the untreated control with a fixed value of one (Fig. 2). The untreated DMSO containing cell line was additionally investigated because little induction effects of DMSO are described in literature as cellular stress response (16). We found no significant changes in the transcription rates in the DMSO containing EGP85–257P cell line. Almost unchanged transcription rates have been found after treatment with the compounds H17 and JW41.

Exposure of the EPG85–257P cell line to daunorubicin led to an increase of mdr1 transcription rates by a factor of 2000 (Fig. 3). Following treatment of this P-gp induced overexpressing gastric carcinoma cell line led to little DMSO effects as described in literature with an insignificant increase

Table II. Fluorescence Uptake of Rhodamine 123 as Control Functionality Assay After Cell Treatment (COLO-320) with Increasing Concentrations of Compounds H17 and JW41, Respectively, Determined *R* Values and After Preincubation with Verapamil Control (100 μM) for Complete P-gp Inhibition (Right Side) Using Flow Cytometry

Compound	Concentration [μM]	*R* values[a]	*R* values preincubated with verapamil[a]
H17	2.5	0.97±0.15	19.25±3.1
	5	1.11±0.17	19.15±3.0
	10	1.25±0.16	20.55±3.2
	20	1.44±0.22	21.88±3.4
JW41	2.5	1.15±0.21	21.40±3.8
	5	1.13±0.21	14.49±2.6
	10	1.22±0.22	27.55±4.9
	20	1.29±0.23	28.57±5.1

Each value indicates the mean±SEM of $n=3$ as number of individual measurements
[a] $p<0.05$, significant difference

of the transcription rates by a factor of about 1.8. The compounds H17 and JW41 practically showed no effects in this cell line.

Finally, the P-gp expressing colon carcinoma cell line COLO-320 was investigated. The quantified mdr1 transcripts of this cell line before treatment are shown in Fig. 3. Referred to the daunorubicin induced cell line the transcription amounts are much lower. However, also in this cell line we found some DMSO stress and an insignificant RNA-increase with a factor similar to the P-gp induced gastric carcinoma cell line. Treatment with the compounds again led to lowered values. Although the differences are insignificant the compounds may neutralize the DMSO stress response.

In addition to the investigations on the RNA level we determined the amounts of expressed P-gp in all cell lines by western blot analyses. We found no detectable P-gp in the parental gastric carcinoma cell line EPG85–257P whereas the induction after treatment with daunorubicin led to increasing protein levels as shown in Fig. 4. Consequent treatment with DMSO and our two cage dimers did not lead to detectable P-gp levels in the parental cell line EPG85–257P. This corresponds to the results given on the RNA level as described. Further investigations of the already P-gp expressing cell line after treatment with our compounds led to protein amounts that practically showed no difference in the resulting blot intensities, so that in agreement with the results given on the RNA level we found no further induction of P-gp which is already expressed on a high protein level according to the blot intensities.

We then investigated the naturally P-gp expressing colon carcinoma cell line which has been suggested to express much lower P-gp protein levels than the induced gastric carcinoma cell line according to the differing mdr1 transcripts as displayed in Fig. 3 (COLO-320) (28). The determined protein amount as shown in Fig. 5 was lower with a thinner blot if compared to the actin control than in the P-gp daunorubicin-induced cell line.

The treatment with DMSO practically led to small insignificant changes in the protein amount as can be derived from the comparison of the blot intensities of P-gp and the actin control. The induction studies with H17 and JW41 led to some differences in the blot intensities. So little differing protein levels have been suggested. Therefore we decided to support the results on the RNA level given with the RTQ PCR which revealed non-inducible P-gp in the colon carcinoma cell line by additional northern blot analysis to visualize the RNA levels. The northern blot analysis practically proved no differences in the RNA amounts after preincubation with the cage dimers as shown in Fig. 6. These results confirmed the results given in the RTQ PCR studies.

Finally, we carried out a functionality assay with the colon carcinoma cell line. Such an assay proves changes in functional P-gp amounts by changing uptake rates of the fluorescent P-gp substrate rhodamine 123 for example (16,24). Although it is a non-direct prove of P-gp induction such an assay may additionally confirm the non-inducible effects of our cage dimers. After preincubation with both of the inhibitors at different concentrations the determined *R* values were similar with almost unchanged ratios (Table II). An induction effect would have given lowered ratios because of increased P-gp amounts and thus lowered intracellular uptake of rhodamine 123. In the control experiment preincubation with high inhibitor dosages of verapamil led to high fluorescence ratios *R* with almost similar values for the H17 treated cell line due to a total P-gp inhibition and thus a maximum rhodamine uptake while in the JW41 treated cell line the values result in a mean value of 23±6.5 (Table II, right).

CONCLUSIONS

Two novel mdr modulators from a series of cage dimers have been investigated in human cancer cell lines for the first time to estimate their clinical potential as effective mdr reversers. The P-gp specific inhibiting effects were proved in competition with daunorubicin as P-gp substrate with an mdr reversal potential superior to cyclosporine A as a known very strong modulator. P-gp induction studies proved no induction effects in the P-gp inducible cell models. So the novel mdr modulators are really perspective candidates for clinical investigations because they have not the unfavourable disadvantages of known mdr modulators which failed in clinical trials.

ACKNOWLEDGEMENTS

The work was financially supported by the EU within the COSTB16 action, the BMBF within a bilateral DAAD exchange program and the DFG within the projects HI 687/5-1 and LA 1039/5-1.

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