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Novel structure–activity relationships and selectivity profiling of cage dimeric 1,4-dihydropyridines as multidrug resistance (MDR) modulators

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ABSTRACT

Synthesized series of cage dimeric 1,4-dihydropyridines have been systematically evaluated as MDR modulators in in vitro assays to investigate structure-dependent selectivity properties of inhibiting most cancer-relevant efflux pump proteins. Structure–activity relationships of each P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) 1 and MRP2 inhibition are discussed and prove to be mainly determined by certain aromatic substitution patterns. The characterization of breast cancer resistance protein (BCRP) inhibition results in the discovery of benzyloxy substituted derivatives as selective P-gp inhibitors.

1. Introduction

The main obstacle in cancer treatment of today is the phenomenon of multidrug resistance (MDR).[1,2] Occurring resistances against anticancer drugs which are caused by single mutations are of less importance because structurally varied drugs that show a different binding mode to the anticancer target protein of the drug may compensate such a single resistance problem. The MDR phenomenon concerns numerous structurally different drug families and makes cancer treatment to a significant problem in these cases.[2,3] The main strategy to overcome MDR has been the development of structurally new drugs that are unlikely to be affected by the MDR phenomenon.[2] Even in the case of newest therapeutics like the tyrosine kinase inhibitors or monoclonal antibodies such resistance developments were observed.[2,4–6] Main causative agents of the MDR phenomenon have been transmembrane efflux pumps which transport drugs out of the cells so that therapeutically necessary intracellular drug levels are not reached.[7,8] Most important cancer-relevant efflux pumps are P-glycoprotein (P-gp), the multidrug resistance associated protein (MRP) 1 and the breast cancer resistance protein (BCRP).[8,9] The development of inhibitors of the efflux pump activities remains the main perspective strategy to overcome MDR because alternative efforts like a transcriptional control of the efflux pump protein formation have not been successful so far.[2,10,11]

Most important problems in the clinical application of such inhibitors have been massive side effects and drug interactions which strongly limit their usage so far.[12,13] The undesired side effects mainly result from a nonselective inhibition of the different naturally occurring efflux pumps by such inhibitors.[14] Most inhibitors affect not only a desired tumour-cell relevant overexpressed efflux pump but also other structurally related ones which protect normal cells from toxification. This nonselective inhibition increases the toxicity of co-applicated anticancer drugs which are usually transported out of those normal cells which express such naturally occurring efflux pumps.[15] So undesired side effects result from the use of nonselective efflux pump inhibitors.

The actual strategy in the development of efflux pump inhibitors concentrates on the development of selective inhibitors in the case of P-gp, MRP1 and MRP2, because especially these efflux pumps occur in many human tissues so that a selective inhibition of one targeted efflux pump will result in reduced side effects in therapy.[8,15] However, the sole inhibition of P-gp will not exclude the observation of side effects.

Actually a target-based development of inhibitors is not possible because of missing highly solved structures of the different efflux pump proteins. The only strategy is the investigation of inhibitor-structure dependent effects on the efflux pump inhibition and

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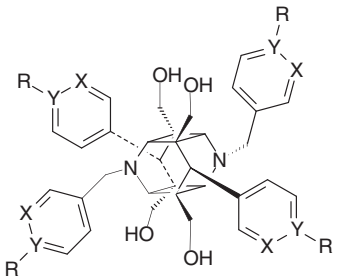


Figure 1.

selectivity to characterize a potential inhibitor binding site of the respective efflux pump protein.

We develop a series of structurally varied cage dimeric 1,4-dihydropyridines as P-gp inhibitors. Similar to recently published quinine and stipiamide homodimers the cage dimers have been derived from monomers, that is, 1,4-dihydropyridines as P-gp inhibitors.[16,17] Monomeric 1,4-dihydropyridines have been early MDR modulators with some P-gp inhibiting activities.[10] They had the disadvantage of retaining calcium antagonistic activities as the original pharmacological activities of this compound class. Therefore, their application led to unfavourable side effects.[10] We varied lipophilic and hydrophilic substituents in our cage dimeric 1,4-dihydropyridines (Fig. 1).

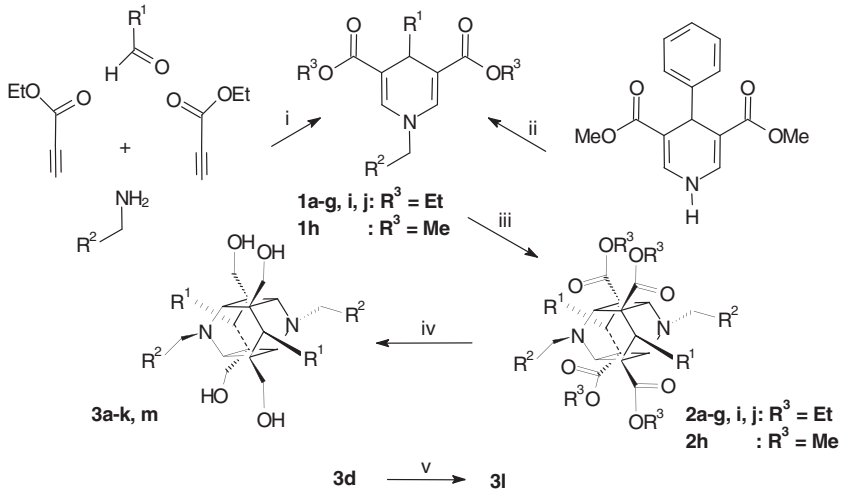
The influence on structure–activity relationships of the P-gp inhibition is discussed. The inhibition of MRP1 and MRP2 has been determined. The selectivity profile of the cage dimers has been strengthened by the characterization of the BCRP inhibition.

2. Results and discussion

2.1. Chemistry

The 1,4-dihydropyridines **1** have been given by a primary cycloaddition reaction of an aromatic aldehyde, ethyl propiolate and, finally, the benzylamine derivative in freshly distiled acetic acid after 2 h of stirring under reflux heating except compound **1h** (Scheme 1). Compound **1h** was yielded by the alkylation of the N-unsubstituted 1,4-dihydropyridine given by the above cyclocondensation reaction with ammonium acetate instead of the benzylamine derivative. The alkylation was carried out in dimethylpropyleneurea. The 1,4-dihydropyridine was treated with a sevenfold excess of sodium hydride and then with a 1.2-fold excess of 4-picolyl bromide.

The cage dimers **2a–j** resulted from a double [2+2]cycloaddition reaction of two corresponding monomers **1** as exclusive reaction products under excitation of the dihydropyridine and the conjugated carbamide ester chromophores, respectively, at irradiation wavelengths >270 nm using Ultra Vitalux® lamps.[18] The cage dimers mainly crystallized from the solutions during the irradiation procedure in THF and gave final yields of about 80% after evaporation



Compd.	R^1	R^2
1a, **2a**, **3a**	phenyl	phenyl
1b, **2b**, **3b**	phenyl	4-methoxyphenyl
1c, **2c**, **3c**	4-methoxyphenyl	4-methoxyphenyl
1d, **2d**, **3d**	4-benzyloxyphenyl	phenyl
1e, **2e**, **3e**	phenyl	4-benzyloxyphenyl
1f, **2f**, **3f**	3-benzyloxyphenyl	phenyl
1g, **2g**, **3g**	phenyl	3-benzyloxyphenyl
1h, **2h**, **3h**	phenyl	4-pyridyl
1i, **2i**, **3i**	4-pyridyl	phenyl
1j, **2j**, **3j**	phenyl	3-pyridyl
1k, **2k**, **3k**	3-hydroxyphenyl	phenyl
1l, **2l**, **3l**	4-hydroxyphenyl	phenyl
1m, **2m**, **3m**	phenyl	4-hydroxyphenyl

Scheme 1. Reagents and conditions: (i) acetic acid, 100 °C, 2 h; (ii) DMPU, NaH, rt, picolylbromide, 30 min; (iii) THF, rt, λ >270 nm; (iv) THF, −18 °C, $LiAlH_4$; (v) TFA, thioanisole, rt.

Table 1
Concentration dependent P-gp, MRP1, MRP2 and BCRP inhibiting properties of target compounds (**3a**–**m**)

Compd	*R* values					
	P-gp		MRP1[a]	MRP2	BCRP	
	1 μM	10 μM	10 μM	10 μM	0.1 μM	1 μM[b]
3a	4.1 ± 0.3	29 ± 3.1[c]	2.3 ± 0.4	1.02 ± 0.14	1.1 ± 0.2	1.9 ± 0.2
3b	3.5 ± 0.6	13 ± 0.3[c]	3.4 ± 0.8	1.10 ± 0.19	1.2 ± 0.2	3.2 ± 0.6
3c	3.1 ± 0.5	46 ± 8.0[c]	4.8 ± 1.0	1.25 ± 0.19	1.5 ± 0.1	6.1 ± 1.7
3d	3.3 ± 1.1	47 ± 2.3	1.0 ± 0.3	1.56 ± 0.66	1.4 ± 0.3	3.5 ± 0.2
3e	2.3 ± 0.3	28 ± 2.5	2.5 ± 0.6	0.75 ± 0.21	n.a.[d]	1.2 ± 0.1
3f	65 ± 8.1	125 ± 16	1.9 ± 0.5	0.97 ± 0.27	1.3 ± 0.3	2.8 ± 0.3
3g	117 ± 1.7	205 ± 6.1	2.8 ± 0.8	0.97 ± 0.26	1.5 ± 0.1	4.2 ± 0.2
3h	1.5 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	0.80 ± 0.23	n.a.[d]	1.3 ± 0.2
3i	2.4 ± 0.1	7.7 ± 0.4	2.2 ± 0.7	0.74 ± 0.29	n.a.[d]	1.2 ± 0.1
3j	1.2 ± 0.1	1.1 ± 0.5	1.1 ± 0.1	0.90 ± 0.29	n.a.[d]	n.a.[d]
3k	1.2 ± 0.1	3.0 ± 0.2	1.3 ± 0.3	1.05 ± 0.43	n.a.[d]	1.1 ± 0.2
3l	1.3 ± 0.4	1.5 ± 0.7	1.8 ± 0.6	0.63 ± 0.18	n.a.[d]	1.5 ± 0.2
3m	1.5 ± 0.1	3.3 ± 0.2	1.6 ± 0.4	0.82 ± 0.06	n.a.[d]	1.1 ± 0.1
Verapamil	4.0 ± 1.4	5.1 ± 0.2	n.a.[d]	n.a.[d]	n.a.[d]	n.a.[d]
Indomethacin	n.d.[e]	n.d.[e]	4.1 ± 0.4	0.75 ± 0.14	n.d.[e]	n.d.[e]
Fumitremogin c	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	1.9 ± 0.4	6.0 ± 1.1

[a] Calculated *R* values are the quotients of each measured fluorescence intensity in the inhibitor-treated cells and of that in the inhibitor-untreated control.
[b] Activity saturation concentration.
[c] Activity saturation effects.
[d] Not active.
[e] Not determined.

of the solvent under atmosphere pressure. The pyridyl-containing monomers **1h** and **j** gave similar higher yields of the corresponding dimers after evaporation whereas the oily compound **1i** resulted in a lower dimer yield with some side products being detectable by tlc.

The alcoholic target compounds **3a–k** and **m** were given by a lithium aluminium hydride reaction of the ester functions to hydroxymethylene groups and a further reductive debenzylation in the case of yielded compounds **3k** and **m**. The reactions were carried out in dried THF at low temperatures of −18 °C. The pyridine nucleus of the pyridyl-containing derivatives remained untouched at that temperature. The total yields of almost about 60% of the target compounds **3** have been isolated from chloroform solutions of the oily residues after hydrolysis and solvent evaporation. The 4-hydroxyphenyl substituted alcoholic target dimer **3l** was yielded from the 4-benzyloxyphenyl derivative **3d** by an acid catalyzed debenzylation procedure using trifluoroacetic acid and thioanisole.

2.2. Structure–activity relationships (SAR) of the P-gp inhibition

The P-gp inhibition was determined as the extent of the cellular uptake of the fluorescent P-gp substrate rhodamine 123 both into the P-gp expressing mouse T lymphoma cell line and the not P-gp expressing parental cell line under inhibitor treatment and with each fluorescence related to the inhibitor-untreated control. The P-gp expression resulted from retrovirus transfection with the human mdr1 gene so that it is assured that an increase in fluorescence uptake can only result from a P-gp inhibition and a consequent lowered efflux from the P-gp expressing cell line compared to the parental cell line.

The only phenyl substituted derivative **3a** was demonstrated to show strong inhibition properties both at the lowered and the higher inhibitor concentrations compared to verapamil used as control (Table 1).[19] Nifedipine as a known P-gp inhibitor of the 1,4-dihydropyridine type practically showed no activities at a concentration of 10 μM with a *R* value of 1.0 ± 0.1. So already the only phenyl substituted dimeric 1,4-dihydropyridine **3a** is much more active than a monomeric 1,4-dihydropyridine.

The introduction of a methoxy function into two phenyl residues of derivative **3a** resulted in similar activities of derivative **3b** at the lower concentrations and some reduced activity at the higher concentrations if compared to the only phenyl substituted derivative **3a**. Two additional methoxy functions in compound **3c** mainly increased the inhibition at the higher inhibitor concentration. So as a final considering of the effect of methoxy functions on the P-gp inhibition properties of the new compound class it can be stated that the introduction of such methoxy functions is of a significant favour for the P-gp inhibition. Methoxy functions in MDR modulators have been discussed to serve as hydrogen bond acceptor functions to amino acid residues of the protein backbone of a potential P-gp binding region.[9,20,21]

Moreover, the observed SAR of increased numbers of methoxy functions in our class of MDR modulators that result in higher activities suggests an important hydrogen binding of the compounds to a certain P-gp binding site.

Another common feature of MDR modulators that has been discussed to be of favour for the biological activities of generally inhibiting transmembrane efflux pumps has been an increased lipophilicity.[9] Such an increased lipophilicity facilitates a membrane penetration of an inhibitor-acting MDR modulator to reach a transmembrane efflux pump protein binding site within the membrane. An increased lipophilicity in P-gp inhibitors has been discussed to directly influence the biological activity by a better P-gp binding to the P-gp binding site which is accessible from the membrane. However, we recently demonstrated that an increased lipophilicity of 3-benzyloxy-1-aza-9-oxafluorenes as a novel class of P-gp inhibitors did not lead to an increased biological activity.[22] Both possible lipophilic interactions and additionally hydrogen bonding via hydrogen bond acceptor functions play a central role in the P-gp binding of inhibitors. 1,4-Dihydropyridines were demonstrated to bind to the P-gp binding site in the cytosolic membrane leafleft.[23] Benzyloxy instead of methoxy functions have been introduced into our cage dimers to investigate the lipophilicity influence on biological activity. However, the exact positioning of the benzyloxy substituent within the phenyl residues was found to play an important role.[19] The positioning of the benzyloxy substituent into the 3-position of the benzyl ring resulted in a main

increase in activity of compound **3g**. A similar increase was also observed for derivative **3f** if compared to **3d** that was found as active as the methoxy derivative **3c** with the double number of methoxy functions. So beside the hydrogen bond acceptor functions also the additional benzyl substituents contribute to the biological activities presumably by a better membrane penetration as discussed above. We also calculated lipophilicity constants as log *P* values of our cage dimers (Table 2). All the four benzyloxy derivatives show a mainly increased lipophilicity with log *P* values of about 6.5. The observed importance of an exact positioning within the molecular scaffold which led to the observed significant differences in the biological activities may be caused by hydrophobic interactions with the amino acid residues of the respective P-gp binding site.[19]

The results clearly show that both lipophilic properties and hydrogen bond acceptor functions mainly attribute to the P-gp inhibiting activities. The more lipophilic substitution of the benzyloxy substituted derivatives **3d**–**g** resulted in higher inhibitory activities than the less lipophilic substitution in the corresponding methoxy substituted derivatives **3b** and **c** with calculated log *P* values of about 3.6. In the case of the less lipophilic methoxy derivatives the increase in methoxy groups as hydrogen bond acceptor functions led to activity data of compound **3c** similar the those of the benzyloxy substituted derivative **3d** with a higher log *P* value.

As the methoxy functions as hydrogen bond acceptor functions were found favourable for the P-gp inhibiting properties we alternatively introduced nitrogen atoms into the phenyl residues to get more insight in the SAR. Moreover, the introduction of the protonable nitrogen functions was suggested to be of favour because most P-gp inhibitors have protonable nitrogen functions within their molecular skeletons.[24] Surprisingly, an introduction of a 4-picolyl residue in derivative **3h** led to a main decrease in activity if compared to the methoxybenzyl substituted derivative **3b**. A movement of the nitrogen atom from the 4- to the 3-position of the benzyl residue practically caused no changes in activity. In the case of the benzyloxy substituents the position within the benzylic residue resulted in main changes in activity. Consequently, it can be concluded from the SAR that a role of the nitrogen atoms as hydrogen bond acceptor functions seems to be unlikely. Furthermore, it can be concluded that the activities of the benzyloxy derivatives are mainly caused by their ability to undergo hydrophobic interactions with amino acid residues of the respective P-gp binding site. When the nitrogen atom was presented in the 4-position of the phenyl residue in compound **3i** instead of the benzyl residue increases in activity were observed. However, the activity was even lower than that of the methoxy substituted derivative **3b**.

Another aspect in the SAR of MDR modulators is the influence of hydrogen bond donator functions on the biological activities. So far the influence of such functional groups has not been systematically investigated within the various MDR modulator classes.[25] We introduced hydroxy functions into both the 3- and the 4-position of the aromatic residues to investigate this influence. The activities of the resulting derivatives **3k–m** were poor and independent from the positioning of the hydroxy functions. The calculated log *P* values of about 3.0 were similar to those of the methoxyphenyl substituted derivatives **3b** and **c** which showed mainly increased activities. These results clearly suggest that hydroxy functions as hydrogen bond donator functions are unfavourable as reported previously.[20]

So finally, it can be concluded that the new compound class of P-gp inhibitors addresses a certain hydrophobic binding site which is sensitive for the exact positioning of the lipophilic aromatic residues for binding interactions.

Table 2
Lipophilicity constant (log *P* value) calculation data of target compounds (**3a**–**m**)

Compd	log *P* value
3a	3.8 ± 0.3
3b	3.6 ± 0.4
3c	3.6 ± 0.6
3d	6.5 ± 1.0
3e	6.5 ± 1.0
3f	6.4 ± 1.0
3g	6.4 ± 1.0
3h	1.5 ± 0.4
3i	1.6 ± 0.3
3j	1.5 ± 0.4
3k	3.0 ± 0.2
3l	3.0 ± 0.2
3m	3.0 ± 0.2

2.3. MDR modulator properties towards MRP1 and MRP2

MRP1 and P-gp have a great overlap in their anticancer drug profiles which means that most P-gp substrates are also MRP1 substrates. Most inhibitors of P-gp also inhibit MRP1 so that a selectivity of inhibition is not given which leads to severe side effects by such dual inhibitors.

So it was of great interest to investigate the ability of our novel cage dimers to inhibit MRP1 to work out structural criteria for a selective P-gp inhibition and no MRP1 inhibition.

Although MRP2 does not play a central role in the MDR it is a naturally spread efflux pump in human tissues that should not be affected by a selective P-gp inhibitor to reduce side effects under co-application of anticancer drugs which are MRP2 substrates.

The MRP inhibiting properties of the cage dimers have been characterized by the determination of the uptake of the MRP specific substrate carboxyfluorescein using the MRP1 overexpressing ovarial carcinoma cell line A2780P and for the determination of the MRP2 inhibiting properties using the MRP2 overexpressing subline A2780RCIS which was induced to overexpress MRP2 after treatment with cisplatin. The MRP substrate specificity of carboxyfluorescein ensured that changes in the extent of the fluorescent carboxyfluorescein uptake exclusively resulted from an inhibition of MRP1 in the parental cell line A2780P whereas the MRP2 specific extent of fluorescence uptake was given by substraction of the value of the MRP1 specific extent of fluorescence uptake into the parental cell line from the whole extent of fluorescence determined as MRP1 and MRP2 inhibition in the induced cell line A2780RCIS.

The only phenyl substituted derivative **3a** was an inhibitor of MRP1 although its activity at 10 μM made only half of the activity than that of the indomethacin control (Table 1). Verapamil is a known inhibitor of both MRP1 and MRP2 at high concentrations >100 μM.[26] We found a consequent increase in the carboxyfluorescein uptake with increasing methoxy functions in compounds **3b** and, finally, **3c** so that compound **3c** was a slightly better inhibitor than indomethacin. So the extent of methoxy functions as hydrogen bond acceptor functions plays a central role in the MRP1 inhibiting properties suggesting a better binding to amino acid residues of the MRP1 binding site with increasing numbers of such hydrogen bond acceptor functions. This is an important finding in the SAR of MRP1 inhibitors because less is known about structural features which strengthen the MRP1 inhibiting properties of inhibitors.

Within the inhibitory activities of the benzyloxy substituted compounds interesting SAR could be found. Both phenyl substituted derivatives **3d** and **f** were practically no MRP1 inhibitors, whereas the benzyl substituted derivatives **3e** and **g** showed weak inhibition of MRP1. Within the group of the benzyloxy substituted compounds the 3-substituted derivatives were more active than the poorly or not active 4-substituted derivatives. In contrast to the P-gp inhibiting properties which were found mainly increased

in case of the lipophilic benzyloxy substituted compounds the more lipophilic substitution in these compounds is less favourable for a binding to the potential MRP1 binding region. This suggests that the MRP1 binding region of our compounds is less lipophilic than the P-gp binding region. Another remarkable feature is the fact that a more lipophilic compound character for a possibly better membrane penetration to reach the potential MRP1 binding site is not of favour for a MRP1 inhibiting compound if compared to the structural necessities of a compound to inhibit P-gp.

From the pyridyl substituted compounds only the 4-pyridyl substituted derivative **3i** showed some MRP1 inhibiting activities whereas the picolyl substituted compounds **3j** and **h** were practically no MRP1 inhibitors. Similar results were found for the hydroxyphenyl derivatives **3k–m** with the 4-hydroxyphenyl substituted derivative **3l** showing weak activities.

So our systematical investigations gave interesting insight in the SAR of MRP1 inhibitors. Methoxy functions as hydrogen bond acceptor functions play an important role in activity whereas more lipophilic substituents are not favourable.

The investigations of the MRP2 inhibitions showed an increase in the MRP2 inhibiting properties from **3a** with no additional methoxy function to **3b** with two methoxy functions and, finally, to **3c** with four methoxy functions. So hydrogen bond acceptor functions also play an important role in the inhibitor binding to the MRP2 binding site. From the four benzyloxy substituted derivatives **3d–g** only the 4-phenyl substituted compound **3d** had some MRP2 inhibiting properties. Within the group of pyridyl substituted derivatives **3h–j** only poor activities were found and within the group of the hydroxyphenyl substituted derivatives only the compound **3k** showed some activities.

The SAR in the MRP2 inhibition profile within our new class of MDR modulators are similar to those of the MRP1 inhibition with an importance of methoxy functions as hydrogen bond acceptor functions and unfavourable more lipophilic substitutions so that generally the benzyloxy substituted derivatives that have been strong P-gp inhibitors are poor or practically no inhibitors of neither MRP1 nor MRP2. Thus they turn out as ideal selective P-gp inhibitors.

2.4. BCRP inhibition profiling

If compared to P-gp or MRP1 the tissue spread of BCRP in humans is limited to liver and intestine.[8,15] The expression of BCRP has been reported in some solid tumours.[27] However, the role of BCRP in the emergence of MDR is yet under discussion.[2] We determined the inhibition profile of our new P-gp inhibitors using the fluorescent BCRP substrate mitoxantrone in comparison of the fluorescence extent uptake into the inhibitor-treated BCRP induced gastric carcinoma cell line EPG85-257RNOV and the non-BCRP-overexpressing parental cell line EPG85-257P each related to the untreated control. We used the fungal toxin fumitremorgin c as inhibitor in relevant BCRP inhibiting concentrations up to saturation effects (Table 2).

The allover phenyl substituted compound **3a** is only a poor BCRP inhibitor at the higher concentrations if compared to the control. Similar to the inhibition of MRP1 and MRP2, respectively, we found an increase of BCRP inhibiting activities with an increasing number of methoxy functions from derivative **3a**–**c**. The benzyloxy substituted compounds show some BCRP inhibiting activities at higher concentrations. Interestingly, the 4-benzyl substituted derivative **3e** is practically no BCRP inhibitor. The positioning of the benzyloxy substituent within the molecular scaffold is also of importance for the BCRP inhibition which will result from an interaction with the amino acids of the potential BCRP binding site. If presented in the 3-position of the benzyl substituent in derivative **3g** we found moderate activities and if moved to the 4-position in compound **3e** the activities decreased. All of the pyridyl substituted derivatives **3h–j** and the hydroxy substituted derivatives **3k–m** were practically no BCRP inhibitors at the given concentrations.

So we found interesting SAR of the BCRP inhibition profile of our novel cage dimeric MDR modulators suggesting hydrogen bonding to the amino acids of the protein backbone via the methoxy functions and to hydrophobic regions via the benzyloxy substituents.

3. Conclusions

We systematically characterized SAR of a novel class of MDR modulators towards P-gp, MRP1, MRP2 and BCRP. As highly solved structures of the various efflux pump proteins do not exist essential SAR for an important selective efflux pump inhibition could only be investigated by a variation of functional groups within the relevant aromatic substituents which were found essential for a good biological activity, that is, methoxy functions and nitrogen atoms which may act as hydrogen bond acceptor functions and hydroxy functions as additional hydrogen bond donator functions.

Methoxy groups as hydrogen bond acceptor functions played an important role in the inhibition activities towards all efflux pump proteins. Whereas the P-gp inhibition was strengthened by more lipophilic benzyloxy substituents, the MRP inhibition was mainly reduced, so that selectively binding compounds **3d–g** were found. The derivatives **3f** and **g** with a 3-positioned benzyloxy substituent showed the comparable strongest differences in activity of P-gp and MRP1/MRP2 inhibition, respectively, and thus are outstanding selective efflux pump inhibitors.

The BCRP profiling suggested a hydrophobic binding site for an exact inhibitor binding and led to the characterization of the 4-benzyloxy derivative **3e** as overall selective P-gp inhibitor. Another selective P-gp inhibitor with no inhibition of MRP1, MRP2 and BCRP was the 4-pyridyl substituted derivative **3i**.

So beside the discovery of important selective P-gp inhibitors, novel insight in the SAR of MDR modulators was given by the inhibitor binding towards the hitherto not characterized binding sites of the efflux pumps MRP1, MRP2 and BCRP.

4. Experimental

4.1. Chemistry

All the chemical agents used were either synthesized or have been commercially available. Melting points were determined using a Boetius melting desk microscope and are uncorrected. Proton NMR spectra were recorded on a Varian Gemini 2000 at 200 or 400 MHz, respectively. Chemical shifts are reported in ppm units with tetramethylsilane as internal reference standard. Mass spectra were recorded on an AMD 402 mass spectrometer named AMD INTEGRA. Elemental analyses (C, H, N) were carried out with a Leco analyzer apparatus (CHN-932) and the results were within ±0.4% of the theoretical values. The synthesis of compounds **1a**,**b**, **d–f**, **2a**,**b**,**d–f** and **3a**,**b**,**d–f** has been demonstrated.[18]

4.1.1. General procedure for the preparation of the 4-aryl- and 4-heteroaryl-1,4-dihydropyridines (1c,i,j)

Either the aromatic or the heteroaromatic aldehyde (10 mmol), ethyl propiolate (20 mmol) and the benzylamine derivative (10 mmol) were dissolved in freshly distiled acetic acid (1 mL) under heating.[18] The solution was then refluxed for 2 h on a steam bath. After cooling to rt sufficient water was added and extraction was carried out with portions of chloroform (50 mL) for several times. After drying over sodium sulfate and filtration the solvent was removed in vacuum. The remaining oil was dissolved in dried ethanol from which the 1,4-dihydropyridine crystallized on cooling.

4.1.1.1. Ethyl 1-(4-methoxyphenymethyl)-4-(4-methoxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylate (1c). Yellow crystals, mp 101–103 °C (ethanol); yield 84%; MS (ESI) *m/z* 474 ($M+Na^+$, 15%), 925 ($2\,M+Na^+$, 100%). ^1H NMR ($CDCl_3$) δ 1.16 (t, J = 7.3 Hz, 6H, $COOCH_2CH_3$), 3.74, 3.81(2 × s, 6H, OCH_3), 4.05 (q, J = 7.3 Hz, 4H, $COOCH_2CH_3$), 4.49 (s, 2H, NCH_2), 4.81 (s, 1H, 4-H), 6.71–6.92 (2 × m, 4H, 3-, 5-H of Ph), 7.14–7.20 (2 × m, 4H, 2-, 6-H of Ph), 7.22 (s, 2H, 2-, 6-H). Anal. Calcd for $C_{26}H_{29}NO_6$: C, 69.16; H, 6.47; N, 3.10. Found: C, 69.39; H, 6.64; N, 3.50.

4.1.1.2. Ethyl 1-benzyl-4-(4-pyridyl)-1,4-dihydropyridine-3,5-dicarboxylate (1i). Brownish oil (petrol ether); yield 8%; MS (ESI) *m/z* 415 ($M+Na^+$, 100%). ^1H NMR ($CDCl_3$) δ 1.15 (t, J = 7.2 Hz, 6H, $COOCH_2CH_3$), 4.04 (q, J = 7.2 Hz, 4H, $COOCH_2CH_3$), 4.57 (s, 2H, NCH_2), 4.88 (s, 1H, 4-H), 7.13–7.41 (m, 9H, 2-, 6-H of pyr, ArH of Ph, 2-, 6-H), 8.45 (m, 2H, 3-, 5-H of pyr). Anal. Calcd for $C_{23}H_{24}N_2O_4$: C, 70.39; H, 6.16; N, 7.13. Found: C, 69.99; H, 5.87; N, 7.49.

4.1.1.3. Ethyl 4-phenyl-1-(3-picolyl)-1,4-dihydropyridine-3,5-dicarboxylate (1j). Brown prisms, mp 161–164 °C (diethyl ether); yield 6%; MS (ESI) *m/z* 393 ($M+H^+$, 100%). ^1H NMR ($CDCl_3$) δ 1.15 (t, J = 7.0 Hz, 6H, $COOCH_2CH_3$), 4.05 (q, J = 7.0 Hz, 4H, $COOCH_2CH_3$), 4.62 (s, 2H, NCH_2), 4.89 (s, 1H, 4-H), 7.12–7.24 (m, 7H, ArH of Ph, 2-, 6-H), 8.41 (m br, 1H, 5-H of pyr), 7.68 (d, J = 8.5 Hz, 1H, 6-H of pyr), 8.61 (m br, 2H, 2-, 4-H of pyr). Anal. Calcd for $C_{23}H_{24}N_2O_4$: C, 70.39; H, 6.16; N, 7.13. Found: C, 70.15; H, 5.95; N, 6.88.

4.1.2. Methyl 4-phenyl-1-(4-picolyl)-1,4-dihydropyridine-3,5-dicarboxylate (1h)

The N-unsubstituted methyl 4-phenyl-1,4-dihydropyridine-3,5-dicarboxylate[28] (0.85 g, 3.1 mmol) was dissolved in a minimum volume of dry dimethylpropyleneurea. A sevenfold molar excess of sodium hydride as a petrol ether (bp 30–50 °C) washed suspension in oil (80%) was added. After stirring at 50 °C, the solution was cooled to rt and a threefold molar excess of 4-picolyl bromide hydrobromide was added in portions over a period of 30 min. After additional stirring at rt the excess of sodium hydride was hydrolysed with ice water. The precipitating oil was filtered and dissolved in chloroform after washing with water for several times. After removal of the dried organic layer the oily residue was purified by column chromatography over silica gel with an eluent mixture of chloroform/acetic acid ethyl ester/methanol (75:25:5). After removal of the eluent mixture in vacuum the compound crystallized from diethyl ether in white prisms, mp 209–211 °C; yield 38%; MS (ESI) *m/z* 365 ($M+H^+$, 100%). ^1H NMR ($CDCl_3$) δ 3.16 (s, 6H, $COOCH_3$), 4.61 (s, 2H, NCH_2), 4.93 (s, 1H, 4-H), 7.14–7.29 (m, 9H, ArH of Ph, 2-, 6-H of pyr, 2-, 6-H), 8.65 (m, 2H, 3-, 5-H of pyr). Anal. Calcd for $C_{21}H_{20}N_2O_4$: C, 69.22; H, 5.53; N, 7.69. Found: C, 69.35; H, 5.83; N, 7.36.

4.1.3. General procedure for the preparation of the cage dimeric alkyl 4-aryl- or 4-heteroaryl-1,4-dihydropyridine-3,5-dicarboxylates (2c,h–j)

Monomeric derivatives **1** (1.2 mmol) have been dissolved in 30 mL of dry THF in a quartz flask. Irradiation of the solutions was carried out under nitrogen atmosphere using Ultra Vitalux lamps® at wavelengths λ >270 nm with an irradiation distance of 60 cm.[18] During irradiation most of the cage dimers crystallized from the solutions and irradiation was continued until no more starting compound could be detected by tlc. During evaporation of the solvent under atmosphere pressure the isolated yields of the cage dimers **2** were collected and recrystallized from alcohol.

4.1.3.1. Tetraethyl 3,9-bis(4-methoxyphenylmethyl)-6,12-bis(4-methoxyphenyl)-3,9-diazahexacyclo[6.4.0.0$^{2.7}$.0$^{4.11}$.0$^{5.10}$]dodecane-1,5,7,11-tetracarboxylate (2c). White solid; mp 238–241 °C (ethanol); yield 75%; MS(ESI) *m/z* 903 ($M+H^+$, 20%), 925 ($M+Na^+$, 100%). ^1H NMR ($CDCl_3$) δ 1.01 (t, J = 7.1 Hz, 12H, $COOCH_2CH_3$), 3.73, 3.78 (2 × s, 12H, OCH_3), 3.97 (q, J = 7.1 Hz, 8H, $COOCH_2CH_3$), 4.21 (s, 2H, 6-, 12-H), 4.22 (s, 4H, 2-, 4-, 8-, 10-H), 4.39 (s, 4H, NCH_2), 6.60–6.82 (2 × m, 8H, 3-, 5-H of ArH), 7.12–7.24 (2 × m, 8H, 2-, 6-H of ArH). Anal. Calcd for $C_{52}H_{58}N_2O_{12}$: C, 69.16; H, 6.47; N, 3.10. Found: C, 69.08; H, 6.54; N, 3.48.

4.1.3.2. Tetramethyl 3,9-bis(4-picolyl)-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0$^{2.7}$.0$^{4.11}$. 0$^{5.10}$]dodecane-1,5,7,11-tetracarboxylate (2h). White solid; mp 255–262 °C (ethanol); yield 83%; MS(ESI) *m/z* 729 ($M+H^+$, 100%). ^1H NMR ($CDCl_3$) δ 3.54 (s, 12H, $COOCH_3$), 4.24 (s, 4H, 2., 4-, 8-, 10-H), 4.25 (s, 2H, 6-, 12-H), 4.48 (s, 4H, NCH_2), 7.11–7.15 (m, 14H, ArH of Ph, 2-, 6-H of pyr), 8.48 (m, 4H, 3-, 5-H of pyr). Anal. Calcd for $C_{42}H_{40}N_4O_8$: C, 69.22; H, 5.53; N, 7.69. Found: C, 69.19; H, 5.55; N, 7.65.

4.1.3.3. Tetraethyl 3,9-dibenzyl-6,12-bis(4-pyridyl)-3,9-diazahexacyclo[6.4.0.0$^{2.7}$.0$^{4.11}$. 0$^{5.10}$]dodecane-1,5,7,11-tetracarboxylate (2i). White solid; mp 225–233 °C (methanol); yield 13%; MS(ESI) *m/z* 785 ($M+H^+$, 100%). ^1H NMR ($CDCl_3$) δ 0.96 (t, J = 7.0 Hz, 12H, $COOCH_2CH_3$), 3.95 (q, J = 7.0 Hz, 8H, $COOCH_2CH_3$), 4.26 (s, 4H, 2-, 4-, 8-, 10-H), 4.27 (s, 2H, 6-, 12-H), 4.47 (s, 4H, NCH_2), 7.19–7.29 (m, 14H, ArH of Ph, 2-, 6-H of pyr), 8.20 (m, 4H, 3-, 5-H of pyr). Anal. Calcd for $C_{46}H_{48}N_4O_8$: C, 70.39; H, 6.16; N, 7.13. Found: C, 70.55; H, 6.48; N, 7.19.

4.1.3.4. Tetraethyl 3,9-bis(3-picolyl)-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0$^{2.7}$.0$^{4.11}$.0$^{5.10}$]dodecane-1,5,7,11-tetracarboxylate (2j). White solid; mp 249–250 °C (ethanol); yield 49%; MS(ESI) *m/z* 785 ($M+H^+$, 100%). ^1H NMR ($CDCl_3$) δ 0.97 (t, J = 7.2 Hz, 12H, $COOCH_2CH_3$), 3.99 (q, J = 7.2 Hz, 8H, $COOCH_2CH_3$), 4.24 (s, 4H, 2-, 4-, 8-, 10-H), 4.25 (s, 2H, 6-, 12-H), 4.48 (s, 4H, NCH_2), 7.10–7.20 (m, 12H, ArH of Ph, 5-H of pyr), 7.59 (d, J = 7.3 Hz, 2H, 6-H of pyr), 8.51 (d, J = 8.5 Hz, 2H, 4-H of pyr), 8.55 (s, 2H, 1-H of pyr). Anal. Calcd for $C_{46}H_{48}N_4O_8$: C, 70.39; H, 6.16; N, 7.13. Found: C, 70.25; H, 6.28; N, 7.33.

4.1.4. General procedure for the preparation of the alcoholic dimeric 4-aryl- and 4-heteroaryl-1,4-dihydropyridines (3c,h–k,m)

Cage dimers **2** (0.07 mmol) were dissolved in 20 mL of dry THF under heating. After stirring at rt for 1 h the solution was cooled down to −18 °C and a 1.1-fold molar excess of lithium aluminium hydride (1 M solution in THF) was added dropwise. After additional stirring at the low temperature the solution was diluted with ice water at 0 °C and extracted with chloroform for several times. The organic layer was then dried over sodium sulfate and filtered. After evaporation to dryness the oily residue was dissolved in chloroform. Then diethyl ether and petrol ether (bp 30–50 °C) were added dropwise until turbidity. The resulting precipitating crystalline alcoholic compounds **3** were recrystallized from methanol.

4.1.4.1. 1,5,7,11-Tetrakishydroxymethyl-3,9-bis(4-methoxyphenylmethyl)-6,12-bis(4-methoxyphenyl)-3,9-diazahexacyclo-[6.4.0.0$^{2.7}$.0$^{4.11}$.0$^{5.10}$]dodecane (3c). White crystals; mp 238–241 °C; yield 75%; MS(ESI) *m/z* 735 ($M+H^+$, 62%), 757 ($M+Na^+$, 100%). ^1H NMR (DMSO-d_6) δ 2.89 (s, 4H, 2-, 4-, 8-, 10-H), 3.06 (ABX, J = 10.2, 4.4 Hz, 4H, CH_BOH), 3.15 (ABX, J = 10.2, 4.4 Hz, 4H, CH_AOH), 3.58 (s, 2H, 6-, 12-H), 3.70, 3.74 (2 × s, 12H, OCH_3), 4.03 (s, 4H, NCH_2), 4.35 (t, J = 4.4 Hz, 4H, OH), 6.51–6.68 (m, 4H, 3-, 5-H of C6-,C12-Ph), 6.86–6.89 (m, 4H, 3-, 5-H of NCH_2Ph), 7.16–7.71 (m, 8H, 2-, 6-H of Ph). Anal. Calcd for $C_{44}H_{50}N_2O_8$: C, 71.91; H, 6.86; N, 3.81. Found: C, 71.68; H, 6.66; N, 3.57.

4.1.4.2. 1,5,7,11-Tetrakishydroxymethyl-3,9-bis(4-picolyl)-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3h). White crystals; mp 291–296 °C; yield 65%; MS(ESI) *m/z* 617 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.94 (s, 4H, 2-, 4-, 8-, 10-H), 3.12–3.20 (m, ABX, 8H, C*H*$_{AB}$OH), 3.66 (s, 2H, 6-, 12-H), 4.17 (s, 4H, *N*CH$_2$), 4.53 (t, *J* = 4.5 Hz, 4H, OH), 7.00–7.17 (m, 8H, 2- or 6-H, 3-, 4-, 5-H of Ph), 7.30–7.50 (m, 4H, 2-, 6-H of pyr), 7.70 (d, *J* = 7.5 Hz, 2H, 2- or 6-H of Ph), 8.84 (m, 2H, 3-, 5-H of pyr). Anal. Calcd for $C_{38}H_{40}N_4O_4$: C, 74.00; H, 6.54; N, 9.08. Found: C, 73.66; H, 6.35; N, 8.84.

4.1.4.3. 3,9-Dibenzyl-1,5,7,11-tetrakishydroxymethyl-6,12-bis(4-pyridyl)-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3i). White solid; mp 228–237 °C; yield 55%; MS(ESI) *m/z* 617 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.94 (s, 4H, 2-, 4-, 8-, 10-H), 3.06 (ABX, *J* = 10.2, 4.5 Hz, 4H, C*H*$_B$OH), 3.16 (ABX, *J* = 10.2, 4.5 Hz, 4H, C*H*$_A$OH), 3.64 (s, 2H, 6-, 12-H), 4.11 (s, 4H, *N*CH$_2$), 4.41 (t, *J* = 4.5 Hz, 4H, OH), 7.21–7.30 (m, 12H, ArH of Ph, 2- or 6-H of pyr), 7.76 (d, *J* = 7.8 Hz, 2H, 2- or 6-H of pyr), 8.28 (d, *J* = 7.6 Hz, 4H, 3-, 5-H of pyr). Anal. Calcd for $C_{38}H_{40}N_4O_4$: C, 74.00; H, 6.54; N, 9.08. Found: C, 73.76; H, 6.66; N, 9.11.

4.1.4.4. 1,5,7,11-Tetrakishydroxymethyl-3,9-bis(3-picolyl)-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3j). White crystals; mp 280–291 °C; yield 68%; MS(ESI) *m/z* 617 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.95 (s, 4H, 2-, 4-, 8-, 10-H), 3.10 (ABX, *J* = 10.4, 4.5 Hz, 4H, C*H*$_B$OH), 3.19 (ABX, *J* = 10.4, 4.5 Hz, 4H, C*H*$_A$OH), 3.67 (s, 2H, 6-, 12-H), 4.14 (s, 4H, *N*CH$_2$), 4.52 (t, *J* = 4.5 Hz, 4H, OH), 6.96 (dd, *J* = 7.2, 5.0 Hz, 2H, 5-H of pyr), 7.04–7.15 (m, 6H, 3-, 4-, 5-H of Ph), 7.29 (d, *J* = 7.9 Hz, 2H, 2- or 6-H of Ph), 7.66 (d, *J* = 7.2 Hz, 2H, 6-H of pyr), 7.71 (d, *J* = 8.1 Hz, 2H, 2- or 6-H of Ph), 8.45 (d, *J* = 5.0 Hz, 2H, 4-H of pyr), 8.51 (s, 2H, 2-H of pyr). Anal. Calcd for $C_{38}H_{40}N_4O_4$: C, 74.00; H, 6.54; N, 9.08. Found: C, 73.86; H, 6.52; N, 8.69.

4.1.4.5. 3,9-Dibenzyl-1,5,7,11-tetrakishydroxymethyl-6,12-bis(3-hydroxyphenyl)-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3k). White crystals; mp 179–184 °C; yield 12%; MS(ESI) *m/z* 647 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.91 (s, 4H, 2-, 4-, 8-, 10-H), 3.07–3.10 (m, ABX, 4H, C*H*$_B$OH), 3.14–3.18 (m, ABX, 4H, C*H*$_A$OH), 3.60 (s, 2H, 6-, 12-H), 4.16 (s, 4H, *N*CH$_2$), 4.32–4.43 (m, 4H, CH$_2$O*H*), 6.44, 6.51 (2 × d, *J* = 8.1 Hz, 2H, 4-H of Ph), 6.71–6.76 (m, 2H, 5-H of Ph), 7.21 (d, *J* = 7.6 Hz, 1H, 6-H of Ph), 7.29–7.32 (m, 11H, ArH of Bz, 2-H of Ph), 7.39 (d, *J* = 7.6 Hz, 1H, 6-H of Ph), 7.46 (s, 1H, 2-H of Ph), 8.78, 8.87 (2 × s, 2H, Ph-OH). Anal. Calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33. Found: C, 73.97; H, 6.35; N, 3.99.

4.1.4.6. 3,9-Bis(4-hydroxybenzyl)-1,5,7,11-tetrakishydroxymethyl-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3m). White crystals; mp 241–246 °C; yield 25%; MS(ESI) *m/z* 647 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.90 (s, 4H, 2-, 4-, 8-, 10-H), 3.05–3.08 (m, ABX, 4H, C*H*$_B$OH), 3.14–3.16 (m, ABX, 4H, C*H*$_A$OH), 3.65 (s, 2H, 6-, 12-H), 4.00 (s, 4H, *N*CH$_2$), 4.38–4.42 (m, 4H, CH$_2$O*H*), 6.68, (d, *J* = 8.4 Hz, 4H, 3-, 5-H of Bz), 6.94–7.01 (m, 2H, 3-, 5-H of Ph), 7.05 (t, *J* = 7.2 Hz, 2H, 4-H of Ph), 7.09–7.14 (m, 6H, 2-, 6-H of Bz, 3-, 5-H of Ph), 7.24 (d, *J* = 7.0 Hz, 2H, 2-, 6-H of Ph), 7.83 (d, *J* = 7.6 Hz, 2H, 2-, 6-H of Ph), 9.17 (s, 2H, Ph-OH). Anal. Calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33. Found: C, 74.33; H, 6.48; N, 4.14.

4.1.5. 3,9-Dibenzyl-1,5,7,11-tetrakishydroxymethyl-6,12-bis(4-hydroxyphenyl)-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (3l)

Cage dimer **3d** (20 mg, 0.02 mmol) was dissolved in a mixture of thioanisole (0.12 mL, 0.1 mmol) and trifluoroacetic acid (0.4 mL) and the solution was stirred at rt until no more of the starting compound was detectable by tlc. Then the solvent was removed in vacuum and the resulting oil was taken up in methanol from which compound **3l** crystallized in yellow prisms under cooling, mp 245–252 °C; yield 32%; MS(ESI) *m/z* 647 (M+H$^+$, 100%). ^{1}H NMR (DMSO-d_6) δ 2.90 (s, 4H, 2-, 4-, 8-, 10-H), 3.09 (ABX, *J* = 10.5, 4.5 Hz, 4H, C*H*$_B$OH), 3.16 (ABX, *J* = 10.5, 4.5 Hz, 4H, C*H*$_A$OH), 3.52 (s, 2H, 6-, 12-H), 4.09 (s, 4H, *N*CH$_2$), 4.33–4.35 (m, 4H, CH$_2$O*H*), 6.35 (d, *J* = 8.6 Hz, 2H, 3-, 5-H of Ph), 6.49 (d, *J* = 8.2 Hz, 2H, 3-, 5-H of Ph), 7.03 (d, *J* = 8.2 Hz, 2H, 2-, 6-H of Ph), 7.23–7.30 (m, 10H, ArH of Bz), 7.54 (d, *J* = 8.6 Hz, 2H, 2-, 6-H of Ph), 8.87 (s, 2H, Ph-OH). Anal. Calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33. Found: C, 74.46; H, 6.23; N, 4.68.

4.2. Cell culture

The L5178Y mouse T lymphoma cell line which was a gift from the National Cancer Institute (NCI) has been infected with the PHA *mdr-1*/A retrovirus as described.[29,30] P-gp expressing cells have been selected by culturing the infected cells in medium which contained 60 ng/mL of colchicine. Both the L5178Y *mdr* subline and the parental cell line L5178 Y were grown in McCoys 5A medium with 10% heat-inactivated horse serum, L-glutamine (2 mM) and antibiotics.[31]

The A2780P cell line was derived from an ovarian cancer[32] and its subline A2780RCIS was the result of treatment with increasing amounts of the anticancer agent and MRP2 substrate cisplatin. The EPG85-257RNOV cell line is a mitoxantrone resistant subcell line derived from EPG85-257 by induction under exposure to increasing concentrations of mitoxantrone.[33]

The human gastric carcinoma cell line EPG85-257RNOV, the ovarian cancer cell line A2780 and the cisplatin-resistant cell line A2780RCIS 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 as described.[34] The cell culture medium for EPG85-257RNOV was supplemented with mitoxantrone (0.2 μg/mL) to ensure BCRP overexpression and for stable MRP2 overexpression the medium for A2780RCIS was supplemented with cisplatin (10 ng/mL).

4.3. MDR reversal assays for P-gp, MRP1, MRP2 and BCRP inhibition

For the reversal of the P-gp-mediated mdr the cultured cells were adjusted to a concentration of 2 × 10^6/mL, then resuspended in serum free McCoys 5A medium and distributed into 0.5 mL aliquots in Eppendorf centrifuge tubes. The test compounds were added from stock solutions (1.0 mg/mL). The samples were incubated for 10 min at rt. After addition of the P-gp substrate rhodamine 123 (5.2 μM final concentration) the cells were incubated for additional 20 min at 37 °C. Then they were washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS). For analysis the fluorescence of 1 × 10^4 cells was measured by flow cytometry with a Becton Dickinson FACScan instrument.

For the reversal of the MRP1- and MRP2-mediated mdr, A2780 cells were adjusted to a concentration of 1 × 10^5 cells per mL and seeded out in 6-well plates to a concentration of 2 × 10^5 cells per mL. The medium was removed after 24 h and fresh medium containing several concentrations of tested compounds was added. After an incubation time of 20 min at 37 °C carboxyfluoresceindiacetat (2 μM final concentration) as a fluorescent substrate for MRP1 and MRP2 was added and cells were incubated for further

40 min at 37 °C. Next, cells were washed for two times with icecold PBS, resuspended in 0.5 mL icecold PBS and stored on ice until measurement.

The BCRP-overexpressing EPG85-257RNOV cells were adjusted to a concentration of 0.5×10^6 cells per mL, distributed into 0.5 mL aliquots in Eppendorf centrifuge tubes and test compounds were added in several concentrations. After an incubation time of 20 min at 37 °C mitoxantrone (20 μM final concentration) was added and cells were incubated for further 60 min at 37 °C. Next, medium was discarded and the cells were trypsinized, washed with icecold PBS for two times and resuspended in 0.5 mL icecold PBS for analysis.

Fluorescence of carboxyfluorescein and mitoxantrone was analysed with a Becton Dickinson FacsCalibur flow cytometer that was equipped with an argon laser. The fluorescence of 10,000 events was logarithmically measured at a laser excitation wavelength of 488 nm through a 530 nm (carboxyfluorescein) and a 670 nm (mitoxantrone) bandpass filter.

Results of flow cytometry were calculated as *R* values. In the case of MRP1 these calculated *R* values each result from dividing the measured fluorescence intensity in the inhibitor-treated cells by the measured fluorescence intensity in the inhibitor-untreated cells. Thus the *R* value means the quotient of the fluorescence intensity in the inhibitor-treated cells and the fluorescence in the inhibitor-untreated cells. In the cases of P-gp and BCRP the resulting quotient has been divided by the quotient of the measured fluorescence intensities in the inhibitor-treated parental cell line and the untreated parental cell line. In the case of MRP2 the *R* values were given by a substraction of the value of the MRP1 specific extent of fluorescence uptake into the parental cell line from the whole extent of fluorescence determined as MRP1 and MRP2 inhibition in the induced cell line A2780RCIS.

4.4. Log *P* value calculations

Molecules were constructed and log *P* values were determined with nine programs (ALOGPs, AC logP, AB/LogP, miLogP, ALOGP, MLOGP, KOWWIN, XLOGP2 and XLOGP3).[35] The resulting mean values were given by the ALOGPS 2.1 program.[35]

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References and notes

1. Gottesman, M. M.; Ling, V. *FEBS Lett.* **2006**, *580*, 998.
2. Lage, H. *Cell. Mol. Life Sci.* **2008**, *65*, 3145.
3. Livingston, D. M.; Silver, D. P. *Nature* **2008**, *451*, 1066.
4. Mahon, F. X.; Belloc, F.; Lagarde, V.; Chollet, C.; Moreau-Gaudry, F.; Reiffers, J.; Goldman, J. M.; Melo, J. V. *Blood* **2003**, *101*, 2368.
5. Matsui, H.; Takeshita, A.; Naito, K.; Shinjo, K.; Shigeno, K.; Maekawa, M.; Yamakaw, Y.; Tanimito, M.; Kobayashi, M.; Ohnishsi, K.; Ohno, R. *Leukemia* **2002**, *16*, 813.
6. Bonavida, B. *Oncogene* **2007**, *26*, 3629.
7. Huang, Y.; Sadee, W. *Cancer Lett.* **2006**, *239*, 168.
8. Choi, C.-H. *Cancer Cell Int.* **2005**, *5*, 30.
9. Sharom, F. J. *Pharmacogenomics* **2008**, *9*, 105.
10. Avendanó, C.; Menéndez, J. C. *Med. Chem. Rev.* **2004**, *1*, 419.
11. Baumert, C.; Hilgeroth, A. *Anticancer Agents Med. Chem.* **2009**, *9*, 415.
12. Sheps, J. A.; Ling, V. *Eur. J. Physiol.* **2007**, *453*, 545.
13. Dean, M.; Rzhetsky, A.; Allikments, R. *Genome Res.* **2009**, *11*, 1156.
14. Leschziner, G. D.; Andrew, T.; Pirnohamed, M.; Johnson, M. R. *Pharmacogenomics J.* **2007**, *7*, 154.
15. Tain, Q.; Zhang, J.; Chan, E.; Duan, W.; Zhou, S. *Drug Dev. Res.* **2005**, *64*, 1.
16. Pires, M. M.; Emmert, D.; Hrycyna, A. Ch.; Chmielewski, J. *Mol. Pharmacol.* **2009**, *75*, 92.
17. Sauna, Z. E.; Andrus, M. B.; Turner, T. M.; Ambudkar, S. V. *Biochemistry* **2004**, *43*, 2262.
18. Hilgeroth, A. *Mini-Rev. Med. Chem.* **2002**, *2*, 235.
19. Coburger, C.; Wollmann, J.; Baumert, C.; Krug, M.; Molnár, J.; Lage, H.; Hilgeroth, A. *J. Med. Chem.* **2008**, *51*, 5871.
20. Seelig, A. *Eur. J. Biochem.* **1998**, *251*, 252.
21. Ferte, J.; Kühnel, J.-M.; Chapuis, G.; Rolland, Y.; Lewin, G.; Schwaller, M. A. *J. Med. Chem.* **1999**, *42*, 478.
22. Krug, M.; Voigt, B.; Baumert, Ch.; Lüpken, R.; Molnár, J.; Hilgeroth, A. *Eur. J. Med. Chem.* **2010**, *45*, 2683.
23. Ferry, D.; Boer, R.; Callaghan, R.; Ulrich, W. R. *Int. J. Clin. Pharmacol. Ther.* **2000**, *38*, 130.
24. Dhainaut, A.; Regnier, G.; Tizot, A.; Pierre, A.; Leonce, S.; Guilbaud, N.; Kraus-Berthier, L.; Atassi, G. *J. Med. Chem.* **1996**, *39*, 4099.
25. Wiese, M.; Pajeva, I. K. *Curr. Med. Chem.* **2001**, *8*, 685.
26. Shuhong, Z.; Morris, M. E.. In *Drug Delivery and Applications*; Wang, B., Siahaan, T., Soltero, R., Eds.; John Wiley & Sons, 2005; Vol. 1, pp 381–411.
27. Diestra, J. E.; Scheffer, G. L.; Català, I.; Maliepaard, M.; Schellens, J. H.; Scheper, R. J.; Germà-Liuch, J. R.; Izquierdo, M. A. *J. Pathol.* **2002**, *198*, 213.
28. Chennat, T.; Eisner, U. *J. Chem. Soc., Perkin Trans. 1* **1975**, *10*, 926.
29. Weaver, J. L.; Szabo, D.; Pine, P. S.; Gottesmann, S.; Goldberg, A.; Aszalos, A. *Int. J. Cancer* **1993**, *54*, 456.
30. Cornwell, M. M.; Pastan, I.; Gottesmann, M. M. *J. Biol. Chem.* **1987**, *262*, 2166.
31. Voigt, B.; Coburger, C.; Molnár, J.; Hilgeroth, A. *Bioorg. Med. Chem.* **2007**, *15*, 5110.
32. Eva, A.; Robbins, K. C.; Andersen, P. R.; Srinivasan, A.; Tronick, S. R.; Reddy, E. P.; Ellmore, N. W.; Galen, A. T.; Lautenberger, J. A.; Papas, T. S.; Westin, E. H.; Wong-Staal, F.; Gallo, R. C.; Aaronson, S. A. *Nature* **1982**, *295*, 116.
33. Dietel, M.; Arps, H.; Lage, H.; Niendorf, A. *Cancer Res.* **1990**, *50*, 6100.
34. Lage, H.; Jordan, A.; Scholz, R.; Dietel, M. *Int. J. Hyperthermia* **2000**, *16*, 291.
35. http://www.vcclab.org/lab/alogps.