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Novel non-substrate modulators of the transmembrane efflux pump P-glycoprotein (ABCB1)

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Novel *N*- and 4-substituted 1,4-dihydropyridines with a C_2-symmetric molecular scaffold have been profiled as highly active modulators of the transmembrane efflux pump P-glycoprotein (P-gp, ABCB1) in an exclusively P-gp overexpressing cell line model. Structure-activity relationships have been discussed for varied substituents of both the *N*- and the 4-residue. The influence of potential hydrogen bond acceptor functions has been characterized in relation to the number and the substituent positioning. Close consideration has been taken into cellular toxicity and suggested P-gp substrate properties as limiting molecular properties of known P-gp modulators. Non-toxicity and non-substrate properties of our novel inhibitors qualify the novel compound class as perspective tool to effectively combate the efflux pump-mediated cellular resistance of anticancer drug substrates as could be demonstrated in first in vitro studies.

Therapeutical cancer treatment remains a great challenge although many promising drugs have been developed in the past.[1] So critical side effects of drugs which target also normal

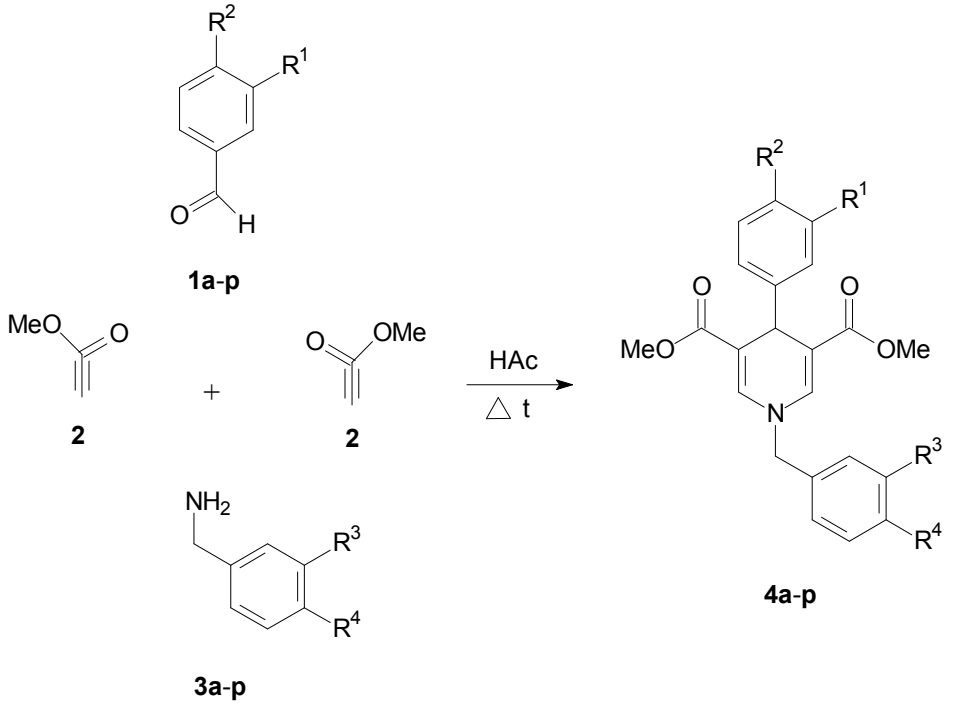
cells can be limited by addressing specific structures in cancer cells like receptor tyrosine kinases.[2-3] Such overexpressed kinases which are found dysregulated in tumor cells can be blocked by monoclonal antibodies or ATP competitive inhibitors.[1,4,5] Moreover, the use of drug carriers like liposomes or nanoparticles is investigated to preferably address the respective tumor cells and avoid effects in normal cells.[6]

The problem of all these novel drugs is that they mostly turned out to be substrates of transmembrane efflux pumps which occur in tumor cells and are partly induced by the drugs themselves.[7-9] Uptaken drugs are transported out of the cells so that the therapeutically necessary drug levels are no longer reached.[7] The tumor cell becomes resistant and, moreover, the resistance mostly includes a great number of anticancer drugs of various drug families.[10] There is still a discussion about the cause of that multidrug resistance (mdr) which may be a multiple binding site for the various drug structures.[11] However, the mode of action of the various transmembrane efflux pumps is still under debate and as threedimensional structures are missing the discussion is concentrated on models of the efflux pump function.[10,12,13]

P-glycoprotein (P-gp, ABCB1) is the most important efflux pump with a wide spread in various types of cancer.[14] P-gp is the longest known efflux pump with occurance in cancer cells and there have been intense efforts to get insight in the structure of this transmembrane protein.[14] P-gp is known to consist of two domains with each six α-helical subunits. A low resolution of the structure using fluorescence spectroscopy suggested a C_2 symmetrical molecular structure with nearly all of the α-helical elements of each domain being located around a C_2 axis which is centered in the middle of the molecule.[15] This closer insight suggested a favour for the development of C_2 symmetric molecules which may serve as modulators of the efflux pump activity. Over the last decades there have been sever efforts to influence the efflux pump activities by the use of such potential inhibitors. Most candidates turned out to be substrates of the efflux pump themselves so that unfavourable high concentrations were necessary to achieve clinical effects.[16-18] However, until now none of the modulators was successful in clinical trials.

Nifidepine was one of the early modulators.[19,20] Structural modifications helped to reduce the calcium-antagonistic properties but although the potential to modulate the P-gp activity increased the derivatives were less active than one best P-gp modulator in such cellular in vitro studies which is verapamil.[21,22] However, the proven P-gp substrate properties of the compounds hampered the further developments and reasoned the clinical ineffectivity of the early investigated nifedipine.[16,20] All the 1,4-dihydropyridines of the series were so far unsubstituted at the nitrogen. We firstly investigated a series of *N*-alkyl substituted 1,4-

dihydropyridines with a maintained C_2 symmetry of the substituted 1,4-dihydropyridine skeleton. Both the *N*-alkyl substituent and the 4-aryl substituent were structurally varied, numbers of hydrogen bond acceptor functions were introduced and the P-gp modulating activities were characterized in a P-gp cell model with exclusively overexpressing P-gp. Finally, a bioanalysis of selected compounds was carried out to provide a compound safety concerning cellular toxicity and to investigate possible P-gp substrate properties. Thus, novel non-substrate modulators could be identified for further preclinical studies.



Compound	R^1	R^2	R^3	R^4
1a, **3a**, **4a**	Cl	H	H	H
1b, **3b**, **4b**	Br	H	H	H
1c, **3c**, **4c**	CF_3	H	H	H
1d, **3d**, **4d**	H	OMe	H	H
1e, **3e**, **4e**	H	H	H	OMe
1f, **3f**, **4f**	H	OMe	H	OMe
1g, **3g**, **4g**	H	OMe	OMe	H
1h, **3h**, **4h**	OMe	H	H	OMe
1i, **3i**, **4i**	OMe	H	OMe	H
1j, **3j**, **4j**	H	OMe	OMe	OMe
1k, **3k**, **4k**	OMe	H	OMe	OMe
1l, **3l**, **4l**	OMe	OMe	H	OMe
1m, 3m, **4m**	OMe	OMe	OMe	H
1n, **3n**, **4n**	OMe	OMe	OMe	OMe
1o, **3o**, **4o**	OBn	OMe	H	H
1p, **3p**, **4p**	OMe	OBn	H	H

Scheme 1. Formation of target structures **4a-p**.

The target compounds **4** were given in a simple one-pot reaction of the respective aromatic aldehyde **1**, methyl propiolate **2** as reacting carbonyl compound and, finally, the aliphatic amine **3** with the various substitutions in glacial acetic acid (Scheme 1). We preferably used methyl propiolate because the resulting target compounds **4** partly crystallized from the reaction mixture after cooling so that extensive purification procedures were not necessary. The simple synthesis work-up procedure is also an advantage of the compound class.

The P-gp modulating properties were determined in a mouse T-lymphoma cell line model. The parental cell line expresses no human P-gp and the subline expresses human P-gp after *mdr1* gene transfection with a retrovirus. This procedure ensured that only P-gp is expressed in the subline and no other transporter is available and detectable. The subline resulted from a consequent collection of surviving cells under colchicine treatment in the cell culture. Only those P-gp expressing cells were able to transport the cell-toxic colchicine out of the cells. We used rhodamine 123 as fluorescent and P-gp specific substrate in our assay and determined the cellular uptake of the substrate in both cell lines with flow cytometry technique at a wavelength of 530 nm using a laser with a fluorescence excitation at 488 nm, so that compound fluorescences at lower wavelengths are not detectable. We then added increasing concentrations of the inhibitor from stock solutions and determined the resulting fluorescence after rhodamine addition and further cell incubation. In those P-gp expressing cells with a P-gp inhibiting effect of the inhibitors the cellular uptake of the fluorescent substrate increased. So called *FAR* (fluorescence activity ratio) values were calculated as a relation of the fluorescence in the P-gp expressing and the non-expressing cell line each under inhibitor treatment and with each fluorescence related to that of the untreated control. Consequently, *FAR* values > 1 proved a P-gp modulating property of the respective investigated compound. We used verapamil as effective in vitro standard inhibitor as well as tariquidar. Although the concentration-dependent effects of the P-gp modulation increased we could not reach a saturation of the effect due to an observed cellular toxicity of both compounds at a higher concentration than 10 μM.[23]

We discuss concentration-dependent *FAR* values similar to all publications in this field so far which report partly different saturation effects for compounds in one compound class. Also in our compound class we reached for only one compound a saturation of the inhibiting effect. In all the other cases of compound inhibition no saturation of the modulating effect was observed, so that it made no sense to determine IC_{50} values. The use of higher concentrations to reach such saturation effects would probably lead to toxic effects. Additionally, a limited compound solubility at high concentrations may prevent the reaching of saturation effects.

Table 1. Concentration dependent P-gp modulating properties as calculated *FAR* values of our target compounds **4a-r**

	FAR value[a]			
Compound	1 µM	2 µM	10 µM	20 µM
4a	2.22 ± 0.47	2.20 ± 0.34	4.05 ± 0.86	8.89 ± 3.61
4b	2.08 ± 0.61	2.36 ± 0.41	5.84 ± 0.81	10.3 ± 3.84
4c	2.14 ± 0.30	2.37 ± 0.41	5.10 ± 1.08	8.92 ± 2.90
4d	2.37 ± 0.47	2.85 ± 0.42	6.23 ± 0.93	6.55 ± 0.73
4e	2.06 ± 0.26	2.55 ± 0.32	5.80 ± 1.03	9.93 ± 3.54
4f	2.96 ± 0.59	3.21 ± 0.91	6.54 ± 0.81	7.48 ± 2.08
4g	2.37 ± 0.42	3.13 ± 0.52	6.50 ± 0.41	7.89 ± 2.08
4h	2.13 ± 0.22	2.83 ± 0.84	5.10 ± 0.69	7.47 ± 1.93
4i	2.14 ± 0.16	2.47 ± 0.56	4.76 ± 0.51	7.21 ± 2.19
4j	3.24 ± 0.31	2.91 ± 0.85	6.93 ± 0.96	9.11 ± 3.13
4k	1.98 ± 0.23	1.93 ± 0.38	4.71 ± 0.61	6.42 ± 0.98
4l	3.48 ± 0.58	3.66 ± 0.48	10.5 ± 2.39	14.1 ± 2.07
4m	3.77 ± 0.50	3.65 ± 0.40	12.6 ± 1.45	14.1 ± 2.49
4n	2.18 ± 0.42	2.25 ± 0.19	4.56 ±0.97	7.24 ± 0.35
4o	3.97 ± 0.40	4.59 ± 0.93	13.1 ± 1.10	11.4 ± 2.06
4p	6.24 ± 0.62	6.74 ± 1.48	15.6 ± 1.56	11.3 ± 3.19
4q	2.42 ± 0.51	2.75 ± 0.37	6.53 ± 1.02	11.6 ± 2.86
4r	2.35 ± 0.83	2.66 ± 0.50	8.19 ± 3.39	13.5 ± 3.98
verapamil	1.34 ± 0.37	n.d.[b]	5.35 ± 0.79	n.d.[b]
tariquidar	5.25 ± 0.58	n.d.[b]	7.24 ± 0.65	n.d.[b]

[a] Mean of three determinations. [b] not determined.

Moreover, the value of 1.1. is the lowest possible *FAR* value which complicated such a calculation. So for the discussion of structure-activity relationships we considered our observed concentration-dependent effects which were found for all investigated compounds.

First, we combined a *N*-benzyl substitution with a 4-(3-halogenphenyl) residue in compounds **4a-c**. Such halogen substituents may undergo halogen bonding to amino acid residues of the potential P-gp binding region. The 3-chlorophenyl compound **4a** was active as P-gp modulator at the lowest concentration of 1 μM with a *FAR* value of 2.22 (Table 1). Similar activities were found for the 3-bromophenyl and the 3-trifluorophenyl compounds **4b** and **4c**. Verapamil was less active at this concentration with a *FAR* value of 1.34, whereas tariquidar showed higher activities with a *FAR* value of 5.25.

Increasing compound inhibitor concentrations led to increased *FAR* values. At a concentration of 10 μM the 3-bromophenyl compound **4b** showed highest activities, again more than verapamil. Also at the used highest concentration of 20 μM compound **4b** remained the most active one suggesting a favourable influence of such a halogen bond substituent on the P-gp inhibitory activity. Attempts to synthesize 2- or 4-halogenphenyl substituted derivatives failed due to a lowered reactivity of the starting aldehyde. An alternative introduction of a 3-halogen substituent into the benzyl residue of the 1,4-dihydropyridine structure failed due to the fact that the corresponding benzylamine compounds were not commercially available. In the case of used 2- as well as 4-chloro substituted benzylamines we could not isolate a respective 1,4-dihydropyridine product, presumably also because of a lowered amine reactivity.

We then introduced a methoxy function into the 4-phenyl as well as into the *N*-benzyl substituent. Methoxy functions are known as favourable hydrogen bond acceptor functions in mdr modulators.[25] Placed into the 4-position of the *N*-benzyl residue of derivative **4e** the activities were almost similar to those of the 4-methoxyphenyl substituted compound **4d** with the activity at the highest concentration being mainly increased with a *FAR* value of 9.93. Next, we investigated combined methoxy substitutions of both the 4-phenyl and the *N*-benzyl residue. A combination of the two methoxy functions in the 4-phenyl and the *N*-benzyl substituent in both 4-positions of compound **4f** led to similar P-gp modulating activities at the given concentrations. A movement of the 4-methoxy function in the *N*-benzyl residue to the 3-position led to almost unchanged activity data of compound **4g**. When instead of this the 4-methoxy function of the 4-phenyl residue was moved into the 3-position and the 4-methoxybenzyl residue remained unchanged the activity of compound **4h** decreased. A combination of both a 3-methoxyphenyl and 3-methoxybenzyl substitution in derivative **4i** led to further decreases in activity. So it may be concluded that the allover symmetric 4-methoxy disubstitution of both residues is most favourable among the varied aromatic monomethoxy substitutions. The 4-methoxyphenyl substitution was most favourable in combination with a second methoxy function.

Next, we tested whether an additional third methoxy function could increase the P-gp-modulating activity and started with a 4-methoxyphenyl group in derivative **4j** combined with a 3,4-dimethoxybenzyl residue. Compared to the disubstituted 4-methoxy derivative **4f** we found increases in activity at almost all the tested concentrations. When the 4-methoxy function in the 4-phenyl residue was moved to the 3-position the activities of the resulting compound **4k** were found mainly lowered at all concentrations by about 30%. The 3-methoxyphenyl function also led to lowered activity data in the series of the phenyl and benzyl dimethoxy-substituted compound series **4f-i**.

We then combined a 3,4-dimethoxyphenyl substitution in compound **4l** with a 4-methoxybenzyl substitution. We found allover main increases in activity if compared to the 3,4-dimethoxybenzyl and 4-methoxyphenyl derivative **4j** up to 50%, even at the higher concentrations. If that most favourable 3,4-dimethoxy function of the 4-phenyl residue is combined with a 3-methoxybenzyl function in derivative **4m** we observed some increases in activity if compared to the 4-methoxybenzyl compound **4l**. The increased *FAR* values at both the 1 μM and the 10 μM concentration were more than the double of the *FAR* value of verapamil and almost the double of the tariquidar activity determined at this concentration.

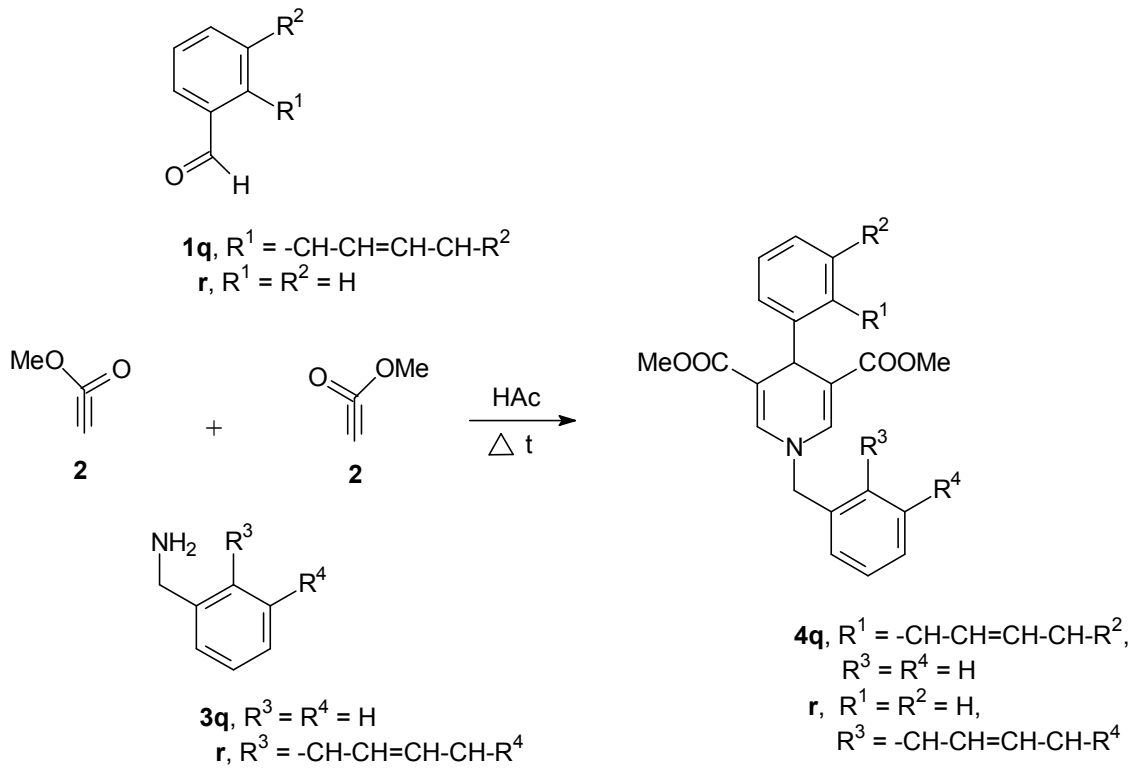
So it can be stated that the trimethoxy substitution is more favourable than the disubstitution with the 3,4-dimethoxyphenyl function being the most favourable substituent combination of highest activities so far.

Surprisingly, a tetramethoxy substitution in compound **4n** was less favourable than the trisubstitution. The activities at all the concentrations were lowered and we found activities in the range of those with the 3-methoxy disubstitution of derivative **4i**. Obviously, the number of methoxy functions to undergo a potential hydrogen bonding is limited. Two methoxy functions are of favour, three methoxy functions give best P-gp modulating results and, finally, four methoxy functions lower the activity data.

We then replaced the 3-methoxy function in the 3,4-dimethoxyphenyl residue with a 3-benzyloxy function in compound **4o** while the *N*-benzyl residue was unsubstituted. The P-gp inhibiting activities were higher than those of the 3,4-dimethoxyphenyl and methoxybenzyl substituted compounds **4l** and **4m** at almost all concentrations. So the more lipophilic 3-benzyloxy substituent is more favourable than the two methoxy functions. A further increase in activity was observed in derivative **4p** with the benzyloxy substituent being located in the 4-position of the 4-phenyl residue and the methoxy function bound in the 3-phenyl position. So the resulting compound had a threefold higher *FAR* value than verapamil at the concentration of 10 μM and was a better modulator than tariquidar at this concentration.

A benzyloxy function however increases the compound lipophilicity beside the ability to serve as potential hydrogen bond acceptor function. Obviously, the increased compound lipophilicity is more important than an increasing number of potential hydrogen bond acceptor functions. We could not alternatively introduce the benzyloxy substituent into the benzyl residue of a respective 1,4-dihydropyridine due to the fact that the corresponding benzylamine compounds were not available. A combination of a benzyloxyphenyl and methoxy substitution in the benzyl residue will lead to similar promising activities than its concentration on the phenyl residue. However, increasing numbers of hydrogen bond acceptor functions have been discussed to be of a limiting favourable effect for activity.

Finally, we prepared a 4-naphthyl and a *N*-naphthylmethyl derivative each combined with an unsubstituted *N*-benzyl and 4-phenyl residue, respectively (Scheme 2).



Scheme 2. Formation of target structures **4q** and **r**.

The *FAR* value of the resulting 4-naphthyl compound **4q** at the highest concentration was even higher than those of the benzyloxy substituted compounds **4o** and **4p**. At the lower concentrations the activity lied in the ranges of the di- and trimethoxy substituted derivatives.

An increase in activity was observed for the *N*-naphthylmethyl derivative **4r** with activity data at the higher concentrations in the range of the most active trimethoxy-substituted derivatives. So beside the importance of a potential hydrogen bonding *via* the introduced methoxy functions which are favourably located at the 4-phenyl residue, lipophilic substituents significantly increase the P-gp modulating properties of the respective compounds.[25]

Toxicity problems occurred with most mdr modulators like tariquidar, at the latest in clinical trials.[26,27] While early mdr modulators were toxic due to their original pharmacological properties as immunosuppressive or antihypertensive agents, even those of the novel generations suffered from toxicity problems which were attributed to the fact that they were substrates of the efflux pumps themselves so that higher concentrations were necessary to achieve the desired cellular effects.[26,28,29] Those concentrations were found partly toxic for normal cells.

We investigated the cellular toxicity of four of our target compounds which showed strongest activities as P-gp modulators as far as evaluated. We determined toxic effects in both the non P-gp expressing parental cell line and the P-gp expressing cell line to have a direct comparison of toxic effects in target cells and the respective normal cells.

We determined the cell viability under increasing modulator concentrations up to 160 μM in the MTT assay. In this assay the mitochondrial toxicity of a compound is determined. That compound toxicity reduces the formation of the fluorescent formazan dye from the MTT reagent under a reduced catalysis of the mitochondrial dehydrogenases as a consequence of the cellular compound toxicity. We calculated the IC_{50} values of the reduced cell viability as shown in Table 2.

The determined IC_{50} values of reduced cell viabilities determined in the non P-gp expressing cell line varied from 84.6 μM for compound **4r** to 197.2 μM for compound **4p**. With these values the compounds are completely non-toxic in the tested concentration ranges starting with 1 μM as effective concentration for all compounds. Even at inhibitor concentrations of 10 μM most compounds caused cell viability rates of more than 90% (data not shown).

Interestingly, we found a partly slightly increased toxicity in the P-gp expressing cell line with IC_{50} values ranging from 71.1 μM for derivative **4r** to 154 μM for compound **4l**. At the concentration of 10 μM the cell viability rates ranged from 78.4% (**4r**) to 95.6% (**4p**).

If a compound would have been a substrate of the efflux pump the toxicity of the compound in the P-gp expressing cell line would have been lower at the given concentrations because parts of the compounds would have been transported out of the cells by the efflux pump activity which is not found in the non-expressing cell line.

Table 2. IC_{50} values of reduced cell viability in the non-P-gp expressing parental cell line (P-cell line) and the P-gp expressing subline (MDR cell line) for selected target compounds **4l**, **4m**, **4p** and **4r** and calculated *FAR* values for fluorescent target compound uptake for relevant concentrations of derivatives **4l** and **4m**

	IC_{50} value (μM)[a]		*FAR* value[a]	
Compound	P-cell line	MDR cell line	P-cell line 50 μM	MDR cell line 100 μM
4l	263 ± 1.22	154 ± 1.32	1.31 ± 0.51	1.14 ± 0.50
4m	133 ± 1.35	122 ± 1.22	0.95 ± 0.47	1.02 ± 0.34
4p	197.2 ± 3.59	> 160[b]	n.d.[c]	n.d.[c]
4r	84.6 ± 1.18	71.1 ± 1.33	n.d.[c]	n.d.[c]

[a] Mean of three determinations. [b] not determinable. [c] not determined.

This would mean an increase of the cell viability rate at the given concentrations and, thus, an increase of the resulting IC_{50} value.
However, we observed partly increased toxicity data as discussed with resulting lowered IC_{50} values in the P-gp-expressing cell line, so that we can exclude such P-gp substrate properties.

This concluded result should be further confirmed in an additional study which we carried out with our most active compounds **4m** and **4l**.
We incubated both the P-gp non-expressing and the P-gp expressing cell line with higher concentrations of both compounds. If one of the compounds would own P-gp substrate properties its uptake into the P-gp expressing cell line would be lower due to a partial cellular efflux. We directly determined the uptake of our compounds by determination of their own fluorescence at 430 nm using flow cytometry technique and a laser with an excitation at 366 nm much lower than that of the laser used for the determination of the P-gp modulating effects. The resulting fluorescence in cells of both cell lines was determined and the ratio was calculated. The calculated *FAR* values are given in Table 2. Compound **4m** showed *FAR* values of 0.95 at 50 μM and of 1.02 at 100 μM. For compound **4l** *FAR* values of 1.31 and

1.14 were found at the respective concentrations. *FAR* values > 1 mean that the compound has completely no P-gp substrate properties. The results of the direct compound measurements correlate with the results from the toxicity studies as discussed. For comparison we determined the *FAR* value for rhodamine 123 as P-gp substrate. At a rhodamine concentration of 5 µM we found a *FAR* value of 0.05 which clearly documents the P-gp substrate properties of the compound.

Table 3. MDR reversal as restored daunorubicin toxicity with determined IC_{50} values of cellular viability for daunorubicin under P-gp modulator application in the P-gp non-expressing and the P-gp overexpressing cell line for compounds **4l**, **4m**, **4p** and **4r**

	IC_{50} value [µM][a]		
Compound	parental cell line	P-gp overexpressing cell line	
		5 µM	10 µM
without inhibitor	0.93 ± 0.09	10.21 ± 0.99	
4l	0.85 ± 0.08[b]]	n.d.[c]	0.97 ± 0.09
4m	0.95 ± 0.06[b]	1.86 ± 0.93	0.85 ± 0.09
4p	0.75 ± 0.06[b]	0.84 ± 0.08	0.61 ± 0.08
4r	0.80 ± 0.04[b]	n.d.[c]	0.93 ± 0.09

[a] Mean of three determinations. [b] determined at a 10 µM modulator concentration. [c] not determined.

Finally, we investigated the ability of our P-gp modulators with highest activities to reverse the mdr of the clinically relevant cytostatic drug daunorubicin in our cell line model. Daunorubicin is a known substrate of P-gp. So we determined the toxicity of daunorubicin in both the mouse T lymphoma cell line without P-gp and the P-gp overexpressing subline (Table 3). The IC_{50} values of daunorubicin for the cellular viability were calculated with 0.93 µM for the parental cell line and with 10.21 µM for the P-gp expressing subline. The lowered daunorubicin toxicity in the subline resulted from the fact that it was transported out of the cells by the P-gp efflux pump activity. We then preincubated cells of the non-P-gp expressing and the P-gp expressing subline with the best of our modulators **4l**, **4m**, **4p** and **4r** at a concentration of each 10 µM and determined the daunorubicin toxicity again. The calculated

IC_{50} values are shown in Table 3. The IC_{50} values in the non-P-gp expressing cell line were found almost unchanged. For compounds **4l** and **4r** we found IC_{50} values in the P-gp expressing cell line almost identically equal to that which we found in the parental cell line without P-gp. The result meant that we had a complete reversal of the mdr in the P-gp expressing subline and a restoration of its daunorubicin sensitivity. Compounds **4m** and **4p** resulted in little lowered IC_{50} values which meant an increased sensitivity of the treated cell line towards daunorubicin. So we lowered the inhibitor concentration of those compounds to 5 μM and determined the daunorubicin toxicity again. While compound **4m** resulted in an IC_{50} value of 1.86 μM, the most active derivative **4p** led to an IC_{50} value of 0.84 μM. So also this lowered concentration was able to reverse the mdr and restore the daunorubicin sensitivity of the P-gp expressing subline.

Our novel and highly active P-gp modulators are no P-gp substrates like rhodamine as shown and are able to reverse the toxicity of a cytostatic drug with clinical relevance. This promising results encourage for further preclinical studies of selected compounds to combate cancer in P-gp overexpressing cells by the inhibition of the causative efflux pump P-gp.

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References

1 M. Dobbelstein and U. Moll, *Nature Rev. Drug Discovery*, 2014, **13**, 179.

2 K. Strebhardt and A. Ullrich, *Nature Rev. Cancer*, 2008, **8**, 473.

3 J. Luo, N. L. Solimini and S. J. Eliedge, *Cell*, 2009, **136**, 825.

4 J. Zhang, P. L. Yang and N. S. Gray, *Nature Rev. Cancer*, 2009, **9**, 28.

5 C. J. Tsai and R. Nussinov, *Sem. Cancer Biol.*, 2013, **23**, 235.

6 F. Perche and V. P. Torchillin, *J. Drug Deliv.*, 2013, ID 507265, http://dx.doi.org/10.1155/2013/705265.

7 P. D. W. Eckford and F. J. Sharom, *Chem. Rev.*, 2009, **109**, 2989.

8 J. P. Gillet and M. M. Gottesman, *Methods Mol. Biol.*, 2010, **596**, 47.

9 A. Hilgeroth, M. Hemmer and C. Coburger, *Mini-Rev. Med. Chem.*, 2012, **12**, 1127.

10 A. Breier, L. Gibalove, M. Seres, M. Barancik, Z. Sulova, *Anti-Cancer Agents Med. Chem.*, 2013, *13*, 159.

11 M. M. Gottesman, *Annu. Rev. Med.*, 2002, **53**, 615.

12 M. M. Gottesman and V. Ling, *FEBS Lett.*, 2006, **580**, 998.

13 St. G. Aller, J. Yu, A. Ward, Y. Weng, S. Chittaboina, R. Zhou, P. M. Harrell, Y. T. Trinh, Q. Zhang, I. L. Urbatsch, G. Chang, *Science*, 2009, **323**, 1718.

14 M. M. Gottesman, T. Fojo and S. E. Bates, *Nature Rev. Cancer*, 2002, **2**, 48.

15 M. F. Rosenberg, R. Callaghan, S. Modok, C. F. Higgins, R. C. Ford, *J. Biol. Chem.*, 2005, **280**, 2857.

16 L. M. S. Chan, S. Lowes and B. Hirst, *Eur. J. Pham. Sci.*, 2004, **21**, 25.

17 R. B. Wang, C. L. Kuo and E. J. Lien, *J. Clin. Pharm. Ther.*, 2003, **28**, 203.

18 C. Cheng, P. M. Bahadduri, J. E. Polli, P. W. Swann, S. Ekins, *Drug. Metab. Disp.*, 2006, **34**, 1976.

19 T. Tsuruo, H. Iida, M. Nojiri, S. Tsukagoshi, Y. Sakurai, *Cancer Res.*, 1983, **43**, 2905.

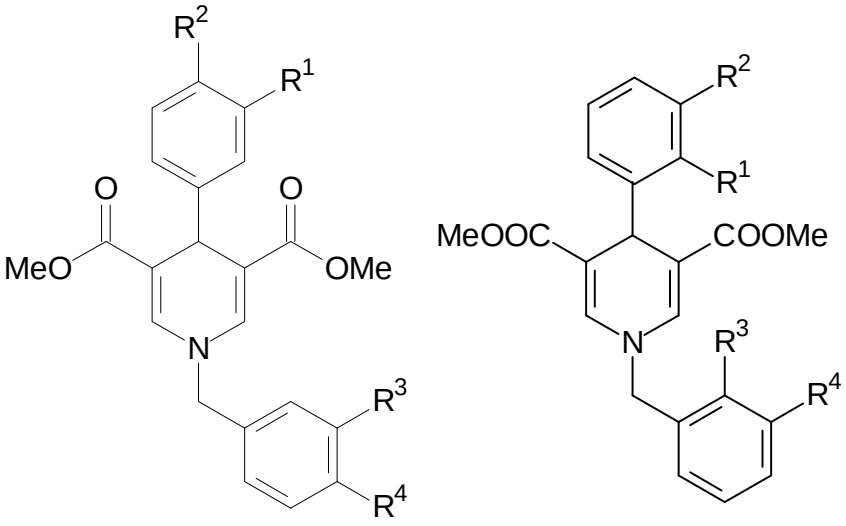
20. P. A. Philip, S. Joel, S. C. Monkman, E. Dolega-Ossowski, K. Tonkin, J. Carmichael, J. R. Idle, A. L. Harris, *Br. J. Cancer*, 1992, **65**, 267.

21 M. Kawase, A. Shah, H. Gaveriya, N. Motohashi, H. Sakagami, A. Varga, J. Molnár, *Bioorg. Med. Chem.*, 2002, **10**, 1051.

22 S. Saponara, M. Kawase, A. Shah, N. Motohashi, J. Molnár, K. Ugocsai, G. Sgaragli, F. Fusi, *Br. J. Pharmacol.*, 2004, **141**, 415.

23. In earlier studies we found similar activities for verapamil and cyclosporine A as P-gp modulators.[24]

24. C. Coburger, H. Lage, J. Molnár, A. Hilgeroth, *Pharm. Res.*, 2009, **26**, 182.
25. We also determined nanomolar activities for our most active P-gp modulating compounds at a concentration of 100 nM **4l** , **4m**, **4p** and **4r** with resulting *FAR* values of 1.23 (**4l**), 1.37 (**4m**), 2.59 (**4p**) and finally 1.19 (**4r**).
26. R. Krishna and L. D. Mayer, *Eur. J. Pharm. Sci.*, 2000*,* **11**, 265.
27. R. J. Kelly, D. Draper, C. C. Chen, *Clin. Cancer Res.*, 2011, **17**, 569.
28. C. Baumert, and A. Hilgeroth, *Anti-Cancer Agents Med. Chem.*, 2009, **9**, 415.
29. Drug-drug interactions have long been discussed to attribute to toxic effects of P-gp inhibitors which may influence the excretion processes of co-administered drugs so that resulting higher intracellular levels of those drugs become toxic. A recent report investigated such possible in vivo effects of digoxin under P-gp inhibitor applications. Digoxin becomes toxic in slightly increased intracellular doses. However, no significant effects on the digoxin toxicity under the P-gp inhibitor applications were observed.[30]
30. K. S. Fenner, M. D. Troutman, S. Kempshall, J. A. Cook, J. A. Ware, D. A. Smith, C. A. Lee, *Clin. Pharmacol. Therap.*, 2009, **85**, 173.



Novel non-substrate modulators of the transmembrane efflux pump P-gp have been profiled as nontoxic and mdr reversing agents.