First Insight into the Symmetry and Flexibility of Membrane Efflux Pump P-Glycoprotein by Novel Bifunctional Modulators

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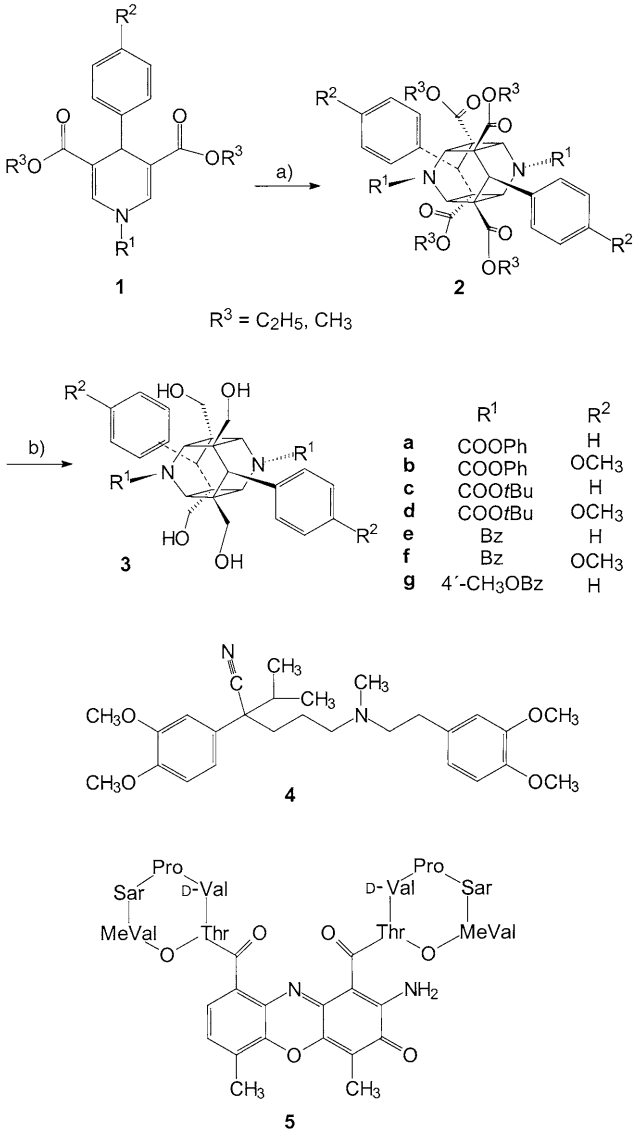
Multidrug resistance (MDR) is an increasingly common problem in the treatment of infectious diseases and cancer.[1–3] Transmembrane efflux pumps play a role in the MDR of bacteria, viruses, fungi and cancer. The MDR efflux pumps mainly found in cancer cells belong to the ABC-transporter family. P-glycoprotein (P-gp) causes the highest incidence of resistance, followed by multidrug resistance-associated protein (MRP) subtypes, such as MRP1 and MRP2.[3,4] P-gp was also discovered to be causative agent for viral resistance to AIDS treatment, because peptidic inhibitors of HIV protease as a therapeutic target in AIDS treatment were found to be P-gp substrates.[2] While recent results of the X-ray crystal-structure analysis of the bacterial efflux transporter AcrB have given insight into the functional aspects of substrate transport,[5] there are, currently, only models for the structure and function of P-gp. While bacterial efflux systems are thought to collect all substrates in a central cavity from which they are actively transported through a pore tunnel,[5] P-gp is assumed to bind substrates first.[6,7] This binding can cause conformational changes in neighboring transmembrane α-helical subunits, and then lead to the entrance of the substrate into the transporter channel.[6]

The binding affinities of various P-gp substrates have been determined in a radioligand-binding assay.[8] These results correlate well with the P-gp-inhibitory properties of the compounds and proved that verapamil was one of the best inhibitors of P-gp function. So far, nothing certain is known about the P-gp-substrate-binding region. We present here structure–activity studies of symmetric and asymmetric modulators of P-gp function that strongly support a C_2-symmetric structure for the P-gp binding region. Activity data suggest, however, that this binding region has a conserved conformational structure than the more flexible one of the second enzymatic target of the molecules, HIV-1 protease, which likewise has a C_2-symmetric structure. Such a conserved binding region might come as a surprise, considering that the P-gp transporter is a model of high flexibility acting as flippase[9] or "vacuum cleaner".[7]

A series of exclusively C_2-symmetric cage compounds **3** (Scheme 1) has been prepared by a primary solution dimerization reaction of monomeric 1,4-dihydropyridines **1** (see Experimental Section). They have been evaluated in a fluorescence-uptake assay in mouse T-lymphoma parental cells and the MDR-1-transfected resistant subline by using rhodamine-123 as a fluorescent P-gp substrate with two inhibitor concentrations (Table 1). As is evident from the calculated inhibitory activity ratios *R* by directly comparing fluorescence-uptake data in relation to an untreated control, an increase in fluorescence uptake unequivocally corresponds to P-gp inhibition. The activity data of all compounds prove that almost all of them are active at a low concentration (1 μM); the exceptions being **3a**[10] and **b**, both of which show activity at 10 μM. Compared to the standard concentration of verapamil (**4**), the other derivatives show up to 50-fold higher activity, with linearity of concentration and biological effect in the lower concentration ranges. With respect to structure–activity relationships (SAR),



Scheme 1. Synthesis of C_2-symmetric homodimers **3**. a) $h\nu$, $\lambda > 270$ nm, MeOH/THF, 4 weeks, 27 °C; b) $LiAlH_4$ (1 equiv), THF, 2 h, −8 °C. Molecular structures of verapamil (**4**) and dactinomycin (**5**).

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Table 1. MDR-modulating properties of cage dimers and verapamil (**4**).

Compound	P-gp-inhibitory activity ratios (R)[a] 1 μM	10 μM	HB functions
3a	0.97	1.81	6
3b	0.38	1.44	8
3c	2.84	33	6
3d	1.13	14.64	8
3e	34.28	50.20	4
3f	34.40	87.01	6
3g	17.50	26.50	6
4	0.66	7.27	
8	2	33	5
9	1.2	12	6
12	1.2	12	6
13	3.7	25.60	6

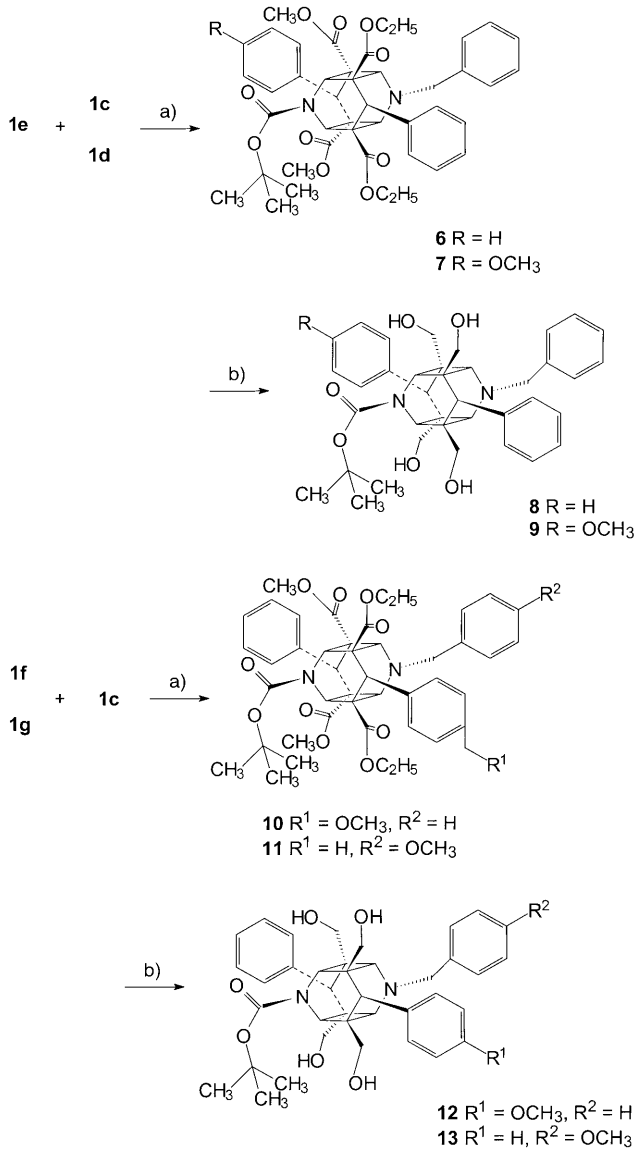
[a] Mean of two determinations.

the phenoxycarbonyl substitution of **3a** and **b** is less favorable, the butoxycarbonyl one of **3c** and **d** is better, and benzylic substitution is the best; this characterizes compound **3f** as one of the strongest P-gp inhibitors so far.

As recent studies have suggested that hydrogen-bond-donor as well as -acceptor functions play an important role in the binding possibilities of P-gp substrates,[11] functional groups for hydrogen bonding (HB) have also been considered in compounds **3a**–**f**. The decrease in biological activity from **3c** to **d** can be correlated to an increasing number of such functional groups (methoxy functions). However, from compound **3e** to **f** the introduction of the same methoxy functions increases biological activity. Trying to correlate the biologically favorable substitutions of butoxycarbonyl, benzylic, and methoxyphenylic groups with the number of HB functions within the new class of highly potent P-gp modulators (Table 1), it was found that six of these functions in compounds **3c** and **f** could be the limit for excellent biological activity as far as evaluated. Fewer HB functions—such as four in compound **3e**—are relatively unfavorable compared to the six in compound **3f**, although the biological activity of **3e** is still impressive. The biological activity of compound **3d**, with eight HB functions, is substantially reduced compared to that of compound **3c**, which has six.

Such a limited number of HB functions as recently suggested for compounds of a differently substituted scaffold contradicts the previous assumption that a high number of HB possibilities ensures good biological activity in known P-gp modulators, which all share a common feature: they are all asymmetric.[10,11] Within our novel class of C_2-symmetric compounds, the highest P-gp inhibitory activities are reached; therefore, we wondered whether their binding region might also have a C_2-symmetric structure. This would suggest that P-gp itself also has a C_2-symmetric structure—in agreement with one early P-gp model.[12] Hints of such a symmetry of the P-gp substrate-binding region could have been seen from the early P-gp inhibitors verapamil (**4**) and dactinomycin (**5**), both of which have an almost symmetrical molecular framework substituted with asymmetrical elements (Scheme 1). However, closer investigations of these compounds as MDR modulators were strongly limited by their toxicity in concentration ranges effective for the reversal of MDR. Thus, we now had the chance to investigate changes in biological activity upon loss of symmetrical elements within our new class of MDR modulators at non-toxic concentrations, as had been evaluated in MTT-cytotoxicity assays in the MDR cell model (MTT = 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetraazolium bromide; data not shown).

We first prepared "mixed" dimers **8** and **9** using a threefold excess of each compound **1c** and **1d** for the solution reaction with **1e** (Scheme 2). Relative to **3c** or **e**, "mixed" compound **8** has lost one pair of symmetric elements, as the butoxycarbonyl substituent has been replaced by a benzylic one. The evaluated P-gp inhibitory activity is found to be significantly reduced at 1 μM compared to that with the double benzylic substitution in **3e** and slightly reduced compared with the bisbutoxycarbonyl substitution in **3c**. As we observed an increase in ac-



Scheme 2. Synthesis of mixed dimers **8**, **9**, **12** and **13**. a) $h\nu$, $\lambda > 300$ nm, MeOH/THF, 4 weeks, 27 °C; b) $LiAlH_4$ (1 equiv), THF, 2 h, −8 °C.

tivity from four to six HB functions in benzylic-substituted compounds **3e** and **f**, the number of five HB functions might suggest improved activity for compound **8**. This degree of activity is not plausible given our present knowledge about the structural requirements for obtaining biological activity within this class of compounds, as we have a C_2-symmetric binding region that hardly tolerates deviations from symmetric substitution patterns without a loss of biological activity.

In the "mixed" chiral compound **9**, we have lost another pair of symmetric elements. Although it combines all the structural elements that we found ensure excellent biological activity in the homodimers **3a**–**f**, such as benzylic, butoxycarbonyl, and methoxyphenyl functions as well as six HB functions, the activity of **9** is reduced relative to that of compound **8**, which has higher symmetry. So we have to conclude that a subsequent loss of C_2-symmetric elements might not be tolerated by the P-gp binding region; this proves that this region is itself C_2-symmetric and has several binding sites for the discussed aromatic substituents. This C_2-symmetric region ensures optimized activity only for compounds of corresponding symmetric structure.

This conclusion was additionally supported by comparing the biological activity of **3g** and "mixed" dimers **12** and **13**, which have a decreasing number of symmetric elements, while maintaining the same number of HB functions. The double 4′-methoxybenzylic substitution in **3g** results in reduced activity compared to the benzylic as well as the butoxycarbonyl substitution of the related symmetric compounds **3e** and **3c**. The exchange of one 4′-methoxybenzyl substituent for the more favorable butoxycarbonyl substituent leads to a decrease in activity in compound **13** at low concentration.

Comparing the most favorable substitution patterns of the completely "mixed" dimer **12** to those of the symmetric derivative **3g**, the more favorable benzyl, 4-methoxyphenyl, and butoxycarbonyl functions in **12** lead to a subsequent decrease in activity, thus supporting the C_2-symmetric character of the P-gp binding region.

Another target that was found to be inhibited by such symmetric cage dimers is the HIV-1 protease, which itself was found to have a C_2-symmetric cavity as binding region with several binding sites for potentially inhibiting compounds.[13,14] Evaluation of HIV-1 protease-inhibiting properties, as determined by the K_i values of our C_2-symmetric cage dimers **3c**–**f**, showed a slight decrease in biological activity on going from bisbenzyl to bisbutoxycarbonyl (presently best inhibitor from a series of *N*-alkoxycarbonyl-substituted compounds[13]) substitution and, finally, methoxyphenyl substitution as least favorable substituent (Table 2). The exchange of one (most favorable) benzyl function for one (less favorable) butoxycarbonyl function in compound **3e** leads to an increased activity for the resulting asymmetric derivative **8** compared with all related C_2-symmetric compounds. The complete loss of symmetric elements in compound **9**, with the introduction of the less favorable methoxyphenyl substituent, leads to the highest biological activity and reveals a more favorable asymmetric binding of the four aromatic substituents to the originally C_2-symmetric enzyme in the substrate-unbound conformation. Comparing the activity of the C_2-symmetric compound **3g**, which was found to be similar to that of the bisbenzyl-substituted derivative **3e**, to that of the "mixed" derivative **13**, the less favorable butoxycarbonyl substituent leads to a significant increase in activity.

Table 2. HIV-protease-inhibitory properties of selected cage dimers.[a]

Compound	K_i [μM]
3c	10
3d	13
3e	7.8
3f	23
3g	5.7
8	3.4
9	0.7
13	0.2

[a] Mean of two determinations.

While HIV-1 protease is evidently able to change its symmetry for a better binding of substrates, the C_2-symmetric binding region of P-gp remains unchanged even if more favorable groups are bound, thus proving a highly conserved character.

We have demonstrated the C_2-symmetric character of the P-gp binding region by analyzing complex features of substrate-binding possibilities for novel modulators. We have proved that symmetric benzylic as well as methoxyphenyl substitution leads to compounds with the highest P-gp-modulating activities known so far. A loss of symmetry leads to a major decrease in P-gp inhibition, while the enzyme-inhibition properties of HIV-1 protease increase to the highest degree within this class of inhibitors. So the suggested C_2-symmetric binding region of P-gp proves to have a conserved character with respect to the binding of substrates. This is a very surprising fact given the discussed high degree of flexibility of the membrane transporter in the models of P-gp function.

Experimental Section

Cage-dimer formation: Monomers **1e** and **f** were given by cyclocondensation reactions of aromatic aldehyde, alkyl propiolate, and amine in acetic acid, as described in ref. [15], or by acylation of *N*-unsubstituted monomers to form **1a**–**d** in dimethylpropylene urea (DMPU) according to literature methods.[16] Irradiation of solutions of monomers **1** in methanol/tetrahydrofuran (THF) carried out at wavelengths >270 nm with Ultra Vitalux lamps led to crystallizing cage compounds **2**, as well as irradiation side products, with isolated yields of about 50–75%. "Mixed" dimers **6** and **7** resulted, with yields of about 80%, from irradiation of mixtures of 1,4-dihydropyridine precursors **1e** plus **1c** or **1d** with relative concentrations of 1:3, so that homodimer formation from the excited compound **1e** with $\lambda_{max} = 360$ nm was strongly repressed by the excess of competing reactant **1c** or **1d**. The preparation of "mixed" dimers **10** and **11** followed the same procedure. Subsequent reduction of the ester groups to give the alcoholic target molecules **3a**–**f**, **8**, **9**, **12**, and **13** was carried out by one equivalent of lithium aluminium hydride in THF hydrolysis, and then final recrystallization from chloroform/methanol/petroleum ether.

MDR modulation: The MDR-resistant mouse T-lymphoma cell line L5178YvMDR was produced by primary infection of the NCI-L5178Y parental cell line with the pHa MDR/A retrovirus and subsequent selection by culturing the infected cells with colchicine (60 ng mL^{-1}—a concentration that does not allow growth of the parental cell line). This L5178YvMDR cell uniformly expresses

Pgp170, as indicated by staining of the cells with MRK-16-FITC monoclonal antibody and detection by flow cytometry. For the fluorescence-uptake assay, 0.5 mL aliquots of cells from both cell lines adjusted to a concentration of $2\times10^6\,mL^{-1}$, were preincubated with varying inhibitor concentrations taken from stock solutions in DMSO. Competition experiments with the fluorescent P-gp substrate rhodamine 123 were carried out as described in ref. [17]. Finally, fluorescence uptake was determined for 1×10^4 cells by flow cytometry by comparing each treasted parental and MDR-resistant cell line to each untreated control. Inhibitory activity ratios (R) were calculated by using Equation (1). Inhibitors with ratios >1.1 were found to be active, and those with ratios >10 were very active.

$$R = \frac{(\text{MDR-treated}/\text{MDR-untreated control})}{(\text{parental treated}/\text{parental untreated control})} \quad (1)$$

Protease inhibition: Preincubation of HIV-1 protease and varying inhibitor concentrations taken from stock solutions in DMSO was carried out in a buffer solution (0.37 M NaCl, 0.1 M MES, 4 mM EDTA, pH 6.25) and followed by addition of fluorogenic hexapeptide Abz (2-aminobenzoic acid)-Thr-Ile-Nle-Nle-pNph (4-nitrophenyl)-Gln-Arg-NH_2.[18] Concentration-dependant substrate cleavage was measured by determining the increased fluorescence of cleaved substrate at 410 nm as reported. Determination of K_i followed ref. [19].

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