Novel Insight in Structure−Activity Relationship and Bioanalysis of P-Glycoprotein Targeting Highly Potent Tetrakishydroxymethyl Substituted 3,9-Diazatetraasteranes

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Novel 3,9-diazatetraasteranes have been synthesized with varied aromatic substitution patterns and evaluated as P-glycoprotein (P-gp) inhibitors. Structure−activity relationships (SAR) are discussed in relation to determined physicochemical properties. The potential to induce P-gp expression has been evaluated in cancer cell lines. The bioanalytical results indicate favorable noninducing properties compared to P-gp inducing drug standard.

Introduction

Over the past decades multidrug resistance (mdr[a]) has been a main problem in cancer treatment with established anticancer drugs like anthracyclines, vinca alkaloides, or topoisomerase inhibitors.[1−4] Also, novel cytostatic agents like monoclonal antibodies, tyrosin receptor kinase inhibitors, or proteasome inhibitors developed the resistance phenomenon under short-time therapies.[5,6] Causative transmembrane efflux pumps like P-glycoprotein (P-gp), which lowered intracellular drug levels, were found in former drug-sensitive cells.[1,3,7,8] Their expression was reported to be induced by the application of the respective cytostatic agent.[9] The use of inhibitors of the efflux pump activities turned out to be the most effective strategy to reverse mdr. However, the use has been limited so far in clinical studies by the fact that the inhibitors themselves were substrates of the efflux pumps and were reported to induce the transporter protein expression.[10−13]

We synthesized a novel series of 3,9-diazatetraasteranes with varied aromatic substitution patterns to investigate the influence on the inhibition of P-gp activities. Mainly improved activities have been found for meta substituted phenyl compounds compared to para substituted derivatives in cancer cell lines. This proved an interesting sensitivity of the potential protein binding site for the positioning of essential functional groups and gave novel insight in the SAR of mdr modulators. We found no P-gp induction in a P-gp inducible cell line in contrast to the P-gp inducing cytostatics, so our novel P-gp inhibitors turn out as perspective mdr reversers for clinical studies.

Chemistry

The *N*-alkyl substituted 1,4-dihydropyridines **2a**−**g** have been synthesized by a cyclocondensation reaction of the amine, the ester, and the aromatic aldehyde in acetic acid under reflux (Scheme 1).[14] The substituted benzylamines **1e** and **1g** have been given by the lithium aluminum hydride reduction of the corresponding nitriles in THF. The nitriles were yielded from the corresponding aldehydes after treatment with hydroxylamine hydrochloride in triethylamine and after abstraction of water by the use of phthalic anhydride in acetonitrile.

The alcoholic target compounds **4a**−**g** were prepared from the ester derivatives **3a**−**g**. The ester derivatives were found as main reaction products from a photodimerization reaction of the 1,4-dihydropyridines **2a**−**g**. They were formed after irradiation of the 1,4-dihydropyridine solutions in THF under excitation of the dihydropyridine chromophores via [2 + 2] cycloaddition reactions with mean yields of about 65%.

Results and Discussion

The evaluation of P-gp inhibiting properties of **4a**−**g** was carried out by comparing the fluorescence uptake of the P-gp specific substrate rhodamine 123 into the inhibitor treated and the untreated controll cell lines. The resulting fluorescence activity ratios (FAR values) are shown in Table 1. The investigated inhibitor concentrations of 1 and 10 μM were much lower than those of reported bishydroxymethyl substituted compounds, which showed poorer activities at 30 μM than our novel compounds at 10 μM, as will be shown.[15] The novel tetrakishydoxymethyl substituted compounds **4a**−**g** have at least two more hydrogen bond donator and acceptor functions. Such functions are discussed to play an important role for the binding to the potential protein binding site of P-gp with which inhibitors primarily interact to inhibit the efflux pump activity.[15−17] It is known from literature that hydrogen bond acceptor functions are essential structural elements in P-gp inhibitors for such binding properties.[16]

Compound **4a** with the *N*-benzyl and 6,12-diphenyl substitutions showed much more increased P-gp inhibiting activities than the usual standard verapamil **5**, especially at the highest inhibitor concentrations. The introduction of the methoxy functions in derivative **4b** mainly increased the activities, which were about more than 10-fold compared to those of verapamil. The additional methoxy functions may serve as additional hydrogen bond acceptor functions toward the potential P-gp binding site of the inhibitors. Corresponding methoxy functions

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[a] Abbreviations: P-gp, P-glycoprotein; SAR, structure−activity relationships; mdr, multidrug resistance; FAR, fluorescence activity ratio; TLC, thin layer chromatography; THF, tetrahydrofuran; rt, room temperature; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; Et_3N, triethylamine; MeCN, acetonitrile.

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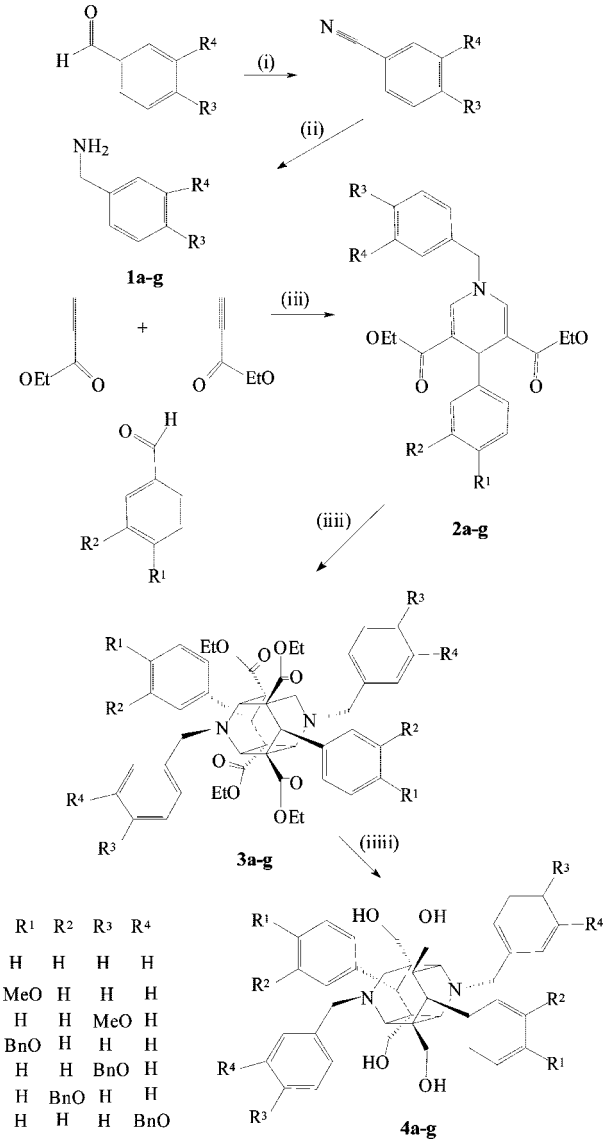
Scheme 1[a]



	R1	R2	R3	R4
a	H	H	H	H
b	MeO	H	H	H
c	H	H	MeO	H
d	BnO	H	H	H
e	H	H	BnO	H
f	H	BnO	H	H
g	H	H	H	BnO

[a] Reagents and conditions: (i) $NH_2OH-HCl$, Et_3N, phthalic anhydride, MeCN, 80 °C; (ii) $LiAlH_4$, THF, 4 °C; (iii) HAc, 100 °C; (iiii) *hν*, THF; (iiiii) $LiAlH_4$, THF, −8 °C.

Table 1. P-gp Inhibition and Lipophilicity Data of 3,9-Diazatetraasterane Target Compounds

	FAR values[a]		
	1 μM	10 μM	log *P*
4a	4.1 ± 0.3	29 ± 2.4	4.3 ± 1.1
4b	4.3 ± 0.4	57 ± 1.4	4.4 ± 1.5
4c	3.5 ± 0.6	13 ± 0.3	4.3 ± 1.3
4d	3.3 ± 1.1	47 ± 2.3	7.4 ± 1.5
4e	2.3 ± 0.2	28 ± 2.5	7.4 ± 1.7
4f	65 ± 8.1	125 ± 16	7.5 ± 1.7
4g	117 ± 1.7	205 ± 6.1	7.4 ± 1.7
5	2.1 ± 0.1	4.1 ± 0.1	

[a] Mean of two determinations.

are also found in verapamil. Thus, the increase of hydrogen bond acceptor functions is found to be favorable.

The introduction of the methoxy functions into the 4-position of the aromatic *N*-benzyl residue in **4c** led to a surprising loss in activity, especially at the highest inhibtor concentrations. We also calculated the lipophilicity-relevant log *P* values with various programs (Table 1). Although it is known from literature that such programs tend to overestimate the lipophilicity, it is possible to compare the resulting log *P* values within one class of considered compounds with respect to the biological activities.[17] High log *P* values of >3 have been reported in literature for good P-gp inhibitors. Correlations between log *P* values and the biological activities were also reported within most P-gp inhibitor classes.[17] The calculated log *P* values for **4a**−**c** are similar to literature values of good P-gp inhibitors but do not explain the differences in activity data of the methoxyphenyl substituted derivatives **4b** and **4c** (Table 1). Obviously the potential P-gp binding site of our compounds shows a strong regiosensitivity for the presentation of the essential functional groups like the methoxy functions with hydrogen bonding properties. Although the molecule could principly change its orientation toward the binding site, which would result in similar activities, the binding of the whole structure is suggested to be fixed within the binding site by the demonstrated different activity data, thus giving novel insight in the SAR binding of mdr modulators.

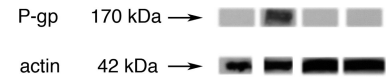


Figure 1. Western blot analyses of the gastric carcinoma cell line (EPG-85) before induction (line 1), after induction with daunorubicin (line 2), after incubation with compounds **4a** (line 3) and **4b** (line 4).

We then replaced the methoxy substituents by benzyloxy substituents in derivatives **4d**−**g**. Such benzyloxy substituents maintained the favorable hydrogen bond acceptor functions and would additionally change the lipophilicity with increased log *P* values.

In **4d** the benzyloxy substituents led to no improvements in the activities if compared to the methoxy substituted derivative **4b**. The replacement of the methoxy by the benzyloxy functions in derivative **4e** led to increases in activity of **4e** compared to **4c** at the highest concentrations. The differences in activity of **4d** and **4e** are comparable to those of **4b** and **4c**, which reflects that the same regiosensitive P-gp binding region is addressed by the inhibitors.

Significant increases in activities were found for different positionings of the benzyloxy substituents within the aromatic residues. The movement of the substituent from the aromatic 4′-para positions in **4d** and **4e** to the 3′-meta positions in derivatives **4f** and **4g** led to main increases in activity, even at the lower inhibitor concentrations with derivative **4g** being about 50-fold more active than verapamil. For all benzyloxy substituted derivatives the calculated log *P* values were similar in range as expected. However, the increased activities of the 3′-benzyloxy substituted derivatives **4f** and **4g** cannot only be reasoned with the increased log *P* values. They are plausible with the presence of the strongly regiosensitive P-gp binding region for the interactions with functional groups, which means a novel aspect in the SAR of mdr modulators.

Because the induction of transmembrane efflux pumps like P-gp is a critical unfavorable property of most active compounds that interact with P-gp as substrates or inhibitors,[18,13] we investigated this potential to induce P-gp expression under usual short-time application regimes for two of our novel inhibitors in a P-gp inducible human cancer cell line.

The gastric carcinoma cell line EPG-85 257P was demonstrated to overexpress P-gp under short-time treatments with the P-gp inducing cytostatic agent daunorubicin.[18] The protein amounts for this induction are shown in the Western blots in Figure 1, lane 2, whereas no P-gp was found in the untreated cell line before induction treatment in lane 1. The

treatment of the P-gp inducible cell model with our inhibitors **4a** and **4b** did not result in detectable P-gp amounts as shown in lanes 3 and 4.

Conclusions

We developed novel P-gp inhibitors with mainly improved activities up to 50-fold compared to verapamil. Methoxy functions as hydrogen bond acceptor functions in compounds **4b** and **4c** improved activities in dependence of their molecular presentation within the aromatic residues. Benzyloxy functions instead of the methoxy functions mainly led to increased activities so that the increased lipophilicity was found favorable. Highest activities were found for meta substituted compounds **4f** and **4g**. This confirmed an unexpected regiosensitivity of the potential P-gp binding site for the presentation of the functionally important groups and thus gives novel insight into the SAR of mdr modulators. The fact that selected compounds **4a** and **4b** did not induce P-gp in the inducible cell model compared to daunorubicin is of favor for the novel P-gp inhibitors with respect to clinical studies and a great advantage compared to known inhibitors.

Experimental Section

General. Commercial reagents were used without further purification. ^{1}H NMR (400 MHz) spectra were run with tetramethylsilane as an internal standard. Mass spectra were recorded with an AMD 402 using EI and a Finnigan-LCQ Classic mass spectrometer using ESI techniques. Elemental analyses were performed with a Leco CHNS-932 apparatus. TLC analyses were carried out with E. Merck 5554 silica gel plates.

The synthesis of the 1,4-dihydropyridines **2a** and **2b** has been reported in ref 19.

General Procedure for the Formation of Benzylamines 1e,g from Corresponding Aldehydes.[20] The aldehyde (1 equiv, 10 mmol) and freshly distilled triethylamine (1.1 equiv, 11 mmol) were added to a cold solution of hydroxylamine hydrochloride (1 equiv, 10 mmol) in anhydrous acetonitrile (50 mL). The mixture was stirred for 1 h. Then phthalic anhydride (1.05 equiv, 10.5 mmol) was added in portions under nitrogen atmosphere. The solution was heated under reflux until nearly no more reaction intermediates were detectable by TLC. Workup procedure included solution concentration, stirring with dichlormethane (60 mL), and filtration followed the washing with 5% ammonia−water for several times, drying over sodium sulfate, filtration, and evaporation to dryness. The residual oil was dissolved in petrol ether/ethylacetate mixtures from which the nitriles crystallized. The crude nitriles (1 equiv, 30 mmol) were dissolved in anhydrous diethyl ether (30 mL), and the solutions were added dropwise to a solution of lithium aluminum hydride (1.2 equiv, 36 mmol) in anhydrous diethyl ether (55 mL) at 4 °C. After the mixture was stirred at low temperature for 2 h, portions of water were added. After separation of the organic layer, drying over sodium sulfate, and filtration, the amines crystallized under evaporation.

General Procedure for the 1,4-Dihydropyridine Formation of 2c−g.[19] Aromatic aldehyde (1 equiv, 10 mmol), ethyl propiolate (2 equiv, 20 mmol), and benzylamine derivatives (1 equiv, 10 mmol) were dissolved in freshly distilled acetic acid (1 mL). The mixture was stirred for 1−2 h at 100 °C. After the mixture was cooled, sufficient water was added (∼10 mL) and then several extractions followed with chloroform (30 mL). After drying of the extracts over sodium sulfate and filtration, the solutions were evaporated and the oily residues were dissolved in ethanol or diethyl ether from which the compounds **2** crystallized.

General Procedure for the 3,9-Diazatetrasterane 3a−g Formation. 1,4-Dihydropyridines **2** (1 equiv, 1.2 mmol) were dissolved in dried THF (30 mL) in a quartz flask. After syringing with argon, the solution was irrdiated with the light of Ultra-Vitalux lamps (λ > 270 nm) from a distance of 60 cm at rt. The product formation was followed by TLC until no more starting compound **2** was detectable. Compounds **3** partly crystallized from the solutions under irradiation and were finally collected after reduction of the THF solvent under cooling.

Tetraethyl 3,9-Dibenzyl-6,12-bis(3-benzyloxyphenyl)-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane-1,5,7,11-tetracarboxylate (3f). Yield 0.275 g (46%); mp 169−178 °C; ^{1}H NMR ($CDCl_3$) δ 7.35−7.17 (m, 20 H), 6.99 (t J = 7.7 Hz, 2 H), 6.96 (s, 2 H), 6.80 (d J = 7.7 Hz, 2 H), 6.72 (d J = 7.7 Hz, 2 H), 4.88 (s, 4 H), 4.46 (s, 4 H), 4.27 (s, 2 H), 4.25 (s, 4 H), 3.97 (q J = 7.0 Hz, 8 H), 0.97 (t J = 7.0 Hz, 12 H); MS (ESI) m/z = 996 (M + H$^+$). Anal. ($C_{62}H_{62}N_2O_{10}$) C, H, N.

Tetraethyl 3,9-Bis(3-benzyloxybenzyl)-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane-1,5,7,11-tetracarboxylate (3g). Yield 0.269 g (45%); mp 119−126 °C; ^{1}H NMR (DMSO-d_6) δ 7.38−7.30 (m, 14 H), 7.22−7.06 (m, 10 H), 6.90−6.87 (m, 4 H), 4.77 (s, 4 H), 4.44 (s, 4 H), 4.27 (s, 2 H), 4.17 (s, 4 H), 3.92 (q J = 7.0 Hz, 8 H), 0.93 (t J = 7.0 Hz, 12 H); MS (ESI) m/z = 996 (M + H$^+$). Anal. ($C_{62}H_{62}N_2O_{10}$) C, H, N.

General Procedure for the Formation of the Alcoholic Target Compounds 4a−g. 3,9-Diazatetrasteranes **3** (1 equiv, 70 μM) were dissolved in dried THF under warming. After syringing with argon, the solution was stirred for 1 h at rt. After the mixture was cooled to 8 °C, lithium aluminum hydride (16 equiv, 1.1 mmol) in a solution in THF (1 M) was added dropwise under stirring, which then continued for 3 h. Then the excess of lithium aluminum was hydrolyzed with portions of ice−water. Extraction followed with chloroform (3 × 60 mL). After drying over sodium sulfate and filtration the solvent was removed in vacuum. The residual oil was taken up in chloroform, and after addition of mixtures of diethyl ether and petrol ether the target compounds **4** crystallized and where recrystallized from methanol/water.

3,9-Dibenzyl-6,12-bis(3-benzyloxyphenyl)-1,5,7,11-tetrakishydroxymethyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (4f). Yield 0.021 g (29%); mp 111−117 °C; ^{1}H NMR (DMSO-d_6) δ 7.63 (d J = 8.7 Hz, 1 H), 7.45−7.20 (m, 21 H), 7.04 (m, 3 H), 6.96 (s, 1 H), 6.78 (d J = 10.8 Hz, 1 H), 6.71 (d J = 10.8 Hz, 1 H), 5.01 (s, 2 H), 4.84 (s, 2 H), 4.44 (m, 4 H), 4.17 (s, 4 H), 3.68 (s, 2 H), 3.15 (m, 8 H), 2.95 (s, 4 H); MS (ESI) m/z = 850 (M + Na$^+$). Anal. ($C_{54}H_{54}N_2O_6$) C, H, N.

3,9-Bis(3-benzyloxybenzyl)-1,5,7,11-tetrahykisdroxymethyl-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane (4g). Yield 0.050 g (69%); mp 219−225 °C; ^{1}H NMR (DMSO-d_6) δ 7.81 (d J = 7.4 Hz, 2 H), 7.40−7.28 (m, 12 H), 7.21 (t J = 8.0, 7.6 Hz, 2 H), 7.12−7.00 (m, 6 H), 6.95 (s, 2 H), 6.89 (dd J = 7.6, 2.4 Hz, 2 H), 6.85 (dd J = 8.0, 2.4 Hz, 2 H), 4.91 (s, 4 H), 4.50 (t J = 4.4 Hz, 4 H), 4.12 (s, 4 H), 3.65 (s, 2 H), 3.16 (dd, J = 10.4, 4.4 Hz, 8 H), 2.96 (s, 4 H); MS (ESI) m/z = 828 (M + H$^+$). Anal. ($C_{54}H_{54}N_2O_6$) C, H, N.

P-gp Inhibiton Assay.[15] Cultured cells from mouse T lymphoma parental cell line L5178 and the P-gp expressing subline L5178Y were adjusted to a concentration of 2 × 10^6 cells/mL in serum-free McCoys 5A medium. Aliquots of 0.5 mL were placed in Eppendorf tubes. Test compounds were added from stock solutions in dmso. After incubation for 10 min at rt, rhodamine 123 was added with a final concentration of 5.2 μM. Incubation continued for 20 min at 37 °C. After washing twice with phosphate-buffered saline (PBS), the samples were resuspended and the 1 × 10^4 cells were measured by flow cytometry. Fluorescence activity ratios were calculated by the relation of the determined fluorescence in the inhibitor treated P-gp expressing subline to the parental cell line with each fluorescence value corrected to that of the untreated control.

Lipophilicity Calculations. Molecules were constructed and log P values were determined with seven calculation programs (ALOGPs, AC_logP, AB/LogP, COSMOFrag, miLogP, KNOWWIN and XLOGP).[21] The resulting mean values were given by the ALOGPS 2.1 program.[21]

Induction Studies. Cells of the gastric carcinoma cell line were cultured as described in six-well plates with a density of 5 × 10^5 cells/well.[22] After addition of the compounds for P-gp induction

the culturing was continued for 72 h. Then the culture medium was removed, the cells were washed twice, and the cell proteins were detached by the use of Triton X-100. The cell suspension was transferred into Eppendorf tubes, and the proteins were denaturated for 10 min at 95 °C. After centrifugation the protein concentration was determined UV spectroscopically at 630 nm after treatment with amido black solution. An amount of 20 μg of the extracted membrane proteins was separated on 4% stacking and 6% resolving SDS−PAGE gels and transferred to a 0.2 μm cellulose nitrate membrane. The membrane was blocked in 5% skim milk in TBS overnight. Then the buffer was removed and the membrane was incubated for 2 h with mouse monoclonal antibody C219, which was diluted 1:100 in 1% skim milk. Afterward the membrane was treated with peroxidase-conjugated antimouse IgG diluted 1:10000 for visualization of the protein−antibody complexes by chemiluminescence. The membrane was also incubated with a mouse monoclonal antibody directed against actin protein diluted 1:50000 as a control for equivalent protein loading.

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Supporting Information Available: Detailed spectroscopic and purity data of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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