Brief Articles

Biological Evaluation of Bishydroxymethyl-Substituted Cage Dimeric 1,4-Dihydropyridines as a Novel Class of P-Glycoprotein Modulating Agents in Cancer Cells

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A series of N-substituted cage dimeric 1,4-dihydropyridines **3a**−**e** was evaluated as inhibitors of membrane efflux pump P-glycoprotein (P-gp) in multidrug resistant (mdr) cancer cells. Structure−activity relationships (SAR) and cytotoxic properties are discussed. Effective concentrations for overcoming mdr have been demonstrated in competition studies with the P-gp substrate epirubicin.

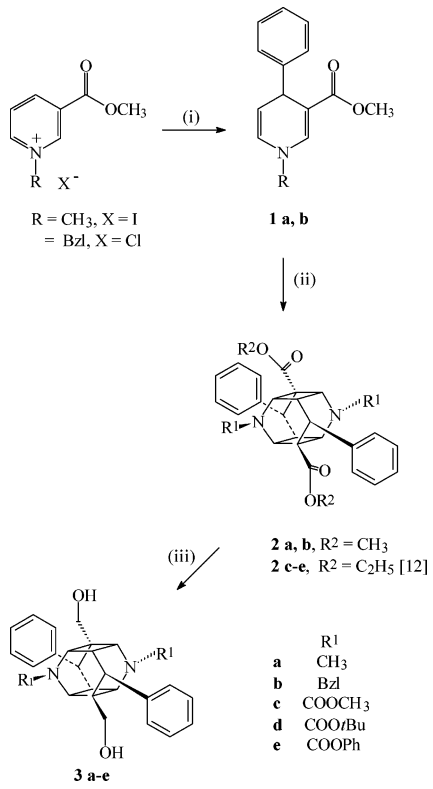
Introduction

Multidrug resistance (mdr) is a main problem in cancer treatment. Membrane efflux pumps such as P-glycoprotein (P-gp) have been identified as causative agents which partly transport cytostatic agents out of the cells before they reach the cytosol.[1,2] With a broad substrate specifity, P-gp was found to transport even novel cytostatics such as tyrosine kinase inhibitor imatinib or monoclonal antibody gemtuzumab ozagamicin.[3,4] Intense efforts to overcome mdr by influencing transporter expressions via signal transduction pathways or by direct transcriptional control have not been successful in clinical trials.[5] The most hopeful alternative to combate mdr is the inhibition of the transporter activity itself. Almost all modulators of mdr have been derived from known drugs such as verapamil with structural modifications which only occasionally led to effective modulators in concentration ranges below their drug-like pharmacological activities.[6] Moreover, most inhibiting agents proved to be transporter substrates themselves, thus requiring high applied concentrations for effectively overcoming mdr.[7,8]

We report a novel series of bishydroxymethyl-substituted cage dimeric 1,4-dihydropyridines **3a**−**e** which prove to be effective and superior P-gp inhibitors compared to verapamil, without showing additional pharmacological activities, as do the structurally related tetrabishydroxymethyl-substituted compounds which have been demonstrated to act as HIV-protease inhibitors.[9] Moreover, the comparison of the cytotoxic properties indicates that they are practically not P-gp substrates and thus form an innovative class of new mdr modulators for further development efforts.

Chemistry. The alcoholic target compounds **3a**−**e** (Scheme 1) have all been prepared from the ester group-substituted cage dimers **2a**−**e** by reduction with lithium aluminum hydride in THF at low temperatures (−8 °C), in effective reaction times of a few hours and overall yields of about 70%. *N*-Alkyl cage dimers **2a**,**b** have been prepared by a solution dimerization reaction of monomeric 1,4-dihydropyridines **1a**,**b** under UV-excitation of the dihydropyridine chromophore with $\lambda_{max} = 346$ nm (**a**) and 348 nm (**b**). Monomeric 1,4-dihydropyridines **1a**,**b** were prepared by a regioselective 4-phenylation of the *N*-alkylpyridinium salt starting compounds by the use of equimolar amounts of phenylmagnesium chloride and catalytic traces of copper(I) iodide.

Scheme 1[a]



[a] Reagents and conditions: (i) CuI (5%), PhMgCl, THF, RT; (ii) hν, 25 °C, THF; (iii) $LiAlH_4$, −8 °C, THF.

Results and Discussion

P-gp inhibiting properties have been evaluated by comparing the concentration-dependent uptakes of P-gp specific fluorescent substrate rhodamine 123 in both mdr1-gene transfected and parental mouse T-lymphoma cell lines as fluorescence activity

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Table 1. P-gp Inhibition, Cytotoxicity and MDR Overcoming Properties of Cage Dimeric 1,4-Dihydropyridines in Tumor Cell Lines

	FAR[a]				cytotoxicity[a]		overcoming MDR,[a] IC_{50} [μM]	
	at 3 μM	at 30 μM	max.	IC_{50} (μM)	Par, IC_{50} (μM)	MDR1, IC_{50} (μM)	modulator	epirubicin
3a	1.2 ± 0.03	34.3 ± 0.8	35	17.6 ± 0.4	7.1 ± 0.3	7.5 ± 0.3	>22.5	10 ± 0.6
3b	24.5 ± 0.5	26.5 ± 0.5	27	<1	11.9 ± 0.8	13.3 ± 0.5	6.5 ± 0.06	1.3 ± 0.5
3c	1.1 ± 0.04	21.6 ± 0.3	22[b]	3.8 ± 0.5	n.d[c]	n.d[c]	n.d[c]	n.d[c]
3d	6.8 ± 0.3	33.0 ± 0.3	33	6.1 ± 0.1	2.5 ± 0.1	3.2 ± 0.2	7.2 ± 0.07	>10
3e	28.1 ± 1.2	30.8 ± 1.3	32	<1	12.7 ± 0.5	21.0 ± 0.9	5.6 ± 0.1	<5
4	0.7 ± 0.03	7.3 ± 0.3[d]	−	n.d[c]				

[a] Mean of two determinations. [b] saturation. [c] n.d., not determined. [d] Determined at the clinically relevant concentration of 11 μM with saturation effects.

ratios (FAR). Within the *N*-acyloxy derivatives **3c**−**e**, an increase in activity is observed at low concentration from methyl to *tert*-butyl and finally phenyl substitution. With increased lipophilicity of the varied substituents at the nitrogen atom, the phenyl-substituted compound **3e** finally reaches a more than 15-fold higher ratio in comparison to verapamil (**4**) as a standard with a FAR value of 7.27 (11 μM) (Table 1). At higher concentrations we reached saturation effects of the P-gp inhibitions with ratios of about 30 for the lipophilic *tert*-butyl and phenyloxycarbonyl substitution.

Within the *N*-alkyl derivatives **3a**,**b**, benzylic substitution leads to a more than 15-fold higher activity than does methyl substitution and both compounds reach final P-gp inhibition saturation effects at higher concentrations. Again, the more lipophilic substituent is found superior at 3 μM.

Comparing equivalents of both *N*-acyloxy and *N*-alkyl compound series in each case, the additional acyl functions may play a role in hydrogen bonding to the P-gp binding region of these compounds. These additional functions lead to a lowered activity in comparison of compounds **3a** and **3c** while they increase activity in comparison of compounds **3b** and **3e**. From these results it may be concluded that both the differing lipophilicity as well as hydrogen bonding patterns will play a complementary role in the biological activity within this class of highly active compounds.

The IC_{50} values for the reduction of fluorescence uptake for compounds of similar comparable P-gp saturation effects (**3a**,**b**,**d**,**e**) with FAR values of about 32, indicate the lowest activity for methyl substitution and highest biological activities for benzyl- and the phenyloxycarbonyl-substituted derivatives **b** and **e**. The structure−activity relationships stress the importance of lipophilic substitution patterns in effective mdr modulators.[10,11]

The cytotoxicity of the comparable compounds **3a**,**b**,**d**,**e** have been determined in both mdr1-gene transfected and parental mouse T-lymphoma cell lines to ascertain whether any potential cytotoxic properties in the parental cell line are reduced in the mdr1 subline by P-gp transport efflux of the compounds out of the cells. Such a reduction of compound cytotoxicity suggests unfavorable P-gp substrate properties so that higher concentrations are necessary to achieve the same cytotoxic effects.[8]

Some cytotoxicity has been found for compound **3d** in the parental cell line, while the cytotoxicity of compounds **b** and **e** are reduced. When both the parental and the mdr1 transfected cell lines are compared, interestingly, only the phenoxycarbonyl-substituted derivative **e** showed differences in cytotoxicity, while for the other three derivatives **a**, **b**, and **d**, almost identical IC_{50} values are found. Only derivative **3e** of this novel series of mdr modulators exhibited some P-gp substrate properties with reduced cytotoxic activity in the mdr1 transfected cell line caused by the efflux from this mdr resistant cell line as a P-gp substrate.

This is an important finding with respect to a potential clinical application of these compounds which allows lowered application rates compared to those mdr1 modulators which are being effluxed from the cells as P-gp substrates themselves and thus require higher unfavorable application rates.[8] We also investigated the concentrations to overcome mdr by comparing the cytotoxicity in mouse T lymphoma parental and mdr1 subline using combinations of each fixed modulator and cytotoxic P-gp-substrate epirubicin concentrations, respectively, in competition with varying epirubicin and modulator concentrations, respectively.

In the study, with varying epirubicin concentrations at each fixed modulator concentration we found the lowest cytotoxicities for the mdr1 subline compared to the parental cell line at the lowest modulator concentrations. These lowest cytotoxicities result from the concentration-dependent low P-gp inhibiting properties. We found subsequent increased cytotoxicities in the mdr1 subline due to increased intracellular epirubicin levels caused by increased P-gp inhibition of increasing modulator concentrations and thus increased cytotoxicity of epirubicin. Increased cytotoxicity was partly found also in the parental cell line due to some increasing cytotoxic effects of the modulators themselves.

With mainly reduced IC_{50} values of cytotoxicity in the mdr1 subline we observed an overcoming of mdr for compounds **3b**,**d**,**e** with equal concentrations in the low range of 5.6 and 7.2 μM in both cell lines but not for the poor mdr modulator **3a** (Table 1).

Using each fixed epirubicin concentration in combination with varied modulator concentrations we observed certain differences in the potential of epirubicin to overcome mdr with equal epirubicin concentrations in both cell lines. While the use of compound **3d** did not overcome mdr within the epirubicin concentration range up to 10 μM, the benzyl- and phenyloxycarbonyl-substituted derivatives **3b** and **3e** were successful competitors that overcame mdr at an epirubicin concentration of 1.3 μM, with compound **3b** as the best derivative.

Conclusion

We report a novel class of mdr modulators which exhibit effective P-gp inhibitory properties significantly superior to that of verapamil. The most active compounds are lipophilic substituted *N*-benzyl and -phenyloxycarbonyl derivatives **3b** and **3e**. Some P-gp substrate properties have been suggested only for the *N*-phenyloxycarbonyl compound **3e**. Competition studies with cytotoxic P-gp substrate epirubicin indicated the overcoming of mdr in comparison of the cell line at concentrations below cytotoxic ranges of the most effective mdr-modulating concentrations of the compounds themselves. The *N*-benzyl compound exhibited the highest activity and practically no P-gp substrate properties. Therefore, it could be a promising candidate for further clinical studies and structural improvements as a novel lead compound for the overcoming of mdr.

Experimental Section

General. Commercial reagents were used without further purification. ^{1}H NMR (500 MHz) spectra were recorded using

tetramethylsilane as an internal standard. TLC was performed on E. Merck 5554 silica gel plates. Mass spectra were measured with an AMD 402 and Finigan-LCQ Classic mass spectrometer, respectively. Elemental analysis was performed using a Leco CHNS-932 apparatus.

The synthesis of cage compounds **2c**−**e** has been reported in ref 12.

General Procedure for the Formation of 4-Phenyl-1,4-dihydropyridines 1a,b from *N*-Alkylpyridinium Salts.[13] The *N*-alkylpyridinium salt (1 equiv, 10 mmol) was suspended in dry THF (100 mL) and anhydrous methyl sulfide (27 mL). After addition of copper(I) iodide (0.095 g, 0.5 mmol), a solution of phenylmagnesium chloride (1 M) in a mixture of THF (10 mL, 10 mmol) was added dropwise. After 2 h of stirring at room temperature, the solution was treated with an aqueous solution of ammonium chloride (60 mL, 113 mmol) and then extracted with diethyl ether (150 mL). The ether phase was washed with 20 mL each of a 20% mixture of ammonia/ammonium chloride (1/1), water, twice with hydrochloric acid (10%), water, and finally saturated aqueous sodium chloride following the described procedure in ref 12. After final removal of the dried ether, the oily residue was solved in ethanol from which the product crystallized.

General Procedure for the Formation of *N*-Alkyl Cage Compounds (2a,b). *N*-Alkyl-1,4-dihydropyridine **1** (1 equiv, 2.18 mmol) was dissolved in dry THF (40 mL) with stirring. The solution was irradiated in a borosilicate flask with an Ultra Vitalux lamp from a distance of 60 cm at 25 °C. After eight weeks, the cage compound **2** precipitated from the solution and the reaction was carried out until no more precipitate was formed. The compound was collected by filtration and dried.

General Procedure for the Formation of Alcoholic Target Cage Compounds (3a−**e)**. Cage compound **2** (1 equiv, 0.04 mmol) was dissolved in dry THF (20 mL). The solution was cooled to −8 °C, and then a solution of lithium aluminum hydride (0.04 mL, 0.04 mmol) in THF (1 M) was added. After 2 h, the reaction mixture was kept overnight at −8 °C and the next day hydrolyzed with 1 mL of cold water at 0 °C. The solution was then extracted with 20 mL of chloroform three times. The combined extracts were then dried over sodium sulfate. After filtration, the organic phase was removed in a vacuum and the remaining oil was dissolved in chloroform/methanol/water from which **3** crystallized after dropwise addition each of diethyl ether and petroleum ether.

Fluorescence Uptake Assay. Cells from L5178Y mouse T-cell lymphoma parental cell line and from its multidrug resistant subline L5178YvMDR which was a gift from the National Cancer Institute (NCI) were adjusted to a concentration of 2×10^6/mL in serum-free McCoys 5A medium and distributed in 0.5 mL aliquots to eppendorf centrifuge tubes. The test compounds were added from stock solutions (1.0 mg/mL) and incubated for 10 min at room temperature. Then rhodamine 123 with 5.2 μM as a final concentration was added and incubation continued for 20 min at 37 °C. After being washed twice with phosphate-buffered saline (PBS), the samples were resuspended in PBS for analysis. The fluorescence of 1×10^4 cells was measured by flow cytometry. Fluorescence activity ratios (FAR) have been calculated from fluorescence uptake relationships of each treated and untreated control cell line. Exact IC_{50} values have been determined from the resulting sigmoidal curves obtained by plotting each concentration and corresponding FAR value.

MTT Cytotoxicity Assay. Cells were cultured for 48 h under 5% CO_2 atmosphere and 37 °C. Then 10 μL of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution with a final concentration of 0.5 mg/mL was added, and incubation was continued for additional 4 h at 37 °C. Precipitated formazane crystals were resolved under addition of 100 μL of SDS solution (10% in 0.01 M HCl), and then samples were left standing overnight under the described conditions. Concentration-dependent UV absorbance of the formazane was corrected by the absorbance of the cell control and the compounds. Plotting of each compound concentration and corrected UV absorbance led to a sigmoidal curve from which the IC_{50} values could be derived. For the determination of the mdr overcoming concentrations, cytotoxicity was determined as described by combining each fixed concentration of epirubicin within a range of 0.02 to 10 μM and modulator within a range of 0.13 (**3d**) to 64.5 (**3e**) μM, respectively, and varying concentrations of modulator and epirubicin in each of both cell lines. Plotting of each fixed concentration and resulting IC_{50} values of cytotoxicity for the varied concentration of the other combined compounds resulted in sigmoid-like curves with an intersection point of the resulting concentration for overcoming of mdr.

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Supporting Information Available: Detailed spectroscopic and purity data of the compounds. Sigmoidal curves of the fluorescence uptake assays are shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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