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Discovery of substituted 1,4-dihydroquinolines as novel promising class of P-glycoprotein inhibitors: First structure–activity relationships and bioanalytical studies

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ABSTRACT

Multidrug resistance (mdr) is the most important problem in the therapeutical treatment of cancer. One central problem in the resistance proceeding is the expression of transmembrane efflux pumps which transport drugs out of the cells. We developed novel substituted 1,4-dihydroquinolines as inhibitors of the transmembrane efflux pump P-glycoprotein. Structure–activity relationships are discussed for this first series. Promising active inhibitors have been identified and first bioanalytical studies have been carried out to address questions of cellular toxicity, P-gp substrate as well as mdr reversal properties.

With the understanding of cell regulating processes in the last decades the number of identified dysregulating agents in cancer cells increased, for example, the role of protein kinases which are partly involved in several cellular pathways.[1] Novel drugs have been developed that address such dysregulated agents and preferably act as protein kinase inhibitors.[2,3] Occurring resistances against protein kinases led to the establishment of multitargeting protein kinase inhibitors which target several protein kinases in the cancer progression.[4] However, successfully acting small-molecule inhibitors like imatinib or monoclonal antibodies were found to be substrates of transmembrane efflux pumps like P-glycoprotein.[5,6] P-glycoprotein is found in cancer cells and its cellular expression is mostly induced by the used anticancer drugs.[7,8] P-gp transports drugs of various structures, so that a multidrug resistance (mdr) of the respective cancer cell results. The occurrence of P-gp makes such a cancer treatment difficult.[9] The inhibition of the efflux pump activity has been studied by the use of potential inhibitors which suffered from cellular toxicity. That toxicity was partly caused by high doses of the inhibitors that were substrates of the efflux pumps.[10,11] Such high doses were required to reach cancer cell-relevant effects, but were toxic for normal cells.

We discovered 1,4-dihydroquinolines as a novel P-gp inhibiting compound class. Related tetrahydroisoquinolines were derived from the bisbenzylisoquinoline alkaloid tetrandrine. They showed cardiovascular effects and some mdr reversal activities that were discussed to result from the affection of the membrane fluidity.[12] Two quinolones isolated from a Chinese medical herb showed some P-gp inhibiting effects, but were not further investigated.[13] We reported some P-gp inhibiting effects for quinolone-related pyridine-2-ones.[14] The structures of both the quinolones and the pyridine-2-ones are shown in Figure 1.

Alkaloids have a complex structure and compounds derived from natural products afford complex isolation and purification processes to provide a suitable drug for therapeutical use.

Our novel 1,4-dihydroquinolines were available in a simple two-step reaction procedure. The first reaction product could be used without purification. We found partly promising P-gp inhibitory activities for our compounds and demonstrate a first bioanalytical compound profile of nontoxicity, no P-gp substrate properties and mdr reversing effects.

Our 1,4-dihydroquinoline synthesis started from ethyl quinoline-carboxylate **1** which was melted in a flask on an oil bath at a temperature of 80–100 °C. Then the respective benzyl bromide **2a**–**c** was added dropwise under stirring at the maintained temperature (Scheme 1).

The noncommercially available 4-methoxy benzyl bromide **2c** was prepared from 4-methoxy benzyl alcohol via treatment with bromic acid in benzole at room temperature. The resulting *N*-benzyl quinolinium salts **3a**–**c** were washed with portions of diethyl ether and dried over phosphorus pentoxide without further

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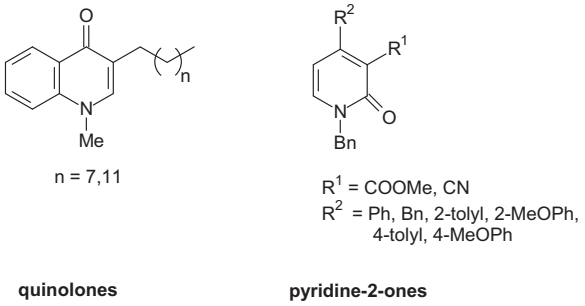


Figure 1. Structures and substitution patterns of cited quinolones and pyridine-2-ones.

purifications. The salts were dissolved in THF for the introduction of the respective 4-phenyl substituent and catalytic amounts of copper(I) iodide were added with lithium chloride to improve the catalyst solubility. The stirred mixture was treated with the respective aryl magnesium chloride as used Grignard reagent at room temperature to give the target compounds **4a–i**[15] which were yielded after the work-up procedure from diethyl ether.

The P-gp inhibiting properties of the compounds were evaluated in a cell line model of a non P-gp expressing mouse T-lymphoma cell line and a respective subline. That subline expresses human P-gp after a retroviral gene transfection and a cell culturing under colchicine supplementation of the medium. That procedure ensured an exclusive survival of the P-gp expressing cells.

We determined the uptake of the fluorescent P-gp substrate rhodamine 123 in both cell lines using flow cytometry technique with and without a P-gp inhibitor addition. The inhibition of P-gp resulted in a higher uptake of the fluorescent substrate in the P-gp expressing cell line.

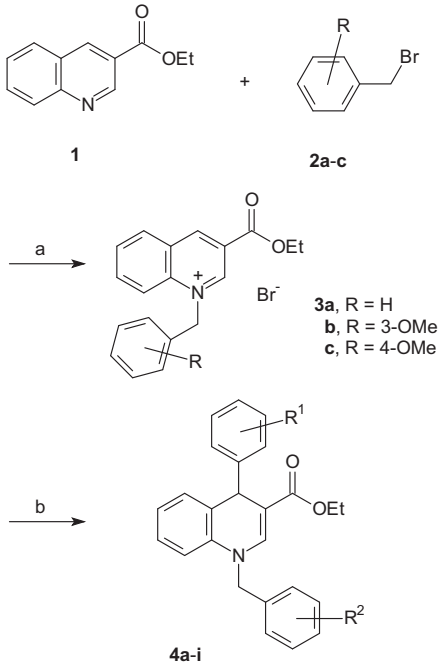
Finally, *FAR* values for the inhibition of P-gp were calculated by a division of the uptaken fluorescence amounts in the P-gp expressing cell line with the fluorescence amounts of the non P-gp expressing cell line each with inhibitor, after both fluorescence amount values have been related to those of the untreated control cell lines without inhibitor.

The given *FAR* values are listed in Table 1. Those calculated *FAR* values >1.1 prove an inhibition of P-gp.

The *N*-benzyl and 4-phenyl compound **4a** showed activity as P-gp inhibitor at the higher concentration of 10 μM with a *FAR* value of 2.86. The introduction of a methoxy function into the 4-position of the phenyl residue in derivative **4b** mainly increased the activity at both concentrations. Methoxy groups in mdr modulators are known to function as hydrogen bond acceptors binding to a potential P-gp binding site.[16] Compound **4b** showed a better activity at the higher concentration than the used standard verapamil which is known to be one of the best in vitro inhibitors.[17] When the methoxy function is moved from the 4- to the 3-position of the phenyl residue we found further increases in the activity of the respective compound **4c**. We then introduced two methoxy functions into the 4-phenyl residue and expected increases in activity provided that such methoxy functions strengthen the bonding of the inhibitor to a potential P-gp binding region. First, we combined a 3- and a 4-methoxy function in compound **4d**. This dimethoxy combination resulted in an increased activity of more than 100 per cent if compared to the monomethoxy substituted compounds **4b** and **4c**, respectively. Compound **4d** showed a more than 2.5-fold higher activity than the standard verapamil. A combination of a 3- and a 5-methoxy substitution in derivative **4e** was not as favourable as the 3- and 4-dimethoxy substitution of compound **4d**. However, if compared to the only 3-methoxy substitution in derivative **4c** we found a higher activity which confirmed that the two potential hydrogen bond acceptor functions are much more favourable than only one of these functional groups.

We then investigated whether the intramolecular positioning of these two methoxy functions would be of importance for the biological activity. So we combined the favourable 3-methoxyphenyl function of derivative **4c** with a 3-methoxybenzyl function in compound **4f**. If compared to the 3- and 5-dimethoxy substitution of derivative **4e** the activities were found little lower at both concentrations. The result indicated that a disubstitution of the 4-phenyl residue in compound **4e** with both methoxy functions is more favourable than the allocation on both aromatic residues in the respective *meta* positions of derivative **4f**.

The combination of the 3-methoxyphenyl substitution of compound **4c** with a 4-methoxybenzyl substitution in derivative **4g** also improved the activity if compared to the monomethoxy phenyl substitution of derivative **4c**. If compared to the 3- and 4-dimethoxy substituted compound **4d** the activity was found lower. Also in this case the allocation of two methoxy functions on both aromatic residues at the respective positioning is less favourable



Scheme 1. Reagents and conditions for the preparation of compounds: (a) 80–100 °C, 20 min, 1 h, rt, 20–85%, (b) aryl magnesium chloride, Cu(I) I, LiCl, THF, rt, 30 min, 18–65%.

Table 1
Concentration dependent P-gp inhibition of target compounds **4a–i**

Compound	R^1	R^2	*FAR* value[a]	
			1 μM	10 μM
4a	H	H	0.79 ± 0.11	2.86 ± 0.33
4b	4-OMe	H	1.27 ± 0.46	5.52 ± 1.22
4c	3-OMe	H	1.55 ± 0.54	7.43 ± 1.14
4d	3-, 4-OMe	H	3.42 ± 0.79	12.77 ± 2.03
4e	3-, 5-OMe	H	2.96 ± 0.52	10.50 ± 1.78
4f	3-OMe	3-OMe	2.26 ± 0.43	9.68 ± 1.12
4g	3-OMe	4-OMe	2.03 ± 0.41	10.34 ± 2.01
4h	4-OMe	3-OMe	2.23 ± 0.40	9.95 ± 1.19
4i	4-OME	4-OMe	2.55 ± 0.26	12.38 ± 0.87
Verapamil			1.46 ± 0.15	4.98 ± 0.82

[a] Mean of three determinations.

than the concentration of both functions at the 4-phenyl residue of the dimethoxy phenyl substituted compound **4d**.

The combination of the 4-methoxyphenyl substitution of compound **4b** with the 3-methoxybenzyl function in derivative **4h** led to almost similar activities than the combination of the 3-methoxyphenyl substitution with the 3-methoxybenzyl substitution of compound **4f**. If combined with the 4-methoxybenzyl function in compound **4i** we found increases in activity reaching the activity of the 3-, 4-dimethoxy substituted compound **4d**.

Next, we investigated the potential of our compounds to reverse the P-gp mediated mdr using our cell line model. We selected daunorubicin as known P-gp substrate and determined the toxicity of the cytostatic agent in both, the P-gp nonexpressing parental cell line and the P-gp expressing subline. The cellular toxicity was determined in the MTT assay for a reduced cell viability resulting from a lowered formazane formation via the mitochondrial dehydrogenases. The resulting IC_{50} values are shown in Table 2.

The IC_{50} value for daunorubicin in the parental cell line was determined with 0.75 μM. In the P-gp expressing subline the IC_{50} value was found mainly increased with 8.26 μM that is a more than tenfold increased value. The higher value results from the fact that the P-gp substrate daunorubicin is transported out of the cell by the P-gp efflux pump activity.

We selected best compounds of our series of mono- and dimethoxy substituted derivatives which has been the 3-methoxyphenyl substituted derivative **4c** and the 3,4-dimethoxy substituted compound **4d**.

We first used the effective 10 μM concentration of both evaluated P-gp inhibitors and determined the daunorubicin toxicity under the given inhibitor concentration. The monomethoxy phenyl substituted derivative **4c** resulted in a mainly reduced IC_{50} value of the daunorubicin toxicity in the P-gp expressing subline with a value of 0.86 μM which is nearly the same value that was found for the daunorubicin toxicity in the non P-gp expressing parental cell line. The reduction meant a complete reversal of the P-gp mediated resistance and a restoration of the daunorubicin toxicity.

The dimethoxy substituted derivative **4d** led to an IC_{50} value of 0.39 in the P-gp expressing subline which meant a slightly increased toxicity in this subline if compared to the non P-gp expressing parental cell line. Beside a complete restoration of the P-gp mediated resistance we found a slightly increased sensitivity of the P-gp expressing subline to the toxic daunorubicin agent. Next, we investigated the effect of a 5 μM concentration application of compound **4d** to reverse the P-gp mediated resistance in the P-gp expressing subline. The resulting IC_{50} value was 4.76 μM, so that this concentration led to a nearly 50% restoration of the daunorubicin toxicity.

We then determined the cellular toxicity of our investigated inhibitors **4c** and **4d** in both cell lines to exclude possible toxic effects of the compounds themselves which may have contributed to the daunorubicin toxicity (Table 3).

We incubated varying concentrations of the inhibitors in the cell lines and found both compounds to be completely nontoxic with IC_{50} values >160 μM. Also after 48 h of incubation the compounds remained nontoxic with unchanged IC_{50} values. So we could exclude any effects of the compounds on the observed daunorubicin toxicity.

Table 2
IC_{50} values of the cellular toxicity of daunorubicin without and with P-gp inhibitor concentrations

Compound	IC_{50} value (μM)	
	P-cell line	mdr cell line
Without inhibitor	0.75 ± 0.61	8.26 ± 0.62
4c (10 μM)	–	0.86 ± 0.24
4d (10 μM)	–	0.39 ± 0.12
4d (5 μM)	–	4.76 ± 0.92

Table 3
IC_{50} values of the cellular toxicity and fluorescence ratio values of cellular uptake rates of **4c** and **4d**

Compound	IC_{50} value (μM)				Fluorescence ratio value[a]	
	P-cell line		mdr cell line			
	24 h	48 h	24 h	48 h	50 μM	100 μM
4c	>160	>160	>160	>160	1.03 ± 0.24	0.99 ± 0.18
4d	>160	>160	>160	>160	1.06 ± 0.24	1.07 ± 0.29

[a] Mean of three determinations.

The observed unchanged toxicity of our compounds in both cell lines indicated that they were no substrates of the efflux pump. In case of such substrate properties the toxicity in the P-gp expressing subline would have been lower due to the fact that the compounds were partly transported out of the cells.

As our 1,4-dihydroquinolines themselves are fluorescent compounds we directly determined their uptake in both cell lines to determine such substrate properties. We calculated fluorescence ratios by relating the determined compound fluorescence uptakes in both cell lines using the flow cytometry technique.[18] A relation of the compound fluorescence's in cells of both cell lines with a value of about 1 meant that the compounds are no substrates of P-gp so that the compound uptake in both cell lines is similar and not affected by the activity of P-g. We used concentrations of our fluorescent compounds of 50 μM and of 100 μM. All resulting fluorescence ratios are displayed in Table 3 and were about a value of 1 which meant that our compounds were really no substrates of the efflux pump because their uptake in both, the nonexpressing parental cell line and the P-gp expressing subline, was almost identical.

In conclusion, we discovered a novel class of promising P-gp inhibitors. First structure–activity relationships prove that methoxy groups as potential hydrogen bond acceptor functions are favourably concentrated at the 4-phenyl residue. The compounds are able to completely reverse the P-gp mediated resistance at the given concentrations. Moreover, they are nontoxic and do not own P-gp substrate properties. Thus, they are a really perspective class of novel mdr modulators for further preclinical studies.

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15. *Spectroscopical data of substituted N-benzyl 4-phenyl 1,4-dihydroquinoline compound class representing compound* **4c**: Yield 0.045 g (18%); mp 100–102 °C; IR (ATR) ν = 1665 (C═O), 1636 (N—C═C), 1596, 1464 (C═C), 1386 (CH_3), 1305 (C—N) cm^{-1}; ^1H NMR ($CDCl_3$) δ = 7.60 (s, 1H, 2-H), 7.22–7.18 (m, 5H, benzylic H), 7.10–7.00 (m, 2H, 6-, 7-H), 6.90 (dt, J = 7.8, 1.6 Hz, 1H, phenylic 5-H), 6.78–6.76 (m, 3H, phenylic 2-, 4-, 6-H), 6.68 (dd, J = 8.2 Hz, 0.8 Hz, 1H, 5-H), 6.58 (dd, J = 8.2, 0.8 Hz, 1H, 8-H), 5.10 (s, 1H, 4-H), 4.78 (AB, J = 16.4 Hz, 2H, N-CH_2), 4.02 (ABX_3, J = 10.9, 7.0 Hz, 2H, $COCH_2CH_3$), 3.63 (s, 3H, OCH_3), 1.12 (ABX_3, J = 7.0 Hz, 3H, CH_3); m/z (EI) 399 (100, M^+). Elemental Anal. Calcd (%) for $C_{26}H_{25}NO_3$: C, 78.17; H, 6.31; N 3.51. Found: C, 78.34; H, 6.25; 3.36.
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