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Dimeric 4-aryl-1,4-dihydropyridines as novel HIV-1 protease inhibitors – affinities to intestinal P-glycoprotein

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P-Glycoprotein (Pgp) is an ATP-dependent, efflux membrane transporter with a broad substrate specifity [1]. Expressed in epithelial cells of the intestinum Pgp lowers the absorption rates of various drugs which are characterized by affinity to Pgp and poor passive membrane permeability [2]. On the other hand the expression at the interface of liver and kidney leads to increased excretion rates of such drugs [1]. Because of low absorption rates of the peptidic HIV-1 protease inhibitors (PI) saquinavir and indinavir, their therapeutical use demands high doses for oral application. They lead to various severe side effects caused by either drugs or their metabolites [3].

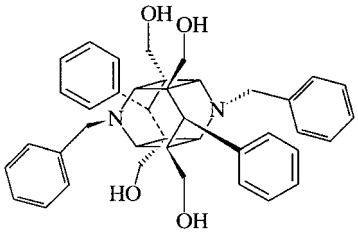
In *in vitro* testing with model Pgp-expressing cell lines L-MDR1 and Caco-2 cells saquinavir and indinavir proved to be Pgp substrates, with increasing concentrations at the basolateral side of the cell monolayers under Pgp inhibition with quinidine or the cyclosporine analog PSC-833 [4]. Furthermore, the limiting role of Pgp in intestinal absorption of those drugs could be demonstrated by oral administration in so-called mdr 1a (~/~)(= knock-out) mice as non Pgp-expressing mice where a 2–5 fold increase of plasma levels was observed [4].

A radioligand-binding assay was recently introduced as appropriate tool to characterize binding affinities of substrates to Pgp in overexpressing Caco-2 monolayers using ^{3}H-verapamil or ^{3}H-vinblastine as radioligand [5–7]. This assay was shown to yield IC50 values of drug affinities to Pgp which are comparable to literature data and is, hence, a suitable experimental model for testing drug affinities to intestinal Pgp [5]. Therefore, this *in vitro* model can be used to evaluate certain drugs with high affinities as potential Pgp inhibitors.

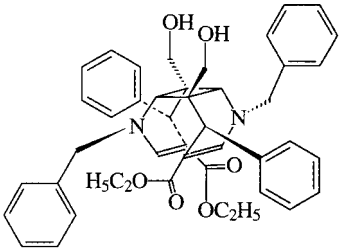
Cage dimeric and *syn* dimeric 4-aryl-1,4-dihydropyridines have been introduced as novel nonpeptidic HIV-1 protease inhibitors with moderate activities of their first representatives [8–11]. The first bioanalytical investigations of H 17 and H 19 in Hep G2 monolayers showed poor metabolism and non-significant protein binding [12–14].

Since they show promising oral bioavailabilities than extensively metabolized peptidic PI's with high protein binding, further drug development of those dimers is encouraged. Since affinities to Pgp may play an important role in limited oral bioavailability of the peptidic PI's, the affinities of the dimeric representatives H 17 and H 19 to Pgp have been evaluated in the radioligand-binding assay and compared to other Pgp inhibitors such as verapamil under equal conditions. Using cultured Pgp expressing Caco-2 monolayers dose-dependent competition experiments of H 17, H 19 and verapamil complexed to Pgp have been made with ^{3}H-verapamil as described below (see Experimental) [15].

From the extent of radioligand binding after incubation with those certain competitor concentrations of H 17 und H 19 the following binding curves (Fig.) were calculated as described previously [5–7]. They represent the specific



H17



H19

binding of ^{3}H-verapamil to Pgp as mean of each two determinations. This specific binding was given by substrating the radioactivity of the radioligand determined as non-specific binding at the highest verapamil concentration from the total radioactivity resulting from both specific and non-specific radioligand binding in each case [5].

With calculated IC50 values for both H 17 and H 19 the compounds exhibit significant affinities to Pgp in comparison to the Pgp inhibitors verapamil or vinblastine.

However, compared to the reported IC50 value of quinidine these affinities suggest certain Pgp inhibitory properties when dosed in combination with the peptidic PI's saquinavir or indinavir.

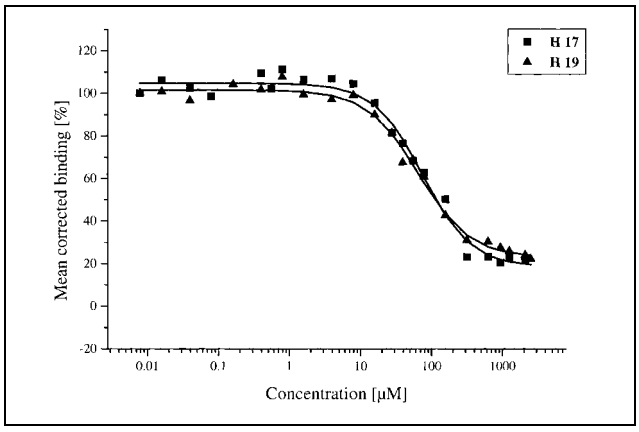


Fig: ^{3}H-verapamil bound as function of concentration of H17 and H19, respectively, and fit as obtained according to the one-affinity model [5]

Table: Calculated IC50-values from competition experiments to ^{3}H-verapamil as radioligand *

Compound	IC50 [µM]	literature data [µM]
Verapamil	1.8	1.5 [5]
H17	74.37	
H19	64.76	
Quinidine		340 [5]
Vinblastine		34 [5]
Talinolol		830 [5]

* IC50 values were determined by fitting the reported Hill-equation for the one-affinity model to the obtained data with Hill-coefficients evaluated by nonlinear regression [5]. In the present study incubations were performed in incubation media containing 10% of DMSO (final concentration).

With respect to the clinically investigated combined HIV-therapy with two PI's [16] both cage and *syn* dimeric HIV-1 protease inhibitors may attract of great interest as their potential Pgp inhibitory activities would suggest an increased intestinal absorption of the peptidic PI's as Pgp substrates in a combined administration as has been demonstrated for the Pgp inhibitor quinindine in the *in vitro* model [4].

Experimental

1. Preparations of P-glycoprotein [5]

Pgp expression was induced in Caco-2 cell lines (passage 72) with vinblastine. Cells were grown in 225 cm^3 flasks at 37 °C in a 5% CO_2 atmosphere using DMEM containing 10 nM vinblastine, 16.5% fetal calf serum, 1% of non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% L-glutamine. Cells were seeded at an initial density of $0.8\text{-}1 \times 10^6$ cells per flask and medium was changed every other day. Monolayers were trypsinized at 90–95% confluence and the cell suspension was then used for radioligand-binding studies.

2. Radioligand-binding assay

Incubations were performed at 37 °C under mild shaking conditions in Hanks Balanced Salt Solution (HBSS) containing 10 mM morpholino ethane sulfonic acid (MES) at pH 7.0 and Pgp preparation of 1.25×10^6 cells per ml with cell membranes permeabilized with lysolecithine-solution (0.01%) as described [5], 24 different dilutions of H 17, H 19 and verapamil within a concentration range of 7.82 nM to 2500 µM in DMSO/HBSS-MES – 1 : 3 and ^{3}H-Verapamil as radioligand in a total volume of 250 µl. The incubation medium was supplemented as described [5]. Incubation was stopped after 30 min by vacuum filtration. Filters were washed twice with ice-cold HBSS containing MES, incubated with scintillation fluid for 12–16 h at RT, and total radioactivity on the filters was counted by liquid scintillation counting. For all calculations Excel 5.0, (Microsoft, USA) was used.

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2-Methoxy-4,5,7-trihydroxy-anthraquinone, a new lichen metabolite produced by *Xanthoria parietina*

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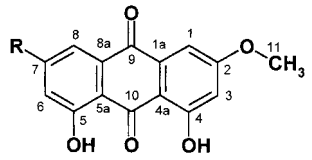
Lichens have been shown, in the past, as a rich source of novel natural products with promising properties for applications as drugs, agricultural agents and cosmetics [1, 2]. Thus, special habitats promoting the growth of lichens such as the Canary Islands were investigated and various lichen compound have been reported [3].

In the course of screening for new natural products we recently investigated an extract of the lichen *Xanthoria parietina* and found it to contain **1** as a new anthraquinone structure. **1** is formed in addition to physcion (**2**) and fallacinol (**3**) as related metabolites [4]. Here we report the isolation and structure elucidation of the new lichen metabolite.

The lichen *Xanthoria parientina* was collected from rocks near Los Llanos (La Palma, Canary Islands, Spain). Compound **1** was isolated by several subsequent chromatographic steps from the evaporated residue of the methanolic extract of this lichen as a yellowish amorphous solid. 250 g of the material were extracted three times by 1 l methanol. The combined extracts were evaporated to dryness on a rotary evaporator. The residue was dissolved in chloroform and chromatographed on a silica gel column (5 cm × 30 cm, silica gel 60, 0.063–0.1 mm, stepwise elution by $CHCl_3$ and $CHCl_3$-MeOH 9 : 1, each 10 column volumes). Thereby **1** was separated from several other anthraquinone components such as physcion (**2**) [4] and fallacinol (**3**) [4]. Final purification was carried out by preparative TLC using silica gel sheets RP_{18} (Merck, acetonitrile/water, 83 : 17, 0.1% trifluoroacetic acid; R_f 0.85) and preparative HPLC on silica gel RP_{18} (1,5 × 25 cm; 5 µm, gradient 95% water to 95% acetonitrile, 25 min, R_t: 17 min).



	R
1	–OH
2	$–CH_3$
3	$–CH_2OH$

Structures of 2-methoxy-4,5,7-trihydroxy-anthraquinone (**1**, R = OH) and coproduced physcion (**2**, R = CH_3) and fallacinol (**3**, R = CH_2OH).

The molecular weight and the elemental composition of **1** were determined by HREI-MS (M^+: m/z 286.1270; calcd. 286.1241 for $C_{15}H_{10}O_6$). The VIS spectrum displayed an absorbance maximum at 435 nm which is the same as was found with **2** and **3**.

The IR spectrum attested to the presence of quinone carbonyles and, respectively, hydroxyl groups due to absorbances at 1704 cm^{-1}, 1736 cm^{-1} and 3438 cm^{-1}.

Conclusive evidence for the structure of **1** was furnished by 1 D and 2 D NMR spectroscopy (^{1}H, ^{13}C, DEPT,