Physicochemical Characteristics Of Novel P-Glycoprotein Inhibitors Of The Cage Dimeric 1,4-Dihydropyridine Type

J. Wollmann[1], J. Molnár[2] and A. Hilgeroth[1,*]

[1]*Institute of Pharmaceutical Chemistry, Martin-Luther-University Halle-Wittenberg, 06120 Halle, Germany,* [2]*Department of Medicinal Microbiology, University of Szeged, 6720 Szeged, Hungary*

Abstract: Physicochemical characteristics of two structurally different cage dimeric 1,4-dihydropyridines **HX** (**1**) and **CC** (**2**) have been determined and compared to their P-glycoprotein inhibiting properties. While the weakly basic compound (**1**) showed pH-dependent apparent partition coefficients (log D), the neutral compound (**2**) proved to have almost identical log D values at varying pH-values. The subsequent determination of partition coefficients (log P) resulted in comparably low log P values revealing a less lipophilic compound character. Determined significantly differing P-glycoprotein (P-gp) inhibitory properties indicated that the lipophilicity of the compounds does not play a decisive role for the P-gp activity.

Key Words: P-glycoprotein, partition coefficient, cage dimer, biological activity.

INTRODUCTION

Multidrug resistance (MDR) is one main problem in the treatment of many infectious diseases and in cancer treatment of today [1-3]. MDR causes a failure of usual therapeutics and thus demands novel innovative alternatives. Multidrug efflux pumps like P-glycoprotein (P-gp) and the multidrug resistance associated proteins (MRP) play an increasing role in the resistance development of cancer cells and also in antiretroviral therapies [3-5]. Even novel drugs like the cytostatically active tyrosine kinase inhibitor imatinib or the monoclonal antibody gemtuzumab ozagamicin are being transported out of the cells by the efflux pump activities and so lead to insufficient intracellular drug levels [6,7]. Inhibitors of the efflux pump activities are continously most hopeful agents to overcome MDR because alternative approaches like influencing of the signalizing pathways or direct transcriptional control *via* antisense oligonucleotides failed [8]. Lipophilicity has been known to be a significant characteristic of MDR-inhibiting agents [9,10]. Lipophilic compounds are known to easily passage the membrane by diffusion process. Authors discussed a transmembrane P-gp binding site for inhibitors within the membrane framework which is accessible by diffusion through the membrane [11,12].

We develop cage dimeric 1,4-dihydropyridines as novel class of P-gp inhibiting compounds [13]. Some of such cage compounds proved to have moderate HIV-1 protease inhibitory activities [14]. With dual P-gp inhibiting properties they may become useful antiretroviral therapeutics because in usually combined drug therapies their ability to inhibit P-gp will help to overcome the P-gp mediated resistance developments against the peptidic protease inhibitors which are P-gp substrates themselves [15].

*Address correspondence to this author at the Institute of Pharmaceutical Chemistry, Martin-Luther-University Halle-Wittenberg, 06120 Halle, Germany; Tel: 49-345-55-25168; Fax: 49-345-55-2726;
E-mail: andreas.hilgeroth@pharmazie.uni-halle.de

Two of these cage dimers **HX** (**1**) and **CC** (**2**) (Fig. (**1**)) have been evaluated as P-gp inhibitors and physicochemically characterized by the determination of the partition coefficients (log P) partly in dependence of the pH as log D values. Beside experimental methods log P values have been calculated using computational methods and results have been compared to one another and with the biological activities.

P-GP INHIBITING PROPERTIES

The determined fluorescence activity ratios (FAR) of compound (**2**) were found almost proportional to the investigated concentrations of 1 μM and 10 μM (Table (**1**)). A similar concentration dependence has also been found for the used standard verapamil, which proved to be not active at 1 μM and half as effective than the P-gp inhibitor **CC** at the higher concentration.

Compound (**1**) proved to be a much stronger inhibitor even at the low concentration. At the high concentration **HX** (**1**) showed improved activities which are not proportional to the increased concentration range so that saturation effects of the P-gp inhibiting properties are likely. Such saturation effects have been recently reported for P-gp inhibitors [13].

Structurally the two compounds differ in the nitrogen substitution which is a Boc residue in compound (**2**) and a benzylic one in compound (**1**). With these differing residues it is suggested that the nature of the nitrogen may play a role for the biological activity. In compound (**2**) there is a pH-neutrally reacting carbamide ester while in compound (**1**) there is tertiary nitrogen atom with suggested basic properties.

PHYSICOCHEMICAL CHARACTERISTICS

In literature partition coefficients are discussed as log P values and frequently they have been calculated by computa-

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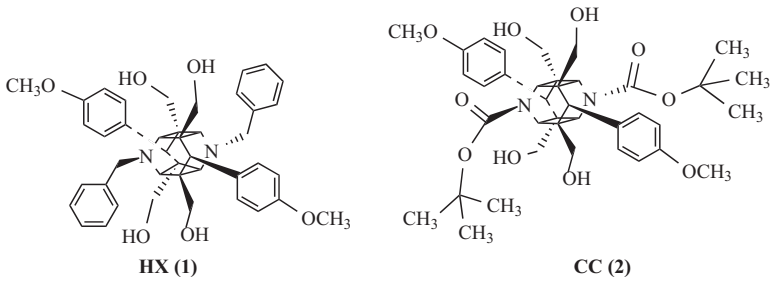

Fig. (1). Structures of compounds.

tional methods. Such a discussion only makes sense for non-ionizable compounds for which a partition coefficient is a constant independently from the pH of the surrounding medium. In the case of basic or acid structural elements within a compound an apparent partition coefficient has to be discussed. This is referred to as log D value and comparable for such compounds at a given pH value. Moreover, the calculated log P values often do not correlate with experimental values which will be dicussed later on.

So we experimentally determined the log D values as octanol/water distribution coefficients at different pH values using weakly acid conditions (pH = 5), neutral condition (pH = 7) and, furthermore, slightly basic conditions (pH = 9) in buffer solutions (see Experimental). From the log D values the log P values have been determined and are listed in Table (**2**).

Table 1. Concentration Dependent P-gp Inhibiting Properties as Determined FAR Values

Compound	FAR [a]	
	1 μM	10 μM
1	34.40 ± 1.10	87.01 ± 2.99
2	1.13 ± 0.04	14.64 ± 0.65
verapamil	0.66 ± 0.03	7.27 ± 0.33

[a] Mean of two determinations.

The determined log D values for compound (**1**) with the benzylic substitution at the tertiary nitrogen atom showed certain differences depending on the pH value. The lowest log D value was found at pH = 5 revealing the comparably highest part of compound with 10.4% found in the water phase. At pH = 7 the log D value increases and at pH = 9 we found the highest log D value with a more than halved part of compound (4%) in the water phase compared to pH = 5. This certain pH-dependence of the log D value resulted from the nitrogen atom with basic properties. We experimentally determined the pK_a value of the compound (**1**) with 4.8 revealing a less basic property for compound (**1**) as has been expected for an alkyl substituted tertiary amine function which normally shows a pK_a value of about 9 [16]. The reduced basicity of the nitrogen atom may result from the shielding of this atom by the neighboring aromatic residues which has also been discussed as reason for lowered metabolic phase-II reaction rates and demonstrated by molecular modeling [17].

Comparably slight differences have been found in the log D values of compound (**2**) at the different pH values. The pH-neutrally reacting carbamide ester function at the tertiary nitrogen leads to a similar octanol/water distribution coefficient at pH = 5 as has been found for the weakly basic compound (**1**) at the highest pH value of 9, so that the resulting log D values are similar.

The calculation of the resulting log P value of derivative (**1**) using the determined D values led to a similar log P value as for compound (**2**). The altogether low difference of the log P value and the log D values of compound (**1**) result from the comparably low pK_a value of the compound. Higher pK_a values in comparably more basic compounds would lead to a higher degree of protonation and lowered log D values and, thus, determine less lipophilic properties as has recently been reported for propranolol derivatives [18].

Both Log P values of 1.38 for compound (**1**) and 1.39 for compound (**2**) reveal only some lipophilic character of the cage compounds compared to literature 1,4-dihydropyridine derivatives which are refered to as P-gp modulating agents with log P values of 3.17 for amlodipine and 4.65 for nicardipine [19].

In general, the allover four hydroxymethylen functions of the cage dimers make the whole compounds rather hydrophilic and so, practically, compensate lipophilic effects of the aromatic residues.

COMPUTATIONAL CALCULATIONS

Two computational methods have been used to calculate the partition coefficients log P. Method LogP (O / W) calculates log P values based on a built linear atom model without considering the atom orientation in space and with reference to 1827 molecules as data base [20]. Method SlogP works with an atom model which considers the orientation of the atoms in space and with reference to 7000 structures as data base [20,21].

Log P (O/W) calculates higher log P values than the experimental values with a difference of log units of 1.4 between the compounds characterizing the Boc substituted derivative (**2**) to be less lipophilic than the benzylic one **HX** (**1**) (Table **2**). A lower difference between the two compounds but each higher log P value have been given by the SlogP method.

Table 2. Partition coefficients

Comp.	log D			log P	LogP (O / W)	SlogP
	pH value					
	5	7	9			
1	0.94 ± 0.01	1.09 ± 0.01	1.38 ± 0.01	1.38 ± 0.01	3.64	4.57
2	1.34 ± 0.02	1.38 ± 0.02	1.46 ± 0.01	1.39 ± 0.02	2.20	3.50

Refering to literature, computational programs have been demonstrated to give to high log P values compared to experimental values within differing compound series like 1,4-dihydropyridines or phenothiazines and thus overestimate the lipophilicity at varying extents [19].

Altogether, our example of the experimental determination of log P and log D values, respectively, which are consistent with the pK_a value strongly supports the necessity to use experimental methods to characterize the physicochemical properties of chemical compounds. Computational methods are not helpful in this case because they result in non-realistic values.

CONCLUSIONS

Cage dimers could be characterized as less lipophilic compounds compared to varying classes of P-gp modulating agents like 1,4-dihydropyridines or phenothiazines with reported log P values beyond 4. However, it may be suggested that a sufficient degree of lipophilicity is not a requirement for a compound to exhibit P-gp inhibiting properties.

Moreover, the less lipophilic cage dimers show strong activities as P-gp inihibitors compared to verapamil with the nitrogen substituted benzylic compound (**1**) being much better than the carbamid ester derivative (**2**). So with similar lipophilicity of the cage dimers it is suggested that the lipophilicity which does not correlate with the biological activities of the compounds is not a decisive feature for the degree of P-gp inhibiting properties within this class of novel P-gp inhibitors.

EXPERIMENTAL

Materials

The syntheses of compounds (**1**) and (**2**) have been described [14,22]. Verapamil was obtained from EGIS Work, Budapest, Hungary. Rhodamine 123 was purchased from Sigma, St. Louis, Montana, USA. 1-Octanol was purchased from Sigma-Aldrich, Steinheim, Germany, citric acid from Grüssing GmbH, Tilsum, Germany, disodium hydrogenphosphate decahydrate from Merck, Darmstadt, Germany, sodium hydrogenphosphate and sodium carbonate from KMF Laborchemie Handels GmbH, Leipzig, Germany.

Fluorescence Uptake Assay

The fluorescence uptake of rhodamine 123 as P-gp specific substrate has been determined as described [13,23]. NCI mouse T lymphoma cell line L5178Y and the P-gp expressing subline L5178YvMDR have been cultured in Mc Coy´s 5A medium (pH = 7.04). After 0.5 mL aliquots of each cell line have been adjusted to a concentration of 2 x 10^6 cells per mL and then preincubated with varying inhibitor concentrations. Inhibitors were added from stock solutions in DMSO. Fluorescence uptake was determined for 1 x 10^4 cells by flow cytometry comparing each treated parental and P-gp expressing cell line with the untreated control. Fluorescence activity ratios (FAR) have been calculated using equation (1) [13,23]. Inhibitors with ratios > 1.1 were found active. Those with ratios > 10 were very active.

$$FAR = \frac{(\text{MDR treated / MDR untreated control})}{(\text{Parental treated / Parental untreated control})} \quad (1)$$

Determination of Distribution Coefficients

Each five different concentrations (3, 6, 8, 10, 15 μM) of both compounds added from stock solutions in DMSO were prepared in the octanol phase which each has been saturated before with buffer solutions (pH = 5, composed of 25 mL of 0.1 M citric acid and 25 mL of 0.2 M disodium hydrogenphosphate solutions, pH = 7, composed of 19 mL of 0.1 M citric acid and 81 mL of 0.2 M disodium hydrogenphosphate solutions, and pH = 9, composed of 10 mL of 0.1 M disodium carbonate and 90 mL of sodium hydrogencarbonate solutions). Then the octanol phase was shaken with the corresponding water phases of given pH values previously saturated with octanol and the concentration dependent remaining amounts of compound in the octanol phase have been determined UV spectroscopically at 225 nm three times for each concentration. The resulting mean compound relations in the octanol and water phases at the given pH values have been the D values and log D values, respectively.

The corresponding P value of compound (**1**) has been given by plotting the reciprocal proton concentrations against the reciprocal values of each product of D value and proton concentration [24]. The reciprocal value of the rise of the resulting straight line corresponds to the P value following equation (2).

$$\frac{1}{D \times [H^+]} = \frac{1}{P} \times \frac{1}{[H^+]} + \frac{K_B}{P} \quad (2)$$

Determination of pK_a Values

Three samples of 100 mL of a 40 μM solution of compound (**1**) added from the stock solution in DMSO were prepared using neutral water which has been distilled twice be-

fore use. PH values of the control without the compound (**1**) and the samples have been determined potentiometrically with resulting pH values of 7.0 for the control and 7.19 ±. 0.02 for the three samples. The pK_a value has been calculated from the determined pK_b values (9.22 ± 0.03) and the proton concentrations of each sample.

Computational Calculation of log P Values

Calculations have been carried out with the MOE (Molecular Operating Environment) program [19]. Before calculation the compound structures have been energetically minimized using Tripos-Force Field with an energy convergence criterion of 0.001 kcal mol^{-1} $Å^{-1}$. LogP (O/W) partition coefficients have been calculated from a linear atom type model with r^2 = 0.931 and RMS = 0.393 as implemented in the MOE program [20]. SlogP is an atomic contribution model which calculates the partition coefficients from the given structures.

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Received: 08 March, 2006 **Revised: 19 May, 2006** **Accepted: 20 May, 2006**

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