European Journal of Medicinal Chemistry 44 (2009) 3060–3063

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Evaluation of substrate and inhibitor properties of a novel MDR modulator H17 towards transmembrane efflux pumps

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ARTICLE INFO

Article history:
Received 8 December 2006
Accepted 11 July 2008
Available online 19 July 2008

Keywords:
Absorption model
Efflux pump
MDR modulator
Substrate properties

ABSTRACT

Substrate and inhibitor properties of H17 as novel modulator of transmembrane efflux pump activities have been characterized in an in situ absorption model. Poor substrate properties towards P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) have been demonstrated. In competition with a MRP substrate H17 proved to have strong MRP-inhibiting properties. The profile of a strong inhibitor with poor substrate properties makes H17 a perspective hopeful candidate for effective therapies of transmembrane efflux pump activities.

1. Introduction

Transmembrane efflux pumps like P-glycoprotein (P-gp) and the multidrug resistance-associated proteins (MRP) play an important role in the resistance development against cytostatics and antiretroviral therapeutics [1–4]. They transport drugs out of the cells and thus lower intracellular therapeutically necessary drug levels so that the P-gp or MRP expressing cell becomes resistant [5]. An additional problem is the fact that the substrate specificity of these efflux pumps is relatively low so that a drug-resistant cell generally is found to be resistant also against other possible therapeutics [5]. This low substrate specificity is discussed to result from several binding sites of the various substrates to the transporter protein [6]. Thus, all the peptidic HIV protease inhibitors used in antiretroviral HIV therapies show cross-resistance as P-gp substrates [5].

Moreover, cells bearing the P-gp encoding gene like *mdr1* are induced to express more P-gp under therapy so that a P-gp overexpression results in these cells [7]. This leads to the development of viral sanctuaries in HIV therapy where P-gp is overexpressed at the blood–brain barrier and the testes [8,9]. In the intestine the expression of both efflux pumps P-gp and MRP leads to lowered absorption rates of orally administered drugs [3,10].

Early inhibition of P-gp by drugs like cyclosporine or verapamil was proved in in vitro cell models, but in in vivo studies the drug use was strongly dose-limited due to toxic pharmacological effects of the inhibitors [11]. In vivo evaluation of stronger inhibitors like valspodar showed dose-limited effects suggested to result from the undesired inhibition of drug uptake transporters [5]. Ritonavir as highly potent in vitro P-gp inhibitor also disappointed in in vivo studies where only poor P-gp inhibiting properties were found [7,12]. As far as investigated most P-gp inhibitors are additionally substrates of the efflux pump so that this property may also play a role in competitional uptake studies of a substrate versus an inhibitor [13]. So the evaluation of both inhibiting as well as substrate properties of a P-gp modulating compound has to be considered in competition with relevant compounds.

Recently, we reported H17 as novel non-peptidic HIV protease inhibitor with P-gp inhibiting affinities in the range of verapamil in an in vitro cell model [14]. To further characterize the new compound and its potential to act as effective inhibitor we evaluated its substrate properties towards transmembrane efflux pumps in an in situ absorption model.

2. Chemistry

H17 has been synthesized as described [15]. Vinblastin has been obtained from Gry-Pharma (Kirchzarten, Germany). Indomethacin was a gift from Germed Berlin-Chemie (Berlin, Germany). Probenecid was provided by Biokanol Pharma GmbH (Rastatt, Germany). AE1 used as internal standard for the HPLC quantification of H17 was given by the acetylation of H17 in acetyl chloride under pyridine base catalysis (Fig. 1).

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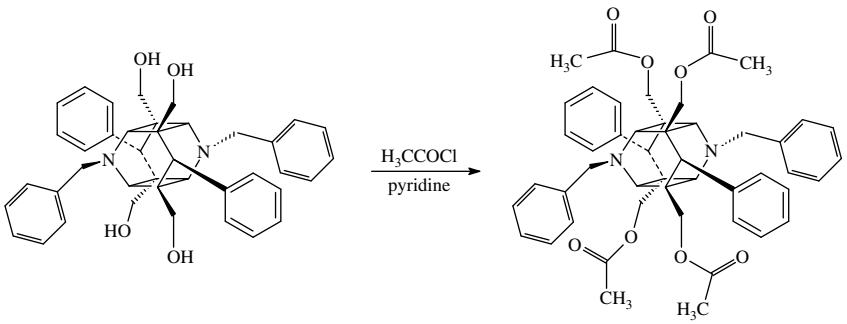
doi:10.1016/j.ejmech.2008.07.014



H17 **AE1**

Fig. 1. H17 and the formation of the internal standard AE1.

3. Pharmacology

Experiments were performed in male White Wistar rats purchased from Charles River Laboratories (Sulzfeld, Germany). Rats were housed individually in a cage under controlled conditions (21 °C, 45% air humidity, 12-h light cycle). They were acclimatized for at least two weeks before experiments and had access to tap water and rodent pellet food from Altromin (Lage, Germany). Animal studies were performed with approval from the Regierungspräsidium Dessau/Sachsen-Anhalt.

Perfusion experiments were performed with weighted rats of 290–350 g. They were anesthesized with ketamine hydrochloride (50 mg/kg) using Ketamine Inresa®, Inresa Arzneimittel GmbH, Freiburg, Germany, and with xylazin hydrochlorid, 10 mg/kg, in Rompun® 2% from Bayer, Leverkusen, Germany. Rats were placed on a heating pad to maintain body temperature at 37 °C. Then the abdomen was opened by a midline longitudinal incision. In concentration dependent competition studies 30 μM of H17 were combined with each inhibitor concentrations of vinblastine (100 μM) and 1 mM of indomethacin, respectively. In the indomethacin study 1 mM of the substrate was combined with 0.25 mM of probenecid and 30 μM of H17, respectively. Three intestinal segements of each rat ($n = 3$) were perfused over a length of each 7–10 cm (jejunum), ~5 cm (ileum) and 2 cm (colon) in a three-step procedure with a perfusate flow rate of 0.5 mL/min using an infusion pump from Ismatec (Wertheim-Mondfeld, Germany). After a preceding period of 30 min for reaching steady state with each substrate and substrate/inhibitor containing tyrode buffer at a temperature of 37 °C, the outflow samples were taken over 30 min collecting in 5 min intervals. So for each perfusate and intestinal segment six samples resulted.

4. Results and discussion

Within the intestine we investigated several rat gut segments by perfusion: jejunum, ileum and colon. P-gp, MRP1 and MRP2 are expressed within these intestinal segments [16,17]. While P-gp is being located at the apical site of the epithelian cells and thus lowers absorption rates from the intestine by the efflux of substrates, MRP1 is located basolaterally and so tends to reduce epithelial drug levels by the efflux into the body. MRP2 is also located at the apical site [10].

In our perfusion studies we used vinblastine as proven P-gp inhibitor at the given concentration which was reported to reach high effects as P-gp inhibitor in the intestinal uptake of P-gp specific substrates like talinolol [18]. As MRP inhibitor we used indomethacin which has been demonstrated to have only MRP-inhibiting properties without affecting P-gp [19].

Similar effective permeabilities have been found for H17 alone in all intestinal segments (Table 1). The combination of H17 with vinblastine as used P-gp inhibitor leads to almost unchanged absorption rates in the colon and the jejunal segment. Also in the ileal segment the increase of H17 uptake is only very poor. For talinolol as P-gp substrate increases of intestinal uptake rates of more than 100% have been reported in combination with P-gp inhibitor vinblastine [18]. So it is obvious that H17 practically is no substrate of P-gp.

By the use of indomethacin as inhibitor of both MRP1 and MRP2 we find a poor increase of the intestinal uptake rate in the colon segment with 24% while in the jejunal and the ileal segment the increases of about 13% are negligible. In order to compare possible increases of intestinal uptake rates of an MRP substrate in combination with a MRP inhibitor we furthermore investigated indomethacin as MRP substrate itself and probenecid as strong inhibitor of MRP [10]. While MRP1 and MRP2 have similar substrate specificities not all of the inhibitors of MRP1 also inhibit MRP2 [10]. Probenecid as MRP1 inhibitor was also shown to inhibit MRP2 and so was a suitable MRP inhibitor model compound. Indomethacin showed poor effective permeabilities in the intestinal segments (Table 2).

However, lowest absorption rates have been observed in the jejunal segment which is known to have high MRP1 expression rates in rats [17]. The combination of indomethacin with probenecid leads to an increase of the indomethacin uptake in all segments. The highest increase of 70% has been found in the jejunal segment due to the highest expression rates of MRP1 in this segment. So compared to the much lower increased H17 uptake rates reported above it can be concluded that H17 is also a poor MRP substrate.

We used this model to additionally evaluate the MRP-inhibiting properties of H17 in combination with the MRP substrate indomethacin. An increase in the effective permeabilities was found in all segments. As has been found for probenecid the relatively highest increase has been found in the jejunal segment with known highest MRP expression rates. Although increases of substrate uptake of 37–54% were lower than those for probenecid, they nevertheless prove strong MRP-inhibiting properties for H17 because it was used in a lower competitor concentration than probenecid due to solubility reasons.

Table 1
Effective permeabilities P_{eff} [10^{-4} cm/s] for H17 alone and in combination with vinblastin and indometacin, respectively

Intestinal segment	H17	H17 + vinblastine	H17 + indomethacin
Jejunum	2.36 ± 0.28	2.73 ± 0.10	2.69 ± 0.16
Ileum	2.40 ± 0.33	3.01 ± 0.28	2.72 ± 0.35
Colon	2.05 ± 0.12	2.27 ± 0.23	2.54 ± 0.21

Table 2
Effective permeabilities P_{eff} [10^{-4} cm/s] for indomethacin alone and in combination with probenecid and H17, respectively

Intestinal segment	Indomethacin	Indomethacin + probenecid	Indomethacin + H17
Jejunum	1.31 ± 0.04	2.22 ± 0.09	2.02 ± 0.07
Ileum	1.84 ± 0.06	2.92 ± 0.06	2.76 ± 0.04
Colon	1.78 ± 0.03	2.73 ± 0.04	2.43 ± 0.04

5. Conclusions

We demonstrated that H17 which belongs to a novel class of non-peptidic HIV protease inhibitors shows poor substrate properties towards transmembrane efflux pumps compared to other substrates discussed. As substrate properties are of great disadvantage with respect to clinical application as efflux pump inhibitors these non-substrate properties of H17 prove this inhibitor to be of favour compared to almost all reported inhibitors. Moreover, within this single in situ model we additionally proved favourable strong inhibitor properties towards efflux pumps. These inhibitor properties give further perspectives for the application in oral regimes with P-gp substrates which show poor oral absorption rates in antiretroviral therapy like the HIV protease inhibitors do.

6. Experimental protocols

6.1. Chemistry

Commercial reagents were used without further purification. The ^{1}H NMR spectrum (400 MHz) was measured using tetramethylsilane as internal standard. TLC was performed on E. Merck 5554 silica gel plates. The mass spectrum was measured with a MAT 710 mass spectrometer. Elemental analysis indicated by the symbols of the elements was within ±0.4% of the theoretical values and was performed using a Leco CHNS-932 apparatus.

6.1.1. 3,9-Dibenzyl-6,12-diphenyl-3,9-diazahexacyclo[6.4.0.0^{2,7}.0^{4,11}.0^{5,10}]dodecane-1,5,7,11-tetrakis(methyleneacetate) (AE1)

H17 (0.009 g, 0.016 mmol) was dissolved in acetyl chloride (0.126 g, 1.6 mmol) under stirring. Catalytic amounts of dried pyridine were added to the mixture and stirring continued for 72 h under TLC control. The reaction mixture was then acidified by the addition of 10% of hydrochloride acid until a pH value of 4–5 was reached.

The water phase was extracted with dichlormethane for several times. The organic layers were dried over sodium sulfate and after filtration the solution volume was reduced in vacuum until the compound crystallized (0.0062 g, 50%). White powder (m.p.: 199–202 °C). ^{1}H NMR ($CDCl_3$): $\delta = 2.04$ (s, 12H, CH_3CO), 3.12 (s, 4H, H-2-, -4-, -8-, -10), 3.63 (s, 2H, H-6, -12), 3.71 (AB, CH_BO, $^2J = 11.2$ Hz), 3.96 (AB, CH_AO, $^2J = 11.2$ Hz), 4.24 (s, 4H, NCH_2), 7.07–7.81 (m, 20H, aromatic H). ESIMS: m/z: 781 [M + H$^+$]. Anal. $C_{48}H_{48}N_2O_8$ (C, H, N).

6.2. HPLC-assay

6.2.1. Sample preparation and analysis

Perfusion samples of H17 (500 μL) were spiked with AE1 as internal standard (50 μM) before extraction with chloroform (3 mL) on a horizontal shaker. The centrifuged organic layer was removed under nitrogen atmosphere at 38 °C and the dried residue was resolved in acetonitril/water (80/20) for HPLC analysis. Analysis of samples before and after perfusion was carried out isocratically on a reverse phase chromatography column RP_{18} (5 μm, 15 cm × 0.5 cm) with the eluent acetonitril/water (80/20) at a flow rate of 1 mL per minute resulting in retention times of 4 min for H17 and 9 min for AE1. Detection was carried out UV-spectroscopically at 240 nm wavelength. The specificity of the assay was investigated by analyzing blank plasma samples. Precision and accuracy were measured as inter- and intraassay precision in three separate runs. Six replicates of five different concentrations of H17 (2500, 5000, 10,000, 20,000 and 30,000 ng/mL) were determined. Analysis of variance (ANOVA) was used to calculate the inter- and intraassay precision. The accuracy was calculated as the average percentage of the nominal concentration. All analyses were within 10% at each concentration. The lower limit of quantification did not exceed 10%. Samples were determined as six replicates. For the limit of detection the difference between a spiked sample and a background sample was tested with a paired t-test. Significance was considered if p was less than 0.05. Quantities that gave a signal-to-noise ratio of three were selected first for the determination of the limit of detection. The value is 1000 ng/mL for the limit of detection and 2500 ng/mL for the limit of quantification. Indomethacin perfusion samples were worked up and analyzed as described in literature [20] using again the reverse phase chromatography column RP_{18} with a phosphate buffer (phosphoric acid, potassium dihydrogen phosphate) pH = 3.5 and methanol mixture (42/58) as described at a flow rate of 1.5 mL per minute resulting in a retention time of 2.4 min for indomethacin. Detection was carried out UV-spectroscopically at 240 nm wavelength. All validation procedures were realized as defined. The values for intra- and interassay precision were within 10% at each concentration. The lower limit of quantification for indomethacin was 180 ng/mL. The value for the limit of detection was 50 ng/mL. Water transport was calculated by weight measurement of each sample before and after perfusion. Steady state existence regarding the analysts and water uptake was examined via the trend test.

$$\text{Water transport}\% = 100 \cdot (w_{in} - w_{out})/w_{in}$$

Where w_{in} and w_{out} represent the solution entering and exiting the intestinal segment. The ratio w_{out}/w_{in} was used to perform given corrections concerning water transport.

7. Data analysis

With the determined mean concentrations of each P-gp substrates in samples before perfusion (C_{in}) and after perfusion (C_{out}) intestinal permeabilities (P_{eff}) have been calculated as previously described using the following equation [18]:

$$P_{eff} = Q^*((C_{in}/C_{out}) - 1)/2\pi rl$$

where Q^* represents the flow rate, r means the radius and l the length of the perfused intestine segment. From all single values, arithmetical means and standard deviations (SD) have been calculated. Statistical significance of correlations was determined by performing linear regression analyses with tested significant correlation coefficients $p < 0.05$. All studies were performed at least in triplicates. Statistical significance was expressed using an unpaired student's t-test (INSTAT, V3.0).

Acknowledgement

The work was financially supported by the DFG.

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