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Research Paper

Multidrug resistance reversal properties and cytotoxic evaluation of representatives of a novel class of HIV-1 protease inhibitors

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Abstract

Objectives P-Glycoprotein (P-gp) plays a central role in the development of resistance against cytostatics in anticancer therapy and against human immunodeficiency virus (HIV) therapeutics of the HIV-1 protease inhibitor type. An approach to reverse the so-called multidrug resistance (MDR) phenomenon by the use of P-gp inhibiting agents is a challenge in the therapy of cancer and AIDS. Effective in-vitro inhibitors have P-gp substrate properties so that the expected in-vivo effects have been disappointing so far. Consequent higher dosages cause toxic effects.

Methods Novel HIV-1 protease inhibitors (H17, JW41, JW33 and JW46) have been evaluated in comparison with ritonavir as P-gp inhibiting agents, in the exclusively P-gp overexpressing model cell line mouse T lymphoma using flow cytometry. The cytotoxic properties against various cell lines were characterized in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to estimate potential toxic effects in therapeutically relevant concentrations in metabolically active HepG2 cells, drug-sensitive Jurkat cells and in gastric carcinoma cells.

Key findings Concentration-dependent effective reversal properties have been discussed in context and proved to be mainly influenced by the number of potential hydrogen bond acceptor functions. The compounds showed no cytotoxic properties in P-gp inhibiting concentration ranges. Ritonavir, a known P-gp substrate, proved to be less toxic in the P-gp expressing cell line than in the nonexpressing cell line at the cell-exposed concentrations and thus showed P-gp substrate properties. Two compounds, H17 and JW41, showed no P-gp substrate properties, with higher toxicity in the P-gp expressing cell line compared with the nonexpressing cell line.

Conclusions The novel compounds have been shown to be prospective AIDS therapeutics, acting as effective and nontoxic P-gp inhibitors compared with ritonavir, which is a known P-gp inhibitor with unfavourable toxic and P-gp substrate properties.

Keywords cytotoxicity; HIV-1 protease inhibitor; multidrug resistance modulators; P-glycoprotein

Introduction

One main problem in the therapy of cancer or infectious diseases is the so-called multidrug resistance (MDR) against structurally different groups of drugs.[1,2] One strategy to overcome this emerging resistance problem has been the development of new drugs with structurally different properties.[1,3] In cancer treatment novel therapeutics have been introduced such as the tyrosine kinase inhibitor imatinib or monoclonal antibodies such as gemtuzumab ozogamicin. Unfortunately, they were also affected by the MDR phenomenon because they were found to be substrates of one MDR-mediating transmembrane efflux pump.[4,5] The most causative agents of the MDR phenomenon are transmembrane efflux pumps which transport drugs out of the cells so that therapeutically necessary drug levels are not reached.[6–8] The alternative strategy to overcome the problem of MDR has been the development of inhibitors of the efflux pump activity.[9–11] Most inhibitors turned out to be substrates of the efflux pumps themselves, so that intracellular effective inhibitor levels were

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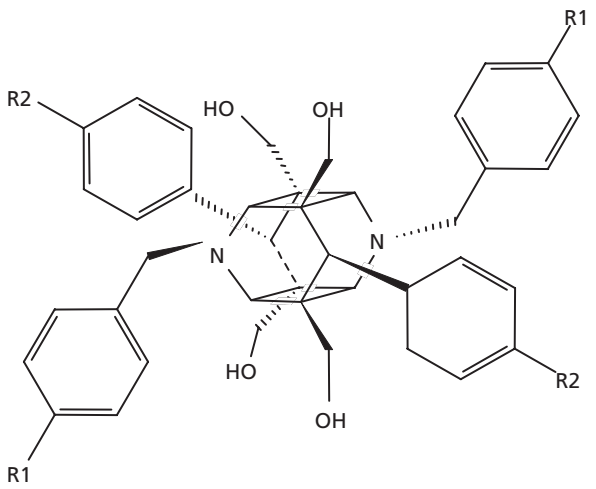

Compound	R1	R2
H17	H	H
JW41	OCH_3	H
JW33	OCH_3	OCH_3
JW46	OBz	OBz

Figure 1 Structures of the investigated compounds except ritonavir.

not reached.[12,13] This fact led to increased application concentrations, which caused critical toxic side effects. Such toxic side effects are one main reason for the clinical failure of the inhibitors which are currently used.[11,14]

P-glycoprotein (P-gp) is the most important efflux pump playing a central role in the MDR of cancer and of human immunodeficiency virus (HIV) infection. In the case of HIV infection the whole drug group of HIV-1 protease inhibitors is subjected to transport out of the cells and thus is affected by the MDR phenomenon.[2,15,16] So far, peptidic and nonpeptidic HIV-1 protease inhibitors are common P-gp substrates.

We have evaluated representatives of a novel class of recently developed HIV-1 protease inhibitors as P-gp inhibitors to determine effective concentrations to overcome MDR (Figure 1). The evaluated compounds have been cytostatically investigated in various human cell lines to judge possible important toxic side effects at therapeutically relevant concentrations. The P-gp substrate properties have been investigated in an additional cell model. We have investigated the significant problems in the development of such inhibitors for the new compound class, i.e. P-gp substrate properties and possible cytotoxic effects. The benefit of the new compounds to act as effective MDR modulators for in-vivo studies has been estimated in a conjoint therapy with a marketed HIV-1 protease inhibitor, ritonavir, which is a P-gp substrate.

Materials and Methods

Reagents

The synthesis of the compounds H17, JW41, JW33 and JW46 has been described.[17] Verapamil as Isoptin injection solution was purchased from Abbott GmbH & Co. KG (Wiesbaden, Germany). Ritonavir was a gift from Abbott GmbH & Co. KG. Other chemicals for cell culture were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise specified. Chemicals (HPLC grade) and columns for HPLC analysis were obtained from Merck (Darmstadt, Germany).

Cell culture

The human gastric carcinoma cell line EPG85-257P and its daunorubicin-resistant subline EPG85-257RDB were cultivated in Leibovitz L15 medium (Bio Whittaker, Verviers, Belgium) supplemented by 10% FCS (fetal calf serum; Biochrom AG, Berlin, Germany), 1 mM Ultraglutamine (Bio Whittaker, Verviers, Belgium), 1.1 g/l $NaHCO_3$, 1% minimal essential vitamins, 0.225 g/l glucose, 80 IU/l insulin (Insuman Rapid, Hoechst Marion Roussell, München, Germany), 5000 kIU Trasylol (Bayer AG, Leverkusen, Germany), 2.5 mg/ml transferrin and 6.25 mg/l fetuin. The daunorubicin-resistant cell line was derived from induction under exposure to increasing concentrations of daunorubicin.[18] Cell culture medium for the P-gp expressing line EPG85-257RDB was supplemented with daunorubicin (2.5 μg/ml) to ensure the MDR phenotype.

The mouse T lymphoma cell line L5178Y and the L5178Y*mdr* subline were grown in McCoy's 5A medium with 10% heat-inactivated horse serum albumin, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) as described.[19] To ensure a stable P-gp expression, the cell culture medium for the MDR-resistant subline was supplemented with colchicine to a final concentration of 60 ng/ml. The hepatic carcinoma cell line HepG2 was purchased from ATCC (Rockville, USA) and was grown in MEM with Earle's salts medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FBS (fetal bovine serum) Gold (PAA Laboratories GmbH, Pasching, Austria), 1 mM sodium pyruvate solution, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, USA) and 2 mM glutamine. The human T cell leukaemia cells Jurkat were purchased from DSMZ (Braunschweig, Germany) and were cultured in RPMI 1640 medium supplemented with 10% FBS Gold and 2 mM glutamine. All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

MDR reversal studies

The cultured mouse T lymphoma cells were adjusted to a density of 2×10^6 cells/ml, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-ml samples into Eppendorf centrifuge tubes. Tested compounds were added in two representative concentrations, 1 and 10 μM. The cells were then incubated for 10 min at room temperature. Rhodamine 123 (10 μl) (5.2 μM final concentration) was added to the samples and cells were incubated for a further 20 min at 37°C. After removal of the medium, cells were washed twice with phosphate-buffered saline (PBS), resuspended in 0.5 ml PBS and stored on ice. The fluorescence of the cell population

(1×10^4 cells) was measured with a Becton Dickinson FACScan flow cytometer. The *R* values were calculated as the ratios of the fluorescence intensities of the treated to the untreated cells. Verapamil was used with the same concentrations as the samples and acted as a positive control. The experiments were performed in three independent replications.

Mitochondrial activity as an index of cell viability

The exponentially growing cells were diluted and placed in 96-well plates to achieve 1×10^4 cells per well. Plated cells were incubated for 24 h before treatment. Cells were individually treated with compounds (0.125–80 μM) incubated for 24 or 48 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 10 μl) was added to each well. After 4 h the formazan crystals, formed in viable cells, were dissolved in dimethyl sulfoxide supplemented with 25% acetic acid and 10% sodium dodecyl sulfate (SDS) and each plate was measured spectrophotometrically with a microplate reader, PolarStar Galaxy (BMG, Offenburg, Germany), at 560 nm. The measured extinction value of the untreated cells was set to a value of 100% and the extinction values of the treated cells were calculated in relation to the untreated cells for each compound. The concentrations required to achieve 50% inhibition (IC50 values) were determined from the mean percentage growth inhibitions after a sigmoid curve fit and all experiments were repeated independently in triplicate. For compounds with nontoxic effects in the cells the IC50 values could not be calculated correctly and have been displayed with a greater than symbol (>) for the highest concentration which was determined.

Metabolism studies

HepG2 cells were cultured to a density of 40–60%. Fresh medium was added containing 20 μM of the cage dimers H17 or JW41. After 48-h incubation the cell medium was collected and cells were treated with 3 ml lysis buffer (0.3% CHAPS, 0.05 M HEPES buffer) for 15 min. Cells and medium were centrifuged for 5 min (2000*g*) and the supernatant was separated into two parts. One part was treated with 1% of β-glucuronidase/arylsulfatase (Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. The second part was treated under the same conditions but without the enzyme mixture. Next, 2 ml of each sample was used for solid phase extraction (SPE) with LiChrolut RP-18 columns (Merck, Darmstadt, Germany). SPE cartridges were activated by aspirating 5 ml acetonitrile, followed by 5 ml HPLC grade water. Samples were then loaded onto the cartridges and aspirated at a reduced vacuum. After aspiration, each cartridge was washed twice with 1 ml HPLC grade water and dried for 15 min under vacuum. In four steps, 2 ml acetonitrile were used to elute the samples. A 20-μl sample of this solution was injected directly into the chromatographic system.

Chromatographic separations were performed on a Merck-Hitachi LaChrom model equipped with a diode array UV detector (L-7455), a quaternary pump (L-7100), a column oven (L-7360) and an automatic injector (L-7200). For compound separation a ChromCart Inertsil ODS-2 reversed phase column (150 × 4.6 mm, 5 μm particle size) from Macherey-Nagel (Düren, Germany) was used and kept under a controlled temperature of 25 ± 0.5°C. The mobile phase consisted of 53% acetonitrile and 47% HPLC grade water and was pumped at a flow rate of 1.5 ml/min.

Table 1 P-glycoprotein inhibiting properties of novel HIV-1 protease inhibitors, ritonavir and verapamil evaluated in the mouse T lymphoma model cell line and the P-glycoprotein expressing subline, determined as *R* values by flow cytometry

Compound	*R* values	
	1 μM	10 μM
H17	4.1 ± 0.3[b]	29 ± 3.1[ab]
JW41	3.5 ± 0.6[b]	13 ± 0.3[ab]
JW33	3.1 ± 0.5[b]	46 ± 8.0[b]
JW46	3.3 ± 1.1[b]	47 ± 2.3[b]
Ritonavir	1.9 ± 0.1	19 ± 0.9[b]
Verapamil (control)	2.1 ± 0.1	4.1 ± 0.3

Each value indicates the mean ± SD of $n = 3$. [a]Saturation. [b]Significant for $P = 0.1$, each compound (1 and 10 μM) vs verapamil (1 and 10 μM).

Statistical analysis

Statistical analysis for MDR reversal studies of reported and novel compounds was conducted using the Mann–Whitney U test. A two-sided alpha level of 0.1 was used to determine statistical significance.

Results

To evaluate the P-gp inhibiting properties of H17, JW41, JW33 and JW46 we used a mouse T lymphoma cell line, and a P-gp expressing subline which had been produced by a primary retroviral transfection with the *mdr1* gene and consequent selection using colchicine as a toxic P-gp substrate.[20] This procedure provided a cell line which exclusively expressed P-gp and no other efflux pump. In the fluorescence uptake assay we used the fluorescent P-gp substrate rhodamine 123. The extent of cellular uptake of this substrate was quantified using flow cytometry in both inhibitor-treated parental and P-gp expressing cell lines (Table 1).

All of the investigated cage dimers showed higher activity at the lower concentration (1 μM) when compared with verapamil (an effective in-vitro P-gp inhibitor). Such high activity has been reported for H17.[21] Ritonavir, reported as a strong P-gp inhibitor in in-vitro studies, displayed poor activity.[22]

JW33, with four methoxy groups, showed the highest activity as compared with reported derivatives with less or no methoxy functions. This observation outlined a significant importance of hydrogen bond acceptor functions in P-gp inhibitors for binding to a potential P-gp binding site. Similar activity was demonstrated for JW46, the bisbenzyloxy substituted compound.

Ritonavir displayed the poorest activity at the higher inhibitor concentrations. Ritonavir was a much better inhibitor compared with verapamil, but when compared with H17, JW41, JW33 and JW46 the activity was poor. Thus it can be stated that all the differently substituted cage dimers showed good P-gp inhibiting properties in the lower micromolar range, superior to verapamil and ritonavir.

Table 2 Effects of various concentrations of novel HIV-1 protease inhibitors and ritonavir on the cell viability of HepG2 cells after incubation

Compound (μM)	Cell viability (%)			
	24 h		48 h	
	Mean	SD	Mean	SD
H17				
20	111.29	8.64	121.05	12.72
40	103.79	7.16	111.84	7.97
80	105.21	10.32	114.30	9.16
JW41				
20	119.95	9.76	108.91	17.40
40	104.56	2.35	83.97	12.26
80	95.55	4.17	69.76	14.52
JW33				
0.5	95.82	1.17	98.02	1.33
1	99.10	1.23	99.72	2.34
2.5	98.25	1.59	96.42	1.65
5	104.44	2.38	103.11	2.28
10	104.69	1.97	101.33	3.09
20	111.29	2.15	98.97	2.28
40	108.47	3.72	94.04	3.95
Ritonavir				
5	121.49	21.64	115.23	10.48
10	112.48	18.21	111.34	12.43
20	108.03	11.70	107.16	8.08
40	96.17	10.89	104.87	6.69
80	109.30	11.13	85.52	8.99
JW46				
0.5	98.61	3.11	100.71	1.26
1	91.90	2.89	82.89	2.57
2.5	73.45	1.42	71.76	3.00
5	75.45	3.15	72.61	2.19
10	77.44	3.08	65.30	1.12
20	82.04	5.42	63.09	1.51
40	80.35	6.59	51.57	0.89

Novel HIV-1 protease inhibitors: H17, JW41, JW33 and JW46. Incubation was for 24 or 48 h. Data displayed as mean ± SD of $n = 3$.

Table 3 Effects of various concentrations of novel HIV-1 protease inhibitors and ritonavir on the cell viability of Jurkat cells after incubation

Compound (μM)	Cell viability (%)			
	24 h		48 h	
	Mean	SD	Mean	SD
H17				
5	93.63	8.79	95.80	6.80
10	89.83	4.32	94.19	3.45
20	9.86	3.94	0.60	2.04
40	1.82	2.43	0.83	3.36
80	4.48	0.45	0.89	4.19
JW41				
5	113.51	5.47	105.18	1.73
10	104.35	4.39	113.08	4.16
20	62.91	12.40	71.63	18.13
40	29.45	2.48	24.42	4.82
80	35.58	3.54	19.53	4.44
JW33				
0.5	96.04	2.34	97.25	3.28
1	102.96	4.54	104.16	4.18
5	108.97	4.44	107.97	2.40
10	100.51	8.70	114.92	3.80
20	92.37	5.13	99.67	4.72
40	101.06	3.80	91.88	6.50
Ritonavir				
5	92.76	2.78	106.54	6.85
10	83.76	8.75	101.54	2.98
20	58.36	5.47	71.91	12.16
40	12.60	2.99	4.40	2.37
80	2.96	0.60	0.97	2.17
JW46				
0.1	91.05	3.74	89.03	2.78
0.5	90.98	6.02	78.66	2.45
1	69.42	3.04	74.29	4.78
5	50.76	4.15	67.92	1.88
10	54.59	2.98	62.77	2.32
20	54.70	2.88	55.20	2.88
40	73.86	2.19	64.10	0.67

Novel HIV-1 protease inhibitors: H17, JW41, JW33 and JW46. Incubation was for 24 or 48 h. Data displayed as mean ± SD of $n = 3$.

To carry out cytotoxic profiling of the new class of strong P-gp inhibitors we used various exclusively human cell lines. First we determined the cytotoxicity in the HepG2 cell line. It was evident from recent studies that HepG2 cells are a suitable human cell model for the qualitative characterization of metabolites formed by oxidative metabolism as well as phase II metabolism.[23] However, with a lowered metabolic activity compared with human hepatocytes HepG2 cells can not be used for a quantitative characterization of drug metabolism. So the use of HepG2 cells in cytotoxic studies gave additional information of possible cytotoxic effects of formed metabolites compared with metabolically inactive cell lines.

After 24-h incubation, compound H17 was found nontoxic in the MTT assay. After 48-h incubation a slight increase in viability was observed. This may have been caused by the inducing effects of H17 on metabolising mitochondrial enzymes (Table 2). JW41 was also nontoxic after 24-h incubation. The viability remained above 80% up to a concentration of 40 μM after 48 h, thus the compound was nontoxic far above the range of P-gp inhibiting activity. After 24- or 48-h incubation, JW33, the tetrakismethoxylated compound, was shown to be nontoxic, like H17, with slight increases in viability after 24-h incubation.

JW46, the bisbenzyloxy substituted derivative, was nontoxic up to a concentration of 40 μM after 24-h incubation. After 48 h some decreases in viability were observed at higher concentrations. Ritonavir also proved to be nontoxic after 24-h incubation. However, longer incubation led to a reduced viability.

To determine the compound toxicity, we used lymphocytes of the Jurkat cell line, which is a frequently used drug-sensitive cell line.[24] H17 was nontoxic after 24- and 48-h incubation up to the tested concentrations of 10 μM, which lay within the concentration range of P-gp inhibiting activity (Table 3). Higher concentrations far beyond the P-gp inhibiting concentration range, however, caused toxic effects. Similar results were observed for compound JW41 which proved to be less toxic than H17 at 20 μM. Interestingly, JW33 was found nontoxic up to a concentration of 40 μM after 24-

Table 4 Effects of various concentrations of novel HIV-1 protease inhibitors and ritonavir on the viability of EPG85-257P cells and the P-glycoprotein expressing subline EPG85-257RDB after incubation

Compound (μM)	Cell viability (%)							
	EPG85-257P				EPG85-257RDB			
	24 h		48 h		24 h		48 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
H17								
5	100.79	3.33	93.87	1.02	130.48	2.08	109.05	5.30
10	112.28	4.03	75.99	1.68	117.57	2.26	81.01	7.93
20	91.90	2.20	73.98	1.31	99.55	1.86	75.76	10.77
40	88.06	7.23	55.82	0.47	74.23	2.03	26.01	1.96
80	81.48	2.81	33.04	4.51	65.27	2.42	20.94	2.18
JW41								
5	110.16	5.69	95.39	5.62	144.17	4.27	120.93	7.94
10	116.00	4.77	78.80	16.25	140.74	7.58	127.19	3.93
20	104.85	0.68	74.78	4.47	116.59	4.83	89.37	4.85
40	110.11	10.00	74.94	6.41	103.34	6.27	60.78	2.49
80	99.48	18.90	50.47	7.04	90.13	9.96	47.47	4.23
JW33								
1.25	96.56	2.38	101.16	15.51	95.46	2.74	86.93	4.22
2.5	96.30	1.34	111.60	16.43	101.97	3.84	95.59	2.15
5	89.73	1.28	100.55	14.78	102.10	4.37	88.05	4.34
7.5	87.87	0.60	102.75	19.86	102.98	5.79	102.52	1.11
10	84.87	1.46	83.12	8.30	102.47	6.54	98.63	4.00
15	85.54	1.55	71.71	5.33	104.92	5.66	106.39	5.55
20	66.63	2.96	58.98	25.91	102.58	13.21	98.34	6.51
40	60.33	7.52	37.27	6.08	95.63	5.65	107.77	4.26
Ritonavir								
5	97.03	4.69	89.97	5.25	121.47	11.71	125.60	9.76
10	90.66	6.93	81.95	5.64	112.26	14.64	118.70	12.45
20	91.30	2.92	83.67	2.53	104.17	6.69	99.13	13.04
40	89.91	11.05	68.43	4.62	99.56	10.11	101.17	7.73
80	73.14	7.34	31.08	5.74	81.41	5.82	69.46	3.26
JW46								
0.625	111.44	3.17	102.96	5.80	116.48	2.94	107.18	10.06
1.25	98.19	1.79	87.75	3.40	120.54	1.67	100.58	10.14
2.5	86.39	9.79	63.60	11.27	108.75	2.02	92.89	10.63
5	89.43	7.92	65.28	3.60	87.83	20.29	87.59	3.04
10	75.61	14.39	2.37	8.57	84.07	17.28	72.03	6.61
20	71.70	13.41	47.04	11.73	95.37	8.63	59.16	15.80
40	69.57	16.05	33.55	1.55	89.80	16.28	60.05	3.28
80	79.39	16.81	32.48	0.42	92.20	23.28	62.43	10.05

Novel HIV-1 protease inhibitors: H17, JW41, JW33 and JW46. Incubation was for 24 or 48 h. Data displayed as mean ± SD of $n = 3$.

and 48-h incubation. JW46 caused reduced cell viability after 24-h incubation and compared with H17 it was found to be less toxic at the higher concentrations. Ritonavir showed remarkable toxic effects at concentrations > 10 μM.

The effects of various concentrations of H17, JW41, JW33 and JW46, and ritonavir on the viability of EPG85-257P cells and the P-glycoprotein expressing subline EPG85-257RDB after incubation is shown in Table 4. After 24-h incubation H17 and JW41 were nontoxic, whereas after 48 h the viability was found reduced for both compounds at concentrations > 10 μM. JW33 showed some toxicity at concentrations > 20 μM after both incubation periods. JW46 was practically nontoxic at a concentration of 80 μM after 24-h incubation. However, after 48 h incubation the toxicity increased. Ritonavir was toxic at concentrations > 40 μM after 24 h and > 20 μM after 48 h. In the P-gp expressing subline EPG85-257RDB, H17 and JW41 had increased toxicity after 24- and 48-h incubation.

Table 5 shows the IC50 values of reduced viability for H17, JW41, JW33 and JW46 in the gastric carcinoma cell lines EPG85-257P and the P-glycoprotein expressing subline EPG85-275RDB, determined in the MTT-assay using UV spectroscopy after incubation.

Discussion

The occurrence of P-gp at the blood–brain barrier and in the testis leads to a resistance in the therapy of HIV infections with HIV-1 protease inhibitors. This is because viral replication, which takes place in both the central nervous system and

Table 5 IC50 values of reduced viability for the novel HIV-1 protease inhibitors in the gastric carcinoma cell lines EPG85-257P and the P-glycoprotein expressing subline EPG85-275RDB determined in the MTT assay using UV spectroscopy after incubation

Compound	IC50 values (μM)							
	EPG85-257P				EPG85-257RDB			
	24 h		48 h		24 h		48 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
H17	>80		45.5	18.9	>80		29.4	7.1
JW41	>80		>80		>80		>80	
JW33	>40		23.8	7.1	>40		>40	
JW46	>80		18.6	8.9	>80		>80	
Ritonavir	>80		55.0	4.8	>80		>80	

Novel HIV-1 protease inhibitors: H17, JW41, JW33 and JW46. Incubation was for 24 or 48 h. Data are displayed as mean ± SD $n = 3$. For compounds with nontoxic effects to the cells the concentrations required to achieve 50% inhibition (IC50 values) could not be calculated correctly and are displayed with a greater than symbol (>) for the highest concentration which was determined.

the testis tissue, cannot be treated effectively with HIV-1 protease inhibitors as they are all P-gp substrates.[2,25,26] The HIV-1 protease inhibitor ritonavir was discovered to be a P-gp inhibitor and was demonstrated to increase intracellular drug levels of co-administered P-gp inhibitors.[16,22] The convincing in-vitro effects of ritonavir were not found in in-vivo studies due to the concentration-limited application of ritonavir, which proved to be an additional P-gp substrate.[16] Thus higher concentrations were needed which led to toxic side effects. The observed nephrotoxicity of ritonavir is problematic in ritonavir co-administered therapies with HIV-1 protease inhibitors.[27]

Recently we developed structurally-varied cage dimeric 1,4-dihydropyridines (H17, JW41, JW33 and JW46; Figure 1) as a novel class of HIV-1 protease inhibitors with a nonpeptidic molecular scaffold.[17] First, representatives of them were systematically evaluated as P-gp inhibitors to estimate their potential to effectively work as P-gp inhibitors in following co-administered therapies with other HIV-1 protease inhibitors.

The observed reduced toxicity at the higher concentrations of H17 and JW41 in the HepG2 cells were closely investigated, although the concentrations lay far above the P-gp inhibiting concentration range. In the HepG2 cell line these compounds were found nontoxic or much less toxic than in the Jurkat cells. We decided to determine the possible metabolites which may have been formed by oxidative metabolism or phase II conjugation, by HPLC after solid-phase extraction of the compound-incubated media. Such metabolites may explain the observed reduced toxicity of H17 and JW41 in the HepG2 cells.

After a solid phase extraction of the incubation media and HPLC analyses of H17 and JW41, we found only the unchanged compounds and no phase I metabolites. To characterize possible phase II metabolites, the incubation media were treated with aryl sulfatase and glucuronidase before the solid phase extraction. Thus, possible phase II conjugation products of the hydroxy functions at the methylene groups could have been identified. However, after the solid phase extractions the detected amounts of the unchanged compounds H17 and JW41 were similar to the studies without enzyme treatment before the cleavage of possible conjugates. Thus the differences in cytotoxicity of the compounds in the HepG2 cells and the Jurkat cells could not be explained by metabolic detoxification because practically no metabolites were found. In the case of detoxification of the compounds in the HepG2 cells we would have expected to find metabolites of either a phase I or a phase II procedure.

Another reason for the reduced cytotoxicity of the two compounds might have been the fact that they were substrates of the efflux pump P-gp, which is expressed in hepatocytes and in HepG2 cells.[28,29] If a compound with a given toxic concentration is presented to both P-gp expressing and non-P-gp expressing cells it will be less toxic in the P-gp expressing cells. This is because in those cells the resulting intracellular concentration of the compound is lower than that in the non-P-gp expressing cells due to the efflux from the P-gp expressing cells. To characterize such potential P-gp substrate properties, we determined the cytotoxicity of our compounds in another human model cell line, a gastric carcinoma cell line EPG85-257 and its P-gp overexpressing subline EPG-257RDB, which has been induced to overexpress P-gp under treatment with the P-gp inducing substrate daunorubicin. First we investigated the cytotoxicity of the compounds in the non-P-gp expressing parental cell line EPG85-257P. After 24-h incubation H17 and JW41 were nontoxic, whereas after 48 h the viability was found reduced for both compounds at concentrations > 10 μM. The cytotoxicity of these two compounds was similar to those in the Jurkat cell line (Table 4). When we determined the cytotoxicity for H17 and JW41 in the P-gp expressing subline EPG85-257RDB, we found increased toxicity after both 24- and 48-h incubations. If H17 and JW41 were P-gp substrates they would cause less toxic effects in the P-gp expressing subline at the given cell-exposed concentrations. The calculated IC50 value of a reduced viability for H17 after 48-h incubation (29.4 ± 7.1 μM) in the P-gp expressing subline was found mainly to be decreased when compared with the IC50 value in the parental cell line (45.5 ± 18.9 μM; Table 5). A P-gp substrate would result in a higher IC50 value in the P-gp expressing cell line. Such a higher IC50 value was found for the known P-gp substrate ritonavir. In the P-gp overexpressing subline EPG85-257RDB we found a mainly reduced toxicity of ritonavir after 24- and 48-h incubation (Table 5). The calculated IC50 value after 48-h incubation in the parental cell line was 55.0 ± 4.8 μM, whereas in the P-gp overexpressing subline the IC50 value was higher than 80 μM. Finally, we characterized the cytotoxicity of JW33 and JW46 and found some reduced viability for JW33 at concentrations > 15 μM in the parental cell line, whereas in the P-gp expressing cell line the compound was found nontoxic at the higher concentrations. These in-vitro results suggested that JW33 had some P-gp substrate properties. For JW46 only the longer incubation in the parental cell line led to some toxic effects, which were reduced in the P-gp expressing cell line. Similar to the results for JW33 the lower toxicity for JW46 in the P-gp expressing cell line may be explained by an efflux out of the P-gp expressing cell line as P-gp substrate.

Conclusions

For the first time cage dimeric 1,4-dihydropyridines, as a novel class of HIV-1 protease inhibitors, have been systematically evaluated as P-gp inhibitors concerning their cytotoxic properties in various human cell lines and additionally considering important P-gp substrate properties. All compounds showed significantly higher P-gp inhibiting properties, even at the lowest inhibitor concentrations, compared with ritonavir. P-gp inhibiting activity proved to be influenced mainly by hydrogen bond acceptor functions, as demonstrated by increasing numbers of methoxy functions within the molecular scaffold. The cytotoxic profiling proved them to be nontoxic in the effectively P-gp inhibiting concentration ranges. Two compounds, H17 and JW41, showed a mainly reduced cytotoxicity in the HepG2 cell line. Comparing the different cytotoxic effects in a P-gp-expressing cell line and a nonexpressing cell line, P-gp substrate properties for the compounds were discussed. Causative P-gp substrate properties were suggested by both the cytotoxic profiles and the IC50 values for reduced viability. The HIV-1 protease inhibitor ritonavir showed higher toxicity in the various cell lines, lower P-gp inhibiting properties and additional P-gp substrate properties by higher toxicity at the cell-exposed concentrations in the P-gp expressing cell line. Our new class of HIV-1 protease inhibitors has been profiled as having excellent potential as HIV-1 therapeutics, with respect to consequent clinical applications by acting as effective P-gp inhibitors in suggested combined therapy regimes with marketed HIV-1 protease inhibitors as P-gp substrates.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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