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New pleconaril and [(biphenyloxy)propyl]isoxazole derivatives with substitutions in the central ring exhibit antiviral activity against pleconaril-resistant coxsackievirus B3

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ABSTRACT

Amino acid 1092 (AA1092) in capsid protein 1 of coxsackievirus B3 (CVB3) is located in close vicinity to the central phenoxy group of capsid binders (i.e. pleconaril). Whereas isoleucine is associated with drug susceptibility, leucine and methionine confer resistance to pleconaril. In the present study, novel analogues with different substitutions in the central phenoxy group were synthesized to study their influence on anti-CVB3 activity with the aim to overcome pleconaril resistance.

Two [(biphenyloxy)propyl]isoxazoles and pleconaril were synthesized without methyl groups in the central phenoxy ring using Suzuki coupling reaction and tested for antiviral activity against the pleconaril-resistant CVB3 Nancy. Furthermore, pleconaril with 3-methyl, 3-methoxy, 3-bromine, 2,3-dimethyl in the central ring as well as the external rings in meta position were synthesized for structure–activity relationship analysis with CVB3 variants containing leucine, methionine or isoleucine at position 1092, other coxsackieviruses B (CVB) as well as several rhinoviruses.

The results demonstrate a high impact of substituents in the central ring of capsid inhibitors for anti-enteroviral activity. Pleconaril resistance of CVB3 based on Leu1092 or Met1092 was overcome by unsubstituted analogues or by monosubstitution with 3-methyl as well as 3-bromine in the central phenyl. The 3-bromine derivative inhibited a broad spectrum of CVB and rhinoviruses.

1. Introduction

Coxsackievirus B3 (CVB3) belongs to the genus enterovirus of the family Picornaviridae (Racaniello, 2007). Like other enteroviruses CVB3 causes a broad spectrum of acute and chronic human diseases including respiratory infections, meningitis, encephalitis, pancreatitis and myocarditis (Melnick, 1996). Up to now, no antiviral agents that are active against enteroviruses are available for clinical use. Besides inhibition of viral RNA and protein synthesis, interference with virus attachment and uncoating is a very promising mechanism to prevent and treat enterovirus-induced diseases (Barnard, 2006; De Palma et al., 2008; Patick, 2006). According to the most recent review published by De Palma et al. (2008), the rhinovirus protease inhibitor rupintrivir reached clinical trials but was recently halted from further development and Biota's HRV drug BTA-798 is scheduled for phase II trials in 2008. BTA-798 is structurally related to pirodavir that binds to the viral capsid (Andries et al., 1992). Compounds binding to the viral capsid, the so-called capsid function inhibitors block virus adsorption, entry and/or uncoating. The most advanced capsid function inhibitor in clinical trials is pleconaril. It exhibits a potent and highly specific in vitro activity against various serotypes of enteroviruses (Pevear et al., 1999). Pleconaril also inhibits a broad spectrum of rhinoviruses in cell culture (Kaiser et al., 2000; Ledford et al., 2005). However, oral pleconaril was not approved by the FDA for the treatment of common cold in adults, primarily based on drug interactions resulting from the activation of cytochrome P-450 3A enzymes (Ma et al., 2006) and marginal treatment effects (Hayden et al., 2002, 2003). In 2007, a phase II clinical study with a pleconaril nasal spray for treatment of common cold symptoms and asthma exacerbations following rhinovirus exposure was completed (De Palma et al., 2008). The results have not yet been reported.

Moreover, it was shown that naturally resistant viruses may exist within an enterovirus serotype and that resistant mutants may emerge rapidly during pleconaril treatment in vitro. For exam-

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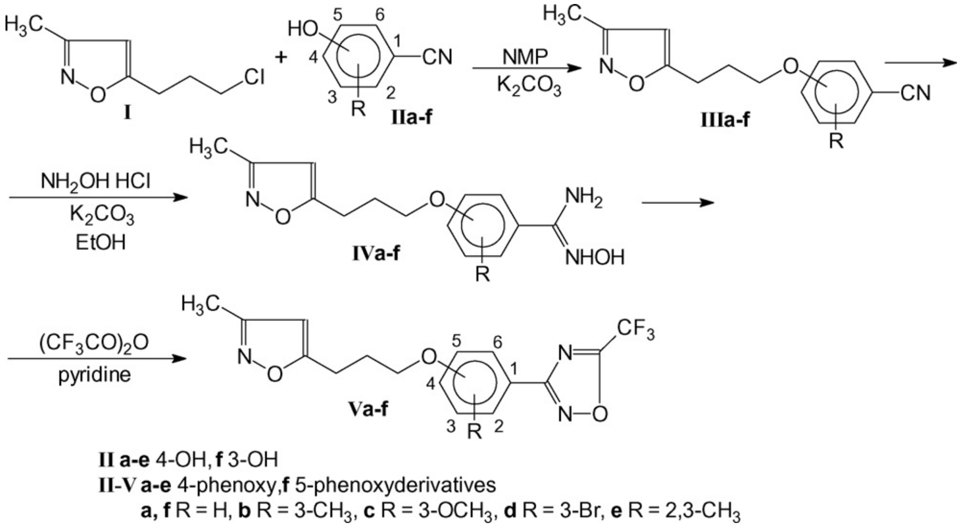

Fig. 1. Synthesis of pleconaril and [(biphenyloxy)propyl]isoxazole analogues.

ple, the prototype strain CVB3 Nancy often used in antiviral studies as well as CVB3 Nancy variants are naturally pleconaril-resistant (Pevear et al., 1999; Schmidtke et al., 2005). Pleconaril-resistant CVB3 mutants were easily selected in vitro (Groarke and Pevear, 1999). The frequency of resistance to pleconaril in the wild type population was $\sim 5 \times 10^{-5}$. CVB3 Nancy as well as in vitro selected resistant mutants carry the amino acid substitution Ile1092 → Leu or Ile1092 → Met in the hydrophobic pocket of viral capsid protein 1. Using recombinant viruses it could be demonstrated that a single amino acid substitution Ile1092 → Leu may induce complete pleconaril resistance (Schmidtke et al., 2005). In vitro selected pleconaril-resistant variants (Leu1092 or Met1092) showed attenuated virulence in a murine model (Groarke and Pevear, 1999). In contrast, the pleconaril-resistant CVB3 31-1-93 (Leu1092) is highly virulent in mice, infects the murine heart and causes chronic myocarditis (Schmidtke et al., 2000, 2007). The possibility of transmission of resistant viruses cannot be excluded. From this perspective, there is a high medical need for improved antiviral agents for either the prevention or therapy of enteroviral diseases.

The correlation between amino acid 1092 in the center of the hydrophobic pocket of viral capsid protein VP1 and pleconaril susceptibility of CVB3 isolates and laboratory strains (Schmidtke et al., 2005) as well as the close vicinity of amino acid 1092 to the central ring of capsid binders (Muckelbauer et al., 1995) suggest an important role of substituents in the central phenyl of these antiviral drugs for anti-CVB3 activity. Methyl groups of capsid binders could be important for compound binding by hydrophobic interactions with the Ile1092 of pleconaril-sensitive CVB3. Alternatively, they may sterically hinder the positioning of capsid binders into the hydrophobic pocket of pleconaril-resistant CVB3 variants (Leu1092 or Met1092). Therefore, pleconaril and [(biphenyloxy)propyl]isoxazoles lacking methyl groups in the central phenyl were synthesized. Furthermore, monosubstitution with 3-methyl as well as with substituents varying in space size or lipophilicity were carried out to acquire new insights into the role of the central part and the strength of its interaction with the hydrophobic pocket of CVB3. After determining cytotoxicity, antiviral assays were performed with 3 pleconaril-resistant (Leu1092 and Met1092) as well 3 pleconaril-sensitive CVB3 variants (Ile1092) to determine the impact of individual substitutions for drug susceptibility and to discover active compounds. The antiviral spectrum of monosubstituted pleconaril analogues was determined with other CVB types and various human rhinoviruses.

2. Materials and methods

2.1. Chemistry

The reference compound pleconaril, as well as the pleconaril derivatives and biphenyl derivatives were synthesized by Dr. Makarov (RAS Institute of Biochemistry, Moscow, Russia). The method of synthesis was the same as described for the pleconaril analogues (Fig. 1). All reagents and solvents were purchased from commercial suppliers and used without further purification. Melting points were determined on Electrothermal 9001 and are uncorrected. ^1H and ^{13}C NMR were measured at 400 MHz on Varian Unity +400. Shifts for NMR are reported in ppm downfield from TMS (σ). A Waters Micromass ZQ detector was used in ESI MS for identification of various products. Elemental analyses were carried out on a Carlo-Erba 5500 elemental analyzer for C,H,F,N. The results are within ±0.3% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC; column chromatography was performed on Merck silica gel 60 (70–230 mesh).

Hydroxybenzonitriles (***IIa–f***) were synthesized from corresponding phenols by NBS bromination (Canibano et al., 2001) and subsequent change bromine atom to cyano group by CuCN (Friedman and Shechter, 1961).

2.1.1. 5-(3-Chloropropyl)-3-methylisoxazole (*I*)

Acetaldoxime (1.65 g, 28.00 mmol) in 10 mL DMF was added to a stirred solution of chlorosuccinimide (3.5 g, 28.08 mmol) in dried DMF (30 mL) with 2 drops of pyridine at 25 °C. After 1 h, chloropentine (1.26 mL, 12.00 mmol) in DMF (30 mL) was added and the reaction mixture was heated to 90 °C. Stirring constantly a solution of Et_3N (3.9 mL, 27.72 mmol) in DMF (10 mL) was added drop by drop. Then the reaction was stirred for an additional 3 h, cooled and diluted by 300 mL water and 50 mL of $CHCl_4$. The layers were separated and the aqueous layer was extracted with $CHCl_4$ (2-times with 75 mL). The combined organic phases were dried (Na_2SO_4), treated with activated carbon, filtered off through coat of SiO_2 and concentrated in vacuo to 1.66 g (91.6%) 5-(3-chloropropyl)-3-methylisoxazole as colourless oil. The physico-chemical data of compound **I** have already been described (Diana et al., 1993a).

*2.1.2. General procedure of reaction of 5-(3-chloropropyl)-3-methylisoxazole I with hydroxybenzonitrils IIa-f: 3-R-4-[3-(3-methylisoxazol-5-yl)propoxy]benzonitriles (**IIIa–e**) and 3-[3-(3-methylisoxazol-5-yl)propoxy]benzonitrile (**IIIf**)*

A mixture of hydroxybenzonitrile **IIa**–**f** (25.00 mmol), finely divided K_2CO_3 (6.9 g, 50.00 mmol), KI (0.42 g, 2.5 mmol), 5-(3-chloropropyl)-3-methylisoxazole (4.45 g, 28.00 mmol) and N-methylpyrrolidinone-2 (25 mL) was heated at 70 °C for 24 h. The cooled mixture was diluted with H_2O (100 mL) and EtOAc (40 mL). The layers were separated. The aqueous layer was extracted with EtOAc (2-times with 50 mL). The combined organic phases were washed with H_2O (50 mL), dried (Na_2SO_4), treated with activated carbon, filtered off, and concentrated in vacuo to an oil, which was dissolved in $CHCl_3$, filtered though a short column of Silicagel 60, and concentrated in vacuo to colourless oil.

*2.1.3. Reaction of benzonitriles (**IIIa–f**) with hydroxylamin hydrochloride: 3-R-N-hydroxy-4-[3-(3-methylisoxazol-5-yl)propoxy]benzenecarboximidamide (**IVa–e**) and N-hydroxy-3-[3-(3-methylisoxazol-5-yl)propoxy]benzenecarboximidamide (**IVf**)*

A mixture of benzonitrile **IIIa**–**f** (16.00 mmol), absolute ethanol (300 mL), finely divided K_2CO_3 (13.25 g, 96.00 mmol), and hydroxylamine hydrochloride (6.66 g, 96.00 mmol) was refluxed for 24 h (for compounds **IIe**—39.75 g (0.28 mol) K_2CO_3 and 20.0 g (0.28 mol) NH_2OH HCl were used, and time of reaction was extended to 7 days). The hot mixture was filtered, and the remaining solids were washed with acetone. The combined filtrates were concentrated in vacuo. The residue was recrystallized from mixture EtOH/H_2O.

*2.1.4. General procedure of amidoximes (**IVa–f**) with trifluoroacetic acid anhydride reaction: 5-(trifluoromethyl)-3-[3-R-4-[[3-(3-methyl-5-isoxazolyl)propyl]oxy]phenyl]-1,2,4-oxadiazoles (**Va–e**) and 5-(trifluoromethyl)-3-[3-[[3-(3-methyl-5-isoxazolyl) propyl]oxy]phenyl]-1,2,4-oxadiazole (**Vf**)*

TFAA (2.00 mL, 14.00 mmol) was added to a solution of amidoxime **IV** (7.00 mmol) in dry pyridine (20 mL) at a rate to maintain a gentle reflux during 10 min. The reaction mixture was stored for 1 h at 90 °C. After cooling to room temperature, the reaction mixture was diluted with water (100 mL) and extracted with EtOAc (3-times with 50 mL). The combined organic phases were washed with H_2O (3-times with 50 mL), dried (Na_2SO_4), treated with activated carbon, filtered off, and concentrated in vacuo. The residue was treated by H_2O (20 mL) and stored in the refrigerator for 12 h. White crystals were collected and recrystallized from the corresponding solvent.

*2.1.5. 5-(Trifluoromethyl)-3-[4-[[3-(3-methyl-5-isoxazolyl) propyl]oxy]phenyl]-1,2,4-oxadiazole (**Va**)*

^1H NMR ($CDCl_3$) σ 8.16 (2H, d, CH), 7.14 (2H, d, CH), 5.90 (1H, s, CH), 3.88 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.28 (3H, s, CH_3), 2.26 (2H, m, CH_2). Anal. $C_{16}H_{14}F_3N_3O_3$, C,H,N.

*2.1.6. 5-(Trifluoromethyl)-3-[3-methyl-4-[[3-(3-methyl-5-isoxazolyl)propyl]-oxy]phenyl]-1,2,4-oxadiazole (**Vb**)*

^1H NMR ($CDCl_3$) σ 7.75 (1H, s, CH), 7.50 (1H, d, CH), 6.86 (1H, d, CH), 5.90 (1H, s, CH), 3.88 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.35 (3H, s, CH_3), 2.28 (3H, s, CH_3), 2.26 (2H, m, CH_2). Anal. $C_{17}H_{16}F_3N_3O_3$, C,H,N.

*2.1.7. 5-(Trifluoromethyl)-3-[3-methoxy-4-[[3-(3-methyl-5-isoxazolyl)propyl]-oxy]phenyl]-1,2,4-oxadiazole (**Vc**)*

^1H NMR ($CDCl_3$) σ 8.05 (1H, d, CH), 7.72 (1H, s, CH), 6.88 (1H, d, CH), 5.90 (1H, s, CH), 3.88 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 3.84 (3H, s, CH_3), 2.28 (3H, s, CH_3), 2.26 (2H, m, CH_2). Anal. $C_{17}H_{16}F_3N_3O_4$, C,H,N.

*2.1.8. 5-(Trifluoromethyl)-3-[3-bromo-4-[[3-(3-methyl-5-isoxazolyl)propyl]-oxy]phenyl]-1,2,4-oxadia-zole (**Vd**)*

^1H NMR ($CDCl_3$) σ 8.30 (1H, s, CH), 8.28 (1H, d, CH), 6.97 (1H, d, CH), 5.90 (1H, s, CH), 3.94 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.30 (3H, s, CH_3), 2.26 (2H, m, CH_2). Anal. $C_{16}H_{13}BrF_3N_3O_3$, C,H,N.

*2.1.9. 5-(Trifluoromethyl)-3-[3-bromo-4-[[3-(2,3-dimethyl-5-isoxazolyl)propyl]-oxy]phenyl]-1,2,4-oxadiazole (**Ve**)*

^1H NMR ($CDCl_3$) σ 7.32 (1H, d, CH), 6.76 (1H, d, CH), 5.90 (1H, s, CH), 3.91 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.35 (3H, s, CH_3), 2.28 (3H, s, CH_3), 2.19 (2H, m, CH_2). Anal. $C_{18}H_{18}F_3N_3O_3$, C,H,N.

*2.1.10. 5-(Trifluoromethyl)-3-[3-[[3-(3-methyl-5-isoxazolyl)propyl]oxy]phenyl]-1,2,4-oxadiazole (**Vf**)*

^1H NMR ($CDCl_3$) σ 8.07 (1H, d, CH), 7.65 (1H, t, CH), 7.36 (1H, s, CH), 6.84 (1H, d, CH), 5.90 (1H, s, CH), 3.88 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.28 (3H, s, CH_3), 2.26 (2H, m, CH_2). Anal. $C_{16}H_{14}F_3N_3O_3$, C,H,N.

*2.1.11. Synthesis of [(biphenlyoxy)propyl]isoxazole derivatives (**VIa** and **b** and **VIIa** and **b**)*

5-{3-[(4′-Fluoro-3,5-dimethylbiphenyl-4-yl)oxy]propyl}-3-methylisoxazole (**VIa**) was synthesized as described previously (Makarov et al., 2005) (Fig. 2).

5-{3-[(4′-Fluorobiphenyl-4-yl)oxy]propyl}-3-methylisoxazole (**VIb**) was synthesized in the same manner. ^1H NMR ($CDCl_3$) σ 7.53–6.88 (8H, m, 8CH), 5.90 (1H, s, CH), 3.91 (2H, t, OCH_2), 3.00 (2H, t, CH_2), 2.26 (3H, s, CH_3), 2.19 (2H, m, CH_2). Anal. $C_{19}H_{18}FNO_2$, C,H,N. The synthesis of 5-{3-[(3′,4′,5′-Trifluoro-3,5-dimethylbiphenyl-4-yl)oxy]propyl}-3-methylisoxazole (**VIIa**) has been published recently (Kuz'min et al., 2007).

5-{3-[(3′,4′,5′-Trifluorobiphenyl-4-yl)oxy]propyl}-3-methylisoxazole (**VIIb**) was synthesized in the same manner. ^1H NMR ($CDCl_3$) σ 7.51 (2H, d, 2CH), 7.11 (2H, d, CH), 6.82–6.92 (2H, m, 2CH), 5.91 (1H, s, CH), 3.91 (2H, t, OCH_2), 3.02 (2H, t, CH_2), 2.26 (3H, s, CH_3), 2.20 (2H, m, CH_2). Anal. $C_{19}H_{16}F_3NO_2$, C,H,N.

2.2. Viruses and cells

Virus stocks of the coxsackievirus B3 prototype strain Nancy (CVB3 Nancy; Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia), the mouse adapted CVB3 Nancy variant 31-1-93 (CVB3 31-1-93 (Merkle et al., 1999)), CVB3 H3 and CVB3 H310A1 (Knowlton et al., 1996), the clinical CVB3 isolate 97–927 (CVB3 97–927; kindly provided by Dr. S. Diedrich, Robert Koch Institute, Berlin, Germany), the in vitro selected pleconaril-resistant CVB3 97927 mutant (CVB3 PR1), coxsackievirus B1 Conn-5 (CVB1), coxsackievirus B2 Ohio-1 (CVB2), coxsackievirus B4 JVB (CVB4), coxsackievirus B5 Faulkner (CVB5) and coxsackievirus B6 Schmitt (CVB6) from the Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia, human rhinoviruses 2, 8, 23, 25, 29, 98 (HRV2, HRV8, HRV23, HRV25, HRV29, HRV98; kindly provided by Dr. J. Seipelt, Greenhills Biotechnology, Ltd., Vienna, Austria), human rhinovirus 14 (HRV14; kindly provided by Dr. H.-P.

Table 1
Physico-chemical properties of pleconaril and [(biphenyloxy)propyl]isoxazole analogues.

Compound	Solvent for recrystallization	Yield (%)	mp (°C)	Formula	MS *m/z* [M⁺]
Va	MeOH	84	78–80	$C_{16}H_{14}F_3N_3O_3$	353
Vb	MeOH	46	67–69	$C_{17}H_{16}F_3N_3O_3$	367
Vc	hexane	75	74–76	$C_{17}H_{16}F_3N_3O_4$	383
Vd	[a]	16	66–69	$C_{16}H_{13}BrF_3N_3O_3$	432
Ve	MeOH/H_2O	51	95–97	$C_{18}H_{18}F_3N_3O_3$	381
Vf	[a]	76	Oil	$C_{16}H_{14}F_3N_3O_3$	353
VIb	MeOH/H_2O	58	67–69	$C_{19}H_{18}FNO_2$	311
VIIb	MeOH/H_2O	42	73–76	$C_{19}H_{16}F_3NO_2$	347

[a] Compounds were purified by column chromatography (SiO_2, eluent: hexane/acetone 4:1) MS *m/z* [M⁺]: molecular weight determined by mass spectrometry.

Grunert, Universitätsklinikum Benjamin Franklin, Berlin, Germany) were prepared in HeLa cells (FlowLabs), aliquoted, and stored at −80 °C until use. HeLa cells were grown in Eagle's minimal essential medium (MEM/E; Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS: HeLa Ohio, PAA, Pasching, Austria), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cambrex, Verviers, Belgium). The test medium contained only 2% of FCS.

2.3. Determination of cytotoxicity

To determine the 50% cytotoxic concentration (CC_{50}), 2-day-old confluent HeLa cell monolayer grown in 96-well plates were incubated with serial 2-fold dilutions of compounds for 72 h (37 °C, 5% CO_2). Then, the cells were fixed and stained with a crystal violet formalin solution as described previously (Schmidtke et al., 2001). After dye extraction, the optical density of individual wells was quantified spectrophotometrically at 550/630 nm with a microplate reader (Dynex, Guernsey, GB). Cell viability of individual compound-treated wells was evaluated as the percentage of the mean value of optical density resulting from six mock-treated cell controls which was set 100%. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration reducing the viability of untreated cell cultures by 50%. It was calculated from the mean dose–response curve of at least 3 tests.

2.4. Cytopathic effect (CPE)-inhibition assay

The CPE-inhibition assays have been performed as described previously (Makarov et al., 2005; Schmidtke et al., 2001). Briefly, the tests were carried out in 1-day-old (all HRV) or 2-day-old (all CVB) confluent HeLa cell monolayers growing in 96-well flat-bottomed microtiter plates. After removal of culture medium, 50 μL of drug solution and a constant amount of virus in a volume of 50 μL were added to the cells. Six wells of non-infected and six wells of infected cells without the test compound served as cell and virus control, respectively, on each plate. The 50% and 100% plaque inhibitory concentrations of guanidine hydrochloride (each 3 wells) were included as positive controls for CVB. Pleconaril was used as reference compound for human rhinoviruses. Using the crystal violet uptake assay described for cytotoxic investigations, the inhibition of the virus-induced CPE was scored 24 h (CVB3 Nancy), 48 h (all other CVB) or 72 h (all HRV) post infection when untreated infected control cells showed maximum cytopathic effect and the positive control compound-treated wells a 50% or 100% protection. The IC_{50} values of the antivirally active compounds were determined from the mean dose response curves of 3 separate experiments.

2.5. Isolation of a drug-resistant mutant and sequencing of viral RNA

Based on the knowledge that pleconaril-resistant mutants can be selected in vitro (Groarke and Pevear, 1999), plaque-reduction assays were performed with CVB3 97927 in HeLa cells as described previously (Schmidtke et al., 2001). Cells were overlaid with agar containing pleconaril (250 ng/mL). Clearly isolated plaques were picked and subjected to 3 further rounds of plaque purification in the presence of increasing noncytotoxic pleconaril concentrations. Thereafter, the pleconaril-resistant phenotype was confirmed by determining the 50% inhibitory concentration in plaque-reduction assays. For genotyping, the region in VP1 that encodes the drug-binding pocket was sequenced as described previously (Schmidtke et al., 2005) and aligned with ClustalW of the program BioEdit.

3. Results

3.1. Synthesis and chemical characterization of new 3-[[3-(3-methyl-5-isoxazolyl)propyl]oxy]phenyl]-1,2,4-oxadiazoles and [(biphenyloxy)propyl]isoxazoles

Several phenyl substituted analogues were synthesized for structure–activity relationship analysis with the aim to clarify the role of substituents of the central phenyl of capsid binders towards inhibition of CVB3 and to discover more active analogues that exhibit antiviral activity against pleconaril-susceptible as well as -resistant virus strains. The method of pleconaril synthesis was used as described previously (Diana et al., 1995). Some modifications were introduced for the preparation of new analogues. One includes the primary synthesis of 5-(3-chloropropyl)-3-methylisoxazole (**I**) from acetaldoxime and chrolopentine followed by its condensation with corresponding hydroxynitriles (**II**) in the presence of K_2CO_3 and KI. The reaction resulted in 65–75% yield. A very slow transformation of the cyano group into the amidoxime fragment was characteristic for the synthesis of N-hydroxy-3-[3-(3-methylisoxazol-5-yl)propoxy]benzenecarboximidamide (**IVf**). Transformation was controlled by TLC. The best results were obtained by daily addition of equal portions of potassium carbonate and hydroxylamine hydrochloride during a week. It resulted in 82% yield of amidoxime. The physico-chemical properties, yields, formula and relative molecular weight of compounds **Va**–**e**, **VIa** and **b**, and **VIIa** and **b** are described in Table 1.

Selected QSAR properties of pleconaril and its derivatives **Va**–**d** substituted in the central ring such as surface area, volume, and log *P* were calculated by using the program Hyperchem 7.5

Table 2
QSAR properties of pleconaril and its analogues.

Compound	Surface area (Å²)	Volume (Å³)	log *P*
Pleconaril	559.18	1029.97	5.15
Va	563.08	912.62	5.31
Vb	573.93	944.68	5.78
Vc	601.80	1015.96	5.05
Vd	575.83	952.52	6.10

log *P*: logarithm lipophilicity (coefficient of distribution between water/octanol).

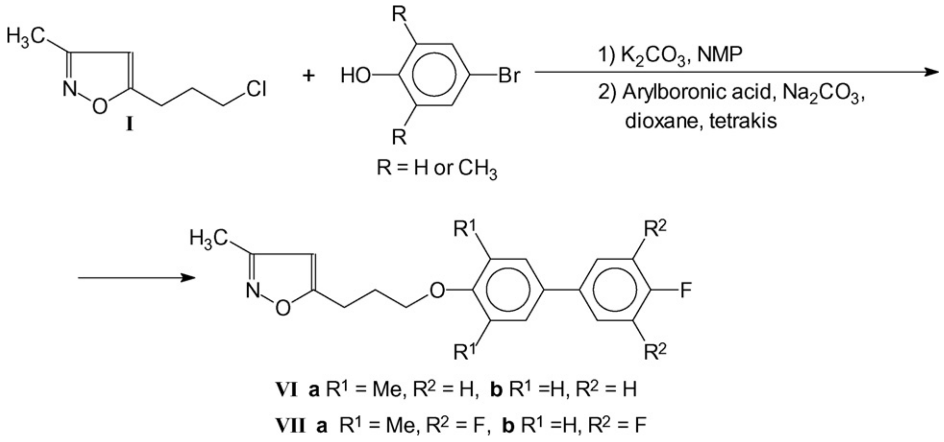

Fig. 2. Structure of [(biphenyloxy)propyl]isoxazole derivatives **VIa** and **b** and **VIIa** and **b**.

(Hypercube Inc., http://www.hyper.com). The data summarized in Table 2 confirm differences in volume and lypophility (log *P*) of pleconaril derivatives synthesized in the present study. This series of compounds was used to study the correlation between these physico-chemical parameters and IC_{50} values obtained with the pleconaril-resistant CVB3 Nancy and CVB3 31-1-93 (both Leu1092) as the activity indicator. The calculated correlation coefficients indicate that there is (i) no correlation between surface area and antiviral activity (−0.04 and 0.07 for CVB3 Nancy and CVB3 31-1-93, respectively) but (ii) an increasing linear relationship between volume and antiviral activity (0.89 and 0.92 for CVB3 Nancy and CVB3 31-1-93, respectively) and a decreasing linear relationship for log *P* (−0.77 and −0.73 for CVB3 Nancy and CVB3 31-1-93, respectively). So, the volume as well log *P* are major determinants of activity for this series of compounds.

3.2. *In vitro selection and characterization of a pleconaril-resistant mutant of CVB3 97927 containing Met1092*

Pleconaril inhibits dose-dependently the plaque formation of the clinical isolate CVB3 97927 in HeLa cells with an IC_{50} of 0.08 μM (Schmidtke et al., 2005). An approximately 10-fold higher drug concentration was used to select resistant CVB3 97927 variants. CVB3 97927 plaques grown in the presence of pleconaril were picked and purified by 3 rounds of plaque purification with increasing noncytotoxic compound concentrations. The resultant virus CVB3 PR1 was completely drug-resistant in a plaque-reduction assay ($IC_{50} > 32.8$ μM, results not shown).

The atomic structure of a CVB3 and the amino acid alignment of the drug-binding pocket have been reported by Muckelbauer et al. (1995). Based on these data, the RNA sequence of the region encoding the drug-binding pocket was determined for CVB3 PR1. The sequence was aligned and compared with that of the parent CVB3 97927, published recently (Schmidtke et al., 2005). Relative to the wild type virus, two nucleotide changes were detected in the partial sequence of CVB PR1. One of these resulted in the amino acid substitution, Ile1092 → Met. All other amino acid residues of the hydrophobic pocket were completely conserved in comparison to CVB3 97927.

3.3. *Cytotoxicity and anti-CVB3 activity of phenyl substituted analogues*

Initially the cytotoxicity and antiviral activity of pleconaril and two [(biphenyloxy)propyl]isoxazole derivatives without methyl groups in the central phenyl (**Va**, **VIb**, and **VIIb**) was examined against the pleconaril-resistant CVB3 Nancy in HeLa cells. Like pleconaril as well as the [(biphenyloxy)propyl]isoxazoles **VIa** and **VIIa** (Makarov et al., 2005; Schmidtke et al., 2005), the 3,5-H derivatives **Va**, **VIb**, and **VIIb** were relatively noncytotoxic. The 50% cytotoxic concentration of compound **Va** was 52.1 μM (Table 3), and that of **VIb** and **VIIb** 269.4 and 42.7 μM, respectively (results not shown). In contrast to pleconaril as well as [(biphenyloxy)propyl]isoxazoles **VIa** and **VIIa**, the analogues **Va**, **VIb**, and **VIIb** exhibited a dose-dependent antiviral activity against CVB3 Nancy at noncytotoxic concentrations (Fig. 3). The antiviral activity of the derivative **Va** was further investigated against other CVB3 variants containing a leucine (CVB3 31-1-93), methionine (CVB3 PR1) or isoleucine (CVB3 H3 and CVB3 H310A1) in position 1092. As a control, pleconaril was included in these studies. The results shown in Table 3 indicate the differential susceptibility of CVB3 to pleconaril and its analogues. Like CVB3 Nancy, CVB3 31-1-93 and CVB3 PR1 were completely pleconaril-resistant. The results obtained with pleconaril-resistant CVB3 31-1-93 and CVB3 PR1 showed that analogue **Va** is more antivirally active than pleconaril. The IC_{50} values of this compound determined with pleconaril-susceptible CVB3

Table 3
Cytotoxicity and anti-CVB3 activity of pleconaril and its analogues.

Compound	CC_{50} (μM)	IC_{50} (μM) towards CVB3					
		Nancy (L1092)	31-1-93 (L1092)	PR1 (Met1092)	97927 (Ile1092)	H3 (Ile1092)	H310A1 (Ile1092)
Pleconaril	72.5	Not active	Not active	Not active	0.021	1.30	0.078
Va	52.1	6.3	2.8	Not active	1.019	1.70	0.057
Vb	50.6	4.1	1.4	17.7	0.005	0.14	0.011
Vc	99.9	20.5	20.3	Not active	0.041	3.13	0.023
Vd	28.5	2.2	1.6	14.8	0.002	0.03	0.001
Ve	24.9	Not active	Not active	24.1	Not active	Not active	6.8
Vf	104.4	Not active	Not tested	Not tested	Not tested	Not tested	Not tested

CC_{50}: 50% cytotoxic concentration; IC_{50}: 50% virus-inhibitory concentration.

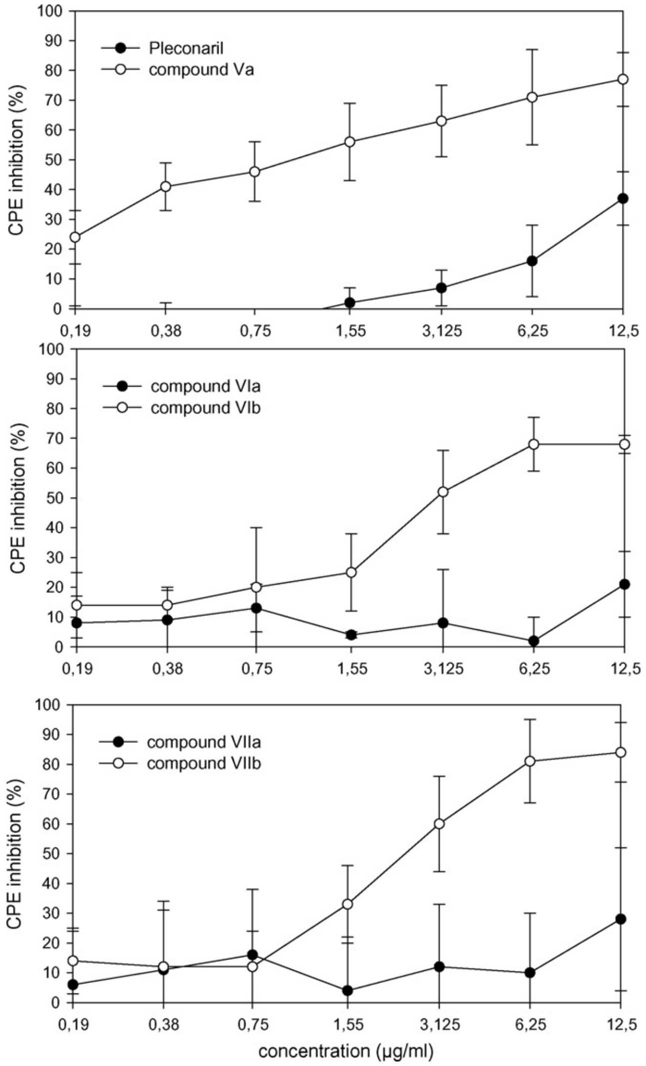

Fig. 3. Comparative antiviral activity of [(biphenyloxy)propyl]isoxazole derivatives and pleconaril with (**VIa** and **VIIa**) and without (**Va**, **VIb** and **VIIb**) methyl groups in the central phenyl towards the pleconaril-resistant CVB3 Nancy. The CPE inhibitory activity of these compounds was determined parallel in 3 independent assays in HeLa cells. Means and standard deviations are shown.

H3 and CVB3 H310A1 were comparable to those obtained for pleconaril. Surprisingly, the replacement of 3,5-methyl by 3,5-H resulted in a 50-fold reduction in activity against CVB3 97927.

Then, the effect of monosubstitution of pleconaril with methyl, methoxy and bromine on anti-CVB3 activity was examined in position 3 of the phenoxy group. When evaluated against the 6 CVB3 variants the 3-bromine analogue **Vd** was the most active of all the compounds examined (Table 3). A very good anti-CVB3 activity was also exhibited by the 3-methyl analogue **Vb** while the 3-methoxy substitution (**Vc**) resulted in a significant reduction in activity. Thus, the analogues **Vb** and **Vd** inhibited the replication of pleconaril-sensitive as well as -resistant CVB3 most effectively.

The analogues with two methyl groups in the central ring (**Ve**) as well as the external rings (**Vf**) in meta position were inactive against most of the tested CVB3 variants.

3.4. Expanded spectra of phenyl substituted pleconaril analogues against the other 5 CVB as well as 7 rhinovirus serotypes

Because the genus enteroviruses includes a broad range of serotypes that often cause similar disease, anti-enteroviral drugs have to possess broad spectrum activity. In this regard, it was important to know the effect of monosubstitution on antiviral activity of capsid-binding compounds against other enteroviruses and rhinoviruses. To investigate an expanded virus spectrum, CPE inhibitory assays were performed with CVB1, CVB2, CVB4, CVB5, and CVB6 as well as 7 rhinovirus serotypes and analogues **Va–d** in HeLa cells. Pleconaril was included as control in these studies.

Table 4
Anti-CVB activity of pleconaril and its analogues.

Compound	IC_{50} (μM) towards				
	CVB1[a]	CVB2[a]	CVB4[a]	CVB5[a]	CVB6[a]
Pleconaril	0.12	0.05	6.3	0.02	28.7
Va	0.03	0.16	7.1	0.23	Not active
Vb	7.22	0.04	15.2	0.04	23.4
Vc	4.57	0.25	20.3	0.21	16.4
Vd	0.06	0.05	9.0	Not active	Not active

[a] Coxsackievirus B serotypes.

Pleconaril exerted a good antiviral activity against CVB1, CVB2 and CVB5 at concentrations between 20 and 120 nM, while the effect on CVB4 and CVB6 replication was markedly lower (Table 4). The 50% inhibitory concentrations were 6.3 and 28.7 μM, respectively. The antiviral activity of analogue **Va** was somewhat better than that of pleconaril against CVB1 and nearly the same against CVB4. While being in the nanomolare concentration range, 3 and 10 times higher compound concentrations were necessary to reduce the CVB2- and CVB5-induced CPE, respectively. The compound was inactive against CVB6. In contrast, the 3-methyl analogue **Vb** inhibited the replication of CVB2 and CVB6 like pleconaril but showed an approximately 2- to 60-fold reduced activity against CVB1, CVB4, and CVB5. The 3-methoxy analogue (**Vc**) was the least active among the compounds studied (except against CVB6). The anti-CVB activity of the 3-Br analogue **Vd** corresponded to pleconaril for CVB1, CVB2, CVB4 as well as CVB5 but there was no activity against CVB6.

The tested rhinoviruses markedly differed in susceptibility towards the pleconaril analogues (Table 5). The lowest antiviral activity against the spectrum of studied rhinovirus serotypes was exhibited by the analogue without any substitute in the central ring (**Va**). Monosubstitution with 3-methyl (**Vb**) as well as 3-bromine (**Vd**) reduced the antiviral activity against HRV2, HRV14, HRV23, and HRV25 in comparison to pleconaril 1.5–85 times. The 3-methoxy analogue **Vc** was the most active against these 5 serotypes. In comparison to pleconaril, it exhibited slightly reduced (HRV2, HRV23, and HRV25) or equal (HRV14) antiviral activity. HRV8 and HRV98 markedly differed from all other HRV studied. But both viruses showed a similar susceptibility pattern. The 4 pleconaril analogues were inactive against these two serotypes regardless of the nature of the substitutions. The HRV29-induced cytopathic effect was 22 times less inhibited by the unsubstituted analogue **Va** in comparison to pleconaril. None of the other substitutions did affect adversely the antiviral activity.

Table 5
Anti-HRV activity of pleconaril analogues **Va–d**.

Compound	IC_{50} (μM) towards HRV						
	2[a]	8[a]	14[a]	23[a]	25[a]	29[a]	98[a]
Pleconaril	0.03	4.17	0.05	0.03	0.01	0.14	0.90
Va	1.13	Not active	0.25	0.76	0.85	3.10	Not active
Vb	0.22	Not active	0.28	0.16	0.07	0.25	Not active
Vc	0.26	Not active	0.04	0.17	0.06	0.23	Not active
Vd	0.12	Not active	0.51	0.11	0.54	0.19	Not active

[a] Human rhinovirus serotypes.

The results of structure–activity relationship analysis demonstrate the crucial role of substitutions in the central phenoxy group for antiviral activity of capsid-binding compounds towards entero- as well as rhinoviruses.

4. Discussion

Capsid binders like pleconaril represent highly potential antiviral drugs for treatment of enterovirus infections. But as shown for pleconaril, a single amino acid substitution in the drug-binding pocket of entero- as well as rhinoviruses may reduce or even completely abolish the antiviral activity of this class of compounds (Groarke and Pevear, 1999; Ledford et al., 2004, 2005; Schmidtke et al., 2005). This was further confirmed for the mouse-adapted CVB3 31-1-93 as well as CVB3 PR1 that was selected in vitro under pleconaril pressure in the present study. Each of these two CVB3 contains one of the known resistance-inducing amino acid substitutions, Ile1092 → Leu and Ile1092 → Met, respectively. They were completely pleconaril-resistant in CPE-inhibition and/or plaque-reduction assay. Therefore, they were included in parallel to the known resistant prototype CVB3 Nancy and 3 pleconaril-susceptible CVB3 in SAR analysis with pleconaril analogues containing substitutions in the central phenyl group.

The results obtained with pleconaril as well as [(biphenyloxy)propyl]isoxazole derivatives lacking 3,5-methyl in the central phenyl provide first evidence for the important role of substituents in the central phenyl for anti-CVB3 activity. Whereas the primary compounds (pleconaril, **VIa**, and **VIIa**) with a 3,5-methyl substituted phenoxy group were completely inactive, 3,5-hydrogen derivatives **Va**, **VIb**, and **VIIb** showed a dose-dependent antiviral activity against CVB3 Nancy. The activity of compound **Va** was comparable with that of pleconaril against drug-susceptible CVB3 H3 and CVB3 H310 but markedly reduced against CVB3 97927 (all Ile1092). Possibly, other amino acids outside the hydrophobic pocket belonging to the coxsackie-adenovirus receptor-binding site that were not investigated in the present study cause this reduction in activity. For example, mutations in the canyon or at the rim of the canyon led to lower susceptibility of rhinoviruses against capsid-binding compounds (Heinz et al., 1989).

The relationship found between monosubstitution (compounds **Vb**–**d**) and anti-CVB3 activity is a further proof of the impact of the central ring for inhibition of viral replication. In particular, monosubstitution with methyl or bromine led to an increase of activity towards pleconaril-sensitive as well as -resistant strains in comparison to pleconaril as well as compound **Va**. Compound **Vd** exhibited the strongest antiviral activity. A markedly lower efficacy against pleconaril-resistant CVB3 was found for the derivative substituted with a 3-methoxy group (**Vc**). In comparison to **Va**, **Vb** and **Vd**, its surface area as well as volume is larger and its lipophilicity ($\log P$) lower. After calculation of correlation coefficients for pleconaril and compounds **Va**–**d**, both volume and lipophilicity were identified as important determinants of anti-CVB3 activity in the present study. These data together with the recent demonstration of the crucial role of amino acid 1092 for pleconaril susceptibility (Schmidtke et al., 2005) confirm the hypothesis that amino acid in position 1092 interacts with the central phenyl ring of capsid-binding compounds or may sterical interfere with this ring during binding into the pocket. Previous studies on cocrystal structure of a structurally related capsid-binding compound complexed with CVB3 showed that such drugs bind with the methylisoxazole ring close to the entrance of the pocket in VP1 and the 3-fluoromethyl oxadiazole ring at the end of the pocket and that the phenyl ring is located in the center of the pocket (Muckelbauer et al., 1995). According to this publication amino acids in positions 1092 and 1180 are situated across the pocket with their side chain opposite to each other. A similar binding of a capsid binder into the pocket of HRV14 and HRV16 was described by Zhang et al. (2004).

A Blast analysis with CVB3 amino acids 1061–1120 of the PubMed GenBank revealed a polymorphism at position 1092 of VP1. From the 92 published sequences, 62 sequences contained an isoleucine, 14 leucine and 16 valine. Although most sequences belong to the pleconaril-susceptible genotype, the circulation of naturally resistant isolates underlines the urgent need for new compounds with improved activity. Therefore, the search for more anti-CVB3 active compounds that act similarly or better than the 3-bromine derivatives (**Vb** and **Vd**) against pleconaril-susceptible as well as -resistant CVB3 is needed. The antiviral results obtained with these compounds provide evidence that resistance of CVB3 can be overcome by substitution of the central phenyl group.

Studies on the spectrum of activity revealed new compounds e.g. **Vb** and **Vd** with broad spectrum activity. In comparison to pleconaril the antiviral spectrum of **Vd** was only marginally affected. CVB1–5 as well as 5 of 7 tested rhinoviruses were highly susceptible to **Vd**. In contrast, **Va** was less or not active against all other CVB variants as well as all HRV variants. The results of the present study are in good agreement with that from previous antiviral studies performed with monosubstituted disoxaril derivatives and rhinoviruses (Diana et al., 1993b). They show that in addition to chain length (Andries et al., 1990, 1991) small differences in the structure of the central ring of capsid-binding compounds may have a strong influence on antiviral activity.

SAR analysis with CVB and HRV further revealed serotype-specific activity pattern e.g. for CVB4, CVB6, HRV8, and HRV98 that quite obviously reflect small differences existing in the capsid structure of these viruses. Amino acid substitutions in the hydrophobic pocket as well as in the receptor-binding region of viral capsid proteins were shown to have an effect on susceptibility of rhinoviruses against capsid-binding antiviral compounds (Hadfield et al., 1995; Kim et al., 1993; Ledford et al., 2004, 2005; Oliveira et al., 1993). Because the sequence of the viral capsid proteins of HRV8 and HRV98 used in the present study was not determined, the molecular base for the different drug susceptibility phenotype remains unclear. This also regards CVB4 and CVB6. A CVB4 with low pleconaril susceptibility was described recently by Berg et al. (2007). No data are available concerning the inhibitory activity of pleconaril towards CVB6.

The obtained information offers a new opportunity for improvement of anti-enterovirus activity of capsid-binding compounds by optimizing the central ring of these inhibitors. They also suggest that it may be necessary to use a combination of two ore more capsid-binding compounds or a combination of drugs with different mode of action to prevent and overcome resistance and to treat entero- and rhinovirus infections effectively.

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