Susceptibility of coxsackievirus B3 laboratory strains and clinical isolates to the capsid function inhibitor pleconaril: antiviral studies with virus chimeras demonstrate the crucial role of amino acid 1092 in treatment

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Objectives: At present, most promising compounds to treat enterovirus-induced diseases are broad-spectrum capsid function inhibitors which bind into a hydrophobic pocket in viral capsid protein 1 (VP1). Coxsackievirus B3 (CVB3) Nancy was the only prototypic enterovirus strain shown to be pleconaril-resistant. This study was designed to better understand the polymorphism of the hydrophobic pocket in CVB3 laboratory strains and clinical isolates and its implications for treatment with the capsid function inhibitor pleconaril.

Methods: Pleconaril susceptibility was determined in cytopathic effect-inhibitory, plaque reduction or virus yield assays. Sequence analysis of the genome region coding for VP1 and/or subsequent alignment of amino acids lining the hydrophobic pocket of five CVB3 laboratory strains and 20 clinical isolates were carried out. Virus chimeras and computational analysis were used to prove the role of amino acid 1092.

Results and conclusions: Despite high conservation of pocket amino acids, polymorphism was detected at positions 1092, 1094 and 1180. Neither Pro-1094 → Thr nor Val-1180 → Ile altered efficacy of pleconaril treatment. But the amino acid at position 1092 was strongly associated with susceptibility of CVB3 to the capsid inhibitor. Whereas leucine was involved in resistance, isoleucine and valine were detected in pleconaril-susceptible CVB3. Results from antiviral assays with hybrid viruses demonstrate the crucial role of amino acid 1092 in pleconaril susceptibility. A resistant cDNA-generated CVB3 became pleconaril-susceptible after accepting parts from the genome region encoding Ile-1092 into its capsid. Computational analysis suggests that conformational changes in the hydrophobic pocket occur when leucine is substituted for isoleucine or valine and that this change leads to susceptibility to pleconaril.

Keywords: enterovirus, sequence, capsid inhibitor

Introduction

Coxsackievirus B3 (CVB3) is one of ~70 human serotypes of the genus enterovirus of the Picornaviridae. This virus family includes small, non-enveloped, single-stranded positive-sense RNA viruses. The viral RNA is protected by a capsid. Like other enteroviruses, CVB3 may cause severe acute and chronic diseases, e.g. encephalitis, meningitis, pancreatitis, gastroenteritis, hand, foot and mouth syndrome, respiratory diseases and non-specific febrile illnesses.1–4 Furthermore, it is the most important agent of virus-induced acute and chronic myocarditis.5–7 At present, the treatment of these diseases is primarily symptomatic.8 There is a considerable interest in a broad-spectrum antiviral compound for the treatment of enteroviral disease for two reasons: firstly, because of the large

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number of enterovirus serotypes and secondly, because no single enterovirus serotype is exclusively associated with any particular disease.

Based on the knowledge of virus capsid structure, broad-spectrum antiviral compounds targeting the viral capsid were developed. The structure of the CVB3 capsid has been solved at 3.5 Å resolution by Muckelbauer et al.9 The CVB3 capsid was shown to be very similar to that of other members of the Picornaviridae, e.g. poliovirus and rhinovirus 14,10,11 The icosahedral capsid consists of 60 identical copies each composed of four structural proteins, designated as VP1, VP2, VP3 and VP4. The five-fold axis is surrounded by a large depression, the so-called canyon. At the base of the canyon, a surface-accessible hydrophobic pocket has been found within VP1. This pocket may be occupied by a pocket factor, which is suggested to be a lipid or a fatty acid. According to the hypothesis of Rossmann et al.,12 specific cell surface molecules, the receptors, interact with the canyon floor. Receptor binding induces both the release of the pocket factor and conformational changes in the capsid which again allow the viral RNA to enter host cells. This step of the viral life cycle can be blocked by capsid inhibitors.13,14 Capsid inhibitors may replace the pocket factor, occupy the hydrophobic pocket and prevent conformational changes in the viral capsid necessary for successful virus attachment to cell receptors and/or uncoating of viral RNA. Various capsid-binding agents were developed.15 But complex drug metabolism, serious side effects or a narrow spectrum of activity hindered their clinical use. Pleconaril, a novel capsid function inhibitor, is considered to be a potential broad-spectrum anti-picornavirus agent because it inhibits the replication of most serotypes of rhinoviruses as well as enteroviruses at nanomolar and micromolar concentrations.16,17 CVB3 Nancy was detected as the only pleconaril-resistant prototype enterovirus strain.18 However, the molecular base of this resistance was not studied. Later on, resistant CVB3 were generated from a pleconaril-susceptible wild-type CVB3 grown in the presence of 1 μg/mL of pleconaril in vitro.18 The resistant mutants contain amino acid substitution Ile-1092→Leu or Ile-1092→Met in the hydrophobic pocket in VP1. Therefore, it was suggested that these amino acid substitutions may be responsible for the observed resistance of these viruses. This hypothesis was not proven until now.

This study was conducted to gain a better understanding of the amino acid polymorphism of the hydrophobic pocket of CVB3 laboratory strains and clinical isolates as well as implications of amino acid substitutions within the pocket for antiviral therapy with capsid inhibitors. The nucleotide sequence of the genome region coding for amino acids lining the hydrophobic pocket was determined and compared with virus susceptibility to pleconaril. Antiviral investigations with hybrid viruses were applied to demonstrate the crucial role of amino acid 1092 in pleconaril susceptibility. Additionally, computational modelling was used to study conformational changes within the hydrophobic pocket as the result of amino acid substitutions.

Materials and methods

**Cell lines and viruses**

HeLa cells and primary human fibroblast line H (HuFi16) were grown in Eagle minimal essential medium (MEM) supplemented with 10% neonatal calf serum (NCS), CHO-K1 cells (DSZM no. ACC-10) were maintained with Dulbecco’s minimal essential medium (DMEM) supplemented with 10% FBS. Penicillin (100 U/mL) and 100 μg/mL of streptomycin were added to all three cell lines. The maintenance medium of HeLa cells (human cervix carcinoma; ATCC no: CCL-2) contained 2% NCS.

The laboratory virus strains used in this study were CVB3 Nancy (Institute of Poliomyelitis and Virus Encephalitides, Moscow, Russia), CVB3 M2 (cDNA generated from plasmid pCVB3-M2 consisting of cDNA of CVB3 Nancy20), CVB3 HA (ATCC: VR-688), CVB3 H3,21 CVB3 P16 and CVB3 PD.22 Furthermore, CVB3 Bgl/Spe, CVB3 Bgl/ Sal and CVB3 Sal/Spe (all three cDNA generated from a pCVB3-M2 clones containing selected sequences coding for VP1 of CVB3 PD; see Figure 3) were included to map pleconaril resistance. The 20 CVB3 isolates were collected and kindly provided by Dr W. Melchers (University Nijmegen, Netherlands) and Dr S. Diedrich (National Reference Laboratory for Polio- and Enteroviruses at the Robert Koch Institute, Berlin, Germany). These CVB3 were isolated from patients suffering from encephalitis, meningitis, gas troenteritis, carditis, pneumonia, herpangina, hand, foot and mouth disease, or morbus bornholm between 1970 and 2000. Virus stocks were prepared in monolayers of CHO-K1 cells (CVB3 PD and Bgl/ Spe), HuFi H (CVB3 P) or HeLa cells (all other laboratory and clinical CVB3). Viral titers were determined on HeLa cell monolayers by end-point titration. Aliquots were stored at −20°C until use.

**Pleconaril susceptibility testing**

The antiviral activity of pleconaril against the CVB3 laboratory strains was determined on confluent HeLa cells using a cytopathic effect (CPE)-inhibitory assay described previously for CVB3 Nancy.24 Briefly, the tests were carried out in 2-day-old confluent HeLa cell monolayers growing in 96-well flat-bottomed microtitre plates (Falcon 3099). After removal of culture medium, 50 μL of twofold pleconaril dilutions and 50 μL of CVB3 suspension containing a certain multiplicity of infection (moi) that guarantees a complete CPE after 24–48 h 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. Using a Crystal Violet uptake assay, the inhibition of viral CPE was scored from 24 to 48 h post-infection when untreated infected control cells showed maximum cytopathic effect. The 50% inhibitory concentration (IC50) of pleconaril was determined from the mean dose–response curves of at least three separate experiments. Two separate experiments were performed with pleconaril-resistant CVB3.

Virus yield reduction assays were performed with CVB3 P and CVB PD in confluent HuFi H monolayers propagated in four-well plates (Greiner, Frickenhausen, Germany). After the cell culture medium was reduced, three wells in each plate were infected with the respective virus at an moi of 1 in the absence (virus control plates) or presence of pleconaril (final concentration in the plate 1 μg/mL). One mock-infected, untreated well was used as cell control on each plate. Following an incubation time of 4 h at 37°C, the supernatant consisting of non-adsorbed virus was removed, the cells were washed three times with maintenance medium and 1 mL of fresh medium without (cell and virus controls) or with pleconaril (1 μg/mL) was added to the wells. Four, 24, 48 and 72 h after virus inoculation, virus was extracted by triple freezing and thawing. The virus yield of untreated and pleconaril-treated wells was determined by end-point titration on HeLa cells. Two independent experiments were performed each with three parallels.

The susceptibility of CVB3 Nancy and clinical isolates was checked with plaque reduction assays. Confluent HeLa cells grown in 12-well plates were inoculated with 0.5 mL of the respective virus suspension in maintenance medium containing between 30 and 60 pfu in the
absence or presence of serial two-fold dilutions of pleconaril. Each compound concentration was tested in duplicate. One uninfected untreated cell control and three untreated virus controls were included per assay. After virus adsorption at 37°C for 1 h, the inoculum was aspirated and the cell monolayers were overlaid with 1 mL of maintenance medium containing 0.4% agar. Following 2–4 days of incubation at 37°C, the plates were fixed and stained with a Crystal Violet formalin solution overnight. Plaques were counted over a light box after removal of the agar overlay and used to calculate the compound-induced plaque reduction. The IC_{50} was calculated from the mean dose–response curve of at least three independent plaque reduction assays.

**RNA isolation**

Total RNA was extracted from HeLa cells infected with clinical CVB3 isolates using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was eluted from the spin column in a total volume of 30 or 50 μL of RNase-free water and stored frozen at –80°C.

**RT–PCR of the hydrophobic pocket-encoding region of VP1 of clinical CVB3 isolates**

The region of viral RNA encoding the complete VP1 was amplified by RT–PCR. RT was conducted with Omniscript RT (Qiagen, Hilden, Germany), a CVB3-specific primer pool containing primers designed for PCR and sequencing, and 3 μg of RNA in a final reaction volume of 20 μL following the manufacturer’s instructions. Two microlitres of the RT reaction was subjected to PCR amplification using Qbiogene molecular biology TaqDNAPol (Heidelberg, Germany) according to the manufacturer’s protocol. The primer pairs utilized for PCR were 5'-ACA TCA GAC GAT CCA TC-3' and 5'-ACG TGT GAC CAG CAG-3', 5'-GGA AGA CGC GAT AAC AGC CGC and 5'-GTC TAG TGA TCC ACC CTT-3', 5'-TGT CCA TYC CRT TTT TGA G-3' or 5'-ATG TCC ATA CCR CTT TT-3' and 5'-CTG TTG TAR TCC CAC AC-3' (20 μmol). The PCR cycling conditions were as follows: 1 cycle of 93°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 50 s and 72°C for 2 min, and a final cycle of 72°C for 20 min, followed by holding at 10°C.

Because in some cases multiple bands were observed when the amplification products were subjected to agarose gel electrophoresis, PCR products of the expected molecular mass were gel-extracted using QIAquick or PCR purification kit (Qiagen, Hilden, Germany) and eluted in 30–50 μL of elution buffer. Purified products were stored at –20°C.

**Sequencing and analysis of RT–PCR amplicons**

Sequencing was performed with fluorescent-labelled nucleotides on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Darmstadt, Germany). Sequencing primers included those used in RT–PCR as well as those derived from genome walking (5'-ATT ACA TGT TGG TAY CAA AC-3', 5'-GGC CCA GTG GAA GAC GCC A-3', 5'-TGY RCK GCT TGG CGW KG T-3', 5'-GAT CAA ACC TCA TGG ATG TGA-3'), each 5 μmol, where K = G and T, R = A and G, W = A and T, and Y = C and T. Twenty-five cycles of 95°C for 20 s, 50°C for 15 s and 60°C for 1 min were run. To confirm each nucleotide assignment, sequencing of both strands of cDNA was performed using the sequencing mix DYEamatic ET terminator kit obtained from Amersham Biosciences (Freiburg, Germany). The GenBank accession numbers are DQ093617 to DQ093636.

**Computational modelling**

The alignment of three neighbouring amino acid residues at positions 1091–1093 of VP1 was predicted using the computer (2400 MHz) based Molecular Modelling System HyperChem 7.01 (Hypercube, Inc., http://www.hyper.com). Additionally, Modified Neglect of Diatomic Overlap (MNDO), a method for semi-empirical quantum mechanics calculations, was used as a semi-empirical method of optimization. MNDO calculates electronic properties, optimized geometries, total energy and heat of formation.

**Results**

**Amino acid polymorphism within the hydrophobic pocket of CVB3 laboratory strains and clinical isolates**

The hydrophobic pocket of enteroviruses plays an important role in receptor-mediated attachment and uncoating of enteroviruses. The high degree of amino acid conservation of this pocket allowed the development of capsid inhibitors such as pleconaril which integrate into this pocket thereby preventing viral attachment and/or uncoating of a broad spectrum of enterovirus serotypes. Because the enterovirus prototype CVB3 Nancy was pleconaril-resistant and resistant CVB3 mutants were also detected in response to drug selection pressure *in vitro*, concern was raised about the amino acid polymorphism of the hydrophobic pocket in VP1 in CVB3 laboratory strains and clinical isolates. The amino acid residues chosen for comparative analysis are those as determined by X-ray crystallography in CVB3.

Prior to this study, amino acid sequences of VP1 of ~10 CVB3 laboratory strains were published. Alignment of amino acid residues in the hydrophobic pocket of the five CVB3 laboratory strains used in this study is shown for example in Table 1. Despite a high degree of amino acid identity, most of these strains possess a leucine at position 1092 of VP1. Because the enterovirus prototype CVB3 Nancy was pleconaril-resistant and resistant CVB3 mutants were also detected in response to drug selection pressure *in vitro*, concern was raised about the amino acid polymorphism of the hydrophobic pocket in VP1 in CVB3 laboratory strains and clinical isolates. The amino acid residues chosen for comparative analysis are those as determined by X-ray crystallography in CVB3.

Additionally, all strains except CVB3 H3 have Val-1180→Ile.

In this study, sequence data of the VP1 genome region encoding the amino acids within the hydrophobic pocket were generated for 20 CVB3 isolates. Alignment of the amino acid residues of these isolates is summarized in Table 1. The data demonstrate strong amino acid conservation within the hydrophobic pocket. The amino acid substitution Ile-1092→Val was detected in one of the 20 clinical CVB3 isolates. Furthermore, the amino acid substitution Pro-1094→Thr was found in all except one (CVB3 99-1000) clinical virus isolate. With the exception of CVB3 H3 which was used in X-ray crystallography by Muckelbauer et al., all CVB3 laboratory strains as well as all clinical CVB3 isolates have the same amino acid at position 1180: Ile-1180→Val.

**Susceptibility of CVB3 laboratory strains to pleconaril**

Studying pleconaril-resistant CVB3 mutants isolated from cell culture under drug selection pressure, Leu-1092 and Met-1092 have been associated with a drug-resistant phenotype. The occurrence of Leu-1092 in most CVB3 laboratory strains (Table 1) suggested a high incidence of resistance. In order to examine the impact of the amino acid residue at position 1092 on pleconaril susceptibility, CPE-inhibitory assays were performed with CVB3 M2, CVB3 HA, CVB3 P and CVB3 PD using non-cytotoxic concentrations of pleconaril on HeLa cells. Because CPE-inhibitory assays were
also applied in the initial studies to determine the activity of pleconaril against enteroviruses, the results of this study obtained with pleconaril-resistant and pleconaril-susceptible control viruses (CVB3 Nancy and CVB3 H3, respectively) are directly comparable to those obtained by Pevear et al.17 The 50% cytotoxic concentration of pleconaril on HeLa cells, determined in our laboratory, amounts to 12.6 μg/mL and was described recently.28 The results shown in Figure 1(a) indicate that all CVB3 laboratory strains containing Leu-1092 were pleconaril-resistant like CVB3 Nancy. The resistant phenotype of CVB3 Nancy was also confirmed in plaque reduction assays (n = 4; results not shown). In addition to CVB3 H3, only CVB3 PD was pleconaril-susceptible (Figure 1b). Like CVB3 H3, CVB3 PD has an isoleucine at position 1092.

Because CPE inhibition does not necessarily mean a reduction in infectious virus, virus yield reduction assays were performed with CVB3 P and CVB PD in diploid human fibroblasts. As shown in Figure 1(c), the virus yield of CVB3 P-infected pleconaril-treated HuFi was only slightly reduced in comparison with the untreated virus control. In contrast, replication of CVB3 PD was nearly completely aborted after treatment with pleconaril. Neither 24 nor 48 nor 72 h after virus inoculation has a rise in viral titres been found in pleconaril-treated, CVB3 PD-infected HuFi whereas the virus yield of untreated virus control increased about four logarithmic steps (Figure 1d). These results confirm the resistant phenotype of CVB3 P as well as pleconaril susceptibility of CVB3 PD found in CPE-inhibitory assays.

### Relationship between pocket amino acid sequence and drug susceptibility of clinical CVB3 isolates

Pleconaril susceptibility of clinical CVB3 isolates was studied by plaque reduction assays in HeLa cells. Based on the mean dose–plaque reduction curves of three separate experiments, the IC50 values were calculated for individual CVB3 isolates (Figure 2). Pleconaril inhibited the virus-induced plaque production of clinical CVB3 isolates by 50% in a concentration range of 13.0–67.4 ng/mL. The mean IC50 calculated from the individual values of all 20 isolates amounted to 38.4–14.7 ng/mL. All of the studied clinical CVB3 isolates were pleconaril-susceptible. The results indicate that none of the observed amino acid substitutions at positions 1092, 1094 and 1180 induced resistance (Table 1).

### Evidence for the crucial role of the amino acid at position 1092

To demonstrate the impact of amino acid 1092 on pleconaril susceptibility, several virus chimeras were used (Figure 3). These viruses were originally generated to study the molecular base of

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**Table 1. Alignment of amino acids lining the β-sandwich pocket of VP1 according to Muckelbauer et al.**

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Sequences of CVB3 laboratory strains used in this study were published by: aKnowlton et al.,21 bKlump et al.25 and cSchmidtke et al.23
the extended cell tropism of CVB3 PD. In addition to amino acid substitutions at the surface of the viral capsid allowing the use of heparan sulphates as additional receptors, CVB3 PD also contains the pocket amino acid Ile-1092. Ile-1092 was suggested to mediate pleconaril susceptibility. All other amino acids lining the hydrophobic pocket of CVB3 PD were completely the same as found in the pleconaril-resistant CVB3 Nancy (Table 1). Three different subgenomic regions of CVB3 PD which included residue

![Figure 1](image1.png)

**Figure 1.** Pleconaril susceptibility of CVB3 laboratory strains. Antiviral activity of pleconaril was determined in CPE-inhibitory assays in HeLa cells (a and b). The mean dose–response curves with SD of resistant viruses were calculated from two (a) and that of susceptible viruses from at least three separate CPE-inhibitory assays in HeLa cells (b). To confirm the pleconaril-resistant phenotype of CVB3 P (c) and the pleconaril-susceptible phenotype of CVB3 PD (d), virus yield assays were performed in HuFi infected at an moi of 1 of respective CVB3. At 4, 24, 48 and 72 h after virus infection, the plates were frozen and thawed three times. Virus yield was determined by end-point titration in HeLa cells. The results are expressed as 50% tissue culture infection dose per 50 µL of virus suspension (TCID50/50 µL). Mean virus yields ± SD of two independent experiments are shown each with three parallels.

![Figure 2](image2.png)

**Figure 2.** Pleconaril susceptibility of clinical CVB3 isolates. The antiviral activity of pleconaril was determined by plaque reduction assays in HeLa cell monolayers grown in 12-well plates. Two-fold dilutions of non-cytotoxic pleconaril concentrations were added to cells during virus adsorption and in the agar overlay. Then, 2–4 days after infection, assays were fixed and stained with Crystal Violet solution, the plaques were counted, and the plaque reduction in compound-treated wells was calculated in comparison with untreated virus-infected wells. Based on the mean dose–response curve of at least three separate experiments, the IC50 values were calculated.

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<th>Clinical CVB3 isolates</th>
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Crucial role of amino acid 1092 in anti-CVB3 activity of pleconaril

Ile-1092 (CVB3 Bgl/Spec and CVB3 Bgl/Sal) or not (CVB3 Sal/Spe) were amplified, digested with the appropriate restriction enzymes, and ligated to the plasmid pCVB3-M2. This plasmid contains the genome encoding CVB3 Nancy. As shown in Figures 1(a) and 3, infectious viruses generated from pCVB3-M2 (Leu-1092) are completely pleconaril-resistant up to a maximum test concentration of 8 \( \mu \text{g/mL} \) like CVB3 Nancy. After replacing the subgenomic regions of the resistant CVB3 M2 encoding Leu-1092 with the respective regions of CVB3 PD encoding Ile-1092 (CVB3 Bgl/Spec and Bgl/Sal) but not when the region corresponding to the CVB3 PD Sal/Spe fragment encoding Leu-1092 like CVB3 M2 was replaced, the replication of the virus chimera was inhibited by the compound as efficiently as the pleconaril-susceptible CVB3 PD. The IC\( _{50} \) values determined for CVB PD, CVB3 Bgl/Spec and CVB3 Bgl/Sal were 0.038, 0.038 and 0.014 \( \mu \text{g/mL} \), respectively. In contrast, inhibition of CVB3 Sal/Spec-induced CPE was not achieved albeit using maximum test concentrations of pleconaril of 8 \( \mu \text{g/mL} \).

Possible changes in the canyon structure after substitution of amino acid 1092

The results obtained with virus chimeras in antiviral assays demonstrate that pleconaril susceptibility of CVB3 depends on one single amino acid in the viral hydrophobic pocket. All of the detected pleconaril-resistant CVB3 contain a leucine at position 1092 of VP1. In contrast, pleconaril-susceptible CVB3 possess an isoleucine or a valine at position 1092. Until now, no atomic structure is available for CVB3 possessing a leucine or valine at position 1092 in VP1. The structure of isoleucine, leucine and valine is very similar. Isoleucine, leucine and valine are non-polar, hydrophobic amino acids differing in the number of hydrocarbons of side chains (four in isoleucine and leucine; three in valine) or in the arrangement of these hydrocarbons (Figure 4a). These structural data suggest that differences in size of hydrophobic side chains alone do not explain pleconaril resistance. To check the hypothesis that the amino acid residue at position 1092 may have an effect on the conformation of the canyon, three-dimensional (3D) conformation modelling of isolated tri-peptides consisting of amino acids 1091–1093 was applied. The proposed computer model considers the conformation of the tri-peptides Val–Ile–Thr, Val–Leu–Thr and Val–Ile–Thr in isolation from the context of the surrounding capsid residues. In this manner, it was possible to show how a minor variation like a single amino acid substitution can change the peptide conformation in a specific position of the hydrophobic pocket. The obtained biological data together with the detected high conservation of the amino acid sequence suggest that only very small changes of the whole peptide conformation may occur, because otherwise the function of the hydrophobic pocket would be destroyed. Surprisingly, 3D images of Val–Ile–Thr and
Val–Val–Thr possess a very similar conformation whereas the introduction of leucine at position 1092 markedly altered the arrangement of the amino acid residues of the peptide Val–Leu–Thr (Figure 4b). To emphasize the difference in amino acid composition, two 3D images, Val–Ile–Thr and Val–Leu–Thr as well as Val–Ile–Thr and Val–Val–Thr, were laid on top of each other to emphasize the difference in amino acid composition. Valine as well as isoleucine contains two alkyl substitutes (methyl and methyl or methyl and ethyl, respectively) at the third carbon atom. As shown in Figure 4(b and c), the presence of a second alkyl substitute at C(3) of the central amino acid residue results in a conformation problem for oxygen of the respective amino acid. This oxygen atom contacts the oxygen of the first amino acid residue which is valine. In contrast to valine as well as isoleucine, leucine has only one alkyl substitute (isopropyl) at the third carbon atom. Because sterical inhibition of the oxygen of leucine due to a second alkyl substitute does not exist, oxygen may remove itself from the oxygen of valine in Val–Leu–Thr. This induces alterations in the conformation of the whole molecule studied. These results indicate that an exchange of isoleucine or valine for leucine may affect the structure of this part of the hydrophobic pocket and that may hinder pleconaril binding.

Discussion

CVB3 is one of about 70 distinct human enterovirus serotypes causing a broad spectrum of human diseases. Because no single enterovirus serotype is exclusively associated with any particular disease, diagnostics as a prerequisite of successful treatment were difficult for a long time. During the last 10 years, PCR methods were developed for group-specific diagnosis of enterovirus infections.30–32 This rapid group-specific diagnosis now enables the use of broad-spectrum anti-enteroviral agents for treatment. Our present knowledge suggests the capsid inhibitor pleconaril as the most promising candidate. This compound integrates into a hydrophobic pocket in VP1 and blocks viral attachment and/or uncoating. Pleconaril inhibited 14 of 15 tested prototypic enterovirus strains as well as 214 of 215 tested clinical enterovirus isolates in vitro.17 The only detected resistant prototype enterovirus was coxsackievirus B3 Nancy. In addition, 10 drug-resistant CVB3 mutants were generated from pleconaril-susceptible wild-type CVB3 under pleconaril treatment by plaque isolation.18 Because all these resistant mutants possess an amino acid substitution Ile-1092 →Leu or Ile-1092 →Met in the hydrophobic pocket of VP1, it was suggested that this amino acid position may be critical to the development of drug resistance. This study reports: (i) the intraserotypic amino acid polymorphism of the hydrophobic pocket of 20 clinical isolates in comparison with already known amino acid sequences of CVB3 laboratory strains; (ii) insight into the range of pleconaril susceptibility of these viruses; and (iii) evidence for the crucial role of a single amino acid in pleconaril treatment. Moreover, results from computational modelling suggest conformational changes within the hydrophobic pocket are the result of this amino acid substitution.

Taken together, there is a high degree of amino acid identity within the hydrophobic pocket of the CVB3 studied (Table 1). Of the 18 amino acid positions, 15 are identical. Val-1180 →Ile was found in all studied CVB3 except CVB H3. Besides CVB3 99-1000, all clinical CVB3 isolates possess Thr-1094 instead of Pro-1094. Leu-1092 but not Met-1092 was found in CVB3 laboratory strains but not in clinical isolates in this study. In addition, CVB3 99-1000 contained a valine at that position. Only Leu-1092, observed in CVB3 Nancy, M2, HA and P, was correlated with resistance to pleconaril (Figure 1a and c). Because these viruses were never treated with pleconaril before, they were called naturally pleconaril-resistant. In contrast, replication of CVB3 containing Ile-1092 or Val-1092 (CVB3 H3, CVB3 PD and all clinical isolates) was strongly inhibited by the drug (Figures 1b and d and 2). The resistant phenotype of CVB3 Nancy as well as susceptibility of CVB3 H3 described previously by Groarke and...
Crucial role of amino acid 1092 in anti-CVB3 activity of pleconaril

Pevear18 were confirmed: IC₅₀ values correspond to previously published data.17 No natural pleconaril-resistant clinical CVB3 isolates were detected. However, neither the existence of resistant strains nor their emergence as the result of drug selection pressure can be excluded. Studying sequences submitted to the NCBI GenBank, we found one recent CVB3 isolate from China (AAD50446) possessing Leu-1092. The majority of the published sequences (more than 20) contained Ile-1092. Several CVB3 isolates possess Val-1092. During clinical studies designed to examine the safety and efficacy of pleconaril for treatment of upper-respiratory-tract disease of picornavirus aetiology, post-baseline non-susceptible rhinoviruses were isolated from 2.7% of treated patients.23 Amino acid substitutions of resistant viruses isolated from pleconaril-treated patients of these clinical studies were different from those found in natural drug-resistant rhinoviruses.34 In CVB3, drug selection pressure resulted in the emergence of pleconaril-resistant variants containing the same amino acid substitutions18 as those observed in drug-resistant CVB3 (Nancy, M2, HA, P) of this study which were never treated with pleconaril before.

When assessed in a murine model in which wild-type infection is lethal, pleconaril-resistant CVB3 that emerged under pleconaril treatment were attenuated and significantly less virulent than drug-susceptible wild-type virus.18 These results indicate that virus–receptor interactions and subsequent viral attachment and entry are also affected by those amino acid substitutions. However, results from our previous studies on replication of CVB3 laboratory strains in cell lines with determined receptor expression indicate no differences between CVB3 laboratory strains containing Ile-1092 or Leu-1092 in regard to CAR-mediated infection (where CAR stands for coxsackievirus-adenovirus receptor) and virulence in vitro.23,29 Moreover, CVB3 with Leu-109223 as well as CVB3 with Ile-1092 (ref. 21 and M. Schmidtke, unpublished data) were successfully used to establish animal models of acute and chronic myocardiitis. Obviously, amino acid variation at position 1092 is not necessarily associated with attenuated virulence in vitro or in vivo. Possibly, other mutations in the 5’ non-coding region or in other virus protein coding regions that were not examined in the respective studies have a potential role in the observed phenotypes.

According to Muckelbauer et al.,9 amino acid residues 1092 and 1180 are situated across the pocket from each other with their side chains extending into the pocket and both are near the substituted phenyl ring of capsid binding compounds. These authors suggested that the change from isoleucine to leucine at 1092, in addition to Val-1180→Leu, may be responsible for the inability of those CVB3 to bind capsid blocking compounds. In this study, hybrid viruses constructed on the genetic background of the pleconaril-resistant CVB3 Nancy (Leu-1092 and Ile-1180) were used to prove the impact of these amino acids on pleconaril susceptibility (Figure 3). Three different genome regions of CVB3 PD coding for parts of VP1 containing Ile-1092 (CVB3 BglSpe and BglSal) or not (CVB3 Sal/Spe) in addition to Ile-1180 (all) were transferred into a plasmid coding for CVB3 Nancy. Viruses obtained after transfection of HeLa cells with the respective plasmids were tested for drug susceptibility. Only the two hybrid viruses containing Ile-1092 resulted in the susceptible phenotype. The CVB3 PD Bgl/Sal fragment is the minimal sequence that results in the transfer of susceptibility to pleconaril to the M2 strain. In addition to Ile-1092, it contains four other mutations (3237, 1078, 1080 and 1091) outside of the binding pocket. These are unique for CVB3 PD (1078, 1080 and 1091) or found also in CVB3 H3 and some of the isolates (3237). The presence or absence of these amino acid substitutions did not correlate with drug susceptibility of CVB3 laboratory strains and isolates. Therefore, a potential role of the other mutations in the observed phenotype can be discarded. Taken together, these data demonstrate that a single amino acid substitution at position 1092 is indeed associated with susceptibility or resistance of CVB3 to pleconaril.

Surprisingly, the substitution of non-polar isoleucine by non-polar leucine but not by non-polar valine induces pleconaril resistance. Both isoleucine and leucine have four hydrocarbon side chains. The difference between isoleucine and leucine is the arrangement of these hydrocarbon chains. As shown by 3D conformation modelling of the amino acid at position 1092 together with its neighbouring amino acids, this small difference has the potential to alter the arrangement of amino acids of peptides as shown for Val–Ile–Thr and Val–Leu–Thr (Figure 4b and c). The results obtained with valine confirm this conclusion. Valine contains three hydrocarbon side chains but their arrangement is comparable to that of isoleucine (Figure 4a). The 3D conformation of Val–Val–Thr was very similar to that of Val–Ile–Thr (Figure 4b and c). These conformational changes may affect the binding of pleconaril in the pocket. However, the atomic structure of CVB3 is only known for a strain containing Ile-1092 at present.9 The correlation between a single amino acid exchange at position 1092 of the hydrophobic pocket and anti-CVB3 activity of pleconaril together with the hypothesis about the structural changes at this position obtained from computational modelling help us to understand the molecular basis of anti-CVB3 activity as well as drug resistance of capsid function inhibitors. Based on these results, new anti-CVB3 active compounds will be designed, synthesized and tested.

In summary, data reported here contribute to the CVB3 database and provide new insight into the polymorphism of the hydrophobic pocket of these viruses. The observed polymorphism can play an important role in binding of capsid inhibitors in the pocket and by this way in treatment. Moreover, these data provide evidence that the amino acid at position 1092 but not at position 1094 or 1180 can predict the susceptibility of pleconaril and could form the basis of rapid resistance monitoring by genotyping.

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References


