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Molecular mechanism of a specific capsid binder resistance caused by mutations outside the binding pocket

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ABSTRACT

Enteroviruses cause various acute and chronic diseases. The most promising therapeutics for these infections are capsid-binding molecules. These can act against a broad spectrum of enteroviruses, but emerging resistant virus variants threaten their efficacy. All known enterovirus variants with high-level resistance toward capsid-binding molecules have mutations of residues directly involved in the formation of the hydrophobic binding site. This is a first report of substitutions outside the binding pocket causing this type of drug resistance: I1207K and I1207R of the viral capsid protein 1 of coxsackievirus B3. Both substitutions completely abolish the antiviral activity of pleconaril (a capsid-binding molecule) but do not affect viral replication rates *in vitro*. Molecular dynamics simulations indicate that the resistance mechanism is mediated by a conformational rearrangement of R1095, which is a neighboring residue of 1207 located at the heel of the binding pocket. These insights provide a basis for the design of resistance-breaking inhibitors.

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1. Introduction

Echoviruses, coxsackie A and B viruses, enteroviruses and rhinoviruses belong to the genus enterovirus (EV) of the *picornaviridae* family (Rollinger and Schmidtke, 2011). They cause a wide range of acute and chronic diseases such as respiratory infections, meningitis, pancreatitis, encephalitis, and myocarditis (Pallansch and Roos, 2007; Turner and Couch, 2007). As of today no therapeutics have been approved for the treatment of these infections (De Palma et al., 2008; Rollinger and Schmidtke, 2011). Several capsid-binding molecules (CIs) are being investigated as promising drug candidates (Andries et al., 1991; Diana, 2003; Makarov et al., 2005; Watson et al., 2003), the most developed ones being pleconaril (Fig. 1) and vapendavir (Diana et al., 1995; Feil et al., 2012).

The architecture of the viral capsid is conserved among enteroviruses (Rossmann et al., 2002), which provides the basis for the design of broad-spectrum CIs (Ledford et al., 2005; Pevear et al., 1999; Schmidtke et al., 2005; Tijsma et al., 2014). The viral capsid consists of 60 protein subunits (Racaniello, 2007), each of them composed of four viral capsid proteins, VP1–4. In VP1, a hydrophobic pocket is present which in most EVs is occupied by a fatty acid. This pocket factor stabilizes the capsid and is released during the viral attachment, thereby facilitating viral uncoating (Rossmann et al., 2002). CIs are known to bind to this hydrophobic pocket and trigger conformational rearrangements in the viral capsid (Grant et al., 1994; Kim et al., 1993; Muckelbauer et al., 1995a; Zhang et al., 2004). Subsequently, attachment of viruses to host cells and/or uncoating is blocked (Diana et al., 1989; Pevear et al., 1989). Studies also suggest that drug integration during assembly additionally contributes to the antiviral activity of CIs (Zhang et al., 2004).

Abbreviations: EV, enterovirus; CI, capsid-binding inhibitors; VP1–4, viral capsid protein 1–4; CVB3, coxsackievirus B3; HPLC, high performance liquid chromatography; MD, molecular dynamics; moi, multiplicity of infection; PME, particle mesh Ewald; RMSD, root-mean-square deviation; RNA, ribonucleic acid; RT, reverse transcriptase; TLC, thin layer chromatography; vdW, van der Waals; VP, viral protein; wt, wild type.

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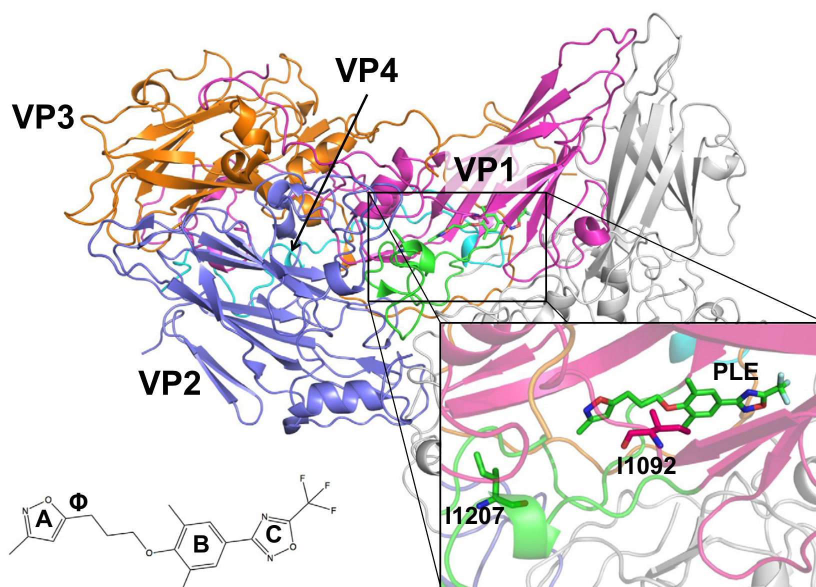

Fig. 1. Overview of the two protomers of the CVB3 97927 virus capsid as used for the MD simulations. VP1 (purple), VP2 (blue), VP3 (orange), VP4 (cyan). The GH loop and pleconaril are green and the second protomer grey. The enlarged section shows VP1 with the location of pleconaril, I1092 and I1207. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Due to the high mutation rates of RNA viruses (Domingo, 1989; Drake et al., 1998) emerging drug resistance poses a threat to efficacy of CIs. All high-level resistance (Heinz et al., 1989) to CIs reported to date involve residues forming the hydrophobic pocket of EVs (Badger et al., 1989; Benschop et al., 2015; Groarke and Pevear, 1999; Ledford et al., 2005, 2004; Schmidtke et al., 2005), hence directly interfering with the binding of CIs. Because of similarities in the binding mode of CIs, cross-resistance may be observed, as reported for pleconaril and vapendavir (Feil et al., 2012).

Here, mutations conferring high-level resistance were further investigated using a clinical coxsackievirus B3 (CVB3) in combination with pleconaril. For the first time substitutions of an amino acid outside the hydrophobic pocket targeted by CIs were shown to cause high-level resistance. A hypothesis of the underlying molecular mechanism was derived from molecular dynamics (MD) simulations.

2. Materials and methods

2.1. Synthesis and chemical analysis

All chemicals and solvents were purchased from Sigma–Aldrich or Alfa Aesar. Pleconaril was synthesized from commercially available starting materials following a previously reported procedure (Diana et al., 1995). The full protocols are provided in the Supporting information.

2.2. Viruses and cells

Virus stock of clinical CVB3 isolate 97927 (CVB3 97927; Robert Koch Institute, Berlin, Germany) was prepared in HeLa Ohio cells (HeLa cell; FlowLabs, USA) and sequenced previously (Schmidtke et al., 2005).

HeLa cells were grown in Eagles minimal essential medium (Lonza, Walkersville, USA) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 100 U/ml penicillin, and 100 U/ml streptomycin (Lonza, Walkersville, USA). The test medium contained only 2% serum.

2.3. Isolation of pleconaril-resistant CVB3 variants

Nine independently prepared pools of wildtype CVB3 97927 (wt-CVB3 97927) were incubated with 1 μg/ml of pleconaril in test medium for 1 h at 37 °C as described previously (Groarke and Pevear, 1999). An untreated virus pool served as control. Then, serial tenfold dilutions of all pools were added to confluent HeLa cell monolayers and overlaid with agar containing 1 μg/ml of pleconaril. After further 48 h of incubation at 37 °C one plaque from each pool was picked. It was further plaque-to-plaque purified for two rounds in the presence of 1 μg/ml pleconaril and propagated in HeLa cells to generate virus stocks.

2.4. Drug susceptibility testing

Plaque reduction assays (with approximately 30–40 plaque-forming units of wt-CVB3 97927 or its variants and up to 8.0 μg/ml of pleconaril) and cytopathic effect (CPE) inhibition assays were performed in HeLa cell monolayers as described previously (Schmidtke et al., 2001).

Additionally, viral protein expression was analyzed in HeLa cells that were infected with wt-CVB3 97927 and its variants (multiplicity of infection (moi) of 10 pfu/cell) in absence or presence of 1 μg/ml pleconaril for 5 h. After fixation, CVB3 antigen was detected with a monoclonal antibody (mAK948, CHEMICON, USA) and the DAKO Real Detection System APAAP Mouse (DAKO, Glostrup, Denmark) as described previously (Zautner et al., 2006).

2.5. RNA isolation, RT-PCR, and sequencing of the capsid protein-encoding region P1

RNA-isolation and RT-PCR of the P1 encoding region of CVB3 97927 variants was performed as described previously (Schmidtke et al., 2005) using primer pairs summarized in Table S1. PCR was carried out with the Taq Core Kit 10 (MP Biomedicals, formerly Qbiogene, France): 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 1 or 3 min, and a final cycle of 72 °C for 20 min.

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