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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.

Cover Letter

AVR-D-15-00087
Antiviral Research

Dear Professor Vasudevan,

please find enclosed the revised version of our manuscript "***Molecular mechanism of a specific capsid binder resistance caused by mutations outside the binding pocket***".

We thank both reviewers for the very constructive comments, which helped us to substantially improve the manuscript. In this revised version we believe that we were able to address all comments and concerns raised by the Reviewers.

Below you will find a point-by-point response to the Editor's and Reviewer's comments.

We hope that you will enjoy this revised version of our manuscript and look forward to your reply.

Kind regards,
Michaela Schmidtke

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- Figure 2 (appears before Figure 1).

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The same moi (10 PFU/cell) for the different viruses was used to infect HeLa cell cultures. It appears that the virus detected at 1 h post-infection differs (more than 1 log) among the viral strains? Were the cultures used at different stages of confluence?

This assumption is incorrect. To confirm this, we added Table S2 in the Supplementary Information that indicates for the wildtype virus as well as all three resistant variants the determined virus titers. As suggested by the reviewer (see answer below), we also performed a statistical analysis (Mann-Whitney-U-test; last sentence in 2.6). No statistically significant differences could be found. We have added a statement to the manuscript text: “Furthermore, no significant difference was detected with the Mann-Whitney-U test, comparing single-step growth curves of wt-CVB3 97927 and high-level resistant variants in HeLa cells (Figure 2B; Table S2).”

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***Response to Reviewers**

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

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- Substitution of I1207 by a more bulky, positively charged residue pushes neighboring R1095 into the hydrophobic pocket, ultimately leading to the abolishment of drug action.

*Manuscript with track changes

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

Heike Braun[a, b#], Johannes Kirchmair[c,d#], Mark J. Williamson[c], Vadim A. Makarov[e], Olga B. Riabova[e], Robert C. Glen[c], Andreas Sauerbrei[a] and Michaela Schmidtke[a*]

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[*] Corresponding author

[#] These authors contributed equally to this work.

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EV, enterovirus; CI, capsid-binding inhibitors; VP1-4, viral capsid protein 1, 2, 3, 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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Abstract

Enteroviruses cause various acute and chronic diseases. ~~Capsid~~The most promising therapeutics for these infections are capsid-binding ~~inhibitors acting~~molecules. These can act against a broad spectrum of enteroviruses~~ represent most promising drug candidates for treatment of these infections today~~, but emerging ~~drug~~ resistant virus variants threaten their ~~therapeutic~~ efficacy. All ~~high resistance~~ known enterovirus variants ~~characterized so far show~~with high-level resistance toward capsid-binding molecules have mutations of residues directly involved in the formation of the hydrophobic ~~drug~~ binding ~~pocket. Here we~~site. This is a first report ~~two new high~~ of substitutions outside the binding pocket causing this type of drug resistance~~ conferring amino acid substitutions in position 1207 (~~: I1207K~~, and~~ I1207R~~)~~ of the viral capsid protein 1 of coxsackievirus B3. Both substitutions completely abolish the antiviral activity of pleconaril (a capsid-binding ~~inhibitor) without affecting virus~~molecule) but do not affect viral replication rates *in vitro*. ~~Importantly, residue 1207 is not part of the drug binding pocket and hence not in direct interaction with pleconaril. Using molecular~~Molecular dynamics simulations ~~a~~ indicate that the resistance mechanism ~~driven~~is mediated by a conformational rearrangement of R1095~~ (~~, which is a neighboring residue of 1207~~)~~ located at the heel of the ~~drug~~ binding pocket~~, was derived~~. These insights ~~on the likely drug resistance mechanism might~~ provide a basis for the design of ~~novel~~ 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 family *picornaviridae* family (Rollinger and Schmidtke, 2011)(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).(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)(De Palma et al., 2008; Rollinger and Schmidtke, 2011). Several capsid-binding inhibitorsmolecules (CIs) are being investigated as promising drug candidates (Andries et al., 1991; Diana, 2003; Makarov et al., 2005; Watson et al., 2003)(Andries et al., 1991; Diana, 2003; Makarov et al., 2005; Watson et al., 2003), the most developed ones being pleconaril and vapendavir (Diana et al., 1995; Feil et al., 2012)(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 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)(Ledford et al., 2005; Pevear et al., 1999; Schmidtke et al., 2005; Tijsma et al., 2014). The viral capsid consists of 60 promoters (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 . 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)~~(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)~~(Diana et al., 1989; Pevear et al., 1989). ~~Study results~~Studies also suggest that drug integration during assembly additionally contributes to the antiviral activity of CIs ~~(Zhang et al., 2004)~~(Zhang et al., 2004).

Due to the high mutation ~~rate~~rates of RNA viruses ~~(Domingo, 1989; Drake et al., 1998)~~(Domingo, 1989; Drake et al., 1998) emerging drug resistance poses a threat to efficacy of CIs. All high-level resistance ~~(HR) (Heinz et al., 1989)~~(Heinz et al., 1989) to CIs reported to date involve residues forming the hydrophobic pocket of ~~EV~~EVs ~~(Badger et al., 1989; Benschop et al., 2015; Groarke and Pevear, 1999; Ledford et al., 2005; Ledford et al., 2004; Schmidtke et al., 2005)~~(Badger et al., 1989; Benschop et al., 2015; Groarke and Pevear, 1999; Ledford et al., 2005; Ledford et al., 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)~~(Feil et al., 2012).

Here, mutations conferring ~~HR~~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 ~~CI~~CIs were shown to cause ~~HR.~~high-level resistance. A hypothesis of the underlying molecular mechanism was derived ~~by~~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)~~(Diana et al., 1995). The full ~~protocol is~~protocols are provided ~~as Supplementary material~~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)~~(Schmidtke et al., 2005).

HeLa cells were grown in Eagles minimal essential medium (Lonza Walkersville) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 100 U/ml penicillin, and 100 U/ml streptomycin (Lonza, Walkersville). 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 two rounds plaque-to-plaque purified in the presence of 1 µg/ml pleconaril and propagated in HeLa cells to get a~~(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 ~~were performed (~~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)~~(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 hours. 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)~~(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)~~(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~~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.

Sequence analysis of the genome region P1 coding for capsid proteins (purified PCR products) was performed with the Beckman Coulter Genom-Lab System (**PubMed GenBank number: JX946654** and **JX946654**) following the manufacturer's instructions. Sequencing primers are summarized in Table S1.

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2.6 Comparison of single-step growth curves

Single-step replication cycles of wt-CVB3 97927 and three selected pleconaril-resistant variants were comparatively studied in two-day-old confluent HeLa cells grown in 4-well tissue culture plates. Three wells of each plate were infected with a moi of 10 pfu/cell for 1 h at 37°C. After three washing steps, addition of fresh medium, and further incubation for 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, and 12 h p.i., supernatant of each of the three infected wells was collected. Virus titers were determined in HeLa cells ~~(Reed and Muench, 1938).~~(Reed and Muench, 1938). The Mann-Whitney-U-test was used for statistical analysis.

2.7 Computational studies

~~Homology models for CVB3 97927 were manually derived from PDB structure 1COV (Muckelbauer et al., 1995b). Molecular dynamics simulations were carried out using the AMBER11 (with patches up to 17 applied) and the AMBERTOOLS version 1.5 suite of programs~~ Homology models for the CVB3 97927 variants were derived using PDB 1cov (Muckelbauer et al., 1995b) as a template. Molecular dynamics simulations were carried out using AMBER11 and the AMBERTOOLS suite of programs ~~(Case et al., 2010)~~(Case et al., 2010). The full ~~protocol is~~protocols are provided ~~as~~in the Supplementary ~~material~~Information.

3 Results

3.1 Selection and characterization of pleconaril-resistant CVB3 variants

The fraction of ~~HR~~high-level resistant mutants present in a population of wt-CVB3 97927 was examined by determining the frequency of survivors that can replicate in presence of the drug ~~(Heinz et al., 1989)~~(Heinz et al., 1989)~~. Pleconaril~~ . In analogy to earlier studies (Groarke and Pevear, 1999), pleconaril was applied at a high concentration of 1 µg/ml ~~in analogy to earlier studies (Groarke and Pevear, 1999).~~ This concentration reduced the plaque titer of wt-CVB3 97927 by approximately four log units (Table 1). Based on these data~~,~~ the frequency of pleconaril resistance in the wildtype population was 1.2×10^{-4}.

~~To~~In order to select ~~HR~~high-level resistant mutants, nine pools of wt-CVB3 97927, treated individually with 1 µg/ml of pleconaril for 1 h at 37°C, were subjected to a plaque assay. Virus from one plaque per pool was collected and ~~two times~~purified twice plaque-to-plaque ~~purified~~ in the presence of 1 µg/ml of pleconaril. For all nine purified isolates ~~HR~~high-level resistance was ~~demonstrated~~confirmed in plaque reduction assays with 8 µg/ml of pleconaril (inactive) and in cytopathic effect inhibition assays for selected variants (one per mutation; Table 1~~.~~). These results were ~~confirmed for three purified isolates~~corroborated by immunohistochemical detection of viral antigen (Figure 2A) with pleconaril in HeLa cells. ~~The~~ wt-CVB3 97927 was included as a control and was found susceptible to pleconaril.

~~No difference in~~Closely related antigen expression profiles of untreated HeLa cells infected with wt-CVB3 97927 and ~~HR~~high-level resistant variants ~~was~~were observed (Figure 2A). The number of infected cells was even slightly higher for the I1092M variant than for the wt-CVB3 97927. Furthermore, no significant difference was detected with the Mann-Whitney-U test, comparing single-step growth curves of wt-CVB3 97927 and ~~HR~~high-level resistant variants in HeLa cells (Figure 2B~~). So~~; Table S2). Hence, replication of the selected ~~HR~~high-level resistant variants was not hampered *in vitro*.

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~~To~~In order to gain insight into the genetic basis of the detected pleconaril resistance, the whole capsid-protein-coding region P1 of all nine plaque-purified~~, HR~~ high-level resistant variants was sequenced and compared with that of wt-CVB3 97927. All ~~HR~~high-level resistant variants showed nucleotide exchanges that result in single-site amino acid substitution in position 1092 or 1207 (Table 1; Figure 1). I1092M substitution was detected for three ~~HR~~high-level resistant variants. Six ~~HR~~high-level resistant variants ~~consisted single~~showed hitherto unknown amino acid substitutions at position 1207 (I1207K, I1207R~~), located outside the drug-binding pocket that were not described before.~~).

3.2 Hypothesis of a specific drug resistance mechanism

~~The~~ A model of the resistance mechanism of ~~HR~~CVB3 to pleconaril ~~caused by I1207K and I1207R~~ was ~~investigated~~developed using MD ~~simulations of a homology model of wt-CVB3 97927. Two copies of VP1-VP4 (one with pleconaril bound and one with the pocket factor bound) of the drug-sensitive and the two HR variants~~simulation techniques. Six systems were simulated~~ for 30 ns each. The~~: The wt-CVB3 97927 and the I1207K and I1207R variants, each in their apo and holo (i.e. bound with pleconaril) states. Each model was simulated for 30 ns; the last 20 ns were used for analysis. ~~Structural~~

All systems were stable for the full duration of the simulations. Commonly observed structural changes were ~~observed~~detected for solvent-exposed areas of the capsid protein, while the protein ~~core and active site~~cores remained close to their start geometries (Figure 3).

The apo protein structures proved to be particularly stable. No significant conformational changes in the region of the binding pocket could be observed. I1207 is part of the GH loop of VP1 ~~in wt-CVB3 97927~~ (Figure 1, 4A). ~~Both I1207K and I1207R lead~~Substitution by Lys or Arg leads to the formation of a new salt bridge with E2131 (Figure 4B,C~~). R1207 approaches~~) and in the case of I1207R also to interactions with E1105 ~~in addition~~ (Figure 4C). Both substitutions appear to have a weak, stabilizing effect on the surrounding region (Figure 3C).

The stability of the apo simulations is a strong indication that both mutations do not induce conformational rearrangements of the capsid pocket that could completely prevent ligands (such as pleconaril) to approach the binding site. The fact that the mutations of I1207 do not affect viral replication rates (indicating an intact function of the pocket factor) supports his model.

In contrast to the apo structure simulations, significant conformational rearrangements were observed for holo structure simulations, for which reason we focused our analysis on these systems.

All MD simulations are based on homology models derived from an X-ray structure of CVB3 with a palmitate bound to the hydrophobic pocket~~, which~~ . This pocket factor forms a salt bridge with R1095~~.~~

~~In~~, which during the MD simulation of the wt-CVB3 97927-pleconaril complex~~, R1095~~ turns away from the hydrophobic methylisoxazole to form hydrogen bonds with E1105 and N1211 (Figure 4A). Interaction with the latter leads to stabilization of the βH strand and a tightening of the hydrophobic pocket. In both ~~HR~~high-level resistant variants, R1095 is pushed toward the hydrophobic pocket because of the additional bulk and charge added by the side chains. Consequently, the polar side chain of R1095 faces the non-polar methylisoxazole group of pleconaril (Figure 4B, C). This leads to destabilization of the protein-ligand interaction and causes the βH and βC strands to drift apart (Figure 5A). In further consequence, hydrogen bonds formed between R1095 and S1190/N1191 increasingly replace the βH strand-stabilizing interactions of R1095 and N1211 (Figure 5B).

The converse shift of both strands results in the dilation of the hydrophobic pocket. For the I1207K variant this translates into RMSDs of up to 1.4 Å for N1211 (Figure 6A). Movement of the βH strand is more pronounced in the I1207R variant, which after 20 ns of simulation time is mostly found in an open conformation (RMSDs up to 1.8 Å for N1211). As a result of this shift, surface contacts between N1211 (also N121 and M1213) and pleconaril (Figure 6B) are diminished. Water molecules fill gaps between the drug and the protein.

Throughout most of the simulation of wt-CVB3 97927, the distance between the polar head of R1095 and the methyl group of pleconaril is about 6 Å or greater (mean 6.5 Å; min. distance 4.7 Å; Figure 6C). While for I1207K the mean distance is comparable to that of wt-CVB3 97927 (6.7 Å), for parts of the trajectory the side chain gets much closer to pleconaril (min. distance 3.9 Å). For I1207R the mean and minimum distances are just 4.6 Å and 3.2 Å, respectively.

The mean RMSD for pleconaril ~~throughout~~in the wt-CVB3 97927 simulation is 0.9 Å (Figure 6D). For I1207K and I1207R these ~~RMSDs~~values are 1.4 Å and 1.1 Å, respectively. The maximum RMSD observed for the I1207K variant (2.3 Å) is significantly higher than for the wt-CVB3 97927 (1.5 Å) and the I1207R variant (1.7 Å). RMSD peaks are a result of the displacement of the methylisoxazolylpropoxy group from the floor of the hydrophobic pocket toward the pore by the charged guanidino group of R1095 (Figure S1).

In the wt-CVB3 97927, the torsion angle Φ (as indicated in the scheme of Figure 1) shows infrequent swaps between two conformations, with Φ around +75 or -120 degrees (Figure ~~S 2A~~S2A). In both conformations the methylisoxazole moiety remains in contact with the hydrophobic part of N1211. In both mutants however, pleconaril shows strong fluctuations for Φ as a result of a loss of surface contacts with N1211, caused by the shift of the βH strand (Figure ~~S 2B~~S2B,C).

A water molecule mediating interactions between the isoxazole nitrogen and the side chains of T1094 and R1095 is part of the protein-ligand interaction network in the sensitive virus (Figure 4A). Even though in the I1207K mutant pleconaril maintains coordination with a water molecule at this position, this variant does not form interactions with R1095. The torsional

fluctuations of the methylisoxazole add to the instability of the water-mediated interactions (Figure ~~S 3A~~S3A). Changes are more significant for the I1207R variant. There, interactions mediated by this water molecule are partially replaced by interactions with T1093 (Figure S3), which, together with the altered conformation of R1095, ~~causes~~cause the conformational shift seen for the βC strand.

4 Discussion

For the first time this work characterizes ~~HR~~high-level resistance to a ~~CI~~CIs of EVs caused by I1207K and I1207R substitution. It also confirms single-site substitution of I1092M as a cause of ~~HR~~high-level resistance ~~(Groarke and Pevear, 1999; Schmidtke et al., 2005)~~(Groarke and Pevear, 1999; Schmidtke et al., 2005). In contrast to I1092, I1207 is not part of the ~~drug~~ligand binding pocket and confers ~~HR~~high-level resistance by an unreported molecular mechanism.

~~The detected frequency of pleconaril resistance of 1.2×10^{-4} corresponds to previously published data for pleconaril-resistant CVB3 (Groarke and Pevear, 1999) and drug-resistant mutants of other ssRNA viruses (frequencies reported are between 10^{-3} and 10^{-5}) The~~ The detected frequency of pleconaril resistance of 1.2×10^{-4} corresponds to previously published data for pleconaril-resistant CVB3 (Groarke and Pevear, 1999) and drug-resistant mutants of other ssRNA viruses (frequencies reported are between 10^{-3} and 10^{-5}) ~~(Heinz et al., 1989; Stech et al., 1999; Wang et al., 1998)~~(Heinz et al., 1989; Stech et al., 1999; Wang et al., 1998). The high mutation rate ~~(Drake et al., 1998; Holland et al., 1982)~~(Drake et al., 1998; Holland et al., 1982) ~~and the existence of quasi species (Domingo, 1992) are caused by (i) integration of one mismatched base per 10^4-10^5 bases by RNA-dependent RNA polymerase of ssRNA viruses and (ii) lack of a proof-reading ability of these enzymes.~~

~~The amino acid substitution I1092M was described earlier by Groarke et al. (Groarke and Pevear, 1999), who also detected a CVB3 variant containing two amino acid substitutions, I1092L as well as L1207V. However, the impact of the L1207V mutation on the resistant phenotype remained unclear. According to the definition of Heinz et al.~~ and the existence of

quasi species (Domingo, 1992) are caused by (i) integration of one mismatched base per 10^4-10^5 bases by RNA-dependent RNA polymerase of ssRNA viruses and (ii) lack of a proof-reading ability of these enzymes.

The amino acid substitution I1092M was described earlier by Groarke et al. (Groarke and Pevear, 1999), who also detected a CVB3 variant containing two amino acid substitutions, I1092L as well as L1207V. However, the impact of the L1207V mutation on the resistant phenotype remained unclear. According to the definition of Heinz et al. ~~(Heinz et al., 1989)~~(Heinz et al., 1989), substitutions in I1092 confer ~~HR~~high-level resistance ~~(Groarke and Pevear, 1999; Schmidtke et al., 2005)~~(Groarke and Pevear, 1999; Schmidtke et al., 2005)~~. This can be explained by the fact that I1092 is one of the 17 amino acids forming the hydrophobic pocket of CVB3 (Muckelbauer et al., 1995a). I1092 is situated in the center of the pocket and interacts with one of the two methyl groups of pleconaril's phenyl ring. Substitutions of amino acids in the center of the pocket that confer HR have also been reported for rhinoviruses~~. This can be explained by the fact that I1092 is one of the 17 amino acids forming the hydrophobic pocket of CVB3 (Muckelbauer et al., 1995a). I1092 is situated in the center of the pocket and interacts with one of the two methyl groups of pleconaril's phenyl ring. Substitutions of amino acids in the center of the pocket that confer high-level resistance have also been reported for rhinoviruses ~~(Badger et al., 1989; Heinz et al., 1989; Ledford et al., 2005; Ledford et al., 2004; Zhang et al., 2004)~~(Badger et al., 1989; Heinz et al., 1989; Ledford et al., 2005; Ledford et al., 2004; Zhang et al., 2004) and echovirus 11 ~~(Benschop et al., 2015)~~(Benschop et al., 2015). The activity of pleconaril derivatives lacking one or both of the methyl groups of the central phenyl ring of pleconaril ~~(Schmidtke et al., 2009)~~(Schmidtke et al., 2009) is less affected by the substitution of I1092.

~~In contrast to I1092, I1207 is not involved in the formation of the drug-binding pocket (Muckelbauer et al., 1995a). Therefore, the observation that mutation of I1207 can lead to a complete loss of drug action is intriguing. MD simulations indicate that substitution of I1207 by a more bulky, positively charged residue pushes R1095 into the hydrophobic pocket toward pleconaril, initiating a cascade of conformational changes, ultimately leading to the~~

~~abolishment of drug action. Both the binding mode of pleconaril and the postulated drug resistance mechanism show similarities with oseltamivir resistance of influenza viruses caused by H274Y (Wang et al., 2002). The active site of influenza neuraminidase is highly polar~~ In contrast to I1092, I1207 is not involved in the formation of the binding pocket (Muckelbauer et al., 1995a). Therefore, the observation that mutation of I1207 can lead to a complete loss of drug action is intriguing. MD simulations indicate that substitution of I1207 by a more bulky, positively charged residue pushes R1095 into the hydrophobic pocket toward pleconaril, initiating a cascade of conformational changes that ultimately lead to the abolishment of drug action. Importantly, the substitutions appear to not lead to conformational rearrangements of the ligand binding site in the apo system, which otherwise could completely impair ligand binding.

The postulated resistance mechanism has similarities with that of influenza virus neuraminidase and oseltamivir. Substitution of H274 by a Tyr leads to a substantial loss of affinity of oseltamivir for neuraminidase (Wang et al., 2002). The pentanyl substituent of oseltamivir forms hydrophobic interactions with the protein. In H274Y variants, the larger Tyr pushes the polar side chain of neighboring E276 farther into the binding site, toward the hydrophobic pentanyl moiety ~~(von Grafenstein et al., 2015; von Itzstein, 2007)~~(Collins et al., 2008)~~. In search for orally effective neuraminidase inhibitors oseltamivir, comprising a pentanyl substituent was designed. This hydrophobic substituent causes the polar side chain of E276 to adopt its conformation and interact with R224. A hydrophobic pocket is created to which oseltamivir binds with high affinity (Kim et al., 1997). In the case of pleconaril it is the polar side chain of R1095 that points into the ligand binding pocket. In CVB3 97927, in presence of pleconaril the side chain of R1095 is reoriented to face away from the ligand and interact with the protein. In influenza neuraminidase, H274Y is located in the second shell of amino acids forming the active site. The larger tyrosine disrupts the interaction of E276 and R224, forcing E276 to point with its polar head group into the binding site, in direction of the hydrophobic substituent of oseltamivir (Russell et al., 2006). In the resistance mechanism postulated herein for CVB3 97927 and pleconaril it is R1095 which is pushed toward the~~

~~hydrophobic ligand by the replacement of I1207 by a larger lysine or arginine. Methods and approaches described can be applied to study similar cases of resistance formation.~~

~~A~~. This leads to high-level resistance against oseltamivir. In the case of the CVB3 97927 I1207 mutants and pleconaril it is the polar side chain of R1095 that is pushed toward the hydrophobic ligand.

An NCBI database survey with standard nucleotide blast demonstrated a polymorphism at position 1092 as well as 1207 in VP1 of CVB3. About 99% of the available CVB3 sequences express I1207, three have a V1207 and each one a T1207 or L1207. With respect to substitutions at position 1092, 30% of available sequences have isoleucine, 3% leucine and 67% valine. Thus, viruses with substitutions in position 1092 or 1207 are circulating in nature. Obviously, they do not hamper viral replication. This is in good agreement with the results of our *in vitro* studies. Neither antigen expression nor one-step growth curves of CVB3 97927 and ~~HR~~high-level resistant mutants showed any differences. In contrast, attenuated virulence of pleconaril-resistant CVB3 with I1092L or I1092M was described *in vivo* ~~(Groarke and Pevear, 1999)~~(Groarke and Pevear, 1999) but highly mouse-pathogenic, pleconaril-resistant CVB3 containing these substitutions also exist ~~(Schmidtke et al., 2005; Schmidtke et al., 2007)~~(Schmidtke et al., 2005; Schmidtke et al., 2007).

The data presented here underline the vulnerability of the therapeutic efficacy of antivirals targeting the hydrophobic pocket of picornaviruses. ~~The identification~~Knowledge of ~~1207 as further~~ the key role of residue ~~for CIs~~1207 in CI activity and the ~~understanding of the underlying drug~~ new model of a specific resistance mechanism are valuable for the rational design of ~~future~~ therapeutic agents with superior resistance profiles.

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Figure captions

Figure 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 ~~indicated in~~ green and the second protomer ~~in~~ grey. The enlarged section shows VP1 with the location of pleconaril, I1092 and I1207.

Figure 2. Replication of wt-CVB3 97927 and three different pleconaril-resistant variants in HeLa cells. HeLa cells were infected at a moi of 10 pfu/cell of the respective virus. (A) The influence of pleconaril treatment (1 µg/ml) on viral antigen expression was comparatively studied by immunohistochemical staining of CVB3-infected HeLa cells 5 h p.i. Virus antigen-positive cells are stained in red color. (B) Comparison of single-step life cycles of the selected CVB3 samples. Values represent the means and SD of 3 parallel measurements per time point.

Figure 3. Backbone RMSD. Development of the backbone RMSD of the (A) ~~whole simulated~~complete apo system ~~and~~, (B) complete holo system, (C) pocket-forming residues~~.~~ of the apo system and (D) pocket-forming residues of the holo system. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue).

Figure 4. Conformational flexibility of VP1 and ~~molecular~~ interactions ~~of VP1~~ with pleconaril. (A) In ~~the~~ wt-CVB3 97927, R1095 forms polar interactions with E1105, N1211 and a water molecule. The polar side chain of R1095 is hence oriented away from pleconaril toward E1105. (B) In the I1207K mutant, K1207 forms a salt bridge with E2131 rather than E1105. R1095 points toward the hydrophobic ligand. (C) An initially observed salt bridge between R1207 and E1105 in the I1207R mutant is weakened by a conformational rearrangement of the βH strand observed during the simulation. In this resistant variant the charged side chain of R1095 points deeply into the hydrophobic pocket. (D) B-factors calculated for VP1 from the MD trajectories. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue). The exposed residues of the GH loop show increased flexibility when compared to the mutants (in particular A1200). B-factor plots for VP2, 3 and 4 are provided in Figure S4.

Figure 5. Conformational shifts observed for the βC and βH strands of the wt-CVB3 97927 and the two mutants. (A) Representative frame of the wt-CVB3 97927 (grey), I1207K (orange) and I1207R (blue) virus strain, selected from the MD trajectories by clustering. (B) Detail of the βH strand of the I1207R variant, before (green) and after (blue) the conformational shift.

Figure 6. Conformational shift of the βH-strand induced by the approach of R1095, repelling the hydrophobic elements of pleconaril and N1211. (A) RMSD plot of N1211. (B) Distance between the aliphatic carbon of the methylisoxazole moiety of pleconaril and the Cα atom of N1211. (C) Distance between the guanidino carbon atom of R1095 and the aliphatic carbon of the methylisoxazole group. (D) RMSD plot of pleconaril. wt-CVB3 97927 (black), I1207K (orange) and I1207R (blue).

Supporting Information

Table S 1. Primers used for PCR amplification and sequencing.

Table S2. Virus titers determined in one-step replication cycle experiments at different hours after infection with CVB3 97-927 variants.

Figure S 1. ConformationConformations observed for pleconaril. Representative conformations selected from the wt-CVB3 97927 (grey), I1207K (orange) and I1207R (blue) trajectories using an average-linkage algorithm for clustering (considering only the conformations of the drug molecule).

Figure S 2. Conformational fluctuations of torsion angle Φ of pleconaril. (A) Fluctuations between two torsion angle states occur infrequently in the wt-CVB3 97927. They are much stronger for the (B) I1207K and (C) I1207R mutants.

Figure S 3. InteractionInteractions of the isoxazole nitrogen of pleconaril with (A) water and (B) T1093. Even though an interaction of pleconaril with a water molecule is maintained in the mutants, the formed interaction network formed is much weaker when compared to thethan that of wt-CVB3 97927 (black). In the I1207R mutant (blue) pleconaril is partially switching from an interaction with water to an interaction with T1093.

Figure S 4. B-factors calculated for (a) VP2, (b) VP3 and (c) VP4. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue).

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

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EV, enterovirus; CI, capsid-binding inhibitors; VP1-4, viral capsid protein 1, 2, 3, 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

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.

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 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).

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; Ledford et al., 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) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 100 U/ml penicillin, and 100 U/ml streptomycin (Lonza, Walkersville). 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 hours. 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.

Sequence analysis of the genome region P1 coding for capsid proteins (purified PCR products) was performed with the Beckman Coulter Genom-Lab System (**PubMed GenBank number: JX946654** and **JX946654**) following the manufacturer's instructions. Sequencing primers are summarized in Table S1.

2.6 Comparison of single-step growth curves

Single-step replication cycles of wt-CVB3 97927 and three selected pleconaril-resistant variants were comparatively studied in two-day-old confluent HeLa cells grown in 4-well tissue culture plates. Three wells of each plate were infected with a moi of 10 pfu/cell for 1 h

at 37°C. After three washing steps, addition of fresh medium, and further incubation for 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, and 12 h p.i., supernatant of each of the three infected wells was collected. Virus titers were determined in HeLa cells (Reed and Muench, 1938). The Mann-Whitney-U-test was used for statistical analysis.

2.7 Computational studies

Homology models for the CVB3 97927 variants were derived using PDB 1cov (Muckelbauer et al., 1995b) as a template. Molecular dynamics simulations were carried out using AMBER11 and the AMBERTOOLS suite of programs (Case et al., 2010). The full protocols are provided in the Supplementary Information.

3 Results

3.1 Selection and characterization of pleconaril-resistant CVB3 variants

The fraction of high-level resistant mutants present in a population of wt-CVB3 97927 was examined by determining the frequency of survivors that can replicate in presence of the drug (Heinz et al., 1989). In analogy to earlier studies (Groarke and Pevear, 1999), pleconaril was applied at a high concentration of 1 µg/ml. This concentration reduced the plaque titer of wt-CVB3 97927 by approximately four log units (Table 1). Based on these data the frequency of pleconaril resistance in the wildtype population was 1.2×10^{-4}.

In order to select high-level resistant mutants, nine pools of wt-CVB3 97927, treated individually with 1 µg/ml of pleconaril for 1 h at 37°C, were subjected to a plaque assay. Virus from one plaque per pool was collected and purified twice plaque-to-plaque in the presence of 1 µg/ml of pleconaril. For all nine purified isolates high-level resistance was confirmed in plaque reduction assays with 8 µg/ml of pleconaril (inactive) and in cytopathic effect inhibition assays for selected variants (one per mutation; Table 1). These results were corroborated by immunohistochemical detection of viral antigen (Figure 2A) with pleconaril in HeLa cells. wt-CVB3 97927 was included as a control and was found susceptible to pleconaril.

Closely related antigen expression profiles of untreated HeLa cells infected with wt-CVB3 97927 and high-level resistant variants were observed (Figure 2A). The number of infected cells was even slightly higher for the I1092M variant than for the wt-CVB3 97927. Furthermore, no significant difference was detected with the Mann-Whitney-U test, comparing single-step growth curves of wt-CVB3 97927 and high-level resistant variants in HeLa cells (Figure 2B; Table S2). Hence, replication of the selected high-level resistant variants was not hampered *in vitro*.

In order to gain insight into the genetic basis of the detected pleconaril resistance, the whole capsid-protein-coding region P1 of all nine plaque-purified high-level resistant variants was sequenced and compared with that of wt-CVB3 97927. All high-level resistant variants showed nucleotide exchanges that result in single-site amino acid substitution in position 1092 or 1207 (Table 1; Figure 1). I1092M substitution was detected for three high-level resistant variants. Six high-level resistant variants showed hitherto unknown amino acid substitutions at position 1207 (I1207K, I1207R).

3.2 Hypothesis of a specific drug resistance mechanism

A model of the resistance mechanism of CVB3 to pleconaril was developed using MD simulation techniques. Six systems were simulated: The wt-CVB3 97927 and the I1207K and I1207R variants, each in their apo and holo (i.e. bound with pleconaril) states. Each model was simulated for 30 ns; the last 20 ns were used for analysis.

All systems were stable for the full duration of the simulations. Commonly observed structural changes were detected for solvent-exposed areas of the capsid protein, while the protein cores remained close to their start geometries (Figure 3).

The apo protein structures proved to be particularly stable. No significant conformational changes in the region of the binding pocket could be observed. I1207 is part of the GH loop of VP1 (Figure 1, 4A). Substitution by Lys or Arg leads to the formation of a new salt bridge with E2131 (Figure 4B,C) and in the case of I1207R also to interactions with E1105 (Figure

4C). Both substitutions appear to have a weak, stabilizing effect on the surrounding region (Figure 3C).

The stability of the apo simulations is a strong indication that both mutations do not induce conformational rearrangements of the capsid pocket that could completely prevent ligands (such as pleconaril) to approach the binding site. The fact that the mutations of I1207 do not affect viral replication rates (indicating an intact function of the pocket factor) supports his model.

In contrast to the apo structure simulations, significant conformational rearrangements were observed for holo structure simulations, for which reason we focused our analysis on these systems.

All MD simulations are based on homology models derived from an X-ray structure of CVB3 with a palmitate bound to the hydrophobic pocket. This pocket factor forms a salt bridge with R1095, which during the MD simulation of the wt-CVB3 97927-pleconaril complex turns away from the hydrophobic methylisoxazole to form hydrogen bonds with E1105 and N1211 (Figure 4A). Interaction with the latter leads to stabilization of the βH strand and a tightening of the hydrophobic pocket. In both high-level resistant variants, R1095 is pushed toward the hydrophobic pocket because of the additional bulk and charge added by the side chains. Consequently, the polar side chain of R1095 faces the non-polar methylisoxazole group of pleconaril (Figure 4B, C). This leads to destabilization of the protein-ligand interaction and causes the βH and βC strands to drift apart (Figure 5A). In further consequence, hydrogen bonds formed between R1095 and S1190/N1191 increasingly replace the βH strand-stabilizing interactions of R1095 and N1211 (Figure 5B).

The converse shift of both strands results in the dilation of the hydrophobic pocket. For the I1207K variant this translates into RMSDs of up to 1.4 Å for N1211 (Figure 6A). Movement of the βH strand is more pronounced in the I1207R variant, which after 20 ns of simulation time is mostly found in an open conformation (RMSDs up to 1.8 Å for N1211). As a result of this shift, surface contacts between N1211 (also N121 and M1213) and pleconaril (Figure 6B) are diminished. Water molecules fill gaps between the drug and the protein.

Throughout most of the simulation of wt-CVB3 97927, the distance between the polar head of R1095 and the methyl group of pleconaril is about 6 Å or greater (mean 6.5 Å; min. distance 4.7 Å; Figure 6C). While for I1207K the mean distance is comparable to that of wt-CVB3 97927 (6.7 Å), for parts of the trajectory the side chain gets much closer to pleconaril (min. distance 3.9 Å). For I1207R the mean and minimum distances are just 4.6 Å and 3.2 Å, respectively.

The mean RMSD for pleconaril in the wt-CVB3 97927 simulation is 0.9 Å (Figure 6D). For I1207K and I1207R these values are 1.4 Å and 1.1 Å, respectively. The maximum RMSD observed for the I1207K variant (2.3 Å) is significantly higher than for the wt-CVB3 97927 (1.5 Å) and the I1207R variant (1.7 Å). RMSD peaks are a result of the displacement of the methylisoxazolylpropoxy group from the floor of the hydrophobic pocket toward the pore by the charged guanidino group of R1095 (Figure S1).

In the wt-CVB3 97927, the torsion angle Φ (as indicated in the scheme of Figure 1) shows infrequent swaps between two conformations, with Φ around +75 or -120 degrees (Figure S2A). In both conformations the methylisoxazole moiety remains in contact with the hydrophobic part of N1211. In both mutants however, pleconaril shows strong fluctuations for Φ as a result of a loss of surface contacts with N1211, caused by the shift of the βH strand (Figure S2B,C).

A water molecule mediating interactions between the isoxazole nitrogen and the side chains of T1094 and R1095 is part of the protein-ligand interaction network in the sensitive virus (Figure 4A). Even though in the I1207K mutant pleconaril maintains coordination with a water molecule at this position, this variant does not form interactions with R1095. The torsional fluctuations of the methylisoxazole add to the instability of the water-mediated interactions (Figure S3A). Changes are more significant for the I1207R variant. There, interactions mediated by this water molecule are partially replaced by interactions with T1093 (Figure S3), which, together with the altered conformation of R1095, cause the conformational shift seen for the βC strand.

4 Discussion

For the first time this work characterizes high-level resistance to a CIs of EVs caused by I1207K and I1207R substitution. It also confirms single-site substitution of I1092M as a cause of high-level resistance (Groarke and Pevear, 1999; Schmidtke et al., 2005). In contrast to I1092, I1207 is not part of the ligand binding pocket and confers high-level resistance by an unreported molecular mechanism.

The detected frequency of pleconaril resistance of 1.2×10^{-4} corresponds to previously published data for pleconaril-resistant CVB3 (Groarke and Pevear, 1999) and drug-resistant mutants of other ssRNA viruses (frequencies reported are between 10^{-3} and 10^{-5}) (Heinz et al., 1989; Stech et al., 1999; Wang et al., 1998). The high mutation rate (Drake et al., 1998; Holland et al., 1982) and the existence of quasi species (Domingo, 1992) are caused by (i) integration of one mismatched base per 10^{4}-10^{5} bases by RNA-dependent RNA polymerase of ssRNA viruses and (ii) lack of a proof-reading ability of these enzymes.

The amino acid substitution I1092M was described earlier by Groarke et al. (Groarke and Pevear, 1999), who also detected a CVB3 variant containing two amino acid substitutions, I1092L as well as L1207V. However, the impact of the L1207V mutation on the resistant phenotype remained unclear. According to the definition of Heinz et al. (Heinz et al., 1989), substitutions in I1092 confer high-level resistance (Groarke and Pevear, 1999; Schmidtke et al., 2005). This can be explained by the fact that I1092 is one of the 17 amino acids forming the hydrophobic pocket of CVB3 (Muckelbauer et al., 1995a). I1092 is situated in the center of the pocket and interacts with one of the two methyl groups of pleconaril's phenyl ring. Substitutions of amino acids in the center of the pocket that confer high-level resistance have also been reported for rhinoviruses (Badger et al., 1989; Heinz et al., 1989; Ledford et al., 2005; Ledford et al., 2004; Zhang et al., 2004) and echovirus 11 (Benschop et al., 2015). The activity of pleconaril derivatives lacking one or both of the methyl groups of the central phenyl ring of pleconaril (Schmidtke et al., 2009) is less affected by the substitution of I1092.

In contrast to I1092, I1207 is not involved in the formation of the binding pocket (Muckelbauer et al., 1995a). Therefore, the observation that mutation of I1207 can lead to a

complete loss of drug action is intriguing. MD simulations indicate that substitution of I1207 by a more bulky, positively charged residue pushes R1095 into the hydrophobic pocket toward pleconaril, initiating a cascade of conformational changes that ultimately lead to the abolishment of drug action. Importantly, the substitutions appear to not lead to conformational rearrangements of the ligand binding site in the apo system, which otherwise could completely impair ligand binding.

The postulated resistance mechanism has similarities with that of influenza virus neuraminidase and oseltamivir. Substitution of H274 by a Tyr leads to a substantial loss of affinity of oseltamivir for neuraminidase (Wang et al., 2002). The pentanyl substituent of oseltamivir forms hydrophobic interactions with the protein. In H274Y variants, the larger Tyr pushes the polar side chain of neighboring E276 farther into the binding site, toward the hydrophobic pentanyl moiety (Collins et al., 2008). This leads to high-level resistance against oseltamivir. In the case of the CVB3 97927 I1207 mutants and pleconaril it is the polar side chain of R1095 that is pushed toward the hydrophobic ligand.

An NCBI database survey with standard nucleotide blast demonstrated a polymorphism at position 1092 as well as 1207 in VP1 of CVB3. About 99% of the available CVB3 sequences express I1207, three have a V1207 and each one a T1207 or L1207. With respect to substitutions at position 1092, 30% of available sequences have isoleucine, 3% leucine and 67% valine. Thus, viruses with substitutions in position 1092 or 1207 are circulating in nature. Obviously, they do not hamper viral replication. This is in good agreement with the results of our *in vitro* studies. Neither antigen expression nor one-step growth curves of CVB3 97927 and high-level resistant mutants showed any differences. In contrast, attenuated virulence of pleconaril-resistant CVB3 with I1092L or I1092M was described *in vivo* (Groarke and Pevear, 1999) but highly mouse-pathogenic, pleconaril-resistant CVB3 containing these substitutions also exist (Schmidtke et al., 2005; Schmidtke et al., 2007).

The data presented here underline the vulnerability of the therapeutic efficacy of antivirals targeting the hydrophobic pocket of picornaviruses. Knowledge of the key role of residue

1207 in CI activity and the new model of a specific resistance mechanism are valuable for the rational design of therapeutic agents with superior resistance profiles.

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Figure captions

Figure 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.

Figure 2. Replication of wt-CVB3 97927 and three different pleconaril-resistant variants in HeLa cells. HeLa cells were infected at a moi of 10 pfu/cell of the respective virus. (A) The influence of pleconaril treatment (1 µg/ml) on viral antigen expression was comparatively studied by immunohistochemical staining of CVB3-infected HeLa cells 5 h p.i. Virus antigen-positive cells are stained in red color. (B) Comparison of single-step life cycles of the selected CVB3 samples. Values represent the means and SD of 3 parallel measurements per time point.

Figure 3. Backbone RMSD. Development of the backbone RMSD of the (A) complete apo system, (B) complete holo system, (C) pocket-forming residues of the apo system and (D) pocket-forming residues of the holo system. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue).

Figure 4. Conformational flexibility of VP1 and interactions with pleconaril. (A) In wt-CVB3 97927, R1095 forms polar interactions with E1105, N1211 and a water molecule. The polar side chain of R1095 is hence oriented away from pleconaril toward E1105. (B) In the I1207K mutant, K1207 forms a salt bridge with E2131 rather than E1105. R1095 points toward the hydrophobic ligand. (C) An initially observed salt bridge between R1207 and E1105 in the I1207R mutant is weakened by a conformational rearrangement of the βH strand observed during the simulation. In this resistant variant the charged side chain of R1095 points deeply into the hydrophobic pocket. (D) B-factors calculated for VP1 from the MD trajectories. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue). The exposed residues of the GH loop show increased flexibility when compared to the mutants (in particular A1200). B-factor plots for VP2, 3 and 4 are provided in Figure S4.

Figure 5. Conformational shifts observed for the βC and βH strands of the wt-CVB3 97927 and the two mutants. (A) Representative frame of the wt-CVB3 97927 (grey), I1207K (orange) and I1207R (blue) virus strain, selected from the MD trajectories by clustering. (B) Detail of the βH strand of the I1207R variant, before (green) and after (blue) the conformational shift.

Figure 6. Conformational shift of the βH-strand induced by the approach of R1095, repelling the hydrophobic elements of pleconaril and N1211. (A) RMSD plot of N1211. (B) Distance between the aliphatic carbon of the methylisoxazole moiety of pleconaril and the Cα atom of N1211. (C) Distance between the guanidino carbon atom of R1095 and the aliphatic carbon of the methylisoxazole group. (D) RMSD plot of pleconaril. wt-CVB3 97927 (black), I1207K (orange) and I1207R (blue).

Supporting Information

Table S1. Primers used for PCR amplification and sequencing.

Table S2. Virus titers determined in one-step replication cycle experiments at different hours after infection with CVB3 97-927 variants.

Figure S1. Conformations observed for pleconaril. Representative conformations selected from the wt-CVB3 97927 (grey), I1207K (orange) and I1207R (blue) trajectories using an average-linkage algorithm for clustering.

Figure S2. Conformational fluctuations of torsion angle Φ of pleconaril. (A) Fluctuations between two torsion angle states occur infrequently in the wt-CVB3 97927. They are much stronger for the (B) I1207K and (C) I1207R mutants.

Figure S3. Interactions of the isoxazole nitrogen of pleconaril with (A) water and (B) T1093. Even though an interaction of pleconaril with a water molecule is maintained in the mutants, the interaction network formed is much weaker than that of wt-CVB3 97927 (black). In the I1207R mutant (blue) pleconaril is partially switching from an interaction with water to an interaction with T1093.

Figure S4. B-factors calculated for (a) VP2, (b) VP3 and (c) VP4. wt-CVB3 97927 (black), I1207K (orange), I1207R (blue).

Table 1. Influence of amino acid substitutions in position 1092 and 1207 on pleconaril susceptibility of CVB3 97927 variants

CVB3 97927	n	1092[a]	1207[a]	plaque reduction [%]	50% inhibitory concentration (µg/ml) [b]
wt	3	I	I	99.99[c]	0.12 ± 0.09
variant 1	3	M	I	no reduction[d]	not active
variant 2	3	I	R	no reduction[d]	not active
variant 3	3	I	K	no reduction[d]	not active

[a] amino acid position in viral protein 1

[b] Mean and standard deviation of at least three cytopathic effect inhibition assays. “Not active” indicates no inhibition was found after treatment with pleconaril in the non-cytotoxic dose range up to 12.5 µg/ml (Makarov et al. 2005).

[c] using 1 µg/ml of pleconaril

[d] using 8 µg/ml of pleconaril

Table 2. Overview of molecular dynamics simulations of the viral protein 1

CVB3 97927	**1092**[a]	**1207**[a]	**state**	**ligand**
wildtype	I	I	apo	none
variant 2	I	R	apo	none
variant 3	I	K	apo	none
wildtype	I	I	holo	pleconaril
variant 2	I	R	holo	pleconaril
variant 3	I	K	holo	pleconaril

[a] amino acid position in viral protein 1

Figure(s)

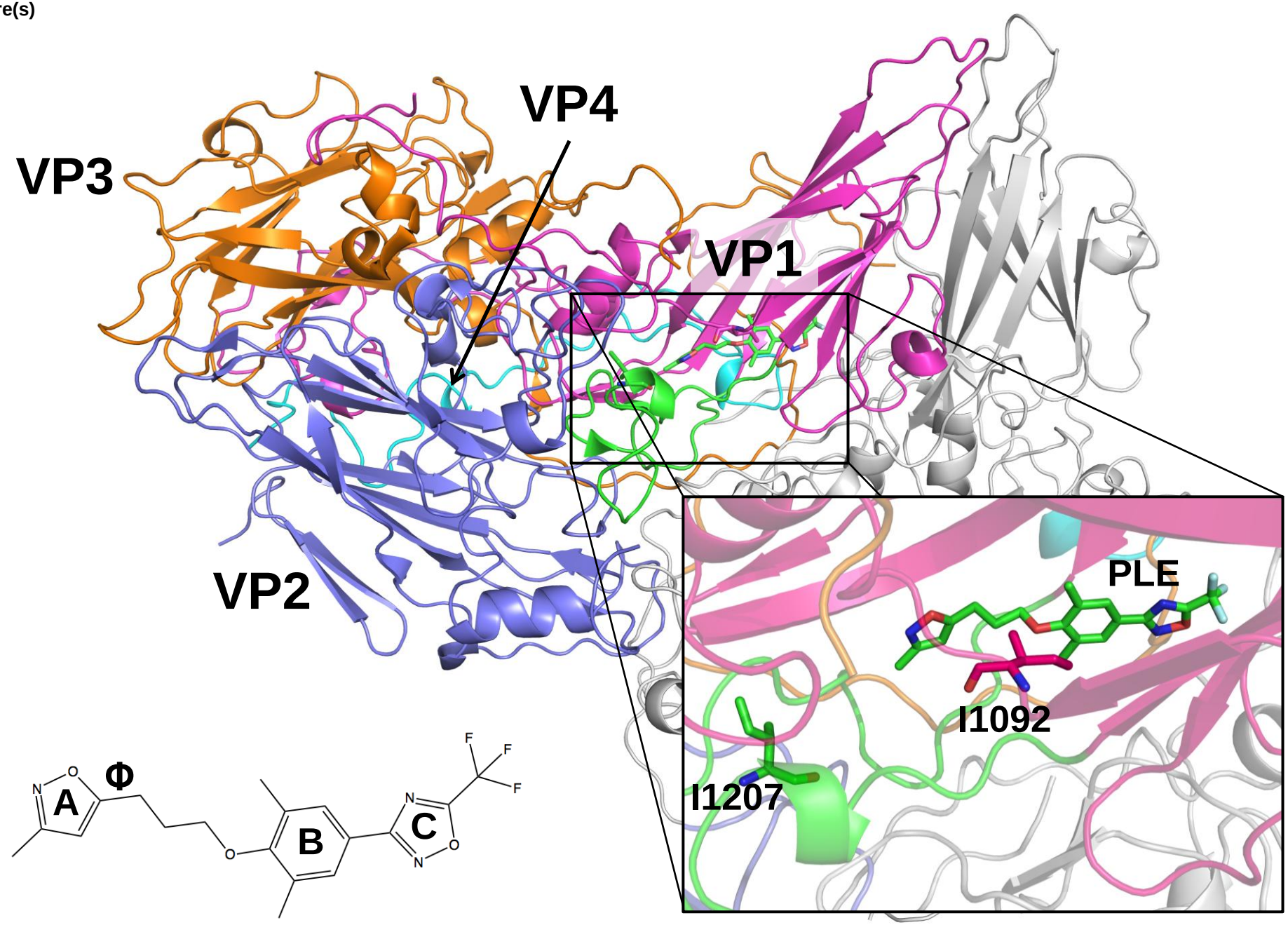

Figure 1

Figure(s)

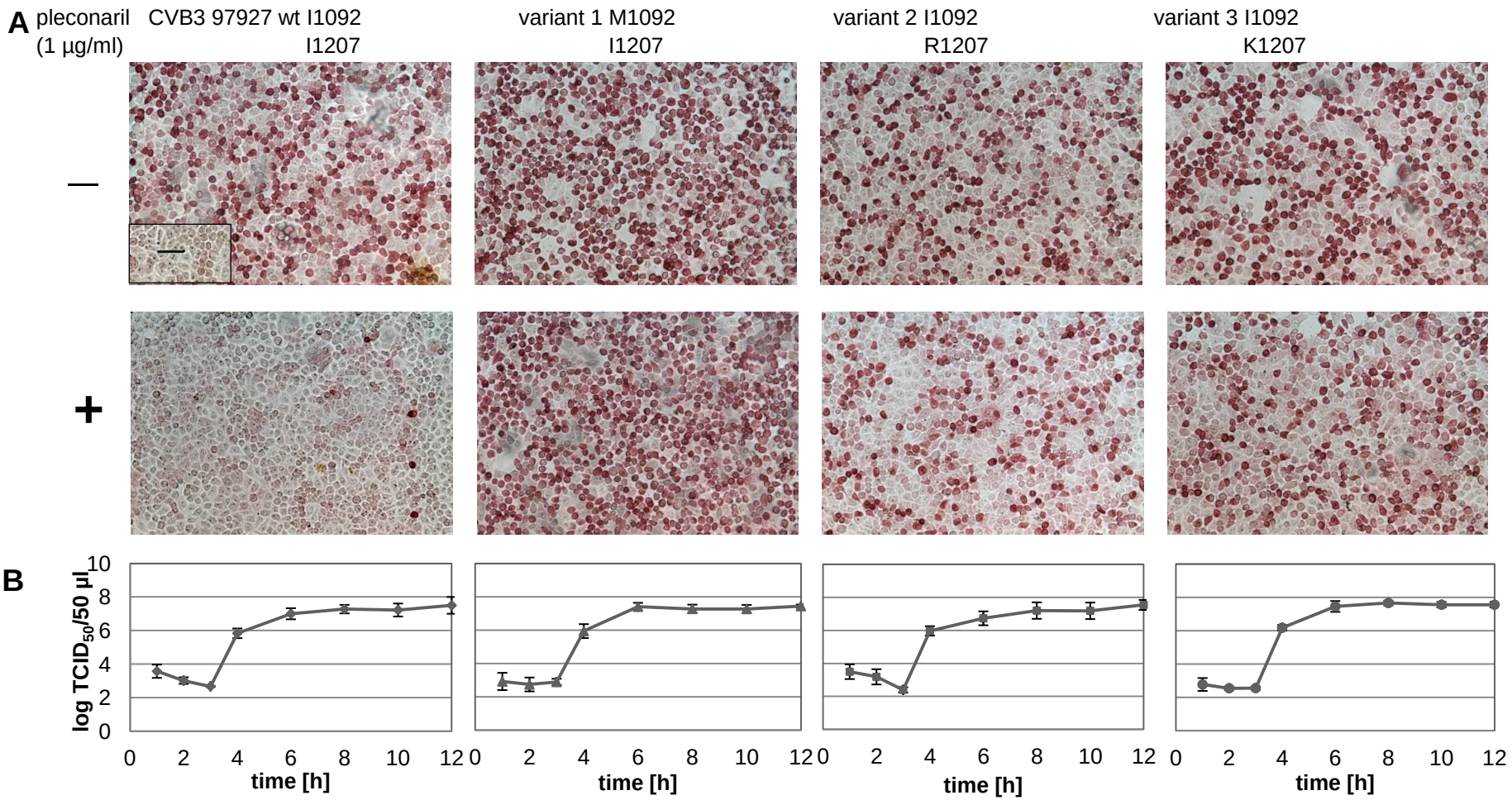

Figure2

Figure(s)

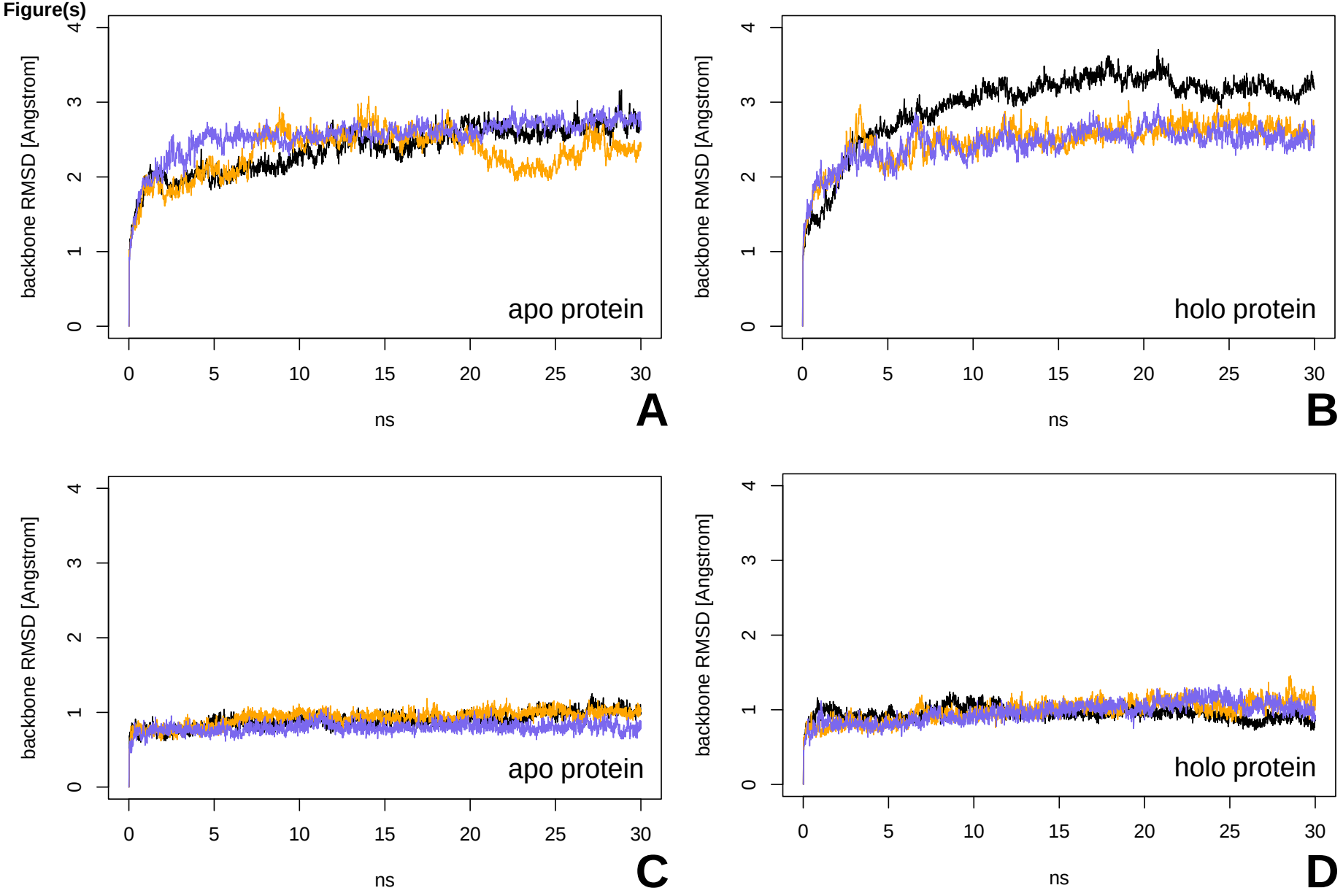

Figure 3

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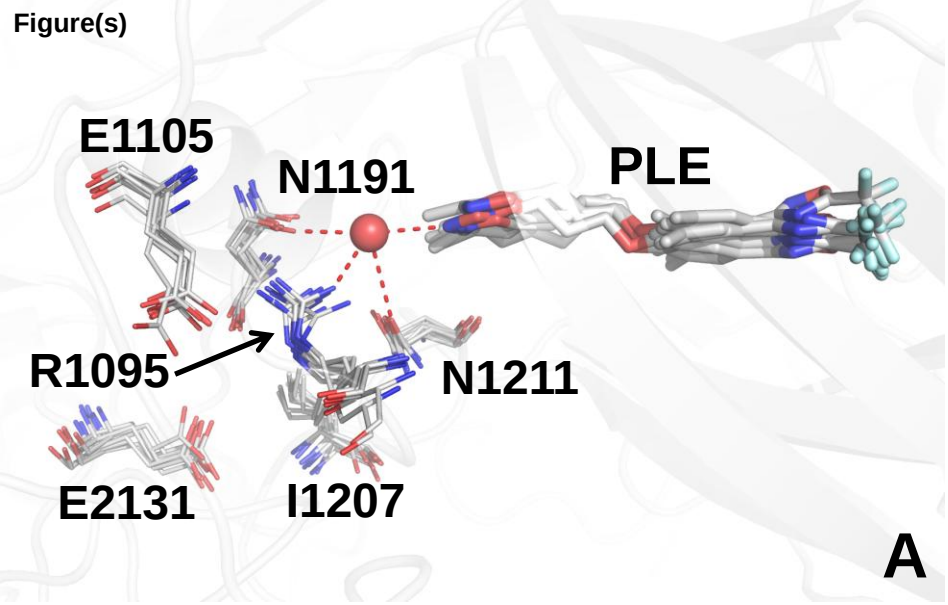
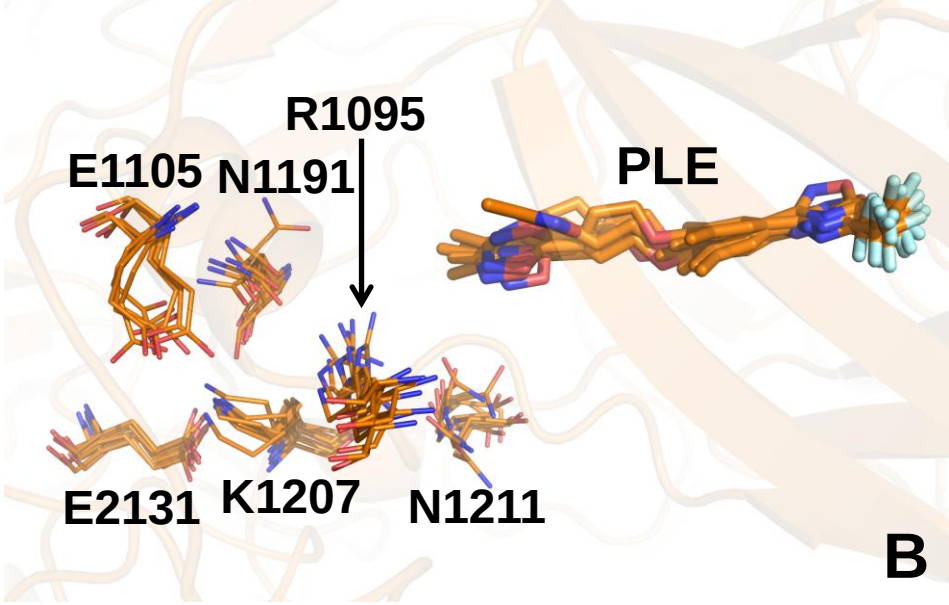
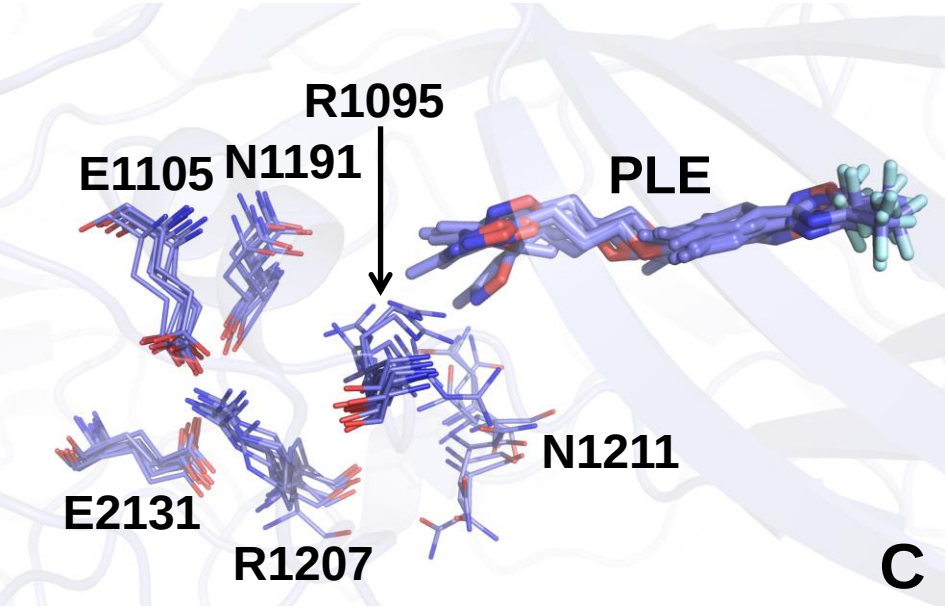






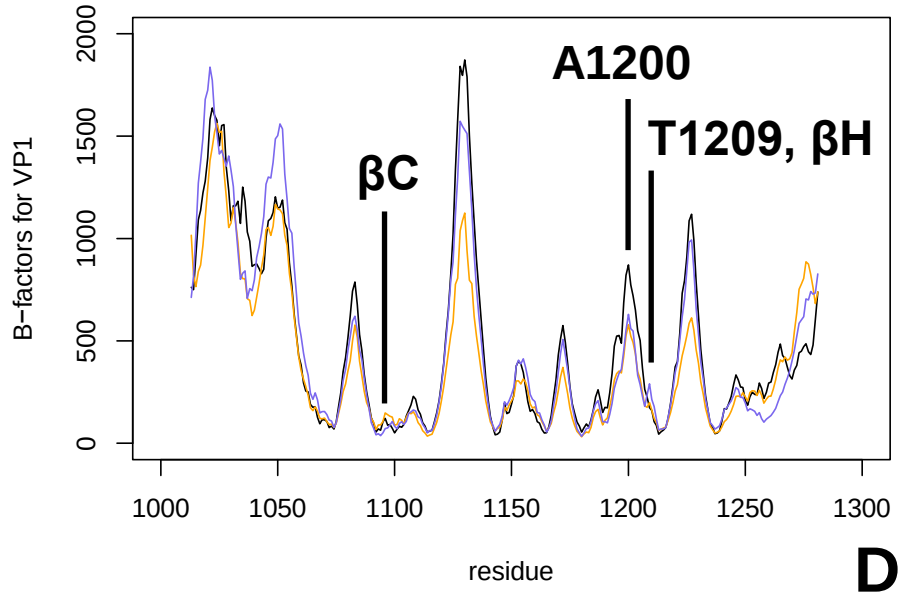
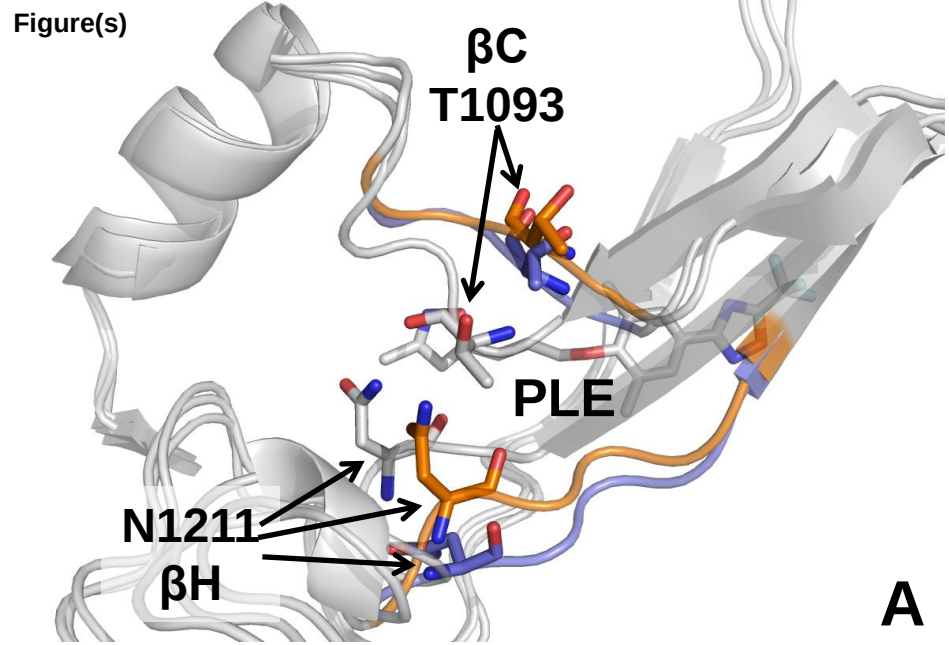


Figure 4

Figure(s)



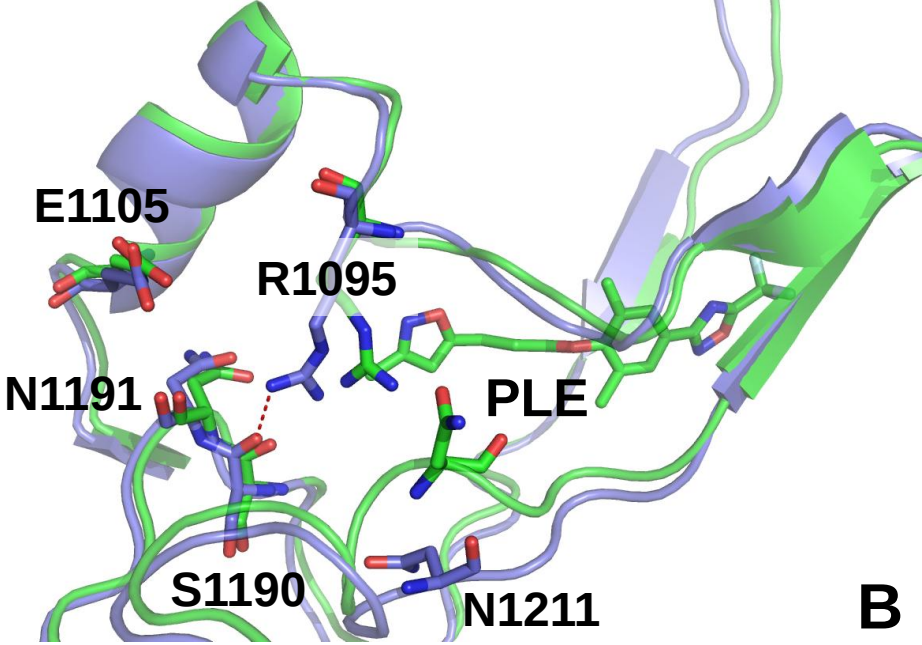
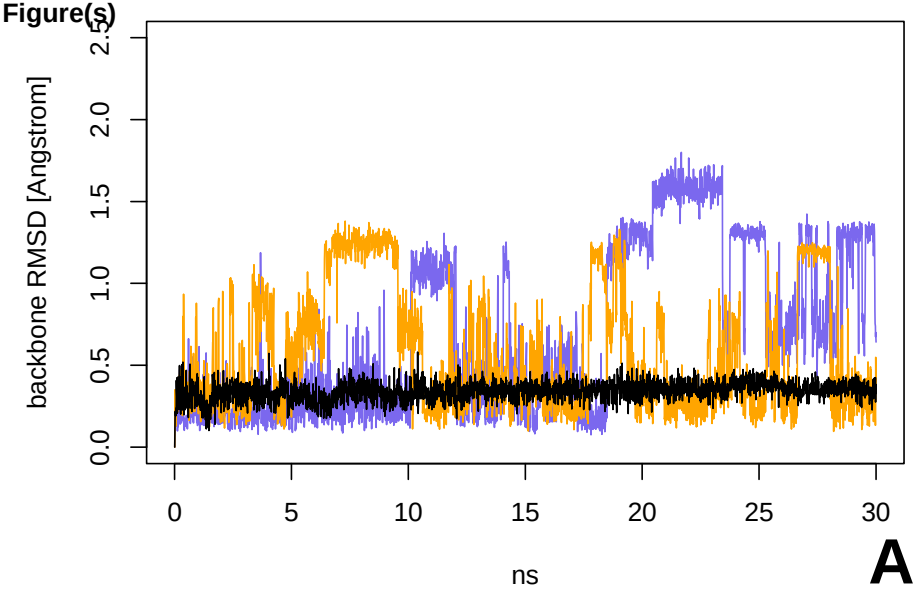
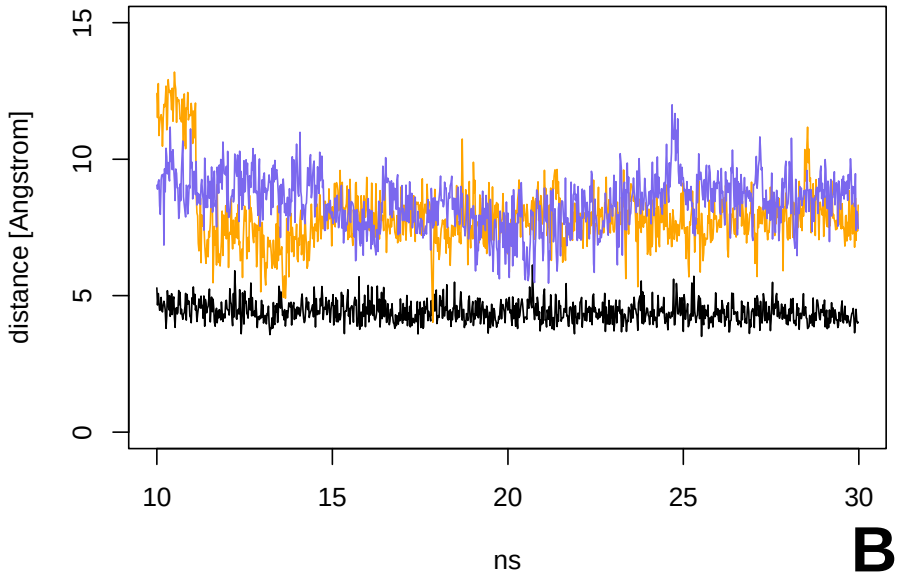
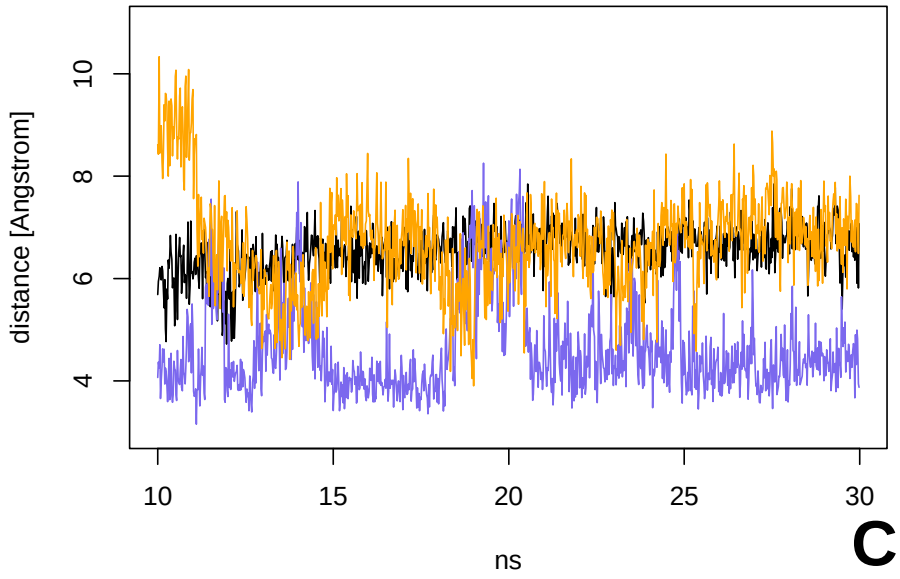


Figure 5

Figure(s)







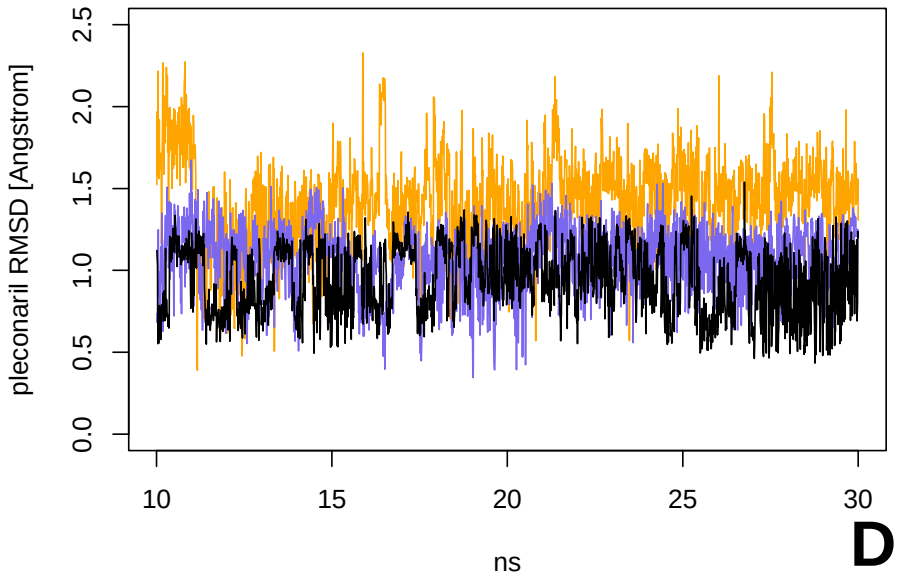


Figure 6

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