

Structural Basis of the Disoxaril Resistance and Dependence of Coxsackievirus B1 Mutants

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Abstract

Disoxaril (WIN) inhibits replication of a broad spectrum of entero- and rhinoviruses through bonding to the hydrophobic pocket within VP1 coat protein. Previously we established Coxsackievirus B1 (CVB1) disoxaril mutants, sensitive (CVB1/SOF), resistant (CVB1/RES) and dependent (CVB1/DEP), which phenotypic characteristics being done. Moreover, analysis of the VP1 gene sequences of CVB1 mutants established an amino acid sequence, highly different at 195-255 in CVB1/RES with two point mutations, M213H and F237L, in the ligand-binding pocket. 3D-alignment of CVA9 over CVB3/B1 allows explicit transferring of two WIN-ligand atomic coordinates into CVB1 “canyon”, site-1 and site-2. The second site is forbidden for ligand in CVB1/SOF. It generated more than 100 models and all of them were treated with ‘clashing analyses’ for side chain rotamers. CVB1/RES has mainly steric and less energetic nature. In CVB1/DEP occupation of site-1 is restricted but site-2 can be filled. WIN molecule in site-2 interacts with the neighboring VP2 protein and all capsomers become chained in the pentamer. This explains the finding that CVB1/DEP mutant needs WIN compound to provoke coating. Analysis of oligomer complexes in the tetramer capsoer resulted in presentation of the whole capsid structure.

Keywords: disoxaril, coxsackievirus B1 mutants

Резюме

Дизоксарил (WIN) инхибира репликацията на широк спектър от ентеро- и риновируси чрез свързване с хидрофобния джоб в VP1 протеина на обвивката. Преди това установихме дизоксарилни мутанти на Coxsackievirus B1 (CVB1), чувствителни (CVB1/SOF), резистентни (CVB1/RES) и зависими (CVB1/DEP), чиито фенотипни характеристики бяха изследвани. Освен това, анализът на генните последователности на VP1 на CVB1 мутанти установи аминокиселинна последователност, силно различна при 195-255 в CVB1/RES с две точкови мутации, M213H и F237L, в лиганд-свързващия джоб. 3D-alignment на CVA9 спрямо CVB3/B1 позволява експлицитно прехвърляне на две атомни координати на WIN-лиганда в „каньона“ на CVB1, сайт-1 и сайт-2. Второто място е забранено за лиганд в CVB1/SOF. Генерирани са повече от 100 модела и всички те са третирани с „сблъскващи анализи“ за ротамери на страничните вериги. CVB1/RES има предимно стеричен и по-малко енергиен характер. В CVB1/DEP заемането на сайт-1 е ограничено, но сайт-2 може да бъде запълнен. Молекулата WIN в сайт-2 взаимодейства със съседния VP2 протеин и всички капсомери се свързват във веригата в пентамера. Това обяснява откритието, че мутантът CVB1/DEP се нуждае от WIN съединение, за да провокира покритие. Анализът на олигомерните комплекси в тетрамерния капсомер доведе до представяне на цялата структура на капсида.

Introduction

Great number of studies have shown that the lack of success in the development of an effective chemotherapy of enteroviral infections is caused by the extraordinarily fast development of drug resistance to each of the known specific inhibitors of the

picornaviral replication no matter of their mechanism of action. The basis of the drug resistance development is the extraordinarily high mutation frequency (10^{-3} - 10^{-4}) observed in this viral family (Richards and Ehrenfeld, 1990), thus defining the

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Acta Microbiol. Bulg. 2026; 42(02) <https://doi.org/10.59393/amb26420205>

contents of the viral population of billions of quasi- or pseudospecies (Domingo *et al.*, 2008). This phenomenon is due to the high infidelity of the viral RNA-dependent RNA polymerase – on average one mutation per each newly synthesized molecule of poliovirus RNA being established (Agol, 2006). It can also explain the vast diversity of clinical manifestations caused by almost each of the *Enterovirus* genus members.

More than thirty years ago was found that WIN-compounds (arildone, disoxaril, pleconaril, etc.) inhibit virus uncoating. Based on direct crystal x-ray analysis of virus-inhibitor complexes it was shown that the primary target structure of their action is the VP1 capsid protein: inhibitors, inserted into a twisted β -sheet formed hydrophobic cleft, increased structural rigidity of VP1 subunit and thus prevent virus “undressing”. Following Darwinian evolution all WIN-treated wild viruses are blocked and the only inhibitor-resistant generation survived.

In our previous research (Nikolova and Galabov, 2003a) we proved the development of resistance to disoxaril (WIN 51711) during experimental infection with CVB1 in newborn mice treated by a 10-day course (25 mg/kg daily dose). Disoxaril is a typical representative of the WIN compounds – highly effective inhibitors of the virus replication stabilizing the virion and blocking its uncoating. The target for these compounds is the hydrophobic pocket located on the so called “canyon” on the VP1 protein (Diana *et al.*, 1989; Rossmann, 1989; Andries *et al.*, 1990). Disoxaril inhibits a broader spectrum of entero- and rhinovirus serotypes. The antiviral effect of disoxaril has been shown to be mediated predominantly through an inhibition of the virion uncoating process (Fox *et al.*, 1986). Enterovirus resistance to disoxaril has been established initially with poliovirus type 3/Sabin (Mosser *et al.*, 1994). Later, this phenomenon was observed in CVB3 to another WIN compound, pleconaril (Cloarke *et al.*, 1999; Schmidtke *et al.*, 2004).

In previous study on the application of treatment course with disoxaril in CVB1 infected mice we established that the virus population in brain samples taken after the 6th day post virus inoculation is fully resistant to disoxaril (Nikolova and Galabov, 2003a). Only three passages of the wild Cocksackievirus B1 (CVB1/SOF) in FL cell cultures in the presence of disoxaril (30 mM/ml) are sufficient for development of disoxaril-resistant Cocksackievirus B1 mutant (CVB1/RES), and nine consecutive passages of CVB1/RES mutant in FL cells in the presence of disoxaril (30mM) gave rise

to a disoxaril-dependent Cocksackievirus B1 mutant (CVB1/DEP) (Nikolova and Galabov, 2003a, b). The phenotype analysis of the disoxaril-resistant mutants manifested almost identical characteristics: size and form of plaques under agar overlay, thermosensitivity (at 50°C) and pathogenicity for newborn mice (increased in comparison to the standard disoxaril-sensitive strain) (Nikolova *et al.*, 2011).

Translation of the VP1 gene of the disoxaril mutants of Cocksackievirus B1 in protein amino acid sequences was carried out (Nikolova *et al.*, 2011). Analysis of the VP1 sequences of CVB1 mutants established an amino acid sequence highly different in the VP1 195-255 peptide of CVB1/RES: two point mutations - M213H and F237L - in the ligand-binding pocket. Upon purification of the VP1 gene of the disoxaril mutants of Cocksackievirus B1, the fragments were sequenced through the dideoxy Sanger’s method (Sanger *et al.*, 1977; Church, 1984). Then, the sequence information obtained was analyzed through comparison to the available online database with the aid of the BLAST® (Basic Local Alignment Search Tool) software package. In order to minimize the relative share of the unidentified nucleotides in the sequences, parallel comparative sequence analysis of both the coding and the matrix chain (Pairwise BLAST) (Tatusova and Madden, 1999) of each of the studied fragments was performed, thus generating a consensual sequence (M 16650) for the VP1 fragments from the genomes of disoxaril mutants of CVB1, respectively CVB1/SOF, CVB1/RES and CVB1/DEP (Nikolova *et al.*, 2011). In particular, VP1 of CVB1/SOF and VP1 of CVB1/DEP demonstrate a high degree of similarity with the referent strain (CVB1/REF) on almost the entire length of the sequence under study (82% and 89%, respectively), while VP1 of CVB1/RES features high (85%) similarity with the referent strain was found in its medium area only – this is the segment between 35th and 197th amino acid residue, i.e. outside the ligand-binding site.

The results of this analysis (Nikolova *et al.*, 2011) provide a basis for fundamentally different interpretation. For comparison, CVB1/REF features Gly on the same position. On position 251, both in the CVB1/SOF strain and in the CVB1/RES, there is a deletion of Arg (the latter is however present in the CVB1/DEP and the CVB1/REF). In the wild mutant there is a deletion of Ser observed outside the ligand-binding site, on position 25, while in the resistant mutant this position is substituted by glutamic acid. Due attention was paid to the fact that

all sequences under study, including the referent one, feature Thr instead of Pro on position 94. The resistant mutant has His instead of Met on position 213. The wild and the resistant mutants feature Leu instead of Phe on position 237.

The main goal in the present study is to construct the atomic model of CVB1 mature virus through explaining the structural basis of producing disoxaril-resistance and to propose a correct working hypothesis for understanding the nature of the disoxaril-dependent mutant. Using the 3D-structures of CVB3 and CVA9 as templates, the 3D-structures of all these proteins with one or two ligands in the “canyon” of VP1 were determined. Both steric restrictions in ‘canyon pocket’ and repulsive interaction between G207H and G207W with positively charged oxazoline groups in case of the resistant mutant were registered. High 3D-homology between CVB3 and CVA9 assumes two disoxaril-binding sites in the CVB3/B1. Site-1 is normal palmitate only site whereas site-2 (as shown in CVA9) cannot be occupied in the wild CVB1/SOF and in CVB1/RES but it is obligatory in the CVB1/DEP. Analysis of oligomer complexes in the tetramer capsomer resulted in presentation of the whole capsid structure.

Materials and Methods

Virus

Coxsackievirus B1 (Connecticut 5 strain), disoxaril-sensitive (CoxB1/SOF), from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in monolayer FL cells; plaque purified, infectious titer 9×10^7 PFU/ml; disoxaril-resistant progeny of Coxsackievirus B1 produced both in cell cultures and in mice (CoxB1/RES) and disoxaril-dependent progeny developed in cell cultures (CoxB1/DEP).

Cells

FL monolayer cultures in Costar (Corning, NY, USA) 96- or 24- well plates grown at 5 % CO₂ or in capped 20-ml scintillation glass vials were used.

Compound tested 5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy]heptyl]-methyl -isoxazole (disoxaril, WIN 51 711) was supplied by Sanofi Winthrop, Inc. (Malverne, Pennsylvania, USA).

RNA extraction and RT-PCR used are described in Nikolova et al. (2011).

Sequencing

Disoxaril mutants of CVB1 were sequenced by the dideoxy Sanger’s method (Sanger et al.,

1977). Sequence comparison of the mutants was done by BLAST® from NCBI (world data base) (Altschul et al., 1997).

Homology 3D-modeling

Five different sequences for each three of them (see below) were superimposed over strict length of 13-288 VP1 frame of VP1 chain with the help of ‘Modeller-4’ (Fox et al., 1991; Sali and Blundell, 1993) and ‘MolIDE’ (Canutescu et al., 2003) program packages. This procedure was especially necessary to obtain correct place of ligand site-2. The resulted models were visualized by programs RasWin (<http://sourceforge.net/projects/rastop>), PyMOL (<http://pymol.sourceforge.net>) and UCSF-Chimera (<http://www.cgl.uscf.edu.chimera>).

Clashing analysis

The clashing analysis was performed using “DeepView”/SPDBV 3.7 (Guex and Peitsch, 1997, 1999) – independent Force Field Energy calculations of non-bonded interactions and visualized by “Select ‘aaMaking Clashes’” as well as from SCWRL 3.0

Results

Analysis of structural differences of the Coxsackievirus B1 strain SOF mutants and ligated forms

The 3D-structure of CVB1 is unknown until now. We disposed the atomic models for CVB3 and CVA9 only (PDB ID 1cox and 1d4m, respectively). Instead of having differences in sequences they differ in occupation of “canyon”: CVB3 has one molecule palmitate (PLM), but CVA9 has two disoxaril molecules – one at virtually the same place site-1 as palmitate in 1cox (Fig. 1) and the second one, tightly fixed to the first, at the internal side of the “canyon”, here named as site-2 (Fig. 2, 3).

Because of the very high sequence homology of CVB1 with both others, we used SCRWL3 of MolIDE and Modeller-4 packages to create its 3D-structure in constant 13-281 frame for all of them. In whole 3D-analysis of five different models were provided: structures based on “B1 PLM” sequence (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “B1 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “E1 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), structures based on “E3 WIN-site-1” (CVB1/REF, CVB1/SOF, CVB1/RES, CVB1/DEP), mutants based on “Del-G WIN-site-2” (CVB1/SOF, CVB1/RES, CVB1/DEP) and mutants based on “Gap WIN-site-2” (CVB1/SOF, CVB1/RES, CVB1/

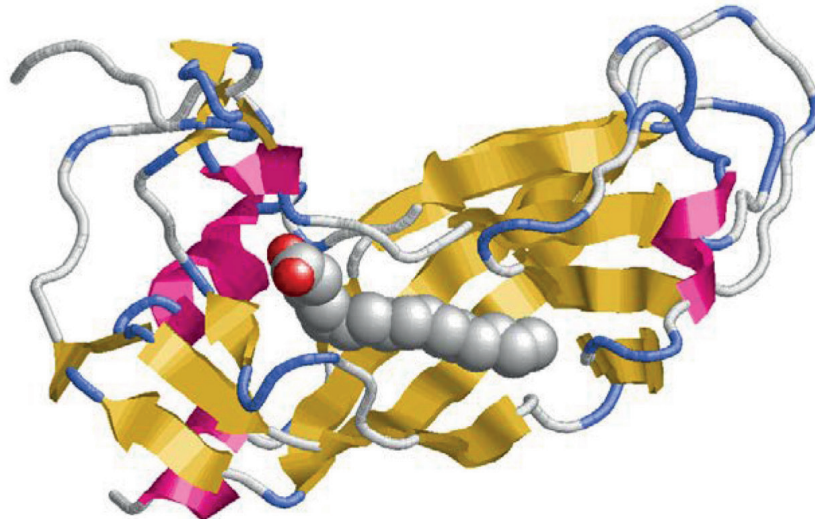


Fig. 1. Natural fulfil on the “canyon” with palmitate (PLM)

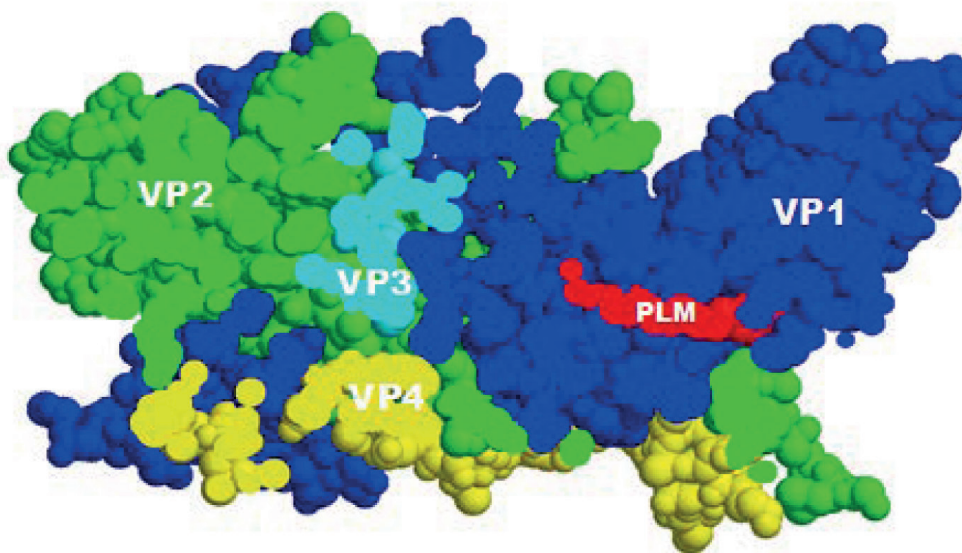


Fig. 2. VP1-4 of CVB1 in “CVA9 configuration” clipped through half-molecule (palmitate ligand in red)

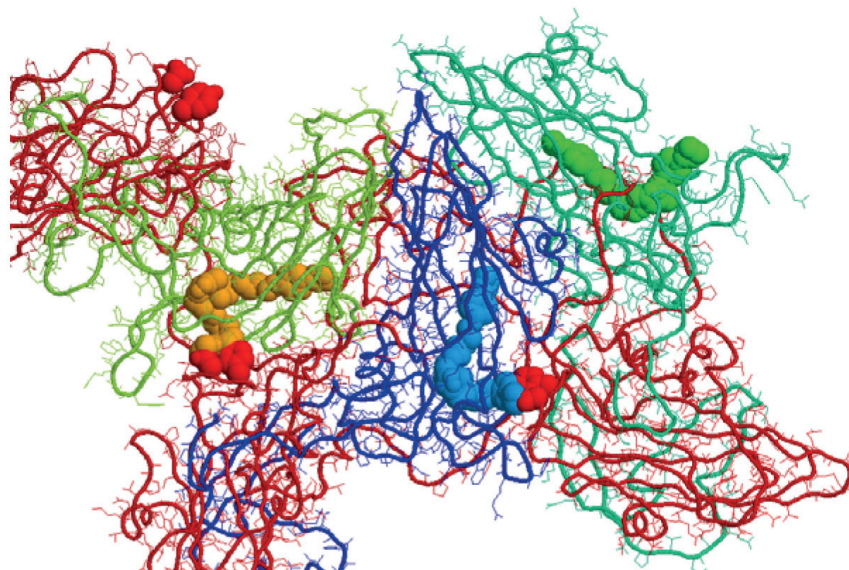


Fig. 3. CVB1 trimer of VP1-VP2 pairs with double ligands. The only site-2 ligands are in contact with VP2-residues (filled – in red)

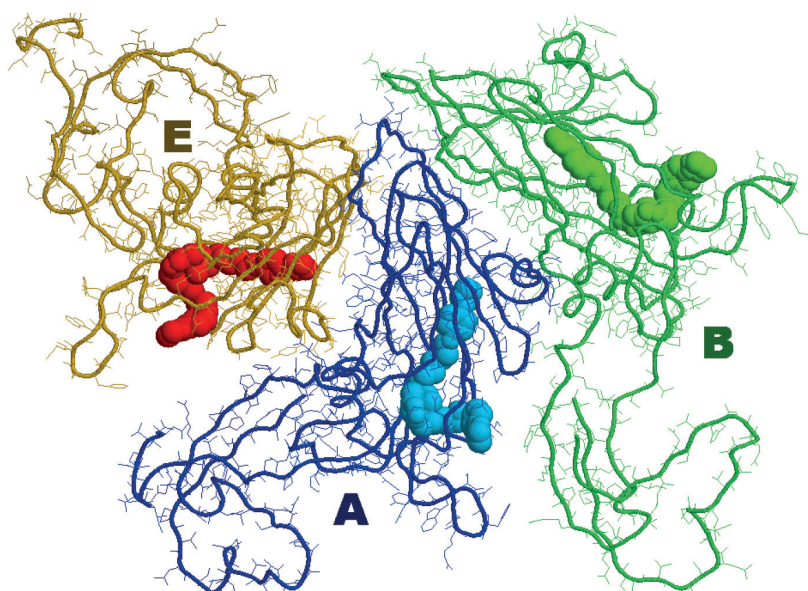


Fig. 3a. CVB1 VP1 trimer (neighbour “ABE subunits”) as a part of the pentamer. Ligands are not in contact to any neighbour VP1 chains.

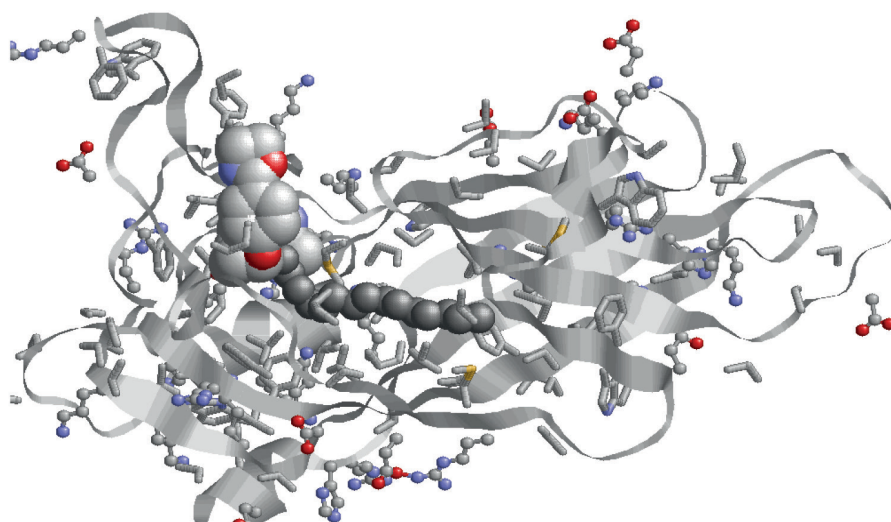


Fig. 3b. VP1 of CVB1/DEP model in CVB3 configuration with two ligands at site-1 (PLM) and at site-2 (WIN) surrounded with ionic groups (ball-and-stick with CPK collared N and O atoms) and nonpolar/hydrophobic groups (grey heavy sticks).

DEP). Differences between each of them arise from optimization procedure. It changes the selected rotamers depending of free space around neighbors. We select the above situations as follows: “B1 PLM” with “canyon” occupied by palmitate, “B1 WIN-site-1” the same but with oxazoline compound transferred from 3D-structure of CVB3 in site-1 (see below), two others “E1 WIN-site-1” and “E3 WIN-site-1” are selected from many totally optimized structures, and the last two with WIN-ligand at site-2 only – models with incorporated Gly-residue at each deletion to not shift the chain (“Del WIN-site-2”) and the sequence with deletions as “gaps” where they are (“Gap WIN-site-2”) (Fig. 4).

The file 1cox from PDB was used as universal template for all structures without and with palmitate (PLM) or disoxaril (WIN) ligands at site-1

and file 1d4m – mainly to generate ligand in both site-1 and site-2. However these two files 1cox and 1d4m have different coordinate origin. Using good overlapping we “transfer” site-2 ligand from superimposed 1d4n to 1cov with reasonable stack to site-1 ligand and without big clashing between them (about clashing see below). With these procedures best fit of WIN-ligands from CVA9 to CVB3 was achieved. Same models were used for “covering” with prepared five CVB1 sequences (see above) and generating their 3D-models.

Thus, we have a set of 22 models each repeated twice (from SCRWL3 and Modeller-4) – in total 44 models. In general, as products of independent procedures, the obtained 3D-models were accepted if their RMSD (root mean standard deviation) was $\pm 0.3\text{\AA}$.

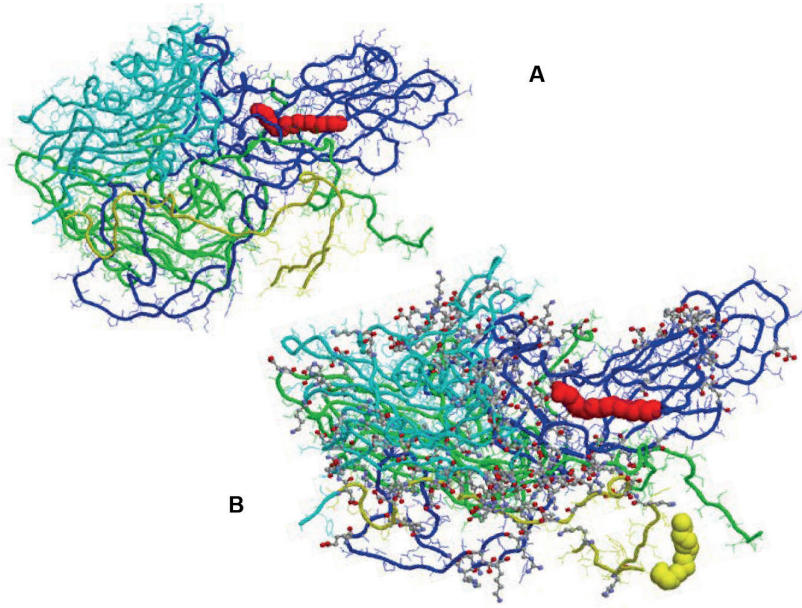


Fig. 4. Cocksackievirus in two conformations on the site-1: ligated A. CVB3 configuration “relaxed conformation”; B. CVA9 configuration “tensed conformation”).

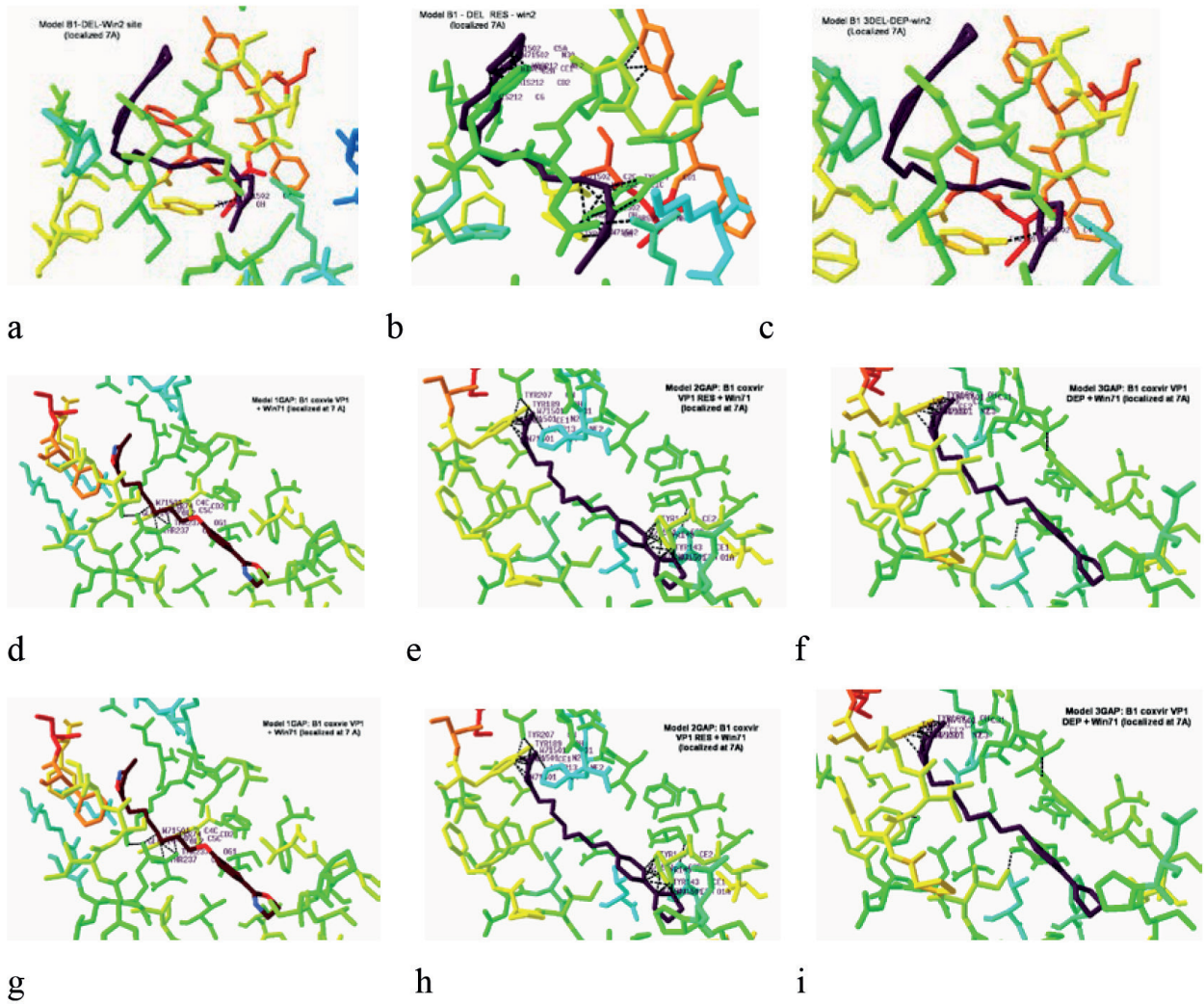


Fig. 5. (a) Model B1-DEL-Win2 site; (b) Model B1-DEL RES-Win2; (c) Model B1 3DEL DEP-Win2; (d) Model 1GAP: B1 CoxVir VP1 + Win71; (e) Model 2GAP: B1 CoxVir VP1 RES + Win71; (f) Model 3GAP: B1 CoxVir VP1 DEP + Win71 ; (g) Model 1GAP: B1 CoxVir VP1 + Win71; (h) Model 2GAP: B1 CoxrVir VP1 RES + Win71; (i) Model 3GAP: B1 CoxVir VP1 DEP + Win71

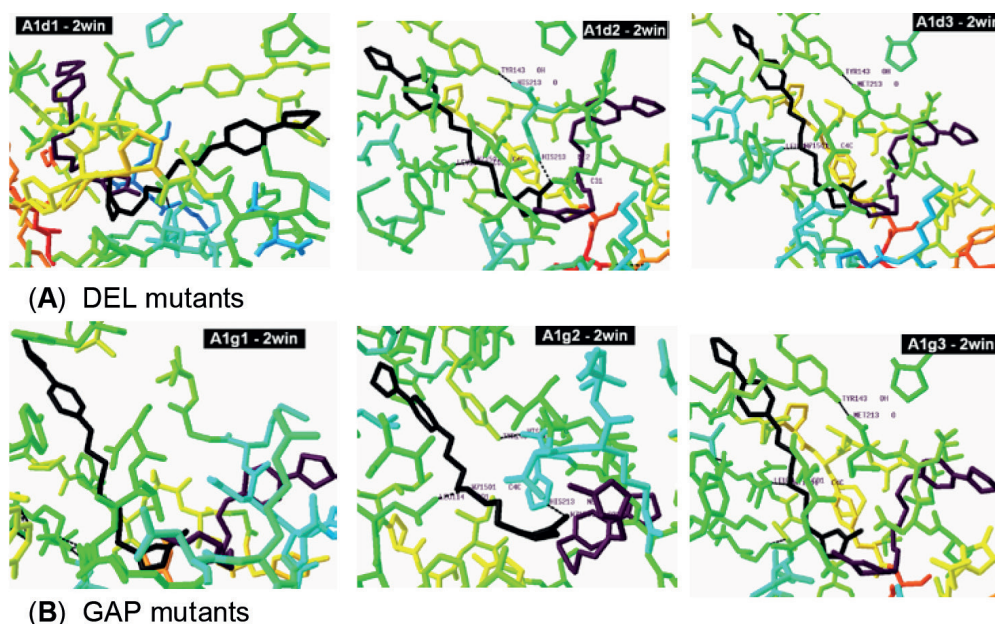


Fig. 6. Comparative clashing analysis (only few of such obtained are here shown).

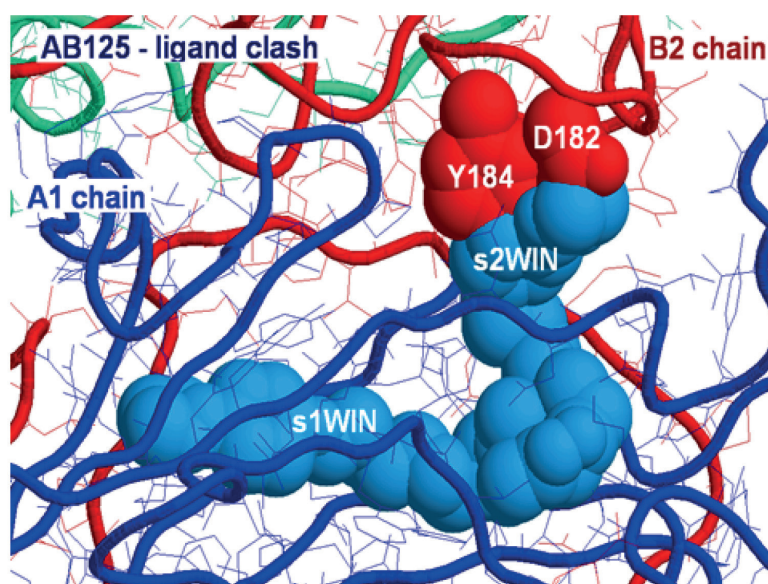


Fig. 7. Why wild CVB1 does not bind with WIN at site-2 - clashing to the neighbour “right sided” VP2.

Clashing analysis

The clashing analysis (Fig. 5, 6) of all generated structures was performed using semi-automatic procedure with and without restricted rotamers. The amino acid residues which are 7 Å from the ligand were visualized and all non-bonded atom-atom distances were checked to be more or equal of the sum of their van der Waals radii. If this sum is smaller, then the program reports “clashing” and/or calculates the energy of local repulsion. As much as 60 mono-ligand models (5x4 for each of CVB1 with PLM at site-1), CVB1 (with WIN at site-1) and CVB1 (with WIN at site-2), as well as 40 di-ligand models (5x4 for each of CVB1 with PLM at site-1 and with WIN at site-2) and CVB1 (with WIN at site-1 and with WIN at site-2) were tested. These data show conformational adaptive structures:

some of them have no clashing features (typical for mono-ligand forms of PLM/WIN at site-1) and others have relatively big number of clashings (such as predominantly mono-ligand of WIN at site-2).

The collected results demonstrated that most clashing sites are at 143, 189, 213 and 237 positions. Disoxaril resistant mutant (CVB1/RES) shows most clashing at Met213His/Arg at site-1 and less clashing of Tyr189Trp at site-2. Disoxaril-dependent mutant (CVB1/DEP) accepts WIN ligand easier than CVB1/RES mutant. It shows definitely clashing with Tyr189Trp, when the WIN-ligand is at site-1 (exclude occupation of this site) and slightly interacts with Thr237His, when it is at site-2. It has to be searched at inter-molecular level of interactions between VP1 and its neighbors (Fig. 8).

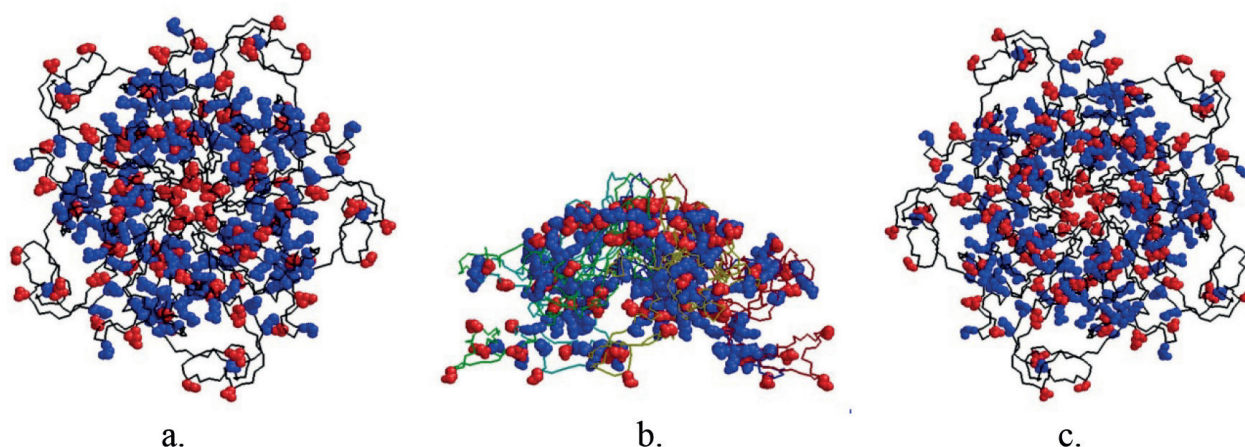


Fig. 8. Cocksackievirus B1 capsid pentamer with all-atomic (spheres) residues Asp/Glu (in red) and Lys/Arg (in blue). Each capsomer contain four VP (1-4). Surface exposed are three of them (VP1, VP2 and VP3); all five VP4 are buried inside the pentamer. Their Ntr NH₂ are amidated by myristic acids and grouped around the central hole. a - Frontal view from top; b - Sidelong view – on the top (the apex) there are 5 identical loops containing Gln125, Pro126, Gly127 and Thr128. Visualised by RasMol

This was confirmed from the data obtained for VP1 ABE trimer generated by VIPER server. From Fig. 8 is visible that the positively charged “oxazoline head” of WIN from VP1(A) (with WIN at site-2) is located between AB-dimer and interact with residues 182 and 184 from the VP2(B)-chain. At CVB1/REF sequence (Iizuka *et al.*, 1987) these two residues are Asp and Tyr, respectively. Even if the mutation does not change the nature of these residues, their slight rotamerization will omit clashing. Then, both attractive interactions between opposite charges of carboxyl and oxazoline, from one side, and aromatic stacking interactions between phenyl and oxazoline rings, from others, will be sufficient to overcome repulsion within VP1 subunit and to stabilize the capsomer.

Oligomer complexes in the tetramer capsomer and in the pentamer

The results mentioned above characterise the oligomer complexes. WIN molecules in site-2 interact the neighboring VP2 protein and all capsomers becomes chained in the pentamer coating. Analysis of oligomer complexes in the tetramer capsomer resulted in presentation of the whole capsid structure. In such a way is explained the finding that CVB1/DEP mutants need WIN compound to provoke replication. This data permits the construction of the position of oligomer complexes in the tetramer capsomer and in the pentamer (Fig. 9, 10, 11, 12).

Discussion

First of all, to understand antiviral properties of disoxaril we have to divide its structure into three parts: two oxazole/iso-oxazoline type “heads”, a phenoxy part and polymethylene - (CH₂)₇ - chain.

In contrast to palmitic acid, which represent

polymethylene chain with one unsaturated bond and one negative charged carboxylic group, disoxaril has two positively charged “heads”, both with o-inductive effects in opposite sign”: a methyl group which tends to increase N-atom pK_a of oxazole ring and a phenoxy group which decreases N-atom pK_a of next iso-oxazoline ring. Thus, both pK_a are 8.3 and 6.2, respectively (Fig. 13, 14). Other difference between palmitate and disoxaril is the relatively increased rigidity of the second if compared to the first. This was marked years ago (Diana *et al.*, 1992) as explanation of the WIN antiviral effect.

The analyses performed so far show that the primary structure of VP1 of CVB1/RES in the segment between 195th and 255th amino acid residue is entirely changed in comparison to all the rest disoxaril mutants.

Molecular basis of drug-resistance and biological properties of the picornavirus (entero- and rhinovirus) strains resistant to VP1-binding WIN compounds were object of a series of investigations. Thus, Mosser *et al.* (1994) found that in the disoxaril-resistant mutant of poliovirus 3/Sabin strain amino acid substitutions were mapped to three distinct loci in VP1 (on the inner capsid surface accompanied most probably with substitution at VP4, in the drug-binding site on the beta barrel and on the receptor-binding site on the canyon edge), while mutations conferring dependence and thermolability occurred in all four capsid proteins (VP1 to VP4).

It was established that pleconaril binds into the CVB3 VP1 hydrophobic pocket and thus stabilizes the capsid resulting in inhibition of the cell attachment and RNA uncoating. In this case RNA

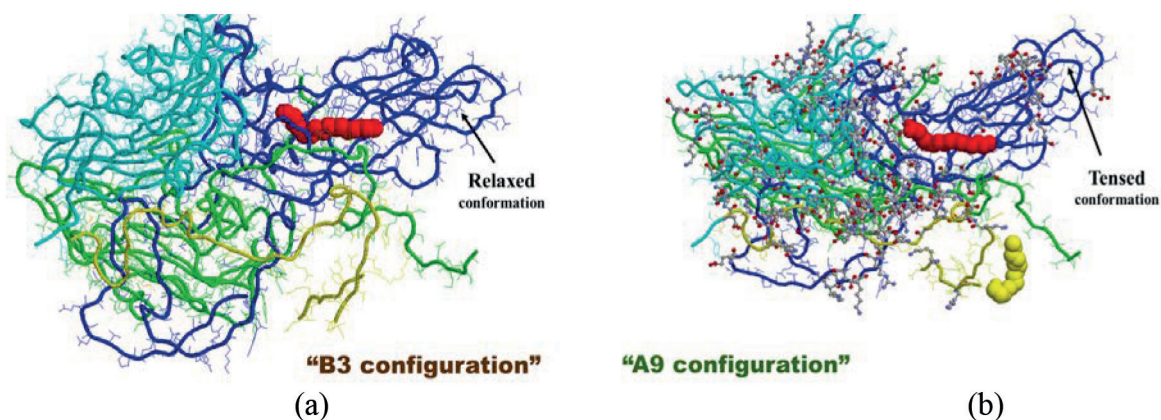
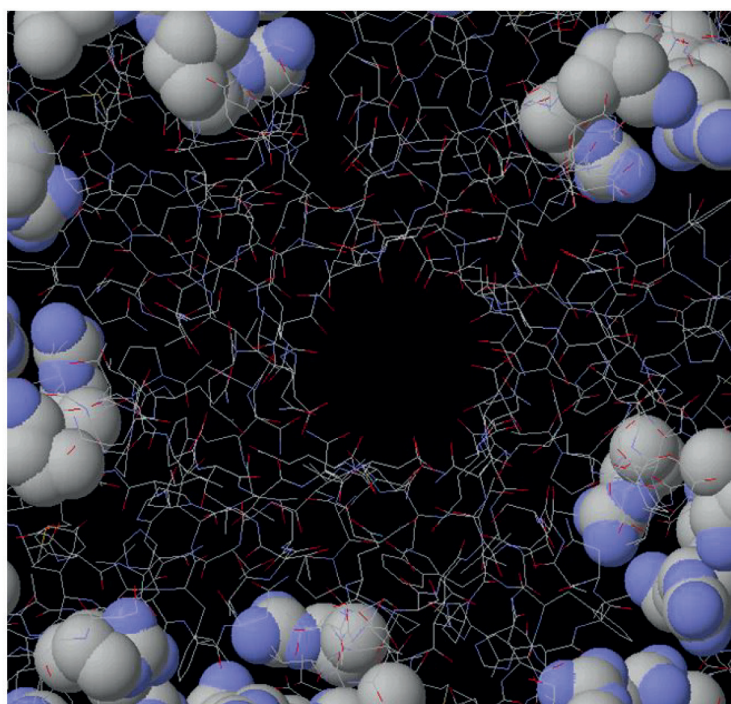


Fig. 9. CoxVir VP1 protein in Relaxed (a) and Tensed (b) configurations as demonstrated in two strains CoxVir B3 and CoxVir A9. In (b) myristyl moiety is visualised in yellow.

Coxsackievirus B1 pentamer (1/12 of full capsid)

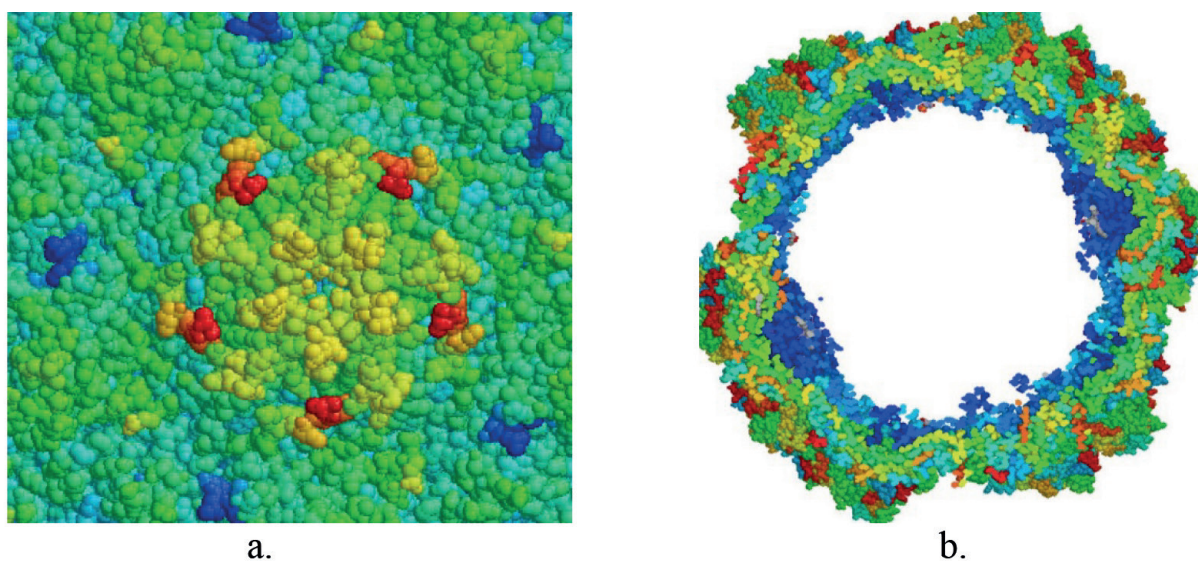
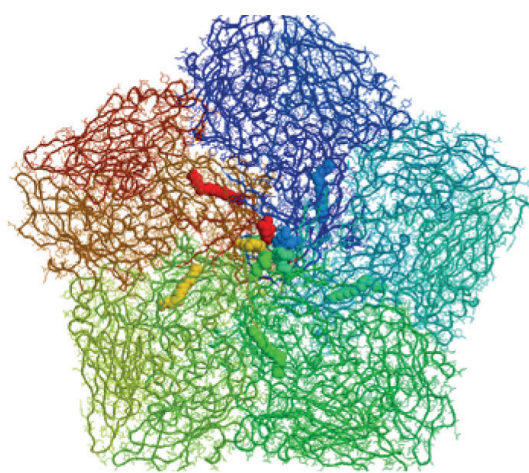
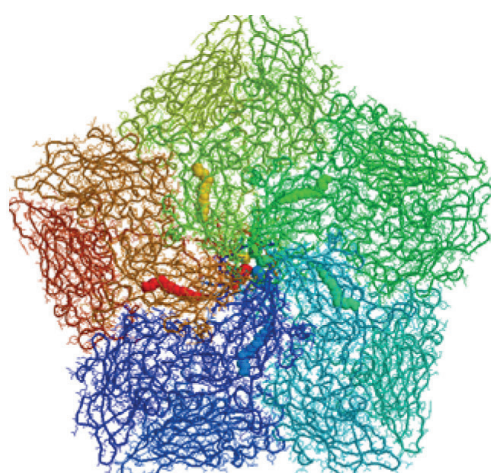


Fig. 10. Coxsackievirus B1 capsid with all-atomic spheres colored by B-factor [1]: **a.** The surface around one capsomer – most fixed in blue and most “moved” in red; **b.** The empty capsid equatorial clipped –the depth of 12 Angstroms. In the formal hole virion mRNA have to be accommodated (see crio-EM picture as in [2]).



inside view

Fig. 11A. The full pentamer



outside view

Fig. 11B. The full pentamer
All ligands are shown, too.

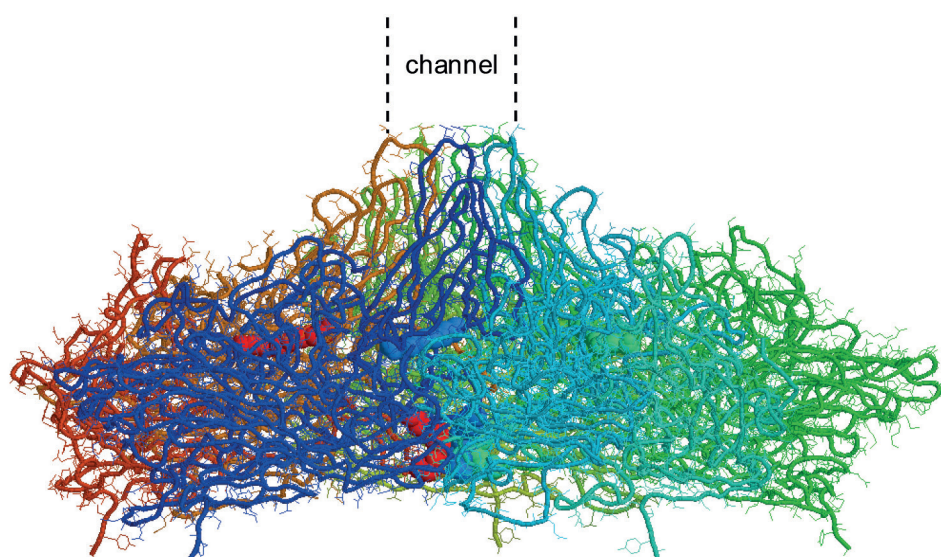


Fig. 12. The full pentamer with ligands – backside view

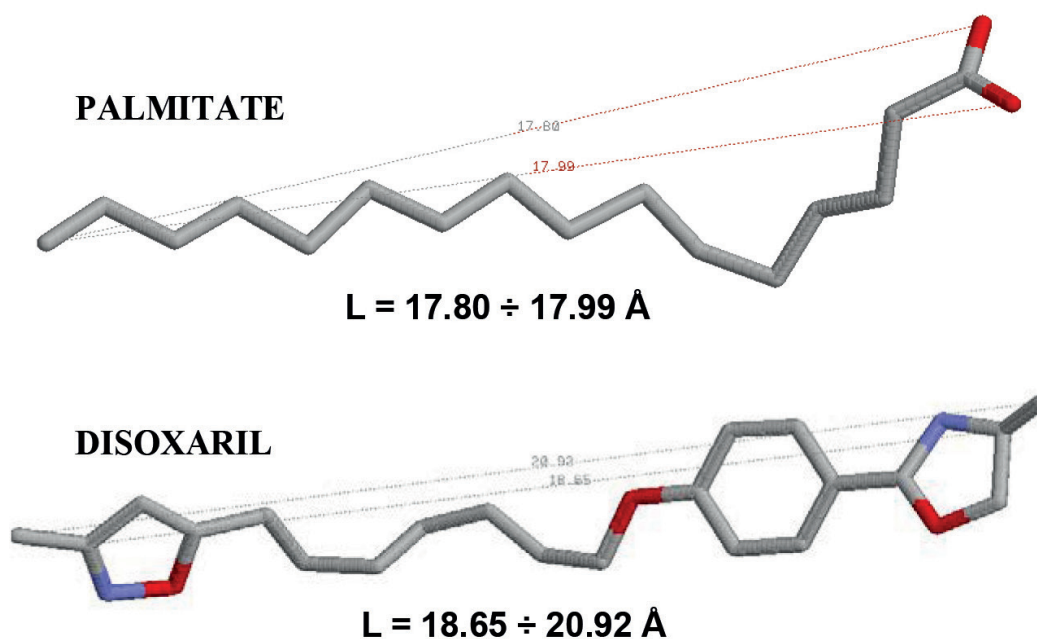


Fig. 13. Ligand length size (optimally extended chain).

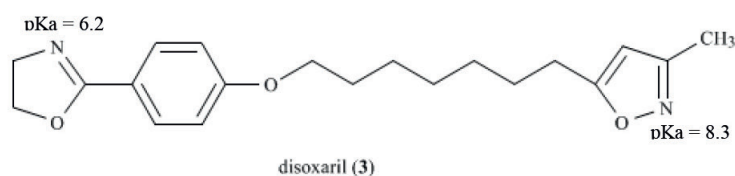


Fig. 14. Disoxaril

sequence analysis revealed amino acid changes in VP1 (Ile to Met at residue 92, and Leu to Val at residue 207) in the pleconaril-binding pocket of the drug-resistant enterovirus mutants (Mosser *et al.*, 1994). Susceptibility of CVB3 to pleconaril was found strongly associated with the VP1 amino acid at position 92. Conformational changes in the hydrophobic pocket leading to susceptibility to pleconaril occur when Leu is substituted for Ile or Val (Schmidtke *et al.*, 2005). Similar data were found in human rhinovirus (HRV) B strains, where in both naturally resistant strains and treatment-emerging resistant strains Phe at residue 152 and Leu at 192 in VP1 “canyon” replace Tyr and Val, respectively (Ledford *et al.*, 2004, 2005). Similar data were registered with HRV14 mutants resistant to WIN 52035 (Shepard *et al.*, 1993).

Our data from the timing-of-addition study with CVB1/DEP mutant showing rapid formation of infectious virus particles when disoxaril is added even at the end of the growth cycle (Nikolova *et al.*, 2011) remind the findings of Lee and Wang (2003) with WIN 52035-dependent HRV16 mutants. The requirement of drug for assembly of mutant pentamers to form provirions and the rapid assembly of preformed subunits (synthesized in the absence of drug) after drug addition suggested that after native pentamers (P5) have been formed they must be converted to an assembly active state, possibly by a conformational change induced by the binding of drug. It was proposed that the pocket factor plays the same role in wild-type virus. Three independent mutations result of VP1 single amino acid substitution conferring drug dependence at M1103T, T1208A, and V1210A were established (Wang *et al.*, 1998).

Single amino acid substitutions located in the hydrophobic pocket of VP1 determined the drug-resistance in poliovirus 2 (Sabin strain) to another VP1 ligand, 3(2H)-isoflavene, by hindering the situation of the isoflavene ring in the pocket. The mutations in the dependent strains registered both in VP1 and in VP4 are located on the surface of the capsid (Salvati *et al.*, 2004). Seven amino acid substitutions in VP1 of enterovirus 71 were revealed in the mutants resistant to pyridyl imidaz-

olidinones (Shih *et al.*, 2004).

In conclusion, the results from our study can give a clear explanation for the established phenotypic characteristic of the mutants. Obviously, in disoxaril-resistant mutants the hydrophobic pocket cavity is empty because the structure is highly restrictive to take any ligand(s); it becomes water accessible and thus – thermolabile (thermounstable). In disoxaril-dependent mutant the hydrophobic pocket is still occupied – it has one (or two) molecule(s) disoxaril and this ensures of that mutant pentamer “cross links” with increased thermostability of the wild disoxaril-sensitive strain. This guarantees a successful virus replication. The new population of disoxaril-dependent mutant needs new molecules of disoxaril for assembling of virions and to continue the virus replication. This study contributes to the capsomer mechanisms of receptor binding and viral uncoating in enteroviruses. The importance of such investigations is of big interest of investigators of enterovirus replication cycle (Zhu *et al.*, 2018).

Acknowledgements

The authors thank Jean Balanant (Pasteur Institute, Paris) for the assistance in the sequencing of the DNA samples.

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