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Characterization of Poliovirus Variants Selected for Resistance to the Antiviral Compound V-073

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V-073, a small-molecule capsid inhibitor originally developed for nonpolio enterovirus indications is considerably more potent against polioviruses. All poliovirus isolates tested to date ($n = 45$), including wild, vaccine, vaccine-derived, and laboratory strains, are susceptible to the antiviral capsid inhibitor V-073. We grew poliovirus in the presence of V-073 to allow for the identification of variants with reduced susceptibility to the drug. Sequence analysis of 160 independent resistant variants (80 isolates of poliovirus type 1, 40 isolates each of types 2 and 3) established that V-073 resistance involved a single amino acid change in either of two virus capsid proteins, VP1 (67 of 160 [42%]) or VP3 (93 of 160 [58%]). In resistant variants with a VP1 change, the majority (53 of 67 [79%]) exhibited a substitution of isoleucine at position 194 (equivalent position 192 in type 3) with either methionine or phenylalanine. Of those with a VP3 change, alanine at position 24 was replaced with valine in all variants ($n = 93$). The resistance phenotype was relatively stable upon passage of viruses in cell culture in the absence of drug. Single-step growth studies showed no substantial differences between drug-resistant variants and the virus stocks from which they were derived, while the resistant viruses were generally more thermally labile than the corresponding drug-susceptible parental viruses. These studies provide a foundation from which to build a greater understanding of resistance to antiviral compound V-073.

In 1988, the World Health Organization (WHO) launched the Global Polio Eradication Initiative (GPEI). The Initiative has relied exclusively on the oral polio vaccine (OPV), an inexpensive and easily administered live, attenuated vaccine. OPV is generally safe and has been highly effective under most circumstances. However, at a low frequency, about 1 per 750,000 vaccinees, OPV itself can cause paralysis (vaccine-associated paralytic poliomyelitis [VAPP]) (1). Normally, OPV viruses are excreted in the stool of healthy vaccinated individuals for several weeks. Should these excreted vaccine-related polioviruses continue to circulate (termed circulating vaccine-derived polioviruses [cVDPVs]), virus reversion to neurovirulence can occur and result in paralytic disease and outbreaks. Moreover, when individuals with a primary immune deficiency, such as agammaglobulinemia, receive OPV, the virus may replicate persistently, accumulating genetic changes associated with reversion to neurovirulence. The resulting immunodeficiency-associated VDPVs can be excreted for years, posing a risk of paralysis for the infected individual and compromising efforts to eradicate the virus. As wild poliovirus transmission is eliminated, and VAPP and VDPV cases continue to occur, the risks of OPV use will outweigh its benefits. Thus, part of the GPEI strategy involves the global cessation of OPV use when it is determined wild poliovirus transmission has ceased (2).

After reviewing current and post-OPV risks, the National Research Council of the National Academies concluded that at least one, preferably two, polio antiviral drugs be developed as a supplement to the tools currently available for control of polio outbreaks posteradication (4, 5).

V-073, a small-molecule capsid inhibitor originally developed for nonpolio enterovirus indications (3), was recently found to be considerably more potent against polioviruses (14). V-073 is currently being advanced for possible use in the management of poliovirus chronic infections and laboratory incidents. While V-073 is active against all poliovirus isolates tested to date (14), due to the quasispecies nature of RNA viruses, there likely exist drug-resistant variants at low levels in drug-susceptible virus populations. Treatment-emergent drug resistance could potentially have medical and public health implications. On the other hand, drug-resistant virus variants may be enfeebled or otherwise more benign. Thus, it is important to understand drug resistance and its potential consequences.

Here, we describe the selection and isolation of V-073-resistant poliovirus variants in cell culture, the genetic basis for resistance, and several features of the variants, including cell culture growth, phenotypic stability in cell culture, and thermal stability of virion infectivity. The antigenic, immunogenic, and pathogenic features of V-073-resistant polioviruses are reported elsewhere (9).

MATERIALS AND METHODS

Viruses. Type 1 VDPV isolates 10235 (cVDPV-1 isolated from the Dominican Republic, GenBank accession no. AF405625) and 10224 (cVDPV-1 isolated from the United States), type 2 isolate 10230 (cVDPV-2 isolated from Egypt, GenBank accession no. AF448783), and type 3 isolate 10805 (cVDPV-3 isolated from Iran, GenBank accession no. EU684056) were obtained from the Centers for Disease Control and Prevention (CDC) Polio and Picornavirus Laboratory collection and propa-

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gated on LLC-MK_2 cells at 37°C (types 1 and 2) or 36°C (type 3) in minimal essential medium (MEM) with Earle's salts (Invitrogen, Carlsbad, CA), supplemented with 2% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, or Thermo Scientific, Lafayette, CO).

Plaque assays. LLC-MK_2 monolayer cells were grown in six-well cell culture plates and washed with MEM (Invitrogen, Grand Island, NY). Tenfold serial dilutions of each virus were prepared in MEM, and 200 μl was inoculated into each well. After infection at room temperature for 45 to 60 min, 2.5 ml of overlay solution was added, consisting of 0.45% agarose (SeaKem LE agarose; Lonza, Rockland, ME) in MEM with 2% FBS. After the agar overlay had gelled, the plates were incubated at 36°C or 37°C, as described above, for 36 to 46 h. The overlay was removed, and the cells were stained with a solution of 0.4% formaldehyde and 0.03% crystal violet for 30 min. The plates were then washed once with water and allowed to dry overnight. Plaques were counted, and virus titer was determined.

Selection of poliovirus variants with reduced susceptibility to V-073. For each virus, 10 independent substrains were obtained by plaque purification. The substrains were amplified in LLC-MK_2 cells in MEM with 2% FBS. For drug-resistant variant selection, serial dilutions of each plaque-purified virus were inoculated into LLC-MK_2 cells in six-well plates and covered under agar overlay with either no drug, drug at a final concentration of 10 times the median effective concentration (EC_{50}) for that particular virus (10× EC_{50}), or drug at 50 times the EC_{50} (50× EC_{50}). Drug (V-073) was provided by ViroDefense, Inc. The susceptibility of these strains to V-073 was reported recently (14). For each parental virus, two plaques from each selection condition were selected and propagated in LLC-MK_2 cells under the selection condition. Drug resistance frequency was calculated by dividing the titer of the surviving plaques in the presence of 50× EC_{50} V-073 by the titer of parental plaque-purified virus in the absence of drug.

Drug susceptibility assay. Susceptibility of parental and drug-resistant variants to V-073 and determination of the EC_{50}s were performed as described previously (15). Briefly, drug and virus were combined with LLC-MK_2 cells in 96-well plates in a cross-titration format to ensure reaching endpoints for both drug and virus titrations, with duplicate wells for each drug-virus concentration. After 3 days of incubation at 37°C, the plates were stained with crystal violet, washed three times with water, and allowed to dry overnight. Viral cytopathic effect was measured by reading the absorbance at 590 nm. EC_{50}s were derived by analyzing dose-response absorbance values by four-parameter curve fitting using Prism 5.04 (GraphPad Software, Inc., La Jolla, CA).

Identification of amino acid changes in V-073-resistant variants. Viral RNA was extracted and purified by using the QiaAmp viral RNA mini-kit (Qiagen, Inc., Valencia, CA). The VP3 and VP1 capsid genes were amplified by reverse transcription-PCR. PCR products were sequenced with the PCR primers and additional primers within each amplicon. The sequences of PCR and sequencing primers used are available upon request. Sequences were assembled using Sequencher (version 4.8; Gene Codes Corp., Ann Arbor, MI) and analyzed using the Wisconsin Sequence Analysis Package, version 11.0 (Accelrys, San Diego, CA). The sequences were submitted to the GenBank sequence database under accession numbers JN105289 to JN105295.

Stability of V-073-resistant phenotype. V-073-resistant viruses representing the two predominant variant classes were subjected to 10 cycles of passage in LLC-MK_2 cell cultures in the absence of drug. Viruses were initially inoculated onto 10^5 LLC-MK_2 cells in 24-well plates at a multiplicity of infection (MOI) of 0.01 and then passaged every 3 days by inoculating 50 μl of a 10^{-4} virus dilution onto 10^5 fresh LLC-MK_2 cells in 0.5 ml of MEM supplemented with 2% FBS. At the tenth passage, the EC_{50} of the virus populations was determined. The distribution of resistant and susceptible viruses was also determined by plaque assay in the presence or absence of V-073 at 10× the respective EC_{50}s, as described above.

Single-step growth curves. Single-step growth of the parental and drug-resistant variants was carried out in LLC-MK_2 cell monolayers in 24-well plates. Cells were washed with MEM and infected at an MOI of 10 with the test virus in replicate plates. After the plates were incubated for 45 min at room temperature, the inoculum was removed, and the cells were washed with MEM without serum, and then 0.5 ml of MEM supplemented with 2% FBS was added (in the absence of V-073), and the plates were incubated at 37°C in an atmosphere of 5% CO_2. At 0, 2, 4, 6, 8, and 10 h postinoculation, a plate was frozen at −70°C. Plates were freeze-thawed three times, and the virus titers in the supernatants were determined by plaque assay in the absence of V-073.

Virion thermal stability. The kinetics of heat inactivation was measured by incubating variant and parental viruses at 46°C for 0, 5, 15, 30, or 60 min. The titers of the treated viruses were determined by plaque assay in the absence of V-073. For each time point, stability at 46°C was expressed as a ratio relative to the titer of virus obtained at 0 min.

Poliovirus sequence analyses. VP1 and VP3 sequences of the drug-resistant variants were compared to a database of sequences composed of circulating wild and vaccine-derived polioviruses using a customized search program implemented in MATLAB R2010a (The MathWorks, Natick, MA).

Structural modeling. Protomer homology models representing each parental VDPV strain were created using the SWISS-model server (http://swissmodel.expasy.org/) (17) using the template PDB coordinate file 1EAH as a reference. Point mutations for each subsequent protomer were created using the "Mutator" plug-in associated with the Visual Molecular Dynamics (VMD) software package (8). Custom parameter data for the associated V-073 molecule and covalently bound myristic acid were obtained using the SWISS-param server (http://swissparam.ch/) (18).

Each protomer was assembled into a pentamer configuration using the biological assembly data contained in the 1EAH crystal structure metadata file. Pentamers were then solvated using a TIP3 water model in a cuboidal system with 12-Å padding on each axis and ionized with sodium and chloride ions at a concentration representing 0.154 M. Energy minimization and molecular dynamics simulation for 1 nanosecond at 37°C and one atmosphere pressure, was performed using the Nanoscale Molecular Dynamics (NAMD) software package (16) on an SGI Altrix supercomputer housed at the Victorian Life Sciences Computation Initiative, Melbourne, Australia. Postsimulation pentamers were deconstructed into five individual protomers and fit-aligned to the original 1EAH coordinates and a final average protomer structure obtained. Simulation data analysis and image generation was achieved using VMD.

RESULTS

Isolation of V-073-resistant poliovirus variants from susceptible virus populations. Previously, we showed that 45 distinct poliovirus isolates, including wild, vaccine, vaccine-derived, and laboratory strains of poliovirus, were all susceptible to the capsid inhibitor V-073, with an MIC for 90% of isolates (MIC_{90}) of 0.076 μM (14). However, due to the quasispecies nature of RNA viruses, there likely exist drug-resistant variants at low levels in drug-susceptible virus populations. Indeed, poliovirus variants with reduced susceptibility to V-073 were isolated from drug-susceptible virus populations by virus growth in cell culture in the presence of V-073. Ten independent wild-type (parental) virus plaques were picked for each of four polioviruses, two VDPV type 1 isolates (strains 10224 and 10235), one VDPV type 2 isolate (strain 10230), and one VDPV type 3 isolate (strain 10805). The plaques were each amplified by a single passage in LLC-MK_2 cells to allow generation of a quasispecies population ("pool") from which drug-resistant variants could be selected. Viruses from each parental pool were propagated in LLC-MK_2 cells in the absence or in the presence of V-073 at either 10× or 50× the EC_{50} for the respective parental virus. Surviving plaques were quantified and their titers compared to that of their parental virus. Under these

TABLE 1 Frequency of V-073-resistant variants in drug-susceptible poliovirus populations

Virus	Parent EC_{50} (μM)	Resistance frequency $\times 10^{-5}$ (95% CI)[a]	
		Selection at 10× EC_{50}	Selection at 50× EC_{50}
VDPV-1 10224	0.069	30.4 (24.0–38.4)	5.61 (4.25–7.41)
VDPV-1 10235	0.018	3.20 (2.24–4.57)	3.31 (2.50–4.37)
VDPV-2 10230	0.036	26.5 (18.2–38.7)	11.8 (7.48–18.6)
VDPV-3 10805	0.029	42.7 (18.2–100)	30.8 (15.6–61.0)

[a] 95% CI, 95% confidence interval.

selection conditions, the frequency of V-073-resistant variants in populations of the polioviruses defined by selection at 10× the parental virus EC_{50} ranged from 3.20 to 42.7 $\times 10^{-5}$ (Table 1). Similar frequencies were observed when selection was conducted at 50× the EC_{50} (3.31 to 30.4 $\times 10^{-5}$).

Genetic basis of resistance. To determine the genetic basis of V-073 resistance, two surviving plaques from each of the parental pools (20 drug-resistant variants from each selection condition [10× and 50× EC_{50}] derived from each of four parental viruses), 160 variants in all, were isolated. Initially, the complete capsid region of two drug-resistant variants from type 1, type 2, and type 3 VDPVs were sequenced and compared to their respective parental virus sequence. Amino acid changes in the variants that correlated with the resistance phenotype were restricted to capsid protein coding regions for VP3 and VP1. This is consistent with the observation that VP3 and VP1 are the only proteins in contact with the drug when bound to the virus (10). Therefore, we subsequently sequenced the VP3 and VP1 coding regions of all variants and their parental virus.

For each independently selected resistant virus, a single amino acid change relative to its parental virus sequence in either VP1 or VP3 was associated with the resistant phenotype (Table 2). Two amino acid positions were predominantly associated with drug resistance and together represented 91% (146 of 160) of all resistant variants. In VP1, the isoleucine at position 194 (equivalent position 192 in type 3 polioviruses) was replaced by either a methionine or phenylalanine in 53 of 160 (33%) of the resistant variants analyzed, with an apparent preference for phenylalanine (38 of 54 [70%]). In VP3, the alanine at position 24 was replaced exclusively with valine in 93 of 160 (58%) of the resistant viruses. Two minor resistant variants were also observed, each representing 4% (6 of 160) of the total viruses analyzed, one found exclusively in the type 1 virus 10224, in which the isoleucine at VP1 position 183 was replaced with threonine, and the other, from the type 3 virus 10805, in which the phenylalanine at VP1 position 237 (position 236 in type 3) was replaced with leucine. The amino acid change at VP1 position 237 was also found in 6 out of 40 resistant variants of another type 2 VDPV (data not shown). There appeared to be no consistent amino acid substitution bias associated with the two drug selection concentrations.

Stability of the resistance phenotype. For further characterization of viruses resistant to V-073, we focused on variants exhibiting the two predominant amino acid substitutions, VP1 I194M/F and VP3 A24V. One representative of each variant for each of the three poliovirus serotypes (6 resistant variants in all), together with their respective drug-susceptible parents, were characterized. The susceptibilities to V-073 of the parental viruses and their resistant variants, expressed as EC_{50}s, are provided in Table 3. The reduction in susceptibility of the variants relative to their parents ranged from 38-fold to >556-fold. To ascertain the stability of the resistance phenotype, the six resistant variants were cultured in the absence of V-073. After 10 passages in the absence of drug selection pressure, the proportion of the virus population

TABLE 2 Amino acid changes associated with resistance to V-073

Virus	EC_{50}	Amino acid change (no. of strains)[a]						No. of viruses analyzed
		VP1					VP3	
		A, 88	P, 161	I, 183	I, 194[b]	F, 237[c]	A, 24	
VDPV-1 10224	10×		S (2)	T (6)	M (1) F (3)		V (8)	20
	50×	T (1)[d]					V (19) V (1)	20
VDPV-1 10235	10×				F (9)		V (11)	20
	50×				F (11) M (1)		V (8)	20
VDPV-2 10230	10×				M (11)		V (9)	20
	50×				M (2)		V (18)	20
VDPV-3 10805	10×				F (3)	L (4)	V (13)	20
	50×				F (12)	L (2)	V (6)	20
Totals	10× (%)		2 (2)	6 (8)	27 (34)	4 (5)	41 (51)	80
	50× (%)	1 (1)	0 (0)	0 (0)	26 (32)	2 (2)	52 (65)	80
	Total (%)	1 (1)	2 (1)	6 (4)	53 (33)	6 (4)	93 (58)	160

[a] Values for VP1 and VP3 are expressed as amino acidchanges (number of changes) except as noted for the totals in column 1. Columnsubheadings indicate the "wild amino acid, residue number."
[b] Equivalent residue 192 in type 3.
[c] Equivalent residue 236 in type 3.
[d] One virus had changes at both residues, $VP1_{88}$and $VP3_{24}$.

TABLE 3 Stability of the V-073 resistance phenotype upon cell culture passage[a]

Virus	Amino acid change	EC_{50} (μM)		Passage 10 (% resistant)[b]
		Passage 1	Passage 10	
VDPV-1 10235 (parent)		0.018	NA[c]	NA
10235.1	VP1 I194F	>10	>10	82
10235.3	VP3 A24V	1.4	0.95	69
VDPV-2 10230 (parent)		0.036	NA	NA
10230.4	VP1 I194 M	1.5	3.0	43
10230.8	VP3 A24V	>10	0.31	93
VDPV-3 10805 (parent)		0.029	NA	NA
10805.1	VP1 I192F	>10	>10	97
10805.5	VP3 A24V	1.1	1.0	37

[a] Passage was performed in the absence of drugselection.
[b] Resistance was determined at 10× the parental EC_{50}.
[c] NA, not applicable.

that maintained the resistance phenotype was determined by plaque assay in both the absence and presence of V-073 at 10× the EC_{50} for the respective virus. As shown in Table 3, after 10 passages in the absence of drug selection pressure, the percentage of the virus population retaining the resistant phenotype ranged from 100% (strains 10230.8 and 10805.1) to 37% (strain 10805.5), suggesting that the resistant phenotype was generally stable in cell culture and not strongly selected against. The EC_{50}s for the V-073-resistant viruses in the passage-10 virus populations were determined and found to be largely the same as the passage 0 virus for five of the six resistant viruses studied (Table 3). However, in the strain 10230.8 passage-10 population, the EC_{50} was reduced from >10 μM to 0.31 μM, suggesting a recovery of drug susceptibility when propagated in the absence of drug selection, but it was still 10-fold less susceptible than its parental virus.

Virus growth in cell culture. To assess the virological consequences of drug resistance, studies were performed in which the resistant variant viruses were compared to their respective drug-susceptible parental virus population. In the first of these, a single step growth curve in cell culture of the predominant VP1 and VP3 variants in the absence of drug was conducted in parallel with the respective parental virus. There appeared to be no substantial difference in the single step growth curve profiles for any of the resistant variants relative to their respective parental virus, using an MOI of 10 (Fig. 1). All variants replicated to approximately the same peak titer and with approximately the same kinetics as their respective parental strain, indicating there was no major deficit in viral replicative capacity in cell culture. Similar results were also observed at MOI of 1 or 0.1 (data not shown).

Thermal stability of virus infectivity. It has been reported previously that drug resistance to enterovirus capsid inhibitors results is a more thermally labile virion (12). Thermolability is thought to be due to the presence of bulkier amino acids in the pocket, impacting the binding of natural factors that bind into the pocket and stabilize the virion in the extracellular space. To investigate whether the changes necessary to confer V-073 resistance affect virion stability, V-073-resistant variants were heated in culture medium without FBS at 46°C for various periods of time, after which the surviving poliovirus infectivity was determined. Four of the six resistant variants were substantially more heat labile than their respective parental virus, while two variants (10235.1 and 10230.4) showed only a slight reduction in thermal stability (Fig. 2).

Structural modeling of drug-resistant variant viruses complexed with V-073. To better understand the mechanism of V-073 resistance, we modeled the amino acid changes responsible for resistance in all three polio serotypes, based on the known crystal structure of V-073 complexed with poliovirus type 2 (Fig. 3). The isoleucine at $VP1_{194\ (192)}$ and alanine at $VP3_{24}$ both have hydrophobic interactions with the dichlorophenyl portion of the drug. The observed substitutions in $VP1_{194\ (192)}$ and $VP3_{24}$ disrupt this interaction and cause deformation of the drug-binding pocket. The amino acid residues of the minor variants (Table 2) are also predicted to impinge on the drug-binding pocket (data not shown). The phenyl group of Phe_{236} residue in VP1 virus appears to interact with the chloro-methoxyphenyl portion of the drug, and this interaction is ablated in the type 3 virus with Leu substituted at residue 236.

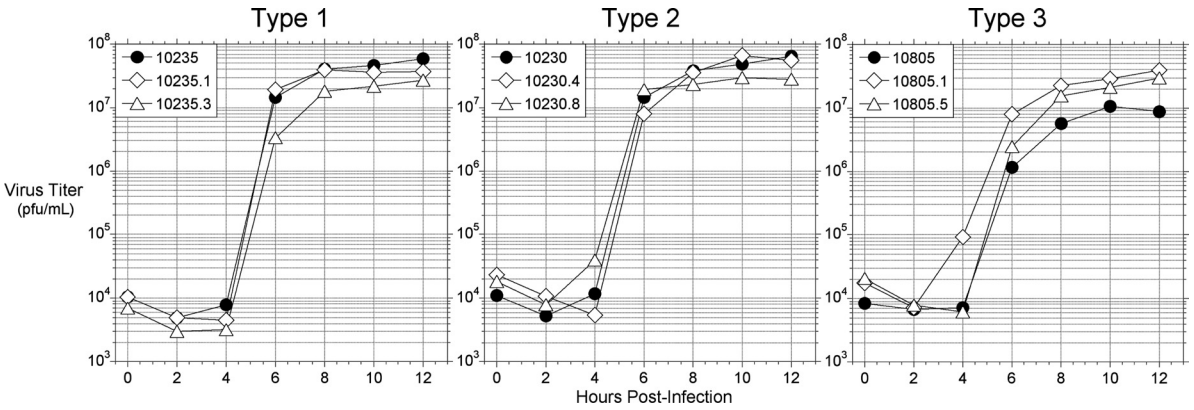

FIG 1 Single-step growth curves. Cultures of LLC-MK_2 cells were infected at an MOI of 10 with drug-susceptible parent virus and two drug-resistant variants for each poliovirus type (types 1, 2, and 3) described in Table 3. The amount of infectious virus present at various times postinfection was quantified by plaque assay. Solid circles, parental viruses; diamonds, VP1 I194 variants (VP1 I192 for type 3); triangles, VP3 A24 variants.

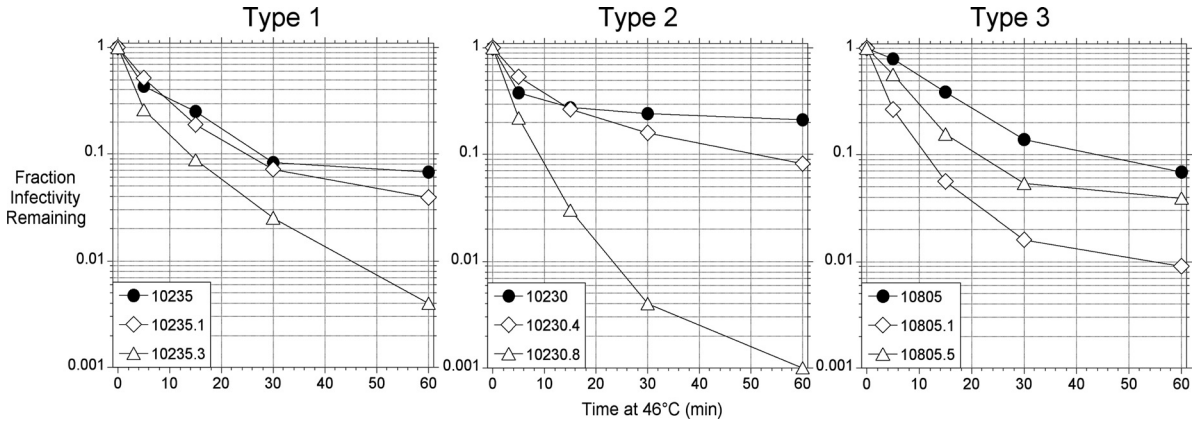

FIG 2 Thermal inactivation of virus infectivity. Drug-susceptible parental virus and two drug-resistant variants for each poliovirus type (types 1, 2, and 3) described in Table 3 were exposed to 46°C for various times, and the remaining infectivity determined by plaque assay. Solid circles, parental viruses; diamonds, VP1 I194 variants (VP1 I192 for type 3); triangles, VP3 A24 variants.

DISCUSSION

The National Research Council of the National Academies has recommended that at least one, preferably two, polio antiviral drugs be developed as a supplement to the tools currently available for control of polio outbreaks posteradication (4, 5). Pursuant to this recommendation, poliovirus-specific capsid inhibitor V-073 is being advanced clinically to assess the potential utility of poliovirus antiviral drugs in the treatment of chronic poliovirus infec-

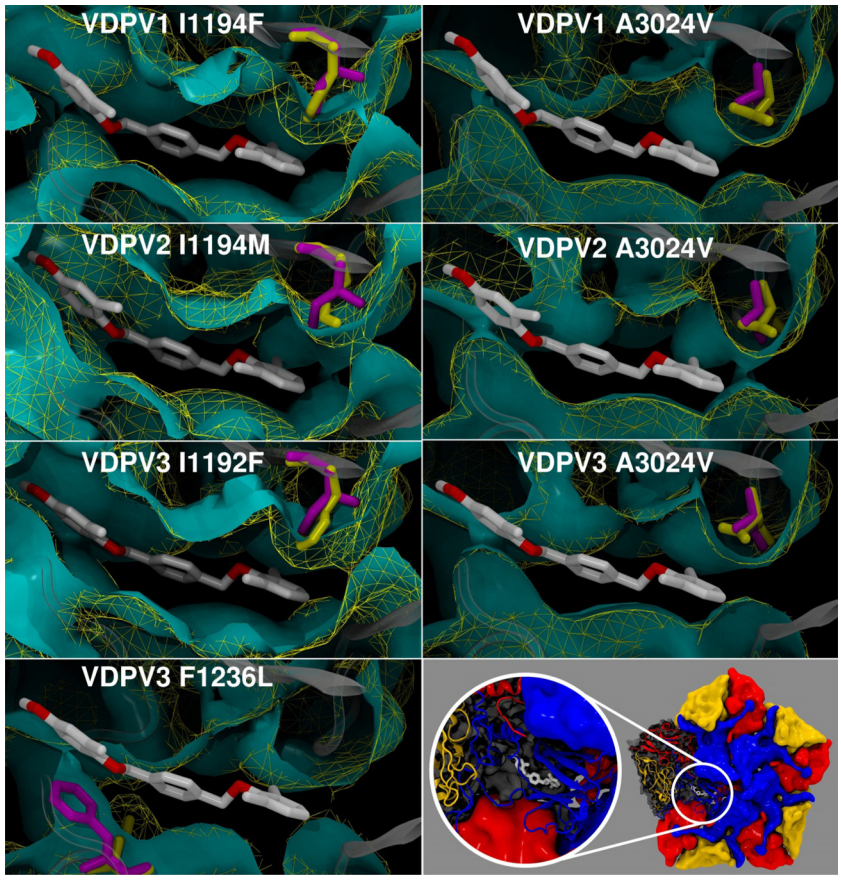

FIG 3 Location in the poliovirus type 2 structure of predominant amino acids substitutions that confer resistance to V-073, based on the crystallographic structure of V-073 bound to poliovirus type 2 (10). Superolateral cutaway view of electron density maps showing V073 bound within the hydrophobic pocket of VP1. Density mapping at 1 Å resolution and 2 Å radius representing the original parental strain (cyan) with a wireframe overlay (yellow) of the resistant virus. Amino acid differences are shown as a liquorice representation with parental (magenta) and variant (yellow). The final frame in the figure shows the position of V073 (white) relative to the overall pentamer structure with VP1 (blue), VP2 (yellow), and VP3 (red); VP4 is not visible in this representation.

tions and management of polio incidents. As with the application of any antiviral drug, the issue of treatment-emergent drug resistance presents a potential obstacle to implementation. It is important to understand the potential for and consequences of antiviral resistance.

We show here that poliovirus variants with reduced susceptibility to V-073 can be isolated in cell culture from otherwise drug-susceptible virus populations. The frequency of these variants in virus populations was estimated at 3.20×10^{-5} to 42.7×10^{-5} (geometric mean, 16.8×10^{-5}). The frequency of drug resistance observed with V-073 is similar to that reported for other capsid inhibitors. For example, the frequency of HRV14 resistance to WIN 52084 was about 4×10^{-5} (7), and that of coxsackievirus B3 to pleconaril was 5×10^{-5} (6).

It was reported previously that poliovirus type 3 variants selected for resistance to capsid inhibitors may also be dependent on drug for their growth (11–13). Drug dependence is characterized by plating indices (ratio of the plaque titer in the presence of drug to the plaque titer in the absence of drug) of about 400 to >2,000, while nondependent drug-resistant variants have values close to 1 (i.e., similar plaque titers with or without drug) (11). The variants studied here had plating indices that ranged from 0.42 to 1.24, indicating a lack of drug dependence. It is unclear why we did not observe drug-dependent viruses in our study; however, it has been reported that the frequency of such viruses may vary widely among different enteroviruses (11). Of course, the physicochemical properties of the drug compound may also influence the type of variants detected and V-073 is chemically distinct from the compounds used to select drug-dependent variants, despite their common mechanism of action.

As described previously for other picornavirus capsid inhibitors, the basis for resistance lies in single amino acid substitutions involving residues that line the drug-binding pocket (6, 7, 12). Here, sequence analysis of 160 independent resistant variants (80 isolates of poliovirus type 1, 40 isolates each of types 2 and 3) established that V-073 resistance also involved single amino acid changes, predominantly at two sites, at isoleucine residue 194 in VP1 (192 in poliovirus type 3) or in VP3 at alanine 24. Minor sites where an amino acid change conferred resistance include proline_{161}, isoleucine_{183}, and $\text{phenylalanine}_{236}$, all in VP1. The locations of the amino acid substitutions are consistent with those observed in polioviruses and rhinoviruses that are resistant to other capsid-binding antiviral compounds (7, 12). For example, the I1192F change confers resistance to WIN 51711 in PV3 (12) and V1188M, lying in a similar position in human rhinovirus 14, confers resistance to the related compound, WIN 52084 (7).

The resistant viruses studied in this report were derived in the laboratory from drug-susceptible virus populations by selection in cell culture. When a collection of naturally occurring polioviruses, including wild polioviruses and VDPVs, were tested for V-073 susceptibility, all of the 42 polioviruses were susceptible, with EC_{50}s ranging from 0.003 to 0.126 μM (14). In that study, all polioviruses possessed the drug-susceptible genotype encoding isoleucine at residue 194 (192 in type 3) in VP1 and alanine at residue 24 in VP3. When the CDC database of 4,500 VP1 sequences and 250 VP3 sequences, representing all known poliovirus genotypes, including sequences for recently circulating lineages, was inspected, there was an absolute conservation of these two residues. Thus, while we are able to select for and isolate V-073-resistant variants from drug susceptible virus stocks in the laboratory, the above observations suggest that changes at these positions are rare in nature.

Our initial cell culture investigations into the stability and fitness of V-073-resistant variants presented here revealed mixed results. Single cycle cell culture growth of resistant variants was indistinguishable from that of their corresponding parental viruses, an observation described previously for other picornavirus capsid inhibitors (6, 11). Furthermore, the drug resistance phenotype appeared relatively stable upon cell culture passage of the resistant viruses studied; however, one of the six variants studied appeared to reacquire some level of drug susceptibility. Finally, the thermal stability data suggested generally reduced virion stability of the resistant viruses compared to their susceptible parents, a feature previously noted for picornavirus capsid inhibitor resistance (6, 11).

To explain the lack of drug-resistant viruses among a broad panel of poliovirus isolates, as well as the lack of genotypic representation in the database, additional assessments of V-073 resistance are necessary. It may be that the parameters of cell culture do not reflect fitness penalties that would be associated with resistance to V-073 in infections in a living host. Indeed, when evaluated in mice, we found that laboratory-derived V-073-resistant variants exhibit clear attenuation of their replicative capacity and neurovirulence (9). Further study of V-073 resistance, particularly in the context of treatment-emergent resistance in clinical studies with V-073, is warranted.

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M.S.C. is a principal in and D.C.P. is a consultant to ViroDefense, Inc., the corporate sponsor of drug candidate V-073. There are no other declarations among the authors.

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