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Anti-poliovirus activity of protease inhibitor AG-7404, and assessment of in vitro activity in combination with antiviral capsid inhibitor compounds ☆

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ABSTRACT

The National Research Council 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 post-eradication. The primary application of such drugs is expected to be the resolution of chronic poliovirus excretion in persons with primary immunodeficiency disorders. We have assessed the in vitro activity of AG-7404 (also known as "compound 1"), an inhibitor of picornaviral 3C protease, against a large panel of programmatically important poliovirus strains and its activity in combination with two poliovirus capsid inhibitors, V-073 and BTA798. AG-7404 was active against all viruses in this panel, with EC_{50} values ranging from 0.080 to 0.674 μM. Similarly, BTA798 was active against all viruses in this panel, with EC_{50} values ranging from 0.003 to 0.591 μM. By comparison, values for V-073 were 0.003–0.126 μM. BTA798 was active against V-073-resistant variants with an alanine to valine change in VP3 at position 24. However, BTA798 was inactive against the V-073-resistant strains with amino acid substitutions at VP1 amino acids 194 (equivalent to 192 in type 3) and 236. As expected from its different mechanism of action, AG-7404 was fully active against all V-073-resistant variants, with EC_{50} values ranging from 0.218 to 0.819 μM, compared to values of 0.202–0.407 μM for the V-073-susceptible parental strains. In vitro drug combination experiments demonstrated synergy between AG-7404 and either V-073 or BTA798, whereas the combination of the two capsid inhibitors acted additively.

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1. Introduction

The Global Polio Eradication Initiative (GPEI) has relied exclusively on the oral polio vaccine (OPV), an inexpensive and easily administered live, attenuated vaccine (Sutter et al., 2008). OPV is generally safe and has been highly effective under most circumstances. Normally, OPV viruses are excreted in the stool of healthy vaccinated individuals for several weeks (Duintjer Tebbens et al., in press). When individuals with a primary immune deficiency with immunoglobulin deficit, such as agammaglobulinemia or combined variable immunodeficiency, receive OPV, the virus may replicate persistently, accumulating genetic changes associated with reversion to neurovirulence and posing a risk of paralysis for the infected individual and compromising efforts to eradicate the virus (Minor, 2009).

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 post-eradication (National Research Council, 2006). The primary application of such drugs is expected to be control of chronic poliovirus excretion in persons with primary immunodeficiency disorders (Collett et al., 2008). Pursuant to the NRC recommendation, poliovirus-specific capsid inhibitor V-073 is being advanced clinically to assess the potential utility of poliovirus antiviral drugs in the management of polio incidents (http://www.taskforce.org/our-work/programs/global-polio-eradication). As with the application of any antiviral drug, treatment-emergent drug resistance may occur, of which the consequences must be understood. While not found in nature, we have recently demonstrated that poliovirus variants with reduced susceptibility to V-073 can be isolated in cell culture at a frequency of 3×10^{-5}–4×10^{-4} from otherwise drug-susceptible virus populations (Liu et al., 2012). Amino acid substitutions conferring resistance to V-073 map to capsid residues that interact directly with the drug and prevent drug entry into the hydrophobic "pocket" below the virion surface (Liu et al., 2012). Therefore, it is important to develop an additional compound(s) with a different site or mechanism of action to complement the use of V-073 in chronic poliovirus excretors. Theoretically, the combination resistance frequency for two drugs with independent sites of action is the

☆ The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of CDC and other contributing agencies.

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product of their individual resistance frequencies, resulting in an extremely low rate of dual resistance.

AG-7404 is an irreversible inhibitor of the picornaviral 3C protease with demonstrated activity against human rhinoviruses and several human enteroviruses, including polioviruses (De Palma et al., 2008a; Dragovich et al., 2003; Patick, 2006; Patick et al., 2005). This compound is attractive for use in combination with V-073 because of its demonstrated anti-poliovirus activity, its distinct mechanism of action, and its preliminary evaluation in a human phase I clinical trial. We have assessed the in vitro activity of AG-7404 against a large panel of programmatically important poliovirus strains and its activity in combination with two poliovirus capsid inhibitors, V-073 and BTA798.

2. Materials and methods

2.1. Antiviral compounds

Capsid inhibitors V-073 (Buontempo et al., 1997) and BTA798 (Watson et al., 2003) were provided by ViroDefense Inc., Rockville, MD, and Biota Holdings Ltd., Notting Hill, Victoria, Australia, respectively. Picornavirus 3C protease inhibitor AG-7404 (Dragovich et al., 2003) was obtained through the Pure Powder Gift Program of Pfizer Inc., Groton, CT.

2.2. Viruses

Antiviral activity was evaluated in a cell culture cytopathic effect (CPE) assay using a panel of 45 polioviruses (Table 1), as described previously (Oberste et al., 2009). The panel consists of viruses of all three poliovirus (PV) serotypes and includes wild reference strains (PV1-Brunhilde, PV1-Mahoney, PV2-Lansing, PV2-MEF-1, PV2-P712, PV3-Leon, and PV3-Saukett), the three Sabin vaccine strains, representative circulating vaccine-derived poliovirus (cVDPV) isolates (12 PV1-cVDPV, nine PV2-cVDPV) and vaccine-derived polioviruses from immunodeficient chronic excretors (iVDPV; eight PV1-iVDPV, four PV2-iVDPV, two PV3-iVDPV) collected from numerous geographic regions over the period 1981–2007 (Table 1). In addition, we assessed antiviral activity of BTA798 and AG-7404 against VDPV strains of all three poliovirus serotypes that had been selected for resistance to V-073 (Liu et al.,

Table 1
Activity of V-073, BTA798, and AG-7404 against a panel of 45 polioviruses.

Strain	Country/Year collected	Serotype	Origin	V-073 EC_{50} ± SD[c]	AG7404 EC_{50} ± SD[c]	BTA798 EC_{50} ± SD[c]
10235	Dominican republic/2000	PV1	cVDPV	0.018 ± 0.003	0.289 ± 0.052	0.202 ± 0.087
10236	Dominican republic/2000	PV1	cVDPV	0.029 ± 0.021	0.199 ± 0.115	0.271 ± 0.087
10238	Dominican republic/2000	PV1	cVDPV	0.014 ± 0.006	0.297 ± 0.070	0.293 ± 0.045
10237	Haiti/2000	PV1	cVDPV	0.011 ± 0.005	0.296 ± 0.028	0.227 ± 0.050
10239	Haiti/2001	PV1	cVDPV	0.014 ± 0.003	0.199 ± 0.023	0.359 ± 0.231
10240	Haiti/2001	PV1	cVDPV	0.015 ± 0.007	0.337 ± 0.028	0.032 ± 0.006
10241	Haiti/2001	PV1	cVDPV	0.083 ± 0.055	0.244 ± 0.099	0.057 ± 0.033
10242	Haiti/2001	PV1	cVDPV	0.017 ± 0.013	0.291 ± 0.017	0.079 ± 0.015
10690	Philippines/2001	PV1	cVDPV	0.029 ± 0.006	0.240 ± 0.176	0.426 ± 0.215
10691	Philippines/2001	PV1	cVDPV	0.018 ± 0.012	0.402 ± 0.030	0.021 ± 0.021
10692	Philippines/2001	PV1	cVDPV	0.029 ± 0.015	0.455 ± 0.143	0.255 ± 0.129
10693	Philippines/2001	PV1	cVDPV	0.008 ± 0.003	0.345 ± 0.050	0.068 ± 0.043
10694	USA/2005	PV1	iVDPV	0.014 ± 0.002	0.446 ± 0.127	0.236 ± 0.205
10695	USA/2005	PV1	iVDPV	0.008 ± 0.005	0.384 ± 0.018	0.129 ± 0.109
10668	Taiwan/2002	PV1	iVDPV	0.021 ± 0.015	0.133 ± 0.059	0.084 ± 0.085
10689	Taiwan/2002	PV1	iVDPV	0.042 ± 0.029	0.236 ± 0.028	0.047 ± 0.020
10223	USA/1982	PV1	iVDPV	0.010 ± 0.008	0.166 ± 0.085	0.066 ± 0.025
10222	USA/1981	PV1	iVDPV	0.015 ± 0.009	0.502 ± 0.172	0.211 ± 0.196
10225	USA/1990	PV1	iVDPV	0.017 ± 0.010	0.349 ± 0.051	0.189 ± 0.104
10224	USA/1987	PV1	iVDPV	0.069 ± 0.064	0.334 ± 0.047	0.039 ± 0.014
Brunhilde	USA/1939	PV1	Wild	0.018 ± 0.009	0.317 ± 0.099	0.052 ± 0.049
Mahoney	USA/1942[b]	PV1	Wild	0.076 ± 0.052	0.321 ± 0.063	0.049 ± 0.010
Sabin 1 OPV[a]	Vaccine strain	PV1	Vaccine	0.017 ± 0.000	0.173 ± 0.137	0.079 ± 0.037
10229	Egypt/1993	PV2	cVDPV	0.020 ± 0.022	0.427 ± 0.088	0.003 ± 0.000
10230	Egypt/1998	PV2	cVDPV	0.036 ± 0.019	0.502 ± 0.152	0.058 ± 0.021
10231	Egypt/1998	PV2	cVDPV	0.028 ± 0.013	0.313 ± 0.206	0.591 ± 0.194
10232	Egypt/1999	PV2	cVDPV	0.019 ± 0.013	0.411 ± 0.174	0.162 ± 0.092
10233	Egypt/1999	PV2	cVDPV	0.024 ± 0.014	0.374 ± 0.247	0.053 ± 0.030
10234	Egypt/1999	PV2	cVDPV	0.009 ± 0.001	0.386 ± 0.185	0.068 ± 0.035
10219	Madagascar/2002	PV2	cVDPV	0.023 ± 0.014	0.427 ± 0.066	0.096 ± 0.024
10220	Madagascar/2002	PV2	cVDPV	0.010 ± 0.007	0.349 ± 0.116	0.174 ± 0.131
10243	Nigeria/2002	PV2	cVDPV	0.038 ± 0.008	0.349 ± 0.228	0.075 ± 0.059
10221	USA/1999	PV2	iVDPV	0.027 ± 0.008	0.278 ± 0.052	0.134 ± 0.054
10228	USA/1992	PV2	iVDPV	0.126 ± 0.063	0.266 ± 0.070	0.005 ± 0.003
10226	USA/1991	PV2	iVDPV	0.019 ± 0.001	0.236 ± 0.134	0.020 ± 0.029
10227	USA/1991	PV2	iVDPV	0.019 ± 0.019	0.367 ± 0.208	0.182 ± 0.095
Lansing	USA/1937	PV2	Wild	0.007 ± 0.000	0.261 ± 0.102	0.067 ± 0.014
MEF-1	Egypt/1942[b]	PV2	Wild	0.026 ± 0.025	0.554 ± 0.100	0.069 ± 0.051
P712	USA/1954	PV2	Wild	0.003 ± 0.000	0.200 ± 0.053	0.109 ± 0.102
Sabin 2 OPV[a]	Vaccine strain	PV2	Vaccine	0.016 ± 0.012	0.305 ± 0.148	0.030 ± 0.034
10804	Egypt/2007	PV3	iVDPV	0.042 ± 0.026	0.305 ± 0.158	0.119 ± 0.047
10805	Iran/2007	PV3	iVDPV	0.029 ± 0.005	0.357 ± 0.133	0.032 ± 0.015
Saukett	USA/1962[b]	PV3	Wild	0.062 ± 0.008	0.240 ± 0.096	0.246 ± 0.038
Leon	USA/1937	PV3	Wild	0.109 ± 0.119	0.270 ± 0.067	0.087 ± 0.025
Sabin 3 OPV[a]	Vaccine strain	PV3	Vaccine	0.020 ± 0.007	0.137 ± 0.007	0.033 ± 0.003

[a] The Sabin type 1, 2, and 3 oral polio vaccine (OPV) strains are PV1-LSc/2ab, PV2-P712, and PV3-Leon, respectively.
[b] PV1-Mahoney, PV2-MEF-1, and PV3-Saukett are seed strains for inactivated polio vaccine.
[c] All values are in μM and based on at least three independent determinations. Values for V-073 (Oberste et al., 2009) are shown for comparison.

2012). All viruses were propagated on HeLa cells in a 5% CO_2 atmosphere at 37 °C 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).

2.3. Drug susceptibility assay

Virus inhibition by drug and determination of the median effective concentration (EC_{50}) were performed as described previously (Pevear et al., 1999). Briefly, drug and virus were combined with HeLa cells in 96-well plates in a cross-titration format (0.5 $\log_{10}$ serial dilutions of both drug and virus) to ensure reaching endpoints for both drug and virus titrations, with duplicate wells for each drug-virus concentration. After three days incubation at 37 °C, plates were stained with crystal violet (0.05% crystal violet, 0.5% Tween-20, 50% ethanol), washed three times with deionized water, and allowed to dry overnight. Viral CPE was measured by reading absorbance at 590 nm. EC_{50} values were derived by analyzing dose–response absorbance values by four-parameter curve fitting using Prism 5.04 (GraphPad Software, Inc., La Jolla, CA).

2.4. Evaluation of AG-7404 activity in combination with V-073 or BTA798

The assay design used a checkerboard dilution matrix (cross-titration) of 0.5 $\log_{10}$ serial dilutions of each of the drugs individually and in pairwise combinations. HeLa cells were seeded in 96-well plates at 2×10^5 cells/mL in 200 μL MEM with 2% FBS. Immediately following addition of drug(s), the cells were infected with 100 $CCID_{50}$ of poliovirus type 1, type 2, or type 3 (all Sabin vaccine strains). The concentration range for AG-7404 was 0.09–5.12 μM, 0.025–0.625 μM for V-073, and 0.20–1.25 μM for BTA798. Theoretical additive interactions of the drugs based on the Bliss Independence mathematical definition of expected effects for drug–drug interactions was calculated by using MacSynergy II (Prichard et al., 1993). Interpretation of significance of the observed volumes of synergy or antagonism, depicted in the differential surface plots, was based upon the guidelines provided in the MacSynergy II documentation. The reproducibility of the peak volumes was assessed using the mean and sample standard deviation of results of at least three replicate experiments. The synergy (or antagonism) volume is the three-dimensional counterpart of the area under a dose response curve, expressed in $\mu M^2\%$, and is a quantitative measure of synergy or antagonism. The volume above the 95% confidence interval for the theoretical additive surface was used to categorize the degree of synergy according to the MacSynergy II user's manual: strong synergy (>100), moderate synergy (50–100), minor synergy (25–50), additive (−25–25), minor antagonism (−25–−50), moderate antagonism (−50–−100), and strong antagonism (<−100) (Prichard et al., 1993; Prichard and Shipman, 1990).

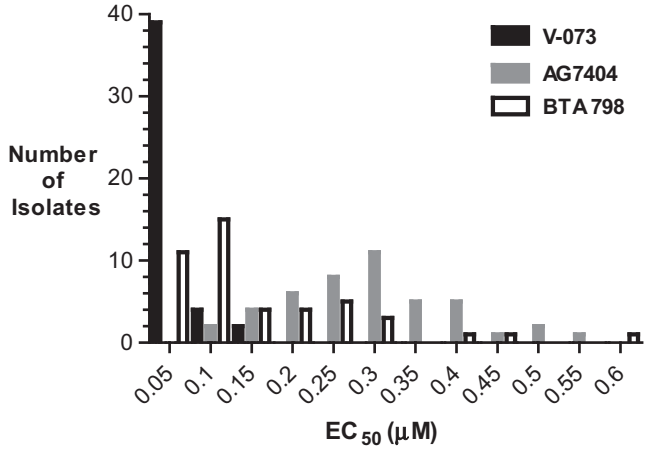

Fig. 1. Distribution of EC_{50} values for V-073, BTA798, and AG7404, against a panel of 45 polioviruses, derived from the data in Table 1.

3. Results

3.1. Anti-poliovirus activity of AG-7404 and BTA798

The spectrum of AG-7404 and BTA798 activity against a panel of 45 polioviruses was evaluated in a cell culture CPE assay. The panel consisted of viruses from all three poliovirus serotypes and included wild reference strains, the three Sabin vaccine strains, and representative circulating vaccine-derived poliovirus (cVDPV) and vaccine-derived polioviruses from immunodeficient chronic excretors (iVDPV) (Oberste et al., 2009). AG-7404 was active against all viruses in this panel, with EC_{50} values ranging from 0.080 to 0.674 μM (Table 1). Similarly, BTA798 was also active against all viruses in the panel, with EC_{50} values ranging from 0.003 to 0.591 μM (Table 1). By comparison, the values for V-073 were 0.003–0.126 μM (Table 1) (Oberste et al., 2009). The distribution of drug susceptibilities of the 45 polioviruses to all three drugs is depicted in Fig. 1. Ninety percent of all polioviruses tested were inhibited by AG-7404 at an EC_{50} value of $\leqslant$0.446 μM (MIC_{90} = 0.446 μM) and by BTA798 at an EC_{50} value of $\leqslant$0.236 μM (MIC_{90} = 0.236 μM), compared to an MIC_{90} of 0.076 μM for V-073 (Oberste et al., 2009).

Table 2
Activity of BTA798 and AG-7404 against V-073 resistant polioviruses.

Strain	Country	Serotype	aa Change[a]	BTA798 EC_{50} ± SD[b]	AG-7404 EC_{50} ± SD[b]
10235	Dominican republic	PV1	–[c]	0.546 ± 0.115	0.202 ± 0.063
10235	Dominican republic	PV1	I1194F	>10[d]	0.218 ± 0.043
10235	Dominican republic	PV1	A3024V	0.681 ± 0.103	0.227 ± 0.026
10230	Egypt	PV2	–[c]	0.182 ± 0.072	0.314 ± 0.044
10230	Egypt	PV2	I1194M	>10[d]	0.318 ± 0.042
10230	Egypt	PV2	A3024V	0.032 ± 0.016	0.269 ± 0.026
10805	Iran	PV3	–[c]	0.028 ± 0.009	0.407 ± 0.079
10805	Iran	PV3	I1192F	>10[d]	0.819 ± 0.205
10805	Iran	PV3	F1236L	>10[d]	0.394 ± 0.020
10805	Iran	PV3	A3024V	0.025 ± 0.004	0.593 ± 0.045

[a] Amino acid residues and positions are indicated by parental amino acid, viral protein affected, position within that protein, and substituted amino acid; e.g. I1194F = isoleucine at VP1 position 194 changed to phenylalanine.
[b] All values are in μM and based on at least three independent determinations.
[c] V-073-sensitive parental virus.
[d] Compound was not active at the highest concentration tested, 10 μM.

3.2. Cross-resistance between capsid inhibitors

To assess potential cross-resistance between V-073 and BTA798, we tested the ability of BTA798 to inhibit poliovirus variants selected in cell culture for resistance to V-073 (Liu et al., 2012). BTA798 was active against V-073-resistant variants with changes at VP3 position 24 (Ala to Val), with EC_{50} values in the same range as its activity against the V-073-susceptible parental strains (0.025–0.681 μM and 0.028–0.546 μM, respectively) (Table 2). However, BTA798 was inactive against V-073 resistant strains with amino acid substitutions at VP1 amino acids 194 (residue 192 in type 3) and 236. As expected from its different mechanism of action, AG-7404 was fully active against all V-073-resistant variants, with EC_{50} values ranging from 0.218 to 0.819 μM, compared to values of 0.202–0.407 μM for the V-073-susceptible parental strains (Table 2).

3.3. Synergistic effect of drug combinations

Potential synergy between AG-7404 and the two capsid inhibitors was assessed by combining AG-7404 with V-073 or BTA798 in varying proportions, with the range of absolute concentrations

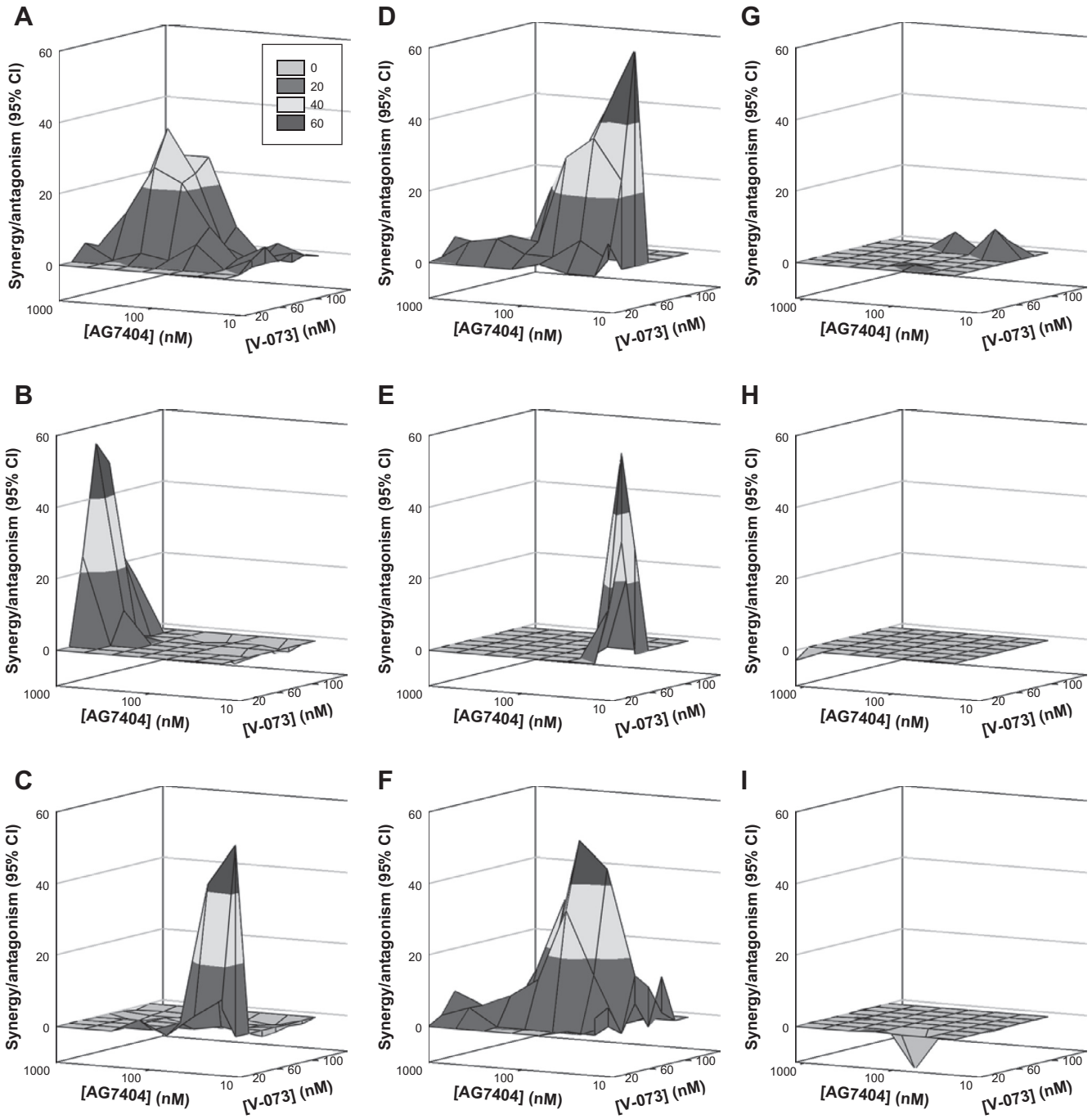

Fig. 2. Effects of AG-7404 in combination with V-073 or BTA798 on CPE induced by Sabin poliovirus strains in HeLa cells. Differential surface plots were derived from 95% confidence interval data generated using MacSynergy II. Volumes of synergy or antagonism that deviate significantly from the expected additive drug interactions are shown. The shading indicates the level of synergy or antagonism; the legend in panel A applies to all panels. Panels A-C, AG-7404 + V-073. Panels D–F, AG-7404 + BTA798. Panels G-!, V-073 + BTA798. A, D, G. Sabin 1. B, E, H. Sabin 2. C, F, I. Sabin 3.

spanning the EC_{50} values for each drug. CPE inhibition was measured for each of the three Sabin poliovirus strains (Fig. 2). For comparison, we also assessed the effect of combining V-073 and BTA798. These two compounds are expected to function additively because they each inhibit CPE by binding to the same site below the virion surface. The degree of synergy was measured by calculating the volume of inhibition above the upper 95% confidence interval for the theoretical surface of additivity (Prichard et al., 1993). The combination of V-073 and AG-7404 exhibited strong synergy for all three Sabin strains, with volume values of 580 $\mu M^2\%$, 459 $\mu M^2\%$, and 288 $\mu M^2\%$, for Sabin 1, Sabin 2, and Sabin 3, respectively (Fig. 2A–C). The combination of BTA798 and AG-7404 also exhibited strong synergy for all three Sabin strains, with volume values of 463 $\mu M^2\%$, 245 $\mu M^2\%$, and 579 $\mu M^2\%$, for Sabin 1, Sabin 2, and Sabin 3, respectively (Fig. 2D–F). As expected, the combined effect of V-073 and BTA798 was additive for all three strains (Fig. 2G–I). Combinations of V-073 with either BTA798 or AG-7404 had no effect on the viability of uninfected HeLa cells (data not shown) and, for all three compounds, the 50% cytotoxic concentration (CC_{50}) was >10 μM, the highest concentration tested.

4. Discussion

Immunodeficient chronic poliovirus excretors represent a community reservoir of virulent poliovirus, and consequently pose a risk to maintenance of a polio-free world. These individuals must be identified and their poliovirus excretion stopped, presumably through the use of antiviral drugs. However, the emergence of resistant variants may compromise drug efficacy should the resistant variants be of sufficient fitness to dominate. Consequently, as recommended by the NRC (National Research Council, 2006), it is prudent that at least two mechanistically distinct anti-poliovirus compounds be developed.

One of the best-studied targets for picornavirus antiviral therapy is a hydrophobic pocket beneath the viral capsid surface formed principally by the VP1 protein (Hogle et al., 1985; Pevear et al., 1989). Capsid inhibitors bind into this pocket, displacing the resident fatty acid molecule (possibly sphingosine) and in doing so, increase virion rigidity, thus inhibiting disassembly (uncoating) of the virion (McKinlay et al., 1992). One such capsid inhibitor, V-073, has been shown to inhibit a wide range of poliovirus strains in vitro (Oberste et al., 2009). V-073 has successfully completed a phase I human safety trial, and a phase II proof-of-concept trial is in progress (https://www.clinicaltrialsregister.eu/ctr-search/search?query=eudract_number:2011-004804-38).
BTA798, another capsid inhibitor, is under development for the treatment of human rhinovirus infections, particularly in high-risk chronic obstructive pulmonary disease and asthma patients (http://www.biota.com.au/?page=1021001&subpage=1021018).
In addition to its activity against human rhinoviruses, BTA798 has been shown to be active in vitro against poliovirus type 1 (De Palma et al., 2008b; Thibaut et al., 2011).

While V-073-resistant variants have not been identified in nature, they can be selected for in cell culture from otherwise drug-susceptible poliovirus populations (Liu et al., 2012). The resistance phenotype correlated with specific amino acid changes in capsid residues that line the hydrophobic pocket. While V-073 and BTA798 target the same site on the capsid, they are chemically distinct and structural differences between the two compounds likely affect the specific interactions with amino acid residues in and around the hydrophobic pocket. Indeed, the differential activity of BTA798 against polioviruses selected for resistance to V-073 (Table 2) supports this assertion. While BTA798 was fully active against V-073-resistant viruses with an Ala-to-Val change in VP3 amino acid 24, V-073-resistant polioviruses with specific changes in VP1, at amino acids 194 (192 in PV3) or 236 were also resistant to BTA798. Because of partial cross-resistance, the two capsid inhibitors are not ideal candidates for combination therapy of poliovirus infection.

AG-7404 (“compound 1”) is an irreversible inhibitor of 3C protease function with improved physicochemical and systemic pharmacokinetic properties over predecessor compounds (e.g., rupintrivir [AG-7088], Pfizer (Patick et al., 2005). While originally being developed for rhinovirus indications (Kaiser et al., 2000; Patick et al., 1999, 2005), both rupintrivir and AG-7404 have activity against the Sabin poliovirus strains (De Palma et al., 2008a; Thibaut et al., 2011). Here, we have expanded the breadth of AG-7404 anti-poliovirus activity to a panel of 45 programmatically relevant polioviruses. We further show that AG-7404 is active against both categories of V-073-resistant virus variants. Preliminary drug resistance studies suggest that the resistance frequency for the combination of AG-7404 and V-073 is significantly lower than for either compound alone (data not shown). These data, together with the synergistic antiviral activity observed when combined, indicate that further investigation of AG-7404-V-073 combination therapy is warranted.

Role of the funding source

This research was funded through federal appropriations in support of global polio eradication. The respective drug sponsors were informed of the study results but had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision of where to submit the manuscript for publication.

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