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H1PVAT is a novel and potent early-stage inhibitor of poliovirus replication that targets VP1

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ABSTRACT

A novel small molecule, H1PVAT, was identified as a potent and selective inhibitor of the *in vitro* replication of all three poliovirus serotypes, whereas no activity was observed against other enteroviruses. Time-of-drug-addition studies revealed that the compound interfered with an early stage of virus replication. Four independently-selected H1PVAT-resistant virus variants uniformly carried the single amino acid substitution I194F in the VP1 capsid protein. Poliovirus type 1 strain Sabin, reverse-engineered to contain this substitution, proved to be completely insensitive to the antiviral effect of H1PVAT and was cross-resistant to the capsid-binding inhibitors V-073 and pirodavir. The VP1 I194F mutant had a smaller plaque phenotype than wild-type virus, and the amino acid substitution rendered the virus more susceptible to heat inactivation. Both for the wild-type and VP1 I194F mutant virus, the presence of H1PVAT increased the temperature at which the virus was inactivated, providing evidence that the compound interacts with the viral capsid, and that capsid stabilization and antiviral activity are not necessarily correlated. Molecular modeling suggested that H1PVAT binds with high affinity in the pocket underneath the floor of the canyon that is involved in receptor binding. Introduction of the I194F substitution in the model of VP1 induced a slight concerted rearrangement of the core β-barrel in this pocket, which disfavors binding of the compound. Taken together, the compound scaffold, to which H1PVAT belongs, may represent another promising class of poliovirus capsid-binding inhibitors next to V-073 and pirodavir. Potent antivirals against poliovirus will be essential in the poliovirus eradication end-game.

1. Introduction

Since the launch of the Global Polio Eradication Initiative (GPEI) in 1988, the number of poliovirus (PV) cases has dropped >99% to a total of 406 in 2013 according to the WHO. Only Pakistan, Afghanistan and Nigeria remain endemic for PV, and India recently celebrated three years since the last polio case. Despite these great achievements, recent events have made it clear that the objective of worldwide PV eradication is far from being achieved. In 2013, a total of 203 wild PV cases were recorded in the Horn of Africa alone, while there were none in 2012. In addition, due to the civil war in Syria, vaccination coverage has dropped, which has set the scene for small PV outbreaks to occur, with at least 17 reported cases in 2013 (Eichner and Brockmann, 2013). Since only 0.5% of those infected will develop acute flaccid paralysis, the real number of infected individuals may be much higher. Moreover, with hundreds of thousands of refugees fleeing from Syria, (asymptomatic) infected individuals will cause the virus to spread again throughout the Middle Eastern region and maybe even beyond. For example in Israel, despite the fact that >95% of its inhabitants are vaccinated with the inactivated polio vaccine (IPV), many dozens of environmental samples taken recently from various sites around the country tested positive for wild poliovirus (Eichner and

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Brockmann, 2013; Roberts, 2013). Since IPV only protects the vaccinated individual from disease, but does not stop intestinal replication op PV, an IPV-only vaccination strategy as employed by Israel and many European countries, may facilitate silent spread of imported polio.

In Western countries, IPV is almost exclusively used as it has a very good safety profile. However, it requires multiple, scheduled-in-time injections to reach the desired protection level, which is a major disadvantage for use in developing countries or during emergency vaccination campaigns. In these latter settings, the oral polio vaccine (OPV) is most frequently used, given the facts that it is stable, relatively cheap, can be administered orally and has a high first immunization rate. However, the use of OPV is not without risks. Although no exact numbers are available, an estimated 250–500 cases of vaccine-associated paralytic poliomyelitis (VAPP) are caused every year (WHO, 2002). Moreover, in rare cases, the live vaccine virus may acquire mutations which cause it to become virulent again. These so-called circulating vaccine-derived polioviruses (cVDPVs) are shed in the environment and may infect non-immunized individuals, causing a PV outbreak. Shedding of VDPVs can be brief, but may also persist for many years in immunodeficient patients (MacLennan et al., 2004). Thus paradoxically, OPV, which greatly contributed to the >99% reduction of the number of PV cases, now impedes total eradication of the virus. Therefore, as soon as transmission of wild PV would have been halted, the use of OPV most likely will be stopped by the WHO. In 2006, the National Research Council recommended that at least one and preferably two anti-PV drugs should be developed, mainly to treat immunodeficient persistent PV shedders and to contain future outbreaks in the post-OPV era.

We here report on a novel inhibitor of poliovirus replication (H1PVAT) that was identified in a PV-1 strain Sabin phenotypic virus-cell-based screen. The antiviral potential and properties of H1PVAT are explored. The mechanism by which the compound exerts its antiviral activity is unraveled and put into in the context of other small-molecule inhibitors that have been described to inhibit poliovirus replication in a similar way.

2. Materials and methods

2.1. Cells and viruses

Buffalo green monkey (BGM) cells (ECACC 90092601), rhabdomyosarcoma (RD) cells and HeLa cells were maintained in minimal essential medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium bicarbonate (Gibco) and 1% L-glutamine (Gibco). Cells were grown at 37 °C and 5% CO_2. The Sabin vaccine strain of poliovirus type 1 was derived from infectious clone pT7/S1F, which was kindly provided by A.J. Macadam (McGoldrick et al., 1995), the vaccine strains of poliovirus type 2 and 3 were kindly provided by J. Martin (NIBSC, Hertfordshire, UK). Enterovirus 71 strain BrCr and Coxsackievirus A21 strain Coe were a kind gift from F. van Kuppeveld (UMC St. Radboud, The Netherlands). Rhinoviruses type 2, 14, 29, 70, 85, Coxsackievirus A9 strain Bozek and echovirus 11 strain Gregory were kindly provided by K. Andries (Janssen Infectious Diseases, Beerse, Belgium). Coxsackievirus B3 strain Nancy was derived from plasmid P53CB3/T7. Coxsackievirus B4 strain Edwards was kindly provided by J.W. Yoon (Yoon et al., 1979). For assays involving virus growth, medium supplemented with 2% FBS instead of 10% was used.

2.2. Compounds

HIPVAT was synthesized using a procedure that has been described previously (Supplemental Data) Dalinger et al., 2005. Pirodavir and V-073 were synthesized by G. Pürstinger. All compounds were dissolved in DMSO at a concentration of 10 mg/ml and were stored at 4 °C between assays. For working solutions, the DMSO stocks were diluted in minimal essential medium (MEM) supplemented with 2% FBS to reach the desired concentration in the assays.

2.3. Multi-cycle CPE reduction assay

The antiviral activity of H1PVAT was initially observed in a phenotypic poliovirus type 1 virus-cell-based assay using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium,innersalt] readout. Briefly, BGM cells were grown at a density of 2.5×10^4 cells per well (100 μl) in 96-well cell culture plates. After 24 h of incubation, the virus and serial dilutions of H1PVAT were added. The cultures were further incubated for 1–2 days until complete virus-induced cytopathic effect (CPE) was observed in the infected and untreated virus control (VC) conditions. After removal of the medium, 100 μl Phenol Red-free MEM (Invitrogen) containing 5% MTS (Promega) was added to each well. Following an incubation period of 1 h at 37 °C, the optical density at 498 nm (OD_{498}) of each well was obtained using a microtiter plate reader (Saffire^2, Tecan). The OD values were converted to percentage of controls and the 50% effective concentration (EC_{50}) was calculated by logarithmic interpolation as the concentration of compound that results in a 50% protective effect against virus-induced CPE. Evaluation of H1PVAT against PV clinical isolates was performed at the CDC and NIBSC.

2.4. Time-of-drug-addition study

BGM cells, grown to confluence in 24-wells assay plates, were treated with H1PVAT (2 μg/ml in 2% FBS culture medium) 0.5 h before or at 1 h post-infection with PV-1 strain Sabin. Virus-infected, untreated cells were used as controls. At 7 h post-infection, intracellular RNA was isolated and viral RNA levels were quantified by means of RT-qPCR (see below).

2.5. RNA isolation

For viral RNA isolation of cell culture supernatant, the RNA isolation kit of Machery-Nagel was used according to the manufacturers' instructions. For total intracellular RNA isolation, the RNeasy mini kit of QIAGEN was used.

2.6. Quantification of PV-1 RNA by RT-qPCR

Per RNA sample, the following reaction mixture was composed: 12.5 μl of 2× reaction mixture (One-Step RT-qPCR mix, Eurogentec), 0.125 μl RT enzyme, 200 nM TaqMan probe (5′-FAM-CCGAC TACTTTGGGTGTCCGTGTTTC-TAMRA-3′), 900 nM forward primer (5′-CCTCCGGCCCCTGAATG-3), 900nM reverse primer (5′-ACCGGAT GGCCAATCCAA-3′), 5 μl of viral RNA extract and RNase/DNase-free water to reach a final volume of 25 μl. The temperature cycling protocol consisted of a 30′ reverse transcription step at 48 °C, 10′ of denaturation at 95 °C followed by 40 cycles of 15″ at 95 °C and 1′ at 60 °C. For this, an ABI 7500 system was used (Applied Biosystems).

2.7. Thermo-stability assay

PV-1 (10^7 $TCID_{50}$/ml) was incubated in the presence of H1PVAT, or V-073 (at a concentration of 50 times the EC_{50}) or an equal volume of DMSO at 12 different temperatures ranging from 42.9 °C to 57.1 °C for 2 min after which the samples were rapidly cooled to