Potent and broad-spectrum cycloheptathiophene-3-carboxamide compounds that target the PA-PB1 interaction of influenza virus RNA polymerase and possess a high barrier to drug resistance

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Abbreviations: cHTC, cycloheptathiophene-3-carboxamide; IAV, influenza A virus; IBV, influenza B virus; HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2; RBV, ribavirin; RdRP, RNA-dependent RNA polymerase; OST, oseltamivir.

Abstract

Influenza viruses are major respiratory pathogens responsible for both seasonal epidemics and occasional pandemics worldwide. The current available treatment options have limited efficacy and thus the development of new antivirals is highly needed. We previously reported the identification of a series of cycloheptathiophene-3-carboxamide compounds as influenza A virus inhibitors that act by targeting the protein-protein interactions between the PA-PB1 subunits of the viral polymerase. In this study, we characterized the antiviral properties of the most promising compounds as well as investigated their propensity to induce drug resistance. Our results show that some of the selected compounds possess potent, broad-spectrum anti-influenza activity as they efficiently inhibited the replication of several strains of influenza A and B viruses, including an oseltamivir-resistant clinical isolate, with nanomolar or low-micromolar potency. The most promising compounds specifically inhibited the PA−PB1 binding *in vitro* and interfered with the influenza A virus polymerase activity in a cellular context, without showing cytotoxicity. The most active PA-PB1 inhibitors showed to possess a drug resistance barrier higher than that of oseltamivir. Indeed, no viral variants with reduced susceptibility to the selected compounds emerged after serial passages of influenza A virus under drug selective pressure. Overall, our studies identified potent PA-PB1 inhibitors as promising candidates for the development of new anti-influenza drugs.

1. Introduction

Influenza A viruses (IAV) are pathogens that infect a variety of bird and mammalian hosts and along with influenza B viruses cause seasonal epidemics of influenza, a contagious respiratory disease, in humans. According to the World Health Organization (WHO), the global burden of seasonal influenza is estimated to be on the order of 3–5 million cases of severe illness annually resulting in 300,000–650,000 deaths (WHO, 2018), especially among people at high risk of developing influenza-related complications (e.g., the elderly, immunocompromised individuals, and patients with underlying chronic respiratory and cardiovascular diseases). IAV is also responsible for recurring human pandemics, which in the past caused devastating effects on the global population in terms of prevalence and mortality rates (Taubenberger and Kash, 2010). In addition, in recent years some subtypes of avian IAV (e.g., H5N1, H7N9) have crossed the species barrier and caused thousands of documented zoonotic outbreaks in humans, leading to severe disease associated with high mortality (Gao et al., 2013; Lai et al., 2016). Although there is no current evidence of a sustained human-to-human spread of these avian influenza virus strains, their pandemic potential is clear and poses a serious and constant threat to global public health.

Vaccination is the main strategy to prevent influenza infections, but current available vaccines fail to provide cross-protection against all subtypes of influenza virus and have several other limitations. Indeed, due to high mutation rates of influenza virus that lead to viral antigenic variability, anti-influenza vaccines must be reformulated on an annual basis and a good antigenic match between circulating strains and vaccine composition is difficult to attain (Salzberg, 2008). Moreover, vaccines have low efficacy in high-risk groups and the lag time needed to produce a new vaccine may not be able to control a potential rapid spread of pandemic strains. For these reasons, antiviral drugs are clearly needed for the management of influenza infections. Since M2 blockers (i.e., amantadine and rimantadine) are no longer recommended in clinical setting due to the widespread circulation of drug-resistant viruses (Loregian et al., 2014; Dong et al., 2015), currently the first-line drugs for the prophylaxis and the treatment of IAV and IBV infections are the neuraminidase (NA) inhibitors, represented by oseltamivir (OST), zanamivir, and peramivir

(Gubareva et al., 2000; Loregian et al., 2014). However, the emergence and spread of drug-resistant IAV strains have been also reported for NA inhibitors, thus limiting their clinical effectiveness (Moscona, 2009; Li et al., 2015). Considering this concern, the development of anti-influenza drugs with different mechanisms of action is highly required.

IAV and IBV RNA-dependent RNA polymerase (RdRP) is a heterotrimeric complex composed of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) subunits. RdRP has a pivotal role in virus replication, as it is responsible for both viral transcription and replication (Fodor, 2013). Due to its multiple and essential enzymatic activities, RdRP represents a promising target for the development of anti-influenza drugs with alternative mechanisms of action (Loregian et al., 2014; Stevaert and Naesens, 2016). Many inhibitors targeting different components and functions of the RdRP have been developed and three of them have been recently approved for clinical use or are in late-phase clinical trials (Koszalka et al., 2017). These compounds are: i) favipiravir (formerly known as T-705), a purine nucleoside analogue; ii) pimodivir (also known as VX-787 or JNJ63623872), a cap-binding inhibitor of the PB2 subunit; and iii) baloxavir marboxil (known as S-033188, the prodrug of S-033447), an enzymatic inhibitor of PA endonuclease. Baloxavir marboxil has been recently approved in US and in Japan for the treatment of IAV and IBV infections (Hayden and Shindo 2019), while favipiravir has been licensed as anti-influenza drug in Japan (Koszalka et al., 2017).

An appealing approach to selectively inhibit RdRP functions is to interfere with proper complex formation. The assembly of the three subunits into a functional trimeric complex is required for the RdRP to exert its functions (Toyoda et al., 1996; Gonzàlez et., 1996; Perez and Donis, 2001). Structural studies have revealed inter-subunit interactions between the C-terminal portion of PA and the N-terminus of PB1 (He et al., 2008; Obayashi et al., 2008, Pflug et al., 2014) as well as between the C-terminal domain of PB1 and the N-terminus of PB2 (Sugiyama et al., 2009; Pflug et al., 2014). Since these interactions are essential for viral replication and their binding interfaces are highly conserved among IAV and IBV strains (Pérez and Donis, 1995; Ghanem et al., 2007; Stevaert and Naesens, 2016), the disruption of such interactions represents an attractive

strategy for the development of anti-influenza drugs with broad activity (Loregian et al., 2002; Loregian and Palù, 2005; Palù and Loregian, 2013; Loregian et al., 2014; Stevaert and Naesens, 2016; Massari et al., 2016). The feasibility and the effectiveness of such an approach have been demonstrated by the increasing number of PA-PB1 dissociative inhibitors developed in the last decade (Wunderlich et al., 2009; Wunderlich et al., 2011; Muratore et al., 2012a; Muratore et al., 2012b; Fukuoka et al., 2012; Massari et al., 2013; Kessler et al., 2013; Lepri et al., 2014; Pagano et al., 2014; Tintori et al., 2014; Massari et al., 2015; Trist et al., 2016; Yuan et al., 2016; Desantis et al., 2017; Watanabe et al., 2017; Massari et al., 2017; Zhang et al., 2018; D'Agostino et al., 2018). Among these, we recently reported the discovery of compounds with a cycloheptathiophene-3-carboxamide (cHTC) scaffold as PA-PB1 inhibitors (Desantis et al., 2017). In preliminary studies, we identified a series of cHTC derivatives able both to disrupt the binding between PA and PB1 *in vitro* as well as to inhibit the replication of IAV A/Puerto Rico/8/1934 (PR/8 strain) in infected cells (Desantis et al., 2017).

In this study, we further characterized the antiviral activity of the most promising cHTC derivatives and assessed their potential to develop drug resistance. Some of the tested cHTC compounds exhibited potent, broad anti-influenza activity and showed to act by interfering specifically with the correct assembly and activity of influenza virus RdRP. Most importantly, we were not able to isolate a viral variant resistant to three different cHTC PA-PB1 inhibitors. Overall, our studies demonstrated cHTC compounds as promising antivirals against IAV and IBV infections.

2. Materials & Methods

2.1. Compounds and peptide

Compounds **21**, **29**, **31**, **32**, **37**, and **54** were synthesized as previously described (Desantis et al., 2017; Desantis et al., 2018). Each test compound was dissolved in dimethyl sulfoxide (DMSO). Ribavirin (RBV, 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and oseltamivir carboxylic acid

(OST) were purchased from Roche; favipiravir (T-705, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was purchased from Selleck Chemicals. The $PB1_{(1–15)}$–Tat peptide was obtained by the Peptide Facility of CRIBI Biotechnology Center (University of Padua, Padua, Italy). This peptide comprises the 15 N-terminal residues of the PB1, i.e. the PA-interacting portion of the protein, conjugated to the amino acid sequence of HIV Tat protein (residues 47−59). RBV, OST, and $PB1_{(1–15)}$–Tat peptide were dissolved in water.

2.2. Cells and virus

Mardin-Darby canine kidney (MDCK) and human embryonic kidney (HEK) 293T cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Life Biotechnologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Life Technologies) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin sulfate, Life Technologies) at 37°C with 5% CO_2. Influenza A/Puerto Rico/8/1934 (PR/8, H1N1, Cambridge lineage) was kindly provided by P. Digard (Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom). Influenza virus strains A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 (Victoria lineage), and B/Bangladesh/333/2007 (Yamagata lineage) were provided by R. Cusinato (Clinical Microbiology and Virology Unit, Padua University Hospital, Padua, Italy). Influenza B/Lee/1940 virus (B/Lee, ancestral lineage) was obtained from W. S. Barclay (Imperial College, London, United Kingdom). The clinical isolate A/Parma/24/2009 (H1N1, OST-resistant) was kindly provided by I. Donatelli (Istituto Superiore di Sanità, Rome, Italy), while a local strain of the 2009 pandemic variant H1N1 IAV, A/Padova/30/2011, was provided by C. Salata and A. Calistri (University of Padua, Padua, Italy).

2.3. Plasmids

The pD15-GST, pD15–$PB1_{1–25}$, and pET28a–$PA_{239–716}$ plasmids, which encode glutathione S-transferase (GST), GST-$PB1_{(1–25)}$, and 6His-$PA_{(239–716)}$ proteins, respectively, were generated as described elsewhere (Loregian et al., 2004; Muratore et al., 2012a). Plasmids pcDNA-PB2, pcDNA-

PB1, pcDNA-PA, and pcDNA-NP, containing cDNA copies of the genomic segments 1, 2, 3, and 5 of the IAV PR/8 strain for the expression of PB2, PB1, PA, and NP proteins, respectively, were kindly provided by P. Digard (Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom). These plasmids were created as previously reported (Mullin et al., 2004). Reporter plasmid pPolIFlu-ffLuc containing an influenza virus-based luciferase minireplicon vRNA under the control of the human RNA polymerase I promoter was provided by L. Tiley (University of Cambridge, Cambridge, United Kingdom). Plasmid pRL-SV40 carrying *Renilla* luciferase reporter gene under the control of the constitutive early SV40 enhancer/promoter region was purchased from Promega.

2.4. Protein expression and purification

6His-PA$_{(239–716)}$, GST, and GST-PB1$_{(1–25)}$ proteins were expressed into *E. coli* strain BL21(DE3)pLysS (Stratagene) and purified as published previously (Loregian et al., 2004; Muratore et al., 2012a).

2.5. PA-PB1 interaction enzyme-linked immunosorbent assay (ELISA)

The PA−PB1 interaction was detected by a procedure previously described (Muratore et al., 2012a), with some modifications. Briefly, 96-well microtiter plates (Nuova Aptca) were coated with 400 ng per well of 6His-PA$_{(239–716)}$ and incubated at 37°C for 3 h. Coated wells were then blocked with 2% BSA (Sigma-Aldrich) in PBS for 1 h at 37°C. After 5 washes with PBS containing 0.3% Tween 20 (Sigma-Aldrich), different concentrations of test compounds or DMSO, as a control, were dissolved in serum-free DMEM and added to the plates along with 200 ng of GST-PB1$_{(1–25)}$, or of GST alone as a background control. Each compound concentration was tested at least in duplicate. Plates were then incubated overnight at room temperature. After 5 washes with 0.3% Tween/PBS, the PA-PB1 interaction was detected with a monoclonal anti-GST antibody conjugated to horseradish peroxidase (GenScript) diluted 1:4,000 in PBS plus 2% FBS. After the final washes, the peroxidase

substrate 3,3′,5,5′tetramethylbenzidine (TMB, KPL) was added to each well and absorbance was read at 450 nm by an ELISA plate reader (Tecan Sunrise™).

2.6. Cytotoxicity assay

Cytotoxicity of test and reference compounds was tested in HEK 293T and MDCK cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) method. Briefly, HEK 293T or MDCK cells seeded in 96-well plates at 3×10^4 per well or 2×10^4 per well, respectively, were incubated at 37°C for 24 or 48 h, respectively, in the presence of compounds serially diluted in DMEM supplemented with 10% FBS and antibiotics. Cell viability was then determined as previously described (Loregian and Coen, 2006).

2.7. Plaque reduction assays

Plaque reduction assays (PRAs) with IAV and IBV were carried out as previously reported (Muratore et al., 2012a), with a few modifications. Briefly, confluent monolayers of MDCK cells in 12-well plates were first washed with serum-free DMEM and then infected with 30-40 Plaque Forming Units (PFU) per well of influenza virus in DMEM supplemented with 2 μg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corporation) and 0.14% BSA, in the presence of different concentrations of test compounds or solvent (DMSO) as a control. After 1 h of incubation at 37°C, cells were incubated with medium containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer), 2 μg/mL of TPCK-treated trypsin, 0.14% BSA, and DMSO or test compounds. At 48 h post-infection (p.i.) for IAV and 48-72 h p.i. for IBV, cell monolayers were fixed with 4% formaldehyde solution and stained with 0.1% toluidine blue.

2.8. Virus yield reduction assay

Virus yield reduction assay (VYRA) was performed as previously described (Muratore et al., 2012a), with some modifications. MDCK cells (2×10^4 per well) were seeded into 96-well plates and

incubated overnight at 37°C. Cells were then infected with the IAV PR/8 strain at a multiplicity of infection (MOI) of 0.001 in DMEM plus 0.14% BSA and 1 μg/mL TPCK-treated trypsin for 1 h at 37°C in the presence of various concentrations of test compounds or RBV as a control.. After 1 h, media were supplemented with fresh DMEM containing 2 μg/ml of TPCK-treated trypsin, 0.14% BSA, and compounds at the same concentrations. The culture supernatants were collected at 24 h p.i. and viral progeny was titrated by plaque assays in fresh MDCK cells.

2.9. Minireplicon assay

The minireplicon assay was performed as described elsewhere (Muratore et al., 2012a). HEK 293T cells (2×10^5 per well) were plated into 24-well plates and incubated overnight at 37°C. The following day, cells were transfected using calcium phosphate co-precipitation method with pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP plasmids (100 ng/well of each) along with 50 ng/well of the pPolI-Flu-ffLuc reporter plasmid and 50 ng/well of pRL-SV40 plasmid as a transfection control. Transfections were performed in the presence of different concentrations of test compounds or DMSO, in duplicate. Cell medium was replaced 4 h post-transfection with DMEM supplemented with 10% FBS and the respective compound concentration or DMSO. At 24 h post-transfection, cells were harvested, lysed and both firefly and *Renilla* luciferase activity were measured using the Dual Luciferase Assay Kit (Promega). In each experiment, firefly luciferase activity was normalized with that of the *Renilla* luciferase and relative luciferase units (RLU) were obtained.

2.10. In vitro serial influenza virus passage experiments

To evaluate the barrier to drug resistance of selected cHTC compounds (**29**, **31**, and **54**) and OST as a reference compound, IAV PR/8 strain was serially passaged in the presence of increasing concentrations of test compounds following a procedure previously described (Zhang et al., 2018). Briefly, confluent MDCK cells were infected with PR/8 at an MOI of 0.001 and then treated with test compounds or DMSO as a control. Cell supernatants were harvested when cytopathic effects

(CPEs) were evident, and the viral progenies titrated by plaque assay. The concentrations of PA-PB1 inhibitors or OST applied to the first passage (P1) corresponded approximately to those of their respective EC_{50} values in PRAs (Table 1); successively, compound concentrations were systematically two-fold increased at each passage until the 6th passage (P6) and then kept constant (32-fold the initial concentration) for four additional passages for OST and for fourteen passages for all tested cHTC compounds (Table S3). For compound **54**, at P21 compound concentration was further increased up to 100-fold the initial concentration and then kept constant for nine additional passages (until P30, Table S4). At selected passages, the drug sensitivity of harvested viruses was determined by PRA as described above, using parental PR/8 virus as a control.

2.11. Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). Data are presented as the means ± standard deviations (SD) of at least two experiments in duplicate. The IC_{50}, EC_{50} and CC_{50} values for the different assays were determined by nonlinear regression curve fitting (inhibitor versus normalized response with variable slope equation) using GraphPad Prism 7.0 software**.**

3. Results

3.1. Potent and broad-spectrum anti-influenza activity of selected cHTC compounds

We previously identified a series of cHTC derivatives as PA-PB1 inhibitors able to inhibit IAV replication without showing significant toxicity in MDCK cells (Desantis et al., 2017). Among them, the most promising derivatives, i.e., **21**, **29**, **31**, **32**, **37**, and **54**, which were previously shown to inhibit the replication of IAV PR/8 in PRA with EC_{50} values <10 μM and with a selectivity index (SI) >25 (Desantis et al., 2017), were selected for further biological characterization. All of them are characterized by a cycloheptathiophene scaffold variously functionalized at the C-2 and C-3 carboxamide moieties (Figure S1). In particular, compounds **21**, **29, 31,** and **32** bear an *o*-

hydroxyphenyl moiety at the C-2 position coupled with various C-3 aromatic rings, i.e., 2-pyridine (**21**), *p*-chlorophenyl (**29**), *p*-fluorophenyl (**31**), and thiazol-2-yl (**32**). In compound **37**, the *o*-hydroxy moiety was moved to C-3 position coupled with a 2-pyridine ring at the C-2, while compound **54** presents the 2-pyridine ring at the C-3 position with a *p*-nitrophenyl ring as C-2 substituent. To evaluate the spectrum of their anti-influenza activity, these compounds were tested by PRA against a panel of IAV strains, belonging to different co-circulating subtypes (i.e., H3N2 and H1N1), as well as against different IBV strains, corresponding to the two distinct lineages (Yamagata and Victoria). RBV and OST were included as positive controls. Results showed that each anti-PA-PB1 compound inhibited all tested IAV and IBV strains with similar potency (Table 1). As reported in Table 1, for compounds **29**, **31**, **32**, and **54** EC_{50}s were within the nanomolar range, while those of compounds **21** and **37** fell in the low-micromolar range. In particular, **54** emerged as the most potent compound, with EC_{50}s ranging from 0.08 to 0.27 μM. Of note, the selected cHTC compounds were found to be also active against a clinical isolate resistant to OST (A/Parma/24/2009 strain), with EC_{50} values comparable to those exhibited against the other tested viral strains. A dose-dependent activity was observed for all compounds against both IAV and IBV (Figure 1A) and EC_{90} values were in the sub-/low-micromolar range for all compounds except **37** (Table S1). Moreover, treatment with compounds **21**, **29**, **31**, **32**, and **54** also resulted in the reduction of plaques size (Figure 1B). In conclusion, some of the selected cHTC compounds showed potent, broad-spectrum anti-influenza activity, providing cross-protection against the two different lineages of IBV and the currently circulating subtypes of human IAV.

3.2. cHTC compounds treatment causes a dose-dependent reduction of infectious viral progeny

To further characterize the anti-influenza activity of the compounds, we assessed the effects of various concentrations of each test compound, and RBV as a control, on the production of infectious progeny. At 24 h p.i., supernatants of infected cell cultures were harvested and titrated by plaque assays. A dose-dependent reduction of the viral progeny was observed with all test compounds (Figure 2). Compounds **21**, **29**, **31**, **32**, and **54** exhibited EC_{50} values in the low-

micromolar range (Table S2). A concentration-dependent reduction of viral progeny titres was also obtained with compound **37** but with higher EC_{50} values (13.2 μM, Table S2). For most compounds, EC_{50} values in VYRA were higher than those obtained in PRA, which might be due to the different MOIs used in the two experimental settings (i.e., ~ 0.00005 in PRA *versus* 0.001 in VYRA), arguing that due to the competitive mechanism of the PA-PB1 inhibitors, at least for some compounds the antiviral activity might be MOI-dependent.

3.3 cHTC compounds specifically disrupt the PA–PB1 interaction in vitro in a dose-dependent manner

The compounds under investigation were previously found to be able to disrupt the physical interaction between the PA and PB1 subunits of IAV RdRP in an ELISA-based interaction assay with the IC_{50} values reported in Figure 3 (Desantis et al., 2017). However, for some cHTC compounds, i.e., **32** and **54**, that were highly active in PRAs, we did not observe a good correlation with their ability to disrupt the PA-PB1 interaction *in vitro*. To investigate whether these discrepancies could be in part due to the experimental conditions, we retested all cHTC compounds by a modified ELISA, wherein compounds were diluted in the same medium used for antiviral assays, i.e., serum-free DMEM instead of PBS. The $PB1_{(1–15)}$-Tat peptide, a peptide interfering with the PA-PB1 interaction (Muratore et al., 2012a), was used as a positive control in this assay. In the modified assay, a moderate (3-fold) and a strong decrease (9-fold) in the IC_{50} values were obtained with compounds **31** and **54**, respectively, when compared to the previous ELISA results (Figure 3). On the contrary, only negligible differences were observed between the two different assay conditions with compounds **21**, **29**, **32**, and **37**. Altogether, these data suggest that the medium composition used in the ELISA could affect the inhibitory activity of some compounds and that compounds **31** and **54** might be soluble in serum-free DMEM than in PBS.

To demonstrate the specificity of the inhibitory activity on the PA-PB1 interaction, the compounds were similarly assayed in an ELISA which we previously used to characterize inhibitors of interactions between the human cytomegalovirus DNA polymerase subunits UL54 and UL44

(Loregian et al., 2003, Loregian and Coen, 2006). None of the compounds caused a dose-dependent reduction of UL54–UL44 binding up to a concentration of 200 μM (data not shown).

Overall, the results from the ELISAs indicated that **21**, **29**, **31**, and **54** were able to inhibit the interaction between PA and PB1 subunits in the low-micromolar concentration range (IC_{50} values ranged from 5.2 μM to 15 μM; Figure 3C), whereas compound **32** and **37** showed only a weak PA-PB1 inhibitory activity *in vitro* (IC_{50} of 81 and 113 μM, respectively; Figure 3C). Thus, for the latter two compounds, **32** and **37**, additional mechanisms of viral inhibition could exist.

3.4 Selected cHTC compounds inhibit the activity of IAV polymerase in minireplicon assays

To evaluate the ability of cHTC compounds to interfere with the IAV polymerase activity in a cellular context, a minireplicon assay was performed. HEK 293T cells were co-transfected with plasmids encoding PA, PB1, PB2, and NP proteins of IAV along with a plasmid containing the firefly luciferase reporter gene flanked by the noncoding regions of A/WSN/1933 segment 8, and treated with test compounds. In addition, a plasmid that constitutively expressed *Renilla* luciferase was also added to normalize variations in transfection efficiency. The firefly luciferase activity values were then normalized to the *Renilla* luciferase activity. RBV and favipiravir were included as positive controls. Each compound was also tested in parallel for cytotoxicity in HEK 293T cells by MTT assays to exclude that the observed inhibitions might be due to toxic side effects of the compounds. All tested compounds, except **37**, were able to strongly inhibit the viral polymerase transcription activity at nontoxic concentrations and in a dose-dependent manner (Figure 4 and Table S3). In keeping with the results from PRAs, compounds **29**, **31**, **32**, and **54** exerted inhibitory activity against IAV RdRP in the submicromolar range (EC_{50}s from 0.27 μM to 1.0 μM), whereas **21** inhibited RdRP activity at low-micromolar concentrations (EC_{50} of 2.8 μM). No/little inhibition was observed in minireplicon assay upon treatment with compound **37** up to 25 μM (Figure 4 and Table S3). Overall, also in this assay compound **54** was the most active, with an EC_{50} of 0.27 ± 0.04 μM (Table S3).

3.5 Selected cHTC compounds have a high barrier to drug resistance in vitro

To evaluate the propensity of this class of inhibitors to induce drug resistance, we tried to select *in vitro* influenza viruses resistant to the most promising compounds under drug selective pressure, following a procedure similar to that previously employed for other anti-influenza compounds, including PA-PB1 inhibitors (Molla et al., 2002; Shih et al., 2010; Ma et al., 2016; Hu et al., 2017; Zhang et al., 2018). The cHTC compounds that both inhibited the viral replication in PRAs with EC_{50} values ≤ 1 μM and disrupted specifically the PA-PB1 interaction in ELISA with IC_{50} values < 20 μM were selected for these studies. Briefly, the IAV PR/8 strain was serially passaged at MOI 0.001 in MDCK cells in the presence of increasing concentrations of **29**, **31**, and **54**, or OST as a reference, starting from their EC_{50} values in PRA. Compound concentrations were serially two-fold increased at each passage (P) reaching concentrations 32-fold higher than the initial concentration and then kept constant until P10. After P10, drug-resistance selection was carried on only for the PA-PB1 inhibitors and the compound concentrations were maintained 32-fold higher than that applied at P0 for further ten passages. In the case of **29** and **31**, these compound concentrations were between the EC_{75} and the EC_{90} values obtained in VYRA at the same MOI (Figure 2 and Table S2), thus enabling a strong selective pressure on virus replication while still allowing the virus to replicate at some extent in order to favor the emergence of compound-related mutations. A similar approach has been used for selecting viral variants resistant to other influenza virus polymerase inhibitors, such as favipiravir (Goldhill et al., 2018) and ribavirin (Cheung et al., 2014). The concentration applied for compound **54** at P11-P20 corresponded approximately to the EC_{75} determined in VYRA, thus the selection studies were continued by performing ten additional passages (P21-P30) of the virus in the presence of 25 μM of compound **54**. A summary of the compound concentrations employed during the whole selection process is reported in Table S4. To select possible cell culture–adapted variants and obtain mock–treated viruses, the same viral strain was passaged in parallel in the presence of DMSO. To monitor the emergence of cHTC compounds- or OST-resistant variants during the *in vitro* passages, the phenotypic susceptibility of passaged viruses to respective compounds was determined by PRA at P0 and then at selected

passages, starting from P3. As summarized in Table 2, the activity of compounds **29**, **31**, and **54** remained unvaried over the whole *in vitro* selection process, as their EC_{50} values were constant from P0 to P20/P30. Moreover, the plaque sizes of the viral progeny harvested at P11 and P20/P30 from cultures passaged in the presence of compounds **29**, **31**, and **54** were comparable to that of parental virus (P0) (Figure 5).

On the contrary, the PR/8 virus sensitivity to OST rapidly changed during the virus passages *in vitro* (Table 2). Indeed, compared with P0 strain, OST-passaged viruses harvested at P5 and P7 showed a slight reduction of susceptibility to OST (6- and 10-fold increase in EC_{50}, respectively), and P9 and P10 viruses exhibited a strong shift in EC_{50} values, corresponding to a 130- and 390-fold resistance to the drug, respectively (Table 2), according to previously reported data (Molla et al., 2002; McKimm-Breschkin et al., 2012). To discriminate if the OST-resistant variants obtained from P5, P7, P9, and P10 were induced by the drug selective pressure or were culture–adapted, OST was tested by PRA also against mock–passaged viruses harvested at the same passages. As expected, the susceptibilities to OST of mock–passaged viruses harvested at various passages (EC_{50}s ranged from 0.007 μM to 0.013 μM, data not shown) did not substantially differ from that of parental virus (EC_{50} of 0.010 μM, Table 2), thus indicating that drug–resistance to OST was induced by the drug selective pressure.

Taken together, these data demonstrated that compounds **29**, **31**, and **54** are considerably less prone than OST to develop drug resistance *in vitro* and overall suggest that dissociative inhibitors of the influenza virus polymerase could have a high barrier to the emergence of resistant viruses.

4. Discussion

A major limitation of the current available anti-influenza drugs is the emergence of drug-resistant viruses. This problem first emerged, and then became more pronounced, with the M2 channel inhibitors adamantanes, so that the use of this drug class is no longer recommended (Dong et al.,

2015). However, there is also an alarming evidence of circulating influenza virus resistant to OST (Moscona, 2009; Li et al., 2015; Hussain et al., 2017). In this scenario, there is an urgent need to develop new anti-influenza drugs that act by new mechanisms of action. In this paper, we report the biological and biochemical characterization of cHTC compounds with potent anti-influenza activity, that act by disrupting the protein-protein interactions between the two subunits PA and PB1 of the viral RdRP. Moreover, we evaluated the barrier to drug resistance of selected cHTC compounds and found that they are impressively refractory to select drug-resistant viral variants under high-dose selective pressure.

We previously reported that some cHTC compounds, i.e., **21**, **29**, **31**, **32**, **37**, and **54**, exhibited antiviral activity against IAV PR/8 with sub- or low-micromolar potency (Desantis et al., 2017). We now demonstrated that they have potent, broad-spectrum activity against a panel of IAV and IBV strains, including clinical isolates. Importantly, these cHTC compounds fully retained activity against an OST-resistant clinical isolate of influenza virus, thus representing a potential alternative for developing rescue therapies to treat infections of influenza virus strains resistant to OST. Noteworthy, compound **54** efficiently inhibited in PRAs all tested influenza virus strains with EC_{50}s ranging from 0.08 μM to 0.27 μM (Table 1), emerging as the most potent compound of the series and also of all PA−PB1 inhibitors developed so far by us. In addition, **54** exhibited an SI up to >3000 (Table 1), which is at least one order of magnitude higher than the values reported for all the other published PA−PB1 inhibitors. The potency and the SI of **54** were also comparable to those observed against IAV and IBV for the RdRP inhibitor favipiravir (EC_{50} values ranged from 0.083 to 3.1 μM, reaching SIs > 2000; Furuta et al., 2002). The other two RdRP inhibitors currently approved or undergoing clinical phase III studies, baloxavir marboxil/S-033188 and pimodivir/VX-787, were found to inhibit multiple IAV strains in CPE reduction assays at sub-nanomolar concentrations (Clark et al., 2014; Noshi et al., 2016). However, the range of antiviral activity of pimodivir is limited to IAV as this compound was found to be ineffective against IBV (Clark et al., 2014).

In addition, a major advantage that the dissociative PA-PB1 inhibitors developed by us could have over the new inhibitors of PB2 and PA is a high barrier to the selection of resistant viruses. In fact, viruses resistant to baloxavir acid, the active form of the drug, were isolated after 6–8 passages *in vitro* (Noshi et al., 2018), and amino acid substitutions in *PB2* gene conferring reduced sensitivity to pimodivir were also reported to emerge *in vitro*, although no detailed analysis of resistance was conducted (Byrn et al., 2015). In contrast, we were not able to isolate viruses with reduced sensitivity to the cHTC compounds **29**, **31**, and **54**. Indeed, IAV isolated after 20/30 serial passages in MDCK cells under increasing concentrations of these PA-PB1 inhibitors (up to 32/100-fold their respective EC_{50}s in PRA against the parental virus) completely retained the susceptibility to the compounds. On the other hand, influenza virus variants with reduced susceptibility to OST arose already after 5 viral passages, and highly OST-resistant viruses (i.e., displaying >100-fold increase in EC_{50} compared to the parent virus) were selected from P9.

During the preparation of this manuscript, a study reporting the identification of a new PA-PB1 inhibitor of influenza virus as well as the evaluation of the barrier to drug resistance of this compound was published (Zhang et al., 2018). In keeping with our findings, Zhang and coworkers showed that OST rapidly induced the selection of resistant viral variants, while no viruses with altered susceptibility to the PA-PB1 inhibitor were isolated. However, they stopped the selection process at P10 (Zhang et al., 2018), while we carried out a prolonged drug-resistance selection for a total of 20/30 viral passages. Altogether, our data and the results obtained by Zhang et al. suggest that drug resistance *in vitro* to dissociative inhibitors targeting the PA-PB1 interactions of influenza virus RdRP appears hard to develop, differently from what happens with the current anti-influenza drugs such as adamantanes and neuraminidase inhibitors and also with PA and PB2 enzymatic inhibitors approved or under clinical development (Hay et al., 1985; Molla et al., 2002; McKimm-Breschkin et al., 2012; Byrn et al., 2015; Noshi et al., 2018). PA–PB1 interaction inhibitors might have a barrier to drug resistance even higher than that observed with the RdRP inhibitor favipiravir (Furuta et al., 2002; Baranovich et al., 2013; Daikoku et al., 2014). In the case of favipiravir, it has been observed that this drug induces lethal mutagenesis in the genome of influenza virus and many

other RNA viruses, causing the accumulation of transition mutations (Goldhill et al., 2019). For this reason, faviripavir-resistant viruses were considered unlikely to arise because this drug induces the generation of defective interfering viral particles and indeed until recently all the attempts to select escape mutants to this drug failed (Furuta et al., 2002; Baranovich et al., 2013; Daikoku et al., 2014). Nonetheless, influenza virus variants resistant to favipiravir were recently isolated under drug selective pressure (Goldhill et al., 2018). Sequencing analysis of the isolated viral variants identified a mutation in the *PB1* gene that conferred resistance to favipiravir and a secondary compensatory mutation in the *PA* gene to restore the viral fitness. In the case of PA-PB1 inhibitors, previous mutational studies showed that only very few amino acids substitutions are tolerated in the key residues of PA-PB1 interaction interface without a loss of binding and such mutations resulted in dramatic impairment of the viral polymerase activity as well as severe attenuation of the viral replication (Mänz et al., 2011). Most importantly, differently from what observed with favipiravir, no compensatory mutations able to restore even partially the viral fitness were identified (Mänz et al., 2011). In this paper, we also confirmed that compounds **21**, **29**, **31**, and **54** act by interfering specifically with the binding of influenza virus polymerase PA-PB1 subunits, as these compounds were able to disrupt this interaction with IC_{50}s in the low micromolar range, but were inactive against an unrelated protein-protein interaction (i.e., the human cytomegalovirus UL54-UL44 interaction). Since the correct assembly of the RdRP complex is essential for its enzymatic activity, the inhibition of the PA-PB1 binding by compounds **21**, **29**, **31**, and **54** also resulted in interference with the enzymatic activities of the IAV polymerase in a cellular context. Compound **54** was the most potent also in these assays.

Given their different mechanism of action, we expect that specific PA-PB1 inhibitors, such as **21**, **29**, **31**, and **54**, could have a synergistic anti-influenza effect if combined not only with NA inhibitors but also with favipiravir, pimodivir, or baloxavir marboxil, and combinations studies will be the subject of our future work. This approach could not only achieve stronger antiviral activity, but also reduce the risk of development of drug-resistance to compounds with lower resistance barrier. In fact, as demonstrated by previous studies, synergistic antiviral effects were obtained by

combining favipiravir, pimodivir, or baloxavir marboxil with various NA inhibitors (Koszalka et al., 2017; Kitano et al., 2017), but also by the combined use *in vitro* of two influenza virus RNA polymerase inhibitors, pimodivir and favipiravir (Bryn et al., 2015).

In conclusion, these results demonstrate that our PA-PB1 inhibitors are potent and broad-spectrum anti-influenza agents endowed with high barrier to drug-resistance *in vitro*. Overall, our findings support the further development of these cHTC compounds, either alone or in combination with other anti-influenza drugs, as promising candidates for the treatment of IAV and IBV infections.

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Declaration of interest

All authors declare no conflicts of interest.

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FIGURE LEGENDS

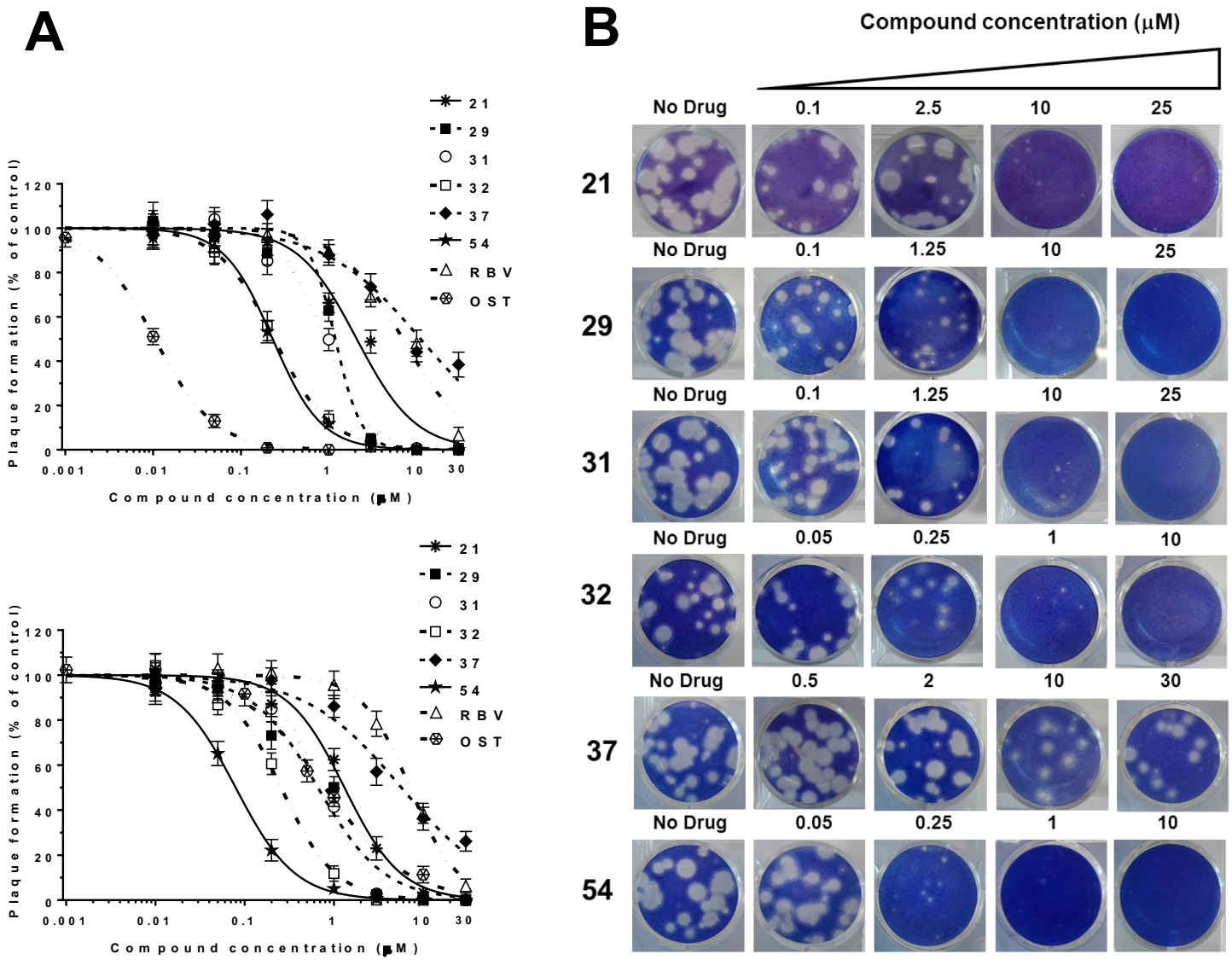
Fig. 1. Dose-dependent inhibition of IAV and IBV replication by cHTC compounds in PRA. (A) Dose-response curves of cHTC compounds, and RBV and OST as controls, on A/PR/8 and B/Lee viruses in MDCK cells. Data shown are the means ± SD of three experiments performed in duplicate. (B) The plaques of IAV PR/8 formed in the absence of compound (Mock) or in the presence of different concentrations of cHTC compounds of a representative experiment are shown.

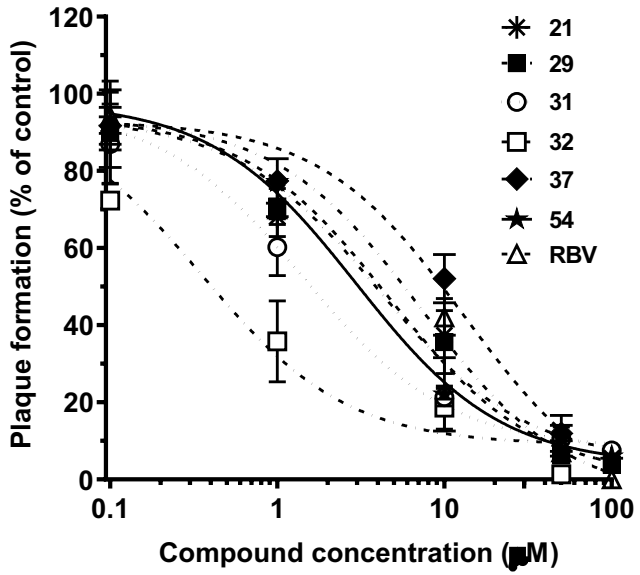
Fig. 2. Dose-dependent inhibition of IAV yield by cHTC compounds in VYRA. MDCK cells infected with IAV PR/8 at MOI = 0.001 were treated with different concentrations of cHTC compounds or RBV as a control. At 24 h p.i., virus progeny titers produced in the presence of test compounds were determined by titration on fresh MDCK cells by plaque assays. Data shown are the means ± SD of three experiments performed in duplicate.

Fig. 3. Comparison of the inhibition of PA–PB1 interaction by cHTC compounds under two different ELISA conditions. (A) Test cHTC compounds or DMSO were dissolved in PBS buffer and added together with GST-PB1$_{(1–25)}$ into wells coated with 6His-PA$_{(239–716)}$. The PA-PB1 binding was then detected by a GST-HRP antibody. Data shown represent the mean ± SD of at least three independent experiments in duplicate. (B) The PA−PB1 interaction assay was performed as in (A), but test compounds were dissolved in serum-free DMEM instead of PBS buffer. Data represent the averages ± SD of data obtained from at least three independent experiments in duplicate. (C) The IC_{50} values, corresponding to the compound concentrations that inhibit by 50% the PA-PB1 interaction, in ELISA performed in PBS buffer (Desantis et al., 2017) or in serum-free DMEM are reported. Values represent the averages ± SD of data derived from at least three independent experiments in duplicate.

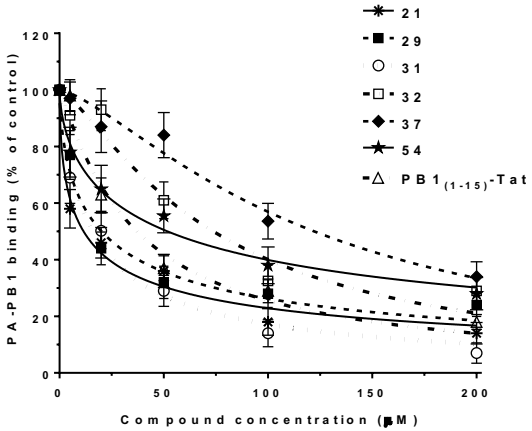
Fig. 4. Effects of cHTC compounds on the activity of IAV polymerase in cells. HEK 293T cells were co-transfected with plasmids expressing IAV PA, PB1, PB2, NP, or the firefly luciferase flanked by the noncoding genomic sequences of IAV in the presence of selected cHTC compounds, or RBV and favipiravir (FPV) as positive controls, or DMSO as a negative control. An additional plasmid, which constitutively expresses *Renilla* luciferase, was included to the transfection mixture. At 24 h post-transfection, luminescent signal of firefly luciferase was measured and normalized against that of *Renilla* luciferase. Values from samples treated with DMSO were set as 100%. All data shown represent the means ± SD of data obtained from at least two independent experiments in duplicate.

Fig. 5. Plaque morphology of IAV PR/8 during serial passages with selected cHTC compounds. Panels show plaques from MDCK cells infected with PR/8 virus passaged in the absence of compound (Mock) or in the presence of compound **29**, **31**, or **54**. After the indicated passage (P), fresh cell cultures were infected with supernatants containing the passaged virus and overlaid with Avicel cellulose containing TPCK-treated trypsin. After 48 h of incubation, cells were fixed with 4% formaldehyde and stained with 0.1% toluidine blue.

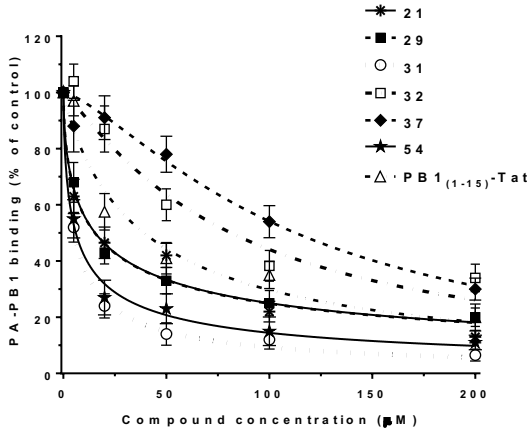

A
21
29
31
32
37
54
RBV
OST
Plaque formation (% of control)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
30
Compound concentration (μM)
21
29
31
32
37
54
RBV
OST
Plaque formation (% of control)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
30
Compound concentration (μM)
B
Compound concentration (μM)
No Drug
0.1
2.5
10
25
21
No Drug
0.1
1.25
10
25
29
No Drug
0.1
1.25
10
25
31
No Drug
0.05
0.25
1
10
32
No Drug
0.5
2
10
30
37
No Drug
0.05
0.25
1
10
54


Plaque formation (% of control)
120
100
80
60
40
20
0
0.1
1
10
100
Compound concentration (μM)
21
29
31
32
37
54
RBV

A

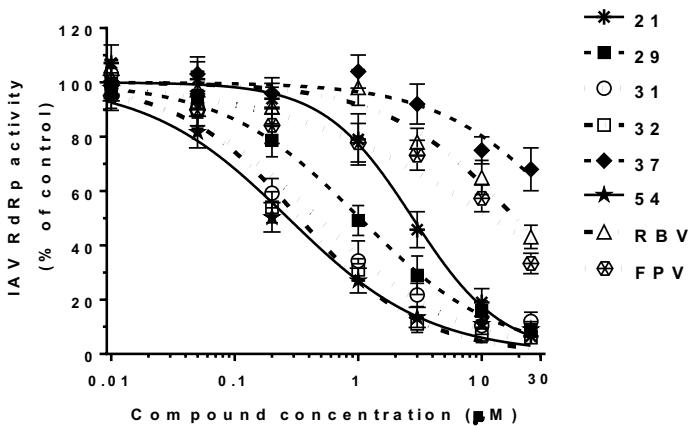


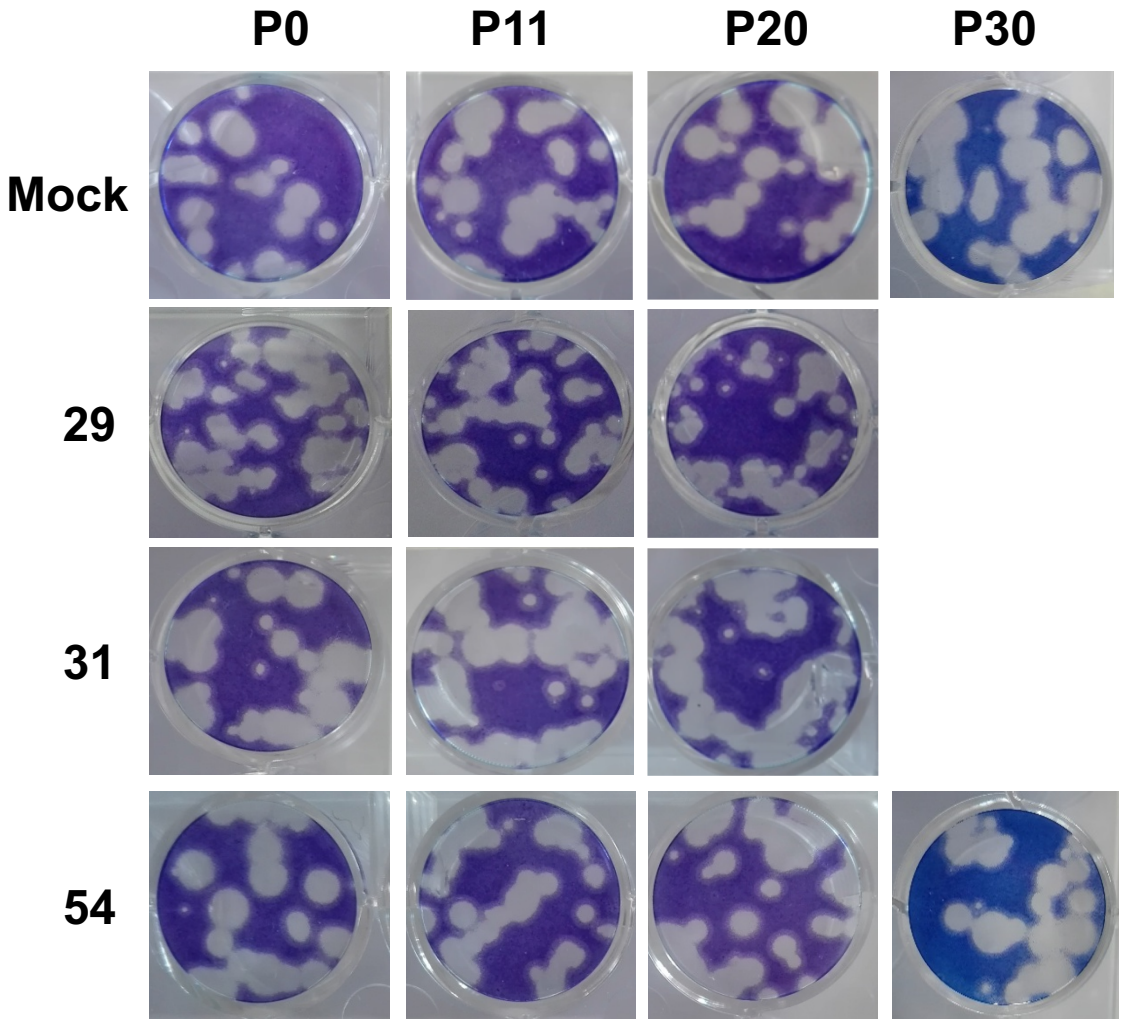
B



C

Compound	PA-PB1 ELISA, IC_{50}, μM	Modified PA−PB1 ELISA IC_{50}, μM
21	12 ± 3	15 ± 3
29	20 ± 4	15 ± 1
31	16 ± 2	5.2 ± 0.5
32	74 ± 11	81 ± 12
37	122 ± 11	113 ± 12
54	52 ± 6	6.0 ± 1.0
$PB1_{(1-15)}$-Tat Peptide	37 ± 5	39 ± 7


IAV RdRp activity
(% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
30
Compound concentration (μM)
21
29
31
32
37
54
RBV
FPV


P0
P11
P20
P30
Mock
29
31
54