An Integrated Biological Approach to Guide the Development of Metal-Chelating Inhibitors of Influenza Virus PA Endonuclease

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Abbreviations:

1: (Z)-4-(1-benzyl-4-(4-chlorobenzyl)piperidin-4-yl)-2-hydroxy-4-oxobut-2-enoic acid; **2:** (Z)-methyl 4-(1-benzyl-4-(4-chlorobenzyl)piperidin-4-yl)-2-hydroxy-4-oxobut-2-enoate; **3:** (Z)-4-(4-(4-chlorobenzyl)-1-(4-fluorobenzyl)piperidin-4-yl)-2-hydroxy-4-oxobut-2-enoic acid; **4:** (Z)-methyl 4-(4-(4-chlorobenzyl)-1-(4-fluorobenzyl)piperidin-4-yl)-2-hydroxy-4-oxobut-2-enoate; **5:** (Z)-4-(4-(4-chlorobenzyl)-1-(cyclohexylmethyl)piperidin-4-yl)-2-

hydroxy-4-oxobut-2-enoic acid; **6**: (Z)-methyl 4-(4-(4-chlorobenzyl)-1-(cyclohexylmethyl)piperidin-4-yl)-2-hydroxy-4-oxobut-2-enoate; **7**: 2,3-dihydroxyfumaric acid; **8**: (Z)-3-hydroxy-1-phenyl-3-(1*H*-1,2,4-triazol-5-yl)prop-2-en-1-one; **9**: (Z)-3-hydroxy-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one; **10**: (Z)-4-(1-(4-fluorobenzyl)-1*H*-pyrrol-2-yl)-2-hydroxy-4-oxobut-2-enoic acid; **11**: (Z)-methyl 4-(1-(4-fluorobenzyl)-1*H*-pyrrol-2-yl)-2-hydroxy-4-oxobut-2-enoate; **12**: (Z)-2-hydroxy-4-oxo-4-phenylbut-2-enoic acid; **13**: (Z)-methyl 2-hydroxy-4-oxo-4-phenylbut-2-enoate; **14**: (Z)-2-hydroxy-4-(2-methoxyphenyl)-4-oxobut-2-enoic acid; **15**: (Z)-methyl 2-hydroxy-4-(2-methoxyphenyl)-4-oxobut-2-enoate; **16**: (Z)-2-hydroxy-4-(4-methoxyphenyl)-4-oxobut-2-enoic acid; **17**: (Z)-4-(4-chlorophenyl)-2-hydroxy-4-oxobut-2-enoic acid; **18**: (Z)-4-(2-chlorophenyl)-2-hydroxy-4-oxobut-2-enoic acid; **19**: (Z)-methyl 4-(2-chlorophenyl)-2-hydroxy-4-oxobut-2-enoate; **20**: (Z)-4-(3,5-bis(benzyloxy)phenyl)-2-hydroxy-4-oxobut-2-enoic acid; **21**: (2*Z*,5*Z*)-2,6-dihydroxy-4-oxo-6-phenylhexa-2,5-dienoic acid; **22**: (2*Z*,5*Z*)-methyl 2,6-dihydroxy-4-oxo-6-phenylhexa-2,5-dienoate; **23**: (2*Z*,5*Z*)-2,6-dihydroxy-6-(2-methoxyphenyl)-4-oxohexa-2,5-dienoic acid; **24**: (2*Z*,5*Z*)-6-(2-chlorophenyl)-2,6-dihydroxy-4-oxohexa-2,5-dienoic acid; **25**: 6-benzyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; **26**: 6-benzyl-1-(2-hydroxyethyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; **27**: 1,6-dibenzyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; **28**: ethyl 1,6-dibenzyl-4-oxo-1,4-dihydroquinoline-3-carboxylate; **29**: *N*-(4-fluorobenzyl)-5-hydroxy-2-isopropyl-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide; **30**: (Z)-2-hydroxy-4-(1-methyl-1*H*-indol-2-yl)-4-oxobut-2-enoic acid; **31**: (Z)-4-(1-benzyl-1*H*-indol-2-yl)-2-hydroxy-4-oxobut-2-enoic acid; **32**: (Z)-methyl 2-hydroxy-4-(1-methyl-1*H*-indol-2-yl)-4-oxobut-2-enoate; **33**: (Z)-methyl 4-(1-ethyl-1*H*-indol-2-yl)-2-hydroxy-4-oxobut-2-enoate; **34**: (Z)-2-hydroxy-4-(5-methyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-6-yl)-4-oxobut-2-enoic acid; **35**:

(Z)-4-(5-ethyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-6-yl)-2-hydroxy-4-oxobut-2-enoic acid; **36**: (Z)-4-(5-benzyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-6-yl)-2-hydroxy-4-oxobut-2-enoic acid; **37**: (Z)-methyl 4-(5-benzyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-6-yl)-2-hydroxy-4-oxobut-2-enoate; **38**: (Z)-methyl 2-hydroxy-4-(5-methyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-6-yl)-4-oxobut-2-enoate; **39**: (Z)-2-hydroxy-4-(1-methyl-1*H*-indol-3-yl)-4-oxobut-2-enoic acid; **40**: (Z)-4-(1-ethyl-1*H*-indol-3-yl)-2-hydroxy-4-oxobut-2-enoic acid; **41**: (Z)-4-(1-benzyl-1*H*-indol-3-yl)-2-hydroxy-4-oxobut-2-enoic acid; **42**: (Z)-2-hydroxy-4-(5-methyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-7-yl)-4-oxobut-2-enoic acid; **43**: (Z)-methyl 2-hydroxy-4-(5-methyl-5*H*-[1,3]dioxolo[4,5-*f*]indol-7-yl)-4-oxobut-2-enoate; **44**: 3-(1-ethyl-1*H*-indol-3-yl)-4-hydroxy-1*H*-pyrrole-2,5-dione; **45**: (*E*)-2-hydroxy-2-(2-oxoindolin-3-ylidene)acetic acid; **46**: (*E*)-methyl 2-hydroxy-2-(2-oxoindolin-3-ylidene)acetate; **47**: (*E*)-methyl 2-hydroxy-2-(2-oxo-1-phenylindolin-3-ylidene)acetate; BHQ2: Black Hole Quencher-2 dye; CC₅₀: 50% cytotoxic concentration; DFO: dragonfly orange dye; DIGE: difference gel electrophoresis; DKA: β -diketo acid; DPBA: 2,4-dioxo-4-phenylbutanoic acid; EC₉₀: compound concentration causing a 1- $\log_{10}$ reduction in virus yield at 24 h p.i.; EC₉₉: compound concentration causing a 2- $\log_{10}$ reduction in virus yield at 24 h p.i.; EGCG: epigallocatechin gallate; FAM: fluorescein amidite dye; HIV IN: HIV integrase; IPTG: isopropylthio- β -galactoside; kb: kilobase; MB: Molecular beacon; MDCK: Madin-Darby canine kidney; ML: metal ligator; MOE: Molecular Operating Environment; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; ND: not done; NTA: nitrilotriacetic acid; p.i.: post infection; PAI: PA inhibitor; PA-Nter: N-terminal part of PA; PPCH: Planar-Polarity-Charge-Hydrophobicity; RT-PCR: reverse transcription polymerase chain reaction; ssDNA: single-stranded DNA; TBE: Tris-Borate-EDTA; V_0 : initial cleavage rate; VC: untreated virus control; vRNP: viral ribonucleoprotein.

ABSTRACT

The influenza virus PA endonuclease, which cleaves capped cellular pre-mRNAs to prime viral mRNA synthesis, is a promising target for novel anti-influenza virus therapeutics. The catalytic center of this enzyme resides in the N-terminal part of PA (PA-Nter) and contains two (or possibly one or three) Mg^{2+} or Mn^{2+} ions which are critical for its catalytic function. There is large interest in PA inhibitors that are optimally designed to occupy the active site and chelate the metal ions. We here focused on a series of β -diketo acid (DKA) and DKA-bioisosteric compounds containing different scaffolds, and determined their structure-activity relationship in an enzymatic assay with PA-Nter, to build a 3D pharmacophore model. Besides, we developed a molecular beacon (MB)-based PA-Nter assay which enables to compare the inhibition versus Mn^{2+} or Mg^{2+} , the latter probably being the biologically relevant cofactor. This real-time MB assay allows to measure the enzyme kinetics of PA-Nter or perform high-throughput screening. Several DKA derivatives were found to cause strong inhibition of PA-Nter with IC_{50} values comparable to that of the prototype L-742,001 (i.e. below 2 μM). Among the different compounds tested, L-742,001 appeared unique in having equal activity against either Mg^{2+} or Mn^{2+} . Three compounds (**10**, with a pyrrole scaffold, and **40** and **41**, with an indole scaffold) exhibited moderate antiviral activity in cell culture (EC_{99} values: 64-95 μM), and were proven to affect viral RNA synthesis. Our approach to integrate complementary enzymatic, cellular and mechanistic assays should guide ongoing development of improved influenza virus PA inhibitors.

INTRODUCTION

Influenza viruses cause significant morbidity and mortality, and vaccination provides only partial protection in some populations such as elderly (Treanor et al., 2012). Antiviral therapy comprises the M2 blockers amantadine and rimantadine, and the neuraminidase inhibitors oseltamivir and zanamivir (Vanderlinden and Naesens, 2014). The global spread of amantadine-resistant influenza viruses and growing resistance to neuraminidase inhibitors (in particular oseltamivir) underline the need for novel therapeutics with a different mode of action.

An attractive target is the influenza virus RNA polymerase complex (Ruigrok et al., 2010), composed of three proteins: PA, PB1 and PB2. The RNA polymerizing activity resides in the PB1 subunit. PB2 and PA carry out the first step of the viral transcription process, referred to as “cap-snatching”. This reaction appears unique for Orthomyxo-, Arena- and Bunyaviridae (Morin et al., 2010; Reguera et al., 2010; Guilligay et al., 2014). In the case of influenza virus, the capped 5' end of a cellular pre-mRNA is bound by PB2 and subsequently cleaved by PA at 10-13 nucleotides from the cap, to serve as primer for viral mRNA synthesis (Bouloy et al., 1978; Plotch et al., 1981; Dias et al., 2009; Yuan et al., 2009). The catalytic site of the PA endonuclease, residing in its N-terminal part (PA-Nter; residues 1-256 or less), is highly conserved in influenza A and B viruses. Several crystallographic studies have revealed the protein structure of PA-Nter which contains two (or possibly one or three) Mn^{2+} or Mg^{2+} ions in its catalytic core, although it is still unresolved which metal ions are present in native enzyme (Yuan et al., 2009; Zhao et al., 2009; Dias et al., 2009; DuBois et al., 2012; Kowalinski et al., 2012; Parhi et al., 2013). In biochemical assays, the enzyme's activity appears higher with manganese, but

magnesium appears more biologically relevant since the intracellular concentration of free Mg^{2+} is >1000-fold higher than that of Mn^{2+} (Maret, 2010). For unrelated nucleases, reduced substrate specificity was observed when tested with manganese instead of magnesium. This is probably due to the less rigid coordination requirements of manganese compared to magnesium (Yang et al., 2006).

The available crystal structures of PA-Nter enable rational design of PA inhibitors (PAIs). The first class of influenza endonuclease inhibitors (Tomassini et al., 1994; Hastings et al., 1996) are 4-substituted-2,4-dioxobutanoic acids with a characteristic β -diketo acid (DKA) motif. Among this series, L-742,001 (**1** in Table 1) was identified as a particularly potent inhibitor, both in enzyme- and cell-based antiviral assays (Hastings et al., 1996; Nakazawa et al., 2008). Several other classes of endonuclease inhibitors have been reported (Tomassini et al., 1996; Cianci et al., 1996; Singh and Tomassini, 2001; Iwai et al., 2010; Baughman et al., 2012; Sagong et al., 2013; Chen et al., 2014). All these compounds bear chelating motifs able to bind the bivalent metal ions in the catalytic core of PA-Nter (Rogolino et al., 2012). A similar concept has led to clinical development of HIV integrase (HIV IN) inhibitors and is also explored to design inhibitors of HIV RNase H or HCV polymerase.

In this report, we investigated a series of DKAs (and DKA-bioisosteric compounds) incorporating different chemical scaffolds, which were synthesized as specific PAIs (**1-6**, Table 1) or originally designed towards HIV IN (**7-47**, Table 1 and 2) (Sechi et al., 2004; Sechi et al., 2005; Sechi et al., 2006; Bacchi et al., 2008; Zeng et al., 2008; Bacchi et al., 2011; Reddy et al., 2011; Maurin et al., 2004). Although these compounds contain relevant scaffolds for PAI development, information on their activity against PA-Nter is lacking.

Hence, their SAR towards PA-Nter was analyzed to build a plausible pharmacophore model. A selection of compounds was also evaluated against untruncated PA.

In the case of HIV IN, the potency of DKAs in enzymatic assays was found to depend on which bivalent metal ion (Mg^{2+} or Mn^{2+}) was used (Marchand et al., 2003). This prompted us to investigate whether a similar metal ion dependency may occur when evaluating PAIs in an enzymatic assay, such as the novel molecular beacon (MB) assay presented here. This real-time MB method, applicable for high-throughput screening, also enabled to compare the kinetic parameters of PA-Nter for different substrates or metal ions.

To validate the inhibition data for PA-Nter, we used two cell culture methods: an influenza virus ribonucleoprotein (vRNP) reconstitution assay and a virus yield replication assay. Furthermore, a basic mechanistic study was performed to verify that the antivirally active DKAs indeed act upon viral RNA synthesis.

MATERIALS AND METHODS

EXPERIMENTAL SECTION

Compounds

The synthetic route to obtain **1-6** (i.e. L-742,001 and derivatives) can be found in the Supplemental Data (Supplemental Methods). Compound **7** was obtained from Sigma-Aldrich (St. Louis, MO). The procedures for chemical synthesis of **8-11** and **30-47** were reported elsewhere (Tomassini et al., 1994; Sechi et al., 2004; Maurin et al., 2004; Sechi et al., 2005; Sechi et al., 2006; Zeng et al., 2008; Bacchi et al., 2008; Bacchi et al., 2011; Reddy et al., 2011). Ribavirin (Virazole; ICN Pharmaceuticals, Costa Mesa, CA) and chloroquine diphosphate salt (Sigma-Aldrich) were included as reference compounds. The test compounds were stored as a 20-100 mM stock solution in DMSO. In all cellular assays, the final DMSO concentration was kept below 0.8%, a concentration that was free of aspecific effects on cell viability or luciferase activity.

Pharmacophore building

Twelve of the more active compounds (**1-6**, **10**, **16**, **34**, **39-41**) were chosen for generating the pharmacophore model. For each compound a full minimized model was built in Molecular Operating Environment software package (MOE, version 2010.11, Chemical Computing Group Inc., Montreal, Canada) using MMFF94x force field with standard bond length and angles. The obtained database was used as starting point for the pharmacophore elucidation procedure implemented in MOE. The selected search scheme

was PPCH_All (Planar-Polarity-Charge-Hydrophobicity), while all settings were kept as default except for conformations (set to stochastic search). Among the proposed pharmacophore queries the best one was chosen on the basis of statistical parameters, i.e. high correlation coefficient and lower RMSD value.

Production of recombinant influenza virus PA-Nter protein

The coding sequence for PA-Nter (i.e., residues 1-217 from the PA protein of influenza virus strain A/X-31) was cloned in the pET28a(+) plasmid (Merck KGaA, Darmstadt, Germany) with an N-terminal 6xHis-tag, and this bacterial expression plasmid was transformed into *E. coli* BL21-CodonPlus cells (Agilent Technologies, Santa Clara, CA). The plasmid was subjected to site-directed mutagenesis (Quikchange II, Agilent Technologies) to prepare the K134A substituted PA-Nter enzyme.

The bacteria were grown to an OD of 0.6, when IPTG was added at a final concentration of 1 mM to induce expression of recombinant proteins for 5 h at 37 °C. The bacterial cells were ruptured using a French press and the proteins were purified by 6xHis-Ni-NTA chromatography (Qiagen, Valencia, CA), followed by buffer exchange using PD-10 desalting columns (GE Healthcare, Diegem, Belgium) to keep the proteins in storage buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM β -mercaptoethanol, 50% glycerol). Protein purity was verified by SDS-PAGE with Coomassie Blue staining and protein concentration was determined by Bradford assay. Finally, the purified proteins were divided in aliquots and stored at -80 °C.

Plasmid-based endonuclease assay

This enzymatic endonuclease activity assay was performed according to a previously published method (Carcelli et al., 2014) with minor modifications. One microgram of recombinant PA-Nter was incubated with 1 μ g (16.7 nM) of single-stranded circular DNA plasmid M13mp18 (Bayou Biolabs, Metairie, Louisiana) in the presence of the test compounds and at a final volume of 25 μ L. The assay buffer contained 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM β -mercaptoethanol and 1 mM $MnCl_2$ (unless stated otherwise). The reaction was incubated at 37 °C for 2 h and then stopped by heat inactivation (80 °C, 20 min). The endonucleolytic digestion of the plasmid was visualized by gel electrophoresis on a 1% agarose gel with ethidium bromide staining. The amount of remaining intact plasmid was quantified by ImageQuant TL software (GE Healthcare). The percentage inhibition of PA endonuclease activity was plotted against the compound concentration on a semi-logarithmic plot, using GraphPad Prism software (GraphPad Software, La Jolla, CA). Values were the mean $\pm$ S.E.M. of three independent experiments. The 50% inhibitory concentrations (IC_{50}) were obtained by nonlinear least-squares regression analysis.

Molecular beacon-based endonuclease assay

The sequences of the eight molecular beacon (MB) substrates are given in Table 3. MB-C₁₅-DFO, MB-A₁₅-DFO, MB-U₆A₂U₇-DFO, MB-OH-DFO and MB-het2-DFO were purchased from Eurogentec (Seraing, Belgium), while MB-het1-FAM, MB-het2-FAM and MB-het3-FAM were synthesized by S.A.E. Marras. The molecular beacons were prepared

by automated solid-phase DNA synthesis on an Applied Biosystems (Forster City, CA) 394 DNA/RNA synthesizer. A controlled-pore glass column (Biosearch Technologies, Novato, CA) was used to incorporate a quencher moiety (dabcyl) at the 3' end and a fluorescein (FAM) phosphoramidite (Glen Research, Sterling, VA) was used to incorporate a fluorophore moiety at the 5' end of the oligodeoxyribonucleotides. The molecular beacons were then purified by high-pressure liquid chromatography on a Beckman Coulter System Gold (Indianapolis, IN) chromatograph through a C-18 reverse-phase column (Waters Corporation, Milford, MA). Concentrations were determined on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

The PA-Nter assay was performed at 37°C in a buffer consisting of 50 mM Tris-HCl pH 8, 100 mM NaCl and 10 mM β -mercaptoethanol, to which either 1 mM MnCl_2 , 1 mM MgCl_2 or no metal was added.

To determine the kinetic parameters for the different MB substrates, the substrate concentrations were varied from 25 nM to 5 μM , while the enzyme concentration was kept constant at 0.04 μg per μL . These tests were performed in the presence of 1 mM MnCl_2 and in 10- μL reaction volumes in a 384-well plate format. MB-het1-FAM, MB-het2-FAM and MB-het3-FAM (FAM-labeled) were excited at 495 nm and emission was recorded at 520 nm, while the DFO-labeled MB molecules MB-C₁₅-DFO, MB-A₁₅-DFO, MB-U₆A₂U₇-DFO, MB-OH-DFO and MB-het2-DFO were read at 554 nm excitation and 576 nm emission wavelength. The increasing fluorescence signal was measured every 14 seconds for 60 minutes using a spectrofluorometer (Safire2, Tecan, Männedorf, Switzerland). All reactions were performed in duplicate. The initial reaction velocity V_0 (determined at concentrations at which no substrate inhibition occurred) was calculated

as the slope for the 5-15 min time interval. To estimate the K_m values, the Michaelis-Menten equation was fitted to these V_0 data, based on nonlinear regression analysis and using GraphPad Prism software.

Similar conditions were used for the inhibition assays. Serial dilutions of the test compounds were added to the reaction mixture containing buffer (with or without added metal ions) and 20 or 100 nM MB, and the reaction was started by adding 0.4 μ g of PA-Nter. All reactions were performed in duplicate. The initial cleavage velocity was plotted against the compound concentration on a semi-logarithmic scale, and for each compound the IC_{50} value was calculated using non-linear regression analysis (Graphpad Prism). Values were the mean of at least two independent experiments.

The cleavage pattern of the molecular beacons was studied in the same buffer conditions, but the reaction was stopped at the indicated time points by heat inactivation (80 °C, 20 min). The samples were loaded on 15% TBE-urea polyacrylamide gels (Criterion, Bio-Rad, Hercules, CA) for denaturing nucleic acid PAGE. The bands were visualized by fluorescent gel imaging using an Ettan DIGE imager apparatus (GE Healthcare).

Cells and media

Madin-Darby canine kidney (MDCK) cells (a kind gift from M. Matrosovich, Marburg, Germany) and human embryonic kidney 293T (HEK293T) cells (purchased from Thermo Fisher Scientific, Waltham, MA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate and 0.075% sodium bicarbonate. Virus experiments were performed in MDCK infection

medium, consisting of Ultra MDCK medium (Lonza, Basel, Switzerland) supplemented with 0.0225% sodium bicarbonate, 2 mM L-glutamine, and 2 µg/mL tosylphenylalanylchloromethylketone (TPCK)-treated trypsin (Sigma-Aldrich). The cells were incubated in a 5% CO₂ humidified atmosphere.

vRNP reconstitution assay

The assay to determine the inhibitory effect of the compounds on reconstituted influenza virus vRNPs is described in more detail elsewhere (Meneghesso et al., 2012; Stevaert et al., 2013). Four sets of vRNP reconstituting plasmids derived from different influenza virus (sub)types were used: [1] four reverse genetics plasmids derived from influenza A/PR/8/34 (encoded pVP-PB1, pVP-PB2, pVP-PA and pVP-NP, which contain the cDNAs in the bidirectional expression cassette of pHH21), kindly given by M. Kim (Korea Research Institute of Chemical Technology, Daejeon) (Kim et al., 2013); [2] four plasmids derived from the avian influenza A/turkey/England/50-92/1991 (H5N1) virus, containing the cDNA sequences in a pol-II driven expression cassette; generously given by W. Barclay (Imperial college London, United Kingdom) (Moncorgé et al., 2010). In this set, the PB2 protein contains the avian influenza Glu627 residue; [3] identical to the previous set, except that the PB2 expressing plasmid was mutated to encode the human influenza Lys627 residue (Moncorgé et al., 2010); [4] four reverse genetics plasmids derived from influenza B/Yamanashi/166/98 (designated pAB251-PB1, pAB252-PB2, pAB253-PA, and pAB255-NP, which contain the cDNAs in a bidirectional expression cassette; generously donated by J. McCullers (St. Jude Children's Research Hospital, Memphis, TN)

(Hoffmann et al., 2002). Set 1 was used in combination with an influenza A-specific firefly luciferase (fluc) reporter plasmid (Kim et al., 2013) that was also given by M. Kim. For sets 2 and 3, the fluc reporter was a kind gift from W. Barclay. For set 4, an influenza B-specific fluc reporter plasmid was used (kindly donated by K. Nagata, University of Tsukuba, Japan) (Wakai et al., 2011).

Briefly, the four relevant plasmids [i.e. the expression plasmids for PB1, PB2, PA and NP] were combined with the corresponding fluc reporter plasmid, and cotransfected into HEK293T cells using Lipofectamin 2000 (Meneghesso et al., 2012; Stevaert et al., 2013). The cells were transferred to a 96-well plate containing serial dilutions of the test compounds and after 24 h incubation at 37 °C, luciferase activity was determined using the One-Glo assay system from Promega (Madison, WI). The 50% effective concentration (EC₅₀) was defined as the compound concentration causing 50% reduction in the vRNP-driven firefly luciferase signal, as compared to cells receiving medium instead of compound. These EC₅₀ values were calculated by interpolation assuming a semi-log dose-response effect. In parallel, compound cytotoxic activity was determined in untransfected HEK293T cells which had been incubated with serial dilutions of the compounds for 24 h, using the MTS cell viability assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega). These spectrophotometric data were used to calculate the 50% cytotoxic concentration (CC₅₀), i.e. the concentration reducing cell viability by 50%, as compared to the wells receiving medium instead of compound. Ribavirin was included as the reference compound.

Virus yield assay

To determine anti-influenza virus activity in infected cell cultures, we performed a virus yield assay (Stevaert et al., 2013). One day prior to infection, MDCK cells were seeded into 96-well plates at 25,000 cells per well. At day 0, serial dilutions of the test compounds were added, immediately followed by infection with influenza A/PR/8/34 virus. The multiplicity of infection (MOI) was 150 CCID₅₀ per well [50% cell culture infectious dose; determined by the method of Reed and Muench]. After 24 h incubation at 35 °C, the supernatants were collected and stored at -80 °C. The virus amount in these samples was estimated by determining the viral genome copy number in a one-step quantitative real-time reverse transcription (qRT)-PCR assay (CellsDirect One-Step qRT-PCR kit; Invitrogen, Life Technologies, Gent, Belgium), with influenza virus M1-specific primers and probe [see (Vanderlinden et al., 2010) for all details]. Absolute quantification of vRNA copies was performed by including an M1-plasmid standard. The EC₉₉ and EC₉₀ values were calculated by interpolation from data of at least three experiments and defined as the compound concentration causing respectively a 2-log₁₀ and 1-log₁₀ reduction in viral RNA (vRNA) copy number, as compared to the virus control receiving no compound. In parallel, the CC₅₀ values after 24 h incubation with compounds were determined in uninfected MDCK cells, using the MTS cell viability assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega). Ribavirin was included as the reference compound.

One-cycle virus replication assay

A simplified time-of-addition experiment was performed as previously described in more detail (Stevaert et al., 2013). The compounds were added to confluent MDCK cells at either 30 min before or 1 h after virus infection with influenza virus A/PR/8/34, and incubated at 35 °C. At 8 h p.i., the supernatant was removed and total cellular RNA extracts were prepared with the RNeasy Mini Kit (Qiagen). In order to quantify the negative-sense vRNA, the samples were analyzed by two-step real-time qRT-PCR (Vanderlinden et al., 2010). cDNA synthesis was performed on 0.5 µg of total cellular RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Life Technologies) and 80 nM M1-FOR primer. Then, real-time PCR was performed, using influenza virus M1-specific primers and probe (Vanderlinden et al., 2010), and quantitative PCR (qPCR) MasterMix (Eurogentec). All samples were analyzed in duplicate. The vRNA copy number was quantified by including an M1-plasmid standard, and the fold increase in vRNA copies was calculated relative to the viral copy number added at time zero. Chloroquine was included as the reference compound.

RESULTS

Selection of DKAs and derivatives

In Tables 1 and 2, the chemical structures are shown of the different series of DKA derivatives that we evaluated for inhibitory activity against PA-Nter. In the known PAI L-742,001 (**1**), included as the reference, the β -diketo acid motif is attached to a piperidine moiety which carries two cyclic substituents. These two ‘wings’ occupy opposite hydrophobic pockets around the catalytic center of PA-Nter and are considered crucial for achieving strong binding to PA-Nter (DuBois et al., 2012; Stevaert et al., 2013). To acquire more information about the structural features of this prototype, we prepared some closely related analogues (**3** and **5**), N-substituted at the piperidine ring, and also considered the diketo methylesters (**2**, **4**, **6**).

Moreover, on the basis of the assumption that the DKA moiety is essential for anti-PA activity, our aim was to explore this structural motif and the related chemical space. First, we selected a series of DKA analogues carrying an indole scaffold (**30-43**; Table 2), where major modifications were made to determine the effect of different substituents on activity. Second, we included other series of compounds where: a) the β -diketo acid group has been replaced with a bioisostere (**44-47**); b) the aromatic scaffold has been replaced with a second DKA functionality (**7**); c) a triazole bioisostere has been incorporated in place of the carboxyl group (**8**); d) an additional phenyl group has been introduced on the 1,3-dioxo-phenylpropane framework (**9**); and e) the DKA motif is connected to a different heterocycle (**10**, **11**). Third, since 2,4-dioxo-4-phenylbutanoic acid (DPBA, **12**, Table 1) represents a suitable platform to examine structural and biological features of DKAs,

several substitutions on the phenyl (**13-20**), as well as on the diketo moiety with an additional keto group (**21-24**), were evaluated.

Finally, inspired by studies on HIV IN (Bacchi et al., 2011), we considered two additional chemotypes as putative DKA-related compounds: the 4-quinolone-3-carboxylic acids **25-27** (and ethyl ester **28**), which bear a monoketo acid motif, and the bioisosteric compound **29**, where the DKA moiety was replaced by a 4,5-dihydropyrimidine-4-carboxamide scaffold.

Activity in the plasmid-based PA endonuclease assay

The PA endonuclease assay that we used is based on end-point gel electrophoretic analysis of substrate cleavage (Fig. 1). Among the various PA-Nter substrates that were reported to work (Dias et al., 2009; DuBois et al., 2012; Kowalinski et al., 2012; Parhi et al., 2013; Bauman et al., 2013; Datta et al., 2013; Chen et al., 2014), we chose the M13mp18 ssDNA plasmid since it is commercially available. The active site substitution K134A in the PA-Nter enzyme completely abolished the endonucleolytic cleavage, in agreement with previous reports (Hara et al., 2006; Yuan et al., 2009; Crépin et al., 2010). At the time of its discovery (Hastings et al., 1996), L-742,001 (**1**) was reported to have an IC₅₀ value of 0.43 µM in an enzymatic endonuclease assay with a capped mRNA substrate and influenza virus ribonucleoprotein complexes, which contain the entire PA protein within the heterotrimeric viral polymerase. We here obtained the same IC₅₀ value (i.e. 0.5 µM; Table 1) against isolated PA-Nter. This supports the view that the endonuclease catalytic site has the same structure in isolated PA-Nter as in native polymerase, and

implicates that enzymatic assays with PA-Nter are relevant for developing catalytic site inhibitors (Crépin et al., 2010; Noble et al., 2012). Regarding the direct analogues of L-742,001 that we tested (Table 1), the IC₅₀ data demonstrate that the activity is unchanged when the phenyl moiety of L-742,001 is replaced by a cyclohexyl group (**5**), and only slightly higher when this phenyl carries a *p*-fluoro substituent (**3**). Also, methyl-esterification of the terminal carboxyl in the DKA part did not affect the inhibitory activity (**2**, **4**, and **6**).

A prototypic inhibitor that has been used in several enzymatic studies with PA-Nter is 2,4-dioxo-4-phenylbutanoic acid (DPBA, **12**). The IC₅₀ value for DPBA in our plasmid assay was 2.7 μ M, which is comparable to the data reported by others (Dias et al., 2009; Kowalinski et al., 2012; Noble et al., 2012). This value was unchanged upon addition of a methoxy in *ortho* (**14**) or *para* position (**16**). Addition of an *ortho*-chlorine had no effect (**18**), but a chlorine in *para* position (**17**) reduced the potency by a factor 4. We also included three methyl esters (**13**, **15** and **19**); their IC₅₀ values were consistently 4- to 5-fold higher than the corresponding carboxylic acids. Also, replacement of the carboxylic functionality with a triazole ring bioisostere (**8**) or a phenyl moiety (**9**) caused a dramatic reduction in inhibitory activity. The length of the DKA arm seems optimal in DPBA, since analogues carrying an extra keto group (**21-24**) were devoid of activity. The critical role for the phenyl ring of DPBA is evident from the fact that unsubstituted dihydroxybutenedioic acid (**7**) was inactive. Introduction of two large aromatic substituents (benzyloxygroups) onto this phenyl ring [**20**; known as the HIV IN inhibitor L-708,906 (Hazuda et al., 2000)] led to a dramatic (50-fold) reduction in activity.

The same DKA fragment as in DPBA and L-742,001 is present in the extended series of indole derivatives that we tested (Table 2). Several of these had strong activity against

PA-Nter with IC₅₀ values below 1 μ M, which is comparable to the value for L-742,001. The following parameters were found to influence the activity. (i) The *N*-substituent of the indole increased the IC₅₀ value in the order methyl < ethyl < benzyl (cf. **30-31**; **34-35-36**; and **39-40-41**). (ii) The location of the DKA arm on the indole moiety (i.e. at position 2 or 3) had variable impact in relationship to the size of the indole moiety. Position 3 represents the optimum geometry in the case of the simple indole ring (compare **39** and **41** with **30** and **31**, respectively). (iii) When the indole part was enlarged by adding an extra dioxole ring, this extension led to increased potency for the 2-diketo derivatives (compare **34** and **36** with **30** and **31**, respectively). However, for the compounds bearing the diketo motif in 3-position, this extra dioxole ring appeared to reduce the activity (cf. **42** and **39**). (iv) The DKA-methyl esters of these indole derivatives (**37** and **43**) were markedly less active than the corresponding analogues containing a free carboxyl group (**36** and **42**, respectively). A favorable IC₅₀ value (0.8 μ M; Table 1 and Fig. 1) was also noted for the pyrrole derivative **10** (known as the Merck compound L-731,988), which was previously discovered as a potent inhibitor of HIV IN (Hazuda et al., 2000). Similar to the data above, its methyl ester proved to be 7-fold less active. This pyrrole derivative can be viewed as an analogue of indole **31**, in which the ring system is “size-reduced” to a pyrrole, and the benzyl is substituted with a *p*-fluoro atom.

Finally, we evaluated several compounds in which the two metal-chelating moiety is incorporated into a cyclic system. This strategy proved highly successful to create potent and selective HIV IN inhibitor (Agrawal et al., 2012). In the case of PA-Nter, neither of the cyclic diketo analogues tested, i.e. the indole derivatives **44-47**, as well as the other DKA bioisosteres, i.e. the quinolones **25-28** and the hydroxypyrimidine carboxamide **29**, had any activity in the enzymatic PA-Nter assay.

Pharmacophore model generation

In order to elucidate the structural requirements for novel PAIs, a 3D pharmacophore model was built (Fig. 2) based on the efficacy data of a selection of tested compounds. Namely, the training set contained twelve molecules with an IC₅₀ value against PA-Nter of 2 μ M or less, i.e. the β -diketo acids or esters **1-6**, **10**, **16**, **34**, and **39-41**, and these were submitted to a pharmacophore generation protocol using the Molecular Operating Environment software package. With the exception of **16**, **34**, and **39**, all compounds also showed antiviral activity in cell culture (see below). Fifteen PAI pharmacophore models were generated and the best hit was evaluated in terms of statistical parameters such as correlation coefficient and RMSD value. This model featured five pharmacophore elements including three metal ligator features (ML), one aromatic or hydrophobic element (Hyd-Aro) and one cyclic aromatic region (Aro) (Fig. 2, panels A and B). This five-points pharmacophore was then validated by screening our *in-house* database of about 150 compounds, chosen on the basis of their previously evaluated chelating ability towards metal cofactors of other metalloenzymes, and considering compounds having molecular diversity. All selected compounds were retrieved from the database and nicely fitted with the chemical features of our hypothesis, thus confirming the reliability of the proposed model.

As shown in Fig. 2A, the overall mapping results indicate that almost all compounds aligned in a similar spatial arrangement within the model, highlighting important structural similarities between compounds bearing piperidine and indole scaffolds. A good fitting was shown for the co-planar diketo acid/ester fragment of all compounds within three

points, whereas the orientation of the substituted heterocyclic portion mapped into the remaining pharmacophore elements, with the exception of the *para*-F-benzyl moiety of the pyrrole scaffold (**10**), which has a different orientation. Moreover, the *N*-methylcyclohexyl substituent of the diketo methyl ester **6** is located in an opposite conformation with respect to the same moiety in the corresponding acid **5**. In L-742,001, the two aromatic moieties attached to the piperidine ring are oriented in opposite directions and perpendicularly to the DKA motif. Tolerance for various orientations of the aromatic and hydrophobic moieties agrees with structural data showing that the catalytic core of PA-Nter is surrounded by different hydrophobic pockets well suited for inhibitor binding (DuBois et al., 2012; Kowalinski et al., 2012). Hence, it is relevant to design DKA derivatives containing hydrophobic substituents in an opportune topological disposition, so as to occupy these alternative pockets in PA-Nter.

With regard to the metal chelating fragment, the 3D spatial arrangement and distance constraints between the chemical features are in agreement with a previously published minimal pharmacophore model (Parkes et al., 2003). Both features ML1–ML2 and ML2–ML3 (i.e. the donor triad atoms chemotype; Fig. 2B) are able to coordinate the two metal ions in the PA-Nter active site. In our pharmacophore model, the interfeature distances between ML2 and ML1, and between ML2 and ML3, are 2.64 and 2.77 Å, respectively (Figure 2C) (distance ML1–ML3 is 4.74 Å). The other two additional features (Aro, Hyd/Aro) would be involved in favorable contacts with the hydrophobic binding pockets around the active center. The distances between the key ML1, ML2 and ML3 features and the aromatic feature “Aro” are 4.41, 6.12 and 6.46 Å, respectively. The distance between

the optimally oriented Aro and Hyd/Aro features is 2.55 Å, whereas that between ML1 and Hyd/Aro is 2.98 Å.

In Fig. 2D, two proposed models for interaction of the β -diketo acid motif with the metal ions are shown (see Figure legend for all details). Importantly, the ML1-ML2-ML3 interfeature distances in our pharmacophoric model are coherent with favorable ligand-metal interactions and in agreement with both binding models (DuBois et al., 2012; Stevaert et al., 2013).

Namely, the Mn^{2+} - Mn^{2+} distance is 3.82 Å in the crystal-based and 3.31 Å in the docking-based model (Fig. 2D). In thermodynamic models using Mg^{2+} as the cofactor, the Mg^{2+} - Mg^{2+} distance was predicted to decrease from 4.91 to 3.85 Å upon RNA binding (Xiao et al., 2014). These changes in the intermetal distance along the catalytic pathway were already demonstrated for *B. halodurans* RNase H (Yang et al., 2006) and *EcoRV* (Horton and Perona, 2004).

The twelve compounds selected for pharmacophore generation were also submitted to basic physicochemical calculations to try to predict their membrane permeating capability, which is required to achieve antiviral activity in cell culture. The calculated atom-based values (Supplemental Table 1) for almost all selected compounds (**1-6**, **10**, **16**, **34**, and **39-41**) fall within the desirable range for good absorption and membrane permeability (i.e. $\log P < 5$; molecular weight < 500 ; H-bond acceptor and donor counts of < 5 and < 10 , respectively). However, three compounds (i.e., **16**, **34** and **39**) showed unfavorable predicted $\log P$ values, which may (at least partially) explain their inactivity in cellular assays (see below). Other studies suggest that compounds with a polar surface area

(PSA) $< 60 \text{ \AA}^2$ are likely to be well absorbed ($> 90\%$), whereas compounds with PSA $> 140 \text{ \AA}^2$ are predicted to be poorly absorbed ($< 10\%$) (Lipinski et al., 2001). All twelve compounds had an intermediate PSA value well below $140 \text{ \AA}^2$.

Development of the molecular beacon-based endonuclease assay

The plasmid-based endonuclease method has the disadvantages of being discontinuous and time consuming, which makes it less suited for screening large series of compounds. We therefore developed an alternative real-time assay which uses a molecular beacon (MB) as the enzyme substrate and is amenable to high-throughput format. A MB is a single-stranded DNA probe that contains a fluorophore moiety at one end and a non-fluorescent quencher moiety at the other. When intact, the MB forms a stem-and-loop structure, bringing the fluorophore and quencher in very close proximity, which results in superior quenching efficiency compared to a linear dual-labeled probe. MB cleavage by recombinant PA-Nter (depicted in Fig. 3A) separates the fluorophore from the quencher, and the evolving fluorescence reveals the cleavage process in real-time (Marras et al., 2006).

First, the real-time MB method was used to determine the kinetic parameters of PA-Nter versus Mn^{2+} or Mg^{2+} , and eight different oligonucleotide substrates (i.e. a MB with a heterogeneous sequence, or containing oligo-C, -A or -U). The K_m values determined for the eight different MB substrates were in the range of $0.09\text{-}0.64 \text{ }\mu\text{M}$ (see Fig. 3B and Table 3, which also contains the signal-to-noise ratio at 100 nM MB). This indicates that, under the conditions of this MB assay, PA-Nter does not possess much preference for specific

dinucleotide linkages. In this context, it is noteworthy that the MB-het2-DFO substrate, which contains two 5'-GC-3' motifs, did not prove to be a better substrate than the dU-rich MB-U₆A₂U₇-DFO. On the contrary, we noted a 6 times higher $V_{\max}$ with MB-U₆A₂U₇-DFO than with MB-het2-DFO. This contrasts with the observation by another group that PA-Nter has a preference for 5'-GC-3' in RNA substrates (Datta et al., 2013). However, efficient cleavage of U-rich RNA substrates was already demonstrated by Dias et al. (Dias et al., 2009). In these two studies, PA-Nter was evaluated against RNA substrates, whereas our MB assay uses DNA-oligonucleotides (which were preferred because of their higher chemical stability). Other groups have successfully developed fluorescent assays for PA endonuclease using totally different DNA or RNA probes (Kowalinski et al., 2012; Noble et al., 2012; Bauman et al., 2013; Datta et al., 2013; Chen et al., 2014), confirming that PA-Nter can cleave different DNA or RNA sequences in enzymatic assays. From a technical viewpoint, our MB assay is superior in having a higher signal-to-noise ratio than fluorescent assays using non-bended oligonucleotide substrates.

By using gel electrophoresis with fluorescence detection (Fig. 3C), we confirmed that the fluorescence enhancement observed by real-time fluorometry was due to MB cleavage and not to mere opening of the stem structure upon binding of the MB to the active site of PA-Nter. An intriguing and unexpected finding relates to the impact of the bivalent metal ion on cleavage efficiency by PA-Nter. While using the 7-kb plasmid substrate (M13mp18), we observed no endonuclease activity when Mn^{2+} was omitted from the reaction mixture or replaced by Mg^{2+} (second and third from last lanes in Fig. 1), in agreement with a previous report (Dias et al., 2009). In contrast, PA-Nter was found to efficiently cleave the MB substrate when magnesium instead of manganese was added,

and even without addition of a metal ion, which is likely due to co-purification of a metal ion (probably Mg^{2+}) (Xiao et al., 2014) during preparation of the PA-Nter enzyme. Significant substrate cleavage in the absence of added salt was also observed by Noble et al. (Noble et al., 2012) in experiments with untruncated PA. The effect of the metal ion depended on which MB sequence was used, since one MB was more rapidly cleaved in the presence of Mg^{2+} than Mn^{2+} , while for another sequence, the opposite was seen (see Fig. 3D for all details).

When analyzing the MB cleavage by gel electrophoresis (Fig. 3C), we observed a difference in the cleavage pattern, dependent on which metal ion was used. This is in accordance with the results obtained by Noble et al (Noble et al., 2012). When using Mn^{2+} as a cofactor, PA-Nter appears able to cleave shorter oligonucleotides than in the presence of Mg^{2+} or without addition of a bivalent metal (i.e. in the presence of the co-purified metal). The Mn^{2+} condition gave rise to cleavage products which increased in function of time and appeared as an extra band on the gel, which migrated slower than intact beacon. Most probably, migration of the shorter oligonucleotides is slowed down by the presence of the positively charged fluorophore. The endonuclease activity resulting from the co-purified metal was completely abrogated upon addition of EDTA to the reaction mixture (Fig. 3C, right panel). The observation that the metal cofactor changes the cleavage pattern suggests that the structure of the active site depends on which metal ions are present. Since this may influence the binding properties of PAIs, we used the MB assay to evaluate a series of representative DKA inhibitors with activity towards PA-Nter in the plasmid-based assay. The MB assay with these compounds was performed in the presence of Mn^{2+} , Mg^{2+} or without addition of a bivalent metal ion, and using either MB-

U₆A₂U₇-DFO or MB-het2-DFO as the substrate. As shown in Table 4, the metal ion and MB substrate had very little, if any, impact on the IC₅₀ values for L-742,001 (**1**). In Fig. 3E, the fluorographs for this compound and derived dose-response curve are shown for the experiments with Mn²⁺; similar curves were obtained in the Mg²⁺ or no added metal conditions. In contrast, other PAIs, i.e. **10**, **31**, **34**, **39**, **40**, and **41**, had up to 61-fold lower IC₅₀ values in the presence of Mn²⁺ compared to Mg²⁺ (Table 4). When the data obtained in the plasmid and molecular beacon assay (both with Mn²⁺) are being compared, both assays gave the same IC₅₀ values for L-742,001, **5**, **10**, **34**, **40**, and **41**. For some compounds, we observed markedly higher IC₅₀ values when using the MB instead of the plasmid substrate (i.e. factor increase of 35 for **39** and 57 for **12**). On the other hand, for **31**, a 7-fold lower IC₅₀ value was obtained in the MB assay than in the plasmid assay.

Finally, we verified that assays using truncated PA-Nter are adequate to investigate PAIs interacting with the catalytic site. Namely, the inhibitory activity of a few DKA compounds was determined in an enzymatic assay with untruncated PA and Mn²⁺ (Noble et al., 2012). The IC₅₀ values for compounds **1**, **30** and **39** (Table 4; right column), were very similar to the values obtained with PA-Nter using the plasmid/Mn²⁺ assay.

Anti-influenza virus activity in cell culture

Besides evaluation in the PA-Nter enzymatic assay, all DKA analogues were examined for anti-influenza virus activity in cell culture. We first performed the influenza vRNP reconstitution assay in HEK293T cells. Using L-742,001, we previously demonstrated that this method is well suited for determining the activity and selectivity of PAIs in cell culture

(Stevaert et al., 2013). In the present study, we further expanded this vRNP assay by including four different vRNP complexes, i.e. derived from a human influenza A or B virus, an avian H5N1 virus containing the PB2-627Glu avian signature, or the PB2-627Lys human adaptation residue (Moncorgé et al., 2010). As shown in Table 5, L-742,001 and its close analogues **2-6** had strong and consistent activity against all four vRNP complexes, which agrees with our previous analysis that the key residues for binding of L-742,001 in PA-Nter show little variation among influenza A (human or avian) or B viruses (Stevaert et al., 2013).

Among our series of DKA analogues with an indole scaffold (Tables 2 and 6), the following were shown to inhibit influenza vRNP activity: **33** and **40**, which have an *N*-ethyl group; and **31**, **36**, **37**, and **41**, which carry an *N*-benzyl substituent (introduced to modulate lipophilicity, thus assumed to improve cellular uptake). Of these, **31**, **36**, **40** and **41** are unesterified DKAs, whereas **33** and **37** are methyl esters. The highest potency was obtained for **36**. Except for **41**, these indole derivatives were not toxic at the highest concentration tested (200 μ M). Importantly, although these compounds displayed relatively low potency in the vRNP assay [i.e. average EC₅₀ values ranging from 14 μ M (**36**) to 118 μ M (**40**)], the same values were obtained for all four vRNP complexes, whether derived from influenza A (human or avian) or B viruses.

Three other analogues in Table 1 had noticeable activity in the vRNP assay: **10** (L-731,988) and its methyl ester **11** (both are strong inhibitors in the enzymatic test) and **20** (L-708,906; a substituted DPBA derivative with weak activity in the enzymatic test). Several DKAs with potent activity in the PA-Nter enzymatic assay (i.e. IC₅₀ values below 5 μ M; Table 1 and 2) were devoid of any activity in the vRNP cell culture assay. This might

be explained by insufficient cellular uptake due to the anionic charge of the DKA moiety. On the other hand, the activity of **9** in the vRNP assay appears unrelated to inhibition of the endonuclease since this compound was inactive in the enzymatic assay.

In a second stage, the compounds showing activity in the vRNP assay, were submitted to a virus yield assay in influenza virus-infected MDCK cells. For L-742,001 and its close analogues (**1-6**), and **10**, **20**, **31**, **40**, and **41**, the inhibitory activity noted in the vRNP reconstitution assay was confirmed in influenza virus-infected cells. Although their inhibitory potency was quite moderate (i.e. antiviral EC₉₉ values of ~80 µM), our cell culture data indicate that these compounds may represent some novel and cell-permeable PAIs. To validate this assumption, we performed a basic time-of-addition experiment (Fig. 4) in which viral RNA synthesis is measured after one virus replication cycle. We previously demonstrated (Stevaert et al., 2013) that the polyphenolic compound EGCG, which acts as a PAI in enzymatic assays (Kuzuhara et al., 2009; Kowalinski et al., 2012), has an unrelated antiviral mode of action in cell culture. Fig. 4 shows that the reference compound chloroquine, which inhibits influenza virus entry by increasing the endosomal pH (Vanderlinden et al., 2012), was devoid of activity when added post-entry, i.e. at 1 h after virus addition. L-742,001 kept its full inhibitory activity when added at +1 h, confirming that its action point is situated after virus entry. For **10**, **40** and **41**, the highest inhibition was seen (i.e. at least 500-fold reduction in viral RNA synthesis) when the compounds were added 30 min prior to the virus. However, the finding that these compounds still produced ~60- to ~300-fold reduction in viral RNA copy number when added at +1 h, indicates that they do inhibit viral RNA synthesis quite potently, which fits with their designation as PAIs.

DISCUSSION

The potential of influenza virus endonuclease inhibitors as an entirely novel concept in antiviral therapy was recognized about twenty years ago, when two prototype inhibitors, L-742,001 and flutimide, were initially described (Hastings et al., 1996; Tomassini et al., 1996). The fact that these compounds were discovered in a screening approach combining enzymatic and cell-based methods probably explains why these molecules still serve as the two main lead PAIs as of today. Their presumed binding mode in the catalytic center of the enzyme was only recently revealed in cocrystallization experiments with PA-Nter (DuBois et al., 2012; Kowalinski et al., 2012). These studies further revealed several hydrophobic pockets surrounding the catalytic core of PA-Nter, and that the binding of flutimide, L-742,001 and its congener DPBA, involves an induced-fit mechanism. Based on these structural insights, several groups have embarked on in silico design of novel PAIs with unrelated chemical structures (Ishikawa and Fujii, 2011; Bauman et al., 2013; Chen et al., 2014).

We here followed another strategy by evaluating, in complementary enzyme- and cell-based biological assays, available scaffolds of DKA derivatives, which were originally developed towards HIV integrase or other metal-dependent enzymes. By determining their inhibitory activity against PA-Nter, we aimed at identifying which structures (and their structural determinants) can serve as the basis for future design of PAIs. For this purpose, we created a 3D pharmacophore model defining the optimal distances between the metal chelating parts of the ligands and the aromatic/hydrophobic moieties.

Efficient development of novel PAIs requires an enzymatic assay that is amenable to high-throughput screening. The plasmid-based electrophoretic assay used here for evaluating the DKA analogues is quite robust, but too labor-intensive to be used in screening programs. In contrast, our real-time MB assay can be performed in miniaturized plate format and at low cost. Although similar fluorescent assays have been described by others (Kowalinski et al., 2012; Noble et al., 2012; Bauman et al., 2013; Datta et al., 2013; Chen et al., 2014), our MB assay is unique in having a superior signal-to-noise ratio. Besides this analytical aspect, we obtained some unanticipated findings that further broaden the relevance of the MB assay. First, the mere observation that a bent molecular beacon is a very efficient substrate for PA-Nter is reminiscent of what has been described for the type II endonuclease *EcoRV*, which requires bending of the nucleic acid substrate for catalysis to occur (Horton and Perona, 2004; Xiao et al., 2014). Second, PA-Nter proved to have low cleavage site specificity, since it was able to cleave molecular beacons with different DNA sequences, cutting not only the beacon itself but also subsequently formed smaller fragments. Third, whereas most enzymatic studies with PA-Nter have indicated that the enzyme is considerably more active in the presence of Mn^{2+} compared to Mg^{2+} (Dias et al., 2009; Crépin et al., 2010; Datta et al., 2013), our molecular beacon assay works equally well with Mg^{2+} as with Mn^{2+} . We speculate that the intrinsic bending of this substrate may bring the scissile phosphodiester group in a pre-reactive conformation and drive catalysis; hence, cleavage occurs with the less reactive Mg^{2+} . On the other hand, with more flexible substrates (such as the M13mp18 plasmid), the softer metal Mn^{2+} seems to be required for optimal enzymatic activity. Mg^{2+} has been proposed as the biologically relevant cofactor, given that its intracellular concentration is at least 1000-fold higher than that of Mn^{2+} (Zhao et al., 2009; Xiao et al., 2014). This may implicate that

PA-Nter assays using Mg^{2+} are more stringent for evaluating potential PAIs. For most of the DKA inhibitors tested here, the inhibitory activity against PA-Nter was far less with Mg^{2+} than with Mn^{2+} . A notable exception is L-742,001 (and its cyclohexyl analogue **5**), for which the activity was similar for both metal ions. It is tempting to speculate that this metal ion-independent behavior of L-742,001 (or, at least, its strong activity versus Mg^{2+}) may contribute to its superior efficacy in cell culture. For comparison, compound **10** had a similarly low IC_{50} as L-742,001 when evaluated against PA-Nter with Mn^{2+} , but was 40-fold less active in the presence of Mg^{2+} . In influenza virus-infected cells, **10** was only moderately active (despite the fact that this molecule is a strong inhibitor of HIV in infected cells, indicating that it enters the cells quite efficiently) (Hazuda et al., 2000). The issue of cell penetration may also explain our observation that the anti-influenza virus activity in cell culture was higher for the DKA-indole derivatives carrying an *N*-benzyl (compared to ethyl or methyl).

We hypothesize that the less rigid coordination requirements of Mn^{2+} may create flexibility and allow binding of structurally diverse inhibitors (Bock et al., 1999). In contrast, Mg^{2+} may result in a tighter catalytic site which only accepts the best fitting compounds. Noble et al. (Noble et al., 2012) showed a change in CD spectrum when comparing PA with Mn^{2+} to PA with Mg^{2+} , which correlates with an increase in helical content. If the secondary structure of PA-Nter changes depending on the metal, it is possible that the metal ion also affects which hydrophobic pockets are available for binding of the inhibitor. Without crystallographic data of PA-Nter crystallized with inhibitors in the presence of only Mg^{2+} and/or more docking studies, this remains speculative. The finding that several DKA inhibitors prefer Mn^{2+} to exert their effect on PA-Nter is reminiscent of what was described

for HIV IN (Marchand et al., 2003). In this case, both the aromatic and DKA part were shown to play a role in this metal selectivity, which was explained by a metal-dependent binding orientation of the inhibitor. Unfortunately, the understanding of how PAIs bind to PA-Nter is still quite limited. For a selection of PAIs (including L-742,001), the binding mode was recently revealed by cocrystallographic analyses (Kowalinski et al., 2012; DuBois et al., 2012). For L-742,001, our resistance studies in cells infected with a series of mutant influenza viruses established the role of several residues in the catalytic center of PA or surrounding hydrophobic pockets, in positioning this inhibitor in the active site of PA-Nter (Stevaert et al., 2013).

Our enzyme inhibition data for PA-Nter convincingly demonstrate that the setup of the enzymatic assay (i.e. substrate, metal cofactor and type of readout) should be carefully chosen when performing a compound screening for PAI development. We provide evidence that inhibitors targeting the catalytic site have similar activity whether assessed with PA-Nter or untruncated PA. Given the uncertainty on which metal ions (i.e. Mg^{2+} or Mn^{2+}) are present in the active site, evaluation of potential PAIs against both metals (as possible with our MB assay) seems recommended. A number of recent reports have described novel PAIs with DKA-unrelated structures (Iwai et al., 2010; Baughman et al., 2012; Chen et al., 2014; Bauman et al., 2013). Only a few of these molecules were subsequently proven to have activity in influenza-virus infected cell cultures. The importance of proof-of-concept cell culture evaluation during early hit discovery of potential PAIs also appears from our cell culture studies using influenza virus vRNP reconstitution and replication assays. The fact that hit compounds with excellent activity against PA-Nter (i.e. comparable to that of L-742,001) may not survive proof-of-concept

evaluation in cell culture can be related to different factors. As suggested above, an enzymatic PA-Nter assay using Mg^{2+} might be more stringent and have a better predictive value. Second, the hydrophilic/lipophilic balance for the chelating fragment and aromatic framework of DKA-based compounds may play an important role in explaining their antiviral potency. Besides, we previously demonstrated that the catechol compound EGCG (epigallocatechin gallate), which is nicely active in an enzymatic PA-Nter assay, owes its excellent activity in virus-infected cells entirely to inhibition of virus entry (Stevaert et al., 2013). To avoid this confusion, we here evaluated the active DKA compounds in a basic time-of-addition experiment. Our data indicate that the pyrrole compound **10** and the indole compounds **40** and **41** do inhibit viral RNA synthesis, besides having a secondary effect on virus entry.

In conclusion, we demonstrated that diverse DKA scaffolds can lead to potent inhibition of PA-Nter in enzymatic assays, which agrees with the notion that the catalytic center of PA-Nter is quite spacious and flexible, and subject to induced-fit inhibitor binding (DuBois et al., 2012; Kowalinski et al., 2012). Our pharmacophore model defines strict structural requirements along with chemical features, and serves as a basis for design of novel PAIs with DKA-based structures or bioisosteres thereof. For high-throughput compound screening, the novel molecular beacon assay appears well suited. This method enables to determine the activity of potential PAIs versus either Mn^{2+} or Mg^{2+} , the latter probably being the biologically relevant cofactor. Any hit-to-lead process on novel PAIs requires rapid progression of potential hit compounds to relevant cell culture assays, such as the vRNP reconstitution, virus yield, and time-of-addition methods that we used to identify some relevant scaffolds for further design of PAIs. Successful development of this new

class of anti-influenza virus therapeutics will require an integrated biological approach such as the one followed here.

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AUTHORSHIP CONTRIBUTIONS

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Illustration of the activity of DKAs in the plasmid-based PA-Nter assay

Recombinant PA-Nter (1 μ g) was incubated with 1 μ g (16.7 nM) of single-stranded circular DNA plasmid M13mp18 in the presence of the test compounds. The endonucleolytic digestion of the plasmid was visualized by gel electrophoresis, and the amount of remaining intact plasmid was quantified. For L-742,001 and **10** (L-731,988), the IC_{50} values were 0.5 μ M and 0.8 μ M, respectively. Wild-type (WT) enzyme showed complete cleavage with Mn^{2+} , but was inactive when Mn^{2+} was omitted from the reaction mixture or replaced by Mg^{2+} . The endonuclease activity was also completely abolished by the active site substitution K134A.

Figure 2. 3D arrangement of five pharmacophore features in the β -diketo acid motif.

A) Twelve active compounds **1-6**, **10**, **16**, **34**, **39-41** (i.e., β -diketo acids or esters with IC_{50} values ≤ 2 μ M) were aligned and mapped to generate a five-points pharmacophore. **B)** The pharmacophore features are depicted as metal ligators (ML1, ML2, and ML3) in cyan, aromatic (Aro) and hydrophobic or aromatic (Hyd-Aro) features in pink. **C)** Interfeature distances are given in angstroms ($\text{\AA}$). **D)** These distances are in agreement with both proposed models for interaction between the β -diketo acid motif and the metal ions. *Left:* in one model [which is based on the cocrystal structure of PA-Nter in complex with L-742,001 (DuBois et al., 2012)], the carboxylate and α -hydroxyl functionalities of the DKA motif coordinate one ion, and the third coplanar oxygen at the γ -position chelates the second metal ion together with the α -hydroxyl group. *Middle:* in an alternative model

[previously proposed by us on the basis of L-742,001 docking within the holo form of PA-Nter, and assuming that L-742,001 mainly binds in its monodeprotonated form (Stevaert et al., 2013)], the γ -oxygen of the DKA motif is not involved. Instead, one metal is coordinated by the lone oxygen pair of the α -hydroxyl group and one oxygen atom of the carboxylate group, while the second metal ion interacts with both oxygens of the carboxylate moiety. *Right*: overlay of both models showing the intermetal distances.

Figure 3. Utility of the MB assay for determination of PA-Nter enzyme properties and activity of PA inhibitors

A) Schematic representation of the MB assay. MB cleavage by recombinant PA-Nter (which can occur at different sites) separates the fluorophore (F) from the quencher (Q). The evolving fluorescence, indicating enzymatic activity, can be monitored in real-time.

B) Michaelis-Menten curve for MB-U₆A₂U₇-DFO. This MB was incubated with PA-Nter and the evolving fluorescence was recorded in function of time and MB substrate concentration. The initial cleavage rate (V_0) was obtained from the slope (fluorescence units per minute) of the best-fit line derived from the 5-15 minutes interval of the reaction. Values shown are the averages $\pm$ S.E.M. of three independent experiments.

C) Gel electrophoretic analysis of the MB cleavage pattern. The amount of intact MB-U₆A₂U₇-DFO substrate decreased over time when incubated with PA-Nter. The first appearing cleavage products migrated faster than the intact MB substrate, while the late cleavage products showed slower migration through the gel. When using Mn²⁺ as a cofactor, PA-Nter appears able to cleave shorter oligonucleotides than in the presence of

Mg²⁺ or without addition of a bivalent metal (i.e. in the presence of the co-purified metal). Addition of 100 mM EDTA completely abrogated the endonuclease activity.

D) Influence of the metal ion on the initial cleavage rate of MB-U₆A₂U₇-DFO and MB-het2-DFO. The initial cleavage rate (V_0 ; based on the real-time fluorographs) for MB-U₆A₂U₇-DFO (*left graph*) was 1.4-fold and significantly higher with Mg²⁺ than with Mn²⁺, and still increased when no metal ion was added. An opposite effect was seen with MB-het2-DFO (*right graph*): in this case, the reaction rate was 2.2-fold higher in the presence of Mn²⁺ compared to Mg²⁺. The statistical significance (****: $P < 0.0001$; ***: $P < 0.001$; ns: not significant) was calculated by one-way ANOVA followed by a Tukey's multiple comparison test using Graphpad Prism.

E) Dose-dependent inhibition of MB cleavage by L-742,001. MB-U₆A₂U₇-DFO (at 20 nM) was incubated with 1 µg PA-Nter and increasing concentrations of L-742,001. The initial cleavage rate (V_0) was obtained from the slope (fluorescence units per minute) of the best-fit line derived from the 5-15 minutes interval of the reaction. These V_0 -values were used to calculate the percentage of inhibition. The inset shows the resulting dose-response curve.

Figure 4. Compounds 10 (L-731,988), 40 and 41 inhibit viral RNA synthesis besides affecting the virus entry process.

Light grey bars: the test compounds L-742,001 (20 µM); **10** (200 µM); **40** (150 µM); **41** (150 µM); or chloroquine (80 µM) were added to MDCK cells, and after 30 min incubation

at 35 °C, influenza virus A/PR/8/34 was added. Dark grey bars: virus was added first and allowed to enter during 1 h incubation, after which the compounds were added at the same concentrations as above. In both conditions, total cellular RNA was extracted at 10 h p.i. (VC: untreated virus control). The number of vRNA copies was quantified by two-step real-time RT-PCR. On the Y-axis, the fold increase in vRNA copies is shown, relative to the viral copy number added at time zero. L-742,001 remains fully effective when added after virus entry. In contrast, the reported entry inhibitor chloroquine (Vanderlinden et al., 2012) is inactive when added at 1 h p.i. Compounds **10**, **40**, and **41** have a dual effect by acting both upon virus entry and viral RNA synthesis. Data shown are the mean $\pm$ S.E.M. of two independent tests.

Table 1. Inhibition of PA-Nter by different DKA or DKA-bioisosteric compounds as determined in the plasmid-based enzymatic assay^a.

Scaffold	Compound	R ₁	R ₂	IC ₅₀ ^b (μM)	Scaffold	Compound	R ₁	R ₂	IC ₅₀ (μM)
PIPERIDINE SCAFFOLD					2,4-DIOXO-4-PHENYLBUTANOIC ACID (DPBA) SCAFFOLD (<i>cont</i>)				
	1 (L-742,001)	H	Ph	0.5		20 (L-708,906)	-	-	137
	2	Me	Ph	0.5					
	3	H	<i>p</i> -F-Ph	1.4	2,4,6-TRIOXO-6-PHENYLHEXANOIC ACID SCAFFOLD				
	4	Me	<i>p</i> -F-Ph	0.4		21	H	H	> 500
	5	H	Cy	0.5		22	Me	H	> 500
	6	Me	Cy	0.6		23	H	<i>o</i> -OMe	> 500
DIHYDROXYBUTENEDIOIC ACID						24	H	<i>o</i> -Cl	> 500
	7			> 500	QUINOLONE SCAFFOLD				
						25	H	H	> 500
1,3-DIOXO-1-PHENYLPROPANE SCAFFOLD						26	H	CH ₂ CH ₂ O	> 500
	8		H	77		27	H	Bn	> 500
	9	Ph	<i>o</i> -OH	> 500		28	Et	Bn	> 500
PYRROLE SCAFFOLD					HYDROXYPYRIMIDINE-CARBOXAMIDE SCAFFOLD				
	10 (L-731,988)	H		0.8		29			> 500
	11	Me		5.5					
2,4-DIOXO-4-PHENYLBUTANOIC ACID (DPBA) SCAFFOLD									
	12	H	H	2.7					
	13	Me	H	14					
	14	H	<i>o</i> -OMe	2.2					
	15	Me	<i>o</i> -OMe	11					
	16	H	<i>p</i> -OMe	2.0					
	17	H	<i>p</i> -Cl	9.8					
	18	H	<i>o</i> -Cl	2.8					
	19	Me	<i>o</i> -Cl	15					

^aOne microgram of PA-Nter was incubated with 1 µg of M13mp18 plasmid substrate and the compounds. After 2 h incubation, cleavage was assessed by gel electrophoresis.

^bIC₅₀: 50% inhibitory concentration. For each compound the IC₅₀ value was calculated using non-linear regression analysis. Values are the mean of at least three independent experiments.

Table 2. Inhibition of PA-Nter by different DKA or DKA-bioisosteric indole derivatives as determined in the plasmid-based enzymatic assay^a.

Indole scaffold	Compound	R ₁	R ₂	IC ₅₀ ^b (μM)
30	H	Me	3.4
	31	H	Bn	22
	32	Me	Me	10
	33	Me	Et	4.1
34	H	Me	0.6
	35	H	Et	5.5
	36	H	Bn	8.9
	37	Me	Bn	204
38	Me	Me	21
	39	H	Me	0.6
	40	H	Et	0.8
	41	H	Bn	1.6
42	H	Me	3.5
	43	Me	Me	22
44		Et	> 500
45	H	H	> 500
	46	Me	H	> 500
	47	Me	Ph	> 500

^{a,b}See legend to Table 1.

Table 3. Endonucleolytic cleavage by PA-Nter of different MB substrates.

MB	Sequence	K_m^a (μ M) $\pm$	Signal-to-noise
		S.E.M.	ratio ^b $\pm$ S.E.M.
MB-C ₁₅ -DFO	5'-[DFO] ^c -GCAGG CCCCCCCCCCCCCC CCTGC-[BHQ2]-3'	0.09 $\pm$ 0.004	8.2 $\pm$ 1.3
MB-A ₁₅ -DFO	5'-[DFO]-GCAGG AAAAAAAAAAAAAA CCTGC-[BHQ2]-3'	0.36 $\pm$ 0.01	9.4 $\pm$ 2.3
MB-U ₆ A ₂ U ₇ -DFO	5'-[DFO]-GCAGG dUdUdUdUdUAA dUdUdUdUdUdUdU CCTGC-[BHQ2]-3'	0.23 $\pm$ 0.02	11.7 $\pm$ 0.6
MB-het1-FAM	5'-[FAM] ^d -GCGAGC TAGGAAACACCAAGATGATATTT GCTCGC-[DABCYL]-3'	0.64 $\pm$ 0.03	4.4 $\pm$ 0.3
MB-het2-FAM	5'-[FAM]-CGCACG TTATGCTAAGCAAGTAAC CGTGCG-[DABCYL]-3'	0.15 $\pm$ 0.01	5.5 $\pm$ 0.4
MB-het3-FAM	5'-[FAM]-CGAGCG GCTGGTAAGGGTTTCCATATAA CGCTCG-[DABCYL]-3'	0.46 $\pm$ 0.06	7.0 $\pm$ 0.6
MB-het2-DFO	5'-[DFO]-CGCACG TTATGCTAAGCAAGTAAC CGTGCG-[BHQ2]-3'	0.46 $\pm$ 0.01	5.7 $\pm$ 0.8
MB-OH-DFO	5'-[DFO]-dUdUdUdUdU GGGGGGGGGGGGGGCCCCCCCCCCCC-[BHQ2]-3'	0.22 $\pm$ 0.01	1.8 $\pm$ 0.1

^aFor each MB, the Michaelis–Menten equation was fitted to the initial reaction rate to estimate the K_m values by nonlinear regression analysis. Values shown are the averages $\pm$ S.E.M. of at least two independent experiments.

^bFold increase at 60 min using 100 nM MB.

^cDFO: Dragonfly Orange.

^dFAM: Fluorescein amidite dye.

Table 4. Inhibitory activity of a selection of DKA derivatives in the MB versus plasmid assay with PA-Nter, or an assay using untruncated PA.

Compound	IC ₅₀ (μM) ^a						Untruncated PA ^e	
	PA-Nter							
	Molecular beacon assay						Plasmid assay ^d	
	MB-U ₆ A ₂ U ₇ -DFO ^b			MB-het2-DFO ^c				
	Mn ²⁺	Mg ²⁺	No metal	Mn ²⁺	Mg ²⁺	No metal		Mn ²⁺
1	1.5	3.1	2.5	3.9	7.1	3.5	0.5	0.5
5	3.9	6.3	9.1				0.5	
10	4.2	177	100	2.4	> 200	> 200	0.8	
12	154	> 200	> 200				2.7	
30	26	41	41				3.4	1.1
31	3.2	44	54				22	
34	3.6	59	180				0.6	
39	21	> 200	> 200				0.6	1.1
40	5.2	> 200	174				0.8	
41	1.4	86	98				1.6	
42	23	65	72				3.5	

^aIC₅₀: 50% inhibitory concentration. For each compound the IC₅₀ value was calculated using non-linear regression analysis. Values are the mean of at least two independent experiments.

^bMB-U₆A₂U₇-DFO: 5'-[DFO]-GCAGG (dU)₆A₂(dU)₆ CCTGC-[BHQ2]-3'.

^cMB-het2-DFO: 5'-[DFO]-CGCACG TTATGCTAAGCAAGTAACC GTGCG-[BHQ2]-3'.

^dSame data as in Tables 1 and 2.

^eSee reference (Noble et al., 2012) for a description of the fluorescence-based enzymatic assay with untruncated PA expressed in insect cells.

Table 5. Activity of compounds **1-29** in two influenza virus cell-based assays.

Compound	Influenza virus ^a vRNP reconstitution assay in HEK293T cells ^b					Virus yield assay in MDCK cells ^b		
	Antiviral activity EC ₅₀ ^c (μM)				Cytotoxicity CC ₅₀ ^d (μM)	Antiviral activity ^e		Cytotoxicity CC ₅₀ (μM)
	Human A/PR/8/34	Human B/Yamanashi	Avian 50-92/ PB2-627E	Avian 50-92/ PB2- 627K		EC ₉₉ (μM)	EC ₉₀ (μM)	
						Human A/PR/8/34		
1	3.6	3.1	4.6	11	≥ 104	8.4	5.4	181
2	2.8	6.9	8.5	20	≥ 102	6.0	3.9	> 100
3	1.8	0.8	6.6	17	≥ 103	2.9	2.0	> 100
4	2.3	0.4	7.6	15	≥ 93	5.6	3.8	> 100
5	1.7	4.4	3.9	8.8	≥ 106	1.5	1.0	> 100
6	2.3	11	7.2	15	≥ 94	2.7	1.9	> 100
7	> 100	ND ^f	ND	ND	> 200	ND	ND	ND
8	> 100	ND	ND	ND	> 200	ND	ND	ND
9	57	25	105	29	117	> 100	> 100	> 200
10	84	116	88	95	> 200	74	58	> 200
11	132	49	47	65	> 200	> 100	≥ 90	≥ 184
12	> 100	ND	ND	ND	> 200	ND	ND	ND
13	> 100	ND	ND	ND	> 200	ND	ND	ND
14	> 100	ND	ND	ND	> 200	ND	ND	ND
15	> 100	ND	ND	ND	> 200	ND	ND	ND
16	> 100	ND	ND	ND	> 200	ND	ND	ND
17	> 100	ND	ND	ND	> 200	ND	ND	ND
18	> 100	ND	ND	ND	> 200	ND	ND	ND
19	> 100	ND	ND	ND	> 200	ND	ND	ND
20	72	97	32	37	> 200	79	55	≥ 194
21	> 100	ND	ND	ND	> 200	ND	ND	ND
22	> 100	ND	ND	ND	> 200	ND	ND	ND
23	> 100	ND	ND	ND	> 200	ND	ND	ND
24	> 100	ND	ND	ND	> 200	ND	ND	ND
25	> 100	ND	ND	ND	> 200	ND	ND	ND
26	> 100	ND	ND	ND	> 200	ND	ND	ND
27	> 100	ND	ND	ND	> 200	ND	ND	ND
28	> 100	ND	ND	ND	> 200	ND	ND	ND
29	> 100	ND	ND	ND	> 200	ND	ND	ND
Ribavirin	16	3.1	50	45	> 200	10	6.7	> 200

^aReconstituted vRNP complexes from: human influenza A virus, strain A/PR/8/34; human influenza B virus, strain B/Yamanashi/166/98; and avian influenza A virus, strain A/turkey/England/50-92/1991 with a PB2-627E or PB2-627K residue.

^bHEK293T cells: human embryonic kidney 293T cells; MDCK cells: Madin-Darby canine kidney cells.

^cEC₅₀: 50% effective concentration (μM), i.e. compound concentration producing 50% reduction in vRNP-driven firefly reporter signal, estimated at 24 h after transfection.

^dCC₅₀: 50% cytotoxic concentration (μM) at 24 h determined by MTS cell viability assay.

^eCompound concentration (μM) causing a 1-log₁₀ (EC₉₀) or 2-log₁₀ (EC₉₉) reduction in virus yield at 24 h p.i., as determined by real-time RT-PCR.

^fND: Not done.

Data shown are the mean ± S.E.M. of at least 3 independent tests.

Table 6. Activity of compounds **30-47** in two influenza virus cell-based assays.

Compound	Influenza virus ^a vRNP reconstitution assay in HEK293T cells					Virus yield assay in MDCK cells ^b		
	Antiviral activity EC ₅₀ ^c (μM)				Cytotoxicity CC ₅₀ ^d (μM)	Antiviral activity ^e		Cytotoxicity CC ₅₀ (μM)
	Human A/PR/8/34	Human B/Yamanashi	Avian 50-92/ PB2-627E	Avian 50-92/ PB2- 627K		EC ₉₉ (μM)	EC ₉₀ (μM)	
						Human A/PR/8/34		
30	> 100	ND ^f	ND	ND	> 200	ND	ND	ND
31	92	59	> 200	87	> 200	85	55	126
32	> 100	ND	ND	ND	> 200	ND	ND	ND
33	77	18	39	62	> 200	> 25	> 25	≥ 169
34	> 100	ND	ND	ND	> 100	ND	ND	ND
35	> 100	ND	ND	ND	> 200	ND	ND	ND
36	16	11	17	12	> 200	> 12.5	> 12.5	> 200
37	48	29	54	30	> 200	> 25	> 25	≥ 184
38	> 100	ND	ND	ND	> 200	ND	ND	ND
39	> 100	ND	ND	ND	> 200	ND	ND	ND
40	166	65	121	121	> 200	95	69	≥ 194
41	45	43	96	62	136	64	38	> 200
42	> 100	ND	ND	ND	> 200	ND	ND	ND
43	> 100	ND	ND	ND	> 200	ND	ND	ND
44	> 100	ND	ND	ND	> 200	ND	ND	ND
45	> 100	ND	ND	ND	> 200	ND	ND	ND
46	> 100	ND	ND	ND	> 200	ND	ND	ND
47	> 100	ND	ND	ND	> 200	ND	ND	ND

^{a,b,c,d,e,f}See legend to Table 5.

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4