**Title:**

Enhanced efficacy of endonuclease inhibitor baloxavir against orthobunyaviruses when used in combination with ribavirin

**Running Title:**

Baloxavir against the orthobunyavirus endonuclease

**Authors:**

Sebastiaan TER HORST[1†], Yaiza FERNANDEZ-GARCIA[2†‡], Marcella BASSETTO[3], Stephan GÜNTHER[2], Andrea BRANCALE[4], Johan NEYTS[1], Joana ROCHA-PEREIRA[1‡*]

[†] These authors contributed equally to this article

[‡] These authors contributed equally to this article

* Corresponding author: J. Rocha-Pereira, +3216321876, KU Leuven, Rega Institute for Medical Research, Herestraat 49 - box 1043, Belgium, joana.rochapereira@kuleuven.be

**Affiliations:**

[1]KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology and Chemotherapy, Leuven, Belgium

[2]Department of Virology, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany

[3]Department of Chemistry, College of Science, Swansea University, United Kingdom

[4]School of Pharmacy and Pharmaceutical Sciences, Cardiff University, United Kingdom

**Abstract**

**Objectives** Baloxavir acid (BXA) is an endonuclease inhibitor approved for use against influenza. We evaluated whether this compound also targets the endonuclease domain of orthobunyaviruses and therefore could potentially be used against orthobunyavirus infections.

**Methods** We performed a thermal shift assay and a FRET-based nuclease-monitoring assay using the LACV endonuclease and BXA to prove their interaction and identify an inhibitory effect. Their interaction was further studied in a docking simulation using Glide SP. We show that BXA inhibits the viral replication of BUNV-mCherry *in vitro* using high content imaging and virus yield assay. Lastly, we investigated the use of BXA in combination with ribavirin *in vitro* by implementing the Zero Interaction Potency (ZIP) response surface model.

**Results** We show that BXA augments LACV enzyme's melting temperature with Δ Tm 9.5 ± 0.4 °C and inhibited substrate cleavage with $IC_{50}$ 0.39 ± 0.03 μM. Moreover, our docking simulation suggests BXA is able to establish an efficient binding with the LACV endonuclease. In the cell based assay we observed BXA and ribavirin inhibited BUNV-mCherry with an $EC_{50}$ of 0.7 ± 0.2 μM and 26.6 ± 8.9 μM, respectively. When used in combination we found a maximum synergistic effect of 8.64.

**Conclusions** The influenza endonuclease inhibitor BXA is able to bind and interfere with the endonuclease domain of orthobunyaviruses and yields a more potent antiviral effect than ribavirin against BUNV-mCherry. The combination of both compounds results in a more potent antiviral effect, suggesting these molecules could potentially be combined to treat orthobunyavirus-infected patients.

## Background

Orthobunyaviruses (Order: *Bunyavirales*, Family: *Peribunyaviridae*) are a large genus of segmented negative single-stranded RNA viruses ((-)ssRNA), of which several members can cause mild to severe disease in humans.[1] *Bunyamwera virus* (BUNV) is the type species and model system for the genus. Additionally, it is considered an emerging pathogen and is known to form reassortments that can be highly pathogenic (i.e. Ngari virus).[2] *La Crosse virus* (LACV) is the leading cause of pediatric arboviral encephalitis in the USA.[3] No specific treatment is available for orthobunyaviruses, but the guanosine analog ribavirin has been used in a clinical trial against LACV.[4] However, this was discontinued due to adverse events caused by the occupied doses.

As all segmented (-)ssRNA viruses, orthobunyaviruses rely on their own transcriptional machinery in the form of an RNA-dependent RNA polymerase (RdRp). The endonuclease domain of this protein cleaves 5'-RNA caps from host cells mRNA, a process dubbed 'cap-snatching'.[5] Cap-snatching is a conserved feature among all (-)ssRNA viruses and therefore a promising antiviral target. The influenza endonuclease domain is a known antiviral target for which multiple antiviral molecules have been identified. Baloxavir marboxil (BXM), the prodrug of baloxavir acid (BXA), is a first-in-class endonuclease-targeting influenza antiviral approved for clinical use by the FDA in 2019.[6,7]

In this work we show the effects BXA has on the stability and enzymatic activity of the endonuclease domain of LACV and investigate the interaction between BXA and the endonuclease through a docking simulation. Furthermore, we show that BXA inhibits the viral replication of BUNV *in vitro* and that its use in combination with ribavirin resulted in a synergistic antiviral effect.

## Methods

### Thermal shift assay

The stability of LACV_$L_{1-184}$_WT, produced as previously reported, was measured using a thermal shift assay (TSA) using a protein concentration of 10 µM in 100 mM Tris pH 7.0, 250 mM NaCl, 5% glycerol complemented or not with 25 µM of BXA in the presence of 10 mM EDTA or 10 mM $MnCl_2$ as described.[8,9]

### FRET-based nuclease-monitoring assay

Serial dilutions of BXA were pre-incubated for 15 min with 0.25 µM of LACV_$L_{1-184}$_WT in a mixture consisting of 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol, 0.05 U/µl RNasin (Promega) and 10 mM $MnCl_2$ or EDTA. The reactions were initiated by the addition of 1 µM of a 12mer poly(A) ssRNA substrate labeled at the 5´ end with fluorophore 6-FAM and the 3´ end with quencher BHQ-1 (Biomers). The fluorescence signal was measured every 10 s for 20 min in a 96-well plate format at wavelengths 490 nm and 515 nm for excitation and emission, respectively. The initial velocity of the reactions ($V_0$) was determined as the slope of the linear phase of the progress curves, and the percentages of the enzymatic activity were plotted against the concentrations of the compounds on a semilogarithmic graph. The half-maximum inhibitory concentration ($IC_{50}$) was calculated by nonlinear regression analysis performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA, www.graphpad.com).

### Molecular Modelling

All molecular docking studies were performed on a Viglen Genie Intel®CoreTM i7-3770 vPro CPU@ 3.40 GHz x 8 running Ubuntu 18.04. Molecular Operating Environment (MOE) 2019.109 and Maestro (Schrödinger Release 2019-3) was used as molecular modelling software.[10,11]

The available X-ray structure of the LACV N-terminal endonuclease domain (PDB: 2XI7) was pre-processed using the Schrödinger Protein Preparation Wizard by assigning bond orders, adding hydrogens and performing a restrained energy minimization of the added hydrogens using the OPLS_2005 force field.[5] BXA was built with MOE and then prepared using the Maestro LigPrep tool by energy minimizing the structures (OPLS_2005 force filed), generating possible ionization states at pH 7 ± 2, generating tautomers and low-energy ring conformers. A 12 Å docking grid (inner-box 10 Å and outer-box 20 Å) was prepared using as centroid the Mn atoms. Molecular docking studies were performed using Glide SP precision keeping the default parameters and setting 5 as number of output poses per input ligand to include in the solution. The output poses were saved as sdf file. The docking results were visually inspected for their ability to bind the active site, using MOE.

Cell based antiviral activity assay

The antiviral activity of BXA and ribavirin against BUNV-mCherry was evaluated in A549 cells (ATCC® CCL-185).[12] 5000 cells/well were seeded in 96-well plates (Greiner Bio-One REF#655090) with DMEM (Gibco REF#41965-039) supplemented with 2% FBS (Gibco REF#10270-106) and 0.075 g/L sodium bicarbonate (Gibco REF#25080) and incubated overnight at 37ºC and 5% $CO_2$. Then a serial dilution of the respective compound was added immediately after infected with 500 $CCID_{50}$ of virus. Both the compound and the virus inoculum were kept on the cells during the incubation period of four days. At 4 days post infection (dpi) the cells were

stained with a 2.5 µg/mL Hoechst (Hoechst 33342, Invitrogen) solution. High content imaging (HCI) analysis was performed on the Arrayscan XTI (Thermofisher) using a custom analysis protocol based on the SpotDetector BioApplication (Cellomics® software). The 50% effective concentration ($EC_{50}$) is the compound concentration that reduces the number of mCherry signal spots by half, compared to the virus control. Cell viability was scored based the number of nuclei-containing objects.

Virus yield assay

Viral RNA was extracted from cell culture supernatant using the NucleoSpin® kit (Macherey-Nagel REF#740955) according to the manufacturer's instructions. Per reaction, a 20 µL RT-qPCR mix was prepared using the ITaq Universal SYBR Green One-Step kit (Bio-Rad CAT#1725151) with 600 nM BUNV-Fw and 900 nM BUNV-Rev primers (Fw: 5'-ACACCACTGGGCTTAG-3', Rev: 5'-CAGCCCCCAAGGTTA-3') on a LightCycler 96 thermocycler (Roche Diagnostics). For absolute quantification, standard curves were generated using 10-fold dilutions of gBlock DNA (IDT) of known concentration.

Synergy scoring

Synergy scoring was determined using "inhibition readout" on the online software of https://synergyfinder.fimm.fi and implementing the Zero Interaction Potency (ZIP) response surface model.[13,14]

**Results**

We show that BXA contributes to the enzyme's melting temperature (Tm) in the presence of $Mn^{2+}$, which is in agreement with the chelating nature of the compound. The addition of BXA raised the

$Mn^{2+}$ baseline Tm by 9.5 ± 0.4 °C (Δ Tm) (Figure 1A). We additionally assessed the impact of BXA on LACV_$L_{1\text{-}184}$_WT catalytic activity via a FRET-based nuclease-monitoring assay, where BXA inhibited the substrate cleavage in a dose-dependent manner with an $IC_{50}$ value of 0.39 ± 0.03 μM (Figure 1B).

To further investigate the binding of BXA within the LACV endonuclease, we performed a molecular docking simulation. The results show a clear coordination of the chelating motif that includes the β-hydroxy ketone and the manganese atoms, with the fluorinated aromatic ring establishing a contact with Lys108 and Thr105 (Figure 1C). These results suggest that, despite the relatively shallow and extensive nature of the endonuclease catalytic site, BXA is able to establish an efficient binding with the enzyme.

To assess the antiviral activity of BXA and ribavirin against orthobunyaviruses we added a serial dilution of the compounds together with BUNV-mCherry onto A549 cells. At 4 dpi BXA and ribavirin inhibited BUNV-mCherry replication in infected cells with an $EC_{50}$ of 0.7 ± 0.2 μM and 26.6 ± 8.9 μM, respectively (Figure 2A, 2B) as determined by HCI analyses. At concentrations of BXA up to 3.13 μM the cell viability remains above 80%. This is in line with cytotoxicity measured in other studies using MDCK cells.[7] We confirmed the effect on the inhibition of virus progeny production by virus yield assay. The resulting $EC_{50}$ values are similar to our fluorescence-based readout with $EC_{50}$ of 0.9 ± 0.3 μM and 11.8 ± 1.8 μM for BXA and ribavirin, respectively (Figure 2A, 2B).

To determine whether the antiviral effect of ribavirin and BXA could be improved by combination, cells were infected with BUNV-mCherry, as in the antiviral activity assay, and treated with multiple concentrations of either one or both inhibitors. We identified a small concentration range

where combination treatment results in a synergistic effect of 8.64 as calculated by the ZIP model (Figure 2C). The area with the highest synergistic score has an average of 3.53.

## Discussion

Taken together, this work shows that cap-snatching endonuclease inhibitors like BXA should be considered as an option to treat a wide range of orthobunyavirus infections. Learning from studies on BXA-treated influenza patients, the highest chance of success will come from starting treatment as early as possible.[6] Although the use of ribavirin is not recommended due to toxicity, the combination with baloxavir would result in a more potent antiviral effect, thus lower (non-toxic) doses of ribavirin might suffice. We here demonstrate that targeting the endonuclease of orthobunyavirus by the influenza compound BXA yields a more potent antiviral effect than ribavirin. Thus, the design of highly potent and selective endonuclease inhibitors hold great promise as drug candidates.

While our results open the door to a possible therapeutic opportunity, further research is needed in small animal models. The effect of the prodrug BXM could then be assessed since this will have favorable pharmacodynamics. BXM is in the greatest part metabolized via UGT1A3, with a minor contribution from CYP3A4. Neither is involved in the metabolism of ribavirin, limiting the risk of an undesirable increase in drug exposure.[15] Consequently, we urge further investigation of the potential benefit of endonuclease targeting compounds in clinical settings, particularly in combination therapies.

## Acknowledgments

We would like to express our gratitude towards Dr. Xiaohong Shi (MRC-University of Glasgow Centre for Virus Research, Glasgow, UK) for kindly providing us with the BUNV-mCherry strain.

We would like to thank Winston Chiu, Stephanie Wurr, Lindsey Bervoets and Jasper Rymenants for their technical assistance.

**Financial support**

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) grant number 01DN17037. StH is supported by a KU Leuven internal project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Transparency declarations**

All authors: None to declare.

**References**


1. Elliot RM. Orthobunyaviruses: recent genetic and structural insights. *Nat Rev Microbiol* 2014; **12**: 673–85.
2. Gerrard SR, Li L, Barrett AD, *et al*. Ngari virus is a Bunyamwera virus reassortant that can be associated with large outbreaks of hemorrhagic fever in Africa. *J Virol*. 2004; **78**: 8922–26.
3. https://www.cdc.gov/lac/tech/epi.html [2020 Mar 02]
4. McJunkin JE, Nahata MC, De Los Reyes EC, *et al*. Safety and pharmacokinetics of ribavirin for the treatment of la crosse encephalitis. *Pediatr Infect Dis J*. 2011; **30**: 860–5.
5. Reguera J, Weber F, Cusack S. *Bunyaviridae* RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription. *PLoS Pathogens*. 2010; **6**: e1001101.
6. Hayden FG, Sugaya N, Hirotsu N, *et al*. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. *N Engl J Med*. 2018; **379**: 913–23.
7. Noshi T, Kitano M, Taniguchi K, *et al*. *In vitro* characterization of baloxavir acid, a first-in-class cap-dependent endonuclease inhibitor of the influenza virus polymerase PA subunit. *Antiviral Res*. 2018; **160**: 109–17.
8. Holm T, Kopicki JD, Busch C, *et al*. Biochemical and structural studies reveal differences and commonalities among cap-snatching endonucleases from segmented negative-strand RNA viruses. *J Biol Chem*. 2018; **293**: 19686-98.
9. Ericsson UB, Hallberg BM, DeTitta GT, *et al*. Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Anal Biochem*. 2006; **357**: 289-98.

10. ULC, C. C. G. Molecular Operating Environment (MOE), 2019.10, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2019.
11. Schrödinger Release 2019-3: Maestro, Schrödinger, LLC, New York, NY, 2019
12. Shi X, van Mierlo JT, French A, *et al*. Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein. *J Virol.* 2010; **84**: 8460–9.
13. Ianevski A, He L, Aittokallio T, *et al*. SynergyFinder: A web application for analyzing drug combination dose-response matrix data. *Bioinformatics*. 2017; **33**: 2413–5.
14. Yadav B, Wennerberg K, Aittokallio T, *et al*. Searching for Drug Synergy in Complex Dose-Response Landscapes Using an Interaction Potency Model. *Comput Struct Biotechnol J*. 2015; **13**: 504–13.
15. Ng KE. Xofluza (Baloxavir Marboxil) for the Treatment Of Acute Uncomplicated Influenza. *P T*. 2019; **44**: 9–11.

**Figure 1A**

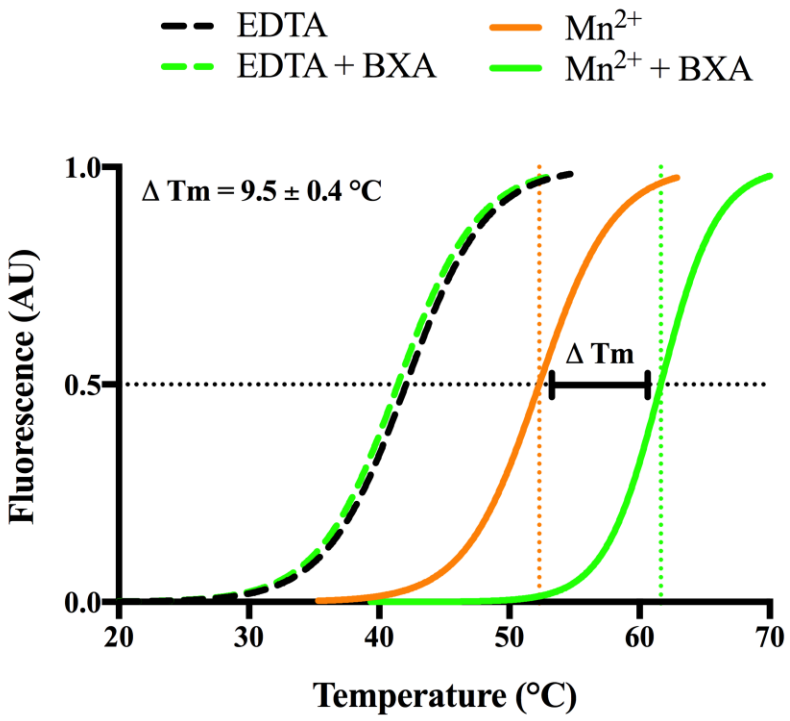



**Figure 1B**

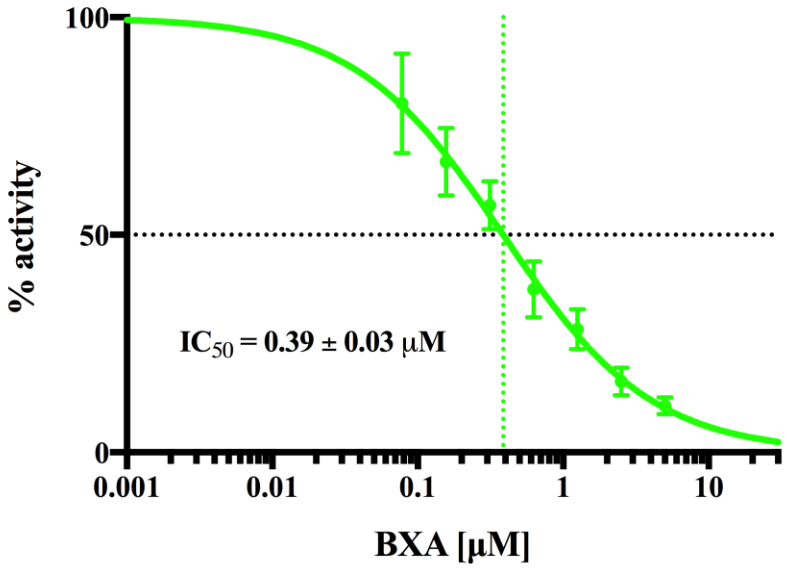


**Figure 1C**

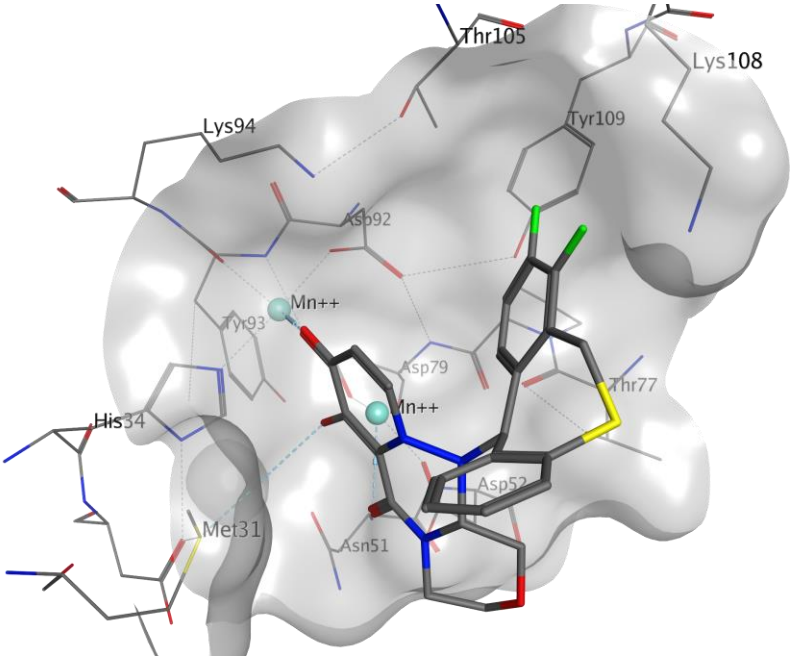



**Figure 2A**

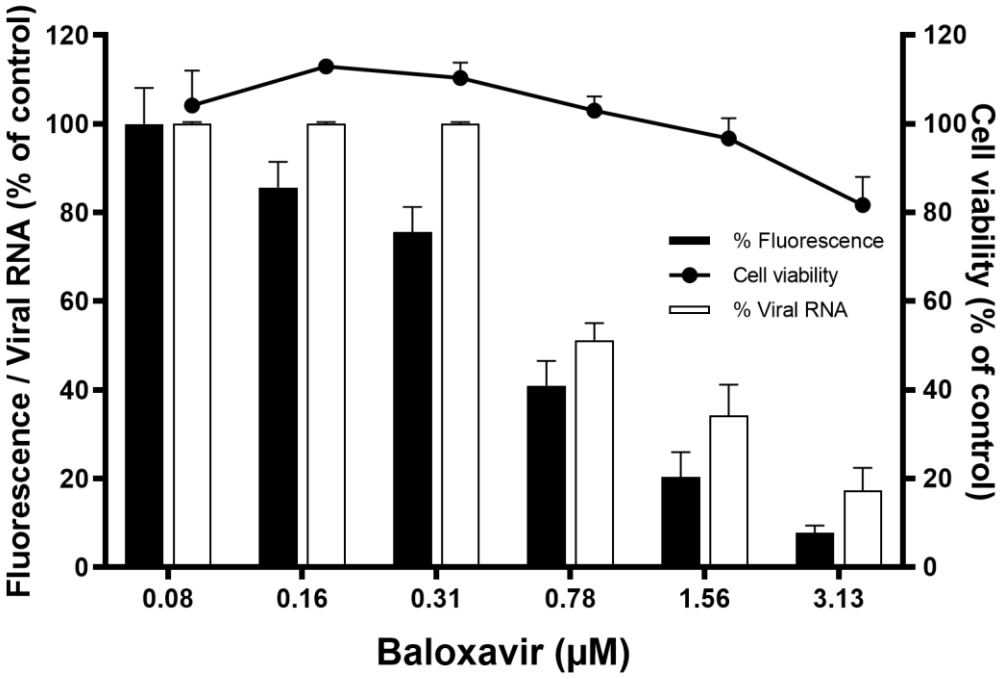


**Figure 2B**

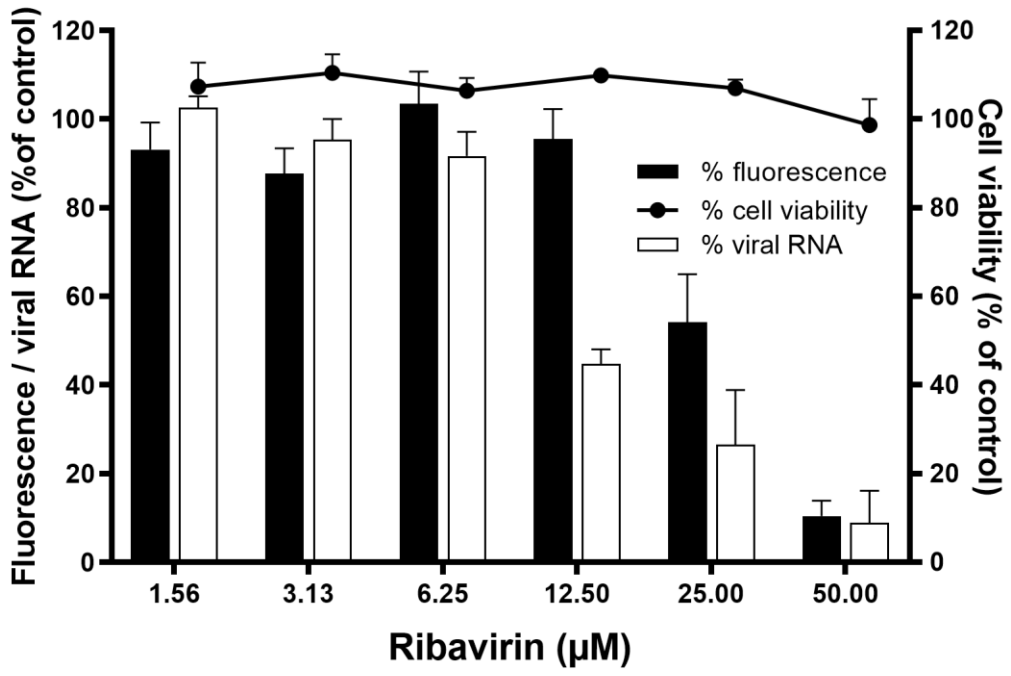
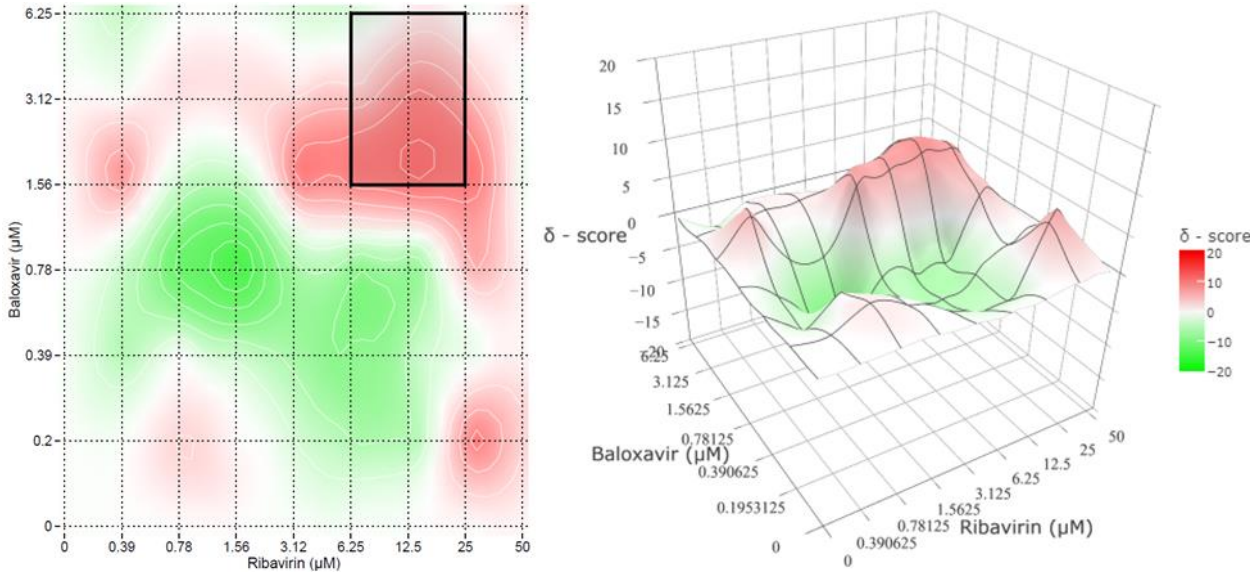



**Figure 2C**



**Legends**

**Figure 1.** Effect of BXA on the Cap-ENDO of LACV. **(A)** Thermal stability of LACV_$L_{1-184}$_WT in the presence of 10 mM EDTA (black dashed line), 10 mM EDTA plus 25 μM BXA (green dashed line), 10 mM $MnCl_2$ (orange solid line) and 10 mM $MnCl_2$ plus 25 μM BXA (green solid line). The compound contribution to the stability of the proteins was determined by the difference in Tm between the samples containing $MnCl_2$ in the presence and absence of BXA (Δ Tm). The lines depict the nonlinear fits of the normalized fluorescence curves to the Boltzmann equation. **(B)** Dose-dependent inhibition of the nuclease activity of LACV_$L_{1-184}$_WT by BXA. The reactions were initiated with the addition of 1 μM of FRET-labeled ssRNA substrate to the pre-incubated mixtures of 0.25 μM of the enzyme with two-fold serial dilutions of BXA. The fluorescent signal arising from the substrate cleavage was measured using a Roche LightCycler 480 II at λex / λem = 490 nm / 515 nm. The $V_0$ of the reactions was used to calculate the percentage of activity at the different concentrations of the inhibitor. The dose-response curves were fit using a four-parameter nonlinear regression. The Δ Tm and the $IC_{50}$ values are the mean and standard deviation from 3 independent experiments. Final 5% DMSO concentration was maintained in all assays to favor compound solubility. **(C)** Proposed binding of BXA within the catalytic site of the LACV endonuclease domain.

**Figure 2.** BXA *in vitro* antiviral activity, alone or in combination with ribavirin, against orthobunyaviruses. **(A)** Antiviral effect of BXA on BUNV-mCherry replication at 4 days post infection in A549 cells. Viral mCherry signal spots (black bars) and cell viability (solid circles) were determined using high-content imaging. Viral RNA yield (white bars) as determined by RT-qPCR, n=5. **(B)** Antiviral effect of ribavirin on BUNV-mCherry replication at 4 dpi in A549 cells. Viral mCherry signal spots (black bars) and cell viability (solid circles) were determined using

high-content imaging. Viral RNA yield (white bars) as determined by RT-qPCR, n=5. **(C)** The interaction surface for combinations of BXA and ribavirin. The square indicates the most synergistic region. The graphics represent the mean of five independent experiments.