

Identification of potent inhibitors of arenavirus and SARS-CoV-2 exoribonucleases by fluorescence polarization assay

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1	Identification of potent inhibitors of arenavirus and SARS-CoV-2
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20	
21	Abstract
22	Viral exoribonucleases are uncommon in the world of RNA viruses. To date, they have only been identified
23	in the Arenaviridae and the Coronaviridae families. The exoribonucleases of these viruses play a crucial role
24	in the pathogenicity and interplay with host innate immune response. Moreover, coronaviruses
25	exoribonuclease is also involved in a proofreading mechanism ensuring the genetic stability of the viral
26	genome. Because of their key roles in virus life cycle, they constitute attractive target for drug design.

27	Here we developed a sensitive, robust and reliable fluorescence polarization assay to measure the							
28	exoribonuclease activity and its inhibition in vitro. The effectiveness of the method was validated on three							
29	different viral exoribonucleases, including SARS-CoV-2, Lymphocytic Choriomeningitis and Machupo							
30	viruses. We performed a screening of a focused library consisting of 113 metal chelators. Hit compounds were							
31	recovered with an IC ₅₀ at micromolar level. We confirmed 3 hits in SARS-CoV-2 infected Vero-E6 cells.							
32								
33	Keywords							
34	Phosphohydrolase activity, Arenaviridae, Coronaviridae, exoribonuclease activity, fluorescence polarization,							
35	screening, inhibitors, IC ₅₀ , cellular assays.							
36								
37	Highlights							
38	• Arenaviridae and Coronaviridae viral families share an exoribonuclease activity of common							
39	evolutionary origin							
40	• Arenaviridae and Coronaviridae exoribonuclease is an attractive target for drug development							
41	• We developed an activity assay using fluorescence polarization in 384 well-plates							
42	• We validated the assay and screened a focused library of 113 metal chelators against SARS-CoV-2,							
43	Lymphocytic Choriomeningitis virus and Machupo virus exoribonucleases							
44	• We selected several hit inhibitors and determined by fluorescence polarization their IC_{50} at micromolar							
45	level.							
46	• We tested hit compounds in cellular assay and we confirmed 3 hits previously detected in the <i>in vitro</i>							
47	screening out of 6 new promising SARS-Cov-2 inhibitors.							
48								
49	1. Introduction							
50	Arenaviruses are emerging RNA viruses associated with fatal neurological and hemorrhagic diseases in hu-							
51	mans. Currently there is only ribavirin as FDA-approved antiviral. For coronaviruses, in the context of the							
52	global SARS-CoV-2 pandemic, it is essential to continue to develop potent antivirals, in support of the vaccine							
53	approach.							

These RNA viruses encode for proteins, forming a replication/transcription complex (RTC) that orchestrates 54 viral replication (Ferron et al., 2017; Knipe and Howley, 2013; Ogando et al., 2019). The peculiarity of 55 Arenaviridae and Coronaviridae families, is the unique viral 3'-5'-exoribonuclease (ExoN) activity described 56 57 so far (Hastie et al., 2011; Snijder et al., 2003), The ExoN activity carried by arenavirus nucleoprotein (N) or the nsp14 protein of coronavirus participates to the suppression of the host innate immune response (Martínez-58 Sobrido et al., 2009, 2007, 2006; Becares et al., 2016; Lei et al., 2020), moreover the nsp14 displays an 59 additional role in maintening the genome integrity (Bouvet et al., 2012; Ferron et al., 2018). The arenavirus 60 61 and coronavirus ExoN belonging to the same DEDD superfamily (DED/EDh subfamily), are structurally 62 homolog (Zuo and Deutscher, 2001), and characterized by the presence of four conserved acidic residues (Asp and Glu) (Bouvet et al., 2012; Yekwa et al., 2019), essential for the binding of two metal cations (Mg²⁺ and/or 63 Mn²⁺) involved in the RNA hydrolysis mechanism (Steitz and Steitz, 1993), always proceeding from the 3'- to 64 the 5'-direction. 65

Because of its biological significance to the viral life cycle, ExoN is an attractive target for drug-design
(Papageorgiou et al., 2020; Subissi et al., 2014).

In drug development efforts using ExoN of mammarenavirus and coronavirus as targets, assays making use of
radiolabeled substrate (Baddock et al., 2022; Bouvet et al., 2012; Ferron et al., 2018; Saramago et al., 2021;
Yekwa et al., 2019, 2017) or mass spectrometry (Scholle et al., 2021) are available but barely adapted to HT
screening. Recently, HTS assays using FRET were applied to screen SARS-CoV-2 ExoN (Canal et al., 2021;
Chen et al., 2021; Rona et al., 2022).

Here we present a convenient method using fluorescence polarization (FP) to assess the ExoN activity and its inhibition. The recorded FP signal is altered proportionally to the size of the fluorescent RNA probe which is hydrolyzed into smaller fragments. We validate the method on SARS-CoV-2, Lymphocytic Choriomeningitis (LCMV) and Machupo viruses (MACV) ExoN. The method is sensitive, robust, amenable to miniaturization (384 well plates) and allowed us to screen a focused library of 113 metal chelators over the three-targeted viral ExoN, validating the proof-of-concept of the assay. We selected several hit inhibitors and determined by FP their IC₅₀. The hit compounds were tested in cellular assays.

80

81 2. Materials and methods

83 2.1 Products and reagents.

Aurintricarboxyclic acid (40) was purchased from Acros Organics. Ribavirin (82) and Favipiravir (83) were 84 purchased from Carbosynth. The library (113 compounds) containing phenyl-DKA, piperidinyl-DKA, indolyl-85 benzofuranyl-DKA, triazolyl-DKA (DKA families), (POP) N-86 DKA, polyphenols family), 87 hydroxyisoquinoline-1,3-diones (HID family) and miscellaneous compounds have been described previously (Saez-Ayala et al., 2019, 2018). The hit compounds 3, 4, 18, 55 and 57 have been synthesized and their 88 synthesis will be published in due course. All compounds were resuspended in 100 % DMSO at 20 mM and 89 stored at - 20 °C. The 22-mer RNA 5'-UGACGGCCCGGAAAACCGGGCC-3' containing FAM dye at the 5'-90 91 end (5'-FAM-RNA) was purchased from Microsynth AG (Switzerland).

92

93 *2.2 Protein expression and purification.*

All proteins were expressed in E. Coli. The LCMV and MACV ExoN plasmids used for this study were described in (Yekwa et al., 2019). For the expression of the SARS-CoV-2 nsp10 and nsp14, the synthetic genes were purchased from Twist Bioscience (USA). The protein production methods are detailed in the supplementary data file (see also figure S1 for elution profiles).

98

99 2.3 Set-up of exonuclease activity conditions based on fluorescent polarization assay

100 2.3.1 Optimization of 5'-FAM-RNA concentration, ExoN/RNA ratio, temperature, metal ion nature and

101 *concentration*

102 Reactions were performed in 20 µl total volume in a buffer containing 40 mM Tris (pH 8), 5 mM DTT and 2

103 or 5 mM MnCl₂. For each 5'-FAM-RNA concentration $(0.05, 0.1, 0.25, 0.5 \text{ and } 1 \mu \text{M})$, a range of concentration

104 of ExoN was tested from a ratio of 5-fold less enzyme up to 10-fold more enzyme (0.01 to 10 µM) than 5'-

105 FAM-RNA. For SARS-CoV-2 ExoN, the molar ratio of nsp14:nsp10 complex in the reactions was always

- 106 kept at 1:4, as optimized previously (Bouvet et al., 2012) to enhance nsp14 activity.
- 107 To find the optimal temperature, metal ion nature and concentration, the 5'-FAM-RNA concentration was kept

108 at 0.1 μ M and ExoN concentration ranged from 0.1 to 1.6 μ M. The activity was tested in the presence of MnCl₂

109 or MgCl₂ at 1, 2 or 5 mM. The FP signal was recorded either at 25°C or at 37°C. Negative controls tested used

- 110 consisted in the replacement of MnCl₂ by CaCl₂ or, depletion of the metal ion or the enzyme. The reactions
- started by the addition of 5'-FAM-RNA and the fluorescence polarization (FP) was read in the Pherastar FSX

(BMG Labtech) using the 480 nm excitation and 520 nm emission filter, during 30 minutes at 25°C or 37°C, every 30 seconds. The gain was set up using the negative control which contained the 5'-FAM-RNA in the presence of heat-denatured nuclease. After 30 minutes, the reactions were stopped by the addition of an equal volume of loading buffer (8M urea containing 10 mM EDTA) and the digestion products were then loaded in 7 M urea containing 20% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio 19:1) buffered with TBE and visualized using a Fluorescent Image Analyzer Typhoon (GE Healthcare).

- 118
- 119 *2.3.3 Time course of ExoN assay*

Reactions of 20 μ l total volume were performed in the buffer mentioned above, with 2 or 5 mM MnCl₂ for SARS-CoV-2 or arenaviruses ExoN, respectively. The 5'-FAM-RNA concentration was fixed at 0.1 μ M. The SARS-CoV-2 nsp14/nsp10 ExoN complex concentration was ranged from 0.2 to 1 μ M and pre-incubation at room temperature for 5 minutes was performed to allow the complex formation. The LCMV and MACV ExoN concentration was ranged from 0.1 to 1.6 μ M. The reaction started with the addition of 5'-FAM-RNA and The FP signal was recorded as mentioned previously. At end point, an aliquot of the sample was examined in urea PAGE as mentioned above. The assays were done in triplicate.

127

128 2.4 Screenings and IC₅₀ determination.

129

130 *2.4.1 Screenings*

131 The screenings at 5 µM and 20 µM inhibitor concentration were performed in 384-wells with flat bottom Nunc 132 plates, in 20 µL total volume reaction. Reactions were performed in a buffer containing 40 mM Tris (pH 8), 5 133 mM DTT, with 2 or 5 mM MnCl₂ for SARS-CoV-2 or arenaviruses ExoN, respectively. The 5'-FAM-RNA and ExoN concentrations were fixed at 0.1 µM. and 0.4 µM, respectively. Compounds (1-113) were added to 134 the reaction with a final concentration of 5 μ M and 20 μ M in 5% DMSO. 8 reactions mix with ExoN were 135 136 used as positive controls and 8 reactions mix with heat-denatured ExoN were used as negative controls. Reactions were initiated by adding the 5'-FAM-RNA and the FP signal was recorded as mentioned previously. 137 To calculate the percentage of inhibition, a time correction was applied for the delayed initiation of the reaction 138 139 due to the use of a multichannel pipette. The percentage of inhibition at a given time was calculated as follows: 140 $inhibition\% = \frac{100 \times (FP-average of positive controls)}{(average negative controls-average positive controls)}$

141 where FP correspond to the fluorescent polarization signal of a compound.

142 The time selected for doing this calculation was the time when the signal of the positive control reached the 143 plateau (30 minutes). To assess the robustness of the assay we calculate the z' value.(Formula and 144 representative scatter plot in supplementary figure S3).

145

146 $2.4.2 IC_{50}$ determination

147 Reactions were performed in the same experimental conditions as used for the screenings. The compound 148 concentrations varied from 0.2 to 12.5 μ M for LCMV ExoN, from 0.4 to 25 μ M for MACV ExoN and from 149 0.5 to 16 μ M for SARS-CoV-2 ExoN nsp14/nsp10 complex. The reaction started with the addition of 5'-FAM-150 RNA and the FP signal was recorded as mentioned previously. The percentage of inhibition was calculated as 151 indicated in the previous section. The curves of the percentage of inhibition with respect to the inhibitor 152 concentration in a logarithmic scale were fitted in Graphpad Prism software using a four parameters equation. 153 The assays were done in triplicate.

154

155 2.6 Cellular assays

LCMV minigenome assays (MG) were adapted from a previously described procedure (Saez-Ayala et al.,
2019, 2018). SARS-CoV-2 cellular assays were performed as previously described (Touret et al., 2021, 2020).
Protocols are detailed in the supplementary data file.

159

160 3. Results and discussion

161

Fluorescence polarization (FP) is a reliable and sensitive tool for monitoring enzymatic reaction, by determining the difference of polarization signals (Zhang et al., 2012). FP - based assay is a robust method commonly used in drug discovery (Lea and Simeonov, 2011; Uri and Nonga, 2020). The FP signal recorded is proportional to the molecular weight (MW) of a fluorescent molecule (Kwok, 2002; Latif et al., 2001). We decided to apply this method to viral ExoN activity, by monitoring the size of a fluorescent-labeled RNA probe which is altered in the course of the nuclease, reflecting the enzymatic activity (Liu et al., 2014; Zhang et al.,
2012). The assay was first optimized.

169

170 3.1 Optimization of experimental conditions of ExoN activity based on FP assay

171

To set-up a screening assay, we explored 5'-FAM-RNA substrate and ExoN concentration and ratio, metal ion co-factors, temperature and reaction duration. Some RNA substrate and optimal conditions were already described (Bouvet et al., 2012; Saramago et al., 2021; Yekwa et al., 2019, 2017). A 22mer RNA that forms a stable hairpin in its 3' end has been reported to be a valuable substrate for both ExoN. Moreover, for SARS-CoV-2 ExoN, nsp10 able to stimulate nsp14 ExoN activity (Bouvet et al., 2012; Saramago et al., 2021) was added in the reaction (ratio 1:4 of nsp14:nsp10).

We first determined the optimal 5'-FAM-RNA concentration that provides sufficient and stable FP signal, and 178 the ratio of ExoN and 5'-FAM-RNA. For each 5'-FAM-RNA concentration (0.05 to 1μ M), single turnover 179 180 (STO) conditions (excess of enzyme versus substrate) and also multiple turnover (MTO) conditions (excess of 181 substrate versus enzyme) were tested. A significant change in the FP signal was observed only under STO 182 conditions. 0.1µM concentration of 5'-FAM-RNA was selected to provide a reproducible and stable FP signal. 183 To optimize the controls, several combinations were tested (Supplementary figure S2). For all negative controls, there was no variation between the initial and final FP values. However, we observe that the initial 184 FP values are lower without MnCl₂. This result is consistent with the study of Liu et al., 2014), who 185 186 reported that cations can affect the FP signal. The negative controls were then prepared by mixing 5'-FAM-187 RNA with MnCl₂ and without ExoN.

Regarding the nature of the metal ion, MnCl₂ and MgCl₂ were tested. An increased activity is observed with MnCl₂ (data not shown). The concentration of MnCl₂ that shows the highest reduction in FP signal is 5 mM for arenaviruses (Yekwa et al., 2019, 2017) and 2 mM for SARS-CoV-2 (Baddock et al., 2020; Saramago et al., 2021). Finally, the temperature of the assay is 25°C since the incubation at 37°C leads to a significant evaporation of the sample.

193

194 3.2 Assessing the SARS-CoV-2, LCMV and MACV ExoN activity by FP

196 While the 5'-FAM-RNA substrate was fixed at 0.1 μ M, 0.1 to 1.6 μ M of ExoN were tested. The FP signal was 197 measured for 30 min. The FP curves and end point RNA degradation products examined in urea PAGE, are 198 gathered in figure 1.

The negative controls (figure 1, brown curves, in panels A, B and C) present flattened curves, as expected. Strikingly, a small increase in the initial FP value is observed, corresponding to the time required for the formation of the catalytic complex. This is particularly visible with SARS-CoV-2 nsp14, which requires its activation by the cofactor nsp10. Indeed, nsp14 ExoN N-terminus is flexible, when nsp10 is not bound the catalytic site of nsp14 is distorted not allowing the catalytic ions to activate the water molecule for the nucleophilic attack (Ferron et al., 2018).

For all tested ExoN, except for the lowest concentration, we observe a reduction of the FP signal with time. The decrease of the FP value is correlated with the hydrolysis of the 5'-FAM-RNA, as confirmed on the urea PAGE analysis (figure 1, panels D, E and F). The difference between initial and final FP value after 30 min is proportional to the number of nucleotides removed from the substrate. By increasing the ExoN concentration, we observe an increase in the FP curves slope but the FP signals reach a plateau, which might be related to the inability to remove any extra nucleotide when the RNA fragments are to shorter.

The method efficiency was validated by the difference of FP values between the negative control (figure 1, brown curves, in panels A, B and C) and one concentration after 30 min reaction. This difference was selected as significant enough, with Δ FP +/- 30 to 60 units of mP. A molar ratio of 1:4 between the 5'-FAM-RNA (0.1 μ M) and ExoN (0.4 μ M) was used for the screening. The robustness of the assay was assessed by calculating the z' factor (supplementary methods and figure S3). For LCMV, MACV and SARS-CoV-2 we obtained z' value of 0.72, 0.74 and 0.69, respectively.

217

218 3.3 Screening of a focused library against the SARS-CoV-2, LCMV, and MACV ExoN using FP

Because ExoN activity is metal-dependent, we screened a focused library of 113 metal chelators that we have previously developed (Saez-Ayala et al., 2019, 2018), in order to demonstrate the robustness of our FP method. All compounds of the library were selected on the basis of competitive inhibition by metal chelation approach. Several patterns were chosen on the chelating scaffolds, in order to explore electronic, structural, and steric features critical for the binding process and active site accommodation. Two screenings were performed using 20 µM and 5 µM of compounds and the percentages of inhibition, deduced from FP curves, are gathered in

figure 2 (panels A, B, and C) and in supplementary figure S3, respectively.

226 Aurintricarboxyclic Acid (ATA, 40), a polyphenol (POP), was included as a positive control as it has been previously described as a nuclease inhibitor (Baddock et al., 2022; Canal et al., 2021; Huang et al., 2016), 227 228 acting as non-specific metal chelator (Liu et al., 2014). Compound 40 inhibits 100% of the different ExoN activity at 20 μ M (Figure 2, Table 1) and 52%, 39% and 23% at 5 μ M (Supplementary figure S3), respectively 229 230 against LCMV, MACV and SARS-CoV-2 ExoN. We identified 5 inhibitors (65, 67, 77, 78 and 91) of LCMV ExoN including 3 common with MACV ExoN 231 (Figure 2 and Table 1) with efficiency between 70 and 100% inhibition at 20 µM. Compounds 65, 67, 77, 78 232 belong to N-hydroxy isoquinoline-1,3-diones (HID) family and compound 91 belongs to β -diketo acid (DKA) 233 family were previously described by us as potent specific metal chelators (Saez-Ayala et al., 2019, 2018). 9 234

hit compounds (18, 40, 46, 55, 68, 77, 78, 91 and 92) inhibiting SARS-CoV-2 ExoN with efficiency between 70 and 100% inhibition at 20 μ M were also identified (Figure 2, Table1, Supplementary figure S3). Compounds 18 and 55 have never been described before. DKA 91 is the more efficient compound against all ExoN, showing inhibition at both 5 μ M and 20 μ M, with 87 to 100% inhibition at 20 μ M and 37 to 100% inhibition at 5 μ M.

240

241 *3.4 IC*₅₀ measurement of hit compounds by FP

IC₅₀ determination was performed for all hit compounds displaying more than 70% inhibition at 20 μ M concentration. The dose-response curves and IC₅₀ values determined by hill plot curve fitting all hit compounds and reference compound **40**, are gathered in figure 3 and table 1. The FP curves and the end point digestion products examined in urea PAGE are gathered in Supplementary Figures S5 to S8. IC₅₀ determination was also performed for compounds **3**, **4**, **44** and **57** (table 1), although they did not display high inhibition during the primary screening.

As expected, the reference compound ATA **40** was a potent inhibitor with IC₅₀ values of 7.05 ± 0.78 , 5.57 ± 0.41 and $5.50 \pm 0.19 \mu$ M, for LCMV, MACV and SARS-CoV-2 ExoN, respectively. The highest inhibition observed with the DKA **91** during the two screenings was confirmed in the IC₅₀ values. DKA **91** displayed equivalent efficiency to inhibit LCMV and MACV ExoN with respectively 5.63 ± 0.35 and $5.42 \pm 0.46 \mu$ M IC₅₀ values. The inhibition is slightly more pronounced for SARS-CoV-2 ExoN with IC₅₀ of $1.86 \pm 0.16 \mu$ M.

For other hit compounds, IC_{50} values ranged between 6 and 24 μ M. Notably, these values correlated perfectly with the inhibition % obtained by both screenings at 20 and 5 μ M, confirming the robustness and reproducibility of the assay. Moreover, The FP signal correlated with the degree of digestion observed upon PAGE analysis (Supplementary Figures S5 to S8). While the HID family (compounds **65**, **67**, **68**, **77** and **78**) showed a rather broad spectrum of efficacy for both arenavirus and coronavirus ExoNs, the DKA family (phenyl-DKA, piperidinyl-DKA and triazolyl-DKA, compounds **3**, **4**, **18**, **44**, **55**, **57**, **91** and **92**) showed a clear specificity for the SARS-CoV-2 ExoN.

260

261 *3.5 LCMV minigenome activity*

262 Arenavirus replication can be modeled using a recombinant plasmid minigenome system comprising the viral RdRp (L), the nucleoprotein (N), and a LCMV genomic RNA (Ortiz-Riaño et al., 2013). Previous studies have 263 shown the efficiency of this method (Saez-Ayala et al., 2019). We tested the hit compounds 40, 65, 67, 77, 78 264 and 91, at 100 and 50 µM. Because ribavirin (82) is the only licensed antiviral agent with activity against 265 arenaviruses (McCormick et al., 1986; Moreno et al., 2011), it was chosen as a reference compound. 266 Favipiravir (83), having demonstrated broad-spectrum activity against a number of RNA viruses, was also 267 268 added in the screening. Since the compounds were supposed to target the polymerase function, the assay was particularly suited by measuring the GLuc reporter gene (Gluc activity %, Supplementary Figure S9). We also 269 tested the cytotoxic effect of the compounds at 50 and 100 µM (Cell viability %, Supplementary Figure S9). 270 The ribavirin (82) resulted in a 76% and 47% inhibition of GLuc activity, at 100 and 50 µM respectively, but 271 272 was associated with important cytotoxic effect with 35 and 50 % cell viability, as described previously 273 (Mendenhall et al., 2011). Unexpectedly, favipiravir (83) displayed weak inhibition (30% at 100 μ M) without 274 cell viability reduction (Mendenhall et al., 2011). ATA (40) displayed no antiviral effect, but, cytotoxicity as described previously (Andrew et al., 1999; Haimsohn et al., 2002). Compounds 65, 67 and 77 displayed slight 275 effect (25 to 50% inhibition) on GLuc expression but associated with cytotoxic effect, as observed for ribavirin 276 (82). Compound 78 displayed weak GLuc expression inhibition (30 % at 50 μM) without cytotoxicity, 277 compared to ribavirin (82). 278

279

280 3.6 SARS-Cov-2 in vitro evaluation

The full library of 113 metal chelators was screened in a SARS-CoV-2 infected cell-based assay. We performed a primary screen for SARS-Cov-2 in VeroE6 cells (Touret et al., 2020) and we used remdesivir as a reference compound (Pruijssers et al., 2020; Wang et al., 2020). This fast and reliable assay, based on the cell viability, measures the ability of a compound to prevent the cytopathic effect induced by the viral replication at 50 µM concentration.

Our primary screen identified 31 hit compounds that prevent cytopathic effect by at least 40 % (Supplemental figure S10). To go further, we next performed dose-response analysis in VeroE6 TMPRSS2 (Touret et al., 2021; Weiss et al., 2021). We used VeroE6/TMPRSS2 cells to avoid off target false-positive compounds as it has been shown for Chloroquine (Hoffmann et al., 2020). Remdesivir, as reference compound, displayed EC₅₀ of $1.5\pm1.1 \mu$ M with CC₅₀ > 10 μ M.

Of the 31 primary hits, 19 compounds displaying between 40 and 100% inhibition were analyzed in doseresponse assays. 7 compounds from the same family and 5 compounds displaying between 40 and 70% inhibition were set aside. Additionally, the 8 hit compounds from the biochemical screening displaying between 0 and 40% inhibition were also analyzed. Here, we confirmed the antiviral activity of ATA (**40**) against SARS-Cov-2 in cell culture (Canal et al., 2021) with EC₅₀ of 27.23 \pm 20.68 µM. We were unable to confirm primary efficiency of 6 potential hit compounds and we identified 5 compounds that displayed significant cytotoxicity.

Finally, we identified 4 new compounds **3**, **4**, **44**, and **57**, that displayed a robust antiviral activity with EC_{50} between 8 and 38 µM without cytotoxicity and 2 compounds **46** and **55** that exert a potent antiviral activity in the same range but also showing signs of cytotoxicity, without reaching the CC_{50} value (table 1 and supplementary figure S11). Compounds **46** and **55** were identified during the biochemical screening on the SARS-CoV-2 ExoN, confirming the ExoN targeting.

303

304 4. Conclusion

We proved in this study that fluorescence polarization (FP) method is reliable, sensitive to monitor nuclease activity and feasible in 384-well plates. The method is robust, rapid and non-destructive allowing the visualization of the RNA substrate degradation using gels as orthogonal method of confirmation. We identified several hit inhibitors of viral arenavirus (LCMV and MACV) and coronavirus (SARS-CoV-2) ExoN activity and determined by fluorescence polarization their IC₅₀ at a micromolar level. Then we validated their activities

310	in cell culture assays and we found compounds displaying efficiency at micromolar level against SARS-CoV-
311	2. These results highlight the effectiveness of fluorescence polarization assay in the screening of compounds
312	libraries for the discovery of antivirals.
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- 328
- 329 Supplementary data associated with this article can be found, in the online version, at
- 330
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Figure 1. ExoN activity measured by FP for LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel C). 484 485 The FP signal variation is recorded with time during 30 min, each 30 secondes, at 25°C. The 5'-FAM-RNA substrate concentration used is 0.1 µM and the ExoN concentration tested ranges 0.1 µM to 1.6 µM. The data 486 represents the average and SEM of three independent experiments. The * shows the enzyme concentrations at 487 which the FP values are significantly different compared to the control condition (no enzyme) * p value < 0.05, 488 489 *** p value < 0.001 (One-way ANOVA). The bottom panels illustrate a representative image of the digestion 490 products analyzed in urea PAGE, of the ExoN activity for LCMV (panel D), MACV (panel E) and SARS-491 CoV-2 (panel F), after recording the FP signal. An aliquot of the sample was loaded into a urea-PAGE 20% 492 and scanned in a fluorescence imager.





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Figure 2. Screening of focused library of metal chelators (113) against LCMV (panel A), MACV (panel B)
and SARS-CoV-2 nsp14:nsp10 complex (panel C) ExoN followed by FP. The dots show the % of inhibition
of the ExoN activity as described in materials and methods. For the screening conditions 0.1 µM 5'-FAMRNA, 0.4 µM ExoN and 20 µM of inhibitor were used.



Figure 3. IC₅₀ measurement of hit compounds by FP on LCMV, MACV and SARS-CoV-2 nsp14:nsp10
complex The graphs show the dose-response curves obtained by extraction of FP data and the corresponding
IC₅₀ values for LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel C). The data represents the
average and SEM of three independent experiments.





- 517 Table 1: List of hits compounds, targets, % of inhibition of the ExoN screening at $20\mu M$, ExoN- IC₅₀ values,
- 518 % of inhibition of the cellular screenings at 50 μ M, EC₅₀ and CC₅₀values and compound structures. * :
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Cmpd N°	Target	% inhib ExoN (20µM)	IC ₅₀ (μM)	% inhib Cellular Assay (50µM)	EC ₅₀ (μM) CC ₅₀ (μM)	structure
3	SARS-CoV-2	5	> 50	50	38 ± 3 > 50	
4	SARS-CoV-2	11	33.8*	70	19±3.7 > 50	
18	SARS-CoV-2	74	20 ± 1.8	15	> 50 > 50	
40	LCMV	102	7.1 ± 0.8	0	ND	от соон
	MACV	111	5.6 ± 0.4	ND	ND	Соон
	SARS-CoV-2	99	5.5 ± 0.2	80	$27\pm20 > 50$	ноос" Ү он
44	SARS-CoV-2	8	> 50	70	26±1.7 > 50	
46	SARS-CoV-2	71	12 ± 0.5	40	12±3.7 > 25	HO
55	SARS-CoV-2	118	14 ± 4	15	23±3.7 > 50	H ₂ N-J O N=N
57	SARS-CoV-2	-5	> 50	64	8.5±1.3 > 50	
65	LCMV	95	16 ± 0.9	33 > 50	ND	
67	LCMV	73	24 ± 0.9	39 > 50	ND	F F ₃ C NH O H O O H

68	SARS-CoV-2	106	14.7 ± 4.6	10	> 50	FNHO
					> 50	
						O ₂ N OH
		00	160 + 1.1	27		Ö
11	LCMV	82	16.8 ± 1.1	27	ND	NH_O
	MACV	102	12.6 ± 1.5	> 30 ND	ND	
	IVIAC V	105	15.0 ± 1.3	ND	ND	O ₂ N OH
	SARS-CoV-2	95	7.8 ± 2	0	> 50	0
					> 50	
78	LCMV	92	13.3 ± 1.3	31	ND	F-NH O
				> 100		
	MACV	113	11.5 ± 0.9	ND	ND	O2N OH
		02	(0 + 2)	0	> 50	0
	SARS-COV-2	93	0.9 ± 2	0	> 50	
		07	5 (+ 0.4	0	> 50	O OH
01	LCMV	87	5.6 ± 0.4	0	ND	но
91	MACV	108	54 ± 05	ND	ND	но
		100	5.4 ± 0.5			
	SARS-CoV-2	97	1.9 ± 0.2	0	> 50	
					> 50	
92	SARS-CoV-2	93	7.1 ± 0.5	0	> 50	но он он
					> 50	но