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

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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 IC₅₀ 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, IC₅₀, 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 IC₅₀ at micromolar
45 level.
- 46 • We tested hit compounds in cellular assay and we confirmed 3 hits previously detected in the *in vitro*
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.

54 These RNA viruses encode for proteins, forming a replication/transcription complex (RTC) that orchestrates
55 viral replication (Ferron et al., 2017; Knipe and Howley, 2013; Ogando et al., 2019). The peculiarity of
56 *Arenaviridae* and *Coronaviridae* families, is the unique viral 3'-5'-exoribonuclease (ExoN) activity described
57 so far (Hastie et al., 2011; Snijder et al., 2003), The ExoN activity carried by arenavirus nucleoprotein (N) or
58 the nsp14 protein of coronavirus participates to the suppression of the host innate immune response (Martínez-
59 Sobrido et al., 2009, 2007, 2006; Becares et al., 2016; Lei et al., 2020) , moreover the nsp14 displays an
60 additional role in maintaining the genome integrity (Bouvet et al., 2012; Ferron et al., 2018). The arenavirus
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
63 and Glu) (Bouvet et al., 2012; Yekwa et al., 2019), essential for the binding of two metal cations (Mg^{2+} and/or
64 Mn^{2+}) involved in the RNA hydrolysis mechanism (Steitz and Steitz, 1993), always proceeding from the 3'- to
65 the 5'-direction.

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

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

73 Here we present a convenient method using fluorescence polarization (FP) to assess the ExoN activity and its
74 inhibition. The recorded FP signal is altered proportionally to the size of the fluorescent RNA probe which is
75 hydrolyzed into smaller fragments. We validate the method on SARS-CoV-2, Lymphocytic Choriomeningitis
76 (LCMV) and Machupo viruses (MACV) ExoN. The method is sensitive, robust, amenable to miniaturization
77 (384 well plates) and allowed us to screen a focused library of 113 metal chelators over the three-targeted viral
78 ExoN, validating the proof-of-concept of the assay. We selected several hit inhibitors and determined by FP
79 their IC_{50} . The hit compounds were tested in cellular assays.

80

81 2. Materials and methods

82

83 *2.1 Products and reagents.*

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

92

93 *2.2 Protein expression and purification.*

94 All proteins were expressed in E. Coli. The LCMV and MACV ExoN plasmids used for this study were
95 described in (Yekwa et al., 2019). For the expression of the SARS-CoV-2 nsp10 and nsp14, the synthetic
96 genes were purchased from Twist Bioscience (USA). The protein production methods are detailed in the
97 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 and 1 µM), 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 µM and ExoN concentration ranged from 0.1 to 1.6 µM. 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
111 started by the addition of 5'-FAM-RNA and the fluorescence polarization (FP) was read in the Pherastar FSX

112 (BMG Labtech) using the 480 nm excitation and 520 nm emission filter, during 30 minutes at 25°C or 37°C,
113 every 30 seconds. The gain was set up using the negative control which contained the 5'-FAM-RNA in the
114 presence of heat-denatured nuclease. After 30 minutes, the reactions were stopped by the addition of an equal
115 volume of loading buffer (8M urea containing 10 mM EDTA) and the digestion products were then loaded in
116 7 M urea containing 20% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio 19:1) buffered with
117 TBE and visualized using a Fluorescent Image Analyzer Typhoon (GE Healthcare).

118

119 *2.3.3 Time course of ExoN assay*

120 Reactions of 20 µl total volume were performed in the buffer mentioned above, with 2 or 5 mM MnCl₂ for
121 SARS-CoV-2 or arenaviruses ExoN, respectively. The 5'-FAM-RNA concentration was fixed at 0.1 µM. The
122 SARS-CoV-2 nsp14/nsp10 ExoN complex concentration was ranged from 0.2 to 1 µM and pre-incubation at
123 room temperature for 5 minutes was performed to allow the complex formation. The LCMV and MACV ExoN
124 concentration was ranged from 0.1 to 1.6 µM. The reaction started with the addition of 5'-FAM-RNA and The
125 FP signal was recorded as mentioned previously. At end point, an aliquot of the sample was examined in urea
126 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
134 and ExoN concentrations were fixed at 0.1 µM. and 0.4 µM, respectively. Compounds (**1-113**) were added to
135 the reaction with a final concentration of 5 µM and 20 µM in 5% DMSO. 8 reactions mix with ExoN were
136 used as positive controls and 8 reactions mix with heat-denatured ExoN were used as negative controls.
137 Reactions were initiated by adding the 5'-FAM-RNA and the FP signal was recorded as mentioned previously.
138 To calculate the percentage of inhibition, a time correction was applied for the delayed initiation of the reaction
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 IC₅₀ 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 μM for LCMV ExoN, from 0.4 to 25 μM for MACV ExoN and from
149 0.5 to 16 μM 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*

156 LCMV minigenome assays (MG) were adapted from a previously described procedure (Saez-Ayala et al.,
157 2019, 2018). SARS-CoV-2 cellular assays were performed as previously described (Touret et al., 2021, 2020).
158 Protocols are detailed in the supplementary data file.

159

160 3. Results and discussion

161

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

167 which is altered in the course of the nuclease, reflecting the enzymatic activity (Liu et al., 2014; Zhang et al.,
168 2012). The assay was first optimized.

169

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

171

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

178 We first determined the optimal 5'-FAM-RNA concentration that provides sufficient and stable FP signal, and
179 the ratio of ExoN and 5'-FAM-RNA. For each 5'-FAM-RNA concentration (0.05 to 1 μ M), single turnover
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. 0.1 μ M 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
184 controls, there was no variation between the initial and final FP values. However, we observe that the initial
185 FP values are lower without MnCl₂. This result is consistent with the study of Liu *et al* (Liu et al., 2014), who
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.

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

193

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

195

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.

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

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

211 The method efficiency was validated by the difference of FP values between the negative control (figure 1,
212 brown curves, in panels A, B and C) and one concentration after 30 min reaction. This difference was selected
213 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
214 μ M) and ExoN (0.4 μ M) was used for the screening. The robustness of the assay was assessed by calculating
215 the z' factor (supplementary methods and figure S3). For LCMV, MACV and SARS-CoV-2 we obtained z'
216 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*

219 Because ExoN activity is metal-dependent, we screened a focused library of 113 metal chelators that we have
220 previously developed (Saez-Ayala et al., 2019, 2018), in order to demonstrate the robustness of our FP method.
221 All compounds of the library were selected on the basis of competitive inhibition by metal chelation approach.
222 Several patterns were chosen on the chelating scaffolds, in order to explore electronic, structural, and steric
223 features critical for the binding process and active site accommodation. Two screenings were performed using
224 20 μ M and 5 μ M of compounds and the percentages of inhibition, deduced from FP curves, are gathered in

225 figure 2 (panels A, B, and C) and in supplementary figure S3, respectively.
226 Aurintricarboxylic Acid (ATA, **40**), a polyphenol (POP), was included as a positive control as it has been
227 previously described as a nuclease inhibitor (Baddock et al., 2022; Canal et al., 2021; Huang et al., 2016),
228 acting as non-specific metal chelator (Liu et al., 2014). Compound **40** inhibits 100% of the different ExoN
229 activity at 20 μ M (Figure 2, Table 1) and 52%, 39% and 23% at 5 μ M (Supplementary figure S3), respectively
230 against LCMV, MACV and SARS-CoV-2 ExoN.

231 We identified 5 inhibitors (**65**, **67**, **77**, **78** and **91**) of LCMV ExoN including 3 common with MACV ExoN
232 (Figure 2 and Table 1) with efficiency between 70 and 100% inhibition at 20 μ M. Compounds **65**, **67**, **77**, **78**
233 belong to *N*-hydroxyisoquinoline-1,3-diones (HID) family and compound **91** belongs to β -diketo acid (DKA)
234 family were previously described by us as potent specific metal chelators (Saez-Ayala et al., 2019, 2018). 9
235 hit compounds (**18**, **40**, **46**, **55**, **68**, **77**, **78**, **91** and **92**) inhibiting SARS-CoV-2 ExoN with efficiency between
236 70 and 100% inhibition at 20 μ M were also identified (Figure 2, Table1, Supplementary figure S3).
237 Compounds **18** and **55** have never been described before. DKA **91** is the more efficient compound against all
238 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%
239 inhibition at 5 μ M.

240

241 3.4 *IC₅₀ measurement of hit compounds by FP*

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

248 As expected, the reference compound ATA **40** was a potent inhibitor with *IC₅₀* values of 7.05 ± 0.78 , $5.57 \pm$
249 0.41 and 5.50 ± 0.19 μ M, for LCMV, MACV and SARS-CoV-2 ExoN, respectively. The highest inhibition
250 observed with the DKA **91** during the two screenings was confirmed in the *IC₅₀* values. DKA **91** displayed
251 equivalent efficiency to inhibit LCMV and MACV ExoN with respectively 5.63 ± 0.35 and 5.42 ± 0.46 μ M
252 *IC₅₀* values. The inhibition is slightly more pronounced for SARS-CoV-2 ExoN with *IC₅₀* of 1.86 ± 0.16 μ M.

253 For other hit compounds, IC₅₀ values ranged between 6 and 24 μM. Notably, these values correlated perfectly
254 with the inhibition % obtained by both screenings at 20 and 5 μM, confirming the robustness and
255 reproducibility of the assay. Moreover, The FP signal correlated with the degree of digestion observed upon
256 PAGE analysis (Supplementary Figures S5 to S8). While the HID family (compounds **65**, **67**, **68**, **77** and **78**)
257 showed a rather broad spectrum of efficacy for both arenavirus and coronavirus ExoNs, the DKA family
258 (phenyl-DKA, piperidinyl-DKA and triazolyl-DKA, compounds **3**, **4**, **18**, **44**, **55**, **57**, **91** and **92**) showed a clear
259 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
263 RdRp (L), the nucleoprotein (N), and a LCMV genomic RNA (Ortiz-Riaño et al., 2013). Previous studies have
264 shown the efficiency of this method (Saez-Ayala et al., 2019). We tested the hit compounds **40**, **65**, **67**, **77**, **78**
265 and **91**, at 100 and 50 μM. Because ribavirin (**82**) is the only licensed antiviral agent with activity against
266 arenaviruses (McCormick et al., 1986; Moreno et al., 2011), it was chosen as a reference compound.
267 Favipiravir (**83**), having demonstrated broad-spectrum activity against a number of RNA viruses, was also
268 added in the screening. Since the compounds were supposed to target the polymerase function, the assay was
269 particularly suited by measuring the GLuc reporter gene (Gluc activity %, Supplementary Figure S9). We also
270 tested the cytotoxic effect of the compounds at 50 and 100 μM (Cell viability %, Supplementary Figure S9).
271 The ribavirin (**82**) resulted in a 76% and 47% inhibition of GLuc activity, at 100 and 50 μM respectively, but
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 μM) without
274 cell viability reduction (Mendenhall et al., 2011). ATA (**40**) displayed no antiviral effect, but, cytotoxicity as
275 described previously (Andrew et al., 1999; Haimsohn et al., 2002). Compounds **65**, **67** and **77** displayed slight
276 effect (25 to 50% inhibition) on GLuc expression but associated with cytotoxic effect, as observed for ribavirin
277 (**82**). Compound **78** displayed weak GLuc expression inhibition (30 % at 50 μM) without cytotoxicity,
278 compared to ribavirin (**82**).

279

280 *3.6 SARS-Cov-2 in vitro evaluation*

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

286 Our primary screen identified 31 hit compounds that prevent cytopathic effect by at least 40 % (Supplemental
287 figure S10). To go further, we next performed dose-response analysis in VeroE6 TMPRSS2 (Touret et al.,
288 2021; Weiss et al., 2021). We used VeroE6/TMPRSS2 cells to avoid off target false-positive compounds as it
289 has been shown for Chloroquine (Hoffmann et al., 2020). Remdesivir, as reference compound, displayed EC₅₀
290 of 1.5 \pm 1.1 μ M with CC₅₀ > 10 μ M.

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

298 Finally, we identified 4 new compounds **3**, **4**, **44**, and **57**, that displayed a robust antiviral activity with EC₅₀
299 between 8 and 38 μ M without cytotoxicity and 2 compounds **46** and **55** that exert a potent antiviral activity in
300 the same range but also showing signs of cytotoxicity, without reaching the CC₅₀ value (table 1 and
301 supplementary figure S11) . Compounds **46** and **55** were identified during the biochemical screening on the
302 SARS-CoV-2 ExoN, confirming the ExoN targeting.

303

304 4. Conclusion

305 We proved in this study that fluorescence polarization (FP) method is reliable, sensitive to monitor nuclease
306 activity and feasible in 384-well plates. The method is robust, rapid and non-destructive allowing the
307 visualization of the RNA substrate degradation using gels as orthogonal method of confirmation. We identified
308 several hit inhibitors of viral arenavirus (LCMV and MACV) and coronavirus (SARS-CoV-2) ExoN activity
309 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.

313

314

315

316

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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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482 Legend to figures

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484 Figure 1. ExoN activity measured by FP for LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel C).

485 The FP signal variation is recorded with time during 30 min, each 30 secondes, at 25°C. The 5'-FAM-RNA

486 substrate concentration used is 0.1 μM and the ExoN concentration tested ranges 0.1 μM to 1.6 μM . The data

487 represents the average and SEM of three independent experiments. The * shows the enzyme concentrations at

488 which the FP values are significantly different compared to the control condition (no enzyme) * p value < 0.05,

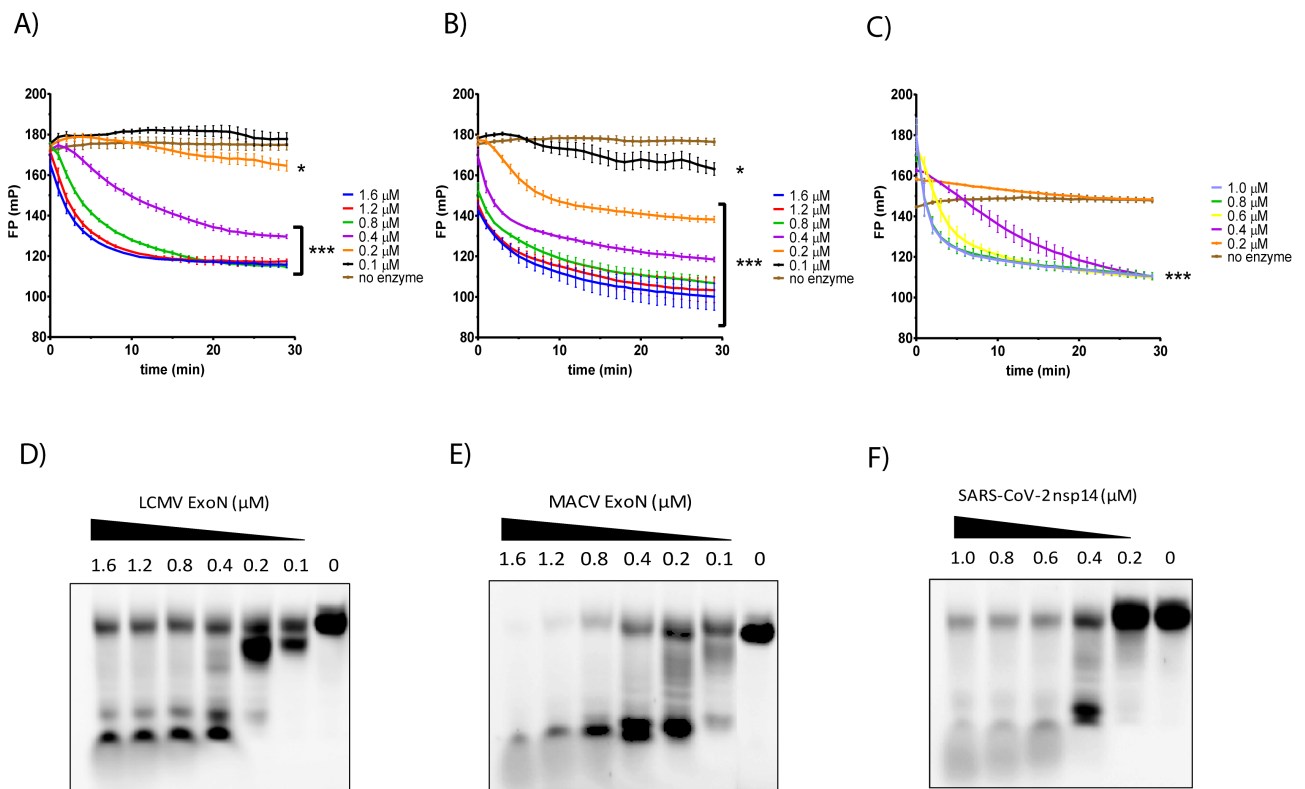
489 *** p value < 0.001 (One-way ANOVA). The bottom panels illustrate a representative image of the digestion

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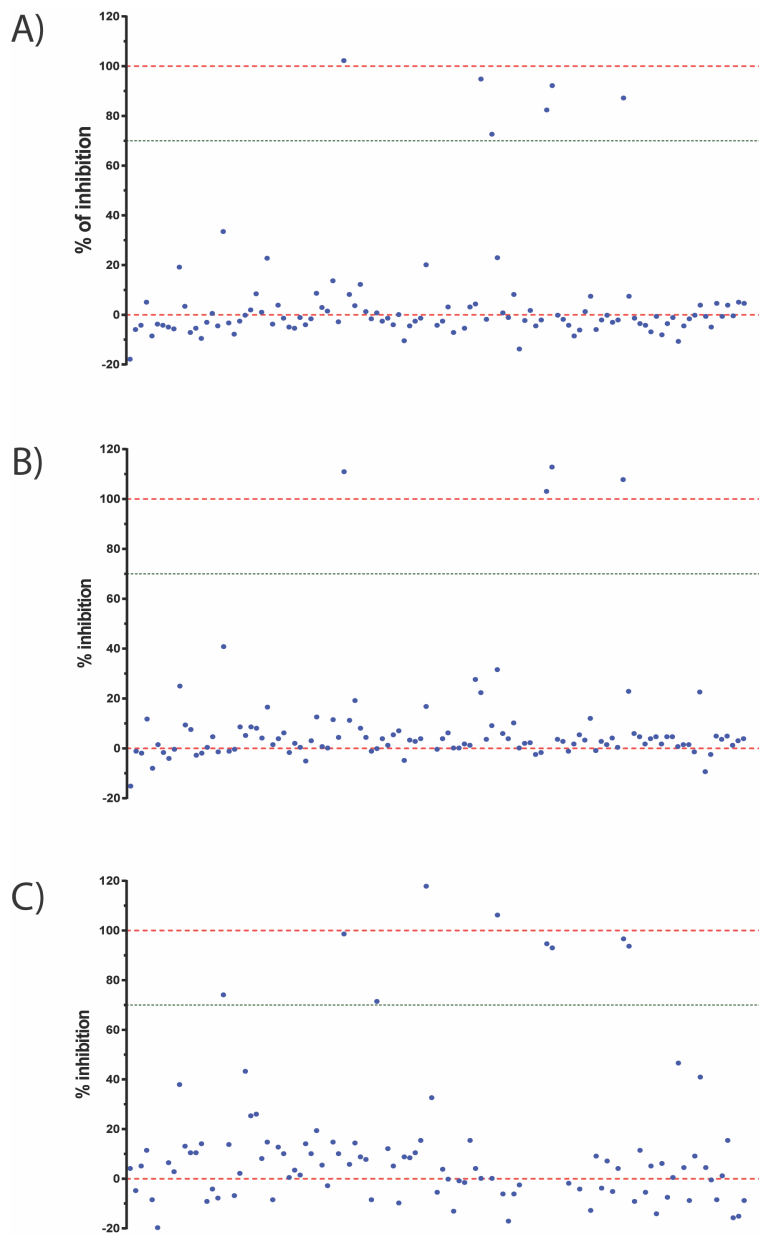
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499 Figure 2. Screening of focused library of metal chelators (113) against LCMV (panel A), MACV (panel B)
500 and SARS-CoV-2 nsp14:nsp10 complex (panel C) ExoN followed by FP. The dots show the % of inhibition
501 of the ExoN activity as described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

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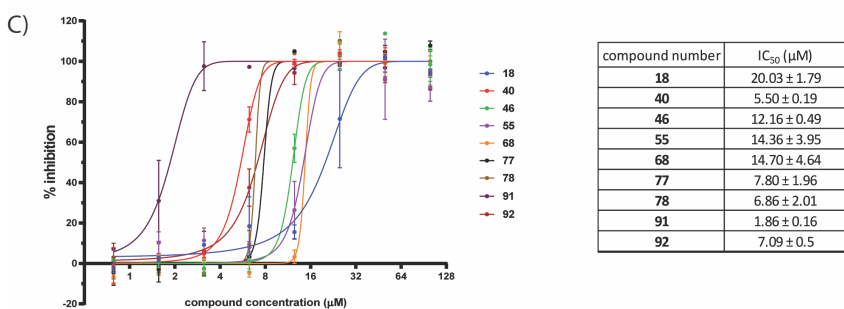
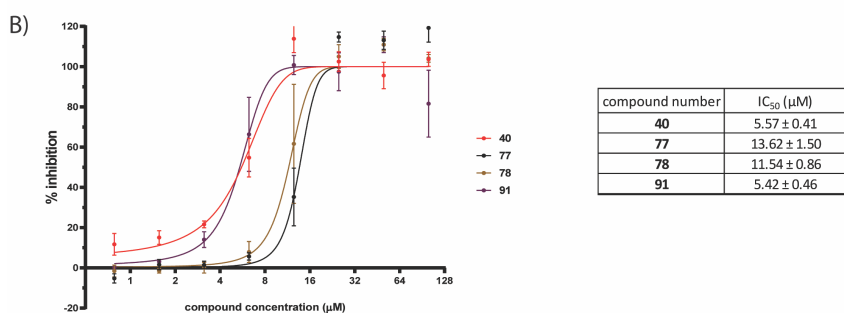
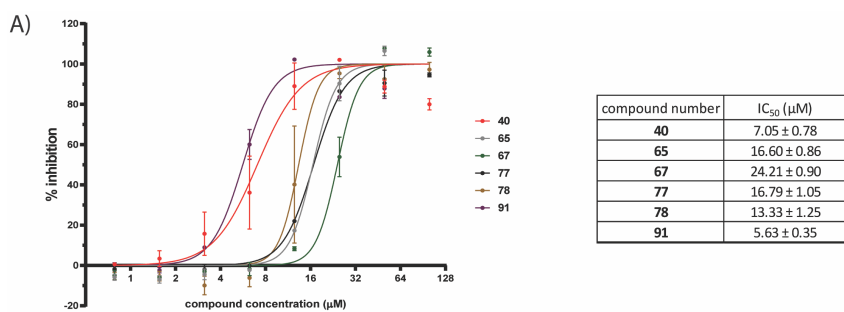
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511 Figure 3. IC₅₀ measurement of hit compounds by FP on LCMV, MACV and SARS-CoV-2 nsp14:nsp10
512 complex The graphs show the dose-response curves obtained by extraction of FP data and the corresponding
513 IC₅₀ values for LCMV (panel A), MACV (panel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

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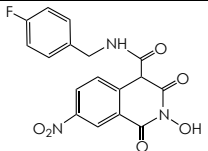
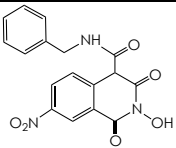
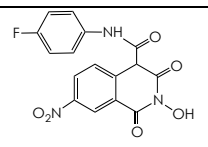
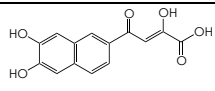
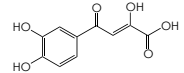


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517 Table 1: List of hits compounds, targets, % of inhibition of the ExoN screening at 20 μ M , ExoN- IC₅₀ values,
 518 % of inhibition of the cellular screenings at 50 μ M, EC₅₀ and CC₅₀ values and compound structures. * :
 519 monuplicate.

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Cmpd N°	Target	% inhib ExoN (20 μ M)	IC ₅₀ (μ M)	% inhib Cellular Assay (50 μ M)	EC ₅₀ (μ M) CC ₅₀ (μ M)	structure
3	SARS-CoV-2	5	> 50	50	38 \pm 3 > 50	
4	SARS-CoV-2	11	33.8*	70	19 \pm 3.7 > 50	
18	SARS-CoV-2	74	20 \pm 1.8	15	> 50 > 50	
40	LCMV MACV SARS-CoV-2	102 111 99	7.1 \pm 0.8 5.6 \pm 0.4 5.5 \pm 0.2	0 ND 80	ND ND 27 \pm 20 > 50	
44	SARS-CoV-2	8	> 50	70	26 \pm 1.7 > 50	
46	SARS-CoV-2	71	12 \pm 0.5	40	12 \pm 3.7 > 25	
55	SARS-CoV-2	118	14 \pm 4	15	23 \pm 3.7 > 50	
57	SARS-CoV-2	-5	> 50	64	8.5 \pm 1.3 > 50	
65	LCMV	95	16 \pm 0.9	33 > 50	ND	
67	LCMV	73	24 \pm 0.9	39 > 50	ND	

68	SARS-CoV-2	106	14.7 ± 4.6	10	> 50 > 50	
77	LCMV	82	16.8 ± 1.1	27	ND	
	MACV	103	13.6 ± 1.5	> 50 ND	ND	
	SARS-CoV-2	95	7.8 ± 2	0	> 50 > 50	
78	LCMV	92	13.3 ± 1.3	31	ND	
	MACV	113	11.5 ± 0.9	> 100 ND	ND	
	SARS-CoV-2	93	6.9 ± 2	0	> 50 > 50	
91	LCMV	87	5.6 ± 0.4	0	ND	
	MACV	108	5.4 ± 0.5	ND	ND	
	SARS-CoV-2	97	1.9 ± 0.2	0	> 50 > 50	
92	SARS-CoV-2	93	7.1 ± 0.5	0	> 50 > 50	

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