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3

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13

14 Abstract

15 Viral exoribonucleases are uncommon in the world of RNA viruses. To date, this activity has been identified
16 only in the *Arenaviridae* and the *Coronaviridae* families. These exoribonucleases play important but
17 different roles in both families: for mammarenaviruses the exoribonuclease is involved in the suppression of
18 the host immune response whereas for coronaviruses, exoribonuclease is both involved in a proofreading
19 mechanism ensuring the genetic stability of viral genomes and participating to evasion of the host innate
20 immunity. Because of their key roles, they constitute attractive targets for drug development. Here we
21 present a high-throughput assay using fluorescence polarization to assess the viral exoribonuclease activity
22 and its inhibition. We validate the assay using three different viral enzymes from SARS-CoV-2, lymphocytic
23 choriomeningitis and Machupo viruses. The method is sensitive, robust, amenable to miniaturization (384
24 well plates) and allowed us to validate the proof-of-concept of the assay by screening a small focused
25 compounds library (23 metal chelators). We also determined the IC₅₀ of one inhibitor common to the three
26 viruses.

27

28 Keywords

29 Phosphohydrolase activity, *Arenaviridae*, *Coronaviridae*, exoribonuclease activity, fluorescence
30 polarization, screening, inhibitors, IC₅₀.

31

32 Highlights

- 33 • *Arenaviridae* and *Coronaviridae* viral families share an exoribonuclease activity of common
34 evolutionary origin
- 35 • *Arenaviridae* and *Coronaviridae* exoribonuclease is an attractive target for drug development
- 36 • We present a high-throughput assay in 384 well-plates for the screening of inhibitors using fluores-
37 cence polarization
- 38 • We validated the assay by screening of a focused library of 23 metal chelators against SARS-CoV-2,
39 Lymphocytic Choriomeningitis virus and Machupo virus exoribonucleases
- 40 • We determined the IC₅₀ by fluorescence polarization of one inhibitor common to the three viruses.

41

42 1. Introduction

43

44 RNA viruses code for a polyprotein, forming the replication/transcription complex (RTC) that orcherstrates
45 viral replication. In this complex process, specific viral enzymatic activities are only found in some RNA
46 viruses families. This is the case for the 3'-5'-exoribonuclease (ExoN) activity identified in *Arenaviridae* and
47 *Coronaviridae* viral families, although these viruses display very different genome organization and
48 replication/transcription strategies. Indeed, arenaviruses have an ambisense bi-segmented negative single-
49 stranded RNA genome whereas the coronavirus genome is composed of a positive single-stranded RNA
50 (Ferron et al., 2017; Knipe and Howley, 2013, Ogando et al., 2019). The ExoN activity identified in these
51 two viral families are involved in different processes (Hastie et al., 2011; Snijder et al., 2003). Indeed, the
52 ExoN carried by arenavirus nucleoprotein (NP) is likely involved in the suppression of the host innate
53 immune response (Martínez-Sobrido et al., 2009, 2007, 2006) while the ExoN carried by the nsp14 protein
54 of coronaviruses allows optimization of the replication fidelity of the viral genome (Bouvet et al., 2012;
55 Ferron et al., 2018) as well as evasion of the host innate immunity (Becares et al., 2016; Lei et al., 2020).

56 The arenavirus and coronavirus ExoN belong to the same DEDD superfamily (DED/EDh subfamily), are
57 structurally similar (Zuo and Deutscher, 2001) and are characterized by the presence of four conserved acidic
58 residues (Asp and Glu) (Bouvet et al., 2012; Yekwa et al., 2019). The catalytic residues are essential for the
59 binding of two metal cations involved in the RNA hydrolysis mechanism (Steitz and Steitz, 1993), always
60 proceeding from the 3'- to the 5'-direction.

61 Because of its biological significance to the viral life cycle and role in infectious processes, arenavirus and
62 coronavirus ExoN is an attractive target for drug development (Papageorgiou et al., 2020; Subissi et al.,
63 2014). Currently, there are almost no FDA-approved antivirals against these viruses, despite the loss of
64 human lives during the short-lived SARS-CoV outbreak in 2002 and the continuing MERS epidemic or
65 regular outbreaks of LASSA virus in West Africa causing several thousand case fatalities. Today, the
66 situation is different, the world is paralyzed by a global SARS-CoV-2 pandemic and this event revealed the
67 urgency to act to develop potent antivirals, in support of a vaccine approach.

68 While millions of molecules are available in countless compounds libraries, drug development efforts are
69 generally restricted by the limitations of validated therapeutic targets or available potent high-throughput
70 screening assays. For mammarenavirus and coronavirus ExoN assay making use of radiolabeled substrate are
71 available but barely adapted to HT screening (Bouvet et al., 2012; Ferron et al., 2018; Yekwa et al., 2019,
72 2017; Baddock et al., 2020; Saramago et al., 2021)).

73 Here we present a method using fluorescence polarization (FP) to assess the ExoN activity and its inhibition.
74 The method relies on the hydrolysis of a fluorescent RNA substrate into smaller fragments which translates
75 in a modification of the FP signal which is measured and recorded. The FP signal is altered proportionally to
76 the size of the fluorescent RNA probe. We validate the method on three different viral enzymes of interest
77 from SARS-CoV-2, Lymphocytic Choriomeningitis and Machupo viruses. The method is sensitive, robust,
78 amenable to miniaturization (384 well plates) and allowed us to screen a focused library of 23 metal
79 chelators over the three targeted viral ExoN, validating the proof-of-concept of the assay. We also
80 determined the IC_{50} of one inhibitor common to the three viruses.

81

82 2. Materials and methods

83

84 2.1 Products and reagents.

85 Raltegravir (**1**) and Baloxavir (**5**) were purchased from Carbosynth. Aurintricarboxylic acid (**11**) was
86 purchased from Acros Organics. Excepting for the compounds **8**, **13**, **14**, **15** and **24**, the other compounds
87 have been described previously (Saez-Ayala et al., 2019, 2018). All compounds were resuspended in 100 %
88 DMSO at 20 mM and stored at - 20 °C. The 22-mer RNA 5'-UGACGGCCCGGAAAACCGGGCC-3'
89 containing FAM dye at the 5'-end (5'-FAM-RNA) was purchased from Microsynth AG (Switzerland).

90

91 *2.2 Protein expression and purification.*

92 The LCMV, MACV, ExoN plasmids used for this study were described in (Yekwa et al., 2019). The protein
93 production method is detailed in the supplementary data file (see also figure S1 for elution profiles). For the
94 expression of the SARS-CoV-2 nsp10 and nsp14, the synthetic genes were purchased from Twist (USA) and
95 were cloned with a hexahistidine tag in the N-terminus of the protein. *E. coli* DE3 cells were transformed
96 with the corresponding expression vectors. Bacteria were grown in TB medium with the corresponding
97 antibiotic and protein expression was induced by addition of IPTG to a final concentration of 500 µM for
98 nsp10 and 50 µM for nsp14 when the OD_{600nm} of the culture reached a value of 0.5. The induction was
99 performed during 16 h at 17°C and 200 rpm. Bacterial cell pellets were frozen and resuspended in lysis
100 buffer (50 mM HEPES, pH 7.5 and 300 mM NaCl) supplemented with 1 mM PMSF, 10 mM imidazole, 10
101 µg/ml DNase I, 0.25 mg/ml of lysozyme and 0.5% Triton X-100. After sonication and clarification, proteins
102 were purified by two steps of chromatography. The first step consisted of an IMAC (Ni Resin). The lysate
103 was passed through the column and washed with lysis buffer supplemented with 20 mM imidazole. The
104 protein was eluted with lysis buffer supplemented with 250 mM imidazole. Protein fractions were then
105 loaded on a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare), and eluted with 10 mM
106 HEPES, pH 7.5, 300 mM NaCl, 1 mM DTT and 5% glycerol. The fraction containing the pure protein, as
107 examined by SDS-PAGE and coomassie staining, were pooled and concentrated in the gel filtration buffer,
108 aliquoted in small volumes, flash frozen in liquid nitrogen and stored at -80°C.

109

110 *2.3 Set-up of exonuclease activity conditions based on fluorescent polarization assay*

111 *2.3.1 Optimisation of 5'-FAM-RNA concentration and SARS-CoV-2, LCMV or MACV ExoN ratio*

112 Reactions were performed in 20 µl total volume in a buffer containing 40 mM Tris (pH 8), 5 mM DTT and 2
113 or 5 mM MnCl₂. The concentration of 5'-FAM-RNA tested varied from 50 to 1000 nM. For each 5'-FAM-

114 RNA concentration (50, 100, 250, 500 and 1000 nM), a range of concentration of ExoN (SARS-CoV-2,
115 LCMV or MACV) was tested from a ratio of 5-fold less enzyme up to 10-fold more enzyme (10 nM to 10
116 μ M) than 5'-FAM-RNA. For SARS-CoV-2 ExoN, the molar ratio of nsp14:nsp10 complex in the reactions
117 was always kept at 1:4, as optimized previously (Bouvet et al., 2012). The reaction started by the addition of
118 5'-FAM-RNA and the fluorescence polarization (FP) was read in the Pherastar FSX (BMG Labtech) using
119 the 480 nm excitation and 520 nm emission filter, during 30 minutes at 25°C, every 30 seconds. The gain
120 was set up using the negative control which contained the fluorescent RNA in the reaction buffer with metal
121 ion and in the presence of heat-denatured nuclease. Other negative controls tested used consisted in the
122 replacement of $MnCl_2$ by $CaCl_2$ or, depletion of the metal ion in the reaction mix and depletion of the
123 enzyme. After 30 minutes, the reactions were stopped by the addition of an equal volume of loading buffer
124 (8M urea containing 10 mM EDTA) and the digestion products were then loaded in 7 M urea containing
125 20% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio 19:1) buffered with TBE and visualized
126 using a Fluorescent Image Analyzer Typhoon (GE Healthcare).

127

128 *2.3.2 Optimisation of temperature, metal ion nature and concentration requirement*

129 To find the optimal temperature, metal ion nature and reagent concentration, reactions were performed in the
130 buffer mentioned above. The 5'-FAM-RNA concentration was kept at 100 nM and ExoN concentration
131 ranged from 100 nM to 1600 nM. The activity was tested in the presence of $MnCl_2$ or $MgCl_2$ at three
132 different concentrations 1, 2 or 5 mM. The FP signal was recorded as mentioned previously either at 25°C or
133 at 37°C. After finishing the recording of the FP signal, an aliquot of the sample was examined in urea PAGE
134 as mentioned above.

135

136 *2.3.3 Time course of ExoN assay*

137 Reactions of 20 μ l total volume were performed in the buffer mentioned above, with 2 mM $MnCl_2$ for
138 SARS-CoV-2 ExoN and 5 mM for LCMV or MACV ExoN. The 5'-FAM-RNA concentration was fixed at
139 100 nM. The SARS-CoV-2 nsp14/nsp10 ExoN complex concentration was ranged from 200 nM to 1 μ M
140 and pre-incubation at RT during 5 minutes was performed to allow the complex formation. The LCMV and
141 MACV ExoN concentration was ranged from 100 nM to 1600 nM. The reaction started by the addition of 5'-
142 FAM-RNA and The FP signal was recorded as mentioned previously. After finishing the recording of the FP

143 signal, an aliquot of the sample was examined in urea PAGE as mentioned above. The assay was done in
144 triplicate for each ExoN.

145

146 *2.4 High throughput screening and IC₅₀ determination.*

147

148 *2.4.1 HTS assay based on fluorescent polarization assay*

149 The screening at 5 μM and 20 μM inhibitor concentration was performed in 384-wells with flat bottom Nunc
150 plates, in 20 μL total volume reaction. Reactions were performed in a buffer containing 40 mM Tris (pH 8),
151 5 mM DTT, with 2 mM MnCl₂ for SARS-CoV-2 and 5 mM MnCl₂ for LCMV or MACV ExoN. The 5'-
152 FAM-RNA concentration was fixed at 100 nM. The ExoN concentration was fixed at 400 nM. Compounds
153 (**1-23**) were added to the reaction with final concentration of 5 μM and 20 μM in 5% DMSO. Reactions were
154 initiated by addition of the 5'-FAM-RNA using a multichannel pipette. 48 positive controls (the reaction mix
155 with 5% DMSO, with ExoN and without compound) have been deposited randomly in the 384-well plate. 48
156 negative controls (the reaction mixture with 5% DMSO, without or with ExoN that has been heat denatured,
157 and without compound), have been deposited randomly in the 384-well plate. The reaction started by the
158 addition of 5'-FAM-RNA and the FP signal was recorded as mentioned previously. To calculate the
159 percentage of inhibition, a time correction was applied for the delayed initiation of the reaction due to the use
160 of a multichannel pipette. The percentage of inhibition at a given time was calculated as follows :

$$\text{Inhibition\%} = \frac{100 \times (\text{FP} - \text{average of positive controls})}{(\text{average negative controls} - \text{average positive controls})}$$

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

162 The time selected for doing this calculation was the time when the signal of the positive control reached the
163 plateau (30 minutes). The Z' factor for the assay was calculated using the following equation:

$$Z' = 1 - \frac{3 \times (\text{SD of negative controls} + \text{SD of positive controls})}{(\text{mean of negative controls} - \text{mean of positive controls})}$$

164 where SD is the standard deviation. 48 positive controls and 48 negative controls were considered to
165 calculate the Z' factor.

166

167 *2.4.2 IC₅₀ determination of compound **II** based on fluorescent polarization assay*

168 Reactions were performed in a buffer containing 40 mM Tris (pH 8), 5 mM DTT, with 2 mM MnCl₂ for
169 SARS-CoV-2 ExoN and 5 mM for LCMV or MACV ExoN. The 5'-FAM-RNA concentration was fixed at
170 100 nM. The ExoN concentration was fixed at 400 nM. The compound **11** concentration varied from 0.2 to
171 12.5 μM for LCMV ExoN, from 0.4 to 25 μM for MACV ExoN and from 0.5 to 16 μM for SARS-CoV-2
172 ExoN nsp14/nsp10 complex. The reaction started by the addition of 5'-FAM-RNA and the FP signal was
173 recorded as mentioned previously. The percentage of inhibition was calculated as indicated in the previous
174 section. The curves of percentage of inhibition respect to the inhibitor concentration in a logarithmic scale
175 were fitted in Graphpad Prism software using a four parameters equation. The assays were done in triplicate.

176

177 3. Results and discussion

178

179 Fluorescence polarization (FP) is a reliable and sensitive tool for monitoring enzymatic reaction
180 progress, by determining the difference of polarization signals during reaction (Zhang et al., 2012). FP -
181 based assays in HTS studies have been used in drug discovery (Lea and Simeonov, 2011; Uri and Nonga,
182 2020, Baughman et al., 2012; Liu et al., 2014)). The FP signal recorded is proportional to the molecular
183 weight (MW) of a fluorescent molecule (Kwok, 2002; Latif et al., 2001). We decided to apply this method to
184 viral ExoN activity, by monitoring the size of a fluorescent labeled RNA probe which is altered in the course
185 of the nuclease activity, reflecting the enzymatic activity (Liu et al., 2014; Zhang et al., 2012). Because
186 several factors can change the FP- monitoring success, the assay was first optimized.

187

188 *3.1 Determination of optimized experimental conditions of the SARS-CoV-2 nsp14/nsp10 complex, LCMV* 189 *and MACV ExoN activity based on FP assay*

190

191 To set-up a HTS assay for the SARS-CoV-2, LCMV and MACV ExoN activity, we explored 5'-FAM-RNA
192 substrate(s) and enzyme(s) concentration, metal ion co-factors, temperature and reaction duration. RNA
193 substrate and optimal conditions for arenavirus and coronavirus ExoN activity are already described (Bouvet
194 et al., 2012; Saramago et al., 2021; Yekwa et al., 2019, 2017). A 22mer RNA that forms stable hairpin in its
195 3' end has been reported to be a valuable substrate for both arenavirus and coronavirus ExoN. Moreover, for
196 SARS-CoV-2 nsp14 ExoN, nsp10 was added in the reaction (ratio 1:4 of nsp14:nsp10) as nsp10 was

197 previously demonstrated to stimulate > 35-fold the nsp14 ExoN activity (Bouvet et al., 2012; Saramago et
198 al., 2021).

199 We first determined the smallest 5'-FAM-RNA concentration that provides sufficient FP signal,
200 concomitantly with the set-up of the ratio of ExoN and 5'-FAM-RNA to obtain a reproducible and stable FP
201 signal. For each 5'-FAM-RNA concentration tested (50 to 1000 nM), single turn over (STO) conditions
202 (excess of enzyme respect to substrate) and also multiple turn over (MTO) conditions (excess of substrate
203 respect to enzyme) were tested. Because the RNA substrate is degraded during the ExoN activity, FP value is
204 altered proportionally. A significant change in the FP signal due to the hydrolysis of the 5'-FAM-RNA was
205 observed only under STO conditions. 100 nM concentration of 5'-FAM-RNA was selected because it was the
206 smallest concentration of substrate providing a reproducible and stable FP signal.

207 We optimized the positive and negative controls of ExoN activity. Several combinations were tested
208 (Supplementary figure S2): 5'-FAM-RNA in reaction buffer with metal ions, 5'-FAM-RNA in reaction buffer
209 with ExoN and without metal ions, 5'-FAM-RNA in reaction buffer with ions and denaturated ExoN and 5'-
210 FAM-RNA in reaction buffer with CaCl₂ instead of MnCl₂. For all the negative controls tested, there was no
211 variation between the initial and final FP values after 30 min incubation, indicating the absence of
212 degradation of the fluorescent probe. However, we observe that the initial FP values are lower without metal
213 ions in the reaction buffer (Supplementary figure S2), or in the presence of EDTA 10 mM (data not shown).
214 This results is in agreement with the study of Liu *et al* (Liu et al., 2014), who carried out the experiments to
215 investigate the influence of metal ions in FP assays, and reported that cations concentration can affect the FP
216 signal by altering the mobility of the fluorophore through stabilizing the RNA secondary structure. Thus, for
217 the rest of the study, the negative controls were prepared by mixing 5'-FAM-RNA in reaction buffer with
218 ions and without ExoN.

219 Regarding the nature of the metal ion, MnCl₂ and MgCl₂ were tested. An increased activity is observed in the
220 presence of MnCl₂ for both arenaviruses and SARS-CoV-2 ExoN (data not shown). The concentration of
221 MnCl₂ that shows the highest reduction in FP signal is 5 mM for arenaviruses as previously reported (Yekwa
222 et al., 2019, 2017) and 2 mM for SARS-CoV-2 as recently reported (Baddock et al., 2020; Saramago et al.,
223 2021). Finally the temperature selected to perform the assay is 25°C since the incubation at 37°C leads to a
224 significative evaporation of the sample that reduces the stability and reproducibility of the FP signal.

225

226 3.2 Assessing the SARS-CoV-2, LCMV and MACV ExoN activity by FP

227

228 While the 5'-FAM-RNA substrate was fixed at 100 nM, which was the lowest concentration fairly detected
229 by the sensitivity of the method, different concentrations of ExoN were tested (100 nM to 1600 nM). The FP
230 signal change was measured during 30 min. The FP curves and digestion products examined in urea PAGE
231 after FP reading, are gathered in figure 1.

232 The negative controls (figure 1, brown curves, in panels A, B and C) present flattened curves, as expected.
233 Remarkably, early in the reaction a small increase in the initial FP value is observed, which could correspond
234 to the time required for the formation of the catalytic complex between the ExoN, the catalytic cations and
235 the 5'-FAM-RNA substrate. This is more visible for the SARS-CoV-2 ExoN, which requires the interaction
236 of nsp14 and nsp10 forming the active complex. This increase of fluorescence occurs for all FP curves but is
237 visible mainly on the FP curves corresponding to the lowest ExoN concentrations when the formation of the
238 pre-hydrolytic complex presumably takes the longest time.

239 For all tested ExoN, excepting for the lowest used concentration, we observe a reduction of the FP signal
240 with time. The decrease of the FP value is correlated with the hydrolysis of the 5'-FAM-RNA substrate by
241 the ExoN, as confirmed on the urea PAGE images (figure 1, panels D, E and F). The difference between
242 initial and final FP value after 30 min is proportional to the number of nucleotides removed from the 5'-
243 FAM-RNA.

244 By increasing the ExoN concentration, we observe an increase in the FP curves slope but the FP signals
245 reach a plateau which might be related to the inability to remove any extra nucleotide after a certain point.

246 The method efficiency was validated by the difference of FP values between the negative control (figure 1,
247 brown curves, in panels A, B and C) and the FP value obtained at one concentration after 30 min reaction.
248 This difference was selected as significant enough, with $\Delta FP \pm 30$ units of mP.

249 To validate this method and use it as screening assay, we decided to use a molar ratio 1:4 between the 5'-
250 FAM-RNA substrate (100 nM) and ExoN (400 nM). With the optimized duration, temperature and
251 experimental conditions, this ratio gives the more stable and reproducible FP signals, correlated with specific
252 5'-FAM-RNA fragments.

253

254 3.3 Screening of a focused library against the SARS-CoV-2, LCMV and MACV ExoN using FP

255 Because ExoN activity is metal dependent, we tested a focused library of 23 metal chelators that we have
256 developed previously (Saez-Ayala et al., 2019, 2018), in order to demonstrate the robustness of our FP
257 method. Two screenings were performed using 5 μ M and 20 μ M of compounds in 5% DMSO in a single
258 assay. The robustness of the assay for HTS was calculated using 48 negative and 48 positive controls. The
259 lowest Z' value is 0.68 indicating that the assay is reliable (see supplementary data figure S3). The
260 percentages of inhibition at 5 μ M and 20 μ M for each ExoN, extracted from FP curves (see supplementary
261 data figure S4), are gathered in supplementary figure S5 and figure 2 (panels A, B and C), respectively. The
262 degree of digestion of the RNA substrate was also controlled by fragments separation on urea-PAGE at the
263 end of the reaction for compounds **4**, **11**, **13**, **18** and **23** (figure 2, panel D).

264 We identified compound **11** which inhibits 100% of the different ExoN activity at 20 μ M and 78%, 76% and
265 100% at 5 μ M, respectively against LCMV, MACV and SARS-CoV-2 ExoN. Compound **11** which is
266 Aurintricarboxylic Acid (ATA) was included as positive control as it has been previously described as
267 nuclease inhibitor (Huang et al., 2016), acting as non-specific metal chelator (Sharma et al., 2000). We also
268 identified inhibitors showing more specific inhibition profile. Compound **4** is active against SARS-CoV-2
269 with 61% inhibition at 20 μ M (less than 10% inhibition of LCMV ExoN) while compound **23** is specific to
270 LCMV ExoN with 71% inhibition (less than 10% inhibition of MACV ExoN). Compounds **13** and **18** are
271 potent inhibitors of SARS-CoV-2 ExoN with more 90% and weak inhibitors of LCMV and MACV ExoN
272 (less than 20% inhibition). The FP method allowed the identification of potent ExoN inhibitors as
273 compounds **4**, **11**, **13**, **18** and **23**, and the absence of RNA degradation was confirmed by urea PAGE analysis
274 (figure 2 - panel D) used as an orthogonal validation assay.

275

276 *3.4 Assessing IC₅₀ measurement of compound 11 by FP*

277 Compound **11** displaying the highest inhibition, at both 5 and 20 μ M, was used as model for IC₅₀
278 determination. The FP curves, the digestion products examined in urea PAGE after FP reading and dose-
279 response curves obtained by extraction of FP data are gathered in figure 3. The IC₅₀ values were determined
280 by hill plot curve fitting (see materials and methods section). The IC₅₀ values for LCMV, MACV and SARS-
281 CoV-2 ExoN were $3.61 \pm 0.20 \mu$ M, $3.46 \pm 0.29 \mu$ M and $1.64 \pm 0.17 \mu$ M, respectively. Notably, these values

282 correlated perfectly with the 80% inhibition obtained by screening at 5 μ M, confirming the robustness and
283 reproducibility of the assay. The FP signal correlates also with the degree of digestion observed by gel.

284

285

286 4. Conclusion

287 The use of fluorescence polarization assay for inhibitors identification was investigated and we prove that
288 this technique is reliable and sensitive to monitor nuclease activity.

289 Our work presents the development of a viral ExoN HTS assay in 384-well plates. Its most valuable feature
290 is that allows a reliable and rapid identification of ExoN inhibitors limiting the ExoN activity of viral
291 enzymes belonging to arenavirus (LCMV and MACV) and coronavirus (SARS-CoV-2).

292 These results make fluorescence polarization assay an important tool in the screening of compounds libraries
293 to discover antivirals.

294

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304

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306

307 References

308 Baddock, H.T., Brolih, S., Yosaatmadja, Y., Ratnaweera, M., Bielinski, M., Swift, L.P., Cruz-Migoni, A.,
309 Morris, G.M., Schofield, C.J., Gileadi, O., McHugh, P.J., 2020. Characterisation of the SARS-CoV-
310 2 ExoN (nsp14ExoN-nsp10) complex: implications for its role in viral genome stability and inhibitor
311 identification. bioRxiv 2020.08.13.248211. <https://doi.org/10.1101/2020.08.13.248211>

- 312 Baughman, B.M., Jake Slavish, P., DuBois, R.M., Boyd, V.A., White, S.W., Webb, T.R., 2012.
313 Identification of influenza endonuclease inhibitors using a novel fluorescence polarization assay.
314 ACS Chem. Biol. 7, 526–534. <https://doi.org/10.1021/cb200439z>
- 315 Becares, M., Pascual-Iglesias, A., Nogales, A., Sola, I., Enjuanes, L., Zuñiga, S., 2016. Mutagenesis of
316 Coronavirus nsp14 Reveals Its Potential Role in Modulation of the Innate Immune Response. J.
317 Virol. 90, 5399–5414. <https://doi.org/10.1128/JVI.03259-15>
- 318 Bouvet, M., Imbert, I., Subissi, L., Gluais, L., Canard, B., Decroly, E., 2012. RNA 3'-end mismatch excision
319 by the severe acute respiratory syndrome coronavirus nonstructural protein nsp10/nsp14
320 exoribonuclease complex. Proc. Natl. Acad. Sci. 109, 9372–9377.
321 <https://doi.org/10.1073/pnas.1201130109>
- 322 Ferron, F., Subissi, L., Silveira De Morais, A.T., Le, N.T.T., Sevajol, M., Gluais, L., Decroly, E., Vonnrhein,
323 C., Bricogne, G., Canard, B., Imbert, I., 2018. Structural and molecular basis of mismatch correction
324 and ribavirin excision from coronavirus RNA. Proc. Natl. Acad. Sci. U. S. A. 115, E162–E171.
325 <https://doi.org/10.1073/pnas.1718806115>
- 326 Ferron, F., Weber, F., de la Torre, J.C., Reguera, J., 2017. Transcription and replication mechanisms of
327 Bunyaviridae and Arenaviridae L proteins. Virus Res. 234, 118–134.
328 <https://doi.org/10.1016/j.virusres.2017.01.018>
- 329 Hastie, K.M., Kimberlin, C.R., Zandonatti, M.A., MacRae, I.J., Saphire, E.O., 2011. Structure of the Lassa
330 virus nucleoprotein reveals a dsRNA-specific 3' to 5' exonuclease activity essential for immune
331 suppression. Proc. Natl. Acad. Sci. U. S. A. 108, 2396–2401.
332 <https://doi.org/10.1073/pnas.1016404108>
- 333 Knipe, D.M., Howley, P.M. (Eds.), 2013. Fields virology, 6th ed. ed. Wolters Kluwer/Lippincott Williams &
334 Wilkins Health, Philadelphia, PA.
- 335 Huang, K.-W., Hsu K.-C., Chu, L.-Y., Yang J.-M., Yuan H. S., Hsiao Y.-Y. 2016. Identification of Inhibitors
336 for the DEDDh Family of Exonucleases and a Unique Inhibition Mechanism by Crystal Structure
337 Analysis of CRN-4 Bound with 2-Morpholin-4-ylethanesulfonate (MES). J. Med. Chem. 59, 8019-
338 8029. <https://doi.org/10.1021/acs.jmedchem.6b00794>
- 339 Kwok, P.-Y., 2002. SNP genotyping with fluorescence polarization detection. Hum. Mutat. 19, 315–323.
340 <https://doi.org/10.1002/humu.10058>
- 341 Latif, S., Bauer-Sardina, I., Ranade, K., Livak, K.J., Kwok, P.Y., 2001. Fluorescence polarization in
342 homogeneous nucleic acid analysis II: 5'-nuclease assay. Genome Res. 11, 436–440.
343 <https://doi.org/10.1101/gr.156601>
- 344 Lea, W.A., Simeonov, A., 2011. Fluorescence polarization assays in small molecule screening. Expert Opin.
345 Drug Discov. 6, 17–32. <https://doi.org/10.1517/17460441.2011.537322>
- 346 Lei, X., Dong, X., Ma, R., Wang, W., Xiao, X., Tian, Z., Wang, C., Wang, Y., Li, L., Ren, L., Guo, F., Zhao,
347 Z., Zhou, Z., Xiang, Z., Wang, J., 2020. Activation and evasion of type I interferon responses by
348 SARS-CoV-2. Nat. Commun. 11, 3810. <https://doi.org/10.1038/s41467-020-17665-9>
- 349 Liu, X., Chen, Y., Fierke, C.A., 2014. A real-time fluorescence polarization activity assay to screen for
350 inhibitors of bacterial ribonuclease P. Nucleic Acids Res. 42, e159.
351 <https://doi.org/10.1093/nar/gku850>
- 352 Martínez-Sobrido, L., Emonet, S., Giannakas, P., Cubitt, B., García-Sastre, A., de la Torre, J.C., 2009.
353 Identification of Amino Acid Residues Critical for the Anti-Interferon Activity of the Nucleoprotein
354 of the Prototypic Arenavirus Lymphocytic Choriomeningitis Virus. J. Virol. 83, 11330–11340.
355 <https://doi.org/10.1128/JVI.00763-09>
- 356 Martínez-Sobrido, L., Giannakas, P., Cubitt, B., García-Sastre, A., Torre, J.C. de la, 2007. Differential
357 Inhibition of Type I Interferon Induction by Arenavirus Nucleoproteins. J. Virol. 81, 12696–12703.
358 <https://doi.org/10.1128/JVI.00882-07>
- 359 Martínez-Sobrido, L., Zúñiga, E.I., Rosario, D., García-Sastre, A., de la Torre, J.C., 2006. Inhibition of the
360 Type I Interferon Response by the Nucleoprotein of the Prototypic Arenavirus Lymphocytic
361 Choriomeningitis Virus. J. Virol. 80, 9192–9199. <https://doi.org/10.1128/JVI.00555-06>
- 362 Ogando, N.S., Ferron, F., Decroly, E., Canard, B., Posthuma, C.C., Snijder, E.J., 2019. The Curious Case of
363 the Nidovirus Exoribonuclease: Its Role in RNA Synthesis and Replication Fidelity. Front.
364 Microbiol. 10, 1813. <https://doi.org/10.3389/fmicb.2019.01813>
- 365 Papageorgiou, N., Spiliopoulou, M., Nguyen, T.-H.V., Vaitsoyopoulou, A., Laban, E.Y., Alvarez, K.,
366 Margiolaki, I., Canard, B., Ferron, F., 2020. Brothers in Arms: Structure, Assembly and Function of
367 Arenaviridae Nucleoprotein. Viruses 12. <https://doi.org/10.3390/v12070772>

- 368 Saez-Ayala, M., Laban Yekwa, E., Mondielli, C., Roux, L., Hernández, S., Bailly, F., Cotelle, P., Rogolino,
369 D., Canard, B., Ferron, F., Alvarez, K., 2019. Metal chelators for the inhibition of the lymphocytic
370 choriomeningitis virus endonuclease domain. *Antiviral Res.* 162, 79–89.
371 <https://doi.org/10.1016/j.antiviral.2018.12.008>
- 372 Saez-Ayala, M., Yekwa, E.L., Carcelli, M., Canard, B., Alvarez, K., Ferron, F., 2018. Crystal structures of
373 Lymphocytic choriomeningitis virus endonuclease domain complexed with diketo-acid ligands.
374 *IUCrJ* 5, 223–235. <https://doi.org/10.1107/S2052252518001021>
- 375 Saramago, M., Bárria, C., Costa, V., Souza, C.S., Viegas, S.C., Domingues, S., Lousa, D., Soares, C.M.,
376 Arraiano, C.M., Matos, R.G., 2021. New targets for drug design: Importance of nsp14/nsp10
377 complex formation for the 3'-5' exoribonucleolytic activity on SARS-CoV-2. *bioRxiv*
378 2021.01.07.425745. <https://doi.org/10.1101/2021.01.07.425745>
- 379 Sharma, R.K., Garg, B.S., Kurosaki, H., Goto, M., Otsuka, M., Yamamoto, T., Inoue, J., 2000. Aurine
380 tricarboxylic acid, a potent metal-chelating inhibitor of NFkappaB-DNA binding. *Bioorg. Med.*
381 *Chem.* 8, 1819–1823. [https://doi.org/10.1016/s0968-0896\(00\)00109-7](https://doi.org/10.1016/s0968-0896(00)00109-7)
- 382 Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L.M., Guan, Y., Rozanov, M.,
383 Spaan, W.J.M., Gorbalenya, A.E., 2003. Unique and Conserved Features of Genome and Proteome
384 of SARS-coronavirus, an Early Split-off From the Coronavirus Group 2 Lineage. *J. Mol. Biol.* 331,
385 991–1004. [https://doi.org/10.1016/S0022-2836\(03\)00865-9](https://doi.org/10.1016/S0022-2836(03)00865-9)
- 386 Steitz, T.A., Steitz, J.A., 1993. A general two-metal-ion mechanism for catalytic RNA. *Proc. Natl. Acad. Sci.*
387 *U. S. A.* 90, 6498–6502. <https://doi.org/10.1073/pnas.90.14.6498>
- 388 Subissi, L., Imbert, I., Ferron, F., Collet, A., Coutard, B., Decroly, E., Canard, B., 2014. SARS-CoV ORF1b-
389 encoded nonstructural proteins 12-16: replicative enzymes as antiviral targets. *Antiviral Res.* 101,
390 122–130. <https://doi.org/10.1016/j.antiviral.2013.11.006>
- 391 Uri, A., Nonga, O.E., 2020. What is the current value of fluorescence polarization assays in small molecule
392 screening? *Expert Opin. Drug Discov.* 15, 131–133. <https://doi.org/10.1080/17460441.2020.1702966>
- 393 Yekwa, E., Aphibanthammakit, C., Carnec, X., Picard, C., Canard, B., Baize, S., Ferron, F., 2019.
394 Arenaviridae exoribonuclease presents genomic RNA edition capacity. *bioRxiv* 541698.
395 <https://doi.org/10.1101/541698>
- 396 Yekwa, E., Khourieh, J., Canard, B., Papageorgiou, N., Ferron, F., 2017. Activity inhibition and crystal
397 polymorphism induced by active-site metal swapping. *Acta Crystallogr. Sect. Struct. Biol.* 73, 641–
398 649. <https://doi.org/10.1107/S205979831700866X>
- 399 Zhang, M., Le, H.-N., Wang, P., Ye, B.-C., 2012. A versatile molecular beacon-like probe for multiplexed
400 detection based on fluorescence polarization and its application for a resettable logic gate. *Chem.*
401 *Commun. Camb. Engl.* 48, 10004–10006. <https://doi.org/10.1039/c2cc35185d>
- 402 Zuo, Y., Deutscher, M.P., 2001. Exoribonuclease superfamilies: structural analysis and phylogenetic
403 distribution. *Nucleic Acids Res.* 29, 1017–1026.
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411 Legend to figures

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413 Figure 1. ExoN activity measured by FP for LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel
414 C). The FP signal variation is recorded with time during 30 min, each 30 seconds, at 25°C. The 5'-FAM-
415 RNA substrate concentration used is 100 nM and the ExoN concentration tested ranges 100 nM to 1600 nM.
416 The data represents the average and SEM of three independent experiments. The bottom panels illustrate a
417 representative image of the digestion products analyzed in urea PAGE, of the ExoN activity for LCMV
418 (panel D), MACV (panel E) and SARS-CoV-2 (panel F), after recording the FP signal. An aliquot of the
419 sample was loaded into a urea-PAGE 20% and scanned in a fluorescence imager.

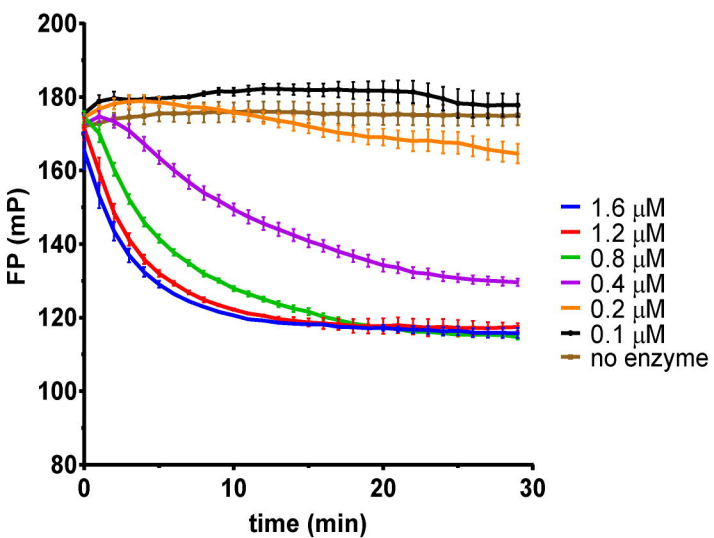
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421 Figure 2. Screening of focused library of metal chelators (23) against LCMV (panel A), MACV (panel B)
422 and SARS-CoV-2 (panel C) ExoN followed by FP. Orthogonal validation assay by analysis of the
423 fluorescent RNA substrate by urea PAGE (panel D) The bars show the % of inhibition of the ExoN activity
424 as described in materials and methods. For the screening conditions 100 nM 5'-FAM-RNA, 400 nM ExoN
425 and 20 μ M of inhibitor were used.

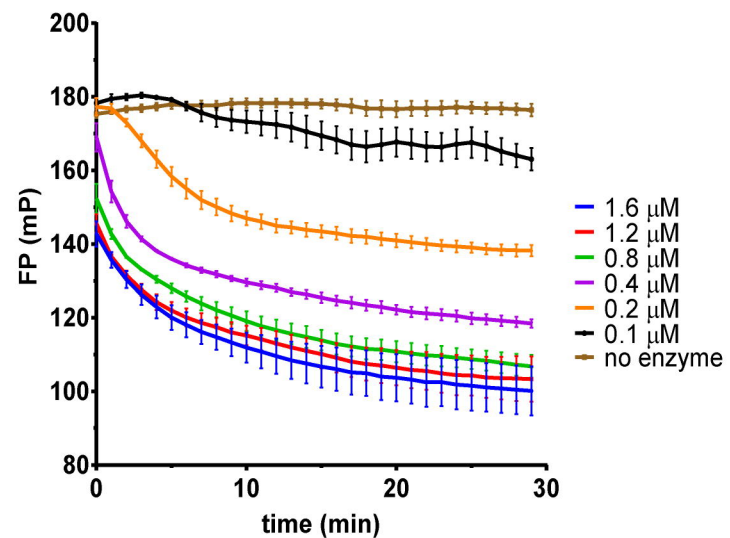
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427 Figure 3. IC₅₀ measurement of compound **11** by FP on LCMV, MACV and SARS-CoV-2 ExoN. In A, B and
428 C the FP signal variation is recorded with time during 30 min, each 30 seconds, at 25°C. The 5'-FAM-RNA
429 substrate and LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel C) ExoN concentration were
430 respectively 100 nM and 400 nM. The data represents the average and SEM of three independent
431 experiments. In the middle panels it is shown a representative image of the digestion products of the LCMV
432 (panel D), MACV (panel E) and SARS-CoV-2 (panel F) ExoN in presence of the different concentrations of
433 compound **11**, visualized in urea AGE after recording the FP signal. In the bottom panels it is shown the
434 dose-response curves obtained by extraction of FP data, the IC₅₀ values for compound **11** with LCMV (panel
435 G), MACV (panel H) and SARS-CoV-2 (panel I). The data represents the average and SEM of three
436 independent experiments.

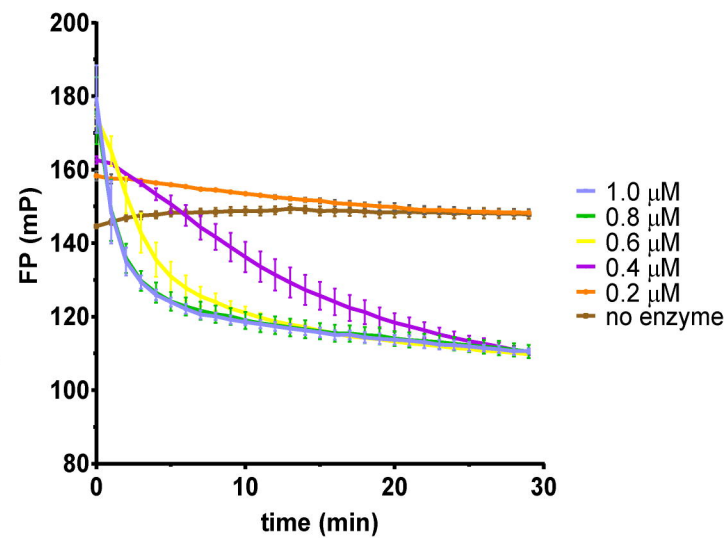
A)



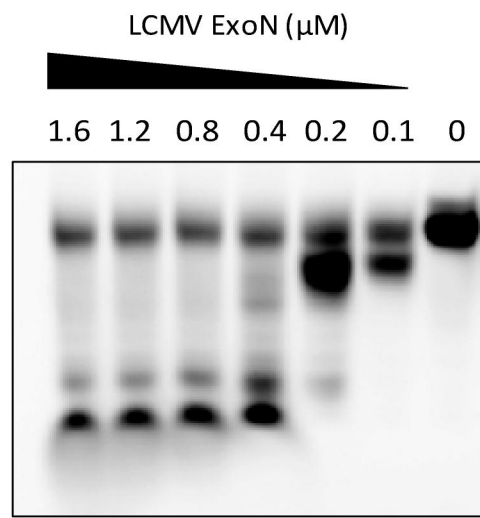
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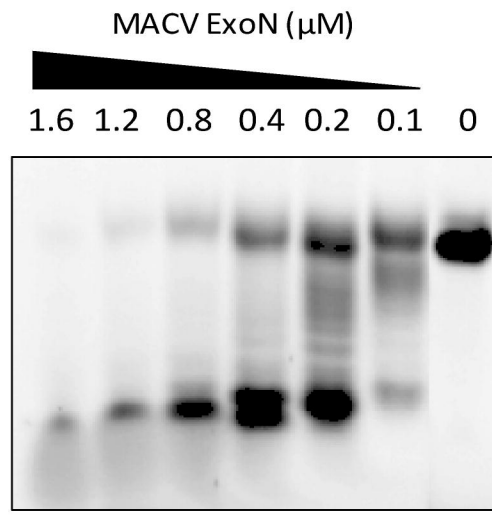
C)



D)



E)



F)

