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# 1 Can Plitidepsin Be Used as an Antiviral Against RSV?

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8

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14

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16

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18

19

## 20 **Abstract**

21 Human respiratory syncytial virus (HRSV) is a main cause of acute lower respiratory tract  
22 infections in infants, the elderly and the immunocompromised patients. Although vaccines  
23 have recently been approved for the elderly and for pregnant women, there is no curative

24 treatment for HRSV. HRSV replicates in the cytoplasm of infected cells, and transcription and  
25 replication of the viral genome depend on the viral RNA polymerase complex, which recruits  
26 cellular factors for RNA synthesis. Among them, the eucaryotic translation elongation factor  
27 1A (eEF1A) was previously shown to be critical for HRSV replication. eEF1A activity can be  
28 inhibited by plitidepsin (Aplidin), a cyclopeptide extracted from ascidian *Aplidium albicans*,  
29 which was shown highly potent against SARS-CoV-2, with a 50% inhibitory concentration  
30 (IC<sub>90</sub>) of 0.70 to 1.62 nM depending on the cell line. Here, we investigated whether  
31 plitidepsin could also inhibit HRSV replication. We found that plitidepsin inhibited HRSV  
32 replication with an IC<sub>50</sub> of ≈3 nM in cell cultures. However, further investigation revealed  
33 that plitidepsin has pleiotropic effects, affecting the translation of both cellular and viral  
34 proteins in a similar manner. Overall, our results show that plitidepsin blocks cellular  
35 translation and indicate that plitidepsin induces a proteasome-mediated degradation of  
36 eEF1A, also showing the dependence of HRSV replication to cellular factors such as eEF1A.  
37 These results thus highlight an original mechanism of action of plitidepsin on eEF1A, which  
38 render the use of this compound for antiviral therapy very risky.

39

## 40 **Introduction**

41 Human respiratory syncytial virus (HRSV) is the commonest cause of lower respiratory tract  
42 infection in young children worldwide and the first cause of their hospitalization (1). HRSV is  
43 estimated to infect about 33 million children leading to more than 3 million hospitalizations,  
44 and 26,000–152,000 deaths in children under 5 years, each year (2). The global healthcare  
45 costs of HRSV-associated infections in young children in 2017 were estimated to be US\$5.45  
46 billion (3). In a systemic multisite study, HRSV was shown to be the first etiological agent

47 responsible for severe pneumonia (more than 30%) in hospitalized children in Asia and Africa  
48 (4). HRSV infections are also associated with significant morbidity and mortality in the elderly  
49 and immunocompromised people (5),(6). The true burden of disease in adults is likely  
50 significantly under-recognized, and recent studies indicate that the HRSV impact is similar to  
51 that of seasonal influenza in adults older than 65 years (7),(8),(9),(10),(11),(12).

52 HRSV vaccine research has been ongoing for nearly 60 years without success, and  
53 only in 2023, two vaccines based on stabilized HRSV prefusion F protein, Arexvy (GSK) and  
54 Abrysvo (Pfizer), were approved for medical use for adults aged 60 or older both in the USA  
55 and Europe. In September 2023, Abrysvo was also approved in the USA for pregnant women  
56 at 32-36 weeks' gestation to prevent HRSV infection in newborn children. Finally, Moderna  
57 received U.S. FDA and E.U. approval for RSV Vaccine mRESVIA (13). The only pharmaceutical  
58 intervention since 1998 has been passive prophylaxis with Palivizumab, a monoclonal  
59 antibody targeting the fusion protein thereby limiting the HRSV entry. However, its use was  
60 limited to high-risk infants because of the elevated cost and moderate efficacy (14). But  
61 recently, nirsevimab (Beyfortus®), a long-acting monoclonal antibody, was approved by  
62 several regulatory agencies around the world for the prevention of HRSV infections in  
63 newborns and infants (15). A single intramuscular injection of this antibody should protect  
64 infants for an entire season compared with monthly doses, and reduced costs (vaccine-like  
65 pricing expected) allowing for administration to all infants. However, there is still no specific  
66 curative treatment against HRSV.

67 HRSV is a nonsegmented single-stranded negative-sense RNA virus of the  
68 *Mononegavirales* order, *Pneumoviridae* family, and *Orthopneumovirus* genus (16). The HRSV  
69 genome is approximately 15.2 kb long, and contains 10 genes encoding 11 proteins (17).  
70 Replication and transcription rely on four of these proteins: the nucleoprotein N involved in

71 genome and antigenome encapsidation, forming the ribonucleoprotein complex (NC), the  
72 RNA-dependent RNA polymerase L which exhibits all the enzymatic activities required for  
73 viral replication, transcription and RNA capping, its cofactor the phosphoprotein P  
74 responsible for recruitment of L on the NC template, and the transcription factor M2-1 that  
75 has been described as an “antiterminating” factor during transcription and interacts with P  
76 and viral mRNAs (18). All viral RNA synthesis take place in viral factories also called  
77 cytoplasmic inclusion bodies (IBs) (19), which concentrate the viral proteins L, N, P, and M2-  
78 1, but also cellular proteins, such as the protein phosphatase 1 (PP1), HSP70 or the human  
79 translation elongation factor eEF1A, all involved in HRVS replication (20),(21),(22).  
80 Interestingly, it was found that many RNA viruses utilize eEF1A for replication, although the  
81 mechanisms by which they do this differ (23). In a previous study in which eEF1A was  
82 knocked down or inhibited with didemnin B, it was suggested that eEF1A plays a key role in  
83 the regulation of F-actin stress fiber formation required for HRSV assembly and release, but  
84 with no effect on HRSV genome replication (24). Furthermore, eEF1A was found associated  
85 with the N and P proteins in infected cells by using a proximity ligation assay and co-  
86 immunoprecipitation (22). Recently, plitidepsin (Aplidin), an analog of didemnin B and a  
87 potent anti-cancer agent targeting eEF1A2 ( $K_D=80\text{ nM}$ ) (25), was shown highly potent  
88 against SARS-Cov-2 by targeting eEF1A, with a 90% inhibitory concentration ( $IC_{90}$ ) of 0.88  
89 nM (26). As suggested by the authors, since HRSV also uses eEF1A for viral replication,  
90 inhibition of eEF1A could be a new strategy to limit HRSV propagation/infection. In this  
91 work, we thus investigated the effect of plitidepsin on HRSV replication. We show that  
92 plitidepsin inhibits HRSV replication in infected cells as well as a minigenome reporter  
93 system with an  $IC_{50}$  of  $\approx 3$  nM in cultured cells. Further mechanistic investigation revealed

94 that plitidepsin induces the degradation of eEF1A, inhibiting the translation of both viral and  
95 cellular proteins in a similar range of concentration.

96

## 97 **Methods**

### 98 **Cells**

99 BSRT7/5 cells (BHK-21 cells that constitutively express the T7 RNA polymerase) (27) and HEp-  
100 2 cells (ATCC: CCL-23) were maintained respectively in DMEM and MEM supplemented with  
101 10% heat-inactivated fetal calf serum (FCS), with 2 mM glutamine, 100 µg/ml penicillin and  
102 100 U/ml streptomycin. Cells were grown in an incubator at 37 °C in 5% CO<sub>2</sub>. Transfection  
103 were performed with 2.5 µL of Lipofectamine 2000 (Thermofisher) per 1 µg of DNA according  
104 to the manufacturer instructions.

105

### 106 **Viruses**

107 Recombinant human RSV rHRSV-mCherry corresponding to HRSV Long strain expressing the  
108 mCherry protein was amplified on HEp-2 cells and titrated using a plaque assay procedure as  
109 previously described (28),(29).

110

### 111 **Plitidepsin antiviral activity**

112 Plitidepsin (MedChemExpress) and Carfilzomib (Cell Signaling #15022) were solubilized in  
113 DMSO at 1 mM and 5 mM as stock solutions, respectively. HEp-2 cells in 96-well plates were  
114 infected for two hours with rHRSV-mCherry at an MOI of 0.2, in the absence of FCS. The  
115 medium was then changed by the same medium with 2% FCS and containing serial dilution  
116 of plitidepsin, with a 1% final concentration of DMSO in the culture medium. At 48 h  
117 postinfection, the red fluorescence intensity of mCherry was quantified using a Tecan infinite

118 M200Pro spectrofluorometer with excitation and emission wavelengths of 580 nm and 620  
119 nm, respectively. Values obtained for nontreated infected and noninfected cells were used  
120 for standardization and normalization. In parallel, the toxicity of the treatment was assessed  
121 on non-infected cells using the CellTiter-Glo Luminescent cell viability assay (Promega). The  
122 half-maximal inhibitory concentration ( $IC_{50}$ ) and cytotoxic concentration ( $CC_{50}$ ) were  
123 determined by fitting the data to the dose-response curve implemented in GraphPad version  
124 8 software.

125

### 126 **Fluorescence microscopy**

127 HEp-2 cells infected with rHRSV-mCherry were fixed with PBS-paraformaldehyde 4% for 20  
128 min at room temperature, rinsed with PBS, and permeabilized with PBS-BSA 1% - Triton X-  
129 100 0.1% for 10 min. Nuclei were stained with Hoechst 33342 (1  $\mu$ g/ml) for 5 min, washed  
130 with PBS, examined under a Nikon TE200 microscope equipped with a CoolSNAP ES2  
131 (Photometrics) camera, and images were processed with Meta-View software (Molecular  
132 Devices).

133

### 134 **Minigenome assay**

135 BSRT7/5 cells at 90% confluence in 96-well dishes were transfected with a plasmid mixture  
136 containing 125 ng of pM/Luc, 125 ng of pN, 125 ng of pP, 62.5 ng of pL, and 31 ng of pM2-1  
137 as well as 31 ng of pRSV- $\beta$ -Gal (Promega) to normalize transfection efficiencies as described  
138 previously (30),(31). After 6 h, the transfection mix was removed and serial dilutions of  
139 plitidepsin were added for 14 h. Transfections were done in triplicate, and each independent  
140 transfection was performed three times. Cells were harvested 24 h post-transfection, then  
141 lysed in luciferase lysis buffer (30 mM Tris pH 7.9, 10 mM  $MgCl_2$ , 1 mM DTT, 1% Triton X-100,

142 and 15% glycerol). The luciferase and  $\beta$ -galactosidase ( $\beta$ -Gal) activities were determined for  
143 each cell lysate with an Infinite 200 Pro (Tecan, Männedorf, Switzerland) and normalized  
144 based on the values obtained for cells treated with DMSO only.

145

#### 146 **Fluorescence-based nucleotide-incorporation assay**

147 Each reaction contained 0.2  $\mu$ M recombinant RSV L-P, 2  $\mu$ M of oligonucleotide template and  
148 2  $\mu$ M of a 5'-6-FAM primer, mixed in a buffer containing 20 mM Tris-HCl pH 7.5, 10 mM KCl,  
149 2 mM DTT, 0.01% Triton X-100, 5% DMSO, 0.2 U/mL RNasin (Ambion), and 6 mM MgCl<sub>2</sub>.  
150 Reactions were started by adding specific NTPs at 100  $\mu$ M and incubated for 2h at 30°C.  
151 Reactions were quenched by adding an equal volume of gel-loading buffer. Samples were  
152 denatured at 70°C for 10 minutes and run on a 20% polyacrylamide urea sequencing gel for  
153 2.5 hours at 45 W. The gel scanned on a Typhoon imager (GE Healthcare). (Unpublished  
154 methodology Sutto et al. 2024)

155

#### 156 **Western blotting**

157 Cells were lysed in 2X Laemmli buffer, run on a 12% polyacrylamide gel and transferred to a  
158 nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad). The blots were  
159 blocked with 5% nonfat milk in PBS Tween20 0.2%, and probed with either a polyclonal  
160 rabbit anti-N serum (32), a rabbit anti-GFP (Invitrogen, Waltham, MA, USA), a mouse  
161 monoclonal anti-EF1A antibody (Santa Cruz G-8, sc377439), a mouse monoclonal anti-  
162 GAPDH antibody (Sigma-Aldrich MAB374) or a mouse anti-alpha-tubulin (DM1A, Sigma) and  
163 further incubated with HRP-coupled anti-mouse or anti-rabbit antibodies (Life Science).  
164 Membranes were revealed with the Clarity Western ECL kit (Bio-Rad) and analyzed with a  
165 ChemiDoc Touch Imaging System and Image Lab software (Bio-Rad).

166

167 **Pulse labelling of newly synthesized proteins with L-azidohomoalanine (AHA)**

168 HEp-2 cells were infected with rHRSV-mCherry at MOI = 1 in 6-wells plates (35 mm in  
169 diameter) and incubated in MEM supplemented with 2% heat-inactivated FCS, 2 mM  
170 glutamine, 100 µg/ml penicillin and 100 U/ml streptomycin. 24 h later, serial dilutions of  
171 plitidepsin or DMSO were added to the cells for 2 h. Cells were washed twice with  
172 methionine-free (Met-) MEM (Gibco), then live-labeled (pulsed) at 37°C for 4 h with 50 µM  
173 Click-IT L-azidohomoalanine (AHA; Jena Bioscience) in Met-MEM supplemented with 5–10%  
174 dialyzed FBS (Gibco) and plitidepsin. Cells were lysed in 1 ml of in RIPA buffer (50 mM Tris-  
175 HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 2 mM, DTT, 10 mM of freshly prepared  
176 iodoacetamide [Merck] and complete protease inhibitor cocktail [Roche]). After  
177 centrifugation for 10 min at 10,000 rpm at 4°C, the supernatants were mixed with 5 µM  
178 AFDye 488-DBCO (Click Chemistry Tools, Jena Bioscience) and incubated for 2 h at 21°C.  
179 Proteins were immunoprecipitated with specific antibodies coupled to protein A sepharose  
180 beads (GE Healthcare) and analyzed by SDS-PAGE, together with the supernatants.  
181 Fluorescence was analyzed with a Fuji FLA-3000 scanner and quantified using the AIDA or Fiji  
182 softwares.

183

184 **RESULTS**

185 **Antiviral potency of plitidepsin against HRSV**

186 By using an siRNA approach, it was demonstrated ten years ago that downregulation of  
187 eEF1A correlates with a reduction in the amount of infectious HRSV release, accompanied by  
188 a reduction in viral genome expression, but not mRNA transcription or protein expression

189 (22). We therefore tested whether plitidepsin, known to inhibit the eEF1A activity, displays  
190 antiviral activity against HRSV. For this purpose, HEp-2 cells were infected with rHRSV-  
191 mCherry at MOI = 0.2 in a culture medium containing increasing concentration of plitidepsin.  
192 Viral replication was quantified by mCherry fluorescence measurement on live cells 48 h  
193 post infection using the Tecan spectrofluorometer. Values were standardized and  
194 normalized by those of obtained for nontreated infected and noninfected cells. The IC<sub>50</sub> was  
195 determined by dose-response curve fitting. In parallel, the toxicity of plitidepsin was  
196 evaluated on non-infected HEp-2 cells. As shown in [Figure 1A](#), plitidepsin inhibited rHRSV-  
197 mCherry replication in a dose-dependent manner, with an IC<sub>50</sub> of 3.5 nM. These data were  
198 confirmed by direct observation of mCherry fluorescence and nuclei staining on fixed rHRSV-  
199 mCherry-infected cells ([Figure 1B](#)). Noteworthy, a cytotoxic activity of plitidepsin was  
200 observed, with a calculated half-maximal cytotoxic concentration (CC<sub>50</sub>) close to 145 nM  
201 ([Figure 1A](#)). With a calculated selective index >30, plitidepsin thus appeared to be a potent  
202 inhibitor of HRSV replication in HEp-2 cells.

203

#### 204 **Effect of plitidepsin on a HRSV minigenome**

205 To determine whether plitidepsin could affect the RNA polymerase replication/transcription  
206 machinery of HRSV, we used a well-established HRSV-specific minigenome assay system  
207 (30). The pM/Luc plasmid, which contains the authentic M/SH gene junction and the Luc  
208 reporter gene downstream of the gene start sequence inserted in this gene junction, was co-  
209 transfected in BSR-T7/5 cells together with p-β-gal, pL, pP, PN, and pM2-1. Luciferase activity  
210 was determined and normalized based on the signal obtained for cells incubated in the  
211 similar media with 1% of DMSO. In parallel, β-galactosidase (β-Gal) activity was determined  
212 for each cell lysate in order to normalize transfection efficiency. [Figure 2A](#) shows that

213 plitidepsin inhibits the function of the HRSV polymerase complex in a dose-dependent  
214 manner with an  $IC_{50}$  of  $\sim 5$  nM while showing no toxicity towards BSR-T7/5 cells. However,  
215 plitidepsin also inhibited, to a lesser extent, the  $\beta$ -Gal activity, the effect being visible from  
216 3.3 nM of plitidepsin.

217         Since plitidepsin targets the eEF1A cellular translation factor, the inhibition of HRSV  
218 RNA polymerase activity could result from either the direct inhibition of polymerase activity  
219 of the HRSV L protein or from the inhibition of translation of the viral mRNAs coding for the  
220 viral proteins. To assess whether the plitidepsin treatment had a specific or global impact on  
221 proteins expression, cells were co-transfected either with the minigenome system or with a  
222 plasmid encoding EGFP, in the presence of increasing concentrations of plitidepsin. The  
223 expression of proteins was analyzed by Western blot. As shown on [Figure 2B](#), a similar  
224 decrease in N and EGFP expression was observed in the presence of plitidepsin, indicating  
225 that plitidepsin had a general inhibitory effect on viral or non-viral proteins expression. In  
226 contrast, the signal corresponding to alpha-tubulin did not decrease significantly, which can  
227 easily be explained by the half-life of this cellular protein ( $\sim 8$  days) (33).

228

### 229 **Effect of plitidepsin on *in vitro* HRSV RdRp activity**

230 To test whether plitidepsin could also affect directly the HRSV RNA polymerase activity, we  
231 performed an *in vitro* RNA polymerase assay using a recombinant L-P complex (34),(35),(36)  
232 in the presence of increasing plitidepsin concentrations. As shown on [Figure 3](#), no inhibitory  
233 effect of plitidepsin on the RNA synthesis was observed, even for a concentration of 100  $\mu$ M  
234 of plitidepsin. These results strongly suggest that the effect of plitidepsin on viral replication  
235 was not due to a direct effect of the RdRp complex but mainly due to its impact on protein  
236 expression.

237

### 238 **Effect of plitidepsin on cellular and viral proteins neosynthesis**

239 To further investigate the impact of plitidepsin on viral and cellular RNA translation, we used  
240 a complementary approach, consisting in a pulse labelling of newly synthesized proteins with  
241 the methionine analog L-azidohomoalanine (AHA) revealed by the fluorescent dye AFDye  
242 488-DBCO based on Click chemistry (37). At 24 h postinfection, HEp-2 cells were incubated  
243 with serial dilutions of plitidepsin and then labelled with AHA for 1 h. Then, cells were lysed  
244 and the HRSV N protein or the cellular  $\alpha$ -tubulin protein were immunoprecipitated.  
245 Immunoprecipitated proteins and total cell lysates were analyzed by SDS-PAGE and the  
246 fluorescence was quantified. As shown on [Figure 4A and 4B](#), a similar drop in protein  
247 synthesis was observed for N and  $\alpha$ -tubulin, but also for the total proteins present in the cell  
248 lysates for concentrations  $\geq 15$  nM.

249

### 250 **Plitidepsin induces proteasome-mediated degradation of eEF1A**

251 The mechanism by which plitidepsin inhibits the activity of the host factor eEF1A is still  
252 debated. It was shown that overexpression of a negative dominant mutant an Ala399  $\rightarrow$  Val  
253 (A399V) of eEF1A reduced sensitivity of cancer cells to didemnin B (38) as well as SARS-CoV-  
254 2 to plitidepsin by a factor  $>10$  (26). Interestingly, this A399V substitution also confers  
255 resistance to the structurally unrelated ternatin-4 (38), suggesting that these molecules  
256 interact with the same binding site on eEF1A (26). However, the mechanism of action of  
257 ternatin-4 was recently studied in more details and revealed that it was mediated by  
258 ubiquitination and proteasome degradation of eEF1A (39). We thus wondered whether  
259 plitidepsin could also induce degradation of eEF1A by a similar mechanism. BSRT7 cells were  
260 treated with serial dilutions of plitidepsin in the presence or absence of the proteasome

261 inhibitor carfilzomib and the expression of eEF1A or the control GAPDH were analyzed by  
262 Western blot. As shown on [Figure 5](#), at the RSV inhibitory concentration of plitidepsin,  
263 minigenome activity or translation inhibition, eEF1A expression was impaired. However,  
264 expression of eEF1A was restored upon treatment in the presence of the proteasome  
265 inhibitor Carfilzomib. These results thus strongly suggest that plitidepsin induces the  
266 degradation of eEF1A by ubiquitination and proteasome degradation in a similar way to  
267 ternatin-4.

268

## 269 **DISCUSSION**

270 The mammalian translation elongation factor eEF1A is an essential GTPase and the second  
271 most abundant intra-cellular protein after actin (3% of the total cellular protein). It is  
272 localized extensively in the cytoplasm and nucleus (40),(41),(23). The canonical function of  
273 eEF1A is to deliver amino acyl tRNAs to the ribosomal A site during the elongation stage of  
274 protein synthesis. In addition to its canonical functions in transporting aa-tRNA to the  
275 ribosome, eEF1A is found to be involved in cellular mechanisms such as regulation of  
276 cytoskeleton organization by interacting with actin and tubulin, protein degradation  
277 mediated by the proteasome, nuclear aa-tRNAs and protein export, signaling transduction  
278 pathway concerning apoptosis and oncogenesis, or binding to viral RNA (42),(41),(43). There  
279 are two variants of eEF1A, eEF1A1 and eEF1A2, that share 92% amino acid identity (44). In  
280 contrast to the ubiquitous expression of eEF1A1 in many cell types, eEF1A2 expression is  
281 limited to the terminally differentiated cells of the brain, heart, and skeletal muscle (45). The  
282 two eEF1A variants have similar translation activity but may differ with respect to their  
283 secondary, “moonlighting” functions. eEF1A1 also plays an important role in the process of

284 heat shock stress response. eEF1A2 activates Akt in a PI3K-dependent fashion, stimulating  
285 cell migration, actin remodeling and invasion, and inhibiting apoptosis (23).

286 Like for many viruses (23),(41), eEF1A has been identified as an important cellular  
287 factor for HRSV replication (22). As an abundant, multifunctional protein, it is not surprising  
288 that many viruses have adapted to use eEF1A as a cofactor for viral transcription,  
289 translation, assembly, and pathogenesis. So, eEF1A was shown to bind to some viral RNA  
290 structures, some viral structural and nonstructural proteins, or to interact with viral  
291 polymerase complexes, such as the one of Vesicular Stomatitis Virus (VSV) (46),(41). Like for  
292 HRSV, VSV forms two different viral RNA polymerase complexes in infected cells: the  
293 transcriptase and the replicase. The transcriptase complex synthesizes capped mRNAs,  
294 whereas the replicase complex initiates genomic minus-strand RNA synthesis at the precise  
295 3' ends of the plus-strand antigenomic and negative-strand genomic RNAs. The transcriptase  
296 was described as a protein complex containing the L and P proteins, the cellular eEF1A and  
297 heat shock protein 60, and a submolar amount of cellular mRNA cap guanylyltransferase  
298 (47). The replicase complex was described as containing the viral proteins L, P, and  
299 nucleocapsid (N) but not eEF1A, heat shock protein 60, or the guanylyltransferase. Hence, it  
300 was proposed that eEF1A is important for transcription of viral mRNAs but not for genomic  
301 RNA replication in VSV.

302 Disentangling the canonical and noncanonical roles of eEF1A and the extent to which  
303 each contributes to viral function is essential if eEF1A is to be targeted therapeutically. It was  
304 previously shown that didemnin B treatment, a drug targeting eEF1A, protected cells from  
305 HRSV-induced cell death (22). Didemnin B did not significantly affect HRSV transcription and  
306 replication, especially at 24 h post-infection, but significantly reduced infectious virus  
307 production and release, possibly as a consequence of changes in actin stress fiber formation

308 (24). More recently, plitidepsin (Aplidin), an analog of didemnin B that was approved in  
309 Australia for the treatment of myeloma (48), was shown to be very efficient against SARS-  
310 CoV-2 in cultured cells as well as in a mice model (26). Since it has entered clinical trials as an  
311 anti-SARS-CoV-2 drug and was shown as a favorable long-term safety profile in adult patients  
312 hospitalized for COVID-19 (49),(50),(51),(52), we wondered whether this compound could  
313 also be efficient against HRSV. Plitidepsin is a cyclic depsipeptide that was first isolated from  
314 a Mediterranean marine tunicate (*Aplidium albicans*) and, at present, is manufactured by  
315 total synthesis and commercialized by PharmaMar, S.A., as Aplidin®(53). Plitidepsin has  
316 antitumoral, and immunosuppressive activities (54). Although not fully clarified, the  
317 molecular mechanisms of action of plitidepsin against tumor cells and SARS-CoV-2 have been  
318 investigated. Plitidepsin induces cell-cycle arrest and apoptosis (55). These effects rely on  
319 the induction of early oxidative stress, the rapid activation of Rac1 GTPase, and activation of  
320 c-Jun N-terminal kinase (JNK), ERK and p38 mitogen-activated protein kinases (p38/MAPK),  
321 which finally result in caspase-dependent apoptosis (56),(57). It was determined that eEF1A  
322 is the primary target of plitidepsin, which can bind to eEF1A at the interface between  
323 domains 1 and 2 of this protein in the GTP conformation with a measured KD of 80 nM (25).  
324 However, it was proposed that the antitumoral effect of plitidepsin is not due to translation  
325 inhibition but inhibition of eEF1A binding to double-stranded RNA-dependent protein kinase  
326 (PKR). In the presence of plitidepsin, PKR would disengage from eEF1A, thereby regaining its  
327 kinase activity to initiate extrinsic apoptosis through activation of MAPK and NF- $\kappa$ B signaling  
328 cascades (58). Plitidepsin was also reported as an ER stress inducer by activating the  
329 unfolded protein response (59). In parallel, plitidepsin was also shown to induce the  
330 phosphorylation of eIF2 $\alpha$ , resulting in the arrest of protein synthesis at the initiation step  
331 (59) ,(60). For SARS-CoV-2, it has been determined that the effect of plitidepsin is also

332 mediated by eEF1A by using a mutated (A399V) version of eEF1A1 in 293T cells (26). It is  
333 likely that this effect is mediated by inhibition of translation of the viral proteins (26),  
334 (61),(55). Very recently, the mechanism of SARS-CoV-2 inhibition was revisited (62). In this  
335 paper, using Vero E6 cells, the authors found that plitidepsin at 50 nM reduced the  
336 translation of RNAs, including cellular RNAs, but with a higher impact on viral mRNAs  
337 translation and without affecting cellular viability. The molecular mechanisms involved cell's  
338 proteostatic response to eEF1A blockade, namely a shift from cap or internal ribosome entry  
339 sites (IRES)-mediated translation towards a N-6-methyladenosine (m6A)-dependent  
340 translation, that could explain cell survival. They also tested plitidepsin against several other  
341 viruses including HRSV, and found an IC<sub>50</sub> of 27 nM in Vero E6 cells, about ten times more  
342 than what we found using HEp-2 cells. For *in vivo* effects, it was also shown that treatment  
343 of a monocyte-derived macrophage cell line by plitidepsin in the same range than ours (2.5-  
344 10 nM) reduced the production of the proinflammatory cytokine IL-6 in the presence of  
345 SARS-CoV-2 virions, and that was associated with a reduction of NF-κB p65 subunit  
346 phosphorylation and of its transcription activity in the inflammatory cascade (63). This effect  
347 was also observed *in vivo* in SARS-CoV-2 or Influenza virus infected mice.

348 In our experiments, we observed that plitidepsin had an inhibitory effect on viral  
349 replication and minigenome activity with an IEC<sub>50</sub> ≈ 3-5 nM, and a toxic effect on cells with  
350 an IC<sub>50</sub> ≈ 150 nM. However, further investigation revealed that this inhibitory effect was due  
351 to a general inhibition of translation in the host cell, with no significant difference between  
352 cellular and viral proteins expression. We then wondered whether eEF1A, which is essential  
353 for cellular translation, could be actively degraded by plitidepsin treatment, as it was  
354 observed recently with ternatin-4, another eEF1A-targeting drug (39). We found that eEF1A  
355 was degraded after plitidepsin treatment for 18H at concentrations as low as 1 nM, which

356 was inhibited by proteasome inhibitor carfilzomib, indicating that a eEF1A ubiquitination  
357 mechanism occurred, that remain to be determined. During this study it was published by  
358 Molina et al. that cellular cap-dependent translation was inhibited by plitidepsin (see above).  
359 However, using pulse-chase experiments, we found that cellular translation was mainly  
360 affected (see [Figure 4](#)).

361 In conclusion, we show here for the first time that treatment of cells with plitidepsin  
362 induces the degradation of eEF1A by the proteasome pathway, which is correlated with a  
363 global extinction of translation. These experiments also show the dependance of HRSV  
364 replication to the cellular factor eEF1A. Although effective concentrations of plitidepsin  
365 against HRSV are in the same range that those found for SARS-CoV-2, the side effects of  
366 plitidepsin on cells and its potential toxicity raises the question of its use *in vivo* for antiviral  
367 assays against HRSV.

368

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## 629 **Figures legend**

630 **Figure 1.** Impact of plitidepsin treatment on HRSV replication on HEp-2 cells. **(A)** Cells were  
631 infected for 2 h with rHRSV-mCherry at MOI 0.2 and the medium was then replaced to  
632 incubate cells in the presence of serial dilutions of plitidepsin for 48 h (red curve). The viral  
633 replication was quantified by measurement of the mCherry fluorescence. In parallel, cell  
634 viability upon treatment with plitidepsin was quantified in non-infected HEp-2 cells (black  
635 curve). Error bars are standard deviations from duplicates. Data are representative of three  
636 experiments. The curves were fitted in Graph Pad 8 software using a four parameters logistic  
637 (4PL) regression. Both  $IC_{50}$  and  $CC_{50}$  are indicated. **(B)** Representative images of HEp-2 cell  
638 cultures infected with rHRSV-mCherry at MOI 0.2, 48 hours post-infection in the absence or  
639 presence of plitidepsin at 12, 4, and 1.4 nM. Nuclei were colored with Hoechst 33432. Scale  
640 bars, 250  $\mu$ m.

641 **Figure 2.** Plitidepsin effect on a HRSV minigenome. **(A)** BSRT7/5 cells were transfected with  
642 plasmids encoding the N, P, M2-1, and L proteins; the pMT/Luc minigenome, together with  
643 pCMV-beta-gal for transfection standardization. Serial dilutions of plitidepsin were added 6 h  
644 post-transfection after removing transfection reagents. Viral RNA synthesis was quantified  
645 by measuring the luciferase activity after cell lysis 24 h post-transfection. Each luciferase  
646 minigenome activity value was normalized based on DMSO treated cells and is the average

647 of three independent experiments performed in triplicate. Error bars represent standard  
648 deviations calculated based on three independent experiments made in triplicate. (B) BSRT7  
649 cells were transfected with the complete minigenome system (see above) or with the pEGFP  
650 plasmid coding for EGFP. Six hours later, the transfection mix was removed and medium  
651 containing plitidepsin at different concentrations was added. Then, 24h post-transfection,  
652 cells were lysed and analyzed by Western blotting using anti-N, anti-GFP or anti-alpha  
653 tubulin antibodies.

654 **Figure 3.** *In vitro* effect of plitidepsin on HRSV RNA polymerase (L-P complex) activity. Primer  
655 extension polymerase assay was performed in the presence of increasing concentration of  
656 plitidepsin. Briefly, 200 nM of RSV L/P was incubated with 2  $\mu$ M of 11 nucleotide length RNA  
657 template and 4 nucleotide primer in presence of increasing concentrations of plitidepsin.  
658 The reactions were incubated for 30' and 120 min at 30°C and the reaction products were  
659 separated on a 20 % UREA-PAGE gel before gel scanning on a Typhoon imager.

660 **Figure 4.** Compared effect of plitidepsin on viral or cellular proteins synthesis. HEp-2 cells in  
661 6 well plates were infected with rHRSV-mCherry at MOI = 1. 24 h later, medium was changed  
662 for protein labelling with AHA with serial dilutions of plitidepsin. After 4 h, cells were lysed  
663 and AHA-labelled proteins were revealed with AFDye 488-DBCO, then immunoprecipitated  
664 with anti-N or anti-alpha-tubulin antibodies. Immunoprecipitated proteins or supernatants  
665 were resolved by SDS-PAGE (A) and fluorescence in the gels was measured using a Fuji FLA-  
666 3000 scanner (B).

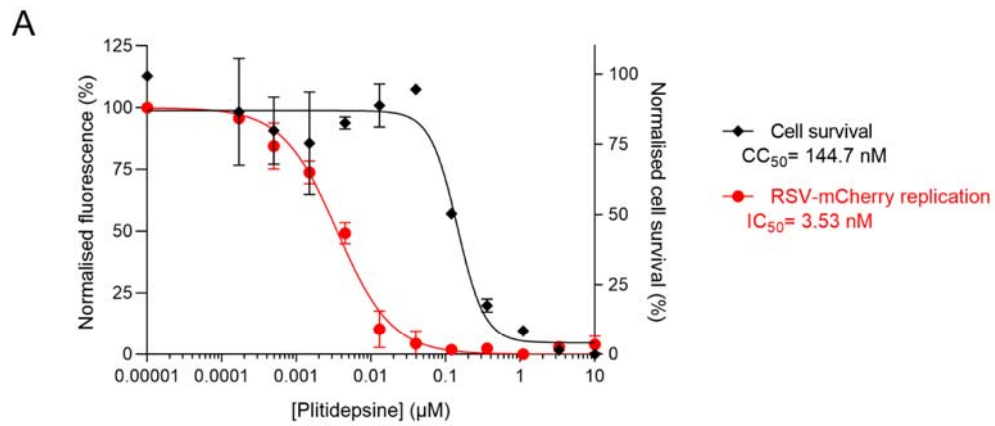
667 **Fig. 5.** Antiviral mechanism of action of plitidepsin is mediated through proteasome-  
668 mediated degradation of eEF1A. BSRT7/5 cells were treated with serial dilutions of

669 plitidepsin or DMSO for 18H in the presence or not of the proteasome inhibitor Carfilzomib

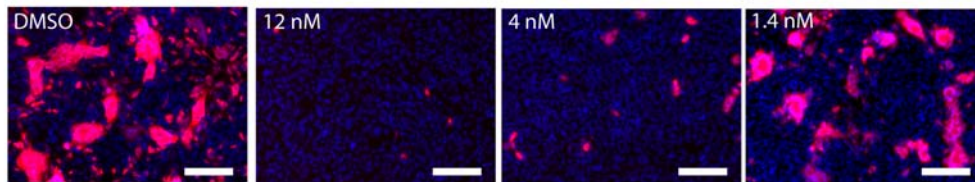
670 (500 nM) and the expression of eEF1A or GAPDH were analyzed by WB.

671

672 Figure 1



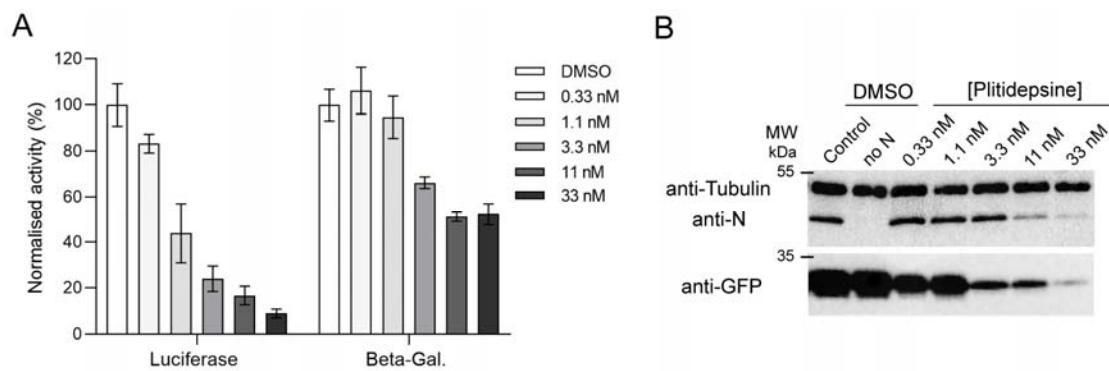
**B**



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675 Figure 2



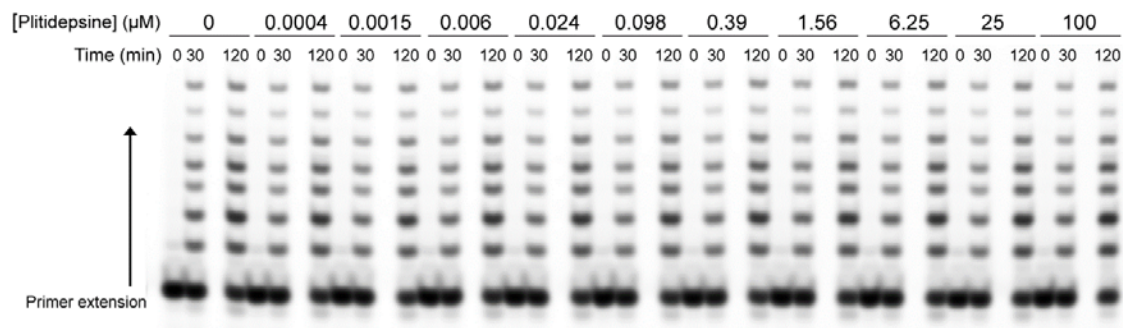
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679 Figure 3

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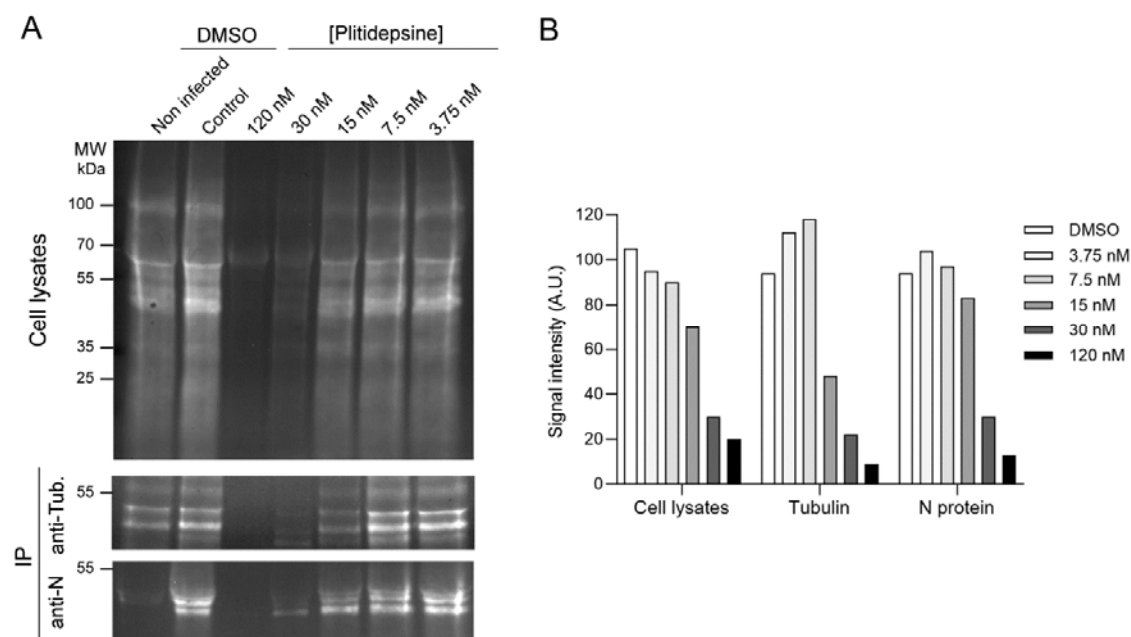
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685 Figure 4

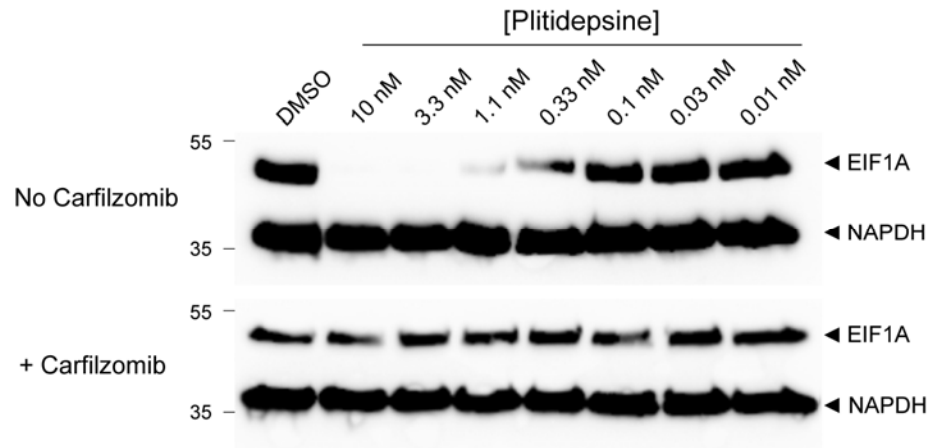


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688 Figure 5

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