1 **Stress granule-inducing eukaryotic translation initiation factor 4A inhibitors block influenza A**
2 **virus replication**
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ABSTRACT

Eukaryotic translation initiation factor 4A (eIF4A) is a helicase that facilitates assembly of the translation preinitiation complex by unwinding structured mRNA 5' untranslated regions. Pateamine A (PatA) and silvestrol are natural products that disrupt eIF4A function and arrest translation, thereby triggering the formation of cytoplasmic aggregates of stalled preinitiation complexes known as stress granules (SGs). Here we examined the effects of eIF4A inhibition by PatA and silvestrol on influenza A virus (IAV) protein synthesis and replication in cell culture. Treatment of infected cells with either PatA or silvestrol at early times post-infection results in SG formation, arrest of viral protein synthesis and failure to replicate the viral genome. PatA, which irreversibly binds to eIF4A, sustained long-term blockade of IAV replication following drug withdrawal, and inhibited IAV replication at concentrations that had minimal cytotoxicity. By contrast, the antiviral effects of silvestrol were fully reversible; drug withdrawal caused rapid SG dissolution and resumption of viral protein synthesis. IAV inhibition by silvestrol was invariably associated with cytotoxicity. PatA blocked replication of genetically divergent IAV strains, suggesting common dependence on host eIF4A activity. This study demonstrates the feasibility of targeting core host protein synthesis machinery to prevent viral replication.

46   **IMPORTANCE**

47   Influenza A virus (IAV) relies on cellular protein synthesis to decode viral messenger RNAs. Pateamine

48   A and silvestrol are natural products that inactivate an essential protein synthesis protein known as eIF4A.

49   Here we show that IAV is sensitive to these eIF4A inhibitor drugs. Treatment of infected cells with

50   pateamine A or silvestrol prevented synthesis of viral proteins, viral genome replication and release of

51   infectious virions. The irreversible eIF4A inhibitor pateamine A sustained long-term blockade of viral

52   replication, whereas viral protein synthesis quickly resumed after silvestrol was removed from infected

53   cells. Prolonged incubation of either infected or uninfected cells with these drugs induced the

54   programmed cell death cascade called apoptosis. Our findings suggest that core components of the host

55   protein synthesis machinery are viable targets for antiviral drug discovery. The most promising drug

56   candidates should selectively block protein synthesis in infected cells without perturbing bystander

57   uninfected cells.

3

58 **INTRODUCTION**

59 Influenza A viruses (IAV) are enveloped viruses with segmented, negative-sense single-stranded RNA

60 genomes (vRNAs) that primarily infect epithelial cells in in a diverse range of hosts. The viruses bind to

61 cell surface sialic acids and are internalized by endocytosis. The virus gains access to the cytoplasm

62 following the fusion of viral and endosome membranes. Viral genome segments are then imported into

63 the cell nucleus, where viral mRNA synthesis is initiated by the viral RNA-dependent RNA polymerase

64 (RdRp) complex, which cleaves nascent host RNA polymerase II (pol II)-transcribed RNAs at sites 10-14

65 nucleotides downstream of 5' 7-methyl-guanosine (m7G) caps (1). These short capped RNAs are used to

66 prime viral mRNA synthesis. Template-directed viral mRNA synthesis is terminated by reiterative

67 decoding of short uridine-rich sequences, which generates 3'-poly-adenylate (polyA) tails. Thus, by

68 coupling a trimeric RdRp complex to each incoming genome segment, the virus ensures faithful

69 generation of mRNAs with 5'-caps and polyA tails that largely resemble host mRNAs.

70 Efficient translation of IAV mRNAs depends on virus-mediated suppression of host gene expression, a

71 process known as host shutoff. Host shutoff gives viral mRNAs priority access to the host protein

72 synthesis machinery. IAV host shutoff mechanisms identified to date encompass different stages of host

73 mRNA biogenesis. The viral PA-X endoribonuclease selectively targets host pol II transcripts while

74 sparing viral mRNAs, as well as pol I and pol III transcripts (2). In addition, the viral nonstructural-1

75 (NS1) protein binds and inhibits cleavage and polyadenylation specificity factor 30 kDa subunit

76 (CPSF30), an essential component of the host 3' end processing machinery for cellular pre-mRNAs that

77 is dispensable for viral mRNA processing (3). NS1 also enhances the association between newly-

78 synthesized viral mRNAs and cellular NXF1/Tap mRNA export factors (4), while simultaneously

79 inhibiting nucleoporin 98 (Nup98)-mediated export of host mRNAs (5). In addition to host shutoff, there

80 is evidence that efficient translation of viral mRNAs depends on short, conserved cis-acting sequences in

4

81   viral 5' untranslated regions (UTRs), downstream of the host-derived leader sequences (6). It is not

82   known precisely how these 5'-UTR sequences assist translation, but recent work has shown that when

83   bound to viral mRNAs, the NS1 protein plays an important role in ribosome recruitment (7). By contrast,

84   recent studies employing RNA-sequencing, ribosomal foot-printing and single-molecule fluorescence in-

85   situ hybridization have suggested that host shutoff is mainly achieved by reduction in cellular mRNA

86   levels, and that IAV mRNAs are not preferentially translated (8). It is clear that viral proteins enable

87   efficient translation of IAV mRNAs, but the precise composition of translation pre-initiation complexes

88   assembled on these mRNAs, and the contribution of diverse host translation initiation factors remains

89   incompletely characterized.

90   Dependence on host protein synthesis machinery makes viral mRNAs sensitive to various stress-induced

91   translation repression mechanisms. A particularly critical checkpoint in translation initiation is the

92   assembly of the ternary complex, comprised of eIF2, GTP and tRNAi^{met}, which allows for incorporation

93   of the tRNAi^{met} into the 40S ribosomal subunit. This step is negatively regulated by serine

94   phosphorylation of eIF2☐ by one of four kinases: heme-regulated translation inhibitor (HRI), general

95   control non-derepressible-2 (GCN2), protein kinase R (PKR) and PKR-like endoplasmic reticulum kinase

96   (PERK) (9). eIF2☐ phosphorylation causes accumulation of stalled 48S preinitiation complexes and

97   associated mRNA transcripts, which are bound by aggregation-prone RNA-binding proteins, including

98   Ras-GAP SH3 domain-binding protein (G3BP), T-cell intracellular antigen-1 (TIA-1), and TIA-1 related

99   protein (TIAR). These complexes nucleate cytoplasmic stress granules (SGs), sites where stalled mRNP

100   complexes are triaged, until stress is resolved and protein synthesis can resume.

101   IAV mRNAs are efficiently translated throughout infection and SGs never form (10). IAV prevents

102   translation arrest, at least in part, through the action of NS1, a double-stranded RNA (dsRNA)-binding

103   protein that prevents PKR-mediated eIF2α phosphorylation. We have shown previously that IAV encodes

104   two additional proteins, PA-X and NP, that block SG formation in an eIF2□-independent manner (11).

105   Precise details of NP and PA-X mechanism of action remain obscure, but SG-inhibition by PA-X is

106   tightly linked to its host shutoff function, as it requires endonuclease activity. This mechanism is

107   reminiscent of the herpes simplex virus-type 2 (HSV-2) virion host shutoff (vhs) protein, which also

108   requires endonuclease activity to prevent SG formation (12). Taken together, these findings suggest that

109   IAV dedicates a significant portion of its small genome to encode proteins with SG-antagonizing activity.

110   SG suppression is maximal at late times post-infection, coinciding with the accumulation of NS1, NP and

111   PA-X proteins. We have shown previously that at early times post-infection, treatment of infected cells

112   with sodium arsenite, which causes HRI activation and eIF2α phosphorylation, triggered SG formation

113   and stalled viral replication (11). Thus, like host mRNAs, at early times post-infection, IAV mRNAs are

114   exquisitely sensitive to eIF2α phosphorylation and ternary complex depletion. We have also shown that

115   IAV mRNAs are sensitive to pateamine A (PatA) (11), a natural product that selectively inhibits the

116   DEAD-box RNA helicase eIF4A which assembles with eIF4E and eIF4G, to form the eIF4F complex

117   (13, 14). By disrupting eIF4A, PatA prevents scanning by the 43S pre-initiation complex, causing arrest

118   of translation initiation, and inducing SG formation (15). Specifically, treatment with PatA caused SG

119   formation at early times post-infection and diminished accumulation of viral proteins.

120   In addition to PatA, eIF4A can be inhibited by a variety of natural compounds currently being

121   investigated for anti-cancer properties, including hippuristanol (16), 15-deoxy-delta 12,14-

122   prostaglandin J2 (15d-PGJ2) (17), silvestrol (18) and synthetic derivatives. Accumulating evidence

123   indicates that many viruses are highly dependent on eIF4A activity for protein synthesis. For example,

124   PatA has been shown to limit human cytomegalovirus infection (19), whereas silvestrol has been shown

125   to inhibit Ebola virus replication (20).

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126   In this study, we methodically documented the antiviral properties of PatA and silvestrol in three

127   workhorse models of IAV infection; A549 human lung adenocarcinoma cells, Madin-Darby canine

128   kidney (MDCK) cells, and African green monkey kidney epithelial cells (Vero). In all cases, dosages of

129   PatA and silvestrol sufficient to elicit SGs concurrently blocked viral protein accumulation and genome

130   replication. PatA, known to bind irreversibly to eIF4A, could sustain long-term arrest of viral protein

131   synthesis following drug withdrawal, whereas the effects of silvestrol were reversible. Finally, PatA could

132   inhibit genetically-divergent strains, suggesting common dependence on eIF4A.

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135 **MATERIALS AND METHODS**

136 **Reagents and cells**

137 Unless specifically indicated, all chemicals were purchased from Sigma. Pateamine A was a kind gift

138 from Dr. Jerry Pelletier (McGill University, Montreal, QC, Canada), silvestrol was obtained from

139 MedChem Express (Princeton, NJ); 1 mM stocks of silvestrol and pateamine A were prepared in DMSO

140 and stored at -80C. A549, Vero and Madin-Darby Canine Kidney (MDCK) cells were maintained in

141 Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% fetal bovine serum

142 (FBS, Life Technologies) and 100 U/ml penicillin + 100 µg/ml streptomycin + 20 µM L-glutamine

143 (Pen/Strep/Gln; Wisent) at 37°C in 5% CO_2 atmosphere. All cell lines were purchased from the American

144 Type Culture Collection (ATCC).

145

146 **Viruses, infections and treatments**

147 Influenza virus strains used in this study include A/PuertoRico/8/34/(H1N1) virus (PR8) and

148 A/Udorn/1972(H3N2) virus (Udorn). Unless specified otherwise, infections were conducted at a

149 multiplicity of infection (MOI) of 1. After inoculation, cells were washed with 1x PBS and incubated

150 with infection medium containing 0.5% bovine serum albumin (BSA) in DMEM and incubated at 37°C in

151 5% CO_2 atmosphere. IAV virions were enumerated by plaque assay using 1.2% Avicel RC 591 (FMC

152 Corporation) overlay on confluent MDCK cells as described in (21). Where indicated, mock and virus-

153 infected cells were treated with 0.156-40 nM pateamine A or 2.5-640 nM silvestrol.

154

155 **Cell viability measurement**

156 Cells were seeded 24 h before drug treatment at a density of 10,000 cells/well in 96-well plates. Drugs

157 were serially-diluted in media to the indicated concentrations and added to cells. After 24-h treatment,

8

158   AlamarBlue assay (Thermo Scientific) was conducted according to manufacturer's protocol using Tecan

159   Infinite M200 PRO microplate reader (excitation = 560 nm, emission = 590 nm). Values were normalized

160   to vehicle control.

161

162   **Immunostaining**

163   For immunofluorescence microscopy cells grown on glass coverslips were fixed and stained as described

164   previously (11) using mouse monoclonal antibody to G3BP (clone 23, BD Transduction Labs), goat

165   polyclonal antibody to influenza virus (ab20841, Abcam), and rabbit monoclonal antibody to TIAR

166   (clone D32D3, Cell Signaling) at manufacturer-recommended dilutions. Donkey Alexa Fluor-conjugated

167   secondary antibodies (Molecular Probes) were used at 1:1000 dilution in combination with 5 ng/ml

168   Hoechst dye. Images were captured using Zeiss Axioplan II microscope or Zeiss LSM 510 laser scanning

169   microscope. For western blot analysis, whole cell lysates were resolved on TGX Stain-Free Precast Gels

170   (BioRad) and analyzed using goat polyclonal antibody to influenza virus described above (recognizes

171   HA1, NP, and M1 proteins), mouse monoclonal antibodies to influenza NS1 (clone 13D8, reference (22)),

172   puromycin (MABE343, Millipore Sigma), and rabbit antibodies to phospho-Ser-51-eIF2α (D9G8, Cell

173   Signaling), PARP (9542S, Cell Signaling), and β-actin (13E5, HRP-conjugated, Cell Signaling).

174

175   **Real time quantitative PCR**

176   Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen) according to manufacturer's protocol.

177   Viral genomic and messenger RNAs were reverse-transcribed as described in (2) using Maxima H Minus

178   Reverse Transcriptase (Thermo Scientific) in separate reactions containing the gene-specific primer for

179   18S rRNA (5'-AGG GCC TCA CTA AAC CAT CC-3') and either the influenza A virus-specific

180   universal primer Uni12 (5'-AGC AAA AGC AGG-3', for vRNA) or the oligo(dT)$_{18}$ primer (for mRNA).

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181   Quantitative PCR analysis was performed using GoTaq PCR master mix (Promega). Relative initial

182   template quantities were determined using the Ct method. Primer sequences and the PCR thermal profile

183   setup are available upon request.

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186   **RESULTS**

187   **Pateamine A and silvestrol induce stress granules and inhibit viral protein accumulation in dose-**

188   **dependent manner.**

189   We previously demonstrated that IAV was sensitive to pharmacologic induction of translation arrest and

190   SG formation in the early stages of infection (11). To gain a fuller understanding of how eIF4A-targeting

191   drugs can trigger SG formation and disrupt viral replication, IAV-infected A549, Vero and MDCK cells

192   were treated at 1 hpi with increasing doses of PatA and silvestrol. In all three cell lines, PatA triggered SG

193   formation and inhibited IAV protein accumulation at concentrations above 2.5 nM (Fig. 1A-D). By

194   contrast, SG formation was triggered in response to 40 nM silvestrol in infected A549 cells, and 160 nM

195   silvestrol in infected MDCK cells. Remarkably, Vero cells were highly resistant to translation arrest and

196   SG induction by silvestrol. For both PatA and silvestrol, SG induction tightly correlated with inhibition of

197   viral protein synthesis across all three cell lines.

198

199   **Pateamine A is more potent than silvestrol at inhibiting viral replication in cultured cells**

200   Previously, we demonstrated that 10 nM PatA blocked IAV replication in A549 cells through sustained

201   total protein synthesis arrest and SG formation (11). To further test the effects of PatA and silvestrol on

202   infectious virus release in the three most common cell culture models of IAV infection, we treated A549,

203   Vero and MDCK cells with increasing concentrations of these drugs at 1 h post-infection with the PR8

204   strain of IAV. At 24 hpi, culture supernatants were harvested and the infectious virus titers were

205   determined using plaque assays in MDCK cells (Fig. 2A, B). In agreement with results presented in Fig.

206   1, 5 nM PatA was sufficient to reduce virus replication 10-fold in all three cell lines while silvestrol had

207   minimal effect on virus replication in Vero cells. In MDCK cells, silvestrol was much less effective at

208   inhibiting infectious virus release;16-times higher drug concentration was required to match the inhibition

11

209   of infectious virion release observed in A549 cells. Overall, these results reveal tight correlation between

210   viral protein accumulation and the infectious virus production in these cell lines, and the unexpected

211   remarkable resistance of Vero cells to silvestrol. Next, we tested the effects of PatA and silvestrol on cell

212   viability after 24 h treatment using the AlamarBlue assay (Fig. 2C, D). Both drugs exhibited notable

213   cytotoxicity at the concentrations required for maximal inhibition of virus replication. However, the

214   cytotoxic effects varied significantly between the cell lines tested. 5 nM PatA had little effect on A549

215   cells, but it caused a sharp decrease in viability of MDCK cells. By contrast, among the three cell types,

216   A549 cells were most sensitive to silvestrol. Taken altogether, these results suggest that PatA is more

217   effective than silvestrol at inhibiting virus replication at sub-cytotoxic concentrations and exhibits a

218   narrow therapeutic index in A549 cells.

219

220   **Silvestrol withdrawal causes SG dissolution and resumption of viral protein accumulation.** PatA and

221   silvestrol have distinct molecular structures and mechanisms of eIF4A disruption. Both molecules induce

222   eIF4A dimerization and force an engagement with RNA, thereby depleting eIF4A from eIF4F complexes

223   and inhibiting cap-dependent translation (14, 23). However, PatA has been shown to be an irreversible

224   translation inhibitor (13), whereas silvestrol-dependent translation inhibition occurs reversibly (16). These

225   distinct properties provided a unique opportunity to investigate whether IAV can 'recover' from SG

226   formation following withdrawal of the SG-inducing drug. To address this directly, IAV-infected A549

227   cells were incubated with 30 nM PatA or 300 nM silvestrol starting from 1 hpi. At 4 hpi, drug was

228   washed off of some infected cells, and SG formation and viral protein accumulation were analyzed using

229   immunofluorescence microscopy at 12 hpi (Fig. 3A). Consistent with our previous observations (11),

230   after withdrawal of PatA, SGs persisted for the remainder of the time-course, and viral proteins did not

231   accumulate beyond the levels observed at 1 hpi. However, silvestrol withdrawal at 4 hpi led to SG

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232   disassembly and robust IAV protein accumulation; thus, 'wash-off' (WO) reversed the effects of

233   silvestrol. To confirm that SG dissolution due to silvestrol withdrawal coincides with the resumption of

234   protein synthesis, we treated A549 cells with silvestrol or PatA and labelled newly synthesized proteins

235   with a 10 min pulse of puromycin at various times post-treatment (24). The newly-synthesized peptides

236   were visualized by western blot (Fig. 3B). Both silvestrol and PatA caused strong inhibition of protein

237   synthesis at 1 h post-treatment, which was sustained throughout the 24 h treatment time-couse. Silvestrol

238   withdrawal at 3 h allowed for a complete restoration of protein synthesis at later times (Fig. 3B, lanes 7

239   and 12). By contrast, protein synthesis was never restored following PatA wash-off (Fig. 3B, lanes 8 and

240   13). Together, these findings demonstrate that IAV is able to recover from transient drug-induced

241   translation arrest and resume the viral replication cycle when eIF4A inhibition is relieved.

242

Prolonged eIF4A inhibition triggers apoptosis

244   SG formation promotes cell survival in response to a variety of environmental stresses (9). However, the

245   SG-inducing eIF4A inhibitors silvestrol and PatA have been shown to promote apoptosis of cancer cells

246   (23, 25–27). Moreover, apoptosis is a well-described feature of late-stage IAV infection, required for

247   efficient virion production (28–31). To determine whether eIF4A disruption affects the fate of IAV-

248   infected cells in our system, both infected and uninfected A549 cells were treated with 300 nM silvestrol

249   or 30 nM PatA, and apoptosis was measured by immunoblotting for PARP cleavage products. Following

250   16 h incubation with drugs, PARP cleavage species accumulated in both mock-infected and IAV-infected

251   cells alike, indicating that sustained eIF4A disruption overcomes any pro-survival effects of SG

252   formation, resulting in apoptosis (Fig. 4A, lanes 5-8). Interestingly, the sustained translation arrest by

253   silvestrol not only diminished viral protein accumulation, but also prevented the RdRp from switching

254   tasks between viral mRNA synthesis and genome replication (Fig. 4B). This finding is consistent with our

13

255   previous observations for PatA (11), suggesting that both drugs affect the viral replication cycle in the

256   same way. This dysregulated RdRp activity and an inability to produce the viral PKR antagonist NS1 due

257   to translation arrest, enables detection of viral pathogen-associated molecular patterns (PAMPs), resulting

258   in eIF2α phosphorylation (Fig. 4, lanes 5-6).

259

260   **Effects of silvestrol and pateamine A on IAV replication are not strain-specific**

261   We have determined that the lab-adapted H1N1 PR8 strain used in this study, which is the preferred

262   backbone for vaccine production worldwide, is sensitive to eIF4A inhibition. To determine whether a

263   genetically-divergent IAV strain could be similarly affected by eIF4A inhibiting drugs, A549 cells were

264   infected with the H3N2 Udorn strain. Infected cells were treated with 300 nM silvestrol at 4 hpi, and the

265   viral RNA and protein accumulation was determined over a 12 h time-course. Consistent with our

266   previous findings using the PR8 strain, silvestrol treatment affected RdRp switching from viral mRNA

267   synthesis to viral genome replication tasks (Fig. 5A, B) due to failure to accumulate viral proteins (Fig.

268   5C). Our findings to date indicate that PatA is better than silvestrol at inhibiting PR8 replication at sub-

269   cytotoxic doses. For this reason, we investigated the effects of PatA on release of Udorn from A549 cells.

270   We observed a similar magnitude of inhibition of Udorn virion production in cells treated with low

271   nanomolar doses of PatA.

14

272   **DISCUSSION**

273   IAV mRNAs generally resemble host mRNAs, which enables efficient translation by host cell machinery.

274   However, these features also make them susceptible to stress-induced arrest of protein synthesis. Host

275   translation initiation requires eIF4A, a helicase that unwinds mRNA secondary structure to permit ternary

276   complex scanning for translation initiation codons. Here, we showed that IAV translation is sensitive to

277   the eIF4A inhibitors PatA and silvestrol. These drugs limited viral protein accumulation and elicited the

278   formation of SGs. Because progression through the viral replication cycle depends on accumulation of

279   key viral proteins, these eIF4A inhibitors prevent the viral polymerase complex from switching from viral

280   mRNA synthesis to viral genome replication. Both molecules could block replication of genetically-

281   divergent IAV strains, PR8 and Udorn, suggesting a potential universal dependence on eIF4A activity.

282   While the effects of silvestrol were reversible, PatA, known to bind irreversibly to eIF4A, sustained long-

283   term arrest of viral protein synthesis following drug withdrawal.

284

285   Because many oncogenes have structured 5'-UTRs, and depend on eIF4A activity for their synthesis,

286   eIF4A inhibitors like PatA and silvestrol, have been extensively studied for anti-cancer activity. Low

287   doses of PatA have been shown to inhibit proliferation of tumor xenografts without appreciable toxicity in

288   murine models (32). Indeed, PatA was able to inhibit oncogene synthesis at low doses that did not

289   impinge on bulk protein synthesis rates, demonstrating that mRNA structure plays a crucial role in

290   determining susceptibility and dose-dependent effects of eIF4A inhibitors. As in cancer cells, efficient

291   virus replication typically demands sustained high rates of protein synthesis, which may likewise be

292   dependent on eIF4A helicase activity. For example, Ebola virus has been shown to be exquisitely

293   sensitive to eIF4A inhibition by silvestrol (20). Ebola virus mRNAs have highly-structured 5'-UTRs (33–

294   35) and require processive helicase activity of eIF4A. By contrast, IAV mRNA 5'-UTRs are relatively

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295   short, and comprised of divergent host-derived mRNA segments fused to conserved viral mRNA

296   segments. The heterogeneous nature of these 5'-UTRs challenges RNA structure prediction algorithms,

297   but the short, conserved regions do not display significant secondary structure that would necessitate high

298   eIF4A activity. Consistent with this, IAV mRNA translation is inhibited by relatively high doses of

299   silvestrol and PatA that would be predicted to deplete eIF4A from translation preinitiation complexes.

300   Our results and previous studies indicate that eIF4A helicase activity is required for translation initiation

301   on IAV mRNAs, but it appears that processive unwinding of long, structured 5'-UTRs is not required.

302   Consistent with this model, IAV infection was shown to deplete the eIF4A processivity factor eIF4B (36).

303   The virus replicates efficiently in eIF4B-depleted cells, and likely benefits from diminished synthesis of

304   eIF4B-dependent interferon-stimulated genes like IFITM3. Viral mRNP complexes likely lack eIF4B, but

305   remain only partially characterized. They have properties that distinguish them from host mRNPs. For

306   example, there is some evidence that eIF4E1 is also dispensable for viral mRNA translation (37).

307   Moreover, IAV NS1 is known to stimulate viral mRNA translation, which may be linked to its ability to

308   bind to viral mRNP complexes through interactions with eIF4G1 and PABP (38). A better understanding

309   of the precise composition of viral mRNP complexes will likely inform our understanding of the role

310   played by eIF4A and other core translation factors. Beyond these considerations of mRNP composition,

311   dependence on eIF4A helicase activity might also be influenced by host shutoff, which is expected to

312   markedly influence the availability of host translation factors.

313

314   We previously established that SGs do not form at any point during IAV infection (10), and that three

315   viral proteins can inhibit SG formation (11). We also demonstrated that an early window of opportunity

316   exists, before sufficient quantities of SG antagonizing viral proteins accumulate, when the virus is

317   exquisitely sensitive to stress-induced translation arrest. Here, we further elucidated the mechanism of

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318   action of SG-inducing eIF4A inhibitors PatA and silvestrol. To date, most of our observations of SGs

319   formed in IAV-infected cells indicate that these granules have canonical composition and properties.

320   Throughout our studies, SG formation in infected cells reliably indicated disruption of viral protein

321   accumulation and viral replication (10, 11). In this study, we observed that, upon withdrawal of silvestrol,

322   SGs rapidly dissolved when viral protein synthesis resumed. At the same time, ongoing translation arrest

323   triggered by PatA resulted in persistence of SGs throughout the observation period. It has been recently

324   demonstrated that PKR is recruited to SGs by direct interaction with G3BP, and that this recruitment

325   results in PKR activation (39, 40). We observe eIF2α phosphorylation in infected cells after prolonged

326   SG induction by PatA or silvestrol. It will be interesting to determine whether this phosphorylation is

327   dependent on PKR activation.

328

329   Our results show the magnitude of the threat that host-targeted translation inhibitors pose for viral

330   replication. Nevertheless, the eIF4A inhibitors studied here have some undesirable properties that may be

331   difficult to surmount. We observed that eIF4A inhibition resulted in cytotoxic effects that largely tracked

332   with viral inhibition in all cell lines examined. The human (A549), dog (MDCK) and green monkey

333   (Vero) cell lines studied here displayed markedly different sensitivities to silvestrol treatment, which

334   cannot be explained by structural differences in eIF4A because all three isoforms of eIF4A (eIF4A-I, -II,

335   and –III) are highly conserved between these species. Interestingly, while silvestrol has been shown to

336   bind to eIF4A-I and eIF4A-II, PatA has been shown to bind to all three isoforms, including eIF4A-III (32,

337   41–43). While eIF4A-I and eIF4A-II function in the cytoplasm, eIF4A-III is localized to the nucleus and

338   thus has no role in translation initiation. Instead, it is a member of the exon junction complex deposited

339   on mRNA post-splicing, where it has been shown to participate in nonsense mediated decay (44).

340   Moreover, eIF4AIII was previously shown to interact with the IAV polymerase complex (45). The

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341   functional significance of this interaction is unknown, and the contribution of eIF4A-III inhibition by

342   PatA to its antiviral effects remain to be determined.

343

344   There is an outstanding need for new antivirals for influenza. Past history has shown that direct-acting

345   antivirals are often rendered ineffective by rapid viral evolution. For this reason, host-targeted antivirals

346   are an attractive alternative approach that should limit the emergence of drug-resistant variants. RNA

347   silencing screens have shown that influenza virus replication depends on thousands of host genes, some of

348   which may be potential candidates for therapeutic intervention. Our data indicates that inhibition of viral

349   protein synthesis potently disrupts the viral replication cycle, and drugs that can block viral protein

350   synthesis may serve as attractive candidates for host-directed antivirals. Despite their antiviral activity

351   against IAV at high doses, PatA and silvestrol appear to lack specificity for viral translation complexes,

352   and impede bulk translation in infected and uninfected cells alike. This distinguishes IAV from other

353   viruses (e.g. Ebola, HCMV) and from cancer models, which were shown to be exquisitely sensitive to

354   much lower doses of eIF4A inhibitor. For these reasons, an effective host-targeted antiviral translation

355   inhibitor for influenza will ideally be specific for infected cells, while sparing uninfected cells.

356   Alternatively, a detailed characterization of IAV mRNP complexes could highlight unique features that

357   could be exploited by future antivirals.

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358 **ACKNOWLEDGEMENTS**

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491

492 **FIGURE LEGENDS**

493 **Figure 1. Concentration-dependent stress granule induction and inhibition of viral protein**

494 **accumulation by pateamine A and silvestrol.** A549, Vero, and MDCK cell lines infected with PR8

495 strain of IAV were treated with the indicated concentrations of pateamine A (PatA) or silvestrol (Sil.) at 1

496 hpi. (A-C) Viral protein accumulation was analysed in whole cell lysates collected at 24 hpi using western

497 blot. (D) Stress granule induction and viral protein accumulation were visualized at 9 hpi by

498 immunofluorescence microscopy using antibody to stress granule marker TIAR (red) and the polyclonal

499 anti-influenza antibody (green). Nuclei were stained with Hoechst dye (blue). Representative images are

500 shown for each cell line and treatment condition.

501

502 **Figure 2. Antiviral and cytotoxic effects of pateamine A and silvestrol vary between cell types.** (A,

503 B) Production of infectious virus progeny (PR8 strain) at 24 hpi was measured using plaque assay. The

504 indicated cell lines were infected with MOI = 0.1 and treated with the increasing concentrations of

505 pateamine A (A) or silvestrol (B) at 1 hpi. (C, D) Cell viability was measured using Alamar Blue assay

506 after 24-h treatment with increasing concentrations of pateamine A (C) or silvestrol (D). Relative

507 fluorescence values are normalized to vehicle control (DMSO). (A-D) Error bars represent standard

508 deviations from 3 independent biological replicates.

509

510 **Figure 3. Translation inhibition by silvestrol is fully reversible.** (A) A549 cells infected with PR8

511 strain of IAV were treated at 1 hpi with 320 nM silvestrol (Sil.) or 20 nM pateamine A (PatA) or left

512 untreated. At 4 hpi, some wells were washed briefly with PBS and received fresh infection media without

513 drugs as shown on the schematic outline of the experiment. At 12 hpi mock and virus-infected cells

514 subjected to continuous incubation with Sil. or PatA or to drug wash off at 4 hpi (WO) were analysed by

26

515 immunofluorescence staining with the polyclonal anti-influenza antibody (IAV, green) and the antibodies

516 to SG markers TIAR (red) and G3BP (blue). (B) Total translation rates in A549 cells were analyzed using

517 metabolic labelling with puromycin and subsequent western blotting with anti-puromycin antibody. In

518 some cases, after initial 3-h treatments, Sil. or PatA were washed off (WO) prior to puromycin labeling.

519 Total protein was visualized using BioRad Stain-Free reagent.
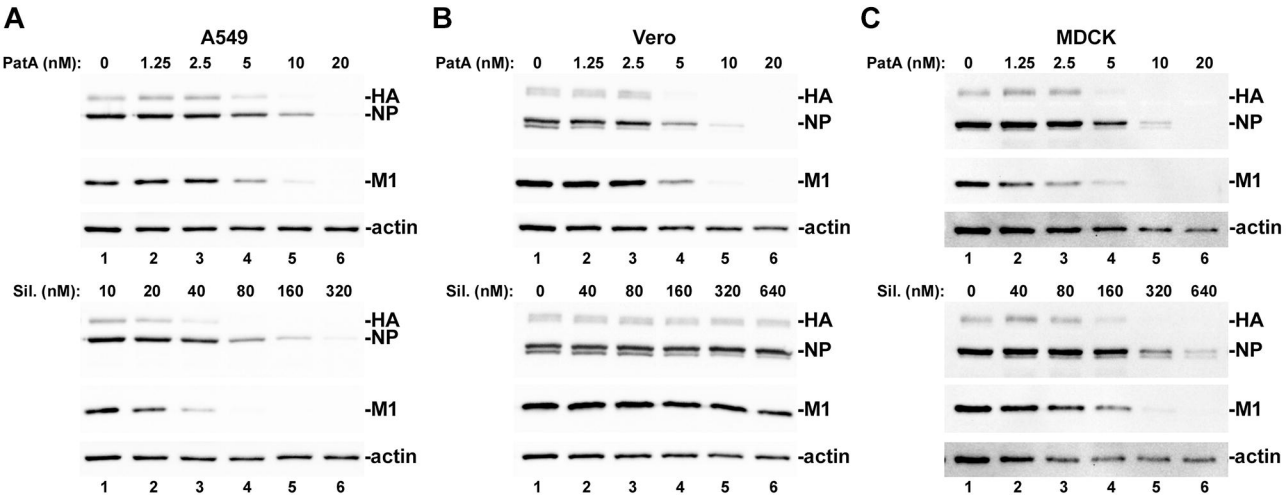
520

521 **Figure 4. Sustained eIF4A inhibition by silvestrol and pateamine A leads to induction of apoptosis.**

522 (A) Western blotting analysis of A549 cell lysates obtained at the indicated times post-infection with the

523 PR8 strain of IAV and treated with 320 nM silvestrol (Sil.) or 20 nM pateamine A (PatA) at 4 hpi or the

524 equivalent time after mock infection. (B) Total RNA was isolated from cells treated with 320 nM

525 silvestrol at 4 hpi and the relative levels of viral NS1 mRNA and vRNA at the indicated times post-

526 infection were determined using RT-qPCR. Error bars represent standard deviations (n=3). P values were

527 calculated using paired Student's t-test.

528

529 **Figure 5. Silvestrol and pateamine A block replication of H3N2 strain of IAV.** (A-C) A549 cells were

530 infected with A/Udorn/72(H3N2) strain of IAV and treated with 320 nM silvestrol at 4 hpi. Total RNA

531 and whole cell protein lysates were collected at 4, 8, and 12 hpi. The accumulation of viral NS segment

532 vRNA (A) and NS1 mRNA (B) was measured using RT-qPCR, and the accumulation of viral proteins

533 was analysed by western blotting (C). (D) Production of infectious virus progeny (Udorn strain) at 24 hpi

534 was measured using plaque assay. A549 cells were infected with MOI = 0.1 and treated with the

535 increasing concentrations of pateamine A at 1 hpi. Error bars represent standard deviations (n = 2).

27

A A549

PatA (nM): 0 1.25 2.5 5 10 20

-HA
-NP
-M1
-actin

1 2 3 4 5 6

Sil. (nM): 10 20 40 80 160 320

-HA
-NP
-M1
-actin

1 2 3 4 5 6

B Vero

PatA (nM): 0 1.25 2.5 5 10 20

-HA
-NP
-M1
-actin

1 2 3 4 5 6

Sil. (nM): 0 40 80 160 320 640

-HA
-NP
-M1
-actin

1 2 3 4 5 6

C MDCK

PatA (nM): 0 1.25 2.5 5 10 20

-HA
-NP
-M1
-actin

1 2 3 4 5 6

Sil. (nM): 0 40 80 160 320 640

-HA
-NP
-M1
-actin

1 2 3 4 5 6

D

mock

untreated

PR8 (9 hpi)

1.25 2.5 5 10 20 - PatA (nM)

A549

Vero

MDCK

100 μm

PR8 (9 hpi)

untreated

PR8 (9 hpi)

40 80 160 320 640 - Silvestrol (nM)

A549

Vero

MDCK

100 μm

TIAR/IAV/hoechst

A Virus yield

PFU/ml

PatA (nM)

A549
Vero
MDCK

B Virus yield

PFU/ml

Silvestrol (nM)

A549
Vero
MDCK

C Cell viability

Relative fluorescence

PatA (nM)

A549
Vero
MDCK

D Cell viability

Relative fluorescence

Silvestrol (nM)

A549
Vero
MDCK

A

mock | PR8 | PR8 + Sil. | PR8 + PatA

100 μm

PR8 → Drug → IF 12 hpi

PR8 → Drug → Wash off → IF 12 hpi (WO)

12 hpi

12 hpi (WO)

TIAR/IAV/G3BP

B

	1 h	12 h	12 h (WO)	24 h	24 h (WO)
	- Sil. PatA	- Sil. PatA	Sil. PatA	- Sil. PatA	Sil. PatA

80-

17-

WB (α-Puro)

80-

17-

Stain-Free

(kDa) 17-

1 2 3 4 5 6 7 8 9 10 11 12 13

A

	PR8 (10 hpi)	PR8 (16 hpi)	mock (16 h)	
	- Sil. PatA	- Sil. PatA	Sil. PatA -	

PARP
cleav. (89)
HA
NP
M1
P-S51 eIF2α
actin

100—
80—
58—
32—

32—
32—
46—

lane: 1 2 3 4 5 6 7 8 9

B

8 hpi   12 hpi

Relative RNA level (vs. untreated)

p<0.05
p<0.05
p<0.005

■ NS1 mRNA
■ NS vRNA

A NS vRNA

B NS1 mRNA

C

	0	4	8		12 (hpi)	
	−	−	−	+Sil.	−	+Sil.

- NP
- M1
- NS1
- actin

1   2   3   4   5   6

D