The PI3K/Akt Pathway Contributes To Arenavirus Budding

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ABSTRACT

Several arenaviruses, chiefly Lassa virus (LASV), cause hemorrhagic fever (HF) disease in humans and pose a significant public health concern in their endemic regions. On the other hand, evidence indicates that the globally distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway participates in many cellular processes, including cell survival and differentiation, and has been also shown to play important roles in different steps of the life cycle of a variety of viruses. Here we report that the inhibition of the PI3K/Akt pathway inhibited budding and to lesser extent RNA synthesis, but not cell entry, of LCMV. Accordingly, BEZ-235, a PI3K inhibitor currently in cancer clinical trials, inhibited LCMV multiplication in cultured cells. These findings, together with those previously reported for Junin virus (JUNV), indicate that targeting the PI3K/Akt pathway could represent a novel antiviral strategy to combat human pathogenic arenaviruses.

INTRODUCTION

Several arenaviruses cause hemorrhagic fever (HF) disease in humans. Thus, Lassa virus (LASV) and Junin virus (JUNV) are the causative agents of Lassa fever (LF) and Argentine HF disease, respectively, which represent significant public health problems within their endemic geographic regions of West Africa (LASV) and Argentina (JUNV). In addition, evidence indicates that the globally distributed prototypic arenavirus LCMV is a neglected human pathogen of clinical significance in congenital viral infections (3, 15, 24). Moreover, LCMV infection of immunosuppressed individuals can result in severe disease and death (13, 30). Public health concerns about arenavirus infections are aggravated by lack of FDA licensed vaccines and limited existing therapeutic options. The only arenavirus vaccine tested in humans is Candid 1, a live attenuated strain of JUNV that is licensed only in Argentina and ineffective against LASV or LCMV. On the other hand, current arenavirus antiviral drug therapy is restricted to the use of the nucleoside analogue ribavirin, which is only partially effective and associated with significant side effects (9, 25, 26). Therefore, it is important to develop novel antiviral strategies to combat human pathogenic arenaviruses, a

task that would be facilitated by a detailed understanding of the molecular and cell biology of arenaviruses.

Arenaviruses are enveloped viruses with a bi-segmented, negative strand (NS) RNA genome and a life cycle restricted to the cell cytoplasm (6). Each RNA genome segment uses an ambisense coding strategy to direct the expression of two gene products in opposite orientation and separated by a non-coding intergenic region (IGR). The Large (L) segment (L; 7.2 kb) encodes the L protein, an RNA-dependent RNA polymerase (RdRp), and the small RING finger protein Z that is the counterpart of the matrix (M) protein found in many enveloped NS RNA viruses. The small (S) segment (S; 3.5 kb) encodes the viral nucleoprotein (NP) and the glycoprotein precursor (GPC) that is post-translationally processed to yield the peripheral virion attachment protein GP1 and the fusion-active transmembrane protein GP2. Trimers of GP1/GP2 form the spikes that decorate the virus surface and mediate cell entry via receptor-mediated endocytosis (6).

Many viruses interfere with signaling pathways in their infected host cells to favor an environment conducive of a productive infection, which can also impact the host physiology and contribute to virus associated pathogenesis and disease. Therefore the identification

66 and targeting of host cell factors and pathways involved in different steps of a virus life
67 cycle may uncover novel antiviral strategies. In this regard, the PI3K /Akt pathway, known
68 to regulate a variety of cellular processes including cell growth, proliferation, survival, and
69 metabolism (14), has also been involved in the regulation of cell entry (34), as well as RNA
70 replication and gene expression (38) for a variety of viruses. Thus, infection with the New
71 World (NW) arenavirus JUNV was shown to activate the PI3K/Akt pathway (20), and
72 inhibition of the PI3K/Akt pathway resulted in decreased production of infectious progeny
73 due to a blockage on the recycling of the transferrin receptor involved in JUNV cell entry
74 (20). Because significant biological differences have been observed among different
75 arenaviruses (17), we examined whether the PI3K/Akt pathway played also a role in the life
76 cycle of LCMV, the prototypic Old World (OW) arenavirus, a group that includes LASV, the
77 HF arenavirus with the highest impact in public health. For this, we tested a variety of
78 commercially available PI3K/Akt inhibitors. The PI3K/Akt signaling pathway is initiated by
79 receptor-mediated recruitment of catalytically active PI3K to the membrane. Active PI3K
80 converts phosphatidylinositol 4, 5-biphosphate to phosphatidylinositol 3, 4, 5-triphosphate
81 (PIP3). PIP3 facilitates co-localization of Akt with its activating kinase PDK1 that mediates

phosphorylation of Akt at residue Thr 308 (T308) resulting in the initial activation of Akt that is subsequent fully activated by a second phosphorylation event at Ser 473 (S473). Consistent with previous findings treatment with the PI3K inhibitor LY294002 (LY) resulted in strong inhibition of Akt phosphorylation (S473) that was associated with a robust inhibitory effect on LCMV multiplication in the absence of cell toxicity. Mechanism of action studies indicated that LY did not affect virus cell entry but rather viral budding and to lesser extent viral RNA synthesis. To our knowledge, this is the first report showing a contribution of the PI3K/Akt pathway to virus budding. The PI3K/Akt pathway is often upregulated in tumors and therefore is being pursued as a target for anti-tumor therapy, and several inhibitors of the PI3K/Akt pathway are currently undergoing clinical trials as potential drugs for treating several different tumors (21). Our finding that BEZ-235, a dual PI3K/mTOR inhibitor currently in cancer clinical trials, inhibited multiplication of LCMV in cultured cells, provided further impetus to explore targeting of the PI3K/Akt pathway as a novel antiviral strategy to combat human pathogenic arenaviruses.

MATERIALS AND METHODS

Plasmids

LCMV-and LASV-Z expressing plasmids have been described (40). These Z constructs were Flag tagged at their C-termini. p-T7, pMG-CAT, pCAGGS-NP, and pCAGGS-L have been described (18, 19).

Chemical Inhibitors

Akt-IV and Akt-VIII were purchased from CALBIOCHEM (#124015 and #124018, respectively). LY294002 was purchased from Cell signaling (#9901). BEZ-235 was purchased from Selleck chemicals (#s1009). Rapamycin was purchased from SIGMA-ALDRICH (# R0395).

Cells and Viruses

293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum, 2mM l-glutamine, 100mg/ml streptomycin, and 100 U/ml penicillin. Recombinant LCMV (rLCMV), Armstrong strain, r3LCMV/GFP as well as rVSVΔG-GFP/VSV-G and rVSVΔG-GFP/LCMV-GP have been described (12, 41). The GP expressed by rVSVΔG-GFP/LCMV-GP corresponded to that of Armstrong strain of LCMV.

Virus titration

LCMV titers were determined using an immunofocus assay (4). Briefly, 10-fold serial virus dilutions were used to infect Vero cell monolayers in a 96-well plate, and at 20 h p.i., cells were fixed with 4% paraformaldehyde (PFA) in PBS. After cell permeabilization by treatment with 0.3% Triton X-100 in PBS containing 3% BSA, cells were stained by using an anti-NP mouse monoclonal antibody and an Alexa Fluor 568-labeled anti-mouse second antibody (Molecular Probes).

Detection of Akt phosphorylation

Cells were treated with the indicated compounds and concentrations. After 4 or 24 h treatment, cells were washed with PBS and cell lysates prepared using a lysis buffer (1% NP-40, 50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA, 0.4% sodium deoxycholate) supplemented with phosphatase inhibitor Cocktail I (P2850 SIGMA) and II (P5726 SIGMA) just before use. Cell lysates were clarified by centrifugation (13000 x g, 5 min at 4^0C). Samples were analyzed by Western Blot (WB) to detect either total or phosphorylated (S473) Akt.

Cytotoxicity assay

129 The effect of compounds tested in 293T cell viability was assessed using the CellTiter-Glo
130 Luminescent Cell Viability Assay (Promega). This method determines the number of viable
131 cells based on levels of ATP (8). Briefly, 5 x 10^4 cells were plated per 96-well and cultured
132 overnight. Cells were treated with the indicated concentrations of each compound, for 24 h
133 before the CellTiter-Glo reagent was added. Thereafter the assay was performed according
134 to the manufacturer's recommendations and readings obtained using a luminometer
135 (Centro LB 960, Berthold technologies). Viability of compound-treated cells was calculated
136 as % of values obtained with DMSO-treated cells (set at 100 %)

137 **Assessment of LCMV GP-mediated cell entry**

138 Recombinant VSV (rVSVΔG-GFP/VSV-G and rVSVΔG-GFP/LCMV-GP) were used to
139 assess the contribution of the PI3K/Akt pathway to cell entry mediated by the GP of
140 Armstrong strain of LCMV. 293T cells were pre-treated with the indicated compounds and
141 concentrations for 1h prior infection (moi = 1) with the indicated rVSV in the presence of
142 drug. At 12 h p.i. cells were fixed (4% PFA/PBS) and numbers of GFP positive cells
143 determined by direct epifluorescence. Total cells and GFP positive cells were counted in
144 four different fields and the average and SD of the % of GFP + cells determined. Results

represent the average +/- SD of the results from the two independent infections for each virus.

LCMV minigenome assay

293T cells were seeded (4.5 x 10^5 per well) on M-12 well plates and the following day transfected with p-T7, pMG-CAT, pCAGGS-NP, and pCAGGS-L using conditions previously described (18, 19, 29, 31, 32). At 5 h post-transfection, the transfection medium was replaced with fresh medium containing the indicated compounds and concentrations. At 24 h post-transfection cell lysates were prepared to determine levels of CAT protein by ELISA using a CAT ELISA kit (Roche, 11363727001). Briefly, cells were lysed with 1 ml of lysis buffer and equal amounts (4 μl) of each sample used for ELISA. Dilutions of a known amount of CAT were used to generate a calibration curve. CAT ELISA plates were incubated for 1 h at 37 ℃, followed by two washes with wash buffer, followed by reaction with an antibody to CAT for 1 h at 37 °C. After reaction with the primary antibody, samples were washed twice and then reacted with the secondary antibody conjugated to peroxidase for 1 h at 37 °C, followed by two washes prior adding the substrate. After 20 min at room temperature samples were analyzed using an ELISA reader (SPECTRA max plus 384,

Molecular Devices) to determine the absorbance (405 nm for samples, 490 nm for the reference).

RNA analysis by Northern blot hybridization

Total cellular RNA was isolated by using TRI Reagent (Molecular Research Center, Inc) according to the manufacturer's instructions and analyzed by Northern blot hybridization. Briefly, RNA samples were fractionated by 2.2 M formaldehyde-agarose (1.2 %) gel electrophoresis followed by transfer (4 h) in 20 X SSC of the RNA to a Magnagraph (0.22 µm) membrane using the rapid downward transfer system (TurboBlotter). Membrane bound RNA was crosslinked by exposure to UV and the membrane was hybridize to a ^{32}P-labeled strand specific probe to the MG-derived CAT mRNA.

Pol-II based transcription assay

293T cells were seeded ($8x10^4$/well) on 96 well plate and next day transfected with pC-Fluc (27.5 ng/well) using Lipofectamine 2000 (Invitrogen). 12 h later, the transfection medium was replaced with fresh medium containing the indicated compounds and concentrations, and 24 h later cell lysates were prepared for firefly luciferase (Fluc) assay using the Steady Glo lysis buffer (Promega) and conditions according to the manufacturer's

recommendations. Values of Fluc activity were obtained using a luminometer (Centro LB 960, Berthold technologies). Level of Fluc activity obtained with lysates from compound-treated cells were determined as % of values obtained with lysates of DMSO-treated control cells (set at 100%).

Budding Assay

293T cells (2.5 x 10^5) were transfected with 0.25 μg of either pC-LCMV-Z or pC-LASV-Z using Lipofectamine 2000 (LF) (2.5 µl LF/µg DNA). At 5 h after transfection, the medium was replaced with fresh medium containing the indicated compounds and concentrations. At 24 h treatment, VLP-containing tissue culture supernatants and cells were collected. After clarification from cell debris (1,500 x g; 5 min), VLPs were collected by ultracentrifugation (100,000 x g; 30 min at 4 °C) through a 20% sucrose cushion. Cells and VLPs were re-suspended in lysis buffer (1% NP-40, 50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA, 0.4% sodium deoxycholate) and analyzed by Western blot.

Immunoblotting

Cell lysates or VLP samples were resolved by SDS-PAGE followed by Western blot (WB) using the indicated antibodies. To detect Flag-tagged Z proteins, we used a rabbit

193 polyclonal serum to Flag (Cayman, 162150) and an anti-rabbit IgG conjugated to HRP as

194 secondary antibody. To detect either total Akt or phosphorylated Akt (S473), mouse

195 monoclonal antibody to Akt (Cell signaling #9272) and to phosphorylated (S473) Akt (Cell

196 signaling #9271), respectively, were used combined with an anti-mouse IgG conjugated to

197 HRP as secondary antibody.

198

199

RESULTS

Effect of PI3K inhibitors on LCMV propagation

We first tested the effect of several commercially available PI3K and Akt inhibitors on LCMV multiplication in cultured cells following infection at both low (0.001) and high (1.0) moi. For this, we infected 293T cells with Armstrong strain of LCMV in the absence of inhibitors during the adsorption time (90 min), followed by addition of each inhibitor tested at the indicated concentrations (**Fig 1A**). At the indicated h p.i. we determined titers of infectious virus in tissue culture supernatants (TCS). The PI3K inhibitor LY inhibited LCMV propagation following infection at both low and high moi, but the drug's inhibitory effect was significantly stronger in cells infected at low moi (**Fig 1A**).

In contrast to the results observed with LY, the Akt inhibitors Akt-IV and Akt-VIII did not affect significantly LCMV propagation following infection at either low or high moi. This finding led us to examine whether under our experimental conditions there was a correlation between the antiviral activity of the compound tested and their ability to inhibit Akt phosphorylation. For this, we treated 293T cells with the different inhibitors at the indicated concentrations and 4 or 24 h later prepared cell lysates to determine levels of Akt

phosphorylation by Western blot (**Fig 1Bi**). Contrary to the commonly assumed properties of Akt-IV (16, 38), but consistent with a recent report (11), we observed that in 293T cells Akt-IV did not inhibit Akt phosphorylation at residue S473. Unexpectedly, under our experimental conditions Akt-VIII treatment also failed to inhibit Akt phosphorylation. In contrast, LY treatment inhibited efficiently phosphorylation of Akt. At the concentrations tested LY and Akt-VIII did not cause significant cell toxicity, whereas treatment with Akt-IV even at the lowest (0.5 µM) concentration tested reduced cell survival by about 60 % as determined by the Cell titer Glo assay (8) (**Fig 1Bii**).

Our finding that LY inhibited very efficiently LCMV multiplication led us to explore the possible repurposing of PI3K inhibitors as anti-arenaviral drugs. For this we used BEZ-235, which is currently in clinical trial for treatment of advanced solid tumors (21). BEZ-235 is a synthetic low molecular mass compound belonging to the class of imidazoquinolines that potently and reversibly inhibits class 1 PI3K catalytic activity by competing at its ATP-binding site. BEZ-235 also inhibits mTOR catalytic activity but does not target other protein kinases (22, 36). Under our experimental conditions BEZ-235 inhibited in a dose-and temporal-dependent manner Akt phosphorylation in 293T cells (**Fig 2A**). Treatment with 1

232 nM BEZ-235 for 24 h resulted in significant reduced levels of phosphorylated Akt at S473,
233 whereas at 50 nM the inhibitory effect was already observed after 4 h treatment. Within the
234 dose range tested BEZ-235 exhibited minimal cell toxicity (**Fig 2B**). To examine the
235 potential antiviral activity of BEZ-235 against LCMV, we infected 293T cells (moi = 0.01)
236 and treated them with BEZ-235 at the indicated concentrations, and at the indicated h p.i.
237 we determined titers of infectious virus in TCS (**Fig 3**). Within the 0.5-5 μM range BEZ-235
238 caused about 2-2.5 logs reduction in production of infectious progeny in the absence of
239 noticeable cell toxicity.

240 **Effect of PI3K inhibitors on LCMV entry**

241 To examine the effect of LY and BEZ-235 on LCMV GP-mediated cell entry we used a
242 recombinant VSV (rVSVΔG-GFP/LCMV-GP) whose cell entry is mediated by the GP of
243 strain Armstrong of LCMV (41). The rational for using this rVSV was that multiplication of
244 VSV was shown not to be affected by LY (10). Therefore, an effect of LY or BEZ-235
245 treatment on rVSVΔG-GFP/LCMV-GP multiplication would reflect the drug's effect on
246 LCMV GP-mediated cell entry. We treated 293T cells with LY (50 μM) or BEZ-235 (5μM)
247 for 1 h prior infection (moi = 1) with rVSVΔG-GFP/LCMV-GP or with the control rVSVΔG-

248 GFP/VSV-G, a rVSV expressing its own glycoprotein (G). At 12 h p.i. cells were fixed and
249 numbers of infected cells determined based on GFP positive cells. Neither LY (**Fig 4A**) nor
250 BEZ-235 (**Fig 4B**) treatment affected significantly the numbers of GFP + cells following
251 infection with either rVSVΔG-GFP/VSV-G or rVSVΔG-GFP/LCMV-GP. These results
252 indicated that the PI3K/Akt pathway does not play a significant role in cell entry mediated
253 by the GP of Armstrong strain of LCMV. We obtained similar results when compounds (LY
254 or BEZ-325) were added 4 h prior starting virus adsorption, or when adsorption was done
255 at 4^0C in the absence of drug, followed by changing to pre-warmed (37^0C) medium
256 containing LY or BEZ-235 and transfer of cells to 37^0C. Together these findings would
257 suggest that an early step of cell entry was unlikely to be affected by LY or BEZ-235.

258 **Effect of PI3K and Akt inhibitors on LCMV RNA replication and gene**
259 **transcription**

260 To examine whether the PI3K/Akt pathway played a role in LCMV RNA replication and
261 gene transcription we used an LCMV minigenome (MG) assay (18, 19, 29, 31, 32) that
262 allowed us to separate the steps involved in virus RNA replication and transcription from
263 those involved in cell entry, as well as virus particle assembly and cell egress. In this MG-

264 based assay expression of the CAT reporter gene was used as a surrogate to measure
265 levels of RNA synthesis by the intracellularly reconstituted LCMV polymerase complex. LY
266 exhibited a dose-dependent inhibitory effect on LCMV MG derived reporter gene
267 expression (30 % and 70 % reduction in CAT expression at 20 μM and 50 μM, respectively),
268 whereas treatment with Akt-VIII had a very modest effect on MG derived CAT expression
269 (**Fig 5A**). Intriguingly, although Akt-IV did not exhibit an inhibitory effect on LCMV
270 propagation, treatment with Akt-IV resulted in a significant (40%) reduction of CAT
271 expression (**Fig 5A**). A plausible explanation for these apparent contradicting findings
272 would be that Akt-IV treatment could have negatively impacted Pol-II based transcription
273 and thereby affecting levels of plasmid supplied LCMV NP and L proteins in the MG-based
274 assay. To evaluate this possibility we determined the effect of LY, Akt-VIII and Akt-IV on
275 firefly luciferase (Fluc) expression mediated by a Pol-II based expression plasmid (**Fig 5B**).
276 LY (20 and 50 μM) and Akt-VIII (2.0 μM) did not affect significantly levels of Fluc
277 expression, whereas treatment with Akt-IV caused a very significant reduction in levels of
278 Fluc expression (50% at 0.5 μM). As with LY, we observed a dose-dependent inhibitory
279 effect of BEZ-235 on MG derived CAT expression (30%, 50%, and 65% reduction, at 50

nM, 500 nM and 5 μM, respectively) (**Fig 5A**). BEZ-235 had a minimal effect on Pol-II mediated expression of Fluc (**Fig 5B**), suggesting that the effect of BEZ-235 on LCMV MG expression was not the result of an overall drug's effect on Pol-II mediated transcription.

To further examine the effects of LY and BEZ-235 in RNA synthesis mediated by the LCMV polymerase, we used Northern blot to determine levels of both RNA replication and transcription in the LCMV MG assay (**Fig 6**). We observed a good correlation between the effect on CAT protein expression caused by either LY or BEZ-235 and the corresponding drug effect on levels of both MG replication and MG-derived CAT mRNA.

Effect of LY and BEZ-235 on LCMV and LASV Z-mediated budding

Arenavirus Z protein has been shown to be the main driving force of virus budding (28, 37, 39). Since the magnitude of the inhibitory effect exerted by LY or BEZ-235 on LCMV multiplication was much higher than their corresponding effects on viral RNA synthesis, we examined whether LY could inhibit Z-mediated budding, a key step of the virus life cycle required for production and propagation of infectious progeny. For this, we transfected 293T cells with either pC-LCMV-Z-FLAG or pC-LASV-Z-FLAG and 12 h later, cells were washed and fresh media containing the indicated concentration of LY or BEZ-235 added.

296 At 24 h post treatment, we collected TCS and prepared cell lysates to detect levels of Flag-
297 tagged Z protein in both cell lysates and VLP recovered from TCS of transfected cells by
298 WB using an anti-FLAG antibody. Both LY (**Fig 7A**) and BEZ-235 (**Fig 7B**) exerted a
299 robust dose-dependent inhibitory effect on Z mediated budding without significantly
300 affecting levels of Z expression in transfected cells. The reasons for the apparent higher
301 inhibitory effect of LY on budding mediated by LASV-Z, compared to LCMV-Z, remain to be
302 determined.

303 **Effect of rapamycin on LCMV multiplication**

304 BEZ-235 was shown to inhibit class 1 PI3K catalytic activity of both class 1 PI3K and
305 mTOR (22, 36). To assess the possible contribution of mTOR inhibition to the antiviral
306 activity associated with BEZ-235 we examined the effect of rapamycin (Rpm), a well
307 characterized mTOR inhibitor, on LCMV multiplication. Production of infectious virus in
308 Rpm-treated cells was not significantly affected (**Fig 8A**). Consistent with this observation,
309 virus cell propagation (**Fig 8B**) and virus RNA replication and gene transcription (**Fig 8C**)
310 were not significantly affected by Rpm treatment.

DISCUSSION

PI3K has been shown to have key regulatory roles in many cellular processes, including cell survival, proliferation and differentiation. PI3K transduce signals from various growth factors and cytokines into intracellular messages by generating phospholipids, which activate the serine-threonine protein kinase Akt (aka protein kinase B (PKB)) and other downstream effector pathways (14). Since many components of the PI3K and the Akt (PI3K/Akt) pathway are frequently targeted by germ line mutations or somatic mutations in a broad range of human cancers, PI3K has become an attractive target for therapeutic intervention in cancer (21). On the other hand, more recent findings have uncovered a relationship between the PI3K/Akt pathway and different steps of the life cycle for a variety of RNA viruses. Thus, recognition of cell surface receptors by a variety of virus surface glycoproteins can result in activation of the PI3K/Akt pathway to promote virus cell entry (34). In addition, some viral proteins like VSV M have been shown to down regulate PI3K/Akt signaling at an early stage of virus replication (10). Likewise, there is evidence that the PI3K/Akt pathway plays an important role in RNA replication of a variety of negative stranded (NS) RNA viruses (38).

327 Infection with JUNV has been shown to activate Akt phosphorylation via a mechanism
328 that did not require active virus replication or gene expression (20). Moreover, LY-
329 mediated inhibition of the PI3K/Akt pathway resulted in decreased production of JUNV
330 infectious progeny, which was proposed to be caused by an LY induced blockage on the
331 recycling of transferring receptor proposed to mediate JUNV cell entry (20). Because
332 significant differences have been reported regarding the biological features displayed by
333 NW and OW arenaviruses, including the use of different receptors for cell entry (7, 17, 33),
334 we investigated whether the PI3K/Akt pathway had also an effect on multiplication of the
335 prototypic OW arenavirus LCMV.
336 The PI3K inhibitor LY, but not the Akt inhibitors Akt-IV and Akt-VIII, inhibited
337 multiplication of LCMV following infection at low moi (**Fig 1A**). Akt-VIII has IC50 of 58 nM,
338 210 nM and 2.12 µM for the Akt isoforms 1, 2 and 3, respectively. Therefore, in cell treated
339 with 2 µM Akt-VIII, Akt3 would be predicted to retain significant activity that could have
340 accounted for the lack of inhibition of LCMV multiplication in cells treated with 2 µM Akt-VIII.
341 Nevertheless, we observed that levels of p27 phosphorylation, known to be mainly an Akt3
342 target (5) were not affected in 293T cells treated with 2 µM Akt-VIII as determined by

343 Western blot using a phospho-p27 (T157)-specific antibody (not shown). In addition,
344 whereas LY treatment inhibited phosphorylation of Akt at S473 (**Fig 1B**), under our
345 experimental conditions Akt phosphorylation at S473 was not significantly affected by
346 treatment with Akt-IV or Akt-VIII (**Fig 1B**). The inability of Akt-IV to inhibit Akt
347 phosphorylation was unexpected based on its published properties (38), but consistent with
348 a recent report showing that Akt-IV blocked VSV multiplication without affecting Akt
349 phosphorylation (11). Akt-VIII has been shown to be a direct inhibitor of the kinase activity
350 of Akt in several cell systems (2) and to have antiviral activity (1). The reason why under
351 our experimental conditions we did not observe an Akt-VIII mediated inhibition of Akt
352 phosphorylation is presently unknown. One possibility is that the inhibitory activity of Akt-
353 VIII may be cell dependent and at 2 μM it could robustly inhibit phosphorylation of Akt in
354 BHK-21 (11) but not in 293T cells used in our studies.
355
356 The lesser inhibitory effect of LY on LCMV multiplication following infection at high
357 compared to low moi suggested a more strict requirement of the integrity of the PI3K/Akt
358 pathway in the late steps of the virus life cycle. Accordingly, LY and BEZ-235 treatment did

359 not affect cell entry mediated by the GP of Armstrong strain of LCMV (**Fig 4**). However, a
360 recent report by Pasqual and colleagues has documented that LASV and LCMV enter host
361 cells via the mutlivesicular body (MVB) pathway (27), and biogenesis and functionality of
362 the MVB requires the lipid PI3P and hence also PI3K activity. Accordingly, treatment with
363 the PI3K inhibitor wortmannin significantly affected cell entry by LASV or LCMV GP (27). A
364 possible explanation for these apparent conflicting findings is that wortmannin is able to
365 rapidly and specifically inhibit some of the non-classical PI3K isoforms that might
366 compensate for LY-mediated inhibition of major PI3K forms. Likewise, differences in cell
367 entry mediated by GPs of Armstrong (our study) and Cl-13 (study by Pasqual and
368 colleagues) strains of LCMV may have also contributed to the differences between our
369 results and those reported by Pasqual and colleague (27). In this regard it should be noted
370 that VSV cell entry was shown to be depended also on the MVB and ESCRT but it was not
371 affected by wortmannin treatment, suggesting that VSV-G mediated fusion with
372 membranes of the intraluminal vesicles (ILV) within the MVB takes place under mildly
373 acidic pH and the virus ribonucleoprotein core is delivered to late endosomes in the lumen

374 of the ILVs (23). Similarly, it is plausible that Armstrong GP2 may promote fusion between
375 viral and cellular membranes at higher pH than GP2 from Cl-13 or LASV.

376 Both LY and BEZ-235 had a significant and specific inhibitory effect on MG-derived
377 CAT expression levels (**Fig 5A**). In contrast, the observed inhibitory effect of Akt-IV on MG-
378 derived reporter gene activity was likely a reflection of a general effect of Akt-IV on cell
379 viability and Pol-II mediated transcription (**Fig 1Bii and 5B**). These results suggested a role
380 the PI3K/Akt pathway in LCMV RNA replication and gene transcription, which was
381 experimentally supported by Northern blot analysis of MG-derived RNA species in the
382 absence or presence of PI3K inhibitors (**Fig 6**). The mechanisms by which either LY or
383 BEZ-235 affect the activity of the arenavirus polymerase remain to be elucidated. Previous
384 studies have shown that Akt directly phosphorylates Parainfluenza virus 5 (PIV5) and
385 Respiratory syncytial virus (RSV) polymerase cofactor P protein, and this phosphorylation
386 of P has a critical role for virus polymerase activity (38). However, arenaviruses do not
387 have a counterpart of the P protein found in many other NS RNA viruses.

388 Consistent with a more pronounced involvement of the PI3K/Akt pathway in late steps
389 of the LCMV life cycle, both LY (**Fig 7A**) and BEZ-235 (**Fig 7B**) exhibited a strong dose-

390 dependent inhibitory effect on Z mediated budding, which documents for the first time a role
391 of the PI3K/Akt pathway in virus budding. The degree of inhibition of Z-mediated budding
392 appeared to be higher in cells treated with BEZ-235 compared to LY-treated cells. It seems
393 however unlikely that a PI3K-independent mTOR activation may have also contributed to Z-
394 mediated budding, as the mTOR inhibitor Rpm did not affect significantly LCMV
395 multiplication in cultured cells (Fig 8). The PI3K/Akt pathway participates in the regulation
396 of many cellular processes including vesicular trafficking (35). PI3P, the product of the PI3K
397 activity, is needed for efficient assembly of the ESCRT complex at the limiting membrane of
398 the early endosome and some ESCRT proteins contain modules that recognize PI3P.
399 Budding of a variety of enveloped viruses, including arenaviruses, involves interactions
400 between viral budding proteins and ESCRT. It is plausible that these interactions may be
401 facilitated by virus induced PI3P-rich “microdomains” at the site of budding and thereby
402 inhibition of PI3K activity will disrupt normal budding. The PI3K/Akt pathway might be
403 directly involved in phosphorylation of LCMV and LASV Z protein, which may be necessary
404 for efficient Z-mediated budding. The use of NetPhos 2.0 software
405 (http://www.cbs.dtu.dk/services/NetPhos/), identified six S residues in both LCMV and

406 LASV-Z, as well as three (LCMV-Z) and one (LASV-Z) Y residues as highly likely
407 substrates for phosphorylation (not shown). Whether Z is target of PI3K/Akt mediated
408 phosphorylation and the possible role of phosphorylation in the regulation of the budding
409 activity of Z are issues that remain to be determined. Likewise, a similar analysis identified
410 twenty one S, four T and four Y residues within NP as highly likely substrates for
411 phosphorylation. Whether PI3K/Akt mediated phosphorylation of NP could contribute to
412 regulation of RNA synthesis by the arenavirus polymerase remains to be determined.
413 Our findings, together with those previously documented for JUNV, suggest that
414 targeting the PI3K/Akt pathway may offer the possibility to inhibit multiplication of both OW
415 and NW HF arenaviruses. It should be also noted that strong evidence indicates that
416 morbidity and mortality of HF arenaviruses correlate with high viral load due to the failure of
417 the host's both innate and adaptive immune responses to restrict virus multiplication.
418 Targeting the PI3K/Akt pathway could reduce virus load and rate of propagation and
419 thereby provide the host with a window of opportunity to mount an efficient anti-viral
420 immune response. Targeting a cellular factor or pathway required for optimal viral growth
421 would offer the advantage to overcome the problem related to the emergence of drug-

422 resistant viral variants commonly observed with antiviral drugs against RNA viruses
423 characterized by their high error prone replication machineries. The detailed mechanisms
424 by which inhibition of the PI3K/Akt pathway affects LCMV RNA synthesis and budding
425 remain to be determined. The identification of specific cellular effectors contributing to
426 impaired LCMV RNA synthesis and budding upon inhibition of the PI3K/Akt pathway,
427 together with current efforts to develop cancer therapies based on targeting of the PI3K/Akt
428 pathway, should facilitate the identification of anti-cancer drugs with potential repurposing
429 value as antiviral drugs to combat human pathogenic arenaviruses. This possibility is
430 illustrated by BEZ-235, a synthetic small molecule that is a dual PI3K and mTOR inhibitor
431 and is currently being tested in clinical trials for solid tumors (21, 22, 36), which also
432 exhibited a potent dose-dependent antiviral activity against LCMV within a range (0.5-5 μM)
433 concentration that had only very modest effects on cell viability.
434

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FIGURE LEGENDS

Figure 1. Effect of PI3K/Akt inhibitors on multiplication of LCMV and Akt phosphorylation. A. LY inhibits multiplication of LCMV in cultured cells. 293T cells were infected with LCMV (moi of 0.001 or 1.0). After 90 min adsorption time, the inoculum was removed, cell monolayers were washed, and fresh medium containing Akt-IV (0.5 μM or 2 μM), Akt-VIII (2 μM), or LY (20 or 50 μM) were added. At the indicated times p.i. virus titers were determined in TCS using an IFF assay (see materials and methods). **B. Effect of LY on Akt phosphorylation.** Bi. 293T cells were treated with Akt-IV (0.5 or 2 μM), Akt-VIII (2 μM), LY (20 or 50 μM) or DMSO as a control. At 4 or 24 h post treatment cell lysates were prepared and total Akt and phosphorylated-Akt (S473) were detected by Western Blot (WB). Bii. Cell toxicity associated with the indicated drug treatments was assessed by determining cell viability after 24 h of treatment using CellTiter-Glo Luminescent Cell Viability Assay (Promega). DMSO treatment was adjusted to 1.0. Data are averages and standard deviations from three independent experiments normalized with respect to DMSO treatment.

Figure 2. Effect of BEZ-235 on Akt phosphorylation in 293T cells. A. 293T cells were treated with BEZ-235 (1, 5, 50, 500 nM or 5 μM) or DMSO as a control. At 4 or 24 h post treatment, cell lysates were prepared and total Akt and phosphorylated-Akt (S473) were detected by Western Blot (WB). B. Cell toxicity associated with BEZ-235 treatment. 293T cells were treated with the indicated BEZ-235 concentrations, or DMSO, for 24 h and then cell viability determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Survival in DMSO-treated cells was adjusted to 1.0. Data are averages and standard deviations from three independent experiments.

Figure 3. BEZ-235 inhibits LCMV multiplication in cultured cells. 293T cells were infected with LCMV (moi=0.01). After 90 min of adsorption time, the inoculum was removed, cell monolayers washed, and fresh medium containing the indicated BEZ-235 concentration added. At the indicated times p.i.virus titers were determined in TCS using an IFF assay (materials and methods).

Figure 4. LY and BEZ-235 do not inhibit LCMV GP-mediated cell entry. 293T cells were pre-treated with LY (50 μM) (A) or BEZ-235 (5 μM) (B) for 1 h prior infection with either VSVΔG-GFP/VSV-G or rVSVΔG-GFP/LCMV-GP (moi =1.0). Infections were done in the

presence of drugs. At 12 h p.i. cells were fixed and for each sample numbers of GFP positive cells in four different fields determined by epifluorescence. Averages and standard deviations were obtained. Numbers of GFP positive cells were normalized with respect to values obtained in non-treated cells that were adjusted to 100%.

Figure 5. Effects of commercially available PI3K/Akt inhibitors and BEZ-235 on LCMV MG derived reporter gene expression. A. Drug effects on MG derived reporter gene expression. 293T cells were transfected with pC-T7, pMG-CAT, pC-NP, and pC-L as described (18, 19, 29, 31, 32). After 5 h transfection medium was replaced with fresh media containing Akt-IV (0.5 µM), Akt-VIII (2.0 µM), LY (20 or 50 µM), or BEZ-235 (0.05, 0.5 or 5 µM). At 24 h post-transfection, cell lysates were prepared for CAT ELISA. CAT expression levels from vehicle (DMSO)-treated cells were set to 1.0 to normalize CAT expression levels from the other samples. B. Drug effects on Pol-II based transcription. 293T cells ($8x10^4$/ 96 well) were transfected with pC-Fluc using Lipofectamine 2000 (Invitrogen), and 12 h later, media was replaced with the fresh media containing the indicated compounds and concentration. At 24 h post-compound treatment levels of Fluc were determined using the Steady Glo assay (Promega) and a luminometer (Centro LB 960, Berthold

613 technologies). Viability of DMSO-treated control cells was set at 1.0. Data are averages

614 and standard deviations from three independent experiments.

615 **Figure 6. Effects of LY and BEZ-235 on LCMV-MG derived RNA synthesis.** 293T cells

616 were transfected with pC-T7, pMG-CAT, pC-NP, and pC-L as described in Fig 5. After 5 h

617 transfection medium was replaced with fresh media containing the indicated drugs and

618 concentrations and 24 h later total cell RNA was isolated and analyzed by Northern blot

619 using a ^{32}P RNA probe that specifically hybridized to the CAT mRNA and the recombinant

620 S antigenome (rSag) RNA species.

621 **Figure 7. Effect of LY and BEZ-235 on Z mediated budding.** 293T cells were transfected

622 with 0.25 µg of either pC-LCMV-Z-FLAG or pC-LASV-Z-FLAG, and 12 h post-transfection,

623 media were replaced with fresh media containing the indicated concentration of LY (A) or

624 BEZ-235 (B), and 24 h later TCS were collected and total cell lysates prepared. VLPs were

625 isolated from TCS as described (40). Levels of Z protein in total cell lysates and VLPs were

626 detected by WB using an antibody to FLAG (Cayman, 162150).

627 **Figure 8. Effect of Rpm on LCMV multiplication**. BHK-21 cells were infected (moi = 0.1)

628 with r3LCMV/GFP (12) and treated with Rpm at the indicated concentrations. At the

629 indicated h p.i., TCS were collected and cell monolayers fixed in 4 % PFA/PBS. In addition,

630 total cellular RNA was isolated from duplicate infections treated with the indicated

631 concentration of Rpm. **A.** Infectious progeny in TCS was determined using an IFF assay. **B.**

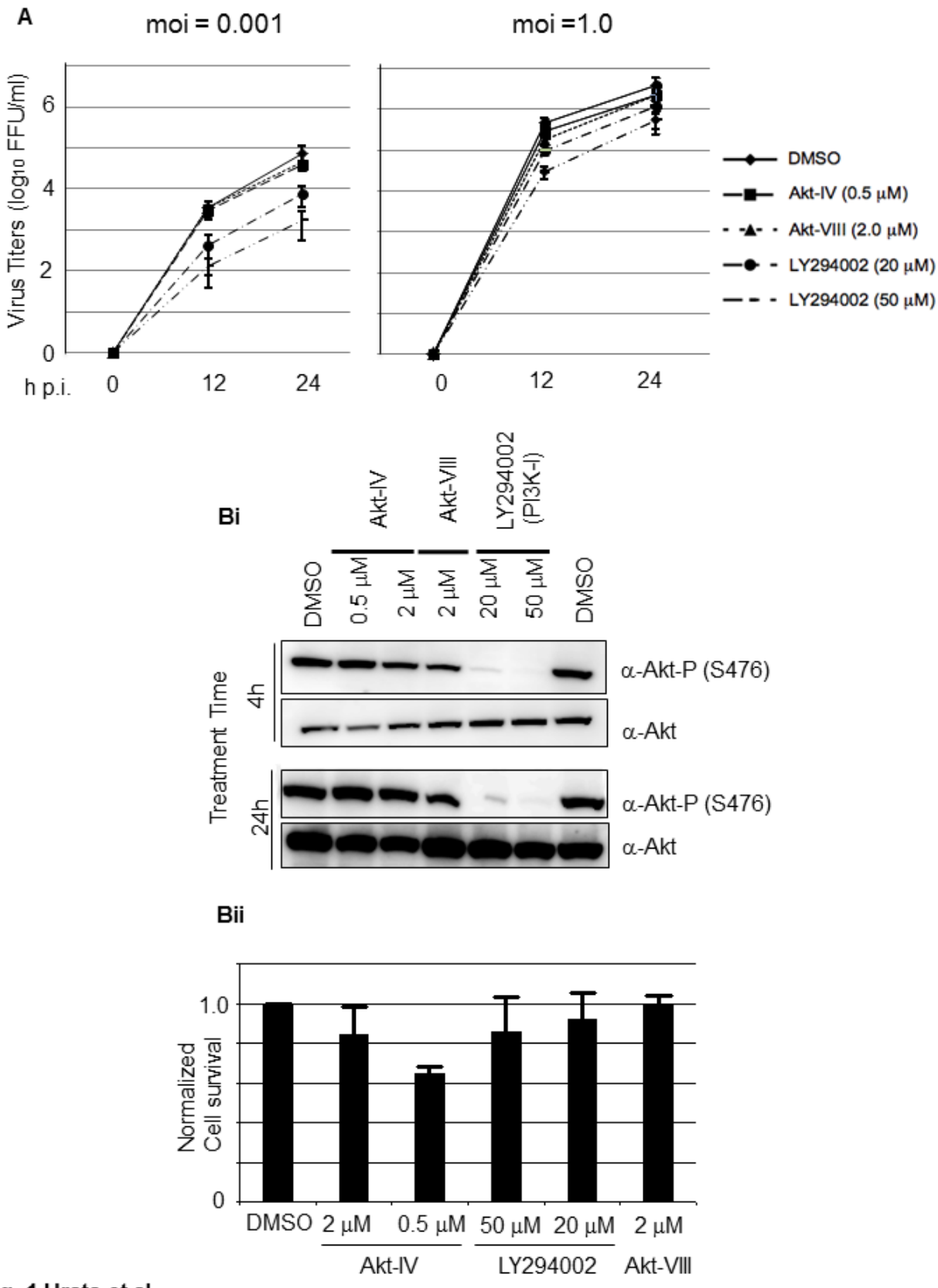
632 Numbers of virus-infected cells in each case were determined based on GFP expression. **C.**

633 Levels of viral RNA synthesis, both replication and transcription, were assessed by

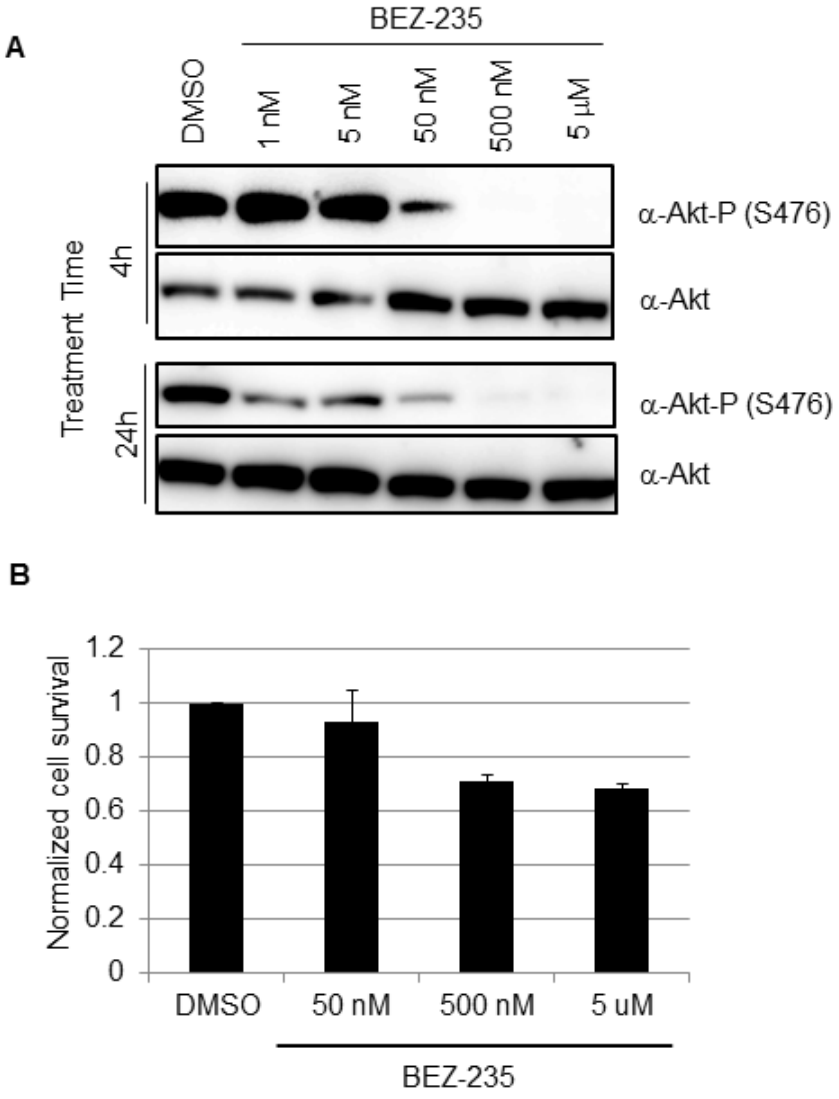
634 Northern blot hybridization using an NP-specific double strand DNA probe that hybridized

635 to the rS (replication) and NP mRNA (transcription) RNA species.

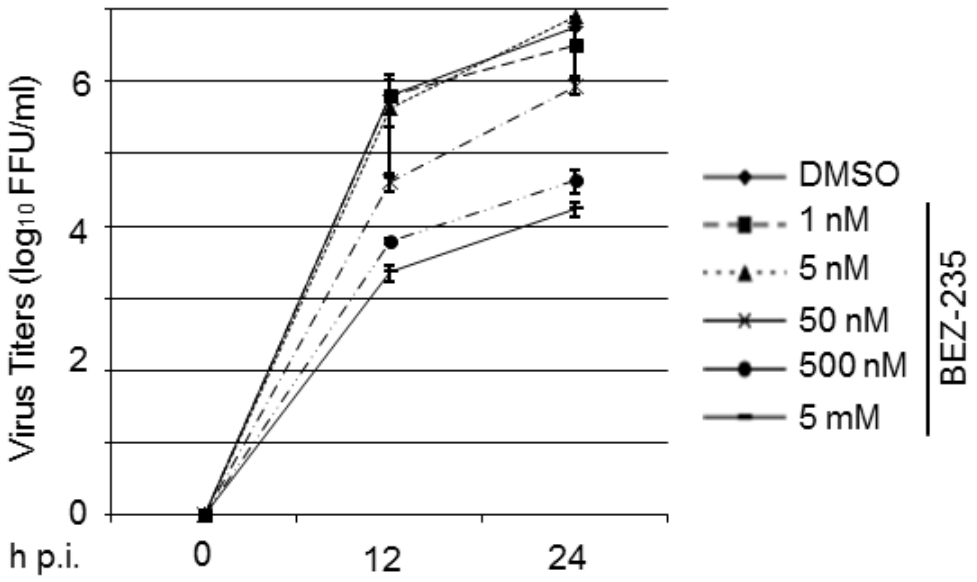
636



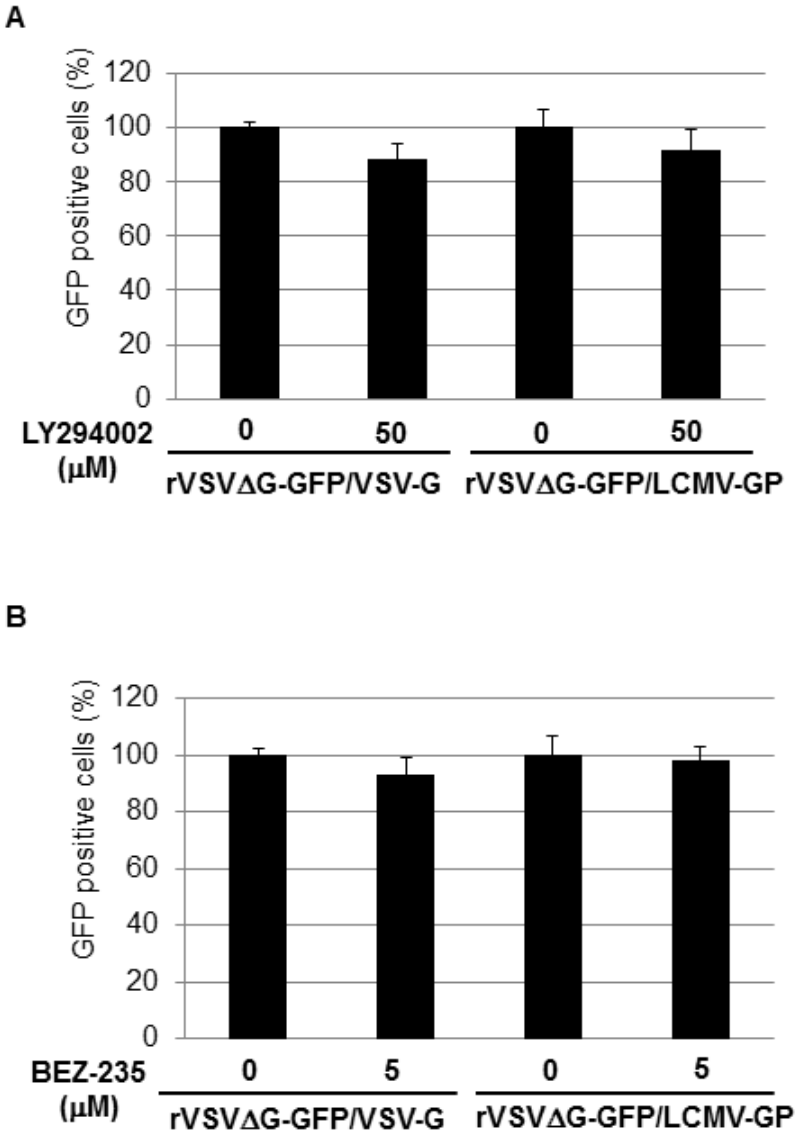
637 **Fig. 1 Urata et al.**



638 **Fig. 2 Urata et al.**



639 **Fig. 3 Urata et al.**



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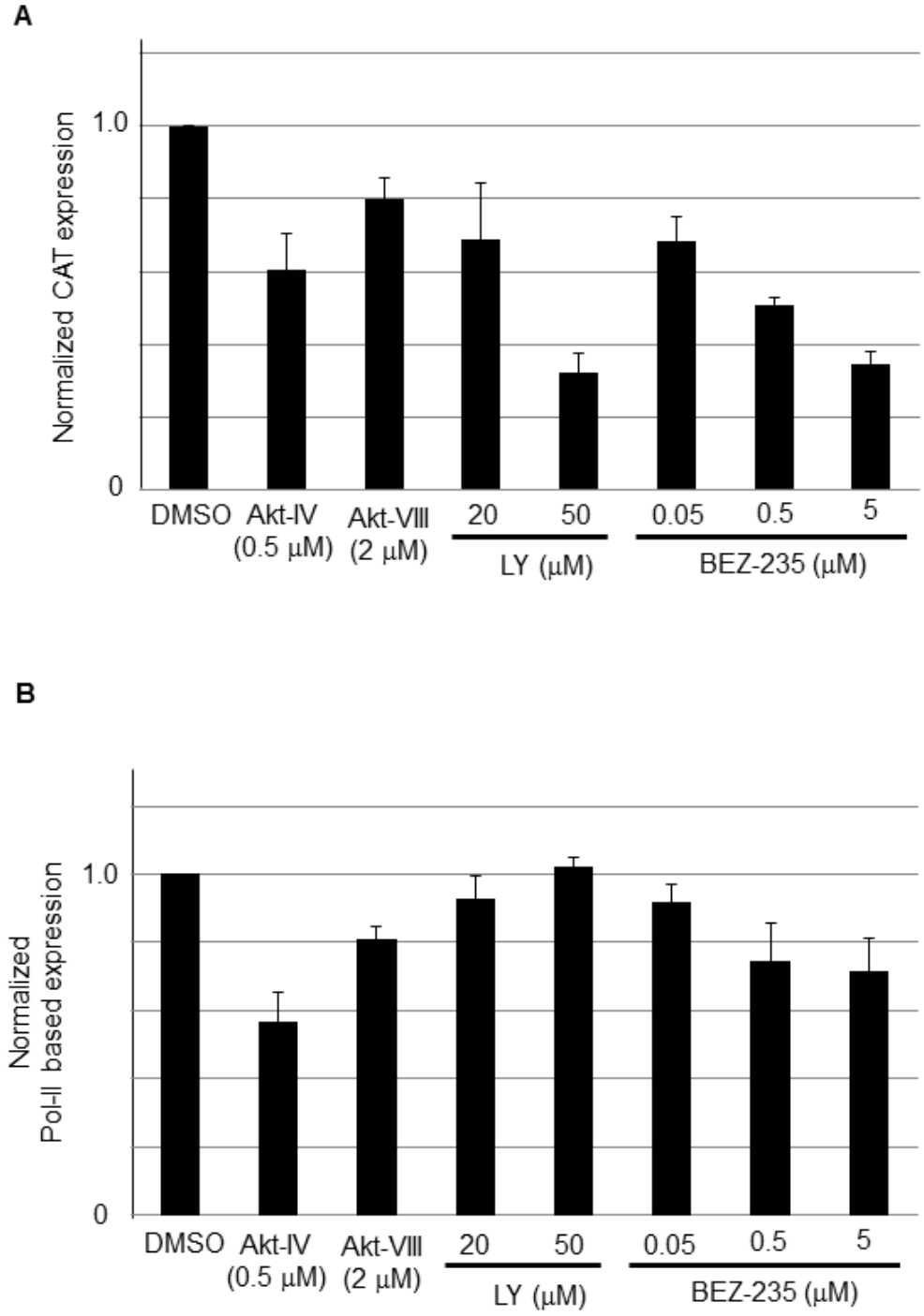

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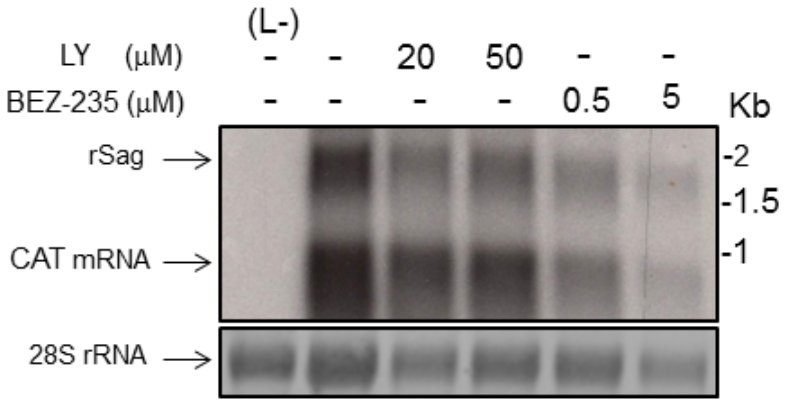

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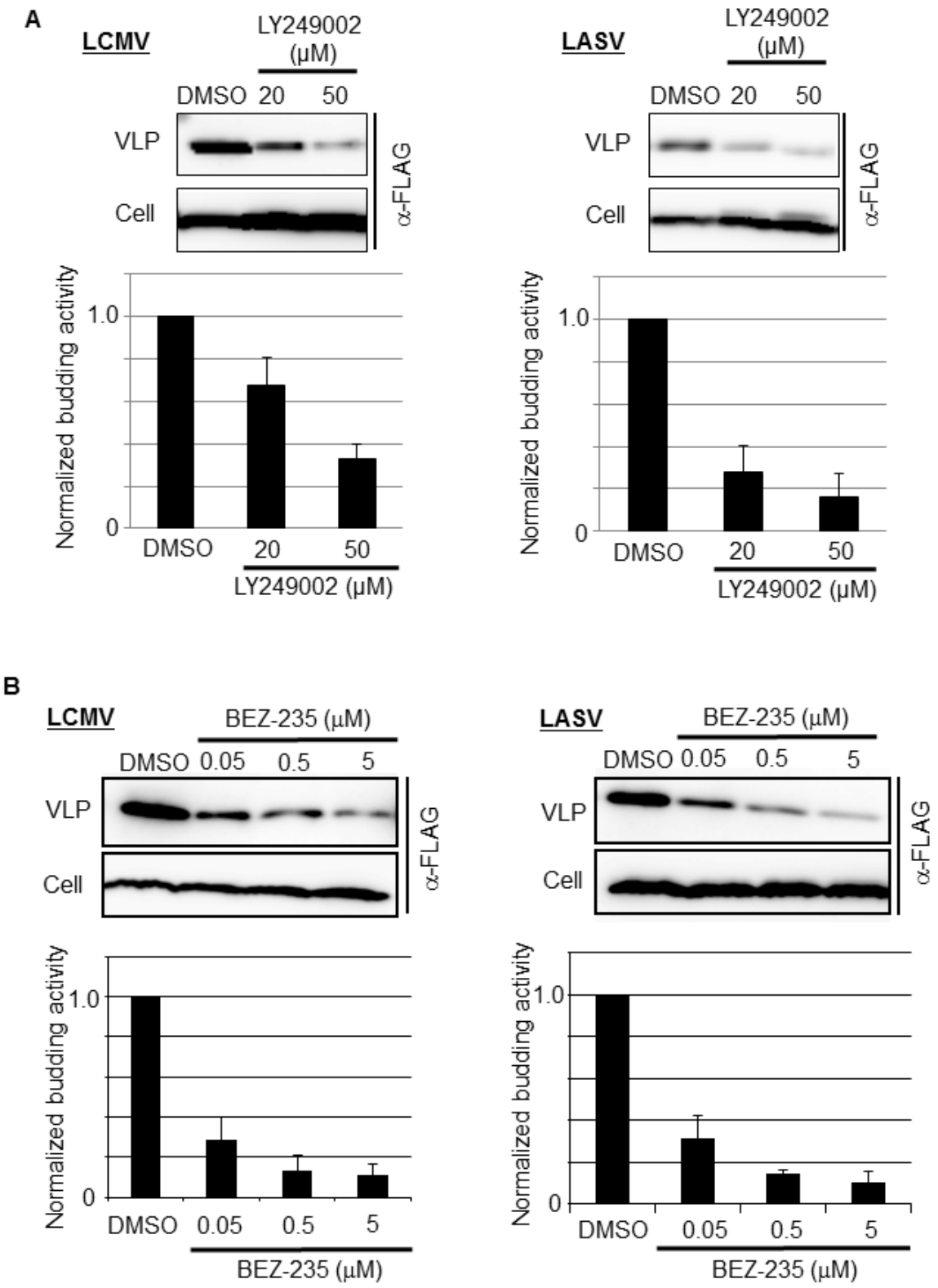

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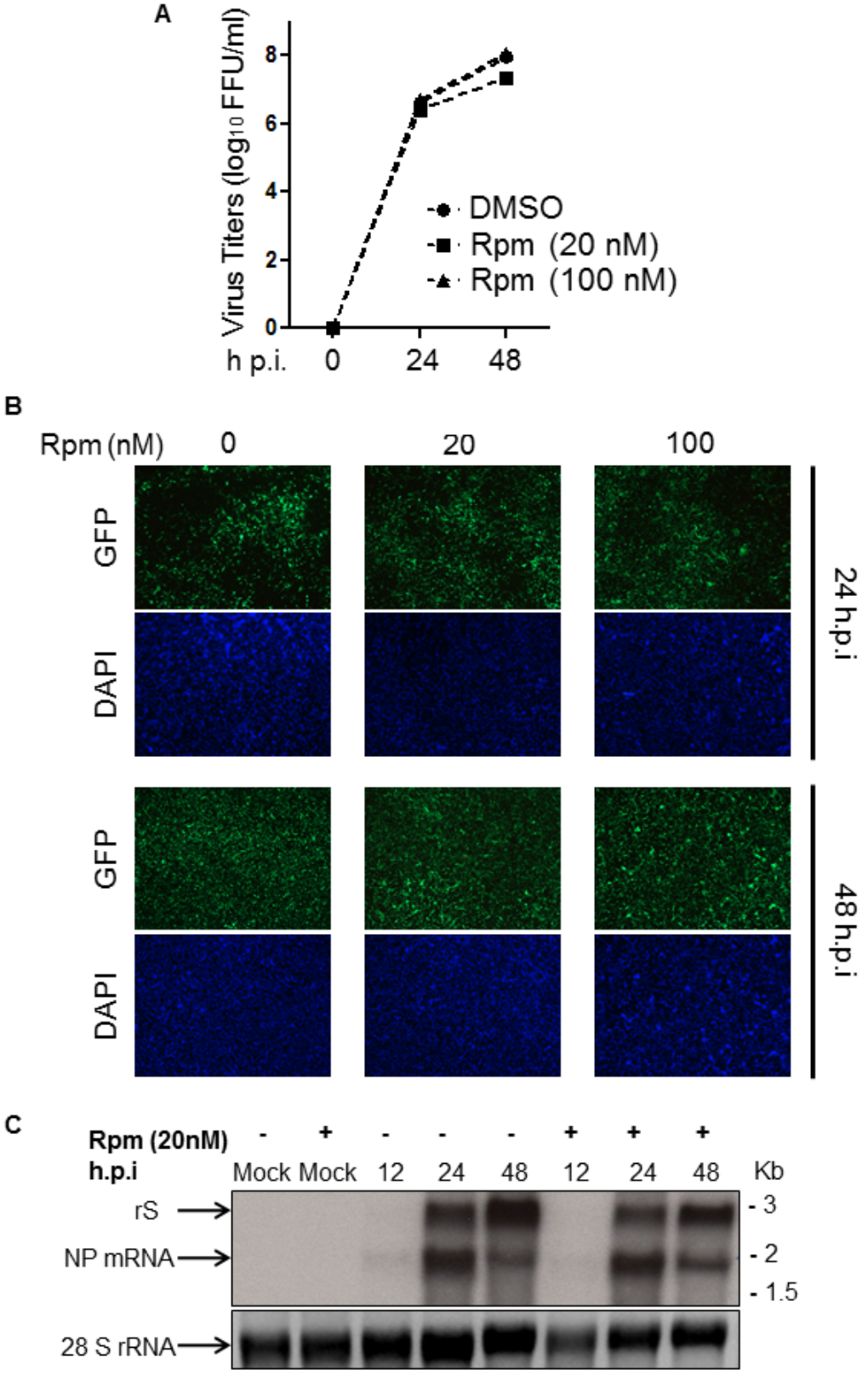

644 **Fig. 8 Urata et al.**