Antiviral Research 137 (2017) 76–81

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

The natural compound silvestrol is a potent inhibitor of Ebola virus replication

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ARTICLE INFO

Article history:
Received 19 August 2016
Received in revised form
10 November 2016
Accepted 11 November 2016
Available online 15 November 2016

Keywords:
Ebola
Silvestrol
eIF4A helicase
Translation inhibition
Primary macrophages
PIM1

ABSTRACT

The DEAD-box RNA helicase eIF4A, which is part of the heterotrimeric translation initiation complex in eukaryotes, is an important novel drug target in cancer research because its helicase activity is required to unwind extended and highly structured 5′-UTRs of several proto-oncogenes. Silvestrol, a natural compound isolated from the plant *Aglaia foveolata*, is a highly efficient, non-toxic and specific inhibitor of eIF4A. Importantly, 5′-capped viral mRNAs often contain structured 5′-UTRs as well, which may suggest a dependence on eIF4A for their translation by the host protein synthesis machinery. In view of the recent Ebola virus (EBOV) outbreak in West Africa, the identification of potent antiviral compounds is urgently required. Since Ebola mRNAs are 5′-capped and harbor RNA secondary structures in their extended 5′-UTRs, we initiated a BSL4 study to analyze silvestrol in EBOV-infected Huh-7 cells and in primary human macrophages for its antiviral activity. We observed that silvestrol inhibits EBOV infection at low nanomolar concentrations, as inferred from large reductions of viral titers. This correlated with an almost complete disappearance of EBOV proteins, comparable in effect to the translational shutdown of expression of the proto-oncoprotein PIM1, a cellular kinase known to be affected by silvestrol. Effective silvestrol concentrations were non-toxic in the tested cell systems. Thus, silvestrol appears to be a promising first-line drug for the treatment of acute EBOV and possibly other viral infections.

1. Introduction

The rocaglate silvestrol, a natural compound with a cyclopenta *[b]*benzofuran skeleton, can be isolated from the plant *Aglaia foveolata* (Kim et al., 2007) and is a potent and highly specific inhibitor of the ATP-dependent DEAD-box RNA helicase eIF4A (Bordeleau et al., 2008). This helicase is part of the heterotrimeric translation initiation complex eIF4F that binds to m^7GpppN mRNA cap structures through its eIF4E subunit, thus enabling the recruitment of ribosomes to the 5′-UTR of mRNAs in eukaryotes (Pelletier et al., 2015). It has been proposed that silvestrol increases the affinity of eIF4A for the bound target mRNA, thereby stalling the helicase on its RNA substrate, which leads to a depletion of eIF4A from mRNA-bound eIF4F complexes (Sadlish et al., 2013). Silvestrol exerts potent and non-toxic antitumor activity *in vitro* and *in vivo* (Lucas et al., 2009; Kogure et al., 2013) by inhibiting translation of short-lived oncoproteins, such as c-MYC and PIM1 (Schatz et al., 2011), whose mRNA 5′-UTRs are extended and include regions of stable RNA secondary structures that require unwinding by eIF4A to create a binding platform for the 43S preinitiation complex (Hinnebusch et al., 2016).

The recent outbreak of the Ebola virus (EBOV) in West Africa caused more than 28,000 cases with at least 11,000 fatalities, revealing that effective viral inhibitors with potential broad-spectrum activity are urgently needed (WHO Ebola Response Team et al., 2015). EBOV is a negative-stranded RNA virus whose 19-kb genome comprises seven protein genes. Transcription of the viral genes is accomplished by the viral polymerase complex which synthesizes monocistronic, 5'-capped and 3'-polyadenylated

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http://dx.doi.org/10.1016/j.antiviral.2016.11.011
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mRNAs (Sanchez et al., 1993; Mühlberger et al., 1999). As for all viruses, EBOV protein synthesis depends on the cellular translation machinery. In this context, antivirals that target host factors are expected to be an advantageous strategy compared to viral targets because escape mutations by the virus are rarer (Müller et al., 2012). However, inhibition of host factors is often a problematic issue in drug development due to pleiotropic unwanted side-effects associated with such strategies (Gerold and Pietschmann, 2013). This concern appears to be mitigated in the case of silvestrol as its inhibition of eIF4A was shown to be highly specific, resulting in efficient and non-toxic antitumor activity in different tumor mouse model systems (Lucas et al., 2009; Kogure et al., 2013). Therefore, eIF4A is discussed as a promising new drug target for cancer treatment (Chu and Pelletier, 2015).

Interestingly, some EBOV mRNAs harbor long 5′-UTRs and all EBOV mRNAs were predicted (Mühlberger et al., 1999) or demonstrated (Weik et al., 2002; Schlereth et al., 2016) to adopt stable RNA secondary structures in their 5′-UTRs. We thus hypothesized that efficient cap-dependent translation of EBOV mRNAs may also require the host helicase activity of eIF4A to unwind such structures during initiation of translation. To test our hypothesis, we analyzed potential inhibitory effects of silvestrol on EBOV-infected Huh-7 cells as well as primary human macrophages, the primary target cells of EBOV in humans.

2. Materials and methods

2.1. Cell culture and preparation of macrophages from PBMCs

Huh-7 (human hepatoma) and VeroE6 (African green monkey, kidney epithelia) cells were cultivated in Dulbecco's modified Eagle medium complemented with penicillin (100 U/ml), streptomycin (100 mg/ml), 5 mM L-glutamine and 10% fetal calf serum at 37 °C and 5% CO_2 in a humidified atmosphere.

Human PBMCs (Peripheral Blood Mononuclear Cells) were isolated from buffy coats by density gradient centrifugation and magnetic CD14 MicroBeads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (3×10^6 cells) were cultivated in 6-well tissue culture plates (Falcon Primaria, Becton Dickinson, Paramus, NJ) at 37 °C in 5% CO_2 using RPMI 1640 medium (GE Healthcare, Freiburg, Germany) supplemented with 2 mM L-Glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. After 2 h, defibrinated human AB serum was added to a final concentration of 2%, and GM-CSF (Biochrom, Berlin, Germany) to a final concentration of 10 ng/ml. Cells were cultivated for 7 days to differentiate them into macrophages. On day 3 post seeding half of the medium was replaced with fresh medium (including all supplements). Alternatively, preparation of monocytes from PBMCs was performed by counterflow centrifugation.

2.2. Ebola virus infection

All work with Zaire Ebola virus (EBOV) strain Mayinga (Acession number AF 086833) was performed at the biosafety level 4 (BSL4) laboratory of the Philipps University Marburg. Briefly, Huh-7 cells (5×10^5 cells) or primary human macrophages (3×10^6 cells) were infected with EBOV at a multiplicity of infection (MOI) of 0.1 for 1 h at 37 °C. Inoculum was removed and cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 3% fetal calf serum supplemented with silvestrol or Dimethylsulfoxid (DMSO) as indicated.

Aliquots of the supernatant were collected at 1–4 days post infection (p.i.) and subjected to the 50% tissue culture infectious dose $TCID_{50}$ assay for determination of viral titers. Cells were lysed with 1% SDS, followed by Western blotting to analyze for expression of viral proteins and the cellular PIM1 kinase.

2.3. Silvestrol treatment of EBOV-infected cells

Silvestrol (Medchemexpress LLc, Princeton, USA; purity >98%) was added to cell cultures as a single dose to final concentrations of 1–50 nM (dissolved from a 6 mM stock in DMSO, dilutions in DMEM). Silvestrol was added (Huh-7: one day post seeding; primary human macrophages: 7 days post seeding) 2 h before infection or directly post infection. As a control, DMSO was added to cells at the same concentration used to dissolve silvestrol. Infected cells were incubated for 1–4 days at 37 °C in growth medium containing the indicated silvestrol concentrations.

2.4. $TCID_{50}$ analysis

Virus titers in the supernatant of infected cells were determined by the 50% tissue culture infectious dose ($TCID_{50}$) assay in VeroE6 cells as described previously (Krähling et al., 2010).

2.5. WST-1 assay of human Huh-7 cells or primary macrophages

Huh-7 cells (7×10^3 cells in 200 μL Iscove's Modified Dulbecco's Medium [IMDM], 10% fetal calf serum [FCS]) were seeded in 96-well microplates and incubated for 24 h (37 °C, 5% CO_2). The medium was replaced with fresh medium, and silvestrol or DMSO was added as indicated for 48 h. The medium was aspirated and 110 μL of 10% WST-1 reagent (Roche, Mannheim, Germany) in PBS were added. Absorbance was measured using a Tecan Safire II (measurement wavelength: 450 nm; reference wavelength: 600 nm).

Monocytes (8×10^4 cells in 100 μL RPMI 1640) were seeded in 96-well microplates and incubated for 2 h (37 °C, 5% CO_2). Then 100 μL RPMI 1640 supplemented with defibrinated human AB serum (final concentration 2%) and GM-CSF (final concentration 10 ng/ml) (Biochrom, Berlin, Germany) were added. After 72 h, half of the medium was removed and replaced with RPMI 1640 containing 4% human serum. After 96 h, the monocytes had differentiated into macrophages. The medium was then replaced with RPMI 1640 containing 2% human serum, and silvestrol or DMSO was added as indicated followed by incubation for another 72 h. The WST-1 assay was then performed after 24, 48, 72 and 96 h as described above for Huh-7 cells.

2.6. Western blot analysis

Cells were lysed in lysis buffer (125 mM Tris/HCl pH 6.8, 4% SDS, 1.4 M 2-mercaptoethanol, 0.05% bromophenol blue) and heated at 95 °C for 5 min. Samples were loaded onto 15% SDS-polyacrylamide gels followed by electrophoresis for 1 h at 180 V. Proteins were transferred onto an Immobilon-P PVDF membrane (Merck Millipore, Darmstadt, Germany) for 30 min at 10 V followed by blocking of the membrane with 5% milk powder dissolved in TBST (10 mM Tris/HCl, 150 mM NaCl, 0,1% Tween 20, pH 7.6). Primary and secondary antibodies were diluted 1:500 in TBST (Pim1, sc-13513, Santa Cruz Biotechnology), 1:5000 (β-Actin, sc-47778, Santa Cruz Biotechnology) and 1:5000 (goat anti-mouse IgG-HRP, secondary antibody). Blots were incubated with Amersham ECL™ or ECL-plus™ Western Blotting Detection Reagents according to the manufacturer's protocol. For detection of chemiluminescence, Kodak® BioMaxTM light films, Kodak GBX Developer and Replenisher and GBX Fixer and Replenisher were used.

Samples from infected cells were lysed in 1% SDS containing sample buffer and subjected to 12% SDS PAGE and semi-dry transfer onto nitrocellulose membranes. Staining of viral proteins from

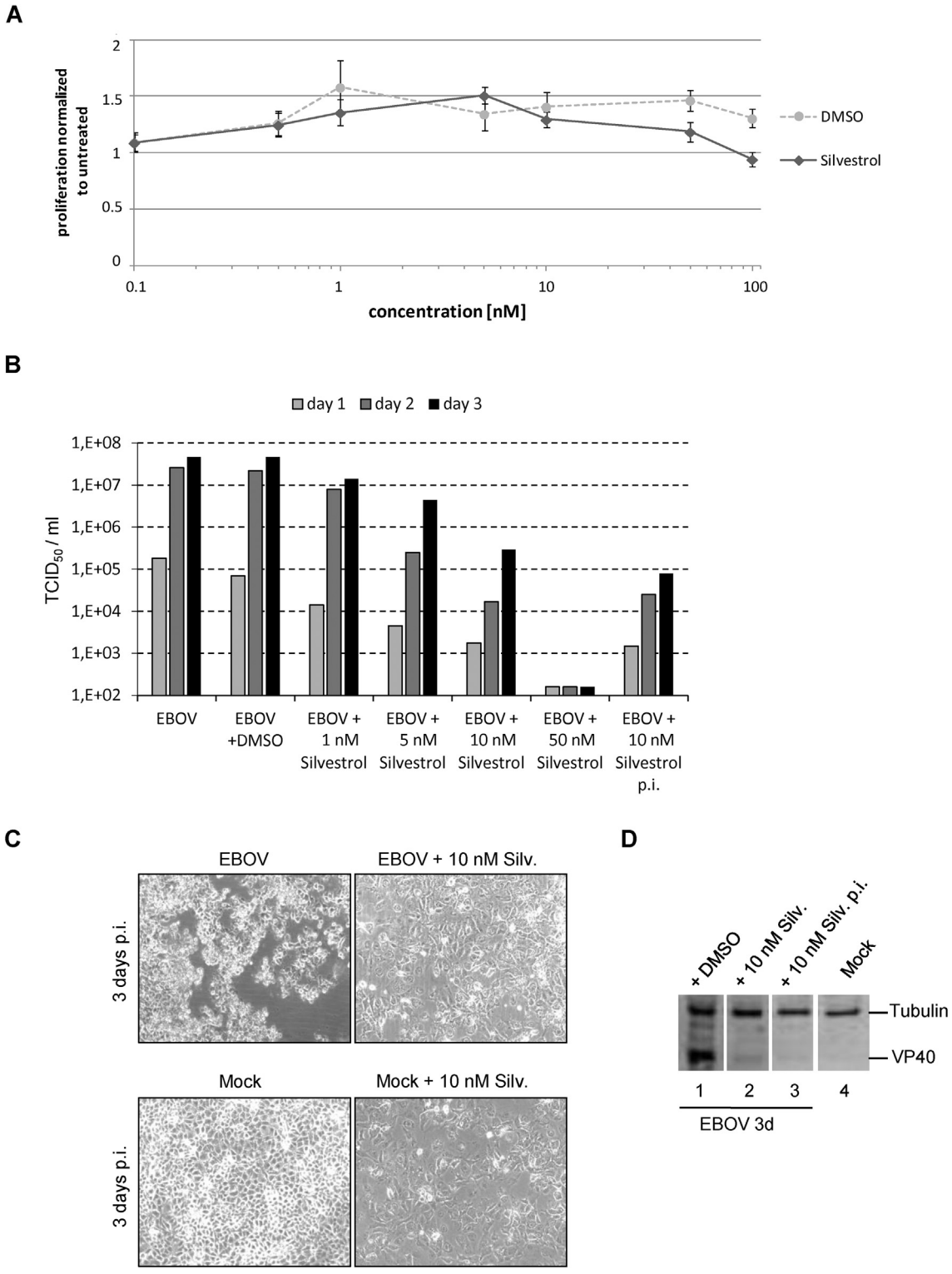

Fig. 1. Antiviral effects of silvestrol on EBOV-infected Huh-7 cells. (**A**) Proliferation of Huh-7 cells measured by the WST-1 assay. Cells were treated with silvestrol at the indicated concentrations for 48 h. No effect of silvestrol on cell proliferation could be observed up to a concentration of 10 nM. A slight reduction in proliferation was observed at concentrations of 50 or 100 nM silvestrol. (**B**) Dose-dependent inhibition of Ebola virus propagation by silvestrol in Huh-7 cells. Addition of silvestrol to a final concentration of 1, 5, 10 or 50 nM 2 h before viral infection, or treatment of cells with 10 nM silvestrol directly after viral infection (p.i), strongly inhibited EBOV replication in Huh-7 cells. Cell supernatants were collected at days 1, 2, and 3 post infection (p.i.) and subjected to $TCID_{50}$ analysis in VeroE6 cells. Viral titers are given as the mean of two or three independent experiments. (**C**) Morphology of Huh-7 cells in the light microscope after three days of silvestrol treatment (10 nM) in the presence or absence of EBOV. Pictures were taken at 10× magnification. (**D**) Silvestrol inhibits the expression of the EBOV protein VP40, whereas the cellular protein tubulin is not affected. EBOV-infected Huh-7 cells were treated with DMSO or 10 nM silvestrol 2 h before (lane 2) or immediately post (lane 3) infection. Shown are the levels of the viral protein VP40 and the cellular protein tubulin in a Western blot analysis from samples obtained at 3 days p.i. Mock: uninfected cells without silvestrol or DMSO addition.

infected Huh-7 cells was performed using a goat anti-VP40 antibody and a donkey anti-goat Alexa680 nm antibody (Thermo Fisher Scientific/Molecular Probes). Detection of antibodies was performed using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). Staining of viral proteins from human primary macrophages was performed using a chicken anti-NP antibody (at a 1:1000 dilution) and a donkey anti-chicken peroxidase-coupled antibody (Dianova). Tubulin was stained using mouse anti-tubulin (Sigma-Aldrich) and goat anti-mouse peroxidase-coupled antibody (Dako). Detection of antibodies was performed using the Chem-iDoc™ XRS + System (Bio-Rad).

3. Results

3.1. Silvestrol inhibits EBOV propagation in Huh-7 cells

First, we analyzed the effects of silvestrol on proliferation of Huh-7 cells, a standard hepatoma cell line to investigate EBOV, by using silvestrol concentrations in a range between 0.1 nM and 100 nM. Up to 10 nM silvestrol no obvious effects on proliferation could be detected in a WST-1 assay. At higher concentrations, cell proliferation was weakly affected compared to the DMSO control (Fig. 1A), which is in line with published data (Cencic et al., 2009).

For testing potential dose-dependent antiviral activity of silvestrol, we preincubated Huh-7 cells for 2 h with 1–50 nM silvestrol and subsequently infected the cells with EBOV at a multiplicity of infection (MOI) of 0.1. Viral titers of supernatants were determined by $TCID_{50}$ analysis in VeroE6 cells at day 1, 2, and 3 (p.i.). Remarkably, even at 1 nM silvestrol we already observed some reduction of the EBOV titer, at 5 nM the inhibitory effect increased to 1-2 orders of magnitude, and upon application of 10 nM silvestrol EBOV titers were reduced by 2-3 orders of magnitude (Fig. 1B) without changing cell morphology at the same concentration as determined by light microscopy (Fig. 1C). At 50 nM silvestrol, where some cytotoxicity became apparent (Fig. 1A), the viral titers dropped to below the detection limit of our assay. We estimated EC_{50}- and EC_{90}-values for the antiviral activity of silvestrol in EBOV-infected Huh-7 cells (see Fig. S1) of ~0.8 and 2 nM, respectively.

We also tested an experimental setup where we applied 10 nM silvestrol immediately after EBOV infection (Fig. 1B, p.i., bars on the right). Again, treatment of Huh-7 cells inhibited EBOV replication by about 2-3 orders of magnitude, thus very similar in effect to the standard setup where we exposed the cells to silvestrol for 2 h before infection (Fig. 1B, third group of bars from the right).

To analyze the specificity of silvestrol on viral mRNA translation, we investigated its impact on expression of the EBOV protein VP40 and the housekeeping protein tubulin by Western blotting. We observed a strong reduction of VP40 levels by treating cells with 10 nM silvestrol 2 h before or post EBOV infection (Fig. 1D). Similar reductions of protein levels could also be seen for the EBOV proteins NP and GP (data not shown). Our findings demonstrate a substantial antiviral effect of silvestrol in EBOV-infected Huh-7 cells at non-toxic concentrations of only 10 nM.

3.2. Antiviral activity of silvestrol on EBOV-infected human primary macrophages

Primary human macrophages, which are one of the first target cells during EBOV infections (Ströher et al., 2001), are a highly relevant cellular system to further investigate the antiviral effects of silvestrol on EBOV propagation. Using the above described silvestrol preincubation setup, we analyzed the impact of 2, 10 and 25 nM silvestrol on EBOV infection of human macrophages isolated from two different donors. We observed a concentration-dependent inhibition of viral titers between 1 and 3 orders of magnitude (Fig. 2A). Since efficient inhibition was accomplished with 10 nM silvestrol, we repeated the experiment with macrophages isolated from more donors to substantiate the antiviral effect. Preincubation

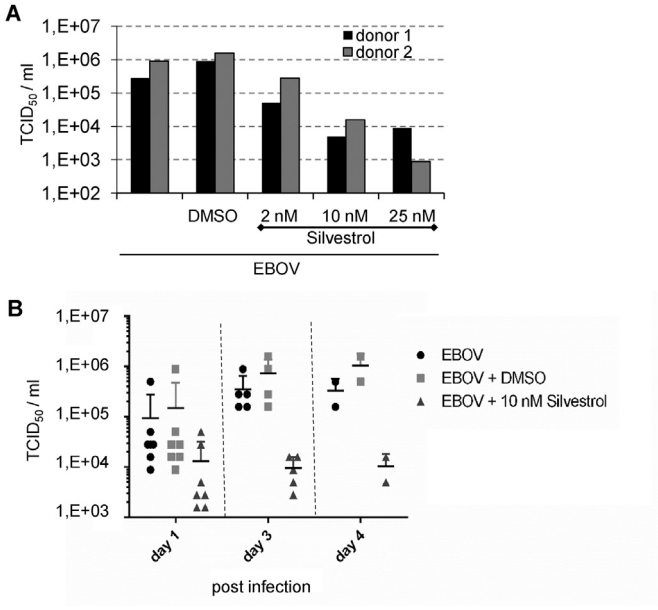

Fig. 2. Antiviral effects of silvestrol in EBOV-infected primary human macrophages. (**A**) Macrophages from two human donors were infected with EBOV and cell culture supernatants subjected to $TCID_{50}$ analysis 3 days post infection. Addition of silvestrol to 2, 10 or 25 nM 2 h before infection resulted in a concentration-dependent inhibition of EBOV propagation between 1 and 3 orders of magnitude compared to DMSO-treated cells. (**B**) Similar results were obtained with an enlarged set of EBOV-infected macrophages from seven human donors upon addition of silvestrol to 10 nM 2 h before infection. Cell culture supernatants were subjected to $TCID_{50}$ analysis 1, 3 and 4 days post infection. Mean values with standard deviation are indicated by horizontal bars.

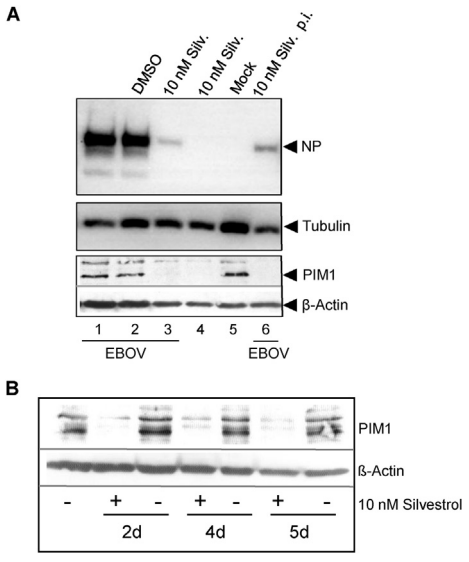

Fig. 3. (**A**) Western blot analysis demonstrating strongly decreased levels of the EBOV protein NP and the proto-oncoprotein PIM1 upon treatment of primary human macrophages with DMSO or 10 nM silvestrol 2 h before or immediately post (lane 6) infection. Tubulin and β-Actin are shown as cellular control proteins. Mock: uninfected cells without silvestrol or DMSO addition. (**B**) Long-term effect of silvestrol analyzed by Western blotting. The inhibitory effect of a single-dose treatment with 10 nM silvestrol on PIM1 levels persisted in macrophages up to at least 5 days without affecting β-Actin levels.

of EBOV-infected macrophages from seven donors with 10 nM silvestrol confirmed the reduction of viral titers by up to two orders of magnitude (Fig. 2B).

We further analyzed the expression level of the EBOV nucleoprotein (NP) by Western blotting and observed a potent down-regulation (Fig. 3A, example of one donor, lane 3). The same was true when silvestrol was administered after EBOV infection (Fig. 3A, lane 6). As mentioned above, silvestrol inhibits translation of highly structured 5′-UTRs of proto-oncogenes and should leave the expression of housekeeping genes largely unaffected (Schatz and Wendel, 2011). Therefore, we tested the impact of silvestrol on the levels of the proto-oncogenic kinase PIM1 in primary macrophages, using β-Actin as a housekeeping control protein. As expected, PIM1 levels were strongly decreased (to almost below detection limits) in the presence of 10 nM silvestrol (Fig. 3A, lanes 3, 4 and 6). Importantly, this inhibitory effect persisted for at least 5 days using a single dose of silvestrol, without affecting cellular β-Actin levels substantially (Fig. 3B). We like to mention in this context that Western blot experiments are a relatively insensitive method to exclude potential general effects of silvestrol on protein synthesis, because housekeeping genes like tubulin or β-Actin are abundant proteins with long half-lives. However, such general effects on housekeeping functions should become evident in toxicity assays (see below).

3.3. *Silvestrol shows no toxicity at low nanomolar concentrations in primary human macrophages*

We quantified silvestrol toxicity on primary macrophages after 48 h in the concentration range between 0.1 nM and 5 μM using the WST-1 viability assay. This revealed an IC_{50} value of about 90.5 nM, with essentially no sign of toxicity up to at least 50 nM silvestrol (Fig. 4). Similar toxicity values were obtained after 72 h (IC_{50} of 81 nM) and 96 h (IC_{50} of 77 nM), whereas no toxicity of silvestrol could be observed after 24 h (Fig. S2). Importantly, the antiviral effect of silvestrol became obvious 24 h after infection in Huh-7 cells as well as primary macrophages (see Figs. 1B and 2B), indicating that the effect of silvestrol on EBOV replication is more rapid than that on cellular functions. In accordance with this result, toxicity of silvestrol at therapeutic doses has not been observed in antitumorigenic animal studies (Kogure et al., 2013; Patton et al., 2015). Our findings predict a therapeutic window within which the eIF4A RNA helicase inhibitor silvestrol can be applied to efficiently inhibit EBOV propagation at non-toxic concentrations by interfering with cap-dependent translation initiation of viral mRNAs.

4. Discussion

Silvestrol, a highly potent antitumor compound with remarkably low toxicity in mouse tumor models (Kogure et al., 2013; Patton et al., 2015), can be isolated from the plant *Aglaia foveolata* and was identified in a screen for eIF4A helicase inhibitors (Bordeleau et al., 2008). The helicase activity seems to be essential for 5′-cap-dependent translation of mRNAs with highly structured 5′-UTRs to enable binding of the translation preinitiation complex. Moreover, eIF4A, a promising tumor drug target already validated *in vitro and in vivo*, seems to be the primary molecular target for silvestrol (Chu et al., 2016).

It was previously demonstrated that translation of oncogenic mRNAs encoding G-quadruplex structures in their 5′-UTRs depends on eIF4A activity (Wolfe et al., 2014). More recently, evidence was presented that rocaglates, the class of compounds to which silvestrol belongs, can clamp eIF4A to its mRNA substrate in an ATP-independent manner due to polypurine sequence elements in their 5′-UTRs (Iwasaki et al., 2016). Thus, future studies need to clarify how mRNA sequence and structure mechanistically relate to eIF4A function. Interestingly, mRNAs transcribed by many different viruses including the highly pathogenic Ebola virus contain structured 5′-UTRs that need to be translated by the cellular translation machinery in order to promote viral protein synthesis (Weik et al., 2002; Schlereth et al., 2016). This prompted us to investigate if cap-dependent translation of EBOV mRNAs may be sensitive towards silvestrol treatment. In two disease-relevant cell types infected with EBOV we observed profound inhibitory effects on virus titer and EBOV protein levels at low nanomolar silvestrol concentrations without concurrent cytotoxicity. The antiviral effect of 10 nM silvestrol was about 2 orders of magnitude in primary human macrophages and 3 orders of magnitude in the tumor cell line Huh-7. This more pronounced effect in cancer cells can be explained by

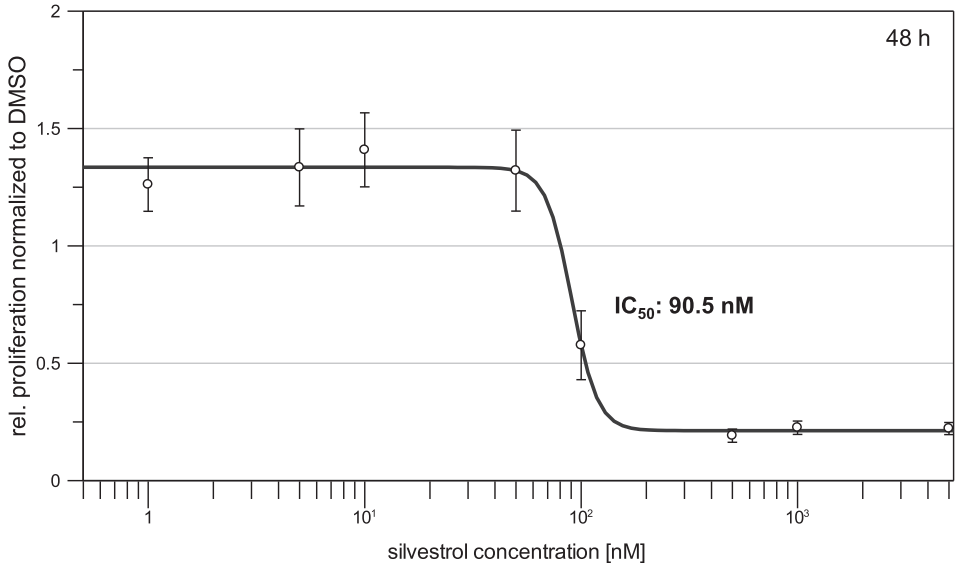

Fig. 4. Silvestrol inhibited proliferation of human primary macrophages with an IC_{50} value of 96 nM, as determined by a WST-1 viability assay. Cells were treated with silvestrol at the indicated concentrations for 48 h.

silvestrol additionally suppressing proto-oncogenes like PIM1, which is expected to negatively affect cell proliferation (Thomas et al., 2012) and thereby also viral propagation. Nonetheless, our data suggest that EBOV translation strongly depends on the cellular helicase eIF4A activity. Future studies will have to address if the silvestrol effect on EBOV affects translation of all EBOV mRNAs or only selected ones. Moreover, it will be intriguing to explore if silvestrol could inhibit also other RNA viruses that depend on cap-dependent mRNA translation, for example (+)-RNA-strand viruses like Coronaviridae, where the infectious RNA serves directly as a template for protein translation.

Our finding that the inhibition of viral propagation by treatment with 10 nM silvestrol occurs more rapidly than any effects on cellular functions by toxic silvestrol concentrations, qualifies silvestrol and related compounds as promising first-line drugs to attenuate EBOV infections, which should in turn increase the chances to develop an effective immune response against the viral infection. In conclusion, our study provides the first proof that inhibition of translation initiation in eukaryotes by specifically targeting the host factor eIF4A with silvestrol is a feasible strategy to combat the large group of viruses that rely on cap-dependent translation initiation such as EBOV.

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by the German Research Foundation (DFG) grant CRC 1021, the German Center for Infection Research (DZIF) and by the LOEWE Research Cluster “Medical RNomics” grant 519/03/00.001-(0003).

Acknowledgments

We thank Andreas Kaufmann and Wilhelm Goswin Bertrams for technical assistance during monocyte preparation from buffy coats. We also like to thank Markus Eickmann, Michael Schmidt, and Gotthard Ludwig for facilitating experiments conducted in the BSL4 laboratory at the Philipps University Marburg, as well as Astrid Herwig for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.11.011.

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