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Sodium phenylbutyrate abrogates African swine fever virus replication by disrupting the virus-induced hypoacetylation status of histone H3K9/K14

Gonçalo Frouco, Ferdinando B. Freitas, Carlos Martins, Fernando Ferreira*

CIISA, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. da Universidade Técnica, Lisboa, Portugal

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ABSTRACT

African swine fever virus (ASFV) causes a highly lethal disease in swine for which neither a vaccine nor treatment are available. Recently, a new class of drugs that inhibit histone deacetylases enzymes (HDACs) has received an increasing interest as antiviral agents.

Considering studies by others showing that valproic acid, an HDAC inhibitor (HDACi), blocks the replication of enveloped viruses and that ASFV regulates the epigenetic status of the host cell by promoting heterochromatinization and recruitment of class I HDACs to viral cytoplasmic factories, the antiviral activity of four HDACi against ASFV was evaluated in this study. Results showed that the sodium phenylbutyrate fully abrogates the ASFV replication, whereas the valproic acid leads to a significant reduction of viral progeny at 48 h post-infection (−73.9%, $p = 0.046$), as the two pan-HDAC inhibitors tested (Trichostatin A: −82.2%, $p = 0.043$; Vorinostat: 73.9%, $p = 0.043$). Further evaluation showed that protective effects of NaPB are dose-dependent, interfering with the expression of late viral genes and reversing the ASFV-induced histone H3 lysine 9 and 14 (H3K9K14) hypoacetylation status, compatible to an open chromatin state and possibly enabling the expression of host genes non-beneficial to infection progression. Additionally, a synergic antiviral effect was detected when NaPB is combined with an ASFV-topoisomerase II poison (Enrofloxacin).

Altogether, our results strongly suggest that cellular HDACs are involved in the establishment of ASFV infection and emphasize that further in vivo studies are needed to better understand the antiviral activity of HDAC inhibitors.

1. Introduction

African swine fever virus (ASFV) is the only member of the *Asfiviridae* family, sharing genomic and structure features with other eukaryotic viruses (e.g. poxviruses and iridoviruses), all belong to the nucleocytoplasmic large DNA viruses (NCLDV) clade (King et al., 2012). It causes a highly lethal disease in swine and is considered one of the most threatening diseases for pig husbandry, since neither a vaccine nor an antiviral treatment are available to control ASFV infection. Originally endemic in most of Sub-Saharan African countries, it was introduced in Transcaucasia (Georgia, Armenia, Azerbaijan) and in the Russian Federation during 2007, where it is maintained (Sánchez-Vizcaíno et al., 2013). More recently, new outbreaks have been reported in Ukraine (2012, 2014 and 2015), Belarus (2013), Lithuania, Estonia, Latvia and Poland (2014 and 2015) and more recently (2016) in Moldova (Gallardo et al., 2015).

To establish a successful infection, ASFV must overcome several host defence mechanisms, such as apoptosis, inflammatory and immune responses (Correia et al., 2013; Galindo et al., 2008; Gil et al., 2008; Granja et al., 2006; Hurtado et al., 2004; Nogal et al., 2001) and, like other viruses, ASFV may alter the epigenetic status of the host chromatin in order to control cellular gene expression to its own benefit (Chiocca et al., 2002; de Souza et al., 2010; Knipe et al., 2013; Placek and Berger, 2010; Punga and Akusjärvi, 2000; Valls et al., 2007, 2003). Although the mechanisms by which viruses elicit this epigenetic reprogramming are not fully understood, their interaction with cellular Histone Deacetylases (HDACs) and Histone Acetyltransferases (HATs) are known to be critical for disrupting the host gene-expression program. Indeed, while HDACs catalyse the removal of the acetyl group from lysines, re-establishing the positive charge on the histones, thus inducing heterochromatinization and gene silencing (Dekker and Haisma, 2009), HATs promote the transfer of an acetyl group from a molecule of Acetyl Coenzyme-A onto the target lysine residue (Herbein and Wendling, 2010; Yang and Seto, 2007). In eukaryotic cells, a significant correlation was found between high acetylation levels of H3K9/K14 and euchromatin formation (an open chromatin state and transcriptionally active), contrasting with low acetylation levels which are associated with a tightly packed and silenced heterochromatin

* Corresponding author at: Faculdade de Medicina Veterinária, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal.
E-mail address: fernandof@fmv.ulisboa.pt (F. Ferreira).

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(Zaratiegui et al., 2007). Recently, eighteen HDACs were identified in mammals and classified as: Class I (HDAC 1, 2, 3 and 8), Class II (subdivided in subgroup IIa – HDAC 4, 5, 7 and 9, and IIb – HDAC 6 and 10), Class III (sirtuins) and Class IV (HDAC 11) (Bolden et al., 2006; de Ruijter et al., 2003). Remarkably, the enzymatic activity of class I and II HDACs can be pharmacologically modulated by inhibitors (HDACi), which have shown potent anti-parasitic (Bougdour et al., 2009; Chaal et al., 2010) and anti-tumour effects (Ahn et al., 2011; Zimmerman et al., 2011). Some of these HDACi are also promising antiviral agents since they activate latent HIV, Epstein-Barr Virus and Human Cytomegalovirus, depleting reservoirs of persistent, quiescent infection (Archin et al., 2012; Ghosh et al., 2012; Huber et al., 2011; Michaelis et al., 2005; Radkov et al., 1999), and reduce replication of enveloped viruses by interfering with the stability of its particles (Vázquez-Calvo et al., 2011).

Considering the above data and knowing that ASFV modifies the epigenetic state of the host chromatin, promoting heterochromatinization of nucleus and recruiting HDAC1, HDAC2 and HDAC3 to viral factories (Simões et al., 2015), we aimed to evaluate the antiviral activity of four HDAC inhibitors: Trichostatin A (TSA), Vorinostat (SAHA), valproic acid (VPA) and sodium phenylbutyrate (NaPB). Since, high acetylation levels of H3K9/K14 (H3K9/K14Ac) is an epigenetic marker for chromosome decondensation and a tell-tale sign of active transcription, we evaluate the dynamics of H3K9/K14 acetylation during ASFV infection and the effect of NaPB in the viral-induced H3K9/K14Ac status.

Finally, taking advantage of previous studies that identified a synergistic effect between HDACi and Topoisomerase's poisons on cancer treatment (Bevins and Zimmer, 2005; Marchion et al., 2005b; Marchion et al., 2005a), the antiviral activity of the NaPB was investigated in the presence of enrofloxacin, a ASFV-topoisomerase II poison (Freitas et al., 2016; Mottola et al., 2013). The results showed that all HDACi inhibit *in vitro* ASFV infection with NaPB completely abrogated viral replication, at non-cytotoxic concentrations and in a dose-dependent manner. Our data also suggest that antiviral effects of NaPB resulted from the drug-induced higher H3 acetylated levels of K9/K14 residues, followed by an inhibition of the late viral protein synthesis and not interfering with the stability of the viral particles before cell entry. Finally, a synergistic antiviral effect was detected when NaPB was combined with enrofloxacin.

2. Material and methods

2.1. Cells and viruses

Vero E6 cells (kidney epithelial cells of African green monkey Cholorocebus aethiops) were obtained from the European Cell Culture Collection (ECACC), and maintained in DMEM (Dulbecco Modified Eagle's minimal essential medium) supplemented with L-Glutamax, 10% heat inactivated fetal bovine serum (FBS), 1X non-essential amino acids and 2 mM L-glutamine (all from Gibco, Life Technologies). All cell cultures were grown at 37 °C in a 5% CO_2 humidified atmosphere.

The Vero adapted ASFV-Ba71V isolate was propagated as previously described (Carrascosa et al., 2011), and viral titers were determined by TCID50 (tissue culture infectious dose 50) titration using Vero E6 and the Spearman-Kärber method (Kärber, 1931).

2.2. Drugs and cytotoxic assay

Trichostatin A (TSA) and vorinostat (SAHA) were dissolved in DMSO, while valproic acid (VPA) and sodium phenylbutyrate (NaPB) were dissolved in water. Enrofloxacin was dissolved in NaOH at 0.1 M. All drugs were purchased from Sigma-Aldrich and conserved according to the manufacturer's recommendations.

Deleterious effects of HDAC inhibitors on cell viability were assessed by trypan blue dye exclusion assay, as previously described (Strober, 2001). Briefly, different concentrations of each drug were added, during 72 h, to 1.0×10^4 cells/cm^2 seeded in 24-well plates. Then, a representative sample of cells from supernatant and adherent monolayer was collected and diluted 1:1 in trypan blue solution (0.4%), with dead cells staining in blue, in contrast to unstained viable cells. To improve assay accuracy, at least 200 cells were analysed in three independent experiments. The cell viability values were normalized against the untreated control group, which was a value of 100%.

2.3. Drug treatment and viral infection

Vero cells were grown in 24-well plates, 1.0×10^4 cells/cm^2, and treated with NaPB (5 mM), VPA (25 mM), TSA (100 nM) and SAHA (1 μM), 12 h before ASFV-Ba71V infection, which was carried out at a MOI of 0.1. At the end of the adsorption period (1 h), the inoculum was removed and cells were washed twice with serum-free medium. Fresh medium was added containing HDACi in the above concentrations and combined or not with enrofloxacin at 25 μg/m and, this time point was considered as 0 h post-infection (hpi).

After cytopathic effect (CPE) observation, infected cultures were subjected to three freeze-thaw cycles and viral yields were determined by plaque assay (Kärber, 1931) at 24 and 48 hpi.

2.4. Antibodies

For immunoblotting analysis, two primary antibodies (anti-α-tubulin, # 2125, 1:200; anti-acetyl histone H3K9/K14, # 9677, 1:100) from Cell Signalling Technology and two HRP-conjugated secondary antibodies (anti-rabbit IgG, 4010-05, 1:100.000 from SouthernBiotech; anti-swine IgG, 114-035-003, 1:100.000 from Jackson ImmunoResearch Lab.) were used. For viral protein detection, an in-house produced swine anti-ASFV serum was used (1:200).

All antibody dilutions were performed in blocking solution (PBS-Tween 20 0.05%, v/v) and BSA (1%, w/v, Sigma-Aldrich, St. Louis, USA) and incubated according to the manufacturers' recommendations.

2.5. Direct immunofluorescence studies

Vero cells treated with NaPB and infected (MOI of 0.1), grown on glass coverslips, in 24-well plates (5.0×10^4 cells/cm^2), were fixed at 24 and 48 hpi, with 3.7% (w/v) paraformaldehyde in HPEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 1 mM $MgCl_2$), during 10 min and permeabilized with PBS/Triton X-100 (0.1%, v/v) for 2 min at room temperature (RT). Then, cells were washed in PBS (Gibco, Life Technologies), blocked with PBS-Tween 20 (PBST, 0.05%, v/v) and BSA (1%, w/v, Sigma-Aldrich) during 30 min, and incubated with an in-house swine anti-ASFV serum conjugated with FITC. All procedures were performed at RT and all antibody incubations were performed in a dark humidified chamber to prevent fluorochrome bleaching. Vectashield mounting medium with DAPI (Vector Laboratories) was used to visualize the nucleus and the viral factories. Fluorescence images were acquired using a Leica DMIRE2 epifluorescence microscope and analysed with ImageJ open source software (version IJ 1.48g, National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012) and Adobe Photoshop CS5 software (Adobe Systems, Inc.).

2.6. Western blotting analysis

Mock-infected and ASFV-infected Vero cells, seeded in P6 multiwell plates, were washed twice with PBS and lysed in ice-cold modified RIPA buffer (25 mM Tris, pH 8.2, 150 mM NaCl, 0.5% NP40, 0.5% sodium deoxycolate, 0.1% SDS) supplemented with both a protease-inhibitor cocktail (cOmplete, Mini, EDTA-free from Roche) and a phosphatase-inhibitor cocktail (PhosStop, Roche). The whole-cell lysates harvested after 0, 1, 12, 24, 48 and 72 h of exposure to NaPB and at indicated time points post-infection, were further subjected to SDS-PAGE gel