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SHORT COMMUNICATION

Amiodarone affects Ebola virus binding and entry into target cells

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SUMMARY

Ebola Virus Disease is one of the most lethal transmissible infections characterized by a high fatality rate. Several research studies have aimed to identify effective antiviral agents. Amiodarone, a drug used for the treatment of arrhythmias, has been shown to inhibit filovirus infection *in vitro* by acting at the early step of the viral replication cycle. Here we demonstrate that amiodarone reduces virus binding to target cells and slows down the progression of the viral particles along the endocytic pathway. Overall our data support the notion that amiodarone interferes with Ebola virus infection by affecting cellular pathways/targets involved in the viral entry process.

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The 2014 outbreak of Ebola Virus Disease in West Africa boosted the search for new antiviral agents active against filovirus. Among chemicals tested, several cationic amphiphilic drugs (CADs) have shown anti-Ebola virus (EBOV) activity (Salata *et al.,* 2017). We and others have demonstrated that the CAD amiodarone, a widely used antiarrhythmic drug, inhibits EBOV infection *in vitro* at an early stage of viral replication (Gehring *et al.,* 2014; Salata *et al.,* 2015). Although the mechanism of antiviral activity still awaits full explanation, it has been suggested that amiodarone, as well as other CADs, might affect diverse cell processes exploited by EBOV during entry (Salata *et al.,* 2017). Previously, we showed that amiodarone modifies the cell distribution of sphingomyelin, glycosphingolipids, cholesterol and its transporter NPC-1. In addition, this molecule blocks the progression of fluid phase endocytosis affecting the late endosomes (LEs) (Piccoli *et al.,* 2011).

Interestingly, it has been shown that the binding efficiency of EBOV to target cells is related to the activity of acid sphingomyelinase (aSMase) and to the presence of plasma membrane sphingomyelin (Miller *et al.,* 2012). Furthermore, EBOV displays a late cell entry kinetics and the transport of viral particles to LEs containing the intracellular viral receptor NPC-1 represents a rate-defining step of the viral entry process (Mingo *et al.,* 2015).

To evaluate whether EBOV binding and internalization were influenced by amiodarone, we used as a viral model fluorescent EBOV-like particles (EBOVLPs) (Martinez *et al.,* 2007). EBOVLPs were produced by transfection of human embryonic HEK293T cells with a plasmid expressing the EBOV matrix protein VP40 fused to the green fluorescent protein GFP (VP40-GFP) along with a construct expressing either the EBOV-GP or the VSV-G envelope glycoprotein. EBOVLPs were incubated for 1 hour at 4°C with Vero cells pre-incubated for 16 hours with 10 μM amiodarone. Next, fluorescent cell-associated VLPs were evaluated by cytofluorimetry. We used an amount of VLPs that led to a similar percentage of positive cells in the no drug controls. As shown in *Figure 1A*, we found a small but significant reduction (13±5.7%) in the cell binding of the VLPs containing the EBOV-GP with respect to VLPs harboring the VSV-G. These results were comparable to those reported for imipramine, a known aSMase inhibitor (Miller *at al.,* 2012). To support this observation, we evaluated the effect of amiodarone on the entry of EBOV-GP pseudotyped VSV. Vero cells, treated for 16 hours with 10 μM amiodarone, were incubated for 3 hours with EBOV-GP-pseudotyped VSV at the MOI of 0.1 Focus Forming Units/cell (Salata *et al.,* 2015). Next, cells were harvested and processed for western blot analysis in order to detect the matrix (M) protein of the VSV. As shown in *Figure 1B*, VSV-M amount was slightly (23.3±6.9%) but reproducibly reduced in drug-treated cells with respect to the control cells, as confirmed by the tubulin loading control. Thus, amiodarone affects both the binding and entry of viral particles harboring the EBOV-GP.

To further characterize the effect of amiodarone on early steps of the EBOV life-cycle, Vero cells, pre-treated for 16 hours with 10 μM amiodarone, were incubated for 1 hour at 4°C with EBOVLPs, washed and incubated at 37°C for 30 to 300 minutes in the presence of 0 (control) or 10 μM amiodarone. At the end of each time point, cells were analyzed by confocal microscopy to study the colocalization of VLPs with markers of the early endosomes (EEA1), LEs/lysosomes (LAMP1) and NPC-1. We found that after 30 minutes in the presence of amiodarone there was a stronger colocalization of EBOVLPs with EEA1 and a lower colocalization with LAMP1 (0.12±0.09 vs 0.07±0.08

Key words:
Amiodarone, Ebola virus, Antivirals, Virus-like particles.

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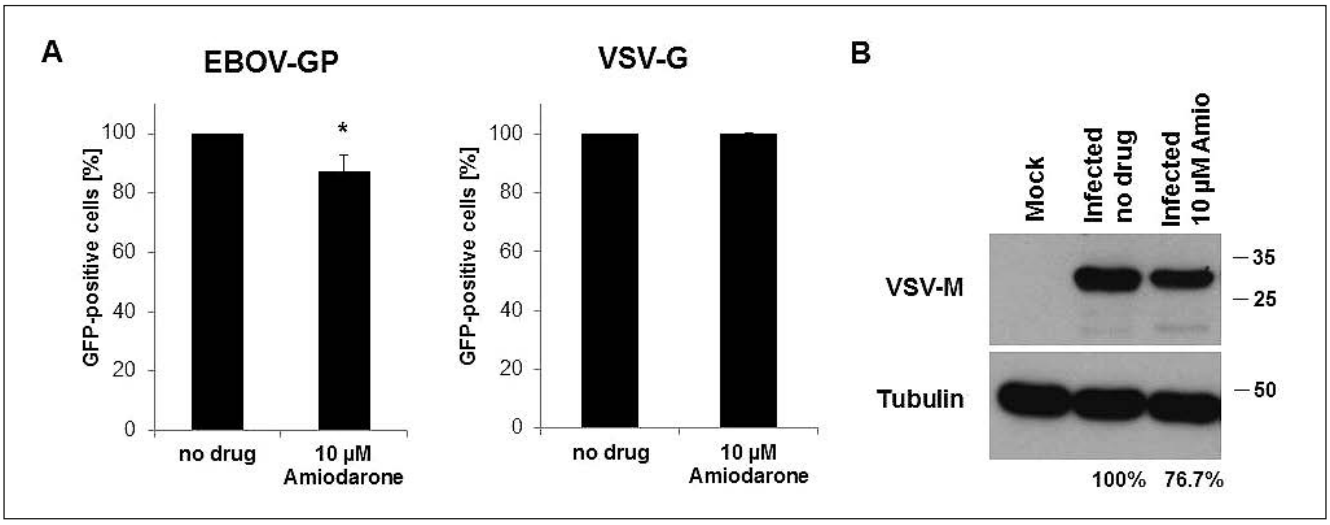

Figure 1 - *Amiodarone inhibits EBOV binding to target cells. (A) Effect of amiodarone on EBOVLPs binding to target cells. Data are presented as % of GFP-positive cells with respect to untreated cells, set as 100%. Data represent mean ± SD of 3 independent experiments performed in duplicate. * stands for different with respect to control (Student's 't' test, p<0.05). (B) Effect of amiodarone on the EBOV-GP pseudotyped VSV entry. The numbers reported on the right side of the gel represent the molecular weight markers (kDa). The numbers below the gel represent the percentage values of VSV-M normalized on the tubulin. Data are representative of results reproduced in three independent experiments.*

and 0.08±0.09 vs 0.15±0.11, respectively). By contrast, after 2 hours the colocalization with LAMP1 was higher (0.19±0.11) in amiodarone-treated than in control cells (0.11±0.06) (*Figure 2*). By analyzing the colocalization of VLPs with NPC-1, we found that:

1) VLPs colocalized with NPC-1 to a greater extent than with the other endocytic markers;
2) the colocalization increased over time;
3) there was no statistically significant difference between control and amiodarone-treated cells (*Figure 2*), indicating that amiodarone does not prevent the arrival of VLPs in a NPC-1 rich compartment.

Thus, amiodarone appears to interfere with the progression of EBOVLPs along the endocytic pathway at discrete steps, by decreasing binding to target cells and by slowing the acquisition of late endocytic markers to the vacuole containing the virus. Since amiodarone stimulates autophagy (Morissette *et al.,* 2009; Piccoli *et al.,* 2011; Salata *et al.,* 2016), we also asked whether amiodarone could favor the autophagy of internalized virions trapped in late endosomes. To this end, after 5 hours of incubation in the presence of amiodarone, colocalization of VLPs with LC3B, a marker of autophagosomes, was investigated (Piccoli *et al.,* 2011). As shown in *Figure 2*, in amiodarone-treat-

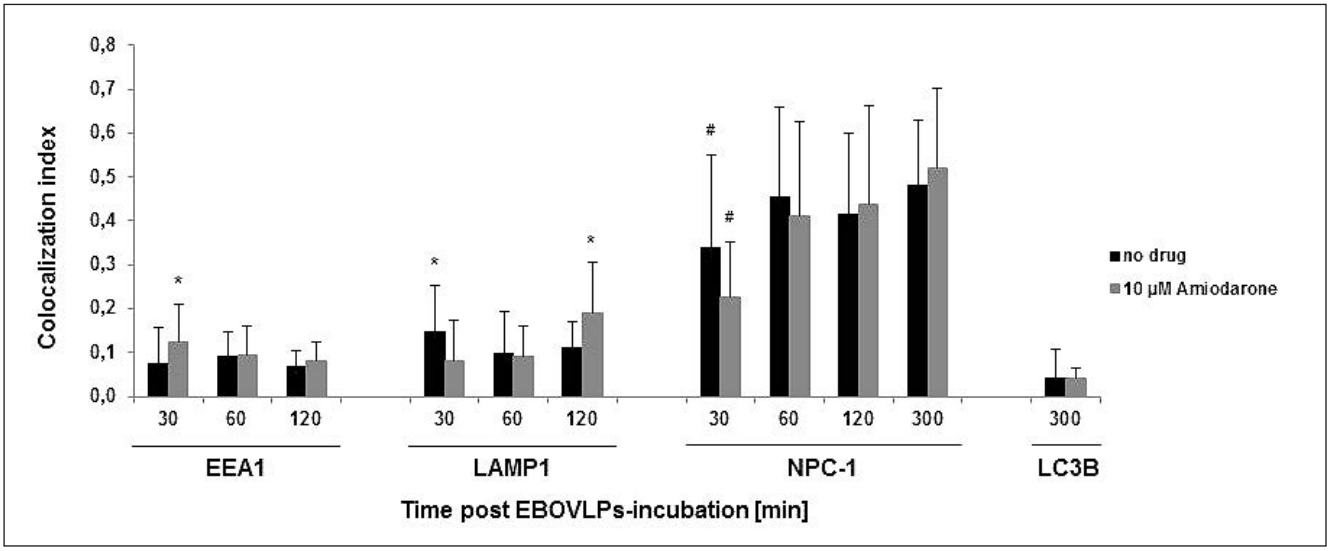

Figure 2 - *Amiodarone interferes with the trafficking of EBOVLPs but does not prevent the colocalization with the intracellular receptor NPC-1. At the indicated time points, Vero cells exposed to EBOVLPs were fixed, stained for different markers, analyzed by confocal microscopy and the colocalization index was calculated. Data represent mean ± SD of at least 20 cells examined in several randomly chosen fields per condition; *different with respect to no drug at the same time; #different from later times with the same treatment (Mann-Whitney Rank Sum test, p<0.05). The colocalization experiment was performed three times.*

ed cells we found that the colocalization between VLPs and LC3B was modest and not statistically different from the control. Similar data were obtained after 2 hours of incubation (data not shown). Thus, increased autophagy does not appear to play a role in the antiviral activity of amiodarone.

We previously showed that amiodarone blocks EBOV entry into target cells at the level of the LE, by inhibiting the fusion of the viral envelope with the LEs membrane (Salata *et al.,* 2015). Here, we show that amiodarone reduces the efficiency of viral binding to the cell surface and slows the progress along the endocytic pathway supporting the view that amiodarone, and likely other CADs with anti-EBOV activity, may act at multiple steps during virus entry.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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