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Receptor tyrosine kinase signaling regulates replication of the peste des petits ruminants virus

K. CHAUDHARY[a], K. K. CHAUBEY[a], S. V. SINGH, N. KUMAR[*]

Virology Laboratory, Division of Animal Health, Central Institute for Research on Goats, Indian Council of Agricultural Research, Makhdoom, Uttar Pradesh 28112, India

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Summary. – In this study, we found out that blocking the receptor tyrosine kinase (RTK) signaling in Vero cells by tryphostin AG879 impairs the *in vitro* replication of the peste des petits ruminants virus (PPRV). A reduced virus replication in Trk1-knockdown (siRNA) Vero cells confirmed the essential role of RTK in the virus replication, in particular a specific regulation of viral RNA synthesis. These data represent the first evidence that the RTK signaling regulates replication of a morbillivirus.

Keywords: PPR virus; receptor tyrosine kinase; RNA synthesis

Introduction

PPR is an acute, highly contagious and fatal disease of small ruminants, characterized by pyrexia, ocular and nasal discharge, erosive lesion on different mucous membranes (particularly in the mouth), enteritis and pneumonia, which ultimately results in death (Shaila *et al*., 1989). It leads to loss of production and high mortality (up to 90%) in small ruminants, therefore increases poverty in some of the poorest parts of the world where it is currently endemic: Africa, the Middle East and Western and Southern Asia (Kumar *et al*., 2014). PPR is caused by a morbillivirus that belongs to the family *Paramyxoviridae* (Gibbs *et al*., 1979).

For the control of PPR, a live attenuated cell culture vaccine is commercially available (Kumar *et al*., 2014). PPRV vaccine is heat sensitive, a serious drawback for the efficient use of the live attenuated vaccine in the endemic areas with hot climate where it is difficult to maintain the cold chain to ensure the potency of the vaccine (Kumar *et al*., 2014). To minimize the impact of the disease, controlling the spread of virus is of utmost importance. The vaccine can not provide instantaneous protection; therefore, antiviral compounds should be developed.

RTK are group of growth factor receptors that, upon ligand binding, undergoes autophosphorylation at Tyr residue. These phosphorylated tyrosines then recruit *Src* homology-2 (SH2) and phosphotyrosine-binding (PTB) domain-containing proteins that activate or link to downstream signaling pathways (Mayer, 2012). There are 58 known RTKs, which form 20 subfamilies (Lemmon and Schlessinger, 2010). All RTKs have a similar structure; a ligand binding domains in the extracellular region, a single transmembrane helix, and a cytoplasmic region containing protein tyrosine kinase (TK) domain and an additional carboxy (C-) terminal and juxtamembrane regulatory regions (Lemmon and Schlessinger, 2010). The RTKs have been extensively used as target for development of anticancer therapeutics (Hopfner *et al*., 2006). Recent studies have also suggested that the RTK signaling regulates replication of influenza and some other viruses (Eierhoff *et al*., 2010; He *et al*., 2010; Inubushi *et al*., 2008; Kumar *et al*., 2011a,b; Stantchev *et al*., 2007). We therefore hypothesized whether Trk1 (RTK) regulates PPR replication so that it can be used as a target to develop antiviral therapeutics against PPRV.

[*]Corresponding author. E-mail: naveenkumar.icar@gmail.com; phone: +91-8881056623. [a]Equal contribution.

Abbreviations: PPRV = Peste des petits ruminants virus; RTK = receptor tyrosine kinase; shRNA = short hairpin RNA; siRNA = small interfering RNA; hpt = hours post-transfection; hpi = hours post-infection; CIRG = Central Institute for Research on Goats

Materials and Methods

Virus. PPRV/Shahjadpur/2013 maintained at Central Institute of Research on Goats (CIRG) was used and has been previously described GenBank Acc. No. KP745466. The virus was amplified in Vero cells and titrated by plaque assay (Kumar *et al.*, 2013).

Cell and reagents. Vero cells have been maintained at CIRG. Tryphostin AG879 was procured from Sigma, USA. Trk1-specific siRNA expressing plasmid, psiRNA-hH1Trk1 and luciferase-specific siRNA (negative control) expressing plasmid, psiRNA-hH1Luc were generated by our group and have been previously described (Kumar *et al.*, 2011a).

Cell viability assay. The cytotoxic concentration of AG879 was determined by a previously described MTT assay (Khandelwal *et al.*, 2014). Briefly, Vero cells were grown in DMEM (Sigma, USA) supplemented with 10% FBS (Sigma, USA) and antibiotics and incubated with increasing concentrations (up to 40 µmol/l) of AG879 or DMSO (control) for 72 hr. An amount of 20 µl of freshly made 5 mg/ml MTT solution was added to each well, and cells were incubated at 37°C for 5 hr. After removal of the medium, 200 µl of DMSO was added to each well to dissolve the purple formazan product. Absorbance of MTT signals was measured at A_{550}.

In vitro efficacy of AG879 against PPRV. The i*n vitro* efficacy of AG879 against PPRV was determined as follows: confluent monolayers of Vero cells (12 well plates) were infected with PPRV at MOI of 1 for 1 hr, washed three times by PBS and grown in DMEM in presence of 0.04–5 µmol/l AG879 or equivalent volume of DMSO (control). The infectious virus released in the supernatants at 24 hrs post-infecion (hpi) was titrated by plaque assay formation.

Real-time RT-PCR assay for viral RNA synthesis. Vero cells were infected with PPRV at an MOI of 5 for 1 hr followed by washing 6 times with PBS and addition of fresh DMEM containing 5 µmol/l AG879 or equivalent volume of control. The cell lysates were prepared at 5 hpi to isolate the total RNA using TRI Reagent according to instructions of manufacturer (Sigma, Germany). The viral and cellular mRNA were quantified by quantitative real-time RT-PCR as follows: the RNA was cleared of possible DNA contamination by incubation for 45 min at 37°C and 80°C for 20 min with DNAse I followed by reverse transcription as described before (Kumar *et al.*, 2013). Real-time RT-PCR was carried out with a 20 µl reaction mixture containing gene specific primers and SYBR Green DNA dye (Promega, USA). The primers for amplification of mRNA of the PPRV N gene have been described previously (Kumar *et al.*, 2013). β-actin was used as a house keeping control gene and was amplified using forward primer: 5'-CCC CAG CCA TGT ACG TTG CTA TCC-3' and reverse primer: 5'-GCC TCA GGG CAG CGG AAC CGC TCA-3'). For PCR amplification of both PPRV N and β-actin mRNA, following thermocycler conditions were used: an initial denaturation of 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec and a final extension step of 72°C for 10 min.

siRNA knockdown of Trk1. ~50% confluent monolayers of Vero cells, in triplicates, in 6 well plates, were either transfected with 2 µg of Trk1 siRNA expressing plasmids (psiRNAhH1-Trk1) or with 2 µg of negative control siRNA expressing plasmid (psiRNAhH1-Luc) using Escort IV transfection reagent according to the instruction of the manufacturer (Sigma, USA). The cell lysates were prepared at 72 hr post-transfection (hpt) and quantification of the Trk1 mRNA expression in control and shRNA-treated cells was performed by quantitative real-time RT-PCR, using following primers: forward primer-5'-CAGTGACCTCAACAGGAAGAAC-3' and reverse primer-5'CATTTGTTGAGCACAAGGAGC-3'. β-actin was used as a house keeping control gene for normalization purpose. The thermocycler conditions were similar as described above for real-time RT-PCR assay for viral RNA synthesis.

Statistical analysis. Pairwise statistical comparisons were performed using Student's t test.

Results

Cytotoxicity and virucidal activity of AG879

The RTK signaling was disrupted using a known RTK inhibitor, tryphostin AG879. The cytotoxic concentration of AG879 was determined by MTT assay. As shown in Fig. 1, no cytotoxicity was observed even after 72 hr of incubation of Vero cells with AG879 at 5 µmol/l. However, a significant loss of viability was observed at >5 µmol/l. Therefore we have decided to use 5 µmol/l of AG879 in subsequent experiments.

In order to demonstrate whether the inhibitory effect of AG879 on PPRV replication is partially due to inactivation of the extracellular virus (virucidal activity), 3-fold serial dilutions of AG879 or equivalent volume of control were incubated with PPRV at 37°C for 1.5 hr and then titrated on Vero cells by plaque formation. No significant inactivation of the PPRV was observed even at 81 µmol/l of AG879 (Fig. 2) suggesting AG879 has no virucidal effect on cell free virion.

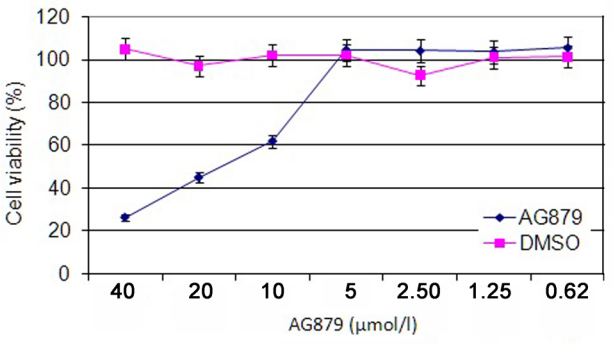

Fig. 1
Cytotoxicity of AG879
Vero cells in microplate wells were treated with 0.62–40 µmol/l inhibitor or DMSO for 72 hr and then assayed for viability.

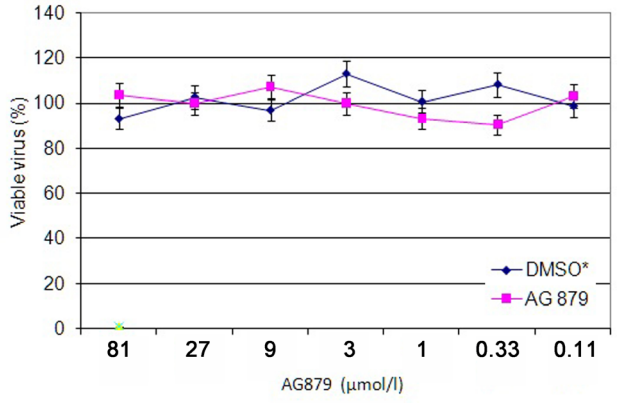

Fig. 2
Virucidal activity of AG879
The virus (~10^6 PFU) was incubated with either serial 3-fold dilutions (0.11–81 µmol/l) of AG879 or DMSO for 1.5 hr at 37°C and then titrated for infectious virus.

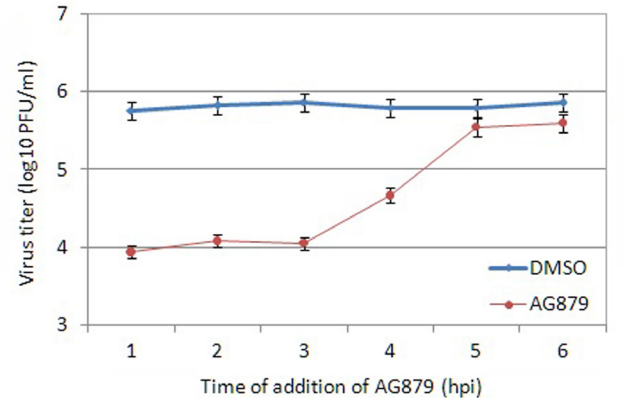

Fig. 4
Time-course of the inhibitory effect of AG879
Vero cells were infected with the PPRV at MOI of 10 for 1hr at 37°C, washed with PBS and refed with DMEM. The inhibitor was applied at indicated time points and 10 hpi the infectious virus was titered.

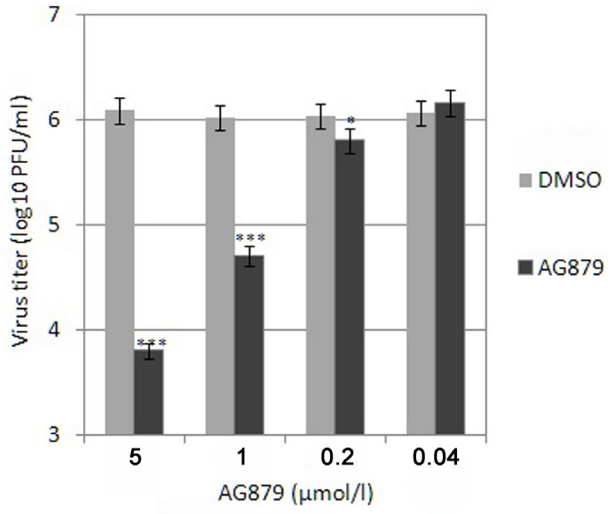

Fig. 3
Inhibition of virus replication with AG879
Vero cells were infected with the PPRV at MOI of 1 for 1 hr, washed with PBS, and refed with fresh DMEM containing either 0.04–5 µmol/l AG879 or DMSO for 24 hr and titrated for infectious virus. * and *** represent statistical significance at P <0.05 and P <0.001, respectively.

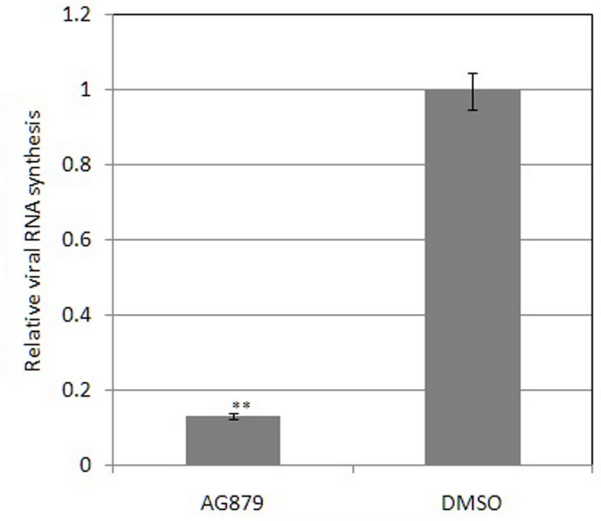

Fig. 5
Inhibition of viral RNA synthesis by AG879
Vero cells were infected with PPRV at MOI of 5 for 1hr, washed with PBS and refed with DMEM and 3 hr later the inhibitor or DMSO were applied. Viral RNA was assayed 5 hpi. β-actin was used as a house keeping control gene for normalization. ** represent statistical significance at P <0.01.

Inhibition of virus replication and viral RNA synthesis by AG879

The *in vitro* efficacy of AG879 against PPRV was determined in Vero cells. As shown in Fig. 3, at a non-cytotoxic concentration (5 µmol/l), as compared to control-treated cells, a significant reduction in viral titers was observed in AG879-treated cells suggesting that RTK supports PPRV replication.

Next, we determined the specific steps of PPRV affected by AG879. PPRV life cycle is 6–8 hr in cultured cells (Kumar *et al.*, 2013). Primarily, a time course assay was performed to evaluate the dynamics of the PPRV replication in presence of AG879. The inhibitor was applied at different time points following infection and the infectious virus released in to the supernatant 10 hpi was titrated by plaque formation. As indicated in Fig. 4, as compared to the control, AG879 inhibited PPRV replication almost at a similar level whether it was applied 1 hpi, 2 hpi or 3 hpi. These results suggest that early steps such as attachment, entry and uncoating which are believed to occur within the first few hours of infection

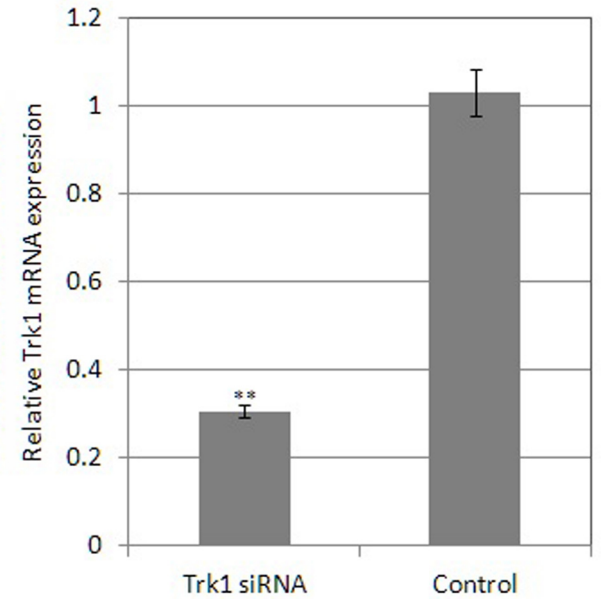

Fig. 6
Knockdown of Trk1 using plasmid expressing Trk1 siRNA
Vero cells were transfected with plasmid psiRNA-hH1Trk1 expressing Trk1-specific siRNA or plasmid psiRNA-hH1Luc expressing luciferase-specific siRNA (negative control) and 72 hpt, assayed for Trk1 mRNA. β-actin was used as a control house keeping gene for normalization. ** represents statistical significance at P <0.01.

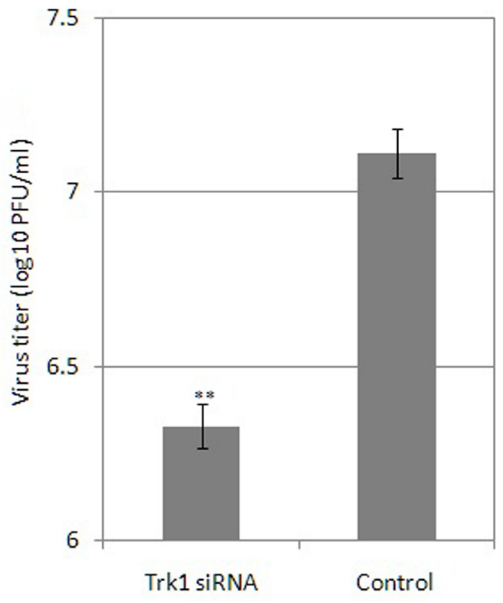

Fig. 7
Inhibition of PPRV replication by Trk1 knockdown
Vero cells were transfected with plasmid psiRNA-hH1Trk1 expressing Trk1-specific siRNA or plasmid psiRNA-hH1Luc expressing luciferase-specific siRNA (negative control), 72 hr later infected with PPRV at MOI of 10 and at 10 hpi, were titered for infectious virus. β-actin was used as a control house keeping gene for normalization. ** represents statistical significance at P <0.01.

are unlikely to be affected. However, there was a significant rise in viral titers in AG879-treated cells, when the inhibitor was applied 4 hpi, a time point around which RNA synthesis is believed to occur (Fig. 4). Further, no significant difference in the viral titers (compared to control) were observed when the inhibitor was applied 5 hpi and 6 hpi suggesting AG879 does not inhibit later stages of PPRV replication (Fig. 4). Taken together, it was concluded from the time-course experiment that neither the early nor the late steps of PPRV life cycle are affected by AG879 and hence it was speculated that the RNA synthesis, which is believed to occur around 3–4 hpi (middle step), is most likely to be affected. As shown in Fig. 5, as compared to control-treated cells, a significant reduction in viral RNA was observed in AG879-treated cells suggesting AG879 inhibits PPRV RNA synthesis.

Role of RTK in PPRV replication

Small interfering RNA (siRNA) or short interfering RNA are a class of double-stranded RNA molecules, 20–25 base pairs in length that interfere with the expression of specific genes with complementary nucleotide sequences (Whitehead *et al.*, 2009). In order to further confirm whether the inhibitory effect of AG879 on PPRV replication is really due to the action on its target molecule (RTK) and not due to off-target effects, a shRNA knockdown experiment was performed.

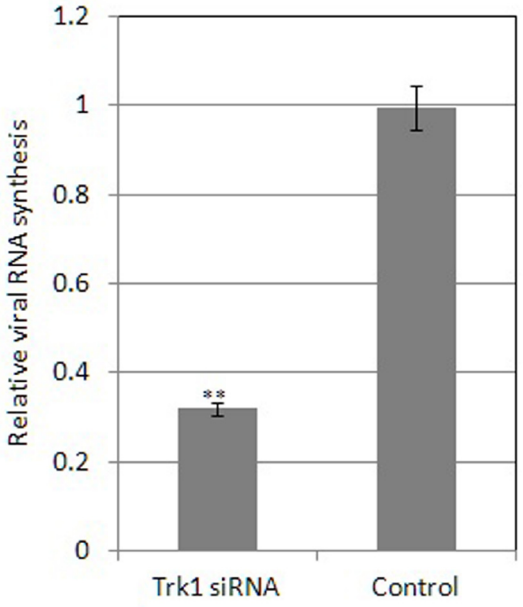

Fig. 8
Inhibition of PPRV RNA synthesis by Trk1 knockdown
Vero cells were transfected with plasmid psiRNA-hH1Trk1 expressing Trk1-specific siRNA or plasmid psiRNA-hH1Luc expressing luciferase-specific siRNA (negative control), 72 hr later infected with PPRV at MOI of 10 and at 10 hpi, cell lysates were assayed for the N gene mRNA. β-actin was used as a control house keeping gene for normalization. **represents statistical significance at P <0.01.

As shown in Fig. 6, at 72 hpt, psiRNAhH1-Trk1 specifically and significantly knocks down the level of Trk1 in Vero cells which was not observed with psiRNAhH1-Luc (negative control). Also, the Trk1 siRNA expressing plasmid did not affect the expression of the off-target genes, as the level of mRNA of the house keeping gene (β-actin) was similar in both Trk1 and negative control siRNA expressing cells. At 72 hpt, when a significant knockdown of Trk1 had already occurred (Fig. 6), cells were infected with PPRV at MOI of 10 and the infectious virus released in the supernatant 10 hpi was titrated by plaque formation. As shown in Fig. 7, as compared to negative control siRNA expressing cells, there was a significant reduction in viral titers from Trk1 siRNA expressing cells. Further, a significant reduction in viral RNA was also observed in Trk1 siRNA expressing cells as compared to negative control siRNA expressing cells (Fig. 8). These findings with siRNA knockdown of Trk1 confirmed the functional role of RTK signaling in PPRV replication.

Discussion

Upon viral infections an array of signal transduction events are initiated by the host cells that are basically antiviral (Ludwig *et al.*, 2006). However there are evidences that the virus has acquired the capability to misuse some of these signaling functions to support its effective replication (Kumar *et al.*, 2008, 2011a). Viruses have been shown to interact with a variety of cell signaling pathways (Kumar and Maherchandani, 2014). Inhibiting such signaling activities by targeting specific host genes leads to impaired virus production suggesting their functional role in virus replication (Kumar *et al.*, 2011b).

Recent studies have also suggested that the RTK signaling regulates replication of influenza and some other viruses (Eierhoff *et al.*, 2010; He *et al.*, 2010; Inubushi *et al.*, 2008; Kumar *et al.*, 2011a,b; Stantchev *et al.*, 2007). Nevertheless, our previous study with influenza virus also suggested that Trk1 regulates viral RNA synthesis. (Kumar *et al.*, 2011a). It seems, therefore, that RTK (Trk1) signaling is a prerequisite for a range of RNA viruses for efficient viral RNA synthesis. However, another study with influenza virus demonstrated that the epidermal growth factor receptor (EGFR, a RTK family member) promotes uptake of influenza A viruses into host cells (Eierhoff *et al.*, 2010) suggesting that RTKs may be involved in regulation of various steps of virus replication. It is a matter of further study to dissect the molecular mechanism of interaction between RTK and downstream signaling components to the virus counterpart.

The cellular factors that are required for the virus replication but at the same time are dispensable for the host cell metabolism may be much better target for antiviral interventions as virus can not easily replace the missing cellular functions by mutations (Kumar *et al.*, 2008; Ludwig *et al.*, 2006). As compared to the viral target, RTK inhibitors (a host target) have a very low tendency to develop drug resistance on sequential high passage (Kumar *et al.*, 2011b) and hence make them more attractive target for the development of antiviral therapeutics. The antiviral strategy may not be cost effective for livestock but could complement emergency vaccination in a previously disease-free setting or be applied to treat valuable zoological collections and breeding stocks (Charleston *et al.*, 2011; Goris *et al.*, 2008; Kumar *et al.*, 2014).

Taken together, it was concluded that RTK regulates PPRV replication at the level of viral RNA synthesis and hence may be proposed as a target to develop novel anti-PPRV therapeutics.

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