Virology 391 (2009) 325–333

Contents lists available at ScienceDirect

Virology

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

The molecular chaperone heat shock protein-90 positively regulates rotavirus infection

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ARTICLE INFO

Article history:
Received 20 May 2009
Returned to author for revision 7 June 2009
Accepted 26 June 2009
Available online 22 July 2009

Keywords:
Rotavirus
NFκB
Akt
HSP90
17-AAG
Geldanamycin analogs

ABSTRACT

Rotaviruses are the major cause of severe dehydrating gastroenteritis in children worldwide. In this study, we report a positive role of cellular chaperone Hsp90 during rotavirus infection. A highly specific Hsp90 inhibitor, 17-allylamono-demethoxygeldanamycin (17-AAG) was used to delineate the functional role of Hsp90. In MA104 cells treated with 17-AAG after viral adsorption, replication of simian (SA11) or human (KU) strains was attenuated as assessed by quantitating both plaque forming units and expression of viral genes. Phosphorylation of Akt and NFκB observed 2–4 hpi with SA11, was strongly inhibited in the presence of 17-AAG. Direct Hsp90–Akt interaction in virus infected cells was also reduced in the presence of 17-AAG. Anti-rotaviral effects of 17-AAG were due to inhibition of activation of Akt that was confirmed since, PI3K/Akt inhibitors attenuated rotavirus growth significantly. Thus, Hsp90 regulates rotavirus by modulating cellular signaling proteins. The results highlight the importance of cellular proteins during rotavirus infection and the possibility of targeting cellular chaperones for developing new anti-rotaviral strategies.

Introduction

Cells induce a stress response to virus infection, which results in the expression of stress response proteins like heat shock proteins (Hsps) (Sullivan and Pipas, 2001). It has been reported that both DNA and RNA viruses induce Hsps, independent of the cytoplasmic or nuclear location of their replication however the type of Hsp induced, depends on the virus or the cell type (Sedger and Ruby, 1994; Sullivan and Pipas, 2001). Hsp family members including Hsp40, 70 and 90 (40-, 70-, 90-kDa heat shock proteins) are also important molecular chaperones which regulate various cellular processes such as protein folding, transport, cell viability etc (Welch, 1991). While some viruses in the *Polyomaviridiae* and *Closteroviridiae* families encode their own viral specific chaperone proteins, most of the viruses have been shown to directly or indirectly depend on one or more cellular chaperones including, Hsp40 (Glotzer et al., 2000; Kumar and Mitra, 2005), Hsp70 (Mayer, 2005), Hsp90 (Hu and Seeger, 1996; Gilmore et al., 1998; Hung et al., 2002; Burch and Weller, 2005; Kampmueller and Miller, 2005; Okamoto et al., 2006; Connor et al., 2007; Naito et al., 2007; Chase et al., 2008; Ujino et al., 2009) and cyclophilins (Franke et al., 1994) to complete their replication. There are overwhelming evidences suggesting the dependence of both DNA and RNA viruses on cellular chaperones however functional mechanism varies among different viruses and host systems (Hu and Seeger, 1996; Hung et al., 2002; Mayer, 2005; Connor et al., 2007).

Hsp90 is a constitutive, atypical member of the molecular chaperone family present in eukaryotes and bacteria. It is an extremely abundant protein, comprising ~1–2% of total cellular protein (Powers and Workman, 2006). Hsp90 displays ATP-dependent folding capacity and has been shown to bind during later stages of protein folding for facilitating the activation-competent state, with the help of its various co-chaperones and ATP (Nollen and Morimoto, 2002; Pearl and Prodromou, 2006; Powers and Workman, 2006). Unlike the other promiscuous cousin chaperone Hsp70, Hsp90 appears to have a specific set of client proteins (Nollen and Morimoto, 2002; Pearl and Prodromou, 2006). The common denominator of the Hsp90 client proteins, is their role as regulators of signal transduction pathways involved in cell cycle or survival, thus maintaining cellular homeostasis (Zhang and Burrows, 2004; Citri et al., 2006). The Hsp90 may contribute to viral replication either by modulating the cellular signaling mechanisms (Sun et al., 2008) or by direct interactions with viral proteins such as hepatitis B (HBV) reverse transcriptase, hepatitis

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doi:10.1016/j.virol.2009.06.044

C NSP2/3 protein or RNA dependent RNA polymerase of Influenza A and vesicular stomatitis viruses (Hu and Seeger, 1996; Connor et al., 2007; Naito et al., 2007; Chase et al., 2008; Ujino et al., 2009).

Rotavirus is a double stranded RNA virus associated with severe dehydrating gastroenteritis in children worldwide. Children from underdeveloped countries, account for >80% of the rotavirus associated deaths (Rossen et al., 2004; Estes and Kapikian, 2007). The infectious virion is a non-enveloped, 11 dsRNA segmented genome surrounded by three concentric layers of protein. Rotavirus generally infects and replicates in mature enterocytes of the small intestine, leading to induction of inflammatory cytokines, reduced expression of enterocyte genes and vacuolization (Estes and Kapikian, 2007). Involvement of Hsc70 (70-kDa heat shock cognate protein) in the multistep entry of RV into intestinal epithelial cells and induction of heat shock protein transcripts following viral infection has been reported previously (Cuadras et al., 2002; Guerrero et al., 2002). However unlike the positive role of Hsps in facilitating replication in case of Influenza or HBV viruses, Hsp70 was found to negatively control RV infection by directing viral proteins towards ubiquitin dependent degradation. Silencing of Hsp70 resulted in increased translation of rotavirus proteins (Broquest et al., 2007). In spite of the induction of Hsps during rotavirus infection, there is no information till date about the role of cellular Hsp90 in regulating rotavirus growth and replication.

To elucidate the role of molecular chaperone Hsp90 in rotavirus replication, we utilized 17-allylamono-demethoxygeldanamycin (17-AAG; NSC 330507), a highly specific but less toxic analogue of the benzoquinone ansamycin antibiotic geldanamycin (Sausville et al., 2003). Geldanamycin and its analogs bind to the ATP-binding pocket on Hsp90 and interferes with its chaperone functions (Stebbins et al., 1997). A significant reduction (~1.5–1.8 log) in viral titers was observed in the presence of 17-AAG in a dose and time dependent manner. To our knowledge this is the first report showing involvement of Hsp90 as a modulator of rotavirus infection. In an attempt to address the possible molecular mechanism of 17-AAG mediated regulation of virus growth we have identified a positive role of Hsp90 client proteins, Akt and NFκB during rotavirus infection. The results emphasize an important role of cellular proteins during infection of rotaviruses.

Results

Inhibition of Hsp90 suppresses the rotavirus replication

The cytotoxicity of 17-AAG was tested in mock-infected MA104 cells treated with increasing concentrations of the inhibitor for 12–48 h in serum free conditions by measuring cell viability using MTS reduction assay measuring metabolic activity of cells. There were 96.5–94.6%; 92.2–88.5% and 87.7–83.3% viable cells at 24–48 h post treatment in the presence of 2.5 μM–5 μM–10 μM of 17-AAG respectively. More than 80% viable cells after 48 h of treatment with 5–10 μM 17-AAG was also confirmed by neutral red dye uptake assay. After 24 and 48 h, 97% and 93.8% viable cells were observed in DMSO (<0.4%) treated control cells in serum free conditions (Fig. 1A). Cell extracts prepared after 48 h were also subjected to immunoblotting using Hsp90 antibody to assess the effects of 17-AAG on Hsp90 expression. A 2–3 fold decrease in Hsp90 expression was observed following treatment with 5–10 μM of 17-AAG for 48 h (Fig. 1A).

To assess whether Hsp inhibitor 17-AAG, could modulate rotavirus growth, MA104 cells were infected with prototype strain SA11 (moi 1). After adsorption, cells were incubated at 37 °C in maintenance media containing either 17-AAG (2.5–10 μM) or DMSO control. Cells were harvested at 20 hour post infection (hpi) and viral titers were determined using plaque assay. SA11, a fast growing strain has high infectivity rate (>90%) in MA104 cells and results in >80% loss of cell viability within 24 h. Compared to no drug controls, 17-AAG reduced viral titers in a dose dependent manner. At 5 μM and 10 μM dose, there was reduction in viral titer by 1.6–1.8 log and 2.1–2.2 log ($p<0.01$) respectively (Fig. 1B). The data suggested that for efficient rotavirus replication, functional Hsp90 chaperone complex is required. Subsequent experiments were carried out with the effective but less toxic dose (5 μM) of 17-AAG. To understand whether 17-AAG affects early or late stages of viral life cycle, a time course study of viral growth was done. The MA104 cells were infected with SA11 (simian) and KU (human) strains (moi 1) and recovered in the absence or presence of 17-AAG. 17-AAG had a significant effect on slowing virus growth and showed noticeable delay in virus growth as early as 8 h in fast growing strain SA11 and 16 h in slow growing human strain KU compared to only virus infected DMSO controls. At the end point, there was 1.6–1.7 log and 1.2–1.4 log reduction in viral titers of SA11 and KU in the presence of 17-AAG (Fig. 1C). To assess whether Hsp90 function is required during early or late stages of infection, MA104 cells were infected with SA11 and 17-AAG was added to the media at 0 hpi, 2 hpi and 4 hpi. After 20 h of infection, cells were harvested and viral titers were estimated. As shown in Fig. 1D, 17-AAG significantly inhibited virus growth even when added 2 hpi (0.8–1.0 log), however the inhibitory effect was reduced when drug was added after 4 h of infection (<0.3 log) indicating the importance of Hsp90 chaperone activity during early stages of infection.

To elucidate the effect of Hsp90 inhibition on expression of viral genes, MA104 cells were infected with strain SA11 (moi 3) in the presence of either 17-AAG or only DMSO. Cells were harvested at 8 hpi and viral VP6 (viral protein 6; inner capsid) and NSP4 transcripts were quantitated in triplicates by real time PCR using gene specific primers and GAPDH as endogenous control. There was 2.2–2.4 fold ($p<0.05$) reduction in VP6 and NSP4 transcripts (Fig. 1E). The decrease in expression of viral genes was further validated by assessing decrease in levels of viral proteins in the presence of 17-AAG by immunoblotting. Cellular extracts prepared at 12 hpi in the presence of 17-AAG showed 2.72, 2.4 and 3.0 fold decrease in expression of VP6, NSP4 and NSP1 proteins respectively compared to only virus treated controls (Fig. 2A).

The specificity of 17-AAG for HSP90 facilitates the study of HSP90-dependent cellular pathways. However, to independently confirm the role of HSP90 in rotavirus replication in MA104 cells using a genetic approach that did not rely on pharmacological inhibitors; we used RNAi to selectively downregulate the expression of HSP90. MA104 cells were transiently transfected with Hsp90 specific or matched negative control siRNAs for two consecutive days. After 24 h of second transfection, a 2 fold reduction in hsp90 was observed compared to controls (Fig. 2B). siRNA transfected MA104 cells were infected with SA11 and rotavirus growth was measured by quantitating VP6 transcript by real time PCR and protein expression by immunoblotting. Compared to only virus controls, more than 2.0 fold decrease in viral transcripts and VP6 protein expression was observed in Hsp90 siRNA transfected cells (Figs. 2C, D) confirming the functional role of Hsp90 during rotavirus growth. A 9–10% decrease ($p>0.05$) observed in the matched negative control siRNA transfected cells could be due to non specific toxicity associated with transfection (Fig. 2D).

Inhibition of rotavirus replication by 17-AAG correlates to downregulation of virus induced Akt phosphorylation

Based on previous reports regarding activation of PI3K/Akt in virus survival (Cooray, 2004; Halasz et al., 2008; Sun et al., 2008) and modulation of Akt by Hsp90 (Fujita et al., 2002; Zhang and Burrows, 2004), the effect of Hsp90 inhibitor on rotavirus-induced Akt infection was analyzed. Cell lysates prepared at 4–12 hpi from cells either mock-infected or infected with SA11 (moi 3), were subjected to western blotting using p-Akt (ser473) and p-GSK-3 β (glycogen synthase kinase-3 β) antibodies. Following rotavirus infection, a 5–7 fold increase in Akt phosphorylation was observed at 4, 8 and 12 hpi compared to 2–3.2 fold increase in p-GSK-3 β. There was no significant change in basal Akt or GSK-3 β levels indicating that rotavirus modulates only the activation of endogenous Akt (Fig. 3A). However, when post infection, cells were recovered in the presence of 17-AAG,

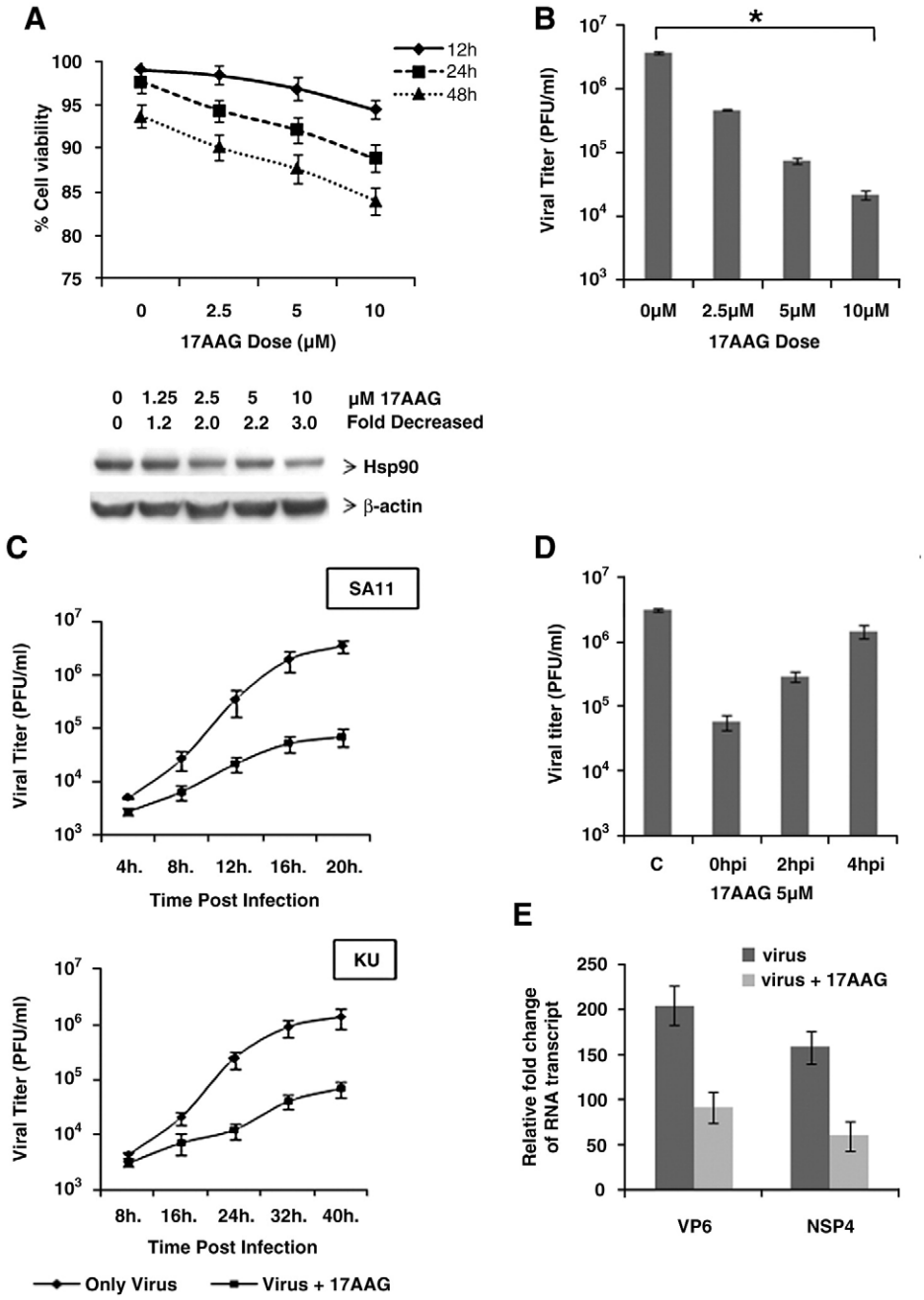

Fig. 1. HSP90 inhibitor 17-AAG inhibits rotavirus multiplication. (A) Viability of MA104 cells cultured in serum free medium supplemented with different concentrations of 17-AAG (0–10 μM) is shown as percent cell viability as determined by MTS reduction assay with Cell titer 96® Aqueous One Solution Cell Proliferation assay kit (Promega) following treatment for 12, 24 and 48 h . After 48 h, cells were lysed and Hsp90 expression was analyzed by immunoblot analysis (0–10 μM). Decrease in Hsp90 expression (2–3 folds) was observed in the presence of 17-AAG. (B) Dose dependent inhibition in viral titers (SA11) by 17-AAG as measured by plaque assay after 20 h post infection. In the presence of 5 μM–10 μM 17-AAG, 1.6–2.2 log inhibition ($p<0.05$; *$p<0.01$) was observed. (C) Time kinetics of inhibition of rotavirus strains (SA11 and KU) growth in the presence (■) or absence (♦) of 17-AAG (5 μM) as measured by plaque assay. At respective end points, a 1.3–1.7 log inhibition was observed for KU and SA11 strains respectively. (D) Role of Hsp90 in initial stages of rotavirus infection was confirmed by addition of 17-AAG at 0 hpi, 2 hpi and 4 hpi. Cells were lysed after 20 h of infection and viral titers were determined by plaque assay. There was an increase in rotaviral titers when 17-AAG was added 2 or 4 h post infection compared to 0 hpi. (E) 17-AAG (grey bar) inhibits expression of *VP6* and *NSP4* gene transcripts (8 hpi) in MA104 cells compared to only virus (black bars) infected cells, as measured by real time PCR. GAPDH was used as endogenous control. The data shown represent mean ± SD of three experiments. The decrease in viral titers and gene expression in the presence of 17-AAG was statistically significant ($p<0.05$).

and significant downregulation of rotavirus-induced phosphorylation of Akt (2.3–2.6 fold) and its substrate GSK-3 β (2.0–2.1 fold), was observed compared to the only virus infected cells (Fig. 3B). Whether the effect of 17-AAG on Akt phosphorylation was due to direct or indirect interactions between Hsp90 and Akt was assessed by immunoprecipitation of cell lysates prepared following rotavirus infection (8 h) in the presence or absence of 17-AAG using Hsp90 antibody. The blots were probed with Akt and Hsp90 antibodies. As shown in Fig. 3C, in cells infected with rotavirus, Akt co-immunoprecipitated with Hsp90 (Fig. 3C, lane 2), which was significantly inhibited in virus infected cells treated with 17-AAG (Fig. 3C, lane 3). At 8 hpi, there was no difference in Hsp90 expression in the presence or absence of 17-AAG.

To confirm whether downregulation of p-Akt by 17-AAG, could be one of the possible mechanism of its antiviral effects, cells were infected with strain SA11 (moi 1) in the presence of p-Akt inhibitor (triciribine 2.5 μM), PI3K inhibitor (LY294002, 10 μM), 17-AAG or DMSO controls. Cells were harvested in a time dependent manner and viral titers were

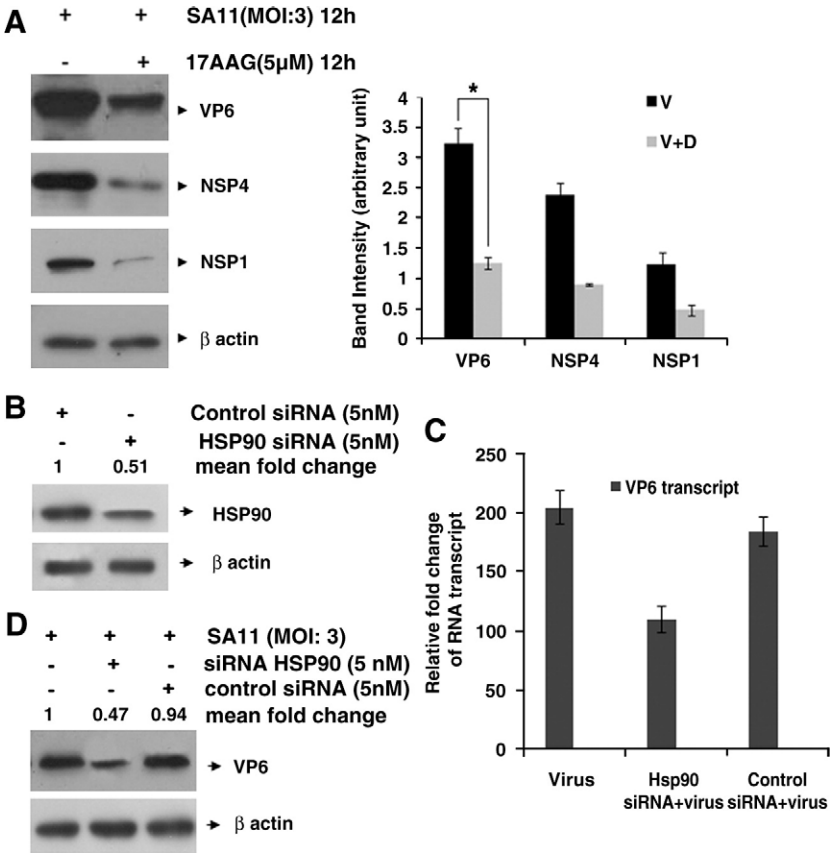

Fig. 2. Effect of Hsp90 inhibition on rotavirus gene expression. (A) Western blot analysis of VP6, NSP4 and NSP1 expression in the presence or absence of 17-AAG (12 hpi). 17-AAG significantly reduced expression of VP6, NSP4 and NSP1 proteins compared to cells infected with virus in the presence of DMSO. The blots were reprobed with β-actin to confirm equal protein loading. Fold change was quantitated by measuring band intensities in respect to β-actin control ($n=3$; $p<0.05$; $^*p\leq 0.01$). (B) Downregulation of HSP90 expression in the presence of HSP90 siRNA (5 nM) as analyzed by western blotting. (C and D) Cells that were mock-transfected or transfected with HSP90 siRNA or matched negative control siRNA were infected with SA11 (moi 3). After 8 hpi or 12 hpi, cells were lysed and viral gene *VP6* transcript or protein expression was measured by RTQ PCR (C) and immunoblotting (D) showing significant reduction in expression of both VP6 transcripts as well as protein expression as a result of HSP90 silencing.

determined. In the presence of triciribine or LY294002, a 1.1–1.3 log reduction in viral titers was observed at 20 h compared to 1.6–1.7 log inhibition with 17-AAG, confirming the importance of virus induced activation of PI3K/Akt during its replication (Fig. 3D). The reduction in viral titers was not due to cytotoxic effects since in serum free conditions mimicking virus infection, there were 92.6–88.8% and 90.1–85.2% cell viability after 24–48 h of triciribine (2.5 μM) and LY294002 (10 μM) treatment (Fig. 3E). The virus induced phosphorylation of Akt was also inhibited, in the presence of both triciribine and LY294002 confirming correlation between inhibition of viral replication and Akt phosphorylation (data not shown). Positive role of Akt was further confirmed using Akt-1 and Akt-2 specific siRNAs. After 24 h of transfection, 60% decrease in Akt expression was observed compared to controls. Following SA11 infection in siRNA transfected MA104 cells, expression of VP6 protein was quantitated by immunoblotting. Compared to only virus controls, more than 3 fold decrease in VP6 protein expression was observed in Akt siRNA transfected cells (Fig. 3F).

17-AAG inhibits Akt regulated mTOR signaling

Based on previous data confirming positive role of Akt, we hypothesized that 17-AAG may effect virus growth by inhibiting the Akt substrates such as regulator of the initiation of cap-dependent translation mTOR (mammalian target of rapamycin). Phosphorylation of mTOR, 4EBP1 (eukaryotic initiation factor 4E binding protein 1) and p70S6K (ribosomal p70 S6 kinase) was analyzed 12 h post infection (SA11) in the presence or absence of 17-AAG by immunoblotting. Rapamycin (100 nM), inhibitor of mTOR, was used as positive control. Rotavirus infection resulted in an increase in phosphorylation of mTOR (>10 fold), 4EBP1 (≥7 fold) and p70S6K (≥5 fold), which was significantly reduced in the presence of 17-AAG (Fig. 4). No change in expression of basal mTOR, 4EBP1 or S6K protein in the presence of 17-AAG was observed (Fig. 4).

17-AAG inhibits activation of rotavirus-induced NFκB, another Hsp90 and Akt modulated protein

Since there are reports of activation of NFκB during rotavirus infection and NFκB is also activated by PI3K/Akt and Hsp90, we analyzed whether rotavirus-induced NFκB activation is modulated by 17-AAG. MA104 cells were infected with SA11 (moi 3) followed by the treatment with either 17-AAG or DMSO, and cell extracts were prepared at 2–8 h post infection. Immunoblotting with phospho-p65 antibody showed increased phosphorylation of p65 subunit of NFκB following rotavirus infection, as early as 2–4 h (2.5–3.5 fold) with subsequent decrease at 8 h (Fig. 5A) confirming previous observations. However there was a significant downregulation of p-p65 at 2 h (1.7 fold) and >4.5 fold inhibition by 4 h in the presence of 17-AAG (Fig. 5A). Inhibition of NFκB activation was further confirmed using a luciferase reporter construct with a 2× NFκB promoter. A strong activation of NFκB promoter as measured by luciferase enzyme activity was observed 4 h and 8 hpi following rotavirus infection, which was inhibited 3–5 fold ($p<0.01$) in the presence of 17-AAG (Fig. 5B).

Discussion

All viruses depend on host cells for a successful infection, thus they need to create a cellular environment which is conducive for their

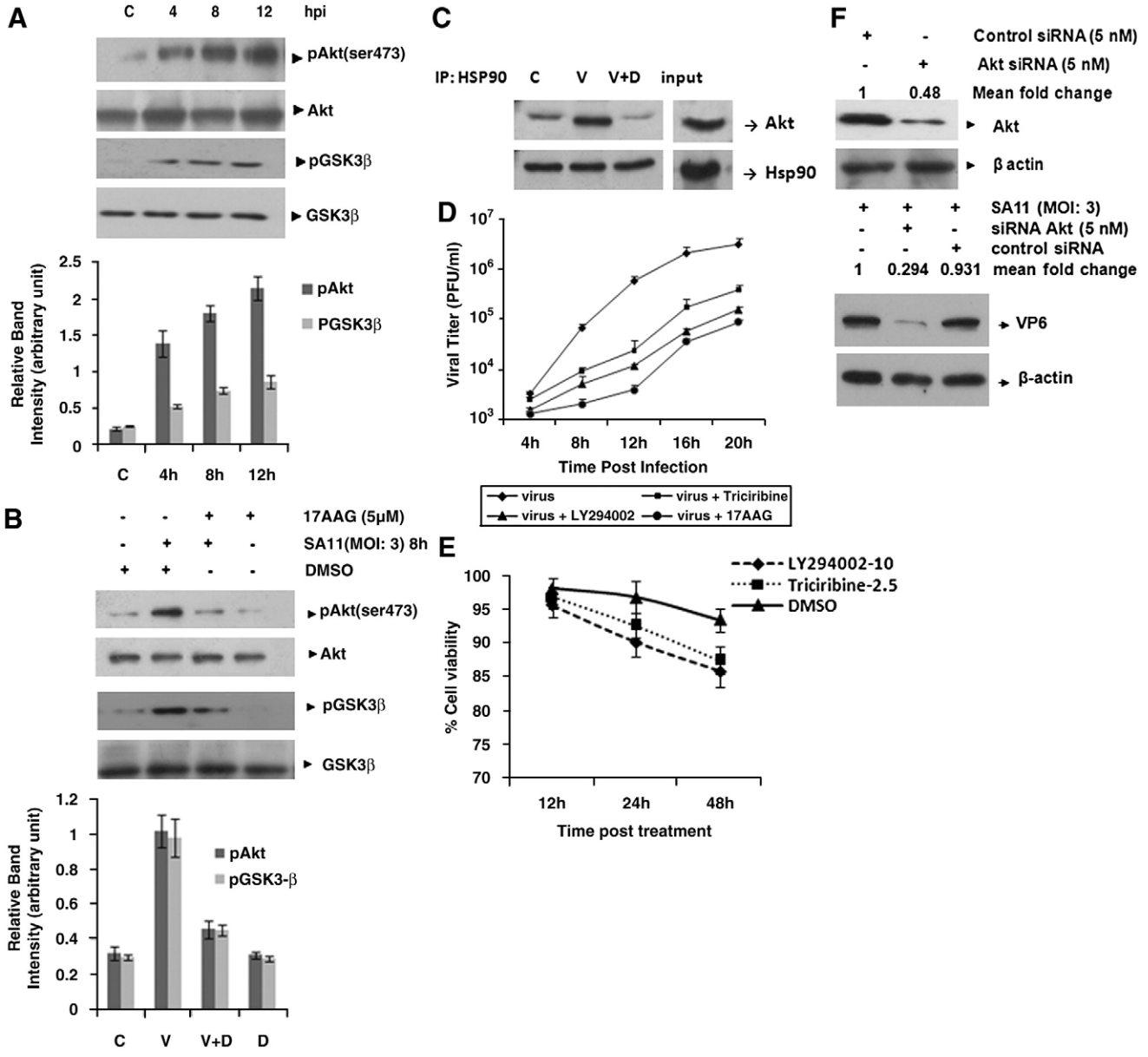

Fig. 3. Effect of 17-AAG on rotavirus-induced Akt phosphorylation. (A) Western blot analysis showing phosphorylation of Akt (ser473) and its downstream substrate GSK-3 β at 4–12 h post SA11 infection. Blots were reprobed to analyze basal expression of Akt or GSK-3 β following rotavirus infection. (B) 17-AAG (5 μM) inhibits the rotavirus (8 hpi) induced Akt and GSK-3 β phosphorylation. No effect of 17-AAG on basal Akt or GSK-3 β expression was observed. (C) Co-immunoprecipitation of Hsp90 with Akt is significantly reduced in the presence of 17-AAG. Cells were either mock-infected or infected with SA11 (moi: 3) in the presence or absence of 17-AAG. Cell lysates prepared at 8 hpi were immunoprecipitated with anti-Hsp90 antibody and separated on a 10% polyacrylamide gel. Blots were probed with Akt and Hsp90 antibody. Whole cell lysates were also loaded as internal control for Akt and Hsp90 protein. (D) Time kinetics of inhibition of SA11 growth in the presence of inhibitors namely, 17-AAG (5 μM ●), triciribine (2.5 μM ■), LY294002 (10 μM ▲) or DMSO control (♦) for 20 h as measured by plaque assay. The data represent the mean ± SD of three experiments ($p<0.05$). (E) Percent cell viability of MA104 cells incubated with triciribine (2.5 μM), LY294002 (10 μM) or DMSO as determined by MTS reduction assay with Cell titer 96® Aqueous One Solution Cell Proliferation assay kit (Promega) following 12, 24 and 48 h of treatment in serum free conditions. Individual data points represent the mean values from three independent experiments. (F) Downregulation of Akt expression by Akt siRNA (5 nM, Ambion) after 24 h of transfection in MA104 cells as confirmed by western blot analysis. Following Akt or negative control siRNA transfection, MA104 cells were infected with SA11 and expression of VP6 protein was quantitated after 12 h by immunoblotting. Significant reduction (3 fold) was observed in the expression of VP6 protein expression in the presence of Akt siRNA ($p<0.05$).

replication. To survive and evade host immune responses, viruses have developed the ability to exploit cellular signaling pathways regulating, the cell survival, homeostasis or apoptosis for their benefit. Thus identification of important cellular factors modulating viral growth is important to not only understand virus–host interactions but pathogenesis of the disease. Moreover the possibility of targeting cellular proteins for developing new generation of low cost antivirals specially in the case of short term viral infections is gaining importance due to its implications in reducing morbidity and mortality in developing countries (Lewis et al., 2000; Geller et al., 2007).

During the last decade, there are lots of reports regarding rotavirus diversity and epidemiology, vaccine development, gene expression, evasion of immune response by modulating IFN regulatory factors etc (Estes and Kapikian, 2007). In a microarray study following RV infection in CaCo2 cells, induction of heat shock protein transcripts was observed (Cuadras et al., 2002), but significance of the Hsp induction has not been studied. There are ample direct or indirect evidences supporting the positive role of heat shock proteins in modulating replication of many viruses (Connor et al., 2007; Geller et al., 2007; Sun et al., 2008; Chase et al., 2008; Ujino et al., 2009). Thus we hypothesized that induction of Hsp90 during rotavirus infection

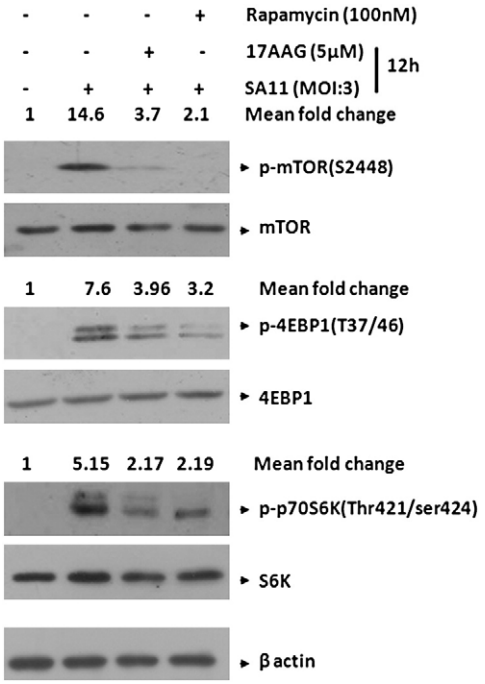

Fig. 4. 17-AAG inhibits rotavirus-induced mTOR-4EBP1-p70S6K activation by Akt regulation. Western blot showing rotavirus (SA11) induced phosphorylation of mTOR, 4EBP1 and p70S6K at 12 hpi. In the presence of 17-AAG, phosphorylation of mTOR-4EBP1-p70S6K was inhibited 3.7, 2.0 and 2.5 fold respectively compared to only virus treated cells. There was no change in expression of basal mTOR, 4EBP1 or p70S6K proteins following treatment with 17-AAG. Rapamycin was used as positive control. The blots were reprobed with β-actin to confirm equal protein loading.

may also have a role in either negative or positive regulation of viral replication. In this report, we establish that rotavirus growth is positively dependent on Hsp90 and its downstream client proteins.

17-AAG, a cell permeable analogue of geldanamycin binds to the ATP-binding pocket within the N-terminal domain of Hsp90 disrupting its chaperone activity (Sausville et al., 2003). Depletion of Hsp90β and modulation of various signaling proteins following treatment with 17-AAG in HT29 has been shown previously by gene expression profiling and proteomic analysis. Downregulation of Hsp90β following 17-AAG treatment for longer periods is probably due to proteosomal degradation of inactive Hsp90 (Clarke et al., 2000). In the presence of 17-AAG, reduction in viral titers was observed irrespective of species or serotype specificity of rotaviral strains. Similar inhibition of rotavirus growth by 17-AAG was also observed in HT29 (24 h) and CaCo2 (40 h) cells (data not shown). At dose or time used in the study (5 μM/24 h), 17-AAG showed minimal cytotoxicity as determined by MTS assay or neutral red uptake, indicating that inhibition of viral growth was not due to cytotoxicity. Moreover the downregulation of Hsp90 expression by RNA interference also resulted in decrease in rotavirus replication. The reduction in viral gene transcripts or protein in the presence of 17-AAG was also not due to global downregulation of cellular gene expression since under similar conditions (8–12 hpi) expression of cellular genes namely *β-actin*, *GAPDH*, *Akt*, *mTOR*, *p70S6K* etc was not altered. Based on the studies where inhibitor was added at different time points after virus infection, it can be deduced that in conditions used during the study the anti-rotavirus effects of 17-AAG were during initial stages of infection since the inhibitory effects of 17-AAG were significantly reduced when a drug was added after 4 h of adsorption compared to 0 hour post adsorption. The reduced viral growth observed during the study is not due to inhibition in virus entry since in all experiments, the inhibitor was added after virus adsorption (45 min). Trypsin activated rotaviruses have been shown to internalize in MA104 cells within 3–5 min of the adsorption (Kaljot et al., 1988).

Based on our observations and lack of any previous reports, we first tried to elucidate the role of Hsp90 in modulating rotavirus-induced intracellular signaling pathways. Hsp90 has been shown to interact with more than 100 protein kinases and associated proteins regulating their biogenesis, stability and activity (Zhang and Burrows, 2004; Citri et al., 2006). Hsp inhibitors have been reported to affect activities of Akt, IkappaB kinase, receptor interacting protein (RIP), NFκB inducing kinase (NIK), Death associated protein kinases (DAPK), cyclin dependent kinases, p53 etc (Lewis et al., 2000; Fujita et al., 2002; Broemer et al., 2004; Zhang and Burrows, 2004; Citri et al., 2006). Activation of the cell survival signals like PI3K/Akt and NFκB has been implicated in maintaining short term cell viability for facilitating pathogen replication during infections. After the production of viral progeny, virus particles are released by downregulation of prosurvival pathways and induction of apoptosis (Cooray, 2004; Sun et al., 2008; Ehrhardt and Ludwig, 2009).

PI3K/Akt activation during rotavirus infection has been shown to modulate integrin expression and positively regulate rotavirus replication (Halasz et al., 2008). In this study too we observed

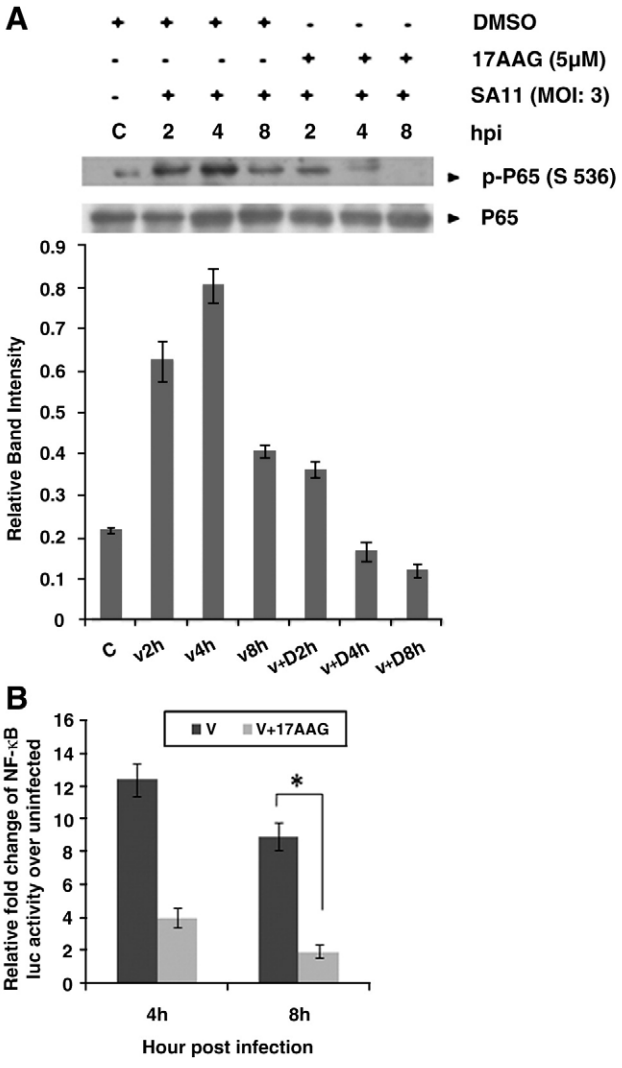

Fig. 5. 17-AAG inhibits rotavirus-induced activation of Hsp90 client protein NFκB. (A) Immunoblotting with phospho-NFκB p65 antibody revealed a significant increase in phosphorylation of p65 subunit (2–4 h), which was inhibited 1.75–4.5 fold ($p<0.05$; $n=3$) in the presence of 17-AAG. There was no change in the expression of p65 in the presence or absence of 17-AAG following 8 h of treatment. (B) NFκB promoter activity was measured in the presence or absence of 17-AAG (4 hpi and 8 hpi) using a 2× NFκB-luc plasmid. Significant inhibition in activation of NFκB promoter was observed at 4 hpi ($p<0.05$; $n=4$) and 8 hpi ($^*p<0.01$) compared to only virus infected cells. Mean relative luciferase activity of NFκB-luciferase was normalized with *Renilla* luciferase.

phosphorylation of Akt and its downstream substrate GSK-3 β, up to 12 h , following rotavirus infection which was inhibited (>2.2 fold) in the presence of 17-AAG. Unlike previous report (Halasz et al., 2008), where Akt activation was observed from 1 h to 8 h depending on cell type with RRV and CRW8 strains, we observed p-Akt until 12 hpi with SA11 strain in MA104 cells, whereas following infection with KU strain, p-Akt was observed only at 2–6 hpi (unpublished observation) indicating strain specific differences. Importance of Akt activation during rotavirus infection was re-confirmed when triciribine, a specific inhibitor of phospho-Akt-1 and Akt-2 (Yang et al., 2004) as well as general PI3K inhibitor LY294002 reduced rotaviral titers in a time dependent manner. The inhibitory effect on viral growth was not due to non specific effects of chemical inhibitors since Akt specific siRNAs, but not control siRNA inhibited the viral gene expression. Based on a previous study by Halasz et al. (2008), and our observations, it is clear that activation of PI3K/Akt positively regulates rotavirus replication. Hsp90 chaperone cycle has been shown to activate associated client protein Akt/PKB by phosphorylation (Citri et al., 2006). Thus 17-AAG mediated inhibition of Hsp90 may be one of the possible mechanisms of inhibition of Akt signaling.

Earlier *in-vivo* study has shown activated p70S6k in crypt cells of rotavirus infected piglets (Rhoads et al., 2007). PI3K/Akt signaling has also been implicated in translational control. Regulation of Tor kinase regulates translation through two independent pathways, involving p70S6 kinase (S6K) and the initiation factor 4E (eIF-4E)-binding protein-1 (4E-BP1). S6K phosphorylation enhances translation of 5′ TOP mRNA, containing oligopyrimidine tract which encodes for ribosomal proteins and elongation factors (Jefferies et al., 1997). Phosphorylation of 4E-BP1, a negative regulator of translation initiation, inhibits its interaction with eIF-4E, which binds to m7GpppN cap of mRNA and directs its correct positioning of ribosomal units to relieve translational block (Gingras et al., 2001). In the presence of 17-AAG, virus induced phosphorylation of mTOR-4EBP1-p70S6K was significantly decreased, which could be partly responsible for inhibition of cap-dependent translation of rotaviral RNAs, further confirming the importance of Akt signaling for virus multiplication.

Other than Akt, Hsp90 inhibition may affect one or more other client proteins associated with modulation of cellular homeostasis. We looked at the effect on NFκB, since previous reports suggested activation of NFκB by rotavirus capsid protein VP4 through interaction with TRAF2 (La Monica et al., 2001), and induction of IL8 in rotavirus infected intestinal epithelial cells (Casola et al., 1998; Rollo et al., 1999). In addition Akt is also implicated in activation of NFκB via IκB kinase pathway (Ozes et al., 1999) and disruption of Hsp90 function has been shown to inhibit NFκB activation by modulating RIP (Lewis et al., 2000). However recent reports have shown rotavirus mediated inhibition of NFκB translocation to nucleus as a mechanism of antagonizing cellular antiviral responses (Holloway et al., 2009) and possible role of NSP1 in the process (Graff et al., 2009). In contrast NFκB specific inhibitors have been shown to inhibit rotavirus replication (Rossen et al., 2004). In these studies too authors have observed activation of NFκB following RRV, Wa or NCDV infection during initial stages but have concluded that during later stages when rotavirus replication is established and rotavirus proteins are expressed, nuclear translocation of p65 NFκB is inhibited to restrict induction of NFκB driven antiviral cytokines like IFNβ or IL8 (Holloway et al., 2009; Graff et al., 2009). A time course study comparing various rotavirus strains from human and animal origin in different cell lines may solve the fine balance of both proviral and antiviral functions of NFκB during rotavirus infection. In this study too, SA11 infection (2–8 hpi), induced both phosphorylation of p65 and NFκB promoter activation confirming previous observations. Increase in IL8 transcript (>50 fold) in MA104 cells following SA11 infection (4–8 hpi), was also observed by real time PCR (data not shown). The activation of virus induced NFκB was significantly reduced in the presence of 17-AAG indicating that NFκB inhibition also partly contributes to antiviral effects of 17-AAG. Hsp90 may also have additional direct effects or may modulate other unknown client proteins during rotavirus infection which requires further studies.

Viral inhibition through regulation of cellular machinery could be exploited further as an antiviral approach with double benefits of avoiding the development of resistant viral strains as well as having a broad spectrum antiviral activity. In case of emergence of new variants of virus causing outbreaks, this would prove to be useful in controlling the infection, before viral target proteins are characterized or vaccines are developed. This report highlights the role of chaperone protein Hsp90 in positive regulation of rotavirus growth possibly by modulating virus induced activation of cellular signaling pathways. Targeting molecular chaperones like Hsp90 for developing antiviral drugs has additional benefits, i) a broad range of DNA and RNA viruses have been reported to be inhibited by geldanamycin analogs and ii) chaperones being evolutionarily conserved proteins, the probability of generation of drug resistant variants is expected to be extremely low (Geller et al., 2007). The high morbidity and mortality associated with rotavirus-induced diarrhea, emphasize the need for evaluating low toxicity inhibitors targeting cellular proteins as future anti-rotaviral strategies.

Materials and methods

Chemicals

Inhibitors for Hsp90 (17-AAG), PI3Kinase (LY294002), phospho-Akt (triciribine), mTOR (rapamycin) were purchased from Invivogen (San Diego, CA) and Biomol (Plymouth Meeting, PA). Other fine chemicals and buffers used in the study were from Sigma-Aldrich (St. Louis, MO).

Cell culture and virus infection

The monkey kidney cell line (MA104) was cultured in minimal essential medium (MEM), supplemented with US certified 10% fetal bovine serum and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA). Cells were maintained in 5% CO_2 at 37 °C humidified incubator. The simian rotavirus SA11 {H[96]} and human rotavirus Ku strains were used in the study. For infection, viruses were activated with acetylated trypsin (10 μg/ml) at 37^0 C for 30 min, diluted as per required multiplicity of infection (moi) and added to the cells for adsorption (45 min) at 37 °C, followed by washing 3× with media to remove unbound virus. Infection was continued in fresh medium. Except for viral growth assays (moi 1), moi 3 was used in the study. The time of virus removal was taken as 0 hour post infection (hpi) for all experiments. At different time points cells were freeze-thawed for cell lysis. Extracted and purified viral preparations by glycerol gradient (30–60%v/v) ultracentrifugation were titrated by plaque assay (Jolly et al., 2000). The end point in experiments were determined based on time required by virus to complete its replication in cells resulting in >80% CPE as determined by neutral dye uptake assay. The end point for SA11 and KU strain varied from 20 to 40 h depending on replication cycle of viruses. In all experiments DMSO (≤0.25%) was added in mock-infected controls to rule out any adverse effects of DMSO. The different inhibitors used in the study were added after adsorption of virus and addition of maintenance media (0 hpi).

Plaque assay

Monolayers of MA104 cells in six well plates were infected with serial dilutions (10^2–10^8) of viral supernatants as described earlier. After 45 min of adsorption, inoculum was removed and cells were overlaid with 0.7% agar in 1×MEM with 1 μg/ml trypsin. After 36–48 h post infection second agar overlay (0.7% agar in 1×MEM with 0.1%

neutral red) was added and plates were incubated at 37 °C until plaques were visualized. Viral plaque forming units were calculated as described previously (Smith et al., 1979). >1 log inhibition in viral titers is equivalent to >90% inhibition.

Cell viability assay (MTS assay)

Cell viability in the presence or absence of different inhibitors (17-AAG, LY294002, triciribine) was determined by Cell titer 96® Aqueous One Solution Cell Proliferation assay kit (Promega, Medison, WI, USA) which contains [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt]; MTS) and an electron coupling reagent (phenazine ethosulphate; PES). MTS gets bioreduced to form soluble coloured formazan by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Briefly, cells with ~80% confluency in 96 well plates were incubated with different inhibitors or DMSO for indicated time periods. At the end of each incubation period cells were treated with 20 µl of the reagent solution in 100 µl serum free medium for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The quantity of the soluble formazan product was measured spectrophotometrically at 490 nm in Varioskan Multimode Reader, Thermofisher. The absorbance was directly proportional to the number of living cells in culture. The percent viability was calculated considering 100% viability for untreated control cells at similar end points.

Neutral dye uptake

Cellular activity in the presence of different inhibitors was determined using uptake of vital stain Neutral Red (NR). The process requires intact membranes and active metabolism in the cells. Based on the principle that living and healthy cells take up more dye, MA104 or HT29 cells were plated on 12 well dishes to 70–80% confluency. Next day, cells were treated with different concentrations of the inhibitors (17-AAG, triciribine or LY294002). The assay was also utilized to determine the end point after virus infection (80% CPE). After the requisite time intervals, plates were washed with PBS 3× and fresh 500 µl media containing neutral red dye (50 µg/ml) was added. After 3 h of incubation, excess dye was washed with PBS 2× and 500 µl of desorbing solution (1% acetic acid, 50% ethanol) was added to extract the absorbed dye. Absorbance was taken at 405 nm and 540 nm in a microplate reader. The % cell viability was calculated considering 100% viability of untreated controls.

Gel electrophoresis and immunoblot analyses

Whole cell lysates were prepared and immunoblotting was done as per standard protocols (Chawla-Sarkar et al., 2002). Rabbit polyclonal antibodies (pAb) to Akt, GSK-3 β, NFκB(P65), 4EBP1, S6K, p-Akt, p-GSK-3 β, p-NFκB(P65), p-mTOR, p-4EBP1(Thr37/46), p-p70S6K (Cell Signaling Inc, Devers, MA), or mouse monoclonal antibodies (mAb) to Hsp90, Hsp70 (BD Pharmingen, San Diego, CA) were used at concentrations recommended by the manufacturer. Rotavirus non structural protein-1 and -4 (NSP1; NSP4) antibodies (polyclonal) were raised in rabbit using standard protocols. Monoclonal mouse VP6 antibody (3C10) was purchased from HyTest Ltd, Turku, Finland. Primary antibodies were detected using HRP-conjugated secondary antibodies (Pierce, Rockford, IL) and chemiluminescent substrate (Millipore, Billerica, MA). Blots were reprobed with anti-β-actin or unphosphorylated proteins to confirm equal protein loading. All immunoblots in this study were repeated ($n \geq 3$) to confirm results. Blots were scanned and quantitated using GelDoc XR system and Quantity One® software version 4.6.3 (BioRad, Hercules, CA).

Dual luciferase NFκB reporter assay

The NFκB-luciferase (NFκB-luc) reporter plasmid has been previously characterized (Elewaut et al., 1999). MA104 cells were co-transfected with 4 µg of NFκB-luc and 0.5 µg of pRL-TK (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h, cells were infected with SA11 for 4 h and 12 h in the presence or absence of 17-AAG, and luciferase activity was measured according to the manufacturer's protocol (Promega, Madison, WI) using a luminometer (Varioskan Multimode Reader, Thermofisher). Relative luciferase activity of NFκB-luciferase was normalized with *Renilla* luciferase.

Quantitative real-time RT-PCR

Confluent monolayers of MA104 cells were mock-infected or infected with SA11 (moi 3). RNA was extracted with TRIZOL™ reagent (Invitrogen, USA) and quantitative PCR was done in triplicates with SYBR Green™ Mastermix using ABI7500 (Applied Biosystems Inc, Foster city, CA). Specific primers for VP6 {VP6-F-5′GCACAGCCATTC-GAACATCATGC-3′; VP6-R5′TGCATCGGCGAGTACAGAC TAC-3′}, Nsp4 {Nsp4-F-5′GACGGTGCAAACGAC AGGCG-3′; Nsp4-R-5′GCTGCAGT-CACTTCTCTTGGTTC-3′}; and GAPDH {G-F-5′GAGA ACGGGAAGCTTGT-CATC-3′; G-R-5′CATGACGAACATGGGGGCATC-3′}, were used. Relative gene expression were normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ [$\Delta\Delta CT = \Delta CT$ (sample) − ΔCT (untreated control)].

RNA interference using siRNAs

Monolayers of MA104 cells (10^6 cells/ml) in 6 well plates were transfected with either *Silencer*® Select Hsp90 (5nM X2;48h) cytosolic [Ambion ID: s6995], or *Silencer*® Select Akt siRNA (5 nM each; 24 h) [Ambion (ID: S659 and ID: S1216)] or 5 nM *Silencer*® Select Negative Control #2 siRNA (Ambion, Foster city, CA) using siPORT™ NeoFX transfection reagent (Ambion) according to the manufacturer's protocol. 24 h post transfection cells were infected with SA11 (moi: 3) for 8 h or 12 h, lysed and *VP6* gene expression was measured by either quantitative PCR or immunoblotting.

Statistical analysis

Data are expressed as mean ± standard deviations of at least three independent experiments ($n \geq 3$). In all tests, $p < 0.05$ was considered statistically significant. Experiments where, $p < 0.01$ are marked (*).

Acknowledgments

The study was supported by financial assistance from the Indian Council of Medical Research (ICMR), New Delhi and the Program of founding research centers for emerging and reemerging infectious diseases (Okayama University-NICED, India) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. D. Dutta, A. Mukherjee and M.K. Nayak are supported by the Senior Research Fellowship from UGC, Govt. of India.

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