Bovine parovirus uses clathrin-mediated endocytosis for cell entry

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Entry events of bovine parovirus (BPV) were studied. Transmission electron micrographs of infected cells showed virus particles in cytoplasmic vesicles. Chemical inhibitors that block certain aspects of the cellular machinery were employed to assess viral dependency upon those cellular processes. Chlorpromazine, ammonium chloride, chloroquine and bafilamicin A1 were used to inhibit acidification of endosomes and clathrin-associated endocytosis. Nystatin was used as an inhibitor of the caveolae pathway. Cytochalasin D and ML-7 were used to inhibit actin and myosin functions, respectively. Nocodazole and colchicine were employed to inhibit microtubule activity. Virus entry was assessed by measuring viral transcription using real-time PCR, synthesis of capsid protein and assembly of infectious progeny virus in the presence of inhibitor blockage. The results indicated that BPV entry into embryonic bovine tracheal cells utilizes endocytosis in clathrin-coated vesicles, is dependent upon acidification, and appears to be associated with actin and microtubule dependency. Evidence for viral entry through caveolae was not obtained. These findings provide a fuller understanding of the early cell-entry events of the replication cycle for members of the genus Bocavirus.

INTRODUCTION

Bovine parovirus (BPV) is a member of the genus Bocavirus, family Paroviridae along with Canine minute virus and Human bocavirus (HBoV). BPV is the prototype virus of this genus. The genome consists of ssDNA that is composed of about 5515 nt (Qiu et al., 2007; Sun et al., 2009). The genome has three ORFs: the left ORF encodes the non-structural proteins NS1 and NS2, the central ORF encodes the non-structural protein NP-1, and the right ORF encodes two or three structural proteins VP1, VP2 and VP3 (Johnson & Hoggan, 1973; Qiu et al., 2007). Replication relies on host cell DNA polymerase and replication factors found in S-phase cells.

Regarding virus–cell interactions, Thacker & Johnson (1998) identified the BPV haemagglutination receptor on erythrocytes as glycoporin A (GPA) and Blackburn et al. (2005) showed BPV binding to α,2,3-linked sialic acid located on the O-linked oligosaccharides of the GPA molecule. Moreover, BPV attachment occurs on both α,2,3-O-linked and α,2,3-N-linked sialic acids on nucleated bovine host cells (Johnson et al., 2004). However, the processes of viral penetration into the cell and trafficking in the cytoplasm have not been previously defined beyond receptor binding. Several possible pathways of receptor-mediated endocytosis are recognized: clathrin- and caveoloae-mediated endocytosis, macropinocytosis and novel non-clathrin/non-caveolae pathways (Dimitrov, 2004; Marsh & Helenius, 2006). During clathrin-mediated endocytosis, transport vesicles contain the cargo and are bound with the clathrin triskelion. Cargo-containing vesicles then mature to early endosomes, which contain an acidic environment (pH 6.5–6.0). Virus-engaged receptors are uncoupled from their ligands in this mildly acidic environment of the early endosomes and are recycled back to the plasma membrane (Sieczkarski & Whittaker, 2002). Acidification of endosomes is required for release of many types of viruses into the cytoplasm. In some instances, such as adenovirus and canine parovirus infections, the genome together with modified capsid components translocate to the nuclear membrane (Leopold & Crystal, 2007; Vihinen-Ranta et al., 2002) where final uncoating occurs. Disassembled clathrin and accessory proteins are recycled in the clathrin-coated vesicle cycle (Edeling et al., 2006). Paroviruses are known to utilize a variety of cell surface molecules as receptor including glycoproteins, glycolipids and glycans. Following attachment, rapid endocytic uptake occurs using clathrin-associated endosomes (Cotmore & Tattersall, 2007; Harbison et al., 2008; Parker & Parrish, 2000; Ros et al., 2002; Vendeville et al., 2009). Changes in parovirus–virion conformation appear to occur in the acidic endosome, but place and time of escape are not clear. Cytoplasmic trafficking may also be associated with actin and microtubules. Viruses that exploit clathrin-dependent (often acid-mediated) entry may be sensitive to the inhibitors of endosomal acidification. Thus, inhibition of virus replication
by endosomal pH inhibitors is taken as evidence for virus tracking through acid-mediated endocytosis pathways.

Studies concerning BPV interactions with its host cell and steps beyond attachment may offer clues in elucidating pathogenesis of human parvoviruses and revealing molecular and cellular mechanisms used by bocaviruses in their replication cycles. In this study, we provide evidence for the functional importance of clathrin-mediated endocytosis in BPV entry into embryonic bovine tracheal (EBTr) cells, demonstrate the pH sensitivity of BPV entry and find evidence implying trafficking in the cytosol associated with actin filaments and possibly microtubules.

RESULTS

Electron microscopic observation of virus in infected cells

BPV-infected EBTr cells were investigated by transmission electron microscopy (TEM) to elucidate endocytosis-associated structural components at early time points after infection. EBTr cells were suspended in PBS, infected with purified BPV virions previously collected on CsCl isopycnic gradients, and incubated at room temperature for 15 min. EBTr cells not infected with virus served as a control. The virus-infected and uninfected cells were then processed for electron microscopy analysis. Virus particles were found extracellularly at thickened, possibly clathrin-associated regions of the plasma membrane after 15 min of incubation (Fig. 1a). These membrane regions appear to be thickened consistent with clathrin recruitment at the cytoplasmic side of virus–receptor complexes although not verified in these images. In clathrin-associated regions of the plasma membrane of BPV-infected cells, some areas invaginated into the cytosol forming pits that appeared to contain virus particles (Fig. 1b). Virus particles were found within completely invaginated vesicles (Fig. 1c). These 100 nm vesicular forms containing virus particles were not observed in cells uninfected with virus.

BPV entry into cells utilizes clathrin and endosomal acidification

Virus binding to its specific receptor on the cell surface triggers cell signalling pathways involved in virus uptake through the plasma membrane. If virus entry is mediated by clathrin-dependent endocytosis, recruitment of clathrin and its adaptor proteins then leads to the formation of clathrin-coated pits along the plasma membrane regions where virus–receptor complexes are bound (Marsh & Helenius, 2006). Thus, we tested whether the depletion of clathrin and its adaptor proteins affect BPV entry into the cells. Chlorpromazine (CHPZ) prevents assembly and disassembly of clathrin lattices at the cell surface and on endosomes, that is, it prevents assembly of coated pits (Blanchard et al., 2006; Brindley & Maury, 2008; Stuart & Brown, 2006). CHPZ was maintained in the cultures for the duration of BPV infection. The drug doses used in all of the tests using inhibitors were chosen based on MTT cell viability tests so that the concentrations were acceptably non-toxic to EBTr cells under the experimental conditions. Microscopic observation of cell morphology compared to control cells was a secondary assessment of drug toxicity. The inhibitory effect of drugs on virus protein synthesis and progeny virion production appeared not to be caused by drug toxicity on the cells. CHPZ treatment of EBTr cells at concentrations of 1–5 μM decreased BPV infectivity (f.f.u. synthesizing protein) from 29 to 70 % in a dose-dependent manner (Fig. 2a). In experiments assessing production of virus...
progeny in the presence of drug. EBTr cells were pre-treated with CHPZ, infected with BPV and then progeny viruses were titrated. Titres of progeny viruses were reduced from 36 to 58% compared with control cultures and inhibition was dose-dependent (Fig. 3a). Transcription of the viral genes NS, NP and VP was tested by quantitative PCR and shown to be inhibited by CHPZ (Fig. 2e).

Blocking acidification of endosomes is a way of showing entry of virus consistent with clathrin-mediated endocytosis (Basak & Turner, 1992). We investigated the role of endosomal acidification in BPV entry into EBTr cells employing NH₄Cl, chloroquine (CHLO) and bafilamicin A1 (Baf A1). Cultures were pre-treated with the drugs and infected with BPV in the presence of the drugs, then tested for viral transcription, capsid protein synthesis and virus yield. NH₄Cl, reduced BPV viral protein-positive cells from 81 to 100% (Fig. 2b) and reduced yields 42–98% (Fig. 3b). Quantitative PCR tests assessing transcription of the viral genes revealed inhibition of NS, NP and VP transcription in the presence of NH₄Cl (Fig. 2f). The second agent, CHLO, suppressed viral protein-positive foci 34–95% (Fig. 2c) and yield 35–61% (Fig. 3c). Baf A1 also diminished BPV protein-positive foci (from 1 to 92%; Fig. 2d) and yield (from 30 to 86%; Fig. 3d). Cultures treated with Baf A1 and CHLO were not tested by PCR. The inhibitory effect of the drugs was dose-dependent, and these concentrations did not demonstrate significant toxicity to EBTr cells. Collectively, these results indicate that BPV infection by EBTr cells is dependent on endosomal acidification.

Fig. 2. Inhibition of viral protein synthesis and transcription in cells treated with inhibitors of acidification and clathrin-mediated endocytosis. EBTr cell cultures were treated with various concentrations of (a) CHPZ, (b) NH₄Cl, (c) CHLO or (d) Baf A1. Infectious centres (generally individual cells) expressing viral protein were detected. The results are expressed as numbers of infectious centres in the drug-treated cultures as a fraction (per cent) of infectious centres in control cultures (bar plots). Cell viability in drug-treated cultures compared with control cells without drug is shown by the data points. The data represent three independent experiments performed with replicate cultures in triplicate per experiment. Error bars show SEM. P-values in this figure and subsequent figures are based on Student’s t-test comparing the experimental datasets with the respective control set. Control bars, dark grey; P < 0.05, white bars; P < 0.01, black bars; P < 0.001, light grey bars. The effects of (e) CHPZ and (f) NH₄Cl on transcription are shown. Quantitative PCR detected changes in transcription of the VP-, NP- and NS-reading frames. A fold change of less than one indicates inhibition of transcription.
BPV uptake by EBTr cells appears to be independent of the caveolae pathway

We also examined the effects of the anti-fungal antibiotic nystatin (NYS) that interferes with the caveolae pathway. NYS depletes cholesterol in lipid domains in the cell membrane (Matveev et al., 2001). EBTr cells were treated with serial concentrations of this drug. Viral protein synthesis and viral transcription were tested. This drug did not affect viral protein synthesis or virus transcription (Fig. 4) as no significant differences from controls were observed.

Test for virucidal activity of the drugs used in BPV entry studies

Tests were carried out for possible direct virucidal activity of the drugs. Viruses were incubated for 30 min at room temperature with the same concentrations of the highest doses of the drugs used in the previous studies, then tested by standard culture for surviving virus. The number of infected cells was scored in comparison to mock-treated virus control cultures. Differences between the control cultures and the cultures of drug-treated virus were not observed (data not shown), confirming that the drugs we used in this study did not have direct virucidal effect on this virus.

Assessment of chemical inhibitor techniques on a non-parvovirus control virus

To determine if the techniques we used in our study could replicate results published for another virus, we used an adenovirus (Ad) model. Ad exploits the clathrin-mediated pathway for cell penetration (Leopold & Crystal, 2007; Marsh & Helenius, 1989). We performed Ad infectivity assays testing for structural protein in HeLa cells employing three inhibitors: NH₄Cl (for acidification dependency), CHPZ (for clathrin involvement) and NYS for caveolae-mediated pathways. HeLa cell cultures were pre-treated with the drugs followed by Ad infection. Following incubation, infected cells were processed for the detection of Ad-infected cells by immunostaining. Ad entry inhibition was scored by the number of virus antigen-positive cells in relation to the number of virus antigen-positive cells in mock-treated cultures and is expressed as a percentage. Acidification- and clathrin-dependent pathway inhibitors reduced Ad infectious centres by 55 and 38 % (NH₄Cl and CHPZ, respectively). NYS did not reduce Ad infectious centres.

Post-entry involvement of cytoskeletal structures

Endosomal vesicles containing the viral cargo may be moved by cytoskeletal structures such as actin (Chu & Ng, 2004). Virus-associated trafficking routes, following release from the endosome, may involve microtubules (Vihinen-Ranta et al., 2004) with their linked dynein and kinesin motors, or virus may possibly associate with actin filaments and myosin motors. To initiate the characterization of intracellular trafficking of BPV, cell cultures were treated with blockers of cell cytoskeleton polymerization or function and infected with virus. Viral protein expression and viral RNA quantification were detected in the drug-treated and mock-treated cells to identify which component of the cell cytoskeleton may contribute to intracellular trafficking of BPV particles. Concerning actin, growing evidence suggests a tight interaction between the actin
network and clathrin-mediated endocytosis (Chu & Ng, 2004; Merrifield et al., 1999). To analyse the involvement of actin filaments on the entry mechanism of BPV, cultures were treated with various concentrations of NYS. Infectious centres expressing viral protein were determined by immunoperoxidase staining (a). Data are expressed as described in the legend for Fig. 2. By Student’s t-test, none of the differences from control was significant. The effect of NYS on transcription (b) was assessed at a drug concentration of 1 μg ml⁻¹. Quantitative PCR analysis detected changes in transcription of the VP-, NP- and NS-reading frames. A fold change of less than one indicates inhibition of transcription.

**DISCUSSION**

Numerous studies on the replication cycles of paroviruses have focused mainly on the middle events of the replication cycle, including genome replication and transcription. Fewer studies have focused on the early events of the replication cycle. There are no reports on the BPV entry route. Therefore, understanding the early events of virus infection, i.e. exploring host factors involved in entry and trafficking processes, will create a clearer understanding of parovirus biology. Additional attention is being given to the bocaviruses, since the identification of the HBoV and BPV is the primary model for the study of bocavirus infection. The aim of this work was to investigate the early cell-associated events that lead to BPV infection.

Images obtained by electron microscopy of virus-infected cells were consistent with virus internalization mediated by a clathrin-associated pathway. Virus particles were seen in association with cytoplasmic vacuoles by 15 min post-infection and virus appeared attached to patches of thickened membrane that were consistent with clathrin-coated regions. Earlier times were not tested, but a report on canine parvovirus (CPV) employing electron microscopy found virus in clathrin-coated vesicles at 5 and 15 min post-infection (Parker & Parrish, 2000). The 100 nm endosomes that contain virus shown in the electron micrographs in our report are very similar in appearance to the endosomes shown by Parker and Parrish in CPV entry and are consistent with the size and morphology of clathrin-associated endosomes (Ehrlich et al., 2004). Tests were designed to block essential cellular functions with pharmacological agents followed by analysis of the effects on virus replication. In this part of the study, a variety of drugs were applied to infected cultures to interrupt viral entry and trafficking at appropriate replication compartments and at various intracellular steps. One of the tests used to determine inhibition was analysis of viral capsid protein synthesis detected by the appearance of antigen-positive cells. This is a sensitive test that can detect individual infected cells. This test was done at 48 h post-infection. This time period was previously...
shown to be before completion of two replication cycles (Abdel-Latif et al., 2006) so that any secondary infected cells do not show as antigen-positive cells. In that study, viral haemagglutinin and infectious virions were not released from the cell until the initial first cycle at 54 h post-infection. In a preponderance of infected cells, the virus must wait for the cell to enter the S phase before viral genome replication and transcription can occur. Moreover, progeny virus is released from infected cells by necrosis (Abdel-Latif et al., 2006), a process during which new virus is waiting to be released. Thus, there were probably no secondary foci of antigen-positive cells to confound the cell-number counts at 48 h post-infection.

Previously, reported studies have demonstrated that the clathrin-dependent endocytic pathway is the primary route for uptake for some members of the family Parvoviridae into cells (Douar et al., 2001; Mani et al., 2006; Ros et al., 2002; Vendeville et al., 2009). To determine BPV entry dependence on clathrin, CHPZ, a cationic amphiphilic drug that inhibits the formation of clathrin-coated pits at the plasma membrane was used. It was found that CHPZ strongly suppressed BPV infection in EBTr cells. Ads are also reported to enter cells through the clathrin pathway. If, in our hands, CHPZ would block Ad entry, such results would lend support to the methods and conditions used in analysing BPV entry. Ad infection in HeLa cells was indeed blocked by CHPZ treatment verifying the activity of CHPZ interference with BPV entry. Moreover, these results tended to validate the use of chemical inhibitors for the study of parvovirus replication biology. Since BPV entry into cells was strongly dependent on clathrin assembly at the plasma membrane; it was then sought to determine the requirement of endosomal acidification for BPV internalization. Among receptor-mediated endocytosis mechanisms, clathrin-mediated endocytosis is a prominent mechanism for introducing cargo into cells and it is often dependent on endosomal acidification to move the cargo from the CCV to the early endosome. Thus, acidification can be essential for viruses which internalize within CCVs (Damm & Pelkmans, 2006; Marsh & Helenius, 2006; Smith & Helenius, 2004). Studies of parvovirus entry reported that minute virus of mice, adeno-associated virus and CPV internalization require endosomal acidification, and the endosomal acidic environment may induce capsid conformational changes vital for viral release from endosomes to the cytoplasm (Basak & Compans, 1989; Douar et al., 2001; Mani et al., 2006). Blocking acidification of endosomes is a way of suggesting involvement of the clathrin system in cargo entry. Inhibitors of endosomal acidification are grouped into three classes: lysosomotropic weak bases (such as NH₄Cl and CHLO), agents altering endosomal pH by exchanging protons for potassium and sodium, and blockers of vacuolar H⁺-ATPases (such as Baf A1). To define the role of pH in the BPV infection process, the effects of these drugs, all inhibitors of endosomal acidification, were analysed. BPV infection was sensitive to these substances in a dose-dependent manner verifying acid pH dependency. The inhibitor of vacuolar H⁺-ATPase,
Baf A1, neutralizes the pH of intracellular vesicles by blocking transport of protons into the endosome. The results obtained with Baf A1 were similar to that obtained with weak bases. Thus, altering the endosomal microenvironmental pH towards alkalinity demonstrated that a low-pH environment plays a role for BPV infection to progress to the next sequential event in the entry process.

One of the well known endocytic pathways is caveolae-dependent endocytosis. The possible role of the caveolae-dependent pathway for BPV uptake was examined by using the inhibitor NYS. This chemical interrupts the caveolae-mediated pathway by disrupting the lipid integrity of the cytoplasmic membrane. NYS did not affect BPV protein synthesis in EBTr cells and it did not inhibit transcription as VP, NP and NS transcription all appeared to be intact after drug treatment. The fact that a subset of virus entry may go through caveolae is not entirely ruled out. However, such a possibility is diminished by virus yield results (not shown) in which NYS failed to block the virus. Overall, the results suggested that caveolae-dependent endocytosis is not a major pathway for BPV entry into EBTr cells.

Virus escape from the endocytic organelles may release it into the cytosolic compartment. Once the virus is released, the virus may target the nucleus by cellular factors that facilitate capsid disassembly, exposure of the genome and movement to the cell nucleus, events not necessarily in that sequence. In some cases, the virus or its genome may converge on the nuclear area right at the time of nuclear membrane dissolution during mitosis and need not wait further for the S phase. The cellular cytoskeleton may play a role in transport of either vesicles containing the virus, or the virus itself after escape to the cytosol. The results of tests using inhibitors of actin (Cyt D and ML-7) indicated a role for actin. The PCR result showed Cyt D inhibition of actin function. The ML-7 results agreed with this result. What the function of actin is in the process of trafficking has not been revealed in this study, only that there seems to be a role for actin. It is possible that actin filaments move the vesicles themselves or possibly move released virus. The virus was also sensitive to the microtubule disrupting agents NOC and COL, and transcription was inhibited by NOC. The results of this aspect of these studies implied that BPV intracellular trafficking is dependent on actin and possibly microtubules. The results of studies using chemical inhibitors must always be interpreted with caution because of pleiotropic effects of the drugs. However, these observations and interpretations are consistent with the literature published for several viruses.

This study elucidated some early BPV entry events and allowed for mapping an entry route used by this virus. It is not yet known whether capsid conformational changes, which may allow endosomal escape, occur in the endosome or whether the genome or an intact virion is released to the cytosol. It is possible that the virion phospholipase activity mediates capsid escape from the endosome by degrading the endosomal membrane as suggested by others (Cohen et al., 2006; Dorsch et al., 2002; Farr et al., 2005). There appears to be a role for the actin and microtubule networks in the entry or trafficking process. Actin, together with myosin motors, is known to perform essential functions for the cell. Myosin type I is involved in membrane binding and endocytic vesicles. Myosin type V operates in vesicle transport and myosin type VI is involved in endocytosis. Thus, actin, together with one or all of these myosin motor types, when inhibited could explain the observations seen in this study. Inhibition of virus replication with blocking

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**Fig. 6.** Effect of microtubule inhibitors on expression of capsid protein production and transcription. Viral protein synthesis was inhibited by (a) NOC and (b) COL. NOC also was shown to reduce transcription (c). Error bars represent SEM. Control bars, dark grey; P<0.01, black bars; P<0.001, light grey bars.
of microtubules may imply movement of viral particles to the nuclear membrane in association with such structures. That dynein may be the motor that moves the particles along micotubular filaments is a possibility but not yet demonstrated.

Future studies will refine existing knowledge of the uncoating process and the process this virus uses to translocate across the nuclear membrane to gain access to the nuclear microenvironment, while the present study shows virus entry through clathrin-associated and acid-mediated endocytosis.

**METHODS**

**Virus, cells and media.** The BPV strain used in this study was the original HADEN isolate obtained from F. R. Abinanti (Abinanti & Warfield, 1961). Virus stocks were prepared from infected EBTtr cells, titres were determined as infected f.f.u., and stocks were stored frozen at −80 °C. EBTtr cells, a diploid cell strain (ATCC NBL-4), were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) containing 0.11% sodium bicarbonate, 10 mM HEPES buffer, 50 μg gentamicin ml⁻¹, and either 5% cosmic calf serum (HyClone) or 1% fetal clone III serum (HyClone). In assays where virus infection was measured, fetal clone III serum was used in the medium as cosmic calf serum contains anti-BPV antibodies. Dilutions of virus were made in serum-free medium, infected in cultures at 70–80% confluency, incubated for 48 h, then stained by an immunoperoxidase technique (see below) to identify infected foci. Titres are expressed as numbers of virus f.f.u. ml⁻¹.

**TEM.** Infected cells were fixed in 2% glutaraldehyde–0.06 M cacodylate for 1 h at 4 °C and then post-fixed for 2 h in osmium tetroxide (1:1 dilution with cacodylate) at room temperature. After dehydration in a grade series of ethanol solutions, cells were infiltrated in Epon resin. Ultrathin sections (0.1 μm) were mounted on copper grids and stained with uranyl acetate and lead citrate. Observations were performed with an FEI T12 electron microscope at 80 kV. Photographic images were taken by a Gaton Multiscan 794 digital camera and Image J software.

**Pharmacological treatment of EBTtr cells during BPV infection.** Cells were treated with inhibitors for 30 min prior to BPV infection at 37 °C. The inhibitors were present in the media for the duration of the infection. The concentrations of the chemical inhibitors in culture media were tested at the working concentrations reported for the infection. The concentrations of the chemical inhibitors in culture media were tested at the working concentrations reported for the chemical inhibitors. These cultures were then incubated at 37 °C for 30 min to allow uptake of the chemical inhibitor and the initiation of its biological activity. The cultures were then infected with virus at three different m.o.i. values, m.o.i. = ≤<1, to ensure countable and significantly valid numbers of infected foci and to ensure each focus was surrounded by a large excess of uninfected cells. The cultures were incubated at 37 °C for 48 h, then fixed with formalin-acetic acid-alcohol (FAA fixative) and stained by immunoperoxidase technique (IP) (Blackburn et al., 2006; Johnson et al., 2004; Luker et al., 1991) using polyclonal guinea pig anti-capsid protein as primary antibody for detection of BPV-infected cells. Dark purple cells were counted as an indicator viral protein. The experiment included cell controls, drug toxicity controls and virus infectivity controls without drug. The effects of each drug on BPV protein synthesis were assessed by three separate and independent experiments with triplicates of each concentration of the drug. The replicate experiments showed similar results.

**Virus production.** Production assays tested for the yield of progeny virions (completion of the replication cycle) in experimental infections in the presence of drug. These assays were carried out in EBTtr cell cultures in 25 cm² culture flasks at a confluency of 70–80%. Cells were washed twice with serum-free medium. The cells were pre-treated with the test drug for 30 min at 37 °C and then infected with virus at an m.o.i. of 2. The infection was allowed to proceed for 3 days at 37 °C in the presence of the drug. The cells were then lysed by three freeze–thaw cycles, then centrifuged at 150 g for 30 min in a tabletop centrifuge to remove cell debris. The supernatants were tested for virus yields that were produced in the presence or absence of the drug. The virus titrations were performed as described above. Virus titres were expressed as virus f.f.u. ml⁻¹. Values are presented as the mean of triplicate samples. The experiments included drug toxicity controls, cell controls and virus infectivity controls.

**Entry of a positive control virus.** Ad is internalized by a clathrin-mediated pH-dependent pathway (Meier et al., 2002). Ad entry into HeLa cells was tested to assess the inhibitory effects of the drugs used for BPV entry. HeLa cells were pre-treated with the drugs and infected with Ad type 2 for 2 days in the presence of the drugs. The infected cells were fixed in FAA fixative and stained with IP as described above. The number of Ad-positive cells in drug-treated cultures was compared to the number of virus-positive cells in inhibitor-negative control cultures.

**Real-time PCR.** Cells were treated with inhibitors 30 min prior to virus infection. Infection proceeded for 24 h at 37 °C in the presence of drugs or with the appropriate controls. The cells were detached by trypsinization and pelleted as recommended in the RNA extraction kit (RNeasy Mini kit; Qiagen). Total RNA was extracted using this spectrophotometrically. These tests on EBTtr cells were performed following the manufacturer’s instructions. Each dose of the inhibitors was also evaluated microscopically on EBTtr cells for visible cytotoxicity.

**Assessment of possible virucidal effects of the inhibitors.** To test for possible direct virucidal effects, media containing the highest concentrations of the chemical inhibitors that were used in any of the test assays were inoculated with virus, incubated at room temperature for 30 min, then surviving virus was detected by standard virus assay. Controls included in these tests were cultures infected with virus not treated with the drugs.

**Viral protein synthesis.** Infectivity assays were designed to test, in the presence of inhibitor, how many cells would be infected, or efficiency of viral protein synthesis, when exposed to a virus stock of known titre. Cells were grown in 24-well plates, washed twice with serum-free medium and renewed in media supplemented with the chemical inhibitors. These cultures were then incubated at 37 °C for 30 min to allow uptake of the chemical inhibitor and the initiation of its biological activity. The cultures were then infected with virus at three different m.o.i. values, m.o.i. = ≤<1, to ensure countable and significantly valid numbers of infected foci and to ensure each focus was surrounded by a large excess of uninfected cells. The cultures were incubated at 37 °C for 48 h, then fixed with formalin-acetic acid-alcohol (FAA fixative) and stained by immunoperoxidase technique (IP) (Blackburn et al., 2006; Johnson et al., 2004; Luker et al., 1991) using polyclonal guinea pig anti-capsid protein as primary antibody for detection of BPV-infected cells. Dark purple cells were counted as an indicator viral protein. The experiment included cell controls, drug toxicity controls and virus infectivity controls without drug. The effects of each drug on BPV protein synthesis were assessed by three separate and independent experiments with triplicates of each concentration of the drug. The replicate experiments showed similar results.
RNA extraction kit following the manufacturer’s instructions. cDNA synthesis was performed with the reverse primer (SuperScript III First-Strand Synthesis system for RT-PCR; Invitrogen). Primers for BPV DNA amplification were designed using Primer-BLAST (Primer-Basic Local Alignment Search Tool by using Primer3 to design PCR primers for user-selected database). Forward and reverse primers were as follows: VP forward, 5’-CCAGTACCGAGAAAAAGGAGA-3’ and reverse, 5’-GGTTTAGGTGATGTAGTG-3’; NP-1 forward, 5’-CGACTACCGCAAAGGA-3’ and reverse, 5’-AACATTCTCACCACCCTTC-3’. NS forward, 5’-CAAGAACATCTGTCCTGGGA-3’ and reverse, 5’-GTTTGGCTGATGAAACTCT-3’. The RPLPO (large ribosomal protein) gene was used as a control gene to normalize DNA quantity between samples and experiments. Amplification and real-time detection of PCR products were performed on the cDNA samples using the Lightcycler system (Roche Diagnostics) with SYBR Green stain (Roche). At the end of the extension step of every cycle, the fluorescence was measured. The cycle number at which the fluorescence starts to increase is related to the initial number of target copies. Cycling conditions consisted of a step at 95 °C for 10 min to activate the polymerase enzyme followed by 35 cycles with the following thermal profile: 94 °C for 15 s, 66 °C for 5 s and 72 °C for 30 s. For each quantitative PCR analysis, 4 μl diluted DNA was deposited in replicates for a 10 μl total reaction volume. The ratio of the amount of amplified gene with the amount of RPLPO control gene allowed us to compare accurately the expression of the gene.

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