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Is the Cytoskeleton Necessary for Viral Replication?

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By

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ABSTRACT

The cytoskeleton plays an important role in trafficking proteins and other macromolecular moieties throughout the cell. Viruses have been thought to depend heavily on the cytoskeleton for their replication cycles. However, studies, including one in our lab, found that some viruses are not inhibited by anti-microtubule drugs. This study was undertaken to evaluate the replication of viruses from several families in the presence of cytoskeleton-inhibiting drugs and to examine the intracellular localization of the proteins of one of these viruses, Sindbis virus, to test the hypothesis that alternate pathways are used if the cytoskeleton is inhibited. We found that Sindbis virus (Togaviridae, positive-strand RNA), vesicular stomatitis virus (Rhabdoviridae, negative-strand RNA), and Herpes simplex virus 1 (Herpesviridae, DNA virus) were not inhibited by these drugs, contrary to expectation. Differences in the localization of the Sindbis virus were observed, suggesting the existence of alternate pathways for intracellular transport.

INDEX WORDS: Cytoskeleton, Microtubules, Virus replication, Cytoskeletal-interfering drugs

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1 INTRODUCTION

The cytoskeletal system of the cell is thought to be a key component in the life cycle of viruses because during every aspect of the viral life cycle, from entry to egress, the virus needs the intracellular transport that the cytoskeleton provides (Radtke *et al*., 2006). There are three major components to the cytoskeleton: the actin filaments, the intermediate filaments, and the microtubules. Of these, the actin filaments, found directly beneath the cellular membrane, and the microtubules, found throughout the cell, are involved in intracellular transport. Although only a relatively small number of the viral families and their interactions with the cytoskeleton have been characterized in detail, it is assumed that all viruses need the actin and/or microtubule networks to actively transport new viruses into the cell, their macromolecular components to appropriate places throughout the cell, and their progeny out. Most studies to date have described specific aspects of viral intracellular trafficking while a few have looked at the role that the microtubules play in viral transcription and gene regulation (Simon *et al*., 2009).

1.1 The cytoskeletal system and its pharmacological inhibitors

The cytoskeleton is composed of three protein filaments: microtubules, microfilaments, and intermediate filaments. These filaments give the cell its shape and structure as well as playing key roles in intracellular transport and cell division. The microtubules are hollow tubes, averaging 25 nm in diameter, composed of α- and β-tubulin dimers (Pollard *et al*., 2008). The tubulin polymerizes with the α-tubulin subunit of one dimer making contact with the β-tubulin of the next dimer. This results in one end of the tubulin having the β-tubulin exposed and the other end having the α-tubulin exposed, which gives the microtubules polarity. The (+) end of the

microtubule has β-tubulin exposed while the (-) end has α-tubulin exposed. The (-) end is almost always capped, so growth normally occurs from the (+) end. The microtubule (+) end usually points towards the plasma membrane where it can interact with the actin cortex (Radtke *et al.*, 2006). The microtubules are nucleated and organized from the microtubule organizing center (MTOC), which in animal cells is called the centrosome (Pollard *et al*., 2008). The MTOC plays two important roles: organizing the microtubules that comprise the flagella and cilia and organizing the mitotic and meiotic spindle apparati during cell division. Contained in the MTOC is another type of tubulin, γ-tubulin. The γ-tubulin forms a complex with other proteins, known as the "γ-tubulin ring complex." The ring complex forms scaffolding for the α- and β-tubulin to build upon. The γ-tubulin also caps and stabilizes the (-) end of the microtubules (Pollard *et al*., 2008). Many proteins bind to the microtubules to facilitate transport and many other proteins associate with microtubules to aid in the maintenance of the cell. For example, the motor proteins, dynein and kinesin mediate transport along the microtubules. Each family of motor proteins walks along the microtubules in one direction; dynein mediates most of the (-) end directed transport while kinesin transports toward the (+) end. However, some members of the kinesin family, such as KIFC2 can also transport toward the (-) end (Radtke et al., 2006).

Microfilaments form the thinnest filaments of the cytoskeleton, averaging 7 nm in diameter (Pollard *et al*., 2008). The filaments are solid rods composed of actin polymers. The microfilaments nucleate from the plasma membrane (so the outer edges of the cell have the greatest concentration of microfilaments) forming a three-dimensional meshwork known as the actin cortex. The actin cortex provides mechanical strength to the plasma membrane and the shape of the cells and enables the cell to move (Radtke et al., 2006). The microfilaments are

built head-to-tail of polar monomeric actin, which result in polar filaments. The microfilaments grow and shrink from both ends, but the (+) end assembles and disassembles faster than does the (-) end. The growth of one end while the other end shrinks allows for cell motility and crawling (Karp, 2009). The microfilaments are flexible, but the cell has accessory proteins which bind to the actin and organize the filaments into stronger, larger structures with various functions. Myosin motor proteins also play a role in cellular transport. There exist different classes of myosin proteins and each move in only one direction along the actin; some classes transport toward the (+) end, while others transport to the (-) end (Radtke et al., 2006).

Intermediate filaments are formed by a group of related proteins and have an average size between that of the microtubules and actin filaments, about 10 nm. About 70 genes control the expression of the intermediate filament proteins. The intermediate filaments have greater tensile strength than do microtubules and actin, so their main functions are structure and support. Each monomer filament has an alpha helical rod domain which connects the amino (head) and carboxyl (tail) terminals. The filaments can form dimers and tetramers. The monomer filaments coil around each other with head regions aligned. Then these dimers can form tetramers by staggering head-tail with other dimers (Karp, 2009). There are several types of intermediate filaments, characterized by the type of proteins that construct them. Type I and Type II are acidic and basic keratin filaments in epithelial cells that form hair, nails, horns, and scales. The most common of the Type III filaments are the vimentin filaments, which can be found in a range of cells including endothelial cells, leukocytes, and fibroblasts. These filaments support the cell membrane and hold organelles in place. Other kinds of Type III filaments are peripherin, desmin, and glial fibrillary acidic protein filaments. Type IV consists of

neurofilaments which are found in the axons of vertebrate neurons. Type V filaments are lamin filaments which have structural functions in the cell nucleus. Type VI filaments are comprised of nestin, which are found in nerve cells (Karp, 2009).

Intracellular trafficking is most commonly completed by vesicular transport from one membrane-bound organelle to another or from the exterior of the cell to a target on the interior or vice versa. Vesicular transport is reliant on the cytoskeletal system as the cargo buds from the donor membrane to form the vesicle and is carried by motor proteins on the microtubules to the target membrane where the vesicle fuses to release its cargo. For example, in most eukaryotic cells, the Golgi body packages and processes proteins for secretion and segregates proteins that are to be delivered to the plasma membrane from those that are to remain inside the cell. The Golgi consists of from four to ten flattened cisternae which together make up the cis-, medial, and trans- Golgi (Karp, 2009). The perinuclear structural organization of the Golgi is dependent on the integrity of the microtubule network and the Golgi is often seen in close association with the MTOC. It has been observed that when the microtubules are depolymerized, the Golgi elements are scattered through the cytoplasm (Storrie *et al*., 1998).

Viral trafficking, as it is currently understood, involves the virus hijacking the cellular transport system and relying on the host's motor proteins associated with the actin filaments and microtubules. Viral proteins often interact with actin/tubulin binding proteins or bind directly to the motor proteins themselves. Some viruses, such as the retroviruses, interact with actin before entering the cell. These viruses can "surf" along filopodia towards the cell body. This "surfing" requires dynamic actin and during the viral "surfing" the virus receptor is most likely coupled with an actin filament inside the filopodia (Radtke et al., 2006). After entry, some

viruses are first transported in vesicles, but leave these vesicles in the cytoplasm. During cytosolic replication, viruses may also interact with the cytoskeleton. For example, the RNA of human parainfluenza virus type 3 is transcribed in association with ribonucleoproteins which are bound to actin filaments (Radtke et al., 2006). Other viruses can reorganize the host cytoskeleton for their own purposes; microtubules and actin have been seen to anchor the viral transcription apparatus and sequester host regulatory factors. After replication and assembly, viruses either have to traffic out individually by budding through host membranes or wait for cell lysis. When trafficking out, the viruses must make use of kinesin and/or myosin. The actin cortex can be a barrier to some viruses, which is why brief periods of actin depolymerization increase the release of some viruses (Radtke et al., 2006).

The cell cycle is highly regulated by many proteins and the microtubules play a key role in correctly aligning and separating the chromosomes during mitosis. The MTOC is the one of the major regulators of cell division. The centriole within the MTOC is responsible for organizing the mitotic spindle in animal cells, although it is not necessary to form the spindle in other cells. During prophase, motor proteins push the centrioles to polar opposite sides of the cell. Once the nuclear membrane breaks down during the late part of prophase, the microtubules attach to kinetochores at the centromere of each chromosome (Pollard *et al*., 2008). Proper chromosome separation requires every kinetochore to be attached to the microtubules. If a kinetochore remains unattached, mitosis will not progress to anaphase. During anaphase, the sister chromatids are separated into daughter chromosomes by the shrinking of microtubules attached to kinetochores. Next, the microtubule bundles not attached to kinetochores lengthen,

pulling the centrosomes to the poles of the cell. Cell division occurs after mitosis is complete (Pollard *et al*., 2008).

Cancer researchers often use mitosis-disrupting drugs to study the role of abnormal cell division in cancer development. In normal cells, factors that interfere with microtubule function cause the mitotic checkpoint to remain unsatisfied and arrest the cell in mitosis, prohibiting cell division. However, some cells are able to override the arrest and keep diving, forming tumor cells. For this reason, mitotic inhibitors are often used in cancer research on one hand as potential therapeutic drugs, but on the other hand to induce the formation of tumor cells. Microtubule dynamics can be impacted in two ways by these inhibitors. The first is to stabilize the microtubules, which makes them resistant to depolymerization and decreases their dynamics. The second is to disrupt the microtubules by fragmenting them. In most cases, the cell can recover a few hours after exposure to the drug has stopped.

Colchicine is the most well-known mitotic inhibitor. It is an alkaloid that is found in the autumn crocus flower (*Colchicum autumnale*) and was first used as a medicine in 16th century BC Egypt. Colchicine interferes with mitosis by binding to tubulin and inhibiting microtubule polymerization, interfering with the development of the spindles as the nuclei are dividing. When colchicine is present in the cell, the spindles don't form and the cell cannot move the chromosomes around, causing the cell to arrest in mitosis. Colchicine is most commonly used to treat gout and familial Mediterranean fever. Despite its potential anti-cancer properties, it is rarely used as a cancer treatment because it has a limited therapeutic range as a chemotherapy agent due to its high toxicity (Molard, 2002).

Paclitaxel is a member of a group of chemotherapy drugs known as taxens, named after the genus of plant, *Taxus*, from which they were first derived. Paclitaxel was first derived from the Pacific yew tree, but is now synthetically made. It is widely used for treatment of a variety of cancers including breast, ovarian, and lung cancers. It is also been used extensively in biomedical research as a microtubule stabilizer. Paclitaxel works by binding to the tubulin and protecting the microtubules from disassembly. With paclitaxel bound, the microtubules are unable to form the proper spindle conformation which arrests the cell in metaphase and leads to apoptosis (Surhone *et al*., 2010).

Noscapine is a non-opiate alkaloid derived from the poppy family (*Papaveraceae*). Since the 1960's it has widely been used as an antitussive and in the late 1990's it was discovered to have anti-cancer properties. Currently, ongoing studies are assessing noscapine's effectiveness in treating stroke as well. Noscapine works by binding to tubulin and altering its conformation, which results in an increase in time that the microtubules spend idle in a paused state, triggering apoptosis and cell death. This is unlike the mechanism of taxanes and vinca alkaloids, which affect microtubule polymerization. Noscapine is also noted for its low toxicity and ease of administration (Mahmoudian, 2009).

Cytochalasin-D is a member of the cytochalasin drug family, potent fungal metabolites that bind to actin to prevent the polymerization of actin filaments. Cytochalasin-D will bind with high affinity to the fast growing plus end of actin, which prevents further growth or disassembly. The disruption of the actin microfilaments causes several side effects for the cell, including the activation of p53-dependent pathways that arrest the cell in mitosis between the G1 and S phases. Protein synthesis is also inhibited. The inhibition of actin polymerization also causes

changes in cell morphology and cell motility. Due to its high toxicity, there are no medical uses for cytochalasin-D (Cooper, 1987).

1.2 Virus replication cycles and their use of the cytoskeletal system

Viruses have a large diversity of virus particle structures, genome organizations, and replication strategies. The different viral families have different genomes, which can be single or double stranded RNA or DNA. Among the viruses with single-stranded genomes, the genome can be positive sense (collinear with mRNA), negative sense (complementary to mRNA), or ambisense (both). Each variation requires a different replication strategy. However, all viruses must be transported to the correct sites within the cell before replication can begin. These sites can be in the nucleus or in the cytoplasm, and in the latter case they are often associated with specific membranes organelles (Fields *et al*., 2007). All viruses follow the same basic steps in their replication cycle: attachment, entry, uncoating, gene expression, replication, assembly, and exit.

The cell membrane is a barrier that all viruses must overcome. In order to do so, they must bind to cellular receptors on the surface of the cell using surface proteins on the outer coat of the virus. The type of cellular receptors that the virus binds determines the specificity of cells, tissues, and organisms that the virus can infect, known as tropism which plays a part in determining the type of disease the virus causes. Many viruses can attach to multiple receptor types, which they can use independently or in series (Fields *et al*., 2007).

Different viral families use different entry pathways once bound to the cellular receptor. Most use endocytic pathways, but a few, such as HIV, can fuse with the plasma membrane and thus penetrate it directly. The most common entry pathway is the clatherin-mediated pathway,

which is used by the cell to internalize a large variety of ligands, proteins, and lipids. Viruses bind to receptors which localize in clatherin-coated pits and are taken into the cell in a matter of minutes. Once in the endosomes which are formed by internalization of these pits, specific virus proteins must undergo conformational changes to mediate penetration of the endosomal membrane. For many viruses, the pH change in the endosome triggers the process. For enveloped viruses, the virus envelope fuses with the endosomal envelope and the capsid is released into the cytoplasm. Nonenveloped viruses have coat proteins which change conformation in response to a trigger, such as low pH, which allows release of the genome across the endosomal membrane into the cytoplasm. For viruses that replicate in the nucleus, following penetration of the cytoplasm the capsid must enter the nucleus through one of two methods. Viruses can use the nuclear pore complex (NPC) which often requires various strategies to overcome the size limit of the pores (35 – 45 nm) including having a genome with multiple segments like influenza virus, or opening the capsid at the pore, which releases the genome into the nucleus, as in the case of HSV-1 and adenovirus. Alternatively, the virus capsid can wait in the cytosol for the dissolution of the nuclear envelope which occurs during cell division.

Although there are many different strategies viruses use to replicate their genome, they all use the conventional base-pairing between template and daughter strands. Most DNA viruses replicate in the nucleus, while most RNA viruses replicate in the cytoplasm, with a few exceptions like the influenza viruses which replicate their RNA in the nucleus and poxviruses which replicate their DNA in the cytoplasm (Fields *et al*., 2007). Regardless of the site of replication, each virus must coordinate the synthesis and trafficking of viral proteins, genome replication, and release of progeny virus. In order for the virus particles and their necessary proteins

to traffic in intracellular space, they must rely on the cellular cytoplasmic transport systems. Most commonly, viruses are thought to use microtubule-mediated mechanisms for long distance transport (Fields *et al*., 2007; Sodeik *et al*., 1997; Luftig, 1982; Radtke *et al*., 2006) while the actin filaments are used for short distances near the cell membrane.

Numerous studies have been done across several virus families that have examined various steps in the replication cycle that rely on the cytoskeletal system (Table 1, Radtke *et al*., 2006). Most of these studies have used immunofluorescence and colocalization techniques to determine the strength of the interaction between virus moieties and the microtubules during early entry and subsequent trafficking. The majority of these studies observed a need for an intact cytoskeleton system for entry and trafficking to the nucleus or perinuclear region. However, a couple of studies have shown that viruses can employ alternate strategies when the cytoskeleton is disrupted (Glotzer et al., 2000; Yea et al., 2007).

Adenovirus is one of the viruses that has been shown to successfully replicate in the absence of an intact cytoskeleton system. Adenovirus is a medium sized, double stranded DNA virus that replicates in the nucleus of the infected cell. While entry mechanisms for human adenoviruses are well understood, the mechanisms for movement to the nucleus are not. The virus particle travels up to 50 μM in the cell within an hour between binding to the plasma membrane and localizing near the nucleus. This is too great of a distance for free diffusion through the cytoplasm, so it suspected that the cytoskeletal system, mainly the microtubules, transports the virus to the nucleus. While some studies have found evidence for the use of microtubules for successful transport, others have suggested that a microtubule-independent pathway exists that is revealed when the microtubules have been depolymerized by cytoskeletal inhibiting

drugs. Glotzer *et al*., 2001, were the first to suggest that adenovirus can utilize microtubule-independent transport and that the mechanisms of cytoplasmic transit and nuclear targeting are more diverse than just the microtubule network. Their study was based on engineered adenovirus particles with fluorescently labeled genomes to study the path the genome followed from entry to nuclear deposition. These infectious adenovirus particles, which were termed AdLite particles, were developed by fluorescently labeling the adenovirus DNA genomes with a green fluorescent protein (GFP)-DNA binding protein fusion. Labeling the genome in this manner was more desirable than labeling the capsid protein by fusion with GFP because the capsid is dismantled upon entry and may not follow the path of the genome. The AdLite particles allowed them to follow the infecting viral DNA using video microscopy in A549 or HeLa cells (both human cell lines) that had been treated with 20 µM nocodazole or 0.5 µM cytochalasin-D. It was found that while intact microtubules were required for the virus to localize in stable clusters around the MTOC, as seen in control cells, the virus particles were still able to reach the nucleus in similar numbers in the drug-treated cells as in the control cells. Genomes in drug-treated cells exhibited similar motility to those in untreated cells, even when the cells were exposed to both nocodazole and cytochalasin-D at the same time. The researchers concluded that the mechanisms of intracellular transport may include both microtubule-dependent and microtubule-independent pathways and that adenovirus may be capable of interacting with different cytoskeletal elements to traffic its particles throughout the cell.

Yea *et al*. also did a study on the trafficking of adenovirus in HeLa, HEp-2, A549, and HEK 293 cells (all human cell lines) and obtained results similar to those of Glotzer *et al*. The goal of their study was to analyze the trafficking patterns in each of these cell lines using fluorescently

labeled virions and to investigate microtubule-mediated transport based on the patterns seen. To test what effect microtubule disruption would have on virion trafficking and genome delivery, A549 and HEK293 cells were treated with 30 µM nocodazole before and during infection with adenovirus. Like Glotzer *et al*., it was found that, while the formation of MTOC-associated clusters was dependent on intact microtubules, the virions were able to reach the nucleus in the absence of them. Adenovirus with a GFP-tagged capsid and expressing X-Gal was then used to determine if infectivity was compromised by the treatment of the cells with nocodazole. It was reasoned that if this drug inhibited infection, then the number of cells with GFP would be lower in the treated cells than the control cells. However, in both A549 and 293 cells the proportion of cells with GFP in treated and non-treated cells was comparable. Genome delivery was monitored microscopically by X-Gal staining (used for the detection of β-galactosidase) treated and non-treated 293 cells. As before, the cells expressing signal was comparable in treated and non-treated cells. It is unknown why virions form stable clusters around the MTOC and spindle poles, but it was concluded these clusters do not appear to be necessary for infection to proceed. The clusters are dependent on intact microtubules, but are not essential to infectivity, as the viruses are able to express their genome in comparable levels in treated cells and control cells. Yea *et al*. concluded, like Glotzer *et al*., that the microtubules are not essential for adenovirus trafficking to the nucleus, but the microtubules may play a role in untreated, infected cells.

Mabit *et al*. also investigated the role of microtubules in adenovirus infection and found through quantitative analysis of adenovirus-mediated gene expression and use of adenovirus tagged with fluorophore that the virus did rely on an intact cytoskeleton system. The goal of

this study was to investigate the findings of Glotzer *et al*. who were the first to suggest adenovirus can infect cells using microtubule-independent transport. First, adenovirus transgene expression was analyzed in A549 cells. The cells either were untreated or had been treated with 20μM nocodazole prior to infection with adenovirus expressing β-Gal. It was found that in the presence of nocodazole, β-Gal expression was reduced by 85% compared to the control cells. Similar results were obtained with an adenovirus expressing luciferase. Consistent with the results with β-Gal, luciferase activity was reduced by 50% to 70%, regardless of MOI used in nocodazole-treated cells. It was determined that this reduction was not due to cell loss, as determined by staining the cells with vital dyes. Mabit *et al*. repeated these experiments using different promoters directing transgene expression (HSV-1 ICP4 and CMV) and different MOIs (2 to 100 PFU/cell) and obtained similar results each time. Mabit *et al*. also used fluorescently labeled adenovirus (Ad2-TR) on A549 cells that were untreated or had been treated with 20 μM nocodazole. Virus was adsorbed to the cells for 1 hour at 4°C and the cells were then shifted to 37°C to achieve a synchronous infection. The cells were fixed at 75 minutes post infection. If was found that while the control cells had high nuclear fluorescent signal, the drug-treated cells had significant increases in signal in the periphery of the cell and cytoplasm, but not the nucleus. Mabit *et al*. thus concluded that microtubules are necessary for the transport of infecting adenovirus to the nucleus and subsequent replication.

Although variable results were obtained, these findings suggest that other cytoskeletal elements play a role in intracellular trafficking and nuclear targeting of adenovirus or that adenovirus can reach the nucleus completely independent of the cytoskeleton, possibly through vacuoles or other vesicles. The vesicles hypothesis was proposed by Yea *et al*. who observed

virions in vesicles and free in the cytoplasm around the nuclei of cells that had been treated with drugs, but the vesicles could also be a defense mechanism of the host. An alternate, less efficient pathway could exist that the viruses are able to use when the microtubules are disrupted. The lack of cell death when the cells are treated with the microtubule inhibiting drugs suggests that the cell could possibly have alternate means of transporting and moving its elements around that the virus is able to exploit.

Reovirus, a double stranded RNA virus, has also been well characterized in terms of the interaction of its proteins with the microtubules. The study, done by Mora *et al*., used gel electrophoresis and sedimentation analysis to analyze the temporal association of the virus proteins to cytoskeletal extracts and IFA to analyze the spatial distribution of the viral proteins. Like two of the adenovirus studies, it was found that exposure to colchicine did not prevent the association of viral proteins or RNA with the intracellular matrix where replication occurs (Mora *et al*., 1987). However, these researchers did not find that the virus relied fully on the cytoskeleton. On the contrary, they found that the virus interacted with the cytoskeleton components at defined times during replication, but the interactions that the virus had with the microtubule and intermediate filaments were not dependent on colchicine-sensitive interactions and thus these interactions were not reflective of active microtubular transport.

1.3 Sindbis virus

Sindbis virus, the model virus used in my research, is a member of the family Togaviridae, whose members are enveloped, plus-strand RNA viruses. The family is divided into two genera: alphaviruses and rubiviruses. Although rubiviruses are classified within the same family as alphaviruses, they are enough differences that the evolutionary relationship between

the two genera is still obscure. *Alphavirus* is a large group, consisting of more than 40 members, while the *Rubivirus* genus only has one member, the rubella virus (Fields *et al*., 2007). Alphavirus members cause a number of human and animal diseases, including encephalitis, fever, and rash. All members of the *Alphavirus* genus are arboviruses, viruses transmitted by arthropods. Sindbis virus is maintained naturally by transmission from birds to mosquitos. Humans are infected when bitten by an infected mosquito. Sindbis virus is the most studied member of the alphaviruses due to its easy growth in the lab and low risk for human disease.

Sindbis virus has an 11.7 kb RNA genome with a capped 5' end and a poly A sequence at the 3' end (Fields *et al*., 2007). The envelope has a spherical shape that is about 70 nm in diameter. The E1 and E2 transmembrane glycoproteins form repeating subunits that give the envelope a rigid icosahedral lattice structure. The envelope is a host-derived lipid bilayer that is rich in cholesterol and sphingolipid. Inside the lipid bilayer is the icosahedral capsid comprised of multiple copies of the capsid protein and containing the single stranded RNA genome (Figure 1).

A cartoon of the Sindbis replication cycle is shown in Fig. 2. The Sindbis virus genome separates expression of the replicative proteins, also known as nonstructural proteins, and virion structural proteins into two open reading frames. The replication protein coding region spans nearly two thirds of the genome, starting at the 5' end. The replicative proteins are translated directly from the genomic RNA while the structural proteins are translated from a subgenomic mRNA. The replicative proteins are translated as a single precursor which is proteolytically cleaved into 4 mature proteins, termed nsP1 through nsP4, while the subgenomic mRNA codes for 5 proteins, the capsid protein, E1, E2, E3, and the 6K protein. The nonstructural proteins are multifunctional: nsP1 codes for a methyl transferase and

guanylytransferase activities which are involved in the capping of viral positive strand RNA; nsP2 contains a helicase and the protease which mediates the cleavage of the nonstructural proteins; the function of nsP3 is poorly understood, but it is thought to be important for minus strand synthesis; and nsP4 codes for the RNA dependent RNA polymerase.

Alphaviruses have a broad host range in both the species of animals and the types of cells. There is speculation that the E2 glycoprotein contains multiple binding sites and uses a cell receptor that is highly conserved across species to make the broad range possible. However, the role for receptors in the entry of the virus is still uncertain (Fields *et al*., 2007). Sindbis virus is internalized once it is bound to receptors in a clathrin-coated pit. Inside the vesicles, exposure to acidic conditions causes the E1 and E2 to undergo conformational changes. The proteins attach to the vesicle membrane and fold back, fusing the viral and cell membrane together and allowing the nucleocapsid to escape into the cytoplasm.

Following the release of the nucleocapsid, it is believed to be disassembled by ribosomes. The genome RNA serves as messenger RNA for the synthesis of the replicative proteins. Two polyproteins are translated from the nonstructural region of the genome, P123 and P1234, the latter being translated by occasional readthrough of a stop codon between nsp3 and nsp4 coding regions. These are subsequently cleaved and processed to form individual nonstructural proteins. P1234 is only made 10 – 20% of the time, and relies on read through of an opal codon between nsP3 and nsP4 (Fields *et al*., 2007). The polyproteins are processed and cleaved by the protease within nsP2. The nonstructural proteins form a replicative enzyme complex (RC) in the cytoplasm contained within vesicles associated with endosomes and lysosomes in the infected cells. The complex functions as the site where genome replication and translation of

subgenomic RNA takes place (Gorchakov et al., 2008). Three species of RNA are produced by the RNA dependent RNA polymerase: genomic RNA, subgenomic RNA, and minus strand RNA, and their abundance is regulated by the availability of the nonstructural proteins. Each species of RNA requires a different configuration of polypeptides. The synthesis of the minus strand is carried out by P123 plus nsP4. Plus strand RNA synthesis is carried out by polyprotein P23 plus nsPs1 and 4, or all four nsPs. Subgenomic RNA synthesis is carried out by all four nsPs. In each case, host factors also likely play a role.

The amount of subgenomic RNA made is roughly three times more than the genomic RNA (Fields *et al*., 2007). The capsid protein is translated first and immediately cleaved by an embedded protease, followed by cleavage of the nascent polypeptide chain by cell signalase to produce E1, pE2 (a precursor of E2 containing E2 and E3) and the 6K protein. The capsid protein associates with the genomic RNA to produce the nucleocapsid. A package sequence in the genomic RNA allows for the internalization of the RNA into the developing capsid, which ensures specificity of packaging (Fields *et al*., 2007). As the capsids are formed the envelope proteins are translocated into the endoplasmic reticulum membrane. From there the glycoproteins are translocated to the Golgi, and then to the plasma membrane, undergoing modifications along the way, including cleavage of pE2 to E2 and E3 by a furin-like enzyme. The final stage in the replication cycle is when the capsids interact with the envelope glycoproteins to promote budding. The capsids assemble close to the plasma membrane and acquire their envelope when they bud out from the cell.

1.4 Goals of the thesis

Our lab has been interested in the use of anti-microtubule drugs effective in cancer therapy against life-threatening acute virus infections, based on the premise that viruses require an intact cytoskeleton for their replication. However, studies in our lab found that rubella virus replication was not inhibited by four anti-microtubule drugs. To follow up on this finding and to test the hypothesis that viruses can replicate in the presence of drugs which compromise the cytoskeletal system, two specific aims were proposed:

1. To test a panel of viruses that represent a variety of families with different replication cycles against a variety of microtubule- and actin filament- interfering drugs to determine if replication is dependent on an intact and dynamic cytoskeletal system.
2. To use Sindbis virus to determine where the viral proteins are localized in infected cells exposed to microtubule and actin interfering drugs to detect if there are changes in the localization and to potentially determine if any alternate localization pathways are employed when the cytoskeletal system is inhibited.

The panel of viruses is made up of viruses from several different viral families that represent a variety of genomic structures and replication strategies, including: Sindbis virus (family Togaviridae, plus strand RNA), VSV (family Rhabdoviridae, minus strand RNA), Dengue virus (family Flaviviridae, plus strand RNA), Herpes Simplex Virus (HSV-1) (family Herpesviridae, DNA), and Adenovirus (family Adenoviridae, DNA). The drugs used were colchicine, noscapine, paclitaxel, and cytochalasin –D. Fluorescence microscopy using viruses expressing fluorescently tagged proteins and immunofluorescence will be employed to examine Sindbis virus -protein and double stranded RNA localization in infected cells.

1.5 Expected Results

Based on the generally accepted dogma that virus replication is dependent on the cytoskeleton system, the expected result of this study were that most, if not all, of the viruses in the panel will exhibit a drop in titer (virus production) when cells exposed to the cytoskeletal inhibiting drugs are infected as compared to untreated control cells. It is also expected that the intracellular localization of the Sindbis virus proteins and dsRNA will be radically altered in the drug infected cells.

2 MATERIALS AND METHODS

2.1 Cell culture, viruses, drugs, and antibodies

Baby hamster kidney (BHK-21) cells and African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (ATCC) and grown and maintained at 35°C in Dulbecco's Modified Eagle Medium (D-MEM, Cellgro) with 5% fetal bovine serum (FBS), essentially as described previously (Frolova, 2010). Adenocarcinomic human alveolar basal epithelial (A549) cells were obtained from ATCC and grown and maintained at 37°C in Ham's F-12, Kaighn's Modification (F-12K, Cellgro) media with 10% FBS and 1% Penicillin/Streptomycin (Pen/Strep), essentially as described previously (Yea *et al*., 2007). Human Embryonic Kidney 293 (HEK 293) cells were obtained from Dr. Sarah Pallas and grown and maintained at 37°C in D-MEM with 10% FBS, essentially as described previously (Yea *et al*., 2007). Colchicine and cytochalasin-D were purchased from Sigma. Noscapine and paclitaxel were gifts from Dr. Ritu Aneja, Department of Biology, Georgia State University, Atlanta, Georgia. The Sindbis virus (SINV, HR strain) and vesicular stomatitis virus (VSV, IND serotype) were laboratory strains rou-

tinely used in the Frey laboratory. Herpes Simplex 1 (HSV-1; KOS-63) virus was a gift from Dr. Richard Dix, Department of Biology, Georgia State University, Atlanta, Georgia. Adenovirus (species C, type 5, dl309) was a gift from Dr. Charlese Benson, Department of Biology, Georgia State University, Atlanta, Georgia. Dengue virus (DENV, serotype 2) was a gift from Dr. Richard Kuhn, Purdue University, West Lafayette, Indiana. Rabbit polyclonal antibody against Sindbis virus was prepared by Dr. Teryl Frey at the University of Pittsburgh. Mouse anti-dsRNA antibody was purchased from Scientific Consulting. Rabbit anti- α-tubulin antibody was purchased from Sigma. Alexa Fluor 568 phalloidin and wheat germ agglutinin Alexa Fluor 594 conjugate were purchased from Molecular Probes. Secondary antibodies, goat anti-mouse TRITC and goat anti-rabbit FITC were purchased from Molecular Probes. Hoechst 33342 was purchased from Molecular Probes.

VSV, SINV, DENV, and HSV-1 were propagated on BHK-21 cells. At 2 days post infection (dpi), the development of cytopathic effects (CPE) was present in nearly 100% of the cells and at that time the supernatant was collected and stored at -80°C, essentially as previously described (Frolova, 2010; Hammonds, 1996). Adenovirus was propagated on 293 cells in 10% D-MEM. At 2 dpi, the development of CPE was present in the majority of the cells and at that time the supernatant was collected, mixed at a 1:1 ratio with sterile glycerol, and stored at -20°C, essentially as previously described (Yea 2007).

2.2 Reconstitution of SINV nsP3-GFP

The pSINV/nsP3GFP plasmid, containing the cDNA of SINV with a GFP insertion in the nsP3-coding sequence, was a gift from Dr. Ilya Frolov, Department of Microbiology, University of Alabama Birmingham, Birmingham, Alabama. The virus was recovered from the plasmid es-

sentially as previously described (Gorchakov, 2008). Briefly, the plasmid was linearized by XhoI digestion and in vitro RNA transcripts were synthesized with SP6 polymerase (Epicentre) in the presence of cap analog (New England Biolabs). RNA yield and integrity was analyzed by gel electrophoresis. In vitro transcripts were used to tranfect BHK-21 cells as previously described (Gorchakov, 2008). Following development of CPE in 100% of the cells, the culture fluid was harvested and stored at -80 C.

2.3 Plaque assay

Plaque assays were carried out essentially as previously described with minor modifications (Tzeng and Frey, 2002). Briefly, plaque assays were performed on monolayers of Vero cells or A549 cells grown in 60 mm cell culture dishes or 6-well plates. The plates were infected with 0.5 ml of virus diluted in PBS-1% FBS. At 1 h post infection, the inoculum was removed and the agar overlay was added to each plate. Plaques were visualized by removing the agar overlay and staining the monolayer with 0.1% crystal violet in 10% formalin. Plaques were counted at 2 days post infection for HSV-1, VSV, and Sindbis, 5 days post infection for adenovirus. DENV failed to form plaques on Vero cells, even after 7 days incubation. Plaque assay of DENV was also attempted on BHK cells, also without success.

2.4 Cytoskeletal drug treatments

For virus replication assays, the drug treatment was performed essentially as previously described with minor modifications (Frolova, 2010). Briefly, a monolayer of cells (BHK-21, 35°C; or 293, 37°C) were infected with the appropriate virus for 1 hr at a multiplicity of infection (MOI) of 0.1 pfu/cell (SINV, VSV, adenovirus) or 0.01 pfu/ml (HSV-1). The cells were then

washed and incubated for 24 hours in media containing drugs at the concentrations indicated in the appropriate figures. The supernatant for virus was collected at 24 hours post-infection for quantification. The supernatant for cells infected with SINV, VSV, and HSV-1 was collected and stored at -80°C. The supernatant for cells infected with adenovirus was mixed 1:1 with sterile glycerol and stored at -20°C. Cell monolayer quality after drug treatment of uninfected cells was assessed at 24 hr with a crystal violet stain.

For microscopy, BHK-21 cells were seeded onto glass cover slips in a 35mm cell culture dish 1 day before infection. The cells were inoculated with SINV at a MOI of 1 in 1% FBS/PBS. At 1 hpi the inoculum was removed and the cells continued to incubate in media with or without colchicine (30 µM) until 5 hpi, at which time the cells were processed for fluorescence or immunofluorescence.

2.5 Immunofluorescence assay

Cytoplasmic localization was determined by fluorescence microscopy with fluorescently tagged stains, fluorescently-tagged viruses, or immunofluorescence and was carried out essentially as previously described with minor modifications (Matthews, 2010). Briefly, the cells were fixed and permeabilized with 100% methanol at -20°C for 10 to 15 min. The cells were rehydrated by an overnight incubation in phosphate-buffered saline (PBS) at 4°C. All antibodies were diluted to 1:1000 in 1% FBS/PBS. The cells were incubated with the primary antibody for at least 1 h, followed by two washes in PBS and application of the secondary antibody. Following 40 min of incubation with the secondary antibody, the cells were washed with PBS, incubated with 10 mg/ml Hoechst for 30 s, washed again with PBS, and mounted. Slides were analyzed

using a Zeiss microscope with epifluorescence capacity and photographed with a Zeiss Axiocam or a Zeiss LSM 700 confocal microscope.

2.6 Golgi fluorescence assay

Golgi staining was performed following the manufacturer's protocol. Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. The wheat germ agglutinin (WGA) Alexa Fluor 594 conjugate was diluted 1:200 in PBS. The cells were incubated with the WGA-conjugate solution for 10 mins, followed by two washes with PBS, and then permeabilized with 1% Triton X-100 for further preparation for immunofluorescence.

3 RESULTS

Determination of the optimal concentrations of drugs to use started with the concentrations used on Vero cells in Matthews et al., 2010, which were 1 µM colchicine, 2.5 µM noscapine, and 7 nM paclitaxel. BHK cells were grown at low density on cover slips for 24 hours and then exposed to cell media containing one of the drugs for a 24 hour period, after which they were fixed with methanol and stained for microtubules and actin filaments. The most effective dose was determined by the highest incidence of morphological change or metaphase cells with the minimal amount of apparent cell death. Ultimately, 30 µM colchicine, 60 µM noscapine, 7 µM paclitaxel and 1 µM cytochalasin-D proved to be the optimal concentrations to use on BHK cells.

The effects of the optimal concentration of drugs on uninfected cells treated for 24 hours is shown in Fig. 3. While all the cells showed effects of the drugs, the two most toxic

drugs, colchicine and cytochalasin-D, produced the greatest morphological changes. The cells treated with colchicine clearly exhibit the depolymerization of the microtubules, turning the long, linear BHK cells into rounded, slightly smaller cells (Figure 3). Noscapine does not change the morphology of the microtubules, so the noscapine treated cells do not exhibit the same morphological changes as do the ones treated with colchicine, but they do have an accumulation of DNA in the nucleus. Cells treated with noscapine are arrested in metaphase, however, they still undergo multiple rounds of DNA synthesis and become multinucleated (Figure 3). Paclitaxel- treated cells also exhibit multinucleated cells. Paclitaxel arrests the cells in metaphase and allows the DNA in the nucleus to undergo multiple rounds of replication, like noscapine. However, unlike noscapine, paclitaxel stabilizes the microtubules and prevents their polymerization, so the cells show some morphological changes, appearing more rounded than the control cells (Figure 3). Cytochalasin-D is the most toxic of the drugs tested. Cytochalasin-D stops mitosis by binding to the actin and not allowing further growth of the filaments. Cells treated with cytochalasin-D show extreme morphological changes, which includes the shrinking and rounding up of cells (Figure 3).

To determine if virus replication was affected by the drug treatments, monolayers of BHK cells were infected and the virus was allowed to adsorb for one hour. After the one hour adsorption, the inoculum was removed and cell media containing one of the drugs was added to the cells and viral replication was allowed to continue for 24 hours, after which time the infected cell medium was collected and the virus titer produced was tittered by plaque assay. A low multiplicity of infection (MOI) was used so the viruses had to undergo multiple rounds of replication in the presence of the drugs, effectively testing all aspects of the viral life cycle (en-

try, gene expression, replication, assembly, and exit). HSV-1 is a large DNA virus that replicates in the nucleus of mammalian cells. We found that none of the drugs tested had an effect on HSV-1 replication. HSV-1 was still able to reach very high titers in 24 hours in the presence of drugs and the viral titers achieved in drug treated cells were similar to those in untreated cells (Figure 4-7, Figure 8). VSV is a negative sense, single strand RNA virus whose replication takes place in the cytoplasm. VSV titers recovered from cells in the presence of all four drugs and the viral titers in untreated cells were similar (Figure 4-7, Figure 8). Sindbis virus is a positive sense single strand RNA virus, whose replication takes place in the cytoplasm. Like the viruses discussed previously, there was no difference in the titers from the drug treated cells and the untreated cells (Figure 4-7, Figure 8).

It was the plan of the study to also test adenovirus and DENV. Even though the DENV received from Dr. Kuhn produced complete CPE on BHK cells within 48 hours of incubation, it would not form plaques on Vero or BHK cells and thus neither input MOI's nor output could be determined. Thus, we did not continue experimentation with DENV. Adenovirus was grown in the absence or presence of drugs on 293 cells and plaque assays were performed on A549 cells. However it was not possible to quantify the virus titers produced by plaque assay. Adenovirus normally takes 48 to 72 hours to produce a high titer, however, the infected cell medium had to be collected after 24 hours due to the toxicity of the drugs on the cells. The infected cell media did contain virus, but at a low titer. Therefore, the residual drug left in the infected cell medium was not diluted out as it was with the other viruses. This, combined with the long period required for adenovirus to form plaques (5 to 7 days), meant that the residual drugs in the media damaged or destroyed the cell monolayers before plaques could form. In particular,

Cytochalasin-D and colchicine completely destroyed the monolayers. Noscapine and paclitaxel did some damage, but plaques were recoverable (data not shown).

Considering the lack of effect of both anti-microtubule and anti-actin filament drugs on virus replication, SINV replication in the presence of these drugs was studied in detail to see if any effect could be discerned. Through fluorescence and immunofluorescence microscopy the localization of dsRNA and both the structural and nonstructural proteins of Sindbis virus were examined. The dsRNA is a marker for locations in the cell where the virus is synthesizing RNA. Only two drugs were used in these studies, colchicine, which had the most profound effect of the anti-microtubule drugs, and cytochalasin-D. For these studies, BHK cells were grown sparsely on coverslips for 24 hours and then infected with SINV for one hour, using an MOI of 1 pfu/cell. At 1 h.p.i., the inoculum was removed and media with one of the drugs was added to the cells. At 5-6 h.p.i., the cells were fixed and stained as necessary. 5 to 6 h.p.i. was determined to be the optimal time through a series of pilot studies that examined the extent of virus replication and the health of the cells at varying time points after infection (data not shown).

dsRNA and its relationship with tubulin was first examined (Figure 9). In untreated, infected cells, the dsRNA did not form any particular pattern and appeared spread out throughout the cytoplasm in infected cells. When the cells were treated with colchicine, the tubulin predictably depolymerized and clustered around the nucleus and the cells became more rounded. However, the cells treated with cytochalasin-D shrank considerably, causing the microtubules to become compacted, although the tubules were not disrupted. Colchicine treatment caused a clustering of dsRNA foci coincident with the depolymerized tubulin while cytochalasin-D did not change the dispersed localization of the dsRNA foci.

The nonstructural proteins were examined using a Sindbis virus expressing a green fluorescent protein (GFP) tagged nsP3 (Figure 10). In infected, untreated cells, the nonstructural proteins were spread uniformly throughout the cytoplasm, although in some cells they clustered in the perinuclear region. In colchicine treated cells, however, the nonstructural proteins formed foci in most of the cells. Although the cytochalasin-D cells were shrunken, the nonstructural proteins appeared uniformly distributed, and not collected into foci. The frequency of cells with the nonstructural proteins distributed uniformly throughout the cytoplasm or clustered in perinuclear foci under the different drug treatments was tabulated as shown in Fig. 11. These cells were co-stained for dsRNA, the distribution of which overlapped with the nonstructural proteins, as expected since these proteins are involved in RNA synthesis.

Next, the localization of the structural proteins was examined (Figure 12). In infected, untreated cells, these proteins localized to the Golgi in the perinuclear region in the majority of the cells observed, as expected since the envelope glycoproteins are processed in the Golgi. In colchicine treated cells, however, the structural proteins were dispersed into foci throughout the cytoplasm. The infected cells treated with cytochalasin-D were markedly shrunken, but the distribution of the structural proteins appeared similar to the control cells, with the structural proteins localizing to the Golgi in perinuclear region. The frequency of cells with the structural proteins coincident with an apparent Golgi apparatus in the perinuclear region under the different drug treatments was tabulated as shown in Fig. 13.

The relationship of localization between the structural and nonstructural proteins was also examined as shown in Fig. 14. There was some overlap in the perinuclear region, but in general the localization of the structural proteins did not appear to coincide with the nonstruc-

tural proteins, either in untreated or drug-treated, infected cells. As seen in the other experiments, colchicine disrupted the patterns of the structural and non-structural proteins while cytochalasin-D did not appear to do so

Given the dispersal of the structural proteins in the colchicine-treated cells, the relationship of the structural proteins and the Golgi was examined using a Golgi-specific stain. As shown in Fig. 15, the structural proteins localized to the Golgi in the infected, untreated cells. As observed previously, the Golgi becomes disrupted and scattered throughout the cytoplasm when the microtubules are depolymerized with colchicine (Storrie *et al*., 1998). In these cells, the structural proteins still localized to the Golgi elements, although they were dispersed (Figure 10). Cytochalasin-D did not disrupt the Golgi, so the relationship between the proteins and the Golgi was similar to that seen in untreated cells.

4 DISCUSSION

This study was initiated based on the findings that treatment with anti-microtubule drugs did not affect rubella virus (RUBV) replication (Matthews *et al*, 2010). In that study, the goal was to determine the role of the fibers made by the replicase proteins during RUBV replication. The fibers appear 24 to 48 h.p.i and their function is unknown. Matthews *et al* took a two-pronged approach to determine the role of the fibers; one was pharmacological and the other through mutagenesis. In the pharmacological approach, Vero cells were treated with colchicine, noscapine, or paclitaxel. In particular, colchicine was shown to disrupt the replicase fibers. The infected Vero cells were treated either early, 4 to 24 h.p.i (before fibers formed), or late, 24 to 48 h.p.i. (when fibers are present). The virus titer, however, was not adversely af-

fected in a meaningful manner during either early or late infection despite the presence of any of these drugs. However, during late infection, a modest (15-25%) decrease in titer was exhibited in the presence of colchicine and vinblastine (used as a control for the toxic effects of colchicine). In the second approach, proline residues were inserted into three α-helices within the N- and C-termini regions of P150, one of the replicase proteins, that had previously been shown to be required for fiber formation (Matthews *et al*., 2009). These mutations showed that the three domains were critical for fiber assembly and the mutations eliminated infectivity when introduced into a RUBV infectious cDNA clone. However, it is likely that the mutations had harmful effects other than fiber formation, so it could not be determined through the mutagenic approach that the fibers are necessary for RUBV infectivity. The importance of the Matthews *et al*., 2010 study, however, was that it indicated that functional microtubules are not important for RUBV infection, which runs counter to the main stream idea that most viruses rely on the cytoskeletal system during replication (Table 1, Radtke *et al*., 2006).

This study found that additional diverse viruses, SINV, VSV, and HSV-1, do not show any sensitivity to cytoskeletal inhibiting drugs as measured by viral titer. A low MOI (0.1 or less) was used for all infections by these viruses. It was reasoned that by so doing, the viruses had to complete the full replication cycle several times in the presence of the anti-cytoskeleton drugs and thus all aspects of the replication cycle would be subjected to treatment by the drugs. The viruses chosen all replicate rapidly and thus can complete multiple replication cycles within 24 hours before the anti-cytoskeleton drugs exert a toxic effect on the cells. A more in-depth analysis of SINV replication using fluorescence microscopy revealed that, while the titers in treated and untreated cells were not different, there was a difference in the localization of the struc-

tural and nonstructural viral proteins when the cells were treated with colchicine, the drug with the most dramatic effect on microtubules, but not cytochalasin. In the presence of colchicine, the nonstructural proteins were concentrated in the perinuclear region of the cytoplasm rather than dispersed throughout the cell. In contrast, the structural proteins were scattered throughout the cell, rather than localized in a perinuclear focus, which was concomitant with dispersion of the Golgi throughout the cytoplasm. Despite the dramatic effect on the Golgi, no effect was seen on virus titer.

DENV and adenovirus were supposed to be included in this study; however, a couple of factors prevented their use. In the case of DENV, we were not able to induce the virus to form plaques, thus obviating the quantitative aspects of the experimental plan of the study. In the case of adenovirus, it replicates at a slower rate than the other viruses used in this study. The slower replication rate meant much lower titers in the 24 hour period allowed for replication. A longer incubation period was not possible due to the cytotoxicity of the drugs. The length of time for these viruses to form plaques was also a problem. Before the time required for plaque formation, the monolayer of cells died due to the toxicity of the drugs remaining in the inoculum. However, some faint plaques were seen in adenovirus assay plates using medium from infected cells that had been treated with some of the less cytotoxic drugs, such as noscapine and paclitaxel. Although these plaques were not quantifiable, their presence supported the hypothesis that adenovirus can replicate in the presence of these drugs.

This study produced similar results as the Matthews *et al*., 2010 study did. Of all the viruses tested, none showed any adverse changes in titer as a result of exposure to the drugs. This study was intended to be a global study encompassing the entire viral replication cycle of a

few viruses which represent a variety of genome types and viral families. Of the three viruses tested, none showed any sensitivity to the cytoskeletal disrupting drugs. A previous study using SINV showed no effect of anti-microtubule drugs on virus titer (Frolova *et al*., 2010). However, previous studies on HSV-1 and VSV showed that these viruses do interact with the microtubules (Das *et al*., 2006, Hammonds *et al*., 1996, Sodeik *et al*., 1997, Mabit *et al*., 2002). However, the studies done on HSV-1 focused on early replication events and transport to the nucleus, rather than the entire replication cycle (Hammonds *et al*., 1996, Sodeik *et al*., 1997, Mabit *et al.*, 2002). Hammonds et al looked at HSV-1 replication in Vero cells pre-treated with podophyllotoxin (which depolymerizes microtubules) or did variable treatment times with podophyllotoxin and colchicine. In both cases, antiviral activity was measured as the percentage inhibition of monolayer destruction, rather than determination of virus titers produced. The authors determined that podophyllotoxin inhibits HSV- 1 replication in stationary monolayers and by the application of multiple concentrations and treatment times showed that the antiviral effects were distinct from the cytostatic effect of the drug. The authors concluded that by breaking down the cytoskeleton via podophyllotoxin, the resting monolayer of cells was protected from viral infection.

Sodiek *et al.* tracked the internalization and movement of the HSV-1 capsids to the nucleus. These researchers first looked at the rate of internalization and determined that nocodazole or cytochalasin-D had no effect on the internalization of the capsids. They then followed the movement of capsids to the nucleus using fluorescent and electron microscopy. Through visualization of the capsids and the microtubules, it was determined that the capsids had a close association with the microtubules. Next, the researchers tried to inhibit the

transport of the capsids by treating the cells with taxol or cytochalasin-D. They found that neither drug affected the transport of the capsids to the nucleus. However, capsid accumulation at the nuclear membrane was significantly reduced by treatments with colchicine, vinblastine, and nocodazole (all of which depolymerize microtubules). This meant that in cells devoid of a functional cytoskeleton, capsid arrival to the nucleus was significantly delayed. Finally, whether nocodazole had an effect on productive infection was examined, for which viral protein synthesis was monitored. It was found that while nocodazole delayed the onset of viral protein synthesis, it ultimately did not prevent viral infection. The authors concluded, that based on their evidence with microscopy and a delay of infection, that HSV-1 was transported to the nucleus during early infection via the microtubules, however their study did not conclude that HSV-1 replication was inhibited in the absence of functional microtubules. In a followup study, Mabit et al, 2002 showed that nocodozole (a microtubule depolymerizing agent) interfered with HSV-1 immediate early reporter gene expression when applied during early infection (through 7 hours post-infection).

The study done with VSV examined both early and late replication events using a mutant expressing GFP fused to the virus P protein, which functions in RNA synthesis (Das *et al*., 2006). The mutant exhibited 10-fold reduced growth in comparison to the wild-type virus. When exposed to nocodazole and colcemid (both of which interfere with the polymerization of the microtubules), the titers produced by this mutant were reduced by ~80%. Fluorescent imaging techniques were used to visualize the nucleocapsids and microtubules in treated and untreated cells. In untreated cells, the nucleocapsids were spread evenly in the cytoplasm while in treated cells, the nucleocapsids formed aggregates in the cytoplasm. The authors also ob-

served a close association of the nucleocapsids with the mitochondria as well, although they stated that the significance of the association was unclear.

In summary of these other studies, the studies on HSV-1 by Sodeik and colleagues (Sodeik *et al.*; Mabit *et al.*) concluded that it is transported to the nucleus via microtubules in normal cells but found that while drug-treated cells may lack an intact cytoskeleton, this delays capsid transport to the nucleus, immediate early promoter-driven reporter gene expression, and the onset of viral protein synthesis. These results are not inconsistent with our results since ultimate virus titers were not measured. Hammonds *et al.* concluded that podophyllotoxin had anti-viral activity, but measured the activity only through monolayer survival measured by a stain, but again virus titers were not measured. The results of Das *et al.* contradicted those of this study, since virus titers were measured, albeit using a high, rather than a low, MOI. Additionally, the results were obtained using the GFP-tagged P protein mutant and no experiments on the anti-viral activity of the anti-cytoskeleton drugs were done on wild type VSV. The study employing SINV (Frolova *et al.*) were entirely consistent with the results of our study.

As stated before, few studies have been done that looked at the full viral replication cycle in the absence of an intact and dynamic cytoskeleton. The studies that have been done have most often looked at early replication events and most concluded that the viruses rely on the microtubules for transport (Table 1, Radtke *et al*., 2006). The adenovirus and reovirus papers discussed previously, as well as the Matthews *et al*., 2010, study, the Frolova *et al*. 2010 study, and this study present evidence that this may not always be the case. The studies of Glotzer and Yea *et al.* on adenovirus both drew the conclusion that while cytoskeletal elements play a

role in intracellular trafficking, specifically nuclear targeting, some viruses can reach the nucleus independent of the cytoskeleton, possibly through vacuoles or other vesicles. The vesicles hypothesis was supported by the observation of Yea *et al*. of virions in vesicles and free in the cytoplasm around the nuclei of cells that had been treated with anti-cytoskeleton drugs. Alternatively, the vesicles could be a defense mechanism of the host. Free diffusion of the capsids through the cytoplasm is thought not to be an option, since the rate of the distance covered is too great and the amount of molecular crowding from high protein concentrations restrict free diffusion of molecules larger than 500kDa (Glotzer *et al*., 2000, Radtke *et al.*, 2006).

It is also possible that alternate, less efficient pathways exist that viruses are able to use for transport when the cytoskeleton is disrupted. Yea *et al*. suggested "cross talk" among the filaments of the cytoskeleton. When one type of filament becomes compromised, it might signal for the use of alternate pathways. It is possible that viruses normally use the microtubules, and much evidence exists to support this, but are able to utilize these putative alternate pathways that the cell has in place when the microtubules become disrupted. Our findings suggest that the use of the cytoskeleton by viruses is more complex than previously thought and brings attention to the necessity for further study. Future studies should include more viruses from other untested viral families, but quantification to determine if virus replication is ultimately hindered despite alterations in specific aspects of the virus replication cycle. Once this is determined, more specific analysis of steps in the virus replication cycle is needed to see if alternative intracellular transport mechanisms are indeed in play. In conclusion, this study, as far as is known, is the first to look at the use of the cytoskeleton by a variety of viruses. Although there have been studies using several different viruses, most have looked at the association the

viruses have with the cytoskeleton during early stages of the replication cycle. While there is evidence to support that the viruses do interact with the cytoskeleton, there is evidence to support the hypothesis that lack of an intact cytoskeleton is not enough to prevent some viruses from completing a productive infection. The exact mechanism that the viruses use in the absence of an intact cytoskeleton is not known, but we and others hypothesize the existence of alternative mechanisms. In this regard, we investigated the replication cycle of SINV in the presence of the anti-cytoskeletal drugs in more detail. In the presence of colchicine, there was a redistribution of the sites of RNA synthesis in which peripheral sites were lost. Most significantly, one organelle critical in virion morphogenesis, namely the Golgi through which the envelope glycoproteins mature during transport to the plasma membrane, was severely compromised by colchicine, concomitantly affecting the distribution of the structural proteins. It will be of interest to study the effect of colchicine treatment on maturation and transport of cell surface proteins, which if alterations are found would indicate that the virus uses an alternate pathway in this step of its replication cycle. This should be a rich area for future research.

REFERENCES

Amorim, M. J., E. A. Bruce, et al. (2011). "A Rab11- and microtubule-dependent mechanism for cytoplasmic transport of influenza A virus viral RNA." J Virol **85**(9): 4143-4156.

Anh-Tuan Dinh, T. T., and Samir Mitragotri (2005). "A Model for Intracellular Trafficking of Adenoviral Vectors." Biophysical Journal **89**: 1574–1588.

Ang, F., et al. (2010). “Small interference RNA profiling reveals the essential role of human membrane trafficking genes in mediating the infectious entry of dengue virus.” J Virol **7**: p. 24.

Arakawa, Y., J. V. Cordeiro, et al. (2007). "F11L-mediated inhibition of RhoA-mDia signaling stimulates microtubule dynamics during vaccinia virus infection." Cell Host Microbe **1**(3): 213-226.

Boisvert, M., S. Fernandes, et al. (2010). "Multiple pathways involved in porcine parvovirus cellular entry and trafficking toward the nucleus." J Virol **84**(15): 7782-7792.

Candurra, N. A., M. J. Lago, et al. (1999). "Involvement of the cytoskeleton in Junin virus multiplication." J Gen Virol **80** (Pt 1): 147-156.

Carter, G. C., G. Rodger, et al. (2003). "Vaccinia virus cores are transported on microtubules." J Gen Virol **84**(Pt 9): 2443-2458

Carvalho, Z. G., A. P. De Matos, et al. (1988). "Association of African swine fever virus with the cytoskeleton." Virus Res **11**(2): 175-192.

Clemente, R. and J. C. de la Torre (2009). "Cell entry of Borna disease virus follows a clathrin-mediated endocytosis pathway that requires Rab5 and microtubules." J Virol **83**(20): 10406-10416.

Cooper, J. A. (1987). "Effects of cytochalasin and phalloidin on actin." The Journal of Cell Biology **105**(4): 1473-1478.

Dales, S. (1963). "Association between the Spindle Apparatus and Reovirus." Proc Natl Acad Sci U S A **50**: 268-275.

Dales, S. (1975). "Involvement of the microtubules in replication cycles of animal viruses." Annals New York Academy of Sciences: 440-444.

Das, S. C., D. Nayak, et al. (2006). "Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells." J Virol **80**(13): 6368-6377

de Matos, A. P. and Z. G. Carvalho (1993). "African swine fever virus interaction with microtubules." Biol Cell **78**(3): 229-234.

Dean, E. E. V. a. D. A. (2005). "Intracellular Trafficking of Plasmids during Transfection Is Mediated by Microtubules." Molecular Therapy **13**(2): 422-428.

Eash, S. and W. J. Atwood (2005). "Involvement of cytoskeletal components in BK virus infectious entry." J Virol **79**(18): 11734-11741.

Fields, B. N., D. M. Knipe, et al. (2007). Fields virology, Lippincott-Raven.

Frolova, E. I., R. Gorchakov, et al. (2010). "Functional Sindbis virus replicative complexes are formed at the plasma membrane." J Virol **84**(22): 11679-11695.

Glotzer, J. B., A. I. Michou, et al. (2001). "Microtubule-independent motility and nuclear targeting of adenoviruses with fluorescently labeled genomes." J Virol **75**(5): 2421-2434.

Kannan, H., S. F., Deendayal Patel, Ioannis Bossis, and Yan-Jin Zhang (2009). "The Hepatitis E Virus Open Reading Frame 3 Product Interacts with
Microtubules and Interferes with Their Dynamics." J Virol: 6375-6382.

Karp, G. (2009). Cell and Molecular Biology: Concepts and Experiments, John Wiley & Sons.

Lai, C. K., K. S. Jeng, et al. (2008). "Association of hepatitis C virus replication complexes with microtubules and actin filaments is dependent on the interaction of NS3 and NS5A." J Virol **82**(17): 8838-8848.

Liu, M., E. E. Schmidt, et al. (2010). "ICP0 dismantles microtubule networks in herpes simplex virus-infected cells." PLoS One **5**(6): e10975.

Luftig, R. B. (1986). "Does the cytoskeleon play a significant role in animal virus replication?" J. theor. Biol. **99**: 173-191.

Mabit, H., M. Y. N., Ute Prank, Bianca Saam, Katinka Do¨hner, and a. U. F. G. Beate Sodeik (2002). "Intact Microtubules Support Adenovirus and Herpes Simplex Virus Infections." J Virol **76**(19): 9962-9971.

Mahmoudian, M. and P. Rahimi-Moghaddam (2009). "The anti-cancer activity of noscapine: a review." Recent Pat Anticancer Drug Discov **4**(1): 92-97.

Martin, D., M. Duarte, et al. (2010). "Sequestration of free tubulin molecules by the viral protein NSP2 induces microtubule depolymerization during rotavirus infection." J Virol **84**(5): 2522-2532.

Martinez, M. G., S. M. Cordo, et al. (2008). "Involvement of cytoskeleton in Junin virus entry." Virus Res **138**(1-2): 17-25.

Matthews, J. D., W. P. Tzeng, et al. (2010). "Analysis of the function of cytoplasmic fibers formed by the rubella virus nonstructural replicase proteins." Virology **406**(2): 212-227.

Matthews, J.D., W.P. Tzeng, and T.K. Frey, (2009). “Determinants of subcellular localization of the rubella virus nonstructural replicase proteins.” Virology, **390**(2): p. 315-23.

Mayuko Nishi, A. R., Naomi Tsurutani, Kenji Ohba, Tatsuya Sawasaki, Ryo Morishita, and I. A. Kilian Perrem, Yuko Morikawa, Naoki Yamamoto (2009). "Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag." FEBS Letters **583**: 1243-1250.

Molad, Y. (2002). "Update on colchicine and its mechanism of action." Current Rheumatology Reports **4**(3): 252-256.

Mora, M., K. Partin, et al. (1987). "Association of reovirus proteins with the structural matrix of infected cells." Virology **159**(2): 265-277.

Nishi, M., et al. (2009). “Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag.” FEBS Lett **583**(8): p. 1243-50.

Ogawa-Goto, K., K. Tanaka, et al. (2003). "Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid." J Virol **77**(15): 8541-8547.

Pollard, T. D., W. C. Earnshaw, et al. (2008). Cell biology, Saunders/Elsevier.

Radtke, K., K. Dohner, et al. (2006). "Viral interactions with the cytoskeleton: a hitchhiker's guide to the cell." Cell Microbiol **8**(3): 387-400.

Ramanathan, H. N. and C. B. Jonsson (2008). "New and Old World hantaviruses differentially utilize host cytoskeletal components during their life cycles." Virology **374**(1): 138-150.

Simon, M., C. Johansson, et al. (2009). "Microtubule-dependent and microtubule-independent steps in Crimean-Congo hemorrhagic fever virus replication cycle." Virology **385**(2): 313-322.

Sodeik, B., M. W. Ebersold, et al. (1997). "Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus." J Cell Biol **136**(5): 1007-1021.

Storrie, B., J. White, et al. (1998). "Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering." J Cell Biol **143**(6): 1505-1521

Surhone, L. M., M. T. Timpledon, et al. (2010). Paclitaxel, VDM Verlag Dr. Mueller e.K.

Tzeng, W. P. and T. K. Frey (2002). "Mapping the rubella virus subgenomic promoter." J Virol **76**(7): 3189-3201.

Xuan, C., W. Qiao, et al. (2007). "Regulation of microtubule assembly and stability by the transactivator of transcription protein of Jembrana disease virus." J Biol Chem **282**(39): 28800-28806.

Yea, C., J. Dembowy, et al. (2007). "Microtubule-mediated and microtubule-independent transport of adenovirus type 5 in HEK293 cells." J Virol **81**(13): 6899-6908.

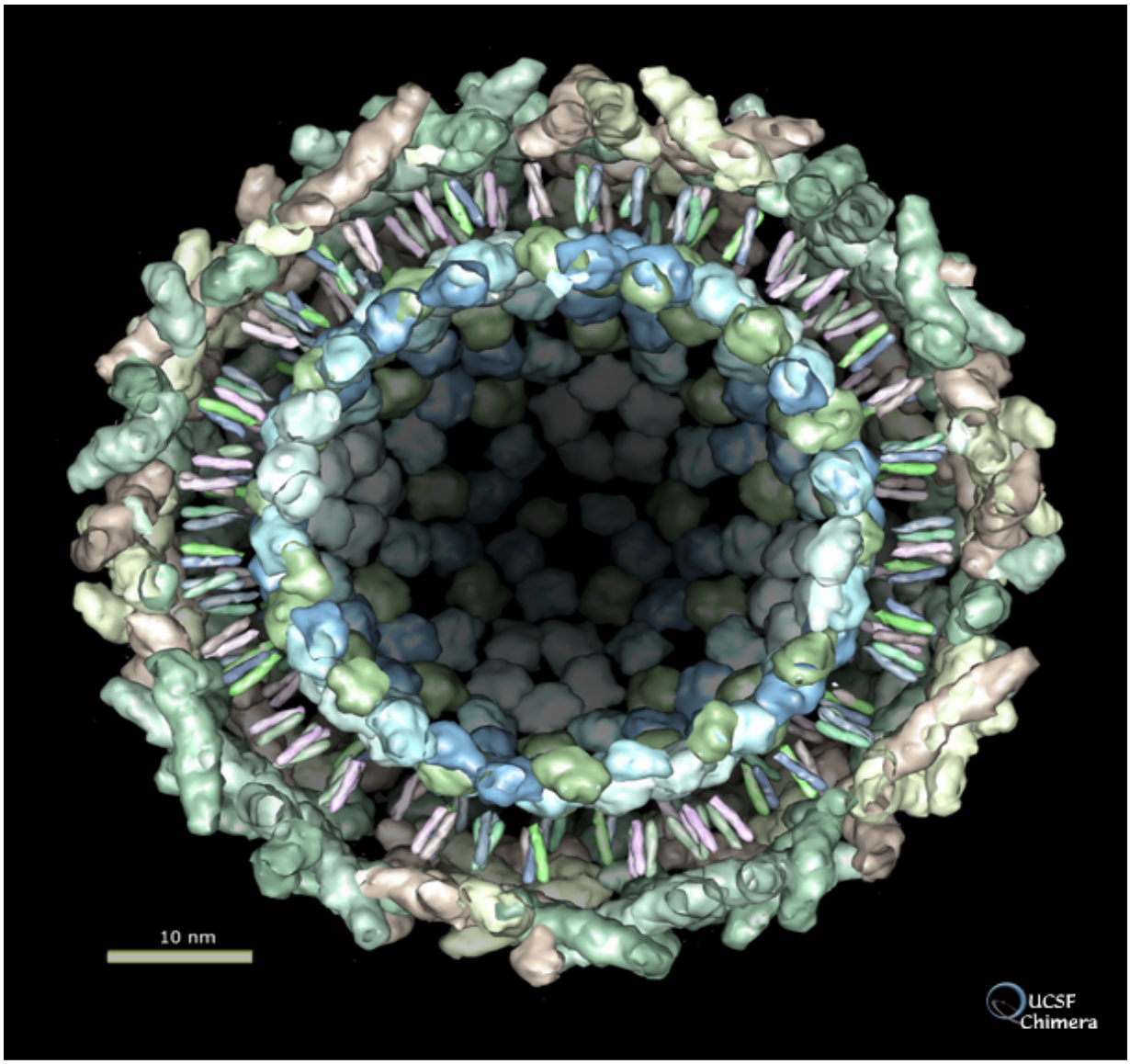


Figure 1. Cross view of Sindbis virion.

The E1 and E2 transmembrane glycoproteins form repeating subunits that give the envelope a rigid lattice structure. Underneath the envelope is a host-derived lipid bilayer that is rich in cholesterol and sphingolipid. Inside the lipid bilayer is the capsid containing the single strand RNA genome. [Wei Zhang, et al., J. Virol., (76) 11645 2002]

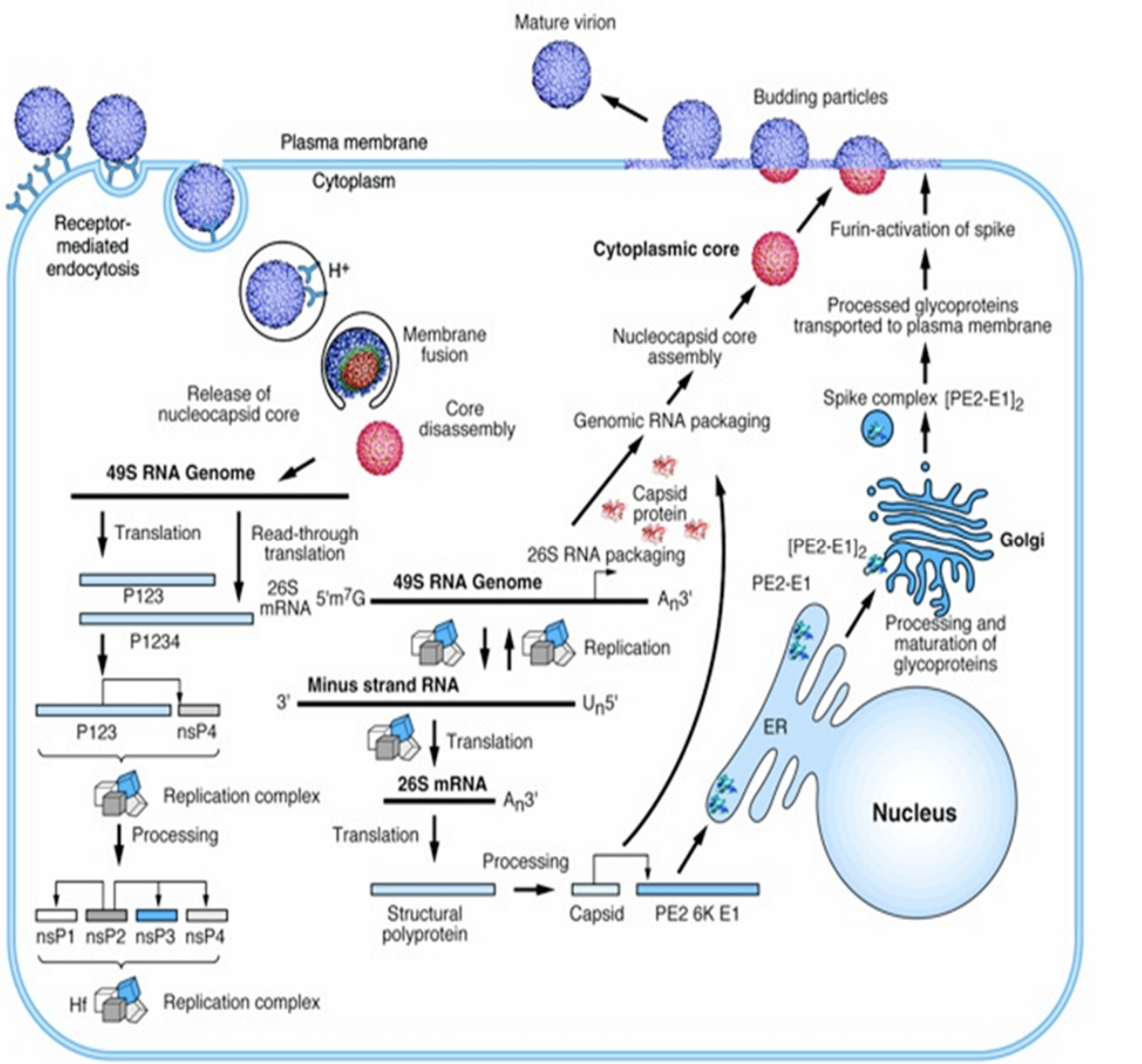


Figure 2. The life cycle of the Sindbis virus.

The virus enters the cell through receptor-mediated endocytosis. Exposure to acidic conditions allows the nucleocapsid to escape into the cytoplasm. Following release, two polyproteins are translated from the nonstructural region of the genome. These are later cleaved into individual nonstructural proteins. The nonstructural proteins form a replicative complex where the genome replication and translation of the subgenome take place. The envelope proteins are translocated into the endoplasmic reticulum membrane. From there the glycoproteins are translocated to the Golgi, and then to the plasma membrane, undergoing modifications along the way. The final stage in the replication cycle is when the capsids interact with the envelope glycoproteins to promote budding. The capsids assemble close to the plasma membrane and acquire their envelope when they bud out from the cell. [Image credit: Kuhn, R. J. 2010. Chapter 13: Togaviruses. In N. A. Acheson (ed.), Fundamentals of Molecular Virology, John Wiley & Sons, Inc. in press.]

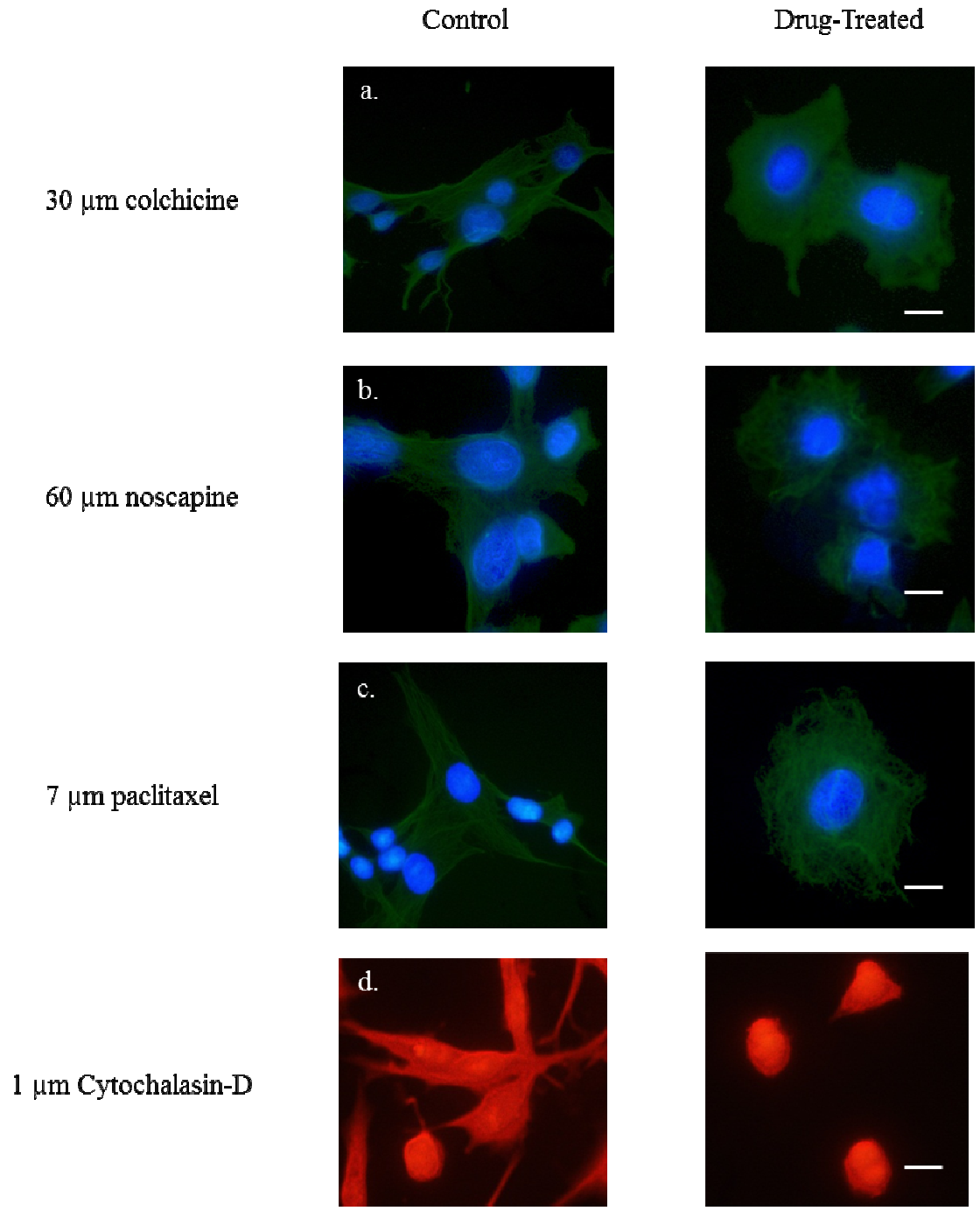


Figure 3. The Effect of the optimal concentration of drugs on uninfected cells.

Uninfected BHK-21 cells were exposed to (a) colchicine, (b) noscapine, (c) paclitaxel, or (d) cytochalasin-D for 24 hours. At 24 hours post-treatment the cells were stained with rabbit anti- α -tubulin or Alexa Fluor 568 phalloidin and visualized with goat anti-rabbit (green) or Alexa Fluor 568 phalloidin (red). Nuclei are stained with Hoechst 33342 (blue) and bars represent 10 µm.

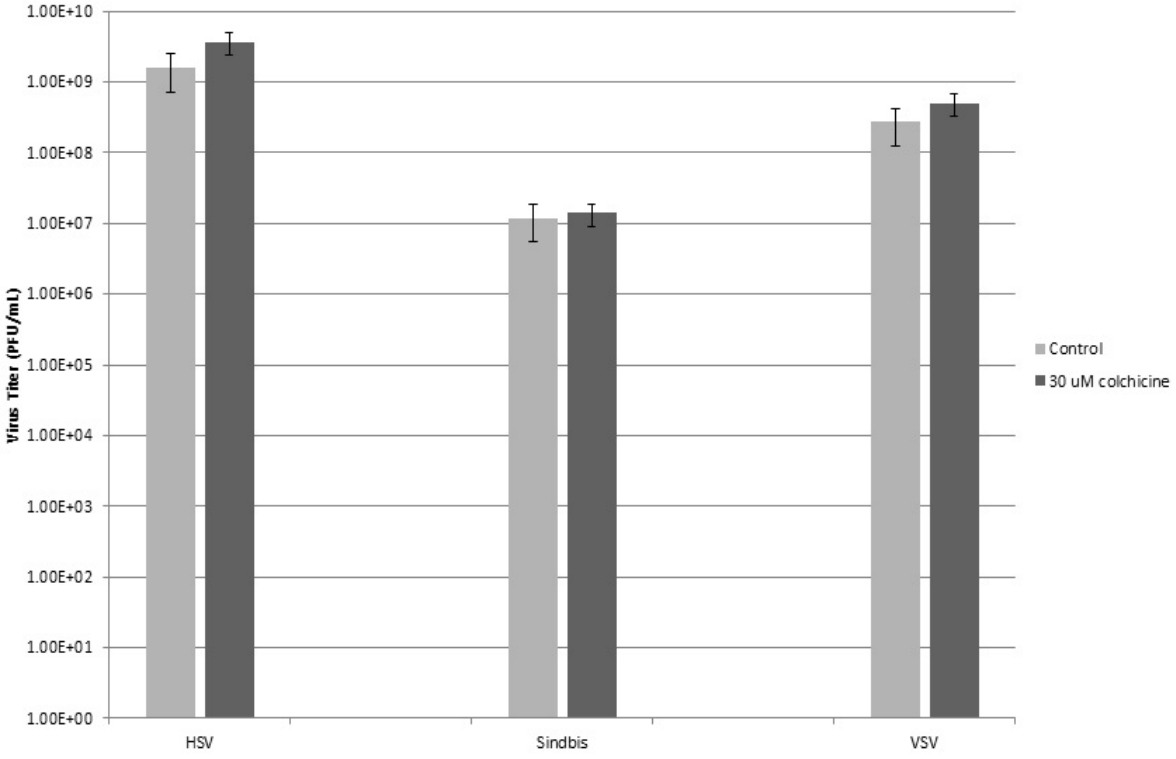


Figure 4. Effect of colchicine treatment on virus replication

BHK cells were infected for 1 hour with either HSV-1 (MOI = 0.01), SINV (MOI = 0.1), or VSV (MOI=0.1) and then incubated for 24 hours in medium containing 30 µM colchicine. At 24 hours post-infection the media were collected and titered by plaque assay. Results, given in log10 PFU/mL, were the average of three independent experiments. Error bars represent the standard deviation from the mean.

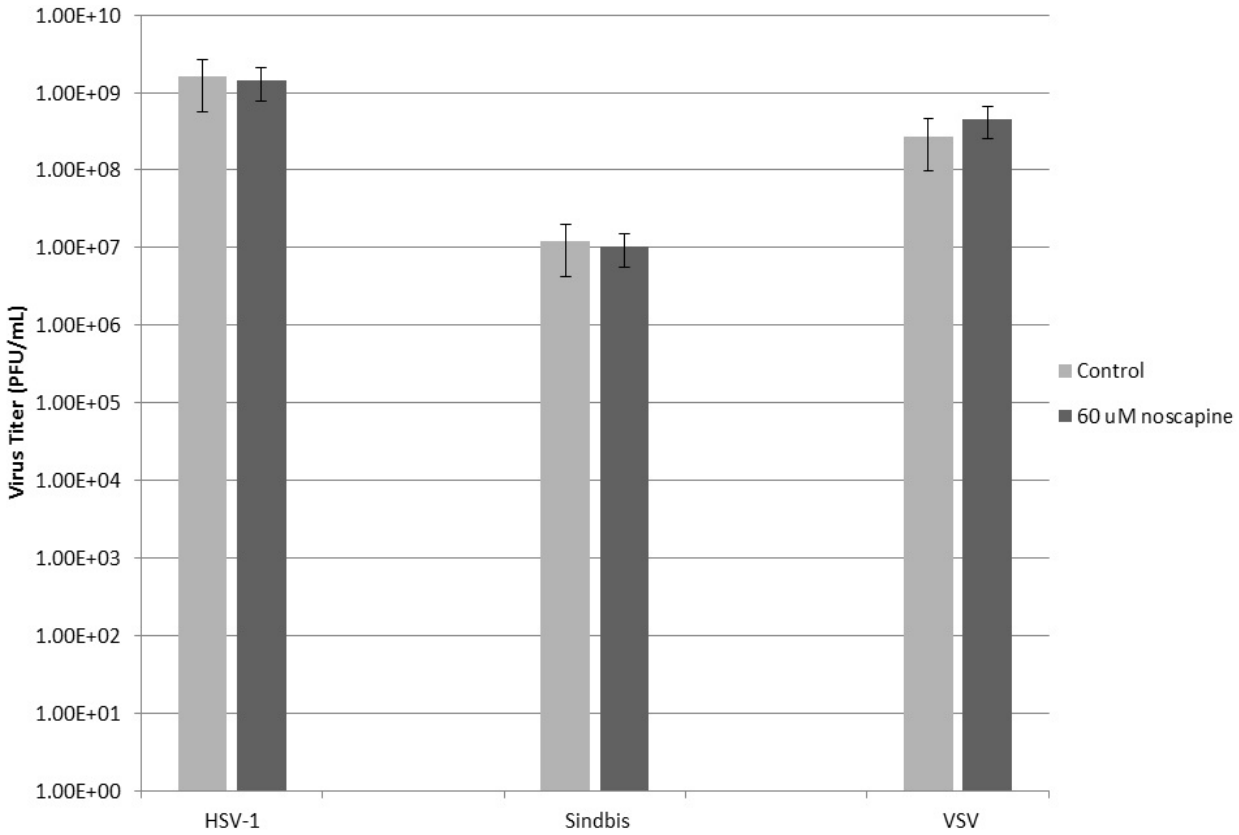


Figure 5. Effect of noscapine treatment on virus replication.

BHK cells were infected for 1 hour with either HSV-1 (MOI = 0.01), SINV (MOI = 0.1), or VSV (MOI=0.1) and then incubated for 24 hours in medium containing 60 µM noscapine. At 24 hours post-infection the media were collected and titered by plaque assay. Results, given in log10 PFU/mL, were the average of three independent experiments. Error bars represent the standard deviation from the mean.

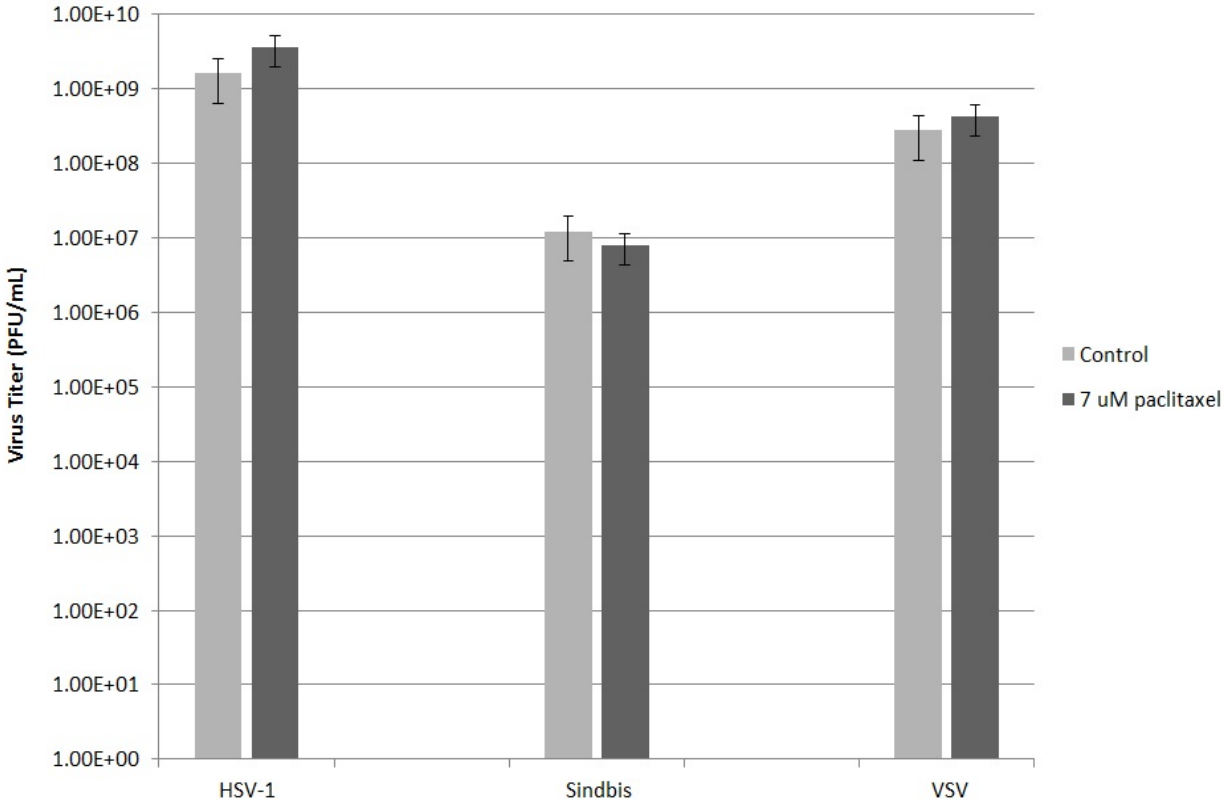


Figure 6. Effect of paclitaxel treatment on virus replication.

BHK cells were infected for 1 hour with either HSV-1 (MOI = 0.01), SINV (MOI = 0.1), or VSV (MOI=0.1) and then incubated for 24 hours in medium containing 7 µM paclitaxel. At 24 hours post-infection the media were collected and titered by plaque assay. Results, given in log10 PFU/mL, were the average of three independent experiments. Error bars represent the standard deviation from the mean.

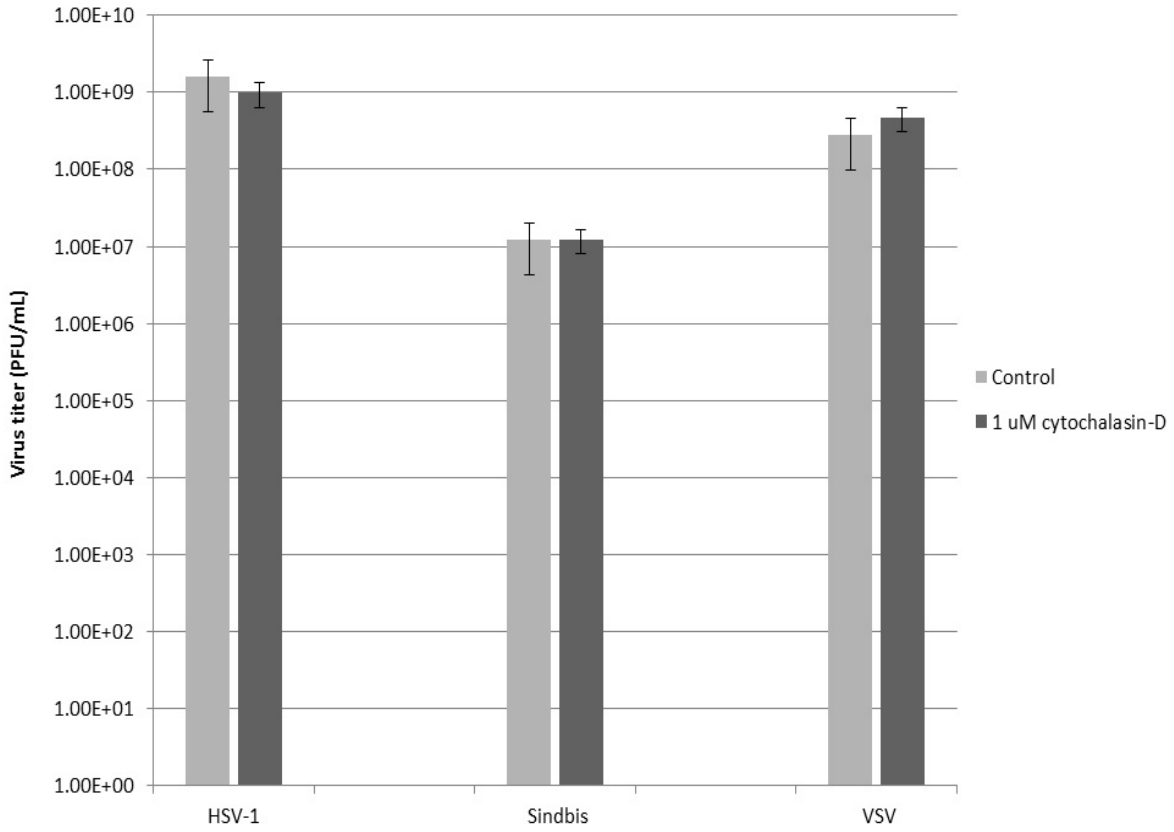


Figure 7. Effect of cytochalasin-D treatment on virus replication.

BHK cells were infected for 1 hour with either HSV-1 (MOI = 0.01), SINV (MOI = 0.1), or VSV (MOI=0.1) and then incubated for 24 hours in medium containing 1 µM cytochalasin-D. At 24 hours post-infection the media were collected and titered by plaque assay. Results, given in log10 PFU/mL, were the average of three independent experiments. Error bars represent the standard deviation from the mean.

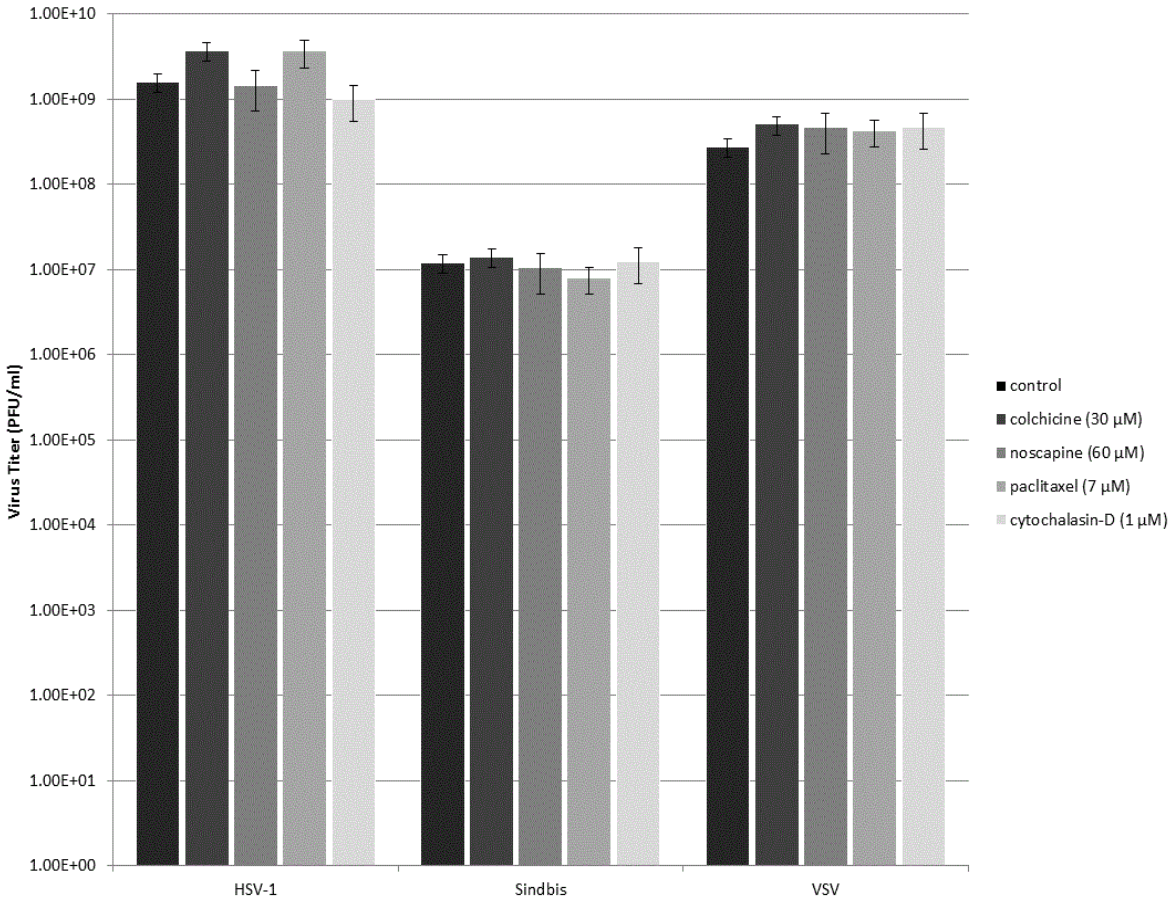
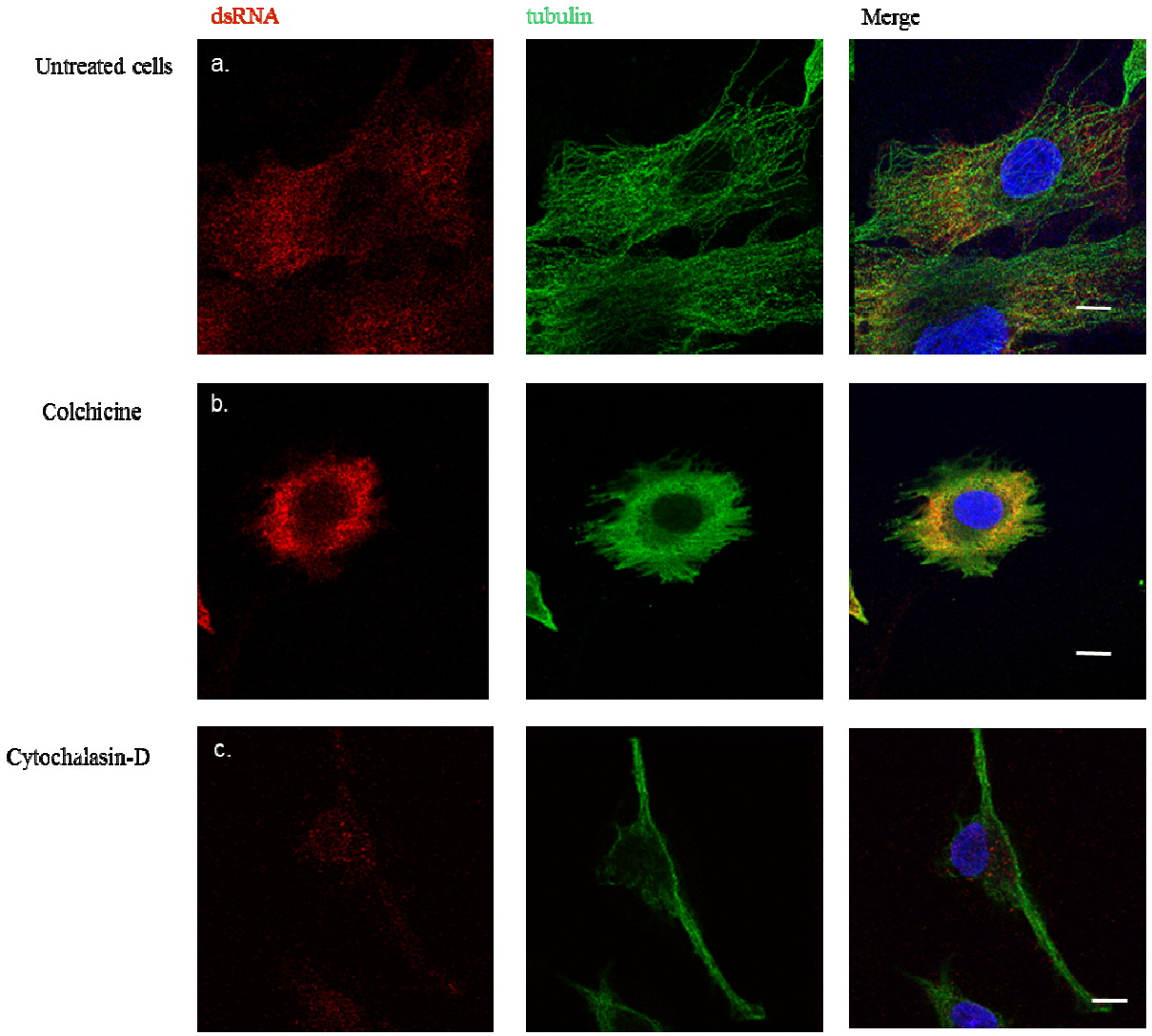


Figure 8. Effect of all drug treatment on virus replication.

BHK cells were infected for 1 hour with either HSV-1 (MOI = 0.01), SINV (MOI = 0.1), or VSV (MOI=0.1) and then incubated for 24 hours in medium containing the indicated drug. At 24 hours post-infection the media were collected and titered by plaque assay. Results, given in log10 PFU/mL, were the average of three independent experiments. Error bars represent the standard deviation from the mean.



ıg treatment.

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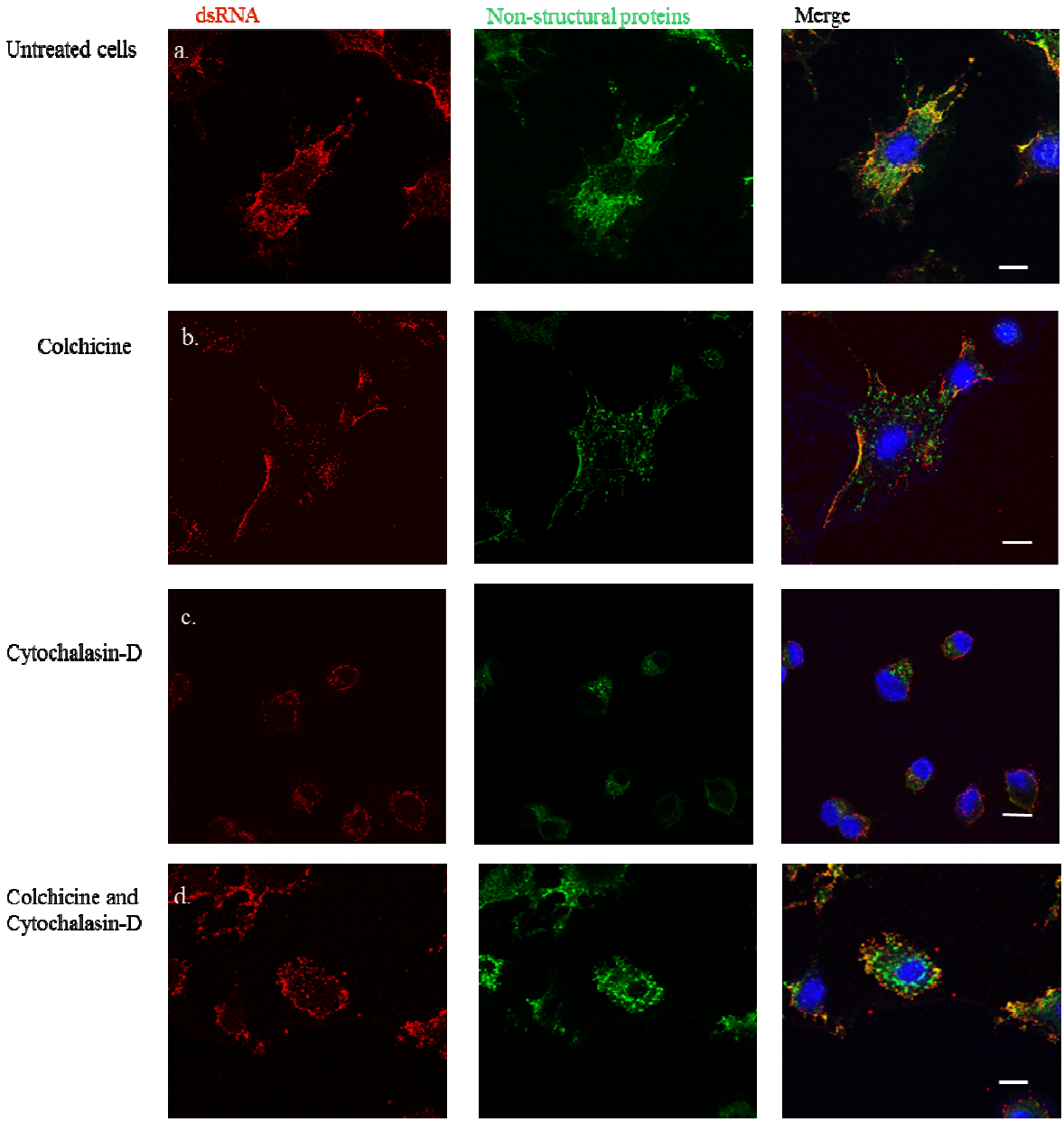

Figure 10. Analysis of location of virus non-structural proteins in SINV-infected cells after cytoskeletal drug treatment.

A recombinant SINV expressing an nsP3 protein fused with GFP fusion (green), called SINV/NSP3-GFP, was used to infect BHK cells (MOI = 1) which were, untreated (a), treated with colchicine (b), cytochalasin-D (c), or treated with both (d) from 1-6 hours post-infection. The cells were stained at 6 hours post-infection with mouse anti-dsRNA and visualized with goat anti-mouse (red). Nuclei are stained with Hoechst 33342 (blue) and bars represent 10 µm. Photographed with a Zeiss LSM 700 confocal microscope.

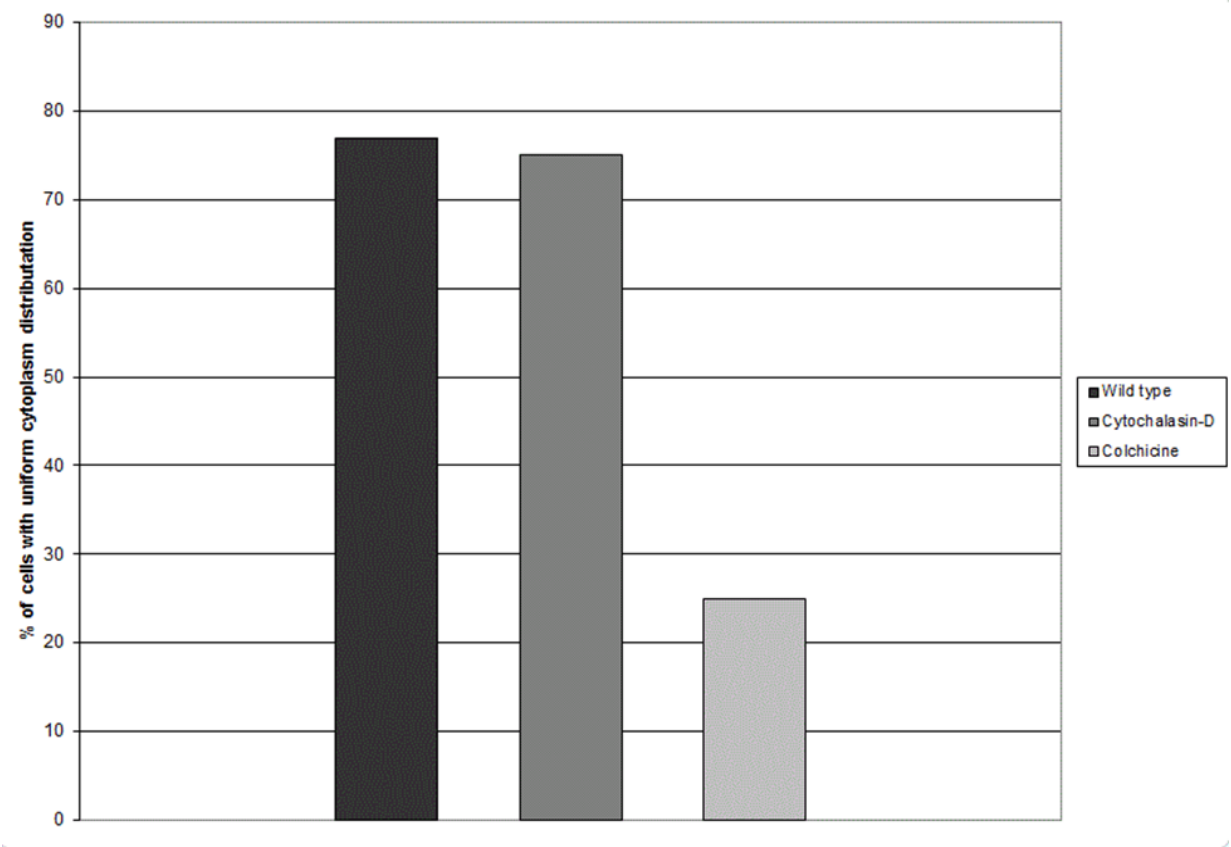

Figure 11. Effect of cytoskeletal drug treatments on localization of virus non-structural proteins.

Cells on previously prepared slides (Figure 10) were counted based on observable location of the virus structural proteins. The cells with a uniform cytoplasmic distribution are given as a percentage out 100 cells. The decrease in cytoplasmic localization of the non-structural proteins in the presence of colchicine was statistically significant (P value less than 0.05).

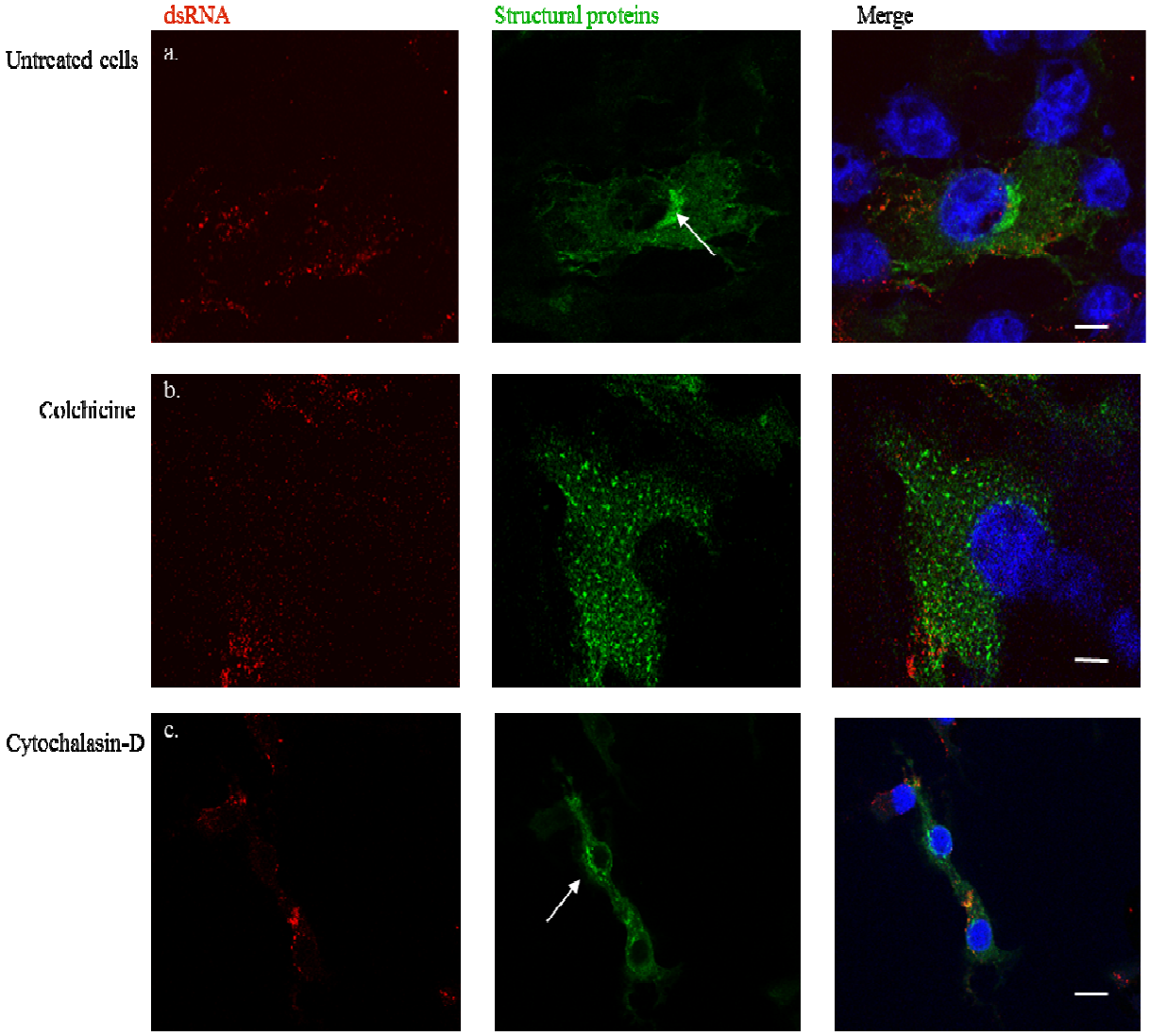

Figure 12. Analysis of location of virus structural proteins in SINV-infected cells after cytoskeletal drug treatment.

SINV-infected BHK cells (MOI = 1), untreated (a) or treated with colchicine (b) or cytochalasin-D (c) from 1-6 hours post-infection were stained at 6 hours post-infection with mouse anti-dsRNA and rabbit anti-Sindbis antibodies and visualized with goat anti-mouse (red) and anti-rabbit antibodies (green). Nuclei are stained with Hoechst 33342 (blue) and bars represent 10 µm. Photographed with a Zeiss LSM 700 confocal microscope.

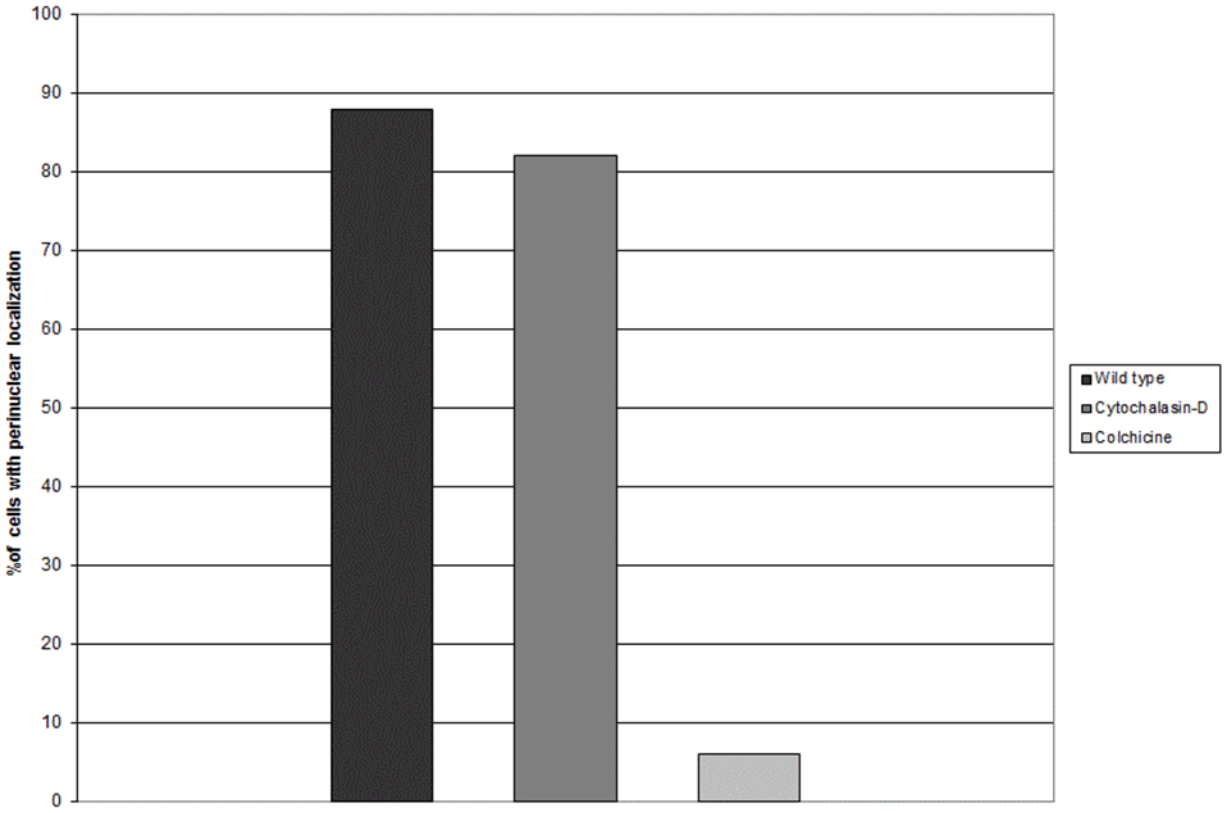

Figure 13. Effect of cytoskeletal drug treatments on localization of virus structural proteins.

SINV-infected cells on previously prepared slides (Figure 12) were counted based on observable location of the virus structural proteins. The decrease in perinuclear localization of the structural proteins in the presence of colchicine was statistically significant (P value less than 0.05).

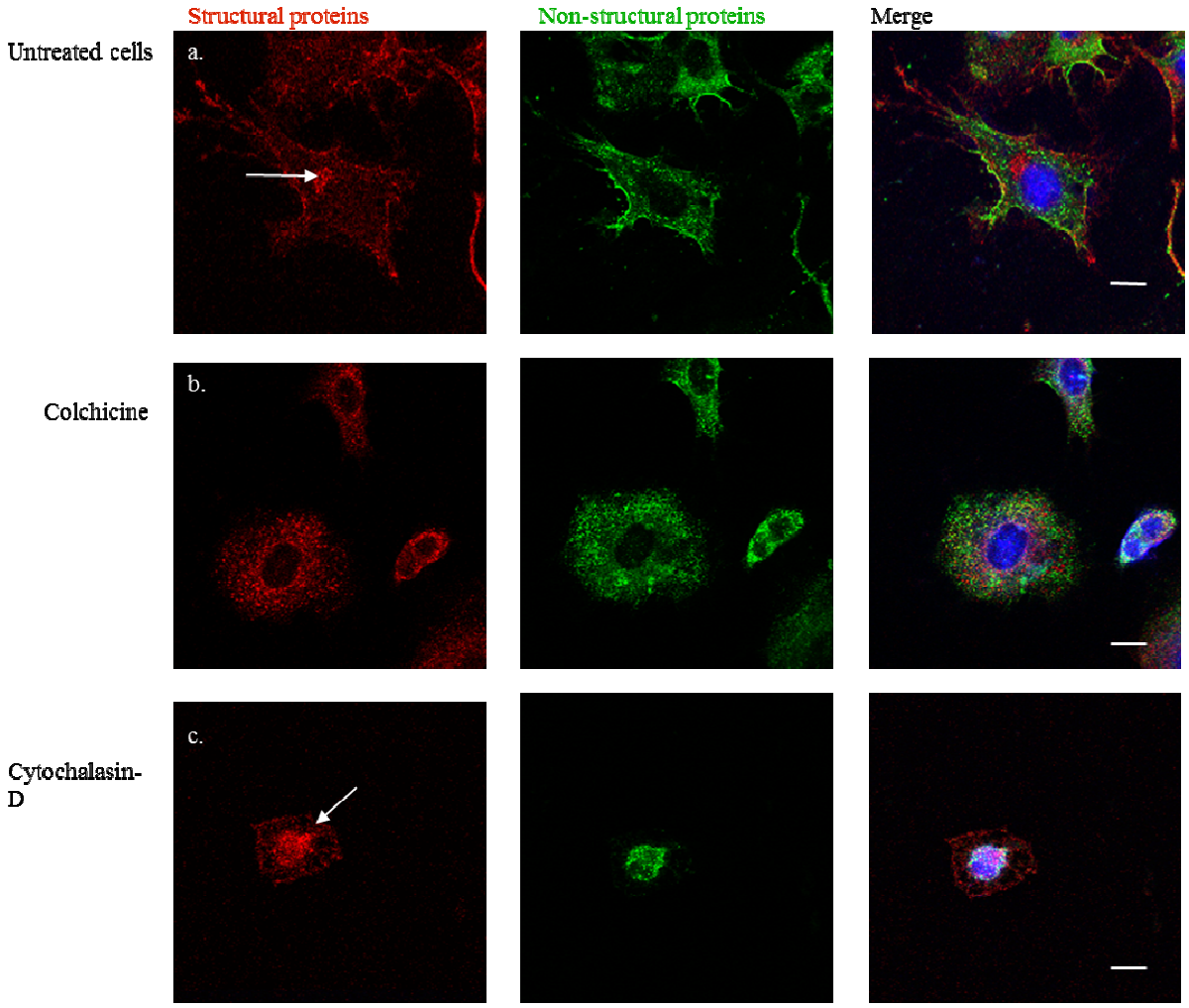

Figure 14. Analysis of location of virus non-structural and structural proteins in SINV-infected cells after cytoskeletal drug treatment.

A recombinant SINV expressing an nsP3 protein fused with GFP fusion (green), called SINV/NSP3-GFP, was used to infect BHK cells (MOI = 1). The cells were untreated (a), treated with colchicine (b), or cytochalasin-D (c) from 1-6 hours post-infection and were stained at 6 hours post-infection with rabbit anti-Sindbis virus antibodies and visualized with goat anti-rabbit (red). Nuclei are stained with Hoechst 33342 (blue) and bars represent 10 µm. Photographed with a Zeiss LSM 700 confocal microscope.

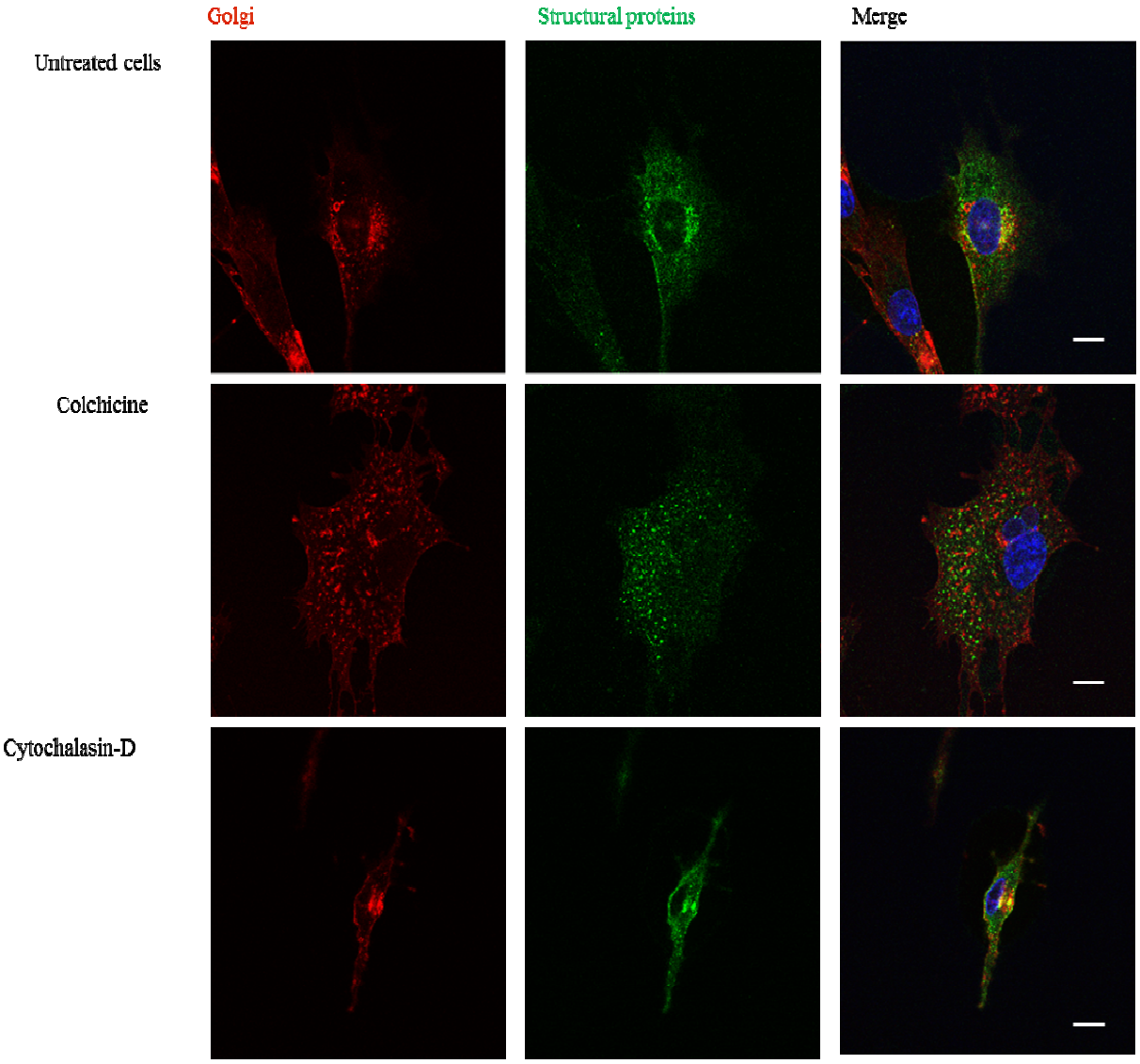

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Table 1. Studies on viral use of the cytoskeleton.

A list of studies on various viruses and their need for the cytoskeleton for successful replication. (+) is required, (-) is not required. Time point refers to events in the virus replication cycle. “Early” events include trafficking to the nucleus, and setting up replication. “Late” events include capsid assembly and exocytosis.

Genome type	Name	Family	MT required	Time point	Reference
dsDNA	Adenovirus (AD)	*Adenoviridae*	+/-	early	Glotzer et al., 2001; Mabit et al., 2002; Yea et al., 2007.
	African Swine Fever (ASFV)	*Asfarviridae*	+	early	Carvalho et al., 1998.
	BK Virus (BKV)	*Polyomaviridae*	+	early	Eash and Atwood, 2005.
	Cytomegalovirus (CMV)	*Herpesviridae*	+	early	Ogawa-Goto et al., 2005.
	Herpes Simplex-1 (HSV-1)	*Herpesviridae*	+	early	Hammonds et al., 1996.; Mabit et al., 2002.
	Murine Polyomavirus (MpyV)	*Polyomaviridae*	+	early	Gilbert et al., 2002.
	Reovirus (RV)	*Reoviridae*	-	early/late	Dales, 1975; Mora et al., 1987.
	Vaccinia Virus (VV)	*Poxviridae*	+	early	Arakawa et al., 2007; Carter et al., 2003.
ssDNA	Parvovirus (ParV)	*Parvoviridae*	+	early	Boisvert et al., 2010.
+ssRNA	Dengue virus (DENV)	*Flaviviridae*	+	early	Ang et al., 2010.
	Hepatitis C (HCV)	*Flaviviridae*	+	early	Lai et al., 2008.
	Hepatitis E (HEV)	*Hepeviridae*	+	early	Kannan et al., 2009.
	Human Immunodeficiency virus (HIV)	*Retroviridae*	+	early	Nishi et al., 2009.
	Jembrana Disease Virus (JDV)	*Retroviridae*	+	early/late	Xuan et al., 2007.
	Rubella (RUBV)	*Togaviridae*	_	early/late	Matthews et al., 2010.
-ssRNA	Andes Hantavirus (ANDV)	*Bunyaviridae*	+	early	Ramanathan and Jonsson, 2008.
	Black Creek Canal virus (BCCV)	*Bunyaviridae*	+	early	Ramanathan and Jonsson, 2008.
	Borna Disease Virus (BDV)	*Bornaviridae*	+	early	Clemente and de la Torre, 2009.
	Crimson-Congo Hemorrhagic Fever (CCHFV)	*Bunyaviridae*	+	early/late	Simon et al., 2009.
	Hantaan (HTNV)	*Bunyaviridae*	+	early	Ramanatan and Jonsson, 2008.
	Influenza A virus	*Orthomyxoviridae*	+	early	Amorim et al., 2011.
	Junin Virus (JUNV)	*Arenaviridae*	+	early/late	Candurra et al., 1999.
	Vesicular stomatitis virus (VSV)	*Rhabdoviridae*	+	early	Das et al., 2006.