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The role of Luteolin, Midostaurin (PKC-412) and PD173074 in Human Cytomegalovirus (HCMV) life cycle

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Jerusalem – Palestine

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Dedication

I dedicate my thesis to soul of my dear mother.

To my father, for his prayers that were always helping me, for his endless support, his love and patience.

To my wife, who encouraged me to pursue the master degree on first place, for her endless support and efforts during this critical stage.

To my brothers (Hazem, Hatem, Moneer and Ahmad) and sisters (Sahar, Bayan and Bushra) for their support and love.

To all of my family and my wife's family, for their encouragement.

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis or any part of the same has not been submitted for a higher degree to any other university or institution.

Signed:

Amer Izzat Mohammad Doufish

Date:2016/4/25

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Finally, I would like to thank my friends and colleagues.

Table content

List of Figures and Tables

Abbreviations

EBV	Epstein–Barr virus
KSHV	Kaposi sarcoma-associated herpesvirus
VZV	Varicellovirus
HSV-1 and HSV-2	Simplexvirus
MCMV	Muromegalovirus
ElHV1	Proboscivirusm
HHV-7	Roseolovirus
EBV	Epstein-Barr virus
HHV4	human herpes virus 4
HHV8	human herpes virus 8
CID	cytomegalic inclusion disease
HCMV	Human cytomegalovirus
HHV5	taxon human herpes virus 5
AC	assembly complex
AC	assembly compartment
PB	peripheral blood
IE	immediate-early
GCV	Gancyclovir
CDK	Cyclin-dependent kinase
PI3	phosphatidylinositide 3
PKC	protein kinase C
INM/ONM	inner/outer nuclear membrane
ER	endoplasmic reticulum
Rb	retinoblastoma
NPC	nuclear pore complex
P	phosphorylation
SES	Socioeconomic status
ELISA	enzyme-linked immunosorbent assay
PCR	polymerase chain reaction
CDV	Cidovir
BDCRB	1H- -d-ribofuranosyl-2-bromo-5,6-dichlorobenzimidazole
MBV	Maribavir
IVIG	immunoglobulins
PTK	protein tyrosine kinase
TPL2	tumor progression locus 2 serine/threonine kinase
RSK	ribosomal S6 kinases
FGFR	fibroblast growth factor receptor
TKI	tyrosine kinase inhibitor
EGFR	epidermal growth factor receptor
CML	chronic myelogenus leukemia
HFF	Human foreskin fibroblasts
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
m.o.i	multiplicity of infection
h p.i	hour post infection
rpm	round per minute
RT	room temperature

PFU	plaque-forming units
IC50	concentration of a drug that gives half-maximal inhibition response
IF	Immunofluorescence
mAbs	monoclonal antibodies
pAbs	Polyclonal antibodies
WGA	Wheat germ agglutinin
VEGFR	vascular endothelial growth factor receptor
FLT	fms-like tyrosine kinase
PDGFR	platelet-derived growth factor receptor
FITC	fluorescein isothiocyanate
SD	Standard deviation

Abstract

The cytoplasmic assembly compartment (AC) in HCMV-infected human foreskin fibroblasts (HFF) is a unique juxtanuclear "bulb"-like structure. Morphology of the AC is dependent on the activity of the viral-encoded serine/threonine kinase, pUL97. The "bulb"-like structure morphology is also altered when wt-HCMV infected cells are treated with kinase inhibitors NGIC-I, a kinase C inhibitor, or Staurosporine, a wide range serine/threonine kinase inhibitor. Infection with a UL97 deletion mutant simulated the inhibition with NGIC-I or Staurosporine of wt-HCMV infection, resulting in a less compact and a vacuole-rich AC. In all three cases viral titers were reduced 2-3 logs. Cellular kinases play central roles in regulation of cell replication and differentiation, making cellular kinase inhibitors attractive antiviral targets. Different protein kinases were identified to affect different stages of HCMV life cycle and infectivity, which prompted us to explore yet not recognized kinase inhibitors for their activity against HCMV infection. In this study, we employed protein kinase inhibitors Luteolin, Midostaurin (PKC-412) and PD173074, and investigated their influence on the assembly compartment, early stages of HCMV infection and viral load. Luteolin at 20 μM did indeed reduce viral load without any detectable effects on the assembly complex. Midostaurin, PKC-412, did not affect viral load or the assembly compartment. The most striking result was observed with the tyrosine kinase inhibitor, PD173074, at 5 μM concentration. Although PD173074 did not affect early stages of HCMV infection, it reduced viral load and induced a specific structure of the assembly compartment we referred to as "vesicles'-rich AC pattern. Hereby, the AC remained its "bulb"-like structure, but was remarkably accompanied with vesicles spread throughout the cytoplasm, which arose at 48 h p.i. and were remarkable by 72 h p.i. and 96 h p.i.. These vesicles stained for markers of the assembly complex; viral tegument proteins pp28 and pp65 as well as WGA Golgi marker and accumulated densely along the cytoplasmic membrane of the infected cells. Taken together, our data provide the first evidence for a role of tyrosine kinase in HCMV assembly.

تأثيرمركبات لتيولين، ميدستيورين(PKC-412) و PD173074 على مراحل حياة فيروس Cytomegalovirus (HCMV) المختلفة.

اعداد: عامر عزت محمد دوفش

اشراف: د. ميساء العزة

الملخص

يحتاج فيروس (HCMV) خلال دورة حياته الى تكوين بناء معين داخل الخلية المصابة وذلك لتجميع الفيروسات الناشئة قبل عميلة الخروج من الخلية يسمى (AC) cytoplasmic assembly compartment ويكون على شكل لامبة، وهذا التكوين يعتمد بالأساس في بناءه على بروتين مفسفر ينتجه الفيروس يدعى pUL 97 الذي يؤثر على وحدات السيرين/ ثيورين، ويكون مظهرالتكوين في خلايا القلفة الليفية المصابة بالفيروس على شكل لامبة تلتصق مباشرة في نواة الخلية، هذا التكوين الفريد (AC) لوحظ انه يتغير عند استخدام فيروس (HCMV) المعدل الخالي من جين UL97 او عند استخدام مثبطات المفسفرات مثل NGIC-I وStaurosporine ومثبطات kinase C protein ، حيث يصبح شكله اقل ترابطا ومليء بالفجوات التي تنتشر داخله، كما انه يقلل كمية الفيروسات داخل الخلية بشكل كبير. من ناحية اخرى تلعب cellular kinases دورا مهما في حياة فيروس(HCMV) وهذا يجعلها هدفا لتطوير علاجات مضادة لهذا الفيروس، وهناك الكثير من cellular kinases لها تأثير معروف على مراحل نمو الفيروس وهذا دفعنا الى الأستمرار بالبحث عن تأثير هذه المفسفرات في حياة الفيروس بأستخدام مثبطات المفسفرات خصوصا التي لم تدرس من قبل.

في هذا البحث تم استخدام ثلاث مثبطات للمفسفرات وهي (PKC412)luteolin, Midostaurin و PD173074 ودراسة مدى تأثير هذه المركبات على (AC) لفيروس (HCMV) وتأثيره على مراحل حياة الفيروس وكمية الفيروسات

الناتجة عن الخلايا المعالجة بهذه المركبات. حيث اظهرت الدراسة ان مركب Midostaurin لم يحدث تأثير يذكر على المجمع الفيروسي(AC) ولا على المراحل الاولى من حياة الفيروس ولم يقلص تركيز الفيروس داخل الخلية المصابة، بينما قلص مركب Luteolin كمية الفيروسات الناتجة بشكل طفيف ولم يحدث تأثير على المجمع الفيروسي والمراحل الاولى من حياة الفيروس كسابقه.

النتيجة الأبرز كانت لمركب PD173074 بتركيز 5 μM وبالرغم انه لم يؤ ثر على المراحل الاولى من حياة الفيروس الا ان هذا المركب نجح في احداث تغير واضح على شكل المجمع الفيروسي حيث اصبح مليء بالحويصلات والتي صبغت بجميع مؤشرات HCMV AC وهي بروتينات الفيروس pp28,pp65 اضافة صبغة WGA الخاصة ب Golgi وبدأت هذه الحويصلات بالظهور بعد 48 ساعة وانتشرت بشكل كبير بعد 72 ساعة من دورة حياة الفيروس والمقدرة ب96 ساعة، بالاضافة الى تاثيره في مراحل حياة الفيروس المختلفة وتقليله لكمية الفيروسات الناتجة.

1. Introduction

1. Introduction

1.1 Herpesviridae background

Herpesviridae is a family of large DNA viruses that causes wide range of human and animal diseases (Mocarski et al., 2007; Pellett and Roizman, 2007). Around 200 herpesviruses have been isolated from deferent hosts include mammals, amphibians, birds, fish and reptiles.

Like opportunistic microorganisms, herpesviruses, specially, Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), and Kaposi sarcoma-associated herpesvirus (KSHV), can cause serious diseases with high morbidity and mortality in patients having an impaired immune system. After primary infection all herpesviruses go through a latent infection. At that period, the herpesviruses remain inactive and have the ability to evade the host immune system. At any time the reactivation of latent infection may occur making the treatment hard (Murray et al., 2002).

1.1.1 Classification

According to their biological features and genome structures, Herpesviruses are assorted into three subfamilies (i.e., the Alphaherpesvirinae, the Betaherpesvirinae, and the Gammaherpesvirinae) (McGeoch et al., 2006). These subfamilies are furthermore divided into different genus based on DNA sequences homology.

1.1.1.1 Alphaherpesvirinae

Alphaherpesvirinae members were classified according to variable host range, short replication cycle, fast spread in culture, efficient destruction of infected cells, and the ability to start latent infections. This subfamily contains the Varicellovirus (VZV), Simplexvirus (HSV-1 and HSV-2), Marek's disease-like virus, and Infectious laryngotracheitis-like virus.

1.1.1.2 Betaherpesvirinae

The main feature of the members of this subfamily is a limited host rang, long replication cycle, infected cells usually enlarged and the infection in cultured cell progresses slowly. The viruses can establish latency in secretory glands, lymohoreticular cells and other tissues. This subfamily includes Cytomegalovirus (HCMV), Muromegalovirus (MCMV), Proboscivirusm (ElHV1), and Roseolovirus (HHV-7).

1.1.1.3 Gammaherpesvirinae

The most common viruses causing human diseases in this subfamily are the genus Lymphocryptovirus represented by the commonly known Epstein-Barr virus (EBV); human herpes virus 4 (HHV4) and genus Rhadinovirus represented by the commonly known Kaposi's Sarcoma-associated herpesvirus (KSHV); human herpes virus 8 (HHV8).

1.1.2 Virion Components

1.1.2.1 The Core

The mature virion core contains viral DNA that is kept in an arranged manner in the form of tours (Furlong et al., 1972; Nazerian, 1974; Zhou et al., 1999). The tours in some herpesvirions appears to be suspended by a proteinaceous spindle composed of fibrils embedded in the base of the capsid and passing through tours .The importance of DNA arrangement in the tours is not known. ATP is required for packaging viral DNA into capsid core.

1.1.2.2 The Capsid

The capsid surrounds and protects the viral DNA; its structural properties (i.e. 100-nm diameter and 162 capsomeres) are typical for all herpesviruses. Three main forms of non-enveloped capsids present in infected cells; A-, B-, and C-capsids (Gibson, 1996). A-capsids are without core, B-capsids have assembly scaffold but no genome, and C-capsids contain viral DNA core. Herpesvirus capsid is composed by four conserved proteins,

which include major capsid protein, the monomer and dimer proteins of the triplex, and the small capsomere interacting protein.

1.1.2.3 The Tegument

The tegument is proteinaceous structure between capsid and the envelop, it has no distinguishing features, it may appears like fibrous on negative staining (Morgan et al., 1959;Wildy and Watson, 1962; Nii et al., 1968). The tegument is occasionally dispersed asymmetrically, which thickness may differ according to the location of the virion in the infected cell (Falke et al., 1959). Tegument may have more than 20 different virus-encoded proteins, some of which are found at hundreds of copies per virus. These proteins play basic roles in virion maturation by inhibiting host protein synthesis, shutting down infection-triggered cell defenses, and inducing viral gene expression.

1.1.2.4 The envelope

The envelope is the outer covering of the virus, derived from patches of altered cellular membranes (Falke et al., 1959; Armstrong et al., 1961; Wildy and Watson, 1962; Nii et al., 1968). The main component of virion envelop is a numerous of virus-encoded glycoproteins. The amounts and the number of viral glycoproteins vary among herpesviruses. The glycoproteins of herpesviruses are more numerous and shorter than those existing on the surface of other enveloped viruses (Wildy and Watson, 1962).

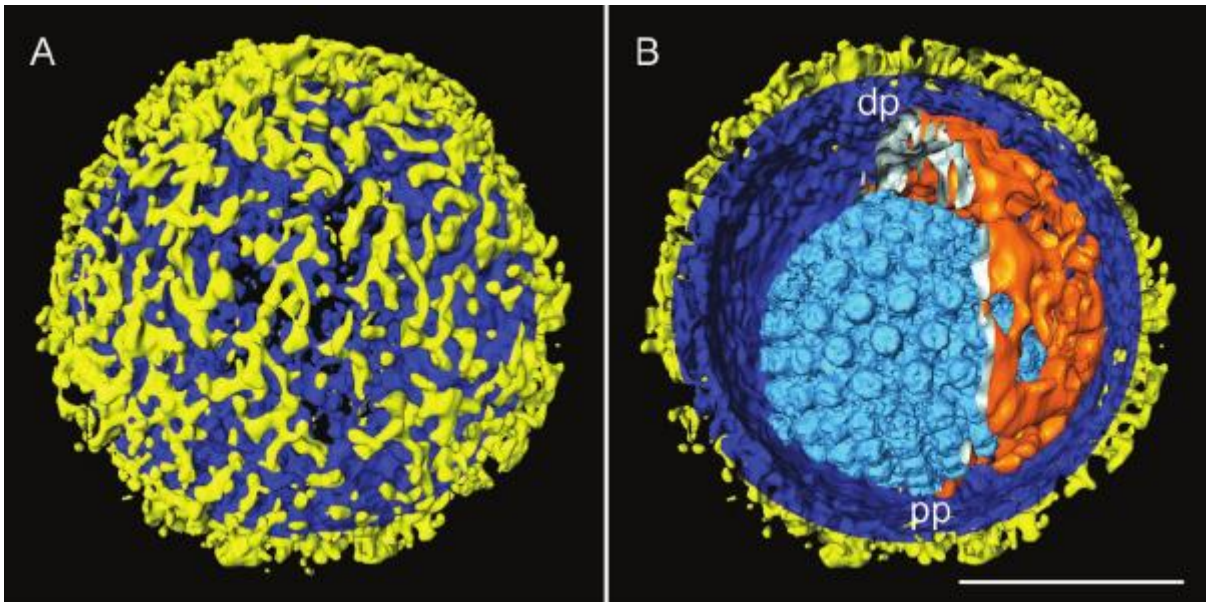


Figure 1.1: A cartoon resembling the 3 dimensional structure of herpes virus of a single virion tomogram after denoising(Grünewald et al., 2003). (**A**): outer surface showing the distribution of glycoprotein spikes (yellow) extended from the membrane (blue). (B): Cutaway view of the virion interior, showing the capsid (light blue) and the tegument "cap" (orange) inside the envelope (blue and yellow).

1.1.3 Biological properties

Members of the family Herpesviridae share four significant biological characteristics:

1. They specify a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase), and processing of proteins (e.g., protein kinases), although the exact array of enzymes may differ from one herpesvirus to another.

2. Virus gene transcription, synthesis of viral DNA, and nucleocapsid assembly occur in the nucleus. Most virions acquire at least part of their tegument and are enveloped in the cytoplasm (Enquist et al., 1998).

3. Production of infectious progeny virus (lytic infection) is generally accompanied by the destruction of the infected cell.

4. The herpesviruses examined to date employ cellular latency as a mechanism for lifelong persistence in their hosts (Figure 1.2).

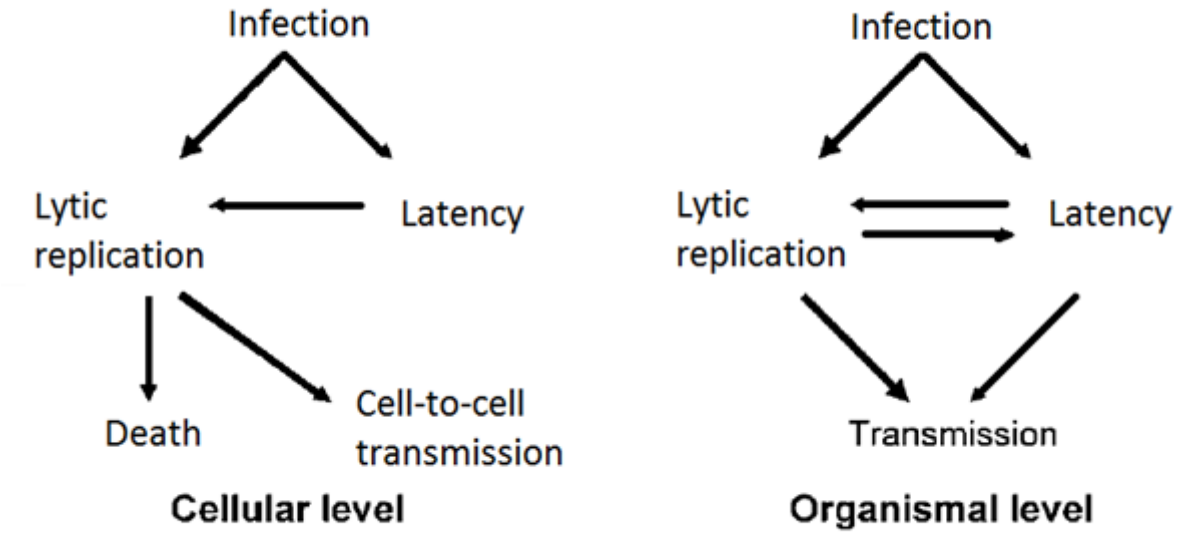


Figure 1.2: Outcomes of lytic and latent infections at the cellular and organismal levels. (Mocarski et al., 2007).

1.2 Human Cytomegalovirus

1.2.1 History

In 1881 Ribbert (Ribbert, 1904) wrote that he saw large cells in sections of new born kidney and in the parotid gland of kids. He could not explain what he saw until he read Jesioneks and Kiolemenoglous (Jesionek and Kiolemenoglou, 1904) report, they described similar cells in liver, lung and kidney of 8 month fetus. They noted the nuclei of these cells contained nuclear body with halo around it. Lòwenstein (Lowenstein, 1907) who also worked with Ribbert observed such cells in the parotid glands of some infants. And this was the first description of cytomegalic cells with intranuclear inclusions.

In 1932, although the viral was not known, Wyatt et al. (Wyatt et al., 1950) described cases of rare fetal congenital infection that had cells with intranuclear inclusions and he named it "generalized cytomegalic inclusion disease (CID)".

In 1953, after using electron microscope, Minder (Ho, 2008) saw particles like a virus with intranuclear inclusion of pancreatic cells. Rowe et al. (Wallace et al., 1956), who studied respiratory viruses isolated of tissue of patient he supposed to be varicella virus, he noticed cells with intranuclear inclusions similar to those described by Weller in cases varicella in 1953 (Weller, 1953) but Weller followed Rowe's results and decided that the virus was not varicella virus but it was similar one and he named the virus "cytomegalovirus".

1.2.2 General Characteristics

Human cytomegalovirus (HCMV), taxon human herpes virus 5 (HHV5), belongs to the beta herpesvirus family and shares common features with other beta herpesviruses like general appearance, long replication cycle in cell culture, hosts specificity, and the tropism for epithelial and hematopoietic cell types. Mammalian cytomegaloviruses are readily propagated in cultured fibroblasts of homologous host species. Through the infection, HCMV binds to endothelial and fibroblasts cells, as well as epithelial and myeloid cells (Revello and Gerna, 2010).

Nuclear and cytoplasmic inclusions are the main cytopathological features of HCMV infected cell and are associated with the viral assembly compartment or assembly complex (AC). The assembly complex is composed of viral tegument and glycoproteins, as well as cellular membranes and organelles and plays a crucial role in final steps of viral maturation and release (Sanchez et al., 2000; Azzeh et al., 2006; Das et al., 2007).

The main specific marker of HCMV infection is the presence HCMV DNA or antigens in peripheral blood (PB) and this is important particularly in immunocompromised patients (Sturgeon et al., 1996) as it allows detection of HCMV viral load in blood.

1.2.3Human cytomegalovirus structure and life cycle

HCMV has similar herpesviruses structure, the viral replication cycle includes nuclear and cytoplasmic stages that controlled by cascade of gene expression (Mocarski et al.,2007) The HCMV composed of an icosahedral protein capsid which contains large double-stranded DNA, a proteinaceous tegument surrounds the capsid and an outer lipid envelope (Mocarski et al., 2007).

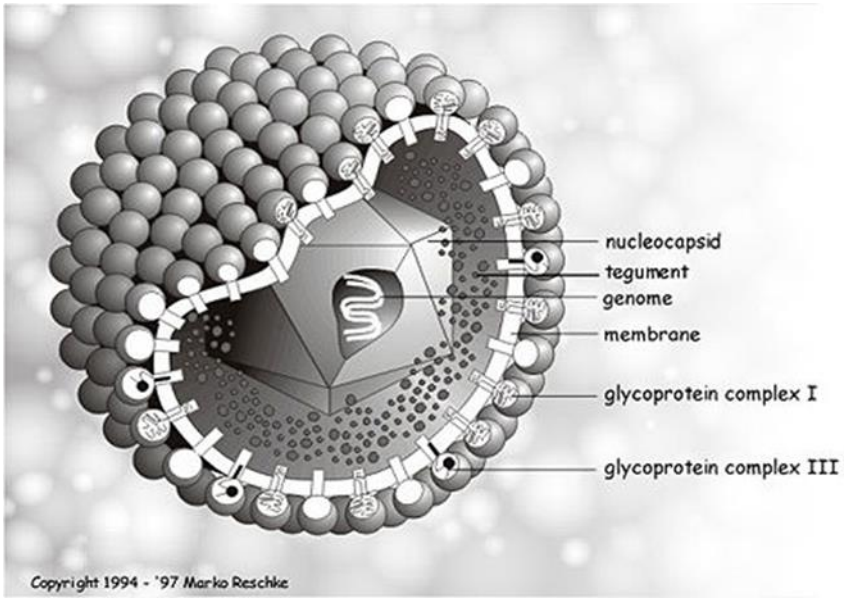


Figure 1.3: The Structure of HCMV virus. The cartoon illustrates the different components of the cytomegalovirus; the DNA embedded in the capsid, which is surrounded by the tegument layer and finally the envelope, which the glycoproteins.

HCMV enters the cell by fusion mechanism, which involves cell membrane and viral glycoproteins, particularly viral glycoprotein C and /or glycoprotein B with cell surface heparin sulfate proteoglycan (Shukla and Spear, 2001). Once the fusion occurs, the viral capsid and tegument proteins are released inside the cell (Stinski and Petrik, 2008), which is followed by a cascade of gene expression. First, viral immediate-early genes are expressed through the lytic infection (Saffert et al., 2010), leading to production of viral proteins that modulate the host cell and motivate the expression of early genes of HCMV(Mocarski et al., 2007). Early genes express proteins that are responsible for replicating the viral DNA. After replication, the immediate-early genes stimulate the expression of late genes, which are responsible for viral assembly and egress (Mocarski et al., 2007).

In certain cells types, HCMV immediate-early genes can be silenced causing latent infection (Sinclair and Sissons, 2006) characterized by the reduction of viral gene expression and suppression of viral assembly and egress (Stinski and Petrik, 2008). Under certain circumstances, latent infection can be reactivated into primary infection and allows viral spread causing disease (Mocarski et al., 2007). Beside infectious viruses, noninfectious enveloped particles and dense bodies are also produced during HCMV infection of mammalian cells (Kalejta, 2008). Noninfectious enveloped particles have structures similar to infectious virions and contain approximately an identical envelope, tegument, and capsid proteins, but lack viral DNA within the capsid (Irmiere and Gibson, 1983). On the other hand dense bodies are enveloped tegument proteins without capsids and are mainly composed of viral pp65 protein (Irmiere and Gibson, 1983). These noninfectious structures contain major antigenic determinants, which are responsible for induction of both the humoral and the cellular immune response against HCMV (Pepperl et al., 2000) and therefore used in vaccine trials against HCMV infection (Pepperl et al., 2002; Cayatte et al., 2013).

The viral DNA production, capsid assembly formation, encapsidation and initial tegumentation occur inside the host cell nucleus (Arvin et al., 2007). The encapsidation of viral DNA forms nucleocapsids, which traffics from the nucleus to the cytoplasm through nuclear egress complex. Nuclear egress is a process, which destabilizes the nuclear lamina barrier enabling the formation of primary nucleocapsids envelopment at the inner nuclear membrane followed by de-envelopment at the outer membrane (Muranyi et al., 2002; Arvin et al., 2007). Arriving the cell cytoplasm, nucleocapsids localize to the cytoplasmic assembly compartment (AC), where it acquires the remaining tegumentation and final envelopment. Cytoplasmic assembly compartment is composed modified host cell organelles, which serve the last steps in viral maturation and egress (Das et al., 2007; Tandon and Mocarski, 2011).

1.2.4 HCMV Tegument proteins

The cytomegalovirus tegument compartment contains most of the virion proteins making up around 40% of virion's mass. There are 27 genes encoding tegument proteins in HCMV (Mocarski et al., 2007). Two of the prominent tegument proteins had been of interest in this work.

The pp65 tegument protein is a phosphprotein and the most abundant component of HCMV particles (Irmiere and Gibson, 1983). After fusion of the viral and cellular membranes, pp65 is delivered immediately to the nucleus of infected cells (Revello et al., 1992). pp65 is the major constituent of capsid less dense bodies and is acquired during envelopment in the AC. This protein is highly phosphorylated and therefore associated with cellular kinases (Gallina et al., 1999). Surprisingly, the UL83 gene that encodes pp65 is unnecessary for HCMV replication in cultured cells (Schmolke et al., 1995), despite the importance of pp65 during infection as pp65 deletion mutants produce successfully infectious progeny viruses, but no dense bodies. On the other hand pp65 is highly immunogenic (Grefte et al., 1992; Wills et al., 1996) due to its ability to modulate multiple levels of immune surveillance (Mocarski et al., 2007). The persistence of this pp65 in the presence of strong cellular immune responses emphasized its importance during the HCMV life cycle. pp65 was also found to protect infected cells from innate immunity by inhibiting natural killer cell cytotoxicity (Arnon et al., 2005).

ppUL99 encodes pp28, the second important tegument protein for this study. pp28 interacts with the virion envelop through a membrane-interactive myristylated domain, which allows tegumented capsids to accumulate in the cytoplasmic assembly compartment. pp28 is a also highly immunogenic phosphoprotein with 190-amino-acids long and is found in virions and dense bodies (Irmiere and Gibson, 1983; Nowak et al., 1984; Re et al., 1985). pp28 is associated with cellular and viral membrane (Sanchez et al., 2000) and is localized with other tegument and viral proteins at the juxtanuclear cytoplasmic assembly compartment, derived from the Golgi apparatus, where final viral assembly and envelopment take place (Sanchez et al., 2000; Azzeh et al., 2006). In contrary to pp65, pp28 is essential for the final envelopment of infectious virions. A pp28 null mutant (pp28

deletion mutant) fails to make infection and results in accumulation of non-enveloped virions in the cytoplasm, despite the lack of defects in gene expression and viral DNA replication (Silva et al., 2003).

1.2.5 HCMV kinase

All Herpes viruses encode kinases, which include thymidine and serine/ threonine kinases (Chee et al., 1990). HCMV have only a serine/threonine kinase. HCMV serine/threonine kinase is encoded by the UL97 gene, which encodes for a tegument protein (van Zeijl et al., 1997; Wolf et al., 1998). UL97 kinase plays a major role in controlling the synthesis of viral DNA (Wolf et al., 2001; Krosky et al., 2003), modification (phosphorylation) of factors that regulate transcriptional and translational processes (Kawaguchi et al., 2003; Baek et al., 2004), and in viral nuclear egress (Wolf et al., 2001; Krosky et al., 2003; Marschall et al., 2005).

The absence of UL97 kinase activity results in a modified subcellular distribution of the viral structural protein assembly sites, from compact structures impacting upon the nucleus to diffuse perinuclear structures punctuated by large vacuoles (Azzeh et al., 2006). pUL97 phosphorylates pUL44 viral DNA polymerase processivity factor (Krosky et al., 2003; Marschall et al., 2003), pp65 major tegument protein (Chevillotte et al., 2009), and others. The phosphorylation activity of pUL97 is the key process for antiviral activity of Gancyclovir (GCV). GCV is a guanosine analog, which phosphorylation by UL97 leads to its integration within the viral replication strand causing termination of the replication process. Therefore, mutations in UL97 gene results in drug resistance to GCV.

pUL97 deletion mutants made it possible to map different activities controlled by pUL97 and lead to the development of novel antiviral drugs, i.e Maribavir (Prichard et al., 1999) . Maribavir is potent inhibitor of UL97 with high selectivity and activity against HCMV in vitro (Zacny et al., 1999; Biron et al., 2002). Interestingly, Maribavir did not show any effect on other herpesviruses, emphasizing its specifity and selectivity (Williams et al., 2003), however it failed in clinical trial phase 3 (ViroPharma, 2009). pUL97 also targets cellular kinases, Cyclin-dependent kinase (CDK)-related targets: retinoblastoma protein (Hume et al., 2008; Hamirally et al., 2009). Generally, as CDKs are involved in cell-cycle dependent checkpoints, UL97 kinases mimics CDK activities for the benefit of the virus.

1.2.6 Interaction of cellular kinases with HCMV

Around 2% of human genes encoded about 500 protein kinases in the body (Manning et al., 2002). Cellular protein kinases are divided into serine/threonine and tyrosine kinases. They phosphorylate cellular amino acids depending on amino acids substrate specificity (Hanks and Hunter, 1995). Kinases regulate up to 30% of human proteins and have crucial role in cellular pathways (Manning et al., 2002).

All viruses interact with the host and mimic cellular proteins in order utilize cellular machinery to supports viral reproduction (Collett and Erikson, 1978). Cyclin dependent kinases CDKs 1, 2, 7 and 9 were found to be important in phosphorylation of viral proteins and participate in viral life cycle (Sanchez et al., 2004; Sanchez and Spector, 2006; Hamirally et al., 2009). HCMV use tyrosine kinases for cell fusion (Keay and Baldwin, 1991) and phosphatidylinositide 3 (PI3) kinase to promote viral replication (Johnson et al., 2001) and inhibit apoptosis (Yu and Alwine, 2002). Furthermore, specific inhibition of UL97 with NGIC-I, a protein kinase C (PKC) inhibitor resulted in structural changes of the viral cytoplasmic assembly compartment in wild type HCMV (Azzeh et al., 2006).

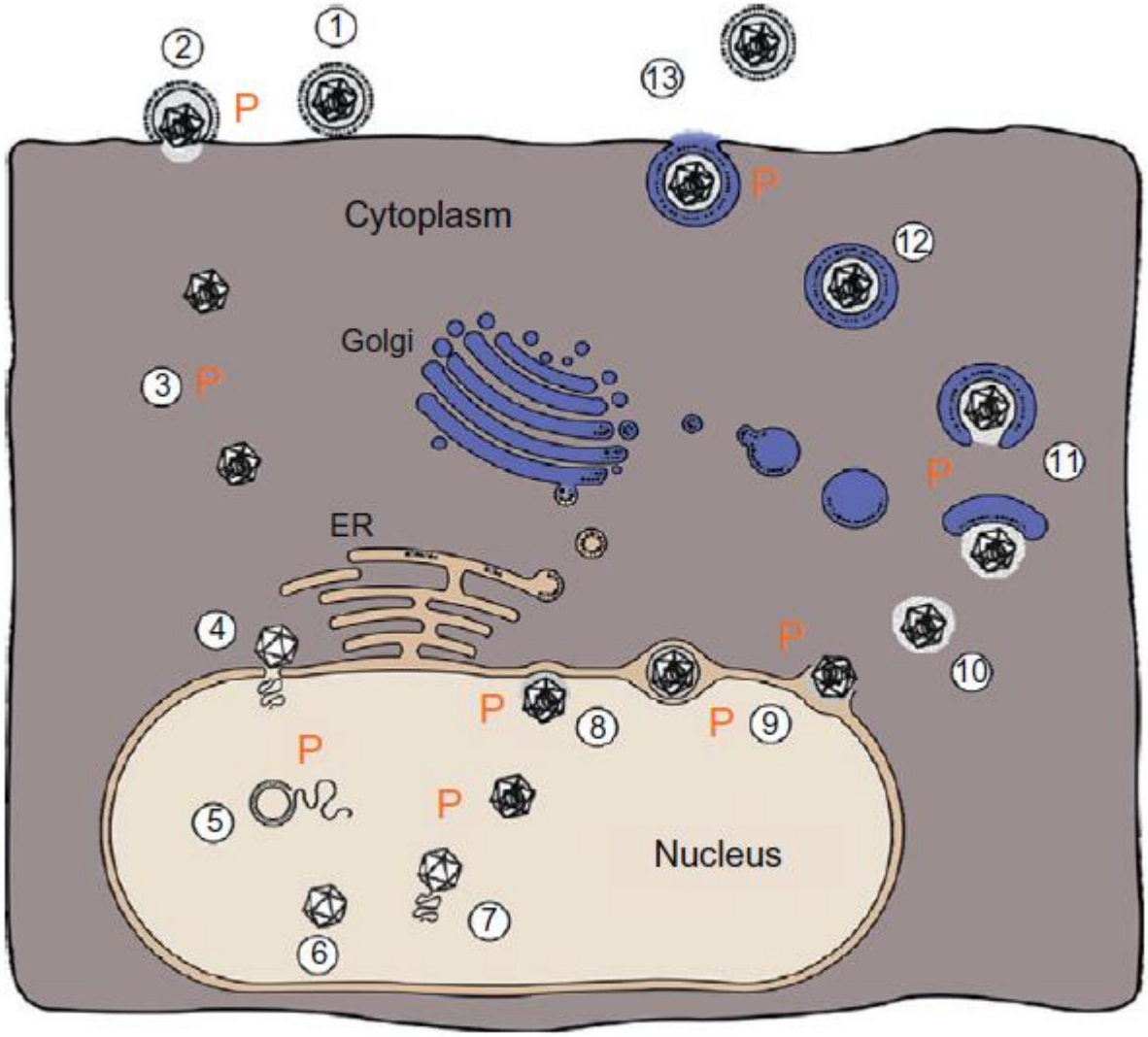


Figure 1.4: The importance of protein kinases in HCMV replication cycle. 1, HCMV receptor binding; 2, membrane fusion and entry; 3, capsid translocation through the cytoplasm; 4, nuclear import of viral genomic DNA; 5, HCMV DNA replication; 6, capsid assembly; 7, DNA loading of preformed capsids; 8, primary budding at the INM; 9, fusion with the ONM; 10, tegumentation; 11, secondary budding at Golgi-derived vesicles; 12, vesicle transport; 13, terminal membrane fusion and virion release; INM/ONM, inner/outer nuclear membrane; ER, endoplasmic reticulum; P, phosphorylation (proven or postulated phosphorylation-regulated steps of viral replication)(Marschall et al., 2011).

1.2.6.1 Functional relationship between cytomegalovirus pUL97 and cellular kinases

HCMV replication cycle is based on interactions between viral and cellular proteins; however both viral and cellular kinases play functional roles in HCMV replication and are involved in many aspects of their regulatory properties (Prichard, 2009; Lee and Chen, 2010). Recently, several studies had shown that pUL97 encoded by HCMV is responsible for phosphorylation of cellular retinoblastoma (Rb) tumor suppressor protein, this protein is usually phosphorylated by cellular cyclin dependent kinases (CDKs) that control cell cycle progression (Hume et al., 2008; Gill et al., 2009; Prichard, 2009).

Interestingly, nuclear lamin proteins that are normally phosphorylated by CDKs (Hamirally et al., 2009) were also found to be phosphorylated by pUL97 (Marschall et al., 2005). In addition, pUL97 can save a G1/S cell cycle defect of a Saccharomyces cerevisiae mutant that absences CDK function (Hume et al., 2008).

These results clarified the potency of pUL97 to cellular CDKs substrates and strongly support the idea that pUL97 share a conserved CDK-like function (Hume et al., 2008; Prichard et al., 2008; Rechter et al., 2009; Kuny et al., 2010).

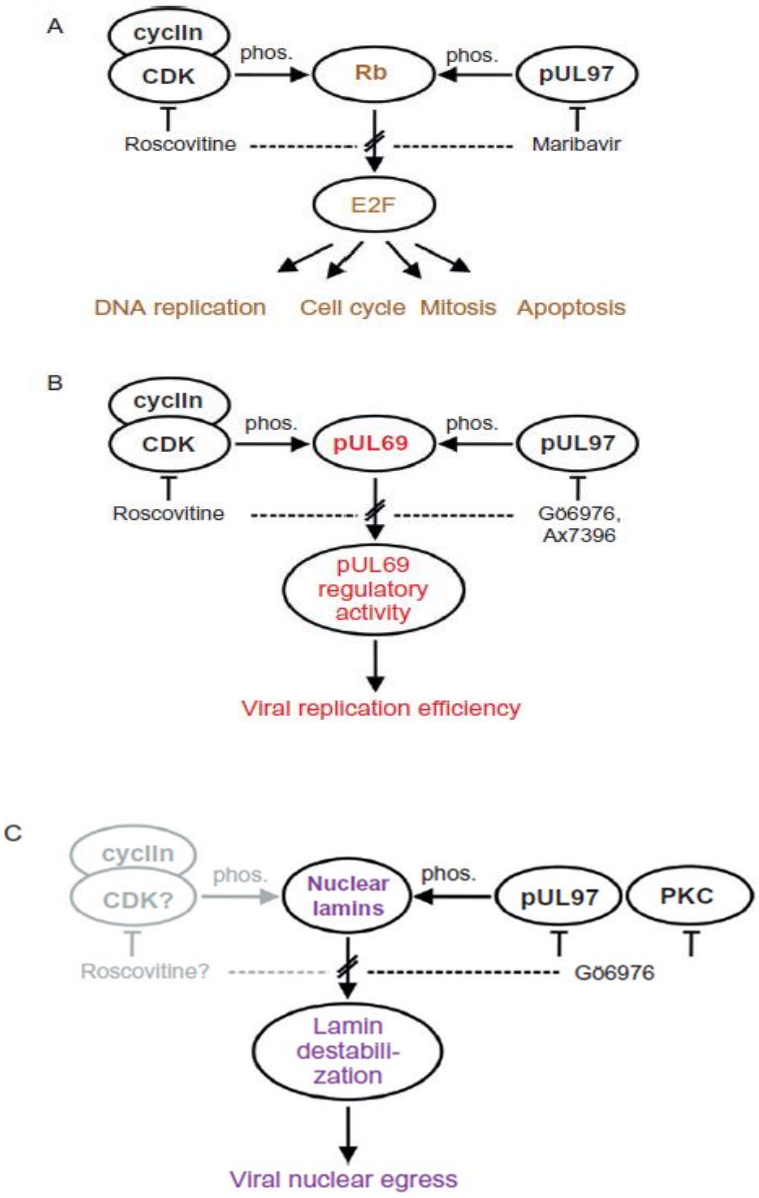


Figure 1.5: Cytomegalovirus pUL97 functional relationship to CDKs and PKC illustrated by dual phosphorylation of three substrate proteins. (A) Cellular retinoblastoma protein (Rb), (B) viral pUL69, and (C) cellular nuclear lamins represent known substrates of both, pUL97 and cellular protein kinases, including cyclin-dependent kinases (CDKs) and protein kinase C (PKC). Specific functional aspects regulated through phosphorylation (phos) of these proteins are delineated schematically and kinase inhibitors interfering with these regulatory processes are indicated. (Prichard, 2009; Marschall et al., 2011).

1.2.6.2 Interaction between pUL97 and CDKs

Because of interregulations between viral and cellular proteins, viral replication is usually connected with host cell pathways. HCMV favors a particular cell cycle phase for the starting of viral gene expression. The cell cycle phases G1, S, G2, and M are controlled by CDKs activities. CDK4 and CDK6 have necessary role in G1 phase which is fundamental for DNA synthesis (Kato et al., 1993; Meyerson and Harlow, 1994; Sherr, 1995), CDK2 is generally associated with the transition from G1 to S phase and regulated DNA replication (Hinds et al., 1992; Dynlacht et al., 1994; Geng et al., 1996; Takeda and Dutta, 2005), while CDK1 is required for cell division (Nurse, 1990; Pines and Hunter, 1991). In addition, numerous cellular processes are regulated by CDKs including transcription, cell differentiation and apoptosis.

In general, HCMV interferes with cell cycle directly after infection (Jault et al., 1995; Bresnahan, et al., 1996) and many reports have illustrated that several regulatory stages of HCMV replication need CDK1,-2,-3.and-9 activities (Jault et al., 1995; Bresnahan et al., 1997; Sanchez et al., 2003; Tamrakar et al., 2005; Sanchez and Spector, 2006; Kapasi and Spector, 2008). Furthermore, CDKs can phosphorylate HCMV pUL69 and modify its nuclear localization and activity (Rechter et al., 2009). pUL69 acts as a posttranscriptional transactivator that enables the nuclear export of mRNAs via its capability to shuttle between the nucleus and the cytoplasm and recruit components of the cellular-mRNA export machinery (Chen et al., 2002; Cullen, 2003; Chen et al., 2005; Williams et al., 2005; Lischka et al., 2006). pUL69 represent as substrate for CDK1,-2,-7, and -9. However, recent report has demonstrated that pUL97 can also phosphorylate pUL69 (Thomas et al., 2009). pUL97 inhibitors as well as CDKs inhibitors produce similar pattern of intranuclear pUL69 aggregates (Marschall et al., 2011). These findings clarified the regulatory roles of cellular CDKs and viral pUL97 for the functionality of pUL69 in HCMV replication.

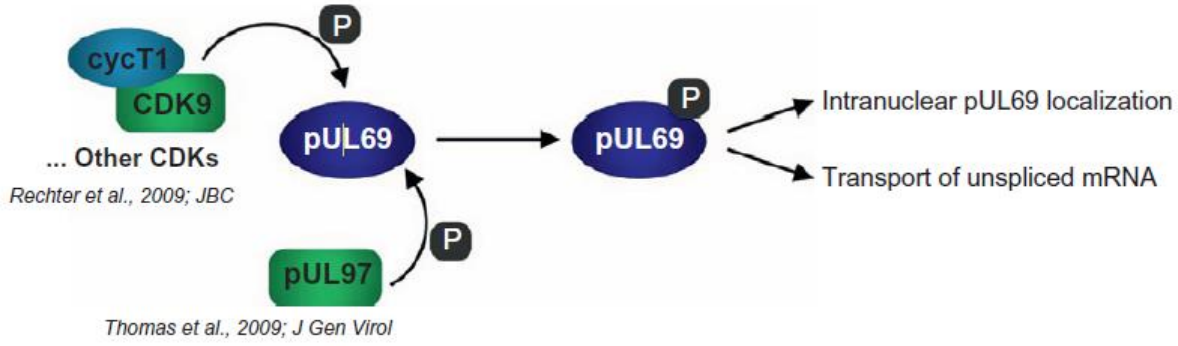


Figure 1.6: Interregulation model between CDKs and pUL97 indicating their cooperative modulation of pUL69 activity. The carton portrays the temporally and synergistically regulated phosphorylation of pUL69 by CDK9/cycT1 (possibly along with further CDK/cyclin complexes) and the viral CDK ortholog pUL97. The state of phosphorylation may influence the intranuclear localization, the mRNA export activity, and possibly other functions of pUL69 during HCMV replication.

1.2.6.3 Interregulation between pUL97 and protein kinase C (viral nuclear egress)

Viral capsid movement from the nucleus to the cytoplasm (nuclear egress) is considered the most important steps during HCMV replication. For efficient nuclear egress the viral components need to overcome the nuclear envelope. The nuclear envelope is composed of nuclear membrane, nuclear pores, and nuclear lamina. HCMV capsids cannot pass through the nuclear pore because of their large size (Mocarski et al., 2007; Pante´ and Kann, 2002). Access of HCMV capsids through nuclear envelope is delayed by network of the nuclear lamina, the main function of the nuclear lamina is to stabilize the structure of the nuclear environment (Dechat et al., 2008), therefor, the nuclear lamina has to be disassembled during cell mitosis and during viral capsids translocation. CDK1 and protein kinase C (PKC) mainly play a major role in laminas phosphorylation resulting in disassembly of nuclear lamina during mitosis (Ward and Kirschner, 1990; Thompson and Fields, 1996; Goldman et al., 2002).

HCMV usually blocks cell cycle through the action of its regulatory proteins (Bain and Sinclair, 2007; Maul and Negorev, 2008). However, it is still unclear if HCMV is able to utilize the CDK1 pathway for destabilizing nuclear lamina that is required for viral egress. It has been reported that viral pUL50 and pUL53 contribute the disassembly of nuclear lamina (Camozzi et al., 2008). Many evidences had shown that transient overexpression of cellular PKC or viral pUL97 can induce particular type of rearrangement of nuclear lamina (Milbradt et al., 2010). Remarkably, pUL50 can recruit both pUL97 and PKC to nuclear lamina (Milbradt et al., 2009). While PKC recruitment occurs by interaction with pUL50, this is not true for pUL97 recruitment. It has been demonstrated that cellular p32 recruits pUL97 to nuclear lamina disassembly (Marschall et al., 2005). In summary, the proper formation and localization of the pUL50–pUL53 complex is highly suggestive to be essential for the regulation of nuclear lamina decomposition by recruiting pUL97 and/or PKC to specific target sites of nuclear capsid egress. The interactions between cellular kinases and viral proteins involved in nuclear egress are illuminated in Figure 1.7.

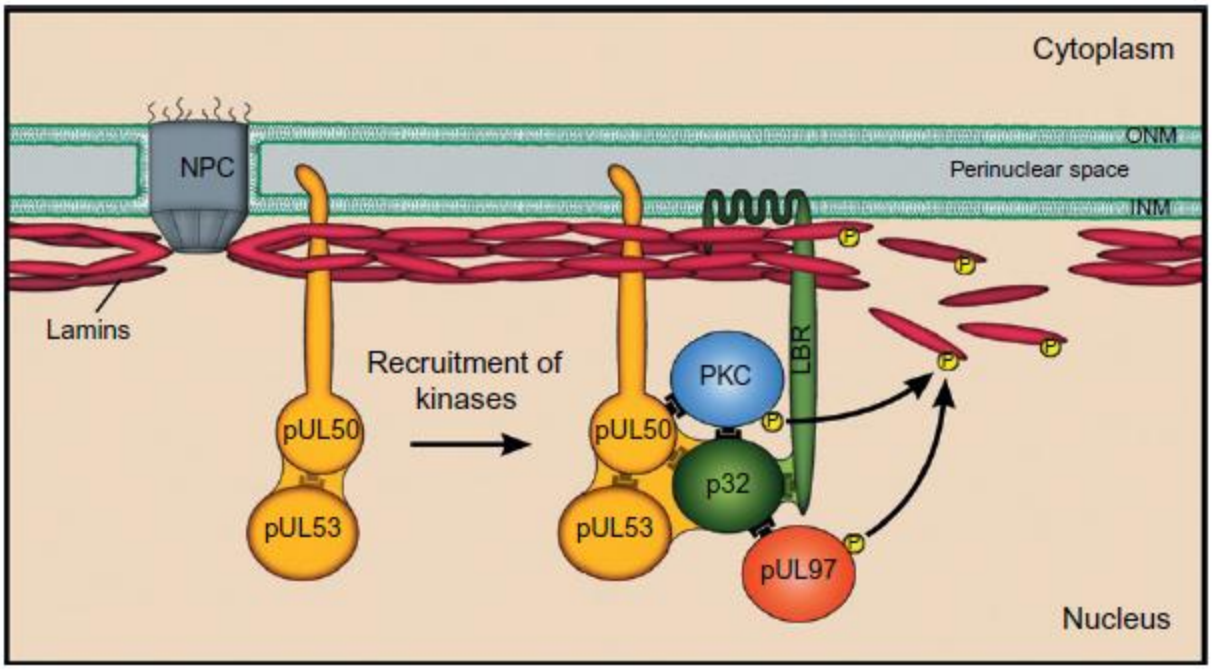


Figure 1.7: Model scheme of the course of events triggering the formation of nuclear lamina-depleted areas, postulated to represent the main sites of HCMV nuclear egress. ONM, outer nuclear membrane; NPC, nuclear pore complex; P, phosphorylation (Marschall et al., 2011).

Overall, pUL97 has a role in nuclear lamin-phosphorylating and contributes to lamina destabilizing during HCMV nuclear egress, a process which is also strongly linked to the activity of cellular protein kinases like PKC and possibly CDKs. Finally, the regulation relationship between HCMV and cellular proteins kinases gives an interesting example for the close interaction of host-viral proteins which may be important for other regulatory processes during viral replication.

1.2.7 Transmission of HCMV

Although the exact mode of HCMV transmission is unknown; direct contact with body fluids from an infected individual is an accepted route of transmission. Breast-feeding, overloaded living conditions, and sexual relationships have all been linked with high rates of HCMV infection. Sources of the HCMV include urine, cervical and vaginal secretions, breast milk, semen, blood, and allograft (Hayes et al., 1972; Reynolds et al., 1973; Lang and Kummer, 1975; Alford et al., 1980). The exposure to saliva and other body fluids containing HCMV virus is a main manner of spread. Infected infants, children and teenagers maintain virus for long periods (>6 months) after primary infection. Furthermore, seropositive individuals continue to shed virus for long period of time, an important cause of the incidence of congenital HCMV infection is the seroprevalence rate in women of reproductive age.

1.2.8 Overview of Human Cytomegalovirus Pathogenesis

Human cytomegalovirus (HCMV) is wide spread contagious agent affecting the health of many people around the world. In a simple words, HCMV pathogenesis can be divided into infection that seen in immunocompetent hosts and that seen in immunocompromised hosts (Britt, 2008). HCMV pathogenesis in persons with normal immunity is usually less serious when compared to the morbidity and mortality observed in persons having an impaired immune system. Severe complications associated HCMV disease such as pneumonia, hepatitis, retinitis, and encephalitis are less frequent in healthy people

(Eddleston et al., 1997; Britt, 2008). Furthermore, HCMV is considered one of the leading causes of congenital infections (Manicklal et al., 2013).

The HCMV infections in the immunocompetent are commonly asymptomatic (Mocarski et al., 2007); however, an active infection can cause a mononucleosis-like syndrome (Bravender, 2010) . HCMV mononucleosis is accounting around 10 % of mononucleosis diagnoses (Mocarski et al., 2007; Bravender, 2010; Klemola et al., 1970). In addition, many studies had shown a strong link between HCMV infection and development of inflammatory cardiovascular diseases and certain types of cancer (Britt, 2008; Söderberg-Nauclér, 2008; Streblow et al., 2008; Michaelis et al., 2009; Caposio et al., 2011). It was shown that patients with coronary artery disease have high levels of C-reactive protein, an indicator of the inflammatory response, which was associated with HCMV seropositivity, proposing that inflammation caused by HCMV infection may act as risk factor cardiovascular disease (Zhu et al., 1999). Finally, the presence of viral nucleic acids and antigens in cancer tissue, as well as Sero-epidemiological studies, indicate the etiologic role for HCMV in the development of numerous types of cancers (Cobbs et al., 2002; Michaelis et al., 2009; Bhattacharjee et al., 2012).

1.2.8.1 Congenital HCMV infection

Congenital infection with HCMV is a major cause of sensorineural hearing loss, vision loss and neurological impairments (Grosse et al., 2008; Dollard et al., 2007; Rosenthal et al., 2009). HCMV congenital infection is a result of vertical transmission, when the virus passes through placental barrier allowing the virus to spread from the mother to the fetus. This can be the consequence of either a primary maternal infection or a recurrent maternal infection (Stagno et al., 1977; Stagno et al., 1986; Huang et al., 1980). Recurrent infection is the result of two possibilities: a reactivation of latent virus acquired prior to pregnancy or a reinfection with a new HCMV strain during pregnancy (Boppana et al., 2001). Primary HCMV infection accounts for the highest transplacental transmission rate to the fetus, resulting in a high likelihood of fetal damage (Stagno et al., 1986; Fowler et al., 1992; Revello and Gerna, 2002; Kenneson and Cannon, 2007), the earlier the primary infection occurs during pregnancy, the more severe the consequences (Stagno et al., 1986; Demmler, 1991; Enders, 2011).

Although it is widely accepted that vertical transmission of HCMV is 15-20% higher in primary infected women compared to women with recurrent infection (Stagno et al., 1986; Fowler et al., 1992; Revello and Gerna, 2002; Fowler et al., 2003), a follow up study demonstrates that the percentage of vertical transmission in women with recurrent infection is 19.6%, which is much higher than earlier estimations of 2.2% (Rahav et al, 2007). In line with this study, a very recent review concluded that maternal HCMV seropositivity is a risk factor for congenital HCMV infection and HCMV-related hearing loss in children due to the ongoing high risk of recurrent infection due to the high percentage of infected population (Mocarski, et al., 2007; Manicklal et al., 2013; Britt, 2008).

Congenital infection can cause pneumonia, neurological disorders, gastrointestinal and retinal diseases (Vancikova and Dvorak, 2001; Mussi-Pinhata et al., 2009), hepatitis, jaundice, hepatosplenomegaly, hemolytic anemia and thrombocytopenia (Mocarski et al., 2007). The symptoms are usually subsiding after few weeks, but the disease can be fetal for some neonates (BOPPANA et al., 1992; Vancikova and Dvorak, 2001; Manicklal et al., 2013).

Newborns can be also be infected with HCMV in utero, via intrapartum transmission, or through breast milk (Nassetta et al., 2009) It is estimated that approximately40–60 % of breast-fed neonates will have HCMV infection in the first year of life if they nursed by seropositive mothers (Dworsky et al., 1983; Nassetta et al., 2009).

1.2.9 Epidemiology of HCMV Infection

HCMV infects between 40-90% of world population, infection rate varies between different countries, ethnic groups, age, sex, profession and health care systems (Alford and Pass, 1981; Pass, 1985). The seroprevalence of HCMV is generally high in developing countries, and among those of lower socio- economic status in developed countries (Mocarski et al., 2007). HCMV seroprevalence status among pregnant and childbearing age women is the main focus of various worldwide studies due to the severe consequences to offspring (see 1.2.8.1). Palestine belongs to the population with high infection rate, around 90% (Neirukh et al., 2013).

As most HCMV infections in immunocompetent individuals are asymptomatic, people are not aware of their infection (Murph et al., 1991; Lasry et al., 1996; Jeon et al., 2006; Ross et al., 2008; Pass et al., 2009). So the only way to evaluate the prevalence of infection among communities is through serological tests (see 1.2.10).

Socioeconomic status (SES) has is proportional with the prevalence of HCMV infection, people of lower socioeconomic status were more likely to have HCMV infection than those with higher SES (Stagno et al., 1986; Kenneson and Cannon, 2007).

1.2.10 Diagnostics of HCMV infection

The most useful method for detecting HCMV infection in patients is serological tests; the presence or absence of HCMV IgG clarifies whether the patient has been infected with HCMV. Several assays have been used for detection of HCMV IgG antibodies such as radioimmunoassay, anti-complement immunofluorescence and enzyme-linked immunosorbent assay (ELISA) (Lennette, 1995). The detection of HCMV IgM is considered as indicator of acute or recurrent infection. Many assays are available like the IgM capture assays, recombinant IgM assays, but Enzyme-linked immunosorbent assay (ELISAs) is the most famous one (Revello and Gerna, 2002). Pregnant women tested positive for IgM and negative for IgG are undergoing a primary infection, those, who test positive for IgM and IgG should test for IgG avidity. IgG avidity is a test which indicated the amount of IgG antibodies to distinguish between recurrent infections and reactivation of latent infection. If the avidity is low, the infection is considered a recurrent infection and therefore similar medical precautions have to be made as in primary infection.

Cell culture is considered as classical way for detecting HCMV. This method is based on utilizing clinical specimens which are injected into human fibroblast cells, after incubation period, HCMV exhibits cytopathic feature characterized by large swollen cells. However, this approach is slow and the results need 2-3weeks to be reported.

On the other hand immediate-early (IE) HCMV antigen might be detected after 16 hours of incubation by using monoclonal antibody and indirect immunofluorescence assay (Reina et

al., 1997). This technique performed in 96-well plates (Boppana et al., 1992) allowing for testing large number of samples.

In antigenemia assay pp65 viral antigen is detected by monoclonal antibodies on blood leukocytes. Besides the qualitative result, this test also gives quantitative results that measure the disease severity in immunocompromised patients (Van der Ploeg et al., 1992; Niubò et al., 1996; Lo et al., 1997).

Currently, real time PCR testing is the most rapid and sensitive method, as it depends on amplification of viral nucleic acids and commonly targets viral immediate early and late genes (Demmler et al., 1988; Chou and Dennison, 1991; Rasmussen et al., 2002). This method can be used for qualitative and quantitative detection. The real time PCR which is quantitative PCR can help in monitoring immunosuppressed patients and identify individuals at risk for HCMV disease for preventive treatment, most importantly, it allows a rapid investigation of the response to the treatment (Seehofer et al., 2004; Kim et al., 2007; Mhiri et al., 2007). This assay is more expensive compared to other methods, but it is faster and can be automated.

1.2.11 Antiviral treatment

1.2.11.1 Approved drugs

The nucleoside analogs have provided the richest source of antiviral agents. These compounds have been highly successful because of the potential for chemical variety within the class, and the differentiation of target viral DNA polymerases or reverse transcriptases from host enzymes. The herpes viruses encoded nucleoside kinases (HSV, VZV and EBV thymidine kinases, and the HCMV protein kinase) gave selectivity in the initial phosphorylation of the nucleoside analogs. The triphoshorylated forms finally played as competitive inhibitors of and substrates for the viral DNA polymerases causing a reduction in the amount of viral DNA synthesized in infected cells.

Acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) was the first nucleoside analog which has selectivity (Elion et al., 1977) and potency against HSV 1 and 2, VZV and EBV, and has moderate activity against HCMV in vitro. Acyclovir (ACV) and its prodrug, the lvalyl

ester valacyclovir, have become the gold standard for prophylaxis and management of HSV and VZV diseases, and also both have provided a benefit in HCMV diseases in transplant patients. However ganciclovir, foscarnet and cidovir are more specific drugs used for treatment of HCMV infections and diseases.

Ganciclovir (GCV) is the backbone of treating and preventing HCMV infection in past two decades. It was the first antiviral drug approved for HCMV infection, and still the drug of choice for treatment of HCMV disease and HCMV infections in organ transplant patients (Razonable and Emery, 2004). Ganciclovir is an acyclic nucleoside analogue, which is converted to ganciclovir triphosphate depending on multistep process that involves both cellular and viral enzymes, and this is the active product against HCMV. Ganciclovir undergo first phosphorylation by protein kinase encoded by the HCMV UL97 gene (Sullivan et al., 1992). Then cellular kinases catalyze the production of diphosphate and the triphosphate gancyclovir that inhibits viral DNA polymerase encoded by UL54 gene. Resistance to gancyclovir appears when mutations occur in either the UL97 or the UL54 genes.

The main side effects of gancyclovir include anemia, thrombocytopenia, primarily neutropenia and reproductive toxicity in long term use. (Biron, 2006).

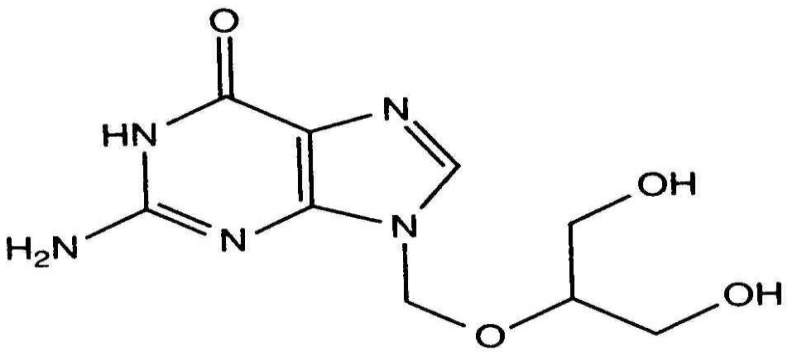


Figure 1.8: Chemical structure of ganciclovir.

Foscarnet, a trisodium salt of phosphormophonic acid, is the second drug approved for treatment HCMV retinitis in patients with weak immune system. It inhibits viral DNA polymerase preventing HCMV replication by binding to the pyrophosphate binding site and blocking cleavage of pyrophosphate from the terminal nucleoside triphosphate added to the growing DNA. Foscarnet is considered a second line of treatment especially in patients, who developed resistance to gancyclovir (Razonable and Emery, 2004). Foscarnet may cause severe side effects like kidney damage and seizers.

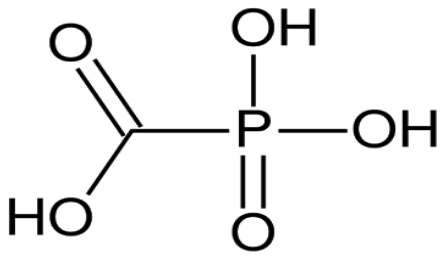


Figure 1.9: Chemical structure of Foscarnet.

Cidovir is acyclic nucleoside phosphate, it is antiviral agent with broad-spectrum activity against herpesviruses and other DNA viruses (De Clercq and Holý, 2005).Cellular kinases convert CDV to diphosphate form which acts as a competitive inhibitor of the viral DNA polymerase, causing premature chain termination in viral DNA synthesis.

Sever renal toxicity and lack of oral bioavailability are the major limitations of using Cidofovir (Safrin et al., 1997; Wood and Jacobson, 1997). However neutropenia is also associated with CDV treatment, and these effects make CDV second line of treatment.

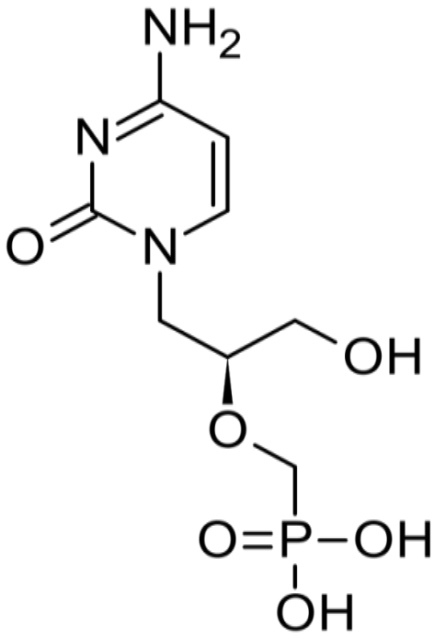


Figure 1.10: Chemical structure of cidovir.

1.2.11.2 Specific inhibitors of HCMV replication

HCMV life cycle is complex and may offers potential targets for novel antiviral agents. Our understanding of the biology of virus opens the door to discover new molecular targets. Here is a summary of compounds that disrupt viral life cycle (illustrated in Figure 1.16):

1.2.11.2.1 Attachment inhibitors

HCMV replication starts when viral glycoproteins bind to host cell surface resulting in membrane fusion and finally penetration of nucleocapsid into cytoplasm (Britt and Mach, 1996). Compounds that inhibit membrane fusion are developed by adding amino acid oligomers that mimic the hepated repeats of HCMV glycoproteins B (English et al., 2005). CFI02 is an example of a small molecule fusion inhibitor that prevents membrane fusion and halts spreading of virus between cells (Jones et al., 2004). Further development of any attachment inhibitor needs clinical trials, as animal models of HCMV infection are insufficient for evaluation studies.

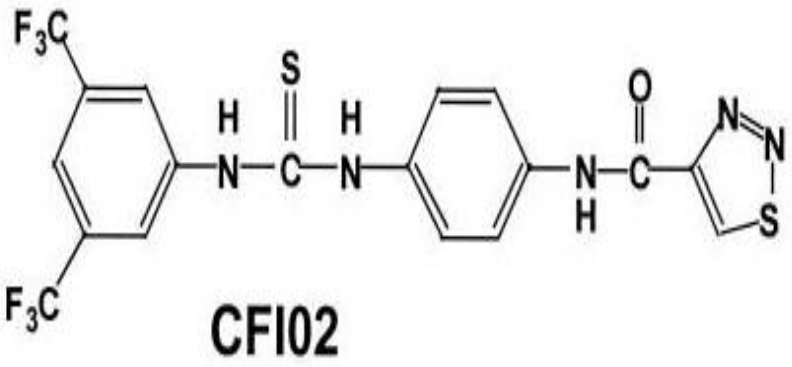


Figure 1.11: Chemical structure of CF102.

1.2.11.2.2 Inhibitors of DNA synthesis

Viral DNA synthesis is the second stage in viral replication cycle, Many active compounds including the approved therapies CDV, GCV and non-nucleoside inhibitors have been described as DNA polymerase inhibitors which is important for DNA synthesis (Wathen, 2002). Also cyclic nucleoside phosphonates compounds have antiviral activity against HCMV (De Clercq and Holý, 2005). Although these analogs exhibit potent antiviral activity against HCMV, some, CDV for example, lack oral bioavailability and has dose limiting toxicity, which may result in severe nephrotoxicity (Safrin et al., 1997; Lalezari et al., 2002).

Synthesis of ether lipid ester prodrugs of CDV, particularly hexadecyloxypropyl CDV (now known as CMX001) is an interesting example to improve CDV bioavailability. CX001 was shown to be 1000- fold potent than CDV against HCMV (Wan et al., 2005).

CMX001 has successfully completed Phase 1 and 2 clinical trials and was well tolerated with low risk of nephrotoxicity (Ciesla et al., 2003; Quenelle et al., 2010). CMX001 is now under evaluation in phase 3 clinical trials for treatment of HCMV infection.

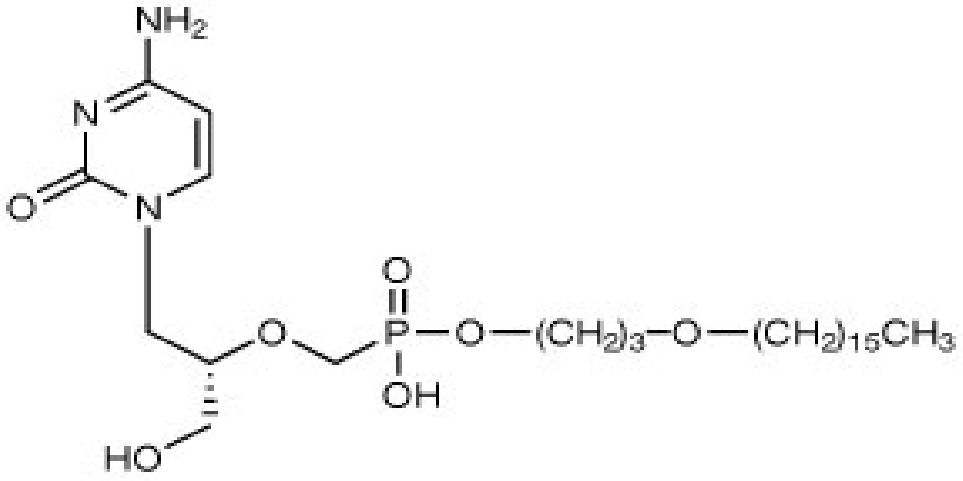


Figure 1.12: Chemical structure of CMX001

1.2.11.2.3 Cleavage/packaging of viral DNA inhibitors

Viral DNA accumulates during HCMV replication and is packaged in capsid inside the nucleus. Terminase subunits encoded by UL89 and UL56 play a key role, together with viral portal protein encoded by UL104 in cleavage/packaging of HCMV genomes (Bogner, 2002). This crucial stage of HCMV replication cycle presents new targets for antiviral treatment. Here are some examples of these agents that inhibit cleavage/packaging of viral DNA:

Benzimidazle analogs were considered the first agents that prevent the cleavage/packaging of viral DNA and have the ability to eliminate formation of monomer viral genomes in infected cells (Underwood et al., 1998). 1H- -d-ribofuranosyl-2-bromo-5,6-dichlorobenzimidazole (BDCRB) is an good inhibitor of HCMV replication both in vitro (Townsend et al., 1995) and in vivo (Kern et al., 2004). This molecule does not need phosphorylation for its activity (Krosky et al., 2002), however it is highly degradable and therefore not subjected to further development (Lorenzi et al., 2006). A rather more stable

analog is GW275175X, which has a mechanism of action similar to BDCRB (Underwood et al., 2004).

BAY 38-4766 was also characterized to inhibit the packaging of HCMV genome (Reefschlaeger, et al., 2001). BAY 38-4766 was shown to be more potent than BDCRB well tolerated in animals with good pharmacokinetic parameters (McSharry et al., 2001).

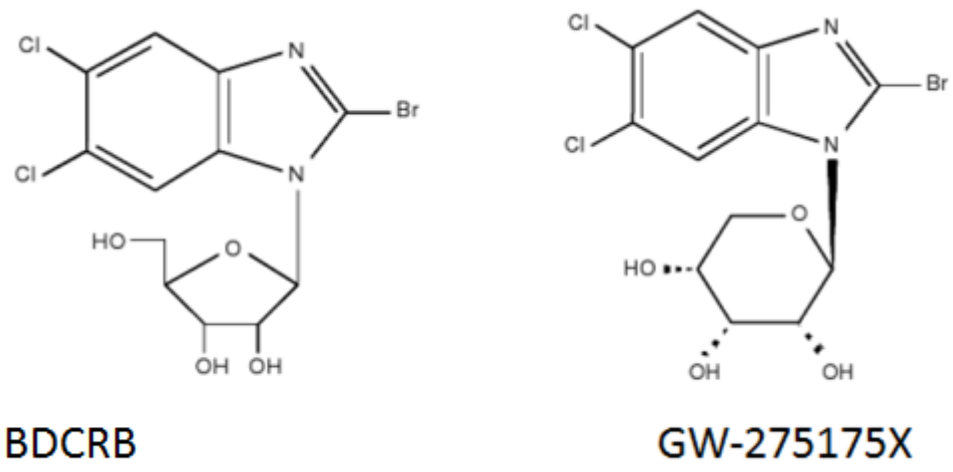
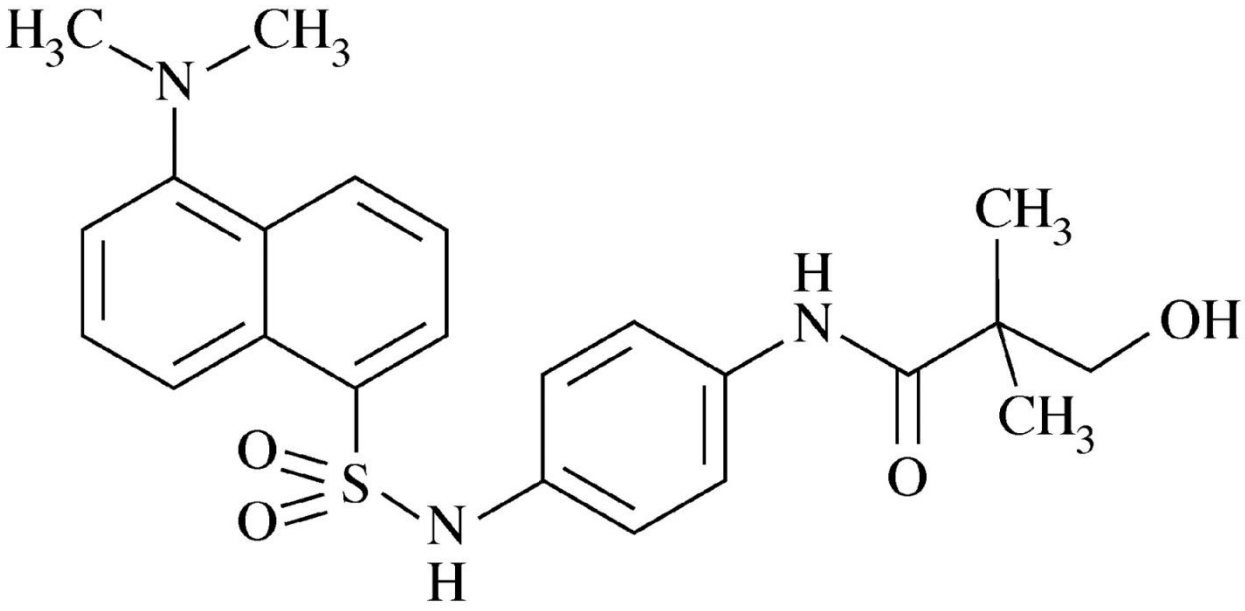


Figure 1.13: Chemical structure of BDCRB and GW-275175X.



BAY 38-4766

Figure 1.14: Chemical structure of BAY 38-4766.

1.2.11.2.4 Protein Kinase Inhibitors

Protein kinases play important role in all HCMV replication stages and this initiated a new field of antiviral targets. The similarity of function between HCMV pUL97 and cellular kinases especially CDKs stimulated many researchers to identify the compounds that can inhibit cellular or viral kinases or both.

pUL97 plays a critical function in HCMV replication and is considered as a promising drug target (Prichard et al., 1999). The deletion of UL97 from HCMV genome reduced viral replication efficiency by 100-1000 fold and strongly decreased viral DNA synthesis (Prichard et al., 1999; Wolf et al., 2001; Marschall et al., 2003; Marschall et al., 2005). Agents that inhibit pUL 97 kinase showed a potent antiviral activities (Wolf et al., 2001; Biron et al., 2002; Marschall et al., 2002; Herget et al., 2004; Marschall et al., 2005; Schleiss et al., 2008).

Despite promising results with pUL97 inhibitors, the efficacy of most of these agents in vivo has been limited so far. Maribavir (MBV) is a highly specific inhibitor of UL97

kinase (Biron, 2006) and its antiviral activity is related to blocking the nuclear egress during HCMV replication cycle (Krosky et al., 2003). MBV showed good pharmacokinetic properties and was considered a promising drug for treatment of HCMV infections (Koszalka et al.,2002; Lalezari et al., 2002; Ma et al., 2006).

Maribavir has undergone phase I and II clinical trials and phase III prophylaxis trials in solid organ and stem cell transplant recipients (Lu and Sun, 2004; Winston et al., 2008). However, it failed in Phase 3 clinical trials, as it did not achieve the clinical end-point goals (Prichard and Kern, 2011). Recently some studies had shown possible clinical profit at higher doses of MBV (Drew et al., 2006) . Therefore, an ongoing phase II dose-ranging trials are examining higher doses of MBV treatment of refractory or resistant HCMV disease (clinicaltrials.gov NCT01611974) and as prophylactic therapy (EudraCT: 2010-024247-32)

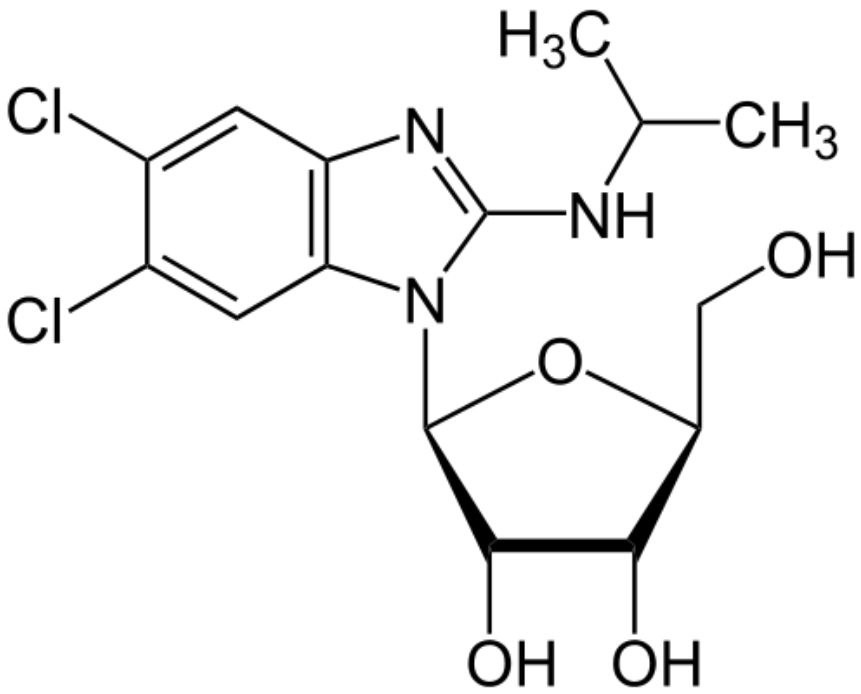


Figure 1.15: Chemical structure of maribavir.

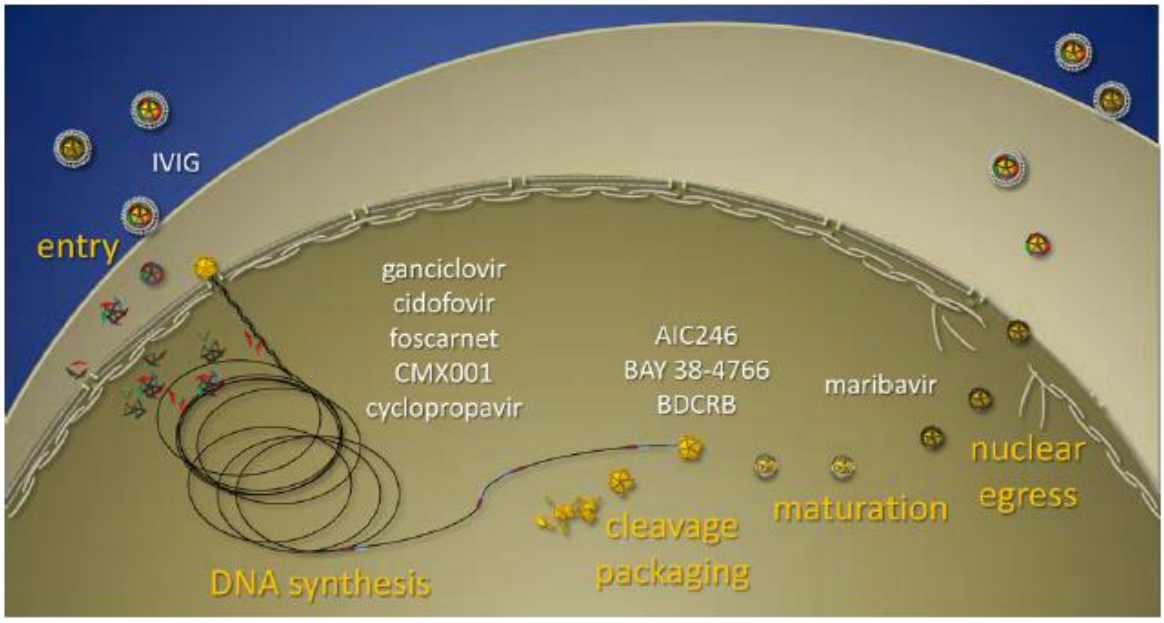


Figure 1.16: Summary of compounds that inhibit various stages of HCMV replication. Infection is began by virions binding to receptors at the cell surface, which can be stopped through the inactivation of virions by purified immunoglobulins (IVIG) or other attachment inhibitors. Several nucleoside inhibitors are capable of inhibiting the subsequent synthesis of viral DNA, including the approved therapies. The coordinated steps of genome cleavage/packaging are inhibited by three separate classes of inhibitors and prevent the stable encapsidation of nascent viral DNA. Inhibitors of the viral UL97 kinase, such as maribavir, impact both early and late events in viral replication and inhibit the egress of mature capsids into the cytoplasm (Prichard et al., 2011).

1.2.12 Commercial kinase inhibitors of interest in this research

1.2.12.1 Luteolin

Luteolin [2-(3, 4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone] (Figure 3) is a flavonoid, found in various fruits and vegetables (Miean and Mohamed, 2001). Flavonoids are polyphenols that play an important role in protecting plant cells against microorganisms, insects, and UV irradiation (Harborne and Williams, 2000).

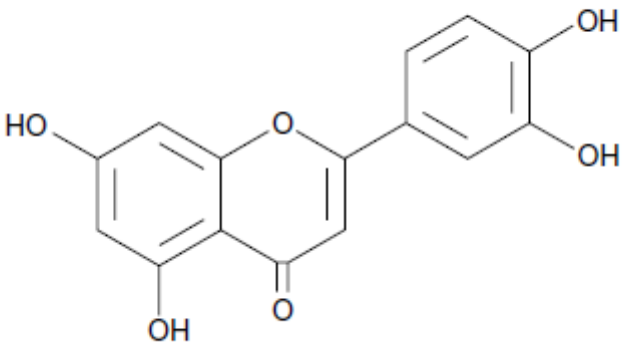


Figure 1.17: Chemical structure of Luteolin.

Flavonoids are broad kinase inhibitors (Kim et al., 2011). Luteolin, like flavonoids, is associated with inhibition of kinases, as many studies have demonstrated that luteolin has anti-proliferative (Huang et al., 1999), anti-metastatic (Huang et al., 1999; Lee et al., 2006), anti-oxidative (Manju et al., 2005), anti-angiogenic (Bagli et al., 2004), and anti-inflammatory (Ueda et al., 2002) effects. Luteolin induces apoptosis of many types of cancer cells and was also shown to inhibit CDK2, protein tyrosine kinase (PTK) (Lee et al., 2001) PKC activities (Shi et al., 2005). Recent studies elucidated that luteolin inhibits serine/threonine kinases such as tumor progression locus 2 serine/threonine kinase (TPL2), required for TNF-induced COX-2 expression (Kim et al., 2011) and p90 ribosomal S6 kinases (RSK), particularly RSK1 and RSK2, which are associated with breast cancer (Reipas et al., 2013).

1.2.12.2 Midostaurin (PKC-412)

Midostaurin is another wide range protein kinase inhibitor. It is a derivative of the alkaloid staurosporine (Meyer et al., 1989), which inhibits several types of kinases such as PKC isoforms (α, β, γ) and c-kit tyrosine kinase and FTL3 tyrosine kinase (Williams et al., 2003). Previous studies at the Virology Research Laboratory showed that staurosporine affected the cytoplasmic assembly compartment (AC) and reduced viral titer (Qawasmi et al., 2011).

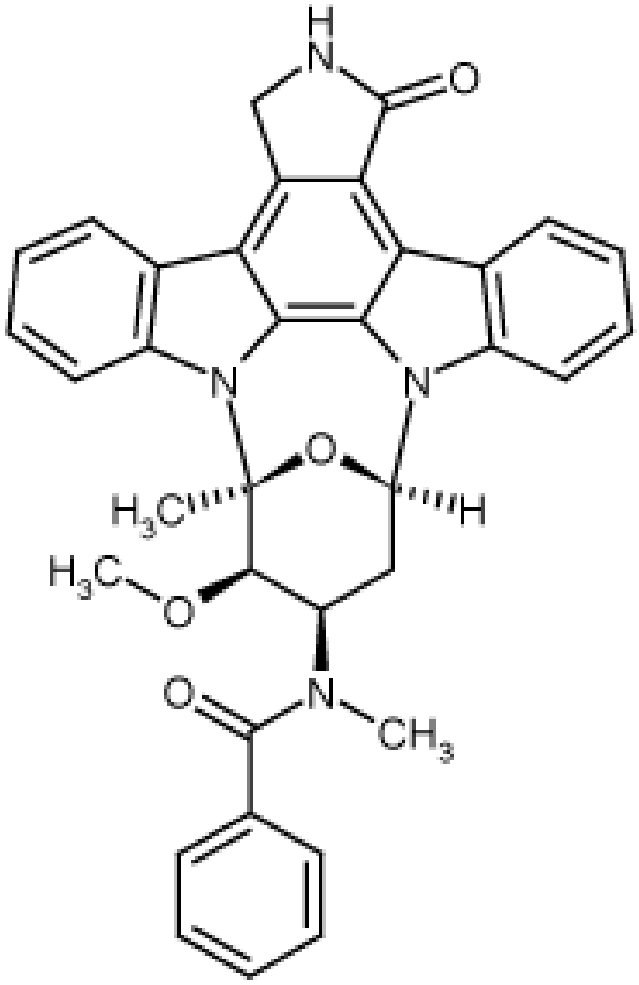


Figure 1.18: Chemical structure of Midostaurin.

1.2.12.3 PD173074

PD173074 is a pyrido-[2,3-d] pyrimidine, which is a tyrosine kinase inhibitor (TKI) that selectively inhibits fibroblast growth factor receptor (FGFR). PD173074 is also found to suppress tumor growth in a wide variety of cancers through its interaction with FGF receptors (Miyake et al., 2010). On the other hand, it is a weak inhibitor of platelet derived growth factor receptors (PDGFR) and c-Src, however, it has no effect on PKC and epidermal growth factor receptor (EGFR) (Mohammadi et al., 1998). FGFRs are cell surface receptors with intrinsic tyrosine kinase activity, which has a role cell signaling (Hamby et al., 1997; Mohammadi et al., 1998).

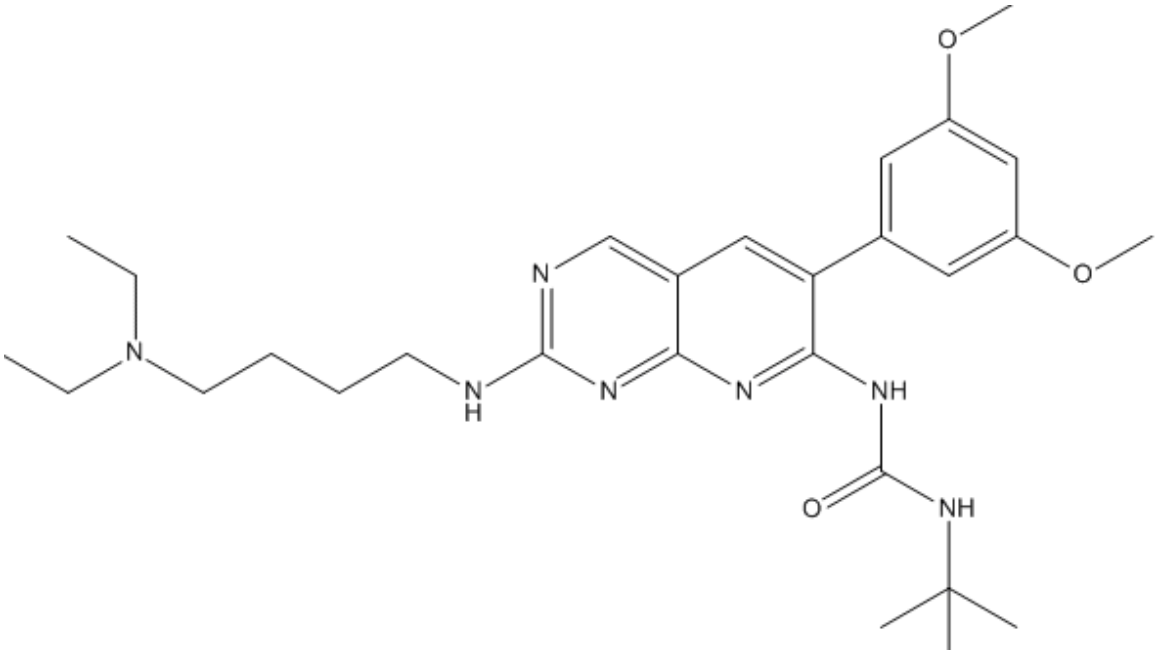


Figure 1.19: Chemical structure of PD173074.

1.2.13 Research significance

The role of protein kinases involved in the regulation of herpesviruses' replication has been proposed to be novel target for new drugs (Herget and Marschall, 2006). There is a need for new antiviral drugs and kinase inhibitors seem to have high potential.

As described above, HCMV pUL97 kinase is involved in phosphorylation of proteins essential for viral life cycle. These kinase activities are also supported by the cellular phosphorylation machinery and different cellular kinases are involved in phosphorylation events of viral and cellular proteins and the deletion of UL97 gene is not crucial for viral survival, therefore, we propose that variable cellular kinases are involved in compensating the activities of the pUL97, which allows the persistence of HCMV infection.

The current use of protein kinases inhibitors especially in cancer therapy (Cohen, 2002; Cruzalegui, 2010) i.e. Gleevac (tyrosine kinase inhibitor used against chronic myelogenus leukemia (CML) is therefore considered a field of interest in treatment of HCMV researches (Andrei et al., 2009; Chou, 2008; Lischka and Zimmermann, 2008; Marschall and Stamminger, 2009). Protein kinase inhibitors can be directed to cellular kinases that

have crucial role in HCMV replication (Filippakis et al., 2010; Schang, 2006; Shugar, 2010).

So far, the role of CDKs in HCMV life cycle was investigated and that of few other kinases, in this work, we aim to explore further cellular kinase inhibitors, which were not yet related to any inhibition activities in HCMV life cycle itself. Some of the inhibitors indicated above were shown to have anti-viral activities against other herpesviruses like Luteolin (Behbahani et al., 2013). On the other hand Luteolin has a serine/threonine inhibition activity (Kim et al., 2011; Reipas et al., 2013) raising a high possibility of inhibition activity against HCMV. Although the other kinases inhibitors mentioned above were not studied for anti-herpes viral activities yet, we believe this is worth our investigations. PKC-412 is a derivate of staurosporine, which was found to inhibit assembly steps in HCMV life cycle (Qawasmi et al., 2011). PD173074 was shown to selectively inhibit FGFR, as HCMV is shown to grow on fibroblasts in vitro and in vivo, we propose that this inhibitor may have an effect on HCMV life cycle. Finally PKC412 is a PKC inhibitor with wide range inhibition activity. Different PKC inhibitors were shown to have anti-HCMV activity, i.e. NGIC-I (Azzeh et al., 2006). We propose that PKC412 may evolve different inhibition activities on HCMV.

1.2.14 Objectives

The overall objective of this work is to study the activity of each kinase inhibitor on HCMV life cycle and this will be accomplished in the following order:

1. To estimate the IC50 for HCMV inhibition *in vitro* of each inhibitor
2. To study the effect of each inhibitor on viral entry and viral exit using plaque titration and viral load assays
3. To delineate the role of each inhibitor on viral life cycle by studying main steps of HCMV life cycle using Immunofluorescence techniques.

2. Materials and Methods

2. Materials and Methods

2.1 Cell culture

All cell culture work was performed in laminar flow purifier safety cabinet (purifier class II biosafety cabinet, Labconco, USA).

Human foreskin fibroblasts (HFF) is a primary cell line (typically have a limited life span) isolated from new born male's foreskin. HFF cells were cultured in complete DMEM medium either in 75 cm^2 cell culture flask or 24 well plates (Nunc, Denmark). Complete DMEM medium contained DMEM (01-055-1A, Beit Haemek), 1:1000 dilution of Pen/Strep (03-031-5C, Penicillin: 100,000 units/ml, Streptomycin: 100 mg/ml, Beit Haemek), 20 mM L-Glutamine (03-020-1A, Beit Haemek) and 10% heat inactivated serum (1:1 mixture of New born bovine serum, 04-121-1A and Fetal bovine serum, 04-122-1A, Beit Haemek). Cells were cultivated in 96% humid CO_2 incubator (5% CO_2) at 37°C (Hera cell incubator).

2.2 Propagation and Passage of HFF Cells

Cells were propagated by splitting into two or more culture flasks, or into different well plates. Cells adhere to the plastic flask and were detached by adding 0.25% Trypsin-EDTA (03-052-1A, Beit Haemek) to the confluent cells for 2 minutes. Flasks were then tapped gently with the hand and finally detached by up and down pipetting using sterile disposable plastic pipette. Complete medium was added to the non-adhering trypsinized cells, gently mixed and distributed to the new flasks. These steps are visualized in Figure 2.1. The new cells are one passage higher than the one they were splited from. Since HFF is a primary cell line, it was propagated up to passage 18. Experiments for this work were performed on passages 10-15.

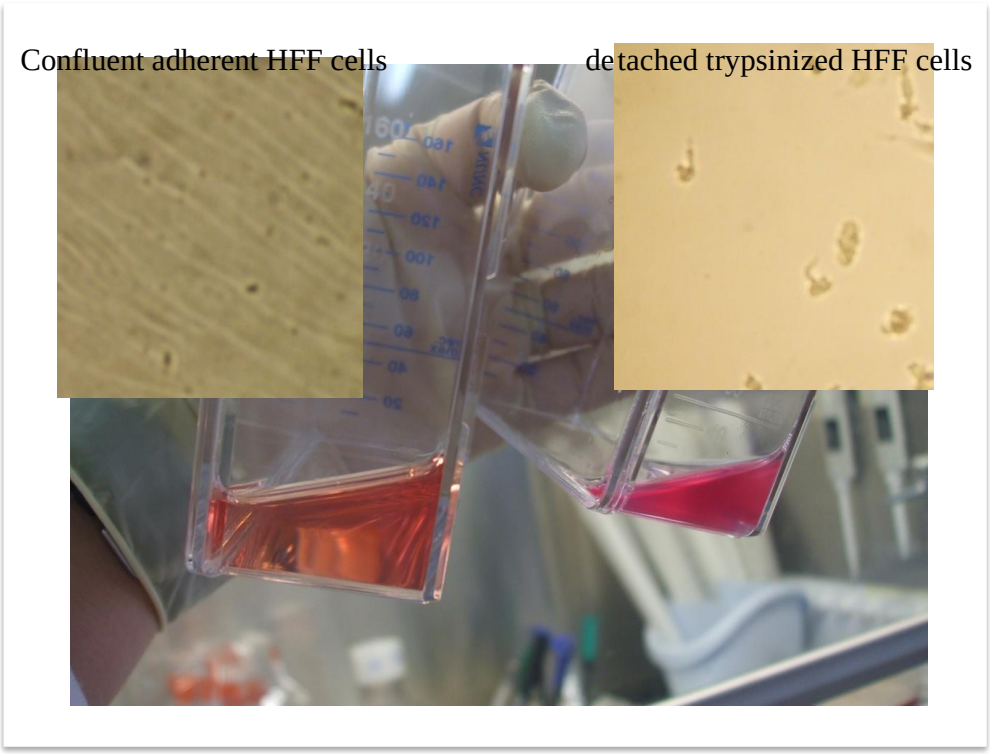


Figure 2.1: Confluent and trypsinized HFF cells. A flask containing confluent cells are shown on the left, with a microscopic photo taken of those confluent HFF cells at 10X magnification. The flask on the right contains trypsinized HFF cells and a microscopic picture visualizing detached cell is shown above. This photo is a property of the Virology laboratory and Dr. Maysa Azzeh.

2.3 Freezing and thawing of HFF Cells

HFF cells can be maintained for long time in liquid nitrogen. HFF cells were frozen according to the following steps:

For a medium flask, 75 cm^2

1- The medium was removed.
2- 3 ml 0.25% of Trypsin was added.
3- Detached cells were pelleted in15 ml tube by centrifuging at 1500 rpm for 10 min.
4- The supernatant was discarded carefully.

5- Pellet was gently re-suspended in 3ml freezing medium (50% complete medium, 40% serum, 10% DMSO, dimethyl sulphoxide, D2650, Sigma-Aldrich, Germany)

6- Re-suspended cells were transferred into pre-cooled (on ice) cryotube (cat#363401, Nunc, Denmark)

7- Cells in cryotubes were first frozen at -70^{o}C and transferred few hours later to liquid nitrogen.

For thawing, cells in cryotubes were taken out of the liquid nitrogen, transferred to the laminar flow, where the screw cover of the cryotube was gently opened to reduce the pressure in the tube and then closed. The tube was warmed shortly in the palm of the hand and cells were finally transferred to a 25 cm^2 culture flask containing 4 ml complete media. The next morning, medium was aspirated to remove DMSO residuals and replaced with fresh complete DMEM medium. Cells are usually ready to split and passage 48-72 hours after thawing.

2.4 Viruses and viral stocks

In this work HCMV AD169 strain (wt-HCMV; American Type Culture Collection) was used, which was kindly provided by Prof. Bodo Plachter, University of Mainz-Germany. All experimental procedures with viruses were performed in laminar flow purifier safety cabinet (purifier class II biosafety cabinet, Labconco, USA).

2.5 Propagation of Viruses

Viruses were propagated in HFF cells. HFF cells were infected with AD169 at low multiplicity of infection (m.o.i). M.o.i is the number of infectious particles per cell. In this work viruses were propagated in 175 cm^2 flasks (contain 3x10^7 HFF cells) as followed:

1- 4 ml viral suspension (virus stock diluted in complete medium) at m.o.i of 0.001 were added to the cells and incubated in 96% humid CO2 incubator (5% CO2) at 37 °C.

2- Viral suspension was removed 2 h later, which is 0 hour post infection (h p.i.) and 30 ml complete DMEM medium was added.

3- 10 days later (10 days p.i.), infected cells were checked for the presence of cytopathogenic effect (see Figure 2.2) caused by the viral infection resulting in massive cell destruction and lyses. If the cytopathogenic effect is clear, viruses were harvested by collecting the 30 ml supernatant in a sterile 50 ml tube and replaced by fresh 30 ml complete medium for further incubation to do a second viral harvest.

4- The 50 ml tubes containing viral harvest were contain cell debris as well, which were pelleted by centrifugation for 10 min at 1500 rpm to allow transfer of the supernatant to a new 50 ml tube.

5- 1% DMSO was added to the viral supernatant and the mixture was aliquoted in cryotubes, and frozen at -70 ^{o}C and in Liquid nitrogen.

6- 14 days post infection (d p.i), the second viral harvest is performed by repeating steps 3-5. The highly infected cells were discarded by autoclaving.

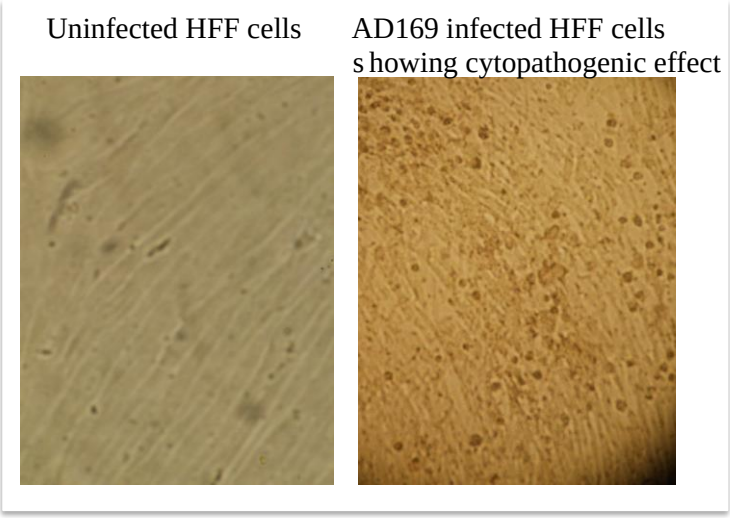


Figure 2.2: Uninfected (mock infected) HFF cells versus infected HFF cells. Mock infected confluent HFF cells are shown on the left, while AD169 infected HFF cells showing cytopathogenic effect are shown on the right. This photo is a property of the Virology laboratory and Dr. Maysa Azzeh.

2.6 Viral Titration and Plaque Assay

Determination of the viral titer is one of the most important procedures in virology, which is performed using plaque assay technique. To perform a plaque assay, an aliquot of frozen viral stock was thawed and viral dilutions of 10^{-1}-10^{-8} (titrations) were prepared step wise (1:10 dilution steps only) in complete medium. 0.1 ml of either the undiluted viral stock, or one of the 10^{-1}-10^{-8} viral dilutions were inoculated onto cultured HF cells in 24 well plates (Nunc, Denmark). Four wells (one column) of the 24 well plates were infected with the same viral dilution to guarantee 4 controls of each dilution. Infected 24 well plates were incubated in 96% humid CO2 incubator (5% CO2) at 37°C for 2h to allow viral absorption to the cells.

2 h after infection (=0 h p.i.), virus-containing media was discarded and replaced with DMEM complete medium containing agarose as followed:

1- 2.5% agarose was dissolved in dH_2O (distilled water) and cooled down to 50°C.
2- Complete medium was also pre-warmed up to 50°C.
3- A 0.25% agarose was prepared in the pre-warmed complete medium.
4- Virus-containing media was removed one column in row and replaced with 1 ml 0.25% agarose containing complete medium. This procedure has to be done fast to avoid pre-polymerization of the agarose.
5- Plates were left at room temperature (RT) for 30 min to allow agarose polymerization. Agarose polymerization at this step is the clue, as it hampers viral spread and allows the formation of plaques.
6- After polymerization, plates were transferred to 96% humid 5% CO_2 cell culture incubator, at 37 °C.
7- One week later (one week p.i.), another 1 ml of agarose-containing medium was added as described above.
8- At 2 weeks p.i., viral plaques were counted in those wells with those viral dilutions, which allowed the formation of countable separate clear plaques. To minimize error, only plates containing between 1-20 plaques were counted. An example of plaques is shown in Figure 2.3.

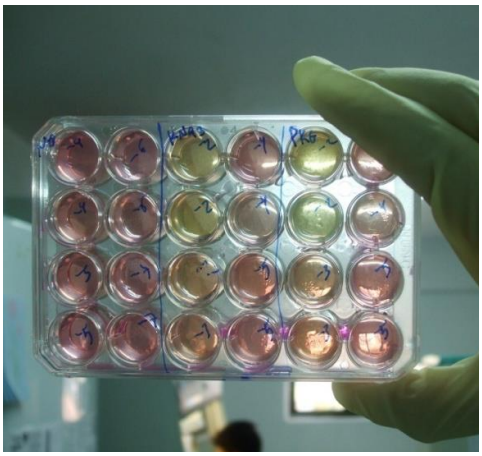
The titer of a virus stock can be calculated in plaque-forming units (PFU) per milliliter

#of plaques / d x V = pfu/ml

d: dilution factor

V: volume of diluted virus added to the well

24 well plate



A plaque

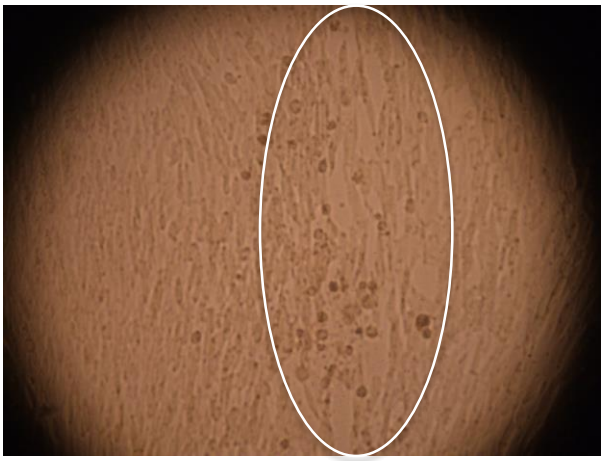


Figure 2.3: A plaque in HFF cells infected with AD169. The 24 well plates used for plaques assay is on the left, while an example of a plaque is shown on the right, the entire circled area resembles one single plaque. This photo is a property of the Virology laboratory and Dr. Maysa Azzeh.

2.7 Viral infection assay

The number of HFF cells in a flask or 24-well plate is known, while the viral titer is determined as described above in 2.6. In this work, HFF cells were always infected with AD169 at m.o.i 0.5, which mean one single viral particle was used per two HFF cells. The suitable amount of virus was always diluted in complete medium to give the desired m.o.i. Complete medium was warmed up to room temperature, while virus stock was used as soon as it was thawed. The virus-containing medium was always prepared directly before infecting cells.

For infection, DMEM medium was replaced with virus-containing medium at the desired m.o.i. and incubated for 2 h. The 2 h infection is the time needed for the viral particles to be attached and absorbed by the cells. After these two hours, virus-containing medium is removed and replaced with wither fresh DMEM medium or drug containing medium, based on the experimental set up and this step is the 0 h p.i. step. The hours post infection (h p.i.) depended on the experimental set up too, 96 h p.i. was used in most experiments. In mock infections, no virus was added at any time point of the experiment.

2.8 Plaque reduction assay (IC50)

Plaque reduction assay determines viral efficiency in forming plaques in the presence of different concentrations of antiviral agent. Drug activity is expressed by its IC50, which represents the compound concentration required to reduce virus plaque formation by 50 %.

Plaque reduction assay was performed as followed:

1- HFF cells were propagated in 25 cm^2 culture flask (Nunc, Denmark).
2- Confluent HFF cells were infected at m.o.i of 0.5 and incubated in 96% humid CO2 incubator (5% CO2) at 37 °C.
3- After 96 h pi, the supernatant was discarded and the cells were washed with DMEM free from bovine serum.
4- Infected HFF cells were harvested using trypsin (see 2.2 above) and DMEM was added in equal amount to trypsin.
5- The infected HFF cells were counted using hemocytometor.

6- 0.5 ml DMEM medium containing 50-70 infected HFF cells were inoculated in 24-well plates containing confluent HFF cells and incubated in 96% humid CO2 incubator (5% CO2) at 37 °C for 2h.
7- After 2h, at 0 h p.i., DMEM containing infected cells was removed and cells in 24-well plate were washed with fresh DMEM medium. Finally, serial dilutions of the compound to be tested were dissolved in DMEM and mixed with agarose to replace the washing medium, one row for each dilution.

The following steps simulate the steps 4-6 of viral plaque assay (see 2.6).

IC50 of a drug was the concentration of that drug, which reduced plaque formation by 50% compared to untreated cells (mock treated cells).

2.9 Immunofluorescence (IF)

Immunofluorescence, also immunostaining, experiments proceeded as followed:

1- HFF cells were either propagated on 12-well plate containing glass slides. 18 mm Ø glass slides were sterilized and placed in each well under sterile conditions in the laminar flow before splitting the cells on them.
2- The HFF cells were infected at m.o.i 0.5, once cells were 90% confluent.
3- 2 h after infection, virus-containing medium was removed and replaced with virus free complete medium.
4- At desired h p.i., mostly 96 h p.i., cells were washed three times with 1X PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1000 ml dH_2O, pH 7.4). This PBS was previously filtered with 04 µm filter to avoid tiny dust or any particle from interfering with the IF staining.
5- The cells were fixed with 3.7% paraformaldehyde (diluted in PBS) for 30 min at RT.
6- After three washes with filtered PBS, cells were permeablized by adding 0.1% Triton X-100 (T8787, Sigma-Aldrich, diluted in PBS) for 1-2 min at RT.
7- Cells were then re-washed for five times with PBS and blocked with 1% bovine serum albumin (BSA, 0175, AMRESCO Inc., USA, prepared in PBS) for 60 min at RT.

8- Primary antibody was diluted in 0.5% BSA and incubated with cells for 2 h at RT or over night at 4 ^{o}C. In case of co-localization experiments of IF, the diluted first antibodies of the different antigens were pre-mixed before adding them to the cells. Note: WGA added for 30 min only and handled as a direct immunofluorescence procedure. Hereby step 10 was skipped, as WGA is a conjugated marker. Figure 2.4 illustrates the difference between direct and indirect immunofluorescence.

9- After incubation with the first antibody, cells were washed 5 times with PBS, while the last wash was left on cells for 30 min.

10- The secondary antibody was diluted at a proper concentration in 0.5% BSA and incubated with the cells 30-60 min in the dark by covering the plates with aluminum paper or by placing them in the dark. In case of co-localization experiments of IF, diluted secondary antibodies were pre-mixed before adding them to the cells.

11- Before the last wash, the nucleus was stained with 1µg/ml 4′, 6-diamidino-2-phenylindole (DAPI, 268298, Calbiochem, Germany) for 10 min at RT.

12- Cells were washed 5 times with PBS.

13- Mounting anti-fading solution (0.5% g n-porpyl gallate; P3130, Sigma-Aldrich, Germany; 100mM Tris pH 9, 70% glycerol), (Giloh and Sedat, 1982) was prepared. 20 µl mounting solution were dropped on an objective slide, pre-cleaned with 70% alcohol on kim wipes, and glasses were taken out of the 12-well plate and placed with cells' face onto the mounting solution drop. The slides were covered with kim wipes and gently pressed with the hand palm to press out the excessive mounting solution. Finally, the cover slips were glued to the slides using regular nail polish.

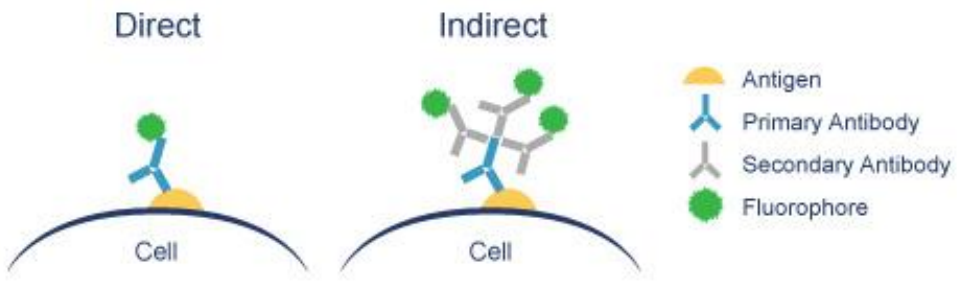


Figure 2.4: Illustration of direct and indirect immunofluorescence (abcam.com). In direct immunofluorescence the primary antibody is conjugated with the fluorophore (left picture), which visualized the target molecule. In indirect immunofluorescence, primary antibody is not conjugated to a fluorophore; the target molecule is visualized using a secondary antibody. The secondary antibody is conjugated to a fluorophore and visualizes the target molecule by binding to the primary antibody.

2.10 Monoclonal, polyclonal antibodies and fluorescing markers

First antibodies:

All HCMV viral antibodies were mouse monoclonal antibodies (mAbs) purchased from Virusys Corporation (MD, USA); pp28 (CA004-100), pp65 (CA003-100), and IE1&IE2 antibody (CH443), and UL84 (CA144). Cellular markers GOLPH4 (ab28049) and TGN46 (ab50595) were purchased from Abcam (Cambridge, UK). Cellular markers were rabbit antibodies, so that colocalization IF experiments with viral anti-mouse antibodies can be performed

Secondary antibodies:

DyLight^{TM}488-conjugated anti-Mouse (715-485-150, Jackson ImmunoResearch, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was used to visualize all viral antibodies, while DyLight^{TM}594-conjugated Anti-Rabbit (111-515-144, Jackson ImmunoResearch, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was used to visualize cellular rabbit antibodies. All secondary antibodies are highly cross-absorbed (they bind specifically to the target, mouse or rabbit). DyLight 488 fluoresces

green, while DyLight 594 fluoresces red and do not cross react, as the 594nm emission is far away from 488nm.

Fluorescing markers:

Golgi marker Wheat germ agglutinin (WGA, L4895 coupled to fluorescein isothiocyanate FITC; green) was obtained from Sigma (St Louis, Missouri, USA).).

DAPI (4′, 6-diamidino-2-phenylindole (268298, Calbiochem, Germany) binds to the DNA in the nucleus and fluoresces blue.

2.11 Immunofluorescence Image captures

An Olympus BX60 and Olympus digital camera DP71 (Olympus, Japan) were used to visualize immunofluorescence staining and capture it. DAPI staining of the nucleus was seen via U-MWU mirror, Cy2 via U-MWIB and Cy5 via U-MWIY2 (all Olympus mirrors, Olympus, Japan) respectively. Image of each fluorescence color was captured in single mode using analysis LS report program (Olympus, Japan).

The same image was captured via the different mirrors in single mode and merged via Picture Merge Genius program (EasyTools Software, Inc., USA) in case of co-localization experiments.

2.12 Recording structures of subcellular distribution

When alteration in subcellular distribution or nuclear shape or was detected via IF, a statistical analysis was done to determine whether this change is statistically relevant or not. For this, structures were recorded in 10 fields of at least 100 cells from 3 different experiments. Alternatively, structures were recorded from captured images.

2.13 Kinetic experiments

Kinetic experiments aim to measure the influence of kinase inhibitors on HCMV infection at different h p.i.. Hereby, infection was performed as detailed in 2.7 or mock infected on 12 well plates for IF analysis. Slides of 12-well plates were removed at different time points; in 24 h p.i. intervals (24 h p.i., 48 h p.i., 72 h p.i. and 96 h p.i.).

2.14 DNA extraction

HCMV DNA was isolated from eukaryotic cells and viral supernatant. First viral supernatants or lysed cells were thawed and exposed to total DNA extraction using QIAamp DNA Mini Kit (cat#51304, Qiagen, Germany). In most cases 200 µl of viral supernatant were aliquoted and subjected directly to DNA extraction, while lysed cells were adjusted to 200 µl using PBS. The experiment steps were preceded according to the manufacturer's instruction. . The DNA was eluted in 200 µl elution buffer and was kept at -20 ºC.

2.15 Real-time PCR method

Polymerase chain reaction (PCR) is a method used to detect a target nucleic acid molecule by amplifying a region of that nucleic acid molecule using two specific primer molecules (regularly 20-30 short nucleic acid molecules complementary to the target region's 5' and 3' ends) and a polymerase enzyme to catalyze the amplification reaction. Real-time quantitative PCR is a technique, which is based on PCR, however allows a quantitative detection of the target nucleic acid amplified since the early phases of the reaction by adding a probe to the amplification reaction. The probe is a short nucleic acid molecule, which is specific for the target nucleic acid to be quantified and has a high-energy reporter dye at the 5' end and a low-energy quencher dye at the 3'end. The reporter dye emits fluorescence when excited by a light source. An intact probe does not produce fluorescence activity, as the reporter dye's emission is suppressed by the quencher dye, however when the probe binds to the target nucleic acid sequence, the probe is cleaved by the polymerase enzyme and the reporter dye is set free, allowing for fluorescence emission, which is proportional to the number of amplicons (amplified region of target nucleic acid) generated.

While regular PCR reaction runs in a PCR machine and the reaction result is detected using gel electrophoresis, a real-time PCR reaction runs in a real-time PCR machine, which captures and analyses the reporter's fluorescence emission reflected by the copy number of the target molecule. If a standard with known concentration is used the quantification is expressed in DNA/RNA copy number/ml. In our experiments, we used comparative $\Delta\Delta C_T$ analysis, which is based on the difference of the cycles ($\Delta\Delta C_T$) needed to amplify the target DNA/RNA reflecting the differences in the copy number of the target DNA/RNA between different samples.

Viral load analysis is a method by which the number or relative number of DNA copies can be detected using real time PCR technique. To detect relative number of DNA copies, no standard is used, but the DNA copies are indirectly expressed in number of cycles needed to amplify detectable amounts of that DNA. In other words, the C_T data is used to determine the amount of each gene/mRNA present relative to each sample.

Viral supernatant were thawed and total DNA was extracted from 200 µl (see 2.14). Real-time PCR was performed using on ABI Real Time PCR 7500 system (Applied Biosystems, USA). All test samples were tested in duplicate manner. A total of 20 µl reaction mixture consisted of 5µl ultra pure water (negative control) or sample's DNA, 10 µl TaqMan universal master mix (4304437, Applied Biosystems, United Kingdom), 1 µl of each primer (forward and reverse, each at 10 pmol/µl, Metabion, Germany CMV –gb-F: 5'-CTA TCG CGT GTG TTC TAT GGC-3' and CMV –gb-R: 5'-CAG GTG ACA TTC TTC TCG TCC-3'), 1 µl probe (5′(FAM) -TGG GCA ACC ACC GCA CTG AGG – (BHQ) 3′ as probe (modified, Boeckh et al., 2004) and 2 µl ultrapure water.

The forward and reverse primers were chosen at the Virology laboratory using the major glycoprotein DNA of HCMV (NC_006273.2, http://www.ncbi.nlm.nih.gov/nuccore) as a template after downloading it into CloneManager program.

The amplification reaction started with 2 min at 50 °C, followed by 10 min at 95°C and final 45 cycles as following: 95°C for 15s and 60°C for 1 min, and finally 15sec at 60˚C.

3. Results

3. Results

3.1 Determination of IC50 of Luteolin, Midostaurin and PD173074 for AD169

To measure the activity of the selected kinase inhibitors against HCMV, a variation of plaque reduction assay was used as described in (2.8). Hereby AD169 infected cells were added to HFF cells in 24-well plates, at 0h p.i., after virus absorption, compounds dissolved in growth medium and mixed with agarose were added to the wells at four to six selected concentrations in duplicate manner and incubated at 37°C, drug containing agarose medium was re-added after one week. Plaques were checked after 10-14 days and if they were clear and countable they were counted as detailed in materials and methods (2.6). The IC50 activity of the drugs was calculated as the compound concentration required for reducing virus plaque formation by 50 % compared to the number observed in the absence of drug.

The three kinase inhibitors compounds showed inhibition activities against HCMV strain AD169, possessing IC50 concentration of 20 µM, 5 µM, 100 nM for Luteolin, PD173074 and Midostaurin respectively (Table 3.1).

Compound	IC50
Luteolin.	20µM
PD173074	5µM
Midostaurin	100 nM

Table 3.1: IC50 values for Luteolin, Midostaurin and PD173074 as determined using plaque reduction assays. (Row data of IC50 was included in the appendix).

3.2 Influence of Luteolin, Midostaurin, and PD173074 compounds on Golgi

The main target of this study is to identify the role of Luteolin, Midostaurin, and PD173074 kinase inhibitors on HCMV life cycle, part of which is the viral assembly, which colocalizes with the Golgi compartment. For initial results, HCMV infected HFF cells well subjected to drug inhibition assays using each compound at the IC50 concentration and finally stained with the Golgi marker WGA at 96h p.i..

FITC conjugated Wheat germ agglutinin (WGA) served as initial marker for the following experiments as it colocalized to the viral cytoplasmic assembly compartment (Azzeh et al., 2006) and is susceptible to kinase inhibitors (Azzeh et al., 2006; Qawasmi et al. 2011). WGA is a carbohydrate that binds to clustered terminal N-acetylneurarminic acid residues and to N-acetylglucosamine-containing oligosaccharides on proteins and decorates distal Golgi cisternae, the trans-Golgi network and the cell surface (Tartakoff and vassalli, 1983; Virtanen et al., 1980).

Subcellular distribution of WGA in infected mock inhibited cells and infected inhibited (with either Luteolin, Midostaurin, or PD173074) cells is demonstrated in Figure 3.1 A. An important line of control for this experiment was to test the subcellular distribution of WGA in uninfected mock inhibited cells as well as uninfected inhibited (with either Luteolin, Midostaurin, or PD173074) cells (Figure 3.2).

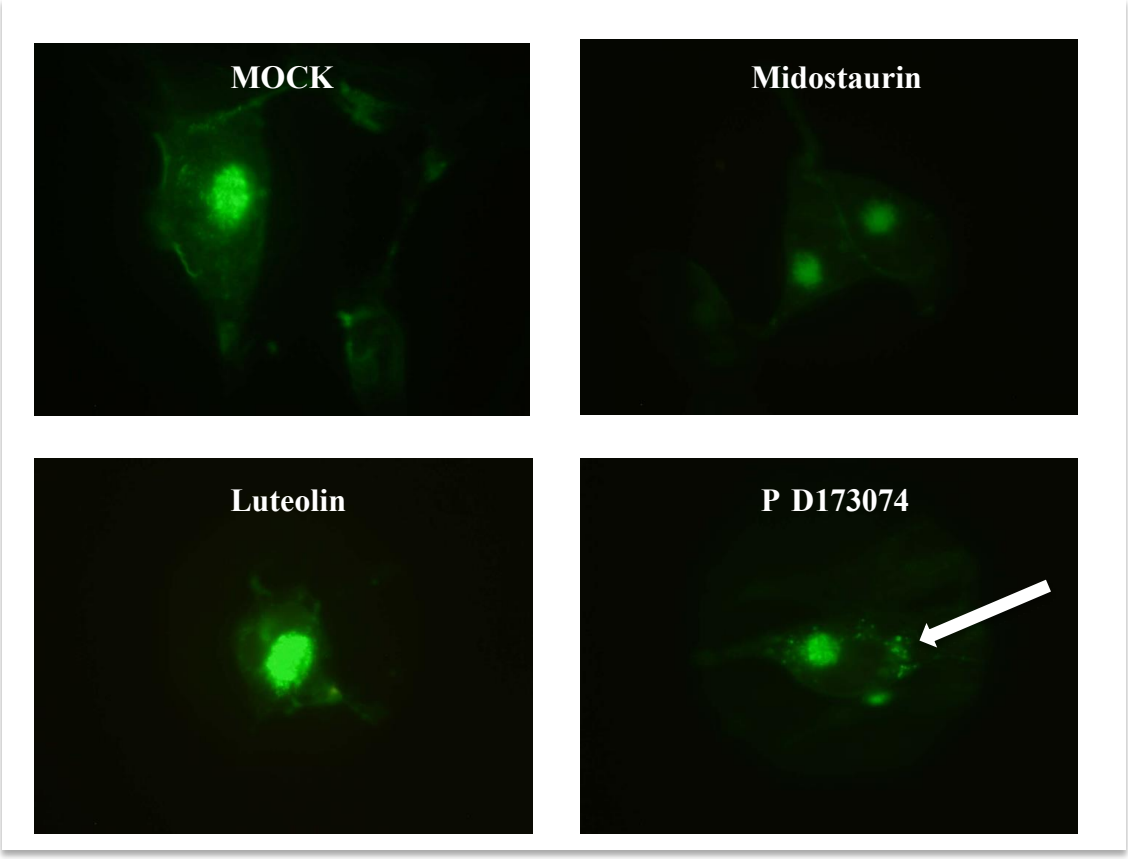


Figure 3.1 A: Subcellular distribution of WGA in AD169 infected cells treated with Midostaurin, Luteolin, and PD173074 kinase inhibitors. HFF cells were infected with AD169 (m.o.i. 0.5) and treated with different cellular kinase inhibitors at 0 h p.i.. At 96 h p.i. cells were stained with FITC conjugated WGA (green). Please see 2.9-2.11 for procedure's details. Inhibitors were used at their IC50 concentrations: Midostaurin (100 nM), Luteolin (20 µM), PD173074 (5 µM). The white arrow indicates the punctuated vesicles in PD173074 treated cells.

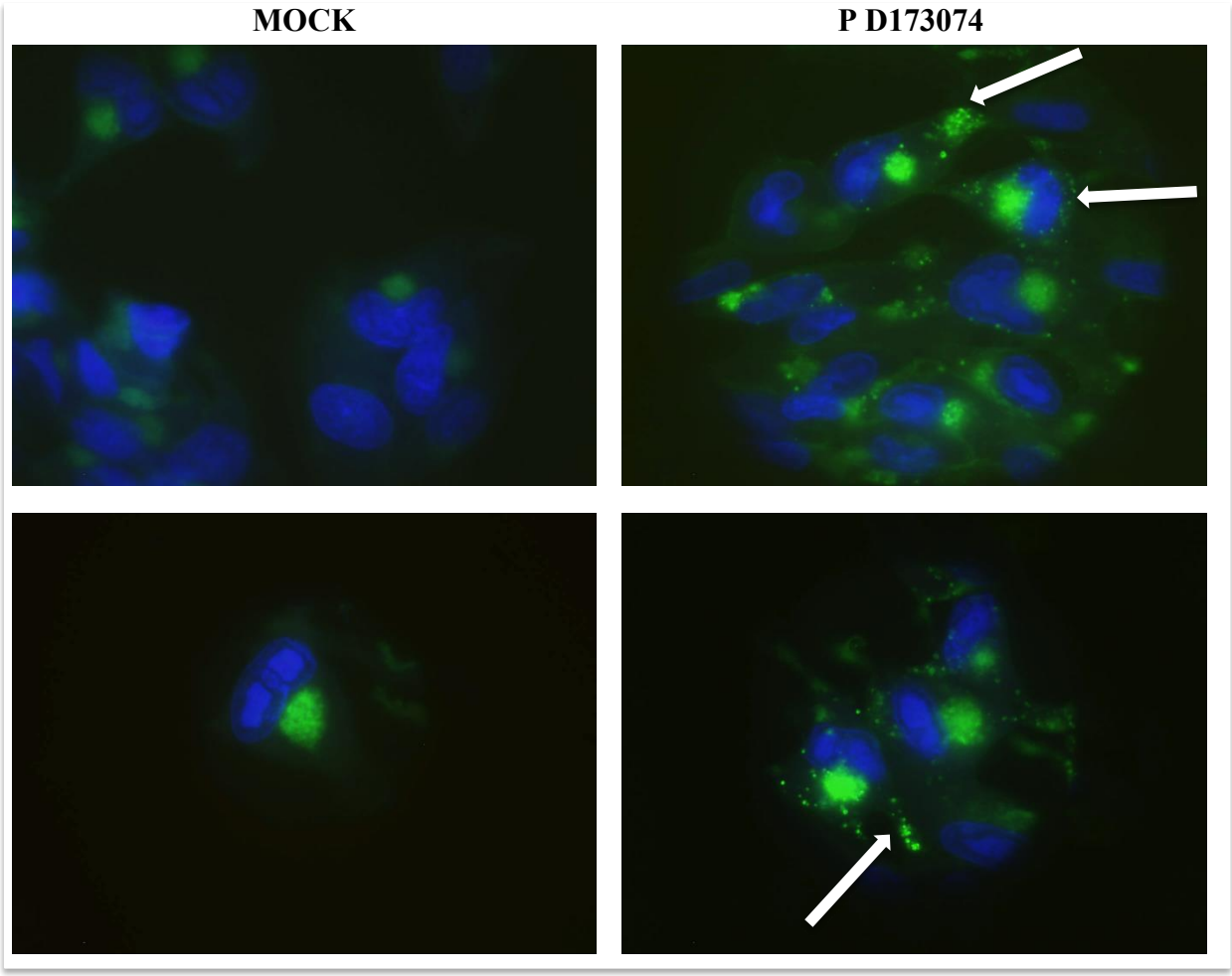


Figure 3.1 B: Subcellular distribution of WGA in AD169 infected cells treated with PD173074 kinase inhibitors. HFF cells were infected with AD169 (m.o.i. 0.5) and treated with 5 µM PD173074 at 0 h p.i.. At 96 h p.i. cells were stained with FITC conjugated WGA (green) and counterstained with DAPI (blue staining of the nucleus). The upper panel is an overview of few cells, while the lower one shows a higher magnification of single cells. The white arrow indicates the punctuated vesicles in PD173074 treated cells.

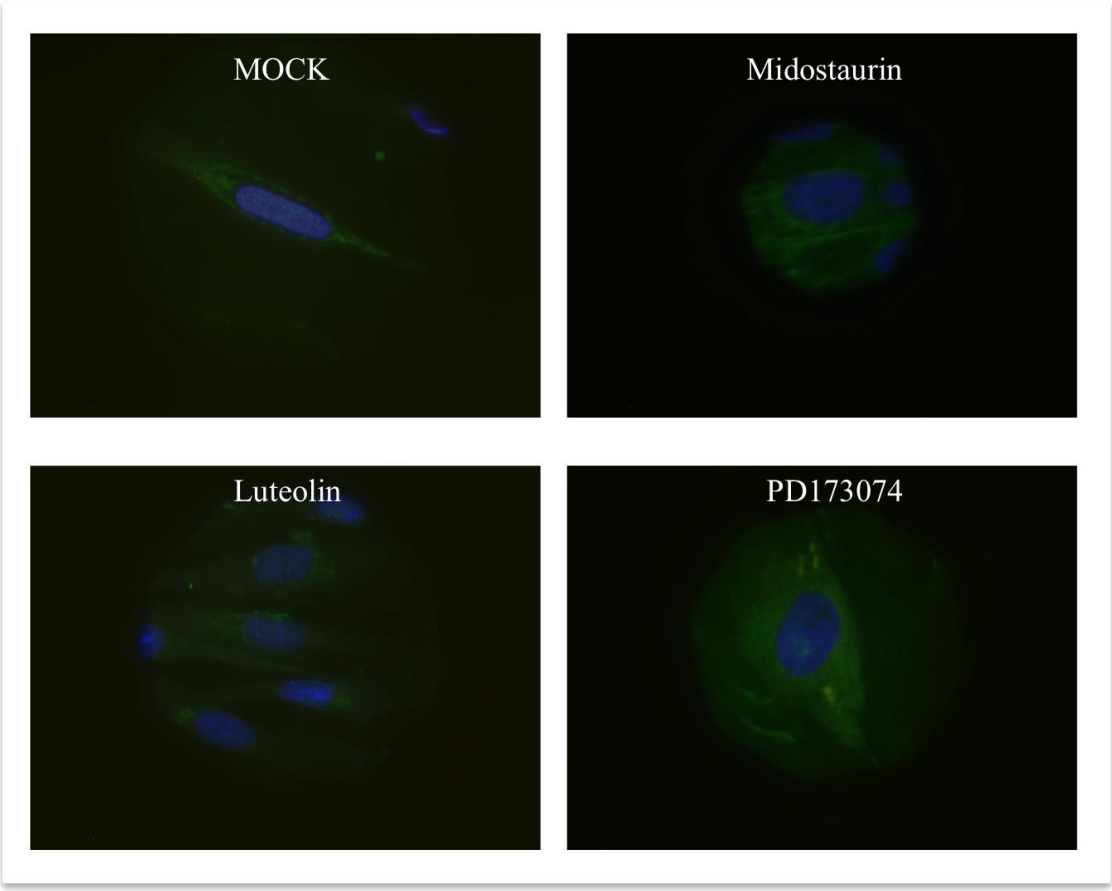


Figure 3.2: Localization of WGA in mock infected cells. HFF cells were either mock treated or treated with the different cellular kinase inhibitors Midostaurin (100 nM), Luteolin (20 µM), and PD173074 (5 µM) for 96h and finally subjected to staining with FITC conjugated WGA (green) and counterstained with DAPI (blue staining of the nucleus).

WGA has a thread like structure surrounding the nucleus in uninfected HFF cells, however it assumes the viral cytoplasmic assembly compartment structure in infected cells, which is the compact "bulb"-like structure (Azzeh et al., 2006). The results presented in Figures 3.1 A and 2 demonstrate that Luteolin had no influence on the subcellular distribution of WGA, while Midostaurin induced a slight distraction of Golgi. Interestingly, PD173074 clearly modified the subcellular distribution of WGA indicating a role in maintaining the integrity of viral cytoplasmic assembly compartment (AC). To verify the structural changes in WGA induced by PD173074, further IF experiments were performed. As clearly accentuated in Figure 3.1 B, remarkably punctuated vesicles distracted from WGA

complex were induced by PD173074 activity and such vesicles were not observed in mock treated cells. These vesicles were not seen in PD173074 treated uninfected cells.

3.3 Only PD173074 inhibition affected AD169 $\Delta\Delta C_T$

PD173074, Luteolin and Midostaurin kinase inhibition activity is not restricted to structural changes in infected cells, therefore it is essential to detect the influence of these compounds on the AD169 comparative $\Delta\Delta C_T$ (see 2.15). AD169 infected cells were subjected to drug inhibition assay using PD173074, Luteolin, or Midostaurin at their IC50 concentrations. At 96 h p.i., cells as well as supernatant were subjected to DNA extraction and comparative $\Delta\Delta C_T$ analysis using real time PCR technique. Detecting comparative $\Delta\Delta C_T$ in supernatant refers to difference in copies of the virus released from the cell, while detecting comparative $\Delta\Delta C_T$ in the cells refers to difference in copies of the yet immature unreleased viruses. The analysis was done in duplicate manner for each concentration and experimental set up and confirmed at least by three independent experiments.

The summary of different experiment is indicated in Figure 3.3 A and B. With the exception of PD173074, endogenous (cells) $\Delta\Delta C_T$ was not affected by the either drug used if compared to mock inhibited infected cells, which means that viral assembly took place successfully and that there are enough immature viruses about to bud (get released) out of the cell. $\Delta\Delta C_T$ in supernatant was not affected by Midostaurin treated infected cells compared to mock treated cells, however it was reduced around 1 cycle in Luteolin and around 3 cycles in PD173074 treated infected cells, respectively.

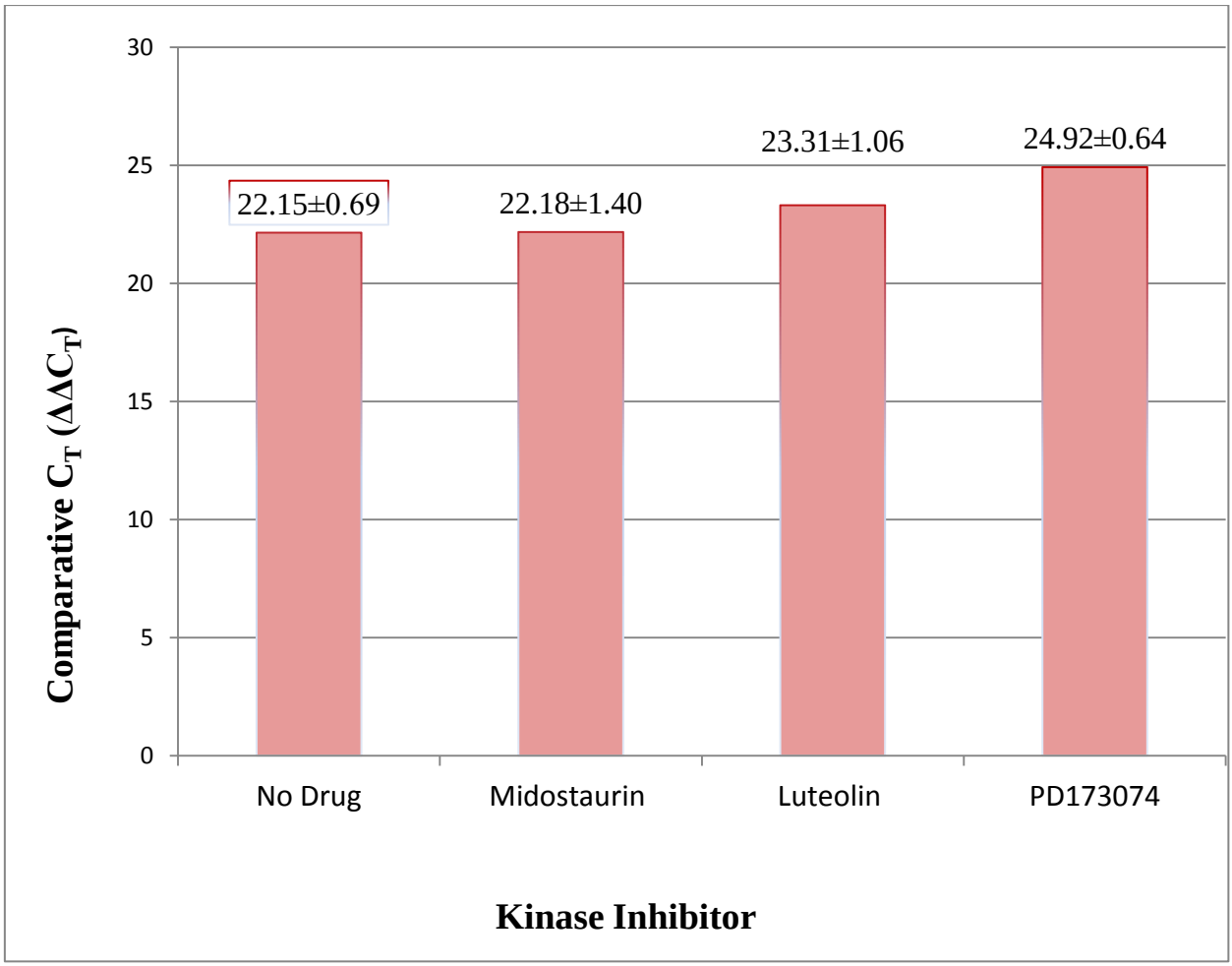


Figure 3.3 A: Comparative $\Delta\Delta C_T$ analysis of AD169 HCMV DNA in supernatant of infected cells after treatment with different kinase inhibitors. AD169 infected cells (m.o.i. 0.5) were treated with Midostaurin (100 nM), Luteolin (20 µM), or PD173074 (5 µM) at 0 h p.i., comparative $\Delta\Delta C_T$ was determined in supernatant at 96 h p.i. respectively. The Y-axis shows the number of cycle (Mean±SD), at which the detection of the AD169 DNA took place, the higher the number of cycle, the lower the number of viral DNA produced. The X-axis shows each compound used for inhibition. $\Delta\Delta C_T$ value for each column is indicated.

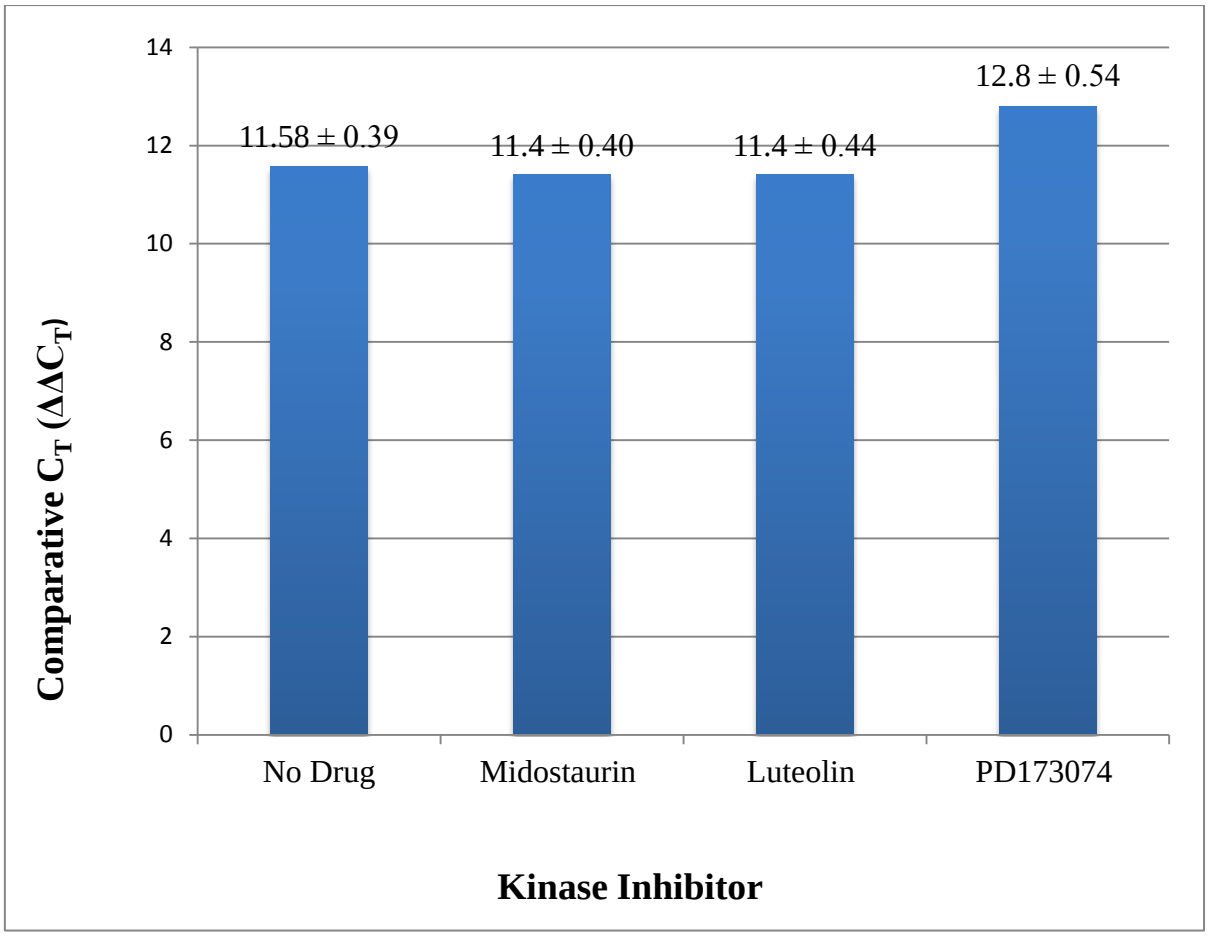


Figure 3.3 B: Comparative $\Delta\Delta C_T$ analysis of AD169 HCMV DNA in infected cells after treatment with different kinase inhibitors. AD169 infected cells (m.o.i. 0.5) were treated with Midostaurin (100 nM), Luteolin (20 µM), or PD173074 (5 µM) at 0 h p.i., comparative $\Delta\Delta C_T$ was determined in infected cells at 96 h p.i. respectively. The Y-axis shows the number of cycle (Mean±SD), at which the detection of the AD169 DNA took place, the higher the number of cycle, the lower the number of viral DNA produced. The X-axis shows each compound used for inhibition. $\Delta\Delta C_T$ value for each column is indicated.

3.4 Comparative $\Delta\Delta C_T$ analysis in AD169 infected cells treated different kinase inhibitors' titers

Further drug inhibition assays were performed using different concentrations of the drugs in order to investigate whether higher or lower concentrations of the kinase inhibitors have a higher impact on comparative $\Delta\Delta C_T$. Drug concentrations were chosen based on the effect of the drug at its IC50 concentration detected in our infection system.

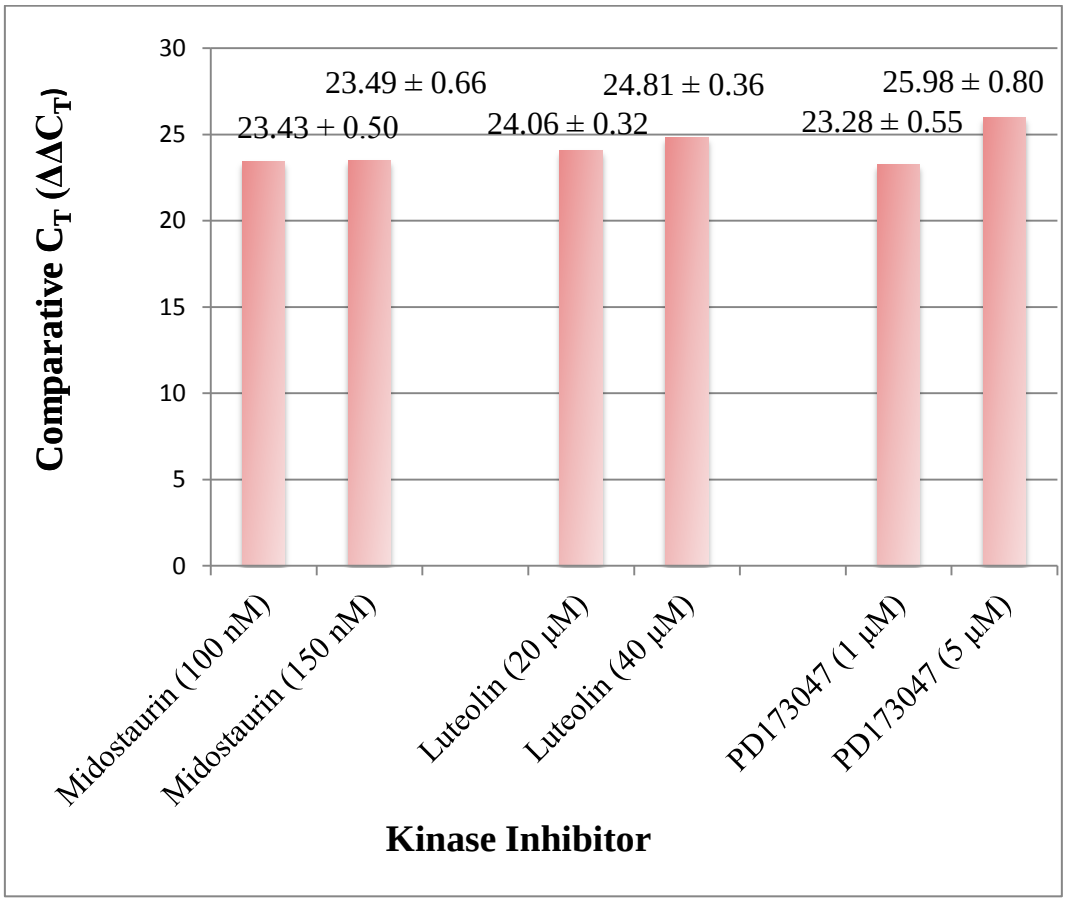


Figure 3.4: Comparative $\Delta\Delta C_T$ analysis of AD169 HCMV DNA in supernatant of infected cells after treatment with different kinase inhibitors' concentrations. AD169 infected cells (m.o.i. 0.5) were treated with Midostaurin (100 nM or150 nM), Luteolin (20 µM or 40 µM), or PD173074 (1 µM or 5 µM) at 0 h p.i., comparative $\Delta\Delta C_T$ was determined in supernatant of infected cells at 96 h p.i..

As PD173074 showed the most significant effect on $\Delta\Delta C_T$ at its IC50 (see Figure 3.3A), a lower concentration was used, while a higher concentration of Luteolin and Midostaurin were used. Interestingly, only PD173074 shows a significant difference in $\Delta\Delta C_T$ at lower concentration, while $\Delta\Delta C_T$ remained unchanged in case of Midostaurin and slightly changed in case of Luteolin. All further experiments were performed using the IC50 of the drugs indicated in Table 3.1.

3.5 PD173074, Luteolin, and Midostaurin did not inhibit released progeny viruses

Determination of comparative $\Delta\Delta C_T$ reflects the total amount of the specific DNA targeted, independent if that DNA came from an infectious viral particle or infection defected ones. Infectious viral particles can be determined using plaque assays. For this, infected cells were subjected to drug inhibition assay and the supernatants were collected at 96 h p.i. and subjected to plaque assay. Supernatants and plaque assays were tested in duplicate manner and the experiments were repeated at least three times for confirmation.

As demonstrated in Figure 3.5, none of the kinase inhibitors tested resulted in any significant reduction of infectious viral titer. This means that none of the kinase inhibitors (Midostaurin, Luteolin, and PD173074) affected the production of infectious progeny viruses.

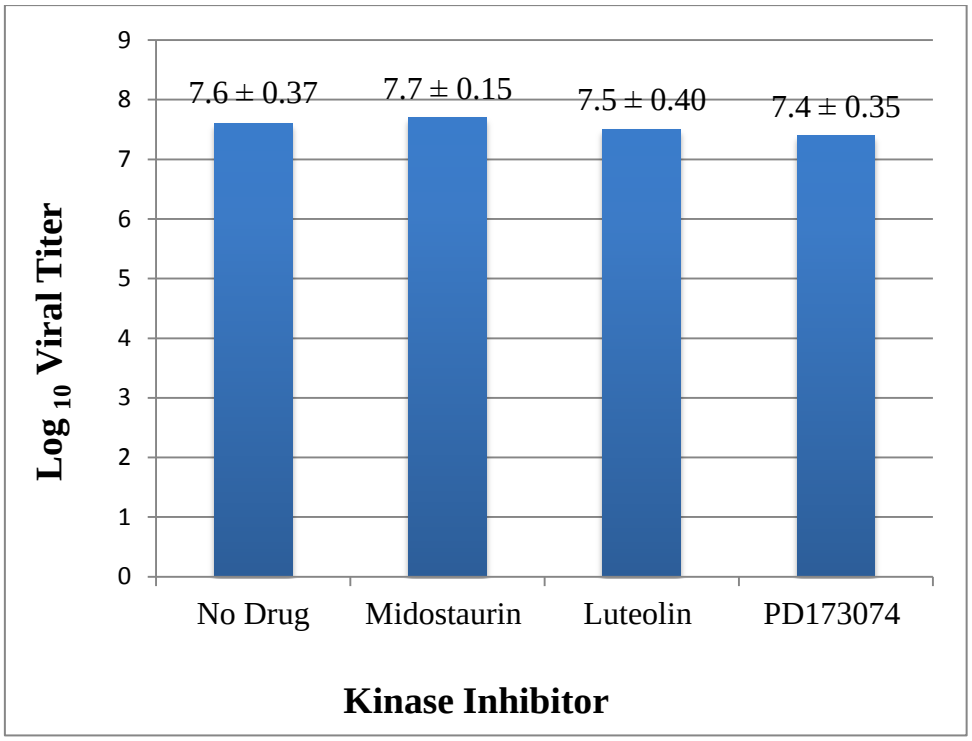


Figure 3.5: AD169 Log_{10} viral titer upon inhibition with Midostaurin, Luteolin, or PD173074. AD169 infected cells (m.o.i. 0.5) were treated with Midostaurin (100 nM), Luteolin (20 µM), or PD173074 (5 µM) at 0 h p.i.. At 96 h p.i. supernatant was subjected to plaque assay analysis. For calculation of viral titer from plaque assay, please see 2.6 for details. The Y-axis shows the Log_{10} viral titer (Mean±SD), the X-axis shows each compound used for inhibition.

3.6 PD173074 inhibition affects viral tegument protein pp28 in assembly compartment

The results presented above showed that PD173074 affected the Golgi apparatus, in order to see whether this is also relevant to viral markers as well, infected inhibited cells were subjected to pp28 HCMV tegument protein immunostaining at 96 h p.i.. pp28 is a true late HCMV protein and localizes to the viral cytoplasmic assembly compartment.

The viral cytoplasmic assembly compartment (AC) in HCMV-infected human foreskin fibroblasts (HFF) is a distinguishable compact “bulb”-like juxtanuclear structure (Azzeh et al., 2006). Morphology of the AC is dependent on the viral-encoded kinase, pUL97.

Infecting HFF cells with the UL97 deletion mutant (ΔUL97) or treating them with kinase inhibitors like NGIC-I alters of the AC morphology of the “bulb”-like structure (Azzeh et al., 2006) to assume a rather distracted “crown"-like structure punctuated with vacuoles (Azzeh et al., 2006).

Similarly to the results obtained from WGA staining, treating AD169 HCMV infected cells with PD173074 resulted in distinguished changes in the subcellular distribution of HCMV tegument protein pp28, which were observed above with WGA staining (Figure 3.1A and B). Hereby PD173074 induced vesicles, which are distracted from the viral cytoplasmic assembly compartment and spread throughout the cytoplasm; however these vesicles accumulated densely along the cytoplasmic membrane of the infected cells. From now on the PD173074 structure will be referred to as “vesicles’-rich” AC. Neither Luteolin, nor Midostaurin inhibition resulted in detectable changes in subcellular distribution of pp28, in line with the WGA staining results for both drug shown in Figure 3.1A and B. In Luteolin or Midostaurin inhibited infected cells, pp28 assumed the same “bulb”-like structure known from infected mock treated cells (Azzeh et al. 2006).

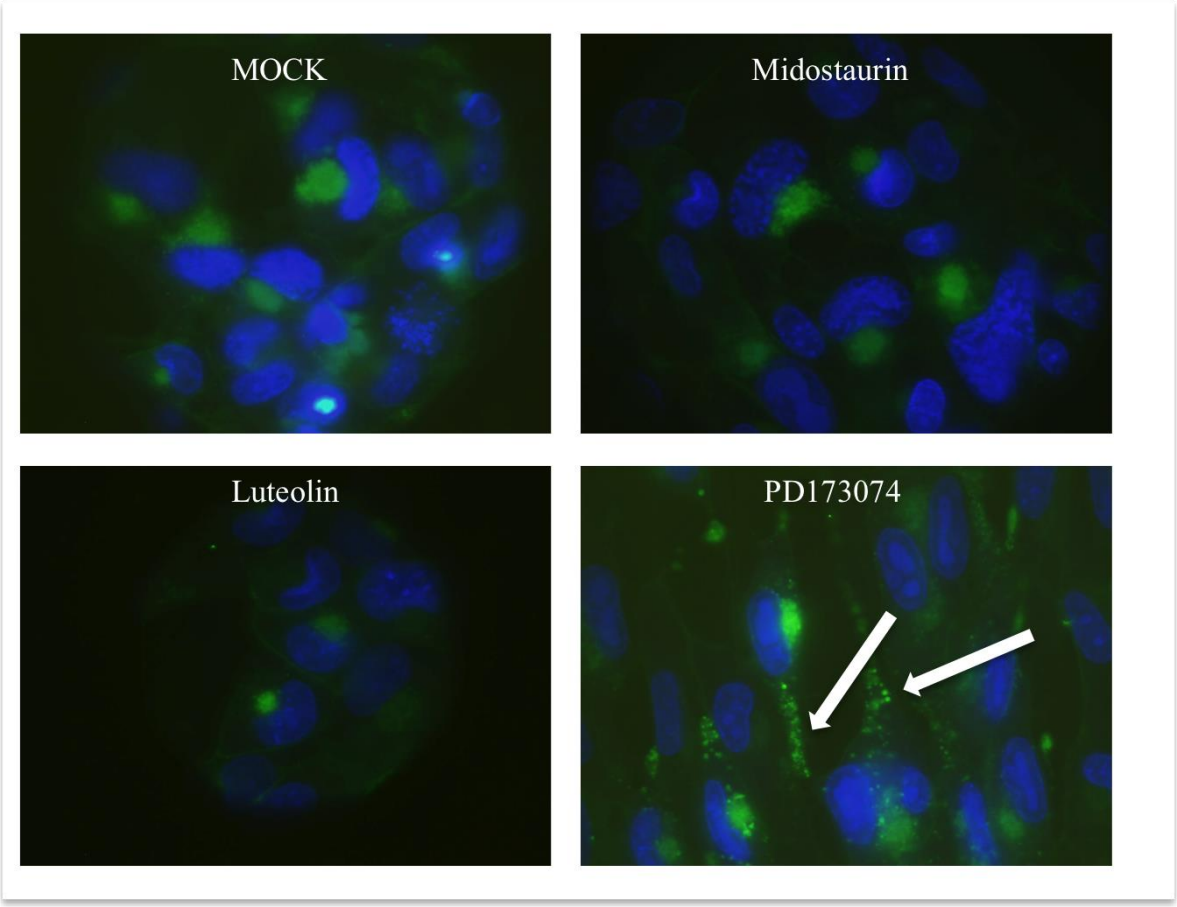


Figure 3.6: Subcellular distribution of pp28 in AD169 infected cells treated with kinases inhibitors. HFF cells were infected with AD169 (m.o.i. 0.5) and treated with Midostaurin (100 nM), Luteolin (20 µM), or PD173074 (5 µM) at 0 h p.i.. At 96 h p.i., cells were immunostained with pp28 (green) and counterstained with DAPI (blue staining of the nucleus), please see (2.9-2.11) for procedure's details. The white arrow indicates the punctuated vesicles in PD173074 treated cells.

3.7 PD173074 impact on HCMV assembly compartment has a kinetic nature

Based on the initial results showed above, PD173074 was the kinase inhibitor affecting AD169 viral cytoplasmic assembly compartment and comparative $\Delta\Delta C_T$. In order to determinate the kinetic activity of the inhibition of PD173074, HFF cells were infected with AD169, PD173074 was added at 0 h p.i., cells were subjected to WGA staining, at 24 h p.i., 48 h p.i., 72 h p.i., and 96 h p.i., all in duplicate manner, respectively.

The results of the kinetic analysis of PD173074 influence on HCMV viral cytoplasmic assembly compartment during its life cycle are summarized in Figure 3.7. At 24 h p.i., viral cytoplasmic assembly compartment was hardly detectable and started assuming its well-recognized “bulb”-like structure at 48 h p.i.. At 72 h p.i., the bulb like structure of the viral cytoplasmic assembly compartment was very clear in both, mock treated and PD173074 treated infected cells. While the distinguished “vesicles’-rich” AC pattern resulting from PD173074 inhibition is clearly visible at 96 h p.i., a deeper look on the staining at 48 h p.i. showed that the vesicles were distracted at the point already, however they were less organized and did not extent to the cytoplasmic membrane yet. Different immunostaining experiments for pp28 and WGA showed that the accumulation of the vesicles was clearly detectable at 72 h p.i.

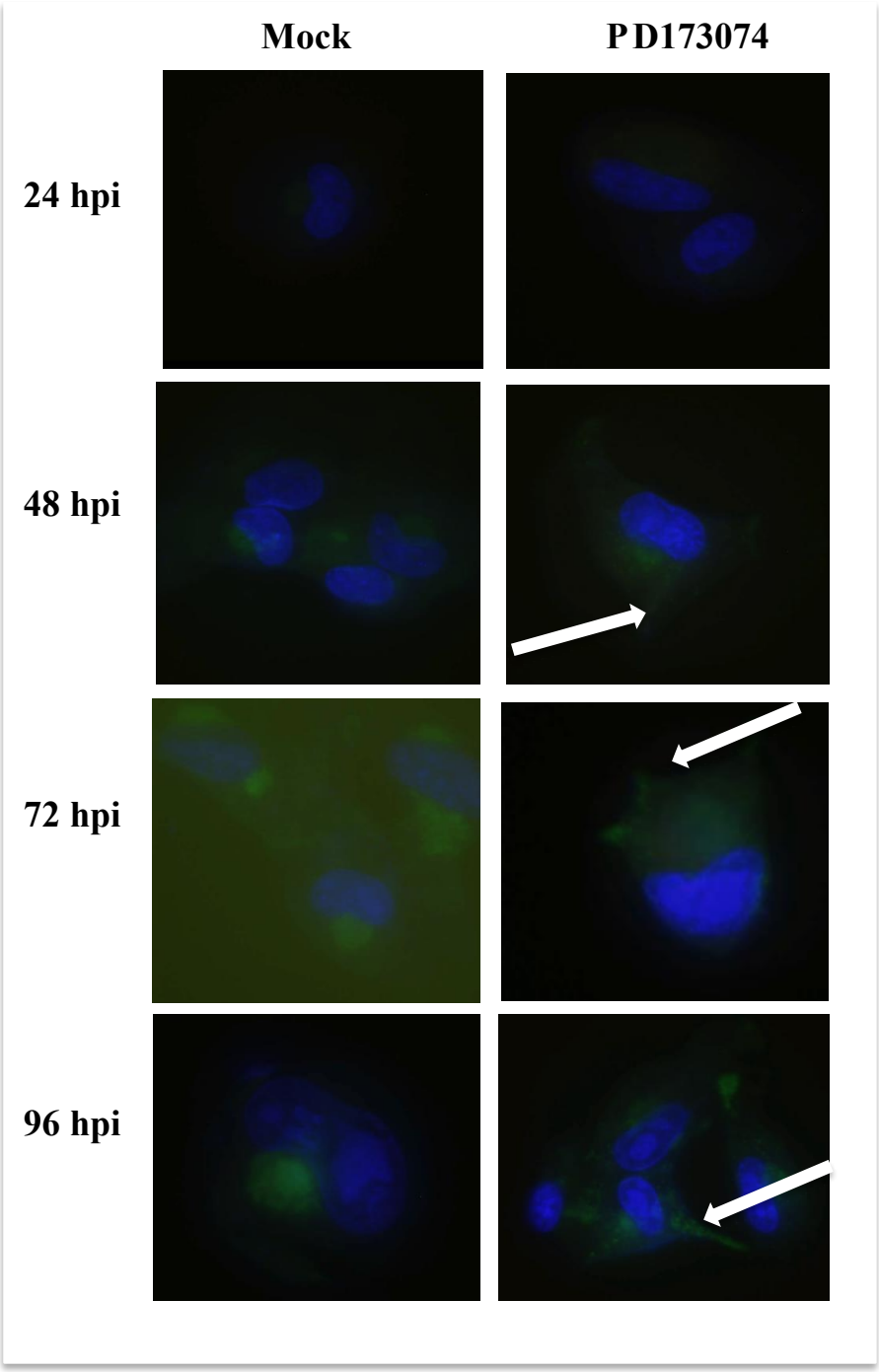


Figure 3.7: Kinetic subcellular distribution of WGA in PD173074 treated infected cells. HFF cells were either mock treated or treated with PD173074 (5 µM) at 0 h p.i. and subjected to WGA staining at 24 h p.i., 48 h p.i., 72 h p.i. and 96 h p.i. (green) and counterstained with DAPI (blue staining of the nucleus). The white arrows indicate the punctuated vesicles in PD173074 treated infected cells.

3.8 PD173074 inhibition causes changes to subcellular distribution of viral, but not cellular markers of AC

The experiments and results shown above illuminated a major effect of PD173074 on HCMV viral cytoplasmic assembly compartment, an essential stage of HCMV life cycle. Therefore, we decided to continue our investigations using PD173074 only. Further experiments were performed to detect the influence of PD173074 on pp65 and pp28, both viral tegument protein involved in building viral cytoplasmic assembly compartment and Golgi markers, which are more specific than WGA. The Golgi markers used were TGN46, which is a trans-Golgi network marker and may be involved in controlling trafficking between membrane and trans-Golgi network. The other Golgi marker used was GOLPH4, which is a Golgi stack membrane marker. GOLPH4 cycles between early Golgi and distal compartment such as endosomes and therefore plays a role in Golgi-endosome trafficking.

Initially, uninfected cells treated with PD173074 for 96 h were immunostained with either Golgi marker, GOLPH4 or TGN46. This immunostaining is a control experiment, to see whether Golgi is affected by PD173046. As demonstrated in Figure 3.8 A, neither Golgi markers was affected by PD173074 in uninfected HFF cells, immunostaining resulted in clear Golgi stack pattern independent from PD173074 treatment.

The next step was to colocalize the viral tegument proteins (pp28 and pp65) residing the viral cytoplasmic assembly compartment with the Golgi markers. Infected HFF cells were subjected to inhibition assay with PD173074 and subjected to immunostaining with pp28 and Golgi marker GOLPH4 (Figure 3.8. B and Figure 3.8 C). Figure 3.8 B illustrates clearly the difference between the subcellular distribution between pp28 in infected mock treated cells (bulb-like structure) and infected PD173074 treated cells (“vesicles’-rich” AC= the bulb-like structure with vesicles distracted throughout the cytoplasm). Images with higher magnification are shown in Figure 3.8 C for better visibility. In line with the results obtained from the uninfected cells, GOLPH4 structure was not affected by PD173074 treatment.

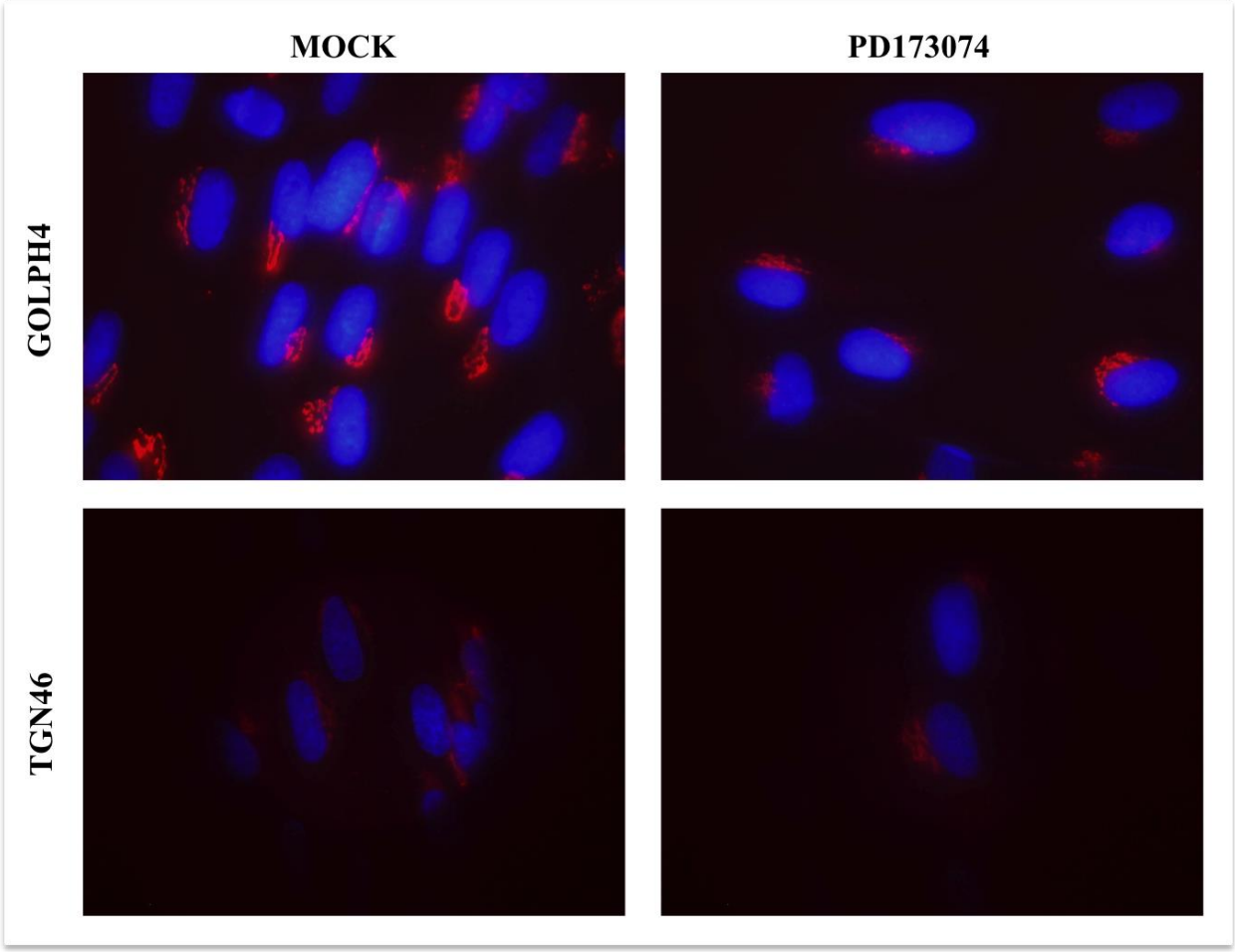
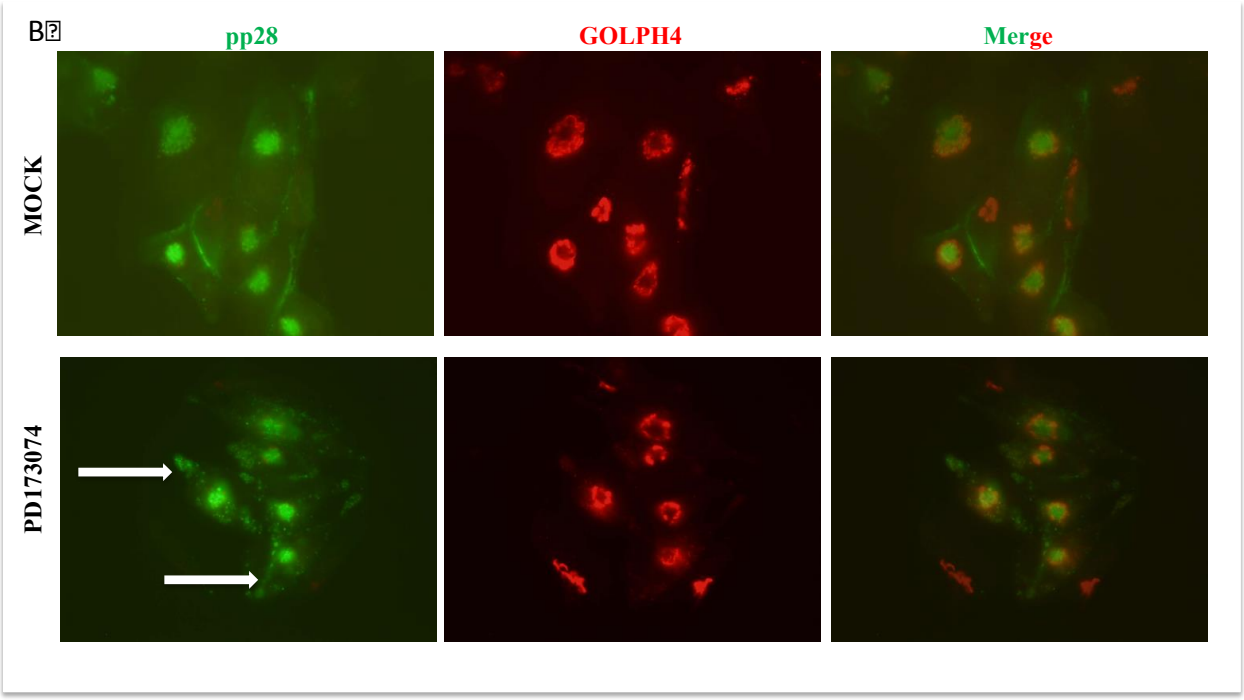


Figure 3.8A: Subcellular distribution of GOLPH4 and TGN46 in mock infected cells. HFF cells were either mock treated or treated with PD173074 (5 µM). 96 h after treatment, cells were subjected to GOLPH4 or TGN46 immunostaining (red) and counterstained with DAPI (blue staining). Please see section (2.9-2.11) for procedure's details.



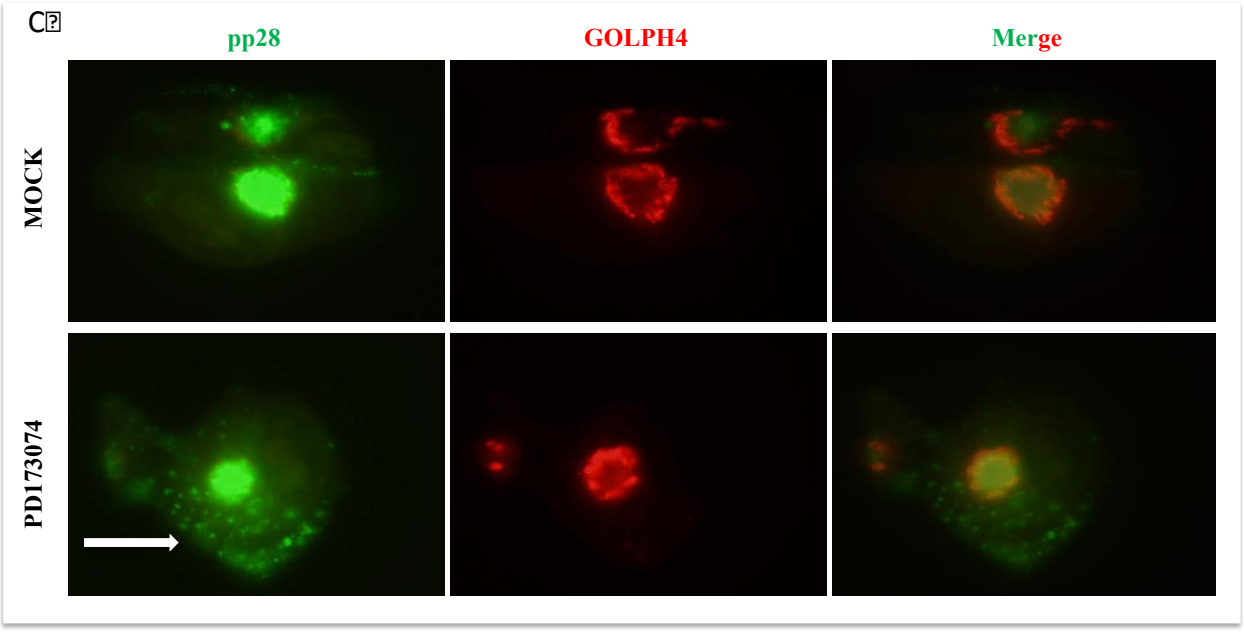


Figure 3.8 B and C: Subcellular distribution of pp28 and GOLPH4 in AD169 infected cells treated with PD173074. AD169 infected HFF cells were either mock treated or treated with PD173074 (5 µM) at 0h p.i.. At 96 h p.i., cells were subjected to immunostaining with pp28 (green) and GOLPH4 (red). The white arrow indicates the vesicles punctuating the cytoplasm and membrane induced by PD173074 activity. Figure 3.8 C shows an image with a higher magnification.

In another experiment set, TGN46 Golgi marker was colocalized with pp65, the most abundant tegument protein in HCMV. pp65 is an early late protein; it localizes to the nucleus in the first 48 h p.i., but then traffics to the cytoplasm and is known to spread equally all over the cytoplasm with higher intensity at the viral cytoplasmic assembly compartment at 72 h p.i. and 96 h p.i.. Immunostaining shown in Figure 3.8 D illuminates the effect of PD173074, as pp65 cytoplasmic distribution is punctuated with vesicles throughout the cytoplasm, similar to those observed with pp28 and WGA staining. In line with the results obtained from the uninfected cells, TGN46 structure remained unchanged despite PD173074 treatment. A detailed image of pp65 immunostaining is shown in figure 3.8 E.

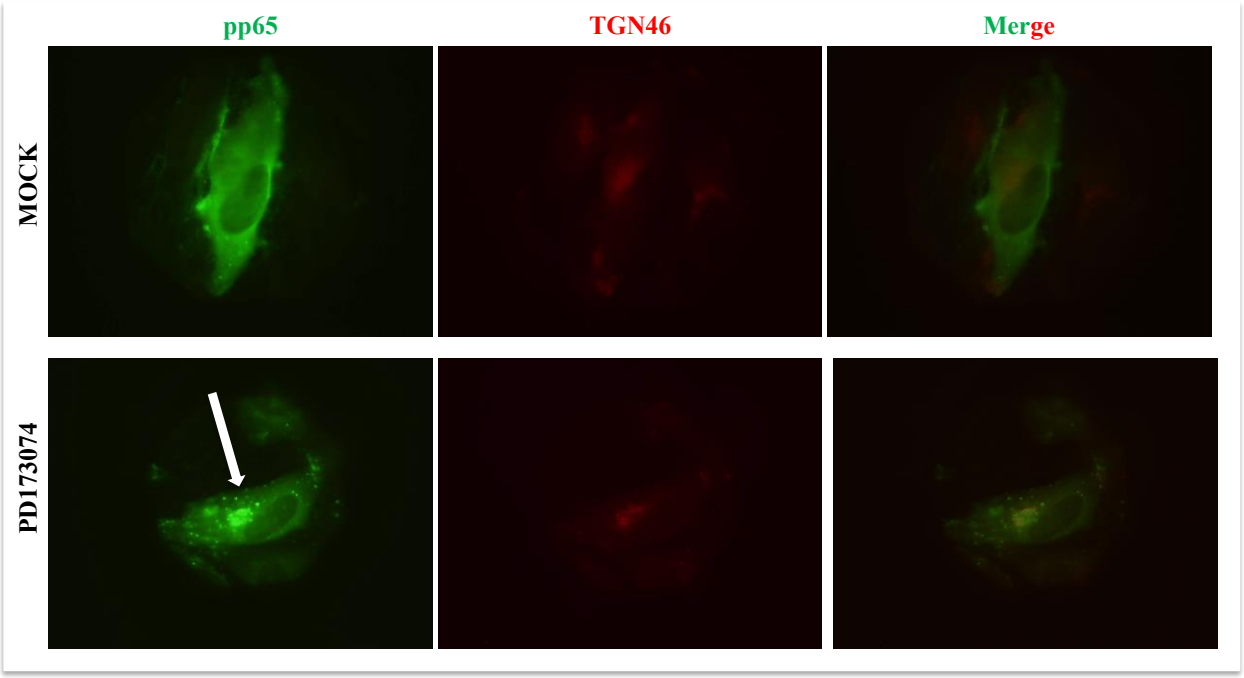


Figure 3.8 D: Subcellular distribution of pp65 and TGN46 in AD169 infected cells treated with PD173074. AD169 infected HFF cells were either mock treated or treated with 5 µM PD173074 at 0 h p.i.. At 96 h p.i., cells were subjected to immunostaining with pp65 (green) and TGN46 (red). The white arrow indicates the vesicles punctuating the cytoplasm. Please see section 2.9-2.11 for procedure's details.

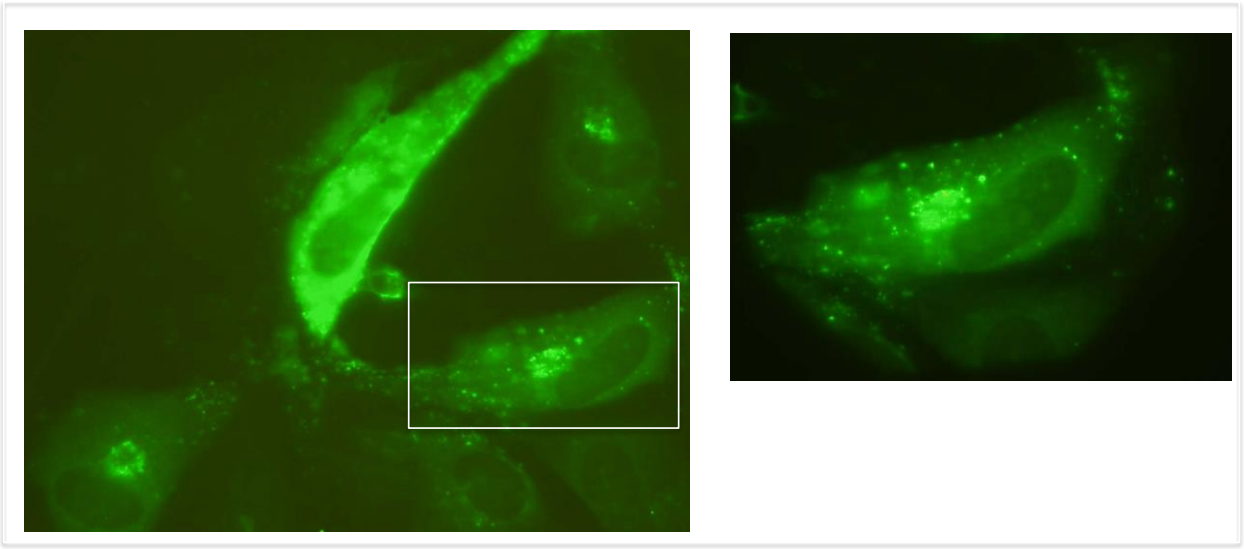


Figure 3.8 E: Subcellular distribution of pp65 in AD169 infected cells treated with PD173074. AD169 infected HFF cells were either mock treated or treated with 5 µM PD173074 at 0 h p.i.. At 96 h p.i., cells were subjected to immunostaining with pp65 (green). The image on the right is a higher magnification capturing of the cell in white rectangle on the left.

3.9 PD173074 inhibition did not affect early stages of HCMV life cycle

As PD173074 influence on the viral cytoplasmic assembly compartment had been established, we tested its influence on early stages of HCMV life cycle. First we tested the influence of PD173074 on subcellular distribution of UL84. UL84 is a very early HCMV protein and was detected 2.5 h p.i. as it plays a role in regulation and replication of HCMV (Xu et al., 2002).

HFF cells were infected with AD169 (0.5 m.o.i) and subjected to PD 173074 drug inhibition at 0 h p.i.. Cells were subjected to immunostaining with UL84 antibody at 96 h p.i.

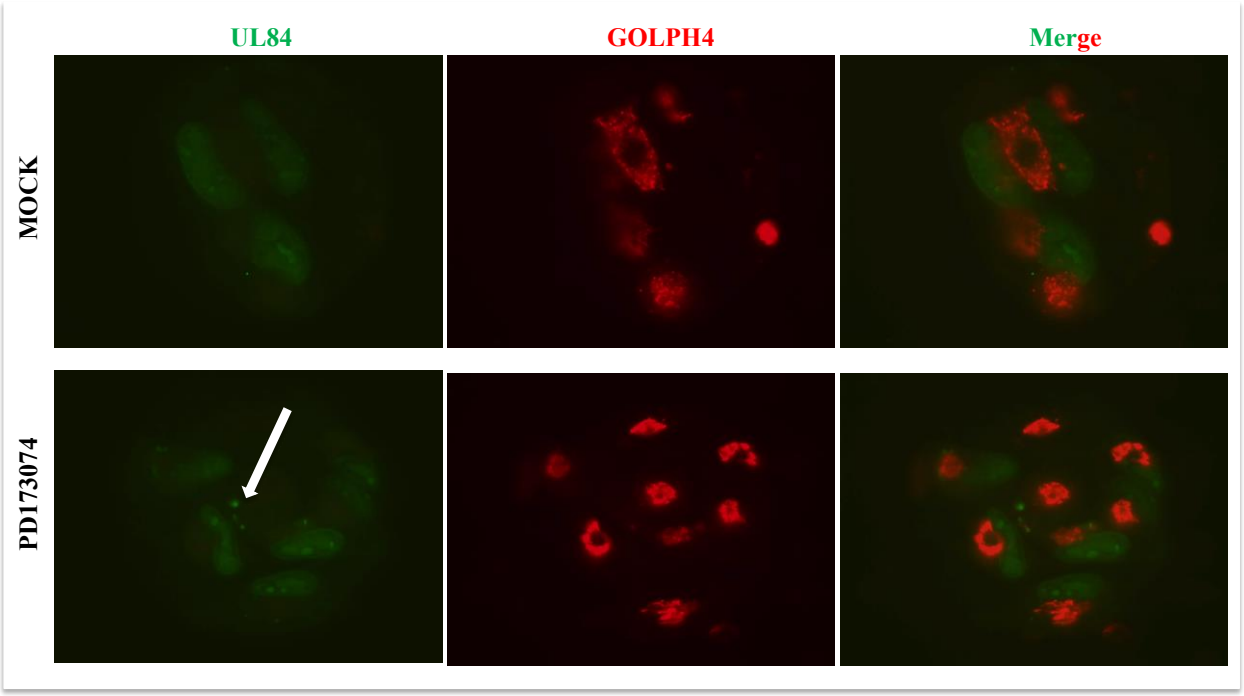


Figure 3.9 A: Subcellular distribution of UL84 early protein. Cells were infected with AD169 and either PD173074 inhibited or mock inhibited. At 96 h p.i., cells were co-immunostained with UL84 (green) and GOLPH4 (red) antibodies. The white arrow shows vesicles in the cytoplasm.

The immunostaining results presented in Figure 3.9 A show that UL84 localized to the nucleus in both, mock treated and PD173074 treated infected cells, however the cytoplasm of some infected cells treated with PD173074 was punctuated with limited number of vesicles, similar to those observed earlier with pp28, pp65 and WGA immunostaining.

IE1 and IE2 are intermediate-early proteins in HCMV life cycle and are known to localize to the nucleus within the first hours of infection. AD169 infected HFF cells were subjected to immunostaining with IE1&2 antibody (a mixture antibody of IE1 and IE2), results are shown in Figure 3.9 B. IE1&2 localized to the nucleus in both mock treated and PD173074 treated infected cells. The only observation we made here was that the rims of the mock treated infected cells were clearly stained; while no such rim staining was identified in PD173074 treated infected cells.

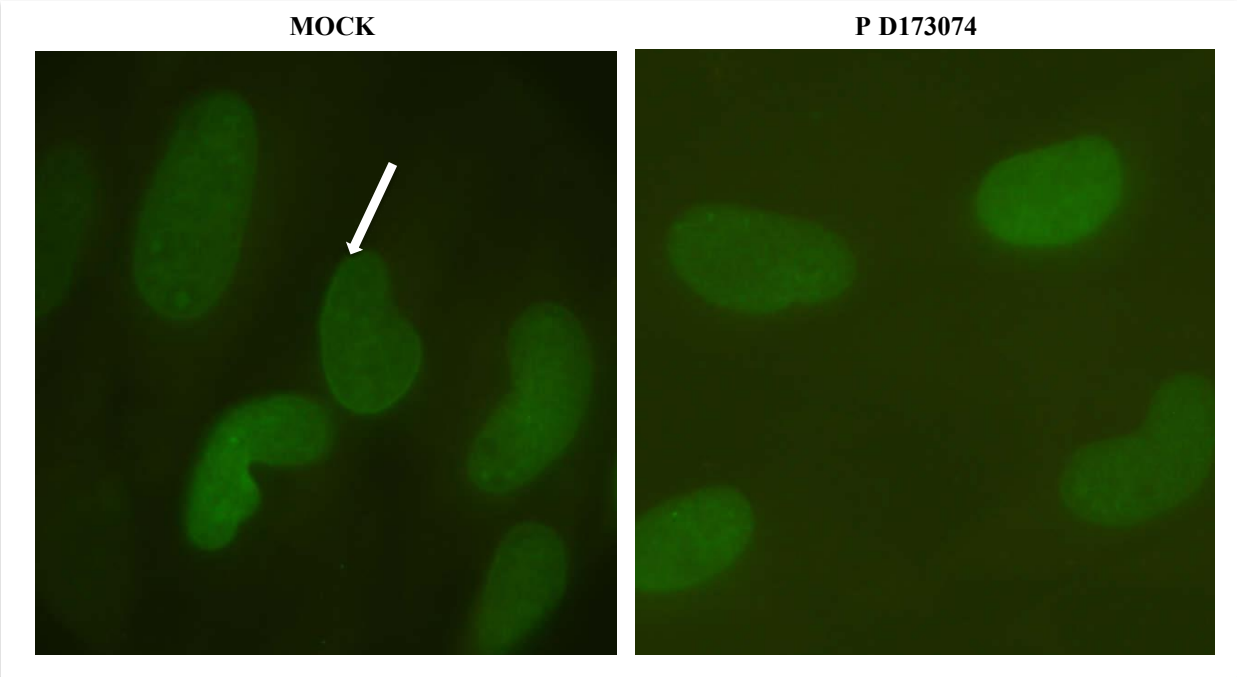


Figure 3.9 B: Subcellular distribution of IE1 and IE2 early proteins. Cells were infected with AD169 and either PD173074 inhibited or mock inhibited. At 96 h p.i., cells were subjected to immunostaining using IE1 and IE2 antibody (green).

3.10 PD173074 inhibition did not affect the morphology of the nucleus of AD169 infected cells.

The typical nucleus of uninfected HFF cell seems as an oval shape, however when infected with AD169 the nucleus morphology turns into kidney shape (Azzeh et al., 2006). Infecting HFF cells with pUL97 deletion mutant of AD169 (ΔUL97) or treating AD169 infected cells with NGIC-I compound restored the rather oval shape of the nucleus (Azzeh et al., 2006). The morphology of the nucleus in infected HCMV cells is associated with the viral cytoplasmic assembly compartment and is essential for virion assembly and egress (reviewed in Alwine, 2012). Therefore, it was noteworthy to see the effect of PD173074 on nucleus of AD169 infected cells. Going through the images taken and different slides, we were unable to see any particular changes in the nuclear morphology of in infected cells treated with PD173074. As in AD169 infected mock treated cells most nuclei showed the kidney shape morphology in AD169 infected cells treated with PD173074 (see previous Figures 3.1 B, 3.6, 3.7, and Figure 3.10 below).

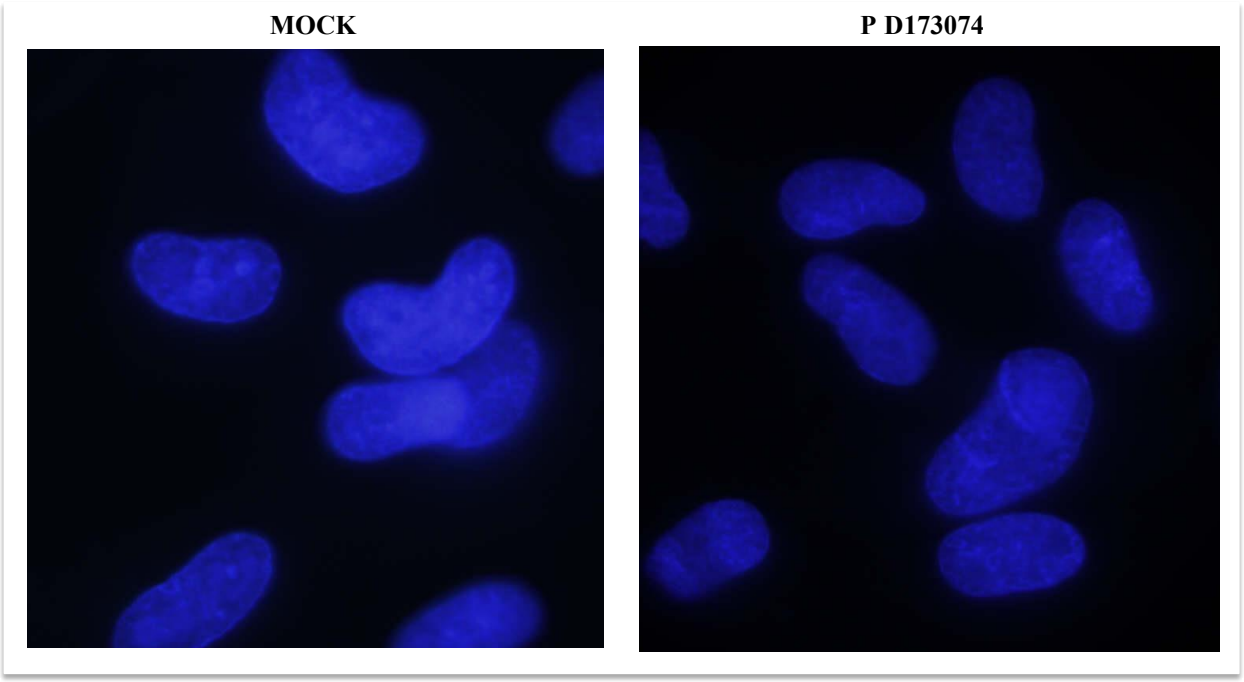


Figure 3.10: Morphology of nuclei in AD169 infected HFF cells. Cells were infected with AD169 and either PD173074 inhibited or mock inhibited. At 96 h p.i., cells were stained with DAPI (blue).

3.11 The abundance of the cells exhibiting the PD173074 induced "vesicles'-rich" AC

The IF figures shown above are the most representative images of different sets of experiments. In order to emphasize this representativeness, we performed statistical analysis to reflect the percentage of the PD173074 typical "vesicles'-rich" AC structure, which is the "new" structure recognized in this study. Going through the images and the original slides, some mock treated AD169 infected cells were indeed punctuated cytoplasmic vesicles, however less abundant than those observed in the PD173074 treated infected cells.

In order to identify the percentage of cells exhibiting "vesicles'-rich" AC, around 300 infected cells and 300 PD173074 treated infected cells were inspected from three different experiments (100 cells each), please see (2.12) for procedure's details. The percentage of cells presented with "vesicles'-rich" AC is shown in Figure 3.11. 92% of the AD169 infected cells treated with PD173074 exhibited "vesicles'-rich" AC, while only 11% showed a similar structure in AD169 infected mock treated cells.

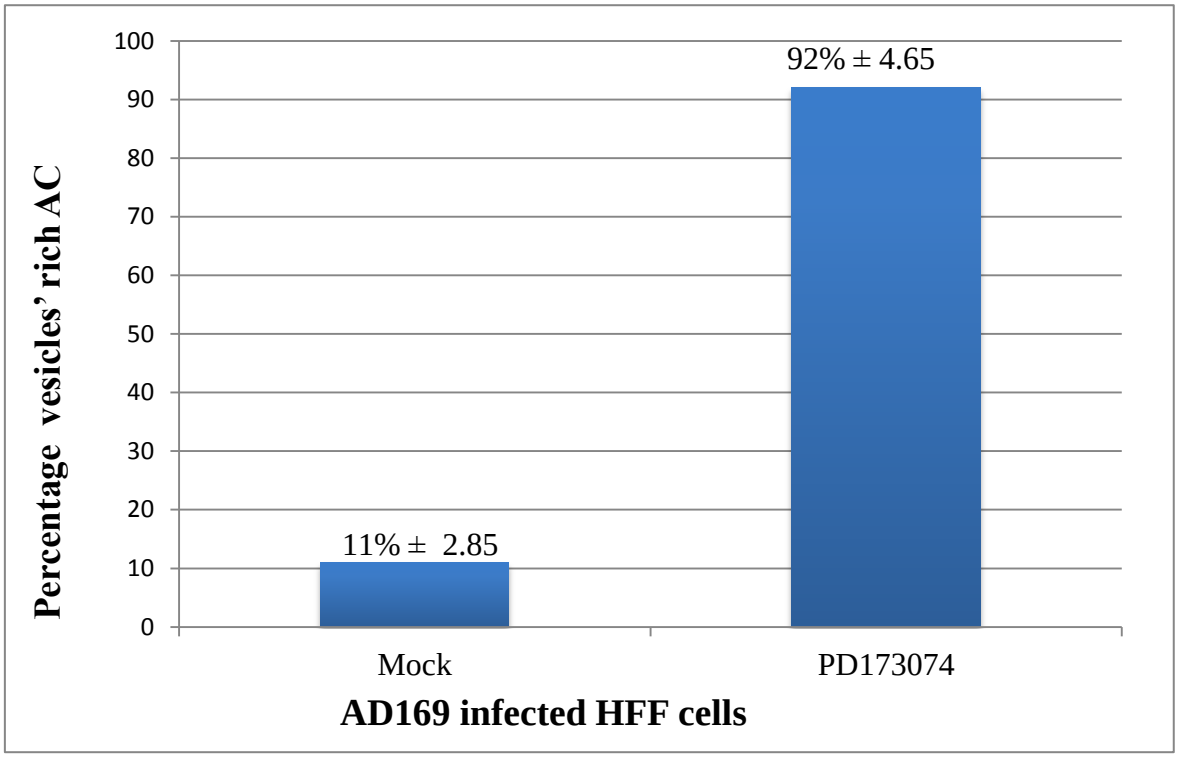


Figure 3.11: "vesicles'-rich" AC abundance in HFF cells infected with AD169. HFF cells were infected with AD169 and either treated with PD173074 or mock treated at 0 h p.i.. Cells were subjected to IF analysis using WGA staining or pp28 immunostaining. This experiment was repeated 3 times. Different fields on the IF slides were eye inspected through the IF microscope, the number of cells exhibiting "vesicles'-rich" AC or not was registered. Percentage calculation was made once a total of 300 cells were inspected (100 cells per experiment) for each experiment set and each treatment (mock or PD173074) respectively.

4. Discussion

4. Discussion

4.1 IC50 of Luteolin, Midostaurin and PD173074, comparative $\Delta\Delta C_T$ and plaque assay

Determination of the IC50 of the different drugs presented in this work is relevant to the *in vitro* experimental approach used. The IC50 of these drugs can vary in different biological systems based on the *in vitro/in vivo* experimental set up (Propper et al., 2001). The IC50 in this work is the amount of the drug, which reduces 50% of the AD169 viral plaque forming units (PFU, see plaque reduction assay, 2.8). This assay however is not equivalent to the chemical determination of IC50. The IC50 determined for these drugs using does not necessary reduce plaque forming units or viral load down to 50% in an infection assay (see viral infection assay 2.7).

The drugs were used at their determined IC50 for all experimental set up. Before testing the influence of these drugs on HCMV life cycle, their effect on its comparative $\Delta\Delta C_T$ and PFU was investigated. During an infection assay, the virus has the chance to mature and bud out of the cell during the 96 h life cycle, the viability of the drug during these 96 h is not guaranteed. Released mature progeny viruses are not necessarily infectious, as the infection cycle may produce defected viruses. A viral load or comparative $\Delta\Delta C_T$ assays using the real-time PCR technique detect all viruses, independent of their ability to cause infection as it only detects the DNA, not the infectivity of the virus. Plaque assay (2.6) is the assay used to detect the viruses that cause infection as only infectious viruses can produce plaque-forming units (PFU). Figures 3.3. A demonstrated that the comparative $\Delta\Delta C_T$ of supernatant AD169 at 96 h p.i. was reduced almost three cycles (C_T) when infection assay was inhibited using PD173074. As three C_T is equal to one fold number wise, PD173074 reduced AD169 viral load 10 times compared to mock inhibition. While Midostaurin did not result in any inhibition effect on C_T, Luteolin caused a one C_T inhibition, which is around three-time inhibition effect. Detecting comparative $\Delta\Delta C_T$ in supernatant reflects the viral DNA from released mature viruses, which bud out of the infected cell. While supernatant contains released viruses, many viruses are still immature and not released yet and therefore can be found in the cell cytoplasm. In order to investigate the status of these immature viruses under drug inhibition, we performed the real-time PCR $\Delta\Delta C_T$ analysis after extracting DNA from the inhibited infected cells. The

results of these experiments are summarized in Figure 3.3. B, which revealed that immature unreleased viruses, were barely affected by the drugs. There is a one C_T reduction in case of PD173074, elaborating that among the three drugs used here, PD173074 is the only one causing HCMV inhibition *in vitro*. The analysis was done in duplicate manner for each drug and experimental set up and confirmed at least by three independent experiments.

Based on these results obtained, we decided to further investigate the effect of the drugs on comparative $\Delta\Delta C_T$ using either a lower drug concentration or a higher one. As Luteolin and Midostaurin showed either a low or no effect on comparative $\Delta\Delta C_T$ at their IC50 concentration, we used higher concentration of both, while a lower concentration of PD173074 was used. As clearly demonstrated in Figure 3.4, using the even higher concentrations of each Luteolin and Midostaurin did not affect the $\Delta\Delta C_T$ at all, while using a lower concentration of PD173074 results in lower effect on the comparative $\Delta\Delta C_T$. These results emphasizes that the IC50 used in our experimental set up was convenient and the results obtained from comparative $\Delta\Delta C_T$ testing are not related to variation in the drugs' concentrations, but rather a true biological effect. Most importantly was the result of the five-time lower PD173074 concentration, which shows clearly that the effect of the drug is not linear and the effect starts at lower concentrations. One could argue that using a higher concentration of PD173074 would reveal a higher effect. Therefore, we tested the effect of 10 µM PD173074, however it resulted in massive destruction of the infected cells and therefore the result of this experiment was neglected. When infected cells are massively destructed due to drug over dose, the supernatant contains both, released and cell-associated viruses and cannot therefore be considered the result of released viruses only.

To test the influence of the drugs on infectious released viruses, plaque assay from supernatant was performed and PFU was determined. None of the drugs, Luteolin, Midostaurin, or PD173074 caused a reduction in infectious viral particles (Figure 3.5). The fact that PD173074 caused three cycles reduction in comparative $\Delta\Delta C_T$ does not necessarily mean that it was expected to cause a reduction in PFU. As mentioned above, a comparative $\Delta\Delta C_T$ assay is based on the detection of the DNA of all viruses, irrespective of their ability to infect, while a plaque assay detects only infectious particles. Therefore, the difference in comparative $\Delta\Delta C_T$ detected with PD173074 inhibition is due to the portion of non-infectious viral particles.

As Midostaurin is a derivate of Staurosporine, we were expecting other results. Staurosporine reduced viral load 100-time and PFU 2.5 logs in a previous study at the Virology Research Laboratory (Qawasmi et al., 2011). Nevertheless, it is very common for analogue compounds not to have the same activity and effect.

4.2 The effect of Luteolin, Midostaurin and PD173074 on HCMV assembly

Initial direct Golgi staining of infected cells inhibited with the different drugs showed a change in the subcellular distribution of WGA in case of PD173074. WGA stains the assembly compartment (AC) of HCMV in infected cells, which is a juxtanuclear "bulb"-like structure (Azzeh et al., 2006). This structure was identified in Figure 3.1 A in infected cells and those inhibited with either Luteolin or Midostaurin. In case of PD173074 staining (3.1 A and B), the juxtanuclear "bulb"-like structure was still clear, however WGA also stained punctuated vesicles throughout the cytoplasm, mainly organized along the cytoplasmic membrane. These initial data proposed that PD173074 tyrosine inhibition activity might have caused a specific destruction of viral assembly compartment (AC) by destructing Golgi or those proteins with N-acetylneurarminic acid residues or N-acetylglucosamine, to which WGA binds (Bhavanandan and Kaltic, 1979).

The next step was to investigate the influence of PD173074 on pp28, an essential HCMV tegument protein, which resides the AC. Interestingly; immunostaining with pp28 specific antibodies elaborates the very same subcellular distribution of AC detailed above for WGA for each drug (Figure 3.6). Mock inhibition of infected cells, inhibition with Midostaurin or Luteolin resulted in the known "bulb"-like structure pp28, while PD173074 caused the very particular subcellular distribution with the vesicles along the cytoplasmic membrane ("vesicles'-rich AC pattern). These data emphasize that the results obtained for WGA are specific and that AC viral and cellular proteins are specifically affected by PD173074 and the nature of the vesicles could be either viral or cellular proteins.

An important aspect of HCMV infection is the long life cycle and the question here was, at which stage was the influence of the tyrosine kinase inhibition of PD173074 visible. For this a kinetic experiment was performed, where infected mock or PD173074 inhibited cells were subjected to staining using WGA at 24 h p.i., 48 h p.i., 72 h p.i. and 96 h p.i. respectively. These experiments showed that by 48 h p.i., vesicles were detectable in PD173074 inhibited infected cells, but the "vesicles'-rich AC pattern was clear by 72 h p.i.

(Figure 3.7). This means that the tyrosine inhibition activity affected rather early late to late stages of AC development and that the "vesicles'-rich AC pattern is very specific for tyrosine kinase inhibition.

The results of AC inhibition detailed above were yet restricted to one viral AC marker and one Golgi marker. For further verifications and manifestation of these data, pp65, another viral tegument protein involved in building AC and other Golgi markers such as GOLPH4 and TGN46 were subjected to immunostaining after inhibition with PD173074. pp65 is the most abundant tegument protein and resides AC as well as the entire cytoplasm in late infection (after 48 h p.i.). GOLPH4 localizes to cis and medial Golgi cisternae and plays a role in endosome to Golgi protein trafficking, while TGN46 resides in trans-Golgi network and is presumably involved in regulating membrane traffic to and from trans-Golgi network.

Initial immunostaining with GOLPH4 and TGN46 of mock infected cells, showed that PD173074 did not affect the subcellular distribution of either Golgi marker (Figure 3.8 A). Similarly, both Golgi markers were not affected by PD173074 inhibition in infected cells (Figures 3.8 B, C and D), although both markers' subcellular distribution was affected by the infection as reported earlier (Qawasmi et al., 2011). Both markers showed a partial co-localization with pp28 and pp65 at the AC (Figures 3.8 B, C and D), which caused the orange color in the merge images. The "vesicles'-rich AC pattern was observed in infected PD173074 inhibited cells immunostained with either pp28 or pp65 (Figures 3.8 B, C and D). The immunostaining with pp65 was very interesting as it seemed like the cytoplasmic distribution of pp65 was less intensive in PD173074 inhibited infected cells and that the staining was intensive at AC and the punctuated vesicles. Our observations for pp65 immunostaining in PD173074 inhibition assay showed that the vesicles were less organized, but rather intensively spread throughout the cytoplasm (Figure 3.8 E). Figure 3.8 E showed an image of six AD169 infected cells immunostained with pp65 and one cell was further shown in higher magnification to visualize the sporadic distribution of the vesicles. We propose that the sporadic distribution of the vesicles in case of pp65 is due to its distribution throughout the cytoplasm emphasizing that the origin of the vesicles is viral proteins.

GOLPH4 is a marker of early Golgi vesicle trafficking, the fact that it did not change accordingly with PD173074 means that the vesicles caused by this inhibition are not

derived from early Golgi or trans Golgi. On the other hand, WGA decorates distal Golgi cisternae (Tartakoff and vassalli, 1983; Virtanen et al., 1980) and stained theses vesicles. In summary, the nature of the vesicles observed under PD173074 inhibition could be derived from either viral tegument proteins pp28 and pp65 or distal Golgi.

The "vesicles'-rich AC pattern was not reported earlier for AC and seems to be unique for PD173074 tyrosine kinase inhibition. Induction of "vesicles'-rich AC pattern by PD173074 was very remarkable as it was observed in most infected cells (Figure 3.11). Taken together, the date presented here illuminate that the AC structure is affected by the tyrosine kinase inhibition activity of PD173074 indicating a role of the tyrosine kinase in maintaining the integrity viral assembly compartment (AC).

While serine/threonine kinase plays a major role in HCMV replication as described earlier (Prichard et al., 1999; Wolf et al., 2001; Marschall et al., 2003; Marschall et al., 2005), Luteolin, which was shown to have a serine/threonine inhibition activity (Kim et al., 2011) failed to induce any remarkable effect on the subcellular distribution of AC or viral replication. Despite the fact that Staurosporine was found to inhibit assembly steps in HCMV life cycle (Qawasmi et al., 2011), Midostaurin, a derivate of Staurosporine lacked the ability to affect the subcellular distribution of AC or the viral replication.

4.3 The effect of PD173074 other stages of HCMV life cycle

To further investigate the consequences of the tyrosine kinase inhibition caused by PD173074, markers of early and intermediate early stages of viral infection were used in immunostaining assays. Immunostaining of very early protein UL84 revealed no major changes in its subcellular distribution when infected cells were treated with PD173074. However we did observe some cytoplasmic vesicle staining in PD173074 inhibited cells (Figure 3.9 A), but cannot verify whether theses vesicles are induced due to PD173074. UL84 plays a role in organizing DNA replication and exhibits nucleocytoplasmic shuttling, but does not localize to the cytoplasm, a fact, which does not approve the stained vesicles shown in (Figure 3.9 A) being attributed to PD173074 staining. An interesting feature was observed under PD173074 activity in IE1/2 immunostaining (Figure 3.9 B). The rims of the nucleus (nuclear lamina) were clearly stained in infected cells, but not in infected PD173074 inhibited cells. We were however unable to relate this feature to nuclear egress

as it is established that tyrosine kinase does not play a role in nuclear egress and that nuclear egress and nuclear lamina are affected by PKC and viral UL97 kinase activity (Milbradt et al., 2010). This fact and conclusion are actually in agreement with the observation we made with DAPI nuclear staining (Figure 3.10). While earlier studies reported structural changes in the nucleus due to PKC kinases inhibitors (Azzeh et al., 2006, Qawasmi et al., 2011), PD173074 did not cause any detectable changes of the nucleus (Figure 3.10).

4.4 The role of tyrosine kinase in HCMV life cycle compared to other kinases

In this work, three cellular kinase inhibitors, Luteolin Midostaurin and PD173074, were tested for their antiviral activity against HCMV. The three kinase inhibitors have not been investigated earlier for their activity in HCMV life cycle, therefore the data presented in this study is novel. Luteolin is a flavonoid compound, which has a serine/threonine inhibition activity (Kim et al., 2011; Reipas et al., 2013), and exhibited antiviral activities against other herpes viruses (Behbahani et al, 2013), but did not show any remarkable effects on HCMV infection in this work. PKC412, known as Midostaurin is the broad spectrum inhibitor of serine-threonine/tyrosine-protein kinases including protein kinase C (PKC), vascular endothelial growth factor receptor (VEGFR), fms-like tyrosine kinase (FLT3), platelet-derived growth factor receptor (PDGFR) and the stem cell factor, c-KIT (Fabbro et al., 2000; Weisberg et al., 2002) also failed to show any detectable antiviral activity in HCMV life cycle. These results however do not exclude a possible inhibition or activation activity of either Luteolin or Midostaurin in other stages of HCMV life cycle, which were not investigated in this work.

Although PD173074, a tyrosine kinase inhibitor, was poorly investigated in HCMV studies, other kinase inhibitors were reported for their role in HCMV infection and life cycle. Tyrosine kinase inhibitors inhibited virus/cell fusion (Keay and Baldwin, 1991). Tyrosine kinase inhibitor Imatinib inhibited primary HCMV infection by inhibiting PDGFR, a critical receptor required for HCMV infection (Soroceanu et al., 2008), other and in vitro studies demonstrated inhibition of HCMV replication by blocking tyrosine kinase activity of PDGFR (Tikkanen et al., 2001). PDGFR is a specific cell surface receptor, which belongs to the tyrosine kinase family of receptors. PD173074 has been shown to effectively inhibit FGFR3 in KMS11, KMS18 and OPM-2 cell lines, as well as in

a xenograft murine model (Grand et al., 2004; Paterson et al., 2004; Trudel et al., 2004). PD173074 is a potent and highly selective inhibitor of the FGFR family that can inhibit autophosphorylation of FGFR1 in a dose-dependent manner with an IC50 in the range 1–5 nM (Mohammadi et al., 1998). Fibroblast growth factor receptor 1 (FGFR1) is receptor tyrosine kinase, and these kinases are known regulators of various cellular processes, including proliferation, migration, survival and angiogenesis (Dailey et al., 2005). As HCMV grows on fibroblasts in vitro and in vivo, we proposed that this inhibitor might have an effect on HCMV life cycle. Indeed, the data presented in this work showed clearly that PD173074 had a major impact on the subcellular distribution of AC markers and affected comparative $\Delta\Delta C_T$. Nevertheless, we cannot verify whether the inhibition activities of tyrosine kinase inhibitor PD173074 observed in our study were specifically related to FGFR or PDGFR. Further studies are needed to elaborate the detailed mechanism of action leading to the changes in AC structure. Inhibition of viral load (as reflected by higher comparative $\Delta\Delta C_T$ number) however, could be related to the tyrosine kinase inhibition activity of PDGFR. Several studies concluded that PDGFR might be a crucial receptor for HCMV infection and therefore an attractive target for new and novel antiviral therapies (Marschall and Stamminger, 2009) making the data presented in this study a respectful contribution to the field of antivirals against HCMV.

Early researches on the AC showed that Golgi markers were re-modulated in HCMV infected cells (Sanchez et al., 2000; Azzeh et al., 2006; Das et al., 2007). These researches had shown that WGA Golgi and trans Golgi markers lost their thread like structure and localized to the rim of the AC structure. PD173074 inhibition altered WGA and viral tegument protein AC markers in infected cells. The vesicles were also clearly seen in cytoplasm and accumulated densely along cytoplasmic membrane, proposing a possible effect of tyrosine inhibition activity on viral or/and cellular trafficking purposes.

Cellular kinases play central roles in regulation of cell replication and differentiation, making cellular kinase inhibitors attractive targets of studies directed specifically against cancer. Protein kinases are essential players in different stages of HCMV life cycle, which supports the idea of searching for new antiviral among specific cellular kinase inhibitors here too. As described earlier HCMV pUL97 mimics cellular kinases especially CDKs and this motivated many researchers to identify the compounds that can inhibit cellular or viral kinases or both. CDKs inhibitors have been considered as highly interesting antivirals targets (Schang, 2001) and many studies have shown the efficacy of these cellular protein

kinases inhibitors against HCMV and other herpesviruses (Schang, 2006). Flavopiridol and roscovitine, which possess broad antiviral activity are examples of these inhibitors and indeed have been involved in several clinical trials (Herget et al., 2006; Schang et al., 2006). Furthermore, specific inhibitors of CDK9, which are under intensive study as potential candidates against HIV (Heredia et al., 2005), may also open potential possibilities for developing novel drugs against HCMV, as CDK9 plays a vital role in HCMV replication (Rechter et al., 2009; Feichtinger et al., 2011).

Although cellular kinase inhibitors were approved for treatments against different types of cancer, those against viruses are still in clinical trials. The literature provides massive evidence that the era of cellular kinase inhibitors against HCMV and other viruses is coming. Many viral infections are induced during cancer therapies; novel therapies using kinase inhibitors targeting both could be the most promising therapy combinations.

Appendix:

IC50 row data.

PD173074 conc.		No	10nM	100nM	1μM	**5μM**	10μM
Relative Plaque number (%)	Experiment 1	100%	93%	75%	69%	**52%**	25%
	Experiment 2	100%	83%	70%	51%	**47%**	30%
	Experiment 3	100%	85%	73%	64%	**51%**	30%
	mean	100%	87%	72.6%	61.3%	**50%**	28.3%

Midostaurin conc.		No	5nM	10nM	50nM	**100nM**	150nM	200 nM
Relative Plaque number (%)	Experiment 1	100%	100%	77%	58%	**58%**	40%	0%
	Experiment 2	100%	88%	70%	67%	**48%**	42%	0%
	Experiment 3	100%	91%	73%	65%	**53%**	41%	0%
	mean	100%	93%	73.3%	63.3%	**53%**	41%	0%

Luteolin conc.		No	1μM	5μM	10μM	15μM	**20μM**	30μM
Relative Plaque number (%)	Experiment 1	100%	85%	70%	70%	63%	**52%**	40%
	Experiment 2	100%	73%	57%	58%	42%	**42%**	35%
	Experiment 3	100%	77%	66%	63%	70%	**60%**	38%
	mean	100%	78.3%	64.3%	63.6%	58.3%	**51.3%**	37.6%

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