Screening and Characterization of Novel Chemical Compounds Inhibiting Cytomegalovirus Infection

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Table of contents

Table of contents I
Summary IV
1. Introduction 1
1.1. Discovery of human cytomegalovirus 1
1.2. Human Cytomegalovirus and disease 1
1.3. Epidemiology and Transmission 1
1.4. Pathogenesis and treatment 2
1.5. Structure of the HCMV particles 3
1.6. The genome structure of HCMV clinical isolates and laboratory strains 4
1.7. The productive, lytic infection cycle of HCMV 5
Virus Entry 6
Viral gene expression 7
Replication, cleavage and packaging of the HCMV DNA genome 8
Capsid assembly and Nuclear egress 9
Secondary envelopment and release of virions from infected cells 9
1.8. Drugs in clinical use for treatment of HCMV disease 10
1.9. Resistance to anti-HCMV drugs 12
1.10. Other drugs with activity against HCMV, currently not in clinical use 13
Maribavir (MBV) 13
Brincidofovir (BCV) 13
Cyclopropavir (CPV) 14
Letermovir (AIC246) 14
1.11. Drugs that are approved for treatment of other diseases and are not specific for CMV 16
Leflunomide 16
Artesunate (ART) 16
Aims of the Study 18
2. Materials and Methods 19
2.1. Materials 19
Buffers and solutions 19
Antibodies 20
Cells 21
Machines and Equipment 22
Compounds 22

2.2. Methods 24
Cell culture 24
Methods for measuring cell viability 24
Screening of the Synthetic compound libraries 25
Virological methods 25
Flow cytometry assay 28
qPCR Method 28
Protein Biochemistry Methods 29
Data analysis 30
3. Results 31
3.1. Establishing a screening assay for identification of CMV inhibitors 31
Optimizing the conditions of the assay and its quality control 31
Randomization of the controls and test run 34
3.2. Results of the first screening rounds 36
Processing of the high throughput screening data 36
Screening of the first compound library 37
Screening of the second and third compound library 37
3.3. Rescreening of the hit substances 39
3.4. Further characterization of the antiviral activity of the identified compounds by GFP reduction assay 42
3.5. Determination of approximate IC_{50} and CC_{50} values 43
3.6. Further cytotoxicity testing of compounds 46
3.7. Discovery of the mechanism of action of the selected compounds inhibiting HCMV 50
Reversibility of the inhibitory activity of the anti-HCMV compounds 50
Inhibition of viral DNA replication by the antiviral compounds 53
Efficacy of the compounds against MCMV 54
Potency of the compounds in inhibiting virus release 55
3.8. Effects of the compounds on expression of viral proteins of different kinetic classes 58
4. Discussion 60
4.1. Experimental design of the High-Throughput Screening 60
4.2. Analysis of the data of the screening assay 61
4.3. Characteristics of the identified compounds 64
4.4. Mode of action of the selected compounds 66
4.5. Outlook 70
References 73
5. Appendices 85

5.1. Supplementary Figures 85
5.2. Abbreviations 105
Acknowledgments 108
Curriculum Vitae 109
Declaration 110

Summary

Human cytomegalovirus (HCMV) is the most frequent viral cause of congenital diseases and a major risk factor for immunocompromised individuals and transplant patients. To date, only a limited number of antivirals against the virus have been developed and are in clinical use. Although these drugs are quite effective in controlling HCMV replication, their toxicity, the emergence of drug-resistance and cross-resistance as well as their common inhibitory mechanism limit their application.

Thus, to discover new substances that inhibit HCMV infection – by a novel mode of action – we screened a large library of small synthetic molecules. A screening assay in 384-well format was established using low multiplicity infection with a GFP-expressing HCMV reporter strain. A multiple step infection procedure was chosen to achieve viral spread in the cultures, thereby allowing to detect all kind of inhibitors independent of which step of the HCMV life cycle is blocked. Calculation of Z-factors – a parameter telling the quality of the screening procedure – indicated that the assay was robust. The platform optimized for cell density and the infectious dose applied was then used to screen 17,640 synthetic compounds. GFP signals were measured on day 6 p.i., and Z-scores were calculated for each compound making use of the median and median absolute deviation of the signals of the entire samples of a plate.

Two hundred four hits were discovered in the primary screen, taking into account the inhibition of virus-driven GFP expression, Z-score values of the compounds and viability measurements of the cell cultures. The activity of the hits was re-checked by a secondary screen and fifty-seven compounds remained as hits after this step. The antiviral activity of the compounds was verified by measuring inhibition of viral GFP expression (i.e. by determining dose-response curves, and by performing time of addition experiments) as well as by measuring the inhibition of virus release by plaque assay. The cytotoxicity of the compounds was re-tested on human fibroblasts and retinal pigmented epithelial cells by applying the MTT assay, as well as employing another method relying on the measurement of ATP levels.

Moreover, several approaches were pursued to narrow down the processes blocked by the hits; for example, effects on the amplification of the viral DNA were checked using real-time qPCR analysis. Reversibility of the activity of the compounds, i.e. restoration of virus production was analysed with a kinetic-block release assay. Finally, the consequences for the

expression of representative members of the different classes of immediate-early, early and late viral proteins were examined after treatment of infected cells with the compounds.

Altogether, nine promising compounds were discovered, which display IC_{50} values in the low micro-molar range, low cytotoxicity, and decent selectivity indices. Based on the results of time-of-addition experiments it was concluded that two compounds inhibit the infection during the early phase. The most promising compound identified displayed an excellent selectivity index (>34), late inhibitory kinetics, and was, moreover, active against both mouse and human CMV. Further studies are aiming at the definition of the target proteins and pathways that are disrupted by this drug, and at the optimization of this lead hit to develop effective therapies against HCMV.

1. Introduction

1.1. Discovery of human cytomegalovirus

In 1881, Ribbert in Germany noticed large cells in sections of kidneys in the stillborn and in the parotid gland of children, which he misinterpreted as protozoa (Ho, 2008). The intracellular inclusions were found in 13% of these infants. Later, Von Glahn and Pappenheimer noticed that the intranuclear inclusions resulted from infections with viruses rather than protozoa (Von Glahn and Pappenheimer, 1925). Farber and Wolbach determined that 14% of children who died from variant causes, showed similar inclusions in the cells of their salivary glands. In 1932, Wyatt et al. found these intranuclear inclusions also in children with rare congenital infections. The name "generalized cytomegalic inclusion disease (CID)" was suggested, due to enlarged cells associated with the disease (Wyatt et al., 1950). Minder was the first person who saw 199-nm large viral particles and suggested that CID is caused by a virus. Subsequently, the virus was called human cytomegalovirus (HCMV).

1.2. Human Cytomegalovirus and disease

The three members of the beta-herpesvirus subfamily, HCMV, HHV-6, and HHV-7, belong to the eight known different herpesviruses for which humans are the natural host (Mocarski et al., 2013). Their common features are narrow host range and slow replication in cell culture which takes at least 48 to 72 hours. HCMV is the prototypical representative of this subfamily. Infection with this virus causes only mild symptoms in immunocompetent individuals. Therefore, HCMV infections are not noticed, and most individuals are able to control the infection efficiently and remain latently infected without overt symptoms. Latent infection with HCMV, which is the most common feature of all herpesviruses, is established in different organs such as kidneys, spleen, liver, lungs, and salivary glands, as well as in different hematopoietic progenitor cells. Some rare cases have shown that HCMV infection of immunocompetent people can lead to mononucleosis-like symptoms with fatigue, lymphocytosis with atypical lymphocytes, and prolonged fever for more than 10 days (Hyde et al., 2010).

1.3. Epidemiology and Transmission

In many countries, HCMV infects the majority of the human population. The HCMV seroprevalence rates vary between different regions around the world and range from 40 –

100% (Adland et al., 2015) depending on the living conditions and socio-economic status of the population. The seroprevalence of HCMV in Germany is ~46%, with more than 75% in elderly people (Hecker et al., 2004).

The main route of transmission is via body fluids containing infectious virus; therefore, it is highly circulated among children in kindergardens, and between children and parents. Transmission via semen or vaginal secretion is common among adolescents (Mocarski et al., 2013; Sohn et al., 1991). The vertical transmission includes mother to fetus, and this can take place congenitally; in fact, HCMV is considered to be the only herpesvirus that is transmitted via the placenta. Transmission can also occur perinatally during birth and postnatally, as well as through breast milk (Bryant et al., 2002).

1.4. Pathogenesis and treatment

Reactivation of HCMV in immunocompromised hosts, such as patients receiving immune-suppressing drugs as part of the transplant therapy, is common. The risk of developing HCMV-associated disease in solid organ transplantation patients is higher when receiving an organ from a seropositive donor. In hematopoietic stem cell transplantation, the situation is reversed and the risk of disease is much higher when the recipient is HCMV seropositive and the donor is seronegative. Often such patients develop signs of pneumonitis and gastrointestinal diseases as a consequence of active CMV infection (Boeckh, 2011). The application of pre-emptive antiviral therapy has reduced the occurrence of acute HCMV infection early after transplantation and improved the survival rate in some high-risk patients; however, late CMV infections still occur frequently. In addition, myelosuppression and fungal infections are major problems in these patients (Capobianchi et al., 2014).

Another common group of immunocompromised hosts affected by CMV reactivation is HIV infected individuals. Around 40% of HIV infected patients receiving highly active antiretroviral therapy (HAART) suffer from HCMV disease (Lin et al., 2002). This is due to the low $CD4^+$ T-cell counts of those HIV infected patients that are also HCMV seropositive. Although the use of HAART led to restored T-cell counts at the normal level (Young et al., 2012), HCMV infection was found to promote directly or indirectly progression to AIDS and death. The major manifestation of HCMV disease in HIV patients is retinitis. Other manifestations include enterocolitis, gastritis, hepatitis and encephalitis (Massimo and Naidu, 2015).

HCMV congenital infection is the most important viral infection associated with birth defects. Primary infections occur in 1 - 4% of HCMV-seronegative pregnant women, and approximately 0.5 % of all pregnancies are fatal or aborted due to congenital HCMV (Buxmann et al., 2017). The rate of congenital CMV infection of newborns in industrialized countries is on average of 0.6 to 0.7% of live births (Dollard et al., 2007; Kenneson and Cannon, 2007). Of those children, who are born with HCMV infection, 10% display a variety of symptoms such as hepatomegaly, splenomegaly, jaundice, pneumonitis, fetal growth retardation, and seizures. Of the 90%, who are born asymptomatically, approximately 10 - 15% will develop symptoms later. These symptoms are vision loss, hearing loss, and mental retardation (Dollard et al., 2007).

1.5. Structure of the HCMV particles

Similar to other members of the Herpesviridae family, the HCMV particles contain a large double-stranded DNA (236 kb), enclosed by an icosahedral capsid, which in turn is surrounded by an amorphous layer of tegument proteins located between the capsid and the lipid envelope (Figure 1) (Mocarski et al., 2013). The tegument layer can be distinguished in a capsid-proximal part (inner tegument) that is well characterized, and a capsid-proximal part (outer tegument) that is closer to the envelope and is less characterized (Chen et al., 1999; Yu et al., 2011). The tegument contains the majority of the virion proteins, and proteomic studies showed that more than 80% of the virion protein content is made of tegument proteins plus of the glycoproteins gB, gM, and gN (Buscher et al., 2015; Varnum et al., 2004). There are several glycoproteins in the virus envelope that are involved in cell attachment and viral entry. The envelope consist of a lipid membrane that is derived from the ER-Golgi compartments of the host cells (Homman-Loudiyi et al., 2003). The virion consists of 71 structural proteins and over 70 associated host cellular proteins, including components of the cytoskeleton such as vimentin and actin, and several enzymes and chaperones (Varnum et al., 2004). The linear double-stranded DNA of the HCMV is tightly packaged within the capsid. The pressure of the viral genome DNA to the inner capsid wall was measured for HSV capsids and is approximately 18 bars (Bauer et al., 2013).

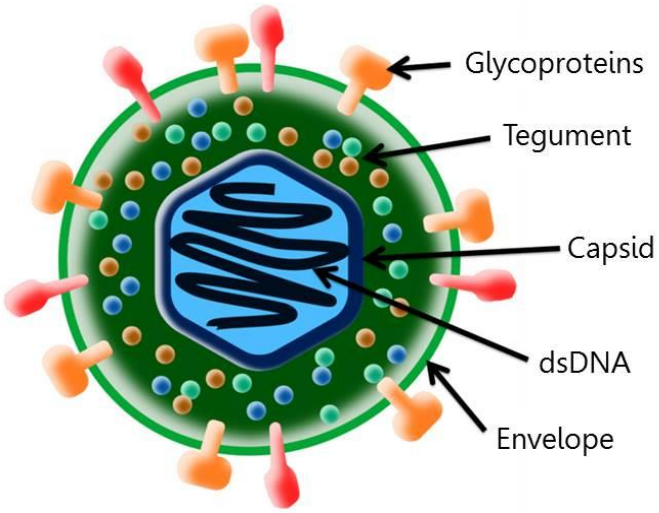


Figure 1. The HCMV virion. The graphical scheme depicts the double-stranded DNA genome contained within the nucleocapsid, which is surrounded by the tegument layer. The viral lipid envelope is derived from host membranes and contains viral glycoprotein complexes.

1.6. The genome structure of HCMV clinical isolates and laboratory strains

The HCMV genome encodes at least 200 open reading frames (ORFs) and consists of two distinct unique regions, the unique long (UL) and the unique short regions (US), which are flanked by repeated sequences. These repeats reside at both ends of the complete genome, and are called terminal repeat long (TRL) and terminal repeat short (TRS), as well as internal repeats at the intersection of the long and short segments (IRL and IRS). This leads to a general configuration of TRL-UL-IRL-IRS-US-TRS (Chee et al., 1990; Cunningham et al., 2010; Dolan et al., 2004).

Of the highest coding capacity among all mammalian DNA viruses, only 41 ORFs are essential for virus growth in vitro, and 88 are nonessential in the laboratory strain AD169 (Yu et al., 2003). In the Towne strain, 45 were found to be essential and 117 nonessential (Dunn et al., 2003). Most of the essential and nonessential genes are located in the middle and towards the ends of the genome, respectively. Interestingly, the UL region is five times larger than the US region and seems to carry more essential genes, whereas deletion of genes within the US region caused only moderate growth defects, indicating that these genes are inessential for virus replication (Dunn et al., 2003).

Continuous passage of HCMV in fibroblasts has led to many genomic mutations within highly passaged laboratory strains (Dargan et al., 2010), therefore; most laboratory strains carry mutations, deletions, and rearrangements throughout their genomes. For instance, the virulent low-passage Toledo strain harbors a 13 kbp segment that is lacking in AD169 (Cha et al., 1996). Towne strain lacks 19 and AD169 strain lack 22 ORFs, in comparison to clinical isolates. Interestingly, the ORFs lost during conversion of low-passage clinical strains to high-passage laboratory strains have an impact on virulence and cell-tropism of HCMV. For

instance, the laboratory strain AD169 can infect and replicate in fibroblasts, but is unable to infect epithelial and endothelial cells (Mocarski et al., 2013). In contrast, TB40/E and Merlin strains have been passaged only to a limited extent, and they can still infect many different cell types. These strains were cloned as bacterial artificial chromosomes (BACs) (Sinzger et al., 2008; Stanton et al., 2010) and have been subjected to genetic manipulations using homologous recombination in E.coli (Borst et al., 2007), a technique initially established for mouse CMV (Messerle et al., 1997), and subsequently for HCMV laboratory strains (Borst et al., 1999).

1.7. The productive, lytic infection cycle of HCMV

There are two states by which CMV can occur – the latent state, where the HCMV genome is dormant, and – the lytic life cycle needed for progeny production. In the lytic life cycle new virus particles are released for the first time after 48 and 72 hours and virus production continues for 6 days or more. The lytic infection cycle consists of several steps: entry of the virus into cells, viral gene expression, viral DNA replication, capsid assembly, genome packaging, nuclear egress of newly produced capsids, secondary envelopment of capsids, and virus release (Figure 2).

HCMV is able to infect and replicate in many cell types such as parenchymal cells and connective tissue cells, as well as epithelial, fibroblasts, endothelial and smooth muscle cells (Sinzger et al., 2008). Since most HCMV laboratory strains lost their tropism for other cell types except fibroblasts, many studies are carried out in fibroblasts. Hence the following chapter describes the HCMV replication cycle in this cell type.

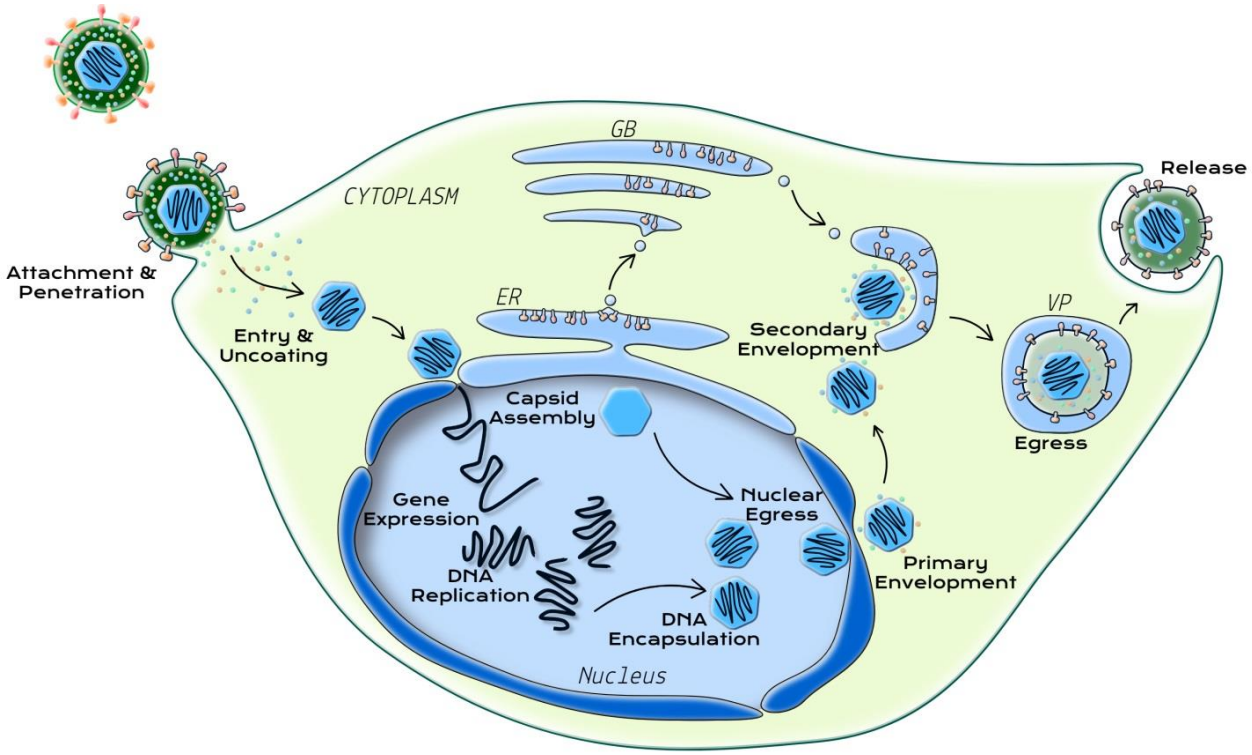


Figure 2. The productive, lytic life cycle of HCMV in the infected cell. HCMV enters the cells either through direct fusion or the endocytic pathway. Virions enter the cells via interaction of the viral envelope glycoproteins (e.g., gB and gH) with a specific cellular receptor (e.g., platelet-derived growth factor receptor α). Following fusion of the envelope, the nucleocapsids and tegument proteins are released into the cytoplasm. Nucleocapsids are then transported to the nuclear envelope, where the viral DNA is released into the nucleus. Subsequently, the IE, E and L viral genes are transcribed. Further, viral DNA is replicated and encapsulated in de novo assembled capsids. The resulting mature capsids are transported from the nucleus to the cytoplasm, where the secondary envelopment occurs at membranes derived from the endoplasmic reticulum (ER)-Golgi intermediate compartment. Final envelopement and egress is a complex two-stage process that leads to release of the virions at the cell surface plasma membrane.

Virus Entry

HCMV entry is a multistep process with a cascade of events including the binding of glycoproteins on the virions surface envelope to cellular surface receptors. Different cell types employ specific molecules for entry of HCMV, and receptor usage depends also on the type of cell in which the virus was produced (Scrivano et al., 2011). In general, there are cellular surface receptors commonly used in HCMV adsorption. Heparan sulfate proteoglycans (HSPG) and on the virus side the complex-forming glycoproteins gB, gN/gM, gH/gL/gO and the pentameric complex gH/gL/UL128/UL130/UL131A (Vanarsdall et al., 2011) are believed to play key roles in the entry process (Compton et al., 1993). Wang et al. reported that the epidermal growth factor receptor (EGFR) can potentially be a cellular co-receptor, which is

recognized by viral gB (Wang et al., 2003). Subsequently, this report was challenged by Fairley et al. who showed downregulation of EGFR by HCMV, and Isaacson et al. concluded that EGFR is not necessary for entry (Fairley et al., 2002; Isaacson, Feire, and Compton, 2007). In addition, cellular integrins were shown to serve as co-receptors in that the $\alpha_V\beta3$, $\alpha2\beta1$ and $\alpha6\beta1$ integrins bind to viral gB or gH (Feire et al., 2004). Other mediators such as Annexin II have been implicated with entry of HCMV into cells, but are not essential for entry into fibroblasts (Pietropaolo and Compton, 1999; Pietropaolo and Compton, 1997). More recently the platelet-derived growth factor receptor-α was reported by Wu et al. to play a key role in the entry process. It binds directly to gH/gL/gO on the surface of the fibroblasts (Wu et al., 2017).

Two possible ways have been described for the penetration of the virions, depending on the type of the target cell and the cell tropism of the virus strain. One way is the direct pH-independent fusion of the viral envelope with the host membrane in fibroblasts (Compton, Nepomuceno, and Nowlin, 1992; Ryckman et al., 2006), and the other way is via endocytosis. Depending on the cell type the HCMV is produced it can enter epithelial cells in two distinct pathways, virions that are produced in epithelial cells enter by fusion and virions that are generated from fibroblasts enter by endocytosis (Bodaghi et al., 1999; Ryckman et al., 2006; Wang et al., 2007). Both processes result in the release of the capsid and tegument proteins into the cytoplasm. Once capsids enter the cytoplasm, they interact with cytoskeletal elements, such as actin filaments, microtubules, and intermediate filaments (Lyman and Enquist, 2009). Capsids with residual tegument proteins attached such as pp150, pUL47, and pUL48 are transported to the nuclear membrane (Ogawa-Goto et al., 2003). The precise mechanism underlying the transportation of these viral proteins and the cellular motor proteins involved is still under investigation, but it has been reported that capsids and tegument proteins interact with cellular transport complexes (Ogawa-Goto et al., 2003; Sodeik et al., 1997).

Viral gene expression

After the release of the viral genome into the nucleus at the nuclear pores, the gene expression starts. Viral gene expression is highly regulated and viral genes are transcribed in a temporary cascade manner (Mocarski et al., 2013). These phases are immediately early (IE), early (E), and late (L). The most abundant gene products that are expressed at immediate-early (IE) times are 72-kDa IE1 and 86-kDa IE2 proteins. Also, it is believed that the IE region is the first to be expressed during acute infection and reactivation from viral latency. IE genes are

usually expressed as early as 2 and 8 hours after virus entry because transcription of these genes does not depend on the other viral gene products. However, the two tegument proteins pp71 and pUL35 have been shown to have a role in enhancing expression of the IE genes (Cantrell and Bresnahan, 2006; Schierling et., 2005). The two highly expressed IE proteins, IE72 and IE86, have been shown to have a key role in optimizing and controlling viral and cellular gene expression, thereby paving the environment for the production of viral DNA (Sinclair and Sissons, 2006). After the expression of IE genes, transcription of early genes takes place, and many of the proteins that are encoded in the early phase have a role in viral DNA replication. Expression of the first late genes begins at the earliest at 24 h after infection. L proteins include tegument proteins and glycoproteins, as well as proteins necessary for capsid assembly, and maturation of capsids (Mocarski et al., 2013).

Replication, cleavage and packaging of the HCMV DNA genome

HCMV DNA replication takes place at later stages of the lytic infection cycle and occurs by the "rolling-circle mechanism", forming head-to-tail concatemeric and branched DNA molecules. Origins of replication (oriLyt) are the regions of the viral genomes, where viral DNA replication of herpesviruses is initiated. The concatemers are then cleaved and genomes inserted into preformed capsids (Lehman and Boehmer, 1999; Masse et al., 1992; Weller and Coen, 2012). Eleven proteins have been implicated with this process: the DNA polymerase catalytic subunit pUL54, the polymerase accessory protein pUL44, the single-stranded DNA binding protein pUL57, the three subunits of the helicase complex pUL70, pUL102 and pUL105, the immediate-early regulator proteins pUL36-38, IRS-1/TRS-1, IE1, IE2, as well as pUL84 and pUL112-113. Disruption of any of these genes stops the viral replication, therefore, proteins encoded by these genes could be potential targets of CMV inhibitors (Mercorelli et al., 2008; Mocarski et al., 2013). Subsequently, the single-strand DNA-binding protein ICP8, which has a role in DNA-dependent ATPase and DNA helicase activities, binds single-stranded DNA rapidly (Kemble et al., 1987; Simonsson et al., 1998). Two main subunits of the terminase complex, pUL56 and pUL89, bind to the concatemeric DNA as well as to preformed capsid. The DNA is spooled into the capsids through a channel formed by the portal protein, pUL104 (Dittmer and Bogner, 2005). pUL89 has a DNA binding domain, and pUL56 binds to the packaging sequence motifs pac1 and pac2 on the viral genome, to pUL104 on the procapsids and to pUL89 (Bogner et al, 1998; Dittmer et al., 2005; Thoma et al., 2006). Hwang and Bogner reported that pUL56 provides ATPase activity for DNA encapsidation (Hwang and Bogner, 2002). Subsequently, the concatemeric DNA is cut by

pUL89 when a full-length genome is inserted into the capsid (Wang et al., 2017), terminating DNA packaging. Neuber et al. have recently discovered that the third terminase subunit pUL51 is needed as well and interacts efficiently with pUL56 and pUL89 when all three are present at the same time and that these proteins regulate the stability of each other upon forming the terminase complex (Neuber et al., 2017).

Capsid assembly and Nuclear egress

In HCMV, procapsids are formed around virus-encoded scaffold proteins. Components are the small capsid protein, the portal protein, the major capsid protein, the minor capsid protein, and the minor capsid protein binding protein (Gibson, 2008). The capsids that have completed the DNA packaging process are called C-capsids. A-capsids are aberrant capsid forms consisting only of the capsid shell and B-capsids contains both scaffold and the capsid shell, but they are noninfectious, because they contain no viral genome (Irmiere and Gibson, 1983; Irmiere and Gibson, 1985; Mocarski et al., 2013). Mature capsids acquire a primary envelope through budding at the inner nuclear membrane, lose that envelope through fusion at the outer nuclear membrane, are released into the cytoplasm, and acquire their final maturation (tegumentation and secondary envelope) in the cytoplasm, at the viral assembly complex (vAC). The process of envelopment/de-envelopment is facilitated by the "nuclear-egress complex" (NEC), proteins which are encoded by all herpesviruses and conserved in HCMV as well (Alwine, 2012; Hagen et al., 2015; Johnson and Baines, 2011). The HCMV nuclear egress complex consists primarily of pUL50 and pUL53, with pUL97 (the viral kinase) (Milbradt et al., 2018; Sharma et al., 2014), some cellular components such as lamin B receptor, protein kinase C (PKC) and PKC chaperone p32 being associated (Marschall et al., 2017; Park and Baines, 2006; Wang et al., 2014) .

Secondary envelopment and release of virions from infected cells

After entering the cytoplasm, capsids are transferred to the viral assembly compartment, probably by utilizing inner tegument proteins such as the *UL32*-encoded pp150 protein to complete their maturation (AuCoin et al., 2006; Fossum et al., 2009). Tegumentation of the capsids begins after nuclear egress, and is highly crucial, because tegument proteins form a network of protein-protein interaction between the capsid and the envelope glycoproteins. Subsequently, these tegumented viral particles bud into vesicle membranes of the viral assembly complex. These trans-Golgi-derived membranes contain virally encoded glycoproteins. The wrapping of the envelope is such that the outer tegument proteins interact

with cytoplasmic tails of the glycoproteins (Mettenleiter, 2002). The formation of the viral assembly compartment is a highly regulated process which involves many cellular and viral interaction partners and is important for generation of infectious virus progeny. Finally, mature, infectious CMV virions, as well as non-infectious particles are released from the cells by fusion of these vesicles with the plasma membrane (Mocarski et al., 2013).

1.8. Drugs in clinical use for treatment of HCMV disease

Several drugs have been used with variable success to treat patients with acute HCMV infection or HCMV disease, particularly immunosuppressed transplant patients. However, the list of approved antivirals is limited, and most of them have severe side effects such as myelosuppression and nephrotoxicity, and can lead to drug-resistant virus strains, because they all target the same viral process, viral DNA replication. The available drugs to treat clinically evident HCMV disease are the nucleoside analogues ganciclovir – for first-line treatment –, valganciclovir for prophylaxis in solid organ transplant (SOT) recipients, as well as the pyrophosphate analogue foscarnet, and nucleotide analogue cidofovir for preemptive therapy (Biron, 2006).

Nucleoside analogues inhibit the viral DNA polymerase. Ganciclovir (GCV) is a deoxyguanosine analogue of acyclovir and upon entry into infected cells it is monophosphorylated by the HCMV protein kinase pUL97. Eventually, GCV monophosphate is converted into GCV triphosphate by cellular kinases (Littler et al, 1992; Sullivan et al., 1992) . In its active form as GCV triphosphate it is a competitive inhibitor of deoxyguanosine triphosphate (GTP) at the nucleotide binding site of the HCMV DNA polymerase (Crumpacker, 1996; Mocarski et al., 2013; Reid et al., 1988). GCV is limited in use because it requires hospitalization for administrating the drug intravenously for up to six weeks of therapy. Additionally, the utility of GCV is restricted due to side effects such as neutropenia and thrombocytopenia, and emergence of GCV-resistant HCMV mutants (Biron, 2006; Chou, 2008). Mutations in UL97 cause GCV-resistance of clinical HCMV strains, while mutations in the viral DNA polymerase (UL54) increases the resistance to GCV and moreover, provides cross-resistance to cidofovir and foscarnet (Lurain and Chou, 2010). The development of valganciclovir (vGCV) in 1990 allowed the oral administration of this prodrug of GCV, because the addition of an L-valyl ester to the deoxyribose ring increases absorption and bioavailability of the drug. Subsequent to uptake, vGCV is metabolized in the intestines and the liver, which leads to a 10 times higher bioavailability of GCV. Resistance development

and toxicity of vGCV is similar to GCV, because of the same mode of action (Biron, 2006; Chou, 2008; Lukacova et al., 2016).

Foscarnet (FOS), which has the generic name phosphonoformic acid, is a pyrophosphate analogue and is a potent inhibitor of the viral DNA polymerase. Due to its toxicity, it is used only as second-line therapy for treatment of patients with HCMV strains resistant to GCV and vGCV. The mechanism of action of FOS differs from the one of GCV and CDV in that it does not require any activation step to perform its antiviral activity. FOS binds to and blocks the pyrophosphate binding site of the viral polymerase, thereby preventing incorporation of deoxynucleoside triphosphates (dNTPs) (Chrisp and Clissold, 1991). This makes FOS useful in treatment of ACV- and GCV-resistant mutants. FOS is only used in myelosuppressed patients, in AIDS patients with associated HCMV retinitis, in hematopoietic stem cell transplant recipients, and in those with leucopenia. Its adverse effects include nephrotoxicity, genital ulceration in men, and electrolyte disturbance, nausea and seizures (Jayaweera, 1997; Torres et al., 2011). Moreover, FOS must be administered intravenously or by intravitreous injection because of its low bioavailability (Gérard and Salmon-Céron, 1995) .

Cidofovir (CDV) is a monophosphate nucleotide analogue, which has to be phosphorylated to its diphosphate form to perform its antiviral effect. Host cellular kinases are responsible for executing the phosphorylations and this is independent of the virus-induced pUL97 enzyme (Lalezari et al., 1995). CDV causes obligatory chain termination, a mechanism identical to the one used by GCV (Erice, 1999).

Fomivirsen is another FDA approved drug against CMV which was successfully applied for the treatment of CMV retinitis in AIDS patients who had failed prior treatment with ganciclovir, foscarnet, or cidofovir. Unlike other drugs against CMV, fomivirsen disrupts the replication of CMV by an antisense mechanism because as an oligonucleotide it is complementary to the mRNA of the viral IE2 gene. It must be administered intravitreally into the eye. Inflammation of the anterior eye chamber and increased intraocular pressure are the major adverse effects (de Smet et al, 1999).

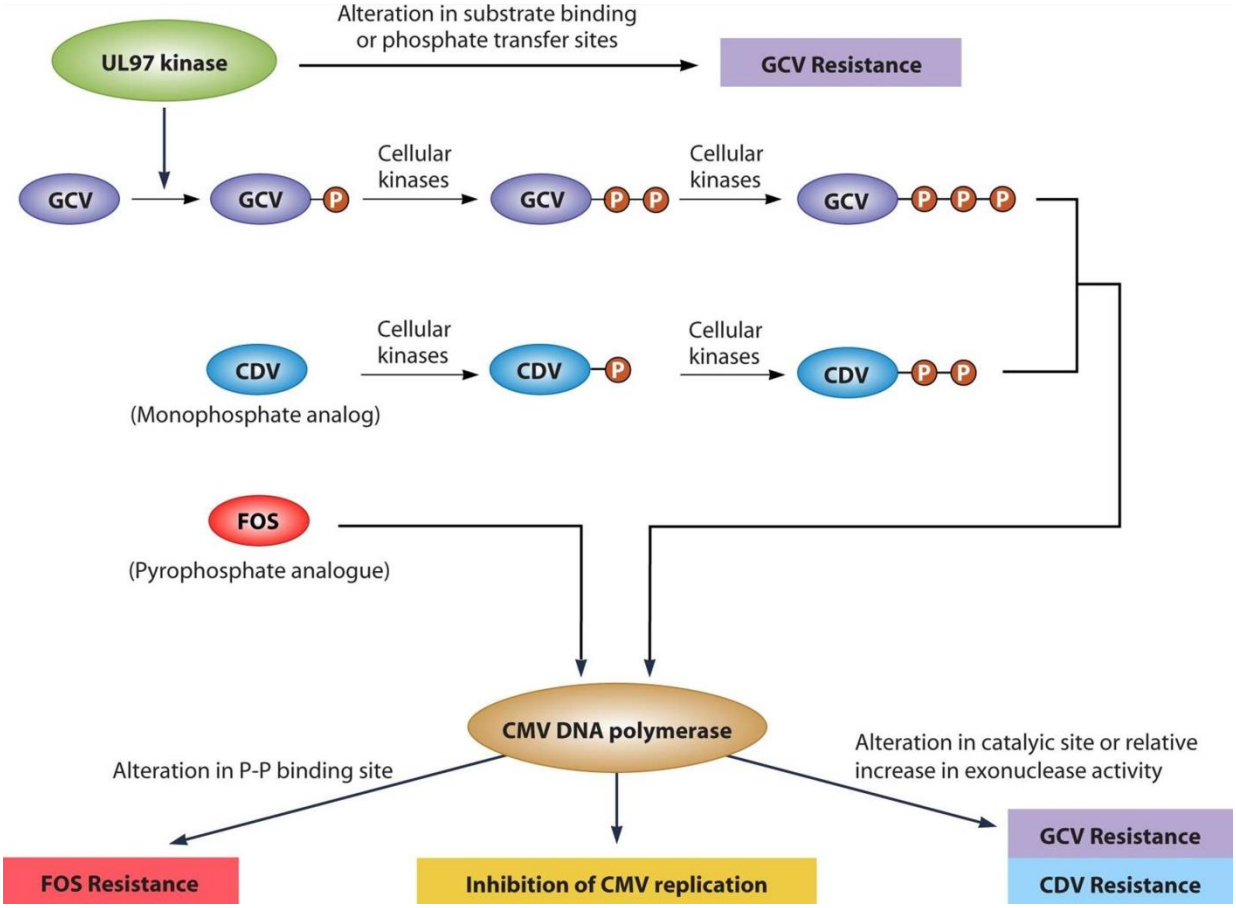


Figure 3. Mode of action of anti-HCMV drugs and development of drug-resistance. Ganciclovir (GCV) and cidofovir (CDV) are phosphorylated by the viral UL97 kinase or cellular kinases or both. In contrast, the pyrophosphate analogue foscarnet (FOS) needs no activation. The CMV DNA polymerase is the target of all three antivirals. Mutations acquired within the DNA polymerase (though at different sites) confer resistance. Image taken from Lurain and Chou (2010) with permission of the publisher (American Society for Microbiology).

1.9. Resistance to anti-HCMV drugs

Following two decades of application of the approved anti-HCMV drugs, many resistant HCMV isolates have been reported. For instance, GCV resistant-HCMV was isolated in vitro after passaging the laboratory strain AD169 in the presence of increasing concentration of GCV (Biron et al., 1986) , and in vivo after first use in immunocompromised patients (Erice et al., 1989). Most of the mutations were detected within the UL97 kinase and UL54 DNA polymerase genes (Chou et al., 2004; Krosky et al, 2003). Through phenotypic and genotypic testing, performed to address the susceptibility of HCMV to antiviral agents, many of the mutations in the UL54 and UL97 genes have been linked to drug resistance. CMV strains with specific mutations in UL97 are resistant to GCV but remain susceptible to FOS and

CDV. Contrary, certain mutations in UL54 provide cross-resistance to GCV and CDV, and CMV strains which contain mutations in both UL97 and UL54 are extremely resistant to GCV (Anwar et al, 1999).

1.10. Other drugs with activity against HCMV, currently not in clinical use

As alternatives, a number of new antivirals with diverse modes of action have been developed and characterized during recent years. Some of these novel CMV agents have proceeded to phase III clinical trials and are actively assessed for treatment of HCMV disease, but are not routinely used in clinics yet. The following are new molecules displaying high efficacy and tolerability.

Maribavir (MBV)

MBV is a benzimidazole molecule which targets the viral pUL97 phosphokinase by competing with ATP binding. As a consequence, viral encapsidation and nuclear egress of viral particles are prevented, meaning that the mechanism of action is not through the viral DNA polymerase (Krosky et al., 2003; Shannon-Lowe and Emery, 2010). Administration of maribavir is oral and it is active against CMV strains resistant to GCV and CDV (Drew et al., 2006). Since the target of MBV is the ATP binding domain of UL97, it may influence the phosphorylation of GCV, which can potentially conflict with co-administration of both drugs at the same time. Interestingly, co-administration of MBV and FOS did not show this effect (Chou and Marousek, 2006). Phase II studies in allograft recipients and phase III trials in allogeneic HSCT recipients were performed and found to be safe (Avery et al., 2010). However, in these studies MBV was apparently used in suboptimal dosing (Frange and Leruez-Ville, 2018). In terms of hematoxicity, it was observed that MBV-treated patients have a lower risk compared to patients receiving GCV or VGCV. Common side effects are altered taste sensation (dysgeusia) at a rate of 40% (Maertens et al., 2016), nausea, and skin disruption; however this occurred only in HIV-infected patients at very high doses (Marty et al., 2011). A study from Winston et al. suggested MBV as a potential drug for prevention of CMV after allogeneic stem cell transplantation (Winston et al., 2008).

Brincidofovir (BCV)

BCV (also called CMX001) is a lipid ester of cidofovir (CDV) and has broad-spectrum antiviral activity in vitro against herpes viruses, adenoviruses, papillomaviruses, smallpox and polyomaviruses (Dropulic and Cohen, 2010). BCV has high oral bioavailability and a long

half-life (4 - 6.5 days), i.e. it remains intact in plasma for a substantial time period (Prasad et al., 2014). The mode of action of BCV and CDV is the same, they compete with the incorporation of deoxycytidine triphosphate (dCTP) into viral DNA, terminating chain elongation. BCV delivers high levels of CDV diphosphate to the cells (up to 1,000-fold compared to CDV) (James et al., 2013). The nephrotoxicity of BCV in patients is lower compared to CDV. HCMV strains carrying mutations in UL97 or UL54 and being resistant against other drugs remain susceptible to BCV in vitro. Therefore it is considered for use against GCV-resistant HCMVs (Price and Prichard, 2011). A phase II study revealed that patients receiving HSC allografts and BCV treatment did not display any risk of nephro- and hematotoxicity and only suffered from diarrhea, but demonstrated acute graft-versus-host disease (GVHD) and a nonsignificantly increased risk of death (Marty et al., 2016, 2013). Other adverse effects included abdominal discomfort and stomatitis (Hostetler, 2009).

Cyclopropavir (CPV)

CPV (or MBX400) is the lead compound of methylenecyclopropane analogues, and is effective against all human herpesviruses (Kern et al., 2005; Zhou et al., 2004). The mechanism of action of this inhibitor was solved upon discovery of a recombinant HCMV strain displaying resistance to CPV, which turned out to carry a large deletion in the UL97 phosphotransferase gene. Not surprising, this virus was also resistant to both GCV and MBV (Kern et al., 2005). CPV has a higher affinity for the UL97 kinase than GCV (Gentry et al., 2010). After phosphorylation of CPV by the UL97 kinase, a guanosine monophosphate kinase phosphorylates CPV monophosphate to CPV triphosphate. Subsequently, the viral DNA polymerase is inhibited (Kern et al., 2005). Most GCV-resistant clinical isolates are susceptible to CPV. Interestingly, a frameshift mutation in the UL27 ORF of a CPV-resistant strain has been identified by deep sequencing suggesting that UL27 can modulate or substitute for the function of the UL97 kinase (James et al., 2011). Pharmacokinetic studies of CPV in vivo revealed good oral bioavailability and favorable toxicology data in dogs and rats (Bowlin, Brooks, and Zemlicka, 2009). A phase 1A study has been performed in normal volunteers receiving various doses of CPV and no considerable side effects have been reported (Komaroff et al., 2016).

Letermovir (AIC246)

Letermovir is a new anti-CMV drug that may prevail the issues of prevention and treatment of CMV. The activity of this drug is highly specific to CMV, and is unique compared to other

currently available antivirals against CMV. It inhibits the viral terminase (Bowman et al., 2017) by specifically interacting with the pUL56 subunit of the terminase complex, at a step subsequent to viral DNA replication (Goldner et al., 2011). The HCMV terminase complex is an interesting target for antiviral therapy because it has a low risk of target-related toxicities in humans as there is no related enzyme in mammalian cells (Ligat et al., 2018). Resistance to letermovir has been studied in vitro and in vivo and was demonstrated to result from amino acid substitution in the terminase subunit UL56 (Goldner et al. 2011, 2015). Notably, letermovir-resistant HCMV mutants did not demonstrate cross-resistance against other terminase inhibitors, e.g. those of the sulfonamide or benzimidazole classes (Goldner et al., 2011). The antiviral efficacy of letermovir has been studied in vitro by several assays using various HCMV variants (Marschall et al., 2012), and was found to be 1000-fold higher than GCV. The approximate 50% effective concentration (EC_{50}) value for letermovir in cell culture was ~4.5 nM versus 2 µM for GCV (i.e. ˜400-fold lower) (Lischka et al., 2010). The EC_{50} values were in a comparable range for various fibroblast lines, for instance NHDF, HFF, NHLF and embryonic lung fibroblasts (Lischka et al., 2010). The CC_{50} values ranged from 27 to 30 µM in liver and kidney epithelial cells, heart muscle cells, fibroblasts derived from embryos and dermis, monocytes, T-lymphocytes, macrophages, and neuroblastoma and hepatoma cells (EPAR_Public_assessment report, 2017). The selectivity index for letermovir was estimated to exceed 15,000, combining great activity with least toxicity. Administration of letermovir is either intravenous or oral, and in both cases, absorption of letermovir in the body is rapid (Bowman et al., 2017). Clinical efficacy of letermovir was studied in clinical phase I, II, IIa and III trails and turned out to be extremely promising when used in patients carrying multidrug-resistant CMVs (Kaul et al., 2011), in CMV-seropositive allogeneic HSCT recipients (Chemaly et al., 2014), and in transplant recipients with active CMV replication (Stoelben et al., 2014). A phase III randomized, placebo-controlled clinical trials confirmed letermovir to be safe and efficient for preventing clinically significant HCMV infection in HSC transplanted adults (Frange and Leruez-Ville, 2018). Two major adverse effects of available CMV antivirals, hepato- and nephrotoxicities, were not documented with letermovir. Only five mild adverse effects including shortness of breath (dyspnea), nasopharyngitis, gastroenteritis and high levels of creatinine were possibly related to letermovir (Bowman et al., 2017).

1.11. Drugs that are approved for treatment of other diseases and are not specific for CMV

Several drugs that were originally developed for treatment of other diseases were found to have activity against HCMV (Chaer et al., 2016). These drugs were only used for patients with multidrug-resistant HCMV strains or patients who could not use one or more of the approved drugs.

Leflunomide

This drug is approved for the treatment of autoimmune conditions such as rheumatoid arthritis, but it has also shown anti-HCMV effects (Lurain and Chou, 2010). It can possibly be used as an add-on therapy to other antiviral therapies (Chaer et al., 2016). The mechanism of action is by inhibition of protein kinase activity and pyrimidine synthesis. Upon treatment of infected cells, the acquisition of tegument proteins to viral nucleocapsids is prevented, thus disrupting virion assembly (Bernhoff et al., 2010; Waldman et al., 1999). In SOT recipients, the results have been positive, but not in HSCT recipients (Battiwalla et al., 2007). However, severe adverse effects include teratogenicity (growth and mental development disorders) and hepatotoxicity are known for this drug (Sudarsanam et al., 2006).

Artesunate (ART)

ART is originally an antimalarial drug which has shown antiviral activity against GCV-resistant and wild-type HCMV in vitro (Kaptein et al., 2006; Schreiber et al., 2009). The mechanism of action was suggested to be via repression of Nf-κB and Sp1 activation pathways, thus inhibiting central regulatory processes of the HCMV infection cycle (Efferth et al., 2008). The drug is currently undergoing clinical phase III trial in stem cell transplant recipients for pre-emptive treatment (Michael Y Shapira, 2010).

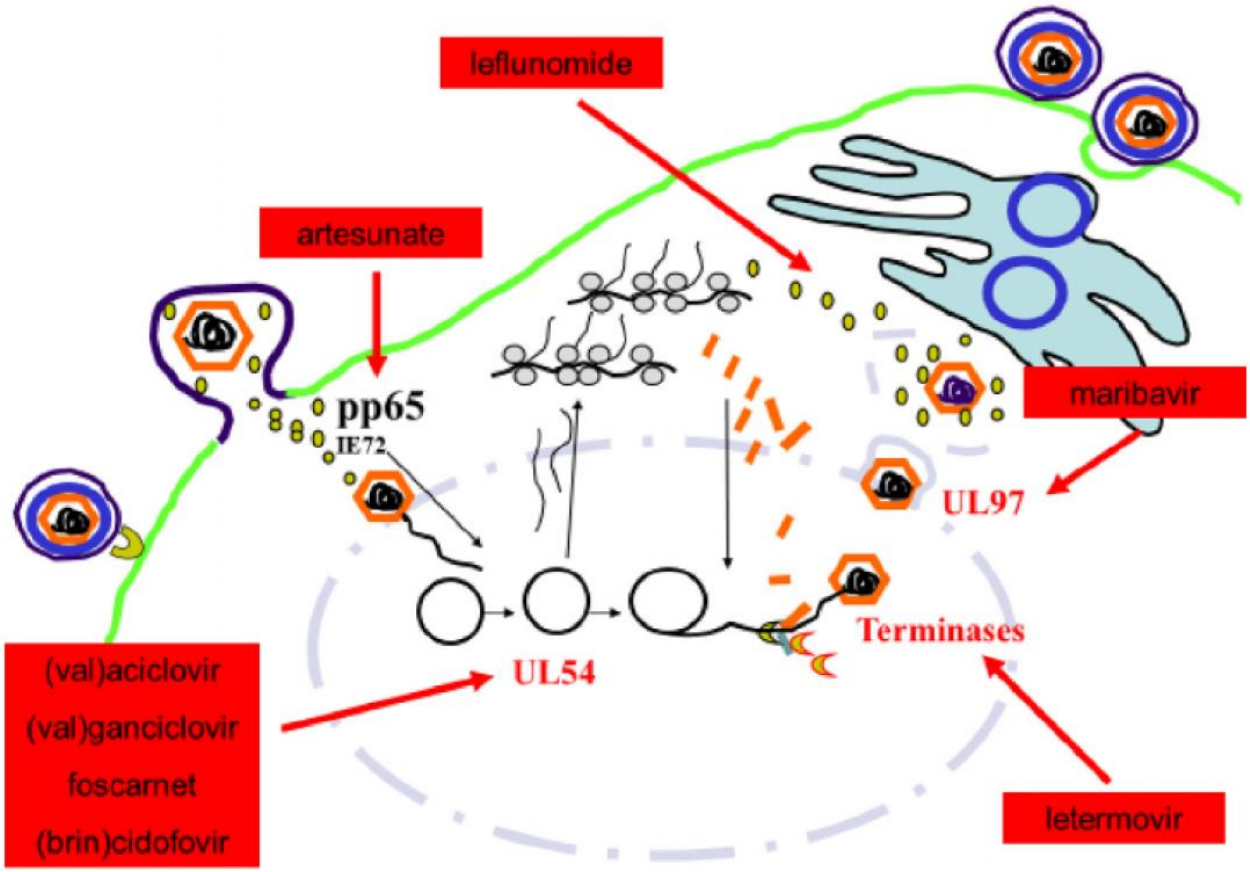


Figure 4. Targets of various drugs inhibiting HCMV. Nucleoside analogue inhibitors act by inhibiting the viral DNA polymerase, maribavir competes with the binding of ATP to the UL97 protein kinase, and letermovir inhibits the viral terminase by targeting the UL56 subunit. Leflunomide treatment blocks virion assembly by preventing the acquisition of tegument proteins to nucleocapsids, and Artesunate interferes with DNA-binding factors such as Nf-κB.

Reproduced from *Frange, P. and M. Leruez-Ville. Maribavir, Brincidofovir and Letermovir: Efficacy and Safety of New Antiviral Drugs for Treating Cytomegalovirus Infections. Med Mal Infect. 2018; 48(8):495-502.*

Taken together, although there are a number of antivirals available against HCMV, none of them seems to be ideal (perhaps with the exception of letermovir), and even the medication in clinical use for treatment of HCMV infection is affected with side effects, limiting their application.

Aims of the Study

The development of novel antiviral drugs for treatment of HCMV infection and disease is still of fundamental importance. All the approved medications that are currently used against HCMV are plagued by adverse effects, either at some point of the clinical application, or concerning the risk of the potential development of antiviral resistance. Therefore, the necessity to identify and characterize newer drug candidates against HCMV is significant. Towards this goal, the following main objectives were addressed in this study:

i. Establishing a highly robust assay allowing to screen for CMV inhibitors
ii. High-content screening of a large library of synthetic compounds to identify hits with antiviral activity against HCMV
iii. To confirm the initial hits, and validate them according to defined criteria in order to select the most promising ones as lead hits
iv. The characterization of the mode of action of the interesting drug candidates, particularly to define the phase of the HCMV infection phase which is blocked.

2. Materials and Methods

2.1. Materials

Buffers and solutions

General buffers

PBS	14 mM NaCl 2.7 mM KCl 2 mM $MgCl_2$ 1.5 mM KH_2PO_4 1.2 mM $CaCl_2$
PBS-T	PBS 0.2% (v/v) Tween-20
Cell lysis buffer	25% (v/v) 4xRoti-Load 1 (Roth) in PBS

Buffers for protein biochemistry

TBS, pH 7.4	50 mM Tris 150 mM NaCl
TBS-T	TBS 0.2% Tween-20
SDS-PAGE Running buffer	24.7 mM Tris pH 8.7 192 mM glycine 1% SDS
Western blot transfer buffer:	24.7 mM Tris 186.6 mM glycine 20% (v/v) methanol
10% Separation gel	12% Acrylamide 400 mM Tris pH 8.8 0,1% SDS 0.1% TEMED 0.5% APS

Stacking gel	3.5% Acrylamide 100 mM Tris pH 6.8 0.1% SDS 0.01% Bromphenole blue 0.1 % TEMED 0.5% APS

Buffers and solutions for cell culture work

Cell freezing medium	80% (v/v) FCS 20% (v/v) DMSO
MTT stock solution (ROTH)	0.2% Thiazolylblue in PBS
Crystal violet stock solution (Sigma)	1% Crystal violet in PBS (w/v) 27% Formaldehyde
PFA (paraformaldehyd [Sigma])	3% (w/v) paraformaldehyde in PBS
Carboxymethyl cellulose (CMC) medium:	1% CMC in ddH_2O 5% (v/v) ml FCS 10% 10x DMEM 10 mM Folic acid 48 mM $NaHCO_3$ 9.7 mM D-Glucose 1% P/S stock solution

Antibodies

Primary antibodies

Specificity [Antigen]	**Origin**	**Dilution used in application**	**Source**
IE1	mouse	1:100	Perkin Elmer
UL44	mouse	1:400	B. Plachter, Mainz
UL52	mouse	1:200	E. Borst, MHH
GAPDH	rabbit	1:1000	Cell Signalling

Secondary antibodies

Antigen	Origin	Dilution/Application	Source
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	rabbit	1:10000	Dako
Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	goat	1:10000	Dako

Cells

Cell Type	Source	Complete medium
Human foreskin fibroblasts (HFF)	provided by Eva Borst	DMEM (Sigma) 10% FCS 100 U/ml penicillin 0.1 mg/ml streptomycin 2 mM L-glutamine
Normal human dermal fibroblasts (nHDF)	Promocell	DMEM (Sigma) 10% FCS 100 U/ml penicillin 0.1 mg/ml streptomycin 2 mM L-glutamine
Retinal pigmented epithelium cells (RPE)	provided by Eva Borst	DMEM - Ham's F12 (Sigma) 10% FCS 41.45 mM sodium bicarbonate
Mouse Embryonic Fibroblasts (MEF) (from Balb/c mice)	provided by Lars Steinbrück	DMEM (Sigma) 10% FCS 100 U/ml penicillin 0.1 mg/ml streptomycin 2 mM L-glutamine

Machines and Equipment

Name	Company
Biomek FX^P Automated Workstation	Beckman Coulter, Indianapolis; IN, USA
BioTek™ Cytation™ 3 Cell Imaging Multi-Mod	BioTek, VT, USA
Synergy™ 2 Multi-Mode Microplate Reader	BioTek, VT, USA
NanoDrop 1000	ThermoScientific, Waltham, MA, USA
LAS 3000 Imager	Fujifilm, Düsseldorf
Optima L-90K Ultracentrifuge	Beckman Coulter, Brea; CA, USA
Avanti-J25 Centrifuge	Beckman Coulter, Brea; CA, USA
qTOWER³	Analytik Jena, Jena
8 Channel Decapper	ThermoScientific, Waltham, MA, USA
Ultrasonics Sonifier S-250A	Branson Ultrasonics, Danbury, CT, USA
Applied Biosystems® 7500 Real-Time PCR	Thermo Scientific, Waltham, MA, USA
HeraeMultifuge 3SR+ Centrifuge	Thermo Scientific, Waltham, MA, USA

Compounds

Three libraries of synthetic compounds were purchased from ChemDiv, Inc., and Enamine as follows:

Company	Number of compounds	Library
ChemDiv	3300	1st Lib.
Enamine	3300	1st Lib.
Enamine	6048	2nd Lib.
ChemDiv	6000	3rd Lib.
ChemDiv	31	1st Lib. : Reordered
Enamine	20	1st Lib. : Reordered
Enamine	79	2nd Lib. : Reordered
ChemDiv	60	3nd Lib.: Reordered

Test inhibitors

Where indicated in experiments, the cells were treated with the test inhibitors at indicated concentrations. The inhibitors were dissolved in the cell growth medium in which the cells were maintained.

Inhibitor	Stock concentration	Supplier	Prepared in
Cycloheximide	10 mg/ml	Sigma	sterile dH_2O
PAA	5 mg/ml	Sigma	DMEM (without serum), pH 7.4, sterilized
Letermovir	10 mM	AiCuris and Merck & Co, Inc	diH_2O
Foscarnet	250 mM	Sigma	diH_2O
Nocodazole	25 mM	Sigma	DMSO

Oligonucleotide primers

Primer	Sequence 5'→3'
UL54 short forward	cggatcttcggctacctcgtag
UL54 short reverse	agaggagagcaacaggagagga

GFP-expressing cytomegalovirus

The GFP-CMV virus was constructed by Eva Borst using a BAC-based mutagenesis technique (Borst and Messerle, 2000), and is called HT8-GFP in this study. The GFP gene was integrated into the genome of the AD169 HCMV strain, replacing the non-essential genes US7 to US12 (Figure1). Expression of GFP is under the control of the major immediate-early promoter/enhancer of CMV (MIEP).

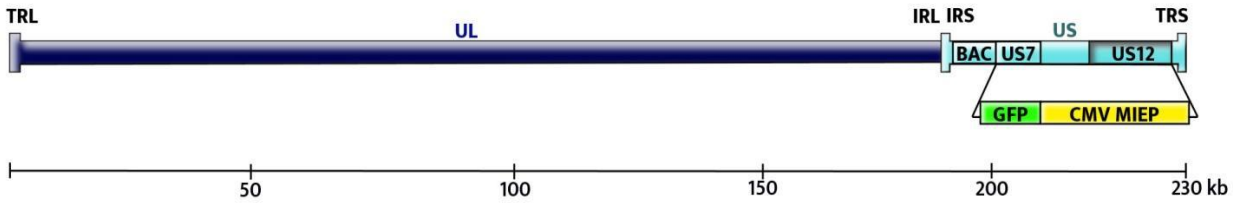


Figure 5. Structure of the HTH8-GFP

2.2. Methods

Cell culture

Cell lines were maintained in appropriate media at 37°C in incubator with humidified atmosphere and 5% CO_2. For cryopreservation, cells were frozen in cell freezing media when they were 80% confluent and stored in liquid nitrogen.

Methods for measuring cell viability

MTT assay

The viability of various cell lines was evaluated using the MTT assay. This assay is based on the ability of cells to metabolize the MTT tetrazolium dye into insoluble formazan crystals, which have a purple color. Compounds were added to the cells and after the incubation period (3-6 days depending on the assay), cells were washed with PBS and MTT solution (0.8 mg/ml) was added to the samples. After 90 min the solution was discarded and cells with formazan crystals were re-suspended in isopropyl alcohol. The absorbance was measured at 570 and 630 nm using a plate reader (BioTek).

Crystal violet staining

This assay is an indirect quantification of cell death and measures the cells that are attached to the plates. Crystal violet solution (1 mg/ml) was added to cells fixed with PFA and incubated for 20 min. The solution was removed and plates were washed gently with water. Plates were dried until the next day. Ethanol was added to dissolve the cells and mixed few times. Finally, the absorbance at 590 nm was measured by the micro-plate reader.

ATP assay

nHDF cells were plated into 96-well plates at a density of 1.5×10^4 cells/well and incubated with different doses of the compounds for 72 h at 37°C under standard growth conditions. Cellular viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The method is based on quantitation of ATP released from live cells upon lysis. Compound doses were evaluated in duplicates, and mean values for duplicate wells were determined and compared to the mean value for the DMSO treated wells.

Assessment of apoptotic cells by DAPI staining

nHDF cells were infected with HT8-GFP and treated with the test substances at various concentrations. At the end of the incubation period, cells were fixed with 3%

paraformaldehyde and permeabilised with 0.1 % Triton X-100 in PBS The cells were then stained with DAPI solution in PBS (10 ng/ml) for 20 min at room temperature protected from light. The fluorescence levels were measured by the plate reader (excitation/emission 360/460 nm), and images of the cells were taken to assess their morphology.

Screening of the Synthetic compound libraries

4000 nHDF cells were seeded into black 384-well plates with transparent bottom (Corning) 24 h before treatment. Using the robots 8 spin head, prepared aliquots of cycloheximide and nocodazole were transferred from the screening tubes (Thermo Matrix 2D) into 96-deep well plates. Master plates were produced by adding the compounds (10 µM) and the virus (MOI 0.05) into the 96-well deep plates. The virus was then mixed with the compounds in the master plate and distributed to the cells seeded in the 384-well plate in 4 replicates (Figure 11). Six days after infection growth media was removed from the cells. For the 1st library the cells were fixed with PFA and the GFP expression was measured at wavelengths of 485 and 516 nm. The viability assay with crystal violet was applied as described above. For the 2nd and 3rd library, as well as the rescreens, cell media were replaced with PBS. The GFP intensity was measured and the MTT viability assay was used to analyze the influence of the compounds on cell viability. The absorbance levels were measured with the plate reader (BioTek) and the data was transferred to CYAME program for data analysis. The screening of the compounds was performed weekly with twelve to eighteen 384-well plates each week.

Virological methods

Preparation of HCMV stocks

Ten to fourteen 15 cm dishes of subconfluent nHDF cells were infected with HCMV at an MOI of 0.1 pfu/cell and maintained under standard growth conditions until the cells reached 100% cytopathic effect. When necessary, fresh medium was added during incubation time. At the time of harvesting, about 90% of the cells were detached. Cells and medium were harvested and spun at 6,400 x g for 20 min at 4°C to pellet cell debris. The supernatant was then transferred into ultracentrifuge buckets and spun at 15,000 x g for 4 h 15 min at 4°C. The virus pellet was re-suspended in DMEM supplemented with 20% FCS. The suspension was dounced 15 times, aliquoted and stored at -80°C.

HCMV-GFP fluorescence reduction assay (GFP-RA)

Cells infected with HT8-GFP were used to perform the GFP based fluorescence reduction assay. nHDF cells were inoculated with HT8-GFP virus at the MOI indicated for the

respective experiments. After 3 h, inocula were removed and cells were incubated with compounds diluted in complete growth media. Depending on the assay, after 3-8 days, the medium was replaced with PBS and GFP fluorescence intensity of the infected cells was measured using a plate reader.

Titer reduction studies

To measure virus titer reduction, 48-well plates with 50,000 nHDF or HFF cells were grown overnight and then infected at MOI of 0.5 with HT8-GFP HCMV for 3 hours. The supernatant was removed and cells were treated with indicated compounds. The progeny virus was harvested after 5 days and titers were calculated using either $TCID_{50}$ assay or plaque assay.

50% tissue culture infective dose ($TCID_{50}$) assay

Viral titers in cell supernatants were determined by $TCID_{50}$. 10-fold serial dilutions of the cell supernatants were prepared in 96-well plates and added to nHDF or HFF cells. $TCID_{50}$/ml was measured by the viral titer of each sample, taking into account the final dilution still expressing GFP. Spearman & Kärber algorithm online platform was used for calculating the $TCID_{50}$/ml (Mahy and Kangro, 1996).

Plaque assay

To determine the number of infectious virus particles in virus stocks or cell supernatants, a standard plaque assay was used. The titration was performed by serial dilutions (factor 10) of the virus in culture medium. nHDF or HFF cells (50,000/well) were seeded into a 48-well plate. The samples were titrated in duplicates and triplicates for plaque assay measuring dose responses and virus stocks, respectively. One day after cell seeding, growth media were removed and 100 µl of the dilution series was added to the cells. After 3 hour incubation at 37°C with gentle shaking, the inoculum was aspirated and replaced with 500 µl of CMC medium to ensure that spread of infection occurs from cell to cell only. The plaques were counted manually after 10-14 days of incubation at 37°C using a fluorescence microscope.
Virus titers were calculated using the following equation:

$$Titer\left(\frac{PFU}{ml}\right) = \frac{M \times dilution\ factor}{volume}$$

M= mean value of the counted plaques

Time-of-addition assay

15,000 nHDF cells/well were seeded on 96-well plate one day prior to the infection. HT8-GFP HCMV was diluted in growth media and added to cells at MOI 0.5. The supernatant was removed and replaced with the indicated compounds either 2 h before infection (-2 h), together with virus (0 h) or 2, 6, 24, 48 h.p.i. as shown in Figure 25. After 72 hours, cells were washed with PBS and GFP signal intensity was measured.

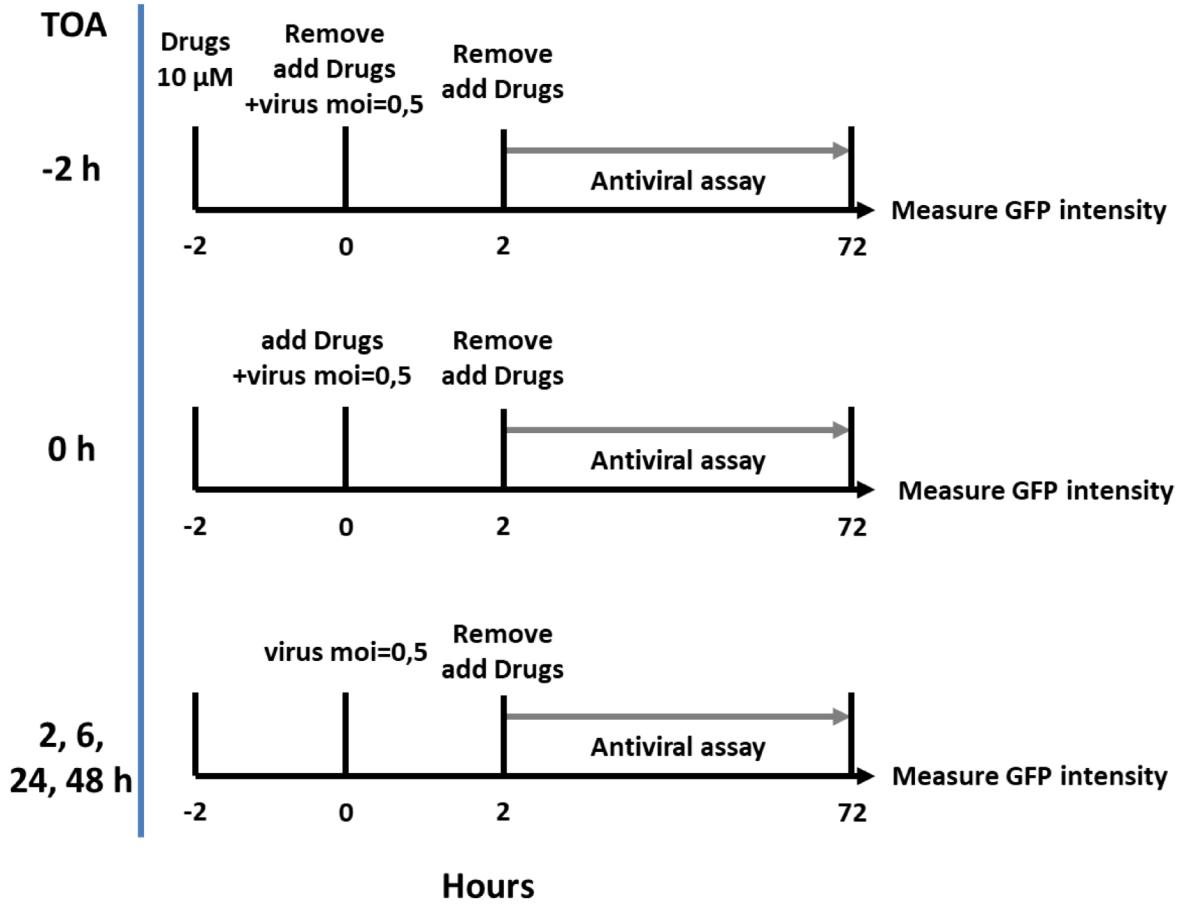


Figure 6. Schematic representation of the time-of-addition (TOA) assay

Kinetic Reverse Block Assay (KRB)

For determination of the reversibility of the effects of compounds, a KRB assay was developed.

nHDF cells (1.5×10^4 cells/well) were seeded in 96-well plates and infected with HT8-GPF at an MOI of 0.5. After virus adsorption for 3 hours, cells were treated with the compounds. Three wells were kept untreated and served as a control. 96 h after treatment, supernatants were removed, cells were washed two times with PBS and further incubated in drug-free medium. Supernatants of infected cells were collected before addition of compounds (0 h) or

24, 48, and 72 h after their removal (post release). Virus titer in collected samples was determined via a GFP-based yield reduction assay. nHDF cells were infected with the collected supernatants for 3 hours, and then the supernatants were removed and replaced with fresh medium. GFP intensity was measured 144 h.p.i.

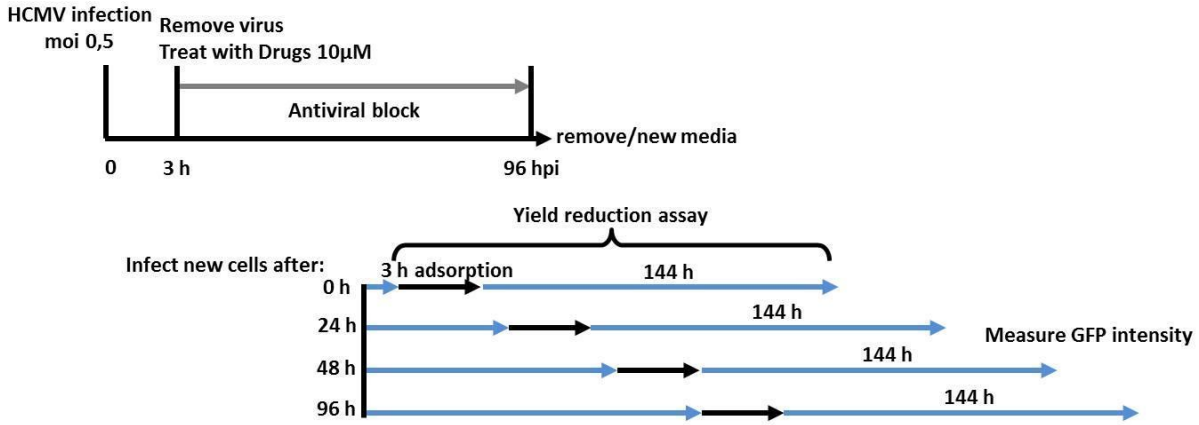


Figure 7. Schematic representation of the KRB assay

Flow cytometry assay

nHDF cells (15 $\times 10^4$ cells/well) were seeded in 12-well plates and infected with HT8-GPF at an MOI of 1. After virus adsorption for 3 hours, cells were treated with the compound. 48 h.p.i the cells were trypsinized, fixed with 3% PFA and resuspended in PBS with 2 mM EDTA and 6 % goat serum. Flow cytometry was performed on a Beckman Coulter FC500 flow cytometer. Number of GFP positive cells was calculated using Kaluza software and application of a gating strategy that excluded 99.9% of the uninfected cells.

qPCR Method

DNA extraction from cells

nHDF cells (1×10^5 cells/well) in 24-well plates were infected with HT8-GPF at an MOI of 0.6 and medium was replaced by compounds after 3 hours. Cells were scraped in 200 µM PBS and DNA purification was performed using the DNeasy Blood & Tissue Kit (Qiagen), following the companies instructions.

DNA quantification by qPCR

Viral DNA amounts were analyzed by quantitative PCR (qPCR). The reaction was performed using 1:100 dilutions of the DNA samples described above and the Luna Universal qPCR master mix (NEB) analyzed kit, and analyzed using the qTower3. Measurement of each

sample was done in triplicates. Amplification of the CRP gene served as an internal control. The data was analyzed using qPCR soft 3.4 and GraphPad Prism 5

qPCR program

50 ºC – 2 minutes

95 ºC – 10 minutes

95 ºC – 15 seconds
60 ºC – 1 minute
} 41×

95 ºC – 30 seconds

60 ºC – 15 seconds

End

Protein Biochemistry Methods

Preparation of cell lysates and SDS-PAGE

Cells were washed with PBS scraped in cell-lysis buffer and placed on ice. Next, lysates were sonicated for 5 seconds, boiled for 5 min at 99 ºC and stored at -20 ºC until needed. After thawing, lysates were vigorously vortexed and precleared by centrifugation. Proteins were loaded on a hand-casted 10% acrylamide gel and separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Electrophoresis was performed using the Mini-PROTEAN electrophoresis cell (Bio-Rad) filled with electrophoresis buffer at a constant voltage setting (100 V). The Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific) was used to follow protein separation.

Protein Transfer (Immunoblotting)

After separation, the wet-method was used to transfer proteins to a nitrocellulose membrane (GE Healthcare). The transfer was applied at constant current setting (250 mA) over a period of 70 min using the Mini-PROTEAN Tetra System Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked in 5% (w/v) skim milk in TBS-T. Subsequently, the membranes were cut and incubated with the indicated primary antibodies overnight at 4°C, followed by the incubation with HRP conjugated rabbit anti-mouse or goat anti-rabbit antibodies for 1 h at room temperature. After incubations with each antibody membranes were washed 3 times in PBS-T buffer for 5 min. The signals were detected by chemiluminescence using the SuperSignal West Femto detection reagent (ThermoFisher) and the LAS3000 imaging system machine.

Data analysis

The relative absorbance and fluorescence units were expressed as ratio of compound treated cells to DMSO treated cells and plotted against the compound concentrations on a logarithmic scale. The 50% effective dose of the compounds that inhibited HCMV replication (IC_{50}) was calculated from dose response curves based on GFP levels. Similarly, the 50% effective dose of the compounds that induces cytotoxicity (CC_{50}) was calculated and compared to the value of DMSO-treated cells. These values were calculated using nonlinear regression curve fitting of the logarithmic values of the inhibitor concentrations and the response values by applying a variable slope model. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) was used for all analyses.

The CYAME platform (Can You Analyze My Experiment) was employed as the main tool for data analysis of screening results. It allowed to calculate the Z' factor, strictly standardized mean difference SSMD (an alternative quality control method), median and mean values for GFP and Crystal violet/MTT measurements. This platform helped to detect and select hits based on infectivity, Z-score, and viability parameters.

3. Results

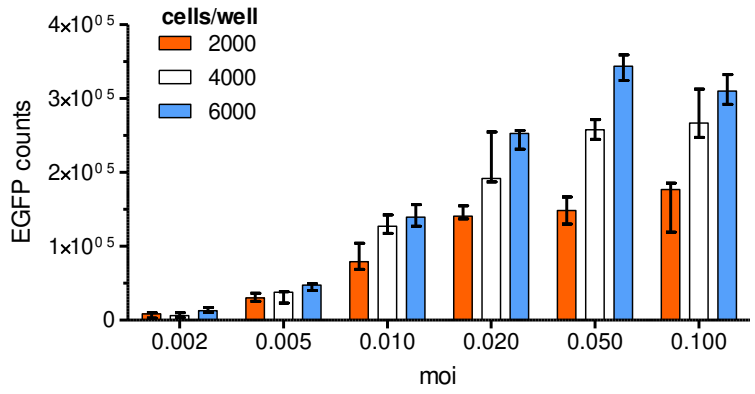
3.1. Establishing a screening assay for identification of CMV inhibitors

In order to identify small molecule inhibitors of HCMV a straightforward assay was designed and optimized in small-scale format useful for "high-throughput". To develop a robust GFP-based CPE-reduction high-throughput assay, two sets of experiments were done in 384-well format plates.

Optimizing the conditions of the assay and its quality control

In order to establish conditions that allow detecting all kind of inhibitors – independent of which step of the infection cycle is blocked, and to avoid too many laborious time-consuming steps, a single step infection approach was used. The laboratory HCMV strain pHG-1 used expresses the green fluorescent protein (GFP) at immediate-early times of infection, and nHDF cells were chosen for *in vitro* infection. To find the optimal virus dose and cell density, infection with HCMV was performed using increasing multiplicities of infection (MOI) and increasing numbers of cells/well. This approach allowed to calculate the Z' factor – a key parameter indicating whether an assay is robust enough to be applied in a large-scale screen (Zhang et al., 1999). It is a statistical parameter that is used in HTS to evaluate if the assay is effective or not and it shows the quality and reproducibility of an assay. If the assay has a Z' factor between 0.5 and 1, it is considered to be excellent, meaning that it has a large dynamic range (difference in average signals between the positive and negative controls) and a very narrow standard deviation (SD); theoretically an assay is perfect when the Z factor is approaching a value of 1. Therefore optimization of the assay was done before proceeding to screening of the compound libraries. As it is shown in Figure 8A, both increasing the cell number as well as increasing the multiplicity of infection led to higher GFP expression, i.e. higher signals that could be measured. The calculated Z' factor from the same experiment showed values of 0.78-0.86, when using MOIs between 0.01 and 0.1, with a maximum of the Z' factor of 0.93 when using MOIs of 0.02 to 0.05 and 4000 to 6000 cells/well. Photos taken with the fluorescence microscope revealed that cultures with 4000 cells/well provided GFP signals which were not completely saturated compared to signals of cultures with 6000 cells/well (Supplementary Figure 1). As a result, 4000 cells/well were chosen for further experiments.

A)



B)

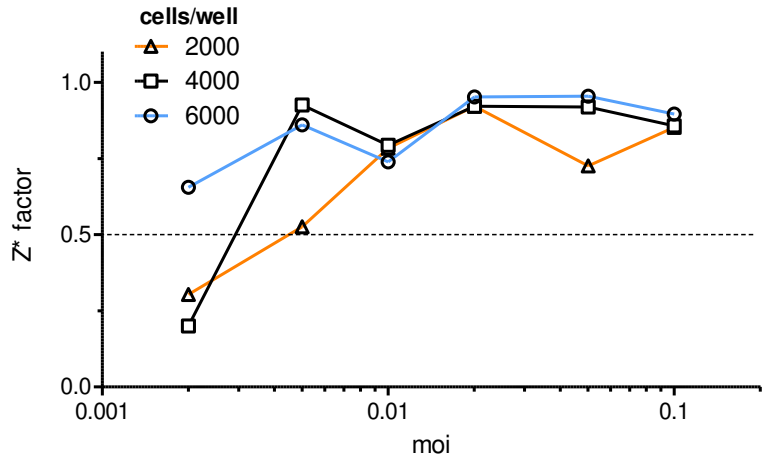
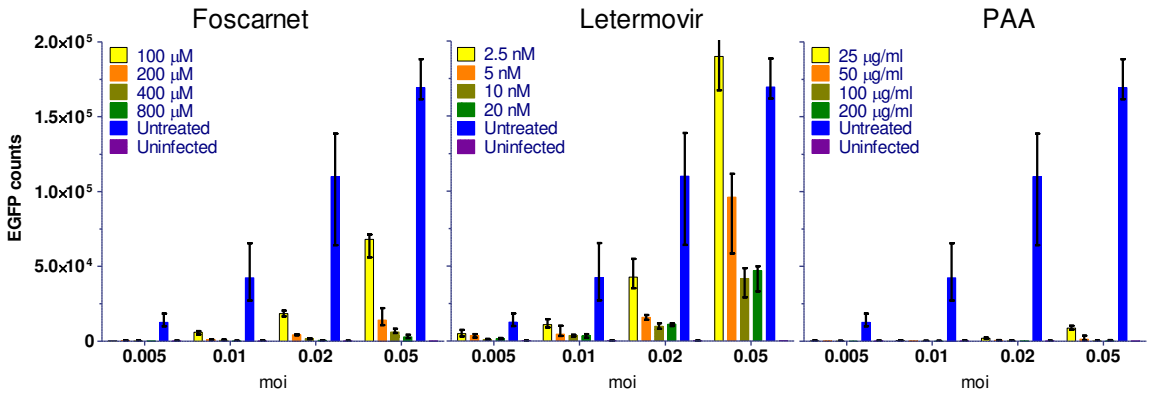


Figure 8. Testing the dependence of the assay fitness on the number of cells seeded and viral dose (MOI) used. A) shows the GFP signals in the infected cultures for 2000 (orange bars), 4000 (white bars) and 6000 (blue bars) cells/well following infection with CMV at increasing MOI (0.002, 0.005, 0.01, 0.02, 0.05 or 0.1 PFU/cell). The error bars indicate standard deviation of 5 biological replicates. B) The Z‘ factors were calculated using the GFP signals from the same experiment.

To test if the determined conditions can be used in the screening for inhibitors and to confirm the multiplicity of infection that should be used in the screen, an experiment was performed using three test substances (foscarnet, letermovir, PAA) with two distinct modes of action (viral DNA polymerase inhibitor and genome encapsidation inhibitor) and various MOIs (Figure 9). The following experiment revealed that using MOI 0.05 led to a larger dynamic range between controls and the treated samples and thus appeared optimal for the screening. Additionally, the calculated Z’ factor values showed excellent robustness of the assay, reaching 0.6 for letermovir and 0.75 for foscarnet and PAA at concentrations that led to effective inhibition of HCMV.

A)



B)

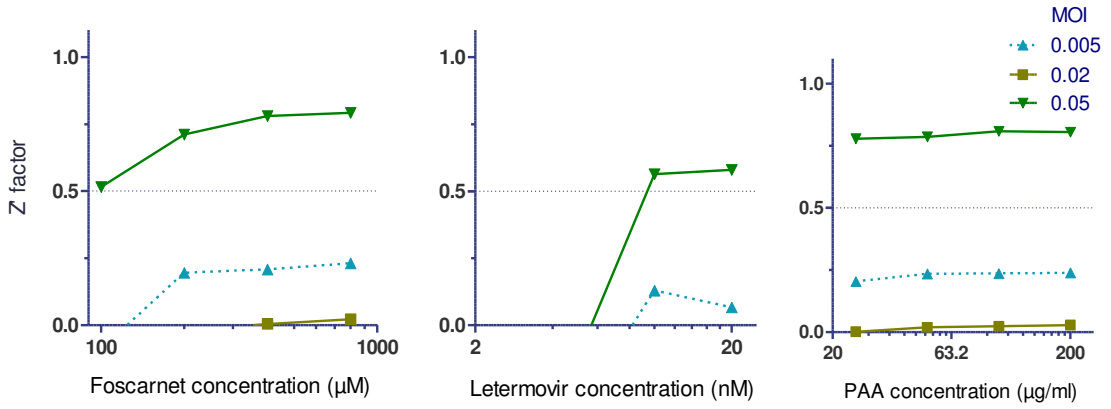


Figure 9. Test of the optimized assay conditions using known inhibitors. A) Counts of GFP expression in CMV infected cells upon treatment with the indicated inhibitor at varying concentrations and dependence from increasing viral dose was measured and plotted. PAA represents phosphonoacetic acid. Results are expressed as means ± standard deviations (SD) of 5 wells for a representative experiment. B) The Z‘ factor was calculated using the GFP signals from the same experiment.

Based on these results the final scheme for the screening of the compound libraries was devised as shown in Figure 10. After infection of the nHDF cells with an MOI of 0.05, the initially infected cells generate new infectious virions at around 3 days post infection. Subsequently, the newly produced viruses spread and infect uninfected neighboring cells. After 6 days two rounds of infection are completed and the number of infected cells reaches its maximum. If a compound is able to inhibit the infection, then there will be no spread to neighboring cells and the GFP signal will be diminished.

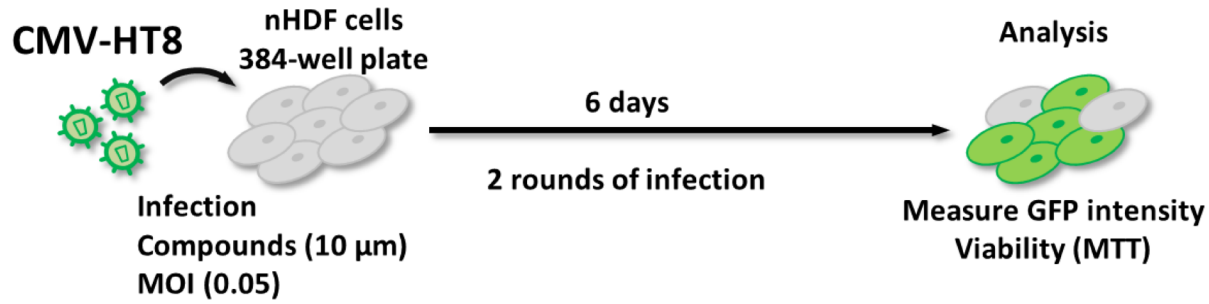


Figure 10. Overview of the CMV inhibitor screening assay. The single step infection approach used for the screening. 4000 NHDF cells were seeded per well on 384-well plates 1 day prior to the infection, then infected with a GFP expressing CMV (MOI: 0.05) and simultaneously incubated with the test compounds for 6 days. Cells were assayed for GFP expression and, in parallel, for compound-related cytotoxicity using the MTT assay.

Randomization of the controls and test run

To decide whether a compound inhibits HCMV infection, positive and negative controls were necessary. Such controls could also tell whether the assay was robust and performed with high quality. Since the screening was performed in parallel with two other groups using two additional viruses (HSV1 and KSHV), we used foscarnet as an efficient drug inhibiting all three viruses by the same mechanism of action (inhibition of viral DNA replication). Cycloheximide - a protein synthesis inhibitor that stops the cell growth and leads to cell death - was employed as a positive control for cytotoxicity. Nocodazole inhibits the polymerization of microtubules and blocks transport of incoming virions, particularly of HSV1, and upon longer treatment it triggers cell death. Further controls included DMSO-treated samples (as the solvent in which compounds were dissolved), untreated samples, where the cells were only infected, and finally uninfected cells.

A parameter that had to be determined was the positioning of the controls and randomization of the controls inside the plate to control for systematic effects (e.g., edge effects) and to secure a robust screen (high Z' factor value). Also, the number of replicates for the controls had to be considered, especially since due to cost and time considerations the initial screening of the compound library could be performed only once, allowing technical replicates, but not biological replicates. We put the positive and negative controls in alternate well locations along the edges of the plate; in this way the controls were equally distributed in each row and each column. Controls with uninfected cells were placed on the column edges, because low signals were expected from these wells and higher edge effects here would have little consequences for data analysis. Moreover, some of the controls, including untreated and uninfected cells as well as foscarnet - and DMSO-treated cells were also randomized inside

the plates, leaving 56 wells for the application of the compounds (Figure 11A). An automated workstation robot from Biomek (Beckman Coulter) was used as a liquid handler to perform the high throughput workflow. The compounds and the virus were mixed in a 96-well "master plate" and then the contents from each well was transferred to four wells in a 384-well plate (Figure 11B). Six days post-infection the plates were measured for GFP intensity.

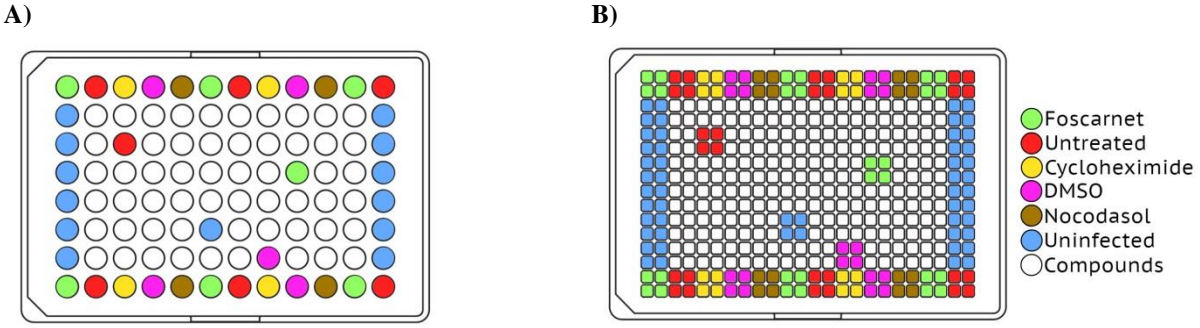


Figure 11. Designated location of the controls within the plates. A) In the 96-well "master plate" compounds were randomized on the edge in a specific fashion. B) Subsequently the compounds were transferred to a 384-well plate by the robot; thereby the contents of each well from the 96-well plate was distributed to 4 replicates.

A pilot experiment was performed to test the randomization of the controls on the edges and inside the plate (Supplementary Figure 2). GFP intensity measured for individual wells at 6 dpi is shown in Figure 12A.

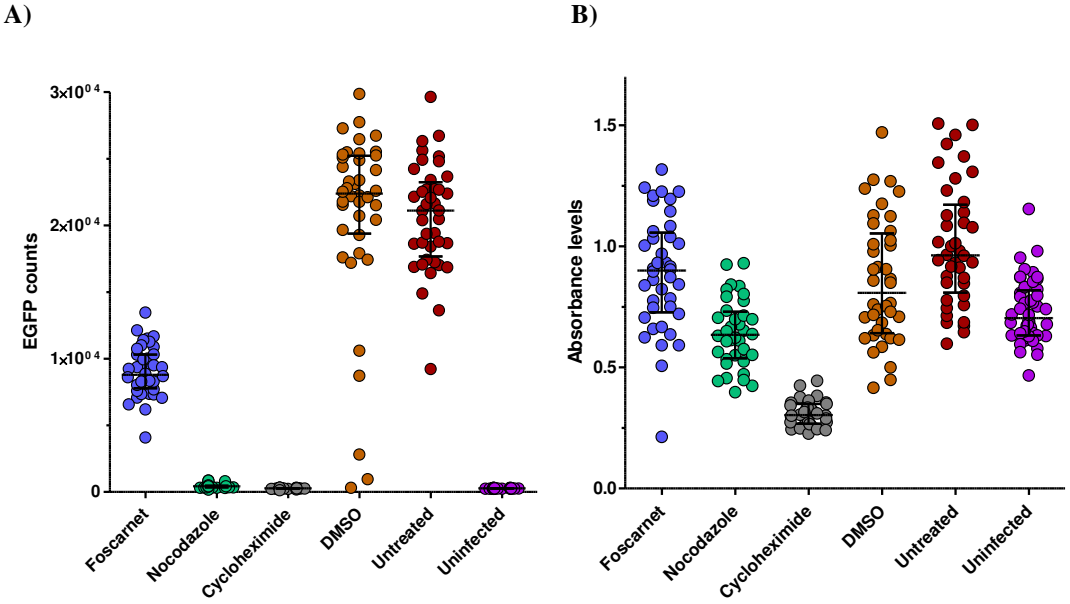


Figure 12. Screening test run performed with the robot. A) EGFP counts measured in the wells of CMV infected cells upon treatment with the indicated inhibitors. B) Absorbance levels were measured by a plate reader

to assess cytotoxicity of the inhibitors. Each point represents the value from a single well in a 384-well format plate. Horizontal bars and error bars are medians ± interquartile range of 36-48 replicates.

Foscarnet reduced the GFP signals by more than 50%, GFP values for cultures treated with nocodazole and cycloheximide were close to zero because of cell death, and DMSO-treated and untreated cultures expressed the highest GFP intensity. For assessing the cytotoxicity of compounds, crystal violet staining of cells was used for the pilot experiment and screening of the first library. Figure 12B shows the absorbance levels of the wells after crystal violet staining. Cells, which did undergo cell death due to inhibition of protein synthesis in cycloheximide-treated wells displayed lower signals compared to other wells. Nocodazole-treated wells showed ca. 30% higher absorbance levels compared to cycloheximide-treated cells, most likely because cells were still attached to the plates, whereas in cycloheximide-treated wells cells detached and floated away (Supplementary Figure 8C). Taken together, the results indicated that cultures which received compounds with an inhibitory effect as strong as foscarnet and most likely also compounds with lower activity could in most cases be clearly discriminated from untreated and DMSO-treated cultures. Using crystal violet staining, cytotoxicity of compounds could be detected for compounds that were as toxic as cycloheximide, whereas this was not reliably possible when compounds were less toxic as in case of nocodazole. This was one of the reasons why other cytotoxicity tests were considered in some of the subsequent screening experiments.

3.2. Results of the first screening rounds

Processing of the high throughput screening data

To exclude false-positive and false-negative hits, it was necessary to perform statistical corrections. Most normalization methods that are used in high throughput screenings rely on non-robust statistics, meaning that they use means and standard deviations of measurements, which are greatly influenced by statistical outliers. Here, we used medians and median absolute deviations (MAD). In addition, by using within-plate references we could control for systematic plate-to-plate variation, which allowed us to make measurements comparable between plates.

A widely used parameter for normalization in HTS is the “Z-score” which is based on the assumption that most compounds are inactive and could serve as controls. It is calculated as

$$Z = \frac{X_i - X^-}{S_x}$$

where X_i is the raw measurement on the *i*th compound, and $\bar{X}$ and S_x are the median and the median absolute deviation, respectively, of all measurements within the plate. All data generated from the screening was analyzed by calculating the robust Z-score based on median and MAD.

For each 384-well plate, the GFP expression of the cells in individual wells as well as absorbance levels following crystal violet staining were measured. The data was then analyzed by the program CYAME (Can You Analyze My Experiment) that was produced and programmed by Dr. Guillaume Beauclair (Department of Virology, MHH).

Screening of the first compound library

After establishing the method for screening, 6656 compounds were screened using the described assay. Me and the colleagues of two other groups started the screening with 12 plates per week and after two weeks the throughput was increased to 18 plates per week. We identified a total of 21 hits active against CMV, and together with the other groups additional 40 hits active against the other viruses tested. For the first library, staining of cells with crystal violet was used as a cheap and fast method to measure cytotoxicity. As expected from the pilot experiment, it turned out that this method did not allow to reliably discriminate between toxic and non-toxic compounds. As a result, the MTT method – a more precise and accurate method for measuring the cytotoxicity - was applied in the initial screening of the second and third library and in the re-screening of the hits from the first library.

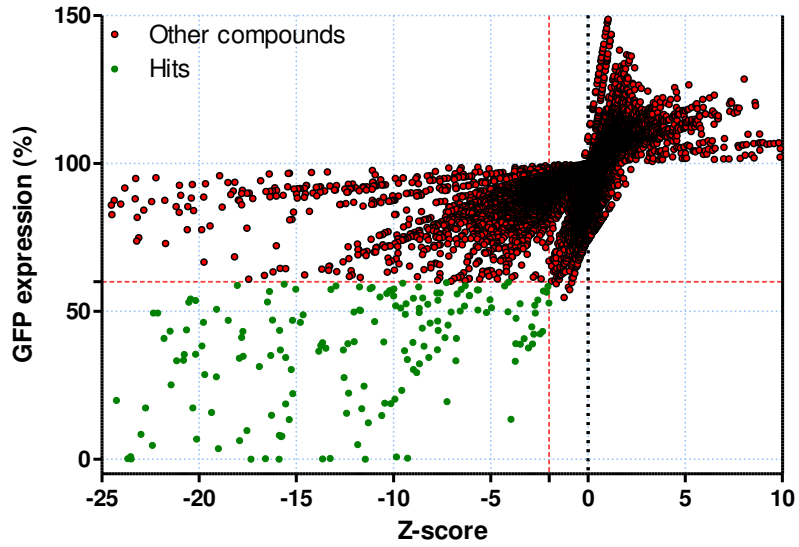
Screening of the second and third compound library

The second library consisted of 5040 compounds, which were screened in the same way except of using the MTT assay for measuring the cytotoxicity as the reason mentioned above. Similarly, 6000 compounds of the third library were analyzed.

In order to map all the data for the total number of compounds tested, and as criterion for the hit identification, the Z-score for each test compound was calculated based on the GFP expression of the respective cell culture well (Figure 13A). For hit identification a Z-score value of -2 or less was chosen in agreement with many HTS methods described in different publications (Malo et al., 2006). A Z-score of -2 means that the GFP expression of the cultures treated with the respective compound is two median absolute deviations lower compared to the GFP expression of the DMSO-treated control samples. Figure 13A shows the GFP expression values versus Z-scores of all compounds from the three libraries that were screened. Hits were defined as compounds with Z-scores less than -2 and GFP expression

values less than 60%. In addition, the treated cells had to present viability of more than 70% compared to the DMSO-receiving control samples (Figure 13B).

A)



B)

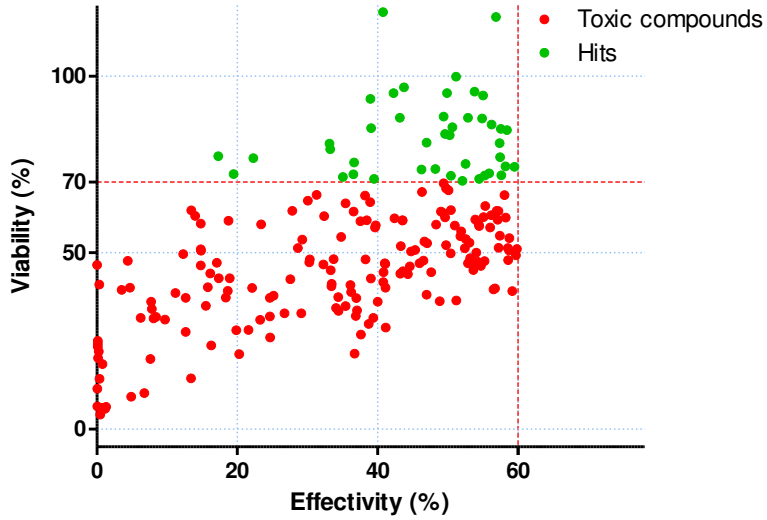


Figure 13. Screen validation and hit identification. The result for the 17640 compounds of the library screening is shown. A) For each test compound, the Z-score was calculated based on the fluorescence intensity data and was plotted against the percentages of GFP expression normalized to the median values of the wells receiving the DMSO control solvent in each plate. The points are medians of quadruple measurements. Hits were defined as compounds inhibiting CMV-driven GFP expression by at least 60% compared to untreated controls and displaying a Z-score value <- 2. The vertical dotted line indicates the chosen Z-score cutoff [-2]. B) The viability of the cell samples represents the median of the data from four wells compared to data of wells receiving DMSO solvent control and was plotted against the GFP expression of the wells treated with the respective compound. Dotted lines indicate the cutoffs of 60% GFP expression and 70% viability of the cells in the cultures.

In parallel – in order to make sure that the overall assay was robust and results reproducible between plates of every week – the Z' factor was calculated by the CYAME program platform, using the GFP expression values of the controls of each plate. The results were plotted in Figure 14. Almost all of the 318 plates had Z' factors larger than 0.5, indicating very good quality of the screening procedure.

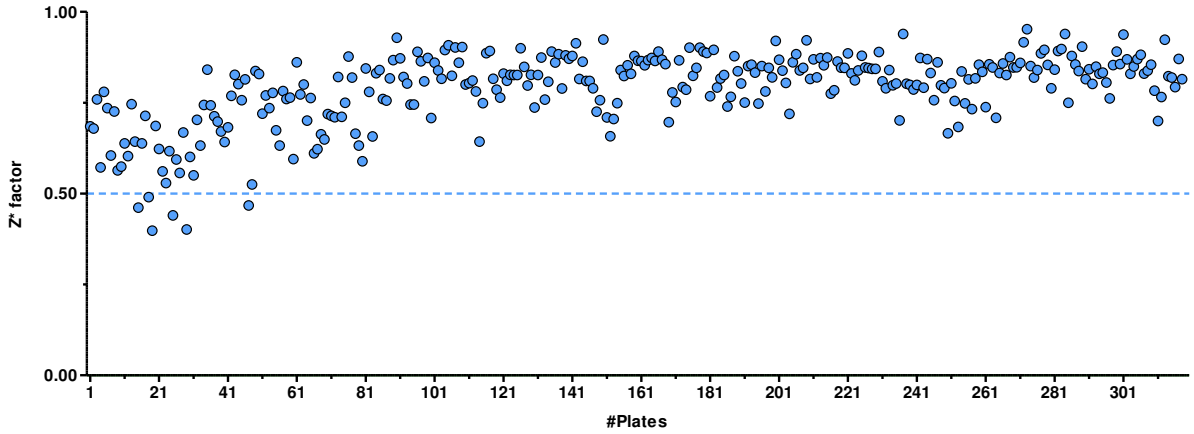


Figure 14. Quality control of the assay and reproducibility of the results. The Z' factor for each plate was calculated based on the GFP expression values of the DMSO-treated controls and of uninfected cells (Median and Median absolute deviation were used). Each dot corresponds to the value of one plate.

By applying the criteria described above, 48 compounds were identified as *bona fide* hits that specifically inhibited the HCMV infection cycle. These compounds represent 0.26 percent of the total number of compounds analyzed in the primary screen. Together with the other groups 204 compounds were discovered in the three libraries, which were active against HCMV, HSV1, KSHV, or RSV or against two (or more) of these viruses. For confirmation of the hits, the compounds displaying activity against HCMV as well as 156 compounds active against the other viruses were included in the re-screening.

3.3. Rescreening of the hit substances

The selected hits were reordered from the companies and analyzed with respect to their inhibitory activity against HCMV using the same assay as for the initial screening. In order to confirm the antiviral activity and to exclude false-positive hits, rescreening was performed 4 times. The complete results for all 204 compounds examined during the rescreen are shown in Supplementary Figure 3. Again, data of the secondary screen was analyzed by the CYAME

program. It turned out that compounds, which showed the best activity in the primary screen, often did not perform as well in the secondary screen. Also, there were considerable differences between the results of the four re-screens (Supplementary Figure 3). “Lead hits” were defined as those compounds inhibiting CMV-driven GFP expression by at least 40% and displaying viability values of the treated cultures of more than 70% compared to DMSO-treated controls, at least in one of the four rescreens (Table 1). In order to not miss an active substance, 57 compounds were selected after the secondary screen for further follow-up studies.

	Final Hits Approved			
Rescreen=>	**1**	**2**	**3**	**4**
PANH_001	Hit	---	---	---
PANH_020	Hit	---	---	---
PANH_021	Hit	---	---	---
PANH_023	Hit	---	---	---
PANH_030	Hit	---	---	---
PANH_031	Hit	---	---	---
PANH_034	Hit	---	---	---
PANH_037	Hit	---	---	---
PANH_039	Hit	---	---	---
PANH_041	Hit	---	---	---
PANH_050	Hit	---	Hit	---
PANH_051	Hit	---	Hit	---
PANH_053	Hit	---	---	---
PANH_057	---	Hit	---	---
PANH_061	---	---	Hit	---
PANH_065	---	---	Hit	Hit
PANH_068	---	Hit	Hit	---
PANH_070	---	---	Hit	---
PANH_080	---	Hit	Hit	---
PANH_097	---	---	Hit	---
PANH_099	---	Hit	---	Hit
PANH_101	Hit	Hit	Hit	---
PANH_102	---	Hit	Hit	Hit
PANH_105	---	---	Hit	---
PANH_111	Hit	Hit	Hit	Hit
PANH_112	Hit	---	Hit	Hit
PANH_124	---	---	Hit	Hit
PANH_125	Hit	---	---	---
PANH_127	---	---	---	---
PANH_129	Hit	Hit	Hit	Hit
PANH_133	---	---	Hit	---
PANH_139	Hit	---	Hit	Hit
PANH_140	---	---	---	Hit
PANH_143	---	---	---	Hit
PANH_144	---	---	---	Hit
PANH_145	Hit	Hit	Hit	Hit
PANH_146	Hit	---	---	Hit
PANH_147	Hit	Hit	Hit	Hit
PANH_148	---	---	---	Hit
PANH_149	---	---	---	Hit
PANH_150	---	---	---	Hit
PANH_154	Hit	Hit	---	Hit
PANH_158	---	---	---	Hit
PANH_159	Hit	---	---	---
PANH_161	---	---	Hit	---
PANH_162	---	---	---	Hit
PANH_168	---	---	---	Hit
PANH_169	---	---	Hit	---
PANH_170	---	---	---	Hit
PANH_183	---	Hit	---	---
PANH_184	---	---	---	Hit
PANH_185	---	---	Hit	Hit
PANH_192	Hit	---	---	---
PANH_195	---	---	---	Hit
PANH_196	Hit	Hit	Hit	Hit
PANH_197	---	---	---	Hit
PANH_201	Hit	---	---	---

Table 1. Lead hits selected after rescreening. 204 compounds were rescreened 4 times independently. Each time hits were defined as compounds inhibiting GFP expression of HCMV by at least 60% and resulting in cell viability of more than 70%. In total 57 compounds were selected as potential hits after the secondary screen. Compounds which were defined as hits in several of the rescreens are marked by darker green.

Interestingly, some compounds that were selected in the first screen as hits to inhibit viruses other than HCMV turned out to be active against HCMV upon re-testing. For instance, compound PANH021 showed a potential inhibitory effect against HCMV as well as HSV-1 and KSHV. Other compounds that were broadly effective against HCMV and other viruses are PANH_023 (KSHV), PANH_099 (RSV), PANH_102 (KSHV), and PANH_196 (KSHV). Thus, it might be that some of the identified compounds target processes in the infected cells, which are important for different viruses.

3.4. Further characterization of the antiviral activity of the identified compounds by GFP reduction assay

The next aim was to select the compounds with the highest effectivity and narrow down the number of the final hits. To define the antiviral activity of the compounds in more detail, a GFP reduction assay was applied in which cells were infected at a low MOI and compounds were added to cells at a concentration of 10 μM. Photos of the cells were taken with a fluorescence microscope each day to follow the inhibition of the viral spread by the compounds. For comparison, the infection of the cells was followed in the respective control cultures. Supplementary Figure 5 shows the fluorescence microscopic photos of the infected cell cultures from day 6 to day 9 post infection. Day 8 post infection was chosen for the measurement of the GFP expression because the untreated cells and DMSO-treated control cells displayed maximal GFP signals at this time point without presenting overt cytopathic effect (CPE). The positive control inhibitor PAA was able to reduce the infection to an appreciable extent (Figure 15, Supplementary Figure 5) when compared to the solvent control DMSO. The same applied to letermovir, although inhibition was not as pronounced as after application of PAA.

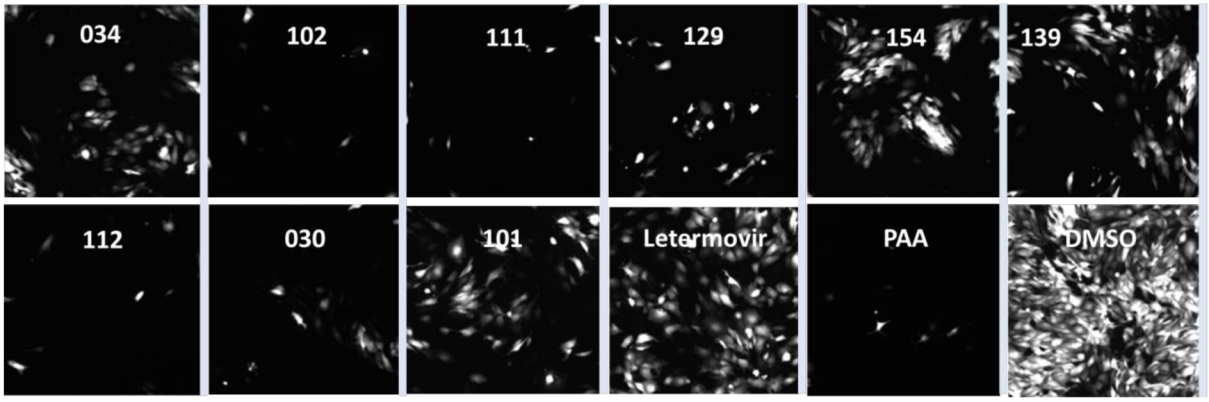


Figure 15. GFP reduction assay for nine selected compounds. Fluorescence microscopic images were taken from the cells at day 8 post infection. Compounds were tested by GFP-based reduction assay using infection with a low viral dose (MOI= 0.05). The concentrations that were applied here for compounds, PAA, letermovir, and DMSO were 10 µM, 180 µM, 10 nM and 0.1%, respectively. The images depicted here are a subset of the data presented in Supplementary Figure 5.

An overview of the different experiments performed with the 57 compounds and the resulting data are shown in Supplementary Figure 4. Based on these results, fourteen compounds (out of the 57 compounds analyzed) were chosen for further characterization, because they appeared the most promising ones at the time point when the analysis was done. These compounds were then tested in an identical manner by applying them in various, decreasing concentrations. The results of the GFP-reduction assay are shown in Supplementary Figure 5 for all compounds (exemplarily those cultures receiving the compounds at the concentration of 10 µM are depicted). Images of cell cultures at day 8 post infection, which displayed effective inhibition of viral spread after treatment with nine of the compounds (at 10 µM) are displayed again in Figure 15. Four compounds inhibited spread of HCMV only slightly (Supplementary Figure 5) and thus, only the nine compounds indicated in Figure 15 were selected for further characterization.

3.5. Determination of approximate IC_{50} and CC_{50} values

In order to define the half maximal inhibitory concentration (IC_{50}) of the compounds, the antiviral activity of the compounds was determined at decreasing concentrations using the same assay as described in chapter 4, followed by calculating dose-response curves. The nonlinear regression curves describing the relationship between the concentration of the compounds and the inhibition of CMV-driven GFP expression are shown in Figure 16. Although there was variability between the results of the three independent biological experiments, for most compounds the data points were within a reasonable range, and

regression curves could readily be fitted and approximate IC_{50} values be determined (Table 2). The graphs clearly indicate the antiviral effects of the respective compounds. An exception was the compound PANH_102, which displayed excellent activity in one experiment, but not in the other two experiments. One explanation is that this compound may have become instable over time.

In a similar manner cytotoxicity of the compounds was assessed by adding increasing concentrations of the compounds (up to 100 µM) to uninfected cells. Viability of the treated cells was measured after 3 days by using the MTT assay (Figure 16, Table 2).

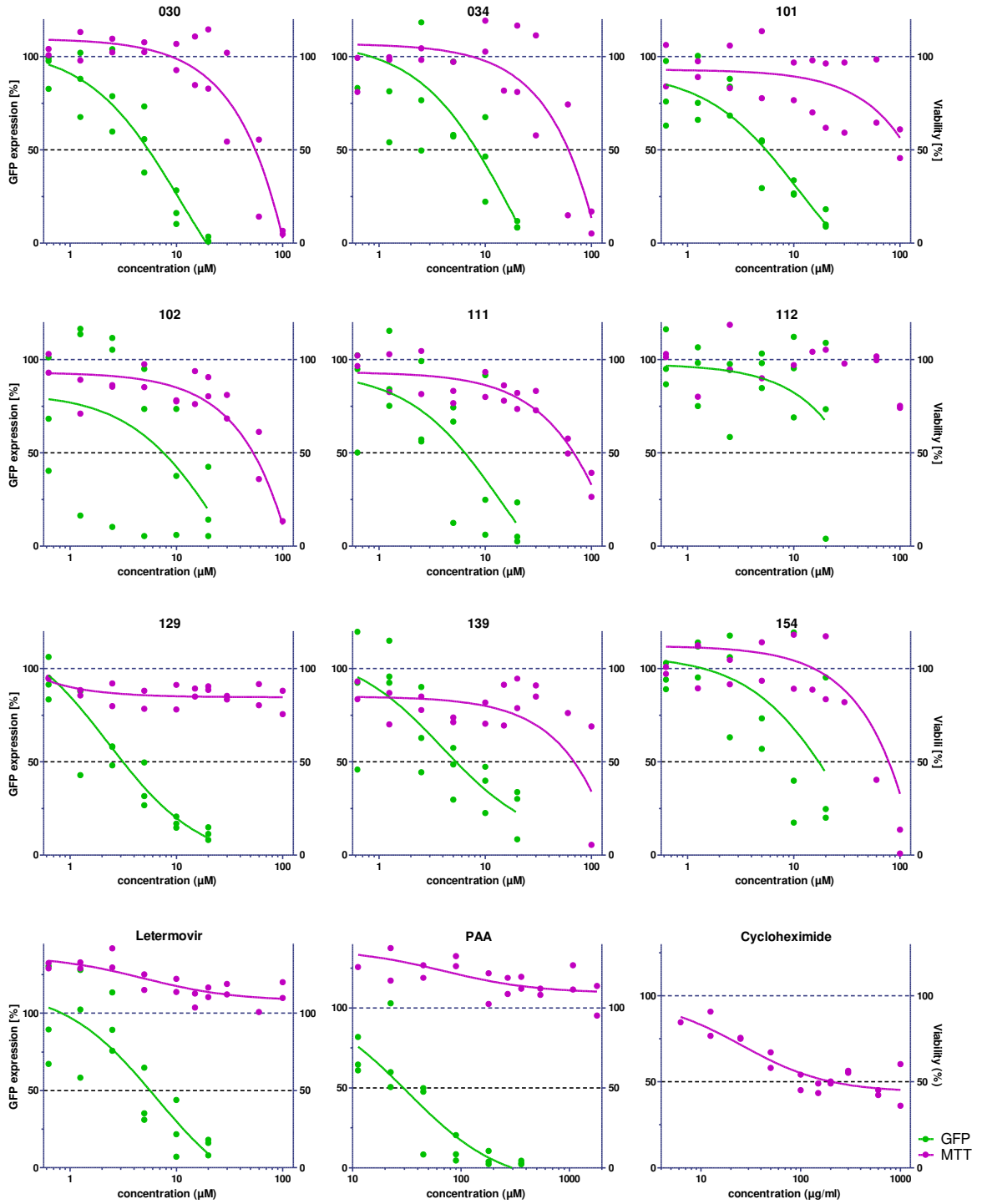


Figure 16. Dose-response curves of the selected compounds for antiviral activity and cytotoxicity. nHDF cells were infected with the GFP expressing CMV at low viral dose (MOI= 0.05). 3 hours after infection, the inocula were replaced with media containing the indicated compounds in 2-fold increasing dilutions. Measurements of GFP signals cells were taken after 8 days (green lines). For determining cell viability, uninfected nHDF cells were exposed to the compounds in 2-fold dilution steps. After 3 days, cells were assayed with MTT reagent for compound-related toxicity (purple lines). Curves show the drug dose vs. the response; values of three and two independent experiments for antiviral activity and cytotoxicity, respectively, are depicted. Data were normalized to the values of cell samples treated with the corresponding DMSO concentrations.

Based on the half maximal cytotoxic concentration (CC_{50}) and the half maximal inhibitory concentrations (IC_{50}) of the individual compounds the selective indices were determined (Table 2), which represent the ratio between these parameters and allow ranking these 9 compounds. For two of the compounds, PANH_129 and PANH_112, no or very little cytotoxicity was observed even when applying a concentration of 100 µM, and PANH_101 reached the CC_{50} value only at this high concentration. Moreover, several compounds displayed IC_{50} values below 5 µM, and PANH_129 had the lowest IC_{50} (2.9 µM). Thus, this compound possessed the highest selectivity index (>34; Table 2) and the most favorable properties with a low IC_{50} and great CC_{50} values.

Compound name	**IC_{50}**	**CC_{50}**	**SI**
PANH_030	5.4	47	8.7
PANH_034	8.8	54	6.1
PANH_101	4.6	100	21.7
PANH_102	6.6	58	8.8
PANH_111	5.1	67	13.1
PANH_112	24	>100	>4
PANH_129	2.9	>100	>34
PANH_139	4.2	51	12.1
PANH_154	16	79	4.9

Table 2. IC_{50} and CC_{50} values were determined based on the results of the virus inhibition and MTT assays. Nonlinear regression analysis was applied to the data depicted in Figure 14, and the resulting graphs were used to determine the respective values. The selectivity index (SI) of each compound was calculated by the ratio of CC_{50}/IC_{50}. For the compounds PANH_129 and PANH_112 the CC_{50} values could not be determined because half maximal values were not reached after applying the highest possible concentration (100 µM). Data were calculated in GraphPad Prism using the measurements from three and two independent experiments for antiviral activity and cytotoxicity, respectively.

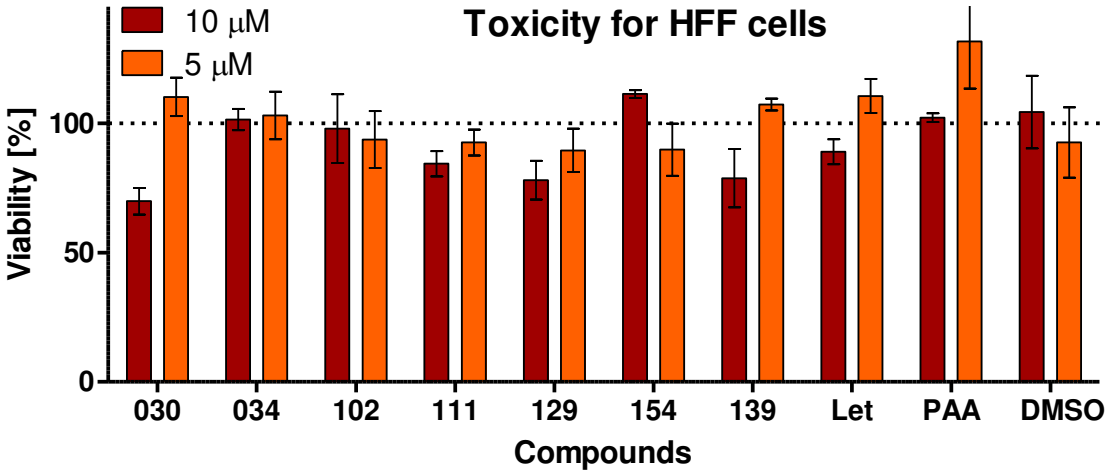
3.6. Further cytotoxicity testing of compounds

In light of the CC_{50} data obtained (as shown in Figure 16 and Table 2), it was important to assess the toxicity of the compounds against several cell types and by using different cytotoxicity assays. Compounds were tested on human foreskin fibroblasts (HFF) and retinal

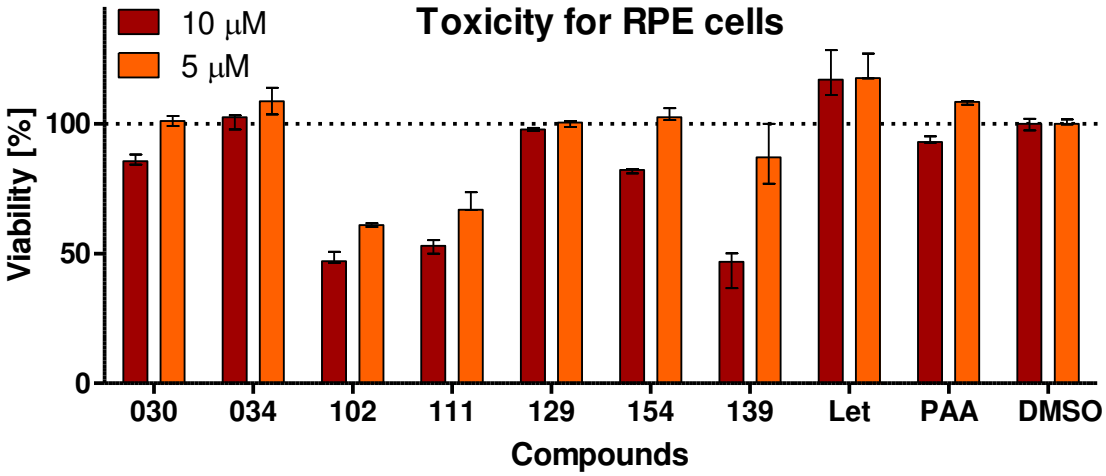
pigmented epithelial (RPE) cells at concentrations of 5 and 10 µM. After 3 days, cells were measured for viability using the MTT assay (Figure 17A, B). At a concentration of 10 µM, compound 030 showed some toxicity in HFF cells. In RPE cells, compounds 102, 111, and 139 displayed substantial toxicity at a concentration of 10 µM, and compounds 102 and 111 were still toxic at 5 µM.

During the re-screening of compounds against HSV-1 and KSHV by the colleagues in our department, the toxicity for HeLa cells and human Burkitt lymphoma B cell line (BJAB) was also analyzed, and these data were included in my analysis. The viability of HSV-1-infected HeLa cells was assessed by DAPI staining one day p.i. (Figure 17C). Similarly, BJAB cells latently infected with KSHV were induced with IgM (to trigger reactivation) and treated with the compounds (at 10 µM), and analyzed after 3 days with the MTT assay. For HeLa cells compound 111 was toxic and 154 showed slight toxicity, whereas no effect was detectable for the other compounds using the DAPI staining assay. Compounds 030, 034, 111 were toxic for BJAB cells and compounds 154 and 196 slightly impaired their viability.

A)



B)



C)

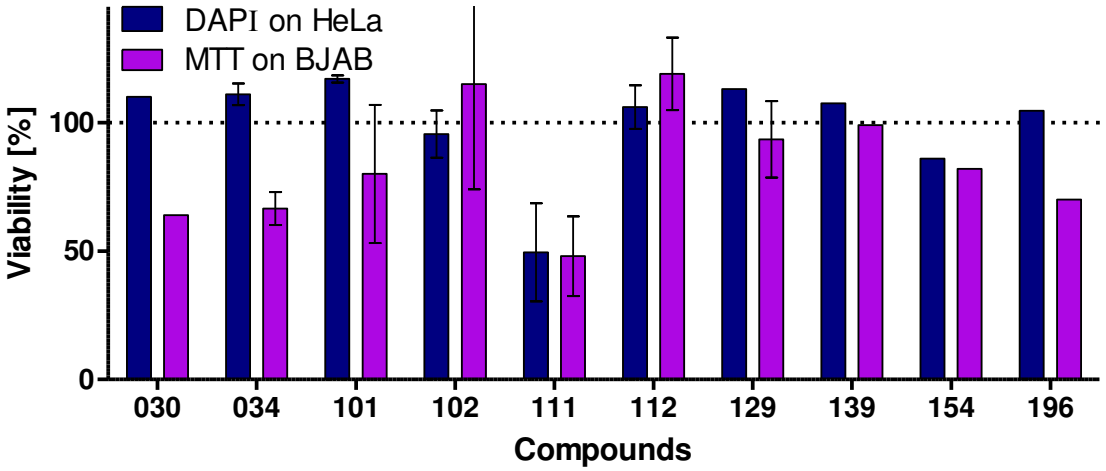


Figure 17. Comparison of cytotoxicity effects of the compounds on different cell lines. A) HFF cells and B) RPE cells were exposed to the two indicated concentrations of the compounds for 3 days. MTT assay was performed to detect the cytotoxicity. The treated cells had to present viability of more than 70%, that the compounds to be considered as non-toxic. Error bars are standard deviations of triplicate measurements from one experiment. C) HeLa cells were infected with HSV1 and treated with the compounds at 10 µM. After 24 h, cells were stained with DAPI, followed by fluorescence measurement. Latently KSHV-infected BJAB cells were exposed to 10 µM of the compounds and treated with IgM, and analyzed by MTT assay after 3 days. Data are either from one experiment or the means of two independent experiments. The data for this analysis (in C) were provided by my colleagues Julio Cesar Villalvazo Guerrero and Jessica Rückert.

In order to assess potential cytotoxic effects of the compounds in an alternative manner, I applied the CellTiter-Glo luminescence assay that quantifies ATP produced by viable cells. Non-infected nHDF cells subjected to increasing concentration of the compounds were analyzed after 3 days. The data in Figure 18 indicate that compound 111 affected the ATP production of cells when applied at 10 µM and higher concentration. Compounds 030 and 034 may affect cell metabolism when applied at 20 µM.

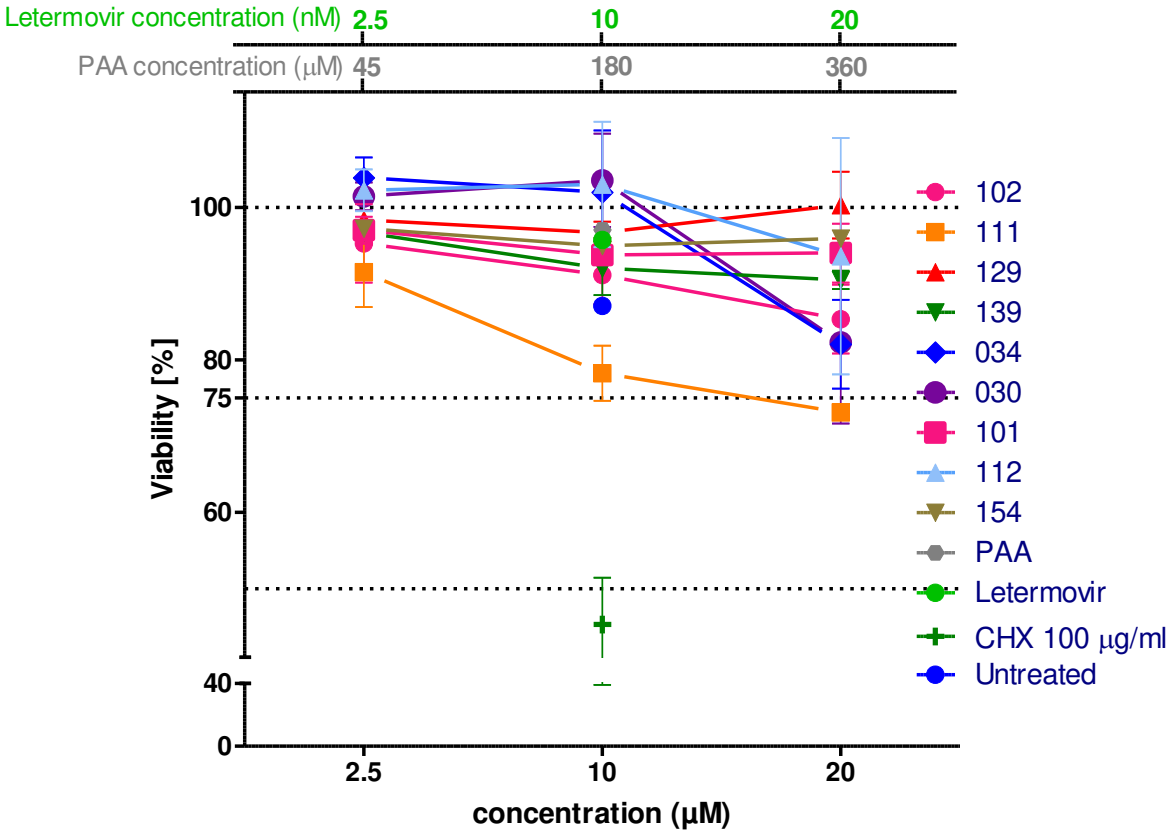


Figure 18. Effects of the compounds on ATP levels of the cells. nHDF cells were tested for their levels of ATP after subjecting them to the compounds at the indicated concentrations. After 3 days ATP levels were quantified by the CellTiter-Glo assay and results were normalized to the value of cells treated with corresponding DMSO concentrations. Data represent mean values + range of duplicate determinations from one representative experiment (of two independent experiments performed).

In summary, these data indicated that the cytotoxicity assays had different detection sensitivities and similarly, different cell types were more or less sensitive against treatment with the compounds. As described before in Figures 17 and 18 for HFF cells, compound 129 was found to be non-toxic for other cells (and when using different assays). In contrast, compound 111 turned out to be rather toxic, and compound 102 displayed considerable toxicity for RPE cells. Several of the other compounds started to become toxic at a

concentration of 10 µM or higher, depending on their selectivity index they may, however, serve as basis for medicine-chemical modification, which may allow reducing their toxicity. Besides compound 129, substances 101, 112 and 154 appeared the most promising ones with respect to low toxicity in the cells.

3.7. Discovery of the mechanism of action of the selected compounds inhibiting HCMV

Measuring the effects of the compounds on inhibition of virus release as well as on inhibition of GFP expression as done in the rescreening of the compounds from the second library provided already hints whether the block is at early or later steps of the viral infection cycle. Moreover, the compounds that exhibited major GFP reduction in the GFP reduction assay performed with low MOI and long incubation times were tested again under infection conditions using a high MOI (Supplementary Figure 9). Two compounds, namely 102 and 111, led to reduction of GFP expression of more than 60%, under these conditions, suggesting that these are early inhibitors. In line with this, a time of addition experiment showed significant reduction of GFP expression when the compounds were applied earlier than 24 h.p.i. (Supplementary Figure 10). As further alternative, the number of infected cells was measured by flow cytometry following treatment with the compounds (Supplementary Figure 11). A substantial reduction of GFP positive cells and of their mean fluorescence intensity was observed for the compounds 034, 102, and 111 from the first library. These data strongly suggested that these compounds act as early inhibitors.

Reversibility of the inhibitory activity of the anti-HCMV compounds

To gain insight into the mechanism of inhibition of the defined compounds and to further confirm the step of the HCMV infection cycle that is blocked, several additional experimental approaches were taken. The first one that I used to characterize the mode of action of the compounds was a kinetic block-release assay. It is based on the assumption that upon withdrawal of the inhibitor restoration of virus production requires different time periods depending on whether an early or late step is blocked. If the compound targets early steps (e.g., the viral DNA polymerase), there will be a significant delay in the release of infectious virions and virus production remains depressed for some time. In contrast, for a compound that acts relatively late in the virus replication cycle (e.g., inhibiting the viral terminase or assembly of virions), a relatively rapid recovery is expected. HCMV-GFP infected nHDF cells were treated with inhibitory concentrations (15 µM) of the compounds or with control

inhibitors (at appropriate concentrations) (Supplementary Figure 14A, and Figure 19). After 96 hours the infected cells were released from the drug block, and the amount of progeny virus present in the supernatant was quantified immediately or after 24, 48, and 72 h, by transferring the supernatant to new cells. Figure 19 clearly demonstrates that the selected compounds led to different profiles of recovery. Upon withdrawal of the control inhibitor letermovir (that inhibits the viral terminase in the late phase), virus replication rapidly resumed and infectious virus particles were detected in the supernatant at 24 h post release from the drug block. Similar profiles were observed for compounds 101, 111, 112, 129, 139, and 154 clearly demonstrating that the antiviral block induced by these compounds was reversible. For compounds 030, 034, and 102 secretion of virus particles into the supernatant occurred after 72 h only, suggesting that early viral steps were blocked. Inhibition by the viral DNA polymerase inhibitor PAA appeared to be irreversible.

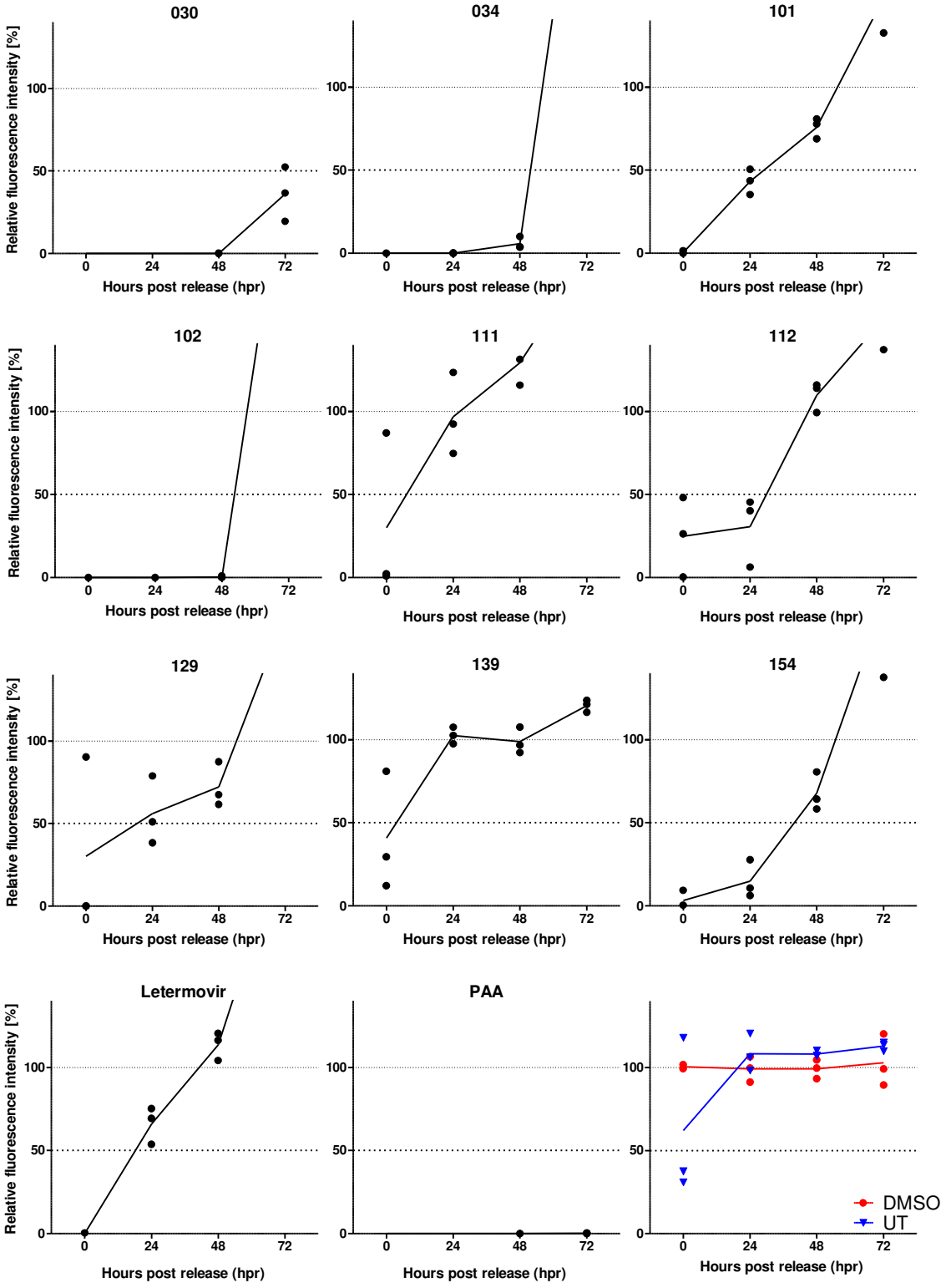


Figure 19. The results of the kinetic block-release assay points to the step of inhibition. HCMV infected nHDF cells were treated for 96 h with the compounds (at 15 µM) (antiviral blocking step). For letermovir and PAA, 15 nM and 270 µM were used, respectively. At 96 h.p.i., the supernatant was removed, cells were washed and new media without inhibitors was added. Immediately (0 h) or 24, 48, and 72 h after drug release (hpr, hours

post release), supernatants were collected and new cells were infected to determine when virus production resumed. The intensity of GFP expression (as an indicator of the amounts of virus present in the supernatants) was measured after 144 h. Data points represent values of triplicate determinations. The lines connect the mean values of the data points which were normalized to the values of DMSO-treated control cells (100%).

Taken together, these data suggest that compounds 030, 034, and 102 inhibit an early step and compounds 101, 111, 112, 129, 139, and 154 at a later point within the HCMV replication cycle.

Inhibition of viral DNA replication by the antiviral compounds

Another possibility to determine whether inhibition by the compounds occurs early or late is by checking the amplification of viral DNA in infected cells, because viral DNA defines the onset of the late phase of herpesviruses. Compounds inhibiting an early step will prevent HCMV DNA replication, whereas this is not the case for late inhibitors. I sought to evaluate the inhibitory effects of the compounds on HCMV DNA replication by real-time qPCR analysis. nHDF cells were infected and treated with the compounds, followed by extraction of total DNA at day 3 p.i. The levels of viral DNA present in the infected cells were measured by qPCR using primers specific for the viral polymerase gene. The data normalized to the amount of viral DNA present in untreated cells are plotted in Figure 20. In cells treated with compounds 129 and 154 similar viral DNA levels were detected like in untreated and letermovir-treated cells, indicating that these compounds do not block viral DNA replication and suggesting that they rather act during the late infection phase.

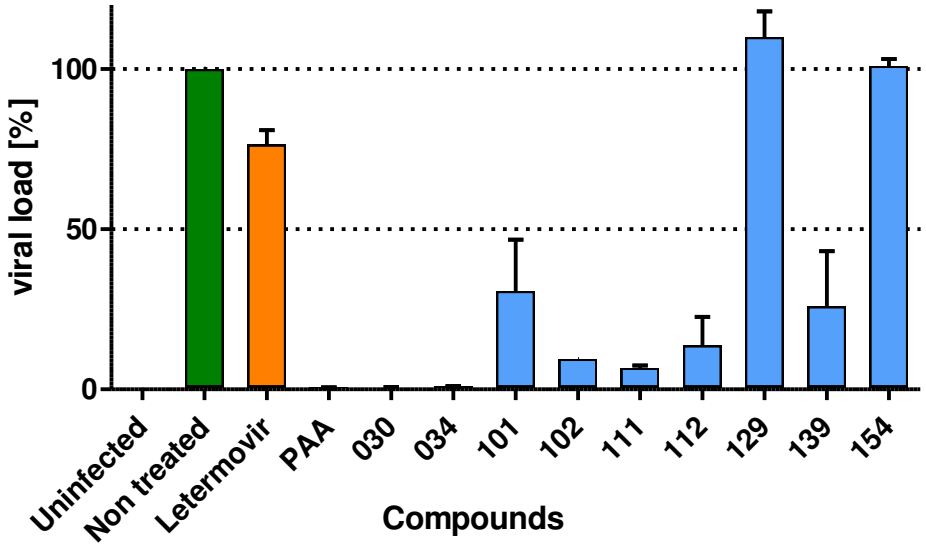


Figure 20. Viral DNA levels present in infected cells after treatment with the indicated compounds. The amounts of HCMV DNA present in nHDF cells 3 days after infection and treatment with 10 µM of the compounds were determined by quantitative real-time-PCR. For controls, PAA (180 µM) and Letermovir (10 nM) were applied. For amplification of HCMV DNA and cellular DNA primers specific for the viral UL54 gene (viral DNA polymerase) and the cellular CRP gene were used. Data were analyzed by using qPCR Software 3.4 and GraphPad Prism. Data shown are averages of 2 wells for one representative experiment (of two independent experiments performed). Data are normalized to the values of the non-treated control. The experiment was performed with support from Lars Steinbrück.

Cells treated with compounds 030 and 034 contained very little amounts of viral DNA comparable to cells treated with the viral DNA polymerase inhibitor PAA, and cells treated with compounds 102, 111 and 112 contained strongly reduced levels of HCMV DNA. These results suggest that the mentioned compounds inhibit either the DNA viral polymerase itself or a step before viral DNA replication. Compounds 101 and 139 reduced the viral DNA levels to a lesser degree, possibly pointing to some leakiness of inhibition, yet again suggesting that these substances act rather early. In contrast, compounds 129 and 154 did not reduce the viral DNA levels, suggesting that they inhibit the viral life cycle after DNA replication.

Efficacy of the compounds against MCMV

Murine cytomegalovirus (MCMV) is widely utilized as a model for understanding CMV infections. Thus, it was tested whether the selected compounds have any antiviral effect on MCMV or whether their effect is restricted to HCMV only. Mouse embryonic fibroblasts were infected with MCMV at a low multiplicity of infection of 0.01 PFU/cell, taking into account that MCMV replicates substantially faster than HCMV. Three hours p.i., the

inoculum was removed and replaced with medium containing increasing concentrations of the compounds. PAA was included in this experiment as a reference compound. As shown in Figure 21, compound 129 significantly reduced the intensity of the GFP expression of the cells, which resulted from infection with the GFP-expressing MCMV mutant. The other compounds had either no effect or at best inhibited the MCMV infection only at the highest concentration tested (10 µM).

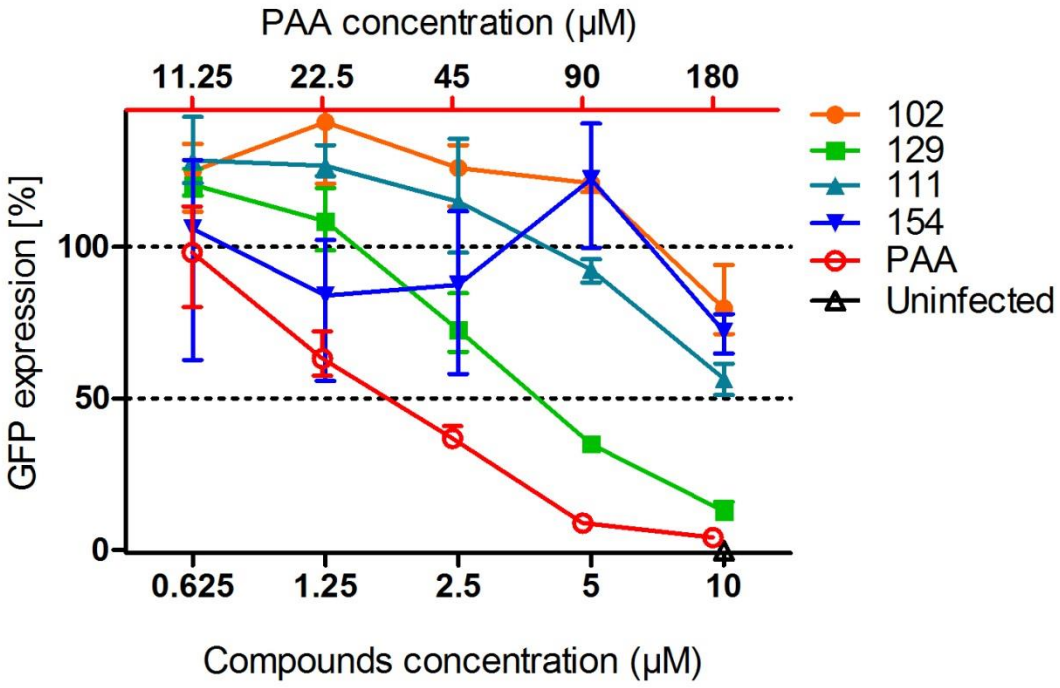


Figure 21. Effect of the compound hits on MCMV infection. MEF cells were infected at MOI=0.01 with an MCMV recombinant that expresses GFP. After 3 hours, inocula were replaced with media containing 2-fold increasing concentrations of the indicated compounds and cells were assayed for GFP expression after 4 days. The labelling at the bottom and top X-axes represent concentrations of the compounds and PAA, respectively. Data were normalized to the GFP expression values of samples treated with respective DMSO concentrations and are plotted as the means and standard deviations of triplicate samples.

The assumption that one or several of the compound hits discovered by screening for HCMV inhibitors might be able to inhibit MCMV as well, turned out to be correct. Interestingly, compound 129 showed inhibition of MCMV infection even at a low concentration of 2.5 µM.

Potency of the compounds in inhibiting virus release

Most of the previous assays were based on the measurement of the GFP signals in infected cells, resulting from a GFP gene in the HCMV genome, which is expressed with immediate

early (IE) kinetics. Although reduction of the viral gene expression was an appropriate and convenient measuring parameter, it was important to get a better understanding of the effect of the compounds on release of infectious HCMV particles. Confluent nHDF cells were infected with a high dose of HCMV (MOI= 0.5) and treated by the compounds in various concentrations. At day 5 p.i. supernatants were harvested and viral titers were determined by standard plaque assay. Figure 22 shows the dose-dependent inhibition on the release of infectious virus mediated by the compounds.

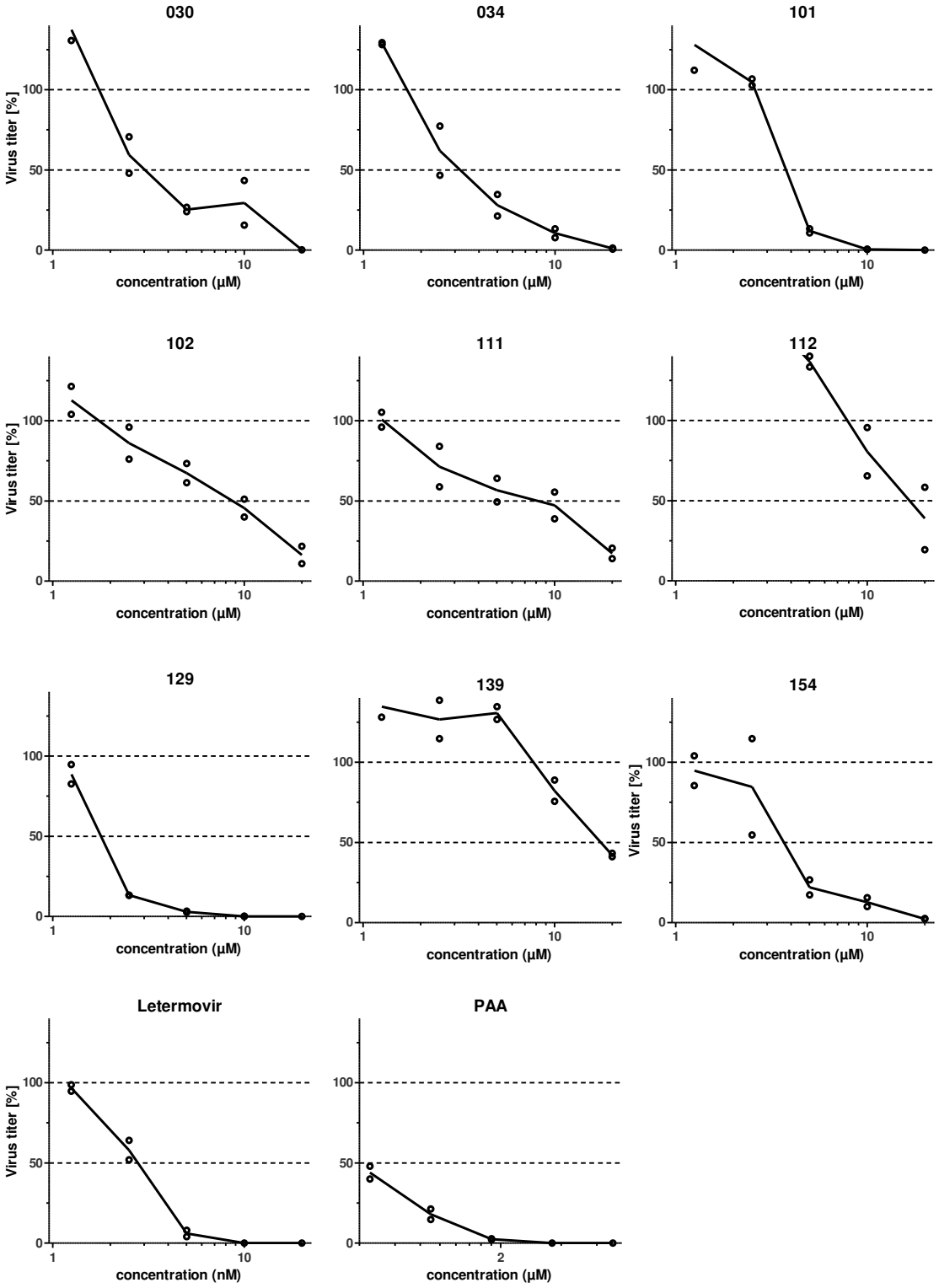


Figure 22. Dose-dependent inhibition of virus release by selected compounds. Confluent nHDF cells were infected with a high viral dose (MOI=0.5) and after 3 hours the inocula were replaced with medium containing increasing concentrations of the compounds. Five days later, the supernatants were serially diluted and plaque assays were performed on new cells. Plaques were counted 14 days later. Calculated titers were normalized to the titers of samples receiving corresponding DMSO concentrations and are shown in percentage of this control. Data are from two technical replicates.

In agreement with the data of the GFP reduction assay, compounds 030, 034, 101, and 129 led to 50% reduction of the viral titers at low one-digit micro-molar concentrations. Notably, the slopes of the dose-response curves of these compounds were relatively steep. As observed before, compound 129 was the most effective one. Compound 154 displayed a different profile here than when tested by the GFP reduction assay, which cannot immediately be explained, requiring additional investigation. Other compounds (102, 111, 112, 139) induced 50% inhibition of virus release at high concentrations (10 μM or more) only, and some of them (compounds 102, 111) had comparably flat dose-response curves. Taken together, for several of the compounds that were initially identified by applying a GFP reduction assay their effectivity could be confirmed when tested for release of infectious virus.

3.8. Effects of the compounds on expression of viral proteins of different kinetic classes

In order to investigate how treatment with the compounds affected the expression of selected proteins of HCMV, nHDF cells infected with HCMV-GFP at MOI 0.5 and treated with either compounds 101, 111, and 129 or control inhibitors, were collected at 24, 48, 72, and 96 hours p.i. Lysates were analyzed by immunoblotting to detect the expression of the IE1, UL44, and UL52 proteins (Figure 23), which are representative members of the classes of immediate-early, early and late viral proteins. PAA was used as a positive control that inhibits HCMV at the step of viral DNA replication. All viral proteins analyzed were highly expressed at 48 hours p.i., and therefore the samples from this time-point are shown exemplarily. Compared to untreated and DMSO-treated cells, in lysates of PAA-treated cells harvested at 48 h p.i., only the 70 kDa, but not the 100 kDa IE1 band was detected and the amounts of the UL44 and UL52 proteins were strongly diminished. It is known that the 100 kDa IE1 protein is a posttranslational modification product of the 70 kDa IE1 protein, which emerges at later stages of the HCMV infection cycle (Elbasani et al., 2014). This pattern of protein expression indicates that PAA blocks viral gene expression at an early stage. In lysates of cells treated with compound 111 a similar pattern was observed, meaning that the inhibition is during an early step of the HCMV infection cycle, as it was expected from the kinetic block-release experiment. Compounds 101 and 129 did not inhibit the expression of the IE1 proteins and affected the levels of the early protein UL44 and of the late protein UL52 only slightly, when compared to lysates of untreated and DMSO-treated control cells.

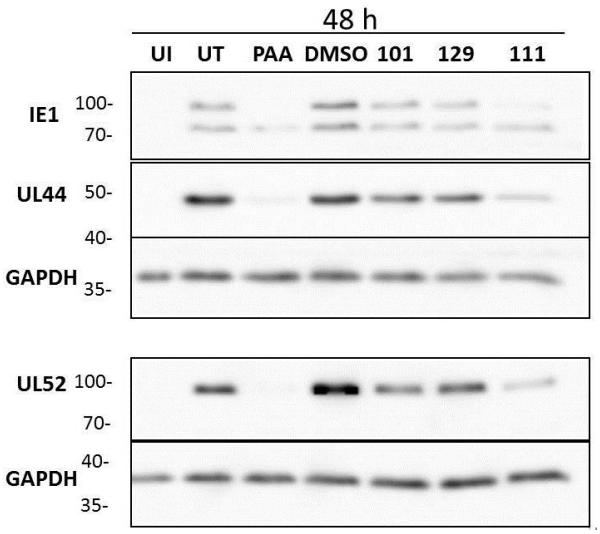


Figure 23. Western blot analysis of compound activity. The expression levels of the indicated immediate-early, early, and late proteins were analyzed by Western blot using lysates of HCMV-GFP-infected nHDF cells treated with the compounds (10 µM) and harvested at 48 h p.i. Treatment with PAA served as positive control, and GAPDH was used as loading control. UI, UT, PAA, and DMSO point to lysates of uninfected, untreated, phosphonoacetic acid-treated and DMSO-treated cells, respectively. Martina Dezeljin supported me in performing the experiment.

These data suggest that compounds 101 and 129 inhibit the infection during the late phase, most likely after the onset of UL52 protein expression. Notably, these data are in agreement with the results of the kinetic block-release and the qPCR assays (compare to Figures 19 and 20).

4. Discussion

4.1. Experimental design of the High-Throughput Screening

Approaches to find novel antivirals against Human Cytomegalovirus (HCMV) have been in development for the past few years. High-throughput screening is the ingredient for drug discovery (Malo et al., 2006) which has made its way into academic settings. In this study, High-Through Screening (HTS) was used to identify antivirals against HCMV. In our screening, I applied strategies to ensure HTS performance and reproducibility. In order to counter the inefficiencies of the performance of the screening, several techniques have been employed to maximize the number of screened targets and to optimize the lead generation and validation process. The screening assay was based on a recombinant HCMV strain, which is tagged with a green fluorescent reporter protein (GFP). The GFP tagged HCMV was employed in the screening and subsequent experiments, instead of a luciferase-expressing HCMV recombinant, because for the latter we faced limitations during the readout (Supplementary Figure 6). Using the luciferase HCMV recombinant led to substantial variability in the measurements within each plate. Therefore, the GFP recombinant HCMV was used. HCMV is a slow replicating virus, and the time for cells to generate new viruses takes at least 72 hours. To find the best conditions that allow a maximum dynamic range of the readout signal between infected cells and uninfected control cells is to either infect cells with a high MOI with less incubation time (single-step infection) or a low MOI with long incubation time (multi-step infection). In the screening, I have used a multi-step infection procedure, where few cells are infected initially using a low MOI. Eventually, these few cells produce viruses, which infects then in a second step, the neighboring cells. This assay allows detecting early, as well as late-step inhibitors. If a compound truly acts on the early steps of the HCMV life cycle, the few cells that are infected with viruses will reduce the GFP signal from those infected cells, and will not produce additional viruses that can infect neighboring cells, which will result in a significant decrease in infection. If a compound acts on late events, the infected cells will produce GFP signal but will reduce the amount of virus produced from these cells and therefore it will show around 40-60% decrease in infection values. This concept was evaluated using letermovir as a terminase inhibitor and foscarnet as a viral DNA polymerase inhibitor. Treatment with letermovir produced only 40% of the GFP signal upon infection with MOI 0.05 and after 6 days of infection (Figure 9). The exact MOI

that was used for the screening was optimized using various test substances and cell numbers. A low MOI will infect a small fraction of cells, therefore even after a long incubation period the GFP signal remains really low and dynamic range is too small to be detected. Using high MOI will lead to higher GFP signals not only from infected cells but also from treated cells in parallel, leading to a small dynamic range. If a compound is toxic for cells, fewer cells produce viruses and the GFP signal decreases. Another parameter that had to be considered was the screening concentration for the compounds applied in HTS. In other studies concentrations between 1 and 10 µM have been used, however, we considered 10 µM concentration. In the meanwhile, activities of compounds of up to 40 µM can be identified (Hughes et al., 2011). In addition, the DMSO concentration in which the chemical library compounds were dissolved, was diluted to 0.1 % in the assay. Typically, cells become intolerant at concentrations above 1% DMSO. The aim was to find all compounds that inhibits any step of the CMV infection cycle. This was challenging in view of systematic errors that can occur, such as potential equipment malfunctions and handling mistakes. In addition, the occurrence of inference decision errors, such as ‘false positives' and ‘false negatives' are common. It is almost impossible to avoid the detection of false-positive hits. Decreasing the threshold value will increase the false-positive rate, but decreases the false-negative rate. Increasing the threshold value will result in the opposite. Using multiple measurements or replicates both reduce the false-negative rate but the false-positive rate will remain (Malo et al., 2006). In general, after the assay was approved, it was successfully employed in the screening of HCMV

4.2. Analysis of the data of the screening assay

Successful identification of hits in an HTS assay is based on several factors. They not only include low volume samples, compound replicates, control positions and replication within the plate, high throughput and robustness, but also sensitivity, reproducibility, and data analysis. The variation of measuring the effect of controls between each plate is common, but in order to get confidence that one specific hit is "real", validation is needed. An evaluation to ensure the confidence of the identified hits is to measure the quality of the screening assay. However, in general there is not an accepted method that can ensure the quality of any HTS. The goal is to identify compounds as hits with a high degree of confidence. To this end, several data analysis methods are applied in this concept. One is the signal window concept, which however lacks uniformity and simplicity in some parameters (Sittampalam et al.,

1997). Signal-to-noise or signal-to-background ratios, are also used as a quality control in HTS assays. Signal-to-noise (S/N) defines whether a signal can be regarded as real, but does not contain information to evaluate the quality of the assay alone. The signal-to-background ratio (S/B) does not contain any information about the data variation. Neither the S/N ratio nor the S/B ratio contains full information about the variability of the samples and background measurements concerning the dynamic range. However, S/B measurements were taken into account in the CYAME programme as an additional measuring factor. It was found that all plates had a 'Good' value for the S/B ratio (Supplementary Figure 13). Additionally, another analytic method called 'strictly standardized mean difference (SSMD)' is used for hit selection and quality control in HTS assays. It measures the effect size for the comparison of two random values in two different groups (Zhang, 2011). SSMD is a stronger parameter than the S/N and S/B ratios and was applied in the calculation of our screening data. As concluding results, all plates were observed to be at least 'Good' after calculating the results (Supplementary Figure 13). By implementing different systematic methods to evaluate the highest quality within a significant degree of confidence in the screening, a statistical parameter, the Z factor, was used and applied in the HTS assays. It defines a screening window coefficient and shows how signals of infected cells (controls without inhibitors) and of positive controls which could be a compound inhibiting the infection (or non-infected cells) are separated. The number of the replicates of each control is important for calculating the standard deviation, a measure that indicates the variation, resulting from technical handling limitations or errors (Zhang et al., 1999). Using this method in the screening assay helped to find the ideal multiplicity of infection, number of cells (Figure 8B), and incubation time period. Since most of the statistical methods used in screenings rely on non-robust statistics, the use of medians and median absolute deviation (MAD) was implemented instead of means and standard deviation (SD). The Z' factor is very sensitive to data variability, for instance, if the addition of standard deviations of the controls is at the lowest or the subtraction of the controls is at the lowest, the Z' factor approaches to its highest value. Thus, a Z' value within the range of 0.5-1 indicates that the assay is excellent, and has a large dynamic range with small data variability. In general, the Z' value can be in a range of $-1 < Z \leq 1$, but $Z < 0$ is at its lowest detection limit, and thus is not recommended. For instance, in the test run using controls, Z' was < 0 for MOI 0.01 for all test substances, and Z' < 0 for MOI 0.02 and letermovir in Figure 9B. Therefore, these MOIs were not applied in the screening. The Z'

factor defines the quality or performance of the HTS. Hence this data analysis method was used to evaluate the robustness of all of the experiments that were performed.

An automated liquid handling robot was used to perform the screening. Controls had to be randomized (positional effects) in the plate in a way to generate strong robustness in terms of Z' factor and decrease the number of false-positive and false-negative hits. This was done by placing the controls on the outer rows of the plate randomly, and the uninfected cells on the outer column of the plates. This strategy can reduce the systematic effects such as edge effects and cell-based biological effects such as cell clumping or evaporation within different areas of the plate. Therefore, the randomization for the screening was done as shown in Figure 11, and Supplementary Figure 2 represents the final version subsequently applied to the test run plate. The results demonstrated a significant confidence interval between the controls themselves and the test substances, and consequently a high Z' factor.

The approach to explore the cytotoxicity of compounds during the screening procedure was challenging. DAPI staining is typically used to stain nuclei in control cells but could be used as an alternative method to detect cell viability (Cummings et al., 2012). In our study, it was found to differentiate poorly between the positive and negative controls. Despite the fact that the method was modified with respect to the staining, fixing, washing, and measuring procedure (Cummings et al., 2012), it nevertheless did not provide meaningful data to be considered in the screening. One other reason that limited the use of DAPI was the time. During performance of the HTS assay, it was difficult to take photos of every well in the 384-well plate for all plates, in order to measure the cells' viability. Although presence of the cells could be confirmed by fluorescence microscopy after the nuclear staining, it did not display huge separation of the data after measurements of fluorescence levels (Supplementary Figure 8A). The method was replaced by crystal violet staining, a method that is used to stain cells because crystal violet binds to proteins and DNA. However, this method had disadvantages too, particularly because it detects any cells that are attached to the surface of the well and cannot distinguish between dead or live cells. The decision to use crystal violet staining in the screening was the rapidity of application since the HTS was simultaneously performed for two other viruses and did not allow sophisticated, laborious procedures. Other reasons were the cost, time, and simplicity of the method exhibiting more advantages over disadvantages (Supplementary Figure 9B). Nevertheless, because of its limitations the crystal violet staining was subsequently replaced by the MTT assay. The MTT assay was used to obtain a more reliable viability measurement (Supplementary Figure 15). The libraries were screened one

after each other to ensure stable robustness of the HTS. Each week, the data was analyzed simultaneously performing the quality control checks and normalization procedures. The next step was to select hits based on three values that were obtained from the screening; a robust Z-score (<-2), the percentage of GFP expression (<60%), and viability of treated cells (>70%). Several compounds were found by me and my colleagues that showed common inhibition of HCMV, HSV, and KSHV. Although later, the compounds with the common property did not turn out as those compounds displaying the most prominent activity.

Subsequently, the hits were tested by a secondary screen in quadruple measurements. The hits that displayed extreme inhibition, viability and Z-score in the initial screen were usually less extreme or only average in the secondary screen, possibly because they were statistical artifacts (outliers) in the initial screen (Campbell and Kenny, 1999; Stigler, 2002). Data were analyzed using the same criteria as the ones that were applied for the initial screen, and at this stage 57 compounds were selected as hits. For rescreening of the hits of the second library, an additional test was performed and the supernatants of the first round of infection (after 6 days) were transferred to new cells, with the aim to identify substances that inhibit late steps of the viral life cycle, e.g. virus particle assembly and release. This led to the identification of three compounds (069, 088, and 124) by setting the criteria for hit characterization to a cell viability of at least 70% and to viral inhibition of at least 80% (Supplementary Figure 3). Subsequently the compounds 069 and 088 were, however, not further followed, because they appeared only once in the four experiments performed to analyze virus release.

4.3. Characteristics of the identified compounds

The antiviral activity of the compounds was determined by determining IC_{50} values upon measuring inhibition of GFP expression (dose response curves) and by measuring the inhibition of virus release. The first part after hit selection was to identify the characteristics of the compounds by a GFP reduction assay. Generally, a dose-response assay of the compounds was applied using infected cells and 2-fold increasing concentrations to determine the IC_{50} values. Initially, upon treatment with most of the compounds, the results were not satisfying since there was no reduction of GFP expression observed. This problem was solved later by infecting cells for 3 hours and replacing the inocula with medium containing the compounds (Figure 15 and Supplementary Figure 5). The IC_{50} values indicate a lot about the effectivity of the compounds, but not without taking into account the slope of the dose-response curves and the maximum effect (E_{max}) of the compounds. A study from Sorger and

colleagues showed that E_{max} is dependent on the cell type used, IC_{50} and E_{max} are associated to cell doubling time, and IC_{50}, the slope of the dose-response curve (HS), and E_{max} are associated to drug class (Fallahi-Sichani et al., 2013). In general, E_{max} is the best indicator for the efficacy of the compounds. Therefore, E_{max} as well as the hill slope (HS) should be considered in future calculations. IC_{50} is dependent on the conditions, which were applied for the measurements. According to the US National Cancer Institute (NCI), in most cases, an IC_{50} of more than 10 µM is usually not acceptable for human tumour cell line (Shoemaker, 2006). Thus, in our screening, we applied the compounds at 10 µM, meaning that compounds that possibly inhibit HCMV at higher concentrations could not be detected. Therefore, the IC_{50} values of the identified hits are below 10 µM, except compound 154, which showed an IC_{50} of 16. The inhibitory activities of the compounds were acceptable, but the related toxicity results were also important for the determination of the selectivity indices.

The cytotoxicity of the compounds was analyzed with or without infection. For all experiments including infection, the MTT assay was used to assess the toxicity of the compounds. However, the actual toxicity was measured in non-infected samples, as shown in Figure 16, because infection of cells can have an impact on the toxicity exerted by the compounds. Primary cells provide effective cytotoxicity results in HTS assays (Eglen and Reisine, 2011). In addition, toxicity data obtained during the rescreening of compounds against HSV1 on HeLa cell lines and against KSHV on the human Burkitt lymphoma B cell line (BJAB) were taken into account (Figure 17). These results provided additional information to define the final hits. Disadvantages of these approaches could be: (i) one-day incubation of the compounds on HeLa cells may be too short to deploy the toxicity, and (ii) Burkitt lymphoma B cell lines might be unusually sensitive to some of the selected hits, considering that KSHV infection of these cells could affect the cytotoxicity.

An alternative method (GLO-TEST) to assess the potential toxicity of some selected hits was the quantification of the ATP levels present in the cells upon treatment. Only one compound exhibited a 25% reduction of ATP levels at concentrations of 10 µM or higher. Thus, these results suggest that most of the selected compounds have a comparably low toxicity, in line with the results of the MTT assays. However, it cannot be excluded that the performance of the GLO-TEST was suboptimal and needs optimization, such as measuring at the right time point, or seeding right number of cells per well. Also, using this assay the dose-response relationship has to be assessed for a broader range of concentrations of the compounds in future experiments.

Ideally, the concentration of IC_{50} should be much lower than CC_{50} so that a compound can inhibit CMV at a concentration, which does not exert cytotoxic effects. Selectivity indices inform about the compounds effectivity in relation to its cytotoxicity, i.e., the higher the value the better. A variety of selectivity indices were observed for the compounds, starting with the lowest value of 4 for compound 112 up to the highest of more than 34 for compound 129, in achieving complete inhibition of CMV. Consequently, compounds 129, 101, and 111 displaying the highest SI were selected for further investigation.

4.4. Mode of action of the selected compounds

In general, several techniques are in use to determine the mechanism of action of antiviral compounds. These techniques can either investigate the particular step of inhibition, or more specifically at the viral gene expression level. To understand the particular step of inhibition of the selected compounds, we sought to employ techniques that engaged the GFP-reduction assay. This included altering the virus input (MOI) level and incubation time (Figure 8 and Supplementary Figures 1 and 9), or quantifying the number of infected cells after treatment with the compounds (Supplementary Figure 11).

The effects of the compounds are different if they target – for instance - the terminase or the viral polymerase and moreover, outcome will be differently, upon withdrawal of the inhibitor from virus-inoculated cells. The kinetic block-release assay checks whether the inhibition by the compounds is reversible or not. Moreover, depending on the time it takes until virus production comes back, one can conclude whether inhibition was early or late. While virus replication remained depressed for a longer period after compounds 030, 034, 102, 112, and PAA have been removed from the cultures, virus replication immediately resumed after removal of compounds 101, 111, 129, 139, and letermovir. An explanation for the latter compounds is that these inhibit processes that occur later than viral DNA replication, therefore progeny viruses are easily assembled and released after drug removal. Notably, these data for compounds 102 and 111 were consistent with the results of the GFP-RA assay and the time-of-addition experiment (Supplementary Figures 9 and 10). There is a possibility that compounds 030, 034, 102, and 112 could have the same mechanism of inhibition based on their profile compared to PAA, because PAA inhibits the viral DNA polymerase. A major issue with using a GFP reduction assay was the accuracy, assuming that the compounds only act on inhibiting the virus (release or replication) and not on the expression of GFP. Compounds with early-step inhibitory effects, have a profound effect on the GFP expression,

leading to low-level GFP production in cells. While compounds acting at later steps, affect the GFP expression driven by the immediate-early promoter to a lesser extent. Possible ways to truly detect a compound that acts on the viral titers are two additional assays, namely $TCID_{50}$ and plaque assay. In several attempts, the $TCID_{50}$ assay led to variable data, which could not always confirm the inhibitory effects of some compounds; therefore this approach was not further followed (Supplementary Figure 12). Instead, a standard plaque assay was applied, revealing that all selected compounds inhibited HCMV release, in a dose-dependent manner. In fact, the results from the standard plaque assay confirmed that the release of the virions from infected cells correlated with the data of the GFP- reduction assay. In conclusion, the two experiments allowed to calculate the dose responses, confirmed and validated one another, and indicated that the compounds inhibit the HCMV infection in a dose-response manner.

Recently, more sophisticated methods to quantify virus particles in liquid samples were invented (Heider and Metzner, 2014), for example, tunable resistive pulse sensing (TRPS), where viruses are driven through a size-tunable nanopore, recognizing the size and concentration of the virus particles. Other methods that aim at virion quantification include: Enzyme-linked immunosorbent assay (ELISA), which uses antibodies to detect virus antigens, new flow cytometry procedures that quantify the number of virus particles after staining with fluorescence dyes specific for proteins and nucleic acids, and quantitative polymerase chain reaction (qPCR) which measures viral DNA. Although these assays are highly accurate, they cannot substitute for the standard plaque assay, because they are unable to differentiate between dead or alive (infectious) particles. Thus, these techniques can only support the biological assays (Plaque assay, $TCID_{50}$).

Based on our previous findings, the approximate step of the inhibition was further characterized by kinetic release block (KBR) and time of addition (TOA) assays. To confirm the hypothesis that the compounds target the HCMV replication cycle before or after DNA synthesis, viral progeny DNA was measured by quantitative real-time PCR. The qPCR results for some compounds were contradictory to the results of the kinetic block-release assay. This includes compound 111, which according to the kinetic release block assay was considered as an inhibitor of late steps of the infection cycle (virus production quickly recovered after removal of the compound), whereas the qPCR data suggested that inhibition was before or at viral DNA replication. Similarly, the results from the time of addition (TOA) assay argued that compound 111 is an early inhibitor (Supplementary Figure 10). A possible explanation

for this discrepancy is the concentration of the compound applied in the KRB assay (15 µM), which may not be sufficient to block the virus completely, causing false results. Usually a concentration of 10 x IC_{50} is recommended to achieve the complete effect, however, higher concentrations were toxic for cells (Supplementary Figure 14B). Similar contradictory results were observed for compound 154, in as much as the KBR assay pointed to an early-step inhibitor and qPCR to a late-step inhibitor. The results of the TOA assay for compound 154 were again consistent with the qPCR data. The rest of the compounds (030, 034, 101, 102, 112, 129, and 139) displayed comparable results in the two assays (KBR and qPCR). Since the data from qPCR assay was confirmed with TOA assay and dose-response, and the KBR assay yielded contradictory results for two compounds, this assay may have to be repeated using more technical replicates.

Besides assessing the inhibitory effect of the compounds on HCMV, their activity against MCMV was also measured. The findings will indicate if the compounds are specific for human CMV or might have inhibitory effects on murine CMV as well and not on HCMV only. Moreover, one may conclude whether the compounds have the same or different modes of action, and most importantly, it can become possible to test the compounds in the mouse model and measure the antiviral activity in vivo. The murine counterpart (MCMV) provides a useful model for studying CMV natural infection in its natural host. Out of the four compounds tested, interestingly compound 129 led to a reduction of MCMV-driven GFP expression in a dose-response manner. The inhibitory effect of other compounds (030, 034, 101, 112, 139) on MCMV remains to be tested. That could help to determine the mode of action of these compounds, because the viruses may use different mechanism for their life cycle and express different viral proteins during their infection.

The analysis of the expression levels of the HCMV IE1, UL44, and UL52 proteins shed light on the compounds' inhibition of different steps of viral infection. In this study, compound 111 and PAA (as a control) displayed similar effects on the expression of UL44, which is a substrate of the UL97 protein kinase that also affects exogenous substrates such as ganciclovir (Krosky et al., 2003). Similarly, the same pattern was observed for PAA and 111 with respect to expression of the UL52 protein, which is known as a 75-kDa protein with late kinetics (Borst et al., 2008). It appeared that compound 111 inhibited CMV infection at a relatively early time point of the expression kinetics, which is consistent with the results of the qPCR assay. In comparison to compound 111, compounds 101 and 129 affected the production of the early and late proteins to a lesser extent. It is known that UL44 has an effect on the

HCMV lytic origin and genome replication (Pari et al., 1993; Silva et al., 2010). As a result, inhibition of UL44 expression by compound 111 will affect the genome replication and later steps of the life cycle. This data for 111 was consistent with the results of the qPCR assay. However, treatment with compound 101 led to lower viral DNA levels than compound 129. An explanation for this might be that compound 101 inhibits other early expressed proteins, whereas compound 129 may exert its effect at a time point later than the onset of UL52 protein expression. Examination of a larger panel of representative virus-encoded proteins such as IE2-p86, IE2-p60, IE2-p40, UL69, UL83/pp65, UL84, and UL86 will led to a clearer picture which of the IE, E, and L class proteins are inhibited.

Compound 129 turned out as the one with the most favorable properties in terms of selectivity index, very low toxicity for cells, inhibition of the HCMV infection cycle at a late step, and activity against MCMV; therefore, it is the most promising compound and will serve as a lead substance for further development. Similarly, compound 101 displayed a decent selectivity index and inhibition of late stages of HCMV cycle. The primary aim is to develop antivirals that ultimately become commercially available for treatment against cytomegalovirus. Another purpose in academia is to define novel targets and pathways in the CMV infection cycle. In this respect compounds that display late inhibitory effects may be more interesting than early inhibitors since many protein-protein interactions involved in assembly and tegumentation of new virions, as well as their egress from cells are still subjects of intense investigation. Hence compound 129 is thought to be the most exciting for continuing the study.

In parallel to the experiments exploring the mode of action, the structure and chemical properties of the hit candidates and their suitability as drug candidates for possible chemical modifications were analyzed by a colleague (Dr. Martin Empting; Helmholtz Centre for Infection Research). The six compounds, which displayed reasonable activity against HCMV, namely 030, 039, 102, 111, 129, and 154, were analyzed and ranked based on criteria of their structure, of Derek warnings, which is a computer-based expert system that predicts compounds toxicity based on their structure (Sanderson and Earnshaw, 1991), of chemical properties, of compliance with Lipinski rules, which predicts a drug's ability to be used actively in humans based on chemical and physiological properties (Lipinski, Lombardo, Dominy, and Feeney, 2012), and on accessibility for chemical modification. On a scale of A (top) to C (moderate), the analyzed compounds ranked either B or C, indicating that they have to be optimized by chemical modification.

In summary, I have accomplished the initial aim of the project to find novel antivirals blocking HCMV, by setting up assays for screening libraries of compounds, confirmation of the initial hits, characterizing and selecting the most promising one, and analyzing the step of inhibition during the lytic HCMV infection cycle.

4.5. Outlook

In view of the potency of the selected compounds, and the fact that some of these compounds have antiviral activity against both HCMV and MCMV, further investigation and development of these compounds is justified.

To identify the exact mode of action of the selected compounds several experiments have to be performed in the future. Primarily, generation of drug-resistant viruses would be helpful and this could be achieved by two approaches: single-step selection method using infected cells in the presence of an excess concentration of the drugs (10-fold EC_{50}), until CPE occurs in the compound-treated cells. If the compound is not toxic for cells at this concentration, the cell-free supernatant virus is used to infect new cells in the presence of the 10-fold EC_{50} (Goldner et al., 2011). The alternative is classical resistance induction using propagation of HCMV in the presence of increasing concentrations of the compounds, until CPE is observed or the drug concentration reaches ≥128-fold EC_{50} (Goldner et al., 2014). This will indicate that the resulting resistant viruses have a specific or multidrug resistance phenotype. Sequencing the genomes of the resistant viruses will reveal the mutation site. As described in chapter 9 of the introduction, several mutations in the UL54 and UL97 genes have been found to be important in drug-resistance of CMV. Unsurprisingly, these genes are essentially related to viral genome replication, however, mutations in other genes are found as well, for instance, mutations in UL27 are important for resistance of viruses against maribavir (Lurain and Chou, 2010). In addition, mutations in the UL56 gene have been confirmed for the terminase inhibitor letermovir (Chou, 2015). Two viruses resistant to letermovir have been selected in vitro, however, such letermovir-resistant viruses did not occur during human studies and moreover, the EC_{50} for these resistant mutants were low (Melendez and Razonable, 2015). This information will be useful in case the mutations of viruses resistant to the compounds show a correlation to these genes.

Furthermore, the dependence of the antiviral effect by the best hits on the multiplicity of infection needs to be explored. Ideally, this should be done after structural modification and optimization. Such an experiment defines an important feature of the potency of an antiviral

compound. If the ratio of the input virus is increased and the antiviral potency decreases only slightly or stays at the same level, the compound is potent to encounter higher viral doses during infection. It would also be of interest to show that the antiviral activity of the hits is not restricted to the HCMV laboratory strain used here, and is seen for ganciclovir- and other drug-resistant viruses or clinical isolates as well. The mutations that provide resistance to ganciclovir are well characterized and map to the viral protein kinase UL97 (Wolf et al., 1995). Identification of compounds active against drug-resistant HCMV would enlarge the treatment options. Therefore, inhibiting these isolates would help to define better candidate drugs against HCMV. Finally, since HCMV has a broad cell tropism, antiviral activity of the compounds has to be assessed for a variety of other HCMV permissive cell lines besides fibroblasts, such as epithelial cells, endothelial cells, and smooth muscle. In addition, besides testing the toxicity of the compounds on normal human dermal fibroblasts, foreskin fibroblasts, normal lung fibroblasts (NHLF, MRC5), and embryonic lung fibroblasts (HEL299) could be used.

Another aspect is the testing of synergic effects of the compounds with each other, as well as with approved drugs against HCMV. Combination therapy is suitable for treatment of multidrug-resistant virus strains in transplant recipients, as well as for patients coinfected with different CMV strains or additional viruses (Wildum et al., 2015). Currently, there is only one drug with a distinct, different mode of action, and this is the recently approved anti-CMV drug letermovir that blocks the viral terminase and thus prevents packaging of the viral genome into capsids. In this respect letermovir represents an exception. The reported antiviral profile of letermovir is excellent with little limitations (Melendez and Razonable, 2015). The focus of the further work will therefore be on compounds that display synergies with ganciclovir or letermovir, suggesting that they target other viral proteins than the viral polymerase or terminase inhibitors, or alternatively on inhibitors with effects on viral attachment or fusion. It would also be of interest to examine whether the new HCMV inhibitors could also be applied to inhibit other herpesviruses (HSV-1, HSV-2, VZV, EBV, KSHV). If the compounds target common cellular pathways used by different viruses, they may even be active against viruses like RSV or HIV. One such example are labyrinthopeptins that have been reported to display a broad effect on different enveloped viruses (Férir et al., 2013; Meindl et al., 2010; Müller et al., 2010).

Overall, the results indicate that nine of the synthetic compounds can reduce HCMV infection in fibroblasts, by different mechanism of actions. Compound 129 demonstrated a more unique

profile than other compounds with a selectivity index of >34 and inhibition with late kinetics rather than interference with the viral DNA polymerase. This mechanism of action is thought to be an advantage compared to that of current polymerase inhibitors. Studies on murine CMV also demonstrated the efficacy of compound 129 on MEFs. One important interest is to find the exact mechanism of action, but additional structural changes of the compound are required first, in order to further decrease the cytotoxicity and also to increase the efficacy. The ultimate aim is the clinical evaluation to provide new treatment options for patients with drug-resistant virus strains.

References

Adland, E., Klenerman, P., Goulder, P., and Matthews, P. (2015). Ongoing Burden of Disease and Mortality from HIV/CMV Coinfection in Africa in the Antiretroviral Therapy Era. *Frontiers in Microbiology*, *6*, 1016.

Alwine, J. C. (2012). The Human Cytomegalovirus Assembly Compartment: A Masterpiece of Viral Manipulation of Cellular Processes That Facilitates Assembly and Egress. *PLoS Pathogens*, *8*(9), e1002878.

Anwar, F., Erice, A., and Jessurun, J. (1999). Are There Cytopathic Features Associated with Cytomegalovirus Infection Predictive of Resistance to Antiviral Therapy? *Annals of Diagnostic Pathology*, *3*(1), 19–22.

AuCoin, D. P., Smith, G. B., Meiering, C. D., and Mocarski, E. S. (2006). Betaherpesvirus-Conserved Cytomegalovirus Tegument Protein PpUL32 (Pp150) Controls Cytoplasmic Events during Virion Maturation. *Journal of Virology*, *80*(16), 8199-8210.

Avery, R. K., Marty, F. M., Strasfeld, L., Lee, I., Arrieta, A., Chou, S., Tatarowicz, W., and Villano, S. (2010). Oral Maribavir for Treatment of Refractory or Resistant Cytomegalovirus Infections in Transplant Recipients. *Transplant Infectious Disease*, *12*(6), 489–496.

Battiwalla, M., Paplham, P., Almyroudis, N. G., McCarthy, A., Abdelhalim, A., Elefante, A., Smith, P., Becker, J., McCarthy, P. L., and Segal, B. H. (2007). Leflunomide Failure to Control Recurrent Cytomegalovirus Infection in the Setting of Renal Failure after Allogeneic Stem Cell Transplantation. *Transplant Infectious Disease*, *9*(1), 28–32.

Bauer, D. W., Huffman, J. B., Homa, F. L., and Evilevitch, A. (2013). Herpes Virus Genome, The Pressure Is On. *Journal of the American Chemical Society*, *135*(30), 11216–11221.

Bernhoff, E., Tylden, G. D., Kjerpeseth, L. J., Gutteberg, T. J., Hirsch, H. H., and Rinaldo, C. H. (2010). Leflunomide Inhibition of BK Virus Replication in Renal Tubular Epithelial Cells. *Journal of Virology*, *84*(4), 2150.

Biron, K. K., Fyfe, J. A., Stanat, S. C., Leslie, L. K., Sorrell, J. B., Lambe, C. U., and Coen, D. M. (1986). A Human Cytomegalovirus Mutant Resistant to the Nucleoside Analog 9-([2-Hydroxy-1-(Hydroxymethyl)Ethoxy]Methyl)Guanine (BW B759U) Induces Reduced Levels of BW B759U Triphosphate. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(22), 8769–8773.

Biron, K. K. (2006). Antiviral Drugs for Cytomegalovirus Diseases. *Antiviral Research*, *71*(2–3), 154–163.

Bodaghi, B., Goureau, O., Zipeto, D., Laurent, L., Virelizier, J. L., and Michelson, S. (1999). Role of IFN-Gamma-Induced Indoleamine 2,3 Dioxygenase and Inducible Nitric Oxide Synthase in the Replication of Human Cytomegalovirus in Retinal Pigment Epithelial Cells. *Journal of Immunology*, *162*(2), 957–964.

Boeckh, M. (2011). Complications, Diagnosis, Management, and Prevention of CMV Infections: Current and Future. *ASH Education Program Book*, *2011*(1), 305–309.

Bogner, E., Radsak, K., and Stinski, M. F. (1998). The Gene Product of Human Cytomegalovirus Open Reading Frame UL56 Binds the Pac Motif and Has Specific Nuclease Activity. *Journal of Virology*, *72*(3), 2259–2264.

Borst, E. M., Hahn, G., Koszinowski, U. H., and Messerle, M. (1999). Cloning of the Human Cytomegalovirus (HCMV) Genome as an Infectious Bacterial Artificial Chromosome in Escherichia Coli: A New Approach for Construction of HCMV Mutants. *Journal of Virology*, *73*(10), 8320.

Borst, E. M., and Messerle, M. (2000). Development of a Cytomegalovirus Vector for Somatic Gene Therapy. *Bone Marrow Transplantation*, *25*(S2), S80.

Borst, E. M., Benkartek, C., and Messerle, M. (2007). Use of Bacterial Artificial Chromosomes in Generating Targeted Mutations in Human and Mouse Cytomegaloviruses. In *Current Protocols in Immunology*. https://doi.org/10.1002/0471142735.im1032s77

Borst, E. M., Wagner, K., Binz, A., Sodeik, B., and Messerle, M. (2008). The Essential Human Cytomegalovirus Gene UL52 Is Required for Cleavage-Packaging of the Viral Genome. *Journal of Virology*, *82*(5), 2065–2078.

Bowlin, T., Brooks, J., and Zemlicka, J. (2009). Preclinical Pharmacokinetic, Toxicokinetic and Toxicology Results for Cyclopropavir, a Promising New Agent for the Treatment of Beta- and Gamma-Herpesviruses. *Antiviral Research*, *82*(2), A46–A47.

Bowman, L. J., Melaragno, J. I., and Brennan, D. C. (2017). Letermovir for the Management of Cytomegalovirus Infection. *Expert Opinion on Investigational Drugs*, *26*(2), 235–241.

Bryant, P., Morley, C., Garland, S., and Curtis, N. (2002). Cytomegalovirus Transmission from Breast Milk in Premature Babies: Does It Matter? *Archives of Disease in Childhood - Fetal and Neonatal Edition*, *87*(2), F75–F77.

Buscher, N., Paulus, C., Nevels, M., Tenzer, S., and Plachter, B. (2015). The Proteome of Human Cytomegalovirus Virions and Dense Bodies Is Conserved across Different Strains. *Medical Microbiology and Immunology*, *204*(3), 285–293.

Buxmann, H., Hamprecht, K., Meyer-Wittkopf, M., and Friese, K. (2017). Primary Human Cytomegalovirus (HCMV) Infection in Pregnancy. *Deutsches Arzteblatt International*, *114*(4), 45–52.

Campbell, D. T., and Kenny, D. A. (1999). *A primer on regression artifacts*. Guilford Publications.

Cantrell, S. R., and Bresnahan, W. A. (2006). Human Cytomegalovirus (HCMV) UL82 Gene Product (Pp71) Relieves HDaxx-Mediated Repression of HCMV Replication. *Journal of Virology*, *80*(12), 6188–6191.

Capobianchi, A., Iori, A. P., Micozzi, A., Torelli, G. F., Testi, A. M., Girmenia, C., Santilli, S., Barberi, W., Antonelli, G. et al. (2014). Cytomegalovirus in Bone Marrow Cells Correlates with Cytomegalovirus in Peripheral Blood Leukocytes. *Journal of Clinical Microbiology*, *52*(6), 2183–2185.

Cha, T. A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996). Human Cytomegalovirus Clinical Isolates Carry at Least 19 Genes Not Found in Laboratory Strains. *Journal of Virology*, *70*(1), 78–83.

Chaer, F. El, Shah, D. P., and Chemaly, R. F. (2016). How I Treat Resistant Cytomegalovirus Infection in Hematopoietic Cell Transplantation Recipients. *Blood*, *128*(23), 2624–2636.

Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A. 3rd, Kouzarides, T., and Martignetti, J. A. (1990). Analysis of the Protein-Coding Content of the Sequence of Human Cytomegalovirus Strain AD169. *Current Topics in Microbiology and Immunology*, *154*, 125–169.

Chemaly, R. F., Ullmann, A. J., Stoelben, S., Richard, M. P., Bornhäuser, M., Groth, C., Einsele, H., Silverman, M., Mullane, K. M. et al. (2014). Letermovir for Cytomegalovirus Prophylaxis in Hematopoietic-Cell

Transplantation. *New England Journal of Medicine*, *370*, 1781–1789.

Chen, D. H., Jiang, H., Lee, M., Liu, F., and Zhou, Z. H. (1999). Three-Dimensional Visualization of Tegument/Capsid Interactions in the Intact Human Cytomegalovirus. *Virology*, *260*(1), 10–16.

Chou, S. (2008). Cytomegalovirus UL97 Mutations in the Era of Ganciclovir and Maribavir. *Reviews in Medical Virology*, *18*(4), 233–246.

Chou, S. (2015). Rapid in Vitro Evolution of Human Cytomegalovirus UL56 Mutations That Confer Letermovir Resistance. *Antimicrobial Agents and Chemotherapy*, AAC-01623.

Chou, S., and Marousek, G. I. (2006). Maribavir Antagonizes the Antiviral Action of Ganciclovir on Human Cytomegalovirus. *Antimicrobial Agents and Chemotherapy*, *50*(10), 3470–3472.

Chou, S., Marousek, G. I., Senters, A. E., Davis, M. G., and Biron, K. K. (2004). Mutations in the Human Cytomegalovirus UL27 Gene That Confer Resistance to Maribavir. *Journal of Virology*, *78*(13), 7124–7130.

Chrisp, P., and Clissold, S. P. (1991). Foscarnet. A Review of Its Antiviral Activity, Pharmacokinetic Properties and Therapeutic Use in Immunocompromised Patients with Cytomegalovirus Retinitis. *Drugs*, *41*(1), 104–129.

Compton, T., Nepomuceno, R. R., and Nowlin, D. M. (1992). Human Cytomegalovirus Penetrates Host Cells by PH-Independent Fusion at the Cell Surface. *Virology*, *191*(1), 387–395.

Compton, T., Nowlin, D. M., and Cooper, N. R. (1993). Initiation of Human Cytomegalovirus Infection Requires Initial Interaction with Cell Surface Heparan Sulfate. *Virology*, *193*(2), 834–841.

Crumpacker, C. S. (1996). Ganciclovir. *The New England Journal of Medicine*, *335*(10), 721–729.

Cummings, B. S., Wills, L. P., and Schnellmann, R. G. (2012). Measurement of Cell Death in Mammalian Cells. *Current Protocols in Pharmacology*, *56*(1), 12–18.

Cunningham, C., Gatherer, D., Hilfrich, B., Baluchova, K., Dargan, D. J., Thomson, M., Griffiths, P. D., Wilkinson, G. W. G., Schulz, T. F., and Davison, A. J. (2010). Sequences of Complete Human Cytomegalovirus Genomes from Infected Cell Cultures and Clinical Specimens. *The Journal of General Virology*, *91*(Pt 3), 605–615.

Dargan, D. J., Douglas, E., Cunningham, C., Jamieson, F., Stanton, R. J., Baluchova, K., McSharry, B. P., Tomasec, P., Emery, V. C. et al. (2010). Sequential Mutations Associated with Adaptation of Human Cytomegalovirus to Growth in Cell Culture. *Journal of General Virology*, *91*, 1535–1546.

de Smet, M. D., Meenken, C., and van den Horn, G. J. (1999). Fomivirsen – a Phosphorothioate Oligonucleotide for the Treatment of CMV Retinitis. *Ocular Immunology and Inflammation*, *7*(3–4), 189–198.

Dittmer, A., and Bogner, E. (2005). Analysis of the Quaternary Structure of the Putative HCMV Portal Protein pUL104. *Biochemistry*, *44*(2), 759–765.

Dittmer, A., Drach, J. C., Townsend, L. B., Fischer, A., and Bogner, E. (2005). Interaction of the Putative Human Cytomegalovirus Portal Protein pUL104 with the Large Terminase Subunit pUL56 and Its Inhibition by Benzimidazole-D-Ribonucleosides. *Journal of Virology*, *79*(23), 14660–14667.

Dolan, A., Cunningham, C., Hector, R. D., Hassan-Walker, A. F., Lee, L., Addison, C., Dargan, D. J., McGeoch, D. J., Gatherer, D., … Davison, A. J. (2004). Genetic Content of Wild-Type Human Cytomegalovirus. *The Journal of General Virology*, *85*(Pt 5), 1301–1312.

Dollard, S. C., Grosse, S. D., and Ross, D. S. (2007). New Estimates of the Prevalence of Neurological and

Sensory Sequelae and Mortality Associated with Congenital Cytomegalovirus Infection. *Reviews in Medical Virology*, *17*(5), 355–363.

Drew, W. L., Miner, R. C., Marousek, G. I., and Chou, S. (2006). Maribavir Sensitivity of Cytomegalovirus Isolates Resistant to Ganciclovir, Cidofovir or Foscarnet. *Journal of Clinical Virology*, *37*(2), 124–127.

Dropulic, L. K., and Cohen, J. I. (2010). Update on New Antivirals under Development for the Treatment of Double-Stranded DNA Virus Infections. *Clinical Pharmacology and Therapeutics*, *88*(5), 610–619.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Zhu, H., and Liu, F. (2003). Functional Profiling of a Human Cytomegalovirus Genome. *Proceedings of the National Academy of Sciences*, *100*(24), 14223–14228.

Efferth, T., Romero, M. R., Wolf, D. G., Stamminger, T., Marin, J. J. G., and Marschall, M. (2008). The Antiviral Activities of Artemisinin and Artesunate. *Clinical Infectious Diseases*, *47*(6), 804–811.

Eglen, R., and Reisine, T. (2011). Primary Cells and Stem Cells in Drug Discovery: Emerging Tools for High-Throughput Screening. *Assay and Drug Development Technologies*, *9*(2), 108–124.

Elbasani, E., Gabaev, I., Steinbrück, L., Messerle, M., Borst, E., Elbasani, E., Gabaev, I., Steinbrück, L., Messerle, M., and Borst, E. M. (2014). Analysis of Essential Viral Gene Functions after Highly Efficient Adenofection of Cells with Cloned Human Cytomegalovirus Genomes. *Viruses*, *6*(1), 354–370.

EPAR_Public_assessment report. (2017). *Prevymis | European Medicines Agency*. London. Retrieved from https://www.ema.europa.eu/medicines/human/EPAR/prevymis

Erice, A. (1999). Resistance of Human Cytomegalovirus to Antiviral Drugs. *Clinical Microbiology Reviews*, *12*(2), 286–297.

Erice, A., Chou, S., Biron, K. K., Stanat, S. C., Balfour, H. H., and Jordan, M. C. (1989). Progressive Disease Due to Ganciclovir-Resistant Cytomegalovirus in Immunocompromised Patients. *New England Journal of Medicine*, *320*(5), 289–293.

Fairley, J. A., Baillie, J., Bain, M., and Sinclair, J. H. (2002). Human Cytomegalovirus Infection Inhibits Epidermal Growth Factor (EGF) Signalling by Targeting EGF Receptors. *Journal of General Virology*, *83*(11), 2803–2810.

Fallahi-Sichani, M., Honarnejad, S., Heiser, L. M., Gray, J. W., and Sorger, P. K. (2013). Metrics Other than Potency Reveal Systematic Variation in Responses to Cancer Drugs. *Nature Chemical Biology*, *9*(11), 708.

Feire, A. L., Koss, H., and Compton, T. (2004). Cellular Integrins Function as Entry Receptors for Human Cytomegalovirus via a Highly Conserved Disintegrin-like Domain. *Proceedings of the National Academy of Sciences*, *101*(43), 15470–15475.

Férir, G., Petrova, M. I., Andrei, G., Huskens, D., Hoorelbeke, B., Snoeck, R., Vanderleyden, J., Balzarini, J., Bartoschek, S., and Brönstrup, M. (2013). The Lantibiotic Peptide Labyrinthopeptin A1 Demonstrates Broad Anti-HIV and Anti-HSV Activity with Potential for Microbicidal Applications. *PLoS One*, *8*(5), e64010.

Fossum, E., Friedel, C. C., Rajagopala, S. V, Titz, B., Baiker, A., Schmidt, T., Kraus, T., Stellberger, T., Rutenberg, C., and Suthram, S. (2009). Evolutionarily Conserved Herpesviral Protein Interaction Networks. *PLoS Pathogens*, *5*(9), e1000570.

Frange, P., and Leruez-Ville, M. (2018). Maribavir, Brincidofovir and Letermovir: Efficacy and Safety of New Antiviral Drugs for Treating Cytomegalovirus Infections. *Médecine et Maladies Infectieuses*, *48*(8), 495–

502.

Gentry, B. G., Kamil, J. P., Coen, D. M., Zemlicka, J., and Drach, J. C. (2010). Stereoselective Phosphorylation of Cyclopropavir by pUL97 and Competitive Inhibition by Maribavir. *Antimicrobial Agents and Chemotherapy*, *54*(8), 3093–3098.

Gérard, L., and Salmon-Céron, D. (1995). Pharmacology and Clinical Use of Foscarnet. *International Journal of Antimicrobial Agents*, *5*(4), 209–217.

Gibson, W. (2008). Structure and Formation of the Cytomegalovirus Virion. *Current Topics in Microbiology and Immunology*, *325*, 187–204.

Goldner, T., Hempel, C., Ruebsamen-Schaeff, H., Zimmermann, H., and Lischka, P. (2014). Geno- and Phenotypic Characterization of Human Cytomegalovirus Mutants Selected in Vitro after Letermovir (AIC246) Exposure. *Antimicrobial Agents and Chemotherapy*, *58*(1), 610–613.

Goldner, T., Hewlett, G., Ettischer, N., Ruebsamen-Schaeff, H., Zimmermann, H., and Lischka, P. (2011). The Novel Anticytomegalovirus Compound AIC246 (Letermovir) Inhibits Human Cytomegalovirus Replication through a Specific Antiviral Mechanism That Involves the Viral Terminase. *Journal of Virology*, *85*(20), 10884–10893.

Goldner, T., Zimmermann, H., and Lischka, P. (2015). Phenotypic Characterization of Two Naturally Occurring Human Cytomegalovirus Sequence Polymorphisms Located in a Distinct Region of ORF UL56 Known to Be Involved in in Vitro Resistance to Letermovir. *Antiviral Research*, *116*, 48–50.

Hagen, C., Dent, K. C., Zeev-Ben-Mordehai, T., Grange, M., Bosse, J. B., Whittle, C., Klupp, B. G., Siebert, C. A., Vasishtan, D. et al. (2015). Structural Basis of Vesicle Formation at the Inner Nuclear Membrane. *Cell*, *163*(7), 1692–1701.

Hecker, M., Qiu, D., Marquardt, K., Bein, G., and Hackstein, H. (2004). Continuous Cytomegalovirus Seroconversion in a Large Group of Healthy Blood Donors. *Vox Sanguinis*, *86*(1), 41–44.

Heider, S., and Metzner, C. (2014). Quantitative Real-Time Single Particle Analysis of Virions. *Virology*, *462*, 199–206.

Ho, M. (2008). The History of Cytomegalovirus and Its Diseases. *Medical Microbiology and Immunology*, *197*(2), 65–73.

Homman-Loudiyi, M., Hultenby, K., Britt, W., and Söderberg-Nauclér, C. (2003). Envelopment of Human Cytomegalovirus Occurs by Budding into Golgi-Derived Vacuole Compartments Positive for GB, Rab 3, Trans-Golgi Network 46, and Mannosidase II. *Journal of Virology*, *77*(5), 3191.

Hostetler, K. Y. (2009). Alkoxyalkyl Prodrugs of Acyclic Nucleoside Phosphonates Enhance Oral Antiviral Activity and Reduce Toxicity: Current State of the Art. *Antiviral Research*, *82*(2), A84–A98.

Hughes, J. P., Rees, S., Kalindjian, S. B., and Philpott, K. L. (2011). Principles of Early Drug Discovery. *British Journal of Pharmacology*, *162*(6), 1239–1249.

Hwang, J. S., and Bogner, E. (2002). ATPase Activity of the Terminase Subunit pUL56 of Human Cytomegalovirus. *Journal of Biological Chemistry*, *277*(9), 6943–6948.

Hyde, T. B., Schmid, D. S., and Cannon, M. J. (2010). Cytomegalovirus Seroconversion Rates and Risk Factors: Implications for Congenital CMV. *Reviews in Medical Virology*, *20*(5), 311–326.

Irmiere, A., and Gibson, W. (1983). Isolation and Characterization of a Noninfectious Virion-like Particle Released from Cells Infected with Human Strains of Cytomegalovirus. *Virology*, *130*(1), 118–133.

Irmiere, A., and Gibson, W. (1985). Isolation of Human Cytomegalovirus Intranuclear Capsids, Characterization of Their Protein Constituents, and Demonstration That the B-Capsid Assembly Protein Is Also Abundant in Noninfectious Enveloped Particles. *Journal of Virology*, *56*(1), 277–283.

Isaacson, M. K., Feire, A. L., and Compton, T. (2007). Epidermal Growth Factor Receptor Is Not Required for Human Cytomegalovirus Entry or Signaling. *Journal of Virology*, *81*(12), 6241–6247.

James, S. H., Hartline, C. B., Harden, E. A., Driebe, E. M., Schupp, J. M., Engelthaler, D. M., Keim, P. S., Bowlin, T. L., Kern, E. R., and Prichard, M. N. (2011). Cyclopropavir Inhibits the Normal Function of the Human Cytomegalovirus UL97 Kinase. *Antimicrobial Agents and Chemotherapy*, *55*(10), 4682–4691.

James, S. H., Price, N. B., Hartline, C. B., Lanier, E. R., and Prichard, M. N. (2013). Selection and Recombinant Phenotyping of a Novel CMX001 and Cidofovir Resistance Mutation in Human Cytomegalovirus. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3321–3325.

Jayaweera, D. T. (1997). Minimising the Dosage-Limiting Toxicities of Foscarnet Induction Therapy. *Drug Safety*, *16*(4), 258–266.

Johnson, D. C., and Baines, J. D. (2011). Herpesviruses Remodel Host Membranes for Virus Egress. *Nature Reviews Microbiology*, *9*(5), 382–394.

Kaptein, S. J. F., Efferth, T., Leis, M., Rechter, S., Auerochs, S., Kalmer, M., Bruggeman, C. A., Vink, C., Stamminger, T., and Marschall, M. (2006). The Anti-Malaria Drug Artesunate Inhibits Replication of Cytomegalovirus in Vitro and in Vivo. *Antiviral Research*, *69*(2), 60–69.

Kaul, D. R., Stoelben, S., Cober, E., Ojo, T., Sandusky, E., Lischka, P., Zimmermann, H., and Rubsamen-Schaeff, H. (2011). First Report of Successful Treatment of Multidrug-Resistant Cytomegalovirus Disease with the Novel Anti-CMV Compound AIC246. *American Journal of Transplantation, 11*(5), 1079–1084.

Kemble, G. W., McCormick, A. L., Pereira, L., and Mocarski, E. S. (1987). A Cytomegalovirus Protein with Properties of Herpes Simplex Virus ICP8: Partial Purification of the Polypeptide and Map Position of the Gene. *Journal of Virology*, *61*(10), 3143–3151.

Kenneson, A., and Cannon, M. J. (2007). Review and Meta-Analysis of the Epidemiology of Congenital Cytomegalovirus (CMV) Infection. *Reviews in Medical Virology*, *17*(4), 253–276.

Kern, E. R., Kushner, N. L., Hartline, C. B., Williams-Aziz, S. L., Harden, E. A., Zhou, S., Zemlicka, J., and Prichard, M. N. (2005). In Vitro Activity and Mechanism of Action of Methylenecyclopropane Analogs of Nucleosides against Herpesvirus Replication. *Antimicrobial Agents and Chemotherapy*, *49*(3), 1039–1045.

Komaroff, A. L., Phan, T., Flamand, L., and Pellett, P. E. (2016). Summary of the 9th International Conference on Human Herpesviruses 6 and 7 (HHV- 6A, HHV- 6B, and HHV- 7). *Journal of Medical Virology*, *88*(12), 2038–2043.

Krosky, P. M., Baek, M.-C., and Coen, D. M. (2003). The Human Cytomegalovirus UL97 Protein Kinase, an Antiviral Drug Target, Is Required at the Stage of Nuclear Egress. *Journal of Virology*, *77*(2), 905–914.

Krosky, P. M., Baek, M.-C., Jahng, W. J., Barrera, I., Harvey, R. J., Biron, K. K., Coen, D. M., and Sethna, P. B. (2003). The Human Cytomegalovirus UL44 Protein Is a Substrate for the UL97 Protein Kinase. *Journal of Virology*, *77*(14), 7720–7727.

Lalezari, J. P., Drew, W. L., Glutzer, E., James, C., Miner, D., Flaherty, J., Fisher, P. E., Cundy, K., Hannigan, J., and Martin, J. C. (1995). (S)-1-[3-Hydroxy-2-(Phosphonylmethoxy)Propyl]Cytosine (Cidofovir): Results of a Phase I/II Study of a Novel Antiviral Nucleotide Analogue. *The Journal of Infectious*

Diseases, *171*(4), 788–796.

Lehman, I. R., and Boehmer, P. E. (1999). Replication of Herpes Simplex Virus DNA. *The Journal of Biological Chemistry*, *274*(40), 28059–28062.

Ligat, G., Cazal, R., Hantz, S., and Alain, S. (2018). The Human Cytomegalovirus Terminase Complex as an Antiviral Target: A Close-up View. *FEMS Microbiology Reviews*, *42*(2), 137–145.

Lin, D. Y., Warren, J. F., Lazzeroni, L. C., Wolitz, R. A., and Mansour, S. E. (2002). Cytomegalovirus Retinitis after Initiation of Highly Active Antiretroviral Therapy in HIV Infected Patients: Natural History and Clinical Predictors. *Retina (Philadelphia, Pa.)*, *22*(3), 268–277.

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2012). Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Advanced Drug Delivery Reviews*, *64*, 4–17.

Lischka, P., Hewlett, G., Wunberg, T., Baumeister, J., Paulsen, D., Goldner, T., Ruebsamen-Schaeff, H., and Zimmermann, H. (2010). In Vitro and in Vivo Activities of the Novel Anticytomegalovirus Compound AIC246. *Antimicrobial Agents and Chemotherapy*, *54*(3), 1290–1297.

Littler, E., Stuart, A. D., and Chee, M. S. (1992). Human Cytomegalovirus UL97 Open Reading Frame Encodes a Protein That Phosphorylates the Antiviral Nucleoside Analogue Ganciclovir. *Nature*, *358*(6382), 160–162.

Lukacova, V., Goelzer, P., Reddy, M., Greig, G., Reigner, B., and Parrott, N. (2016). A Physiologically Based Pharmacokinetic Model for Ganciclovir and Its Prodrug Valganciclovir in Adults and Children. *The AAPS Journal*, *18*(6), 1453–1463.

Lurain, N. S., and Chou, S. (2010). Antiviral Drug Resistance of Human Cytomegalovirus. *Clinical Microbiology Reviews*, *23*(4), 689–712.

Lyman, M. G., and Enquist, L. W. (2009). Herpesvirus Interactions with the Host Cytoskeleton. *Journal of Virology*, *83*(5), 2058–2066.

Maertens, J., Cordonnier, C., Jaksch, P., Poiré, X., J Wu, J., Wijatyk, A., Saliba, F., Witzke, O., and Villano, S. (2016). Maribavir Versus Valganciclovir for Preemptive Treatment of Cytomegalovirus (CMV) Viremia: A Randomized, Dose-Ranging, Phase 2 Study Among Hematopoietic Stem Cell Transplant (SCT) and Solid Organ Transplant (SOT) Recipients. *Open Forum Infectious Diseases* (Vol. 3).

Mahy, B. W. J., and Kangro, H. O. (1996). *Virology methods manual*. (Hierholzer. J.C. and Killington. R.A., Ed.) (1st ed.). Academic Press.

Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J., and Nadon, R. (2006). Statistical Practice in High-Throughput Screening Data Analysis. *Nature Biotechnology*, *24*(2), 167.

Marschall, M., Muller, Y. A., Diewald, B., Sticht, H., and Milbradt, J. (2017). The Human Cytomegalovirus Nuclear Egress Complex Unites Multiple Functions: Recruitment of Effectors, Nuclear Envelope Rearrangement, and Docking to Nuclear Capsids. *Reviews in Medical Virology*, *27*(4), e1934.

Marschall, M., Stamminger, T., Urban, A., Wildum, S., Ruebsamen-Schaeff, H., Zimmermann, H., and Lischka, P. (2012). In Vitro Evaluation of the Activities of the Novel Anticytomegalovirus Compound AIC246 (Letermovir) against Herpesviruses and Other Human Pathogenic Viruses. *Antimicrobial Agents and Chemotherapy*, *56*(2), 1135–1137.

Marty, F. M., Ljungman, P., Papanicolaou, G. A., Winston, D. J., Chemaly, R. F., Strasfeld, L., Young, J.-A. H.,

Rodriguez, T., Maertens, J. et al. (2011). Maribavir Prophylaxis for Prevention of Cytomegalovirus Disease in Recipients of Allogeneic Stem-Cell Transplants: A Phase 3, Double-Blind, Placebo-Controlled, Randomised Trial. *The Lancet Infectious Diseases*, *11*(4), 284–292.

Marty, F. M., Winston, D. J., Chemaly, R. F., Boeckh, M. J., Mullane, K. M., Shore, T. B., Papanicolaou, G. A., Morrison, M. E., Brundage, T. M., and Mommeja-Marin, H. (2016). Brincidofovir for Prevention of Cytomegalovirus (CMV) after Allogeneic Hematopoietic Cell Transplantation (HCT) in CMV-Seropositive Patients: A Randomized, Double-Blind, Placebo-Controlled, Parallel-Group Phase 3 Trial. *Biology of Blood and Marrow Transplantation*, *22*(3), S23.

Marty, F. M., Winston, D. J., Rowley, S. D., Vance, E., Papanicolaou, G. A., Mullane, K. M., Brundage, T. M., Robertson, A. T., Godkin, S. et al. (2013). CMX001 to Prevent Cytomegalovirus Disease in Hematopoietic-Cell Transplantation. *New England Journal of Medicine*, *369*(13), 1227–1236.

Masse, M. J., Karlin, S., Schachtel, G. A., and Mocarski, E. S. (1992). Human Cytomegalovirus Origin of DNA Replication (OriLyt) Resides within a Highly Complex Repetitive Region. *Proceedings of the National Academy of Sciences*, *89*(12), 5246–5250.

Massimo, C., and Naidu, K. (2015). HIV/AIDS and the Oral Manifestations Associated with the Disease. *Occup Med Health Aff*, *3*(2), 1000e108.

Meindl, K., Schmiederer, T., Schneider, K., Reicke, A., Butz, D., Keller, S., Gühring, H., Vértesy, L., Wink, J., and Hoffmann, H. (2010). Labyrinthopeptins: A New Class of Carbacyclic Lantibiotics. *Angewandte Chemie International Edition*, *49*(6), 1151–1154.

Melendez, D. P., and Razonable, R. R. (2015). Letermovir and Inhibitors of the Terminase Complex: A Promising New Class of Investigational Antiviral Drugs against Human Cytomegalovirus. *Infection and Drug Resistance*, *8*, 269.

Mercorelli, B., Sinigalia, E., Loregian, A., and Palù, G. (2008). Human Cytomegalovirus DNA Replication: Antiviral Targets and Drugs. *Reviews in Medical Virology*, *18*(3), 177–210.

Messerle, M., Crnkovic, I., Hammerschmidt, W., Ziegler, H., and Koszinowski, U. H. (1997). Cloning and Mutagenesis of a Herpesvirus Genome as an Infectious Bacterial Artificial Chromosome. *Proceedings of the National Academy of Sciences*, *94*(26), 14759–14763.

Mettenleiter, T. C. (2002). Herpesvirus Assembly and Egress. *Journal of Virology*, *76*(4), 1537–1547.

Michael Y Shapira, D. G. W. (2010). *Artesunate in Preemptive Treatment of Human Cytomegalovirus (CMV) in Stem Cell Transplant Recipients*.

Milbradt, J., Sonntag, E., Wagner, S., Strojan, H., Wangen, C., Lenac Rovis, T., Lisnic, B., Jonjic, S., Sticht, H. et al. (2018). Human Cytomegalovirus Nuclear Capsids Associate with the Core Nuclear Egress Complex and the Viral Protein Kinase pUL97. *Viruses*, *10*(1). 35.

Mocarski, E. S., Shenk, T., and Pass, R. F. (2013). *Cytomegaloviruses*. (B. N. Fields, D. M. Knipe, & P. M. Howley, Eds.) (6th ed.). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Müller, W. M., Schmiederer, T., Ensle, P., and Süssmuth, R. D. (2010). In Vitro Biosynthesis of the Prepeptide of Type-III Lantibiotic Labyrinthopeptin A2 Including Formation of a C-C Bond as a Post-translational Modification. *Angewandte Chemie International Edition*, *49*(13), 2436–2440.

Neuber, S., Wagner, K., Goldne, T., Lischka, P., Steinbrück, L., Messerle, M., and Maria Borst, E. (2017). Mutual Interplay between the Human Cytomegalovirus Terminase Subunits pUL51, pUL56, and pUL89

Promotes Terminase Complex Formation. *Journal of Virology*, *91*(12), e02384-16.

Ogawa-Goto, K., Tanaka, K., Gibson, W., Moriishi, E., Miura, Y., Kurata, T., Irie, S., and Sata, T. (2003). Microtubule Network Facilitates Nuclear Targeting of Human Cytomegalovirus Capsid. *Journal of Virology*, *77*(15), 8541–8547.

Pari, G. S., Kacica, M. A., and Anders, D. G. (1993). Open Reading Frames UL44, IRS1/TRS1, and UL36-38 Are Required for Transient Complementation of Human Cytomegalovirus OriLyt-Dependent DNA Synthesis. *Journal of Virology*, *67*(5), 2575–2582.

Park, R., and Baines, J. D. (2006). Herpes Simplex Virus Type 1 Infection Induces Activation and Recruitment of Protein Kinase C to the Nuclear Membrane and Increased Phosphorylation of Lamin B. *Journal of Virology*, *80*(1), 494–504.

Pietropaolo, R., and Compton, T. (1999). Interference with Annexin II Has No Effect on Entry of Human Cytomegalovirus into Fibroblast Cells. *Journal of General Virology*, *80*(7), 1807–1816.

Pietropaolo, R. L., and Compton, T. (1997). Direct Interaction between Human Cytomegalovirus Glycoprotein B and Cellular Annexin II. *Journal of Virology*, *71*(12), 9803–9807.

Prasad, V. K., Grimley, M., Papanicolaou, G., Yu, L., Florescu, D., Brundage, T., Mommeja-Marin, H., and Kurtzberg, J. (2014). Brincidofovir (CMX001) Is Well Tolerated in Highly Immunocompromised Pediatric Patients. *Biology of Blood and Marrow Transplantation*, *20*(2), S93.

Price, N. B., and Prichard, M. N. (2011). Progress in the Development of New Therapies for Herpesvirus Infections. *Current Opinion in Virology*, *1*(6), 548–554.

Reid, R., Mar, E. C., Huang, E. S., and Topal, M. D. (1988). Insertion and Extension of Acyclic, Dideoxy, and Ara Nucleotides by Herpesviridae, Human α and Human β Polymerases. A Unique Inhibition Mechanism for 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine Triphosphate. *Journal of Biological Chemistry*, *263*(8), 3898–3904.

Ryckman, B. J., Jarvis, M. A., Drummond, D. D., Nelson, J. A., and Johnson, D. C. (2006). Human Cytomegalovirus Entry into Epithelial and Endothelial Cells Depends on Genes UL128 to UL150 and Occurs by Endocytosis and Low-PH Fusion. *Journal of Virology*, *80*(2), 710–722.

Sanderson, D. M., and Earnshaw, C. G. (1991). Computer Prediction of Possible Toxic Action from Chemical Structure; the DEREK System. *Human & Experimental Toxicology*, *10*(4), 261–273.

Schierling, K., Buser, C., Mertens, T., and Winkler, M. (2005). Human Cytomegalovirus Tegument Protein pUL35 Is Important for Viral Replication and Particle Formation. *Journal of Virology*, *79*(5), 3084–3096.

Schreiber, A., Härter, G., Schubert, A., Bunjes, D., Mertens, T., and Michel, D. (2009). Antiviral Treatment of Cytomegalovirus Infection and Resistant Strains. *Expert Opinion on Pharmacotherapy*, *10*(2), 191–209.

Scrivano, L., Sinzger, C., Nitschko, H., Koszinowski, U. H., and Adler, B. (2011). HCMV Spread and Cell Tropism Are Determined by Distinct Virus Populations. *PLoS Pathogens*, *7*(1), e1001256.

Shannon-Lowe, C. D., and Emery, V. C. (2010). The Effects of Maribavir on the Autophosphorylation of Ganciclovir Resistant Mutants of the Cytomegalovirus UL97 Protein. *Herpesviridae*, *1*(1), 4.

Sharma, M., Kamil, J. P., Coughlin, M., Reim, N. I., and Coen, D. M. (2014). Human Cytomegalovirus UL50 and UL53 Recruit Viral Protein Kinase UL97, Not Protein Kinase C, for Disruption of Nuclear Lamina and Nuclear Egress in Infected Cells. *Journal of Virology*, *88*(1), 249–262.

Shoemaker, R. H. (2006). The NCI60 Human Tumour Cell Line Anticancer Drug Screen. *Nature Reviews*

Cancer, *6*(10), 813.

Silva, L. A., Loregian, A., Pari, G. S., Strang, B. L., and Coen, D. M. (2010). The Carboxy-Terminal Segment of the Human Cytomegalovirus DNA Polymerase Accessory Subunit UL44 Is Crucial for Viral Replication. *Journal of Virology*, *84*(21), 11563–11568.

Simonsson, S., Samuelsson, T., and Elias, P. (1998). The Herpes Simplex Virus Type 1 Origin Binding Protein. *Journal of Biological Chemistry*, *273*, 24633–24639.

Sinclair, J., and Sissons, P. (2006). Latency and Reactivation of Human Cytomegalovirus. *Journal of General Virology*, *87*(7), 1763–1779.

Sinzger, C., Digel, M., and Jahn, G. (2008). Cytomegalovirus Cell Tropism. *Current Topics in Microbiology and Immunology*, *325*, 63–83.

Sinzger, C., Hahn, G., Digel, M., Katona, R., Sampaio, K. L., Messerle, M., Hengel, H., Koszinowski, U., Brune, W., and Adler, B. (2008). Cloning and Sequencing of a Highly Productive, Endotheliotropic Virus Strain Derived from Human Cytomegalovirus TB40/E. *The Journal of General Virology*, *89*(Pt 2), 359–368.

Sittampalam, G. S., Iversen, P. W., Boadt, J. A., Kahl, S. D., Bright, S., Zock, J. M., Janzen, W. P., and Lister, M. D. (1997). Design of Signal Windows in High Throughput Screening Assays for Drug Discovery. *Journal of Biomolecular Screening*, *2*(3), 159–169.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-Mediated Transport of Incoming Herpes Simplex Virus 1 Capsids to the Nucleus. *The Journal of Cell Biology*, *136*(5), 1007–1021.

Sohn, Y. M., Oh, M. K., Balcarek, K. B., Cloud, G. A., and Pass, R. F. (1991). Cytomegalovirus Infection in Sexually Active Adolescents. *Journal of Infectious Diseases*, *163*(3), 460–463.

Stanton, R. J., Baluchova, K., Dargan, D. J., Cunningham, C., Sheehy, O., Seirafian, S., McSharry, B. P., Neale, M. L., Davies, J. A., … Wilkinson, G. W. G. (2010). Reconstruction of the Complete Human Cytomegalovirus Genome in a BAC Reveals RL13 to Be a Potent Inhibitor of Replication. *The Journal of Clinical Investigation*, *120*(9), 3191–3208.

Stigler, S. M. (2002). *Statistics on the table: The history of statistical concepts and methods*. Harvard University Press.

Stoelben, S., Arns, W., Renders, L., Hummel, J., Mühlfeld, A., Stangl, M., Fischereder, M., Gwinner, W., Suwelack, B., … Budde, K. (2014). Preemptive Treatment of Cytomegalovirus Infection in Kidney Transplant Recipients with Letermovir: Results of a Phase 2a Study. *Transplant International*, *27*(1), 77–86.

Sudarsanam, T. D., Sahni, R. D., and John, G. T. (2006). Leflunomide: A Possible Alternative for Gangciclovir Sensitive and Resistant Cytomegalovirus Infections. *Postgraduate Medical Journal*, *82*(967), 313–314.

Sullivan, V., Talarico, C. L., Stanat, S. C., Davis, M., Coen, D. M., and Biron, K. K. (1992). A Protein Kinase Homologue Controls Phosphorylation of Ganciclovir in Human Cytomegalovirus-Infected Cells. *Nature*, *358*(6382), 162–164.

Thoma, C., Borst, E., Messerle, M., Rieger, M., Hwang, J.-S., and Bogner, E. (2006). Identification of the Interaction Domain of the Small Terminase Subunit pUL89 with the Large Subunit pUL56 of Human Cytomegalovirus. *Biochemistry*, *45*(29), 8855–8863.

Tischer, B. K., Smith, G. A., and Osterrieder, N. (2010). En Passant Mutagenesis: A Two Step Markerless Red Recombination System. *Methods in Molecular Biology (Clifton, N.J.)*, *634*, 421–430.

Torres, T., Fernandes, I., Sanches, M., and Selores, M. (2011). Foscarnet-Induced Penile Ulceration. *Acta Dermatovenerologica Alpina, Pannonica, et Adriatica*, *20*(1), 39–40.

Vanarsdall, A. L., Chase, M. C., and Johnson, D. C. (2011). HCMV Glycoprotein GO Complexes with GH/GL Promoting Interference with Viral Entry into Human Fibroblasts, but Not Entry into Epithelial Cells. *Journal of Virology*, *85*(22), 11638–11645.

Varnum, S. M., Streblow, D. N., Monroe, M. E., Smith, P., Auberry, K. J., Pasa-Tolic, L., Wang, D., Camp, D. G. 2nd, Rodland, K., … Nelson, J. A. (2004). Identification of Proteins in Human Cytomegalovirus (HCMV) Particles: The HCMV Proteome. *Journal of Virology*, *78*(20), 10960–10966.

Von Glahn, W. C., and Pappenheimer, A. M. (1925). Intranuclear Inclusions in Visceral Disease. *The American Journal of Pathology*, *1*(5), 445–466.3.

Waldman, W. J., Knight, D. A., Lurain, N. S., Miller, D. M., Sedmak, D. D., Williams, J. W., and Chong, A. S. F. (1999). Novel Mechanism of Inhibition of Cytomegalovirus by the Experimental Immunosuppressive Agent Leflunomide. *Transplantation*, *68*(6), 814–825.

Wang, D., Yu, Q.-C., Schröer, J., Murphy, E., and Shenk, T. (2007). Human Cytomegalovirus Uses Two Distinct Pathways to Enter Retinal Pigmented Epithelial Cells. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(50), 20037–20042.

Wang, X., Huong, S.-M., Chiu, M. L., Raab-Traub, N., and Huang, E.-S. (2003). Epidermal Growth Factor Receptor Is a Cellular Receptor for Human Cytomegalovirus. *Nature*, *424*(6947), 456.

Wang, Y., Mao, L., Kankanala, J., Wang, Z., and Geraghty, R. J. (2017). Inhibition of Human Cytomegalovirus pUL89 Terminase Subunit Blocks Virus Replication and Genome Cleavage. *Journal of Virology*, *91*(3), e02152-16.

Wang, Y., Yang, Y., Wu, S., Pan, S., Zhou, C., Ma, Y., Ru, Y., Dong, S., He, B., … Cao, Y. (2014). P32 Is a Novel Target for Viral Protein ICP34.5 of Herpes Simplex Virus Type 1 and Facilitates Viral Nuclear Egress. *The Journal of Biological Chemistry*, *289*(52), 35795–35805.

Weller, S. K., and Coen, D. M. (2012). Herpes Simplex Viruses: Mechanisms of DNA Replication. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a013011.

Wildum, S., Zimmermann, H., and Lischka, P. (2015). In Vitro Drug Combination Studies of Letermovir (AIC246, MK-8228) with Approved Anti Human Cytomegalovirus (HCMV) and HIV Compounds in Inhibition of HCMV and HIV Replication. *Antimicrobial Agents and Chemotherapy*, AAC-00114.

Winston, D. J., Young, J.-A. H., Pullarkat, V., Papanicolaou, G. A., Vij, R., Vance, E., Alangaden, G. J., Chemaly, R. F., Petersen, F. et al. (2008). Maribavir Prophylaxis for Prevention of Cytomegalovirus Infection in Allogeneic Stem Cell Transplant Recipients: A Multicenter, Randomized, Double-Blind, Placebo-Controlled, Dose-Ranging Study. *Blood*, *111*(11), 5403–5410.

Wolf, D. G., Smith, I. L., Lee, D. J., Freeman, W. R., Flores-Aguilar, M., and Spector, S. A. (1995). Mutations in Human Cytomegalovirus UL97 Gene Confer Clinical Resistance to Ganciclovir and Can Be Detected Directly in Patient Plasma. *The Journal of Clinical Investigation*, *95*(1), 257–263.

Wu, Y., Prager, A., Boos, S., Resch, M., Brizic, I., Mach, M., Wildner, S., Scrivano, L., and Adler, B. (2017). Human Cytomegalovirus Glycoprotein Complex GH/GL/GO Uses PDGFR-α as a Key for Entry. *PLoS Pathogens*, *13*(4), e1006281.

Wyatt, J. P., Saxton, J., Lee, R. S., and Pinkerton, H. (1950). Generalized Cytomegalic Inclusion Disease. *The*

Journal of Pediatrics, *36*(3), 271–294.

Young, J., Psichogiou, M., Meyer, L., Ayayi, S., Grabar, S., Raffi, F., Reiss, P., Gazzard, B., … Bucher, H. (2012). CD4 Cell Count and the Risk of AIDS or Death in HIV-Infected Adults on Combination Antiretroviral Therapy with a Suppressed Viral Load: A Longitudinal Cohort Study from COHERE. *PLoS Medicine*, *9*(3), e1001194–e1001194.

Yu, D., Silva, M. C., and Shenk, T. (2003). Functional Map of Human Cytomegalovirus AD169 Defined by Global Mutational Analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(21), 12396–12401.

Yu, X., Shah, S., Lee, M., Dai, W., Lo, P., Britt, W., Zhu, H., Liu, F., and Zhou, Z. H. (2011). Biochemical and Structural Characterization of the Capsid-Bound Tegument Proteins of Human Cytomegalovirus. *Journal of Structural Biology*, *174*(3), 451–460.

Zhang, J.-H., Chung, T. D. Y., and Oldenburg, K. R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of Biomolecular Screening*, *4*(2), 67–73.

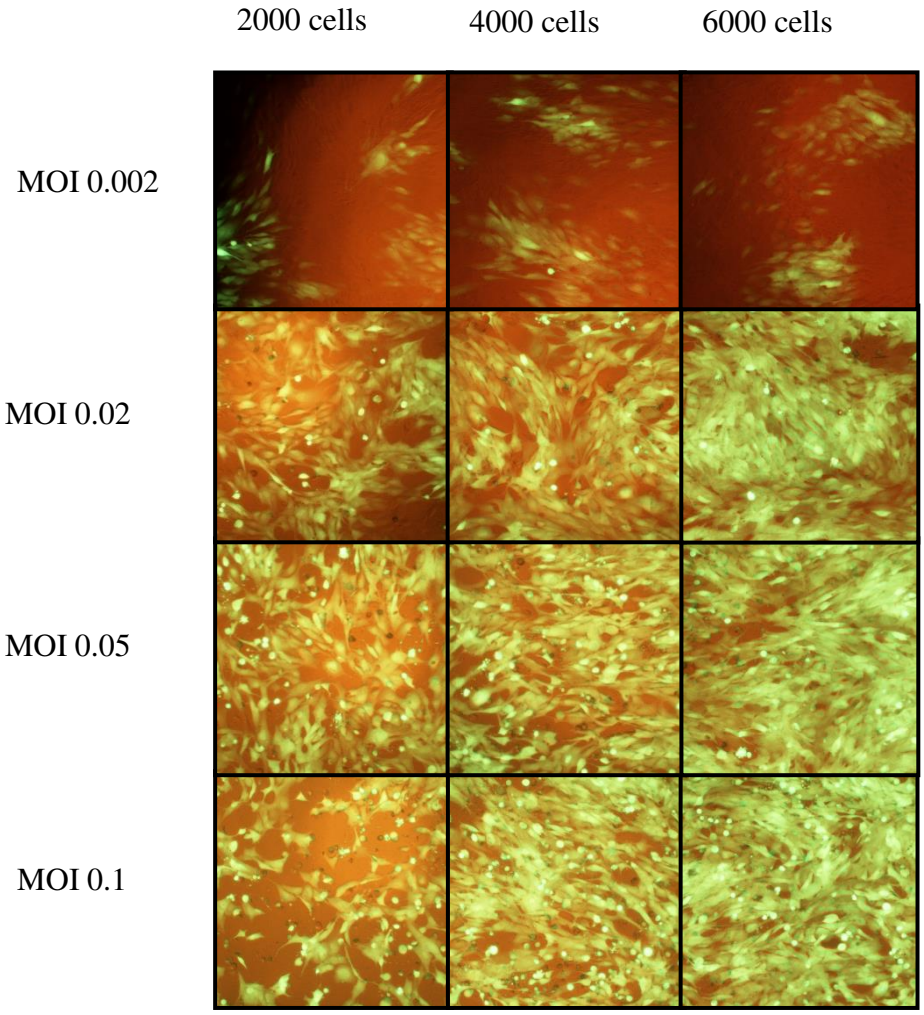
Zhang, X. D. (2011). Illustration of SSMD, z Score, SSMD*, Z* Score, and t Statistic for Hit Selection in RNAi High-Throughput Screens. *Journal of Biomolecular Screening*, *16*(7), 775–785.

Zhou, S., Breitenbach, J. M., Borysko, K. Z., Drach, J. C., Kern, E. R., Gullen, E., Cheng, Y.-C., and Zemlicka, J. (2004). Synthesis and Antiviral Activity of (Z)- and (E)-2,2-[Bis(Hydroxymethyl)Cyclopropylidene]Methylpurines and -Pyrimidines: Second-Generation Methylenecyclopropane Analogues of Nucleosides. *Journal of Medicinal Chemistry*, *47*(3), 566–575.

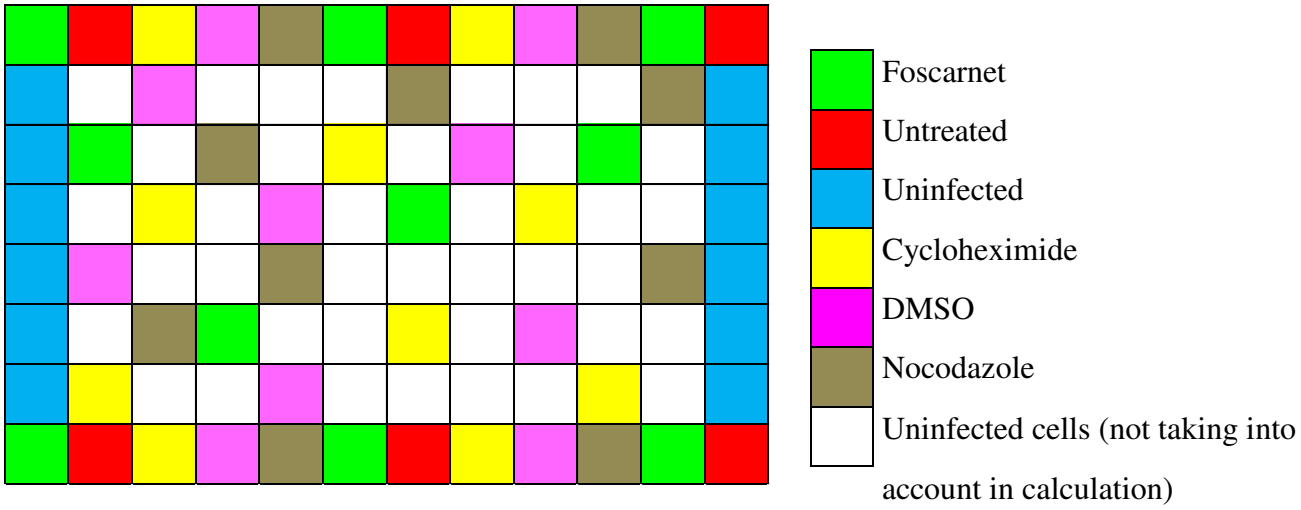
5. Appendices

5.1. Supplementary Figures

Supplementary Figure 1. Images of the cell cultures infected with the GFP-HT8 virus. Cell cultures at the indicated confluency were infected at increasing viral doses. At 6 dpi cells were exposed to fluorescence microscopy and photos were taken.



Supplementary Figure 2. Randomization of the test compounds within the 96-well plate used for the screening test.



Supplementary Figure 3. Results of the rescreen of the hits that showed activity against HCMV, HSV-1, KSHV, or RSV as defined in the primary screen. Each compound was tested 4 times independently (4 biological replicates). Numbers in the table indicate the percentage of the GFP expression in wells treated with the compounds (10 µM) normalized to the GFP expression in DMSO-treated controls. Similarly, the absorbance measurements for the MTT assay in relation to the DMSO control are depicted. Green highlighted rows indicate compounds chosen as lead hits after rescreening. Bright and saturated highlights indicate compounds which were selected as hits in one and several of the biological replicates of the rescreen, respectively. GFP 1 represents the effect of the compounds on the infection cycle after 6 d.p.i., and GFP 2 represents the effect of the compounds on virus release, measured by using the supernatants for infection of new cultures and determining GFP signals after additional 6 days.

														Final Hits approved
1st Library	GFP 1				MTT				Hits					No. times
Rescreen=>	1	2	3	4	1	2	3	4	1	2	3	4		GFP1
PANH_001	41	62	39	53	118	89	60	53	Hit	---	---	---		1
PANH_002	95	90	104	88	75	81	72	88	---	---	---	---		
PANH_003	93	98	107	101	116	98	72	101	---	---	---	---		
PANH_004	99	95	91	87	115	78	66	87	---	---	---	---		
PANH_005	68	79	57	75	75	83	55	75	---	---	---	---		
PANH_006	13	61	19	34	62	97	45	34	---	---	---	---		
PANH_007	77	97	110	103	79	91	61	103	---	---	---	---		
PANH_009	81	107	101	97	153	98	85	97	---	---	---	---		
PANH_010	131	99	106	109	108	94	78	109	---	---	---	---		
PANH_011	114	91	100	101	123	88	82	101	---	---	---	---		
PANH_012	124	93	101	115	109	88	106	115	---	---	---	---		
PANH_013	138	96	113	123	80	84	77	123	---	---	---	---		
PANH_014	103	83	106	105	90	76	72	105	---	---	---	---		
PANH_015	95	89	95	89	75	80	80	89	---	---	---	---		
PANH_016	85	100	117	111	81	78	53	111	---	---	---	---		
PANH_017	100	91	105	96	68	97	90	96	---	---	---	---		
PANH_019	113	98	114	110	107	82	74	110	---	---	---	---		
PANH_020	54	93	77	97	96	98	75	97	Hit	---	---	---		1
PANH_021	52	72	67	89	70	79	44	89	Hit	---	---	---		1
PANH_022	76	90	108	99	91	82	87	99	---	---	---	---		
PANH_023	49	74	68	74	89	112	63	74	Hit	---	---	---		1
PANH_024	80	82	91	100	97	98	62	100	---	---	---	---		
PANH_025	85	99	113	106	121	87	67	106	---	---	---	---		
PANH_026	101	102	92	99	80	87	69	99	---	---	---	---		
PANH_027	98	99	112	94	77	67	42	94	---	---	---	---		
PANH_028	94	109	113	95	95	91	72	95	---	---	---	---		

PANH_030	39	35	36	21	85	65	50	21					Hit	---	---	---					1	
PANH_031	33	0	0	0	81	3	7	0					Hit	---	---	---					1	
PANH_033	69	101	99	102	67	87	86	102					---	---	---	---						
PANH_034	39	61	49	44	94	74	61	44					Hit	---	---	---					1	
PANH_036	83	71	77	75	73	60	37	75					---	---	---	---						
PANH_037	19	25	13	14	72	18	19	14					Hit	---	---	---					1	
PANH_038	67	98	93	93	99	113	107	93					---	---	---	---						
PANH_039	53	64	63	68	88	89	57	68					Hit	---	---	---					1	
PANH_040	93	101	107	96	114	99	69	96					---	---	---	---						
PANH_041	22	102	115	113	77	97	95	113					Hit	---	---	---					1	
PANH_042	110	96	113	106	85	78	66	106					---	---	---	---						
PANH_043	91	99	111	114	105	101	59	114					---	---	---	---						
PANH_044	107	103	107	97	85	102	73	97					---	---	---	---						
PANH_045	72	90	70	85	83	101	77	85					---	---	---	---						
PANH_046	80	90	87	84	100	94	75	84					---	---	---	---						
PANH_047	62	80	73	74	89	84	66	74					---	---	---	---						
PANH_048	90	104	118	104	109	103	88	104					---	---	---	---						
PANH_049	84	106	105	100	116	107	74	100					---	---	---	---						
PANH_050	42	86	48	53	95	118	93	53					Hit	---	Hit	---					2	
PANH_051	43	76	52	57	88	120	90	57					Hit	---	Hit	---					2	
PANH_052	62	84	87	79	87	92	71	79					---	---	---	---						
PANH_053	50	85	64	70	95	99	101	70					Hit	---	---	---					1	
PANH_054	79	95	91	85	89	105	66	85					---	---	---	---						
PANH_055	105	84	106	95	92	81	46	95					---	---	---	---						
PANH_056	74	86	78	72	68	82	54	72					---	---	---	---						
2nd Library	GFP 1				MTT				GFP2				Hits GFP1				Hits GPF2				GFP1	GFP2
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		
PANH_057	33	52	51	58	49	70	62	67	0	6	2	4	---	Hit	---	---	---	Hit	---	---	1	1
PANH_058	42	87	69	84	58	87	88	99	53	96	37	76	---	---	---	---	---	---	---	---		
PANH_059	83	86	62	82	71	86	64	72	102	89	93	87	---	---	---	---	---	---	---	---		
PANH_060	90	81	94	76	74	85	71	77	28	71	66	89	---	---	---	---	---	---	---	---		
PANH_061	83	80	58	80	78	85	79	89	34	110	8	51	---	---	Hit	---	---	---	Hit	---	1	1
PANH_062	69	77	102	73	54	84	66	61	67	98	57	85	---	---	---	---	---	---	---	---		
PANH_063	66	98	82	92	64	107	90	107	9	89	25	55	---	---	---	---	---	---	---	---		
PANH_064	88	62	41	36	67	62	65	54	100	22	8	0	---	---	---	---	---	---	---	---		
PANH_065	7	69	57	59	53	84	73	78	0	27	9	18	---	---	Hit	Hit	---	---	Hit	Hit	2	2
PANH_066	95	105	95	94	74	105	86	74	87	107	65	105	---	---	---	---	---	---	---	---		
PANH_067	98	107	98	85	82	102	94	91	94	92	70	94	---	---	---	---	---	---	---	---		

PANH_068	5	18	12	13	64	82	70	54	0	0	0	0	---	Hit	Hit	---	---	Hit	Hit	---	2	2
PANH_069	92	96	89	87	80	92	74	81	39	88	20	85	---	---	---	---	---	---	Hit	---		1
PANH_070	48	81	59	88	46	81	75	86	6	36	17	39	---	---	Hit	---	---	---	Hit	---	1	1
PANH_071	95	90	92	104	73	89	82	90	79	115	37	86	---	---	---	---	---	---	---	---		
PANH_072	27	32	18	49	44	32	59	55	0	2	0	16	---	---	---	---	---	---	---	---		
PANH_073	81	89	78	96	72	89	73	68	93	113	37	102	---	---	---	---	---	---	---	---		
PANH_074	97	93	78	86	81	85	69	81	53	89	30	84	---	---	---	---	---	---	---	---		
PANH_075	103	91	86	97	94	91	83	89	88	88	71	90	---	---	---	---	---	---	---	---		
PANH_076	110	100	96	84	76	90	78	69	87	109	33	105	---	---	---	---	---	---	---	---		
PANH_077	103	98	96	91	79	81	77	83	95	177	48	106	---	---	---	---	---	---	---	---		
PANH_078	105	93	91	82	86	96	91	94	76	104	54	58	---	---	---	---	---	---	---	---		
PANH_079	93	92	93	77	82	88	73	81	70	97	69	86	---	---	---	---	---	---	---	---		
PANH_080	53	57	53	60	41	72	75	66	2	8	2	15	---	Hit	Hit	---	---	Hit	Hit	---	2	2
PANH_081	97	96	104	97	77	97	103	92	70	122	57	116	---	---	---	---	---	---	---	---		
PANH_082	88	94	79	92	80	94	73	74	35	58	50	67	---	---	---	---	---	---	---	---		
PANH_083	87	104	89	84	82	95	65	81	64	122	32	79	---	---	---	---	---	---	---	---		
PANH_084	95	79	95	76	64	68	56	58	86	137	54	98	---	---	---	---	---	---	---	---		
PANH_085	87	80	72	46	60	63	60	43	35	127	6	4	---	---	---	---	---	---	---	---		
PANH_086	93	88	92	74	83	86	74	79	71	108	58	83	---	---	---	---	---	---	---	---		
PANH_087	93	84	95	77	88	91	88	76	59	56	47	61	---	---	---	---	---	---	---	---		
PANH_088	64	77	84	87	88	100	89	95	12	49	28	50	---	---	---	---	Hit	---	---	---		1
PANH_089	90	96	103	97	80	96	74	77	70	92	30	75	---	---	---	---	---	---	---	---		
PANH_090	79	95	79	89	72	95	79	84	67	84	83	107	---	---	---	---	---	---	---	---		
PANH_091	101	101	95	99	81	101	80	79	97	104	57	112	---	---	---	---	---	---	---	---		
PANH_092	84	84	64	66	76	89	73	70	72	84	33	61	---	---	---	---	---	---	---	---		
PANH_093	91	99	91	89	84	99	79	83	58	113	34	64	---	---	---	---	---	---	---	---		
PANH_094	94	97	106	97	74	97	73	73	124	163	127	133	---	---	---	---	---	---	---	---		
PANH_095	72	88	84	70	60	76	60	57	75	107	39	83	---	---	---	---	---	---	---	---		
PANH_096	92	98	99	74	87	90	73	76	146	141	80	110	---	---	---	---	---	---	---	---		
PANH_097	25	75	33	65	50	91	71	83	1	123	16	100	---	---	Hit	---	---	---	Hit	---	1	
PANH_098	96	105	86	99	89	112	96	118	29	57	41	69	---	---	---	---	---	---	---	---		
PANH_099	33	49	36	54	51	84	65	80	0	1	1	3	---	Hit	---	Hit	---	Hit	---	Hit	2	2
PANH_100	88	92	95	99	77	83	77	85	62	134	83	126	---	---	---	---	---	---	---	---		
PANH_101	55	51	55	39	76	75	73	63	0	1	1	0	Hit	Hit	Hit	---	Hit	Hit	Hit	---	3	3
PANH_102	25	27	8	12	67	98	70	82	0	0	0	0	---	Hit	Hit	Hit	---	Hit	Hit	Hit	3	3
PANH_103	108	101	86	73	92	91	81	91	86	107	22	24	---	---	---	---	---	---	---	---		
PANH_104	97	105	106	93	82	91	90	89	100	95	62	110	---	---	---	---	---	---	---	---		
PANH_105	75	93	60	90	59	93	73	91	29	70	28	56	---	---	Hit	---	---	---	---	---	1	

PANH_106	98	104	89	92	93	82	72	83	72	89	29	86	---	---	---	---	---	---	---	---		
PANH_107	92	89	80	90	70	89	90	88	77	70	78	99	---	---	---	---	---	---	---	---		
PANH_108	76	97	86	100	68	97	84	85	89	88	112	85	---	---	---	---	---	---	---	---		
PANH_109	100	99	100	95	86	97	91	90	97	103	59	108	---	---	---	---	---	---	---	---		
PANH_110	86	76	73	39	60	79	59	54	46	49	5	5	---	---	---	---	---	---	---	---		
PANH_111	15	20	19	15	74	78	75	75	0	0	0	0	Hit	Hit	Hit	Hit	Hit	Hit	Hit	Hit	4	4
PANH_112	17	80	39	44	83	80	92	78	0	21	1	1	Hit	---	Hit	Hit	Hit	---	Hit	Hit	3	3
PANH_113	101	98	105	91	84	94	74	91	102	110	112	115	---	---	---	---	---	---	---	---		
PANH_114	101	105	98	98	78	105	95	87	91	110	113	109	---	---	---	---	---	---	---	---		
PANH_115	107	108	97	97	51	108	39	59	71	130	106	112	---	---	---	---	---	---	---	---		
PANH_116	115	106	104	112	105	106	99	98	101	100	61	89	---	---	---	---	---	---	---	---		
PANH_117	66	84	77	83	32	84	41	54	111	115	175	130	---	---	---	---	---	---	---	---		
PANH_118	88	85	84	85	103	119	92	111	1	25	3	18	---	---	---	---	Hit	---	Hit	Hit		
PANH_119	98	96	88	94	87	106	81	104	65	85	37	83	---	---	---	---	---	---	---	---		
PANH_120	105	103	89	87	93	99	82	81	57	100	40	79	---	---	---	---	---	---	---	---		
PANH_121	86	68	61	76	63	71	56	59	18	48	3	27	---	---	---	---	---	---	---	---		
PANH_122	93	94	101	82	80	88	70	82	102	64	51	77	---	---	---	---	---	---	---	---		
PANH_123	11	21	9	12	55	64	58	56	0	0	0	0	---	---	---	---	---	---	---	---		
PANH_124	64	69	38	58	82	85	71	85	13	27	4	14	---	---	Hit	Hit	Hit	---	Hit	Hit	2	3
PANH_125	60	73	63	74	81	86	64	74	4	26	3	22	Hit	---	---	---	Hit	---	---	---	1	1
PANH_126	117	110	123	85	87	83	78	71	70	116	107	137	---	---	---	---	---	---	---	---		
PANH_127	82	96	67	86	80	90	82	81	18	49	18	68	---	---	---	---	Hit	---	Hit	---		2
PANH_128	96	90	83	79	85	83	73	82	81	123	29	73	---	---	---	---	---	---	---	---		
PANH_129	51	45	48	46	73	83	79	79	0	0	0	0	Hit	Hit	Hit	Hit	Hit	Hit	Hit	Hit	4	4
PANH_130	86	81	72	62	69	74	63	62	45	91	47	68	---	---	---	---	---	---	---	---		
PANH_131	99	99	99	88	98	86	93	96	88	106	67	89	---	---	---	---	---	---	---	---		
PANH_132	67	73	68	85	68	77	76	75	41	84	40	79	---	---	---	---	---	---	---	---		
PANH_133	74	89	51	83	67	89	82	90	23	38	20	41	---	---	Hit	---	---	---	Hit	---	1	1
PANH_134	98	88	103	82	60	71	72	74	136	186	175	145	---	---	---	---	---	---	---	---		
PANH_135	68	99	89	104	74	99	85	81	111	147	190	165	---	---	---	---	---	---	---	---		
DMSO	100	100	100	100	100	100	100	100	100	100	100	100	---	---	---	---	---	---	---	---		
Let	51	63	50		108	118	107		0	0	1		Hit	---	Hit	---	Hit	Hit	Hit	---	2	3
CHX	0	0	0	0	6	8	10	9	0	0	0	0	---	---	---	---	---	---	---	---		
Fos	28	13	9	4	107	97	99	94	0	0	0	0	Hit	Hit	Hit	Hit	Hit	Hit	Hit	Hit	4	4
UT	104	100	101	97	100	107	100	115	85	91	70	93	---	---	---	---	---	---	---	---		
UI	0	0	0	0	72	88	82	80	0	0	0	0	Hit	Hit	Hit	Hit	Hit	Hit	Hit	Hit	4	4

3rd Library	GFP 1				MTT				Hits				GFP1
	1	2	3	4	1	2	3	4	1	2	3	4	
PANH_136	185	183	142	75	64	77	36	50	---	---	---	---	
PANH_138	95	106	60	96	99	76	68	103	---	---	---	---	
PANH_139	55	44	33	13	98	59	71	73	Hit	---	Hit	Hit	3
PANH_140	73	68	81	40	118	72	103	102	---	---	---	Hit	1
PANH_142	73	59	78	42	66	63	54	55	---	---	---	---	
PANH_143	42	30	34	23	70	59	55	79	---	---	---	Hit	1
PANH_144	65	99	89	55	102	97	117	130	---	---	---	Hit	1
PANH_145	44	29	56	30	84	80	82	135	Hit	Hit	Hit	Hit	4
PANH_146	53	52	54	13	79	70	67	79	Hit	---	---	Hit	2
PANH_147	48	54	48	28	83	78	74	116	Hit	Hit	Hit	Hit	4
PANH_148	31	35	28	20	66	58	64	87	---	---	---	Hit	1
PANH_149	91	101	110	49	91	81	75	73	---	---	---	Hit	1
PANH_150	91	101	42	32	72	60	69	70	---	---	---	Hit	1
PANH_151	36	39	47	28	62	48	68	43	---	---	---	---	
PANH_152	72	79	68	50	57	47	58	60	---	---	---	---	
PANH_154	30	33	36	30	71	84	58	99	Hit	Hit	---	Hit	3
PANH_155	86	92	95	90	73	61	69	83	---	---	---	---	
PANH_156	32	19	38	14	44	54	53	51	---	---	---	---	
PANH_157	127	163	144	98	75	82	40	63	---	---	---	---	
PANH_158	80	67	61	54	93	78	83	106	---	---	---	Hit	1
PANH_159	34	20	37	23	76	54	68	59	Hit	---	---	---	1
PANH_160	29	58	44	69	68	68	60	58	---	---	---	---	
PANH_161	74	92	56	53	66	61	86	57	---	---	Hit	---	1
PANH_162	77	57	74	45	90	62	98	78	---	---	---	Hit	1
PANH_163	83	103	122	88	71	68	59	56	---	---	---	---	
PANH_164	76	89	66	65	83	79	35	56	---	---	---	---	
PANH_165	108	101	146	83	88	82	47	95	---	---	---	---	
PANH_166	-2	0	26	7	2	25	27	28	---	---	---	---	
PANH_167	72	62	100	75	67	64	51	74	---	---	---	---	
PANH_168	15	-2	29	16	63	24	68	95	---	---	---	Hit	1
PANH_169	48	-5	19	2	69	2	82	36	---	---	Hit	---	1
PANH_170	64	119	112	44	77	71	86	78	---	---	---	Hit	1
PANH_171	43	51	58	36	59	58	59	49	---	---	---	---	
PANH_172	-1	-1	4	1	39	10	46	30	---	---	---	---	
PANH_173	136	86	130	76	80	65	74	74	---	---	---	---	
PANH_174	95	93	124	88	71	61	61	83	---	---	---	---	

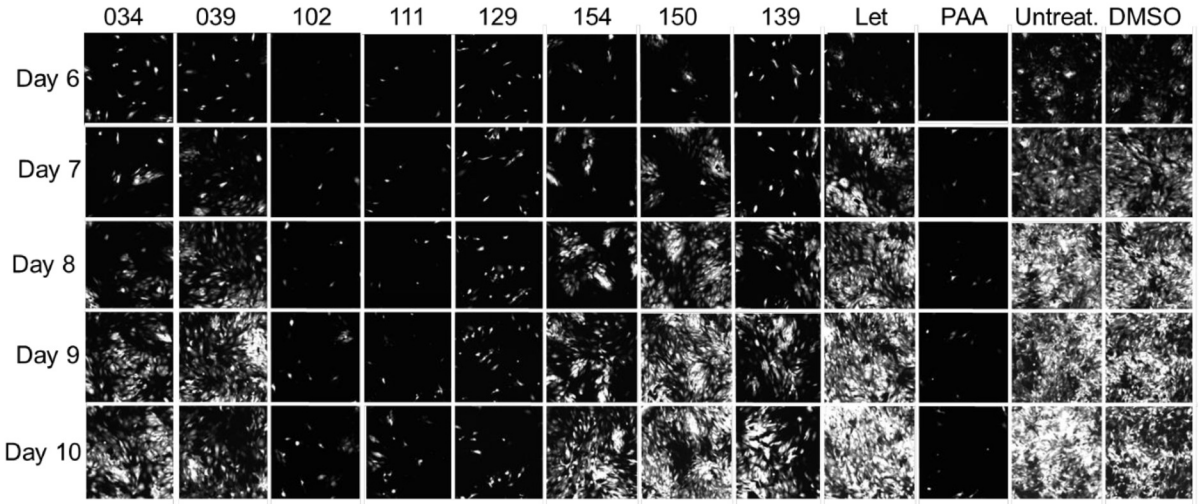
PANH_175	93	141	105	111	93	82	69	75	---	---	---	---		
PANH_176	83	120	114	78	75	65	65	75	---	---	---	---		
PANH_177	98	72	96	45	76	50	77	61	---	---	---	---		
PANH_178	46	-5	-3	13	69	3	5	45	---	---	---	---		
PANH_179	11	8	15	3	36	30	38	23	---	---	---	---		
PANH_181	51	6	42	33	63	48	39	56	---	---	---	---		
PANH_182	98	105	77	67	59	62	29	58	---	---	---	---		
PANH_183	86	50	82	60	92	75	38	69	---	Hit	---	---		1
PANH_184	20	6	8	9	64	59	67	82	---	---	---	Hit		1
PANH_185	12	24	25	16	65	66	73	87	---	---	Hit	Hit		2
PANH_189	20	0	22	13	53	31	42	36	---	---	---	---		
PANH_190	10	2	-3	3	66	37	52	34	---	---	---	---		
PANH_192	43	9	35	26	73	58	68	66	Hit	---	---	---		1
PANH_194	61	-5	1	73	70	3	12	71	---	---	---	---		
PANH_195	71	-5	25	40	78	7	45	74	---	---	---	Hit		1
PANH_196	31	36	44	22	86	80	76	99	Hit	Hit	Hit	Hit		4
PANH_197	68	32	68	42	69	35	51	77	---	---	---	Hit		1
PANH_198	-2	-5	-3	0	26	3	23	9	---	---	---	---		
PANH_199	13	-4	6	6	48	48	60	44	---	---	---	---		
PANH_200	22	7	0	5	52	40	50	59	---	---	---	---		
PANH_201	58	23	19	26	72	51	57	65	Hit	---	---	---		1
PANH_202	26	17	10	14	58	44	60	53	---	---	---	---		
PANH_203	65	38	35	14	67	57	52	44	---	---	---	---		
PANH_204	1	-3	-2	2	46	46	55	40	---	---	---	---		

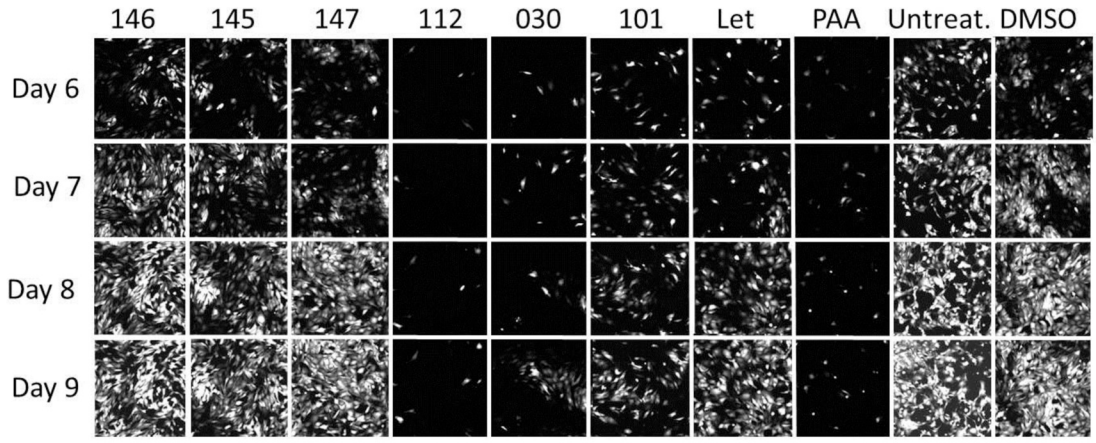
Supplementary Figure 4. Overview of all experiments that were performed for the selected lead hits. Compounds which were selected as the 14 lead hits are marked in green. Blue marked cells represent values that meet the selected criteria and were used to define hits, namely inhibiting GFP expression of HCMV by at least 60% (GFP1) or virus release by at least 20% (GPP2) and cell viability of more than 70%. Orange marked cells are compounds which led to lower viability at 60 µM. Yellow marked cells are the controls. The number of experiments that are performed for each assay is labelled with n.

	nHDF moi 0.05, 6 dpi			nHDF moi 0.5, 3dpi		nHDF moi0.5, 5dpi		cytotoxicity assay		cytotoxicity assay 3 days			Time of addition assay	
	GFP1	MTT	GFP2 10% 6dpri	GFP	MTT	GFP	MTT	MTT 6 days n=1-4		n=1	n=2	n=3	10 µM	
	10 µM n=3-4		10 µM n=1-2	10 µM n=2		10 µM n=3-5	10 µM n=3-4	20 µM	10 µM	60 µM	20 µM	10 µM	>24 hpi	48 hpi
PANH_001	80	123						57	58					
PANH_020	106	121						61	84					
PANH_021	84	89	26					56	107					
PANH_023								62	82					
PANH_030	41	84	0			86	98	70	99	35	99	106		
PANH_031								6	25					
PANH_034	78	89	6			90	110	74	102	45	103	108		
PANH_037	16	34	1					37	46					
PANH_039	72	112	1			84	134	86	94					
PANH_041								105	87					
PANH_050								112	85					
PANH_051	84	133	45					109	103					
PANH_053	96	115						89	90					
PANH_057	112	95	88	86	100								102	72
PANH_061	118	110	95	93	110								108	108
PANH_065								90	94					
PANH_068														
PANH_070	108	100	89											
PANH_080														
PANH_097														
PANH_099	54	90	4	75	94			90	123					
PANH_101	37	62	0	97	86	72		79	80	81	89	94		
PANH_102	37	67	1	33	114	94	151	97	109	49	95	97	18	85
PANH_105														
PANH_111	18	85	29	39	97	66	114	105	118	54	101	99	38	96
PANH_112	64	105	7	78	100	140		107	109	101	109	112		
PANH_124	50	76	25	83	85			85	90					
PANH_125	73	79	15					86	95					
PANH_127	81	86	85											
PANH_129	43	75	0	96	93	83	107	107	102	86	103	100		
PANH_133	69	94	74			62	109							
PANH_139						87	95	77	88	37	99	87		
PANH_140						80	95							
PANH_143														
PANH_144						84	140							
PANH_145						88	111	2	126					
PANH_146						65	103							
PANH_147						85	101	111	113					
PANH_148														
PANH_149														
PANH_150						79	94							
PANH_154						75	95	107	109	92	118	118		
PANH_158						107	103							
PANH_159														
PANH_161														
PANH_162						91	95							
PANH_168														
PANH_169														
PANH_170						77	129							
PANH_183														
PANH_184						75	100							
PANH_185						91	109							
PANH_192						86	117							
PANH_195														
PANH_196						82	40	111	113					
PANH_197														
PANH_201														
DMSO				95	95	99	100	92	100		94	100	100	100
Letermovie				102	96	102	99	107	109		95	98	110	113
PAA						39	158	103	103		99	104	31	87
Untreated				107	94	104	92							
Foscarnet				9	134									
Cycloheximide								6	9		43	44		
Uninfected														

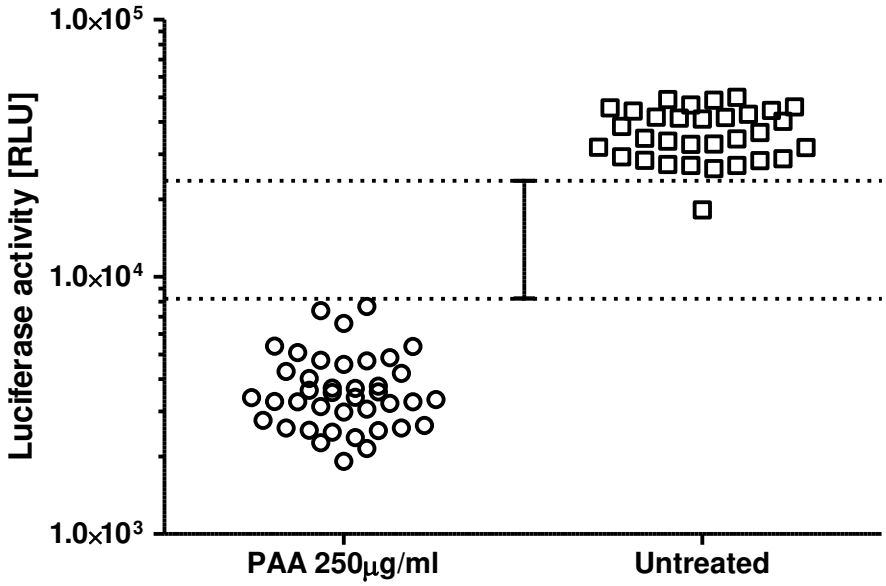
Toxicity assay (without infection)			MCMV, MEF, moi0.01, 5dpi	qPCR	nHDF moi0.05, 8dpi	cytotoxicity Cell-titer GLO		Plaque assay
	HFF	RPE	GFP	viral load	GFP n=2	n=2		virus titer n=1
	10 µM n=1		10 µM	10 µM	10 µM	20 µM	10 µM	10 µM
PANH_001								
PANH_020								
PANH_021								
PANH_023								
PANH_030	68	86		1	10	82	104	29
PANH_031								
PANH_034	100	103		1	22	82	102	11
PANH_037								
PANH_039	108	72			53			
PANH_041								
PANH_050								
PANH_051								
PANH_053								
PANH_057								
PANH_061								
PANH_065								
PANH_068								
PANH_070								
PANH_080								
PANH_097								
PANH_099								
PANH_101				31	27	94	94	1
PANH_102	108	47	80	9	6	85	91	46
PANH_105								
PANH_111	83	53	56	7	6	73	78	47
PANH_112				14	5	94	103	81
PANH_124								
PANH_125								
PANH_127								
PANH_129	77	98	13	110	15	100	97	0
PANH_133								
PANH_139	78	47		26	23	90	92	82
PANH_140								
PANH_143	91	62						
PANH_144								
PANH_145	93	46			90			
PANH_146					112			
PANH_147	110	105			109			
PANH_148								
PANH_149								
PANH_150								
PANH_154	112	82	72	101	40	96	95	13
PANH_158								
PANH_159								
PANH_161								
PANH_162								
PANH_168								
PANH_169								
PANH_170								
PANH_183								
PANH_184								
PANH_185								
PANH_192								
PANH_195								
PANH_196								
PANH_197								
PANH_201								
DMSO			100		100			
Letermovie				76	44	96		0
PAA			1	0	4	97		0
Untreated			82	100				
Foscarnet								
Cycloheximide						45		
Uninfected						87		

Supplementary Figure 5. GFP reduction assay to determine the antiviral activity of 14 selected compounds. Fluorescence microscopic images taken of cells at various days post infection. The activity of compounds was tested by GFP reduction assay using a low viral dose (MOI= 0.05). The concentrations that were used here for compounds, PAA, letermovir, and DMSO were 10 µM, 180 µM, 10 nM and 0.1%, respectively.

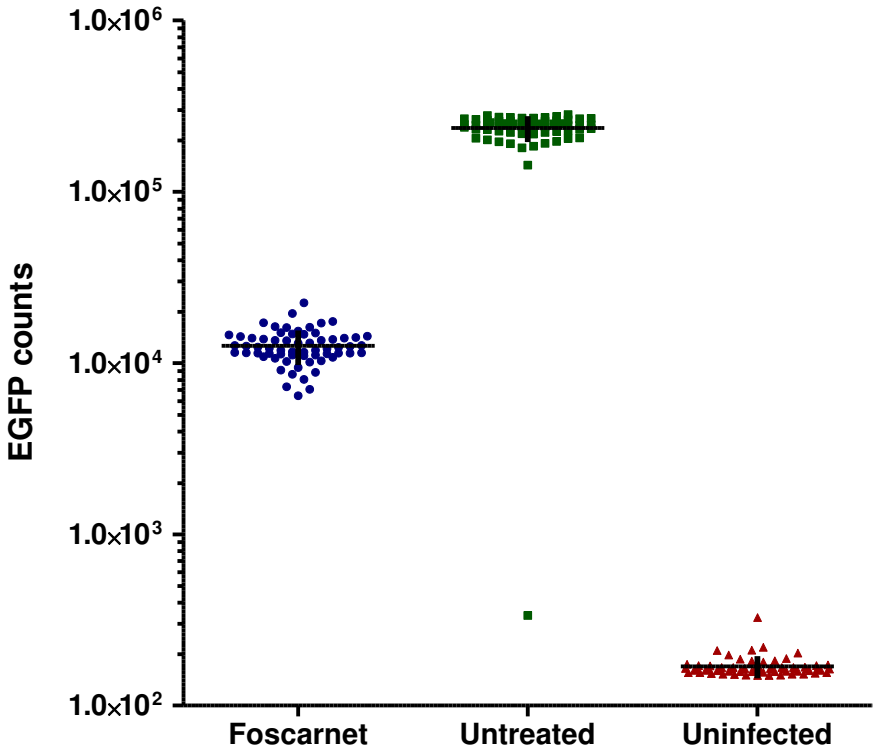




Supplementary Figure 6. Variability of the readout within a plate using a luciferase-expressing recombinant HCMV. nHDF cells were infected with the luciferase-expressing recombinant HCMV and treated either with PAA (250 µg/ml) or left untreated. Cells were measured for the infection efficiency at 4 days post infection using a luciferase assay. Each point represents a single well measurement from a 96-well format plate (40 replicates for PAA, 32 replicates for untreated). Vertical line represents the delta between the two samples.

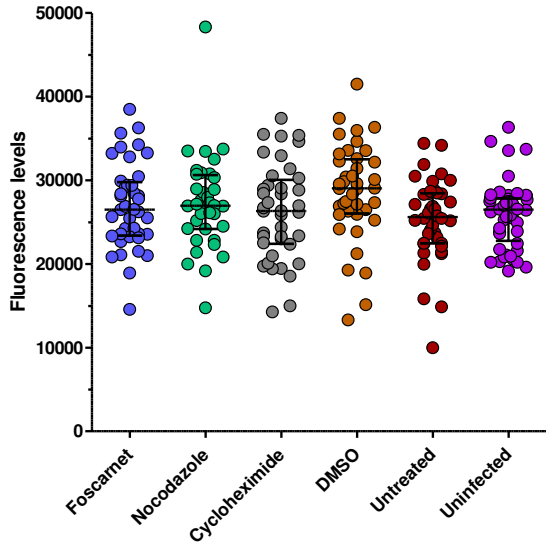


Supplementary Figure 7. Edge effects of the controls for experimental design of the HTS. nHDF cells were seeded on the edges of the 384-well plates. They were either left without infection or were infected at MOI 0.05 with a GFP tagged HCMV and were treated with foscarnet (180 μM) or left without treatment. Cells were measured for their GFP expression after 6 days. Each point represents the value from a single well in a 384-well format plate. Horizontal bars represent means and the error bars are the standard deviation of 36-48 replicates.

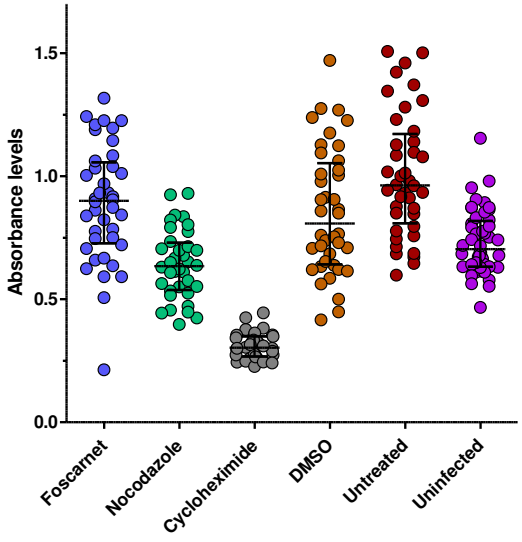


Supplementary Figure 8. Comparison of the DAPI staining and crystal violet staining. A pilot experiment was performed using the automated robot before the screening of the second library. Test inhibitors were randomized according to the design that was previously described. nHDF cells were infected with an MOI 0.05 and treated with foscarnet (180 µM), nocodazole (50 µM), cycloheximide (10 µg/ml), and DMSO (0.1%) or remained untreated. A) Fluorescence levels were measured by a plate reader after DAPI staining at day 6. B) Subsequently cells were stained with crystal violet and absorbance levels were measured by a plate reader. C) Fluorescence microscopic images taken of cells after DAPI staining, but before crystal violet staining. Cell nuclei are shown as blue signals, and cells that express GFP after infection are shown in green. Each point represents the value from a single well in a 384-well format plate. Horizontal bars represent medians and the error bars are the interquartile range of 36 (nocodazole), 40 (foscarnet, cycloheximide, DMSO, and untreated), and 48 (uninfected) replicates.

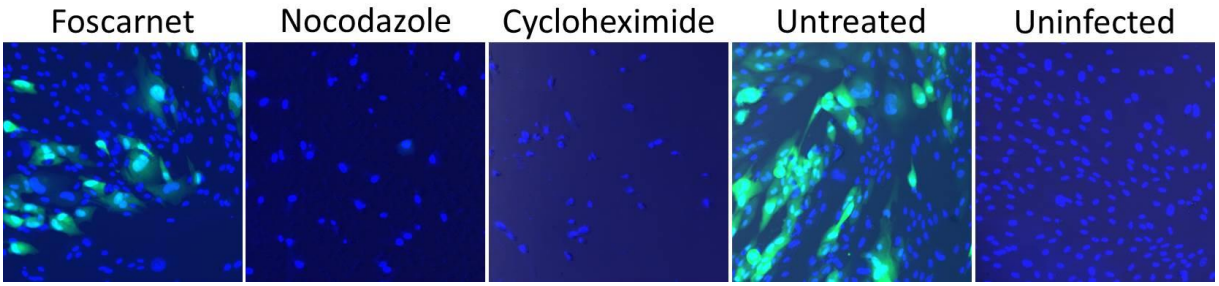
A)



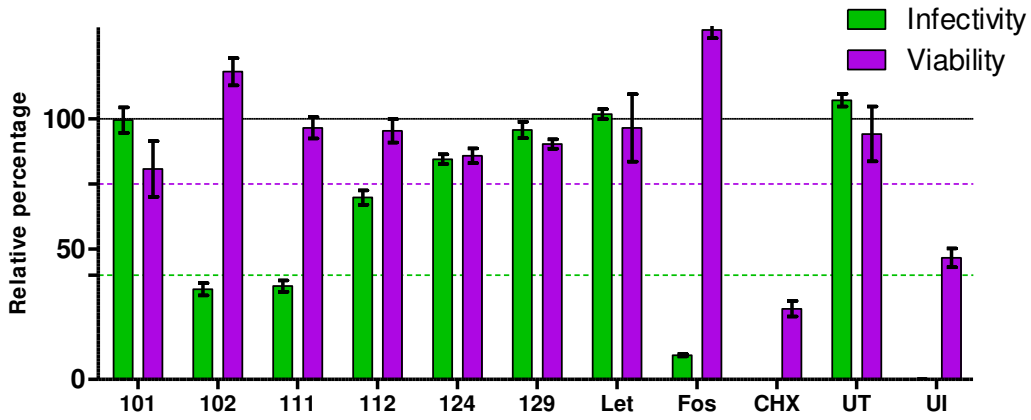
B)



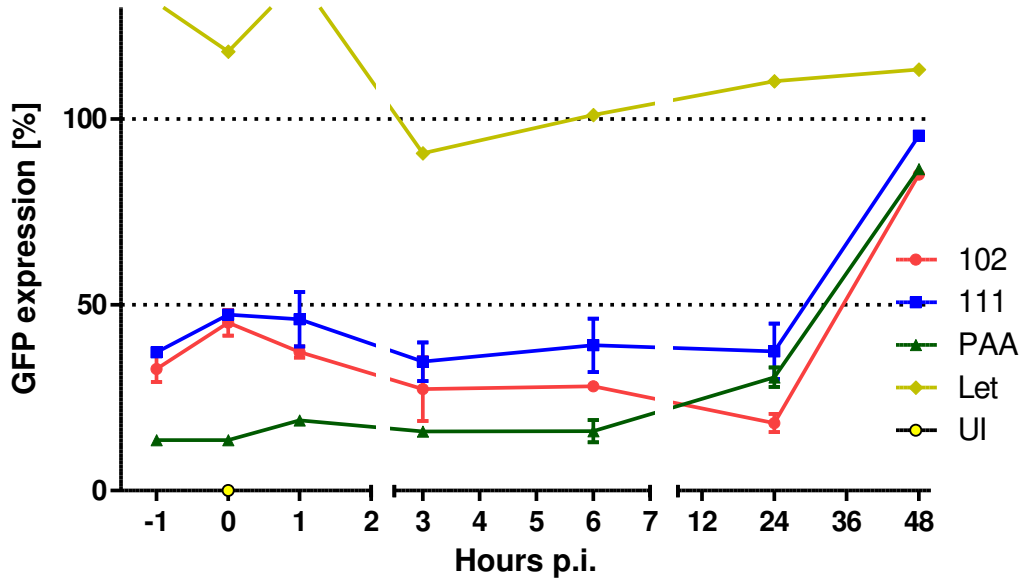
C)



Supplementary Figure 9. Early step inhibitors reduce the infectivity by more than 60% using a GFP reduction assay with high MOI and short incubation time. nHDF cells were infected with a high viral dose (MOI=0.5) and then inoculated with the compounds hits (10 µM), or cycloheximide (10 µg/ml), or foscarnet (180 µM), or left untreated (UT) or UI (uninfected). Cells were assayed for GFP expression and relative cytotoxicity 3 days later. Bars represent means ± SD of 4 replicates.

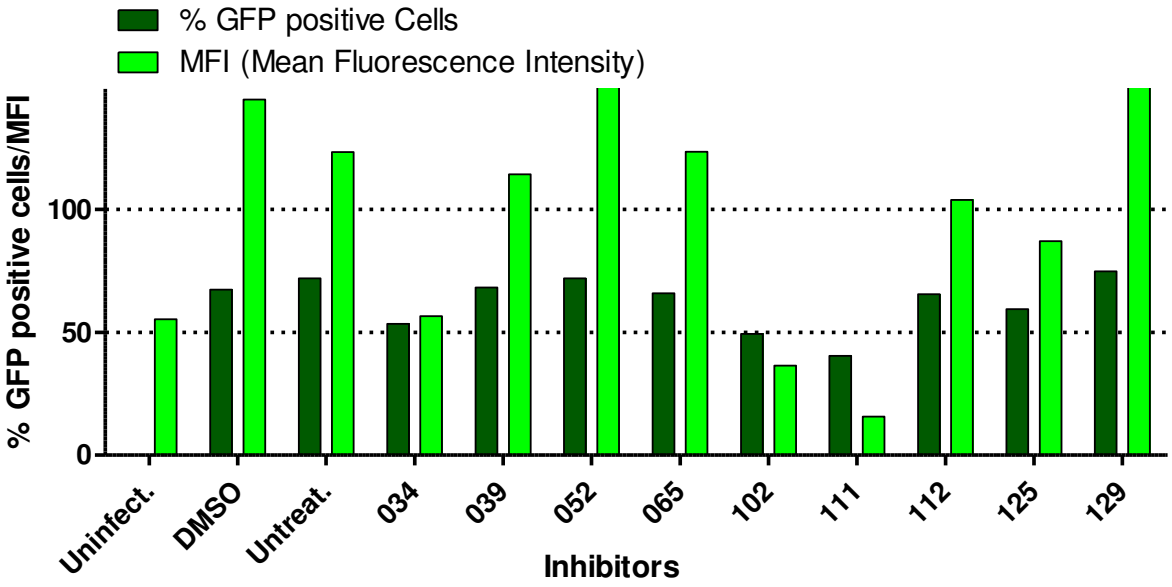


Supplementary Figure 10. Effect of time-of-addition of inhibitors on HCMV replication. nHDF cells were inoculated with GFP-tagged HCMV and treated with 10 µM of the compounds, 10 nM of letermovir, and 180 µM of PAA as controls at the indicated time points postinfection (hpi). Cells were measured for their GFP expression after 72 h. The figure shows the inhibition of HCMV replication (relative GFP fluorescence intensity) by the compounds compared to DMSO-treated control. The connected lines represent the mean values of two replicates for compounds 102, 111, PAA, and a single replicate for letermovir.

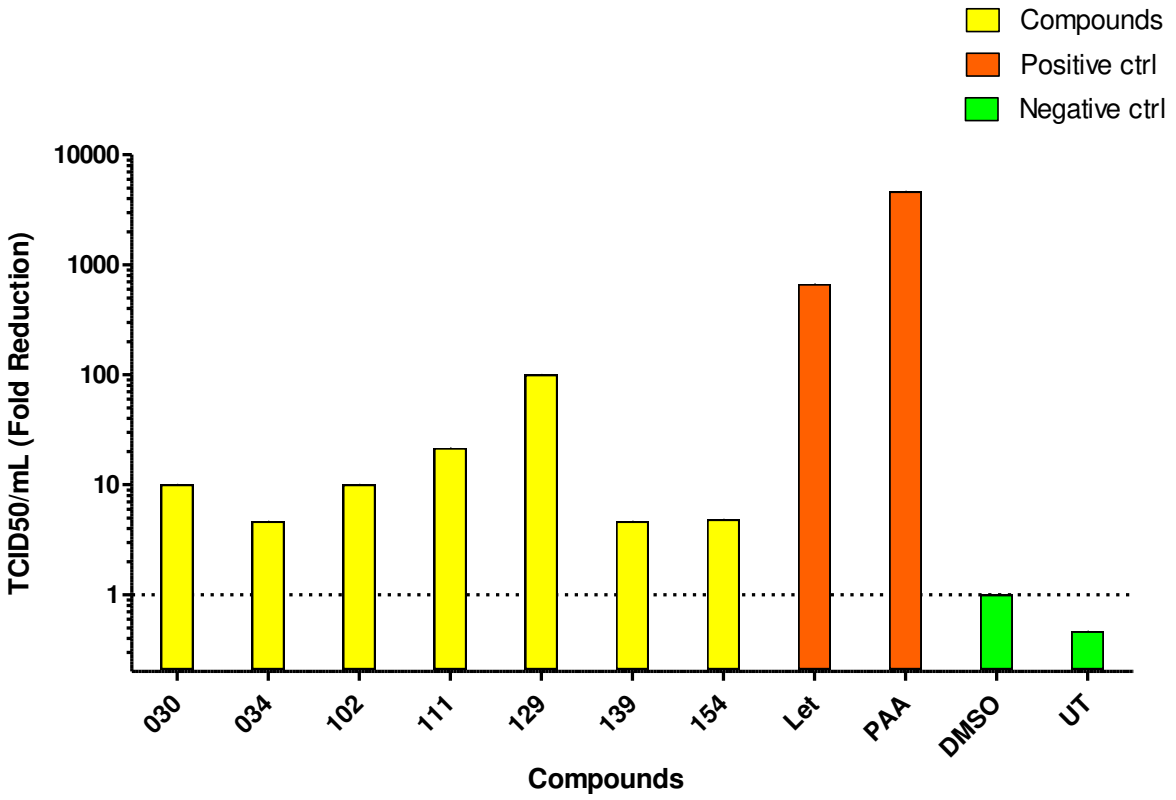


Supplementary Figure 11. FACS based assay for detection of early inhibitors. HCMV-GFP-inoculated nHDF cells (MOI=1) were treated for 24 h with the compounds at 40 µM concentrations. After 48 h cells were fixed, and GFP+ cells were counted with the flow cytometer. Uninfected cells were 99.9% GFP-negative. The percentage of the GFP positive cells is indicated in dark green. Mean fluorescence intensity were calculated based by gating on GFP positive cells.

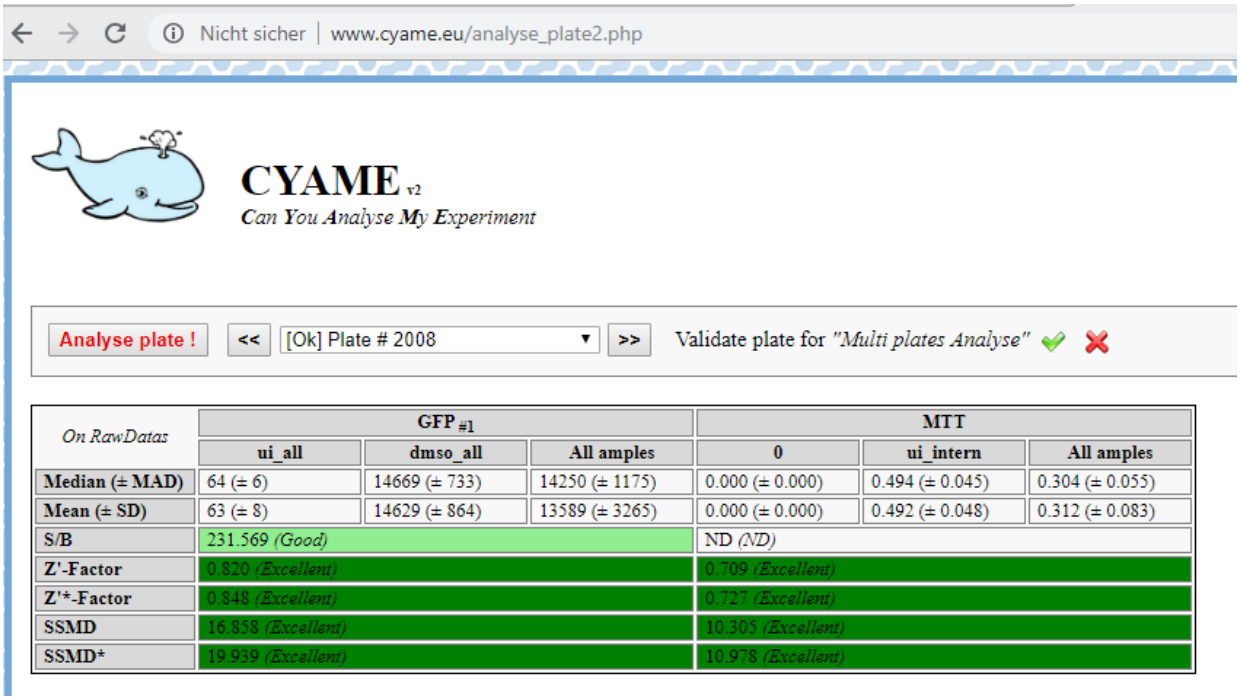
	Uninfect.	DMSO	Untreat.	34	39	52	65	102	111	112	125	129
% GFP+ Cells	0	67	72	54	68	72	66	49	40	66	59	75
Mean Fluorescence Intensity	55	145	123	57	114	155	124	36	16	104	87	172



Supplementary Figure 12. Measuring of HCMV inhibition by the compounds using the $TCID_{50}$ assay. nHDF cells were infected with high viral dose (MOI=0.5) and then inoculated with the compounds hits (10 µM), or letermovir (10 nM), or PAA (180 µM) for 5 days. The supernatants were serially diluted and $TCID_{50}$ assay were performed on new cells. Cells were assayed for the GFP expression 6 days later. Calculation of the $TCID_{50}$ was done using an online platform based on the Spearman & Kärber algorithm described in Hierholzer & Kilington (1996). Data was normalized to the $TCID_{50}$/ml of samples receiving corresponding DMSO concentration and are shown in logarithmic fold change reduction of this control.



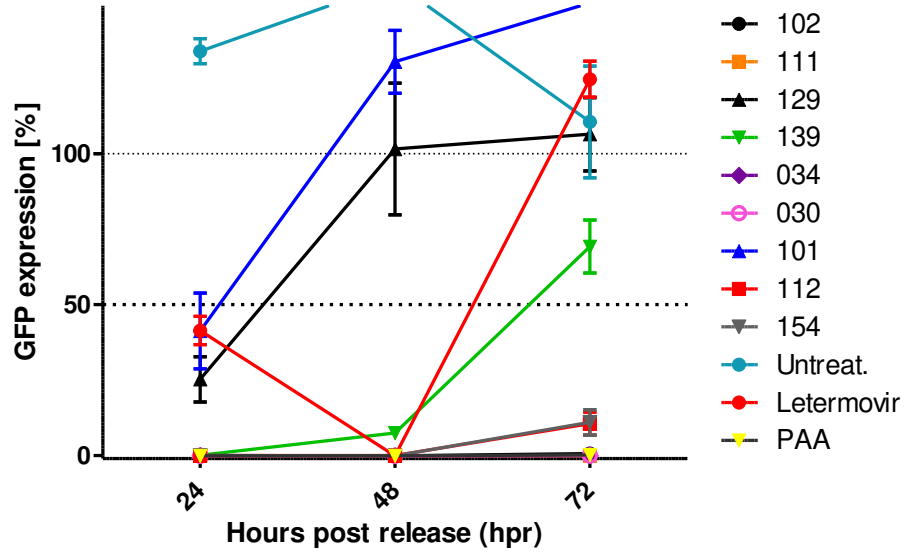
Supplementary Figure 13. A Screenshot of the CYAME programme used for plate analysis. Signal to background ratio (S/B), Z'-Factor, and strictly standardized mean difference (SSMD) methods were calculated and shown for each plate. The data is shown as an example for plate 8 in the second library.



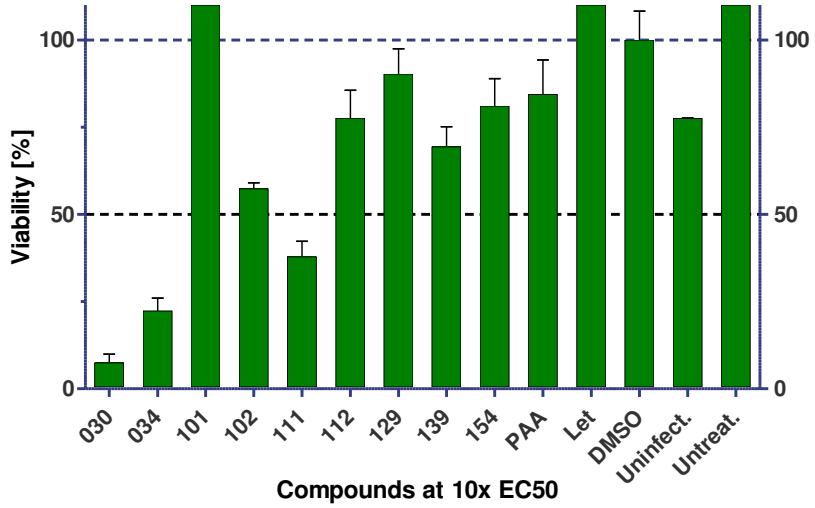
On RawDatas	GFP #1			MTT		
	ui_all	dmso_all	All amples	0	ui_intern	All amples
Median (± MAD)	64 (± 6)	14669 (± 733)	14250 (± 1175)	0.000 (± 0.000)	0.494 (± 0.045)	0.304 (± 0.055)
Mean (± SD)	63 (± 8)	14629 (± 864)	13589 (± 3265)	0.000 (± 0.000)	0.492 (± 0.048)	0.312 (± 0.083)
S/B	231.569 *(Good)*			ND *(ND)*		
Z'-Factor	0.820 *(Excellent)*			0.709 *(Excellent)*		
Z'*-Factor	0.848 *(Excellent)*			0.727 *(Excellent)*		
SSMD	16.858 *(Excellent)*			10.305 *(Excellent)*		
SSMD*	19.939 *(Excellent)*			10.978 *(Excellent)*		

Supplementary Figure 14. Kinetic block release assay using high concentrations of the compounds. HCMV infected nHDF cells were treated for 96 h with the compounds (at 40 µM) (10 x IC50 for most of the compounds). For Letermovir and PAA, 40 nM and 720 µM were used, respectively. At 96 hpi, the supernatant was removed, cells were washed and new media without inhibitors was added. 24, 48, and 72 h after drug release (hpr, hours post release), supernatants were collected and new cells were infected to determine when virus production resumed. A) The intensity of GFP expression (as an indicator of the amounts of virus present in the supernatants) was measured after 96 h. B) Cells were assayed for their viability at 96 h post infection after transferring supernatant using the MTT assay. Data points represent values of triplicate determinations from a representative experiment. The connected lines represent the mean values of the data points which were normalized to the values of DMSO-treated control cells (100%).

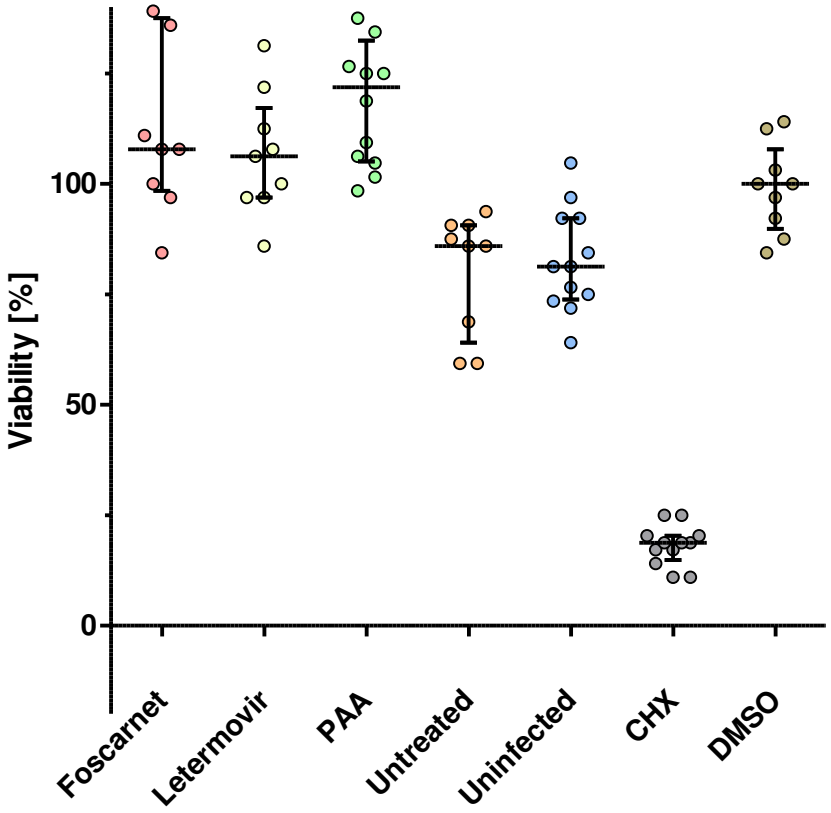
A)



B)



Supplementary Figure 15. Employing MTT as more reliable cytotoxicity assay. nHDF cells were infected with MOI 0.05 and treated with the test compounds for 6 days. This experiment was performed with the automated robot. Cells were assayed with the MTT reagent for compound-related toxicity. Each point represents the value from a single well in a 384-well format plate. Horizontal bars represent medians and the error bars are the interquartile range of 12 replicates for PAA, uninfected, and cycloheximide; 9 replicates for foscarnet, letermovir, untreated, and DMSO.



5.2. Abbreviations

AIDS	acquired immunodeficiency syndrome
APS	ammoniumperoxodisulfat
BJAB	human burkitt lymphoma b cell line
CaCl2	calcium chloride
CC50	half-maximal cytotoxic concentration
CDV	cidofovir
CMC	carboxymethyl cellulose
CPE	cytopathic effect
CV	crystal violet
CYAME	can you analyze my experiment
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein-Barr-virus
EC50	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FDA	food and drug administration
FOS	foscarnet
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCV	ganciclovir
GVHD	graft-versus-host disease
HCMV	human cytomegalovirus
HEL299	human embryo lung fibroblasts derived from embryonic lung tissue
HFF	human foreskin fibroblasts
HIV	human immunodeficiency virus
HS	hill slope

HSC	hematopoietic cell transplantation
HSCT	hematopoietic stem cell transplantation
HSPG	heparan sulfate proteoglycans
HSV	herpes simplex virus
HTS	high throughput screening
IC50	half maximal inhibitory concentration
ICP8	single-strand DNA-binding protein
KBR	kinetic block release
kDa	kilo-dalton
KSHV	kaposi's sarcoma-associated herpesvirus
MAD	median absolute deviation
MCMV	murine cytomegalovirus
MEF	mouse embryonic fibroblast
MOI	multiplicity of infection
NEC	nuclear-egress complex
Nf-κB	nuclear factor 'kappa-light-chain-enhancer' of activated b-cells
NHDF	normal human dermal fibroblasts
NHLF	normal human dermal fibroblasts
ORF	open reading frame
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PFU	plaque-forming unit
PKC	protein kinase c
qPCR	quantitative polymerase chain reaction
RPE	retinal pigment epithelium
S/B	signal-to-background
S/N	signal-to-noise
SDS	sodium dodecyl sulfate
SOT	solid organ transplant
SSMD	strictly standardized mean difference
TCID50	tissue culture infection dose50
TEMED	tetramethylethylendiamin

TOA	time of addition
TRPS	tunable resistive pulse sensing
U	units
vAC	viral assembly complex
vGCV	valganciclovir
VZV	varizella zoster virus
w/v, v/v	weight/volume, volume/volume

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Declaration

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