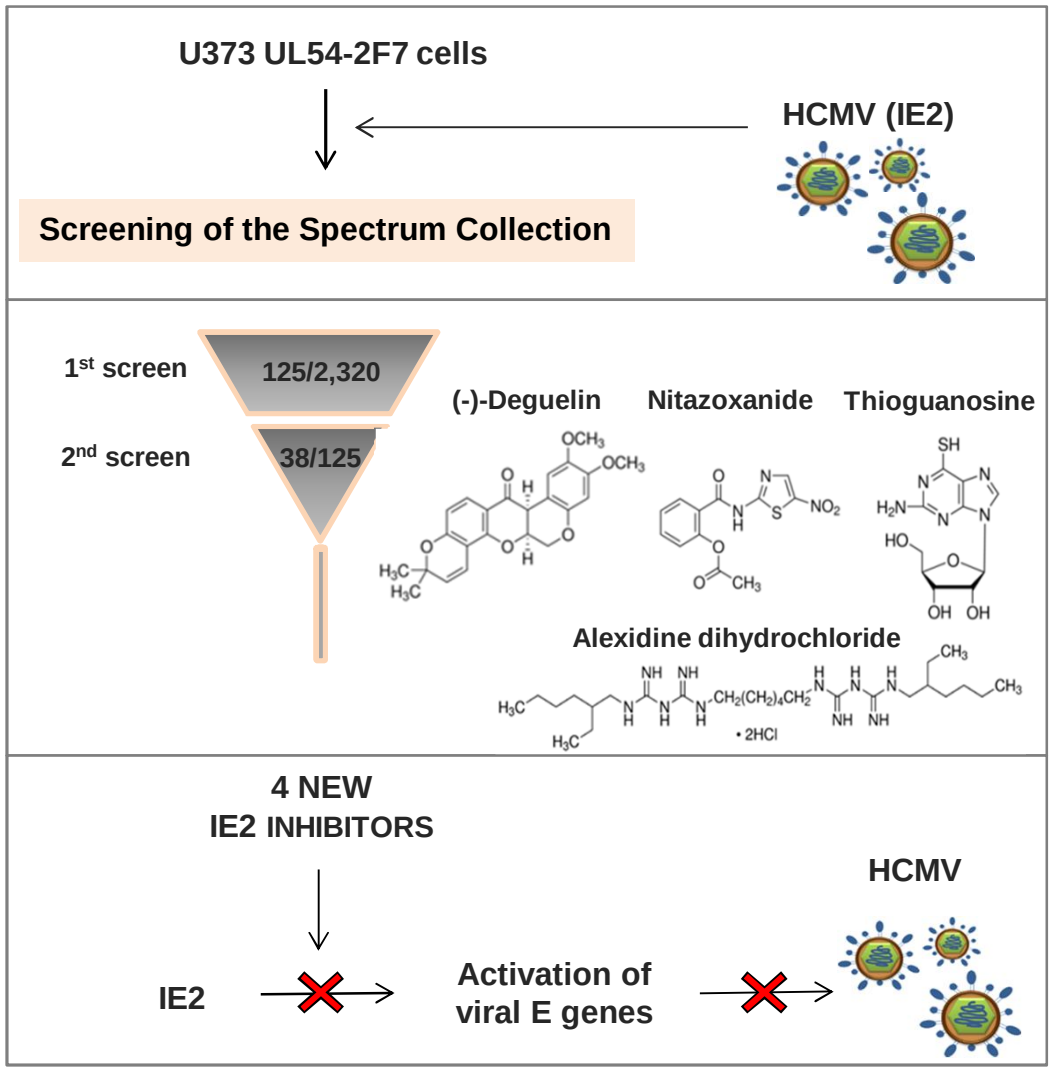
Graphical abstract



Inhibitors of the Prototypic Transcription Factor IE2 of Human Cytomegalovirus Identified by Cell-Based Screening

Beatrice Mercorelli,[1,4] Anna Luganini,[2,4] Giulio Nannetti,[1] Oriana Tabarrini,[3] Giorgio Palù,[1] Giorgio Gribaudo,[2,5] and Arianna Loregian,[1,5,*]

[1]Department of Molecular Medicine, University of Padua, 35121 Padua, Italy.

[2]Department of Life Sciences and Systems Biology, University of Turin, 10123 Turin, Italy.

[3]Department of Pharmaceutical Sciences, University of Perugia, 06123 Perugia, Italy.

[4]Co-first author.

[5]Co-senior author.

[*]Correspondence: arianna.loregian@unipd.it

SUMMARY

New targets for antiviral strategies are needed against human cytomegalovirus (HCMV), a major human pathogen. A cell-based screen aimed at finding inhibitors of the viral transcription factor Immediate-Early 2 (IE2) was performed in HCMV-infected cells expressing EGFP under the control of an IE2-inducible viral promoter. Screening of a library of bioactive small molecules led to the identification of several compounds able to inhibit EGFP expression and also HCMV replication with potency in the low-micromolar range. Follow-up studies with four selected hits indicated that they all block viral DNA synthesis as well as viral Early and Late genes expression. Furthermore, mechanistic studies confirmed that the compounds specifically act via inhibition of IE2 transactivating activity, thus blocking viral Early gene expression and the progression of virus replication. These results provide a proof of concept for identifying small molecules that modulate the activity of a microbial transcription factor to control pathogen replication.

INTRODUCTION

Viral transcription factors (vTFs) play pivotal roles not only in virus replication but also in virus-induced immunopathogenesis. This is achieved by their ability to both activate viral gene expression and to reprogram host pathways by interacting with cellular partners. For these reasons, vTFs represent attractive targets for drug discovery. However, traditionally, the identification of inhibitors of both cellular and microbial TFs activity has been considered very challenging, if not impossible. Indeed, TFs have been long considered "undruggable" targets since they lack of intrinsic enzymatic activity, but in the recent years this idea has evolved and some modulators of TFs involved in pathogenic processes such as cancer progression and virus replication have been identified (Fletcher and Prochownik, 2015; Illendula et al., 2015; Makley and Gestwicki, 2013; Mousseau et al., 2012; Yan and Higgins, 2013).

The Immediate-Early 2 (IE2) protein encoded by human cytomegalovirus (HCMV) represents a prototypic vTF. In fact, it is an essential, multi-tasking protein that is involved in many events of HCMV replication cycle (Mocarski et al., 2013). IE2 is required to activate the expression of viral Early (E) and Late (L) genes, negatively regulates its own expression by acting on the Major Immediate Early promoter, and both activates and inhibits cellular gene expression (Marchini et al., 2001; Stinski and Petrik, 2008). Importantly, IE2 stimulates the expression of several pro-inflammatory genes in infected cells, deregulates the host cell cycle, first by inducing its progression and then by blocking it before S phase, and hampers the host immune responses (Stinski and Petrik, 2008; Taylor and Bresnahan, 2005, 2006). Taken together, there is compelling evidence that IE2 is crucial for the progression of HCMV replicative cycle and plays a major role in HCMV pathogenesis and host immunomodulation (Mocarski et al., 2013). Thus, it represents a promising target for the development of new anti-HCMV agents (Mercorelli et al., 2011; Scholz et al., 2001); however, the identification and development of anti-IE2 molecules is hampered by the lack of a crystal structure of the protein, making the rational design of drugs an unfeasible strategy.

HCMV is a ubiquitous beta-herpesvirus that infects more than 60% of human population worldwide. HCMV is also a major human pathogen, which greatly affects HIV/AIDS patients, immunocompromised subjects, and transplant recipients, for whom consequences of HCMV infection can cause severe diseases and be life-threatening (Nogalski et al., 2014). Moreover, HCMV is one of the major causes of congenital defects in newborns (Manicklal et al., 2013). The available anti-HCMV drugs are used as both prophylactic and preemptive agents and are all targeted at the viral DNA polymerase, which is responsible of the replication of the viral genome (Boeckh et al., 2015). However, they suffer from several drawbacks including long-term toxicity, unfavorable pharmacokinetic properties, and emergence of viral resistance. None of them has been licensed for the treatment of congenital infection; in addition, second-line therapeutics to manage drug-resistant viruses are inadequate. Despite the great efforts spent in the last years, also an effective and preemptive vaccine is still lacking (Fu et al., 2014); for all these reasons, there is a strong need for new anti-HCMV agents. Blocking the viral replication before E gene expression has been proposed to be a valid antiviral approach that can also lead to the repression of a series of proinflammatory effects due to HCMV infection and responsible for virus-induced pathogenesis (Mercorelli et al., 2011; Scholz et al., 2001).

To find inhibitors of the vTF IE2, we decided to apply a strategy of drug repurposing and to use a cell-based approach that selects compounds acting prior to or during the E gene expression. We exploited an engineered HCMV permissive cell line that stably expresses EGFP under the control of a prototypic E gene promoter of HCMV, i.e., the DNA polymerase *UL54* gene promoter (Luganini et al., 2008; Mercorelli et al., 2014a). This strategy led to the identification of a series of small molecules (one already FDA-approved) that block the replication of HCMV by inhibiting the activity of IE2 and could represent new candidates for anti-HCMV drug discovery. The described approach validates a cell-based screening strategy to find small-molecule modulators of the activity of a TF that could be potentially exploited for the identification of inhibitors of other TFs involved in pathogenic processes and for which ~~a~~ ~~solved~~ a crystal structure does not yet exist.

RESULTS

Optimization of a Cell-Based Assay for Small-Molecule Screening Targeting IE2

The U373-pUL54-EGFP (named UL54-2F7) and U373-pUL112-113-EGFP (named UL112-113-1B4) cell lines stably express EGFP under the control of the IE2-dependent *UL54* and *UL112-113* E promoters of HCMV, respectively (Luganini et al., 2008; Mercorelli et al., 2014a). We adapted this cell-based system to find compounds that act early in HCMV replication cycle, using the IE2-dependent transactivation of an E promoter as a readout. A series of experiments were performed in order to determine the optimal cell line/cell density/multiplicity of infection (MOI) combination that allowed to obtain a substantial and reliable induction of EGFP expression and to detect a significant reduction in the presence of an active compound. The antisense oligodeoxynucleotide fomivirsen, which blocks IE2 expression (Azad et al., 1993), served as a positive control for the optimization and throughout the screening. As reported in Figure S1 in the Supplemental Information (SI), the maximal activation of EGFP expression was obtained with the combination of the UL54-2F7 cell line infected with HCMV at MOI of 0.5 Plaque Forming Units (PFU)/cell; under these conditions, EGFP expression levels in the presence of fomivirsen were comparable to those of the mock-infected cells (Figure S1). The calculated Z' factor from the positive and negative controls was 0.79 (Zhang et al., 1999); thus, we concluded that this experimental setting could be suitable for the screening and identification of inhibitors of IE2-dependent activation of viral E promoters. Moreover, to verify the progression of HCMV replication beyond the IE2-induced EGFP expression, we analyzed by Western Blot (WB) the expression of representative IE, E, and L HCMV proteins in U373 UL54-2F7 cells under the same conditions used for the screening. The results presented in Figure S2 in the SI clearly demonstrate that in HCMV-infected U373 UL54-2F7 cells representative viral proteins are expressed, thus indicating that the progression of HCMV replication cycle occurs under the conditions used for of the screening.

Screening of the Spectrum Collection

The UL54-2F7 cell line was used for a primary screening of a total of 2,320 compounds, each at the concentration of 10 µM. As a positive control for the inhibition of EGFP expression, samples treated with fomivirsen were included in each plate. The Z' factors ranged between 0.59 and 0.76 and negligible interplate variability was observed. The criterion for an active compound was one that exhibited ≥ 50% reduction of the Fluorescence Units (FU) value measured in the infected and DMSO-treated cells. In the primary screening, 125 compounds were identified that met this criterion (Figure 1A). These compounds were then retested by the same cell-based assay; however, when considering the secondary screen results, we excluded those compounds that resulted clearly toxic (i.e., induced cell detachment) upon visual inspection of cell monolayers before lysis at 72 h post-infection (p.i.). This was done because the reduction in EGFP expression in those cases could most likely be due to cytotoxicity rather than to a real inhibitory activity. Taking this into account, 38 molecules were reconfirmed as active in inhibiting EGFP expression (Figure 1B). Overall, we obtained a 1.6% hit rate (38/2,320) when the limits of 50% inhibition in all the replicates and the absence of obvious toxicity were applied. This rate is higher than that expected for random high-throughput screenings (<0.1-0.3%); however, this is most likely due to the fact that the Spectrum Collection is not a random library but is composed only by bioactive molecules. In Table S1 in the SI is reported the list of the 38 compounds identified by the cell-based screens as able to inhibit HCMV-mediated induction of EGFP expression in UL54-2F7 cells with their PubChem Compound Identification number and known bioactivities.

Hit Compounds Inhibit the Replication of HCMV in a Dose-Dependent Manner

To validate the anti-HCMV activity of the hits, plaque reduction assays (PRA) with HCMV AD169 were performed with the 38 molecules from the library, along with the reference compound ganciclovir (GCV) as a control, and in parallel their cytotoxicities were tested by MTT assays. As reported in Table S1, 27 compounds exhibited an EC_{50} < 10 µM in PRAs and 14 of them emerged

with a Selectivity Index (SI) ≥ 50. To select the compounds to be further characterized, a high SI value was then combined with information present in PubMed and other sources about: i) compound toxicological profile, ii) previous use in clinical trials in humans and in pediatric setting, iii) pharmacokinetic properties, and iv) commercial availability. In addition, compounds with a previously reported anti-HCMV activity likely not involving the inhibition of IE2 activity, i.e., berberine, clotrimazole, and papaverine, were excluded (Isler et al., 2005; Hayashi et al., 2007; Albrecht et al., 1987). This review process also revealed that some compounds selected by the screening may be useful as investigational tools but not for anti-HCMV drug repurposing. In fact, two of the most potent inhibitors, i.e., isorotenone and hexachlorophene (Table S1), have been reported to be associated with long-term toxicity (Evangelista de Duffard and Duffard, 1996; Moretto and Colosio, 2011), which is incompatible with their development as anti-HCMV therapeutics. At the end of this process, four hit compounds (Table 1), i.e., deguelin (DGN), nitazoxanide (NTZ), thioguanosine (TGN), and alexidine dihydrochloride (AXN), were selected and purchased for follow-up testing. PRAs and cytotoxicity assays were repeated with the selected hits and obtained EC_{50} curves are reported in Figure 2. As reported in Table 1 and Figure 2, the anti-HCMV activity and the lack of significant toxicity were reconfirmed for all of them obtaining EC_{50} values in the low-micromolar range. Since we observed that there was no difference between the EC_{50} values obtained in PRAs performed pretreating the cells for 2 h with test compounds before virus infection and EC_{50} values obtained in assays performed by adding the compounds 2 h p.i. (data not shown), in all the experiments for follow-up testing the compounds were added p.i..

PRAs were then repeated to assess the antiviral activity of the four selected hits against different strains of HCMV (including a clinical isolate) and viral strains resistant to ganciclovir or foscarnet (two drugs currently used in anti-HCMV therapy targeted at the viral DNA polymerase). As reported in Table S2, the EC_{50} values determined in these experiments were comparable to those obtained with the AD169 strain, suggesting that all the selected hits are broad-spectrum inhibitors

of HCMV and likely block virus replication with a mechanism that differs from that of the viral DNA polymerase inhibitors.

Given the readout of the expression of EGFP at 72 h p.i. in the screening, we performed time-of-addition studies in HCMV-infected U373 UL54-2F7 cells to determine which phase of the virus replication cycle was affected by the hit compounds. As shown in Figure S3 in the SI, all the hit compounds act at a step subsequent to that affected by fomivirsen (an IE2 expression inhibitor), since their addition started losing inhibitory effects from 8 to 24 h p.i., a time frame compatible with the transition from IE to E phase during HCMV replication cycle.

Hit Compounds Block the Onset of HCMV DNA Synthesis

To investigate deeper the antiviral mechanism of the selected compounds, we next tested the effects of the selected hits on viral DNA synthesis in HCMV-infected cells, a step subsequent to viral E gene expression. To this aim, quantitative PCR (qPCR) was performed from HCMV-infected Human Foreskin Fibroblast (HFF) cells treated with DGN, NTZ, TGN, AXN, or WC5, a 6-aminoquinolone already reported to inhibit HCMV DNA synthesis, as a control (Loregian et al., 2010; Massari et al., 2013). As reported in Figure 3, HCMV DNA synthesis was inhibited by the treatment with all the tested hits and with WC5, as expected (Loregian et al., 2010). Thus, all the selected hits prevent the onset of HCMV DNA synthesis, demonstrating that they target an event in the HCMV replication cycle that precedes or takes place during virus DNA replication.

Hit Compounds Halt HCMV Replication Cycle Beyond the IE Phase

To support further the above conclusion, we investigated the effects of the hit compounds on the expression of newly synthesized HCMV proteins by WB. To this aim, total cell extracts were prepared at various times p.i. from HCMV-infected HFF cells treated with DGN, NTZ, TGN, and AXN. The general transcription inhibitor actinomycin D (ActD) and WC5 were included as controls. Then, the extracts were analyzed for their content of representative Immediate-Early (IE1

and IE2), Early (UL44) and Late (UL55 and UL99) proteins. As expected from its mode of action, no viral proteins were detected in the ActD-treated cells (Figure 4). In keeping with previous reports (Loregian et al., 2010; Mercorelli et al., 2014b), at 48 h p.i. the expression of the E protein UL44 was strongly reduced by WC5 (Figure 4), while it had no effect on IE proteins synthesis. When the selected hits were considered, none of the compounds significantly affected the expression of IE proteins at any of the time points considered (Figure 4, panels A to D). In contrast, treatment with DGN, NTZ, TGN, and AXN reduced UL44 expression in a manner similar to WC5 (Figure 4). Moreover, a strong inhibitory effect on the expression of L proteins (UL55 and UL99) was observed for all of the tested molecules at 72 h p.i. (Figure 4). Overall, these results showed that DGN, NTZ, TGN, and AXN affect HCMV replication by acting at an E phase of the viral replicative cycle after the IE proteins expression, but before the E and L proteins expression, in keeping with the results obtained from the time-of-addition experiments (Figure S3) and the studies on viral DNA synthesis (Figure 3).

Hit Compounds Inhibit IE2-Dependent Transactivation of HCMV E Promoters

The results presented above and the features of cell-based assay employed for the screening suggested that the selected hit compounds could act by inhibiting the IE2-mediated E gene transactivation. To test this hypothesis, the indicator cell line UL54-2F7, already used for the screening, was transduced with adenoviral vectors either expressing IE2 (AdVIE2) or *E. coli* β-galactosidase (AdVLacZ) as a control (Mercorelli et al., 2014b; Supplemental Figure S4). Subsequently, cells were treated with DNG, NTZ, TGN, and AXN to examine their effects on EGFP expression. WC5 and fomivirsen were included as controls. As shown in Figure 5A (left panel), IE2 overexpression resulted in EGFP expression; in contrast, a strong inhibition of EGFP expression was observed in the cells treated with all test compounds. These results were confirmed by a quantitative microscopic analysis of EGFP-positive cells that showed a 70-80% reduction in EGFP expression upon treatment of IE2-overexpressing cells with test compounds (Figure 5A, right

panel), similar to that obtained with WC5 and fomivirsen. A comparable inhibitory activity of the hit compounds on the IE2-dependent transactivation was also observed with the *UL112-113* E gene promoter (Figure 5B), thus indicating an interference with the IE2-dependent transactivation of two different E gene promoters of HCMV.

To support further this conclusion, we examined the effects of the four selected compounds on the *UL54* gene promoter activity in a different format of cell-based assay. To this end, a luciferase reporter plasmid containing the entire *UL54* promoter (pUL54-0.4) was first transfected into HFFs that were then transduced with AdVIE2 or AdVLacZ as a control. Two hours later, cells were treated with the selected hits (and WC5 and fomivirsen as controls) and the luciferase activity was assessed after 48 h. As reported in Figure 5C, in agreement with the results described above (Figure 5A), the IE2-dependent transactivation of the *UL54* promoter was inhibited by the treatment with all the tested compounds. Noteworthy, similarly reduced levels of reporter gene activity were observed when the transfection-transduction assays were performed using a luciferase reporter construct driven by a minimal *UL54* promoter (pUL54-0.15, with 0.15 kbp upstream from the transcription start site, Figure 5C). Altogether, these results indicate that the selected hits target the IE2-dependent transactivation of viral E gene promoters.

IE2 Inhibitors Do Not Interfere with the Transactivating Activity of Another HCMV TF

To examine the specificity of the four hits' inhibitory effect on the IE2 transactivating activity, we investigated their effects on the activity of another HCMV-encoded vTF, i.e., the Immediate-Early 1 (IE1) protein. IE1 is the other major protein product of the HCMV *MIE* locus and shares with IE2 the first 85 amino acids (Stinski and Petrik, 2008). In a previous study, we demonstrated that the cellular human thymidylate synthase (*TS*) gene promoter is transactivated by both HCMV and the IE1 protein in quiescent fibroblasts (Gribaudo et al., 2002). Thus, we investigated the effects of the hit compounds on the IE1-dependent transcriptional activation of *TS* promoter. As shown in Figure 6, in transient transfection experiments performed in HFFs, all the tested compounds did not affect

the IE1-dependent transactivation of human *TS* promoter (Gribaudo et al., 2002). The IE2-specific inhibitor WC5, used as a control, was ineffective in interfering with the IE1-mediated activation of the cellular gene promoter, as expected (Mercorelli et al., 2014b). Together, these results indicated that the four selected hit compounds are endowed with an inhibitory activity that is specific for IE2 over IE1.

DISCUSSION

In this paper, we describe a target-based drug repurposing screen to identify molecules able to inhibit the activity of an essential transcription factor encoded by HCMV and hence pathogen replication. IE2 has emerged as an attractive antiviral target for new anti-HCMV drug discovery (Mercorelli et al., 2011; Scholz et al., 2001) and we recently identified a small molecule, the 6-aminoquinolone WC5, as a specific inhibitor of IE2 functions essential for optimal viral replication (Mercorelli et al., 2009; Loregian et al., 2010; Mercorelli et al., 2014b). The identification of the mechanism of action of this compound gave us the proof of principle that IE2 is a druggable target and that the modulation of its activity by small molecules is possible. Not only IE2 is the major transactivator of viral E gene expression, but it also establishes a strict interplay with several cellular pathways that result modulated and hijacked to promote HCMV replication in the host cell. For this reason, we thought that a drug repurposing approach could successfully lead to the discovery of new IE2 inhibitors and therefore we screened a small-molecule library that consists of 2,320 bioactive molecules including all FDA-approved drugs.

The screening assay was based on the exploitation of a HCMV-permissive cell line stably transfected with an EGFP reporter under the control of the promoter of the prototypic viral E gene *UL54*, which encodes the DNA polymerase. Upon infection with HCMV, EGFP expression is induced by the IE2-dependent transactivation of *UL54* promoter. Treatment with compounds able to interfere with this event or upstream viral functions leads to a quantifiable reduction in EGFP expression. From the initial screening, 125 compounds emerged as able to reduce the IE2-induced EGFP-expression from the *UL54* promoter. We then retested these compounds by the same assay and finally obtained 38 molecules to be evaluated for their antiviral activity against HCMV. Therefore, plaque reduction assays and in parallel cytotoxicity assays were performed in HFF cells. Interestingly, we noticed that some of the compounds showing anti-HCMV activity belong to the same chemotype (as an example, isorotenone and its derivatives DGN, apotoxicarol and alpha-toxicarol, Table S1). Moreover, we also found compounds with an already reported early anti-

HCMV activity, such as berberine (Hayashi et al., 2007), clotrimazole (Isler et al., 2005), and papaverine (Albrecht et al., 1987) (Table S1), thus validating our experimental approach. The fact that other known anti-HCMV drugs included in the library that have a target other than IE2 (e.g., DNA polymerase inhibitors) were not selected by our screening indicates that the strategy was specific for the identification of IE2 inhibitors (or compounds acting upstream) and not of any HCMV inhibitor.

For the characterization of the antiviral activity, we selected only four hits, an FDA-approved drug (NTZ) and three drugs under development (DGN, TGN, and AXN). Other compounds with a very promising SI (e.g., hexachlorophene and isorotenone, Table S1) were excluded since they are not suitable for clinical use in anti-HCMV therapy due to their long-term toxicity. The possibility to exclude *a priori* some compounds, although very active, based on the available information is one of the advantages of the use of the drug repurposing approach. It is also important to underline, however, that the active compounds that were not selected as drug candidates could anyway represent useful research tools to uncover molecular mechanisms of HCMV biology, since they point out possible cellular pathways that might be involved in virus replication and thus be a starting point also for other researchers in the scientific community. All the four selected hits were reordered separately and confirmed their antiviral activity not only against the HCMV AD169 strain, but also against a panel of other HCMV strains, suggesting for all of them a broad-spectrum anti-HCMV activity. When the effects on viral DNA synthesis and viral proteins expression were analyzed, we observed that all the four hits did not affect the expression of IE proteins, while viral DNA synthesis as well as the E and L gene products were significantly reduced. These results suggested that the IE2-dependent transactivation of E genes could be a target of the four compounds. Indeed, we provided strong evidence that the four hits act via the inhibition of an IE2-dependent activity (i.e., transactivation of viral E genes), thus preventing the progression of HCMV replication cycle. Figure 5 summarizes the mechanistic analysis of the anti-IE2 activity of the four hits. We clearly demonstrated by means of transfection-transduction experiments with a

recombinant adenovirus expressing IE2 alone that the hits block the IE2-dependent transactivation of E genes in the absence of any other HCMV protein and in various experimental settings. Importantly, the lack of interference with the transactivating activity of another vTF encoded by HCMV, i.e., IE1, which shares 85 N-terminal residues with IE2 (Stinski and Petrik, 2008), strongly supports the specificity of the hit compounds towards the IE2-dependent transactivation. During the preparation of this manuscript, a paper reporting the screening of an older, smaller version of the Spectrum Collection to find early inhibitors of HCMV was published (Gardner et al., 2015). Our assay was conceived to find inhibitors of IE2-dependent E gene activation, while the assay described by Gardner et al. was designed to find compounds that act by inhibiting IE2 expression, and therefore an event that precedes viral E gene expression. This difference can thus explain why the hits identified in our screening were not selected by the other approach (Gardner et al., 2015). On the other hand, the two hits reported by Gardner et al., convallatoxin and podofillox, fall into the list of the compounds that we excluded due to their toxicity at 10 µM. This is in agreement with the fact that these two compounds were reported to have CC_{50} values around 5 µM (Gardner et al., 2015), thus our test concentration was 2-fold higher that their CC_{50}.

The results presented here provide evidence of the feasibility of targeting the activity of a virus-encoded TF to block HCMV replication. This represents a significant conceptual advance over previously identified anti-HCMV compounds, since they were targeted mainly at viral enzymes. We have employed and successfully validated an assay for small-molecule screening that uses the transactivation of an essential viral gene by a specific virus-encoded TF as readout for the screening. Although the library used in this study has a small size, our assay is potentially amenable also for high-throughput screening campaigns, since it resulted relatively rapid, robust, easy to perform, and reliably quantitative. A similar strategy could be applied for the identification of inhibitors of other TFs involved in microbial pathogenic processes. TFs have long been considered "undruggable" targets for therapeutic development; however, this paper and others (Fletcher and

Prochownik, 2015; Illendula et al., 2015; Mousseau et al., 2012; Yan and Higgins, 2013) provide the evidence that small-molecule modulators of TFs activity can be identified by screening.

Small molecules that modulate IE2 activity might act by different mechanisms, for example by disrupting the interaction between IE2 and its cellular/viral partners, by interfering with IE2 post-translational modifications required for its transactivating functions, and by indirectly affecting host molecular pathways intercepted by IE2 and essential to create a cellular environment conducive to HCMV replication. Since all the four hits have been already reported to have effects on cellular pathways , their antiviral activity could be due most likely to the interference with those pathways that in HCMV-infected cells are required for the switch from IE to E phase. For instance, DGN, a natural rotenoid derivative, is a known inhibitor of activated Akt, Hsp90 and client proteins and is under investigation as an antitumoral agent (Wang et al., 2013; Oh et al., 2007). Being a multifunctional signal transduction inhibitor, DGN could interfere with several cellular and viral pathways required for the correct progression of HCMV replication. To corroborate this, it has been demonstrated that PI3K/Akt inhibitors can block herpesvirus infection (Liu and Cohen, 2015). TGN is an analog of guanosine and has been reported to inactivate cellular GTPases activity by depleting GTP levels. GTP-binding partners such as Rheb and Rac proteins are required for efficient HCMV replication and indeed VX-497, an inhibitor of GTP/dGTP biosynthesis, demonstrated anti-HCMV activity (Markland et al., 2000). Moreover, GTP-Rheb activity is necessary for mTOR activation, which is required for efficient HCMV replication (Moorman and Shenk, 2010). AXN is an antibiotic and a selective inhibitor of the mitochondrial phosphatase Ptpmt1. It was reported that treatment with AXN reprograms cellular metabolism from aerobic activity to glycolysis leading to hyperactivation of AMPK (Liu et al., 2015). The hyperactivation of AMPK could be detrimental for HCMV replication, since it counteracts the PI3K/Akt/mTOR pathway. The only FDA-approved drug among the selected hits, NTZ, was initially developed as an oral antiparasitic agent against protozoan and helmintic infections, but it also demonstrated antibacterial activity including *Mycobacterium tuberculosis*. More recently, NTZ emerged as a first-in-class broad-spectrum

antiviral agent, since it showed activity against 20 different RNA and DNA viruses - but up to this report not against HCMV - by interfering with cellular functions necessary for viral replication (Rossignol et al., 2009; Stachulski et al., 2011; La Frazia et al., 2013; Rossignol, 2014). Currently, NTZ is in Phase 3 clinical trials for treating influenza virus infection (Haffizulla et al., 2014). Moreover, NTZ promotes autophagy, activates AMPK, and interferes with several kinases activity such as PI3K/Akt/mTOR and mitogen-activated protein kinases (MAPKs) signaling pathways (Senkowski et al., 2015; Lam et al., 2012; Fan-Minogue et al., 2013). Furthermore, it depletes ATP-sensitive intracellular Ca^{2+} inducing mild endoplasmic reticulum stress (Ashiru et al., 2014). HCMV, like other viruses, induces viral and cellular kinases and phosphatases at appropriate times in order to mediate a correct temporal expression of viral genes during the course of infection. The activity of TFs is regulated within the cell by different mechanisms such as intracellular localization and post-translational modifications that can induce conformational changes and/or modification of local charge and affect the ability of TFs to interact with other proteins. We thus investigated whether the antiviral effect of NTZ against HCMV IE2 could be due to an altered localization of the TF, since following NTZ treatment no difference in IE2 expression was detected (Figure 4), while the inhibition of IE2-dependent transactivation of viral E promoters was observed (Figure 5). By immunofluorescence analysis, we observed that in infected cells treated with NTZ IE2 nuclear localization was altered since the protein was partially retained into the cytoplasm (Figure S5). A possible explanation of the effect of NTZ on IE2 nuclear localization could be that it interferes with the correct and time-regulated phosphorylation status of IE2, resulting in an altered conformation of the protein and/or impairment in protein-protein interactions. In fact, the nuclear localization of some HCMV proteins, such as the DNA polymerase processivity factor UL44, is regulated by phosphorylation (Alvisi et al., 2011). Since NTZ is known to intercept several kinase pathways including MAPKs, that have been suggested to be involved in IE2 phosphorylation (Harel and Alwine, 1998; Barrasa et al., 2005), it could be possible that the anti-HCMV activity of NTZ might be due, at least in part, to altered post-translational modifications of IE2, but this hypothesis has to

be tested in the future. In keeping with this, the major protein-protein interactions domains of IE2 colocalize with domains of IE2 that are phosphorylated (Lukac et al., 1997) and mutations of certain phosphorylated residues of IE2 or *in vitro* phosphorylation of IE2 have been related to the modulation of its transactivating properties and its ability to interact with protein partners (Barrasa et al., 2005; Waheed et al., 1998).

The dissection of the mode of action of the hits against IE2 and HCMV replication will be object of our future work. Regardless, the IE2 inhibitors that we identified, as well as the other compounds with anti-HCMV activity reported in this study, could be starting points for new anti-HCMV drug discovery. In particular, NTZ, which is already used in clinical practice, could be rapidly considered for clinical evaluation for the treatment of HCMV-associated pathogenic conditions as well as prophylactic agents to prevent HCMV reactivation in high-risk patients.

SIGNIFICANCE

HCMV is a major human pathogen and new drugs are needed to manage congenital infections and the emergence of strains resistant to the currently available anti-DNA polymerase drugs. Using a target-based screening assay, a few small-molecule inhibitors of HCMV-encoded IE2 have been discovered. These HCMV inhibitors prevent viral DNA synthesis, E and L gene expression as well as the IE2-dependent transactivation of viral E promoters. These compounds could be exploited in anti-HCMV drug development but also could represent useful tools in elucidating molecular mechanisms of HCMV replication. Importantly, the drug repurposing approach that we applied resulted in the identification of a molecule - NTZ - that is an already FDA-approved therapeutic; moreover, NTZ is also currently being evaluated in phase III clinical trials as a new anti-influenza candidate drug. Thus, its further clinical evaluation is strongly recommended. The inhibitors described here could be the first anti-HCMV small molecules specifically targeting IE2 functions and could be able to fill the gap left in the current anti-HCMV therapeutic options by the DNA polymerase inhibitors. Our results, furthermore, open the way to the study of new cellular pathways that could be essential for virus replication and eventually reveal new drug targets.

EXPERIMENTAL PROCEDURES

Cells and Viruses

U373-pUL54-EGFP (clone 2F7) and U373-pUL112-113 (clone 1B4) cells, that stably express EGFP under the control of the IE2-dependent early *UL54* and *UL112-113* promoters of HCMV, respectively (Luganini et al., 2008), were grown in Dulbecco Modified Eagle's Medium (DMEM, Life Technologies) with 10% fetal bovine serum (FBS; Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin sulfate (P/S, Life Technologies), and 750 µg/ml of G418 (Life Technologies). HFF cells were cultured in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, and P/S. HCMV strain AD169 (VR-538) was purchased from American Type Culture Collection (ATCC, Manassas, VA). HCMV strains resistant to antiviral drugs (all obtained from the NIH AIDS Research and Reference Reagent Program, Rockville, MD) were previously described (Mercorelli et al., 2009). HCMV TB40-UL32-EGFP (kindly provided by C. Sinzger, University of Ulm, Germany) was previously described (Sampaio et al., 2005) as well as HCMV VR1814 (kindly provided by G. Gerna, IRCCS Policlinico San Matteo, Pavia, Italy) recovered from a cervical swab from a pregnant woman (Revello et al., 2001). Recombinant adenoviruses expressing HCMV IE2 or *E. coli* β-galactosidase (LacZ) were previously described (Mercorelli et al., 2014b).

Plasmids

The pUL54-luciferase indicator plasmids pUL54-0.4 and pUL54-0.15, containing *UL54* promoter-derived sequences were previously reported (Gariano et al., 2012). The pSGIE72, an IE1 expression construct, was generated as described (Klucher et al., 1993). The luciferase indicator construct phTS-243/+30 contains a portion of the promoter of the human thymidylate synthase gene (*TS*) (-243 and +30 relative to the AUG start codon) (Gribaudo et al., 2002). The pRL-TK vector expressing *Renilla* luciferase was purchased from Promega.

Spectrum Collection and Chemical Substances

The Spectrum Collection was purchased from Microsource Discovery System (Gaylordsville, CT). It consists of 2,320 bioactive molecules, including 1,040 FDA-approved drugs, dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM and was stored at -20°C. Fomivirsen (ISIS 2922) was synthesized by Metabion International AG (Martinsried, Germany). The anti-HCMV 6-aminoquinolone WC5 was previously described (Mercorelli et al., 2009). All the other chemical substances including the hits for the follow-up studies were purchased from Sigma-Aldrich (St. Louis, MO).

Set Up of a Cell-Based EGFP-Expression Reporter Assay

The detailed description of the optimization of the cell-based assay for the screening is reported in the Supplemental Experimental Procedures.

Screening Protocol and Data Analysis

U373 UL54-2F7 cells seeded the day before at 2×10^4 cells per well in 96-well plates were pretreated with test compounds for 2 h prior to infection with HCMV AD169 at MOI of 0.5 PFU/cell. The compounds of the Spectrum Collection were screened in duplicate in two independent plates at a fixed concentration of 10 µM. Control wells with mock-infected cells and infected cells treated with either 0.01% DMSO or 5 µM fomivirsen were included in each plate during the screening. After the infection, cells were incubated with the compounds for 72 h at 37°C. Finally, cells were lysed and EGFP expression was determined as described in the Supplemental Experimental Procedures. The total screening time for each compound was approximately 5 days. The quality of the screening assay was determined as described in (Zhang et al., 1999) by calculating the Z' score of each plate according to Equation 1:

$$Z' = 1 - (|3\sigma_{C+} + 3\sigma_{C-}| \ / \ |3\mu_{C+} - 3\mu_{C-}|) \qquad \text{(Equation 1)}$$

where C+ are the Fluorescence Units (FU) in the cells treated with fomivirsen and C- are the FU in the DMSO-treated cells. For hits selection, a cut-off of 50% of inhibitory activity was arbitrarily set. The inhibitory activity of each compound was determined according to Equation 2.

$$\% \text{ Activity} = (FU_{Compound} / FU_{C-}) \times 100 \qquad \text{(Equation 2)}$$

Nontoxic compounds with ≥ 50% inhibitory activity in two rounds of screening were selected for further characterization.

Antiviral and Cytotoxicity Assays

Plaque reduction assays and cytotoxicity assays were performed as previously described in Loregian and Coen, 2006.

Quantitative Real-Time PCR (qPCR)

To analyze the effects of the hits on viral DNA synthesis, HFFs were seeded at a density of 1.5×10^5 per well in 24-well plates. The next day, cells were infected with HCMV AD169 at a MOI of 0.5 PFU/cell and following virus adsorption (2 h at 37°C), they were incubated with 20 μM DGN, 30 μM NTZ, 15 μM TGN, 2 μM AXN, or 50 μM WC5 as a control. At 24 and 72 h p.i., cells were collected and total DNA and the levels of viral DNA were then determined by qPCR and were normalized to the cellular *β-globin* gene copies (Loregian et al., 2010; Mengoli et al., 2004). The oligonucleotide sequences used for the qPCR experiments are reported in the Supplemental Information (Table S3).

Western Blot Analysis

HFFs were seeded in 6-well plates at a density of 3×10^5 cells per well. The next day, cells were infected with HCMV AD169 at an MOI of 0.5 PFU/cell and following virus adsorption, cells were treated with 20 μM DGN, 30 μM NTZ, 15 μM TGN, 2 μM AXN, or with equal volume of DMSO (0.1% v/v). Infected samples treated with either 5 μg/ml ActD or 50 μM WC5 were also included. Cells extracts collected at 24, 48, and 72 h p.i. were prepared and analyzed as previously described (Bronzini et al., 2012). The list of the primary antibodies used in this study is reported in the Supplemental Information (Table S4).

Cell Transfections and Adenoviral Transductions

For transduction experiments, UL54-2F7 and UL112-113-1B4 cells were seeded at 4.5×10^4 cells per well onto glass coverslips in 24-well plates. Cells were transduced with either AdVIE2 or AdVLacZ (MOI of 40 PFU/cell) for 2 h at 37°C, and then treated with 20 μM DGN, 30 μM NTZ,

15 μM TGN, 2 μM AXN, 50 μM WC5, 5 μM fomivirsen, or 0.1% v/v of DMSO. At 72 h p.i., cells were fixed and mounted with mounting medium (Sigma). Samples were observed with a 20× objective under a Olympus IX70 inverted laser scanner confocal microscope equipped with FluoView 300 software.

For transfection and transduction experiments, HFFs were seeded on 24-well plates at 4.5×10^4 cells per well and the following day co-transfected using calcium phosphate (CellPhect Transfection Kit, GE Healthcare) with 0.9 μg of pUL54-0.4 or pUL54-0.15 plasmid, and with 0.1 μg of pRL-TK plasmid as a control to assess transfection efficiency. At 24 h post-transfection, cells were transduced with either AdVIE2 or AdVLacZ at an MOI of 10 PFU/cell for 2 h at 37°C, and then treated with 20 μM DGN, 30 μM NTZ, 15 μM TGN, 2 μM AXN, 50 μM WC5, or 0.1% DMSO (v/v) for 48 h before determination of luciferase activity.

For transfection experiment in HFFs, cells were co-transfected with calcium phosphate with 0.3 μg of phTS-243/+30 plasmid, 0.6 μg of pSGIE72 or pSG5 empty vector, and with 0.05 μg of pRL-TK plasmid. At 18 h post-transfection, cells were washed and then maintained in low-serum medium and treated with 20 μM DGN, 30 μM NTZ, 15 μM TGN, 2 μM AXN, 50 μM WC5, or 0.1% v/v of DMSO for 48 h before determination of luciferase activity. For all the experiments, the values were normalized by dividing the values obtained for firefly luciferase by the values obtained for *Renilla* luciferase and expressed as relative luciferase units (RLU).

Statistical Analysis

All statistical tests were performed using GraphPad Prism version 5.0 for Windows. Data are presented as the means ± standard deviations (SD) of at least two experiments in duplicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and four tables and can be found with this article online.

AUTHOR CONTRIBUTION

B.M. and A.L. conceived and performed experiments, analyzed data, wrote the paper, and edited the paper. G.N. performed experiments. O.T. provided reagents. G.P., G.G., and A.L., designed experiments, wrote the paper, and edited the paper.

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FIGURE LEGENDS

Figure 1. Cell-Based Screening Data. Each circle represents the mean % of EGFP expression for a given compound tested at 10 µM in duplicate during the primary (A) and secondary screen (B). Infected and DMSO-treated cells were considered as exhibiting 100% of EGFP expression. The dashed line represents the arbitrary hit cutoff (50% of EGFP expression). In panel B, among the compounds with two replicates below 50% of EGFP expression threshold, active (green) compounds were distinguished from toxic (red) compounds. Compounds with the means above 50% were considered inactive (black).

Figure 2. Dose-Dependent Inhibition of HCMV Replication by Hit Compounds Identified in the Cell-Based Screening. EC_{50} curves were obtained by infecting HFF cells with HCMV AD169 and then treating them with five different concentrations of DGN (A), NTZ (B), TGN (C), and AXN (D). Chemical structures of the hits are also reported. Calculated EC_{50}s are indicated. Data shown are the means ± SD of three experiments performed in duplicate.

Figure 3. Treatment with the Hit Compounds Blocks Viral DNA Synthesis in HCMV-Infected Cells. HFFs were infected with HCMV and then treated with test compounds or DMSO as a control. At 24 and 72 h p.i., total DNA was extracted and qPCR was performed with appropriate *IE2* and *β-globin* primers. HCMV genomic copies were normalized to the cellular *β-globin* gene copies. Data shown are the means ± SD of three independent experiments performed in duplicate. (*$p<0.05$; **$p<0.001$, *versus* calibrator sample [HCMV-infected cells + DMSO] in a paired *t* test).

Figure 4. Effects of Hit Compounds on the Expression of Representative HCMV IE, E, and L Proteins. HFFs were infected with HCMV and then treated with test compounds or DMSO as a control. At the indicated times p.i., total cell extracts were prepared and analyzed by WB with anti-

IEA (IE1 and IE2), anti-UL44, anti-UL55, and anti-UL99 antibodies. Samples treated with either ActD or WC5 were also included as controls for inhibition of protein expression at 24 h and 48 h p.i., respectively. Tubulin immunodetection served as a loading control.

Figure 5. Hit Compounds Block the IE2-Dependent Transactivation of HCMV E Promoters. UL54-2F7 (A) or UL112-113-1B4 (B) cells were transduced with AdVIE2 or with AdVLacZ and then treated with test compounds or DMSO as a control. Representative confocal microscopy images (EGFP) acquired at 72 h post-transduction (p.t.) are shown (left panel). At the same time, confocal microscopy was used to quantify the percentage of EGFP-expressing cells in 20 different fields. DMSO-treated transduced cells were considered 100% (right panel). C) HFFs were transfected with luciferase reporter plasmids pUL54-0.4 or pUL54-0.15 and after 24 h were transduced with AdVIE2 or AdVLacZ. Then, cells were treated with test compounds or DMSO as a control. At 48 h p.t., luciferase expression was determined and the transcriptional activity was expressed as relative luciferase units (RLU). Data shown are the means ± SD of three experiments performed in duplicate. The results presented in all panels were analyzed with Bonferroni post-test correction for multiple comparisons. (***$p<0.001$ *versus* calibrator sample [AdVIE2 + DMSO]).

Figure 6. Hit Compounds Do Not Interfere with the IE1-Dependent Transactivation of the Cellular Thymidylate Synthetase (*TS*) Gene Promoter. HFFs were transfected with a luciferase reporter plasmid containing a portion of the human thymidylate synthetase promoter (phTS-243/+30) alone, or co-transfected with a plasmid expressing HCMV IE1 (pSGIE72), or the pSG5 empty vector, and then treated with test compounds or DMSO as a control. At 48 h post-transfection, the luciferase activity was determined. Data shown are the means ± SD of three independent experiments performed in duplicate.

Table 1. Antiviral Activity and Cytotoxicity of the Hit Compounds in Follow-Up Testing

Compound (cat. # Sigma)	PubChem CID[a]	Antiviral Activity EC_{50} (µM)[b]	Antiviral Activity EC_{90} (µM)[c]	Cytotoxicity CC_{50} (µM)[d]	SI[e]
(-)-Deguelin (D0817)	107935	1.3 ± 1.0	6.5 ± 1.6	> 500	> 357
Nitazoxanide (N0290)	41684	3.2 ± 0.6	11.8 ± 4.4	285 ± 86	102
Thioguanosine (858412)	2724387	1.2 ± 0.7	6.0 ± 2.1	> 500	> 357
Alexidine dihydrochloride (A8986)	102678	0.17 ± 0.04	0.44 ± 0.04	14.0 ± 8.5	88
Ganciclovir (G2536)	3454	3.4 ± 1.1	19.3 ± 1.1	>1000	> 294

[a]PubChem Compound ID. Compounds data are available in PubChem Compound Database (http://pubchem.ncbi.nlm.nih.gov/compound).
[b]EC_{50}, compound concentration that inhibits 50% of plaque formation, as determined by PRAs against HCMV AD169 in HFF cells. Reported values represent the means ± the SD of data derived from three experiments in duplicate.
[c]EC_{90}, compound concentration that inhibits 90% of plaque formation.
[d]CC_{50}, compound concentration that produces 50% of cytotoxicity, as determined by MTT assays in HFF cells. Reported values represent the means ± the SD of data derived from two experiments in duplicate.
[e]SI, selectivity index (determined as the ratio between CC_{50} and EC_{50}).
See also Table S2.

Figure 1

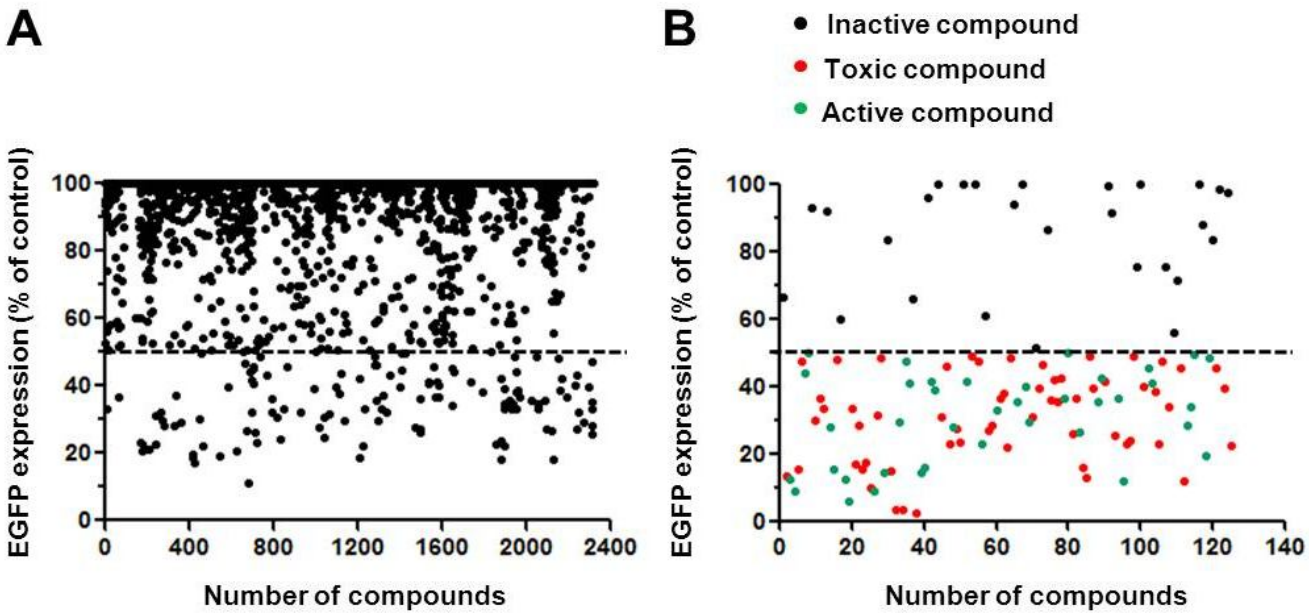

A
B
Inactive compound
Toxic compound
Active compound
EGFP expression (% of control)
Number of compounds
0
20
40
60
80
100
120
140
400
800
1200
1600
2000
2400

Figure 2

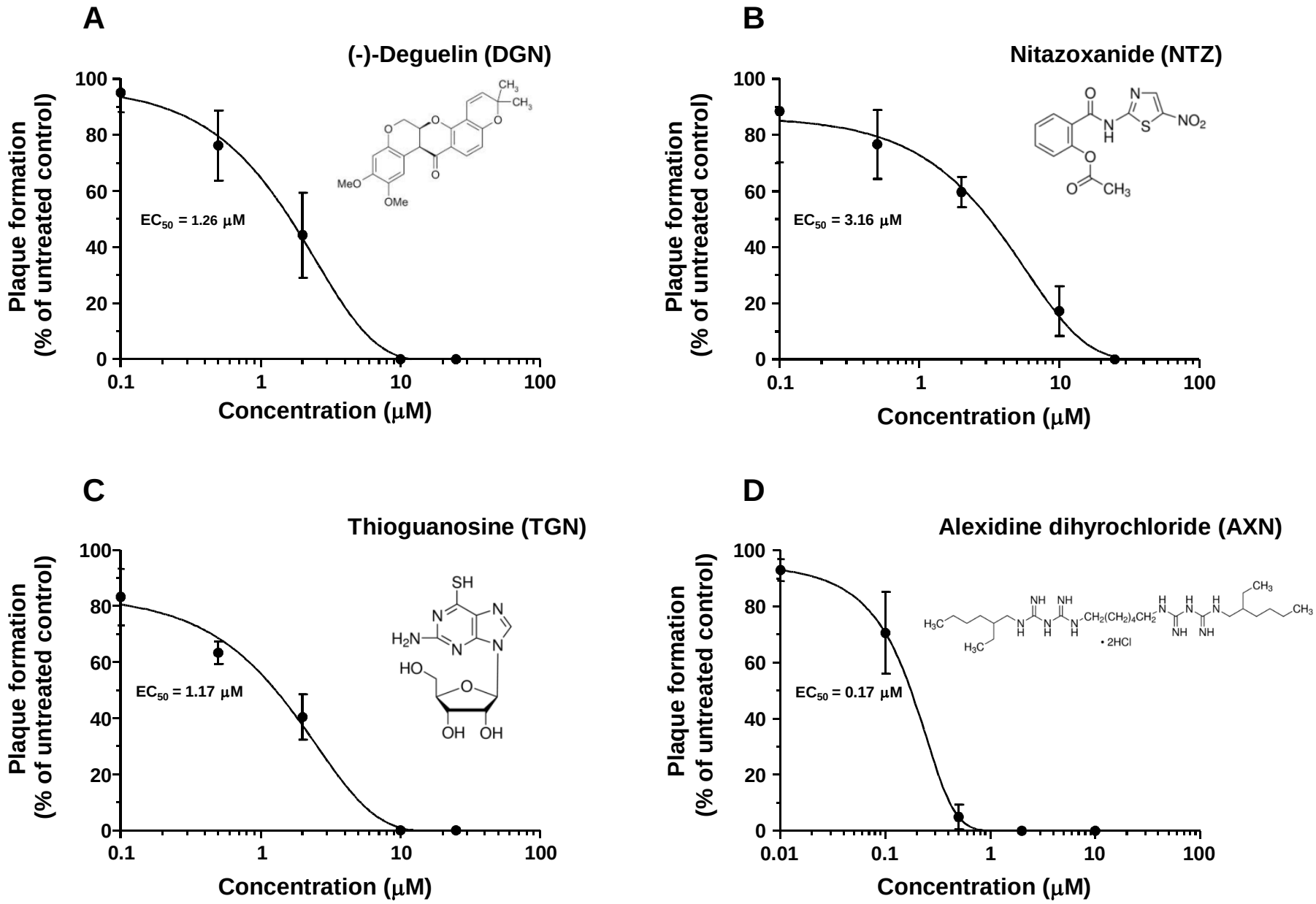

A
(-)-Deguelin (DGN)
Plaque formation (% of untreated control)
EC50 = 1.26 μM
Concentration (μM)
B
Nitazoxanide (NTZ)
EC50 = 3.16 μM
C
Thioguanosine (TGN)
EC50 = 1.17 μM
D
Alexidine dihyrochloride (AXN)
EC50 = 0.17 μM
• 2HCl

Figure 3

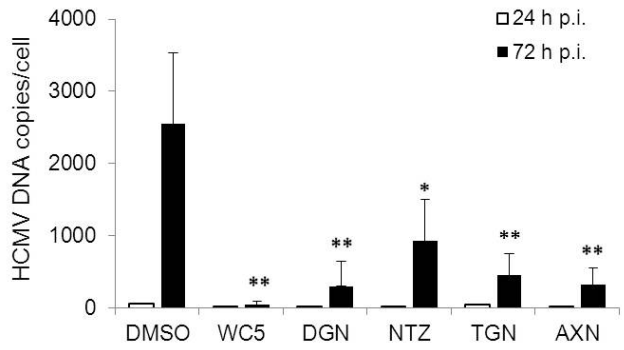

HCMV DNA copies/cell
4000
3000
2000
1000
0
24 h p.i.
72 h p.i.
**
**
*
**
**
DMSO
WC5
DGN
NTZ
TGN
AXN

Figure 4

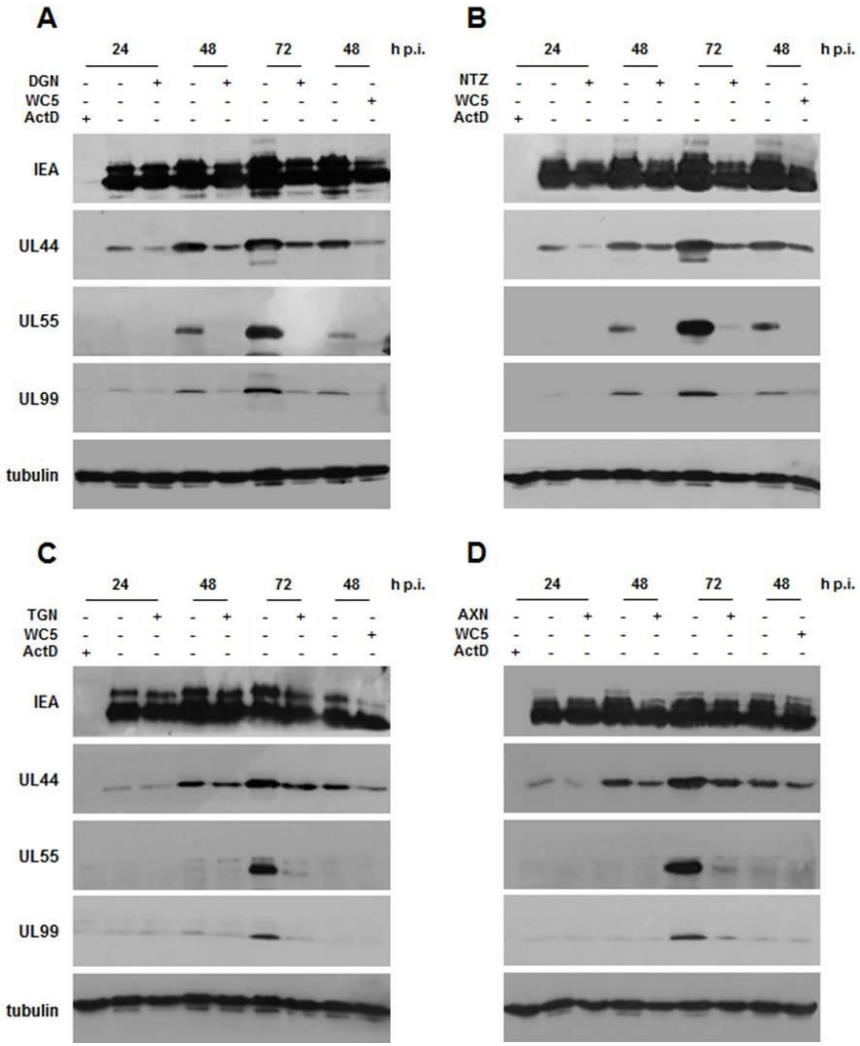

A
24
48
72
48
h p.i.
DGN
WC5
ActD
IEA
UL44
UL55
UL99
tubulin
B
24
48
72
48
h p.i.
NTZ
WC5
ActD
C
24
48
72
48
h p.i.
TGN
WC5
ActD
IEA
UL44
UL55
UL99
tubulin
D
24
48
72
48
h p.i.
AXN
WC5
ActD

Figure 5

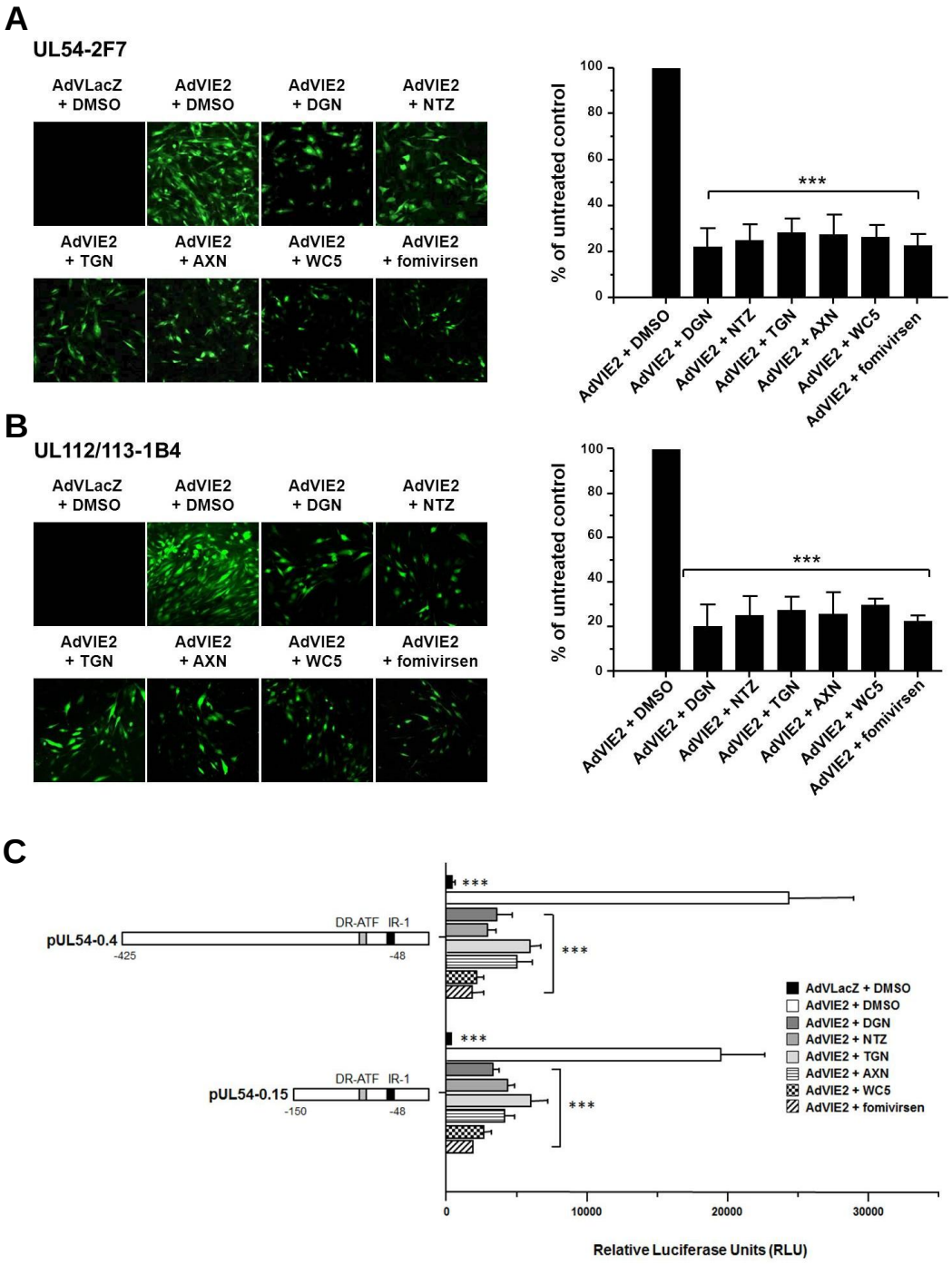
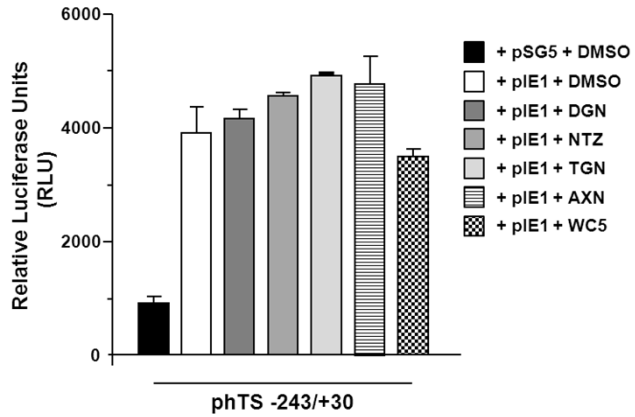

A
UL54-2F7
AdVLacZ + DMSO
AdVIE2 + DMSO
AdVIE2 + DGN
AdVIE2 + NTZ
AdVIE2 + TGN
AdVIE2 + AXN
AdVIE2 + WC5
AdVIE2 + fomivirsen
% of untreated control
***
B
UL112/113-1B4
C
pUL54-0.4
DR-ATF IR-1
-425
-48
pUL54-0.15
-150
AdVLacZ + DMSO
AdVIE2 + DMSO
AdVIE2 + DGN
AdVIE2 + NTZ
AdVIE2 + TGN
AdVIE2 + AXN
AdVIE2 + WC5
AdVIE2 + fomivirsen
Relative Luciferase Units (RLU)

Figure 6


6000
4000
2000
0
Relative Luciferase Units (RLU)
phTS -243/+30
+ pSG5 + DMSO
+ pIE1 + DMSO
+ pIE1 + DGN
+ pIE1 + NTZ
+ pIE1 + TGN
+ pIE1 + AXN
+ pIE1 + WC5

Supplemental Figure S1, Related to Figure 1 and Supplemental Experimental Procedures

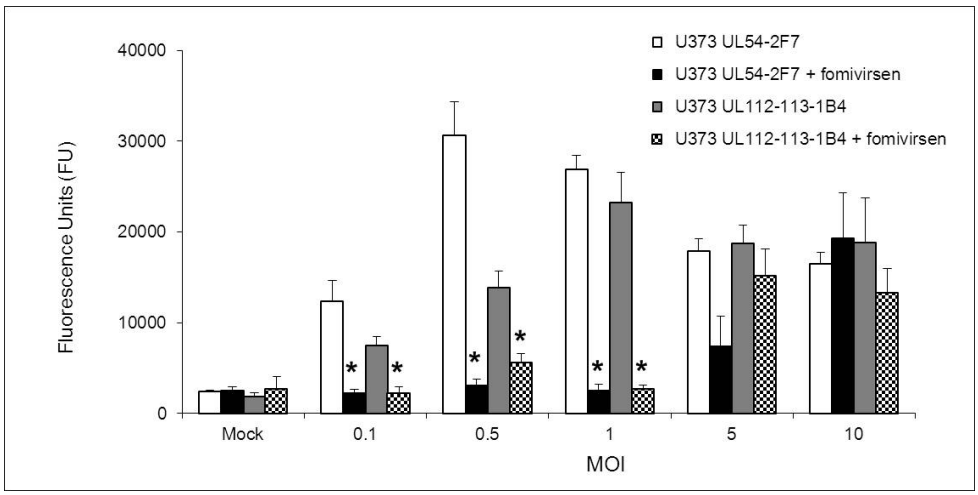


Figure S1. Optimization of the Cell-Based Assay for the Screening.
U373 UL54-2F7 and UL112-113-1B4 cell lines were either mock-infected or infected with HCMV AD169 at different multiplicity of infection (MOI) and treated with 5 μM fomivirsen as a positive control or untreated. EGFP levels (expressed as Fluorescence Units [FU]) in cell-free supernatants were determined at 72 h p.i. after cell lysis. All data are represented as mean ± SD of three independent experiments; (*$p<0.01$ *versus* calibrator sample [HCMV-infected untreated U373 UL54-2F7 and UL112-113-1B4 cell lines]).

Supplemental Figure S2, Related to Figure 1 and Supplemental Experimental Procedures

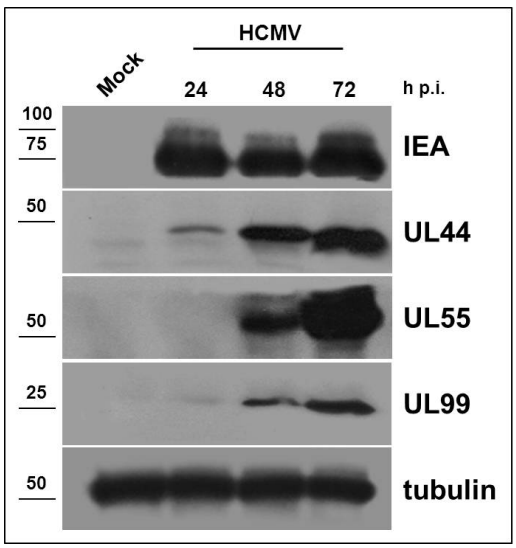


Figure S2. Expression of Representative Viral IE, E, and L Proteins in HCMV-Infected U373 UL54-2F7 Cells.
U373 UL54-2F7 cells were either mock-infected or infected with HCMV AD169 (MOI 0.5 PFU/cell). At the indicated times p.i., whole-cell extracts were prepared and analyzed by Western Blot (WB) with antibodies specific for IE (IE1 and IE2), E (UL44), and L (UL55 and UL99) proteins. Tubulin immunodetection served as an internal control.

Supplemental Figure S3, Related to Figures 3 and 4 and Supplemental Experimental Procedures

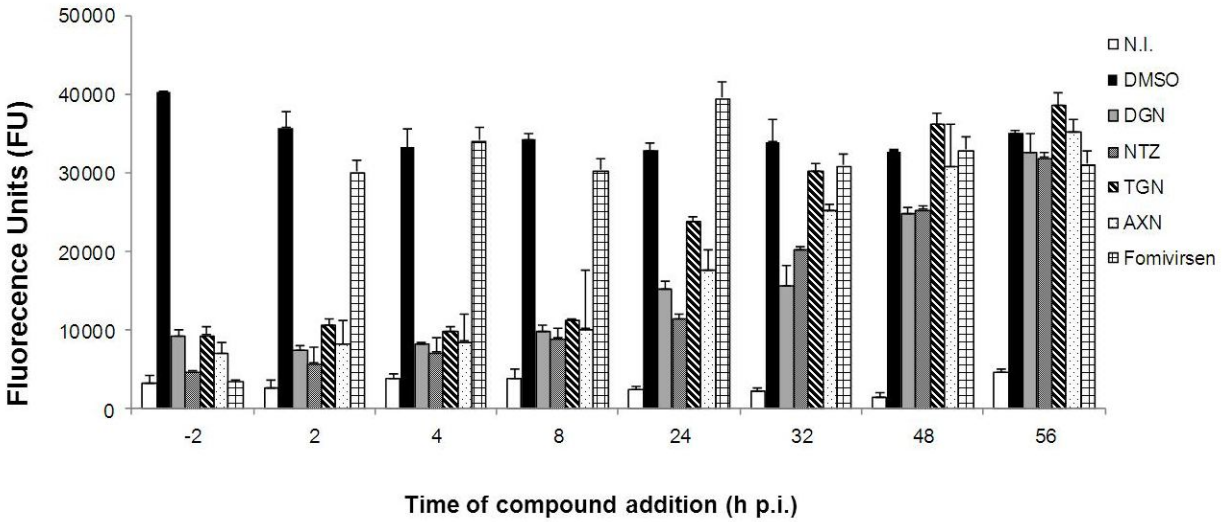


Figure S3. Effects of Addition of Hit Compounds and Fomivirsen on EGFP Expression at Different Time-Points after HCMV Infection of U373 UL54-2F7 Cells.
U373 UL54-2F7 cells were either mock-infected (N.I.) or infected with HCMV AD169 (MOI 0.5) and incubated with 10 μM hit compounds, or DMSO or 5 μM fomivirsen as controls, at the indicated times prior or after virus infection. EGFP levels (expressed as Fluorescence Units [FU]) in cell-free supernatants were determined at 72 h p.i. after cell lysis. All data are represented as mean ± SD of three independent experiments.

Supplemental Figure S4, Related to Figure 5 and Supplemental Experimental Procedures

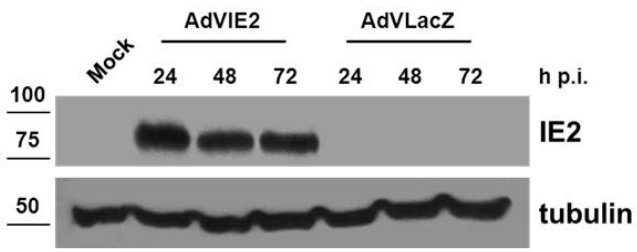


Figure S4. Expression of the IE2 Protein in U373 UL54-2F7 Cells Transduced with AdVIE2.
U373 UL54-2F7 cells were not transduced (mock) or transduced with AdVIE2 or AdVLacZ at MOI of 40 PFU/cell. At the indicated times p.i., whole-cell extracts were analyzed by WB with an anti-IE2 antibody. Tubulin immunodetection served as an internal control.

Supplemental Figure S5, Related to Supplemental Experimental Procedures

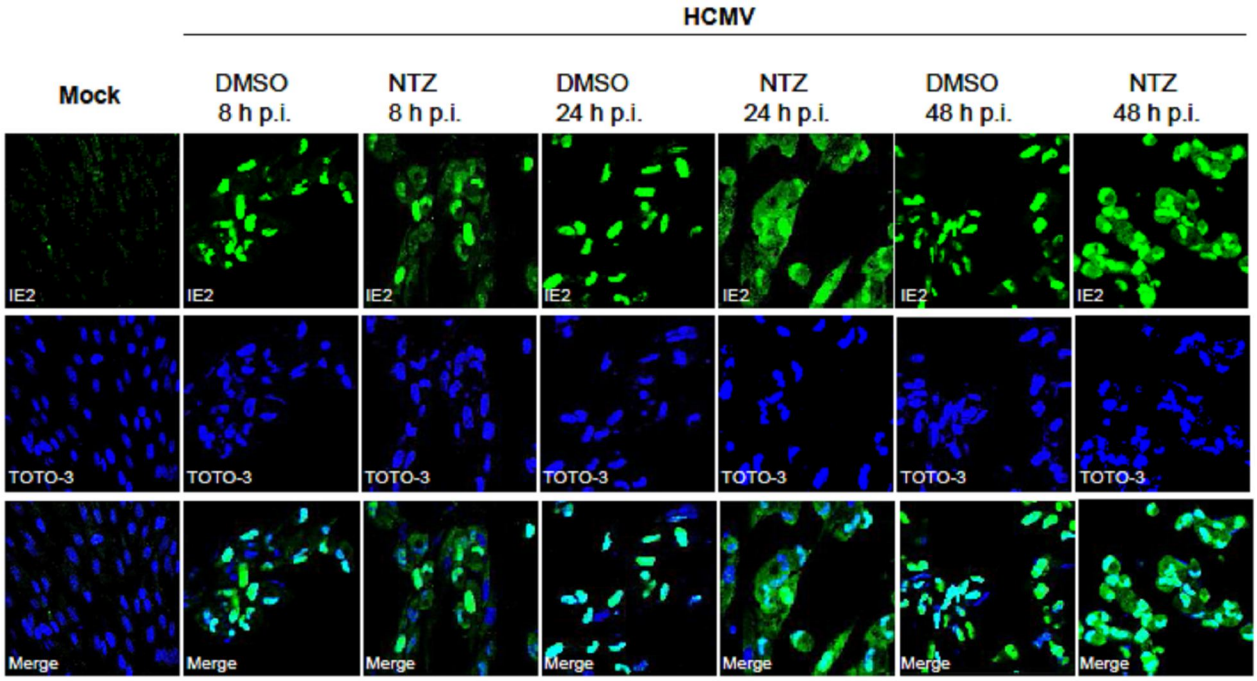


Figure S5. Intracellular Localization of IE2 in Infected, NTZ-treated HFF Cells.
The intracellular localization of IE2 in HFF cells infected with HCMV and treated with NTZ or DMSO as a control was analyzed by immunofluorescence with an anti-IE2 antibody at 8, 24, and 48 h p.i.. Nuclei were counterstained with TOTO-3 stain. Individual green (IE2) and blue (TOTO-3) channels and merged images are shown. Representative images of four independent experiments performed in duplicate are shown.

Supplemental Table S1. Table of the Hit Compounds Identified by Cell-based Screens, Related to Figure 1 and Experimental Procedures.

Compound	PubChem CID[a]	Bioactivity	Antiviral Activity EC_{50} (μM)[b]	Cytotoxicity CC_{50} (μM)[c]	SI[d]
Clotrimazole	2812	Antifungal	0.8 ± 0.6	40 ± 28	50
Hexachlorophene	3598	Antiinfective	0.3 ± 0.8	150 ± 68	500
Clioquinol	2788	Antiseptic, antiamebic	4.8 ± 3.6	42 ± 35	9
Edoxudine	66377	Antiviral	> 20	> 200	>10
Pararosaniline pamoate	11292	Antihelmintic, antischistosomal	5.5 ± 1.4	100 ± 36	18
Nitazoxanide	**41684**	**Antiparasitic**	**3.9 ± 1.6**	**>200**	**>51**
Nisoldipine	4499	Vasodilator (coronary)	3.8 ± 2.6	80 ± 21	21
Manidipine hydrochloride	4008	Antihypertensive	2.2 ± 2.8	>200	>91
Miconazole nitrate	68553	Antifungal	1.2 ± 0.6	55 ± 16	46
Azacitidine	9444	Antineoplastic, antimetabolite	> 20	> 200	>10
Papaverine hydrochloride	6084	Muscle relaxant, cerebral vasodilator	7.0 ± 2.9	>200	>29
Hydroxyprogesterone caproate	169870	Progestogen	> 20	> 200	>10
Cycloheximide	6197	Antipsoriatic, protein synthesis inhibitor	6.0 ± 1.3	>200	>33
Pentamidine isethionate	8813	Antiprotozoal	ND	< 20	ND
Oligomycin A	5281899	Antibacterial, antifungal	ND	< 20	ND
Econazole nitrate	68589	Antifungal	4.9 ± 2.3	85 ± 13	17
Nitroxoline	19910	Antibacterial	4.5 ± 2.4	100 ± 27	22
Salinomycin, sodium	53487842	Antibacterial	7.5 ± 1.0	50 ± 18	7
Dequalinium chloride	10649	Antiinfectant	ND	< 20	ND
Berberine chloride	12456	Antiarrhythmic, α2 agonist, anticonvulsant	1.3 ± 0.8	> 200	>160
Ethaverine hydrochloride	13796	Antispasmodic	5.0 ± 1.7	> 200	>50
Tioxolone	72139	Antiseborrhoic	> 20	> 200	>10
Alexidine dihydrochloride	**102678**	**Antibacterial**	**0.22 ± 0.2**	**15 ± 5**	**68**
Isorotenone	6453092	Natural substance	< 0.2	> 200	>1000
Antimycin A	3084471	Antifungal, antiviral	1.4 ± 0.9	> 200	>143
Pyrromycin	196990	Antibacterial	0.2 ± 0.6	30 ± 8	137
Mundulone acetate	4587968	Natural substance	> 20	> 200	>10
Ergosterol acetate	444679	Provitamin	> 20	> 200	>10
Deguelin(-)	**107935**	**Antineoplastic, antiviral, insecticide**	**0.6 ± 0.9**	**> 200**	**> 333**
Perseitol	441436	Natural substance	6.0 ± 1.5	>200	>33
Duartin(-)	3666064	Natural substance	0.9 ± 0.4	> 200	> 235
Mundoserone	3083809	Natural substance	6.1 ± 2.5	>200	>33
Rotenonic acid	4303568	Natural substance	> 20	> 200	>10
Thioguanosine	**2724387**	**Antineoplastic**	**0.7 ± 1.2**	**>200**	**>308**
2,4-dinitrophenol	1493	Uncouples oxidative phosphorylation	5.8 ± 1.7	100 ± 18	18
Apotoxicarol	ND	Natural substance	15.2 ± 8.6	> 200	> 13
Alpha-toxicarol	442826	Natural substance	3.2 ± 0.6	110 ± 27	31
Pentachlorophenol	992	Insecticide, herbicide	6.3 ± 1.1	75 ± 8	137

[a]PubChem Compound ID. Compounds data are available in PubChem Compound Database (http://pubchem.ncbi.nlm.nih.gov/compound).

[b]Compound concentration that inhibits 50% of plaque formation, as determined by plaque reduction assays against HCMV AD169 in HFF cells. Reported values represent the means ± the SD of data derived from two independent experiments in duplicate.

[c]Compound concentration that produces 50% of cytotoxicity, as determined by MTT assays in HFF cells. Reported values represent the means ± the SD of data derived from two independent experiments in duplicate.

[d]SI, Selectivity Index (determined as the ratio between CC_{50} and EC_{50}).

In boldface are highlighted the compounds selected for follow-up studies; ND, not determined because of cytotoxicity.

For a comparison with the reference compound GCV, please see the values reported in Table 1 in the main text.

Supplemental Table S2. Table of the Antiviral Activity of Selected Hits Against a Panel of Different HCMV Strains, Related to Figure 2 and Experimental Procedures.

		Antiviral Activity EC_{50} (μM)[a]				
HCMV strain	Resistance	Control[b]	DGN	NTZ	TGN	AXN
TB40	None	2.7 ± 1.2	1.1 ± 0.5	3.1 ± 0.1	1.4 ± 0.9	0.16 ± 0.03
VR1814	None	6.6 ± 0.5	1.4 ± 0.9	3.5 ± 0.6	1.6 ± 1.7	0.23 ± 0.05
759^{r}D100	GCV	54.2 ± 4.6	2.5 ± 1.9	3.7 ± 0.2	1.5 ± 0.2	0.80 ± 0.07
PFA^{r}D100	FOS, ACV	200 ± 25	1.9 ± 0.1	3.0 ± 0.1	1.9 ± 0.1	0.20 ± 0.08

[a]EC_{50}, compound concentration that inhibits 50% of plaque formation, as determined by plaque reduction assays. Reported values represent the means ± the SD of data derived from three independent experiments in duplicate.
[b]Ganciclovir (GCV) was used as a control in all plaque reduction assays, except for PFA^{r}D100 strain, where foscarnet (FOS) was used as a control. ACV: acyclovir.

Supplemental Table S3. List of the Oligonucleotides Used in This Study, Related to Figure 3 and Experimental Procedures.

UL122 qPCR primer set	Primer FOR (CM-5T)	TCATCCACACTAGGAGAGCAGACT
	Primer REV (CM-3T)	GCCAAGCGGCCTCTGAT
	Probe	ACTGGGCAAAGACCTTCATGCAGATCTC
β-globin qPCR primer set	Primer FOR	AGGGCCTCACCACCAACTT
	Primer REV	GCACCTGACTCCTGAGGAGAA
	Probe	ATCCACGTTCACCTTGCCCCACA

Supplemental Table S4. List of the Primary Antibodies Used in This Study, Related to Figures 4, S4, and S5 and Experimental Procedures.

Antigen	Supplier	Clone ID	Usage
Tubulin	Sigma	TUB 2.1	WB (1:2,000)
IE1/IE2	Argene-Biosoft	E13	WB (1:400)
UL44	Virusys	CH16	WB (1:1,000)
UL55	Virusys	CH28	WB (1:1,000)
UL99	Virusys	CH19	WB (1:2,000)
IE2	Santa Cruz Biotech.	12E2	WB (1:1000); IF (1:100)

WB: Western Blot; IF: immunofluorescence.

Supplemental Experimental Procedures

Optimization of the Cell-Based Assay for the Screening, Related to Figure S1.

U373 UL54-2F7 and UL112-113-1B4 cells were seeded in 96-well plates at a density of 2×10^4/well and allowed to grow for 16 h. The next day, cells were pretreated for 2 h with 5 μM fomivirsen, and then infected with HCMV AD169 at different multiplicities of infection (MOI, from 0.1 to 10 PFU/cell). After a 2-h incubation at 37°C, viral inocula were removed and cells were incubated with 5 μM fomivirsen for 72 h at 37°C. Thereafter, cells were lysed in 0.1 ml of lysis buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 0.15% NP-40) for 30 min at 4°C and centrifuged. Finally, 90 μl of cell-free supernatants were transferred into 96-well black plates (Nunc) to quantify EGFP expression by fluorescence detection with Victor X2 (Perkin Elmer) after 5 min equilibration.

Analysis of HCMV Proteins Expression in U373 UL54-2F7 Cells by Western Blot, Related to Figure S2.

To verify the completion of the HCMV replication cycle in U373 cells, U373 UL54-2F7 cells were seeded in 6-well plates at a density of 3×10^5/well. The following day, cells were infected with HCMV AD169 at MOI of 0.5 PFU/cell for 2 h at 37°C. Whole-cell extracts were then prepared at 24, 48, and 72 h p.i. as described in the main text, fractionated by 8.5% SDS-PAGE, and then analyzed by WB with anti-IEA (IE1/IE2), anti-UL44, anti-UL55, anti-UL99, and anti-tubulin MAbs (Table S4).

Time-of-Addition Studies, Related to Figure S3.

For time-of-addition studies, U373 UL54-2F7 cells were seeded at a density of 2×10^4/well in 96-well plates and incubated overnight at 37°C. The next day, cells were infected with HCMV AD169 at MOI 0.5 PFU/cell for 2 h at 37°C. At the end, the viral inocula were removed and at different times (-2, 2, 4, 8, 24, 32, 48, 56 h p.i.) the cell medium was replaced with medium containing 10 μM of the hit compounds or 0.1% DMSO as a control. Cells were incubated at 37°C for a total of 72 h after infection; at this time point, cells were lysed and EGFP expression was determined as previously described.

Analysis of IE2 Protein Expression in AdVIE2 Transduced-U373 UL54-2F7 Cells by Western Blot, Related to Figure S4.

To verify the expression of the IE2 protein following transduction with AdVIE2, U373 UL54-2F7 were seeded at a density of 3×10^5/well in 6-well plates, and then transduced the following day with AdVIE2, or AdVLacZ, as a control, at an MOI of 40 PFU/cell. At different times post-infection, whole-cell extracts were prepared as described in the main text and analyzed by 8.5% SDS-PAGE and WB using anti-IE2 and anti-tubulin MAbs (Table S4).

Analysis of IE2 Nuclear Localization by Immunofluorescence, Related to Figure S5.

To analyze the intracellular localization of IE2, HFF cells at a density of 5×10^5/well seeded on coverslips were infected with HCMV AD169 (MOI 0.5) for 2 h at 37°C. At the end of the incubation, medium containing 30 μM NTZ, or DMSO as a control, was added and cells were incubated at 37°C. At 8, 24, and 48 h p.i., cells were fixed with 4% (v/v) formaldehyde in PBS for 20 min. After permeabilization with 0.3% Triton X-100 in PBS for 15 min at RT, cells were first incubated at RT for 1 h with 4% FBS in PBS, followed by incubation for 1 h at 37°C with an anti-IE2 Mab (Table S4). Following washes, an anti-mouse IgG FITC-conjugated antibody (Santa Cruz

Biotechnology) was added for 1 h at 37°C. Finally, cells were incubated for 20 min at RT with TOTO-3 iodide (Molecular Probes) and coverslips were mounted. Cells were imaged with a Leica TCS-NT/SP2 confocal microscope with a 40× objective and the images were digitally analyzed with Leica software.