Evasion of a human cytomegalovirus entry inhibitor with potent cysteine reactivity is concomitant with the utilisation of a heparan sulfate proteoglycan independent route of entry

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Running title: HCMV anti-viral resistance triggers immune sensitivity

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Abstract

The dependence of viruses on the host cell to complete their replicative cycle renders cellular functions potential targets for novel anti-virals. We screened a panel of broad acting cellular ion channel inhibitors for activity against human cytomegalovirus (HCMV) and identified the voltage-gated chloride ion channel inhibitor 4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid (DIDS) as a potent inhibitor of HCMV replication. Time of addition studies demonstrated that DIDS inhibited entry via a direct interaction with the virion that impeded binding to the plasma membrane. Synthesis and analysis of pharmacological variants of DIDS suggested that intrinsic cysteine, and not lysine, reactivity was important for activity against HCMV.

Although sequencing of a DIDS-resistant HCMV revealed enrichment of a mutation within UL100 (encoding for glycoprotein M) and a specific truncation of glycoprotein RL13, these did not explain the DIDS resistance phenotype. Specifically, only the introduction of the RL13 mutant partially pheno-copied the DIDS resistance phenotype. Serendipitously, the entry of DIDS-resistant HCMV also became independent of heparan sulfate proteoglycans (HSPGs) suggesting that evasion of DIDS lowered dependence on an initial interaction with HSPGs. Intriguingly, the DIDS-resistant virus demonstrated increased sensitivity to antibody neutralisation, which mapped, in part, to the presence of the gM mutation.

Taken together the data characterise the anti-viral activity of a novel HCMV inhibitor that drives HCMV infection to occur independent of HSPGs and the generation of increased sensitivity to humoral immunity. The data also demonstrate that compounds with cysteine reactivity have the potential to act as anti-viral compounds against HCMV via direct engagement of virions.
Importance

Human cytomegalovirus (HCMV) is major pathogen of non-immunocompetent individuals which remains in need of new therapeutic options. Here we have identified a potent antiviral compound (4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid, DIDS), its mechanism of action and the chemical properties required for its activity. In doing so, the data argue that cysteine-reactive compounds could have the capacity to be developed for anti-HCMV activity. Importantly, the data show that entry of DIDS resistant virus became independent of heparan sulfate proteoglycans (HSPGs) but, concomitantly, became more sensitive to neutralising antibody responses. This serendipitous observation suggests that retention of an interaction with HSPGs during the entry process in vivo may be evolutionarily advantageous through better evasion of humoral responses directed against HCMV virions.

Introduction

Human cytomegalovirus (HCMV) entry involves a complex series of events requiring the interaction of virally encoded glycoproteins with cell membrane receptors (1–10). The ability of HCMV to utilize multiple entry pathways likely underpins an ability to infect a number of different cell types. As such, HCMV entry has been reported to proceed via pH-dependent fusion, endocytosis and macropinocytosis in a cell type and virus strain specific manner (11–14). Prior to the cell type specific events that dictate HCMV tropism the virus engages with the cell via a low affinity interaction with heparan-sulfate proteoglycans (HSPGs) (1, 2, 15). The importance of this interaction for HCMV entry is emphasized by the ability of heparin to act as a potent inhibitor of HCMV infection (2). Indeed, many viruses utilize an interaction with HSPGs presumably as a mechanism to establish initial contact with the plasma membrane prior to triggering the complicated entry process (16–18).
The requirement for virally encoded glycoproteins to drive entry exposes the virus to humoral immunity. Antibodies that recognize glycoproteins B, H/L/O and the pentameric complex have all been shown to be potent inhibitors of viral infection in vitro (19–26). Furthermore, promising data from clinical studies demonstrate passive immunization with antibodies directed against viral complexes incorporating gH/gL can reduce HCMV viraemia in vivo (27). Antibody mediated neutralization of HCMV can occur either via the recruitment of complement to promote pathogen lysis or through steric hinderance of the interactions of these glycoproteins with their cell surface receptors (28). Although the specific interactions that are occurring at the cell surface are not fully understood a substantial body of work points towards cell type specific roles for the gH/gL complexes whereas gB is involved in entry into all cell types with reported roles in initial attachment, fusion and receptor binding, and activation of signalling pathways (4, 29–34).

The other angle of viral entry is the role of host cell functions in the process. Recently, a number of studies point to the importance of ion channel activity in the entry of a range of viruses, with roles in entry and uncoating being reported for diverse viruses such as Influenza A, Ebola virus and Bunyamwera (35–38). Furthermore, the impact of ion channels is not restricted to the viral entry process with roles for potassium channels Kv2.1 and TASK-1 being demonstrated in the activation of apoptosis induced by hepatitis C virus and the budding of HIV-1 respectively (39, 40). Given that a number of ion channel inhibitors are used to treat non-viral disorders in the clinic it has raised the possibility that a number of licensed compounds with good safety profiles may be re-purposed for use in viral infections (41).

Here we report the unexpected outcome of a compound screen of known ion channel inhibitors. Further characterization of a lead hit (4,4’-diisothiocyano-2,2’-stilbenedisulfonic acid, DIDS) revealed that the inhibitory activity was not related to inhibition of its canonical
ion channel target but instead, upon characterization, revealed that the compound directly bound the virus to inhibit initial viral engagement at the stage of HSPG interactions. Further characterisation of the mechanism of action revealed DIDS inhibition was dependent on a reversible cysteine-dependent interaction with the virion to prevent entry. Generation of a DIDS resistant mutant revealed that DIDS resistance was concomitant with resistance to heparin and an entry pathway independent of HSPGs. Surprisingly, the acquisition of a DIDS/heparin resistant phenotype resulted in an increased sensitivity to neutralizing antibody responses present in sera when tested in vitro. Sequence analysis of the mutant revealed an enrichment for mutations in two genes – RL13 and UL100 – which encode for glycoproteins present in the virion envelope (42, 43). Interestingly, re-engineering of these mutations into the Merlin BAC revealed a complicated phenotype. A partial resistance to DIDS was imparted by the RL13 truncation but, intriguingly, this was reduced in the presence of the gM mutation. However, it was the gM and not the RL13 mutation that appeared to be the major contributor to the antibody sensitivity phenotype of the DIDS resistant virus. In summary, the data also suggest that resistance to the action of the anti-viral entry inhibitor, DIDS, may be countered by increased sensitivity to humoral immunity which could have positive implications for the potential development and use of DIDS like molecules against HCMV in vivo.

Materials & Methods

Cells and virus propagation

Human fetal foreskin fibroblasts (HFFs, SCRC-1041) and retinal pigment epithelial cells (RPE-1, CRL-4000) were purchased from ATCC. U373 cells were a kind gift of John Sinclair, University of Cambridge. HFF-Tet cells were a kind gift from Richard Stanton,
Cardiff University. Murine 3T3 fibroblasts were kindly provided by Ian Humphreys, Cardiff University. All cells were grown in high glucose Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (ThermoFisher), 100U/ml penicillin and 100µg/ml streptomycin. Merlin, TB40/E-UL32-GFP (a kind gift from Christian Sinzger), Towne and AD169 stocks of HCMV were propagated in HFFs. HSV-VP26-GFP was a kind gift from Richard Milne. Stocks of murine CMV strain Smith were kindly provided by Ian Humphreys. All experiments were performed with Merlin HCMV unless otherwise stated. BAC recombinant viruses were propagated in HFF-Tet cells to prevent mutations in the RL13 (under the control of a Tet repressor in BAC_RL13wt, BAC_RL13trunc and BAC_RL13trunc+gMmut, defective in BACgMwt, BACgMmut and BAC_RL13neg) and UL128-131 loci (under the control of a Tet repressor in BACgMwt and BACgMmut, defective in all other BAC viruses) and thus retain a wildtype genotype and maintained as the seed stocks (44). Prior to their use the virus seed stocks encoding either wildtype or truncated RL13 were then amplified in HFFs to allow expression of their respective RL13, whilst RL13 negative variants were generated from HFF-Tet cells.

Supernatants from cells infected with BAC recombinant viruses were purified using sorbitol gradient (20% D-sorbitol, 50mM pH7.4 Tris, 1mM MgCl₂) centrifugation (65,000g for 90 minutes at 4°C) using a SW32Ti rotor (Beckman Coulter) in an Optima XE ultracentrifuge (Beckman Coulter).

Inhibitors and chemicals

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), apamin, α-Pompilidotoxin (α-PMTX) and tetraethylammonium (TEA) were gifts from Jamel Mankouri (University of Leeds). GaTx2 was purchased from Tocris, whilst 4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid
(DIDS), 4-Acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid (SITS), N-ethylmaleimide (NEM), 2-Iminothiolane (2-IT), 4-chloro-7-nitrobenzofurazan (Nbd-Cl), 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs₂) and heparin were all purchased from Sigma Aldrich and used at the concentrations described in the figures. Concentrations of NPPB and GaTx2 used were based on the concentrations previously established to be required for inhibition of chloride ion channels (45, 46). For beta mercaptoethanol (β2M) experiments, HCMV was pre-treated with DIDS or DMSO for 1 hour and then subsequently incubated with β2M for 10 minutes, diluted and used to infect HFFs by standard procedure. For enzymatic removal of heparin sulfates, HFFs were treated with heparinase I (New England Biolabs) at 30°C for 1 hour prior to infection with HCMV.

Synthesis of 4,4’-dimaleimidylazobenzene (DMIA)

4-[(E)-(4-aminophenyl)azo]aniline (200mg, 0.94mmol) was dissolved in DMF (8 mL) in a flask wrapped in foil and to this maleic anhydride (214mg, 2.07mmol) dissolved in DMF (8 mL) was added portion wise. The reaction gradually turned a dark black/orange and the reaction was stirred for 2 hours. The reaction was then filtered and the solid was washed with THF (20 mL) and DCM (100 mL) and dried under vacuum to give (Z)-4-[(E)-4-[[4-[[[(Z)-3-carboxyprop-2-enoyl]amino]phenyl]azo]anilino]-4-oxo-but-2-enoic acid (251mg, 66% yield).

A solution of (Z)-4-[(E)-4-[[[(Z)-3-carboxyprop-2-enoyl]amino]phenyl]azo]anilino]-4-oxo-but-2-enoic acid (251mg, 0.62mmol) in acetic anhydride (10mL) was heated to 95°C and then sodium acetate (202mg, 2.46mmol) was added. The reaction was heated at 95°C for 2h and then allowed to cool to room temperature. The mixture was poured onto ice water and stirred for 30 minutes. The mixture was then neutralised with aqueous sodium hydrogen carbonate solution, filtered and the solid was air-dried to give 4,4’-
dimaleimidylazobenzene (128mg, 56% yield). 1H NMR (DMSO-d6, 400MHz) 8.04 (4H, d), 7.63 (4H, d) and 7.26 (2, 4H).

Synthesis of 4,4'-dimaleimidylstilbene (DMIS)

4-[(E)-2-(4-aminophenyl)vinyl]aniline (200mg, 0.95mmol) was dissolved in DMF (8 mL) in a flask wrapped in foil and to this maleic anhydride (216mg, 2.09mmol) dissolved in DMF (8 mL) was added portion wise. The reaction gradually turned a dark black/orange and the reaction was stirred for 2 hours. The reaction was then filtered and the solid was washed with THF (20 mL) and DCM (100 mL) and dried under vacuum to give (Z)-4-[(E)-2-[4-[[Z]-3-carboxyprop-2-enoyl]amino]phenyl]vinyl]anilino]-4-oxo-but-2-enoic acid (250mg, 65% yield).

A solution of (Z)-4-[(E)-2-[4-[[Z]-3-carboxyprop-2-enoyl]amino]phenyl]vinyl]anilino]-4-oxo-but-2-enoic acid (250mg, 0.62mmol) in acetic anhydride (10mL) was heated to 95°C and then sodium acetate (202mg, 2.46mmol) was added. The reaction was heated at 95°C for 2h and then allowed to cool to room temperature. The mixture was poured onto ice and stirred for 30 minutes. The mixture was then neutralised with sodium hydrogen carbonate solution and then filtered and air-dried to give 4,4’-dimaleimidylstilbene (174mg, 76% yield).

1H NMR (DMSO-d6, 400MHz) 7.74 (4H, d), 7.38 (4H, d), 7.37 (2H, s) and 7.21 (2, 4H).

Virus binding and entry assays

To study virus binding and entry by detection of viral DNA HFFs were infected at +4°C for 1 hour to allow virus binding. Cells were washed in ice cold PBS and then either lysed immediately for analysis by qPCR for genomes (using UL138 primers as noted below) or temperature shifted to 37°C to promote viral entry. Fifteen minutes post entry DNA was
harvested from cells and analysed by qPCR. To assess virus binding by direct visualisation of virus particles, HFFs were first labelled with 1µg/ml Hoechst 33342 (Tocris) for 10 minutes at 37°C. Cells were then washed in ice cold PBS and infected at +4°C at MOI=5 with TB40/E-UL32-GFP HCMV for 1h. Cells were washed twice with ice cold PBS and live cells visualised directly by confocal imaging using a 60x objective on a Nikon Ti inverted microscope, with a C2 confocal scan head (Nikon). Z-stacks were acquired at high zoom to create representative images, whilst Z-stacks of multiple low zoom fields were acquired to allow for quantification. ImageJ was used to process images for display.

Fixation and immunostaining

Human cells were fixed by treatment with 100% ice-cold ethanol for >20 minutes at -20°C. Murine 3T3 cells were fixed by treatment with 100% ice-cold methanol for 30 minutes at -20°C. To detect viral proteins cells were then washed in PBS and incubated with mouse anti-IE (6F8.2, Merck Millipore; 1:2000 dilution), mouse anti-pp28 (5C3, Santa Cruz Biotechnology; 1:1000 dilution), mouse anti-ICP4 (abcam, 1:1000 dilution), mouse anti-MCMV IE (Genetex, 1:1000 dilution) or mouse anti-pp65 (abcam; 1:500 dilution) for 1 hour, followed by incubation with Goat anti-Mouse IgG-Alexa-fluor-568 (Life Technologies; 1:2000 dilution) plus 0.5µg/ml DAPI for 1 hour.

Innate and humoral immunity studies and quantification of infection

To measure the impact of humoral immunity on HCMV infection, all viruses were pre-incubated with pooled sera from seropositive or seronegative donors for 1 hour at dilutions from 1:5-800. After 1 hour, cells were infected with these HCMV preparations and fixed and stained 24hpi for IE gene expression as a marker of lytic infection.
For studies with IFN, cells were pre-treated with a combination of IFNα/β (Peprotech) for 3 hours and then infected with HCMV at an MOI of 1. After 16 hours cells were fixed and stained for IE gene expression as a marker of lytic infection.

Cells stained for viral IE protein expression were quantified by automated fluorescence microscopy and image recognition. A Hermes WiScan (IDEA Bio-Medical) automated microscope was used to image the central 25% of each well (20 images/well), and the resultant images processed via MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices).

Oligonucleotide design for shRNA production

Sequences for shRNA production were designed using the RNAi Consortium from the Broad Institute (https://portals.broadinstitute.org/gpp/public/). The selected siRNA sequence for CLCN2 knockdown was CCTTTGCTGTCATTGGTATTG. To engineer the shRNA vectors the following oligonucleotides were used: CLCN2 (F: GAT CCG CCT TTG CTG TCA TTG GTA TTG TTC AAG AGA CAA TAC CAA TGA CAG CAA AGG TTT TTT G, R: AAT TCA AAA AAC CTT TGC TGT CAT TGG TAT TGT CTC TTG AAC AAT ACC AAT GAC AGC AAA GGC G), Scramble (F: GAT CCG TTC TAA CAT GAC TCT AGT AAT TCA AGA GAT TAC TAG AGT CAT GTT AGA ACT TTT TTG, R: AAT TCA AAA AAG TTC TAA CAT GAC TCT AGT AGT CAT GTT AAG TTC TAA CAT GAC TCT AGT AAT CTC TTG AAT TAC TAG AGT CAT GTT AGA ACG).

Cloning of shRNA-encoding vector

pSIREN-RetroQ vector (a gift from Paul Lehner, Cambridge University), was digested with BamHI-HF and EcoRI-HF in CutSmart buffer and dephosphorylated with Antarctic...
phosphatase as per manufacturer’s instructions (New England Biolabs). Product was subjected to gel electrophoresis and the linear plasmid purified using the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer’s instructions.

Oligonucleotides for cloning into the pSIREN-RetroQ vector were annealed and phosphorylated using T4 polynucleotide kinase (T4 PNK, New England Biolabs). Annealed oligonucleotides were diluted 1:20 with ddH2O. Ligation of annealed primers into linearised plasmid was performed using T4 DNA Ligase (New England Biolabs) as per manufacturer’s instructions. 2μl of the ligation reaction used to transform 25μl of Alpha-Select silver efficiency competent *E. coli* (Bioline) as per manufacturer’s instructions and plated onto LB agar supplemented with 100 μg/ml ampicillin. Single colonies were inoculated into LB media supplemented with 100μg/ml ampicillin. Insertion of desired sequence was confirming by Sanger sequencing (Eurofins).

Production of shRNA-encoding lentiviruses

3μl TransIT-293T (Mirus) was mixed with 0.75μg shRNA-encoding pSIREN-RetroQ vector, 0.5μg pCMV-dR8.91 and 0.25μg pCMV-VSV-G in 100μl Opti-MEM for 20 minutes at room temperature. Mixture was then added dropwise to 70% confluent 293T cells in a 12 well plate with 500μl DMEM+10% FBS. 8 hours later, 500μl DMEM+10% FBS was added. Two days post transfection, supernatant was clarified by centrifugation and stored.

Transduction of HFFs with lentiviral vectors and selection of transduced cells

500μl of lentivirus was added to 60% confluent HFFs in a 12 well plate in 1.5ml total volume. Plates were then spun at 200g for 30 minutes. Two days post transduction, transduced HFFs were selected with 1μg/ml puromycin for two days. Successful knockdown of CLCN2 was confirmed by qRT-PCR.
Nucleic acid isolation and analysis

DNA was extracted by proteinase K digestion. Cells were treated with 100mM KCl, 10mM Tris-HCl and 2.5mM MgCl₂ for 5 minutes prior to the addition of an equal volume of 10mM Tris-HCl, 2.5mM MgCl₂, 1% v/v Tween-20, 1% v/v Nonidet P-40 (Santa Cruz Biotechnology) and 0.4mg/ml Proteinase K (Sigma-Aldrich) for a further 5 minutes. Resultant mixture was heated for 1 hour at 60°C followed by 10 minutes at 95°C.

RNA was extracted using the Qiagen RNeasy kit, with columns from Epoch Life Sciences, as per manufacturer’s instructions. cDNA was then synthesised from 250ng total RNA using the Qiagen Quantitect Reverse Transcription Kit as per manufacturer’s instructions.

Relative quantification by quantitative PCR (qPCR) was performed using PowerUp SYBR green Master mix (ThermoFisher), with forward and reverse primers (250nM), as per manufacturer’s instructions using an Applied Biosystems 7500 Real-Time PCR System (50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, 72°C for 1 minute). Data were analysed by the ddCt method using 18S RNA as a housekeeping gene. The following gene specific primers (Invitrogen) were used: 18S (F: GTA ACC CGT TGA ACC CCA, R: CCA TCC AAT CGG TAG TAG CG), UL138 (F: GAG CTG TAC GGG GAG TAC GA, R: AGC TGC ACT GGG AAG ACA CT), IFIT2 (F: ACT GCT GAA AGG GAG CTG AA, R: TGC ACA TTG TGG CTT TGA AT), IFIT3: (F: AGA AAT GAA AGG GCG AAG GT, R: ATG GCC TGC TTC AAA ACA TC), CXCL10: (F: TGG CAT TCA AGG AGT ACC TC, R: TTG TAG CAA TGA TCT CAA CAC G), CLCN2: (F: GAT CGT GTT CGA GCT CAC AG, R: GCA GGT AGG GCA GTT TCT TG). Absolute quantification by qPCR for viral genome replication was performed using TaqMan Fast Advanced Master Mix (ThermoFisher) with cycling conditions of 50°C for 2
minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Primers against gB were used (F: GAG GAC AAC GAA ATC CTG TTG GGC A
R: TCG ACG GTG GAG ATA CTG CTG AGG) at 100nM final concentration. Product was
detected by TaqMan probe (CAA TCA TGC GTT TGA AGA GGT AGT CCA CG) labelled
with 6-FAM and TAMRA fluorophores. Samples containing known quantities of viral
genomes included to allow for absolute quantification.

Construction of HCMV mutants

HCMV mutants were made by BAC recombineering based on the method of Stanton et al
(44). BACgMmut was generated from BACgMwt (RCMV1778), whilst BAC_RL13trunc
was generated from BAC_RL13wt (RCMV1516). BAC_RL13trunc+gMmut was then
generated from BAC_RL13trunc. Oligo sequences for amplification of rpsL selection
cassette for insertion of gM mutation were: F: GCT CAC TCT TTT TCT TCT CGC GTC
TGC ACC CCA AGC TCA AAG GTA CGG TGC AGT TCC GCA CGC TCA TCG TCA
ACC TGG CCT GTG ACG GAA GAT CAC TTC G and R: CGC ACG AAA AAG TTG
TTT CCG AAG CCG TAG CAC AGG GCC ATG GCT ACC ACG GTG GTG TTG AAA
CCA AGC GCT ACC TCT ACT GAG GTT CT T ATG GCT CTT G. For amplification of
cassette for insertion of RL13 truncation F: ACG CGA TTT GAA TAT AAT ATC ACG
GGA TAT GTT GGC CAA GAA GTG ACT CTA AAC TTT ACT GGA TCA TGG AAT
TAC ATT CCT GTG ACG GAA GAT CAC TTC G R: GTA TGA TAA TTA TTG CTG
GTA ACG GTG CAT ATT GTT GCC GAA TAA AGC CAG CCT GGA GAA CCG
TAC CGG AAC CAT TCT GAG GTT CTT ATG GCT CTT G. Oligo for insertion of gM
mutation was: CCC CAA GCT CAA AGG TAC GGT GCA GTT CCG CAC GCT CAT
CGT CAA CCT GCT AGA GTG AGC GCT TGG TTT CAA CAC CAC CGT GTT AGC
CAT GGC CCT GTG C and for RL13: ATG TTG GCC AAG AAG TGA CTC TAA ACT
Library preparation, deep sequencing and assembly

Illumina sequence libraries were constructed by combining NEBNext® Ultra™ II DNA library prep kit (NEB, E7645) and Agilent SureSelect XT kits. Here 200ng of total DNA, derived from original DNA extracts and supplemented with human DNA (Promega, G1471), was used as input for each sample. DNA was sheared using a Covaris E220 (Peak Incident Power - 175, Duty Factor - 5, Cycle per Burst - 200, Treatment time = 150s) and fragmented DNA used as input for NEBNext End Prep, as described in the manufacturer’s instructions. Adaptor ligation was performed as described in the instructions except for using a 1:10 dilution of the SureSelect Adapter and omitting the USER™ enzyme step. 12 cycles of PCR-based amplification of the end-repaired and adapter-ligated library was carried out, as described in the SureSelect XT Protocol (Version C0, December 2016). Hybridisation-based enrichment of HCMV sequences was undertaken using a custom HCMV bait set (based on HCMV reference strain Merlin) for 24 hrs, as described in the instructions. Sequence libraries were multiplexed and run on an Illumina MiSeq (2x300bp paired-end reads).

Following the sequencing run, paired-end sequence data were demultiplexed and sequence reads trimmed to remove low quality 3’ based and adapter sequences using TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Read pairs were subsequently aligned against the HCMV reference strain Merlin (NC_006273.2) using BBMap (http://sourceforge.net/projects/bbmap/) while duplicate read pairs were removed using MarkDuplicates (http://broadinstitute.github.io/picard) and local realignment performed using IndelRealigner (47). SAM, BAM and mpileup files were parsed using SAMTools v1.0.
(48), VarScan 2 (49) and custom PERL scripts to produce both a consensus sequence for each sample and frequency profile for sites with two or more alleles present.

Generation of DIDS-resistant virus

To generate a DIDS-resistant strain of HCMV, HFFs were infected with the clinical Merlin strain at low MOI in the presence of 10µM DIDS. The virus was allowed to propagate through the cell layer, with the media and DIDS being refreshed every 3-4 days, until all cells exhibited cytopathic effect. Supernatants or physically disrupted cell layers were transferred onto fresh HFFs in the continued presence of DIDS. The process was repeated over a period of 6 months, with the concentration of DIDS gradually escalating to a maximum of 100µM. The concentration of DIDS was then reduced to 25µM to allow for production of sufficient levels of virus for assessment.

Results

On initiation of these studies, our original remit was to test whether cellular ion channel activity played an important role in HCMV replication. A small-scale analysis of broad acting ion channel inhibitors in a single round infection assay revealed one compound, DIDS, was a potent inhibitor of HCMV replication when added 6hpi (Fig. 1A). To identify the basis for the DIDS anti-viral effect we analysed different aspects of the HCMV lifecycle. Surprisingly, this revealed that DIDS was not inhibitory to viral DNA replication nor the formation of putative virion assembly compartments (Fig. 1B,C). Thus by these analyses the viral lifecycle appeared normal yet infectious virion production appeared impaired. Serendipitously, a time of addition study revealed that the pre-treatment of cells with DIDS prior to infection potently inhibited viral infection at the level of initiation of IE gene expression (Fig. 1D,E). Thus, we
considered whether a potential explanation for the apparent lack of infectivity observed in the supernatants analysed in the original screen (Fig. 1A) could be due to the persistence of DIDS in the culture media in the original assays impairing our assay for measuring for the presence of infectious virus in our supernatants. Consistent with this, a dilution of the supernatants from the DIDS treated cells (tested in Fig. 1A) revealed the presence of infectious virus (Fig. 1F) – suggesting that the original effect of DIDS observed was not an impact on replication per se but long term persistence of the inhibitor in the media impacting on the ability of the replicated virus to infect new target cells (which was our surrogate measure of viral replication). We further went on to confirm that DIDS was capable of inhibiting multiple strains of HCMV at the level of IE gene expression, with AD169, Towne (RC256) and TB40/E-UL32-GFP strains all strongly inhibited by DIDS (Fig. 2A). Additionally, this inhibition was not specific to HCMV, with HSV-VP26-GFP infection of HFFs powerfully inhibited by DIDS whether by assessment of ICP4+ cells 4hpi (Fig. 2B) or VP26-GFP+ cells 24hpi (Fig 2C). DIDS additionally inhibited the infection of murine cells by murine cytomegalovirus as quantified 24hpi by staining for IE proteins (Fig 2D).

Having established that DIDS was most active against the early stages of viral entry and the initiation of viral IE gene expression we next sought to understand why it was inhibitory. DIDS is a well-established chloride ion channel inhibitor and thus we tested the ability of other known chloride channel inhibitors to prevent HCMV infection. Unlike DIDS, NPPB and GaTx2 had no impact on infection in the entry assay (Fig. 3A). Furthermore, depletion of the canonical target of DIDS (CIC-2, encoded by the cldn2 gene) from cells by shRNA (validated in Fig. 3B) was similarly not inhibitory to HCMV infection. Moreover, the infection of cells depleted of CIC-2 was still prevented by DIDS (Fig. 3C).
The data pointed towards an event prior to IE gene expression that was targeted by DIDS activity and possibly independent of ion channel activity. To begin identification of the stage of the viral entry pathway impacted by DIDS we analysed the delivery of virion components to the cells (Fig. 4). Immunofluorescent analysis revealed pp65 tegument failed to translocate to the nucleus in the presence of DIDS, demonstrating that an event prior to IE expression was indeed being targeted by DIDS (Fig. 4A,B). Furthermore, fluorescently labelled viruses failed to bind to the surface of the cells in the presence of DIDS (Fig. 4C,D) during infection at +4°C, which otherwise allows for binding of the virus to the plasma membrane but not for efficient entry into the cell. The microscopy data was augmented by an assessment of binding and internalisation of virions by detection of viral DNA by qPCR revealed that not only was viral DNA not internalised into the cell following the promotion of viral entry by warming to 37°C after infection at 4°C in the presence of DIDS (Fig. 4E), but detection of viral DNA bound to the cell surface was greatly reduced by DIDS (Fig. 4F), reinforcing the evidence that DIDS prevents the virus from binding to the cell surface. In support of limited contact between the virion and the plasma membrane was the observation that HCMV induction of interferon stimulated gene expression was absent in cells infected in the presence of DIDS (Fig. 4G). Taken together, these data pointed to an event during the very early stages of viral entry possibly during the initial stages of virus binding. Furthermore, DIDS inhibited the infection of HCMV into multiple cell types arguing the activity of DIDS was against a common event in the entry process (Fig. 5A,B).

An inherent assumption of the study thus far was that DIDS was inhibiting the activity of a cellular function required for entry. However, if cells were pre-treated with DIDS for 24 hours, but then washed, and infected for 1 hour in the absence of DIDS, DIDS’ inhibitory activity on HCMV infection was abolished (Fig. 6A). Two explanations were plausible: the
inhibition was rapidly reversible, or the target was virally encoded. We noted that the DIDS molecule carries a negative charge and thus clear parallels with heparin – a known inhibitor of HCMV entry – were evident. To investigate the impact of charge we took the charged molecule SITS, which is highly related to DIDS (Fig 6B,C) except one isothiocyanate reactive groups is exchanged for an acetamide group and asked if this inhibited HCMV (Fig. 6D). Even extremely high concentration of SITS (~45 times greater than IC₅₀ of DIDS) had only a minor effect on HCMV infection, rendering it far less potent than DIDS, arguing that the basis of DIDS inhibition was not due to charge alone. Furthermore, it suggested the reactivity of the isothiocyanate groups may be responsible as the loss of this group represented the only difference between the two molecules. Biochemical studies have suggested that the presence of dual isothiocyanate groups would render DIDS reactive with lysine and cysteine whereas SITS displays preferential activity towards lysine residues (50).

Thus we hypothesized that the reactive isothiocyanate groups may promote an interaction of DIDS with the virus via cysteine interactions. To test for a virus:DIDS interaction, we incubated HCMV with DIDS prior to infection for 1 hour. The virus was then purified by centrifugation and re-suspended in fresh, DIDS-free media and used to infect cells. The data show that the entry of HCMV was still dramatically reduced (Fig. 6E). Consistent with cysteine reactivity underpinning the impact of DIDS on the virus we observed that the treatment of the DIDS:virus mix with reducing agent β-mercaptoethanol could partially reverse the effects of DIDS and restore infectivity of the DIDS-treated virus (Fig. 6F). Furthermore, the ability of β-mercaptoethanol to reverse the effects of DIDS also demonstrated that DIDS was not anti-viral due to toxicity against the virion. The virions clearly remained viable as they were infectious if the interaction with DIDS was reversed.
Thus the data pointed towards cysteine reactivity in the DIDS molecule as important. The ideal experiment was to generate a version of DIDS with charged sulphonated groups removed. However, the resultant compound proved highly insoluble. An alternative approach used the exchange of the isothiocyanate groups with similarly cysteine reactive maleimide groups which allowed the generation of non-charged, isothiocyanate-like derivatives (Fig. 7A,B). Both these compounds remained inhibitory to HCMV infection although DMIA appeared to display better potency than DMIS (Fig. 7C). Further support for cysteine reactivity being anti-viral was established through the testing of a panel of compounds previously characterized for cysteine and lysine reactivity (Fig 8A). The data show that compounds with cysteine reactivity inhibited HCMV infection under the same experimental conditions as that observed for DIDS (Fig. 8B-E). It was also noteworthy that compounds with dual reactivity (i.e. DIDS and Nbd-Cl) appeared more potent against HCMV than solely cysteine reactive compounds.

To try and derive some biological insight from these studies of DIDS, a DIDS resistant strain was generated with a view to the identification of a viral target for DIDS. Wild type Merlin was passaged in the presence of increasing concentrations of DIDS to isolate a resistant strain. After 6 months a mixed population of Merlin containing a highly DIDS-resistant fraction was produced (Fig. 9A). Interestingly, the DIDS resistant virus population was also resistant to the inhibitory effects of heparin (Fig. 9B) and could infect heparinase treated cells (Fig. 9C), suggesting that the generation of DIDS resistance had altered the entry pathway by circumventing the initial steps of viral entry and HSPG dependence. It was clear that the infectivity of the resistant virus never reached 100% in the presence of DIDS or heparin and thus the heparin and DIDS sensitivity could conceivably be due to different mutants in the virus stock. However, when DIDS and heparin were used in combination no additive impact
on infection with the DIDS resistant virus was observed suggesting that the same fraction was resistant to both (Fig. 9D).

The resistant population of HCMV was sequenced and revealed an enrichment for two mutations – a change from valine to leucine at position 206 in UL100 (gM) and an enrichment for a population containing a premature stop codon at residue 129 in RL13, in comparison to the original wild type virus that contained a raft of different RL13 mutations (Fig. 9E). Given the role of gM in entry and, particularly, events associated with HSPGs this was investigated first. However, the introduction of the gM mutation into a Merlin BAC lacking RL13 had no impact on the sensitivity of the virus to DIDS (Fig. 9F). In contrast, introduction of the mutation that generated the truncation in RL13 identified in the sequencing did provide partial evidence of a resistance phenotype although only a minor impact was observed in comparison to the original DIDS resistant virus (Fig. 9G). However, it was intriguing that both RL13+ and RL13- viruses were similarly sensitive to DIDS whereas the RL13 truncation did have slightly more resistance to the effects of DIDS (Fig. 9F) at specific DIDS concentrations suggesting the specific expression of the truncated form of RL13 may play a role. Finally, we generated the double mutant but yet again no overt phenotype was observed that would explain the DIDS resistance of the original DIDS resistance strain (Fig. 9H). However, we were intrigued to note that any benefit regarding resistance to DIDS proffered by the RL13 truncation were abrogated when the gM mutation was introduced concomitantly (Fig. 9H).

Given that the genetic mutants made in the Merlin BAC viruses did not map the generation of DIDS-resistant virus to a specific gene, we tested the hypothesis that the virus could acquire
resistance to DIDS by altering the particle:infectious virus ratio. The rationale being that the provision of more targets for DIDS to bind to may thus lower the effective DIDS concentration available for inhibiting the infectious particles. Consequently, we compared the DNA content:infectious virus ratio for the DIDS-resistant virus against the wildtype, as well as comparing the ratios for BAC_RL13wt, BAC_RL13trunc and BAC_RL13neg. Although no dramatic differences in the ratios were observed we note that the data suggest that the DIDS resistant virus displayed an approximately 2 fold increase in infectivity (Fig. 10A). In contrast, in the BAC mutants the ratios did not show any consistent variation that correlated with DIDS resistance (Fig. 10B).

Although the genetic basis of the resistance was not unequivocally revealed we were intrigued by the observation that HCMV could be forced by DIDS to mutate in vitro to enrich for a fraction of virions that are HSPG independent for entry. This led us to consider the molecular and evolutionary basis for the retention of this interaction in wild type HCMV strains in vitro and, more pertinently, in vivo. Complexity in the entry pathways of a number of viruses has been hypothesized to provide a mechanism of immune evasion. Essentially, there is an evolutionary trade-off between efficient and rapid delivery of the viral cargo versus the effective evasion of humoral immune responses. Consequently, the concept of conformational masking – whereby the virus only exposes key epitopes required for entry at specific stage of the process – has developed. The key tenet is that conserved epitopes essential for entry cannot tolerate extensive mutation as a mechanism to evade immune surveillance. These epitopes are instead hidden from the immune response for as long as possible to limit opportunity for immune recognition. Thus we wished to test whether resistance to DIDS, and by extension heparin, was met with a selective disadvantage for the virus via a failure to evade humoral immunity. Both wild type and DIDS-resistant virus was
incubated with pooled sera from seropositive or seronegative donors and then infectivity measured. The data show that whilst both wild type and resistant viruses are sensitive to antibody neutralisation, a titration of seropositive sera reveals an increased sensitivity of the original DIDS resistant virus to neutralization at higher dilutions of sera (Fig. 11A,B). Next, the sensitivity of the mutant BAC viruses was tested to identify whether increased sensitivity could be explained by RL13 or gM (Fig. 11C,D). Here the BAC generated mutants presented with a complicated phenotype. The expression of the gM mutant in a RL13 null background rendered the virus more sensitive to neutralization (Fig. 11D). The introduction and expression of the RL13 truncation did not have a consistent effect on the antibody sensitivity phenotype on the wild type virus (Fig. 11C) and also did not additionally contribute the gM phenotype (Fig. 11D).

To test the hypothesis that the DIDS resistant virus was intrinsically more sensitive to immune responses we asked whether the DIDS resistant virus was rendered generally more susceptible to additional immune activity that is active during the early phases of viral infection. However, in contrast to the studies with humoral immunity, both wild type and DIDS resistant viruses were equally sensitive to the anti-viral activity of type I interferons in IFN pre-treated HFFs (Fig. 12).

**Discussion**

HCMV entry is a complex process that requires a concert of viral glycoprotein:cell surface interactions to occur. Although the list of specific receptors that facilitate HCMV entry continues to grow the initial interaction of HCMV with HSPGs is a well-established interaction important for viral entry (2). This initial binding via HSPGs is a common event
for the entry process of many viruses and has long been considered a mechanism to promote co-localisation of the virion with the plasma membrane which potentially could enhance infectivity (16–18). Thus an anti-viral strategy that prevents this initial step represents an attractive strategy particularly given the role of this interaction for the infectivity of many enveloped viruses. However, it is less well understood whether the virus garners any additional benefits from this interaction over and above the concept that HSPGs provide a means to promote initial virus engagement with the cell and thus could mutate rapidly into a HSPG-independent strain of virus as a mechanism to evade the activity of anti-virals.

Here we report that DIDS is inhibitory to HCMV infection through a blockade of the ability of the virus to bind and engage with the plasma membrane. From a translational standpoint, inhibitors of viral entry have long represented an attractive focus for new anti-virals. Although DIDS carries a negative charge it became clear that the cysteine reactivity played an important role in its anti-viral activity. Indeed, a number of cysteine reactive compounds appeared to inhibit HCMV infection. A simple explanation could be the binding of DIDS (and other cysteine reactive compounds) to the virions provides steric hinderance. In that case, it is perhaps surprising that lysine reactive compounds cannot achieve the same effect since viral glycoproteins encode for multiple lysine residues. The relative accessibility of the lysines versus cysteines may explain the difference although lysine is an amphipathic amino acid and thus can be found buried within the protein structure and simultaneously can decorate the protein surface (51, 52).

Studies of hepatitis delta virus (HDV, which incorporates HBV glycoproteins in the virion) have shown that cysteine residues play an important role in infection due to conformational
changes they promoted via an ability to catalyse thiol/disulfide exchange (53). As such, compounds that prevented thiol/disulfide activity blocked HDV infection. A number of HCMV glycoproteins contain substantial numbers of cysteines and thus this may invoke a model whereby DIDS is decorating the virion surface and possibly preventing similar cysteine driven activities. HCMV glycoproteins have been shown to undergo conformational changes during the entry process and possibly cysteine residue activity plays a role in this rendering cysteine reactive compounds more potent as anti-virals (30).

However, any cysteine targeted activity does not rule out that the charge associated with DIDS could also play a role to augment its activity against HCMV especially since charge is considered the basis of the anti-viral activity of heparin against HCMV. Binding of DIDS to the virus may introduce a localized negative charge that contributes to an inhibition of infection. Furthermore, it is likely that the charge carried by the DIDS molecule is responsible for the lack of cellular and in vivo toxicity observed with DIDS (54). It was evident in cultures that a number of cysteine reactive derivatives which did not carry charge were far more toxic than DIDS – despite the fact DIDS also carries highly reactive isothiocyanate groups. We hypothesise that DIDS is poor at crossing cell membranes due to the presence of the charged sulphonated groups. This prediction would be borne out by the original screen that suggested DIDS persisted in the extracellular media for 5 days evidenced by its ability to diminish the infectivity of newly released viruses after a single round of replication. Of course, inability to cross membranes comes with a cost for any potential therapeutic derivatives of DIDS as their delivery would likely require strategies that facilitate transport across the epithelial cell membranes if administered orally, for example. Furthermore, it may limit the impact of DIDS-like compounds against cell-associated HCMV if DIDS enters the cell inefficiently. On the other hand, a large difference between CC₅₀ and
EC\textsubscript{50} provides a good therapeutic index which is always a desirable property in any pharmacological intervention particularly when the transitioning from cell culture to in vivo models.

It was disappointing that a single mutation was not evident to fully explain the resistance phenotype. The RL13 truncation had a partial phenotype but did not fully re-capitulate the original DIDS resistant virus phenotype. It is important to note that the partial phenotype may have been influenced by the requirement to grow RL13trunc- or RL13wt-expressing viruses in HFF cells, in order to allow for expression of RL13. One potential issue is the backbone of the viruses used. The original resistant virus was generated using wild type Merlin which had already been passaged in the lab and is likely more heterogeneous as a virus stock. In contrast, the RL13 truncation was generated in the BAC Merlin which is likely to be genetically more homogeneous than the original Merlin virus used. For example, the RL13 phenotype maybe enhanced by minor mutations accumulating in the Merlin through culture in vitro and thus resistance via RL13 is enhanced by other, yet unidentified, polymorphisms. Essentially, the accumulation of the RL13 and gM mutations in the original resistant virus suggests these are important for resistance but their impact is underpinned by fixed mutations present in our passaged Merlin stocks that have been acquired over time and are absent from the BAC virus (which has been repaired to reflect the original consensus sequence derived from clinical studies). Certainly, the data do suggest that the BAC derived viruses are intrinsically more sensitive to DIDS with inhibition of infection seen at much lower concentrations of DIDS when compared to non-BAC derived Merlin stocks. This would argue that the original Merlin is carrying fixed SNPs that contribute to the resistance phenotype. It also raises a tangential but important question when considering potential potency of new therapeutics. Specifically, how diverse are HCMV populations in vivo and,
consequently, is the relative genetic homogeneity associated with BAC viruses completely
reflective of the in vivo situation and, potentially, do they give unfair or even
unrepresentative measures of potency in vitro.

An important aspect of the pharmacological data is that the EC\textsubscript{50} for both resistant and wild
type virus were very similar. The reason for this is that it was a fraction of virus within in the
DIDS resistant virus that was responsible for the resistance rather than an overall increase in
the resistance of the HCMV viruses to DIDS. This would support a concept that a single
mutant identified in >90% of viruses cannot re-capitulate the phenotype and partially explain
the lack of phenotype in the re-engineered RL13 and gM viruses. Indeed, we revisited the
sequencing data to identify whether there was evidence of enrichment for another genetic
mutation around 10-15% but this was not evident. Thus we hypothesise this may point to the
acquisition of multiple mutations alongside pre-determined SNPs that generate the DIDS
resistant sub-population that accumulates in the culture. Indeed, attempts to expand the
frequency of resistance in the DIDS resistant virus have not resulted in the accumulation of a
higher frequency of DIDS resistant viruses.

Finally, a serendipitous aspect of the data was the suggestion HCMV can be driven to mutate
to infect cells in an HSPG-independent manner yet, to date, all wild type strains of HCMV
tested utilize HSPG binding during the entry process. Thus the retention of this activity
argues that, from an evolutionary aspect, HSPG interactions are indeed important. One
hypothesis we tested was whether increased complexity in the entry process via engagement
with multiple receptors may promote reduced recognition by the humoral immune response.
This idea, termed conformational masking, is not unique to HCMV and was established
through studies of HIV whereby the virus engages with CD4 before secondary interaction with CCR5 or CXCR4 with the important consequence of reducing antibody recognition and neutralisation of CXCR4/CCR5 binding epitopes of HIV gp120 (55). Although preliminary data did suggest increased sensitivity to humoral immunity and this did map functionally to a mutation in the HSPG binding protein, gM, whether this represents increased sensitivity to anti-gM antibodies or just changes in the virion proteome induced by the gM mutation alone is not clear. Paradoxically, this mutation alone did not explain the DIDS resistance so it is not clear whether the two events (DIDS resistance and antibody sensitivity) are inherently linked. However, it is possible if the mutation is one of multiple events that contribute to the DIDS resistance then it would provide the link. Experimentally, repairing the gM mutant in the original DIDS resistant virus would address this but first requires a complete understanding of the genetic basis of the DIDS phenotype in the first place.

In summary, we report a novel anti-viral activity of putative chloride ion channel inhibitor active against HCMV during the initial stages of entry. DIDS’ inhibitory activity is dependent on a reversible cysteine reactivity driven by isothiocyanate chemical groups and not through the targeting of host chloride ion channel function. Intriguingly, identification of a DIDS resistant virus revealed that HCMV can mutate to utilize an HSPG independent mechanism of entry and results in a concomitant increase in sensitivity to antibody neutralisation. However, it remains to be determined whether the genetic basis for DIDS resistance is responsible for the acquired antibody sensitivity. The data also illustrate that cysteine-reactive compounds have potential as novel antivirals through an ability to bind and inhibit HCMV virions to prevent the binding and entry into target cells.
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**Figure 1: DIDS inhibits HCMV prior to IE gene expression.** (A): HFFs were infected at MOI=5 and treated with ion channel inhibitors 24hpi. Five dpi, supernatants were used to inoculate fresh HFF monolayers, which were immunostained for viral IE expression 24h later. Infection was assessed by automated fluorescence microscopy and IE forming units/ml calculated (n=3). (B): HFFs were infected at MOI=3. 24hpi, cells were either harvested for DNA (Input) or treated with indicated compounds. At 96hpi, DNA was harvested from all conditions and HCMV genome copies/ml assessed by qPCR (n=2). (C,D): HFFs were infected at MOI=3 and treated with indicated compounds 24hpi. Three dpi, cells were fixed and stained for viral pp28 expression (C) and pp28+ foci counted manually (D) by fluorescence microscopy (n=2). (E,F): HFFs were treated for 1h at a range of DIDS concentrations or controls prior to MOI=3 infection. Cells were fixed 24hpi, stained for viral IE (E) and counted (F) by automated fluorescence microscopy (n=3). (G): HFFs infected at MOI=3 were treated with DMSO, 50µM DIDS or 10µM DIDS 24hpi. Five dpi, supernatants were used to infected fresh HFF monolayers at varying dilutions, which were fixed 24hpi, immunostained for IE proteins and IE forming units/ml calculated as previously (n=2). P values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test where appropriate. *** = p < 0.001, ** = p < 0.01, * = p < 0.05, ns = p > 0.05.

**Figure 2: DIDS inhibits multiple HCMV strains in addition to HSV-1.** (A): HFFs were treated for 1h at a range of DIDS concentrations or controls prior to MOI=3 infection with Merlin, RC256 (Towne), AD169 or TB40/E-UL32-GFP strains of HCMV. Cells were fixed 24hpi, stained for viral IE and counted by automated fluorescence microscopy (n=3). (B,C): HFFs were treated for 1h with 50µM DIDS or DMSO prior to MOI=1 infection with HSV-1-VP26-GFP. Cells were fixed either (B) 4 hpi and stained for ICP4 expression and ICP4+ cells
quantified (n=1) or (C) 24hpi and VP26-GFP+ cells quantified (n=1). (D) 3T3 cells were treated for 1h at a range of DIDS concentrations or controls prior to MOI=3 infection with mCMV stain Smith in the absence of serum. Cells were washed 1hpi and DIDS-containing media refreshed. Cells were fixed 24h, stained for viral IE proteins and counted by automated fluorescence microscopy (n=1).

**Figure 3: Alternative chloride ion channel inhibitors or knockdown of the major target of DIDS do not inhibit HCMV infection.** (A): HFFs were treated with NPPB or GaTx2 at a range of concentrations for 1h prior to infection at MOI=3. After 24h, cells were fixed and immunostained for viral IE proteins and infection quantified as previously (n=3). (B): Levels of CLCN2 RNA in HFFs transduced with shScramble or shCLCN2-expressing lentiviral vectors were assessed by qRT-PCR. (C): shScramble or shCLCN2 transduced HFFs were treated with DMSO or 20µM DIDS for 1h prior to infection at MOI=3. Infection was assessed by IE immunostaining 24hpi as previously (n=2). P values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test or Mann-Whitney test where appropriate. * = p < 0.05, ns = p > 0.05.

**Figure 4: DIDS prevents engagement of HCMV with the cell surface, and the infection of multiple cell types.** (A,B): HFFs were treated with indicated compounds prior to infection at MOI=2. 30 minutes post infection, cells were fixed and immunostained for viral pp65. Representative images are shown in (A), and enumeration by automated fluorescence microscopy in (B) (n=2). (C,D): HFFs pre-labelled with Hoechst were infected at MOI=5 with TB40/E-UL32-GFP in the presence of 50µM DIDS for 1h at 4°C. Cells were then washed and visualised by confocal microscopy. Representative images (C) and quantification (D) are provided. (E,F,: HFFs were infected at MOI=2 at 4°C for 1h in the presence of the...
indicated compounds. Cells were then washed and DNA extracted immediately (E) or were returned to 37°C for 30 minutes prior to DNA extraction (F). Relative levels of HCMV DNA were assessed by qPCR (n=3). (G): HFFs were infected at MOI=2 in the presence of indicated compounds. Six hpi, RNA was extracted and induction of CXCL10, IFIT2 and IFIT3 assessed by qRT-PCR (n=3). P values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test or Mann-Whitney test where appropriate. *** = p < 0.001, ** = p < 0.01, * = p < 0.05.

Figure 5: DIDS inhibits the infection of multiple cell types by HCMV. (A,B): U373 cells (A) or RPE-1 cells (B) were pretreated for 1h with a range of concentrations of DIDS or controls prior to MOI=3 infection. 24hpi, cells were fixed and infection assessed by IE immunostaining as previously (n=3).

Figure 6: DIDS inhibits HCMV infection by targeting the virion. (A): HFFs were either untreated or treated with DMSO or 50µM DIDS for 24h. Compound was removed from pretreated cells, untreated cells had either 50µM DIDS or DMSO added and all cells were infected at MOI=2 for 1h. Cells were then washed, and media only added to all cells. Infection was quantified by IE immunostaining 24hpi as previously (n=2). (B): Structure of DIDS, 4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid. (C): Structure of SITS, 4-acetamido-4’-isothiocyanato-2,2’-stilbenedisulfonic acid. (D): HFFs were treated with 25µM DIDS or a range of concentrations of SITS for 1h prior to MOI=2 infection. Infection was quantified by IE immunostaining 24hpi as previously (n=3). (E): HCMV was treated with indicated compounds for 1h. Samples were then pelleted by centrifugation, resuspended in media only and used to inoculate HFFs. Eight hpi, cells were immunostained for IE proteins and infection quantified as previously (n=3). (F): Virus was treated with 25µM DIDS, DMSO or
media only for 1 hour. DIDS/DMSO treated virus was then either treated with 100mM βME or left untreated (βME-) for 10 minutes. All preparations were then diluted, and previously untreated virus had sufficient DIDS or DMSO added (‘Spike’) to create the same final concentration as present in those samples pretreated with either DIDS or DMSO. Virus was then used to inoculate HFFs at MOI=1, with cells stained 24hpi and infection quantified as previously (n=2). *P* values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test or Mann-Whitney test where appropriate. *** = *p* < 0.001, ** = *p* < 0.01, * = *p* < 0.05, ns = *p* > 0.05.

**Figure 7:** Cysteine-reactive compounds structurally related to DIDS inhibit HCMV.  
(A): Structure of DMIS, 4,4’-dimaleimidylstilbene. (B): Structure of DMIA, 4,4’-dimaleimidylazobenzene. (C): HCMV was treated with indicated compounds for 1h. Samples were then pelleted by centrifugation, resuspended in media only and used to inoculate HFFs. Twenty-four hpi, cells were immunostained for IE proteins and infection quantified as previously. *P* values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test. *** = *p* < 0.001, * = *p* < 0.05, ns = *p* > 0.05.

**Figure 8:** HCMV infection is sensitive to the activity of structurally unrelated cysteine-reactive compounds. (A): Summary of reactivities of tested compounds. (B,C,D,E): HCMV was treated with indicated compounds (B: 2-Iminothiolane, 2-IT, C: 5,5’-dithiobis(2-nitrobenzoic acid), Nbs₂, D: 4-chloro-7-nitrobenzofurazan, Nbd-Cl, E: N-ethylmaleimide, NEM) for 1h. Virus was then pelleted, resuspended in media only and used to inoculate HFFs. Twenty-four hpi, cells were immunostained for IE proteins and infection enumerated as previously. *P* values calculated by Kruskal-Wallis test with Dunn’s multiple comparison’s test where appropriate. *** = *p* < 0.001, ** = *p* < 0.01, * = *p* < 0.05, ns = *p* > 0.05.
Figure 9: Virus passaged in the presence of DIDS additionally develops resistance to heparin and cellular heparan sulfate depletion. (A,B): DIDS-passaged HCMV (‘Resistant’) and the virus from which it was derived (‘Wildtype’) were used to infect HFFs at MOI=2 in the presence of either DIDS (A) or heparin (B). Cells were immunostained for IE 24hpi and infection enumerated as previously (n=3). (C): HFFs were treated with heparinase I or buffer only for 1h at 37°C. Cells were then infected at MOI=2 with ‘Resistant’ or ‘Wild type’ HCMV, followed by IE immunostaining 24hpi and infection was enumerated as previously (n=2). (D): HFFs were infected with ‘Resistant’ HCMV at MOI=2 in the presence of increasing concentrations of heparin in addition to either 10µM DIDS or DMSO. Infection was quantified 24hpi by IE immunostaining as previously (n=2). (E): Summary of mutations found to be enriched within DIDS-resistant HCMV by sequencing. (F,G,H): BAC-derived viruses lacking RL13 with wildtype gM (BACgMwt), lacking RL13 with mutant gM (BACgMmut), possessing wildtype gM with either wildtype RL13 (BAC_RL13wt), truncated RL13 (BAC_RL13trunc) or lacking RL13 (BAC_RL13neg), or possessing both truncated RL13 and mutant gM (BAC_RL13trunc+gMmut) were used to infected HFFs at MOI=1 in the presence of DIDS. After 24h, cells were fixed and immunostained for viral IE proteins and infection quantified as previously (n=3). P values calculated by Mann-Whitney or Kruskal-Wallis test with Dunn’s multiple comparison’s test where appropriate. *** = p < 0.001, ** = p < 0.01, * = p < 0.05, ns = p > 0.05.

Figure 10: DIDS resistant viruses display a moderate increase in infectivity
HFFs were infected with serial dilutions of (A) wildtype and DIDS-resistant HCMV or (B) BAC_RL13wt, BAC_RL13trunc and BAC_RL13neg in the presence of media only. Simultaneously, samples of virus were pelleted by centrifugation and DNA extracted. HFFs
were immunostained at 24hpi for IE proteins, infection was enumerated and IE forming units/ml calculated for each virus. Quantitative PCR for viral genomes was performed on the viral DNA samples and DNA (arbitrary units)/ml calculated. DNA/ml was then divided by IE forming units/ml to produce a ratio of genomes:infectious units for each virus.

**Figure 11: The DIDS-resistant virus is sensitive to humoral immunity.** (A,B): DIDS-resistant or wildtype HCMV was treated with the indicated dilution of either pooled seropositive (A) or pooled seronegative (B) serum for 1h. Treated virus was then used to infect HFFs at MOI=1, and cells were immunostained 24hpi for IE proteins and infection enumerated as previously (n=2). *** = p < 0.001. (C,D): BAC-derived viruses expressing variations of RL13 (C) or the RL13 truncation & gM mutation in all combinations (D) were treated with the indicated dilution of pooled seropositive serum for 1h. Treated virus was used to infect HFFs at MOI=1, and cells immunostained 24hpi for IE proteins and infection enumerated as previously (n=3). P values calculated by 2-way ANOVA with Tukey’s multiple comparisons test. ### = p < 0.001 for BACgMwt vs BACgMmut, * = p < 0.05 for BACgMwt vs BAC_RL13trunc+gMmut.

**Figure 12: DIDS-resistant virus is not sensitised to anti-viral type I interferons activity.** HFFs were treated for 3h with the indicated quantity of IFNα/β prior to infection at MOI=1. Twenty-four hpi, cells were fixed and immunostained for IE, and infection quantified as previously (n=2).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
A

| Compound | Cysteine Reactive | Lysine Reactive |
|----------|------------------|-----------------|
| DIDS     | Yes              | Yes             |
| SITS     | No               | Yes             |
| 2-IT     | No               | Yes             |
| Nbs$_2$  | Yes              | No              |
| Nbd-Cl   | Yes              | Yes             |
| NEM      | Yes              | No              |

B

Figure 8
Figure 9
Figure 12