Identification of lead anti-human cytomegalovirus compounds targeting MAP4K4 via machine learning analysis of kinase inhibitor screening data

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Abstract

Chemogenomic approaches involving highly annotated compound sets and cell based high throughput screening are emerging as a means to identify novel drug targets. We have previously screened a collection of highly characterized kinase inhibitors (Khan *et al.*, Journal of General Virology, 2016) to identify compounds that increase or decrease expression of a human cytomegalovirus (HCMV) protein in infected cells. To identify potential novel anti-HCMV drug targets we used a machine learning approach to relate our phenotypic data from the aforementioned screen to kinase inhibition profiling of compounds used in this screen. Several of the potential targets had no previously reported role in HCMV replication. We focused on one potential anti-HCMV target, MAPK4K, and identified lead compounds inhibiting MAP4K4 that have anti-HCMV activity with little cellular cytotoxicity. We found that treatment of HCMV infected cells with inhibitors of MAP4K4, or an siRNA that inhibited MAP4K4 production, reduced HCMV replication and impaired detection of IE2-60, a viral protein necessary for efficient HCMV replication. Our findings demonstrate the potential of this machine learning approach to identify novel anti-viral drug targets, which can inform the discovery of novel anti-viral lead compounds.

Introduction

Identification of viral and cellular proteins required for virus replication can be a critical step in the discovery of novel anti-viral targets. A number of genetic methods are available to screen infected cells to identify proteins required for virus replication. These include the screening of infected cells using siRNA [1-7] or CRISPR/Cas9 [8-10] and analysis of infected haploid cells treated with "gene trap" retroviruses [11-17]. In genetic experiments, knock down or knock out of a factor in a screen can directly identify the factor required for virus replication. However, the factors required for viral replication identified in these screens may not be pharmacologically tractable ("druggable") with small molecules. Also, genetic depletion of a protein and pharmacological inhibition of a single catalytic domain in that protein may have divergent phenotypic consequences [18].

Screening collections of compounds can directly identify small molecules with anti-viral activity. However, if the target of the compound "hits" from these screens is unknown, it is not always possible to either effectively utilize medicinal chemistry to develop more effective compounds that share the same target, or not always possible to directly identify known drugs with the same target. This can be further complicated if the compounds screened display promiscuity, as is the case of most kinase inhibitor compounds. This complexity often makes it difficult to provide novel observations regarding mechanisms of virus replication from analysis of the biochemical profiles of screened compounds.

Genetic and compound screening has been extensively used to find drug targets and drugs for viruses of clinical importance that have few therapeutic options. An

example of this is human cytomegalovirus (HCMV). HCMV is a prominent cause of morbidity and mortality in a number of patient populations [19]. There is currently no widely available vaccine [20] and available anti-HCMV drugs (such as ganciclovir, valganciclovir and foscarnet) have many short-comings, including toxicity and viral drug resistance [21,22]. Several anti-HCMV drugs are in clinical trials, but may have similar shortcomings to those anti-HCMV drugs currently available [23-27]. Therefore, there is a necessity to identify and develop novel anti-HCMV compounds to improve patient outcomes.

Previously, we and others have used large scale genetic screening of siRNAs to identify factors required for HCMV replication [28-31]. This approach has had limited success in identifying pharmacologically tractable anti-viral targets. Therefore, as an alternative to genetic screening for drug targets we pursued a chemogenomic approach and screened collections of kinase inhibitors to identify those with anti-HCMV activity [32-34]. Many of the active kinase inhibitors typically displayed promiscuity [32-35] and often it was not possible to efficiently mine the data from our screens to understand which inhibited kinases were driving anti-viral activity. To identify drug targets from our screening data we revisited our analysis of a screen [33] using the GlaxoSmithKline (GSK) Published Kinase Inhibitor Set (PKIS) [36] and employed a machine learning approach [37] to analyze the relationship between the phenotypic data from our screen and the kinase inhibition profiles of the compounds used in the screen. From this analysis we identified a number of potential drug targets and investigated lead compounds targeting the kinase MAP4K4, whose function in HCMV replication was unknown.

Materials and methods

Machine learning analysis of kinase inhibitor screening data

Each component of the machine learning analysis described in the Results section has been previously reported [37] and was carried out at the University of Miami using Support Vector Machines. Please contact Hassan Al-Ali for information on all aspects of the machine learning analysis.

Viruses and Cells

HCMV strains AD169 and Merlin (RCMV1111) [38] were generously provided by Don Coen (Harvard Medical School) and Richard Stanton (Cardiff University), respectively. Human foreskin fibroblast (HFF) cells (clone Hs29) were obtained from the American Tissue Culture Collection.

Western blotting

HFF cells were infected at the MOI indicated in each Figure or prepared for analysis at the time of infection. After washing with PBS, cells were resuspended in Laemmli buffer containing 5% β-mercaptoethanol. Proteins were separated on 8% polyacrylamide gels. Membranes were probed with antibodies recognizing IE1/2, (Virusys, 1:1000 dilution), IE2 proteins (clone 5A8.2, Millipore, 1:1000 dilution),

MAP4K4 (ab155583, Abcam, 1:500 dilution) and □-actin (SIGMA, 1:5000 dilution). All primary antibodies were detected using anti-mouse- or anti-rabbit-horseradish peroxidase (HRP) conjugated antibodies (Millipore and Cell Signaling Technology, respectively). Chemiluminescence solution (GE Healthcare) was used to detect secondary antibodies on film. Where necessary blots were striped and re-probed. Relative band intensity (band intensity relative to β-actin signal in the same lane) was analyzed using ImageJ software obtained from the National Institutes of Health (USA). Thusly, in lanes where relative band intensity was analyzed, densitometry was used to calculate the percentage difference in band intensity between β-actin bands in those lanes. The percentage difference in band intensity for specific proteins in those lanes was then calculated. Specific protein band intensity was divided by β-actin intensity to calculate relative band intensity.

Treatment of cells with siRNA and infection of transfected cells

Twenty four hours before transfection 1 x 10^5 HFF per well were seeded in 12-well plates in media with no antibiotics. siControl Non targeting siRNA #3 (D-001810-03-05) or ON-TARGETplus or SMARTpool MAP4K4 siRNA (L-003971-00-0005) (both Dharmacon/GE) were used. Per well, 113 µl of 1 µM siRNA and 2 µl Dharmafect2 (Dharmacon/GE) were diluted in 93 µl and 146 µl OptiMEM (Invitrogen), respectively. After 5 mins at room temperature, both solutions were combined. After 20 mins, media was removed from each well and replaced with the siRNA/Dharmafect mixture, then

500 µl of media with no antibiotics was added to each well. Transfected cells were incubated at 37°C for 72 hours then used as indicated in the text.

Compounds

PF06260933 dihydrochloride [39] was purchased from Bio-Techne (Minneapolis, MN, USA). Ganciclovir was purchased from SIGMA, UK). JNK-8-IN was a kind gift from Nathanael Gray (Harvard Medical School). A 4-Amino-pyridopyrimidine compound, here designated CA409, was synthesized as previously reported [40]. All compounds were resuspended in dimethyl sulfoxide (DMSO).

Viral yield reduction assays

HFF cells (5×10^4 per well) were incubated overnight and infected at an MOI of 1. Virus was adsorbed to cells for 1 hour at 37°C and then infected cells were incubated with 0.5 ml of media containing DMSO or compound at a range of concentrations in duplicate. Plates were incubated for 72 hours at 37°C. The final concentration of DMSO in all samples was maintained at <1% (v/v).Viral titre (plaque forming units (p.f.u.) per ml) was determined by titration of viral supernatants on HFF monolayers. The mean value of duplicate plaque counts was determined and the percentage of viral titre in the presence of compound compared to control was calculated. To determine ED_{50} values, parentage inhibition versus compound concentration was plotted using Microsoft Excel

and a linear fit model was used to determine the concentration at which virus yield was reduced by 50%.

MTT assays

HFF cells (1×10^4 per well) were incubated overnight and then treated for 72 hours with either DMSO or compound at range of concentrations (2 fold dilution series starting at 50 μM) in duplicate. Relative cell number was then determined with an MTT assay according to the manufacturer's instructions (GE Healthcare). The mean value of duplicate readings was determined and the percentage of assay output in the presence of compound compared to DMSO was calculated. The final concentration of DMSO in all samples was maintained at <1% (v/v). As a positive control, in all experiments a 2-fold dilution series of HFF cells starting at 1×10^4 cells per well was included. In each experiment we found a linear relationship between the number of cells per well and output from the MTT assay (data not shown).

Results

Collection and organization of kinase inhibitor screening data for machine learning analysis

Previously, we devised a cell based high throughput methodology [33] to screen the GSK PKIS collection of kinase inhibitors [35,36] for their ability to increase or decrease the expression of a viral protein, pp28, in cells infected with HCMV high passage strain AD169. After excluding screened compounds for toxicity effects [33], we interpreted the results of our screen [33] as a z-score [41,42], where a positive or negative z-score represented an increase or decrease, respectively, in the number of pp28 positive cells in the presence of each compound. The z-score for each compound is shown in Fig 1A, where each bar represents the z-score of a single compound..

Fig 1. Analysis of hit and anti-hit classes of screening data. (A) z-scores from screening of GSK PKIS collection (version 1) [33], where each bar represents a single compound. (B) Heatmaps of kinase inhibition profiling of compounds grouped from Hit and Anti-Hit classes. The potency of each compound at 1 □M concentration against a particular kinase is represented in colour (less than 0% inhibition – blue, 0-50% inhibition – green, 51-75% inhibition – yellow, 76-90% inhibition – orange, greater than 91% inhibition – red). Each row represents a kinase tested and each column represents a compound. (C) Schematic of machine learning analysis of Hit and Anti-hit kinase inhibition profiles. (D) Table of machine learning outputs in which pharmacologically

190 linked kinase groups are listed with their MAXIS and Bk scores. Abbreviations in the
191 table: ALK: Anaplastic lymphoma kinase, CAMK2: Calcium/calmodulin-dependent
192 protein kinase type II subunit, CHEK2: Checkpoint Kinase 2, CLK: CDC-like kinase,
193 CSNK1G1: Casein Kinase 1 Gamma 1, DYRK: Dual specificity tyrosine-
194 phosphorylation-regulated *kinase,* EPHA: Ephrin type-A receptor, HIPK4:
195 Homeodomain Interacting Protein Kinase 4, IGF1R: Insulin-like growth factor 1
196 receptor, INSR: Insulin receptor, INSRR: Insulin Receptor Related Receptor, *JNK:* c-Jun
197 N-terminal *kinase,* LTK: Leukocyte Receptor Tyrosine Kinase, MAPK: Mitogen-
198 activated protein kinase, MAP4K4: Mitogen-Activated Protein Kinase Kinase Kinase
199 Kinase 4, MINK1: Misshapen Like Kinase 1, NTRK: TKR receptor kinase, PHKG:
200 Phosphorylase Kinase Catalytic Subunit Gamma, PRKD: Serine/threonine-protein
201 kinase, TNIK: TRAF2 And NCK Interacting Kinase, TRAF2: *TNF Receptor Associated*
202 *Factor 2,* TSSK: Testis Specific Serine Kinase.
203
204 The GSK PKIS collection has been extensively characterized [35], including each
205 compounds kinase inhibition profile; biochemical analysis of each compounds ability to
206 inhibit 224 human kinases using *in vitro* assays. The kinase inhibition profiles of PKIS
207 compounds demonstrated that nearly every compound in this collection displayed some
208 degree of promiscuity [33,35]. To deconvolute the kinase inhibition profiles of
209 compounds and identify kinases that inhibit HCMV replication, we subjected our GSK
210 PKIS screen data to a machine learning algorithm that was previously developed and
211 validated in a mammalian screening system [37].

The kinase inhibition profiles of compounds with z-scores between <-0.75 ("hit class") and >0.25 ("anti-hit class") were selected. Profiles of compounds with z-scores between < -0.75 and > 0.25 were chosen to ensure a separation of at least 1 between the hit and anti-hit classes and to ensure the profiles that potentially had the most information were analyzed. The aforementioned kinase inhibition profiles are shown in Fig 1B, where the heatmap indicated the potency of kinase inhibition (each row represents a kinase tested and each column represents a compound). The selected kinase profiles in Fig 1B were analyzed using a maximum relevance (MR) algorithm [37] to identify kinases whose inhibition in both classes had the highest information content (Fig 1C). Thus, the MR analysis was able to produce a list of kinase proteins most likely related to either inhibition or promotion of HCMV protein production.

Identification of potential drug targets within pharmacologically linked kinase groups

From the kinases selected by MR analysis, a greedy backwards feature selection algorithm using support vector machines (SVM) [37] was then used to identify the minimum number of kinases whose inhibition was highly predictive of HCMV protein production inhibition (Fig 1C). These kinases were referred to as the Maximum Information Set (MAXIS). Closely related kinases can have similar inhibition profiles, termed "pharmacological linkage". Therefore, the MAXIS kinase proteins were grouped as pharmacologically linked kinases (Fig 1D) (Analysis of sequence homology and pharmacological similarity that identified the pharmacologically relationship between kinases has been previously described [37].) Each group was given a MAXIS score to

indicate the number of times kinase proteins within each group had been analyzed by SVM [37] (Figs 1C and 1D). The greater the number of times a kinase is selected by the selection algorithm increases the MAXIS score. To determine whether kinase groups with MAXIS scores were acting as targets (inhibition resulted in suppression of HCMV protein production) or anti-targets (inhibition resulted in promotion of HCMV protein production), we used a previously developed inhibition bias metric, Bk [37]. A positive Bk score indicated that the MAXIS kinase was a candidate target, while a negative Bk score indicated that a kinase was a candidate anti-target (Fig 1D). Therefore, the analysis of our GSK PKIS screening data yielded 15 groups of pharmacologically related kinases with positive Bk scores, indicating one or more members of each groups was a potential target for inhibiting HCMV protein production (Fig 1D).

Many of the kinase proteins shown in Fig 1D had no known role in HCMV replication. To elucidate which members of each pharmacologically linked group were relevant to HCMV replication and, therefore, potential drug targets, we sought to understand which proteins were present in HCMV infected cells and which facilitated HCMV replication.

We compared proteins in each group (Fig 1D) to a proteomics dataset listing proteins that have previously been found in human fibroblasts infected with HCMV [43] (Table 1). Nearly every group contained at least one kinase protein found in this proteomic dataset. We then compared the proteins in each group to datasets in which collections of siRNAs had been used to understand the requirement for kinase proteins in HCMV replication [30] or HCMV protein production [28] (Table 1). Many of the siRNA had no obvious effect in the siRNA screen, or were toxic to infected cells in the screen.

Table 1. Scores of pharmacologically linked kinase proteins and analysis compared to other datasets.

MAXIS score	Kinase Group	Proteomic Analysis[1]	siRNA Dataset 1[2]	siRNA Dataset 2[3]
100	DYRK1A	+		NC
	DYRK1B	ND	NC	T
99	MAP4K4	+	NC	NC
	TNIK	ND	NC	T
	MINK1	+	NC	NC
83	MAPK14	+	NC	T
	MAPK11	ND	NC	T
	MAPK13	+	NC	T
	MAPK12	ND	NC	
82	PRKD1	+	NC	T
	PRKD2	ND	NC	T
	PRKD3	ND	NC	T
80	EPHA2	+	NC	NC
	EPHA3	ND	NC	T
	EPHA4	+	NC	T
	EPHA5	ND	NC	T
	EPHA7	ND	NC	T
	EPHA8	ND	NC	T
	EPHB1	ND	NC	T
	EPHB2	+	NC	
	EPHB3	+	NC	T
	EPHB4	+	NC	
70	NTRK1	+	NC	NC
	NTRK2	+	NC	T
	NTRK3	+	NC	T
63	PHKG1	ND	NC	T
	PHKG2	ND	NC	T
62	HIPK4	ND	NC	T
60	TSSK2	ND	NC	T
	TSSK1B	ND	NC	T
	IGF1R	+	NC	T
	INSR	+		T
	INSRR	ND		T

54	CLK1	+	NC	T
	CLK2	+	NC	T
	CLK3	+	NC	T
	CLK4	ND	NC	T
46	ALK	ND	NC	T
	LTK	ND	NC	
43	CHEK2	+		NC
30	CAMK2A	ND	NC	T
	CAMK2B	ND	NC	NC
	CAMK2D	+	NC	T
	CAMK2G	+	NC	NC
29	CSNK1G2	ND	NC	NC
	CSNK1G3	+	NC	T
	CSNK1G1	+		T
25	MAPK8	+	NC	NC
	MAPK9	+	NC	T
	MAPK10	ND	NC	T

[1]Data from reference [43]. Black box with plus=detected, ND=not detected.

[2]Data from reference [30]. Green box=decrease in HCMV replication, red box=increase in HCMV replication, NC=no change in HCMV replication.

[3]Data from reference [28]. Green box=decrease in HCMV protein pp28 production, red box=increase in HCMV protein pp28 production, NC=no change in HCMV protein pp28 replication, T=toxic.

DYRK1A, CHEK2 and CSNK1G1 were present in the proteomic analysis and were found to be necessary for HCMV protein production or HCMV replication in siRNA screens (Table 1). It has been demonstrated that inhibitors of DYRK1A prevent HCMV replication [44]. Although CHEK2 (also known as Chk2) was found to be required for HCMV replication in one siRNA based study [30], it has also been reported that signaling involving CHEK2 is inhibited in HCMV infected cells [45]. Thus, the

277 requirement for CHEK2 in HCMV replication was unclear. There was no other
278 information on the requirement of CSNK1G1 in HCMV infected cells. Although
279 inhibitors that specifically inhibit other casein kinase isozymes have been reported [46],
280 there is no selective and potent inhibitor of CSNK1G1. Therefore, our analysis of
281 proteins from the pharmacologically linked groups showed that a known anti-HCMV
282 drug target, DYRK1A, could be identified. However, it was unclear if CHEK2 and
283 CSNK1G1 could be considered as anti-HCMV targets.
284

285 MAP4K4 was present in HCMV infected cells and was
286 required for efficient HCMV replication and protein
287 production

288 We noted that one group of kinase proteins including MAP4K4, TNIK and
289 MINK1, had a high MAXIS score (Fig 1C). MAP4K4 and MINK1 were thought to be
290 present in HCMV infected cells (Table 1). However, our analysis of siRNA did not
291 indicate a role for any of these proteins in HCMV replication or identity a lead compound
292 for any of these proteins (Table 1). Given the high MAXIS score of this group, we
293 decided to investigate if one or more of the aforementioned proteins were necessary for
294 HCMV replication.
295 TNIK was not reported to be found in HCMV infected cells (Table 1) and the
296 functional role of MINK1 is unclear and may be restricted to T cells [47]. It has been
297 reported that MAP4K4 is required for production of the IE proteins of another

herpesvirus, Kaposi's sarcoma herpesvirus (KSHV) [48,49]. Therefore, we focused on investigation of MAP4K4.

Using western blotting, we confirmed the presence of MAP4K4 in HCMV infected cells. In this assay MAP4K4 was found in HFF cells infected with AD169 at 48-72 h.p.i. (Fig 2A, lanes 3 and 4). In this and subsequent western blotting, the presence of □-actin was assayed to determine the amount of cell lysate in each sample. We noted that detection of MAP4K4 was co-incident with the production of the late viral protein pp28 (Fig 2A, lanes 3 and 4). Production of late viral proteins, including pp28, requires DNA replication [19]. However, in the presence of HCMV DNA replication inhibitor ganciclovir we found a decrease in pp28 production, but no obvious defect in production of MAP4K4 using western blotting (Fig 2B), indicating MAP4K4 production was not dependent on HCMV DNA synthesis.

Fig 2. Treatment of HCMV infected cells with siRNA. (A, B and D) Western blotting of uninfected and HCMV infected cells. As outlined in the text, HFF cells were either (A) infected with AD169 (MOI of 1), (B) infected with AD169 (MOI of 1) and treated at the time of infection with either 10μM ganciclovir (GCV) or the equivalent volume of DMSO, or (D) treated with siRNA and infected with AD169 (MOI of 1). Cell lysates were prepared for western blotting at the time points (hours post infection (h.p.i.)) indicated above the Figure (A) or at 72 h.p.i. (B and D). In (A) uninfected cells harvested at the time of infection are shown as 0 h.p.i.. Proteins recognized by the antibodies used are indicated to the right of each figure. Also indicated are the IE antibodies used (IE1/2, recognizing IE1 and IE2-86, and IE2, recognizing all IE2 proteins). The positions of

molecular mass markers (kDa) are indicated to the left of each figure. The numbers in white represent the relative band intensity relative to the β-action band in the same lane. (C) Production of HCMV in cells treated with siRNA. HFF cells were treated with siRNA then infected with AD169 (MOI of 1). At 72 h.p.i. the virus released into the cell supernatant was quantified as plaque forming units (p.f.u.)/ml. The figure shows the average and standard deviation of data from three independent experiments. The result of an unpaired *t* test is shown above the data. (E) HCMV sequences encoding IE1/2 proteins and IE1/2 proteins produced during HCMV replication. Five exons of the HCMV UL122-123 locus that encode IE1 and IE2 proteins are shown in grey. Black arrows in exons 2 and 5 represent start codons. Below the exons IE1 and IE2 proteins are shown (white boxes), as are IE2 proteins IE2-60 and IE2-40 produced from internal start codons in exon 5 (white boxes). The alternative spicing of RNAs is also indicated. The molecular weight of each protein is shown to the right of the figure.

To investigate if MAP4K4 was necessary for HCMV replication, we treated HFF cells with siRNA targeting production of MAP4K4 or a control siRNA, then challenged those cells with high passage HCMV strain AD169. Virus released into the supernatant of infected cells was quantified (Fig 2C). In parallel, AD169 infected cells treated with siRNA were prepared for western blotting to analyze the presence of viral and cellular proteins (Fig 2D). We hypothesized that in our previous siRNA screening experiments (Table 1, [28]), the concentration of siRNA targeting MAP4K4 used was too low to see effects in our screen. Therefore, in this study we increased the concentration of siRNA used in transfections by approximately 4-fold and observed no obviously harmful effects

344 to transfected cells. Assays were carried out at 72 hours post infection, as at this time
345 point HCMV virus production from infected cells should be underway and all HCMV
346 proteins should be produced.

347 Compared to production of HCMV from cells treated with control siRNA,
348 treatment of cells with siRNA targeting MAP4K4 production resulted in a more than 3-
349 fold decrease in HCMV production (Fig 2C), indicating that MAP4K4 was required for
350 efficient HCMV replication. The production of MAP4K4 was examined using western
351 blotting. MAP4K4 was robustly detected in HCMV infected cells treated with control
352 siRNA (Fig 2D, lane 2), but no other sample, resulting in an approximately 5-fold
353 decrease in MAP4K4 detection (as determined by relative band intensity of bands
354 compared to β-actin in the same lane) in cells treated with siRNA targeting MAP4K4
355 production. Further analysis of infected cells by western blotting was carried out to
356 understand HCMV protein production. HCMV replication is dependent upon the
357 production of Immediate Early (IE) proteins IE1 and IE2, which antagonize innate
358 immunity and promote viral transcription, respectively. IE1 and IE2-86 are produced by
359 alternative splicing of the same RNA (Fig 2E). At late time points, two other IE2
360 proteins, IE2-60 and IE2-40, are produced from translation initiation start codons in RNA
361 from exon 5 (Fig 2E). IE2-60 and IE2-40 are essential for efficient HCMV replication
362 [50].

363 Western blotting for IE proteins revealed that treatment of cells with siRNA
364 targeting MAP4K4 production resulted in an approximately 2-fold decrease in IE2-60
365 and IE2-40 detection (Fig 2D), but no obvious defect in detection of either IE1 or IE2-86.
366 Thus, a reduction in the presence of MAP4K4 in HCMV infected cells was associated

with a loss of HCMV replication and impaired detection of IE2-60 and IE2-40 and a corresponding inhibition of HCMV replication.

Lead compounds targeting MAP4K4 inhibited HCMV replication

There has been little development of compounds targeting MAP4K4. However, we identified two structurally unrelated lead compounds, PF06260933 and CA409, reported to inhibit MAP4K4. PF06260933 (Fig 3A) strongly inhibited MAP4K4 and a number of other kinase proteins including MINK1 and TNIK [39]. CA409 (Fig 3B) was a potent and selective inhibitor of MAP4K4 and MINK1 [40].

We investigated the ability of PF06260933 and CA409 to inhibit HCMV replication in virus replication assays using the high passage HCMV strain AD169. It was observed that both PF06260933 and CA409 could inhibit HCMV replication with a 50% effective dose (ED_{50}) value of approximately 10μM (Table 2). To exclude the possibility that cellular cytotoxicity was responsible for the anti-HCMV effects of PF06260933 and CA409, we tested uninfected cell viability in the presence of PF06260933 and CA409 using an MTT assay to measure the activity of the mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes. We found no defect in cell viability at concentrations below 50 μM (Table 2), which was well above the ED_{50} value we had observed of 10 μM. This result indicated the anti-HCMV effects of PF06260933 and CA409 were unlikely to be due to cytotoxicity in the presence of these compounds.

Table 2. Anti-HCMV activity and cytotoxicity of compounds.

Compound	HCMV strain	EC_{50}[1]	CC_{50}[2]
PF06260933	AD169	9.6 ± 0.5	<50
CA409	AD169	12.3 ± 2.5	<50
PF06260933	Merlin(R1111)	13.3 ± 5.7	<50
CA409	Merlin(R1111)	9.6 ± 2.0	<50

[1] 50% Effective Dose (ED_{50}). Data shown is the mean ± standard deviation values (μM) from three independent experiments.

[2] 50% Cytotoxic concentration (CC_{50}). Data shown is the mean value from two independent experiments (μM).

Low passage strains of HCMV have a genomic content comparable to primary HCMV strains [51]. Therefore, we also tested the ability of PF06260933 and CA409 to inhibit replication of the low passage HCMV virus Merlin(R1111) [38] (Table 2). Similar results to those found when using AD169 were observed. Therefore, protein kinases inhibited by PF06260933 and CA409 were required for replication of both high and low passage HCMV viruses.

Lead compounds targeting MAP4K4 inhibited HCMV protein production

Next, we investigated how PF06260933 and CA409 inhibited HCMV replication. Based on experiments using siRNA shown in Figure 2, we hypothesized that treatment of

411 infected cells with inhibitors of MAP4K4 would inhibit production of IE2 proteins. Using
412 western blotting, we assayed the production of IE2 proteins at 72 hours post infection in
413 cells infected with AD169 and treated with the ED_{50} dose of either PF06260933 or
414 CA409 (10μM). Compared to infected cells treated with DMSO (Fig 3A, lane 2),
415 treatment of HCMV infected cells with PF06260933 resulted in a decrease in production
416 of all three IE2 proteins (Fig 3A, lane 3). Reduction in IE2-86 and IE2-40 production was
417 less than 2-fold, but the reduction in IE2-60 production was approximately 2-fold.
418 Compared to infected cells treated with DMSO (Fig 3B, lane 2), when HCMV infected
419 cells were exposed to CA409 we observed an approximately 2-fold decrease (data not
420 shown) in detection of IE2-86 and IE2-60 and a defect in IE2-40 production that was less
421 than 2-fold (Fig 3B, lane 3). We found no obvious decrease in IE1 production in the
422 presence of either PF06260933 or CA409 (data not shown). Therefore, treatment of
423 infected cells with ED_{50} dose of either PF06260933 or CA409 resulted in an
424 approximately 2-fold decrease in production of IE2-60.
425
426 *Fig 3. Use of PF06260933 and CA409 in HCMV infected cells.* (A and B) Structure of
427 PF06260933 and CA409, respectively. (C and D) Western blotting of PF06260933 and
428 CA409 treated infected cells. HFF cells were infected with AD169 at an MOI of 1, then
429 treated with either 10 □M PF06260933, CA409 or the equivalent volume of DMSO at
430 the time of infection. Uninfected cell lysate (lane 1) was prepared for western blotting at
431 the time of infection and infected cell lysate was prepared at 72 hours post infection
432 (h.p.i.) (lanes 2 and 3). Treatment of cells is indicated above the figure. Proteins
433 recognized by the antibodies used are indicated to the right of the figure. The positions of

molecular mass markers (kDa) are indicated to the left of the figure. The numbers in white represent the relative band intensity relative to the β-action band in the same lane. In (B) different panels originate from the same exposure of a single membrane to film.

Discussion

We demonstrate how a machine learning approach can be applied to reveal new insights into data from high throughput compound kinase inhibitor screening. Examination of machine learning results identified potential anti-HCMV drug targets. Many of these potential targets had no previously reported roles in HCMV replication or pathogenesis. Thus, the methodology used here also has the potential to uncover hitherto unappreciated aspects of HCMV biology. Further analysis of machine learning resulted in the identification of lead compounds targeting MAP4K4 that had anti-HCMV activity.

Given the benefits of the methods we use here, we propose that our study and others will stimulate renewed interest in screening of kinase inhibitors for anti-viral effects and support the production of highly characterized kinase inhibitor collections for screening. There are, however, several points surrounding validation of machine learning results that should be addressed. We attempted to validate machine learning results by comparing our data to previously reported siRNA datasets. There was no overlap in the two datasets of siRNAs that had either positive or negative effects. However, it has been reported that there is only limited overlap in the effects of orthologous siRNAs [52]. Many of the siRNA examined in our analysis had no obvious effect in siRNA screens, or were toxic to infected cells in the screen in which they were used. The paucity of data from siRNA screens meant that several kinases could not be directly validated as drug targets. However, it is possible that the lack of effect in an siRNA screen could be the result of inefficient knockdown of protein or the statistical method of analysis used in the siRNA screening process scored an siRNA as a false negative [41,52]. Therefore, many

461 of the kinase proteins identified in our machine learning results could be required for
462 HCMV replication and could be anti-HCMV drug targets. Indeed, we went on to
463 demonstrate using siRNA that MAP4K4 had effects on HCMV replication and protein
464 production even though it had been previously reported that siRNA targeting MAP4K4
465 had no obvious effects in two different siRNA screens [28,30]. Thus, in future, siRNA
466 screening data should be cautiously interpreted during validation of machine learning
467 results.

468 In this study we examined the role of MAP4K4 in HCMV replication and sought
469 to identify lead compounds targeting MAP4K4 that had anti-HCMV activity. The use of
470 siRNA or compounds inhibiting MAP4K4 all result in a reduction in the detection of IE2-
471 60. Thus, we propose that there is an association between the function of MAP4K4 and
472 production of IE2-60. It is interesting to note that use of either siRNA or different
473 compounds had different effects on production of IE2-86 and IE2-40. We propose that
474 this may be due to different off-target effects or lack of potency of the siRNA and
475 compounds we have used. Our observation using siRNA that knockdown of MAP4K4
476 leads to a reduction in IE2-60 and IE2-40 production is consistent with the somewhat
477 limited reduction in HCMV replication in the presence of siRNA targeting MAP4K4
478 production. IE2-60 and IE2-40 are not essential for HCMV replication, but their
479 expression is required for optimal HCMV replication [50]. Therefore, it is perhaps to be
480 expected that loss of either IE2-60 or IE2-40 did not lead to a drastic reduction in HCMV
481 replication. This leads to the question of should factors non-essential for virus replication
482 be targeted in anti-viral strategies? We would argue that this should be considered, as
483 there has been previous success in targeting proteins non-essential for HCMV replication.

For example, the HCMV kinase protein UL97 is non-essential for HCMV replication [53], but an inhibitor of UL97, maribavir, has been used in phase III clinical trials in humans [54].

Our screen of GSK compounds was based upon inhibition of HCMV pp28 production [33]. However, it has been noted that deletion of IE2-60 or IE2-40 from the HCMV genome had no effect on pp28 production [50]. Thus, we propose that the machine learning approach used here is able to identify factors required for virus replication that were not directly related to production of pp28. We suggest that in our screen compounds that were assigned negative z-scores had inhibition profiles that contained MAP4K4 and kinase proteins that were able to inhibit pp28 production.

It remains unknown how inhibition of MAP4K4 leads to a reduction in production of IE2 proteins, as there is little understanding of MAP4K4 function. A canonical view of MAP4K4 signaling in human cells involves activation of a phosphorylation cascade that includes MAP4K4 which results in that leads to activation of the kinase JNK1 and transcriptional activation [55,56]. This may involve upstream regulation of MAP4K4 by TRAF2 [55]. We have observed an increase in TRAF2 production late in HCMV replication, similar to that which we observed with MAP4K4 in this study (data not shown). It has been reported that activation of JNK1 is inhibited in HCMV infected cells [57]. However, JNK proteins JNK1-3 (MAPK8-10) were identified in our machine learning analysis (Fig 1C). We treated HCMV infected cells with a potent inhibitor of JNK1-3, JNK-IN-8 [58], and found that this compound had very little or no effect on production of infectious HCMV (data not shown). Thus, activation of signaling that

506 leads to JNK1 function, including that involving MAP4K4, was unlikely to be required
507 for HCMV replication.

508 Other intracellular signaling pathways involving MAP4K4 have been reported
509 [55,56], but are less well characterized. These data suggest STAT3 and NF-□B proteins
510 are substrates of MAP4K4 [55,56]. However, we have previously demonstrated that
511 neither canonical nor non-canonical NF-□B signaling was active in HCMV infected cells
512 [59]. While inhibition of STAT3 can influence HCMV replication [60], we found that
513 treatment of HCMV infected cells with either PF06260933 or CA409 had no obvious
514 effect on STAT3 phosphorylation (data not shown). Thus, it was unlikely that inhibition
515 of MAP4K4 in our experiments was related to the function of either STAT3 or NF-□B
516 proteins.

517 Emerging evidence places MAP4K4 directly or indirectly in a number of other
518 intracellular signaling pathways in a number of human pathologies [55,56]. Thus, it is
519 possible that further study of MAP4K4 will uncover poorly understood, or as yet
520 unrecognized, intracellular signaling pathways required for HCMV replication.
521 Alternatively, we hypothesize that IE2-60 could have been a substrate of MAP4K4 in
522 HCMV infected cells and lack of phosphorylation could have resulted in lack of protein
523 production or detection during western blotting. As it is unclear what dictates how a
524 protein serves as a substrate for MAP4K4. Further study of HCMV infected cells could
525 reveal novel insights into a protein that appears to be widely used in a number of contexts
526 [55,56].

527 We identify PF06260933 and CA409 as lead compounds that could be developed
528 to be become highly active anti-HCMV compounds. This will be necessary as the ED_{50}

for both compounds were in the high micromolar concentrations range despite high affinity on target results in *in vitro* binding assays [39,40]. It is possible that the weak ED_{50} of both PF06260933 and CA409 could be attributed to poor solubility, poor cell permeability and the dynamic environment in HCMV infected cells. We observed MAP4K4 production increased over time. However, we found no obvious decrease in MAP4K4 production in HCMV infected cells treated with either PF06260933 or CA409 (data not shown). This suggested that inhibition of MAP4K4 had no effect on MAP4K4 production. Regardless, these observations imply that production of proteins thought to be novel drug targets in HCMV infected cells should be assayed to investigate a potential relationship between production of protein and anti-viral effects of a compound. We argue that increased production of a protein thought to be a drug target in infected cells should not preclude development of compounds against that target, as many effective anti-viral compounds target viral proteins whose production increases over time.

Furthermore, we argue that the seemingly high ED_{50} concentrations recorded here for PF06260933 and CA409 should not preclude the development of these compounds. It is not unusual that lead compounds have somewhat high ED_{50} values before development using medical chemistry approaches. Medicinal chemistry approaches to modifying CA409 have been reported [61] and may have potential to produce a novel anti-HCMV compound with a more potent ED_{50} value. Also, maribavir, an HCMV inhibitor that has been used in human clinical trials [54], can display ED_{50} values in excess of 10μM in virus yield reduction assays [62]. Thus, there is precedent for continued study of compounds that otherwise might be discarded due to somewhat limited performance in anti-viral assays.

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Figure 1

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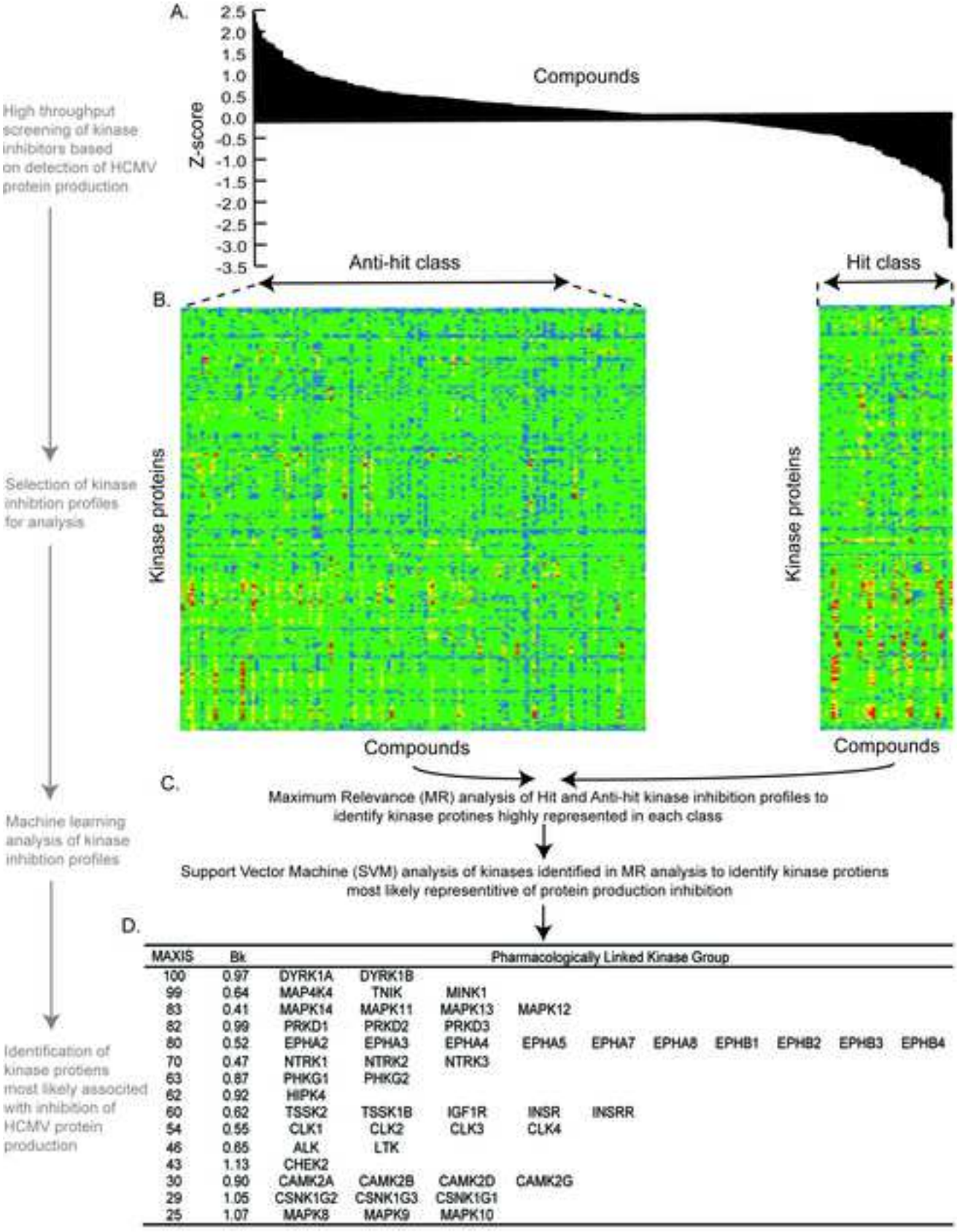

MAXIS	Bk	Pharmacologically Linked Kinase Group									
100	0.97	DYRK1A	DYRK1B								
99	0.64	MAP4K4	TNIK	MINK1							
83	0.41	MAPK14	MAPK11	MAPK13	MAPK12						
82	0.99	PRKD1	PRKD2	PRKD3							
80	0.52	EPHA2	EPHA3	EPHA4	EPHA5	EPHA7	EPHA8	EPHB1	EPHB2	EPHB3	EPHB4
70	0.47	NTRK1	NTRK2	NTRK3							
63	0.87	PHKG1	PHKG2								
62	0.92	HIPK4									
60	0.62	TSSK2	TSSK1B	IGF1R	INSR	INSRR					
54	0.55	CLK1	CLK2	CLK3	CLK4						
46	0.65	ALK	LTK								
43	1.13	CHEK2									
30	0.90	CAMK2A	CAMK2B	CAMK2D	CAMK2G						
29	1.05	CSNK1G2	CSNK1G3	CSNK1G1							
25	1.07	MAPK8	MAPK9	MAPK10							

Figure 1

Figure 2

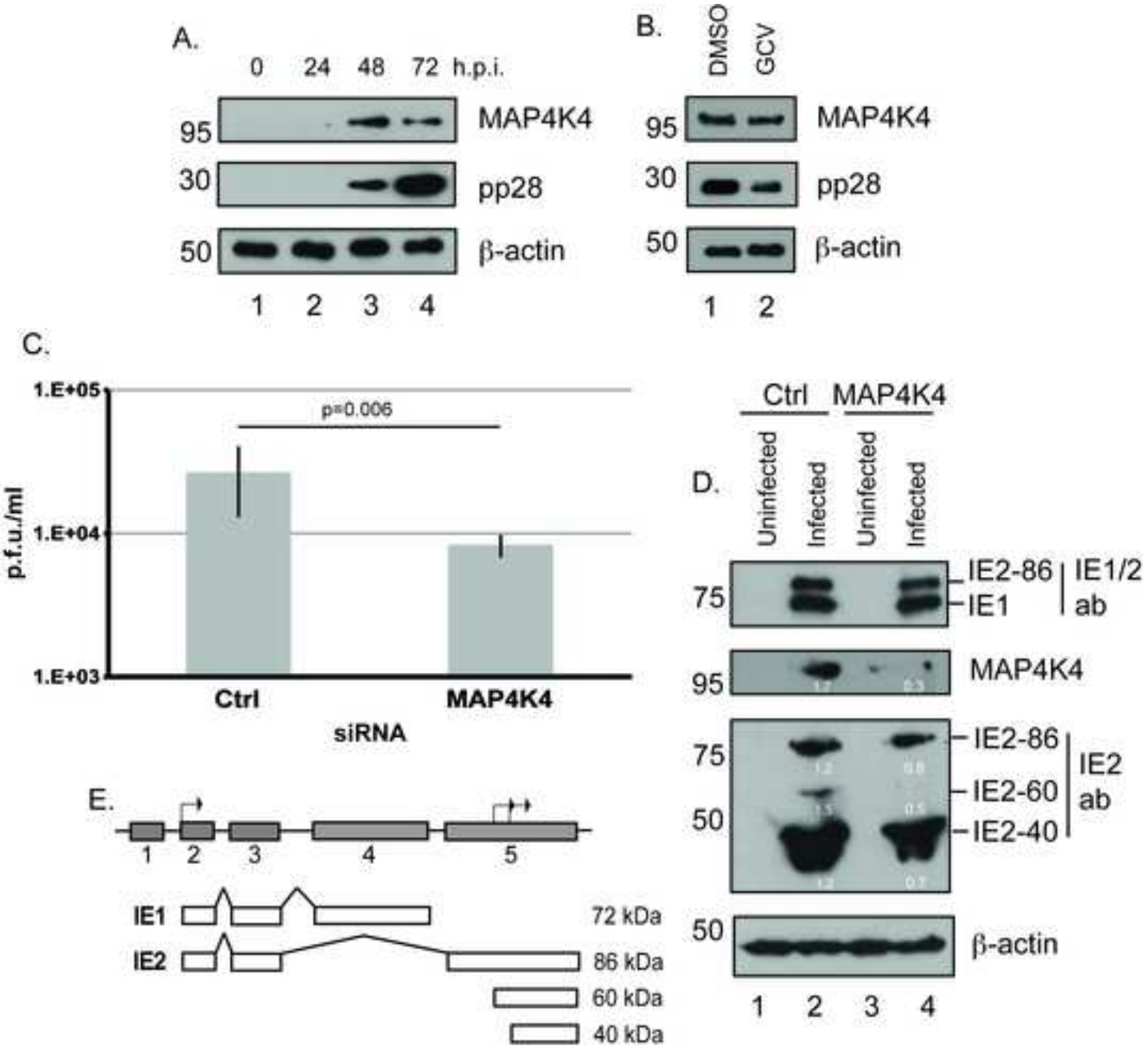

Figure 2

Figure 3

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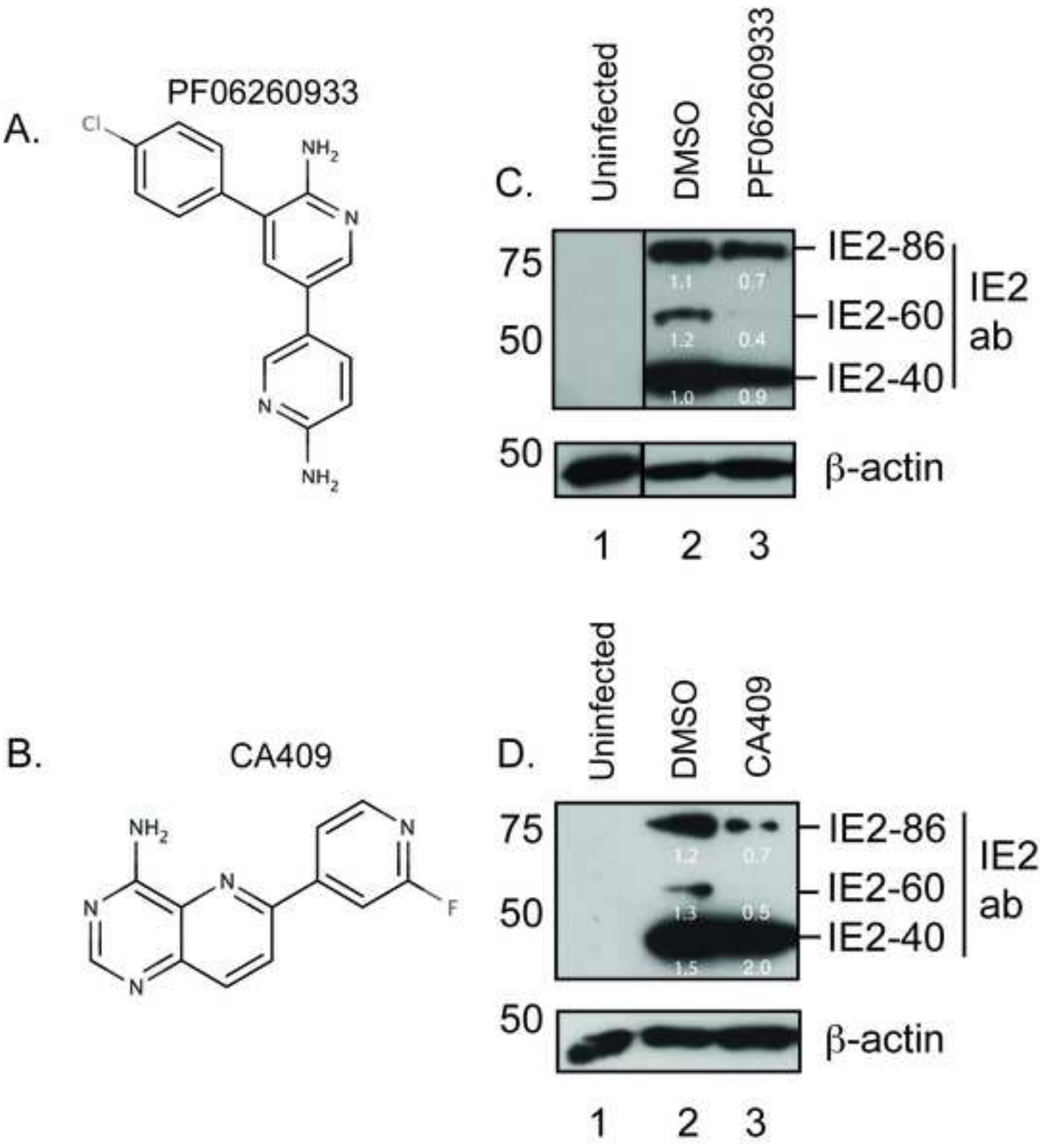

Figure 3