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High Throughput Screening of a GlaxoSmithKline Protein Kinase Inhibitor Set Identifies an Inhibitor of Human Cytomegalovirus Replication that Prevents CREB and Histone H3 Post-Translational Modification

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Abstract:	To identify new compounds with anti-human cytomegalovirus (HCMV) activity and new anti-HCMV targets, we developed a high throughput strategy to screen a GlaxoSmithKline (GSK) Published Kinase Inhibitor Set (PKIS). This collection contains a range of extensively characterized compounds grouped into chemical families (chemotypes). From our screen we identified compounds within chemotypes that impede HCMV replication and identified kinase proteins associated with inhibition of HCMV replication that are potential novel anti-HCMV targets. We focused our study on a top "hit" in our screen, SB-734117, which we found inhibits productive replication of several HCMV strains. Kinase selectivity data indicated that SB-734117 exhibits polypharmacology and is an inhibitor of several proteins from the AGC and CMCG kinase groups. Using western blotting we found that SB-734711 inhibited accumulation of HCMV immediate-early proteins, phosphorylation of cellular proteins involved in immediate-early protein production (CREB and histone H3) and histone H3 lysine 36 trimethylation (H3K36me3). Therefore, we identify SB-734117 as a novel anti-HCMV compound and find that inhibition of AGC and CMCG kinase proteins during productive HCMV replication is associated with inhibition of viral protein production and prevents post-translational modification of cellular factors associated with viral protein production.

STANDARD RESEARCH ARTICLE

High Throughput Screening of a GlaxoSmithKline Protein Kinase Inhibitor Set Identifies an Inhibitor of Human Cytomegalovirus Replication that Prevents CREB and Histone H3 Post-Translational Modification

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Running Title: Screening Kinase Inhibitors Targeting HCMV

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ABSTRACT

To identify new compounds with anti-human cytomegalovirus (HCMV) activity and new anti-HCMV targets, we developed a high throughput strategy to screen a GlaxoSmithKline (GSK) Published Kinase Inhibitor Set (PKIS). This collection contains a range of extensively characterized compounds grouped into chemical families (chemotypes). From our screen we identified compounds within chemotypes that impede HCMV protein production and identified kinase proteins associated with inhibition of HCMV protein production that are potential novel anti-HCMV targets. We focused our study on a top “hit” in our screen, SB-734117, which we found inhibits productive replication of several HCMV strains. Kinase selectivity data indicated that SB-734117 exhibits polypharmacology and is an inhibitor of several proteins from the AGC and CMCG kinase groups. Using western blotting we found that SB-734711 inhibited accumulation of HCMV immediate-early proteins, phosphorylation of cellular proteins involved in immediate-early protein production (CREB and histone H3) and histone H3 lysine 36 trimethylation (H3K36me3). Therefore, we identify SB-734117 as a novel anti-HCMV compound and find that inhibition of AGC and CMCG kinase proteins during productive HCMV replication is associated with inhibition of viral protein production and prevents post-translational modification of cellular factors associated with viral protein production.

INTRODUCTION

Disease associated with human cytomegalovirus (HCMV) infection affects a range of immunodeficient individuals [1]. As yet, there is no widely available vaccine against HCMV [2] and disease management largely rests on the use of anti-HCMV drugs [1, 3]. The most widely used anti-HCMV drugs (including the frontline drug ganciclovir) target the viral DNA polymerase, thereby inhibiting HCMV replication [3]. However, there are drawbacks to the use of ganciclovir and other currently available anti-HCMV drugs, including the development of drug resistant virus [3]. Furthermore, HCMV not only undergoes productive replication but can also enter a latent state from which the virus can reactivate. Currently, there is no effective treatment to clear latent HCMV infection.

Several novel anti-HCMV drugs are under development [1, 3, 4]. One strategy to expand the range of anti-HCMV drugs available is to identify existing compounds with hitherto unappreciated anti-HCMV activity. A large number of currently available compounds inhibit protein kinases in each of the groups that comprise the human kinome. Protein kinases are involved in many aspects of HCMV replication and pathogenesis, including intracellular signaling that results in transcription from the HCMV major immediate early promoter (MIEP), which stimulates a transcriptional cascade (*immediate-early* to *early* to *late* gene transcription) required for productive HCMV replication and reactivation from latency [1]. Therefore, protein kinase inhibitors could inhibit productive HCMV replication or reactivation from latency and a number of kinase inhibitors with

anti-HCMV activity have been identified [3]. Furthermore, it is possible that the full complement of protein kinases that are required for HCMV replication have yet to be identified. Therefore, kinase inhibitors could be used as chemical probes to identify kinase proteins required for HCMV replication, many of which could be novel anti-HCMV drug targets. However, an important consideration when using kinase inhibitors is that compounds targeting the conserved ATP-binding site of a kinase protein can display polypharmacology and are capable of inhibiting several kinase proteins or proteins outside the kinome, such as G-protein coupled receptors (GPCRs) [5-7]. Therefore, knowledge of kinase selectivity is important when discussing the use of kinase inhibitors as drugs or chemical probes [5].

We utilized a high throughput screening methodology to assess the ability of compounds within a GlaxoSmithKline (GSK) Published Kinase Inhibitor Set (PKIS) [8] to inhibit HCMV protein production. This compound library contained a range of extensively characterized compounds organized into structurally related collections of compound families (chemotypes) [7, 8]. Known characteristics of compounds within this GSK PKIS collection include kinase selectivity, compound structures and off-targets effects. Therefore, screening of this compound library allowed identification of both compounds and chemotypes with anti-HCMV activity, identification of novel anti-HCMV drug targets and permitted the on and off-targets effects of compounds identified in the screening process to be considered. These data lead to investigation of the anti-HCMV activity of a top "hit" in our screen, SB-734117.

RESULTS

High throughput screening of a GSK PKIS library to identify protein kinase inhibitors with anti-HCMV activity. To identify compounds with anti-HCMV activity we utilized a high throughput screening methodology (Fig. 1(a)), similar to the approach that we have previously used to screen siRNAs in HCMV infected cells [9]. Briefly, high passage HCMV strain AD169 and compounds from the GSK PIKS collection (listed in Table S1) were added to duplicate 384-well plates seeded with human foreskin fibroblast (HFF) cells. As negative and positive controls for compound treatment several wells in each plate were treated with either DMSO or heparan sulphate (a small molecule that inhibits HCMV entry into cells [10]), respectively. At 72 hours post infection (h.p.i.) cells were stained with Hoescht 33342 to detect nuclear DNA and CellMask to detect the area of the cell, plus were treated with antibodies to detect the cytoplasmic HCMV antigen pp28. An automated microscopy system was then used to assay both the number of cells in each well and the number of infected cells in each well expressing pp28. An image of infected cells treated as described above and captured using automated microscopy is shown in Fig. 1(b).

The mean number of cells in each well per plate was determined. Where the number of cells in any well was less than 2-fold below the mean number of cells of the plate, the compound in that well was judged to be cytotoxic (listed by chemotype in Table 1 and by compound in Table S2). Data from the remaining wells on duplicate plates were combined and converted to a z-score (the number

of standard deviations from the mean of the data [11, 12]) to demonstrate the increase (positive z-score) or decrease (negative z-score) in the number of pp28 positive cells in presence of each compound (shown in Fig. 1(c), listed by chemotype in Table 1 and by compound in Table S3).

Analysis of cytotoxic compounds. We first investigated what compounds within chemotypes were judged to be cytotoxic. Approximately 40-50% of compounds in the benzimidazole N-thiophene, 2H-3 pyrimidinyl pyrazolopyridazine, 3-amino pyrazolopyridines and 6-phenyl isoquinolines chemotypes contained compounds judged to be cytotoxic to HCMV infected cells (Table 1 and Table S2). Therefore, compounds from these chemotypes are generally not suitable for further use.

We then utilized kinase selectivity data to investigate which kinase proteins were inhibited by each compound judged to be cytotoxic (Table S4). Kinase selectivity data [7] lists the ability of each compound to inhibit a panel of 224 kinase proteins from several protein groups of the human kinome (including TK, STE, AGC, S-T-PK, CAMK and CMCG groups). We found that all cytotoxic benzimidazole N-thiophenes were potent inhibitors of PLK-1, whose inhibition can lead to apoptosis, and nearly all other cytotoxic compounds from a number of chemotypes were potent inhibitors of a range of CDK proteins, which are involved in regulation of the cell cycle. In our screen cytotoxicity was judged by the number of cells detected in each well of the screening plate. Therefore, we concluded that compounds were generally judged to be cytotoxic due to

apoptosis associated with inhibition of PLK-1 or due to lack of cell division associated with inhibition of CDK function.

Analysis of compounds assigned z-scores. Next, we analyzed those compounds assigned z-scores to determine which compounds and chemotypes should be considered for further study as anti-HCMV compounds and chemical probes. We found that nearly all chemotypes contained compounds that had both positive and negative effects on pp28 production. Notably, however, the 2-aryl 3-pyridimidinyl pyrazolopyridazine and furopyrimidine chemotypes and the 3-vinyl pyridine, 2,4-diamino pyrimidine, maleimide, phenyl carboxamide and indazole-5-carboxamide chemotypes contained a large number of compounds with negative and positive effects on pp28 production, respectively (Tables 1 and S3). We sought to further characterize the results of our screen and judged any compound with a z-score between 1 to -1 to have little or no effect on pp28 production, whereas compounds with z-scores of -1 to -2 and 1 to 2 had modest negative or positive effects on pp28 production, respectively. Thusly, compounds with z-scores of less than -2 or more than 2 had the greatest negative or positive effect on pp28 production, respectively. Therefore, three compounds (GW575808A, GW874091X and GW627512B) from three different chemotypes (2,4-diamino pyrimidines, imidazotriazines, 2-amino oxazoles, respectively) had strong positive effects on pp28 production, while four compounds (GW297361X, SB-734117, SB-220025-R and GW795493X) from four chemotypes (oxindoles, furazan benzimidazoles, 4-pyrimidinyl ortho-aryl azoles, furopyrimidines,

160 respectively) had strong negative effects on pp28 production (Figs. 1(c) and
161 1(d)).
162
163 **Examination of kinase protein inhibition by compounds assigned z-**
164 **scores.** We then examined kinase selectivity data of compounds assigned z-
165 scores (Table S5). The data from Table S5 is presented in Figure 2 as a
166 “heatmap” of kinase inhibition. Nearly all compounds assigned z-scores exhibited
167 polypharmacology and could inhibit more than one kinase protein. Consistent
168 with our analysis of compounds judged to be cytotoxic (Table S2), we found that
169 less than 5% of all compounds either potently inhibited PLK-1 or were potent
170 inhibitors of several different CDK proteins (Table S5). Compounds with positive
171 or negative z-scores were inhibitors of a wide range of kinase proteins in the TK
172 kinase group (Fig. 2 and Table S5). Therefore, inhibition of TK kinases alone was
173 unlikely to positivity or negatively influence pp28 production. However, a number
174 of kinases in the STE (including MAP4K4 and MNK), CAMK (including PRKD1,
175 PRKD2 and PRKD3) and CMCG (including CLK2, HIPK1, HIPK4, DYRK1A,
176 DYRK1B, DYRK2) kinase groups were inhibited by compounds assigned z-
177 scores of less than -1 from 8, 4 and 3 different chemotypes, respectively (Figs.
178 2(a)-(c), respectively, and Table S5). Therefore, these kinase proteins, alone or
179 in combination, were likely to be important for HCMV replication and could
180 represent future anti-HCMV drug targets. A number of compounds from two
181 different chemotypes that inhibited kinases in AGC kinase family (including
182 PRKG1, PRKG2, PRKX, PKA, ROCK1, ROCK2) were assigned z-scores over 1

183 (Fig. 2(d)). Therefore, these kinase proteins, alone or in combination, were likely
184 to be inhibitory to HCMV replication. Compounds targeting these kinase proteins
185 are likely to be off little value as anti-HCMV drugs.

186 Compounds assigned z-scores of less than -2 (GW297361X, SB-734117,
187 SB-220025-R and GW795493X) each had a distinct kinase selectivity profile
188 (Fig. 2 and Table S5). Therefore, it was likely each compound inhibited pp28
189 production by a different mechanism. Each was a potent inhibitor of several
190 kinase proteins from several groups, except for SB-220025-R, which was a
191 potent inhibitor of only 2 kinase proteins: CK1a and p38α (Fig. 2 and Table S5).

192 Kinase inhibition of compounds assigned z-scores of greater than 2 was
193 also examined. GW575808A and GW627512B had similar kinase selectivity
194 profiles and were inhibitors of several TK and S-T-PK group kinases (Fig. 2 and
195 Table S5). As inhibition of these TK and S-T-PK kinases can result in either
196 positive or negative z-scores (Fig. 2 and Table S5), it was unlikely that inhibition
197 of these TK and S-T-PK kinase proteins had a direct effect on pp28 production.
198 Moreover, we found that GW874091X was not a potent inhibitor of any kinase
199 assayed (Fig. 2 and Table S5). Therefore, GW874091X was either an inhibitor of
200 kinase proteins not assayed in the kinase selectivity data or exerted an effect on
201 pp28 production that did not involve inhibition of cellular kinase proteins. It,
202 therefore, remains unclear from this analysis which kinase proteins should not be
203 targeted in the development of future anti-HCMV drugs.

204 We further considered the polypharmacology of compounds tested in our
205 screen. It has been reported that ATP-competitive kinase inhibitors can inhibit the

function of proteins other than kinases, including aminergic GPCRs [6]. GPCR agonism and antagonism of the compounds in the GSK PKIS collection has been investigated elsewhere [7]. No compound within the GSK PKIS collection is a GPCR agonist, but several are GPCR antagonists [7]. However, we observed no correlation between compounds judged to be cytotoxic, compounds assigned a z-score and GPCR antagonism (data not shown).

Inhibition of HCMV replication by SB-734117. We chose to focus our studies on SB-734117, a compound from the furazan benzimidazole chemotype that had a low z-score in our screen (Figs. 1(c) and 1(d)). First, we used viral yield reduction assays to assess the ability of SB-734117 to inhibit replication of HCMV strain AD169 compared to the frontline anti-HCMV drug ganciclovir (GCV) (Table 2, experiment 1) at up to 96 h.p.i. The 50% effective dose (ED50) of both SB-734117 and GCV was 0.5μM, indicating that SB-734117 inhibit AD169 replication as efficiently as the current frontline anti-HCMV drug. To complement and confirm this data we analyzed AD169 replication over time and found an approximately 2-fold decrease in AD169 replication from 72-96 h.p.i. in the presence of 1μM SB-734117 (Fig. 3(a)).

We also found that SB-734117 could inhibit replication of a ganciclovir resistant virus (AD169-P53) and a low passage HCMV strain (Merlin(RCMVR1111)), whose genomic content is similar to a clinical sample [13], at low or sub-micromolar ED50 values (Table 2, experiments 2 and 3,

228 respectively) at up to 96 h.p.i.. Therefore, SB-734117 was an effective inhibitor of
229 different HCMV strains.

230 To ensure that the anti-HCMV activity of SB-734117 was not due to
231 cellular cytotoxicity we used an MTT dye-uptake assay to assess cell viability and
232 cell division in uninfected cells in the presence of SB-734117. We found that the
233 50% cellular cytotoxicity (CC50) of SB-734117 after 96 hours treatment with SB-
234 734117 was greater than 10μM (data not shown). Thus, the CC50 values in
235 uninfected cells were greater than 10μM at the ED50 values for all HCMV strains
236 tested. Therefore, inhibition of HCMV replication by SB-734117 observed in
237 experiments shown in Table 2, or in the other experiments presented here, was
238 unlikely to be the result of cellular cytotoxicity or inhibition of cell division.

239

240 **Examination of HCMV immediate early protein and mRNA production**
241 **in HCMV infected cells treated with SB-734117.** We next sought to understand
242 how HCMV replication was inhibited by SB-734117. Therefore, western blotting
243 was used to analyze the accumulation of HCMV proteins in the presence or
244 absence of SB-734117 (Fig. 3(b)). Compared to treatment of infected cells with
245 DMSO (Fig. 3(b), lanes 2-4), the treatment of infected cells with SB-734117 (Fig.
246 3(b), lanes 5-7) reduced the accumulation of immediate early proteins IE1 and
247 IE2, and IE2 proteins expressed late in infection (IE2-60 and IE2-40 [14]). In this
248 and subsequent western blots the amount of β-actin in each sample was also
249 assayed, which demonstrated equivalent loading of samples in each lane.

We then quantified the relative density of the western blotting bands shown in Fig. 3(b), by determining the band intensity of bands corresponding to viral proteins relative to the intensity of the β-actin band in the same lane (Fig. 3(c)). We found an approximately 2-4 fold decrease in the accumulation of IE1 in the presence of 1 μM SB-734117, which is consistent with an ED50 of 0.5-1 μM shown in Table 2. The loss of IE2 protein production (approximately 2- to 20-fold, depending on which antibody was used (Fig. 3(c))) was greater than IE1. Consistent with loss of IE protein production and our screening results, we also observed using western blotting that treatment of cells with SB-734117 resulted reduced accumulation of the HCMV early and late proteins UL44 and pp28, respectively, compared to infected cells treated with DMSO (data not shown). To compliment these findings we assayed for differences in IE1 and IE2 mRNA expression in infected cells treated with SB-734117 compared to infected cells treated with DMSO using quantitative PCR against the two major IE RNA species (Fig. 3(d)). This analysis revealed that no obvious defect in IE1 mRNA levels was evident in the presence of SB-734117. The analysis of IE2 mRNA again did not show any overt phenotype although typically a 2 fold reduction in IE2 mRNA was observed in SB-734117 treated cells when compared with DMSO control. However, taken together the data suggest that the decrease in IE1 and IE2 protein production shown in Figs. 3(b) and 3(c) was unlikely to be the result of decreased *IE* gene expression.

Our studies thus far could not rule out that SB-734117 impacted events occurring prior to IE gene expression. Thus we addressed whether the presence

of SB-734117 may affect virus entry into the cell or translocation of the HCMV genome to the nucleus. Pre-exposure of cells to SB-734117 before infection or incubation of virus with SB-734117 before infection did not increase the inhibitory effects of the compound (data not shown). However, when we treated AD169 infected HFF cells with 1μM SB-734117 at 24 h.p.i. we found a 2-fold decrease in HCMV replication at 120 h.p.i., compared to infected cells treated with DMSO at 24 h.p.i. (Fig. 4(a)). Quantitative analysis of western blotting of infected cells treated as described above (Figs 4(a) and 4(b)) showed that, similar to data presented in Fig. 3, treatment of infected cells with SB-734117 resulted in approximately 2-fold decrease in IE2 protein production depending on which antibody was used. However, there was no obvious decrease in production of IE1 protein.

Therefore, SB-734117 had no obvious effect on cells or virus before infection, but could inhibit HCMV replication after entry of the HCMV genome and did so by reducing IE2 protein production. Thus, SB-734117 may not inhibit events during infection before expression of IE proteins, but could have inhibitory effects on HCMV replication after the initiation of IE protein production.

Inhibition of AGC and CMCG kinase proteins by SB-734711. Next, we investigated what kinases proteins are inhibited by SB-734117. SB-734117 has been reported to inhibit MSK1 [15]. However, using the kinase selectivity data shown in Fig. 2 and Table S5, we found that at SB-734117 inhibits several AGC kinase group proteins, including MSK1 (MSK1, MSK2, RSK1, RSK2, RSK3,

296 p70S6K1, PCK-η, PRKG2, ROCK1, ROCK2), and several CMCG kinase group
297 proteins (GSK3A, GSK3B, DYRK1A and DYRK1B). However, in our screen
298 potent and selective inhibitors of GSK3A and GSK3B had no obvious negative
299 effect on pp28 production (Table S5) and compounds with either positive or
300 negative z-scores were potent inhibitors of PKC-η, PRKG2, ROCK1 and ROCK2
301 (Table S5). Therefore, inhibition of these kinases proteins may not be related to
302 inhibition of pp28 production. Rather, analysis of SB-734117 kinase selectivity
303 data compared to other assigned z-scores argued that potent inhibition of MSK1,
304 MSK2, RSK1, RSK2, RSK3, p70S6K1, DYRK1A and DYRK1B was related to
305 inhibition of pp28 production.

306 A kinase inhibitor that is structurally unrelated to SB-734117, H-89, inhibits
307 a similar range of AGC and CMCG kinase proteins [16]. We found that H-89
308 inhibited productive HCMV replication and immediate-early protein production
309 (data not shown). Therefore, inhibition of AGC and CMCG kinase proteins, not
310 an unknown function of SB-734117, is likely to be responsible for the observed
311 defects in HCMV replication and protein production. Furthermore, using western
312 blotting [17], we found that SB-734117 did not inhibit autophosphorylation of the
313 HCMV encoded kinase UL97 (data not shown). Therefore, the anti-HCMV effects
314 of SB-734117 were unlikely to be due to inhibition of UL97.

315

316 **Analysis of CREB and histone H3 phosphorylation in HCMV infected**
317 **cells.** We then considered how inhibition of AGC and CMCG kinase proteins by
318 SB-734114 would affect post-translational modification of cellular proteins

thought to be involved in HCMV replication. We focused our investigation on phosphorylation of the cellular transcription factor CREB and histone H3.

CREB is thought to directly or indirectly facilitate transcription from the MIEP [18, 19] and other viral promoters [20] during productive HCMV replication and it has been reported that phosphorylation of CREB at serine residue 133 (CREB-Ser133) by MSK1 is involved in promoting changes to chromatin required for activation of the MIEP during HCMV reactivation from latency [21]. In preliminary experiments we could not detect either total cellular CREB or CREB-Ser133 before 72 h.p.i. using western blotting (data not shown). However, both proteins could only be detected at 72 h.p.i. when we increased the amount of cell lysate assayed (see Materials & Methods). Therefore, we used western blotting to assay total cellular CREB and CREB-Ser133 phosphorylation in HCMV infected cells treated with either DMSO or SB-734117 at 72 h.p.i. (Fig. 5(a)). We observed a decrease in accumulation of CREB-Ser133 and an increase in the accumulation of CREB in the presence of SB-734117 (Fig. 5(a), lane 5), compared to infected cells treated with DMSO (Fig. 5(a), lane 3). Analysis of relative band intensities (Fig. 5(b)), indicated that there was approximately a 2-fold decrease in CREB-Ser133 in infected cells treated with SB-734117, compared to those treated with DMSO and a modest increase in CREB. The 2-fold decrease in the accumulation of CREB-Ser133 in the presence of 1μM SB-734117 was consistent with the observed ED50 of 0.5-1 μM and 2-4 fold decrease in production of immediate-early HCMV protein production (Table 2 and Fig. 3). Therefore, the effect of SB-734117 on HCMV replication correlated

342 with a loss of CREB-Ser133 phosphorylation. Similar observations were made
343 when infected cells were treated with H89 (data not shown), indicating the AGC
344 and CMCG kinase proteins were involved in phosphorylation of CREB.

345 Phosphorylation of histone H3 by MSK1 or another kinase, IKKα is
346 required for binding of transcription factors to DNA in uninfected cells [22, 23].
347 We have previously demonstrated that phosphorylation of histone H3 at serine
348 residue 10 (H3S10p) by IKKα is associated with immediate-early protein
349 production during productive HCMV replication [17]. Also, it has been
350 demonstrated that H3S10 phosphorylation by MSK1 is associated with
351 immediate-early gene expression during reactivation of HCMV from latency [21].
352 We decided to assay H3S10 phosphorylation in the presence of SB-734117.
353 Using western blotting we analyzed accumulation of H3 and H3S10
354 phosphorylation in uninfected HFF cells (Fig. 5(c), lane 1) and AD169 infected
355 HFF cells treated with either DMSO or SB-734117 (Fig. 5(c), lanes 2-4 and 5-7,
356 respectively, at 24-72 h.p.i.). Similar levels of H3 were found in each sample,
357 however, over time we observed a decrease in H3S10p in infected cells treated
358 with SB-734117 to near undetectable levels, compared to infected cells treated
359 with DMSO. Therefore, inhibition of H3S10 phosphorylation during productive
360 HCMV replication may have contributed to the anti-HCMV activity of SB-734117.
361 Similar results were found when infected cells were treated with H89 (data not
362 shown), indicating that AGC and CMCG kinase proteins were involved in
363 phosphorylation of H3S10.

364 Phosphorylation of histone H3 at serine residue 28 (H3S28) by MSK1 is
365 also known to be associated with gene expression in uninfected cells [22]. We
366 also used western blotting to investigate H3S28 phosphorylation during HCMV
367 replication. However, we could not detect H3S28p in either uninfected HFF cells,
368 AD169 infected HFF treated with either DMSO or SB-734117, or uninfected HFF
369 cells treated with either anisomycin, which can stimulate H3S28 phosphorylation,
370 or phosphatase inhibitor okadaic acid, which can prevent dephosphorylation of
371 histones (data not shown). Therefore, we suggest that inhibition of H3S28
372 phosphorylation did not contribute to the anti-HCMV activity of SB-734117.
373
374 **Analysis of histone H3 post-translational modifications in HCMV**
375 **infected cells.** H3S10 phosphorylation by either MSK1 or IKKα is associated
376 with the presence of acetyl modifications of H3, including at acetylation (ac) of
377 lysine 14 (H3K14ac) [24-26]. There is a relationship during transcriptional
378 activation between the presence of H3S10p and H3K14ac and the association of
379 transcription factors with DNA [23, 27]. As the presence of H3K14ac is
380 associated with transcriptional activation in HCMV infected cells [28], H3K14ac
381 may be required for HCMV replication. We have previously demonstrated that
382 during HCMV replication inhibition or depletion of IKKα leads to loss of total
383 cellular H3S10 phosphorylation, but not loss of total cellular H3K14ac [17]. Thus,
384 we assayed whether treatment of HCMV infected cells by SB-734117 would lead
385 to loss of H3S10p or H3K14ac. We used western blotting to assay total cellular
386 levels of H3, H3S10p and acetylation of H3 on a number of commonly studied H3

lysine residues including K14 (H3K9ac, H3K14ac, H3K18ac, H3K27ac) in either uninfected HFF cells (Fig. 5(c), lane 1) or HFF cells infected with HCMV and treated with either DMSO or SB-734117 (Fig. 5(c), lanes 2-4 and 5-7, respectively). Treatment of infected cells with SB-734117 had a slight effect (less than 2-fold (data not shown)) on accumulation of H3K14ac and no detectable effect on detection of H3K9ac, H3K18ac, or H3K27ac. Therefore, treatment of infected cells with SB-734117 was associated with loss of total cellular H3S10p, but not loss of the total cellular H3 acetylation modifications we assayed, including H3K14ac.

The relationship between H3S10p and dimethylation (me2) and trimethylation (me3) of H3 and H3 phosphorylation is not well characterized, but it has been reported that in a murine model there is a relationship between the presence of H3S10p and the presence of H3K36me3 [29] and in *Drosophila melanogaster* loss of the MSK1/2 homologue JIL-1 results in loss of H3S10p, H3 acetylation and H3 methylation, including H3K36me3 [30]. Therefore, we asked if loss of total cellular H3S10p in HCMV infected cells was associated with me2 and me3 modification of H3 lysine residues. Western blotting was used to assay the presence of H3 and H3S10p, plus me2 (H3K4me2, H3K27me2, H3K36me2) or me3 (H3K4me3, H3K9me3, H3K27me3, H3K36me3) modifications of H3 in uninfected HFF cells (lane 1, Figs. 5(d) and 5(e), respectively) or HFF cells infected with HCMV and treated with either DMSO or SB-734117 (lanes 2-4 and 5-7, Figs. 5(d) and 5(e), respectively). We observed that SB-734117 had no effect on total cellular accumulation of any me2 modification of H3 (Fig. 5(d)) or

410 accumulation of H3K4me3, H3K9me3 or H3K27me3 (Fig. 5(e)). However, we
411 observed a near total loss of detectable H3K36me3 over time in infected cells
412 treated with SB-734117 (Fig. 5(e), lane 7) compared to infected cells treated with
413 DMSO (Fig. 5(e), lane 4). Similarly, a near total loss of detectable H3K36me3
414 was observed in infected cells treated with H89 (data not shown), suggesting that
415 loss of H3K36me3 is related to inhibition of AGC and CMCG kinase proteins.
416 Thus, inhibition of H3K36me3 was associated with loss of total cellular H3S10p
417 and was likely the result of inhibition of AGC and CMCG kinase proteins inhibited
418 by SB-734117. Loss of both total cellular H3S10p and total cellular H3K36me3
419 may have contributed to the anti-HCMV activity of SB-734117.
420
421 **Investigation of HCMV MIEP transcriptional activation.** Loss of CREB
422 and H3S10 phosphorylation (Figs. 5(a)-(e)) suggested that SB-734117 acted by
423 inhibiting activation of the HCMV MIEP. However, our analysis of IE1 and IE2
424 gene expression (Fig. 3(d)) indicated that transcription from the MEIP was not
425 obviously compromised in the presence of SB-734117. To investigate this in
426 more detail we utilized chromatin immunoprecipitation (ChIP) to assay the
427 presence of H3K14ac, a marker of MIEP transcriptional activation [28], at the
428 MIEP in the presence of either DMSO or SB-734117 (Fig. 6). The data showed
429 that no overt impact on H3K14ac at the MIEP between 24-72hpi in DMSO or SB-
430 734117 treated cells. Indeed, we noted that SB-734117 treated cells actually
431 showing higher levels of H3K14ac at the MIEP at late times post infection when
432 compared to control. Therefore, in agreement with our analysis of IE1 and IE2

433 gene expression (Fig. 3(d)), there was no obvious defect in MIEP transcriptional
434 activation in the presence of SB-734177. Thus, the observed defects in HCMV
435 immediate-early protein production (Figs 3 and 4) could not be explained by
436 defects in transcription from the MIEP.
437
438

DISCUSSION

Our overall analysis of the chemotypes screened indicated that each chemotype contained compounds with anti-HCMV activity, however modest that anti-HCMV activity may have been. As the structure of each compound in each chemotype is known, structure-activity relationships derived from our data could form the basis of future studies in the discovery of compounds with anti-HCMV activity from each chemotype.

Our survey of the GSK PKIS kinase selectivity data argued that several proteins from several kinase groups, alone or in combination, were required for pp28 production. These proteins kinases include those from the STE (including MAP4K4 and MNK), CAMK (including PRKD1, PRKD2 and PRKD3) and CMCG (including CLK2, HIPK1, HIPK4, DYRK1A, DYRK1B, DYRK2) kinase groups. The function of these protein kinases in productive HCMV replication is unclear or unknown. Therefore, it is possible that our data identified novel cellular factors required for productive HCMV replication. However, the polypharmacology of the compounds tested makes it difficult to identify specific kinases required for productive HCMV replication. Thus, each of the aforementioned kinases will have to be tested individually to identify their roles in HCMV infected cells. With this information these kinases could be exploited as novel anti-HCMV drug targets.

We chose to pursue studies of SB-734117 as this compound had one of the greatest negative effects on pp28 production in our screen, with no obvious cytotoxic effects, and was an effective inhibitor of a number of HCMV strains.

462 Moreover, the function of the AGC and CMCG kinase proteins inhibited by SB-
463 734117 in productive HCMV replication was unknown or unclear.

464 SB-734117 was originally described as an inhibitor of MSK1 [15].
465 However, like other MSK1 inhibitors [16], SB-734117 displays polypharmacology
466 and can inhibit several kinases whose roles in productive HCMV replication are
467 unknown or unclear. Thus, to understand how SB-734117 inhibits HCMV
468 replication it will be necessary to understand if a particular kinase or a
469 combination of kinase proteins is required for productive HCMV replication. A
470 truly selective inhibitor of MSK1 has yet to be found. Structure-activity
471 relationships involving SB-734117 and other furazan benzimidazole compounds
472 could be explored to generate compounds with improved anti-HCMV activity and
473 kinase selectivity. However, we could discern no obvious relationship between
474 inhibition of HCMV replication, kinase selectivity and the structures of
475 compounds within the furazan benzimidazole chemotype analyzed here due to
476 the small number of furazan benzimidazole compounds that returned low
477 negative z-scores in our screen (data not shown). An improved compound
478 related to SB-734117 would have value as an anti-HCMV drug, as it would have
479 the potential to inhibit both productive HCMV replication and reactivation of
480 HCMV from latency. Plus, based on our observations, an improved compound
481 should be as effective an inhibitor of HCMV replication as ganciclovir and be able
482 to inhibit replication of ganciclovir resistant HCMV strains.

483 Perhaps the most intriguing observations we make concern modification of
484 histone H3 in the presence of SB-734117. Previous observations from our

485 laboratory have indicated that IKKα was required for H3S10 phosphorylation in
486 AD169 infected HFF cells [17], which is consistent with data presented
487 elsewhere indicating that H3S10 is substrate of IKKα [24, 25, 31-33]. We note
488 that inhibition of IKKα results in loss of H3S10p early in HCMV replication (24
489 h.p.i. onwards) [17], whereas treatment with SB-734117 leads to a loss of
490 H3S10p later in HCMV replication (48-72 h.p.i.). SB-734117 does not inhibit
491 IKKα (Table S5). Therefore, we propose that in HCMV infected cells a
492 mechanism exists wherein IKKα does not phosphorylate H3S10 during treatment
493 with SB-734117. Conversely, kinases inhibited by SB-734117 do not
494 phosphorylate H3S10 when IKKα is inhibited or depleted. This mechanism may
495 ensure appropriate regulation of H3S10 phosphorylation that is necessary for
496 productive HCMV replication.

497 It has been reported that in uninfected cells from humans and mice loss of
498 H3S10p can lead to loss of H3K14ac [24-26]. However, we did not observe loss
499 of total cellular H3K14ac upon treatment of HCMV infected cells with SB-734117
500 or in our previous study where inhibition or depletion of IKKα resulted in loss of
501 H3S10p [17]. We speculate, as we have done previously [17], that an as yet
502 unrecognized mechanism exists in HCMV infected cells that maintains total
503 H3K14ac when total H3S10p levels are lowered to near undetectable levels.

504 We observed that treatment of HCMV infected cells with SB-734117
505 resulted in loss of H3K36me3. We have previously found that depletion of IKKα
506 in HCMV infected cells leads to loss of H3S10p, but not H3K36me3 [17].
507 Therefore, the loss of H3K36me3 in HCMV infected cells is related to loss of

508 H3S10p during inhibition of AGC and CMCG kinases, but not during inhibition of
509 IKKα. This may be related to regulation of H3S10 phosphorylation by different
510 kinases, as we discuss above. We propose that, as in mice and *Drosophila* [29,
511 30], the presence of H3K36me3 in HCMV infected cells is related to the presence
512 of H3S10p, potentially via phosphorylation of H3 by MSK1. Alternatively, there
513 may be a substrate of kinase proteins inhibited by SB-734117 whose
514 phosphorylation directly or indirectly mediates H3K36 tri-methylation.

515 Treatment of HCMV infected cells with SB-734117 impacted on immediate
516 early protein production and caused loss of total cellular levels of post-
517 translational modification of cellular factors potentially involved in transactivation
518 of the HCMV MIEP. However, in the presence of SB-734117 we did not find
519 obvious defects in activation of transcription from the HCMV MIEP or defects in
520 immediate-early gene transcription. Therefore, the loss of total cellular levels of
521 CREB and H3S10 phosphorylation or H3K36me3 had no direct impact on
522 transcription from the HCMV MIEP. This interpretation would be consistent with
523 previous studies that have shown that the deletion of CREB binding sites from
524 the MIEP has little impact on productive HCMV replication [21, 34]. During
525 reactivation, it is hypothesized the H3S10p is important as it drives the transition
526 of the MIEP from a repressed to active promoter, a mechanism also proposed for
527 HSV reactivation [35] Thus, during productive HCMV replication at high MOI,
528 where the MIEP is associated with active, not repressed, chromatin very early
529 post infection [28], H3S10p may not be essential for transcription. However, it
530 remains possible that H3S10p has a role in the release of Early and Late HCMV

promoters from repression as infection proceeds. Thus, future challenges will include mapping of CREB, H3S10p and H3K36me3 to viral and cellular promoters to understand in more detail to understand their possible involvement in productive HCMV replication.

We propose that the greatest impact of SB-734117 on productive HCMV replication is on production of IE proteins. It will be important to investigate if SB-734117 impacts on additional phosphorylation events that are required for production of both proteins. Post-translational modification of both IE1 and IE2 is not yet completely characterized. Thus, SB-734117 could directly or indirectly inhibit phosphorylation of these proteins, which leads to their loss. Further investigation of IE protein production and the roles of IE post-translational modification are required in order to fully understand the mechanism of action of SB-734117 during productive HCMV replication.

MATERIALS & METHODS

Compounds. The GSK PIKS library (version 1) was supplied to the Institute of Chemistry and Chemical Biology-Longwood at Harvard Medical School by GSK. SB-734117 was a kind gift from GlaxoSmithKline. Ganciclovir was obtained from SIGMA. H89 and heparan sulphate were obtained from Calbiochem. All drugs were resuspended in dimethyl sulfoxide (DMSO).

Cells and viruses. Human foreskin fibroblast (HFF) cells (clone Hs29) were obtained from American Type Culture Collection no. CRL-1684 (ATCC, Manassas, VA) and maintained in Dulbeccos Modified Eagles Medium (DMEM) (Gibco) containing 5% fetal bovine serum (FBS) (Gibco), plus penicillin and streptomycin. High passage HCMV strain AD169 was a gift from Don Coen (Harvard Medical School). Low passage strain Merlin R1111 (derived from BACmid pAL1111, which does not express RL13 and UL128)[36] was a gift from Richard Stanton (Cardiff University). Ganciclovir resistant virus AD169-P53 was supplied by the National Institute of Health (NIH) AIDS Reagent Program.

Screening of GSK PKIS collection and analysis of screening data. See supplementary material.

Characterization of compounds within the GSK PKIS collection. See supplementary material.

568

569 **Viral yield reduction assays.** Assays were performed essentially as described
570 in [37]. HFF cells were plated at 5 × 10^4 cells per well in 24-well plates. After
571 overnight incubation, cells were infected with HCMV at a multiplicity of infection
572 (MOI) of 1. After virus adsorption for 1 hour at 37°C, cells were washed and
573 incubated with 0.5 ml of media containing DMSO or compounds at a range of
574 concentrations in duplicate. Plates were incubated for 4 days at 37°C. Titers
575 were determined by serial dilution of viral supernatant onto HFF monolayers
576 which were covered in DMEM containing 5% FBS, 0.6% methylcellulose and
577 antibiotics. Cultures were incubated for 14 days, cells were stained, with crystal
578 violet and plaques were counted. Data shown represents the mean value of
579 duplicate plaque counts. The final concentration of DMSO in all samples was
580 maintained at <1% (v/v).

581

582 **MTT cytotoxicity assays.** Assays were performed essentially as described [37].
583 HFF cells were seeded at 1 × 10^4 cells per well into 96-well plates. After
584 overnight incubation to allow cell attachment, cells were treated for the time
585 indicated in the text with at range of concentrations concentrations in duplicate.
586 The highest concentration of compound tested was 10 μM. Cell viability was then
587 determined with an MTT assay according to the manufacturer's instructions (GE
588 Healthcare). Data shown represents the mean value of duplicate readings. The
589 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).

Western blotting. At time points indicated in the text cells were washed once with PBS and resuspended in 100 μl Laemmli buffer containing 5% β-mercaptoethanol. Proteins were separated on 8% or 10% polyacrylamide gels. Typically, a volume of cell lysate corresponding to 1 x 10^4 HFF cells was analyzed, except when blotting for CREB or CREB-Ser133 when a volume of cell lysate corresponding to 5 x 10^5 HFF cells was analyzed. Antibodies used are listed in the supplementary material. 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).

RNA analysis and Quantitative PCR. Quantitative PCR was performed using a SYBR green qPCR kit (Qiagen) and analysed using the ΔΔCT method to compare DMSO versus SB-734117 treated cells. Briefly, cDNA was prepared from RNA extracted from infected cells at timepoints indicated in the Figure. cDNA and no RT controls were amplified in technical duplicates from multiple experiments using a constant primer in exon 3 (UL122-123) and a primer from

611 exon 4 (UL123) or exon 5 (UL122). Cellular RNA was amplified using 18S RNA
612 primers.
613 Exon 3: ACG AGA ACC CCG AGA AAG ATG; exon 4: CGC CAG TGA ATT
614 TCT CTT C; exon 5: CCG GGG AGA GGA GTG TTA GT; 18S for: GTA ACC
615 CGT TGA ACC CCA; 18S rev: CCA TCC AAT CGG TAG TAG CG.
616 qPCR was performed using cycling conditions: 95^{o}C (15s) then 40 cycles of 94^{o}C
617 (15s), 55^{o}C (30s) and 72^{o}C (30s).
618
619 **Chromatin immunoprecipitation.** All procedures were performed
620 essentially as previously described [21]. Briefly, HFFs were fixed with 1%
621 formaldehyde (10 mins) and then enzymatically digested to fragment DNA as
622 described by manufacturer (Pierce chromatin preparation kit). DNA associated
623 with histones was immunoprecipitated with control serum (Sigma) or anti-acetyl-
624 histone H3-lysine 14 (1:150 dilution of antibody – see supplementary material).
625 For detection of the HCMV MIEP, DNA from disrupted nucleosomes was
626 precipitated and amplified by SYBR green qPCR kit (Qiagen) using 5′ - TGG
627 GAC TTT CCT ACT TGG (sense) and 5′ - CCA GGC GAT CTG ACG GTT (anti-
628 sense) primers. Specific immuno-precipitation of sequences was expressed as
629 enrichment from Input.
630

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ABBREVIATIONS

AGC: containing PKA, PKG, PKC families group, **CAMK**: calcium/calmodulin-dependent protein kinase group, **CDK**: cyclin-dependent kinase, **CLK2**: cyclin-dependent kinase-like kinase 2, **CK**: casein kinase, **CMCG**: containing CDK, MAPK, GSK3, CLK families group, **CREB**: cAMP response element-binding protein, **GCV**: ganciclovir, **DYRK**: dual-specificity tyrosine phosphorylation-regulated kinase, **GSK**: glycogen synthase kinase, **HCMV**: human cytomegalovirus, **HIPK**: homeodomain interacting protein kinase, **MAPK4K4**: mitogen-activated protein kinase kinase kinase kinase 4, **MNK1**: MAP kinase-interacting serine/threonine-protein kinase 1, **MSK**: mitogen and stress kinase, **PCK-η**: protein kinase C-η, **PLK-1**: polo-like kinase 1, **PRKD**: protein kinase D, **PRKG**: protein kinase, cGMP-Dependent, **PRKX**: protein kinase, x-linked, **p70S6K1**: ribosomal protein S6 kinase beta-1, **ROCK**: rho-associated, coiled-coil-containing protein kinase 1, **RSK**: ribosomal s6 kinase, **STE**: homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases group, **S-T-PK**: serine/threonine protein kinase group, **TK**: tyrosine kinase group, **TKL**: tyrosine kinase-like group.

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FIGURE LEGENDS

Fig. 1 High throughput screening of the GSK PKIS collection. (a) Diagram of screening process. (b) A representative example of a microscopy image from an Image Express Micro microscope of infected HFF cells treated with Hoecsht 33342 (blue), Deep Red Cell Mask (Red) and primary and secondary antibodies to detect HCMV pp28 (green). The large white box is a magnified image of the area identified by the small white box. (c) Plot of z-scores where each data point represents a single compound. The compounds with highest and lowest z-scores are identified and their z-scores are stated in parentheses. (d) Structures of compounds with z-scores ((i)-(iv)) lower than -2 and ((v)-(vii)) greater than 2.

Fig. 2 Kinase selectivity of compounds assigned z-scores. The full list of kinase selectivity data is shown in Table S5. Table S5 is shown here as a "heatmap" of kinase selectivity wherein the potency of each compound at 1 μM concentration against a particular kinase is represented in colour as indicated at the bottom of the figure (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 compound and each column represents the kinase tested. The z-scores of each compound are indicated to the left of the figure. The kinase groups of each kinase tested are indicated above the figure. (a)-(c) Kinase inhibition of compounds with z-scores of less than -1 not found in

804 compounds with z-scores greater than 1. (d) Kinase inhibition of compounds with
805 z-scores greater than 1 not found in compounds with z-scores of less than -1.
806
807 **Fig. 3 Analysis of HCMV replication and protein production in infected HFF**
808 **cells treated with DMSO or SB-734117 at the time of infection.** (a) HFF cells
809 were infected at MOI1 with AD169 then treated with 1μM SB-734117 or the
810 equivalent volume of DMSO. Viral titre (plaque forming units (p.f.u.)/ml)) was
811 determined at the indicated time points (hours post infection (h.p.i.)). Data points
812 and error bars represent the mean and standard deviation, respectively, from
813 three experiments. (b) HFF cells were infected with AD169 at an MOI of 1, then
814 treated with either 1μM SB-734117 or the equivalent volume of DMSO. Cell
815 lysates were prepared for western blotting at the time points (hours post infection
816 (h.p.i.)) indicated above the figure. Uninfected cells harvested at the time of
817 infection are shown as 0 h.p.i.. Proteins recognized by the antibodies used are
818 indicated to the right of each figure. The positions of molecular mass markers
819 (kDa) are indicated to the left of each figure. (c) Relative band intensity of
820 immediate-early protein bands relative to β-actin signal in the same lane in
821 Figure 3A, as quantified using ImageJ. Band intensities of 0-1, 1-2 and greater
822 than 2 are highlighted in light grey, dark grey and black, respectively. (d) HFF
823 cells were infected at MOI1 with AD169 then treated with 1μM SB-734117 or the
824 equivalent volume of DMSO. Samples were prepared for quantitative PCR
825 analysis of *IE1* and *IE2* mRNA expression, respectively, at the time points
826 indicated in the figures. Data and error bars represent the mean and standard

827 deviation of three PCR replicates from each sample, respectively (n=2). Change
828 in gene expression relative to DMSO is shown for each timepoint using ΔΔCT
829 method.
830
831 **Fig. 4 Analysis of HCMV replication and protein production in infected HFF**
832 **cells treated with DMSO or SB-734117 at 24 hours post infection.** (a) HFF
833 cells were infected at MOI1 with AD169 then treated with 1μM SB-734117 or the
834 equivalent volume of DMSO at 24 hours post infection. Viral titre (plaque forming
835 units (p.f.u.)/ml)) was determined at 120 hours post infection (h.p.i.). (b) HFF
836 cells were infected with AD169 at an MOI of 1, then treated with either 1μM SB-
837 734117 or the equivalent volume of DMSO at 24 hours post infection. Cell
838 lysates were prepared for western blotting at the time points (hours post infection
839 (h.p.i.)) indicated above the figure. Infected cells harvested at 24 h.p.i. that were
840 not treated with either SB-734117 or DMSO were also assayed. Proteins
841 recognized by the antibodies used are indicated to the right of each figure. The
842 positions of molecular mass markers (kDa) are indicated to the left of each figure.
843 (c) Relative band intensity of immediate-early protein bands relative to β-actin
844 signal in the same lane in Figure 3A, as quantified using ImageJ. Band intensities
845 of 0-1, 1-2 and greater than 2 are highlighted in light grey, dark grey and black,
846 respectively.
847
848 **Fig. 5 Inhibition of phosphorylation of cellular proteins by SB-734117** ((a),
849 (c)-(3)) HFF cells were infected with AD169 at an MOI of 1, then treated with

either 1μM SB-734117 or the equivalent volume of DMSO (as indicated above each Figure). Cell lysates were prepared for western blotting at (a) 72 h.p.i. or ((c)-(e)) as indicated above the figure. Uninfected cells harvested at the time of infection are shown as 0 h.p.i. in (c)-(e). In (a) no lysate was analyzed in lanes 2 and 4. Proteins recognized by the antibodies used are indicated to the right of each figure. The positions of molecular mass markers (kDa) are indicated to the left of each figure. (b) Relative band intensity of CREB and CREB-Ser133 bands relative to β-actin signal in the same lane in Fig. 4(a), as quantified using ImageJ. Band intensities of 0-1, 1-2 and greater than 2 are highlighted in light grey, dark grey and black, respectively.

Fig. 6 H3K14ac at the MIEP is unaffected in SB-734117 treated cells. DNA was immune-precipitated from infected cells (24-72hpi) treated with either DMSO or SB-734117 using an anti-H3K14ac antibody (K14) or isotype control (C), then amplified in an MIEP qPCR. Enrichment of MIEP sequences was expressed relative to amplification in Input sample. Data and error bars represent the mean and standard deviation of three PCR replicates from each sample, respectively

TABLES

Table 1

Chemotype	Total no. of compounds in chemotype	No. of compounds excluded due to cytotoxicity	No. of compounds assigned z-scores.
4-pyrimidinyl ortho-aryl azoles	31	0	31
Oxindoles	30	3	27
Furazan benzimidazoles	25	1	24
4-anilino quinazolines and related	25	0	25
Benzimidazole N-thiophenes	21	9	12
4-pyridyl ortho-aryl azoles	18	0	18
2H-3 pyrimidinyl pyrazolopyridazines	16	7	9
2-amino oxazoles	15	0	15
4-hydrazinly pyrazolopyrimidines	15	0	15
2,4-dianilino pyrrolopyrimidines	15	1	14
Biaryl amides	14	0	14
3-vinyl pyridines	13	0	13
Anilino thienopyrimidines	12	0	12
Benzimidazolyl diaryl ureas	12	2	10
2-aryl 3-pyridimidinyl pyrazolopyridazines	12	0	12
2,4-diamino pyrimidines	12	0	12
Maleimide	11	0	11
Furopyrimidines and related	9	0	9
Indazole-3-carboxamides	7	1	6
3-amino pyrazolopyridines	7	3	4
2-pyridinyl imidazoles and related	7	0	7
4-anilino 5-alkynyl pyrimidines	7	0	7
3-cyano thiophenes	6	2	4
Phenyl carboxamides	6	0	6
Indazole-5-carboxamides	6	0	6
3-amino pyrazolopyridazines	4	1	3
3-amino pyrazoles	3	0	3
Imidazotriazine	3	0	3
4-anilino quinolones	2	0	2
6-phenyl isoquinolines	2	1	1
3-benzyl pyrimidines	1	0	1

874 Table 2

875

Experiment	Viral Strain	Compound	ED50* (μM)
1	AD169	SB-734117	0.5
	AD169	GCV†	0.5
2	AD169	SB-734117	0.4
	AD169-P53	SB-734117	1.3
3	AD169	SB-734117	1
	Merlin(RCMV1111)	SB-734117	2

876 *50% effective dose
877 †Ganciclovir

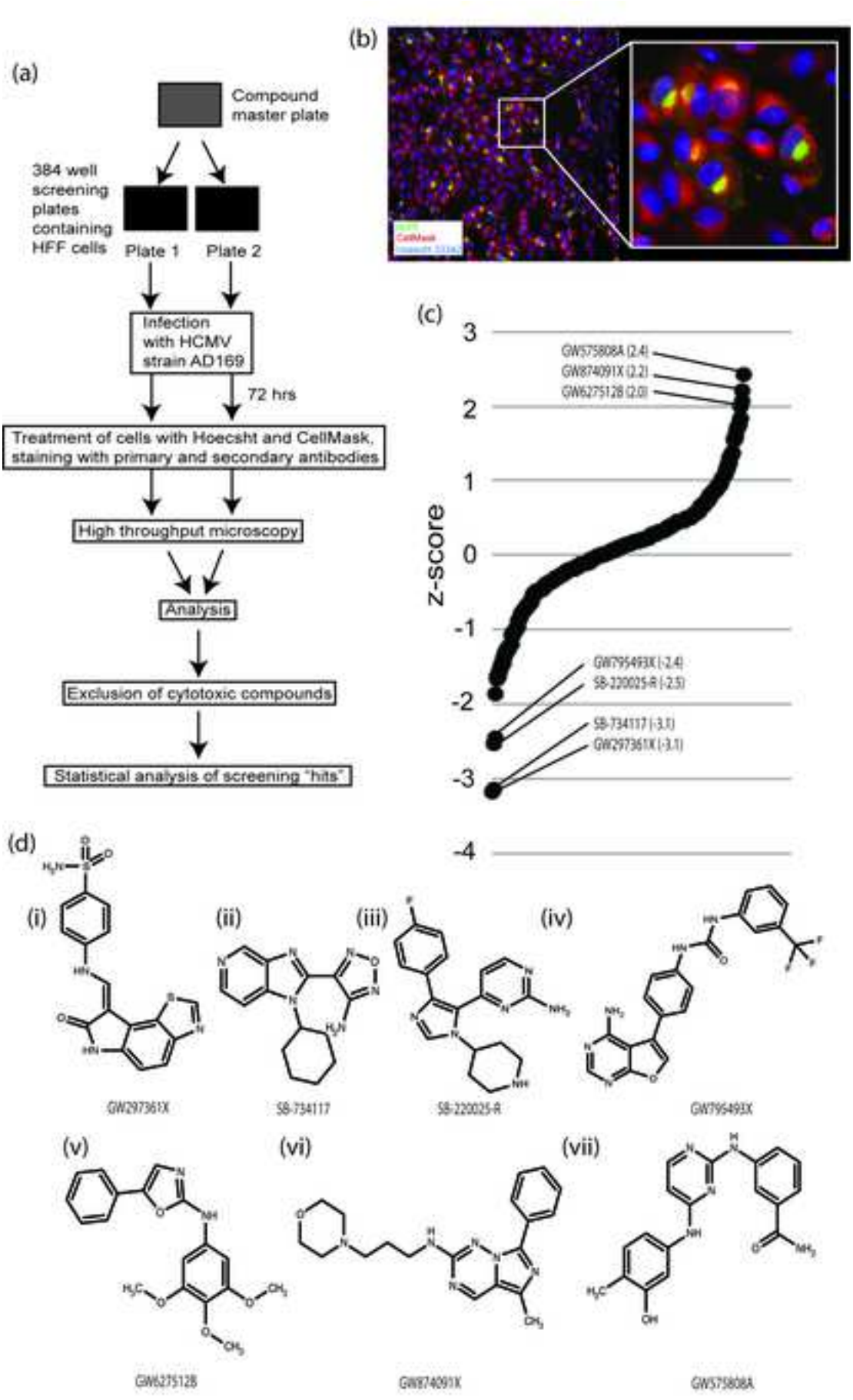


Fig. 1

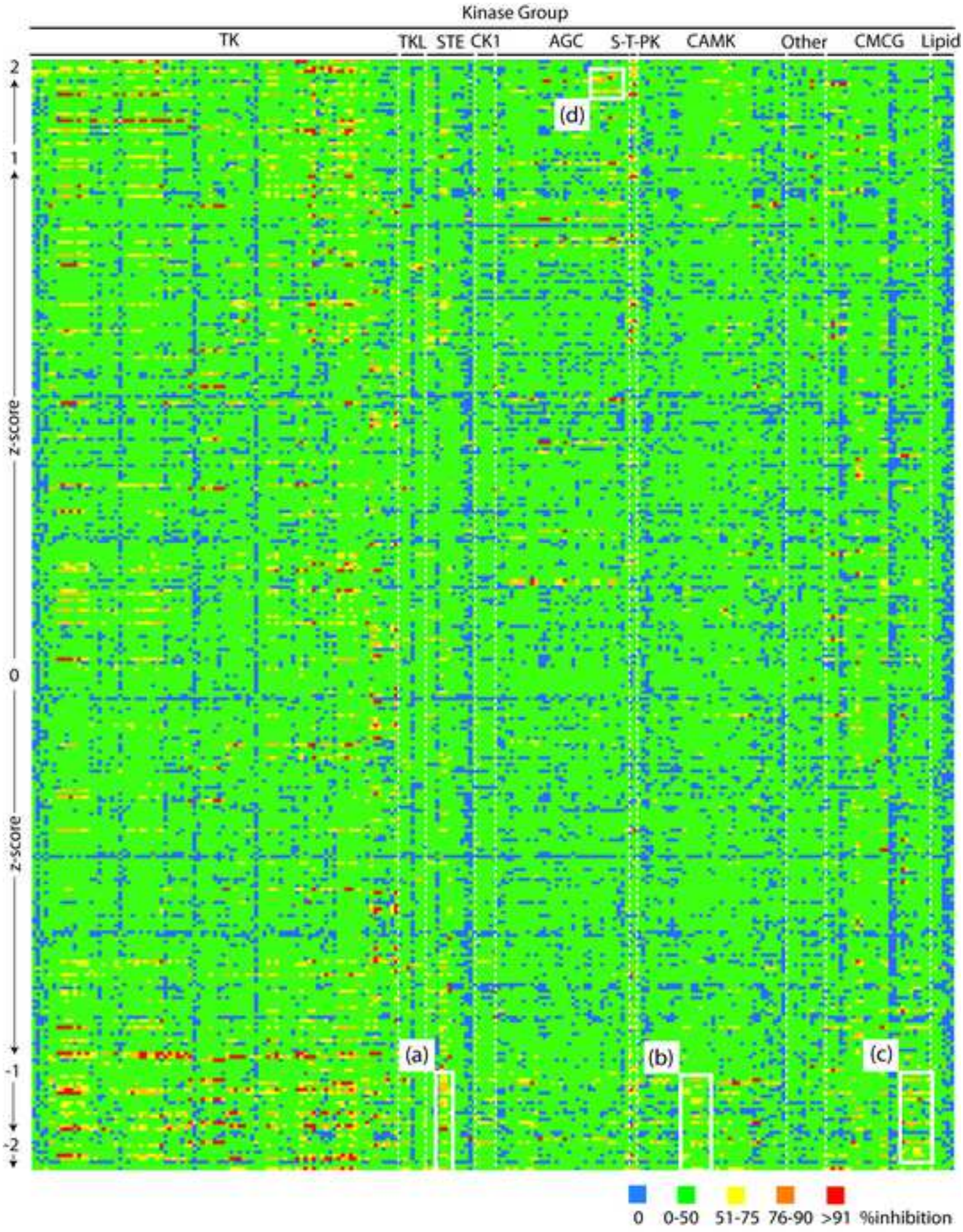
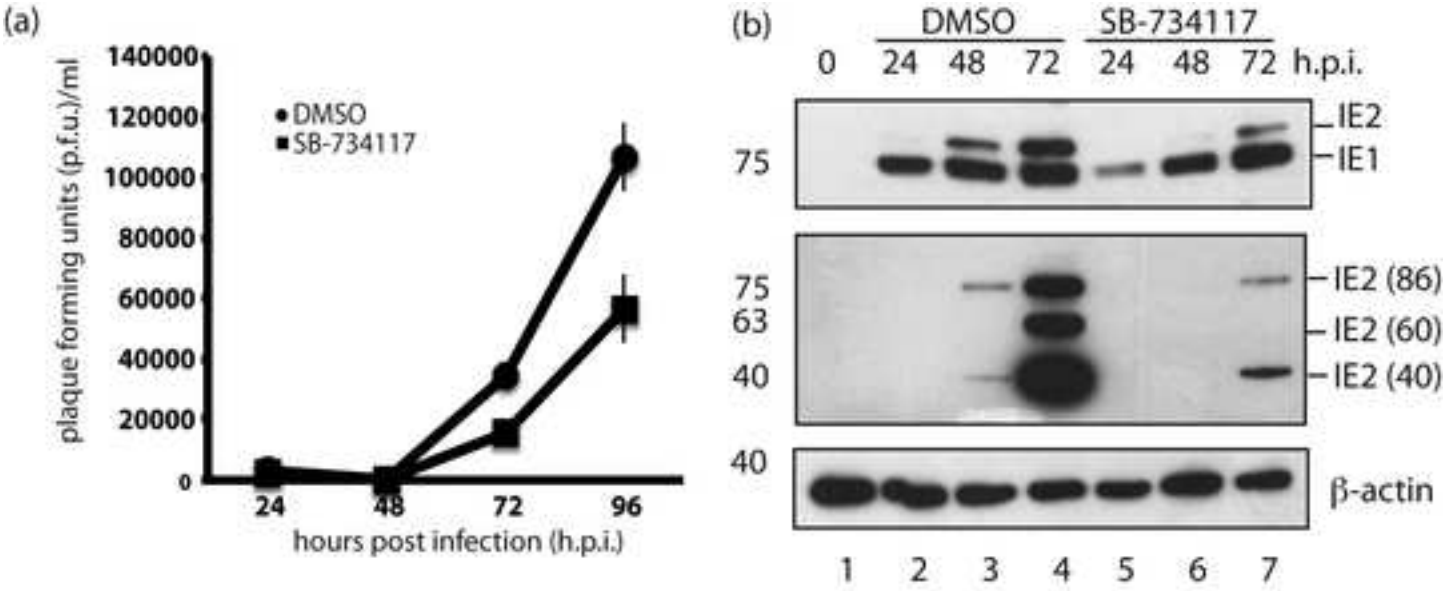


Fig. 2



(c)

	DMSO			SB-734117		
	h.p.i.			h.p.i.		
Protein	24	48	72	24	48	72
IE1	0.51	1.65	2.25	0.16	0.52	1.38
IE2	0	1.46	4.19	0	0	1.50
IE2(86)	0	0.21	6.28	0	0	0.37
IE2(60)	0	0	6.98	0	0	0
IE2(40)	0	0.03	5.72	0	0	1.27

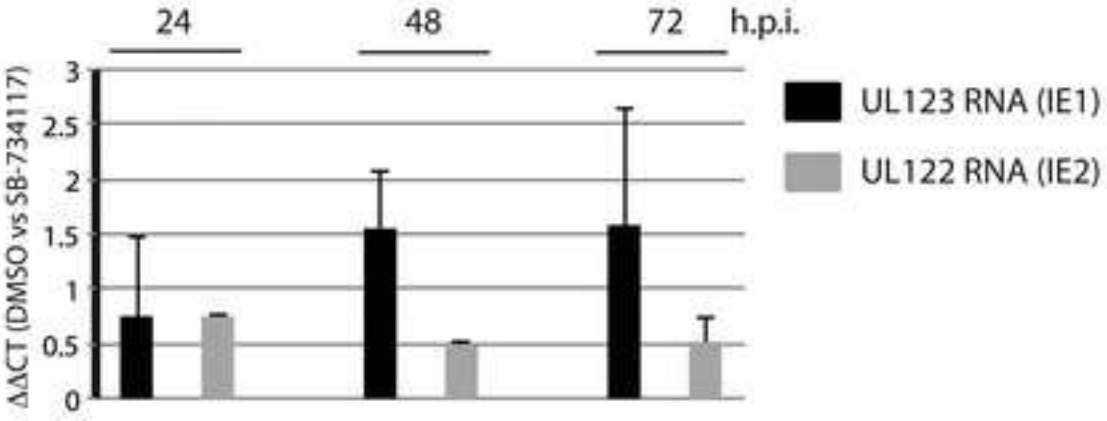
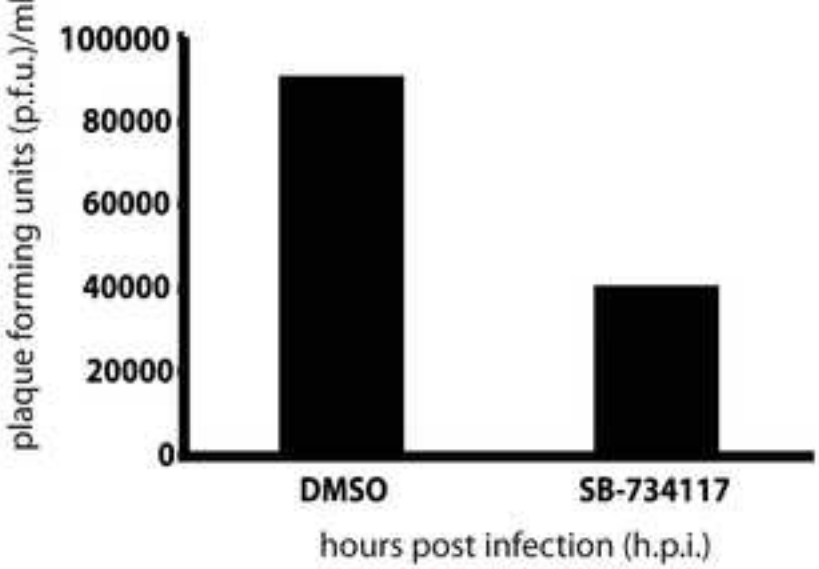
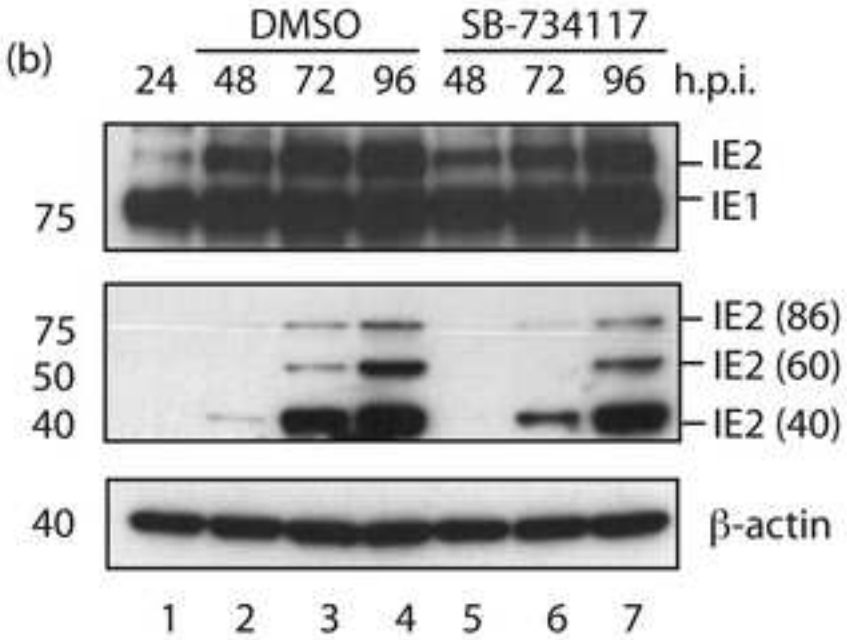


Fig. 3

(a)



(b)



(c)

Protein	h.p.i.	DMSO h.p.i.			SB-734117 h.p.i.		
	24	24	48	72	24	48	72
IE1	1.10	1.10	1.00	1.02	1.02	0.81	0.87
IE2	0.11	0.81	1.32	1.08	0.62	1.32	1.74
IE2(86)	0	0	1.10	2.06	0	0.61	2.64
IE2(60)	0	0	0.23	3.66	0	0	2.92
IE2(40)	0	0.09	1.86	2.06	0	0.90	2.14

Fig. 4

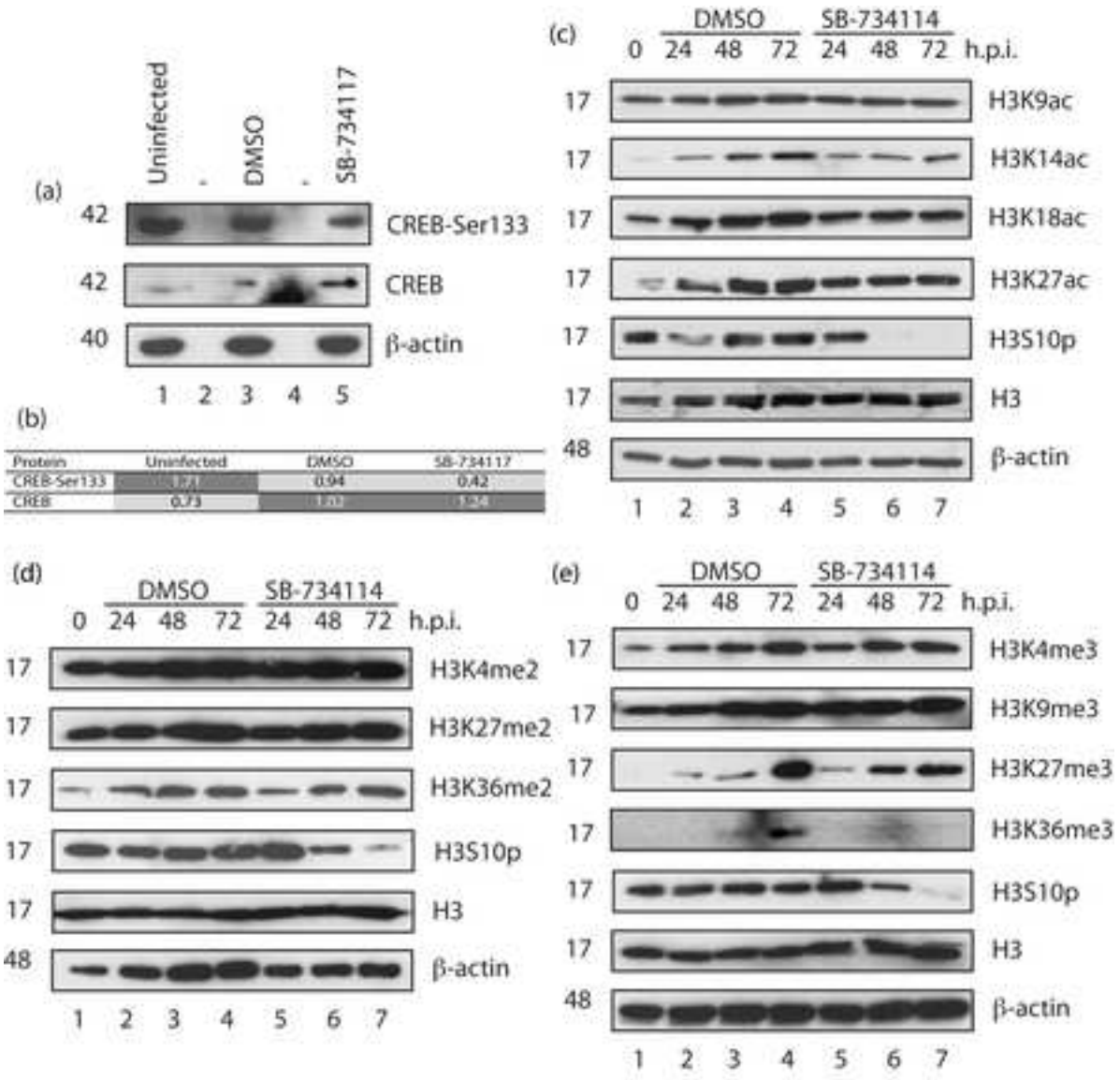


Fig. 5

Figure 5

Figure6

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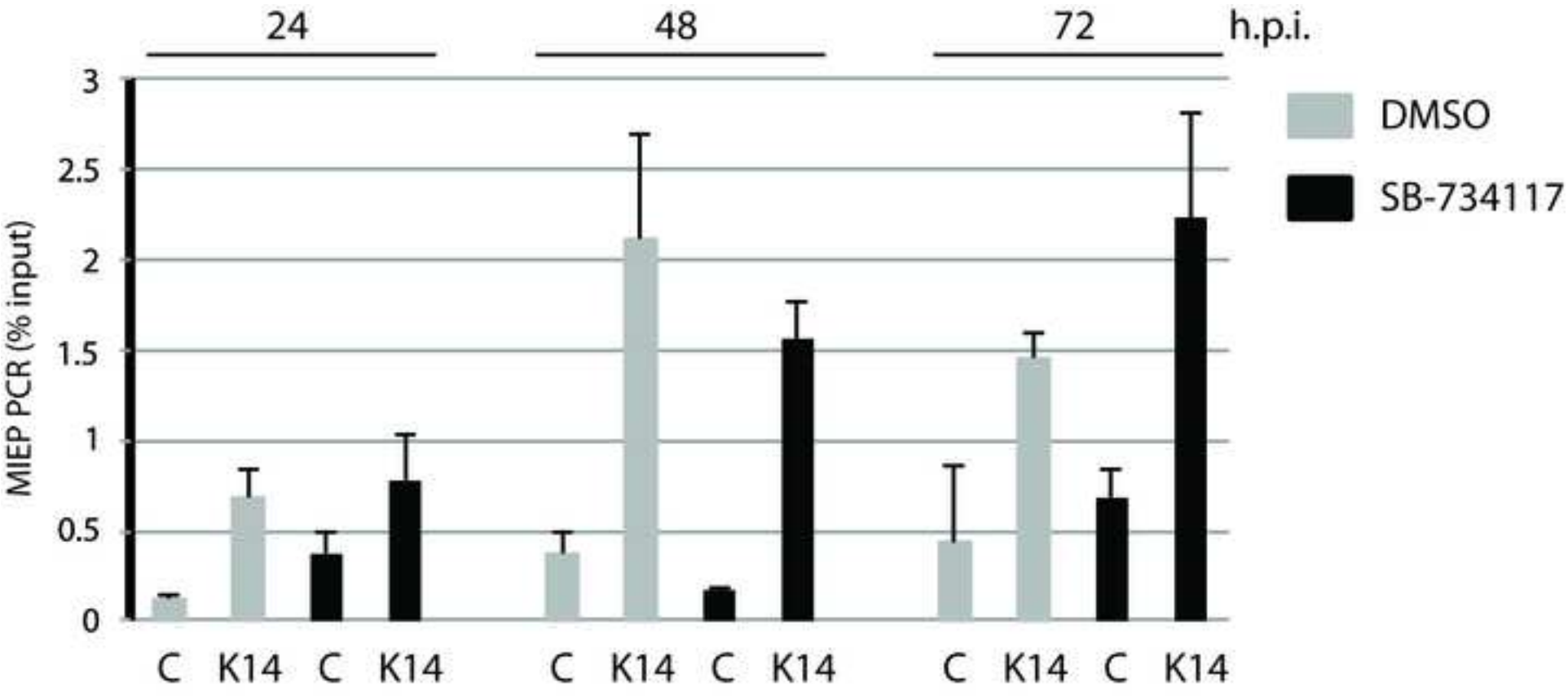

Fig. 6

SUPPLEMENTARY MATERIAL

High Throughput Screening of a GlaxoSmithKline Protein Kinase Inhibitor Set Identifies an Inhibitor of Human Cytomegalovirus Replication that Prevents CREB and Histone H3 Post-Translational Modification

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Matthew B Reeves[2] & Blair L Strang[1,4]

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Compound treatment and infection of cells for high throughput screening. The GSK PKIS library [1] (stock concentration of 3.3 mM of each compound in DMSO) was screened in duplicate. Twenty four hours before infection 2000 HFF cells were seeded in each well of each Corning 384 plate. Unless stated otherwise, liquid was added to wells using a WellMate apparatus. At the time of infection, media was removed with a suction manifold and 30 μl of complete media was added to each well. Compounds were added to the plate containing HFF cells using a 100 nl pin transfer on a liquid handling robot. Negative and

positive controls (water+0.3% DMSO or heparin sulfate (5 μg/ml) + 0.3% DMSO, respectively) were added to plates by hand (12 wells of each). Cells were then infected with HCMV strain AD169 (MOI 1) in a total volume of 5 μl. Thus, the final concentration of compound in each well was 9.4 μM. Infected cells were incubated for 72 hours at 37°C, then prepared for analysis.

Preparation of screening plates for high throughput microscopy analysis. Cell culture media was removed from infected cells and replaced with 20 μl Hoecsht 33342 (SIGMA) diluted in PBS to a final concentration of 10 μg/ml. After incubation for 1 hour at 37°C, 20 μl of Deep Red Cell Mask (Invitrogen) (diluted in PBS to a concentration of 5 μg/ml) was added to each well. Cells were incubated for a further 5 min at 37°C. Cells were then fixed by removing PBS containing Hoescht and Cell Mask and adding 50 μl of 3.5%Formaldyhyde (SIGMA) in PBS to each well. After incubating at room temperature for 10 min, fixative was removed and 50 μl of PBS containing 0.5% TritonX-100 was added per well to permeablize cells. After 10 min incubation at room temperature, PBS containing detergent was removed, and cells was washed once with PBS. PBS was removed and replaced with 20 μl MAb P207 recognizing pp28 (Virusys) (dilution 1:1000) and anti-mouse secondary antibody conjugated to flurophore Alexa488 (Molecular Probes) (dilution 1:1000). Plates were incubated at 37°C for 1 hour. After incubation, PBS containing antibodies was removed and replaced with 50 μl of PBS. Plates were then analyzed using automated microscopy for the presence of pp28 protein.

Microscopy analysis of screening plates. Infected cells stained with antibody to detect pp28 were imaged on an Image Express Micro (IXM) microscope (Molecular Devices) at 10x magnification to detect 3 wavelengths; 488 nm to detect antibody recognizing pp28, 568nm to detect Deep Red CellMask and 350 nm to detect Hoescht 33342 stain bound to nuclear DNA. Three images were captured from each wavelength in each well of the 384-well plate. The number of cells positive at all 3 wavelengths and percentage of pp28 positive cells in each well was determined using the Metamorph Multiwavelength Cell Scoring software (Molecular Devices). Typically, approximately 60% of cells were infected in wells treated with negative control, DMSO (data not shown).

Analysis of screening results. To assess the quality of data that could be returned from the screening protocol we calculated the Z'-factor [2, 3] derived from the positive (heparan sulphate treated infected cells) and negative (DMSO treated infected cells) control wells. The screening controls returned Z'-factors of greater than or equal to 0.5, indicating a robust separation of difference in the data derived from positive and negative controls (data not shown). Thus, the screening protocol could be reliably used to screen the compound collection.

After screening of the compound collection, data was discarded from any well in which the number of cells stained with Hoescht 33342 fell below 2-fold of the mean of the number of cells in each well of the plate. The data from the remaining wells from each plate was converted to a z-score (the number of

standard deviations from the mean of the data [2, 3]) and the average z-score from data in duplicate plates was determined. Images chosen at random were visually inspected throughout image capture and analysis to ensure raw data was consistent with z-scores.

Characterization of compounds within the GSK PKIS collection. Characterization of compounds has been reported by Elkins and co-workers [4]. Kinase profiling was previously performed by using the Nanosyn microfluidics capillary electrophoresis technology (based on the change in electrophoretic mobility of a substrate upon phosphorylation) to determine each compounds ability to inhibit a panel of 224 recombinant kinase proteins. GPCR screening using calcium mobility assays was carried out as previously described [4]. The structure of each compound is available at ChEMBL (https://www.ebi.ac.uk/chembl/) [1].

Primary and secondary antibodies used in western blotting. Membranes were probed with antibodies recognizing IE1/2, UL44, pp28, UL84 (all Virusys, 1:1000 dilution), IE2 proteins (clone 5A8.2, Millipore, 1:1000 dilution), β-actin (SIGMA, 1:5000 dilution), CREB (product no. 06-863) or CREB-Ser133 (product no. 06-519) (both Millipore, 1:500 dilution). Antibody recognizing UL97 [5] was a kind gift from Donald Coen (Harvard Medical School, USA), respectively. All antibodies recognizing histone proteins were obtained from Cell Signaling Technology (products #9927, #9847, #9783) and used as per suppliers instructions. 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 in each case to detect secondary antibodies using film.

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