Cell-impermeable staurosporine analog targets extracellular kinases to inhibit HSV and SARS-CoV-2

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Herpes simplex virus (HSV) receptor engagement activates phospholipid scramblase triggering Akt translocation to the outer leaflet of the plasma membrane where its subsequent phosphorylation promotes viral entry. We hypothesize that this previously unrecognized outside-inside signaling pathway is employed by other viruses and that cell-impermeable kinase inhibitors could provide novel antivirals. We synthesized a cell-impermeable analog of staurosporine, CIMSS, which inhibited outer membrane HSV-induced Akt phosphorylation and blocked viral entry without inducing apoptosis. CIMSS also blocked the phosphorylation of 3-phosphoinositide dependent protein kinase 1 and phospholipase C gamma, which were both detected at the outer leaflet following HSV exposure. Moreover, vesicular stomatitis virus pseudotyped with SARS-CoV-2 spike protein (VSV-S), but not native VSV or VSV pseudotyped with Ebola virus glycoprotein, triggered this scramblase-Akt outer membrane signaling pathway. VSV-S and native SARS-CoV-2 infection were inhibited by CIMSS. Thus, CIMSS uncovered unique extracellular kinase processes linked to HSV and SARS-CoV-2 entry.
Hepatitis B virus serotypes 1 and 2 (HSV-1 and HSV-2) are major global health problems, representing leading causes of infectious corneal blindness, sporadic fatal encephalitis, and significant perinatal disease. The public health impact of HSV-2, in particular, is magnified because it is a key coinfection fueling the HIV epidemic. The epidemiology of HSV highlights the need to identify novel strategies for treatment. The molecular complexity of HSV entry has impeded the development of antivirals targeting this process. We previously demonstrated that HSV enters most human epithelial cells through a complex calcium-dependent signaling pathway. Specifically, binding of HSV envelope glycoprotein C (gC) (HSV-1) or glycoprotein B (gB) (HSV-2) to cellular heparan sulfate proteoglycans and engagement between glycoprotein D (gD) and a cellular receptor, most commonly nectin-1, triggers intracellular calcium ion (Ca^{2+}) transients near the plasma membrane. These transients activate phospholipid scramblase 1 (PLSCR1), a Ca^{2+}-responsive enzyme responsible for the bidirectional translocation of phospholipids, including phosphatidylserine (PtdSer), between the inner and outer leaflets of the plasma membrane. Notably, we found that these lipid movements are also associated with the translocation of Akt to the outer leaflet of the plasma membrane where it is phosphorylated by yet to be determined kinases. Extracellular phosphorylation of Akt is associated with downregulation of antiviral genes and upregulation of proinflammatory genes. This process may be a general mechanism for HSV to evade the host immune response.

Results

Design, synthesis and characterization of a cell-impermeable staurosporine analog. Hypothesizing that inhibition of Akt and other upstream or downstream activating kinases that have been translocated to the outer leaflet of the plasma membrane would modulate viral entry, we selected the well-studied broad spectrum kinase inhibitor staurosporine as a scaffold to develop an inhibitor that would selectively target extracellular kinases. As there was no extant structure of an Akt:staurosporine complex, we generated a model by overlaying the coordinates of Akt1 (one of three Akt isoforms) bound to a small molecule inhibitor (Protein Data Bank (PDB) entry 3MVH) with those of the related ribosomal protein S6 kinase beta-1, p70(S6K1), bound to staurosporine (>46% sequence identity, PDB entry 3A60). The structural alignment revealed close agreement between the protein coordinates (RMSD = 1.0 Å for 211 aligned Ca pairs). The 2nd and 3rd moieties of staurosporine appeared to be solvent accessible and unhindered in the resulting model and thus represented a candidate for synthetic elaboration to introduce a solvent exposed polar group, which would impair cell entry while maintaining Akt binding and inhibitory properties.

Based on this modeling, we devised a synthetic scheme to install a moeity bearing a sulfonate functionality at the 2nd amine of staurosporine, yielding a cell-impermeable analogue of staurosporine (Fig. 1a). We hypothesized that CIMSS would inhibit HSV entry without inducing apoptosis or interfering with intracellular Akt signaling pathways triggered by other stimuli such as activation of insulin receptors. In an in vitro kinase inhibitor assay performed against a panel of 393 kinases with 10 μM ATP, CIMSS (10 μM) retained the pleiotropic inhibitory property of staurosporine including inhibition of all three Akt isoforms (Akt1, Akt2, Akt3) and PDK1, the enzyme responsible for cytoplasmic phosphorylation and activation of Akt isoforms, by >75% (Supplementary Data 1). Similar levels of inhibition were observed for a range of Src family kinases, Aurora kinases, JAK kinases, and receptor tyrosine kinases. The 50% inhibitory concentration (IC_{50}) for CIMSS and staurosporine were comparable for PDK1, for example, but CIMSS was less effective than staurosporine for other kinases, including insulin receptor, Akt1 and PKA (~40, ~230, and ~46-fold less effective, respectively). The altered inhibitory profile of CIMSS is consistent with a previous report, in which acylation of the 4'-methylamine negatively impacted IC_{50}s, with increases of 1–2 orders of magnitude for diverse kinases, including PIM1 (>200-fold), CHK1 (84-fold), CDK2 (50-fold), and PKA (14-fold).

The low permeability of CIMSS compared to control compounds was demonstrated by measuring the apparent permeability coefficient (Papp) with confluent MDCK cells as well as in an artificial membrane permeability assay (PAMPA) (Supplementary Table 2). This behavior was further assessed in cell viability studies, which demonstrated that unmodified staurosporine significantly inhibited HaCat (human keratinocyte) cell growth at concentrations as low as 1 μM at 24 h (h), and 0.1 μM at 72 h, while no significant inhibition was observed with CIMSS except when cells were cultured for 72 h in media containing 100 μM CIMSS (Fig. 1b). Similarly, little or no cytotoxicity was observed when fully confluent HaCat cells were incubated with 10 μM CIMSS or when ~50% confluent primary vaginal epithelial cells were cultured with 10 or 50 μM CIMSS for up to 120 h (Fig. 1c, d).
Fig. 1 Synthesis of cell impermeable staurosporine analog (CIMSS) and the effects of the parental drug and the analog on cell proliferation and viability. a Scheme illustrating the synthetic route to the sodium salt of CIMSS: the secondary amine of staurosporine was derivatized via amidation of succinic anhydride and the resulting carboxylate (compound 5) was condensed with aminomethyl-triazol propane-sulfonate (compound 4, which was obtained in four steps from NBoc-propargylamine and azidopropanol) to afford CIMSSNa in modest yield as a white powder. b Confluent HaCat cells (~50% confluence) were cultured in media containing increasing concentrations of staurosporine or CIMSS (0.1–100 µM) or the equivalent concentration of DMSO (0.1, 0.5, or 1%) and cell proliferation and viability (optical densitometry units, odu) quantified after 24 and 72 h. c Confluent HaCat cells were cultured in media containing 10 µM CIMSS, 10 µM staurosporine or 0.1% DMSO (control) and viability assessed after 24, 72 and 120 h. Note that staurosporine was completely cytotoxic after 72 and 120 h of exposure and thus no bar is visible. d Primary vaginal epithelial cells (~50% confluent) were cultured in media containing 0.5% DMSO, 10 or 50 µM CIMSS or 1 or 10 µM staurosporine for 120 h and cell proliferation and viability monitored. Results are presented as mean ± SEM odu as a percentage of the DMSO control (n = 2 independent experiments each conducted in duplicate for e and 1 experiment conducted in duplicate for d). Results in b–d were compared by ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Consistent with its cell impermeability, 10 µM CIMSS did not induce apoptosis when assessed by SYTOX Green and anti-caspase antibody staining (Fig. 2a, b). Similarly, compared to cells treated with 0.1 µM DMSO, CIMSS at doses of 0.1, 1, or 10 µM did not induce PARP-1 or caspase 8 cleavage whereas cleavage of both was observed with staurosporine at concentrations as low as 0.1 µM (Fig. 2c and Supplementary Fig. 1). Furthermore, CIMSS did not block Akt phosphorylation in response to insulin. HaCat cells were treated with 10 µM insulin in the absence or presence of CIMSS (10 or 100 µM) or staurosporine (0.1 µM), fixed with or without Triton X-100 permeabilization and stained with conjugated antibodies to detect phosphorylated Akt (pAkt 308) (red) or total Akt (green); nuclei were stained with DAPI (blue). We used higher doses of CIMSS than staurosporine based on the in vitro kinase activity (Supplementary Data 1). Following insulin treatment, Akt was only visualized in permeabilized cells and its intracellular phosphorylation was inhibited by staurosporine at doses as low as 0.1 µM, but not by 100 µM CIMSS (Fig. 2c). These findings indicate that engagement of the insulin receptor by insulin does not trigger translocation of Akt to the outer leaflet of the plasma membrane and that phosphorylation of cytoplasmic Akt is inhibited by staurosporine but is insensitive to CIMSS. These observations, together with direct permeability measurements and lack of apoptotic induction, are consistent with our proposition that CIMSS is membrane impermeable.

CIMSS inhibits HSV infection and blocks the viral induced phosphorylation of Akt. Given the association of Akt translocation and its phosphorylation with HSV cell entry, we examined whether CIMSS would inhibit HSV infection by plaque assay and found that it inhibited HSV-2 infection of HaCat and primary vaginal epithelial cells in a dose dependent manner with greater than 75% inhibition at a dose of 10 µM (Fig. 3a). Based on these results and the cell viability data, 10 µM CIMSS was used for subsequent studies. The effects of CIMSS or antibodies to Akt on HSV entry were assessed using complementary assays. First, the kinetics of viral entry were compared using a synchronized infection assay. HaCat cells were exposed to 150–200 pfu/well of HSV-2(G) at 4 °C for 4 h to allow virus to bind, washed, transferred to 37 °C (a temperature permissive for viral entry) in fresh media containing 10 µM CIMSS or DMSO. At the indicated times post-temperature shift (0.5, 1, 1.5, or 2 h), the cells were treated with a low pH buffer to inactivate any extracellular virus, washed, overlaid with methylcellulose and viral plaques counted at 48 h. The addition of CIMSS at each timepoint during the viral entry period resulted in a significant reduction in viral plaque formation (Fig. 3b, p < 0.001). Second, the effects of CIMSS or anti-Akt antibodies on nuclear transport of the viral tegument protein, VP-16, a surrogate marker for viral entry, were assessed by preparing immunoblots of nuclear extracts 1 h post-infection14.
Fig. 2 CIMSS does not induce apoptosis or block the intracellular phosphorylation of Akt in response to insulin. HaCat cells were exposed to 0.1% DMSO, 10 µM CIMSS, or 10 µM staurosporine and after 6 or 24 h of incubation, the cells were fixed and stained for activated caspases (red), integrity of plasma membrane with SYTOX Green, and nuclei (Hoechst stain, blue). a Representative images taken with ZeissLive/DuoScan (objective 100×1.4, bar = 10 µm) and b the percentage of cells positive for activated caspase and SYTOX Green at 24 h was quantified after counting ~100 cells from four independent fields, n = 3 experiments; asterisks indicate significance relative to DMSO (unpaired t-test, ***p < 0.001; ****p < 0.0001). c HaCat cells were exposed to 0.1% DMSO, 0.1, 1, or 10 µM CIMSS or 0.01, 0.1, 1, or 10 µM staurosporine and after 8 h of incubation, lysates were prepared and analyzed by western blotting for cleaved PARP-1 or cleaved caspase 8. The intensity of cleaved PARP-1 or caspase 8 (relative to β-actin) is indicated below each lane. The immunoblot is representative of two independent experiments. d HaCat cells were exposed to insulin (10 µM) in the absence or presence of 10 or 100 µM CIMSS or 0.1µM staurosporine for 30 and 120 min, fixed with or without Triton X-100, stained for nuclei (blue), pAktT308 (red), or total Akt (green). Representative images from two independent experiments obtained with Leica SP8 microscope equipped with objective 63×1.4 are shown (bar = 10 µm).

The blots were also stained with an antibody to histone 1 (nuclear marker) and anti-golgin-97 (cytoplasmic marker). CIMSS and anti-Akt IgG (but not control IgG) inhibited the nuclear transport of VP-16 in HaCat and primary vaginal cells (Fig. 3c and Supplementary Fig. 2). The effects of CIMSS or anti-Akt antibodies on viral entry were also assessed by quantifying viral capsid transport to the nuclear pore by confocal imaging12. Cells were synchronously infected with HSV-1 VP2613, and entry monitored by confocal microscopy in non-permeabilized cells within 15 min of exposure to HSV, but were no longer detected on the cell exterior at 120 min in DMSO treated cells, in agreement with the previously described kinetics and restoration of the membrane lipid distribution in response to HSV entry2. CIMSS did not block the translocation of PtdS and Akt to the outer leaflet of the plasma membrane (Fig. 4d and Supplementary Fig. 5a). PtdS and Akt were detected by microscopy in non-permeabilized cells within 15 min of exposure to HSV, but were no longer detected on the cell exterior at 120 min in DMSO treated cells, in agreement with the previously described kinetics and restoration of the membrane lipid distribution in response to HSV entry2. CIMSS did not block the translocation of PtdS and Akt to the outer leaflet, but did reduce levels of phosphorylated Akt (serine 473 and threonine 308) detected in non-permeabilized cells, indicating that these phosphorylation events (unlike PLS1 phosphorylation) occur extracellularly (Fig. 4e and Supplementary Fig. 5b). Treatment with CIMSS also resulted in a significant reduction in the extended Ca2+ release (quantified for the first hour p.i.) (Fig. 4f) as well as a decrease in phosphorylated Akt detected in permeabilized cells (Fig. 4e)-findings reflective of our prior observations that these cellular responses are triggered by viral entry2–4. Notably, Akt (but not PtdS) was still detected in non-permeabilized cells at 120 min in CIMSS but not DMSO treated cells (Fig. 4d). Using a small molecular PLS1 inhibitor, we previously demonstrated that PtdS relocalization is a PLS1-dependent process5. The differences in the repartitioning of PtdS and Akt might reflect different internalization mechanisms. Akt colocalizes with HSV gB in immunoprecipitation studies2 and could be retained on the outside if viral entry does not occur whereas reinternalization of PtdS may occur independent of viral entry.
HSV also triggers translocation of PDPK1 and PLCγ and their subsequent phosphorylation is inhibited by CIMSS. We hypothesized that other kinases typically associated with the inner leafllet might also translocate to the outer leafllet of the plasma membrane in response to PLSCR1 activation and be susceptible to the inhibitory effects of CIMSS. We focused on PDPK1, which activates Akt and other kinases, and PLCγ, which is involved in activating intracellular Ca^{2+} signaling pathways and may be a substrate for phosphorylated Akt.\ref{3,14,15}. Prior to HSV exposure, neither PDPK1, PLCγ or Akt were detected in the membrane fraction following biotinylation of cell surface proteins, precipitation with streptavidin beads, and immunoblotting of the precipitated proteins with antibodies for the respective proteins. The proteins were detected in the whole cell lysates. However, within 15 min of HSV exposure, total and phosphorylated PDPK1, PLCγ and Akt were detected in the membrane fraction but their phosphorylation was reduced when the cells were treated with CIMSS (Fig. 5a and Supplementary Fig. 6). As an additional control, blots were probed for FIC-1 (floppase), a cytosolic protein, which was only detected in the whole cell lysates, but not in the membrane fraction. Similar results were obtained by confocal microscopy. No phosphorylated PDPK1 or PLCγ signal was detected in cells prior to HSV exposure, but both phosphorylated proteins were detected in images obtained 15 and 30 min following viral infection and their phosphorylation was inhibited by CIMSS (Fig. 5b).

Transfection of HaCat cells with siRNA targeting Akt1 (the dominant isoform) or PDPK1 reduced protein expression as assayed by immunoblot to 14% and 24%, respectively, relative to cells transfected with a control siRNA (Fig. 6a and Supplementary Fig. 7a). The silencing of Akt1 had no effect on HSV-triggered phosphorylation of PDPK1, but resulted in a reduction in phosphorylated PLCγ in non-permeabilized cells. Conversely, silencing of PDPK1 was associated with a reduction in phosphorylated Akt (Fig. 6b). These findings demonstrate that PDPK1 and PLCγ are also translocated to the outer leafllet of the plasma membrane in response to HSV. They further suggest a model, similar to intracellular signaling pathways, in which PDPK1 is activated upstream and PLCγ downstream of Akt phosphorylation when these kinases are translocated to the outer leafllet of the plasma membrane. Silencing of Akt1 and PDPK1 were each associated with a significant reduction in HSV infection by plaque assay (Fig. 6c). To further assess the importance of these extracellular ATP-dependent processes on HSV infection, we conducted studies in the presence of apyrase, a cell-impermeable enzyme that hydrolyzes extracellular ATP to AMP. Apyrase had no discernible effect on the detection of PtdS or PLSCR1 at the outer leafllet by fluorescence microscopy, consistent with intracellular activation of PLSCR1, but inhibited the HSV-induced phosphorylation of Akt and PDPK1 in non-permeabilized cells (Fig. 6d).

CIMSS blocks viral entry mediated by SARS-CoV-2 Spike protein. In addition to providing a tool to identify proteins that undergo phosphorylation at the outer leafllet of the plasma membrane, susceptibility to CIMSS may identify other viruses that exploit PLSCR1-dependent signaling pathways to promote viral entry. To test this hypothesis, we evaluated the inhibitory effects of CIMSS on vesicular stomatitis virus (VSV) expressing
its native glycoprotein G (VSV-G), the Ebola virus glycoprotein (VSV-EBOV-GP) or SARS-CoV-2 spike protein (VSV-S); each of these also express enhanced GFP for tracking nano, VSV and EBOV enter cells by endocytosis, whereas SARS-CoV-2 enters by fusion of the viral envelope with the cell plasma membrane as well as by endocytosis. CIMSS (10 µM) had no effect on VSV-G or VSV-EBOV-GP, but significantly reduced VSV-S infection of Vero cells as monitored by quantifying the percentage of GFP⁺ cells (Fig. 7a). We extended the studies with VSV-S to include the human cell lines, Huh7 and Calu-3. SARS-CoV-2 infection of Calu-3 cells has been shown to be highly dependent on expression of the cellular protease TMRPSS2, which triggers the cleavage of spike to release the S2 fusion subunit and is insensitive to chloroquine, an inhibitor of endocytosis. Western blots demonstrated that Huh7 and Vero cells also express TMRPSS2 (Fig. 7b and Supplementary Fig. 7b). Both CIMSS and casomaltose, a protease inhibitor that blocks TMRPSS2 activity, individually inhibited VSV-S infection of all 3 cell lines (Calu-3, Vero and Huh7) in a dose-dependent manner (Fig. 7c, d). Importantly, CIMSS exhibited inhibitory activity against authentic SARS-CoV-2 (WA1/2020) infection of human Huh-7.5 cells at 24 and 72 h. The percentage of infected cells was determined by automated microscopy after staining for viral nucleoprotein to identify infected cells and for total cell number by nuclear staining with Hoechst 33342 (Fig. 7e).

To directly address the role of the PLSCR1 on entry mediated by the SARS-CoV-2 spike protein, we assessed phosphorylation of immunoprecipitated PLSCR1 following exposure of Vero cells to the VSV pseudotyped viruses. As observed with HSV, VSV-S, but not VSV-G or VSV-EBOV-GP triggered phosphorylation of PLSCR1. Phosphorylation of PLSCR1 was preserved in the presence of CIMSS, but was inhibited by staurosporine, as well as by treatment with anti-ACE2 or anti-Spike antibodies (Fig. 8a and Supplementary Fig. 8). VSV-S triggered PLSCR1 phosphorylation was associated with translocation of PtdS to the outer leaflet as assessed by confocal microscopy, which peaked at 30 min with restoration of lipid distribution by 4 h (Fig. 8b and
DuoScan, objective 100 × 1.4) or pPLC were biotinylated and precipitated with streptavidin magnetic beads and analyzed by immunoblotting with Abs to pPDPK1S241 and total PDPK1, pPLC are shown (bars with control siRNA, as judged by western blot (Fig. 9a and Supplementary Fig. 11). Silencing of PDPK1, Akt1 and PLC γ1 indicated time post-infection cells were permeablized in response to VSV-S and their phosphorylation is inhibited by CIMSS. a HaCat cells were mock-infected or infected with HSV-2(G) in the absence or presence of 10 μM CIMSS. After 15 min incubation, cell surface proteins were biotinylated and precipitated with streptavidin magnetic beads and analyzed by immunoblotting with Abs to pPDPK1S241 and total PDPK1, pAktT308 and total Akt, and FIC-1 (cytosolic protein); controls include whole cell lysates. Results are representative of two independent experiments. b HaCat cells were mock-infected or infected with HSV-2(G) (MOI = 10 PFU/cell) in the presence of 0.1% DMSO or 10 μM CIMSS and at the indicated time post-infection were fixed with or without Triton X-100 permeabilization and stained for pPDPK1S241 (green, lower panel, LeicaSP8, objective 63 × 1.4). Representative images from 2–3 independent experiments are shown (bars = 10 μm).

Supplementary Fig. 5c). Moreover, VSV-S (but not VSV-G or VSV-EBOV-GP) triggered extracellular Akt translocation and phosphorylation as assessed following biotinylation of cell surface proteins, precipitation with streptavidin beads, and immunoblotting for pAktT308 or total Akt; the extracellular phosphorylation of Akt was inhibited by CIMSS (Fig. 8c and Supplementary Fig. 9). Similar results were obtained by confocal microscopy; Akt and pAkt as well as PDPK1 and pPDPK1 were detected in non-permeabilized cells in response to VSV-S and their phosphorylation was inhibited by CIMSS (Supplementary Fig. 10).

To evaluate whether this pathway contributed to VSV-S entry, Vero cells were transfected with siRNA targeting Akt1, PDPK1, PLCγ1, or FIC-1 (negative control). Targeted protein expression was reduced to 29–40% of expression detected in cells transfected with control siRNA, as judged by western blot (Fig. 9a and Supplementary Fig. 11). Silencing of PDPK1, Akt1 and PLCγ1 resulted in significant reduction of VSV-S but not VSV-G infection (Fig. 9b). Furthermore, silencing of PDPK1 prevented VSV-S induced phosphorylation of Akt following VSV-S infection as evidenced by microscopy of non-permeabilized and permeabilized cells (Fig. 9c). To further assess the role of this signaling pathway in VSV-S entry, Vero cells were infected with virus in the presence of antibodies that target Akt, PDPK1, the human ACE2 receptor, or an isotype control for 1 h, washed, and infection monitored by counting plaques 24 h pi. Anti-ACE2 (murine mAb), anti-Akt (rabbit polyclonal), and anti-PDPK1 (rabbit polyclonal) significantly inhibited VSV-S, but not VSV-G infection (Fig. 9d).

**Discussion**

We synthesized a cell impermeable analog of staurosporine, CIMSS, which represents the prototype of a new class of tool compounds (to the best of our knowledge). Impermeability of this analog was demonstrated by its partitioning properties, lack of cytotoxicity, inability to induce apoptosis, and failure to inhibit intracellular Akt phosphorylation in response to insulin at concentrations 100–1000-fold greater than the parental drug. The higher concentrations of CIMSS were used in these assays because the analog is generally not as potent and exhibits reduced IC50 relative to staurosporine for a range of kinases. This behavior is not unexpected, as the amide formed by acylation possesses altered hydrogen bonding capabilities relative to the initial secondary amine, and in some cases may impose unfavorable steric10. This argument has been invoked to explain why a staurosporine derivative acylated at the 4′-methylamine exhibits an ~80-fold reduction in affinity for ASK1/MAP3K5 relative to staurosporine22, although there is not complete concordance in the literature as a different acylated staurospine analog exhibited an IC50 similar to that of staurosporine23.

Importantly, CIMSS retains the broad inhibitory profile characteristic of staurosporine. This promiscuity, coupled with impermeability, makes CIMSS an excellent tool compound for examining unique kinase-dependent processes that are occurring at the outer leaflet of the plasma membrane or extracellularly. CIMSS enabled the identification of other cellular proteins (PDPK1 and PLCγ) that are translocated to the extracellular milieu following activation of PLCγ1 by HSV triggered calcium transients. Moreover, CIMSS also

**Fig. 5 HSV triggers translocation of PDPK1 and PLCγ1 to the outer leaflet and their subsequent phosphorylation is inhibited by CIMSS.** a HaCat cells were mock-infected or synchronously infected with HSV-2(G) in the absence or presence of 10 μM CIMSS. After 15 min incubation, cell surface proteins were biotinylated and precipitated with streptavidin magnetic beads and analyzed by immunoblotting with Abs to pPDPK1S241 and total PDPK1, pAktT308 and total Akt, and FIC-1 (cytosolic protein); controls include whole cell lysates. Results are representative of two independent experiments. b HaCat cells were mock-infected or infected with HSV-2(G) (MOI = 10 PFU/cell) in the presence of 0.1% DMSO or 10 μM CIMSS and at the indicated time post-infection were fixed with or without Triton X-100 permeabilization and stained for pPDPK1S241 (green, lower panel, LeicaSP8, objective 63 × 1.4). Representative images from 2–3 independent experiments are shown (bars = 10 μm).
uncovered the ability of SARS-CoV-2 spike protein to activate this outside-in PLSCR1-Akt signaling pathway. This process is distinct from the previously described intracellular activation of the phosphatidylinositol 3-kinase/Akt signaling pathway, which among other intracellular processes, regulates clathrin-mediated endocytosis of SARS-CoV-2 and other viruses. However, the intracellular activation of Akt signaling would not be susceptible to CIMSS as illustrated by the studies with insulin. Notably, HIV also triggers the externalization of PtdS to promote membrane fusion through the activation of a different phospholipid scramblase, TMEM16F, but whether this is associated with externalization of cellular kinases and thus whether HIV entry would be inhibited by CIMSS is not yet known.

Specifically, using CIMSS to discriminate whether a phosphorylation event occurs intracellularly or in association with the outer leaflet of the plasma membrane, we demonstrated that in response to canonical activation of the insulin receptor, Akt is phosphorylated only intracellularly, whereas HSV and VSV-S infection also induce extracellular Akt phosphorylation. In contrast, PLSCR1 itself was phosphorylated intracellularly in response to virally induced Ca^{2+} transients as evidenced by its inhibition by unmodified staurosporine but its insensitivity to CIMSS. Focusing on proteins associated with intracellular Akt signaling, we demonstrated that PLSCR1 activation also resulted in the translocation of PDPK1 and PLCγ to the outer leaflet of the plasma membrane where they are subsequently phosphorylated, as evidenced by confocal imaging, biotinylation of cell membranes, and susceptibility to blockade of these processes by CIMSS. By analogy with the canonical cytoplasmic signaling pathways, we propose that PDPK1 participates in extracellular autophosphorylation and that the resulting activated phosphorylated PDPK1 is likely responsible for the subsequent phosphorylation of outer leaflet Akt since silencing of PDPK1 prevented outer leaflet plasma membrane Akt phosphorylation. Furthermore, activated phosphorylated Akt likely elicits PLCγ phosphorylation, as silencing of Akt results in a reduction in PLCγ phosphorylation at the outer leaflet of the plasma membrane. Notably, CIMSS inhibited PDPK1, Akt, and PLCγ phosphorylation and reduced HSV entry and infection, highlighting the importance of extracellular kinase function/phosphorylation events in viral infection. Evidence for a role of extracellular kinase activity in HSV entry is further provided by the studies with apyrase, which inhibited the phosphorylation of Akt and PDPK1, and with our studies showing that antibodies targeting Akt also inhibit HSV entry and subsequent infection.

CIMSS also served as an effective tool to identify other viruses that might exploit a similar kinase-dependent signaling pathway to promote viral entry. Specifically, we found that VSV, a close relative of HSV, infected cells with the typical morphologic phenotype expected of HSV, but not of VSV-S. As observed with HSV, VSV-S activated phospholipid scramblase to promote translocation of PtdS, PDPK1, and Akt to the outer leaflet and the latter two...
kinases were subsequently phosphorylated in a CIMSS-sensitive manner. Silencing of PDPK1, Akt1, or PLCγ resulted in a reduction in VSV-S infection supporting the importance of this signaling pathway. Additional support for the role of extracellular kinases in VSV-S comes from observation that polyclonal or monoclonal Abs targeting Akt or PDPK1 inhibited VSV-S infection. While the antibody studies do not distinguish between mechanisms involving reductions in catalytic activity or steric blockade of protein-protein interactions involving these kinases, these results, combined with the observation that CIMSS inhibits VSV-S, provide strong evidence that both Akt and PDPK1 extracellular signaling are directly involved in HSV and VSV primary entry. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here. We found that all three cell types, our studies support a role for direct fusion pathway for viral entry in the cells studied here.
After 15, 30, or 60 min, the cell surface proteins were biotinylated and precipitated with streptavidin magnetic beads and analyzed by immunoblotting with Abs or an isotype control IgG (10 μg/ml of each immunoglobulin). The cells were lysed and incubated with rabbit anti-PLSCR antibody and immune complexes precipitated with protein A-agarose and analyzed by western blotting with a mouse anti-phosphotyrosine (PY20) or mouse anti-PLSCR mAb. The blot is representative of results obtained in two independent experiments.

Methods

Synthesis of CIMSS. All chemical reagents and solvents were obtained from commercial sources and used without further purification. Microwave reactions were performed using an Anton Paar Monowave 300 reactor. Chromatography was performed on a Teledyne ISCO CombiFlash Rf 200i using disposable silica cartridges. Analytical thin layer chromatography (TLC) was performed on Merck silica gel plates and compounds were visualized using UV or CAM stain. NMR spectra were recorded on a Bruker 600 spectrometer. 1H chemical shifts (δ) are reported relative to tetramethyl silane (TMS, 0.00 ppm) as internal standard or relative to residual solvent signals.

**Step 1. tert-Butyl ((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)carbamate (1):** N-Boc-propargylamine (3.1 g, 20 mmol), 3-azidopropan-1-ol (2.0 g, 20 mmol) and THF (50 mL) were combined in a flask which was then purged with Ar. (PPh3)3CuBr (150 mg, 0.16 mmol, 0.8 mol%) was added and the reaction mixture was stirred at room temperature for 3 days. Evap of volatiles gave a precipitate which was then purified by flash chromatography (24 g silica, 0–25% Ultra [CH2Cl2:MeOH:NH4OH 75:23:2] in DCM). Minor impurities were removed by a second chromatographic separation (0–100% acetone in hexanes) to give the pure product as an oil (4.9 g, 20 mmol, 97%).

1H NMR (600 MHz, CDCl3) δ 7.57 (s, 1H), 5.21 (bs, 1H), 4.51 (t, 6.8 Hz, 2H), 4.38 (d, 5.1 Hz, 1H), 2.12 (p, 6.3 Hz, 2H), 2.34 (t, 6.5 Hz, 2H), 2.46 (p, 6.4 Hz, 2H), 1.44 (s, 9H). 13C NMR (151 MHz, CDCl3) δ 143.93, 145.43, 122.43, 79.77, 58.67, 46.92, 36.86, 32.56, 28.39. ESI-MS: calc’d for C14H20BrC4N4O2 (M+H)+ 319.0766 found 319.0764.

**Step 2. tert-Butyl ((1-(bromomethyl)-1H-1,2,3-triazol-4-yl)methyl)carbamate (2):** a flask containing alcohol 1 (2.90 g, 11 mmol, 1.0 equiv.) was purged with argon and closed with a septum/Ar balloon. THF (100 mL) was added followed by PPh3 (5.94 g, 22.6 mmol, 2.0 equiv.) and CBr4 (7.50 g, 22.6 mmol, 2.0 equiv.). A precipitate formed within 15 min. The resulting mixture was stirred at room temperature for 17 h, before being diluted with Et2O (50 mL) and filtered. The solids were rinsed with Et2O (50 mL). Volatiles were removed and the residue purified by column chromatography (24 g silica, 0–40% acetone in hexanes). The product was obtained as an oil that crystalized upon standing (2.53 g, 7.93 mmol, 70%).

**TLC:** Rf = 0.29 (hexanes:acetonate 1:1, I2 then CAM). 1H NMR (600 MHz, CDCl3) δ 8.57 (s, 1H), 5.11 (bs, 1H), 4.53 (t, 6.6 Hz, 2H), 4.40 (d, 6.0 Hz, 2H), 3.36 (t, 6.2 Hz, 2H), 2.46 (p, 6.4 Hz, 2H), 1.44 (s, 9H). 13C NMR (151 MHz, CDCl3) δ 155.85, 145.53, 122.49, 79.75, 48.10, 36.09, 32.55, 29.32, 28.37. ESI-MS: calc’d for C15H24BrC4N4O2 (M+H)+ 319.0764 found 319.0766.

Other inhibitors of endocytosis at inhibiting SARS-CoV-2 infection20,21. It should be noted that CIMSS did not abolish VSV or HSV infection, which may reflect the ability of both viruses to use alternative endocytic pathways for entry.

In summary, we have explored the unique properties of CIMSS to examine viral entry mechanisms of HSV and SARS-CoV-2, and to identify unique extracellular interactions and catalytic contributions to these processes (Fig. 10). Future efforts to define the contribution of extracellular kinase activities to viral entry will benefit from more selective reagents, including mechanistically defined antibodies and impermeable analogs of selective kinase inhibitors. While we focused on CIMSS as a tool compound, our results suggest that cell impermeable kinase inhibitors may also be relevant to other viral infections because they target host proteins and not the virus. The previously unappreciated translocation of kinases to viral escape mutants because they target host proteins and not the virus.

**Methods**

**Synthesis of CIMSS.** All chemical reagents and solvents were obtained from commercial sources and used without further purification. Microwave reactions were performed using an Anton Paar Monowave 300 reactor. Chromatography was performed on a Teledyne ISCO CombiFlash Rf 200i using disposable silica cartridges. Analytical thin layer chromatography (TLC) was performed on Merck silica gel plates and compounds were visualized using UV or CAM stain. NMR spectra were recorded on a Bruker 600 spectrometer. 1H chemical shifts (δ) are reported relative to tetramethyl silane (TMS, 0.00 ppm) as internal standard or relative to residual solvent signals.

**Step 1. tert-Butyl ((1-(3-hydroxypropyl)-1H-1,2,3-triazol-4-yl)methyl)carbamate (1):** N-Boc-propargylamine (3.1 g, 20 mmol), 3-azidopropan-1-ol (2.0 g, 20 mmol) and THF (50 mL) were combined in a flask which was then purged with Ar. (PPh3)3CuBr (150 mg, 0.16 mmol, 0.8 mol%) was added and the reaction mixture was stirred at room temperature for 3 days. Evap of volatiles gave a precipitate which was then purified by flash chromatography (24 g silica, 0–25% Ultra [CH2Cl2:MeOH:NH4OH 75:23:2] in DCM). Minor impurities were removed by a second chromatographic separation (0–100% acetone in hexanes) to give the pure product as an oil (4.9 g, 20 mmol, 97%).

1H NMR (600 MHz, CDCl3) δ 7.57 (s, 1H), 5.21 (bs, 1H), 4.51 (t, 6.8 Hz, 2H), 4.38 (d, 5.1 Hz, 1H), 2.12 (p, 6.3 Hz, 2H), 2.34 (t, 6.5 Hz, 2H), 2.46 (p, 6.4 Hz, 2H), 1.44 (s, 9H). 13C NMR (151 MHz, CDCl3) δ 143.93, 145.43, 122.43, 79.77, 58.67, 46.92, 36.86, 32.56, 28.39. ESI-MS: calc’d for C14H20BrC4N4O2 (M+H)+ 319.0766 found 319.0764.

**Step 2. tert-Butyl ((1-(bromomethyl)-1H-1,2,3-triazol-4-yl)methyl)carbamate (2):** a flask containing alcohol 1 (2.90 g, 11 mmol, 1.0 equiv.) was purged with argon and closed with a septum/Ar balloon. THF (100 mL) was added followed by PPh3 (5.94 g, 22.6 mmol, 2.0 equiv.) and CBr4 (7.50 g, 22.6 mmol, 2.0 equiv.). A precipitate formed within 15 min. The resulting mixture was stirred at room temperature for 17 h, before being diluted with Et2O (50 mL) and filtered. The solids were rinsed with Et2O (50 mL). Volatiles were removed and the residue purified by column chromatography (24 g silica, 0–40% acetone in hexanes). The product was obtained as an oil that crystalized upon standing (2.53 g, 7.93 mmol, 70%).

**TLC:** Rf = 0.29 (hexanes:acetonate 1:1, I2 then CAM). 1H NMR (600 MHz, CDCl3) δ 8.57 (s, 1H), 5.11 (bs, 1H), 4.53 (t, 6.6 Hz, 2H), 4.40 (d, 6.0 Hz, 2H), 3.36 (t, 6.2 Hz, 2H), 2.46 (p, 6.4 Hz, 2H), 1.44 (s, 9H). 13C NMR (151 MHz, CDCl3) δ 155.85, 145.53, 122.49, 79.75, 48.10, 36.09, 32.55, 29.32, 28.37. ESI-MS: calc’d for C15H24BrC4N4O2 (M+H)+ 319.0764 found 319.0766.
cells were infected with VSV-S or VSV-G in the presence of antibodies that target Akt (rabbit polyclonal), PDPK1 (rabbit polyclonal), the ACE2 receptor (murine monoclonal), or a murine monoclonal isotype control for 1 h, washed, and infection monitored by counting plaques 24 h pi. Results are presented stained with DAPI. Images (Leica SP8, objective 63 × 1.4) are representative of results obtained in two independent experiments (bar indicated; blots are representative of two independent experiments.

Fig. 9 Silencing of PDPK1, PLCγ1, and Akt inhibits VSV-S infection. a Vero cells were transfected with control siRNA or siRNA targeting Akt1, PDPK1, PLCγ1, or FIC-1 and after 72 h, cell lysates were assayed by preparing western blots and probing for respective proteins. Cell lysates were probed for β-actin on separate blots. Blots were scanned and the percent reduction in protein expression of the silenced protein relative to siControl-transfected cells is indicated; blots are representative of two independent experiments. b Silenced cells were infected in duplicate with VSV-G or VSV-S and plaques quantified after 48 h incubation. Results are presented as mean ± SD and asterisks indicate significance relative to plaques formed on siControl wells (ANOVA, ***p < 0.001). c Vero cells were transfected with siPDPK1 or control siRNA as in a and then infected with VSV-G or VSV-S. Following incubation for 30, 60, or 120 min, cultures were fixed without or with Triton X-100 permeabilization and stained with antibodies to pAktT308 (red) or PDPK1 (green); nuclei were stained with DAPI. Images (Leica SP8, objective 63 × 1.4) are representative of results obtained in two independent experiments (bar = 10 μm). d Vero cells were infected with VSV-S or VSV-G in the presence of antibodies that target Akt (rabbit polyclonal), PDPK1 (rabbit polyclonal), the ACE2 receptor (murine monoclonal), or an murine monoclonal isotype control for 1 h, washed, and infection monitored by counting plaques 24 h pi. Results are presented as mean ± SD and asterisks indicate significance relative to plaques formed on isotype control antibody treated wells (**p < 0.01, ***p < 0.001).

Step 3. Sodium 3-[(tert-butoxycarbonyl)amino(methyl)-1H-1,2,3-triazol-1-yl]propane-1-sulfonate (3). Bromide 2 (330 mg, 1.66 mmol, 1.0 equiv.), ethanol (2 mL), water (1 mL) and sodium sulfite (532 mg, 4.15 mmol, 2.5 equiv.) were added to a microwave vial. The solids were collected by filtration (glasswool) and rinsed with ethanol. The liquid phase was concentrated almost to dryness and the product was precipitated by addition of acetone (5 mL). The solids were collected by filtration and rinsed with acetone and CH2Cl2 to give the sulfonate salt in good purity (310 mg, 0.91 mmol, 55%).

1H NMR (600 MHz, D2O) δ 7.92 (s, 1H), 4.57 (t, J = 6.8 Hz, 2H), 4.34 (s, 2H), 2.87 (t, J = 7.7 Hz, 2H), 2.34 (p, J = 7.6 Hz, 2H), 1.43 (s, 9H). 13C NMR (151 MHz, D2O) δ 181.36, 157.97, 123.52, 81.36, 48.72, 47.61, 30.21, 27.57, 25.15. ESI-MS: calc’d for C11H20N4O5Na (M + H)+ 343.1047 found 343.1044.

Step 4. Sodium 3-[(aminomethyl)-1H-1,2,3-triazol-1-yl]propane-1-sulfonate (4): Boc-protected amine 4 (53 mg, 0.15 mmol) was dissolved in water and heated to 150 °C for 30 min in a microwave vial. The product was obtained as a white powder after freeze-drying (38 mg, 0.15 mmol, >99%).

1H NMR (600 MHz, D2O) δ 8.03 (s, 1H), 4.60 (t, J = 6.7 Hz, 2H), 4.08 (s, 2H), 2.93–2.80 (m, 2H), 2.36 (p, J = 7.0 Hz, 2H). 13C NMR (151 MHz, D2O) δ 145.38, 123.99, 48.74, 47.58, 34.99, 25.11. ESI-MS: calc’d for C6H12N4O3S (M + H)+ 243.0523 found 243.0524.

Step 5. Staurosporine 4-oxobunanoic acid (5). Staurosporine (16 mg, 34 mmol, 1.0 equiv.), succinyl anhydride (10 mg, 0.10 mmol, 2.9 equiv.), DMAP (8.4 mg, 69 mmol, 2.0 equiv.) and DMSO (1 mL) were combined in a vial, and the resulting solution was stirred overnight while being protected from light. The reaction mixture was diluted with EtOAc (5 mL) and transferred to a Falcon tube containing water (5 mL) and 1 M HCl (1 mL). The phases were separated and the aqueous layer was extracted with EtOAc (3 × 2 mL). The combined organic layers containing water (5 mL) and 1 M HCl (1 mL). The phases were separated and the aqueous layer was extracted with EtOAc (3 × 2 mL). The combined organic layers were combined, dried (Na2SO4), and rinsed with ethanol. The liquid phase was concentrated almost to dryness and the product was precipitated by addition of acetone (5 mL). The solids were collected by filtration and rinsed with acetone and CH2Cl2 to give the sulfonate salt in good purity (310 mg, 0.91 mmol, 55%).

ESI-MS: calc’d for C11H20N4O5Na (M + H)+ 343.1047 found 343.1044.
$\delta$ 172.62, 172.36, 171.81, 145.36, 139.36, 136.73, 133.12, 126.11, 125.78, 125.46, 124.20, 123.37, 123.07, 121.88, 120.74, 119.89, 119.83, 115.65, 114.56, 114.17, 109.51, 95.13, 83.70, 82.72, 60.96, 55.39, 48.97, 48.57, 48.51, 45.88, 34.79, 31.28, 30.58, 29.96, 29.05, 27.24, 27.07, 27.05. ESI-MS: calc’d for $C_{38}H_{40}N_8O_8S$ (M + H)$^+$ 791.2582 found 791.2513.

**In vitro kinase assays studies.** Kinase activity were performed by Reaction Biology Corporation using the “HotSpot” assay platform modified from the published procedure. Briefly, into a base reaction buffer (20 mM Hepes (pH 7.5), 10 mM MgCl$_2$, 1 mM EGTA, 0.01% Brij35, 0.02 mg/mL BSA, 0.1 mM Na$_3$VO$_4$, 2 mM DTT, 1% DMSO) containing substrate were delivered sequentially (1) required cofactors, (2) kinase enzyme (followed by gentle mixing), (3) compound (CIMSS or controls) in 100% DMSO (introduced by acoustic technology (Echo550; nanoliter range, followed by incubation at 20 °C for 20 min), and finally (4) $[^{33}]P$-ATP to initiate the reaction. After two hours, kinase activity was detected by the P81 filter-binding method. The concentration of drug that inhibited 50% of kinase activity (IC$_{50}$) was calculated relative to controls.

**Cell permeability.** MDCK Transport analysis was performed by Quintara Discovery (Hayward, CA). MDCK-MDR1 cell plates were maintained for 3 days at 37 °C with 5% CO$_2$. Cells were washed with Hank’s Balanced Salt Solution (HBSS) with 5 mM HEPES for 30 min before starting the experiment. Test compound (CIMSS and controls digoxin and propranolol) solutions were prepared by diluting from DMSO stock into HBSS buffer to a final concentration of 5 μM. Prior to the experiment, cell monolayer integrity was verified by transendothelial electrical resistance (TEER). All of the wells had high resistance above the acceptance cut-off (1 kΩ). The test compounds were added to the apical (75 μL) side with blank buffer on the basal side (250 μL). A P-glycoprotein (gp) inhibitor (GF120918, 10 μM) was maintained in the transport buffer to block the active P-gp transporter. Transport plates were incubated at 37 °C in a humidified incubator with 5% CO$_2$. A sample was obtained from the donor compartment at time zero and from donor and acceptor compartments after 1 h and analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS). Apparent permeability (Papp) values were calculated using the equation: Papp = ($dQ/dt$)/A/C$_0$ where $dQ/dt$ is the initial rate of amount of test compound transported across cell monolayer, A is the surface area of the filter membrane, and C$_0$ is the concentration of the test compound at time zero. All samples were analyzed on LC/MS/MS using an AB Sciex API 4000 instrument, coupled to a Shimadzu LC-20AD LC Pump system. Analytical samples were separated using a Waters Atlantis T3 4C18 reverse phase HPLC column (20 mm × 2.1 mm) at a flow rate of 0.5 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). Elution conditions are detailed below.

![Fig. 10 Model of how cell-impermeable kinase inhibitors block viral entry. a Binding of HSV to cellular receptors (1) triggers intracellular calcium transients (2), which activate phospholipid scramblase-1 (3) leading to the translocation of phosphatidylserines and cellular proteins (PDPK1, Akt, PLCγ and possibly others) to the outer leaflet of the plasma membrane (4) where, analogous to the the canonical cytoplasmic signaling pathways, autophosphorylation of PDPK1 triggers phosphorylation of outer leaflet Akt, which, in turn, phosphorylates PLCγ (5). This signaling pathway is required for HSV entry and is associated with subsequent restoration of phospholipid distribution (6). b Cell-impermeable kinase inhibitors (e.g. CIMSS) or antibodies to Akt block the activation of this extracellular signaling pathway (5) and prevent HSV entry resulting in (6) persistence of extracellular PtdS, which may lead to apoptosis.](https://doi.org/10.1038/s42003-022-04067-4)
PAMP analysis was performed by Quintara Discovery (Hayward, CA). Pre-coated 96-well PAMPA plate system was purchased from Corning and left to thaw to the room temperature for at least 30 min before use. Compounds (CIMSS and staurosporine) were dissolved at 100 μM in PBS with 1.2% microcrystalline cellulose (Avicel). Cells were exposed to culture media alone, media containing increasing concentrations of compounds, and 0.05% for 50–200 μM, and 1% DMSO for 100 μM. The day prior to infection Huh-7.5 cells were seeded into 96-well plates at two densities: 1.25 × 10^{4} cells/well and at 5 × 10^{3} cells/well for fixation with 7% formaldehyde and cryostat sections. Caco-2 cells were infected at a MOI of 0.25 PFU/cell (6 h timepoint) and 0.01 PFU/cell (24 h timepoint). Cells were then incubated at 37 °C for 48 h and at 33 °C for 72 h. At the respective timepoints, cells were fixed by 3% glutaraldehyde in PBS for 2 h and subsequently permeabilized with 0.1% Triton X-100 for 10 min. After extensive washing, Caco-2 virus-infected cells were incubated for 1 h at room temperature with blocking solution of 5% goat serum in PBS (catalog no. 005–000–121; Jackson ImmunoResearch). A rabbit polyclonal antibody to SARS-CoV-2 nucleocapsid protein (catalog no. GTX133557; GeneTex) was added to the wells at 1:1000 dilution in blocking solution and incubated at 4 °C overnight. Goat anti-rabbit AlexaFlour 594 (catalog no. A-11012; Life Technologies) was used as a secondary antibody at a 1:2000 dilution. Nuclei were stained with Hoechst 33342 (catalog no. 62249; Thermo Scientific) at a 1 μg/ml dilution. Images were acquired with a laser microscopy system and analyzed using ImageXpress Micro XLS (Molecular Devices, Sunnyvale, CA). All SARS-CoV-2 experiments were performed in a biosafety level 3 laboratory.

**VSV pseudotyped virus infection assays.** Infection of Vero or HepG2 cells by VSV-G, VSV-EBOV-GP, and VSV-S was monitored by plaque assay after 48 h culture and staining with crystal violet. Calu-3 cells were infected with virus and after 96 h culture, supernatants were harvested and viral yields quantified by titrating on Vero cells.

**SARS-CoV-2 assays.** The day prior to infection HepH-7.5 cells were seeded into 96-well plates at two densities: 1.25 × 10^{4} cells/well and at 5 × 10^{3} cells/well for fixation at 24 h and 72 h, respectively. The next day, serially diluted CIMSS (or DMSO control) was added to the wells, followed by infections with SARS-CoV-2 at MOIs of 0.25 PFU/cell (24 h timepoint) and 0.01 PFU/cell (72 h timepoint). Cells were then incubated at 37 °C for 24 h and at 33 °C for 72 h. At the respective timepoints, cells were fixed by 3% glutaraldehyde in PBS for 2 h and subsequently permeabilized with 0.1% Triton X-100 for 10 min. After extensive washing, SARS-CoV-2 infected cells were incubated for 1 h at room temperature with blocking solution of 5% goat serum in PBS (catalog no. 005–000–121; Jackson ImmunoResearch). A rabbit polyclonal antibody to SARS-CoV-2 nucleocapsid protein (catalog no. GTX133557; GeneTex) was added to the cells at 1:1000 dilution in blocking solution and incubated at 4 °C overnight. Goat anti-rabbit AlexaFlour 594 (catalog no. A-11012; Life Technologies) was used as a secondary antibody at a 1:2000 dilution. Nuclei were stained with Hoechst 33342 (catalog no. 62249; Thermo Scientific) at a 1 μg/ml dilution. Images were acquired with a laser microscopy system and analyzed using ImageXpress Micro XLS (Molecular Devices, Sunnyvale, CA). All SARS-CoV-2 experiments were performed in a biosafety level 3 laboratory.
blots were horseradish peroxidase-conjugated goat anti-mouse (170-5047, Bio-Rad, Hercules, CA), goat anti-rabbit (170-5064, Bio-Rad), and donkey anti-goat 1:1000 (sc-2365, Santa Cruz) antibody. All secondary antibodies were diluted in 1% BSA-PBS at 1:1000. Images were captured using a Bio-Rad ChemiDoc Imaging System equipped with GelDoc2000 software using Chemiluminescent Western Blotting Substrates (Bio-Rad). The mean fluorescence intensity ratios were calculated using ImageJ version 9.0 software (GraphPad Software Inc., San Diego, CA). A Student’s t test was considered statistically significant. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Biotinylation of cell surface proteins. Cells were exposed to HSV-2 (MOI = 10 PFU/cell) in the presence of control buffer (0.1% DMSO) or CIMSS (10 µM) for 15 and 30 min, washed four times with ice-cold PBS and then immediately placed on ice. The cells were then lysed by sonication in RIPA buffer (Thermo Scientific). The precipitated complexes (pellet) were collected by centrifugation and resuspended in cold lysis buffer (1% NP-40, 0.5% Tween-20, 50 mM Tris pH 7.0, 150 mM NaCl, 1 mM EDTA, 2 µM aprotinin, 2 µM leupeptin, 10 mM NaF, 100 µM Na3VO4) and incubated at 4°C for 30 min. Immunoprecipitation was performed using the Invitrogen afﬁnity puriﬁed IgG agarose beads (I34406, W11261, Invitrogen Molecular Probes, Carlsbad, CA, USA) overnight at 4°C. The immunoprecipitates were washed four times with RIPA buffer and then incubated with 25 µg/ml of Protein A/G agarose beads (sc-2007, Santa Cruz) for 1 h at 4°C. The beads were washed four times with RIPA buffer and then resuspended in 50 mM Tris pH 7.5, 50 mM NaCl, 0.1% SDS, and 5% glycerol before analysis by SDS-PAGE and Western blotting with anti-actin antibodies. Western blots were analyzed using the ChemiDoc Imaging System (Bio-Rad) and quantiﬁed using the ImageJ software (NIH, USA).

Statisitics and reproducibility. Analyses were performed using GraphPad Prism version 9.0 software (GraphPad Software Inc., San Diego, CA). A p value of 0.05 was considered statistically signiﬁcant. Results were compared using unpaired Student’s t tests or one-way ANOVA with correction for multiple comparisons as indicated. The number of biological and technical replicates is indicated for each figure and presented as dot plots to show data distribution.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
11. John, M. et al. Cervicovaginal secretions contribute to innate resistance to herpes simplex virus infection. J. Infect. Dis. 192, 1731–1740 (2005).
12. Cheshenko, N., Liu, W., Satlin, L. M. & Herold, B. C. Focal adhesion kinase plays a pivotal role in herpes simplex virus entry. J. Biol. Chem. 280, 31116–31125 (2005).
13. Desai, P. & Person, S. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. J. Virol. 72, 7563–7568 (1998).
14. Wang, Y., Wu, J. & Wang, Z. Akt binds to and phosphorylates phospholipase C-gamma1 in response to epidermal growth factor. Mol. Biol. Cell 17, 2267–2277 (2006).
15. Hoffmann, M. et al. Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. Nature 585, 588–590 (2020).
16. Kawaguchi, M. et al. Development of a novel fluorescent probe for fluorescence correlation spectroscopic detection of kinase inhibitors. Bioorg. Med. Chem. Lett. 18, 3752–3755 (2008).
17. Shi, H., Cheng, X., Sze, S. K. & Yao, S. Q. Proteome profiling reveals potential cellular targets of staurosporine using a clickable cell-permeable probe. Chem. Commun. 47, 11306–11308 (2011).
18. Basile, M. S. et al. The PI3K/Akt/mTOR pathway: a potential pharmacological target in COVID-19. Drug Discov. Today 27, 848–856 (2022).
19. Cheng, C. Y., Huang, W. R., Chi, P. I., Chiu, H. C. & Liu, H. J. Cell entry of bovine ephemeral fever virus requires activation of Src-JNK-AP1 and PI3K-Akt-NF-kappaB pathways as well as Cox-2-mediated PGE2 /EP receptor signalling to enhance clathrin-mediated virus endocytosis. Cell. Microbiol. 17, 967–975 (2015).
20. Zaitseva, E. et al. Fusion stage of HIV-1 entry depends on virus-induced cell surface exposure of phosphatidylserine. Cell Host Microbe 22, 99–110.e117 (2017).
21. Stewart, C. M. et al. Ebola virus triggers receptor tyrosine kinase-dependent signaling to promote the delivery of viral particles to entry-conducive intracellular compartments. PLoS Pathog. 17, e1009275 (2021).
22. Wittels, M. & Spear, P. G. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res. 18, 271–290 (1991).
23. Meyer, S. C., Shomim, C. D., Gaj, T. & Ghosh, I. Tethering small molecules to a phage display library: discovery of a selective bivalent inhibitor of protein kinase A. J. Am. Chem. Soc. 129, 13812–13813 (2007).
24. Anastassiadi, T., Deacon, S. W., Devarajan, K., Ma, H. & Peterson, J. R. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. Nat. Biotechnol. 29, 1039–1045 (2011).
25. Blight, K. J., McKeating, J. A. & Rice, C. M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76, 13001–13014 (2002).
26. Herold, B. C., WuDunn, D., Solty, N. & Spear, P. G. Glycoprotein B of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. 65, 1090–1098 (1991).
31. Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H. & Peterson, J. R. Proteome profiling reveals potential cellular targets of staurosporine using a clickable cell-permeable probe. Chem. Commun. 47, 11306–11308 (2011).
32. Basile, M. S. et al. The PI3K/Akt/mTOR pathway: a potential pharmacological target in COVID-19. Drug Discov. Today 27, 848–856 (2022).
33. Cheng, C. Y., Huang, W. R., Chi, P. I., Chiu, H. C. & Liu, H. J. Cell entry of bovine ephemeral fever virus requires activation of Src-JNK-AP1 and PI3K-Akt-NF-kappaB pathways as well as Cox-2-mediated PGE2 /EP receptor signalling to enhance clathrin-mediated virus endocytosis. Cell. Microbiol. 17, 967–975 (2015).
34. Cheshenko, N. & Herold, B. C. Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread. J. Gen. Virol. 83, 2247–2255 (2002).

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**Author contributions**

N.C., S.C.A., and B.C.H. designed the experiments. N.C., J.B.B., H.-H.H., and R.K.J. conducted experiments. N.C., H.-H.H., S.C.A., and B.C.H. analyzed data and wrote the manuscript. K.C. and C.M.R. provided key reagents and edited the manuscript.

**Competing interests**

The authors (N.C., B.C.H., J.B.B., and S.A.) have a pending patent application related to the development of cell-impermeable kinase inhibitors as antivirals.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s42003-022-04067-4.

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