Identification of novel antivirals inhibiting recognition of Venezuelan equine encephalitis virus capsid protein by the Importin α/β1 heterodimer through high-throughput screening

David R. Thomas, Lindsay Lundberg, Chelsea Pinkham, Sharon Shechter, Aaron DeBono, Jonathan Baell, Kylie M. Wagstaff, Caroline A. Hick, Kylene Kehn-Hall, David A. Jans

Keywords: VEEV, Importins, High-throughput screen, Antivirals, Quantitative microscopy

Abstract

Although the alphavirus Venezuelan equine encephalitis virus (VEEV) has been the cause of multiple outbreaks resulting in extensive human and equine mortality and morbidity, there are currently no anti-VEEV therapeutics available. VEEV pathogenicity is largely dependent on targeting of the viral capsid protein (CP) to the host cell nucleus through the nuclear transporting importin (Imp) α/β1 heterodimer. Here we perform a high-throughput screen, combined with nested counterscreens to identify small molecules able to inhibit the Impα/β1:CP interaction for the first time. Several compounds were able to significantly reduce viral replication in infected cells. Compound G281-1564 in particular could inhibit VEEV replication at low μM concentration, while showing minimal toxicity, with steady state and dynamic quantitative microscopic measurements confirming its ability to inhibit CP nuclear import. This study establishes the principle that inhibitors of CP nucleocytoplasmic trafficking can have potent antiviral activity against VEEV, and represents a platform for future development of safe anti-VEEV compounds with high efficacy and specificity.

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is a single stranded RNA virus of the genus Alphavirus. Although first identified as the causative agent of an equine disease in 1938, it was later also found to be a human pathogen (Casals et al., 1943). In the early 1960s, a large outbreak in Panama and Venezuela resulted in tens of thousands of human cases, with further outbreaks in 1969 and 1995 highlighting the damage that the virus, through its high infectivity and stability, can cause in human and equine populations (Navarro et al., 2005; Weaver et al., 2004). This, combined with its ease of manipulation and ability to grow to high titres in host cells, makes VEEV attractive as a potential bioweapon (Reichert et al., 2009). Beyond bioterrorism, a decline in equine vaccination rates and the presence of circulating virus in wild animals is creating a significant risk of another natural outbreak (Paniz-Mondolfi et al., 2017). There are currently no anti-viral treatments targeting VEEV, and no licenced vaccines approved for general human use. In humans, the TC-83 live attenuated strain is used for at risk personnel, including military and researchers; however, it provides limited protection and high rates of adverse effects (Pittman et al., 1996). Clearly, there is a need to develop new treatments to combat future outbreaks.

The RNA genome of VEEV encodes four structural and four non-structural proteins. In addition to its structural role in binding viral RNA and forming viral particles, the capsid protein (CP) plays a key role in viral pathogenicity (Lundberg et al., 2013), where it is responsible for the down-regulation of host-cell transcription (Garmashova et al., 2007a, 2007b). It has been reported to inhibit the active transport of large proteins into the nucleus through the nuclear pore, potentially thereby interrupting signal cascades and inhibiting the cells ability to mount an effective antiviral response (Atasheva et al., 2008). Host cell factors that interact with CP include those from the Importin (Imp) superfamily of nuclear transporters. CP's nuclear localisation signal (NLS) is recognised by the Impα/β1 heterodimer, and its nuclear export...
signal (NES) recognised by the IMPβ family member exportin-1 (CRM1). Both the NLS and NES are required for CP’s pathogenic effects on infected host cells (Atasheva et al., 2010, 2015), where it appears CP simultaneously binds both Impα/β1 and CRM1 (Atasheva et al., 2010). This tetrameric complex then accumulates at or within the nuclear pore, preventing the active transport of host molecules into the nucleus. Reduced binding of CP to either Impα/β1 or CRM1 through mutations in the NLS or NES, or treatment of cells with small molecules inhibiting binding, results in reduced pathogenicity of the virus (Lundberg et al., 2013, 2016), validating nucleocytoplasmic trafficking of CP as a target for antivirals against VEEV.

Targeting host components in the quest for antiviral effects can lead to unintended side-effects, whereas targeting viral proteins can result in rapid selection for resistance mutations (Caly et al., 2012; Wagstaff et al., 2011; Fraser et al., 2014). In contrast, targeting the host: virus interface limits the scope for viral resistance and minimizes the potential for unwanted side-effects on the host. We set out here to perform a high throughput screen for small molecules able to inhibit the Impα/β1:CP interaction for the first time, with the ultimate aim of identifying new chemical scaffolds with antiviral activity. From a library of 14,468 compounds, we were able to identify compounds that showed strong activity against the Impα/β1:CP interaction, while minimally inhibiting Impα/β1 binding to other cargoes. Several of these compounds were able to significantly reduce viral replication in infected cells; one compound, G281-1564, could inhibit VEEV replication at low μM concentration, while showing minimal toxicity, with FRAP experiments demonstrating its ability to inhibit CP nuclear import. This study thus proves the principle that selective inhibitors of CP nucleocytoplasmic trafficking can have potent antiviral activity against VEEV, and represents a platform for future development of safe anti-VEEV compounds with high efficacy and specificity.

Fig. 1. HTS Pipeline. Stage 1) The 33,999 compounds in the Open Scaffold library were filtered to select 14,468 compounds amenable to drug development, and screened for their ability to inhibit the Impα/β1:CP Alphascreen, then counter-screened to remove those showing significant off-target effects (inhibition of Impα/β1:GFP-T-agNLS Alphascreen) or assay interference (inhibition of His6-GST AlphaScreen). Stage 2) Compounds that were structurally similar to selective hits identified in step one were tested using the same screen/counter-screen procedure. Stage 3) Compounds were selected for further analysis based on their compatibility with drug-development, and subjected to IC50 analysis and cell-based experiments.
2. Results

2.1. Selection of screening library

A set of compounds was chosen from Queensland Compound Library’s (QCL) Open Scaffolds collection (Simpson and Poulsen, 2014), consisting of 33,999 compounds grouped into 1226 scaffold families (chemotypes) based on structural similarities. After converting the simplified molecular-input line-entry (SMILES) into SYBYL line notation (SLN (Homer et al., 2008)), compounds were filtered to remove pan assay interference compounds (Baell, 2016; Baell and Hollaway, 2010), and compounds selected that were likely to be soluble and permeate cells, while being optimizable as drug candidates. Specific criteria were: mixtures, metals, isotopes = 0; minimum number of rings = 1; maximum number of rings = 4; minimum molecular weight = 1500a; maximum molecular weight = 4000a; hydrogen bond donors = 3; hydrogen bond acceptors ≤ 6; maximum number of chiral centres = 3; and maximum number of rotatable bonds = 10. This reduced the number of compounds to 19,408. Finally, compounds whose structures were > 90% similar were removed, selecting a final library of 14,468 compounds for HTS.

2.2. HTS pipeline

The AlphaScreen binding assay has previously been used to identify small molecule inhibitors of protein–protein interactions (Wagstaff et al., 2011, 2012; Fraser et al., 2014; Wagstaff and Jans, 2006; Glickman et al., 2002). AlphaScreen was applied here to identify compounds able to inhibit CP binding to Impα/β1. Briefly, 30 nM CP was added to 15 nM pre-dimerised Impα/β1 in the presence of 25 μM of each compound. The signal above background was then standardised using DMSO-only positive controls to determine the percent binding inhibition due to each compound. Counterscreens included those that replaced CP with a His6-tagged GFP peptide fused to an optimised NLS from the SV40 large T-antigen (GFP-T-agNLS; a well-characterised Impα/β1 cargo), and a second that excluded both CP and Impα/β1, instead using a his6-GST peptide to produce a signal without requiring protein:protein binding. These counterscreens identified compounds that reduced the AlphaScreen signal through general Impα/β1 inhibition or assay inhibition, respectively.

2.3. Screening

The HTS was performed as shown in Fig. 1. Subsequent to optimisation of the assay conditions (Supp. Fig. 1), screening of the 14,468 compound library for the ability to inhibit Impα/β1:CP binding was performed over 2 days using 82 384-well plates (Fig. 2). To minimise well location effects, inhibition in each well was standardised using the average of that well position over the plates tested that day. The assays provided robust data (Z’s > 0.75 throughout, with positive CV’s < 8). The 332 top compounds (≥ 42% inhibition) were retested twice in duplicate at 25 μM (Fig. 3), with 73% of them confirmed to inhibit the AlphaScreen signal by > 50%. The compounds were then counter-screened at 25 μM in the AlphaScreen system for the ability to inhibit the Impα/β1:GFP-T-agNLS interaction and/or to inhibit the signal generated by His6-GST (Fig. 3); these assays again provided robust data (Z’s > 0.82 and 0.75, and positive CV’s of < 5.8 and 7.45 for the Impα/β1:GFP-T-agNLS, and His6-GST assays, respectively). The active compounds were generally specific to the Impα/β1:CP interaction, with < 30% of compounds inhibiting the Impα/β1:GFP-T-agNLS AlphaScreen signal by > 40%, whilst the majority did not have a significant effect on the His6-GST AlphaScreen signal. Compounds were considered active and specific, and hence worthy of further study, if: 1) they inhibited the Impα/β1:CP assay > 50%; 2) they inhibited the Impα/β1:CP assay > 2-fold the extent of inhibition of the Impα/β1:GFP-T-agNLS assay (specificity > 2); 3) they inhibited the His-GST assay by < 40%; and 4) they inhibited the Impα/β1:CP assay > 2-fold the extent of inhibition of the His-GST assay. 32 compounds belonging to 24 different scaffold families (Supp. Table S1) were identified in the initial screen that met these criteria.

2.4. Extended screen of scaffold families

Based on the first stage of screening, 352 additional compounds from the Open Scaffolds collection belonging to scaffold families of compounds fulfilling the criteria above were also screened, initially for the ability to inhibit Impα/β1:CP binding (Fig. 4). Following counter-screening using the His-GST and Impα/β1:GFP-T-agNLS assays, 22 additional compounds were identified which met the criteria above; this corresponded to a hit rate of 6.2%, ~30-fold higher than in the first HTS stage (Supp. Table S2).

2.5. IC50 analysis

Of 54 compounds identified in total from the first 2 stages of screening, those likely to be difficult to synthesise or modify, contain promiscuous domains, or be otherwise suboptimal for drug development were excluded, reducing the list to 38 compounds from 12 scaffold families. Of these, 18 compounds from 12 different scaffold families representing the inhibitors with the highest inhibition activity (> 63%) or highest specificity (> 2.9) were selected for IC50 analysis. IC50 analysis was performed in the AlphaScreen system using 15 nM pre-dimerised Impα/β1 and 30 nM of CP together with increasing concentrations of compounds, with, signal inhibition standardised to the DMSO control. IC50 ranged from c. 8–48 μM, with two compounds (295856735 and 270927013) from the same scaffold family possessing the lowest IC50 (Table 1, Fig. 5).
2.6. Lead compounds inhibit viral replication

The top five compounds as well as four related compounds were tested for their toxicity and ability to inhibit replication of VEEV TC83 expressing the firefly luciferase reporter gene (VEEV-luc) (Table 1). Cells were pre-treated with compounds 2 h before infection, compounds added again post-infection, and luciferase activity measured 16 h later. Three compounds were found to possess robust anti-VEEV activity, with EC50s of 7.5–27 μM; two of these belonged to the same (CL6662) scaffold family. The most active compound (G281-1485, EC50 of 7.5 μM) showed cytotoxicity (CC50 of 55 μM - Table 1), as did the closely related compound G281-1481 (CC50 of 33 μM). In contrast, G281-1564, which possessed antiviral activity (EC50 of 10.8 μM) similar to that of G281-1485, lacked significant toxicity (Table 1). Since the structure of G281-1564 differs from that of G281-1485 in having 2 fewer methyl groups (highlighted in Fig. 6), it seems reasonable to speculate that these groups may be linked to toxicity. Representative EC50 and CC50 curves of the non-toxic active compounds G281-1564 and Z1139230913 are shown in Fig. 7AB. The lack of direct correlation between in vitro IC50 values and in vivo antiviral activity is not unexpected when considering unpredictable limiting factors such as compound stability and availability in the medium/intracellular environment, and cell permeability.

In addition to testing the compounds ability to inhibit viral replication through the luciferase assay, inhibition of viral production was also assessed via plaque assay (Fig. 7C). While the luciferase reporter assay gives a measure of viral protein production, the plaque assay quantifies infectious virus; although the plaque assay indicated lower inhibition at 10 μM, both assays were in strong agreement at 50 μM. Cells were pre-treated with DMSO with or without 10 or 50 μM G281-1564, Z113923091 or Z70927013 before infection with VEEV TC83, and then post-treated for a further 16 h. At 10 μM, there was no strong inhibition from any compounds, with G281-1564 and Z1139230911 reducing plaque formation by > 95%, while Z70927013 resulted in only c. 25% fewer. As in the luciferase assay, Z1139230911 had intermediate activity, reducing the viral titre by c. 75%.

---

Fig. 3. Differential selectivity of hit compounds. Distribution of the average inhibition of signal compared to positive control at 25 μM in the Impα/β1:CP, Impα/β1:GFP-T-agNLS, and His6-GST AlphaScreens as indicated, for all 332 compounds rescreened (n = 2).

Fig. 4. Compounds belonging to active scaffold families were screened for activity in the Impα/β1:CP Alphascreen. Distribution of the average inhibition of signal at 25 μM compared to positive control of 352 compounds related to hits from the initial screen (n = 2).
Table 1
Summary of structure and activities of top hit compounds. The 18 compounds possessing either the top 10 inhibition of Impα/β1:CP binding or specificity from the selected screen compounds were tested in Alphascreen to determine their IC\text{50} as per Fig. 5. CC\text{50} (concentration yielding 50% cytotoxicity) and EC\text{50} (concentration yielding 50% reduction of luminescence signal from VEEV-luc infected cells) were determined as per Fig. 6. Specificity is the ratio of inhibition of Impα/β1:CP binding compared to inhibition of IMPα/β1 GFP-T-agNLS binding (AlphaScreen results from HTS). SI: Selective index, calculated as CC\text{50}/EC\text{50}. Scaffold families are grouped by colour for identification in Supplementary Figs. S3 and S4.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Scaffold family</th>
<th>Compound</th>
<th>IC\text{50} (μM)*</th>
<th>Specificity</th>
<th>Screen</th>
<th>EC\text{50} (μM)*</th>
<th>CC\text{50} (μM)*</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC006675</td>
<td></td>
<td>Z95856735</td>
<td>7.8 ± 2.2</td>
<td>2.44</td>
<td>initial</td>
<td>&gt;12.5</td>
<td>45.0 ± 11.2</td>
<td>&lt;3.6</td>
</tr>
<tr>
<td>SC006675</td>
<td></td>
<td>Z70927013</td>
<td>9.0 ± 2.0</td>
<td>2.16</td>
<td>initial</td>
<td>&gt;50</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>SC007512</td>
<td></td>
<td>Z1333601597</td>
<td>10.4 ± 1.3</td>
<td>4.48</td>
<td>initial</td>
<td>&gt;50</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>CL6662</td>
<td></td>
<td>G281-1485</td>
<td>12.2 ± 0.3</td>
<td>3.04</td>
<td>scaffold</td>
<td>7.5 ± 4.6</td>
<td>55.1 ± 11.5</td>
<td>7.3</td>
</tr>
<tr>
<td>SC015096</td>
<td></td>
<td>Z1139583813</td>
<td>12.7 ± 0.9</td>
<td>2.62</td>
<td>scaffold</td>
<td>&gt;50</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>CL6662</td>
<td></td>
<td>G281-1481</td>
<td>17.9 ± 3.2</td>
<td>3.36</td>
<td>scaffold</td>
<td>&gt;12.5</td>
<td>32.7 ± 14.6</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>CL3458</td>
<td></td>
<td>C351-0352</td>
<td>20.3 ± 2.7</td>
<td>3.71</td>
<td>initial</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SC015096</td>
<td></td>
<td>Z1139230991</td>
<td>20.7 ± 1.3</td>
<td>2.09</td>
<td>scaffold</td>
<td>26.9 ± 8.0</td>
<td>&gt;100</td>
<td>&gt; 3.7</td>
</tr>
<tr>
<td>CM1159</td>
<td></td>
<td>T503-0529</td>
<td>21.6 ± 1.0</td>
<td>4.16</td>
<td>initial</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SC00912</td>
<td></td>
<td>Z48847300</td>
<td>21.8 ± 2.5</td>
<td>2.00</td>
<td>initial</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(continued on next page)
The nested counterscreens performed in stages 1 and 2 of our HTS (see Fig. 1) were designed to identify compounds possessing activity specific to the Impα/β1:CP interaction, with limited inhibition of Impα/β1 binding to an unrelated cargo (GFP-T-agNLS). To confirm selectivity in a cellular context, HeLa cells were transfected to express either CP-GFP or GFP-T-agNLS fusion proteins and incubated for 20 h in media supplemented with 50 μM compounds, before Hoechst staining and quantitative imaging by confocal laser scanning microscopy.

### Table 1 (continued)

| CL9247  | D475-0080 | 23.1 ± 4.3 | 2.25 | scaffold | - | - | - |
| SC012104 | Z1180083001 | 25.0 ± 1.8 | 2.74 | initial | - | - | - |
| CL6662  | C281-1564 | 25.0 ± 2.2 | 6.41 | initial | 10.8 ± 4.3 | >100 | >9.3 |
| CL9247  | D475-0017 | 27.3 ± 0.7 | 2.07 | initial | - | - | - |
| SC008115 | Z643995494 | 33.3 ± 4.1 | 4.45 | initial | - | - | - |
| CM1028  | T480-0135 | 42.5 ± 8.4 | 5.17 | initial | - | - | - |
| SC006675 | Z118580606 | 47.5 ± 0.4 | 3.25 | scaffold | >50 | >100 | ND |
| SC003131 | Z55426342 | 48.1 ± 0.81 | 2.94 | initial | - | - | - |

#### 2.7. Lead compounds target the Impα/β1:CP interaction

The nested counterscreens performed in stages 1 and 2 of our HTS (see Fig. 1) were designed to identify compounds possessing activity specific to the Impα/β1:CP interaction, with limited inhibition of Impα/β1 binding to an unrelated cargo (GFP-T-agNLS). To confirm selectivity in a cellular context, HeLa cells were transfected to express either CP-GFP or GFP-T-agNLS fusion proteins and incubated for 20 h in media supplemented with 50 μM compounds, before Hoechst staining and quantitative imaging by confocal laser scanning microscopy.

![Representative inhibition curves of the top 5 compounds.](image-url)
(CLSM) (Fig. 8). Nuclear accumulation of the respective proteins was quantified by measuring the nuclear to cytoplasmic fluorescence ratio (Fn/c; see Methods), with the established Impα/β1 import inhibitor ivermectin (Lundberg et al., 2013; Wagstaff et al., 2011, 2012) used as a positive control. As expected, ivermectin, but none of our selective inhibitors reduced the nuclear accumulation of GFP-T-agNLS (Fig. 8; Fn/c analysis right). In similar fashion to ivermectin, however, both 1139230991 and G281-1564 significantly reduced the nuclear accumulation of CP-GFP, correlating with their robust anti-VEEV activity (see Fig. 7). In contrast, 70927013, which lacks anti-VEEV activity, had no effect. These results confirm the target specificity of the selected compounds, validate the counter-screening approach taken in the HTS, and indicate the correlation between inhibition of CP nuclear accumulation in a cellular context, and anti-VEEV activity.

2.8. G281-1564 inhibits the kinetics of CP nuclear import

To confirm that the reduction in nuclear CP caused by lead compound G281-1564 is a result of perturbing CP nuclear import by Impα/β1, fluorescence recovery after photobleaching (FRAP) assays were performed in live HeLa cells. Briefly, 24 h prior to FRAP, HeLa cells were transfected to express a GFP-capsid fusion protein (CP-GFP). 4 h before imaging, fresh media containing DMSO with or without 100 μM compound was added. Nuclear fluorescence was irreversibly bleached by high intensity laser scanning (bleached region indicated by dashed lines in Fig. 9A), and the recovery of nuclear fluorescence (Fn(rec)), which can only occur through nuclear import from the cytoplasm of unbleached, cytoplasmic CP-GFP by Impα/β1) was tracked for almost 10 min. Consistent with the luciferase and plaque assays, G281-1564 significantly inhibited the nuclear import of CP-GFP, while 70927013 had no effect (Fig. 9). G281-1564 not only reduced the initial rate of nuclear import by c. 30% (Fig. 9D), but also reduced the maximal amount of CP-GFP fluorescence that returned to the nucleus by c. 45%. These experiments in living cells are conclusive evidence that G281-1564 reduces CP nuclear accumulation by restricting the nuclear import process itself.

3. Discussion

This study is the first to perform HTS to identify compounds able to selectively inhibit the Impα/β1:VEEV CP interaction, with our nested counterscreening strategy of particular utility in helping prioritise compounds specific to the Impα/β1:CP interaction, rather than those inhibiting Impα/β1 or the screening assay itself. The most active compounds possessed IC50 below 10 μM, with three of our compounds (G281-1485, Z1139230991, and G281-1564) ultimately shown to be able to reduce viral proliferation in Vero cells infected with VEEV TC83; two of these belonged to the CL6662 scaffold family (Fig. 6). A third CL6662 compound was also identified as able to inhibit the Impα/β1:VEEV CP interaction, but proved to be cytotoxic. In contrast to G281-1481 and G281-1485, G281-1564 was not toxic (CC50 > 100 μM) and possessed robust antiviral activity (EC50 = 10.8 μM). Further, the activity of this compound was found to be specific to the Impα/β1:CP interaction, since it had no effect on nuclear accumulation of an alternative Impα/β1 cargo. It seems likely that G281-1564 may bind directly to CP to mask the binding site for Impα/β1, although further study is needed to demonstrate this directly using a biophysical or other approach (Gras et al., 2012; Atkinson et al., 2018). Given that the CP sequence is highly conserved amongst New World alphaviruses (Atasheva et al., 2010), it would also be exciting to test our compounds against the related Eastern and Western Equine Encephalitis viruses, as well as wild type strains of VEEV.

The CL6662 compounds are extremely similar, sharing a complex backbone of aromatic and non-aromatic hetero/carbocycles attached to a central diazepine core. Compounds G281-1481 and G281-1485 share an identical isopropyl substituent on the central diazepine core, and only differ by a methyl group on the carboxamide side chain, possessing methoxypropyl or ethyloxypropyl carboxamide, respectively (Fig. 6); both show significant cytotoxicity, unlike the related G281-1564. G281-1564 differs at only one position compared to G281-1481, carrying an ethyl substituent on the diazepine core rather than an isopropyl group. The SAR relating to compound G281-1481 provides evidence that cytotoxicity could be linked to the alkyl substituent attached to the diazepine core, with initial results demonstrating branched alkyl groups could lead to cytotoxicity of otherwise efficacious compounds and non-toxic leads. Our limited SAR analysis thus suggests that the CL662 scaffold, and the G281-1564 compound in particular, may represent a solid starting point for medicinal chemistry approaches to develop more efficacious, non-toxic anti-VEEV agents.

In conclusion, this study validates the important principle that targeting nuclear import of VEEV CP, and in particular the host Impα/β1:VEEV CP interface, represents a viable antiviral strategy, with the compounds identified here providing a foundation for the development of highly active and safe anti-VEEV agents. Most importantly, our HTS approach, based on nested counterscreens designed to enable the rapid identification of selective, active inhibitors, represents a platform that can easily be used for various other viruses, and indeed other pathogens. Although the platform as used here and previously (Wagstaff et al., 2011; Fraser et al., 2014) is optimised for host-pathogen interactions reliant on Impα/β1, it is easily adaptable to other host-pathogen interfaces that are critical to pathogenesis.
4. Methods

4.1. CP expressing constructs

The protein coding sequence for VEEV CP was PCR-amplified using plasmid pTC83 as a template (bp7563-8387) and inserted upstream of a Gly4 linker region and the eGFP sequence using overlap PCR. It should be noted that the CP sequence of TC-83 is identical to that of the virulent TrD strain of VEEV (Kinney et al., 1993). This fragment was then inserted into pcDNA3.1 using restriction enzymes BamHI and NotI, cleavage sites for which were included in the 5' and 3' primer sequences respectively to create pcDNA3.1_VEEVC-GFP for transfection.
experiments. Plasmid pcDNA3.1_VEEVC-GFP was then used as a template for PCR amplification of the C coding sequence using primers to incorporate an in-frame His6 tag in the 5′ coding sequence, as well as flanking NdeI and HindIII restriction sites. This fragment was then inserted into plasmid vector pCOLDIV (Takara Bio, Japan) to create pColdIV-C for bacterial expression of CP.

4.2. Protein expression in bacteria

NICO21 (DE3) cells carrying plasmid pColdIV-C were grown overnight in Overnight Express TB (Merck, Germany) at 28 °C, followed by 24 h at 30 °C. Cells were harvested by centrifugation at 3500 rpm, re-suspended in 100 mM Na2HPO4, 150 mM NaCl, pH 7.4, and immediately frozen at −20 °C. Upon thaw, 1 ml of popculture lysis solution (Merck), 1 protease inhibitor cocktail tablet and 10 μg/ml DNase I was added and the cells sonicated at 10 hz for 6 × 30 s cycles of sonication followed by 30 s of cooling on ice. Lysates were cleared by centrifugation (16,000 rpm, 4 °C) and then incubated with Ni-NTA agarose resin (Qiagen, Germany) for 3 h at 4 °C on a rotator. The resin was washed with 30x resin volume in 100 mM Na2HPO4, 300 mM NaCl, pH 7.4, and eluted in 100 mM Na2HPO4, 150 mM NaCl, 500 mM imidazole, pH 7.4. The eluate was further puriﬁed by gel ﬁltration using a S200 column (100 mM Na2HPO4, 150 mM NaCl, pH 7.4). Enriched fractions were pooled and concentrated using an Amicon centrifuge ﬁlter device with a 10 kDa MWCO (Merck Millipore, USA). IMPα2 and IMPβ1 were expressed as GST-tagged proteins in bl21(pREP4) E. coli cells and puriﬁed under native conditions as previously (Hubner et al., 1997; Xiao et al., 1997). Prior to use, IMPβ1 and IMPα2 were dimerised at a 1:1 ratio in intracellular buffer (110 mM KCl, 5 mM NaHCO3, 5 mM MgCl2, 1 mM EGTA, 0.1 mM CaCl2, 20 mM HEPES, pH 7.4) at a concentration of 13.6 μM for 15 min at room temperature (Baliga et al., 2003). The optimised GFP-T-agNLS fusion protein was puriﬁed as a His6-tagged peptide under denaturing conditions from bl21(pREP4) as previously (Wagstaff and Jans, 2006). His6-GST was purchased from Abcam (UK). Protein concentrations were estimated by SDS-PAGE and Coomassie staining, and/or quantiﬁed by Bradford Protein Assay (Bio-Rad Laboratories, USA).

4.3. AlphaScreen assay

The AlphaScreen technology was used largely as previously (Wagstaff et al., 2011; Fraser et al., 2014). Briefly, 10 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) was added to each well, followed by 5 μl of either CP or GFP-T-agNLS (ﬁnal concentration of 30 nM), and 5 μl of pre-dimerised IMPα/β1, followed incubation for 30 min at room temperature. Finally, 5 μl AlphaScreen bead mix (1/250 dilution of nickel NTA donor beads, 1/250 dilution of GST acceptor beads, and 0.1% BSA in PBS) was added and the plates incubated in the dark at room temperature for 16 h. Assays from the HTS or IC50 analysis were then read on Envision or Enspire plate readers (PerkinElmer), respectively.

For HTS, compounds were provided in plates by Compound Fig. 8. Lead compounds selectively target the Impα/β1:CP interaction. HeLa cells transfected to express CP-GFP or GFP-T-agNLS fusion proteins were treated with 50 μM compound or DMSO as indicated for 20 h prior to imaging. Fn/c ratios of transfected cells were determined and compared to DMSO treated controls. Data represent the mean ± SEM (n ≥ 28). *p < .05; **p < .01; ***p < .001.
Australia in 125 nl of DMSO to a final concentration of 25 μM which were stored at −20 °C until use. 5 μl of PBS (or 10 μl for His₆-GST plates) was added to each well using a Multidrop liquid dispenser (Thermo Scientific), then 10 μl of either CP or GFP-T-agNLS (to 30 nM final concentration), or His₆-GST (to 1.25 nM final concentration) was added to sample wells using JANUS Modular Dispense technology (MDT, PerkinElmer), while 10 μl of PBS was added to negative control wells manually. 5 μl of pre-dimerised Impα/β₁ was then added to CP or GFP-T-agNLS assay plates to a final concentration of 15 nM using the Multidrop dispenser. The Impα/β₁-cargo mix was then left to bind for 30 min, before 5 μl of bead mix (final concentration of 1/250 NiNTA donor beads and GSH acceptors beads, and 0.1% BSA in PBS) was added to all wells by multidrop. Plates were incubated at room temperature overnight, and read on an Enspire plate reader the next morning. DMSO alone positive controls were included on each assay plate, along with negative controls in which one of the proteins was excluded. The signal from the negative control wells was subtracted from all samples, including the positive control, and the % inhibition determined in relation to the positive control.

IC₅₀ values were determined from AlphaScreen binding assays essentially as above, but with binding assessed in the presence of a range of compound concentrations (0.2–200 μM). Assays were performed in triplicate, and all additions were performed manually. Data was analysed using Prism software (GraphPad Software, Inc.) and IC₅₀s determined by fitting one-phase dissociation curves to the data. Compounds were purchased as powder from established suppliers Enamine (Kyiv, Ukraine) and Chemdiv (CA, USA).

**4.4. Fluorescence microscopy**

HeLa cells were maintained in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% fetal calf serum (FCS), 1 x GlutaMAX...
(Thermofisher, USA), and 1 x non-essential amino acids (NEA, Thermofisher) at 37 °C with 5% CO2. Cells grown on glass coverslips in 6-well plates were transfected with 1 μg of DNA using lipofectamine 3000 (Invitrogen) as per the manufacturer’s instructions. For steady state analysis, HeLa cells were transfected to express either the Cp-GFP or GFP-T-ag-NLS fusion proteins. After 6 h, the transfection media was replaced with media containing 50 μM compound, and cells were Hoechst stained and imaged 20 h later using an Olympus FV1000 microscope equipped with temperature control and a 100x oil immersion lens. Digitised images were analysed using the ImageJ software (National Institute of Health, Bethesda, MD) to measure the Fn/c ratio as previously (Wagstaff et al., 2011, 2012), using the formula; Fn/c = (Fn-Fb)/(Fc-Fb), where Fn is the nuclear fluorescence, Fc, the cytoplasmic fluorescence, and Fb the background fluorescence. Statistical analyses were carried out using one-way ANOVA.

For FRAP experiments, HeLa cells were transfected, and medium was replaced with medium containing DMSO ± 100 μM compound before incubating the cells at 37 °C for 4 h, prior to CLSM imaging. FRAP analysis was performed as previously (Kuusisto et al., 2012; Ng et al., 2014; Roth et al., 2011), whereby nuclei were selectively bleached (12 scans at 100% laser power, 488 nm laser) and recovery monitored every 20 s for 580 s. Digitised images were analysed using ImageJ to determine the % recovery of nuclear fluorescence (F(Ren)), calculated using the formula; F(en)(en) = {F(e)-F(n)/[F(e)-F(n)]}, where F(n) is the nuclear fluorescence above background at x seconds post-bleach, and F(n) is the nuclear fluorescence above background pre-bleach. One-phase association curves were fitted using Prism to quantify recovery. Statistical analyses were performed using Students t test.

4.5. CC50 and EC50 assays

Cells were cultured at 37 °C in a humidified 5% CO2 atmosphere. All compounds were dissolved in DMSO, with 0.1% DMSO used as the vehicle control. CC50s were measured by addition of serially diluted compounds or DMSO to vero cells. 24 h later, cytotoxicity was assessed using Promega’s CellGlo Cell Viability assay according to the manufacturer’s specifications. EC50s were determined using the VEEV-luc reporter virus, which encodes in the VEEV TC-83 backbone the firefly luciferase gene under control of a second sub-genomic promoter (Lundberg et al., 2016; Patterson et al., 2011). Serial dilutions of each compound were used to pre-treat Vero cells for 2 h prior to infection with the reporter virus. After infection, cells were washed and the drug media replaced. Inhibition of viral replication was measured 16 h post-infection using Promega’s BrightGlo Luciferase assay according to the manufacturer’s specifications.

4.6. Plaque assays

Compounds were diluted in DMEM (supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin), and used to pre-treat Vero cells for 2 h. Supernatants were removed, and virus diluted in supplemented DMEM was added to the cells for 1 h. Virus inoculum was removed, cells were washed once with sterile 1X PBS, and compound media replaced. Supernatants were then collected at 16 h post-infection (hpi).

To determine plaque titre, crystal violet plaque assays were performed as previously (Lundberg et al., 2013). Briefly, infected supernatants were serially diluted 1:10 in supplemented DMEM then placed over confluent Vero cells. After 1 h incubation, a 1:1 mixture of 2 x Eagle’s Minimal Essential Medium (EMEM) (supplemented with 5% FBS, 1% minimum essential amino acids, 1% sodium pyruvate, and 2% penicillin/streptomycin) and 0.6% agarose in purified water was added to each well. After 48 h, plates were fixed with 10% formaldehyde for at least 1 h. Once the agarose plugs were removed, cells were stained with 1% crystal violet in 20% ethanol and purified water. Plaques were counted, and triplicate samples averaged. Statistical analyses were carried out using two-way ANOVA.

Author contributions

DT, LL, and CP performed the experiments, DT, KK, and DJ wrote the manuscript, KW, AD, JB, KK, CH, SS, and DJ helped design experiments, and all authors read and approved the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This work was funded through a Defense Threat Reduction Agency grant, HDTRA1-15-1-0014, to KK, DJ, JB and KMW. DTRA does not have any role in the design of the study and collection, analysis, and interpretation of data and nor in writing the manuscript. DJA is an National Health and Medical Research Council Senior Principal Research Fellow (APP1103050).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2018.01.007.

References


