Identification of a small molecule inhibitor of Ebola virus genome replication and transcription using in silico screening

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\textbf{ABSTRACT}

Ebola virus (EBOV) causes a severe haemorrhagic fever in humans and has a mortality rate over 50%. With no licensed drug treatments available, EBOV poses a significant threat. Investigations into possible therapeutics have been severely hampered by the classification of EBOV as a BSL4 pathogen. Here, we describe a drug discovery pathway combining in silico screening of compounds predicted to bind to a hydrophobic pocket on the nucleoprotein (NP); with a robust and rapid EBOV minigenome assay for inhibitor validation at BSL2. One compound (MCCB4) was efficacious (EC\textsubscript{50} 4.8μM), exhibited low cytotoxicity (CC\textsubscript{50} > 100μM) and was specific, with no effect on either a T7 RNA polymerase driven firefly luciferase or a Bunyamwera virus minigenome. Further investigations revealed that this small molecule inhibitor was able to outcompete established replication complexes, an essential aspect for a potential EBOV treatment.

1. Introduction

The \textit{Ebola} virus genus, alongside \textit{Cuevavirus} and \textit{Marburgvirus}, is classified within the \textit{Filoviridae} family within the order \textit{Mononegavirales}. There are 5 species within this genus: \textit{Bundibugyo} (BDBV), \textit{Reston} (RESTV), \textit{Sudan} (SUDV), \textit{Taï Forest} (TAFV) and \textit{Zaire} (previously known as ZEBOV) the type species now referred to as \textit{Ebola virus} (EBOV) (Amarasinghe et al., 2017; Kuhn et al., 2010). Marked differences can be seen between the different species with regard to geographical spread and pathogenicity. For example EBOV can exhibit disease mortality rates of up to 90% in humans (Rollin, 2009), while RESTV is not known to cause disease in humans (Miranda and Miranda, 2011).

The high pathogenicity of EBOV, the ease of transmission via bodily fluids (Bausch et al., 2007), the rapid infection progression (CDC, 2014), and the current lack of licenced treatments has resulted in its classification as a Biosafety Level 4 (BSL4) pathogen, hampering development of effective therapies. Hence, despite much research on EBOV replication and potential therapeutics there are currently no licenced treatments for infection.

EBOV is a filamentous enveloped virus with a non-segmented, negative sense single stranded RNA (-ssRNA) genome of ~19 kb (Geisbert and Jahrling, 1995; Kiley et al., 1982). The genome encodes 7 proteins: a nucleoprotein (NP), a glycoprotein, 4 viral proteins (VP24, VP30, VP35 and VP40) and the L protein (RNA-dependent RNA-polymerase) (Mühlberger et al., 1999).

The NP forms a complex with VP35, VP30 and L which is essential for genome replication and transcription (Ruigrok et al., 2011; Sun et al., 2012; Zhou et al., 2013). This complex is the basis for the EBOV minigenome system (MG) (Mühlberger et al., 1999) where plasmids expressing these 4 proteins under the control of a T7 promoter are transfected into cells constitutively expressing T7 RNA polymerase, together with a plasmid with a T7 promoter driving production of an RNA containing the reverse complement of a reporter gene (firefly luciferase) flanked by EBOV genome recognition sequences. A functional replication complex will recognise these sequences, transcribe and translate luciferase which provides an indirect measurement of EBOV-specific gene expression. Because the complete genome is not present and therefore no infectious virus can be produced, this system allows for the investigation of EBOV genome replication and transcription at BSL2.
Recently, the structure of the NP and the interactions with VP35 have been characterised (Dong et al., 2015; Leung et al., 2015). A hydrophobic pocket on NP either binds intramolecularly with a flexible arm of NP (helix-20), or with an NP binding peptide of VP35 (NPBP, residues 20–48). The two binding states control the binding of NP and release of RNA and oligomerisation – essential to viral replication (Kirchdoerfer et al., 2015). For other negative-strand viruses, it has been shown that NP is a valid target for small molecule inhibitors (SMIs), exemplified by the influenza inhibitor Nucleozin, which triggers aggregation of NP with an EC<sub>50</sub> in the nM range (Kao et al., 2010), and the < 60 nM EC<sub>50</sub> reported for a series of inhibitors which promote NP oligomerisation (Gerritz et al., 2011). Another reason why NP is an attractive target for possible inhibitors is the VP35 binding pocket is highly conserved between EBOV and the related Marburgviruses (Zhu et al., 2017). Although VP35 NPBP bind with a stronger affinity to their own NPs, they are able to bind to the NP of other filoviruses.

Although the MG system has been used recently to identify small molecule inhibitors of EBOV replication (Edwards et al., 2015; Luthra...
2. Materials and methods

2.1. Cell lines and plasmids

BSR-T7 cells are derived from BHK-21 cells and stably express T7 RNA polymerase (Buchholz et al., 1999). Huh7-Lunet-T7 cells are a derivative of Huh7 cells and also express T7 RNA polymerase (Backes et al., 2010). Cells were grown in DMEM (Sigma-Aldrich), 10% FBS (Sigma-Aldrich), 1% non-essential amino acids (Lonza), penicillin-streptomycin (100 units/ml) (Life Technologies) (BSR-T7) or Zeocin (Invitrogen) (5 μg/ml) (Huh7-Lunet-T7) added at every second passage. Cells were maintained at 37 °C with 5% CO2.

The minigenome system for EBOV (Makona strain, GenBank KJ660347.2) (García-Dorival et al., 2016) expressing Firefly luciferase, used in this study was kindly donated by Julian Hiscox (University of Liverpool) following the minigenome system model developed by Mühlenber et al. (1999) (see Supplementary Fig. 1). The control plasmid pT7FFLuc contains a Firefly luciferase under the direct control of a T7 promoter. The plasmids for the Bunyamwera minigenome express a Renilla luciferase and were described previously (Barr et al., 2003; Walter et al., 2011).

2.2. Compounds

These were initially selected from our Medicinal Chemistry/Chemical Biology (MCCB) group in-house compound library in the School of Chemistry, University of Leeds. Compounds are stored at 14 °C in 100% DMSO stock solutions, within 0% humidity cabinets. Dry stocks of the original compounds were later purchased from the original supplier (ChemDiv) to confirm initial biological activity. Follow-up compounds were purchased from ChemBridge or ChemDiv and shipped as dry stocks. Compound purity was assessed by LCMS using standard methods and a cut-off of > 95% purity was used.

2.3. Virtual screening cascade

The in-house library containing 48,750 small molecules was docked to both crystal structures of Ebola NP (4Z9P and 4YPI) using the exhaustive docking algorithm eHiTs (Zsoldos et al., 2007).

The small molecules were then ranked according to predicted binding affinity (eHiTs score). The top 5000 scoring molecules for each protein structure were re-docked using an alternative docking software, AutoDock 4.2 (Morris et al., 2009). The 5000 molecules were clustered by energy (predicted binding affinity) and an arbitrary cut-off of −7.5 was chosen for visually inspection. Since 20 conformations are produced for each structure, visual inspection allowed us to ascertain good clustering (> 10/20 conformations were of similar energy, i.e. a highly populated pose), and identify predicted hydrogen bonds and steric clashes with the protein. Compounds that satisfied this criteria were cross-referenced with the top ranking molecules from eHiTs docking to identify those which were predicted to bind favourably in both crystal structures and both docking software. We envisioned that a consensus docking approach (and orthogonal scoring functions) with both crystal structures would increase our hit-finding. Additional in silico analysis was performed using Maestro 10.2 (Maestro, Schrödinger, LLC, New York, NY, 2017).

2.4. EBOV and BUNV minigenome and pT7FFLuc transfections

1 × 10^5 cells were seeded in each well of a 24-well plate and allowed to adhere overnight to achieve a desired cell density of ~90% confluency. Per well, cells were transfected with EBOV-MiniG 0.5 μg, NP 0.25 μg, VP30 0.125 μg, VP35 0.125 μg and L 0.125 μg; the pT7FFLuc 0.3 μg; or BUNV(S) RN 0.4 μg, L-sup 0.1 μg and S-sup 0.1 μg. Transfections were performed with Lipofectamine (ThermoFisher...
2.5. Luciferase and MTT assays

Cells were harvested 24 hpt by washing in PBS then lysing in Passive Lysis Buffer (PLB, Promega) (See Fig. 3B legend for deviations in the timings of cell harvest). Samples were freeze/thawed at −20 °C and read using the Firefly or Renilla luciferase reporter assay systems (Promega) and a FLUOStar optima microplate reader.

MTT assays were performed alongside luciferase assays. After washing the cells in PBS, the MTT reagent (1 mg/mL in H2O) (Alfa Aesar) was added for 1 h. DMSO was used to lyse cells prior to being read on a Tecan Infinite F50 microplate reader. All plates contained a transfection only control and a cell only control to show 100% luciferase signal and background signal, respectively. 116 compounds were tested for effect against EBOV replication.

2.6. Site-Directed mutagenesis

Mutagenesis was performed on the NP plasmid, using a Q5 Site-Directed mutagenesis kit (NEB). Primers were designed using NEBaseChanger™ (sequences available on request) and contained silent AccI and SacI restriction sites, respectively, for identification.
3.2. MCCB4 inhibits EBOV genome replication and transcription

In order to assess whether MCCB4 was able to inhibit EBOV genome replication and transcription, we utilised a minigenome (MG) system (Supplementary Fig. 1). As negative controls we also used a Bu-nyamwera virus (BUNV) MG system, and a plasmid in which FF-luc expression was directly driven by a T7 promoter. All EC50 and CC50 data combinations of plasmids to account for any additional toxicity due to expression of exogenous proteins (in particular those of EBOV).

To characterise the behaviour of MCCB4 further, a time course was performed. BSR-T7 cells were transfected in the presence or absence of 4.8 μM MCCB4 (EC50) and harvested at the time points indicated. These data are shown as percentages of the DMSO only transfection control and demonstrated a statistically significant inhibition of luciferase from 12 h post transfection (hpt), with a 50% inhibition at 24 and 48 hpt (Fig. 3A). Between 0 and 6 hpt the luciferase signal was indistinguishable from background levels.

To further probe the potential of MCCB4 as a drug lead for the treatment of EBOV infection, the activity of MCCB4 on established NP-VP35 complexes was investigated as an indication of possible post-exposure treatment. MCCB4 at a concentration equivalent to the EC50 (49 μM) was added to BSR-T7 cells at different time points pre- (−24–0 hpt), during (0–48 hpt & 0–24 hpt) and post-transfection (24–48 hpt) (Fig. 3B, top panel). Cells were harvested at 48 hpt and the data shown as a percentage of the DMSO only control. Pre-treatment of cells with MCCB4 followed by removal at transfection did not reduce the luciferase signal detected (Fig. 3B, −24–0 hpt). As expected, the addition of MCCB4 at transfection was able to disrupt EBOV replication complex formation, as demonstrated by the 85% reduction in luciferase (Fig. 3B and 0–48 hpt). The addition of MCCB4 post-transfection (Fig. 3B and 24–48 hpt) also reduced luciferase signal by 84%. This suggested that MCCB4 was able to effectively compete for binding to NP in established replication complexes. Interestingly, while the addition of MCCB4 at transfection and subsequent removal (Fig. 3B and 0–24 hpt) revealed a 65% reduction in luciferase compared to the DMSO only control – this reduction was significantly less than that displayed by both the sustained addition (0–48 hpt) and the post transfection addition (24–48 hpt).

3.3. MCCB4 activity is observed in two different cell types

To ensure that the effect of MCCB4 was not specific to BSR-T7 cells, EC50 and CC50 analyses were performed in Huh7-Lunet-T7 cells, a human hepatocellular carcinoma cell line which also expressed the T7 RNA polymerase. Both fibroblasts and hepatocytes are capable of supporting Ebola replication (Baskerville et al., 1985). The transfection efficacy of the EBOV MG and the T7FFLuc control in the different cell lines were similar (data not shown). In Huh7-Lunet-T7 cells, MCCB4 was shown to be effective with an EC50 1.5 μM (Fig. 4A). MCCB4 exhibited a low level of cytotoxicity (CC50 43.2 μM) and a low level of activity against the T7FFLuc control (EC50 30.8 μM) in the Huh7-Lunet-T7 cells (Fig. 4B).

3.4. Validation of MCCB4-NP interaction through mutation of the NP hydrophobic pocket

Although MCCB4 was predicted using eHiTs and AutoDock software to bind to hotspots one and two (Fig. 1), in a similar manner to the other active compounds.

MCCB4 is a linear hydrophobic molecule containing an ene-thiazolidinedione group. This molecule has the potential to act as a Michael acceptor via covalent bond formation to nucleophilic residues within proteins, and display ‘promiscuous’ behaviour in biological assays (Baell and Walters, 2014). We were, therefore cautious concerning any biological activity observed for this molecule and wished in particular to probe the selectivity of the activity displayed by this molecule.

of well-to-low variation in transfection efficiency.

Transfected cells were treated with MCCB4 over a 10-fold range of concentrations from 10 nM to 100 μM. As shown in Fig. 2A, MCCB4 exhibited a dose-dependent inhibition of the EBOV MG with an EC50 of 4.8 μM. This was specific to EBOV as the EC50 for both BUNV MG and the T7FFLuc controls was > 100 μM. In parallel, we confirmed that MCCB4 did not exhibit any significant toxicity (CC50 > 100 μM: Fig. 2B). This was assessed in cells transfected with the various combinations of plasmids to account for any additional toxicity due to expression of exogenous proteins (in particular those of EBOV).

As negative controls we also used a Bu-nyamwera virus (BUNV) MG system, and a plasmid in which FF-luc expression was directly driven by a T7 promoter. All EC50 and CC50 data...
Mutagenic analysis of NP confirms the mode of action of MCCB4. A. The EBOV NP hotspot 2 residues Phe280 and Leu284 were mutated to alanine (F280A and L284A, respectively). B. EC$_{50}$ curve of percentage reduction in luciferase. MCCB4 was added to cells at 100, 50, 10, 5, 1, 0.5, 0.1, and 0.01 μM immediately before transfection with either wildtype EBOV NP, mutant F280A or L284A. Cells were harvested at 24 hpt and analysed for Firefly luciferase. Values are represented as percentages of a transfection only control. C. Comparison of EC$_{50}$ values generated from the wildtype NP or mutant NP transfections. EC$_{50}$ values from 3 independent experiments performed in triplicate were plotted and compared using an unpaired two-tailed t-test. Error bars show SD. *** P Value < 0.001.
over a 10-fold range of concentrations. Of note, although the two mutations resulted in a 10-fold reduction in RLU compared to wildtype, they were replication competent (Supplementary Fig. 2). The low levels of RLU were not due to residual unspecific reporter gene activity as they were significantly higher than the values obtained in the absence of the plasmid expressing the L polymerase. These data were compared to the relevant transfection-only control and shown as a percentage of this to take into account any discrepancy between absolute units of luciferase. As a control, cells were treated with mycophenolic acid (MPA), an inosine monophosphate dehydrogenase inhibitor which inhibits RNA virus replication (Khan et al., 2011), including EBOV (Johansen et al., 2015). As expected, MPA at the EC₉₀ concentration affected all three

Fig. 6. SAR analysis of MCCB4-8. The MCCB4 derivative MCCB4-8 was tested for effect against the T7FFLuc control, and EBOV minigenome. The compounds were added to BSR-T7 cells at 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 μM immediately before transfection. Cells were harvested at 24 hpt and analysed for Firefly luciferase. Values are represented as percentages of a transfection only control. A. Structure of the MCCB4-8 compound. B. EC₅₀ curve of percentage reduction in luciferase. EBOV, red circles. T7FFLuc control, blue squares. C. CC₅₀ curve of percentage reduction in cell viability. EBOV, red circles. T7FFLuc control, blue squares. Averages plotted from 3 independent experiments performed in triplicate. Error bars show SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

NP(s) equally.

While MCCB4 inhibited both EBOV NP mutants at high concentrations, the EC₅₀ values were significantly higher than those shown by the wildtype NP (F280A 15.5 μM, L284A 13.8 μM Fig. 5B and C), demonstrating that these mutations resulted in partial resistance to MCCB4. MCCB4 cytotoxicity (CC₅₀ > 100 μM, Fig. 5B) was not affected by the substitution of wildtype NP with either of the mutants. These data are consistent with the predicted binding of MCCB4 to the hydrophobic pocket within NP.
Table 1

| COMPOUND | TARGET | CELL   | EC_{50} | CC_{50} | SI^a |
|----------|--------|--------|---------|---------|------|
| MCCB4    | WT NP  | BSR-T7 | 4.8 μM  | > 100 μM| > 20.8|
| MCCB4    | WT NP  | Huh-Lunet-T7 | 1.5 μM | 43.2 μM | 28.8 |
| MCCB4    | NP F280A | BSR-T7 | 15.5 μM | > 100 μM| > 6.5 |
| MCCB4    | NP L284A | BSR-T7 | 13.8 μM | > 100 μM| > 7.2 |
| MCCB4-8  | WT NP  | BSR-T7 | 11.2 μM | 44.9 μM | 4    |
| MCCB4-12 | WT NP  | BSR-T7 | 8.8 μM  | 57.8 μM | 6.5  |

^a Selective Index (CC_{50}/EC_{50}).

3.5. Structure-activity relationships of MCCB4 and related analogues

In order to rapidly ascertain structure-activity relationship (SAR) data for MCCB4, five close structural derivatives, where particular functional groups had been modified, were purchased (from ChemDiv Inc) and a further eight ChemBridge analogues were identified using the programme ROCS (rapid overlay of chemical structures) (OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com) (Hawkins et al., 2007), a program which identifies compounds based on 3D shape similarity. Initially compounds were screened for efficacy compared to the MCCB4 parent compound. Two MCCB4 derivatives, MCCB4-8 and MCCB4-8 (Fig. 6) and MCCB4-12 (Fig. 7) were identified as effective and so further investigated. Transfected cells were treated with the analogues over a range of concentrations (10 nM–100 μM). As shown in Figs. 6 and 7, both MCCB4-8 and MCCB4-12 exhibited a dose-dependent inhibition of the EBOV MG.

MCCB4-8 is identical to the parent compound MCCB4 except for a fluorine at the 3-position of the terminal benzene ring (Fig. 6A, red box). MCCB4-8 has an EBOV specific EC_{50} of 11.2 μM (Fig. 6B) and a CC_{50} of 45 μM (Fig. 6C EBOV). MCCB4-12 is identical to the parent compound MCCB4 except for a fluorine at the 4-position of the terminal benzene ring (Fig. 7A, red box). MCCB4-12 has an EBOV specific EC_{50} of 8.8 μM (Fig. 7B) and a CC_{50} of ~58 μM (Fig. 7C EBOV and T7FFLuc). The higher EC_{50} values and the lower CC_{50} values for these derivatives demonstrate that these structural changes are not advantageous.

4. Discussion

The data presented here outlines an *in silico* screen of SMIs which are predicted to bind in the hydrophobic pocket on the EBOV NP. MCCB4 is predicted to bind to hotspots 1 and 2 within the hydrophobic pocket on the NP structure (Fig. 1). The benzyl group of MCCB4 fills the deep hydrophobic hotspot 2 with the pyridyl group located in hotspot 1. From our computational analysis, compounds predicted to bind to NP had structural features that allowed the molecule to traverse both these hotspots. MCCB4 contains five ring motifs and also an enethiazolinediene group (which is structurally similar to the rhodamine class of promiscuous inhibitors (Baell and Walters, 2014), which potentially decrease its attractiveness for further drug development. Whilst we were mindful of the potential for promiscuous inhibition of additional cellular processes, we did not observe any additional toxic effects in our cellular assays compared with other compounds which were identified as non-selective and toxic. MCCB4 is present in screening collections but has not been reported in the scientific literature or in Chembl, the bioactive screening database.

MCCB4 was shown to be efficacious in reducing the luciferase signal detected in EBOV MG transfected BSR-T7 cells (EC_{50} 4.8 μM) and Huh7-Lunet-T7 cells (EC_{50} 1.5 μM). This effect was specific against EBOV replication as shown by high EC_{50} values (EC_{50} > 100 μM) demonstrated by both the T7FFLuc control transfection and the BUNV MG transfection. MCCB4 was well tolerated by cells, exhibiting a CC_{50} > 100 μM in BSR-T7 cells, although the compound appears to have a greater cytotoxicity in Huh7-Lunet-T7 cells (CC_{50} 43.2 μM). The reduction in luciferase signal presented by the T7FFLuc and BUNV MG control transfections tracks closely to cell viability so most likely reflects a reduction in the viability of transfected cells.

There are two distinct paths for potential EBOV treatments: post-exposure prophylaxis and treatment of symptomatic patients. Both have different challenges but a common strategy might be to limit virus replication to allow the adaptive and innate immune systems time to fight infection (Bray and Paragas, 2002; Feldmann et al., 2005). In this regard we demonstrated that MCCB4 can inhibit EBOV replication with a single dose at the time of transfection, but also was able to effectively inhibit replication when added at 24 h post, supporting the possible use of MCCB4 as a post-exposure drug.

In order to confirm that MCCB4 interacts with EBOV NP at the targeted hydrophobic binding pocket, two alanine substitution mutants were generated (NP L284A and NP F280A) in this region. These changes were predicted to reduce binding efficiency. This pocket is the binding site of NP-VP35 during EBOV replication so a balance between retaining replicative function and disrupting the compound binding, must be met. As expected, the mutants exhibited a 10-fold reduction in replication (Supplementary Fig. 2). The mutations did not have any effect on the sensitivity to the non-specific antiviral compound MPA which was used as a control, nor the CC_{50} value of MCCB4 but did cause a significant EC_{50} shift between wildtype and mutant in response to MCCB4. This reduction was consistent with the hypothesis that MCCB4 binds to NP as predicted using *in silico* screening.

The selective index (SI = CC_{50}/EC_{50}) (Table 1) is a numerical method of comparing the therapeutic window of potential drugs, with a higher selective index being preferable. The EC_{50} values of the two MCCB4 analogues, MCCB4-8 and MCCB4-12, are higher than the parental compound (Table 1). In addition they exhibited lower CC_{50} values. Compounds MCCB4-8 and MCCB4-12, were predicted to enhance binding to EBOV NP via an enhanced hydrophobic effect, with the additional fluorine atom filling the deep hydrophobic hotspot 2. However, it is clear that the structural changes present in MCCB4-8 and MCCB4-12 adversely affected the efficacy of the compounds, pointing to a key role for the terminal benzene ring as a key player in the activity of MCCB4. This observation will inform future SAR that will focus on this part of the molecule in an attempt to improve efficacy.

5. Conclusions

Much research has been performed to investigate Ebola replication with a view to developing this knowledge into therapeutic strategies. Many SMIs have been tested *in vitro* but this has yet to be translated into successful clinical studies and licenced drugs. Using the structure of NP, *in silico* screening was performed to identify SMIs including MCCB4. MCCB4 is efficacious, selective and well tolerated by cells. Limited SAR has been performed and it is our opinion that an extended investigation into the structure of MCCB4 could generate a superior compound suitable for *in vivo* investigations. As the NP protein is conserved and essential for replication, this drug discovery pipeline could generate a drug not only for EBOV but also related Filoviruses.

Declarations of interest

None.

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