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Structure-based discovery of clinically approved drugs as Zika virus NS2B-NS3 protease inhibitors that potently inhibit Zika virus infection *in vitro* and *in vivo*

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ABSTRACT

Zika virus (ZIKV) infection may be associated with severe complications in fetuses and adults, but treatment options are limited. We performed an *in silico* structure-based screening of a large chemical library to identify potential ZIKV NS2B-NS3 protease inhibitors. Clinically approved drugs belonging to different drug classes were selected among the 100 primary hit compounds with the highest predicted binding affinities to ZIKV NS2B-NS3-protease for validation studies. ZIKV NS2B-NS3 protease inhibitory activity was validated in most of the selected drugs and *in vitro* anti-ZIKV activity was identified in two of them (novobiocin and lopinavir-ritonavir). Molecular docking and molecular dynamics simulations predicted that novobiocin bound to ZIKV NS2B-NS3-protease with high stability. Dexamethasone-immunosuppressed mice with disseminated ZIKV infection and novobiocin treatment had significantly ($P < 0.05$) higher survival rate (100% vs 0%), lower mean blood and tissue viral loads, and less severe histopathological changes than untreated controls. This structure-based drug discovery platform should facilitate the identification of additional enzyme inhibitors of ZIKV.

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1. Introduction

Zika virus is an emerging human-pathogenic flavivirus that has caused an unprecedented large-scale epidemic of congenital microcephaly and malformations in the Americas (Chan et al., 2017a; Zhu et al., 2016). Initially thought to be a completely self-limiting illness in infected adults, an increasing number of serious complications were recently reported among adult patients as the epidemic expanded in the Americas and other regions (Duffy et al., 2009; Musso and Gubler, 2016). These included severe neurological complications, such as Guillain-Barré syndrome, menigoencephalitis, and myelitis, thrombocytopenia and disseminated intravascular coagulation with hemorrhagic complications, hepatic dysfunction, acute respiratory distress syndrome, shock, multi-organ dysfunction syndrome, and death (Arzuza-Ortega et al., 2016; Azevedo et al., 2016; Cao-Lormeau et al., 2016; Carteaux et al., 2016; Chraibi et al., 2016; Mecharles et al., 2016; Sarmiento-Ospina et al., 2016; Soares et al., 2016). Alarmingly, human cases of hematospermia and mouse models of orchitis with possible long-term effects on male fertility were also described (Chan et al., 2016c; Foy et al., 2011; Govero et al., 2016; Ma et al., 2016; Musso et al., 2015). Currently, treatment options for ZIKV infection in pregnant patients and severe ZIKV-associated complications remain limited.

To identify immediately available anti-ZIKV treatment options, a number of drug repurposing programmes have been conducted by screening drug libraries using cell culture-based antiviral assays (Barrows et al., 2016; Retallack et al., 2016; Xu et al., 2016). However, most of these clinically approved drugs found to have *in vitro* anti-ZIKV activity are anti-cancer or immunomodulating agents which are immunosuppressive or contraindicated in pregnancy (FDA pregnancy category D). Moreover, such screening approach does not elucidate the anti-ZIKV mechanisms of these drugs which are important for further development of safer and more effective drug analogues than the lead drug compound. An alternative approach to discover other potential anti-ZIKV treatment options is by repurposing clinically approved drugs which inhibit the key enzymes of ZIKV, including protease, helicase, and/or polymerase. In this study, we performed an *in silico* structure-based virtual screening of a large chemical library consisting >8000 drug compounds to identify potential ZIKV NS2B-NS3 protease inhibitors. Among the validated ZIKV NS2B-NS3 protease inhibitors, novobiocin and lopinavir-ritonavir were verified to inhibit virus replication *in vitro*. Importantly, treatment with novobiocin significantly improved the clinical outcome of mice with disseminated ZIKV infection. These results illustrated the capability of our systematic *in silico, in vitro,* and *in vivo* approach to expand the treatment options for ZIKV infection. Our findings provided a new avenue for the development of novel anti-ZIKV agents.

2. Materials and methods

2.1. In silico structure-based virtual screening of chemical library and molecular docking

All compounds deposited in the DrugBank v5.0.1 were set up for docking simulations by using AmberTools (AMBER 2017; University of California, San Francisco) (Case et al., 2017). The crystal structure of ZIKV NS2B-NS3 protease (Protein Data Bank (PDB) code 5LC0) was used to build up the protein model system (Lei et al., 2016). Other details of virtual screening calculations are specified in the Supplementary Methods.

2.2. Molecular dynamics simulations

Molecular dynamics simulations were conducted to predict the stability of the novobiocin-ZIKV NS2B-NS3 complex. Protocols are fully outlined in the Supplementary Methods.

2.3. Virus strain and titration

A clinical isolate of ZIKV (Puerto Rico strain PRVABC59) obtained from a patient in the recent South American epidemic was kindly provided by Brandy Russell and Barbara Johnson, Centers for Disease Control and Prevention, USA. The virus was cultured and titrated as we previously described with slight modifications (Chan et al., 2016b; Zhou et al., 2014) (Supplementary methods).

2.4. Cell lines and drug compounds

Vero and Huh-7 cell lines were obtained from American Type Culture Collection and JCRB cell bank of Okayama University, Japan, respectively, as we previously described (Chan et al., 2013a, 2016b). Aprotinin (Sigma-Aldrich, Missouri, USA), desmopressin acetate (Ferring Pharmaceuticals, Saint Prex, Switzerland), lopinavir-ritonavir (Abbott Laboratories, Illinois, USA), montelukast (Merck & Co., Inc., New Jersey, USA), novobiocin sodium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), octreotide acetate (Novartis, Basel, Switzerland), rifampicin (Gruppo Lepetit Srl, Milan, Italy), sirolimus (Pfizer, New York city, USA), tacrolimus (Astellas Pharma, Tokyo, Japan) were used for the *in vitro* and/or *in vivo* studies.

2.5. Cell viability assay and CPE inhibition assay

The 50% effective cytotoxic concentration (CC_{50}) of the selected drugs in Vero and Huh-7 cells were determined by thiazolyl blue tetrazolium bromide (MTT) assay as we previously described with modifications (Chan et al., 2017a; Yuan et al., 2016). To confirm the antiviral activity of novobiocin, the MTT-based CPE inhibition assay was also performed as previously described with slight modifications (Chan et al., 2017a). The half maximal inhibitory concentration (IC_{50}) and CC_{50} were calculated using Sigma plot (SPSS) in an Excel add-in ED50V10. Other details are described in the Supplementary Methods.

2.6. Viral load reduction and plaque reduction assays

Viral load reduction assay and plaque reduction assay was performed for the evaluation of antiviral activity. Experimental protocols are specified in supplementary methods as previously described with modifications (Chan et al., 2013b, 2016b, 2017a, 2017b). Both assays were performed in triplicate and repeated twice for confirmation.

2.7. Time-of-drug-addition assay

Time-of-drug-addition assay was performed for novobiocin to determine which phase(s) of virus cycle the drug interfered with (Chan et al., 2017a; Kato et al., 2016). Details are described in the Supplementary Methods.

2.8. Fluorescence-based protease inhibition assay

The recombinant ZIKV NS2B-NS3 protease was produced as previously described with some modifications (Lei et al., 2016). Experimental conditions are described in supplementary methods. To detect ZIKV NS2B-NS3 protease activity, a fluorescence-based enzymatic assay was conducted in 96-well black micro-plates