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TARGETING THE PROTEASES OF ARBOVIRUSES WITH CYCLIC AND BICYCLIC PEPTIDES

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ABSTRACT: Arthropod-borne (arbo) viruses infect hundreds of millions of people annually. Many arboviruses and their mosquito vectors circulate in Australia and neighbouring regions. Recent years have shown that explosive outbreaks accompanied by life-threating symptoms such as encephalitis are unpredictable and increasingly common, particularly due to the effects of climate change. Two major genera of pathogenic viruses are flaviviruses and alphaviruses. Both require their own viral proteases to replicate and infect new cells. Viral proteases can be considered the Achilles heel of replication and are already long-established drug targets in the fight against chronic viral infectious diseases, for example, HIV/AIDS. We developed a large variety of high-affinity and proteolytically stable peptide inhibitors of flaviviral proteases using innovative chemical approaches. Our chemical strategies proceed under biocompatible conditions, enabling in situ access to constrained peptide ligands in presence of proteases and other drug targets. While cyclic and bicyclic peptides offer many advantages over small molecules used in conventional drug discovery, their limited bioavailability is a major challenge that still needs to be overcome.

Keywords: flavivirus, alphavirus, antivirals, peptides, proteases

INTRODUCTION

The presentation at the nineteenth Biennial Conference of the Australian and New Zealand Associations of von Humboldt Fellows in Geelong, Victoria, entitled ‘Cyclic and Bicyclic Peptides as Next-Generation Antiviral Drug Candidates’ was mainly concerned with SARS-CoV-2 as the most topical example. This paper is extended to cover arboviruses, which have received much less attention during the last couple of years. Over the past decades the increasing prevalence of arbovirus infections has led to hundreds of millions of annual cases of disease. Australia has been largely unaffected in the past, but with the geographic expansion of mosquito vectors and epidemics in other parts of Oceania and Southeast Asia, due to climate change and other factors, this might change soon. It is a genuine concern, as Australia is a significant reservoir of relatively unique arboviruses, including Ross River, Barmah Forest, Murray Valley Encephalitis, and others. Neglected circulating arboviruses may cause epidemic outbreaks at any time, as highlighted by numerous Ross River and Japanese encephalitis virus cases throughout Australia triggered by the wet La Niña conditions. The importance of the existing danger and growing threat of arboviruses is significant and emphasises the need for basic research to develop specific antiviral treatments.

ARBOVIRUSES

The growing threat of arboviruses

Arboviruses are transmitted to humans via biting insects like mosquitos, ticks, sand flies and midges. They can cause explosive epidemic outbreaks and are a permanent global health concern (Gould et al. 2017; Young 2018). For example, the dengue virus alone is estimated to cause up to 390 million infections per year (Bhatt et al. 2013) and, despite a vaccine, 78,000 people are estimated to die each year from Yellow Fever in Africa (Garske et al. 2014). The past decades have seen a significant expansion of arbovirus distribution driven by urbanisation, population growth, water management, climate change, travel and trade (Young 2018). Over six billion people now live within regions that common arbovirus vectors, such as *Aedes albopictus* and *Aedes aegypti*, can survive in for at least one month per year, including most of Australia (Ryan et al. 2019). Recent outbreaks of Ross River, Barmah Forest and Japanese encephalitis viruses across Australia underpin that the Australian population is at risk of arbovirus infections.

Viral evolution can have dramatic consequences and transform a neglected virus into a global health-threatening pathogen. For example, a single amino acid mutation in the E1 glycoprotein of the Chikungunya virus facilitated its spread into a new mosquito vector, *Aedes albopictus*, which consequently triggered its distribution to new parts of the world (Her et al. 2009). Another more recent example is the Zika virus. Although known to circulate in

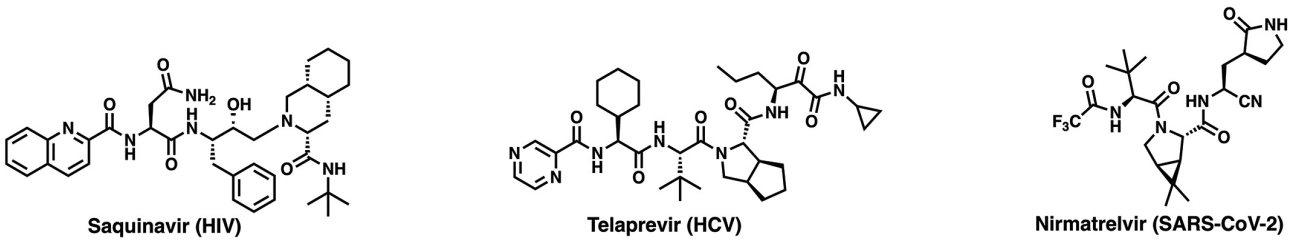

Figure 1: Viral protease inhibitors in clinical use. All compounds are derived from peptide substrates.

Africa since the 1940s, it has not been considered a health concern until it spread via Southeast Asia and Polynesia into Brazil, where it caused an epidemic in 2015–2016 associated with congenital microcephaly (Musso et al. 2019). There is a genuine risk of adaptation to new vectors and spread to currently non-endemic regions (Gould et al. 2017; Young 2018). This article deals with the two most important pathogenic arbovirus genera for humans, which are flaviviruses and alphaviruses.

Flaviviruses

Despite widespread vaccine use, 70,000 cases of Japanese encephalitis (JEV) and 130,000 cases of Yellow Fever (YFV) are estimated annually, resulting in a total loss of approximately 100,000 lives due to the absence of specific antiviral treatments (Pierson & Diamond 2020). Other well-characterised flaviviruses that have caused major epidemic outbreaks in the twenty-first century include dengue (DENV), West Nile (WNV) and Zika (ZIKV) viruses (Gould et al. 2017; Pierson & Diamond 2020; Young 2018). There are many additional flaviviruses that are far less prominent, for example, Murray Valley encephalitis (MVEV), Powassan (POWV), Usutu (USUV), Ilhéus (ILHV), Wesselsbron (WSLV), Spondweni (SPOV), Rocio (ROCV), Kunjin (KUNV), Kyasanur forest disease (KFDV) viruses and many more (Pierson & Diamond 2020). MVEV, JEV and KUNV circulate in Australia. Australia is in the midst of a JEV outbreak with notified cases in humans across New South Wales, Victoria, South Australia, Queensland and the Northern Territory (Australian Department of Health and Aged Care, health alerts, Japanese encephalitis virus, 24 February 2023).

Alphaviruses

In contrast to flaviviruses, no alphavirus vaccines are currently available. Although fatality is generally low, many alphaviruses can cause severe debilitating arthritic disease which can become chronic (Zaid et al. 2020). The explosive outbreaks of Chikungunya (CHIKV) virus between 2006 and 2019, with millions of cases, have caught global attention (Zaid et al. 2020). However, there are other (re)emerging alphaviruses such as Ross River (RRV), Barmah Forest (BFV), Sindbis (SINV), O'nyong-nyong (ONNV), Mayaro (MV), Eastern equine encephalitis (EEEV), Venezuelan equine encephalitis (VEEV) and Semliki forest (SFV) viruses that cause disease and can spread to new regions (Zaid et al. 2020). RRV, BFV and SINV are endemic to Australia.

PROTEASES

Viral proteases are established drug targets

Drugs are an alternative and/or complementary intervention to vaccination for viral infection. Many viral pathogens use the host-cell ribosomal machinery to translate their genome but employ viral proteases to cleave the polyproteins into their active constituents (Sharma and Gupta 2017). Therefore, viral proteases are essential for viral replication and thus excellent drug targets. Examples, shown in Figure 1, are approved drugs against HIV (Agbowuro et al. 2018), hepatitis C virus (Agbowuro et al. 2018), and the recently approved SARS-CoV-2 main protease inhibitor nirmatrelvir (Ullrich et al. 2022a).

The flavivirus protease NS2B-NS3

Flaviviruses have a single-stranded positive-sense RNA genome, which is translated into a single polyprotein (Barrows et al. 2018). Proteolytic cleavage is performed by host proteases and the flavivirus protease NS2B-NS3, which consists of the serine protease NS3 and the small cofactor NS2B (Nitsche et al. 2014). A superimposition of available structures of NS2B-NS3 (Noble et al. 2012; Noske et al. 2020; Lei et al. 2016; Nitsche et al. 2017) in their active conformation is shown in Figure 2a.

The alphavirus protease nsP2

Like flaviviruses, alphaviruses have a single-stranded positive-sense RNA genome (Jose et al. 2009; Schwartz & Albert 2010). The first open reading frame is translated into a polyprotein, which is processed by the viral protease, located on the C-terminus of the non-structural protein nsP2. Structures of the protease domain of CHIKV and VEEV reveal that nsP2 is a cysteine protease, is highly conserved, and comprises two sub-domains (Narwal et al. 2018; Russo et al. 2006). A superimposition of the nsP2 proteases from CHIKV and VEEV are shown in Figure 2b.

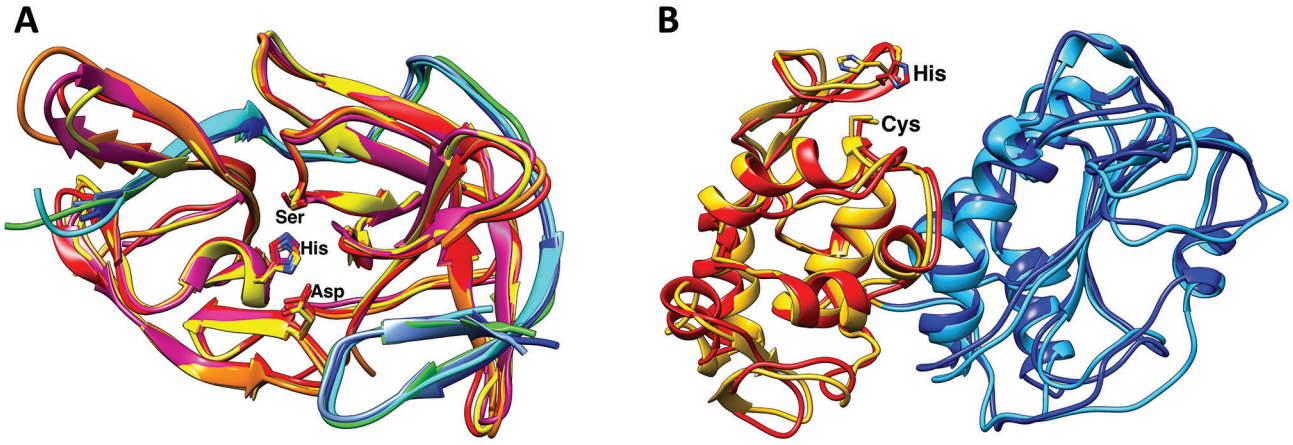

Figure 2: (a) Superimposition of NS2B-NS3 structures of YFV, DENV, WNV and ZIKV. Residues of the catalytic triad are highlighted. NS2B is green/blue. (b) Superimposition of nsP2 protease structures of CHIKV and VEEV. Both domains and residues of the catalytic dyad are highlighted.

A pan-genus drug discovery approach

The superimpositions shown in Figure 2 demonstrate that viral proteases are often structurally conserved within their respective genus, making it potentially possible for pan-genus inhibitors to be developed. However, in addition to the protein structure, it is important to assess further parameters such as the substrate specificity of each viral protease to understand the function and mechanism of action. The pan-genus approach is distinct to typical virus-specific approaches in conventional drug discovery. While many of the alphaviruses and flaviviruses are neglected pathogens because they either affect predominantly developing nations or are not always associated with high degree of mortality, together these arboviruses comprise a group that places extraordinary strain on healthcare systems. Tackling the problem of all viruses within the genus may allow us to have compounds ready for the next outbreak or epidemic. The recent past has shown that whenever arboviruses (re)emerge, the international research efforts increase; however, this is often followed by a rapid decline in attention and funding.

CYCLIC PEPTIDES

Cyclic peptides are privileged structures for protein binding

Small cyclic peptides (1000–2000 Da) can deliver outstanding binding affinities to protein targets (Morrison 2018). Cyclisation and other noncanonical modifications of peptides can enhance their metabolic stability by greater resistance towards proteolysis, promote their uptake across cell membranes, and decrease the entropic penalty of binding by locking the peptides in their active conformations. Bicyclic peptides offer even greater conformational rigidity, metabolic stability, and antibody-like affinity and specificity. Furthermore, high-affinity peptides can be identified for nearly any drug target in fast display screening approaches (Passioura 2020). Combined with biocompatible chemistry and genetic code reprogramming techniques, exceptionally large peptide libraries of highly modified cyclic peptides can be generated and screened, which include features such as backbone N-methylation, side chain modifications or D-stereochemistry. These modifications render the resulting compounds better starting points for drug discovery campaigns than peptides built of canonical amino acids (Passioura et al. 2014).

Cyclic peptides as antiviral agents

Cyclic peptides are increasingly turning into clinically approved drugs (Vinogradov et al. 2019). Natural cyclic peptides with noncanonical features are well-known for their antimicrobial activity (Abdalla & McGaw 2018). Although cyclic peptides have received increasing attention as potential antiviral agents (Kadam et al. 2017), their potential in antiviral therapy and diagnostics remains underappreciated (Vilas Boas et al. 2019).

Cyclic and bicyclic peptide inhibitors of flaviviral proteases

While strong cyclic peptide inhibitors of the alphavirus protease nsP2 remain so far elusive, we were able to develop a range of cyclic and bicyclic inhibitors of the flavivirus protease NS2B-NS3, some of which are shown in Figure 3 (Morewood & Nitsche 2021; Morewood & Nitsche 2022; Nitsche et al. 2019; Patil et al. 2021; Ullrich et al. 2022b; Voss et al. 2022). We developed various unnatural amino acids functionalised with cyanopyridine and 1,2-aminothiol groups which can be directly incorporated into peptides by standard Fmoc solid-phase peptide synthesis. In addition, we explored bismuth as a new reagent to generate bicyclic peptides. Cyclisation and stapling reactions proceed under biocompatible conditions in presence of the viral proteases of interest. Using these

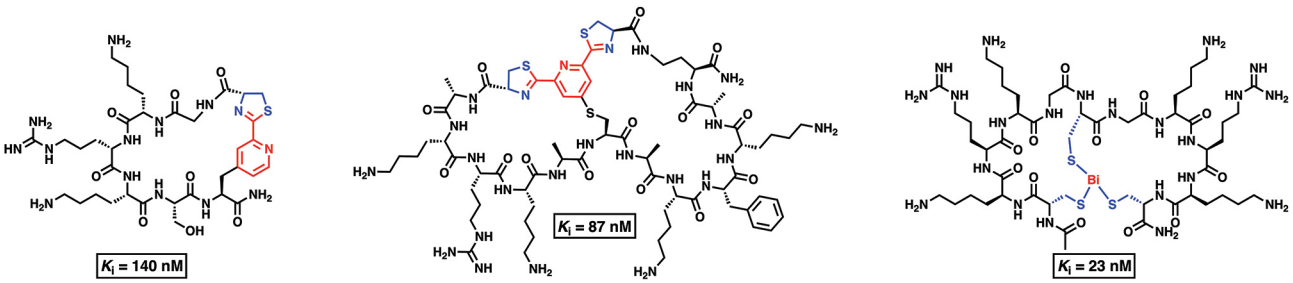

Figure 3: Monocyclic and bicyclic ZIKV NS2B-NS3 protease inhibitors with nanomolar affinity.

approaches in small screening campaigns, we were able to identify various macrocyclic peptide inhibitors of viral proteases. Bicyclic peptides showed higher affinity (lower inhibition constant K_i) than monocyclic peptides (Figure 3). Our peptides displayed not only high protease affinity but also promising proteolytic and plasma stability.

CONCLUSION

Arboviruses are already a major health concern and have the potential to cause epidemics. The variation in the receptor binding domains makes it difficult to develop broadly effective vaccines. However, their highly conserved proteases are a common Achilles heel and thus an attractive drug target for broad-spectrum antiviral drugs. Cyclic and bicyclic peptides can achieve extraordinary affinity to viral proteases. The major current challenge is to translate this target affinity into antiviral activity in infected cells.

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Conflict of interest

The author declares no conflict of interest.

References

Abdalla, M.A. & McGaw, L.J., 2018. Natural cyclic peptides as an attractive modality for therapeutics: a mini review. *Molecules* 23: 2080

Agbowuro, A.A., Huston, W.M., Gamble, A.B. & Tyndall, J.D.A., 2018. Proteases and protease inhibitors in infectious diseases. *Medical Research Reviews* 38: 1295–1331

Barrows, N.J., Campos, R.K., Liao, K.-C., Prasanth, K.R., Soto-Acosta, R., Yeh, S.-C., Schott-Lerner, G., Pompon, J., Sessions, O.M., Bradrick, S.S. & Garcia-Blanco, M.A., 2018. Biochemistry and molecular biology of flaviviruses. *Chemical Reviews* 118: 4448–4482

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R.W., Simmons, C.P., Scott, T.W., Farrar, J.J. & Hay, S.I., 2013. The global distribution and burden of dengue. *Nature* 496: 504–507

Garske, T., Van Kerkhove, M.D., Yactayo, S., Ronveaux, O., Lewis, R.F., Staples, J.E., Perea, W. & Ferguson, N.M., 2014. Yellow Fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and serological data. *PLoS Medicine* 11: e1001638

Gould, E., Pettersson, J., Higgs, S., Charrel, R. & de Lamballerie, X., 2017. Emerging arboviruses: Why today? *One Health* 4: 1–13

Her, Z., Kam, Y.-W., Lin, R.T.P. & Ng, L.F.P., 2009. Chikungunya: a bending reality. *Microbes and Infection* 11: 1165–1176

Jose, J., Snyder, J.E. & Kuhn, R.J., 2009. A structural and functional perspective of alphavirus replication and assembly. *Future Microbiology* 4: 837–856

Kadam, R.U., Juraszek, J., Brandenburg, B., Buyck, C., Schepens, W.B.G., Kesteleyn, B., Stoops, B., Vreeken, R.J., Vermond, J., Goutier, W., Tang, C., Vogels, R., Friesen, R.H.E., Goudsmit, J., van Dongen, M.J.P. & Wilson, I.A., 2017. Potent peptidic fusion inhibitors of influenza virus. *Science* 358: 496–502

Lei, J., Hansen, G., Nitsche, C., Klein, C.D., Zhang, L. & Hilgenfeld, R., 2016. Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor. *Science* 353: 503–505

Morewood, R. & Nitsche, C., 2021. A biocompatible stapling reaction for in situ generation of constrained peptides. *Chemical Science* 12: 669–674

Morewood, R. & Nitsche, C., 2022. Bioinspired peptide stapling generates stable enzyme inhibitors. *Chemical Communications* 58: 10817–10820

Morrison, C., 2018. Constrained peptides' time to shine? *Nature Reviews Drug Discovery* 17: 531–533

Musso, D., Ko, A.I. & Baud, D., 2019. Zika virus infection: after the pandemic. *The New England Journal of Medicine* 381: 1444–1457

Narwal, M., Singh, H., Pratap, S., Malik, A., Kuhn, R.J., Kumar, P. & Tomar, S., 2018. Crystal structure of Chikungunya virus nsP2 cysteine protease reveals a putative flexible loop blocking its active site. *International Journal of Biological Macromolecules* 116: 451–462

Nitsche, C., Holloway, S., Schirmeister, T. & Klein, C.D., 2014. Biochemistry and medicinal chemistry of the dengue virus protease. *Chemical Reviews* 114: 11348–11381

Nitsche, C., Onagi, H., Quek, J.-P., Otting, G., Luo, D. & Huber, T., 2019. Biocompatible macrocyclization between cysteine and 2-cyanopyridine generates stable peptide inhibitors. *Organic Letters* 21: 4709–4712

Nitsche, C., Zhang, L., Weigel, L.F., Schilz, J., Graf, D., Bartenschlager, R., Hilgenfeld, R. & Klein, C.D., 2017. Peptide–boronic acid inhibitors of flaviviral proteases: medicinal chemistry and structural biology. *Journal of Medicinal Chemistry* 60: 511–516

Noble, C.G., Seh, C.C., Chao, A.T. & Shi, P.Y., 2012. Ligand-bound structures of the dengue virus protease reveal the active conformation. *Journal of Virology* 86: 438–446

Noske, G.D., Gawriljuk, V.O., Fernandes, R.S., Furtado, N.D., Bonaldo, M.C., Oliva, G. & Godoy, A.S., 2020. Structural characterization and polymorphism analysis of the NS2B-NS3 protease from the 2017 Brazilian circulating strain of Yellow Fever virus. *Biochimica et Biophysica Acta — General Subjects* 1864: 129521

Passioura, T., 2020. The road ahead for the development of macrocyclic peptide ligands. *Biochemistry* 59: 139–145

Passioura, T., Katoh, T., Goto, Y. & Suga, H., 2014. Selection-based discovery of druglike macrocyclic peptides. *Annual Review of Biochemistry* 83: 727–752

Patil, N.A., Quek, J.-P., Schroeder, B., Morewood, R., Rademann, J., Luo, D. & Nitsche, C., 2021. 2-Cyanoisonicotinamide conjugation: a facile approach to generate potent peptide inhibitors of the Zika virus protease. *ACS Medicinal Chemistry Letters* 12: 732–737

Pierson, T.C. & Diamond, M.S., 2020. The continued threat of emerging flaviviruses. *Nature Microbiology* 5: 796–812

Russo, A.T., White, M.A. & Watowich, S.J., 2006. The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure* 14: 1449–1458

Ryan, S.J., Carlson, C.J., Mordecai, E.A. & Johnson, L.R., 2019. Global expansion and redistribution of *Aedes*-borne virus transmission risk with climate change. *PLoS Neglected Tropical Diseases* 13: e0007213

Schwartz, O. & Albert, M.L., 2010. Biology and pathogenesis of Chikungunya virus. *Nature Reviews Microbiology* 8: 491–500

Sharma, A. & Gupta, S.P. 2017. Fundamentals of viruses and their proteases. In *Viral Proteases and Their Inhibitors,* S.P. Gupta, ed. Academic Press, pp. 1–24

Ullrich, S., Ekanayake, K.B., Otting, G. & Nitsche, C., 2022a. Main protease mutants of SARS-CoV-2 variants remain susceptible to nirmatrelvir. *Bioorganic & Medicinal Chemistry Letters* 62: 128629

Ullrich, S., George, J., Coram, A., Morewood, R. & Nitsche, C., 2022b. Biocompatible and selective generation of bicyclic peptides. *Angewandte Chemie International Edition* 61: e202208400

Vilas Boas, L.C.P., Campos, M.L., Berlanda, R.L.A., de Carvalho Neves, N. & Franco, O.L., 2019. Antiviral peptides as promising therapeutic drugs. *Cellular and Molecular Life Sciences* 76: 3525–3542

Vinogradov, A.A., Yin, Y. & Suga, H., 2019. Macrocyclic peptides as drug candidates: recent progress and remaining challenges. *Journal of the American Chemical Society* 141: 4167–4181

Voss, S., Rademann, J. & Nitsche, C., 2022. Peptide–bismuth bicycles: in situ access to stable constrained peptides with superior bioactivity. *Angewandte Chemie International Edition* 61: e202113857

Young, P.R. 2018. Arboviruses: a family on the move. In *Dengue and Zika: Control and Antiviral Treatment Strategies*, R. Hilgenfeld & S.G. Vasudevan, eds. Singapore: Singapore, pp. 1–10

Zaid, A., Burt, F.J., Liu, X., Poo, Y.S., Zandi, K., Suhrbier, A., Weaver, S.C., Texeira, M.M. & Mahalingam, S., 2020. Arthritogenic alphaviruses: epidemiological and clinical perspective on emerging arboviruses. *The Lancet Infectious Diseases* 21: e123–e133