

Combating mosquito-transmitted diseases

Professor Alexander Khromykh is actively involved in projects examining the molecular mechanisms of replication and pathogenesis of mosquito-transmitted West Nile and Chikungunya viruses, which have been the source of deadly outbreaks in recent years. Here, he discusses his research and progress to date

The main focus of your current work is on the West Nile Virus (WNV). Could you explain how your previous research into mechanisms of Kunjin virus RNA replication led you to this area of research?

I was very fortunate to begin my research career in the flavivirus field under the mentorship of a great flavivirus researcher, the founder of the *Flaviviridae* virus family, Edwin Westaway (deceased). My initial task was to establish key molecular techniques and generate essential reagents for analysis of the mechanisms of flavivirus RNA replication using an Australian naturally attenuated strain of WNV, Kunjin virus (KUNV), as a model for more pathogenic WNV.

These productive studies resulted in a number of crucial discoveries published in the leading virological journals and set the scene for the majority of my further studies and successful NHMRC Senior Research Fellowship application.

Of the combination of classical and modern approaches you have been employing, which have you found to be particularly successful?

The most successful approaches have been molecular manipulations with viral infectious cDNA clones and replicons, transient and stable expression of viral proteins and non-coding RNAs, viral replication and virulence assays, microarrays, mass spectrometry, siRNA knockdowns and knockout cells and mice. More recently we have also embarked on the use of next generation sequencing analysis of changes in the profile and the levels of cellular RNAs (both messenger RNAs and small RNAs) caused by infections with natural and mutated WNV strains. Together, these approaches allow us to perform detailed analysis of the effects of changes in the viral and host genomes that are likely to have a profound impact on WNV replication and pathogenesis.



Can you explain your research into the development of live attenuated WNV vaccine candidates?

We have been exploring the potential of a relatively benign KUNV as a vaccine candidate against infection with a more pathogenic WNV strain, NY99V. We have shown that infection of adult mice with KUNV results in effective protection against challenge with a lethal dose of NY99V. As part of an NIH-funded project aimed at developing a safer live-attenuated WNV vaccine, we have identified a number of mutations in the KUNV genome that make it even more attenuated. These include single amino acid mutations in two of the viral proteins as well as nucleotide mutations in the 3' non-coding region. Incorporation of these mutations individually or in combination resulted in higher attenuation but still allowed effective protection against NY99V.

To simplify vaccine production and improve vaccine stability we constructed a DNA version of the KUNV vaccine that allows generation of infectious KUNV in the immunised organism from injection with a single dose of plasmid DNA encoding

the full-length viral cDNA. The DNA showed promising results in mice either via conventional needle-based injection (PNAS, 2003) or when delivered by a novel needle-free miniature device, nanopatches (Small, 2011). A safer DNA version of the vaccine that allows only limited (one round) infection *in vivo*, developed based on our novel split-genome strategy, was also shown to be highly effective in mice and to induce high level of WNV-neutralising antibodies in horses (*Nature Biotechnology*, 2008).

Could you discuss your laboratory's recent attempts to clarify mechanisms of rheumatic disease caused by the mosquito-transmitted Chikungunya virus (CHIKV)?

CHIKV is the mosquito-transmitted alphavirus which causes painful inflammation of joints that may persist for months after initial infection. The largest outbreak ever known for this disease occurred in 2004-11 with 1.4-1.6 million cases, with imported cases reported in nearly 40 countries. Our aim is to understand what causes the disease following the initial infection and how virus is able to persist for so long after initial infection.

This is a collaborative project with Andreas Suhrbier from Queensland Institute of Medical Research (QIMR) and my former postdoctoral fellow Gorben Piljman (now at Wageningen University, The Netherlands). The main research focus in my laboratory is to characterise viral determinants leading to disease and persistence using molecular manipulations with viral infectious clone. So far our collaborative efforts resulted in showing the role for one of the viral proteins, nsP2, in inhibition of host interferon response (*Journal of Virology*, 2011) and demonstrating an essential role for interferon induction by interferon regulatory factors 3 and 7 in virus-induced disease (*Journal of Virology*, 2012).

Elucidating West Nile Virus pathogenesis

Research underway at the **University of Queensland**, Brisbane, examining the pathogenicity of different West Nile Virus strains may help determine the viral and host factors responsible for disease outcome

WEST NILE VIRUS (WNV), a mosquito-transmitted flavivirus spread to humans and animals, has the potential to cause serious illness. Although it was first isolated in Africa in 1937, the virus was largely ignored as a dangerous pathogen until a number of epidemics occurred in Europe in the 1990s and later in the US. Since its emergence in New York in 1999, the virus has spread rapidly across the continent and infected 1.8 million people, causing 360,000 people to fall ill and killing an estimated 13,000. Most recently, the national Centres for Disease Control and Prevention (CDC) in the US reported the biggest increase of WNV infection rate since 2004, with 3,963 cases and 163 deaths from the disease identified so far this year.

Fortunately, the Australian strain of the disease, Kunjin, is naturally attenuated and to date is not responsible for causing major health problems in humans. Despite the pathogen being so closely related to its American relative, only a handful of cases of noticeable infections in humans have been found, and fortunately no deaths, since its first discovery in the region in 1960. Why the two pathogens behave so differently is unclear and of keen interest to research scientists. Researchers are also curious to understand the origin and the reasons for higher pathogenicity of the recently emerged new virulent strain of Kunjin virus (KUNV) that broke out in New South Wales last year, causing neurological disease in horses with an estimated mortality rate of 10-15 percent.

LEADING THE RESEARCH

One scientist attempting to understand what determines the pathogenicity of a particular WNV strain, both at the viral genome and at the host anti-viral response levels, is Professor Alexander Khromykh at the University of Queensland (UQ) in Australia. The foundations of his studies derive from his initial work examining viral ribonucleic acid (RNA) – a nucleic acid molecule encoding the viral genetic information – that led to the establishment of key molecular techniques and reagents, such as infectious clones and replicons, and antibodies to all viral proteins. After the success of Khromykh's earlier work, whereby he defined the composition and intracellular localisation of the viral RNA replication complex and proposed a model for viral RNA replications, Khromykh was awarded a highly prestigious Australian NHMRC Senior Research Fellowship.

With a significant number of researchers investigating various aspects of infectious diseases caused by viral, bacterial and fungal pathogens at the UQ, Khromykh initiated the establishment of

the Centre for Infectious Disease Research (CIDR) in 2009, before being elected its founding Director. Following a successful application for expansion to include other research groups at UQ, a new centre, Australian Infectious Disease Research Centre (AID), was opened in 2011 which has recently expanded even further to become a joint centre between the UQ and the Queensland Institute for Medical Research (QIMR).

One of Khromykh's current projects aims to build on his existing findings on identifying the pathogenic determinants of the WNV pathogenic strain through generating infectious cDNA clones of the highly pathogenic WNV strain circulating in America, attenuated Australian KUNV strains and more recent pathogenic for horses KUNV strain emerged in New South Wales. "These infectious clones are being used to exchange genomic regions between the viral strains in order to identify which regions in the viral genome determine pathogenicity," he explains. "To identify which factors in the host restrict WNV replication and thus determine outcome of WNV infection we employed mice and cells defective in

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production of various genes involved in the host innate immune response and monitor the effect of these deficiencies on the severity of infection with different WNV strains." Khromykh also intends to identify which combinations of viral and host genes determine outcome of infection.

As part of his NHMRC Fellowship, Khromykh is also engaged in employing his previous and current findings to the development of safe and effective vaccines against WNV and other medically important flaviviruses. In addition, Khromykh has developed the first flavivirus replicon expression and delivery system based on Kunjin replicon and applied it extensively for developing vaccines against other medically important viral pathogens, such as human immunodeficiency (HIV), Ebola and respiratory syncytial viruses, as well as for therapy against cancers. Unfortunately, failure to secure funding for this translational research meant the four patents – commercialised by a start-up biotech

INTELLIGENCE

ELUCIDATING WEST NILE VIRUS PATHOGENESIS

OBJECTIVES

- To identify viral and host factors regulating replication and pathogenesis of West Nile Virus infection that lead to different disease outcomes for viral strains circulating in different parts of the world
- To unravel the mechanisms by which this encephalitic virus evades host antiviral response and causes disease

KEY COLLABORATORS

Roy Hall; **Paul Young**; **Mario Lobigs**; **Helle Bielefeldt-Ohmann**; **Sassan Asgari**; **Mark Kendall**, University of Queensland • **Andreas Suhrbier**, Queensland Institute of Medical Research • **Peter Kirkland**, Department of Primary Industries, New South Wales • **Jason Mackenzie**, La Trobe University, Melbourne • **Michael Diamond**, Washington University School of Medicine, USA • **David Barton**, University of Colorado, USA • **Yutaro Kumagai**; **Osamu Takeuchi**; **Shizuo Akira**, Osaka University, Japan • **Sonia Best**, National Institutes of Health, USA • **Viktor Volchkov**, INSERM, France • **Philippe Despres**, Pasteur Institute, France • **Jeffrey Wilusz**; **Richard Bowen**, Colorado State University, USA • **Aaron Brault**, CDC, USA • **Gorben Pijlman**, Wageningen University, The Netherlands

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PROFESSOR ALEXANDER KHROMYKH

received his PhD in Molecular Virology. He was awarded an NHMRC Senior Research Fellowship in 2004 and 2009. Khromykh moved his laboratory to the University of Queensland's St Lucia campus in 2005. He was the founding Director of the SCMB's Centre for Infectious Disease Research and is currently the Deputy Director of the Australian Infectious Disease Research Centre.

company before going bankrupt – for this research were discontinued but Khromykh continues to use replicons and replicon-like technology to further advance vaccine development.

A HELPING HAND

Key to Khromykh's success is the role international and national collaborations have played throughout the research. "I strongly believe that collaboration with researchers around the world that have strong expertise in complementary areas of research is the quickest way to advance knowledge," he affirms. "In this respect, I have been very lucky to collaborate with some of the world's most respected researchers in the fields of innate immunity, RNA and protein structure, flaviviruses and filoviruses." For instance, Roy Hall from UQ, Richard Randal from St Andrew's University in the UK, Philip Despres from the Pasteur Institute in Paris, Sonia Best from the National Institutes of Health in the US, Shizuo Akira from Osaka University in Japan, Michael Diamond from the Washington School of Medicine in St Louis, US, and David Barton from the University of Colorado in Denver all provided substantial contributions to the studies on the interplay between virus infections and host innate immune response. This research was instrumental in identifying WNV proteins involved in viral evasion of host response and provided the basis for his recent research regarding the role in pathogenesis of viral non-coding RNA.

His collaboration with Michael Diamond in particular allowed the Senior Fellow to further identify host factors preferentially restricting replication of attenuated KUNV strain. Long-term collaboration with Andreas Suhrbier from QIMR has been instrumental in evaluating KUNV replicon-based vaccine and cancer therapy

vectors in animal models, and more recently together with Gorben Pijlman from Wageningen University in The Netherlands in studying pathogenesis of CHIKV.

KEY RESULTS

To date, his most significant findings include defining molecular mechanisms of viral RNA replication, demonstration of direct correlation between the ability of different WNV strains to overcome host innate immune response and their pathogenicity, as well as identification of interferon regulatory factors 3 and 7 as the key components of host anti-viral response, determining the outcome of an infection with a particular WNV strain.

Most recently, the Senior Research Fellow has focused his research on the role of WNV non-coding RNAs in virus-host interactions and viral pathogenesis. Working in collaboration with research groups in the US and Japan, Khromykh discovered that small but highly-structured viral non-coding RNA (sfRNA) contributes to viral evasion of host innate immune response and therefore viral pathogenesis

In collaboration with Jeffrey Wilusz at Colorado State University in the US and Gorben Pijlman at Wageningen University in The Netherlands, the roles for sfRNA in modulating cell mRNA degradation and RNA interference pathways have been recently demonstrated. One of his most recent findings produced as a result of successful collaboration with Sassan Asgari's group at UQ is the discovery of WNV micro RNA, the first micro RNA produced by cytoplasmic RNA viruses, and its role in regulating expression of a host transcription factor in order to facilitate virus replication. The success of all these findings ensured by financial support from NHMRC, Australian Research Council and National Institutes of Health continues to attract significant competitive funding which progresses these studies forward.

Khromykh continues to use both infectious clones and replicons to unravel various aspects of the WNV replication cycle, identify functions of viral proteins and RNAs and discover new features in viral genome and their functions, as well as to develop vaccine candidates against flaviviruses and other pathogens. Looking ahead, he anticipates further expansion of his work: "For the future I intend to integrate classical molecular virology approaches with more modern 'omics' technologies to gain an even deeper understanding into the mechanisms of WNV replication and pathogenesis, both at the viral and the host levels, and apply obtained knowledge to the development of effective vaccines and anti-virals".

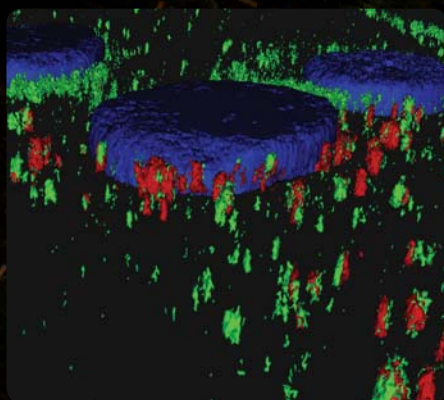


FIGURE 1. Slide showing processing and accumulation of KUNV non-coding RNA (red) in the cytoplasmic stress granules (green) of virus-infected cells.