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Meeting report: 30th International Conference on Antiviral Research, in Atlanta, GA, USA

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Abstract

The 30th International Conference on Antiviral Research was held in Atlanta, GA, USA, from 21 to 25 May 2017. Each year, the International Society for Antiviral Research (ISAR) presents three major awards, this year to Mike Sofia (Elion award), David Chu (Holý award) and Maaike Everts (Prusoff award). Also this year, the inaugural ISAR Women in Science award lecture was presented by Priscilla Yang. For several years, International Conference on Antiviral Research (ICAR) has included at least one Keynote lecture, this year there were four. Although there are accounts of only these eight lectures, they reflect the diversity that is characteristic of ICAR – employment (academia, industry, public health), type of research (virus biology, potential antiviral targets, antiviral drugs, research organisation) and a range of viruses. For example, the viruses included were hepatitis C virus and hepatitis B virus (Mike Sofia), HIV and hepatitis B virus (David Chu), multiple antiviral projects (Maaike Everts), dengue (Priscilla Yang), rhinovirus C (Ann Palmenberg), polio (Mark Pallansch), HIV (Eric Hunter) and Zika virus (Pei-Yong Shi). This report ends with my personal comments giving examples in which this diversity can bring benefits. The 31st ICAR will be in Porto, Portugal, 11–15 June 2018.

Keywords

Antiviral therapy, HIV, hepatitis C virus, rhinovirus C, Zika virus

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Introduction

This article provides an overview of the invited lectures at the 30th International Conference on Antiviral Research (ICAR), sponsored by the International Society for Antiviral Research (ISAR), which was held in Atlanta, GA, USA, from 21 to 25 May 2017. Published shortly after ICAR, a multi-author report entitled 'Highlights of the 30th International Conference on Antiviral Research'[1] gave an overview of a broad range of the ICAR presentations. In contrast, this meeting report gives detailed accounts of the lectures by the recipients of ISAR's three major awards, held in memory of Gertrude (Trudy) Elion, Antonín (Tony) Holý and William (Bill) Prusoff, the presentation by the first ISAR Women in Science (WIS) awardee and the four Keynote addresses. Following the suggestion by Joe Colacino, a previous ISAR President, I have added my own personal comments within the 'My personal comments and Conclusion' section.

Because this review article simply provides accounts of oral presentations, it is not generally accompanied by references to the scientific literature. Any descriptions of favourable treatment outcomes should not be taken as a recommendation for clinical use. Generally, I have added my personal comments on the meeting within the conclusion. In a few instances, I have added my own comment within the main text, indicated either by the wording or by the use of italics within square brackets. Although this meeting report covers only eight of the presentations, the topics range greatly in diversity and illustrate how ICAR brings together knowledge and expertise from a wide spectrum of antiviral research.

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Gertrude Elion Memorial Award Lecture: Viral hepatitis – The search for a cure

Michael (Mike) J Sofia, Arbutus Biopharma, Inc., Doylestown, PA, USA

Michael Sofia (Mike) (Figure 1) first paid tribute to Trudy Elion. He had not known her personally but felt that he had gained an appreciation of her work on acyclovir (ACV) through his time working closely with Phil Furman (a Past-President of ISAR) who was part of Trudy's team.

Viral hepatitis is a major, worldwide clinical problem, with about 170 million chronically infected with hepatitis C virus (HCV) and 350 million with hepatitis B virus (HBV). In the USA, during the period 1999–2007, the burden of mortality with viral hepatitis due to HBV had been falling steadily (from six to four per 100,000 each year) whereas that due to HCV was rising (three to over four per 100,000 each year). In the same period, the mortality due to HIV was less than one per 100,000 each year. HCV has genotypes 1–6, genotype 1 being the most common in North and South America, Europe, Australasia and some parts of Asia, genotype 3 being most common in South Asia and all genotypes being found in differing proportions through Africa. When considering the prospects for finding a cure for HCV-infected patients, the high rates of HCV replication ($10e^{12}$ virions/day) and viral mutation (0.1–1 error per viral RNA synthesised) were seen as presenting a huge challenge. However, there was one aspect which offered hope; there was no known viral reservoir.

In Europe, after HCV therapy with interferon (IFN), 530 patients were followed prospectively for a median of 8.4 years. Of these, 192 (36%) had achieved sustained virological response (SVR). Whereas liver-related mortality rose to 30% in those who did not achieve SVR, there were only a handful of deaths in the SVR group. Mike summarised the outlook for an IFN-free cure 'Many of the thought leaders said that the challenge was so great, it would never be overcome until the mid-2020's'.

The HCV NS5B polymerase is an attractive drug target. It is highly conserved across all HCV genotypes – enhancing the chance of discovering a pan-genotype drug. Also, the highly active, critical nature of the polymerase active site means that any mutations resistant to nucleoside/tide drugs are likely to reduce polymerase efficiency and hence the fitness of the mutated virus and hence reduce the probability of generating highly resistant virus. When Mike joined Pharmasset, the standard-of-care HCV therapy was IFN together with ribavirin (RBV) which could not be used without IFN. The cost was high, in terms of dollars, time (one year) and in severe restrictions on patient lifestyle. Also, the outcome was uncertain, the cure rate being around 40%. But Pharmasset had an early lead compound, PSI-6130 (Figure 2).

Figure 1. The Gertrude Elion Memorial Award being presented to Michael Sofia by ISAR President, José Esté.

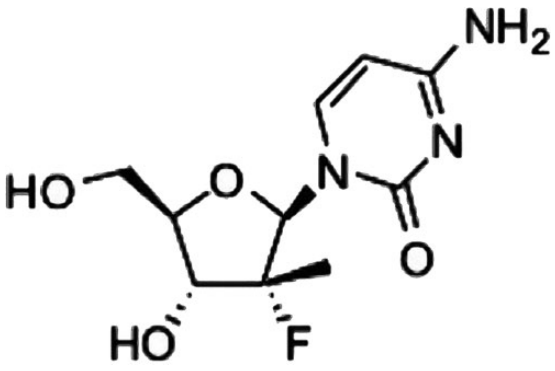

Figure 2. Structure of 2′-α-F-2′-β-C-methylcytidine (PSI-6130).

PSI-6130 was highly selective for HCV versus other viruses, it was active *in vitro* against HCV polymerases from genotypes (GT) 1, 2, 3 and 4, was a non-obligate chain terminator of viral RNA and it had at least additive activity with other anti-HCV drugs. After passaging in cell culture for six months, a resistant mutant (S282T) was obtained but it was of low viral fitness and with no known pre-existing prevalence in the clinic. In rats and monkeys, the oral bioavailability was ~20%. Of concern, there was deamination to give the uridine metabolite which was inactive against HCV. In monkeys, there was 40–70% conversion to the uridine metabolite.

In a Phase I clinical study, PSI-6130 was well tolerated, there being no serious adverse events up to a dose of 9000 mg. The bioavailability was modest (~ 25%) and, encouragingly, there was only 10% conversion to the inactive uridine metabolite. In collaboration with Roche, a prodrug approach was started with the aim to

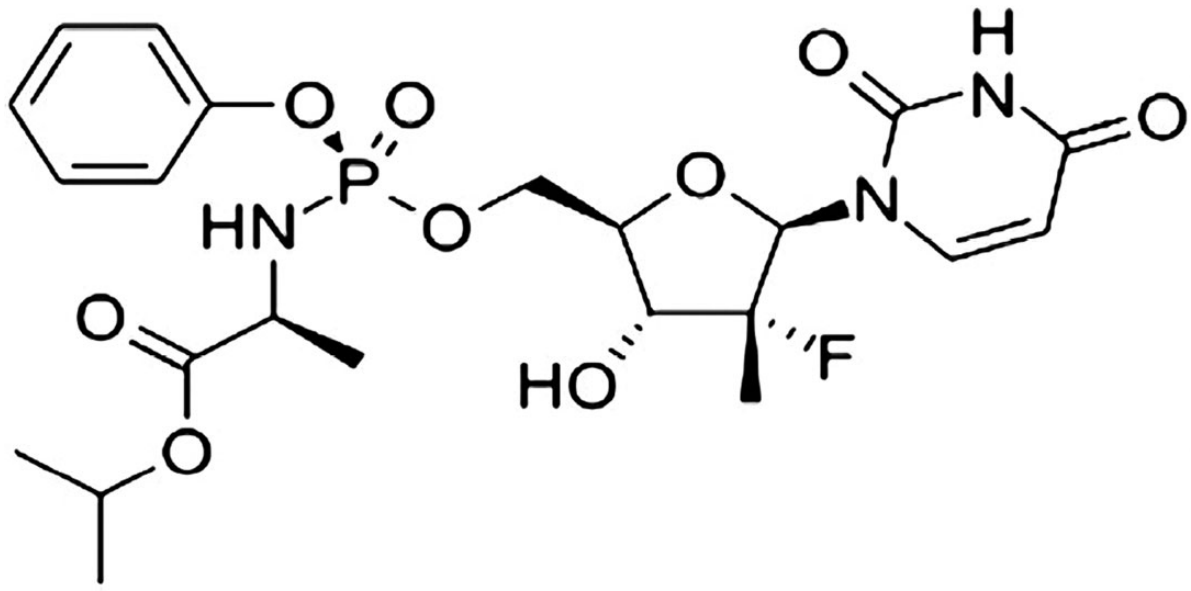

Figure 3. Structure of sofosbuvir (PSI-7977).

increase bioavailability and, if possible, decrease the conversion to the uridine metabolite. This programme led to RG7128. In a 14-day monotherapy Phase II study in HCV GT1 non-responder patients, there was a 2.7 $\log_{10}$ reduction in HCV RNA levels. In a four-week combination study (RG7128 (1000 or 1500 mg bid) with pegylated IFN and RBV), there was $\sim$90% response rate with safety comparable to the corresponding placebo control. Certainly, this was an encouraging result but the high doses and the twice-daily dosing regimen were of concern which were unlikely to be overcome by the prodrug approach. So was there another option? Why was the uridine metabolite (PSI-6206) inactive? The Pharmasset team decided to try a different approach.

In cell cultures, PSI-6206 was not converted to the corresponding uridine monophosphate (PSI-6206-MP) but that human kinases were able to convert PSI-6206-MP to the di- and tri-phosphate (PSI-6206-TP). Both the cytidine and the uridine TPs inhibited the HCV polymerase but the uridine TP was the less potent as assessed by their inhibition constants (Ki), 0.42 and 0.06 μM, respectively. However, the half-life ($T_{1/2}$) of PSI-6206-TP was much longer than that of PSI-6130-TP (38 and 5 h, respectively). The 38 h half-life would enable PSI-6206 to be administered once daily.

The next key concept was to synthesise a PSI-6206 phosphoramidate prodrug, which would be able to enter liver cells and be metabolised to the monophosphate which is then further phosphorylated to the di- and tri-phosphate. Because the phosphates carry a charge, they remain trapped within the cell. [*Phosphoramidate prodrugs were first introduced by Chris McGuigan, a former President of ISAR.*] Although the phosphoramidate approach is well known, there are side chains which can influence the properties of the prodrug. In addition, this approach in the past never demonstrated clinical efficacy and the concept of liver targeting using phosphoramidates was never contemplated. [*In his Elion Award lecture at the 2015 ICAR, Phil Furman described how over 140 phosphoramidate prodrugs of PSI-6206 were synthesised and how PSI-7977 was selected to become sofosbuvir (Figure 3). An account of this work was included in the 2015 ICAR meeting report.*[2]]

The ELECTRON Phase II clinical study became known as 'The Game Changer' because it was the first ever study in which HCV therapy, without IFN, had given 100% sustained viral response for 24 weeks (SVR_{24}) (Table 1).

In sofosbuvir Phase III studies, four separate trials recruited patients with different genotypes, 1 and 4, 2, 3 and 5 and 6, respectively. By this time, the SVR for 12 weeks (SVR_{12}) had become the accepted measure for an HCV cure. The SVR_{12} were 90, 97, 93 and 100%, respectively. To further optimise the cure rates, Gilead combined sofosbuvir with an NS5A inhibitor, ledipasvir, which became Harvoni®. However, ledipasvir has now been largely replaced by velpatasvir, another NS5A inhibitor but with an improved genetic barrier to resistance. This combination is Epclusa® which gives >96% cure rate for all genotypes 1–6. Sofosbuvir-based cure regimens have been shown to be effective in patients co-infected with HIV, in previously difficult-

Table 1. The ELECTRON Phase II study in HCV GT 2/3.

n	Treatment (randomised1:!:1:1)	SVR_{24} (%)
10	PSI-7977 + RBV 12 weeks	100
9	PSI-7977 + RBV + Peg IFN four weeks, then PSI-7977 + RBV eight weeks	100
10	PSI-7977 + RBV + Peg IFN eight weeks, then PSI-7977 + RBV four weeks	100
11	PSI-7977 + RBV + Peg IFN 12 weeks	100
10	Additional 10 patients enrolled for PSI-7977 monotherapy 12 weeks	60

to-treat patients – African Americans, in cirrhotic patients, in those with decompensated liver disease and in liver transplant patients. To date, there have been no cases of clinically observed viral resistance. [*After ICAR (July 2017) Gilead obtained approval for Vosevi® (sofosbuvir–velpatasvir–voxilaprevir combination). I include further details within my conclusions.*]

Sofosbuvir (Sovaldi®) received approval from the USA Food and Drug Administration (FDA) in December 2013. Since then, more than a million patients have been cured on sofosbuvir-based regimens. Although at least 15 nucleoside/tide drugs have entered clinical development, sofosbuvir remains the only approved nucleoside/tide drug for HCV therapy. It is the drug of choice to act as the 'backbone' of HCV combination therapies – only sofosbuvir has a sufficiently high genetic barrier to viral resistance combined with an excellent safety profile. HCV could become a rare disease if the issues regarding patient access can be resolved.

The focus switched from HCV to HBV, with Mike describing his recent work at Arbutus. Long-term (many years) therapy with nucleoside/tide analogues has given good control of HBV replication. The preferred therapies have been entecavir and tenofovir – the latter now being replaced by the new prodrug tenofovir alafenamide which provides the same efficacy but improved safety profile. Although the circulating HBV DNA can remain below detectable levels for years, the viral reservoir, i.e. covalently closed circular DNA (cccDNA), remains. Preventing the cccDNA from being transcriptionally active would reduce the levels of circulating HBV antigens and thereby may restore the host immune activity. The Arbutus strategy for an HBV cure is to minimise the levels of HBV DNA and antigens and to restore the host immune response.

HBV replication is dependent on capsid assembly around pregenomic RNA (pgRNA) prior to synthesis of a partially double-stranded relaxed circular DNA genome and subsequent cccDNA synthesis. AB-423 inhibits HBV pgRNA encapsidation in a cell culture assay, maintains activity across genotypes A–D, against strains resistant to nucleoside/tide drugs and is highly selective for HBV versus other viruses. AB-423 also inhibits cccDNA synthesis, presumably by inhibiting the capsid uncoating process. There is at least additive activity in cell culture assays when combined with nucleoside/tide drugs and with ARB-1740 (see below). Similar results were obtained in a mouse model.

ARB-1467 is a combination of three siRNAs packaged in lipid nanoparticles. By targeting all four viral RNA transcripts, ARB-1467 inhibits the production of all four HBV antigens. In a mouse model, ARB-1467 gives a rapid reduction of HBV virions and antigens in both liver and blood samples. ARB-1740 has improved *in vivo* potency and longer duration of activity relative to ARB-1467. ARB-1740 has modified oligonucleotide chemistry and changes in the HBV sites targeted. In a dose escalation Phase II trial, ARB-1467 doses increased from 0.2 mg/kg monthly to 0.4 mg/kg biweekly, with a possible extension up to a year based on the outcome after three months. In both HBeAg positive and negative patients, with the last dose (0.4 mg/kg) being on day 57, there was $\sim$0.5 $\log_{10}$ reduction in HBsAg levels. Maybe, will ARB 1740 be more effective?

Using the HBV-infected chimeric mouse, the humanised liver supports the complete HBV life cycle. This model is proving useful to assess various combination of drugs. So far, the greatest reductions in serum HBsAg loads have been achieved by combining AB-423 + ARB-1740 + PegIFN. Slightly less reductions were obtained with AB-423 + ARB-1740 + entecavir. Neither of these regimens seemed to reduce the pre-established levels of cccDNA but both reduced the transcriptional activity of cccDNA. In human hepatocytes, AB-423 + ARB-1740 + PegIFN gave >90% reduction of HBsAg levels which seemed to correlate with the best stimulation of human IFN-α levels.

To date, only a small proportion of patients have achieved a functional cure (anti-HBsAg positive) while on nucleoside/tide therapy, even after several years. Mike concluded that there is still no effective cure but that there is now light at the end of the tunnel.

The Antonín Holý Memorial Award Lecture: Nucleosides: A rich source of antiviral agents

C. K. (David) Chu, University of Georgia, Athens, GA, USA

Figure 4. The Antonín Holý Memorial Award being presented to David Chu by ISAR President, José Esté.

David (Figure 4) had known Antonín (Tony) Holý, meeting him at ICAR over many years. David commented that Tony's work continues to touch the lives of millions of people. In his introduction, David said that he would briefly summarise work on HIV and VZV, then switch focus to HBV. Multistep enantiomeric syntheses were devised for D- and L-1,3-dioxolane nucleosides, starting from D-mannose and L-glucose, respectively. The D-dioxolanes led to two compounds (DAPD and DOT) with activity against HIV and the L-dioxolanes included a compound (L-BHDU) active against herpesviruses.

Amdoxovir (DAPD, (−)-β-D-2,6-diaminopurine-dioxolane) had activity against both HIV and HBV. DAPD is converted by adenosine deaminase to the corresponding guanosine anaologue, DXG. At that time (late 1990s), AZT- and 3TC-resistant strains of HIV were limiting the effectiveness of therapy. In cell culture assays using resistant clinical strains, DXG retained good activity, there being only a 2.3-fold decrease with the double AZT/3TC-resistant mutations (67N/70R/184V/215Y/219E). DAPD was progressed to Phase IIa trials in HIV patients. DAPD (500 mg twice daily for two weeks) gave modest activity as monotherapy but, when added to current therapy, there was about 2 $\log_{10}$ reduction in plasma HIV-1 RNA levels.

Interest in 1-(β-D-dioxolane)thymine (DOT) was stimulated when it was discovered that it retained its anti-HIV activity when tested using resistant strains. David showed the nine-step synthesis of DOT, starting from 1,6-anhydro-D-mannose, reported in 1991. The resistance profile for DOT was done in human peripheral blood mononuclear cells (PBMCs) using cloned HIV with known resistance mutations. Against AZT, 3TC and AZT/3TC mutants, DOT retained full activity, even a marginal trend to being more active. Of particular note, DOT was active against the AZT-resistant strain with four mutations (K67N, K70R, T215Y, K219Q), (1.1-fold increase in EC_{50}), and against tenofovir-resistant strain (K65R) (0.5-fold). Although the active moiety, DOT-triphosphate was prepared to investigate its interaction with HIV reverse transcriptase (RT), DOT was not progressed to clinical trials.

In collaboration with Jennifer Moffat, who was also attending this ICAR, L-BHDU (β-L-1-(E-2-bromo-vinyl)-2-1,3-(dioxolan-4-yl)uracil) was discovered to be active against varicella zoster virus (VZV, chicken-pox/shingles) both *in vitro* and *in vivo*. Although with slightly less activity, L-BHDU inhibited two other herpesviruses, Epstein–Barr virus (EBV, glandular fever/infectious mononucleosis) and herpes simplex virus type 1 (cold sores). Against these herpesviruses, L-BHDU was more active than the positive control, ACV. In contrast to the known anti-VZV compound, bromovinyl-deoxyuridine (BVdU), L-BHDU does not interfere with 5-fluorouracil (5-FU) metabolism. This became an important toxicity issue when BVdU and 5-FU were co-administered to cancer patients. In a VZV animal model, L-BHDU (150 mg/kg) was markedly more effective than valacyclovir (200 mg/kg).

The focus of David's lecture then switched to HBV. David commented that two nucleotide analogues, used widely for HBV therapy, came from Tony Holý – adefovir and tenofovir. Initially, lamivudine was approved for HBV therapy but viral resistance became a major problem, about two-thirds of the patients having resistant virus after four years of treatment. With adefovir, the resistance was much less, at about 30% after five years but a better treatment was needed. With entecavir and tenofovir, the resistance levels remain very low, just a handful of patients having resistant virus. Recently, tenofovir alafenamide (a new prodrug) has been approved – the efficacy is comparable to that with tenofovir but the safety profile is significantly improved.

D-FMAU was known to be very active against herpesviruses but it was highly toxic. Therefore, the enantiomer, L-FMAU was synthesised – the synthesis, starting from either L-xylose or L-arabinose, was shown. Although L-FMAU had only modest activity against EBV, it had good activity against HBV in cell culture assays (EC_{50}, 0.1 μM) and did not inhibit cell replication at >100 μM. Against woodchuck hepatitis virus (WHV) in woodchucks (n = 4/group), L-FMAU (clevudine) at various doses was administered for 28

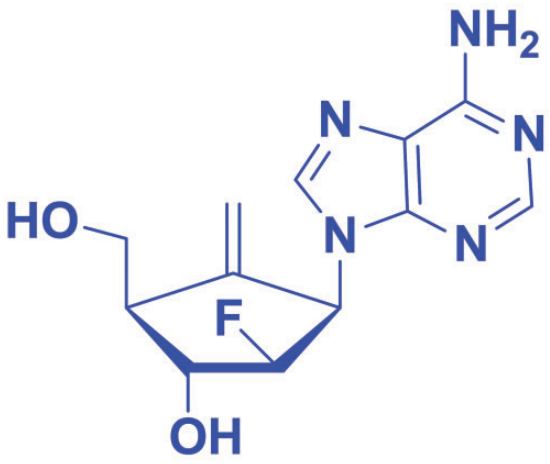

Figure 5. 2′-Deoxy-2′-fluoro-3′-hydroxy-4′-(hydroxymethyl)-5′-methylene-carbocyclic adenosine (FMCA).

days and the levels of serum WHV DNA (pg/ml) were followed for 16 weeks. Very quickly, the WHV DNA levels decreased to below the detection limit and remained so for the rest of the dosing period. Soon after dosing with nucleoside/tide analogues, it is usual for HBV DNA levels to return to baseline. With the lower doses of clevudine (0.3 and 1 mg/kg), there appeared to be a slow return to baseline levels. With the higher doses (3 and 10 mg/kg), WHV DNA remained undetectable to week 14, then low levels were detected at week 16.

In a Phase II/III trial clevudine was dosed (30 mg once daily) for 24 weeks. In HBeAg(+) (hepatitis B antigen positive) patients, HBV DNA levels continued to decrease during the dosing period to 5.1 $\log_{10}$ copies/ml below baseline. During the 24 weeks after dosing, HBV DNA levels rose slowly but were still 2 $\log_{10}$ copies/ml below baseline. In HBeAg(−) patients, the decrease in HVB DNA levels was similar (4.25 $\log_{10}$ copies/ml) but then rose very slowly to 3.1 $\log_{10}$ copies/ml below baseline at 24 weeks after dosing. Presently, clevudine is marketed by EISAI (Japan) in South Korea, Thailand and the Philippines. NDAs (new drug applications) have been filed in various other Asian countries. In the USA, Phase III trials were discontinued by Pharmasset.

The final section of David's lecture described the investigation of a new carbocyclic nucleoside, 2′-Deoxy-2′-fluoro-3′-hydroxy-4′-(hydroxymethyl)-5′-methylene-carbocyclic adenosine (FMCA) (Figure 5). The 14-step synthesis was shown. FMCA was tested in a cell culture HBV DNA replication assay against both wild-type (wt), lamivudine-resistant strains (M204V, M204I, L180M and L180M/M204V) and adefovir-resistant strain (M236T). FMCA, retained good activity against all these strains, the change in EC_{50} values versus the wt virus was between 1.0- and 1.2-fold. In a separate cell culture assay, FMCA and its phosphoramidate prodrug (FMCA-P) were tested against an entecavir-resistant strain (L180M/M204V/S202G). Both FMCA and its prodrug retained good activity (1.2- and 0.9-fold). Although the data were not shown, FMCA also retains activity against tenofovir-resistant strains.

In collaboration with Mike Sofia (this ICAR's Elion awardee), FMCA-P was tested in an immunodeficient mouse model of HBV. Entecavir was the positive control. The drugs were administered by hydrodynamic injection (this technique enables larger injection volumes to be administered via the tail vein; prior to injection, the mice are placed in a warm box to enlarge the tail vein). David kindly shared some unpublished, preliminary results; FMCA-P had sufficient activity to encourage further work.

In summary, FMCA and its phosphoramidate prodrug, FMCA-P, were fully active against HBV strains resistant to lamivudine, entecavir, adefovir and tenofovir. FMCA appears to have a good safety profile, having minimal inhibitory effects on cellular and mitochondrial DNA replication. Compared to entecavir, the good safety profile enables higher doses of FMCA-P to be used in efficacy studies using a mouse model. Further *in vivo* evaluation of FMCA-P is ongoing. Although pre-existing HBV cccDNA is not eliminated by therapy with nucleoside/tide analogues, such drugs will play a vital role in controlling HBV replication while minimising drug resistance.

The William Prusoff Young Investigator Award Lecture: Collaborating in drug discovery: Challenges and solutions

Maaike Everts, University of Alabama at Birmingham, AL, USA

Maaike (Figure 6) said that she admired William (Bill) Prusoff for his ability to establish collaborations that enable him to discover the anti-herpesvirus activity of idoxuridine (IDU). She showed a photo of Bill with the comment 'Early innovator in antiviral drugs'. Then she recalled that IDU was the first FDA-approved antiviral (in 1962) following the collaboration with Herb Kaufman (NIH) who had demonstrated efficacy in a rabbit eye model.

It has always been true that drug discovery and development (DD&D) depend on the skills of many different disciplines. However, the best combination of skills may be found in different institutions, each being critical to different parts of the DD&D pipeline. To illustrate the potential advantages and pitfalls associated with collaborations, Maaike chose two

Figure 6. The William Prusoff Young Investigator Awardee, Maaike Everts.

examples, the Alabama Drug Discovery Alliance (ADDA) and the Antiviral Drug Discovery and Development Consortium (AD3C).

The ADDA has two participants, the University of Alabama at Birmingham (UAB) and Southern Research (SR). It was Richard (Rich) Whitley and John (Jack) Secrist who initiated (2008) this collaboration. (Both were Past Presidents of ICAR and regular ICAR attendees – was that a factor?) UAB identified the targets, SR did the high throughput screening (HTS) and medicinal chemistry, both UAB and SR evaluated leads through cell culture and animal studies, with UAB progressing clinical trials. Intellectual property (IP) was jointly owned.

In 2009, Maaike was recruited to act as a coordinator, helping to build multidisciplinary projects. To date, more than 30 projects have been initiated, of these, 15 have been closed due to the chance of success being assessed as low. Such closures have been done in a considered, respectful way. More than 20 projects are still ongoing.

There are eight partners in AD3C: UAB, SR, Oregon Health & Science University, Vanderbilt University, Washington University in St Louis, University North Carolina at Chapel Hill, University of Colorado Denver and Gilead Sciences, Inc. The consortium was set up in 2014 with funding (U19) from National Institute of Allergy and Infectious Diseases. The general aims are HTS, chemistry and *in vivo* evaluation of antiviral compounds. Gilead has been a supplier of some of the compounds for testing. The four projects are on flaviviruses, alphaviruses, coronaviruses and influenza. There are monthly teleconferences and face-to-face meeting at least once, sometimes twice, a year. Also, there are ad hoc meetings, for example at ICAR.

The success factors are:

1. Strong team of biologists, experienced principal investigators.
2. Experienced DD&D talent.
3. An external advisory board, committed to success but remaining independent.
4. The 'glue' which holds the whole project together, Rich Whitley.

The potential challenges are:

1. Lack of trust
2. There needs to be a legal contract but trust is a key element.
3. IP inventorship
4. To be included in the patent, there must be a contribution to a claim or to have conceived at least one claim.
5. Motivation – money or status (publications)
6. Premature publication of a structure will destroy chance of obtaining a patent.

The administration core includes Rich Whitley, Mary Wyatt Bowers and Maaike herself. Whereas Rich is the 'glue', Maaike sees herself as the 'glue applicator', able to understand the languages of different disciplines, writing clear meeting notes, identifying action items and arranging for an outside party to interview all interested parties when deciding on inventorship. Also, the need for data has to be explained – data on ALL compounds are required to determine structure–activity relationships. What measures should be used, 50%, 90% or $\log_{10}$ reduction of virus replication? What serotype? There must be free exchange of data and engagement of the whole team. When misconceptions arise, it is important to rectify the problem in an open, transparent way. Above all else, it is not sufficient just to safeguard trust but also it is necessary to promote trust by face-to-face meetings which should include fun, social events.

ICAR has been used by AD3C to report recent progress, presentations 68, 70, 85, 155 and 172.

The ISAR WIS Award Lecture: Small molecule inhibitors of viral entry inspired by the humoral immune response to viral infection

Priscilla L. Yang, Harvard Medical School, Boston, MA, USA

The underlying aim of Priscilla Yang (Figure 7) and her laboratory is to use small molecules to inhibit or control protein function in order to probe virological processes and validate new antiviral targets. Using viral proteins (VPs) may open up new avenues for antiviral drug discovery. This presentation will focus on a specific target and why it was chosen. Along the way, that target's biochemical and biological function may be better understood.

Our humoral immune response is usually effective in stopping an infection developing to clinical symptoms. The antibodies prevent viral entry into target cells, the earliest step in the viral life cycle. Could small molecules mimic this activity by binding to the viral glycoprotein? The flavivirus entry viral glycoprotein (E) has clear biochemical functions, mediating attachment and membrane fusion. Is it possible to validate E as a suitable antiviral target for drugs *in vivo*? The laboratory has chosen to work with dengue virus because it is the most widespread mosquito-borne viral pathogen; it is closely related to West Nile, Zika and other pathogenic flaviviruses. The medical need is great, there being a vaccine with only modest activity and no approved drugs.

The flavivirus E protein, which exists as a pre-fusion dimer on the surface of virions, attaches to the host cell through interactions with host factors on the plasma membrane. This triggers clathrin-dependent uptake of the virion. Acidification of the endosomal compartment triggers structural changes in E leading to its reorganisation and refolding as a post-fusion trimer (E_3). These structural changes in E bring the endosome and viral membranes in close enough proximity to fuse, leading to formation of a fusion pore through which the viral genome can escape into the cytoplasm. In this fusion process, E acts as a catalyst in that it lowers the energy barrier for membrane fusion, but it has no classical active site to which an inhibitor can be rationally targeted. In addition, although each virion has 180 copies of E (90 pre-fusion dimers) that can reorganise as 60 post-fusion trimers, it has been unclear how many of these copies of E must be bound by an inhibitor to prevent fusion. Is it possible to inhibit this process with drugs? Earlier structural studies in which pre-fusion E co-crystallised with a detergent, β-octoglucoside, identified a potential drug-binding pocket located between domains I and II. Does that give a binding location for potential inhibitors?

Figure 7. The inaugural ISAR WIS Award being presented to Priscilla Yang by Rhonda Cardin, chair of the WIS committee.

To identify E inhibitors, phenotypic screens were used to find inhibitors of dengue virus entry and these were then screened for compounds likely to affect this process through interactions with E. For the primary antiviral assay, cells were infected with dengue virus serotype 2, the compound added 1 h after infection and the assay read at three days, to allow inhibitors of every step in the viral life cycle, including viral spread, to be identified. In secondary screens, time-of-addition experiments were performed, including experiments in which compound treatment was restricted to a preincubation of the viral inoculum and the initial 1 h infection period. This enabled identification of three chemical series, 2,4-diaminopyrimidines, 4,6-disubstituted pyrimidines and cyanohydrazones that all appeared to inhibit dengue virus entry via binding to a target present in the viral inoculum. Medicinal chemistry efforts were used to improve the initial lead compounds, leading to inhibitors including 1-100-1 (4,6-disubstituted pyrimidine), 7-148-6, 2-12-2 (2,4-diaminopyrimidine) and 3-110-22 (cyanohydrazone) that had 90% effective concentration (EC_{90}) values in cell culture between 2 and 10 μM. Medicinal chemistry was also used to generate derivatives containing biotin and fluorophores, which were used to demonstrate binding to pre-fusion, E_2, rather than to post-fusion, E_3. An *in vitro* fusion assay monitoring pH-triggered fusion of virions to synthetic liposomes was used to demonstrate that all three compound series inhibit E-mediated fusion. To establish where these compounds bind on E_2, virus was serially passaged in the presence of inhibitors to select for resistance mutations. Up to passage 9, no resistance

was detected with three of the compounds but resistance to 7-148-6 was obtained at passage 4, due to a single mutation, E-M196V, located in the detergent-binding pocket. This mutation reduces the affinity of recombinant E for all three series of inhibitors. The mutation T171A located in domain I away from the pocket also confers resistance to compound 3-110-22, but does so without affecting the affinity of the E-3-110-22 interaction. Additional point substitutions in the pocket were chosen from naturally occurring E sequences identified in public databases. These were introduced to the pocket (Q52A, F193L, M272S, L277M and F279S) by site-directed mutagenesis and the impact of each of these substitutions was assessed by measurement of Kd values against each of four compounds (3-110-22, 7-148-6, 2-12-2, GNF-2 and 1-100-1). The compounds were all affected by mutations in the pocket but 'footprints' of inhibitors in the same structural class were more similar to each other than to compounds in the other structural classes. Collectively, these results provide strong evidence that all three inhibitor series bind in pocket between domains I and II.

As there is considerable sequence similarity in the pocket region of E from dengue, Zika, West Nile and Japanese encephalitis viruses, the compounds were tested for activity against these other viruses. Whereas compound 3-110-22 was equipotent against all four viruses, inhibitors of the both pyrimidine series appeared to be five- or more fold selective for dengue over other flaviviruses. This is consistent with the idea that 3-110-22 and other cyanohydrazones target the same pocket as the pyrimidines, but interact with the pocket in qualitatively different manner leading to different viral selectivities.

The existing inhibitors have been used to develop a competitive amplified luminescent proximity homogeneous assay screen (ALPHAscreen), which was then used for a target-based screening campaign. From an initial screen of ~20,000 compounds, eight new scaffolds that inhibit dengue virus by binding to E and blocking fusion were discovered. IC_{50} values for inhibition of the ALPHAscreen assay were well correlated with EC_{90} values for antiviral activity, validating the target-based assay.

The fusion inhibitors have also been used to define the stoichiometry for inhibiting E-mediated fusion of virus-like particles (VLPs) using a single-particle fusion assay. In this experimental model, VLPs with 120 copies of E (60 pre-fusion dimers) can be induced to fuse with a supported lipid bilayer upon exposure to acidic pH. Using a derivative of 3-110-22 modified with a fluorophore, binding of 10–12 copies of the inhibitor (~10% of sites) was shown to be sufficient to prevent viral fusion.

Figure 8. Ann Palmenberg, a Keynote speaker at the 2017 ICAR.

In conclusion, it has been confirmed that small molecules can interact with E to give activity in both a target-based assay and a cell culture antiviral assay. The antiviral activity may be extended to other flaviviruses. These inhibitors have been used to give more information about the fusion process and to develop a target-based assay currently being used to drive medicinal chemistry efforts aimed at generating compounds suitable for *in vivo* validation studies. Still to come, can E be validated as an antiviral target *in vivo*?

Keynote addresses

The elusive rhinovirus C (RV-C): Historical context and biological enigma of RV-C

Ann Palmenberg, University of Wisconsin at Madison, Madison, WI, USA

Ann Palmenberg (Figure 8) received her BSc degree (Chemistry) from St Lawrence University in 1970, and her PhD from the University of Wisconsin-Madison (Biochemistry, 1975). Following two postdoctoral posts (Zurich and Madison), Ann became the Principal Investigator for a NIH-funded research programme at University of Wisconsin-Madison since 1978. Ann is now a Professor in the Department of Biochemistry and the Institute for Molecular Virology. During her work on RNA viruses, including rhinoviruses (RVs), Ann has developed a wide network

of collaborators. Her ICAR Keynote lecture focused on the fast developing story of RV-C species which were discovered only recently.

RVs are traditionally associated with upper respiratory tract infections, particularly the 'common cold'. First discovered in the 1950s, two types of RV were recognised, RV-A and RV-B, each type having many serotypes. RVs have single-stranded positive (ss+) RNA genomes with one long open reading frame encoding all the VPs. There are four capsid proteins (VP-1, 2, 3 and 4).

Following the outbreak of a severe acute respiratory syndrome, which was shown to be caused by a coronavirus and is now known as SARS-CoV, the SARS surveillance programme monitored the viral RNA sequences in clinical samples. Unexpectedly in 2006, a newly discovered viral RNA sequence seemed to be a third type of RV, designated RV-C. Initially, RV-C could not be replicated in cell cultures, thus giving an explanation for it remaining 'hidden' for five decades. When growth in culture was achieved, it was shown that, unlike RV-A and RV-B, it uses the cellular receptor, cadherin-related protein 3 (CDHR3). Just as there are many serotypes of RV-A (78) and RV-B (30), there are 55 serotypes of RV-C. Since 2006, RV-C has been shown to be the primary cause of asthma exacerbations and viral pneumonia in infants. In a clinical study, RV-C was detected in 60% of children with asthma exacerbations versus 24% with either RV-A or RV-B, 14% with another virus without RV and 8% with no virus detected. RV-C was associated with children who had more severe asthma.

When examining the structure of RV-C virions, about 70% of the particles contained RNA (full), the other particles being empty. This proportion is consistent for all serotypes and for virus obtained from cell cultures or clinical specimens. In contrast to RV-A and RV-B, both full and empty particles of RV-C had spikes formed by VP-1. The full and empty particles of RV-C are similar externally but the inclusion of RNA gives a more ordered structure internally. Antibodies to VP-1 are neutralising. RV-A and RV-B have a VP-1 pocket into which drugs, such as pleconaril, can bind, but this 'pocket' has collapsed in RV-C. However, RCV-C does have a glycan binding surface which is conserved among all 55 strains. Sialic acid (N-acetylneuraminic acid, Neu5Ac) binds to this site.

The normal role of cadherins is to hold cells together. They have a domain, which is anchored into the cell surface, and a long elongated part, which extends into the intercellular space and binds to the cadherins anchored to the neighbouring cells. Because cadherin-1 binds only with cadherin-1 and not to other variants, like cells bind to each other. CDHR3 has six extracellular domains, of which domains 1, 2 and 3 contain the RV-C contact sites.

In 2014, CDHR3 was reported to be an important susceptibility gene for asthma exacerbations, which are one of the most frequent causes of hospitalisation during childhood.[3] Although CDHR3 is highly expressed in airway epithelium, there is another important factor. In modern humans, there are two forms of CDHR3, Tyr_{529} and Cys_{529} (CDHR3 Y_{529} and CDHR3 C_{529}, respectively). Those children, who have Tyr_{529} encoded in the corresponding genes from both parents (homozygous for Tyr_{529}), have 5–10-fold higher risk for asthma exacerbations and hospitalisation than those with only Cys_{529} (homozygous for Cys_{529}). Children with genes encoding Tyr/Cys CDHR3 had intermediate risk. In this study, the proportions of patients with asthma were 67, 3 and 29%, respectively.

The CDHR3 Tyr_{529} is the ancestral protein. Although a few mammals (e.g. horse and mouse) have CDHR3 His_{529}, most mammals, and even some birds and reptiles, have Tyr_{529}. All non-human primates are Tyr_{529}. The early humans, Neanderthal and Denisovan, are thought to have diverged from the modern human lineage about 300,000 years ago. It is a remarkable achievement of recent scientific discovery that their DNA sequence is now known – they have the ancestral Tyr_{529} protein. In contrast, the Cys_{529} protein appears to be unique to our human race. However, the Cys_{529} variant first appeared in modern humans a long time ago, being detected in some ancient modern human remains which were discovered in Africa. The dates showed that Cys_{529} was already present within the human population before the 'out of Africa' event, around 50,000 years ago.[4] Incidentally, Otzi, the 5000-year-old 'Ice-man' found in the Alps, had the Tyr_{529} protein.

Dating back to within the Neolithic (late stone age) period, a large DNA data set includes the genomes of 230 West Eurasians who lived between 6500 and 300 BC, including 163 with newly reported data.[5] To the authors' knowledge, the new samples included the first genome-wide ancient DNA (~8500–3000 years before present) from Anatolian Neolithic farmers, who became Europe's first farmers. [*Anatolia corresponds to that part of modern Turkey east of the Bosphorus and bordered by the Black Sea to the north and the Mediterranean to the south*.] Within that Anatolian population, 25% had the Cys_{529} variant and 75% had the Tyr_{529} form. In samples from central Europe dating about 1000 years before present, the proportions had reversed (85 and 15%, respectively). In modern times, the majority of humans have both genes for the Cys_{529} protein, ranging from 53% in Africa to 87% in Asia, with the Americas (75%) and Europe

Figure 9. Mark Pallansch, a Keynote speaker at the 2017 ICAR.

(68%) having intermediate values. Could it be that RV-C had evolved and was putting pressure on human evolution?

To answer this question, a phylogenetic tree was created in collaboration with Nels Elde and Alesia McKeown (University of Utah) from many clinical samples, most of which were unpassaged clinical isolates of RVs, types A, B and C. Samples from all serotypes were represented. Preliminary information suggested an emergence of RV-C from the RV-A, about 3000–5000 years ago. Ann generously added interesting unpublished data which has led to a probable date for this divergence. The emergence of RV-C may explain why the Cys_{529} protein has become so dominant in the modern human population but it does not account for the early appearance of this variant in ancient human DNA prior to 'out of Africa'.

In answer to a question from the audience, Ann replied that it was not known if the switch from Tyr, which is so highly conserved, to Cys has involved a cost to modern humans.

Antivirals at the interface with public health: A case study of polio

Mark A. Pallansch, Centers for Disease Control and Prevention, Atlanta, GA, USA

Mark Pallansch (Figure 9) received his BS in Biochemistry (1976) from Virginia Tech and PhD in Biochemistry (1982) from the University of Wisconsin-Madison. For his postdoctoral fellowship (1984), he studied persistent measles infections at the Rockefeller University in New York. Currently, he is Director of the Division of Viral Diseases at the Centers for Disease Control and Prevention, in Atlanta, Georgia. The polio laboratory at CDC serves as the lead centre for the WHO Global Polio Laboratory Network supporting the Global Polio Eradication Initiative (GPEI).

In 1988, GPEI was started with four partners, the World Health Organisation, Rotary International, CDC and UNICEF. In 1988, the World Health Assembly passed a resolution that set out their aim, to eradicate polio by 2000. Although 17 years later, that target has still not been achieved, the burden of disease has been successfully reduced. In the 1988 baseline year, there were about 350,000 cases/year in 125 countries – by the 2000 target year, there were less than 3000 cases. Each year, this low number of cases has continued to decline, there being just five cases in two countries (Afghanistan and Pakistan) so far this year (April 2017). Initially, the oral vaccine (OPV), containing attenuated viruses of the three serotypes, was widely used because its administration was so easy. It was highly effective in reducing the number of new cases. Although most vaccinated subjects clear the attenuated virus, a small reservoir of virus was identified in immunocompromised subjects. As the number of polio cases have declined in a particular area, the oral vaccine has been replaced by the inactivated poliovirus vaccine (IPV), thus avoiding the creation of potential new virus reservoirs. However, IPV supply shortages are a problem. OPV remains in use in most countries.

During prolonged replication of OPV in immunocompromised subjects, polioviruses can evolve rapidly, the RNA replication error rate being $\sim 10^{-4}$/cycle. In an individual, there are about 1 or 2 base substitutions/week, giving an overall evolution rate of about 1%/year. For comparison, this mutation rate is faster than that for HIV, influenza viruses and HCV, viruses generally considered to be rapidly evolving. Most mutations will generate synonymous codons that do not alter the protein coding. However, the few, critical, attenuating mutations in OPV are under negative selection pressure to revert towards the wt. Vaccine-derived polioviruses (VDPVs) have been defined on the basis of threshold divergence in the VP1 capsid protein, There have been a few vaccine-associated paralytic polio cases, about 1–2/1,000,000 (cf. 1/200 from wt virus). Various types of immune deficiency can lead to prolonged virus shedding (>6 months) but severe combined immunodeficiency (SCID) and common variable immunodeficiency (CVID) can result in chronic shedding (>5 years). One individual has been

shedding poliovirus for >30 years. Regardless of paralysis status, VDPV in immunodeficient subjects (iVDPV) has been identified as a major threat to polio eradication, especially in those countries that do not have a good medical system. In countries from which poliovirus has been eliminated for many years, the chronic excretors have largely either cleared the virus or died. For example, in the UK there is just one individual alive and still excreting virus after 32 years. In the USA, there may be none remaining, with one potential case lost to follow-up.

Since 1995, surveillance has been expanded to create a registry which includes all known iVDPV cases, initially mainly in well-resourced countries but increasingly now worldwide. Encouragingly, iVDPV shedders, not just those showing symptoms of paralysis, are being identified at a greater rate due to improved surveillance – in the period 2010–2016, over 50 cases were reported (cf. <10 in 1980–1989). The majority of shedders are derived from the type 2 attenuated virus within the OPV, 67% only type 2 and 72% when including co-infection with either type 1 or 3.

Following the declared eradication of type 2 poliovirus, it was decided to include only types 1 and 3 in the OPV from May 2016. Hopefully, this will reduce the number of new cases of prolonged and chronic poliovirus shedders.

The objective of an iVDPV prevalence study was to assess the frequency of poliovirus excretion in patients with B cell immunity defects. Enrolment (n = 647) closed in November 2015, 636 stool samples were submitted and 635 laboratory results were obtained. Of the enrolled subjects, 19% were <6 years old, 46% were 6–19 years and 35% were >19 years. Most (51%) had CVID with other subjects having agammaglobulinaemia (39%), SCID (9%) or major histocompatibility complex Class II deficiency (1%); nearly all patients had treatment, either intravenous immunoglobulin (88%) or subcutaneous immunoglobulin (5%), just 7% having no treatment. Only 13/636 (2%) patients excreted poliovirus; of these, five were classified as iVDPV, all five being patients receiving treatment. As may be expected, the majority (four) of these patients had SCID. The majority of the 13 excretors, including 4/5 of iVDPV patients, had poliovirus type 2.

Considering the public health perspective, the primary risks associated with continued OPV use are two-fold, to the individual (prolonged poliovirus replication may progress to paralysis and even to death) and to the community (iVDPV excretors may seed outbreaks). Therefore, the potential role for antiviral therapy should be assessed.

The Polio Antivirals Initiative was set up with the aim to select promising candidate compounds and seek sponsors to progress these compounds. Just two compounds, pocapavir (V-073, capsid inhibitor, preventing viral RNA release) and V-7404 (3C protease inhibitor (PI)), have been identified as having the potential to be progressed quickly. Both these compounds have known cell culture activity against poliovirus, preclinical bioavailability and safety profile data.

Pocapavir was evaluated in a volunteer challenge study conducted in Swedish IPV vaccinated population. The study was randomised, double-blind placebo-controlled (96 active, 48 placebo). All subjects were challenged with OPV type1 and housed within the clinic in groups of 18 (active: placebo, 2:1) for 14 dosing days and then followed to day 45. Safety, pharmacokinetics, amounts of virus excreted, time to virus clearance and drug susceptibility were assessed. The subjects were assessed each day during the dosing period and on days 18, 22, 29 and 43.

In the placebo group, most subjects had cleared the poliovirus by day 18, although two, three and two had cleared the virus by days 22, 29 and 43, respectively. Within the treated group, there appeared to be 'responders' (n = 36) who cleared the virus early, within the period day 2–8. The remainder (n = 54) cleared the virus in the same time span as for placebo, with some clearing virus late (three, five and four by days 22, 29 and 43, respectively). None of the 'responders' had drug-resistant virus but most of the remainder (38/54, 70%) did have resistant virus. This resistant virus was transmitted to 10% (5/48) of the placebo group. The conclusions arising from this challenge study were that pocapavir was well tolerated and had significant activity but that there was a high incidence of drug resistance which appeared rapidly and was transmitted to some of the placebo group. Therefore, it is necessary to determine the resistance profile in the target iVDPV population and to urgently develop another drug for combination therapy.

Having distinct mechanisms of action, pocapavir and V-7404 were tested in cell culture assays, comparing the drugs singly and in combination. It was confirmed that the combination results in a marked reduction of viral resistance frequency. However, in these immunodeficient subjects, will this drug combination have sufficiently high barrier to viral resistance?

Impact of transmitted HIV phenotype on host–virus interactions and disease progression – Implications for treatment and cure

Eric Hunter, Emory Vaccine Center, Atlanta, GA, USA.

Eric Hunter (Figure 10) received his BSc degree (bacteriology) from Birmingham University, England (1969) and his PhD from Brunel University, London, England (1972) while working on tumour immunology

Figure 10. Eric hunter, a Keynote speaker at the 2017 ICAR.

at Imperial Cancer Research Fund. He started his work on retroviruses during his postdoctoral studies at the University of Southern California. In 1976, he joined UAB. While at UAB, Eric was the founding Director of the UAB Center for AIDS Research. In 2004, Eric moved to Emory – he is now professor at Emory Vaccine Center and Co-Director of Emory Center for AIDS Research.

Eric's current research interests include HIV transmission studies among stable heterosexual couples living in Rwanda and Zambia. The aim of this review was to show how the transmitted virus is not representative of the broad range of quasi-species present in the infecting individual but is highly selected, and how specific features of this transmitted virus can profoundly impact HIV disease progression. These clinical studies recruited stable couples, in which one is HIV positive and the other HIV negative, so-called discordant couples for prevention research. In the few couples where infection of the negative partner occurred it was possible to examine both the 'donor' and 'recipient' viruses. Newly infected partners have been followed for up to eight years. This NIH-funded work is also supported by the International AIDS Vaccine Initiative (IAVI), which is a global not-for-profit organisation whose mission is to ensure the development of a safe, effective, accessible, HIV vaccine.

When recruiting couples into the study, counselling and supply of condoms reduces the expected transmission rate by more than two-thirds but some transmissions do occur. In approximately three-fourths of these transmissions, the newly infecting virus can be genetically linked to the partner's virus. HIV transmission involves a severe genetic bottleneck, especially when it is the male partner who is newly infected. However, if the male has genital infections with lesions, then the bottleneck is less severe, comparable to that when the female partner is infected. HIV transmission involves both selection and chance. Despite this selection, individual virions differ in their replicative capacities (vRC) and in the epitopes, which may, or may not, be detected by the new host's immune system. How do these factors influence disease progression?

Of all the viruses in the quasi-species present in the transmitting partner, there is a selection during transmission for virions that are closer to the consensus; this selection is statistically highly significant. Especially in men without ulcers, these virions are likely to represent some of the fitter viruses. The consequences of this virion selection have been followed in the newly infected partner for up to eight years with longitudinal CD4 and viral load (VL) following IAVI protocol C.

For years, it has been known that asymptomatic, or set point, VL is correlated with disease progression, but little is known about the impact of the virus itself, or the speed with which it reproduces (replicative capacity or vRC). For example, individuals were followed for five years, starting with widely varying set point VLs and measuring for how long the individuals have CD4 counts remaining >300. For individuals with low VL (10^2–10^3), intermediate VL (10^3–10^5) and high VL ($>10^5$), the proportion retaining more than 300 CD4 cells/mm^3 were 100%, about 25% and virtually 0%, respectively. Indeed, the high VL group reached the end point within three years ($p = <0.001$). In a similar study comparing individuals infected with low vRC, intermediate vRC and high vRC virus, the latter also lost CD4 cells more rapidly with only ~25% with CD4 count > 300. In contrast, 50% low vRC individuals retained CD4 cells above 300. To determine if this detrimental effect of higher vRC was simply an effect on VL, individuals infected with low and high vRC viruses, but with similar set point VLs (3.8 and 4.0 $\log_{10}$), respectively, were compared. About 50 and 25%, respectively, retained CD4 counts (>300) ($p = <0.0001$). Therefore, the impact of vRC is independent of the effect of set point VL.

During the primary infection (about five weeks), the levels of HIV RNA rise rapidly(about 10^7 copies/ml plasma) at which time the anti-HIV cytotoxic T-lymphocytes appear and the HIV RNA levels start to decline to the set point VL. The transmitted variants with high vRC appear to initiate crucial events early in

infection that dictate both the acute and chronic stages of the disease, regardless of the ability of the immune system to limit HIV RNA levels to the set point. It seems that irreversible damage is done very early in infection.

To investigate this idea experimentally, at a median of 45 days after the estimated date of infection, the viral burden, levels of inflammatory cytokines, cellular immune activation and exhaustion and depletion of different CD4+ memory T cells were measured. It was shown that high vRC virus is linked to enhanced levels of inflammatory cytokines, driving T cell dysfunction and increased viral burden in naïve CD4+ T cells and CD4+ memory T cells. This finding is linked to significantly faster decline of CD4 cells. Therefore, what happens early in an infection can only be modulated by the adaptive immune system, reversal is not possible.

The transmitted virus, while distinct, is derived from a viral quasi-species that has evolved in its chronically infected host. This is because in the infecting partner, the HLA-directed cellular immune response leads to the selection of escape mutations. How does the transmission of such HLA-associated mutations effect the immune recognition of the transmitted founder virus in its new host? Because of successive selection in previous hosts, it is possible that some of the transmitted virus will already be 'adapted' to the immune system of the newly infected person. On the other hand, virions, which retain the consensus epitopes, are likely to be more easily recognised by the immune system. Therefore, samples of PBMCs were taken from 20 newly infected individuals (68 days after estimated infection) and stimulated with peptides corresponding to adapted (42) and non-adapted (61) epitopes in their founder virus. This work confirmed that prior immune selection history could render the transmitted virus either more or less visible to the new host's immune system. Adaption of the virus can reduce the protective effects of potentially favourable HLA alleles and impact the ability of the newly infected individual's immune system to control the virus.

In conclusion, transmitted polymorphisms can affect HIV disease progression. With low vRC and original non-adapted epitopes, the disease will progress more slowly. High vRC and adapted epitopes will aid rapid disease progression. Alternatively, these functions can oppose each other, the balance defining viral virulence.

Zika antiviral and vaccine development

Pei-Yong Shi, University of Texas Medical Branch, Galveston, TX, USA.

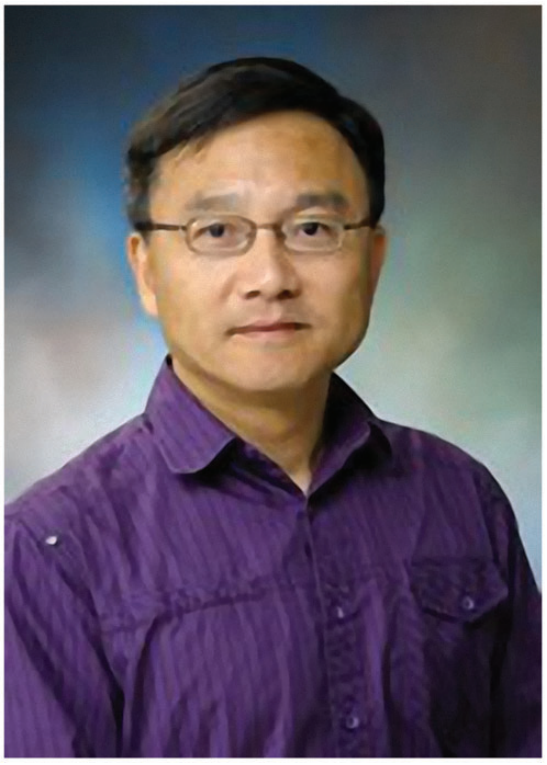

Figure 11. Pei-Yong Shi, a Keynote speaker at the 2017 ICAR.

Pei-Yong Shi (Figure 11) received his PhD in virology from Georgia State University (1996) and then moved to Yale University for postdoctoral training. He moved to Bristol-Myers Squibb, working on HIV and HCV therapeutics, from 1998 to 2000. He was at Wadsworth Center, New York State Department of Health, to study West Nile virus (2000–2008). At Novartis Institute for Tropical Diseases, he was Dengue Unit Head and Executive Director to lead drug discovery (2008–2015). Currently, Pei-Yong is I. H. Kempner Professor of Human Genetics, University of Texas Medical Branch, Galveston, Texas, USA. Having worked for many years with flaviviruses, he is now developing a vaccine for Zika virus.

Although there are no approved antiviral therapies for flaviviruses, there are some vaccines, for example, yellow fever virus vaccine. This suggests that a vaccine for Zika virus may be possible – certainly, the medical need is high. Generally, inactivated vaccines and subunit vaccines have a better safety profile than live attenuated vaccines. However, the later may give lifetime immunity after a single dose, a feature that Pei-Yong considered critical for a successful Zika vaccine. He has considered two possible approaches to produce an attenuated vaccine with a good safety profile; to knock-out a key viral enzyme or to delete sections of the three prime untranslated region (3′UTR). His target enzyme

was methyltransferase, which enables the Zika virus to acquire a cap to protect the viral RNA. The active site was inactivated by changing two amino acids. In the 3′UTR approach, he compared vaccines with 10-, 20- and two different 30-nucleotide deletions. Rather unexpectedly, the 10-nucleotide deletion (10-del ZIKV) seemed to be more attenuated than both a 20- or 30-nucleotide deletion vaccines. This may be due to the 10-del ZIKV being more susceptible to IFN than the other deletion vaccines – the reason for the differing IFN responses is not known. The 10-del ZIKA vaccine was the focus of the remainder of his ICAR presentation. The initial evaluation was done in a mouse model using type 1 IFN receptor-deficient A129 mice. To study the protective effect provided by vaccination in pregnant mice, C57BL/6 mice were used. Studies on male mice used A129 mice. Then safety and efficacy were confirmed in rhesus macaques.

The 10-del ZIKA vaccine was administered subcutaneously (sc). Mice were administered vaccine or wt virus (10^4 plaque-forming units (PFU)) on day 0. Viraemia and weight were measured on days 2, 3 and 4. On day 28, the neutralising titre was measured and mice challenged with wt virus (10^6 PFU). Subsequent viraemia and weight were followed. As expected, there were high levels of viraemia following infection with wt virus, the viral titres being about 5, 6 and 5 $\log_{10}$ PFU/ml on days 2, 3 and 4, respectively. In contrast, the corresponding titres for the vaccine were about 2, 2.5 and 3, respectively. Also, there was no disease or weight loss in the vaccinated mice. The vaccinated mice were fully protected from detectable viraemia after the wt virus challenge (10^6 PFU). Antibody titres, at 28 days after vaccination, were nearly 4 $\log_{10}$ in both groups and the titres were no higher at 28 days after virus challenge. Such effective antibody titres are sometimes referred to as 'sterilising antibodies', there being no detectable viraemia and not even a rise in antibody levels after a virus challenge.

After such an encouraging first result, further evaluation was continued. Having shown that the vaccine at a lower dose (100 PFU) was effective, it was tested at 10 PFU. In mice infected with wt virus, the viraemia was over 6 $\log_{10}$ PFU/ml at day 4, reducing to about 4 on day 5 and about 2, near the limit of detection (LOD), on days 6 and 7. In the vaccinated mice, the viraemia was undetectable on day 4 but similar to the control group on days 5, 6 and 7. This low-dose vaccine group had similar antibody titres at day 28 and provided protection after virus challenge, there being no detectable viraemia.

The T cell response was investigated by infecting A129 mice with 10^4 infectious units (IFU) of wt virus or vaccine. On day 28 after infection, mouse spleens were harvested, stimulated and assayed for T cell response. The vaccinated mice developed a robust T cell response.

To study the VLs in various tissues (heart, lung, liver, spleen, kidney, muscle, brain, testis and eye), three-week-old A129 mice were infected with wt virus (10^4 IFU) or vaccine. VLs were measured on days 6 and 10. In the wt group on day 6, virus was present in all tissues, the viral titre being about 4–6 IFU/g tissue. By day 10, virus was detected in kidney, brain and eye at low levels (about 4 IFU/g tissue) but at a high level in testis (8 IFU/g tissue). In the vaccine group at day 6, low levels of virus were detected in most tissues but notably not in brain. At day 10, virus was not detected in any tissue. To investigate the potential for neurovirulence, wt virus or vaccine was administered to one-day-old mice by intracerebral inoculation using a range of doses (10, 100, 1000 and 10,000 IFU). In the wt groups, there was a dose–response but there were some deaths even at the lowest dose. In the vaccine groups, there were no deaths, even at the highest dose.

To evaluate vaccine efficacy in pregnant mice, C57BL/6 mice were administered vaccine (10^5 IFU) or placebo on day 0. Day 28, antibody levels were measured – as expected all the vaccinated group had high levels, the placebo group having none. On day 35, the mice were mated, then challenged with 10^6 focus-forming units mouse-adapted Dakar ZIHV on day 41, at embryonic day 6 (E6). On day 48 (E13), various tissues were harvested and assayed for virus. The virus levels, in maternal spleen tissue taken from the vaccinated group, were undetectable in most mice, with a few having virus titres close to the LOD, whereas the placebo group had high levels, mostly between 4 and 7 $\log_{10}$ IFU/g. In maternal brain tissues from the vaccinated group, virus levels were undetectable or at the LOD except for one mouse but, from the placebo group, ranged widely from low to high. In placenta, 9/30 had low levels in the vaccinated group, all high in the placebo group. In fetal head, again 9/30 had low levels in the vaccinated group, but a wide range from low to high in the placebo group. In all these tests, the differences between vaccinated and placebo groups were highly significant.

The vaccine efficacy was evaluated in male A129 mice. On day 0, one group were vaccinated (sc, 10^4 IFU) and two groups given placebo. On day 28, it was confirmed that all the vaccinated mice had high levels of antibodies but undetectable in the two placebo groups; that day, the vaccinated group and one of the placebo groups were challenged with ZIKV (strain from Puerto Rico, PRVABC59, 10^5 IFU). Viraemia on day 30 (two days after challenge) were high in the challenged-placebo group but undetectable in the other two groups. Tissues were harvested on day 49 (21 days after virus challenge). The virus levels in the testis tissue

were high in the challenged-placebo group (~ 4 $\log_{10}$ IFU/g) but undetectable in the other two groups. The sperm counts were low in the challenged-placebo group ($\sim 10 \times 10^6$/ml) but equally high ($\sim 20 \times 10^6$/ml) in the other two groups. The difference was even more marked when measuring motile sperm, being undetectable in the challenged-placebo group but equal (up to 10×10^6/ml) in the other two groups. In all these tests using male mice, the differences between the challenged-placebo group and the other two groups were highly significant but the results for the vaccinated group and the double-placebo (healthy mice) group were similar, the differences being not significant.

The efficacies of the 10-del ZIKA and 20-del ZIKA vaccines were compared in a study using rhesus macaques. There were four groups, a positive control with wt ZIKV administered on day 0 (n = 4), active groups vaccinated (10^3 IFU sc) with 10-del ZIKA (n = 4) and 20-del ZIKA (n = 3) vaccines, respectively, and a placebo control (n = 2). Viraemia was measured from day 2 to 10 and antibody levels from day 2 to 56. On day 56, all groups were challenged with ZIKV (PRVABC59, 10^3 PFU). Viraemia was measured from day 58 to 66 and antibody levels from day 70 to 98. As expected, the infected control group had high viraemia, high antibody levels from day 14 and had no viraemia after virus challenge. The placebo group had neither viraemia nor antibodies until after the virus challenge. Following vaccination with 10-del ZIKA vaccine, only 1/4 animals had detectable viraemia, 4/4 had moderate levels of antibodies which rose more than four-fold on virus challenge. In spite of the suboptimal levels of antibodies, no viraemia was detected after the virus challenge. In the 20-del ZIKA vaccine group, 2/3 had low levels of viraemia and 3/3 with high antibody levels from day 14 onwards. After virus challenge, there was minimal increase in antibody levels (0/4 with a four-fold rise) and there was no viraemia. Both deletion vaccines appeared to have a good safety profile.

Another aspect of vaccine safety is its infectivity for mosquitoes. Therefore, mosquitoes were allowed to feed on blood containing either wt ZIKV or 10-delZIKA vaccine (10^6 PFU/ml blood). The vaccine did not infect any mosquitoes (0/40) whereas the wt virus infected over half (20/36) of the mosquitoes.

In summary, the 10-del ZIKV vaccine was well tolerated in mice and non-human primates. A single dose gave protective immunity within 14 days after vaccination in both mice and rhesus macaques. The vaccine prevented in utero transmission of Zika virus in pregnant mice. In male mice, the vaccine prevented prolonged infection in testis and protected against testis damage. These studies have addressed two major concerns arising from clinical studies.

My personal comments and conclusions

Each year, ISAR presents three major awards, this year to Mike Sofia (Elion award), David Chu (Holý award) and Maaike Everts (Prusoff award). Also this year, the inaugural ISAR WIS award lecture was presented by Priscilla Yang – it is intended that this WIS award will become an annual event. For several years, ICAR has included at least one Keynote lecture, this year there were four. As these four are all leaders in their respective fields, I have introduced each with a short biography. All eight speakers gave excellent presentations, each on a different topic, each of such interest to me that I wanted to prepare a detailed account of their lectures. I could not have prepared this report without the active support of each of these presenters – I most gratefully thank them for their help. In this section, I express my personal opinions.

Although there are accounts of only eight lectures, these reflect the diversity that is characteristic of ICAR – employment (academia, industry, public health), type of research (virus biology, potential antiviral targets, antiviral drugs, research organisation) and a range of viruses. For example, the viruses included were HCV and HBV (Mike Sofia), HIV and HBV (David Chu), multiple antiviral projects (Maaike Everts), dengue (Priscilla Yang), RV-C (Ann Palmenberg), polio (Mark Pallansch), HIV (Eric Hunter) and Zika virus (Pei-Yong Shi). The following comments give examples in which this diversity can bring benefits.

Mike Sofia described his past work on sofosbuvir and his current work with HBV. His talk on sofosbuvir followed on from that of Phillip (Phil) Furman, the Elion Awardee at the 2015 ICAR. Both Phil and Mike worked at Pharmasset Inc. until it was bought by Gilead Inc. Then Mike continued at Gilead to assist in the development of sofosbuvir. The clinical introduction of sofosbuvir transformed the therapy of HCV, becoming the 'backbone' of various combination therapies. When so many firms were searching for anti-HCV drugs, what special factors enabled Pharmasset to discover such a uniquely active drug? My comments are based on both Phil's and Mike's lectures.

The founding culture of Pharmasset included a focus on nucleoside/tide analogues and selecting only those compounds that have a good safety profile. Pharmasset understood that all the drugs, with the highest genetic barriers, were nucleoside/tide analogues (but, of course, not all nucleoside/tide analogues have a high genetic barrier). For all viruses which have high mutation rates (e.g. HIV and HCV), combination therapy needs at least one drug with a high genetic barrier. A good safety profile is just as important as a good activity – at Pharmasset, compounds were assessed in a cascade, which started with a test for activity

(an HCV replicon assay), but this assay also included a ribosomal-RNA cytotoxicity test. The next steps in the cascade were all aimed at assessing the safety profile – cytotoxicity evaluations in a panel of five cell lines with replicating cells, mitochondrial toxicity and bone marrow toxicity. To my knowledge, it is rare to find a compound-screening strategy giving so much emphasis to the safety profile at such an early stage in the evaluation of a novel compound. This strategy led to PSI-6130, being identified as a compound with potential, when their competitors had compounds which may have had comparable activities but not the same safety profile. The Pharmasset selection strategy had led to a compound having a 'hidden' advantage over all the competitor compounds.

The next two decisions were also crucial. A prodrug approach improved bioavailability but did not enable once-daily dosing. Appreciating that an apparent problem (partial metabolism to an inactive compound) was a potential opportunity, it was decided to investigate the uridine analogue. This led to the testing of the cytosine and uridine triphosphate analogues versus the viral polymerase. Although the uridine analogue (PSI-6206-TP) was less potent than the cytidine analogue (PSI-6130-TP) as an inhibitor of the HCV polymerase, the half-life ($T_{1/2}$) of PSI-6206-TP was much longer than that of PSI-6130-TP (38 and 5 h, respectively). The 38 h half-life would enable PSI-6206 to be administered once daily. The second crucial decision was to select the half-life as the more important parameter, the reduced activity as being of less importance. A prodrug approach was initiated to maximise activity, synthesising and evaluating many phosphoramidate prodrugs in order to select the best candidate drug. Each of these steps was important for the discovery of sofosbuvir which happened much earlier than expected.

Mike emphasised the importance of teamwork which was another crucially important factor. Very correctly, he was too modest to mention that the name of the new compound, sofosbuvir, was based on his surname. That is such a great compliment to Mike, his colleagues must have valued Mike's contribution to this project.

At the 25th ICAR at Sapporo, Japan (2012), Mike gave an update on GS-7977 (formerly PSI-7977), including reporting the results of the ELECTRON trial.[6] This must be one of only a few ICAR presentations which foretold such a great improvement in clinical therapy. In my 2016 ICAR meeting report,[7] Jerome Deval (Alios BioPharma) and his colleagues chose to screen only nucleoside analogues for activity against RSV. Now, ALS-8176 (lumicitabine) looks set to become the game-changer for RSV therapy as sofosbuvir was for HCV therapy. Their success follows in the footsteps of Pharmasset. At this 2017 ICAR, the Zika vaccine (Pei-Yong Shi) may be another game-changing therapy – I understand that, since ICAR, this vaccine has been selected for progression.

Standard HBV therapy is with TAF, an excellent nucleotide-analogue drug which has a high genetic barrier to resistance. Although it limits HBV DNA effectively, it neither eliminates viral cccDNA nor the production of VPs transcribed from cccDNA. In this situation, compounds targeting VP production, and thereby allowing the restoration of the host immune response, require compounds with good activity and safety profile but not necessarily with a high genetic barrier because such compounds will always be used with TAF.

Switching focus in his Elion lecture, Mike summarised his recent work at Arbutus. Their strategy for an HBV cure is to minimise the levels of HBV antigens and thereby to restore the host immune response. There has been good progress setting up in vitro and *in vivo* screens in which novel approaches are being evaluated. Mike is evaluating David Chu's novel nucleoside analogue, FMCA.

Mike concluded that a cure has not yet been discovered but there is light at the end of the tunnel. It seems likely that patients with chronic HBV infection will be functionally cured, but only by combining drugs with differing modes of action so that both HBV replication and the production of VPs from HBV reservoirs are controlled.

Priscilla Yang is investigating the potential of the entry viral glycoprotein (E), mediating attachment and membrane fusion, as a suitable antiviral target for drugs against dengue. It has been confirmed that small molecules can interact with E to give activity in both a target-based assay and a cell culture antiviral assay. The next step is to validate E as an antiviral target *in vivo*. Although it is necessary to confirm antiviral activity, it is also necessary to show that this approach can lead to antiviral compounds having a high genetic barrier to resistance. It was an encouraging start to find that some compounds did not show resistance up to passage 9. However, I think that Sophie Duraffour holds the record for ICAR's longest resistance test, with resistance to (S)-HPMPDAP appearing in two strains of camelpox virus after passaging 30 times, taking 18 months, before resistant clones could be obtained. (Sophie Duraffour, 22nd ICAR, 3–7 May in Miami Beach, FL, USA). To investigate resistance to dengue, Priscilla's group prepared various dengue mutant viruses containing point substitutions, which were chosen from naturally occurring E sequences identified in public databases. None of the current compounds were active against all these mutant strains. Surely, that suggests that, for these

compounds, resistance will be a critical factor to be investigated.

I have mentioned above that Pharmasset understood that all the drugs with the highest genetic barriers were nucleoside/tide analogues – why should that be so? Hugh Field[8] and I devised the term 'back door escape route'. We illustrated the concept using the HIV protease. When treating an HIV patient with a single PI, the first (primary) resistance mutation will decrease the inhibition by the antiviral drug but perhaps by a small degree. Then other mutations (secondary) will further decrease the inhibition by the drug. These mutations are usually near the active centre of the protease. Further mutations (tertiary) may be remote from the active centre and may not decrease the inhibition of the drug but restore some of the lost functionality of the protease. By carful combination of PIs with different resistance mutations, it may be that none of the spontaneously arising mutant proteases can be both resistant to the drugs and be a competent enzyme – the 'front door is locked'. However, there is a 'back door escape route' – the viral RNA encoding both the protease and that for the cleavage sites (the protease substrate) can co-mutate so that both the enzymic activity and the substrate change. The altered protease is no longer inhibited by the PIs, it has 'escaped through the back door'. The 'back door escape route' is not available to the viral polymerases because the natural nucleotides are absolutely required for building the viral DNA/RNA. Hence, careful combinations of nucleoside/tide analogues have given the drug therapies with the highest genetic barriers.

Ann Palmenberg described how RV-C had remained undetected for many years after the discovery of RV-A and RV-B and that this delay was due to RV-C using a different cellular receptor. I consider it optimistic to expect the inhibition or blocking of a host protein will give an antiviral therapy that is free from viral resistance problems. Although the target host protein may not be subject to change, the virus may well be able to use a 'back door escape route'. Priscilla Yang's team may find that is so for their antiviral approach. To my mind, the important result from her work is a good demonstration that antiviral compounds, which maybe not good enough for clinical use, are effective tools to learn more about viral replication. Her compounds have given more information about the dengue virus fusion process. I recall discussing with my friends from Roche at the 1999 ICAR (Jerusalem) the use of their HSV thymidine kinase inhibitors to investigate how an initially (immature) latently infected cell can mature to become a competent latently infected cell from which HSV can reactivate. Shortly afterwards, that work was closed down but the opportunity still remains. I ought to declare an interest in such an investigation into herpesvirus latency – famciclovir appears to act like a thymidine kinase inhibitor, preventing immature latently infected cells maturing[8] (and references therein).

Mark Pallansch described the need for an effective anti-poliovirus compounds and to the ongoing work investigating resistance to pocapavir (V-073, capsid inhibitor, preventing viral RNA release) and V-7404 (3C PI). The greatest medical need is to treat those SCID patients in whom poliovirus infection has continued for many years. Experience with other long-lasting chronic infections indicates that drug therapy requires a combination of drugs, which includes at least one nucleoside/tide inhibitor with a high genetic barrier to resistance. Therefore, it may be timely to prepare a 'plan B', approaching companies with a good panel of nucleoside/tide compounds.

Clearly, the continuing transmission of HIV is of great medical concern. Eric Hunter presented work providing insights into that transmission process. Although this work was done within the programme seeking an HIV vaccine, it also gives baseline data for preventing HIV transmission using drugs. The trial using Truvada (FTC/TDF) proved that preventing transmission was an achievable aim but that once-daily dosing was not an acceptable option for most of the trial participants. Douglas (Doug) Richman, in his Elion Lecture at the 29th ICAR in La Jolla,[7] reported clinical studies which indicated that both EFdA (a nucleoside analogue inhibiting HIV RT) and cabotegravir (an HIV integrase inhibitor) could be administered by injection at long time intervals. If women can have a combined injection of EFdA/cabotegravir with their monthly contraceptive injection, preventing HIV transmission may become a practical reality, even in countries with limited medical resources.

I choose to end my comments with the Prusoff Award lecture by Maaike Everts because it illustrates the tremendous breadth of topics which have been presented at ICAR. ISAR was founded on the concept that successful antiviral research needs a multidimensional approach and every ICAR has promoted that aim. Maaike described the pros and cons of various collaborations involving different organisations. Her talk reminds me of the time when I started work at Beechams in 1969. Beechams had just switched from a Department system to Project-based format. I joined a newly formed multidisciplinary antiviral team. Many of us felt that the Project system worked well. I am interested in Maaike's work helping to re-creating a Project system. She mentioned many of the pros and cons which we faced during my time at Beechams.

I hope these comments help to illustrate the value of ICAR in bringing many different aspects of antiviral research together. I thank the ICAR Program committee, especially the co-chairs Mark Prichard and Justin Julander, for organising such a good conference. I am looking forward to the next ICAR at Porto, Portugal (11–15 June 2018).

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