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Report of the third VIRGIL annual international symposium on antiviral drug resistance

Three years after its creation, the VIRGIL European network has confirmed the pioneer role of its multidisciplinary approach to antiviral drug resistance. This was highlighted by research reported at the 3rd annual VIRGIL international symposium in Lyon, France, on May 23, 2007.

VIRGIL aims to improve the surveillance, diagnosis and management of antiviral drug resistance by integrating resources and skills throughout Europe to achieve common research objectives. It functions as a "virtual institute" organised into seven complementary technological and clinical platforms, with research focusing mainly on hepatitis B, hepatitis C and influenza.

Highlighting major achievements of the network over the past 3 years, **Fabien Zoulim**, VIRGIL network co-ordinator, said that these include:

- Development of a centralised clinical virology platform
- On-line viral resistance register
- Bank of antivirals for *in vitro* studies
- Cellular immunology studies
- HBV and influenza phenotyping assays for clinical studies
- Cross-resistance data for HBV, HCV and influenza

VIRGIL teams were the first to precisely characterise resistance to the hepatitis B drugs adefovir and entecavir. In hepatitis C, *in vitro* studies have identified synergies and antagonisms between antiviral molecules and new viral targets for treatment. Also important is the surveillance by VIRGIL groups of influenza drug resistance — this will have immediate benefit on seasonal influenza while skills developed in this programme will be able to be mobilised in the event of an influenza H5N1 pandemic.

Many aspects of the VIRGIL research programmes were outlined in presentations throughout the symposium.

Finding new hepatitis C drug targets

The HCV non-structural protein NS5A is a potential new drug target, said **Ralf Bartenschlager**, from the University of Heidelberg. He believes that this RNA-binding phosphoprotein could be a key regulator of the HCV replication complex.

Non-structural proteins are required for viral assembly and release, and Dr Bartenschlager said that new data point to NS5A being the most important: for example, enhancing mutations cluster in NS5A, replication activity is regulated by phosphorylation, and mutations in NS5A can block assembly without affecting replication.

Also, the x-ray crystal structure of the NS5A binding domain was recently solved [Tellinghuisen et al. Nature, 2005] and shows this to be a dimer with a "claw-like" shape which would be a perfect binding site for single stranded RNA.

NS5A is usually present in infected cells as a basally phosphorylated and a hyperphosphorylated form. Several cell culture adaptive mutations which enhance viral RNA replication interfere with production of the hyperphosphorylated form. Also, pharmacological inhibition of the kinase responsible for phosphorylation of NS5A enhances RNA replication.

"We propose that replicase itself, and most importantly NS5A, decides which part of the replication cycle is favoured — translation, replication or assembly," Dr Bartenschlager said This might be linked to differential phosphorylation of NS5A, with the level of hyperphosphorylation favouring different parts of the replication cycle.

In a related presentation, **Francois Penin**, from IBCP CNRS, Lyon, took delegates through recent work on the structure and membrane associations of HCV proteins. He explained how increasing knowledge of the 3D structures of the HCV enzymes has clear implications for the identification of new targets and design of new drugs for antiviral therapy.

New step in HCV entry

Several known cellular factors are involved in HCV entry into host cells. But cells expressing these factors are not always susceptible to infection, suggesting that others are still missing. **Matthew Evans**, from Rockefeller University, New York, reported experiments indicating that claudine-1 (CLDN1) is one such entry factor.

Claudin proteins form the backbone of the tight junction strands in epithelial tissues. They are most highly expressed in hepatocytes. Dr Evans presented data showing that all HCV susceptible cell lines express CLDN1 and some (not all) HCV resistant cells become susceptible when expressing CLDN1. Silencing CLDN1 by RNA interference renders Hep38 cells uninfectable by HCV.

"We believe that CLDN-1 might act at a post-binding, post CD81, pre-fusion step in the HCV entry process," Dr Evans said.

Combination therapies

Discussing potential combination therapies for hepatitis C infection, **Johan Neyts**, from the Rega Institute, KU Leuven, Belgium, said that studies suggest that Debio-025 might delay or prevent development of HCV resistance to other drugs.

This trial drug is a ciclosporin analogue, with no immunosuppressant activity. It has potent anti-HCV activity in cell culture and has been shown to prevent emergence of drug-escape mutants when used in combination with either telaprevir (VX-950, protease inhibitor) or R1479 (4-azidocytidine, an HCV polymerase inhibitor).

Dr Neyts also described an *in vitro* study which indicated that combining two non-nucleoside polymerase inhibitors acting at different binding sites —a thumb- and a palm-site polymerase binding inhibitor — might reduce the frequency of resistance development [Le Pogom et al. J Virol, 2006]. "It may be important to explore such combinations," he said.

He warned that some combinations can be detrimental. For example, his group recently reported that ribavirin antagonises the *in vitro* anti-HCV activity of the nucleoside analogue 2'-C-methylcytidine, the active component of the trial drug valopicitabine [Coelmont et al. Antimicrob Ag Chemother, 2006].

Future drugs for HCV

While response to peginterferon/ribavirin can be improved with further optimisation and individualisation of therapy, future treatment of HCV is likely to involve use of direct antivirals, according to **Markus Cornberg**, from Hannover Medical School, Germany. "Every step in the HCV life cycle is a potential target of a specific antiviral treatment for hepatitis C," he said.

The pipeline of protease inhibitors and polymerase inhibitors is growing. The problem is that use of direct antiviral drugs leads to selection of resistant strains: this has been seen with HIV and HBV and, said Dr Cornberg, will be the major challenge of the future for HCV.

Since resistance can be associated with low drug exposure it is important to maintain effective plasma levels and to ensure good adherence, Dr Cornberg noted. In this regard, protease inhibitors that have to be given three times a day can be a problem for patients. Interestingly, he said, ritonavir boosting has been shown to improve plasma levels of the protease inhibitor boceprevir (SCH-503034) by inhibiting cytochrome P450 3A.

Combination therapies can help avoid resistance and early studies showed that resistant mutants developing with telaprevir were very sensitive to interferon. However, first results from the PROVE 1 study of telaprevir plus peginterferon/ribavirin in treatment-naïve HCV genotype 1 patients show only 35% of patients to have SVR with 12 weeks' therapy. Boceprevir combined with peginterferon/ribavirin was effective in some non-responders.

Other new types of drugs will be needed to combine with protease and polymerase inhibitors, Dr Cornberg said.

Therapeutic vaccine trial

Matti Sallberg, from the Karolinska Institute, Stockholm, described how his team is planning phase I/II testing of a therapeutic vaccine in HCV infected patients using non-structural protein 3/4A as the target.

He explained that HCV NS3/4A protein appears to be involved in immune evasion and undoubtedly participates in viral persistence in hepatocytes. Outside the liver, it is highly immunogenic, at least in mice, and is a potent inducer of cytotoxic T-lymphoctyes that enter the liver and eliminate NS3/4A expressing cells.

The planned clinical work involves delivery of codon optimised NS3/4A DNA by *in vivo* electroporation. This technique – which Dr Sallberg emphasised "is not as painful as it sounds" — is a way of enhancing uptake of plasmid DNA by generating temporary pores in the cell membrane.

Treating HCV in HIV co-infected patients

One of the complex areas of HCV treatment is co-infection with HIV. As **Vicente Soriano**, from Hospital Carlos III, Madrid, explained, several large clinical trials have shown that response to peginterferon/ribavirin is lower in co-infected patients, with SVRs of 27-40% compared with 55-60% in monoinfected patients.

But there is now evidence that response can be improved by increasing the ribavirin dose. Dr Soriano outlined the results of the new 400-patient PRESCO trial which used a weight-based ribavirin dosage of 1000mg or 1200mg. To minimise toxicity, treatment was only started if the CD4 count was >300 cells/mm^3, concurrent didanosine was contraindicated and zidovudine was avoided where possible.

SVR was seen in 50% of patients. "It looks as if with higher dose ribavirin we can improve response in co-infected patients, regardless of genotype," Dr Soriano said. He emphasised that discontinuation due to severe anaemia was similar to that seen in HIV-negative patients, despite the high ribavirin dose.

Since PRESCO showed response at week 4 to be a good predictor of relapse, Dr Soriano said that one current approach to optimise treatment is to use high dose ribavirin and to taper duration of therapy as follows:

- 6 months' treatment for genotype 2/3 patients negative for HCV RNA at week 4 — an estimated 15% of patients
- 12 months' treatment for genotype 2/3 patients positive for HCV-RNA at week 4, or genotype 1/ 4 patients negative for HCV RNA at week 4 — 50% of patients
- 18 months' treatment for genotype 1/4 patients positive for HCV-RNA at week 4 — 35% of patients.

Be aware of risk of HBV reactivation

Clinicians need to be more aware of the risk of hepatitis B reactivation in immunosuppressed patients, said **Wolfram Gerlich**, from the Institute for Medical Virology, Giessen, Germany.

He suggested that reactivation is well known in inactive HBsAg carriers. However, the problem is underestimated in anti-HBc and/or anti-HBs positive patients: while it occurs rarely with moderate immunosuppression (for example after renal translantation), it is common in patients undergoing bone marrow/stem cell transplantation and lymphoma therapy. In these situations, pre-testing of the patient for anti-HBc and anti-HBs is necessary.

Monitoring for reactivation is important but Dr Gerlich cautioned that HBsAg monitoring can be unreliable because of escape mutations. Sensitive HBV DNA assays and "early or premptive therapy" are needed, he emphasised.

New tool for HBV analysis

Hepatitis B virus genotyping and mutation detection is of interest both for clinical research and for patient management. **Guy Vernet**, from BioMérieux, Lyon, described the characteristics and validation of a DNA chip that can be used as a tool for this analysis.

The chip determines HBV genotype and known polymorphisms along the complete HBV genome from a single serum assay, making it simpler and quicker than sequencing techniques. Nearly 400 mutations can be detected. The chip was validated in serum samples from 170 patients, with close correlation between sequencing and chip analysis [Tran et al. J Clin Microbiol, 2006].

As an illustration of the use of the genotyping assay, Dr Vernet described how a recent analysis helped to identify HBV genotype G as a major determinant of liver fibrosis in HIV/HBV co-infected patients [Lacombe et al. AIDS 2006]

In a second version of the chip, currently being validated, the number of analysed positions/mutations has increased: "With version 2 we can detect nearly 1000 mutations at nearly 300 positions on the genome," Dr Vernet noted.

Managing lamivudine resistance

The Italian experience of managing HBV resistance to lamivudine was outlined by **Pietro Lampertico**, from the University of Milan. He identified the key issues as being:

- To choose a drug with no cross resistance
- To add-on rather than use sequential monotherapy
- To start rescue therapy early.

A multicentre Italian study in HBeAg-negative patients with lamivudine resistance confirmed previous reports that adding adefovir (the only drug available for rescue therapy in Italy) to lamivudine is better than swapping to adefovir. "The data suggest that combination therapy can protect against adefovir resistance," Dr Lampertico said.

He added that early rescue is critical. Rather than waiting until clinical resistance occurs, it is better to start at the first phase of lamivudine resistance — the virological breakthrough phase — when there is rebound viraemia but no change in ALT.

Dr Lampertico told delegates : “Early add-on suppresses HBV replication and ALT levels in most patients for at least 6 years, preventing clinical decompensation and progression of portal hypertension.” However, there has not been any prevention of hepatocellular cancer.

The “roadmap” now used in his centre for HBeAg negative patients is based on viral response at week 24. Patients with low viraemia (<2 log cp/ml) at week 24 have a low risk of lamivudine resistance (5% at week 104) and carry on with monotherapy, but with monitoring for secondary failure and rescue with early add-on adefovir. Patients with a poor response at week 24 (>2 log cp/ml) are at high risk of resistance (39% at week 104) and start adefovir.

In the future, combination therapy from the start might be useful for some patients but long-term efficacy and safety data will be needed, Dr Lampertico concluded.

Advances in understanding HBV replication

Finding new targets for chronic hepatitis B drugs will depend on improved understanding of the virus replication processes.

Michael Nassal, from the University of Freiburg, Germany, gave an update on developments in this area gained from experiments with his group’s cell-free reconstitution model of duck HVB polymerase activity.

He explained that the cellular chaperones that have been shown to be necessary for initiation of viral reverse transcriptase (P protein) activity are heat shock protein (Hsp) 40 and Hsp 70. These are ATP-dependent folding catalysts and they produce an activated, RNA binding form, of P protein.

In recent experiments the researchers have been looking at how this activation occurs. It appears that the chaperones “open up” the inactive form of P protein. Dr Nassal commented that while many questions remain to be answered, it is clear that the activation process involves the transient exposure of an epsilon RNA binding site involving the terminal protein region.

Current picture on influenza drug resistance

A series of presentations gave delegates an update on the current picture on resistance to the neuraminidase inhibitor (NI) influenza drugs.

Maria Zambon, from the Health Protection Agency, London, emphasised that resistance is rare and, generally speaking, associated with compromised virus.

She reported that global surveillance carried out by the Neuraminidase Inhibitor Susceptibility Network (NISN) in the first 3 years of NI use [Monto et al. Antimicrob Ag Chemother, 2006] shows a low level of resistant variants, and no evidence of increased frequency over time, or of a shift in susceptibility profile.

Within the Virgil/EISS network 25 countries provide isolates for analysis, and over 2004/7 there has been emergence of occasional resistant virus but no overall trends in mean IC50.

Since Kiso's 2004 report from Japan of a high level of oseltamivir resistance in viruses isolated from children post-treatment, NISN has undertaken enhanced surveillance in Japan to assess resistance in this highly treated population. [An estimated 5% of the population took oseltamivir in 2004/5.] The results were little different from the global surveillance [WHO Weekly Epi Record, 2007]. "The very low frequency of resistance in community isolates, despite substantial oseltamivir use, is encouraging," Dr Zambon commented. But she added that it is not known if the unselected surveillance is detecting low level transmission of resistant variants or primary NI resistance: "Knowledge of transmissibility of resistance is a major gap in our understanding."

Noting some concern about emergence of oseltamivir resistance in H5N1 influenza, Dr Zambon said there are several possible explanations for the reports of treatment failure in H5N1, including inadequate dose regime or host factors, but there are also structural reasons why resistance in the N1 subtype might emerge more easily.

John Oxford, from Retroscreen Virology Ltd, London, described a series of experiments in the ferret model showing reduced pathogenicity and virulence of an oseltamivir-resistant mutant.

Their studies involved the arginine to serine 294 mutant, one of the drug-resistant H3N2 mutants isolated by Kiso. "The data are very clear. We saw a marked difference between fully virulent wild type virus and the less virulent, less pathogenic drug resistant mutant," he said. The mutant virus was also much less transmissible.

Jennifer McKimm-Breschkin, from CSIRO Molecular and Health Technologies, Melbourne, made the point that resistance to neuraminidase inhibitors is both drug specific and N1 and N2 sub-type specific.

Drug specificity relates to differences in the chemical structures of the two drugs. To accommodate oseltamivir's branched side chain the neuraminidase active site has to change shape; zanamivir is more closely related to the natural substrate and binds without the need for structural change. Mutations that inhibit change in shape can prevent oseltamivir binding but it is hard for the virus to mutate to become resistant to zanamivir without compromising its ability to bind to natural substrate, Dr McKimm-Breschkin commented.

She added that not all mutations are predictable: "We thought we could predict what mutations we get and why the virus is resistant. However, we are now starting to see mutations outside the active site."

It also appears from *in vitro* studies that haemagglutinin mutations can confer resistance to NIs.
'We can get both drug resistance and drug dependence through haemagglutinin mutations," Dr McKimm-Breschkin observed. In the latter case, the viruses need NI to infect efficiently.

There is as yet no validated assay for screening for haemagglutinin resistance.

Expanding further on sub-type specific mutations, **Alan Hay,** from the National Institute for Medical Research, London, explained that influenza A neuraminidases are now known to differ in their active site: group 2 neuraminidases (N2, N9) have the standard previously understood closed conformation while group 1 neuraminidases (N1, N4, N8) have an additional “150 cavity” (an open conformation). Oseltamivir can bind to both the open and closed conformation. This provides the basis for further structure-based design of antineuraminidase drugs.

Differences in structure can explain the sub-type specific differences that are seen in the principal resistance mutations to oseltamivir. But Dr Hay commented that it is not yet clear whether they affect the frequency of resistance emergence, since studies have shown that oseltamivir resistance can occur in H1N1 viruses as frequently as in H3N2 viruses.

New inhibitor tested against avian flu viruses

Oliver Planz, from the Federal Research Institute for Animal Health, Tübingen, gave an update on VIRGIL work on the NF kappa B (NFkB) signalling pathway as a drug target for influenza. His team has been working with an NFkB inhibitor called SC75741.

In cell culture, the drug was effective against avian influenza viruses (H7N7 and H5N1) without producing resistant strains. “We can reduce infectivity by 4 logs using this inhibitor,” Dr Planz reported.

For assessing clinical efficacy, they are using an H5N1 virus isolated from a deceased Mallard that is only passaged twice in eggs. “So we have a natural field isolate which is highly pathogenic in a mammalian model without adaptation,” Dr Planz explained. In a mouse model, SC75741 has been tested as influenza treatment (given on day 4, when activated T cells might start migrating to the lung) and shows a significant effect against the H5N1 virus.

Studies on mechanism of action indicate marked reduction in viral specific messenger RNA in the lung, and an associated reduction in IL-6 and IP-10 in treated animals.

“Super interferon” strategy

A “super interferon” could be one way forward for treating acute viral infection, **Eleanor Fish**, from Toronto General Research Institute, believes. As a complementary strategy to development of specific antivirals, her research group is focusing “on the host not the pathogen” to develop broad-spectrum antivirals.

One of the strands of this strategy is to develop new interferons. Dr Fish explained that induction of type 1 interferon is key to the body’s response to virus infection, with interferon orchestrating a broad spectrum of antiviral effects through both direct antiviral activity and various immunomodulatory responses.

She pointed out that viruses have evolved to evade this interferon response. For example, the SARS coronavirus encodes a factor that blocks transcription activation of

interferon. However, their experiments have shown that this inhibition can be overridden by administering interferon. During the SARS outbreak, patients' lungs cleared quickly when interferon treatment was given. Similarly, in a mouse model of influenza, viral inhibition of the interferon response can be overridden.

Their strategy is to develop small molecule non-peptide interferon-mimetics with enhanced receptor affinities. Current lead compounds have been shown to outbind interferon alfacon-1 at receptor sites, Dr Fish reported.

Fighting drug resistance in design of new HIV drugs

Delegates were also given an insight into approaches that are being taken to reduce resistance problems with HIV.

"In HIV we are looking at lifelong therapy. It is clear that the number of antiviral targets is not endless, so we have to make a strong effort to optimise compounds for the targets we have," said **Rudi Pauwels**, from Federal Institute of Technology, Lausanne, who outlined the development of the newly approved HIV protease inhibitor TMC 114 (darunavir).

He explained that first generation PIs and non-nucleoside reverse transcriptase inhibitors were optimised using wild-type virus. But in the 1990s massive resistance development became apparent, and considerable cross-resistance between different classes of agents. Structural studies have shown that cross-resistance is due to variability of the drug binding site.

"The logical conclusion was that the wild-type drug binding sites we, and others, were using in studies did not fully represent the *in vivo* challenge in the clinic. So the first thing was to map the genetic diversity," Dr Pauwels said.

The researchers reasoned that it should be possible to find drugs that share the same binding site as first generation inhibitors but bind differently and to more conserved regions of the binding site, and to various forms of the binding site, ie, broad spectrum target inhibitors.

The approach they took was to optimise interactions with the backbone (to delay breakthrough of resistant virus) and to find compounds that bind tightly to highly conserved side chains only. The final drug, TMC 114, is effective in multiple PI-experienced HIV patients, Dr Pauwels noted.

He concluded: "HIV displays an extraordinary genetic diversity and its replicative enzymes have a large functional plasticity. Mapping of this diversity and a more detailed understanding of the mechanisms of drug resistance is paving the way for new generations of anti-HIV drugs."

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