Editorial

Current viral infections and epidemics of *flaviviridae*; lots of grief but also some hope

Dimitrios Vlachakis[1], Spyridon Champeris Tsaniras[2] and Sophia Kossida[1]

[1] Biomedical Research Foundation, Academy of Athens, Athens, Greece

[2] Department of Physiology, Medical School, University of Patras, Rio, Patras, Greece

Correspondence should be addressed to Sophia Kossida; Phone: +30 210 6597199, Fax: +30 210 6597545, Email: skossida@bioacademy.gr

Abstract

Flaviviridae is a family of RNA viruses that includes numerous important human and animal pathogens. Recent studies on subgenomic flaviviridae replicons have revealed that the non-structural (NS) proteins, which are encoded by the C-terminal part of the polyprotein, play a crucial role in viral RNA replication. Accordingly, these proteins are assumed to form replication complexes in conjunction with genomic RNA and possibly with other cellular factors. One the most important non-structural enzymes that plays a key role in the life cycle of flaviviridae viruses is the viral helicase. Sequence alignments of the viral helicases from this family identified several conserved sequence motifs that are important for biological functions. Herein, an effort is made to summarize the current epidemics associated with the flaviviridae family worldwide, the potential of helicase enzymes as a promising pharmacological target and the use of nucleoside analogs as simple, efficient and rather versatile antiviral agents.

The flaviviridae viral family

Flaviviridae is a family of viruses that infect vertebrates (Neyts et al. 1999). The small, enveloped virions of flaviviridae contain a single positive-sense RNA genome, in a long open reading frame (ORF), which is flanked by untranslated regions (UTRs) at the 5' and 3' ends. Virions are spherical in shape, usually between 40-60 nm in diameter and are slightly pleomorphic during their life cycle. Their nucleocapsids are isometric and sometimes penetrated by stain. The usual size of the nucleocapsids is 25-30 nm in diameter and they have polyhedral symmetry (Guzmán & Kourí 2004). *Flaviviridae* consists of three characterized genera and certain unclassified members (Courageot et al. 2003). The main representatives of each family are summarized in Figure 1. What is remarkable is that even though the above viruses are separated to different genera, do not have common biological properties and do not show serological cross-reactivity, they manage to retain high similarity in the morphology of the virion, the organization of the viral genome, and the estimated life cycles and replication patterns they follow (Wang & Fikrig 2004, Calisher & Gould 2003, Collett 1992).

Current epidemics and necessity

More than 170 million people worldwide are currently chronically infected with the Hepatitis C virus (Avalos -Ramirez *et al* 2001). They are all considered to be at risk of developing cirrhosis and some of them will progress to liver cancer. Hepatitis C has spread all over the world and causes ten thousand deaths per year. It is the main cause for more than half of the four thousand liver transplantations performed annually (Degos 1994).

Infections carried by mosquitoes or, in more general terms, arthropod-borne *flaviviridae* infections have reached epidemic dimensions in some parts of the world. Dengue fever infects 50 million people per year in central Africa. According to the World Health Organization (WHO) there are 6.5 billion inhabitants on this planet that live in areas of high risk of acquiring the dengue virus (Figure 2). For example, in 2006 alone, the Philippines reported 197 deaths and 14,738 cases of dengue fever (Kadare & Haenni 1997). Indonesia's Dengue fever deaths reached 634 and Malaysia has already confirmed 74 deaths in the first 9 months. In Thailand more than 32,000 Thais have been infected with the virus and currently Singapore is going

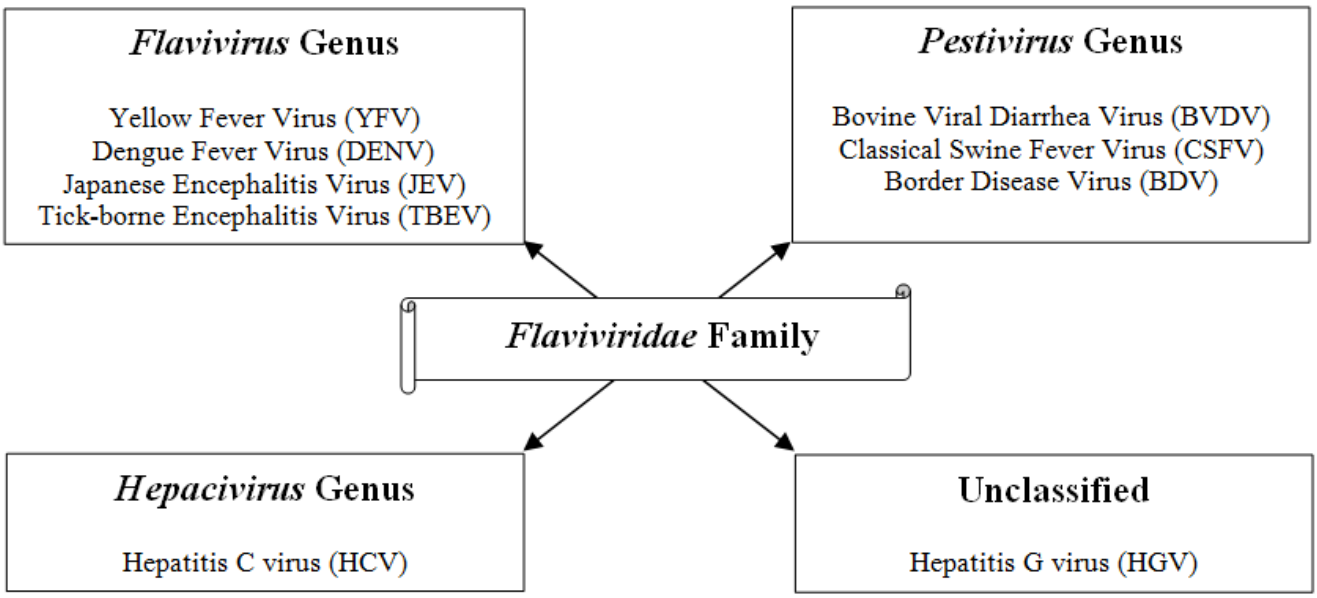

Figure 1. The three genera that constitute the viral family of *flaviviridae*; Hepatitis C virus was recently discriminated from the rest of the Flaviviridae due to its distinct properties and clinical manifestations.

through its worst dengue fever outbreak ever on record, since the officially reported Dengue cases are nearing 11,000 (Pasta et al. 2005). According to the WHO of South-East Asia, since November 2005, a total of 5,737 cases of Japanese Encephalitis with 1,334 deaths (a fatality rate of 23.3%) have been reported in Uttar, India since the outbreak started in July 2005. Moreover, since January 2006, a total of 2,824 individuals have been infected with Japanese Encephalitis and 316 of these have resulted in death (a fatality rate of 11.2%). In response, the Japanese Government has employed both anti-larval and anti-adult measures by distributing 200,000 mosquito nets and passing legislation making vaccination and immunization against the virus compulsory for all children aged between 1-12 years (Allain 2005).

West Nile Virus (WNV) first hit New York with 77 deaths in 1999. The United-States were alarmed and action was taken to stop the virus from spreading. Nevertheless, in 2002, WNV hit Illinois harder than any other state, with 399 cases, 21 of which resulted in death. The humid and full of swamps Louisiana came second with 11 deaths in 2006 (Gilbert et al. 2005).

In addition, farming and agriculture have both seriously suffered in the past from the impact that Pestiviruses have had on livestock. Many economies depend on primary production that comes from farming and agriculture and as a result most of these countries take preventative action against these viruses (Keeffe 2005, Shepard et al. 2005). Despite the severity of the infection with almost all members of *flaviviridae*, no specific antiviral therapy is available today (Courageot et al. 2003, Wang & Fikrig 2004, Calisher & Gould 2003, Collett 1992).

NS3 Helicase is a promising pharmacological target; Insights by X-ray crystallography

All of the non-structural viral proteins have had their time in the spotlight. A lot of research has been conducted on the NS2 Protease or the NS5 RNA-dependent RNA enzyme that is responsible for the unwinding of the viral genome and the subsequent propagation and proliferation of the virus. However, X-ray crystallography work on the HCV helicase (Kim et al. 1998) has shed light to the structure of this mysterious protein that has now become one of the 'hottest' viral pharmacological targets for major US pharmaceutical companies.

The Hepatitis C helicase consists of a 456 amino acid poly-peptide. It has three domains in total, which are separated by two channels (Figure 3). The first and third domains interact much more strongly with each other than each of them does with domain two. As a result of this, the channel between domains 1-2 and 2-3 is larger than the channel between domains 1-3 and 2-3. Domain two is supposed to undergo significant movements compared to the other two domains, during the unwinding of double-stranded nucleic acids. The position of domain two is therefore far more flexible relatively to the other two domains and the Helicase and can acquire the form of a dynamic "hinge" that moves accordingly to the needs of the protein and the process it is involved into (Kim *et al.* 1998).

The topology of the first and the second domain is very similar. These two domains contain the structurally conserved regions of helicases of this family. This is confirmed by the superimposition of the two domains, which gives an RMSd of 2.0 Å for 76 Ca atoms. The third domain consists mostly of α-helices and is linked to domain two with a set of antiparallel β-strands.

Domain 3 includes a 40 amino-acid long region, just before the α-helix in the C terminus that does not contain any secondary structures. This may contribute to the flexibility required by the protein during its cleavage from the NS4A domain during polyprotein processing. On the other hand, towards the N -terminus of the protein there is the highly conserved "Walker A box" or "P-loop" motif (Kim *et al.* 1998). This motif is very often found among helicases and is basically a glycine rich region of the protein that provides a quite flexible loop between beta-strands and alpha-helices. The "Walker A box" has got phosphate-binding properties and is found in most ATPases (Kim *et al.* 1998). The sulfate ion interacts with the Nitrogens Gly207 and Gly209, and the side-chains of Ser208, Lys210 and Ser211. Lys210 establishes a H2O mediated interaction with As290 of the DExH motif (Asp-Glu-x-His). The position of the sulphate ion was found to be very similar to the position that the β-phosphate of ADP would take in the PcrA helicase–ADP complex. So, it is suggested that β-phosphate should occupy this area when NTP or nucleotide diphosphate (NDP) is bound to the HCV helicase. The residues Gln460, Arg464 and Arg467 are highly conserved residues from domain 2 that are exposed to solvent in the major channel of the Helicase. Arg461 and Arg462 are buried amino-acids in the core of the second domain.

The single strand of DNA is located in the main channel of the helicase between domains 3 and 1-2 (Figure 3). The size of the channel is approximately 16 Å in diameter. The 5′ end of the oligonucleotide is towards the part of the channel formed between domains 2 and 3 and the 3′ end towards the part of the channel formed between domains 1 and 3.

The interaction between the ssDNA and the Helicase is essentially between the backbone of the DNA, since it is nonspecific protein-DNA interaction. The majority of the established interactions are located towards the two ends of the oligonucleotide. From the protein's point of view most interactions arise from regions lacking secondary structures in domains one and two. The positioning of the interaction-participating amino acids is symmetric and as a result there appears to be a symmetric distribution of the interactions between the DNA and protein. After superimposing the first and the second domains it was proven that the residues involved in the phosphate contacts are structurally equivalent (Kim *et al.* 1998). Furthermore the phosphate-binding amino acid series of Ser231, Thr269, Ser370 and Thr411 are conserved in NS3 domains and this provides evidence that these two domains may descend from a gene duplication event. Val432 and Trp501 are also highly conserved residues among HCV NS3 sequences; nevertheless neither seems to play any role in nucleic acid binding or duplex unwinding.

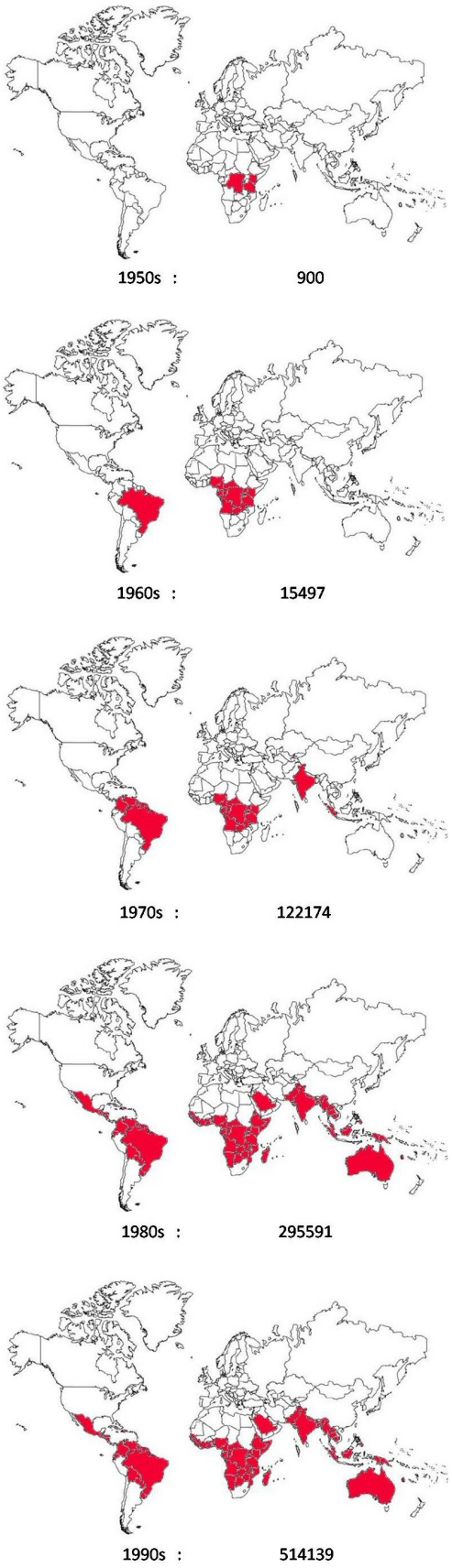

Figure 2. The spread of the Dengue Virus from the 50's until today, showing the casualties per decade. Even highly-developed areas of the planet are under threat.

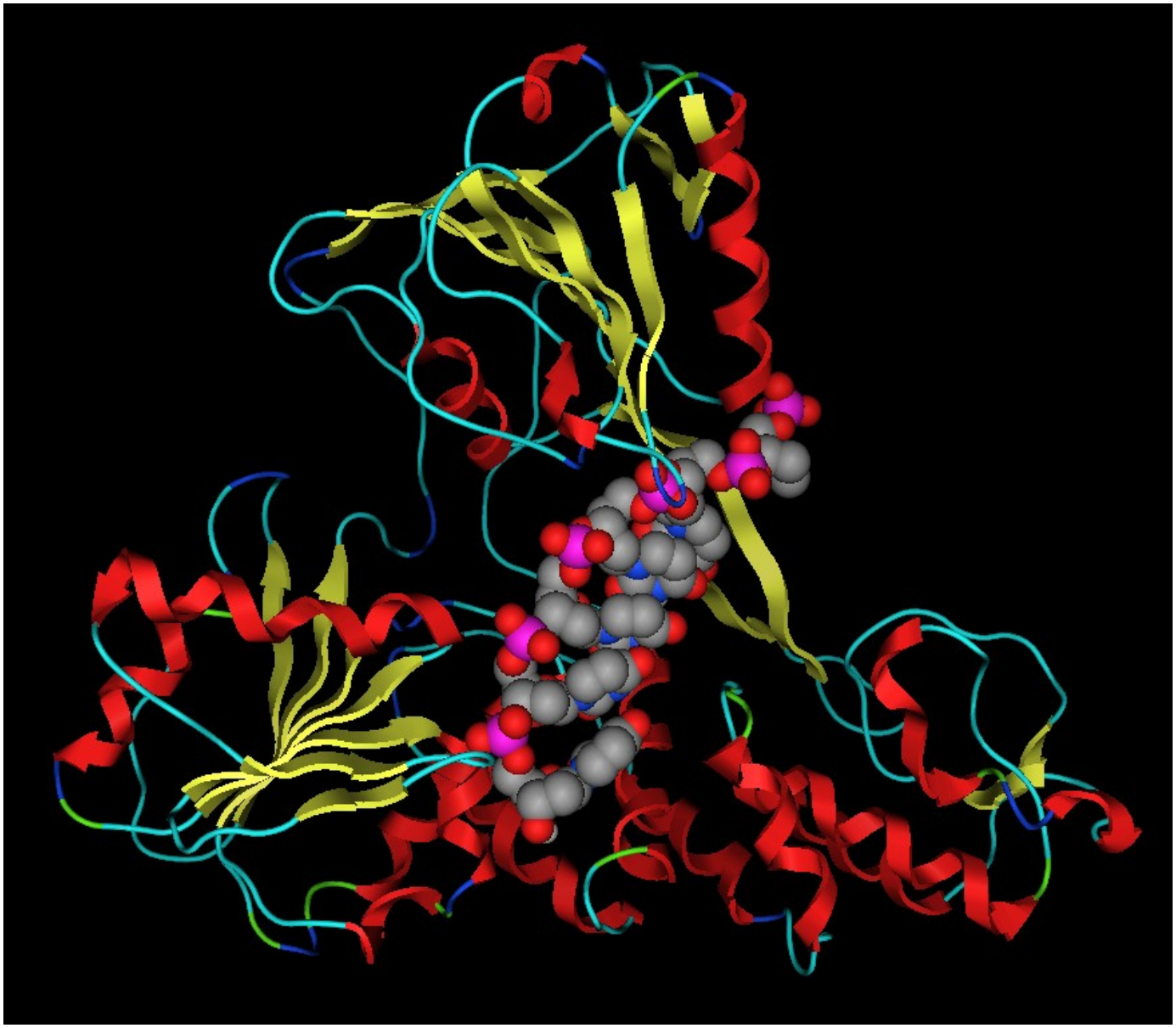

Figure 3. The HCV Helicase co-crystallised with a ssDNA fragment. The enzyme is shown in ribbon representation while the oligonucleotide in CPK – ball and sticks representation.

Rationalized use of nucleoside analogues as modern antiviral agents

Viruses are very simple in structure. They contain a limited package of nucleic acid that is encased by a rather basic protein coat. They are primarily intracellular parasites that lack any craft in catering for themselves as they cannot even produce their own metabolic energy. The machinery that they use in terms of their proliferation comes explicitly from the host cell. Once inside the cell, they are exceedingly good at abusing and exploiting their host in order to produce the viral proteins and nucleic acid that they require for the assembly of new virions. Eventually the host cell will become exhausted and destroyed by releasing numerous copies of the virus (Levine 1992). Basic biochemistry has revealed that all those viral proteins are being produced soon after the virus has penetrated into the host cell and that makes specific targeting of the viral machinery quite challenging (Neyts *et al.* 1999). A virion in the blood stream is quite 'invisible' and only when found within its host it is somehow exposed and vulnerable. However, separating and targeting those viral proteins in the ocean of host cell enzymes, proteins and cytoplasm is not an easy task. The goal of chemotherapy and antiviral research is to block such viral enzymes using non-toxic doses of drugs for the host cell.

Antiviral research must therefore be cunning. Extra effort must be made to identify those steps and proteins that are involved with the replication of the viral genetic material. It is no coincidence that most of

the current antiviral drugs target enzymes involved in the handling of the genome. In this regard, one of the most crucial enzymes is the viral helicase as a target for antiviral chemotherapy. It goes without saying that due to its preference in natural substrates, nucleoside analogues constitute a very important family of antiviral compounds (Levine 1992, Eriksson & Wang 1997). Next to the viral helicases are the viral polymerases, which in turn utilise nucleotide triphosphates as substrates for the building of new viral genetic material. Consequently, nucleoside analogues must be provided in their phosphorylated form when interacting with those catalytic enzymes. On the other hand, a triphosphate nucleoside is not of much use as it is very polar and heavily charged, making it incapable of crossing through cellular membranes. Therefore nucleoside analogues aim for kinases (Eriksson & Wang 1997) which are the key proteins that phosphorylate nucleosides and convert them into their active form. Still this is tricky too, as the structural similarity of these antiviral agents with the natural nucleosides gives rise to toxicity issues. Things get more complicated if one considers that certain viruses encode their own kinases. Should the nucleoside analogue in this case act on the activation and phosphorylation stage or at the actual mechanism of the involved enzyme (e.g. dsDNA unwinding of viral helicases)? Special efforts are made in this direction, in order to develop a platform where the nucleoside compound gets phosphorylated by the viral kinases while the host kinases do not detect it at all. By this approach, we can achieve some kind of selectivity between infected and uninfected cells through the preferential phosphorylation by viral kinases as a key proliferative and metabolic step. Inhibition at the enzyme level will be achieved by the misplacement or misuse of our introduced nucleoside analogue which can act as a faulty substrate and block the viral enzyme. For instance, if a non-natural nucleoside is introduced into the viral genome, it will immediately disrupt the base pairing of the viral genetic material and result in the composition of new DNA containing mutations in its sequence. Alternatively, if the incorporated nucleoside analogue has a modified sugar moiety lacking the necessary 3'- hydroxyl group, it will inevitably result in a sudden stop and terminate the chain generation.

In conclusion, we need to emphasize that epidemiologically *flaviviridae* are currently affecting every corner of the globe. Extensive antiviral research has focused on the viral polymerase and protease while the helicase enzyme has been purposely neglected due to its dynamic structure. However, recent advances in crystallography and bioinformatics have shed light to the function and mechanism of action of the viral helicase. Therefore there is an on-going battle in the designing of potent antiviral agents, where much potential and hope is expected by nucleoside analogues, whether they are acting on the kinase level, the viral enzyme level or both.

Conflicts of interest

The authors have no conflicts of interest.

References

Allain JP 2005 Hepatitis C virus in blood donation. *Lancet* **365** 276-278.

Avalos-Ramirez R, Orlich M, Thiel HJ & Becher P 2001 Evidence for the Presence of Two Novel Pestivirus Species. *Virology* **286** 456-465.

Calisher CH & Gould EA 2003 Taxonomy of the virus family Flaviviridae. *Adv Vir Res* **59** 1-19.

Collett MS 1992 Molecular genetics of pestiviruses. *Comp Immunol Microbiol Infect Dis* **15** 145-154.

Courageot MP, Catteau A & Desprès P 2003 Mechanisms of Dengue virus-induced cell death. *Adv Vir Res* **60** 157-186.

Degos F 1994 Epidemiology of hepatitis C virus in Europe. *FEMS Microbiol Rev* **14** 267-271.

Eriksson S & Wang L 1997 Substrate specificities, expression and primary sequences of deoxynucleoside kinases; implications for chemotherapy. *Nucleos Nucleot* **16** 653-659.

Gilbert LK, Bulger J, Scanlon K & Moyer L 2005 Viral hepatitis prevention education: what do people and providers need to know? *Pat Educ Couns* **59** 46-55.

Guzmán MG & Kourí G 2004 Dengue diagnosis, advances and challenges. *Int J Infect Dis* **8** 69-80.

Kadare G & Haenni AL 1997 Virus-encoded RNA helicases. *J Virol* **71** 2583-2590.

Keeffe EB 2005 Chronic Hepatitis C: Management of Treatment Failures. *Clin Gastroenterol Hepatol* **3** S102-S105.

Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C & Caron PR 1998 Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **6** 89-100.

Levine AJ 1992 The Human Herpesviruses. In: Viruses. Scientific American Library, pp. 67-85

Neyts J, Leyssen P & De Clercq E 1999 Infections with flaviviridae. *Verh K Acad Geneeskd Belg* **61** 661-697.

Pasta L, Marrone C, D'Amico M, Bevacqua EA, D'Amico G & Pagliaro L 2005 Familial clustering of HCV-related liver cirrhosis and hepatocellular carcinoma. *Dig Liv Dis* **37** 716-717.

Shepard CW, Finelli L & Alter MJ 2005 Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* **5** 558-567.
Wang T & Fikrig E 2004 Immunity to West Nile virus. *Curr Opin Immunol* **16** 519-523.