ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 4/2010
541–546
on-line at: www.actabp.pl

Regular paper

Effect of tunicamycin on the biogenesis of hepatitis C virus glycoproteins

Natalia Reszka[1]✉, Ewelina Krol[1], Arvind H. Patel[2] and Boguslaw Szewczyk[1]

[1]Department of Molecular Virology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland; [2]MRC — University of Glasgow Centre for Virus Research, University of Glasgow, Glasgow, United Kingdom

Hepatitis C virus (HCV) infects humans, with a prevalence around 3 % of population, causing acute and chronic hepatitis and hepatocellular carcinoma. We studied the effect of inhibition of glycosylation on the assembly of the HCV particle. HCV possesses two envelope glycoproteins E1 and E2 that are highly modified by N-glycans. These glycan residues are crucial for viral entry and maturation of the progeny. Here, we examined the influence of inhibition of N-glycosylation on expression of E1 and E2. Since the propagation of HCV in cell culture is limited, we used a recombinant baculovirus producing viral-like particles in insect cells. Our data showed that blocking of N-glycan transfer to the nascent polypeptide chain with the antibiotic tunicamycin resulted in the loss of E1 and E2. We also found that a dose of tunicamycin that did not influence the cell viability significantly reduced the E2 level in infected cells. The results indicate that blocking of glycosylation at an early step efficiently reduces the assembly of HCV virions. Thus, we suggest that derivatives of tunicamycin that preferentially block glycosylation of viral proteins may become potential therapeutic agents against HCV.

Keywords: hepatitis C virus, glycoproteins, glycosylation inhibition, tunicamycin

Received: 10 May, 2010; revised: 18 October, 2010; accepted: 27 November, 2010; available on-line: 06 December, 2010

INTRODUCTION

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family that primarily infects hepatocytes causing acute and chronic hepatitis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with the virus (World Health Organization, 1997; Shepard *et al.*, 2005). The acute infection is often asymptomatic making early diagnosis difficult. HCV is relatively noncytopathic and the liver disease is likely immune-mediated (Su *et al.*, 2002; Thimme *et al.*, 2002; Lindenbach & Rice, 2005). In about 70–80 % of patients, the infection becomes chronic, which can eventually progress to liver cirrhosis and hepatocellular carcinoma (Bowen & Walker, 2005).

HCV is an enveloped virus with a single-stranded RNA (ssRNA) genome of positive polarity. The genome carries a single open reading frame (ORF) encoding a polyprotein that is cleaved into three structural and seven nonstructural (NS) proteins. The structural proteins (core and envelope glycoproteins E1 and E2) are components of viral particle, while the NS gene products (p7, NS2, NS3, NS4, NS4B, and NS5B) mediate replication (Bartenschlage & Lohmann, 2000). The ORF is flanked by untranslated regions (UTRs) which contain control elements required for viral RNA translation and replication. Several studies provide evidence that host cell infection is initiated by binding of the HCV glycoproteins (gps) to cell surface receptors, followed by clathrin-mediated endocytosis. It is suggested that low pH in endosome induces fusion of viral and cell membranes, and release of nucleocapsid into the cytosol (Tscherne *et al.*, 2006; Blanchard *et al.*, 2006; Meertens *et al.*, 2006). The HCV life cycle is restricted to the cytoplasm. The released positive-strand RNA serves multiple roles within the virus life cycle. First, it acts as a messenger RNA (mRNA) for translation of the viral polyprotein. Second, it is a template for RNA replication, carried out by viral NS proteins. Nascent plus strand RNA is packaged into the virions. The progeny viral particles are then thought to bud into the endoplasmic reticulum (ER) and leave the cell through the secretory pathway (von Hahn & Rice, 2008).

HCV exhibits a high degree of genetic diversity with six distinct genotypes which are further divided into subtypes (Simmonds *et al.*, 1993). Furthermore, due to the error-prone nature of its RNA-dependent RNA polymerase, high replication rate *in vivo* and immune selective pressure, HCV persists in an infected individual as a diverse population of viral variants known as quasispecies (Gomez *et al.*, 1999; Simmonds, 2004). The genetic diversity together with the possibility of re-infection of re-exposed individuals and the propensity of HCV to chronic infection, have hampered the development of effective vaccine against HCV. Numerous studies over the last 20 years indicate that preventive and therapeutic vaccine may be within reach (Bassett *et al.*, 2001; Seeff, 2002; Nevens *et al.*, 2003; Lanford *et al.*, 2004; review in Houghton & Abrignani, 2005). Nevertheless, there is no vaccine against HCV yet and the standard treatment of HCV is based on ribavirin and pegylated INF-α (McHutchison & Fried, 2003). This therapy is expensive, toxic and effective in only 50–60 % of patients treated (Pearlman, 2004; Strader *et al.*, 2004). Therefore it is clear that less toxic and more effective treatments are needed. Most of the approaches to address this issue are focused on identifying agents that inhibit specific steps

✉e-mail: natalia.reszka@gmail.com

Abbreviations: aa, amino acid; CSFV, classical swine fever virus; FCS, fetal calf serum; gps, glycoproteins; HCV, hepatitis C virus; hpi, hours post infection; MOI, multiplicity of infection; NS, non structural proteins; ORF, open reading frame; PBS, phosphate-buffered saline; rbac, recombinant baculovirus; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* insect cell line; VLPs, virus-like particles; WT, wild type.

in the life cycle of the virus. These include small-molecule inhibitors of viral enzymes (Lamarre *et al.*, 2003; Lin *et al.*, 2004; Chen & Tan, 2005; Reiser & Timm, 2009), nucleic-acid-based antiviral agents (Foster, 2004; Kronke *et al.*, 2004) and novel immunomodulatory agents (Hahn, 2003). Another target for HCV drug discovery is the glycosylation pathway involved in the biogenesis of viral glycoproteins (Durantel *et al.*, 2001; Chapel *et al.*, 2007).

HCV glycoproteins E1 and E2 are the major components of the viral envelope involved in the viral entry. E1 and E2 are type 1 transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor. They assemble as a non-covalent heterodimer and are highly modified by N-linked glycans (Op De Beeck *et al.*, 2001; Dubuisson *et al.*, 2002). E1 contains five potential N-linked glycosylation sites and E2 may contain up to eleven glycosylation sites. The N-glycans are believed to be responsible for correct folding of E1 and E2, they are essential for the entry functions of HCV and play a role in the assembly and release of the viral particle (Goffard & Dubuisson, 2003).

In this report, we investigated the significance of blocking the incorporation of glycans into E1 and E2. Specifically, we were interested in testing whether tunicamycin, an early-step inhibitor of N-glycosylation, affects the synthesis and stability of E1 and E2 and hence the formation of the viral particle. Since propagation of HCV in a cell culture system is limited and a small-animal model does not exist, several other methods to study HCV are used (reviewed in Rychlowska & Bienkowska-Szewczyk, 2007). In our experiments we used a recombinant baculovirus (rbac-B45) expressing aa 1 to 836, representing the entire structural region (core, E1 and E2) plus p7 and the N-terminal 27 aa of NS2 of the infectious clone of HCV genotype 1a strain H77c (Clayton *et al.,* 2002). It was previously shown that insect cells infected with rbac-B45 produce viral-like particles (VLPs) that are empty virions of HCV (Owsianka *et al.*, 2001; Clayton *et al.*, 2002). Our experiments showed that in the presence of tunicamycin the synthesis of E1 and E2 glycoproteins in insect cells is restrained and consequently the assembly of VLPs is blocked. Therefore, we speculate that tunicamycin can control the HCV by blocking the formation of viral particles. Our results call for thorough studies of tunicamycin and its non-toxic derivatives as potential therapeutic agents for HCV infections.

MATERIALS AND METHODS

Cell cultures. The insect cell line *Spodoptera frugiperda* (Sf9) was grown in TC-100 medium (Gibco) supplemented with penicillin-streptomycin and 10% FCS, or in HyQ medium (HyClone) at 27 °C.

Production and purification of virus-like particles (VLPs). Approximately 2×10^8 Sf9 cells were infected at a multiplicity of infection (MOI) of 3 with the recombinant baculovirus rbac-B45 expressing the genotype 1a HCV strain H77c (Yanagi *et al.*, 1997) core, E1, E2, p7 and the N-terminal 27 aa of NS2 (Owsianka *et al.*, 2001; Clayton *et al.*, 2002). At 96 h post-infection cells were centrifuged, washed with PBS and lysed with lysis buffer (20 mM Tris/HCl, pH 7.4, 20 mM iodoacetamide, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). The VLPs were partly purified by sucrose density gradient centrifugation. Fractions were collected from the top of centrifuged samples and analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE) for the presence of VLP proteins as described by Baumert *et al.* (1998).

Expression of the HCV E2 gene in insect cells. Monolayer culture of Sf9 cells was infected with rbac-B45 at an MOI of 1 and incubated for 24, 48 and 96 h at 27 °C. Cells were lysed and proteins were separated by SDS/PAGE under reducing conditions and then transferred to Immobilon P membranes. Monoclonal anti-E2 HCV AP33 (Clayton *et al.*, 2002) was used as the primary antibody. Anti-mouse alkaline phosphatase (AP)-conjugated antibody was used as secondary species-specific antibodies. Nitrotetrazolium blue (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as substrates.

Inhibition of glycoprotein synthesis. Stock solution of tunicamycin (2 mM) (Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO). Sf9 cells were infected with rbac-B45 at an MOI of 1 or 3 for 1 h. Then the viral inoculum was replaced with medium containing tunicamycin at a concentration of 2 μg/ml, 1 μg/ml or 0.5 μg/ml. Cells were harvested 24, 48 and 96 hpi and subjected to SDS/PAGE followed by Western blotting.

Indirect immunofluorescence assay. Sf9 cells (1.5×10^6) in monolayer culture were grown on cover slips in TC-100 medium with 10% FCS and then infected with rbac-B45. One hour post infection the viral inoculum was replaced with medium with or without tunicamycin (0.5 μg/ml). At 48 h post infection, the cells were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. Cells were incubated with rabbit polyclonal anti-core serum or mouse monoclonal anti-E2 AP33 antibody for 1 h, washed and incubated with anti-rabbit or anti-mouse IgG conjugated with Alexa Fluor 488 or Alexa Fluor 546 for an additional hour. After three washes the cells were analyzed using a fluorescence microscope.

SDS/PAGE and Western blotting. Protein samples were resolved by SDS/PAGE in reducing conditions and processed for Western blotting as described previously (Tyborowska *et al.*, 2006).

Serological reagents. The anti-E1 and anti-E2 mouse monoclonal antibodies, 21.010 and AP33, respectively, and the rabbit polyclonal anti-core serum R526 have been described previously (Clayton *et al.*, 2002).

Endo H treatment. Samples were digested with endoglycosidase H (Endo H; Calbiochem) according to the manufacturer's instructions or left untreated (negative control) for 24 h at 37 °C, mixed with an equal volume of 2× sample reducing buffer and analyzed by SDS/PAGE, followed by Western blotting.

RESULTS AND DISCUSSION

Effect of tunicamycin on the synthesis of HCV core, E1 and E2 glycoproteins in insect cells

With the exception of HCV strain JFH-1 (Kato *et al.*, 2001) and a few chimeric genotypes based on this strain that can be propagated in human hepatoma cells (Huh-7), there is no cell culture system suitable for *in vitro* propagation of HCV (Pietschmann *et al.*, 2006). Therefore other *in vitro* models are used to study HCV infection. Several reports have shown that HCV structural proteins expressed in recombinant baculovirus-infected cells can assemble into enveloped viral capsid described as virus-like particles (VLPs) (Baumert *et al.*, 1998; 1999;

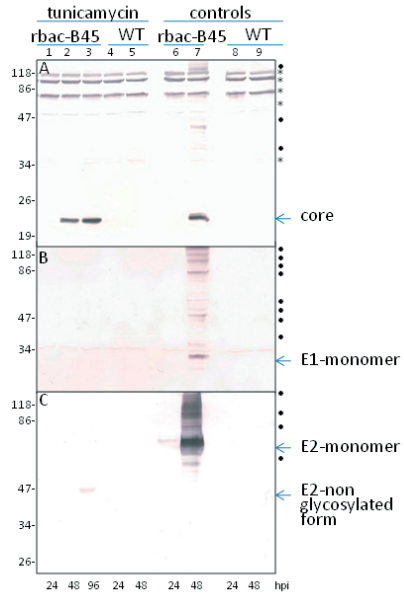

Figure 1. Western blot analysis of HCV structural proteins expressed in insect cells in the presence of tunicamycin
Insect cells were infected with rbac-B45 or with wild type baculovirus (WT) and cultured with tunicamycin at 2 μg/ml (lane 1, 2, 3, 4, 5) or without it (lane 6, 7, 8, 9). Cells were harvested at 24, 48 and 96 hpi and Western blot analysis was performed using anti-core rabbit polyclonal serum R526 (A), anti-E1 monoclonal antibody 21.010 (B) or anti-E2 monoclonal antibody AP33 (C). Monomeric forms of these two glycoproteins are marked with arrows. Oligomeric forms of E1 and E2 are marked with dots.

Clayton *et al.*, 2002). Such particles have been used to study the structure and function of viral glycoproteins and the virus-receptor interaction (Baumert *et al.*, 1998; Owsianka *et al.*, 2001; Clayton *et al.*, 2002; Triyatni *et al.*, 2002). In this study, we used the previously described recombinant baculovirus rbac-B45 expressing core, E1, E2, p7 and the N-terminal 27 aa of NS2 of the infectious HCV genotype 1a strain H77c (Yanagi *et al.*, 1997; Owsianka *et al.*, 2001; Clayton *et al.*, 2002). Previous studies have shown that infection of insect cells with rbac-B45 results in assembly of HCV VLPs (Owsianka *et al.*, 2001; Clayton *et al.*, 2002).

We used the rbac-B45 baculovirus to study the effect of tunicamycin (Elbein, 1987) on the synthesis of viral glycoproteins E1 and E2. Tunicamycin blocks N-glycosylation of the translated peptide at a very early step, during the transfer of a 14-residue oligosaccharide core unit (Glc3Man9GlcNAc2) from dolichol pyrophosphate to the asparagine moiety of the sequence N-X-S/T (Imperiali *et al.*, 1999). This antibiotic has been shown to efficiently inhibit the expression of glycoproteins of classical swine fever virus (CSFV), also a member of the *Flaviviridae* family, that is often used as a surrogate model to study HCV (Tyborowska *et al.*, 2007).

To study the effect of inhibition of glycosylation on the assembly of VLPs, we cultured rbac-B45 in the presence of tunicamycin. Interestingly, when we fractionated the lysate of infected cells on a sucrose gradient, followed by SDS/PAGE analysis (as described in Materials and Methods), the bands corresponding to VLP proteins were not visible in contrast to cells cultured without tunicamycin (not shown). That observation suggested that progeny VLPs were not assembled. Thus, we performed a more detailed analysis of core, E1 and E2 expression in the presence of tunicamycin in insect cells infected with rbac-B45. Lysates of insect cells infected with rbac-B45 in the presence or absence of tunicamycin were analyzed for HCV core, E1 and E2 by Western blotting. HCV core protein is a 21 kDa polypeptide that is known not to be modified by glycosylation, therefore it served as a control in those experiments. The expression of core protein in rbac-B45-infected cells was monitored from 24 until 96 hpi. As expected, the core protein pattern from insect cells grown with or without tunicamycin was identical (Fig. 1A, lane 2, 3 and 7, core marked with arrow), indicating that this antibiotic had no effect on the synthesis of core in the insect cells. The 118–80 kDa bands (marked with stars) were most likely cellular proteins which cross reacted with the anti-core polyclonal serum, as they were also seen in WT baculovirus-infected cells (Fig. 1A, lanes 8 and 9) (these bands provided loading control on the gel blot). The same amounts of infected cell lysates were analyzed with the E1 and E2 antibodies. Both E1 and E2 are highly glycosylated in their native forms; E1 contains five potential glycosylation sites, while E2 may contain up to eleven glycosylation sites (Op De Beeck *et al.*, 2001; Voisset & Dubuisson, 2004). As shown in Fig. 1B, E1 was expressed in rbac-B45, but not in WT baculovirus-infected cells (lane 6 and 7; E1 monomer marked with arrow), however, E1 was not detectable when tunicamycin was added to the medium (Fig. 1B line 1 to 3). Similarly, E2 was expressed by the rbac-B45-infected cells between 24–48 hpi, while in the presence of tunicamycin the protein was not visible (Fig. 1C). The faint band observed in the sample cultured with tunicamycin harvested at 96 hpi (Fig. 1C, lane 3) was presumably an insignificant amount of non-glycosylated form of E2. E1–E2 complexes as well as the E2–E2 and E1–E1 interaction are known to be very stable, therefore these proteins are not fully dissociated even in SDS/PAGE lysis buffer.

Our results show that tunicamycin specifically abrogates the synthesis of E1 and E2, but does not inhibit core protein expression. Indeed, as shown before with CSFV (Tyborowska *et al.*, 2007), almost no non-glycosylated forms of HCV glycoproteins were observed in the presence of tunicamycin. This effect of tunicamycin on flavivirus glycoproteins is unusual because in the vast majority of viruses a lack of glycan chains on glycoproteins does not lead to a rapid disappearance of glycan-free polypeptide chains (Stohrer & Hunter, 1979; Pizer *et al.*, 1980; Norrild & Pedersen, 1982; Lu *et al.*, 1995; Nakhasi *et al.*, 2001).

N-linked glycosylation occurs co-translationally and influences the maturation of the nascent peptide. Blocking of the transfer of N-glycans to the nascent peptides of HCV gps may either hamper their full translation or prevent the maturation of E1 and E2 which may result in their quick degradation immediately after synthesis. We suggest that the second scenario is more likely to occur but future experiments are needed to confirm our hypothesis. Also, the faint band of E2 detected in cells cultured with tunicamycin indicated that at least a vestigial amount of the non-glycosylated protein was expressed and this protein was stable even late after infection.

Loss of glycans does not influence the reactivity of E2 and E1 with anti-E2 and anti-E1 antibodies

A lack of glycans in a nascent peptide chain often causes the loss of its immunodominant epitopes. This could result in weaker reactivity of antibodies that have been raised to the glycosylated product. Here, we asked

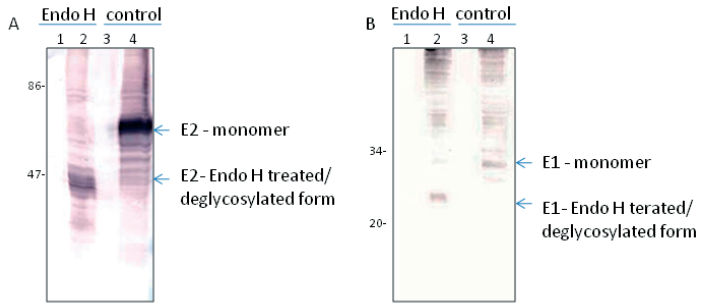

Figure 2. Anti-E2 and anti-E1 monoclonal antibodies recognize both glycosylated and deglycosylated forms of E2 and E1
Insect cells were infected with wild type baculovirus (lane 1, 3) or rbac-B45 (lane 2, 4). At 48 hpi insect cell extracts were treated (lane 1, 2) or not (control, lane 3, 4) with Endo H. Western blot analysis was performed using anti-E2 monoclonal antibody AP33 (panel A) and anti-E1 monoclonal antibody 21.010 (panel B).

if the disappearance of HCV gps expressed in the presence of tunicamycin was or was not related to the lack of reactivity with specific antibodies. As shown in Fig. 2, insect cell-expressed E2 and E1 retained their reactivity with the anti-E2 monoclonal antibody AP33 (panel A) and the anti-E1 monoclonal antibody 21.010 (panel B) following enzymatic deglycosylation with Endo H (Fig. 2 panels A, B, lanes 2). Thus, we exclude the possibility that the tunicamycin-mediated E1 and E2 disappearance in insect cells observed above (Fig. 1) is related to the loss of epitopes recognized by the antibodies used.

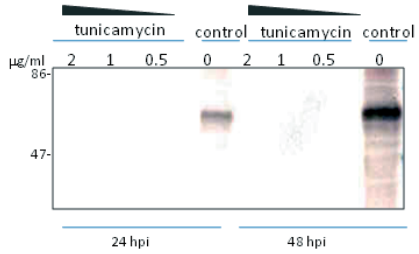

Figure 3. Tunicamycin blocks the E2 synthesis in insect cells at concentration as low as 0.5 mg/ml
rbac-B45-infected insect cells were either treated with different doses of tunicamycin (2 µg/ml, 1 µg/ml or 0.5 µg/ml) or not treated with tunicamycin (control). Cells were harvested and analysed by Western blot at 24 and 48 hpi with anti-E2 monoclonal antibody AP33.

Tunicamycin blocks E2 synthesis in insect cells at a concentration that does not significantly influence cell viability

Tunicamycin is toxic at high concentrations and significantly decreases cell viability. In mammalian cells, we found that concentrations of tunicamycin between 62.5–125 ng/ml result in cell viability between 90–98% (Tyborowska *et al.*, 2007). Insect cells are less susceptible to tunicamycin treatment than mammalian cells. At the concentration of 1 µg/ml, tunicamycin efficiently blocks N-glycosylation but not protein synthesis in insect cells (Sf9) (Webb *et al.*, 1989; Jarvis & Summers, 1989; Richardson, 1995). Our results indicate that at 0.5 µg/ml of tunicamycin the cell viability varies between 90 and 100% (not shown). Therefore we checked whether the concentration of tunicamycin as low as 0.5 µg/ml can lead to the disappearance of HCV glycoproteins. The highly glycosylated E2 served as a model glycoprotein in the experiment. In the presence of 0.5 µg/ml of tunicamycin, cells infected with rbac-B45 exhibited similar behavior with respect to E2 as in the presence of 1–2 µg/ml of tunicamycin (Fig. 3). This indicated that the inhibition of E2 glycosylation by tunicamycin leads to the disappearance of this protein even at concentrations that have little or no effect on cell survival.

Quantitative estimation of tunicamycin effect on E2

Our experiments showed a significant loss of HCV glycoproteins in insect cells cultured with tunicamycin. Using immunofluorescence microscopy, which is more sensitive than classical biochemical methods, we tried to quantify the degree of inhibition of HCV gps synthesis in insect cells by tunicamycin. Cells were infected

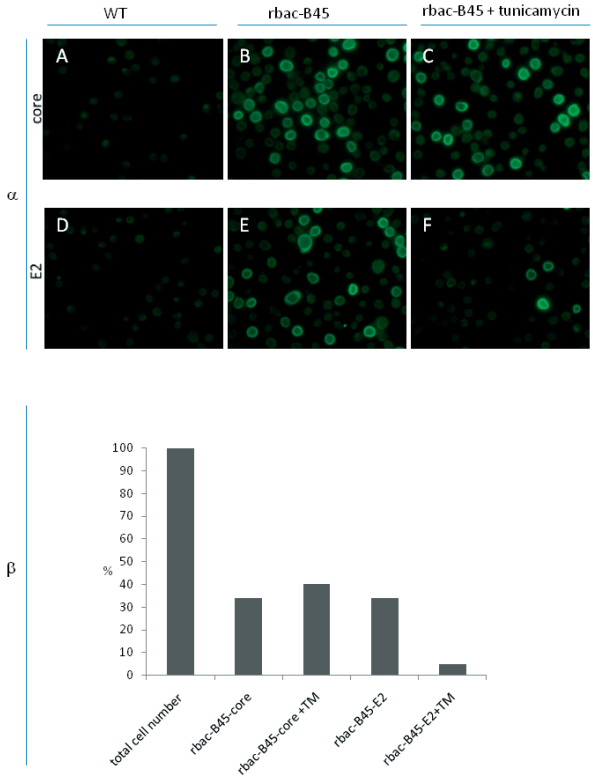

Figure 4. Effect of tunicamycin on core and E2 protein expression in insect cells
Indirect immunofluorescence microscopy analysis (panel α). Insect cells were infected with WT (A, D) or rbac-B45 and cultured without (B, E) and with (C, F) tunicamycin. At 48 h post infection, cells were fixed and analyzed for the presence of core (A, B, C) and E2 (D, E, F) with specific antibodies. Quantitative estimation of E2 synthesis inhibition by tunicamycin (panel β). Insect cells were infected with WT or rbac-B45 and treated with 0.5 µg/ml of tunicamycin. Forty-eight hours post infection cells were fixed and analyzed for the presence of core and E2 with specific antibodies. Positive cells were scored three times from three independent areas to calculate average. Graph represents percentages of infected cells positive for expression of core and E2.

with rbac-B45 with and without tunicamycin and were studied for the presence of E2 and core. As seen with Western blots (Fig. 1, panel A and C), we observed a loss of E2 in cultures with tunicamycin, when compared with cells cultured without this antibiotic (Fig. 4, panel α, photo F). In contrast, tunicamycin had no influence on core expression (photo C). Figure 4 panel β shows the percentage of infected cells cultured with and without tunicamycin expressing a given protein. The expression of core and E2 in cells cultured without tunicamycin, and also core expression in culture with tunicamycin, reached 30–40% of infected cells. The synthesis of E2 in the presence of tunicamycin was detected in less than 5% of insect cells.

CONCLUSIONS

In this report we have shown that blocking N-glycosylation with tunicamycin results in a loss of the HCV gps E1 and E2 expressed in insect cells. Obviously, due to the disappearance of E1 and E2, the formation of HCV VLPs is blocked. We suggest two scenarios that may take place during the translation of HCV gps in the presence of tunicamycin. First, E1 and E2 synthesis is blocked at the level of extension of the nascent polypeptides, and/or second, the non-glycosylated proteins are directed for rapid degradation. Although we favor the second option, further experiments using proteasome inhibitors are necessary to differentiate between these possibilities.

Our results indicate that tunicamycin has antiviral effects and may block the assembly of HCV. This is consistent with the results of Tyborowska *et al.* (2007) who showed a similar effect of this antibiotic on CSFV gps. The obvious disadvantage of the use of tunicamycin in antiviral therapy is its toxicity. Although we showed that tunicamycin actively blocks E2 production at a concentration at which cell proteins synthesis is not inhibited, we have to take into account that insect cells are less susceptible to tunicamycin treatment than are mammalian cells. Our preliminary results should lead to more detailed studies of tunicamycin and its antiviral effects. Currently, we are testing other derivatives of tunicamycin for their effect in mammalian cells expressing HCV gps, which gives several advantages over insect cell systems because the mammalian cell glycosylation machinery is similar to the one found in human cells which are permissive for HCV growth. Two mammalian cell-culture models (HCV JFH1 strain in Huh7.5 cells, and infectious HCV pseudotyped particles (HCVpp) produced in HEK-293T cells) have already been used to study the replication and infection of HCV in the presence of derivatives of deoxynojirimycin (DNJ) iminosugars which are potent endoplasmic reticulum α-glucosidase inhibitors. Treatment with DNJ derivatives resulted in misfolding of HCV gps, reduced incorporation of E1–E2 complexes into HCVpp and eventually reduced infectivity of HCVpp (Chapel *et al.*, 2007). Such studies have not been performed for tunicamycin yet and we plan to perform such experiments in the future. The results presented in this report suggest that it is likely that tunicamycin may have a similar effect as iminosugars.

In conclusion, we suggest that the development of tunicamycin derivatives and other small-molecule inhibitors of glycosylation that are less toxic than tunicamycin and preferentially block the synthesis of viral glycans, may aid in the discovery of drugs against HCV infection.

Acknowledgements

We thank Dr. Anna Owsianka from the MRC – University of Glasgow Centre for Virus Research, University of Glasgow for her great help in performing the experiments. This work was supported by the Ministry of Science and Higher Education (Poland) grants 2P04B 012 26 and 564/6PR UE/2008/7.

REFERENCES

Bartenschlager R, Lohmann V (2000) Replication of hepatitis C virus. *J Gen Virol* **81:** 1631–1648.

Bassett SE, Guerra B, Brasky K, Miskovsky E, Houghton M, Klimpel GR, Lanford RE (2001) Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* **33:** 1479–1487.

Baumert TF, Ito S, Wong DT, Liang TJ (1998) Hepatitis C virus structural proteins assemble into virus-like particles in insect cells. *J Virol* **72:** 3827–3836.

Baumert TF, Vergalla J, Satoi J, Thomson M, Lechmann M, Herion D, Greenberg HB, Ito S, Liang TJ (1999) Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. *Gastroenterology* **117:** 1397–1407.

Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, Rouille Y (2006) Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* **80:** 6964–6972.

Bowen DG, Walker CM (2005) The origin of quasispecies: cause or consequence of chronic hepatitis C viral infection? *J Hepatol* **42:** 408–417.

Chapel C, Garcia C, Bartosch B, Roingeard P, Zitzmann N, Cosset FL, Dubuisson J, Dwek RA, Trepo C, Zouli F, Durantel D (2007) Reduction of the infectivity of hepatitis C virus pseudoparticles by incorporation of misfolded glycoproteins induced by glucosidase inhibitors. *J Gen Virol* **88:** 1133–1143.

Chen SH, Tan SL (2005) Discovery of small-molecule inhibitors of HCV NS3-4A protease as potential therapeutic agents against HCV infection. *Curr Med Chem* **12:** 2317–2342.

Clayton RF, Owsianka A, Aitken J, Graham S, Bhella D, Patel AH (2002) Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. *J Virol* **76:** 7672–7682.

Dubuisson J, Penin F, Moradpour D (2002) Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol* **12:** 517–523.

Durantel D, Branza-Nichita N, Carrouee-Durantel S, Butters TD, Dwek RA, Zitzmann N (2001) Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. *J Virol* **75:** 8987– 8998.

Elbein AD (1987) Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu Rev Biochem* **56:** 497–534.

Foster GR (2004) Past, present, and future hepatitis C treatments. *Semin Liver Dis* **24:** 97–104.

Goffard A, Dubuisson J (2003) Glycosylation of hepatitis C virus envelope proteins *Biochimie* **85:** 295–301.

Gomez J, Martell M, Quer J, Cabot B, Esteban JI (1999) Hepatitis C viral quasispecies. *J Viral Hepat* **6:** 3–16.

Hahn YS (2003) Subversion of immune responses by hepatitis C virus: immunomodulatory strategies beyond evasion? *Curr Opin Immunol* **15:** 443–449.

Houghton M, Abrignani S (2005) Prospects for a vaccine against the hepatitis C virus. *Nature* **18:** 961–966.

Imperiali B, O'Connor SE, Hendrickson T, Kellenberger (1999) Chemistry and biology of asparagine-linked glycosylation. *Pure Appl Chem* **71:** 777–787.

Jarvis DL, Summers MD (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol Cell Biol* **9:** 214–223.

Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, Nagayama K, Tanaka T, Wakita T (2001) Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* **64:** 334–339.

Kronke J, Kittler R, Buchholz F, Windisch MP, Pietschmann T, Bartenschlager R, Frese M (2004) Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. *J Virol* **78:** 3436–3446.

Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Bös M, Cameron DR, Cartier M, Cordingley MG, Faucher AM, Goudreau N, Kawai SH, Kukolj G, Lagacé L, LaPlante SR, Narjes H, Poupart MA, Rancourt J, Sentjens RE, St George R, Simoneau B, Steinmann G, Thibeault D, Tsantrizos YS, Weldon SM, Yong CL, Llinàs-Brunet M (2003) An NS3 protease inhibitor with anti-

viral effects in humans infected with hepatitis C virus. *Nature* **13:** 186–189.
Lanford RE, Guerra B, Chavez D, Bigger C, Brasky KM, Wang XH, Ray SC, Thomas DL (2004) Cross-genotype immunity to hepatitis C virus. *J Virol* **78:** 1575–1581.
Lin C, Lin K, Luong YP, Rao BG, Wei YY, Brennan DL, Fulghum JR, Hsiao HM, Ma S, Maxwell JP, Cottrell KM, Perni RB, Gates CA, Kwong AD (2004) *In vitro* resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem.* **23:** 17508–17514.
Lindenbach BD, Rice CM (2005) Unravelling hepatitis C virus replication from genome to function. *Nature* **436:** 933–938.
Lu X, Mehta A, Dwek R, Butters T, Block T (1995) Evidence that N-linked glycosylation is necessary for hepatitis B virus secretion. *Virology* **213:** 660–665.
McHutchison JG, Fried MW (2003) Current therapy for hepatitis C**:** pegylated interferon and ribavirin. *Clin Liver Dis* **7:** 149–161.
Meertens L, Bertaux C, Dragic T (2006) Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. *J Virol* **80:** 11571–11578.
Nakhasi HL, Ramanujam M, Atreya CD, Hobman TC, Lee N, Esmaili A, Duncan RC (2001) Rubella virus glycoprotein interaction with the endoplasmic reticulum calreticulin and calnexin. *Arch Virol* **146:** 1–14.
Nevens F, Roskams T, Van Vlierberghe H, Horsmans Y, Sprengers D, Elewaut A, Desmet V, Leroux-Roels G, Quinaux E, Depla E, Dincq S, Vander Stichele C, Maertens G, Hulstaert F (2003) A pilot study of therapeutic vaccination with envelope protein E1 in 35 patients with chronic hepatitis C. *Hepatology* **38:** 1289–1296.
Norrild B, Pedersen B (1982) Effect of tunicamycin on the synthesis of herpes simplex virus type 1 glycoproteins and their expression on the cell surface. *J Virol* **43:** 395–402.
Op De Beeck A, Cocquerel L, Dubuisson J (2001) Biogenesis of hepatitis C virus envelope glycoproteins. *J Gen Virol* **82:** 2589–2595.
Owsianka A, Clayton RF, Loomis-Price LD, McKeating JA, Patel AH (2001) Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. *J Gen Virol* **82:** 1877–1883.
Pearlman B (2004) Hepatitis C treatment update. *Am J Med* **117:** 344–352.
Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* **103:** 7408–7413.
Pizer LI, Cohen GH, Eisenberg RJ (1980) Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J Virol* **34:** 142–153.
Reiser M, Timm J (2009) Serine protease inhibitors as anti-hepatitis C virus agents. *Expert Rev Anti Infect Ther* **7:** 537–547.
Richardson ChD (1995) *Baculovirus expression protocols*; pp 324. Humana Press, Totowa, N.J.
Rychlowska M, Bienkowska-Szewczyk K (2007) Hepatitis C-new developments in the studies of the viral life cycle. *Acta Biochim Pol* **54:** 703–715.
Seeff LB (2002) Natural history of chronic hepatitis C. *Hepatology* **36:** S35–46.
Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* **5:** 558–567.
Simmonds P (2004) Genetic diversity and evolution of hepatitis C virus — 15 years on. *J Gen Virol* **85:** 3173–3188.
Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS (1993) Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* **74:** 2391–2399.
Stohrer R, Hunter E (1979) Inhibition of Rous sarcoma virus replication by 2-deoxyglucose and tunicamycin**:** identification of an unglycosylated *env* gene product. *J Virol* **32:** 412–419.
Strader DB, Wright T, Thomas DL, Seeff LB (2004) Diagnosis, management, and treatment of hepatitis C. *Hepatology* **39:** 1147–1171.
Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV (2002) Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci USA* **99:** 15669–15674.
Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV (2002) Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 99**:** 15661–15668.
Triyatni M, Saunier B, Maruvada P, Davis AR, Ulianich L, Heller T, Patel A, Kohn LD, Jake Liang T (2002) Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. *J Virol* **76:** 9335–9344.
Tscherne DM, Jones CT, Evans MJ, Lindenbach BD, McKeating JA, Rice CM (2006) Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J Virol* **80:** 1734–1741.
Tyborowska J, Reszka N, Kochan G, Szewczyk B (2006) Formation of Pseudorabies virus glycoprotein E/I complex in baculovirus recombinant system. *Acta Virol* **50:**169–74.
Tyborowska J, Zdunek E, Szewczyk B (2007) Effect of N-glycosylation inhibition on the synthesis and processing of classical swine fever virus glycoproteins. *Acta Biochim Pol* **54:** 813–819.
Voisset C, Dubuisson J (2004) Functional hepatitis C virus envelope glycoproteins. *Biol Cell* **96:** 413–420.
von Hahn T, Rice CM (2008) Hepatitis C virus entry. *J Biol Chem* **283:** 3689–3693.
World Health Organization (1997) Hepatitis C**:** global prevalence. *Wkly Epidemiol Rec* **72:** 341–348.
Webb NR, Madoulet C, Tosi PF, Broussard DR, Sneed L, Nicolau C, Summers MD (1989)Cell-surface expression and purification of human CD4 produced in baculovirus-infected insect cells. *Proc Natl Acad Sci USA* **86:** 7731–7735
Yanagi M, Purcell RH, Emerson SU, Bukh J (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci USA* **94:** 8738–8743.