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The use of AlphaLISA assay technology to detect interaction between hepatitis C virus-encoded NS5A and cyclophilin A.

Helen Waller¹, Udayan Chatterji², Philippe Gallay², Tanya Parkinson¹, and Paul Targett-Adams^{1,*}

¹Pfizer Global Research & Development, Antivirals Research Unit, Sandwich Laboratories, Sandwich, Kent, CT13 9NJ, UK.

²Department of Immunology and Microbial Science, IMM-9, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA.

Abstract

Cyclosporine A (CsA) is an immunosuppressive molecule that also impedes replication of hepatitis C virus (HCV). CsA inhibits isomerase activity of cellular-encoded cyclophilin proteins, of which cyclophilin A (CypA) in particular is required for HCV replication. Evidence suggests that the HCV-encoded NS5A and NS5B proteins may govern dependence of the virus on CypA-mediated isomerase activity, although the molecular mechanisms involved are unclear. However, association of NS5A, and NS5B, with CypA has been reported, raising the possibility that direct interaction between these proteins facilitates HCV replication. In the present study, mammalian two-hybrid and AlphaLISA assay technologies were utilized to detect interactions between NS5A, and NS5B, with CypA. AlphaLISA assays revealed associations between NS5A and CypA using purified proteins, and in cell lysates prepared from co-transfected cells. Importantly, the NS5A-CypA interactions were sensitive to CsA in a dose-responsive manner and an isomerase mutant of CypA interacted with NS5A less efficiently than wild type CypA. These findings correlate the anti-HCV properties of CsA with an ability of the compound to disrupt NS5A-CypA interactions in vitro and in vivo, whilst providing the basis for development of assay platforms suitable to screen compound libraries for novel inhibitors of the NS5A-CypA interaction.

Keywords

Hepatitis C virus; NS5A; Cyclophilin A; Cyclosporine A; Interaction; AlphaLISA

Introduction:

Hepatitis C virus (HCV) is a global health burden and a primary causative agent of chronic liver diseases including liver cirrhosis and hepatocellular carcinoma (Pawlotsky, 2006). Current treatment regimens focus on combination therapy using interferon alpha and ribavirin but are not universally effective and often associated with serious side effects

*Corresponding author: Paul Targett-Adams, Pfizer Global Research & Development, Antivirals Research Unit (IPC424), Sandwich Laboratories, Sandwich, Kent, CT13 9NJ, UK. Phone: +44 (0)1304 648416. Fax: +44 (0)1304 651819. paul.targett-adams@pfizer.com.

(Hayashi and Takehara, 2006). Thus, there is an urgent unmet medical need for the development of specific and safe anti-HCV therapies.

Cyclosporine A (CsA) is an immunosuppressive drug that also exhibits clinical efficacy against HCV (Akiyama et al., 1997; Inoue et al., 2003; Inoue and Yoshida, 2005). Additional studies have further demonstrated that CsA also inhibits HCV RNA replication in vitro (Ishii et al., 2006; Nakagawa et al., 2004; Watashi et al., 2003), and that non-immunosuppressive CsA derivatives display anti-HCV properties both in vitro (Coelmont et al., 2009; Goto et al., 2006; Ma et al., 2006; Mathy et al., 2008; Paeshuyse et al., 2006) and in vivo (Flisiak et al., 2009; Flisiak et al., 2008). Unlike CsA, these molecules do not display calcineurin affinity and specifically inhibit the peptidylprolyl cis-trans isomerase (PPIase) activity of the cyclophilin (Cyp) proteins (Gallay, 2009). Inhibition of the Cyp proteins is believed to account for the anti-HCV activity of CsA and its analogues, suggesting one/more of the Cyp family of proteins are required by HCV for replication of its genome and, in this context, roles for CypA, B, and C have been proposed (Nakagawa et al., 2004; Watashi et al., 2005; Yang et al., 2008). However, findings from recent studies have indicated that inhibition of CypA PPIase activity is primarily responsible for the anti-HCV activity of CsA and its analogues (Chatterji et al., 2009; Kaul et al., 2009; Liu et al., 2009). Thus, small molecule-mediated inhibition of CypA could form the basis for development of novel anti-HCV therapies. The exact role of Cyps in the HCV life cycle remain to be elucidated but current hypotheses suggest that Cyps, through interaction with NS5B (the virus encoded RNA-dependant RNA polymerase), increase the affinity of the polymerase for the viral RNA to enhance levels of genome synthesis (Rice and You, 2005; Yang et al., 2008). HCV resistance to CsA is linked to the emergence of resistance mutations that map to the HCV NS5B and NS5A open reading frames (ORFs) (Fernandes et al., 2007; Robida et al., 2007), highlighting a genetic link between the antiviral properties of CsA and both proteins. Additionally, NMR spectroscopy has revealed a direct interaction between domain 2 of NS5A and CypA and B (Hanouille et al., 2009); further supporting the notion that the requirement of HCV for Cyps may also involve the NS5A protein. The precise roles of NS5A in the HCV life cycle are unknown and are likely related to its numerous reported biological properties (Huang et al., 2005; Huang et al., 2007; Macdonald and Harris, 2004; Penin et al., 2004). NS5A is, however, essential for genome replication of the virus (Lindenbach and Rice, 2005; Moradpour et al., 2007), and can also influence virion morphogenesis (Appel et al., 2008; Hughes et al., 2009; Tellinghuisen et al., 2008). A greater understanding of the NS5A-CypA interaction would undoubtedly lead to an enhanced comprehension of the NS5A role in the HCV life cycle.

Thus, the aim of the current study was to investigate potential for the application of readily available assay systems, designed for convenient evaluation of protein-protein interactions, to detect the association of NS5A with CypA. The purpose of this approach was *i)* to develop assays whereby the molecular mechanisms involved in the interaction between NS5A and CypA could be probed to increase our understanding of the nature and significance of the association and *ii)* to identify the basis for a future assay platform that could be used to identify new/different inhibitors of the NS5A-CypA interaction.

2. Materials and Methods:

2.1 Plasmid construction:

2.1.1 Plasmids for mammalian-2-hybrid analysis: ORFs encoding the HCV non-structural (NS) proteins NS3, NS4A, NS5A, and NS5B were generated by PCR amplification from HCV genotype 1b (con1 strain) cDNA and subcloned into pACT and pBIND plasmids (Checkmate Mammalian Two-Hybrid System; Promega) to give pACT-NS3, pBIND-NS3 etc. Constructs pACT-CypA and pBIND-CypA were generated by PCR amplification using template DNA pcDNA3.1/Hygro(+)-CypA_{WT}-HA (Chatterji et al., 2009), followed by ligation of the resulting CypA ORF with pACT or pBIND. The nucleotide sequence of all constructs generated was verified prior to use.

2.1.2 Plasmids for AlphaLISA analysis of transfected cell lysates: HCV NS protein ORFs were generated as described in section 2.1.1 and subcloned into pFLAG-CMV-5.1 (Sigma). Specific reverse primer design resulted in the generation of expression constructs that encoded the HCV NS proteins His-tagged at their C-termini; plasmids were termed pCMV-NS3-His, pCMV-NS5A-His etc. Plasmids pcDNA3.1/Hygro(+)-CypA_{WT}-HA and pcDNA3.1/Hygro(+)-CypA_{H126Q}-HA were used to express C-terminally HA-tagged wild type (WT) CypA, and CypA H126Q species respectively, as described previously (Chatterji et al., 2009). The nucleotide sequence of all constructs generated was verified prior to use.

2.2 Cell culture and transfection:

CHO cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1x penicillin/streptomycin, and 1x glutamine. For transfection, cells seeded in either 24-well (for mammalian 2-hybrid [M2H] analysis) or 6-well (for AlphaLISA assays) plates were transfected with appropriate plasmid DNAs using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Where appropriate, CsA (Calbiochem) prepared in 100% DMSO was added to medium to achieve the desired molarity with a final DMSO concentration of 1% v/v.

2.3 Protein purification:

To express C-terminally His-tagged NS5A protein in bacteria, an ORF corresponding to amino acids 25–447 of genotype 1b NS5A (strain con1) was amplified by PCR and subcloned into pET30b to generate pET30b-UbY-NS5A(25–447)His6 Clone 263C. NS5A expression was induced in BL21(DE3) bacteria using IPTG and His-tagged protein was purified by affinity chromatography using His-Select resin (Sigma) followed by S-200 size exclusion chromatography. Recombinant GST-CypA protein was produced and purified as described previously (Saphire et al., 1999). GSTCypA H126Q was created by PCR mutagenesis from the wild-type GST-CypA bacterial expression construct and protein was purified in an analogous manner.

2.4 Mammalian 2-hybrid analysis:

Evaluation of intracellular protein-protein interactions using 2-hybrid screening technology was accomplished using the 'Checkmate Mammalian Two-Hybrid System' according to the

manufacturer's instructions (Promega). Briefly, pACT- and pBIND-based plasmids were cotransfected (using Lipofectamine 2000) with the pG5*luc* reporter construct into mammalian (CHO or Huh-7) cells and incubated for 37°C. After 48 hours, cell lysates were prepared and assayed for luciferase activity using the 'Steady-Glo Luciferase Assay System' according to the manufacturer's instructions (Promega).

2.5 In vitro transcription/translation:

Coupled in vitro transcription/translation (IVT/T) was performed using the 'TnT High Yield Wheat Germ Protein Expression System' according to the manufacturer's instructions (Promega). The system was primed with DNA fragments generated by PCR amplification of pCMV-NS5A-His and pcDNA3.1/Hygro(+)-CypA_{WT}-HA using primers designed to insert a SP6 promoter sequence at the 5' end of the amplicons while maintaining the NS5A 3' polyhistidine tag and inserting a 3' FLAG sequence onto the CypA ORF. The resulting PCR fragments, encoding NS5A-His and CypA-FLAG, were gel purified prior to use in IVT/T reactions.

2.5 AlphaLISA assays:

2.5.1 Using IVT/T reactions: Samples (9µl each) of NS5A-His and CypA-FLAG IVT/T reactions were mixed and incubated together for 2 hours at 25°C in a 384-well AlphaPlate (Perkin Elmer). For controls, 18µl of blank IVT/T was used or 9µl of NS5A-His / CypA-FLAG IVT/T was combined with 9µl blank IVT/T. Following incubation, 6µl of 100ng/ml Anti-FLAG AlphaLISA Acceptor beads (Perkin Elmer) were added to each well and incubation was continued. After 1 hour, 6µl of 100ng/ml Nickel Chelate Alpha Donor beads (Perkin Elmer) were added to the reactions and incubation was allowed to continue for another hour. Plates were then read on a PE Envision plate reader using an excitation wavelength of 680nm and emission detection was set at 615nm.

2.5.2 Using purified proteins: Purified NS5A-His and GST-CypA proteins were diluted in Protein Buffer (100mM HEPES pH7.5, 1mM EDTA, 5mM DTT, 0.1% CHAPS, 5% glycerol) and 9µl of each were combined in wells of a 384-well AlphaPlate (to give a final assay concentration of 150nM each). Where appropriate, CsA (prepared in 100% DMSO) was added to the reactions to achieve the desired molarity with a final DMSO concentration of 1% v/v. Reactions were subsequently incubated for 2 hours with agitation. Concomitantly, 5µl of 5mg/ml AlphaLISA Protein G Acceptor beads (Perkin Elmer) were incubated with 15µl of 1mg/ml mouse anti-NS5A monoclonal antibody (Meridian Life Science). The antibody was allowed to bind to the beads for 2 hours followed by bead retrieval using low-speed centrifugation. Beads were washed and resuspended in Bead Buffer (20mM HEPES pH7.5, 125mM NaCl, 0.1% BSA, 0.1% CHAPS) to 100ng/ml; 6µl was then added to each well containing the purified proteins. Following incubation for a further hour, 6µl of 100ng/ml Glutathione Alpha Donor beads (Perkin Elmer) were added to the reactions, which were incubated for a final hour with agitation. Plates were read as described in section 2.5.1.

2.5.3 Using transfected cell lysates: CHO cells were seeded into 6-well plates. The next day, cells were transfected with plasmids encoding His-tagged HCV NS proteins and

HA-tagged CypA species (plasmids described in section 2.1.2) using Lipofectamine 2000 (section 2.2). Following transfection, fresh tissue culture medium containing CsA diluted to appropriate molarity was added to the cells (final DMSO concentration of 1% v/v). Control transfections received medium containing 1% DMSO only. The following day, the cells were washed in PBS and lysed in 100µl Lysis Buffer (1% Triton X-100, 200mM Tris-HCl pH 7.5, 50mM NaCl) per well. The cell lysates were incubated for 30 minutes on ice and then centrifuged at 13.5K in a microcentrifuge for 5 minutes at 4°C. The resulting supernatant was transferred to a fresh tube and used for AlphaLISA analysis; 18µl was transferred to each well and 6µl of 100ng/ml Anti-HA AlphaLISA Acceptor beads (PerkinElmer) were added. Plates were incubated for 1 hour with agitation followed by the addition of 6µl Nickel Chelate Alpha Donor beads (100ng/ml) to each well. Incubation continued for a further hour and plates were read as described in section 2.5.1.

2.6 Indirect Immunofluorescence:

Cells, grown on 13mm glass coverslips, were processed for observation as described previously (Targett-Adams et al., 2008) and images were captured using a Leica TCS SP5 inverted confocal microscope and associated software. Antibodies specific for NS5A and CypA were purchased from Meridian Life Science and Abcam respectively.

3. Results:

3.1 Mammalian 2-hybrid analysis of NS5A/NS5B-CypA interactions:

Two-hybrid systems represent powerful methods for detecting protein-protein interactions in vivo. Originally developed in yeast (Chien et al., 1991; Fields and Song, 1989), the technology has been adapted for use in mammalian cells (Dang et al., 1991; Fearon et al., 1992). The principles for M2H analysis remain identical to those of the yeast system (Fig. 1A); ‘bait’ consists of a protein of interest fused to the GAL4 DNA-binding domain (expressed from the pBIND plasmid) and ‘prey’ is composed of a second protein of interest fused to the VP16 transcriptional activation domain (encoded by the pACT vector). Interaction between the two proteins of interest bring the GAL4 and VP16 domains into close proximity to create a functional transcriptional unit (Sadowski et al., 1988), which subsequently drives transcription of the firefly luciferase gene from a reporter plasmid (pG5*luc*) (Fig. 1A). Thus, following co-transfection of all 3 plasmid species into mammalian cells, luciferase activity provides a quantitative measure of the propensity of the two proteins of interest to interact with each other.

M2H constructs, designed to analyse interactions between NS5A/CypA and NS5B/CypA, were generated and transfected into CHO cells (Fig. 1B). Constructs designed to reveal the previously established interaction between HCV-encoded NS3 (the viral helicase/protease) and NS4A (the NS3 cofactor) served as a positive control (Bartenschlager et al., 1994; Failla et al., 1995). Transfection of pACT-NS3 and pBIND-NS4A resulted in the production of over 10 000 relative light units (RLU); a 12-fold increase over RLU generated from the negative control (pACT/pBIND) (Fig 1.B). These findings indicated that the M2H assay was capable of detecting interactions between HCV-encoded proteins. However, transfection with another set of positive control plasmids (pACT-MyoD and pBIND-ID; supplied with

the M2H system) produced RLU of over 120 000 (data not shown). Therefore, even established and robust interactions involving HCV proteins only produced a signal corresponding to approximately 8% that of an 'ideal' positive control using the M2H system. Transfection of pACT-NS5A/pBIND-CypA and pACT-NS5B/pBIND-CypA resulted in RLU of 4.4- and 6-fold higher than those resulting from transfection of pACT/pBIND alone, and indicated detection of NS5A/NS5B-CypA interactions (closed arrows, Fig. 1B). However, control reactions revealed VP16 fusion proteins generated elevated background levels; probably as a result of low-level, non-specific interaction of NS5A and NS5B with the GAL4 domain (open arrows, Fig. 1B). Therefore, the window of specific interaction detected between NS5A-CypA and NS5B-CypA was 2.9- and 2.6-fold respectively (Fig. 1B). Interestingly, the NS5A/NS5B-CypA interactions displayed vector directionality (Finkel et al., 1993), and positive results were detected only when the HCV proteins were fused to the VP16 domain, and not when fused to the GAL4 domain (Fig. 1B). The low RLU produced when using the M2H system to detect NS5A/NS5B-CypA interactions were likely due to the requirement for interactions to occur in nuclei in order to stimulate transcription of the luciferase reporter gene. However, HCV-encoded proteins are bound to the membrane of the endoplasmic reticulum in the cytoplasm (Moradpour et al., 2007). Therefore, weak/low-level interactions may have been a consequence of insufficient quantities of fusion proteins in nuclei. In support of this hypothesis; pACT-NS5A-transfected CHO cells were analysed using confocal microscopy, which revealed co-localization of NS5A and CypA in the cytoplasm (arrows, Fig. 1C), with only minor levels of NS5A observed in nuclei (Fig. 1C). Removal of the NS5A and NS5B membrane-targeting sequences did not result in an appreciable increase in RLU in the M2H assay (data not shown), suggesting that the low RLU initially observed were probably due to a reduced capacity of nuclear-localized fusion proteins to adopt appropriate conformations required for interaction rather than from an inability of the fusion proteins to localize to nuclei per se. Therefore, in light of these limitations, alternative methods to examine the NS5A-CypA interaction were sought.

3.2 Use of AlphaLISA assay technology to detect interaction between NS5A and CypA proteins:

Amplified luminescent proximity homogeneous assay (Alpha) technology is a Perkin Elmer proprietary bead-based assay format designed to measure a wide range of biological molecules and interactions in custom or high-throughput formats. AlphaLISA assays rely on simple principles involving matching Alpha Donor and Acceptor beads. The beads are approximately 250nm in diameter and are coated with a layer of hydrogel to promote bioconjugation of different functional groups e.g. Glutathione or specific antibodies (Sehr et al., 2007). A biological reaction of interest brings specific assay-tailored Alpha Donor and Acceptor beads into close proximity and laser excitation excites a photosensitizer inside the Donor bead, which converts oxygen from ambient to singlet state (Fig. 2A). The singlet state oxygen molecules diffuse to produce a chemiluminescent reaction in the nearby Acceptor bead, leading to light emission (Fig. 2A). In the absence of a specific biological interaction, singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead.

To evaluate the potential application of Alpha technology to detect NS5A-CypA interactions, IVT/T reactions were used to produce FLAG-tagged CypA and His-tagged NS5A. IVT/T lysates were examined for the presence of relevant proteins (using Western analysis; data not shown) and Nickel Chelate Alpha Donor/Anti-FLAG AlphaLISA Acceptor beads were used to probe for specific interaction between NS5A and CypA (Fig. 2B). Control reactions (mock, CypA, NS5A) produced RLU of below 700, whereas incubation of CypA-FLAG- and NS5A-His-containing lysates generated RLU 2.3-fold higher (Fig. 2B). Little variation was seen between replicates (Fig. 2B). These observations suggested that interactions between CypA and NS5A were responsible for the elevated RLU generated in the presence of both protein species. To confirm these findings, the potential for interaction between purified GST-CypA and NS5A-His was assessed using the AlphaLISA assay format (Fig. 2C). Compared to buffer- or NS5A-His-only controls, higher RLU were produced in AlphaLISA assays containing only purified GST-CypA; probably as a consequence of low-level cross-reactivity with the NS5A monoclonal antibody attached to the Acceptor beads (Fig. 2C). However, incubation of purified GST-CypA with NS5A-His generated robust RLU of over 13 000; 2.9-fold higher than those obtained using GST-CypA alone (Fig. 2C). These findings confirmed those obtained using IVT/T reactions; AlphaLISA assay technology detected interaction between NS5A and CypA. As an additional measure, a mutant species of CypA (H126Q) was also included in the AlphaLISA analysis of interactions between purified protein species (Fig. 2C). The H126Q mutation abrogates the isomerase activity of CypA, which is required by HCV for replication of its genome (Chatterji et al., 2009; Zydowsky et al., 1992). RLU generated following incubation of GST-CypA H126Q with NS5A-His were 2.7-fold higher than those observed using GST-CypA H126Q alone (Fig. 2C). However, in the presence of NS5A, the signal obtained with the mutant CypA was only 45% of that seen with WT CypA (Fig. 2C). These findings suggested that while interaction between mutant CypA and NS5A was detected, it was not as efficient as that of WT CypA-NS5A. Importantly, these results demonstrated that the requirement for CypA PPIase activity by HCV correlated with the capacity of CypA to bind NS5A.

3.3 AlphaLISA-measured CsA inhibition of the NS5A-CypA interaction using purified proteins:

CsA inhibits HCV replication in tissue culture cells and exhibits an IC_{50} of 100–400nM (Ishii et al., 2006; Nakagawa et al., 2004; Robida et al., 2007). To determine whether the anti-HCV properties of CsA correlated with a capacity of the molecule to block the in vitro NS5A-CypA interaction, the NS5A-CypA AlphaLISA interaction assay was performed in the presence of CsA using WT and mutant CypA (Fig. 3A). The addition of 10 μ M CsA to the NS5A-WT CypA reaction reduced the RLU generated in the AlphaLISA assay by 65%; in fact, values were reduced to those obtained using WT GST-CypA alone and indicated near total inhibition of the NS5A-WT CypA interaction was achieved using 10 μ M CsA (Fig. 3A). A subsequent three-fold stepwise dilution of CsA added to the NS5A-WT CypA reaction resulted in alleviation of the inhibition in a dose-responsive manner (Fig. 3A). The addition of CsA to the GST-CypA H126Q-NS5A reaction had no effect on the RLU produced in the AlphaLISA assay indicating that the low-level interaction detected between NS5A and the mutant CypA was not sensitive to CsA (Fig. 3A). Use of the dose-response data described in Fig. 3A to calculate an IC_{50} for CsA in the in vitro assay generated a value

of 132nM (Fig. 3B), which is within the range of published values for CsA-mediated inhibition of HCV replication in tissue culture cells (Ishii et al., 2006; Nakagawa et al., 2004; Robida et al., 2007). Taken together, these findings highlight a correlation between the biological property of CsA to inhibit HCV propagation in vivo and the capacity of the molecule to inhibit the NS5A-CypA interaction in vitro. Moreover, these results reveal that a functional CypA isomerase active site is required for efficient association of CypA with NS5A.

3.4 AlphaLISA-mediated detection of NS5A-CypA interactions in transfected cells:

Having established that the AlphaLISA assay reliably detected interaction between NS5A and CypA in vitro, the capacity of the technology to measure the NS5A-CypA interaction in cells was ascertained. Accordingly, lysates prepared from CHO cells transfected with plasmids expressing CypA-FLAG and His-tagged HCV NS proteins were probed using appropriate AlphaLISA Donor/Acceptor beads (Fig. 4A). Results indicated that, consistent with previous findings; higher RLU were observed in lysates prepared from cells containing NS5A-His or NS5B-His, together with CypA-FLAG, compared to lysates containing CypA-FLAG alone (Fig. 4A). However, although little variation was seen between replicates, the window of specific interaction seen above the CypA-FLAG control was low; 1.7- and 1.1-fold for NS5A-His/CypA-FLAG and NS5B-His/CypA-FLAG respectively (Fig. 4A). This was probably a consequence of the relatively low proportion (<5%, data not shown) of cells that were received both plasmid species following co-transfection procedures. However, despite the low RLU, the intracellular interaction between NS5A-His and CypA-FLAG was sensitive to CsA (Fig. 4B). RLU generated from NS5A-His/WT CypA cotransfected cells decreased in a dose-responsive manner following treatment with CsA (Fig. 4B). WST-1 cellular toxicity assays revealed use of CsA above 3 μ M was detrimental to the health of the cells (data not shown), thus, CsA treatment of cells did not exceed this concentration. Nevertheless, 3 μ M CsA was sufficient to reduce the RLU, produced following AlphaLISA analysis of cell lysates containing NS5A-His and CypA-FLAG, to background levels (Fig. 4B). In agreement with results described in section 3.3, a low-level of interaction between NS5A and CypA H126Q was detected, which was not sensitive to CsA treatment (Fig. 4B). RLU values indicated that, as expected, no interaction was formed between NS3-His and WT CypA or CypA H126Q (Fig. 4B). Dose-response data from a separate experiment were used to calculate an IC₅₀ of 141nM for CsA in the cell-based AlphaLISA NS5A-WT CypA interaction assay (Fig. 4C); consistent with the IC₅₀ value obtained in the in vitro assay (section 3.3) and published data (Ishii et al., 2006; Nakagawa et al., 2004; Robida et al., 2007).

In summary, the capacity of M2H and Alpha technology-based assays to detect protein-protein interactions, primarily involving HCV NS5A and CypA, was assessed by the present study. Taken together, results indicated that AlphaLISA assay technology provided a more suitable platform to examine the NS5A-CypA association, both in vitro; using purified proteins, and in vivo; using transfected cell lysates. Furthermore, the use of AlphaLISA assays to examine the NS5A-CypA interaction provided insight into the potential mechanisms related to the anti-HCV properties of CsA.

4. Discussion:

Protein-protein interactions are central to the vast majority of biological processes that occur within cells and the identification and understanding of specific protein-protein interactions has the capacity to greatly further knowledge of particular molecular mechanisms. Alteration of protein-protein interactions are known contributory factors for many diseases; therefore, the understanding, and manipulation, of interactions that contribute to disease may represent potential strategies for therapeutic interventions. Many viruses, including HCV, are heavily dependant on host cell machinery in order to produce progeny virus. Consequently, identification of interactions between virus-encoded proteins and host cell factors can provide valuable insight into the means through which host cell metabolism is parasitized by viruses. Yeast two-hybrid technology has been successfully used to identify and characterize a plethora of different protein-protein interactions; indeed, interactions between HCV-encoded NS proteins have also been identified in this manner (Dimitrova et al., 2003). However, yeast two-hybrid analysis is not a suitable basis for convenient and routine detection of protein-protein interactions with a view to establishing medium- to high-throughput screening platforms for small molecule libraries. To this end, the current study selected the M2H system for evaluation of the NS5A-CypA interaction since the assay end-point is luciferase activity and M2H protocols can be executed with relative ease compared to analogous yeast-based approaches. Furthermore, M2H systems have been used previously to identify and examine interactions involving HCV- and cellular-encoded proteins (Chen et al., 2009; Ghosh et al., 2003; Ghosh et al., 2000; Itsui et al., 2009; Majumder et al., 2001). In the present study, positive interaction between NS5A (and NS5B) with CypA was detected using the M2H assay. However, the assay window resulting from specific interaction was low. Indeed, the poor assay window resulted in ambiguous data when attempting to examine the effects of CsA upon the NS5A-CypA interaction using the M2H system (data not shown). Therefore, analysis of NS5A-CypA interaction by the M2H system did not produce data robust enough for detailed analysis into the nature of the interaction. This was probably a consequence of the relatively low proportion of cells receiving all 3 plasmid species by transfection and the reduced ability of the fusion proteins to adopt specific conformations required for interaction in nuclei of transfected cells. Thus, the suitability of additional assays to examine protein-protein interactions was subsequently investigated.

The Alpha-based assay system is a simple, convenient, high throughput-ready screening tool that can be used to search for inhibitors of specific protein-protein interactions in vitro. For example, inhibitors of the human immunodeficiency virus-encoded integrase interaction with cellular LEDGF (Hou et al., 2008), the human papillomavirus-encoded E6-E6AP interaction (Sehr et al., 2007), and the HSP90-cochaperone interaction (Yi and Regan, 2008; Yi et al., 2009), have been identified using Alpha technology. Consequently, the current study assessed the use of Alpha-based technology for the capacity to examine the interaction between HCV NS5A and CypA. Of the three conditions chosen for analysis (IVT/T, purified protein, and transfected cell lysates), results obtained using purified proteins were more robust; as judged by generation of highest RLU. Since the AlphaLISA assay signal is proportional to the number of interaction events occurring between two interaction partners, the high RLU observed using purified proteins presumably resulted from increased amount

of NS5A and CypA present in the reactions compared to IVT/T samples and transfected cell lysates. Additionally, the lack of contaminating proteins present in purified protein preparations compared to those derived from IVT/T reactions or transfected cell lysates may have also contributed to the generation of high RLU. Nevertheless, specific interaction between NS5A and CypA was also detected in lysates generated from transfected cells and provided confidence that interactions demonstrated using purified proteins were physiologically relevant. Moreover, the present study revealed that the AlphaLISA-based NS5A-CypA interaction assays were sensitive to CsA. This finding was important for two reasons; firstly, evidence for compound-mediated inhibition of the interaction indicated that the assays could be suitable for medium-high throughput screening of small molecule libraries. The high assay signal and relatively technical simplicity of AlphaLISA set-up using purified proteins make this approach particularly attractive for adaptation to automated screening protocols. Secondly, inhibition of the NS5A-CypA interaction by CsA suggested a potential mode of action for the anti-HCV properties of CsA.

CsA is a molecule that suppresses the isomerase activity of cellular cyclophilin proteins; the activity of one such family member, CypA, is required for HCV propagation in tissue culture (Chatterji et al., 2009; Kaul et al., 2009; Liu et al., 2009). Since HCV is dependent on CypA activity, inhibition of CypA PPIase by CsA impedes HCV replication in tissue culture (Ishii et al., 2006; Nakagawa et al., 2004; Watashi et al., 2003), and in patients (Akiyama et al., 1997; Inoue et al., 2003; Inoue and Yoshida, 2005). The molecular mechanisms that serve to govern the dependence of HCV on CypA activity have been linked to HCV-encoded proteins NS5A (Goto et al., 2009; Kaul et al., 2009), NS5B (Fernandes et al., 2007; Rice and You, 2005; Robida et al., 2007; Watashi et al., 2005; Yang et al., 2008), and NS2 (Ciesek et al., 2009). The emergence of resistance mutations in NS5A following prolonged exposure of replicating HCV genomes to CsA provides genetic evidence implicating NS5A in CsA mode of action (Fernandes et al., 2007; Goto et al., 2009; Kaul et al., 2009). Findings from the current study now contribute biochemical evidence in support this hypothesis. Specifically, interaction between NS5A and CypA, as revealed by AlphaLISA, was inhibited by CsA in a dose responsive manner and exhibited IC_{50} values in accordance with published values for CsA-mediated inhibition of HCV replication in tissue culture cells (Ishii et al., 2006; Nakagawa et al., 2004; Robida et al., 2007). Hence, results from the present study suggest inhibition of the NS5A-CypA interaction by CsA could account for the anti-HCV properties of the molecule. Exactly how the NS5A-CypA interaction serves to enhance HCV replication is unclear at present; it's possible that CypA binding to NS5A modulates the association of NS5A with the HCV polymerase, NS5B (Gallay, 2009), thereby regulating activity of the NS5B polymerase complex. Alternatively, CypA association with NS5A may be required for NS5A to accomplish one of its many reported biological functions e.g. binding to HCV RNA (Huang et al., 2005). However, it's clear that NS5B also serves a role in the requirement of HCV for CypA since CsA resistance mutations also occur in NS5B (Fernandes et al., 2007; Robida et al., 2007), and interactions between CypA and NS5B have been reported (Chatterji et al., 2009; Yang et al., 2008). There probably exists complex interplay between NS5A, NS5B, and CypA that is required by HCV for one/more stage(s) in its life cycle. Dissecting the mechanisms through which this requirement is realised will be a future challenge.

This study demonstrated an isomerase mutant of CypA did not bind NS5A as efficiently as WT CypA and that, in contrast to the NS5A-WT CypA interaction, binding of mutant CypA to NS5A was not sensitive to CsA. These findings suggested that a functional CypA isomerase active site is required for efficient binding to NS5A and that altering the active site, through mutagenesis or pharmacological inhibition, reduced the ability of CypA to associate with NS5A. The amino acids of CypA responsible for PPIase activity are located in a hydrophobic pocket. The residues within this hydrophobic pocket are also required for interaction with the human immunodeficiency virus Gag polyprotein (Franke et al., 1994; Thali et al., 1994), and for mediating association with the HCV NS5B protein (Chatterji et al., 2009; Yang et al., 2008). Therefore, it is possible that CypA PPIase activity is required to isomerase peptidyl-prolyl bonds within certain virus-encoded proteins. Alternatively, the NS5A/NS5B binding region of CypA may only be coincident with the isomerase active site. More in-depth studies will determine the exact role of CypA PPIase activity during HCV replication.

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Fig. 1A **Fig. 1B** **Fig. 1C** **Figure 1 - Mammalian 2-hybrid analysis of NS5A/NS5B-CypA interactions.**

(A) Schematic representation of the mammalian 2-hybrid system. ‘Protein A’ is expressed as a fusion with the GAL4 DNA-binding domain, and ‘Protein B’ is fused to the VP16 activation domain. Interaction of Proteins ‘A’ and ‘B’ bring the GAL4 and VP16 domains into close proximity, which activate transcription of the luciferase reporter gene located downstream from GAL4 and TATA sequences. (B) Two-hybrid analysis of NS5A/NS5A-CypA interactions in mammalian cells. Reporter plasmid pG5/luc was transfected into CHO cells together with plasmid combinations indicated by the ‘x’ axis. Luciferase activity in subsequent cell lysates is expressed as relative light units (RLU) by the ‘y’ axis. Representative data from at least 3 independent repeats are presented as mean RLU +/- standard error of the mean (SEM) calculated from 3 replicates. Open arrows illustrate background levels seen with VP16 fused to NS5A/NS5B; closed arrows highlight RLU generated from VP16-NS5A/NS5B interactions with GAL4-CypA. Mock refers to blank

transfection. (C) Indirect immunofluorescence analysis of pACT-NS5A-transfected CHO cells; white arrows indicate co-localization of VP16-NS5A and CypA in the cytoplasm.

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Fig. 2A **Fig. 2B** **Fig. 2C** **Figure 2 - AlphaLISA assay technology identifies interaction between NS5A and CypA.**

(A) Alpha assay technology utilizes specific Donor and Acceptor beads and a fluorescence output to reveal protein-protein interactions (see section 3.2). (B) AlphaLISA assessment of the potential to detect interaction between NS5A-His and CypA-FLAG produced by coupled in vitro transcription/translation (IVT/T). Mock refers to blank IVT/T i.e. no DNA added to the reaction. (C) AlphaLISA detection of interaction between purified NS5A-His and GST-CypA WT / GSTCypA H126Q proteins. Reactions were carried out as described in section 2.5.2. Representative data from at least 3 independent repeats are presented as mean RLU +/- SEM calculated from a minimum of 3 replicates.

Fig. 3A

Fig. 3B

Figure 3 - In vitro interaction between NS5A and CypA is sensitive to CsA.

(A) NS5A-His and GST-CypA WT or GST-CypA H126Q proteins were incubated together with varying concentrations of CsA followed by analysis with specific Alpha Donor/Acceptor beads (see section 2.5.2). Data are presented as mean RLU $\pm$ SEM calculated from a minimum of 3 replicates. Representative data from at least 3 repeats are shown. (B) The IC₅₀ of CsA in the in vitro NS5ACypA AlphaLISA interaction assay was calculated using LabStats add-in for Microsoft Excel and data described in (A).

Fig. 4A

Fig. 4B

Fig. 4C

Figure 4 - Detection of NS5A-CypA interactions in transfected CHO cell lysates using AlphaLISA.

(A) and (B) Cell lysates prepared from CHO cells transfected with combinations of pcDNA3.1/Hygro(+)-CypA_{WT}-HA, pcDNA3.1/Hygro(+)-CypA_{H126Q}-HA, pCMV-NS5A-His, pCMV-NS5B-His, and pCMV-NS3-His, were assessed for the presence of specific protein complexes using AlphaLISA. CsA was included where indicated and mock refers to blank transfection. Data are presented as mean RLU $\pm$ SEM calculated from a minimum of 3 replicates and representative data from at least 3 repeats are shown. (C) Results from a similar experiment to (B) were used to calculate the IC₅₀ of CsA in the cell-based NS5A-CypA AlphaLISA interaction assay.