Albecka, A., Belouzard, S., De Beeck, A. O., Descamps, V., Goueslain, L., Bertrand-Michel, J., Terce, F., Duverlie, G., Rouille, Y., and Dubuisson, J. (2012). Role of LDL receptor in the hepatitis c virus life cycle. *Hepatology*. doi: 10.1002/hep.25501

Allain, F., Denys, A., and Spik, G. (1994). Characterization of surface binding sites for cyclophilin B on a human tumor T-cell line. *J. Biol. Chem.* 269, 16537–16540.

Amako, Y., Sarkeshik, A., Hotta, H., Yates, J. III, and Siddiqui, A. (2009). Role of oxysterol binding protein in hepatitis C virus infection. *J. Virol.* 83, 9237–9246.

Amako, Y., Syed, G. H., and Siddiqui, A. (2011). Protein kinase D negatively regulates hepatitis C virus secretion through phosphorylation of oxysterol-binding protein and ceramide transfer protein. *J. Biol. Chem.* 286, 11265–11274.

Anderson, L. J., Lin, K., Compton, T., and Wiedmann, B. (2011). Inhibition of cyclophilins alters lipid trafficking and blocks hepatitis C virus secretion. *Virol. J.* 8, 329.

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J. L., Sodoyer, M., Pol, S., Brechot, C., Paranhos-Baccala, G., and Lotteau, V. (2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J. Virol.* 76, 6919–6928.

Banerji, S., Ngo, M., Lane, C. F., Robinson, C. A., Minogue, S., and Ridgway, N. D. (2010). Oxysterol binding protein-dependent activation of sphingomyelin synthesis in the golgi apparatus requires phosphatidylinositol 4-kinase IIalpha. *Mol. Biol. Cell* 21, 4141–4150.

Barba, G., Harper, F., Harada, T., Kohara, M., Goulinet, S., Matsuura, Y., Eder, G., Schaff, Z., Chapman, M. J., Miyamura, T., and Brechot, C. (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1200–1205.

Barth, H., Schafer, C., Adah, M. I., Zhang, F., Linhardt, R. J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T. H., Depla, E., Von Weizsacker, F., Blum, H. E., and Baumert, T. F. (2003). Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J. Biol. Chem.* 278, 41003–41012.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A., and Cosset, F. L. (2003). Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J. Biol. Chem.* 278, 41624–41630.

Benga, W. J., Krieger, S. E., Dimitrova, M., Zeisel, M. B., Parnot, M., Lupberger, J., Hildt, E., Luo, G., Mclauchlan, J., Baumert, T. F., and Schuster, C. (2010). Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. *Hepatology* 51, 43–53.

Berger, K. L., Cooper, J. D., Heaton, N. S., Yoon, R., Oakland, T. E., Jordan, T. X., Mateu, G., Grakoui, A., and Randall, G. (2009). Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7577–7582.

Berger, K. L., Kelly, S. M., Jordan, T. X., Tartell, M. A., and Randall, G. (2011). Hepatitis C virus stimulates the phosphatidylinositol 4-kinase III alpha-dependent phosphatidylinositol 4-phosphate production that is essential for its replication. *J. Virol.* 85, 8870–8883.

Bigger, C. B., Guerra, B., Brasky, K. M., Hubbard, G., Beard, M. R., Luxon, B. A., Lemon, S. M., and Lanford, R. E. (2004). Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. *J. Virol.* 78, 13779–13792.

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

Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* 290, 1972–1974.

Boguszewska-Chachulska, A. M., Krawczyk, M., Stankiewicz, A., Gozdek, A., Haenni, A. L., and Strokovskaya, L. (2004). Direct fluorometric measurement of hepatitis C virus helicase activity. *FEBS Lett.* 567, 253–258.

Borawski, J., Troke, P., Puyang, X., Gibaja, V., Zhao, S., Mickanin, C., Leighton-Davies, J., Wilson, C. J., Myer, V., Cornellataracido, I., Baryza, J., Tallarico, J., Joberty, G., Bantscheff, M., Schirle, M., Bouwmeester, T., Mathy, J. E., Lin, K., Compton, T., Labow, M., Wiedmann, B., and Gaither, L. A. (2009). Class III phosphatidylinositol 4-kinase alpha and beta are novel host factor regulators of hepatitis C virus replication. *J. Virol.* 83, 10058–10074.

Borowski, P., Deinert, J., Schalinski, S., Bretner, M., Ginalski, K., Kulikowski, T., and Shugar, D. (2003). Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur. J. Biochem.* 270, 1645–1653.

Boson, B., Granio, O., Bartenschlager, R., and Cosset, F. L. (2011). A concerted action of hepatitis C virus p7 and nonstructural protein 2 regulates core localization at the endoplasmic reticulum and virus assembly. *PLoS Pathog.* 7, e1002144. doi:10.1371/journal.ppat.1002144

Bradley, D., Mccaustland, K., Krawczynski, K., Spelbring, J., Humphrey, C., and Cook, E. H. (1991). Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. *J. Med. Virol.* 34, 206–208.

Brodsky, J. L., Gusarova, V., and Fisher, E. A. (2004). Vesicular trafficking of hepatic apolipoprotein B100 and its maturation to very low-density lipoprotein particles; studies from cells and cell-free systems. *Trends Cardiovasc. Med.* 14, 127–132.

Carabaich, A., Ruvoletto, M., Bernardinello, E., Tono, N., Cavalletto, L., Chemello, L., Gatta, A., and Pontisso, P. (2005). Profiles of HCV core protein and viremia in chronic hepatitis C: possible protective role of core antigen in liver damage. *J. Med. Virol.* 76, 55–60.

Carrere-Kremer, S., Montpellier-Pala, C., Cocquerel, L., Wychowski, C., Penin, F., and Dubuisson, J. (2002). Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J. Virol.* 76, 3720–3730.

Cerutti, A., Maillard, P., Minisini, R., Vidalain, P. O., Roohvand, F., Pecheur, E. I., Pirisi, M., and Budkowska, A. (2011). Identification of a functional, CRM-1-dependent nuclear export signal in hepatitis C virus core protein. *PLoS ONE* 6, e25854. doi:10.1371/journal.pone.0025854

Chang, K. S., Jiang, J., Cai, Z., and Luo, G. (2007). Human apolipoprotein E is required for infectivity and production of hepatitis C virus in cell culture. *J. Virol.* 81, 13783–13793.

Chatel-Chaix, L., Melancon, P., Racine, M. E., Baril, M., and Lamarre, D. (2011). Y-box-binding protein 1 interacts with hepatitis C virus NS3/4A and influences the equilibrium between viral RNA replication and infectious particle production. *J. Virol.* 85, 11022–11037.

Chatterji, U., Bobardt, M., Selvarajah, S., Yang, F., Tang, H., Sakamoto, N., Vuagniaux, G., Parkinson, T., and Gallay, P. (2009). The isomerase active site of cyclophilin A is critical for hepatitis C virus replication. *J. Biol. Chem.* 284, 16998–17005.

Chen, B. J., Takeda, M., and Lamb, R. A. (2005). Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 proteins of two subtypes differ in their ability to support assembly. *J. Virol.* 79, 13673–13684.

Chen, X., Barton, L. F., Chi, Y., Clurman, B. E., and Roberts, J. M. (2007). Ubiquitin-independent degradation of cell-cycle inhibitors by the REGgamma proteasome. *Mol. Cell* 26, 843–852.

Cheng, G., Zhong, J., and Chisari, F. V. (2006). Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8499–8504.

Codran, A., Royer, C., Jaeck, D., Bastien-Valle, M., Baumert, T. F., Kieny, M. P., Pereira, C. A., and Martin, J. P. (2006). Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis. *J. Gen. Virol.* 87, 2583–2593.

Counihan, N. A., Rawlinson, S. M., and Lindenbach, B. D. (2011). Trafficking of hepatitis C virus core protein during virus particle assembly. *PLoS Pathog.* 7, e1002302. doi:10.1371/journal.ppat.1002302

Cun, W., Jiang, J., and Luo, G. (2010). The C-terminal alpha-helix domain of apolipoprotein E is required for interaction with nonstructural protein 5A and assembly of hepatitis C virus. *J. Virol.* 84, 11532–11541.

De Francesco, R., Tomei, L., Altamura, S., Summa, V., and Migliaccio, G. (2003). Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase. *Antiviral Res.* 58, 1–16.

Dentzer, T. G., Lorenz, I. C., Evans, M. J., and Rice, C. M. (2009). Determinants of the hepatitis C virus nonstructural protein 2 protease domain required for production of infectious virus. *J. Virol.* 83, 12702–12713.

Dhanak, D., Duffy, K. J., Johnston, V. K., Lin-Goerke, J., Darcy, M., Shaw, A. N., Gu, B., Silverman, C., Gates, A. T., Nonnemacher, M. R., Earnshaw, D. L., Casper, D. J., Kaura, A., Baker, A., Greenwood, C., Gutshall, L. L., Maley, D., Delvecchio, A., Macarron, R., Hofmann, G. A., Alnoah, Z., Cheng, H. Y., Chan, G., Khandekar, S., Keenan, R. M., and Sarisky, R.

T. (2002). Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J. Biol. Chem.* 277, 38322–38327.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D., and Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* 76, 5974–5984.

Evans, M. J., Von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., Mckeating, J. A., Bieniasz, P. D., and Rice, C. M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446, 801–805.

Failla, C., Tomei, L., and De Francesco, R. (1994). Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* 68, 3753–3760.

Failla, C., Tomei, L., and De Francesco, R. (1995). An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. *J. Virol.* 69, 1769–1777.

Ferraris, P., Blanchard, E., and Roingeard, P. (2010). Ultrastructural and biochemical analyses of hepatitis C virus-associated host cell membranes. *J. Gen. Virol.* 91, 2230–2237.

Ferreon, J. C., Ferreon, A. C., Li, K., and Lemon, S. M. (2005). Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J. Biol. Chem.* 280, 20483–20492.

Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989). Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* 337, 476–478.

Foy, E., Li, K., Sumpter, R. Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M. Jr. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2986–2991.

Gao, L., Aizaki, H., He, J. W., and Lai, M. M. (2004). Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J. Virol.* 78, 3480–3488.

Gastaminza, P., Cheng, G., Wieland, S., Zhong, J., Liao, W., and Chisari, F. V. (2008). Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J. Virol.* 82, 2120–2129.

Giese, S. I., Woerz, I., Homann, S., Tibroni, N., Geyer, M., and Fackler, O. T. (2006). Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1(SF2) Nef. *Virology* 355, 175–191.

Gosert, R., Jendrsczok, W., Berke, J. M., Brass, V., Blum, H. E., and Moradpour, D. (2005). Characterization of nonstructural protein membrane anchor deletion mutants expressed in the context of the hepatitis C virus polyprotein. *J. Virol.* 79, 7911–7917.

Goto, K., Watashi, K., Murata, T., Hishiki, T., Hijikata, M., and Shimotohno, K. (2006). Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. *Biochem. Biophys. Res. Commun.* 343, 879–884.

Grakoui, A., Mccourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993a). Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* 67, 2832–2843.

Grakoui, A., Mccourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993b). A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. U.S.A.* 90, 10583–10587.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993c). Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* 67, 1385–1395.

Hamamoto, I., Nishimura, Y., Okamoto, T., Aizaki, H., Liu, M., Mori, Y., Abe, T., Suzuki, T., Lai, M. M., Miyamura, T., Moriishi, K., and Matsuura, Y. (2005). Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* 79, 13473–13482.

Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M., and Nishijima, M. (2003). Molecular machinery for non-vesicular trafficking of ceramide. *Nature* 426, 803–809.

Harada, S., Watanabe, Y., Takeuchi, K., Suzuki, T., Katayama, T., Takebe, Y., Saito, I., and Miyamura, T. (1991). Expression of processed core protein of hepatitis C virus in mammalian cells. *J. Virol.* 65, 3015–3021.

Harris, C., Herker, E., Farese, R. V. Jr., and Ott, M. (2011). Hepatitis C Virus Core Protein Decreases Lipid Droplet Turnover: a mechanism for core-induced steatosis. *J. Biol. Chem.* 286, 42615–42625.

Havel, R. J. (2000). Remnant lipoproteins as therapeutic targets. *Curr. Opin. Lipidol.* 11, 615–620.

Herker, E., Harris, C., Hernandez, C., Carpentier, A., Kaehlcke, K., Rosenberg, A. R., Farese, R. V. Jr., and Ott, M. (2010). Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. *Nat. Med.* 16, 1295–1298.

Herz, J., Chen, Y., Masiulis, I., and Zhou, L. (2009). Expanding functions of lipoprotein receptors. *J. Lipid Res.* 50(Suppl.), S287–S292.

Hicham Alaoui-Ismaili, M., Gervais, C., Brunette, S., Gouin, G., Hamel, M., Rando, R. F., and Bedard, J. (2000). A novel high throughput screening assay for HCV NS3 helicase activity. *Antiviral Res.* 46, 181–193.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 5547–5551.

Hijikata, M., Shimizu, Y. K., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H., and Yoshikura, H. (1993). Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J. Virol.* 67, 1953–1958.

Hishiki, T., Shimizu, Y., Tobita, R., Sugiyama, K., Ogawa, K., Funami, K., Ohsaki, Y., Fujimoto, T., Takaku, H., Wakita, T., Baumert, T. F., Miyanari, Y., and Shimotohno, K. (2010). Infectivity of hepatitis C virus is influenced by association with apolipoprotein E isoforms. *J. Virol.* 84, 12048–12057.

Hofmann, W. P., and Zeuzem, S. (2011). A new standard of care for the treatment of chronic HCV infection. *Nat. Rev. Gastroenterol. Hepatol.* 8, 257–264.

Hope, R. G., and McLauchlan, J. (2000). Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. *J. Gen. Virol.* 81, 1913–1925.

Hope, R. G., Murphy, D. J., and Mclauchlan, J. (2002). The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J. Biol. Chem.* 277, 4261–4270.

Hopkins, S., Scorneaux, B., Huang, Z., Murray, M. G., Wring, S., Smitley, C., Harris, R., Erdmann, F., Fischer, G., and Ribeill, Y. (2010). SCY-635, a novel nonimmunosuppressive analog of cyclosporine that exhibits potent inhibition of hepatitis C virus RNA replication in vitro. *Antimicrob. Agents Chemother.* 54, 660–672.

Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* 109, 1125–1131.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., Rice, C. M., and Mckeating, J. A. (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7271–7276.

Hsu, N. Y., Ilnytska, O., Belov, G., Santiana, M., Chen, Y. H., Takvorian, P. M., Pau, C., Van Der Schaar, H., Kaushik-Basu, N., Balla, T., Cameron, C. E., Ehrenfeld, E., Van Kuppeveld, F. J., and Altan-Bonnet, N. (2010). Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell* 141, 799–811.

Huang, H., Sun, F., Owen, D. M., Li, W., Chen, Y., Gale, M. Jr., and Ye, J. (2007). Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5848–5853.

Hussy, P., Langen, H., Mous, J., and Jacobsen, H. (1996). Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* 224, 93–104.

Ikeda, M., Yi, M., Li, K., and Lemon, S. M. (2002). Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J. Virol.* 76, 2997–3006.

Ilyin, G. P., Rialland, M., Glaise, D., and Guguen-Guillouzo, C. (1999). Identification of a novel Skp2-like mammalian protein containing F-box and leucine-rich repeats. *FEBS Lett.* 459, 75–79.

Inoue, K., Sekiyama, K., Yamada, M., Watanabe, T., Yasuda, H., and Yoshiba, M. (2003). Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. *J. Gastroenterol.* 38, 567–572.

Ishii, N., Watashi, K., Hishiki, T., Goto, K., Inoue, D., Hijikata, M., Wakita, T., Kato, N., and Shimotohno, K. (2006). Diverse effects of cyclosporine on hepatitis C virus strain replication. *J. Virol.* 80, 4510–4520.

Ito, M., Masumi, A., Mochida, K., Kukihara, H., Moriishi, K., Matsuura, Y., Yamaguchi, K., and Mizuochi, T. (2010). Peripheral B cells may serve as a reservoir for persistent hepatitis C virus infection. *J. Innate Immun.* 2, 607–617.

Jacobson, I. M., Cacoub, P., Dal Maso, L., Harrison, S. A., and Younossi, Z. M. (2010). Manifestations of chronic hepatitis C virus infection beyond the liver. *Clin. Gastroenterol. Hepatol.* 8, 1017–1029.

Jiang, J., and Luo, G. (2009). Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. *J. Virol.* 83, 12680–12691.

Jirasko, V., Montserret, R., Appel, N., Janvier, A., Eustachi, L., Brohm, C., Steinmann, E., Pietschmann, T., Penin, F., and Bartenschlager, R. (2008). Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. *J. Biol. Chem.* 283, 28546–28562.

Jones, C. T., Murray, C. L., Eastman, D. K., Tassello, J., and Rice, C. M. (2007). Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J. Virol.* 81, 8374–8383.

Kapadia, S. B., and Chisari, F. V. (2005). Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2561–2566.

Kasama, Y., Sekiguchi, S., Saito, M., Tanaka, K., Satoh, M., Kuwahara, K., Sakaguchi, N., Takeya, M., Hiasa, Y., Kohara, M., and Tsukiyama-Kohara, K. (2010). Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas in vivo. *Blood* 116, 4926–4933.

Kaul, A., Stauffer, S., Berger, C., Pertel, T., Schmitt, J., Kallis, S., Zayas, M., Lohmann, V., Luban, J., and Bartenschlager, R. (2009). Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics. *PLoS Pathog.* 5, e1000546. doi:10.1371/journal.ppat.1000546

Kawano, M., Kumagai, K., Nishijima, M., and Hanada, K. (2006). Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT. *J. Biol. Chem.* 281, 30279–30288.

Koch, J. O., Lohmann, V., Herian, U., and Bartenschlager, R. (1996). In vitro studies on the activation of the hepatitis C virus NS3 proteinase by the NS4A cofactor. *Virology* 221, 54–66.

Kockx, M., Jessup, W., and Kritharides, L. (2010). Cyclosporin A and atherosclerosis – cellular pathways in atherogenesis. *Pharmacol. Ther.* 128, 106–118.

Koike, K., Tsutsumi, T., Yotsuyanagi, H., and Moriya, K. (2010). Lipid metabolism and liver disease in hepatitis C viral infection. *Oncology* 78(Suppl. 1), 24–30.

Krieger, N., Lohmann, V., and Bartenschlager, R. (2001). Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J. Virol.* 75, 4614–4624.

Kukihara, H., Moriishi, K., Taguwa, S., Tani, H., Abe, T., Mori, Y., Suzuki, T., Fukuhara, T., Taketomi, A., Maehara, Y., and Matsuura, Y. (2009). Human VAP-C negatively regulates hepatitis C virus propagation. *J. Virol.* 83, 7959–7969.

Kumagai, K., Yasuda, S., Okemoto, K., Nishijima, M., Kobayashi, S., and Hanada, K. (2005). CERT mediates intermembrane transfer of various molecular species of ceramides. *J. Biol. Chem.* 280, 6488–6495.

Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M. Jr., and Lemon, S. M. (2005a). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2992–2997.

Li, X. D., Sun, L., Seth, R. B., Pineda, G., and Chen, Z. J. (2005b). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17717–17722.

Li, Q., Brass, A. L., Ng, A., Hu, Z., Xavier, R. J., Liang, T. J., and Elledge, S. J. (2009). A genome-wide genetic screen for host factors required for hepatitis C virus propagation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16410–16415.

Li, X., Lonard, D. M., Jung, S. Y., Malovannaya, A., Feng, Q., Qin, J., Tsai, S. Y., Tsai, M. J., and O'malley, B. W. (2006). The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. *Cell* 124, 381–392.

Lim, Y. S., and Hwang, S. B. (2011). Hepatitis C virus NS5A protein interacts with phosphatidylinositol 4-kinase type IIIalpha and regulates viral propagation. *J. Biol. Chem.* 286, 11290–11298.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., Mckeating, J. A., and Rice, C. M. (2005). Complete replication of hepatitis C virus in cell culture. *Science* 309, 623–626.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., Mckeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E., Leroux-Roels, G., and Rice, C. M. (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3805–3809.

Liu, J., Farmer, J. D. Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66, 807–815.

Loewen, C. J., Roy, A., and Levine, T. P. (2003). A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. *EMBO J.* 22, 2025–2035.

Lohmann, V., Korner, F., Dobierzewska, A., and Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* 75, 1437–1449.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285, 110–113.

Loo, Y. M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M. A., Garcia-Sastre, A., Katze, M. G., and Gale, M. Jr. (2008). Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J. Virol.* 82, 335–345.

Loo, Y. M., Owen, D. M., Li, K., Erickson, A. K., Johnson, C. L., Fish, P. M., Carney, D. S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., Lee, W. M., Hagedorn, C. H., Lau, D. T., Weinman, S. A., Lemon, S. M., and Gale, M. Jr. (2006). Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6001–6006.

Lorenz, I. C., Marcotrigiano, J., Dentzer, T. G., and Rice, C. M. (2006). Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* 442, 831–835.

Ma, S., Boerner, J. E., Tiongyip, C., Weidmann, B., Ryder, N. S., Cooreman, M. P., and Lin, K. (2006). NIM811, a cyclophilin inhibitor, exhibits potent in vitro activity against hepatitis C virus alone or in combination with alpha interferon. *Antimicrob. Agents Chemother.* 50, 2976–2982.

Maga, G., Gemma, S., Fattorusso, C., Locatelli, G. A., Butini, S., Persico, M., Kukreja, G., Romano, M. P., Chiasserini, L., Savini, L., Novellino, E., Nacci, V., Spadari, S., and Campiani, G. (2005). Specific targeting of hepatitis C virus NS3 RNA helicase. Discovery of the potent and selective competitive nucleotide-mimicking inhibitor QU663. *Biochemistry* 44, 9637–9644.

Mannova, P., Fang, R., Wang, H., Deng, B., Mcintosh, M. W., Hanash, S. M., and Beretta, L. (2006). Modification of host lipid raft proteome upon hepatitis C virus replication. *Mol. Cell Proteomics* 5, 2319–2325.

Masaki, T., Suzuki, R., Murakami, K., Aizaki, H., Ishii, K., Murayama, A., Date, T., Matsuura, Y., Miyamura, T., Wakita, T., and Suzuki, T. (2008). Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. *J. Virol.* 82, 7964–7976.

Matto, M., Rice, C. M., Aroeti, B., and Glenn, J. S. (2004). Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J. Virol.* 78, 12047–12053.

McLauchlan, J., Lemberg, M. K., Hope, G., and Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* 21, 3980–3988.

Meertens, L., Bertaux, C., and 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.

Merz, A., Long, G., Hiet, M. S., Brugger, B., Chlanda, P., Andre, P., Wieland, F., Krijnse-Locker, J., and Bartenschlager, R. (2011). Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. *J. Biol. Chem.* 286, 3018–30132.

Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., and Shimotohno, K. (2007). The lipid droplet is an important organelle for hepatitis C virus production. *Nat. Cell Biol.* 9, 1089–1097.

Miyoshi, H., Moriya, K., Tsutsumi, T., Shinzawa, S., Fujie, H., Shintani, Y., Fujinaga, H., Goto, K., Todoroki, T., Suzuki, T., Miyamura, T., Matsuura, Y., Yotsuyanagi, H., and

Koike, K. (2011). Pathogenesis of lipid metabolism disorder in hepatitis C: polyunsaturated fatty acids counteract lipid alterations induced by the core protein. *J. Hepatol.* 54, 432–438.

Monazahian, M., Bohme, I., Bonk, S., Koch, A., Scholz, C., Grethe, S., and Thomssen, R. (1999). Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *J. Med. Virol.* 57, 223–229.

Moriishi, K., Mochizuki, R., Moriya, K., Miyamoto, H., Mori, Y., Abe, T., Murata, S., Tanaka, K., Miyamura, T., Suzuki, T., Koike, K., and Matsuura, Y. (2007). Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1661–1666.

Moriishi, K., Okabayashi, T., Nakai, K., Moriya, K., Koike, K., Murata, S., Chiba, T., Tanaka, K., Suzuki, R., Suzuki, T., Miyamura, T., and Matsuura, Y. (2003). Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. *J. Virol.* 77, 10237–10249.

Moriishi, K., Shoji, I., Mori, Y., Suzuki, R., Suzuki, T., Kataoka, C., and Matsuura, Y. (2010). Involvement of PA28gamma in the propagation of hepatitis C virus. *Hepatology* 52, 411–420.

Moriya, K., Nakagawa, K., Santa, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishibashi, K., Horie, T., Imai, K., Todoroki, T., Kimura, S., and Koike, K. (2001). Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res.* 61, 4365–4670.

Moriya, K., Yotsuyanagi, H., Shintani, Y., Fujie, H., Ishibashi, K., Matsuura, Y., Miyamura, T., and Koike, K. (1997). Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 78 (Pt 7), 1527–1531.

Najda-Bernatowicz, A., Krawczyk, M., Stankiewicz-Drogon, A., Bretner, M., and Boguszewska-Chachulska, A. M. (2010). Studies on the anti-hepatitis C virus activity of newly synthesized tropolone derivatives: identification of NS3 helicase inhibitors that specifically inhibit subgenomic HCV replication. *Bioorg. Med. Chem.* 18, 5129–5136.

Nielsen, S. U., Bassendine, M. F., Burt, A. D., Martin, C., Pumeechockchai, W., and Toms, G. L. (2006). Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J. Virol.* 80, 2418–2428.

Nishimura, Y., Hayashi, M., Inada, H., and Tanaka, T. (1999). Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. *Biochem. Biophys. Res. Commun.* 254, 21–26.

Nunez, O., Fernandez-Martinez, A., Majano, P. L., Apolinario, A., Gomez-Gonzalo, M., Benedicto, I., Lopez-Cabrera, M., Bosca, L., Clemente, G., Garcia-Monzon, C., and Martin-Sanz, P. (2004). Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut* 53, 1665–1672.

Ogino, T., Fukuda, H., Imajoh-Ohmi, S., Kohara, M., and Nomoto, A. (2004). Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. *J. Virol.* 78, 11766–11777.

Ohata, K., Hamasaki, K., Toriyama, K., Matsumoto, K., Saeki, A., Yanagi, K., Abiru, S., Nakagawa, Y., Shigeno, M., Miyazoe, S., Ichikawa, T., Ishikawa, H., Nakao, K., and Eguchi, K. (2003). Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. *Cancer* 97, 3036–3043.

Okamoto, K., Mori, Y., Komoda, Y., Okamoto, T., Okochi, M., Takeda, M., Suzuki, T., Moriishi, K., and Matsuura, Y. (2008). Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. *J. Virol.* 82, 8349–8361.

Okamoto, K., Moriishi, K., Miyamura, T., and Matsuura, Y. (2004). Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78, 6370–6380.

Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., and Matsuura, Y. (2006). Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* 25, 5015–5025.

Okuda, M., Li, K., Beard, M. R., Showalter, L. A., Scholle, F., Lemon, S. M., and Weinman, S. A. (2002). Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 122, 366–375.

Oomens, A. G., Bevis, K. P., and Wertz, G. W. (2006). The cytoplasmic tail of the human respiratory syncytial virus F protein plays critical roles in cellular localization of the F protein and infectious progeny production. *J. Virol.* 80, 10465–10477.

Peretti, D., Dahan, N., Shimoni, E., Hirschberg, K., and Lev, S. (2008). Coordinated lipid transfer between the endoplasmic reticulum and the Golgi complex requires the VAP proteins and is essential for Golgi-mediated transport. *Mol. Biol. Cell* 19, 3871–3884.

Perlemuter, G., Sabile, A., Letteron, P., Vona, G., Topilco, A., Chretien, Y., Koike, K., Pessayre, D., Chapman, J., Barba, G., and Brechot, C. (2002). Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J.* 16, 185–194.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F. L., and Bartenschlager, R. (2006). Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7408–7413.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D., and Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J. Virol.* 76, 4008–4021.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G., and Abrignani, S. (1998). Binding of hepatitis C virus to CD81. *Science* 282, 938–941.

Ploss, A., Evans, M. J., Gaysinskaya, V. A., Panis, M., You, H., De Jong, Y. P., and Rice, C. M. (2009). Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457, 882–886.

Popescu, C. I., Callens, N., Trinel, D., Roingeard, P., Moradpour, D., Descamps, V., Duverlie, G., Penin, F., Heliot, L., Rouille, Y., and Dubuisson, J. (2011). NS2 protein of hepatitis C virus interacts with structural and non-structural proteins towards virus assembly. *PLoS Pathog.* 7, e1001278. doi:10.1371/journal.ppat.1001278

Pumeechockchai, W., Bevitt, D., Agarwal, K., Petropoulou, T., Langer, B. C., Belohradsky, B., Bassendine, M. F., and Toms, G. L. (2002). Hepatitis C virus particles of different density in the blood of chronically infected immunocompetent and immunodeficient patients: implications for virus clearance by antibody. *J. Med. Virol.* 68, 335–342.

Rahfeld, J. U., Schierhorn, A., Mann, K., and Fischer, G. (1994). A novel peptidyl-prolyl cis/trans isomerase from *Escherichia coli*. *FEBS Lett.* 343, 65–69.

Randall, G., Panis, M., Cooper, J. D., Tellinghuisen, T. L., Sukhodolets, K. E., Pfeffer, S., Landthaler, M., Landgraf, P., Kan, S., Lindenbach, B. D., Chien, M., Weir, D. B., Russo, J. J., Ju, J., Brownstein, M. J., Sheridan, R., Sander, C., Zavolan, M., Tuschl, T., and Rice, C. M. (2007). Cellular cofactors affecting hepatitis C virus infection and replication. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12884–12889.

Reiss, S., Rebhan, I., Backes, P., Romero-Brey, I., Erfle, H., Matula, P., Kaderali, L., Poenisch, M., Blankenburg, H., Hiet, M. S., Longerich, T., Diehl, S., Ramirez, F., Balla, T., Rohr, K., Kaul, A., Buhler, S., Pepperkok, R., Lengauer, T., Albrecht, M., Eils, R., Schirmacher, P., Lohmann, V., and Bartenschlager, R. (2011). Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell Host Microbe* 9, 32–45.

Ridgway, N. D., Dawson, P. A., Ho, Y. K., Brown, M. S., and Goldstein, J. L. (1992). Translocation of oxysterol binding protein to Golgi apparatus triggered by ligand binding. *J. Cell Biol.* 116, 307–319.

Rubbia-Brandt, L., Quadri, R., Abid, K., Giostra, E., Male, P. J., Mentha, G., Spahr, L., Zarski, J. P., Borisch, B., Hadengue, A., and Negro, F. (2000). Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. *J. Hepatol.* 33, 106–115.

Sainz, B. Jr., Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., Marsh, K. A., Yu, X., Chayama, K., Alrefai, W. A., and Uprichard, S. L. (2012). Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat. Med.* doi: 10.1038/nm.2581

Sakamoto, H., Okamoto, K., Aoki, M., Kato, H., Katsume, A., Ohta, A., Tsukuda, T., Shimma, N., Aoki, Y., Arisawa, M., Kohara, M., and Sudoh, M. (2005). Host sphingolipid biosynthesis as a target for hepatitis

C virus therapy. *Nat. Chem. Biol.* 1, 333–337.

Santolini, E., Migliaccio, G., and La Monica, N. (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J. Virol.* 68, 3631–3641.

Santolini, E., Pacini, L., Fipaldini, C., Migliaccio, G., and Monica, N. (1995). The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J. Virol.* 69, 7461–7471.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R., and Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* 21, 5017–5025.

Schiene-Fischer, C., Habazettl, J., Schmid, F. X., and Fischer, G. (2002). The hsp70 chaperone DnaK is a secondary amide peptide bond cis-trans isomerase. *Nat. Struct. Biol.* 9, 419–424.

Shavinskaya, A., Boulant, S., Penin, F., Mclauchlan, J., and Bartenschlager, R. (2007). The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly. *J. Biol. Chem.* 282, 37158–37169.

Shi, S. T., Lee, K. J., Aizaki, H., Hwang, S. B., and Lai, M. M. (2003). Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* 77, 4160–4168.

Siekierka, J. J., Hung, S. H., Poe, M., Lin, C. S., and Sigal, N. H. (1989a). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* 341, 755–757.

Siekierka, J. J., Staruch, M. J., Hung, S. H., and Sigal, N. H. (1989b). FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. *J. Immunol.* 143, 1580–1583.

Skehel, P. A., Martin, K. C., Kandel, E. R., and Bartsch, D. (1995). A VAMP-binding protein from Aplysia required for neurotransmitter release. *Science* 269, 1580–1583.

Soriano, V., Peters, M. G., and Zeuzem, S. (2009). New therapies for hepatitis C virus infection. *Clin. Infect. Dis.* 48, 313–320.

Spik, G., Haendler, B., Delmas, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H. P., Keller, R., Hiestad, P. C., and Movva, N. R. (1991). A novel secreted cyclophilin-like protein (SCYLP). *J. Biol. Chem.* 266, 10735–10738.

Steinmann, E., Penin, F., Kallis, S., Patel, A. H., Bartenschlager, R., and Pietschmann, T. (2007). Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog.* 3, e103. doi:10.1371/journal.ppat.0030103

Sumpter, R. Jr., Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M. Jr. (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* 79, 2689–2699.

Suzuki, R., Moriishi, K., Fukuda, K., Shirakura, M., Ishii, K., Shoji, I., Wakita, T., Miyamura, T., Matsuura, Y., and Suzuki, T. (2009). Proteasomal turnover of hepatitis C virus core protein is regulated by two distinct mechanisms: a ubiquitin-dependent mechanism and a ubiquitin-independent but PA28gamma-dependent mechanism. *J. Virol.* 83, 2389–2392.

Tai, A. W., Benita, Y., Peng, L. F., Kim, S. S., Sakamoto, N., Xavier, R. J., and Chung, R. T. (2009). A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. *Cell Host Microbe* 5, 298–307.

Tai, A. W., and Salloum, S. (2011). The role of the phosphatidylinositol 4-kinase PI4KA in hepatitis C virus-induced host membrane rearrangement. *PLoS ONE* 6, e26300. doi:10.1371/journal.pone.0026300

Takikawa, S., Ishii, K., Aizaki, H., Suzuki, T., Asakura, H., Matsuura, Y., and Miyamura, T. (2000). Cell fusion activity of hepatitis C virus envelope proteins. *J. Virol.* 74, 5066–5074.

Targett-Adams, P., Hope, G., Boulant, S., and Mclauchlan, J. (2008). Maturation of hepatitis C virus core protein by signal peptide peptidase is required for virus production. *J. Biol. Chem.* 283, 16850–16859.

Thomssen, R., Bonk, S., Propfe, C., Heermann, K. H., Kochel, H. G., and Uy, A. (1992). Association of hepatitis C virus in human sera with beta-lipoprotein. *Med. Microbiol. Immunol.* 181, 293–300.

Thomssen, R., Bonk, S., and Thiele, A. (1993). Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. *Med. Microbiol. Immunol.* 182, 329–334.

Tory, R., Sachs-Barrable, K., Hill, J. S., and Wasan, K. M. (2008). Cyclosporine A and rapamycin induce in vitro cholesteryl ester transfer protein activity, and suppress lipoprotein lipase activity in human plasma. *Int. J. Pharm.* 358, 219–223.

Tripathi, L. P., Kataoka, C., Taguwa, S., Moriishi, K., Mori, Y., Matsuura, Y., and Mizuguchi, K. (2010). Network based analysis of hepatitis C virus core and NS4B protein interactions. *Mol. Biosyst.* 6, 2539–2553.

Trotard, M., Lepere-Douard, C., Regeard, M., Piquet-Pellorce, C., Lavillette, D., Cosset, F. L., Gripon, P., and Le Seyec, J. (2009). Kinases required in hepatitis C virus entry and replication highlighted by small interference RNA screening. *FASEB J.* 23, 3780–3789.

Tscherne, D. M., Jones, C. T., Evans, M. J., Lindenbach, B. D., Mckeating, J. A., and Rice, C. M. (2006). Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *J. Virol.* 80, 1734–1741.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., and Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* 66, 1476–1483.

Tsutsumi, T., Suzuki, T., Shimoike, T., Suzuki, R., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K., and Miyamura, T. (2002). Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. *Hepatology* 35, 937–946.

Tu, H., Gao, L., Shi, S. T., Taylor, D. R., Yang, T., Mircheff, A. K., Wen, Y., Gorbalenya, A. E., Hwang, S. B., and Lai, M. M. (1999). Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 263, 30–41.

Vaillancourt, F. H., Pilote, L., Cartier, M., Lippens, J., Liuzzi, M., Bethell, R. C., Cordingley, M. G., and Kukolj, G. (2009). Identification of a lipid kinase as a host factor involved in hepatitis C virus RNA replication. *Virology* 387, 5–10.

Vieyres, G., Thomas, X., Descamps, V., Duverlie, G., Patel, A. H., and Dubuisson, J. (2010). Characterization of the envelope glycoproteins associated with infectious hepatitis C virus. *J. Virol.* 84, 10159–10168.

Voisset, C., Lavie, M., Helle, F., Op De Beeck, A., Bilheu, A., Bertrand-Michel, J., Terce, F., Cocquerel, L., Wychowski, C., Vu-Dac, N., and Dubuisson, J. (2008). Ceramide enrichment of the plasma membrane induces CD81 internalization and inhibits hepatitis C virus entry. *Cell. Microbiol.* 10, 606–617.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11, 791–796.

Wang, C., Gale, M. Jr., Keller, B. C., Huang, H., Brown, M. S., Goldstein, J. L., and Ye, J. (2005a). Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol. Cell* 18, 425–434.

Wang, P. Y., Weng, J., and Anderson, R. G. (2005b). OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science* 307, 1472–1476.

Watashi, K., Hijikata, M., Hosaka, M., Yamaji, M., and Shimotohno, K. (2003). Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 38, 1282–1288.

Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y., and Shimotohno, K. (2005). Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19, 111–122.

Wolins, N. E., Brasaemle, D. L., and Bickel, P. E. (2006). A proposed model of fat packaging by exchangeable lipid droplet proteins. *FEBS Lett.* 580, 5484–5491.

Wolk, B., Sansonno, D., Krausslich, H. G., Dammacco, F., Rice, C. M., Blum, H. E., and Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* 74, 2293–2304.

Wozniak, A. L., Griffin, S., Rowlands, D., Harris, M., Yi, M., Lemon, S. M., and Weinman, S. A. (2010). Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production. *PLoS Pathog.* 6, e1001087. doi:10.1371/journal.ppat.1001087

Yamaga, A. K., and Ou, J. H. (2002). Membrane topology of the hepatitis C virus NS2 protein. *J. Biol. Chem.* 277, 33228–33234.

Yang, F., Robotham, J. M., Nelson, H. B., Irsigler, A., Kenworthy, R., and Tang, H. (2008). Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. *J. Virol.* 82, 5269–5278.

Ye, J., Wang, C., Sumpter, R. Jr., Brown, M. S., Goldstein, J. L., and Gale, M. Jr. (2003). Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15865–15870.

Zhang, Z., and Zhang, R. (2008). Proteasome activator PA28 gamma regulates p53 by enhancing its MDM2-mediated degradation. *EMBO J.* 27, 852–864.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T., and Chisari, F. V. (2005). Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9294–9299.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 December 2011; accepted: 31 January 2012; published online: 14 February 2012.

Citation: Moriishi K and Matsuura Y (2012) Exploitation of lipid components by viral and host proteins for hepatitis C virus infection. Front. Microbio. 3:54. doi: 10.3389/fmicb.2012.00054

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

European Journal of Medicinal Chemistry 49 (2012) 191–199

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Inhibition of hepatitis C virus NS5B polymerase by S-trityl-L-cysteine derivatives

Daniel B. Nichols [a], Guy Fournet [b], K.R. Gurukumar [a], Amartya Basu [a], Jin-Ching Lee [c], Naoya Sakamoto [d], Frank Kozielski [e], Ira Musmuca [f], Benoît Joseph [b,**], Rino Ragno [f,***], Neerja Kaushik-Basu [a,*]

[a] Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, USA
[b] Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, UMR-CNRS 5246, Université de Lyon, Université Claude Bernard – Lyon 1, Bâtiment Curien, 43 Boulevard du 11 Novembre 1918, F-69622 Villeurbanne, France
[c] Department of Biotechnology, College of Life Science, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo 113-8519, Japan
[e] The Beatson Institute for Cancer Research, Molecular Motors Laboratory, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK
[f] Rome Center for Molecular Design, Dipartimento di Chimica e Tecnologie del Farmaco, Sapienzà Universita id Roma, P.le A. Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Received 20 December 2011
Received in revised form
3 January 2012
Accepted 5 January 2012
Available online 12 January 2012

Keywords:
Antiviral agents
Hepatitis C
HCV NS5B polymerase
Inhibitors
STLC derivatives

ABSTRACT

Structure-based studies led to the identification of a constrained derivative of S-trityl-L-cysteine (STLC) scaffold as a candidate inhibitor of hepatitis C virus (HCV) NS5B polymerase. A panel of STLC derivatives were synthesized and investigated for their activity against HCV NS5B. Three STLC derivatives, **9**, F-3070, and F-3065, were identified as modest HCV NS5B inhibitors with IC_{50} values between 22.3 and 39.7 μM. F-3070 and F-3065 displayed potent inhibition of intracellular NS5B activity in the BHK-NS5B-FRLuc reporter and also inhibited HCV RNA replication in the Huh7/Rep-Feo1b reporter system. Binding mode investigations suggested that the STLC scaffold can be used to develop new NS5B inhibitors by further chemical modification at one of the trityl phenyl group.

1. Introduction

Hepatitis C virus (HCV) infection represents a major public-health concern. It is estimated that over 200 million people, ~3% of the world population, are chronically infected with the virus [1–3]. HCV has an array of immune evasion strategies and can persist in the host for years. Individuals with chronic HCV infection are at increased risk of developing cirrhosis and hepatocellular carcinoma [3–7]. Currently, HCV infections are treated by a combination of pegylated-interferon, the nucleoside analog ribavirin, and one of two recently approved HCV protease inhibitors, Boceprevir or Telaprevir [8–13]. However, this therapy is limited in efficacy against the various HCV genotypes. Furthermore, in addition to its high cost, the current treatment is associated with severe side effects and a complicated dosing regimen that may limit patient compliance [11,12]. Also the possibility of selecting drug resistant HCV variants remains [12,13]. Therefore, the development of additional, efficacious and more cost effective HCV antiviral therapies that target viral proteins and have limited effects on host biological processes is a priority.

HCV is a member of the *Flaviviridae* family. The positive sense, 9.6 kb RNA genome is translated into a single 3000 amino acid polyprotein via an IRES sequence located within the 5′ non-translated region (NTR) of the viral genome [14,15]. The viral polyprotein is processed by both host and viral proteases into individual viral proteins consisting of four structural (core, E1, E2, and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [16]. HCV replicates exclusively in the cytoplasm of host cells. Replication of the viral RNA genome is mediated by the RNA-dependent RNA polymerase (RdRp) activity of the HCV nonstructural protein NS5B [17–19]. Because of the absolute requirement of NS5B to synthesize nascent HCV RNA, NS5B represents an attractive target for the development of anti-HCV inhibitors [20,21]. Furthermore, host cells lack RdRp. Therefore, an inhibitor that blocks RdRp activity should, in theory, have minimal or no effect on host biological processes. Though, a number of NIs and NNIs with potent in vitro anti-NS5B activity have been identified in recent years, they have presented challenges of toxicity and selection of resistant viruses, thus necessitating identification of better NS5B inhibitor scaffolds.

* Corresponding author. Tel.: +1 973 972 8653; fax: +1 973 972 5594.
** Corresponding author. Tel.: +33 4 72448135; fax: +33 4 72431214.
*** Corresponding author. Tel.: +396 4991 3937; fax: +396 4991 3627.
E-mail addresses: benoit.joseph@univ-lyon1.fr (B. Joseph), rino.ragno@uniroma1.it (R. Ragno), kaushik@umdnj.edu (N. Kaushik-Basu).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2012.01.010

The structure of NS5B has been extensively characterized. The 66 kDa viral polymerase resembles a "right hand" with the active site contained in the palm domain and the RNA interacting region in the finger and thumb domains [22–25]. Current NS5B inhibitors can be divided into two classes, nucleoside inhibitors (NI) and non-nucleoside inhibitors (NNI). Once converted by host proteins into nucleotides, NIs cause RNA-chain termination upon incorporation by NS5B into the nascent RNA chains. NNIs bind to one of the five allosteric sites on NS5B and inhibit the initiation step of RNA synthesis.

Recently, we reported on the utility of three-dimensional quantitative structure-activity relationship (3D-QSAR) in combination with ligand-based and structure-based alignment procedures for in silico screening of new HCV NS5B polymerase inhibitors [26]. This investigation identified four new NS5B inhibitors from forty candidates examined from the NCI diversity set [26]. The most interesting hit, NSC123526 (Fig. 1), has been reported to be active against other viruses [27] and can be simply viewed as a constrained derivative of the S-trityl-L-cysteine (STLC) scaffold. STLC derivatives are versatile compounds endowed with antileukemic activity [28] and are also reported to inhibit the human mitotic kinesin Eg5 (HMKEg) by a non-competitive mechanism [29].

Herein, we describe molecular modeling studies that led us to explore the potential of STLC and its derivatives to inhibit HCV NS5B RdRp activity in vitro. Further, we examined the effect of STLC derivatives on intracellular HCV NS5B RdRp activity and on HCV RNA replication. Among the tested STLC derivatives, we identified three compounds as novel HCV NS5B inhibitor leads. These compounds merit further optimization through classical medicinal chemistry and virtual screening.

2. Results and discussion

2.1. Molecular modeling

Recently, we utilized structure-based 3-D QSAR modeling to identify NS5B thumb-binding inhibitors and reported on the identification of NSC123526 as a modest HCV NS5B inhibitor [26]. NSC123526 can be considered as a constrained STLC derivative (Fig. 1). Since STLC derivative NSC123139 (Fig. 1) was found to be most potent in inhibiting HMKEg, we performed cross-docking experiments to investigate whether it could also bind the HCV-NS5B thumb domain [26,29]. Fig. 2 depicts the docked conformation of NSC123139 in HCV-NS5B and HMKEg.

The activity of the docked NSC123139 (Predicted $pIC_{50} = 5.64$) was predicted by our 3-D QSAR model in the same range of NSC123526 (Experimental $pIC_{50} = 4.33$, predicted $pIC_{50} = 5.4$) [26]. However, NSC123139 exhibited a much weaker inhibition of NS5B RdRp activity in vitro (Table 1), compared to NSC123526, as previously reported [26].

Based on the above partial results, we tested a series of STLC derivatives for their ability to inhibit NS5B, with the objective of identifying new lead scaffolds. While our investigations with additional STLC derivatives were still ongoing, the co-crystal structures of HMKEg with NSC123139 (pdb entry code 2wog and 2xae) and other STLCs (2xr2 and 3ken) were released [30]. Nevertheless, docking calculations performed through Autodock Vina, were in good agreement with the experimental results (rmsd = 0.44) and with similar docking calculations previously reported [31], thus supporting our protocol.

The above docking protocol was also applied to the other STLCs. In addition, we analyzed the Autodock Vina proposed binding

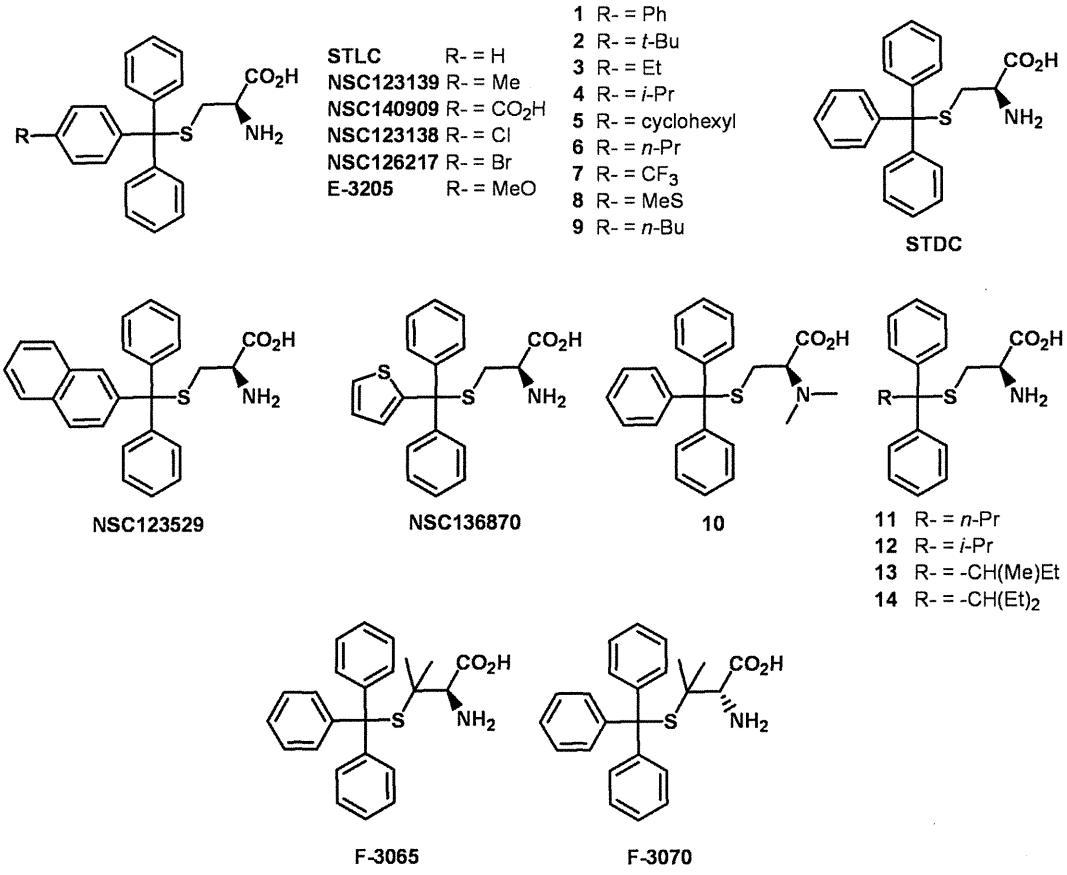


Fig. 1. Structures of NSC123526, STLC, STDC and STLC derivatives.

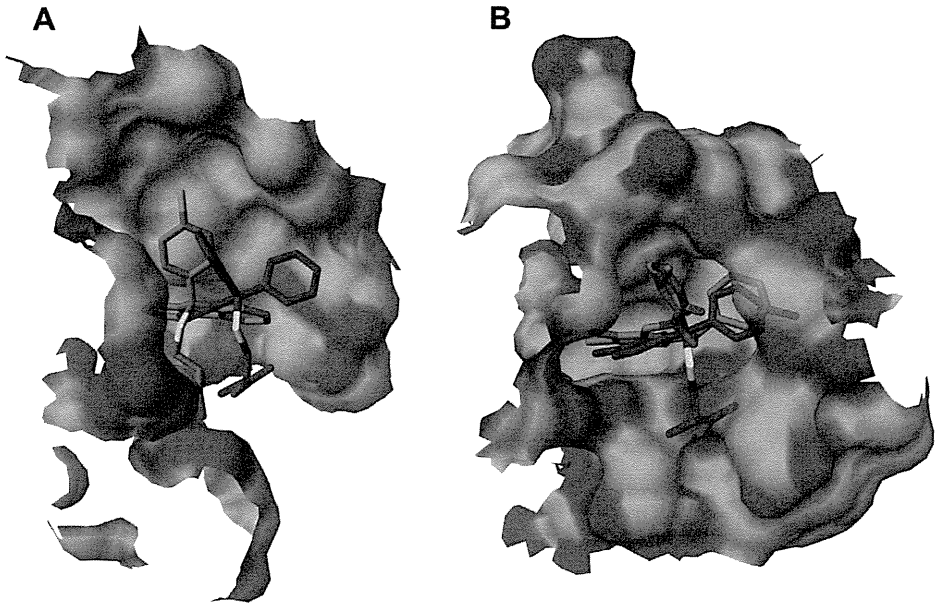


Fig. 2. Molecular docking of STLC derivatives in NS5B. Panel A: Docked conformations of NSC123139 (red-colored carbon atoms) and NSC123526 (green-colored carbon atoms) within NS5B thumb domain. Panel B: HMKEg (PDB entry code 2fme) with docked conformation of NSC123139 (red-colored carbon atoms) and the experimental bound NSC123526 (green-colored carbon atoms). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mode of the two most active compounds, F-3065 and F-3070, cross-docked into the 15 NS5B-NNI co-crystal structures as previously described by us [26]. As expected, docked conformations of F-3065 and F-3070 ((*R*) and (*S*) enantiomers of the same compound, respectively) exhibited the lowest binding energy in the PDB entry 2d3u. Further, the bound conformations of F-3065 and F-3070, in agreement with the biological data, revealed that the cysteine stereocenter does not affect the overall binding mode, wherein the terminal amino acid group is involved in a hydrogen bonding network, as shown in the ligplot diagrams in Fig. 3. In particular the α-amino acid portion of F-3065 makes two hydrogen bonds, one between its amino group and the carbonyl group of Trp528 (N···O distance = 3.01 Å) and the other between a carboxy oxygen and the ε-amino group of Lys533 (O···N distance = 2.98 Å) (Fig. 3A). The ligplot diagram of the (*S*) enantiomer F-3070, that forms two hydrogen bonds with its two carboxy oxygens, one with the guanidinic nitrogen of Arg501 (O···N distance = 3.21 Å) and the other with ε-amino group of Lys533 (O···N distance 3.05–3.22 Å), is shown in Fig. 3B. This type of hydrogen bonding network was observed in all other STLC derivatives (Fig. 5) suggesting that hydrogen bonds are the leading interactions.

Other notable interactions are hydrophobic in nature, and the trityl moieties are buried in the thumb allosteric binding side (Fig. 4). For both F-3065 and F-3070, one phenyl is placed in a pocket formed by Leu419, Arg422, Met423 and Trp528, while the other two benzenes fill-up two depressions on the enzyme surface. By comparing the binding mode of the most active STLCs with that of the experimental co-crystallized compound found in 2d3u and considering the conserved binding modes shown in Fig. 5, we believe the STLC can be used as a starting scaffold, whose activity could be improved by inserting a side chain in one of the two surface bound benzene rings to better fill the binding cleft formed by Leu419, Met423, Ile482, Val485, Ala486, Leu489 and Leu497 (Fig. 6) and occupied by a 2-(4-cyanophenyl)thiophene group in the original complex (PDB ID 2d3u). As expected and within the limit of any predictive model, the application of our 3-D QSAR to all the new STLCs, predicted these compounds to have activities between the 10–100 μM range (data not shown).

2.2. Chemistry

A total of 35 STLC derivatives were utilized in this study (Fig. 1 and Scheme 1). STLC and 14 derivatives (**1**–**14**) have been reported previously [29,31]. Compounds F-3070 and F-3065 were purchased from Bachem, while STDC (NSC123676), NSC123139, NSC136870, NSC140909, NSC123529, NSC123138, and NSC126217 were procured from NCI/NIH. In accordance with published literature, 12 STLC derivatives were newly synthesized for this investigation (Scheme 1). Starting aryldiphenylmethanol compounds **15** were prepared in good yields from appropriate esters ($ArCO_2Me$) and phenylmagnesium chloride (data not shown) [32]. Condensation of cysteamine.HCl (**16**) with $Ar(Ph)_2COH$ (Ar = 4-Me–Ph, 4-Et–Ph, 4-*n*-Pr–Ph, 4-MeS–Ph, 4-I–Ph, 4-(Ph)–Ph and 2-naphthyl) **15a**–**g** in TFA gave final compounds **17a**–**g** in 29–47% yield (Scheme 1). Treatment of L-cysteine (**18**) or L-penicillamine (**19**) with $Ar(Ph)_2COH$ (Ar = 4-(n-C_5H_{11})-Ph, 4-(n-C_6H_{13})-Ph, 4-PrO-Ph and 4-*n*-Bu-Ph) **15h**–**k** in the presence of $BF_3 \cdot Et_2O$ afforded target compounds **17h**–**l** in 30–55% yield (Scheme 1).

2.3. Biological studies

With the objective of identifying novel HCV NS5B inhibitors, we investigated STLC and its derivatives employing the in vitro NS5B RdRp inhibition assay as described previously [33–35]. Recombinant HCV NS5B (genotype 1b) carrying an N-terminal His-tag and C-terminal 21-amino acid truncation (NS5BCΔ21) was purified to homogeneity by Ni-NTA chromatography and used as a source of enzyme [33–35]. Wedelolactone, a documented NS5B inhibitor, was employed as an internal reference standard, and yielded an IC_{50} value of 36.0 μM (data not shown), consistent with our previously reported value [34]. In order to identify a wider range of NS5B inhibitor candidates, preliminary screening of STLC and its derivatives was conducted at 100 μM compound concentration. While the parent STLC molecule yielded only ~12% inhibition of NS5B RdRp activity during preliminary screening, its thirty-five derivatives, with the exception of **17l**, exhibited a much higher inhibition ranging from 14 to 83% (Table 1). Of these, three compounds **9**, F-

Table 1
Anti-HCV NS5B RdRp activity of STLC derivatives.

Compound	% Inhibition[a]	IC_{50} (μM)[b]
STLC	12.6 ± 2.3	n.d.
STDC	17.0 ± 0.6	n.d.
NSC123139	23.1 ± 1.6	n.d.
NSC136870	23.4 ± 2.9	n.d.
NSC140909	30.9 ± 3.7	n.d.
NSC123529	14.7 ± 1.5	n.d.
NSC123138	28.3 ± 5.9	n.d.
NSC126217	20.2 ± 2.5	n.d.
1	22.4 ± 5.4	n.d.
2	22.6 ± 2.0	n.d.
3	36.8 ± 2.4	n.d.
4	31.2 ± 1.5	n.d.
5	20.5 ± 1.1	n.d.
6	36.9 ± 2.5	n.d.
7	43.5 ± 0.8	n.d.
8	44.3 ± 3.0	n.d.
9	60.0 ± 3.4	39.7 ± 0.9
10	17.2 ± 2.9	n.d.
11	19.1 ± 1.7	n.d.
12	22.5 ± 2.2	n.d.
13	34.0 ± 1.1	n.d.
14	33.1 ± 0.7	n.d.
17a	31.7 ± 1.8	n.d.
17b	28.7 ± 2.1	n.d.
17c	27.4 ± 4.2	n.d.
17d	24.0 ± 4.5	n.d.
17e	36.7 ± 2.1	n.d.
17f	36.0 ± 1.0	n.d.
17g	33.3 ± 2.3	n.d.
17h	28.3 ± 5.9	n.d.
17i	16.1 ± 3.0	n.d.
17j	14.0 ± 3.3	n.d.
17k	22.0 ± 1.4	n.d.
17l	n.i.	n.d.
F-3070	82.8 ± 1.3	22.3 ± 5.9
E-3205	40.2 ± 0.7	n.d.
F-3065	76.7 ± 2.4	24.6 ± 6.0

n.d., not determined.
n.i., no inhibition.
[a] Percent inhibition was determined at 100 μM concentration of the indicated compound and represents an average of at least two independent measurements in duplicate.
[b] The IC_{50} values of the compounds were determined from dose-response curves employing 8–12 concentrations of each compound in duplicate in two independent experiments. Curves were fitted to data points using nonlinear regression analysis and IC_{50} values were interpolated from the resulting curves using GraphPad Prism 3.03 software.

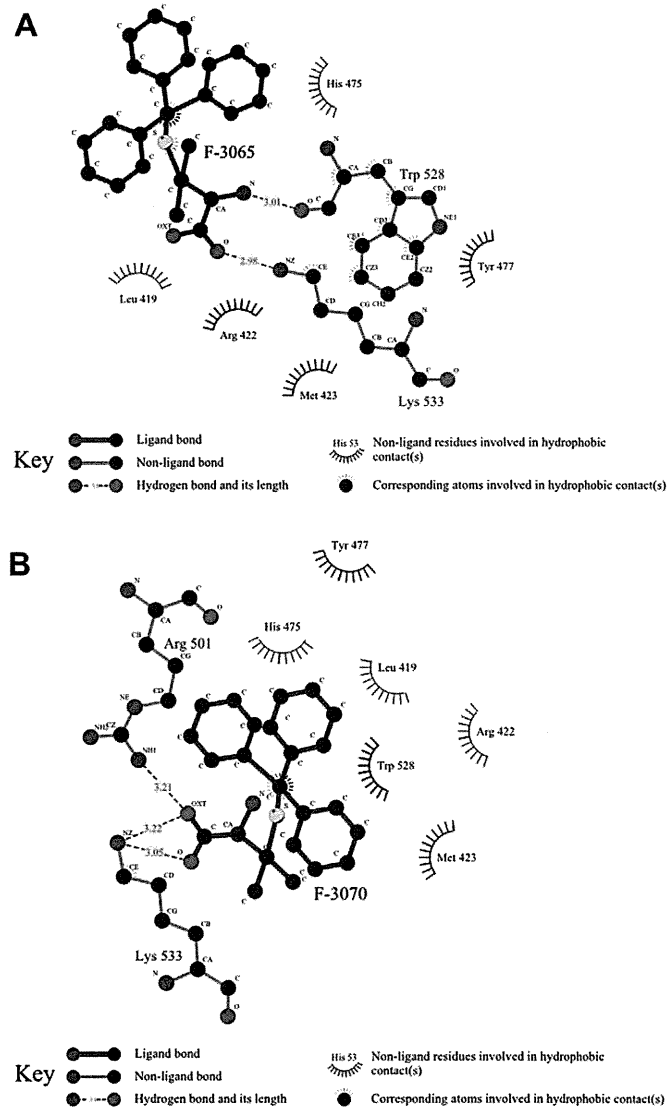


Fig. 3. Panel A: Ligplot diagram for F-3065 docked in NS5B (PDB ID 2d3u). Panel B: Ligplot diagram for F-3070 docked in NS5B (PDB ID 2d3u).

3070, and F-3065 having ≥60% anti-NS5B activity at 100 μM were further pursued for their IC_{50} value determination. This analysis resulted in the identification of F-3070 and F-3065 with near similar IC_{50} values, as the two most potent of the 36 STLC derivatives examined in this investigation, while **9** exhibited ~1.6–1.8-fold higher IC_{50} value compared to the two afore-mentioned compounds. Together, these data suggest that STLC scaffold may offer further scope for improvement of its anti-NS5B activity.

To evaluate the anti-HCV activity of STLC compounds in a more biologically relevant setting, we employed the BHK-NS5B-FRLuc reporter and the Huh7/Rep-Feo1b reporter systems [36,37]. The former reporter system carries stably transfected NS5B and a bicistronic reporter gene, (+)FLuc-(−)UTR-RLuc for cell based investigations of HCV NS5B RdRp inhibitors [36]. The advantage of this system is that it can simultaneously measure intracellular HCV NS5B RdRp activity as reflected by the ratio of *Renilla to* firefly luciferase luminescence and cellular viability which is reflected by the firefly luciferase luminescence, thus enabling the identification of potent non-toxic inhibitors. The Huh7/Rep-Feo1b reporter system, on the other hand, autonomously replicates the subgenomic HCV genotype 1b replicon RNA carrying the firefly luciferase reporter as an indicator of HCV RNA replication, and has been widely employed to identify inhibitors of HCV RNA replication [37].

Only three STLC derivatives F-3070, F-3065, and E-3205 inhibited intracellular NS5B RdRp activity in the BHK-NS5B-FRLuc reporter at 100 μM concentration (Table 2). The two more potent of these, F-3070 and F-3065 exhibited ≥84% inhibition while E-3205 displayed only ~44% inhibition of NS5B RdRp activity, consistent with the in vitro data. In terms of their cytotoxicity parameters, F-3070 and F-3065 did not affect cell viability at 100 μM, as was evident from equivalent levels of firefly luciferase luminescence in compound treated cells versus DMSO controls. Treatment with E-3205 however, decreased cell viability by ~70% at 100 μM concentration. The remaining thirty-three STLC derivatives as well as the parent molecule, exhibited ≥50% reduction in cell viability at 100 μM, with only a marginal 15–30% decrease in intracellular NS5B activity (data not shown), consistent with the in vitro RdRp data.

In the Huh7/Rep-Feo1b reporter system, compounds F-3070 and F-3065 exhibited an overall similar pattern of cell viability and HCV RNA replication inhibition, corresponding to ~73–74% and ~89–91%, respectively at 100 μM concentration (Table 2). E-3205,

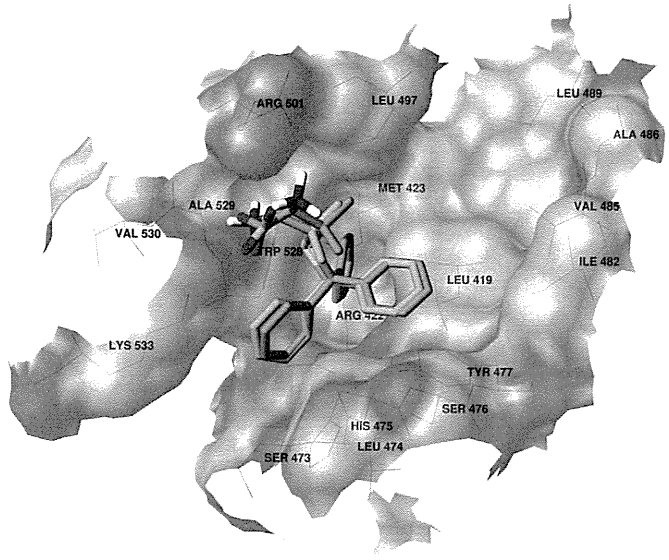


Fig. 4. Docked conformation of F-3065 (orange) and F-3070 (cyan) in NS5B (PDB ID 2d3u). The enzyme surface is shown in atom type color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

however, exhibited decreased cell viability (44%) compared to the other two compounds, though its inhibition of HCV RNA replication (~89%) was similar. It is worth noting here that the inhibition observed in this system may be partly attributed to the cellular toxicity effects of these compounds.

The results in this present study suggest that STLC derivatives inhibit HCV RNA replication by targeting the NS5B polymerase. It is possible that other host factors such as HMKEg are also targeted by STLCs in the HCV replicase complex and needs to be elucidated. These studies provide a platform to optimize the STLC scaffold as a potent anti-NS5B inhibitor. An extensive focused virtual screening approach is ongoing on a database constituted of more than 500 K trityl cysteine analogs to optimize the newly reported lead compounds.

3. Conclusion

In summary, STLC derivatives were identified as novel inhibitors of HCV NS5B polymerase activity in vitro and in cell based assays.

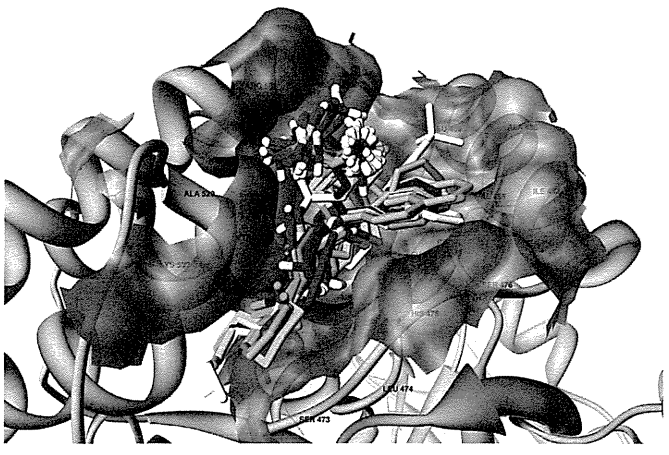


Fig. 5. STLC analogues docked within the HCV NS5B (in pink ribbon) thumb allosteric surface. The compounds overlap in this pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

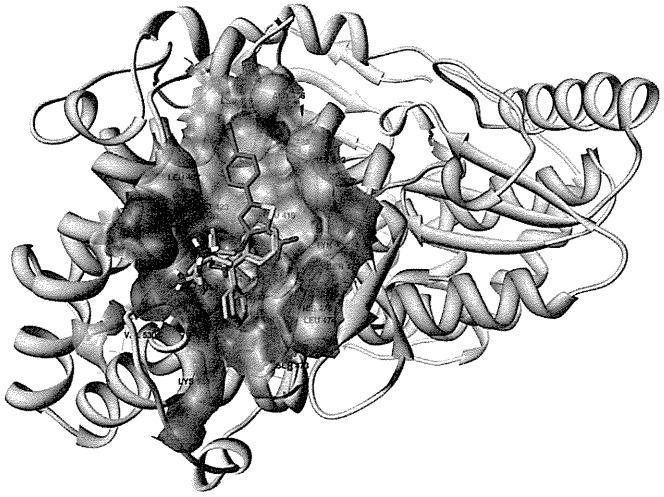


Fig. 6. F-3065 (orange) and F-3070 (cyan) overlapped on the 2d3u co-crystallized ligand (green). HCV NS5B (PDB ID 2d3u, in pink ribbon) and the thumb allosteric surface (in atom type color) are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This study validates structure-based molecular modeling coupled with 3-D QSAR prediction, as a viable strategy for identification of new structural scaffolds targeting NS5B. STLC binding mode analysis revealed a common way by which STLCs bind to the HCV NS5B thumb allosteric site and further suggested that improved STLC derivatives may be achieved by chemical modification at one of the trityl phenyl ring.

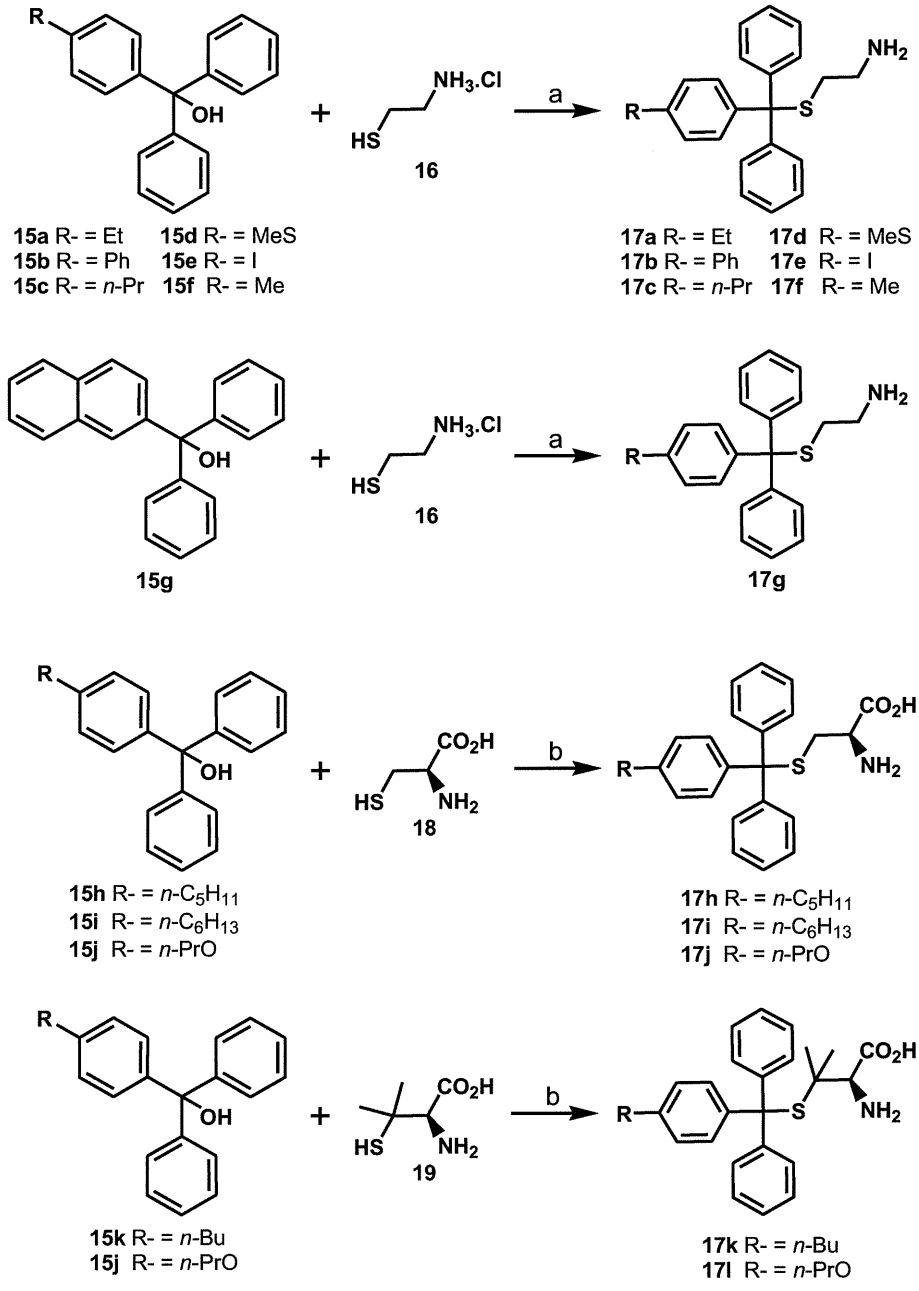
4. Experimental section

4.1. Molecular modeling

All molecules were generated by means of molecular mechanics of Chemaxon Marvin software (http://www.chemaxon.com/). Molecular graphics images were produced using UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco on a 3 GHz AMD CPU equipped IBM-compatible workstation with the Debian 5.0 version of the Linux operating system. Different from the previous protocol, the faster Autodock Vina [38] docking program was used in place of Autodock for all docking studies. Docking assessment was conducted via re-docking, re-docking modeled, cross-docking and cross-docking modeled as previously reported [26]. Autodock Vina proved to be as good as Autodock (data not shown), but much faster in calculations. The compounds were then submitted for structure-based molecular alignment through cross-docking protocols as previously reported [26]. For activity predictions, the previously developed SB 3-D QSAR model was applied without any modification [26]. The program ligplot v. 4.0 was used [39] to generate the ligand/NS5B interaction maps.

4.2. Chemistry

General methods: Melting points were determined using a Büchi capillary instrument and are uncorrected. Optical rotations were measured at the sodium D line (589 nm) at 25 °C with a Perkin–Elmer 241 polarimeter using a 1 dm path length cell. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300, 400 or 500 MHz spectrometers. Chemical shifts (δ) are in parts per million. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Mass spectra were recorded with a Perkin–Elmer SCIEX



Scheme 1. Synthesis of STLC derivatives **17a–l**. Reagents and conditions: (a) TFA, rt, 3 h; (b) $BF_3 \cdot Et_2O$, AcOH, rt, 2 h.

API spectrometer. Elemental analyses were performed on a Thermoquest Flash 1112 series EA analyzer. Elemental analyses were found to be within ±0.4 of the theoritical values. Purity of tested compounds was >95%. All commercially available reagents and solvents were used without further purification. STLC and derivatives **1–14** have been previously described [29]. E-3205, F-3070 and F-3065 were purchased from Bachem. STDC (NSC124676), NSC123139, NSC136870, NSC140909, NSC123529, NSC123138, and NSC126217 were procured from NCI/NIH.

*4.2.1. General procedure for preparation of compounds **17a–g***

At 0 °C and under argon atmosphere, a solution of cysteamine HCl (**16**) (1.33 mmol) was added dropwise to a solution of appropriate alcohol **15** (1.33 mmol) in TFA (5 mL). The reaction mixture was stirred at room temperature for 1 h, evaporated and extracted with a saturated solution of $NaHCO_3$(aq) and EtOAc. The organic

Table 2
Anti-HCV effects of STLC derivatives in cell based reporter assay.

Compound	BHK-NS5B-FR Luc[a]		Huh7/Rep-Feo1b[b]	
	Viability (%)	Inhibition (%)	Viability (%)	Inhibition (%
F-3070	100.0	85.4	72.6	89.5
E-3205	30.2	44.4	44.2	88.6
F-3065	100.0	84.3	74.1	91.2

[a]BHK-NS5B-FRLuc and [b]Huh7/Rep-Feo1b reporter cells were treated with the indicated compounds at 100 μM concentration for 42 h. Cell viability in the BHK-NS5B-FRLuc reporter[a] was estimated as the relative levels of Firefly luciferase in compound treated cells versus DMSO controls, while that in the Huh7/Rep-Feo1b cells[b] was evaluated by the MTS assay. The inhibitory effect of the compounds on NS5B RdRp activity[a] and HCV RNA replication[b] is presented as percent of DMSO treated controls. Data represents an average of three independent experiments in duplicate.

phase was dried over $MgSO_4$, filtered, and evaporated under vacuum. The oil was crystallized from Et_2O or Et_2O/pentane 1:1. The desired compounds **17a–g** were obtained by filtration in the range of 29–47% yield.

*4.2.1.1. 2-[1-(4-Ethylphenyl)-1,1-diphenylmethylthio]ethanamine (**17a**).* Starting alcohol = 1-(4-ethylphenyl)-1,1-diphenylmethanol (**15a**). Yield: 31%; mp 138–140 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 1.23 (t, 3H, J = 7.5 Hz, CH_3), 2.45–2.59 (m, 4H, 2 CH_2), 2.58 (q, 2H, J = 7.5 Hz, CH_2), 7.17 (d, 2H, J = 8.5 Hz, H_{Ar}), 7.22–7.35 (m, 8H, H_{Ar}), 7.43–7.46 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.3 (CH_3), 27.6 (CH_2), 28.6 (CH_2), 37.7 (CH_2), 66.2 (Cq), 126.9 (2× CH), 127.5 (2× CH), 128.2 (4× CH), 129.0 (6× CH), 141.3 (Cq), 142.3 (Cq), 144.2 (2× Cq); MS (ESI): m/z 370 $[M + Na]^+$; Anal. Calcd for $C_{23}H_{25}NS$: C 79.49, H 7.25, N 4.03, found: C, 79.47, H 7.20, N 3.97.

*4.2.1.2. 2-[1,1-Diphenyl-4-(phenyl)phenylmethylthio]ethanamine (**17b**).* Starting alcohol = 1-(4-phenylphenyl)-1,1-diphenylmethanol (**15b**). Yield: 30%; mp 160–162 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 2.50–2.62 (s, 4H, 2 CH_2), 7.27–7.63 (m, 19H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6): δ 28.7 (CH_2), 37.8 (CH_2), 66.2 (Cq), 126.4 (2× CH), 126.6 (2× CH), 127.0 (2× CH), 127.6 (CH), 128.3 (4× CH), 128.9 (2× CH), 129.0 (4× CH), 129.6 (2× CH), 138.6 (Cq), 139.2 (Cq), 143.2 (Cq), 143.9 (2× Cq); MS (ESI): m/z 418 $[M + Na]^+$; Anal. Calcd for $C_{27}H_{25}NS$: C 81.98, H 6.37; N 3.54, found: C 82.26, H 6.44, N 3.73.

*4.2.1.3. 2-[1,1-Diphenyl-4-(propyl)phenylmethylthio]ethanamine (**17c**).* Starting alcohol = 1,1-diphenyl-1-(4-propylphenyl) methanol (**15c**). Yield: 45%; mp 133–135 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 0.94 (t, 3H, J = 7.3 Hz, CH_3), 1.58–1.70 (m, 2H, CH_2), 2.45–2.49 (m, 2H, CH_2), 2.50–2.60 (m, 4H, CH_2), 7.14 (d, 2H, J = 8.3 Hz, H_{Ar}), 7.22–7.34 (m, 8H, H_{Ar}), 7.42–7.45 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.8 (CH_3), 23.9 (CH_2), 28.6 (CH_2), 36.7 (CH_2), 37.7 (CH_2), 66.2 (Cq), 126.9 (2× CH), 128.1 (2× CH), 128.2 (4× CH), 128.9 (2× CH), 129.0 (4× CH), 140.8 (Cq), 141.3 (Cq), 144.2 (2× Cq); MS (ESI): m/z 384 $[M + Na]^+$; Anal. Calcd for $C_{24}H_{27}NS$: C, 79.73, H 7.53, N 3.87, found: C 79.55, H 7.40, N 3.82.

*4.2.1.4. 2-[1,1-Diphenyl-4-(methylthio)phenylmethylthio]ethanamine (**17d**).* Starting alcohol = 1,1-diphenyl-1-(4-methylthiophenyl)methanol (**15d**). Yield: 40%; mp 142–144 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 2.47 (s, 3H, CH_3), 2.50–2.59 (m, 4H, CH_2), 7.20–7.37 (m, 10H, H_{Ar}), 7.43–7.46 (m, 4H, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.3 (CH_3), 28.6 (CH_2), 37.7 (CH_2), 66.0 (Cq), 125.3 (2× CH), 127.0 (2× CH), 128.2 (4× CH), 128.9 (4× CH), 129.6 (2× CH), 137.0 (Cq), 140.3 (Cq), 143.9 (2× Cq); MS (ESI): m/z 388 $[M + Na]^+$; Anal. Calcd for $C_{22}H_{23}NS_2$: C 72.28, H 6.34, N 3.83, found: C 72.00, H 6.35, N 3.77.

*4.2.1.5. 2-[1-(4-Iodophenyl)-1,1-diphenylmethylthio]ethanamine (**17e**).* Starting alcohol = 1-(4-iodophenyl)-1,1-diphenylmethanol (**15e**). Yield: 47%; mp 150–152 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 2.54 (s, 4H, 2 CH_2), 7.21–7.36 (m, 8H, H_{Ar}), 7.41–7.44 (m, 4H, H_{Ar}), 7.68 (d, 2H, J = 10.8 Hz, H_{Ar}); ^{13}C NMR (125 MHz, DMSO-d_6): δ 28.8 (CH_2), 37.7 (CH_2), 66.0 (Cq), 93.4 (Cq), 127.1 (2× CH), 128.3 (4× CH), 128.9 (4× CH), 131.4 (2× CH), 137.0 (2× CH), 143.5 (2× Cq), 143.8 (Cq); MS (ESI): m/z 468 $[M + Na]^+$; Anal. Calcd for $C_{21}H_{20}INS$: C 56.63, H 4.53, N 3.15, found: C 56.60, H 4.61, N 3.22.

*4.2.1.6. 2-[1-(4-Methylphenyl)-1,1-diphenylmethylthio]ethanamine (**17f**).* Starting alcohol = 1-(4-methylphenyl)-1,1-diphenylmethanol (**15f**). Yield: 29%; mp 138–140 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 2.32 (s, 3H, CH_3), 2.48–2.55 (m, 4H, 2 CH_2), 7.13 (d, 2H, J = 8.1 Hz, H_{Ar}), 7.24–7.34 (m, 8H, H_{Ar}), 7.41–7.45 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.5 (CH_3), 29.0 (CH_2), 37.9 (CH_2), 66.2 (Cq), 126.9 (2× CH), 128.2 (4× CH), 128.7 (2× CH), 129.0 (6× CH), 136.1 (Cq), 141.1 (Cq), 144.2 (2× Cq); MS (ESI): m/z 356 $[M + Na]^+$; Anal. Calcd for $C_{22}H_{23}NS$: C 79.23, H 6.95, N 4.20, found: C 78.88, H 7.03, N 4.19.

*4.2.1.7. 2-[1-(2-Naphthyl)-1,1-(diphenyl)methylthio]ethanamine (**17g**).* Starting alcohol = 1-(2-naphthyl)-1,1-diphenylmethanol (**15g**) [32,40]. Yield: 32%; mp 126–128 °C; ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 2.34–2.47 (s, 4H, 2 CH_2), 7.21–7.34 (m, 6H, H_{Ar}), 7.43–7.49 (m, 6H, H_{Ar}), 7.55 (dd, 1H, J = 1.9, 8.9 Hz, H_{Ar}), 7.70–7.83 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, DMSO-d_6): δ 35.6 (CH_2), 40.8 (CH_2), 66.0 (Cq), 126.4 (CH), 126.5 (CH), 126.8 (2× CH), 127.2 (CH), 127.3 (CH), 127.5 (CH), 128.1 (5× CH), 128.2 (CH), 129.1 (4× CH), 131.6 (Cq), 132.3 (Cq), 141.9 (Cq), 144.5 (2× Cq); MS (ESI): m/z 392 $[M + Na]^+$; Anal. Calcd for $C_{25}H_{23}NS$: C 81.26, H 6.27, N 3.79, found: C 81.38, H 6.31, N 3.89.

*4.2.2. General procedure for preparation of compounds **17h–l***

At 0 °C and under argon atmosphere, a solution of $BF_3 \cdot Et_2O$ (1.33 mmol) was added dropwise to a solution of appropriate alcohol **15** (0.86 mmol), L-cysteine (**18**) or L-penicillamine (**19**) (0.77 mmol) in AcOH (1 mL). The reaction mixture was stirred at room temperature for 3 h. Addition of 10% solution of NaOAc (2 mL), then H_2O (2 mL) led to the formation of a gum. After elimination of the supernatant, the final compound was precipitated by addition of pentane or Et_2O. The desired compounds **17h–l** were obtained by filtration in the range of 30–55% yield.

*4.2.2.1. S-[1-(4-Pentylphenyl)-1,1-diphenylmethyl]-L-cysteine (**17h**).* Starting alcohol = 1-(4-pentylphenyl)-1,1-diphenylmethanol (**15h**). Yield: 55%; mp 127–129 °C; $[\alpha]_{589}^{25}$ = +61 (c = 0.52 in MeOH); ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 0.91 (t, 3H, J = 6.7 Hz, CH_3), 1.32–1.39 (m, 4H, 2 CH_2), 1.56–1.66 (m, 2H, CH_2), 2.59 (broad t, 2H, J = 7.9 Hz, CH_2), 2.70 (dd, 1H, J = 9.2, 13.5 Hz, CH_2), 2.82 (dd, 1H, J = 4.2, 13.5 Hz, CH_2), 3.04 (dd, 1H, J = 4.2, 9.2 Hz, CH), 7.13 (d, 2H, J = 8.5 Hz, H_{Ar}), 7.20–7.35 (m, 8H, H_{Ar}), 7.43–7.46 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, CD_3OD): δ 14.4 (CH_3), 23.6 (CH_2), 32.3 (CH_2), 32.7 (CH_2), 34.0 (CH_2), 36.4 (CH_2), 54.6 (CH), 68.0 (Cq), 128.0 (2× CH), 129.1 (6× CH), 130.6 (2× CH), 130.7 (4× CH), 142.8 (Cq), 143.0 (Cq), 145.8 (2× Cq), 172.0 (CO); MS (ESI): m/z 456 $[M + Na]^+$; Anal. Calcd for $C_{27}H_{31}NO_2S$: C 74.79, H 7.21, N 3.23, found: C 74.79, H 7.17, N 3.25.

*4.2.2.2. S-[1-(4-Hexylphenyl)-1,1-diphenylmethyl]-L-cysteine (**17i**).* Starting alcohol = 1-(4-hexylphenyl)-1,1-diphenylmethanol (**15i**). Yield: 40%; mp 129–131 °C; $[\alpha]_{589}^{25}$ = +53 (c = 0.54 in MeOH). ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 0.90 (t, 3H, J = 6.7 Hz, CH_3), 1.30–1.42 (m, 6H, 3 CH_2), 1.54–1.66 (m, 2H, CH_2), 2.59 (broad t, 2H, J = 7.9 Hz, CH_2), 2.70 (dd, 1H, J = 9.2, 13.5 Hz, CH_2), 2.82 (dd, 1H, J = 4.2, 13.5 Hz, CH_2), 3.03 (dd, 1H, J = 4.2, 9.2 Hz, CH), 7.12 (d, 2H, J = 8.3 Hz, H_{Ar}), 7.21–7.35 (m, 8H, H_{Ar}), 7.43–7.46 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, CD_3OD): δ 14.4 (CH_3), 23.7 (CH_2), 30.1 (CH_2), 32.5 (CH_2), 32.8 (CH_2), 34.0 (CH_2), 36.4 (CH_2), 54.7 (CH), 68.0 (Cq), 128.0 (2× CH), 129.1 (6× CH), 130.6 (2× CH), 130.7 (4× CH), 142.8 (Cq), 143.0 (Cq), 145.8 (2× Cq), 172.0 (CO); MS (ESI): m/z 470 $[M + Na]^+$; Anal. Calcd for $C_{28}H_{33}NO_2S$: C 75.13, H 7.43, N 3.13, found: C 74.87, H 7.30, N 3.02.

*4.2.2.3. S-[1,1-Diphenyl-1-(4-propoxyphenyl)methyl]-L-cysteine (**17j**).* Starting alcohol = 1,1-diphenyl-1-(4-propoxyphenyl)methanol (**15j**) [41]. Yield: 43%; mp 144–146 °C; $[\alpha]_{589}^{25}$ = +60 (c = 0.51 in MeOH); ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 1.03 (t, 3H, J = 7.3 Hz, CH_3), 1.72–1.84 (m, 2H, CH_2), 2.68 (dd, 1H, J = 9.2, 13.4 Hz, CH_2), 2.83 (dd,

1H, J = 4.0, 13.4 Hz, CH_2), 3.04 (dd, 1H, J = 4.0, 9.2 Hz, CH), 3.92 (t, 2H, J = 6.4 Hz, CH_2), 6.92 (d, 2H, J = 8.8 Hz, H_{Ar}), 7.22–7.33 (m, 8H, H_{Ar}), 7.43–7.45 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, CD_3OD): δ 10.8 (CH_3), 23.6 (CH_2), 34.2 (CH_2), 55.0 (CH), 67.7 (Cq), 70.5 (CH_2), 114.9 (2× CH), 127.9 (2× CH) 129.1 (4× CH), 130.5 (4× CH), 132.0 (2× CH), 137.2 (Cq), 146.0 (Cq), 146.1 (Cq), 159.3 (Cq), 172.3 (CO); MS (ESI): m/z 444 $[M + Na]^+$; Anal. Calcd for $C_{25}H_{27}NO_3S$: C 71.23, H 6.46, N 3.32, found: C 71.44, H 6.54, N 3.30.

*4.2.2.4. S-[1-(4-Butylphenyl)-1,1-diphenylmethyl]-L-penicillamine (**17k**).* Starting alcohol = 1-(4-butylphenyl)-1,1-diphenylmethanol (**15k**). Yield: 30%; mp 123–125 °C; $[\alpha]_{589}^{25}$ = +171 (c = 0.54 in MeOH); ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 0.93 (t, 3H, J = 7.3 Hz, CH_3), 1.30 (s, 3H, CH_3), 1.32–1.40 (m, 2H, CH_2), 1.42 (s, 3H, CH_3), 1.54–1.64 (m, 2H, CH_2), 1.85 (s, 1H, CH), 2.59 (t, 2H, J = 7.5 Hz, CH_2), 7.14 (d, 2H, J = 8.3 Hz, H_{Ar}), 7.19–7.34 (m, 6H, H_{Ar}), 7.56 (d, 2H, J = 8.5 Hz, H_{Ar}), 7.67–7.70 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, CD_3OD): δ 14.3 (CH_3), 23.4 (CH_2), 25.9 (CH_3), 27.9 (CH_3), 34.7 (CH_2), 36.1 (CH_2), 53.5 (Cq), 61.9 (CH), 69.2 (Cq), 127.9 (2× CH), 129.0 (6× CH) 130.7 (2× CH), 130.8 (2× CH), 130.9 (2× CH), 142.9 (Cq), 143.2 (Cq), 146.0 (Cq), 146.1 (Cq), 170.6 (CO); MS (ESI): m/z 470 $[M + Na]^+$; Anal. Calcd for $C_{28}H_{33}NO_2S$: C 75.13, H 7.43, N 3.13, found: C 75.45, H, 7.53, N 3.32.

*4.2.2.5. S-[1,1-Diphenyl-1-(4-propoxyphenyl)methyl]-L-penicillamine (**17l**).* Starting alcohol = 1,1-diphenyl-1-(4-propoxyphenyl)methanol (**15j**). Yield: 34%; mp 133–135 °C; $[\alpha]_{589}^{25}$ = +69 (c = 0.15 in MeOH); ^{1}H NMR (300 MHz, $CD_3OD + D_2O$): δ 1.03 (t, 3H, J = 7.1 Hz, CH_3), 1.31 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.74–1.81 (m, 2H, CH_2), 1.92 (s, 1H, CH), 3.93 (t, 2H, J = 6.0 Hz, CH_2), 6.87 (d, 2H, J = 8.4 Hz, H_{Ar}), 7.20–7.30 (m, 6H, H_{Ar}), 7.56 (d, 2H, J = 8.4 Hz, H_{Ar}), 7.64–7.72 (m, 4H, H_{Ar}); ^{13}C NMR (100 MHz, CD_3OD): δ 10.8 (CH_3), 23.7 (CH_2), 25.9 (CH_3), 28.4 (CH_3), 53.2 (Cq), 62.0 (CH), 69.2 (Cq), 70.5 (CH_2), 114.6 (2× CH), 127.8 (2× CH) 128.7 (4× CH), 129.5 (4× CH), 131.6 (2× CH), 137.3 (Cq), 146.0 (2× Cq), 159.6 (Cq), 172.6 (CO); MS (ESI): m/z 472 $[M + Na]^+$; Anal. Calcd for $C_{27}H_{31}NO_3S$: C 72.13, H 6.95, N 3.12, found: C 71.99, H 7.00, N 3.11.

4.3. Biological studies

4.3.1. NS5B inhibition assay

Recombinant NS5B carrying the N-terminal histidine-tag was purified from the plasmid pThNS5BCΔ21 expressed in *Escherichia coli* DH5α by Ni-NTA chromatography [33,34]. The compounds were dissolved in dimethylsulfoxide (DMSO) as a 10 mM stock solution and stored at −20 °C. Serial dilutions were made in DMSO immediately prior to the assay. The activity of the compounds against HCV NS5B was evaluated by the standard primer dependent elongation assay as previously described [33,34]. Briefly, preliminary screening was performed in the presence or absence of 100 μM STLC or the indicated derivative in a reaction buffer containing 20 mM Tris–HCl (pH 7.0), 100 mM NaCl, 100 mM Na-glutamate, 0.1 mM DTT, 0.01% BSA, 0.01% Tween-20, 5% glycerol, 20 U/mL of RNasin, 20 μM UTP, 2 μCi [α-^{32}P]UTP, 0.25 μM polyrA/U_{12}, 100 ng NS5BCΔ21 and 1 mM $MnCl_2$. Following 60 min incubation at 30 °C, reactions were terminated by the addition of chilled 5% trichloroacetic acid (TCA) containing 0.5 mM sodium pyrophosphate. Reaction products were precipitated on GF-B filters and quantified on a liquid scintillation counter. NS5B activity in the presence of DMSO control was set at 100% and that in the presence of the STLC derivatives was determined relative to this control. Compounds exhibiting greater than 50% inhibition at 100 μM were evaluated for their IC_{50} values from dose-response curves employing 8–12 concentrations of the compounds in duplicate in two independent experiments. Curves were fitted to data points using nonlinear regression analysis and IC_{50} values were interpolated from the dose-response curves using GraphPad Prism 3.03 software.

4.3.2. Cell culture

BHK-NS5B-FRLuc reporter cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 5% antibiotic-antimycotic, 5% nonessential amino acid, 1 mg/mL G418 and 10 μg/mL blasticidin. Huh7/Rep-Feo1b replicon reporter cells were cultivated in DMEM containing 10% fetal calf serum, 5% antibiotic and 0.5 mg/mL G418. All cell lines were incubated at 37 °C in the presence of 5% CO_2 supplement.

4.3.3. BHK-NS5B-FRLuc reporter assay

The effect of the compounds on intracellular NS5B RdRp activity was screened employing the BHK-NS5B-FRLuc reporter system as previously described [36]. Briefly, BHK-NS5B-FRLuc reporter cells were plated at a confluence of 1×10^4 cells/well in 96 well plates and incubated with DMSO (1%) or the indicated compound (100 μM) for 42 h. Reporter gene expression was measured with a Dual-Glo Luciferase Assay Kit (Promega, USA) in accordance with the manufacturer's instructions. Effect of the compounds on cell viability was estimated as the relative levels of firefly luciferase in compound treated cells versus DMSO controls. The inhibitory effect of the compounds on the intracellular NS5B RdRp activity was evaluated from the percent reduction in RLuc to FLuc luminescence signal in compound treated cells versus DMSO controls.

4.3.4. Huh7/Rep-Feo1b reporter system

The effect of the compounds on HCV RNA replication was screened employing the Huh7/Rep-Feo1b replicon reporter cells as previously described [42]. Briefly, 1×10^4 Huh7/Rep-Feo1b cells were plated in 96 well plates and treated with 100 μM concentration of the indicated compound or DMSO for 42 h. The concentration of DMSO in cell culture was kept constant at 1.0%. Cell viability was measured by the colorimetric MTS assay employing the CellTiter $96AQ_{ueous}$ one solution assay reagent (Promega, USA). Inhibitory effect of the compounds on HCV RNA replication was measured as the relative levels of firefly luciferase signals in compound treated cells versus DMSO controls.

Acknowledgments

This work was supported by the National Institute of Health Research Grants DK066837 and CA153147 to N.K.-B. We would like to thank Prof. Garland R. Marshall (Washington University of St. Louis - MO) for critical reading of the manuscript.

References

[1] A. Wasley, M.J. Alter, Epidemiology of hepatitis C: geographic differences and temporal trends, Semin. Liver Dis. 20 (2000) 1–16.
[2] A. Grakoui, H.L. Hanson, C.M. Rice, Bad time for Bonzo? Experimental models of hepatitis C virus infection, replication, and pathogenesis, Hepatology 33 (2001) 489–495.
[3] G.M. Lauer, B.D. Walker, Hepatitis C virus infection, N. Engl. J. Med. 345 (2001) 41–52.
[4] I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al., Hepatitis C virus infection is associated with the development of hepatocellular carcinoma, Proc. Natl. Acad. Sci. USA 87 (1990) 6547–6549.
[5] J. Ruiz, B. Sangro, J.I. Cuende, O. Beloqui, J.I. Riezu-Boj, J.I. Herrero, J. Prieto, Hepatitis B and C viral infections in patients with hepatocellular carcinoma, Hepatology 16 (1992) 637–641.
[6] J.H. Hoofnagle, A.M. di Bisceglie, The treatment of chronic viral hepatitis, N. Engl. J. Med. 336 (1997) 347–356.
[7] J.M. Pawlotsky, Pathophysiology of hepatitis C virus infection and related liver disease, Trends Microbiol. 12 (2004) 96–102.

[8] J.J. Feld, T.J. Liang, HCV persistence: cure is still a four letter word, Hepatology 41 (2005) 23–25.
[9] P. Ferenci, Pegylated interferon plus ribavirin for chronic hepatitis C: the role of combination therapy today, tomorrow and in the future, Minerva Gastroenterol. Dietol. 52 (2006) 157–174.
[10] J.M. Pawlotsky, Virology of hepatitis B and C viruses and antiviral targets, J. Hepatol. 44 (2006) S10–S13.
[11] C. Sheridan, New Merck and Vertex drugs raise standard of care in hepatitis C, Nat. Biotechnol. 29 (2011) 553–554.
[12] C. Rice, Perspective: miles to go before we sleep, Nature 474 (2011) S8.
[13] K. Garber, Hepatitis C: move over interferon, Nat. Biotechnol. 29 (2011) 963–966.
[14] Q.L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359–362.
[15] K. Tsukiyama-Kohara, N. Iizuka, M. Kohara, A. Nomoto, Internal ribosome entry site within hepatitis C virus RNA, J. Virol. 66 (1992) 1476–1483.
[16] V. Brass, D. Moradpour, H.E. Blum, Molecular virology of hepatitis C virus (HCV): 2006 update, Int. J. Med. Sci. 3 (2006) 29–34.
[17] S.E. Behrens, L. Tomei, R. De Francesco, Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus, EMBO J. 15 (1996) 12–22.
[18] C.H. Hagedorn, E.H. van Beers, C. De Staercke, Hepatitis C virus RNA-dependent RNA polymerase (NS5B polymerase), Curr. Top. Microbiol. Immunol. 242 (2000) 225–260.
[19] D. Moradpour, V. Brass, E. Bieck, P. Friebe, R. Gosert, H.E. Blum, R. Bartenschlager, F. Penin, V. Lohmann, Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication, J. Virol. 78 (2004) 13278–13284.
[20] R. De Francesco, C.M. Rice, New therapies on the horizon for hepatitis C: are we close? Clin. Liver Dis. 7 (2003) 211–242 (xi).
[21] A.D. Kwong, L. McNair, I. Jacobson, S. George, Recent progress in the development of selected hepatitis C virus NS3.4A protease and NS5B polymerase inhibitors, Curr. Opin. Pharmacol. 8 (2008) 522–531.
[22] S. Bressanelli, L. Tomei, A. Roussel, I. Incitti, R.L. Vitale, M. Mathieu, R. De Francesco, F.A. Rey, Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus, Proc. Natl. Acad. Sci. USA 96 (1999) 13034–13039.
[23] C.A. Lesburg, M.B. Cable, E. Ferrari, Z. Hong, A.F. Mannarino, P.C. Weber, Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site, Nat. Struct. Biol. 6 (1999) 937–943.
[24] H. Ago, T. Adachi, A. Yoshida, M. Yamamoto, N. Habuka, K. Yatsunami, M. Miyano, Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus, Structure 7 (1999) 1417–1426.
[25] S. Bressanelli, L. Tomei, F.A. Rey, R. De Francesco, Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides, J. Virol. 76 (2002) 3482–3492.
[26] I. Musmuca, A. Caroli, A. Mai, N. Kaushik-Basu, P. Arora, R. Ragno, Combining 3-D quantitative structure-activity relationship with ligand based and structure based alignment procedures for in silico screening of new hepatitis C virus NS5B polymerase inhibitors, J. Chem. Inf. Model. 50 (2010) 662–676.
[27] J.E. Silverman, M. Ciustea, A.M. Shudofsky, F. Bender, R.H. Shoemaker, R.P. Ricciardi, Identification of polymerase and processivity inhibitors of vaccinia DNA synthesis using a stepwise screening approach, Antivir. Res. 80 (2008) 114–123.
[28] K.Y. Zee-Cheng, C.C. Cheng, Experimental antileukemic agents. Preparation and structure-activity study of S-tritylcysteine and related compounds, J. Med. Chem. 13 (1970) 414–418.
[29] S. Debonis, D.A. Skoufias, R.L. Indorato, F. Liger, B. Marquet, C. Laggner, B. Joseph, F. Kozielski, Structure-activity relationship of S-trityl-L-cysteine analogues as inhibitors of the human mitotic kinesin Eg5, J. Med. Chem. 51 (2008) 1115–1125.
[30] H.Y. Kaan, D.D. Hackney, F. Kozielski, The structure of the kinesin-1 motor-tail complex reveals the mechanism of autoinhibition, Science 333 883–885, New York, N.Y.
[31] H.Y. Kaan, J. Weiss, D. Menger, V. Ulaganathan, K. Tkocz, C. Laggner, F. Popowycz, B. Joseph, F. Kozielski, Structure-activity relationship and multidrug resistance study of new S-trityl-L-cysteine derivatives as inhibitors of Eg5, J. Med. Chem. 54 (2011) 1576–1586.
[32] E. Weber, K. Skobridis, A. Wierig, I. Goldberg, Triarylmethanol host compounds. Synthesis, crystalline complex formation and X-ray crystal structures of three inclusions species, J. Inclus. Phenom. Mol. 28 (1997) 163–179.
[33] N. Kaushik-Basu, A. Bopda-Waffo, T.T. Talele, A. Basu, Y. Chen, S.G. Kucukguzel, 4-Thiazolidinones: a novel class of hepatitis C virus NS5B polymerase inhibitors, Front. Biosci. 13 (2008) 3857–3868.
[34] N. Kaushik-Basu, A. Bopda-Waffo, T.T. Talele, A. Basu, P.R. Costa, A.J. da Silva, S.G. Sarafianos, F. Noel, Identification and characterization of coumestans as novel HCV NS5B polymerase inhibitors, Nucleic Acids Res. 36 (2008) 1482–1496.
[35] Y. Chen, A. Bopda-Waffo, A. Basu, R. Krishnan, E. Silberstein, D.R. Taylor, T.T. Talele, P. Arora, N. Kaushik-Basu, Characterization of aurintricarboxylic acid as a potent hepatitis C virus replicase inhibitor, Antivir. Chem. Chemother. 20 (2009) 19–36.
[36] J.C. Lee, C.K. Tseng, K.J. Chen, K.J. Huang, C.K. Lin, Y.T. Lin, A cell-based reporter assay for inhibitor screening of hepatitis C virus RNA-dependent RNA polymerase, Anal. Biochem. 403 (2010) 52–62.
[37] Y. Itsui, N. Sakamoto, S. Kakinuma, M. Nakagawa, Y. Sekine-Osajima, M. Tasaka-Fujita, Y. Nishimura-Sakurai, G. Suda, Y. Karakama, K. Mishima, M. Yamamoto, T. Watanabe, M. Ueyama, Y. Funaoka, S. Azuma, M. Watanabe, Antiviral effects of the interferon-induced protein guanylate binding protein 1 and its interaction with the hepatitis C virus NS5B protein, Hepatol. 50 (2009) 1727–1737 Baltimore, Md.
[38] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J. Comput. Chem. 31 (2010) 455–461.
[39] A.C. Wallace, R.A. Laskowski, J.M. Thornton, LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions, Protein Eng. 8 (1995) 127–134.
[40] J. Barluenga, F.J. Fananas, R. Sanz, C. Marcos, M. Trabada, On the reactivity of o-lithioaryl ethers: tandem anion translocation and Wittig rearrangement, Org. Lett. 4 (2002) 1587–1590.
[41] Y. Kawakami, Y. Sakai, A. Okada, Synthesis and polymerization of mono-p-alkoxy-substituted triphenylmethyl acrylate, Polym. J. 22 (1990) 705–718.
[42] K. Kim, K.H. Kim, H.Y. Kim, H.K. Cho, N. Sakamoto, J. Cheong, Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway, FEBS Lett. 584 (2010) 707–712.

PLOS ONE

A Subclone of HuH-7 with Enhanced Intracellular Hepatitis C Virus Production and Evasion of Virus Related-Cell Cycle Arrest

Asako Murayama[1], Nao Sugiyama[1], Seiko Yoshimura[2], Mitsuko Ishihara-Sugano[2], Takahiro Masaki[1], Sulyi Kim[1], Takaji Wakita[1], Shunji Mishiro[3], Takanobu Kato[1]*

1 Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, **2** Corporate Research and Development Center, Toshiba Corporation, Kanagawa, Japan, **3** Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan

Abstract

Hepatitis C virus (HCV) cell culture system with JFH-1 strain and HuH-7 cells enabled us to produce infectious HCV particles *in vitro*, and such system is useful to explore the anti-HCV compounds and to develop the vaccine against HCV. In the present study, we describe the derivation of a cell line that permits improved production of HCV particles. Specifically, we characterized several subclones that were isolated from the original HuH-7 cell line by limiting dilution. These HuH-7 subclones displayed a notable range of HCV production levels following transfection by full-genome JFH-1 RNA. Among these subclones, HuH-7T1 produced HCV more efficiently than other subclones and Huh-7.5.1 that is known to be highly permissive for HCV replication. Upon transfection with full-genome RNA, HCV production was increased ten-fold in HuH-7T1 compared to Huh-7.5.1. This increase in viral production correlated with increased efficiency of intracellular infectious virus production. Furthermore, HCV replication did not induce cell cycle arrest in HuH-7T1, whereas it did in Huh-7.5.1. Consequently, the use of HuH-7T1 as host cells could provide increased population of HCV-positive cells and elevated viral titer. In conclusion, we isolated a HuH-7 subclone, HuH-7T1, that supports efficient HCV production. High efficiency of intracellular infectious virus production and evasion of cell cycle arrest were important for this phenotype. We expect that the use of this cell line will facilitate analysis of the underlying mechanisms for HCV particle assembly and the cell cycle arrest caused by HCV.

Citation: Murayama A, Sugiyama N, Yoshimura S, Ishihara-Sugano M, Masaki T, et al. (2012) A Subclone of HuH-7 with Enhanced Intracellular Hepatitis C Virus Production and Evasion of Virus Related-Cell Cycle Arrest. PLoS ONE 7(12): e52697. doi:10.1371/journal.pone.0052697

Editor: Kui Li, University of Tennessee Health Science Center, United States of America

Received July 25, 2012; **Accepted** November 19, 2012; **Published** December 20, 2012

Funding: This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan, and from the Ministry of Education, Culture, Sports, Science and Technology. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: SY, MIS and SM are employees of Toshiba Corporation. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: takato@nih.go.jp

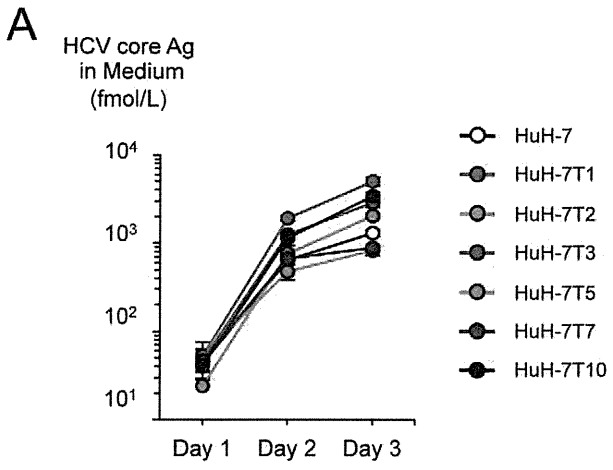
Introduction

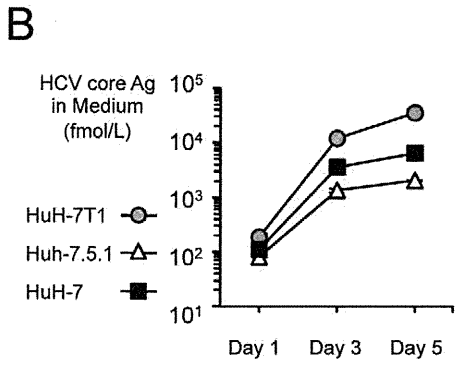
Hepatitis C virus (HCV) is a major cause of chronic liver disease [1,2]. Currently, approximately 200 million people are infected with HCV worldwide and are at continued risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3,4]. Historically, the lack of a cell culture system capable of producing virus particles hampered progress in the field of HCV research. Subsequently, a robust HCV cell culture system was developed using HCV JFH-1 strain that had been cloned from a fulminant hepatitis patient [5,6,7]. JFH-1 was the first HCV strain that could replicate and produce HCV particles autonomously *in vitro*, thereby facilitating investigation of the entire life cycle of the virus. This HCV cell culture system employed HuH-7 cell line, which was established from a hepatocellular carcinoma [5,8], as a host. Since the HCV replicon system enabling HCV subgenomic RNA replication was originally developed using HuH-7 [9], this cell line has been used in the research field of HCV most frequently. However, HuH-7 is known to be heterogeneous. Notably, Saintz et al. reported that HuH-7 cell lines obtained from various laboratories exhibit distinct morphological, cell growth, and HCV susceptibility properties [10]. We also found that single-cell cloning of HuH-7 maintained in our laboratory yielded multiple subclones that exhibited different characteristics of HCV infection and replication [11]. In the present study, we derived cell lines from original HuH-7 obtained from the cell bank and screened to identify a cell line with improved production of infectious HCV particles. As we report here, we obtained one such clone (HuH-7T1) and performed an initial characterization of the HCV life cycle in this host.

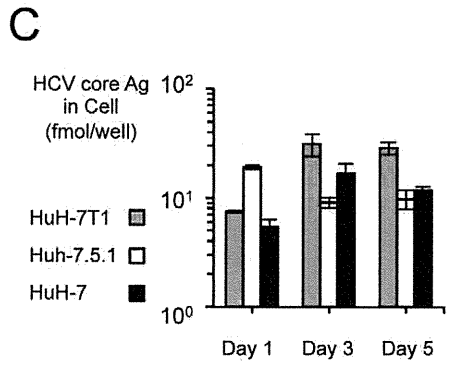
Materials and Methods

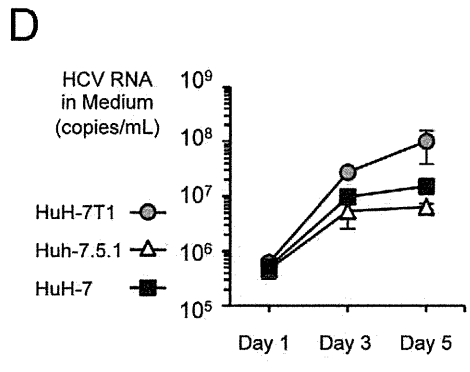
Cell culture

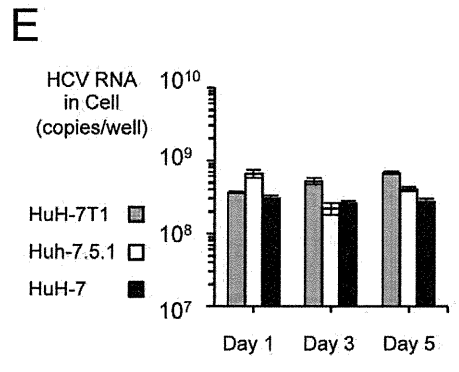
The original HuH-7 cell line (catalog number; JCRB0403) was purchased from Health Science Research Resources Bank (Osaka, Japan). The cured cell line, Huh-7.5.1, was a kind gift from Dr. Francis V. Chisari (Scripps Research Institute, La Jolla, CA) [6]. These cell lines were cultured at 37°C in a 5% CO_2 environment using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum.

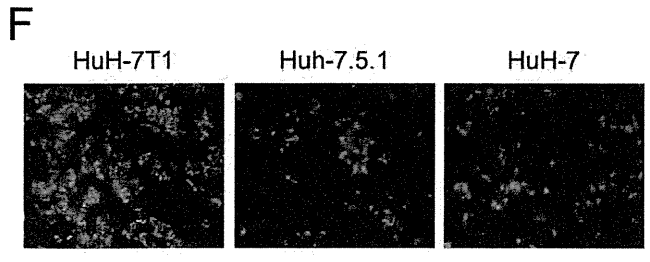

A
HCV core Ag
in Medium
(fmol/L)
10⁴
10³
10²
10¹
Day 1
Day 2
Day 3
HuH-7
HuH-7T1
HuH-7T2
HuH-7T3
HuH-7T5
HuH-7T7
HuH-7T10


B
HCV core Ag
in Medium
(fmol/L)
10⁵
10⁴
10³
10²
10¹
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


C
HCV core Ag
in Cell
(fmol/well)
10²
10¹
10⁰
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


D
HCV RNA
in Medium
(copies/mL)
10⁹
10⁸
10⁷
10⁶
10⁵
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


E
HCV RNA
in Cell
(copies/well)
10¹⁰
10⁹
10⁸
10⁷
HuH-7T1
Huh-7.5.1
HuH-7
Day 1
Day 3
Day 5


F
HuH-7T1
Huh-7.5.1
HuH-7

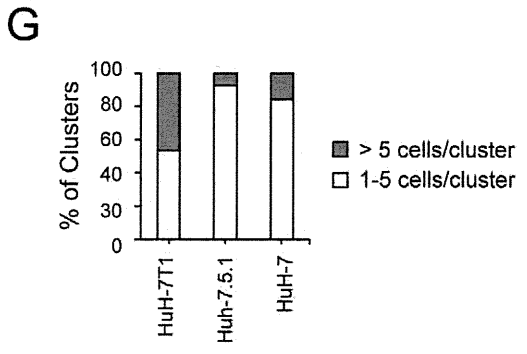

G
% of Clusters
100
80
60
40
30
0
HuH-7T1
Huh-7.5.1
HuH-7
> 5 cells/cluster
1-5 cells/cluster

Figure 1. HCV production in HuH-7 subclones. (A) Two micrograms of JFH-1 RNA were electroporated into the HuH-7 subclones. Culture medium was harvested at Days 1, 3, and 5, and HCV core protein levels in the culture medium were measured. Assays were performed three times independently, and data are presented as mean ± standard deviation. (B–D) Comparison of HCV production among HuH-7T1, Huh-7.5.1 and HuH-7. HCV core protein (B and C) and HCV RNA (D and E) levels in cells and culture medium were measured. Assays were performed three times independently, and data are presented as mean ± standard deviation. (F) HCV-positive cells at Day 3 post-transfection were visualized with anti-core antibody (green); nuclei were visualized with DAPI (blue). (G) The number of HCV positive cells within a cluster were counted and classified into 2 groups (>5 cells/cluster and 1–5/cluster). More than 100 foci were counted. The percentages of each group are shown.
doi:10.1371/journal.pone.0052697.g001

Single cell cloning by limiting dilution

The original HuH-7 cell line was diluted with medium at 1 cell/mL and seeded at 100 μL/well in 96-well plates. Six subclones were obtained and resulting subclones were expanded and stored at −80°C pending further characterization. The characteristics of obtained subclones were maintained after passages over several months.

HCV constructs and RNA transfection

pJFH1 is a full-length JFH-1 clone whose construction was reported previously [5]. pSGR-JFH1-Luc (a JFH-1 subgenomic replicon construct containing a firefly luciferase-encoding reporter gene) and pSGR-JFH1/GND-Luc (a replication-defective mutant construct) also were described previously [12]. pH77S.2, a full-length H77S.2 construct, was a kind gift from Dr. Stanley M Lemon (University of North Carolina at Chapel Hill, Chapel Hill, NC). This construct is a derivative of strain H77S (genotype 1a) harboring an additional mutation, and produces infectious virus in cultured cells after full-genome RNA transfection [13]. RNA synthesis and transfection were performed as described previously [14,15].

Quantification of HCV core protein and RNA

The concentration of HCV core protein in the culture medium and cell lysate was measured using a chemiluminescent enzyme immunoassay (Lumipulse Ortho HCV antigen, Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions. The concentration of HCV RNA was measured as described previously [16].

Determination of infectivity titers

To determine the intracellular infectivity of the HCV RNA-transfected cells, a cell lysate of HCV RNA-transfected cells cultured in a 10 cm dish was generated by subjecting the cells to four freeze-thaw cycles. The culture supernatant and cell lysate were serially diluted and inoculated into naive Huh-7.5.1 seeded at 1×10^4 cells/well in poly-D-lysine-coated 96-well plates (BD, Franklin Lakes, NJ), and the inoculated plates were incubated for another 3 days at 37°C. The cells were then fixed with methanol, and the infected foci were visualized by staining with anti-core antibody (clone 2H9 [5,8] for JFH-1 and c7-50 (Abcam, Cambridge, MA) for H77S.2) and Alexa Fluor 488 Goat Anti-mouse IgG (Invitrogen, Carlsbad, CA). The infectivity titer was quantified by counting the stained foci and expressing the value as the number of focus-forming units (FFU).

Table 1. Infectivity titers in culture medium and cells of HuH-7T1 and Huh-7.5.1 transfected with JFH-1 RNA.

Cell Line	Infectivity		Secretion Rate
	Medium (FFU/dish)	Cells (FFU/dish)	
HuH-7T1	$2.23\times10^6\pm3.15\times10^5$ *	$1.11\times10^4\pm1.15\times10^3$ *	$2.00\times10^2\pm1.98\times10^1$ *
Huh-7.5.1	$9.92\times10^4\pm2.98\times10^4$	$1.34\times10^2\pm1.42\times10^1$	$7.30\times10^2\pm1.40\times10^2$

*$P<0.05$ as compared with Huh-7.5.1.
doi:10.1371/journal.pone.0052697.t001

Flow cytometric analysis

For cell cycle distribution analyses, cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU) for 4 h prior to harvest. The harvested cells were fixed in 4% paraformaldehyde, permeabilized, and stained with anti-nonstructural (NS) 5A antibody (clone KS0265-1; raised by immunization with JFH-1 NS5A) and Alexa Fluor 647 Goat Anti-mouse IgG (Invitrogen). Incorporated EdU was stained with Alexa Fluor 488 azide by using the Click-iT EdU flow cytometry kit (Invitrogen) according to the manufacturer's instructions. Following treatment with RNase A, 7-aminoactinomycin D (7-AAD) was added. Samples were analyzed using a FACS Calibur flow cytometer. The population of cells in G0/G1, S, or G2/M phases of the cell cycle was determined using FlowJo software (Tree Star, Inc., Ashland, OR).

Immunostaining

Infected cells were cultured on glass cover slips in a 12-well plate. Cells were fixed in 4% paraformaldehyde and permeabilized. After blocking, HCV-positive cells were visualized by staining with anti-core antibody (clone 2H9) and Alexa Fluor 488 Goat Anti-mouse IgG, and nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI).

Virus entry assay

HCV pseudo type virus (HCVpp) harboring the JFH-1 E1 and E2 glycoprotein was prepared as described previously [11]. Target

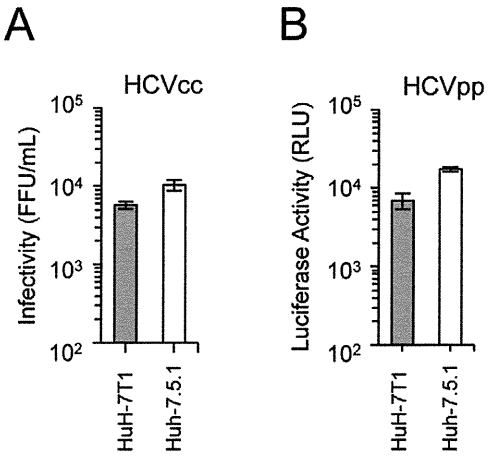


Figure 2. Comparison of infection in HuH-7T1 and Huh-7.5.1. (A) Infection of HCVcc into HuH-7T1 and Huh-7.5.1. The cells were fixed 3 days after infection and infected foci were counted. (B) Infection of HCVpp into HuH-7T1 and Huh-7.5.1. The cells were harvested 3 days after infection, and the luciferase activity in the cell lysate was measured.
doi:10.1371/journal.pone.0052697.g002

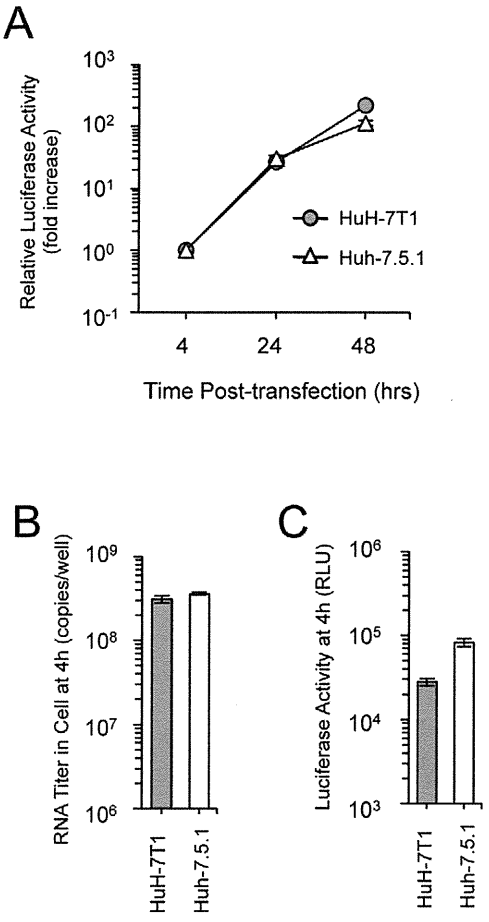


Figure 3. Comparison of replication in HuH-7T1 and Huh-7.5.1. (A) Five micrograms of JFH-1 subgenomic replicon RNA was electroporated into HuH-7T1 and Huh-7.5.1. The cells were harvested at indicated time points. The luciferase activity in the cell lysates was normalized to the data at 4 h after transfection; values are expressed as fold increases. (B and C) Comparison of transfection and translation efficiencies. Five micrograms of JFH-1/GND-Luc RNA was transfected into HuH-7T1 and Huh-7.5.1. The cells were harvested at 4 h after transfection, and the amount of transfected RNA in cells (B) and luciferase activity in the cell lysates (C) were measured.
doi:10.1371/journal.pone.0052697.g003

cells were seeded into 48-well plates at a density of 2×10^4 cells/well. On the following day, a 100-μL aliquot of each diluted supernatant containing HCVpp was added to each well and incubated for 3 h. The supernatants were replaced with fresh medium, and the cells were incubated for 72 h at 37°C. Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI). Luciferase activities were quantified using a luciferase assay system (Promega). Assays were performed in triplicate; data are presented as mean ± standard deviation.

Cell culture-generated HCV JFH-1 virus (HCVcc) was prepared as follows: culture medium from JFH-1 RNA-transfected cells was collected and 40-times concentrated using Amicon Ultra-15 filter units (100-kDa cutoff; Millipore, Bedford, MA) and stored at −80°C until use. HCVcc was inoculated into target cells, and infectivity titer was determined as described.

Luciferase assay

Luciferase activity of subgenomic reporter replicon RNA-transfected cell lysate was measured as described previously [14,15].

Statistical analysis

Significant differences were evaluated using the Student's t-test. $P<0.05$ was considered significant.

Results

Isolation of HuH-7 subclones with improved HCV production

To obtain cell lines with improved HCV production potential, we used limiting dilution to establish six subclones (HuH-7T1, HuH-7T2, HuH-7T3, HuH-7T5, HuH-7T7, and HuH-7T10) from the original HuH-7 purchased from the cell bank. We transfected JFH-1 RNA into each of these subclones and measured the level of core protein in the culture medium. These subclones displayed a range of core protein production levels. (Fig. 1A). Compared to the original HuH-7, four (HuH-7T1, HuH-7T3, HuH-7T5 and HuH-7T10) and two (HuH-7T2 and HuH-7T7) subclones produced higher or lower amounts of HCV core protein, respectively. Among these subclones, we chose HuH-7T1 for further characterization because this subclone produced HCV core protein at the highest level (Fig. 1A). Then, we compared core protein production of HuH-7T1 with Huh-7.5.1, a cell line reported to be highly permissive for HCV replication [6]. After JFH-1 RNA transfection, HCV core protein level in the culture medium of HuH-7T1 was 17.6-fold higher than that seen with Huh-7.5.1 (Fig. 1B). HCV core protein levels in cell lysate of HuH-7T1 were lower at Day 1, but higher at Days 3 and 5 after transfection, compared to Huh-7.5.1 (Fig. 1C). HCV RNA levels in the culture medium and cell lysates of these cells showed similar tendencies (Fig. 1D and 1E). The infectivity titer in culture medium of HuH-7T1 at Day 5 was 22.5-fold higher than that of Huh-7.5.1 (Table 1), indicating that HuH-7T1 supported production of infectious HCV particles to levels higher than those seen in Huh-7.5.1. The number of HCV-positive cells of HuH-7T1 at Day 5 also was higher than that seen with Huh-7.5.1 (Fig. 1F). The percentage of HCV positive cell clusters consisting of more than 5 cells was higher in HuH-7T1 than in Huh-7.5.1 (Fig. 1G). We also assessed if HuH-7T1 produced higher amount of core protein after infection of HCVcc. HuH-7T1 produced higher amount of HCV core protein than Huh-7.5.1 after JFH-1 virus infection at the same multiplicity of infection (Fig. S1A), and HCV core protein levels in cell lysate of HuH-7T1 were also higher than that of Huh-7.5.1 (Fig. S1B). These data indicated that HuH-7T1 produced infectious HCV particles more efficiently than Huh-7.5.1 after JFH-1 RNA transfection and JFH-1 virus infection.

The original HuH-7 could produce higher amount of HCV core protein than Huh-7.5.1 after JFH-1 RNA transfection (Fig. 1B). However, in the experiment of HCVcc infection, HuH-7 produced lower amount of HCV core protein than Huh-7.5.1 in culture medium (Fig. S1A) and in cell lysate (Fig. S1B).

Analysis of HCV life cycle in HuH-7T1

To clarify the underlying mechanism of the enhanced virus production in HuH-7T1, we assessed the efficiencies of each step in the HCV life cycle. The viral infection step was assessed by using HCVcc and HCVpp. The HCVcc system uses cell culture-generated HCV and detects steps from viral attachment through replication. On the other hand, the HCVpp system uses the

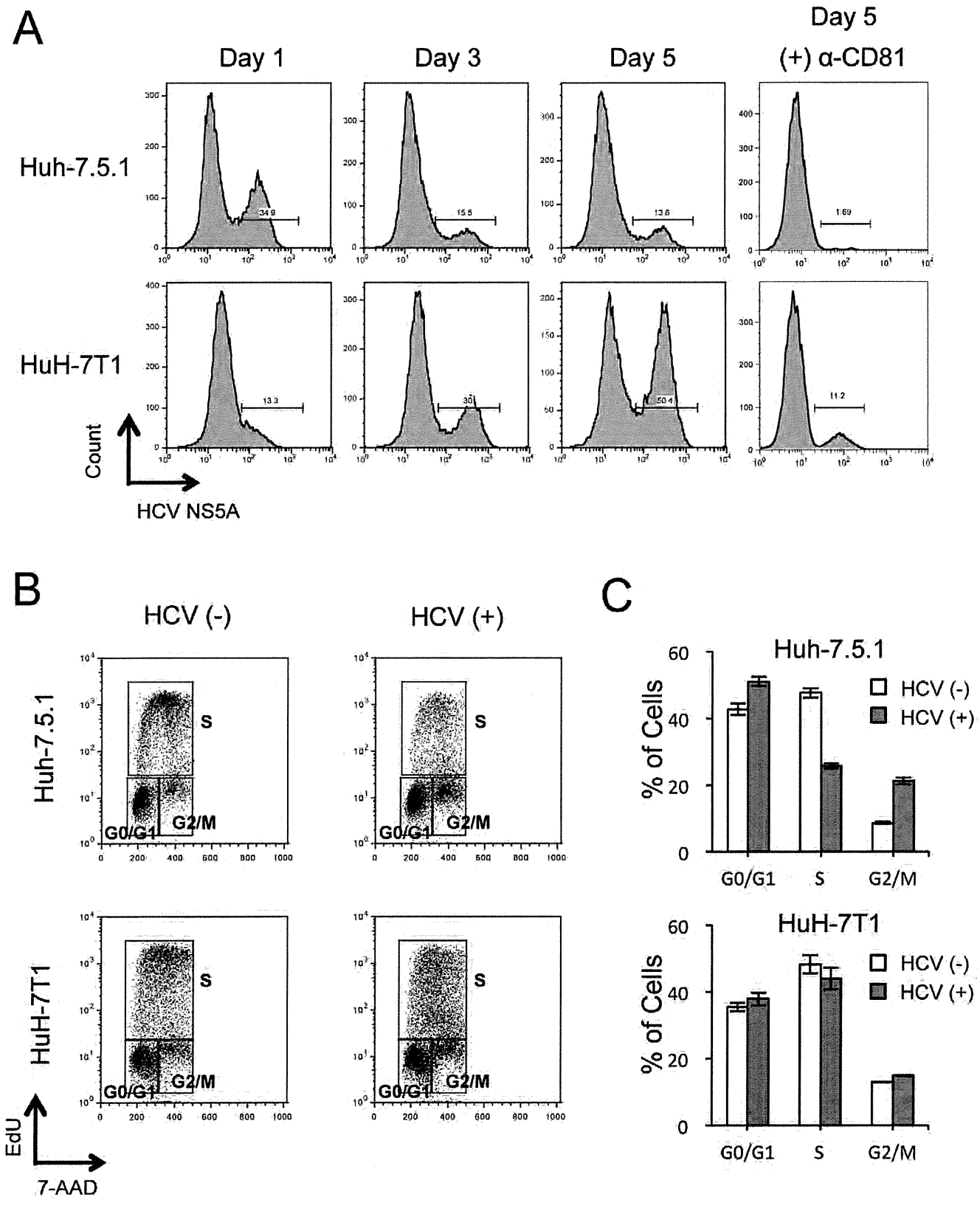


Figure 4. Effects of HCV replication on cell proliferation of Huh-7.5.1 and HuH-7T1. (A) Population of HCV-positive cells after JFH-1 RNA transfection. Two micrograms of JFH-1 RNA was electroporated into Huh-7.5.1 and HuH-7T1 and cultured with or without 10 mg/mL of anti-CD81 antibody (clone JS-81, BD). Cells were harvested at Days 1, 3, and 5. After fixing, cells were stained with anti-NS5A antibody and analyzed by flow cytometry. (B, C) Cell cycle distribution of HCV-positive and -negative cells after JFH-1 RNA transfection. Two micrograms of JFH-1 RNA was electroporated into Huh-7.5.1 and HuH-7T1. Cells were pulse-labeled with EdU and analyzed for cell cycle distribution. The percentages of cells in G0/