Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 76 (2007) 93–97
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

Identification and characterization of mutations conferring resistance to an HCV RNA-dependent RNA polymerase inhibitor *in vitro*

Liangjun Lu*, Tatyana Dekhtyar, Sherie Masse, Ron Pithawalla, Preethi Krishnan, Wenping He, Teresa Ng, Gennadiy Koev, Kent Stewart, Dan Larson, Todd Bosse, Rolf Wagner, Tami Pilot-Matias, Hongmei Mo, Akhteruzumman Molla

Abbott Laboratories, Global Pharmaceutical Research and Development, Abbott Park, IL 60064, United States

Received 22 September 2006; accepted 26 April 2007

Abstract

Compound A-837093, a non-nucleoside HCV RNA-dependent RNA polymerase inhibitor, displayed nanomolar potencies against HCV genotypes 1a and 1b replicons. It also exhibited an excellent metabolic profile and achieved high plasma and liver concentrations in animals. In order to characterize the development of resistance to this anti-HCV agent, HCV subgenomic 1b strain N replicon cells were cultured in the presence of A-837093 with G418. Mutations S368A, Y448H, G554D, Y555C, and D559G in the NS5B polymerase gene were identified that led to substantial decreases in the susceptibilities of 1b genotype replicons to the inhibitor A-837093. However, the resistant mutants remained susceptible to HCV protease inhibitor BILN-2061 and alpha interferon as well as to a different class of non-nucleoside HCV polymerase inhibitor. In addition, each single resistant mutation identified significantly reduced the replication capacity of mutant compared to wild-type replicon. These findings provide a strategic guide for the future development of non-nucleoside inhibitors of HCV NS5B polymerase.

Keywords: Antiviral; HCV; Polymerase inhibitor; Resistance

The current standard of therapeutic care for HCV genotype 1 involves 48 weeks of dosing with pegylated alpha interferon (PEG-IFN-α) and ribavirin (RBV). With these regimens, approximately 50% of genotype 1 patients achieve sustained virologic response (SVR) whereas the other 50% of patients are null responders, slow responders or relapsers (viral load rebound during treatment) (Davis et al., 1998; Davis, 1999; McHutchison et al., 1998; Fried et al., 2002). Importantly, genotype 1 patients who do not achieve an early virologic response (EVR, defined as >2 log viral load decline at week 12) are highly unlikely to achieve SVR. Because of the poor tolerability and the limited efficacy of IFN-based regimens, much effort has been focused on the development of inhibitors of virally encoded enzymes essential for HCV replication.

HCV RNA-dependent RNA polymerase (NS5B) plays an essential role in viral replication. Several classes of HCV polymerase inhibitors binding to either the active site or allosteric sites have been identified (for review: Carroll and Olsen, 2006; Koch and Narjes, 2006). Previously, we have reported the antiviral activity and resistance profile of A-782759, a member of the benzothiadiazine series of NS5B polymerase inhibitors (Mo et al., 2005). Further optimization of the A-782759 series has led to the identification of the inhibitor A-837093 (Fig. 1) that showed impressive potency against both 1a strain H77 and 1b strain N replicon cells with a 50% effective concentration (EC_{50}) of 11 or 6 nM, respectively *in vitro*. This is approximately a 12-fold improvement in potency compared with A-782759 (EC_{50} = 77 nM against 1b strain N replicons). The 50% cytotoxic concentration (CC_{50}) of A-837093 was about 32 μM, yielding a therapeutic window (CC_{50}/EC_{50}) of greater than 5000 for this inhibitor. In addition, A-837093 showed significantly improved metabolism and pharmacokinetic (PK) properties compared to A-782759, and achieves high concentrations in liver, the primary site of HCV infection (data not shown).

HCV has a high replication rate *in vivo* and its polymerase has poor fidelity, thus, it is highly likely that drug-resistant HCV variants will emerge in patients treated with HCV polymerase

* Corresponding author at: Department R4CQ, Building AP52-N, 200 Abbott Park Road, Abbott Park, IL 60064-6217, United States. Tel.: +1 847 935 8314; fax: +1 847 938 2756.
E-mail address: liangjun.lu@abbott.com (L. Lu).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.04.005

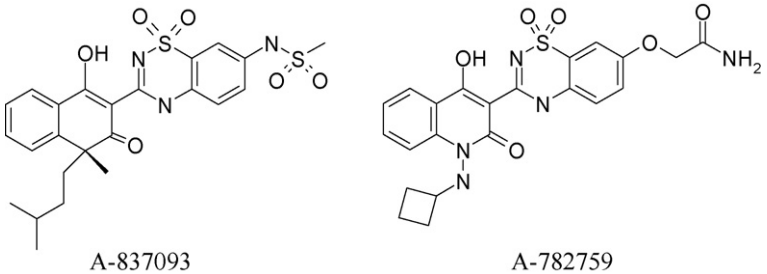

Fig. 1. Chemical structures of A-837093 and A-782759.

inhibitors. In fact, *in vitro* selection of HCV replicons resistant to a number of polymerase inhibitors has been reported (Migliaccio et al., 2003; Nguyen et al., 2003; Tomei et al., 2003, 2004; Chan et al., 2004; Ludmerer et al., 2005; Mo et al., 2005). It was found that HCV replicons resistant to polymerase inhibitors contain specific amino acid substitutions within the NS5B polymerase. For example, a single S282T mutation in NS5B polymerase conferred resistance to 2′-C-MeATP and other 2′-methyl-nucleotides (Migliaccio et al., 2003), whereas substitutions at residues 495 or 496 of NS5B were responsible for significant resistance to benzimidazoles (Tomei et al., 2003). Similarly, we and others have found that specific mutation(s) (H95R, N411S, M414L, M414T, Y448H, C451R, and/or G558R) in the NS5B gene led to decreased susceptibility to benzothiadiazines (Nguyen et al., 2003; Mo et al., 2005). In order to identify mutants resistant to A-837093, a genotype 1b strain N replicon cell line obtained and licensed from Dr. Stanley Lemon (Yi et al., 2002) was used. 2×10^4 replicon-containing cells were plated in a 10-cm diameter cell culture dish and cultured in the presence of selection medium containing Dulbecco's modified eagle medium (DMEM, Invitrogen) plus 10% fetal bovine serum (FBS, Atlanta Biologicals), 400 μg/ml G418, 2 μg/ml blasticidin and A-837093 at a concentration 10 times above its EC_{50} (final concentration was 60 nM). Under these conditions, cells harboring drug-resistant replicons formed colonies after approximately 2–3 weeks. Replicon cells incubated in the selection medium without inhibitor were used for the parental cell control. Selection with A-837093 yielded 48 resistant colonies from 20,000 replicon cells thus yielding a frequency of resistant colony formation of about 0.24%.

Ten replicon colonies from the selection 3 weeks post-treatment were randomly picked and expanded for genotypic characterization (Table 1). Total cellular RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions and subjected to RT-PCR as described before (Mo et al., 2005). Amplified DNA fragments were purified using the QIAquick PCR purification kit (Qiagen) and nucleotide sequences were determined by automated sequencing using BigDye terminator v3.1 (Applied Biosystems). Compared to the parental wild-type 1b strain N replicon, each colony contained one to three mutations in the NS5B polymerase gene and a majority of the colonies (7 out of 10) were found by population sequencing to result in a single mutation in NS5B (either S368A, G554D, Y555C, or D559G) (Table 1). This is the first time each of these mutations has been reported to confer resistance to a benzothiadiazine inhibitor. One of the selected colonies contained two mutations in NS5B: Y448H and Y586C. The mutation Y448H has already been reported as conferring resistance to A-782759 (Mo et al., 2005). The most frequent mutation detected was D559G, which appeared in 4 of 10 colonies analyzed.

In order to characterize the resistant replicon colonies for their susceptibilities to A-837093, 3000 replicon-containing cells per well were seeded into a 96-well plate and grown in DMEM supplemented with 5% FBS. The culture medium was removed the following day and replaced with fresh medium containing A-837093 in a series of dilutions. Replicon cells were incubated 3 days with inhibitor, after which total cellular RNA was extracted using RNeasy-96 (Qiagen) and the copy number of the HCV RNA was determined by a quantitative, real time RT-PCR (Taqman) assay. The EC_{50} was then calculated by non-linear regression analysis using Prism (GraphPad Software, Inc.). Results from representative colonies each containing a single NS5B mutation except colony 3 which showed two mutations are shown in Table 2. The highest levels of resistance to A-837093 (~800-fold) were observed with a colony containing the G554D mutation. The colony that contained the S368A mutation exhibited approximately 500-fold changes in EC_{50}. Other colonies with either Y448H, Y555C or D559G mutations were ~100–200-fold resistant to A-837093 (Table 2). All of these mutants remained highly susceptible to the protease inhibitor BILN-2061 (Table 2).

The positions of the mutations were mapped onto the three-dimensional structure of NS5B polymerase along with the locations of several inhibitor classes (Fig. 2). Residues 368, 448, 554, 555 and 559 are located within or close to the known bind-

Table 1
Genotype of lb-N replicon colonies selected by A-837093

Colony	Mutation(s) in NS5B gene
1	S368A
2	S368A
3	Y448H, Y586C
4	G554D
5	S121A, G554D
6	Y555C
7	D559G
8	D559G
9	D559G
10	K155R, D177D/Y, D559G

Table 2
Phenotype of representative lb-N replicon colonies selected by A-837093

NS5B mutation (colony)	EC_{50} (μM) (fold change[a])	
	A-837093	BILN-2061
1b-N	0.006	0.002
S368A (1)	3.40 ± 1.53 (567)	0.004 ± 0.001
Y448H, Y586C (3)	0.84 ± 0.29 (140)	0.004 ± 0.001
G554D (4)	4.94 ± 0.94 (823)	0.005 ± 0.003
Y555C (6)	1.28 ± 0.42 (213)	0.002 ± 0.002
D559G (7)	0.77 ± 0.40 (192)	0.002 ± 0.001

Data are shown as mean of three different experiments.
[a] Fold changes in EC_{50} = EC_{50} of the mutant tested/EC_{50} of the wild-type replicon.

Table 3
Susceptibilities of resistant mutant clones to HCV inhibitors

Mutant clone	Fold change in EC_{50} vs. wild type[a]			
	A-837093	Shire 2	BILN-2061	IFN-α
wt 1b-N	1 (1 nM)	1 (2 μM)	1 (9 nM)	1 (1 IU/ml)
S368A	122 ± 64	3.3 ± 1.0	1.1 ± 0.1	3.0 ± 1.1
Y448H	21 ± 10	1.0 ± 0.3	0.7 ± 0.4	1.9 ± 0.5
G554D	430 ± 177	3.2 ± 1.5	1.2 ± 0.2	1.7 ± 0.2
D559G	328 ± 52	4.0 ± 17	1.1 ± 0.4	1.8 ± 1.2

EC_{50} of inhibitors against wt are shown in parentheses. We were unable to measure the EC_{50} against mutant Y555C due to very poor replication capacity.
[a] Data are shown as average ± S.D. of four different experiments.

ing site of benzothiadiazine inhibitors (Tedesco et al., 2006). Residues 121, 155 and 177 are far away from any known inhibitor binding site and likely confer minor, if any, resistance to A-837093. Interestingly, mutation M414T, which was identified as a major resistance mutation against A-782759 (Mo et al., 2005), was not found in this study. To confirm this observation, we have tested susceptibility of replicon cells carrying the M414T mutant to A-837093. These replicon cells displayed about a twofold change in EC_{50} relative to wild type. We speculate that this is due to a diminished interaction between residue 414 and the A-ring or isopentyl group of A-837093 (the portions of A-837093 expected to be in direct contact with residue 414), relative to A-782759, potentially due to altered orientation from the presence of the "dialkyl" moiety.

To further characterize the mutations that are responsible for resistance to A-837093 in HCV replicon cell culture, we have introduced single mutations into replicon constructs containing a firefly luciferase reporter gene and determined their susceptibility to A-837093 as well as other HCV inhibitors in a 4 days transient transfection assay as previously described (Mo et al., 2005). Luciferase activity was also measured 4 h post-transfection in the absence of any inhibitor to determine the efficiency of transfection. Replicon constructs containing single NS5B mutations are shown in Table 3. All mutant clones tested displayed significantly reduced susceptibility to A-837093 with EC_{50} values increased by 21–430-fold. Due to the poor replication capacity of the Y555C mutant clone (<1% of wild type), we could not obtain accurate data on the phenotype of this mutant when treated with inhibitors.

In order to assess cross-resistance to other classes of HCV inhibitors, we evaluated the susceptibility of the mutant clones against a polymerase inhibitor (Shire 2), a protease inhibitor (BILN-2061) and IFN-α. Not surprisingly, none of the mutations (S368A, Y448H, G554D, or D559G) had an effect on susceptibility to BILN-2061 and IFN-α (Table 3). Compound Shire 2 was identified by Shire Pharmaceuticals Group plc. (Wang et al., 2003) as a non-nucleoside polymerase inhibitor that bound to an allosteric site of NS5B polymerase (Fig. 2). This compound also retained its potency against these mutants (Table 3). Previously, we have shown that mutations M414T and Y448H displayed

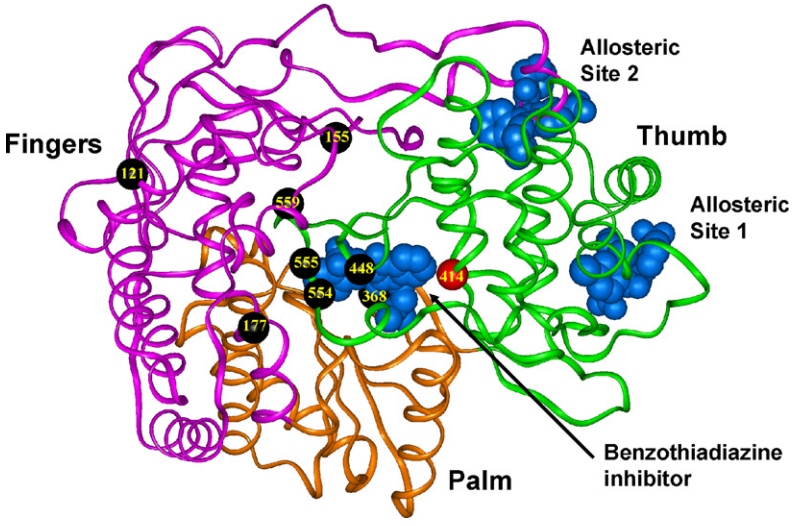

Fig. 2. Crystal structure of HCV polymerase (PDB entry 1C2P, Lesburg et al., 1999) showing the locations of inhibitor binding sites and mutation positions. A ribbon representation of the protein in three colors shows the fingers (pink), palm (brown) and thumb (green) domains. Mutations (from Table 1) are shown as black balls at the alpha carbons of the specified residues. Residue 414, not identified as a mutation position in this study, is shown as a red ball for discussion purposes. Residue 586 is located in a C-terminal membrane anchor domain and is not shown. Space-filling representations of various inhibitors (blue) are also shown: benzothiadiazine (Tedesco et al., 2006); allosteric site 1 inhibitor (Wang et al., 2003); allosteric site 2 inhibitor (Di Marco et al., 2005). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

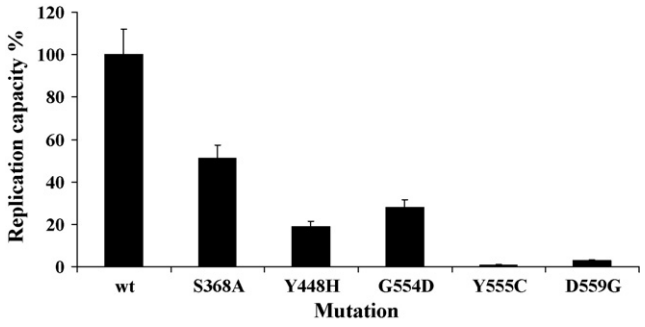

Fig. 3. Comparison of replication capacity of wild-type replicon with the recombinant mutant replicons. Cells were transfected either with wild-type or with mutant replicon RNA and firefly luciferase activities were measured at 4 h and 4 days after transfection. The replication capacity for each mutant was calculated by comparing the luciferase activity generated by the mutants to that generated by wild-type replicon at day 4, after adjusting for minor differences in transfection efficiencies (the 4 h luciferase activity). The data are average of at least two separate experiments with six replicates in each experiment. The error bars represent the S.D.

reduced replication capacity. To understand the effect of the newly identified polymerase mutations on replication capacity, RNAs from each mutant replicon clone were transfected in the absence of inhibitor into Huh7 cells that had been "cured" of the replicon (Lu et al., 2004), and luciferase activity was measured at 4 days post-transfection. All mutant clones exhibited reduced replication capacity compared to their parent wild-type clone. The S368A, Y448H, G554D, Y555C and D559G mutants retained 51%, 19%, 28%, 0.9%, and 3% of wild-type replication capacity, respectively (Fig. 3).

In conclusion, we have selected HCV replicons *in vitro* that have increased resistance to A-837093. Single amino acid substitutions at residues 368, 448, 554, 555, and 559 of NS5B polymerase resulted in high levels of resistance to A-837093. Furthermore, lack of cross-resistance may allow this class of polymerase inhibitor to be used in combination with other classes of polymerase inhibitors, and/or protease inhibitors and/or IFN for treatment of HCV infection.

Acknowledgments

We thank Stanley Lemon and Minkyung Yi for providing replicon cell lines. We also thank John Randolph, Keith McDaniel, David Betebenner and Larry Klein for providing compounds as well as Dale Kempf, Bill Kohlbrenner and Shing Chang for providing continuous support.

References

Carroll, S.S., Olsen, D.B., 2006. Nucleoside analog inhibitors of hepatitis C virus replication. Infect. Disord. Drug Targets 6 (1), 17–29.

Chan, L., Pereira, O., Reddy, T.J., Das, S.K., Poisson, C., Courchesne, M., Proulx, M., Siddiqui, A., Yannopoulos, C.G., Nguyen-Ba, N., Roy, C., Nasturica, D., Moinet, C., Bethell, R., Hamel, M., L'Heureux, L., David, M., Nicolas, O., Courtemanche-Asselin, P., Brunette, S., Bilimoria, D., Bedard, J., 2004. Discovery of thiophene-2-carboxylic acids as potent inhibitors of HCV NS5B polymerase and HCV subgenomic RNA replication. Part 2. Tertiary amides. Bioorg. Med. Chem. Lett. 14, 797–800.

Davis, G.L., 1999. Combination treatment with interferon and ribavirin for chronic hepatitis C. Clin. Liver Dis. 3, 811–826.

Davis, G.L., Esteban-Mur, R., Rustgi, V., Hoefs, J., Gordon, S.C., Trepo, C., Shiffman, M.L., Zeuzem, S., Craxi, A., Ling, M.H., Albrecht, J., 1998. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. N. Engl. J. Med. 339, 1493–1499.

Di Marco, S., Volpari, C., Tomei, L., Altamura, S., Harper, S., Narjes, F., Koch, U., Rowley, M., De Francesco, R., Migliaccio, G., Carfi, A., 2005. Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. J. Biol. Chem. 280, 29765–29770.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.

Koch, U., Narjes, F., 2006. Allosteric inhibition of the hepatitis C virus NS5B RNA dependent RNA polymerase. Infect. Disord. Drug Targets 6 (1), 31–41.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6, 937–943.

Lu, L., Pilot-Matias, T.J., Stewart, K.D., Randolph, J.T., Pithawalla, R., He, W., Huang, P.P., Klein, L.L., Mo, H., Molla, A., 2004. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro. Antimicrob. Agents Chemother. 48, 2260–2266.

Ludmerer, S.W., Graham, D.J., Boots, E., Murray, E.M., Simcoe, A., Markel, E.J., Grobler, J.A., Flores, O.A., Olsen, D.B., Hazuda, D.J., LaFemina, R.L., 2005. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. Antimicrob. Agents Chemother. 49 (5), 2059–2069.

McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S., Albrecht, J.K., 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N. Engl. J. Med. 339, 1485–1492.

Migliaccio, G., Tomassini, J.E., Carroll, S.S., Tomei, L., Altamura, S., Bhat, B., Bartholomew, L., Bosserman, M.R., Ceccacci, A., Colwell, L.F., Cortese, R., De Francesco, R., Eldrup, A.B., Getty, K.L., Hou, X.S., LaFemina, R.L., Ludmerer, S.W., MacCoss, M., McMasters, D.R., Stahlhut, M.W., Olsen, D.B., Hazuda, D.J., Flores, O.A., 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. J. Biol. Chem. 278 (49), 49164–49170.

Mo, H., Lu, L., Pilot-Matias, T., Pithawalla, R., Mondal, R., Masse, S., Dekhtyar, T., Ng, T., Koev, G., Stoll, V., Stewart, K.D., Pratt, J., Donner, P., Rockway, T., Maring, C., Molla, A., 2005. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. Antimicrob. Agents Chemother. 49, 4305–4314.

Nguyen, T.T., Gates, A.T., Gutshall, L.L., Johnston, V.K., Gu, B., Duffy, K.J., Sarisky, R.T., 2003. Resistance profile of a hepatitis C virus RNA-dependent RNA polymerase benzothiadiazine inhibitor. Antimicrob. Agents Chemother. 47, 3525–3530.

Tedesco, R., Shaw, A.N., Bambal, R., Chai, D., Concha, N.O., Darcy, M.G., Dhanak, D., Fitch, D.M., Gates, A., Gerhardt, W.G., Halegoua, D.L., Han, C., Hofmann, G.A., Johnston, V.K., Kaura, A.C., Liu, N., Keenan, R.M., Lin-Goerke, J., Sarisky, R.T., Wiggall, K.J., Zimmerman, M.N., Duffy, K.J., 2006. 3-(1,1-Dioxo-2H-(1,2,4)-benzothiadiazin-3-yl)-4-hydroxy-2(1H)-quinolinones, potent inhibitors of hepatitis C virus RNA-dependent RNA polymerase. J. Med. Chem. 49 (3), 971–983.

Tomei, L., Altamura, S., Bartholomew, L., Biroccio, A., Ceccacci, A., Pacini, L., Narjes, F., Gennari, N., Bisbocci, M., Incitti, I., Orsatti, L., Harper, S., Stansfield, I., Rowley, M., De Francesco, R., Migliaccio, G., 2003. Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 77, 13225–13231.

Tomei, L., Altamura, S., Bartholomew, L., Bisbocci, M., Bailey, C., Bosserman, M., Cellucci, A., Forte, E., Incitti, I., Orsatti, L., Koch, U., De Francesco, R., Olsen, D.B., Carroll, S.S., Migliaccio, G., 2004. Characterization of the

inhibition of hepatitis C virus RNA replication by nonnucleosides. J. Virol. 78, 938–946.

Wang, M., Ng, K.K., Cherney, M.M., Chan, L., Yannopoulos, C.G., Bedard, J., Morin, N., Nguyen-Ba, N., Alaoui-Ismaili, M.H., Bethell, R.C., James, M.N., 2003. Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. J. Biol. Chem. 278, 9489–9495.

Yi, M., Bodola, F., Lemon, S.M., 2002. Subgenomic hepatitis C virus replicons inducing expression of a secreted enzymatic reporter protein. Virology 304, 197–210.