Bioluminescence imaging of Hepatitis C virus NS3/4A serine protease activity in cells and living animals

Licui Wang, Qiuxia Fu, Yafeng Dong, Yong Zhou, Shuaizheng Jia, Juan Du, Fang Zhao, Yingli Wang, Xiaohui Wang, Jianchun Peng, Shuhua Yang, Linsheng Zhan

Laboratory of Blood-Borne Virus, Beijing Institute of Transfusion Medicine, 27(9) Tai Ping Road, Beijing 100850, China

1. Introduction

Hepatitis C virus (HCV) chronically infects approximately 170 million people worldwide, increasing the risk of developing cirrhosis and hepatocellular carcinoma (Alter et al., 1999; Conry-Cantilena et al., 1996; Shepard et al., 2005). The current combination therapy of pegylated-interferon-alpha and ribavirin is not adequate and many patients are discouraged from IFN-based treatment because of severe side-effects (Husa and Husova, 2001; Manns et al., 2001). New and better therapeutic strategies are therefore needed.

Replication of the HCV largely depends on the maturation of the viral polyprotein precursor encoded in the positive-sense single-stranded RNA of the HCV NH2–C–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH, which is cleaved into 10 viral proteins. Associated with the cofactor NS4A, the serine protease domain of non-structural protein 3 (NS3) cleaves the viral polyepitope at four sites to liberate the functional forms of NS3, NS4A, NS4B, NS5A and NS5B proteins that are required for viral replication (Moradpour et al., 2007; Penin et al., 2004; Tomei et al., 1993). For this reason, HCV NS3/4A serine protease becomes a promising target for developing new therapeutics.

The development of new HCV NS3/4A protease inhibitors has been hampered by the lack of appropriate model systems in vivo. Cell culture systems and several alternative cell-based systems for monitoring the activity of HCV NS3/4A protease have played crucial roles in the development of new HCV NS3/4A protease inhibitors (Boonstra et al., 2009; Chung et al., 2005; Lee et al., 2003, 2004; Martinez and Clotet, 2003; Pan et al., 2009; Sabariegos et al., 2009). The cell-based system, however, cannot serve to assess the efficacy, toxicity, and bioavailability of the new therapeutics in vivo. The chimpanzee model is, although considered to be the most valuable, of limited application due to the expense and short supply (Liu, 2006). Thus, development of a new strategy for monitoring the HCV NS3/4A protease activity in vivo would contribute significantly to the development of new anti-HCV therapies.

New imaging tools, many based on optical signals and capable of quantifying protein interactions in vivo, have advanced the study of protein activities in cells and living animals. In particular, luciferase fragment complementation provides a platform for near real-time detection and characterization of protein–protein interactions and enables a wide range of novel applications in drug discovery (Coppola et al., 2008; Villalobos et al., 2007; Binkowski et al., 2009). The strategy monitors protein interactions as an increase in reporter activity caused by reversible proximity of two
fragments, which are inactive when separate, so are reconstitute activity when brought together. Here, we describe an HCV NS3/4A protease reporter system using luciferase fragment complementation strategy for the first time. Our data show that this HCV NS3/4A protease reporter system is a sensitive, dynamic, and quantitative reporter of HCV NS3/4A protease activity in both cells and living animals. This system will constitute a new tool to allow the efficient screening of HCV NS3/4A protease inhibitors.

2. Materials and methods

2.1. Plasmid construction

The plasmid ANLuc(NS5A/B)BCLuc encoding peptide A fused in-frame with the NLuc(1-1245 bp) followed by the NS5A/5B protease recognition sequence, peptide B and CLuc(1197–1653 bp) were assembled by PCR and cloned into the pCI Mammalian Expression Vector as described previously (Coppola et al., 2008). pepA (with linker) was added to the NH2-terminal fragment of luciferase (1-1245 bp) using primer 5’-CTGAGATATGGACACATGAATGACGGTCCTGTACCCTGACCGCCGGACAGTATAGAACAAGAAGCCGAAAACATAAAAA-3’ by PCR. The NS5A/B cleavage site (with triglycine linkers on each side) was added to the COOH-terminal end of pepANLuc fragment using primer 5’-CAACAGCCCTGGATGAGAAGGAGGAGGAGGAGGCGATGAGATGGAAGAGTGCGCCTCACACCTG-3’. Primers for NS3/4A were 5’-TGCGAAGGGCCAAGGAAAGGAGGAGGAGGAGGAGGCGATGAGATGGAAGAGTGCGCCTCACACCTG-3’. PepB (with linker) was added NH2 terminally to the COOH-terminal fragment of luciferase (1197–1653 bp) using primer 5’-CTGACAGGCCACAGAAGGAAAGGAGGAGGAGGAGGAGGCGATGAGATGGAAGAGTGCGCCTCACACCTG-3’. The full-length NS5A/B cleavage site was 5’-GAATTC-3’ and 5’-CGGACGCGGCCGACGCACTCTGCAAGACCGACGGCCGAGCAGACAAGCTTACCCGCAATT-3’. The complete plasmid was constructed by ligation of Xhol-pepANLuc fragment using 5’-GAATTC-3’ and 5’-CGGACGCGGCCGACGCACTCTGCAAGACCGACGGCCGAGCAGACAAGCTTACCCGCAATT-3’. Primers for NS3A/B cleavage site were 5’-GAATTC-3’ and 5’-CGGACGCGGCCGACGCACTCTGCAAGACCGACGGCCGAGCAGACAAGCTTACCCGCAATT-3’. The plasmid ANLuc(NS5A/B)BCLuc was constructed under the same conditions as described above. Primers for NS4A/4B cleavage site were 5’-GAATTC-3’ and 5’-CGGACGCGGCCGACGCACTCTGCAAGACCGACGGCCGAGCAGACAAGCTTACCCGCAATT-3’.

2.2. Cell culture and transfection

Hepa1-6, HepG2, Huh-7 and HCV full-length replicon (FL) cells were maintained in Dulbecco’s medium Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and penicillin(100 μg/ml)/streptomycin (100 μg/ml). In 24-well plates, cells were seeded at a density of 6 x 10⁴ cells well per well in 500 μl of DMEM/10% FBS. After incubation at 37 °C overnight or until cells were approximately 70% confluent, cells were transfected with various plasmid constructs using Lipofectamine™ 2000 reagent (Invitrogen). As an internal control for transfection efficiency, 0.02 μg Renilla luciferase plasmid, pRL-TK (Promega) was cotransfected with 1 μg of each expression vector in each experiment.

2.3. Generation of HCV replicon cells

Plasmid pCon1-FL containing full-length HCV sequences of genotype 1b was kindly provided by Charles M. Rice of the Rockefeller University. In vitro synthesis of HCV RNA, electroporation, and G418 selection were performed as described previously (Lohmann et al., 1999). Stable cells containing the self-replicating HCV replicons were selected and maintained in the presence of 500 μg of G418 (Invitrogen) per ml. NS3-specific antibodies (US Biological) were used for Western Blotting detection of HCV proteins.

2.4. Treatment of HCV replicon cells with IFN-α

HCV replicon cells were seeded in 24-well plates at a density of 2 x 10⁴ cells per well. After incubation at 37 °C overnight, cells were treated with various concentrations of IFN-α (0, 20, 100, 200, 500 units/ml Schering-Plough Corp., Kenilworth, NJ, USA). Three days later, cells were transfected with ANLucΔNS5A/B/BCLuc at the same concentration of drugs, while cells were incubated for two more days. Before they were collected from each well, the luciferase activity and NS3/4A expression level were measured by in vivo imaging and Western Blotting, respectively.

2.5. Mice

Specific-pathogen-free (SPF) 8-week-old female C57BL/6 mice were obtained from the Animal Center of the Academy of Military Medical Sciences and were housed under SPF conditions at National Beijing Center for Drug Safety Evaluation and Research (NBDSER). Animals were treated according to the guidelines of NBDSER and Beijing Institute of Transfusion Medicine.

2.6. Hydrodynamic tail vein injections

In each experiment, 20 μg of plasmid DNA in 2 ml of 0.9% NaCl solution was injected rapidly, in less than 5–8 s, into the tail vein of the mouse, using a 27-gauge needle.

2.7. Luciferase activity monitoring by the IVIS camera in cells and living animals

For bioluminescence imaging (BLI) in cells, the cells were harvested and resuspended in 100 μl PBS, 8 min prior to monitoring light emission. 100 μl (1 mg/ml in PBS) D-luciferin was added to the cell suspension. Cells were then scanned using the Xenogen IVIS-50 optical imaging system (Xenogen Corporation, Alameda, CA). For in vivo bioluminescence imaging, mice which were transfected with firefly luciferase reporter gene were anesthetized, and 8 min before monitoring light emission, the animals were injected with 100 μl (15 mg/ml in PBS) of D-luciferin i.p. Mice were then scanned using the Xenogen IVIS-50 optical imaging system. Regions of interest (ROI) were drawn by the instrument automatically. As a result, 90% of the bioluminescence signal was contained inside ROI. The quantification was reported as the maximum photon flux within ROI. The bioluminescence signal was represented as photons/s/cm²/sr.

2.8. Western blotting

Protein was estimated by BCA™ Protein Assay Kit from Pierce. Cell lysates were separated by SDS-PAGE, and protein expression was detected by Western blotting analysis using antibodies. ANLucBCLuc was detected using a goat polyclonal antibody to luciferase (Promega), actin was detected using a rabbit polyclonal antibody (Sigma), with horseradish peroxidase–conjugated secondary antibody followed by detection by chemiluminescent horseradish peroxidase substrate (Millipore).

2.9. Generation of shRNA

We selected three NS3-specific siRNAs, NS3-1948, NS3-2052 and NS3-3532 (named on the basis of their location
and nucleotide start site in the HCV subgenome), as they had previously been reported to have much specific effect on HCV RNA replication (Kapadia et al., 2003; Takigawa et al., 2004). The NS3-specific siRNAs target sequences of NS3-1948 were 5′-AAGACAGTCAACACCCGCACTTCTC-3′ (sense) and 5′-AATTGCGTGTGACTCTCCCTGCTCTC-3′ (antisense), NS3-2052 5′-CAAGGACCTCTGCGCCGTG (sense) and CCAGCCGAGGTGCCGCTG (antisense), NS3-3532 5′-CGAGGTATTACACCACAC (sense) and GTGTGTGTAACCTCG (antisense), respectively. Chemically synthesized and annealed DNA fragments were cloned into the unique BamHI–HindIII site of plasmid pSilencer™ 2.1-U6 neo (Ambion). The resulting plasmids were named sh-1948, sh-2052 and sh-3492, respectively.

2.10. Statistical analysis

All the results were expressed as mean ± s.d. Statistical comparisons between two groups were made using Student’s t-test after analysis of variance. The level of significance was set to α = 0.05.

3. Results

3.1. Correlation between HCV NS3/4A protease and luciferase reporter activity in hepatoma cells

To develop an efficient fusion protein-based split-FLuc complementation system for studies on NS3/4A activity, two substrates for HCV NS3/4A protease were designed (Fig. 1). NLuc- and Cluc- containing pepA and pepB at their respective NH2 termini were fused head to tail, and two different NS3/4A cleavage sites of NS4A/B and NS5A/B were separately fused between NLuc and pepB. The resulting plasmids were named ANLuc(NS4A/B)BCLuc and ANLuc(NS5A/B)BCLuc, respectively.

Next, the validity of the two assays was compared by cotransfecting NS3/4A expression plasmid with ANLuc(NS4A/B)BCLuc or ANLuc(NS5A/B)BCLuc plasmid in Huh7 cells. Luciferase activity was measured by BLI 48 h post-transfection. A significantly high level of signal (P < 0.05) from both systems was observed when the cells were cotransfected with NS3/4A compared to the cells cotransfected with control empty vector. The system with the NS5A/B cleavage site showed a very low background activity while giving a higher complementation signal (Fig. 2A). Expression by this construct showed a 2- to 3-fold signal versus the background ratio. The specificity of this reaction was demonstrated by using a mutated form of the NS3/4A protease (NS3-S139A). No difference was observed with mutant NS3/4A-S139A protease compared with the cells cotransfected with control empty vector (Fig. 2B and C). Similar results were obtained in HepG2 and Hepa1–6 cells (data not shown).

To further confirm these results, cells were subjected to Western blotting analysis using anti-FLuc antibody and anti-NS3 antibody to detect the expression of fusion protein ANLuc(NS5A/B)BCLuc and the NS3 protein levels, respectively. The expression of NS3/4A protease in transfected cells resulted in the expected cleavage as shown in Fig. 2D. A distinct band corresponding to the size of free ANLuc was evident in lane 3. The free ANLuc presumably resulted from the cleavage of the ANLuc(NS5A/B)BCLuc by NS3/4A protease. The full-length fusion (∼69 kDa) remained intact when a mutant NS3/4A-S139A or an empty control vector was used. No free ANLuc could be detected (lane 2 versus lane 3), although the expression level of NS3/4A-S139A protease was comparable to NS3/4A protease. In cells that expressed wild-type NS3/4A (lane 3), the molecular weight of the detected band was clearly lower than that in mutant NS3/4A (lane 4). This result was likely due to the fact that active NS3/4A will autocleave the junction between NS3 and NS4A protease. The replicon cell lines were transfected with ANLuc(NS5A/B)BCLuc, and naive Huh7 cells were simultaneously transfected to serve as a control. Cells were evaluated for luciferase activity 48 h after transfection. Luciferase activity in HCV replicon cells was approximately 12 times higher than that in control group (Fig. 3A). Also, western blotting analysis detected free ANLuc in HCV replicon cells only (Fig. 3B). These results indicated that this reporter system could be used for quantitative analysis of NS3/4A protease activity and its inhibiting compounds in cell culture systems.

3.3. Validation of the ANLuc(NS5A/B)BCLuc reporter in vivo

Further, this assay was applied for assessing NS3/4A protease activity in Huh7 cells that stably express full-length HCV replicons. The replicon cell lines were transfected with ANLuc(NS5A/B)BCLuc, and naive Huh7 cells were simultaneously transfected to serve as a control. Cells were evaluated for luciferase activity 48 h after transfection. Luciferase activity in HCV replicon cells was approximately 12 times higher than that in control group (Fig. 3A). Also, western blotting analysis detected free ANLuc in HCV replicon cells only (Fig. 3B). To confirm the feasibility of this report system for characterizing HCV NS3/4A inhibitors, we examined the sensitivity of ANLuc(NS5A/B)BCLuc with HCV replicon cells. Replicon cells incubated at various concentrations of IFN-α ranging from 0 to 500 unit(s)/ml for 72 h were transfected with ANLuc(NS5A/B)BCLuc. Luciferase activity which was evaluated 48 h after transfection proved to be inversely proportional to the extent of anti-HCV treatment (Fig. 4A). Meanwhile, western blotting analysis was employed to examine whether the amount of NS3/4A protease encoded by HCV replicon changed to the same way as luciferase. In Fig. 4B, levels of the NS3 protein were proportionally reduced by treatment with increasing concentrations of IFN-α. The increase in luciferase activity was proportional to the dose of active NS3/4A protease. These results indicated that this report system could be used for quantitative analysis of NS3/4A protease activity and its inhibiting compounds in cell culture systems.

Fig. 1. Strategy for noninvasive imaging of NS3/4A protease activity. The substrate construct ANLuc(NS5A/B)BCLuc constitutes the split N- and C-terminal fragments of luciferase, fused to interacting peptides, pepA and pepB, respectively, with an intervening NS3/4A cleavage motif. When coexpressed with NS3/4A proteins, the reporter molecule is proteolytically cleaved by NS3/4A at the NS5A/B motif. Association of pepA and pepB brings inactive fragments of luciferase into close proximity, thereby producing bioluminescence activity.

To evaluate the applicability of the reporter in vivo, the hydrodynamic injection procedure was used to deliver...
Fig. 2. In vitro validation of NS3/4A protease reporter. (A) Comparison of the validity of the two assays. ANLuc(NS5A/B)BCLuc and ANLuc(NS4A/B)BCLuc were separately cotransfected with NS3/4A containing plasmid, or a control empty vector in Huh7 cells. The cells were harvested 48 h after transfection, and luciferase activities were measured by bioluminescence imaging. (B, C) Specificity of ANLuc(NS5A/B)BCLuc responses to NS3/4A protease. Huh7 cells were cotransfected with ANLuc(NS5A/B)BCLuc reporter plasmid and indicated NS3/4A or mNS3/4A containing plasmid, or a control empty vector. The cells were harvested 48 h after transfection, and luciferase activities were measured by bioluminescence imaging. (C) A representative Western blotting analysis of ANLuc(NS5A/B)BCLuc cleaved by NS3/4A. Lysates of transfected cells treated as above were harvested at 48 h post-transfection and then analyzed by Western blotting using anti-Fluc and anti-NS3 antibodies (lane 1: Huh-7 cells cotransfected with ANLuc(NS5A/B)BCLuc and pCIneo; lane 2: Huh-7 cells cotransfected with ANLuc(NS5A/B)BCLuc and pmNS3/4A; lane 3: Huh-7 cells cotransfected with ANLuc(NS5A/B)BCLuc and pNS3/4A). Arrows indicate the positions of ANLuc(NS5A/B)BCLuc, pepANFluc, mNS3/4A, NS3 and β-actin, respectively. In this experiment, pRL-TK was cotransfected to normalize transfection efficiency. Three independent experiments were done and error bars reflect standard deviations.

ANLuc(NS5A/B)BCLuc with pNS3/4A or pCIneo simultaneously into mouse livers at a ratio of 1:1. Bioluminescence imaging was done 24 and 96 h post-injection. Compared with the control group, a 10- to 40-fold increase in bioluminescence activity was observed in animals co-delivered with pNS3/4A (Fig. 5), suggesting the applicability of the ANLuc(NS5A/B)BCLuc reporter to studies of NS3/4A protease activity in vivo.

3.4. Application of this system to reflect the activity of NS3/4A protease inhibitors

To confirm that this reporter system can be used to reflect the activity of NS3/4A protease inhibitors, the shRNAs expression plasmids or the control empty vector were cotransfected with ANLuc(NS5A/B)BCLuc into HCV full-length replicon cells. Examina-
Fig. 4. Luciferase reporter correlates with the anti-HCV activity of IFN-α. (A) Reduction of luciferase activity in ANLuc(NS5A/B)BCLuc transfected replicon cells treated with IFN-α. Before transfection, replicon cells were incubated in the absence or in the presence of 20, 100, 200, and 500 IU of IFN-α/ml for 72 h. The luciferase activity was measured by bioluminescence imaging at 48 h post-transfection in the presence of IFN-α. The percentage decrease of luciferase activity relative to the untreated control was plotted. Bars indicate standard deviation (n = 3). (B) A representative Western blotting of replicon cell lysates treated as above and probed with luciferase, NS3- and β-actin-specific antibodies.

Fig. 5. In vivo validation of NS3/4A protease reporter. Bioluminescence imaging of representative animals both 24 and 96 h post-hydrodynamic injection with ANLuc(NS5A/B)BCLuc reporter plasmid and NS3/4A containing plasmid, or a control empty vector.

4. Discussion

The HCV NS3/4A serine protease has been explored as a target for the inhibition of viral replication in preclinical models and in HCV-infected patients. A number of potent and efficacious active-site inhibitors have been described (De Francesco and Carfì, 2007). Despite many years of effort by the pharmaceutical industry, only few NS3-specific antiviral compounds have been developed for clinical use. For a long time, the discovery and development of potent competitive inhibitors of the HCV serine protease has been hampered by the lack of appropriate model systems.

Numerous attempts in the development of various cell culture systems and small animal models for HCV NS3/4A serine protease have been undertaken in the past few years, each with their own advantages and disadvantages. The establishment of in vitro biochemical assays has allowed the discovery of anti-NS3/4A protease compounds (Berdichevsky et al., 2003). But these assays depend on the expression and purification of large amounts of the active enzyme for biochemical characterization, and in most cases chemical modification of the substrate peptide or of the cleavage product is required for its detection. Also, these assays made it impossible to determine whether inhibitors were able to penetrate the cell membrane and exert their activity in the appropriate cellular environment. The development of the replicon system can be considered a big stride that may have great impact on the development of high throughput assays for the identification of novel antiviral compounds that specifically target the protease activity of NS3/4A, as well as for studies on the process of HCV replication (Lohmann et al., 1999; Blight et al., 2002, 2003; Krieger et al., 2001; Pietschmann et al., 2001). However, current subgenomic or full-length genomic replicons are restricted to a human hepatoma Huh-7 cell line and genotype 1 isolates, making difficult the assessment of how genetic variation within the viral population of an HCV-infected patient may affect response to an antiviral therapy. The recent potential of growing the virus in cell culture can shed light on virus tropism, virus–host interactions and virus life cycle, while boosting the search for NS3/4A-specific antiviral compounds (Lindenbach et al., 2006; Pietschmann et al., 2006; Wakita et al., 2005; Yi et al., 2007; Zhong et al., 2005). A major obstacle is that the host cell is generally limited to Huh7-derived hepatoma cell lines, and that the in vitro-reduced infectious HCV is currently restricted to the JFH-1 isolate. Several alternative cell-based systems for monitoring the activity of NS3/4A protease have been reported, mostly based on reporter substrates fused to a cleavage sequence. But no cell-based system can serve to assess the efficacy, toxicity, and bioavailability of the new therapeutics in vivo.
When it comes to the in vivo models, the chimpanzee is still the best available animal to study antiviral therapy against HCV (Abe et al., 1992; Shimizu et al., 1990). However, experiments are generally performed on a limited number of animals for ethical reasons, plus their outbred nature as an endangered species and high costs. There have been some reports on the infection of the mice with HCV (Galun et al., 1995; Ilan et al., 2002). These models allow the assessment of the in vivo efficacy of anti-HCV drugs. But because of the extremely narrow species tropism of HCV, all rodent models require xenografting of human cells and a constitutive lack of immune rejection toward these engrafted cells.

Therefore, more research and progress is needed to establish simple, accurate non-primate systems for drug development. Here, we describe of experiments using split firefly luciferase complementation strategy to real-time, noninvasive, whole-body imaging of HCV NS3/4A protease activity and apply it to monitoring NS3/4A protease activity for drug discovery. The development of the reporter gene, ANLuc(ΔNS5A/B)BCLuc, makes it possible to screen anti-HCV NS3/4A protease compounds by simply measuring the luciferase activity both in cells and in living mice. When coexpressed with NS3/4A protease in cells or mice livers, ANLuc(ΔNS5A/B)BCLuc has been shown to be able to serve as a valid reporter of anti-HCV activity both in HCV subgenomic replicon cells and in mice livers. Our data show that this NS3/4A reporter system is a sensitive, dynamic, and quantitative reporter of NS3/4A protease activity both in cells and in living mice. It should be pointed out that in our reporter system the activity of luciferase cannot be completely silenced. Firefly luciferase fragments with novel split sites enabling more effective silencing of luciferase have been reported (Paulmurugan and Gambhir, 2007). We will construct new reporters using these improved split sites to further reduce the background signal of our reporter system.

The distinct advantage of the model described in this report lies in user-friendliness and economy. The use of firefly luciferase fragment complementation as a reporter strategy can accurately and rapidly dissect HCV NS3/4A protease activity by fluorescence and bioluminescence both qualitatively and quantitatively, in different mice using an easy, quick and inexpensive system (Fu et al., 2009). In addition, this approach enables us to perform ongoing monitoring of HCV NS3/4A protease activity through repeated bioluminescence imaging. It will constitute a new tool to allow the efficient screening of HCV NS3/4A protease inhibitors.

Acknowledgements

We thank Dr Tao Zhou and Ms Weili Gong for performing in vivo BLI and Dr. Charles M. Rice of Rockefeller University for the plasmid pcOn1-FL used in this work. This work was partially supported by the Natural Science Foundation of China (#30600330, #30671842, #30672488, #30700475, #30771919, #30700757), the National High Technology Research and Development Program of China (#2008AA022132), Beijing, Municipal Natural Science Foundation (#5082016) and Mega-projects of Science Research for the 11th Five-Year Plan (#2009ZX10004-4001).

References


Fig. 6. Bioluminescence activity was coincident with NS3/4A protease activity both in cells and in living mice. (A and B) Inhibitory effect of shRNA on NS3 expression in HCV replicon cells. Sh-2052 (lane 2), sh-1948 (lane 3), sh-3532 (lane 4) or control empty plasmid (lane 1) was cotransfected with ANLuc(ΔNS5A/B)BCLuc into cells containing HCV replicons. Cells were evaluated for luciferase activity 72 h after transfection. (C and D) Inhibitory effect of shRNA on NS3 expression in vivo. 10 μg sh-2052 vector or the control empty vector was co-injected with 10 μg ANLuc(ΔNS5A/B)BCLuc and 10 μg pNS3/4A into the mice tail vain under hydrodynamic pressure. Luciferase activity in the mouse liver was monitored 24 and 96 h post-injection. Compared with the negative control of RNA inhibitors, the inhibitory ratio was 90% for shNS3-2052 24 h post-injection.


