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A homogeneous, solid-phase assay for hepatitis C virus RNA-dependent RNA polymerase

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Abstract

Discovery and development of effective antiviral agents to combat hepatitis C virus (HCV) is the focus of intensive research both in academia and in pharmaceutical companies. One of the HCV nonstructural proteins, NS5B (an RNA-dependent RNA polymerase), represents an attractive target in light of the clinical success of human immunodeficiency virus reverse transcriptase inhibitors. To identify and evaluate NS5B inhibitors, we developed a homogeneous, solid-phase, high-throughput biochemical assay for detecting NS5B enzymatic activity. In this assay, a biotinylated oligo(dT_{12}) primer was immobilized onto streptavidin-coated scintillation proximity assay (SPA) beads, and after addition of homopolymeric A template, NS5B enzyme, and radiolabeled uridine 5′-triphosphate, the primer-dependent RNA synthesis occurred on beads. Optimization of the on-bead reaction resulted in the use of significantly less RNA template and NS5B enzyme while producing a faster steady state reaction rate compared to the solution-phase or off-bead SPA. The newly developed solid-phase assay is functionally comparable to the solution-phase assay as similar potencies of HCV NS5B inhibitors tested were obtained with the two assays. Furthermore, the solid-phase assay offers the advantage of delaying initiation, mimicking a physical preincubation step required for evaluating time-dependent inhibitors.

Keywords: Hepatitis C virus; HCV; RNA-dependent RNA polymerase; RdRp; NS5B; High-throughput screening; Scintillation proximity assay; SPA; Solid-phase assay

The hepatitis C virus (HCV)[1] nonstructural protein NS5B is an RNA-dependent RNA polymerase (RdRp) that is responsible for catalyzing the replication of the HCV RNA genome [1]. Purified HCV NS5B is capable of catalyzing a primer-dependent or primer-independent (de novo) synthesis of RNA by using a variety of homo- or heteropolymeric RNA templates [2–4]. While the template is RNA, the primer can be either DNA (for example, oligo(dT_{12})) or RNA (for example, oligo(U_{18})) [3]. Identification of small-molecule inhibitors for HCV NS5B activity is one of the many approaches being taken to develop effective anti-HCV therapies [5]. Several different types of biochemical assays have been developed and successfully used in high-throughput screening (HTS) for HCV NS5B inhibitors [6,7].

Both radioactive and nonradioactive HTS biochemical assays have been reported. Among the radioactive assays is the solution-phase reaction using the scintillation proximity assay (SPA) technology where a biotinylated primer (typically oligo(G) or oligo(dT)) is used for the primer-dependent polymerization with either poly(C) or poly(A) as the template. The newly synthesized RNA containing radiolabeled nucleotides is captured onto streptavidin-coated SPA beads via biotin–streptavidin linkage, followed by detection of the radioactivity as a measure of product formation [6–8]. Two different types of nonradioactive polymerase assays were recently described. Both use coupled-enzyme systems to measure the pyrophosphate (PPi) released from the incorporation of NTPs. In the first coupled-enzyme

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[1] *Abbreviations used:* HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; HTS, high-throughput screening; SPA, scintillation proximity assay; PPi, pyrophosphate; PNP, *p*-nitrophenyl; DTT, dithiothreitol; DMSO, dimethyl sulfoxide.

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assay, the incorporation of a specially modified ATP or GTP (*p*-nitrophenyl–NTP or PNP–NTP) to the elongated RNA chain releases PNP–PPi, which is then hydrolyzed by calf intestinal phosphatase to generate the colorimetrically measurable chromophore *p*-nitrophenylate [9]. The other nonradioactive assay measures PPi release in a two-step coupled-enzyme system involving ATP sulfurylase and firefly luciferase [10]. In this system, the free PPi is converted by ATP sulfurylase to ATP, which in turn provides energy for a luciferase-catalyzed reaction, producing a chemiluminescent signal.

All HTS polymerase assays have limitations. Although widely used, the solution-phase SPA polymerase assay is inherently an end-point assay, which prevents its use in monitoring reactions in real time. On the other hand, nonradioactive HTS assays using coupled-enzyme systems could be complicated by the presence of multiple enzymes. A major concern or issue with coupled-enzyme assays in HTS screening is the false positive hits by compounds that inhibit the secondary enzyme(s) instead of the primary enzyme. Here we report a robust, solid-phase SPA for the detection of HCV NS5B enzymatic activity. The on-bead reaction permits efficient detection of radiolabeled product, leading to the use of significantly less RNA template and enzyme. This solid-phase assay is as sensitive for evaluating HCV NS5B inhibitors as the solution-phase assay and offers an advantage for evaluating time-dependent inhibitors.

Materials and methods

HCV NS5B expression and purification

HCV NS5B protein, genotype 1b (con1) with an 18-amino-acid C-terminal truncation, was expressed in *Escherichia coli* and purified. Briefly, cultures of BL21(DE3) cells were grown at 37 °C for ~4 h until the cultures reached an optical density of 2.0 at 600 nm. The cultures were cooled to 20 °C and induced with 1 mM isopropyl β-D-thiogalactoside overnight at 20 °C. Cell pellets (3 L) were resuspended using a tissue homogenizer in a lysis buffer consisting of 20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, 20% glycerol, 0.5 mg/ml lysozyme, 10 mM $MgCl_2$, 15 μg/ml deoxyribonuclease I, and Complete protease inhibitor tablets (Roche). To reduce the viscosity of the sample, aliquots of the lysate were sonicated on ice using a microtip attached to a Branson sonicator. The sonicated lysate was centrifuged at 100,000*g* for 1 h at 4 °C and filtered through a 0.2-μm filter unit (Corning). The protein was purified using two sequential chromatography steps: heparin sepharose CL-6B and poly(U) sepharose 4B (Pharmacia). The chromatography buffers were identical to the lysis buffer but contained no lysozyme, deoxyribonuclease I, $MgCl_2$, or protease inhibitors with concentration of NaCl specified below. Using a 30-ml heparin sepharose CL-6B column, the protein was eluted with a 20-column volume gradient from 0.2 to 1 M NaCl. Peak fractions were pooled, buffer-exchanged, and applied to a 10-ml poly(U) sepharose 4B column. The proteins were eluted with a 5-column volume gradient from 0.15 to 1 M NaCl. After the final chromatography step, the resulting purity of the enzyme is >90% based on SDS–PAGE analysis with a typical yield of 8.3–16.7 mg/L. The enzyme was aliquoted and stored at −80 °C.

Standard poly(AdT) solution-phase assay for HCV NS5B

Activity of HCV NS5B enzyme was measured using a homopolymeric A (poly(A)) template and a biotinylated oligo(dT_{12}) primer in a final volume of 60 μl in 96-well plates (Corning Costar 3912). The assay buffer is composed of 20 mM Hepes, pH 7.5, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 1.6 U RNase inhibitor (Promega N2515), 0.1 mg/ml bovine serum albumin (Promega R3961), and 2% glycerol. Typical reactions contain 11–28 nM HCV NS5B enzyme, 4–11 nM poly(A) template, 20–3000 nM biotinylated oligo(dT_{12}) primer, and 0.6 μCi [^{3}H] UTP (0.29 μM total UTP, 37.4 Ci/mmol; Amersham NET380) or 0.5 μCi [^{33}P] UTP (3000 Ci/mmol; Amersham NEG-607H) with 25 μM cold UTP (See figure legends for exact condition used). The poly(A) template was obtained commercially (Amersham 27-4110), and the biotinylated primer was prepared by SigmaGenosys. Where compounds were evaluated, a 3-fold serial dilution was made in DMSO with subsequent dilution in water such that the final concentration of DMSO in the assay was 2% (a concentration also used in no-compound control reactions). The order of assembly was (1) compound or DMSO, (2) template/primer/UTP, and (3) enzyme. If a preincubation was included, the order of addition changed to (1) compound, (2) enzyme with up to 1 h incubation at room temperature, and (3) template/primer/UTP label to initiate the reaction. Reactions were incubated at 30 °C for 60 min and stopped with EDTA (final concentration 25 mM). The newly synthesized RNA product was quantified by one of two different methods. In the first method, straptavidin-coated SPA beads (4 mg/ml, Amersham RPNQ 0007) were added to the terminated reactions to capture the radiolabeled RNA. After incubating at room temperature for ⩾1 h, plates were read on a Packard Top Count NXT. The second method used 10% trichloroacetic acid to precipitate the RNA product. After 30 min on ice, the precipitated RNA was harvested onto Unifilter GF/B plates (PerkinElmer 6005177) that were presoaked with 0.1 M sodium pyrophosphate. The plates were washed 16 times with water (~200 μl per wash), then with a final wash of ethanol (200 μl), and air-dried for at least 10 min. MICROSCINTO (40 μl, PerkinElmer 6013611) was added, and plates were read on the Packard Top Count after ~10 min.

Solid-phase or on-bead NS5B enzyme assay

The assay is performed in two-steps: immobilization of the biotinylated oligo(dT_{12}) primer on SPA beads and