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(−)-Epigallocatechin gallate inhibits hepatitis C virus (HCV) viral protein NS5B

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ABSTRACT

In this study, we elucidated a small molecule inhibitor on viral protein NS5B identified through a high-throughput screening strategy using optical nanoparticle-based RNA oligonucleotide. We have previously shown that quantum dots (QDs)–RNA oligonucleotide can specifically recognize the HCV viral proteins. We have also demonstrated that conjugated QDs–RNA oligonucleotide can specifically and sensitively interact with designed biochips [1,2]. Among the flavonoids examined, (−)-epigallocatechin gallate (EGCG) demonstrated a remarkable inhibition activity on HCV viral protein, NS5B. (−)-Epigallocatechin gallate, at 0.005 μg mL^{-1} or more, concentration-dependently attenuated the binding affinity on a designed biochip as evidenced by QDs–RNA oligonucleotide. At a concentration of 0.1 μg mL^{-1}, (−)-epigallocatechin gallate showed a 50% inhibition activity on QDs–RNA oligonucleotide biochip assay. We screened a small molecule inhibitor on the viral protein, NS5B, identified through a high-throughput screening strategy using on-chip optical nanoparticle-based RNA oligonucleotide on chip. In this designed strategy, the convenient and efficient screening and development of an on-chip viral protein inhibitor using a QDs–RNA oligonucleotide assay is achievable with high sensitivity and simplicity. In addition, this platform is expected to be applicable toward the inhibitor screening of other types of diseases.

1. Introduction

Globally, over 170 million people (ca. 3%) are infected with the hepatitis C virus (HCV) and the rate of global death from liver-related mortality to HCV has remarkably increased. And, its infection causes serious liver diseases such as acute and chronic hepatitis, cirrhosis, and the development of hepatocellular carcinoma [3–5]. HCV is a positive single-stranded RNA virus whose 9.6 kb genome organized to contain a single, large translational, open-reading frame that encodes a large polyprotein precursor (3010–3030 a.a.). HCV is flanked by 5′ and 3′ untranslated regions (UTRs) that are required for replication and translation initiation [6–9]. HCV RNA replication was catalyzed using the viral polymerase nonstructural protein 5B (NS5B). This RNA-dependent RNA polymerase synthesizes a negative-strand RNA that serves as a template for the synthesis of new positive RNA strands. The RNA-dependent polymerase of the hepatitis C virus (HCV) plays a pivotal role in the life cycle of the virus, standing in as a crucial and unique component of the viral replication machinery [10–12]. Due to its essential role in viral replication, HCV NS5B viral protein is mainly regarded as a prime target for antiviral therapy [13,14]. For this reason, the HCV NS5B is an attractive and crucial target for anti-HCV therapeutic drug discovery. Currently, small molecular targets for HCV direct-acting antiviral agents in drug screening are focused on the non-structural proteins required for replication, such as the NS3 protease, NS5A, and the NS5B RNA-dependent RNA polymerase.

Flavonoids are phytochemical compounds found in numerous plants and fruits [15–17]. They have been reported to act as antioxidants, free radical scavengers, metal chelators, anti-allergic, anti-cancer, anti-oxidant, anti-inflammatory, anti-fungal, anti-viral, and anti-bacterial agents. In general, these compounds are known to have medicinal and chemopreventive activities in human health. Flavonoids are becoming very popular due to their many health promoting effects [18–21]. Flavonoids such as catechins are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants [22]. In particular, (−)-epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the ester of epigallocatechin and gallic acid, and is a type of catechin. (−)-Epigallocatechin gallate is the most abundant catechin, most notably in tea and other plants, and is also a potent antioxidant [23] with possible therapeutic properties for many disorders, including cancer [24,25]. The Shearer group reported the benefit of (−)-epigallocatechin gallate from green tea in the treatment of HIV infection, where (−)-epigallocatechin gallate has been shown to reduce plaques related to AIDS-related dementia in the laboratory, as well as block gp120 [26–28]. There have also been reports showing that EGCG can be beneficial in

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treating brain [29], prostate [30,31], cervical [32], bladder, and other types of cancers [33].

Quantum dots (QDs), which are colloidal nanoparticles of semiconductor materials, have attracted remarkable attention in the various fields of nanotechnology and biotechnology, especially in the biological imaging applications due to possess remarkable optical characteristics compared with conventional organic fluorophores in terms of being bright, tunable and having narrow fluorescence emission, and broad absorption spectra [34,35].

In this study, we elucidated a novel approach for the inhibitor screening of the viral protein using a QDs-based system with wide applicability for reliable and effective imaging analysis. We screened a small molecule inhibitor on viral protein NS5B identified through a high-throughput screening strategy using an on-chip optical nanoparticle-based RNA oligonucleotide. To the best of our knowledge, this is the first report on the inhibition effect of (−)-epigallocatechin gallate on HCV viral protein NS5B using an optical nanoparticle-based RNA oligonucleotide platform.

2. Material and methods

2.1. Chemicals

EDC (N-(3-dimethylaminopropyl)-N′ ethylcarbodiimide hydrochloride), cyclosporine A, (−)-epigallocatechin gallate and kanamycin were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Quantum dots (QDs605) were purchased from Invitrogen Corporation (Carlsbad, CA). Prolinker^{TM}-terminated glass slide were purchased from Proteogen (Seoul, Republic of Korea). All other chemicals were of the highest grade.

2.2. Conjugation of quantum dots and RNA oligonucleotide

An amine group with a terminal modification of the NS5B RNA oligonucleotide was synthesized by BIONEER Co. Ltd. (Seoul, Republic of Korea) and carboxyl-terminated QDs605 was purchased from Invitrogen (Carlsbad, CA). The amino group of the NS5B RNA oligonucleotide (H2N-5′-GGCCACAUUGUGAGGGGCUC-3′) was first covalently conjugated onto the surface of the carboxyl-terminated QDs (10 pM, 1.25 μL). That is, 10 pM of QDs were conjugated with 400 pM of oligonucleotide with 1 μL of 40 nM EDC, to activate amide bond formation to produce QDs-conjugated oligonucleotide (QDs–NS5B oligonucleotide) at a QDs:RNA oligonucleotide molar ratio of 1:40 for 1 h at room temperature. Thereafter, the QDs–oligonucleotide conjugate was collected using centrifugal filtration at 15,000 rpm for 30 min, followed by several washing steps with a Tris buffer (50 mM Tris–HCl pH 7.4, 5 mM KCl, 100 mM NaCl, 1 mM MgCl2, and 0.1% NaN_3). After centrifugal filtration and washing, a pellet of the QDs-conjugated RNA oligonucleotide was dispersed through a brief sonication (22 kHz, amplitude of 12 μm, and sonication time of 120 s) using a sonic dismembrator (Model F60 Sonic Dismembrator; Fisher Scientific, Fair Lawn, NJ).

2.3. Subcloning, expression and purification of viral protein NS5B

The HCV NS5B gene [11], except for the hydrophobic C terminus 21 amino acids, was amplified using polymerase chain reaction (PCR) with a primer set, sense: 5′-cgcgaattcatgtcctacacatggacagg-3′; antisense: 5′-tttctcgagtcggttggggagcaggta-3′, containing restriction enzyme sites of EcoRI/XhoI. PCR was run with following conditions on a thermal cycler: denaturation at 94 °C for 1 min, annealing at 65 °C for 30 s, and an extension step at 72 °C for 2 min and 30 s, respectively. The sequence was repeated 35 times followed by a 7 min final extension step at 72 °C. The PCR product was digested with an EcoRI/XhoI, and then ligated into EcoRI/XhoI digested expression vector pET 28a+ (Novagen, Madison, WI), and transformed into *E. coli* DH5α (Stratagene, La Jolla, CA). The right colony, transformed with an inserted gene, was transformed into *E. Coli* BL21 (*DE3*) (Stratagene, La Jolla, CA). and plated on LB agar containing 50 μg mL^{-1} kanamycin. The transformant was grown in a 250 mL flask containing 50 mL of a Luria–Bertani (LB) medium supplemented with 50 μg mL^{-1} of kanamycin at 37 °C until the cell concentration reached OD600 nm of 0.6, and isopropyl-thio-β-D-galactopyranoside (IPTG) with a final concentration of 0.1 mM, followed by growing for additional growth overnight at 25 °C with shaking at 180 rpm. Cells were harvested through centrifugation at 4000 rpm for 30 min at 4 °C and resuspended in a 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed using a Sonicator (W250 Sonifier, Branson, Dietzenbach, Germany). The cell debris was removed through centrifugation at 15,000 rpm for 30 min. The supernatant was collected, and the recombinant viral protein was purified using a Ni–nitrilotriacetic acid (Ni–NTA) affinity chromatography column (Qiagen, Germany). The supernatant was equilibrated with buffer A (10 mM Tri–HCl, 500 mM NaCl, 5 mM imidazole, pH 8.0). The bound protein was eluted with buffer B (10 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0) at 4 °C. The purity of the purified protein was estimated using SDS-PAGE in the eluted fractions, using 10% polyacrylamide running gels [36]. The purity of the enzyme was also estimated using SDS-PAGE. The protein concentration was determined as described by Bradford with bovine serum albumin (BSA) as the standard [37]. The HCV NS5B viral protein was purified using a single chromatography step on a Ni^{2+} affinity column. The C-terminally his-tagged HCV viral protein NS5B was visualized with a molecular mass of approximately 66 kDa on the SDS-PAGE gel. The purified protein was supplemented with 50% glycerol and stored at −20 °C until used.

2.4. Fluorescent assay in a confocal laser-scanning microscope

The recombinant viral protein NS5B was immobilized directly onto the functional ProLinker-terminated surface. For the inhibition activity, the QDs-conjugated RNA oligonucleotide and inhibitor were facilitated through by spotting on an immobilized HCV viral protein glass chip. After incubation for 1 h at 25 °C, the glass chip was then washed three times with the phosphate buffer (pH 7.2) for 1 min. The glass chip was analyzed using a confocal laser-scanning microscope, an LSM 510 META (Carl Zeiss, Jena, Germany). The signal intensity was determined using software for the LSM510 (LSM Image Browser). A histogram of the intensity was obtained from the region of the spotted chip. The value of the signal intensity was obtained by calculating and expressing it as the mean intensity.

3. Results and discussion

3.1. Scheme for inhibitor screening of viral protein NS5B on chip

For the inhibitor screening of viral protein, we designed the QDs-based specific RNA oligonucleotide for on-chip HCV-specific protein targeting: (i) immobilization of viral protein NS5B (1 μL) on a glass chip, (ii) binding of QDs–RNA oligonucleotide conjugates (1 μL) on an immobilized chip, (iii) inhibitor treatment on the conjugated RNA oligonucleotide and viral protein, (iv) washing and unspecific binding removal, and (v) detection to directly show the specific recognition of the on-chip inhibition effect of viral protein. The design of the inhibitor screening for effective on-chip monitoring of viral protein inhibition is illustrated in Fig. 1. To accomplish the feasibility of targeting and imaging, we used QDs605

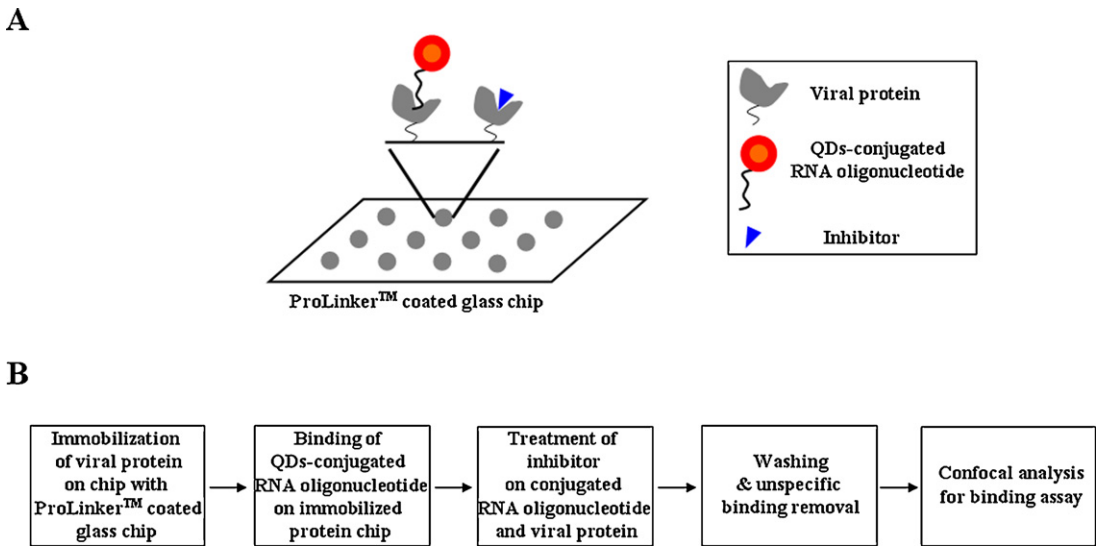

Fig. 1. A representative scheme for the inhibitor screening of viral protein using QDs-based RNA oligonucleotide on a biochip.

conjugates having an RNA oligonucleotide for viral protein NS5B with an emission wavelength as the fluorescent imaging probe.

3.2. Expression and purification of viral protein NS5B

The recombinant HCV NS5B viral protein in *E. Coli* expression system was expressed and purified using Ni–NTA affinity chromatography. In the purification step, the protein was eluted in a 1 mL fraction with a 250 mM imidazole buffer. To verify the purity and homogeneity of the eluted NS5B, aliquots of eluted fractions were analyzed through Coomassie blue staining on SDS-PAGE. Eluates with the highest purity of 95% were pooled, dialyzed and stored with 50% glycerol in aliquots at −80 °C. The HCV NS5B viral protein was purified using a single chromatography step on a Ni^{2+} affinity column. The C-terminally his-tagged HCV NS5B was purified with a molecular mass of approximately 66 kDa (data not shown).

3.3. Inhibitory effect of viral protein NS5B

In Table 1, the effects of flavonoid compounds and cyclosporine A on the inhibition of the viral protein NS5B used in this study are described. The chemical structure of (−)-epigallocatechin gallate is shown in Fig. 2. Many researchers have reported that cyclosporine A is one of the remarkable drugs for viral protein NS5B [34]. To evaluate the effects of cyclosporine A on viral protein NS5B, we used cyclosporine A as a positive control for elucidation of our designed biochip platform. In Fig. 3(A), we confirmed cyclosporine A concentration-dependent inhibition or antiviral activity against viral protein NS5B. Among the flavonoids screened, (−)-epigallocatechin gallate showed high antiviral activity. (−)-Epigallocatechin gallate, at 0.005 μg mL^{-1} or more, concentration-dependently attenuated the binding affinity on the designed biochip as evidenced by QDs–RNA oligonucleotide (Fig. 3(B)). At a concentration of 0.1 μg mL^{-1}, (−)-epigallocatechin gallate showed 50% inhibition activity on a QDs–RNA oligonucleotide biochip assay. As shown in Fig. 3(A and B), cyclosporine A and (−)-epigallocatechin gallate showed a similar pattern when compared to the concentration-dependent antiviral activity. The half-maximal inhibitory concentration (IC_{50}) values of cyclosporine A and (−)-epigallocatechin gallate were found to be approximately 0.5 and 0.1 μg mL^{-1}, respectively (Fig. 3(A and B)). To perform high-throughput screening of the inhibitors, it would be efficient to be able to measure the antiviral activity from optical images of a biochip containing multiple reaction compounds. This system may

Table 1
The effects of flavonoid compounds and cyclosporine A on the inhibition of viral protein NS5B used in this study.

Compounds	Inhibition effect
Naringenin	–
Hesperetin	–
Apigenin	–
Luteolin	–
Quercetin	–
Rutin	–
Fisetin	–
Myricetin	–
(−)-Catechin	–
(−)-Epigallocatechin 3-gallate	+
Biochanin A	–
Formononetin	–
Daidzein	–
Genistein	–
Glycitein	–
Cyclosporine A	+

(+) It means this compound has inhibition activity on viral protein NS5B.
(−) It means this compound has no inhibition activity on viral protein NS5B.

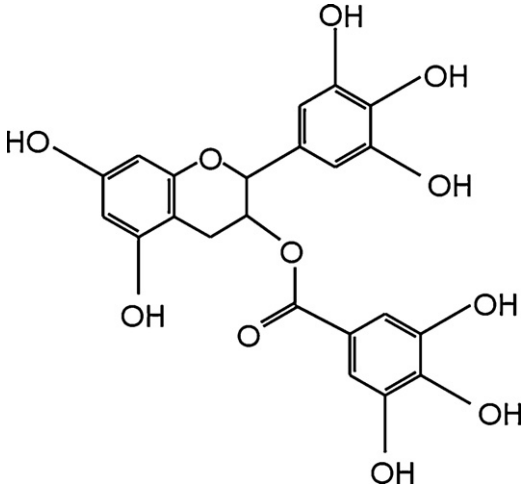

Fig. 2. Chemical structure of inhibitory compound (−)-epigallocatechin gallate with regard to viral protein NS5B.

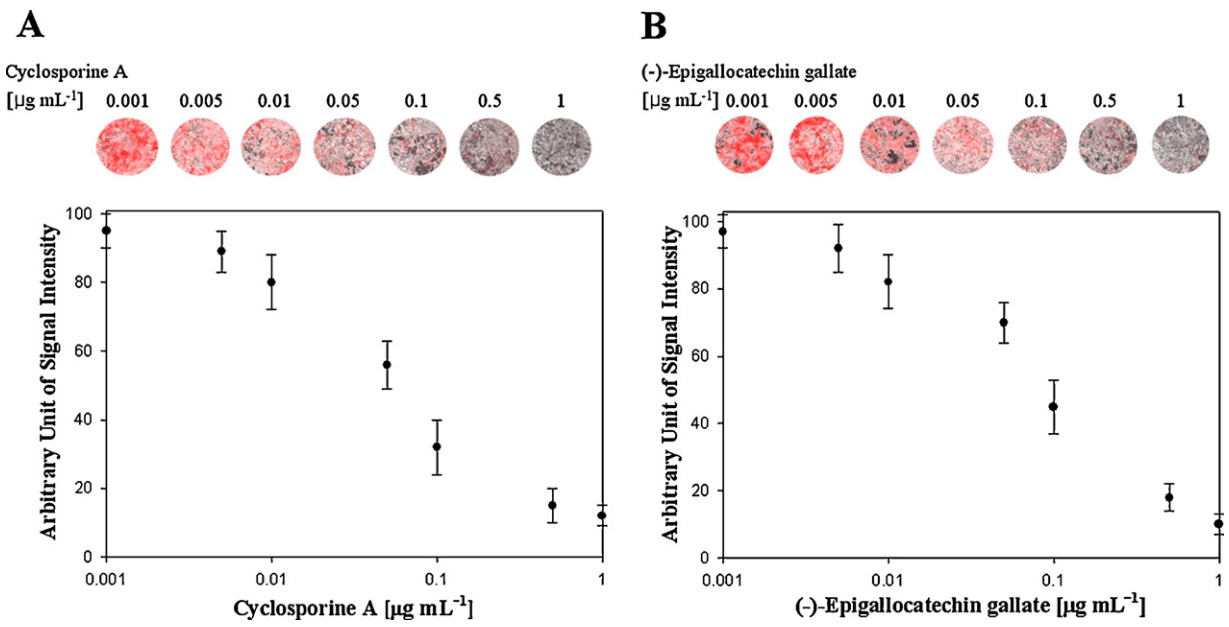

Fig. 3. Inhibitory effect of (A) cyclosporine A and (B) (−)-epigallocatechin gallate on viral protein NS5B.

be useful for rapid detection of inhibitor of viral protein within 60 min. The inhibition of the antiviral activity from cyclosporine A and (−)-epigallocatechin gallate were clearly illustrated, and dose dependency was distinctly observable in the optical images. These results suggested that the QDs-conjugated RNA oligonucleotide could be effective for the quantification of viral protein detection and inhibitor screening. The remarkable imaging by semiconductor nanocrystals QDs allows for monitoring with the intense light emission and accuracy.

4. Conclusions

In summary, we demonstrated inhibitor screening on a designed biochip platform using a QDs–RNA oligonucleotide. We discovered a novel function of (−)-epigallocatechin gallate as an antiviral agent. The discovery of antiviral drugs has been of considerable interest in developing efficient and effective methods for high-throughput screening in medicine. Our main goal in this study is to demonstrate a proof-of-concept that viral protein can be inhibited and detected with remarkable sensitivity and simplicity. For application, this designed platform for a novel inhibitor assay possesses significant potential as a target screening.

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