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Identifying Inhibitors of the HBx–DDB1 Interaction Using a Split Luciferase Assay System

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Dear Editor-in-Chief,
JoVE
RE: JoVE60652R1

We appreciate the comments by the editors. We herewith submit our revised manuscript along with a letter containing point-by-point responses to the comments.

As noted in the cover letter for the original manuscript, we described a convenient method to identify HBx–DDB1 interaction inhibitor. This method may become a key assay to discover novel therapeutic agents for chronic HBV hepatitis.

We have addressed all the critical issues raised by the editors and hope that the manuscript is now suitable for publication in ***JoVE***.

The revised manuscript contains:

- # Text: 2,515 words (including captions and references)
- # 2 Figures
- # Supporting information (Table of Materials)

Thank you for your time and consideration.

Sincerely yours,

Kazuma Sekiba, M.D.
Department of Gastroenterology, Graduate School of Medicine,
The University of Tokyo, Japan.

TITLE:

Identifying Inhibitors of the HBx–DDB1 Interaction Using a Split Luciferase Assay System

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KEYWORDS:

hepatitis B virus, drug screening, high-throughput, virus–host interaction, Smc5/6, protein–protein interaction, nitazoxanide

SUMMARY:

Here, we present a method for screening anti-hepatitis B viral agents that inhibit the HBx–DDB1 interaction using a split luciferase assay system. This system allows easy detection of protein–protein interactions and is suitable for identifying inhibitors of such interactions.

ABSTRACT:

There is an urgent need for novel therapeutic agents for hepatitis B virus (HBV) infection. Although currently available nucleos(t)ide analogs potently inhibit viral replication, they have no direct effect on the expression of viral proteins transcribed from a viral covalently closed circular DNA (cccDNA). As high viral antigen load may play a role in this chronic and HBV-related carcinogenesis, the goal of HBV treatment is to eradicate viral proteins. HBV regulatory protein X (HBx) binds to the host DNA damage-binding protein 1 (DDB1) protein to degrade structural maintenance of chromosomes 5/6 (Smc5/6), resulting in activation of viral transcription from cccDNA. Here, using a split luciferase complementation assay system, we present a comprehensive compound screening system to identify inhibitors of the HBx–DDB1 interaction. Our protocol enables easy detection of interaction dynamics in real time within living cells. This technique may become a key assay to discover novel therapeutic agents for treatment of HBV infection.

INTRODUCTION:

Hepatitis B virus (HBV) infection is a major public health concern worldwide, with annual estimates of 240 million people chronically infected with HBV and 90,000 deaths due to complications from the infection, including cirrhosis and hepatocellular carcinoma (HCC)[1].

Although the current anti-HBV therapeutic agents, nucleos(t)ide analogues, sufficiently inhibit viral reverse transcription, they rarely achieve elimination of viral proteins, which is the long-term clinical goal. Their poor effect on viral protein elimination is due to their lack of direct effect on viral transcription from episomal viral covalently closed circular DNA (cccDNA) minichromosomes in the hepatocyte nucleus[2].

HBV transcription is activated by HBV regulatory X (HBx) protein[3]. Recent studies revealed that HBx degrades structural maintenance of chromosomes 5/6 (Smc5/6), a host restriction factor that blocks HBV transcription from cccDNA, via hijacking a DDB1-CUL4-ROC1 E3 ubiquitin ligase complex[4–6]. Therefore, a crucial step in promoting viral transcription from cccDNA is thought to be the HBx–DDB1 interaction. Compounds capable of inhibiting the binding between HBx and DDB1 may block viral transcription, and indeed nitazoxanide was identified as an inhibitor of the HBx–DDB1 interaction via a screening system developed in our laboratory[7].

Here, we present our convenient screening system used to identify inhibitors of the HBx–DDB1 interaction, which utilizes a split luciferase complementary assay[7,8]. Split luciferase subunits are fused to HBx and DDB1, and the HBx–DDB1 interaction brings the subunits into close proximity to form a functional enzyme that generates a bright luminescent signal. As the interaction between the subunits is reversible, this system can detect rapidly dissociating HBx–DDB1 proteins (**Figure 1**). Using this system, a large compound library can be easily screened, which may result in the discovery of novel compounds capable of efficiently inhibiting the HBx–DDB1 interaction.

PROTOCOL:

NOTE: A schematic representation of the split luciferase assay is shown in **Figure 1A**, and the assay process is outlined in **Figure 1B**. The interaction dynamics can be measured in real time without cell lysis.

1. Cell preparation

1.1. Maintain cultured HEK293T cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 1x penicillin/streptomycin at 37 °C in 20% O_2 and 5% CO_2.

1.2. Seed 5 x 10^6 cells into a 100 mm dish with 10 mL of DMEM and incubate at 37 °C overnight.

1.3. Transiently transfect 1 µg of HBx and DDB1 with split luciferases into the cells according to the following method.

NOTE: The amount of the plasmid DNA transfected may depend on the transfection regent used. The optimal position of the split luciferase fused to the target protein must be determined beforehand. In this case, HBx fused to LgBit at the C-terminus of HBx (HBx–LgBit) and DDB1 fused to SmBit at the N-terminus of DDB1 (SmBit–DDB1) provided the best results (i.e., the brightest

luciferase signals). This process has been reported previously in detail[7].

1.3.1. Dilute 1 µg of HBx–LgBit expressing DNA plasmid and 1 µg of SmBit–DDB1 expressing DNA plasmid (**Table of Materials**) in DNA condensation buffer (**Table of Materials**) to a total volume of 300 µL.

1.3.2. Add 16 µL of enhancer solution (**Table of Materials**) and mix by vortexing for 1 s.

1.3.3. Incubate the sample at room temperature for 3 min.

1.3.4. Add 60 µL of transfection reagent (**Table of Materials**) to the sample and mix by vortexing for 10 s.

1.3.5. Incubate the sample at room temperature for 8 min.

1.3.6. During incubation, aspirate the culture medium from the dish (prepared in step 1.2), and wash cells with 5 mL of phosphate-buffered saline (PBS). Remove PBS by aspiration and add 7 mL of DMEM.

1.3.7. Add 3 mL of DMEM to the tube containing the transfection complexes. Mix by pipetting and add the transfection complexes onto the cell in the 10 cm dish.

1.4. Incubate the cells at 37 °C in an incubator under 5% CO_2 for 10 h.

1.5. Reseed the cells into a white 96 well plate at 5 x 10^4 cells/well in 50 µL of medium/well according to the following method.

1.5.1. Remove the spent cell culture medium and wash cells with 5 mL of PBS.

1.5.2. Remove PBS by aspiration, add 1 mL of 0.25% trypsin-EDTA, and incubate at 37 °C for 5 min to detach cells.

1.5.3. Add 4 mL of DMEM and disperse the medium by pipetting over the surface of the cell layer several times. Transfer the cell suspension to a tube.

1.5.4. Centrifuge cells at 500 x *g* for 5 min at room temperature.

1.5.6. Discard supernatant and resuspend the cell pellet in 1 mL of PBS.

1.5.7. Centrifuge the cell suspension at 500 x *g* for 5 min at room temperature and discard supernatant.

1.5.8. Dilute the cell pellet with buffered cell culture medium (**Table of Materials**) supplemented with 10% FBS to a seeding density of 1.0 x 10^6 cells/mL.

1.5.9. Pipette 50 µL of cell suspension into each well of a 96 well plate and return the cells to the incubator.

1.6. Incubate cells at 37 °C under 5% CO_2 for 10 h.

2. Compound screening

2.1. During the incubation, dilute the screening compounds (**Table of Materials**) and solvent (dimethyl sulfoxide [DMSO]) at 13.5x concentration. For example, if the stock is 10 mM and the screening concentration is 10 µM, add 1 µL of stock solution to 73.1 µL of buffered cell culture medium.

2.2. Add 12.5 µL of luminescent substrate (**Table of Materials**) to each well and incubate for 5 min at room temperature.

NOTE: As negative controls, the wells at both ends of the plate (i.e., columns 1 and 12) should contain no luminescent substrate.

2.3. Measure the baseline luminescence using a luminometer (**Table of Materials**).

2.4. Immediately after the initial measurement, add 5 µL of compounds and control DMSO diluted in step 2.1 to each well.

NOTE: The final concentration will be 10 µM.

2.5. Measure luminescence values every 30 min for 2 h.

NOTE: The plate should be incubated in the dark at room temperature.

2.6. Calculate the inhibitory effects by comparison with control DMSO treatment after normalization to the baseline signals.

NOTE: Screening each compound in duplicate or triplicate can reduce variation.

REPRESENTATIVE RESULTS:

Representative outcomes following the use of this protocol are shown in **Figure 2A,B**. The signal-to-background ratio was greater than 80 and the Z' factor[9] (the gold standard quality index for high-throughput screening) was greater than 0.5, indicating that this assay system was acceptable for high-throughput screening. With the threshold set to >40% inhibition compared with the control (DMSO only), we identified nitazoxanide as a candidate drug[7]. Using this system, better candidate drugs can be found by screening other, larger compound libraries.

FIGURE LEGENDS:

Figure 1: Schematic representation of split luciferase analysis of the HBx–DDB1 interaction. (**A**) The principle underlying detection of HBx–DDB1 binding using the split luciferase complementary assay system. The separated luciferase subunits, LgBit and SmBit, are fused to HBx and DDB1, respectively. The HBx–DDB1 interaction brings the subunits into close proximity to form a functional enzyme that generates a luminescent signal. The interaction between the subunits is reversible. (**B**) Split luciferase assay. After co-transfection of plasmids for expression of HBx fused to LgBit and DDB1 fused to SmBit, cells were re-seeded into a 96 well plate. The addition of luminescent substrate enables measurement of luciferase activity without a cell lysis step. Luciferase activities can be measured after adding the screening compounds.

Figure 2: Successful results of the split luciferase assay. (**A**) Representative baseline luminescent signals from a 96 well plate. Luciferase intensity is represented by numbers and colors. Columns 1 and 12 are controls in which the luminescent substrate was not added. The Z' factor was greater than 0.5. (**B**) Representative time-series result of relative luciferase activity levels after addition of screening compounds to a 96 well plate. The x-axis represents the inhibitory effects calculated compared to control (DMSO) after standardization to the baseline luciferase activity. The most effective compound was nitazoxanide.

DISCUSSION:

We developed a convenient screening method using a split luciferase assay to find HBx–DDB1 binding inhibitors. The interaction dynamics can be detected in real time in living cells without the need for cell lysis. Inhibition of the HBx–DDB1 interaction leads to restoration of Smc5/6, which results in suppression of viral transcription, protein expression, and cccDNA production[7]. This novel mechanism of antiviral action may overcome the inadequacies of current HBV therapies.

Although a number of methods are available to investigate protein–protein interactions in living cells, examining these interactions remain difficult[10]. Our procedure is simple and requires only a short time to screen one 96 well plate. Moreover, the screening quality was satisfactory with a high Z' score, the gold standard quality index for high-throughput screening[9]. Our assay may be suitable for robotic automation[11] and is an efficient assay for drug discovery.

While the protocol described here used the HEK293T cell line because its high transfection efficacy and high proliferation ability are suitable for high-throughput screening, this screening method can be performed using other cell lines (e.g., HepG2) without modifications[7]. As a realistic strategy for screening compounds, HEK293T cells may be used in the first screening followed by HepG2 cells in a second validation screening. Some compounds may not show significant results in different cell lines when the effects are dependent on indirect mechanisms.

As our intention was to develop a high-throughput screening method, subsequent validation studies are necessary to confirm whether the identified compounds function as interaction inhibitors. Decreased levels of luminescent signals in this assay do not always correspond to inhibition of the HBx–DDB1 interaction. Cytotoxicity tests, co-immunoprecipitation studies, and

further anti-HBV experiments are important to confirm the effects[7].

Although we previously identified nitazoxanide as an inhibitor of the HBx–DDB1 interaction by screening a relatively small-scale compound library[7], further studies involving screening of much larger compound libraries can be easily performed to identify novel compounds that are capable of inhibiting protein–protein interactions more efficiently. When performing such further screening, nitazoxanide can be used as a positive control for the assay. Furthermore, the system described here can be applied to other protein–protein interactions. Protein–protein interactions are an important class of drug targets[12]. Indeed, many other viruses interact with host factors to replicate or express their pathogenicity[13,14]. The split luciferase-based assay described here, which targets the interactions between viral and host proteins, may provide a new strategy to develop cures for HBV and other infectious diseases.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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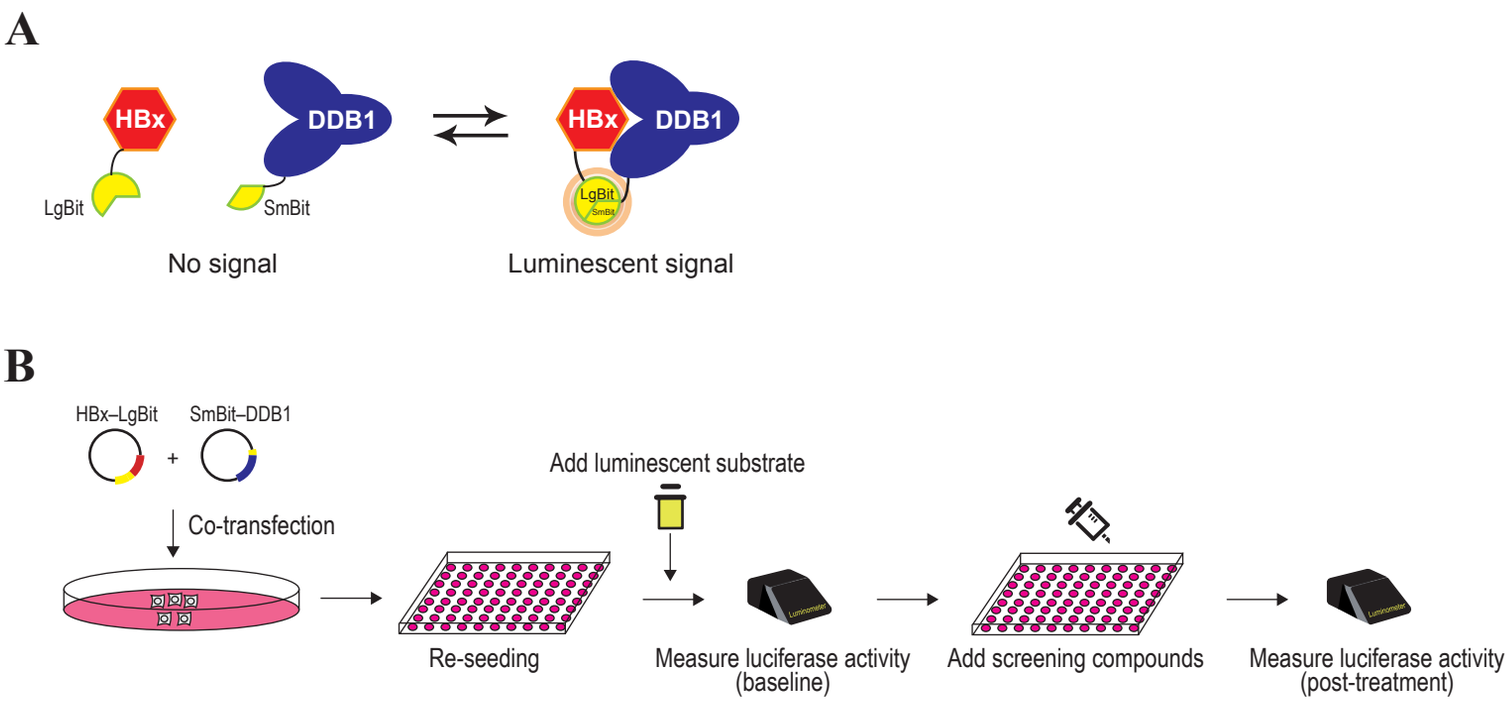
Figure 1

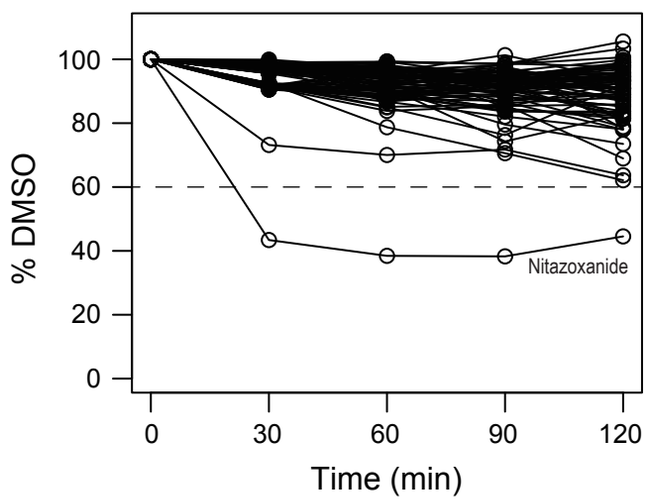


A

	1	2	3	4	5	6	7	8	9	10	11	12
A	960	81682	66406	84060	88125	87465	92649	80154	71803	65048	72324	1056
B	948	84797	77934	88290	91496	81893	88520	70566	68833	88646	74109	1067
C	821	86086	82925	84243	78209	87857	101554	77191	73762	77057	74447	1115
D	941	86607	81489	96475	90441	91143	88745	86454	83801	78995	72944	1148
E	1013	83261	76210	89203	83419	88553	78937	86855	74143	84940	79169	1152
F	952	79986	79040	92939	85163	96844	90605	88856	86771	78361	71446	1129
G	930	86678	83490	82842	79736	93943	83535	87004	73982	77854	82483	1127
H	944	77300	84411	93199	93500	87535	92483	83089	90766	72791	76006	934

Z' factor = 0.72 > 0.5

B



Name of Material/Equipment	Company	Catalog Number
Cell culture microplate, 96 well, PS, F-BOTTOM	Greiner-Bio-One GmbH	655098
DMEM	Sigma Aldrich	D6046
DMSO	Tocris Bioscience	3176
Effectene transfection reagent	Qiagen	301425
FBS	Nichirei	175012
GloMax 96 microplate luminometer	Promega	E6521
HBx–LgBit expressing DNA plasmid	Our laboratory	
HEK293T cells	American Type Culture Collection	CRL-11268
NanoBiT PPI starter systems	Promega	N2015
Opti-MEM	Thermo Fisher Scientific	11058021
PBS	Takara	T900
Penicillin-Streptomycin	Sigma Aldrich	P0781
Screen-Well FDA-approved drug library V2 version 1.0	Enzo Life Sciences	BML-2841
SmBit–DDB1 expressing DNA plasmid	Our laboratory	
Trypsin-EDTA	Sigma Aldrich	T4049

Comments/Description
Includes DNA-condensation buffer, enhancer solution and transfection reagent
Available upon request
Includes Nano-Glo Live Cell Reagent Described as "buffered cell culture medium" in the manuscript
Compounds used here were as follows: mequinol, mercaptopurine hydrate, mesna, mestranol, metaproterenol hemisulfate, metaraminol bitartrate, metaxalone, methacholine chloride, methazolamide, methenamine hippurate, methocarbamol, methotrexate, methoxsalen, methscopolamine bromide, methsuximide, methyclothiazide, methyl aminolevulinate·HCl, methylergonovine maleate, metolazone, metyrapone, mexiletine·HCl, micafungin, miconazole, midodrine·HCl, miglitol, milnacipran·HCl, mirtazapine, mitotane, moexipril·HCl, mometasone furoate, mupirocin, nadolol, nafcillin·Na, naftifine·HCl, naratriptan·HCl, natamycin, nebivolol·HCl, nelarabine, nepafenac, nevirapine, niacin, nicotine, nilotinib, nilutamide, nitazoxanide, nitisinone, nitrofurantoin, nizatidine, nortriptyline·HCl, olsalazine·Na, orlistat, oxaprozin, oxtriphylline, oxybutynin Chloride, oxytetracycline·HCl, paliperidone, palonosetron·HCl, paromomycin sulfate, pazopanib·HCl, pemetrexed disodium, pemirolast potassium, penicillamine, penicillin G potassium, pentamidine isethionate, pentostatin, perindopril erbumine, permethrin, perphenazine, phenelzine sulfate, phenylephrine, phytonadione
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We would like to appreciate editor's valuable comments on the manuscript. We have responded to all of the concerns raised as indicated below. Changes to the text are underlined and highlighted in red.

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Response: *We would like to thank the editor for formatting the manuscript. We used this version to incorporate the changes requested.*

2. Please revise lines 29-31, 33-36, 52-56, and 188-190 to avoid previously published text.

Response: *We would like to thank the editor for raising this matter. We revised the manuscript as requested.*

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Response: *We would like to appreciate the editor's specific comments. We revised the manuscript as requested with turning on Track Changes.*

4. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

Response: *We would like to thank the editor for raising this matter. We revised the Table of Materials as requested.*

We greatly thank the editor for the constructive comments, which have helped us to substantially improve our manuscript.

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