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Evaluation of Inhibitory Effects of Thiobarbituric Acid Derivatives Targeting HCV NS5B Polymerase

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A series of thiobarbituric acid derivatives were constructed and evaluated for inhibitory activity on hepatitis C virus NS5B polymerase. In biochemical assays using purified viral polymerase and RNA template, the IC_{50} value was improved to 0.41 μM from the original compound's 1.7 μM value. In HCV subgenomic replicon assay, the EC_{50} value was improved to 3.7 μM from the original compound's 12.3 μM value. CC_{50} was higher than 77 μM for all compounds tested, suggesting that they are useful candidates for anti-HCV therapy.

Keywords: Hepatitis C virus, NS5B polymerase, inhibitor, thiobarbituric acid

Viral polymerases have been attractive targets in antiviral development since they play an essential role in genomic replication during viral multiplication. Hepatitis C virus (HCV), the causative agent of chronic hepatitis, hepatic cirrhosis, and hepatocellular carcinoma, has a nonstructural 5B (NS5B) protein as the RNA polymerase among 10 viral proteins encoded in the positive-sense RNA genome [1, 5, 8]. It has been one of the major therapeutic targets since the virus was identified in 1989 [3]. In spite of ceaseless efforts for development of HCV antivirals, there is no vaccine and effective therapeutics so far. Patients infected with HCV are estimated at over 170 million, which is 3% of the whole world population [9]. Therefore, development of effective antivirals against HCV is urgently demanded and continuously required.

We previously reported screening of a chemical library searching for compounds inhibiting HCV NS5B and selection of a series of thiobarbituric acids using a system for partial reconstitution of HCV polymerization in a bacterial cell [4, 6]. In this study, we examined the inhibitory effects of derivatized chemicals by determining IC_{50} values in a biochemical enzyme reaction and EC_{50} values in HCV subgenomic replicon cells [7].

Chemical structures of selected thiobarbituric acid derivatives (#30, #35, #39, #46, and #53) are shown in Table 1. IC_{50} and EC_{50} values of the compounds were evaluated by [^{32}P]-incorporation assay and real-time PCR, respectively. For IC_{50} evaluation, 20 μl of reaction mixture containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 μCi of ^{32}P-UTP (GE Healthcare), 50 μM UTP, 130 g/ml poly(A)-oligo(dT) template (GE Healthcare), and 1 μg of recombinant NS5B was incubated at 30°C for 90 min. To stop the reaction, EDTA was added in the reaction to the final concentration of 100 mM. Unincorporated ^{32}P-UTP was removed using a QIA Quick Nucelotide Removal kit (Qiagen) and the incorporated radioactivity was measured in a liquid scintillation counter (Perkin Elmer Life Sciences). For EC_{50} evaluation, we performed real-time PCR using a Taqman probe specific to the positive-sense HCV RNA strand. Approximately 6×10^5 subgenomic replicon-harboring Huh7 cells [7] were seeded in a 6-well culture plate and incubated in a CO_2 incubator at 37°C for 24 h. Chemicals were added at various concentrations and the cells were incubated for another 72 h. The cells were recovered with trypsin treatment, and total RNA was isolated using TRIzol reagent (Invitrogen). The RNA was treated with DNase I (Promega) to remove any contaminating DNA and was subjected to a real-time PCR using an iCycler MyiQ system (BioRad). A Taqman probe method of real-time PCR was used to determine the HCV RNA level, which was calculated by the delta/delta C_t method [2] with a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers specific to the genotype 1b HCV replicon (GenBank AJ242654) were 5'-ttccatgctcaccgaccc-3' (sense), 5'-cgccc

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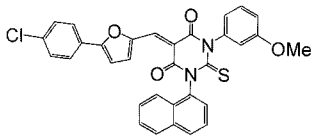
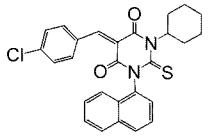
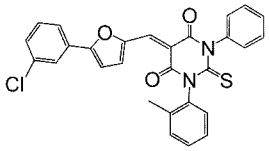
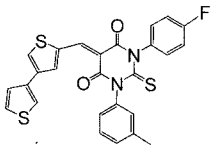
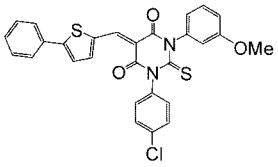
Table 1. Inhibitory effects of derivative compounds.

Derivatives	Structure	IC_{50}*,a (μM)	EC_{50}#,a (μM)
#30		0.43±0.03	18.7±1.2
#35		0.41±0.13	6.2±1.5
#39		0.55±0.08	3.7±0.6
#46		0.92±0.17	5.6±0.5
#53		0.78±0.05	5.1±0.5

*The IC_{50} was measured by a [^{32}P]-UTP incorporation assay using poly(A)-oligo(dT) template and recombinant NS5B, and represents the concentration of the inhibitor showing a 50% reduction in the recombinant NS5B polymerase activity.

#The EC_{50} was measured by real-time PCR analysis, and represents the concentration of the inhibitor showing 50% reduction in the RNA level in a Huh7 cell harboring the HCV subgenomic replicon.

[a]Values are reported as means±standard deviation.

atctcctgccg-3' (antisense), and 5'-ccacattacggcggagacggct-3' (probe). The primers specific to GAPDH (GenBank NM002046) were 5'-aaacctgccaaatatgatgacat-3' (sense), 5'-gcccaggatgcccttga-3' (antisense), and 5'-ccgacgcctgcttcacc acctt-3' (probe).

Five compounds with improved activity were selected based on the performance in the bacterial cell-based assay [4]. The original compound (Fig. 1) showed an IC_{50} of 1.7 μM. IC_{50} values of the five compounds were improved to the range of 0.41 μM~0.92 μM (Table 1). EC_{50} values of five derivatives were improved to the range of 3.7 μM~18.7 μM as compared with the EC_{50} of the original compound (12.3 μM). Since cytotoxicity is an important characteristic for inhibitory compounds, we measured the cytotoxicity of the five derivatives using MTT assay in Huh7 cells. CC_{50} values of all compounds were higher than 77 μM (data not shown).

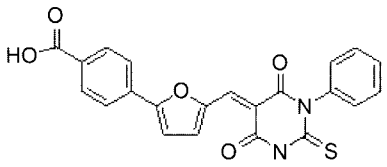


Fig. 1. The structure of the original hit compound (G05). A series of derivatives were synthesized based on this structure.

When compared with the initial hit compound, placing phenyl or naphthyl on the ring side chain for more hydrophobicity improved the potency. However, the comparable high activity of the five-membered-ring compound with the six-membered thiobarbiturate revealed that the side chain on the ring nitrogen was not necessary to hold the activity as long as the proper core ring sits on the proper site of the enzyme. Therefore, this suggests that identifying the exact location of the active molecule in the enzyme is necessary to understand the high activity of these thiobarbituric acid derivatives.

In summary, we confirmed derivatized thiobarbituric acids as potent inhibitors of HCV NS5B polymerase, using three methods. With IC_{50} values at the submicromolar range and CC_{50} values at > 77 μM, the compounds are good candidates for anti-HCV therapy.

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