RESEARCH PAPER

Isoindole-1,3-dione-based α,γ-diketo acid bioisosteres as hepatitis C virus NS5B polymerase inhibitors

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Keywords
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Abstract

Integration of 2-N-hydroxyl- and 2-N-benzoyl-1,3-diketo acid moieties into isoindole-1,3-dione led to the development of two series of hepatitis C virus NS5B polymerase inhibitors. Structural optimization to translate enzyme inhibitory activity to cellular cytotoxicity yielded compound 16c, a moderate enzyme inhibitor (IC50 = 27.3 μM) with selective toxicity to hepatitis C virus 1b replicon-containing Ava5 cells (EC50 = 18.0 μM). Binding experiments indicated that 16c (Kd = 1.25 μM) not only competed with fluorescein-labeled GTP for NS5B binding, but also displaced bound GTP from the polymerase active site.

Introduction

Hepatitis C virus (HCV) infections are steadily increasing and have affected approximately 170 million people worldwide (Weiss, 2005). Chronic HCV infections are the major cause of liver fibrosis, cirrhosis, and hepato-cellular carcinoma (Quer et al, 2010). The current therapeutic approach for HCV infections is a combination of the broad spectrum antiviral ribavirin and pegylated interferon-α. However, poor sustained virological response and severe side effects limit the effectiveness of this treatment (Bjornsson et al, 2009); therefore, there is an urgent need for more effective treatment options for HCV infection.

HCV is an enveloped linear single-stranded RNA virus in the hepacivirus

genus of the flaviviridae family (Choo et al, 1989). The RNA-dependent RNA polymerase of HCV, NS5B, plays a crucial role in viral replication and it has no counterpart in mammalian cells, making it a common target in the design of potential drugs to treat HCV infection (Walker et al*,* 2002).

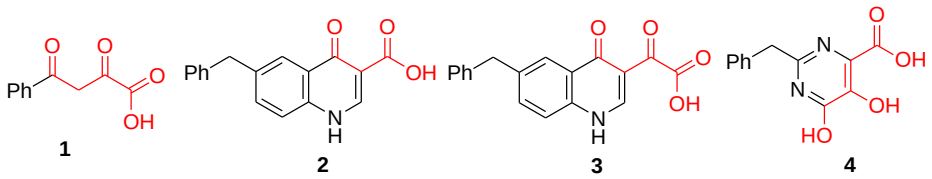
A number of structurally diverse scaffolds have been identified that can inhibit NS5B (Deore et al*,* 2010). Although many potent allosteric inhibitors of NS5B have been developed and reached clinical trials (Fansciscus, 2013), relatively few active site inhibitors, including α,γ-diketo acid (DKA) derivatives (Summa et al, 2004), dihydroxy-pyrimidine (Stansfield et al, 2004), meconic acid (Pace et al*,* 2004), β-*N*-hydroxy-γ-keto acid (Deore et al*,* 2010), and β-*N*-hydroxy-γ-keto carboxamide (Deore et al*,* 2010), have been examined despite the fact that the active site of NS5B is highly conserved among all HCV subtypes (Wu et al, 2003).

DKA has served as a starting point for the design of several divalent metal ion chelators to inhibit influenza endonuclease, human immunodeficiency virus (HIV) integrase, and flap endonuclease (Kirschberg et al, 2007). Mechanis-tically, DKA derivatives bind to the two magnesium ions involved in coordinating the β- and γ-phosphate groups of the reacting ribonucleotide triphosphate (NTP) and thus are also referred to as pyrophosphate mimics (Summa et al, 2004). At physio-logical pH, the carboxylic acid of DKA is deprotonated and the acidity of the 1,3-dicarbonyl scaffold generates dianions that can coordinate the divalent metal ion species (Kirschberg et al, 2009). Based on the structural features and divalent metal ion chelating ability, we have classified DKA bioisosteres into three categories (Fig. 1), as follows: those containing a free carboxylate moiety, those containing an aromatic ring-bound hydroxamate moiety, and those containing two hydroxyl groups within the isoindole core.

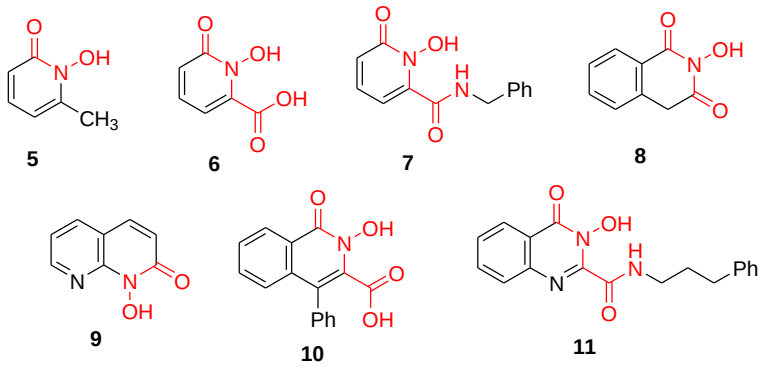
HIV integrase inhibitors DKA **1**, 4-quinolone-3-carboxylic acid **2**, and and 4-quinolone-3-glyoxalic acid **3** maintain coplanar conformations with three functional groups that mimic ketone, enolizable ketone, and carboxyl oxygen (Sato et al, 2006), whereas the pyrimidinol-containing carboxylic acid **4** is an HIV-1 RNase H inhibitor (Kirschberg et al, 2009).

Small molecules containing hydroxamate within their aromatic scaffold can also chelate divalent metal ions, and compounds **5** and **6** have been shown to specifically sequester tetravalent actinides (Uhlir et al*,* 1993; White et al*,* 1988). Zinc-binding hydroxamate compound **7** can act as anthrax lethal factor inhibitors (Agrawal et al*,* 2009) and others, such as *N*-hydroxyimide **8** (Billamboz et al*,* 2008), *N*-hydroxy-naphthyridinone **9** (Williams et al, 2010), *N*-hydroxy-isoquinoline **10** (Deore et al, 2012), and *N*-hydroxy-quiazolinone **11** (Deore et al, 2012), inhibit magnesium-dependent enzymes. DKA isosteres with dihydroxyl within their isoindole core, including dihydroxy-isoindol-1-one **12** and dihydroxy-isoindole-1,3-dione **13**, inhibit Mg^{2+} dependent strand transfer by HIV-1 integrase (Zhao et al, 2009).

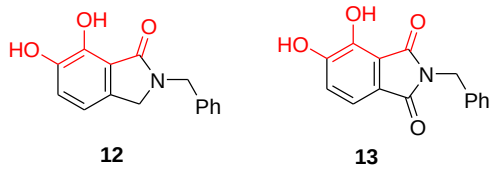
In a continued effort to identify new pharmacophores for NS5B inhibition, we developed two isoindole-1,3-dione–based series of compounds, **15a-n** and **16a-f** starting from compound **14** (Fig. 2).



(a) DKA isosters with free carboxylate



(b) DKA isosters with ring inbuilt hydroxamate



(c) DKA isosters with dihydroxy function on isoindole core

Figure 1 Classes of divalent metal ion-chelating DKA isosteres. (a) DKA isosteres containing a free carboxylate moiety (**1**-**4**), (b) an aromatic ring-bound hydroxamate moiety (**5**-**11**), and (c) two hydroxyl groups within the isoindole core (**12** and **13**).

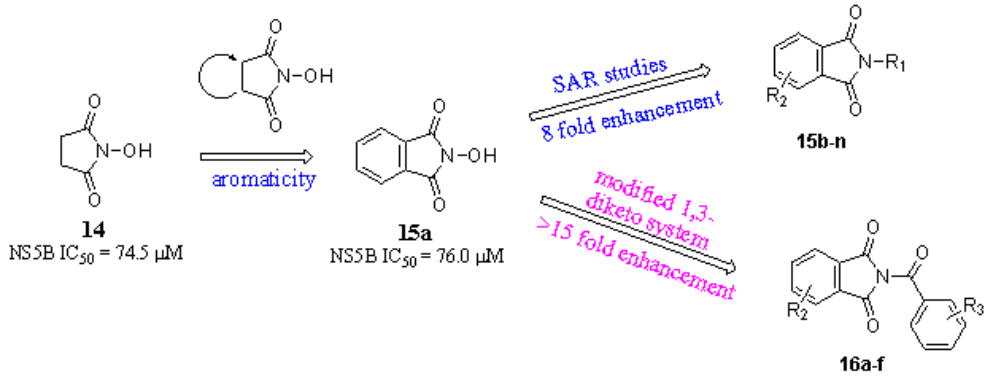
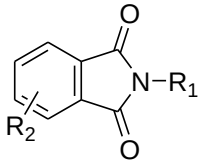


Figure 2 Design of NS5B polymerase inhibitors**15a-n** and **16a-f**. The chemical moieties at R_1, R_2, and R_3 in the various derivatives are listed in Tables 1 and 2.

Table 1 2-Hydroxyisoindoline-1,3-diones as anti-HCV agents

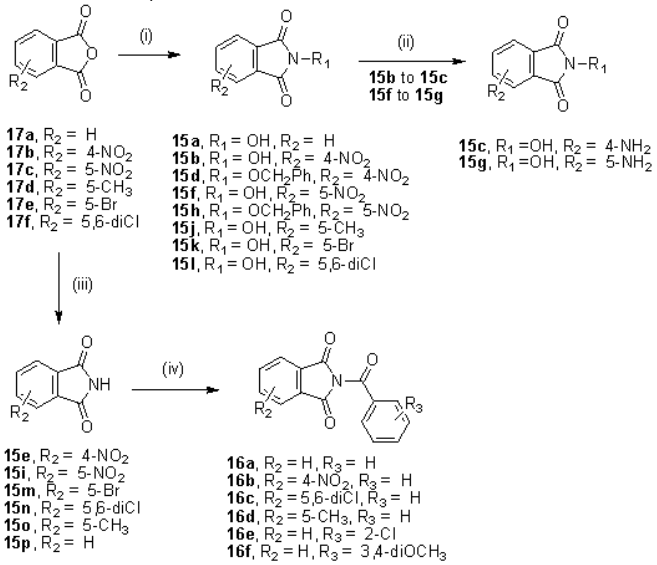


15a-n

Compound	R_1	R_2	IC_{50}[a,b] (μM)	CC_{50}[c] (μM)	EC_{50}[d] (μM)
14			74.5 ± 3.8	> 50.0	> 50.0
15a	OH	H	76.0 ± 7.5	> 50.0	> 50.0
15b	OH	4-NO_2	9.5 ± 5.4	> 50.0	> 50.0
15c	OH	4-NH_2	23.0 ± 2.5	49.4 ± 5.7	> 50.0
15d	OCH_2Ph	4-NO_2	32.9 ± 3.8	> 50.0	> 50.0
15e	H	4-NO_2	> 50.0	> 50.0	> 50.0
15f	OH	5-NO_2	18.5 ± 2.1	> 50.0	> 50.0
15g	OH	5-NH_2	30.8 ± 4.3	> 50.0	> 50.0
15h	OCH_2Ph	5-NO_2	27.3 ± 1.9	> 50.0	26.1 ± 4.2
15i	H	5-NO_2	43.1 ± 6.7	> 50.0	> 50.0
15j	OH	5-CH_3	42.6 ± 5.5	35.5 ± 2.6	> 50.0
15k	OH	5-Br	49.1 ± 3.7	38.7 ± 4.9	> 50.0
15l	OH	5,6-diCl	25.2 ± 2.9	> 50.0	> 50.0
15m	H	5-Br	> 50.0	> 50.0	> 50.0
15n	H	5,6-diCl	> 50.0	> 50.0	> 50.0
1			19.2 ± 2.4	> 50.0	> 50.0

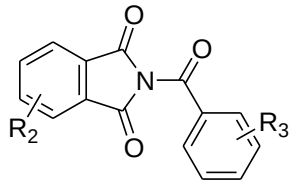
[a]NS5B inhibitory activity based on pyrophosphate generation
[b]IC_{50} (concentration required for 50% inhibition of NS5B activity)
[c]CC_{50} (concentration required for 50% cytotoxicity of Huh7 cells)
[d]EC_{50} (half maximal effective cytotoxic concentration in Ava5 cells containing the HCV replicon)
[e]DKA 1 was synthesized as a reference compound
Values represent the mean ± standard deviation of three independent experiments, each carried out in triplicate.

Scheme 1 Synthesis of Isoindole-1,3-dione derivatives[a]



[a]Reagents and conditions: (i) $PhCH_2O\text{-}NH_2.HCl$/$HO\text{-}NH_2.HCl$, triethylamine, pyridine; microwave, 200W, 150°C, 10 to 20 min. (ii) H_2, Pd/C, methanol, room temperature, 2 h.(iii) formamide; microwave, 200W, 200°C, 10 min.(iv) aroyl chloride, triethylamine, tetrahydrofuran, reflux, 24 to 60 h

Table 2 2-Aroylisoindoline-1,3-diones as anti-HCV agents



16a-f

Compound	R_2	R_3	IC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)
16a	H	H	6.8 ± 1.9	> 50.0	37.2 ± 6.2
16b	4-NO_2	H	4.9 ± 2.2	> 50.0	> 50.0
16c	5,6-diCl	H	27.3 ± 5.1	> 50.0	18.0 ± 3.1
16d	5-CH_3	H	10.8 ± 1.7	> 50.0	> 50.0
16e	H	2-Cl	16.2 ± 4.6	> 50.0	26.1 ± 2.5
16f	H	3,4-diOCH_3	12.3 ± 2.2	> 50.0	> 50.0

Materials and Methods

All solvents were ACS grade, obtained from Merck, ECHO, or Mallinckrodt, and used without further purification. An SP-1 stand-alone solvent purification system (LC Technology Solutions) was used to dry the solvents, which then contained <10 ppm water as determined by Karl-Fischer moisture analysis. Other chemicals were purchased from Acros, Aldrich, Alfa Aesar, Carbosynth, Matrix, Ryss, and Zealing and used without additional purification.

TLC was performed using precoated silica gel plates (60 F254, Merck), and separated compounds were visualized under UV light.

Flash column chromatography used Merck silica gel 60 (40–63 μm). ^{1}H- and ^{13}C-NMR spectra were obtained using a Bruker Avance 400-MHz or 200-MHz

NMR spectrometer, each equipped with a quadruple nucleus probe. Chemical shifts were referenced to the central peak of the DMSO-d_6 spectrum (2.49 ppm for ^{1}H NMR and 39.4 ppm for ^{13}C NMR). Elemental analyses for C, H, and N used a Heraeus VariaEL-III elemental analyzer.

Melting points were determined using a Mel-Temp melting-point apparatus (Laboratory Devices Inc.) and are reported uncorrected.

Chemistry: Compounds **15a**, **15b**, **15d**, **15f**, **15h**, and **15j-l** were synthesized from commercially available phthalic anhydrides by treatment with hydroxylamine HCl and triethylamine in pyridine under microwave (200W) irradiation at 150 °C for 10 to 20 min. Nitro analogs **15b** and **15f** were reduced to amino analogs **15c** and **15g**, respectively, by addition of hydrogen and Pd on carbon in methanol at room tempera-ture for 2h. Treatment of the phthalic anhydrides with formamide under microwave irradiation resulted in **15e**, **15i**, and **15m-p**, which upon addition of aroyl chloride, triethyl-amine, and tetrahydrofuran under reflux condition for 24 to 60 h yielded **16a-f**. Synthetic procedures and charac-terization of all compounds are described in supplementary information.

General procedure for synthesis of 15a, 15b, 15d, 15f, 15h, 15j-l: A mixture of suitable phthalic anhydride, hydroxylamine/benzyloxy-amine hydro-chloride (2 equivalents) and pyridine (as a solvent) was subjected to microwave

irradiation (200 W, 150°C, 10-20 min).

Reaction progress was monitored by TLC. The reaction mixture was acidified with 1 N HCl followed by extraction using ethyl acetate. The combined organic extracts were dried using anhydrous sodium sulfate, filtered, and concentrated. Either derived solid was filtered and washed with hexane to provide final compounds or were passed through flash silica gel column chromatography (chloroform/ methanol, 100:0 to 95:5) followed by evaporation of the solvents and recrystallization.

2-Hydroxy-isoindole-1,3-dione (**15a**): A white solid (Yield: 80.4%); Mp: 231-233 °C [230-238 °C (Kebber et al*,* 1972)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 10.81 (s, 1H, OH), 7.82 (s, 4H, ArH).

2-Hydroxy-4-nitro-isoindole-1,3-dione (**15b**): A yellowish white solid (Yield: 23.2%); Mp: 208-210 °C [209-213 °C (Wentzel et al*,* 2000)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 11.77 (bs, 1H, OH), 8.25 (dd, 1H, ArH, J = 7.7 Hz, 1.2 Hz), 8.13-7.98 (m, 2H, ArH).

2-Benzyloxy-4-nitro-isoindole-1,3-dione (**15d**): A yellowish white solid (Yield: 54.2%); Mp: 189-192 °C; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 8.30 (d, 1H, ArH, J = 8 Hz), 8.18-8.02 (m, 2H, ArH), 7.53-7.41 (m, 5H, ArH), 5.17 (s, 2H, CH_2). Anal. Calcd for $C_{15}H_{10}N_2O_5.0.2H_2O$: C, 59.68; H, 3.47; N, 9.28. Found: C, 59.47; H, 3.53; N, 9.41.

2-Hydroxy-5-nitro-isoindole-1,3-dione (**15f**): A yellowish white solid (Yield: 61.2%); Mp: 170-172 °C [168-170 °C (Chan et al*,* 1987)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 11.83 (s, 1H, OH), 8.59 (dd, 1H, ArH, J = 8.2 Hz, 2 Hz), 8.41 (d, 1H, ArH, J = 1.4 Hz), 8.05 (d, 1H, ArH, J = 8.2 Hz).

2-Benzyloxy-5-nitro-isoindole-1,3-dione (**15h**): A yellowish white solid (Yield: 56.0%); Mp: 190-191 °C [188-190 °C (Kerrigan et al*,* 1996)]; ^{1}H NMR (DMSO-d_6), 400 MHz: δ 8.62 (dd, 1H, ArH, J = 8 Hz, 2 Hz), 8.48 (d, 1H, ArH, J = 2 Hz), 8.12 (d, 1H, ArH, J = 8.4 Hz), 7.54-7.50 (m, 2H, ArH); 7.44-7.39 (m, 3H, ArH), 5.19 (s, 2H, OCH_2), ^{13}C NMR (DMSO-d_6), 100 MHz: δ 161.9, 161.7, 151.9, 134.4, 133.9, 130.5, 130.2, 130.0, 129.6, 128.9, 125.1, 118.3, 79.8.

2-Hydroxy-5-methyl-isoindole-1,3-dione (**15j**): A white solid (Yield: 49.1%); Mp: 202-203 °C [202 °C (Wentzel et al*,* 2000)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ10.7 (s, 1 H, OH), 7.72-7.59 (m, 3 H, ArH), 2.45 (s, 3 H, CH_3).

5-Bromo-2-hydroxy-isoindole-1,3-dione (**15k**): A white solid (Yield: 37.0%); Mp: 215-217 °C; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 10.93 (bs, 1H, OH), 8.04-7.99 (m, 2H, ArH), 7.77-7.72 (m, 1H, ArH). Anal. Calcd for $C_8H_4BrNO_3.H_2O$: C, 36.95; H, 2.33; N, 5.39. Found: C, 37.11; H, 2.42; N, 5.22.

5,6-Dichloro-2-hydroxy-isoindole-1,3 -dione (**15l**): A white solid (Yield: 76.0%); Mp: 194-195 °C [195-197 °C (Villiger*,* 1909)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ11.05 (bs, 1H, OH), 8.14 (s, 2H, ArH).

General procedure for synthesis of 15c and 15g: A mixture of nitro compound **15b**/**15f** and 10% palladium on carbon in EtOH and THF was stirred under hydrogen gas at room temperature for 2 hours. The mixture was passed through a plug of celite before being concentrated to yield crude mixture. Purification by flash silica gel chromatography (chloroform/methanol, 100:0 to 95:5) provided amino compound **15c/15g**.

4-Amino-2-hydroxy-isoindole-1,3-dione (**15c**): A yellow solid (Yield: 28.0%); Mp: 248-250 °C [247-249 °C (Elke*,* 2001)]; ^{1}H NMR (DMSO-d_6), 200 MHz: δ10.49 (s, 1H, OH), 7.45-7.37 (m, 1H,

ArH), 6.94 (t, 2H, ArH, J = 8 Hz), 6.45 (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6), 50 MHz: δ 165.7, 164.3, 146.2, 135.1, 129.2, 121.9, 110.7, 105.3.

5-Amino-2-hydroxy-isoindole-1,3-dione (**15g**): A yellow solid (Yield: 35.5%); Mp: 270-272°C [272-273 °C (Chan et al*,* 1987)]; ^1H NMR (DMSO-d_6), 200 MHz: δ 10.42 (s, 1H, OH), 7.43 (d, 1H, ArH, J = 8.2 Hz), 6.88 (d, 1H, ArH, J = 2 Hz), 6.75 (dd, 1H, ArH, J = 8.2 Hz, 2.2 Hz), 6.50 (s, 2H, NH_2).

General procedure for synthesis of 15e, 15i, 15m-p: Appropriate phthalic anhydrides were reacted with formamide (4 ml) under microwave condition at 150 ºC for 10 minutes. The reaction mixture was mixed with water, the crystals were filtered off and washed with water. Isoindole-1,3-diones **15e**, **15i**, **15m-p** were recrystallized using hexane and ethyl acetate.

4-Nitro-isoindole-1,3-dione (**15e**): A white crystalline solid (Yield: 80.6%); Mp: 205-206°C [205-208 °C (Caswell et al*,* 1964)]; ^1H NMR (DMSO-d_6), 200 MHz: δ 11.76 (s, 1H, NH), 8.25 (dd, 1H, ArH, J = 7.6 Hz, 1.2 Hz); 8.13-7.98 (m, 2H, ArH).

5-Nitro-isoindole-1,3-dione (**15i**): A white crystalline solid (Yield: 85.0%); Mp: 198-200°C [199-201 °C (Bailleux et al*,* 1994)]; ^1H NMR (DMSO-d_6), 400 MHz: δ 8.63 (s, 1H, NH), 8.59 (dd, 1H, ArH, J = 8 Hz, 2 Hz), 8.41 (d, 1H, ArH, J = 2 Hz), 8.05 (d, 1H, ArH, J = 8 Hz).

5,6-Dichloro-isoindole-1,3-dione (**15 m**): A white crystalline solid (Yield: 90.5%); Mp: 192-193 °C [193-195 °C (Wohrle et al*,* 1993)]; ^1H NMR (DMSO-d_6), 200 MHz: δ 11.62 (s, 1H, NH), 8.10 (s, 2H, ArH).

5-Methyl-isoindole-1,3-dione (**15n**): A white crystalline solid (Yield: 88.0%); Mp: 196-198 °C [196 °C (Smith et al*,* 1964)]; ^1H NMR (DMSO-d_6), 200 MHz: δ 9.81 (s, 1H, NH), 7.54-7.31 (m, 3H, ArH), 2.39 (s, 3H, ArH, J = 2 Hz).

General Procedure for 16a-f: A mixture of appropriate isoindole1,3-dione **15**, triethylamine (1.5 equivalents) and aroyl chloride/ benzyl bromide (1.5 equivalents) in THF was stirred under reflux condition for 24-60 h (reactions were monitored by TLC for completion). After removal of the solvent under reduced pressure, water was added to the residue, acidified with 2 N HCl followed by extraction using ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated. Purification of crude product was performed by chromategraphy on silica gel column (hexane/ethyl acetate, 1:1) to afford compound **16**.

2-Benzoyl-isoindole-1,3-dione (**16a**): A white solid (Yield: 85.4%); Mp: 167-169 ºC [167-168 ºC (Kaiser et al*,* 1970)]; ^1H NMR (DMSO-d_6), 200 MHz: δ 8.02-7.93 (m, 6H, ArH), 7.77-7.69 (m, 1H, ArH), 7.58-7.50 (m, 2H, ArH).

2-Benzoyl-4-nitro-isoindole-1,3-dione (**16b**): A yellowish white solid (Yield: 80.5%); Mp: 160-162 ºC; ^1H NMR (DMSO-d_6), 200 MHz: δ 8.40 (dd, 1 H, ArH, J = 7.84 Hz, 0.88 Hz), 8.28 (d, 1 H, ArH, J = 6.6 Hz), 8.15 (t, 1H, ArH), 8.05 (d, 2 H, ArH, J = 7.14 Hz), 7.77 (t, 1 H, ArH), 7.57 (t, 2 H, ArH); Anal. Calcd for $C_{15}H_8N_2O_5$: C, 60.82; H, 2.72; N, 9.46. Found: C, 60.98; H, 2.87; N, 9.36.

2-Benzoyl-5,6-dichloro-isoindole-1,3-dione (**16c**): A white solid (Yield: 60.4%); Mp: 198-200 ºC; ^1H NMR (DMSO-d_6), 400 MHz: δ 8.33 (s, 2H, ArH), 8.00 (d, 2H, ArH, J = 8 Hz), 7.74 (t, 1H, ArH, J = 7.6 Hz), 7.55 (t, 2H, ArH, J = 7.6 Hz), ^{13}C NMR (DMSO-d_6), 100 MHz: δ 166.5, 163.7, 138.1, 134.5, 132.3, 131.3, 130.3, 128.6, 126.0; Anal. Calcd for $C_{15}H_7Cl_2NO_3$: C, 56.28;

H, 2.20; N, 4.38. Found: C, 56.18; H, 2.48; N, 4.48.

2-Benzoyl-5-methyl-isoindole-1,3-dione (**16d**): A white solid (Yield: 69.7%); Mp: 143-145 ºC; ^{1}H NMR (DMSO-d_6), 400 MHz: δ 7.96 (d, 2H, ArH, J = 8 Hz), 7.87 (d, 1H, ArH, J = 7.6 Hz), 7.82 (s, 1H, ArH), 7.77-7.68 (m, 2H, ArH), 7.54 (t, 2H. ArH, J = 7.8 Hz), 2.52 (s, 3H, CH_3), ^{13}C NMR (DMSO-d_6), 100 MHz: δ 167.1, 165.5, 165.3, 146.6, 135.9, 134.3, 132.7, 131.6, 130.2, 128.6, 128.5, 124.3, 123.9, 21.3; Anal. Calcd for $C_{16}H_{11}NO_3$: C, 72.45; H, 4.18; N, 5.28. Found: C, 72.32; H, 4.19; N, 5.23.

2-(2-Chloro-benzoyl)-isoindole-1,3-dione (**16e**): A white solid (Yield: 68.4%); Mp: 182-183 ºC; ^{1}H NMR (DMSO-d_6), 200 MHz: δ 8.10-7.94 (m, 4H, ArH), 7.78 (d, 1H, ArH, J = 7 Hz), 7.65-7.43 (m, 3H, ArH); ^{13}C NMR (DMSO-d_6), 50 MHz: δ 165.0, 164.4, 136.4, 134.4, 133.4, 131.3, 130.8, 130.7, 130.3, 127.9, 124.8; Anal. Calcd for $C_{15}H_8ClNO_3$: C, 63.06; H, 2.82; N, 4.90. Found: C, 63.03; H, 2.82; N, 5.00.

2-(3,4-Dimethoxy-benzoyl)-isoindole -1,3-dione (**16f**): A yellowish white solid (Yield: 65.4%); Mp: 199-202 ºC; ^{1}H NMR (DMSO-d_6), 400 MHz: δ 8.00-7.93 (m, 4H, ArH), 7.68 (dd, 1H, ArH, J = 8 Hz, 2 Hz), 7.53 (d, 1H, ArH, J = 2 Hz) 7.07 (d, 1H, ArH, J = 8.4 Hz); 3.88 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6), 100 MHz: δ 166.7, 166.2, 154.9, 149.0, 135.7, 131.9, 126.5, 125.1, 124.3, 113.0, 11.4, 56.3, 56.2; Anal. Calcd for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.21; N, 4.50. Found: C, 65.37; H, 4.24; N, 4.43.

Biology: The detailed protocols for expression and purification of recombinant His-tagged NS5B polymerase, NS5B polymerase assay based on inorganic pyrophosphate (PPi) generation, and MTT based cytotoxic analysis are described in our previous publications (Deore et al*,* 2012; Deore et al*,* 2012). The recombinant His-tagged NS5B polymerase was confirmed by using anti-NS5B antibody with western blotting analysis (Fig. S1).

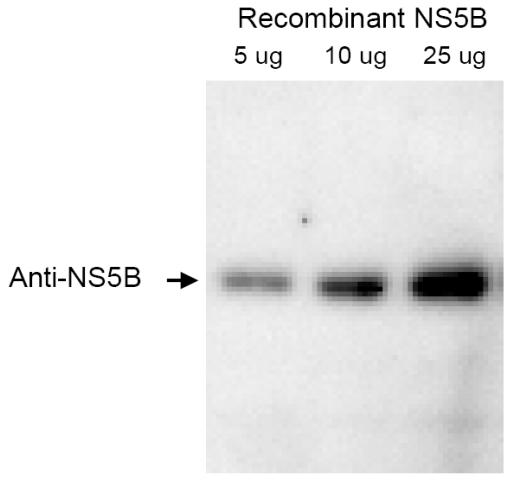


Figure S1 Identification of NS5B by using western blotting with specific anti-NS5B antibody.

NS5B polymerase assay based on inorganic pyrophosphate (PPi) generation: For PPi assay, HCV NS5B activity was performed in Luimtrac 96-well microplate (Greiner Bio-one) as the followings: Reaction conditions for NS5B polymerase were HEPES (50 mM, pH 8.0), $MgCl_2$ (2.5 mM), RNAsin (20 U/mL, Promega), Random heteropolymer primer (0.5 μg/mL, MDbio), RNA template isolated from HepG2 cells (5 μg/mL), NTP mixture (1 μM, Invitrogen), DTT (4 mM, Sigma), and recombinant His-tagged NS5B polymerase (1 μg/mL) in the presence or absence of compounds (0, 1, 10, 25, 50 μM, but for 14 and 15a the concentration was up to 100 μM). The reaction mixture was supplemented with ATP sulfuryl-ase and luciferase-coupled enzyme (each 30 μL, Cambrex Bio Science Roackland) after incubation at 37°C for 60 min. The initiated

sulfurylase/ luciferase based reactions were incubated at 37°C for 60 min and transferred to the Luminometer Orion II (Berthold DS, Germany) for detection of the light signal gen-erated over time at a 0.2 s reading every 30-60 s. Readings were monitored and compared with NS5B reaction without treatment as positive control (100% NS5B activity), and no NS5B reaction as negative control (0% NS5B activity means no inorganic pyrophosphate generation). For replicon-based anti-viral activity, it was assessed in a 3 day assay using human hepatoma cell lines, Ava5 (Huh7 cells containing subgenomic HCV replicon, genotype 1b) and parent Huh7 cells, maintained as sub-confluent cultures on 96-well plates. The cytotoxic effects triggered by compounds were assessed for 48 h. Huh7 and Ava5 cells were grown at a density of 5×10^3 cells per well in 96-well plates. After 12 h, the cells were treated with compounds (0, 1, 10, 25, 50 μM) for an additional 48 h. Cell survival in 96 well plates was assessed by reduction. Cells were then incubated with 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (250 μM) at 37 °C for 3 h, and the absorbance was measured ($^{570}A_t$). Values of CC_{50} and EC_{50} were the cytotoxicities against the parent Huh7 cells and HCV replicon Ava5 cells, respectively. Standard devia-tions for CC_{50} or EC_{50} values were calculated from standard errors generated by regression analyses. Experiments were performed in quadruplicate.

Morphological investigation of Huh7 cells and Ava5 cells: The morphologies of Huh7 cells and Ava5 cells after treating with compound **16c** were investigated by Giemsa staining. Briefly, 50,000 cells were grown on glass coverslips in a 6-well tissue-culture plate (Corning, MI) in FBS (10%) and antibiotic supple-mented media, and treated with compound **16c** (20 μM) for 48 h. Then, the slides were washed with distilled water and then stained with Giemsa's solution (0.38 g of Giemsa powder in 15% methanol and 5% glycerin) followed by rinsing with distilled water and differentiated with aqueous acetic acid (0.5%), finally dehydrate rapidly and cleared and mounted. Images were taken randomly in at least 5 fields of vision under phase-contract microscopy.

Binding assays using QCM: The binding analysis of the interactions between recombinant NS5B protein and NS5B inhibitors was performed with a QCM (AffinixQμ, Initium Inc., Tokyo, Japan). The recombinant NS5B protein was absorbed onto the gold surface of QCM crystal (QN sensor microsystem, Initium Inc., Tokyo, Japan) directly.

Firstly, the gold surface was cleaned by dipping in 30% H_2O_2/H_2SO_4 (1:3) for 10 min followed by rinsing thoroughly in deionized water and then blowing dry in N_2 gas. This gold substrate was further used for the absorption of recombinant NS5B. NS5B proteins were spon-taneously adsorbed onto the evaporated gold surface of the quartz crystal from 30 μg/mL aqueous solution. After 20 min, the sensor was immersed, rinsed successively with deionized water and dried shortly in N_2 gas. This immobilized NS5B generated a final signal shifting of about 500 Hz, indicating that about 24 ng of recombinant NS5B protein was absorbed. The immobilized NS5B was confirmed (Fig. S2) by anti-NS5B antibody (1:2000) and also negative control antibody (anti-actin antibody, 1:2000).

The NS5B immobilized quartz crystal was further used for the following successive experiments. To evaluate the binding affinity of compound on NS5B, PBS (0.5 mL) with 2 mM $MgCl_2$ was added, and then compound was added to the reaction chamber by the titrant injections (0, 5, 10, 50, or 200 μM).

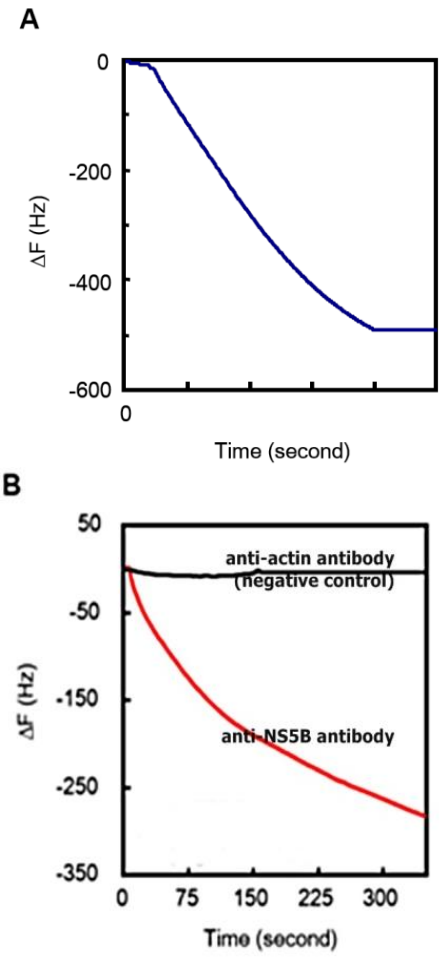


Figure S2 A) The level of absorbed NS5B onto the gold surface of QCM crystal. B) Identification of NS5B by using QCM with specific anti-NS5B antibody (also negative control antibody).

The interactions was monitored by alterations in frequency (dF) resulting from changes in mass at the electrode surface. The validation of the non-specific binding of compounds on both control (non-modified) and reference-protein (BSA) modified sensors were also carried out. The kinetic parameter was then determined by using AffinixQμ software and GraphPad Prism.

NTP competition and displacement studies and direct interactions of NS5B inhibitors with NS5B polymerase by QCM were conducted as described earlier[13]. Briefly, for competition assay, NS5B (5 μg/mL) and fluorescin-GTP (500 μM) were incubated at 25 °C for 30 min in the presence of varying concentrations of **16c** in binding buffer (containing 50 mM HEPES pH 8.0, 2.5 mM $MgCl_2$, 20 U/mL RNAsin, and 4 mM DTT).in 3kDa-cutoff 96-well filtration plate. Then, the unbound fluorescin-GTP was removed through filtration. The fluorescent intensity of the re-suspended NS5B-bound GTP was measured. For displacement assay, the NS5B was first incubated with fluorescin-GTP at 25 °C for 30 min, and the unbound GTP was subsequently removed by filtration. Then, **16c** was added into re-suspended GTP-NS5B complex for an additional incubation at 25 °C for 30 min. The rest procedures were following as described above. Both assays were taken with fluorescin-GTP as the positive control (100% GTP bound) and fluorescin alone as the negative control (0% GTP bound).

Results and Discussion

In this proof-of-concept study, we proposed that the 2-*N*-hydroxyl-1,3-diketo moiety would act as a metal-chelating pharmacophore which could serve as an isosteric replacement for DKA. The inhibitory effects of these derivatives were compared to the commercially available parent compound, *N*-hydroxysuccinimide **14** and to a reference compound DKA **1** in a non-radioactive assay based on inorganic pyrophosphate generation using recombinant, purified His-tagged NS5B, and cytotoxicity was evaluated in the Huh7 and Ava5 cell lines (Tables 1 and 2) (Deore et al, 2012; Deore et al, 2012; Lahser et al, 2004). Ava5 cells contain the replicon for the most prevalent HCV genotype, 1b. N-hydroxysuccinimide inhibited NS5B with an IC_{50} value of 74.5 μM, which is ~4

fold lower than DKA **1**(IC_{50} = 19.2 μM). 2-Hydroxyisoindoline-1,3-dione **15a** generated by fixing the aromatic ring at the 4 and 5 positions with an IC_{50} value of 76.0 μM was almost equipotent to *N*-hydroxysuccinimide. Neither compound exhibited cytotoxic properties in either of the cell lines at concentrations up to 50 μM.

We compared the activity of compounds with additional modifications to that of **15a** (Table 1). The 4-nitro analog **15b** exhibited an 8-fold enhancement in inhibitory activity (IC_{50} = 9.5 μM) over the unsubstituted analog (**15a**), comparable to our previously reported NS5B inhibitors *N***-**hydroxy-isoquinoline **10** (IC_{50} = 9.5 μM) and *N*-hydroxyquiazolinone **11**(IC_{50} = 8.8 μM) (Deore et al*,* 2012; Deore et al*,* 2012). The 4-amino analog **15c** exhibited a ~3-fold increase in anti-NS5B activity (IC_{50} = 23.0 μM) over **15a**, whereas the 2-*O*-benzyl–protected analog **15d** was ~3-fold less potent (IC_{50} = 32.9 μM) than **15b**. This reduction in the activity of the *N*-*O*-benzyl analog may be attributable to the absence of a free *N*-hydroxyl at position 2 of the scaffold. To further evaluate the importance of the *N*-hydroxyl group, we replaced the hydroxyl at position 2 with the hydrogen. The resulting compound, **15e**, was completely inactive against NS5B, confirming that the *N*-hydroxyl group is essential for activity and suggesting that it may chelate at least one of the two magnesium ions present in the active site of NS5B. Further, the 5-nitro analog **15f** was a moderate inhibitor of NS5B (IC_{50} = 18.5 μM) with a 2-fold loss of activity compared with the 4-nitro analog **15b**. Replacement of the 5-nitro group (**15f**) with a 5-amino group (**15g**) proved detrimental to inhibition (IC_{50} = 30.8 μM). Similarly, absence of the free *N*-hydroxyl group in **15h** and **15i** also resulted in loss of inhibition (IC_{50} = 27.3 μM and IC_{50} = 43.1 μM, respectively). Furthermore, the 5-methyl (**15j**), 5-bromo (**15k**), and 5,6-dichloro (**15l**) substitutions were moderate inhibitors of NS5B, though less potent than the 5-nitro derivative (**15f**), but none of them showed cytotoxic activity. However, among the compounds **15a-n**, only **15h** demonstrated cytotoxic activity.

Interestingly, although it is also only a moderate inhibitor of NS5B, the *O*-benzyl-protected analog **15h** demonstrated selective cytotoxicity against HCV genotype 1b-containing Ava5 cells, with an EC_{50}value of 26.1 μM. The benzyl group is known to improve cellular activity of isoindole-based HIV integrase inhibitors **12** and **13** (Zhao et al*,* 2009), suggesting that the benzyl group of **15h** may be directly responsible for its increased cytotoxic activity, possibly because of better cell penetration due to the relatively high lipophilicity of the *O*-benzyl group. Additionally, **15h** was not cytotoxic to Huh7 cells at concentrations up to 50 μM, indicating that the cellular effects of **15h** in Ava5 cells are HCV replicon-specific. These results suggest**,** therefore, that translating NS5B inhibitory activity into specific toxicity at the cellular level should be a primary objective of further structural modifications in isoindole-1,3-dione based NS5B inhibitors.

To explore the effects of substitutions at the other side of the scaffold and to further optimize the inhibitory and cellular activity, the 2-*N*-hydroxyl in **15a** was replaced with a 2-*N*-benzoyl group to generate the modified 1,3-diketo scaffold **16a** (Fig 2, Table 2). The additional carbonyl group in **16a** was expected to assist in chelating interactions with divalent metal ions. Compound **16a** strongly inhibited generation of pyrophosphate by NS5B (IC_{50} = 6.8 μM), showing a greater than 10-fold enhancement over the parent compound **15a**.

Moreover, **16a** demonstrated moderate cytotoxicity in the HCV replicon-containing Ava5 cells (EC_{50} = 37.2 μM), but was not toxic to the parent Huh7 cells. The 4-nitro substitution **16b** led to a remarkable increase in inhibitory activity (IC_{50} = 4.9 μM), but was inactive in the cell-based assay. On the other hand, the 5,6-dichloro substitution **16c**, was only moderately inhibitory to NS5B (IC_{50} = 27.3 μM), but was selectively cytotoxic to Ava5 cells (EC_{50}= 18.0 μM).

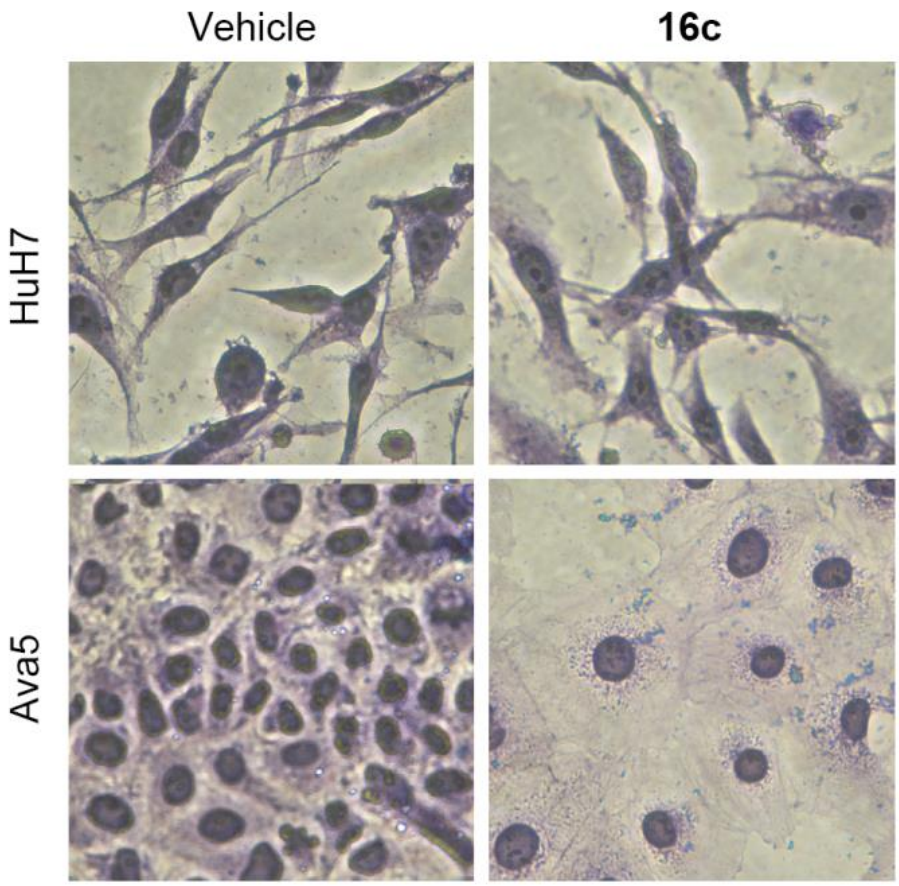


Figure 3 Effects of compound **16c** on Huh7 and Ava5 cells. Representative morphologies of Huh7 and Ava5 cells after treatment with 20 μM compound **16c** or a vehicle control (40× magnification, Giemsa staining). Similar results were obtained from at least three independent experiments for each treatment and cell type.

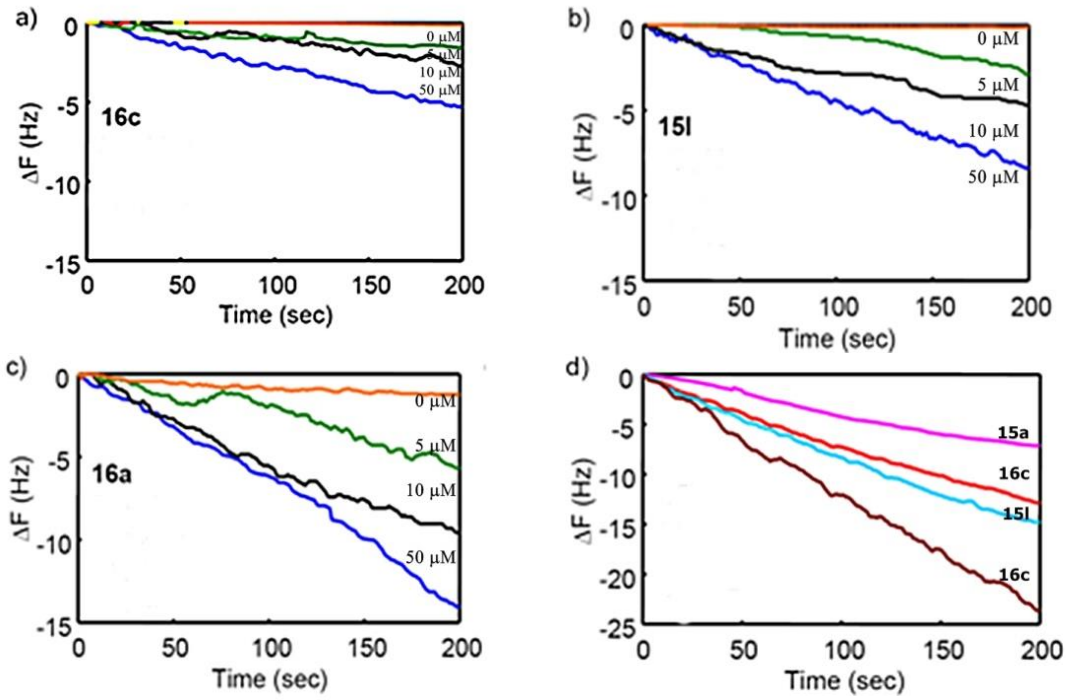


Figure 4 Binding of inhibitors to an NS5B-coated (5 μg/mL) QCM sensor chip. The changes in frequency (**Δ**F) due to interactions between immobilized NS5B and 0 to 50 μM **16c** (a), **15l** (b), and **16a** (c) and between immobilized NS5B and 200 μM **15a**, **16c**, **15l**, and **16a** (d) are shown. Similar frequency shifts were obtained from triplicate experiments

We hypothesize that the cytotoxic activity of **16c** may also be attributable to enhanced lipophilicity of the dichloro group. As shown in Fig 3, **16c** triggered a significant decrease in cell number and detectable morphological changes in Ava5 cells but not in Huh7 cells. The **16c**-treated Ava5 cells were characterized by apoptotic cell swelling and nucleus fragmentation. Although the 5-methyl substitution **16d** was active against NS5B (IC_{50} = 10.8 μM), it was not toxic to either of the cell lines.

To gain further insight into the effects of substitutions in the benzoyl portion of the scaffold, we prepared the 2′-chloro (**16e**) and 3′,4′-dimethoxy (**16f**) analogs. Although **16f** exhibited better NS5B inhibitory potential (IC_{50} = 12.3 μM) than **16e**(IC_{50} = 16.2 μM), it failed to produce any cytotoxic effects. In contrast, **16e** was moderately cytotoxic to Ava5 cells (EC_{50} = 26.1 μM), and this toxicity was 2-fold higher than in Huh7 cells.

A quartz crystal microbalance (QCM) system was used to monitor the binding interactions between selected NS5B inhibitors and recombinant NS5B (Deore et al, 2012). The concentration range (0 μM to 50 μM) allowed the determination of the dissociation constant (K_d) of compounds **16c** (Fig. 4a), **15l** (Fig. 4b), and **16a** (Fig. 4c) with NS5B. The *N*-benzoyl compound **16c**, which was the most cytotoxic in the cell-based assay, showed binding characteristics comparable to its *N*-hydroxyl counterpart **15l** (K_d= 1.25 μM and K_d= 1.45 μM, respectively). Importantly, compound

16a, which was the most potent NS5B inhibitor in the *in vitro* inhibition assay, exhibited an excellent concentration-dependent binding profile, with a K_d value of 0.85 μM. To further compare the NS5B-interactions of **15l**, **16a**, and **16c** with that of parent compound **15a**, we investigated the binding of compounds **15a**, **15l**, **16a**, and **16c** (Fig. 4d) at higher concentration (200 μM). The binding profiles of **15l** and **16c** were comparable, whereas **16a** showed stronger interaction for NS5B than the other compounds. Compound **15a** displayed weaker binding than the other derivatives. The results of the QCM binding studies were consistent with the *in vitro* enzymatic inhibition profiles. None of the compounds showed absorption with a control (non-immobilized) and reference (bovine serum albumin-immobilized) sensor.

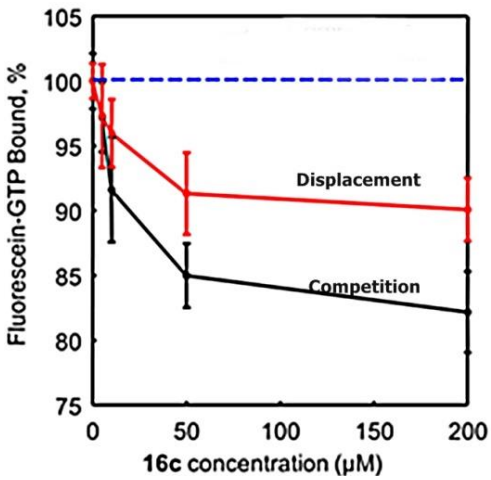


Figure 5 Effects of **16c** on the formation and stabilization of GTP (fluorescein labeled)-NS5B complexes. The black line represents **16c** competition with GTP for NS5B binding, and the red line represents **16c** displacement of GTP from preformed GTP-NS5B complexes. The fluorescence intensity of NS5B-bound GTP was measured. The fluorescence in the absence of inhibitors was taken as 100% and the fluorescence in the absence of fluorescein-GTP was set at 0%. Values are the mean± standard deviation of three independent experiments, each performed in triplicate.

Although the above results do not necessarily indicate that binding occurs solely in the active site, similar DKA bioisosteres are known to occupy the active site of NS5B (Deore et al, 2012, Deore et al, 2012). Because of the promising inhibitory and binding profiles of **16c**, we further examined effects of this compound on the NS5B active site by measuring formation and stabilization of complexes between NS5B and fluorescein-labeled GTP. Compound **16c** decreased the amount of GTP-NS5B complexes in a concentration-dependent manner, both through binding competition and displace-ment of bound GTP (Fig. 5). Thus, compound **16c** appears to be a potentially very useful active-site inhibitor of NS5B.

Conclusion

In summary, a new series of *N*-aroylisoindole-based NS5B inhibitors have been identified through sequential structural optimization of an isoindole-1,3-dione core. Com-pounds **16c** and **16e** are moderate NS5B inhibitors, but they displayed selective cytotoxic activity against HCV replicon-containing Ava5 cells. Binding profiles of **16c** strongly suggest that this series of inhibitors target the active site of NS5B. In addition, none of these compounds produced cytotoxic effects in the parent Huh7 cells, suggesting that they have the potential to be safer anti-HCV agents than those currently available. Our continued detailed mechanistic investigations and efforts to improve potency and selectivity will further optimize these compounds for potential clinical use.

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