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

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Keywords
Hepatitis C virus
NS5B polymerase
α,γ-diketo acid
bioisostere
isoindole-1,3-dione

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). Mechanistically, 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 physiological 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 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-quinazolinone 11 (Deore et al., 2012), inhibit magnesium-dependent enzymes. DKA isosteres with dihydroxy within their isoindole core, including dihydroxy-isoindol-1-one 12 and dihydroxy-isoindole-1,3-dione 13, inhibit Mg\textsuperscript{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).
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 R1, R2, and R3 in the various derivatives are listed in Tables 1 and 2.
Table 1 2-Hydroxyisooindoline-1,3-diones as anti-HCV agents

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ᵇ</th>
<th>CC₅₀ᶜ</th>
<th>EC₅₀ᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>74.5 ± 3.8</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15a</td>
<td>OH</td>
<td>H</td>
<td>76.0 ± 7.5</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15b</td>
<td>OH</td>
<td>4-NO₂</td>
<td>9.5 ± 5.4</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15c</td>
<td>OH</td>
<td>4-NH₂</td>
<td>23.0 ± 2.5</td>
<td>49.4 ± 5.7</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15d</td>
<td>OCH₂Ph</td>
<td>4-NO₂</td>
<td>32.9 ± 3.8</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15e</td>
<td>H</td>
<td>4-NO₂</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15f</td>
<td>OH</td>
<td>5-NO₂</td>
<td>18.5 ± 2.1</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15g</td>
<td>OH</td>
<td>5-NH₂</td>
<td>30.8 ± 4.3</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15h</td>
<td>OCH₂Ph</td>
<td>5-NO₂</td>
<td>27.3 ± 1.9</td>
<td>&gt; 50.0</td>
<td>26.1 ± 4.2</td>
</tr>
<tr>
<td>15i</td>
<td>H</td>
<td>5-NO₂</td>
<td>43.1 ± 6.7</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15j</td>
<td>OH</td>
<td>5-CH₃</td>
<td>42.6 ± 5.5</td>
<td>35.5 ± 2.6</td>
<td>&gt; 50.0</td>
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<tr>
<td>15k</td>
<td>OH</td>
<td>5-Br</td>
<td>49.1 ± 3.7</td>
<td>38.7 ± 4.9</td>
<td>&gt; 50.0</td>
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<tr>
<td>15l</td>
<td>OH</td>
<td>5,6-diCl</td>
<td>25.2 ± 2.9</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15m</td>
<td>H</td>
<td>5-Br</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15n</td>
<td>H</td>
<td>5,6-diCl</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>15k</td>
<td>H</td>
<td>5-NO₂</td>
<td>19.2 ± 2.4</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
</tbody>
</table>

*NSSB inhibitory activity based on pyrophosphate generation

²IC₅₀ (concentration required for 50% inhibition of NSSB activity)

³CC₅₀ (concentration required for 50% cytotoxicity of Huh7 cells)

⁴EC₅₀ (half maximal effective cytotoxic concentration in Ava5 cells containing the HCV replicon)

⁵DKA 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

Reagents and conditions: (i) PhCH₂O-NH₂, HCl/HO-NH₂, HCl, triethylamine, pyridine; microwave, 200W, 150°C, 10 to 20 min. (ii) H₂, 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM)</th>
<th>CC₅₀ (µM)</th>
<th>EC₅₀ (µM)</th>
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<tr>
<td>16a</td>
<td>H</td>
<td>H</td>
<td>6.8 ± 1.9</td>
<td>&gt; 50.0</td>
<td>37.2 ± 6.2</td>
</tr>
<tr>
<td>16b</td>
<td>4-NO₂</td>
<td>H</td>
<td>4.9 ± 2.2</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>16c</td>
<td>5,6-diCl</td>
<td>H</td>
<td>27.3 ± 5.1</td>
<td>&gt; 50.0</td>
<td>18.0 ± 3.1</td>
</tr>
<tr>
<td>16d</td>
<td>5-CH₃</td>
<td>H</td>
<td>10.8 ± 1.7</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
<tr>
<td>16e</td>
<td>H</td>
<td>2-Cl</td>
<td>16.2 ± 4.6</td>
<td>&gt; 50.0</td>
<td>26.1 ± 2.5</td>
</tr>
<tr>
<td>16f</td>
<td>H</td>
<td>3,4-diOCH₃</td>
<td>12.3 ± 2.2</td>
<td>&gt; 50.0</td>
<td>&gt; 50.0</td>
</tr>
</tbody>
</table>

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). ¹H- and ¹³C-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-d₆ spectrum (2.49 ppm for ¹H NMR and 39.4 ppm for ¹³C 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 temperature for 2h. Treatment of the phthalic anhydrides with formamide under microwave irradiation resulted in 15e, 15f, and 15m–p, which upon addition of aroyl chloride, triethylamine, and tetrahydrofuran under reflux condition for 24 to 60 h yielded 16a–f. Synthetic procedures and characterization 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 hydrochloride (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-isooindole-1,3-dione (15a): A white solid (Yield: 80.4%); Mp: 231-233 °C [230-238 °C (Keber et al, 1972)]; 1H NMR (DMSO-d6), 200 MHz: δ 10.81 (s, 1H, OH), 7.82 (s, 4H, ArH).

2-Hydroxy-4-nitro-isooindole-1,3-dione (15b): A yellowish white solid (Yield: 23.2%); Mp: 208-210 °C [209-213 °C (Wentzel et al, 2000)]; 1H NMR (DMSO-d6), 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-Benzoyl-4-nitro-isooindole-1,3-dione (15d): A yellowish white solid (Yield: 54.2%); Mp: 189-192 °C; 1H NMR (DMSO-d6), 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, CH2). Anal. Calcd for C15H10N2O5: C, 59.68; H, 3.47; N, 9.28. Found: C, 59.47; H, 3.53; N, 9.41.

2-Hydroxy-5-nitro-isooindole-1,3-dione (15f): A yellowish white solid (Yield: 61.2%); Mp: 170-172 °C [168-170 °C (Chan et al, 1987)]; 1H NMR (DMSO-d6), 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-Benzoyl-5-nitro-isooindole-1,3-dione (15h): A yellowish white solid (Yield: 56.0%); Mp: 190-191 °C [188-190 °C (Kerrigan et al, 1996)]; 1H NMR (DMSO-d6), 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, OCH3), 13C NMR (DMSO-d6), 100 MHz: δ 161.9, 161.7, 151.9, 134.4, 133.9, 130.5, 130.2, 129.6, 128.9, 125.1, 118.3, 79.8.

2-Hydroxy-5-methyl-isooindole-1,3-dione (15j): A white solid (Yield: 49.1%); Mp: 202-203 °C [202 °C (Wentzel et al, 2000)]; 1H NMR (DMSO-d6), 200 MHz: δ 10.7 (s, 1 H, OH), 7.72-7.59 (m, 3 H, ArH), 2.45 (s, 3 H, CH3).


5,6-Dichloro-2-hydroxy-isooindole-1,3-dione (15l): A white solid (Yield: 76.0%); Mp: 194-195 °C [195-197 °C (Villiger, 1909)]; 1H NMR (DMSO-d6), 200 MHz: δ11.05 (bs, 1H, OH), 8.04 (s, 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-isooindole-1,3-dione (15c): A yellow solid (Yield: 28.0%); Mp: 248-250 °C [247-249 °C (Elke, 2001)]; 1H NMR (DMSO-d6), 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, NH₂); 13C NMR (DMSO-d₆), 50 MHz: δ 165.7, 164.3, 146.2, 135.1, 129.2, 121.9, 110.7, 105.3.

5-Amino-2-hydroxy-isooindole-1,3-dione (15g): A yellow solid (Yield: 35.5%); Mp: 270-272°C [272-273°C (Chan et al., 1987)]; 1H NMR (DMSO-d₆), 200 MHz: δ 10.42 (s, 1H, OH), 7.43 (d, 1H, ArH, J = 8 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, NH₂).

General procedure for synthesis of 15e, 15i, 15m-p: Appropriate phthalic anhydrides were reacted with formamide (4 ml) under microwave condition at 150°C 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-isooindole-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-d₆), 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-isooindole-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-d₆), 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-isooindole-1,3-dione (15m): A white crystalline solid (Yield: 90.5%); Mp: 192-193°C [193-195°C (Wohrle et al., 1993)]; 1H NMR (DMSO-d₆), 200 MHz: δ 11.62 (s, 1H, NH), 8.10 (s, 2H, ArH).

5-Methyl-isooindole-1,3-dione (15n): A white crystalline solid (Yield: 88.0%); Mp: 196-198°C [196°C (Smith et al., 1964)]; 1H NMR (DMSO-d₆), 200 MHz: δ 8.91 (s, 2H, NH₂), 7.54-7.31 (m, 3H, ArH), 8.05 (d, 1H, ArH, J = 8 Hz).

General Procedure for 16a-f: A mixture of appropriate isoindole1,3-dione 15, triethylamine (1.5 equivalents) and aryl 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 chromatography on silica gel column (hexane/ethyl acetate, 1:1) to afford compound 16.

2-Benzoyl-isooindole-1,3-dione (16a): A white solid (Yield: 85.4%); Mp: 167-169°C [167-168°C (Kaiser et al., 1970)]; 1H NMR (DMSO-d₆), 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-isooindole-1,3-dione (16b): A yellowish white solid (Yield: 80.5%); Mp: 160-162°C; 1H NMR (DMSO-d₆), 200 MHz: δ 8.40 (dd, 1H, ArH, J = 7.84 Hz, 0.88 Hz), 8.28 (d, 1H, ArH, J = 6.6 Hz), 8.15 (t, 1H, ArH), 8.05 (d, 2H, ArH, J = 7.14 Hz), 7.77 (t, 1H, ArH), 7.57 (t, 2H, ArH); Anal. Calcd for C₁₅H₁₄N₂O₃: C, 60.82; H, 2.72; N, 9.46. Found: C, 60.98; H, 2.87; N, 9.36.

2-Benzoyl-5,6-dichloro-isooindole-1,3-dione (16c): A white solid (Yield: 60.4%); Mp: 198-200°C; 1H NMR (DMSO-d₆), 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), 13C NMR (DMSO-d₆), 100 MHz: δ 166.5, 163.7, 138.1, 134.5, 132.3, 131.3, 130.3, 128.6, 126.0; Anal. Calcd for C₁₅H₂Cl₂N₂O₃: C, 56.28;
H, 2.20; N, 4.38. Found: C, 56.18; H, 2.48; N, 4.48.

2-Benzoyl-5-methyl-isooindole-1,3-dione (16d): A white solid (Yield: 69.7%); Mp: 143-145 °C; $^1$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, 136.3, 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)-isooindole-1,3-dione (16e): A white solid (Yield: 68.4%); Mp: 182-183 °C; $^1$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$_8$ClNO$_3$: 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 °C; $^1$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).

**NS5B polymerase assay based on inorganic pyrophosphate (PPI) generation:** For PPI assay, HCV NS5B activity was performed in LumiTrac 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 sulfurylase and luciferase-coupled enzyme (each 30 μL, Cambrex Bio Science Rockland) after incubation at 37°C for 60 min. The initiated

![Figure S1](image-url)
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 x 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 nm). Values of CC50 and EC50 were the cytotoxicities against the parent Huh7 cells and HCV replicon Ava5 cells, respectively. Standard devia-tions for CC50 or EC50 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 (AffinixQ, 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% H2O2/H2SO4 (1:3) for 10 min followed by rinsing thoroughly in deionized water and then blowing dry in N2 gas. This gold substrate was further used for the absorption of recombinant NS5B. NS5B proteins were spontane-ously 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 N2 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 MgCl₂ was added, and then compound was added to the reaction chamber by the titrant injections (0, 5, 10, 50, or 200 μM).
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.

**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).

NTP competition and displacement studies and direct interactions of NS5B inhibitors with NS5B polymerase by QCM were conducted as described earlier\textsuperscript{13}. Briefly, for competition assay, NS5B (5 µg/mL) and fluorescein-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\textsubscript{2}, 20 U/mL RNAsin, and 4 mM DTT). in 3kDa-cutoff 96-well filtration plate. Then, the unbound fluorescein-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 fluorescein-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 fluorescein-GTP as the positive control (100% GTP bound) and fluorescein 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 \textsuperscript{14} and to a reference compound DKA \textsuperscript{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\textsubscript{50} value of 74.5 µM, which is \textasciitilde 4
fold lower than DKA 1 (IC\textsubscript{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\textsubscript{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\textsubscript{50} = 9.5 µM) over the unsubstituted analog (15a), comparable to our previously reported NS5B inhibitors N-hydroxy-isoquinoline 10 (IC\textsubscript{50} = 9.5 µM) and N-hydroxyquiazolinone 11 (IC\textsubscript{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\textsubscript{50} = 23.0 µM) over 15a, whereas the 2-O-benzyl-protected analog 15d was 3-fold less potent (IC\textsubscript{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\textsubscript{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\textsubscript{50} = 30.8 µM). Similarly, absence of the free N-hydroxyl group in 15h and 15i also resulted in loss of inhibition (IC\textsubscript{50} = 27.3 µM and IC\textsubscript{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\textsubscript{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 replication-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\textsubscript{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.
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. 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 displacement 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-aroylisooindole-based NS5B inhibitors have been identified through sequential structural optimization of an isoindole-1,3-dione core. Compounds 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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