Synthesis and SAR studies of 3,6-disubstituted indazole derivatives as potent hepcidin production inhibitors

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Abstract
Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis. Inhibition of hepcidin could be a strategy favorable to treating anemia of chronic disease (ACD). We report herein the synthesis and structure-activity relationships (SARs) of a series of indazole compounds as hepcidin production inhibitors. The optimization study of compound 1 led to a potent hepcidin production inhibitor 45, which showed serum hepcidin lowering effects in a mouse IL-6 induced acute inflammatory model.

Hepcidin is a peptide hormone, and known as the master regulator of systemic iron mobilization.1 The maintenance of serum iron level is important since a high iron concentration induces oxidative organ damage, and a low iron concentration results in iron deficiency anemia.2 As hepcidin was originally discovered as an antibacterial peptide,3 this hormone is inducible by inflammatory cytokines, such as IL-6,4 in addition to iron signaling. Based on hepcidin promoter analysis, it is well known that hepcidin promoter contains BMP-responsive elements and STAT-binding sites,5 which explains clearly the presence of the iron signaling. The signal is converted as BMP6 ligand production6 occurs by using hepatocytes as a response to organ iron storage levels, and the presence of inflammation signaling for induction of this hormone.

Anemia of chronic disease (ACD), which includes anemia of inflammation, is a heterogenic anemic condition due to chronic inflammation from a basic disease, such as rheumatoid arthritis.7 ACD patients are known to present iron deficiency despite abundant body iron stores (termed “functional iron deficiency”).8 Recently, high hepcidin induction based on inflammatory status was recognized as the cause of functional iron deficiency. Hepcidin blocks iron recycling from reticuloendothelial macrophages (iron recycles from senescent red blood cells, which are the main source for daily hemoglobin synthesis) and blocks dietary iron from enterocytes by internalization of ferroportin, the iron exporter important for maintenance of iron influx into the blood. This deregulation of hepcidin production results in the immobilization of organ iron storage for hemoglobin synthesis. Thus, a hepcidin controlling therapy would be promising for the treatment of functional iron deficiency.

Hepcidin expression deficiency is a common phenotype of hereditary hemochromatosis. Among several causative genes for hereditary hemochromatosis, HFE2, which codes hemojuelin, is known as the key molecule for hepcidin induction signaling.9 Hemojuelin, a member of the RGM family,9 alternatively known as RGMc, is a co-receptor for BMP receptor coupling. From the study of HFE2 null mice,10 it was shown that hemojuelin is involved in both iron- and inflammatory-induced signaling for hepcidin induction. The controlling of hepcidin level would be a promising therapeutic strategy for treating ACD caused by consistent overexpression of hepcidin. And indeed, to this end a few such biologics are entering clinical trials for treatment of anemia.

Based on this knowledge, we hypothesized that inhibitors of the BMP6/hemojuvelin signaling pathway would inhibit hepcidin...
production induced by BMP and/or inflammatory cytokines, and identified compound 1 through our screening.

Herein we describe the lead optimization of 1 to discover N-[6-(4-hydroxyphenyl)-1H-indazol-3-yl]-4-[2-(morpholin-4-yl)ethoxy]benzamide 45, a potent hepcidin production inhibitor.

Through the screening of our compound library, we identified a 5-substituted indazole compound 1 as a hepcidin production inhibitor, but compound 1 showed weak activity in vitro (Fig. 1, IC50 = 3.1 μM).1

To begin its optimization, we first investigated the derivatization of a 5-isooindoline moiety.

In the results from three simplified phenyl derivatives, the substituent at the 6-position was favored (compounds 8–10, Table 1).

Next, various substituents at the 6-position of the indazole were examined.

The 5-substituted indazole derivatives 8–20 were synthesized as illustrated in Scheme 1.

1-Boc-indazole intermediate 4 was synthesized via phthalimide 3 with deprotection under hydrazine monohydrate. Continuously, amidation with cyclopropanecarbonyl chloride and a subsequent Suzuki-coupling reaction with the appropriate boronic acid, boronic acid ester, or halide yielded a derivative at the 6-position. Finally, deprotection of the Boc group with an acidic condition gave the objective compounds.

The results are summarized in Table 2.

The ortho-hydroxyphenyl group 11 showed no inhibitory activity. On the other hand, the meta-hydroxyphenyl group 12 showed moderate activity. What is more, the para-hydroxyphenyl group 13 effectively enhanced the inhibitory activity.

The benzyl alcohol 14 and aniline 15 were found to be weaker inhibitors than 13. Contrastingly, benzoic acid 16 and methanesulfonylamide 17 as acidic substituents exhibited lost activity. Furthermore, aliphatic alcohol 18, pyridone 19 and phenol with a methylene linker 20 also exhibited lost activity.

After determining that the para-hydroxyphenyl group enhanced the inhibitory activity, we next turned our attention to derivatizing the substituent at the 3-position on the indazole ring.

The preparation of 3-substituted indazole derivatives listed in Tables 3 and 4 is shown in Scheme 2.

1-Boc indazole intermediate 4 reacted with O-TBDMS protected phenylboronic acid gave the key intermediate 21 in a moderate yield. Amidation with various acid chlorides yielded 3-amide indazole derivatives. Finally, deprotection of the Boc and TBDMS groups with an acidic condition gave the objective compounds. Using isocyante, aldehyde/sodium triacetoxylborohydride and sulfonyl chloride in place of acid chloride gave urea, secondary amine and sulfonamide derivatives.

The SAR results of the indazole derivatives on the 3-position are summarized in Table 3.

The compound 25 without a substituent at the 3-position dramatically lost activity. The amine derivatives (27, 31) and the urea derivative 32 exhibited a slightly weak activity. On the other hand, transformation to a reverse amide 30 and sulfonamide 33 would not be acceptable.

After determining that the amine group enhanced inhibitory activity at the 3-position, we next turned our attention to derivatizing the substituent at the 3-position on the indazole ring. Small acetamide 34 and sterically bulky cyclohexanecarboxamide 35 maintained a moderate activity, but benzamide 36 showed a similar activity to compound 13.

Judging from the results of compounds 13 and 36, sp2-carbon at the α-position of the amide was important to the enhancement of the activity.

Moreover, the substituent on the phenyl ring of benzamide was examined. The SAR results of the 3-benzamide indazole derivatives are summarized in Table 4.

Installation of substituents at the ortho-position 37 produced lost activity. In contrast, substituents introduced at the meta- or para-positions were acceptable (compounds 38, 39). Electron-withdrawing groups at the para-position deteriorated the activity, but electron-donating groups slightly improved the activity (compounds 40–42).

The results of the para-substituent optimizations, 2-(piperidin-1-yl)ethoxy group 44, 2-(morpholin-4-yl)ethoxy group 45 and 4-methylpiperazon-1-yl group 46, displayed a significant leap in the in vitro activity.

Pharmacokinetic (PK) parameters of compound 45 are summarized in Table 5.

Compound 45 possessed good metabolic stability in liver microsomes and showed a high plasma exposure in mice with intraperitoneal administration.

Next, the hepcidin lowering effect of compound 45 was evaluated by a mouse IL-6 induced acute inflammatory model.
As hepcidin is known as an acute-phase protein responding to chronic inflammatory conditions, with intravenous injection we evaluated the acute-phase induction of hepcidin in serum levels in response to mouse interleukin-6 (IL-6). Beforehand, we conducted a time course study in which serum hepcidin levels began increasing as early as 1 h after injection of IL-6, reached plateau at 4 h, and stayed constant until 6 h after injection (data not shown). Compound was administered intraperitoneally to 9-week-old Male C57BL/6J mice 30 min before IL-6 administration. Blood was collected at 4 h after the IL-6 injection and determined of serum hepcidin concentration.

Administration of 30 mg/kg doses of compound 45 inhibited hepcidin production triggered by IL-6 (Fig. 2).

In conclusion, we discovered a series of indazole derivatives as lead structures for potent hepcidin production inhibitors. Starting from indazole compound 1, introduction of a para-hydroxyphenyl group at the 6-position and [2-(morpholin-4-yl)ethoxy]benzamide group at the 3-position provided a significant leap in the in vitro activity. Compound 45 inhibited hepcidin production from HepG2 cells and lowered serum hepcidin level in IL-6 induced acute inflammatory model mice.

### Table 2
SAR of 6-substituted indazole derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>O.H</td>
<td>&gt;30</td>
<td>16</td>
<td>O.H</td>
<td>&gt;10</td>
</tr>
<tr>
<td>12</td>
<td>O.H</td>
<td>4.9</td>
<td>17</td>
<td>O.SQMe</td>
<td>&gt;30</td>
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<tr>
<td>13</td>
<td>O.H</td>
<td>0.40</td>
<td>18</td>
<td>O.H</td>
<td>&gt;30</td>
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<tr>
<td>14</td>
<td>O.H</td>
<td>1.4</td>
<td>19</td>
<td>O.H</td>
<td>&gt;30</td>
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<tr>
<td>15</td>
<td>NH$_2$</td>
<td>4.5</td>
<td>20</td>
<td>O.H</td>
<td>&gt;30</td>
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### Table 3
SAR of 3-substituted indazole derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
<th>Compound</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
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<tbody>
<tr>
<td>13</td>
<td>O.H</td>
<td>0.40</td>
<td>30</td>
<td>O.H</td>
<td>4.2</td>
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<tr>
<td>25</td>
<td>O.H</td>
<td>&gt;3.0</td>
<td>33</td>
<td>O.H</td>
<td>&gt;10</td>
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<tr>
<td>31</td>
<td>H$_2$N</td>
<td>1.0</td>
<td>34</td>
<td>O.H</td>
<td>1.1</td>
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<td>27</td>
<td>H$_2$N</td>
<td>1.6</td>
<td>35</td>
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<td>0.93</td>
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<tr>
<td>32</td>
<td>O.N</td>
<td>0.94</td>
<td>36</td>
<td>O.H</td>
<td>0.47</td>
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</table>

As hepcidin is known as an acute-phase protein responding to chronic inflammatory conditions, with intravenous injection we evaluated the acute-phase induction of hepcidin in serum levels in response to mouse interleukin-6 (IL-6). Beforehand, we conducted a time course study in which serum hepcidin levels began increasing as early as 1 h after injection of IL-6, reached plateau at 4 h, and stayed constant until 6 h after injection (data not shown). Compound was administered intraperitoneally to 9-week-old Male C57BL/6J mice 30 min before IL-6 administration. Blood was collected at 4 h after the IL-6 injection and determined of serum hepcidin concentration.

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The target molecule of the mechanism of action is not known exactly, yet 45 was found to be a promising compound showing in vivo efficacy. Further optimization of this series, and the target identification to understand the mechanisms of action of these compounds are ongoing and will be reported in due course.

Table 4
SAR of 3-benzamide indazole derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ (µM)</th>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ (µM)</th>
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<tr>
<td>36</td>
<td>H</td>
<td>0.47</td>
<td>42</td>
<td>4'-NMe₂</td>
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<tr>
<td>37</td>
<td>2'-OMe</td>
<td>&gt;30</td>
<td>43</td>
<td>(4'-)</td>
<td>0.16</td>
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<tr>
<td>38</td>
<td>3'-OMe</td>
<td>0.70</td>
<td>44</td>
<td>(4'-)</td>
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</tr>
<tr>
<td>39</td>
<td>4'-OMe</td>
<td>0.42</td>
<td>45</td>
<td>(4'-)</td>
<td>0.13</td>
</tr>
<tr>
<td>40</td>
<td>4'-Cl</td>
<td>0.98</td>
<td>46</td>
<td>(4'-)</td>
<td>0.085</td>
</tr>
<tr>
<td>41</td>
<td>4'-CO₂Me</td>
<td>1.1</td>
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Scheme 2. Synthesis of 3-substituted indazole derivatives. Reagents and conditions: (a) 4-(tert-Butyldimethylsiloxy)phenylboronic acid pinacol ester, Pd(dppf)Cl₂CH₂Cl₂, K₂PO₄·nH₂O, 1.2-dimethoxyethane/H₂O, 56%; (b) BOC₂Cl, pyridine, CH₂Cl₂, 93–95%; (c) 4N-HCl/dioxane, 90–94%; (d) Boc₂O, Et₃N, DMAP, CH₃CN, 50–59%; (e) 4-Hydroxyphenylboronic acid pinacol ester, Pd(dppf)Cl₂CH₂Cl₂, K₂PO₄·nH₂O, 1.2-dimethoxyethane/H₂O, 57–97%; (f) Cyclopropene carbonyl chloride, Sodium triacetoxyborohydride, CH₂Cl₂, 62%; (g) Cyclopropylamine, CDI, Et₃N, N,N-dimethylformamide, 64%.
Acknowledgments

We would like to thank Dr. Nakayama and Dr. Machinaga for their technical assistance and helpful discussions.

References


Table 5

<table>
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<th>Physicochemical property and PK parameters of 45.</th>
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<td>LogD</td>
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<td>3.4</td>
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</table>

* Remaining (%) of the tested compound after 0.5 h incubation with mouse liver microsome (0.5 mg/mL).

b Average of two values administered at 30 mg/kg i.p. to C57BL/6j mice.

Fig. 2. Effect of compound 45. The compound was administered to an IL-6 pretreated mouse at doses of 30 mg/kg (n = 4). **, p < 0.01 vs saline treated group (t-test), , , p < 0.001 vs 0.5% MC treated group (t-test).