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Discovery of DS28120313 as a potent orally active hepcidin production inhibitor: Design and optimization of novel 4,6-disubstituted indazole derivatives

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ABSTRACT

Hepcidin has emerged as the central regulatory molecule in systemic iron homeostasis, and its inhibition could be a favorable strategy for treating anemia of chronic disease (ACD). Here, we report the design, synthesis and structure-activity relationships (SAR) of a series of 4,6-disubstituted indazole compounds as hepcidin production inhibitors. The optimization study of multi-kinase inhibitor **1** led to the design of a potent and bioavailable hepcidin production inhibitor, **32** (DS28120313), which showed serum hepcidin-lowering effects in an interleukin-6-induced acute inflammatory mouse model.

The maintenance of serum iron levels is important since high and low iron concentrations induce oxidative organ damage and iron deficiency anemia, respectively.[1]

Anemia of chronic disease (ACD), is the second most prevalent form after that caused by iron deficiency and occurs in patients with acute or chronic immune activation. ACD, which includes inflammation-associated anemia, is a heterogenic anemic condition caused by chronic inflammation from a basic disease such as rheumatoid arthritis.[2] Some patients with ACD are known to present with iron deficiency despite abundant body iron stores (termed *functional iron deficiency*).

Hepcidin was originally discovered as an antibacterial peptide,[3] and this hormone is inducible by inflammatory cytokines such as interleukin (IL)-6,[4] in addition to iron signaling. This peptide hormone is the homeostatic regulator of intestinal iron absorption, iron recycling by macrophages, and iron mobilization from hepatic stores.[5]

Recently, high hepcidin induction based on inflammatory status was recognized as the cause of functional iron deficiency.[6] Hepcidin expression deficiency is a common phenotype of hereditary hemochromatosis. Therefore, controlling hepcidin levels would be a promising therapeutic strategy for treating hepcidin-induced functional iron deficiency. Indeed, a few biologics (e.g., NOX-H94, LY2928057, and LY2787106) are proceeding to clinical trials for the treatment of anemia. Here, we describe the design and optimization process aimed at lowering the multi-kinase inhibitory activity of **1** to discover methyl [6-(3-cyclopropyl-5-methyl-1*H*-

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pyrazol-4-yl)-1*H*-indazol-4-yl]carbamate (DS79182026, **32**), a potent orally available hepcidin production inhibitor.

As previously reported,[7,8] we identified the indazole derivative **1** as a potently active lead compound with a half-maximal inhibitory concentration (IC_{50}) of 0.23 μM.[9]

Although compound **1** showed a hepcidin-lowering effect following oral administration, it inhibited numerous kinases at double the concentration of the IC_{50} value (Figure 1).

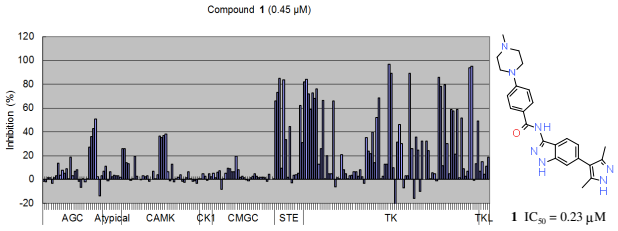
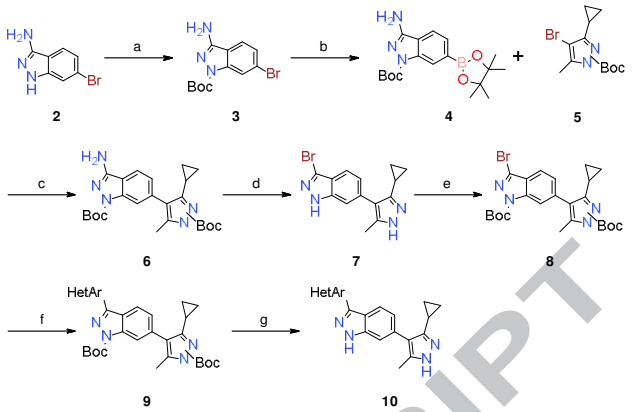


Figure 1. IC_{50} values and kinase inhibitory profiles of compound **1**

Multi-kinase inhibitors are not considered suitable as medicines for chronic diseases and, therefore, we decided to carry out a derivatization to reduce the kinase inhibitory activity of our lead compound. Hydrogen bonds are among the most important specific interactions in biological recognition processes. In particular, for kinases, hydrogen bonds with hinge backbone residues are considered the anchors of ligand binding, a generally indispensable interaction for potent enzyme inhibition.[10] We considered that the kinase inhibitory potency of **1** was probably due to its 3-aminoindazole moiety acting as a hinge binding site.[11] Therefore, we designed a scaffold, which transformed the amide moiety into a heteroaromatic ring. We thought that we could reduce the kinase inhibitory potency by transforming the NH group at 3-position of the indazole, which is thought to play the role of a hydrogen bond donor.

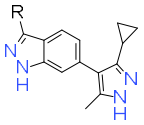
3-Heteroaryl substituted indazole derivatives were synthesized using the process illustrated in Scheme 1. The results of the analysis of the indazole derivatives with various functional groups on the 3-position are summarized in Table 1.

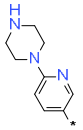
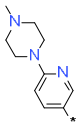


Scheme 1. Synthesis of 3-heteroaryl indazole derivatives. Reagents and conditions: (a) Boc_2O, Et_3N, DMAP, CH_3CN, 63%; (b) Bis(pinacolato)diboron, $Pd(dppf)Cl_2$-CH_2Cl_2, KOAc, 1,4-dioxane, 97%; (c) $Pd(dppf)Cl_2$-CH_2Cl_2, K_3PO_4-nH_2O, 1,2-dimethoxyethane/H_2O, 91%; (d) CuBr, $NaNO_2$, HBr aq., AcOH/H_2O, 29%; (e) Boc_2O, Et_3N, DMAP, CH_3CN, 89%; (f) Heteroarylboronic acid, $Pd(dppf)Cl_2$-CH_2Cl_2, K_3PO_4-nH_2O, 1,2-dimethoxyethane/H_2O, 46-83%; (g) 4N-HCl/1,4-dioxane, 71-97%. (The Boc protected compounds were isolated as a single regioisomer, but the regio of Boc groups were not determined.)

The transformation of amide substituents to simple heteroaromatic rings deteriorated the inhibitory activity (compounds **12**-**14**). However, the activity was effectively enhanced by introducing a cyclic amine moiety at the pyridine ring. Compounds **15** and **16** showed a significantly enhanced *in vitro* activity.

Table 1. SAR of 3-heteroaryl-substituted indazole derivatives



compound	R	IC_{50} (μM)	compound	R	IC_{50} (μM)
11		0.41	**14**		3.1
12		2.6	**15**		0.17
13		2.3	**16**		0.12

The 4-methylpiperazin-1-yl-pyridine derivative (**16**) showed good hepcidin inhibitory activity.

Although it improved slightly, compound **16** inhibited numerous kinases at a concentration that was 2-fold the IC_{50} value (Figure 2).

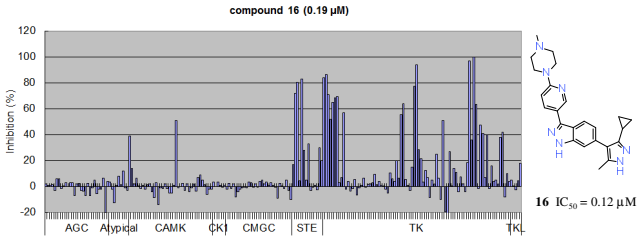
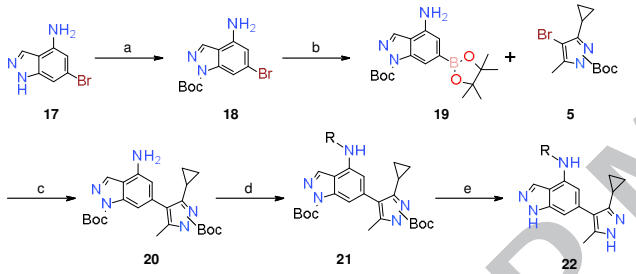


Figure 2. IC_{50} values and kinase inhibitory profiles of compound **16**

We estimated that the hydrogen atom of the newly introduced pyridine ring functioned as a hydrogen bond donor. Therefore, we designed a new scaffold, which transferred the substituent from the 3- to 4-position of indazole. We hypothesized that the kinase inhibitory potency could be decreased by removing substituents that could function as hydrogen bond donors.

The 4-substituted indazole derivatives were synthesized using the process illustrated in Scheme 2.



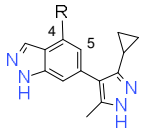
Scheme 2. Synthesis of 4-substituted indazole derivatives. Reagents and conditions: (a) Boc_2O, Et_3N, DMAP, CH_3CN, 55%; (b) Bis(pinacolato)diboron, Pd(dppf)Cl_2-CH_2Cl_2, KOAc, 1,4-dioxane, 99%; (c) Pd(dppf)Cl_2-CH_2Cl_2, K_3PO_4-nH_2O, 1,2-dimethoxyethane/H_2O, 72%; (d) acyl chloride, pyridine, CH_2Cl_2 or alkyl chloroformate, pyridine, CH_2Cl_2 or isocyanate, Et_3N, THF, 27-61%; (e) 4N-HCl/1,4-dioxane, 16-90%. (The Boc protected compounds were isolated as a single regioisomer, but the regio of Boc groups were not determined.)

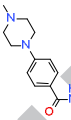
The results of the analysis of the indazole derivatives with various functional groups on the 4-position are summarized in Table 2.

Compound **23** that lacked a substituent at the 3-position showed a considerably weaker activity than its parent compound, but compound **24** in which the amide moiety migrated to the 4-position showed moderate activity. The compounds with sterically smaller substituents on the amide group showed higher activity (**25** and **26**). The introduction of the 4-piperidynylaryl group, which showed high activity at the 3-position, limited the enhancement of activity (compound **27**).

The reverse amide, primary amine, and methane sulfonamide derivatives (**28**–**30**) exhibited weaker activity than that of the parent compound.

Table 2. SAR of 4-substituted indazole derivatives



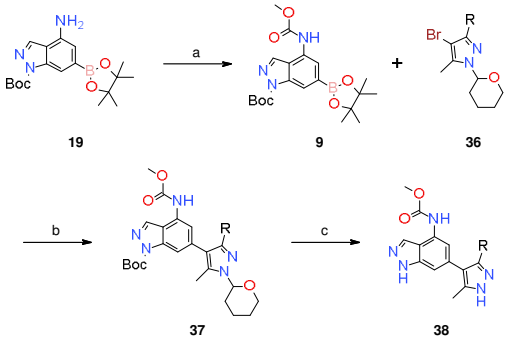
compound	R	IC_{50} (μM)	compound	R	IC_{50} (μM)
23	H	4.1	**29**		2.7
24		1.0	**30**		1.2
25		0.45	**31**		0.34
26		0.19	**32**		0.093
27		1.9	**33**		0.66
			34		3.0
28		2.2	**35**	(5-) 	>10

Furthermore, the methyl urea group showed good activity and, surprisingly, the methyl carbamate group showed a significant increase in the *in vitro* activity (compounds **31** and **32**).

The steric tolerance on the carbamate group was narrow, and the conversion to an ethyl carbamate group (**33**) considerably attenuated the activity.

In addition, transferring the substituent to the 5-position of indazole completely abrogated the *in vitro* activity.

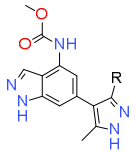
Next, the effects of various substituents on the pyrazole were examined. The 4-methoxycarbonyl aminoindazole derivatives with various substituents on the pyrazole ring were synthesized using the process illustrated in Scheme 3.[12]



Scheme 3. Synthesis of 6-substituted indazole derivatives. Reagents and conditions: (a) Methyl chloroformate, pyridine, CH_2Cl_2, 92%; (b) Pd(dppf)Cl_2-CH_2Cl_2, K_3PO_4-nH_2O, 1,2-dimethoxyethane/H_2O, 31-90%; (c) 4N-HCl/1,4-dioxane, 20-70%.

The results of the analysis of the substituted pyrazole derivatives are summarized in Table 3.

Table 3. SAR of 6-substituted indazole derivatives



compound	R	IC_{50} (μM)	compound	R	IC_{50} (μM)
32	c-Pr	0.093	**43**	CO_2Me	0.13
39	Me	0.15	**44**	CO_2H	>10
40	Et	0.18	**45**	CONHMe	2.0
41	i-Pr	0.19	**46**	Ph	1.1
42	CH_2OMe	3.9	**47**		0.32

The alkyl groups showed high activity, but the ether substituent deteriorated the inhibitory activity (compounds **39**–**42**). In addition, the compound with the ester group showed high activity, but that of compounds with a carboxylic acid or amide group was greatly attenuated (compounds **43**–**45**). The introduction of a phenyl or 2-pyridyl group, which produced compounds that showed high activity in the derivatization of benzisoxazole scaffold, limited the enhancement of activity (compounds **46** and **47**).[8]

Since we acquired compound **32** that possessed high inhibitory activity, we performed a kinase profiling assay of this candidate. As expected, the kinase inhibitory potency of **32** was significantly reduced at double the concentration of its IC_{50} value (Figure 3).

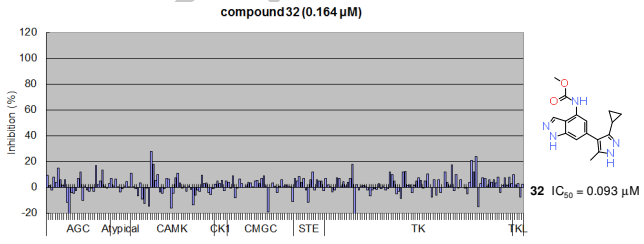


Figure 3. IC_{50} values and kinase inhibitory profiles of compound **32**

Thus, the potently active hepcidin production inhibitor **32**, named DS28120313, was selected for further investigations.

To evaluate the *in vivo* efficacy, we assessed the pharmacokinetic (PK) parameters of DS28120313 (Table 4).

Table 4. Physicochemical properties and pharmacokinetic (PK) parameters of DS28120313

LogD	MS[a] (%)	PB (free %)	Cmax[b] (μg/mL)	Tmax[b] (h)	AUC[b] (h*μg/mL)
3.1	84	24	4.94	0.67	14.7

[a] Remaining (%) test compound after 0.5 h incubation with mouse liver microsomes (0.5 mg/mL).
[b] Average of two values dosed at 30 mg/kg orally (p.o.) in C57BL/6J mice (0.5% methylcellulose suspension).

The appropriately lipophilic compound DS28120313 possessed good metabolic stability and low plasma protein binding (*fu,p* = 24%). Further, DS28120313 showed high plasma exposure in mice and was considered to have a suitable profile as an oral agent. Next, the hepcidin-lowering effect of DS28120313 was evaluated using an IL-6-induced acute inflammatory mouse model.

Hepcidin is known as an acute-phase protein produced in response to chronic inflammatory conditions. Therefore, we evaluated the acute-phase induction of hepcidin in serum in response to intravenous injection of mouse IL-6. Prior to this evaluation, we conducted a time-course study where serum hepcidin levels began increasing as early as 1 h after the injection of IL-6, plateaued at 4 h, and then remained constant until 6 h after the injection (data not shown). DS28120313 was administered orally to 9-week-old male C57BL/6J mice 30 min before IL-6 administration. Blood samples were collected 4 h after the IL-6 injection, and the hepcidin concentration was determined.

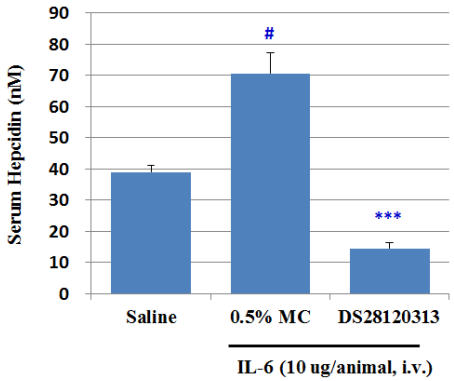


Figure 4. Effect of compound DS28120313. The compound was administered to mice at dose of 30 mg/kg (p.o., 0.5% methylcellulose, suspension, $n = 4$) before IL-6 treatment. #, $p < 0.05$ vs Saline treated group (t-test), ***, $p < 0.001$ vs 0.5% MC treated group (t-test).

DS28120313 significantly reduced the blood hepcidin level at an oral dose of 30 mg/kg (Figure 4).

In conclusion, we discovered a series of 4,6-disubstituted indazole derivatives as potent and

orally bioavailable inhibitors of hepcidin production. Starting from the multi-kinase inhibitor **1**, we developed a 4,6-disubstituted indazole as a scaffold with a lower kinase inhibitory activity than its parent compound by transforming the hinge binding site.

The optimization of substituents at the 4- and 6-positions produced DS28120313, which possessed potent *in vitro* activity. Furthermore, DS28120313 inhibited hepcidin production in HepG2 cells and lowered the serum hepcidin levels in an IL-6 induced acute inflammatory mouse model.

The exact target molecule underlying the mechanism of action is not known, but DS28120313 was found to be a promising compound as an oral agent with *in vivo* efficacy.

Both DS28120313 and DS79182026[8] showed potent *in vitro* activity and strong ability to inhibit hepcidin production in IL-6-induced high-hepcidin model mouse by oral administration, we consider both as promising compounds. We are planning to evaluate in higher order *in vivo* model(e.g. anemia amelioration models) and we will comprehensively assess the qualities of both compounds.

And, the target identification to elucidate the mechanism of action of these compounds is ongoing and will be reported in due course.

Acknowledgments

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Graphical Abstract

Discovery of DS28120313 as a potent orally active hepcidin production inhibitor: Design and optimization of novel 4,6-disubstituted indazole derivatives

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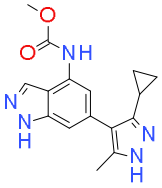

32 (DS28120313)

Hepcidin mRNA expression
IC_{50} = 0.093μM