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Design, synthesis, and evaluation of 4,6-diaminonicotinamide derivatives as novel and potent immunomodulators targeting JAK3

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Abstract

In organ transplantation, T cell-mediated immune responses play a key role in the rejection of allografts. Janus kinase 3 (JAK3) is specifically expressed in hematopoietic cells and associated with regulation of T cell development via interleukin-2 signaling pathway. Here, we designed novel 4,6-diaminonicotinamide derivatives as immunomodulators targeting JAK3 for prevention of transplant rejection. Our optimization of C4- and C6-substituents and docking calculations to JAK3 protein confirmed that the 4,6-diaminonicotinamide scaffold resulted in potent inhibition of JAK3. We also investigated avoidance of human ether-a-go-go related gene (hERG) inhibitory activity. Selected compound **28** in combination with tacrolimus prevented allograft rejection in a rat heterotopic cardiac transplantation model.

Keywords: JAK3 (Janus kinase 3), immunomodulator, transplant rejection, hERG (human ether-a-go-go related gene)

1. Introduction

Prevention of transplant rejection is urgently required for patients with end-stage organ failure.[1] Transplant rejection is triggered by T cell recognition of allografts, resulting in production of interleukin-2 (IL-2) and further T cell differentiation and proliferation.[1] The immune responses injure allografts, resulting in graft loss. In current immunotherapy, calcineurin inhibitors (CNIs) such as tacrolimus and cyclosporin A are used to treat transplant rejection. CNIs inhibit IL-2 production and the following T cell activation, and are therefore highly effective in preventing acute rejection. However, use of CNIs require constant monitoring of drug levels to manage the related nephrotoxic and neurotoxic side effects.[2-4] Co-administration with a drug working via a distinct mechanism of action will likely reduce the drug levels required and side effects of CNIs.

Janus kinases (JAKs) are a family of cytoplasmic protein tyrosine kinases consisting of JAK1, JAK2, JAK3, and TYK2, each of which are associated with corresponding cytokine receptors to mediate signal transduction.[5-9] JAK3 is involved in the signaling pathways of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. IL-2 stimuli induce JAK3 activation following the phosphorylation of signal transducers and activators of transcription protein 5 (STAT5) in the cytoplasm. Phosphorylated STAT5 then undergoes dimerization and translocation to the nucleus, where it activates the transcription of specific genes related to the differentiation, proliferation, and survival of T cells. In contrast to the ubiquitous expression of other JAKs throughout the body, JAK3 is predominantly expressed in hematopoietic cells.[10,11] The phenotype of JAK3 knockout mice is defined by immunodeficiency with a marked reduction in the numbers of functional T cells.[12,13] JAK3 inhibitors might therefore be novel and safe immunomodulators for the prevention of organ transplant rejection. Regarding other JAKs, JAK1 is associated with IL-2

receptors and the regulation of T cell function in concert with JAK3, and is also involved in the signaling pathways of IL-6 and interferon (IFN)-γ in inflammatory responses.[5-9] JAK2 participates in the differentiation and proliferation of erythrocytes, neutrophils, and thrombocytes by mediating the signaling of hematopoietic growth factors such as erythropoietin, colony-stimulating factor, and thrombopoietin.[5-9]

A number of JAK inhibitors have been studied,[14-18] and tofacitinib (compound **1**, Figure 1) is approved to use for the treatment of rheumatoid arthritis (RA). This compound exhibited *in vivo* efficacy in various animal models of organ transplantation, RA, and psoriasis.[19-21] In our laboratory, we identified a series of 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide derivatives to show JAK3 inhibitory activity.[22,23] Docking calculations to JAK3 indicated that the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide scaffold interacts with the hinge region in the ATP-binding site and that the C4-amino substituent interacts with the hydrophobic cavity. The chemical modification of C4-substituents of lead compound **2** was investigated to enhance the affinity of the hydrophobic cavity, and compound **3** was found to have increased JAK3 inhibitory activity over **2** (Figure 2). However, **3** exhibits undesirable human ether-a-go-go related gene (hERG) inhibitory activity associated with cardiotoxicity, such as QT interval prolongation.[24] Our optimization study of the C4-substituent led to the identification of compound **4**, which exhibited potent JAK3 inhibitory activity and low hERG inhibitory activity (Figure 2).

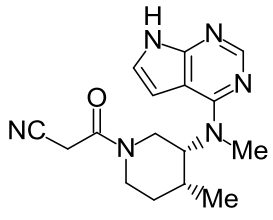

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Figure 1. Chemical structure of tofacitinib.

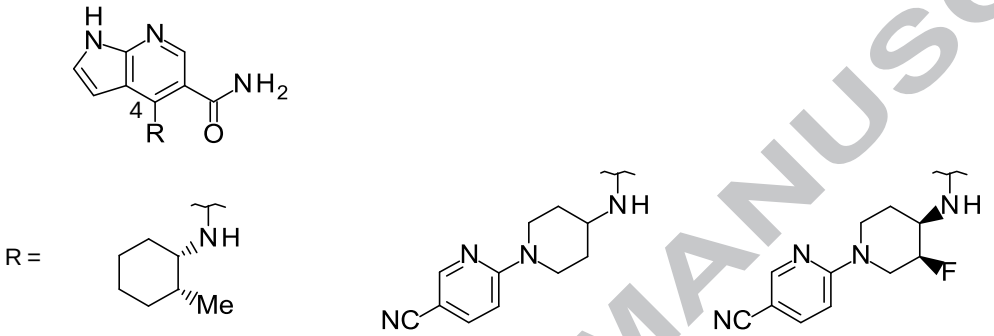


Figure 2. Profiles of 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide derivatives.

As mentioned above, our previous study revealed that conversion of the C4-substituent was important for interaction with the hydrophobic cavity and increase of JAK3 inhibitory activity. On the other hand, the binding to the hinge region is generally critical for kinase inhibitory activity, and modification of the scaffold moiety around hinge region may increase the affinity to JAK3. Although many other JAK inhibitors have bicyclic fused heteroaryl scaffold comprised of 5- and 6-membered rings (e.g., pyrrolopyrimidine of **1**),[16-18] little studies are known about effect of modification of the 5-membered ring moiety on JAK3 inhibitory activity. We therefore designed and focused on a novel single pyridine ring of 4,6-diaminonicotinamide scaffold

mimicking the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide. In our previous study, the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide moiety occupied the ATP-binding site to form a hydrogen bond with Glu903 and Leu905 in the hinge region, with the N1-hydrogen atom serving as a hydrogen donor and N7-nitrogen atom as an acceptor.[22] As shown in Figure 3, a 4,6-diaminonicotinamide scaffold was designed to retain two hydrogen bond interactions with the hinge region, as this interaction is considered important for JAK3 inhibitory activity. In addition, as the bicyclic aromaticity of the 1*H*-pyrrolo[2,3-*b*]pyridine ring contributed to CH-π interaction with the Leu828 and Val836, introduction of a phenyl ring in C6-position of the 4,6-diaminonicotinamide was considered effective for holding the scaffold around the hinge region.

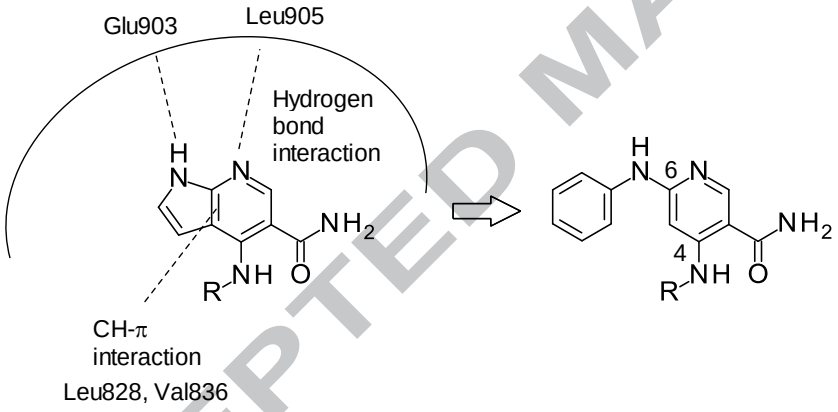
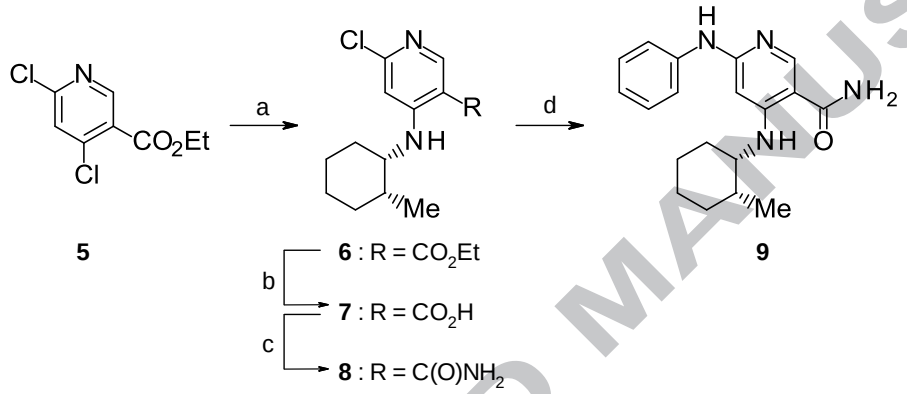


Figure 3. Design of 4,6-diaminonicotinamide derivatives.

Here, we describe our structure-activity relationship (SAR) study of the 4,6-diaminonicotinamide derivatives for JAK3 inhibitory activity. In addition, reduction of hERG inhibition was investigated via modulation of molecular lipophilicity and basicity. Further, the *in vivo* efficacy of this series of compounds on a rat heterotopic cardiac transplant model[25] was also investigated.

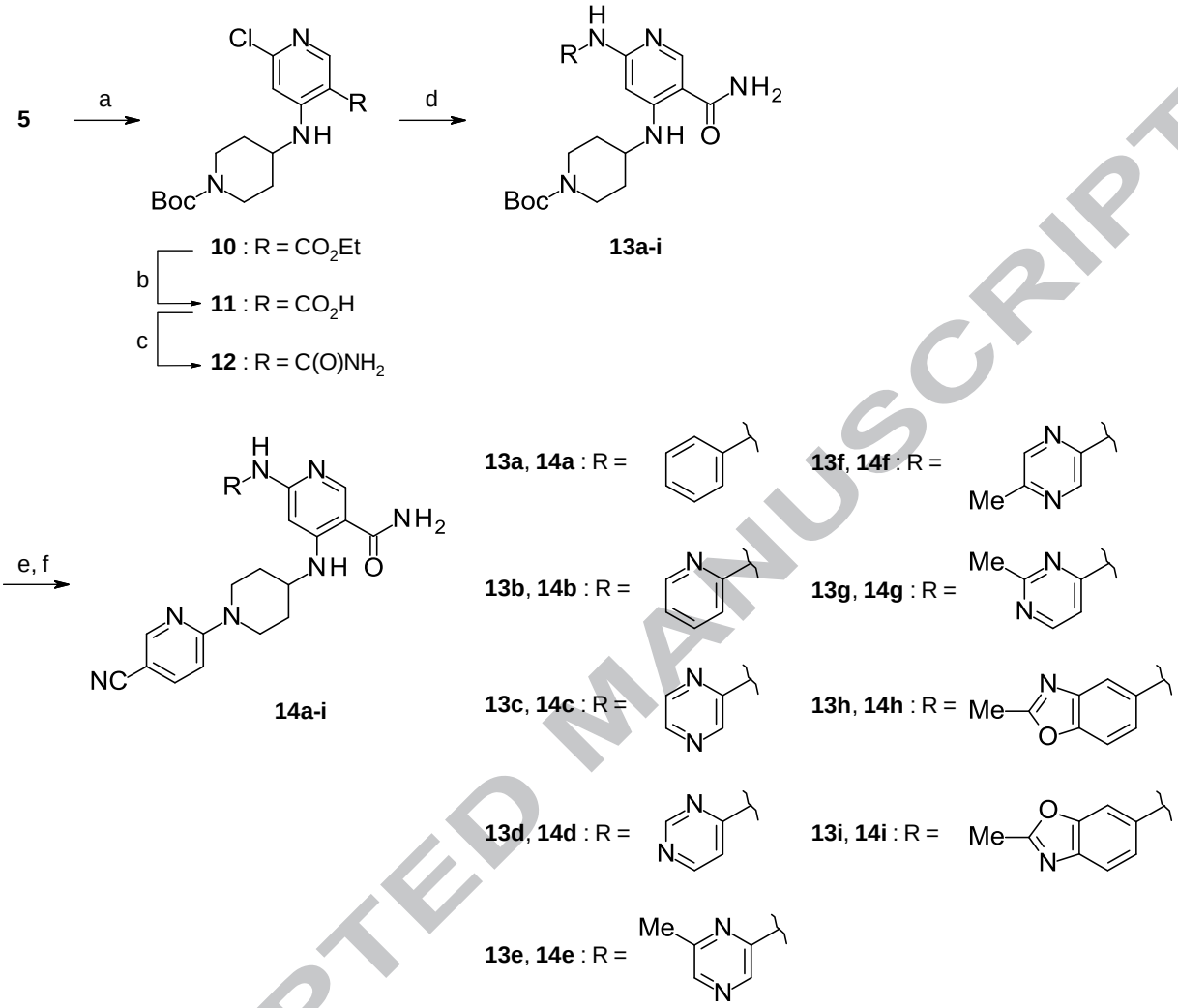
2. Chemistry

Scheme 1 shows the general synthesis of 4,6-diaminonicotinamide derivatives. Ethyl 4,6-dichloronicotinate (**5**) was reacted with (1*S*,2*R*)-2-methylcyclohexanamine, and nucleophilic substitution gave compound **6**. The ester group of **6** was hydrolyzed to give **7**, and successive condensation with aqueous ammonia gave carboxamide **8**. Treatment of **8** with aniline under a Pd-catalyzed condition gave the 4,6-diaminonicotinamide derivative **9**.



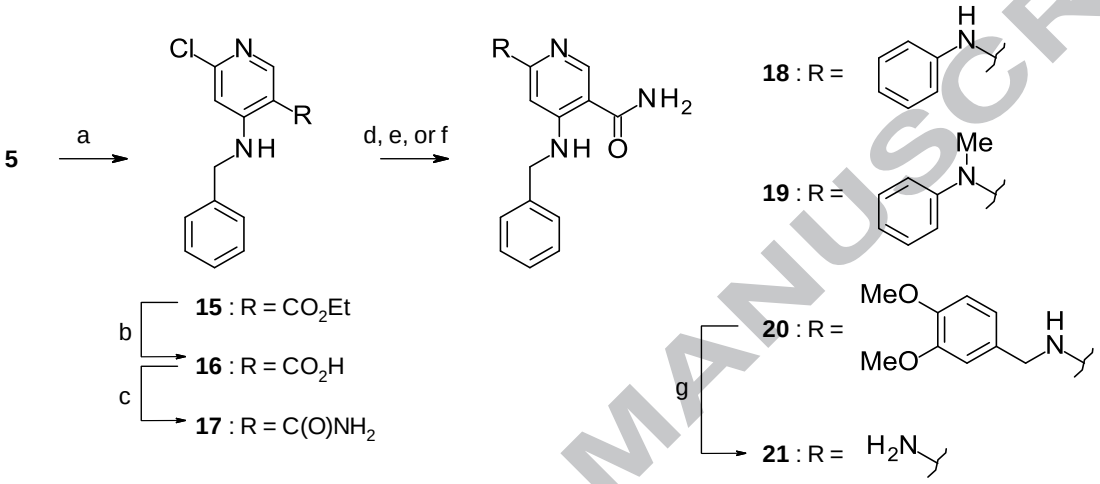
Scheme 1. Reagents and conditions: (a) (1*S*,2*R*)-2-methylcyclohexanamine hydrochloride, DIPEA, *n*-BuOH, 120 °C; (b) 2 M NaOH, dioxane, 110 °C; (c) CDI, DMF, room temperature, then 28% NH_4OH, room temperature; (d) aniline, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl, *t*-BuONa, *t*-BuOH, microwave, 130 °C.

Scheme 2 shows synthesis of the derivatives having *N*-cyanopyridyl-4-aminopiperidine in C4-position. Similar to the synthesis of **8**, compound **12** was prepared using **5** and *N*-Boc-4-aminopiperidine. The Pd-catalyzed coupling reaction of **12** with a variety of arylamines gave compound **13a-i**. The Boc group of **13a-i** was deprotected under acidic condition, followed by reaction with 2-chloro-5-cyanopyridine to give compound **14a-i**.



Scheme 2. Reagents and conditions: (a) *N*-Boc-4-aminopiperidine, DIPEA, *n*-BuOH, 120 °C; (b) 2 M NaOH, dioxane, 110 °C; (c) CDI, DMF, room temperature, then 28% NH_4OH, room temperature; (d) amines, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl, *t*-BuONa (or K_2CO_3), *t*-BuOH, microwave, 130-140 °C; (e) 4 M HCl, dioxane, room temperature; (f) 2-chloro-5-cyanopyridine, K_2CO_3, DMSO, 100 °C (or Et_3N, NMP, microwave, 150 °C).

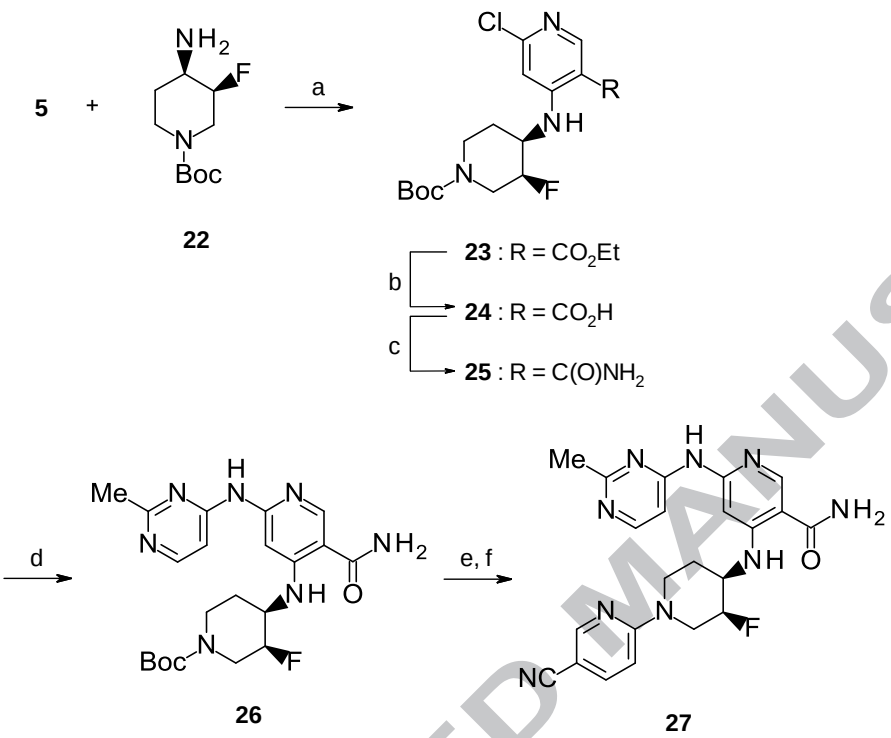
Scheme 3 shows the synthesis of benzylamine derivatives. Coupling reaction of **17** with aniline gave **18**, and nucleophilic substitution of **17** with *N*-methylaniline gave **19** and with 3,4-dimethoxybenzylamine gave **20**. Deprotection of the 3,4-dimethoxybenzyl group of **20** by TFA gave compound **21**.



Scheme 3. Reagents and conditions: (a) benzylamine, DIPEA, *i*-PrOH, 80 °C; (b) 6 M NaOH, EtOH, 70 °C; (c) CDI, DMF, room temperature, then 28% NH_4OH, room temperature; (d) aniline, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl, *t*-BuONa, *t*-BuOH, NMP, dioxane, microwave, 140 °C (for **18**); (e) *N*-methylaniline, tetraethylammonium chloride, NMP, 180 °C (for **19**); (f) 3,4-dimethoxybenzylamine, DIPEA, NMP, microwave, 180 °C (for **20**); (g) TFA, microwave, 100 °C.

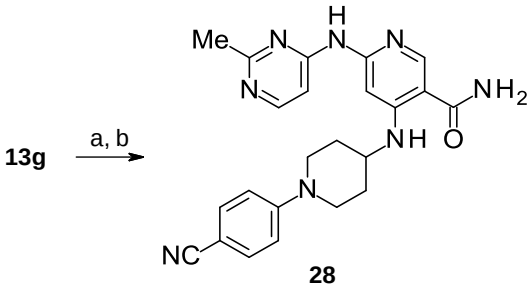
Scheme 4 shows the synthesis of compound **27**. Compound **23** was prepared by reacting **5** with chiral 4-amino-3-fluoropiperidine **22**, which was synthesized as previously described.[23] The ester group of **23** was converted to carboxamide to give compound **25**, followed by a coupling

reaction with 2-methylpyrimidine-4-amine to give **26**. After deprotection of the Boc group, **26** was reacted with 2-chloro-5-cyanopyridine to give **27**.



Scheme 4. Reagents and conditions: (a) DIPEA, DMF, 130 °C; (b) 2 M NaOH, dioxane, 110 °C; (c) CDI, DMF, room temperature, then 28% NH_4OH, room temperature; (d) 2-methylpyrimidin-4-amine, $Pd_2(dba)_3$, 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl, *t*-BuONa, *t*-BuOH, 90 °C; (e) 4 M HCl, dioxane, room temperature; (f) 2-chloro-5-cyanopyridine, Et_3N, NMP, 100 °C.

Scheme 5 shows the synthesis of compound **28**. After deprotection of the Boc group under acidic condition, **13g** was reacted with 4-fluorobenzonitrile to give **28**.



Scheme 5. Reagents and conditions: (a) 4 M HCl, dioxane, room temperature; (b) 4-fluorobenzonitrile, K_2CO_3, DMSO, microwave, 140 °C.

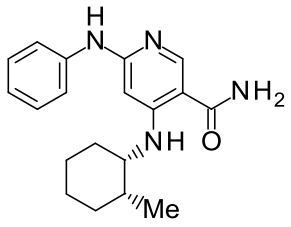
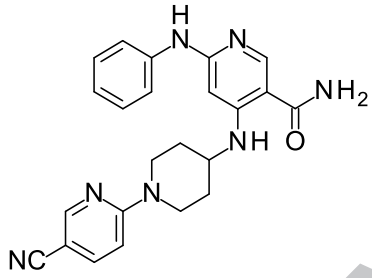
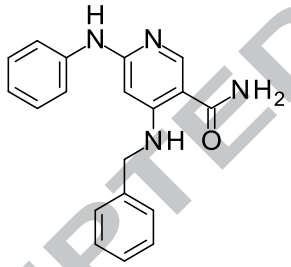
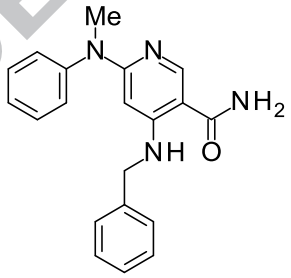
3. Results and discussion

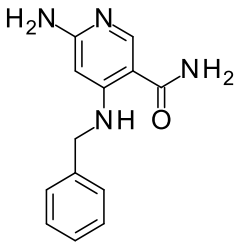
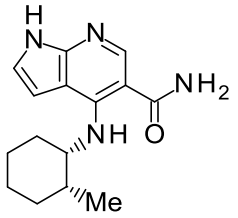
We evaluated human JAK3 inhibitory activity of synthesized compounds, and JAK1 inhibitory activity was also evaluated, as JAK3 and JAK1 collaboratively regulate the IL-2 signaling pathway for T cell-mediated immune responses. JAK2 inhibitory activity was then investigated, as JAK2 inhibition might be related to adverse hematopoietic effects such as anemia.[26] To determine the *in vivo* effect, a rat heterotopic cardiac transplantation model was investigated using ACI rats as donors and Lewis rats as recipients, and median survival times (MST) of grafts were analyzed. In the model, we evaluated immunomodulating effect to prevent transplant rejection via JAK3 pathway and following IL-2 mediated T cell development.

Table 1 shows the fundamental SAR study results for the 4,6-diaminonicotinamide derivatives. In the C4-position, a methylcyclohexylamine corresponding to the C4-substituent of **2** and an *N*-cyanopyridyl-4-aminopiperidine corresponding to that of **3** were introduced to interact with the hydrophobic cavity. The resulting respective compounds **9** and **14a** exhibited moderate JAK3 inhibitory activity (IC_{50} = 37 and 18 nM, respectively). These results suggest that chemical modification of C4-position of the 4,6-diaminonicotinamide derivatives maintains JAK3 inhibitory activity. Conversion to a simple aliphatic group such as a benzylamine retained moderate JAK3 inhibitory activity (**18**; IC_{50} = 19 nM). To then validate the effect of the C6-substituent on JAK3 inhibitory activity, modification of the C6-position of **18** was investigated. N-methylation and deletion of the phenyl group resulted in loss of JAK3 inhibitory activity, indicating that hydrogen bond donor and aromaticity around the hinge region were critical for JAK3 inhibitory activity (**19** and **21**; IC_{50} >1000 nM, respectively).

Table 1

SARs of 4,6-diaminonicotinamide derivatives

Compound	Structure	JAK3 IC_{50}[a] (nM)
9		37
14a		18
18		19
19		>1000 (40% inh.@ 1 μM)

21		>1000 (41% inh.@1 μM)
2		5.1

[a]IC_{50} values are mean of duplicate experiments.

Optimization of the C6-substituent of 4,6-diaminonicotinamide derivatives is shown in Table 2. Regarding the C4-substituent, the *N*-cyanopyridyl-4-aminopiperidine group was selected as **3** showed preferable pharmacokinetic (PK) profiles such as metabolic stability in our previous study.[23] Compared to the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide derivative **3**, the 6-anilino-nicotinamide derivative **14a** have more flexible structure around the hinge region of JAK3 due to the rotation of C6-substituent. We considered that mimicking the planar bicyclic scaffold of **3** was effective for JAK3 inhibitory activity, because the aromaticity of 1*H*-pyrrolo[2,3-*b*]pyridine contributed to CH-π interaction with the Leu828 and Val836 (Figure 3). Therefore, conversion of the aniline group of **14a** to a 2-aminopyridine in the C6-position was attempted to maintain the scaffold in a planar orientation by reducing steric hindrance (Figure 4).

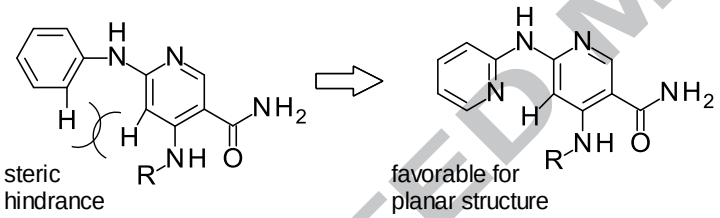


Figure 4. Modification of C6-substituent.

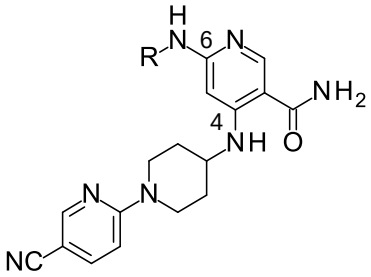
In addition, a polar functional group such as pyridine in the C6-position might be more favorable in the hydrophilic hinge region. Results showed that **14b** achieved a 40-fold increase in JAK3 inhibitory activity (IC_{50} = 0.46 nM) over **14a**. Regarding the potency of JAK3 inhibitory activity, the 4,6-diaminonicotinamide scaffold was superior to the 1*H*-pyrrolo[2,3-*b*]pyridine (**3**; IC_{50} = 1.3 nM vs. **14b**; IC_{50} = 0.46 nM). Further, **14b** maintained moderate selectivity for JAK3 over JAK1 (6-fold selectivity) and JAK2 (11-fold selectivity). The potent JAK3 inhibitory

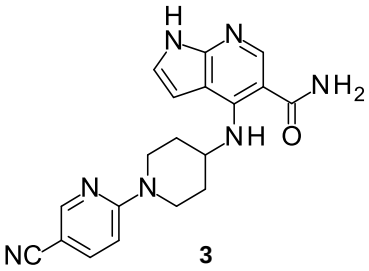
activity of **14b** suggested that the 4,6-diaminonicotinamide was a potential scaffold to form suitable binding to the hinge region.

However, despite the promising profile of **14b** as a JAK3 inhibitor, this compound also inhibited hERG with an IC_{50} value of 0.36 μM. As binding to the hERG channel is related to hydrophobic interactions[23,24], the pyridine in the C6-position of **14b** was therefore converted to another hetero aromatic ring to reduce molecular lipophilicity. Results showed that 6-pyrazylamine analogue **14c** and 6-pyrimidylamine analogue **14d** maintained potent JAK3 inhibitory activity (IC_{50} = 0.72 and 0.61 nM, respectively). Substitution of pyrazine and pyrimidine was then investigated, and introduction of a methyl group to the pyrazine (**14e** and **14f**) was tolerable for JAK3 inhibitory activity (IC_{50} = 1.0 and 1.1 nM, respectively). In the case of methylation of the pyrimidine, **14g** exhibited increased JAK3 inhibitory activity over **14d** (IC_{50} = 0.36 nM). Further, introduction of a bicyclic hetero ring in the C6-position was examined, and benzoxazole analogues **14h** and **14i** showed moderate JAK3 inhibitory activity (IC_{50} = 22 and 25 nM, respectively). Regarding hERG inhibition of the 4,6-diaminonicotinamide derivatives, the 6-pyrimidylamine analogues **14d** and **14g** with lower *C*LogP value (= 4.0 and 4.3, respectively) tended to have reduced hERG inhibitory activity (IC_{50} = 17 and 8.1 μM, respectively) compared to **14b** while retaining potent JAK3 inhibitory activity.

Table 2

SARs of *N*-cyanopyridyl-4-aminopiperidine derivatives





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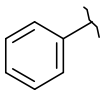
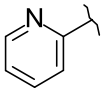
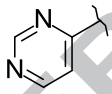
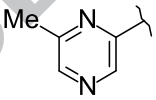
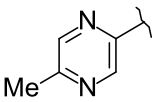
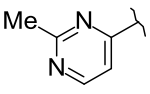
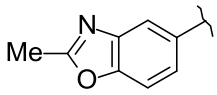
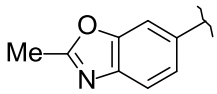
Compound	R	JAK3 IC_{50}[a] (nM)	JAK1 IC_{50}[a] (nM)	JAK2 IC_{50}[a] (nM)	hERG IC_{50}[b] (μM)	*C*LogP[c]
14a		18	NT[d]	190	NT[d]	5.1
14b		0.46	2.9	4.9	0.36	4.7
14c		0.72	2.5	4.1	7.7	4.2
14d		0.61	3.5	5.5	17	4.0
14e		1.0	1.9	4.0	5.4	4.7
14f		1.1	6.7	5.3	4.8	4.7
14g		0.36	5.4	3.2	8.1	4.3

14h		22	NT[d]	400	6.6	4.7
14i		25	NT[d]	130	NT[d]	4.7
3		1.3	16	18	13.4	3.4

[a]IC_{50} values are mean of duplicate experiments.

[b]Inhibitory activity in Rb efflux assay (n = 2).

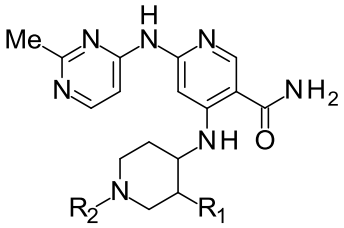
[c]*C*LogP values calculated using ACD/Percepta 14.0.0.

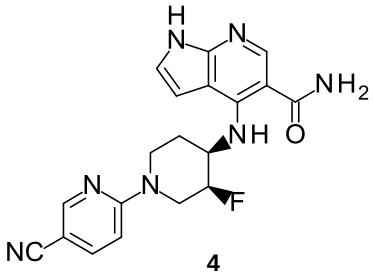
[d]NT = not tested.

Table 3 shows modification of the C4-substituent of the 6-pyrimidylaminonicotinamide derivatives. Findings from our previous study suggested that the molecular basicity around the *N*-cyanopyridyl-4-aminopiperidine may have been involved in hERG inhibitory activity, and decreasing the basicity in the C4-substituent was effective for reduction of this activity.[23] The introduction of a fluoro group to the piperidine of **14g** was therefore investigated. The basicity of the 3-fluoropiperidine is considered to be reduced by electron withdrawing effect due to the fluorine atom,[27,28] which effectively reduces hERG inhibitory as shown in the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide derivatives (**4**; hERG IC_{50} >100 μM). As expected, the 3-fluoropiperidine analogue **27** showed substantially decreased hERG inhibitory activity (IC_{50} >100 μM) while the JAK3 inhibitory activity was maintained (IC_{50} = 0.39 nM). The pyridine of **14g** was then converted to a phenyl ring to remove the basicity of pyridine, and **28** exhibited a large decrease in hERG inhibitory activity (IC_{50} >100 μM) with potent JAK3 inhibitory activity (IC_{50} = 0.80 nM). Despite an increase in molecular lipophilicity, conversion of the pyridyl piperidine structure to the phenyl piperidine in the C4-substituent was mainly effective to reduce hERG inhibitory activity. JAK3 selectivity of **28** toward JAK1 and JAK2 was slightly increased compared to **27**. In addition, **28** exhibited weak inhibitory activity of 19 representative kinases in a kinase panel assay (see Supplementary data). In a series of the 4,6-diaminonicotinamide derivatives, **28** was identified as a potent JAK3 inhibitor without hERG inhibitory activity.

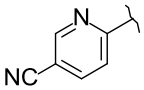
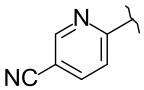
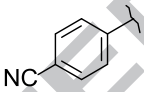
Table 3

SARs of 6-pyrimidylaminonicotinamide derivatives





4

Compound	R1	R2	JAK3 IC_{50}[a] (nM)	JAK1 IC_{50}[a] (nM)	JAK2 IC_{50}[a] (nM)	hERG IC_{50}[b] (μM)	*C*LogP[c]
14g	H		0.36	5.4	3.2	8.1	4.3
27	F (3*S*, 4*R*)		0.39	0.24	1.1	>100	4.0
28	H		0.80	4.2	3.5	>100	4.9
4			0.30	4.1	3.2	>100	3.1

[a]IC_{50} values are mean of duplicate experiments.

[b]Inhibitory activity in Rb efflux assay (n = 2).

[c]*C*LogP values calculated using ACD/Percepta 14.0.0.

To clarify the binding mode of the 4,6-diaminonicotinamide derivatives to JAK3, docking calculations were conducted for **28**. Figure 5 shows superposition of binding mode of **28** and **4**. Docking calculations were performed for the crystal structure of **1** bound to JAK3 (PDB code: 3LXK[29]) using the XP mode in Glide (version 5.8; Schrödinger, LLC, New York, NY, 2012). Around the hinge region, the 4,6-diaminonicotinamide scaffold of **28** maintained a planar conformation, and the N1-nitrogen group formed a hydrogen bond with Leu905 via a hydrogen acceptor while the C6-amino group formed one via a hydrogen donor. The C3-carbamoyl group of **28** was located in the back of the hinge region and formed an additional hydrogen bond interaction with Glu903. Further, the C3-carbamoyl group interacted with the gatekeeper amino acid Met902. Interestingly, the position of the carbamoyl group of **28** was different from that of **4**, in spite of structural similarity between the 4,6-diaminonicotinamide scaffold and the 1*H*-pyrrolo[2,3-*b*]pyridine-5-carboxamide. Although the pyridine ring of **28** was located in the position of the corresponding pyridine ring of **4**, the C3-carbamoyl group of **28** was directed to narrow space in the back side, occupied by the pyrrole ring in the case of **4**. The different orientation of C3-carbamoyl group of **28** is likely due to the spatial capacity in the back of the hinge region. The pyrrolopyridine ring of **4** was acceptable in the narrow area, while the pyrimidinylaminopyridine moiety of **28** was not allowed. It was suggested that the bulky C6-substituent of 4,6-diaminonicotinamide scaffold of **28** was directed to solvent exposure space, and the small C3-carbamoyl group was turned to the back side to form hydrogen bond interaction with Glu903 and Met902. The novel hydrogen bond of C3-carbamoyl group of **28** may retain the 4,6-diaminonicotinamide scaffold around the hinge region and contribute to potent JAK3 inhibitory activity. Each of the aromaticity of pyridine and pyrimidine ring of **28** enabled a CH-π interaction with Leu828. The SAR results in Table 2 suggested that introduction

of a heteroaryl ring such as pyridine, pyrazine, and pyrimidine in C6-position was favorable for the planar conformation to form the CH-π interaction, leading to potent JAK3 inhibitory activity. Regarding the C4-substituent, the *N*-cyanophenyl-4-aminopiperidine group of **28** occupied the hydrophobic cavity. In the back of the hydrophobic cavity, the cyanophenyl group formed a CH-π interaction with Gly831 and a hydrogen bond with Arg953. These findings indicate that **28** was effectively positioned in the JAK3 binding pocket and achieved high affinity to JAK3.

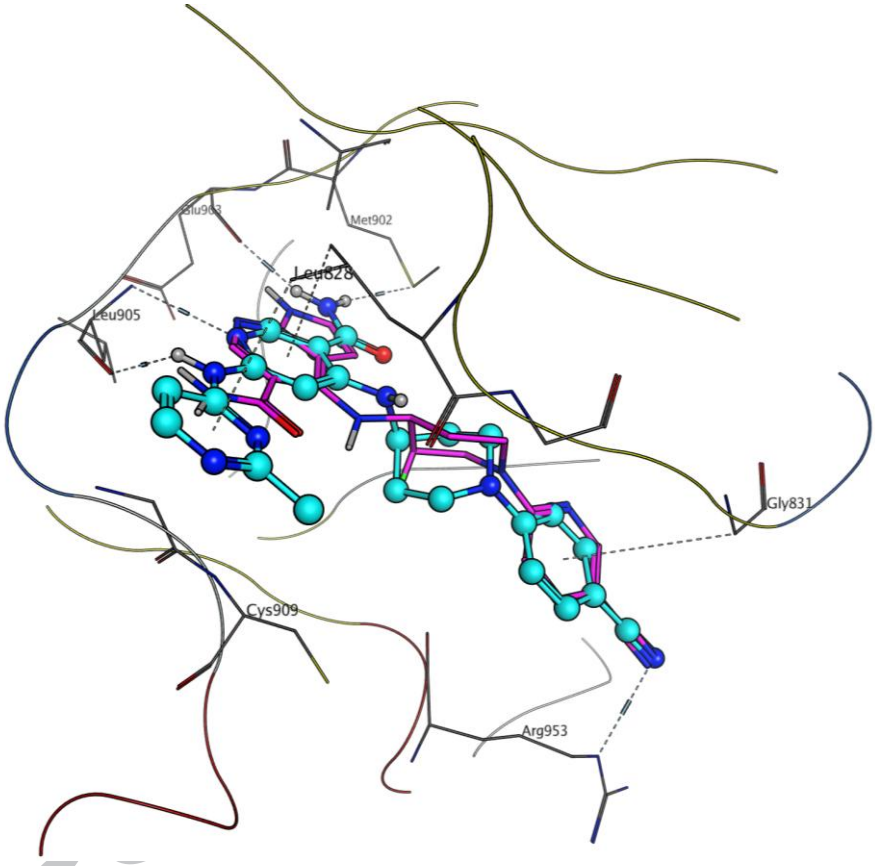


Figure 5. Predicted binding mode of compound **28** (blue) and **4** (pink) to human JAK3.

In vitro profiles of the selected compound **28** are shown in Table 4. The metabolic stability of **28** in rat liver microsomes was acceptable, with an *in vitro* intrinsic clearance (CL_{int}) value of 146 mL/min/kg, and no species difference was shown between rats and humans (human CL_{int} =

109 mL/min/kg). In a parallel artificial membrane permeability assay (PAMPA), **28** exhibited a high effective permeability value (P_e >30 × 10^{-6} cm/sec). Table 5 shows PK parameters of **28** after oral (p.o.) and intravenous (i.v.) administration, and the oral bioavailability was 23.6%, suggesting moderate oral exposure in rats.

Table 4

In vitro profiles of compound **28**

Metabolic stability CL_{int}[a] (mL/min/kg)		Membrane permeability P_e[b] (× 10^{-6} cm/sec)
Rat	Human	
146	109	>30

[a]*In vitro* metabolism with liver microsomes in the presence of NADPH-generating system (n =2).

[b]PAMPA Evolution™ (pION Inc., USA), donor buffer pH: 6.5 (n = 3).

Table 5

Pharmacokinetic parameters after single administration of **28** to rats

Species	i.v. (1 mg/kg, n=3)				p.o. (1 mg/kg, n=3)			
	AUC_{0-24} (ng·h/mL)	$t_{1/2}$ (h)	V_{dss} (L/kg)	CL_{tot} (mL/min/kg)	C_{max} (ng/mL)	t_{max} (h)	AUC_{0-24} (ng·h/mL)	F[a] (%)
Rat	1177	3.4	3.5	14.3	52.9	0.4	278	23.6

[a]F = bioavailability.

Selected compound **28** exhibited potent JAK3 inhibitory activity and acceptable oral exposure, and therefore **28** was evaluated in an ACI-to-Lewis rat heterotopic cardiac transplant model to validate the *in vivo* efficacy as an immunomodulator. In the model, allografts were ultimately rejected by approximately 5 days after transplantation in untreated rats. In addition, tacrolimus monotherapy at a dose of 0.02 mg/kg via intramuscular injection exhibited only marginal efficacy in preventing allograft rejection (MST = 6.5 days).[25] As shown in Table 6, combination therapy of **28** with tacrolimus prevented allograft rejection and prolonged graft survival (MST = 22.5 days). This result demonstrates the additive effect of JAK3 inhibitors on current CNI-based immunosuppressive therapy, and **28** might therefore be an effective immunomodulator for preventing transplant rejection.

Table 6

Effect of compound **28** on graft survival in a rat cardiac transplantation model[a]

Treatment	Median survival times (days)
28, 10 mg/kg (p.o.) with tacrolimus, 0.02 mg/kg (i.m.)[b]	22.5

[a] ACI rat hearts were heterotopically transplanted into Lewis rats (n = 4).

[b] **28** and tacrolimus were administered from day of transplantation for 14 days or by day of graft rejection; i.m., intramuscular.

4. Conclusion

We designed 4,6-diaminonicotinamide derivatives to modify interaction with hinge region, and conducted an SAR study of JAK3 inhibitory activity. In the C6-position of the 4,6-diaminonicotinamide derivatives, the introduction of a heteroaryl ring such as pyridine, pyrazine, and pyrimidine was important for achieving potent JAK3 inhibitory activity, while *N*-cyanopyridyl-4-aminopiperidine was acceptable as a C4-substituent (**14b**, **14c,** and **14d**). Although a number of 4,6-diaminonicotinamide derivatives exhibited undesirable hERG inhibitory activity, decreasing the molecular lipophilicity in the C6-position and modulating the molecular basicity in the C4-position effectively reduced this inhibitory activity (**27** and **28**). A docking calculation of **28** to JAK3 indicated the predicted binding mode around the hinge region and hydrophobic cavity, suggesting that the 4,6-diaminonicotinamide derivative formed not only conventional interaction of our previous series of compounds, but also novel hydrogen bond interaction of C3-carbamoyl group and CH-π interaction of aryl ring around the hinge region. In a rat heterotopic cardiac transplantation model, the combination of **28** and tacrolimus resulted in prolonged graft survival. These findings suggest that the 4,6-diaminonicotinamide derivatives are novel immunomodulators targeting JAK3 for treating organ transplant rejection.

5. Experimental Section

5.1. Chemistry

^{1}H NMR spectra were recorded on a Bruker Biospin AV400 or AV400M spectrometer. Chemical shifts are expressed in δ units using tetramethylsilane as an internal standard (NMR peak description: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; and br = broad peak). Mass spectra (MS) were recorded on a Hitachi LC/3DQMS M8000 or an Agilent HP1100 LC/MCD spectrometer. High-resolution mass spectroscopy (HRMS) spectra were recorded on a Waters LCT Premier XE. Column chromatography was conducted using silica gel 60N (Kanto Chemical, 63-210 μm) or a Yamazen HI-FLASH^{TM} Column. The purity of all compounds screened in biological assays was given by HPLC analysis with a ZORBAX Eclipse Plus C18 column (detection at 254 nm). Elemental analysis values were recorded on a YANACO JM-10 and were within 0.4% of the theoretical values calculated for C, H, and N.

5.1.1. Ethyl 6-chloro-4-{[(1*S*,2*R*)-2-methylcyclohexyl]amino}nicotinate (6)

(1*S*,2*R*)-2-Methylcyclohexanamine hydrochloride (510 mg, 3.41 mmol) and DIPEA (0.59 mL, 3.41 mmol) were added to a solution of ethyl 4,6-dichloronicotinate (**5**) (500 mg, 2.27 mmol) in *n*-BuOH (5 mL), and the mixture was stirred at 120 °C for 3 h. After adding (1*S*,2*R*)-2-methylcyclohexanamine hydrochloride (102 mg, 0.68 mmol), the mixture was stirred at the same temperature for additional 1 h. After cooling to room temperature, the mixture was extracted with EtOAc, and washed with H_2O. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc = 90/10 to 80/20) to give the title compound (660 mg, 98%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, d, J = 6.8 Hz), 1.17-1.39 (8H, m), 1.43-1.70 (3H,

m), 1.82-1.93 (1H, m), 3.83-3.89 (1H, m), 4.32 (2H, q, *J* = 7.2 Hz), 6.91 (1H, s), 8.37 (1H, d, *J* = 8.8 Hz), 8.54 (1H, s). MS (ESI) *m/z*: 297 $(M + H)^+$.

5.1.2. 6-Chloro-4-{[(1*S*,2*R*)-2-methylcyclohexyl]amino}nicotinic acid (7)

NaOH aqueous solution (2 M; 4.45 mL, 8.89 mmol) was added to a solution of **6** (660 mg, 2.22 mmol) in dioxane (6.6 mL), and the mixture was stirred at 110 °C for 2 h. After cooling to 4 °C, the mixture was acidified with 1 M HCl aqueous solution. The precipitate was filtrated and washed with H_2O to give the title compound (348 mg, 58%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, d, *J* = 7.2 Hz), 1.21-1.69 (8H, m), 1.82-1.93 (1H, m), 3.78-3.85 (1H, m), 6.85 (1H, s), 8.50 (1H, s), 8.63 (1H, d, *J* = 8.8 Hz), 13.38 (1H, brs). MS (ESI) *m/z*: 269 $(M + H)^+$.

5.1.3. 6-Chloro-4-{[(1*S*,2*R*)-2-methylcyclohexyl]amino}nicotinamide (8)

Carbonyldiimidazole (CDI) (463 mg, 2.86 mmol) was added to a solution of **7** (384 mg, 1.43 mmol) in DMF (3.84 mL), and the mixture was stirred at room temperature for 1 h. After the addition of 28% NH_4OH aqueous solution (0.89 mL, 7.15 mmol), the mixture was stirred at room temperature for 1 h. H_2O was added to the mixture, and the precipitate was filtrated and washed with H_2O and IPE to give the title compound (350 mg, 91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.83 (3H, d, *J* = 6.8 Hz), 1.20-1.67 (8H, m), 1.79-1.89 (1H, m), 3.69-3.76 (1H, m), 6.73 (1H, s), 7.46 (1H, br), 8.09 (1H, br), 8.41 (1H, s), 9.22 (1H, d, *J* = 8.8 Hz). MS (ESI) *m/z*: 268 $(M + H)^+$.

5.1.4. 6-Anilino-4-{[(1*S*,2*R*)-2-methylcyclohexyl]amino}nicotinamide (9)

In the vessel of a microwave reactor, **8** (30 mg, 0.112 mmol), aniline (31.3 mg, 0.336 mmol), $Pd_2(dba)_3$ (5.1 mg, 5.6 μmol), 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl (2.7 mg, 5.7 μmol), and *t*-BuONa (16.2 mg, 0.169 mmol) were suspended in *t*-BuOH (0.75 mL). The vessel was then sealed, and the mixture was reacted at 130 °C for 30 min under microwave irradiation. After cooling to room temperature, the mixture was extracted with $CHCl_3$/MeOH (4/1), and washed with brine. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by NH silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 90/10) to give the title compound (20 mg, 55%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, d, J = 7.0 Hz), 1.30-1.61 (7H, m), 1.67-1.73 (1H, m), 1.87-1.94 (1H, m), 3.44-3.49 (1H, m), 5.96 (1H, s), 6.84-6.88 (1H, m), 6.99 (1H, br), 7.19-7.25 (2H, m), 7.57-7.60 (2H, m), 7.74 (1H, br), 8.36 (1H, s), 8.86 (1H, s), 8.88 (1H, d, J = 8.2 Hz). MS (ESI) *m/z*: 325 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{19}H_{24}N_4O$: 324.1950. Found: 325.2031.

5.1.5. Ethyl 4-{[1-(*tert*-butoxycarbonyl)piperidin-4-yl]amino}-6-chloronicotinate (10)

The title compound was prepared from **5** and *N*-Boc-4-aminopiperidine in accordance with the procedure for preparing **6** in 99% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.28-1.43 (14H, m), 1.85-1.93 (2H, m), 2.88-3.06 (2H, m), 3.74-3.90 (3H, m), 4.29 (2H, q, J = 7.2 Hz), 6.99 (1H, s), 8.05 (1H, d, J = 8.0 Hz), 8.54 (1H, s). MS (ESI) *m/z*: 384 $(M + H)^+$.

5.1.6. 4-{[1-(*tert*-Butoxycarbonyl)piperidin-4-yl]amino}-6-chloronicotinic acid (11)

The title compound was prepared from **10** in accordance with the procedure for preparing **7** in 88% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.24-1.45 (11H, m), 1.86-1.94 (2H, m), 2.88-

3.07 (2H, m), 3.72-3.90 (3H, m), 6.94 (1H, s), 8.31 (1H, d, *J* = 8.0 Hz), 8.51 (1H, s), 13.39 (1H, br). MS (ESI) *m/z*: 356 $(M + H)^+$.

5.1.7. *tert*-Butyl 4-[(5-carbamoyl-2-chloropyridin-4-yl)amino]piperidine-1-carboxylate (12)

The title compound was prepared from **11** in accordance with the procedure for preparing **8** in 90% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: *1*.21-1.32 (2H, m), 1.40 (9H, s), 1.81-1.92 (2H, m), 2.88-3.08 (2H, m), 3.64-3.74 (1H, m), 3.76-3.86 (2H, m), 6.82 (1H, s), 7.48 (1H, br), 8.09 (1H, br), 8.41 (1H, s), 8.86 (1H, d, *J* = 8.0 Hz). MS (ESI) *m/z*: 377 $(M + Na)^+$.

5.1.8. *tert*-Butyl 4-[(2-anilino-5-carbamoylpyridin-4-yl)amino]piperidine-1-carboxylate (13a)

The title compound was prepared from **12** and aniline in accordance with the procedure for preparing **9** in 48% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.37 (2H, m), 1.41 (9H, s), 1.89-1.98 (2H, m), 2.91-3.10 (2H, m), 3.40-3.49 (1H, m), 3.76-3.87 (2H, m), 5.99 (1H, s), 6.84-6.89 (1H, m), 7.03 (1H, br), 7.21-7.26 (2H, m), 7.59-7.63 (2H, m), 7.74 (1H, br), 8.37 (1H, s), 8.65 (1H, d, *J* = 7.2 Hz), 8.91 (1H, brs). MS (ESI) *m/z*: 412 $(M + H)^+$.

5.1.9. *tert*-Butyl 4-{[5-carbamoyl-2-(pyridin-2-ylamino)pyridin-4-yl]amino}piperidine-1-carboxylate (13b)

In the vessel of a microwave reactor, **12** (400 mg, 1.13 mmol), 2-aminopyridine (318 mg, 3.38 mmol), $Pd_2(dba)_3$ (51.6 mg, 56.4 μmol), 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-biphenyl (26.9 mg, 56.4 μmol), and K_2CO_3 (312 mg, 2.25 mmol) were suspended in *t*-BuOH (3 mL). The vessel was then sealed, and the mixture was reacted at 130 °C for 1 h under microwave

irradiation. After cooling to room temperature, the mixture was extracted with $CHCl_3$/MeOH (4/1), and washed with brine. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by NH silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 90/10) to give the title compound (362 mg, 78%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.27-1.38 (2H, m), 1.41 (9H, s), 1.95-2.02 (2H, m), 2.96-3.10 (2H, m), 3.43-3.53 (1H, m), 3.80-3.88 (2H, m), 6.83-6.87 (1H, m), 7.07 (1H, br), 7.22 (1H, s), 7.62-7.66 (2H, m), 7.80 (1H, br), 8.20-8.22 (1H, m), 8.40 (1H, s), 8.70 (1H, d, J = 7.2 Hz), 9.47 (1H, s). MS (ESI) *m/z*: 413 $(M + H)^+$.

5.1.10. *tert*-Butyl 4-{[5-carbamoyl-2-(pyrazin-2-ylamino)pyridin-4-yl]amino}piperidine-1-carboxylate (13c)

The title compound was prepared from **12** and 2-aminopyrazine in accordance with the procedure for preparing **13b** in 87% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.28-1.39 (2H, m), 1.41 (9H, s), 1.93-2.02 (2H, m), 2.97-3.10 (2H, m), 3.44-3.53 (1H, m), 3.78-3.88 (2H, m), 7.11 (1H, s), 7.14 (1H, br), 7.85 (1H, br), 8.07 (1H, d, *J* = 2.8 Hz), 8.23-8.24 (1H, m), 8.45 (1H, s), 8.77 (1H, d, *J* = 7.2 Hz), 9.01 (1H, d, *J* = 4.0 Hz), 9.84 (1H, s). MS (ESI) *m/z*: 414 $(M + H)^+$.

5.1.11. *tert*-Butyl 4-{[5-carbamoyl-2-(pyrimidin-4-ylamino)pyridin-4-yl]amino}piperidine-1-carboxylate (13d)

The title compound was prepared from **12** and 2-aminopyrimidine in accordance with the procedure for preparing **13b** in 92% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.28-1.38 (2H, m), 1.41 (9H, s), 1.94-2.01 (2H, m), 2.98-3.11 (2H, m), 3.44-3.52 (1H, m), 3.80-3.88 (2H, m), 7.12

(1H, s), 7.19 (1H, br), 7.74 (1H, d, *J* = 5.2 Hz), 7.89 (1H, br), 8.39 (1H, d, *J* = 6.0 Hz), 8.45 (1H, s), 8.70 (1H, s), 8.76 (1H, d, *J* = 7.2 Hz), 9.98 (1H, s). MS (ESI) *m/z*: 414 $(M + H)^+$.

5.1.12. *tert*-Butyl 4-({5-carbamoyl-2-[(6-methylpyrazin-2-yl)amino]pyridin-4-yl}amino)piperidine-1-carboxylate (13e)

The title compound was prepared from **12** and 2-amino-6-methylpyrazine in accordance with the procedure for preparing **13b** in 50% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.28-1.45 (11H, m), 1.98-2.06 (2H, m), 2.40 (3H, s), 2.92-3.12 (2H, m), 3.45-3.53 (1H, m), 3.84-3.92 (2H, m), 7.11 (1H, br), 7.39 (1H, s), 7.85 (1H, br), 7.96 (1H, s), 8.44 (1H, s), 8.64 (1H, s), 8.77 (1H, d, *J* = 6.8 Hz), 9.84 (1H, s). MS (ESI) *m/z*: 428 $(M + H)^+$.

5.1.13. *tert*-Butyl 4-({5-carbamoyl-2-[(5-methylpyrazin-2-yl)amino]pyridin-4-yl}amino)piperidine-1-carboxylate (13f)

The title compound was prepared from **12** and 2-amino-5-methylpyrazine in accordance with the procedure for preparing **13b** in 93% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.44 (11H, m), 1.92-2.02 (2H, m), 2.38 (3H, s), 2.95-3.11 (2H, m), 3.42-3.51 (1H, m), 3.78-3.89 (2H, m), 7.03 (1H, s), 7.11 (1H, br), 7.83 (1H, br), 8.13 (1H, s), 8.43 (1H, s), 8.75 (1H, d, *J* = 7.2 Hz), 8.91-8.92 (1H, m), 9.68 (1H, brs). MS (ESI) *m/z*: 428 $(M + H)^+$.

5.1.14. *tert*-Butyl 4-({5-carbamoyl-2-[(2-methylpyrimidin-4-yl)amino]pyridin-4-yl}amino)piperidine-1-carboxylate (13g)

The title compound was prepared from **12** and 4-amino-2-methylpyrimidine in accordance with the procedure for preparing **13b** in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.44

(11H, m), 1.98-2.06 (2H, m), 2.48 (3H, s), 2.93-3.08 (2H, m), 3.45-3.54 (1H, m), 3.83-3.92 (2H, m), 7.17 (1H, br), 7.33 (1H, d, *J* = 5.6 Hz), 7.41 (1H, s), 7.86 (1H, br), 8.28 (1H, d, *J* = 5.6 Hz), 8.44 (1H, s), 8.77 (1H, d, *J* = 6.8 Hz), 9.93 (1H, s). MS (ESI) *m/z*: 428 $(M + H)^+$.

5.1.15. *tert*-Butyl 4-({5-carbamoyl-2-[(2-methyl-1,3-benzoxazol-5-yl)amino]pyridin-4-yl}amino)piperidine-1-carboxylate (13h)

The title compound was prepared from **12** and 2-methyl-1,3-benzoxazol-5-amine in accordance with the procedure for preparing **13b** in 69% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.44 (11H, m), 1.90-1.99 (2H, m), 2.57 (3H, s), 2.94-3.12 (2H, m), 3.40-3.51 (1H, m), 3.74-3.90 (2H, m), 5.97 (1H, s), 7.04 (1H, br), 7.38 (1H, dd, *J* = 2.4, 8.8 Hz), 7.50 (1H, d, *J* = 8.8 Hz), 7.77 (1H, br), 8.10 (1H, d, J = 2.4 Hz), 8.39 (1H, s), 8.66, (1H, d, *J* = 7.2 Hz), 9.01 (1H, s). MS (ESI) *m/z*: 467 $(M + H)^+$.

5.1.16. *tert*-Butyl 4-({5-carbamoyl-2-[(2-methyl-1,3-benzoxazol-6-yl)amino]pyridin-4-yl}amino)piperidine-1-carboxylate (13i)

The title compound was prepared from **12** and 2-methyl-1,3-benzoxazol-6-amine in accordance with the procedure for preparing **13b** in 29% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.44 (11H, m), 1.91-1.99 (2H, m), 2.56 (3H, s), 2.95-3.08 (2H, m), 3.40-3.50 (1H, m), 3.78-3.86 (2H, m), 6.00 (1H, s), 7.06 (1H, br), 7.30 (1H, dd, *J* = 2.0, 8.8 Hz), 7.48 (1H, d, *J* = 8.8 Hz), 7.78 (1H, br), 8.26 (1H, d, *J* = 2.0 Hz), 8.41 (1H, s), 8.66 (1H, d, *J* = 7.2 Hz), 9.15 (1H, s). MS (ESI) *m/z*: 467 $(M + H)^+$.

5.1.17. 6-Anilino-4-{[1-(5-cyanopyridin-2-yl)piperidin-4-yl]amino}nicotinamide (14a)

HCl in dioxane (4 M; 6 mL) was added to a solution of **13a** (138 mg, 0.335 mmol) in dioxane (2 mL), and the mixture was stirred at room temperature overnight. After adding IPE to the mixture, the precipitate was filtrated and washed with IPE to give 6-anilino-4-(piperidin-4-ylamino)nicotinamide dihydrochloride (114 mg, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.63-1.75 (2H, m), 2.05-2.13 (2H, m), 2.94-3.05 (2H, m), 3.22-3.31 (2H, m), 3.66-3.76 (1H, m), 6.17 (1H, s), 7.22-7.28 (1H, m), 7.32-7.36 (2H, m), 7.43-7.48 (2H, m), 7.70 (1H, br), 8.34 (2H, brs), 8.93 (1H, br), 9.05 (1H, br), 9.55 (1H, br), 10.15 (1H, br), 12.92 (1H, br). MS (ESI) *m/z*: 312 (M + H)$^+$.

2-Chloro-5-cyanopyridine (32.4 mg, 0.234 mmol) and K_2CO_3 (48.6 mg, 0.351 mmol) were added to a solution of 6-anilino-4-(piperidin-4-ylamino)nicotinamide dihydrochloride (45 mg, 0.117 mmol) in DMSO (0.72 mL), and the mixture was stirred at 100 °C for 45 min. After cooling to room temperature, the mixture was purified by silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 88/12) to give the title compound (39.5 mg, 82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.36-1.46 (2H, m), 2.01-2.10 (2H, m), 3.25-3.35 (2H, m), 3.54-3.64 (1H, m), 4.25-4.31 (2H, m), 6.04 (1H, s), 6.87 (1H, t, J = 7.2 Hz), 6.99 (1H, d, J = 9.2 Hz), 7.03 (1H, br), 7.24 (2H, t, J = 7.2 Hz), 7.60-7.64 (2H, m), 7.74 (1H, br), 7.85 (1H, dd, J = 2.4, 9.2 Hz), 8.38 (1H, s), 8.49 (1H, d, J = 2.4 Hz), 8.69 (1H, d, J = 7.2 Hz), 8.93 (1H, s). MS (ESI) *m/z*: 414 (M + H)$^+$. HRMS (M + H)$^+$ calcd for $C_{23}H_{23}N_7O$: 413.1964. Found: 414.2050.

5.1.18. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-(pyridin-2-ylamino)nicotinamide (14b)

HCl in dioxane (4 M; 3 mL) was added to a solution of **13b** (360 mg, 0.873 mmol) in dioxane (1.5 mL), and the mixture was stirred at room temperature for 2 h. The mixture was concentrated under reduced pressure to give 4-(piperidin-4-ylamino)-6-(pyridin-2-ylamino)nicotinamide dihydrochloride (315 mg, 94%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.70-1.81 (2H, m), 2.14-2.21 (2H, m), 3.00-3.10 (2H, m), 3.29-3.35 (2H, m), 3.68-3.76 (1H, m), 6.71 (1H, s), 7.17-7.23 (2H, m), 7.80 (1H, br), 7.90-7.95 (1H, m), 8.35-8.38 (1H, m), 8.40 (1H, br), 8.47 (1H, s), 8.94-9.10 (2H, m), 9.30 (1H, d, J = 7.2 Hz), 11.95 (1H, s), 14.50 (1H, br).

In the vessel of a microwave reactor, 2-chloro-5-cyanopyridine (27 mg, 0.195 mmol) and Et_3N (90 μL, 0.649 mmol) were added to a solution of 4-(piperidin-4-ylamino)-6-(pyridin-2-ylamino)nicotinamide dihydrochloride (50 mg, 0.130 mmol) in NMP (1 mL). The vessel was then sealed, and the mixture was reacted at 150 °C for 30 min under microwave irradiation. After cooling to room temperature, the mixture was purified by aminosilica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 90/10) to give the title compound (17 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.38-1.48 (2H, m), 2.07-2.14 (2H, m), 3.27-3.35 (2H, m), 3.60-3.68 (1H, m), 4.27-4.33 (2H, m), 6.84-6.88 (1H, m), 7.00 (1H, d, J = 9.0 Hz), 7.07 (1H, br), 7.26 (1H, s), 7.61-7.68 (2H, m), 7.80 (1H, br), 7.85 (1H, dd, J = 2.3, 9.0 Hz), 8.21-8.23 (1H, m), 8.41 (1H, s), 8.49-8.50 (1H, m), 8.74 (1H, d, J = 7.1 Hz), 9.49 (1H, s). MS (ESI) m/z: 415 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{22}H_{22}N_8O$: 414.1917. Found: 415.1997.

5.1.19. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-(pyrazin-2-ylamino)nicotinamide (14c)

The title compound was prepared from **13c** in accordance with the procedure for preparing **14b** in 90% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.40-1.49 (2H, m), 2.06-2.12 (2H, m),

3.28-3.37 (2H, m), 3.62-3.68 (1H, m), 4.26-4.32 (2H, m), 7.00 (1H, d, *J* = 9.1 Hz), 7.11 (1H, br), 7.14 (1H, s), 7.85 (1H, dd, *J* = 2.4, 9.1 Hz), 7.87 (1H, br), 8.07 (1H, d, *J* = 2.7 Hz), 8.23-8.24 (1H, m), 8.46 (1H, s), 8.48-8.51 (1H, m), 8.81 (1H, d, *J* = 7.2 Hz), 9.03 (1H, d, *J* = 1.5 Hz), 9.86 (1H, s). MS (ESI) *m/z*: 416 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{21}H_{21}N_9O$: 415.1869. Found: 416.1954.

5.1.20. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-(pyrimidin-4-ylamino)nicotinamide (14d)

The title compound was prepared from **13d** in accordance with the procedure for preparing **14b** in 28% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.45-1.54 (2H, m), 2.05-2.12 (2H, m), 3.24-3.40 (2H, m), 3.67-3.76 (1H, m), 4.28-4.35 (2H, m), 6.94 (1H, br), 7.01 (1H, d, *J* = 9.1 Hz), 7.35-7.66 (2H, m), 7.86 (1H, dd, *J* = 2.4, 9.1 Hz), 8.11 (1H, br), 8.46 (1H, s), 8.50 (1H, d, *J* = 2.4 Hz), 8.51-8.54 (1H, m), 8.83 (1H, s), 9.10 (1H, br), 10.77 (1H, br). MS (ESI) *m/z*: 416 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{21}H_{21}N_9O$: 415.187. Found: 416.1945.

5.1.21. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-[(6-methylpyrazin-2-yl)amino]nicotinamide (14e)

The title compound was prepared from **13e** in accordance with the procedure for preparing **14a** in 52% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.39-1.50 (2H, m), 2.10-2.17 (2H, m), 2.42 (3H, s), 3.23-3.37 (2H, m), 3.60-3.70 (1H, m), 4.30-4.40 (2H, m), 7.00 (1H, d, *J* = 9.2 Hz), 7.14 (1H, br), 7.42 (1H, s), 7.78-7.90 (2H, m), 7.97 (1H, s), 8.45 (1H, s), 8.50 (1H, d, *J* = 2.2 Hz), 8.63 (1H, s), 8.83 (1H, brd), 9.88 (1H, br). MS (ESI) *m/z*: 430 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{22}H_{23}N_9O$: 429.2026. Found: 430.2094.

5.1.22. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-[(5-methylpyrazin-2-yl)amino]nicotinamide (14f)

The title compound was prepared from **13f** in accordance with the procedure for preparing **14a** in 30% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.42-1.54 (2H, m), 2.04-2.13 (2H, m), 2.42 (3H, s), 3.26-3.52 (2H, m), 3.63-3.75 (1H, m), 4.26-4.36 (2H, m), 6.85-6.95 (1H, m), 7.01 (1H, d, J = 9.2 Hz), 7.34 (1H, br), 7.85 (1H, dd, J = 2.4, 9.2 Hz), 8.00 (1H, br), 8.18 (1H, s), 8.42 (1H, s), 8.50 (1H, d, J = 2.4 Hz), 8.69-8.82 (1H, m), 8.94-9.08 (1H, m), 10.19 (1H, br). MS (ESI) *m/z*: 430 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{22}H_{23}N_9O$: 429.2026. Found: 430.2104.

5.1.23. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-[(2-methylpyrimidin-4-yl)amino]nicotinamide (14g)

The title compound was prepared from **13g** in accordance with the procedure for preparing **14a** in 57% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.38-1.50 (2H, m), 2.09-2.18 (2H, m), 2.50 (3H, s), 3.24-3.44 (2H, m), 3.60-3.70 (1H, m), 4.30-4.40 (2H, m), 7.00 (1H, d, J = 9.0 Hz), 7.16 (1H, br), 7.34 (1H, d, J = 5.8 Hz), 7.46 (1H, s), 7.82-7.92 (2H, m), 8.29 (1H, d, J = 5.9 Hz), 8.44 (1H, s), 8.49 (1H, d, J = 2.3 Hz), 8.79 (1H, d, J = 6.9 Hz), 9.94 (1H, s). MS (ESI) *m/z*: 430 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{22}H_{23}N_9O$: 429.2026. Found: 430.2104.

5.1.24. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-[(2-methyl-1,3-benzoxazol-5-yl)amino]nicotinamide (14h)

The title compound was prepared from **13h** in accordance with the procedure for preparing **14a** in 31% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.36-1.48 (2H, m), 2.02-2.10 (2H, m), 2.58

(3H, s), 3.25-3.36 (2H, m), 3.56-3.66 (1H, m), 4.20-4.30 (2H, m), 6.02 (1H, s), 6.97-7.18 (2H, m), 7.39 (1H, dd, *J* = 2.1, 8.8 Hz), 7.52 (1H, d, *J* = 8.8 Hz), 7.72-7.89 (2H, m), 8.10 (1H, br), 8.39 (1H, s), 8.49 (1H, d, *J* = 2.1 Hz), 8.74 (1H, br), 9.08 (1H, br). MS (ESI) *m/z*: 469 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{25}H_{24}N_8O_2$: 468.2022. Found: 469.2104.

5.1.25. 4-{[1-(5-Cyanopyridin-2-yl)piperidin-4-yl]amino}-6-[(2-methyl-1,3-benzoxazol-6-yl)amino]nicotinamide (14i)

The title compound was prepared from **13i** in accordance with the procedure for preparing **14a** in 26% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.36-1.48 (2H, m), 2.01-2.10 (2H, m), 2.56 (3H, s), 3.25-3.36 (2H, m), 3.56-3.66 (1H, m), 4.24-4.32 (2H, m), 6.05 (1H, s), 6.96-7.16 (2H, m), 7.31 (1H, dd, *J* = 2.0, 8.6 Hz), 7.49 (1H, d, *J* = 8.6 Hz), 7.70-7.88 (2H, m), 8.28 (1H, d, *J* = 1.9 Hz), 8.42 (1H, s), 8.49 (1H, d, *J* = 2.2 Hz), 8.70 (1H, d, *J* = 7.2 Hz), 9.18 (1H, s). MS (ESI) *m/z*: 469 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{25}H_{24}N_8O_2$: 468.2022. Found: 469.2102.

5.1.26. Ethyl 4-(benzylamino)-6-chloronicotinate (15)

Benzylamine (23.8 mL, 218 mmol) and DIPEA (47.5 mL, 273 mmol) were added to a solution of **5** (40.0 g, 182 mmol) in *i*-PrOH (400 mL), and the mixture was stirred at 80 °C for 2 h. After cooling to room temperature, the mixture was concentrated under reduced pressure. EtOAc and H_2O were added to the residue. The organic layer was separated, washed with 10% citric acid aqueous solution, saturated $NaHCO_3$ aqueous solution, and brine, and dried over $MgSO_4$. The extract was concentrated under reduced pressure to give the title compound (51 g, 97%), which was used in the next reaction without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.39 (3H, t, *J* = 7.2 Hz), 4.35 (2H, q, *J* = 7.2 Hz), 4.43 (2H, d, J = 5.6 Hz), 6.55

(1H, s), 7.29-7.34 (3H, m), 7.35-7.40 (2H, m), 8.52-8.60 (1H, m), 8.70 (1H, s). MS (ESI) *m/z*: 291 $(M + H)^+$.

5.1.27. 4-(Benzylamino)-6-chloronicotinic acid (16)

NaOH aqueous solution (6M; 112 mL, 674 mmol) was added to a solution of **15** (49.0 g, 169 mmol) in EtOH (392 mL), and the mixture was stirred at 70 °C for 1.5 h. After cooling to room temperature, H_2O and 2 M HCl aqueous solution were added, and the mixture was stirred for 0.5 h. The precipitate was filtrated and washed with H_2O to give the title compound (40.2 g, 91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.55 (2H, d, J = 6.0 Hz), 6.71 (1H, s), 7.25-7.40 (5H, m), 8.53 (1H, s), 8.71-8.80 (1H, m), 13.40 (1H, brs). MS (ESI) *m/z*: 285 $(M + Na)^+$.

5.1.28. 4-(Benzylamino)-6-chloronicotinamide (17)

CDI (54.0 g, 333 mmol) was added to a solution of **16** (35.0 g, 133 mmol) in DMF (280 mL), and the mixture was stirred at room temperature for 1 h. After cooling in an ice bath, 28% NH_4OH aqueous solution (45 mL, 666 mmol) was added, and the mixture was stirred at room temperature for 0.5 h. H_2O was added to the mixture, and the precipitate was filtrated and washed with H_2O to give the title compound (29.7 g, 85%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.48 (2H, d, J = 6.0 Hz), 6.64 (1H, s), 7.25-7.40 (5H, m), 7.53 (1H, brs), 8.12 (1H, brs), 8.42 (1H, s), 9.14-9.20 (1H, m). MS (ESI) *m/z*: 284 $(M + Na)^+$.

5.1.29. 6-Anilino-4-(benzylamino)nicotinamide (18)

In the vessel of a microwave reactor, **17** (126 mg, 0.483 mmol), aniline (102 mg, 1.10 mmol), $Pd_2(dba)_3$ (44.2 mg, 0.048 mmol), 2-dicyclohexylphosphino-2',4',6'-triisopropyl-1,1'-

biphenyl (46.2 mg, 0.097 mmol), and *t*-BuONa (111 mg, 1.15 mmol) were suspended in *t*-BuOH/dioxane/NMP (1.5 mL/0.4 mL/0.15 mL). The vessel was then sealed, and the mixture was reacted at 140 °C for 45 min under microwave irradiation. After cooling to room temperature, the mixture was filtrated through a pad of Celite®. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 90/10) to give the title compound (55 mg, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.35 (2H, d, J = 6.0 Hz), 5.58 (1H, br), 5.92 (1H, s), 6.65 (1H, br), 6.96-7.07 (3H, m), 7.15-7.39 (8H, m), 8.21 (1H, s), 8.90-8.96 (1H, m). MS (ESI) *m/z*: 319 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{19}H_{18}N_4O$: 318.1481. Found: 319.1552.

5.1.30. 4-(Benzylamino)-6-[methyl(phenyl)amino]nicotinamide (19)

N-methylaniline (62 μL, 0.573 mmol) and tetraethylammonium chloride (6.3 mg, 0.038 mmol) were added to a solution of **17** (100 mg, 0.382 mmol) in NMP (0.5 mL), and the mixture was stirred at 180 °C for 3 h. After cooling to room temperature, H_2O and EtOAc were added to the mixture. The organic layer was separated, washed with H_2O, and dried over $MgSO_4$. The extract was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (*n*-hexane/EtOAc) to give the title compound (20 mg, 16%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.32 (3H, s), 4.14 (2H, d, J = 6.0 Hz), 5.50 (1H, s), 7.02 (1H, br), 7.07-7.12 (4H, m), 7.18-7.35 (6H, m), 7.72 (1H, br), 8.35 (1H, s), 8.95-9.00 (1H, m). MS (ESI) *m/z*: 333 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{20}H_{20}N_4O$: 332.1637. Found: 333.1716.

5.1.31. 4-(Benzylamino)-6-[(3,4-dimethoxybenzyl)amino]nicotinamide (20)

In the vessel of a microwave reactor, 3,4-dimethoxybenzylamine (767 mg, 4.59 mmol) and DIPEA (0.80 mL, 4.59 mmol) were added to a solution of **17** (200 mg, 0.764 mmol) in NMP (1 mL). The vessel was then sealed, and the mixture was reacted at 180 °C for 4 h under microwave irradiation. After cooling to room temperature, the mixture was purified by silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 70/30) to give the title compound (190 mg, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.69 (3H, s), 3.71 (3H, s), 4.26-4.32 (4H, m), 5.53 (1H, s), 6.73-7.00 (5H, m), 7.22-7.35 (5H, m), 7.56 (1H, br), 8.24 (1H, s), 8.87-8.92 (1H, m). MS (ESI) *m/z*: 393 $(M + H)^+$.

5.1.32. 6-Amino-4-(benzylamino)nicotinamide (21)

In the vessel of a microwave reactor, **20** (100 mg, 0.255 mmol) was dissolved with TFA (1 mL). The vessel was then sealed, and the mixture was reacted at 100 °C for 1 h under microwave irradiation. The mixture was neutralized with saturated $NaHCO_3$ aqueous solution, and EtOAc and THF were added. The organic layer was separated, washed with brine, and dried over $MgSO_4$. The extract was concentrated under reduced pressure to give the title compound (20 mg, 32%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.45 (2H, d, J = 6.0 Hz), 5.72 (1H, s), 7.22-7.40 (7H, m), 7.57 (1H, br), 8.08 (1H, br), 8.15 (1H, s), 9.50-9.55 (1H, m). MS (ESI) *m/z*: 243 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{13}H_{14}N_4O$: 242.1168. Found: 243.1244.

5.1.33. Ethyl 4-{[(3*S*,4*R*)-1-(*tert*-butoxycarbonyl)-3-fluoropiperidin-4-yl]amino}-6-chloronicotinate (23)

The title compound was prepared from **5** and *tert*-butyl (3*S*,4*R*)-4-amino-3-fluoropiperidine-1-carboxylate (**22**) in accordance with the procedure for preparing **6** in 71%

yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.31 (3H, t, J = 7.2 Hz), 1.40 (9H, s), 1.47-1.59 (1H, m), 1.77-1.84 (1H, m), 2.81-3.17 (2H, m), 3.94-4.18 (2H, m), 4.20-4.34 (3H, m), 4.74-4.90 (1H, m), 7.10 (1H, s), 8.30 (1H, d, J = 8.8 Hz), 8.57 (1H, s). MS (ESI) *m/z*: 424 $(M + Na)^+$.

5.1.34. 4-{[(3*S*,4*R*)-1-(*tert*-Butoxycarbonyl)-3-fluoropiperidin-4-yl]amino}-6-chloronicotinic acid (24)

The title compound was prepared from **23** in accordance with the procedure for preparing **7** in 84% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.40 (9H, s), 1.45-1.58 (1H, m), 1.76-1.84 (1H, m), 2.82-3.38 (2H, m), 3.93-4.35 (3H, m), 4.73-4.90 (1H, m), 7.05 (1H, s), 8.50-8.55 (2H, m), 13.47 (1H, brs). MS (ESI) *m/z*: 396 $(M + Na)^+$.

5.1.35. *tert*-Butyl (3*S*,4*R*)-4-[(5-carbamoyl-2-chloropyridin-4-yl)amino]-3-fluoropiperidine-1-carboxylate (25)

The title compound was prepared from **24** in accordance with the procedure for preparing **9** in 80% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.40 (9H, s), 1.43-1.56 (1H, m), 1.73-1.80 (1H, m), 2.82-3.23 (2H, m), 3.90-4.06 (2H, m), 4.14-4.32 (1H, m), 4.70-4.87 (1H, m), 6.94 (1H, s), 7.51 (1H, br), 8.10 (1H, br), 8.44 (1H, s), 9.08 (1H, d, J = 8.8 Hz). MS (ESI) *m/z*: 395 $(M + Na)^+$.

5.1.36. *tert*-Butyl (3*S*,4*R*)-4-({5-carbamoyl-2-[(2-methylpyrimidin-4-yl)amino]pyridin-4-yl}amino)-3-fluoropiperidine-1-carboxylate (26)

The title compound was prepared from **25** in accordance with the procedure for preparing **9** in 46% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.41 (9H, s), 1.48-1.61 (1H, m), 1.91-1.98 (1H, m), 2.81-3.25 (2H, m), 3.32 (3H, s), 3.64-3.79 (1H, m), 3.98-4.13 (1H, m), 4.19-4.38 (1H, m),

4.79-4.98 (1H, m), 7.19 (1H, br), 7.33 (1H, d, *J* = 6.0 Hz), 7.41 (1H, brs), 7.89 (1H, br), 8.29 (1H, d, *J* = 6.0 Hz), 8.46 (1H, s), 8.99 (1H, d, *J* = 7.6 Hz), 9.93 (1H, brs). MS (ESI) *m/z*: 446 $(M + H)^+$.

5.1.37. 4-{[(3*S*,4*R*)-1-(5-Cyanopyridin-2-yl)-3-fluoropiperidin-4-yl]amino}-6-[(2-methylpyrimidin-4-yl)amino]nicotinamide (27)

The title compound was prepared from **26** in accordance with the procedure for preparing **14a** in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.58-1.69 (1H, m), 2.05-2.12 (1H, m), 3.12-3.20 (1H, m), 3.32 (3H, s), 3.40-3.51 (1H, m), 3.81-3.95 (1H, m), 4.58-4.64 (1H, m), 4.85-4.94 (1H, m), 4.97-5.12 (1H, m), 7.06 (1H, d, *J* = 9.0 Hz), 7.19 (1H, br), 7.34 (1H, d, *J* = 5.9 Hz), 7.47 (1H, s), 7.87 (1H, dd, *J* = 2.3, 9.0 Hz), 7.90 (1H, br), 8.30 (1H, d, *J* = 5.9 Hz), 8.47 (1H, s), 8.49 (1H, d, *J* = 2.3 Hz), 9.01 (1H, d, *J* = 7.9 Hz), 9.95 (1H, brs). MS (ESI) *m/z*: 448 $(M + H)^+$. HRMS $(M + H)^+$ calcd for $C_{22}H_{22}FN_9O$: 447.1931. Found: 448.2009.

5.1.38. 4-{[1-(4-Cyanophenyl)piperidin-4-yl]amino}-6-[(2-methylpyrimidin-4-yl)amino]nicotinamide (28)

HCl in dioxane (4M; 110 mL) was added to a solution of **13g** (3.19 g, 7.46 mmol) in dioxane (14.7 mL), and the mixture was stirred at room temperature overnight. IPE was added to the mixture, and the precipitate was filtrated to give 6-[(2-methylpyrimidin-4-yl)amino]-4-(piperidin-4-ylamino)nicotinamide trihydrochloride (3.03 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.72-1.84 (2H, m), 2.13-2.23 (2H, m), 2.67 (3H, s), 2.99-3.11 (2H, m), 3.27-3.36 (2H, m), 3.69-3.81 (1H, m), 7.19-7.32 (1H, m), 7.68-7.81 (1H, m), 8.31-8.45 (1H, m), 8.58 (1H, d, *J* = 6.8 Hz), 8.64 (1H, s), 9.14-9.52 (3H, m), 12.44 (1H, br). MS (ESI) *m/z*: 328 $(M + H)^+$.

In the vessel of a microwave reactor, 4-fluorobenzonitrile (1.66 g, 13.7 mmol) and K_2CO_3 (3.80 g, 27.5 mmol) were added to a solution of 6-[(2-methylpyrimidin-4-yl)amino]-4-(piperidin-4-ylamino)nicotinamide trihydrochloride (3.0 g, 6.87 mmol) in DMSO (60 mL). The vessel was then sealed, and the mixture was reacted at 140 °C for 1 h under microwave irradiation. After cooling to room temperature, the mixture was poured into H_2O, and the precipitate was filtrated and washed with H_2O and EtOAc. The precipitate was purified by silica gel column chromatography ($CHCl_3$/MeOH = 100/0 to 80/20). The residue was dissolved in DMF (8 mL), and H_2O (50 mL) was added. The precipitate was filtrated to give the title compound (2.9 g, 57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.44-1.56 (2H, m), 2.08-2.16 (2H, m), 2.54 (3H, s), 3.10-3.20 (2H, m), 3.55-3.65 (1H, m), 3.85-3.93 (2H, m), 7.07 (2H, d, J = 9.1 Hz), 7.16 (1H, br), 7.34 (1H, d, J = 5.8 Hz), 7.46 (1H, s), 7.58 (2H, d, J = 9.0 Hz), 7.86 (1H, br), 8.29 (1H, d, J = 5.9 Hz), 8.45 (1H, s), 8.80 (1H, d, J = 6.8 Hz), 9.94 (1H, s). MS (ESI) m/z: 429 $(M + H)^+$. Anal. calcd for $C_{23}H_{24}N_8O \cdot 1.4H_2O$: C, 60.89; H, 5.95; N, 24.70. Found: C, 60.94; H, 6.07; N, 24.44.

5.2. Computational analysis

5.2.1 Docking calculation

Docking calculations were performed for the crystal structure of compound **1** bound to JAK3 (PDB code: 3LXK[29]), using the Protein Preparation Wizard in Maestro,[30] with impref applying the appropriate side-chain protonation states, refinement, and structure minimization. Docking grids were generated and defined based on the centroid of compound **1** in the ATP binding site with incorporation of hydrogen bond constraints to the hinge region and hydrophobic region constraints. Compound **28** and **4** were prepared using LigPrep[31] and Confgen[32], and the energy-minimized conformation was used to input molecules into docking

calculations. Ligand receptor docking was conducted using the XP mode in Glide[33]. The top-scoring pose, as assessed by GlideScore, was employed for discussions.

5.3. Biology

5.3.1. Human JAK assay

Human JAK1, JAK2, and JAK3 kinase-domains were purchased from Carna Biosciences, Inc. (Kobe, Japan), and the assay was conducted on streptavidin-coated 96-well plates. The final 50-μL reaction mixture contained 15 mM Tris-HCl (pH 7.5), 0.01% Tween 20, 2 mM DTT, 10 mM $MgCl_2$, 250 nM Biotin-Lyn-Substrate-2 (Biotin-XEQED EPEGD YFEWL EPE, X = ε-Acp; Peptide Institute, Inc., Osaka, Japan) and ATP. Final concentrations of ATP were 200 μM (JAK 1), 10 μM (JAK 2), and 8 μM (JAK 3). Test compounds were dissolved in DMSO. The reaction was initiated by adding the kinase domain (JAK1: 60 ng/mL, JAK2: 20 ng/mL, JAK3: 16 ng/mL), followed by incubation at room temperature for 1 h. Kinase activity was measured as the rate of phosphorylation of Biotin-Lyn-Substrate-2 using HRP-conjugated anti-phosphotyrosine antibody (HRP-PY-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using a phosphotyrosine-specific ELISA. Duplicate experiments were conducted for test compounds, and the IC_{50} value of each experiment was calculated using linear regression analysis.

5.3.2. Rat cardiac transplantation model

ACI rats (Japan SLC, Inc., Japan) and Lewis rats (Charles River Japan, Inc., Japan) were used as donors and recipients, respectively. Compound **28** was orally administered once daily for 14 consecutive days from the day of operation. In the combination study, tacrolimus was

administered intramuscularly once daily. Cardiac allograft survival was assessed by daily palpation for 28 days.

5.3.3. hERG inhibition (Rb efflux assay)

CHO (CHO-K1) cells stably expressing the hERG potassium channel were constructed and maintained in alpha-minimum essential medium (MEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% Geneticin® in a humidified atmosphere at 37 °C with 5% CO_2. On the day before experiments, stable clones were seeded at 6×10^4 cells/well in 96-well plate and cultured for 24 h. The culture medium was exchanged to the Rb loading medium and incubated for 1.5 h. Medium was discarded, the plate was washed with pH 7.3 buffer, and high K^+ buffer (pH = 7.3) containing test substances were added to initiate the Rb efflux through the potassium channel. After 10 min incubation, the supernatant was transferred to a new plate (Plate [A]). The plate containing the cells was lysed with 1% Triton-X 100 in the pH 7.3 buffer (Plate [B]). Rb in Plates (A) and (B) was detected using an Ion Channel Reader 8000 (Aurora Biomed Inc.), and the Rb efflux rate (RE) and the efflux inhibition rate were calculated using the following equations:

Rb efflux rate (RE) (%) = remained Rb (B)/total Rb (A + B) × 100

Efflux inhibition (%) = RE (test compound) – RE (vehicle) / RE (positive control) – RE (vehicle) × 100

The IC_{50} value of the efflux inhibition was calculated via nonlinear regression analysis.

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Conflicts of Interest

All authors were employees of Astellas Pharma Inc. when this study was conducted and have no further conflicts of interest to declare.

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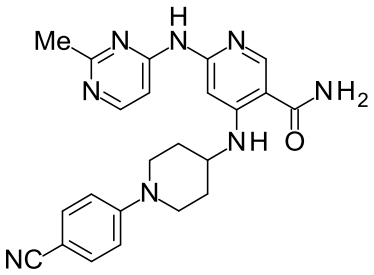
Graphical Abstract



28

JAK3, IC_{50} = 0.80 nM

JAK1, IC_{50} = 4.2 nM

JAK2, IC_{50} = 3.5 nM

hERG, IC_{50} >100 μM