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Article

Discovery of 5-Chloro-1-(5-chloro-2-(methylsulfonyl)benzyl)-2-imino-1,2-dihydropyridine-3-carboxamide (TAK-259), as a Novel, Selective, and Orally Active #1D Adrenoceptor Antagonist with Anti-urinary Frequency Effects: Reducing Human Ether-a-go-go-Related Gene (hERG) Liabilities

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Discovery of 5-Chloro-1-(5-chloro-2-(methylsulfonyl)benzyl)-2-imino-1,2-dihydropyridine-3-carboxamide (TAK-259), as a Novel, Selective, and Orally Active α_{1D} Adrenoceptor Antagonist with Anti−urinary Frequency Effects: Reducing Human Ether−a−go−go−Related Gene (hERG) Liabilities

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ABSTRACT

A novel structural class of iminopyridine derivative **1** was identified as a potent and selective human α_{1D} adrenoceptor (α_{1D} adrenergic receptor; α_{1D}-AR) antagonist against α_{1A}- and α_{1B}-AR through screening of an in-house compound library. From initial structure-activity relationship studies, we found lead compound **9m** with hERG K^+ channel liability. To develop analogues with reduced hERG K^+ channel inhibition, a combination of site-directed mutagenesis and docking studies was employed. Further optimization led to the discovery of (*R*)-**9s** and **9u**, which showed antagonistic activity by a bladder strip test in rats with bladder outlet obstruction, as well as ameliorated cystitis-induced urinary frequency in rats. Ultimately, **9u** was selected as a clinical candidate. This is the first study to show the utility of iminopyridine derivatives as selective α_{1D}-AR antagonists and evaluate their effects in vivo.

KEYWORDS iminopyridine, α_{1D} adrenergic receptor antagonist, hERG K^+ channel inhibition

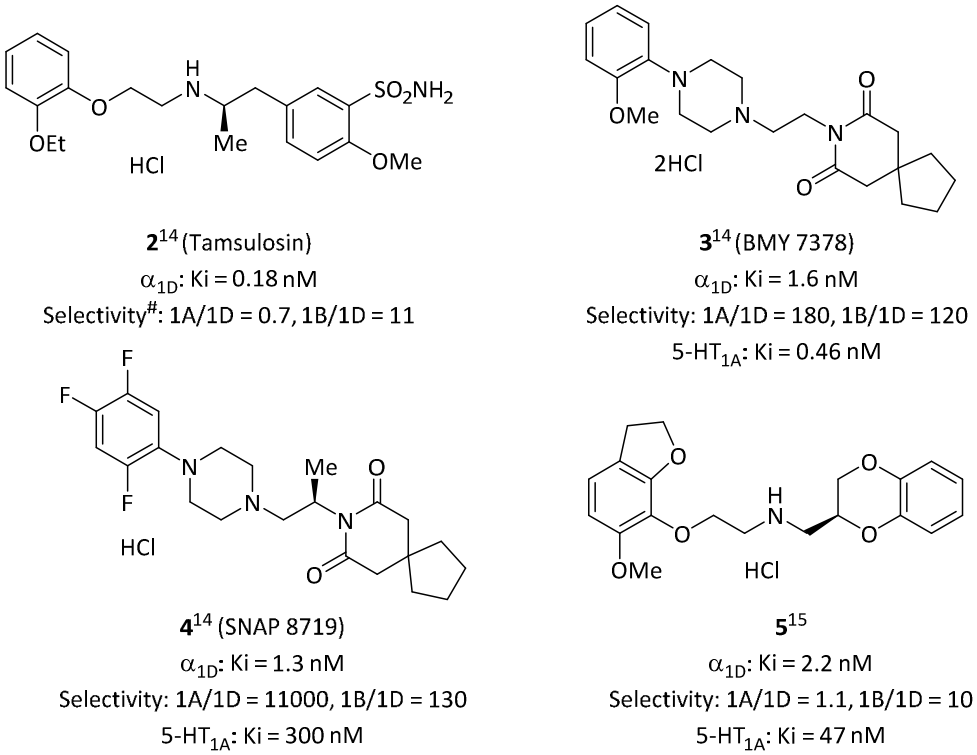
Introduction

Overactive bladder (OAB) is defined as urinary urgency, typically with urinary frequency and with or without urinary incontinence.[1] More than 10% of the population over 40 years old in Japan, the U.S., and Europe suffers from OAB, with worldwide numbers steadily increasing.[2] Antimuscarinic agents that inhibit detrusor muscle contraction are widely used to treat OAB.[3] However, these agents have several issues, including mechanism based-adverse events, such as dry mouth, constipation, stomach discomfort, and voiding difficulty in patients with benign prostatic hyperplasia.[4] Alternative therapeutic approaches are sought to both avoid side effects and provide more potent therapeutic options, leading to the development of new drug classes with different mechanisms.[5]

The α_1 adrenergic receptors (α_1 adrenoceptors, α_1-ARs) are widely distributed in many organs, including the cardiovascular system and lower urinary tract, and are involved in sympathetic responses. Moreover, α_1-ARs have been an active target of drug discovery research for more than three decades because of their significant relationship with diseases, including hypertension, cardiomegaly, and dysuria. α_1-ARs are classified into three subtypes, α_{1A}, α_{1B}, and α_{1D}-ARs, which have different tissue distribution profiles.[6] Among these, α_{1D}-AR is expressed at higher levels in the bladder and parasympathetic nucleus of the sacral cord, as compared to other subtypes.[7,8] Furthermore, it is known to be involved in urinary tract function. For example, α_{1D}-AR knockout mice show significantly increased bladder capacity and single urine volume per void than wild type mice.[9] Additionally, an α_{1A}- and α_{1D}-AR antagonist, tamsulosin, improves OAB symptoms in patients with benign prostatic hyperplasia.[10-12] Naftopidil monotherapy, which has greater affinity for α_{1D}-AR than for α_{1A}-AR, is more effective for storage symptoms, whereas tamsulosin monotherapy is more effective for voiding symptoms.[13] Thus, selective α_{1D}-AR antagonists could represent a novel therapeutic treatment for OAB.

The phenylpiperazine derivatives **3** (BMY 7378) and **4** (SNAP 8719), as well as phenoxyethylamine derivative **5**, are reported to be selective α_{1D}-AR antagonists (Figure 1).[14, 15] Although **3** is a reference selective α_{1D}-AR ligand, it has high binding affinity for the serotonin type

1A (5-HT_{1A}) receptor. Furthermore, **4** showed better selectivity for α_{1D}-AR, with lower binding affinity for the 5-HT_{1A} receptor. Nevertheless, there are no selective α_{1D}-AR antagonists in clinical trials. Hence, the identification of selective α_{1D}-AR antagonists would provide a useful treatment strategy and clarify the functional roles of α_{1D}-AR in disease pathogenesis. Accordingly, we sought to identify novel and selective α_{1D}-AR antagonists.



2[14] (Tamsulosin)
α_{1D}: Ki = 0.18 nM
Selectivity[#]: 1A/1D = 0.7, 1B/1D = 11

3[14] (BMY 7378)
α_{1D}: Ki = 1.6 nM
Selectivity: 1A/1D = 180, 1B/1D = 120
5-HT_{1A}: Ki = 0.46 nM

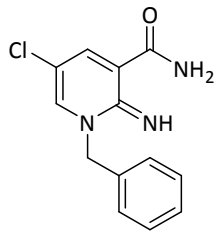
4[14] (SNAP 8719)
α_{1D}: Ki = 1.3 nM
Selectivity: 1A/1D = 11000, 1B/1D = 130
5-HT_{1A}: Ki = 300 nM

5[15]
α_{1D}: Ki = 2.2 nM
Selectivity: 1A/1D = 1.1, 1B/1D = 10
5-HT_{1A}: Ki = 47 nM

Figure 1. Structures and biological activity of reported α_1-ARs antagonists (literature data).[14, 15]

In order to develop novel and selective α_{1D}-AR antagonists, we conducted a high-throughput screen of our chemical library. This led to the identification of the iminopyridine derivative **1**, a known compound for which biological activity has not been described previously in the literature (Figure 2).[16] Compound **1** has a different structure from the known α_1-AR antagonists shown in Figure 1. Additionally, **1** showed low binding affinity for 5-HT_{1A} receptors, while the reported selective α_{1D}-AR antagonist, **3**, showed high affinity. Hence, iminopyridine derivative **1** was considered to be an attractive starting point for lead optimization as a selective α_{1D}-AR antagonist.

In this report, we describe the discovery of a highly potent, selective, and orally active α_{1D}-AR antagonist, **9u** (TAK-259), as a clinical development candidate with anti-urinary frequency effects.



1

α_{1D}: Ki = 30 nM

α_{1A}: Ki = >2700 nM, α_{1B}: Ki = >1200 nM

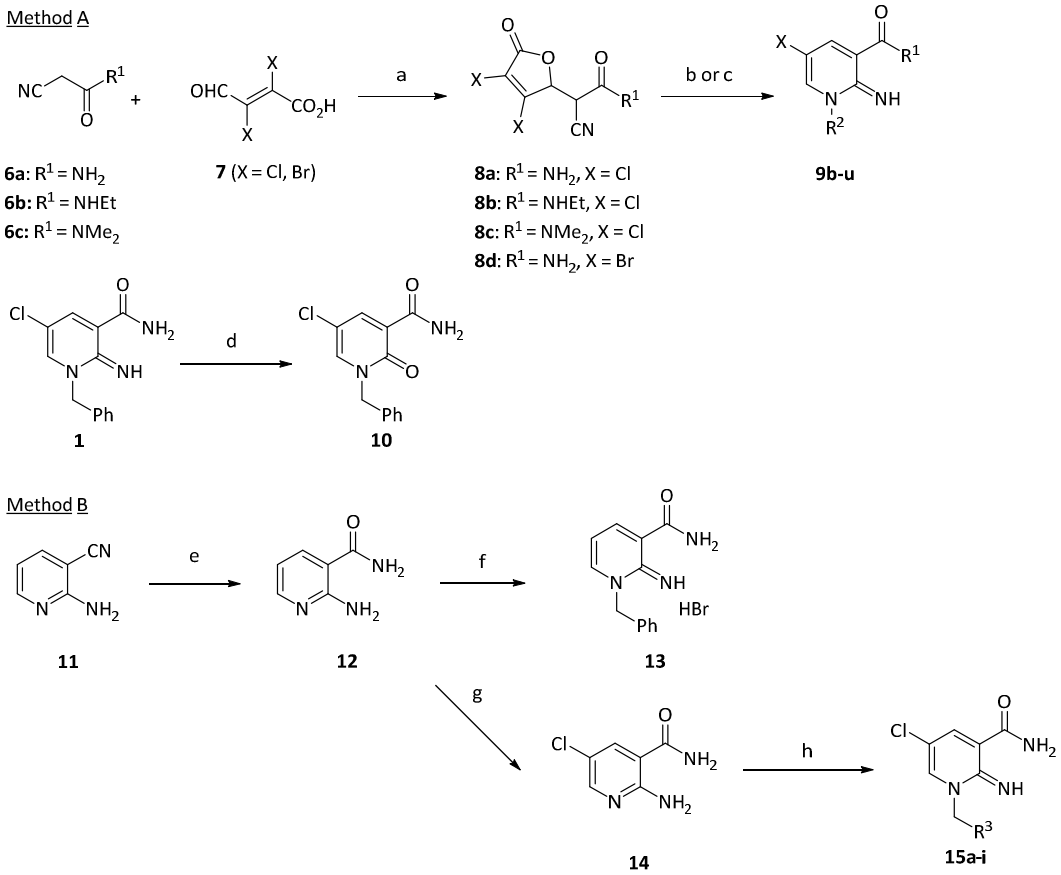
5-HT_{1A}: IC_{50} = >1000 nM

Figure 2. Structure of **1**. Ki and IC_{50} values shown are in-house data.

Chemistry

The synthesis of various iminopyridine derivatives was conducted by two main routes, Method A and B, as shown in Scheme 1. Cyclization is a key reaction in Method A, using the appropriate amine and dihydrofuran **8**.[16] Acetamide **6a-c** was treated with mucochloric acid or mucobromic acid **7** in the presence of aqueous sodium hydroxide (NaOH) to afford dihydrofuran-2-one **8a-d**, followed by amine treatment to obtain iminopyridine derivatives **9b-u**. Hydrolysis of iminopyridine **1**[16] with 6 M hydrochloric acid (HCl) afforded pyridone **10**. Alkylation is a key reaction in Method B, using 2-aminopyridine **12** or **14** and appropriate benzylbromide. Iminopyridine **13** was prepared by acidic hydrolysis of cyanopyridine **11** to afford pyridine carboxamide **12**, followed by alkylation with benzylbromide. The other method used to synthesize iminopyridine derivatives **15a-i** is oxidative chlorination of **12** with 30% hydrogen peroxide in the presence of concentrated HCl, which afforded aminopyridine **14**. Iminopyridine derivatives **15a-i** were then prepared from **14** by alkylation with the corresponding benzyl bromide.

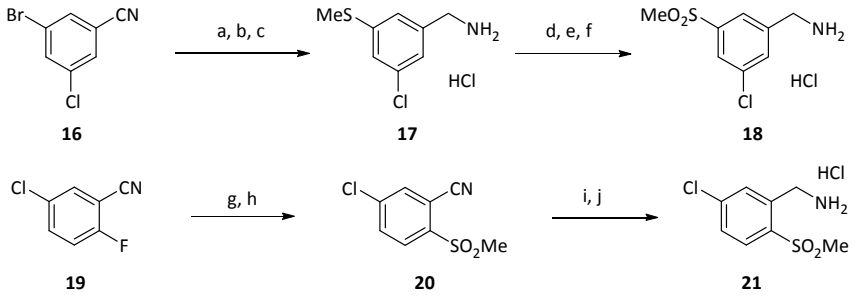
Scheme 1. Synthesis of iminopyridine derivatives[a]



[a]Reagents and conditions: (a) aqueous NaOH, MeOH; (b) amine, Et_3N, EtOH, or THF, rt, then DMSO, 80 °C; (c) amine, K_2CO_3, EtOH, 80 °C; (d) 6 M HCl, reflux; (e) conc. H_2SO_4, 100 °C; (f) benzylbromide, DMF, 80 °C; (g) 30% H_2O_2, conc. HCl, 0 to 60 °C; (h) R^3CH_2Br, DMF, 100 °C.

Benzylamines **18** and **21,** which contain methylsulfonyl groups for the synthesis of **9u** and **9v** in Method A, were prepared as depicted in Scheme 2. Substitution of benzonitrile derivative **16** with sodium thiomethoxide, followed by reduction with lithium aluminum hydride and treatment with 4 M HCl in ethyl acetate (AcOEt) gave methylsulfanyl derivative **17**. Benzylamine **18** was obtained by oxidation of the sulfanyl group with *m*-chloroperoxybenzoic acid (mCPBA). 5-Chloro-2-fluorobenzonitrile **19** was converted to methylsulfonyl derivative **20** through the introduction of a methylsulfanyl moiety, followed by oxidation with Oxone. Reduction of **20** with Raney-Co, followed by salt formation with 2 M HCl in methanol (MeOH), provided benzylamine hydrochloride **21**.

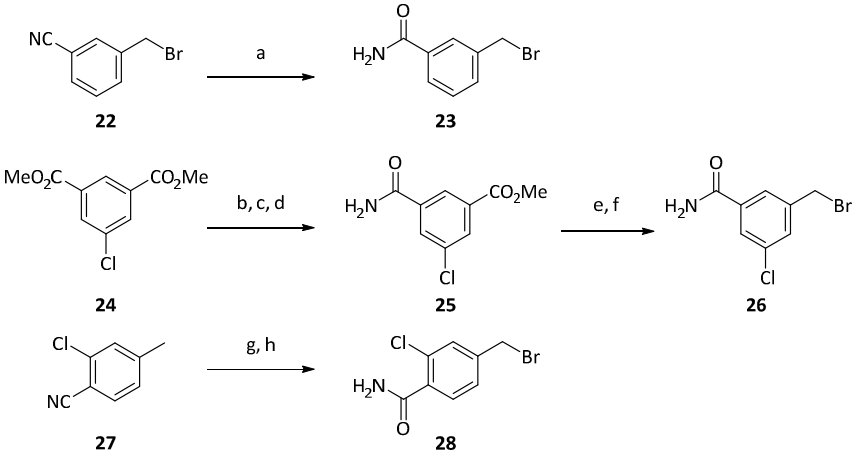
Scheme 2. Synthesis of intermediates **18** and **21**[a]



[a]Reagents and conditions: (a) NaSMe, i-Pr_2NEt, $Pd_2(dba)_3$, Xantphos, toluene, 90 °C; (b) $LiAlH_4$, THF, 0 °C to rt; (c) 4 M HCl in AcOEt, MeOH, rt; (d) Boc_2O, Et_3N, THF, rt; (e) mCPBA, AcOEt, rt; (f) 4 M HCl in AcOEt, MeOH, 60 °C; (g) NaSMe, DMSO, rt; (h) Oxone, CH_3CN, H_2O, rt; (i) Raney-Co, NH_4OH, H_2, EtOH, MeOH, rt; (j) 2 M HCl in MeOH, EtOH, rt.

Benzylbromides **23**, **26**, and **28**, which contained a carboxamide moiety for the synthesis of **15b**, **15d**, and **15e** in Method B, were synthesized as follows (Scheme 3). Hydrolysis of the cyano group with concentrated sulfuric acid for benzonitrile **22** afforded benzylbromide **23**. Methylbenzoate **25** was obtained by selective mono-hydrolysis of dimethyl ester group for dimethyl isophtalate **24** and amidation of the carboxyl group. Benzylbromide **26** was prepared by reduction of the remaining methyl ester to an alcohol and bromination. Hydrolysis of the cyano group with concentrated sulfuric acid for benzonitrile **27**, followed by bromination of the methyl group with *N*-bromosuccinimide, afforded benzylbromide **28**.

Scheme 3. Synthesis of intermediate **23**, **26** and **28**[a]



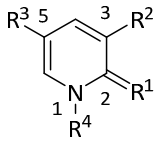
[a]Reagents and conditions: (a) conc. H_2SO_4, 50 °C; (b) 1 M NaOH, MeOH, rt; (c) $(COCl)_2$, DMF, THF, 0 °C; (d) 8 M NH_3 in MeOH, EtOH, 0 °C; (e) $LiAlH_4$, THF, EtOH, rt to 60 °C; (f) Br_2, PPh_3, CH_3CN, rt; (g) conc.H_2SO_4, 80 °C; (h) NBS, AIBN, AcOt-Bu, 80 °C.

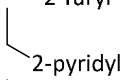
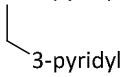
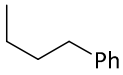
Results and Discussion

All compounds were evaluated for their affinity for cloned human α_{1D}-AR in binding studies, and the results are expressed as Ki values. The selectivity for α_{1D}-AR over α_{1A}- and α_{1B}-AR was also evaluated. Inhibition of the human ether-a-go-go related gene (hERG) channel was evaluated using an *in vitro* Ion Works Quattro study.[17]

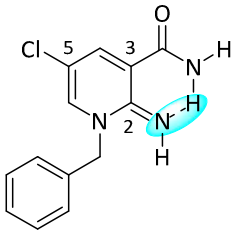
We conducted structure-activity relationship (SAR) studies for various substituents on the dihydropyridine ring (Table 1). *N*-Ethyl carboxamide derivative **9b**, *N*,*N*-dimethyl carboxamide derivative **9c**, and pyridine-2-one derivative **10** had low binding affinity for cloned human α_{1D}-AR. The X-ray crystal structure of **1**, shown in Figure 3, revealed that hydrogen bond formation between the hydrogen atom of the carboxamide moiety at 3-position on the dihydropyridine ring and the nitrogen atom on the imino moiety at 2-position was observed. Immobilization between the 2-position and 3-position due to the hydrogen bonding was found to be important for compound potency, based on the SAR analysis. As for the 5-position on the iminopyridine ring, **1**, which contains chlorine, was found to be more potent than **9d**, containing bromine, and **13**, which contains hydrogen. Next, the effects of modifications as the 1-position on the iminopyridine ring were investigated. **9e**, which has a *tert*-butylmethyl moiety at this position, showed decreased potency, whereas **9g** (2-furylmethyl), **9h** (2-pyridylmethyl), and **9i** (3-pyridylmethyl) exhibited a 5 to 14-fold decrease in the potency versus **1**. Regarding the length of the methylene linker, compound **9j** (phenethyl) and **9k** (3-phenylpropyl) showed inhibitory activity equipotent to **1**, although introduction of a phenyl group in **9f,** which did not contain a methylene linker, resulted in decreased potency. These results indicate that a phenyl ring with a methylene-based linker (length: 1 to 3) was indispensable for potency.

Table 1. Affinity of dihydropyridine derivatives for human α_{1D}-AR



Compound	R^1	R^2	R^3	R^4	Ki (nM)[a] α_{1D}
1	NH	$CONH_2$	Cl		30 [26-35]
9b	NH	CONHEt	Cl		>940
9c	NH	$CONMe_2$	Cl		>940
10	O	$CONH_2$	Cl		>940
9d	NH	$CONH_2$	Br		160 [130–190]
13	NH	$CONH_2$	H		880 [630–1200]
9e	NH	$CONH_2$	Cl		820 [670–1040]
9f	NH	$CONH_2$	Cl		>940
9g	NH	$CONH_2$	Cl		160 [120–220]
9h	NH	$CONH_2$	Cl		420 [360–500]
9i	NH	$CONH_2$	Cl		250 [210–320]
9j	NH	$CONH_2$	Cl		16 [14–19]
9k	NH	$CONH_2$	Cl		54 [43-67]

[a]Receptor binding affinity of human α_{1D}-AR. Ki value is presented as the mean of triplicate experiments with 95% confidence intervals in parentheses.



1

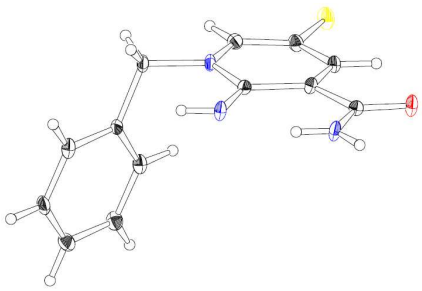
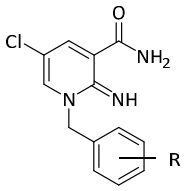


Figure 3. Chemical structure and single crystal structure of compound **1**

Based on the SAR study summarized in Table 1, compounds **9j** and **9k** were found to possess potency similar to **1**. Among these, **1** was selected for further optimization, because it demonstrated the highest ligand efficiency score (**1**: 0.570, **9j**: 0.559, **9k**: 0.495).[18] The effects of introducing substituents into the benzyl group at the iminopyridine 1-position on α_{1D}-AR binding affinity are displayed in Table 2. Substitution with a chlorine atom on the phenyl ring, 3-chloro derivative **9m**, exhibited the highest inhibitory activity, as compared to the 2- or 4-chloro derivatives (**9l**, **9n**). Furthermore, this compound was found to be 10-fold more potent than **1**. These results indicate that the introduction of a substituent at 3-position on the phenyl ring was important to increase potency. Next, we evaluated the effect of a substituent at 3-position. Introduction of methyl (**15a**), methoxy (**9o**), carboxamide (**15b**), cyano (**15c**), or methylsulfonyl (**9p**) group resulted in equipotent binding affinity compared to **1**. These results indicated that introduction of a substituent on the phenyl ring resulted in potency retention.

Table 2. Affinity of 5-chloro-3-carboxamide-2-iminopyridine derivatives with a substituted benzyl group for human α_{1D}-AR



Compound	R	Ki (nM)[a] α_{1D}
1	H	30 [26–35]
9l	2–Cl	39 [33–45]
9m	3–Cl	2.6 [2.3–3.0]
9n	4–Cl	23 [19–26]
15a	3-Me	7.5 [6.3–9.0]
9o	3-OMe	8.7 [7.3–10]
15b	3-$CONH_2$	26 [20–34]
15c	3-CN	10 [8.8–12]
9p	3-SO_2Me	52 [44–61]

[a]Receptor binding affinity of human α_{1D}-AR. Ki value is presented as the mean of triplicate experiments with 95% confidence intervals in parentheses.

Based on the results shown in Table 2, we selected 3-chloro derivative **9m**, which exhibited the highest potency among the mono-substituted compounds, and evaluated its absorption, distribution, metabolism, excretion, toxicity (ADMET) profile (Figure 4). Compound **9m** displayed favorable properties in studies investigating cytochrome P450 3A4 (CYP3A4) inhibition, parallel artificial membrane permeability assay (PAMPA) permeability,[19] and metabolic stability in human liver microsomes. However, this compound exhibited moderate hERG inhibitory activity (47% inhibition at 10 μM). Accordingly, we focused on the identification of analogues with reduced hERG liability.

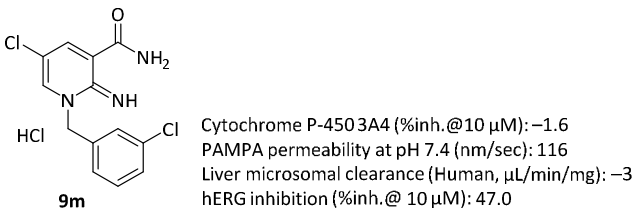
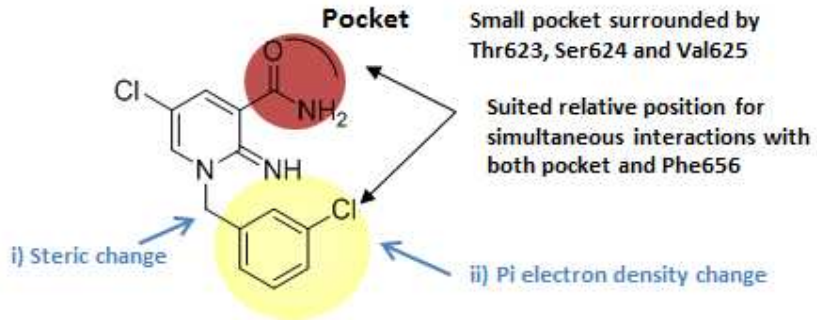


Figure 4. ADMET profile of **9m**.

Several reports indicate that hERG inhibition can be reduced by lowering compound lipophilicity.[20] An effective method to reduce the hERG K^+ channel binding affinity might be to lower lipophilicity by the introduction of hydrophilic substituents. Hence, we sought to find appropriate substituents for reducing hERG inhibition. A docking study using **9m** and the hERG K^+ channel was carried out using the results of a tandem site-directed mutagenesis approach: a tandem dimer of the hERG K^+ channel was constructed, wherein mutation was introduced into one subunit to determine the residues that bind to **9m**. We have previously reported the use of this approach.[21] The presumed **9m** binding mode to the hERG K^+ channels is shown in Figure 5. Our docking study suggested that the terminal phenyl ring at the iminopyridine ring 1-position and the carboxamide group at the 3-position adopted relative positions suitable for simultaneous interactions with both Phe656 and the adjacent small pocket. The small pocket is surrounded by three amino acid residues (Thr623, Ser624, and Val625), and is occupied by a carboxamide moiety. The initial SAR study

using substituents on the dihydropyridine derivative (Table 1) indicated that the carboxamide group at 3-position was requisite for potency. Thus, we decided to avoid this unfavorable interaction with Phe656 by further modifying 1-position of the iminopyridine ring and/or lowering the overall compound lipophilicity. Our approaches to attenuate **9m**–induced hERG inhibition are described in Figure 5: (i) steric changes through the introduction of a methyl group at the benzyl position to avoid interaction with Phe656; (ii) changes in pi electron density on the phenyl ring by introduction of a polar moiety into the phenyl ring of 3-chlorobenzyl derivative **9m**, as well as modification of the overall compound lipophilicity, as needed.

A)



B)

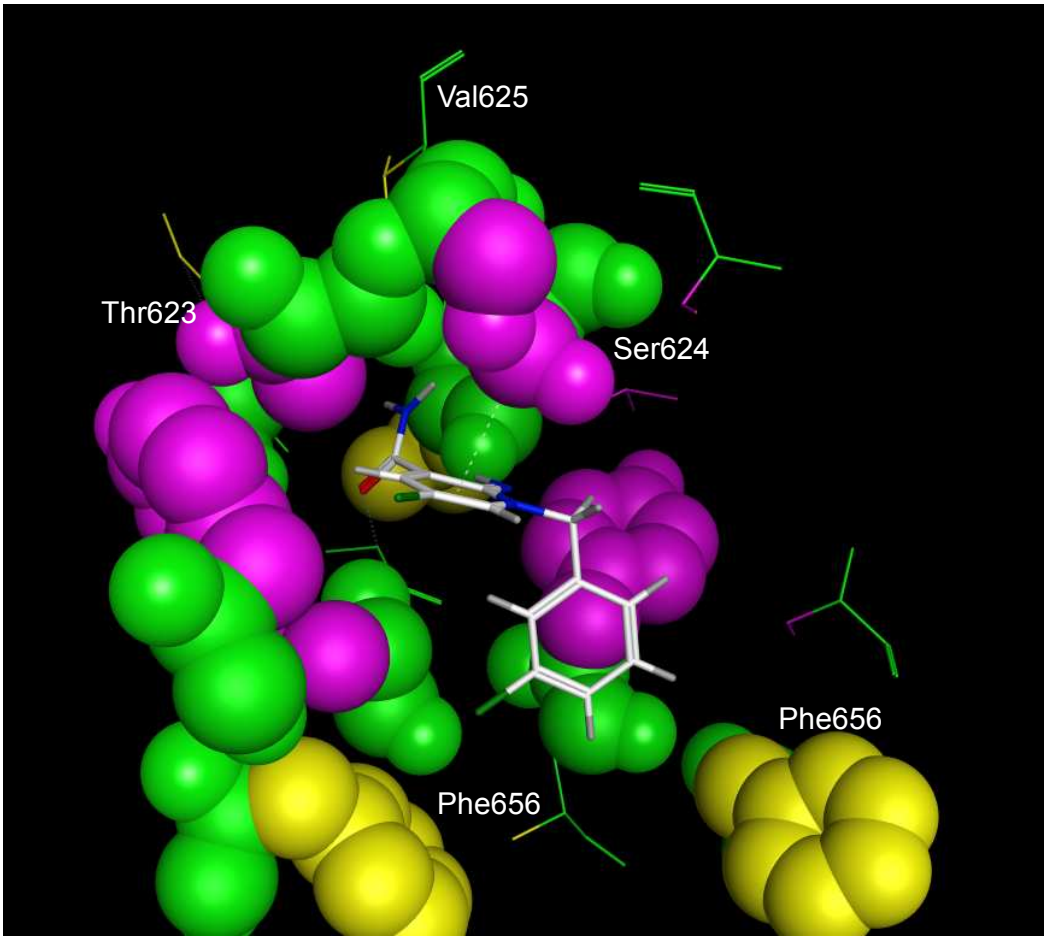
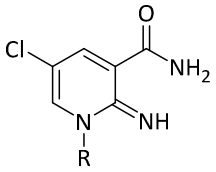


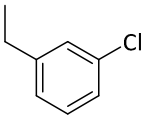
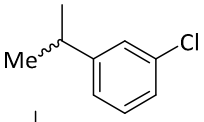
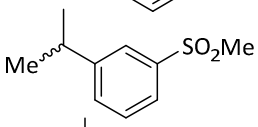
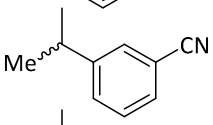
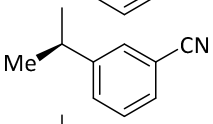
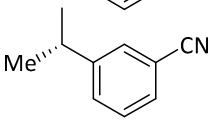
Figure 5. A) Presumed **9m** (black) binding to both hERG K^+ channel models and hypothetical modifications to avoid hERG binding (blue). B) The binding mode for **9m** shown by MOE.[22]

To attenuate hERG inhibition by **9m**, we prepared the compounds shown in Table 3. We first introduced a methyl group at the benzylic position, thereby inducing a steric change. The 3-chlorophenethyl moiety **9q** showed 4-fold higher binding affinity for α_{1D}-AR, but similar hERG inhibitory activity to **9m**. Interestingly, these results indicated that the introduction of a methyl group at the benzyl position was effective at enhancing α_{1D}-AR affinity, without increasing hERG inhibition, despite the higher lipophilicity of **9q**. Thus, we postulated that the introduction of a polar substituent in place of chlorine on the phenyl ring of **9q** would be result in retained potency and altered hERG inhibition. Indeed, 3-methylsulfonylphenethyl derivative **9r** and 3-cyanophenethyl derivative **9s** (clogP = -0.25 for **9r**, clogP = 0.82 for **9s**)[23] both exhibited a lower clogP values than **9q** (clog P = 2.10), and tended to have low hERG inhibitory activity. Additionally, we evaluated the difference between the two enantiomers 3-cyanophenethyl derivative **9s**. The eutomer, (*R*)-**9s**,

showed retention of in vitro activity with decreased hERG inhibition. Other optically resolved chiral derivatives tended to yield similar results (data not shown). On the other hand, the calculated pKa values for these compounds did not correlate with the hERG inhibitory activity.[23]

Table 3. Affinity for human α_{1D}-AR and hERG inhibitory activity of 1-phenethyl derivatives



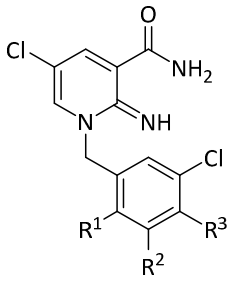
Compound	R	Ki (nM)[a] / α_{1D}	hERG inhibition[b] % inh. @10 μM	clog P[c]	pKa[d]
9m		2.6 [2.3–3.0]	47 ± 2	1.79	11.99
9q		0.66 [0.57–0.75]	48 ± 5	2.10	11.64
9r		15 [13–18]	19[e]	-0.25	11.64
9s		3.4 [2.9–3.9]	32 ± 3	0.82	11.64
(*R*)-**9s**		1.6 [1.4–1.9]	25 ± 4	0.82	11.64
(*S*)-**9s**		130 [110–160]	57 ± 23	0.82	11.64

[a]Receptor binding affinity of human α_{1D}-AR. Ki value is presented as the mean of triplicate experiments with 95% confidence intervals in parentheses. [b]Percent inhibition is represented as mean ± standard deviation. [c]cLogP value determined using Daylight software. [d]pKa value calculated by using Accelrys software. [e]Percent inhibition is represented as the average of duplicate measurements (n = 2).

A second approach to attenuate **9m**–induced hERG inhibition was the introduction of a polar moiety in the *m*-chlorophenyl ring to modulate the π-electron density (Table 4). Carboxamide (**15d**,

15e) and methylsulfonyl derivatives (**9t**, **9u** and **15f**) showed reduced hERG inhibition, while the the corresponding cyano derivatives demonstrated increased hERG inhibiton. These data indicated that the type of polar substituent selected would have an effect on the hERG inhibitory activity of these 3-chlorobenzyl derivatives. Furthermore this effect did not depend strongly on the substituted position. In addition, the benzonitrile derivatives possessed potential hERG liabilities, despite the clogP values being lower than **9m**. Regarding the binding affinity, **15d** (R^3 = carboxamide) exhibited lower potency for α_{1D}-AR and carboxamide derivative **15e** (R^2 = carboxamide) showed equipotent affinity for α_{1D}-AR compared to **9m**. Compound **15h** (R^2 = cyano) showed higher potency than **9m**, whereas **15g** (R^3 = cyano) or **15i** (R^1 = cyano) possessed lower binding affinity for α_{1D}-AR. Methylsulfonyl groups at R^2 or R^3 (**9t** and **15f**, respectively) resulted in decreased binding affinity, while compound **9u** (R^1 = methylsulfonyl) showed α_{1D}-AR potency retention.

Table 4. Affinity for α_{1D}-AR and hERG inhibitory activity of 2-iminopyridine derivatives containing a substituted 3-chlorobenzyl moiety



Compound	R^1	R^2	R^3	Ki (nM)[a] / α_{1D}	hERG inhibition[b] % inh. @10 μM	clogP[c]
9m	H	H	H	2.6 [2.3–3.0]	47 ± 2	1.79
15d	H	H	$CONH_2$	65 [52–81]	10 ± 3	-0.36
15e	H	$CONH_2$	H	3.7 [3.0–4.5]	13 ± 3	0.47
15f	H	H	SO_2Me	9.4 [8.5–11]	13 ± 4	0.28
9t	H	SO_2Me	H	4.8 [4.1–5.7]	14 ± 2	0.28
9u	SO_2Me	H	H	1.1 [0.91–1.3]	16 ± 3	0.28
15g	H	H	CN	11 [9.1–13]	74 ± 2	1.10
15h	H	CN	H	0.86 [0.76–0.94]	71 ± 12	1.23
15i	CN	H	H	8.5 [7.3–10]	59 ± 4	1.37

[a]Receptor binding affinity of human α_{1D}-AR. Ki value is presented as the mean of triplicate experiments with 95% confidence intervals in parentheses. [b]Percent inhibition is represented as mean ± standard deviation. [c]cLogP value determined using Daylight software.

Based on the in vitro study results shown in Tables 3 and 4, we selected (*R*)-**9s**, **9u**, and **15e** for further evaluation. The AR-subtype selectivity and pharmacokinetic parameters were investigated, and the results are shown in Table 5. These compounds showed higher selectivity for α_{1D}-AR over α_{1A}- and α_{1B}-ARs. Indeed, the selectivity of each compound over both α_{1A}- and α_{1B}-ARs was more than 200-fold. In terms of pharmacokinetic parameters, the 3-cyanophenethyl derivative (*R*)-**9s** and 2-methylsulfonylbenzyl derivative **9u** showed higher plasma concentrations and better oral bioavailability at a dose of 1 mg/kg than **15e**. In particular, **9u** exhibited the longest mean residence time (MRT)po value and would be promising in terms of duration of action.

Table 5. Selectivity and pharmacokinetic profiles for (*R*)-**9s**, **9u** and **15e**

comp.	Binding[a]	Selectivity		Pharmacokinetic parameters in rat[b, c]					
	Ki (nM)	1A/1D	1B/1D	Vdss[b] (mL/kg)	CL_{total}[b] (mL/h/kg)	C_{max}[c] (ng/mL)	AUC_{po}[c] (ng·h/mL)	MRT_{po}[c] (h)	*F*[c] (%)
(*R*)-**9s**	1.6 (1.4–1.9)	>1700	>750	4676	2350	142	368	3.14	86.0
9u	1.1 (0.91–1.3)	200 (190–250)	800 (690–1100)	4432	2395	60.2	358	4.53	84.5
15e	3.7 (3.0–4.5)	>730	>320	3212	2830	3.20	10.8	2.47	3.0

[a]Receptor binding affinity of human α_{1D}-AR. Ki value is presented as the mean of triplicate experiments with 95% confidence intervals in parentheses.

[b]0.1 mg/kg, iv. Solvent: DMA/1,3-butanediol = 1:1 (v/v).

[c]1 mg/kg, po (fed). Solvent: 0.5% methylcellulose suspension. All values are the average of three rats.

Given the selectivity and pharmacokinetic profiles (Table 5), **9u** and (*R*)-**9s** were chosen for in vitro and in vivo evaluation (Table 6). The effects of these compounds on phenylephrine-induced bladder contraction in isolated bladder strips taken from rats with bladder outlet obstruction (BOO) were measured, and the results were expressed as IC_{30} values. Phenylephrine-induced bladder contractions were obvious in strips from rats with BOO, but not in normal rats. Both **9u** and (*R*)-**9s** dose-dependently inhibited bladder contractions, with IC_{30} values of 12 nM and 15 nM, respectively. In rats with BOO, the non-voiding bladder contractions observed during urinary storage phase were dose-dependently decreased by both **9u** and (*R*)-**9s**, with ID_{50} values of 1.3 μg/kg, i.v. and 4.4 μg/kg, i.v., respectively. In rats with cyclophosphamide-induced cystitis, which induces significantly higher urinary frequency, the compounds increased voiding intervals, with similar minimum effective doses (10 μg/kg, p.o.). These results demonstrated that 1) both the phenylephrine-induced contractions of rat bladder strips in vitro and the non-voiding bladder contractions in vivo were mediated by stimulation of α_{1D}-ARs, and that 2) α_{1D}-AR antagonists effectively reduced urinary frequency. As phenylephrine induces similar contractions in bladder strips taken from patients with benign prostatic hyperplasia[24], and involuntary bladder contractions are observed during the urinary storage phase in some OAB patient populations,[25] α_{1D}-AR antagonists may inhibit these contractions in patients. Thus, selective α_{1D}-AR antagonists may alleviate the symptoms of OAB. However, as OAB is a disease induced by various causes, the percentage of patients who could be treated by this drug remains uncertain. Clinical studies with a selective α_{1D}-AR antagonist are needed to clarify this point.

Table 6. In vivo pharmacological data for **9u** and (*R*)-**9s**

compound	Phenylephrine-induced contractions[a] IC_{30} (nM)	Non-voiding contractions[b] ID_{50} (μg/kg, iv)	Urinary frequency[c] MED[d] (μg/kg, po)
9u	12	1.3 [0.79-2.1]	10
(*R*)-**9s**	15	4.4 [0.80-16]	10

[a]Effects on the phenylephrine-induced bladder contractions in rats with BOO (n = 6 − 8).

[b]Effects on non-voiding bladder contractions in rats with BOO (n = 5). ID_{50} values are presented as the dose required to induce a 50% reduction in non-voiding bladder contractions with 95% confidence intervals in parentheses.

[c]Cyclophosphamide induced urinary frequency in rats (n = 5 − 10).

[d]MED = minimum effective dose

Conclusion

We identified iminopyridine derivative **1** as a novel, selective α_{1D}-AR antagonist. Initial SAR studies revealed that the 2-iminopyridine-3-carboxamide structure was critical for binding affinity, and that substitution of the benzyl moiety at the iminopyridine ring 1-position was tolerated in terms of potency. In order to enhance α_{1D}-AR binding activity, we modified the benzyl group, and found that 3-chlorobenzyl derivative **9m** was more potent than **1**. However, this compound showed moderate hERG inhibitory activity. To attenuate the hERG inhibition, a docking study of compound **9m** and the hERG K^+ channel was performed to characterize the binding site and binding mode using a tandem site-directed mutagenesis approach. The docking study indicated that the carboxamide group at 3-position and phenyl group at the 1-position interacted with the hERG K^+ channel. Because initial SARs suggested that the carboxamide group was important for inhibitory activity against α_{1D}-AR, we decided to weaken the interaction with the phenyl group at 1-position. Thus, we introduced polar substituents into the phenyl ring to decrease hERG inhibitory activity. We thus identified lead compounds **15e**, **9u**, and (***R***)-**9s**. **9u** and (***R***)-**9s** showed higher selectivity for

α_{1D}-AR over α_{1A}- and α_{1B}-AR, preferable pharmacokinetic parameters, and in vitro and in vivo effects in rats, suggesting potential efficacy in patients with urinary frequency. In particular, **9u** exhibited a larger MRTpo value and more potent anti-urinary frequency efficacy than (***R***)-**9s**. We selected **9u** as a clinical candidate and our results indicated that **9u** represents a promising novel therapeutic agent for the treatment of OAB symptoms.

Experimental Section

Chemistry

Melting points were determined on a BÜCHI B-545 melting point apparatus and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker Ultra Shield-300 (300 MHz) instruments. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Bruker Avance II 600 (600 MHz) instruments. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet of doublets, br = broad. Coupling constants (*J* values) are given in hertz (Hz). Elemental analyses were carried out by Takeda Analytical Laboratories, Ltd., and were within ±0.4% of the theoretical values unless otherwise noted. Low-resolution mass spectra (MS) were determined on a Shimadzu UFLC/MS (Prominence UFLC high pressure gradient system/LCMS-2020) with an L-column 2 ODS (3.0 x 50 mm I.D., CERI, Japan), and Waters Liquid Chromatography–Mass Spectrometer System (MS), using a CAPCELL PAK UG-120 ODS (Shiseido Co., Ltd.) column (2.0 mm i.d. × 50 mm) with aqueous CH_3CN (10–95%) containing 0.05% trifluoroacetic acid (TFA), or an HP-1100 (Agilent Technologies) apparatus for monitoring at 220 nm. All MS experiments were performed using electrospray ionization (ESI) in positive ion mode. HPLC separation was carried out using a Gilson system employing the following conditions; Column: Shiseido Capcelpak C18 UG-120, S-5 μM, 20 x 50 mm or YMC CombiPrep Hydrosphere C18 HS-340-CC, S-5 μM, 20 x 50 mm; Mobile phase: A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in acetonitrile; gradient cycle: 0.00 min (A/B＝95/5), 1.10 min (A/B＝95/5), 5.00 min (A/B＝0/100), 6.40 min (A/B＝0/100),

6.50 min (A/B＝95/5); flow rate; 20 ml/min; detection UV 200 nm. After purification by preparative HPLC, the solutions were neutralized by PL-HCO_3 MP tube (200 mg cartridge, Polymer Laboratories Ltd.). Reagents and solvents were obtained from commercial sources and used without further purification. Reaction progress was determined by thin layer chromatography (TLC) analysis on Merck Kieselgel 60 F254 plates or Fuji Silysia NH plates. Chromatographic purification was carried out on silica gel columns [(Merck Kieselgel 60, 70–230 mesh or 230–400 mesh, Merck) or (Chromatorex NH-DM 1020, 100–200 mesh)] or on Purif-Pack (SI ϕ 60 μM or NH ϕ 60 μM, Fuji Silysia Chemical, Ltd.). Each compound was confirmed to be ≥95% purity by either LC/MS or elemental analysis. Yields are not optimized.

2-Cyano-2-(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl)acetamide (8a). To an ice-cooled mixture of 2-cyanoacetamide (**6a**) (25.3 g, 0.30 mol) and mucochloric acid (**7**) (50.4 g, 0.30 mol) in MeOH (180 mL) was added a solution of sodium hydroxide (18.0 g, 0.45 mol) in water (180 mL) at 0 °C, and the resulting mixture was stirred at room temperature for 3 h. The mixture was poured into ice and 1 M HCl (200 mL), concentrated in vacuo and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was crystallized from EtOH-*i*Pr_2O to give the title compound (14.3 g, 20%) as pale brown solids. ^{1}H-NMR (DMSO-d_6) δ 4.84 (d, *J* = 3.2 Hz, 1H), 5.91 (d, *J* = 4.0 Hz, 1H), 7.85 (br. s., 1H), 8.03 (br. s., 1H). mp 162–163 °C.

2-Cyano-2-(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl)-*N*-ethylacetamide (8b). To an ice-cooled mixture of mucochloric acid (3.00 g, 17.8 mmol) and 2-cyano-*N*-ethylacetamide (**6b**) (2.22 g, 19.8 mmol) in MeOH (10 mL) was added dropwise a solution of sodium hydroxide (1.08 g, 27.0 mmol) in water (10 mL), and the resulting mixture was stirred at room temperature for 18 h. The mixture was poured into ice and 1 M HCl (20 mL), and concentrated in vacuo. The residue was dissolved in AcOEt. The solution was washed with brine and dried over $MgSO_4$. The solvent was evaporated in vacuo to give the title compound as a pale brown amorphous solids. This product was used for the next reaction without further purification.

2-Cyano-2-(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl)-*N*,*N*-dimethylacetamide (8c). To an ice-cooled mixture of mucochloric acid (5.00 g, 29.6 mmol) and 2-cyano-*N*,*N*-dimethylacetamide

(**6c**) (3.70 g, 33.0 mmol) in MeOH (18 mL) was added dropwise a solution of sodium hydroxide (1.80 g, 45.0 mmol) in water (18 mL), and the resulting mixture was stirred at room temperature for 3 h. The mixture was poured into ice and 1 M HCl (20 mL), concentrated in vacuo and dissolved in AcOEt. The solution was washed with brine and dried ($MgSO_4$). The solvent was evaporated in vacuo to give the title compound as a brown syrup. This material was used for the next reaction without further purification.

2-Cyano-2-(3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl)acetamide (8d). To an ice-cooled mixture of mucobromic acid (5.00 g, 19.4 mmol) and 2-cyanoacetamide (**6a**) (1.63 g, 19.4 mmol) in MeOH (18 mL) was added a solution of sodium hydroxide (1.16 g, 29.0 mmol) in water (18 mL), and the resulting mixture was stirred at room temperature for 4 h. The mixture was poured into ice and 1 M HCl (200 mL), concentrated in vacuo and extracted with AcOEt. The extract was washed with brine and dried over $MgSO_4$. The solvent was evaporated in vacuo to give the title compound as a dark brown tar. This material was used for the next reaction without further purification.

1-Benzyl-5-chloro-*N*-ethyl-2-imino-1,2-dihydropyridine-3-carboxamide (9b). To a solution of **8b** (1.00g, 3.80 mmol) in EtOH (10 mL) was added benzylamine (0.61 g, 5.69 mmol). After stirring at room temperature for 8 h, Et_3N (0.58 g, 5.73 mmol) was added. The resulting mixture was stirred at room temperature for further 48 h and concentrated in vacuo. The residue was dissolved in DMSO (10 ml) and stirred at 80 °C for 1 h. The reaction mixture was concentrated in vacuo, and the residue was purified by preparative HPLC to afford the title compound (0.21 g, 19%) as a pale yellow solid. ^{1}H-NMR ($CDCl_3$): δ 1.13 (t, *J* = 7.3 Hz, 3H), 3.35–3.46 (m, 2H), 4.89 (s, 2H), 7.20 (d, *J* = 7.2 Hz, 2H), 7.24 (d, *J* = 2.6 Hz, 1H), 7.33–7.46 (m, 3H), 8.26 (d, *J* = 2.6 Hz, 1H), 11.1 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 14.0, 34.2, 56.0, 117.4, 117.5, 127.4, 128.5, 128.8, 132.5, 140.8, 142.4, 151.8, 163.1. LC-MS (ESI): m/z [M + H^+] calcd, 290.1; found, 290.2. purity: 100%. mp 140–143 °C.

1-Benzyl-5-chloro-2-imino-*N*,*N*-dimethyl-1,2-dihydropyridine-3-carboxamide (9c). To a solution of **8c** (3.95 g, 15.0 mmol) in EtOH (50 mL) was added benzylamine (4.82 g, 45.0 mmol). After stirring at room temperature for 18 h, Et_3N (4.55 g, 45.0 mmol) was added. The resulting mixture was stirred at room temperature for further 5 h and concentrated in vacuo. A solution of the

residue in DMSO (20 mL) was stirred at 60 °C for 1 h. The mixture was concentrated in vacuo, and the residue was purified by preparative HPLC to afford the title compound (0.12 g, 3%) as a yellow amorphous solid. ^{1}H-NMR ($CDCl_3$): δ 3.03 (br. s., 6H), 5.10 (s, 2H), 6.86 (d, J = 2.3 Hz, 1H), 7.02 (d, J = 2.3 Hz, 1H), 7.28–7.41 (m, 5H). ^{13}C-NMR (DMSO-d_6): δ 34.7, 37.8, 56.2, 118.2, 124.1, 127.4, 128.5, 128.9, 132.6, 138.7, 140.7, 149.5, 162.3. LC-MS (ESI): m/z [M + H^+] calcd, 290.1; found, 290.0. purity: 98.0%.

1-Benzyl-5-bromo-2-imino-1,2-dihydropyridine-3-carboxamide (9d). To a solution of **8d** (15.0 mmol) in EtOH (50 mL) was added benzylamine (4.82 g, 45.0 mmol). After stirring at room temperature for 18 h, Et_3N (4.55 g, 45.0 mmol) was added. The resulting mixture was stirred at room temperature for further 5 h and concentrated in vacuo. A solution of the obtained compound in DMSO (20 mL) was stirred at 60 °C for 1 h. The mixture was concentrated in vacuo, and the residue was purified by preparative HPLC to afford the title compound (10 mg, 0.34%) as a pale orange amorphous solid. ^{1}H-NMR ($CDCl_3$): δ 4.90 (s, 2H), 5.84 (br. s., 1H), 7.21 (d, J = 7.2 Hz, 1H), 7.30–7.46 (m, 5H), 8.33 (br. s., 1H), 10.8 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 55.8, 103.6, 116.7, 127.3, 128.5, 128.8, 132.6, 143.1, 145.0, 152.2, 165.6. LC-MS (ESI): m/z [M + H^+] calcd, 306.0; found, 306.2 . purity: 98.4%.

5-Chloro-2-imino-1-(3-chlorobenzyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (9m). To an ice-cooled solution of **8a** (0.50 g, 2.10 mmol) in THF (10 mL) was added a mixture of 3-chlorobenzylamine (0.45 g, 3.20 mmol) and Et_3N (0.32 g, 3.20 mmol) in THF (3 mL). The reaction mixture was stirred for 18 h at room temperature and concentrated in vacuo to give a crystalline product. The product was added DMSO (5 mL) and water (5 mL) at room temperature. The mixture was stirred for 1 h at 80 °C and concentrated in vacuo. The residue was purified by preparative HPLC to give as a pale yellow amorphous. This amorphous was dissolved in MeOH (1 mL), and 4 M HCl in AcOEt (1 mL) was added at room temperature. The resulting precipitate was collected by filtration and recrystallized from EtOH and AcOEt to give the title compound (48.2 mg, 7%) as a colorless solid. ^{1}H-NMR (DMSO-d_6) δ 5.61 (s, 2H), 7.23–7.30 (m, 1H), 7.43–7.47 (m, 2H), 7.50 (s, 1H), 8.19 (br. s., 1H), 8.67–8.74 (m, 2H), 8.83 (d, J= 1.8 Hz, 1H), 9.55 (br. s., 2H). ^{13}C-

NMR (DMSO-d_6): δ 55.3, 116.9, 117.4, 125.9, 127.5, 128.4, 130.7, 133.4, 135.0, 141.3, 142.9, 152.3, 165.8. LC-MS (ESI): m/z [M + H^+] calcd, 296.0; found, 296.0. purity: 100%. mp 160−163 °C.

Compounds **9e-l** and **9n-p** were prepared in a manner similar to that described for the synthesis of **9m**, and salt formation conducted as needed to provide crystalline solid.

5-Chloro-1-(2,2-dimethylpropyl)-2-imino-1,2-dihydropyridine-3-carboxamide (9e). Yield 3%. ^{1}H-NMR ($CDCl_3$): δ 1.05 (s, 9H), 3.54 (s, 2H), 5.82 (br. s., 1H), 6.36 (br. s., 1H), 7.08 (d, *J* = 2.6 Hz, 1H), 8.20 (d, *J* = 2.6 Hz, 1H), 11.1 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 26.5, 34.2, 61.6, 116.1, 116.2, 141.9, 142.7, 152.8, 165.9. LC-MS (ESI): m/z [M + H^+] calcd, 242.1; found, 242.3. purity: 99.2%.

5-Chloro-2-imino-1-phenyl-1,2-dihydropyridine-3-carboxamide (9f). Yield 10%. ^{1}H-NMR ($CDCl_3$): δ 5.82 (br. s., 1H), 6.17 (br. s., 1H), 7.19 (d, *J* = 2.6 Hz, 1H), 7.31–7.36 (m, 2H), 7.53–7.65 (m, 3H), 8.29 (d, *J* = 2.8 Hz, 1H), 10.9 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 103.5, 108.1, 117.2, 123.6, 127.5, 129.3, 129.7, 130.5, 139.2, 140.1, 154.0, 164.3. LC-MS (ESI): m/z [M + H^+] calcd, 248.1; found, 248.2. purity: 98.6%.

5-Chloro-1-(furan-2-ylmethyl)-2-imino-1,2-dihydropyridine-3-carboxamide (9g). Yield 8%. ^{1}H-NMR ($CDCl_3$): δ 4.82 (s, 2H), 5.83 (br. s., 1H), 6.40–6.42 (m, 1H), 6.43–6.46 (m, 1H), 7.21 (d, *J* = 2.6 Hz, 1H), 7.24–7.29 (m, 1H), 7.46 (d, *J* = 1.3 Hz, 1H), 8.18 (d, *J* = 2.4 Hz, 1H), 10.81 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 49.1, 110.3, 111.4, 115.9, 116.6, 140.3, 142.5, 144.1, 144.6, 151.2, 165.1. LC-MS (ESI): m/z [M + H^+] calcd, 252.1; found, 252.2. purity: 99.5%.

5-Chloro-2-imino-1-(pyridin-2-ylmethyl)-1,2-dihydropyridine-3-carboxamide (9h). Yield 9%. ^{1}H-NMR ($CDCl_3$) δ 4.98 (s, 2H), 5.79 (br. s., 1H), 6.59 (br. s., 1H), 7.22 (d, *J* = 7.8 Hz, 1H), 7.30 (dd, *J* = 5.0, 7.5 Hz, 1H), 7.39 (d, *J* = 2.4 Hz, 1H), 7.70–7.78 (m, 1H), 8.24 (br. s., 1H), 8.63 (d, *J* = 4.9 Hz, 1H), 10.94 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 55.9, 116.4, 116.9, 123.5, 124.4, 140.1, 142.1, 143.2, 147.0, 150.1, 152.7, 165.7. LC-MS (ESI): m/z [M + H^+] calcd, 263.1; found, 262.7. purity: 100%.

5-Chloro-2-imino-1-(pyridin-3-ylmethyl)-1,2-dihydropyridine-3-carboxamide (9i). Yield 17%. ^{1}H-NMR ($CDCl_3$): δ 4.95 (s, 2H), 5.84 (br. s., 1H), 6.22 (br. s., 1H), 7.26–7.28 (m, 1H), 7.36 (dd, *J* = 4.7, 7.9 Hz, 1H), 7.52 (d, *J* = 7.3 Hz, 1H), 8.23 (br. s., 1H), 8.58 (d, *J* = 2.3 Hz, 1H), 8.62–8.65 (m, 1H), 10.84 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 53.1, 116.6, 117.5, 126.9, 132.6, 141.0, 141.2, 141.5, 143.1, 145.0, 152.7, 165.7. LC-MS (ESI): m/z [M + H^+] calcd, 263.1; found, 263.2. purity: 100%.

5-Chloro-2-imino-1-(2-phenethyl)-1,2-dihydropyridine-3-carboxamide (9j). Yield 23%. ^{1}H-NMR ($CDCl_3$): δ 3.03 (t, *J* = 7.2 Hz, 2H), 3.95 (t, *J* = 7.2 Hz, 2H), 5.86 (br.s., 1H), 6.80 (d, *J* = 2.6 Hz, 1H), 7.12–7.17 (m, 2H), 7.19–7.48 (m, 4H), 8.14 (d, *J* = 2.4 Hz, 1H), 10.96 (br.s., 1H). ^{13}C-NMR (DMSO-d_6): δ 32.3, 54.6, 115.9, 116.5, 126.9, 128.3, 129.2, 136.1, 141.3, 142.4, 151.7, 165.8. LC-MS (ESI): m/z [M + H^+] calcd, 276.1; found, 276.2. purity: 99.5%.

5-Chloro-2-imino-1-(2-phenylpropyl)-1,2-dihydropyridine-3-carboxamide (9k). Yield 21%. ^{1}H-NMR ($CDCl_3$): δ 2.05–2.15 (m, 2H), 2.74 (t, *J* = 7.3 Hz, 2H), 3.67 (d, *J* = 7.0 Hz, 2H), 5.86 (br. s., 1H), 7.06 (d, *J* = 2.6 Hz, 1H), 7.16–7.29 (m, 4H), 7.30–7.37 (m, 2H), 8.18 (d, *J* = 2.6 Hz, 1H), 10.97 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 28.4, 31.3, 54.0, 115.9, 116.8, 125.9, 128.1, 128.2, 140.6, 141.4, 142.3, 151.7, 165.8. LC-MS (ESI): m/z [M + H^+] calcd, 290.1; found, 290.1. purity: 97.9%.

5-Chloro-2-imino-1-(2-chlorobenzyl)-1,2-dihydropyridine-3-carboxamide trifluoroacetate (9l). Yield 5%. To a solution of **8a** (23.5 mg, 0.10 mmol) in EtOH (1 mL) was added a mixture of 2-chlorobenzylamine (17.0 mg, 0.12 mmol) and triethylamine (25 μL, 0.18 mmol) in EtOH (0.5 mL) and stirred at room temperature for 18 hr. The mixture was purified by preparative HPLC to give the title compound (2.1 mg, 5%). ^{1}H-NMR (DMSO-d_6): δ 5.53 (s, 2H), 6.79–6.86 (m, 1H), 7.28－7.38 (m, 1H), 7.44 (td, *J* = 7.7, 1.5 Hz, 1H), 7.58–7.65 (m, 1H), 8.25 (s, 1H), 8.62 (br.s., 1H), 8.66 (q, *J* = 2.4 Hz, 2H), 9.22–9.90 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 54.8, 116.3, 117.0, 117.2, 118.3, 126.3, 127.6, 129.7, 130.2, 132.0, 141.1, 142.9, 152.7, 157.5, 165.8. LC-MS (ESI): m/z [M + H^+ – (CF_3CO_2H)] calcd, 296.0; found, 296.0. purity: 100%.

5-Chloro-2-imino-1-(4-chlorobenzyl)-1,2-dihydropyridine-3-carboxamide trifluoroacetate (9n). Yield 7%. ^{1}H-NMR (DMSO-d_6) δ ppm 5.52 (s, 2H), 7.30–7.36 (m, 2H), 7.47–7.52 (m, 2H), 8.21 (s, 1H), 8.56–8.62 (m, 2H), 8.78 (d, *J* = 2.2 Hz, 1H), 9.21–9.60 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 55.3, 113.3, 115.3, 117.0, 117.2, 117.3, 128.8, 129.2, 131.4, 133.2, 141.2, 142.8, 152.2, 157.9, 165.7. LC-MS (ESI): m/z [M + H^+ – (CF_3CO_2H)] calcd, 296.0; found, 296.0. purity: 100%.

5-Chloro-2-imino-1-(3-methoxybenzyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (9o). Yield 16%. ^{1}H-NMR (DMSO-d_6): δ 3.76 (s, 3H), 5.53 (s, 2H), 6.79–6.87 (m, 1H), 6.92–7.00 (m, 2H), 7.28–7.39 (m, 1H), 8.19 (s, 1H), 8.66 (d, *J* = 1.9 Hz, 2H), 8.79 (d, *J* = 2.3 Hz, 1H), 9.47 (br. s., 2H). ^{13}C-NMR (DMSO-d_6): δ 55.1, 55.8, 113.6, 113.8, 116.7, 117.2, 119.2, 130.1, 134.0, 141.1, 142.9, 152.2, 159.4, 165.8. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 292.1; found, 292.2. purity: 98.9%.

5-Chloro-2-imino-1-[3-(methylsulfonyl)benzyl]-1,2-dihydropyridine-3-carboxamide hydrochloride (9p). Yield 10%. ^{1}H-NMR (DMSO-d_6): δ 3.24 (s, 3H), 5.66 (s, 2H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.88–8.05 (m, 2H), 8.20 (s, 1H), 8.55–8.70 (m, 2H), 8.85 (d, *J* = 2.3 Hz, 1H), 9.33–9.68 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 43.4, 55.4, 116.9, 117.3, 126.2, 126.9, 130.0, 132.0, 134.1, 141.2, 141.4, 143.0, 152.4, 165.8. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 340.0; found, 339.8. purity: 100%.

5-Chloro-1-(1-(3-chlorophenyl)ethyl)-2-imino-1,2-dihydropyridine-3-carboxamide (9q). Yield 6%. ^{1}H-NMR ($CDCl_3$): δ 1.78 (d, *J* = 6.8 Hz, 3H), 5.32 (br. s., 1 H), 5.79 (br. s, 1H), 6.35 (br. s., 1H), 7.08–7.12 (m, 1 H), 7.19 (d, *J* = 2.4 Hz, 1H), 7.22 (s, 1H), 7.33–7.38 (m, 2H), 8.16 (br.s., 1H), 10.94 (br. s., 1H). ^{13}C-NMR (DMSO-d_6): δ 19.0, 59.2, 116.7, 117.9, 126.0, 127.4, 128.8, 130.8, 133.5, 137.1, 139.4, 142.7, 152.3, 165.9. LC-MS (ESI): m/z [M + H^+] calcd, 310.0; found, 310.0. purity: 97%.

5-Chloro-2-imino-1-(1-(3-(methylsulfonyl)phenyl)ethyl)-1,2-dihydropyridine-3-carboxamide hydrochloride (9r). Yield 40%. ^{1}H-NMR (DMSO-d_6): δ 1.95 (d, *J* = 6.7 Hz, 3H), 3.26 (s, 3H), 6.24 (q, 1H, *J* = 6.7 Hz), 7.69-7.76 (m, 2H), 7.94–8.00 (m, 1H), 8.04 (br. s., 1H), 8.23

(br. s., 1H), 8.47 (d, *J* = 2.2 Hz, 1H), 8.59 (d, *J* = 2.2 Hz, 1H), 8.66 (br. s., 1H), 9.80 (br. s., 2H). ^{13}C-NMR (DMSO-d_6): δ 19.1, 43.3, 59.13, 116.9, 117.7, 125.9, 127.3, 130.1, 132.3, 137.2, 138.5, 141.4, 142.6, 152.6, 166.0. LC-MS (ESI) m/z [M + H^+ – (HCl)] calcd, 354.1; found, 354.0. purity: 100%.

5-Chloro-1-(1-(3-cyanophenyl)ethyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride (9s). Yield 8%. ^{1}H-NMR (DMSO-d_6): δ 1.92 (d, *J* = 6.7 Hz, 3H), 6.19 (q, *J* = 6.7 Hz, 1H), 7.61–7.69 (m, 1H), 7.71–7.79 (m, 1H), 7.89 (d, J = 7.6 Hz, 1H), 7.97 (s, 1H), 8.22 (s, 1H), 8.43 (d, J = 2.3 Hz, 1H), 8.59 (d, J = 2.3 Hz, 1H), 8.65 (s, 1H), 9.79 (br.s., 2H). ^{13}C-NMR (DMSO-d_6): δ 19.0, 58.9, 111.8, 116.9, 117.8, 118.4, 130.0, 131.2, 132.3, 132.5, 137.2, 138.5, 142.5, 152.7, 166.1. LC-MS (ESI): m/z [M + H^+] calcd, 301.1; found, 301.0. purity: 100%.

5-Chloro-1-((1*R*)-1-(3-cyanophenyl)ethyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride ((*R*)-9s). To an ice-cooled solution of **8a** (6.42 g, 27.3 mmol) in MeOH (60 mL) was added a mixture of (*R*)-3-(1-aminoethyl)benzonitrile (8 g, 54.7 mmol) and Et_3N (7.6 mL, 54.5 mmol) in MeOH (10 mL). The reaction mixture was stirred for 18 h at 45 °C and concentrated in vacuo to give a crystalline product. To the residue was added acetic acid (50 mL) at room temperature. The mixture was stirred for 4 h at 50 °C and concentrated in vacuo. The residue was basified by 8 M NaOH and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (AcOEt:hexane = 7:3–1:0) to give a pale brown oil. To this oil in EtOH (3 mL) was added 4 M HCl in AcOEt (3 mL) at room temperature. The resulting precipitate was collected by filtration, recrystallized from EtOH, H_2O and washed with AcOEt to give the title compound (5.32 g, 58%, 98.9% e.e.) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 1.92 (d, *J* = 6.8 Hz, 3H), 6.24 (q, *J* = 6.7 Hz, 1H), 7.60–7.71 (m, 1H), 7.71–7.80 (m, 1H), 7.85–7.93 (m, 1H), 7.98 (s, 1H), 8.22 (s, 1H), 8.43 (d, *J* = 2.3 Hz, 1H), 8.62 (d, *J* = 2.3 Hz, 1H), 8.69 (s, 1H), 9.71–9.96 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 19.0, 59.0, 111.8, 116.9, 117.8, 118.4, 130.0, 131.2, 132.3, 132.4, 137.2, 138.6, 142.6, 152.7, 166.11. LC-MS (ESI): m/z [M + H^+] calcd, 301.1; found, 301.2. purity: 100%. Anal. Calcd for $C_{15}H_{14}Cl_2N_4O$: C, 53.43; H, 4.18; N, 16.62. Found: C, 53.33; H, 4.39; N, 16.59. mp 191–193 °C. $[\alpha]^{25}_D$ +157.0 (c 0.526, MeOH).

5-Chloro-1-((1*S*)-1-(3-cyanophenyl)ethyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride ((*S*)-9s). Optical resolution of **9s** (174 mg, 0.516 mmol) was conducted by HPLC (CHIRALCEL OJ (JL001), 50 mmID×500 mmL, solvent phase: hexane/EtOH/diethylamine = 700/300/1) after desalting. The desired fraction with larger retention time was separated and concentrated in vacuo. The residue (66.9 mg, 98.3% e.e.) in EtOH (1 mL) was added 4 M HCl in AcOEt (1 mL) at room temperature. The resulting precipitate was collected by filtration, recrystallized from EtOH, H_2O and washed with AcOEt to give the title compound (52.9 mg, 30%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 1.92 (d, *J* = 6.6 Hz, 3H), 6.12–6.27 (m, 1H), 7.60–7.69 (m, 1H), 7.71–7.78 (m, 1H), 7.86–7.93 (m, 1H), 7.97 (s, 1H), 8.24 (s, 1H), 8.44 (d, *J* = 2.1 Hz, 1H), 8.57–8.73 (m, 2H), 9.66–9.97 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 19.0, 58.9, 111.8, 116.9, 117.8, 118.4, 130.0, 131.2, 132.3, 132.5, 137.1, 138.5, 142.5, 152.7, 166.1. LC-MS (ESI): m/z [M + H^+] calcd, 301.1; found, 301.2. purity: 100%. mp 195–199 °C. $[\alpha]^{25}_D$ −145.4 (c 0.921, MeOH).

5-Chloro-1-[3-chloro-5-(methylsulfonyl)benzyl]-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride (9t). A mixture of **7a** (1.68 g, 7.16 mmol), 1-[3-chloro-5-(methylsulfonyl)phenyl]methanamine hydrochloride (**20**) (2.20 g, 8.59 mmol) and K_2CO_3 (2.97 g, 21.5 mmol) in EtOH (30 mL) was stirred overnight at 85 °C. To the reaction mixture was added 1 M NaOH until the mixture was made basic and the product was extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on NH silica gel (AcOEt:MeOH = 1:0-17:3) to give a pale yellow amorphous. This amorphous was dissolved in MeOH, and 4 M HCl/AcOEt (3 mL) was added at room temperature. The resulting precipitate was collected by filtration and recrystallized from EtOH and AcOEt to give the title compound (630 mg, 21%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 3.31 (s, 3H), 5.65 (s, 2H), 7.78 (t, *J* = 1.7 Hz, 1H), 7.92 (t, *J* = 1.5 Hz, 1H), 8.03 (t, *J* = 1.7 Hz, 1H), 8.21 (s, 1H), 8.65 (s, 1H), 8.67 (d, *J* = 2.1 Hz, 1H), 8.83 (d, *J* = 2.1 Hz, 1H), 9.56 (s, 2H). ^{13}C-NMR (DMSO-d_6): δ 43.1, 55.0, 116.9, 117.3, 124.9, 126.8, 132.2, 134.3, 136.3, 141.4, 142.8, 143.0, 152.6, 165.8. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 374.0; found, 373.8. purity: 100%. Anal. Calcd for $C_{14}H_{14}Cl_3N_3O_3S$: C, 40.94; H, 3.44; N, 10.23. Found: C, 40.96; H, 3.46; N, 10.11. mp 276–277 °C.

5-Chloro-1-[5-chloro-2-(methylsulfonyl)benzyl]-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride (9u). A mixture of **8a** (7.65 g, 32.5 mmol), 1-[5-chloro-2-(methylsulfonyl)phenyl]methanamine hydrochloride (**21**) (10.0 g, 39.0 mmol) and potassium carbonate (11.9 g, 85.9 mmol) in EtOH (100 mL) was stirred overnight at 85 °C. To the reaction mixture was added 1 M sodium hydroxide until the mixture was made basic and the product was extracted with AcOEt and THF. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on NH silica gel (AcOEt:MeOH = 4:1) to give a pale yellow amorphous solid. This amorphous solid was dissolved in MeOH, and 4 M HCl in AcOEt (15 mL) was added at room temperature. The resulting precipitate was collected by filtration and recrystallized from MeOH, H_2O and AcOEt to give the title compound (3.80 g, 28%) as a colorless solid. ^{1}H-NMR (DMSO-d_6) δ 3.43 (s, 3H), 5.82 (br. s., 2H), 7.14 (d, *J* = 1.9 Hz, 1H), 7.78 (dd, *J* = 8.5, 2.1 Hz, 1H), 8.07 (d, *J* = 8.5 Hz, 1H), 8.22 (br. s., 1H), 8.66 (br. s., 3H), 9.65 (br. s., 2H). ^{13}C-NMR (DMSO-d_6): δ 44.5, 54.5, 116.8, 117.3, 126.6, 129.3, 132.5, 134.2, 136.9, 139.3, 141.0, 143.0, 153.2, 166.0. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 374.0; found, 374.2. purity: 99.4%. Anal. Calcd for $C_{14}H_{14}Cl_3N_3O_3S$: C, 40.94; H, 3.44; N, 10.23. Found: C, 40.93; H, 3.56; N, 10.15. mp 256 °C.

1-Benzyl-5-chloro-2-oxo-1,2-dihydropyridine-3-carboxamide (10). A mixture of **1** (0.25 g, 0.96 mmol) and 6 M HCl (10 mL) was stirred under reflux for 15 h. The mixture was adjusted to pH 5 with ammonium hydroxide. The resulting precipitate was collected by filtration and washed with AcOEt to give the title compound (0.21 g, 83.3%) as a pale brown solid. ^{1}H-NMR (DMSO-d_6): δ 5.43 (s, 2H), 7.22–7.29 (m, *2*H), 7.33–7.46 (m, 3H), 8.34 (d, *J* = 2.5 Hz, 1H), 8.38–8.71 (m, 1H), 8.48 (d, *J* = 2.5 Hz, 1H). ^{13}C-NMR (DMSO-d_6): δ 55.0, 116.9, 123.6, 127.1, 128.3, 128.9, 133.0, 138.2, 142.7, 154.0, 162.9. LC-MS (ESI): m/z [M + H^+] calcd, 263.1; found, 263.2. purity: 97.2%. mp 216−219°C.

2-Aminopyridine-3-carboxamide (12). A solution of 2-aminopyridine-3-carbonitrile (**11**) (0.50 g, 4.20 mmol) in concentrated sulfuric acid (5 mL) was stirred at 100 °C for 5 h. The mixture was poured into ice-water and adjusted to pH 8 with saturated sodium hydrogen carbonate solution. The

precipitate was collected by filtration and washed with water to give the title compound (0.29 g, 50%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 6.55 (dd, J = 4.8, 7.6 Hz, 1H), 7.19 (br. s., 2H), 7.32 (br. s., 1H), 7.92 (dd, J = 1.7, 7.6 Hz, 1H), 7.94 (br. s., 1H), 8.06 (dd, J = 1.7, 4.8 Hz, 1H). mp 197−198 °C.

1-Benzyl-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (13). A mixture of **12** (0.10 g, 0.73 mmol) and benzylbromide (0.25 g, 1.46 mmol) in DMF (3 mL) was stirred at 80 °C for 18 h. After addition of toluene to the mixture, the resulting precipitate was collected by filtration and washed with AcOEt to give the title compound (0.10 g, 45%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 5.56 (s, 2H), 7.13 (t, J = 7.1 Hz, 1H), 7.26 (d, J = 7.1 Hz, 2H), 7.34–7.47 (m, 3H), 8.10 (br. s., 1H), 8.40 (d, J = 6.6 Hz, 1H), 8.49 (d, J = 7.6 Hz, 1H), 8.56 (br. s., 1H), 9.30 (br. s., 2H). ^{13}C-NMR (DMSO-d_6): δ 55.5, 111.9, 115.8, 127.3, 128.4, 128.9, 132.8, 142.8, 143.5, 153.1, 166.9. LC-MS (ESI): m/z [M + H^+] calcd, 228.1; found, 228.2. purity: 100%. mp 226−228 °C.

2-Amino-5-chloropyridine-3-carboxamide (14). To a mixture of **12** (2.0 g, 14.6 mmol) and conc. HCl (12 mL) was added 30% hydrogen peroxide (1.2 mL) at 0°C. After stirring at 60°C for 1 h, the mixture was diluted with water. The reaction mixture was basified with 1 M NaOH and extracted with THF and AcOEt. The organic layer was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography (AcOEt:hexane = 4:1–1:0) to give the title compound (0.94 g, 38%) as colorless crystals. ^{1}H-NMR (DMSO-d_6) δ 7.35 (s, 2H), 7.46 (s, 1H), 7.97–8.07 (m, 2H), 8.09 (d, J = 2.7 Hz, 1H). mp 226–228 °C.

1-(3-Carbamoyl-5-chlorobenzyl)-5-chloro-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15e). A mixture of **14** (150 mg, 0.87 mmol) and 3-carbamoyl-5-chlorobenzyl bromide (258 mg, 1.04 mmol) in DMF (3 mL) was stirred at 100 °C for 3 h, cooled to room temperature and diluted with AcOEt. The resulting precipitate was collected by filtration and recrystallized from MeOH and AcOEt to give the title compound (110 mg, 30%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 5.57 (s, 2H), 7.61 (s, 2H), 7.67 (s, 1H), 7.94 (s, 1H), 8.14 (s, 1H), 8.20 (s, 1H), 8.59 (s, 1H), 8.63 (d, J = 1.5 Hz, 1H), 8.81 (d, J = 1.1 Hz, 1H), 9.42 (s, 2H). ^{13}C-NMR

(DMSO-d_6): δ 48.5, 55.3, 117.0, 125.0, 127.1, 129.9, 133.4, 135.0, 136.5, 141.4, 142.9, 152.4, 165.7. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 339.0; found, 338.8. purity: 95.8%. mp 237–239 °C.

Compounds **15a-d** and **15f-i** were prepared in a manner similar to that described for the synthesis of **15e**, and desalting, purification and salt formation conducted as needed to provide crystalline solids.

5-Chloro-2-imino-1-(3-methylbenzyl)-1,2-dihydropyridine-3-carboxamide hydrobromide (15a). Yield 41%. ^{1}H-NMR (300 MHz, DMSO-d_6): δ 2.31 (s, 3H), 5.50 (s, 2H), 7.00–7.24 (m, 3H), 7.31 (t, *J* = 7.6 Hz, 1H), 8.20 (s, 1H), 8.58 (s, 1H), 8.62 (d, *J* = 1.9 Hz, 1H), 8.76 (d, *J* = 1.9 Hz, 1H), 9.38 (s, 2H). ^{13}C-NMR (DMSO-d_6): δ 20.9, 55.9, 116.8, 117.2, 124.2, 127.8, 128.8, 129.1, 132.3, 138.2, 141.2, 142.8, 152.2, 165.8. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 276.1; found, 276.2. purity: 96.9%.

1-(3-Carbamoylbenzyl)-5-chloro-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15b). Yield 42%. ^{1}H-NMR (DMSO-d_6): δ 5.59 (s, 2H), 7.39–7.55 (m, 3H), 7.76 (s, 1H), 7.88 (d, *J* = 8.0 Hz, 1H), 8.03 (s, 1H), 8.20 (s, 1H), 8.59 (s, 1H), 8.64 (d, *J* = 2.3 Hz, 1H), 8.81 (d, *J* = 2.3 Hz, 1H), 9.40 (s, 2H). ^{13}C-NMR (DMSO-d_6): δ 55.8, 116.9, 117.3, 126.4, 127.3, 128.8, 129.9, 132.7, 134.7, 141.3, 142.8, 152.3, 165.7, 167.1. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 305.1; found, 305.2. purity: 100%.

5-Chloro-1-(3-cyanobenzyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15c). Yield 28%. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 5.60 (2 H, s), 7.64 (2 H, d, *J* = 4.9 Hz), 7.79–7.90 (2 H, m), 8.21 (1 H, s), 8.60 (1 H, s), 8.65 (1 H, d, *J* = 2.3 Hz), 8.80 (1 H, d, *J* = 2.3 Hz), 9.44 (2 H, s). ^{13}C-NMR (DMSO-d_6): δ 55.3, 111.7, 117.0, 117.3, 118.3, 129.9, 130.7, 132.1, 132.1, 134.1, 141.3, 142.8, 152.4, 165.8. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 287.1; found, 287.0. purity: 100%.

1-(4-Carbamoyl-3-chlorobenzyl)-5-chloro-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15d). Yield 30%. ^{1}H-NMR (DMSO-d_6): δ 5.55 (s, 2H), 7.24 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.47 (d, *J* = 7.9 Hz, 1H), 7.52 (d, *J* = 1.5 Hz, 1H), 7.65 (s, 1H), 8.20 (s, 1H), 8.57 (s, 1H), 8.61

(d, J = 2.1 Hz, 1H), 8.82 (d, J = 2.1 Hz, 1H), 9.28 (s, 2H). ^{13}C-NMR (DMSO-d_6): δ 55.1, 117.1, 117.4, 125.7, 128.6, 129.0, 130.0, 135.0, 137.0, 141.2, 142.9, 152.2, 165.7, 167.5. LC-MS (ESI): m/z: [M + H^+ – (HBr)] calcd, 339.1; found, 338.8. purity: 95.4%.

5-Chloro-1-[3-chloro-4-(methylsulfonyl)benzyl]-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride (15f). Yield 12%. ^{1}H-NMR (DMSO-d_6): δ 3.38 (s, 3H), 5.68 (s, 2H), 7.46 (dd, J = 8.1, 1.5 Hz, 1H), 7.78 (1H, s), 8.04 (d, J = 8.3 Hz, 1H), 8.22 (s, 1H), 8.62–8.72 (m, 2H), 8.82 (d, J = 1.3 Hz, 1H), 9.54 (br. s., 2H). ^{13}C-NMR (DMSO-d_6): δ 42.4, 55.0, 117.0, 117.3, 126.4, 130.5, 130.7, 131.4, 137.6, 140.4, 141.4, 143.0, 152.6, 165.8. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 374.0; found, 374.2. purity: 97.2%.

5-Chloro-1-(3-chloro-4-cyanobenzyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15g). Yield 27%. ^{1}H-NMR (DMSO-d_6): δ 5.60 (s, 2H), 7.39 (dd, J = 8.0, 1.6 Hz, 1H), 7.75 (d, J = 1.6 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 8.20 (s, 1H), 8.52–8.67 (m, 2H), 8.74 (s, 1H), 9.24–9.64 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 55.2, 111.6, 115.6, 117.1, 117.4, 126.4, 128.6, 134.8, 135.5, 140.1, 141.3, 143.0, 152.6, 165.8. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 321.0; found, 321.0. purity: 100%.

5-Chloro-1-(3-chloro-5-cyanobenzyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrobromide (15h). Yield 23%. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 5.56 (s, 2H), 7.74–7.80 (m, 1H), 7.81–7.88 (m, 1H), 8.02–8.14 (m, 1H), 8.21 (s, 1H), 8.53–8.70 (m, 2H), 8.71–8.81 (m, 1H), 9.21–9.66 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 54.9, 113.3, 117.0, 117.1, 117.5, 129.5, 131.7, 132.4, 134.1, 136.3, 141.4, 142.9, 152.6, 165.8. LC-MS (ESI): m/z [M + H^+ – (HBr)] calcd, 321.0; found, 321.0. purity: 100%.

5-Chloro-1-(5-chloro-2-cyanobenzyl)-2-imino-1,2-dihydropyridine-3-carboxamide hydrochloride (15i). Yield 9%. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 5.76 (s, 2H), 7.04 (d, J = 8.5 Hz, 1H), 7.74 (dd, J = 8.5, 2.3 Hz, 1H), 8.20–8.28 (m, 2H), 8.62–8.81 (m, 3H), 9.38–9.85 (m, 2H). ^{13}C-NMR (DMSO-d_6): δ 54.7, 112.3, 115.5, 116.9, 117.3, 127.2, 132.9, 133.0, 133.5, 135.2, 141.3, 143.2, 152.9, 165.8. LC-MS (ESI): m/z [M + H^+ – (HCl)] calcd, 321.0; found, 321.0. purity: 100%.

1-[3-Chloro-5-(methylsulfanyl)phenyl]methanamine hydrochloride (17). A mixture of 3-bromo-5-chlorobenzonitrile (**16**) (4.00 g, 18.5 mmol), sodium thiomethoxide (1.42 g, 20.3 mmol), tris(dibenzylideneacetone)dipalladium (0) (84.0 mg, 0.09 mmol), Xantphos (106 mg, 0.18 mmol) and diisopropylethylamine (4.78 g, 37.0 mmol) in toluene (100 mL) was stirred at 90 °C for 9 h. The reaction mixture was poured into 1 M HCl and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (AcOEt:hexane = 1:20) to give 3-chloro-5-(methylsulfanyl) benzonitrile (2.60 g, 72%) as colorless solids. To a suspension of lithium aluminium hydride (0.79 g, 20.8 mmol) in THF (200 mL) was added 3-chloro-5-(methylsulfanyl)benzonitrile (3.20 g, 17.4 mmol) at 0 °C. The reaction mixture was stirred for 3 h at room temperature, and sodium sulfate decahydrate was added thereto. The mixture was stirred for further 0.5 h at room temperature and filtered. The filtrate was concentrated in vacuo. The residue was dissolved in MeOH (3 mL) and 4 M HCl in AcOEt (10 mL) was added at room temperature. The resulting precipitate was collected by filtration and recrystallized from MeOH and AcOEt to give the title compound (2.80 g, 72%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 2.52 (s, 3H), 4.00 (s, 2H), 7.31 (t, J = 1.8 Hz, 1H), 7.36 (s, 1H), 7.41 (s, 1H), 8.51 (s, 3H). LC-MS (ESI): m/z [M + H^+] calcd, 187.0; found, 186.9. mp 185−186 °C.

1-[3-Chloro-5-(methylsulfonyl)phenyl]methanamine hydrochloride (18). To a mixture of **17** (2.60 g, 11.6 mmol) and Et_3N (2.35 g, 23.2 mmol) in THF (100 mL) was added di-*tert*-butyl dicarbonate (3.80 g, 17.4 mmol) at room temperature. The reaction mixture was stirred for 4 h at room temperature, then 1 M HCl was added, and the mixture was extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was dissolved in AcOEt (100 mL) and *m*CPBA (7.15 g, 41.4 mmol) was added at room temperature. The reaction mixture was stirred for 2 h at room temperature and aqueous $NaHCO_3$ was added. The organic layer was separated and washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on NH silica gel (AcOEt:hexane = 1:1) to give colorless oil. To a solution of this oil in MeOH (70 mL) was added 4 M HCl in AcOEt (10 mL) at room temperature,

the mixture was stirred for 0.5 h at 60 °C and the reaction mixture was concentrated in vacuo. The resulting precipitate was collected by filtration and washed with AcOEt to give the title compound (2.41 g, 81%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 3.30 (s, 3H), 4.17 (s, 2H), 8.01 (d, J = 1.5 Hz, 2H), 8.09 (d, J = 1.3 Hz, 1H), 8.55 (s, 3H). LC-MS (ESI): m/z [M + H^+] calcd, 220.0; found, 219.9. mp 239−241 °C.

5-Chloro-2-(methylsulfonyl)benzonitrile (20). To a solution of 5-chloro-2-fluorobenzonitrile (**19**) (5.00 g, 32.1 mmol) in DMSO (100 mL) was added 21% sodium thiomethoxide in water (15.0 mL, 45.0 mmol) at room temperature. After stirring for 2 h at room temperature, water (200 mL) was added to the reaction mixture, which was stirred for 0.5 h. The resulting precipitate was collected by filtration and was washed with water to give 5-chloro-2-(methylsulfanyl)benzonitrile (4.60 g, 78%) as a colorless solid. To a solution of 5-chloro-2-(methylsulfanyl)benzonitrile (1.00 g, 4.54 mmol) in acetonitrile (15 mL) was added dropwise a solution of Oxone® (6.98 g, 11.4 mmol) in water (20 mL) over 2 h, and the mixture was stirred overnight at room temperature. To the reaction mixture was added water (25 mL), and the mixture was stirred for 3 h at 0 °C. The resulting precipitate was collected by filtration and washed with water (40 mL) to give the title compound (915 mg, 93%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 3.41 (s, 3H), 8.08–8.13 (m, 2H), 8.44 (d, J = 1.3 Hz, 1H). LC-MS (ESI): m/z [M + H^+] calcd, 216.0; found, 215.9. mp 164−166 °C.

1-[5-Chloro-2-(methylsulfonyl)phenyl]methanamine hydrochloride (21). To a solution of **20** (3.82 g, 17.7 mmol) in EtOH (25 mL) were added 8 M ammonia in MeOH (5 mL, 40.0 mmol) and Raney Co (19.0 g, 17.7 mmol), and the mixture was stirred overnight under hydrogen atmosphere. The mixture was filtered through Celite, and the filtrate was concentrated in vacuo. To the residue were added EtOH (20 mL) and 2 M HCl/MeOH (10 mL). The mixture was stirred for 1 h at room temperature and concentrated in vacuo. The resulting precipitate was collected by filtration and washed with AcOEt to give the title compound (3.61 g, 80%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 3.37 (s, 3H), 4.44 (s, 2H), 7.65–7.89 (m, 1H), 7.89–8.27 (m, 2H), 8.61 (br. s., 3H). LC-MS (ESI): m/z [M + H^+] calcd, 220.0; found, 219.9. mp 248−250 °C.

3-(Bromomethyl)benzamide (23). A mixture of 3-(bromomethyl)benzonitrile (**22**) (2.00 g, 10.2 mmol) and conc. H_2SO_4 (5 mL) was stirred at 50 °C for 30 min. The reaction was quenched with ice and the mixture was extracted with AcOEt and THF. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo to give the title compound (200 mg, 9%) as a colorless solid. ^{1}H-NMR (DMSO-d_6): δ 4.74 (s, 2H), 7.35–7.49 (m, 2H), 7.56–7.63 (m, 1H), 7.77–7.84 (m, 1H), 7.91–8.07 (m, 2H). LC-MS (ESI): m/z [M + H^+] calcd, 214.0; found, 214.0. mp 140−143 °C.

3-(Bromomethyl)-5-chlorobenzamide (26). A mixture of dimethyl 5-chlorobenzene-1,3-dicarboxylate (**24**) (1.00 g, 4.37 mmol) and 1 M sodium hydroxide (4.37 mL, 4.37 mmol) in MeOH (15 mL) was stirred overnight at room temperature. The reaction mixture was acidified with 1 M HCl and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo to give 3-chloro-5-(methoxycarbonyl)benzoic acid (640 mg, 68%) as colorless solids. To a solution of 3-chloro-5-(methoxycarbonyl)benzoic acid (500 mg, 2.33 mmol) and DMF (1 drop) in THF (20 mL) was added oxalyl chloride (444 mg, 3.50 mmol) at 0 °C. After stirring at room temperature for 1 h, the mixture was concentrated in vacuo. The residue was dissolved in THF (20 mL), and 8 M ammonia in MeOH (2 mL) was added at 0 °C. After stirring at room temperature for 14 h, the reaction mixture was poured into saturated sodium hydrogen carbonate and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo to give methyl 3-carbamoyl-5-chlorobenzoate (**25**) (350 mg, 70%) as colorless solids. To a solution of **25** (2.20 g, 10.3 mmol) in THF (30 mL) and EtOH (3 mL) was added lithium borohydride (335 mg, 15.4 mmol) at room temperature. After stirring at 60 °C for 2 h, the reaction was quenched with ice, and the mixture was extracted with AcOEt. The extract was washed with 1 M HCl and brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (AcOEt:hexane = 5:1) to give 3-chloro-5-(hydroxymethyl) benzamide (950 mg, 50%) as a colorless solid. To a suspension of triphenylphosphine (1.27 g, 4.86 mmol) in acetonitrile (50 mL) was added bromine (0.25 mL, 4.86 mmol) at room temperature. After stirring at room temperature for 30 min, 3-chloro-5-(hydroxymethyl)benzamide (900 mg, 4.86 mmol) was added to the reaction mixture. After stirring at 85 °C for 2 h, the reation mixture was poured into water and extracted with AcOEt.

The extract was washed with saturated sodium hydrogen carbonate and brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (AcOEt:hexane = 1:1) to give the title compound (540 mg, 45%) as a amorphous solid.

^{1}H-NMR (DMSO-d_6): δ 4.74 (s, 2H), 7.57 (s, 1H), 7.71 (t, J = 1.7 Hz, 1H), 7.86 (t, J = 1.7 Hz, 1H), 7.91 (t, J = 1.5 Hz, 1H), 8.11 (s, 1H). LC-MS (ESI): m/z [M + H$^+$] calcd, 247.9; found, 247.9.

4-(Bromomethyl)-2-chlorobenzamide (28). A mixture of 2-chloro-4-methylbenzonitrile (**27**) (2.00 g, 13.2 mmol) and conc. sulfuric acid (5 mL) was stirred for 10 h at 80 °C. The reaction mixture was cooled to 0 °C, basified with aqueous sodium hydroxide and extracted with THF and AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo to give 2-chloro-4-methylbenzamide (1.25 g, 56%) as a colorless solid. A mixture of 2-chloro-4-methylbenzamide (1.05 g, 6.19 mmol), *N*-bromosuccinimide (1.32 g, 7.43 mmol) and AIBN (50.0 mg, 0.30 mmol) in *tert*-butyl acetate (25 mL) was stirred for 1 day at 80 °C. The reaction mixture was poured into water and extracted with AcOEt. The extract was washed with brine, dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (AcOEt:hexane = 5:2) to give a mixture of the title compound and 2-chloro-4-methylbenzamide as a colorless solid (620 mg). This mixture was used to the next step without further purification.

Biology.

α_1 Binding Assay

Membranes of human α_{1A}-, α_{1B}-, α_{1D}-adrenoceptors were prepared from CHO-K1 cells stably expressing each α_1-adrenoceptor. Binding assay for adrenergic α_1 receptor was performed in 200 μL of α_1 binding assay buffer (50 mmol/L Tris-HCl pH7.5, 10 mmol/L $MgCl_2$, 5 mmol/L EDTA and 0.5% BSA) containing membrane protein (10 μg for each α_1 receptor) and 2.5 nmol/L 7-methoxy-[^{3}H]-prazosin in the presence of compound at 12 different concentrations. Following incubation at room temperature for 60 min, the membranes were filtered through GF/C filter plates (Perkin Elmer

Life and Analytical Sciences) and washed with 50 mmol/L Tris-HCl (pH7.5). The membrane-associated radioactivity was determined using TopCount liquid scintillation counter (Perkin Elmer Life and Analytical Sciences). Non-specific binding was defined as binding in the presence of 10 μmol/L phentolamine. IC_{50} values and 95 % confidence intervals were calculated by logistic regression analysis. The Kd values of α_1 receptor subtypes (α_{1A}, α_{1B} and α_{1D}) were 0.93, 0.35 and 0.26 nmol/L, respectively. Ki values were calculated as Ki= IC_{50}/{1+(^{3}H-ligand concentration)/ Kd}.[26]

hERG inhibition assay

hERG/CHO cells stably expressing hERG channel were purchased from Millipore (UK) Ltd. (cat. #CYL3038). Cells were cultured at 32 ºC, 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum, 500 μg/mL Geneticin (Invitrogen). The hERG inhibition assay was performed on the IonWorks Quattro (Molecular Devices) system in population patch clamp (PPC) mode. The extracellular solution was phosphate-buffered salines (PBS) with calcium and magnesium (Cat. #14040, Invitrogen). The intracellular solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM HEPES, pH 7.3 with KOH. After perforation using 100 μg/mL amphotericin B (Sigma-Aldrich), hERG current was measured under the potential-clamp protocol (Holding potential −80 mV, the first voltage 40 mV: 2 sec, the second voltage −50 mV: 2 sec). The peaktail current before addition of the compounds was measured as the pre hERG current. Test compounds were incubated on the cells for a period of 5 min. The peaktail current after addition of the compounds was measured as the post hERG current. %hERG inhibition was calculated (n = 3 or 4) to the following.

%hERG inhibition = 100 − (post hERG current / pre hERG current) × 100.

Evaluation of reversible inhibition of Cytochrome P450 3A4.

Human liver microsomes were purchased from Xenotech, LLC (Lenexa, KS). Inhibition activity of a test compound of cytochrome P450 3A4 was evaluated by incubating midazolam with 0.1 mg/mL human microsomes in the presence of 10 μM test compound. The incubation mixture was allowed to stand for 10 min at 37 °C and then the incubation was terminated by addition of

acetonitrile/water. After centrifugation, the supernatant was subjected to LC/MS/MS analysis to measure the peak of 1'-hydroxymidazolam.

In vitro metabolic clearance in human hepatic microsomes.

Human liver microsomes were purchased from Xenotech, LLC (Lenexa, KS). An incubation mixture consisted of microsomal protein in 50 mM KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4) and 1 μM test compound. The concentration of microsomal protein was 0.2 mg/mL. An NADPH-generating system containing 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM β-$NADP^+$, and 1.5 units/mL glucose-6-phosphate dehydrogenase were added to the incubation mixture to initiate the enzyme reaction. The reaction was terminated 15 and 30 min after the initiation of the reaction by mixing the reaction mixture with acetonitrile, followed by centrifugation. The supernatant was subjected to LC/MS/MS analysis. The metabolic velocity was calculated as the slope of the concentration-time plot.

Pharmacokinetic analysis in rat cassette dosing.

Test compounds were administered intravenously (0.1 mg/kg, solvent: DMA/1,3-butanediol = 1:1) or orally (1 mg/kg, solvent: 0.5% methylcellulose suspension) by cassette dosing to fed rats. After administration, blood samples were collected and centrifuged to obtain the plasma fraction. The plasma samples were deproteinized followed by centrifugation. The compound concentrations in the supernatant were measured by LC/MS/MS.

Associated content

Supporting Information

The Supporting Information is available free of charge on the http://pubs.acs.org/.

Biological procedures of tested compounds not described in the manuscript. Method and instrumentation used to the X-ray crystal structure for compound **1**

Accession code

CDCC code for compound **1** is CDCC 1058155.

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Notes

The authors declare no competing financial interest.

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Abbreviations used

AIBN, 2,2'-azobis(isobutyronitrile); AUC, area under the blood concentration time curve; CLtotal, total clearance; Cmax, maximum concentration in plasma; *m*CPBA, *m*-chloroperoxybenzoic acid; DMF, *N*,*N*-dimethylformamide; DMA, *N*,*N*-dimethylacetamide; DMSO, dimethylsulfoxide; DPPA, diphenylphosphoryl azide; Et_3N, triethylamine; EtOH, ethanol; F, rat bioavailability; HCl, hydrochloric acid; iv, intravenous; MeOH, methanol; MRT, mean residence time; Pd/C, palladium on carbon; $i Pr_2O$, diisopropylether; THF, tetrahydrofuran; Vdss, volume of distribution at steady state.

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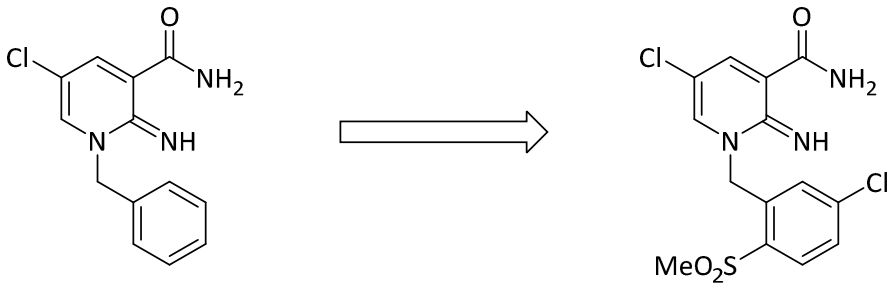
Table of Contents graphic



1

α_{1D}: Ki = 30 nM

9u

α_{1D}: Ki = 1.1 nM