Regular Article

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Novel Indirect AMP-Activated Protein Kinase Activators: Identification of a Second-Generation Clinical Candidate with Improved Physicochemical Properties and Reduced hERG Inhibitory Activity

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This study reports the synthesis and evaluation of novel indirect AMP-activated protein kinase (AMPK) activators. The series of compounds selectively inhibited cell growth in several human breast cancer cell lines by activating AMPK. We performed back-up medicinal chemistry synthetic research on ASP4132, a previously reported as a compound for clinical development that acts as an indirect AMPK activator. This led to the successful identification of 4-({4-[5-({1-[(5-ethoxypyrazin-2-yl)methyl]-4-fluoropiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]piperazin-1-yl}methyl)benzonitrile succinate (27b), a potent, highly aqueous soluble and metabolically stable compound in human hepatocytes. Compound 27b also showed weaker human Ether-a-go-go Related Gene (hERG) inhibitory activity than that of compound 13 and ASP4132. Therefore, 27b was a promising AMPK activator and a second-generation clinical candidate for treatment for human cancer.

Key words AMP-activated protein kinase; human breast cancer; precision medicine; human ether-a-go-go related gene; aqueous solubility; metabolic stability

Introduction

AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine protein kinase, was first described for its important role in lipid metabolism and regulation of cholesterol and fatty acid levels.[1,2] Since then, AMPK has been known as a master player in metabolism because it maintains energy homeostasis during metabolic stress both at the cellular and physiological levels.[1,3] AMPK is generally activated in response to a negative energy balance by sensing increases in the ratios of AMP to ATP and adenosine ADP to ATP, and regulates energy balance by inhibiting consumption of ATP while promoting generation of ATP.[1,4]

Disruption of cellular energetics is a core feature of cancer.[5–7] Therefore, AMPK activation may lead to metabolic tumor suppression as a result of its regulation of energy balance, enforcement of metabolic checkpoints and inhibition of cell growth.[8] In fact, a number of studies have demonstrated that AMPK exerts its tumor suppressor function in human cancers *via* several downstream effectors, such as mammalian target of rapamycin (mTOR),[9] p53[10,11] and acetyl-CoA carboxylase (ACC).[12,13] Thus, AMPK activators may be suitable therapeutic targets for the treatment of cancer.

We recently reported ASP4132, (5-{1-[(6-methoxypyridin-3-yl)methyl]piperidin-4-yl}-1*H*-benzimidazol-2-yl)(4-{[4-(trifluoromethyl)phenyl]methyl}piperazin-1-yl)methanone ditosylate, as a compound for clinical trial and an indirect AMPK activator that shows potent and selective cell growth inhibitory activity against human breast cancer cell lines, and suitable *in vivo* anti-tumor efficacy in a xenograft mouse model following oral administration[14] (Fig. 1).

While ASP4132 showed potent *in vitro* and *in vivo* anti-tumor activity, good *in vitro* metabolic stability in human hepatocytes and favorable *in vivo* animal pharmacokinetics (PK) profiles, its aqueous solubility at gastrointestinal pH was moderate. Considering the potential issues that may arise due to its moderate aqueous solubility, which may lead to limited oral absorption at high doses in clinical dose escalation studies, we planned to identify a second generation clinical candidate with good aqueous solubility at gastrointestinal pH while maintaining the pharmacological and absorption, distribution, metabolism, excretion (ADME) profiles of ASP4132.

Previously, a study of GlaxoSmithKline reported that oral drug candidates with more than three aromatic rings typically have poorer compound developability.[15] According to this review, aqueous solubility dramatically decreases with increasing aromatic ring count (the number of aromatic and heteroaromatic rings). ASP4132 contains four aromatic rings, suggesting that decreasing the number of aromatic rings is one strategy to improve aqueous solubility. A structure–activity relationship study of ASP4132 and its derivatives indicated that both of the terminal aromatic moieties were important for their AMPK activation activities. Therefore, we considered that the benzimidazole moiety of ASP4132 should be replaced with a monocyclic aromatic moiety.

We previously reported that anilide linkage compound **1**, which has three aromatic rings, was a potent AMPK activator[14] (Fig. 2). While the benzimidazole scaffold of ASP4132 was effective for producing good animal PK profiles, probably due to elimination of the need for metabolic hydrolysis of an anilide linkage,[14] we decided to conduct optimization using a different approach, one that does not increase the aromatic ring count, to acquire a compound with both good aqueous solubility and animal PK profiles.

Herein, we report the discovery of a second generation orally active, potent and selective AMPK activator. Our backup medicinal chemistry strategy using com-

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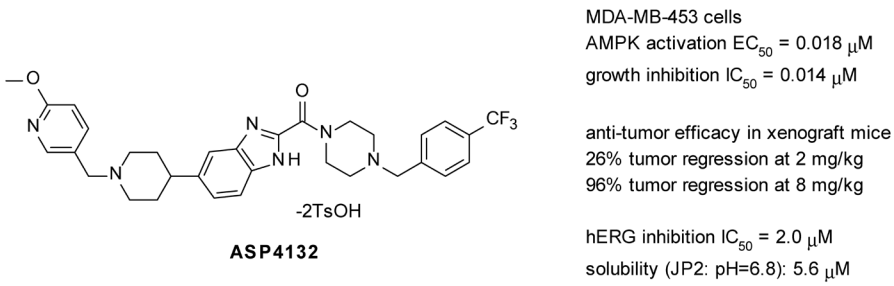


Fig. 1. Chemical Structure, Pharmacological Properties and Aqueous Solubility of ASP4132

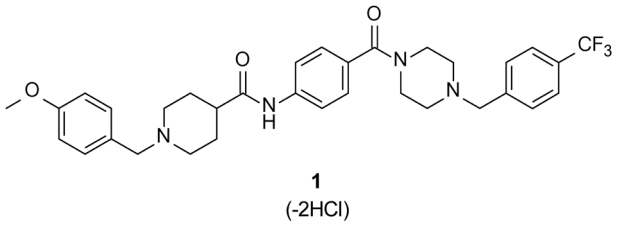


Fig. 2. Chemical Structure of Compound **1**

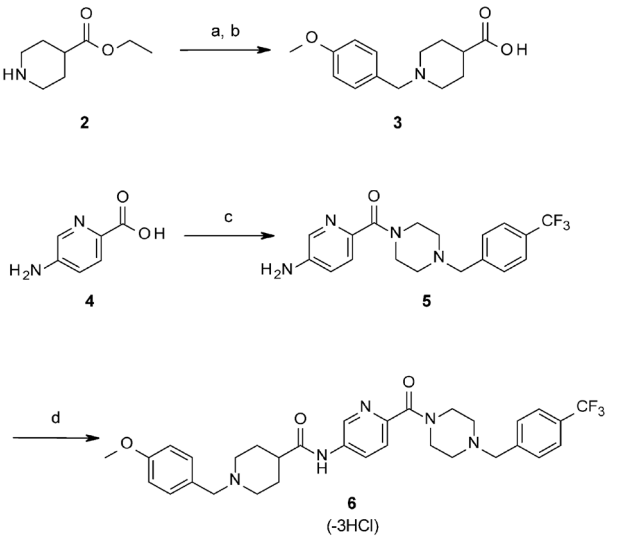
pound **1** as the lead compound led to the identification of 4-({4-[5-({1-[(5-ethoxypyrazin-2-yl)methyl]-4-fluoropiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]piperazin-1-yl}-methyl)benzonitrile succinate (**27b**). Compound **27b** showed good aqueous solubility, excellent animal PK and weak human ether-a-go-go related gene (hERG) inhibitory activity.

Chemistry

Chart 1 illustrates the synthesis of compound **6**. Benzylation of commercially available ethyl piperidine-4-carboxylate (**2**) and subsequent ester hydrolysis yielded carboxylic acid **3**. Next, commercially available 5-aminopyridine-2-carboxylic acid (**4**) was condensed with 1-{[4-(trifluoromethyl)phenyl]-methyl}piperazine to obtain compound **5**. Finally, condensation of **3** with **5** yielded compound **6**.

The synthetic route for **13**–**16** is described in Chart 2. Esterification of commercially available 5-hydroxypyridine-2-carboxylic acid (**7**) with methanol yielded **8**. Alkylation of **8** with *tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate by Mitsunobu reaction, and subsequent ester hydrolysis yielded **9**. For the synthesis of **13** and **14**, condensation of **9** with 1-{[4-(trifluoromethyl)phenyl]methyl}piperazine and a subsequent deprotection reaction produced **10**. Alkylation of **10** with the requisite commercially available aryl aldehydes by reductive amination reaction yielded **13** and **14**. For the synthesis of **15** and **16a**–**16c**, **9** was condensed with benzyl piperazine-1-carboxylate, the benzyloxycarbonyl group was removed using palladium-catalyzed hydrogenation. The obtained amine was alkylated with the requisite commercially available aryl aldehydes and the Boc protecting group was removed to give **11** and **12**. Finally, alkylation of **11** or **12** with the requisite aryl aldehydes by reductive amination reaction yielded **15** and **16a**–**16c**.

Analogs **21a**–**21c** were synthesized as shown in Chart 3. Dealkylation of the methyl ether of **17**[16] using $AlCl_3$ gave **18**. As with **10**, described above, **18** was alkylated with *tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate, hydrolyzed, condensed with **31**, and subsequent deprotection of the Boc group yielded **20**. Compound **20** was coupled with aldehyde **29** or



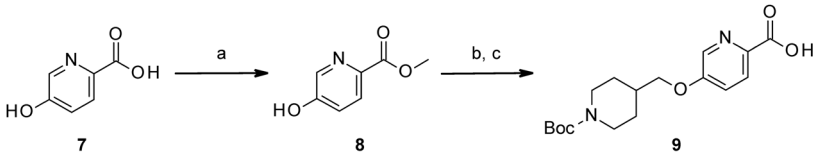
Reagents and conditions: (a) 4-methoxybenzyl chloride, DMF, room temperature (r.t.); (b) 4.0 mol/L NaOH*aq.*, THF, EtOH, r.t., 63% for 2 steps; (c) 1-{[4-(trifluoromethyl)phenyl]methyl}piperazine, WSC·HCl, HOBt, DMF, r.t., 79%; (d) **3**, $(COCl)_2$, DMF, CH_2Cl_2, 0°C to r.t., then **5**, pyridine, CH_2Cl_2, 0°C to r.t., then 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., 40%.

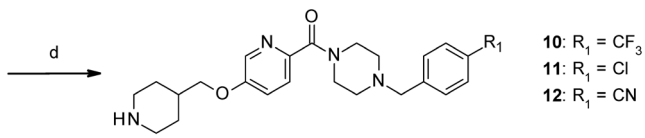
Chart 1.

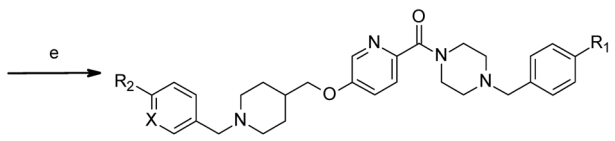
benzyl chloride **35** by reductive amination reaction or alkylation reaction to give **21a**–**21c**.

Analogs **26** and **27** were generated using the synthetic route outlined in Chart 4. Alkylation of **18** with the requisite 4-(hydroxymethyl)piperidine derivatives by Mitsunobu reaction using cyanomethylenetributylphosphorane (Tsunoda's reagent),[17] and subsequent ester hydrolysis gave **22** and **23**. Compounds **22** and **23** were condensed with **31** and subsequent removal of the Boc protecting group gave **24** and **25**. As with **21** in Chart 3, compounds **24** and **25** were coupled with aldehyde **29** or benzyl chloride **35b** to yield **26a**, **26b**, **27a** and **27b**.

The synthetic routes of the intermediates derived in Chart 1–4 are summarized in Chart 5. Compound **29** was synthesized by lithiation of commercially available bromide **28** with *n*-BuLi, and subsequent trapping with DMF. *tert*-Butyl piperazine-1-carboxylate (**30**) was alkylated with 4-formylbenzonitrile, and subsequent deprotection of the Boc group yielded **31**. For the synthesis of **35**, ipso-substitution of commercially available **32** with methanol or ethanol gave **33a** and **33b**. Reduction of the ester group of **33a** and **33b** using $NaBH_4$, and subsequent chlorination of the benzyl alcohol group gave **35a** and **35b**. Compound **37** was synthesized by *p*-toluenesulfonic acid-catalyzed epoxide opening of known **36**[19] with methanol.



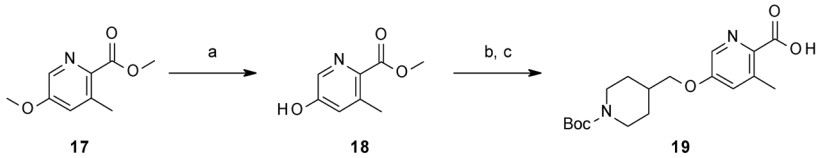


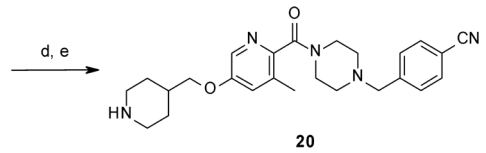


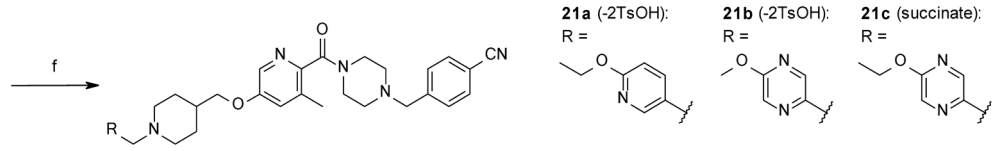
compound	X	R_2	R_1
13 (-3HCl)	CH	OMe	CF_3
14 (-2TsOH)	N	OMe	CF_3
15 (-2TsOH)	N	OMe	Cl
16a (-2TsOH)	N	OMe	CN
16b (-4HCl)	N	Me	CN
16c (-2TsOH)	N	OEt	CN

Reagents and conditions: (a) c.H_2SO_4, MeOH, 85°C, 76%; (b) *tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate, 2.2 mol/L DEAD in toluene, PPh_3, THF, 0°C to r.t.; (c) 1.0 mol/L NaOH*aq*., THF, MeOH, 60°C, 99% for 2 steps; (d) **10**: 1-{[4-(trifluoromethyl)phenyl]methyl}piperazine, WSC·HCl, HOBt, CH_2Cl_2, r.t., then 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., 88%; **11**, **12**: benzyl piperazine-1-carboxylate, WSC·HCl, HOBt, CH_2Cl_2, r.t., then 10% Pd/C (wetted with approx. 50% water), H_2 (1.0 kgf/cm^2), EtOH, r.t., then ArCHO, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t., then 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., 33%; (e) **14**, **15**, **16a**, **16c**: ArCHO, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t., then *p*-TsOH·H_2O, acetone, r.t., 53–72%; **13**, **16b**: ArCHO, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t., then 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., 38–51%.

Chart 2.







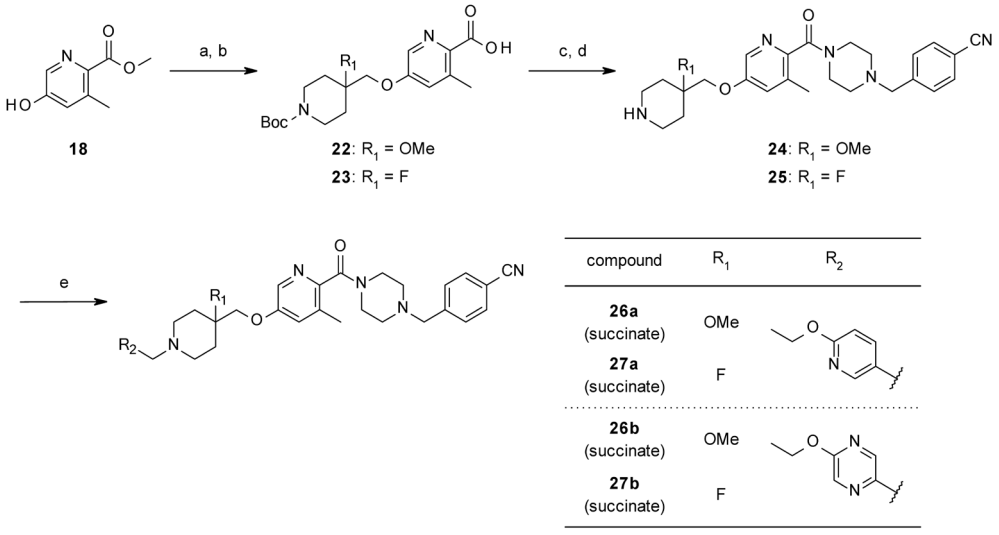
Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, 55°C, 80%; (b) *tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate, 2.2 mol/L DEAD in toluene, PPh_3, THF, 0°C to r.t.; (c) 1.0 mol/L NaOH*aq*., THF, MeOH, 60°C, 69% for 2 steps; (d) **31**, WSC·HCl, HOBt, Et_3N, CH_2Cl_2, r.t.; (e) 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., quant. for 2 steps; (f) **21a**: **29**, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t., then *p*-TsOH·H_2O, acetone, r.t., 56%; **21b**: **35a**, K_2CO_3, MeCN, r.t., then *p*-TsOH·H_2O, MeOH, r.t., 59%; **21c**: **35b**, DIPEA, MeCN, r.t., then succinic acid, EtOH, r.t., 74%; Structures of compounds **29**, **31**, **35a** and **35b** were indicated in Chart 5.

Chart 3.

Results and Discussion

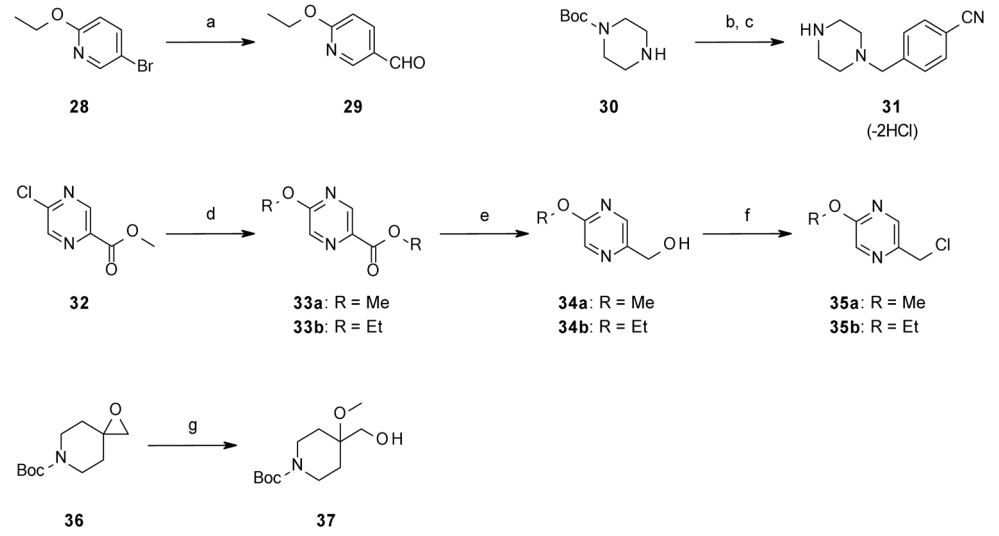
The AMPK activation activity of the synthesized compounds was measured by phospho-ACC whole cell Enzyme-Linked ImmunoSorbent Assay (ELISA) using the MDA-MB-453 human breast cancer cell line. Selected compounds were also evaluated for aqueous solubility in the Japanese Pharmacopoeia 2nd fluid for disintegration test (JP2; pH = 6.8), *in vitro* metabolic stability in human liver microsomes (HLM) and hERG inhibitory activity.

First, we introduced nitrogen atoms into the central phenyl moiety to improve the aqueous solubility of **1** (Table 1). Introducing hetero atoms into compounds to reduce the hydrophobicity is a widely used strategy for improving aqueous solubility[20] because a compound's aqueous solubility generally depends on its hydrophobicity.[21] Compound **6** exhibited good aqueous solubility at gastrointestinal pH (≥100 μM) with 6-fold lower AMPK activating activity than that of **1**. Next, we attempted to replace the anilide linkage with an ether linkage, which we expected would eliminate the potential for metabolic hydrolysis. Surprisingly, compound **13** with an ether linkage maintained good aqueous solubility, despite showing similar hydrophobicity to compound **1**. Recently, Walker sug-



Reagents and conditions: (a) **22**: **37**, cyanomethylenetributylphosphorane, toluene, 100°C; **23**: *tert*-butyl 4-fluoro-4-(hydroxymethyl)piperidine-1-carboxylate,[18] cyanomethylenetributylphosphorane, toluene, 110°C; (b) 1.0 mol/L NaOH*aq*., MeOH, 60°C, 54–91% for 2 steps; (c) **31**, WSC·HCl, HOBt, Et_3N, CH_2Cl_2, r.t.; (d) 4.0 mol/L HCl in 1,4-dioxane, MeOH, r.t., quant. for 2 steps; (e) **26a**, **27a**: **29**, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t., then succinic acid, 61–68%; **26b**, **27b**: **35b**, DIPEA, MeCN, r.t. to 50°C, then succinic acid, 65–68%; Structures of compounds **29**, **31** **35b** and **37** were indicated in Chart 5.

Chart 4.



Reagents and conditions: (a) 1.7 mol/L *n*-BuLi in hexane, THF, −68°C then DMF, −68°C to r.t., 59%; (b) 4-formylbenzonitrile, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t.; (c) 4.0 mol/L HCl in EtOAc, EtOAc, $CHCl_3$, r.t., 77% for 2 steps; (d) **33a**: MeONa, MeOH, 0°C to r.t., 81%; **33b**: *t*-BuOK, EtOH, 0°C, 82%; (e) $NaBH_4$, MeOH, 0°C to r.t., 75–77%; (f) $SOCl_2$, CH_2Cl_2, 0°C to r.t., quant.; (g) *p*-TsOH·H_2O, MeOH, r.t., 52%.

Chart 5.

gested that intermolecular hydrogen-bonding in the solid state may lead to increased crystalline stability and reduced aqueous solubility.[22] Compound **13** possesses no hydrogen bond donors, suggesting that it may have lower crystalline stability than **6**, which would contribute to the maintenance of good aqueous solubility. Furthermore, compound **13** showed about 10-fold more potent cellular activity than that of **6**.

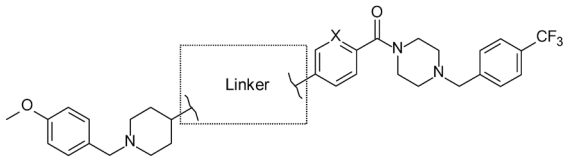
Given that compound **13** showed both good aqueous solubility and potent AMPK activating activity, we subjected it to further *in vitro* profiling studies. The results are summarized in Table 2. Compound **13** showed good metabolic stability in human liver microsomes. In contrast, potent inhibitory activity for the hERG channel was confirmed in the auto patch clamp hERG blockade assay ($IC_{50} = 0.74\,\mu M$). Inhibition of the hERG channel can cause delayed ventricular cell repolarization, which results in prolongation of the QT interval, leading to serious risk of arrhythmias and sudden death.[24,25] In general, the pharmacophore of hERG blockers contains a basic center flanked by a hydrophobic group.[26] As shown in Table 2, compound **13** showed high basicity and hydrophobicity. Therefore, we attempted to decrease both the basicity and hydrophobicity of **13** to reduce the hERG inhibitory activity.

First, we introduced another nitrogen atom into the left aromatic moiety to reduce its hydrophobicity and basicity. Resulting compound **14** showed good aqueous solubility and metabolic stability, but continued to show moderate inhibitory activity against the hERG channel. Next, we replaced the hydrophobic CF_3 moiety with a less hydrophobic chloro group (**15**). Compound **15** exhibited good aqueous solubility and potency, but dramatically elevated *in vitro* CL_{int} in HLM. Pre-

viously, a study of Pfizer reported that the nitrile group was a less hydrophobic isostere of the chloro group, and that such modification led to improved medicinal chemistry properties, such as physicochemical and ADME properties.[27] In reference to this report, we attempted to replace the chloro group in **15** with a cyano group (**16a**). While compound **16a** showed 7-fold less potent cellular activity ($EC_{50} = 0.036\,\mu M$) than the CF_3 congener (**14**), the metabolic stability of **16a** was dramatically improved (HLM $CL_{int,\ vitro} = 58$ mL/min/kg) compared to that of **15**. Furthermore, **16a** showed a 4-fold reduction in hERG channel inhibitory activity compared to **14**. The hydrophobicity and basicity of **16a** was the lowest among the compounds in Table 3. This suggests that the favorable physical properties led to improved metabolic stability and reduced hERG inhibitory activity as expected.

While compound **16a** showed moderate AMPK activating activity, its other *in vitro* properties were promising, including excellent aqueous solubility, good *in vitro* metabolic stability and weak hERG inhibitory activity. Therefore, we tried to further optimize substituents of the center and left aromatic moiety of **16a** to improve its *in vitro* cellular activity (Table 4). Replacement of the methoxy group on the left moiety with a methyl group (**16b**) produced a compound with slightly weaker potency ($EC_{50} = 0.054\,\mu M$) than that of **16a**. However, replacement with an ethoxy group (**16c**) resulted in a slight improvement in potency ($EC_{50} = 0.019\,\mu M$). Further improvement in cellular activity was observed when a methyl group was introduced into the central pyridine moiety (**21a**), with the resulting compound showing an EC_{50} of $0.0093\,\mu M$. Replacement of the left pyridine moiety with a pyrazine moiety (**21b** and **21c**) led to further improvement in the metabolic stability (HLM $CL_{int,\ vitro} = 46$ and 44 mL/min/kg, respectively) probably due to decreased hydrophobicity. However, cellular activity was reduced in methoxy analog **21b** ($EC_{50} = 0.043\,\mu M$). Similar to the pyridine series, ethoxy analog **21c** showed

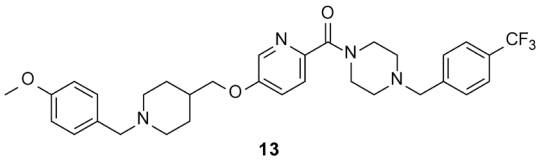
Table 1. AMPK Activation Activity and Aqueous Solubility of **6** and **13** Compared to **1**



Compound	X	Linker	AMPK activation EC_{50} (μM)[a]	JP2 (μM)[b]	$c\mathrm{Log}P$[c]
1[d]	CH		0.011	<1	4.9
6[e]	N		0.089	≥100	3.6
13[e]	N		0.0083	≥100	4.8

[a] The EC_{50} values were examined by whole cell ELISA using the MDA-MB-453 cell line and determined in triplicate in one experiment. [b] Aqueous solubility in JP2 (pH = 6.8). [c] $c\mathrm{Log}P$ values were calculated using ACD Log*P* prediction software.[23] [d] Dihydrochloride. [e] Trihydrochloride.

Table 2. AMPK Activation Activity, Aqueous Solubility, Metabolic Stability and hERG Inhibitory Activity of **13**

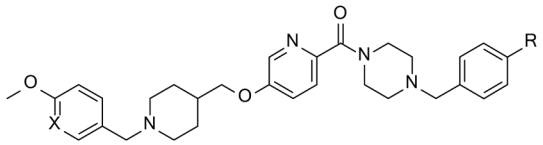


13

Compound	AMPK activation EC_{50} (μM)[a]	JP2 (μM)[b]	HLM $CL_{int,\ vitro}$ (mL/min/kg)	hERG inhibition IC_{50} (μM)	$c\mathrm{Log}P$[c]	Calculated most basic pK_a[c]
13[d]	0.0083	≥100	97	0.74	4.8	9.2

[a] The EC_{50} value was examined by whole cell ELISA using the MDA-MB-453 cell line and determined in triplicate in one experiment. [b] Aqueous solubility in JP2 (pH = 6.8). [c] $c\mathrm{Log}P$ and pK_a values were calculated using ACD Log*P* prediction software.[23] [d] Trihydrochloride.

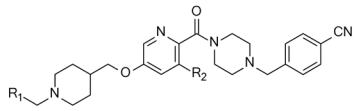
Table 3. AMPK Activation Activity, Aqueous Solubility, Metabolic Stability and hERG Inhibitory Activity of **13**–**16a**

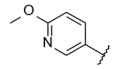
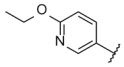
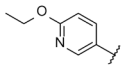
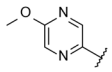
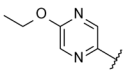


Compound	X	R	AMPK activation EC_{50} (μM)[a]	JP2 (μM)[b]	HLM $CL_{int,\ vitro}$ (mL/min/kg)	hERG inhibition IC_{50} (μM)	$c\mathrm{Log}P$[c]	Calculated most basic pK_a[c]
13[d]	CH	CF_3	0.0083	≥100	97	0.74	4.8	9.2
14[e]	N	CF_3	0.0051	≥100	94	3.4	3.8	8.0
15[e]	N	Cl	0.018	≥100	523	NT[f]	3.4	8.0
16a[e]	N	CN	0.036	≥100	58	13	2.5	8.0

[a] The EC_{50} values were examined by whole cell ELISA using the MDA-MB-453 cell line and determined in duplicate or triplicate in one experiment. [b] Aqueous solubility in JP2 (pH = 6.8). [c] $c\mathrm{Log}P$ and pK_a values were calculated using ACD Log*P* prediction software.[23] [d] Trihydrochloride. [e] Ditosylate. [f] NT: not tested.

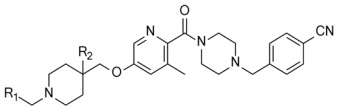
Table 4. AMPK Activation Activity, Aqueous Solubility and Metabolic Stability of **16a–c** and **21a–c**

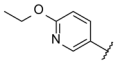
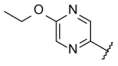


Compound	R_1	R_2	AMPK activation EC_{50} (μM)[a]	JP2 (μM)[b]	HLM $CL_{int, vitro}$ (mL/min/kg)	$c\mathrm{Log}P$[c]
16a[d]		H	0.036	≥100	58	2.5
16b[e]		H	0.054	≥100	NT[g]	2.7
16c[d]		H	0.019	≥100	61	2.7
21a[d]		Me	0.0093	≥100	83	3.2
21b[d]		Me	0.043	≥100	46	2.7
21c[f]		Me	0.025	≥100	44	2.9

[a] The EC_{50} values were examined by whole cell ELISA using the MDA-MB-453 cell line and determined in duplicate or triplicate in one experiment. [b] Aqueous solubility in JP2 (pH = 6.8). [c] $c\mathrm{Log}P$ values were calculated using ACD Log$P$ prediction software.[23] [d] Ditosylate. [e] Tetrahydrochloride. [f] Succinate. [g] NT: not tested.

Table 5. AMPK Activation Activity, Aqueous Solubility and Metabolic Stability of **26a–b** and **27a–b**



Compound	R_1	R_2	AMPK activation EC_{50} (μM)[a]	JP2 (μM)[b]	HLM $CL_{int, vitro}$ (mL/min/kg)	$c\mathrm{Log}P$[c]
21a[d]		H	0.0093	≥100	83	3.2
26a[e]		OMe	0.035	≥100	NT[f]	3.2
27a[e]		F	0.0038	≥100	168	3.2
21c[e]		H	0.025	≥100	44	2.9
26b[e]		OMe	0.082	≥100	NT[f]	2.8
27b[e]		F	0.011	≥100	89	2.7

[a] The EC_{50} values were examined by whole cell ELISA using the MDA-MB-453 cell line and determined in duplicate or triplicate in one experiment. [b] Aqueous solubility in JP2 (pH = 6.8). [c] $c\mathrm{Log}p$ values were calculated using ACD Log$P$ prediction software.[23] [d] Ditosylate. [e] Succinate. [f] NT: not tested.

Table 6. hERG Inhibitory Activity of **21a** and **27b** Compared to **13** and ASP4132

Compound	hERG inhibition IC_{50} (μM)	$c\mathrm{Log}P$[a]	Calculated most basic pK_a[a]
13[b]	0.74	4.8	9.2
21a[c]	16	3.2	8.0
27b[d]	> 30	2.7	7.1
ASP4132[c]	2.0	3.5	8.3

[a] $c\mathrm{Log}P$ and pK_a values were calculated using ACD Log$P$ prediction software.[23] [b] Trihydrochloride. [c] Ditosylate. [d] Succinate.

slightly more potent cellular activity ($EC_{50} = 0.025\,\mu$M) than **21b** in the pyrazine series. Based on these results, we chose compound **21a** and **21c** as a promising compound and conducted further modifications.

To further improve the AMPK activating activity, we focused on introducing substituents into the 4-position of the piperidine linker, into which we had yet to introduce any substituents (Table 5). Introduction of a methoxy group into the 4-position of the piperidine linker (**26a** and **26b**) produced compounds with 3-fold weaker potency ($EC_{50} = 0.035$ and $0.082\,\mu$M, respectively) than that of non-substituted analogs. Surprisingly, introduction of a fluoro group (**27a** and **27b**) resulted in 2- to 3-fold more potent cellular activity ($EC_{50} = 0.0038$ and $0.011\,\mu$M, respectively) than that of non-substituted analogs. Unfortunately, ethoxypyridine analog **27a** showed high *in vitro* metabolic clearance (HLM $CL_{int,\ vitro} = 168$ mL/min/kg). However, its pyrazine analog **27b** showed significant metabolic stability (HLM $CL_{int, vitro} = 89$ mL/min/kg) that was similar to that of **21a**.

Based on their pharmacological, physicochemical and ADME properties, compounds **21a** and **27b** were selected for further profiling. The hERG inhibitory activity of these compounds is summarized in Table 6. Compound **21a** showed 22-fold lower inhibitory activity ($IC_{50} = 16\,\mu$M) than that of **13**. Compound **27b** showed even lower hERG inhibitory activity, with an IC_{50} over $30\,\mu$M. In addition, the hERG inhibitory activity of **27b** was significantly weaker than ASP4132. A clear reverse correlation was observed between the hERG inhibitory activity and physical properties; that is, decreasing hydrophobicity and basicity was effective for weakening a compound's hERG inhibitory activity.

Overall, **27b** displayed promising AMPK activating activity, aqueous solubility, metabolic stability and hERG inhibitory activity, which reflect the fact that it had the lowest hydrophobicity and basicity among the series of compounds. Next, we evaluated the pharmacological, pharmacokinetic and physicochemical properties of **27b** *in vitro* and *in vivo* (Table 7). Compound **27b** showed comparable AMPK activating activity and cell growth inhibitory activity against MDA-MB-453 to that of ASP4132. Furthermore, **27b** showed relatively weak antiproliferative activity against SK-BR-3, suggesting that it maintained the selective cell growth inhibitory activity observed in ASP4132. In addition, the aqueous solubility of **27b** at gastrointestinal pH was dramatically improved over that of ASP4132.

Next, we conducted a pharmacokinetic study of compound **27b** in Sprague-Dawley (SD) rats. The PK parameters of **27b** are shown in Table 8. Compound **27b** showed good oral bioavailability (F = 83%) and relatively low total body clearance ($CL_{tot} = 2.5$ mL/min/kg) compared to the hepatic blood flow rate in rats (55.2 mL/min/kg).[28]

To evaluate the *in vivo* efficacy of **27b**, MDA-MB-453 xenografts were established in nude mice. Upon tumor establishment, dosing of compound **27b** was initiated with *per os* (*p.o.*) doses of 1 to 4 mg/kg, twice daily (BID). The results are summarized in Fig. 3. The tumor growth inhibition (TGI) rate was 83% at 1 mg/kg (BID), and the tumor regression rates

Table 7. Summary of *In Vitro* Profiles of **27b** Compared to ASP4132

Compound	AMPK activation EC_{50} (μM)	MDA-MB-453 growth inhibition IC_{50} (μM)	SK-BR-3 growth inhibition IC_{50} (μM)	HLM $CL_{int,\ vitro}$ (mL/min/kg)	Aqueous solubility (μM)[a] JP1/JP2/JP2 + TC[b]
ASP4132	0.018[d]	0.014[d]	>3[d]	61	≥100/5.6/≥100
27b[c]	0.011[e]	0.026[f]	21% inh. at 3 μM[f]	89	≥100/≥100/≥100

[a] Aqueous solubility in JP1 (pH = 1.2) and JP2 (pH = 6.8). [b] TC: taurocholic acid. [c] Succinate. [d] The geometric mean of the EC_{50} and IC_{50} values from 3 independent experiments are shown. [e] The EC_{50} value was examined by whole cell ELISA using the MDA-MB-453 cell line and determined in triplicate in one experiment. [f] The IC_{50} value was determined in triplicate in one experiment.

Table 8. Pharmacokinetic Properties of **27b** in SD Rats

Compound	i.v.[b] (1 mg/kg)				*p.o.*[b] (1 mg/kg)			
	AUC_{24h}[c] (ng·h/mL)	$t_{1/2}$[d] (h)	V_{ss}[e] (L/kg)	CL_{tot}[f] (mL/min/kg)	AUC_{24h}[c] (ng·h/mL)	C_{max}[g] (ng/mL)	t_{max}[h] (h)	F[i] (%)
27b[a]	6580	2.7	0.42	2.5	5470	1090	1.3	83

[a] Succinate. [b] i.v.: intravenous, *p.o.*: oral administration. [c] Area under the plasma concentration *versus* time curve from time zero to 24 h after dosing. [d] Elimination half-life from plasma. [e] Volume of distribution at steady state. [f] Total body clearance. [g] Maximum plasma concentration. [h] Time to reach maximum plasma concentration. [i] Absolute oral bioavailability.

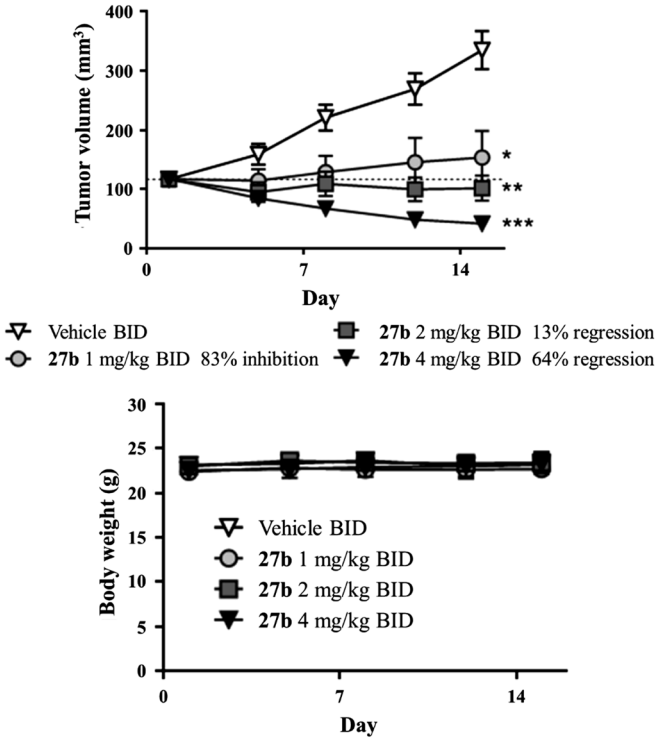


Fig. 3. Antitumor Efficacy of **27b**[a] at 1, 2 and 4 mg/kg by Twice-Daily Dosing in the MDA-MB-453 Xenograft Mouse Model (Top)[b] and the Effect on Body Weight (Bottom)[c]

[a] Succinate. [b] Mice were treated with **27b** from Days 1 to 14. Each point represents the mean ± S.E.M. ($N = 5$). Statistical analysis was performed the values on Day 15. *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$ compared with the value of vehicle on Day 15 (Dunnett's multiple comparison test). [c] Mice were treated with **27b** from Days 1 to 14. Each point represents the mean ± S.E.M. ($N = 5$).

were 13% and 64% at 2 and 4 mg/kg (BID), respectively. All doses of **27b** were well tolerated over the 14-d dosing window without body weight loss.

Conclusion

We reported the medicinal chemistry study behind the development of the second-generation clinical candidate, 4-({4-[5-({1-[(5-ethoxypyrazin-2-yl)methyl]-4-fluoropiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile succinate (**27b**), as an indirect AMPK activator. We conducted a structure–activity relationship study with the aim of identifying a compound with improved aqueous solubility at gastrointestinal conditions (JP2; pH = 6.8) compared to ASP4132. As a result, we identified the compound **13**, which showed good aqueous solubility at pH = 6.8 but potent inhibition of hERG potassium channels. Further optimization efforts along with strategies to decrease hydrophobicity and basicity resulted in the identification of compound **27b**, which had potent cellular activity, good metabolic stability and aqueous solubility, favorable animal PK properties and desirable *in vivo* anti-tumor efficacy on oral dosing. Our findings suggested that **27b** was a promising AMPK activator and a second-generation clinical candidate for treatment for human cancer.

Experimental

Chemistry ^{1}H-NMR spectra were recorded on a Varian VNS-400, Varian 400-MR, JEOL JNM Lambda-300, JEOL JNM Lambda-400 or Bruker AVANCE III-HD500 spectrometer. Chemical shifts were expressed in δ values (ppm) using tetramethylsilane as the internal standard (s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, ddd = double double doublet, m = multiplet and br = broad peak). MS were recorded on a JEOL GC Mate II, Waters SQD, Waters ZQ-2000, Thermo Fisher LCQ Advantage or Thermo Fisher Exactive Plus Orbitrap. All reactions were performed using commercially available reagents and solvents without further purification. The following abbreviations are used: AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; BuLi, butyllithium; *t*-BuOK, potassium *tert*-butoxide; DEAD, diethyl (*E*)-diazene-1,2-dicarboxylate; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; Et_3N, triethylamine; Et_2O, diethylether; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1*H*-benzotriazol-1-ol; IPA, isopropyl alcohol; MeCN, acetonitrile; MeOH, methanol; $NaBH(OAc)_3$, sodium triacetoxyborohydride; PPh_3, triphenylphosphine; THF, tetrahydrofuran; *p*-TsOH·H_2O, 4-methylbenzenesulfonic acid monohydrate; WSC·HCl, *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride.

1-[(4-Methoxyphenyl)methyl]piperidine-4-carboxylic Acid (3) To a solution of 4-methoxybenzyl chloride (1.1 mL,

7.7 mmol) in DMF (10 mL) was added ethyl piperidine-4-carboxylate (**2**; 3.0 mL, 19 mmol). After stirring at room temperature for 16 h, the mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with H_2O, saturated $NaHCO_3$ aqueous solution and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in THF (15 mL) and EtOH (10 mL) was added 4.0 mol/L NaOH aqueous solution (13 mL, 52 mmol). After stirring at room temperature for 3 d, the mixture was acidified with concentrated HCl*aq.* (approximately pH 5) and concentrated *in vacuo*. To the residue was added $CHCl_3$/MeOH (2 : 1), the mixture was filtered and the filtrate was concentrated *in vacuo*. To the residue was added EtOAc, and the precipitated solid was collected by filtration to obtain the product (1.2 g, 63%). ^{1}H-NMR (DMSO-d_6) δ: 1.46–1.63 (2H, m), 1.69–1.88 (2H, m), 1.90–2.30 (3H, m), 2.64–2.88 (2H, m), 3.08–3.63 (2H, m), 3.73 (3H, s), 6.88 (2H, d, J = 8.4 Hz), 7.21 (2H, d, J = 8.4 Hz); MS *m/z*: 250 $(M+H)^+$.

(5-Aminopyridin-2-yl)(4-{[4-(trifluoromethyl)phenyl]-methyl}piperazin-1-yl)methanone (5) To a solution of 5-aminopyridine-2-carboxylic acid (**4**; 200 mg, 1.4 mmol) in DMF (3.3 mL) were added 1-{[4-(trifluoromethyl)phenyl]-methyl}piperazine (360 mg, 1.5 mmol), WSC·HCl (330 mg, 1.7 mmol) and HOBt (250 mg, 1.9 mmol). After stirring at room temperature overnight, the mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with H_2O, saturated $NaHCO_3$ aqueous solution and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH) to obtain the product (420 mg, 79%). ^{1}H-NMR ($CDCl_3$) δ: 2.32–2.69 (4H, m), 3.58 (2H, s), 3.72–3.84 (4H, m), 3.90 (2H, s), 7.01 (1H, dd, J = 2.7, 8.4 Hz), 7.46 (2H, d, J = 8.0 Hz), 7.54–7.60 (3H, m), 7.98 (1H, d, J = 2.7 Hz); MS *m/z*: 365 $(M+H)^+$.

1-[(4-Methoxyphenyl)methyl]-*N*-[6-(4-{[4-(trifluoromethyl)phenyl]methyl}piperazine-1-carbonyl)pyridin-3-yl]piperidine-4-carboxamide Trihydrochloride (6) To a solution of 1-[(4-methoxyphenyl)methyl]piperidine-4-carboxylic acid (**3**; 110 mg, 0.45 mmol) in CH_2Cl_2 (4.0 mL) were added $(COCl)_2$ (44 μL, 0.51 mmol) and DMF (1 drop) at 0°C. After stirring at room temperature for 1 h, to the mixture were added a solution of (5-aminopyridin-2-yl)(4-{[4-(trifluoromethyl)phenyl]methyl}piperazin-1-yl)-methanone (**5**; 140 mg, 0.37 mmol) in CH_2Cl_2 (2.0 mL) and pyridine (76 μL, 0.95 mmol) at 0°C. After stirring at room temperature for 4 h, the mixture was diluted with H_2O and extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a mixture of the residue in MeOH (3.0 mL) was added 4.0 mol/L HCl in 1,4-dioxane (0.50 mL, 2.0 mmol). After stirring at room temperature for 15 min, the mixture was concentrated *in vacuo*. To the residue was added Et_2O, and the precipitated solid was collected by filtration to obtain the product (110 mg, 40%). ^{1}H-NMR (CD_3OD) δ: 1.93–2.36 (4H, m), 2.73–2.86 (1H, m), 3.03–3.16 (2H, m), 3.20–3.74 (10H, m), 3.83 (3H, s), 4.28 (2H, s), 4.52 (2H, s), 7.00–7.07 (2H, m), 7.43–7.48 (2H, m), 7.77–7.86 (5H, m), 8.28 (1H, dd, J = 2.4, 8.6 Hz), 8.94 (1H, d, J = 2.4 Hz); MS *m/z*: 596 $(M+H)^+$; ESI-MS *m/z*: 596.2836 $(M+H)^+$ (Calcd for $C_{32}H_{37}O_3N_5F_3$: 596.2843).

Methyl 5-Hydroxypyridine-2-carboxylate (8) To a solution of 5-hydroxypyridine-2-carboxylic acid (**7**; 10 g, 72 mmol) in MeOH (120 mL) was added concentrated H_2SO_4 (8.0 mL, 150 mmol). The mixture was stirred at 85°C overnight. After cooling to 0°C, the mixture was basified with 1.0 mol/L NaOH aqueous solution (approximately pH 9), and then acidified with 10% citric acid aqueous solution (approximately pH 5). The mixture was extracted with $CHCl_3$/IPA (4 : 1), the organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (8.4 g, 76%). ^{1}H-NMR (DMSO-d_6) δ: 3.82 (3H, s), 7.27 (1H, dd, J = 2.8, 8.6 Hz), 7.94 (1H, dd, J = 0.5, 8.6 Hz), 8.22 (1H, dd, J = 0.5, 2.8 Hz), 10.81 (1H, s); MS *m/z*: 154 $(M+H)^+$.

5-{[1-(*tert*-Butoxycarbonyl)piperidin-4-yl]methoxy}pyridine-2-carboxylic Acid (9) To a solution of methyl 5-hydroxypyridine-2-carboxylate (**8**; 2.0 g, 13 mmol), *tert*-butyl 4-(hydroxymethyl)piperidine-1-carboxylate (3.0 g, 14 mmol) and PPh_3 (5.0 g, 19 mmol) in THF (40 mL) was added dropwise 2.2 mol/L DEAD in toluene (9.0 mL, 20 mmol) at 0°C. After stirring at room temperature overnight, the mixture was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc). To a solution of the residue in THF (20 mL) and MeOH (20 mL) was added 1.0 mol/L NaOH aqueous solution (40 mL, 40 mmol). The mixture was stirred at 60°C for 1 h. After cooling to room temperature, the mixture was acidified with 10% citric acid aqueous solution (approximately ~pH 5). The mixture was extracted with $CHCl_3$/IPA (4 : 1), the organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (4.4 g, 99%). ^{1}H-NMR (DMSO-d_6) δ: 1.10–1.25 (2H, m), 1.40 (9H, s), 1.70–1.81 (2H, m), 1.91–2.05 (1H, m), 2.60–2.91 (2H, m), 3.91–4.09 (4H, m), 7.50 (1H, dd, J = 2.8, 8.6 Hz), 8.01 (1H, d, J = 8.6 Hz), 8.36 (1H, d, J = 2.8 Hz), 8.97 (1H, s); MS *m/z*: 337 $(M+H)^+$.

{5-[(Piperidin-4-yl)methoxy]pyridin-2-yl}(4-{[4-(trifluoromethyl)phenyl]methyl}piperazin-1-yl)methanone (10) To a mixture of 5-{[1-(*tert*-butoxycarbonyl)piperidin-4-yl]-methoxy}pyridine-2-carboxylic acid (**9**; 1.4 g, 4.2 mmol), 1-{[4-(trifluoromethyl)phenyl]methyl}piperazine (0.85 mL, 4.3 mmol) and CH_2Cl_2 (25 mL) were added WSC·HCl (1.1 g, 5.7 mmol) and HOBt (750 mg, 5.6 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in MeOH (10 mL) was added 4.0 mol/L HCl in 1,4-dioxane (10 mL, 40 mmol). After stirring at room temperature for 2.5 h, the mixture was basified with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$/IPA (4 : 1). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (1.7 g, 88%). ^{1}H-NMR (DMSO-d_6) δ: 1.10–1.24 (2H, m), 1.64–1.74 (2H, m), 1.77–1.93 (1H, m), 2.24–2.57 (6H, m), 2.91–3.02 (2H, m), 3.32–3.73 (6H, m), 3.92 (2H, d, J = 6.4 Hz), 7.47 (1H, dd, J = 2.9, 8.8 Hz), 7.52–7.58 (3H, m), 7.69 (2H, d, J = 8.2 Hz), 8.24 (1H, dd, J = 0.7, 2.9 Hz); MS *m/z*: 463 $(M+H)^+$.

{4-[(4-Chlorophenyl)methyl]piperazin-1-yl}{5-[(piperidin-4-yl)methoxy]pyridin-2-yl}methanone (11) To a mixture of 5-{[1-(*tert*-butoxycarbonyl)piperidin-4-yl]methoxy}-

pyridine-2-carboxylic acid (**9**; 3.0 g, 8.8 mmol), benzyl piperazine-1-carboxylate (1.8 mL, 9.3 mmol) and CH_2Cl_2 (50 mL) were added WSC·HCl (2.3 g, 12 mmol) and HOBt (1.6 g, 12 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in EtOH (40 mL) was added 10% Pd/C (wetted with approx. 50% water, 1.0 g). After stirring under a hydrogen atmosphere (1.0 kgf/cm^2) at room temperature for 3 d, to the mixture was added 10% Pd/C (wetted with *ca*. 50% water, 1.5 g). After stirring under a hydrogen atmosphere (1.0 kgf/cm^2) at room temperature for 2 d, the mixture was filtered through a Celite pad and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH) to obtain the intermediate (1.2 g). To a solution of the obtained intermediate (600 mg, 1.5 mmol) in CH_2Cl_2 (12 mL) were added 4-chlorobenzaldehyde (240 mg, 1.7 mmol), acetic acid (10 μL, 0.17 mmol) and $NaBH(OAc)_3$ (800 mg, 3.8 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in MeOH (6.0 mL) was added 4.0 mol/L HCl in 1,4-dioxane (6.0 mL, 24 mmol). After stirring at room temperature for 1 h, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$/IPA (4 : 1). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (640 mg, 33%). ^{1}H-NMR (DMSO-d_6) δ: 1.10–1.25 (2H, m), 1.64–1.72 (2H, m), 1.76–1.91 (1H, m), 2.26–2.55 (6H, m), 2.91–3.00 (2H, m), 3.41–3.69 (6H, m), 3.91 (2H, d, J = 6.4 Hz), 7.29–7.42 (4H, m), 7.46 (1H, dd, J = 2.9, 8.8 Hz), 7.55 (1H, dd, J = 0.7, 8.8 Hz), 8.24 (1H, dd, J = 0.7, 2.9 Hz); MS *m/z*: 429 $(M+H)^+$.

4-[(4-{5-[(Piperidin-4-yl)methoxy]pyridine-2-carbonyl}-piperazin-1-yl)methyl]benzonitrile (12) Compound **12** was prepared from **9** and 4-formylbenzonitrile in 33% yield using a similar approach to that described for **11**. ^{1}H-NMR (DMSO-d_6) δ: 1.10–1.29 (2H, m), 1.64–1.73 (2H, m), 1.75–1.92 (1H, m), 2.30–2.56 (6H, m), 2.90–3.01 (2H, m), 3.48–3.68 (6H, m), 3.92 (2H, d, J = 6.4 Hz), 7.44–7.59 (4H, m), 7.76–7.83 (2H, m), 8.24 (1H, dd, J = 0.4, 2.4 Hz); MS *m/z*: 420 $(M+H)^+$.

[5-({1-[(4-Methoxyphenyl)methyl]piperidin-4-yl}-methoxy)pyridin-2-yl](4-{[4-(trifluoromethyl)phenyl]-methyl}piperazin-1-yl)methanone Trihydrochloride (13) To a solution of {5-[(piperidin-4-yl)methoxy]pyridin-2-yl}(4-{[4-(trifluoromethyl)phenyl]methyl}piperazin-1-yl)methanone (**10**; 300 mg, 0.65 mmol) in CH_2Cl_2 (6.0 mL) were added 4-methoxybenzaldehyde (90 μL, 0.74 mmol), acetic acid (5.0 μL, 87 μmol) and $NaBH(OAc)_3$ (300 mg, 1.4 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in MeOH was added excess 4.0 mol/L HCl in 1,4-dioxane. The mixture was concentrated *in vacuo*. To the residue was added Et_2O, and the precipitated solid was collected by filtration to obtain the product (230 mg, 51%). ^{1}H-NMR (DMSO-d_6) δ: 1.54–2.15 (5H, m), 2.75–3.73 (10H, m), 3.78 (3H, s), 3.91–4.06 (2H, m), 4.11–4.37 (3H, m), 4.38–4.68 (3H, m), 7.01 (2H, d, J = 8.8 Hz), 7.43–7.61 (3H, m), 7.68 (1H, d, J = 8.8 Hz), 7.78–7.97 (4H, m), 8.27 (1H, d, J = 2.9 Hz), 10.52 (1H, brs), 11.89 (1H, brs); MS *m/z*: 583 $(M+H)^+$; ESI-MS *m/z*: 583.2888 $(M+H)^+$ (Calcd for $C_{32}H_{38}O_3N_4F_3$: 583.2891).

[5-({1-[(6-Methoxypyridin-3-yl)methyl]piperidin-4-yl}-methoxy)pyridin-2-yl](4-{[4-(trifluoromethyl)phenyl]-methyl}piperazin-1-yl)methanone Ditosylate (14) To a solution of {5-[(piperidin-4-yl)methoxy]pyridin-2-yl}(4-{[4-(trifluoromethyl)phenyl]methyl}piperazin-1-yl)-methanone (**10**; 300 mg, 0.65 mmol) in CH_2Cl_2 (6.0 mL) were added 6-methoxypyridine-3-carbaldehyde (100 mg, 0.73 mmol), acetic acid (5.0 μL, 87 μmol) and $NaBH(OAc)_3$ (300 mg, 1.4 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue (230 mg, 0.40 mmol) in acetone was added *p*-TsOH·H_2O (150 mg, 0.79 mmol). The mixture was concentrated *in vacuo*. To the residue was added Et_2O, and the precipitated solid was collected by filtration to obtain the product (320 mg, 53%). ^{1}H-NMR (DMSO-d_6) δ: 1.38–1.65 (2H, m), 1.78–2.17 (3H, m), 2.29 (6H, s), 2.85–3.56 (10H, m), 3.89 (3H, s), 3.99 (2H, d, J = 6.0 Hz), 4.12–4.73 (6H, m), 6.93 (1H, d, J = 8.4 Hz), 7.07–7.16 (4H, m), 7.43–7.60 (5H, m), 7.70 (1H, d, J = 8.6 Hz), 7.75 (2H, d, J = 7.9 Hz), 7.80–7.93 (3H, m), 8.24–8.31 (2H, m), 9.32 (1H, brs), 10.07 (1H, brs); MS *m/z*: 584 $(M+H)^+$; ESI-MS *m/z*: 584.2840 $(M+H)^+$ (Calcd for $C_{31}H_{37}O_3N_5F_3$: 584.2843).

{4-[(4-Chlorophenyl)methyl]piperazin-1-yl}[5-({1-[(6-methoxypyridin-3-yl)methyl]piperidin-4-yl}methoxy)pyridin-2-yl]methanone Ditosylate (15) Compound **15** was prepared from **11** in 72% yield using a similar approach to that described for **14**. ^{1}H-NMR (DMSO-d_6) δ: 1.39–1.65 (2H, m), 1.81–2.19 (3H, m), 2.29 (6H, s), 2.77–3.60 (10H, m), 3.70–4.74 (11H, m), 6.93 (1H, d, J = 9.0 Hz), 7.12 (4H, dd, J = 0.7, 8.4 Hz), 7.45–7.59 (9H, m), 7.62–7.75 (1H, m), 7.80–7.87 (1H, m), 8.18–8.36 (2H, m), 9.27 (1H, brs), 9.89 (1H, brs); MS *m/z*: 550 $(M+H)^+$; ESI-MS *m/z*: 550.2584 $(M+H)^+$ (Calcd for $C_{30}H_{37}O_3N_5Cl$: 550.2579).

4-({4-[5-({1-[(6-Methoxypyridin-3-yl)methyl]piperidin-4-yl}methoxy)pyridine-2-carbonyl]piperazin-1-yl}methyl)-benzonitrile Ditosylate (16a) Compound **16a** was prepared from **12** in 66% yield using a similar approach to that described for **14**. ^{1}H-NMR (DMSO-d_6) δ: 1.35–1.68 (2H, m), 1.78–2.14 (3H, m), 2.29 (6H, s), 2.77–3.60 (10H, m), 3.89 (3H, s), 3.99 (2H, d, J = 6.0 Hz), 4.09–4.71 (6H, m), 6.93 (1H, d, J = 8.4 Hz), 7.12 (4H, d, J = 7.9 Hz), 7.46–7.60 (1H, m), 7.49 (4H, d, J = 7.9 Hz), 7.65–7.76 (3H, m), 7.83 (1H, dd, J = 2.4, 8.6 Hz), 7.97 (2H, d, J = 8.4 Hz), 8.20–8.35 (2H, m), 9.27 (1H, brs), 10.01 (1H, brs); MS *m/z*: 541 $(M+H)^+$; ESI-MS *m/z*: 541.2925 $(M+H)^+$ (Calcd for $C_{31}H_{37}O_3N_6$: 541.2922).

4-({4-[5-({1-[(6-Methylpyridin-3-yl)methyl]piperidin-4-yl}methoxy)pyridine-2-carbonyl]piperazin-1-yl}methyl)-benzonitrile Tetrahydrochloride (16b) Compound **16b** was prepared from **12** and 6-methylpyridine-3-carbaldehyde in 38% yield using a similar approach to that described for **13**. ^{1}H-NMR (DMSO-d_6) δ: 1.54–2.22 (5H, m), 2.57–4.86 (21H,

m), 7.54 (1H, dd, $J=2.9$, 8.8 Hz), 7.68 (1H, d, $J=8.8$ Hz), 7.79–8.04 (4H, m), 8.27 (1H, d, $J=2.9$ Hz), 8.38–8.68 (2H, m), 8.86–9.08 (1H, m), 11.34 (1H, brs), 11.91 (1H, brs); MS *m/z*: 525 $(M+H)^+$; ESI-MS *m/z*: 525.2971 $(M+H)^+$ (Calcd for $C_{31}H_{37}O_2N_6$: 525.2973).

4-({4-[5-({1-[(6-Ethoxypyridin-3-yl)methyl]piperidin-4-yl}methoxy)pyridine-2-carbonyl]piperazin-1-yl}methyl)-benzonitrile Ditosylate (16c) Compound **16c** was prepared from **12** and **29** in 59% yield using a similar approach to that described for **14**. ^{1}H-NMR (DMSO-d_6) δ: 1.33 (3H, t, $J=7.1$ Hz), 1.41–1.63 (2H, m), 1.76–2.17 (3H, m), 2.29 (6H, s), 2.84–3.54 (10H, m), 3.99 (2H, d, $J=6.0$ Hz), 4.12–4.77 (8H, m), 6.89 (1H, d, $J=9.0$ Hz), 7.11 (4H, d, $J=8.2$ Hz), 7.41–7.59 (1H, m), 7.48 (4H, d, $J=8.2$ Hz), 7.65–7.75 (3H, m), 7.82 (1H, dd, $J=2.4$, 8.6 Hz), 7.97 (2H, d, $J=8.4$ Hz), 8.22–8.34 (2H, m), 9.25 (1H, brs), 10.01 (1H, brs); MS *m/z*: 555 $(M+H)^+$; ESI-MS *m/z*: 555.3079 $(M+H)^+$ (Calcd for $C_{32}H_{39}O_3N_6$: 555.3078).

Methyl 5-Hydroxy-3-methylpyridine-2-carboxylate (18) To a solution of methyl 5-methoxy-3-methylpyridine-2-carboxylate[16] (**17**; 2.4 g, 13 mmol) in CH_2Cl_2 (140 mL) was added $AlCl_3$ (20 g, 150 mmol). The mixture was stirred at 55°C overnight under an argon atmosphere. After cooling to 0°C, the mixture was diluted with 1.0 mol/L HCl aqueous solution and stirred at room temperature. The mixture was basified with 1.0 mol/L NaOH aqueous solution and acidified with 10% citric acid aqueous solution. The mixture was extracted with $CHCl_3$/IPA (4 : 1), and the organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (1.8 g, 80%). ^{1}H-NMR (DMSO-d_6) δ: 2.44 (3H, s), 3.79 (3H, s), 7.08 (1H, dd, $J=0.8$, 2.6 Hz), 8.02 (1H, dd, $J=0.4$, 2.6 Hz), 10.56 (1H, brs); MS *m/z*: 168 $(M+H)^+$.

5-{[1-(*tert*-Butoxycarbonyl)piperidin-4-yl]methoxy}-3-methylpyridine-2-carboxylic Acid (19) Compound **19** was prepared from **18** in 69% yield using a similar approach to that described for **9**. ^{1}H-NMR (DMSO-d_6) δ: 1.03–1.27 (2H, m), 1.40 (9H, s), 1.66–1.82 (2H, m), 1.86–2.06 (1H, m), 2.46–2.54 (3H, m), 2.61–2.92 (2H, m), 3.92–4.03 (2H, m), 3.99 (2H, d, $J=6.4$ Hz), 7.36 (1H, d, $J=2.7$ Hz), 8.16 (1H, d, $J=2.6$ Hz), 12.57 (1H, brs); MS *m/z*: 351 $(M+H)^+$.

4-[(4-{3-Methyl-5-[(piperidin-4-yl)methoxy]pyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (20) To a solution of 5-{[1-(*tert*-butoxycarbonyl)piperidin-4-yl]-methoxy}-3-methylpyridine-2-carboxylic acid (**19**; 600 mg, 1.7 mmol), 4-[(piperazin-1-yl)methyl]benzonitrile dihydrochloride (**31**; 480 mg, 1.8 mmol), Et_3N (0.49 mL, 3.5 mmol) in CH_2Cl_2 (9.0 mL) were added WSC·HCl (450 mg, 2.3 mmol), HOBt (320 mg, 2.4 mmol). After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in MeOH (5.0 mL) was added 4.0 mol/L HCl in 1,4-dioxane (5.0 mL, 20 mmol). After stirring at room temperature for 1.5 h, the mixture was basified with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$/IPA (4 : 1). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to obtain the product (790 mg, quantitative yield). ^{1}H-NMR (DMSO-d_6) δ: 1.07–1.33 (2H, m), 1.62–1.73 (2H, m), 1.75–1.91 (1H, m), 2.21 (3H, s), 2.25–2.37 (2H, m), 2.38–2.55 (4H, m), 2.89–3.04 (2H, m), 3.06–3.16 (2H, m), 3.58–3.61 (2H, m), 3.61–3.69 (2H, m), 3.88 (2H, d, $J=6.6$ Hz), 7.32 (1H, d, $J=2.7$ Hz), 7.52 (2H, d, $J=8.4$ Hz), 7.79 (2H, d, $J=8.4$ Hz), 8.06 (1H, d, $J=2.6$ Hz); MS *m/z*: 434 $(M+H)^+$.

4-({4-[5-({1-[(6-Ethoxypyridin-3-yl)methyl]piperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile Ditosylate (21a) Compound **21a** was prepared from **20** and **29** in 56% yield using a similar approach to that described for **14**. ^{1}H-NMR (DMSO-d_6) δ: 1.33 (3H, t, $J=7.1$ Hz), 1.41–1.59 (2H, m), 1.78–2.14 (3H, m), 2.26 (3H, s), 2.29 (6H, s), 2.79–3.78 (10H, m), 3.95 (2H, d, $J=6.0$ Hz), 4.06–4.82 (8H, m), 6.90 (1H, d, $J=8.6$ Hz), 7.07–7.15 (4H, m), 7.37 (1H, d, $J=2.4$ Hz), 7.48 (4H, d, $J=7.9$ Hz), 7.61–7.76 (2H, m), 7.82 (1H, dd, $J=2.4$, 8.6 Hz), 7.91–8.02 (2H, m), 8.08 (1H, d, $J=2.4$ Hz), 8.26 (1H, d, $J=2.4$ Hz), 9.27 (1H, brs), 10.04 (1H, brs); MS *m/z*: 569 $(M+H)^+$; ESI-MS *m/z*: 569.3238 $(M+H)^+$ (Calcd for $C_{33}H_{41}O_3N_6$: 569.3235).

4-({4-[5-({1-[(5-Methoxypyrazin-2-yl)methyl]piperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile Ditosylate (21b) To a mixture of 4-[(4-{3-methyl-5-[(piperidin-4-yl)methoxy]-pyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (**20**; 410 mg, 0.94 mmol) and MeCN (8.0 mL) were added 2-(chloromethyl)-5-methoxypyrazine (**35a**; 170 mg, 1.1 mmol) and K_2CO_3 (390 mg, 2.8 mmol). After stirring at room temperature overnight, the mixture was diluted with H_2O and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue (380 mg, 0.68 mmol) in MeOH was added *p*-TsOH·H_2O (240 mg, 1.3 mmol). The mixture was concentrated *in vacuo*. To the residue was added Et_2O, and the precipitated solid was collected by filtration to obtain the product (500 mg, 59%). ^{1}H-NMR (DMSO-d_6) δ: 1.42–1.65 (2H, m), 1.76–2.16 (3H, m), 2.26 (3H, s), 2.29 (6H, s), 2.81–3.82 (10H, m), 3.85–4.05 (5H, m), 4.34–4.75 (4H, m), 4.41 (2H, d, $J=4.9$ Hz), 7.07–7.15 (4H, m), 7.37 (1H, d, $J=2.7$ Hz), 7.48 (4H, d, $J=7.9$ Hz), 7.70 (2H, d, $J=8.2$ Hz), 7.97 (2H, d, $J=8.2$ Hz), 8.09 (1H, d, $J=2.7$ Hz), 8.38 (1H, d, $J=1.3$ Hz), 8.43 (1H, d, $J=1.3$ Hz), 9.66 (1H, brs), 9.78–10.19 (1H, m); MS *m/z*: 556 $(M+H)^+$; ESI-MS *m/z*: 556.3030 $(M+H)^+$ (Calcd for $C_{31}H_{38}O_3N_7$: 556.3031).

4-({4-[5-({1-[(5-Ethoxypyrazin-2-yl)methyl]-piperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile Succinate (21c) To a solution of 4-[(4-{3-methyl-5-[(piperidin-4-yl)methoxy]-pyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (**20**; 3.2 g, 7.3 mmol), DIPEA (4.0 mL, 23 mmol) and MeCN (30 mL) was added 2-(chloromethyl)-5-ethoxypyrazine (**35b**; 1.5 g, 8.4 mmol) in MeCN (20 mL). After stirring at room temperature for 14 h, the mixture was concentrated *in vacuo*. The residue was purified by column chromatography on amino functionalized silica gel (hexane/EtOAc). The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue (3.2 g, 5.7 mmol) in EtOH (12 mL) was added succinic acid (670 mg, 5.7 mmol). After stirring at room temperature for 0.5 h, the mixture was concentrated *in vacuo*. To the residue was added heptane. After stirring at room temperature overnight, the precipitated solid was collected by filtration to obtain the product (3.7 g, 74%). ^{1}H-NMR (DMSO-

d_6) δ: 1.19–1.43 (2H, m), 1.34 (3H, t, J = 7.0 Hz), 1.64–1.85 (3H, m), 1.98–2.17 (2H, m), 2.20 (3H, s), 2.25–2.36 (2H, m), 2.39–2.45 (2H, m), 2.41 (4H, s), 2.79–2.96 (2H, m), 3.03–3.21 (2H, m), 3.54–3.69 (6H, m), 3.91 (2H, d, J = 6.0 Hz), 4.33 (2H, q, J = 6.9 Hz), 7.32 (1H, d, J = 2.7 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.79 (2H, d, J = 8.4 Hz), 8.06 (1H, d, J = 2.7 Hz), 8.16 (1H, d, J = 1.3 Hz), 8.21 (1H, d, J = 1.3 Hz), 12.10 (2H, brs); MS m/z: 570 $(M+H)^+$; ESI-MS m/z: 570.3185 $(M+H)^+$ (Calcd for $C_{32}H_{40}O_3N_7$: 570.3187).

5-{[1-(*tert*-Butoxycarbonyl)-4-methoxypiperidin-4-yl]-methoxy}-3-methylpyridine-2-carboxylic Acid (22) To a mixture of methyl 5-hydroxy-3-methylpyridine-2-carboxylate (**18**; 960 mg, 5.7 mmol), *tert*-butyl 4-(hydroxymethyl)-4-methoxypiperidine-1-carboxylate (**37**; 1.4 g, 5.7 mmol) and toluene (20 mL) was added cyanomethylenetributylphosphorane (2.2 mL, 8.3 mmol). The mixture was stirred at 100°C overnight under an argon atmosphere. After cooling to room temperature, the mixture was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc). To a solution of the residue in MeOH (20 mL) was added 1.0 mol/L NaOH aqueous solution (20 mL, 20 mmol). The mixture was stirred at 60°C for 1 h. After cooling to room temperature, the mixture was washed with Et_2O and the aqueous layer was acidified with 10% citric acid aqueous solution. The mixture was extracted with $CHCl_3$, and the organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. To the residue was added Et_2O, and the precipitated solid was collected by filtration to obtain the product (2.0 g, 91%). ^{1}H-NMR (DMSO-d_6) δ: 1.40 (9H, s), 1.45–1.60 (2H, m), 1.73–1.88 (2H, m), 2.51 (3H, s), 2.85–3.16 (2H, m), 3.19 (3H, s), 3.61–3.82 (2H, m), 4.11 (2H, s), 7.42 (1H, d, J = 2.4 Hz), 8.20 (1H, d, J = 2.4 Hz), 12.60 (1H, brs); MS m/z: 381 $(M+H)^+$.

5-{[1-(*tert*-Butoxycarbonyl)-4-fluoropiperidin-4-yl]-methoxy}-3-methylpyridine-2-carboxylic Acid (23) Compound **23** was prepared from **18** and *tert*-butyl 4-fluoro-4-(hydroxymethyl)piperidine-1-carboxylate[18] in 54% yield using a similar approach to that described for **22**. ^{1}H-NMR (DMSO-d_6) δ: 1.41 (9H, s), 1.55–1.82 (2H, m), 1.83–2.01 (2H, m), 2.51 (3H, s), 2.89–3.18 (2H, m), 3.73–3.93 (2H, m), 4.27 (2H, d, J = 21.2 Hz), 7.42 (1H, d, J = 2.4 Hz), 8.21 (1H, d, J = 2.4 Hz), 12.64 (1H, brs); MS m/z: 369 $(M+H)^+$.

4-[(4-{5-[(4-Methoxypiperidin-4-yl)methoxy]-3-methylpyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (24) Compound **24** was prepared from **22** in quantitative yield using a similar approach to that described for **20**. ^{1}H-NMR (DMSO-d_6) δ: 1.41–1.62 (2H, m), 1.64–1.83 (2H, m), 2.22 (3H, s), 2.26–2.36 (2H, m), 2.37–2.48 (2H, m), 2.63–2.81 (4H, m), 3.06–3.18 (2H, m), 3.16 (3H, s), 3.60 (2H, s), 3.60–3.70 (2H, m), 4.00 (2H, s), 7.37 (1H, d, J = 2.4 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.80 (2H, d, J = 8.4 Hz), 8.11 (1H, d, J = 2.4 Hz); MS m/z: 464 $(M+H)^+$.

4-[(4-{5-[(4-Fluoropiperidin-4-yl)methoxy]-3-methylpyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (25) Compound **25** was prepared from **23** in quantitative yield using a similar approach to that described for **20**. ^{1}H-NMR (DMSO-d_6) δ: 1.52–1.90 (4H, m), 2.21 (3H, s), 2.26–2.35 (2H, m), 2.37–2.48 (2H, m), 2.63–2.87 (4H, m), 3.05–3.19 (2H, m), 3.60 (2H, s), 3.61–3.69 (2H, m), 4.14 (2H, d, J = 21.0 Hz), 7.38 (1H, d, J = 2.4 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.79 (2H, d, J = 8.4 Hz), 8.12 (1H, d, J = 2.4 Hz); MS m/z: 452 $(M+H)^+$.

4-({4-[5-({1-[(6-Ethoxypyridin-3-yl)methyl]-4-methoxypiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile Succinate (26a) To a solution of 4-[(4-{5-[(4-methoxypiperidin-4-yl)methoxy]-3-methylpyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (**24**; 370 mg, 0.79 mmol) in CH_2Cl_2 (6.0 mL) were added 6-ethoxypyridine-3-carbaldehyde (**29**; 120 mg, 0.79 mmol), acetic acid (5.0 μL, 87 μmol) and $NaBH(OAc)_3$ (450 mg, 2.1 mmol). After stirring at room temperature for 4 h, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue (370 mg, 0.62 mmol) in MeOH was added succinic acid (72 mg, 0.61 mmol). The mixture was concentrated *in vacuo*. To the residue was added heptane, and the precipitated solid was collected by filtration to obtain the product (340 mg, 61%). ^{1}H-NMR (DMSO-d_6) δ: 1.30 (3H, t, J = 7.1 Hz), 1.50–1.70 (2H, m), 1.73–1.87 (2H, m), 2.14–2.63 (8H, m), 2.21 (3H, s), 2.41 (4H, s), 3.05–3.16 (2H, m), 3.15 (3H, s), 3.44 (2H, s), 3.54–3.72 (2H, m), 3.59 (2H, s), 4.00 (2H, s), 4.28 (2H, q, J = 7.1 Hz), 6.75 (1H, d, J = 8.6 Hz), 7.37 (1H, d, J = 2.2 Hz), 7.52 (2H, d, J = 8.2 Hz), 7.61 (1H, dd, J = 2.4, 8.6 Hz), 7.79 (2H, d, J = 8.2 Hz), 8.03 (1H, d, J = 2.2 Hz), 8.10 (1H, d, J = 2.4 Hz), 12.22 (2H, brs); MS m/z: 599 $(M+H)^+$; ESI-MS m/z: 599.3348 $(M+H)^+$ (Calcd for $C_{34}H_{43}O_4N_6$: 599.3340).

4-({4-[5-({1-[(6-Ethoxypyridin-3-yl)methyl]-4-fluoropiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]piperazin-1-yl}methyl)benzonitrile Succinate (27a) Compound **27a** was prepared from **25** in 68% yield using a similar approach to that described for **26a**. ^{1}H-NMR (DMSO-d_6) δ: 1.30 (3H, t, J = 7.1 Hz), 1.55–2.06 (4H, m), 2.14–2.48 (6H, m), 2.21 (3H, s), 2.42 (4H, s), 2.58–2.74 (2H, m), 3.03–3.18 (2H, m), 3.46 (2H, s), 3.59 (2H, s), 3.60–3.70 (2H, m), 4.15 (2H, d, J = 20.5 Hz), 4.28 (2H, q, J = 7.1 Hz), 6.75 (1H, d, J = 8.5 Hz), 7.37 (1H, d, J = 2.2 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.62 (1H, dd, J = 2.4, 8.5 Hz), 7.79 (2H, d, J = 8.4 Hz), 8.03 (1H, d, J = 2.2 Hz), 8.11 (1H, d, J = 2.4 Hz), 12.17 (2H, brs); MS m/z: 587 $(M+H)^+$; ESI-MS m/z: 587.3137 $(M+H)^+$ (Calcd for $C_{33}H_{40}O_3N_6F$: 587.3140).

4-({4-[5-({1-[(5-Ethoxypyrazin-2-yl)methyl]-4-methoxypiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]piperazin-1-yl}methyl)benzonitrile Succinate (26b) Compound **26b** was prepared from **24** and **35b** in 65% yield using a similar approach to that described for **21c**. ^{1}H-NMR (DMSO-d_6) δ: 1.34 (3H, t, J = 7.1 Hz), 1.53–1.72 (2H, m), 1.72–1.88 (2H, m), 2.21 (3H, s), 2.25–2.47 (6H, m), 2.41 (4H, s), 2.53–2.65 (2H, m), 3.05–3.18 (2H, m), 3.15 (3H, s), 3.59 (4H, s), 3.61–3.69 (2H, m), 4.01 (2H, s), 4.33 (2H, q, J = 7.1 Hz), 7.37 (1H, d, J = 2.4 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.79 (2H, d, J = 8.4 Hz), 8.10 (1H, d, J = 2.4 Hz), 8.17 (1H, d, J = 1.3 Hz), 8.21 (1H, d, J = 1.3 Hz), 12.22 (2H, brs); MS m/z: 600 $(M+H)^+$; ESI-MS m/z: 600.3292 $(M+H)^+$ (Calcd for $C_{33}H_{42}O_4N_7$: 600.3293).

4-({4-[5-({1-[(5-Ethoxypyrazin-2-yl)methyl]-4-fluoropiperidin-4-yl}methoxy)-3-methylpyridine-2-carbonyl]-piperazin-1-yl}methyl)benzonitrile Succinate (27b) To a solution of 4-[(4-{5-[(4-fluoropiperidin-4-yl)methoxy]-3-methylpyridine-2-carbonyl}piperazin-1-yl)methyl]benzonitrile (**25**; 2.0 g, 4.4 mmol) in MeCN (20 mL) were added DIPEA (2.7 mL, 16 mmol) and 2-(chloromethyl)-5-ethoxypyrazine (**35b**; 960 mg, 5.5 mmol) in MeCN (5.0 mL). After stirring at 50°C for 18 h, the mixture was concentrated *in vacuo*. The

residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). The residue was purified by column chromatography on amino functionalized silica gel (hexane/EtOAc). To the residue was added Et_2O. After stirring at room temperature overnight, the precipitated solid was collected by filtration. The obtained solid was purified by column chromatography on silica gel ($CHCl_3$/MeOH/EtOAc). To the residue was added Et_2O, and the precipitated solid was collected by filtration. To a mixture of the obtained solid (2.0 g, 3.3 mmol), acetone (15 mL) and EtOH (15 mL) was added succinic acid (390 mg, 3.3 mmol). After stirring at room temperature 15 min, to the mixture was added EtOH (15 mL). After stirring at room temperature 0.5 h, the mixture was stirred at 50°C for additional 15 min. The mixture was filtered and the filtrate was concentrated *in vacuo*. To the residue was added heptane. After stirring at room temperature for 0.5 h, the precipitated solid was collected by filtration to obtain the product (2.1 g, 68%). ^{1}H-NMR (DMSO-d_6) δ: 1.34 (3H, t, J = 7.1 Hz), 1.61–2.01 (4H, m), 2.21 (3H, s), 2.26–2.47 (6H, m), 2.42 (4H, s), 2.62–2.81 (2H, m), 3.05–3.19 (2H, m), 3.53–3.72 (2H, m), 3.59 (2H, s), 3.62 (2H, s), 4.16 (2H, d, J = 20.7 Hz), 4.34 (2H, q, J = 7.1 Hz), 7.37 (1H, d, J = 2.4 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.79 (2H, d, J = 8.4 Hz), 8.12 (1H, d, J = 2.4 Hz), 8.18 (1H, d, J = 1.3 Hz), 8.22 (1H, d, J = 1.3 Hz), 12.16 (2H, br s); MS *m/z*: 588 $(M+H)^+$; ESI-MS *m/z*: 588.3092 $(M+H)^+$ (Calcd for $C_{32}H_{39}O_3N_7F$: 588.3093).

6-Ethoxypyridine-3-carbaldehyde (29) To a solution of 5-bromo-2-ethoxypyridine (**28**; 5.0 g, 25 mmol) in THF (70 mL) was added dropwise *n*-BuLi (16 mL, 26 mmol, 1.7 mol/L in hexane) at −68°C under a nitrogen atmosphere. After stirring at the same temperature for 0.5 h, to the mixture was added dropwise DMF (10 mL, 130 mmol). The mixture was allowed to warm gradually to room temperature and stirred for 3 h. The mixture was diluted with 10% citric acid aqueous solution and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to obtain the product (2.2 g, 59%). ^{1}H-NMR (DMSO-d_6) δ: 1.35 (3H, t, J = 7.1 Hz), 4.43 (2H, q, J = 7.1 Hz), 6.96 (1H, ddd, J = 0.7, 0.7, 8.7 Hz), 8.11 (1H, dd, J = 2.4, 8.7 Hz), 8.75 (1H, dd, J = 0.7, 2.4 Hz), 9.96 (1H, d, J = 0.7 Hz); MS *m/z*: 152 $(M+H)^+$.

4-[(Piperazin-1-yl)methyl]benzonitrile Dihydrochloride (31) To a solution of *tert*-butyl piperazine-1-carboxylate (**30**; 7.0 g, 38 mmol) in CH_2Cl_2 (47 mL) were added 4-formylbenzonitrile (5.9 g, 45 mmol) and acetic acid (4.3 mL, 75 mmol). After stirring at room temperature for 0.5 h, to the mixture was added $NaBH(OAc)_3$ (16 g, 75 mmol). After stirring at room temperature for 3 h, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH). To a solution of the residue in EtOAc (60 mL) and $CHCl_3$ (60 mL) was added 4.0 mol/L HCl in EtOAc (60 mL, 240 mmol). After stirring at room temperature for 3 d, the mixture was diluted with hexane, and then the precipitated solid was collected by filtration to obtain the product (8.0 g, 77%). ^{1}H-NMR (D_2O) δ: 3.59 (8H, s), 4.52 (2H, s), 7.70 (2H, d, J = 8.2 Hz), 7.90 (2H, d, J = 8.2 Hz); MS *m/z*: 202 $(M+H)^+$.

Methyl 5-Methoxypyrazine-2-carboxylate (33a) To a solution of methyl 5-chloropyrazine-2-carboxylate (**32**; 10 g, 58 mmol) in MeOH (100 mL) was added portion-wise sodium methoxide (5.0 g, 93 mmol) at 0°C under a nitrogen atmosphere. After stirring at room temperature for 3 h, the mixture was diluted with 10% citric acid aqueous solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (7.9 g, 81%). ^{1}H-NMR (DMSO-d_6) δ: 3.88 (3H, s), 4.00 (3H, s), 8.41 (1H, d, J = 1.3 Hz), 8.84 (1H, d, J = 1.3 Hz); MS *m/z*: 169 $(M+H)^+$.

Ethyl 5-Ethoxypyrazine-2-carboxylate (33b) To a solution of *t*-BuOK (2.3 g, 21 mmol) in EtOH (30 mL) was added portion-wise methyl 5-chloropyrazine-2-carboxylate (**32**; 3.0 g, 17 mmol) at 0°C. After stirring at the same temperature for 1 h, the mixture was neutralized with 1.0 mol/L HCl aqueous solution and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel ($CHCl_3$) to give the product (2.8 g, 82%). ^{1}H-NMR (DMSO-d_6) δ: 1.33 (3H, t, J = 7.1 Hz), 1.37 (3H, t, J = 7.1 Hz), 4.34 (2H, q, J = 7.1 Hz), 4.44 (2H, q, J = 7.1 Hz), 8.37 (1H, d, J = 1.3 Hz), 8.81 (1H, d, J = 1.3 Hz); MS *m/z*: 197 $(M+H)^+$.

(5-Methoxypyrazin-2-yl)methanol (34a) To a mixture of methyl 5-methoxypyrazine-2-carboxylate (**33a**; 2.3 g, 14 mmol) and MeOH (45 mL) was added $NaBH_4$ (1.6 g, 42 mmol) at 0°C. After stirring at the same temperature for 15 min, the mixture was stirred at room temperature for an additional 2 h. The mixture was acidified with 1.0 mol/L HCl aqueous solution and basified with 1.0 mol/L NaOH aqueous solution. The mixture was extracted with $CHCl_3$/IPA (4 : 1), and the organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to obtain the product (1.5 g, 77%). ^{1}H-NMR (DMSO-d_6) δ: 3.90 (3H, s), 4.55 (2H, d, J = 5.7 Hz), 5.40 (1H, t, J = 5.7 Hz), 8.20–8.22 (1H, m), 8.22–8.24 (1H, m); MS *m/z*: 141 $(M+H)^+$.

(5-Ethoxypyrazin-2-yl)methanol (34b) Compound **34b** was prepared from **33b** in 75% yield using a similar approach to that described for **34a**. ^{1}H-NMR (DMSO-d_6) δ: 1.34 (3H, t, J = 7.1 Hz), 4.34 (2H, q, J = 7.1 Hz), 4.54 (2H, d, J = 5.7 Hz), 5.39 (1H, t, J = 5.7 Hz), 8.07–8.32 (2H, m); MS *m/z*: 155 $(M+H)^+$.

2-(Chloromethyl)-5-methoxypyrazine (35a) To a solution of (5-methoxypyrazin-2-yl)methanol (**34a**; 700 mg, 5.0 mmol) in CH_2Cl_2 (10 mL) was added $SOCl_2$ (1.0 mL, 14 mmol) at 0°C. After stirring at room temperature for 0.5 h, the mixture was concentrated *in vacuo* to obtain the product (800 mg, quantitative yield). ^{1}H-NMR ($CDCl_3$) δ: 3.98 (3H, s), 4.65 (2H, s), 8.19–8.20 (1H, m), 8.20–8.21 (1H, m); MS *m/z*: 159 $(M+H)^+$.

2-(Chloromethyl)-5-ethoxypyrazine (35b) Compound **35b** was prepared from **34b** in 100% yield using a similar approach to that described for **35a**. ^{1}H-NMR (DMSO-d_6) δ: 1.35 (3H, t, J = 7.1 Hz), 4.36 (2H, q, J = 7.1 Hz), 4.80 (2H, s), 8.29 (1H, d, J = 1.3 Hz), 8.33 (1H, d, J = 1.3 Hz); MS *m/z*: 173 $(M+H)^+$.

***tert*-Butyl 4-(Hydroxymethyl)-4-methoxypiperidine-1-carboxylate (37)** To a solution of *tert*-butyl 1-oxa-6-azaspiro[2.5]octane-6-carboxylate[19] (**36**; 10 g, 47 mmol) in MeOH (150 mL) was added *p*-TsOH·H_2O (300 mg, 1.6 mmol).

After stirring at room temperature overnight, the mixture was diluted with saturated $NaHCO_3$ aqueous solution and H_2O. The mixture was extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (6.0 g, 52%). ^{1}H-NMR (DMSO-d_6) δ: 1.23–1.43 (2H, m), 1.39 (9H, s), 1.53–1.67 (2H, m), 2.77–3.06 (2H, m), 3.13 (3H, s), 3.30–3.34 (2H, m), 3.53–3.73 (2H, m), 4.54 (1H, t, $J = 5.5$ Hz); MS *m/z*: 246 $(M + H)^+$.

Biology

Cell Lines The breast cancer cell lines MDA-MB-453 and SK-BR-3 were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). MDA-MB-453 was cultured in Leibovitz's L-15 Medium (Life Technologies, Carlsbad, CA, U.S.A.) under CO_2-free conditions at 37°C. SK-BR-3 was cultured in RPMI 1640 medium (Sigma-Aldrich Co. LLC., St. Louis, MO, U.S.A.) at 37°C in 5% CO_2. All media were supplemented with 10% fetal bovine serum (Sigma-Aldrich Co. LLC.) and 1% penicillin/streptomycin (Life Technologies [Cat. No. 15070-063]).

Whole Cell ELISA MDA-MB-453 cells were seeded onto 384-well clear flat plates at 1.5×10^4 cells/well. The following day, the cells were treated with the test compound at final concentrations of 0 (DMSO only), 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, 3000 and 10000 nmol/L. The final concentration of DMSO in each well was 0.1% (v/v). Two hours after addition of the test compound, the cells were fixed in 40% glyoxal solution (Nacalai Chemical Ltd., Kyoto, Japan) for 0.5 h at room temperature. For probing, the supernatant was discarded and the cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature. Subsequently, the supernatant was discarded and the cells were blocked with ODYSSEY Blocking solution (Li-Cor Biosciences, Lincoln, NE, U.S.A.) for 1 h at room temperature. After discarding the supernatant, a primary phospho- Acetyl-CoA Carboxylase (Ser79) antibody (1 : 500 in ODYSSEY Blocking solution; Cat. No. 3661, Cell Signaling Technologies, Danvers, MA, U.S.A.) was added. After incubating overnight at 4°C, the plates were washed 3 times with Tris-buffered saline containing 0.05% Tween 20 (TBS/Tween). Goat anti-rabbit IRDye 800CW (Li-Cor Biosciences; 1 : 1000 in ODYSSEY Blocking solution) was added, and the plates were incubated for 1 h at room temperature. After the incubation, the plates were washed 3 times with TBS/Tween and dried for at least 3 h at room temperature. Fluorescence signals were quantified using the Aerius automated infrared imaging system (Li-Cor Biosciences). The assays were performed in duplicate or triplicate, and the data were analyzed using Prism5 software (GraphPad Software Inc., San Diego, CA, U.S.A.). The EC_{50} value of each test compound was calculated using Sigmoid-Emax model non-linear regression analysis. The average signal was normalized by regarding the signal in the DMSO-treated group as 0% in each experiment.

Cell Growth Inhibition Assay Each breast cancer cell line above was seeded onto non-adherent 384-well white plates at 500 cells/well or 96-well white plates at 1000 cells/well. The following day, the test compound was added to each well at final concentrations of 0 (DMSO only), 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nmol/L. The final concentration of DMSO in each well was 0.1% (v/v). Four days after addition of the test compound, cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, U.S.A.). CellTiter-Glo® Reagent was added to each well, and luminescence was measured using ARVO SX® (PerkinElmer, Inc., Waltham, MA, U.S.A.). The assay was performed in triplicate. Cell viability according to luminescence intensity was normalized by regarding the average luminescence intensity following treatment with DMSO only as 100% and no luminescence intensity as 0%. The IC_{50} value of each test compound was calculated by Sigmoid-E_{max} non-linear regression analysis using Prism5 software (GraphPad Software Inc., San Diego, CA, U.S.A.).

Animals Five-week-old male nude mice (CAnN.Cg-Foxn1nu/CrlCrlj[nu/nu]) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were maintained on a standard diet and water throughout the experiments under specific-pathogen-free conditions.

***In Vivo* Antitumor Evaluation** MDA-MB-453 cells were suspended in a 1 : 1 BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, U.S.A.) to PBS solution at 3×10^7 cells/mL. The cell suspension was subcutaneously implanted at 3×10^6 cells/animal/100 μL around the lumbar region and allowed to grow. Mice were divided into 4 groups ($N = 5$) approximately 3 weeks after inoculation such that the mean tumor volume was comparable among the groups. The first day of administration was designated Day 1, and observation was continued until Day 15. **27b** was dissolved in 6% (2-hydroxypropyl)-β-cyclo-dextrin (Sigma-Aldrich Co. LLC.) solution as the vehicle, to obtain concentrations of 1, 2 and 4 mg/10 mL. Vehicle or 1, 2 or 4 mg/kg of **27b** was orally administered (10 mL/kg) to nude mice twice-daily for 14 d. All dose levels of **27b** are expressed as the free base. Tumor diameter and body weight were measured on Days 0, 5, 8, 12 and 15. Tumor volume was calculated as follows:

$$\begin{aligned}&\text{Tumor volume [mm}^3\text{]}\\&= (\text{Length of tumor long axis [mm]})\\&\quad\times(\text{Length of tumor short axis [mm]})^2 \times 0.5\end{aligned}$$

Dunnett's multiple comparisons test was used to compare the tumor volume and body weight the day after the last treatment (Day 15) between the vehicle- and **27b**-treated groups. $p < 0.05$ was used to indicate statistical significance.

The tumor growth inhibition rate was calculated as follows:

$$\begin{aligned}&\text{Tumor growth inhibition rate [\%]}\\&= 100 \times \left\{ 1 - \frac{\begin{array}{c}[(\text{Mean tumor volume of each group on Day 15 [mm}^3\text{]})\\ -(\text{Mean tumor volume of each group on Day 0 [mm}^3\text{])}]\end{array}}{\begin{array}{c}[(\text{Mean tumor volume of the vehicle group on Day 15 [mm}^3\text{])}\\ -(\text{Mean tumor volume of the vehicle group on Day 0 [mm}^3\text{])}]\end{array}} \right\}\end{aligned}$$

The tumor regression rate was calculated in groups whose tumor growth inhibition exceeded 100% as follows:

$$\begin{aligned}&\text{Tumor regression rate [\%]}\\&= 100 \times \left[1 - \frac{(\text{Mean tumor volume of each group on Day 15 [mm}^3\text{]})}{(\text{Mean tumor volume of each group on Day 0 [mm}^3\text{])}} \right]\end{aligned}$$

***In Vitro* hERG Inhibition Assay** CHO cells that stably expressed hERG channels were cultured in Ham's F12 containing 10% FBS, 5% ganeticin and 5% hygromycin B. Prior

to experiment, the culture medium was removed and the cells were washed with PBS. Cell was suspended with Serum Free Media containing CHO-S-SFM II, 25 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 units/mL P/S at concentration of $2–5 \times 10^6$ cells/mL. Cell suspension, test compounds solution on plate, and extracellular and intracellular solutions were set at Qpatch system. Used solutions are followings:

Extracellular (mM): 145 NaCl, 4 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 10 D(+)-Glucose, adjust pH to 7.4 with NaOH

Intracellular (mM): 120 KCl, 4 Na_2-ATP, 10 ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)·tetraacetic acid, 10 HEPES, 1.75 $MgCl_2$, 5.374 $CaCl_2$, adjust pH to 7.2 with KOH

The application of pressure for forming gigaseals and the whole-cell patch clamp configuration were established using QPatch Assay Software (Sophion Bioscience).

Addition of test compounds was also set up using the QPatch Assay Software. Compounds were added to the cells with the eight pipettes *via* the QPlate integrated glass microfluidic pathways. hERG inhibition was evaluated using a stimulus voltage protocol consisting of a 4.8 s activating pulse to 20 mV from a holding potential of −80 mV and a 5 s test pulse to −50 mV (tail current). The pulse pattern was repeated continuously at 15 s intervals. All QPatch data were analyzed and IC_{50}s were calculated using Sophion's QPatch Assay software.

***In Vitro* Intrinsic Clearance with Human Liver Microsomes** The test compounds (0.1–0.2 μmol/L) were incubated with pooled human liver microsomes (0.2 mg protein/mL), reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1 mmol/L) and ethylenediaminetetraacetic acid (EDTA) (0.1 mmol/L) in pH 7.4 phosphate buffer (100 mmol/L) at 37°C for 30 min. The peak area of the test compound was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to calculate the *in vitro* intrinsic clearance ($CL_{\mathrm{int,\ vitro}}$).

Pharmacokinetic Study Male SD rats were administered orally and intravenously with compound **27b**. Compound **27b** was dissolved in 30–40% PEG400 for oral and intravenous administration. Blood samples were collected using syringes containing heparin sodium at multiple time points up to 24 h after oral or intravenous administrations. Compound concentrations were determined using LC-MS/MS. Pharmacokinetic parameters were calculated by non-compartment analysis.

Aqueous Solubility The test compounds in 10 mmol/L DMSO solution (13 μL) were diluted to 130 μmol/L by adding the fluid for disintegration test (JP1: pH = 1.2, JP2: pH = 6.8, JP2 + TC). After incubation at 25°C for 20 h, precipitates were separated by filtration. The filtrate and a standard solution comprising a 100 μmol/L DMSO solution of the test compound were examined using liquid chromatography. The ratio of the peak area of the sample solution to the peak area of the standard solution was calculated to determine the aqueous solubility.

All animal experiments were performed in accordance with the regulation of the Animal Ethics Committee of Astellas Pharma Inc.

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

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