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Design and synthesis of pyrrolo[3,2-*d*]pyrimidine HER2/EGFR dual inhibitors: Improvement of the physicochemical and pharmacokinetic profiles for potent in vivo anti-tumor efficacy

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ABSTRACT

During the course of our studies on a novel HER2/EGFR dual inhibitor (TAK-285), we found an alternative potent pyrrolo[3,2-*d*]pyrimidine compound (**1a**). To enhance the pharmacokinetic (PK) profile of this compound, we conducted chemical modifications into its N-5 side chain and conversion of the chemically modified compounds into their salts. Among them, **2cb**, the tosylate salt of compound **2c**, showed potent HER2/EGFR kinase inhibitory activity (IC_{50}: 11/11 nM) and cellular growth inhibitory activity (BT-474 cell GI_{50}: 56 nM) with a good drug metabolism and PK (DMPK) profile. Furthermore, **2cb** exhibited significant in vivo antitumor efficacy in both mouse and rat xenograft models with transplanted 4-1ST gastric cancer cell lines (mouse, T/C = 0%, **2cb** po bid at 100 mg/kg; rat, T/C: -1%, **2cb** po bid at 25 mg/kg).

1. Introduction

In recent years, molecular targeted cancer therapies have attracted the attention of researchers because they are more effective and safer than conventional cytotoxic chemotherapies, due to these therapies having well-defined targets and mechanisms of action (MOAs).[1] The epidermal growth factor receptor (EGFR or erbB1) and human epidermal growth factor receptor 2 (HER2 or erbB2) are members of the erbB family of receptor tyrosine kinases, which have been validated as target molecules for cancer therapy.[2] The stimulation of ligands such as EGF causes the erbB receptors to either homodimerize or heterodimerize with the other erbB family receptors, thus inducing elevation of tyrosine kinase activity. These events activate downstream signaling pathways and eventually promote tumor cell growth.[3] Therefore, HER2/EGFR inhibitors can inhibit tyrosine kinase phosphorylation and block elevated intracellular signaling pathways in cancer cells, resulting in loss of regulatory function of the tumor. The significant clinical efficacy of small-molecule EGFR inhibitors, such as gefitinib[4] (Iressa™) and erlotinib[5] (Tarceva™), provided the proof of concept (POC) for their efficacy in lung cancer therapy. More recently, a small-molecule HER2/EGFR dual inhibitor, lapatinib[6] (Tykerb™) was launched for the treatment of advanced metastatic breast cancer.[7]

Most reported small-molecule inhibitors of HER2/EGFR contain a quinazoline ring as the core molecular scaffold.[4–6,8] In a previous study, we reported the discovery of a novel pyrrolo[3,2-*d*]pyrimidine derivative compound **A** (TAK-285) as a potent and orally active HER2/EGFR dual inhibitor (Fig. 1).[9] During our research on **A**, we also searched for another compound that could replace **A** if any unpredictable issues occurred during the clinical studies. Here, we describe the discovery of an alternative preclinical candidate, compound **2cb**, with an improved physicochemical and pharmacokinetic (PK) profile.

Although compound **1a** showed potent cellular antiproliferative activity (BT-474 cell GI_{50}: 5.2 nM), we did not select it as a candidate compound based on its poor cell membrane permeability and PK profile (see below, Table 1). Therefore, we initiated program to

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Abbreviations: HER2 (erbB2)/EGFR (erbB1), human epidermal growth factor receptor 2/epidermal growth factor receptor; PK profile, pharmacokinetic profile; IC, inhibitory concentration; GI, growth inhibitiory; DMPK, drug metabolism and pharmacokinetic profile; MOA, mechanism of action; GCDC, sodium glycochenodeoxycholic acid; FISA, hydrophilic component of solvent accessible surface area; POC, proof of concept; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole monohydrate; NMM, 4-methylmorpholine; *m*-CPBA, *m*-chloroperbenzoic acid; BsOH, benzenesulfonic acid; TsOH, *p*-toluenesulfonic acid.

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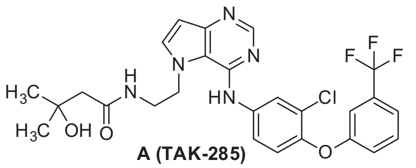


A (TAK-285)

Figure 1. Structure of compound **A** (TAK-285).

identify alternative compounds with both potent cellular growth inhibitory (GI) activity and good oral bioavailability. On the basis of our prior experience during research on **A**, we conducted studies focused on (a) introduction of alkyl groups into the N-5 sulfonylamide side chain (**R^2**, **X**) and (b) optimization of substituents on the 4-anilino group (**R^1** = CF_3, Cl) to enhance the membrane permeability and PK profile of **1a** (Fig. 2). Further, we expected that the conversion of **1a** into its respective salts after chemical modification could lead to the preparation of candidate compounds with high solubility and enhanced PK profiles.

2. Chemistry

Synthesis of compounds **1a–c** and **2a–c** was conducted as outlined in Scheme 1. Condensation of compound **3**[9] with anilines was carried out in 2-propanol, followed by removal of the terminal Boc group of the resulting compound **4** or **5** with 10% w/w HCl/MeOH solution to afford key intermediates (**6** or **7**) in greater than 94% yield. Amidation of **6** or **7** with a variety of carboxylic acids in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole monohydrate (HOBT) provided the corresponding desired carboxamides (**1a**, **2a** and **2c**) in approximately 70–80% yield. Conversion of 2-methyl-2-(methylsulfonyl)propanoic acid[10] to the corresponding acid chloride using thionyl chloride followed by treatment with **6** provided **1c** in 99% yield. In addition, mono-methylated derivatives (**1b, 2b**) were prepared by condensation of **6** or **7** with 2-chloropropionic acid in the presence of EDC and HOBT followed by nucleophilic displacement of the chloro group with sodium methanesulfinate in 68% and 43% yield over two steps, respectively.

Chemical modification of the terminal sulfonyl alkyl groups was carried out as shown in Scheme 2. Reaction of **6** with chloroacetylchloride in the presence of 4-methylmorpholine (NMM) in THF and subsequent displacement with the appropriate sodium alkylthiolates provided **9–11**. The resulting sulfides **9–11** were converted to the corresponding sulfones **1d–f** by oxidation in two steps with *m*-chloroperbenzoic acid (*m*-CPBA) followed by titanium tetraisopropoxide/*tert*-butylperoxide (Kagan's method[11]) in 42–58% overall yield.

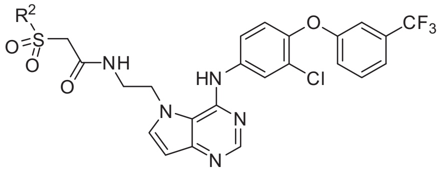
3. Results and discussion

To analyze effects of modification of the N-5 side chain of pyrrolo[3,2-*d*]pyrimidine, we replaced the 3-hydroxy-3-methylbutanamide moiety of **A** with a 2-(methylsulfonyl)acetamide moiety (**1a**). Compound **1a** showed potent HER2/EGFR kinase activity and inhibition of cell growth (HER2/EGFR IC_{50}: 6.3/15 nM, BT-474 cell GI_{50}: 5.2 nM), results which were superior to those obtained for **A**. However, the PK of **1a** in mice and the low A-B permeability of **1a** across Caco-2 membrane (B-A/A-B: 82.9/5.2) were issues that required further evaluation. We assumed that **1a** was a P-glycoprotein (P-gp) substrate, whereas **A** is not. We envisioned that masking of the polar sulfonyl group of **1a** by introducing **R^2** into the vicinal position should improve the A-B permeability, because it is known that molecules with a highly polar substituent such as sulfonamide, phenol, or a secondary amine group have a tendency to behave as P-gp substrates.[12] In addition, a preliminary physicochemical study indicated that introduction of various alkyl groups into **1a** could increase the lipophilicity of the molecule (*c*Log*D*) and improve its physicochemical profile. All the compounds thus synthesized (**1d**–**f**) showed potent HER2/EGFR kinase inhibitory activities, although their cell growth inhibitory activities were weaker than that of **1a** (Table 1). The HER2/EGFR kinase and cell growth inhibitory activities of **1d**–**f** show a trend towards lower potencies for compounds with larger **R^2** substituents. Furthermore, it was apparent that the introduction of alkyl groups at **R^2** would not improve the PK profile, although the *c*Log*D* (3.32–4.02) values of **1d–f** were close to that of **A**. On the other hand, calculation of the hydrophilic component of the solvent-accessible surface area (FISA: See Table 1 footnote) [13] values for this series suggested that we should seek compounds with lower FISA values, as well as higher *c*Log*D*.

Next, we examined the effect of introducing alkyl groups into the linker (**X**) between the sulfonyl and carbonyl groups of **1a**. Methyl (**1b**) and dimethyl (**1c**) groups were selected as representative alkyl substituents (Table 2). In addition, the corresponding derivatives **2a–c** were prepared because they were expected to exhibit good HER2/EGFR inhibitory activity according to our previous research on **A**. We also performed a physicochemical calculation, which suggested that replacement of the 3-trifluoromethyl

Table 1

Evaluation of N-5-substituted pyrrolo [3,2-*d*] pyrimidine derivatives



Compound	R^2	Enzyme IC_{50} (nM)		Cell growth GI_{50} (nM)	Metabolic stability (μL/min/mg)		Caco-2 (nm/sec)	Mouse PK $AUC_{0–8\ h}$ (μg h/mL)	*c*Log*D*	FISA[a] Å^2
		HER2	EGFR	BT-474	Human	Mouse	B–A/A–B	(10 mg/kg)		
1a	Me	6.3	15	5.2	18	11	82.9/5.2	0.947	2.79	147
1d	Et	8.5	31	36	26	22	NT	0.647	3.32	135
1e	^{i}Pr	11	53	48	42	35	NT	1.275	3.67	131
1f	^{t}Bu	14	56	36	45	31	NT	1.07	4.02	127
A		17	23	17	35	28	68.7/50.5	1.923	3.47	97

[a] FISA is the hydrophilic component of the solvent accessible surface area using a probe with a 1.4-Å radius. Compounds computationally found to be outside of the range for 95% of all drugs based on QikProp 2.1 are indicated in boldface.

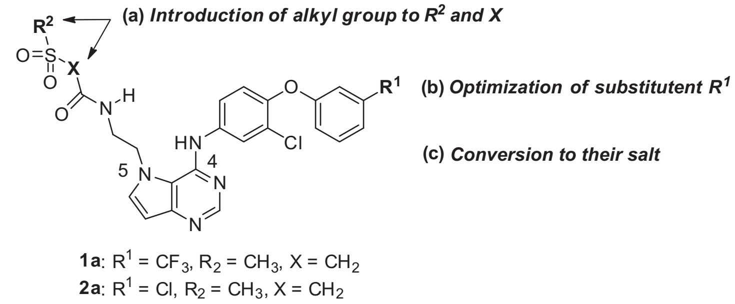
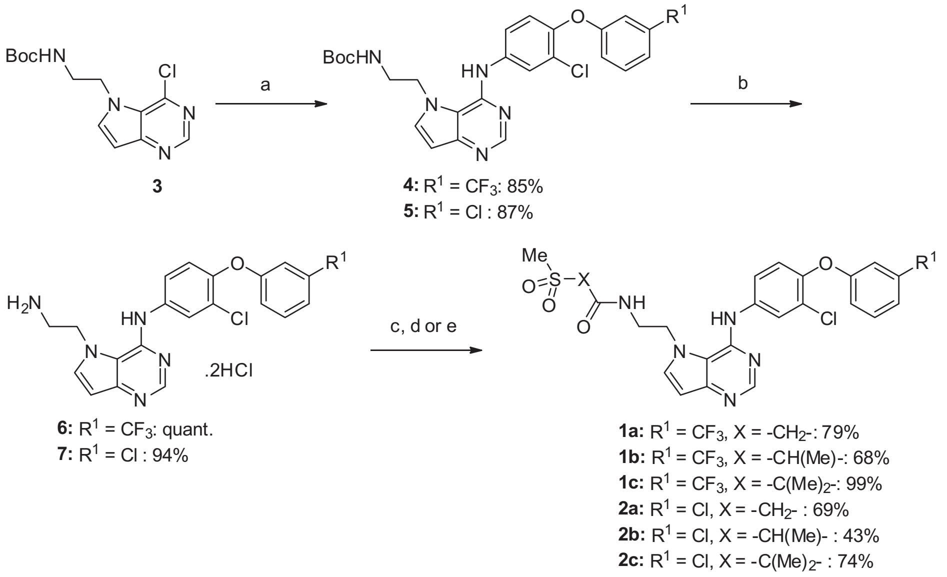
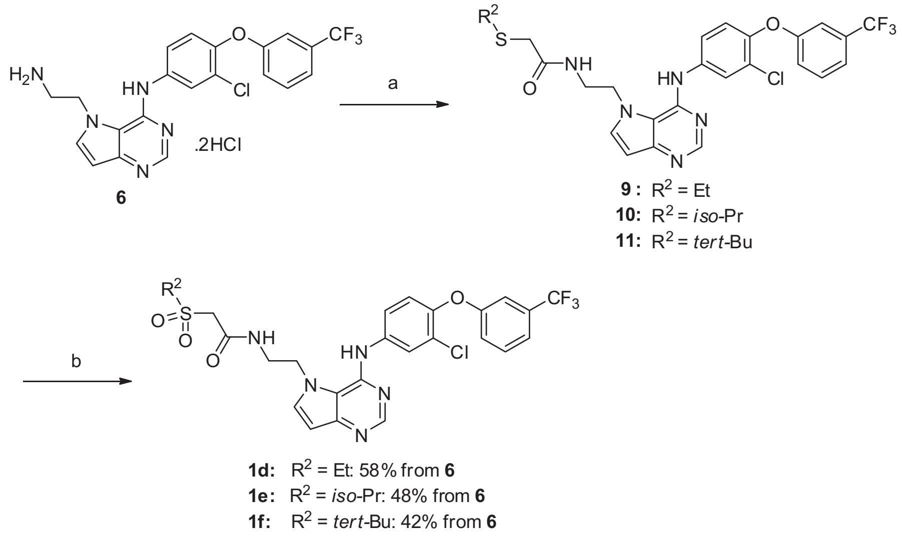


Figure 2. Synthetic strategies for improvement of membrane permeability and PK.



Scheme 1. Reagents: (a) 3-Chloro-4-[3-(trifluoromethyl)phenoxy]aniline or 3-chloro-4-(3-chlorophenoxy)aniline, 2-propanol; (b) 10% HCl/MeOH; (c) $MeSO_2$-X-CO_2H, EDC, HOBT, Et_3N, DMF; (d) (i) $MeCH(Cl)CO_2H$, EDC, HOBT, Et_3N, DMF, (ii) $MeSO_2Na$, pyridine, DMF; (e) (i) $MeSO_2C(Me)_2COOH$, $SOCl_2$, cat.DMF, (ii) Et_3N, THF.

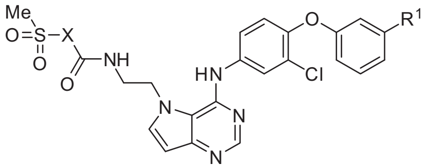


Scheme 2. Reagents: (a) (i) $ClCH_2COCl$, NMM, THF, (ii) R^2SNa, DMF/THF; (b) (i) *m*-CPBA, CH_2Cl_2, (ii) $Ti(OPr^i)_4$, *tert*-butylperoxide, $MeOH/CH_2Cl_2$.

moiety with a 3-chloro moiety could provide more favorable profiles for **2a–c** with higher *c*Log*D* and lower FISA values than those of the corresponding derivatives of **A** (**1a–c**).

The mono- and dimethylated compounds (**1b** and **1c**, respectively) exhibited good in vitro HER2/EGFR kinase activity and BT-474 cell growth inhibitory activity, although their activities were

Table 2
Evaluation of C4- and N5-substituted pyrrolo[3,2-*d*]pyrimidine derivatives



Compound	X	R^1	Enzyme IC_{50} (nM)		Cell growth GI_{50} (nM)	Metabolic stability (μL/min/mg)		Caco-2 (nm/sec)	Mouse PK $AUC_{0-8\ h}$ (μg h/mL)	In vivo efficacy[b] T/C (%)	*c*Log*D*	FISA[a]
			HER2	EGFR	BT-474	Human	Mouse	B–A/A–B	(10 mg/kg)	Mice		
1a	CH_2	CF_3	6.3	15	5.2	18	11	82.9/5.2	0.947	80	2.79	147
1b	CH(Me)	CF_3	7.1	18	34	30	33	NT	0.882	NT	3.14	133
1c	$C(Me)_2$	CF_3	17	47	55	38	23	42.6/28.7	2.289	43	3.49	122
2a	CH_2	Cl	2.9	6.7	3.9	11	NT	67.9/6.7	0.936	NT	3.52	148
2b	CH(Me)	Cl	4.1	9.8	32	4	−12	NT	1.547	NT	3.86	111
2c	$C(Me)_2$	Cl	11	11	56	21	−3	76.9/28.5	3.506	24	4.21	78
A			17	23	17	35	28	68.7/50.5	1.923	29	3.47	97

[a] See Table 1 footnote.
[b] Compounds suspended in 0.5% (w/v) methylcellulose solution, administered po b.i.d. (100 mg/kg) in BT-474 xenograft mice for 14 days.

Table 3
Physicochemical and pharmacokinetics properties of **2c**

2c (FormA)	55	0.56	1.46
2c (FormB)	28	0.33	–
2ca (BsOH salt)	620	9.47	1.71
2cb (TsOH salt)	620	7.58	3.39
A	>1000	5.80	2.87

$$\mathbf{2c} \xrightarrow{\text{a or b}} \begin{matrix}\mathbf{2ca}(\textbf{BsOH salt})\\ \mathbf{2cb}(\textbf{TsOH salt})\end{matrix}$$

Reagents: (a) benzenesulfonic acid monohydrate, EtOAc/EtOH; (b) *p*-toluenesulfonic acid monohydrate, EtOAc/EtOH.

weaker than those of **1a**, without a substituent at **X**. On the other hand, in mouse models, the introduction of a dimethylmethylene group (**1c**) at **X** was found to be the most effective for improving the PK profile and the Caco-2 membrane A-B permeability (B–A/A–B: 42.6/28.7). In addition, as anticipated, the 3-chloro anilino derivatives (**2**) showed similar structure–activity relationships (SAR) to the 3-trifluoromethyl derivatives (**1**). In particular, **2c** exhibited good in vitro activity (HER2/EGFR IC_{50}: 11/11 nM, BT-474 cell GI_{50}: 56 nM) and improved A-B permeability across Caco-2 membrane (B-A/A-B: 76.9/28.5). Furthermore, **2c** showed favorable PK properties (AUC: 3.506 μg h/mL), which can be explained by the optimal combination of *c* Log*D* and FISA values. As a reflection of the good PK profile of **2c**, the compound showed potent efficacy (T/C: 24%) in a BT-474 tumor xenograft model in mice, efficacy that was comparable to that observed for **A**.

Next, we examined the polymorphism of **2c** and identified multiple crystal forms (forms A–C): the most stable form was B, whereas form A was used for PK evaluation in mouse and other in vivo studies. We presumed that form B may yield a poor PK profile due to its low solubility and solubility rate compared to form A (Table 3). Therefore, to improve these physical properties, we initiated a study on the conversion of the salt-free form of **2c** into its salt form. Although we examined a variety of acid salts, only two salts of **2c** were obtained in crystalline form: **2ca**, which was obtained using benzenesulfonic acid (BsOH), and **2cb**, which was obtained using *p*-toluenesulfonic acid (TsOH). The physicochemical and PK properties of **2ca** and **2cb** are given in Table 3. As expected, **2ca** and **2cb** showed higher solubility and solubility rate than the salt-free form B of **2c**; the AUC value of the tosylate form (**2cb**), in particular, was better than that of the salt-free form A of **2c** in rats. Since we did not observe the formation of polymorphic forms for **2cb**, we selected **2cb** as the best salt form of inhibitor **2c**.

The in vivo antitumor efficacy of **2cb** in mouse and rat xenograft models with transplanted 4-1ST gastric cancer cell lines is shown

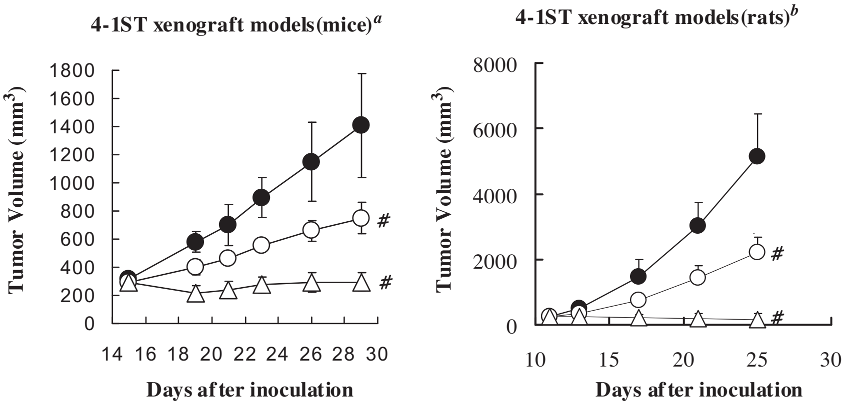


Figure 3. Anti-tumor activity of **2cb** in 4–1ST xenograft models. [a]Values are means ± S.D. of five mice. (●): Control, (○): 65.3 mg/kg and (Δ): 130.6 mg/kg of **2cb** twice daily from 15 to 28 days after tumor inoculation. Anti-tumor activity of **2cb** was evaluated by comparison of the T/C (%) values at 29 days after tumor inoculation. [#]: $p \leqslant 0.025$ versus control (one-tailed Shirley–Williams test). [b]Values are means ± S.D. of eight rats. (●): Control, (○): 16.4 mg/kg and (Δ): 32.8 mg/kg of **2cb** twice daily from 11 to 24 days after tumor inoculation. [#]: $p \leqslant 0.025$ versus control (one-tailed Shirley–Williams test).

Table 4
In vivo anti-tumor efficacy of **2cb** and compound **A** in mouse and rat

Compound	In vivo anti-tumor efficacy in mouse 4–1ST xenograft model T/C (%)		In vivo anti-tumor efficacy in rat 4-1ST xenograft model T/C (%)	
	100 mg/kg, bid (as salt free)	50 mg/kg, bid (as salt free)	25 mg/kg, bid (as salt free)	12.5 mg/kg, bid (as salt free)
2cb	0	41	−1	41
A	11	44	−12	14

Table 5
Kinase inhibition profile of **2cb**

Enzyme	IC_{50} (nM)	Enzyme	IC_{50} (nM)
HER2	11	Lyn B	3300
EGFR	11	Lyn A	3600
HER4	160	c-met	3900
AuroraB	500	CSK	5100
MEK1	980	28 Other kinases[a]	>10,000
LCK	1300		

[a] Other kinases: VEGFR1, VEGFR2, PDGFRalpha, PDGFRbeta, InsR, TIE2, c-kit, IGF-1R, FAK, ASK1, MEK5, MEKK, TTK, TAK1, JNK1, IKKbeta, ERK1, P38alpha, PKA, PKCtheta, PLK, GSK3beta, B-rafw, FGFR1, FGFR3, Src, ZAP70, BMX.

in Fig. 3. As a reflection of the good PK profile of **2cb**, we observed significant in vivo antitumor efficacy in both mouse and rat models (mouse T/C: 0%, **2cb** po b.i.d at 100 mg/kg as free salt form; rat T/C: −1%, **2cb** po b.i.d at 25 mg/kg as free salt form); these efficacies were comparable to those obtained with **A** (Table 4).

We also investigated the kinase selectivity of **2cb** against 30 different kinases. Except for EGFR/HER2/HER4 (IC_{50} = 11/11 nM/155 nM, respectively), no other tyrosine and serine/threonine kinases were significantly inhibited by **2cb**. Thus, we demonstrated that **2cb** is a HER family-selective inhibitor and is as potent as **A** (Table 5).

4. Conclusion

We performed chemical modification of **1a** to obtain a compound with both high HER2/EGFR inhibitory potency and a good PK profile. Introduction of a dimethyl group into the N-5 linker (**X**) of the pyrrolo[3,2-*d*]pyrimidine ligand was found to be the most effective method to improve the A–B permeability and PK profile with good *c*Log*D* and FISA values. We then converted **2c** into its tosylate salt (**2cb**) to enhance the solubility and PK profile. The salt **2cb** exhibited significant in vivo antitumor efficacy in both mouse and rat models (mouse T/C: 0%, **2cb** po b.i.d at 100 mg/kg; rat T/C: -1%, **2cb** po b.i.d at 25 mg/kg), with results comparable to those obtained for **A**. Consequently, we selected **2cb** as an alternative candidate for preclinical development.

5. Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus or SRS OptiMelt melting point apparatus, and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer or Varian Mercury-300 (300 MHz) spectrometer. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard, and coupling constants (*J* values) are given in Hertz (Hz). Splitting patterns and apparent multiplicities are designated as s (singlet), d (doublet), dd (double doublet), t (triplet), dt (double triplet), q (quartet), m (multiplet), br s (broad singlet). Elemental analyses were carried out by Takeda Analytical Research Laboratories, Ltd., and the results obtained were within ±0.4% of the theoretical values. MS spectra were collected with a Waters LC–MS system (ZMD-1) and were used to confirm $\geqslant$95% purity of each compound. The column used was an L-column 2 ODS (3.0 × 50 mm I.D., CERI, Japan) with a temperature of 40 °C and a flow rate of 1.2 mL/min. Mobile phase A was 0.05% TFA in ultrapure water. Mobile phase B was 0.05% TFA in acetonitrile which was increased linearly from 5% to 90% over 2 min, 90% over the next 1.5 min, after which the column was equilibrated to 5% for 0.5 min. Column chromatography was carried out on a silica gel column (Kieselgel 60, 63–200 mesh, Merck or Chromatorex® NH-DM1020, 100–200 mesh, Fuji Silysia chemical). Yields were not optimized.

Commercial reagents and solvents were used without additional purification. Abbreviations are used as follows: $CDCl_3$, deuterated chloroform; DMSO-d_6, dimethyl sulfoxide-d_6; AcOEt, ethyl acetate; DMF, *N*,*N*-dimethylformamide; MeOH, methanol; THF, tetrahydrofuran; EtOH, ethanol; DMSO, dimethyl sulfoxide; NMP, *N*-methylpyrrolidone.

5.1. *tert*-Butyl{2-[4-({3-chloro-4-[3-(trifluoromethyl)phenoxy]phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}carbamate (4)

A mixture of *tert*-butyl [2-(4-chloro-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]carbamate[9] (**3**, 0.71 g, 2.39 mmol) and 3-chloro-4-[3-(trifluoromethyl)phenoxy]aniline[9] (0.83 g, 2.79 mmol) in 2-propanol (7.1 mL) was stirred at 80 °C for 12 h. To the reaction mixture was added saturated sodium hydrogen carbonate (50 mL) under ice-cooling, and the mixture was extracted with ethyl acetate (200 mL). The organic layer was washed with brine and dried over anhydrous magnesium sulfate. The solvent was concentrated in vacuo, and the residue was purified by silica gel column chromatography (AcOEt/hexane eluent, 1:1 to 1:0) to give 1.12 g (85%) of **4** as white solid. ^{1}H NMR ($CDCl_3$) δ: 1.49 (9H, s), 3.43–3.54 (2H, m), 4.43–4.51 (2H, m), 5.10 (1H, t, *J* = 5.6 Hz), 6.60 (1H, d, *J* = 3.3 Hz), 7.07 (1H, m), 7.09–7.14 (1H, m), 7.16–7.22 (2H, m), 7.25–7.30 (1H, m), 7.37–7.45 (1H, m), 7.89 (1H, dd, *J* = 8.7, 2.4 Hz), 8.02 (1H, d, *J* = 2.4 Hz), 8.50 (1H, s), 8.64 (1H, br s).

The following compound **5** was prepared from **3** by a method similar to that described for **4** using 3-chloro-4-(3-chlorophenoxy)aniline.[9]

5.2. 2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethylcarbamate (5)

Yield 87%, white solid. ^{1}H NMR ($CDCl_3$) δ: 1.50 (9H, s), 3.40–3.60 (2H, m), 4.40–4.60 (2H, m), 5.00–5.10 (1H, m), 6.61 (1H, d, *J* = 2.6 Hz), 6.85–7.05 (2H, m), 7.07 (2H, d, *J* = 8.8 Hz), 7.18 (1H, d, *J* = 2.6 Hz), 7.20–7.30 (1H, m), 7.85–7.95 (1H, m), 8.00–8.05 (1H, m), 8.52 (1H, s), 8.62 (1H, br s).

5.3. 5-(2-Aminoethyl)-*N*-{3-chloro-4-[3-(trifluoromethyl)phenoxy]phenyl}-5*H*-pyrrolo[3,2-*d*]pyrimidin-4-amine dihydrochloride (6)

A mixture of **4** (1.12 g, 2.04 mmol), 2 N HCl (15 mL) and THF (30 mL) was stirred at 60 °C for 20 h. After the mixture was concentrated in vacuo, EtOH (50 mL) was added to the concentrate, and the mixture was further concentrated in vacuo. The residual solid was collected by filtration and washed with AcOEt (30 mL) to give 1.07 g (quant.) of **6** as pale-yellow solid. ^{1}H-NMR (DMSO-d_6) δ: 3.21–3.35 (2H, m), 4.92–5.02 (2H, m), 6.71–6.76 (1H, m), 7.24–7.32 (2H, m), 7.37 (1H, d, J = 9.0 Hz), 7.50–7.56 (1H, m), 7.64–7.71 (2H, m), 7.91–7.97 (1H, m), 7.98–8.06 (1H, m), 8.13–8.26 (3H, m), 8.71 (1H, br s), 9.90 (1H, br s).

The following compound **7** was prepared from **5** by a method similar to that described for **6**.

5.4. 5-(2-Aminoethyl)-*N*-[3-chloro-4-(3-chlorophenoxy) phenyl]-5*H*-pyrrolo[3,2-d]pyrimidin-4-amine dihydrochloride (7)

Yield 94%, pale-yellow solid. ^{1}H NMR (95%$CDCl_3$ + 5%DMSO-d_6) δ: 3.30–3.60 (4H, m), 5.00–5.15 (2H, m), 6.71 (1H, d, J = 3.2 Hz), 6.90–7.00 (2H, m), 7.10–7.20 (1H, m), 7.22 (1H, d, J = 8.8 Hz), 7.30–7.45 (1H, m), 7.60–7.70 (1H, m), 7.87 (1H, d, J = 2.6 Hz), 8.05 (1H, d, J = 2.4 Hz), 8.20–8.40 (2H, m), 8.71 (1H, s).

5.5. *N*-{2-[4-({3-Chloro-4-[3-(trifluoromethyl)phenoxy] phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}-2-(methylsulfonyl)acetamide (1a)

A mixture of **6** (150 mg, 0.288 mmol), 2-(methylsulfonyl)acetic acid (79.6 mg, 0.576 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (166 mg, 0.870 mmol), 1-hydroxybenzotriazole monohydrate (HOBt) (133 mg, 0.870 mmol) and triethylamine (0.40 mL) in *N*,*N*-dimethylformamide (DMF) (5.0 mL) was stirred at room temperature for 20 h. Water (50 mL) was added to the reaction mixture and the mixture was extracted with AcOEt (100 mL). The organic layer was washed with water (30 mL) and brine (30 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/AcOEt eluent, 0:1 to 1:4) to give 129 mg (79%) of **1a** as colorless crystals. mp 177–178 °C. ^{1}H NMR ($CDCl_3$) δ: 3.12 (3H, s), 3.64–3.75 (2H, m), 3.98 (2H, s), 4.43–4.53 (2H, m), 6.62 (1H, d, J = 3.0 Hz), 7.07 (1H, d, J = 9.0 Hz), 7.09–7.15 (1H, m), 7.18–7.33 (4H, m), 7.40–7.45 (1H, m), 7.77 (1H, dd, J = 9.0, 2.7 Hz), 7.96 (1H, d, J = 2.7 Hz), 8.19 (1H, s), 8.51 (1H, s). Anal. Calcd for $C_{24}H_{21}ClF_3N_5O_4$: C, 50.75; H, 3.73; N, 12.33. Found: C, 50.85; H, 3.71; N, 12.38.

The following compound **2a** was prepared from **7** by a method similar to that described for **1a**.

5.6. *N*-[2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]-2-(methylsulfonyl)acetamide (2a)

Yield 69%, pale-yellow crystals. Mp 206–207 °C. ^{1}H NMR ($CDCl_3$) δ: 3.13 (3H, s), 3.60–3.80 (2H, m), 3.99 (2H, s), 4.40–4.60 (2H, m), 6.62 (1H, d, J = 3.4 Hz), 6.85–6.95 (2H, m), 7.00–7.10 (2H, m), 7.20–7.30 (2H, m), 7.70–7.80 (1H, m), 7.95–8.00 (1H, m), 8.19 (1H, s), 8.52 (1H, s). Anal. Calcd for $C_{23}H_{21}Cl_2N_5O_4S$: C, 51.69; H, 3.96; N, 13.10. Found: C, 51.72; H, 3.92; N, 13.01.

5.7. *N*-{2-[4-({3-Chloro-4-[3-(trifluoromethyl)phenoxy] phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}-2-(methylsulfonyl)propanamide (1b)

To a mixture of **6** (150 mg, 0.288 mmol), triethylamine (0.39 mL, 2.80 mmol) and THF (5.0 mL) was added 2-chloropropionyl chloride (54 µL, 0.550 mmol) at room temperature. The mixture was stirred at room temperature for 72 h, and then water (20 mL) was added to the mixture at room temperature. The mixture was extracted with AcOEt (50 mL). The organic layer was washed with saturated brine (20 mL) and dried over anhydrous magnesium sulfate. After concentration in vacuo, to a solution of the residue in DMF (5.0 mL) were added sodium methanesulfinate (85 mg, 0.833 mmol) and pyridine (67 µL, 0.828 mmol) at room temperature, and the mixture was stirred at 70 °C for 48 h. Water (20 mL) was added to the reaction mixture and the mixture was extracted with AcOEt (50 mL). The organic layer was washed with saturated brine (20 mL) and dried over anhydrous magnesium sulfate. After concentration under reduced pressure, the residue was purified by basic silica gel column chromatography (AcOEt/MeOH, 1:0 to 9:1) and recrystallized from AcOEt/diisopropyl ether (10:2 mL) to give 114 mg (68%) of **1b** as colorless crystals. mp 169–171 °C. ^{1}H NMR ($CDCl_3$) δ: 1.71 (3H, d, J = 7.2 Hz), 2.98 (3H, s), 3.63–3.75 (2H, m), 3.81 (1H, q, J = 7.2 Hz), 4.44–4.55 (2H, m), 6.64 (1H, d, J = 3.0 Hz), 7.09 (1H, d, J = 8.7 Hz), 7.11–7.18 (2H, m), 7.19–7.25 (2H, m), 7.30–7.36 (1H, m), 7.40–7.47 (1H, m), 7.85 (1H, dd, J = 8.7, 2.7 Hz), 8.01 (1H, d, J = 2.7 Hz), 8.30 (1H, s), 8.54 (1H, s). Anal. Calcd for $C_{25}H_{23}ClF_3N_5O_4S$: C, 51.59; H, 3.98; N, 12.03. Found: C, 51.66; H, 3.98; N, 12.17.

The following compound **2b** was prepared from **7** by a method similar to that described for **1b**.

5.8. *N*-[2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]-2-(methylsulfonyl) propanamide (2b)

Yield 43%, colorless crystals. Mp 169–171 °C. ^{1}H NMR ($CDCl_3$) δ: 1.71 (3H, d, J = 7.2 Hz), 2.98 (3H, s), 3.65–3.75 (2H, m), 3.81 (1H, q, J = 7.2 Hz), 4.45–4.55 (2H, m), 6.61 (1H, d, J = 3.3 Hz), 6.85–6.90 (1H, m), 6.90–6.95 (1H, m), 7.00–7.10 (2H, m), 7.20–7.30 (1H, m), 7.30–7.40 (1H, m), 7.75–7.85 (1H, m), 7.97 (1H, d, J = 2.4 Hz), 8.28 (1H, s), 8.51 (1H, s). Anal. Calcd for $C_{24}H_{23}Cl_2N_5O_4S\cdot 0.2H_2O$: C, 52.22; H, 4.27; N, 12.69. Found: C, 52.06; H, 4.24; N, 12.72.

5.9. *N*-{2-[4-({3-Chloro-4-[3-(trifluoromethyl)phenoxy]phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}-2-methyl-2-(methylsulfonyl)propanamide (1c)

To a solution of 2-methyl-2-(methylsulfonyl)propanoic acid (115 mg, 0.690 mmol) and DMF (20 µL) in THF (5.0 mL) was added thionyl chloride (0.10 mL, 1.37 mmol) at room temperature. After being stirred at room temperature for 3 h, the mixture was concentrated in vacuo. The residue was dissolved in THF (10 mL) and the solution was added dropwise to a suspension of **6** (180 mg, 0.346 mmol) and triethylamine (0.48 mL, 3.44 mmol) in THF (10 mL) at room temperature. After being stirred at room temperature for 20 h, water (30 mL) was added to the reaction mixture. The mixture was extracted with AcOEt (50 mL). The organic layer was washed with saturated brine (30 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt/MeOH eluent, 1: 0 to 9: 1) to give 205 mg (99%) of **1c** as colorless crystals. Mp 167–168 °C. ^{1}H NMR ($CDCl_3$) δ: 1.70 (6H, s), 2.93 (3H, s), 3.63–3.73 (2H, m), 4.43–4.52 (2H, m), 6.64 (1H, d, J = 3.3 Hz), 7.09 (1H, d, J = 8.7 Hz), 7.10–7.16 (1H, m), 7.18–7.24 (2H, m), 7.27–7.35 (2H, m), 7.40–7.47 (1H, m), 7.90 (1H, dd, J = 8.7, 2.7 Hz), 8.05 (1H, d, J = 2.7 Hz), 8.38 (1H, s), 8.54 (1H, s). *Anal.* Calcd for $C_{26}H_{25}ClF_3N_5O_4S$: C, 52.39; H, 4.23; N, 11.75. Found: C, 52.33; H, 4.18; N, 11.85.

5.10. *N*-[2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]-2-methyl-2-(methylsulfonyl)propanamide (2c)

A mixture of **7** (974 mg, 2 mmol), 2-methyl-2-(methylsulfonyl)propanoic acid (499 mg, 3 mmol), EDC (633 mg, 3.3 mmol), HOBt (450 mg 3.33 mmol) and triethylamine (1.39 mL, 10 mmol) in DMF (10 mL) was stirred at room temperature for 15 h. Water (100 mL) was added to the reaction mixture and the mixture was extracted with AcOEt (200 mL). The organic layer was washed with water (50 mL) and brine (50 mL), dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (AcOEt/MeOH eluent, 1:0 to 20:1) and recrystallized from AcOEt/EtOH-diisopropyl ether (30 mL/6 mL/30 mL) to give 838 mg (74%) of **2c** as colorless crystals. mp 178–180 °C. ^{1}H NMR (CDCl$_3$) δ: 1.70 (6H, s), 2.93 (3H, s), 3.60–3.80 (2H, m), 4.40–4.60 (2H, m), 6.46 (1H, d, J = 2.8 Hz), 6.85–7.00 (2H, m), 7.00–7.15 (2H, m), 7.15–7.30 (2H, m), 7.30–7.40 (1H, m), 7.85–7.95 (1H, m), 8.00–8.05 (1H, m), 8.36 (1H, br s), 8.54 (1H, s). Anal. Calcd for $C_{25}H_{25}Cl_2N_5O_4S$: C, 53.38; H, 4.48; N, 12.45. Found: C, 53.40; H, 4.54; N, 12.36.

5.11. *N*-{2-[4-({3-Chloro-4-[3-(trifluoromethyl)phenoxy] phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}-2-(ethylsulfonyl)acetamide (9)

Under ice-cooling, to a solution of **6** (222 mg, 0.497 mmol) and 4-methylmorpholine (3.0 mL, 27.3 mmol) in THF (7.0 mL) was added chloroacetyl chloride (0.7 mL, 8.78 mmol), and the mixture was stirred at 0 °C for 5 h. To the reaction mixture was added saturated aqueous sodium hydrogen carbonate (100 mL) under ice-cooling, and the mixture was extracted with AcOEt (100 mL). The extract was dried over anhydrous magnesium sulfate and concentrated in vacuo, and then the residue was dissolved in a mixed solvent of DMF (3.5 mL) and THF (6.0 mL). To the mixture was added sodium ethanethiolate (630 mg, 7.49 mmol), and the mixture was stirred at room temperature for 2 h. To the reaction mixture was added saturated aqueous sodium hydrogen carbonate (100 mL) under ice-cooling and the mixture was extracted with AcOEt (100 mL). The extract was dried over anhydrous magnesium sulfate and concentrated in vacuo, and the residue was purified by silica gel column chromatography (AcOEt/MeOH eluent, 100:0 to 90:10) to give **9** as oil. **9** was dissolved in dichloromethane (7.0 mL), and to the solution were added *m*-chloroperbenzoic acid (162 mg, 0.939 mmol) at room temperature. The mixture was stirred at room temperature for 1 h. Saturated aqueous sodium thiosulfate solution (100 mL) was added to the reaction mixture under ice-cooling, and then the mixture was stirred at room temperature for 1 h. The mixture was extracted with dichloromethane (100 mL) and the extract was dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was dissolved in dichloromethane (5.0 mL), and to the solution were added titanium tetraisopropoxide (0.9 mL, 3.06 mmol), MeOH (0.50 mL) and 70% aqueous *tert*-butyl hydroperoxide solution (10 mL, 72.2 mmol), and the mixture was stirred at room temperature for 1 h. Saturated aqueous sodium thiosulfate solution (100 mL) was added to the reaction mixture under ice-cooling, and the mixture was stirred at room temperature for 1 h. The mixture was extracted with dichloromethane (100 mL) and the extract was dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography AcOEt/MeOH eluent, 100:0 to 90:10) and crystallization from diethyl ether–AcOEt–hexane to give 167 mg (58%) of **1d** as colorless crystals. Mp 196–198 °C. ^{1}H NMR (DMSO-d_6) δ: 1.22 (3H, t, J = 7.5 Hz), 3.25 (2H, q, J = 7.5 Hz), 3.40–3.51 (2H, m), 4.03 (2H, s), 4.48–4.63 (2H, m), 6.51 (1H, d, J = 3.0 Hz), 7.17–7.27 (2H, m), 7.31 (1H, d, J = 8.9 Hz), 7.47 (1H, d, J = 7.9 Hz), 7.58–7.68 (2H, m), 7.78 (1H, dd, J = 8.9, 2.5 Hz), 7.99 (1H, d, J = 2.5 Hz), 8.36 (1H, s), 8.72 (2H, s). Anal. Calcd for $C_{25}H_{23}ClF_3N_5O_4S$: C, 51.59; H, 3.98; N, 12.03. Found: C, 51.72; H, 3.99; N, 12.11.

The following compounds (**1e, 1f**) were prepared from **6** and the corresponding sodium thiolate by a method similar to that described for **1d**.

5.12. *N*-{2-[4-({3-Chloro-4-[3-(trifluoromethyl)phenoxy] phenyl}amino)-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}-2-(isopropylsulfonyl)acetamide (1e)

Yield 48%, colorless crystals. Mp 188–191 °C. ^{1}H NMR (DMSO-d_6) δ: 1.24 (6H, d, J = 7.0 Hz), 3.41–3.59 (3H, m), 4.03 (2H, s), 4.48–4.61 (2H, m), 6.51 (1H, d, J = 3.0 Hz), 7.16–7.25 (2H, m), 7.31 (1H, d, J = 8.9 Hz), 7.47 (1H, d, J = 7.7 Hz), 7.57–7.69 (2H, m), 7.78 (1H, dd, J = 8.9, 2.5 Hz), 7.98 (1H, d, J = 2.5 Hz), 8.36 (1H, s), 8.72 (2H, s). Anal. Calcd for $C_{26}H_{25}ClF_3N_5O_3S$: C, 52.39; H, 4.23; N, 11.75. Found: C, 52.54; H, 4.20; N, 11.71.

5.13. 2-(*tert*-Butylsulfonyl)-*N*-{2-[4-({3-chloro-4-[3-(trifluoromethyl)phenoxy]phenyl}amino)-5H-pyrrolo[3,2-*d*]pyrimidin-5-yl]ethyl}acetamide (1f)

Yield 42%, colorless crystals. Mp 172–173 °C. ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s), 3.42–3.51 (2H, m), 3.96 (2H, s), 4.50–4.61 (2H, m), 6.51 (1H, d, J = 3.0 Hz), 7.12–7.36 (3H, m), 7.45–7.55 (1H, m), 7.58–7.67 (2H, m), 7.74 (1H, dd, J = 8.9, 2.5 Hz), 8.00 (1H, d, J = 2.5 Hz), 8.35 (1H, s), 8.53–8.63 (1H, m), 8.71 (1H, s). Anal. Calcd for $C_{27}H_{27}ClF_3N_5O_4S$: C, 53.16; H, 4.46; N, 11.48. Found: C, 53.16; H, 4.38; N, 11.42.

5.14. *N*-[2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]-2-methyl-2-(methylsulfonyl)propanamide benzenesulfonate monohydrate (2ca)

Compound **2c** (400 mg, 0.711 mmol) was dissolved in AcOEt (12 mL) and EtOH (4 mL) by heating at 60 °C, and to the solution was added benzenesulfonic acid monohydrate (132 mg, 0.747 mmol). The mixture was left at room temperature for 17 h under light shielding, and then concentrated in vacuo. To the residue was added AcOEt (10 mL), and the mixture was stood at room temperature for 17 h under light shielding. The resulting crystals were collected by filtration, washed with diisopropyl ether to give 447 mg (87%) of **2ca** as colorless crystals. Mp 142–144 °C. ^{1}H NMR (DMSO-d_6) δ: 1.41 (6H, s), 2.93 (3H, s), 3.50–3.60 (2H, m), 4.65–4.75 (2H, m), 6.65 (1H, d, J = 3.0 Hz), 6.95–7.00 (1H, m), 7.00–7.05 (1H, m), 7.20–7.25 (1H, m), 7.25–7.35 (3H, m), 7.35 (1H, d, J = 8.4 Hz), 7.45 (1H, t, J = 8.4 Hz), 7.55–7.65 (2H, m), 7.67 (1H, dd, J = 8.7, 2.4 Hz), 7.88 (1H, d, J = 3.0 Hz), 7.93 (1H, d, J = 2.4 Hz), 8.20–8.25 (1H, m), 8.73 (1H, s), 9.74 (1H, br s). *Anal.* Calcd for $C_{31}H_{31}Cl_2N_5O_7S_2 \cdot 1.0H_2O$: C, 50.41; H, 4.50; N, 9.48. Found: C, 50.53; H, 4.43; N, 9.48.

5.15. *N*-[2-(4-{[3-Chloro-4-(3-chlorophenoxy)phenyl]amino}-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)ethyl]-2-methyl-2-(methylsulfonyl)propanamide *p*-toluenesulfonate (2cb)

Compound **2c** (9.0 g, 16.0 mmol) was dissolve in ethyl acetate (200 mL) and EtOH (70 mL) by heating at 65 °C, and to the solution was added *p*-toluenesulfonic acid monohydrate (3.04 g, 16.0 mmol). The mixture was left at room temperature under light shielding for 23 h and the resulting crystals were collected by filtration, washed with AcOEt (30 mL) and diisopropyl ether (30 mL) to give 11.5 g (98%) of **2cb** as colorless crystals. mp 217–218 °C. ^{1}H NMR (DMSO-d_6) δ: 1.40 (6H, s), 2.28 (3H, s), 2.93 (3H, s), 3.50–3.60 (2H, m), 4.65–4.75 (2H, m), 6.65 (1H, d, J = 3.0 Hz), 6.90–7.00 (1H, m), 7.00–7.05 (1H, m), 7.10 (2H, d, J = 7.8 Hz), 7.20–7.25 (1H, m), 7.35 (1H, d, J = 9.0 Hz), 7.40–7.50 (3H, m), 7.60–7.70 (1H, m), 7.89 (1H, d, J = 3.0 Hz), 7.91 (1H, d, J = 1.8 Hz), 8.15–8.25 (1H, m), 8.74 (1H, s), 9.80 (1H, br s). *Anal.* Calcd for $C_{32}H_{33}Cl_2N_5O_7S_2$: C, 52.32; H, 4.53; N, 9.53. Found: C, 52.35; H, 4.54; N, 9.49.

5.16. HER2 and EGFR kinase assay

The cytoplasmic domain (amino acids 676–1255) of human HER2 and the cytoplasmic domain (amino acids 669–1210) of human EGFR were expressed as N-terminal peptide (DYKDDDD)-tagged protein using baculovirus expression system. The expressed HER2 kinase and EGFR kinase were purified by using *anti*-FLAG M2 affinity gel (Sigma–Aldrich, USA).

The HER2 and EGFR kinase assays were performed using radio labeled [γ-^{32}P] ATP (GE Healthcare, USA) in 96 well plates. The kinase reactions were performed in 50 mmol/L Tris–HCl, pH7.5, 5 mmol/L $MnCl_2$, 0.01% Tween-20 and 2 mmol/L DTT containing 0.9 μCi of [γ-^{32}P] ATP per reaction, 50 μmol/L ATP, 5 μg/mL poly-Glu-Tyr (4:1) and 0.25 μg/mL of the purified Her2 or EGFR cytoplasmic domain in a total volume of 50 μL. To measure the IC_{50} value for enzyme inhibition, the compounds were incubated with the enzyme for 5 min prior to the reaction at room temperature. The kinase reactions were initiated by adding ATP. After the kinase reaction for 10 min at room temperature, the reactions were terminated by the addition of 10% (final concentration) trichloroacetic acid. The [γ-^{32}P]-phosphorylated proteins were filtrated in Harvest plate (Millipore, USA) with a Cell harvester (PerkinElmer, USA) and washed free of [γ-^{32}P] ATP with 3% phosphoric acid. The plates were dried, followed by the addition of 25 μL of MicroScint0 (PerkinElmer, USA). The radioactivity was counted by a Topcount scintillation counter (PerkinElmer, USA). IC_{50} values and 95% confidence intervals were calculated by nonlinear regression analysis.

5.17. Cell lines and culture

The cell lines BT-474 (HER2 over-expressing human breast cancer) were obtained from American Type Culture Collection. BT-474 cell was cultured in RPMI 1640, and mediums supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Human gastric cancer, 4-1ST was obtained from Central Institutes for Experimental Animals (Kawasaki, Japan). These tumors are maintained in vivo in mice.

5.18. Cell growth assay

HER2 over-expressing human cancer cell (BT-474) was used for the cell growth assays. BT-474 cells were seeded into 48-multiwell plates (3×10^4 cells/well) and allowed to attach overnight. The cells were treated continuously with compounds for 5 days and then the live cell numbers were counted with a particle analyzer (CDA-500; Sysmex Corporation).

5.19. Animals

Female BALB/c nu/nu mice, 5 weeks old, were obtained from Clea Japan Inc. All animals were housed in rooms maintained at 24 ± 1 °C with a 12-h light/12-h dark photo-cycle. Food (CE-2, Clea Japan Inc.) and tetracyclin including water (5% w/v) was provided ad libitum during the experiment period.

Female nude rats (F344/N Jcl-rnu), 5 weeks old, were obtained from Clea Japan Inc. All animals were housed in rooms maintained at 24 °C with a 12-h light/12-h dark photo-cycle. Food (CE-2, Clea Japan Inc.) and water was provided ad libitum.

5.20. Inoculation of BT-474 cells in nude mice

Anti-tumor studies were conducted according to the following procedures. BT-474 xenografts were initiated by subcutaneous implantation of 1×10^7 cells, suspended in 100 μL of MATRIGEL (Becton Dickinson, USA) solution, into the right flank of nude mice and allowed to develop to measurable size. On day 0 and day 7 after inoculation, each mouse was administered intramuscularly with 0.25 mg estradiol dipropionate (ASKA Pharmaceutical Co., Ltd.). The tumor-bearing animals were inspected for physical abnormalities and tumor growth two or three times per week, and animals bearing tumor volume between 200 and 300 mm^3 were used for the further study. At 21 days after the tumor cell inoculation, selected mice were separated into 3 groups and comparisons between the volumes of each group were carried out using the one-way analysis of ANOVA for values of $P < 0.05$.

5.21. Inoculation of 4–1ST in nude mice or rats

Anti-tumor studies were conducted according to the following procedures. Human gastric cancer, 4-1ST tumor tissues were excised from donor nude mice or rats bearing tumors and minced to small pieces. The tumor fragments were implanted subcutaneously and allowed to develop to measurable size. Mice or rats bearing tumor volume between 200 and 300 mm^3 were used for the further study. At 16 days after tumor inoculation, selected mice or rats were separated into 3 groups and comparisons between the volumes of each group were carried out using the one-way analysis of ANOVA for values of $p < 0.05$.

5.22. Antitumor activity in vivo

Selected compounds suspended in a 0.5% (w/v) methylcellulose solution were orally twice daily administered to the mice or rats at the indicated dose (10 mL/kg) for 14 days. For control group, 0.5% (w/v) solution was administered. Tumor volume was assessed in a blind manner and measured at two or three times per week throughout the dosing period using digital calipers. The volume was calculated using the following formula.

$$\text{Tumor volume } (\text{mm}^3) = (\text{a}) \times (\text{b})^2/2$$

(a) longer diameter and (b) shorter diameter.

Drug effects were expressed as (each growth volume of treated tumor/average of growth volume of control tumor) × 100 (T/C (%)). Anti-tumor activities of selected compounds were evaluated by comparison of the T/C (%) values at Day 31, the day following the last dosing day.

5.23. Mouse cassette dosing study

The animals used in the study were female BALB/cAJcl mice (7-weeks old; CLEA Japan, Inc.). Mixture of 5 test compounds were suspended in 0.5 w/v% methylcellulose solution for oral administration at a dose of 10 mg each/10 mL/kg. The concentrations of compounds in the plasma were determined by LC/MS/MS.

5.24. Metabolic stability assay

Human hepatic microsomes were purchased from Xenotech, LLC (Lenexa, KS). An incubation mixture with a final volume of 0.1 mL consisted of microsomal protein in 50 mmol/L phosphate buffer (pH 7.4) and 1 μmol/L test compound. The concentration of hepatic microsomal protein was 0.2 mg/mL. An NADPH-generating system containing 50 mmol/L $MgCl_2$, 50 mmol/L glucose-6-phosphate, 5 mmol/L β-NADP$^+$ and 15 unit/mL glucose-6-phosphate dehydrogenase was prepared and added to the incubation mixture with a 10% volume of the reaction mixture. After the addition of the NADPH-generating system, the mixture was incubated at 37 °C for 0 and 20 min. The reaction was terminated by the addition of acetonitrile equivalent to the volume of the reaction mixture. All incubations were made in duplicate. The test compound in the reaction mixture was measured by HPLC. The Waters Alliance HPLC system was equipped with a separation module, a thermostatted column compartment, and a photodiode array detector (Waters, Milford, MA). The column was a Capcell Pak MG (75 × 4.6 mm I.D., Shiseido, Japan). The column temperature and the flow-rate were 40 °C and 1.2 mL/min, respectively. The mobile phase A was 0.01 mol/L ammonium acetate and the mobile phase B was methanol. The time program for the gradient elution was as follows: the concentration of mobile phase B was linearly increased from 42% to 90% over a period of 8.5 min, 90% over a period of the next 4 min, after which the column was equilibrated to 42% for 5 min. For metabolic stability determinations, chromatograms were analyzed for parent compound disappearance from the reaction mixtures.

5.25. Permeability of test compounds across Caco-2 monolayers

Caco-2 monolayers were grown to confluence on collagen-coated, microporous, polycarbonate membranes in 12-well Costar Transwell® plates. The permeability assay buffer was Hanks' Balanced Salt Solution containing 10 mmol/L HEPES, 15 mmol/L glucose, and 1% bovine serum albumin at a pH of 7.3–7.5. The test compounds dosing concentrations were 10 μmol/L in the assay buffer. The cells were dosed on the apical side (A-to-B) or basolateral side (B-to-A) and incubated at 37 °C with 5% CO_2 in a humidified chamber. At each time point, 1 and 2 h, 200 μL were taken from the A-to-B receivers, and 50 μL were taken from the B-to-A receivers. Fresh assay buffer was added to the receivers after the 1-hour sampling. Also, at 2 h, 50 μL of the donors were taken. Each determination was performed in duplicate. The permeability through a cell-free (blank) membrane was studied to determine non-specific binding and free diffusion of the test compounds through the device. The lucifer yellow flux was also measured for each monolayer after being subjected to the test compounds to ensure no damage was inflicted to the cell monolayers during the flux period. All samples were assayed by LC/MS/MS using electrospray ionization. The apparent permeability, P_{app}, and percent recovery were calculated as follows:

$$\mathrm{P_{app}} = (d\mathrm{C_r}/d\mathrm{t}) \times \mathrm{V_r}/(\mathrm{A} \times \mathrm{C_0}) \tag{1}$$

$$\text{Percent Recovery} = 100 \times ((\mathrm{V_r} \times \mathrm{C_r^{final}}) + (\mathrm{V_d} \times \mathrm{C_d^{final}}))/(\mathrm{V_d} \times \mathrm{C_0}) \tag{2}$$

where, *d*Cr /*d*t is the slope of the cumulative concentration in the receiver compartment versus time in μmol/L s^{-1}; V_r is the volume of the receiver compartment in cm^3; V_d is the volume of the donor compartment in cm^3; A is the area of the cell monolayer (1.1 cm^2 for 12-well Transwell®); C_0 is the nominal concentration of the dosing solution in μmol/L; C_r^{final} is the cumulative receiver concentration in μmol/L at the end of the incubation period; C_d^{final} is the concentration of the donor in μmol/L at the end of the incubation period.

5.26. Kinase selectivity assay

The HER4 kinase assay was performed in the same method as the HER2 and EGFR kinase assays described above using 0.125 μg/mL of HER4 cytoplasmic domain purchased from Upstate (USA).

Assays for the other 17 tyrosine kinases were performed using the Alphascreen® system (Perkin Elmer, USA) in 384-well plates. The cytoplasmic domains of VEGFR2 were expressed as N-terminal FLAG-tagged proteins using a baculovirus expression system. The full-length proteins of FAK and BMX were expressed as N-terminal FLAG-tagged proteins using a baculovirus expression system. FGFR3, VEGFR1(Flt1), PDGFRα, PDGFRβ, TIE2, c-Met, c-kit, Src, Lck, ZAP70, and InsR were purchased from Upstate (USA). FGFR1 was purchased from ProQinase (Germany). IGF-1R and CSK were purchased from BIOMOL (USA). Lyn A and Lyn B were purchased from Invitrogen (USA). The reaction conditions were optimized for each kinase: VEGFR2 (19 ng/mL of enzyme, 10 μmol/L ATP, 10 min reaction, PY-100 conjugated acceptor beads [PY-100]); VEGFR1 (20 ng/mL of enzyme, 0.5 μmol/L ATP, 5 min reaction, PT66 conjugated acceptor beads [PT-66]); FGFR1 (10 ng/mL of enzyme, 0.2 μmol/L ATP, 10 min reaction, PY-100); FGFR3 (20 ng/mL of enzyme, 20 μmol/L ATP, 10 min reaction, PY-100); PDGFRα (50 ng/mL of enzyme, 10 μmol/L ATP, 30 min reaction, PT66); PDGFRβ (50 ng/mL of enzyme, 20 μmol/L ATP, 60 min reaction, PT66); InsR (100 ng/mL of enzyme, 10 μmol/L ATP, 60 min reaction, PT66); TIE2 (20 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PT66); c-Met (1 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PT66); c-kit(10 ng/mL of enzyme, 20 μmol/L ATP, 20 min reaction, PT66); IGF-1R (10 ng/mL of enzyme, 10 μmol/L ATP, 20 min reaction, PT66); Src (0.33 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); Lck (100 ng/mL of enzyme, 2 μmol/L ATP, 30 min reaction, PY-100); BMX (6.6 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); ZAP70 (30 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); CSK(3.2 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); FAK (62 ng/mL of enzyme, 2 μmol/L ATP, 60 min reaction, PT66); Lyn A (2 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100); Lyn B (2.7 ng/mL of enzyme, 2 μmol/L ATP, 10 min reaction, PY-100). The tyrosine kinase reactions were performed in 50 mmol/L Tris–HCl, pH 7.5, 5 mmol/L MnCl2, 5 mmol/L $MgCl_2$, 0.01% Tween-20 and 2 mmol/L DTT, 0.1 μg/mL biotinylated poly-Glu-Tyr (4:1) containing optimized concentration of enzyme, ATP as described above in a total volume of 25 μL. To determine IC_{50} values, the remaining kinase activities at 7 concentrations (0.01, 0.1, 1, 10, 100, 1000, and 10000 nmol/L) of compound were measured. Prior to the kinase reaction, test compound and enzyme were incubated for 5 min at room temperature. The reactions were initiated by adding ATP. After the reaction period as described above at room temperature, reactions were stopped by the addition of 2tμL of 100 mmol/L EDTA, 10 μg/mL Alphascreen streptavidine donor beads and 10 μg/mL acceptor beads described above in 62.5 mM HEPES, pH7.4, 250 mmol/L NaCl, and 0.1% BSA. The plates were incubated in the dark for more than 12 h and read by an EnVision 2102 Multilabel Reader (PerkinElmer, USA) or a Fusion α Plate Reader (Packard, USA). Wells containing the substrate and the enzyme without the compound were used as total reaction control. Wells containing the biotinylated poly-Glu-Tyr (4:1) and the enzyme without ATP were used as basal control.

Assays for 16 serine/threonine kinases were performed using radiolabeled [γ-33P] ATP (GE Healthcare, USA) in 96-well plates. p38α, ERK1, TAK1, ASK1, PKCθ, JNK1, MEK5, GSK3β, B-raf, PLK1, and TTK were expressed as N-terminal FLAG tagged protein using a baculovirus expression system. IKKβ and MEKK1 were expressed as C-terminal FLAG tagged protein using a baculovirus expression system. Aurora-B was expressed as N-terminal 6× His tagged protein using a baculovirus expression system. MEK1 was expressed as N-terminal GST fusion protein using freestyle293 (Invitrogen, USA) expression. PKA was expressed using E.coli expression. The reaction conditions were optimized for each kinase: p38α (100 ng/well of enzyme, 1 μg/well of MBP (Wako, Japan), 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); ERK1 (100 ng/well of enzyme, 2 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); MEKK1 (25 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); TAK1 (30 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); ASK1 (30 ng/well of enzyme, 1 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at

30 °C); PKCθ (25 ng/well of enzyme, 2 μg/well of MBP, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); JNK1 (10 ng/well of enzyme, 1 μg/well of c-Jun, 0.1 μCi/well of [γ-33P] ATP, 60 min reaction at 30 °C); MEK5 (3 ng/well of enzyme, 1 μg/well of GST-ERK5(K83 M), 0.3 μCi/well of [γ-33P] ATP, 30 min reaction at 30 °C); GSK3β (100 ng/well of enzyme, 0.2 μg/well of pGS peptide, 0.1 μCi/well of [γ-33P] ATP, 30 min reaction at room temperature); IKKβ (20 ng/well of enzyme, 1 μg/well of IκBα, 0.1 μCi/well of [γ-33P] ATP, reaction at room temperature); B-raf (25 ng/well of enzyme, 1 μg/well of GST-MEK1(K96R), 0.1 μCi/well of [γ-33P] ATP, 20 min reaction at room temperature); MEK1 (100 ng/well of enzyme, 0.3 μg/well of GST-ERK1(K71A) 0.2 μCi/well of [γ-33P] ATP, 20 min reaction at room temperature); Aurora-B (50 ng/well of enzyme, 30 μmol/L of Aurora substrate peptide, 0.2 μCi/well of [γ-33P] ATP, 60 min reaction at room temperature); PLK1 (80 ng/well of enzyme, 3 μg/well of α-casein (usb, USA), 0.2 μCi/well of [γ-33P] ATP, 40 min reaction at room temperature); TTK (120 ng/well of enzyme, 0.3 μg/well of GST-MOBK1B, 0.2 μCi/well of [γ-33P] ATP, 10 min reaction at room temperature); PKA (3 nmol/L of enzyme, 1 μmol/L of PKA substrate peptide (Upstate, USA), 0.2 μCi/well of [γ-33P] ATP, 10 min reaction at room temperature). Except for the PKCθ reaction, the serine/threonine kinase reactions were performed in 25 mmol/L HEPES, pH 7.5, 10 mmol/L magnesium acetate, 1 mmol/L DTT and 0.5 μmol/L ATP containing optimized concentration of enzyme, substrate and radiolabeled ATP as described above in a total volume of 50 μL. For the PKCθ reaction, enzyme reactions were performed in 25 mmol/L HEPES, pH 7.5, 10 mmol/L magnesium acetate, 1 mmol/L DTT, lipid activator (Upstate, USA) and 0.5 μmol/L ATP containing optimized concentration of enzyme, substrate and radiolabeled ATP as described above in a total volume of 50 μL. To determine IC_{50} values, the remaining kinase activities at 5 concentrations (1, 10, 100, 1000, and 10000 nmol/L) of compound were measured. Prior to the kinase reaction, compound and enzyme were incubated for 5 min at reaction temperature. The kinase reactions were initiated by adding ATP. After the reaction period as described above, the reactions were terminated by the addition of 10% (final concentration) trichloroacetic acid. The [γ-33P]-phosphorylated proteins were filtrated in Harvest Plate (Millipore, USA) with a Cell Harvester (PerkinElmer, USA) and then free of [γ-33P] ATP was washed out with 3% phosphoric acid. The plates were dried, followed by the addition of 40 μL of MicroScintO (PerkinElmer, USA). Radioactivity was counted by a TopCount scintillation counter (PerkinElmer, USA). Wells containing the substrate and the enzyme without the compound were used as total reaction control. Wells containing the substrate and radiolabeled ATP without the enzyme were used as basal control. IC_{50} values were calculated by nonlinear regression analysis.

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