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Research paper

Replacing the terminal piperidine in ceritinib with aliphatic amines confers activities against crizotinib-resistant mutants including G1202R

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ABSTRACT

The piperidine fragment in ceritinib was replaced with diverse aliphatic amines to improve inherent resistance issues of ceritinib. While most of the prepared compounds exhibit as similar in vitro activities as ceritinib, compound **10** shows encouraging activities against wild-type ALK as well as crizotinib-resistant mutants including extremely resistant G1202R mutant with an IC_{50} of 1.8 nM. Furthermore, pharmacokinetic profiles of **10** is apparently better than that of ceritinib. In murine xenograft studies, compound **10** turns out to be as active as ceritinib, suggesting that further optimization of **10** may lead to clinical candidates overcoming ALK mutant issues.

1. Introduction

Anaplastic lymphoma kinase (ALK), one of the receptor tyrosine kinases, was first identified as a fused form with nucleophosmin (NPM) in anaplastic large cell lymphoma (ALCL) and a subclass of T-cell non-Hodgkin lymphomas [1]. In 2007, echinoderm microtubule-associated protein-like-4 (EML4)-ALK, found in over 6% of non-small cell lung cancers (NSCLC), was recognized as a promising oncogenic drug target [2]. Although the specific physiological function of ALK in cancer remains unclear, ALK fusion protein has been found in various human cancers [3], such as breast, colorectal, inflammatory myofibroblastic tumors (IMT), diffuse large B-cell lymphoma (DLBCL), and most notably in 2–7% of NSCLC and 70% of ALCL [4]. Moreover, it has been reported that the amplification or point mutations of ALK is involved in the development of neuroblastoma (NB), anaplastic thyroid cancer, and ovarian cancer [5].

In 2011, crizotinib was approved as the first drug to treat ALK-positive advanced NSCLC patients (Fig. 1) [6]. It is remarkable in a sense that the clinical development of crizotinib took only six years from the ALK rearrangement identification in NSCLC patients. However, relapse inevitably occurs usually in a year of crizotinib treatment. It has been known that crizotinib resistance occurs through various mechanisms, such as secondary mutations, amplification of the EML4-ALK fusion gene, and EGFR activation [7,8]. To overcome the advent of crizotinib-resistant mutants, second-generation ALK inhibitors were developed, such as ceritinib and alectinib. Ceritinib is known to be active against most of the crizotinib-resistant mutants except G1202R and F1174C [9]. Considering ALK mutations occurred in 5–7% of NB cases, one of

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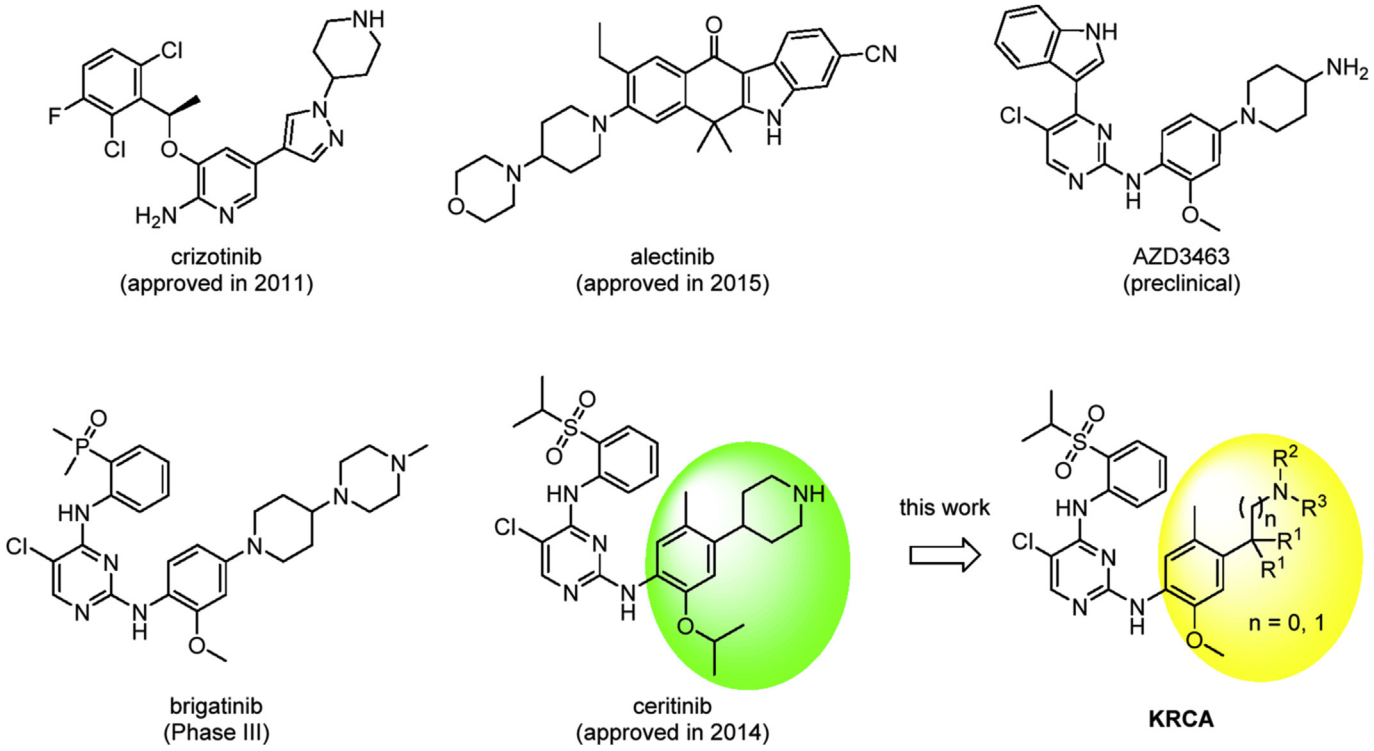


Fig. 1. Known ALK inhibitors and design strategies.

the most significant drawbacks of ceritinib is unfavorable blood-brain barrier (BBB) penetration abilities. On the other hand, alectinib has superb BBB penetration properties as well as activities against the ALK mutants except G1202R [10,11]. It is noteworthy to mention that relapsed tumors from ceritinib and alectinib often express the G1202R mutant [9,11]. Lately, tremendous efforts were devoted to discover ALK inhibitors fortified with G1202R activities, giving rise to the discovery of a macrocyclic molecule mimicking crizotinib (PF-06463922; Clinical Phase II) [12] and an alectinib analog (JH-VIII-157-02) [13].

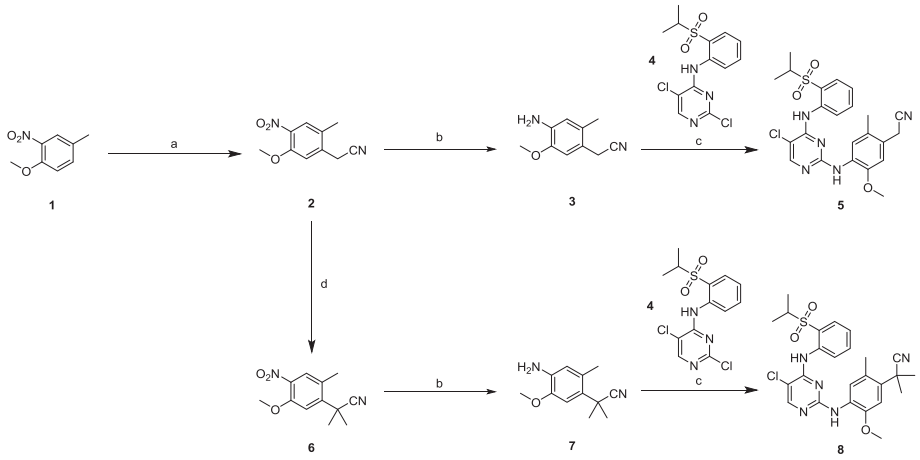
The 2-position of pyrimidine in ceritinib has been explored extensively to find out novel ALK inhibitors by introducing either bicyclic scaffolds, such as tetrahydrothiazoloazepine [14], benzazepines [15], tetrahydroisoquinolines [16], and tetrahydronaphthalenes [17], or flexible side chains, such as amino acid analogs [18], phosphamides or carbamates [19], and hydrazones [20]. Ceritinib has a secondary amine at the piperidine part as a key functional group known to interact with Glu1210 of ALK [21]. A recent literature suggested that modification of the terminal secondary amine in ceritinib could be tolerable to maintain efficacies [19], as one may envision flexible accommodation nature of this terminal amine pocket from the structures of AZD3463 [22] and brigatinib [5,23], recently developed ALK inhibitors with terminal primary and tertiary amines, respectively (Fig. 1). A previous report revealed that presence of a methyl group *para* to the alkoxy group on the aromatic ring helps slow down the metabolism of the phenyl group by blocking the formation of reactive metabolites [24]. With the above information in mind and aiming to achieve challenging G1202R mutant activities, **KRCA** compounds were designed and synthesized in which the methyl group was retained while the piperidine was replaced with diverse aliphatic amine groups.

2. Chemistry

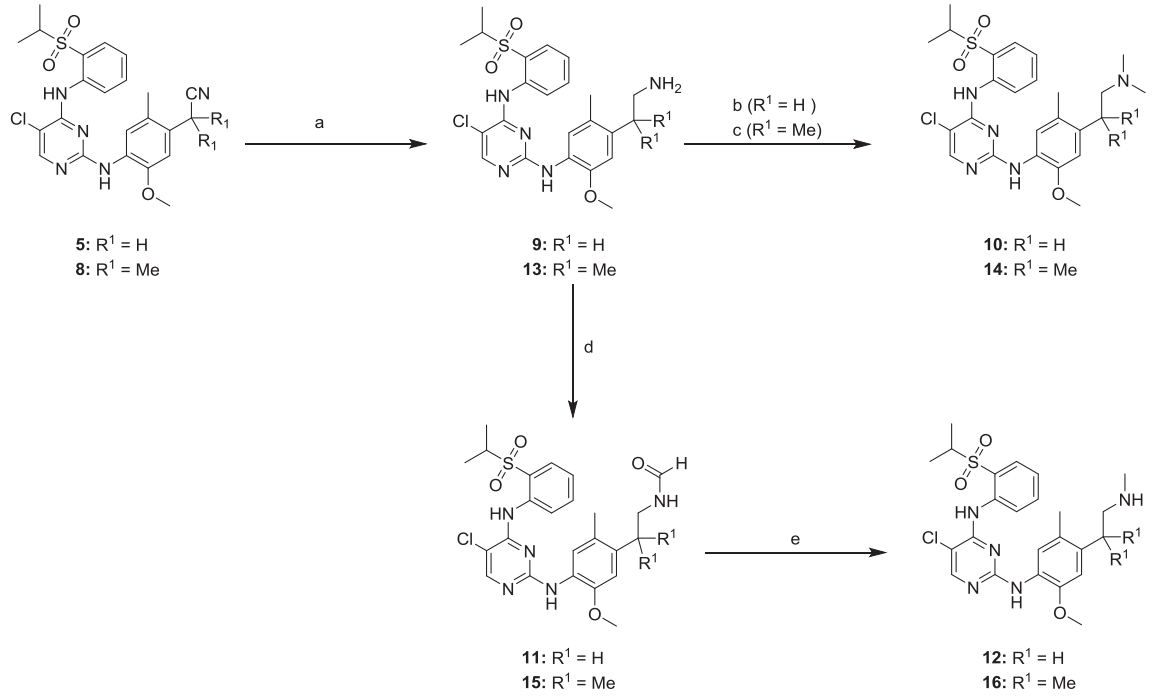
Compounds **5** and **8** were synthesized as outlined in Scheme 1, starting with 4-methyl-2-nitroanisole **1**. Introduction of acetonitrile group *para* to the nitro group of anisole **1** was carried out using phenylthioacetonitrile and powdered sodium hydroxide in DMSO to afford nitrile **2** [25]. Nitro group of **2** was reduced under hydrogenation conditions using Pd/C in MeOH to facilitate amine **3**. Commercially available pyrimidine **4** was coupled with amine **3** to facilitate compound **5** in the presence of diluted HCl [16]. Similarly, compound **8** was prepared from dimethylated nitrile **7**, which was generated from tandem NaH-mediated dimethylation of the active methylene group of **2** and subsequent reduction of the nitro group in **6**.

To examine substitution effects of the terminal amine group, primary, secondary, and tertiary amines were prepared (Scheme 2). Reduction of the nitrile group in **5** and **8** with $BH_3 \cdot THF$ proceeded effectively to give primary amines **9** and **13**, respectively. Tertiary amines were prepared by using two different methods, *viz.* reductive alkylation of **9** with formaldehyde and $NaBH_3CN$ for **10**, and *N*-alkylation of **13** using iodomethane and DIPEA for **14**. Primary amines **9** and **13** were also transformed to afford formamides **11** and **15** under refluxing ethyl formate conditions [26], and subsequent reduction with $LiAlH_4$ gave secondary amines **12** and **16** in low yields, respectively.

With a view to understanding the effect of carbon chain length, compounds **22**–**23** and **29**–**30**, one carbon shorter version of **9**–**10** and **13**–**14**, respectively, were prepared (Scheme 3). Nitrile **2** was treated with conc. HCl to facilitate amide **17**. While acid-catalyzed hydrolysis of **2** in H_2SO_4 (aq) under refluxing conditions resulted in the complete hydrolysis of nitrile to acid, the same reaction condition with nitrile **6** delivered amide **24**. Treatment of **17** and **24** with (bis(trifluoroacetoxy)iodo)benzene gave Hofmann rearrangement products **18** and **25**, respectively [27]. Tandem protection of amines **18** and **25** with trifluoroacetyl and reduction of the nitro groups afforded amides **19** and **26**, and anilines **20** and **27**, respectively. Anilines **20** and **27** were coupled with **4** in the presence of HCl to give compounds **21** and **28**, respectively. Compounds



Scheme 1. Reagents and conditions: (a) $PhSCH_2CN$, NaOH, DMSO, 0 °C to rt, 1 h (78%); (b) Pd/C, H_2 (g), MeOH, rt, 12 h (91% for **3**; 89% for **7**); (c) **4**, 0.08 M HCl in EtOEtOH, 80 °C, 12 h (81%); (d) NaH, MeI, DMF, 0 °C to rt, 8 h (94%).



Scheme 2. Reagents and conditions: (a) 1.0 M BH_3 in THF, THF, rt, 12 h (45%); (b) formaldehyde (37 wt. % in H_2O), AcOH (cat.), $NaBH_3CN$, MeOH, rt, 2 h (77%); (c) MeI, DIPEA, DMF, μW, 80 °C, 10 min (47%); (d) HCO_2Et, 60 °C, 4 h (76% for **11**; 94% for **15**); (e) $LiAlH_4$, THF, 60 °C, 12 h (17% for **12**; 20% for **16**).

22 and **29** were obtained from hydrolysis of **21** and **28** with LiOH (aq), respectively. The *N*,*N*-dimethylation was achieved using formaldehyde for **22** and iodomethane for **29** to furnish tertiary amines **23** and **30**, respectively.

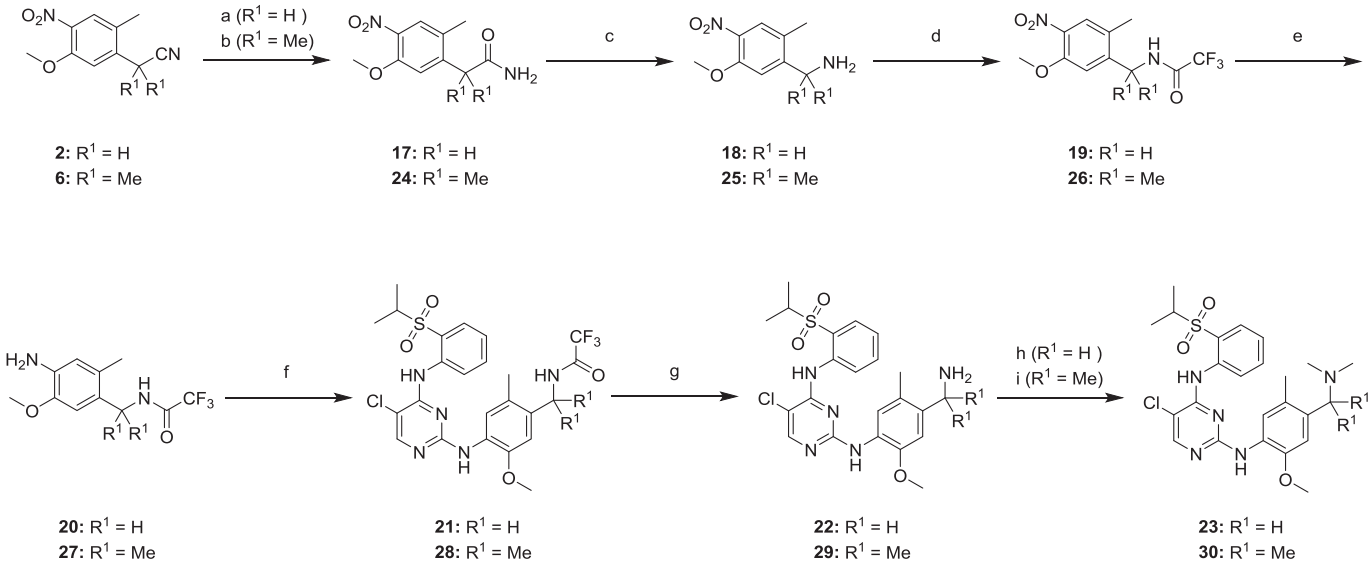
With **18** and **25** in hand, tandem formylation, nitro reduction, coupling with **4**, and amide reduction gave rise to **31**–**34** and **35**–**38**, respectively (Scheme 4).

3. Results and discussion

3.1. In vitro biological evaluations

Overall 18 KRCA compounds were synthesized with varying alkyl chain lengths and substitutions. To investigate the anti-proliferative activities, these compounds were evaluated for their ALK inhibitory activity in both enzymatic and cell-based assays. Enzymatic assays include the ALK wild-type and crizotinib-resistant L1196M mutant enzymes. The cell lines harboring EML4-ALK fusion genes were utilized for the assay, such as H3122, Ba/F3 L1196M, and Ba/F3 EML4-ALK wild-type.

Nitriles **5** and **8** are not active most likely due to the absence of terminal amines. All of the other compounds tested exhibited activities against both ALK wild-type and crizotinib-resistant L1196M mutant enzymes with IC_{50} values of single-digit nano molars. In cell assays, non-substituted primary amines, **9** and **22**, are less active than ceritinib against at least two of the three cell lines tested. Among phenethylamines without substitution at the benzylic carbon (**9**–**12**), tertiary amine **10** is more active than primary (**9**) and secondary (**12**) amines as well as formamide **11**. Likewise, when the benzylic position was substituted with dimethyl (**13**–**16**), tertiary



2: R^1 = H
6: R^1 = Me

17: R^1 = H
24: R^1 = Me

18: R^1 = H
25: R^1 = Me

19: R^1 = H
26: R^1 = Me

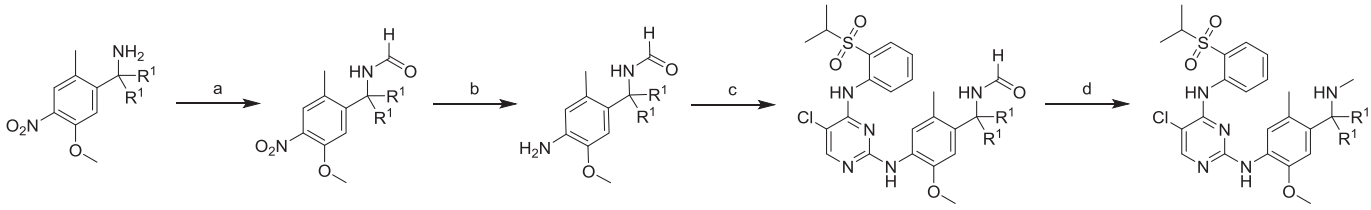
20: R^1 = H
27: R^1 = Me

21: R^1 = H
28: R^1 = Me

22: R^1 = H
29: R^1 = Me

23: R^1 = H
30: R^1 = Me

Scheme 3. Reagents and conditions: (a) conc. HCl, 30 °C, 48 h (70%); (b) H_2SO_4, H_2O, 100 °C, 4 h (78%); (c) $PhI(OCOCF_3)_2$, CH_3CN/H_2O (1/1), rt, 12 h (76% for **18**; 89% for **25**); (d) TFAA, Et_3N, DCM, 0 °C to rt, 30 min (76% for **19**; 83% for **26**); (e) Pd/C, H_2 (g), MeOH, rt, 2 h (82% for **20**; 63% for **27**); (f) **4**, 0.08 M HCl in EtOEtOH, 80 °C, 12 h (82% for **21**; 74% for **28**); (g) $LiOH{\cdot}H_2O$, THF/MeOH/H_2O (4/2/1), rt, 12 h (78% for **22**; 40% for **29**); (h) formaldehyde (37 wt. % in H_2O), AcOH (cat.), $NaBH_3CN$, MeOH, rt, 2 h (57%); (i) MeI, DIPEA, DMF, μW, 80 °C, 10 min (47%).



18: R^1 = H
25: R^1 = Me

31: R^1 = H
35: R^1 = Me

32: R^1 = H
36: R^1 = Me

33: R^1 = H
37: R^1 = Me

34: R^1 = H
38: R^1 = Me

Scheme 4. Reagents and conditions: (a) HCO_2Et, 60 °C, 12 h (52% for **31**; 78% for **35**); (b) Pd/C, H_2 (g), MeOH, rt, 12 h (85% for **32**; 87% for **36**); (c) **4**, 0.08 M HCl in EtOEtOH, 80 °C, 12 h (42% for **33**; 55% for **37**); (d) $LiAlH_4$, THF, 60 °C, 12 h (17% for **34**; 27% for **38**).

amine **14** was the most active. Substitution at the benzylic position does not seem go give significant influences on the activity. Compound **10** is 2- to 7-fold more active than ceritinib in the cell assays. In case of the benzyl amines prepared (**22**–**38**), all of them showed moderate to good activities in the cell assays except primary amine **22**. Compound **29**, primary amine with dimethyl substituted at the benzylic position, turns out to be the most active among the benzyl amines. Considering both enzyme and cell assay results, compound **10** (KRCA-764) was eventually selected for further in-depth investigation.

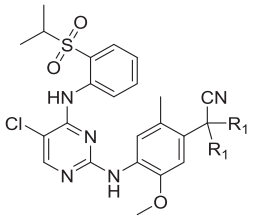
G1202R is one of the most crucial mutants to overcome in patients with crizotinib- or ceritinib-resistant NSCLC [9,11]. Due to the incapability of first- and second-generation ALK inhibitors against G1202R, discovery of novel ALK inhibitors conferring G1202R activities is urgently needed. Thus, the activity of selected compound **10** was further evaluated using several crizotinib-resistant ALK mutant enzymes (Table 2). Overall, compound **10** exhibited similar or better activities than those of ceritinib against the mutants tested. In case of G1202R, compound **10** showed seven-fold higher activity than that of ceritinib. Additionally, the activity of compound **10** against G1269A, one of the most frequently observed secondary mutations along with L1196M [9], is three-fold more active than ceritinib.

3.2. Docking studies

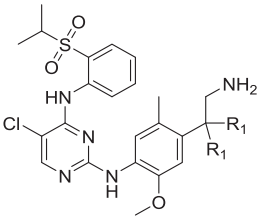
To decipher the binding mode of **10** for wild-type and L1196M mutant ALKs, docking studies were performed using the x-ray co-crystal structure of wild-type ALK and ceritinib (PDB code 4MKC) [9] as well as L1196M mutant ALK complexed with crizotinib (PDB code 2YFX) [28]. In case of wild-type ALK, the proposed binding mode of **10** is similar to that of ceritinib (Fig. 2a). Compound **10** has hinge region interactions with Met1199 backbone amide as ceritinib does. Moreover, oxygen of the sulfonyl group in **10** has a hydrogen bond with Lys1150 through water-mediated interactions. 5-Chloropyrimidine core structure, located between Ala1148 and Leu1256 at the active site, is bound to a hydrophobic pocket composed of gatekeeper residue Leu1196. Methoxyphenyl group of **10** forms hydrophobic interactions with Leu1122. In particular, the tertiary amine group of **10**, heading for the solvent exposed area, makes a hydrogen bond with Ser1206 as well as electrostatic interactions with Asp1203 and Glu1210.

In Fig. 2b, predicted binding modes of both ceritinib and **10** in L1196M mutant ALK appear to be virtually identical, as observed in wild-type ALK. It is worthwhile to point out that there is a weak steric repulsion adjacent to Met1196 in case of **10**. Using the x-ray structure of ALK with ceritinib (PDB code 4MKC), a G1202R mutant ALK model was generated (Fig. 2c). The G1202R mutant model shows that the bulky isopropoxy group of ceritinib has a significant amount of steric collision interactions with Arg1202 side chain [29], while the methoxy group of **10** has much less steric effect, providing a rationale for the improved G1202R mutant activity of **10** over ceritinib [13,16].

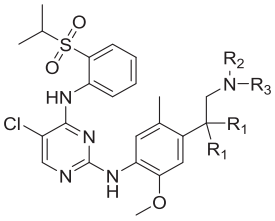
Table 1
ALK inhibitory activities against enzymes and cells.



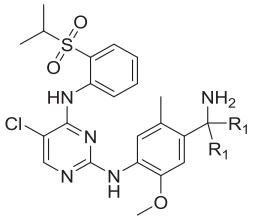
5: R^1 = H
8: R^1 = Me



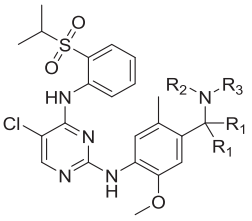
9: R^1 = H
13: R^1 = Me



10: R^1 = H; R^2 = R^3 = Me
11: R^1 = R^2 = H; R^3 = CHO
12: R^1 = R^2 = H; R^3 = Me
14: R^1 = R^2 = R^3 = Me
15: R^1 = Me; R^2 = CHO ; R^3 = H
16: R^1 = R^2 = Me; R^3 = H



22: R^1 = H
29: R^1 = Me



23: R^1 = H; R^2 = R^3 = Me
30: R^1 = R^2 = R^3 = Me
33: R^1 = R^2 = H; R^3 = CHO
34: R^1 = R^2 = H; R^3 = Me
37: R^1 = Me; R^2 = CHO; R^3 = H
38: R^1 = R^2 = Me; R^3 = H

Compounds	Enzymes (IC_{50} nM)		Cells (CC_{50} nM)		
	ALK (wt)	ALK L1196M	H3122	Ba/F3 L1196M	Ba/F3 EML4-ALK wt
ceritinib	14	29	25	75	75
5	21	36	74	190	61
8	200	570	250	440	250
9	2.7	1.7	60	300	68
10	1.4	1.3	13	35	10
11	4.0	4.4	68	260	60
12	1.5	1.9	20	58	13
13	4.9	3.4	53	96	36
14	2.2	3.6	26	66	14
15	7.8	6.1	63	99	38
16	1.5	1.7	41	68	19
22	1.9	2.1	110	39	120
23	3.4	2.1	37	66	21
29	1.5	1.6	12	55	12
30	2.8	1.5	35	91	20
33	4.7	2.8	28	62	15
34	1.7	2.2	25	54	15
37	2.1	2.5	52	81	24
38	1.2	1.4	29	66	15

Table 2
Inhibitory activities against ALK mutant enzymes.

Compounds	G1202R (nM)	G1269A (nM)	T1151-L1152 insT (nM)	F1174L (nM)	C1156Y (nM)
ceritinib	13	1.4	0.63	1.5	2.0
10	1.8	0.4	0.63	0.56	1.1

3.3. Pharmacokinetic profiles

Pharmacokinetic profiles of compound **10** and ceritinib was investigated using male rats with a dose of 10 mg/kg. Compared with ceritinib, compound **10** exhibited over two times of improved C_{max} and AUC values (Fig. 3).

3.4. In vivo xenograft evaluations

Female nude mice were utilized for the xenograft assay using H3122 cells which were inoculated subcutaneously into the right flanks of the mice. When the tumor volume became as big as 200 mm^3, drug treatment began. Ceritinib and **10** were orally administered with a dose of 50 mg/kg each for 14 days (Fig. 4). Although compound **10** exhibited better in vitro activities and PK profiles, compared with ceritinib, xenograft assay results showed that **10** and ceritinib have similar potency, indicating that drug-like properties of **10** should be improved to escalate its efficacy. Furthermore, no substantial body weight changes were observed during this xenograft studies (Fig. 5).

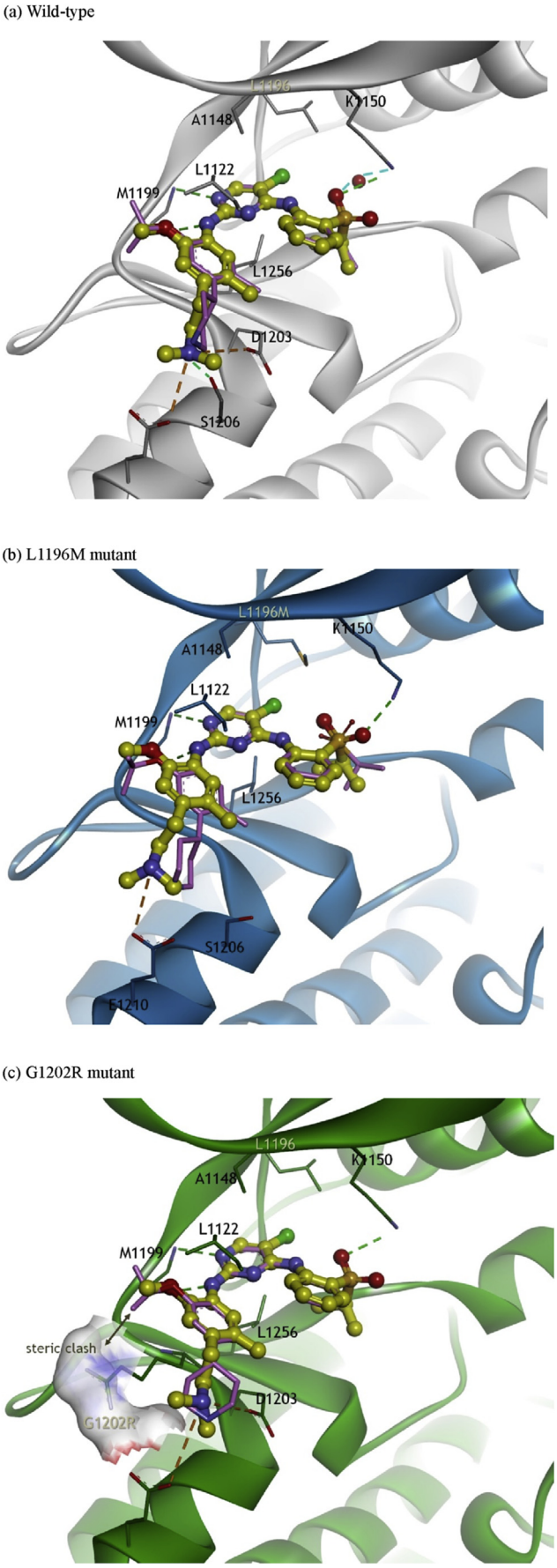
4. Conclusion

Considerably potent ALK inhibitors against wild-type as well as crizotinib-resistant mutants were discovered through replacing piperidine in ceritinib with various alkyl amines. In particular, compound **10** displayed significantly better ALK inhibitory activities in both enzymes and cell assays, compared to those of ceritinib. Intriguingly, highly resistant G1202R mutant is also sensitive to compound **10**. Docking studies support that more compact methoxy group in **10** could be favorable for more effective binding to the active site of G1202R mutant. With these encouraging biological data in hand, further optimization of **10** is ongoing and the progress will be reported shortly.

5. Experimental

5.1. Materials and methods for chemistry

Solvents and reagents were obtained from commercial vendors and used as received. Compound **4** was acquired from Chemlin



(Nanjing, China). TLC was carried out on precoated silica gel F_{254} plate (Merck, art. 5715) and column chromatography was performed using silica gel (Merck, mesh 230–400 μm). Melting point was detected on OptiMelt-Automated Melting Point System. ^{1}H and ^{13}C NMR spectra were recorded with Bruker Avance 300 and 500, using $CDCl_3$ or other deuterated solvents as an internal standard. LC/MS analysis was performed on the Waters Acquity UPLC system with electrospray ionization in positive ion mode. HRMS was detected on JMS-700 (JEOL, Tokyo, Japan). Most of the final compounds in Table 1 have purities over 95% based on LC/MS.

*5.1.1. 2-(4-Nitro-5-methoxy-2-methylphenyl) acetonitrile (**2**)*

To a suspension of powdered sodium hydroxide (5.74 g, 144 mmol) in anhydrous DMSO (15 mL) was added a mixture of 4-methyl-2-nitroanisole (**1**, 2.40 g, 14.4 mmol) and phenylthioacetonitrile (2.14 g, 14.4 mmol) in anhydrous DMSO (10 mL). The reaction mixture was stirred at rt for 1 h, and then poured into ice water and 6 N HCl (aq) solution. The mixture was extracted with DCM (2 × 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give the crude mixture which was purified by silica gel column chromatography using EtOAc/hexanes (2/3) to afford the title compound (2.31 g, 11.2 mmol, 78%) as a yellow solid. Mp 126.9–129.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.15 (s, 1H), 3.99 (s, 3H), 3.73 (s, 2H), 2.32 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.57, 138.75, 135.14, 128.20, 127.41, 116.44, 113.88, 56.80, 22.23, 18.21; LC/MS *m/z* 207.1 [M+H$^+$].

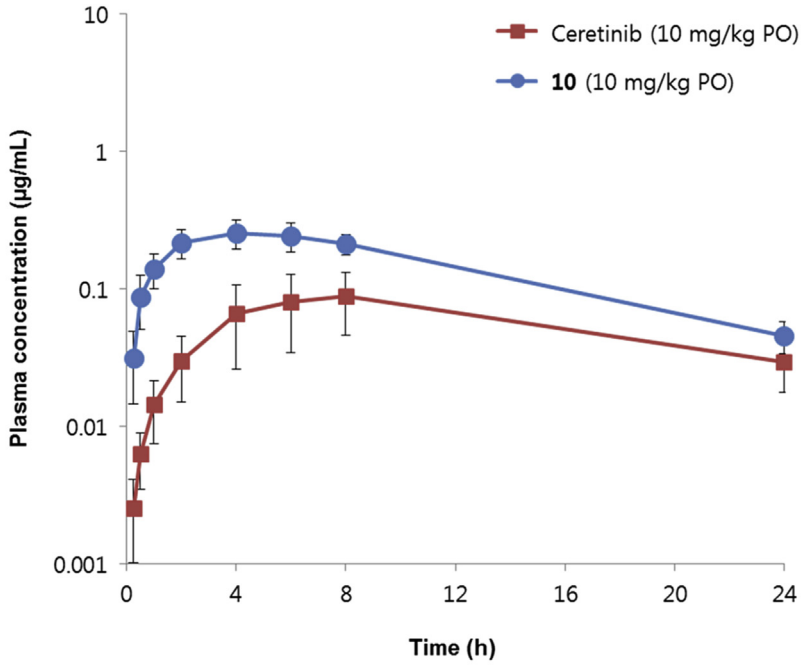
*5.1.2. 2-(4-Amino-5-methoxy-2-methylphenyl) acetonitrile (**3**)*

To a solution of **2** (450 mg, 2.18 mmol) in MeOH (25 mL) was added Pd/C (45.0 mg, 0.422 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 12 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (350 mg, 1.98 mmol, 91%) as an off white solid. Mp 86.5–88.1 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (s, 1H), 6.53 (s, 1H), 3.83 (s, 1H), 3.77 (s, 1H), 3.55 (s, 1H), 2.18 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.68, 136.07, 128.38, 118.37, 117.31, 117.07, 111.11, 55.76, 21.36, 18.50; LC/MS *m/z* 177.1 [M+H$^+$].

*5.1.3. 2-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino) pyrimidin-2-yl)amino)-5-methoxy-2-methylphenyl)acetonitrile (**5**)*

To a solution of **3** (200 mg, 1.13 mmol) in 0.08 M HCl in ethoxyethanol (1.0 mL) was added 2,5-dichloro-*N*-(2-(isopropylsulfonyl) phenyl)pyrimidin-4-amine (**4**, 472 mg, 1.36 mmol) at rt and the reaction mixture was stirred at 80 °C for 12 h. The solid precipitated in the reaction mixture was filtered and washed with ethanol (2.0 mL). The filtered solid was then dissolved in EtOAc (50 mL) and washed with sat. $NaHCO_3$ (aq). The combined organic layers were concentrated under reduced pressure to give the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (450 mg, 0.926 mmol, 81%) as a white solid. Mp 152.8–154.6 °C; ^{1}H NMR

Fig. 2. Molecular docking studies of ceritinib and **10** with wild-type, L1196M, and G1202R mutant ALKs. (a) Proposed binding mode of **10** (yellow, ball and stick style) and overlapped x-ray co-crystal structure of ceritinib (pink, stick style) in wild type ALK (grey ribbon); (b) Predicted binding modes of both ceritinib and **10** in L1196M mutant ALK (blue ribbon); (c) Overlapping **10** and ceritinib docking model in G1202R mutant ALK generated (green ribbon). *Notation: water (red sphere), hydrogen bonds (green dashed lines), water-mediated interactions (sky blue dashed lines), electrostatic interactions (orange dashed lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



compounds	T_{max} (h)	C_{max} (μg/mL)	$T_{1/2}$ (h)	AUC_t (μg•hr/mL)	AUC_∞ (μg•hr/mL)
ceritinib	7.33	0.0898	10.3	1.38	2.65
10	4.00	0.263	7.48	3.75	4.25

Fig. 3. Pharmacokinetic profiles of ceritinib and compound **10** in male rats.

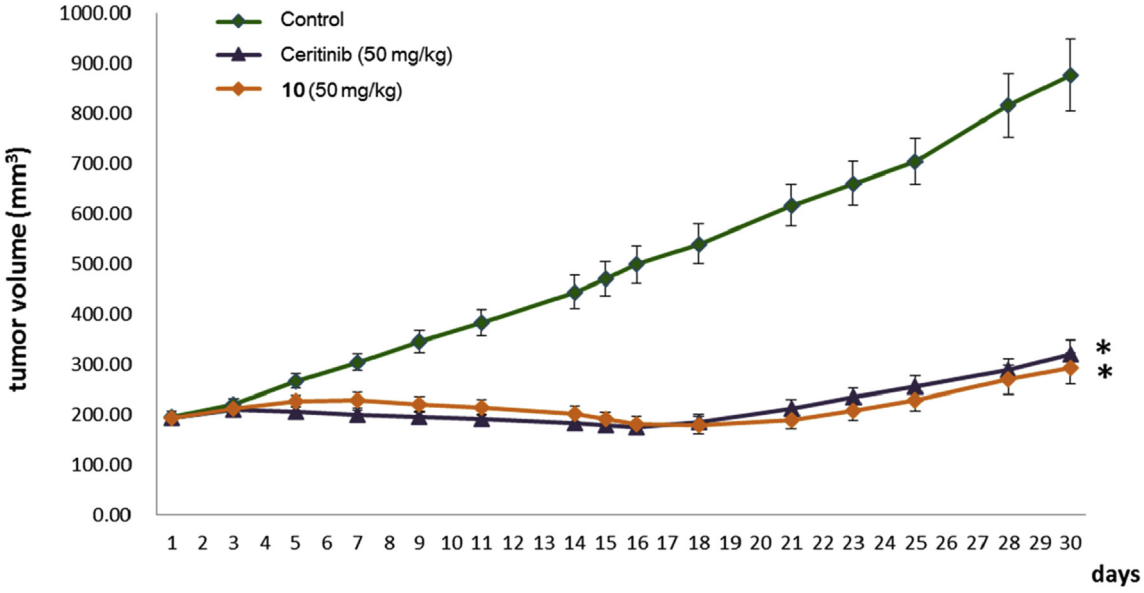


Fig. 4. Effects of compounds on growth of H3122 human lung cancer in nude mice. *$P < 0.001$ vs. control group on the final day using Student's *t*-test.

(300 MHz, $CDCl_3$) δ 9.53 (s, 1H), 8.53 (d, $J = 8.3$ Hz, 1H), 8.18 (s, 1H), 8.13 (s, 1H), 7.94 (d, $J = 7.9$ Hz, 1H), 7.65 (t, $J = 7.9$ Hz, 1H), 7.55 (s, 1H), 7.32–7.25 (m, 1H), 6.86 (s, 1H), 3.91 (s, 3H), 3.64 (s, 2H), 3.26 (sept, $J = 6.9$ Hz, 1H), 2.18 (s, 4H), 1.32 (d, $J = 6.9$ Hz, 7H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.29, 155.37, 155.25, 146.54, 138.35, 134.64, 131.35, 128.68, 127.90, 125.01, 123.59, 123.33, 121.17, 120.62, 117.84,

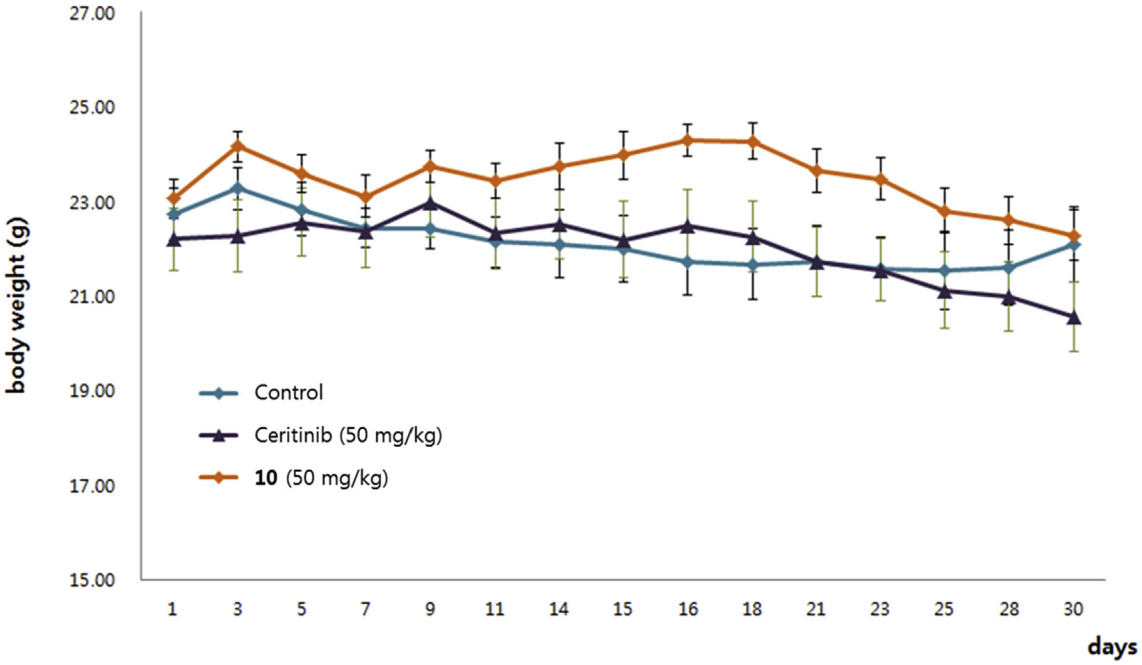


Fig. 5. Effects of ceritinib and **10** on body weight of nude mice bearing H3122.

110.48, 106.49, 56.02, 55.53, 21.69, 18.94, 15.37; LC/MS *m/z* 485.9 [M+H$^+$]; HRMS (EI) *m/z* calcd for $C_{23}H_{24}ClN_5O_3S$ [M$^+$] 485.1288, found 485.1289.

5.1.4. 2-(5-Methoxy-2-methyl-4-nitrophenyl)-2-methylpropanenitrile (*6*)

To a suspension of 60% NaH in mineral oil (0.804 g, 20.2 mmol) in DMF (15 mL) was added a solution of **2** (1.66 g, 8.05 mmol) in DMF (10 mL) at 0 °C. After stirring for 20 min, to the mixture was added CH_3I (1.10 mL, 17.7 mmol) at 0 °C. The reaction mixture was stirred at rt for 8 h, quenched with ice, extracted with EtOAc (2 × 150 mL). The combined organic layers were dried over anhydrous Na_2SO_4 to get the crude mixture which was recrystallized using water. The solid precipitated was filtered to afford the title compound (1.77 g, 7.57 mmol 94%) as an off white solid. Mp 116.5–118.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (s, 1H), 7.06 (s, 1H), 3.97 (s, 3H), 2.62 (s, 3H), 1.83 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.16, 144.51, 129.46, 128.67, 123.20, 111.09, 56.67, 35.84, 27.85, 20.23; LC/MS *m/z* 235.1 [M+H$^+$].

5.1.5. 2-(4-Amino-5-methoxy-2-methylphenyl)-2-methylpropanenitrile (*7*)

To a solution of **6** (450 mg, 1.92 mmol) in MeOH (25 mL) was added Pd/C (45.0 mg, 0.422 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 12 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (350 mg, 1.71 mmol, 89%) as an off white solid. Mp 87.2–88.9 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.75 (s, 1H), 6.59 (s, 1H), 3.86 (s, 3H), 3.78 (s, br, 2H), 2.51 (s, 3H), 1.78 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.22, 135.66, 128.79, 127.46, 125.01, 119.06, 108.00, 55.77, 34.78, 28.45, 20.30; LC/MS *m/z* 205.1 [M+H$^+$].

5.1.6. 2-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-methoxy-2-methylphenyl)-2-methylpropanenitrile (*8*)

To a solution of **7** (200 mg, 0.979 mmol) in 0.08 M HCl in ethoxyethanol (1.0 mL) and 2,5-dichloro-*N*-(2-(isopropylsulfonyl) phenyl)pyrimidin-4-amine (**4**, 407 mg, 1.17 mmol) was added at rt and the reaction mixture was stirred at 80 °C for 12 h. The solid precipitated was filtered and washed with ethanol (2 mL). The solid filtered was then dissolved in EtOAc (50 mL) and washed with sat. $NaHCO_3$ (aq). The combined organic layers were concentrated under reduced pressure to obtain the crude product which was purified by silica gel column chromatography using MeOH/DCM (1:9) to afford the title compound (433 mg, 0.875 mmol, 94%) as a white solid. Mp 207.4–209.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.53 (s, 1H), 8.53 (d, J = 8.3 Hz, 1H), 8.18 (s, 1H), 8.12 (s, 1H), 7.94 (dd, J = 7.9, 1.3 Hz, 1H), 7.66 (t, J = 7.3 Hz, 1H), 7.53 (s, 1H), 7.30 (t, J = 7.9 Hz, 1H), 6.83 (s, 1H), 3.90 (s, 3H), 3.26 (sept, J = 6.9 Hz, 1H), 2.45 (s, 3H), 1.79 (s, 6H), 1.32 (d, J = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.32, 155.40, 155.33, 146.15, 138.34, 134.76, 131.36, 131.11, 128.33, 125.00, 124.58, 123.55, 123.38, 122.45, 107.24, 106.48, 55.93, 55.52, 35.00, 28.27, 20.78, 15.37; LC/MS *m/z* 513.8 [M+H$^+$]; HRMS (EI) *m/z* calcd for $C_{25}H_{28}ClN_5O_3S$ [M$^+$] 513.1601, found 513.1607.

5.1.7. N^2-(4-(2-Aminoethyl)-2-methoxy-5-methylphenyl)-5-chloro-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (*9*)

To a solution of **5** (200 mg, 0.411 mmol) in THF (2 mL) was added 1.0 M BH_3·THF in THF (2.06 mL, 2.06 mmol) at 0 °C. The reaction mixture was stirred at rt for 12 h, quenched with methanol, and concentrated to dryness. To the mixture was added 1.5 N HCl (aq) (25 mL) and extracted with EtOAc. The aqueous layer was basified with NaOH (aq) and extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (90.0 mg, 0.185 mmol, 45%) as a pale yellow solid. Mp 94.1–96.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.57 (d, J = 8.4 Hz, 1H), 8.15 (s, 1H), 8.01 (s, 1H), 7.92 (dd, J = 8.0, 1.3 Hz, 1H),

7.63 (t, J = 7.8 Hz, 1H), 7.49 (s, 1H), 7.29–7.22 (m, 1H), 6.70 (s, 1H), 3.87 (s, 3H), 3.26 (sept, J = 6.8 Hz, 1H), 2.94 (t, J = 6.8 Hz, 2H), 2.75 (t, J = 7.0 Hz, 2H), 2.18 (s, 5H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS m/z 489.8 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{23}H_{28}ClN_5O_3S$ [M$^+$] 489.1601, found 489.1601.

5.1.8. 5-Chloro-N^2-(4-(2-(dimethylamino)ethyl)-2-methoxy-5-methylphenyl)-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**10**)

To a solution of **9** (25.0 mg, 0.051 mmol) in MeOH (2.0 mL) was added acetic acid (catalytic), formalin (35%) (4.37 mg, 0.051 mmol) at 0 °C. After 30 min, $NaBH_3CN$ (4.80 mg, 0.076 mmol) was added to the mixture and stirred at rt for 30 min. The reaction mixture was quenched with $NaHCO_3$ (aq) and extracted with EtOAc (2 × 15 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (20.0 mg, 0.039 mmol, 77%) as a white solid. Mp 140.4–142.1 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.54 (s, 1H), 8.59 (d, J = 8.3 Hz, 1H), 8.18 (s, 1H), 8.05 (s, 1H), 7.95 (dd, J = 8.0, 1.5 Hz, 1H), 7.66 (t, J = 8.5 Hz, 1H), 7.52 (s, 1H), 7.33–7.28 (m, 1H), 6.73 (s, 1H), 3.91 (s, 3H), 3.28 (sept, J = 6.9 Hz, 1H), 2.86 (dd, J = 10.2, 6.5 Hz, 2H), 2.68 (dd, J = 10.2, 6.4 Hz, 2H), 2.52 (s, 6H), 2.20 (s, 3H), 1.34 (d, J = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.57, 155.33, 155.32, 146.49, 138.47, 134.68, 131.96, 131.25, 127.66, 126.68, 124.80, 123.62, 123.11, 120.78, 111.33, 105.87, 60.43, 55.87, 55.45, 45.43, 31.73, 18.91, 15.37; LC/MS m/z 518.0 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{25}H_{32}ClN_5O_3S$ [M$^+$] 517.1914, found 517.1909.

5.1.9. N-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-methoxy-2-methylphenethyl)formamide (**11**)

A solution of **9** (250 mg, 0.510 mmol) in ethyl formate (10 mL) was heated at 60 °C for 4 h. The reaction mixture was concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (200 mg, 0.386 mmol, 76%) as a white solid. Mp 145.5–147.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 9.52 (s, 1H), 8.57 (d, J = 8.4 Hz, 1H), 8.18 (s, 1H), 8.16 (s, 1H), 8.07 (s, 0.2H), 8.05 (s, 0.8H), 7.94 (dd, J = 7.9, 1.3 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.49 (s, 1H), 7.31–7.22 (m, 1H), 6.68 (s, 0.8H), 6.61 (s, 0.2H), 5.54 (s, br, 1H), 3.87 (s, 3H), 3.53 (q, J = 6.8 Hz, 1.7H), 3.44–3.42 (m, 0.4H), 3.26 (sept, J = 6.8 Hz, 1H), 2.82 (t, J = 7.2 Hz, 2H), 2.18 (s, 3H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 517.7 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{24}H_{28}ClN_5O_4S$ [M$^+$] 517.1551, found 517.1551.

5.1.10. 5-Chloro-N^4-(2-(isopropylsulfonyl)phenyl)-N^2-(2-methoxy-5-methyl-4-(2-(methylamino)ethyl)phenyl)pyrimidine-2,4-diamine (**12**)

To a solution of **11** (180 mg, 0.347 mmol) in THF (15 mL) was added $LiAlH_4$ (132 mg, 3.47 mmol) at 0 °C. The reaction mixture was stirred at 60 °C for 12 h, quenched with water and NaOH (aq), and filtered. The filtrate was extracted with EtOAc (2×) and the combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (30.0 mg, 0.059 mmol, 17%) as a pale yellow solid. Mp 123.2–125.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.57 (d, J = 8.3 Hz, 1H), 8.16 (s, 1H), 8.00 (s, 1H), 7.93 (d, J = 7.9 Hz, 1H), 7.63 (t, J = 7.9 Hz, 1H), 7.48 (s, 1H), 7.34–7.21 (m, 1H), 6.70 (s, 1H), 3.87 (s, 3H), 3.26 (sept, J = 6.8 Hz, 1H), 2.80 (s, 4H), 2.48 (s, 3H), 2.18 (s, 3H), 1.78 (s, br, 1H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 504.0 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{24}H_{30}ClN_5O_3S$ [M$^+$] 503.1758, found 503.1729.

5.1.11. N^2-(4-(1-Amino-2-methylpropan-2-yl)-2-methoxy-5-methylphenyl)-5-chloro-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**13**)

To a solution of **8** (100 mg, 0.194 mmol), 1.0 M BH_3·THF in THF (0.972 mL, 0.972 mmol) was added at 0 °C. The reaction mixture was stirred at rt for 12 h, quenched with methanol, and concentrated to dryness. To the mixture was added 1.5 N HCl (aq) (25 mL) and extracted with EtOAc. The aqueous layer was basified with NaOH (aq) and extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (45.0 mg, 0.087 mmol, 45%) as a white solid. Mp 152.3–154.1 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.57 (d, J = 8.4 Hz, 1H), 8.16 (s, 1H), 8.00 (s, 1H), 7.93 (dd, J = 8.0, 1.4 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.47 (s, 1H), 7.31–7.21 (m, 1H), 6.87 (s, 1H), 3.89 (s, 3H), 3.26 (sept, J = 6.9 Hz, 1H), 2.97 (s, 2H), 2.34 (s, 3H), 1.40 (s, 6H), 1.32 (d, J = 6.9 Hz, 6H), 1.26 (s, br, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.40, 155.36, 155.19, 146.08, 138.41, 136.19, 134.76, 131.29, 128.14, 127.05, 124.80, 123.46, 123.33, 123.20, 110.49, 106.16, 55.82, 55.49, 50.43, 40.44, 27.35, 22.80, 15.37; LC/MS m/z 517.7 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{25}H_{32}ClN_5O_3S$ [M$^+$] 517.1914, found 517.1916.

5.1.12. 5-Chloro-N^2-(4-(1-(dimethylamino)-2-methylpropan-2-yl)-2-methoxy-5-methylphenyl)-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**14**)

To a solution of **13** (40.0 mg, 0.077 mmol) in DMF (1.0 mL) was added DIPEA (24.9 mg, 0.192 mmol) and CH_3I (21.9 mg, 0.154 mmol) at rt and the reaction mixture was heated under microwave at 80 °C for 10 min. The reaction was quenched with water (10 mL) and extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with brine solution, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (20.0 mg, 0.036 mmol, 47%) as a pale yellow solid. Mp 134.2–136.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.49 (s, 1H), 8.57 (d, J = 8.3 Hz, 1H), 8.15 (s, 1H), 7.98–7.88 (m, 2H), 7.62 (t, J = 7.8 Hz, 1H), 7.47 (s, 1H), 7.32–7.21 (m, 1H), 6.94 (s, 1H), 3.89 (s, 3H), 3.26 (sept, J = 6.9 Hz, 1H), 2.60 (s, 2H), 2.36 (s, 3H), 2.11 (s, 6H), 1.44 (s, 6H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS m/z 545.6 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{27}H_{36}ClN_5O_3S$ [M$^+$] 545.2227, found 545.2225.

5.1.13. N-(2-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)-5-methoxy-2-methylphenyl)-2-methylpropyl)formamide (**15**)

A solution of **13** (80.0 mg, 0.154 mmol) in ethyl formate (3.0 mL) was heated at 60 °C for 4 h. The reaction mixture was concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (80.0 mg, 0.146 mmol, 94%) as a white solid. Mp 89.4–91.3 °C; ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 9.54 (s, 0.7H), 9.50 (s, 0.2H), 8.56 (d, J = 8.4 Hz, 1H), 8.17 (s, 1H), 8.12 (s, 1H), 8.05 (s, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.64 (t, J = 7.9 Hz, 1H), 7.48 (s, 1H), 7.36–7.21 (m, 1H), 6.86 (s, 0.8H), 6.79 (s, 0.2H), 5.20 (s, 1H), 3.89 (s, 2H), 3.88 (s, 1H), 3.67 (d, J = 5.9 Hz, 1.6H), 3.48 (d, J = 6.6 Hz, 0.4H), 3.26 (sept, J = 6.9 Hz, 1H), 2.37 (s, 2.3H), 2.34 (s, 0.7H), 1.44 (s, 6H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 546.2 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{26}H_{32}ClN_5O_4S$ [M$^+$] 545.1864, found 545.1866.

5.1.14. 5-Chloro-N^4-(2-(isopropylsulfonyl)phenyl)-N^2-(2-methoxy-5-methyl-4-(2-methyl-1-(methylamino)propan-2-yl)phenyl)pyrimidine-2,4-diamine (**16**)

To a solution of **15** (54.0 mg, 0.098 mmol) in THF (5.0 mL) was

added $LiAlH_4$ (37.5 mg, 0.988 mmol) at 0 °C. The reaction mixture was stirred at 60 °C for 12 h, quenched with water and NaOH (aq), and filtered. The filtrate was extracted with EtOAc (2×) and the combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (10.0 mg, 0.018 mmol, 20%) as a white solid. Mp 124.9–127.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.57 (d, J = 8.3 Hz, 1H), 8.16 (s, 1H), 7.98 (s, 1H), 7.92 (dd, J = 7.9, 1.3 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.46 (s, 1H), 7.32–7.19 (m, 1H), 6.90 (s, 1H), 3.88 (s, 3H), 3.26 (sept, J = 6.8 Hz, 1H), 2.86 (s, 2H), 2.39 (s, 3H), 2.35 (s, 3H), 1.45 (s, 6H), 1.32 (d, J = 6.9 Hz, 7H), 1.25 (s, br 1H); LC/MS m/z 531.7 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{26}H_{34}ClN_5O_3S$ [M$^+$] 531.2071, found 531.1993.

5.1.15. 2-(5-Methoxy-2-methyl-4-nitrophenyl)acetamide (**17**)

To a solution of **2** (900 mg, 4.36 mmol) was added HCl (10 mL) at rt and the reaction mixture was stirred at 30 °C for 48 h. The reaction mixture was diluted with water and extracted with EtOAc (2 × 150 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude product which was recrystallized using EtOAc to afford the title compound (690 mg, 3.08 mmol, 70%) as a white solid. Mp 188.1–189.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (s, 1H), 6.98 (s, 1H), 5.53 (s, br, 1H), 5.37 (s, br, 1H), 3.97 (s, 3H), 3.65 (s, 2H), 2.33 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.35, 150.62, 143.22, 137.55, 129.83, 126.16, 116.62, 56.99, 40.64, 18.37; LC/MS m/z 224.9 [M+H$^+$].

5.1.16. (5-Methoxy-2-methyl-4-nitrophenyl)methanamine (**18**)

To a solution of **17** (600 mg, 2.68 mmol) in CH_3CN/H_2O (1/1) was added [bis(trifluoroacetoxy)iodo]benzene (1.15 g, 2.67 mmol) at rt. The reaction mixture was stirred at rt for 12 h, diluted with water (5.0 mL) and $NaHCO_3$ (aq), and extracted with EtOAc (2 × 75 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude product which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (400 mg, 2.04 mmol, 76%) as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.23 (s, 1H), 3.98 (s, 3H), 3.91 (s, 2H), 2.27 (s, 3H), 1.46 (s, br, 2H); LC/MS m/z 197.1 [M+H$^+$].

5.1.17. 2,2,2-Trifluoro-N-(5-methoxy-2-methyl-4-nitrobenzyl) acetamide (**19**)

To a solution of **18** (150 mg, 0.764 mmol) in DCM (15 mL) was added trifluoroacetic anhydride (193 mg, 0.917 mmol) and Et_3N (193 mg, 1.91 mmol) dropwise 0 °C. The reaction mixture was stirred at rt for 30 min, quenched with water (15 mL), and extracted with DCM (2 × 30 mL). The combined organic layers were washed with water and brine solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to obtain the crude product which was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (150 mg, 0.581 mmol, 76%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (s, 1H), 6.96 (s, 1H), 6.53 (s, br, 1H), 4.56 (d, J = 5.9 Hz, 2H), 3.94 (s, 3H), 2.32 (s, 3H); LC/MS m/z 293.0 [M+H$^+$].

5.1.18. N-(4-Amino-5-methoxy-2-methylbenzyl)-2,2,2-trifluoroacetamide (**20**)

To a solution of **19** (150 mg, 0.513 mmol) in MeOH (20 mL) was added Pd/C (15.0 mg, 0.140 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 2 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (3/7) to afford the title compound (110 mg, 0.419 mmol, 82%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.68 (s, 1H), 6.58 (s, 1H), 6.25 (s, br, 1H), 4.45 (d, J = 5.2 Hz, 2H), 3.85 (s, 3H), 3.83 (s, br, 2H), 2.21 (s, 3H); LC/MS m/z 263.1 [M+H$^+$].

5.1.19. N-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino) pyrimidin-2-yl)amino)-5-methoxy-2-methylbenzyl)-2,2,2-trifluoroacetamide (**21**)

To a solution of **20** (100 mg, 0.381 mmol) in 0.08 M HCl in ethoxyethanol (1.0 mL) was added **4** (145 mg, 0.419 mmol) at rt and the reaction mixture was stirred at 80 °C for 12 h. The reaction mixture was then basified with sat. $NaHCO_3$ (aq) and extracted with EtOAc (2 × 25 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using EtOAc/Hexane (3/7) to afford the title compound (180 mg, 0.314 mmol, 82%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.54 (s, 1H), 8.54 (d, J = 8.3 Hz, 1H), 8.18 (s, 1H), 8.14 (s, 1H), 7.94 (dd, J = 7.9, 1.5 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.56 (s, 1H), 7.35–7.22 (m, 1H), 6.76 (s, 1H), 6.30 (s, 1H), 4.49 (d, J = 5.3 Hz, 2H), 3.89 (s, 3H), 3.25 (sept, J = 6.9 Hz, 1H), 2.18 (s, 3H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 572.1 [M+H$^+$].

5.1.20. N^2-(4-(Aminomethyl)-2-methoxy-5-methylphenyl)-5-chloro-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**22**)

To a solution of **21** (170 mg, 0.297 mmol) in THF (4.0 mL), MeOH (2.0 mL) and H_2O (2.0 mL) was added $LiOH\cdot H_2O$ (62.4 mg, 1.49 mmol) at rt. The reaction mixture was stirred at rt for 12 h and concentrated. The reaction was quenched with water (15 mL) and extracted with EtOAc (2 × 35 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (110 mg, 0.231 mmol, 78%) as an off white solid. Mp 91.6–93.8 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.53 (s, 1H), 8.59 (d, J = 8.3 Hz, 1H), 8.18 (s, 1H), 8.05 (s, 1H), 7.95 (dd, J = 7.9, 1.4 Hz, 1H), 7.66 (t, J = 7.8 Hz, 1H), 7.54 (s, 1H), 7.32–7.25 (m, 1H), 6.92 (s, 1H), 3.92 (s, 3H), 3.85 (s, 2H), 3.28 (sept, J = 6.8 Hz, 1H), 2.21 (s, 3H), 1.68 (s, br 2H), 1.34 (d, J = 6.9 Hz, 6H); LC/MS m/z 475.9 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{22}H_{26}ClN_5O_3S$ [M$^+$] 475.1445, found 475.1438.

5.1.21. 5-Chloro-N^2-(4-((dimethylamino)methyl)-2-methoxy-5-methylphenyl)-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**23**)

To a solution of **22** (50.0 mg, 0.105 mmol) in MeOH (2.0 mL) was added acetic acid (catalytic), formalin (35%) (19.8 mg, 0.051 mmol) at 0 °C. After 30 min, $NaBH_3CN$ (9.90 mg, 0.157 mmol) was added to the mixture and stirred at rt for 30 min. The reaction mixture was quenched with $NaHCO_3$ (aq) and extracted with EtOAc (2 × 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (30.0 mg, 0.059 mmol, 57%) as a white solid. Mp 148.1–150.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.50 (s, 1H), 8.57 (d, J = 8.4 Hz, 1H), 8.16 (s, 1H), 8.02 (s, 1H), 7.93 (d, J = 7.9 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.53 (s, 1H), 7.38–7.19 (m, 1H), 6.87 (s, 1H), 3.89 (s, 3H), 3.36 (s, 2H), 3.25 (sept, J = 6.9 Hz, 1H), 2.27 (s, 6H), 2.19 (s, 3H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS m/z 503.9 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{24}H_{30}ClN_5O_3S$ [M$^+$] 503.1758, found 503.1732.

5.1.22. 2-(5-Methoxy-2-methyl-4-nitrophenyl)-2-methylpropanamide (**24**)

To a solution of **6** (500 mg, 2.13 mmol) in H_2O (8.0 mL) was added H_2SO_4 (7.0 mL) at rt. The reaction mixture was stirred at 100 °C for 4 h and extracted with EtOAc (2 × 100 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude product which was purified by silica gel column

chromatography using MeOH/DCM (1/9) to afford the title compound (420 mg, 1.66 mmol, 78%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.14 (s, 1H), 5.33 (s, br, 1H), 5.18 (s, br, 1H), 3.99 (s, 3H), 2.34 (s, 3H), 1.62 (s, 6H); LC/MS *m/z* 253.1 [M+H$^+$].

5.1.23. 2-(5-Methoxy-2-methyl-4-nitrophenyl)propan-2-amine (**25**)

To a solution of **24** (100 mg, 0.396 mmol) in CH_3CN/H_2O (1/1) was added [bis(trifluoroacetoxy)iodo]benzene (171 mg, 0.396 mmol) at rt. The reaction mixture was stirred at rt for 12 h, diluted with water (5.0 mL) and $NaHCO_3$ (aq), and extracted with EtOAc (2 × 25 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude product which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (80.0 mg, 0.356 mmol, 89%) as an off white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.46 (s, 1H), 3.96 (s, 3H), 2.57 (s, 3H), 1.58 (s, 6H), 1.54 (s, br, 2H); LC/MS *m/z* 225.1 [M+H$^+$].

5.1.24. 2,2,2-Trifluoro-N-(2-(5-methoxy-2-methyl-4-nitrophenyl) propan-2-yl)acetamide (**26**)

To a solution of **25** (80.0 mg, 0.356 mmol) in DCM (5 mL) was added trifluoroacetic anhydride (89.9 mg, 0.428 mmol) and Et_3N (90.1 mg, 0.892 mmol) dropwise 0 °C. The reaction mixture was stirred at rt for 30 min, quenched with water (5 mL), and extracted with DCM (2 × 10 mL). The combined organic layers were washed with water and brine solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to obtain the crude product which was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (95.0 mg, 0.296 mmol, 83%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.10 (s, 1H), 6.48 (s, br, 1H), 3.96 (s, 3H), 2.41 (s, 3H), 1.83 (s, 6H); LC/MS *m/z* 321.1 [M+H$^+$].

5.1.25. N-(2-(4-Amino-5-methoxy-2-methylphenyl)propan-2-yl)-2,2,2-trifluoroacetamide (**27**)

To a solution of **26** (95.0 mg, 0.296 mmol) in MeOH (10 mL) was added Pd/C (10.0 mg, 0.094 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 2 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (54.0 mg, 0.186 mmol, 63%) as an off white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.82 (s, 1H), 6.53 (s, 1H), 6.37 (s, br, 1H), 3.84 (s, 3H), 3.74 (s, 2H), 2.33 (s, 3H), 1.83 (s, 6H); LC/MS *m/z* 291.1 [M+H$^+$].

5.1.26. N-(2-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino) pyrimidin-2-yl)amino)-5-methoxy-2-methylphenyl)propan-2-yl)-2,2,2-trifluoroacetamide (**28**)

To a solution of **27** (54.0 mg, 0.186 mmol) in 0.08 M HCl in ethoxyethanol (0.5 mL) was added **4** (77.3 mg, 0.223 mmol) at rt and the reaction mixture was stirred at 80 °C for 12 h. The reaction mixture was then basified with sat. $NaHCO_3$ (aq) and extracted with EtOAc (2 × 25 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (83.0 mg, 0.138 mmol, 74%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.54 (s, 1H), 8.55 (d, J = 8.4 Hz, 1H), 8.20 (s, 1H), 8.09 (s, 1H), 7.96 (dd, J = 7.9, 1.5 Hz, 1H), 7.64 (t, J = 7.2 Hz, 1H), 7.54 (s, 1H), 7.37–7.23 (m, 1H), 6.94 (s, 1H), 6.43 (s, 1H), 3.93 (s, 3H), 3.28 (sept, J = 6.9 Hz, 1H), 2.30 (s, 3H), 1.87 (s, 6H), 1.34 (d, J = 6.9 Hz, 6H); LC/MS *m/z* 599.7 [M+H$^+$].

5.1.27. N^2-(4-(2-Aminopropan-2-yl)-2-methoxy-5-methylphenyl)-5-chloro-N^4-(2-(isopropylsulfonyl)phenyl)pyrimidine-2,4-diamine (**29**)

To a solution of **28** (73.0 mg, 0.121 mmol) in THF (4.0 mL), MeOH (2.0 mL) and H_2O (2.0 mL) was added $LiOH\cdot H_2O$ (127 mg, 3.03 mmol) at rt. The reaction mixture was stirred at rt for 12 h and concentrated. The reaction was quenched with water (5 mL) and extracted with EtOAc (2 × 15 mL). The combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (25.0 mg, 0.049 mmol, 40%) as an off white solid. Mp 178.8–181.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.50 (s, 1H), 8.57 (d, J = 8.3 Hz, 1H), 8.16 (s, 1H), 8.00 (s, 1H), 7.92 (d, J = 7.9 Hz, 1H), 7.64 (t, J = 7.2 Hz, 1H), 7.49 (s, 1H), 7.31–7.22 (m, 1H), 7.15 (s, 1H), 3.89 (s, 3H), 3.26 (sept, J = 6.9 Hz, 1H), 2.43 (s, 3H), 1.76 (s, br, 2H), 1.58 (s, 6H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS *m/z* 504.0 [M+H$^+$]; HRMS (EI) *m/z* calcd for $C_{24}H_{30}ClN_5O_3S$ [M$^+$] 503.1758, found 503.1758.

5.1.28. 5-Chloro-N^2-(4-(2-(dimethylamino)propan-2-yl)-2-methoxy-5-methylphenyl)-N^4-(2-(isopropylsulfonyl)phenyl) pyrimidine-2,4-diamine (**30**)

To a solution of **29** (10.0 mg, 0.019 mmol) in DMF (1.0 mL) was added DIPEA (6.41 mg, 0.049 mmol) and CH_3I (5.63 mg, 0.039 mmol) at rt and the reaction mixture was heated under microwave at 80 °C for 10 min. The reaction was quenched with water (10 mL) and extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with brine solution, concentrated to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (5.0 mg, 0.0090 mmol, 47%) as a pale yellow solid. Mp 141.9–144.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.50 (s, 1H), 8.59 (d, J = 8.3 Hz, 1H), 8.15 (s, 1H), 7.92 (d, J = 8.3 Hz, 2H), 7.63 (t, J = 7.9 Hz, 1H), 7.46 (s, 1H), 7.32–7.18 (m, 1H), 6.91 (s, 1H), 3.87 (s, 3H), 3.26 (sept, J = 6.9 Hz, 1H), 2.47 (s, 3H), 2.16 (s, 6H), 1.38 (s, 6H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS *m/z* 532.3 [M+H$^+$]; HRMS (EI) *m/z* calcd for $C_{26}H_{34}ClN_5O_3S$ [M$^+$] 531.2071, found 531.2070.

5.1.29. N-(5-methoxy-2-methyl-4-nitrobenzyl)formamide (**31**)

A solution of **18** (250 mg, 1.27 mmol) in ethyl formate (10 mL) was heated at 65 °C for 4 h. The reaction mixture was concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (150 mg, 0.669 mmol, 52%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 8.34 (s, 0.86H), 8.20 (d, J = 11.8 Hz, 0.1H), 7.70 (s, 0.12H), 7.68 (s, 0.82H), 7.00 (s, 0.85H), 6.97 (s, 0.12H), 5.88 (s, 1H), 4.50 (d, J = 6.0 Hz, 1.78H), 4.44 (d, J = 6.6 Hz, 0.25H), 3.94 (s, 0.35H), 3.93 (s, 2.59H), 2.31 (s, 2.46H), 2.30 (s, 0.43H); LC/MS *m/z* 225.1 [M+H$^+$].

5.1.30. N-(4-Amino-5-methoxy-2-methylbenzyl) formamide (**32**)

To a solution of **31** (150 mg, 0.668 mmol) in MeOH (15 mL) was added Pd/C (15.0 mg, 0.140 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 12 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (110 mg, 0.566 mmol, 85%) as an off white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (s, 1H), 6.67 (s, 1H), 6.54 (s, 1H), 5.54 (s, br, 1H), 4.37 (d, J = 5.3 Hz, 2H), 3.82 (s, 3H), 3.78 (s, br, 2H), 2.20 (s, 3H); LC/MS *m/z* 194.1 [M+H$^+$].

5.1.31. N-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino) pyrimidin-2-yl)amino)-5-methoxy-2-methylbenzyl)formamide (**33**)

To a solution of **32** (100 mg, 0.514 mmol) in 0.08 M HCl in

ethoxyethanol (1.0 mL) was added **4** (214 mg, 0.617 mmol) at rt and the reaction mixture was stirred at 80 °C for 12 h. The solid precipitated in the reaction mixture was filtered and washed with ethanol (2.0 mL). The filtered solid was then dissolved in EtOAc (50 mL) and washed with sat. $NaHCO_3$ (aq). The combined organic layers were concentrated under reduced pressure to give the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (110 mg, 0.218 mmol, 42%) as an off white solid. Mp 94.8–97.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.53 (s, 1H), 8.54 (d, J = 8.3 Hz, 1H), 8.25 (s, 1H), 8.17 (s, 1H), 8.09 (s, 1H), 7.93 (d, J = 7.9 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.53 (s, 1H), 7.29–7.24 (m, 1H), 6.78 (s, 1H), 5.60 (s, br, 1H), 4.44 (d, J = 5.5 Hz, 2H), 3.88 (s, 3H), 3.25 (sept, J = 6.9 Hz, 1H), 2.19 (s, 3H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS m/z 503.6 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{23}H_{26}ClN_5O_4S$ [M$^+$] 503.1394, found 503.1398.

5.1.32. 5-Chloro-N^4-(2-(isopropylsulfonyl)phenyl)-N^2-(2-methoxy-5-methyl-4-((methylamino)methyl)phenyl)pyrimidine-2,4-diamine (**34**)

To a solution of **33** (50 mg, 0.099 mmol) in THF (15 mL) was added $LiAlH_4$ (37.6 mg, 0.992 mmol) at 0 °C. The reaction mixture was stirred at 60 °C for 12 h, quenched with water and NaOH (aq), and filtered. The filtrate was extracted with EtOAc (2×) and the combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (8.0 mg, 0.016 mmol, 17%) as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.56 (d, J = 8.4 Hz, 1H), 8.16 (s, 1H), 8.04 (s, 1H), 7.93 (d, J = 6.7 Hz, 1H), 7.64 (t, J = 7.9 Hz, 1H), 7.52 (s, 1H), 7.38–7.17 (m, 1H), 6.91 (s, 1H), 3.88 (s, 3H), 3.73 (s, 2H), 3.25 (sept, J = 6.8 Hz, 1H), 2.52 (s, 3H), 2.19 (s, 3H), 2.02 (s, br, 1H), 1.31 (d, J = 6.9 Hz, 6H); LC/MS m/z 490.1 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{23}H_{28}ClN_5O_3S$ [M$^+$] 489.1601, found 489.1595.

5.1.33. N-(2-(5-methoxy-2-methyl-4-nitrophenyl)propan-2-yl) formamide (**35**)

A solution of **25** (250 mg, 1.11 mmol) in ethyl formate (10 mL) was heated at 65 °C for 12 h. The reaction mixture was concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (220 mg, 0.872 mmol, 78%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 8.15 (s, 0.6H), 7.99 (d, J = 12.4 Hz, 0.3H), 7.74 (s, 0.4H), 7.70 (s, 0.6H), 7.15 (s, 1H), 6.09 (d, J = 12.4 Hz, 0.3H), 5.73 (s, br, 0.6H), 3.99 (s, 1H), 3.98 (s, 2H), 2.50 (s, 1H), 2.47 (s, 2H), 1.80 (s, 4H), 1.79 (s, 2H); LC/MS m/z 253.1 [M+H$^+$].

5.1.34. N-(2-(4-Amino-5-methoxy-2-methylphenyl)propan-2-yl) formamide (**36**)

To a solution of GM-15-029 **(35)** (220 mg, 0.872 mmol) in MeOH (25 mL) was added Pd/C (25.0 mg, 0.234 mmol) at rt. The reaction mixture was stirred under a hydrogen balloon at rt for 12 h, filtered through a bed of celite, and evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography using EtOAc/Hexane (2/3) to afford the title compound (170 mg, 0.764 mmol, 87%) as an off white solid. ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 8.11 (s, 0.3H), 7.99 (d, J = 12.4 Hz, 0.7H), 6.84 (s, 0.3H), 6.82 (s, 0.7H), 6.54 (s, 0.7H), 6.52 (s, 0.3H), 5.90 (d, J = 12.6 Hz, 0.7H), 5.56 (s, 0.3H), 3.84 (s, 3H), 3.73 (s, br, 2H), 2.37 (s, 1H), 2.33 (s, 2H), 1.81 (s, 1.9H), 1.69 (s, 4.1H); LC/MS m/z 223.2 [M+H$^+$].

5.1.35. N-(2-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino) pyrimidin-2-yl)amino)-5-methoxy-2-methylphenyl)propan-2-yl) formamide (**37**)

To a solution of **36** (150 mg, 0.674 mmol) in 0.08 M HCl in ethoxyethanol (1.0 mL) was added **4** (280 mg, 0.809 mmol) at rt and the reaction mixture was stirred at 80 °C for 12 h. The solid precipitated in the reaction mixture was filtered and washed with ethanol (2.0 mL). The filtered solid was then dissolved in EtOAc (50 mL) and washed with sat. $NaHCO_3$ (aq). The combined organic layers were concentrated under reduced pressure to give the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (197 mg, 0.371 mmol, 55%) as an off white solid. Mp 113.4–115.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$, rotameric mixture) δ 9.52 (s, 0.6H), 9.50 (s, 0.3H), 8.53 (t, J = 7.7 Hz, 1H), 8.17 (s, 0.6H), 8.16 (s, 0.4H), 8.13 (s, 0.4H), 8.09 (s, 0.6H), 8.02 (d, J = 6.6 Hz, 0.7H), 7.97 (s, 0.3H), 7.93 (d, J = 8.0 Hz, 1H), 7.63 (t, J = 7.9 Hz, 1H), 7.53 (s, 0.6H), 7.51 (s, 0.4H), 7.29–7.28 (m, 0.5H), 7.24–7.23 (m, 0.5H), 6.94 (s, 0.4H), 6.93 (s, 0.6H), 6.03 (d, J = 12.4 Hz, 0.6H), 5.63 (s, br, 0.4H), 3.90 (s, 1.9H), 3.89 (s, 1.1H), 3.26 (sept, J = 6.8 Hz, 1H), 2.32 (s, 1H), 2.30 (s, 2H), 1.81 (s, 2H), 1.73 (s, 4H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 532.0 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{25}H_{30}ClN_5O_4S$ [M$^+$] 531.1707, found 531.1708.

5.1.36. 5-Chloro-N^4-(2-(isopropylsulfonyl)phenyl)-N^2-(2-methoxy-5-methyl-4-(2-(methylamino)propan-2-yl)phenyl)pyrimidine-2,4-diamine (**38**)

To a solution of **37** (170 mg, 0.319 mmol) in THF (15 mL) was added $LiAlH_4$ (121 mg, 3.19 mmol) at 0 °C. The reaction mixture was stirred at 60 °C for 12 h, quenched with water and NaOH (aq), and filtered. The filtrate was extracted with EtOAc (2×) and the combined organic layers were concentrated under reduced pressure to obtain the crude mixture which was purified by silica gel column chromatography using MeOH/DCM (1/9) to afford the title compound (45.0 mg, 0.086 mmol, 27%) as a pale yellow solid. Mp 170.2–172.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.51 (s, 1H), 8.58 (d, J = 8.3 Hz, 1H), 8.16 (s, 1H), 7.98 (s, 1H), 7.93 (d, J = 7.9 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.48 (s, 1H), 7.30–7.20 (m, 1H), 6.93 (s, 1H), 3.88 (s, 3H), 3.26 (sept, J = 6.8 Hz, 1H), 2.41 (s, 3H), 2.17 (s, 3H), 1.75 (s, br, 1H), 1.52 (s, 6H), 1.32 (d, J = 6.9 Hz, 6H); LC/MS m/z 520.0 [M+H$^+$]; HRMS (EI) m/z calcd for $C_{25}H_{32}ClN_5O_3S$ [M$^+$] 517.1914, found 517.1915.

5.2. In vitro enzyme assays

Experimental procedure was followed by the manufactured instruction (Cisbio, France). The reaction was initiated by ATP addition to a mixture containing the kinases, peptide substrates, and inhibitors. After 30 min, EDTA containing solution was added to stop the reaction mixture. EDTA containing solution has europium conjugated *anti*-phospho residue antibody and streptavidin-XL665 (SA-XL665) for the detection of the phosphorylated peptide product. After 1 h incubation, fluorescence was measured with excitation at 337 nm and dual emission at 665 and 620 nm of the Envision reader. IC_{50} was calculated using GraphPad Prism version 5 for Windows. The curves were fit using a nonlinear regression model with a log (inhibitor) versus response formula.

5.3. Cell cytotoxicity assays

For viability experiments, Ba/F3 cells were tested by WST-1 assay. Cells were seeded in 96-well plates at 30% confluency and exposed to chemicals the next day. After 72 h, WST-1 reagent was added and absorbance at 450 nm was measured on a Spectramax spectrophotometer (Molecular Devices, US) according to the manufacturer's instructions. H3122, Ba/F3 L1196M, and Ba/F3

EML4-ALK wild-type were tested by SRB (SulfoRhodamine B) assay. Cells were seeded in 96-well plates at 30% confluency and exposed to chemicals the next day. After 72 h, cells were fixed with 10% CCl_3CO_2H and stained with 0.05% SRB solutions. Then, 10 mM Tris base was added to solubilize the protein-bound SRB dye. Absorbance at 564 nm was measured on each well. IC_{50} and curve fitting were performed at the methods described above.

5.4. Molecular docking

A flexible docking study was performed using the Schrödinger Glide v.7.0 program with standard precision settings (Schrödinger, LLC, http://www.schrodinger.com). The x-ray crystal structure of the human wild type ALK (PDB code 4MKC) and L1196M mutant ALK (PDB code 2YFX) were obtained from the Protein Data Bank (http://www.rcsb.org). The G1202R mutant ALK model was built using 4MKC PDB as a template. The ligands were minimized using a MMFF force field with a dielectric constant of 80.0 using the MacroModel v.11.1 program. The binding models were visualized using Discovery Studio 2016 (Biovia, http://www.accelrys.com).

5.5. PK studies

In vivo PK of compounds was examined in male rats (6–7 weeks, 250–280 g). The rats were cannulated with polyethylene tubing (PE-50, Intramedic, BD Bioscience, MD) in the femoral vein under anesthesia by a mixture of zoletil and rompun solution (3:1, 1 mL/kg). Animals were fasted overnight after the cannulation surgery. Then, the test compound was administered orally using oral gavage. Dosing vehicles were composed of 5% DMSO and 40% PEG400 in water and dosing volume was 2 mL/kg in p.o. administration. Blood samples were collected at different time points ($n = 3$ rats per time point) from the femoral vein. After centrifugation (13000 g, 3 min, 37 °C), the plasma samples were obtained and stored at −20 °C until the analysis. The area under the plasma concentration-time curve (AUC_∞) was calculated by the trapezoidal rule with extrapolation to time infinity. The maximum plasma concentration (C_{max}) was obtained by visual inspection of the plasma concentration-time curve. The concentrations of test compounds were determined by LC/MS/MS on an Agilent 1200 HPLC system coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with ESI source (Agilent, Santa Clara, CA, USA) using imipramine as an internal standard. The peak areas for all components were automatically integrated using Agilent 6460 Quantitative Analysis processing software.

5.6. In vivo xenograft assay

Six week-old female athymic BALB/c (nu/nu) mice were obtained from Charles River of Japan. Animals were maintained under clean room conditions in sterile filter top cages and housed on high efficiency particulate air-filtered ventilated racks. Animals were received sterile rodent chow and water ad libitum. All of the procedures were conducted in accordance with guidelines approved by the Laboratory Animal Care and Use Committee of Korea Research Institute of Chemical Technology. H3122 cells were implanted subcutaneously into the right flank region of each mouse and allowed to grow to the designated size. Once tumors reached an average volume of about 190 mm^3, mice were dosed via oral gavage daily with the indicated doses of compounds **10** and ceritinib for 14 days. Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula: length × $width^2$ × 0.5. Body weight was also assessed twice weekly. Statistical significances were evaluated by using Mann-Whitney *U* test ($a = 0.01$).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.11.046.

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