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Novel 2,4-diaminopyrimidines bearing tetrahydronaphthalenyl moiety against anaplastic lymphoma kinase (ALK): synthesis, *in vitro*, *ex vivo*, and *in vivo* efficacy studies

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Abstract

A series of novel 2,4-diaminopyrimidines bearing tetrahydronaphthalenyl moiety were synthesized and evaluated for their anti-anaplastic lymphoma kinase (ALK) activities using enzymatic and cell-based assays. Amongst the compounds synthesized, compound **17b** showed promising pharmacological results in *in vitro*, *ex vivo*, and pharmacokinetic studies. An *in vivo* efficacy study with compound **17b** demonstrated highly potent inhibitory activity in H3122 tumor xenograft model mice. A series of kinase assays showed that compound **17b** inhibited various kinases including FAK, ACK1, FGFR, RSK1, IGF-1R, among others, thus demonstrating its potential for synergistic anti-tumor activity and development as a multi-targeted non-small cell lung cancer (NSCLC) therapy.

Keywords

Anaplastic lymphoma kinase, cancer, 2,4-diaminopyrimidine, tetrahydronaphthalenyl

Anaplastic lymphoma kinase (ALK) belongs to the insulin receptor family of receptor tyrosine kinases (RTK) and is responsible for many cancers including anaplastic large-cell lymphomas (ALCL), diffuse large B-cell lymphoma (DLBCL), inflammatory myofibroblastic tumors (IMT), and a variety of solid tumors [1-4]. ALK gene is fused to various partner genes, such as NPM-ALK, EML4-ALK, KIF5-ALK, and the fusion genes are detected in approximately 3-7 % of non-small cell lung cancer (NSCLC) in humans [4]. The constitutive kinase activity associated with ALK fusions seems to play an essential role in the growth and survival of cancer cells [5]. Crizotinib bearing 2-aminopyridine structure is the first-in-class drug approved in 2011 for the treatment of ALK-positive lung cancer patients (Figure 1). Although crizotinib displayed impressive anti-ALK efficacy, patients eventually developed resistance owing to ALK mutations including L1196M and C1156M [6, 7]. Ceritinib (LDK378), bearing 2,4-diaminopyrimidine, was approved in 2014 for the treatment of ALK-positive cancer following treatment with crizotinib [8, 9]. Ceritinib could inhibit crizotinib-resistant mutants, L1196M, G1269A, and S1206Y, but failed to inhibit mutant G1202R and 1151Tins. Alectinib having a novel benzo[*b*]carbazole moiety is also active against the crizotinib-resistant ALK mutations L1196M, C1156Y, and F1174L in preclinical studies [10]. A Phase 3 clinical trial study of alectinib is currently ongoing for ALK-positive NSCLC patients [11]. Other inhibitors including AP26113 (Phase 2), [12] PF-06463922 (Phase 1/2), [13] and CEP-37440 (Phase 1) [14] are currently undergoing evaluation in clinical trials.

In our previous effort to discover potent ALK inhibitors [15-17], we synthesized LDK378 and CEP-37440 with hybridized structures, thereby leading to a novel structure, KRCA-0445, which is a 2,4-diaminopyrimidine bearing 4-(isopropylsulfonyl)anilino and bicyclic benzazepine moiety as shown in Figure 2 [18]. In further optimization to improve the anti-ALK potency of KRCAs and to increase structural diversity, we devised novel compounds bearing tetrahydronaphthalenyl (THN) moiety (Figure 2). Particularly, we incorporated a germinal dimethyl substituent in the benzylic methylene of THN since it was found to be easily oxidized to hydroxyl group by Cytochrome P450 [19]. Herein, we report the synthesis of THN analogs and their anti-ALK activities in biochemical and cellular assays as well as pharmacokinetic (PK) and *in vivo* xenograft data of the selected compounds.

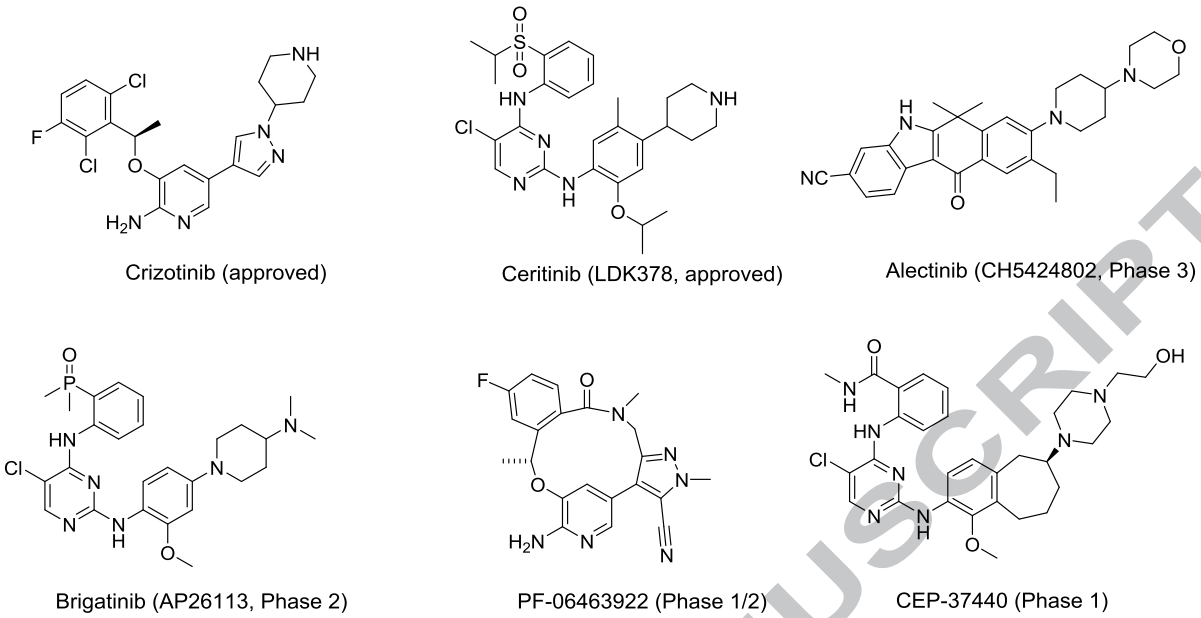


Figure 1. Chemical structures of currently available ALK inhibitors.

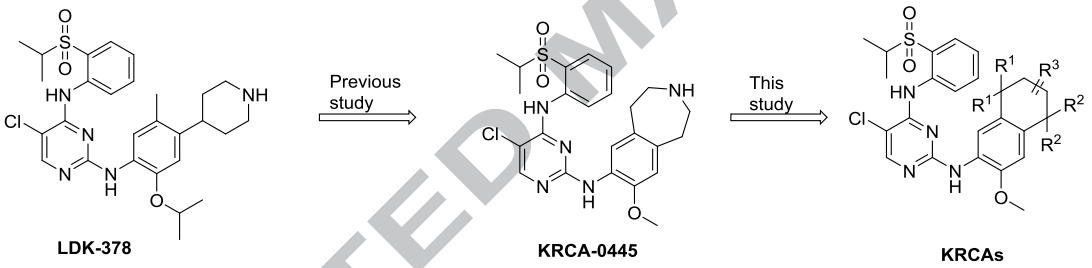
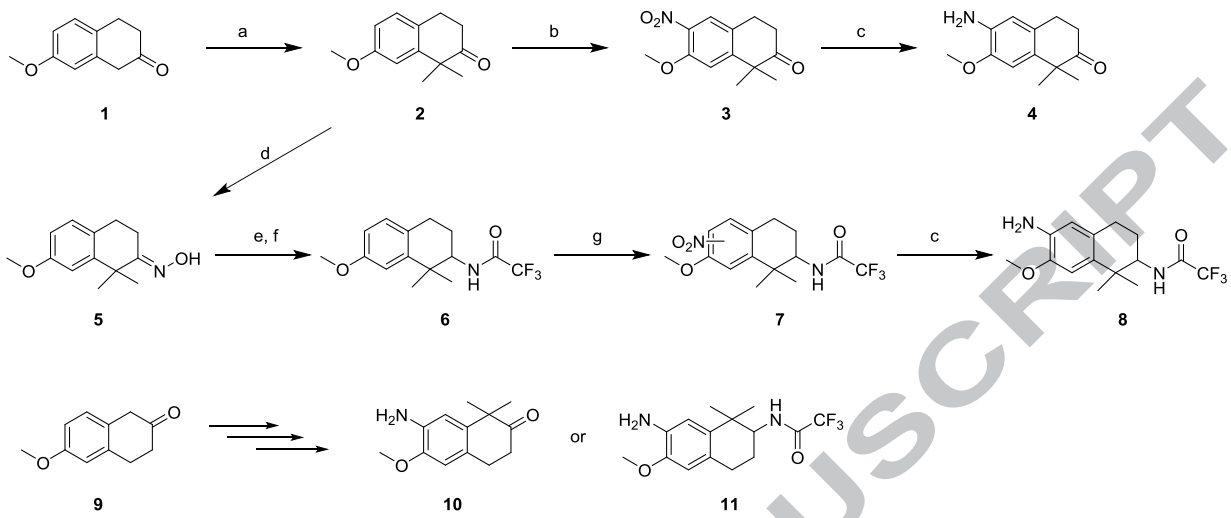


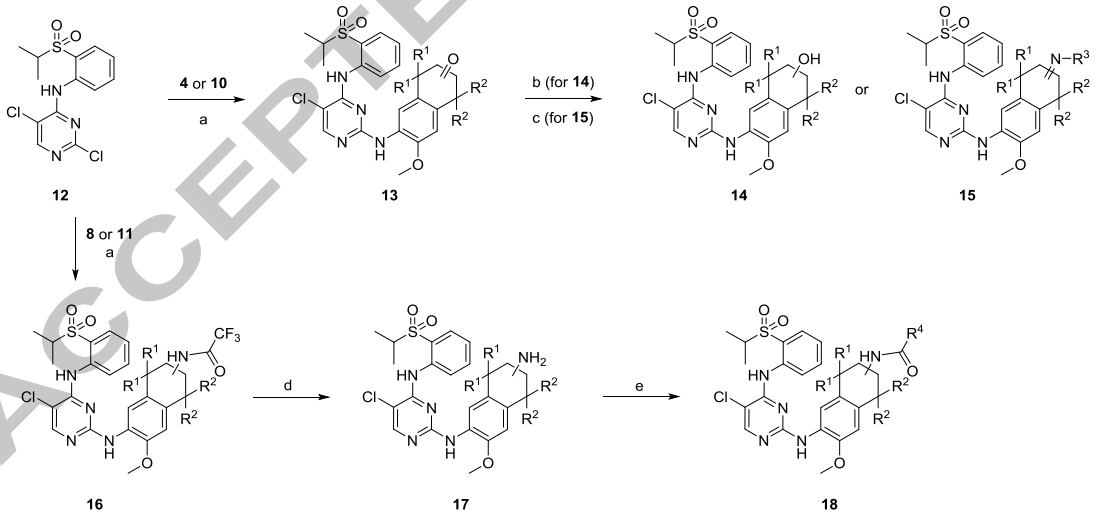
Figure 2. Rational design of KRCAs in this study.

To synthesize novel KRCAs with THN moiety, key THN intermediates **4**, **8**, **10**, and **11** were generated as shown in Scheme 1. 7-Methoxy-2-tetralone (**1)** was treated with MeI in the presence of tetrabutylammonium sulfate (TBAS) and KOH to yield 1,1'-dimethyltetralone (**2)** [19]. Nitration of **2** with KNO_3 afforded 6-nitrotetralone (**3)**. Reduction of the nitro group in the assistance of Pd/C gave 6-aminotetralone (**4)**. Dimethyltetralone (**2)** was converted to oxime (**5)** by treatment of hydroxylamine [20]. Reduction of **5** by treatment of BH_3/THF and subsequent addition of trifluoroacetic anhydride (TFAA) afforded THN (**6)** protected with trifluoroacetyl group. Nitration and reduction of **6** provided the desired amino-THN (**8)**. Compounds **10** and **11** were also prepared

from the 6-methoxy-2-tetralone (**9**) by same synthetic manner described above.



Scheme 1. Synthesis of compound **4**, **8**, **10**, and **11**. Reagents and conditions: (a) MeI, TBAS, KOH, THF, rt, 2 h; (b) KNO_3, TFA, 0 °C to rt, 3 h; (c) H_2, 10% Pd/C, MeOH, rt, 2 h; (d) $NH_2OH{\cdot}HCl$, AcONa, H_2O, MeOH, 90 °C, reflux, overnight; (e) BH_3, THF, reflux, 3 h; (f) TFAA, TEA, DCM, rt, 4 h; (g) KNO_3, TFAA, MeCN, 0 °C to rt, 4 h.



Scheme 2. General synthesis of KRCAs. Reagents and conditions: (a) 4 N HCl in dioxane, 2-ethoxyethanol, 100 °C, overnight; (b) $NaBH_4$, MeOH, rt, 2 h; (c) Nucleophiles, conc. H_2SO_4, EtOH, 80 °C, overnight; (d) LiOH, MeOH, THF, H_2O, rt, overnight; (e) acids, EDCI, HOBt, TEA, DCM, rt, overnight.

The synthesis of KRCA analogs is shown in Scheme 2. Commercially available 4-amino-2,5-dichloropyrimidine **12** was coupled with amino-THNs **4**, **8**, **10**, and **11** under acidic conditions to afford compounds **13** and **16** [21, 22]. The ketone of **13** was reduced by $NaBH_4$ to afford hydroxyl compound **14**. Condensation with various nucleophiles such as hydroxylamine or hydrazines including acetyl hydrazide, semicarbazide, aminoguanidine, 1-amino-4-methylpiperazine, and 1-aminomorpholine gave hydrazone compound **15**. Compound **16** were converted to compound **17** by hydrolysis of trifluoroacetamide group with K_2CO_3 in MeOH. Compound **17** were reacted with glycolic acid or *N*,*N*-dimethylglycine in the presence of 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC) and hydroxybenzotriazole (HOBt) to yield compound **18**.

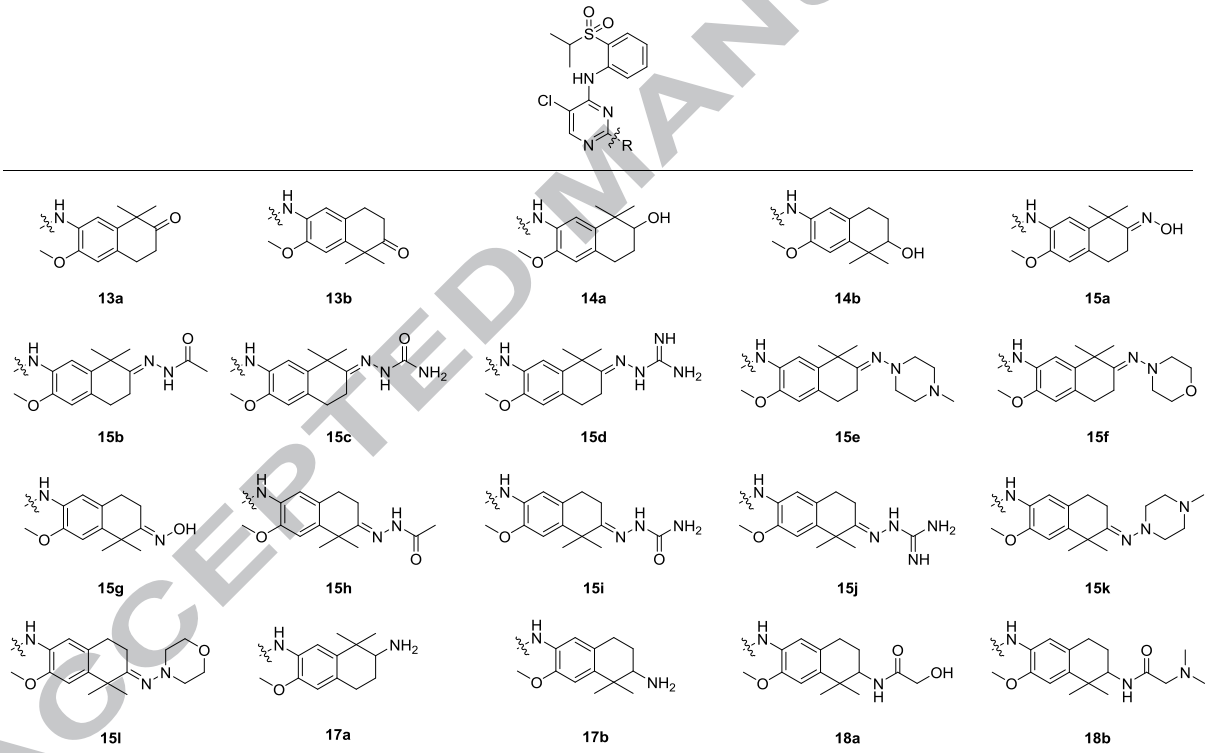


Figure 3. List of KRCAs synthesized in this study.

Table 1. Activity profiles of compounds **13-18**.

No.	ALK (wt.) IC_{50} (nM)	L1196M IC_{50} (nM)	H3122 CC_{50} (nM)	BaF3 L1196M CC_{50} (nM)

LDK378	14 ± 4	29 ± 10	38 ± 7	75 ± 9
13a	342 ± 158	1376 ± 1111	1310 ± 140	1995 ± 49
13b	43 ± 23	207 ± 164	100 ± 7	432 ± 3
14a	34 ± 17	109 ± 47	726 ± 72	1600 ± 87
14b	21 ± 12	56 ± 16	99 ± 7	299 ± 11
15a	424 ± 339	600 ± 30	1220 ± 100	1363 ± 358
15b	52 ± 32	201 ± 121	146 ± 17	640 ± 32
15c	16 ± 14	97 ± 70	146 ± 17	400 ± 20
15d	16 ± 9	100 ± 25	2404 ± 343	2092 ± 190
15e	44 ± 22	200 ± 98	2828 ± 478	2601 ± 400
15f	122 ± 57	222 ± 100	732 ± 123	1643 ± 47
15g	212 ± 154	N.D.	276 ± 24	1528 ± 104
15h	21 ± 11	40 ± 15	128 ± 37	314 ± 2
15i	29 ± 7	42 ± 11	117 ± 10	334 ± 10
15j	18 ± 6	37 ± 10	235 ± 69	1584 ± 23
15k	0.5 ± 0.2	8.4 ± 0.9	14 ± 2	66 ± 6
15l	40 ± 22	40 ± 22	30 ± 6	416 ± 165
17a	27 ± 10	94 ± 40	2094 ± 379	1748 ± 47
17b	0.7 ± 0.2	1.5 ± 0.3	16 ± 4	62 ± 2
18a	6.1 ± 2.5	9.9 ± 2.6	44 ± 6	86 ± 5
18b	4.2 ± 1.1	3.6 ± 0.9	39 ± 4	71 ± 2

The IC_{50} values are for the inhibition of ALK wild type and ALK L1196M mutant using homogenous time resolved fluorescence (HTRF) KinEASE-TK assay in a 384-well plate. The CC_{50} values are for cellular proliferation inhibition. Data are fitted to a four-parameter sigmoidal dose response for determination of IC_{50} and CC_{50} values. The errors are reported as the 95% confidence interval. N.D.: not determined.

All final compounds synthesized were evaluated for their anti-ALK activities in biochemical and cell-based assays and summarized in Table 1. For kinase inhibition studies, ALK wild type (*wt.*) and ALK L1196M homogenous time resolved fluorescence (HTRF) KinEASE-TK assays were performed in a 384-well plate. The cytotoxicity assay was conducted with H3122 (*wt.*) cells, which are EML4-ALK addicted non-small cell lung cancer cells [23]. For ALK mutant cells, Ba/F3 cells transfected with ALK L1196M gene were used [24]. Most of the compounds in this study exhibited excellent ALK inhibitory activities with IC_{50} values in low double-digit nanomolar ranges in wild type ALK enzymatic assays, except for compounds **13a**, **15a**, **15f**, and **15g**. Interestingly, compounds **15k**

and **17b** were highly potent (IC_{50} = 0.5 nM and 0.7 nM, respectively), and showed 88-fold and 39-fold more potency than their structural isomers **15e** and **17a**, respectively. Although most of the compounds showed good inhibitory activity against mutant L1196M ALK in a similar pattern observed in wild type, the mutant enzyme was relatively less sensitive, about 2~6-fold against inhibitors in comparison with wild type. Among the compounds synthesized, **15k** and **17b** were the most promising in both wild and mutant ALK enzyme assays.

In the cell-based assay with H3122 cells, five compounds **15k**, **15l**, **17b**, and **18a-18b** showed excellent anti-ALK activities with CC_{50} values in low double-digit nanomolar ranges (14-44 nM). These compounds also exhibited moderate inhibitory potencies against L1196M BaF3 mutant cell lines. Remarkably, **15d**, **15e**, and **17a,** which showed excellent activity in the enzyme assays, exhibited weak cytotoxicity with IC_{50} values in micromolar ranges. It is worthwhile to note that compounds **13b**, **14b**, **15g-15l**, and **17b** derived from THN intermediates **4** and **8** are generally more potent than compounds **13a**, **14a**, **15a-15f**, and **17a** derived from THNs **10** and **11**. It is reported that the terminal piperidine in LDK378 fits closely to the protein surface and plays an important role in the interaction with E1210 in ALK [24]. The activity differences observed might be related to the interaction with E1210. Amongst the compounds tested in this study, **15k** and **17b** were the most potent in both enzymatic and cell-based assays.

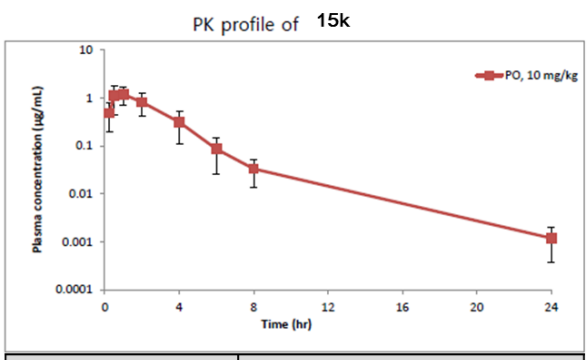
Two highly active compounds (**15k** and **17b**) were tested in the human and mouse liver microsomal models to aid in the prediction of metabolic stability (Table 2). The hERG inhibition with patch clamp experiment and CYP inhibition assay were also conducted for predicting *in vivo* toxicity. Compound **17b** displayed moderate stability (56 % remaining at 30 min) in human microsomal models but relatively short half-life in mouse microsomal models (23 % remaining at 30 min). Compound **15k** was rapidly metabolized in both mouse and human microsomal models (14 % in mouse and 20 % in human). In hERG assay, compounds **15k** and **17b** exhibited weak hERG inhibition with an IC_{50} value of 24 and 44 μM, respectively, thus posing weak or no cardiac toxicity in relation to potassium channel blockade. In case of CYP inhibition, compound **15k** showed no or poor

inhibition against a panel of five CYP isozymes at 10 μM, while compound **17b** inhibited 97 % and 72 % of CYP3A4 and CYP2C19 isozymes, respectively.

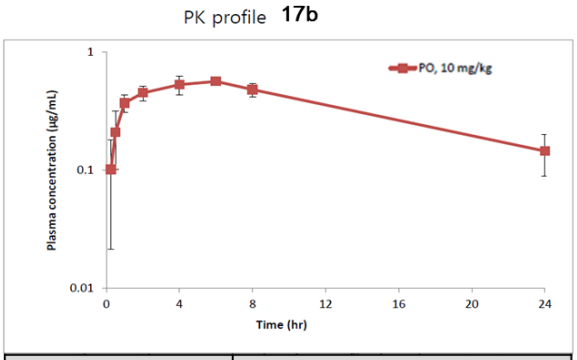
Table 2. Metabolic stability, hERG, and CYP inhibition of **15k** and **17b**

Compound	Metabolic stability Mouse / Human (% remaining at 30 min)	hERG patch clamp (IC_{50}, μM)	CYP inhibition at 10 μM (%)				
			1A2	2C9	2D6	3A4	2C19
15k	14 / 20	24	7.2	17.1	11.4	47.5	43.4
17b	23 / 56	44	9.5	38.0	31.0	97.0	72.0

Next, pharmacokinetic studies were performed with compounds **15k** and **17b**. Serum concentrations were determined using LC-MS/MS after oral administration (PO) of compounds (10 mg/kg) and the data is summarized in Figure 4. Interestingly, compounds **15k** and **17b** showed promising pharmacokinetic parameters in rats although these had a short half-life in the *ex vivo* mouse microsomal model. Particularly, compound **17b** showed very a long half-life ($T_{1/2}$) with 9.5 h and large AUC values, thus could exhibit long drug exposure time in the *in vivo* study.



Parameters	PO, 10 mg/kg
T_{max} (hr)	0.833 ± 0.289
C_{max} (μg/mL)	1.27 ± 0.57
$T_{1/2}$ (hr)	3.04 ± 0.07
AUC_t (μg·hr/mL)	3.75 ± 1.76
AUC_∞ (μg·hr/mL)	3.76 ± 1.76



Parameters	PO, 10 mg/kg
T_{max} (hr)	5.33 ± 1.15
C_{max} (μg/mL)	0.582 ± 0.036
$T_{1/2}$ (hr)	9.5 ± 2.9
AUC_t (μg·hr/mL)	8.75 ± 0.94
AUC_∞ (μg·hr/mL)	10.9 ± 1.8

Figure 4. Pharmacokinetic parameters of **15k** and **17b** in male rats.

Both **15k** and **17b** exhibited excellent anti-ALK activities and moderate pharmacological properties; hence, their *in vivo* antitumor efficacy was assessed in H3122 tumor xenograft-bearing SCID mouse. Compounds and controls (DMSO and LDK378) were administered to SCID mice orally in 20 % PEG400 and 3 % Tween 80 in DDW at doses of 50 mg/kg or 25 mg/kg q.d. for the 14-day duration of the study (Figure 5). Tumor growth was inhibited and regression was observed in all conditions during drug treatment for 14 days. Although slight rebound of tumor growth was observed after discontinuation of dose as observed in LDK378, compound **17b** exhibited better tumor growth inhibition than LDK378, while compound **15k** was less potent. No changes in body weight or side effects were observed during the study (data not shown).

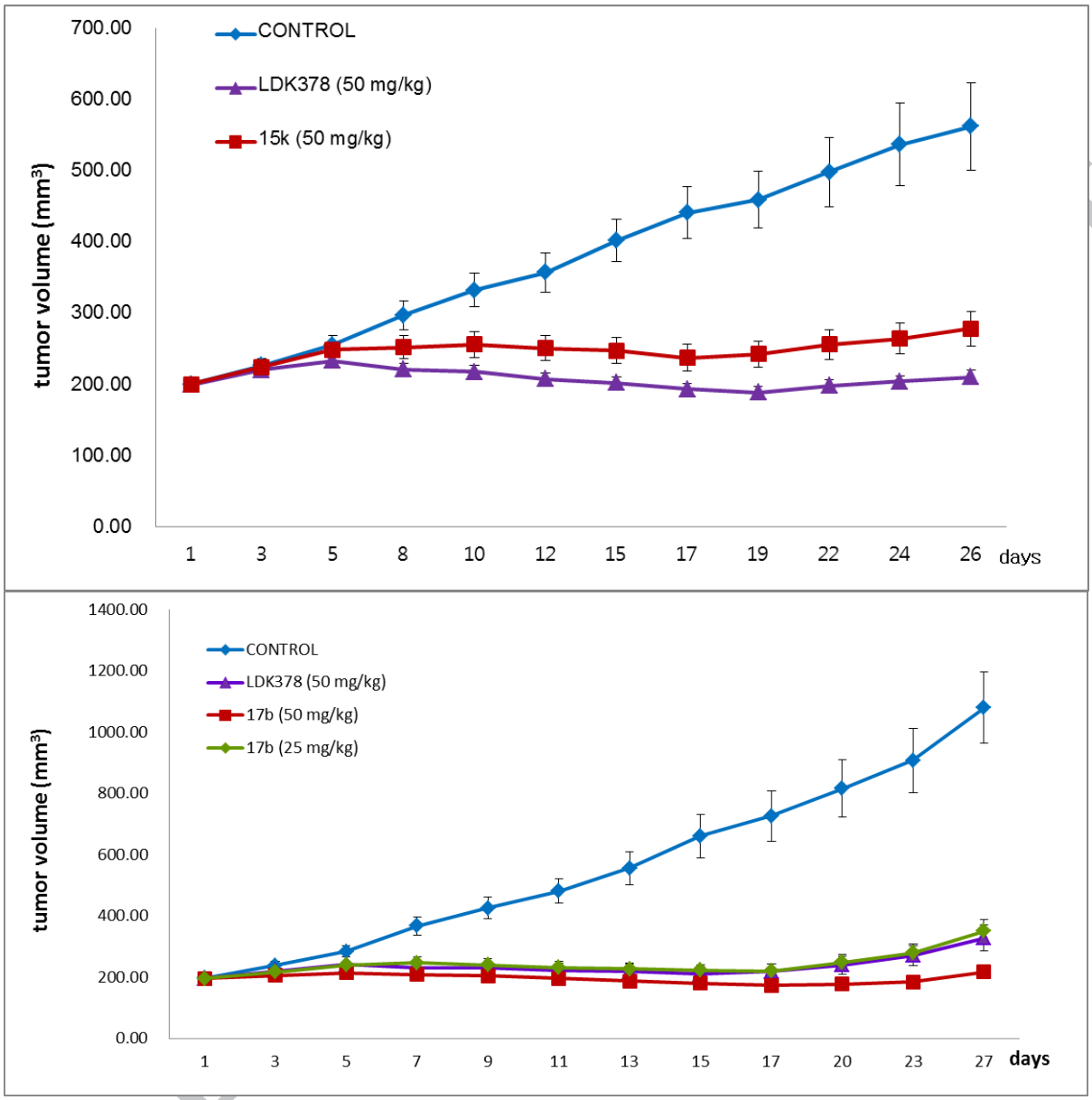
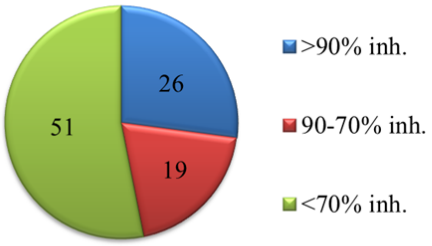


Figure 5. Antitumor activity of compounds **15k** and **17b** in H3122 xenograft model. Compounds were administered to SCID mice orally at doses of 50 mg/kg q.d. for 14 days after the tumor volume reached 200 mm^3. Each group consisted of 8 mice. The results are shown as the mean ± standard error.

A



B

Kinases	Inhibition at 1 μM (%)	Kinases	Inhibition at 1 μM (%)
FAK(h)	100	Aurora-B(h)	97
IR(h)	100	Axl(h)	97
ACK1(h)	100	ARK5(h)	96
FGFR1(h)	100	LOK(h)	96
Flt3(h)	100	Ros(h)	96
Mer(h)	100	TAO1(h)	96
Rsk1(h)	100	TSSK1(h)	96
ALK(h)	100	TrkA(h)	95
IGF-1R(h)	100	Yes(h)	95
Aurora-A(h)	99	PRK2(h)	93
CHK2(h)	99	EphB1(h)	92
EGFR (h)	99	ROCK-II(h)	92
Pyk2(h)	98	EphA1(h)	91

Figure 6. Kinase selectivity of compound **17b** A, Pie chart for kinase inhibition by compound **17b**; B, list of kinases with >90% inhibition.

Kinase selectivity of compound **17b** was evaluated with a set of 96 kinases and is summarized in Figure 6 and Supplementary data. Interestingly, 26 out of 96 kinases were strongly inhibited by compound **17b** at 1 μM (>90% inhibition) and 19 kinases were moderately inhibited (90%-70%), while 45 kinases were weakly inhibited (<70%). Although IC_{50} values of compound **17b** against those kinases showing >70% inhibition were not determined, it seems to be a multi-targeting inhibitor. It was reported that ceritinib (LDK378) inhibited both ALK and IGF-1R, thereby showing better potency in *in vivo* study than crizotinib [24]. Many ALK inhibitors including crizotinib, ceritinib, and PF-06463922 are also currently under development as ROS1 inhibitor [25-30]. In this context, compound **17b** targeting ALK and other kinases may provide clinical benefits for the treatment of NSCLC in the future.

In conclusion, the design, synthesis, and anti-ALK activity of novel 2,4-diaminopyrimidines bearing THN moiety were investigated. Compound **17b** showed the most potent anti-ALK activity in enzymatic and cell-based assays. In addition, compound **17b** showed reasonable pharmacological

properties such as PK, hERG, and CYP inhibition. An *in vivo* efficacy study with compound **17b** demonstrated highly potent inhibitory activity against H3122 tumor xenograft model in mice. Kinase selectivity assay showed that compound **17b** is a multi-targeting inhibitor; hence, it could possess synergistic effect in anti-tumor activity and possibility for multi-targeted NSCLC drug development. The compound **17b** is currently undergoing further optimization to develop novel ALK inhibitors.

Acknowledgements

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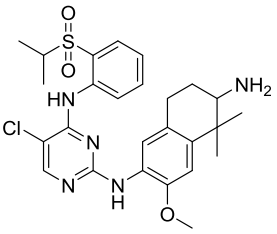
Graphical Abstract



17b

ALK wt	IC_{50} = 0.7 nM
L1196M	IC_{50} = 1.5 nM
H3122	CC_{50} = 16 nM
Ba/F3 L1196M	CC_{50} = 62 nM