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Synthesis and structure–activity relationship study of pyrazolo[3,4-*d*] pyrimidines as tyrosine kinase RET inhibitors

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ABSTRACT

Three series of pyrazolo[3,4-*d*]pyrimidine derivatives were synthesized and evaluated as RET kinase inhibitors. Compounds **23a** and **23c** were identified to show significant activity both in the biochemical and the BaF3/CCDC6-RET cell assays. Compound **23c** was found to significantly inhibit RET phosphorylation and down-stream signaling in BaF3/CCDC6-RET cells, confirming its potent cellular RET-targeting profile. Different from other RET inhibitors with equal potency against KDR that associated with severe toxicity, **23c** did not show significant KDR-inhibition even at the concentration of 1 μM. These results demonstrated that **23c** is a potent and selective RET inhibitor.

The transmembrane receptor tyrosine kinase RET (REarranged during Transfection) is mainly expressed in both central and peripheral nervous systems.[1,2] Collective studies have shown that activated RET oncogenes *via* mutations, amplifications or fusions are involved in the pathogenesis of many human cancers.[3] For example, gain of function mutation in RET is relevant to multiple endocrine neoplasia (MEN 2A and 2B), familial medullary thyroid carcinoma (FMTC), and papillary thyroid carcinoma (PTC).[4–7] Recently, several chimeric fusion partners of RET (KIF5B-RET, CCDC6-RET, TRIM33-RET NCOA4-RET) have been identified in 1–2% of non-small cell lung cancers (NSCLC) that led to abnormal activation of RET transcription and subsequent tumorigenesis.[8] Therefore, RET has been recognized as an emergent molecular target for cancer treatment.[9,10] Small molecule RET-targeting inhibitors cabozantinib (XL-184)[11] and vandetanib[12] have been granted FDA's approval recently for the treatment of MTC. In addition, ponatinib from Ariad is another RET inhibitor that recently undergoing phase II clinical trials to treat RET-rearranged NSCLC.[14,15] Notably, all these RET inhibitors are nonselective with high potency against several kinases other than RET, especially VEGFR-2 that correlates to many off-target effects such as rash, diarrhea, hypertension and others.[13,16–18]

Therefore, new inhibitors selectively targeting RET are highly needed both to validate the sole contribution of RET-targeting to the clinical antitumor efficacy and to minimize the off-target side effects. Recently, pyrazolo[3,4-*d*]pyrimidines have been reported to show selective inhibitory activity against RET, and the representative compounds **1** (**7a** in Ref. 20) and **2** (**6i** in Ref. 21) both showed single-digit nanomolar potency against RET and much improved selectivity against VEGFR-2. Unfortunately, these compounds failed to show similar high potency in the cellular assay.[20,21] In this regard, we conducted a structure-activity relationship study based on the pyrazolo[3,4-*d*]pyrimidin skeleton, by ring-closing to form a tricyclic center, insertion of an amido moiety to fit the hydrophobic pocket and attachment of a functional group to form additional interactions (Fig. 1). Herein, we report our design and pharmacological investigation on these new derivatives (**3**–**5**, Fig. 1).

The synthesis of compounds **10**–**12** and **18** was outlined in Scheme 1. Commercially available iodide **6** was used as the starting material to synthesize intermediate **7** by following literature procedures.[20] *N*-Alkylation with 4-bromobut-1-ene in the presence of potassium carbonate yielded **8**. Bromination of **8** with NBS at

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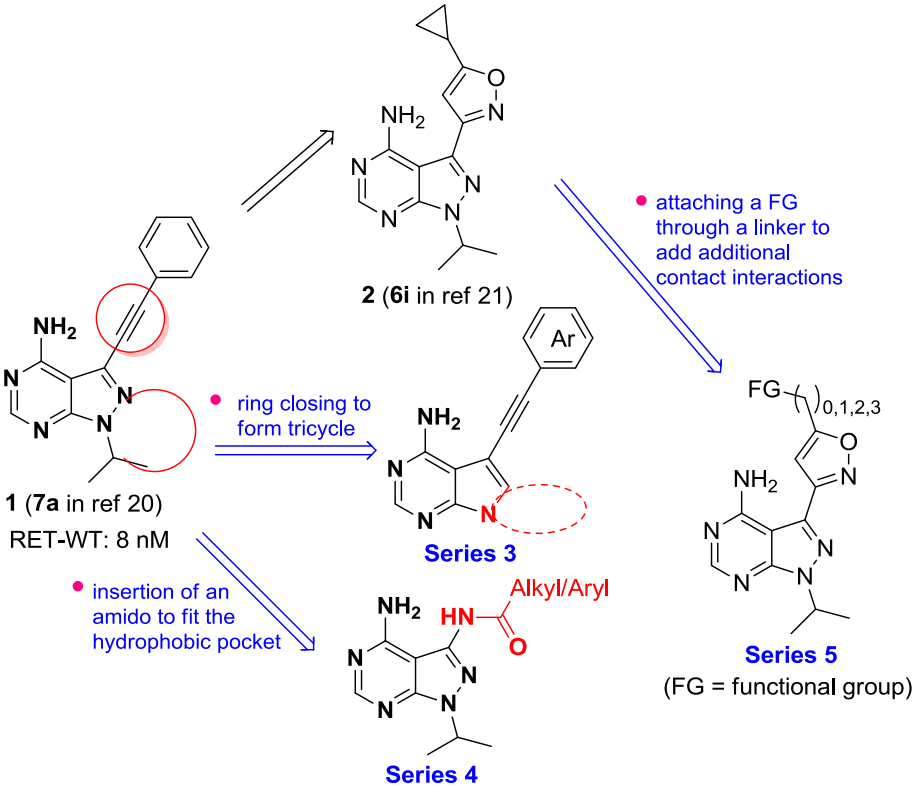


Fig. 1. Known RET inhibitors **1**–**2** and our designed new inhibitors **3**–**5**.

−20 °C produced **9**. Compounds **10** and **11** were obtained via Heck reaction from **9i** and **9ii**. Meanwhile, compound **12** was prepared from **9ii** by following literature procedures.[23] In addition, treating **13** with but-3-en-2-ol via Mitsunobu reaction provided precursor **14**. Subsequent Sonagashira coupling of **14** with 1-ethyl-4-ethynylbenzene in the presence of $Pd(PPh_3)_2Cl_2$/CuI provided **15**, which then underwent amination reaction with ammonium hydroxide at 120 °C to yield **16**.[22] Compound **18** was obtained by bromination of **16** to give **17**, followed by hydroboronation with 9-BBN and subsequent Suzuki coupling in the presence of Pd(dppf)Cl_2.[23]

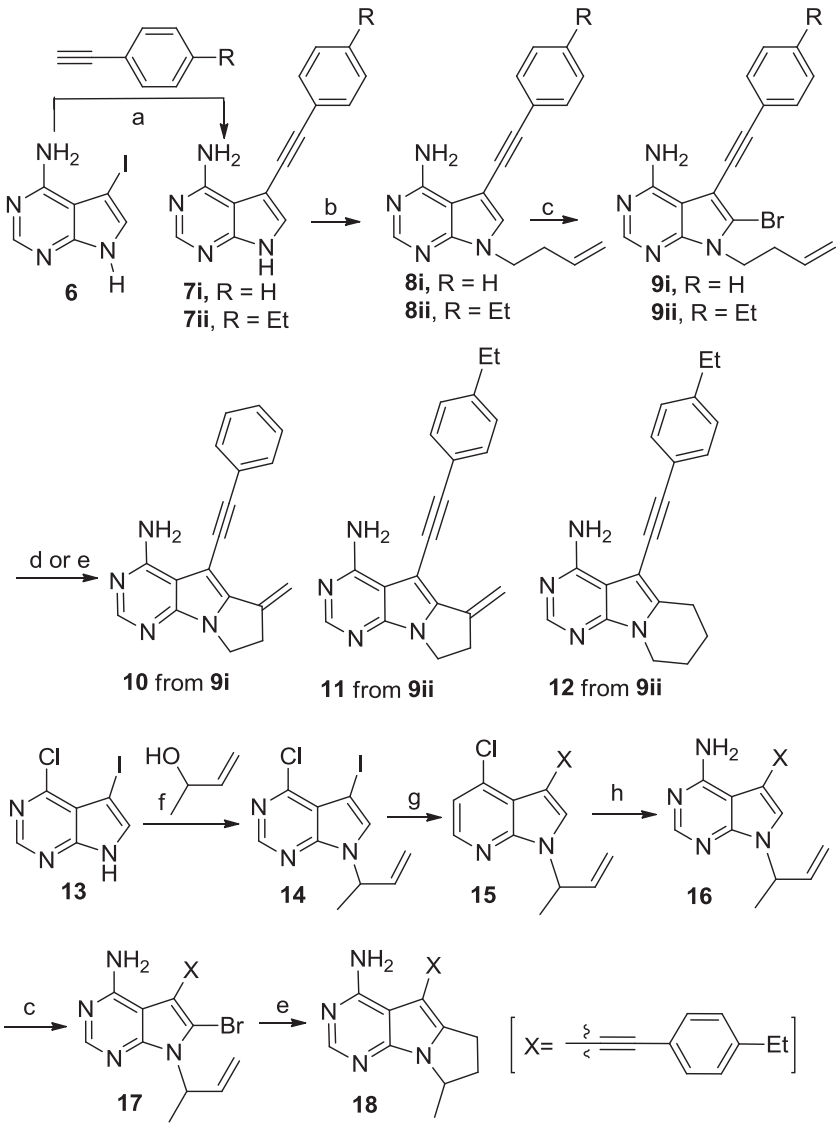
Preparation of compounds **21a**–**o** was described in Scheme 2. Reaction of 4-amino-6-chloropyrimidine-5-carbonitrile with isopropyl-hydrazine hydrochloride produced **20**.[24] Acylation of **20** with corresponding acyl chlorides provided **21a**–**o**. As shown in Scheme 3, following the literature procedure,[21] 5-substituted isoxazolines **23a**–**i** were obtained from appropriate terminal alkynes with the key intermediate **22**.

All the prepared new compounds were screened against RET kinase. As summarized in Table 1, the tricyclic compounds exhibited moderate to good potency against RET. Compounds **10** and **11** bearing a 6-methylene-7,8-dihydro-6H-pyrimido[5,4-*b*]pyrrolizine tricyclic center showed reduced potency compared to reference **1** (>100 nM *vs* 56 nM). The 6,7,8,9-tetrahydropyrimido[5,4-*b*]indolizine **12** also displayed slightly reduced potency with an IC_{50} value of 102 nM. However, compound **18** bearing the 7,8-dihydro-6*H*-pyrimido[5,4-*b*]pyrrolizine center showed an IC_{50} value of 62.5 nM, which is comparable to that of reference **1**.

Compounds **21a**–**o** represent a series of 1*H*-pyrazolo[3,4-*d*]pyrimidines bearing a C3-amido moiety. As shown in Table 2, cyclopropanecarboxamides **21a**–**c** displayed negligible activities against RET. Benzamide **21d** also lost the potency, whereas heterocyclic amides **21e**–**i** exhibited moderate potency with inhibitory rates around 50% at the concentrations of 10 nM. The five-membered heterocycle-substituted carboxamides were slightly more potent than the six-membered congeners (**21f**,**g**,**i**,**j** *vs* **21a**–**e**). 5-Cyclopropylisoxazole **21j** and furan-isoxazole **21l** retained good RET inhibition. The reduced activity of benzo[*d*]isoxazole **21k** and 5-phenylisoxazoles **21m**–**o** revealed that the hydrophobic pocket of RET kinase was slightly narrow and only limited substituents were tolerant.

Compounds **23a**–**i** are a series of analogues of **2** but bearing a more flexible side chain on the isoxazoline motif. As showed in Table 3, hydroxymethyl substituted compound **23a** turned out as the most potent with an IC_{50} value less than 10 nM against RET kinase. Capping the free hydroxyl (**23b**) or extending the carbon chain (**23c**,**d**) of **23a** led to reduced inhibitory activity. Esteric substituent (**23e**), or alkyl substituents bearing a terminal morpholinyl (**23f**–**h**) or pyrrolyl (**23i**) moieties nearly lost the inhibitory potency at the tested concentrations.

In view of the high inhibitory potency against RET at the concentration of 100 nM, compounds **23a** and **23c** were further evaluated. As shown in Table 4, high was observed for **23c** with an IC_{50} value of 61 nM, which is >6-fold more potent than **23a**. In the antiproliferative effect assay against the BaF3 cell harboring the fusion gene CCDC6-RET, the reference **1**, though quite potent in the biochemical assay, did not show significant antiproliferative effect in our cellular assay, with IC_{50} values greater than 1000 nM. Our new compounds **23a** and **23c** showed moderate cellular potency against BaF3/CCDC6-RET cells with an IC_{50} value of 454 and 433 nM respectively.



Scheme 1. Reagent and conditions: (a) $Pd(PPh_3)_4$ amberlitelR-67, CuI, THF, 120 °C, 30 min, 65%; (b) 4-bromobut-1-ene, K_2CO_3, DMF, 30 °C, 6 h, 83%; (c) NBS, −20 °C 1 h, 38–47%; (d) NaOH, $Pd(dppf)Cl_2$ 70–80%, 12 h, ~14%; (e) 1) 9-BBN, THE, 0 °C – rt, 6 h; 2) NaOH, $Pd(dppf)Cl_2$, 70–80 °C, 12 h, ~11% over two steps; (f) PPh_3, DIAD, THF 0 °C – rt, 2 h, 94%; (g) $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, N_2, THF, 70 °C, 10 h; (h) ammonia hydrate, 1,4-dioxane, 120 °C,18 h, 56–64% over two steps.

Western blotting analysis of **23c** was evaluated in BaF3/CCDC6-RET cells. We found that **23c** inhibited the phosphorylation of RET and the phosphorylation of STAT3 and AKT, main downstream effectors of RET signaling, in a dose-dependent manner in the tested cell line (Fig. 2). These results suggested that **23c** exhibited an effective inhibition of RET signaling. In contrast to its high potency against RET kinase, **23c** did not show significant inhibitory effect against VEGFR-2 kinase even at the concentration of 1 μM. These results demonstrated that **23c** is a potent and selective RET inhibitor.

The docking study of **23c** was described in Fig. 3. In addition to the essential interactions formed between RET kinase and the isoxazol-3-yl-pyrazolo[5,4-*d*]pyrimidine scaffold, an additional hydrogen bond was formed between the hydroxyl and Glu775 (1.8 Å), which was not observed in the biding mode of **1**. Such additional hydrogen bonding may partially contribute to the higher potency of **23c** than **1**.

In summary, we have designed and synthesized three series of pyrazolo[3,4-*d*]pyrimidine derivatives and tested their potency against RET. Compounds **23a** and **23c** were identified to show significant inhibition against both RET kinase and the proliferation of BaF3/CCDC6-RET cells. Compound **23c** significantly inhibited RET phosphorylation and down-stream signaling in BaF3/CCDC6-RET cells confirming its RET-selective targeting profile. More importantly, different from other RET inhibitors with equal potency against KDR that associated with severe toxicity, **23c** did not show significant KDR-inhibition even at the concentration of 1 μM. These results demonstrated that **23c** is a potent and selective RET inhibitor.

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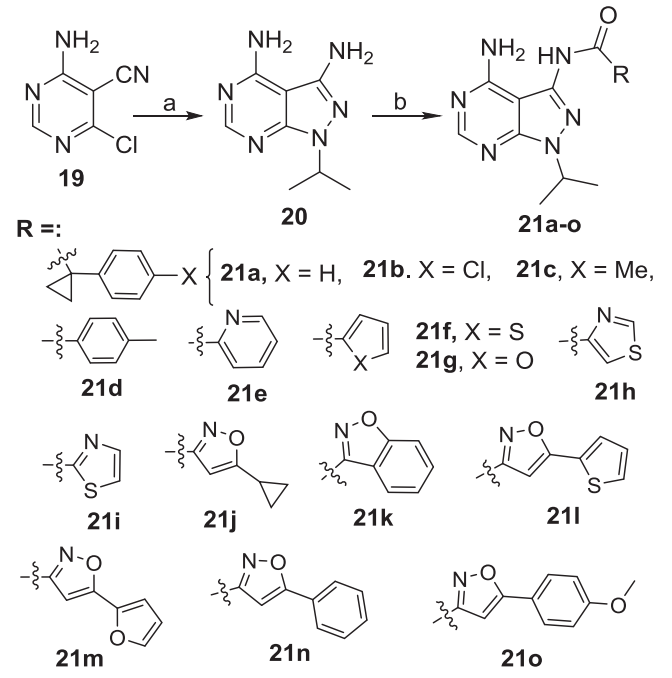
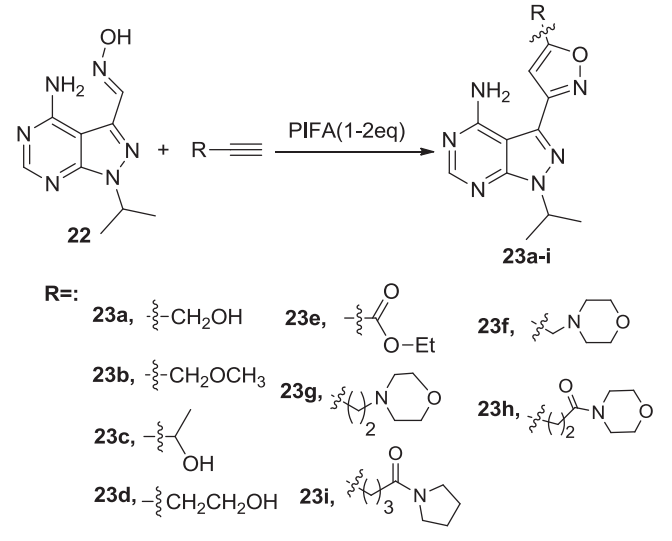


Scheme 2. Reagent and conditions: (a) isopropylhydrazine hydrochloride, MeOH, TEA, 60 °C, 4 h, 75%; (b) 1) RCOOH, oxalylchloride, *cat.* DMF, DCM, N_2, 0 °C, 3 h; 2) DIPEA, THF, N_2, −10 °C, 3 h, 25–65% in two steps.



Scheme 3. Reagent and conditions: MeOH/H2O (5/1), N_2, 0 °C, -rt, 15–24 h, 15–30%; PIFA = phenyliodine(III)bis(trifluoroacetate).

Table 1
Biochemical assays of tricyclic compounds against RET kinase.[a]

Compd	Inhibition, %[b]	IC_{50}, nM
10	49.0%@100 nM	104 ± 39
11	23.0%@100 nM	ND
12	63.6%@100 nM	102 ± 21
18	77.6%@100 nM	62.5 ± 0.7
1	75.1%@100 nM	56.5 ± 2.1

[a] The IC_{50} values are shown as the mean ± SD (nM) or estimated values from two separate experiments.
[b] Inhibition % means inhibition rate against enzyme concentration (nM).

Table 2
Biochemical assays against RET kinase.[a]

Compd	Inhibition, %	
	100 nM	10 nM
21a	4%	−4%
21b	−15%	3%
21c	−16%	9%
21d	4%	11%
21e	53%	44%
21f	67%	57%
21g	63%	56%
21h	61%	48%
21i	51%	51%
21j	60%	57%
21k	25%	1%
21l	57%	54%
21m	44%	13%
21n	30%	16%
21o	13%	3%

[a] Inhibition % means inhibition rate against enzyme with the concentration (nM) of corresponding compound.

Table 3
Biochemical assays against RET kinase.[a]

Compd	Inhibition, %	
	100 nM	10 nM
23a	43%[b]	18%[c]
23b	51%	22%
23c	73%	45%
23d	42%	34%
23e	37%	5.4%
23f	-21%	0.8%
23g	42%	24%
23h	23%	7%
23i	29%	11%

[b] and [c]Inhibition rates at the concentration of 250 nM and 15 nM respectively.
[a] Inh% means inhibition rate against enzyme with the concentration (nM) of corresponding compound.

Table 4
Antiproliferative effects against BaF3/CCDC6-RET cells.[a]

Compd	RET (IC_{50}, nM)	BaF3/CCDC6-RET cell (IC_{50}, nM)
23a	410 ± 195	454 ± 7.4
23c	61.2 ± 35.5	433 ± 15.4
1	56.5 ± 2.1	>1000

[a] Cell proliferation was detected by microculture tetrozolium (MTT) as described in Experimental Section. IC_{50} values are given as the mean ± SD (nM) from two separate experiments.

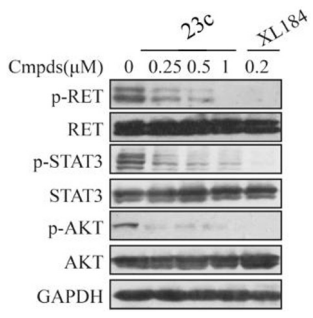


Fig. 2. 23c suppressed RET phosphorylation and down-stream signaling in BaF3/CCDC6-RET Cells.[a]

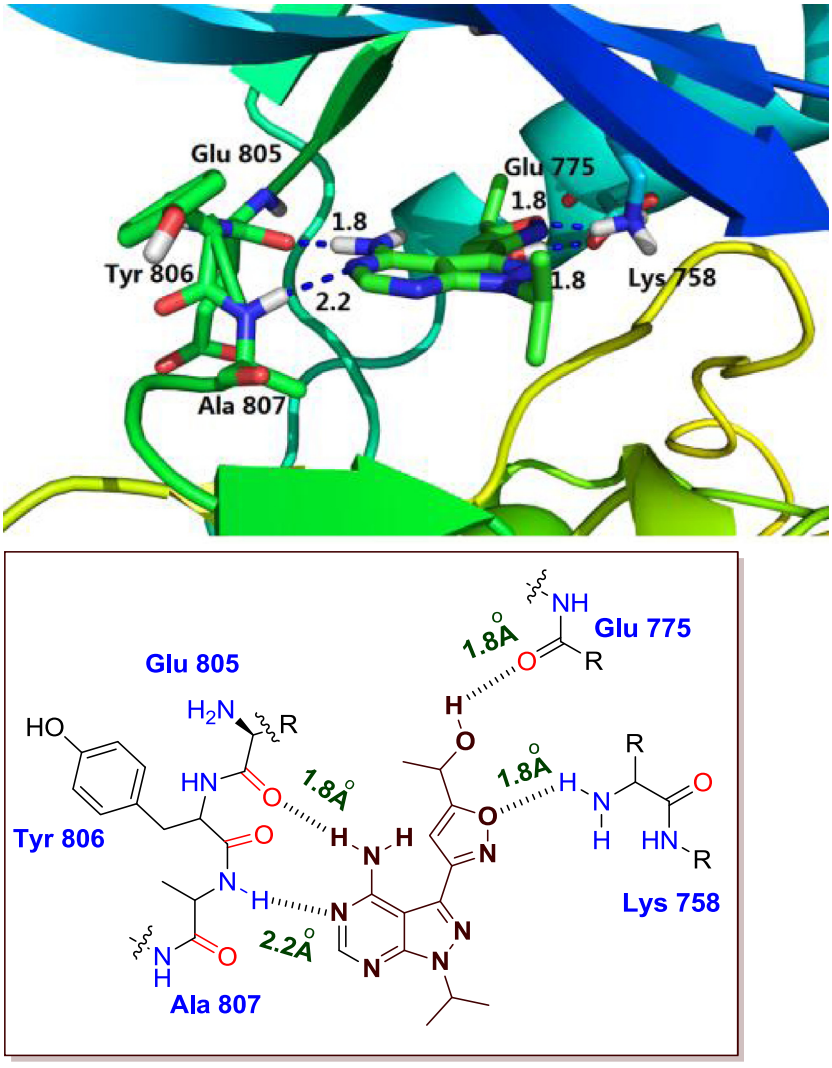


Fig. 3. Docking of **23c** with RET catalytic domain (PDB code 2IVV).

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.03.088.

References

1. Attié-Bitach T, Abitbol M, Gérard M, et al. *Am J Med Genet*. 1998;80:481.
2. Durbec PL, LarssonBlomberg LB, Schuchardt A, Costantini F, Pachnis V. *Development*. 1996;122:349.
3. de Groot JW, Links TP, Plukker JT, Lips CJ, Hofstra RM. *Endocr Rev*. 2006;27:535.
4. Gallel P, Pallares J, Dolcet X, et al. *Hum Pathol*. 2008;39:994.
5. Blume-Jensen P, Hunter T. *Nature*. 2001;411:355.
6. Lodish MB, Stratakis CA. *Expert Rev Anticancer Ther*. 2008;8:625.
7. Santoro M, Grieco M, Melillo RM, Fusco A, Vecchio G. *Eur J Endocrinol*. 1995;133:513.
8. Drilon A, Wang L, Hasanovic A, et al. *Cancer Discovery*. 2013;3:630.
9. Horiike A, Takeuchi K, Uenami T, et al. *Lung Cancer*. 2016;93:43.
10. Barabé F, Gil L, Celton M, et al. *Leukemia*. 2016;1.
11. Hart CD, De Boer RH. *Oncol Targets Ther*. 2013;6:1.
12. Fallahi P, Ferrari SM, Baldini E, et al. *Expert Rev Anticancer Ther*. 2016;16:1109.
13. Perez CA, Arango BA, Velez M, Raez LE, Santos ES. *Biologics*. 2012;6:257.
14. Mologni L, Redaelli S, Morandi A, Plaza-Menacho I, Gambacorti-Passerini C. *Mol Cell Endocrinol*. 2013;377:1.
15. Cain CRET, set, go. *SciBX*. 2013;6:318.
16. Zhang X, Shao Y, Wang K. *Expert Rev Clin Pharmacol*. 2016;9:1109.
17. Zhang ZF, Wang T, Liu LH, Guo HQ. *PLoS One*. 2014;9:e90135.
18. Qi WX, Shen Z, Lin F, et al. *Br J Clin Pharmacol*. 2012;75:919.
20. Dinér P, Alao JP, Söderlund J, Sunnerhagen P, Grøtli M. *J Med Chem*. 2012;55:4872.
21. Yoon H, Kwak Y, Choi S, Cho H, Kim ND, Sim T. *J Med Chem*. 2016;59:358.
22. Perez L, Sheng Tao. WO2006/102079 A1.
23. Miyaura N, Ishiyama T, Sasaki H, Ishikawa M, Sato M, Suzuki A. *J Am Chem Soc*. 1989;111:314.
24. Neidlein R, Wang Z. *Heterocycles*. 1997;45:1509.