Discovery of 4-chloro-3-(5-(pyridin-3-yl)-1,2,4-oxadiazole-3-yl)benzamides as novel RET kinase inhibitors

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ABSTRACT

A series of novel 4-chloro-benzamides derivatives containing substituted five-membered heteroaryl ring were designed, synthesized and evaluated as RET kinase inhibitors for cancer therapy. Most of compounds exhibited moderate to high potency in ELISA-based kinase assay. In particular, compound **I-8** containing 1,2,4-oxadiazole strongly inhibited RET kinase activity both in molecular and cellular level. In turn, **I-8** inhibited cell proliferation driven by RET wildtype and gatekeeper mutation. The results implied that 4-chloro-3-(5-(pyridin-3-yl)-1,2,4-oxadiazole-3-yl)benzamides are promising lead compounds as novel RET kinase inhibitor for further investigation.

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The RET receptor tyrosine kinase is crucial for normal development but also contributes to pathologies that reflect both the loss and the gain of RET function. Activation of RET *via* oncogenic mutations is involved in the development of several human cancers.^{1,2} Activation of the RET point mutation is associated with medullary thyroid carcinoma, and RET rearrangement has been detected in papillary thyroid carcinoma.³ More importantly, RET fusion genes have also recently been identified as driver oncogenes in non-small cell lung cancer (NSCLC), with the associated fusion genes present in approximately 1% to 2% of cases.⁴ The response of cabozantinib as multikinase inhibitor targeting RET in RET fusion-positive NSCLC provides early clinical validation of RET fusions as drivers in lung cancers and suggests that RET inhibition may represent a new treatment paradigm in this molecular cohort.^{5,6} Thus, the genetic alterations of RET are considered to be promising targets for anticancer therapy.

Due to the structural similarity between RET and other RTKs in functional domains, especially the ATP-binding site, it has been proved that many small-molecule RTK inhibitors (TKIs) possess RET inhibitory activity.⁷ Among them, VEGFR2/EGFR

inhibitor Vandetanib,⁸ VEGFR2/MET inhibitor Cabozantinib,⁹ VEGFR2/3 inhibitor Lenvatinib¹⁰ and multi-targeted inhibitor Sorafenib¹¹ have been approved for the treatment of thyroid cancer. Ponatinib, a drug that is approved as a therapy for chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia, is a potent inhibitor of both RET^{WT} and Vandetanib/Cabozantinib-resistant RET^{V804M} kinase.^{12,13} The phase II clinical trials of ponatinib in treating non-small cell lung carcinoma (NSCLC) harboring RET rearrangements is now in progress, in treating advanced or metastatic MTC has been terminated this year (**Scheme 1**).

Our group has previously synthesized an array of Ponatinib analogues and identified that 4-chloro-3-(pyridin-3-ylethynyl) benzamide derivative **Hu1-117** exhibited potential RET enzyme inhibitory activity with IC₅₀ value of 2.5 ± 0.2 nM. Since five-membered heteroaromatic rings is an ubiquitous and privileged scaffold in drug design as a linker for its rigid structure, which enable the substituent groups on it stretching to the appropriate orientation, we envision that replacing the alkynyl linker of compd **Hu1-117** with different five-membered rings might keep the RET inhibitory activity and generate the novel scaffold for RET inhibitor. Herein, we reported the design, synthesis and biological evaluation of these compounds.

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Scheme 1. Small-molecule RET inhibitors

Compounds containing 1,2,4-oxadiazole, 1,3,4-oxadiazole and oxazole rings were synthesized by the following routes outlined in **Scheme 2**. Commercially available 4-chloro-3-iodobenzoic acid (**1**) as the starting material was esterified with MeOH and then cyanided by Pd-catalyst with $\text{Zn}(\text{CN})_2$ ¹⁴ to generate compound **3**. After addition reaction of **3** with hydroxylamine,

compound **4** was condensed with nicotinic acid to afford novel 3-phenyl-5-pyridin-1,2,4-oxadiazole intermediate **5**, which was hydrolyzed and then condensed with 4-((4-methylpiperazin-1-yl)methyl)-3-(trifluoromethyl)aniline to provide desired products **I-1** (**Scheme 2**, route A). 5-Phenyl-3-pyridin-1,2,4-oxadiazole derivative **II-1** and 1,3,4-oxadiazole-containing compound **III-1** were prepared from similar route by the different cyclization in **Scheme 2** (route B and C).

Oxazole-containing intermediate **16** was obtained from 2-bromo-1-(pyridin-3-yl)ethan-1-one (**14**) by the substitution with 2-chloro-5-(methoxycarbonyl)benzoic acid and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ -catalyzed cyclization. After the hydrolysis of **16** and then condensation with 4-((4-methylpiperazin-1-yl)methyl)-3-(trifluoromethyl)aniline, oxazole-containing compound **IV-1** was generated (**Scheme 2**, route D). Reversed oxazole-containing compound **V-1** was prepared by the key step of $\text{Pd}(\text{OAc})_2$ -catalyzed direct C-H activation of methyl 4-chloro-3-(oxazol-5-yl)benzoate (**19**) with 3-iodopyridine¹⁵ (**Scheme 2**, route E).

Scheme 2. Reagents and conditions: (a) SOCl_2 , CH_3OH , reflux; (b) $\text{Zn}(\text{CN})_2$, $\text{Pd}(\text{PPh}_3)_4$, DMF, N_2 , 80 °C; (c) 50% NH_2OH solution, $\text{C}_2\text{H}_5\text{OH}$, reflux; (d) nicotinic acid, EDCI, HOBt, DMF, 120 °C; (e) LiOH , $\text{THF}/\text{H}_2\text{O}$, r.t.; (f) HATU, DIPEA, DMF, r.t.; (g) 2-chloro-5-(methoxycarbonyl)benzoic acid, EDCI, HOBt, DMF; (h) 2-chloro-5-(methoxycarbonyl)benzoic acid, POCl_3 , reflux; (i) Cs_2CO_3 , EtOH, r.t.; (j) CH_3CONH_2 , $\text{BF}_3 \cdot \text{Et}_2\text{O}$, xylene, reflux; (k) Tosmic, K_2CO_3 , MeOH, reflux; (l) 3-iodopyridine, K_2CO_3 , $\text{Pd}(\text{OAc})_2$, CuI, DMF, 150 °C, sealed tube.

The effects of the target products **I-1**~**V-1** on the activity of RET were determined using enzyme-linked immunosorbent assay (ELISA). As shown in **Table 1**, all the synthetic compounds exhibited moderate to potent RET inhibitory

activities, which have slightly decreased comparing with **Hu1-117**. Among them, compound **I-1** with IC_{50} value of 7.3 nM indicated that 1,2,4-oxadiazole ring as the core structure is worthy for the further investigation.

Table 1 RET enzymatic activities of the designed different five-membered heteroaryl derivatives

Compd.	A ring	IC ₅₀ (nM) ^a
I-1		7.3 ± 1.7
II-1		137.8 ± 12.8
III-1		12.4 ± 1.6
IV-1		21.4 ± 2.7
V-1		19.4 ± 3.3
Hu1-117		2.5 ± 0.2
Ponatinib		0.9 ± 0.2

^a IC₅₀ values are given as the mean ± SD (nM) from two separate experiments.

In order to obtain more potent RET inhibitors, further modification on the phenyl and pyridine ring of **I-1** was proceeded. All new products containing 1,2,4-oxadiazole were synthesized by the following routes in **Scheme 3**.

The RET enzyme inhibition of the compounds **I-2~I-8** were outlined in **Table 2**. Firstly, replacing the chloro- by H or CH₃ (**I-2** and **I-3**) attenuated RET inhibitory activity with IC₅₀ value of 17.9 and 107.6 nM accordingly, indicated that chloro- is crucial to RET inhibitory activity. The weaker activity of compound **I-4** compared to **I-1** revealed that the nitrogen on pyridine at the *meta*-position is more favorable than at the *para*-position. Compounds **I-5** to **I-8** were prepared to investigate the influence of substitutions on pyridine ring for RET inhibitory activity. Obviously, the 2-methoxyl group on the pyrimidine will decrease the activity. The compounds with methoxyethoxy (**I-6**), morpholine (**I-7**) or 1-methyl-pyrazole (**I-8**) substitutions at the 3-position of the pyrimidine exhibited good to excellent RET inhibition. In particular, compound **I-8** is the most efficient RET inhibitor with a comparable IC₅₀ values of 1.8 nM.

Table 2. Effects of R and B ring on RET inhibitory activity

Compd.	B ring	R	RET IC ₅₀ (nM) ^a
I-1		Cl	7.3 ± 1.7
I-2		H	17.9 ± 1.2
I-3		CH ₃	107.6 ± 7.3
I-4		Cl	26.6 ± 13.7
I-5		Cl	341.9 ± 34.3
I-6		Cl	11.4 ± 2.6
I-7		Cl	2.1 ± 0.1
I-8		Cl	1.8 ± 0.01

Ponatinib 0.9 ± 0.2

^a IC₅₀ values are given as the mean ± SD (nM) from two separate experiments.

Western blotting analysis for the cellular kinase-targeting activity of **I-8** was carried out using BaF3/CCDC6-RET cells (**Figure 1**). The results showed that **I-8** inhibited the phosphorylation of RET and the phosphorylation of STAT3 and ERK, key downstream molecules of RET in a dose-dependent manner, with a complete abolishment at 1 μM. These findings demonstrate that **I-8** could significantly block the RET signaling pathway.

Figure 1. **I-8** suppressed RET phosphorylation and downstream signaling in BaF3/CCDC6-RET Cells. Cells were treated with indicated concentrations of **I-8** for 1 hour and analyzed by immunoblotting.

To investigate the sensitivity of RET fusion-positive cells to **I-8**, we evaluated **I-8**'s capacity to inhibit cell growth in BaF3/CCDC6-RET cells, which growth was driven by CCDC6-RET fusion. **I-8** inhibited BaF3/CCDC6-RET cell proliferation, with IC₅₀ value of 406.9 ± 12.2 nM (**Table 3**). More importantly, compound **I-8** showed similar potency against the proliferation of gatekeeper mutant V804M-driven cell proliferation, with an IC₅₀ value of 441.8 ± 108.5 nM, further indicating that compound **I-8** is active in cancers driven by RET wildtype and gatekeeper mutation.

Table 3. Effect of **I-8** against BaF3 cells expressing CCDC6-RET and gatekeeper mutation (V804M).

IC ₅₀ (nM) ^a	Cell Line	
	BaF3/CCDC6-RET	BaF3/CCDC6-RET-V804M
I-8	406.9 ± 12.2	441.8 ± 108.5
Ponatinib	180.4 ± 12.2	136.0 ± 8.9

^a IC₅₀ values are given as the mean ± SD (nM) from two separate experiments.

With the aim to further probe the binding mode of the bioactive compounds, molecular modeling was proposed by constructing RET^{WT/V804M} DFG-out homology model with Discovery studio 2.5 (see details in supporting information). The reliability of this model has been verified by the molecular docking of a novel RET inhibitor **Pz-1** reported by Brendan Frett¹⁶ (**Figure S1**). Computational modeling results showed that the oxygen and nitrogen atoms on 1,2,4-oxadiazole of **I-8** form hydrogen bond interaction with Asp892, meanwhile fluorine atom on trifluoromethyl group interact with hydrogen bond of Ala807 (**Figure 2A, B**). Besides, the phenyl ring on 3-position of 1,2,4-oxadiazole could generate cation-π interaction with Lys758, and the phenyl ring linked to trifluoromethyl group could form π-σ interaction with Phe893. Although **I-5** formed the similar hydrogen bonds interaction with RET^{V804} homology

model (Figure 2C, D), no cation- π interactions were found. In addition, the LibDock score of **I-5** was 32.18, which is lower than **I-8** (LibDock Score = 70.62). Molecular superposition (Figure 2E) exhibited that the bioactive compounds **I-6**, **I-7**, and **I-8** had similar molecular conformation to bond with RET active site.

In summary, we have designed and synthesized a new class of 4-chloro-3-(5-(pyridin-3-yl)-1,2,4-oxadiazol-3-yl)benzamides as novel RET kinase inhibitors. The most potent compound **I-8**

showed the comparable RET kinase activity with Ponatinib. Moreover, **I-8** significantly inhibited RET signaling *in vitro*, in turn inhibited cell proliferation driven by RET wildtype and gatekeeper mutant. These results indicated that the substitution of alkynyl by 1,2,4-oxadiazole is feasible in drug discovery and optimization, and compound **I-8** could act as a promising lead compound in the further development of new RET inhibitors containing 1,2,4-oxadiazole core.

Scheme 3. Reagents and conditions: (a) Zn(CN)₂, Pd(PPh₃)₄, DMF, N₂, 80 °C; (b) 50% NH₂OH solution, C₂H₅OH, reflux; (c) B ring-COOH, EDCI, HOBt, DMF, 120 °C; (d) LiOH, THF/H₂O, r.t.; (e) HATU, DIPEA, DMF, r.t..

Figure 2. Molecular modeling of compound **I-8** (A, B) and **I-5** (C, D) in RET^{WT} DFG-out homology model; (E) Molecular superposition and the docking mode of **I-6**, **I-7**, **I-8** in RET^{WT} DFG-out homology model. Hydrogen bond interaction was highlighted using red dash line, π - π interaction and cation- π interaction was highlighted using orange dash line.

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Graphical Abstract

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