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PII: S0960-894X(17)30986-1
DOI: https://doi.org/10.1016/j.bmcl.2017.10.012
Reference: BMCL 25341

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 18 August 2017
Revised Date: 6 October 2017
Accepted Date: 7 October 2017

Please cite this article as: Koda, Y., Kikuzato, K., Mikuni, J., Tanaka, A., Yuki, H., Honma, T., Tomabechi, Y., Kukimoto-Niino, M., Shirouzu, M., Shirai, F., Koyama, H., Identification of pyrrolo[2,3-*d*]pyrimidines as potent HCK and FLT3-ITD dual inhibitors, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: https://doi.org/10.1016/j.bmcl.2017.10.012

Identification of pyrrolo[2,3-*d*]pyrimidines as potent HCK and FLT3-ITD dual inhibitors

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Key words
Acute myeloid leukemia (AML)**,** FMS-like tyrosine kinase 3 with internal tandem duplication mutations (FLT3-ITD), Hematopoietic cell kinase (HCK), Pyrrolo[2,3-*d*]pyrimidine.

Abstract

A series of novel pyrrolo[2,3-*d*]pyrimidines were synthesized by introducing 15 different amino acids to 7-cyclohexyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-amine. Compounds with potent activities against HCK and FLT3-ITD were evaluated in viability studies with acute myeloid leukemia cell line MV4-11. Our structure activity relationship analyses lead to the identification of compound **31**, which exhibited potent HCK and FLT3-ITD inhibition and activity against the MV4-11 cell line.

Acute myeloid leukemia (AML) is one of the most common and aggressive forms of acute leukemia in adults. While approximately 40-50% of AML patients successfully enter complete remission with initial combination chemotherapy, the majority of patients experience relapses with poor prognosis[1,2]. Recent studies have revealed that overexpression and/or mutation of certain kinases were identified among AML patients and that inhibitors of several kinases have become drug discovery targets for the treatment of AML.[3] For example, FMS-like tyrosine kinase 3 with internal tandem duplication mutations (FLT3-ITD) is associated with karyotype AML in approximately 30% of AML patients.[4, 5] More recent studies indicated that hematopoietic cell kinase (HCK), a non-receptor protein-tyrosine kinase belonging to the Src family, is highly expressed in chemotherapy-resistant human AML stem cells.[6] Both HCK and FLT3-ITD are associated with

irregular signaling pathways leading to uncontrolled cancer cell proliferation and differentiation in AML. Based on the hypothesis that inhibition of both FLT3-ITD and HCK might offer a comprehensive treatment of relapse-prone AML, we set out to find potent HCK and FLT3-ITD dual inhibitors.

We recently reported the identification of a highly potent HCK inhibitor RK-20449.[7] Herein we would like to report our progress toward structurally-diverse HCK and FLT3-ITD dual inhibitors. Given the impressive potency of RK-20449 (HCK IC_{50}=0.43 nM), we decided to start our structure-activity relationship (SAR) studies by modifying the structure of RK-20449. Since the piperazine moiety of RK-20449 was reported to contribute to the increased binding affinity to HCK by interacting with Asp348 of HCK,[7] we sought to find an alternative structure to piperazine with the help of *in silico* docking and X-ray crystallographic studies (Fig. 1). We hoped that replacement of the piperazine moiety with an acyclic amino group would result in interaction with different amino acid residue(s) of the enzyme, due to its increased rotational freedom.

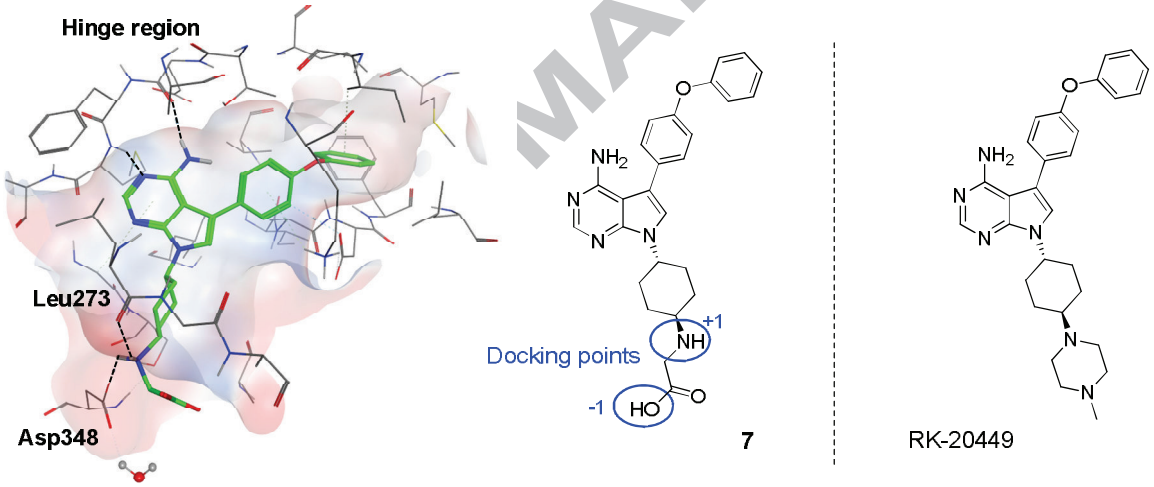
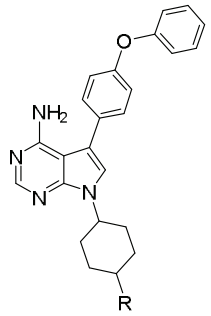


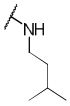
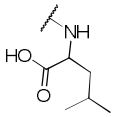
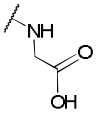
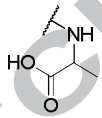
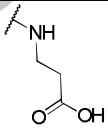
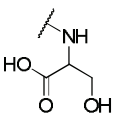
Figure 1. X-ray co-crystal structure of **7** with HCK (resolution: 2.85 Å unpublished). Based on this crystal structure, derivatives incorporated with various amino acids (Ala, Phe, Pro, and Tyr replacing Gly of **7**) were assessed for hydrogen bonding potential with Asp348 and other residues in HCK by *in silico* docking studies. Docking scores were summarized in Table 1.

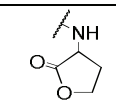
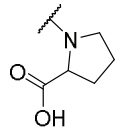
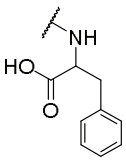
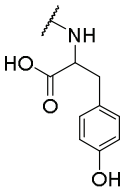
When an acyclic version of the methyl piperazine moiety of RK-20449 was synthesized (**1** and **2** in Table 1), major activity decreases were observed with HCK (IC_{50}=226 nM and 119 nM for *trans* and *cis* isomers, respectively) while the FLT3-ITD activities decreased only modestly (IC_{50}=56 nM and 44 nM, respectively). Since **1** and **2** became quite lipophilic as indicated by their cLogP values, we decided to introduce hydrophilic substituents to attenuate the high lipophilicity.[8] When a carboxylic acid group was introduced to the aminoalkyl moiety of **1** and **2**, remarkable

improvement in the HCK activity was observed (**3**-**6**). These findings prompted us to further explore the SAR by introducing various amino acids, first through *in silico* docking simulation studies (Fig. 1), and then through actual synthesis and activity measurements. Thus, four different amino acids (Ala, Phe, Pro, and Tyr) including their enantiomers were incorporated into the structure of 7-cyclohexyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine-4-amine. Then the conceived compounds were subjected to docking simulation studies using the X-ray co-crystal structure of HCK and **7**, where potentials for hydrogen bond formation with Asp348 and other residues of HCK were assessed. The results of the docking simulation studies are shown as a docking score (kcal/mol) in Table 1, where many of the compounds were expected to have favorable HCK binding affinities. The phenoxyphenyl moiety at the 5-position of pyrrolo[2,3-*d*]pyrimidine was left unchanged since our previous SAR studies had indicated this group was critically necessary for FLT3-ITD activity.

Table 1. HCK and FLT3-ITD IC_{50} values of pyrrolo[2,3-*d*] pyrimidine derivatives.



#	R		Docking score with HCK (kcal/mol) [a]	IC_{50} (nM) [b]		GI_{50} (nM) [b]	cLogP [c]
				HCK	FLT3-ITD	MV4-11	
	RK-20449 	- *trans*	-	0.4	24	40	4.9
1		- *trans*	-	226	56	-	6.0
2		- *cis*	-	119	44	-	
3		L-Leu *trans*	-	4.4	14	21	2.9
4		*cis*	-	105	23	-	
5		D-Leu *trans*	-	27	53	-	
6		*cis*	-	137	35	-	
7		Gly *trans*	-	3.7	28	98	1.2
8		*cis*	-	12	32	-	
9		L-Ala *trans*	-11.27	6.9	39	-	1.7
10		*cis*	-10.58	73	20	-	
11		D-Ala *trans*	-11.23	36	39	-	
12		*cis*	-10.62	36	23	-	
13		*β*Ala *trans*	-	15	33	-	1.2
14		L-Ser *trans*	-	1.8	18	>100	0.8
15		*cis*	-	40	13	-	
16		D-Ser *trans*	-	6.3	17	-	
17		*cis*	-	23	12	-	

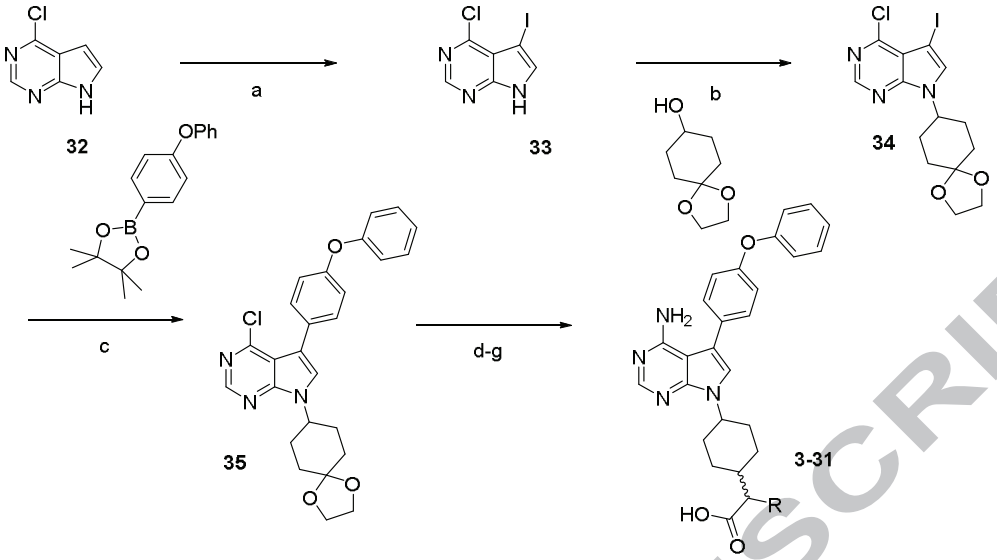
18		L-Hsl	*trans*	-	28	25	-	4.1
19			*cis*	-	5.4	27	29	
20		L-Pro	*trans*	-10.93	52	26	-	2.0
21			*cis*	-11.05	79	20	-	
22		D-Pro	*trans*	-11.34	200	126	-	
23			*cis*	-11.08	65	16	-	
24		L-Phe	*trans*	-11.30	7.5	24	-	3.2
25			*cis*	-11.33	74	3.1	-	
26		D-Phe	*trans*	-11.40	24	27	-	
27			*cis*	-11.30	18	2.6	26	
28		L-Tyr	*trans*	-11.36	7.4	28	-	3.0
29			*cis*	-11.70	39	5.0	-	
30		D-Tyr	*trans*	-11.61	15	11	-	
31			*cis*	-11.08	13	5.0	18	

[a] Docking scores were determined by hydrogen bonds between each derivative and HCK residues. Hydrogen bonds whose energy magnitude is >2 kcal/mol were detected and calculated by Molecular Operating Environment (MOE), (ver. 2016.0802, Chemical Computing Group Inc., Montreal, QC, Canada, 2016.)

[b] Data represents an average of at least two separate determinations

[c] Dassault Systemes BIOVIA, BIOVIA Pipeline Pilot, Version 9.0.2, San Diego: Dassault Systemes, 2013.

The actual synthesis was carried out as described in Scheme 1. First, a commercially available pyrrolopyrimidine **32** was iodinated to give **33**. Then the cyclohexanone and the phenoxyphenyl moieties were successively introduced to **33** by the Mitsunobu reaction and the Suzuki coupling to yield **35**. [9] Introduction of the amino group, deprotection of the ketal group, and reductive amination with corresponding amino acid esters followed by ester hydrolysis furnished the desired products **3** to **31**. Compounds **1** and **2** were synthesized from **35** in a similar fashion employing isoamylamine instead of an amino acid ester.[9] The *cis* and *trans* isomers of the 1,4-positions of the cyclohexyl group were formed during the reductive amination step and chromatographically separated. The *cis* isomers were obtained as major products in most cases.



Scheme 1. Reagents and conditions: (a) NIS, DMF, rt, 95%; (b) DIAD, PPh_3, THF, 0-4° C to rt, 53 %; (c) $Pd(PPh_3)_4$, *sat*-Na_2CO_3, DME, 80° C, >95%; (d) NH_4OH, dioxane, 120° C, 96%; (e) 6N-HCl, acetone, 40° C, 88%; (f) amino acid esters, $NaBH(OAc)_3$, DCM, rt, *cis* and *trans* ratio=3:1; (g) 2M NaOH, EtOH, 70° C.

Enzymatic activities[10] against HCK and FLT3-ITD of the newly synthesized compounds **1**-**31** are summarized in Table 1. It is interesting to note that the HCK activities are generally sensitive to the *cis*/*trans* conformation of the cyclohexane ring, with the *trans* isomers showing more potent activities in most cases, while the FLT3-ITD activities are less sensitive. It appears that the results of the docking studies with HCK are generally consistent with the *cis* and *trans* isomers' activities, while activities between compounds with different amino acids are not. Some of the differences in HCK activity between the *cis* and *trans* isomers can be explained by X-ray co-crystal structure analyses; the amino group of the *trans* isomers **3** and **5** interacts with the backbone carbonyl of Asp348, whereas the amino group of the corresponding *cis* isomers **4** and **6** does not interact with the enzyme (Fig. 2). The crystal structure of HCK and **31** revealed an interesting binding motif where the amino group and the tyrosine carbonyl group of **31** interact with the backbone carbonyl of Ala390 via a water molecule, and that the phenol hydroxyl group of **31** interacts with the backbone carbonyl of Gly279 (Fig. 3). Introduction of amino acids with aromatic substituents such as phenylalanine and tyrosine improved the FLT3-ITD activities (**24**-**31**).

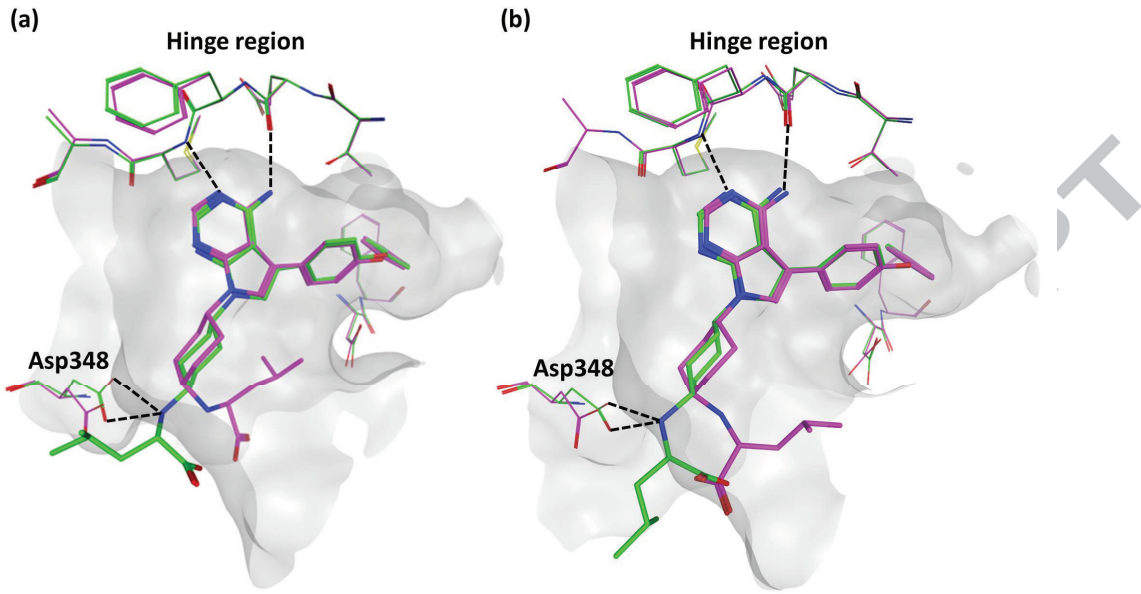


Figure 2. Crystal structures of **3**-**6** bound to HCK (grey area) in stick model. The nitrogen (blue), oxygen (red), sulfur (yellow) atoms and hydrogen bonds (dotted lines) were shown. (a) **3** (green, PDB ID: 5H0B) and **4** (magenta, PDB ID: 5H0A); (b) **5** (green, PDB ID: 5H0D) and **6** (magenta, PDB ID: 5H0C).

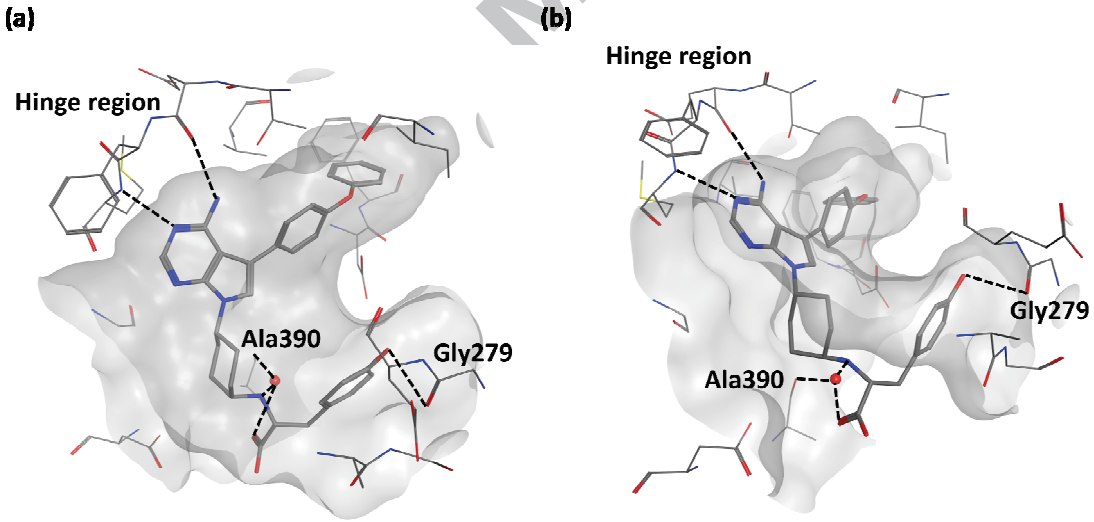


Figure 3. (a) Crystal structure of **31** bound to HCK (grey area) in stick model (PDB ID: 5H0F). The carbon atoms (grey), water oxygen atom (red ball) and hydrogen bonds (dotted lines) were shown. (b) An alternative view (45° rotation of (a)).

Compounds with good enzymatic activities were tested in the AML-derived MV4-11 cell viability studies. Based on the structural diversity and potent activities against HCK and FLT3-ITD, compounds **3**, **7**, **14**, **19**, **27** and **31** were chosen for the study. The results of the study are

summarized in Table 1. While compounds **3**, **19**, **27** and **31** exhibited good anti-leukemic activities, compounds **7** and **14** showed only weak activities despite their good kinase activities. This apparent discrepancy may be attributed to cell membrane penetration potential. As indicated by their cLogP values, relatively low lipophilicity of compounds **7** and **14** may make cell penetration difficult.

In conclusion, we have demonstrated that potent HCK and FLT3-ITD inhibition activities can be incorporated in a series of novel pyrrolo[2,3-*d*]pyrimidines with the help of *in silico* docking and X-ray crystallographic studies. Among the compounds prepared, compound **31** showed potent activity in the AML-derived MV4-11 cell viability study. Some of the HCK binding motifs revealed by the crystallographic studies may provide valuable information for the development of potent HCK and FLT3-ITD dual inhibitors.

Acknowledgments

We would like to thank Drs. Fumihiko Ishikawa and Yoriko Saito of RIKEN Center for integrative medical sciences and Dr. Takehiro Fukami of RIKEN Program for drug discovery and medical technology platforms for helpful discussions, and Dr. Hiroaki Ohtake of Nard Institute for technical support.

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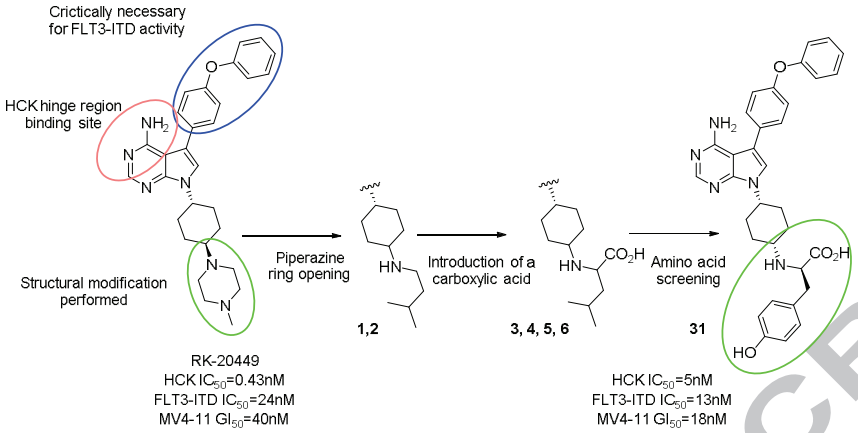
Mori, M.; Ogawa, N.; Tomabechi, Y.; Handa, N.; Honma, T.; Yokoyama, S.; Kojima, H.; Okabe, T.; Nagano, T.; Tanaka, A. *J Mol Biol* **2012**, *417*, 240.



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