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Discovery of quinolinone derivatives as potent FLT3 inhibitors

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ABSTRACT

Recently some fms-like tyrosine kinase 3 (FLT3) inhibitors have shown good efficacy in acute myeloid leukemia (AML) patients. In an effort to develop anti-leukemic drugs, we investigated quinolinone derivatives as novel FLT3 inhibitors. Two substituted quinolinones, KR65367 and KR65370 were subjected to FLT3 kinase activity assay and showed potent inhibition against FLT3 kinase activity *in vitro*, with IC_{50} of 2.7 and 0.57 nM, respectively. As a measure of selectivity, effects on the activity of other kinases were also tested. Both compounds have negligible activity against Met, Ron, epidermal growth factor receptor, Aurora A, Janus kinase 2, and insulin receptor; with IC_{50} greater than 10 μM. KR compounds showed strong growth inhibition in MV4;11 AML cells and increased the apoptotic cell death in flow cytometric analyses. A decrease in STAT5 phosphorylation by KR compounds was observed in MV4;11 cells. Furthermore, *in vitro* evaluation of compounds structurally related to KR65367 and KR65370 showed a good structure-activity relationship.

1. Introduction

Fms-like tyrosine kinase 3 (FLT3), also known as fetal liver kinase 2, is a receptor tyrosine kinase involved in proliferation and differentiation of hematopoietic stem cells [1]. FLT3 is activated by binding of FLT3 ligand, and results in activation of tyrosine kinase activity and downstream signal transduction pathways [2]. FLT3 is overexpressed in most acute myeloid leukemia (AML) cases and activating mutations are found in about one third of AML patients [3]. There are two classes of activating mutations. One is internal tandem duplication (ITD) in the juxtamembrane domain, and the other is point mutation in the tyrosine kinase domain of FLT3. The mutant form of FLT3 aberrantly activates downstream effectors such as STAT5 in a manner different from wild type FLT3 [4]. FLT3 has emerged as an important target for molecular therapy and ITD mutations in FLT3 have been well documented as a drug target in leukemia [5].

AML, most common type of acute leukemia, is characterized by blocking differentiation, and uncontrolled proliferation of cells inside the bone marrow [6]. There are many reports which show that AML patients with mutated FLT3 genes show poor prognosis [7]. Currently, a combination of cytarabine and anthracycline is mainly used for chemotherapy of AML patients. However, there are high remission rates after the chemotherapy, resulting in a high unmet medical need for a new therapeutic strategy [8,9]. Quite a few small molecules that act as FLT3 inhibitors have been identified for the purpose of developing anti-leukemic agents. Small molecules tested in clinical trials include lestaurtinib [10], midostaurin [11], tandutinib [12], sunitinib [13], and quizartinib [14].

In this study we present two quinolinone derivatives as potent FLT3 inhibitors. The compounds KR65367 and KR65370 inhibited FLT3 activity and downstream STAT5 phosphorylation. Both of the compounds exhibited cytotoxicity against AML cells and induced apoptosis in flow cytometric analysis.

2. Materials and methods

2.1. Cell culture and reagents

Human MV4;11 and RS4;11 leukemic cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1%

Abbreviations: FLT3, fms-like tyrosine kinase 3; AML, acute myeloid leukemia; ITD, internal tandem duplication; HTRF, homogeneous time-resolved fluorescence; TR-FRET, time resolved-fluorescence resonance energy transfer; VEGFR-2, vascular endothelial cell growth factor receptor 2; STAT5, Signal transducer and activator of transcription 5.

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Table 1
In vitro inhibition of KR65367 and KR65370 against activity of selected kinases.

Kinase	IC_{50} (μM)	
	KR65367	KR65370
FLT3	2.7×10^{-3}	5.7×10^{-4}
Met	>10	>10
Ron	>10	>10
EGFR	>10	>10
Aurora A	>10	>10
Jak2	>10	>10
Insulin Receptor	>10	>10

EGFR, epidermal growth factor receptor; Jak2, Janus kinase 2.

penicillin/streptomycin at 37 °C with 5% CO_2 in an incubator. Cells were cultured for preselected time periods for different experiments. Lestaurtinib was obtained from LC laboratories (Woburn, MA), and cytosine-D-arabinofuranoside (Ara-C) from Sigma Co. (St. Louis, MO).

2.2. In vitro kinase assay

Inhibition of kinase activity against a variety of recombinant kinases was measured using homogeneous, time-resolved fluorescence (HTRF) assays. Briefly, such assays are based on the phosphorylation of peptide substrates in the presence of ATP. The resulting phosphorylated substrates are detected by a TR-FRET (time resolved-fluorescence resonance energy transfer) signal. Recombinant proteins containing a kinase domain were purchased from Millipore (Billerica, MA). Optimal enzyme, ATP, and substrate concentrations were established for each enzyme using an HTRF KinEASE kit (Cisbio, France) according to manufacturer instructions. Assays consist of enzymes mixed with serially diluted compounds and peptide substrates in a kinase reaction buffer (250 mM HEPES (pH 7.0), 0.5 mM orthovanadate, 0.05% BSA, 0.1% NaN_3). Following the addition of reagents for detection, the TR-FRET signal was measured with a Victor multi-label reader (Perkin Elmer, Waltham, MA). IC_{50} was calculated by nonlinear regression using Prism (GraphPad, La Jolla, CA).

2.3. In vitro cytotoxicity assay

Cells were seeded in a 96-well plate (10,000 cells per well) and incubated with compounds for 72 h. As a negative control, cells were treated with vehicle, dimethyl sulfoxide (DMSO). Cell viability was measured by a tetrazolium-based assay using EZ-Cytox Cell Viability Assay kit (DaeilLab, Korea). IC_{50} was calculated by nonlinear regression using Prism version 5.01 [15].

2.4. Flow cytometric analysis

Cells were grown in 24-well plates (500,000 cells per well) and treated with compounds for 48 h. Cells were then fixed and stained with propidium iodide (PI) (Sigma) and subjected to flow cytometry using FACSCalibur™ (BD Biosciences). Data were analyzed by CellQuest Pro (BD Biosciences). For Annexin V staining, cells were

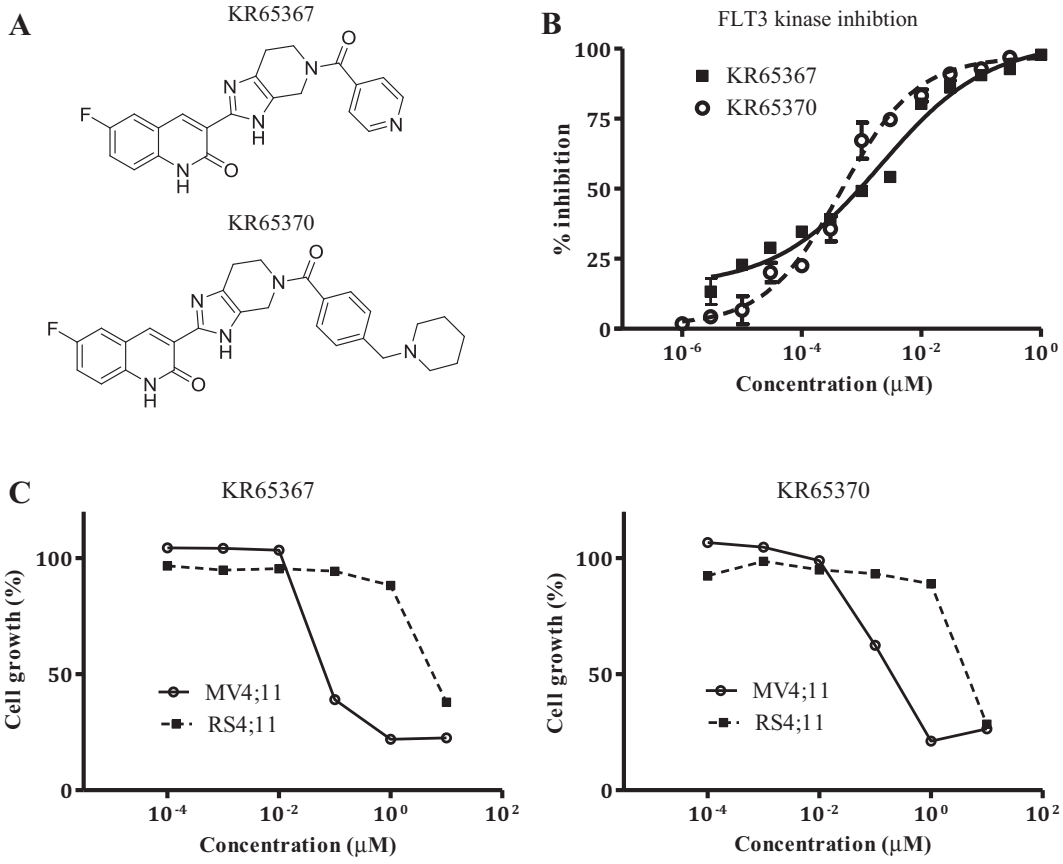

Fig. 1. Effects of KR65367 and KR65370 on FLT3 kinase and AML cell proliferation. (A) Structures of KR65367 and KR65370. (B) Effects of KR65367 and KR65370 on the kinase activity of FLT3. Direct inhibition of kinase activity was measured using purified recombinant FLT3 enzyme using the TR-FRET method. (C) Effects of KR65367 and KR65370 on the proliferation of MV4;11 and RS4;11 cells. Cells were seeded in 96-well plates (10,000 cells per well) and treated with compounds in a series of increasing concentration. Cells were incubated for 72 h and cell viability was measured using a tetrazolium dye assay using. Cell growth (%) was calculated using 0.5% DMSO treatment as a negative control.

seeded in 24-well plates (500,000 cells per well) and treated with compounds for 24 h. Cells were then stained with dead cell apoptosis kit with Alexa Flour 488 Annexin V (Life Technologies, Carlsbad, CA) and subjected to flow cytometry (Accuri C6, BD Biosciences).

2.5. Western blot analysis

Cells were lysed with SDS lysis buffer (12 mM Tris–Cl, pH 6.8, 5% glycerol, 0.4% SDS) and samples were incubated at −70 °C for 10 min, then boiled at 90 °C for 5 min. Total protein isolated by centrifugation at 13,000 rpm for 2 min at 4 °C, was quantified by BCA protein assay kit (iNtRON, Korea). Protein (20 μg) was run by electrophoresis in 8% SDS–polyacrylamide gel followed by its transfer to a PVDF membrane. Blots were blocked with 5% skim milk in Tris-buffered saline (10 mM Tris–Cl, pH 7.4, 140 mM NaCl) containing 0.1% Tween-20 (TBST), and were incubated overnight with p-STAT5, STAT5 or β-actin antibody (Cell Signaling Technology, USA) at 4 °C. Next day, the blots were washed in TBST, incubated with horseradish peroxidase conjugated secondary antibody for an hour at room temperature, again washed with TBST; then visualized using enhanced chemiluminescent reagent (Thermo Scientific Pierce, USA).

3. Results

3.1. Effects of KR65367 and KR65370 on the kinase activity

In the course of the discovery process for FLT3 inhibitors as anti-leukemic agents, KR65367 and KR65370 were tested against FLT3 activity. Using the purified recombinant FLT3 protein, inhibition by small molecules was measured using the TR-FRET method as shown in Materials and methods. KR65367 inhibited FLT3 kinase activity potently (IC_{50} of 2.7 nM). Similarly, KR65370 exhibited even stronger inhibition of FLT3 activity (IC_{50} of 0.57 nM). Other receptor tyrosine kinases (Met, Ron, EGFR, Janus kinase 2, Insulin receptor) and serine/threonine kinase (Aurora A) were not significantly inhibited by KR compounds, and exhibited IC_{50} greater than 10 μM, as shown in Table 1.

3.2. Effects of KR65367 and KR65370 on the AML cells

In order to see the effects of KR65367 and KR65370 on cell proliferation, a cytotoxicity assay was performed using AML cells. KR65367 and KR65370 showed selectivity in terms of cytotoxicity. For MV4;11 cells with the constitutive active form of FLT3, GI_{50} was in the submicromolar range (0.11 μM for KR65367 and 0.26 μM for KR65370). However, for RS4;11 cells with wild type FLT3, KR65367 and KR65370 showed weak cytotoxic activity as shown in Fig. 1 (GI_{50} of 6.3 μM for KR65367 and 4.86 μM for KR65370).

MV4;11 cells were treated with different concentrations of KR65367 and KR65370, and then subjected to cell cycle analysis. Both compounds increased cell populations in the sub-G_1 phase, indicating cell death by KR compounds. The sub-G_1 population of MV4;11 cells was increased by KR65367 from 9% to 56% at 1 μM, and KR65370-treated cells showed similar results (Fig. 2). The sub-G_1 population of MV4;11 cells was 60% at 1 μM of KR65370 treatment, while 9% of MV4;11 cells of the DMSO control was sub-G_1 population. Dose-dependent increase of sub-G_1 population was observed with KR compounds treatment (data now shown).

In order to detect apoptotic cells, Annexin V staining was performed. As shown in Fig. 3, cells were treated with KR65367 and KR65370 for 24 h and analyzed with flow cytometry after staining with Alexa Flour 488 Annexin V and PI. Dose-dependent increase of Annexin V-positive cells was observed by KR65367 and KR65370 treatment. Percentage of early apoptotic cells (bottom right quadrant) increased slightly at 1 μM KR65367 and KR65370 treatment. Cell fraction in top right quadrant (cells undergoing late apoptosis or already dead cells) changed approximately two-to-four fold at 1 μM concentration of KR65367 and KR65370. These results are

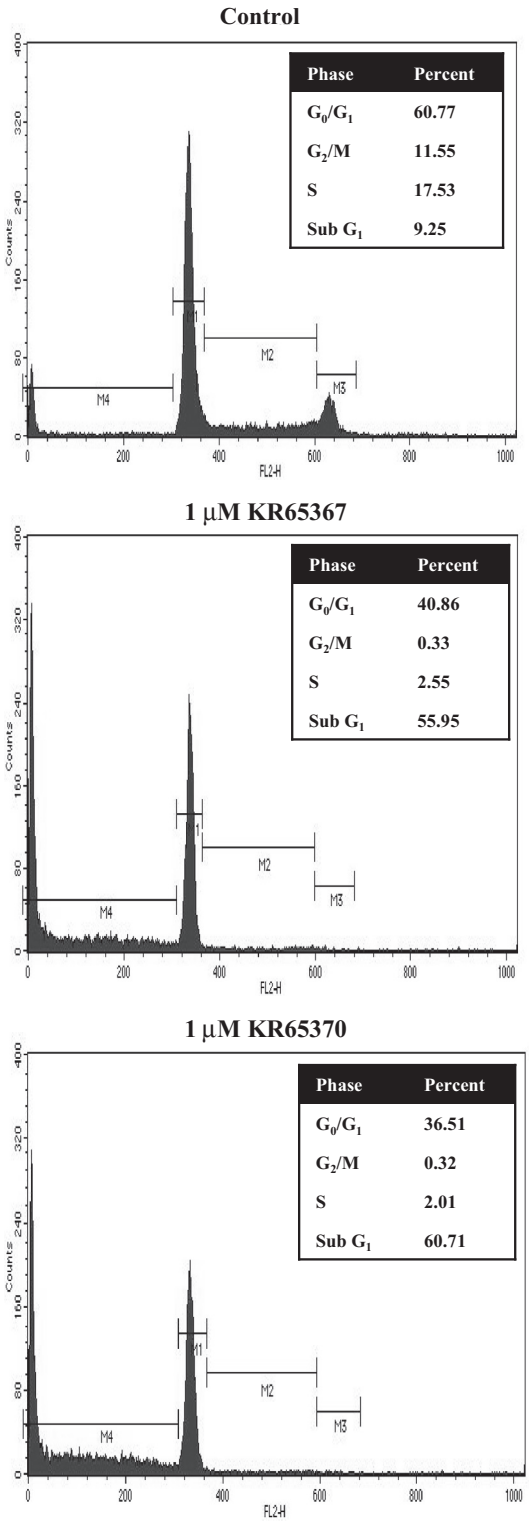

Fig. 2. Effect of KR65367 and KR65370 on cell cycle. MV4;11 cells were treated with KR65367 and KR65370 at 1 μM for 48 h and stained with PI for cell cycle analysis. M1: G_0/G_1 phase, M2: S phase, M3: G_2/M phase and M4: sub-G_1 phase. The charts show a representative experiment ($n = 3$).

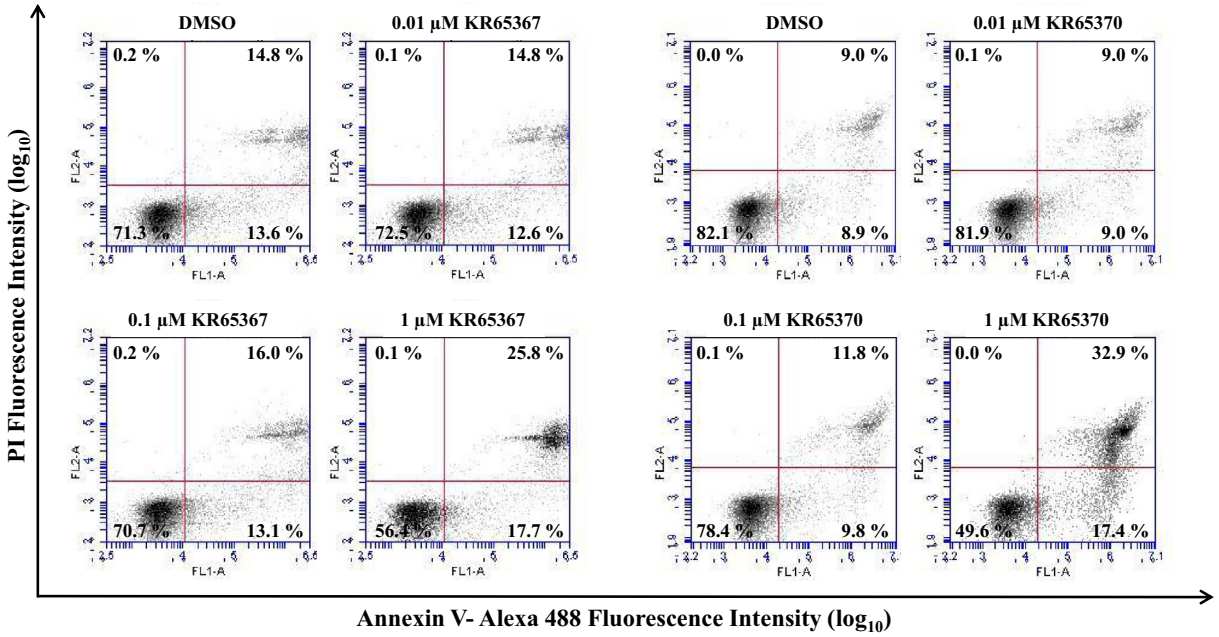

Fig. 3. Effect of KR65367 and KR65370 on the apoptosis. MV4;11 cells were treated with KR65367 and KR65370 at the indicated concentration for 24 h and stained with Alexa Flour 488 Annexin V and propidium iodide (PI). Bottom right quadrant (Annexin V+ and PI–): early apoptotic cells; top right quadrant (Annexin V+ and PI+): late apoptotic and already dead cells. The charts show a representative experiment ($n = 3$).

consistent with growth inhibition (Fig. 1C) and cell cycle analyses (Fig. 2) and indicate apoptotic cell death by KR compounds.

3.3. Effects of KR65367 and KR65370 on STAT5 signaling

Activation of FLT3 induces phosphorylation of STAT5 protein. Inhibition of STAT5 phosphorylation by these compounds was examined using antibody directed against the phosphorylated form of STAT5 (p-STAT5). The MV4;11 cells were treated with KR65367 or KR65370 for 2 h and subjected to western blot analysis. As shown in Fig. 4A, KR65367 decreased p-STAT5 level at a dose of 1 μM. KR65370 showed a stronger effect than KR65367 in downregulation of p-STAT5. Inhibition of p-STAT5 was observed from 0.01 μM of KR65370 (Fig. 4B). At 1 μM of KR65370, STAT5 phosphorylation was completely inhibited.

3.4. Structure-activity relationship of substituted quinolinones

In order to investigate structure-activity relationship, structurally related compounds previously reported [16] were tested for FLT3 enzyme activity and cytotoxicity. The results are summarized in Supplemental Table 1. All the compounds bearing amide or urea type substituents at the R_1 position in the tetrahydroimidazopyridine ring showed a submicromolar range of inhibitory activity against FLT3, and some of them showed single-digit nanomolar activity. Substitution of hydrogen at the R_2 position in the quinolinone ring with halides or a methoxy group, increased inhibitory potency. It was observed that inhibitory potency was better improved when halides or a methoxy group was replaced in the 6-position of the quinolinone ring than in the 5-position. Furthermore, all the compounds inhibited proliferation of MV4;11 cells, while less inhibition was observed for RS4;11 cells.

4. Discussion

In order to find anti-leukemic agents for treatment of AML patients, small molecules inhibiting FLT3 kinase activity were studied. We previously reported KR65367 and KR65370 as vascular endothelial cell growth factor receptor 2 (VEGFR-2) inhibitors with anticancer activity [16]. KR65367 and KR65370 showed good inhibitory activity in a kinase activity assay using purified VEGFR-2 enzyme. However, both of the compounds showed weak anti-proliferative activity against human umbilical vein endothelial cells (HUVEC). When the same compounds were applied to

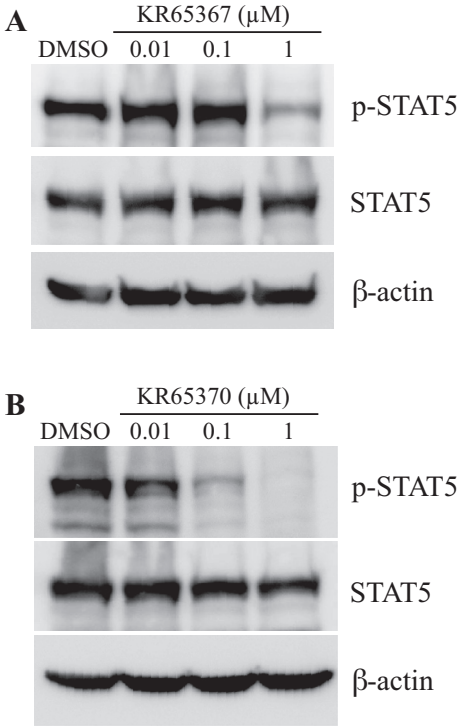

Fig. 4. Effect of KR65367 and KR65370 on the STAT5 phosphorylation. MV4;11 cells were treated with KR65367 (A) and KR65370 (B) for 2 h at the indicated concentration. Cell extracts were subjected to western blotting using antibody against phosphorylated STAT5 (p-STAT5) and STAT5. As loading controls, western blots using β-actin antibody were performed.

cells of the AML cell line MV4;11, KR65367 and KR65370 exhibited strong cytotoxic effect. Along with potent *in vitro* FLT3-inhibitory activity and strong inhibition of STAT5 phosphorylation, KR compounds have potential for development as anti-leukemic agents.

KR compounds inhibited STAT5 phosphorylation in MV4;11 cells, and the KR65370 compound in particular, showed strong effect on down regulation of phosphorylated STAT5 (Fig. 4). There are reports that STAT5 is the preferred second messenger of FLT3, and expression of the phosphorylated form of STAT5 is important in malignant phenotype of AML, rather than in other signaling pathways [17,18]. Considering the importance of STAT5 phosphorylation in AML, the decrease of STAT5 phosphorylation by KR compounds is consistent with growth inhibition in MV4;11 cells.

As shown in Fig. 1, KR compounds exhibited cytotoxic effect preferentially to MV4;11 cells. MV4;11 cells express mutant-type FLT3 (FLT3-ITD), while RS4;11 cells contain wild type FLT3. MV4;11 cells are 'addicted' to FLT3 activation, meaning cell growth and survival are dependent on FLT3 signaling [19]. Thus, these cells are widely utilized for development of FLT3 inhibitors. Low GI_{50} values in the MV4;11 cells, compared to RS4;11 cells, indicate that the cytotoxic effect by KR compounds is mediated by FLT3 inhibition. However, the anti-proliferative effect by KR compounds is also observed in RS4;11 cells; although it is weak. Considering that RS4;11 cells are not addicted to FLT3 signaling, growth inhibition in these may be mediated by inhibition of VEGFR-2 kinase. Our results provide valuable chemical and pharmacological information useful in the field of FLT3 research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.029.

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