## Title

N-(3-Methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-a]pyridin-3-yl)pyridin-2-amine is an inhibitor of the FLT3-ITD and BCR-ABL pathways, and potently inhibits FLT3-ITD/D835Y and FLT3-ITD/F691L secondary mutants.



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# N-(3-Methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridin-3-yl)pyridin-2-amine is an inhibitor of the FLT3-ITD and BCR-ABL pathways, and potently inhibits FLT3-ITD/D835Y and FLT3-ITD/F691L secondary mutants

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## Abstract

Activating mutations within FLT3 make up 30 % of all newly diagnosed acute myeloid leukemia (AML) cases, with the most common mutation being an internal tandem duplication (FLT3-ITD) in the juxtamembrane region (25 %). Currently, two generations of FLT3 kinase inhibitors have been developed, with three inhibitors clinically approved. However, treatment of FLT3-ITD mutated AML is limited due to the emergence of secondary clinical resistance, caused by multiple mechanism including on-target FLT3 secondary mutations – FLT3-ITD/D835Y and FLT3-ITD/F691L being the most common, as well as the off-target activation of alternative pathways including the BCR-ABL pathway. Through the screening of imidazo[1,2-*a*]pyridine derivatives, N-(3-methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridin-3-yl)pyridin-2-amine (**compound 1**) was identified as an inhibitor of both the FLT3-ITD and BCR-ABL pathways. **Compound 1** potently inhibits clinically related leukemia cell lines driven by FLT3-ITD, FLT3-ITD/D835Y, FLT3-ITD/F691L, or BCR-ABL. Studies indicate that it mediates proapoptotic effects on cells by inhibiting FLT3 and BCR-ABL pathways, and other possible targets. **Compound 1** is more potent against FLT3-ITD than BCR-ABL, and it may have other possible targets; however, **compound 1** is first step for further optimization for the development of a balanced FLT3-ITD/BCR-ABL dual inhibitor for the treatment of relapsed FLT3-ITD mutated AML with multiple secondary clinical resistant subtypes such as FLT3-ITD/D835Y, FLT3-ITD/F691L, and cells co-expressing FLT3-ITD and BCR-ABL.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Keywords**

FLT3-ITD; BCR-ABL; Secondary mutants; Cancer; AML

## 1. Introduction

Activating mutations in FLT3 are detected in about 30 % of all acute myeloid leukemia (AML) cases [1,2], most commonly consisting of internal tandem duplication (FLT3-ITD) mutations in the juxtamembrane region (25 %) [3]. To date, two generations of FLT3 kinase inhibitors have been developed and assessed in clinical trials, with three inhibitors clinically approved (Fig. 1). Midostaurin, a first generation FLT3 kinase inhibitor, is able to inhibit FLT3 autophosphorylation, but its lack of selectivity limits its clinical use [4–6]. Therefore, midostaurin was only approved by the US-FDA for use in combination with other chemotherapy agents. Second generation FLT3 kinase inhibitors such as quizartinib [7–13] and gilteritinib [14,15] were improved in potency and selectivity, and were approved for single agent therapy of FLT3-ITD mutated AML. However, the clinical efficacy of quizartinib and gilteritinib is short-lived due to the emergence of drug-resistant secondary mutations within FLT3 after 3–4 months of treatment. The most clinically important resistance mutations are FLT3-ITD/D835Y and FLT3-ITD/F691L [16–18]. FLT3-ITD/D835Y is resistant to quizartinib [19,20] while FLT3-ITD/F691L is resistant to both quizartinib and gilteritinib [21]. Therefore, new FLT3 kinase inhibitors which retain inhibitory effects on both FLT3-ITD/D835Y and FLT3-ITD/F691L are needed [22].

Besides on-target FLT3 secondary mutations, off-target secondary clinical resistance was also observed in patients after treatment with FLT3 kinase inhibitors, mainly due to mutations that activate the PI3K/AKT and/or RAS/MAPK pathways [23,24]. Recently, the BCR-ABL gene fusion has emerged as another cause of off-target secondary clinical resistance in AML after FLT3 inhibitor treatment. BCR-ABL arises as a consequence of a chromosomal translocation between chromosome 9 and 22 - t(9, 22), resulting in the fusion of the BCR and ABL genes [25,26]. The BCR-ABL gene fusion causes unregulated ABL activity, and is found in >95 % of chronic myelogenous leukemia (CML) [27,28], 20–30 % of acute lymphoblastic leukemia (ALL) [29], and a minority of primary AML cases. Notably, AML with the BCR-ABL genetic abnormality is stratified as an “adverse risk” group in European LeukemiaNet (ELN) [30,31]. FLT3-ITD/BCR-ABL was recently reported as a new secondary clinical resistant subtype emerges after gilteritinib treatment and is detected in 5 % of relapsed FLT3 mutated patients [23,32–34]. However, there is no FLT3-ITD/BCR-ABL dual inhibitor which also inhibits the problematic FLT3-ITD/D835Y and FLT3-ITD/F691L mutations. Therefore, there is an unmet clinical need for an inhibitor that simultaneously blocks FLT3-ITD and BCR-ABL pathways to treat relapsed AML patients with FLT3-ITD/BCR-ABL secondary resistance subtype.

Imidazo[1,2-*a*]pyridine was previously identified as a promising scaffold for the development of FLT3 inhibitors [35,36]. In this study, in order to discover an inhibitor that simultaneously blocks FLT3-ITD and BCR-ABL pathways, we screened a newly synthesized library of >60 imidazo[1,2-*a*]pyridine derived compounds (Table

S1). Through screenings, we identified N-(3-methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridin-3-yl)pyridin-2-amine (**compound 1**) as an inhibitor of both the FLT3-ITD and BCR-ABL pathways; in addition, it also potently inhibits FLT3-ITD/D835Y and FLT3-ITD/F691L secondary mutants. **Compound 1** may serve as a promising start compound for further optimization to develop a balanced FLT3-ITD/BCR-ABL dual inhibitor to treat FLT3-ITD mutated AML with multiple secondary clinical resistant subtypes, including FLT3-ITD/D835Y, FLT3-ITD/F691L, and FLT3-ITD/BCR-ABL.

## 2. Results and discussion

### 2.1. Cell antiproliferative effects

In order to discover a small molecule inhibitor to treat AML patients with secondary clinical resistant subtypes, we screened a FLT3 inhibitor compounds library synthesized in a previous study for their antiproliferative effects on a few selected clinically relevant cell lines. MOLM14 is a patient-derived AML cell line driven by a FLT3-ITD mutation. MOLM14-D835Y and MOLM14-F691L are sublines of MOLM14 that were derived after long-term selection in quizartinib and carry drug-resistant FLT3 secondary mutations - either FLT3-ITD/D835Y or FLT3-ITD/F691L, respectively. In order to verify the compounds' inhibitory effects on the BCR-ABL pathway, we also screened the compounds on K562, a patient-derived CML cell line driven by the BCR-ABL gene fusion.

The abovementioned cell lines were treated with various concentrations of **compound 1,** as well as several other FLT3 inhibitors, to assess their inhibitory effects (Table 1). **Compound 1** was observed to have slightly better antiproliferative effects on MOLM14 cells than gilteritinib, with an $IC_{50}$ of (3.65 ± 0.56) nM versus (5.47 ± 0.25) nM. Importantly, **compound 1** also retains very comparable antiproliferation effects on MOLM14-D835Y and MOLM14-F691L cells, with very close $IC_{50}$ values in all three MOLM14 cell lines (Fig. 2). In contrast, although gilteritinib has similar inhibitory effects on both MOLM14-D835Y and MOLM14 cells, MOLM14-F691L cells are relatively resistant. K562 cells, which harbor a BCR-ABL mutation, are also sensitive to **compound 1** treatment, with an $IC_{50}$ of (8.43 ± 2.75) nM (Table 1). In order to verify that the antiproliferative effects of **compound 1** are specific, we also assessed its activity in MDA-MB-231, MDA-BM-468, and HCC1806 cells, which are all triple-negative breast cancer cell lines with no known FLT3 and ABL mutations. No antiproliferative effects were observed in MDA-MB-231 or MDA-BM-468 cells, although HCC1806 cells show some sensitivity at higher concentrations, which indicates that **compound 1** is not generally cytotoxic.

We compared the antiproliferative effects of **compound 1** with its close analogue **compound 2**. The structural difference between **compounds 1** and **2** is the location of their methoxy groups. The methoxy group in **compound 1** is at the *meta*-position of the aniline, while the methoxy group in **compound 2** is at the *para*-position (Fig. 3). **Compound 1** and **2** act similarly in FLT3 mutant cell lines such as MOLM14, MOLM14-D835Y, and MOLM14-F691L; but they act differently in the BCR-ABL driven K562 cell line (Table 1). Therefore, the 3-methoxy aniline at the end of **compound 1** is structurally important for the

target inhibition in K562 cells. It might either have crucial interactions with specific amino acid residues or have location-sensitive steric effects in the binding pocket.

### 2.2. Western blots

To determine the molecular effects of **compound 1** on FLT3 signaling, we treated MOLM14, MOLM14-D835Y, and MOLM14-F691L cells with **compound 1** and gilteritinib at 1/10X, 1X, 10X, and 100X of their $IC_{50}$ values in MOLM14 cells for 2 h and looked at activation of FLT3 and its downstream mediator ERK by western blot analysis (Fig. 4, **Panel A, B, and C**). In MOLM14 cells, **compound 1** starts reducing the phosphorylation of FLT3 and ERK at 1X $IC_{50}$, and causes complete inhibition at 10X $IC_{50}$. **Compound 1** works very similarly in MOLM14-D835Y cells, although in MOLM14-F691L cells there is some residual signal at 10X $IC_{50}$ suggesting that MOLM14-F691L cells are slightly resistant to **compound 1**.

To determine the molecular effects of **compound 1** on BCR-ABL signaling, we treated K562 cells with **compound 1** and dasatinib, a second-generation ABL inhibitor, at 1/10X, 1X, 10X, and 100X of their $IC_{50}$ values in K562 cells for 2 h and looked at activation of BCR-ABL and its downstream mediators STAT5 and ERK by western blot analysis (Fig. 4, **Panel D**). **Compound 1** is able to inhibit the phosphorylation of BCR-ABL and downstream targets, STAT5 and ERK. However, its inhibitory effects are observed to be about 10-fold less potent than dasatinib. For example, the reduction of phosphorylation signals of BCR-ABL, STAT5, and ERK are observed starting at 100X, 10X, and 100X of $IC_{50}$s treatment of **compound 1**, respectively. In comparison, when treated with dasatinib, the reduction of phosphorylation signals of BCR-ABL, STAT5, and ERK are observed starting at 10X, 1X, and 10X of $IC_{50}$s respectively. Therefore, although **compound 1** is able to inhibit the BCR-ABL pathway, it is not as potent as dasatinib.

The western blot results of K562 cells shows that **compound 1** does require higher concentrations to inhibit the BCR-ABL pathway. Therefore, the antiproliferative effects of **compound 1** on K562 cells may not be primarily from the inhibition of BCR-ABL pathway, but possibly from other targets that enhance the antiproliferation effects of **compound 1** on the K562 cell line. Simultaneous inhibition of two or more kinase targets has been shown to enhance antitumor effects [37], therefore, there might be other kinase pathway targets besides FLT3-ITD and BCR-ABL that **compound 1** also inhibit, such as CDK9 [38,39], PI3K/AKT [40], PDGF/PDGFR [41], KIT [42], etc. Till now, we have ruled out the possibility of PI3K/AKT pathway inhibition (Fig. S1). Future investigations on other possible targets of **compound 1** will be necessary to further dissect its mechanisms of action in these cells.

### 2.3. Kinase selectivity profiling

To evaluate the inhibition properties of **compound 1** on other kinases, we conducted a site-directed competition binding assay for 97 kinases (Fig. 5). The results revealed that at 8.4 nM concentration, **compound 1** only exhibited interactions (>65 % wash-off) with FLT3 and ABL1 (phosphorylated and unphosphorylated). The result high-lighted the selectivity of **compound 1** among human kinome. Therefore, the antiproliferative effects of **compound 1**

on cells may hardly be due to a combinative inhibition of kinases; instead, there might be non-kinase targets.

### 2.4. Apoptosis assay

Both FLT3 gain-of-function mutations [43–45] and the BCR-ABL gene-fusion [46,47] mediate anti-apoptotic effects through downstream cascades of phosphorylation reactions and their associated signaling pathways such as RAS/RAF/MEK/ERK, JAK/STAT5 and PI3K/AKT/mTOR. In order to verify that **compound 1**′s anti-proliferative effects are mediated by apoptosis, we performed apoptosis assays in MOLM14 cells 24, 48 and 72 h after treatment with **compound 1** (Fig. 6). In the representative figures below, cells in the lower left quadrant (Q1-LL) are nonapoptotic cells while cells in the lower right quadrant (Q1-LR) and upper right quadrant (Q1-UR) are early and late apoptotic cells, respectively. No notable difference was observed between DMSO and 1 nM **compound 1** treatments at any time points, indicating that this low dose, which is below the $IC_{50}$ value of 3.65 nM, did not induce apoptosis. However, 10 nM treatment of **compound 1** showed a substantial increase in both early and late apoptotic cells at all timepoints, when compared with the DMSO treatment. Specifically, after 24 h of treatment with 10 nM **compound 1**, early apoptotic cells make up 9.5 % of cells, while late apoptotic cells make up of 6.1 % of cells. After 48 h, early apoptotic cells decreased to 4.7 % while late apoptotic cells increased to 24.0 %. Finally, after 72 h, early apoptotic cells further decreased to 4.3 % while late apoptotic cells continued to increase to 45.5 % of cells. In conclusion, **compound 1** induces proapoptotic effects in MOLM14 cells in a dose- and time-dependent manner.

## 3. Conclusion

**Compound 1** was discovered to be an inhibitor of the FLT3-ITD and BCR-ABL pathways in a library screen for FLT3 kinase inhibitors. It is observed to have potent antiproliferative effects on a variety of clinically relevant leukemia cell lines driven by FLT3-ITD, FLT3-ITD/D835Y, FLT3-ITD/F691L, or BCR-ABL, which do not appear to be due to a general mechanism of toxicity. Its inhibitory effects on FLT3-ITD, FLT3-ITD/D835Y, FLT3-ITD/F691L, and their downstream targets, are verified by loss of phosphorylation on FLT3 and its downstream signaling molecules by western blot analysis in MOLM14, MOLM14-D835Y, MOLM14-F691L cells, respectively. From the western blots of K562 cells, **compound 1** is also identified as an inhibitor of the BCR-ABL pathway, although its inhibitory effects are not as potent as dasatinib. In addition, **compound 1** may have other possible targets. In summary, **compound 1** inhibits cell proliferation by mediating proapoptotic effects on cells through inhibiting FLT3 and BCR-ABL pathways, and other possible targets. **Compound 1** is not a perfectly balanced dual inhibitor of FLT3-ITD and BCR-ABL pathways, and it may have other possible targets; however, it represents an important initial compound for further optimization toward the development of FLT3-ITD/BCR-ABL dual inhibitors to treat relapsed AML with FLT3-ITD and multiple secondary clinical resistant subtypes such as FLT3-ITD/D835Y, FLT3-ITD/F691L, and FLT3-ITD/BCR-ABL. Ongoing studies are directed toward investigating other possible targets of **compound 1** and further optimizing the structure of **compound 1** to achieve a more balanced FLT3-ITD/BCR-ABL dual inhibitor.

# 4. Materials and methods

## 4.1. Chemistry

The synthesis of 3-(6-chloropyridin-2-yl)-7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridine was described in another report [48]. The other chemical reagents were obtained from commercial suppliers and used without further purification. Thermo Finnigan LCQ Deca with Thermo Surveyor LCMS System at variable wavelengths of 254 nm and 214 nm was used to monitor the reaction and test the purity of the compounds. The purity of all the final compounds is >95 %. The water-methanol gradient buffered with 0.1 % formic acid was used as the mobile phase for the HPLC system. NMR spectra were completed on a Varian 400 MHz instrument. The $^{1}$H NMR spectra and $^{13}$C spectra were recorded at 400 MHz and 101 MHz, respectively. The final compound was purified using Silica gel (0.035e0.070 mm, 60 Å) flash chromatography.

### 4.1.1. Synthesis and Characterization of N-(3-methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-a]pyridin-3-yl)pyridin-2-amine, compound 1—

3-(6-Chloropyridin-2-yl)-7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridine (100 mg, 322.83 umol), 3-methoxyaniline (79.52 mg, 645.67 umol), cesium carbonate (157.78 mg, 434.25 umol) were dissolved into 5 mL Dioxane. The resulting reaction mixture was degassed with nitrogen for 5–10 min. After that, $Pd(PPh_3)_4$ (37.31 mg, 32.28 umol) was added. The resulting mixture was stirred at 100 °C under $N_2$ atmosphere overnight (sealed bottle). After completion of reaction, the reaction mixture was filtered and concentrated in vacuo. Silica flash chromatography (MeOH/DCM 0—7 %) followed by C18 flash chromatography (MeOH/$H_2O$ 10—80 %) yielded **compound 1** as a black solid (47 mg, 36.72 %). MS *m/z* $[M + 1]^+$ 397.2. $^{1}$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.74 (d, $J$ = 7.3 Hz, 1H), 9.15 (s, 1H), 8.36 (s, 1H), 8.25 (s, 1H), 8.09 (s, 1H), 7.88 (d, $J$ = 0.9 Hz, 1H), 7.66 (t, $J$ = 7.9 Hz, 1H), 7.34 (d, $J$ = 7.6 Hz, 1H), 7.26 (t, $J$ = 8.1 Hz, 1H), 7.15 (dt, $J$ = 12.9, 5.4 Hz, 3H), 6.74 (d, $J$ = 8.2 Hz, 1H), 6.58 (dd, $J$ = 8.1, 2.1 Hz, 1H), 3.89 (s, 3H), 3.72 (s, 3H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 159.86, 155.04, 147.61, 147.22, 142.38, 138.34, 136.75, 135.41, 129.76, 128.80, 127.88, 123.38, 120.03, 111.75, 111.00, 110.65, 110.43, 107.53, 107.39, 106.61, 104.94, 54.97, 38.68. HRMS *m/z* $[M + H]^+$ Calculated: 397.1771, Found: 397.2397.

### 4.1.2. Synthesis and characterization of N-(4-methoxyphenyl)-6-(7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-a]pyridin-3-yl)pyridin-2-amine, compound 2—

3-(6-Chloropyridin-2-yl)-7-(1-methyl-1H-pyrazol-4-yl)imidazo[1,2-*a*]pyridine (100 mg, 322.83 umol), 4-methoxyaniline (645.67 umol), cesium carbonate (157.78 mg, 434.25 umol) were dissolved into 5 mL Dioxane. The resulting reaction mixture was degassed with nitrogen for 5–10 min. After that, $Pd(PPh_3)_4$ (37.31 mg, 32.28 umol) was added. The resulting mixture was stirred at 100 °C under $N_2$ atmosphere overnight (sealed bottle). After completion of reaction, the reaction mixture was filtered and concentrated in vacuo. Silica flash chromatography (MeOH/DCM 0–7 %) followed by C18 flash chromatography (MeOH/$H_2O$ 10–80 %) yielded **compound 2** as a yellow solid (42 mg, 32.81 %). MS *m/z* $[M + 1]^+$ 397.2. $^{1}$H NMR (400 MHz, CDCl3) $\delta$ 9.70 (d, $J$ = 7.4 Hz, 1H), 8.06 (s, 1H), 7.83 (d, $J$ = 0.5 Hz, 1H), 7.70 (s, 2H), 7.53 – 7.48 (m, 1H), 7.34 – 7.29 (m, 2H),

7.13 (d, $J$ = 7.6 Hz, 1H), 6.97 – 6.92 (m, 2H), 6.90 (dd, $J$ = 7.3, 1.8 Hz, 1H), 6.53 (d, $J$ = 8.2 Hz, 1H), 6.49 (s, 1H), 3.97 (s, 3H), 3.85 (s, 3H). $^{13}$C NMR (101 MHz, CDCl3) $\delta$ 156.62, 156.54, 148.87, 138.37, 137.10, 134.76, 133.28, 130.20, 128.44, 127.65, 124.78 (2C), 121.36, 114.66 (2C), 111.60, 111.50, 111.03, 104.90, 55.73, 50.93, 39.41, 29.84. HRMS $m/z$ $[M + H]^+$ Calculated: 397.1771, Found: 397.1760.

### 4.2. Cell culture

MV4-11 cells were obtained from the laboratory of Dr. Farrar in Arkansas Children's Hospital and cultured in IMDM (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Gibco, USA). MOLM14, MOLM14-D835Y, MOLM14-F691L cells were obtained from the laboratory of Dr. Shah in University of California San Francisco and cultured in RPMI (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Gibco, USA). K562 cells were obtained from the laboratory of Dr. Shah in University of California San Francisco and cultured in IMDM (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Gibco, USA). MDA-MB-231 cells were obtained from the laboratory of Dr. Qin in University of Arkansas for Medical Sciences and cultured in DMEM (Gibco, USA) containing 10 % fetal bovine serum (FBS) (Gibco, USA). All media contained 100 units/mL penicillin (Gibco, USA), and 100 μg/mL streptomycin (Gibco, USA). Other cell lines were cultured in appropriate media according to provider's instructions. Cells were incubated at 37 °C in a humidified atmosphere of 5 % $CO_2$.

### 4.3. Cell viability assays

Cells in logarithmic phase were seeded into 96-well culture plates at 5000 cells per well. Then, after 24 h of incubation, cells were treated in triplicate with various concentrations of compounds for 72 h in final volumes of 200 μL. Upon end point, 20 μL Resazurin solution (Biotium, USA) was added to each well, and the cells were incubated for an additional 4–6 h. Fluorescence values at a wavelength of 590 nM were taken on a spectrophotometer (BioTek, USA). $IC_{50}$ values were calculated using percentage of growth versus vehicle (DMSO) treated control. The data were finally fitted in GraphPad Prism V9.0 software to obtain $IC_{50}$ values using [inhibitor] vs. normalized response (variable slope) model. Each compound was tested at least three times.

### 4.4. FLT3 kinase inhibition assay

Kinase assays were conducted using the bioluminescent ADP-Glo™ kinase assay (Promega, USA), following the manufacturer's instructions. Assay was performed with the test compounds at 8-point half log dilutions (1 uM to 0.316 nM). Luminescence signal was measured on a spectrophotometer (BioTek, USA), and $IC_{50}$ values reported are based on the dose–response curve fitted in GraphPad Prism V9.0 using [inhibitor] vs. normalized response model. Each compound was tested at least three times.

### 4.5. Western blot

MOLM14, MOLM14-D835Y and MOLM14-F691L cells, at $5x10^5$ cells/ml, were exposed to compound 1 or gilteritinib for 2 h at 37 degrees at 1/10X, 1X, 10X, and 100X of their $IC_{50}$ values in Molm14 cells (3.65 nM and 5.47 nM, respectively). K562 cells, at $5x10^5$

cells/ml, were exposed to compound 1 or dasatinib for 2 h at 37 degrees at 1/10X, 1X, 10X, and 100X of their $IC_{50}$ values in K562 cells (8.43 nM and 0.56 nM, respectively). Total cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then probed with primary antibodies purchased from Cell Signaling (Danvers, MA, USA) [pFLT3 Y842 (#4577), FLT3 (#3462), pABL Y245 (#2861), pSTAT5 Y694 (#9351), STAT5 (#9363), pERK (#4370), ERK (#9107) and GAPDH (#5174)] and from BD Biosciences (San Jose, CA, USA) [ABL (#554148)] followed by secondary antibodies purchased from Licor (Lincoln, NE, USA). Immunoblots were then visualized by a Biorad ChemiDoc MP Imagine System (Hercules, CA, USA).

### 4.6. Kinase selectivity profiling

Compound 1 was dissolved in DMSO to required concentrations and sent to Eurofins DiscoverX Corporation located in San Diego, CA USA for KINOMEscan™ Profiling Service. The TREEspot™ Interaction Maps was generated online based on the testing results.

### 4.7. Apoptosis assay

MOLM14 cells were cultured in 2 mL of growth medium in 6-well plates for 24 h. Then the cells were treated with DMSO or various concentrations of compound 1. After a 72 h exposure to the compound, the cells were collected and centrifuged for 5 min at 1200 rpm, re-suspended in PBS and counted using a Countess II Automated Cell Counter (Applied Biosystems, Foster City, USA). The cells were then washed with cold PBS two more times and resuspended in binding buffer (BD Bioscience, San Jose, USA) at a concentration of $1 \times 10^6$ cells/ml. Then, 100 μL of the cell suspension was transferred to FACS tubes and 5 μL of FITC Annexin V (BD Bioscience, San Jose, USA) and 5 μL PI (BD Bioscience, San Jose, USA) was added as the manufacture's instruction stated. 400 μL of binding buffer was added to each sample after 15 min incubation at room temperature in the dark. Lastly, all the samples were analyzed by flow cytometry using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, USA).

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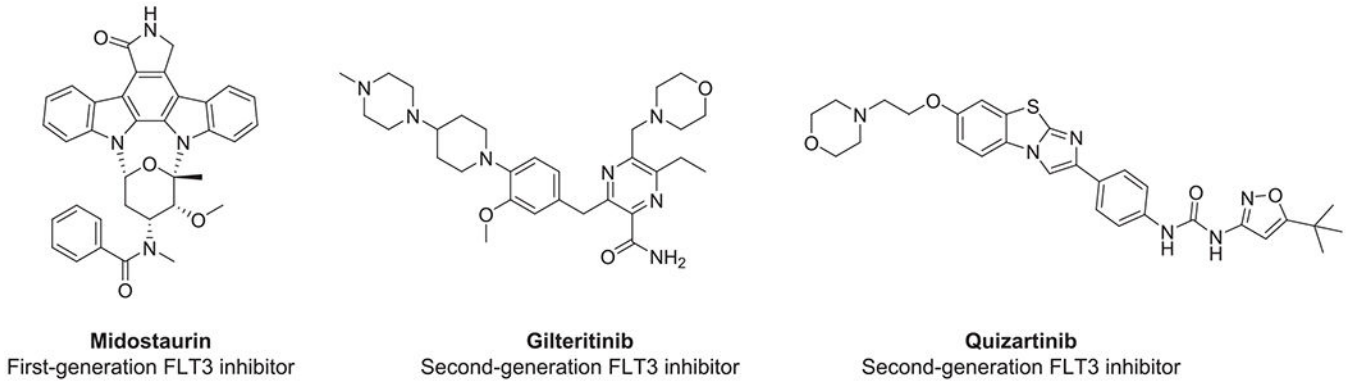


**Fig. 1.**
Chemical structures of clinically approved FLT3 inhibitors.

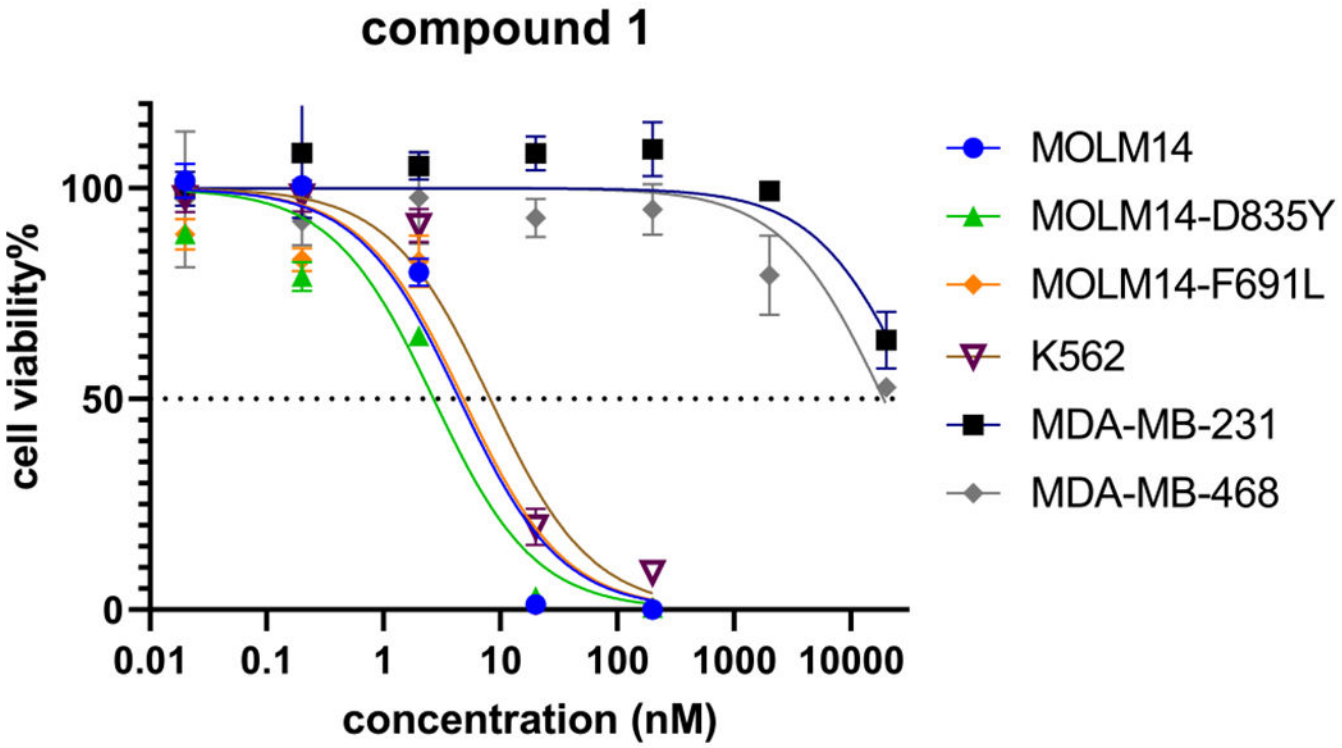


**Fig. 2.**
Cell-based viability curves of compound 1.

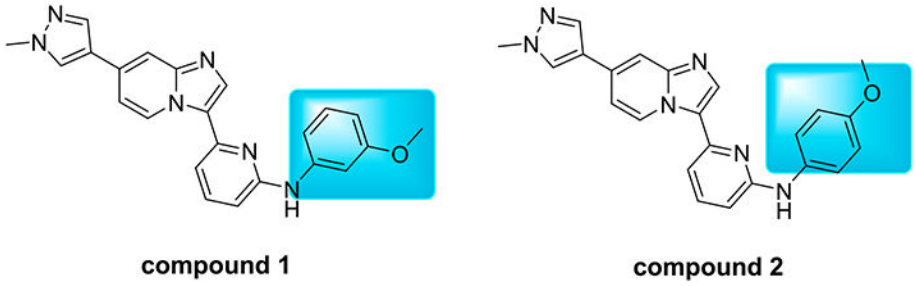


**Fig. 3.**
Chemical structures of compound 1 and 2.

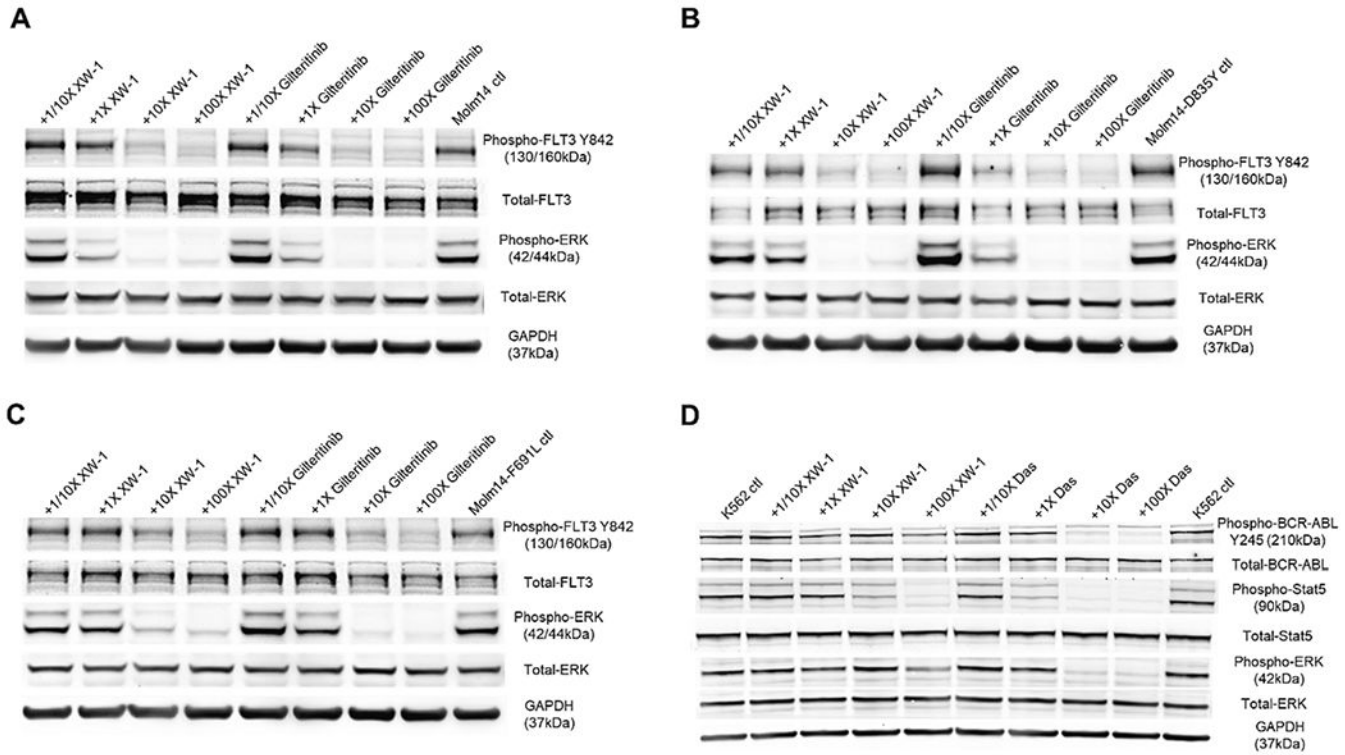


**Fig. 4.**

Western blot of compound 1 on MOLM14 (A), MOLM14-D835Y (B), MOLM14-F691L (C), and K562 (D).

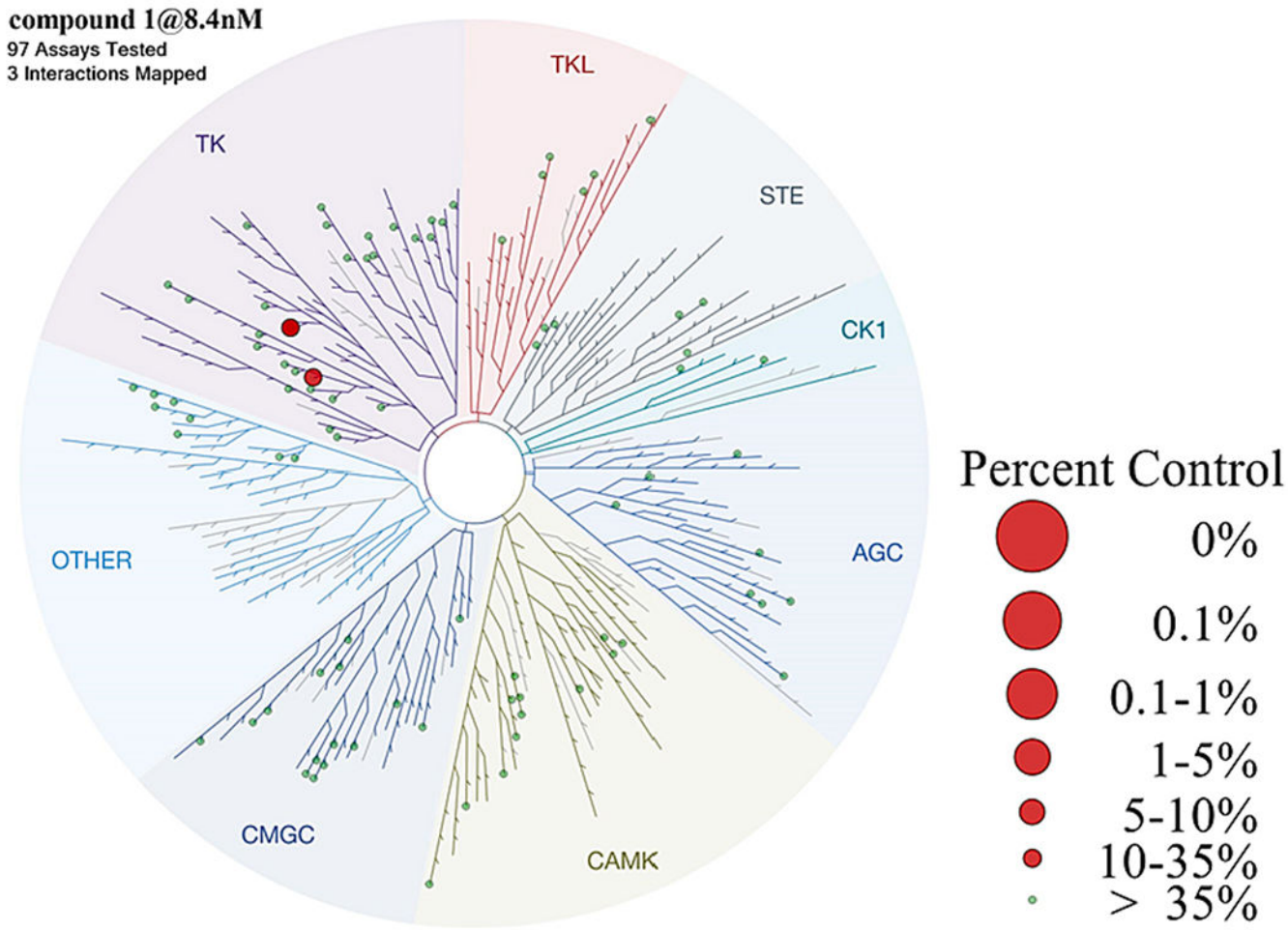


**Fig. 5.**
Kinase selectivity profiling of compound 1.

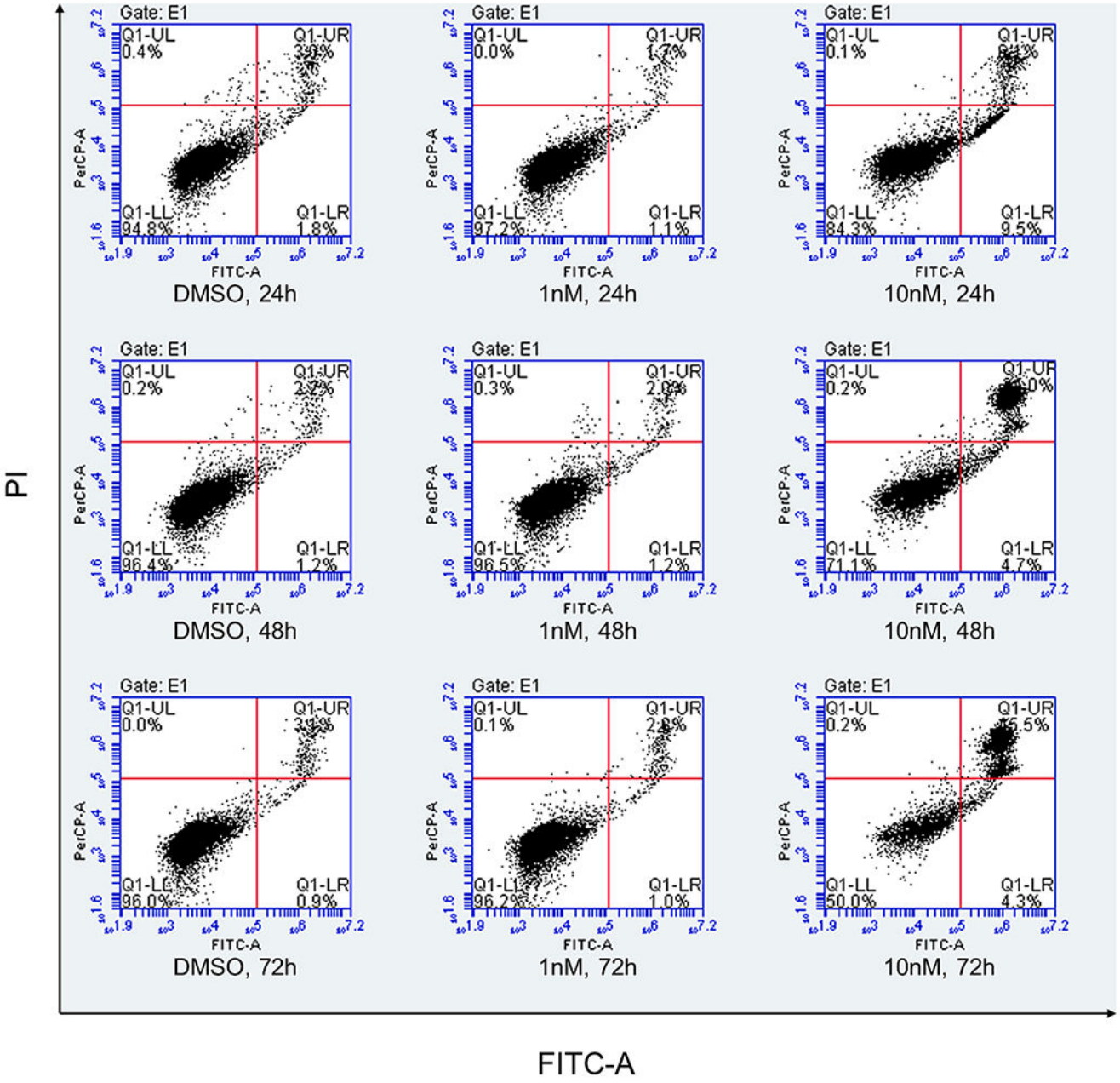


**Fig. 6.**

Apoptosis assays of MOLM14 cells treated with 1 nM and 10 nM of compound 1 for 24 h, 48 h, or 72 h, respectively, DMSO as control.

**Table 1**

$IC_{50}$s (nM) of compounds 1 and 2 on FLT3 kinase[a] and multiple cell lines[b], gilteritinib and vandetanib as control.

| $IC_{50}$ | compound 1 | compound 2 | gilteritinib | vandetanib |
|---|---|---|---|---|
| MOLM14 | 3.65 ± 0.56 | 3.20 ± 0.13 | 5.47 ± 0.25 | 1108 ± 84 |
| MOLM14-D835Y | 2.08 ± 0.49 | 2.45 ± 0.27 | 7.83 ± 0.03 | 2805 ± 139 |
| MOLM14-F691L | 4.23 ± 0.50 | 19.82 ± 4.38 | 58.8 ± 1.0 | >10.000 |
| K562 | 8.43 ± 2.75 | 7786 ± 2727 | 13100 ± 2532 | 1535 ± 150 |
| MDA-MB-231 | >20,000 | – | – | – |
| MDA-MB-468 | >20,000 | – | – | – |
| HCC1806 | 114.7 ± 2.5 | – | – | – |

[a] $IC_{50}$ on FLT3 kinase, compound concentration required to inhibit 50% enzymatic activity of FLT3.

[b] $IC_{50}$ on cells, compound concentration required to inhibit tumor cell proliferation by 50%.