Identification of New FLT3 Inhibitors That Potently Inhibit AML Cell Lines via an Azo Click-It/Staple-It Approach

Xiaochu Ma,^{†,‡,||,#} Jie Zhou,^{†,||,#} Changhao Wang,^{‡,#} Brandon Carter-Cooper,[§] Fan Yang,[‡] Elizabeth Larocque,^{†,||} Jonathan Fine,^{†,||} Genichiro Tsuji,^{†,||} Gaurav Chopra,^{†,||} Rena G. Lapidus,[§] and Herman O. Sintim^{*,†,‡,||,⊥}

[†]Institute for Drug Discovery, ^{||}Department of Chemistry, [⊥]Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907, United States

[‡]Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, United States

[§]Translational Core Laboratory, University of Maryland Greenebaum Cancer Center, 655 West Baltimore Street, Baltimore, Maryland 21201, United States

S Supporting Information

ABSTRACT: Acute myeloid leukemia (AML) is an aggressive malignancy with only a handful of therapeutic options. About 30% of AML patients harbor mutated FLT3 kinase, and thus, this cancer-driver has become a hotly pursued AML target. Herein we report a new class of FLT3 inhibitors, which potently inhibit the proliferation of acute myeloid leukemia (AML) cells at nanomolar concentrations.

KEYWORDS: FMS-like tyrosine kinase 3 (FLT3), acute myeloid leukemia (AML), inhibitor, azo, click-it/staple-it

Cancer continues to claim the lives of millions of patients annually, despite the great advances that have been made in cancer therapeutics in the last few decades. Whereas the five year survival rates of cancer patients who are diagnosed with thyroid, prostate, skin, and hormone responsive breast are now over 90%, those with pancreatic, liver, lung, and esophageal have less than 20% chance of surviving in five years.¹ Even for certain types of cancers such as breast, which generally has a good prognosis, there are still certain subtypes that record low five-year survival rates. For example, the five-year survival rate for hormone responsive ER+ breast cancer is over 90%,¹ but for inflammatory breast cancer is at a miserly 44%² and for metastatic triple negative breast cancer the median survival is less than two years.³ Another example is leukemia, which has a general five-year survival rate of 59%,¹ but acute myeloid leukemia (AML) registers a disappointing five-year survival rate of 26%.⁴ Clearly there is a need for new chemotypes that kill cancers that are associated with poor survival rates.

We have focused on finding new chemical entities that inhibit the proliferation of AML because there has not been a breakthrough therapeutic against AML for over two decades, and cytarabine (araC), which is commonly used for treating AML patients, is the most effective when used in combination with other drugs. Resistance to araC has also been well documented, and 30–50% of patients relapse upon treatment

with araC alone or in combination with other drugs.⁵ Arguably there is a need for new chemical entities that are effective against AML. Recently there has been interest in targeting FMS-like tyrosine kinase 3 (FLT3), a tyrosine receptor kinase that is commonly mutated (activating mutations) in AML patients (~30%).⁶ The FLT3 protein is a member of the class III receptor-tyrosine kinase (RTK) family.⁷ Upon binding of FLT3 ligand, FLT3 receptor dimerizes, resulting in phosphorylation, followed by activation of downstream signaling cascades, including the RAS/MEK, PI3K/AKT/mTOR, and STAT5 pathways, which in turn regulate cell cycle progression, apoptosis, and other important cellular processes.⁸

Mutations in FLT3 lead to constitutive autophosphorylation of FLT3 and thus uncontrolled activation of downstream signaling.⁹ Examples of FLT3 inhibitors that have been developed^{10–13} and are in clinical trials for treating AML are sorafenib, quizartinib, crenolanib, and midostaurin.^{14–16} Unfortunately many of the tested FLT3 inhibitors have experienced limited clinical efficacy, especially on patients who harbor FLT3 D835Y/V/I/F mutations (these mutations

Received: November 18, 2016

Accepted: April 14, 2017

Published: April 14, 2017

are particularly resistant to type II inhibitors, which target the inactive state of the active ATP binding site).¹⁷ Therefore, despite the availability of several FLT3 inhibitors, there is still a need for new-generation kinase inhibitors that could lead to a durable disease-free survival.

We have developed a simple strategy to identify and develop FLT3 kinase inhibitors by first making compound libraries, using the facile azo coupling (which is tolerant of many functional groups and operationally simple) and testing the “azo” library against MV4–11 or Molm-14 cell lines, which are driven by FLT3 ITD mutation (Figure 1). Compounds that inhibit these FLT3-driven cell lines but not non-FLT3 driven cell lines, such as THP-1 are likely FLT3 inhibitors (Figure 1). We were aware that azo moieties are generally not stable in biological environments¹⁸ but the successful development of the drug eltrombopag¹⁹ from an azo hit encouraged us to pursue the “azo library” strategy, with an eye toward replacing the azo group with a more stable moiety after a hit molecule had been found (see Figures 1 and 2). We call this approach “Azo-Click-it/Staple-it” drug discovery strategy. The “Azo-Click-it” term denotes the fact that azo coupling is facile and falls within Sharpless’ definition of a Click reaction,²⁰ and the “Staple-it” term reflects the fact that for practical application one has to change the “labile” azo group with a more stable unit. A first-generation library of potential kinase inhibitors was therefore made by conjugating various aromatic diazonium compounds (generated from the aromatic amines) with quinolines and isoquinolines (privileged pharmacophores) (see SI, Figure S1), and the compounds were screened against MV4–11 (SI, Table S1).

To confirm that compounds that were active against MV4–11 and Molm-14 were also FLT3 inhibitors, we screened (using Reaction Biology service) the compounds against FLT3 at concentration of 500 nM, in the presence of 100 μ M radiolabeled ATP (SI, Figure S2). Compounds containing benzamidines and isoquinolin-3-amine moieties had superior FLT3 inhibition profile than those containing naphthalen-2-

Figure 1. “Click it and staple it” strategy for compound library construction and hit optimization. X = CH or N; Y and R₁ are substituents. See Figure S1 for details.

amine, quinolin-7-amine, quinolin-6-amine, isoquinolin-6-amine, or quinolin-8-amine groups (SI, Figure S2), suggesting that the position of the ring nitrogen on the isoquinoline or quinoline and also the substitution pattern of the benzene ring

Figure 2. Replacement of azo moiety in hit compound with alkyne and alkene units.

Figure 3. Western Blot analysis of phospho-FLT3 and total FLT3 protein expression in MV4–11 after treatment with HSW630-1. MV4–11 cells were treated with DMSO vehicle (V) and HSW630-1. Scanned images were analyzed using ImageJ software.

Figure 4. Antiproliferative effects of HSW630-1 against leukemia and normal lung (MRC-5A) cell lines.

(denoted as Y in Figure 1) were critical for FLT3 inhibition. The best azo inhibitor, HSW630-1 (see Figure 2) inhibited FLT3 and FLT3 ITD (internal tandem duplications mutation) with IC₅₀ values of 18.6 and 8.77 nM, respectively (SI, Figure S2C). As stated earlier, several FLT3 point mutations, such as D835Y, confer resistance to FLT3 inhibitors (especially type II inhibitors). Interestingly, HSW630-1 could inhibit FLT3 D835Y,²¹ with IC₅₀ of 28 nM (SI, Figure S2C). Docking of HSW630-1 against FLT3, using the CANDOCK program, suggested that HSW630-1 binds to a site near the ATP binding site (see SI, Figure S3). Other FLT3 inhibitors that are currently in clinical trials, such as quizartinib and crenalonib, also inhibit c-Kit and TrkC in addition to FLT3 inhibition, see SI, Table S2. Analogously, HSW630-1 could also inhibit TrkC (IC₅₀ = 5.9 nM) and c-Kit (IC₅₀ = 6.9 nM). The effect of HSW630-1 on FLT3-mediated signaling in leukemic cells was investigated using Western blot analysis (Figure 3). In agreement with the *in vitro* FLT3 inhibition data, HSW630-1

Table 1. FLT3 Inhibition Profile and Antiproliferative Activities of Alkyne Analogues of HSW630-1 against Different Leukemia Cancer Cell Lines

Entry	Structure	% Inhibition of kinases FLT3 at 500 nM	Anti-proliferative effects in leukemia panel (μM) MV4-11 ^a MOLM14 ^b THP-1 ^c K-562 ^d HL60 ^e
1		92	0.15 ± 0.01^a 0.15 ± 0.01^b 1.20 ± 0.20^c 3.00 ± 0.96^d
2		60	0.05 ± 0.01^a 0.11 ± 0.02^b 1.85 ± 0.92^c 1.39 ± 0.01^d 3.05 ± 0.07^e
3		43	0.30 ± 0.20^a 0.10 ± 0.02^b
4		51	0.08 ± 0.02^a 0.30 ± 0.08^b
5		56	0.07 ± 0.02^a 0.30 ± 0.01^b
6		1	$> 5^a$ $> 5^b$
7		34	$> 5^a$ $> 5^b$
8		6	$> 5^a$ $> 5^b$
9		21	$> 5^a$ $> 5^b$
10		36	$> 5^a$ $> 5^b$
11		61	0.04 ± 0.01^a 0.05 ± 0.03^b
12		54	0.02 ± 0.01^a 0.06 ± 0.01^b
13		41	0.35 ± 1.17^a
14		98	0.001 ± 0.001^a 0.001 ± 0.001^b
15		99	0.005 ± 0.001^a 0.004 ± 0.001^b

^{a–e}These correlate the cell lines tested to respective IC₅₀.

Figure 5. (A) Dose–response curves of HSW1651 against FLT3 and important FLT3 mutants. (B,C) Western Blot analyses of p-FLT3/total FLT3 (B) and p-STAT5/STAT5 (C) protein expression in MV4–11 after treatment with HSM1651 and DMSO vehicle (V) control. Scanned images were analyzed using ImageJ software.

could inhibit FLT3 phosphorylation in a dose-dependent manner (see Figure 3).

Having established that HSW630-1 and analogues are FLT3 inhibitors (Figure 3 and SI, Figure S2) and inhibit leukemia cell line MV4–11 (FLT3-driven) (Figure 4 and SI Table S1), we also tested the HSW630-1 against MOLM-14 (also FLT3-driven), K-562 (chronic myelogenous leukemia, CML, which is not driven by FLT3), and THP1 (non-FLT3-driven), using Alamar Blue assay (see Figure 4). The effect of HSW630-1 was also evaluated against MRC-5A (lung), which is a control cell line that is typically used to evaluate the effect of a cytotoxic agent against normal cells. Excitingly, and in agreement with the FLT3 inhibition profile, HSW630-1 showed potent inhibition of FLT3-driven MV4–11 and MOLM-14 cancer cells (IC₅₀ ≈ 150 nM), but not THP1 (IC₅₀ > 1000 nM) or K-562 (IC₅₀ = 3000 nM). Compounds that did not potently inhibit FLT3 also did not inhibit the proliferation of MV4–11 cells (FLT3-driven) (see Figure S2 and Table S1 for details). Since HSW630-1 (a FLT3-inhibitor) could slow down the proliferation of MV4–11 and MOLM-14 but not K-562 or THP1 (both are not FLT3-driven), it is likely that HSW630-1 and analogues thereof that inhibit FLT3 also inhibit MV4–11 and MOLM-14 proliferation via FLT3 inhibition.

Having achieved our goal of identifying an azo compound (HSW630-1) that could potentially inhibit FLT3 kinase and also inhibit AML proliferation, we proceeded to identify linkers that could replace the potentially problematic azo moiety in HSW630-1.

We designed few analogues that we thought could be readily synthesized, where the azo moiety was replaced with a more “stable” group (alkene, alkyne, ether, and amines), and used computational methods (Gaussian²² at B3LYP/6-31+G(d) level of theory) to identify the most stable conformers of these analogues. From these calculations, it appeared that the alkene and alkyne moieties were better mimics of the azo unit than the rest (see SI Figure S4 for discussion). Alkyne and alkene analogues of HSW630-1 were readily prepared via Sonogashira or Suzuki coupling of iodo arenes with alkynes or alkene boronates, catalyzed by Pd(PPh₃)₂Cl₂ (Sonogashira) or Pd(PPh₃)₄ (Suzuki) (see SI Scheme S1). In our hands it was easier to make the Sonogashira products than the Suzuki products; therefore, the majority of the stable analogues that were synthesized contained the alkyne unit (see Table 1). The nonazo compounds were also FLT3 inhibitors (see Table 1 for percentage enzymatic inhibition at 0.5 μM compounds), albeit not as potent enzyme inhibitors as the azo compounds. Detailed characterization of one of the nonazo analogues, HSM1651, revealed that it inhibited FLT3, FLT3 ITD, and FLT3 D835Y with IC₅₀ values of 40, 100, and 56 nM, respectively (see Figure 5A).

As was in the HSW630-1 case, the “stable” analogues also inhibited c-Kit and TrkC enzymes (see SI Table S3). Encouragingly, the “stable” HSW630-1 analogues could also potentially inhibit AML cell lines (see Table 1). Western analysis of MV4–11, treated with HSM1651, revealed that FLT3 phosphorylation as well as the downstream STAT5 phosphorylation^{23,24} were reduced in the presence of HSM1651 (Figure 5B,C), and so it appears that the stable analogues act analogously to the azo lead compound, HSW630-1. A few of the alkyne analogues have respectable antiproliferative properties (HSM1819 has an IC₅₀ value of 20 nM, which is only four times less potent than crenolanib (IC₅₀ = 5 nM), which proceeded to clinical trials). Regarding structure–activity relationship studies of the alkyne/alkene analogues, it appears that modifications to the isoquinoline ring greatly affected both kinase inhibition and antiproliferative activity, which is similar to what was observed in the azo series (see SI, Figures S1 and S2 and Table S1). In the azo series, it was observed that substitution of the isoquinoline 6-position with halogen or alkyl groups was beneficial for both kinase inhibition and inhibition of MV4–11 proliferation. For example HSW 1107-2 (Cl at 6-position of isoquinoline) inhibited proliferation of MV4–11 with IC₅₀ of 60 nM, whereas HSW308-4 (H at 6-position of isoquinoline) inhibited the proliferation of MV4–11 with IC₅₀ of 900 nM (see SI, Figure S1 and Table S1). For the alkyne series, HSM1813 (H at 3-position and also lacking Cl at 6-position) was not a potent inhibitor of MV4–11 proliferation (see Table 1, entry 10). HSM1651 (amino at 3-position and Cl at 6-position) is a good inhibitor of MV4–11 and MOLM-14 proliferation, whereas compounds HSM1781, 1798, and 1796, which differed from HSM1651 at the isoquinoline part, are ineffective antiproliferative agents in AML cell lines. The amidine group also appears to be important for antiproliferative

activity. The cyano analogue HSM1611 is ineffective against MV4-11 and MOLM-14, whereas the hydroxyamidine analogue (HSM1819), which is derived from HSM1611, potentially inhibits the proliferation of FLT3-driven AML cell lines. The hydroxyamidine HSM1819 inhibits FLT3 and FLT3 ITD with IC_{50} of 217 and 240 nM, respectively, *in vitro*. HSM1820, another hydroxyamidine analogue, inhibits FLT3 and FLT3 ITD with IC_{50} of 359 and 350 nM, respectively, *in vitro*.

The azo unit is not strictly forbidden in a drug, and an example of an azo containing drug is phenazopyridine, which is an analgesic extensively used to alleviate pain or irritation associated with the urinary tract.²⁵ Drugs containing azo moiety can, however, cause hemolysis in patients with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, and this patient group is excluded from taking azo-containing drugs.²⁶ Therefore, it is a good measure to replace the azo unit in drug candidates when possible. Azo-containing compounds are easy to synthesize so the "Azo-Click-It/Staple-It" approach is a powerful (yet an underutilized strategy in drug discovery and optimization) means to explore chemical space quickly and then convert "hits" into drug-like molecules. Future work aims to determine the ADME and *in vivo* efficacy properties of our new FLT3 inhibitors. These ongoing studies will be reported in a full paper in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00468.

Detailed synthesis strategies and chemical characterization as well as biological assays (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: hsintim@purdue.edu.

ORCID

Herman O. Sintim: 0000-0002-2280-9359

Author Contributions

[#]These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Purdue University for financial support. NMR and MS data were acquired by the NMR and MS facilities supported by NIH P30 CA023168. X.C.M. thanks China Scholarship Council (No. 201406140119) for financial support.

■ ABBREVIATIONS

ITD, internal tandem duplication; PI3K, phosphatidylinositol 3-kinases; mTOR, mechanistic or mammalian target of rapamycin; STAT5, signal transducer and activator of transcription 5; DMSO, dimethyl sulfoxide; AKT, v-akt murine thymoma viral oncogene homologue 1; TrkC, tropomyosin receptor kinase C; FMS, feline McDonough sarcoma viral oncogene homologue; c-Kit, CD117; RAS, guanine-nucleotide

binding protein; MEK, mitogen-activated protein kinase; ADME, absorption, distribution, metabolism, and excretion

■ REFERENCES

- (1) American Cancer Society. Cancer Facts & Figures 2014. <http://www.cancer.org/acs/groups/content/@research/documents/webcontent/acspsc-042151.pdf>.
- (2) Dawood, S.; Merajver, S. D.; Viens, P.; Vermeulen, P. B.; Swain, S. M.; Buchholz, T. A.; Dirix, L. Y.; Levine, P. H.; Lucci, A.; Krishnamurthy, S.; Robertson, F. M.; Woodward, W. A.; Yang, W. T.; Ueno, N. T.; Cristofanilli, M. International expert panel on inflammatory breast cancer: consensus statement for standardized diagnosis and treatment. *Ann. Oncol.* **2011**, *22*, 515–523.
- (3) Ovaricek, T.; Frkovic, S. G.; Matos, E.; Mozina, B.; Borstnar, S. Triple negative breast cancer - prognostic factors and survival. *Radiol. Oncol.* **2011**, *45*, 46–52.
- (4) Leukemia—Acute Myeloid—AML: Statistics. <http://www.cancer.net/cancer-types/leukemia-acute-myeloid-aml/statistics>.
- (5) Cai, J.; Damaraju, V. L.; Groulx, N.; Mowles, D.; Peng, Y.; Robins, M. J.; Cass, C. E.; Gros, P. Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells. *Cancer Res.* **2008**, *68*, 2349–2357.
- (6) Levis, M.; Small, D. FLT3: It does matter in leukemia. *Leukemia* **2003**, *17*, 1738–1752.
- (7) Matthews, W.; Jordan, C. T.; Wiegand, G. W.; Pardoll, D.; Lemischka, I. R. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell* **1991**, *65*, 1143–1152.
- (8) Konig, H.; Levis, M. Targeting FLT3 to treat leukemia. *Expert Opin. Ther. Targets* **2015**, *19*, 37–54.
- (9) Small, D. FLT3 mutations: biology and treatment. *Hematology Am. Soc. Hematol. Educ. Program* **2006**, *2006*, 178–184.
- (10) Sun, D.; Yang, Y.; Lyu, J.; Zhou, W.; Song, W.; Zhao, Z.; Chen, Z.; Xu, Y.; Li, H. Discovery and rational design of pteridin-7(8H)-one-based inhibitors targeting FMS-like tyrosine kinase 3 (FLT3) and its mutants. *J. Med. Chem.* **2016**, *59*, 6187–6200.
- (11) Wander, S. A.; Levis, M. J.; Fathi, A. T. The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond. *Ther. Adv. Hematol.* **2014**, *5*, 65–77.
- (12) Warkentin, A. A.; Lopez, M. S.; Lasater, E. A.; Lin, K.; He, B. L.; Leung, A. Y.; Smith, C. C.; Shah, N. P.; Shokat, K. M. Overcoming myelosuppression due to synthetic lethal toxicity for FLT3-targeted acute myeloid leukemia therapy. *eLife* **2014**, *3*, e03445.
- (13) Park, S.; Chapuis, N.; Bardet, V.; Tamburini, J.; Gallay, N.; Willems, L.; Knight, Z. A.; Shokat, K. M.; Azar, N.; Viguié, F.; Ifrah, N.; Dreyfus, F.; Mayeux, P.; Lacombe, C.; Bouscary, D. PI-103, a dual inhibitor of class IA phosphatidylinositol 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia* **2008**, *22*, 1698–1706.
- (14) Grunwald, M. R.; Levis, M. J. FLT3 tyrosine kinase inhibition as a paradigm for targeted drug development in acute myeloid leukemia. *Semin. Hematol.* **2015**, *52*, 193–199.
- (15) Kadia, T. M.; Ravandi, F.; Cortes, J.; Kantarjian, H. New drugs in acute myeloid leukemia. *Ann. Oncol.* **2016**, *27*, 770–778.
- (16) Levis, M. FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? *Hematology Am. Soc. Hematol. Educ. Program* **2013**, *2013*, 220–226.
- (17) Smith, C. C.; Lin, K.; Stecula, A.; Sali, A.; Shah, N. P. FLT3 D835 mutations confer differential resistance to type II FLT3 inhibitors. *Leukemia* **2015**, *29*, 2390–2392.
- (18) Ueda, T.; Yamaoka, T.; Miyamoto, M.; Kim, S.-I. Bacterial reduction of azo compounds as a model reaction for the degradation of azo-containing polyurethane by the action of intestinal flora. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 1139–1142.
- (19) Weisberg, E.; Sattler, M.; Ray, A.; Griffin, J. D. Drug resistance in mutant FLT3-positive AML. *Oncogene* **2010**, *29*, 5120–5134.
- (20) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.

- (21) Smith, C. C. Disease diversity and FLT3 mutations. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 20860–20861.
- (22) Frisch, M. J.; et al. *Gaussian 09*, revision D.01; Gaussian, Inc.: Wallingford, CT, 2013.
- (23) Benekli, M.; Baer, M. R.; Baumann, H.; Wetzler, M. Signal transducer and activator of transcription proteins in leukemias. *Blood* **2003**, *101*, 2940–2954.
- (24) Choudhary, C.; Brandts, C.; Schwable, J.; Tickenbrock, L.; Sargin, B.; Ueker, A.; Böhmer, F. D.; Berdel, W. E.; Müller-Tidow, C.; Serve, H. Activation mechanisms of STAT5 by oncogenic Flt3-ITD. *Blood* **2007**, *110*, 370–374.
- (25) Deepalatha, C.; Deshpande, N. A comparative study of phenazopyridine (pyridium) and cystone as short-term analgesic in uncomplicated urinary tract infection. *Int. J. Pharm. Pharm. Sci.* **2011**, *3*, 224–226.
- (26) Mercieca, J. E.; Clarke, M. F.; Phillips, M. E.; Curtis, J. R. Acute hemolytic anaemia due to phenazopyridine hydrochloride in G-6-PD deficient subject. *Lancet* **1982**, *2*, 564.