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Indirubin derivatives as potent FLT3 inhibitors with anti-proliferative activity of acute myeloid leukemic cells

Soo Jeong Choi [a], Myoung Ju Moon [a], So Deok Lee [a], Sang-Un Choi [b], Sun-Young Han [b,*], Yong-Chul Kim [a,*]

[a] Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea
[b] Pharmacology Research Center, Korea Research Institute of Chemical Technology, Yuseong, Daejeon 305-600, Republic of Korea

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ABSTRACT

Indirubin derivatives were identified as potent FLT3 tyrosine kinase inhibitors with anti-proliferative activity at acute myeloid leukemic cell lines, RS4;11 and MV4;11 which express FLT3-WT and FLT3-ITD mutation, respectively. Among several 5 and 5′-substituted indirubin derivatives, 5-fluoro analog, **13** exhibited potent inhibitory activity at FLT3 (IC_{50} = 15 nM) with more than 100-fold selectivity versus 6 other kinases and potent anti-proliferative effect for MV4;11 cells (IC_{50} = 72 nM) with 30-fold selectivity versus RS4;11 cells. Cell cycle analysis indicated that compound **13** induced cell cycle arrest at G_0/G_1 phase in MV4;11 cells.

FLT3 (FMS-like receptor tyrosine kinase-3), a member of the class III tyrosine kinase receptor family, is predominantly expressed in hematopoietic progenitor cells, playing an important role in the pathogenesis of acute myeloid leukemia (AML).[1] FLT3 is expressed in blast cells of most of patients with AML, in which wild-type (WT) form is overexpressed or activating mutated forms are present.[2] Two classes of activating FLT3 mutations have been detected in AML patients: internal tandem duplication (ITD) mutations[3] in the juxtamembrane domain (15–35% of AML patients), and point mutations (TKD)[4] in heavily conserved areas of the intracellular tyrosine kinase domain (5–10% of AML patients). Both types of FLT3 mutations have been reported to be responsible for the ligand-independent dimerization, auto-phophorylation, and constitutive activation of the FLT3 receptor.[5] In the case of the patients with AML containing FLT3/ITD mutations, the relapse rates have been significantly increased and overall survival rates decreased compared with the patients without such mutations of FLT3.[6] In addition, the overexpression or constitutive activation of WT-FLT3 display an important role in the development of AML, not yet completely elucidated.[7] Therefore, inhibition of both the mutated form and WT-FLT3 kinase could provide an effective strategy for the treatment of AML.

Several small-molecule FLT3 tyrosine kinase inhibitors are currently under clinical investigations (Fig 1). The staurosporine structural analogues, PKC412 (midostaurin) and CEP-701 (lestaurtinib) are well known multi-targeted tyrosine kinase inhibitors.[8]

MLN-518 (tandutinib), a piperazinyl-quinazoline derivative was reported to inhibit FLT3 with an IC_{50} value of 30 nM with similar inhibitory potency at c-Kit and PDGFR.[9] An indazoyl-diphenyl urea inhibitor, ABT-869 (linifanib) is known to excellently inhibit not only FLT3 (IC_{50} = 4 nM) but also VEGFR-2 (IC_{50} = 4 nM).[10] Despite of the strong inhibitory potency of the current FLT3 inhibitors, their multi-targeted and non-selective tyrosine kinase inhibitory actions may display side effects. Therefore, development of new potent and selective FLT3 kinase inhibitors is required.

Since indirubin has been reported to be the active constituent of a traditional Chinese medicine, Danggui Longhui Wan, for the treatment of chronic myelocytic leukemia, it is possible that indirubin might interact with a target molecule related with leukemia such as FLT3 kinase. Therefore, a series of indirubin derivatives, previously reported to potentially inhibit CDK2 by our group,[11] was evaluated and several hit compounds displayed potent FLT3 inhibitory activities. Interestingly, Zhou et al. recently reported that another indirubin derivative, which was not an inhibitor of FLT3, abolished resistance to FLT3 inhibitor, ABT-869 by inhibiting STAT signaling activity and destroying survivin expression.[12] Also, the combination therapy of the indirubin analog with ABT-869 provided a potent in vivo activity in the FLF3/ITD mutated resistance xenograft model.[12] Herein, we report the detailed study of indirubin analogs on the direct inhibitory activity against FLT3 kinase, anti-proliferative activity of AML cells with structure–activity relationships, cell cycle arrest, and selectivity profile among a few other kinases, tyrosine kinase and serine/threonine kinase.

Indirubin derivatives were evaluated for FLT3 tyrosine kinase inhibitory activity[13] as well as for anti-proliferative activity[14] against two AML cell lines: an RS4;11, MV4;11, which are human

* Corresponding authors. Tel.: +82 42 860 7416; fax: +82 42 861 4246 (S.-Y.H.), tel.: +82 62 970 2502; fax: +82 62 970 2484 (Y.-C.K.).
E-mail addresses: syhan@krict.re.kr (S.-Y. Han), yongchul@gist.ac.kr (Y.-C. Kim).

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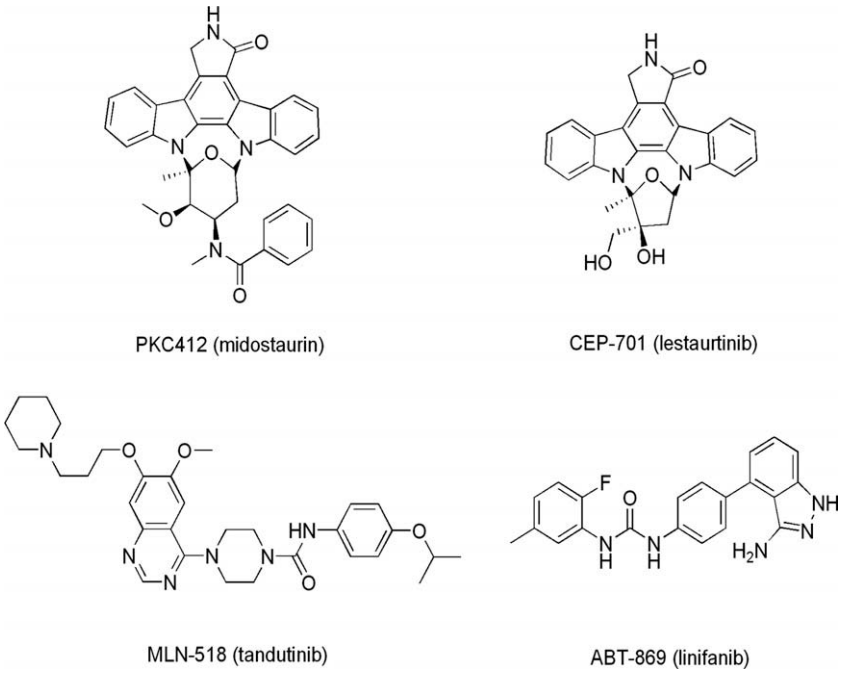
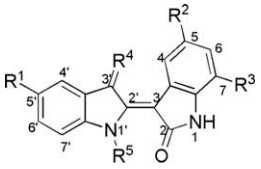


Figure 1. FLT3 inhibitors in clinical development.

leukemia cell lines that express FLT3-WT and a FLT3-ITD mutation, respectively.[15] MV4;11 cells have an ITD mutation in the FLT3 receptor, resulting in constitutive activation FLT3. A widely used drug in AML therapy, cytosine β-D-arabinofuranoside (AraC)[16], and new drug candidate under clinical trial, Lestaurtinib[17,18] were tested together as a positive control for comparison.

The results of biological activities of indirubin analogs were summarized in Table 1. In general, oxime substituted compounds at R^4 position showed more potent FLT3 enzyme inhibition and

Table 1
Effect of indirubin derivatives on the FLT3 activity and cytotoxicity of AML cell



Compound	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)		
						FLT3[a]	MV4;11[b]	RS4;11[b]
1	H	H	H	O	H	>10	>10	>10
2	H	H	H	O	$COCH_3$	>10	>10	>10
3	H	NO_2	H	O	H	>10	>10	>10
4	H	F	H	O	H	10.07	>10	>10
5	H	Na^+SO_3	H	O	H	0.280	>10	>10
6	H	CH_3	H	O	H	0.209	1.54	>10
7	H	Cl	H	O	H	0.212	7	>10
8	H	I	H	O	H	4.722	4.79	>10
9	H	NH_2	H	O	H	0.359	4.3	4.67
10	H	H	H	NOH	H	0.033	0.15	3.56
11	H	CF_3O	H	NOH	H	>10	3.55	3.36
12	H	NO_2	H	NOH	H	0.062	0.45	0.56
13	H	F	H	NOH	H	0.015	0.072	2.21
14	H	CH_3	H	NOH	H	0.028	0.26	8.56
15	H	Cl	H	NOH	H	0.082	0.22	1.58
16	H	I	H	NOH	H	0.418	0.36	1.45
17	H	CH_3	CH_3	NOH	H	0.952	2.4	>10
18	H	Cl	CH_3	NOH	H	>10	>10	>10
19	H	Na^+SO_3	H	NOH	H	0.009	>10	>10
20	Br	NO_2	H	NOH	H	>10	0.27	0.16
21	H	NH_2	H	NOH	H	0.128	1.65	3.34
AraC							3.36	0.26
Lestaurtinib						0.005	0.009[c]	0.12[d]

[a] See Ref. 13 for assay details.
[b] See Ref. 14 for assay details.
[c,d] See Refs. 17,18, respectively.

good anti-proliferative activity compared with corresponding indirubin derivatives (R^4 = O).

Next, the effects of substitutions at R^2, R^3 positions of indirubin oxime analogs were studied. Compounds **11, 12, 15, 16,** and **21** of which R^2 and R^3 positions are substituted with relatively bulky groups (CF_3O, NO_2, Cl, I, and NH_2) could not improve the inhibitory activities of unsubstituted indirubin-3′-oxime, **10** (R^2 = H, IC_{50} = 33 nM for FLT3 and 150 nM for MV4;11) either in FLT3 kinase assay as well as cell-based assay with MV4;11 cells. However, compounds **13, 14** with F and Me groups at R^2 position were more potent than **10** in FLT3 kinase assay. Particularly, R^2–F substituted compound **13** showed the most potent FLT3 inhibitory activity (IC_{50} = 15 nM) as well as anti-proliferative activity against MV4;11 cells (IC_{50} = 72 nM) in this series while maintaining moderate activity against the FLT3-WT cell line. The order of inhibitory activity for R^2-halogen-substituted indirubin oxime analogs **(13, 15,** and **16)** is F > Cl > I.

Despite of weak activity for R^2 substituted derivatives for FLT3 inhibition, the R^2-sulfonate indirubin oxime compound, **19** showed the most potent FLT3 kinase inhibitory activity (IC_{50} = 9 nM) while displaying no anti-proliferative activity in cell-based assay, presumably due to the low membrane permeability of ionic sulfonate group. Interestingly, R^1-bromo-substituted compound **20** displayed potent anti-proliferative activity in both cell lines than would have been expected from its FLT3 kinase inhibitory activity. This result indicated that compound **20** might take another mechanism of anti-proliferation of these cell lines.

In the analysis of the effects of R^3-substitutions, the introduction of an additional methyl group at R^3 position reduced FLT3 inhibition activity as well as anti-proliferative activity compared with R^3-unsubstituted compounds (compare **15** vs **18**, **14** vs **17**). Especially, R^2–Cl, R^3–Me substituted indirubin oxime derivative, **18** was at least 100-fold less active at FLT3 than R^3–Me-unsubstituted derivative **15**. A similar tendency was also observed in cell-based assay, resulting in no anti-proliferative activity of compound **18** against MV4;11 and RS4;11.

Taken together, R^1, R^3-substitutions of indirubin oxime derivatives decreased the inhibitory activities and particular size at R^2 position could be tolerated in FLT3 kinase as well as MV4;11 cell proliferation. Thus, the counter part of R^1, R^2 and R^3 position of indirubin oxime structure in FLT3 binding site might be relatively tight only to accommodate minimum size of functional groups.

Attention should be drawn that most of indirubin oxime derivatives exhibited good inhibitory potency for MV4;11 cell line having FLT3-ITD mutation, while weak or no inhibition for RS4;11 cells expressing wild-type FLT3. For example, MV4;11 cells harbouring constitutively activated form of FLT3 are more likely to respond to the treatment of **13** compared to FLT3-WT controls at the concentration range of 10 nM–1 μM (Fig 2a). In contrast to **13**, the responses of AraC, a standard chemotherapeutic drug, were similar at the concentration range of 10 nM–100 nM against two cell lines (Fig 2b).

The most potent FLT3 kinase and AML cell growth inhibitor, **13** was further examined on the cell cycle distribution[19] against MV4;11 cell line (Fig 3). As a result of cell cycle analysis, treatment of **13** induced cell cycle arrest at G_0/G_1 phase. Cell population at G_2/M phase decreased from 13% (control) to 1% (1 μM), and cells at S phase were also reduced from 19% (control) to 3% (1 μM). Apoptotic cell death was detected at concentrations starting at 0.1 μM as evaluated by quantifying sub-G_1 DNA content. Cells distributed at sub-G_1 phase increased from 10% (control) to 38% (1 μM), indicating cell death by compound **13**. Recently, a study of combination effect of FLT3 inhibitors with standard AML chemotherapeutic agents (AraC or daunorubicin) was reported to provide synergistic activity with the importance of

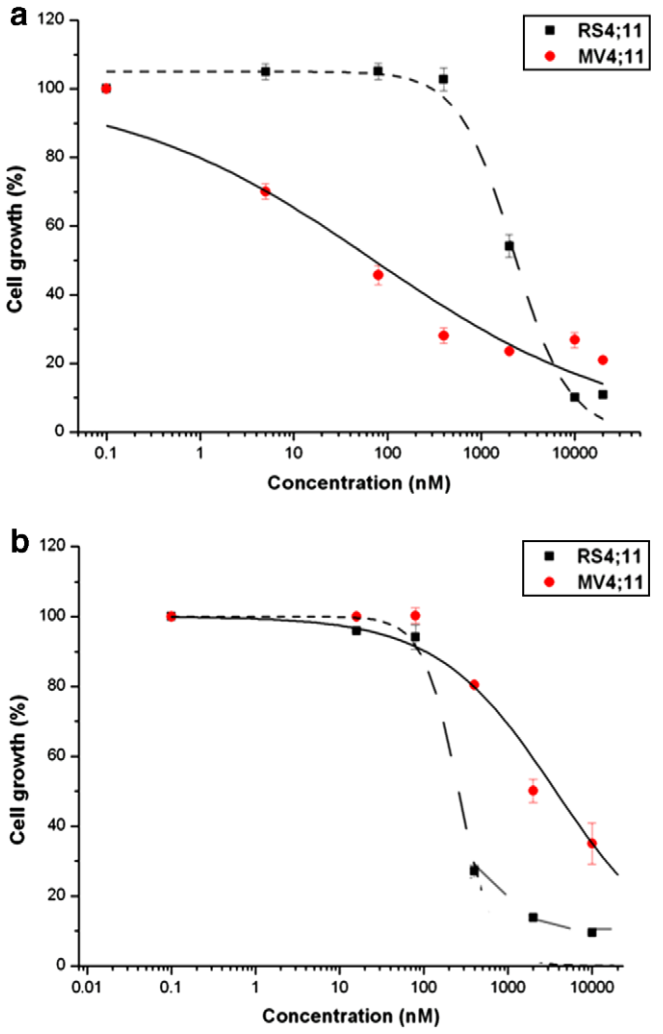


Figure 2. (a) Anti-proliferative activities of compound **13** against MV4;11 and RS4;11 cell lines. (b) Anti-proliferative activities of AraC against MV4;11 and RS4;11 cell lines.

administration sequence of two drugs because of the cell cycle dependent effect of each drug.[17] Therefore, compound **13** could be used in combination therapy with chemotherapeutic agent by applying its characteristics of cell cycle arrest induction at G_0/G_1 phase to obtain synergistic effect.

In order to determine kinase selectivity, compound **13** was tested against a diverse panel of receptor tyrosine kinases comprising of VEGFR2, Met, Ron, EGFR, and insulin receptor and a serine/threonine kinase, Aurora A (Table 2). Compound **13** turned out to be a selective inhibitor for FLT3 kinase, displaying negligible inhibitory activities against selected kinase panel. Although compound **13** showed some degree of inhibitory activities against VEGFR2 (IC_{50} = 1.53 μM) and Aurora A (IC_{50} = 1.27 μM), the activity was 100-fold less potent compared with FLT3 inhibitory activity (IC_{50} = 15 nM).

In summary, we have demonstrated indirubin derivatives as potent inhibitors of FLT3 kinase. SAR studies showed that it was preferable to restrict the size of substituted groups at 5 and 7 positions for the inhibitory activity. Compound **13**, 5-fluoro substituted indirubin oxime analog, displayed potent and selective FLT3 kinase inhibitory activities versus 6 other kinases and selective anti-proliferative activities against MV4;11 cell line having FLT3/ITD mutation, with weak inhibition of FLT3-WT-expressing cells, RS4;11.

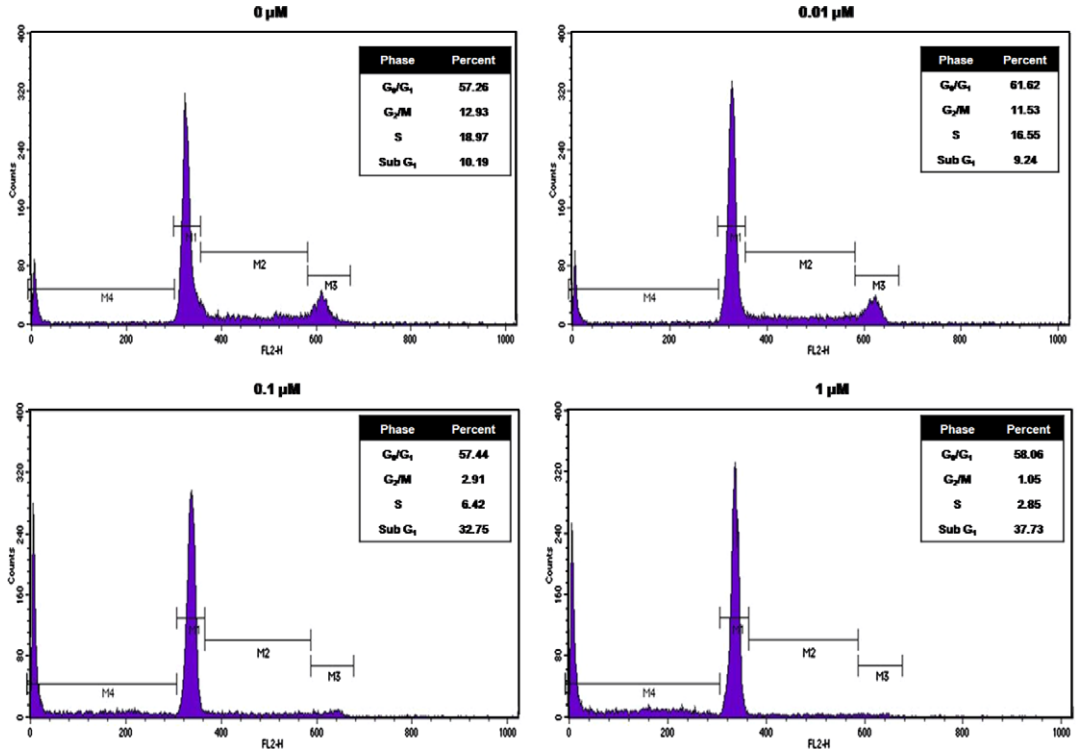


Figure 3. Effect of compound **13** on the cell cycle of MV4;11 cells. Cells were treated with **13** at the indicated concentration for 48 h and stained with propidium iodide for cell cycle analysis. M1: G_0/G_1 phase, M2: S phase, M3: G_2/M phase and M4: sub-G_1 phase. The charts show a representative experiment ($n = 3$).

Table 2
In vitro activity of compound **13** against select kinase

Kinase	IC_{50} (μM)
VEGFR2[a]	1.53
Met	>10
Ron	>10
EGFR[b]	>10
Insulin Receptor	>10
Aurora A	1.27

[a] Vascular endothelial growth factor receptor 2.
[b] Epithelial growth factor receptor.

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13. *In vitro kinase assays*. Inhibition of kinase activity against a variety of recombinant kinases was measured using homogeneous time-resolved fluorescence (HTRF) assays. Briefly, assays are based on the phosphorylation of peptide substrates in the presence of ATP. Resulting phosphorylated substrates are detected by TR-FRET (Time Resolved-Fluorescence Resonance Energy Transfer) signal. Recombinant proteins containing kinase domain were purchased from Millipore (Billerica, MA). Optimal enzyme, ATP, and substrate concentrations were established for each enzyme using HTRF KinEASE kit (Cisbio) according to the manufacturer's instruction. Assays are consisted of enzymes mixed with serially diluted compounds and peptide substrates in 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, TR-FRET signal was measured using EnVision multi-label reader (Perkin Elmer, Waltham, MA). IC_{50} was calculated by nonlinear regression using Prism version 5.01 (GraphPad).
14. *Cytotoxicity assay protocol*. Cells were plated in 96-well plates (10,000 cells per well) and serial dilutions of compounds were added. At the end of the incubation period (72 h), cell viability was measured by tetrazolium dye assay using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] (Dojindo, Japan). IC_{50} was calculated by nonlinear regression using Origin version 7.5.
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19. *Cell cycle analysis protocol*. MV4;11 cell 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 (Sigma) and subjected to flow cytometry using FACSCalibur™ (BD Biosciences). Data were analyzed by CellQuest Pro (BD Biosciences).