

Discovery of oxindole-based FLT3 inhibitors as a promising therapeutic lead for acute myeloid leukemia carrying the oncogenic ITD mutation

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Abstract

FMS-like tyrosine kinase 3 (FLT3) mutations occur in approximately 30% of acute myeloid leukemia (AML) patients. In the current study, the oxindole chemotype is employed as a structural motif for the design of new FLT3 inhibitors as potential hits for AML irradiation. Cell-based screening was performed with 18 oxindole derivatives and **5a–c** inhibited 68%–73% and 83%–91% of internal tandem duplication (ITD)-mutated MV4-11 cell growth for 48- and 72-h treatments while only 0%–2% and 27%–39% in wild-type THP-1 cells. The most potent compound **5a** inhibited MV4-11 cells with IC₅₀ of 4.3 μM at 72 h while it was 8.7 μM in THP-1 cells, thus showing two-fold selective inhibition against the oncogenic ITD mutation. The ability of **5a** to modulate cell death was examined. High-throughput protein profiling revealed low levels of the growth factors IGFBP-2 and -4 with the blockage of various apoptotic inhibitors such as Survivin. p21 with cellular stress mechanisms was characterized by increased expression of HSP proteins along with TNF-β. Mechanistically, compounds **5a** and **5b** inhibited FLT3 kinase with IC₅₀ values of 2.49 and 1.45 μM, respectively. Theoretical docking studies supported

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the compounds' ability to bind to the FLT3 ATP binding site with the formation of highly stable complexes as evidenced by molecular dynamics simulations. The designed compounds also provide suitable drug candidates with no violation of drug likeability rules.

KEYWORDS

anticancer, FLT3, ITD, molecular modelling, oxindoles

1 | INTRODUCTION

Cancer is one of the major challenges globally for the scientific community. It is a group of highly complicated diseases and is the main clinical burden on the general population. It has become the main focus of research on both pathological understanding and contemporary treatment strategies.^[1–4] Acute myeloid leukemia (AML) is a type of cancer with proliferative mutations and defects in myeloid differentiation. It is the most common malignant disorder of progenitor cells in myeloid hematopoiesis.^[5] The subjects suffering from AML demonstrate a heterogeneous response to therapies. The present care method is the application of intense chemotherapy initially followed by posttreatment remission consisting of additional chemotherapy cycles or stem cell transplantation.^[5] There is a rapid proliferation of immature leukemia cells in AML and the presence of aggressive malignancy.^[6,7] In most patients, it's reported that this disease consists of malignant cells in bone marrow, extramedullary and peripheral blood.^[8] Specific genetic defects characterize the acute promyelocytic leukemia variant of AML in the leukemic

cells and severe coagulopathy, and the treatment options also differ from other variants of AML.^[7,9,10] The intensive treatment option is the only way to treat AML, and allogeneic or autologous stem cell transplantation can be part of this intensive treatment.^[11] The most common survival pathway, PI3K-Akt-mTOR, is highly upregulated in AML, and responsible for metabolic reprogramming. For example, constitutively active mutant Akt demonstrates increased glycolysis.^[12] There are reports in AML due to oncogenic amplification and mutations that lead to dysregulation of the PI3K-Akt-mTOR axis, causing inactivation of tumor suppressor genes and upstream activation of receptor tyrosine kinases (RTKs).

The dysregulated PI3K-Akt-mTOR axis results in malignant transformation and uncontrolled proliferation in AML.^[13,14] The signaling via FLT3 in AML is one of the most common causes responsible for dysregulation of the PI3K-Akt-mTOR axis because the oncogenic mutations in the FLT3 gene lead to abnormal activation of the PI3K-Akt-mTOR axis.^[15,16] In AML, the most frequent mutations have been observed in the FLT3 gene.^[17] The ITD or the TKD are the common mutations in AML.^[18] The juxta-membrane domain of the RTK is the site where FLT3-ITDs are

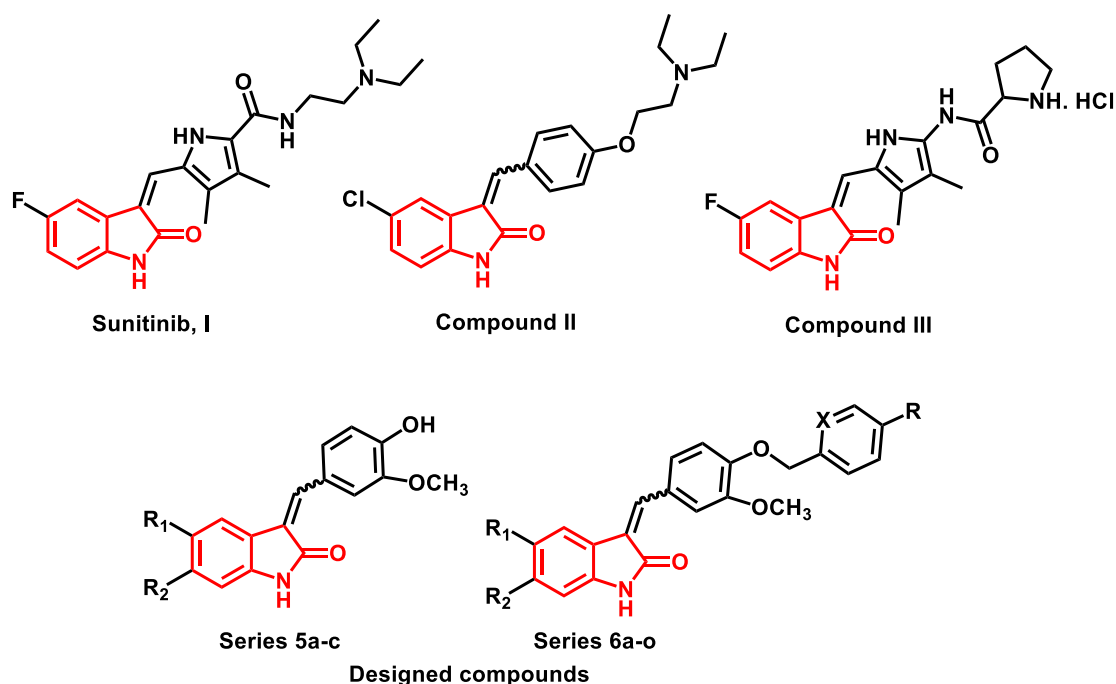


FIGURE 1 Structure of the kinase inhibitor sunitinib, the reported oxindole FMS-like tyrosine kinase 3 (FLT3) inhibitors II and III, and the designed compounds 5a–c and 6a–o.

located, and they affect several processes within the activation loop of TKD. The other mutations are effectively isolated, leading to the single amino acid substitution in the loop.^[18]

Oxindoles constitute a class of endogenous heteroaromatic agents existing in mammals' tissues and body fluids. They are also present in plants as natural products.^[19] There are several reports where oxindoles have demonstrated anticancer activities.^[20] Oxindoles, as a core structure of the multikinase inhibitor sunitinib **I** (Figure 1), were also used for the design of several kinase inhibitors, including FLT-3,^[21,22] where the ring could occupy the hinge region of the FLT3 kinase enzyme.^[23] For example, compounds **II** and **III** (Figure 1) were developed as FLT3 inhibitors with compound **II** inducing 100% inhibition of FLT3 in in vitro settings. Compound **III** demonstrated high selectivity towards FLT3 among other kinases with high potency as IC₅₀ 5.3 nM.^[22] Compounds **5** and **6** were designed to carry the oxindole nuclei of sunitinib along with the long chain a side carrying multiple hydrogen bond acceptors that might aid in binding to the ATP binding site of the FLT3 enzyme.

So herein, synthesis of the designed compounds **5a–c** and **6a–o** was performed, followed by comprehensive analyses for the synthesized oxindole compounds against FLT3 expressed AML cells (THP-1 and MV4-11) to investigate their potential use as candidates for designing effective and potent anti-AML agents. We explored the oxindole compound-mediated apoptotic, molecular docking, molecular dynamics, and computational ADME profiles against FLT3 mutated AML.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The route for synthesizing target compounds **5a–c** and **6a–o** is outlined in Scheme 1. Alkylated vanillins **3a–e** were achieved via S_N2 substitution reaction on various alkyl halides **1a–e** using vanillin's nucleophilic hydroxyl group. Vanillin or alkylated vanillin derivatives were integrated into the oxindole ring via a condensation reaction yielding compounds **5a–c** and **6a–o**, respectively. NMR data supported the success of the used route. ¹H NMR of all target compounds showed a characteristic singlet signal at ~7.6 parts per million (ppm) for the ethylenic proton. An additional singlet

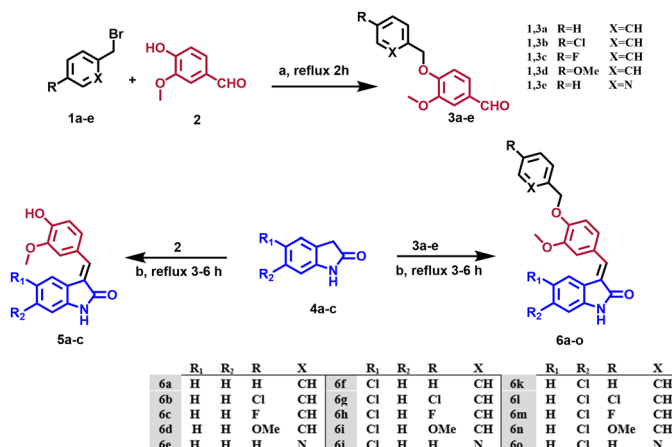
is observed at ~5.1–5.2 ppm for the CH₂ protons found in compounds **6a–o**.

Similarly, ¹³C NMR confirmed the formation of the target compounds showing characteristic ethylenic carbons at ~140 ppm and the aliphatic carbon at ~69 ppm. The purity of the compounds reported was evaluated using HPLC. Compounds were mixtures of *E* and *Z* isomers (see Supporting Information: S1). No separation was made since previous literature reported that the *E* isomer is mainly the major isomer^[24–26] and acknowledged the interconversion between the two isomers in methanol within 2 days.^[21,27]

2.2 | Pharmacology/biology

2.2.1 | One-dose screening against FLT3 ITD-mutated MV4-11 and FLT3 WT THP-1 acute myeloid leukemia cells

Research studies have synthesized diverse compounds like oxindole ring core molecules, which are among the popular candidates against cancer.^[19,28,29] Several groups have screened the anticancer potential of oxindole derivatives.^[19,20] The synthesized oxindole compounds carrying a vanillin functionality were tested on cell lines (MV4-11/THP-1) expressing FLT3 with different mutational characteristics. THP-1 cell line was chosen which represents the model that expresses only FLT3 and does not contain any variation. Quentmeier et al.^[30] characterized the acute myeloid leukemia cell lines for FLT3 mutations. Of the 69 AML-derived cell lines ITD-mutated MV4-11 cells contained mutated alleles and constitutively phosphorylated receptor protein. It also had the highest level of FLT3 expression. For these reasons, we chose MV4-11 cells, believing that the potential efficacy of the compounds in this biology would provide the most valuable output. For this purpose, MV4-11 or THP-1 cells were treated with 10 μM compounds and then incubated for 48 or 72 h before cellular inhibition measurements were performed. After 48 h of treatments, the highest inhibition was observed with compound **6a** with 20% in FLT WT THP-1 cells (Figure 2c), while various and high



SCHEME 1 Synthesis of the target oxindole derivatives **5a–c** and **6a–o**. Reagents: (a) K₂CO₃, acetonitrile; (b) EtOH, piperidine

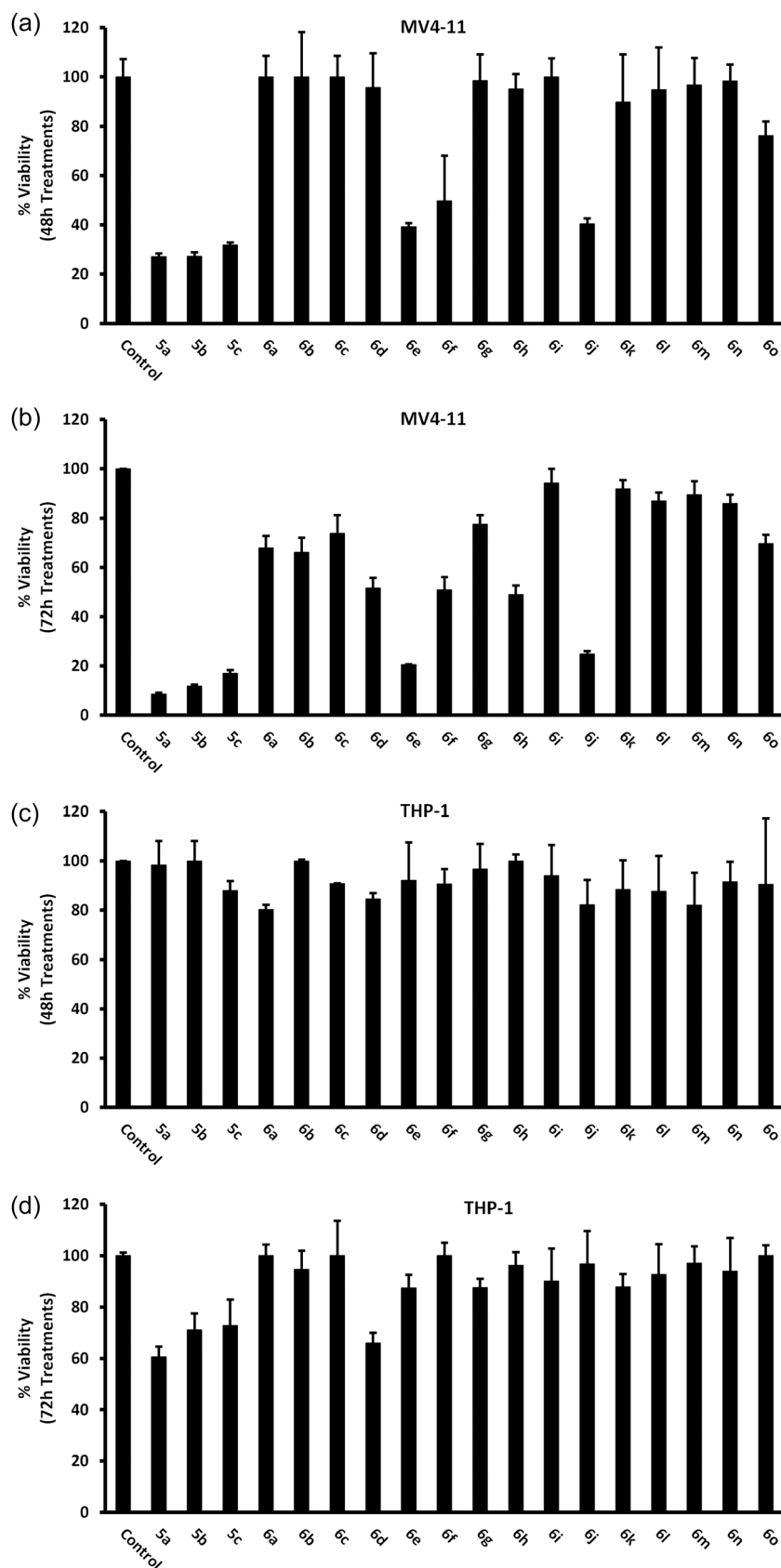


FIGURE 2 Ten micromolar single dose screening against MV4-11 and THP-1 cells for 48 or 72 h. (a) MV4-11 cells treated with compounds for 48 h; (b) MV4-11 cells treated with compounds for 72 h; (c) THP-1 cells treated with compounds for 48 h; (d) THP-1 cells treated with compounds for 72 h.

levels of inhibition were observed in FLT3 ITD-mutated MV4-11 cells (Figure 2a). Compounds **5a** and **5b** with 73% and **5c** with 68% were the most active ones. Compounds **6e**, **6f**, and **6j** followed with 61%, 60%, and 50% respectively. In 72-h treatments, the most active compounds on THP-1 cells were determined as **5a** (39% inhibition), **6d** (34% inhibition), and **5b** (29% inhibition) (Figure 2d). All remaining compounds had inactive on THP-1 cells. On the other hand, remarkable results were seen with MV4-11 cells with **5a** showing the highest inhibition activity at 91%, while **5b** (88%) and **5c** (83%) (Figure 2b). Compounds **6e** and **6j** also had 79% and 75% levels. Compounds other than these were considered ineffective (viability >50%). Considering all these data, it was determined that these oxindole compounds were more effective on MV4-11 cells than THP-1 cells, thus showing more selective inhibition against ITD mutation. Of the 18 compounds, the three most potent compounds in both cell lines were **5a**–**c**, and the remaining compounds were excluded from the study set. Inhibition values of these three potent compounds at different time periods in both cell lines are summarized in Table 1. Compound **5a** was selected as the most potent compound for further in vitro analyses, while compounds **5a**–**c** were used for in silico analyses.

2.2.2 | Time and dose-dependent effects of compound **5a** on MV4-11 and THP-1 cells

Compound **5a**, being the most potent, was selected for dose-dependent cytotoxic activities using the WST-1 cell proliferation assay system. MV4-11 and THP-1 cells were treated with serial dilutions of compound **5a** from 0.625 to 50 μ M for 48 or 72 h. As seen in Figure 3, no noticeable activity was observed on THP-1 cells during both incubation times except at the highest dose of 50 μ M (Figure 3c,d). On the other hand, a dose-dependent increase in activity was observed on MV4-11 cell lines (Figure 3a,b). While 0.25

TABLE 1 Cell growth inhibition ratios of potent compounds against MV4-11 and THP-1 cells for 48 or 72 h at a 10 μ M single dose

Compound name	MV4-11 cells (ITD+)		THP-1 cells (Wild type)	
	48 h	72 h	48 h	72 h
5a	73 $\pm$ 1.3%	91 $\pm$ 0.6%	2 $\pm$ 9.7%	39 $\pm$ 3.9%
5b	73 $\pm$ 1.6%	88 $\pm$ 0.5%	0 $\pm$ 8.1%	29 $\pm$ 6.3%
5c	68 $\pm$ 1.0%	83 $\pm$ 1.3%	2 $\pm$ 3.8%	27 $\pm$ 10%

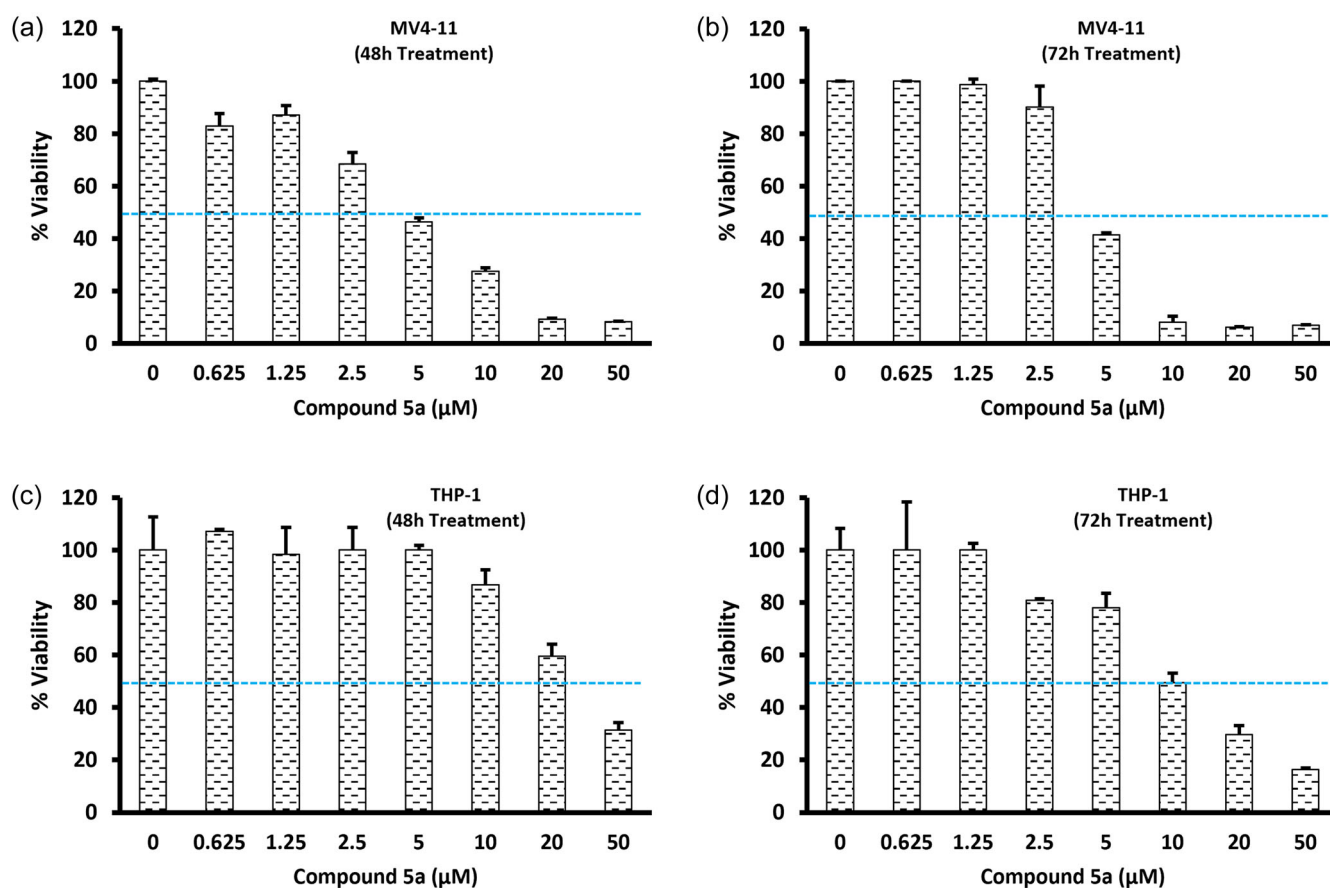


FIGURE 3 The influence of compound **5a** at different concentrations on FMS-like tyrosine kinase 3 (FLT3) internal tandem duplication (ITD)-mutated MV4-11 and FLT3 WT THP-1 acute myeloid leukemia cells viability. (a) MV4-11 cells, 48 h treatment; (b) MV4-11 cells, 72 h treatment; (c) THP-1 cells, 48 h treatment; (d) THP-1 cells, 72 h treatment.

and 2.5 μM doses did not show an inhibition effect of 50% or less on MV4-11 cells, as seen from the turquoise lines, a 10 μM treatment of compound **5a** decreased the viability of MV4-11 cells by 72% at 48 h, while this rate was 92% at 72 h. No viability was observed in MV4-11 cells after 20 and 50 μM dose treatments, both 48 and 72 h, with inhibition values of 91% and 94%, respectively (Figure 3a,b). IC_{50} calculations (Table 2) confirmed the data obtained with compound **5a** inhibits MV4-11 cells with 4.5 μM while IC_{50} was 17 μM for THP-1 cells. The results obtained went in accordance with those obtained with single-dose screening. Taken together, compound **5a** showed a dose-dependent inhibition character in FLT3-expressing cells. Its superior efficacy on FLT3 ITD-mutated MV4-11 cells compared to FLT WT THP-1 cells at both 48 and 72 h reveals its selectivity against the mutational character. Therefore, further analyzes were performed on MV4-11 cells based on a potential agent specific to the ITD mutation. Additionally, the results were consistent with similar studies by Song et al. claimed that indole-3-ylidene acetate analogs are highly antiproliferative drugs.^[31] Sharma et al. also investigated indolin-2-ones derivatives with eight members possessed significant cytotoxic activity against breast cancer cell lines,^[32] while Kumar et al. synthesized oxindole derivatives as antimitotic agents against lung cancer cell line

A549.^[33] Such results support that the antiproliferating and cytotoxic activities of the new compound **5a** make it a potential candidate for FLT3-mutated AML.

2.2.3 | Compound **5a** induced morphological changes in MV4-11 cells

Microscopic analysis was performed to record morphological changes induced to MV4-11 cell lines by compound **5a**. MV4-11 cells were photographed after 48 h of treatment at doses between 2.25 and 18 μM , which is around the IC_{50} values. 0.01% DMSO was used as a control standard in the whole study. In Figure 4, treating cells with 2.25 μM **5a**, cells continued their normal proliferation and had the same phenotype as the control cells. While there was a noticeable decrease in the amount of cells treated with an IC_{50} value (4.5 μM) of compound **5a**, no serious irritation was observed. It was observed that the membrane structures of the cells were preserved, and an antiproliferation state was observed due to the cessation of division rather than a cytotoxic effect (Figure 4, top right panel). After the IC_{100} dose (9 μM) of compound **5a** treatment, no cell debris or heterogeneous distribution was observed on a more general view (100X objective), as seen in Figure 4 bottom left panel. However, stress onset was observed in cells that had stopped dividing at a higher magnification (200X) of the square area in Figure 4. bottom middle panel. Also, no toxic death cell residue was found in the examined areas. Cell membrane structures were disrupted in cells treated with 18 μM compound **5a**, and cell remnants lost their structural integrity (Figure 4 bottom right panel). All these morphological findings show that the selective inhibition effect is up to 9 μM

TABLE 2 IC_{50} values of compound **5a** in two different cell lines for each time

Cell line (mutation status)	48 h (μM)	72 h (μM)
MV4-11 (FLT3 ITD+)	4.5 $\pm$ 0.4%	4.3 $\pm$ 0.6%
THP-1 (FLT3 WT)	17.9 $\pm$ 3.0%	8.7 $\pm$ 2.2%

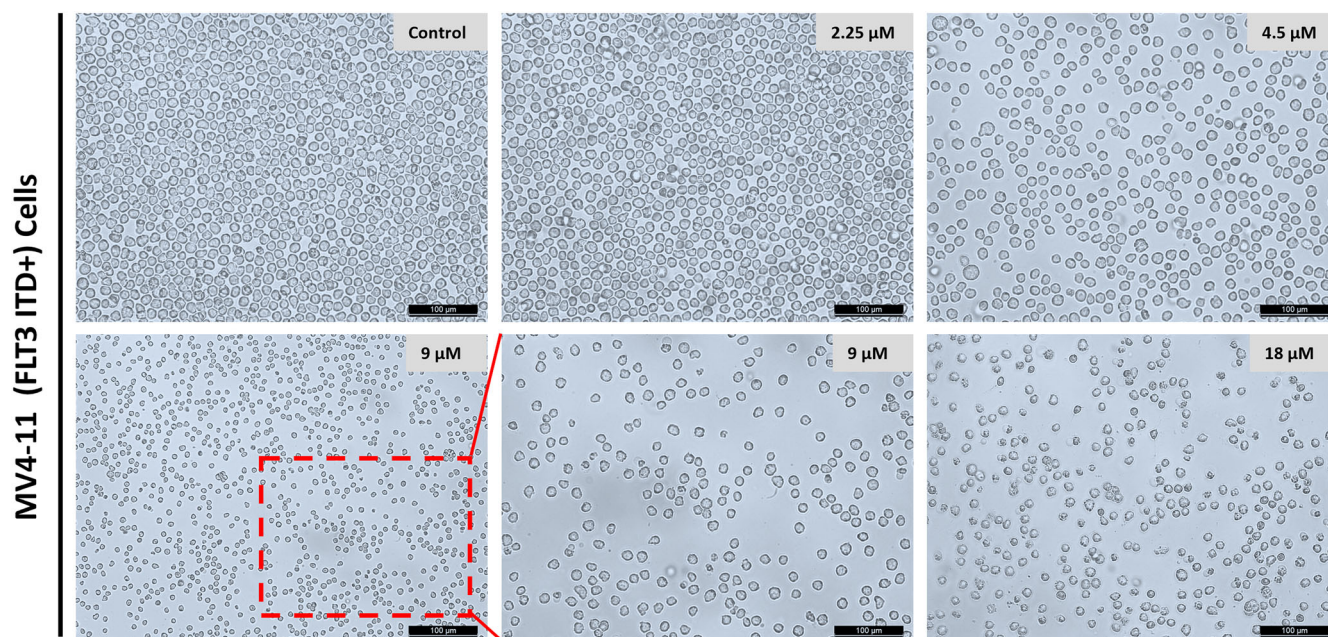


FIGURE 4 Alteration of cell morphology after treatments of compound **5a** on MV4-11 cells. Magnification: 200X; red square area magnification: 100X; scale bar: 100 μm .

dose. This selectivity at IC_{50} and IC_{100} values reflects the potential of compound **5a** to be an FLT3-ITD+ agent. The dose-dependent cytotoxicity of oxindole derivatives was also carried by Jia et al., who found that 3-amino-3-hydroxymethyl oxindole derivatives demonstrated significant G2/M cell cycle arrest, time and dose-dependent cellular apoptosis in SJSA-1 cells.^[34]

2.2.4 | High-throughput protein profiling against cell death, survival, and resistance targets

The quantitative real-time RT-PCR data gave us preliminary insight into a possible mechanism of apoptotic resistance and cell death on MV4-11 cells after the treatment of compound **5a**. To elucidate this mechanism in-depth, an analysis of high-throughput protein targets, including cell death, survival, and resistance, was performed. Human Apoptosis Antibody Array was used, which allows simultaneous processing and analysis of 43 different targets, including all system checks. In this dot-based system, target antibodies are attached to ready-to-use membranes and are based on the principle of starting with protein lysate treatment. In this study, MV4-11 cells were treated with 9 μ M compound **5a** for 48 h, while cells cultured with a medium containing 0.01% DMSO were used as an untreated control. Chemiluminescence images of the membranes were obtained after a strict follow-up of kit procedures (Figure 5a). Endogenous protein expression levels were determined in cells expressing FLT3 and having ITD mutation with the control membrane (Figure 5a, left panel) in this study. Varying expressions of 42 of 43 proteins were detected, and some were highly expressed compared to others. CD40, p21, SMAC, Survivin, sTNF-R1, and XIAP protein expressions were high, mirroring the aggressiveness of the disease. When the normalized protein expressions of control cells and compound **5a**-treated cells were compared, it was determined that 12 of the 43 targets changed significantly. When the Bcl-2 family was examined, only a significant increase in proapoptotic Bad expression was observed. Contrary to the decrease in mRNA level, there was no significant change in protein regulation in Bax. A nonsignificant increase was observed in the caspase-3 level, which was consistent with the mRNA data. The conclusion drawn from this data set was that although the mitochondrial pathway was desired to be affected by a BH3-only proapoptotic mechanism-mediated system, the pro-survival guardians-mediated apoptotic machine was more actively engaged and balanced. As a result, no intrinsic death mechanism emerged. This was also confirmed by the unchanged cytoC level. On the other hand, when focusing on cell death receptors, Fas and DR6 levels increased but did not change significantly. This was also confirmed by the levels of caspase-3 and caspase-8. Thus, it was found that the death receptor-mediated mechanism was not affected by the compound **5a** treatment. Significant increases were observed in three different heat-shock proteins, HSP27, HSP60, and HSP70 indicating stress is being induced in the cells, which can be another reason for undergoing apoptosis.^[35,36] In addition, they caused the inhibition of the mitochondrial pathway-mediated apoptosis. Another significantly increased target was the serine protease HTRA. This target, released from mitochondria to the

cytosol, contributes to apoptosis by degrading various apoptosis inhibitors.^[36] The HTRA level increased by the treatment of compound **5a** prevented the activation of inhibitors such as cIAP-2 and XIAP. It also contributed to caspase activation in maintaining the balance observed in this study. When it comes to the IGF family, the most striking result was the significantly reduced level of IGFBP-2. As widely documented in the literature,^[37] IGFBP-2 has roles in cell proliferation and migration, whether dependent or independent of IGF. IGFBP-2 contributes to cell survival by affecting downstream cell signaling pathways. The dramatic reduction of IGFBP-2 level by compound **5a** appears to be a unique effect in this disease. Another data supporting this situation is a similar decrease in IGFBP-4, the paralog of IGFBP-2. In summary, we can say that compound **5a** significantly reduces IGFBP-2 and 4 levels and affects cell proliferation. Another change contributing to this mechanism of action is Survivin, whose expression was significantly reduced after treatment with compound **5a**. The decrease in the level of this endogenously high expressed apoptosis inhibitor contributed which is another supportive finding in inducing cell growth inhibition in the AML cells.^[38] Other contributors were found as p27 and TNF- β . The significant increase in these targets, which are known for their roles in cell cycle inhibition and apoptosis induction, played a critical role in breaking the total resistance.^[39,40] In the TRAIL-R series, however, no significant change was observed, thus confirming that there was no mechanism mediated by death receptors. From these results, we can say that compound **5a** is inducing cell death via multiple mechanisms and is following Bad, HSP27/HSP60/HSP70, HTRA, IGFBP-2/4, Survivin/p27 and TNF- β signaling pathways, with significant changes in the key protein levels decipher the involvement of cell death pathways. Considering high FLT3 expression and ITD-based internal growth signaling of MV4-11, we think that compound **5a** provides this effect, especially with inhibition of IGFBP-2 and Survivin. At the same time, significant increases in sets of heat shock proteins also presented a unique value indicating cell stress induced by **5a**. However, while these changes in cell death signaling pathways are very promising, alterations in downstream targets linked to FLT3 kinase domains are also very important. Activation of STAT5 on the Kinase 1 domain and the Ras/Raf/MEK/ERK cascade from the Kinase II domain should also be investigated for the effects on cell growth and metabolic activity of compound **5a**.

2.2.5 | Relationship between FLT3 kinase and potent compounds

The inhibition effect of potent compounds on the FLT3 kinase enzyme was measured with the ADP-Glo™, a luminescent kinase assay. For this purpose, **5a–c** compounds were studied by titration against 100 ng FLT3 kinase and 100 μ M ATP up to a concentration of 5 μ M with twofold serial dilutions starting at 1.22 nM. In Figure 6, the capacities of all three compounds to inhibit FLT3 kinase are given at doses ranging from 1.22 nM to 5 μ M. No significant inhibition was observed up to 156 nM compound doses. Regularly increasing FLT3 kinase inhibition was observed for

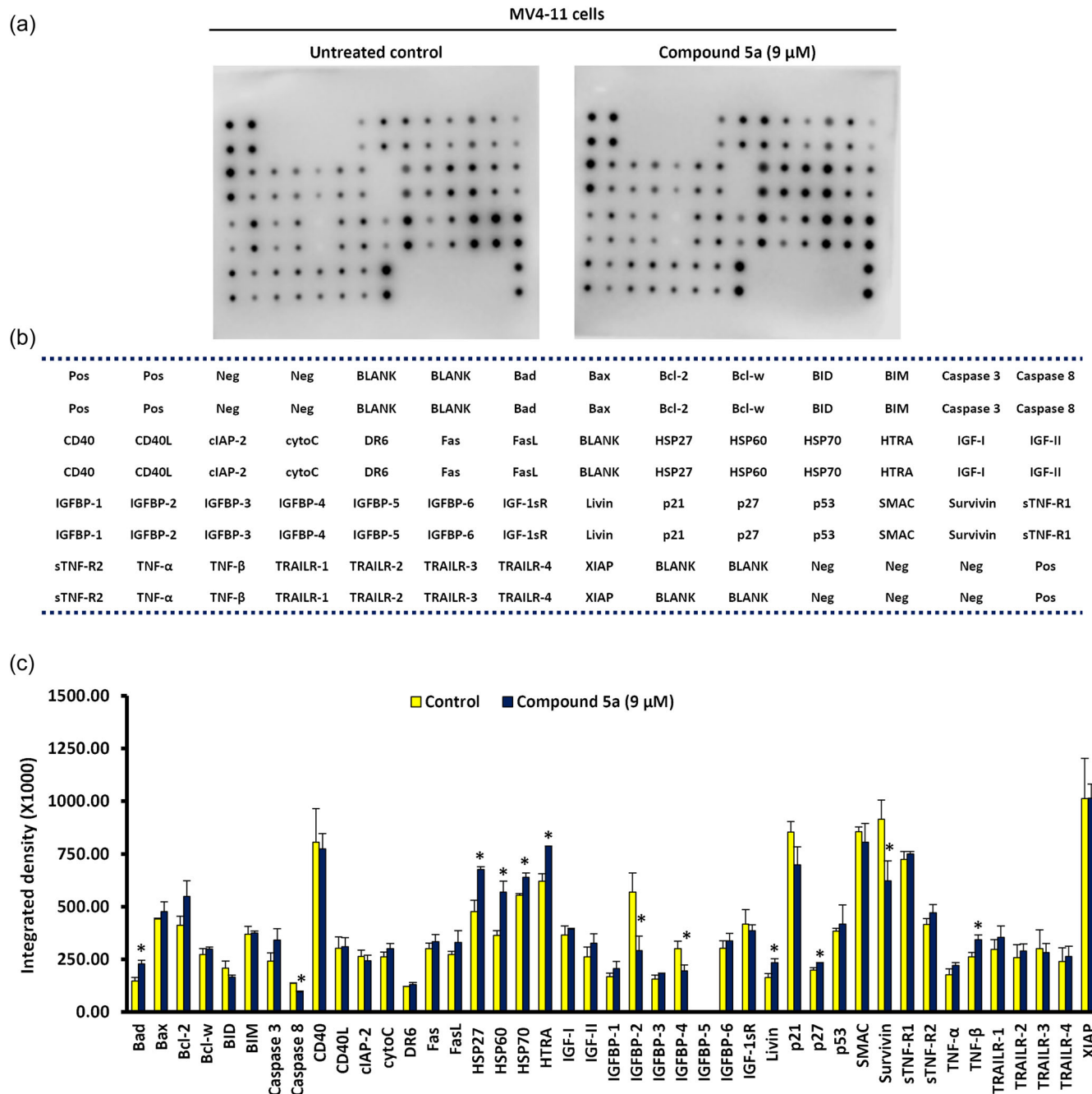


FIGURE 5 High-throughput protein profiling against cell death, survival, and resistance markers. MV4-11 cells were treated with 9 μ M compound **5a** or %0.01 DMSO as an untreated control for 48 h. (a) Chemiluminescence images of protein targets. The protein expression levels of cells treated with **5a** in the right panel, while the left panel demonstrates the untreated control. (b) Array reference map. Pos means positive control spots which use relative quantification. Neg means negative control spots contain antibody dilutions buffer, and blank means empty spots. The other spots contain target proteins. (c) Quantification of target proteins. * $p < 0.05$.

compounds **5a** and **5b** with IC₅₀s of 2.49 and 1.45 μ M respectively. At the highest dose examined of 5 μ M, **5a** showed 67% inhibition, and **5b** showed 74% inhibition. Nevertheless, **5c** did not show noticeable inhibition with only 27% inhibition observed at 2.5 μ M. With these data, it was determined that the three compounds which showed the best effect in AML cells expressing FLT3 inhibited the FLT3 kinase enzyme in vitro.

2.3 | Molecular modeling

2.3.1 | Molecular docking analysis

The FLT3 kinase is constitutively activated in AML due to mutations. The activation loop and mutations in the FLT3 domain can be predicted to result in the loss of regulatory functionality. The

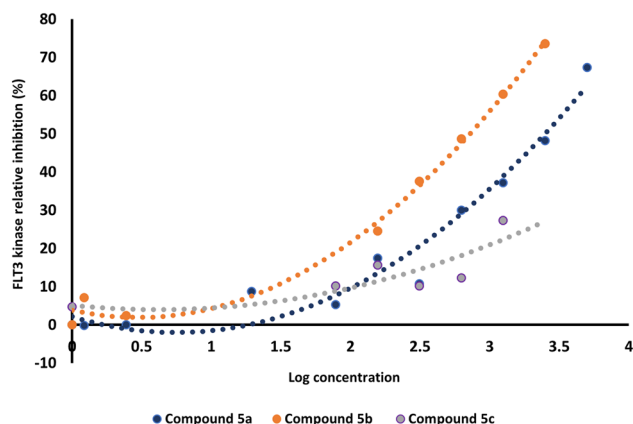


FIGURE 6 FMS-like tyrosine kinase 3 (FLT3) kinase inhibition activity against compounds **5a–c** (1.22–5000 nM) in the presence of 100 ng FLT3 kinase and 100 μ M ATP using ADP-Glo assay.

activation of FLT3 kinase leads to downstream survival signaling pathways like ERK/MEK/MAP-Kinase- and Akt/PI3k/mTOR survival signaling.^[8] To better understand the molecular bases of potent oxindole compounds' bioactivity as FLT3 inhibitors, *in silico* docking experiments were carried out. Our experiments generally demonstrated that the *cis* compounds have shown a better docking score compared to the *trans* analogs, also the ΔG -binding energy is better for the *cis*-analogues having a more efficacious positioning in the enzymatic pocket on FLT3 (Table 3); moreover, among the three selected compounds the docking score of compounds *cis*-**5b** and *cis*-**5c** were the highest and the free binding energy also follow this path. The best poses of each *cis* compound generated by Glide are depicted in Figure 7. The key interactions observed in the crystal complex FLT3 were well represented in the docking pose of *cis*-**5a**, -**5b**, and -**5c** (Figure 7b–d) with two hydrogen bonds respectively to Glu692 and Cys694, which accommodate the amide moiety of the β -lactam. The two aromatic moieties are accommodated in the enzymatic pocket similarly to the crystallographic ligand. Indeed, the compounds of the oxindole series are able to occupy efficiently the enzymatic pocket occupied by the piranyl-aminopyrazine-carboxamide portion of the crystallographic ligand, as depicted in Figure 7a and Supporting Information: Figure S1b (superimposing with *cis*-**5b**).

2.3.2 | Molecular dynamics (MD) simulations

MD simulations are used to predict the movements of the protein-ligand complex at the atomic level. In this section, the stability and movements of FLT3 docked protein-ligand complexes of compounds **5a–c** were analyzed for 300 ns. To validate the MD simulation, MD was performed on the cocrystal ligand gilteritinib under the same conditions. root mean square deviation (RMSD) analyses were performed to analyze the shifts of compound **5a–c** complexes with FLT3, and root mean square fluctuation (RMSF) to analyze their fluctuations. A stable RMSD value

indicates that the system has reached stability, and analysis can be performed. As given in Figure 8a, the gilteritinib, **5a**, and **5b** complex of FLT3 remained stable around 0.35 nm after the peak at 50 nm, while the FLT3-**5c** complex remained stable below 0.5 nm. RMSF measurements are another method that shows flexibility and conformation changes in protein structure. After the complex structure is formed by the ligand with the protein, the fluctuation of the active site residues should decrease and reach a more stable structure. As shown in Figure 8b, residues of Glu692 and Cys694 fluctuated around 0.1 nm at the site of H bond formation. The highest fluctuation was measured at loop residues between 765 and 775. While **5c** gave the highest fluctuation, gilteritinib, compounds **5a**, and **5b** produced lower fluctuations.

Time-dependent measurement of the binding-free energy MMPBSA between protein-ligand complexes is another important approach to evaluate stability. Binding free energy is obtained by averaging the sum of van der Waals, electrostatic, polar solvation, and SASA energies. For this purpose, MMPBSA was calculated from 250 frames between 250 and 300 ns. Compounds **5a–c**, cocrystal ligand gilteritinib, and FLT3 complexes were measured as -63.336 ± 12.153 , -61.832 ± 12.544 , -64.755 ± 11.981 , and -201.339 ± 17.859 kJ/mol, respectively. Compounds **5a–c** gave close MMPBSA values, while cocrystal gilteritinib produced a very high binding free energy. This difference may be due to the fact that gilteritinib interacts more with residues due to the length of its structure. Finally, the binding modes at 300 ns were analyzed to show the variation of the H bond and other interactions where compounds **5a–c** form residues of Glu692 and Cys694. Figure 9a–c shows the 3D binding pose and 2D protein-ligand interaction diagrams of compounds **5a–c**. After 300 ns, all three compounds remained fairly stable at the active site. As in the pose obtained from molecular docking, the H bond between NH of the indole ring and Glu692, and the H bonds between indol-2-one and Cys694 remained stable with compounds **5a** and **5c**. Compound **5b** retained its H bond with Cys694. In addition, the motions of compounds **5a–c**, and gilteritinib with FLT3 for 300 ns were given in

TABLE 3 Docking scores of the best pose of selected compounds, ΔG free binding energy calculated by MM-GBSA method.

Compound	Docking score	ΔG binding energy (kcal/mol)	Root mean square deviation (RMSD) (Å)
Gilteritinib	-11.5	-75.0	0.30**
<i>cis</i> - 5a	-11.6	-58.2	0.76*
<i>trans</i> - 5a	-11.6	-54.0	0.82*
<i>cis</i> - 5b	-11.9	-60.7	0.91*
<i>trans</i> - 5b	-11.2	-51.3	0.92*
<i>cis</i> - 5c	-11.9	-56.7	0.80*
<i>trans</i> - 5c	-11.4	-57.4	0.70*

*Calculated versus the crystallographic ligand.

**Calculated on self docking.

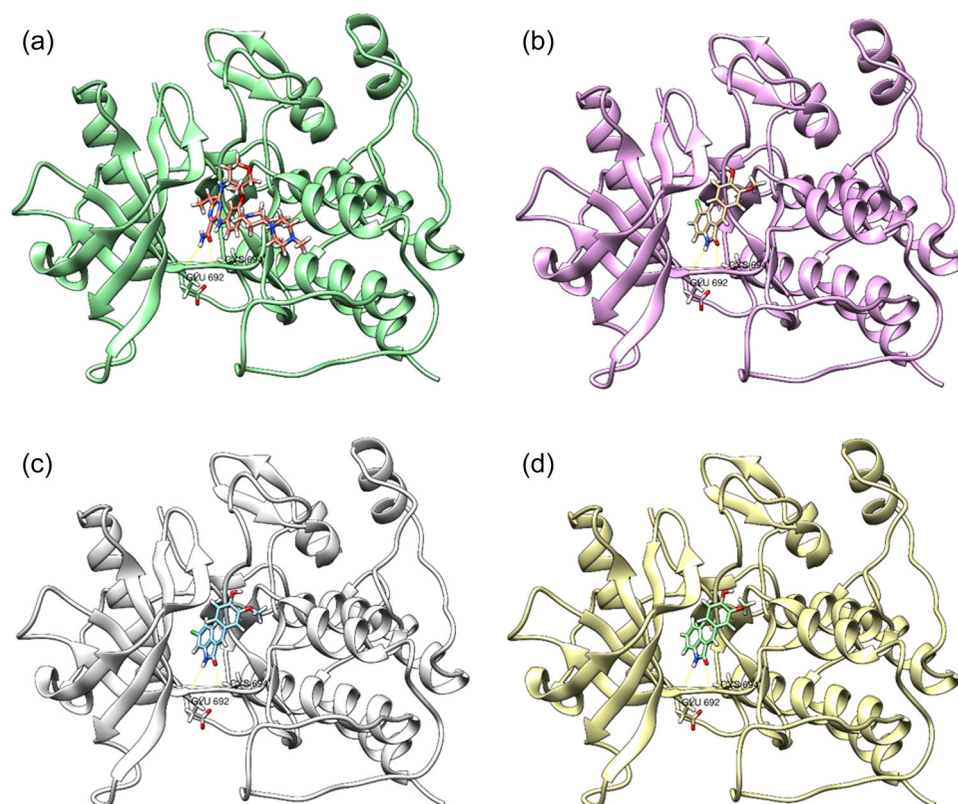


FIGURE 7 (a) Crystallographic inhibitor; (b) best pose of *cis*-**5b** docked to FMS-like tyrosine kinase 3 (FLT3); (c) best pose of *cis*-**5c** docked to FLT3; (d) best pose of *cis*-**5a** docked to FLT3.

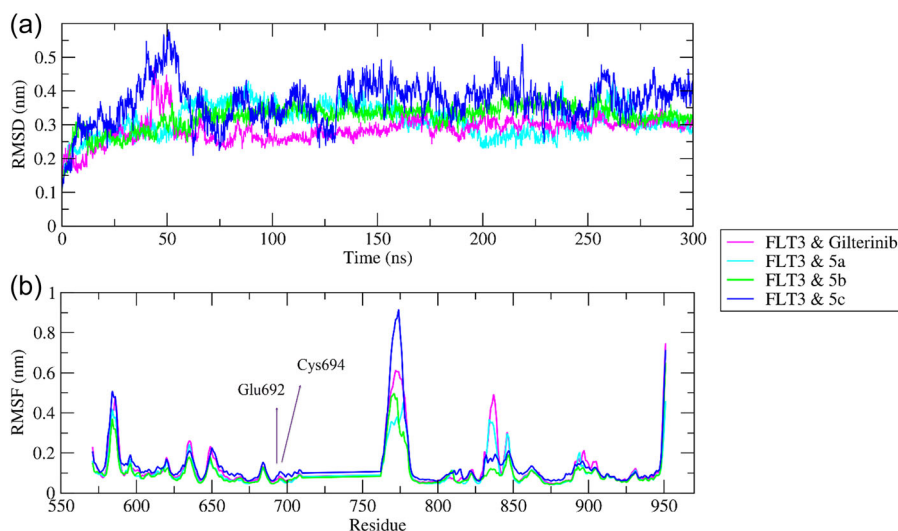


FIGURE 8 Molecular dynamics simulations trajectory analysis of compounds **5a–c**, cocrystal ligand gilteritinib, and FLT3 complexes (PDB ID: 6JQR). (a) Root mean square deviation (RMSD) and (b) root mean square fluctuation (RMSF) of protein-ligand complexes throughout 300 ns.

Supporting Information 3: Video 1–4. The compounds **5a–c** maintained their stable interactions.

2.3.3 | Computational ADMET analysis

Estimating ADMET properties in lead compound designs and designing for these calculations can enable the active compound to proceed

without problems in in vivo and clinical phase studies stages.^[41] For this purpose, the estimation of ADMET properties of compound active compounds **5a–c** was performed. Data obtained from SwissADME and pkCSM servers are given in Table 4. Colored zones of the bioavailability radar plot from SwissADME indicate suitability for oral bioavailability.^[42] There are no substituents on the indole ring for compounds **5a–c** are structural isomers of each other and carry the chlorine substituent at positions 5 and 6 of the indole ring. Thus,

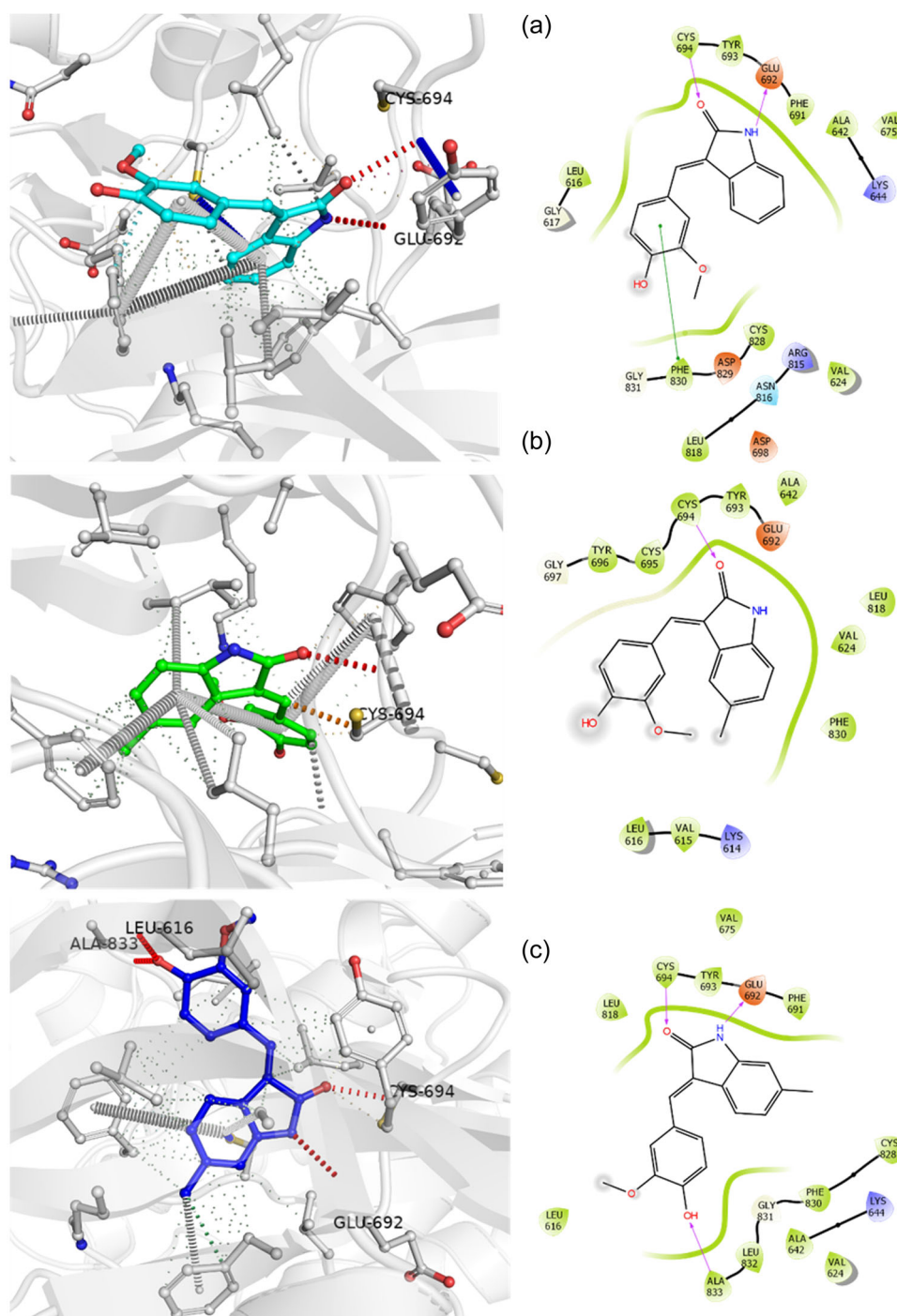


FIGURE 9 Binding poses (left) and schematic interactions diagrams (right) of (a) compound **5a**, (b) **5b**, and (c) **5c** end of the 300 ns molecular dynamics simulations

compounds **5b** and **5c** exhibit similar ADME properties. As given in Figure 10, compounds **5b** and **5c** have more lipophilic properties and higher insoluble properties due to the Cl atom they contain. The unsaturation feature of all three compounds is outside the colored zone. The fraction Csp3 value of compounds **5a–c** was calculated as 0.06. For saturation, the ratio of sp3 hybridized carbons over the total carbon count of the molecule (Fraction Csp3) should be at least 0.25. The solubility of the compounds in water is between soluble and

moderately soluble. It has high GI absorption, BBB permeant, and CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 inhibitory properties. It conforms to the limiting rules of Lipinski, Ghose, Veber, Egan, and Muegge. Like Stilbene, it has a Michael acceptor unit, lead likeness yes, and synthetic accessibility is in the easy class. According to the toxicity parameters, the compound with the best profile is **5c**, then **5a** and the highest estimated toxicity is **5b**. The compounds are in the class of non-HERG I inhibitors, nonhepatotoxicity, and nonskin

TABLE 4 Physicochemical properties, lipophilicity, water-solubility, pharmacokinetics, drug-likeness, medicinal chemistry, and toxicity parameters that were obtained from SwissADME and pkCSM server of compounds **5a**, **5b**, and **5c**

Physicochemical properties	5a	5b	5c
Formula	C ₁₆ H ₁₃ NO ₃	C ₁₆ H ₁₂ ClNO ₃	C ₁₆ H ₁₂ ClNO ₃
Molecular weight	267.28 g/mol	301.72 g/mol	301.72 g/mol
Num. heavy atoms	20	21	21
Num. arom. heavy atoms	12	12	12
Fraction Csp ³	0.06	0.06	0.06
Num. rotatable bonds	2	2	2
Num. H-bond acceptors	3	3	3
Num. H-bond donors	2	2	2
Molar refractivity	80.65	85.66	85.66
TPSA (topological polar surface area)	58.56 Å ²	58.56 Å ²	58.56 Å ²
<i>Lipophilicity</i>			
Log P _{o/w} (iLOGP)	2.44	2.62	2.67
Log P _{o/w} (XLOGP3)	2.51	3.14	3.14
Log P _{o/w} (WLOGP)	2.21	2.87	2.87
Log P _{o/w} (MLOGP)	1.85	2.36	2.36
Log P _{o/w} (SILICOS-IT)	2.97	3.60	3.60
Consensus Log P _{o/w}	2.40	2.92	2.93
<i>Water solubility</i>			
Log S (ESOL)	−3.39	−3.98	−3.98
Solubility	1.09e-01 mg/ml; 4.07e-04 mol/L	3.16e-02 mg/ml; 1.05e-04 mol/l	3.16e-02 mg/ml; 1.05e-04 mol/L
Class	Soluble	Soluble	Soluble
Log S (Ali)	−3.39	−4.04	−4.04
Solubility	1.10e-01 mg/ml; 4.12e-04 mol/l	2.76e-02 mg/ml; 9.13e-05 mol/L	2.76e-02 mg/ml; 9.13e-05 mol/l
Class	Soluble	Moderately soluble	Moderately soluble
Log S (SILICOS-IT)	−4.99	−5.59	−5.59
Solubility	2.74e-03 mg/ml; 1.02e-05 mol/L	7.70e-04 mg/ml; 2.55e-06 mol/L	7.70e-04 mg/ml; 2.55e-06 mol/L
Class	Moderately soluble	Moderately soluble	Moderately soluble
<i>Pharmacokinetics</i>			
GI absorption	High	High	High
BBB permeant	Yes	Yes	Yes
P-gp substrate	No	No	No
CYP1A2 inhibitor	Yes	Yes	Yes
CYP2C19 inhibitor	No	Yes	Yes
CYP2C9 inhibitor	No	Yes	Yes
CYP2D6 inhibitor	Yes	Yes	Yes
CYP3A4 inhibitor	Yes	Yes	Yes
Log K _p (skin permeation)	−6.15 cm/s	−5.91 cm/s	−5.91 cm/s

TABLE 4 (Continued)

Physicochemical properties	5a	5b	5c
<i>Druglikeness</i>			
Lipinski	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation
Ghose	Yes	Yes	Yes
Veber	Yes	Yes	Yes
Egan	Yes	Yes	Yes
Muegge	Yes	Yes	Yes
Bioavailability score	0.55	0.55	0.55
<i>Medicinal chemistry</i>			
PAINS	0 alert	0 alert	0 alert
Brenk	2 alerts: michael_acceptor_1, stilbene	2 alerts: michael_acceptor_1, stilbene	2 alerts: michael_acceptor_1, stilbene
Leadlikeness	Yes	Yes	Yes
Synthetic accessibility	2.43	2.47	2.49
<i>Toxicity</i>			
AMES toxicity (Yes/No)	Yes	No	Yes
Max. tolerated dose (human) (log mg/kg/day)	0.089	-0.074	0.123
hERG I inhibitor (Yes/No)	No	No	No
hERG II inhibitor (Yes/No)	Yes	Yes	Yes
Oral rat acute toxicity (LD50) (mol/kg)	2.148	2.186	2.003
Oral rat chronic toxicity (LOAEL) (log mg/kg_bw/day)	1.57	1.588	1.511
Hepatotoxicity (Yes/No)	No	No	No
Skin sensitisation (Yes/No)	No	No	No
<i>T. pyriformis</i> toxicity (log µg/L)	1.292	0.879	1.09
Minnow toxicity (log mM)	0.745	0.693	0.979

Abbreviation: TPSA, topological polar surface area.

sensitization. In contrast, compounds with AMES toxicity are the carcinogenic effect of chemicals (except compound **5b**), and hERG II is potassium ion channel inhibitory properties.

3 | CONCLUSION

Presently approximately 10 million new cancer cases are diagnosed every year.^[28] Cancer has remained a dreadful disease so far, despite the advancements in the medical field, which is difficult to diagnose and treat.

However, chemotherapy pays an effort to treat the milder cases and buys more time for cancer patients. New drugs against cancer are in full demand globally. Research studies are being consciously performed to find new promising candidates that can better treat cancer patients.

To contribute to these efforts, in this study, synthesis of the designed compounds **5a–c** and **6a–o** was performed, followed by comprehensive analyses for the synthesized oxindole compounds against FLT3 expressed AML cells to investigate their potential use as candidates for designing effective and potent anti-AML agents. In conclusion, the synthesized oxindole compounds tested against

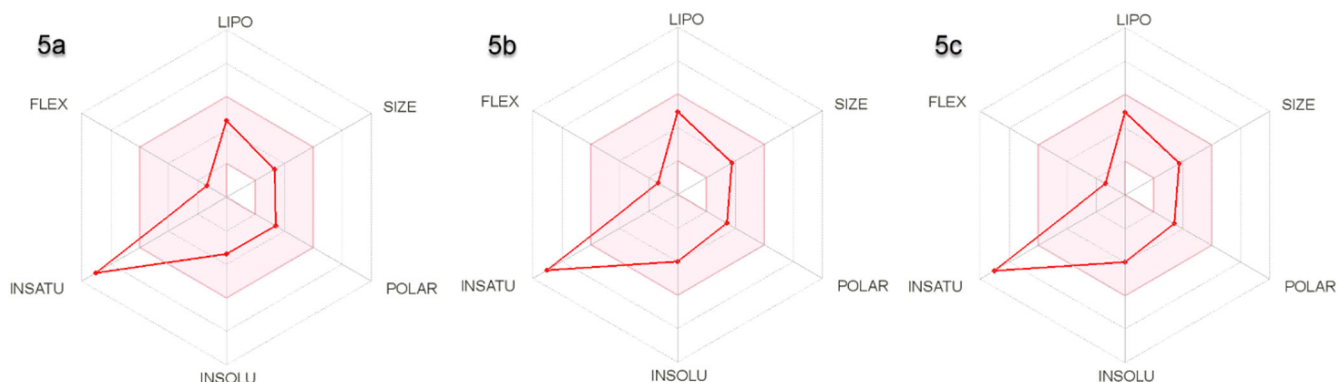


FIGURE 10 Bioavailability radar plot obtained from SwissADME server tool of compounds **5a–c**

FLT3-expressed AML have novel and promising modes of action. Compounds **5a** and **5b** are potent inhibitors of FLT-3 and demonstrate a selective FLT3 ITD-mutation profile. Their ability to induce cytostatic activity at lower doses and cytotoxic ($9\mu\text{M}$) at higher doses. Additionally, they showed a crucial impact on significant proteins affecting cell growth. The oxindole ring generally is of great biological potential due to the presence of two H-bond forming functionalities (N, C=O). The results point out that compounds **5a–c** are the most active pointing at the fact that the smaller-sized oxindoles might be advantageous in certain cases. This fact makes these compounds excellent candidates for drug design as they allow more structural versatility and the possibility of incorporating a variety of heterocycles that might contribute to additional receptor bonding. Additionally, the selectivity observed with certain cell lines adds to the nobility of these compounds though we do recognize that this needs to be addressed in detail such as exploring the FLT3 signaling pathway specifically STAT5 phosphorylation in the kinase I domain.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General remarks

All chemical compounds and organic solvents employed in the synthesis of the target compounds were of commercial grade and used without further purification. Reaction progress was monitored using pre-coated TLC plates (Kieselgel 60 F254, Merck), utilizing UV lamp at a wavelength of 254 nm for spot visualization. Melting points were measured on Stuart Electro-Thermal apparatus and recorded uncorrected. ^1H NMR was run on a JEOL JNM-ECA400 (^1H : 400 MHz) spectrophotometer at Zagazig University, Egypt. ^{13}C NMR was executed on a JEOL JNM-ECA400 (^{13}C : 100 MHz) spectrophotometer at Zagazig University, Egypt. $\text{DMSO}-d_6$ (ppm $\delta = 2.5$) was used as a solvent, and chemical shifts are reported in ppm correlated to TMS as a standard. Coupling constants (J) are

computed in hertz (Hz). Multiplicity were appointed as: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; dd, doublet of doublet; and m for multiplet. Sample purity was examined on HPLC (Agilent 1260, USA) at Deraya University, Minia, Egypt. LC/MS data were recorded on the LC/MS/MS (Agilent 1260 Infinity II with 6420 Triple Quad LC/MS detector) at Nawah Centre.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure for the synthesis of compounds **3a–e**^[43]

A mixture of the appropriate alkyl halide (**1a–e**, 1mmol), vanillin **2** (1mmol, 152mg), and potassium carbonate (1mmol, 138mg) was heated to reflux in acetonitrile (20ml) for 2h. The mixture is allowed to cool and concentrate under vacuum. The precipitate formed was filtered off and washed with acetonitrile to yield compounds **3a–e** that were used for the following steps without additional purification.

4.1.3 | General procedure for the synthesis of compounds **5a–c** and **6a–o**^[21]

Oxindoles (**4a–c**, 1mmol) were added to a solution of either compound **2** (1mmol, 152mg) or appropriate alkylated vanillin (**3a–e**, 1mmol) in EtOH (10ml). A catalytic amount of piperidine was added, and the reaction mixture was heated at reflux for 3–6h. Reaction progress was monitored using TLC. After completion, the reaction mixture is allowed to cool. The formed precipitate was filtered and washed with EtOH to yield compounds **5a–c** or **6a–o**.

4.1.3.1 | 3-(4-Hydroxy-3-methoxybenzylidene)indolin-2-one (**5a**)

Yellow powder; mp = 222–223°C; yield: 85%; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.60 (s, 1H), 8.67 (s, 1H), 7.83 (t, $J = 8.6$ Hz, 2H), 7.61 (s, 1H), 7.32 (s, 1H), 7.23 (t, $J = 8.4$ Hz, 1H), 7.16 (d, $J = 7.6$ Hz, 1H), 6.90

(t, $J = 7.6$ Hz, 1H), 6.80 (d, $J = 7.6$ Hz, 1H), 3.83 (s, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 169.00, 149.10, 147.56, 143.83, 141.03, 137.75, 133.28, 128.27, 125.10, 124.01, 123.47, 120.78, 120.27, 115.70, 113.69, 109.86, 55.62; ESI-MS (m/z): calcd for $\text{C}_{16}\text{H}_{13}\text{NO}_3$: 267.09; found: 265.9 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{16}\text{H}_{13}\text{NO}_3$: C, 71.90; H, 4.90; N, 5.24. Found: C, 72.05; H, 4.97; N, 5.18. Major isomer (HPLC, RT: 1.857 min): 93.41%.

4.1.3.2 | 5-Chloro-3-(4-hydroxy-3-methoxybenzylidene)indolin-2-one (5b)

Orange powder; mp = 233–234°C; yield: 80%; ^1H NMR (400 MHz, DMSO- d_6) δ 10.60 (s, 1H), 8.67 (s, 1H), 7.83 (t, $J = 8.6$ Hz, 1H), 7.61 (s, 1H), 7.32 (s, 1H), 7.23 (t, $J = 8.4$ Hz, 1H), 7.16 (d, $J = 7.6$ Hz, 1H), 6.90 (t, $J = 7.6$ Hz, 1H), 6.80 (d, $J = 7.6$ Hz, 1H), 3.83 (s, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 167.33, 147.42, 141.11, 138.31, 128.49, 126.63, 125.10, 124.71, 123.20, 121.44, 118.64, 115.88, 115.62, 113.47, 110.30, 55.38; ESI-MS (m/z): calcd for $\text{C}_{16}\text{H}_{12}\text{ClNO}_3$: 301.05; found: 299.8 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{16}\text{H}_{12}\text{ClNO}_3$: C, 63.69; H, 4.01; N, 4.64. Found: C, 63.74; H, 4.12; N, 4.69. Major isomer (HPLC, RT: 1.968 min): 67.98%.

4.1.3.3 | 6-Chloro-3-(4-hydroxy-3-methoxybenzylidene)indolin-2-one (5c)

Yellow powder; mp = 230–231°C; yield: 85%; ^1H NMR (400 MHz, DMSO) δ 10.68 (s, 1H), 9.85 (bs, 1H), 8.66 (d, $J = 1.8$ Hz, 1H), 7.81–7.71 (m, 1H), 7.68 (d, $J = 8.2$ Hz, 1H), 7.59 (s, 1H), 7.31–7.25 (m, 1H), 7.02 (d, $J = 8.1$ Hz, 1H), 6.99–6.81 (m, 1H), 3.83 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.03, 149.10, 147.56, 143.83, 137.75, 133.28, 128.27, 125.10, 124.01, 123.47, 120.78, 120.27, 115.70, 113.69, 109.86, 55.62; ESI-MS (m/z): calcd for $\text{C}_{16}\text{H}_{12}\text{ClNO}_3$: 301.05; found: 299.9 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{16}\text{H}_{12}\text{ClNO}_3$: C, 63.69; H, 4.01; N, 4.64. Found: C, 63.47; H, 4.14; N, 4.75. Major isomer (HPLC, RT: 1.972 min): 68.13%.

4.1.3.4 | 3-[4-(Benzyloxy)-3-methoxybenzylidene]indolin-2-one (6a)

Yellow powder; mp = 240–241°C; yield: 90%; ^1H NMR (400 MHz, DMSO) δ 10.55 (s, 1H), 8.67 (s, 1H), 7.83 (dd, $J = 8.6, 1.9$ Hz, 1H), 7.74 (s, 1H), 7.67 (d, $J = 7.5$ Hz, 1H), 7.47 (d, $J = 8.4$ Hz, 2H), 7.41 (dd, $J = 10.9, 4.8$ Hz, 2H), 7.34 (d, $J = 7.2$ Hz, 1H), 7.19 (d, $J = 7.6$ Hz, 1H), 7.17–7.13 (m, 1H), 6.98 (t, $J = 7.6$ Hz, 1H), 6.83 (d, $J = 7.6$ Hz, 1H), 5.19 (s, 2H), 3.85 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 167.44, 150.09, 148.24, 140.20, 137.33, 136.70, 128.47, 128.21, 128.00, 127.94, 127.49, 127.19, 125.42, 124.08, 120.92, 119.19, 115.27, 112.55, 109.23, 69.78, 55.46; ESI-MS (m/z): calcd for $\text{C}_{23}\text{H}_{19}\text{NO}_3$: 357.14; found: 355.8 $[\text{M}-\text{H}]^-$. Anal. calcd. for $\text{C}_{23}\text{H}_{19}\text{NO}_3$: C, 77.29; H, 5.36; N, 3.92. Found: C, 77.03; H, 5.29; N, 4.03. Major isomer (HPLC, RT: 1.925 min): 52.36%.

4.1.3.5 | 3-[4-[(4-Chlorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6b)

Yellow powder; mp = 248–249°C; yield: 92%; ^1H NMR (400 MHz, DMSO) δ 10.56 (s, 1H), 8.67 (s, 1H), 7.83 (d, $J = 8.5$ Hz, 1H), 7.74

(s, 1H), 7.67 (d, $J = 7.5$ Hz, 1H), 7.56–7.43 (m, 4H), 7.16 (d, $J = 28.3$ Hz, 2H), 6.98–6.94 (m, 1H), 6.83 (d, $J = 7.6$ Hz, 1H), 5.19 (s, 2H), 3.85 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 169.03, 159.21, 149.46, 148.85, 142.74, 140.22, 137.47, 136.38, 129.89, 129.78, 128.63, 126.98, 125.67, 124.04, 121.22, 119.24, 115.27, 113.92, 110.15, 69.77, 55.19; ESI-MS (m/z): calcd for $\text{C}_{23}\text{H}_{18}\text{ClNO}_3$: 391.10; found: 389.9 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{23}\text{H}_{18}\text{ClNO}_3$: C, 70.50; H, 4.63; N, 3.57. Found: C, 70.73; H, 4.60; N, 3.68. Major isomer (HPLC, RT: 2.036 min): 54.36%.

4.1.3.6 | 3-[4-[(4-Fluorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6c)

Yellow powder; mp = 235–236°C; yield: 95%; ^1H NMR (400 MHz, DMSO) δ 10.55 (s, 1H), 8.67 (d, $J = 1.8$ Hz, 1H), 7.84 (dd, $J = 8.5, 1.7$ Hz, 1H), 7.74 (s, 1H), 7.67 (d, $J = 7.6$ Hz, 1H), 7.52–7.45 (m, 2H), 7.24–7.21 (m, 2H), 7.17–7.11 (m, 2H), 6.98 (t, $J = 7.5$ Hz, 1H), 6.83 (d, $J = 7.6$ Hz, 1H), 5.17 (s, 2H), 3.84 (s, 3H). ESI-MS (m/z): ^{13}C NMR (100 MHz, DMSO) δ 167.44, 149.95, 148.24, 140.22, 137.31, 132.96, 132.39, 130.25, 130.17, 128.23, 127.57, 127.16, 125.41, 124.13, 120.93, 119.20, 115.41, 113.24, 109.24, 69.06, 55.60; ESI-MS (m/z): calcd for $\text{C}_{23}\text{H}_{18}\text{FNO}_3$: 375.13; found: 373.9 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{23}\text{H}_{18}\text{FNO}_3$: C, 73.59; H, 4.83; N, 3.73. Found: C, 73.78; H, 4.48; N, 3.71. Major isomer (HPLC, RT: 1.925 min): 52.36%.

4.1.3.7 | 3-[3-Methoxy-4-[(4-methoxybenzyl)oxy]benzylidene]indolin-2-one (6d)

Yellow powder; mp = 239–240°C; yield: 85%; ^1H NMR (400 MHz, DMSO) δ 10.55 (s, 1H), 8.66 (d, $J = 1.9$ Hz, 1H), 7.84 (dd, $J = 8.6, 1.9$ Hz, 1H), 7.74 (s, 1H), 7.67 (d, $J = 7.5$ Hz, 1H), 7.43–7.40 (m, 2H), 7.22–7.17 (m, 2H), 6.96–6.89 (m, 3H), 6.83 (d, $J = 7.7$ Hz, 1H), 5.10 (s, 2H), 3.83 (s, 3H), 3.76 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 167.44, 149.83, 148.25, 140.22, 137.28, 135.79, 132.57, 129.70, 128.49, 128.24, 127.65, 127.12, 125.40, 124.18, 120.93, 119.21, 115.30, 112.65, 109.24, 68.92, 55.49, 48.60; ESI-MS (m/z): calcd for $\text{C}_{24}\text{H}_{21}\text{NO}_4$: 387.15; found: 385.9 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{24}\text{H}_{21}\text{NO}_4$: C, 74.40; H, 5.46; N, 3.62. Found: C, 74.28; H, 5.54; N, 3.69. Major isomer (HPLC, RT: 1.934 min): 84.02%.

4.1.3.8 | 3-[3-Methoxy-4-(pyridin-2-ylmethoxy)benzylidene]indolin-2-one (6e)

Orange powder; mp = 217–218°C; yield: 83%; ^1H NMR (400 MHz, DMSO) δ 10.57 (s, 1H), 8.70 (d, $J = 1.8$ Hz, 1H), 8.60 (d, $J = 4.1$ Hz, 1H), 7.88–7.85 (m, 1H), 7.83 (dd, $J = 8.6, 1.8$ Hz, 1H), 7.75 (s, 1H), 7.68 (d, $J = 7.5$ Hz, 1H), 7.55 (d, $J = 7.8$ Hz, 1H), 7.38 (d, $J = 6.6$ Hz, 1H), 7.19 (t, $J = 7.6$ Hz, 1H), 7.15 (d, $J = 8.5$ Hz, 1H), 6.99 (t, $J = 7.5$ Hz, 1H), 6.84 (d, $J = 7.7$ Hz, 1H), 5.27 (s, 2H), 3.88 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 167.43, 156.37, 149.82, 149.15, 148.21, 140.23, 137.06, 136.14, 128.25, 127.71, 127.14, 125.39, 124.22, 123.09, 121.84, 120.93, 119.22, 115.32, 112.59, 109.24, 70.77, 55.52; ESI-MS (m/z): calcd for $\text{C}_{22}\text{H}_{18}\text{N}_2\text{O}_3$: 358.13; found: 356.8 $[\text{M}-\text{H}]^-$; Anal. calcd. for $\text{C}_{22}\text{H}_{18}\text{N}_2\text{O}_3$: C, 73.73; H, 5.06; N, 7.82.

Found: C, 73.54; H, 5.14; N, 7.89. Major isomer (HPLC, RT: 1.963 min): 92.5%.

4.1.3.9 | 3-[4-(Benzyloxy)-3-methoxybenzylidene]-5-chloroindolin-2-one (6f)

Yellow powder; mp = 220–221°C; yield: 82%; ¹H NMR (400 MHz, DMSO) δ 10.69 (s, 1H), 8.69 (s, 1H), 7.88–7.83 (m, 2H), 7.81 (s, 1H), 7.48–7.44 (m, 2H), 7.42–7.39 (m, 2H), 7.37 (d, *J* = 7.0 Hz, 1H), 7.20–7.16 (m, 2H), 6.84 (d, *J* = 8.2 Hz, 1H), 5.20 (s, 2H), 3.85 (s, 3H). ¹³C NMR (100 MHz, DMSO) δ 167.21, 150.52, 148.24, 139.35, 138.79, 136.61, 129.10, 128.47, 128.00, 127.30, 126.58, 125.29, 124.81, 123.50, 122.90, 119.22, 115.40, 112.57, 110.56, 69.79, 55.43. ESI-MS (*m/z*): calcd for C₂₃H₁₈ClNO₃: 391.13; found: 389.9 [M–H][–]; Anal. calcd. for C₂₃H₁₈ClNO₃: C, 70.50; H, 4.63; N, 3.57. Found: C, 70.68; H, 4.60; N, 3.76. Major isomer (HPLC, RT: 2.289 min): 49.35%.

4.1.3.10 | 5-Chloro-3-[4-[(4-chlorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6g)

Yellow powder; mp = 221–222°C; yield: 85%; ¹H NMR (400 MHz, DMSO) δ 10.69 (s, 1H), 8.69 (d, *J* = 1.9 Hz, 1H), 7.89 (s, 1H), 7.86 (d, *J* = 2.0 Hz, 1H), 7.81 (d, *J* = 2.0 Hz, 1H), 7.57–7.42 (m, 4H), 7.21 (dd, *J* = 8.2, 2.1 Hz, 1H), 7.17 (d, *J* = 8.5 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 5.21 (s, 2H), 3.85 (s, 3H). ¹³C NMR (100 MHz, DMSO) δ 167.21, 150.28, 148.26, 139.30, 138.82, 135.71, 132.60, 129.71, 129.14, 128.50, 127.65, 127.45, 126.77, 125.31, 123.01, 119.25, 115.44, 112.67, 110.58, 68.93, 55.46. ESI-MS (*m/z*): calcd for C₂₃H₁₇Cl₂NO₃: 425.06; found: 423.8 [M–H][–]; Anal. calcd. for C₂₃H₁₇Cl₂NO₃: C, 64.80; H, 4.02; Cl, 16.63; N, 3.29. Found: C, 65.01; H, 4.09; N, 3.73. Major isomer (HPLC, RT: 1.936 min): 64.86%.

4.1.3.11 | 5-Chloro-3-[4-[(4-fluorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6h)

Yellow powder; mp = 227–228°C; yield: 94%; ¹H NMR (400 MHz, DMSO) δ 10.69 (d, *J* = 8.6 Hz, 1H), 8.68 (d, *J* = 1.9 Hz, 1H), 7.88 (s, 1H), 7.83 (dd, *J* = 21.6, 2.0 Hz, 1H), 7.71–7.63 (m, 1H), 7.58–7.49 (m, 2H), 7.40–7.31 (m, 1H), 7.27–7.23 (m, 3H), 6.86 (dd, *J* = 24.5, 8.3 Hz, 1H), 5.18 (s, 2H), 3.83, 3.81 (2s, 3H). ¹³C NMR (100 MHz, DMSO) δ 167.23, 149.55, 148.26, 141.47, 139.35, 132.90, 130.29, 129.15, 127.71, 127.39, 126.69, 125.33, 124.84, 123.47, 119.25, 115.22, 113.12, 112.64, 110.60, 69.10, 55.45. ESI-MS (*m/z*): calcd for C₂₃H₁₇ClFNO₃: 409.09; found: 407.7 [M–H][–]; Anal. calcd. for C₂₃H₁₇ClFNO₃: C, 67.40; H, 4.18; N, 3.42. Found: C, 67.66; H, 4.28; N, 3.51. Major isomer (HPLC, RT: 1.936 min): 67.38%.

4.1.3.12 | 5-Chloro-3-[3-methoxy-4-[(4-methoxybenzyl)oxy]benzylidene]indolin-2-one (6i)

Yellow powder; mp = 241–242°C; yield: 82%; ¹H NMR (400 MHz, DMSO) δ 10.68 (s, 1H), 8.68 (s, 1H), 7.89 (s, 1H), 7.86 (d, *J* = 1.8 Hz, 1H), 7.81 (d, *J* = 2.0 Hz, 1H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.20–7.14 (m, 2H), 6.97 (d, *J* = 8.7 Hz, 2H), 6.84 (d, *J* = 8.2 Hz, 1H), 5.11 (s, 2H), 3.83 (s, 3H), 3.77 (s, 3H). ¹³C NMR (100 MHz, DMSO) δ 167.22, 159.16,

150.64, 148.24, 139.40, 138.78, 133.36, 129.83, 128.45, 127.76, 127.44, 127.18, 125.30, 122.81, 119.21, 115.37, 113.85, 112.56, 110.56, 69.60, 55.39, 55.12. ESI-MS (*m/z*): calcd for C₂₄H₂₀ClNO₄: 421.11; found: 419.9 [M–H][–]; Anal. calcd. for C₂₄H₂₀ClNO₄: C, 68.33; H, 4.78; N, 3.32. Found: C, 68.07; H, 4.85; N, 3.53. Major isomer (HPLC, RT: 1.935 min): 62.71%.

4.1.3.13 | 5-Chloro-3-[3-methoxy-4-(pyridin-2-ylmethoxy)benzylidene]indolin-2-one (6j)

Yellow powder; mp = 230–231°C; yield: 83%; ¹H NMR (400 MHz, DMSO) δ 10.69 (s, 1H), 8.71 (s, 1H), 8.60 (d, *J* = 6.3 Hz, 1H), 7.89 (s, 1H), 7.87 (d, *J* = 1.9 Hz, 1H), 7.85–7.77 (m, 1H), 7.71–7.63 (m, 1H), 7.56–7.52 (m, 1H), 7.40 (dd, *J* = 11.0, 3.9 Hz, 1H), 7.22 (dd, *J* = 11.3, 3.0 Hz, 1H), 7.19 (dd, *J* = 11.2, 5.3 Hz, 1H), 6.87 (dd, *J* = 24.2, 8.3 Hz, 1H), 5.28 (s, 2H), 3.87, 3.84 (2s, 3H). ¹³C NMR (100 MHz, DMSO) δ 167.20, 156.32, 150.25, 149.16, 148.23, 139.27, 138.03, 137.06, 129.14, 127.51, 127.35, 125.27, 124.82, 123.49, 123.11, 121.91, 119.26, 115.46, 112.62, 110.58, 70.77, 55.49. ESI-MS (*m/z*): calcd for C₂₂H₁₇ClN₂O₃: 392.09; found: 390.7 [M–H][–]; Anal. calcd. for C₂₂H₁₇ClN₂O₃: C, 67.26; H, 4.36; N, 7.13. Found: C, 66.99; H, 4.31; N, 7.21. Major isomer (HPLC, RT: 1.936 min): 57.29%.

4.1.3.14 | 3-[4-(Benzyloxy)-3-methoxybenzylidene]-6-chloroindolin-2-one (6k)

Yellow powder; mp = 213–214°C; yield: 92%; ¹H NMR (400 MHz, DMSO) δ 10.71 (s, 1H), 8.65 (s, 1H), 7.83 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.80 (s, 1H), 7.69 (d, *J* = 8.1 Hz, 1H), 7.52–7.45 (m, 2H), 7.41 (t, *J* = 7.2 Hz, 2H), 7.35 (t, *J* = 6.2 Hz, 1H), 7.17 (d, *J* = 8.5 Hz, 1H), 7.03 (d, *J* = 6.2 Hz, 1H), 6.84 (s, 1H), 5.19 (s, 2H), 3.82, 3.80 (2s, 3H). ¹³C NMR (100 MHz, DMSO) δ 168.93, 148.87, 144.03, 136.71, 128.52, 127.99, 126.81, 124.54, 123.36, 120.87, 113.22, 69.96, 55.63. ESI-MS (*m/z*): calcd for C₂₃H₁₈ClNO₃: 391.10; found: 389.8 [M–H][–]; Anal. calcd. for C₂₃H₁₈ClNO₃: C, 70.50; H, 4.63; N, 3.57. Found: C, 70.23; H, 4.62; N, 3.77. Major isomer (HPLC, RT: 1.936 min): 57.33%.

4.1.3.15 | 6-Chloro-3-[4-[(4-chlorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6l)

Yellow powder; mp = 235–236°C; yield: 88%; ¹H NMR (400 MHz, DMSO) δ 10.72 (s, 1H), 8.65 (s, 1H), 7.83 (dd, *J* = 8.6, 1.8 Hz, 1H), 7.70 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.56–7.42 (m, 4H), 7.35 (dd, *J* = 13.2, 4.3 Hz, 1H), 7.17 (dd, *J* = 11.9, 8.5 Hz, 1H), 7.00 (dd, *J* = 32.5, 9.2 Hz, 1H), 6.87 (dd, *J* = 18.1, 1.9 Hz, 1H), 5.20 (s, 2H), 3.85 (s, 3H). ¹³C NMR (100 MHz, DMSO) δ 168.93, 149.56, 148.87, 144.03, 137.21, 136.71, 133.64, 128.52, 128.06, 127.99, 127.55, 126.81, 124.54, 123.54, 123.36, 120.87, 120.16, 113.22, 110.02, 69.96, 55.63. ESI-MS (*m/z*): calcd for C₂₃H₁₇Cl₂NO₃: 425.06; found: 423.7 [M–H][–]; Anal. calcd. for C₂₃H₁₇Cl₂NO₃: C, 64.80; H, 4.02; N, 3.29. Found: C, 64.61; H, 4.13; N, 3.48. Major isomer (HPLC, RT: 1.936 min): 62.17%.

4.1.3.16 | 6-Chloro-3-[4-[(4-fluorobenzyl)oxy]-3-methoxybenzylidene]indolin-2-one (6m)

Yellow powder; mp = 224–225°C; yield: 95%; ¹H NMR (400 MHz, DMSO) δ 10.72 (s, 1H), 8.65 (s, 1H), 7.84 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.71

(dd, $J = 8.2$, 3.0 Hz, 1H), 7.54–7.50 (m, 2H), 7.35 (dd, $J = 8.5$, 6.0 Hz, 1H), 7.26 (dd, $J = 9.0$, 2.7 Hz, 1H), 7.23–7.14 (m, 2H), 7.04 (dd, $J = 8.1$, 1.9 Hz, 1H), 6.87 (dd, $J = 18.2$, 1.9 Hz, 1H), 5.18 (s, 2H), 3.83, 3.80 (2s, 3H). ESI-MS (m/z): calcd for $C_{23}H_{17}ClFNO_3$: 409.09; found: 407.7 $[M-H]^-$; Anal. calcd. for $C_{23}H_{17}ClFNO_3$: C, 67.40; H, 4.18; N, 3.42. Found: C, 67.28; H, 4.30; N, 3.47. Major isomer (HPLC, RT: 1.935 min): 54.31%.

4.1.3.17 | 6-Chloro-3-[3-methoxy-4-[(4-methoxybenzyl)oxy]benzylidene]indolin-2-one (6n)

Yellow powder; mp = 243–244°C; yield: 90%; ^1H NMR (400 MHz, DMSO) δ 10.72 (s, 1H), 8.65 (d, $J = 1.9$ Hz, 1H), 7.90–7.78 (m, 1H), 7.71 (dd, $J = 8.2$, 3.0 Hz, 1H), 7.63 (s, 1H), 7.60–7.47 (m, 2H), 7.39–7.31 (m, 1H), 7.26 (dd, $J = 9.0$, 2.7 Hz, 1H), 7.03–6.98 (m, 2H), 6.87 (dd, $J = 18.2$, 1.9 Hz, 1H), 5.18 (s, 2H), 3.83, 3.81 (2s, 3H), 3.76 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 168.91, 159.17, 149.64, 148.84, 144.01, 137.23, 133.60, 129.84, 128.54, 128.49, 126.65, 124.45, 123.50, 123.37, 120.84, 120.16, 113.88, 113.18, 113.09, 69.73, 55.57, 55.14. ESI-MS (m/z): calcd for $C_{24}H_{20}ClNO_4$: 421.11; found: 419.9 $[M-H]^-$; Anal. calcd. for $C_{24}H_{20}ClNO_4$: C, 68.33; H, 4.78; N, 3.32. Found: C, 68.09; H, 4.88; N, 3.47. Major isomer (HPLC, RT: 1.933 min): 64.83%.

4.1.3.18 | 6-Chloro-3-[3-methoxy-4-(pyridin-2-ylmethoxy)benzylidene]indolin-2-one (6o)

Yellow powder; mp = 210–211°C; yield: 93%; ^1H NMR (400 MHz, DMSO) δ 10.71 (s, 1H), 8.66 (d, $J = 2.0$ Hz, 1H), 8.59 (d, $J = 4.0$ Hz, 1H), 7.86 (dd, $J = 16.2$, 10.2 Hz, 1H), 7.81 (d, $J = 8.8$ Hz, 1H), 7.70 (dd, $J = 8.2$, 4.4 Hz, 1H), 7.58–7.52 (m, 1H), 7.39–7.31 (m, 2H), 7.17 (dd, $J = 12.9$, 8.5 Hz, 1H), 7.06–6.95 (m, 1H), 6.87 (dd, $J = 17.8$, 2.0 Hz, 1H), 5.26 (s, 2H), 3.85, 3.82 (2s, 3H). ESI-MS (m/z): calcd for $C_{22}H_{17}ClN_2O_3$: 392.09; found: 390.9 $[M-H]^-$; Anal. calcd. for $C_{22}H_{17}ClN_2O_3$: C, 67.26; H, 4.; N, 7.13. Found: C, 67.46; H, 4.44; N, 7.20. Major isomer (HPLC, RT: 1.934 min): 76.62%.

4.2 | Pharmacological/biological assays

4.2.1 | Cells and culture conditions

MV4-11 (Cat. no. CRL-9591) (biphenotypic B myelomonocytic leukemia) and THP-1 (Cat. no. TIB-202) (acute monocytic leukemia) cell lines were purchased from American Type Culture Collection (ATCC). MV4-11 cells were cultured in IMDM-Iscove's Modified Dulbecco's Medium (Cat. no. 01-058-1A, Biological Industries) supplemented with 10% heat-inactivated fetal bovine serum (Biowest), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco) and 2.5 μ g/ml plasmocin (Invivogen) in a 5% CO_2 humidified incubator at 37°C. THP-1 cells were maintained in RPMI 1640 medium (ATCC modification) (Cat. no. A10491-01) with 10% heat-inactivated fetal bovine serum (Biowest), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco) and 2.5 μ g/ml plasmocin (Invivogen) in a 5% CO_2 humidified incubator at 37°C. Cells were regularly cultured in cell

culture flasks and passaged when they reached a density above 70%. Before the main experiments, cell counts were made using a hemocytometer, and cell viability was also measured by the trypan blue (Gibco) exclusion method.

4.2.2 | Compound screening against AML cells with WST-1 assay

Single-dose screening of compounds against MV4-11 and THP-1 cells was performed by the Premix WST-1 Cell Proliferation Assay System (Takara Bio). Cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates with 50 μ l growth media. 24 h after cell seeding, 50 μ l of each oxindole compound was applied to the cells at a final concentration of 10 μ M. 48 or 72 h after treatments, 10 μ l of WST-1 reagent was added to the cells and incubated for 3 h at 37°C and 5% CO_2 . After incubation, the absorbance was measured at 450 nm and a reference wavelength of 600 nm using a microplate reader (Infinite® 200 PRO) (Tecan Life Sciences). For cell culture experiments, compounds were dissolved in DMSO (Sigma) at a stock concentration of 20 mM. For treatments, doses were prepared from 20 mM stocks with cell growth medium with a final DMSO concentration of 0.1%. For the negative control, 0.1% DMSO-containing media was used.

4.2.3 | Dose-dependent cell proliferation assay

Dose-dependent inhibition profiles of the potent compound were performed by Premix WST-1 Cell Proliferation Assay System (Takara Bio) as we had previously described with slight modifications.^[44] MV4-11 or THP-1 cells were seeded 1.0×10^4 cells/well with 50 μ l growth mediums in 96-well plates. 24 h after seeding, cells were treated with compound OV in two-fold serial dilution doses between 2.25 and 18 μ M. 0.1% DMSO-containing media was used as a negative control. Cells were incubated with increasing doses of compound 5a for 48 or 72 h. After treatments, 10 μ l of WST-1 reagent was added to the cells and incubated for 3 h under cell growth conditions. Absorbance values were obtained by taking readings at a wavelength of 450 nm with a microplate reader (Infinite® 200 PRO) (Tecan Life Sciences). The 600 nm wavelength was used as a reference reading. IC_{50} values were calculated and analyzed using GraphPad Prism 9.2.0. software.

4.2.4 | Cell morphology

Morphological analysis was performed to observe the phenotypic effects of compound 5a on MV4-11 cells, as we had previously reported.^[44] For this purpose, 5.0×10^5 cells were seeded in 6-well plates in a 1 ml growth medium. After 24 h, 2.25, 4.5, 9, and 18 μ M of compound 5a were applied to the cells. 0.1% DMSO-containing media was used as a negative control. After 48 h of incubation with

compounds, cells were photographed under an inverted microscope, Leica DM IL LED with a DFC-290 camera (Leica).

4.2.5 | High-throughput protein profiling

Human Apoptosis Antibody Array—Membrane 43 Targets (Abcam) was used to determine the apoptotic profiling of compound **5a** on MV4-11 cells. For this purpose, 5.0×10^5 cells were seeded in each well of the 6-well cell culture plate. After 24 h of incubation from seeding, 9 μ M of compound **5a** was applied to the cells. Medium containing 0.01% DMSO was used as the untreated control. After 48 h of treatment, cells were collected in a 2 ml Eppendorf tube and centrifuged at 1500 rpm for 5 min. The cell pellet was washed with PBS and then centrifuged at 1500 rpm for 10 min. Four hundred microliter of 1 $\times$ Lysis Buffer was added to the cell pellet. It was incubated for 30 min at 2–8°C on a shaker (0.5–1 cycle/s). The lysate was centrifuged at 14,000 rpm for 15 min. The supernatant was collected, and total protein quantification was performed with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Meanwhile, each Array membrane was blocked by incubating with 2 ml of 1X Blocking Buffer for 30 min at RT. Cell lysates diluted with 1X Blocking Buffer were then added onto the membranes and incubated overnight at 4°C. Membranes were treated with 1 ml of 1 $\times$ Biotin-conjugated Anti-Cytokines after serial washings with Wash Buffer I and Wash Buffer II and incubated overnight at 4°C. After serial washings were performed in the same way, 2 ml of 1X HRP-Streptavidin was pipetted into the membranes and incubated at RT for 2 h. After a final series of washes, the membranes were labeled with Detection Buffer C and D, and chemiluminescence imaging was performed on the Odyssey[®] XF Imaging System (LI-COR Biosciences). The relative intensities were evaluated with Image J software (National Institutes of Health). Normalization of specific protein expressions in each membrane was done according to the following formula:

$$X(Ny) = X(y) * P1/P(y),$$

P1 = mean signal density of positive control spots on reference array,

P(y) = mean signal density of positive control spots on array "y,"

X(y) = mean signal density for spot "X" on array for sample "y,"

X(Ny) = normalized signal intensity for spot "X" on array "y."

4.2.6 | FLT3 kinase inhibition assay

Determination of the FLT3 kinase inhibition effects of potent compounds was performed with the ADP-Glo[™] Assay using the FLT3 Kinase Enzyme System (Promega) according to the manufacturer's instructions. First, ATP to ADP conversion study was performed. FLT3 kinase was titrated against 50 or 100 μ M ATP with two-fold serial dilutions at concentrations of 200 to 3.1 ng. As a

result of titration, 100 ng FLT3 enzyme concentration was selected. Potent compounds (**5a–c**) were titrated against 100 ng of FLT3 enzyme in two-fold serial dilutions from 5 μ M to 0.625 nM in the presence of 100 μ M ATP. One microliter of the compound, 2 μ l of FLT3 enzyme, and 2 μ l of substrate/ATP mix were added to the wells of 384-well plates and incubated at RT for 120 min. Then 5 μ l of ADP-Glo[™] Reagent was added. Incubation was done for 40 min at RT. Finally, 10 μ l of Kinase Detection Reagent was added and incubated at RT for 30 min. Luminescence measurement was taken using a microplate reader (Infinite[®] 200 PRO) (Tecan Life Sciences).

4.3 | Molecular modeling

4.3.1 | Molecular docking

Receptor preparation

The 3D structure of the human Fms-like tyrosine kinase 3 (FLT3) co-crystallized with its inhibitor Gilteritinib was downloaded from the protein RSCB database (PDB id: 6JQR).^[45] The structure was prepared for the docking investigations by the PrepWizard module of Maestro 11.1.^[46] The missing chains were automatically added by Prime [Prime]. The protonation state was next estimated by PropKa at pH = 7.4 followed by minimization of the receptor-ligand complex by the OPLS-3 force field employing a well-established protocol described by our research group.^[47–49]

Docking grid generation

The Glide module of Maestro was employed for generating the docking grid.^[46] The grid was centered on the crystallographic inhibitor found in the crystal structure with an extended space of 25 $\times$ 25 $\times$ 25 Angstrom. The generated grid was used for the docking experiments.

Docking validation protocol

A self-docking experiment was performed to validate the docking protocol used. The originally separated inhibitor was removed from the receptor, prepared, and minimized by the LigPrep module^[46] of Maestro using EpiK at pH = 7.4.^[47] and used for the self-docking protocol. The obtained ligand was presented at the first round of docking using Glide at Standard Precision (Glide-SP) accuracy; then, the best-ranked pose was exposed to the second round of docking by Glide in eXtra Precision (Glide-XP) mode. The best-ranked pose (RMSD = 0.3 Å) is superimposed on the original crystallographic pose. Thus, the validated protocol pertained to and was employed for the docking experiments of the novel compounds **5a–c**. The picture of superimposing selfdocked Gilteritinib with its crystallographic pose and with the best pose of compound *cis-5b* are depicted in Supporting Information: Figures S2.

Ligands preparation

Compounds **5a–c** were drawn by the 2D editor embedded in Maestro; both geometric isomers have been taken into consideration; thus, the

ligands tested were named *cis*-5a, *trans*-5a, *cis*-5b, *trans*-5b, *cis*-5c, and *trans*-5c and prepared by the LigPrep module following the same procedure applied to Gilteritinib. Then, the minimized structures were submitted to the docking experiments without further modifications.

Ligand docking experiments

The prepared molecules *cis* and *trans* 5a–c were docked to the FLT3 enzyme. The first round of docking was performed by Glide in Standard Precision mode. The best pose generated from the first step was then submitted to the second round of docking using Glide in eXtra Precision accuracy. The best-ranked poses are depicted in Figure 8 as three-dimensional pictures (for 2D interaction diagrams of the crystallographic ligand, see Supporting Information: Figure S2).

Binding energy estimation

All best-ranked docking poses of *cis* and *trans* compounds 5a–c were submitted to the binding energy estimation by the MM/GBSA approach in the Prime program of the Schrodinger software suite.^[48] MM/GBSA protocol has been applied using default parameters to calculate the free binding energy of the ligand–enzyme complex in a water environment, employing the variable dielectric surface generalized Born model (VSGB),^[49] with empirical corrections to hydrogen-bond and π -stacking interactions, to give favorable binding free energies for different protein–ligand complexes.^[50] The calculated free binding energy is reported in Table 4 as kcal/mol.

4.3.2 | MD simulations

MD simulation was executed with Gromacs version 2020.4 to analyze the stability of the 5a–c, and Gilteritinib & FLT3 complex (PDB ID: 6JQR) to validate and compare the MD simulation.^[51] The molecular dynamic system required input files are created by the solution builder tool of the CHARMM-GUI web server.^[52] The topology file of the compound 5a–c, Gilteritinib, and FLT3 was generated with the TIP3 water model with an amber Charmm36m^[53] force fields. The rectangular system size for simulation was defined as 10 Å from the surface of the protein center. The MD simulations system was neutralized by adding 0.15 M KCl with the Monte-Carlo ion placing method. Molecular dynamics simulations are performed in the periodic boundary condition. The system was minimized and then equilibrated with 0.5 ns NVT and 0.5 ns NPT stages at 1 atm pressure and 303.15 K temperature according to Nose-Hoover^[54] thermostat and Parrinello-Rahman^[55] barostat. The 300 ns MD simulation was performed with the leap-frog MD integrator to 2fs. Long-range electrostatic interactions and covalent bond constraints were estimated using Particle Mesh Ewald (PME) method and linear constraint (LINCS) algorithm, respectively. Trajectory analysis was performed with Gmx scripts for RMSD and RMSF measurements. Molecular mechanical Poisson-Boltzmann surface area (MM-PBSA) and binding free energy calculations were performed using RashmiKumari's^[56] g_mmpbsa package. MD trajectory analysis results were generated with PyMOL Molecular Graphics System version 2.4.1, and graphics were produced with QtGrace v0.2.6 software.

4.3.3 | ADMET Estimation

The physicochemical properties, lipophilicity such as logP value, water solubility calculations, pharmacokinetics such as GI absorption and CYP enzyme inhibitor, drug-likeness such as Lipinski, and medicinal chemistry properties such as the lead likeness of the compounds 5a–c were performed with the SwissADME online tool (<http://www.swissadme.ch/>).^[42] Toxicity parameters of the compounds were calculated via the pkCSM-pharmacokinetics server (<http://biosig.unimelb.edu.au/pkcsm/>).^[57]

4.4 | Statistical analysis

The statistical analyses were performed using the student *t*-test to compare differences in results between the control and treatment groups for the related part of the study. $p < 0.05$ was considered to be statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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